UNIT-I

SYLLABUS

Introduction, Extraction and Separation of Proteins: Introduction - Amino acids and their properties - hydrophobic, polar and charged amino acids. Biologically important peptides - hormones, antibiotics and growth factors. Multimeric proteins, conjugated proteins and metallo proteins. Diversity of function. Extraction of proteins for downstream processing - Solubilization of proteins from their cellular and extracellular locations. Use of simple grinding methods, homogenization, ultrasonication, French press and centrifugation. Separation techniques - Ammonium sulphate fractionation, solvent fractionation, dialysis and lyophilization.

Amino Acids and Proteins

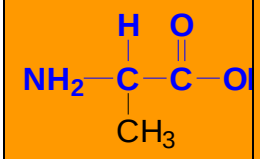
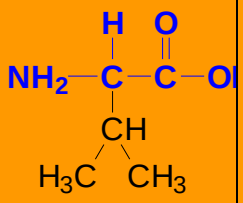
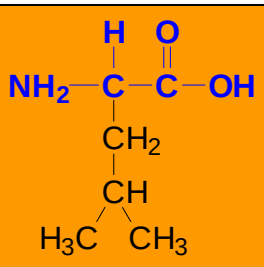
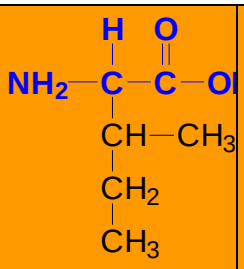
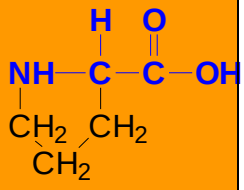
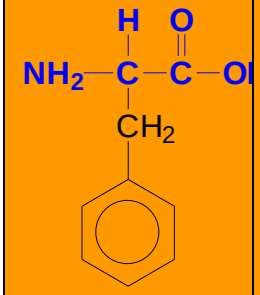
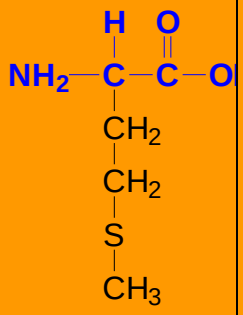
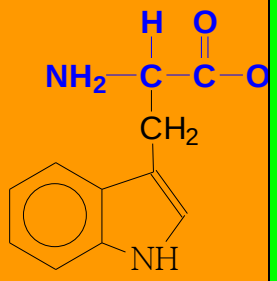
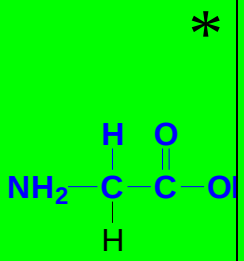
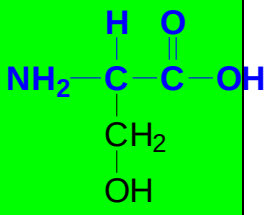
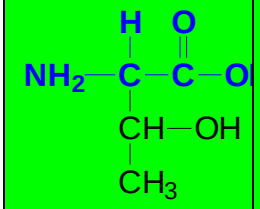
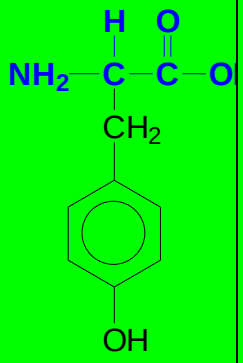
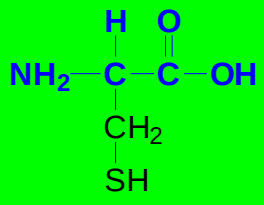
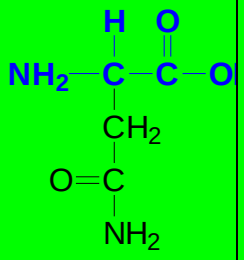
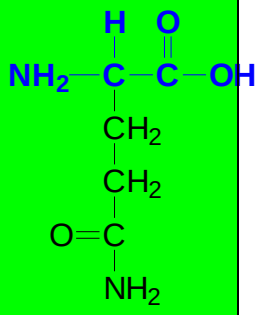
Proteins are about 50% of the dry weight of most cells, and are the most structurally complex molecules known. Each type of protein has its own unique structure and function. Polymers are any kind of large molecules made of repeating identical or similar subunits called monomers. The starch and cellulose we previously discussed are polymers of glucose, which in that case, is the monomer. Proteins are polymers of about 20 amino acids monomers.

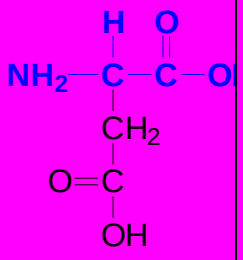
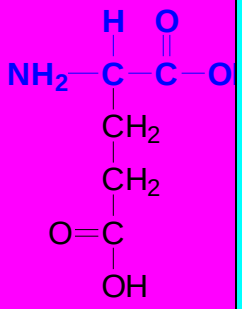
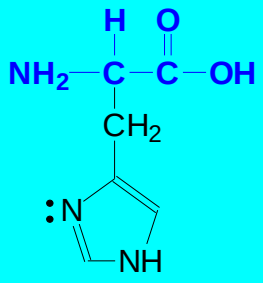
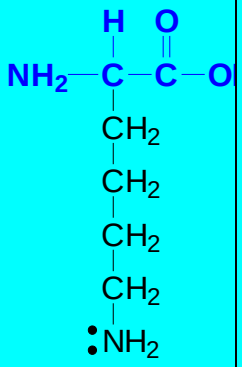
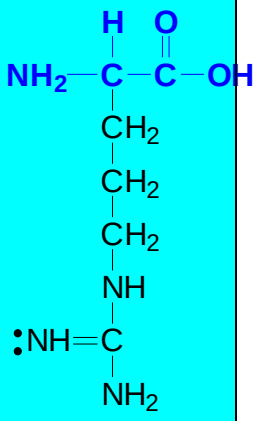
The amino acids all have both a single and triple letter abbreviation. Here is an example.

Alanine = A = Ala

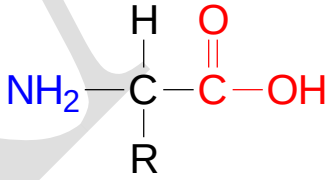
- Each amino acid contains an "amine" group, (NH_2) and a "carboxylic acid" group (COOH) (shown in **black** in the diagram).
- The amino acids vary in their side chains (indicated in **blue** in the diagram).
- The eight amino acids in the **orange** area are nonpolar and hydrophobic.
- The other amino acids are polar and hydrophilic ("water loving").
- The two amino acids in the **magenta** box are acidic ("carboxylic" group in the side chain).
- The three amino acids in the **light blue** box are basic ("amine" group in the side chain).

Amino Acids: shown in another structural formula format

				
alanine	valine	leucine	isoleucine	proline
ala – A	val – V	leu – L	ile – I	pro – P
				
phenylalanine	methionine	tryptophan	glycine*	serine
phe – F	met – M	trp – W	gly – G	ser – S
				

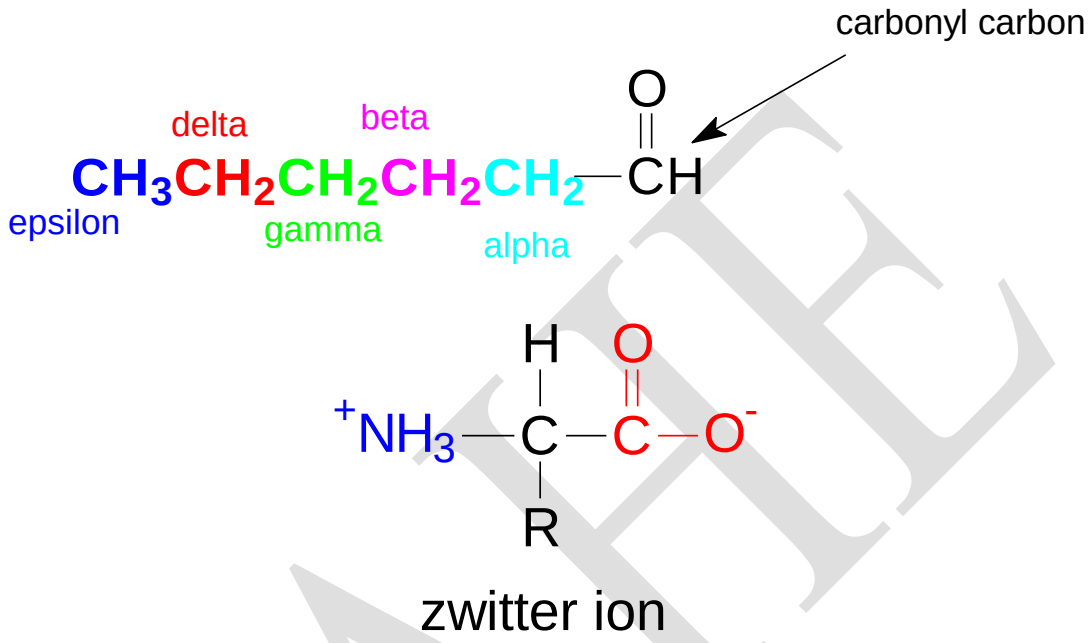
threonine	tyrosine	cysteine	asparagine	glutamine
thr – T	tyr – Y	cys – C	asn – N	gln – Q
				
aspartic acid	glutamic acid	histidine	lysine	arginine
asp – D	glu – E	his – H	lys – K	arg – R

Amino acids are named as such because each amino acid consists of an **amine** portion and a **carboxylic acid** part, as seen below.



Compare this structure to the above structures of each of the amino acids. Each amino acid has this general structure. The side chains are sometime shown as R-groups when illustrating the backbone. In the approximately 20 amino acids found in our bodies, what varies is the side chain. Some side chains are hydrophilic while others are hydrophobic. Since these side chains stick out from the backbone of the molecule, they help determine the properties of the protein made from them. The amino acids in our bodies are referred to as alpha amino acids. The reason is that the central carbon is in an alpha position in relation to the carbonyl carbon. The carbon

adjacent to the carbonyl carbon is designated the alpha carbon. Each carbon in the chain will be designated with a different letter of the Greek alphabet. See the example below.

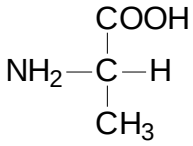


You may have noticed that the general form for the amino acid is often drawn with the acidic hydrogen attached to the amine group. This occurs because amine groups are basic. So, the amino acid has performed an acid-base reaction on itself. When the amino acid is in this form it is referred to as a Zwitter ion. When amino acids are in solution this is the form that they will be found.

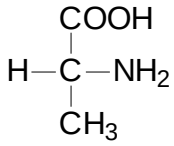
Chirality:

A chiral compound must contain a carbon that is bonded to four different atoms/groups. If you look at the above amino acids you will see that, with the exception of glycine, each structure is chiral around the carbon with the R group. Each amino acid will come in two structural formats, called enantiomers, an L and a D. You were given two tables of all the amino acids, the tables

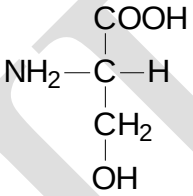
are only different in the format that the amino acids are drawn. The first format is the one used to show the chirality of the amino acid. To determine which enantiomer you have you look to the location of the hydrogen on the chiral carbon. If the hydrogen is on the left, then the amine group is on the right, this is the D enantiomer. If the hydrogen is on the right, then the amine group is on the left, this is the L enantiomer. See the below structural diagrams:



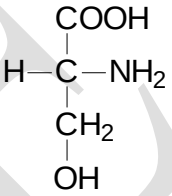
L-alanine



D-alanine



L-serine

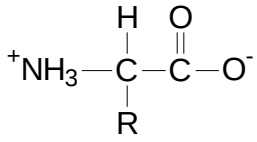


D-serine

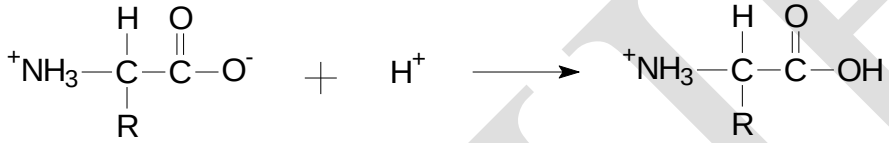
The importance of chiral compounds is that their chemical reactivity is different. Sometimes the difference means the compound will have an adverse effect on a person. Sometimes the difference means the person simple can not metabolize the compound. The latter is the case with amino acids. Meaning we can consume both L and D amino acids, but our bodies will only metabolize the D form. The enzymes used in the metabolism of amino acids are built to fit this D form but not the L form. The L form will pass through your body unused.

Buffering:

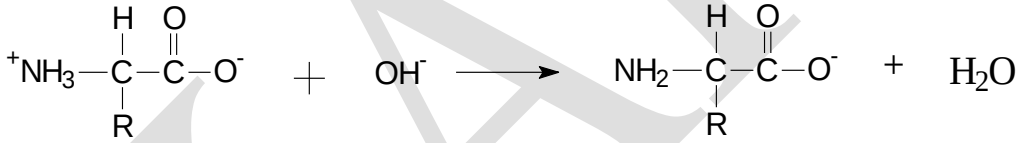
α-amino acids contain both acidic -COOH and the basic $–NH_2$ groups, these two functional groups allow them to act as a buffer. Unfortunately, though, the picture is not as simple as this. In the solid crystalline state the a-amino acids exist as Zwitter ions, as discussed before they are formed by the transfer of protons, H^+ from the -COOH to the $–NH_2$ groups. For α-amino acids without acidic or basic side chains these zwitter ions have charged groups but are neutral overall. Amino acids are found in the Zwitter form even as solids, they form an ionic matix similar to salts.



Zwitter ions remain when the α-amino acid is dissolved in water at pH 7. Addition of an acid, supplying more protons, produces ions with an overall positive charge. The amino acid forms the below structure in an acid environment.



Addition of a base, removes the acidic hydrogens, producing ions with an overall negative charge. The amino acid forms the below structure in a basic environment.



We can describe α-amino acids as amphoteric as they can react with both acid and alkali. They are effective buffers in biological systems. The situation is more complicated in a-amino acids that have acidic or basic R groups , e.g. glu or lys.

At very low pH all α-amino acids exist as ions with an overall positive charge, while at high pH they exist as ions with an overall negative charge. For each α-amino acid there is a pH between these extremes at which its molecules are neutral overall. This value is called the **isoelectric point** for the α-amino acid. At its isoelectric point the α-amino acid molecules will not move when placed in an electric field. The separation technique called

electrophoresis relies on molecules with different isoelectric points moving at different speeds when kept at a fixed pH and placed in an electric field.

The isoelectric point is calculated by averaging the pKa values for the carboxylic acid and the amine group. pK_1 applies to the carboxylic acid and pK_2 applies to the amine.

$$pI = \frac{pK_1 + pK_2}{2}$$

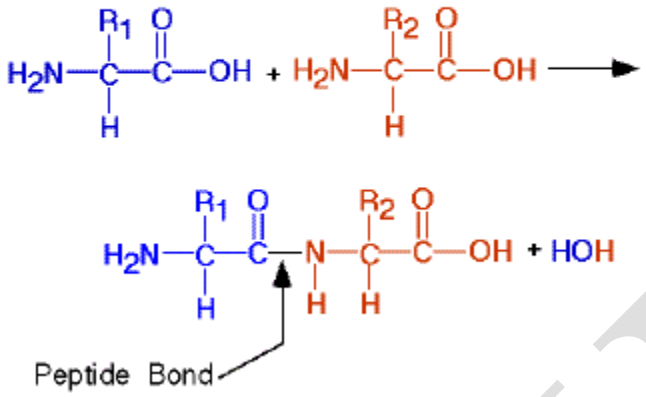
This formula does not take into account acidic or basic side chains. If an acidic side chain is present you average the side chain pK_a and pK_1. For a basic side chain you average the side chain pK_a and pK_2.

Protein Functions:

Type of Protein	Function	Examples
Structure	structural support	collagen in tendons and cartilage keratin in hair and nails
Contractile	muscle movement	actin, myosin, tubulin and kinesin proteins
Transportation	movement of compounds	hemoglobin carries O_2 and lipoproteins carry lipids
Storage	nutrient storage	ferritin stores iron is spleen and liver casein stores proteins in milk
Hormone	chemical communication	insulin regulates blood sugar
Enzyme	Catalyze biological reactions	lactase breaks down lactose trypsin breaks down proteins
Protection	Recognized and destroy foreign substances	immunoglobulins stimulate immune system

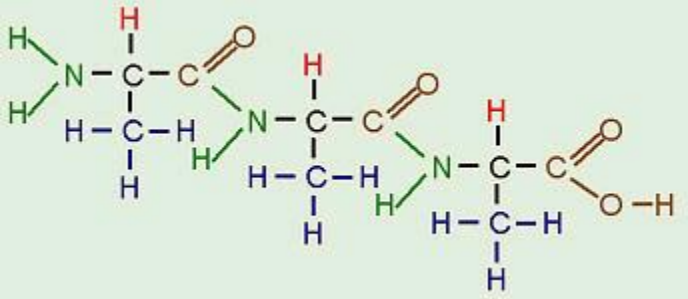
Reactions of Amino Acids: To form protein, the amino acids are linked by dehydration synthesis to form peptide bonds. The chain of amino acids is also known as a polypeptide.

Peptide Bond Formation



Protein Structure Types:

Some proteins contain only one polypeptide chain while others, such as hemoglobin, contain several polypeptide chains all twisted together. The sequence of amino acids in each polypeptide or protein is unique to that protein, this is call the **primary structure**. If even one amino acid in the sequence is changed, that can potentially change the protein's ability to function. For example, sickle cell anemia is caused by a change in only one nucleotide in the DNA sequence that causes just one amino acid in one of the hemoglobin polypeptide molecules to be different. Because of this, the whole red blood cell ends up being deformed and unable to carry oxygen properly. The **primary structure** is created through the linking of amino acids. This linking is accomplished by the formation of a peptide bond. This is a dehydration reaction. In other words the peptides combine and lose a water molecule. The peptide bonding of three alanine amino acids are shown below.



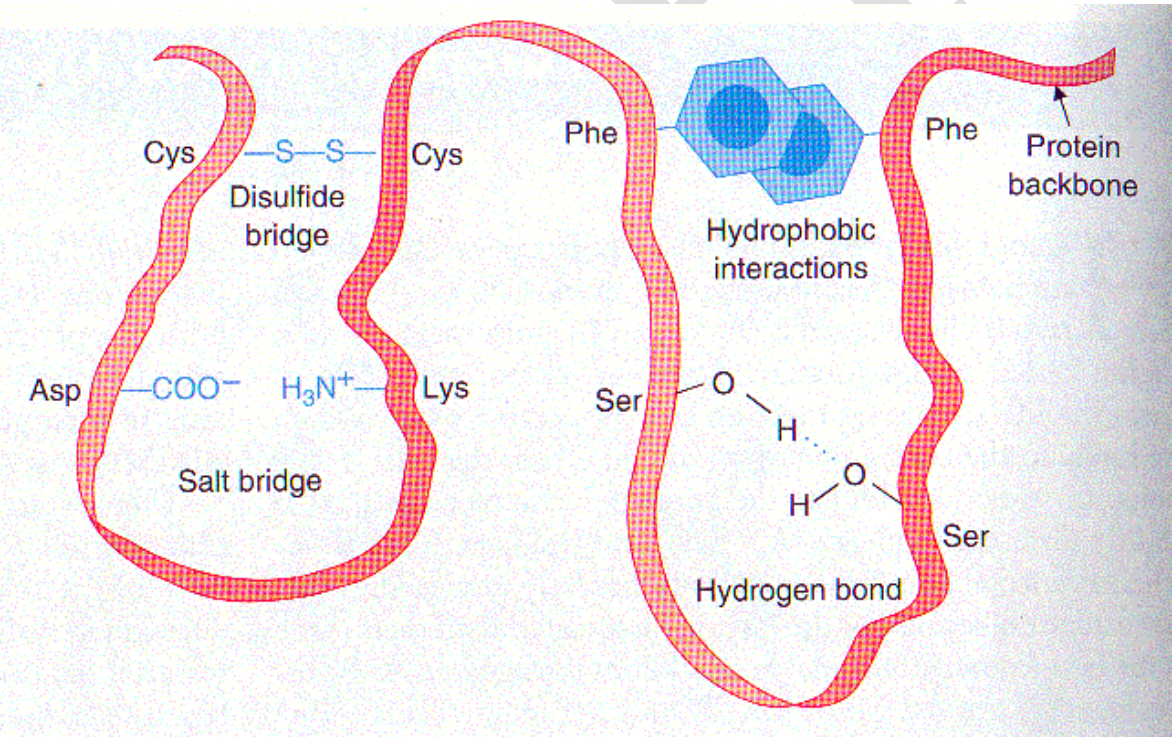
A longer polypeptide is shown above. Each peptide chain will have an amine end and a carboxylic acid end and each amino acid is referred to as a residue. So, the ends are named, n-terminal residue and c-terminal residue or the n-terminus and c-terminus.

The sequence of amino acids will cause the protein to have areas of its shape conforming to one of 3 shapes, alpha-helical, beta-pleated sheet or woven (sometimes called turns). These are **secondary structures.**

The two most common secondary structures are shown above, alpha helical and beta sheet.

These structures are created by molecular interactions between amino acids. Normally the interactions are hydrogen bonds. Other interactions will form as well.

- Hydrogen bonds, in the most simple explanation, from between hydrogens attached to an oxygen or nitrogen and the lone pairs found on an oxygen or nitrogen.
- Disulfide bridges form between cysteine and methionine amino acids.
- Salt bridges are interactions between the ends of the Zwitter ion, the NH_3^+ and the COO^-.
- Hydrophobic interactions are formed between those amino acids with hydrophobic R groups.

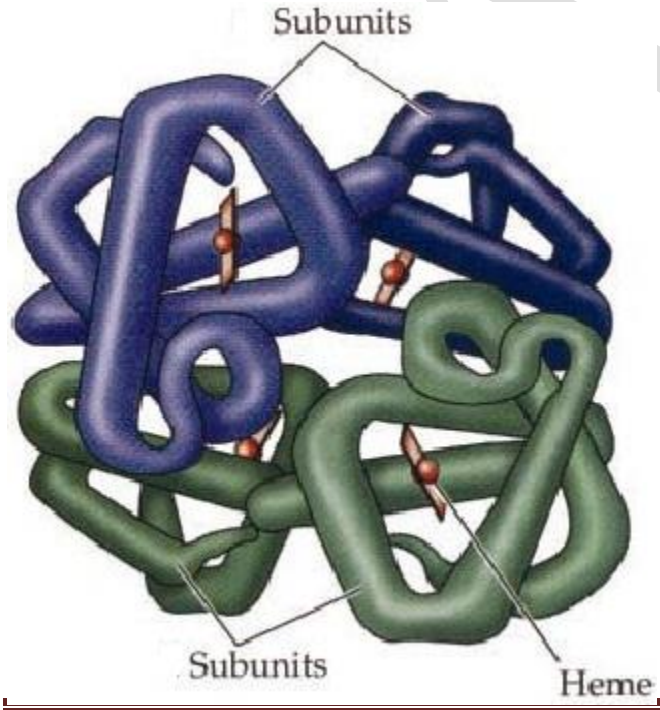


Overall Tertiary Structure showing:

Pink = alpha-helical area

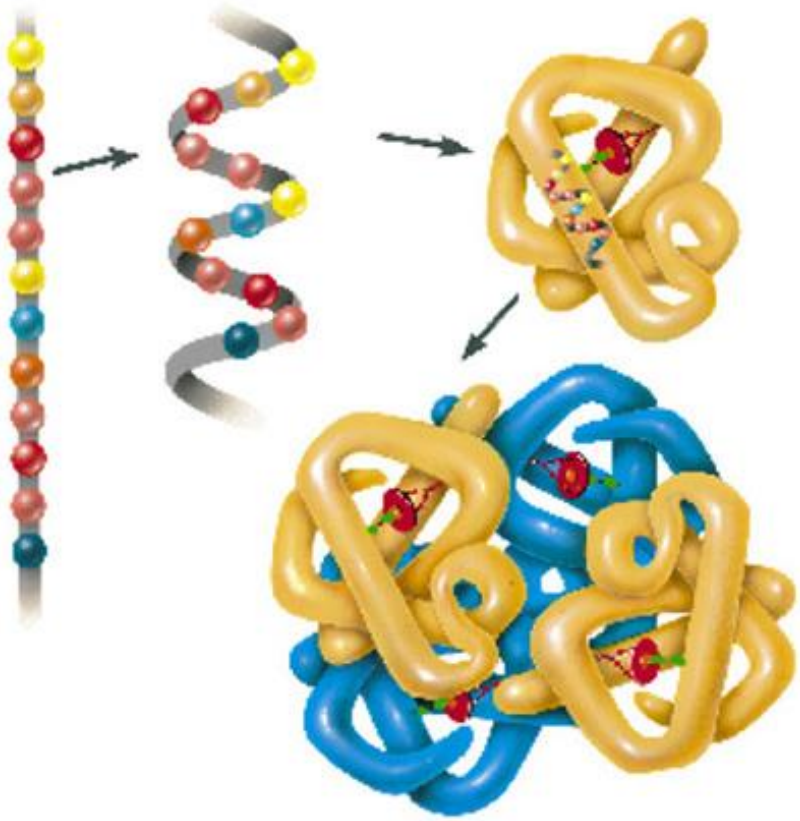
Yellow = beta-sheet area

The above picture shows an entire protein, this is called its **tertiary structure**. The **tertiary structure** gives the protein its function. If the tertiary structure is deformed the protein **will not** function. The primary structure is sequenced in a way as to form the tertiary structure. The side chains of the amino acids cause them to interact with the other parts of the chain. These interactions include hydrogen bonding, hydrophobic interactions, electrostatic interactions and van der Waals forces. An egg white is all protein, when it comes out of the shell it is clear, when you cook the egg you destroy its Tertiary Structure and the protein unfolds and becomes white, this destroys the proteins secondary, tertiary and quaternary structures. The primary structure will normally stay intact if the food is cooked..



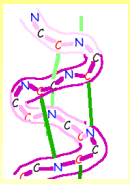
Some proteins have a quaternary structure. The quaternary structure occurs in proteins composed of more than one peptide chain. Meaning two or more proteins come

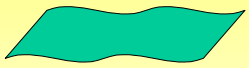
together to form one large protein. We have mentioned hemoglobin a couple of time. This large protein has a quaternary structure as it is composed of four myoglobin subunits. Each subunit is a separate polypeptide chain.



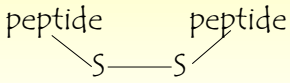
Examining Proteins

- Primary structure
  - amino acid sequence SNHEEVADLLAQIQ
- Secondary structure
  - alpha helices or beta sheets

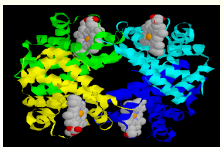




- Tertiary structure
  - disulfide bonds, H-bonds, salt bridges



- Quaternary structure
  - dimers, polymers, complexes



This picture is showing each of the four structure types and the order of their formation. First the primary structure is formed, as this structure forms secondary structures take shape. Once the protein sequence is completed the protein folds into its tertiary structure. If the protein has a quantanary structure, two or more proteins will come together to complete the quantanary structure.

Destroying a Protein:

When a protein is destroyed it is said to be denatured. Remember that the purpose of a primary structure is so the secondary and tertiary structures will form. Certain conditions will cause the protein to unfold, leaving only the primary structure.

- heat – breaks hydrogen bonds by causing the atoms to vibrate too radically
- UV light – breaks hydrogen bonds by exciting bonding electrons

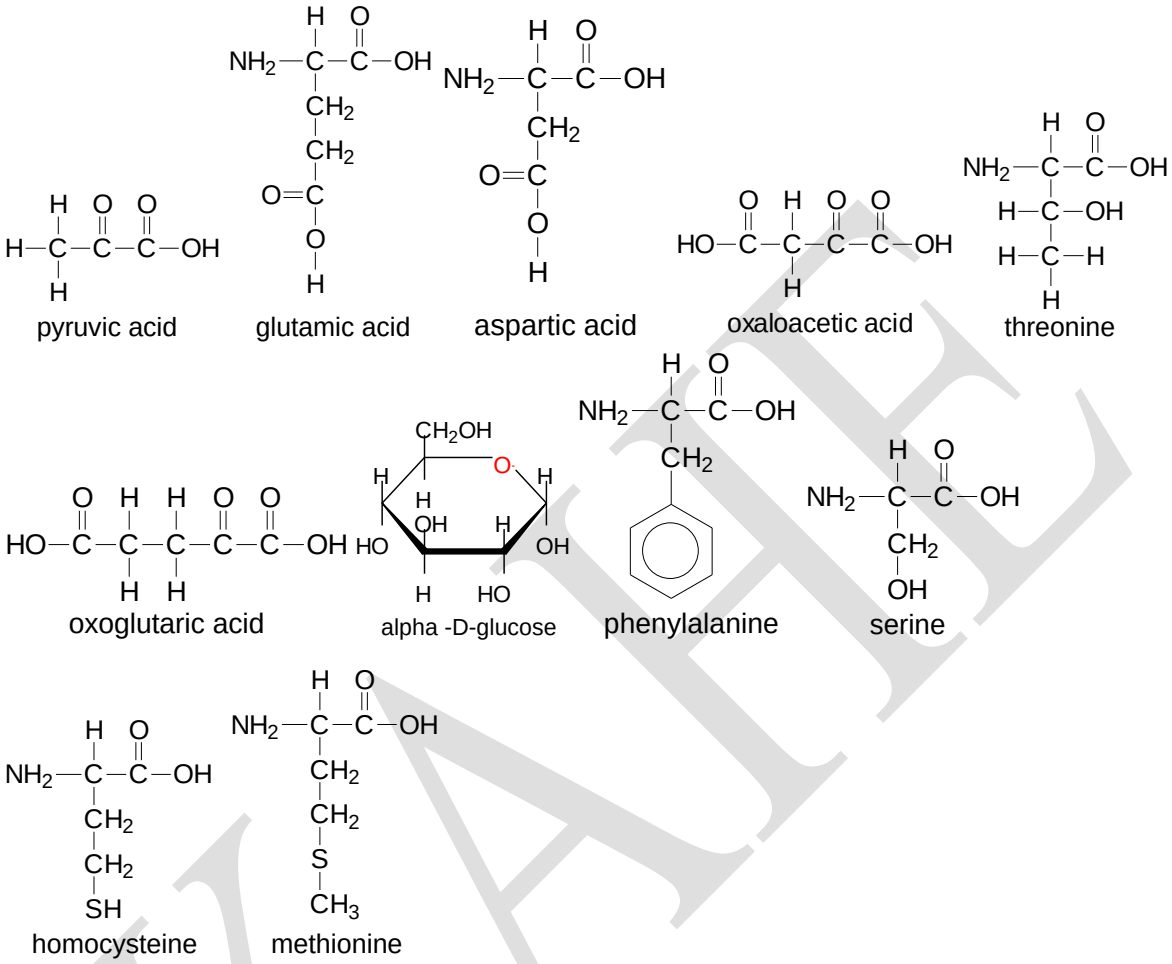
- organic solvents – breaks hydrogen bonds
- strong acids and bases – breaks hydrogen bonds and can hydrolyze the peptide bonds, breaking the primary structure
- detergents – disrupt hydrophobic interactions
- heavy metal ions – forms bonds to sulfur groups and can cause proteins to precipitate out of solution.

Essential and Non-Essential Amino Acids:

As far as your body is concerned, there are two different types of amino acids: essential and non-essential. Non-essential amino acids are amino acids that your body can create out of other chemicals found in your body. Essential amino acids cannot be created, and therefore the only way to get them is through food. Here are the different amino acids:

Non-Essential Amino Acids:

1. Alanine (synthesized from pyruvic acid)
2. Arginine (synthesized from glutamic acid)* essential for infants and young children
3. Asparagine (synthesized from aspartic acid)
4. Aspartic Acid (synthesized from oxaloacetic acid)
5. Cysteine (synthesized from homocysteine, which comes from methionine)
6. Glutamic Acid (synthesized from oxoglutaric acid)
7. Glutamine (synthesized from glutamic acid)
8. Glycine (synthesized from serine and threonine)
9. Proline (synthesized from glutamic acid)
10. Serine (synthesized from glucose)
11. Tryosine (synthesized from phenylalanine)



Essential Amino Acids:

1. Histidine
2. Isoleucine
3. Leucine
4. Lysine
5. Methionine
6. Phenylalanine
7. Threonine
8. Tryptophan
9. Valine

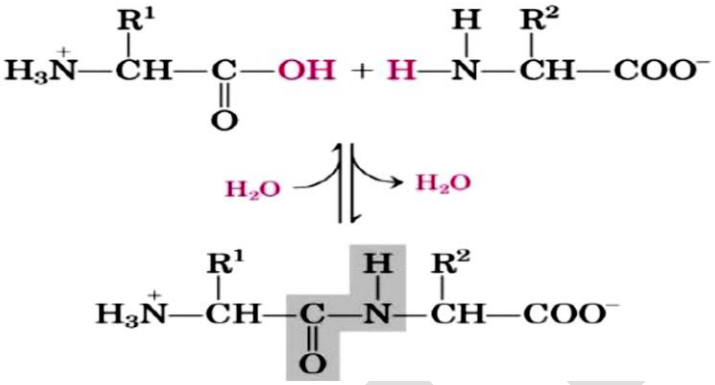
must be consumed

Peptides

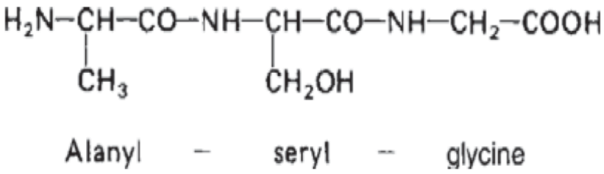
Peptides (from the Greek word means "digested") are short polymers of amino acid (monomers) linked by peptide bonds, the covalent chemical bonds formed between two molecules when the carboxyl group of one molecule reacts with the amino group of the other molecule. Peptides are distinguished from proteins on the basis of size, typically containing fewer than 50 monomer (AA) units. The shortest peptides are dipeptides, consisting of two amino acids joined by a single peptide bond. There are also tripeptides, tetrpeptides, etc. Amino acids which have been incorporated into a peptide are termed "residues"; every peptide has a N-terminus and C-terminus residue on the ends of the peptide.

A polypeptide is a long, continuous, and unbranched peptide. -Proteins consist of one or more polypeptides arranged in a biologically functional way and are often bound to cofactors, or other proteins. Long peptides such as amyloid beta can be considered proteins, whereas small proteins such as insulin can be considered peptides.

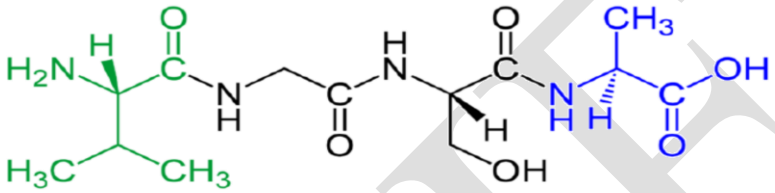
Peptide Bond Formation - Amino acids are linked together by condensation reaction between carboxylic and amino groups from two different amino acids (with elimination of water). The amide bond formed is called peptide bond. The product is called a peptide, and named according to the number of amino acids involved: e.g. dipeptide, tripeptide, decapeptide. Big peptides (> 50 amino acids) are called polypeptides. Peptide bonds are formed by a condensation reaction of carboxylic group of an amino acid and amino group of another amino acid with removal of water molecule.



Characteristics of Peptide Bonds - Peptide bonds are strong with partial double bond character: They are not broken by usual denaturing agents like heating or high salt concentration. They can be broken by: Prolonged exposure to strong acid or base at elevated temperatures. Specific enzymes such as digestive enzymes. Peptide bonds are rigid and planner resisting free rotation, therefore they stabilize protein structure. Peptide bonds are uncharged but polar: Peptide bonds contain polar hydrogen atoms of amino groups (with a partial positive charge) and polar oxygen atoms of carboxyl groups (with a partial negative charge). This allows hydrogen bonds to form between peptide bonds in different parts of the chain. Peptides are formed by binding amino acids together through an amide linkage. On the other hand, peptide hydrolysis results in free amino acids. Functional groups not involved in the peptide synthesis reaction should be blocked. The protecting or blocking groups must be removed after synthesis under conditions which retain the stability of the newly formed peptide bonds. Peptides are denoted by the number of amino acid residues as di-, tri-, tetrapeptides and the term "oligopeptides" is used for those with 10 or less amino acid residues. Higher molecular weight peptides are called polypeptides. The transition of "polypeptide" to "protein" is rather undefined, but the limit is commonly assumed to be at a molecular weight of about 10 kdal, i.e., about 100 amino acid residues are needed in the chain for it to be called a protein. The first three letters of the amino acids are used as symbols to simplify designation of peptides. Thus, the peptide shown can also be given as:

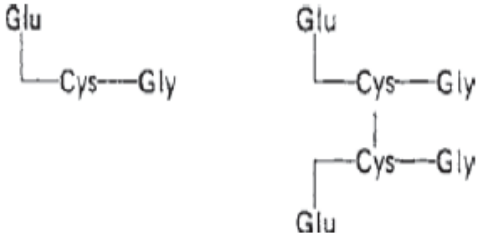


Ala Ser Gly or ASG



A tetrapeptide (example Val-Gly-Ser-Ala) with green marked amino end (L-Valine) and blue marked carboxyl end (L-Alanine).

One-letter symbols are used for amino acid sequences of long peptide chains. In compounds in which a functional group of the side chain is involved, the bond is indicated by a perpendicular line. The tripeptide glutathione (glutamyl-cysteinyl-glycine) is given as an illustration along with its corresponding disulfide, oxidized glutathione.



The amino acid residue with the free amino group is always placed on the left. The amino acids of the chain ends are denoted as N-terminal and C-terminal amino acid residues.-The peptide linkage direction in cyclic peptides is indicated by an arrow, i.e., CO → NH-

Peptide classes

Peptides are divided into several classes, depending on how they are produced:

1- **Milk peptides** - Milk peptides are formed from milk proteins by enzymatic breakdown by digestive enzymes or by the proteinases formed by *lactobacilli* during the fermentation of milk.

2-**Ribosomal peptides** - -Ribosomal peptides are synthesized by translation of mRNA. They are often subjected to proteolysis to generate the mature form. These function, typically in higher organisms, as hormones and signaling molecules. Some organisms produce peptides as antibiotics, such as microcins. Since they are translated, the amino acid residues involved are restricted to those utilized by the ribosome. However, these peptides frequently have post-translational modifications, such as hydroxylation, sulfonation, and disulfide formation.

3- **Nonribosomal peptides** - These peptides are assembled by enzymes that are specific to each peptide, rather than by the ribosome. The most common non-ribosomal peptide is glutathione, which is a component of the antioxidant defenses of most aerobic organisms. Other non-ribosomal peptides are most common in plants, and fungi and are synthesized by enzyme complexes called *nonribosomal peptide synthetases*. These peptides are often cyclic and can have highly-complex cyclic structures, although linear non-ribosomal peptides are also common.

4- **Peptones** - Peptones are derived from animal milk or meat digested by proteolytic digestion.

-In addition to containing small peptides, the resulting spray-dried material includes fats, metals, salts, vitamins and many other biological compounds. Peptone is used in nutrient media for growing bacteria and fungi.

5-**Peptide fragments** - -Peptide fragments refer to fragments of proteins that are used to identify or quantify the source protein. Often these are the products of enzymatic degradation performed in the laboratory on a controlled sample, but can also be samples that have been degraded by natural effects.

HORMONES

A hormone is a chemical that acts as a messenger transmitting a signal from one cell to another. When it binds to another cell which is the target of the message, the hormone can alter several aspects of cell function, including cell growth, metabolism, or other function. Hormones can be

classified according to chemical composition, solubility properties, location of receptors, and the nature of the signal used to mediate hormonal action within the cell. Hormones that bind to the surfaces of cells communicate with intracellular metabolic processes through intermediary molecules called second messengers (the hormone itself is the first messenger), which are generated as a consequence of the ligandreceptor interaction. The second messenger concept arose from an observation that epinephrine binds to the plasma membrane of certain cells and increases intracellular cAMP. This was followed by a series of experiments in which cAMP was found to mediate the effects of many hormones. To date, only one hormone, atrial natriuretic factor (ANF), uses cGMP as its second messenger.

CHARACTERIZING HORMONE - The first way of characterizing a hormone is by looking at the distance over which the hormone acts. Hormones can be classified on three primary ways as following:

Autocrine: An autocrine hormone is one that acts on the same cell that released it.

Paracrine: A paracrine hormone is one that acts on cells which are nearby relative to the cell which released it. An example of paracrine hormones includes growth factors, which are proteins that stimulate cellular proliferation and differentiation. Specifically, consider the binding of white blood cells to T cells. When the white blood cell binds to a T cell, it releases a protein growth factor called interleukin-1. This causes the T cell to proliferate and differentiate.

Endocrine: An endocrine hormone is one that is released into the bloodstream by endocrine glands. The receptor cells are distant from the source. An example of an endocrine hormone is insulin, which is released by the pancreas into the bloodstream where it regulates glucose uptake by liver and muscle cells. There are three major classifications one should be aware of:

Steroids: Steroid hormones are for the most part derivatives of cholesterol.

Amino acid derivatives: Several hormones (and neurotransmitters) are derived from amino acids.

Polypeptides: Many hormones are chains of amino acids.

FUNCTIONAL IMPORTANCE OF HORMONES

Insulin - Insulin is a polypeptide hormone synthesized in the pancreas by β-cells, which construct a single chain molecule called proinsulin. Enzymes excise a portion of the proinsulin molecule called the C peptide, producing the actual insulin molecule. When in demand, the β-cells will release insulin together with the c peptide into the blood stream via exocytosis. The role of insulin in the body is well known, with its primary role being to control the uptake of glucose by liver and muscle cells and also the storage of glucose in the form of glycogen.

Diabetes results from a lack of insulin secretion by the pancreas. Without insulin, cells take up glucose very slowly. The lack of insulin results in an inability to use blood glucose for fuel. Insulin is a signal for high blood glucose levels and increases glucose transport into cells. It stimulates synthesis of glycogen, fat, and protein. It inhibits breakdown of glycogen, fat, and protein. Insulin, secreted by the β-cells of the pancreas in response to rising blood glucose levels, is a signal that glucose is abundant. Insulin binds to a specific receptor on the cell surface and exerts its metabolic effect by a signaling pathway that involves a receptor tyrosine kinase phosphorylation cascade. Note that insulin stimulates storage processes and at the same time inhibits degradative pathways.

The pancreas secretes insulin or glucagon in response to changes in blood glucose

When glucose enters the bloodstream from the intestine after a carbohydraterich meal, the resulting increase in blood glucose causes increased secretion of insulin (and decreased secretion of glucagon). Insulin release by the pancreas is largely regulated by the level of glucose in the blood supplied to the pancreas. The peptide hormones insulin, glucagon, and somatostatin are produced by clusters of specialized pancreatic cells, the islets of Langerhans. Each cell type of the islets produces a single hormone: α-cells produce glucagon; β-cells, insulin; and δ-cells, somatostatin.

Insulin secretion - When blood glucose rises, GLUT2 transporters carry glucose into the b-cells, where it is immediately converted to glucose 6-phosphate by hexokinase IV (glucokinase) and enters glycolysis. The increased rate of glucose catabolism raises [ATP], causing the closing of ATP-gated K+ channels in the plasma membrane. Reduced efflux of K+ depolarizes the

membrane, thereby opening voltage-sensitive Ca2+ channels in the plasma membrane. The resulting influx of Ca2+ triggers the release of insulin by exocytosis. Stimuli from the parasympathetic and sympathetic nervous systems also stimulate and inhibit insulin release, respectively. Insulin lowers blood glucose by stimulating glucose uptake by the tissues; the reduced blood glucose is detected by the β-cell as a diminished flux through the hexokinase reaction; this slows or stops the release of insulin. This feedback regulation holds blood glucose concentration nearly constant despite large fluctuations in dietary intake.

Insulin counters high blood glucose - Insulin stimulates glucose uptake by muscle and adipose tissue, where the glucose is converted to glucose 6-phosphate. In the liver, insulin also activates glycogen synthase and inactivates glycogen phosphorylase, so that much of the glucose 6-phosphate is channelled into glycogen. Insulin also stimulates the storage of excess fuel as fat. In the liver, insulin activates both the oxidation of glucose 6-phosphate to pyruvate via glycolysis and the oxidation of pyruvate to acetyl-CoA. If not oxidized further for energy production, this acetyl- CoA is used for fatty acid synthesis in the liver, and the fatty acids are exported as the triglyerides to the adipose tissue. These fatty acids are ultimately derived from the excess glucose taken up from the blood by the liver. In summary, the effect of insulin is to favor the conversion of excess blood glucose to two storage forms: glycogen (in the liver and muscle) and triacylglycerols (in adipose tissue).

Diabetes Mellitus arises from defects in insulin production or action - Diabetes mellitus, caused by a deficiency in the secretion or action of insulin, is a relatively common disease. There are two major clinical classes of diabetes mellitus: type I diabetes, or insulin-dependent diabetes mellitus (IDDM), and type II diabetes, or non-insulin-dependent diabetes mellitus (NIDDM), also called insulin-resistant diabetes. In type I diabetes, the disease begins early in life and quickly becomes severe. This disease responds to insulin injection, because the metabolic defect stems from the pancreatic β-cells and a consequent inability to produce sufficient insulin. IDDM requires insulin therapy and careful, lifelong control of the balance between dietary intake and insulin dose. Characteristic symptoms of type I (and type II) diabetes are excessive thirst and

frequent urination (polyuria), leading to the intake of large volumes of water (polydipsia) ("diabetes mellitus" means "excessive excretion of sweet urine"). These symptoms are due to the excretion of large amounts of glucose in the urine, a condition known as glucosuria. Type II diabetes is slow to develop (typically in older, obese individuals), and the symptoms are milder and often go unrecognized at first. This is really a group of diseases in which the regulatory activity of insulin is defective: insulin is produced, but some feature of the insulin-response system is defective. These individuals are insulin-resistant. Individuals with either type of diabetes are unable to take up glucose efficiently from the blood; recall that insulin triggers the movement of GLUT4 glucose transporters to the plasma membrane of muscle and adipose tissue. Another characteristic metabolic change in diabetes is excessive but incomplete oxidation of fatty acids in the liver. Insulin secretion reflects both the size of fat reserves (adiposity) and the current energy balance (blood glucose level). Insulin acts on insulin receptors in the hypothalamus to inhibit eating. Insulin also signals muscle, liver, and adipose tissues to increase catabolic reactions, including fat oxidation, which results in weight loss.

Glucagon - Glucagon, a peptide hormone synthesized and secreted from the α-cells of the islets of Langerhans of pancreas, raises blood glucose levels. Its effect is opposite that of insulin, which lowers blood glucose levels. The pancreas releases glucagon when blood sugar (glucose) levels fall too low. Glucagon causes the liver to convert stored glycogen into glucose, which is released into the bloodstream. High blood glucose levels stimulate the release of insulin. Insulin allows glucose to be taken up and used by insulin-dependent tissues. Thus, glucagon and insulin are part of a feedback system that keeps blood glucose levels at a stable level.

Regulation and function - Secretion of glucagon is stimulated by hypoglycemia, epinephrine, arginine, alanine, acetylcholine, and cholecystokinin. Secretion of glucagon is inhibited by somatostatin, insulin, increased free fatty acids and keto acids into the blood, and increased urea production. Glucose is stored in the liver in the form of glycogen, which is a starch-like polymer chain made up of glucose molecules. Liver cells (hepatocytes) have glucagon receptors. When glucagon binds to the glucagon receptors, the liver cells convert the glycogen polymer into

individual glucose molecules, and release them into the bloodstream, in a process known as glycogenolysis. As these stores become depleted, glucagon then encourages the liver and kidney to synthesize additional glucose by gluconeogenesis. Glucagon turns off glycolysis in the liver, causing glycolytic intermediates to be shuttled to gluconeogenesis. Glucagon is a signal for low blood glucose levels. It stimulates breakdown of glycogen, fat, and protein. It inhibits synthesis of glycogen, fat, and protein. Several hours after the intake of dietary carbohydrate, blood glucose levels fall slightly because of the ongoing oxidation of glucose by the brain and other tissues. Although its primary target is the liver, glucagon (like epinephrine) also affects adipose tissue, activating TAG breakdown by activating triacylglycerol lipase and liberates free fatty acids, which are exported to the liver and other tissues as fuel, sparing glucose for the brain. The net effect of glucagon is therefore to stimulate glucose synthesis and release by the liver and to mobilize fatty acids from adipose tissue, to be used instead of glucose as fuel for tissues other than the brain.

During fasting and starvation, metabolism shifts to provide fuel for the brain - The fuel reserves of a healthy adult human are of three types: glycogen stored in the liver and, in relatively small quantities, in muscles; large quantities of triacylglycerols in adipose tissues; and tissue proteins, which can be degraded when necessary to provide fuel. In the first few hours after a meal, the blood glucose level is diminished slightly, and tissues receive glucose released from liver glycogen. There is little or no synthesis of lipids. By 24 hours after a meal, blood glucose has fallen further, insulin secretion has slowed, and glucagon secretion has increased.

Thyroid Hormones -Thyroid hormones (T4 and T3) are tyrosine-based hormones produced by the follicular cells of the thyroid gland and are regulated by TSH made by the thyrotropes of the anterior pituitary gland, are primarily responsible for regulation of metabolism. Iodine is necessary for the production of T3 (triiodothyronine) and T4 (thyroxine). A deficiency of iodine leads to decreased production of T3 and T4, enlarges the thyroid tissue and will cause the disease known as goitre. The thyronines act on nearly every cell in the body. They act to increase the basal metabolic rate, affect protein synthesis, help regulate long bone growth (synergy with

growth hormone) and neural maturation, and increase the body's sensitivity to catecholamines (such as adrenaline). The thyroid hormones are essential to proper development and differentiation of all cells of the human body. These hormones also regulate protein, fat, and carbohydrate metabolism, affecting how human cells use energetic compounds. They also stimulate vitamin metabolism. Numerous physiological and pathological stimuli influence thyroid hormone synthesis. Thyroid hormone leads to heat generation in humans. However, the thyronamines function via some unknown mechanism to inhibit neuronal activity; this plays an important role in the hibernation cycles of mammals and the moulting behaviour of birds.

Iodine is essential for thyroid hormone synthesis - Iodide is actively absorbed from the bloodstream by a process called iodide trapping. In this process, sodium is cotransported with iodide from the basolateral side of the membrane into the cell and then concentrated in the thyroid follicles to about thirty times its concentration in the blood. Via a reaction with the enzyme thyroperoxidase, iodine is bound to tyrosine residues in the thyroglobulin molecules, forming monoiodotyrosine (MIT) and diiodotyrosine (DIT). Linking two moieties of DIT produces thyroxine. Combining one particle of MIT and one particle of DIT produces triiodothyronine. If there is a deficiency of dietary iodine, the thyroid will not be able to make thyroid hormone. The lack of thyroid hormone will lead to decreased negative feedback on the pituitary, leading to increased production of thyroid-stimulating hormone, which causes the thyroid to enlarge (the resulting medical condition is called endemic colloid goiter).

Thyroid hormones are transported by Thyroid-Binding Globulin - Thyroxinebinding globulin (TBG), a glycoprotein binds T4 and T3 and has the capacity to bind 20 μg/dL of plasma. Under normal circumstances, TBG binds – noncovalently – nearly all of the T4 and T3 in plasma, and it binds T4 with greater affinity than T3. The small, unbound (free) fraction is responsible for the biologic activity. Thus, in spite of the great difference in total amount, the free fraction of T3 approximates that of T4, and given that T3 is intrinsically more active than T4, most biologic activity is attributed to T3. TBG does not bind any other hormones.

Circulation and transport - Most of the thyroid hormone circulating in the blood is bound to transport proteins. Only a very small fraction of the circulating hormone is free (unbound) and biologically active, hence measuring concentrations of free thyroid hormones is of great diagnostic value. When thyroid hormone is bound, it is not active, so the amount of free T3/T4 is what is important. For this reason, measuring total thyroxine in the blood can be misleading.

Related diseases - Both excess and deficiency of thyroxine can cause disorders. z Hyperthyroidism (an example is Graves Disease) is the clinical syndrome caused by an excess of circulating free thyroxine, free triiodothyronine, or both. It is a common disorder that affects approximately 2% of women and 0.2% of men. z Hypothyroidism (an example is Hashimoto's thyroiditis) is the case where there is a deficiency of thyroxine, triiodiothyronine, or both.

Parathyroid Hormone - Parathyroid hormone (PTH), parathormone or parathyrin, is secreted by the chief cells of the parathyroid glands. It acts to increase the concentration of calcium (Ca^{2+}) in the blood, whereas calcitonin (a hormone produced by the parafollicular cells of the thyroid gland) acts to decrease calcium concentration. PTH acts to increase the concentration of calcium in the blood by acting upon the parathyroid hormone 1 receptor (high levels in bone and kidney) and the parathyroid hormone 2 receptor (high levels in the central nervous system, pancreas, testis, and placenta). PTH was one of the first hormones to be shown to use the Gprotein, adenylyl cyclase second messenger system. Parathyroid hormone regulates serum calcium through its effects.

Regulation of PTH secretion - Secretion of parathyroid hormone is controlled chiefly by serum [Ca^{2+}] through negative feedback. Calcium-sensing receptors located on parathyroid cells are activated when [Ca^{2+}] is low. Hypomagnesemia inhibits PTH secretion and also causes resistance to PTH, leading to a form of hypoparathyroidism that is reversible. Hypermagnesemia also results in inhibition of PTH secretion. Stimulators of PTH includes decreased serum [Ca^{2+}], mild decreases in serum [Mg^{2+}], and an increase in serum phosphate. Inhibitors include increased serum [Ca^{2+}], severe decreases in serum [Mg^{2+}], which also produces symptoms of hypoparathyroidism (such as hypocalcemia), and calcitriol.

Clinical significance

- Hyperparathyroidism, the presence in the blood of excessive amounts of parathyroid hormone, occurs in two very distinct sets of circumstances. Primary hyperparathyroidism is due to autonomous, abnormal hypersecretion of PTH in the parathyroid gland while secondary hyperparathyroidism is an appropriately high PTH level seen as a physiological response to hypocalcemia
- A low level of PTH in the blood is known as hypoparathyroidism and is most commonly due to damage to or removal of parathyroid glands during thyroid surgery.
- There are a number of rare but well-described genetic conditions affecting parathyroid hormone metabolism, including pseudohypoparathyroidism, familial hypocalciuric hypercalcaemia, and autosomal dominant hypercalciuric hypocalcaemia.

Growth hormone - Growth hormone (GH or HGH), also known as somatotropin or somatropin, is a peptide hormone that stimulates growth, cell reproduction and regeneration in humans. It is a type of mitogen which is specific only to certain kinds of cells. Growth hormone is a single-chain polypeptide that is synthesized, stored, and secreted by somatotropic cells within the lateral wings of the anterior pituitary gland. The anterior pituitary gland secretes hormones that tend to elevate the blood glucose and therefore antagonize the action of insulin. Growth hormone secretion is stimulated by hypoglycemia; it decreases glucose uptake in muscle. Some of this effect may not be direct, since it stimulates mobilization of free fatty acids from adipose tissue, which themselves inhibit glucose utilization. Growth hormone increases amino acid transport in all cells, and estrogens do this in the uterus. A deficiency of this hormone produces dwarfism, and an excess leads to gigantism.

Regulation of growth hormone secretion - Secretion of growth hormone (GH) in the pituitary is regulated by the neurosecretory nuclei of the hypothalamus. These cells release the peptides Growth hormone-releasing hormone (GHRH or somatocrinin) and Growth hormone-inhibiting hormone (GHIH or somatostatin) into the hypophyseal portal venous blood surrounding the

pituitary. GH release in the pituitary is primarily determined by the balance of these two peptides, which in turn is affected by many physiological stimulators (e.g., exercise, nutrition, sleep) and inhibitors (e.g., free fatty acids) of GH secretion. A number of factors are known to affect GH secretion, such as age, sex, diet, exercise, stress, and other hormones.

Regulation - Stimulators of growth hormone (GH) secretion include peptide hormones, ghrelin, sex hormones, hypoglycemia, deep sleep, niacin, fasting, and vigorous exercise. Inhibitors of GH secretion include somatostatin, circulating concentrations of GH and IGF-1 (negative feedback on the pituitary and hypothalamus), hyperglycemia, glucocorticoids, and dihydrotestosterone.

Effects of growth hormone - Increased height during childhood is the most widely known effect of GH. In addition to increasing height in children and adolescents, growth hormone has many other effects on the body such as:

- Increases calcium retention, and strengthens and increases the mineralization of bone z Increases muscle mass through sarcomere hypertrophy
- Promotes lipolysis
- Increases protein synthesis
- Stimulates the growth of all internal organs excluding the brain
- Plays a role in homeostasis z Reduces liver uptake of glucose
- Promotes gluconeogenesis in the liver
- Contributes to the maintenance and function of pancreatic islets
- Stimulates the immune system

Clinical significance - Excess - The most common disease of GH excess is a pituitary tumor composed of somatotroph cells of the anterior pituitary. These somatotroph adenomas are benign and grow slowly, gradually producing more and more GH. For years, the principal clinical problems are those of GH excess. Eventually, the adenoma may become large enough to cause headaches, impair vision by pressure on the optic nerves, or cause deficiency of other pituitary hormones by displacement. Prolonged GH excess thickens the bones of the jaw, fingers and toes.

Resulting heaviness of the jaw and increased size of digits is referred to as acromegaly. Accompanying problems can include sweating, pressure on nerves (e.g., carpal tunnel syndrome), muscle weakness, excess sex hormone-binding globulin (SHBG), insulin resistance or even a rare form of type 2 diabetes, and reduced sexual function. GH-secreting tumors are typically recognized in the fifth decade of life. It is extremely rare for such a tumor to occur in childhood, but, when it does, the excessive GH can cause excessive growth, traditionally referred to as pituitary gigantism.

Deficiency - The effects of growth hormone deficiency vary depending on the age at which they occur. In children, growth failure and short stature are the major manifestations of GH deficiency, with common causes including genetic conditions and congenital malformations. It can also cause delayed sexual maturity. In adults, deficiency is rare, with the most common cause a pituitary adenoma, and others including a continuation of a childhood problem, other structural lesions or trauma, and very rarely idiopathic GHD. Adults with GHD "tend to have a relative increase in fat mass and a relative decrease in muscle mass and, in many instances, decreased energy and quality of life".

CLASSIFICATION BASED ON COMPOSITION AND SOLUBILITY

This is nowadays the most accepted system of classification and is based on the proposals made by the committees of *British Physiological Society* (1907) and the *American Physiological Society* (1908). The system divides the proteins into 3 major groups, based on their composition *viz*., simple, conjugated and derived.

A. Simple Proteins or Holoproteins.

These are of globular type except for scleroproteins which are fibrous in nature. This group includes proteins containing only amino acids, as structural components. On decomposition with acids, these liberate the constituent amino acids. These are further classified mainly on their solubility basis as follows:

1. Protamines and histones. These are basic proteins and occur almost entirely in animals, mainly in sperm cells; possess simplest structure and lowest molecular weight (approximately

5,000); soluble in water; unlike most other proteins, not coagulated by heat; strongly basic in character owing to high content of basic amino acids (lysine, arginine); form salts with mineral acids and nucleic proteins. Protamines are virtually devoid of sulfur and aromatic amino acids. Histones are somewhat weaker bases and are, therefore, insoluble in NH4OH solution, whereas the protamines are soluble. *e.g.,* protamines—*clupeine* from herring sperm, *salmine* from *salmon* sperm, *sturine* from sturgeon and *cyprinine* from carp, histones—*nucleohistones* of nuclei; *globin* of hemoglobin.

2. Albumins. These are widely distributed in nature but more abundant in seeds; soluble in water and dilute solutions of acids, bases and salts; precipitated with a saturated solution of an acid salt like (NH4)2.SO4 or a neutral salt like Na2.SO4; coagulated by heat. *e.g.*, *leucosine* in cereals, *legumeline* in legumes, *ovalbumin* from white of egg, *serum albumin* from blood plasma, *myosin* of muscles and *lactalbumin* of milk whey.

3. Globulins. These are of two types— pseudoglobulins and euglobulins*. Euglobulins are more widely distributed in nature than the pseudoglobulins; either soluble (pseudoglobulins) or insoluble (euglobulins) in water; precipitated with half saturated solution of (NH4)2.SO4; coagulated by heat. *e.g.,* pseudoglobulins— *pseudoglobulin* of milk whey. euglobulins— *serum globulin* from blood plasma, *ovoglobulin* from eggwhite; *myosinogen* from muscle; globulins of various plant seeds like hemp (*edestin*), soybeans (*glycinine*), peas (*legumine*), peach (*amandine*), oranges (*pomeline*); also potato (*tuberin*).

4. **Glutelins.** These have been isolated only from plant seeds; insoluble in water, dilute salt solutions and alcohol solutions but soluble in dilute acids and alkalies; coagulated by heat. *e.g.*, *glutenin* from wheat, *glutelin* from corn, *oryzenin* from rice, etc.

5. **Prolamines.** These have also been isolated only from plant seeds; insoluble in water and dilute salt solutions but soluble in dilute acids and alkalies and also in 60 – 80% alcohol solutions; not coagulated by heat *e.g.*, *gliadin* from wheat, *zein* from corn, *hordein* from oat, etc. Some biochemists like Karlson (1968) are of the viewpoint that glutelins and prolamines should

not be granted the status of exclusive classes since they are small groups of vegetable proteins occurring in grain kernels.

6. **Scleroproteins or Albuminoids.** These occur almost entirely in animals and are, therefore, commonly known as the '*animal skeleton proteins*'; insoluble in water, dilute solution of acids, bases and salts and also in 60–80% alcohol solutions; not attacked by enzymes. *e.g.*, collagen of bones, *elastin* in ligaments, *keratin* in hair and horry tissues and *fibroin* of silk.

B. Conjugated or Complex Proteins or Heteroproteins.

These are also of globular type except for the pigment in chicken feathers which is probably of fibrous nature. These are the proteins linked with a separable nonprotein portion called *prosthetic group*. The prosthetic group may be either a metal or a compound. On decomposition with acids, these liberate the constituent amino acids as well as the prosthetic group. Their further classification is based on the nature of the prosthetic group present. The various divisions are metalloproteins, chromoproteins, glycoproteins, phosphoproteins, lipoproteins and nucleoproteins. (Instead of metalloproteins, chromoproteins etc., the terms metalloproteids, chromoproteids etc., are sometimes used.)

1. **Metalloproteins.** These are the proteins linked with various metals. These may be of stable nature or may be more or less labile. Based on their reactivity with metal ions, the metalloproteins may be classified into 3 groups:

I. *Metals strongly bound by proteins.* Some heavy metals (Hg, Ag, Cu, Zn) become strongly binded with proteins like *collagen, albumin, casein* etc., through the —SH radicals of the side chains. Some other proteins have strong binding affinities for Fe (*siderophilin**) and Cu (*ceruloplasmin***). In these cases, the following pattern of binding may be present: **Siderophilin**, also called as **transferrin**, is an important metalloprotein and constitutes about 30% of the total plasma protein. It has a molecular weight of about 90,000 and is capable of binding 2 atoms of iron per mole. It facilitates iron transport. **Ceruloplasmin** is an important blue copper-binding protein in the blood of humans and other vertebrates. This protein contains about 90% of copper in serum. It has a molecular weight of about 150,000 and contains 8 atoms

of copper per mole. Ceruloplasmin is only one of the many sialoglycoproteins whose removal from the bloodstream is triggered by the loss of sialic acid units. It probably functions by reversibly releasing and binding copper at various sites in the body, whereby regulating copper absorption. In its deficiency, the Wilson's disease develops in man which is characterized by hepatolenticular degeneration.

II. *Metals bound weakly by proteins.* Ca belongs to this category. Here the binding takes place with the help of radicals possessing the electron charge.

III. *Metals which do not couple with proteins.* Na and K belong to this group. These form compounds with nucleic acids where apparently electrostatic bonds are present.

2. **Chromoproteins.** These are proteins coupled with a coloured pigment. Such pigments have also been found among the enzymes like catalase, peroxidase and flavoenzymes. Similarly, chlorophyll is present in leaf cells in the form of a protein, the chloroplastin. The chloroplastin dissolves in water as a colloid and is readily denatured. *e.g., myoglobin, hemoglobin, hemocyanin, hemoerythrin, cytochromes, flavoproteins, catalase, etc.*

3. **Glycoproteins and Mucoproteins.** These are the proteins containing carbohydrate as prosthetic group. Glycoproteins contain small amounts of carbohydrates (less than 4%), whereas mucoproteins contain comparatively higher amounts (more than 4%). *e.g.,* glycoproteins— *egg albumin,* elastase certain *serum globulins* and also certain *serum albumins*. mucoproteins— *ovomucoid* from eggwhite, *mucin* from saliva and *Dioscorea* tubers, *osseomucoid* from bone and *tendomucoid* from tendon.

4. **Phosphoproteins.** These are proteins linked with phosphoric acid; mainly acidic. *e.g., casein* from milk and *ovovitellin* from egg yolk.

5. **Lipoproteins.** Proteins forming complexes with lipids (cephalin, lecithin, cholesterol) are called lipoproteins; soluble in water but insoluble in organic solvents. *e.g., lipovitellin* and *lipovitellenin* from egg yolk; lipoproteins of blood. The lipoproteins are in reality the temporary intermediates in the process of transfer of lipids from the site of absorption to the site of utilization. The classification of lipoproteins is frequently based on an operational definition, *i.e.*,

the migration of the fraction in a density gradient separation. On this basis, the lipoproteins have been classified into following 4 categories: (*a*) Very high density lipoproteins (VHDLs). These have densities greater than 1.21. (*b*) High density lipoproteins (HDLs). These possess density range of 1.063 to 1.21. (*c*) Low density lipoproteins (LDLs). Their densities range between 1.05 and 1.063. (*d*) Very low density lipoproteins (VLDLs). Their density range is from 0.93 to 1.05.

6. **Nucleoproteins.** These are compounds containing nucleic acid and protein, esp., protaminesand histones. These are usually the salt-like compounds of proteins since the two components have opposite charges and are bound to each other by electrostatic forces. They are present in nuclear substances as well as in the cytoplasm. These may be considered as the sites for the synthesis of proteins and enzymes. *e.g., nucleoproteins* from yeast and thymus and also viruses which may be regarded as large molecules of nucleoproteins; *nucleohistones* from nuclei-rich material like glandular tissues; *nuclein.*

C. Derived Proteins.

These are derivatives of proteins resulting from the action of heat, enzymes or chemical reagents. This group also includes the artificially-produced polypeptides.

I. *Primary derived proteins.* These are derivatives of proteins in which the size of protein molecule is not altered materially.

1. **Proteans.** Insoluble in water; appear as first product produced by the action of acids, enzymes or water on proteins. *e.g., edestan* derived from edestin and *myosan* derived from myosin.

2. **Metaproteins or Infraproteins.** Insoluble in water but soluble in dilute acids or alkalies; produced by further action of acid or alkali on proteins at about 30–60°C. *e.g., acid* and *alkali metaproteins.*

3. **Coagulated Proteins.** Insoluble in water; produced by the action of heat or alcohol on proteins. e.g., *coagulated eggwhite.*

II. *Secondary derived proteins.* These are derivatives of proteins in which the hydrolysis has certainly occurred. The molecules are, as a rule, smaller than the original proteins.

1. **Proteoses.** Soluble in water; coagulable by heat; produced when hydrolysis proceeds beyond

the level of metaproteins; *primary proteoses* are salted out by half saturation with (NH4)2.SO4 and precipitated by HNO3 and picric acid; *secondary proteoses* are salted out only by complete saturation with (NH4)2.SO4 but are not precipitated by HNO3 or picric acid. *e.g.*, *albumose* from albumin; *globulose* from globulin.

2. **Peptones.** Soluble in water; noncoagulable by heat; produced by the action of dilute acids or enzymes when hydrolysis proceeds beyond proteoses; neither salted out by (NH4)2SO4 nor precipitated by HNO3 or picric acid.

3. **Polypeptides.** These are combinations of two or more amino acid units. In fact, *the proteins are essentially long chain polypeptides*. *Drawbacks.* Although widely accepted, the system outlined above has certain discrepancies: 1. The classification is arbitrary. 2. The criterion of solubility is not well demarcated as some globulins (pseudoglobulins) are also soluble in water. 3. Protamines and histones should have been kept under derived proteins. 4. The group metaproteins is an artificial assemblage.

Separation of Proteins

How individual proteins can be isolated in homogeneous form from biological samples - especially from tissues of multicellular organisms. Naturally, the more information we have on the protein to be investigated, the more straightforward it is to establish a well-suited and efficient isolation protocol. The first question to consider is the distribution of the given protein among the various tissues of the organism. Obviously, the tissue in which the given protein is the most abundant should be used as the starting point of the isolation protocol. The next question is whether the protein is intracellular, extracellular (secreted) or membrane-bound. If it is intracellular, the subcellular distribution of the protein should be considered. If the protein is associated to one of the many organelles of the eukaryotic cell, that organelle should be first isolated. The process through which individual organelles (plasma membrane, nucleus, mitochondria, etc.) are separated from one another is called cell fractionation. The process of cell

fractionation starts with the disruption of the tissue and its cell constituents by a homogenisation procedure performed as gently as possible. Once the cells are opened up, individual organelle types can be separated from each other by various types of centrifugation techniques.

Cell disruption

The technical details of the cell disruption procedure largely depend on the type of tissue or cells to be homogenised. In the case of multicellular organisms, the first aim is to disintegrate the tissue into individual cells by abolishing the connections that organise the cells into the given tissue. Then the plasma membrane and, in case of plants, fungi and bacteria, the cell wall need to be ruptured. The harshness of the treatment can greatly vary depending on the tissue and cell type. For example, in the case of blood cells that do not need to be disintegrated from a solid tissue, even a mild osmotic shock using a hypotonic solution can lead to the rupture of the cell membrane. In the case of cells having a cell wall or tissues stabilised by a strong extracellular matrix, simple osmosis-based treatments are inefficient. In such cases various mechanical methods applying shearing force on the cells can be used. The two most frequently applied tools are high-speed laboratory blenders and ultrasonic cell disruptors. While blenders can disrupt even highly structured strong tissues, ultrasonic cell disruptors applying ultrasound (~ 20–50 kHz) to the sample (sonication) are used mostly in the case of cell suspensions. The ultrasonic cell disruptor generates the high-frequency waves electronically. These shock waves are transmitted to the cell suspension via an oscillating metal probe. The oscillation causes large localised pressure inhomogeneity resulting in cavitation eventually disrupting the cells.

There are several other procedures that also use shearing force to open up cells. Some of these apply high pressure to pump the cell suspension through a very narrow channel or orifice into a low pressure container. Due to the sudden drop of pressure the cells "explode" in the container. Shearing force can be also generated by a pair consisting of a carefully designed glass tube and a tightly fitting glass pestle, called the Potter-Elvehjem homogeniser, or a potter in short. The diameter of the tube is just a little larger than that of the pestle. The sample is pushed into the very narrow space between the sides of the tube and the pestle. The shearing force is

generated as the cell suspension squeezes up and past the pestle. This method is applied on the suspension of individual cells (already dissociated tissues, blood cells etc.). Plant cells protected by cell wall are most often disrupted by various grinding methods. Manual grinding is the most common method. The tissue is usually frozen in liquid nitrogen and then crushed using a mortar and pestle.

Optimal cell disruption methods open up a high percentage of the cells in the sample while preserving the organelles or molecules to be investigated in their native state. This is not a trivial task. In order to preserve the native state of most organelles and molecules, the procedure should be quick and the heat generated by the disruption method should be dissipated by intensive cooling. This helps to avoid heat denaturation of proteins and also lowers the rate of unwanted chemical reactions such as oxidation or proteolytic cleavage of proteins. To further suppress these chemical reactions, oxidation can be prevented by the addition of reducing agents and proteolysis can be controlled by the addition of a mixture of protease inhibitors, often referred to as a protease inhibitor cocktail. In order to extract the content of the cell into a native-like solution, the buffers used for cell disruption often mimic the cytosol in terms of pH and ionic strength. The sample might contain trace amounts of heavy metal ions. Such ions can form complexes with various amino acid residues of proteins. To prevent such complex formation, a chelating agent, most often ethylene-diamine-tetraacetic acid (EDTA), is added to the homogenisation buffer to sequester the heavy metal ion components.

Cell fractionation

The major goal of cell fractionation is to separate the various types of cell organelles from each other and from the cytosol. As mentioned, cell disruption should be intensive enough to open up a large fraction of the cells but still gentle enough to preserve the native state of the organelles and the soluble components of the cytosol. Upon cell disruption, the plasma membrane and the endoplasmic reticulum become disintegrated into small membrane vesicles. These vesicles—along with the native organelles of the cell and soluble molecules of the cytosol—compose the mixture from which the components need to be separated into different fractions. The most

important fractionation technique applied to this mixture is centrifugation using specialised laboratory equipment. The following section reviews the principles and major types of laboratory centrifugation.

Centrifugation

When an object attached to a rope is whirled around, one can feel that the rope must be pulled inward towards the centre of the rotation in order to keep the object on the orbit. This force prevents the object from getting away and move with a constant speed along a straight tangential line. The inward force with which one has to pull the rope is called the centripetal force. One can also define the outward force, the centrifugal force, by which the object pulls the rope. This force is equal in magnitude to the centripetal force but has the opposite direction. The centrifugal force (F_c) is a virtual, so-called fictional force emerging due to the inertia of the object. Yet, because it leads to a simpler mathematical formalism, equations describing the processes when solutions are centrifuged use the F_c force.

According to the well-known Newton equation:

$$F = m \times a$$

Upon centrifugation, acceleration equals the product of the radius of the orbit and the square of the angular velocity:

$$a = \omega^2 \times r$$

The fictive centrifugal accelerating force in vacuum is therefore:

$$F_c = m \times \omega^2 \times r$$

The product of the radius and the square of the angular velocity equals the centrifugal accelerating potential. Traditionally, and perhaps somewhat misleadingly, the magnitude of this

potential is compared to the Earth's gravitational accelerating potential (*g*), and has been expressed in "*g*" units. The reason is quite simple. Earth's gravitational potential, similarly to the accelerating potential provided by centrifugation, can also sediment particles dispersed in solution. This type of quantitation shows how many times centrifugation is more effective to sediment particles compared to the gravitational effect of Earth. In the fastest laboratory ultracentrifuges the applied accelerating potential can exceed 1 000 000 *g*.

When solutions are centrifuged, the particles are not in vacuum but in a solvent having a given density (mass/volume). Importantly, the centrifugal force acts not only on the particles, but on the solvent too. If the density of the particle equals the density of the solvent, the particle will not move relative to the solvent, and its velocity along the radius will be zero. If the density of the particle exceeds that of the solvent, the particle sediments (sinks), i.e. it moves outwards along the radius, while the displaced solvent molecules move inwards. In the opposite case when the density of the particle is lower than that of the solvent, the particle floats—it moves inwards while the displaced solvent molecules move outwards.

In order to provide a simple mathematical description of this phenomenon, the buoyancy factor has been defined as follows:

$$(1-\frac{\rho}{\rho_r})$$

The numerator of the fraction contains the density of the medium (solvent) while the denominator contains the density of the particle.

Introduction of the buoyancy factor leads to the following equation:

$$F_c = m \times (1-\frac{\rho}{\rho_r}) \times \omega^2 \times r$$

This equation clearly shows that, upon centrifugation, the force acting on a given particle is a function of the mass of the particle, the relative density of the particle (compared to that of the medium), the angular velocity of the rotation and the distance of the particle from the centre of the rotation (i.e. from the spindle of the centrifuge).

The first two of these parameters, namely the mass and the density, are characteristic of the particle and differences in these parameters can allow for the physical separation of different types of particles. As we will see, there are two major types of centrifugation-based separation techniques. In one technique called differential centrifugation, the separation is based on both particle mass and density. In the case of the other, called equilibrium density-gradient centrifugation, the separation is based strictly on the density of the particles.

As soon as the particles are accelerated by the centrifugal force and start moving towards the spindle, a dragging force (F_d) called friction is exerted on them by the medium. This force, which has a direction opposite to the direction of the particle movement, is proportional to the velocity of the particle. At the typically very low speed of the sedimentation process, the F_d force is a linear function of the velocity. The ratio of the force and the velocity is defined as the frictional coefficient (*f*). The value of *f* is a function of the viscosity of the medium and of the size and shape of the particle as described below by Stokes' law:

$$f = 6 \times r \times \pi \times \mu$$

In this equation "r" denotes the Stokes radius. If the particle is spherical, this equals the radius of the particle. If the particle is not spherical, "r" (a virtual value) denotes the radius of a spherical particle that has identical diffusion properties as the non-spherical particle in question and μ denotes viscosity of the medium. Note that the value of the frictional coefficient is proportional to the radius of the particle. The larger the particle, the higher dragging force is exerted on it by the medium.

In the course of centrifugation, the velocity of each particle is increasing due to the accelerating force F_c. However, as the velocity increases, the dragging force also increases. Therefore, the velocity of each particle can increase to a given value where the value of the dragging force F_d reaches the value of the accelerating F_c value. The magnitude of the two opposing forces becomes equal in a very short time:

$$F_c = F_d = f \times v$$

Once the magnitude of the two opposing forces becomes equal, the resultant force becomes zero. Therefore, the particle will move with a constant velocity characteristic to that particle at the given accelerating potential and medium. (A similar phenomenon is described in Chapter 7 on electrophoresis. There, the accelerating force is proportional to the charge instead of the mass of the particle, but the friction force and the phenomenon of two opposing forces leading to a characteristic particle velocity is analogous.) Substituting F_c into the previous equation leads to the following equation:

$$f \times v = m \times (1 - \frac{\rho}{\rho_r}) \times \omega^2 \times r$$

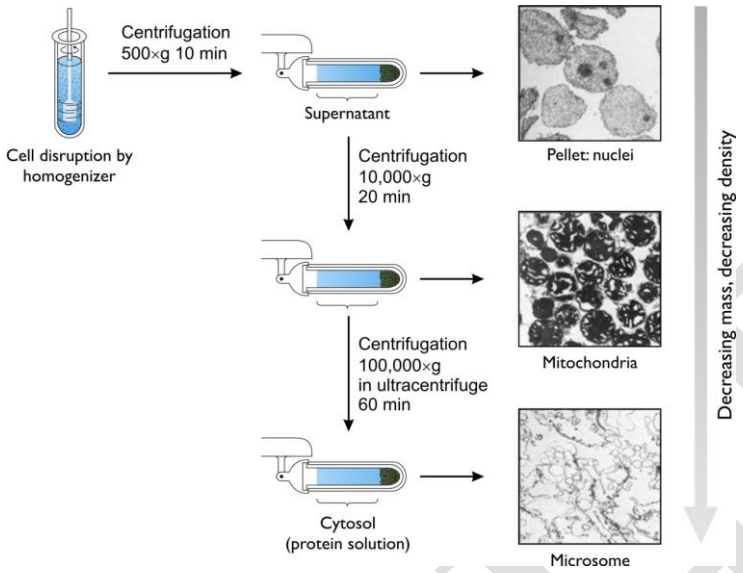
If the above equation is rearranged by dividing particle velocity with the centrifugal acceleration potential, the resulting equation will lead to a useful parameter. This is the sedimentation coefficient (its unit of measure is one over seconds), which is usually expressed in Svedberg units. This coefficient describes the sedimentation propensity of the particle. It provides the characteristic sedimentation velocity of a particle triggered by a unit level of accelerating potential.

$$S = \frac{v}{\omega^2 \times r} = \frac{m \times (1 - \frac{\rho}{\rho_r})}{f} = \frac{M \times (1 - \frac{\rho}{\rho_r})}{Nf}$$

The numerator of the equation contains all parameters that favour sedimentation. The higher the mass and relative density (compared to the medium) of the particle, the higher its sedimentation velocity will be when unit accelerating potential is applied. The mass of the particle of a given density, of course, is linearly proportional to its volume. In other words, the mass is a linear function of the cube of the particle radius. The denominator contains the parameter that negatively influences sedimentation speed. The larger the frictional coefficient, the lower velocity will be triggered by unit level acceleration potential. As we have seen, the frictional coefficient is a linear function of the particle radius. As the accelerating force is a linear function of the third power of the radius, while the dragging force is a linear function of the first power of the radius, the velocity of the particle will ultimately be proportional to the second power of the particle radius. *If two particles have identical density, the larger particle will sediment faster and the ratio of the velocities will follow a square law with respect to the ratio of the particle radii.* This relationship provides the basis for the so-called differential centrifugation methods.

Differential centrifugation: cell fractionation based primarily on particle size

The density of the various organelles differs on a smaller scale than their size. Therefore, while both size and density affect sedimentation velocity, their size difference dominates when organelles are separated by centrifugation. In the procedure of differential centrifugation, cell constituents are separated from each other by their Svedberg value. Several consecutive centrifugation steps are applied in the order of increasing accelerating potential. Each individual centrifugation step relies on the different sedimentation speed of the different cell constituents at the given acceleration potential. At a properly chosen acceleration potential, almost 100 % of the largest component will sediment in the time span of the centrifugation. The sedimented organelles form a pellet at the bottom of the centrifuge tube. The potential should be set so that in the same period of time only a small portion of all smaller constituents latch on to the pellet.



Differential centrifugation. In the course of differential centrifugation, consecutive centrifugation steps are applied. The consecutive centrifugation steps follow each other in the order of increasing centrifugal acceleration potential. During the first centrifugation, only the largest and/or heaviest cell constituents sediment in the time frame of the centrifugation. Typically, only nuclei and undisrupted whole cells form the pellet. The supernatant of the first centrifugation step is further centrifuged in the consecutive step at higher acceleration potential and typically for a longer period of time. Following this scheme, ever smaller and/or lower-density cell constituents can be sedimented.

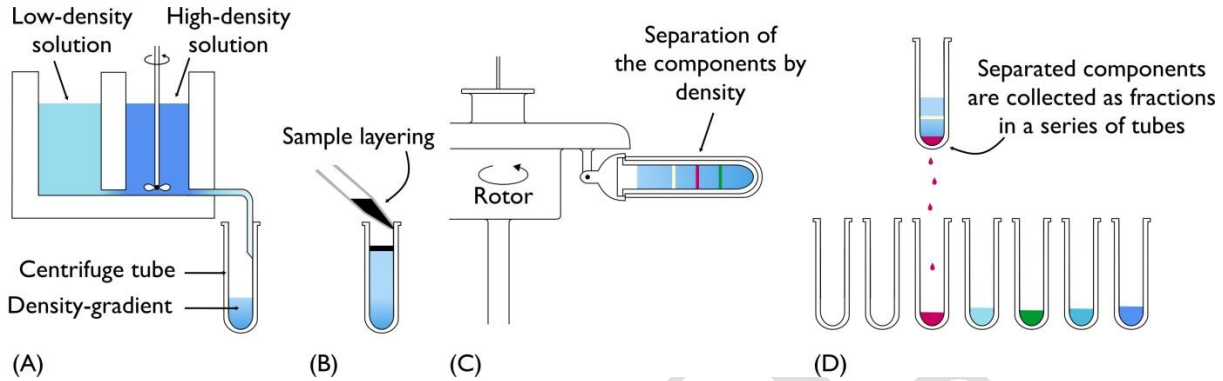
The disrupted cell homogenate is centrifuged first at a relatively low accelerating potential of 500 g for 10 minutes. Under these conditions, only particles having the highest Svedberg value, intact cells and nuclei will form the pellet. All other cell constituents will sediment at a much lower rate and remain in the homogenate. The supernatant of the first centrifugation is transferred into an empty centrifugation tube and is subjected to another centrifugation step, now at a significantly higher accelerating potential of 10,000 g and for 20 minutes. These conditions favour sedimentation of mitochondria, lysosomes and peroxisomes

having lower Svedberg values than nuclei. Many cell constituents still remain in the supernatant, which is again transferred into an empty tube. This tube is placed into an ultracentrifuge and, with an accelerating potential of 100 000 g in one hour, the so-called microsomal fraction sediments. This fraction contains mostly artificial vesicles with a diameter of 50-150 nm that originate mostly from the endoplasmic reticulum and are generated by the cell disruption procedure. Other natural cell constituents of the same size range will also contribute to this fraction. After this third centrifugation step, the supernatant contains mostly macromolecules and supramolecular complexes such as ribosomes. By applying an accelerating potential as high as several hundred thousand *g*, ribosomes and large proteins can also be sedimented.

Equilibrium density-gradient centrifugation: fractionation based on density

In the previous section we introduced the method of differential centrifugation. For simplicity, we stated that the constituents of the sample were separated in a medium of homogeneous density. This first approximation has didactical advantages as it makes the basic principle of differential centrifugation easier to comprehend. Nevertheless, it is sometimes advantageous to use a very shallow density gradient in the medium during differential centrifugation. This is done only to suppress convectional flows in the medium that could unsettle and mix layers of already separated cell constituents.

The essence of equilibrium density-gradient centrifugation is principally different. In this case, a rather steep density gradient is created in the medium—in such a manner that the density of the medium gradually increases towards the bottom of the centrifuge tube. This is achieved by using a very high-density additive, for example caesium chloride (CsCl). The density gradient is created as follows. When the centrifuge tube is filled with the medium, a high concentration CsCl solution is added first. Subsequently, in the process of filling the tube, the concentration of CsCl is gradually decreased resulting in a CsCl gradient and, as a consequence, a density gradient in the tube. The sample is layered on the top of this special medium.

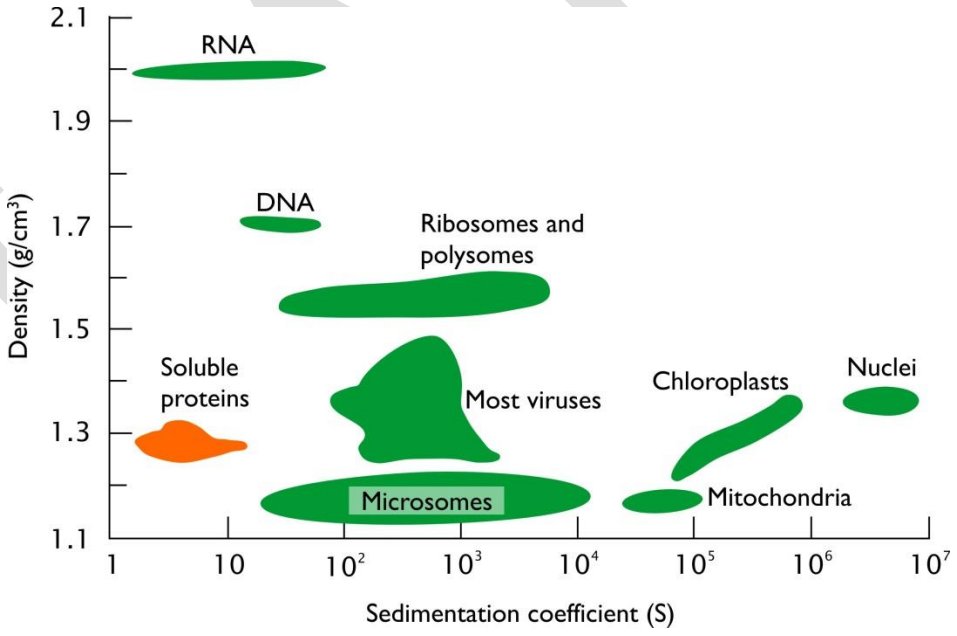


Equilibrium density-gradient centrifugation. In the course of equilibrium density-gradient centrifugation, a concentration gradient of a high density compound such as caesium chloride is generated. (The compound should not react with the biological sample.) The concentration gradient of this special additive creates a density gradient in the centrifuge tube. The density gradually increases toward the bottom of the centrifuge tube. The sample is layered on the low-density top of this gradient. As the centrifugation begins, each compound of the sample starts to sediment. By doing so, the compounds travel through layers of increasing density. As soon as a compound reaches the layer where the density equals its own density, the compound stops sedimenting. At this layer, no resultant force is exerted on the particle and thus it will float. As a result, equilibrium density-gradient centrifugation separates compounds from each other independently of their size, solely by their density, in a single run.

In the course of centrifugation, particles start to sediment moving towards the bottom of the centrifuge tube. By doing so, they travel through an increasing density medium. Each particle sediments to a section of the medium where its own density equals the density of the medium. At this section, the buoyancy factor becomes zero and, as a consequence, the accelerating force acting on the particle also becomes zero. The particle stops sedimenting. If it moved further towards the bottom of the tube, it would meet a higher density medium and a force opposing to its moving direction would be exerted on it, turning the particle back. If, by travelling

backwards, it would meet a density lower than its own density, it would sediment again. As a consequence, this method separates particles exclusively based on their density. It is an equilibrium method in which, by the end of the separation, the system reaches a constant state. (In this aspect, this method shows an interesting analogy to the isoelectric focusing (IEF) method reviewed earlier. The two methods separate particles by entirely different characteristics (density versus isoelectric point), but in both cases, the separation leads to an equilibrium state. Both methods apply a gradient, but in the case of IEF a pH gradient is created.)

Note that the two centrifugation approaches introduced above separate particles by partially different characteristics. Consecutive combination of the two methods can lead to a more efficient separation than achieved by any of the methods alone. Therefore, to increase separation efficiency, fractions generated by differential centrifugation can be subjected to a subsequent density-gradient centrifugation step to further separate individual components.



Combination of differential centrifugation and density-gradient centrifugation. Differential centrifugation separates compounds primarily based on their size, while density-gradient centrifugation separates compounds exclusively based on their density. Compounds that have different density but sediment in the same fraction during differential centrifugation can be separated by a subsequent step of density-gradient centrifugation. Two such consecutive steps of the two centrifugation methods can provide significantly higher separation efficiency than either procedure alone.

Low-resolution, large-scale protein fractionation

Once individual cell constituents have been separated to different fractions, proteins of these fractions can be further fractionated. The ultimate goal is to isolate the protein to be investigated in a homogeneous and functional form. In order to obtain a homogeneous protein solution, typically several purification procedures have to be combined consecutively. For each novel protein, the optimal isolation protocol needs to be established in an iterative trial and error process.

These multi-step protocols usually start with low-cost, large-scale but low-resolution fractionation steps. These steps are based mostly on characteristically different solubility of individual proteins depending on pH, ionic strength, temperature etc. The applied techniques usually include centrifugation or filtering to remove precipitated insoluble proteins. The components of the usually still crude fractions are then further separated by various smaller-scale but higher-resolution chromatography techniques.

Obviously, individual proteins are separated from each other based on characteristic differences in their physicochemical properties. Mostly, the following characteristics are important from this aspect. What is the temperature and pH range where the protein to be isolated preserves its native state? What is the isoelectric point (pI) of the protein? What is the molecular mass of the protein? Is the protein composed of a single polypeptide chain, or it is a

multi-subunit protein? If it is composed of more than one polypeptide chain, what is the molecular mass of the individual chains? What is the subunit composition and the native molecular mass of the protein?

In order to establish an optimised protocol, one should be able to determine the quantity and, in an optimal case, the functionality of the protein to be investigated in the presence of an excess of irrelevant 'contaminating' proteins. If the protein to be isolated is an enzyme, a specific chemical reaction catalysed by the enzyme can be used for functional testing. If certain conditions apply (see later), this enzymatic reaction can accurately measure the amount of the functional enzyme molecules. With such a quantitation method, one can measure the initial quantity of the enzyme in the first sample and the actual quantity of the enzyme after each fractionation step. By dividing the quantity after fractionation with that before fractionation, we can obtain the yield of the given purification step. In general, one aims to choose purification methods that provide a high yield. It is equally important to apply fractionation techniques that, while retain a large proportion of the protein to be isolated, remove a large proportion of the 'contaminating' proteins. Thus, it is also important to measure the total amount of proteins in the sample after each purification step and to calculate the target protein versus total protein ratio. Dividing the ratio after fractionation by that before fractionation provides a useful descriptor of the purification called enrichment. An efficient purification method has a high yield (retains a large percentage of the target protein) and, at the same time, provides high enrichment (removes a large proportion of the contaminating proteins).

If additional purification steps do not further increase the enrichment value, this can be an indication that the sample has become homogeneous and contains only the target protein. If the target protein is not an enzyme, or it is an enzyme for which no selective activity assay exists, other specific detection methods can also be used. All of these rely on a selective binding interaction between the target protein and another—usually, labelled—molecule. If the target

protein is an enzyme, the interacting molecule can be its specific inhibitor. If the target protein is a receptor, the interacting molecule can be a specific ligand. In a more general case, the interacting molecule can be a monoclonal antibody raised specifically against the target protein. The interacting molecule can be labelled with a coloured or fluorescent dye to permit its detection by spectrophotometry or spectrofluorimetry. It can also be covalently linked to an enzyme for which sensitive spectrometric detection assays are available.

If no specific interacting molecule is available but the molecular mass of the target protein is known, a less reliable but almost universal method, SDS-PAGE (see Chapter 7) can be applied to monitor the purification process. As already mentioned, the low-resolution, high-scale initial purification steps rely mostly on solubility differences and, in some cases, on size differences.

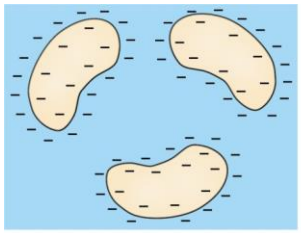
Fractionation methods based on solubility

The solubility of a protein is a measure of the maximal quantity of the given protein that a unit volume of solvent can keep in solution. Solubility is often expressed in units of mg/mL. Solubility depends largely on the composition of the solvent. Before going into details about the influence of solvent composition, it is important to clarify whether solubility refers to native or denatured proteins. A popular misconception based on oversimplification is that native proteins are always soluble while denatured proteins always precipitate from the solution. The following section focuses on the solubility of native proteins but later we will discuss the case of denatured proteins as well.

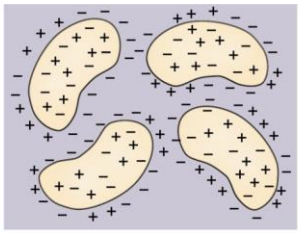
pH-dependence of solubility

The solubility of native proteins depends primarily on the pH and ionic strength of the solvent. It is widely known that proteins have the lowest solubility when the pH equals their pI value. At such pH value, the number of positive charges on the protein equals that of negative charges.

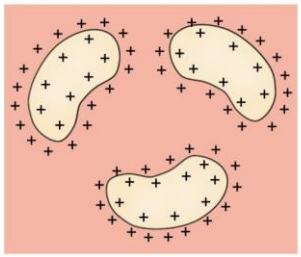
Consequently, in this state the level of ionic repulsion between the protein molecules is minimal, while the number of possible intermolecular attracting ionic interactions is at its maximum.



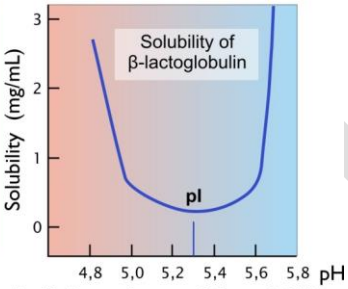
a) At pH values above the isoelectric point the protein is negatively charged



b) pH=pI, the number of negative and positive charges is equal



c) At pH values below the isoelectric point the protein is positively charged



d) pH-dependence of the solubility of the β-lactoglobulin protein

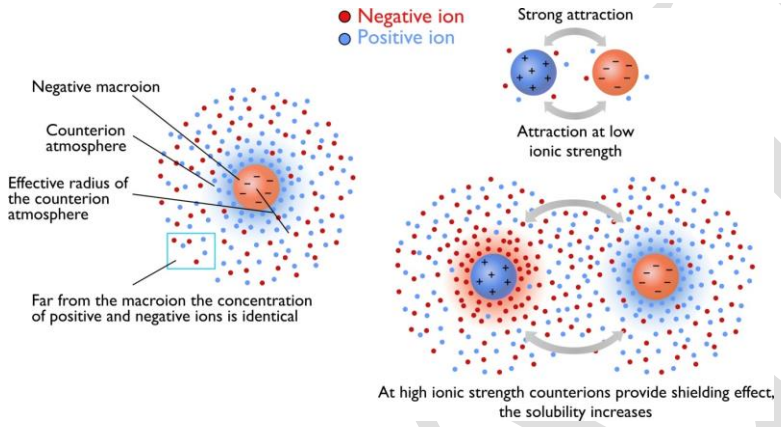
pH dependence of protein solubility. The solubility of proteins is at its minimum when the pH of the solution equals the pI value of the protein. At this pH, the number of the positive and negative charges of the protein is identical. As a consequence, the number of potential attracting intermolecular ionic interactions is at a maximum, which facilitates aggregation of the protein molecules. At pH values below or above the pI, the protein molecules have a net charge and so they tend to repel each other.

Naturally, a minimum in solubility does not mean that all molecules of a given protein precipitate when the pH equals their pI value. When the concentration of the given protein is below the solubility value, all of its molecules still remain in solution. When the protein concentration exceeds the solubility value, a dynamic equilibrium exists between the precipitated and the soluble set of molecules. Note that both the soluble as well as the insoluble subset contains native proteins—precipitation is not linked to denaturation. If one aims to precipitate native proteins, it is advisable to perform the experiment at a pH that equals the pI of the protein, since proteins are the most stable at pH values equal to their pI's.

Dependence of solubility on ionic strength

Solubility of proteins in distilled (deionised) water is significantly lower than in solutions containing ions. Accordingly, when salt is added to an ion-free protein sample in which the

protein is dissolved above its solubility, the originally precipitated insoluble fraction becomes soluble. This phenomenon is called 'salting-in' (Figure 5.5). Apparently, ions added at a small concentration increase the solubility of proteins. As it has been discussed in the previous paragraph on pH-dependence, the solubility of proteins is lowered by attracting intermolecular ionic interactions between opposite surface charges of the protein molecules. Ions incorporated by the solvent act as counter ions and efficiently shield the intermolecular attraction between proteins. This effect readily explains the salting-in phenomenon.



The 'salting-in' effect. The solubility of proteins is usually low in deionised water, but it increases with increasing ionic strength of the solution, up to a certain point. As the figure illustrates, dissolved salt (ions) facilitates solvation of proteins by shielding the exposed charges of protein molecules. This shielding effect hinders intermolecular attracting interactions between opposite charges of individual protein molecules and, by doing so, it also hinders protein aggregation.

When the concentration of ions is increased above a critical level, the solubility of proteins gradually decreases. Most importantly, the critical ion concentration level is characteristic of the identity of the protein. Accordingly, at high protein concentration, if the ionic strength of the solvent is increased, the excess of the proteins above the solubility level will precipitate while preserving their native state. This phenomenon is called salting-out. The

salting-out effect can be explained as follows. When ions (salt) are added to aqueous solutions, ions become solubilised such that a multilayer water boundary, a hydration shell is formed around them. In chemically pure water, the concentration of water molecules is ~ 55.6 M. When the concentration of ions exceeds several mol/litre, a very high percentage of the originally free water molecules will become engaged in the hydration shell of ions. Formation of more and more new hydration shells around ions competes for water molecules hydrating proteins. As the hydration shells around proteins decrease intermolecular attracting forces, mostly apolar interactions between protein molecules take over leading to precipitation of native protein molecules.

Generally, various salts of univalent positive ions are used for this purpose, with the most common salt being ammonium sulphate. As the solubility of different proteins depends differently on salt concentration, the salting-out phenomenon can be applied as a protein fractionation method. Let us consider the following ammonium sulphate fractionation scheme. Let us suppose that we have a protein mixture and the protein to be isolated has a medium sensitivity to ammonium sulphate precipitation. When—very slowly and gradually, e.g. by adding with mild stirring a saturated ammonium sulphate solution—ammonium sulphate is introduced in the solution, many proteins precipitate. The ammonium sulphate concentration can be increased up to a level where the target protein remains in the solution while many other, more sensitive proteins precipitate. At this point, the precipitated proteins can be removed simply by centrifugation. (Clumps of precipitated proteins represent an enormous particle size and a very high corresponding Svedberg value. Therefore they sediment at a very high rate.) The supernatant will contain our target protein and many other proteins. The ammonium sulphate concentration can then be further increased to a level where the target protein and some other proteins precipitate, while many other proteins still remain in solution. After a second centrifugation step the supernatant can be disposed of and the pellet containing the target protein can be collected. The solution of the resolubilised pellet will contain a protein mixture in which

the target protein will be enriched compared to the starting sample. Then the high concentration of ammonium sulphate can be decreased by dialysis or size exclusion chromatography.

Irreversible precipitation methods

As already mentioned, the relationship between solubility and native/denatured state of proteins is much more complex than usually considered. Native globular proteins have most of their apolar residues buried in the hydrophobic core of the molecule. Nevertheless, the dominantly polar surface of the protein still contains hydrophobic patches of apolar residues. The size and distribution of these hydrophobic spots widely varies with individual proteins. Upon unfolding, all apolar residues become solvent-accessible. Water molecules form a highly structured, low-entropy clathrate structure around apolar residues. The free enthalpy level of the thermodynamic system can decrease if the amount of the clathrate structure decreases. This indeed happens, because this way the entropy of the system increases. The amount of the clathrate structures can decrease if the apolar residues form short-range, weak, apolar-apolar interactions with each other. These interactions are weaker than the apolar-polar interactions between the apolar residues and the water molecules, but the newly formed polar-polar interactions between water molecules partially balance this loss of interaction energy. As already mentioned, the breakdown of the clathrate structures has a major contribution to lowering the free energy of the system. As a result, the apolar residues are expelled from water and interact with each other rather than with water molecules. This phenomenon is denoted as the hydrophobic effect. Due to the hydrophobic effect, denatured globular proteins tend to precipitate from solution. However, it might be confusing that many popular denaturing agents such as urea or sodium dodecyl sulphate (SDS) are highly effective solubilisers of denatured proteins. When urea or SDS is used as denaturing agents, denaturation is not accompanied by precipitation.

Denaturation can be triggered by increasing the temperature or by applying extreme pH values. If the target protein is unusually resistant against certain denaturing effects (e.g. it is

thermostable, acid-stable etc.), this property can be a good starting point for establishing an effective fractionation protocol. After high-temperature or extreme-pH treatment, many proteins unfold and precipitate, while the target protein (and, usually, several other resistant proteins) remains folded and stays in the solution. After a centrifugation step, the target protein will be in the supernatant and can be easily separated from the pellet of denatured 'contaminating' proteins.

Protein fractionation based on particle size

Several low-resolution fractionation methods separate molecules from each other based on their size. The two methods reviewed below apply semipermeable membranes to separate the sample in two fractions by size. Semipermeable membranes are special 'sieves' having pores with diameters in the size range of molecules. Molecules that are smaller than the pores of the membrane can permeate the membranes while molecules larger than the pores are retained.

In the course of *dialysis*, the sample is poured in a tube made of a semipermeable membrane. The two ends of the tube are then sealed, and the resulting sachet is immersed into a container containing an appropriate buffer. All molecules that are smaller than the pores of the membrane start to diffuse across the membrane in both directions. This process leads to an equilibrium. At equilibrium, the concentration of the small molecules on the two sides of the membrane becomes identical, while large molecules remain in the dialysis sachet. The time required for reaching the equilibrium state can be shortened by stirring the buffer in the container. Dialysis is usually applied in order to remove small molecules from the sample and/or to replace the buffer around the retained large molecules (usually proteins) in the sample. The equilibrium concentration of the small molecules can be calculated based on their original concentrations inside and outside the sachet and the ratio of the volumes of the sachet and the container. The larger the container, the lower the concentration of the small molecules to be removed will be in the sachet. However, instead of increasing the volume of the container to an impractical volume, a better strategy is to apply a relatively small container and change the

buffer in the container several times. This way, the concentration of the small molecule in the sample will decrease exponentially. At the end of the previously mentioned salting-out experiment, the protein sample will contain high concentration of ammonium and sulphate ions. These ions are typically removed by dialysis.

Semipermeable membranes are applied in the course of *ultrafiltration*, too. However, in this case the sample solution is forced to flow through the membrane. This comes with two advantages. First, the process is faster than dialysis. The second, more important difference is that by applying an external driving force, the method does not rely on equilibrium. This way, the concentration of the molecules that are retained by the membrane can be increased tremendously. This method is often applied to concentrate protein samples. Furthermore, the solvent of the large molecules can also be easily changed by this method by adding the appropriate buffer to the sample between several consecutive ultrafiltration steps. The external force can be generated either by centrifugation of the sample in Centricon vials, or by a high-pressure inert gas (usually nitrogen) in Amicon systems.

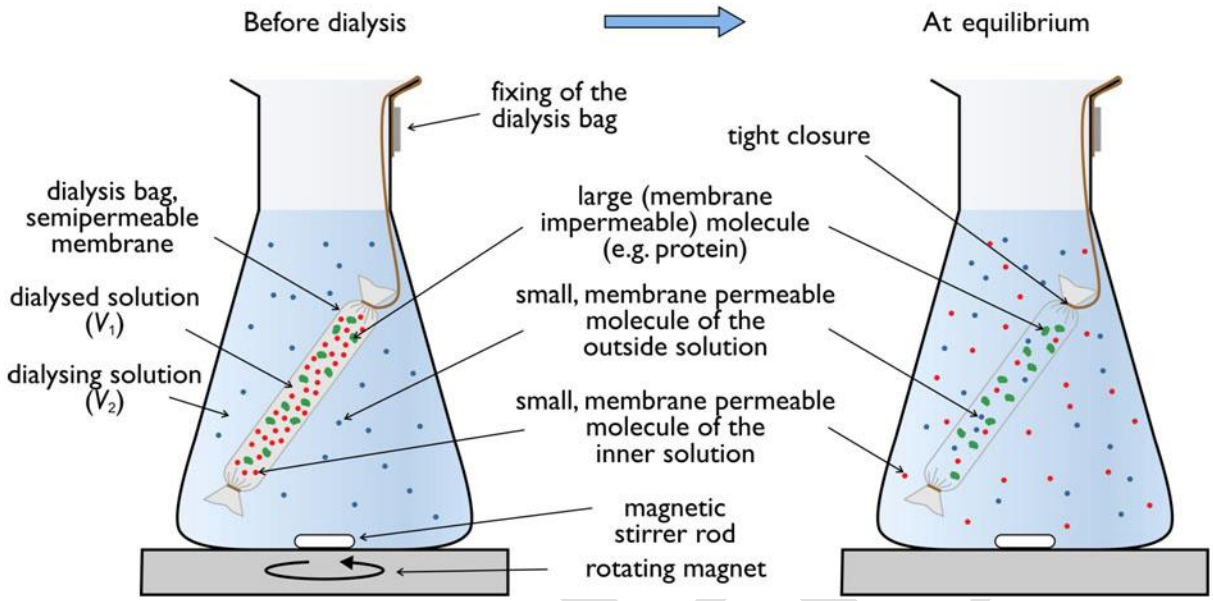
Dialysis

The principle of dialysis

Dialysis is a procedure employed in a number of cases when a change in the concentration or composition of solutes is necessary. In the biochemical practice, dialysis is often used to alter the concentration of salts and/or small molecules in protein solutions—usually aimed at decreasing the concentration of these solutes. However, the composition of the solution can also be changed in additional ways.

Dialysis is based on diffusion during which the mobility of solute particles between two liquid spaces is restricted, mostly according to their size. (In rarely used versions of dialysis,

restriction of diffusion via polarity or charge is also possible.) Size restriction is achieved by using a porous material, usually a semi-permeable membrane called dialysis membrane. This membrane is permeable only for particles below a certain size. In the biochemical laboratory, this membrane is mostly a hose made from transparent material (also called dialysis bag) that can be tightly closed (tied) at its ends. The solution to be dialysed (with a volume V_1) is loaded into the dialysis bag. The dialysis bag is then placed into a dialysis solution (with a volume V_2) that is stirred slowly to aid the diffusion of the subset of solutes that can be released through the bag membrane, in order to achieve equilibrium between solute concentrations in the two liquid spaces. If the difference in volume between the two spaces is large ($V_2 >> V_1$, e.g. V_2 = 10 L and V_1 = 0.1 L, a 100-fold difference), the onset of the equilibrium will lead to a very significant dilution of the small solutes that were initially inside the bag (their concentration will change by a factor $V_1/(V_1+V_2)$, in this case << 1), with only a slight change in the concentration of small solutes in the outside solution (by a factor $V_2/(V_1+V_2) \approx 1$), whereas the concentration of the molecules inside the bag that cannot penetrate the membrane remains almost completely unchanged (see in details below).



Dialysis in the biochemical laboratory practice

Practical aspects and applications of dialysis

The efficiency of dialysis, i.e. the extent to which the concentration and composition of the inside solution can be changed, is an important aspect. It follows from the above description of dialysis that the efficiency of dialysis largely depends on the difference between the volumes of the inside and outside liquid spaces. This is why we generally seek to use as large volume (V_2) of the dialysing solution as possible. However, the efficiency of dialysis can be further increased by performing multi-step dialysis by exchanging the outer solution after the equilibrium has been reached. In this case, the attainable dilution of the inside solution will be $[V_1/(V_1+V_2)]^n$ where n is the number of steps. It is easy to see that efficiency that can be achieved by applying a two-step dialysis at a 50-fold volume difference is much higher than the efficiency of a single-step dialysis at a 100-fold volume difference.

The speed of dialysis can be increased not only by stirring the outside solution but also by increasing the surface/volume ratio of the inside solution, as the flux of diffusion is linearly proportional to the cross-section. It is, therefore, more practical to choose a narrower and longer tube than a wider and shorter one.

The semi-permeable membrane can be crossed not only by salts and small molecules but also by solvent particles (in most cases, water). The direction and extent of the net solvent flow is determined by the difference between the total concentration of solutes in the inside and outside solutions such that the solvent migrates from the less to the more concentrated solution (with regard to solutes). This way the equilibrium concentration of the solute(s) of the inside solution that cannot cross the membrane will be influenced also by the diffusion of the solvent. As the solute(s) that cannot cross the membrane also contribute to the total concentration of the inside solution, the net direction of solvent migration will almost always point towards the inside solution. Therefore, the volume of the inside solution will increase, thereby selectively decreasing the concentration of the membrane-impermeable solute(s)—but not that of the membrane-permeable ones, even if the relative increase in the volume is large. However, the relative increase in the volume is generally not large because (*i*) the concentration of the large impermeable solutes is low (much lower than that of the small permeable ones) (*ii*) the dialysis tube is largely unable to increase its volume. The occasional small (5-20 %) volume increase of the inside solution is associated with the compression of air above the liquid phase that was originally enclosed in the bag. Taken together, the decrease in the concentration of the large solutes (proteins) is usually negligibly small. The increase in the volume of the inside solution is remarkable from a technical point of view because it is accompanied by a (sometimes substantial) elevation of the pressure. Therefore, if there is a hidden "weakness" somewhere in the material of the membrane, the elevation of pressure may lead to bursting of the bag and, as a consequence, the complete loss of the dialysed material (e.g. protein preparation). To avoid this "catastrophe", it is recommended to perform a pressure test on the bag in its water-filled state.

The other risk associated with pressure elevation occurs during the opening of the bag after completion of dialysis. In the absence of necessary care, the pressurised inside solution can sprinkle out, causing loss of material.

In the biochemical laboratory practice, solutions of proteins are generally dialysed following fractioned ammonium sulfate precipitation as well as before or after ion exchange chromatography. A size selectivity (size exclusion or cut-off) specified as 4 or 11 kDa means that the pores of the dialysis membrane are impermeable for particles larger than 4 or 11 kDa, respectively.

Besides the biochemical laboratory, dialysis is utilised in the field of life sciences also for therapeutic purposes during haemodialysis, i.e. in artificial kidneys. The principal difference between these two applications is that, in the artificial kidney, dialysis is executed under continuous counter-flow of the two solution spaces: both the inside solution (the blood of the patient) and the outside solution are pumped. Thus, in such a setting, also the inside liquid space is "open": it is not in a "bag" but flows inside a tube. Moreover, in order to increase the flux of diffusion, a large number of capillary tubes are employed in a bundle (which is actually the artificial kidney) by which the surface/volume ratio is increased enormously. The composition of the outside dialysing solution is very special as it must meet special requirements. In addition, the artificial kidney equipment is a very special apparatus because it must be able to ensure the appropriate pressure and temperature while the blood entering the body of the patient must be free of entrapped air bubbles that could lead to lethal consequences.

Lyophilisation (freeze-drying)

Protein (or any other non-volatile molecule) samples can be concentrated by evaporating water and other volatile compounds from the sample. In principle, this could be achieved by simply heating the sample. However, most proteins would unfold during such a simple evaporation process. In order to prevent the denaturation of proteins, the sample is transferred into a glass

container and is frozen as quickly as possible, usually by immersing the outside of the container into liquid nitrogen. Moreover, the container is rotated in order to spread and freeze the sample on a large surface area. The glass container with the sample is then placed into an extremely low-pressure space (vacuum) that contains a cooling coil as well. The cooling coil acts as a condenser. The temperature of the coil is usually lower than -50°C. Volatile compounds of the frozen sample will evaporate (sublimate) in the vacuum. The process of evaporation (in this case, sublimation) absorbs heat. This effect keeps the sample frozen. Evaporated molecules are captured from the gas phase by the cooling coil, forming a frozen layer on it. At the end of the process, proteins and other non-volatile compounds of the sample remain in the container in a solid form. This process does not cause irreversible denaturation of proteins. Thus, it is a method frequently used not only to concentrate but also to preserve proteins or other sensitive biomolecules for long-term storage. Such samples can usually be stored for years without a significant deterioration of quality. However, before lyophilisation it is very important to carefully consider all non-volatile compounds of the initial sample as these will concentrate along with the proteins. Non-volatile acids or bases can cause extreme pH, and the presence of salts can result in very high ionic strength when the sample is resolubilised.

UNIT-II

SYLLABUS

Purification and Characterization of proteins: Chromatographic Techniques - Ion-exchange chromatography, molecular sieve chromatography, hydrophobic interaction/reverse phase chromatography, affinity chromatography, HPLC (Normal and Reverse phase) and FPLC. Characterization of proteins - Determination of purity, molecular weight, extinction coefficient and sedimentation coefficient, IEF, SDS-PAGE and 2-D electrophoresis.

Chromatography is the collective term for a set of separation techniques that operate based on the differential partitioning of mixture components between a mobile and a stationary phase. The mobile phase (a liquid or a gas) travels through the stationary phase (a liquid or a solid) in a defined direction. The distribution of components between the two phases depends on adsorption, ionic interactions, diffusion, solubility or, in the case of affinity chromatography, specific interactions. Depending on the experimental design, the separation in a liquid mobile phase may be carried out via column or planar chromatography, on analytical or preparative scales.

Chromatographic methods are important in the analytical and preparative separation of biological samples. Gel filtration chromatography (size exclusion chromatography) is often the method of choice to purify macromolecules, taking advantage of their different sizes and shapes. Ion exchange chromatography is also useful for the separation of macromolecules, operating based on the various net charges on their surface, which can be tuned via the pH of the medium. Biological specificity in enzyme-substrate, enzyme-inhibitor, receptor-ligand, antigen-antibody (and other) interactions is utilised in affinity chromatography. In this method, one interaction

partner is immobilised on a solid surface (stationary phase) and can selectively bind its interacting partner from a mixture in the mobile phase. The other components of the mixture can then be removed by replacing the mobile phase (washing). The pure material is then eluted by applying a mobile phase that disrupts the specific interaction.

Of the quasi-infinite possibilities of analytical applications of liquid chromatography, only a few relevant ones will be mentioned in this chapter. The analysis of amino acid mixtures and that of the products of Edman degradation in the process of amino acid sequencing are both carried out using chromatography. Chromatographic methods coupled to on-line mass spectrometry are instrumental in current proteomics research.

Quantification of separation

(1) Resolution (R_S)

Resolution is a number describing the separation of chromatographic peaks. By definition, resolution is the distance between peak maxima (elution volumes) divided by the average of peak widths (Figure 6.1). The elution volumes and the peak widths are to be measured in identical units. R_S is therefore a dimensionless number. In the case of a constant flow rate, the quantification of volumes can be replaced with the more convenient measurement of time.

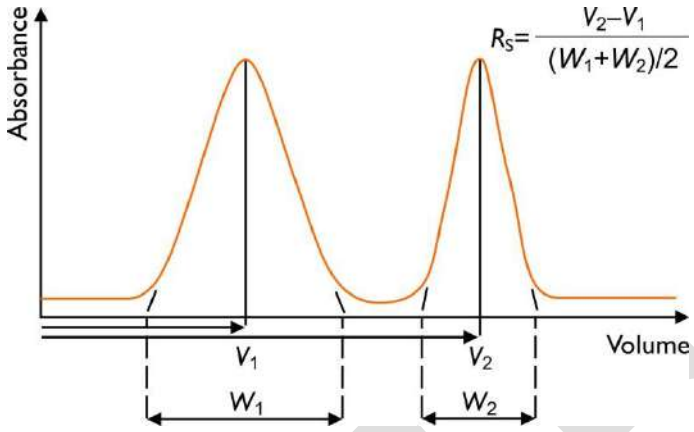


Figure 6.1. Determination of the resolution. R_S = resolution; V_1 = elution volume at the maximum of the first peak; W_1 = base width of the first peak; V_2 = elution volume at the maximum of the second peak; W_2 = base width of the second peak. In the case of a constant flow rate, it is more convenient to measure time instead of elution volumes. (In this case, time will be directly proportional to the elution volume.) Thus, $R_S = 2(t_2 - t_1 / W_2 + W_1)$, where t_2 and t_1 are elution times corresponding to V_2 and V_1, respectively.

The R_S value defines the extent of separation. The larger the R_S between two peaks, the more ideal the separation (Figure 6.2). (Note that even perfectly separated peaks may comprise impure materials. In many cases, two or more components may co-elute under a given set of chromatographic conditions.)

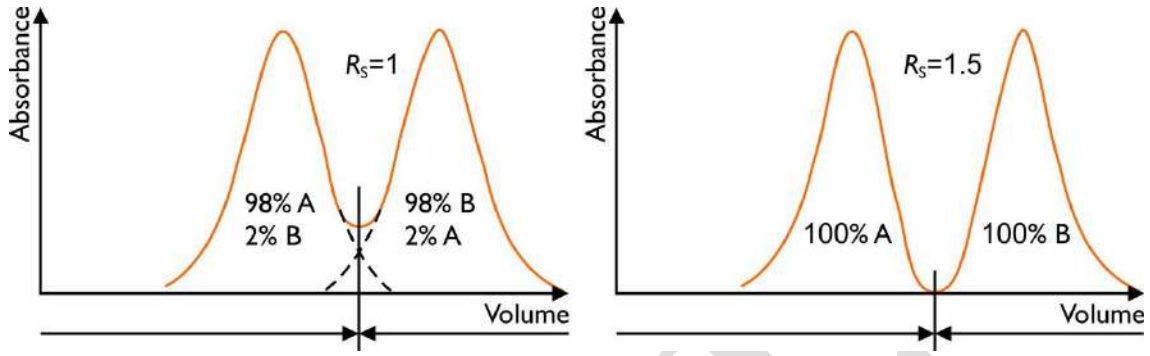


Figure 6.2. Relation between resolution and separation. It can be proven by calculation that, if R_S = 1, and both peaks have ideal shapes (i.e. Gauss curves) and identical sizes, then the two components can be isolated at 98 % purity. Perfect, so-called base-line separation can be achieved in cases where R_S > 1.5.

The chromatographic separation, the behaviour of the different peaks, and the efficiency of the chromatographic column can be described using the following parameters.

(2) Retention

The retention factor or capacity factor, $k`_n$, is defined for the extent of retention of a compound under a given set conditions. The retention factor can be calculated for each peak.

$$k'_n = V_n - V_0 / V_0 \text{ or } k'_n = t_R - t_0 / t_0 \quad (6.1)$$

where V_n is the elution volume of component *n*, V_0 is the elution volume of a component proceeding through the column without any interaction, and t_R and t_0 are the corresponding times, respectively (Figure 6.3).

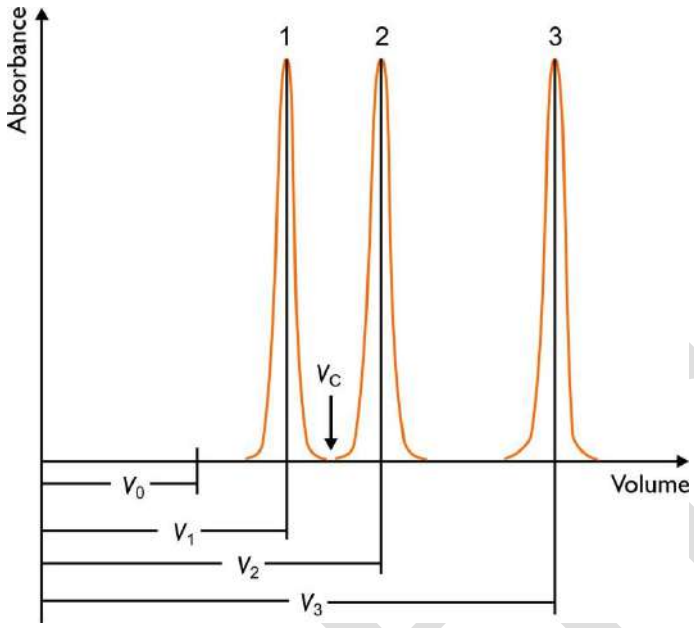


Figure 6.3. Idealistic chromatogram to demonstrate the calculation of the retention factor. V_0 = elution volume of a material passing through the column without any interaction; V_C = total volume of the column; V_1-V_3 = elution volumes corresponding to individual peaks. In the case of gel filtration, V_0 equals the exclusion volume of the column, and separation takes place in the range between V_0 and V_C. In other cases where the materials of interest bind to the stationary phase (based on ion exchange, adsorption, affinity etc.), the elution volumes can exceed the total volume of the column (V_C) by several times.

The retention factor is characteristic of a component in a given composition of the mobile phase, in a given type of column, and at a given temperature. It is independent of the size of the column and of the flow rate of the mobile phase.

(3) Efficiency (N)

The efficiency, N, or in other words, the number of theoretical plates characterises the spreading of the eluted compound. It can be calculated as follows:

$$N = (V_e/\sigma)^2 = 16(V_e/w)^2 = 5{,}54^*(V_e/w_{1/2})^2 \quad (6.2)$$

or

$$N = (t_R/\sigma)^2 = 16(t_R/w)^2 = 5.54^*(t_R/w_{1/2})^2 \quad (6.3)$$

where V_e and t_R are the elution volume and the retention time of the peak, respectively; σ is band broadening (to be determined by measuring the peak width, $W = 4\sigma$); W is peak width measured at the baseline; $W_{1/2}$ is peak width measured at 50 % of the maximal peak depth. The sign * marks a coefficient calculated from the ratio of W and $W_{1/2}$ if the peak follows a Gaussian distribution.

The value of N is used to characterise the chromatographic column. As N largely depends on the experimental conditions including the flow rate of the mobile phase, the quantity and quality of the loaded sample, the determination of N is carried out in a standardised manner.

The main reason behind the spreading of a compound and the broadening of chromatographic bands is the longitudinal diffusion of molecules within the column. The effect of diffusion may be decreased by using smaller chromatographic beads and by enhancing the homogeneity of their size distribution.

Besides the particle size, efficiency is significantly influenced by the method of packing of the chromatographic media, especially when the column is home-made. Any inhomogeneity in the sedimented matrix (e.g. due to air bubbles or tunnels) will result in poorer efficiency and, in consequence, imperfect separation.

(4) Selectivity (α)

The selectivity (α) for two neighbouring elution peaks at V_1 and V_2 is characterised by the quotient of their retention factors:

$$\alpha = k'_2/k'_1 \qquad (6.4)$$

The overall quality of the chromatographic separation is influenced by both selectivity and efficiency. Nevertheless, selectivity is more definitive (Figure 6.4).

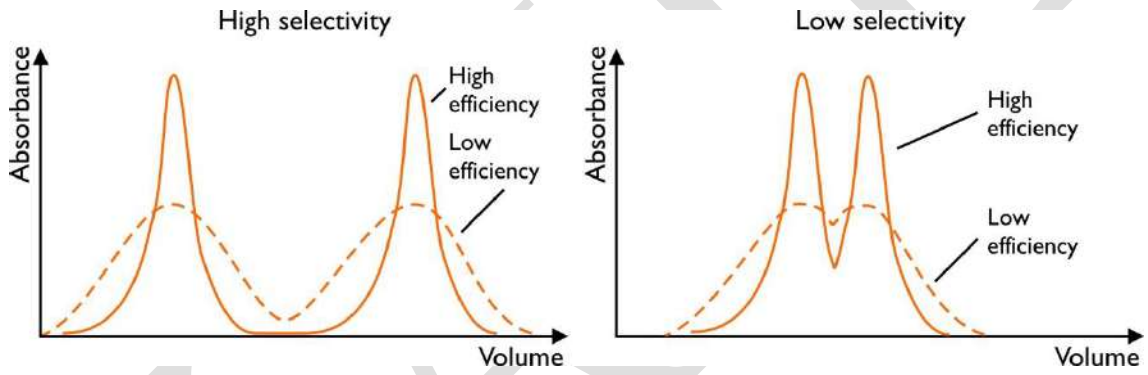


Figure 6.4. The effect of efficiency and selectivity on separation. The efficiency of a chromatographic column (N) is the qualitative property that reflects how thin and symmetrical the peaks are during elution. The separation of two materials is determined by the extent of selectivity. At high selectivity, even a smaller efficiency will provide sufficient separation. In an ideal case, a high selectivity is accompanied by a high efficiency.

6.1. Gel filtration chromatography

The chromatographic medium for gel filtration is a hydrophilic gel made up from porous, fine-grain spheres of 10-300 µm diameter. This type of medium defines two solution compartments

within the column: one is the freely moving mobile phase outside the gel particles, while the other is the restricted liquid compartment inside the porous particles (Figure 6.5).

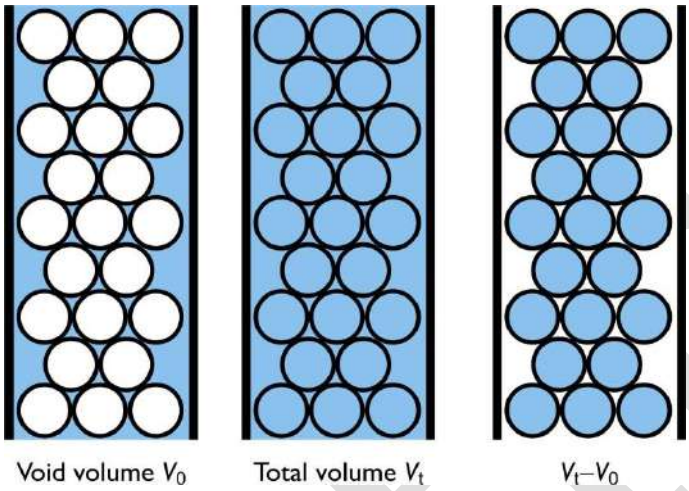


Figure 6.5. Liquid compartments inside a column packed with a porous gel. The individual liquid compartments are depicted as blue areas. V_0 = exclusion volume (void volume); V_t = total volume of the column; $V_t - V_0$ = combined volume of the liquid inside the gel particles and the material of the gel.

When a solution is moving through the gel filtration column, the movement of the solutes depends on two factors: the flow rate of the mobile phase and diffusion. Diffusion enables the molecules to explore the inside of the gel particles if their size so permits. The separation of a molecular mixture is based on the phenomenon that some molecules are excluded from the inside of the gel particles due to their size. These molecules travel quickly in the mobile phase of the column, which is the only compartment available to them. Smaller molecules, on the other hand, spend various amounts of time inside the particles (stationary phase) and flow through the column slower (Figure 6.6).

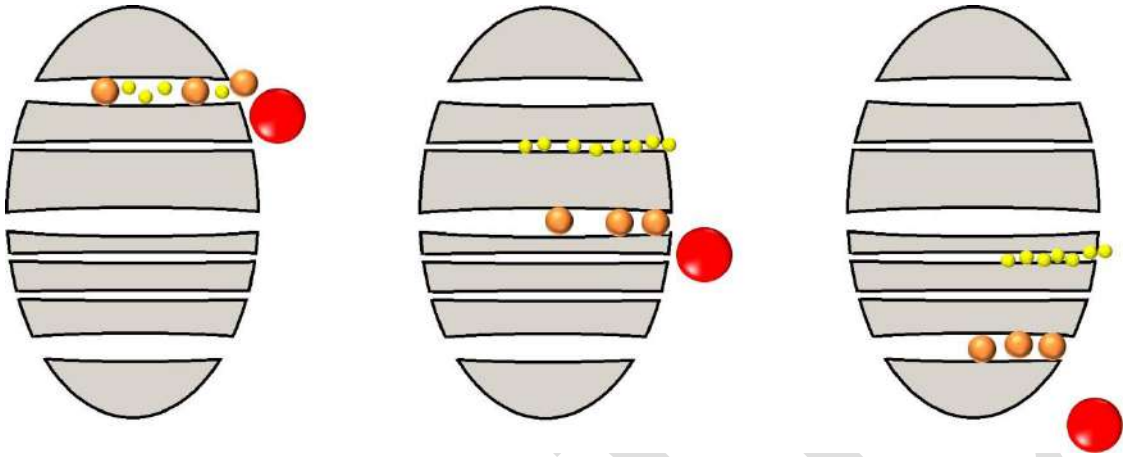


Figure 6.6. Travel of variably-sized molecules through a porous gel. During gel filtration (size exclusion) chromatography, molecules of different sizes will explore the available liquid spaces via diffusion. The largest molecules (red in the figure), due to their size, cannot enter the pores of the matrix at all. The movement of molecules with medium sizes (orange) is confined to the larger pores. The smallest molecules (yellow) can enter the gel particles through all pores. Therefore, the largest molecules will advance most rapidly through the column, whereas the smaller molecules will be retarded.

The result of a gel filtration experiment is usually depicted as an elution diagram. In this diagram, the concentration of the eluted compound is plotted against the volume of the eluent. The appearance of a given compound occurs at its elution volume (V_e). As in other distribution chromatographic methods, the elution of a compound is best characterised by its distribution coefficient (Kd):

$$K_d = (V_e - V_o)/V_s \qquad (6.5)$$

where V_o equals the exclusion volume, i.e. the elution volume of a molecule that is larger than the largest pore size of the separating gel. Such a molecule therefore explores only the mobile

phase, and is entirely excluded from the gel. V_s equals the volume of the stationary phase, i.e. the volume of the liquid inside the gel particles that is fully accessible only to molecules small enough to travel smoothly even through the smallest pores of the gel. V_s itself is difficult to determine. Therefore, in practice, it is replaced by the V_t-V_o term, also accounting for the non-negligible volume of the gel itself. As a result, a constant pertinent to an apparent volume (K_{av}) is used instead of K_d (the latter would be valid only for real liquid volumes):

$$K_{av} = (V_e - V_o)/(V_t - V_o) \quad (6.6)$$

where K_{av} represents the portion of the gel volume that is accessible to a molecule of a given size. For a totally excluded macromolecule, $K_{av} = 0$; whereas, for small molecules diffusing freely in the entire volume of the gel, $K_{av} = 1$.

Planning a gel filtration experiment

(1) Choosing the gel type

Several different gel filtration media are available, which should be chosen according to the substance to be separated. These media differ in the chemical properties of the gel matrix, the pore size, the particle size, as well as the physical and chemical stability of the gel. The first developed and still widely used gel matrix is made of crosslinked dextran. Polymer beads made of dextran are known by the trade name Sephadex.

The pore size of the various Sephadex media is controlled by the number of crosslinks. The most popular ones are the entirely hydrophilic G-type gels. Numbers accompanying the G-type mark refer to the pore size and indicate the approximate molecular mass of excluded molecules in kDa. For example, Sephadex G-25 is used to separate relatively small molecules in the molecular mass range of 1000-5000 Da, including peptides. Alternatively, it can also be used to desalt

larger proteins. To fractionate larger macromolecules up to 200-300 kDa, the G-150 or G-200 Sephadex gels are to be used. The mechanical properties of dextran gels having large pores are unfavourable due to the low density of crosslinks. These gels are easily compressible. Therefore, more rigid gels made of synthetic polymers are used to separate very large or elongated molecules.

If the size difference between the compounds to be separated is relatively large, e.g. during desalting of a macromolecule, it is practical to choose a gel in which the large-sized compound is eluted in the excluded volume (V_o; thus $K_{av} = 0$), while the small component elutes around V_t (thus $K_{av} = 1$). In this case, the fraction containing the macromolecules appears sharply, with minimal band broadening and dilution, in the shortest possible elution time.

In case of fractionating macromolecules and if the molecular weight of the compound of interest is known, the gel should be chosen so that the component of interest will elute approximately at the half of the entire fractionation range. For example, if a 100-kDa protein is to be isolated from a protein mixture, the use of a gel that spans the 10-250 kDa fractionation range is recommended.

(2) Choosing the particle size of the gel

Fine-sized beads fill the available space within the chromatographic column more efficiently. Therefore, the volume of the mobile phase will be reduced. This will result in a similar reduction in dilution and band broadening and, in turn, will yield a better resolution. On the other hand, the flow rate in a compact gel column is also reduced. Therefore, larger pressure should be applied when using super-fine beads. Indeed, special pumps are needed below a particle size of 10 µm. Naturally, only rigid, non-compressible gel types can be used in these cases.

For most purposes, the Fine and Medium type particle sizes (20-150 µm) are suitable. For preparative purposes and desalting, where high flow rates are required and the compounds of interest separate well even at a poor resolution, Coarse type gels can be used too.

(3) Choosing the size of the column

During gel filtration, the distance between two zones of separation increases proportionally to the square root of the column length. Long columns (> 100 cm) are used when a high resolution is required, while shorter (< 50 cm) columns are more practical when the aim is desalting or the separation of compounds that can be eluted at markedly different volumes.

Columns with diameters of around 1 cm are used for analytical purposes. By increasing the diameter, the amount of the applied sample, i.e. the capacity of the column, can be increased.

(4) Choosing the sample volume

A narrow start zone (relative to the column length) is sought if maximal resolution is to be achieved, e.g. for analytical purposes or in the case of compounds whose separation is difficult. Therefore, the sample volume in this case should be chosen to be 1-5 % of the column volume. The resolution cannot be further increased using smaller sample volumes, while the dilution will be greater. The sample volume can be increased to as much as 15-20 % of the column volume if the compounds are readily separable, especially when working on a large scale.

(5) Choosing the eluent

The composition of the eluent does not directly influence the resolution of gel filtration. However, all components that have an effect on the molecules to be separated may influence the separation. The pH, ionic strength or the presence of detergents may influence the molecular state of the solutes. For instance, changes in molecular shape or the dissociation of multimeric

proteins and enzyme-inhibitor complexes will change their chromatographic behaviour. In general, dilute (0.01-0.1 M) buffers are used that do not influence the structure of the compounds to be separated, but restrict the unwanted adsorption interactions between the gel matrix and the molecules of interest.

When the fractions containing the separated compounds are to be later concentrated, volatile buffers (e.g. ammonium bicarbonate) are practical to use that easily disappear during lyophilisation or film evaporation. The same considerations apply when the salt content of the gel filtration buffer should be subsequently eliminated.

(6) Choosing the flow rate of the eluent

During gel filtration, increasing the flow rate will deteriorate the resolution, because it prevents the formation of equilibrium between the mobile and the stationary phases. Generally, 5-10 mL/cm^2x hour is recommended as an optimal flow rate, but in most cases, a several times excess of this will not deteriorate the separation significantly. When doing preparative work, or if the operation must be performed quickly for some reason, the advantage conferred by the higher flow rate may compensate for the deterioration of separation.

To achieve a higher flow rate, of course, a larger pressure must be applied. Therefore, in these cases, the mechanical stability of the gel matrix must be taken into consideration. Non-rigid gels may be compressed at pressures higher than allowed, which may lead to the complete clogging of the chromatographic column.

Ion exchange chromatography

Ion exchange chromatography is one of the most efficient methods for the separation of charged particles.

Ion exchange chromatography is most often performed in the form of column chromatography. However, there are also thin-layer chromatographic methods that work basically based on the principle of ion exchange. In the following, we will exclusively deal with column chromatographic applications.

Column materials used for ion exchange chromatography contain charged groups covalently linked to the surface of an insoluble matrix. When suspended in an aqueous solution, the charged groups of the matrix will be surrounded by ions of the opposite charge. In this "ion cloud", ions can be reversibly exchanged without changing the nature and the properties of the matrix.

The charged groups of the matrix can be positively or negatively charged. A positively charged matrix will bind negatively charged ions from the solution. Therefore, it is called an anion exchanger. Cation exchanger matrices have negative charges.

Based on the structure of the ion exchange matrix, we distinguish ion exchangers with hydrophobic and hydrophilic matrices. Ion exchangers with a hydrophobic matrix are most often highly substituted polystyrene resins. These are suitable for the binding of inorganic ions, e.g. in water softening applications. However, they tend to denature proteins due to the high hydrophobicity of their matrix and their high surface charge density.

Ion exchangers with hydrophilic matrices were first produced from modified cellulose. However, cellulose has disadvantageous mechanical properties: cellulose fibres are prone to break, making it difficult to create a well-utilisable column. This disadvantage has been partially remedied in Sephadex (dextran-based) ion exchange matrices.

In recent years, regular spherical and monodisperse matrices have been produced from synthetic hydrophilic polymers. The best known of such resins is the MonoBead-based ion exchange matrix.

Table 6.I summarises the charged groups linked to ion exchange matrices.

Anion exchangers	**Functional group**
diethyl-aminoethyl (DEAE)	$-OCH_2CH_2N^+H(CH_2CH_3)_2$
quaternary aminoethyl (QAE)	$-OCH_2CH_2N^+(C_2H_5)_2CH_2CH(OH)CH_3$
quaternary ammonium (Q)	$-CH_2N^+(CH_3)_3$
Cation exchangers	**Functional group**
carboxymethyl (CM)	$-OCH_2COO^-$
sulfopropyl (SP)	$-CH_2CH_2CH_2SO_3^-$
methylsulfonate (S)	$-CH_2SO_3^-$

Table 6.I. Functional groups of ion exchangers

Ion exchangers containing sulfonyl and quaternary ammonium groups are called strong ion exchangers. These are practically completely charged between pH 3.0 and 11.0. The degree of dissociation of DEAE and CM groups—and thus their ion exchange capacity—depends on the pH of the medium.

The theory of ion exchange

Most ion exchange experiments comprise five different phases (Figure 6.7).

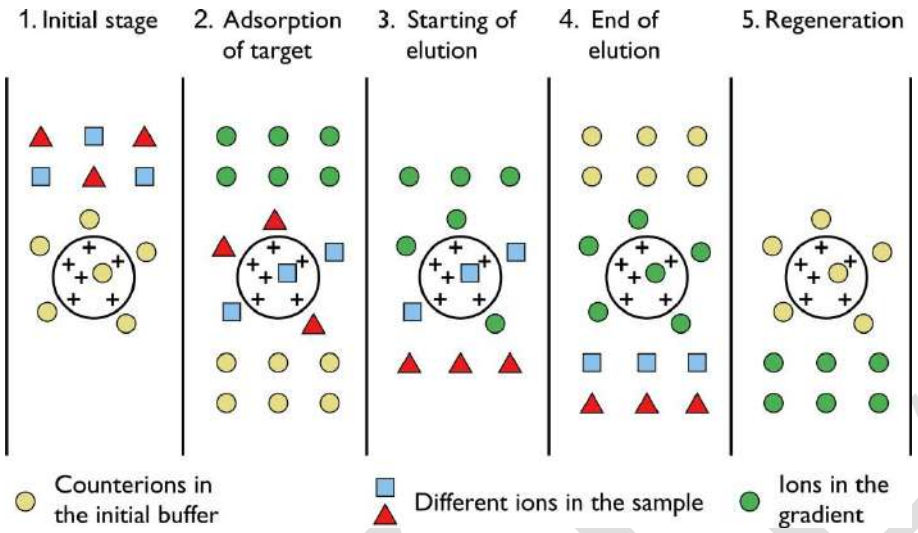


Figure 6.7. Phases of ion exchange chromatography (salt gradient elution). In the figure, a positively charged anion exchanger particle is shown, with counterions on its surface in the starting state (first phase). In the second phase, the binding of the ions to be separated takes place. At the start of the elution (third phase), weaker-binding ions are desorbed. At the end of the desorption, the stronger-binding ions are also desorbed (fourth phase). During regeneration (fifth phase), the starting state can be reconstituted via washing the column with the starting buffer.

The first phase is the equilibration of the ion exchange column with a so-called starting buffer, setting the conditions of the experiment (pH and ionic strength). In this phase, the charged groups of the ion exchanger will bind easily replaceable simple ions (e.g. chloride or sodium).

The second phase is the loading of the sample and its reversible binding to the column. If some of the contaminating materials do not bind to the column, these can be removed via washing the column with the starting buffer.

The third and fourth phases comprise the elution, i.e. the desorption of the bound molecules, which can be achieved via changing the composition of the elution buffer. The simplest form of elution is achieved via an increase in ionic strength, i.e. in the concentration of the counterions present. Another means of desorption is the change of the pH of the medium. The most effective method is the continuous change of the ionic strength or the pH, i.e. the application of a gradient elution. During gradient elution, molecules with smaller net charges (i.e. the weaker-binding ones) will be the first to leave the column.

An important property of an ion exchange column medium is its *ion exchange capacity*. This parameter reflects the amount of counterions that can be bound to the column. Three types of capacity can be distinguished:

Total capacity: The number of charged groups per gram dry weight of the ion exchanger or per millilitre of swollen gel. This can be determined by titration with a strong acid or base.

Free capacity: Due to steric reasons, only a part of the full capacity is accessible for macromolecules (proteins, nucleic acids). This is the free capacity.

Dynamic capacity: The so-called dynamic capacity is determined when the binding of the given macromolecule to the column is measured during buffer flow.

The free and the dynamic capacity values are dependent on the properties of the material to be separated, the properties of the ion exchanger, and the applied experimental conditions.

With regard to separation, the important properties of the material to be separated are the size of the molecules and the pH dependence of their charge. This implies that the capacity of ion exchangers will de different for different proteins.

Frequently used Sephadex-based ion exchangers

Via the modification of the Sephadex G-25 and G-50 gel filtration matrices with four different groups, eight different ion exchangers were created (Table 6.II). Matrices derived from G-25 (A-25 and C-25) contain more crosslinks. Therefore, they are more rigid and swell to a smaller extent than the ones derived from G-50.

Ion exchanger	**Total capacity**	**Total capacity**	**Functional group**	**Counterion**
	µmol/mg	**µmol/mL**		
DEAE Sephadex A-25	3.5 ± 0.5	500	diethyl-aminoethyl	chloride
DEAE Sephadex A-50		175	diethyl-aminoethyl	chloride
QAE Sephadex A-25	3.0 ± 0.4	500	diethyl-(2hydroxypropyl)aminoethyl	chloride
QAE Sephadex A-50		100	diethyl-(2hydroxypropyl)aminoethyl	chloride
CM Sephadex C-25	4.5 ± 0.5	550	carboxymethyl	sodium
CM Sephadex C-50		170	carboxymethyl	sodium
SP Sephadex C-25	2.3 ± 0.3	300	sulfopropyl	sodium
SP Sephadex C-50		90	sulfopropyl	sodium

Table 6.II. Properties of Sephadex-based ion exchangers

With the use of specially-treated polysaccharide and synthetic polymer matrices that are more pressure-resistant than Sephadex, it was possible to develop fine-grained regular spherical polymer beads for ion exchangers with significantly increased efficiency. Such matrices include monodisperse MonoBeads with a bead size of 10 μm, or MiniBeads with a 3-μm bead size. Such ion exchangers are used in FPLC techniques (see below).

Important parameters to consider during the planning of ion exchange-based separation

1. *The charge of components present in the sample to be separated*

In the case of proteins, this will depend on the isoelectric point. Below the isoelectric point, proteins are cations; above the isoelectric point, they are anions. The applied chromatographic buffer should be chosen in a way that the proteins to be separated should bind to the given anion or cation exchange column, from which they can be eluted after the washout of the non-binding components. More rarely, it can occur that the unwanted contaminants are bound to the column, whereas the component to be isolated will freely flow through.

2. *The amount of the sample*

The size of the column should be chosen so that the dynamic capacity of the medium should somewhat exceed the amount of the sample. If the column is too small, the sample will saturate it, and part of the sample will not bind to the column. If the column is too large, a significant loss can occur during elution.

3. *The molecular mass of the protein to be isolated*

The pore size of the ion exchange matrix should be chosen in a way that gel filtration effects—i.e. size-based separation—do not occur during ion exchange.

Hydrophobic interaction chromatography

The method of hydrophobic interaction chromatography (HIC) is based on the observation that protein molecules can interact with fully hydrophobic adsorbents, and this interaction is dependent on the salt concentration of the solution. Similarly to the salting-out of proteins (see in details in Chapter 5) where the increasing salt concentration will lead to the aggregation and precipitation of protein molecules via the rearrangement of their hydrate shell, during HIC chromatography a high salt concentration (1-1.5 M neutral salt) facilitates the interaction between the hydrophobic chromatographic medium and the hydrophobic patches present on protein molecules. During separation, the decrease in salt concentration will lead to the elution of bound molecules.

Based on these observations, bead polymers were created that are suitable for HIC chromatographic purposes. The surface of the beads was modified by hydrophobic alkyl or aryl groups. Such media include the polysaccharide-based Butyl-Sepharose, Octyl-Sepharose and Phenyl-Sepharose materials that are derived from polymers that had proven to be suitable for chromatographic separation of proteins.

Here we draw attention to the fact that the so-called reverse-phase chromatography (RPC, see below) is in principle very similar to HIC chromatography. In both cases, the separation is based on the strength of interaction forming between hydrophobic surfaces. The main difference is that, in the case of media used in HIC chromatography, the concentration of the hydrophobic ligand bound to the solid matrix is 10-20 μmol/mL of column volume, whereas in RPC, the hydrophobic ligand concentration on the matrix surface is several 100 μmol/mL. Therefore, in the case of reverse-phase chromatography, the binding between the adsorbent and the molecules to be separated is very strong. Thus, for RPC elution, it is necessary to use solvents that are less polar than water (methanol, acetonitrile, etc.). Therefore, the isolation of native proteins is not always possible with RPC—the proteins can denature on the column as the amino acids forming

their hydrophobic core can also strongly bind to the hydrophobic matrix. However, due to its high resolution, the RPC technique is very advantageous for the qualitative analysis of complex protein or peptide mixtures. During HIC, we can work in an aqueous, non-denaturing medium throughout the chromatographic procedure, from loading to elution.

Factors affecting HIC chromatography

1. *The type and density of the hydrophobic ligand on the matrix surface*

The immobilised hydrophobic ligands will primarily determine the selectivity of the HIC adsorbent. Alkyl chains show purely hydrophobic interactions. In the case of aromatic ligands, beyond the hydrophobic effect, specific interactions between aromatic groups are also present. The choice between alkyl-containing versus aromatic-liganded matrices is mainly empirical and can be made based on preliminary binding experiments.

In the case of alkyl matrices, the protein-binding capacity of the HIC adsorbent will increase with the length of alkyl chains. In addition to alkyl chain length, the binding capacity will, of course, also depend on the concentration of these chains on the matrix surface. In the case of HIC, the optimal hydrophobic ligand concentration is around 20 μmol/mL of medium. The behaviour of the medium is, to some extent, also affected by the hydrophobicity of the polymer matrix. For instance, even in the case of the same hydrophobic ligands, the selectivity will differ in the case of agarose and synthetic polymer matrices.

2. *The quality of the salt used and its concentration*

The effect of salts used in HIC is similar to their salting-out efficiency. Both effects are associated with the effect of the given salt on the surface tension of water, which can be arranged in the following order:

$LiCl < NaCl < Na_2HPO_3 < (NH_4)_2SO_4 < Na_2SO_4$

Most often, $(NH_4)_2SO_4$ is used, as the salting-out efficiency of this salt is about four times that of NaCl.

The initial salt concentration should be selected so that the protein to be isolated should bind to the column with sufficent efficiency. The determination of the salt concentration is empirical—a good approach may be testing the use of a 1-M $(NH_4)_2SO_4$ solution.

We must also ensure that, at the applied initial salt concentration, protein precipitation does not occur due to salting-out.

3. *The effect of pH*

The pH of the medium changes the charge of ionisable groups of protein molecules. This effect will obviously affect the separation based on hydrophobic interactions.

It is found that, in general, the retention of protein samples changes dramatically below pH 5 and above pH 8.5. Thus, the states close to the zwitterionic state are advantageous for HIC. Therefore, similarly to the salt concentration, the pH of the solvent should also be optimised.

4. *The effect of temperature*

The hydrophobic interaction is temperature dependent. Increasing temperature will increase the strength of the interaction in most cases, but this phenomenon is complex—the opposite effect has also been observed. In practical terms, it must be taken into account that the outcome of a method developed at room temperature may not be reproducible in the cold room (at 4°C).

5. *The effect of additives*

Water-miscible organic solvents (alcohols, acetonitrile, dimethyl formamide, etc.) or added detergents can reduce the binding of proteins to be separated, even when present at low concentrations. These compounds "compete" with the protein for the adsorption sites on the matrix surface.

Therefore, when added in low concentrations, these substances can increase the efficiency of the elution. They can be thus applied e.g. when the decrease in salt concentration alone does not lead to satisfactory results. One must ensure, however, that the additives used do not denature the protein to be isolated. Additives can be used even at high concentrations for the purification or regeneration of HIC columns.

Affinity chromatography

By affinity chromatography, high-selectivity separation of biomolecules can be achieved through their specific interactions. This separation technique is special because it is based on the biological function or the unique chemical structure of a given biomolecule. During affininty chromatography, the interacting partner of the biomolecule is immobilised on a chromatographic resin. This ligand, fixed to the stationary phase, reversibly binds the desired biomolecule present in the multi-component mobile phase. The materials can be eluted from the column by changing the composition of the mobile phase.

The technique provides high selectivity, high resolution and generally high capacity for the desired protein. The degree of purification can be thousands of times, and the achieved yield can also be usually very good.

Affinity chromatography, as already mentioned, is unique in the sense that it is based on the specific biological function of the biomolecule of interest. This feature also makes affinity

chromatography suitable for the selective separation of active biomolecules, and their isolation from the inactive or denatured forms.

Another significant advantage of the method is that, in many cases, it allows for single-step isolation of the desired biomolecule. However, it is required that the sample to be separated should be a clear solution free of large particles. It is often advisable to prepare the sample for affinity chromatography via an initial partial separation. For instance, in the case of affinity isolation of very scarce components of the blood serum, it is advisable to perform an initial separation to eliminate serum albumin (which makes up more than 50 % of the serum protein content).

Affinity chromatographic purification is frequently of great importance in the case of recombinant proteins. Recombinant proteins are often produced in a way that they contain a fused "label" at their N- or C-terminus, resulting from genetic engineering. This way, if the label endows the protein to enter into affinity binding, the recombinant protein can be simply "fished out" of the cell extract via affinity chromatography

One of the most widely used of such labels fused to protein termini is the oligo-histidine tag (His-tag), which binds reversibly to metal chelates (e.g. Ni chelate immobilised on the stationary phase). Another frequently applied tag is glutathione S-transferase (GST), a fusion protein that can be used to isolate the protein of interest using a glutathione-conjugate matrix. These specific affinity matrices are commercially available as pre-packed columns.

In other cases, the specific ligand is to be linked by the user to the chromatographic matrix. Various activated reactive chromatographic matrices are available for this purpose.

Some of the commonly used interactions in affinity chromatography are listed in Table 6.III.

Enzyme	Substrate analogue or inhibitor
Antibody	Antigen (virus, cells)
Nucleic acid	Complementary nucleic acid
Nucleic acid	Histone or other nucleic acid binding protein
Hormone	Hormone receptor
Glutathione	Glutathione S-transferase (GST) fusion protein
Metal chelate	His-tag fusion protein

Table 6.III. Commonly used interactions in affinity chromatography

The phases of affinity chromatography are shown in Figure 6.8.

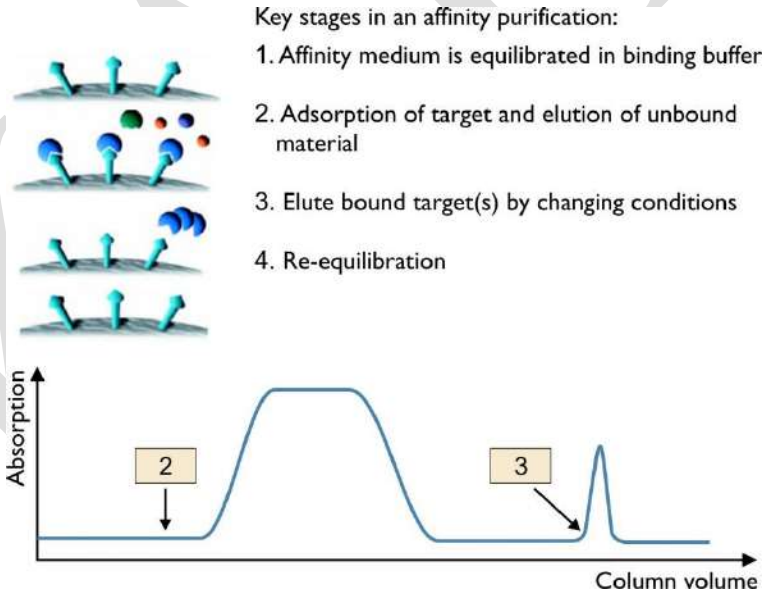


Figure 6.8. The phases of affinity chromatography. In the upper left side of the figure, an illustrative representation of affinity chromatography is shown. Of the molecules present in the

sample, only the ones having a "matching" binding site can bind to the matrix-conjugated specific ligand molecules. Other molecules can be readily washed off. By changing the composition of the mobile phase, the molecules of interest can be isolated in a pure form. The lower part of the figure shows a typical affinity chromatographic elution profile.

1. *Sample preparation*

The sample must be a clear solution free from solid particles. This can be achieved by centrifugation or filtration. Protein solutions should be centrifuged at at least 10000 g. Cell lysates should be centrifuged at 40-50000 g. A 0.45-µm pore size filter can be used for filtration. (Such preparation of samples is also necessary in FPLC and HPLC methods.)

One must also consider how the solubility and stability of the sample or the desired protein can be influenced by the pH, the salt concentration, or the presence of any organic solvent. The factors affecting the interactions between the desired target protein and the matrix-bound ligand (pH, salt concentration, temperature) should also be determined. The composition of the initial binding buffer must be adjusted accordingly.

Sample components interfering with the target protein and/or the ligand (e.g. metabolites in cell lysates) should be removed before loading onto the column.

2. *Equilibration with a buffer facilitating the specific interaction*

The chromatographic column is washed with 3-4 column volumes of the starting (binding) buffer. The sample must also be equilibrated with this starting binding buffer (if necessary, via solvent exchange or dialysis).

3. *Binding of the molecule of interest and wash-out of the unbound material*

During sample loading, consider the strength of the interaction. In case of high-affinity samples, a high flow rate may be applied. In case of a weak interaction and/or a slow equilibration process, reduce the rate of sample loading. After sample application, the column should be further washed with binding buffer until all unbound components are removed.

4. *Elution of the molecules of interest by changing the composition of the mobile phase*

Elution via pH and/or ionic strength changes: One possible and simple means of elution is achieved through decreasing the interaction strength between the ligand and the target protein. Changes in the pH will change the ionisation state of charged groups of the ligand and/or the target protein, thereby changing the strength of the interaction. Similarly, increasing the ionic strength (usually by raising the NaCl concentration) will generally reduce the interaction strength. In either case, the solubility and stability of the target protein should be considered.

Competitive elution: For competitive elution, materials are applied that react with the target protein or the ligand, competing for the pre-existing interaction. For instance, His-Tag fusion proteins can be readily displaced from the metal chelate matrix by imidazole buffer (Figure 6.9). GST-tagged target proteins will detach from their column-conjugated glutathione ligand upon mixing excess glutathione into the elution buffer.

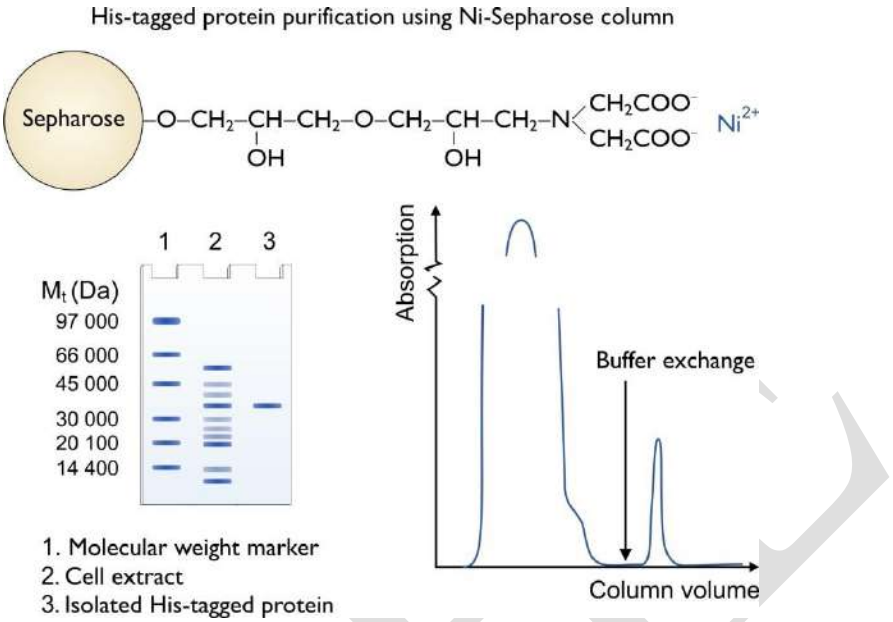


Figure 6.9. Isolation of a His-Tag fusion protein. The cell extract containing the His-Tag fusion protein is purified using a Ni^{2+} chelate column. The upper panel shows the structure of the immobilised Ni^{2+}chelate ligand. The sample is loaded onto the column in a neutral buffer. The fusion protein containing the His-Tag binds the Ni^{2+} chelate ligand. After washing off other proteins, competitive elution with imidazole buffer can be applied to isolate the pure fusion protein (lower right panel). Lower left panel shows the purity of fractions assessed by SDS gel electrophoresis, discussed in detail in Chapter 7.

In all cases, the flow rate of the buffer should be reduced during elution, thereby avoiding excessive dilution of the target protein.

In cases when the target-ligand interaction is very strong, the above elution methods may turn out insufficient for eluting the protein of interest. In these cases, chaotropic agents (urea, guanidine) can be used to wash off the target protein from the column. This naturally will

involve the denaturation of the protein, which can then be renatured in some (lucky) cases under suitable conditions—in the case uf urea or guanidine there is a good chance for this.

5. *Regeneration*

After successful completion of the elution, the column can be washed with several column volumes of binding buffer, and it can then be reused. For long-term storage, one must ensure that the column is not exposed to bacterial or fungal infection. The toxic compound sodium azide can be used to prevent such infections.

High performance (high pressure) liquid chromatography (HPLC)

In the discussion of gel filtration and ion exchange chromatography, we saw that the efficiency of chromatography increases with reducing the particle size of the gel matrix and with enhancing its size homogeneity. The efficiency reaches a new level of quality when a matrix grain size of 3-10 μm is applied. Liquid chromatography performed using such resins is called high performance liquid chromatography, abbreviated as HPLC.

However, at such particle sizes, the sufficient flow of the mobile phase (eluent) can be achieved only by applying a high pressure of around 10 MPa by using special precision pumps (Figure 6.10). HPLC thus also stands for high pressure LC—according to many researchers, the abbreviation also refers to the high price of the specialised equipment.

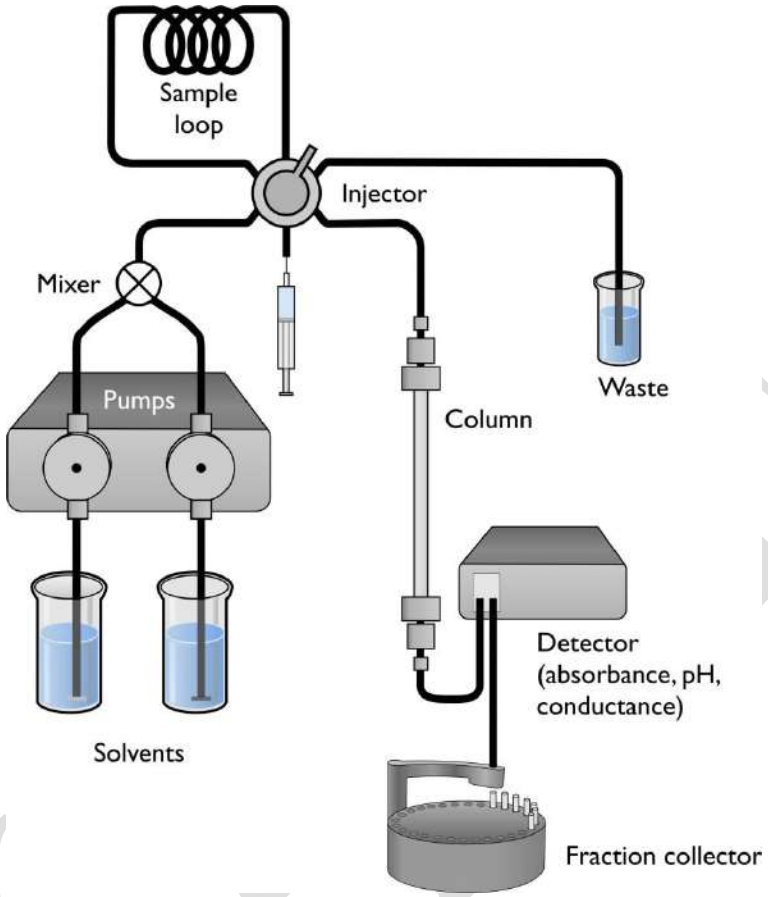


Figure 6.10. Schematic layout diagram of the HPLC equipment. The system consists of pumps that ensure the high-pressure delivery of a two-component mobile phase, a mixing unit, a sample injector, a chromatographic column and a detector.

Due to the application of high pressure, the primary requirement regarding HPLC columns is that they should be incompressible. Silica is predominantly used for this purpose. Under appropriate conditions, silica can be used to create homogeneous column media of sufficient strength and with a well-controlled particle size. For the hydrophilic silica stationary phase, only hydrophobic mobile phases can be applied. Therefore, HPLC was primarily suitable for the separation of

hydrophobic organic solvent-soluble materials. Later, the chemical modification of the silica surface made possible the creation of hydrophobic silica gels. In this case, the hydrophilic-hydrophobic relation of the stationary and mobile phases became reversed, hence the term reverse-phase chromatography (RPC). Reverse-phase chromatography opened up the possibility of the separation of water-soluble substances, including the majority of molecules of biological origin. Large pore-size gels also allowed the separation of macromolecules. The hydrophobic surface is formed by long alkyl chains linked to the silica. These include octadecyl, octyl, butyl (labelled as C18, C8, C4) and also phenyl groups (see above at the description of HIC chromatography). Furthermore, gels containing charged groups can be used for ion exchange.

If the composition of the mobile phase is constant during chromatography, we speak of isocratic elution. Gradient elution is achieved via applying a linear or non-linear concentration gradient. In many cases, isocratic and gradient sections are combined in the elution profile. The gradient is most often created by using microprocessor-controlled, variable-speed pumps (two at least). In this case, the mobile phase components are mixed at the high-pressure side of the pumps.

With the help of precision valves, the gradient can also be made by mixing the buffers at low pressure. This way, the eluent can be transmitted onto the column by using only one pump. If unmodified silica is used, the mobile phase can be created by mixing organic solvents of different hydrophobicity.

In reverse-phase chromatography, a dilute aqueous solution (e.g. 0.1 % trifluoroacetic acid, few mM phosphate buffer) and a water-miscible organic solvent (e.g. acetonitrile, methanol, propanol) can be mixed to prepare a mobile phase for isocratic or gradient applications.

In the biochemical practice, light absorption and fluorescence detectors are most commonly used. However, refractive index, conductivity, optical rotation and electrochemical detectors are also frequently applied. For photometric detection, the eluent must be optically clear and must

not absorb light in the applied wavelength range. The use of extremely pure solvents is generally required anyway in order to avoid both the contamination of the column media and the appearance of unexpected materials during chromatography.

A very important development is the appearance of mass spectrometric (MS) detectors. These are used to determine the mass of the separated components, which is a decisive parameter of a given substance. Actually, MS detectors do not determine the mass directly, but determine the mass/charge (m/z) ratio. However, the mass can be easily derived for single- and multiple-charged values. In the past two decades, ionisation techniques have been introduced in MS measurement systems. Ionisation of high molecular weight biopolymers (such as proteins) can be efficiently achieved via ESI (electrospray ionisation) or MALDI (matrix-assisted laser desorption).

As a result, chromatographic methods play an increasingly important role in protein research. One- or multi-dimensional high performance liquid chromatography combined with in-line mass spectrometry allows the targeted identification of all proteins expressed in a cell at a given time, i.e. the “proteome”; or a specific subset of these proteins. This is significant because, unlike the genome, the proteome is not constant: it may vary by tissue or cell type and may also depend on the physiological state or developmental stage of the individual. The detection of post-translational modifications is also of importance. For instance, the specific detection of phosphorylated proteins in the proteome aids the understanding of various biological regulatory processes.

All components of the HPLC equipment must be pressure-resistant and chemically resistant. Thus, stainless steel and, in more special cases, resistant titanium alloys are applied. Moving parts, including pistons and valves, are also made of highly mechanoresistant materials (special ceramic, glass, industrial ruby etc.). Recently, the investigation of samples sensitive to trace

metal contamination (e.g. some enzymes) has necessitated the application of particularly pressure-resistant plastic parts.

The FPLC (fast protein liquid chromatography) system was developed for the separation of native proteins. FPLC differs from the above-described HPLC chromatography in that the column resins are specially-treated dextran-based or synthetic polymeric materials (Superdex, Superose, Sephacryl etc.), which are, due to their hydrophilic character and high porosity, particularly useful for the separation of biopolymers. These particles have a slightly larger size and lower pressure resistance than the silica-based HPLC media. However, FPLC media are suitable for ensuring sufficient fluid flow at pressures in the range of 0.5-1 MPa, and the efficiency of FPLC also meets most requirements of protein purification applications. FPLC columns are made of pressure-resistant borosilicate glass. Exposed metal parts are also avoided in the pumps and the piping systems.

Both HPLC and FPLC provide better efficiency and sensitivity as well as lower time requirement compared to conventional chromatographic applications.

Some practical considerations

During sample preparation, one must take it seriously that the solution should be clear and free of dust or other particles. Otherwise, the apparatus may become blocked and the chromatographic column may become contaminated. The sample can be centrifuged at 20-40000 g and/or filtered through a 0.45-µm filter. The sample is preferably dissolved in the starting mobile phase. In must be ensured that the sample is fully dissolved. The volume of the sample depends on the diameter of the column used (see below).

1. *Selection of the stationary phase (column medium)*

In the case of nonpolar, water-insoluble materials, unmodified silica, or possibly diol media should be used. In the case of amino group-containing polar, water-soluble substances, reverse-phase C18, C8 or C4-modified silica media can be applied. Larger hydrophobic peptides and proteins bind too strongly to the C18 solid phase. In this case it is advisable to use C4 or C8 matrices.

For analytical purposes, smaller particle size matrices (around or less than 3 µm) should be used. This will increase the efficiency of separation, but has the disadvantage of increasing the pressure in the system. For semi-preparative and preparative purposes, 5-10-µm particle sizes are suitable. One must also consider the porosity of matrix particles. In the case of low molecular weight metabolites, amino acids and small peptides, the commonly-used 100-Å pore-size media are suitable. In the case of macromolecules, high porosity (wide pore), 300-Å pore-size media are to be chosen (1 Å = 0.1 nm).

2. *Selection of the mobile phase*

The most important characteristics of the mobile phase include purity, viscosity, UV transparency and miscibility with other solvents. HPLC techniques require special-purity ("HPLC grade") solvents, including water specially purified for this purpose. The selection of the correct column size is important in terms of the economic use of materials. High-viscosity solvents should be generally avoided as their use increases the system pressure.

In terms of detectability, it is important that the mobile phase should be optically pure. Given that the most commonly used chromatographic detectors operate in the UV range, the UV absorption of the mobile phase should be considered. The most commonly used HPLC reagents (e.g. acetonitrile) are available in various qualities. Highest-quality reagents enable photometric detection even at a wavelength of 200 nm. In the range of 280-340 nm, it is sufficient to use less expensive grades of acetonitrile.

In reverse-phase chromatography, the initial mobile phase is a dilute aqueous solution. The organic component used for the reduction of solvent polarity can be e.g. methanol, ethanol, propanol or acetonitrile. The initial aqueous solution can be, for instance, 0.1 % formic acid, acetic acid or few mM phosphate buffer. For ion pair formation, the commonly used agent is 0.1 % trifluoroacetic acid. Ion pair formation enhances the retention of highly charged molecules due to charge compensation. However, if chromatography is coupled to on-line MS measurements, trifluoroacetic acid should be avoided as reduces the ionisability of molecules. In this case, the use of dilute formic acid or ammonium formate is recommended.

It must be taken into consideration that the mixing of the solvents during gradient elution will result in changes in the solubility of air in the mobile phase. This effect may result in air bubble formation when the solution leaves the column and the pressure is reduced. This will severely interfere with photometric detection. Mobile phase components must therefore be degassed prior to usage. Some chromatographic instruments contain a so-called degasser unit, which applies a slight vacuum to keep the concentration of dissolved air continuously low. If no degasser unit is attached to the chromatographic instrument, mobile phase components should either be degassed by vacuum, or the very poorly water-soluble helium gas should be bubbled through the solutions to expel the dissolved air.

Before use, filtering of the mobile phase through a fine (0.45-µm) filter is recommended in order to get rid of fine dust contamination.

3. *Selection of column size*

With regard to column size selection, it is crucially important whether the chromatographic column will be used for analytical or preparative purposes. For analytical purposes, microbore or minibore columns with an internal diameter of 1-3 mm should be used. In the case of microbore/minibore columns, the applicable sample volume is 5-25 µL, which may contain 0.01-

0.1 mg of material. In such applications, the amount of solvents used can be reduced significantly, which is advantageous for both financial and environmental reasons. The use of such thin columns is also recommended when only a small amount of sample is available. If a small sample is applied to a large column, the sample may "disappear".

The most commonly used type of column is the standard column with a 4.6-mm diameter. These columns can be used for both analytical and semi-preparative purposes. The volume of standard columns is 10-50 µL in the case of analytical uses; whereas for semi-preparative work, column volumes up to 1000 µL can be used. The amount of material that can be applied is in the range of 0.1-2 mg.

Columns with a diameter of 10-20 mm can be used for preparative purposes. In such columns, the amount of material that can be applied may be 20-200 mg, and the sample volume can reach 5-10 mL.

Columns are expensive. It is advisable to insert a protective, few-millimetre-long "pre-column" before the main column, with the two columns having identical column media. The pre-column can be replaced at low cost if blockage occurs.

Principles of electrophoresis

Electrophoresis is a method used to separate charged particles from one another based on differences in their migration speed. In the course of electrophoresis, two electrodes (typically made of an inert metal, e.g. platinum) are immersed in two separate buffer chambers. The two chambers are not fully isolated from each other. Charged particles can migrate from one chamber to the other (Figure 7.1). By using an electric power supply, electric potential (E) is generated between the two electrodes. Due to the electric potential, electrons move by a wire between the two electrodes. More specifically, electrons move from the anode to the cathode. Hence, the

anode will be positively charged, while the cathode will be negatively charged. As mentioned above, the two electrodes are immersed in two buffer chambers. Electrons driven to the cathode will leave the electrode and participate in a reduction reaction with water generating hydrogen gas and hydroxide ions. In the meantime, at the positive anode an oxidation reaction occurs. Electrons released from water molecules enter the electrode generating oxygen gas and free protons (which immediately form hydroxonium ions with water molecules). The amount of electrons leaving the cathode equals the amount of electrons entering the cathode. As mentioned, the two buffer chambers are interconnected such that charged particles can migrate between the two chambers. These particles are driven by the electric potential between the two electrodes. Negatively charged ions, called anions, move towards the positively charged anode, while positively charged ions, called cations, move towards the positively charged cathode.

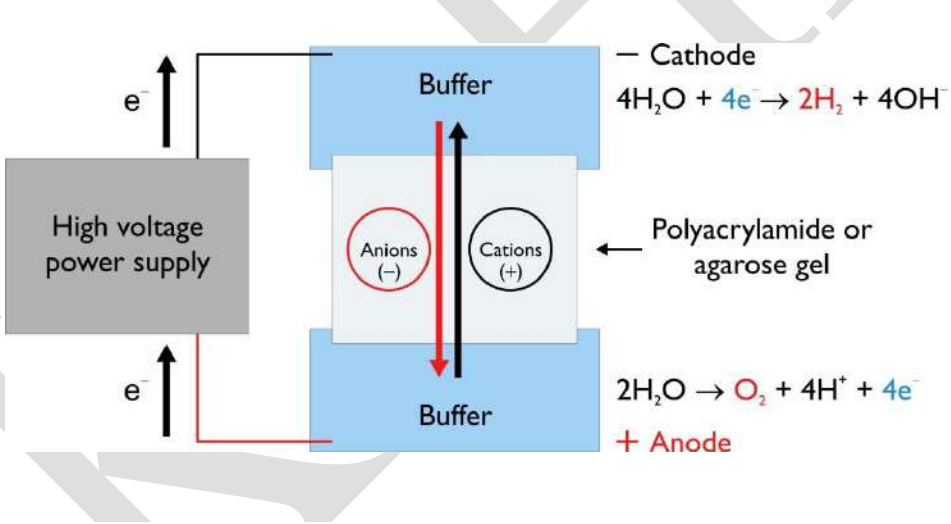


Figure 7.1. The principle of electrophoresis. In the course of electrophoresis, two electrodes are immersed in two separate buffer chambers. The two chambers are connected such that charged particles can migrate from one chamber to the other. By using a power supply, electric potential difference is generated between the two electrodes. As a result, electrons flow from one of the electrodes, the anode, towards the other electrode, the cathode. Electrons from the cathode are taken up by water molecules of the buffer, resulting in a chemical reaction which generates

hydrogen gas and hydroxide ions. In the other buffer chamber, water molecules transfer electrons to the anode an in another chemical reaction that generates oxygen gas and protons. (Protons are immediately taken up by water molecules to form hydroxonium ions.) As charged particles can migrate between the two chambers due to the electric potential difference, positive ions (cations) move towards the negatively charged cathode while negatively charged ions (anions) move towards the positively charged anode.

Different ions migrate at different speeds dictated by their sizes and by the number of charges they carry. As a result, different ions can be separated from each other by electrophoresis. It is very important to understand the basic physics describing the dependence of the speed of the ion as a function of the number of charges on the ion, the size of the ion, the magnitude of the applied electric field and the nature of the medium in which the ions migrate. By understanding these basic relationships, the principles of the many different specific electrophoresis methods become comprehensible. The fundamental principle of electrophoresis is illustrated in Figure 7.1.

The mathematical description of the force during electrophoresis is simple. An electric force F_e is exerted on the charged particle. The magnitude of the electric force equals the product of the charge q of the particle and the electric field E generated between the two electrodes:

$$F_e = q \times E \qquad (7.1)$$

Dimensions of the electric field E are defined either in newton/coulomb or volt/cm units. During electrophoresis, the magnitude of the electric field E is defined in volt/cm units. It can be easily calculated using the value of the voltage (volt) set by the electric power supply and the distance of the two electrodes (cm).

As soon as the electric field is applied and the charged particles are accelerated by the electric force, a drag force (F_d) called friction will also be immediately exerted on the particles by the

medium. This force, whose direction is opposite to the direction of particle movement, is proportional to the velocity of the particle. At the typically very low speed of particle migration during electrophoresis, the force $F_d$is a linear function of the velocity (v) of the particle, as described by Equation 7.2:

$$F_d = f \times v \qquad (7.2)$$

The ratio of the force and the velocity is defined as the frictional coefficient (f). The value of f is a function of the size and shape of the particle and the viscosity of the medium. The larger the particle and the more obstructing the medium, the higher the value of f.

When electrophoresis is started, particles accelerate instantaneously to a velocity (v) at which the magnitude of the drag force equals the magnitude of the (opposite) accelerating electric force:

$$q \times E = f \times v \qquad (7.3)$$

Once the magnitude of the two opposing forces becomes equal, the resultant force becomes zero. Therefore, each particle will move at a constant velocity characteristic of the given particle at the given accelerating potential and medium. (A similar phenomenon is described in Chapter 5 for centrifugation. There, the accelerating force is unrelated—being proportional to the mass instead of the charge of the particle—but the frictional force and the phenomenon of two opposing forces leading to a characteristic particle velocity is analogous.) A useful parameter, the electrophoretic mobility (μ) of the particle, defines the velocity of the particle in a given medium when one unit of electric field is applied. (This parameter is analogous to the Svedberg units defined for centrifugation.) Electrophoretic mobility is a linear function of the charge of the particle and it is a reciprocal function of the frictional coefficient (which depends on both the size of the particle and the nature of the medium):

$$\mu = \frac{v}{E} = \frac{q}{f} \qquad (7.4)$$

Particles having different electrophoretic mobility, i.e. those that migrate at different speeds in the same medium and electric field, can be separated by electrophoresis.

In biochemical and molecular biological studies, the most typical charged molecules that are analysed and separated by electrophoresis are proteins and nucleic acids. Electrophoresis is always performed by using a special medium, most often a gel. The corresponding methods are therefore denoted as gel electrophoresis.

About gel electrophoresis

The principle of electrophoresis does not assume any particular requirements about the nature of the liquid medium in which the ions are separated. Yet, in the great majority of currently used electrophoretic applications, the medium has a three-dimensional network structure, i.e. the medium is a gel.

At the very beginning when the technique was invented, electrophoresis was performed without using a gel matrix. Charged particles were migrated in a homogeneous liquid phase. However, it soon became apparent that the use of a liquid medium raises at least three major difficulties.

One is that the separation of different ions in an ordinary liquid is rather inefficient. It is so because a significant factor of an effective separation should be a marked size-dependent drag force exerted by the medium on the particles. Although even ordinary liquids do interfere with the migration speed of the particles in a size-dependent manner, this size dependence is quite moderate.

The other big problem has a simple technical origin. In liquid phase, even very small levels of temperature inhomogeneity trigger convection that significantly compromises the resolution of the separation. Finally, in an ordinary liquid phase, the extent of diffusion is high and, in the typical timeframe of the generally slow electrophoresis experiments, diffusion decreases the efficiency of the separation.

All three problems had been dealt with when, instead of ordinary liquids, gels were introduced as a medium.

The gel provides a three-dimensional molecular network structure to the liquid medium. It prevents convections and also lowers the rate of diffusion. Moreover, perhaps the most dramatic advantageous effect of the gel is that it acts as a molecular sieve: it interferes only slightly with the movement of small molecules, but drastically slows down the motion of large molecules. All gels are characterised by an average pore size. Molecules much smaller than the mean pore diameter are almost unaffected by the presence of the gel, while those that are larger than the pores practically do not migrate in the gel. When ions with sizes in the range of the pore size are migrated through the gel by electrophoresis, the gel exerts a pronounced size-dependent dragging force on them.

As a consequence, the pore size distribution of the gel determines an operational size range in which different ions can be separated. Looking at this from the opposite point of view, each separation problem defines an optimal pore size to be applied.

The gel has to fulfil several general criteria to be applicable for biochemical electrophoresis. It needs to be hydrophilic, chemically stable (should not participate in chemical reactions during electrophoresis), neutral (free of electric charges, otherwise it would act as an ion exchanger) and mechanically resistant (should not be too elastic or too rigid as such gels would be difficult to handle). Furthermore, as the separated ions (mostly proteins and nucleic acids) need to be

visualised in the gel by some kind of staining procedure, the gel should be transparent, and should not strongly bind the dyes used for staining. Finally, and very importantly, the experimenter should be able to adjust the pore size during the preparation of the gel.

The size range of molecules (ions) studied in molecular biology is extremely broad. No single gel-forming compound is known that could cover the entire corresponding range of applicable pore sizes. Two compounds are dominantly used for gel electrophoresis: polyacrylamide and agarose. Polyacrylamide gels typically provide much smaller pores than do agarose gels. The polyacrylamide gel is formed by the radical polymerisation of acrylamide monomers. This process alone would lead to very long polymer chains instead of a three-dimensional gel. The three-dimensional network is brought about by the incorporation of *N*,*N*′-methylenebisacrylamide into the polymerising chains, which results in crosslinks between the long chains. The polyacrylamide gel is held together by covalent bonds. The pore size of polyacrylamide gels can be adjusted via the concentration of the acrylamide monomer and the ratio of the crosslinking agent, *N*,*N*′-methylenebisacrylamide. The pore size of polyacrylamide gels corresponds to a relatively low value (compared to that of agarose gels). Polyacrylamide gels are used typically for the electrophoresis of proteins and relatively small nucleic acids.

In comparison, the agarose gel is formed via non-covalent interactions between long polysaccharide chains. The pore size of agarose gels is much larger than that of acrylamide gels. Accordingly, agarose gels are used typically for the electrophoresis of large nucleic acids. The pore size of the agarose gel can be controlled via the concentration of the agarose solution. As the interaction between agarose molecules is non-covalent, the gel is formed by a physical (in contrast to a chemical) process. A suspension of agarose is heated up until the system reaches a sol state and then it is left to cool down to room temperature to reach the gel state. The following sections review the various polyacrylamide- and agarose-based electrophoresis methods.

Polyacrylamide gel electrophoresis (PAGE)

7.3.1. About the PAGE method in general

As mentioned previously, polyacrylamide gels can be used for the separation and analysis of proteins and relatively small nucleic acid molecules. For example, when it was first invented, Sanger's DNA sequencing method (see in details in Chapter 10) applied PAGE to separate linear single-stranded DNA molecules based on their length. The resolution of the PAGE method is so high that, in the size range of about 10-1000 nucleotide units, it is capable of separating DNA molecules that differ in length only by a single monomer unit. In the case of single-stranded DNA, individual molecules are separated solely based on their length. This is due to the fact that, in the case of DNA (or RNA), the number of negative charges is a simple linear function of the number of monomer units (i.e. the length of the molecule). In other words, the specific charge (number of charges per particle mass) is invariant, i.e. it is the same for all DNA molecules. It is so because each monomer unit has one phosphate moiety that carries the negative charge. When an appropriate denaturing agent, such as urea, is added to the DNA sample and the gel is heated, the shape of the varying-length linear DNA molecules becomes identical. As a consequence, denatured molecules will be separated exclusively based on their size. (We will see the same principle at the SDS-PAGE method that separates denatured proteins almost exclusively based on their size (molecular weight)). There are several PAGE methods (SDS-PAGE, isoelectric focusing, 2D PAGE) that can be applied mostly for the separation of proteins based on distinct molecular properties.

At a given pH, different proteins carry different amounts of electric charge. Moreover, different proteins have different shapes and sizes, too. Consequently, during electrophoresis, proteins are separated by a complex combination of their charge, shape and size. PAGE separation of proteins provides high resolution. However, as three independent molecular properties simultaneously influence electrophoretic mobility, it will provide limited room for precise

interpretation. For example, when two proteins are compared, it remains hidden what makes one of them migrate faster: a larger number of electric charges, a smaller size, or a more spherical shape. Nevertheless, even the simplest PAGE method, which will be referred to as native PAGE, provides many particular advantages (see below).

In order to increase the analytical applicability of the PAGE technology, several variations of the method have been established to separate proteins based on a single molecular property. As we will see, SDS-PAGE separates proteins based primarily on molecular weight, while isoelectric focusing separates proteins exclusively based on isoelectric point.

In the presence of suitable initiator and catalyst compounds, acrylamide can readily polymerise in a radical process. (Acrylamide is harmful by inhalation or skin contact, and thus it should be handled with care.) This reaction would lead to very long polyacrylamide chains, yielding a highly viscous liquid instead of a gel. As already mentioned, these long chains need to be cross-linked to form a three-dimensional network. This is achieved by mixing *N*,*N*′-methylenebisacrylamide into the acrylamide solution. In essence, *N*,*N*′-methylenebisacrylamide is composed of two acrylamide molecules covalently interconnected via a methylene moiety. When, during the polymerisation reaction, the acrylamide groups of *N*,*N*′-methylenebisacrylamide molecules become incorporated in the long polyacrylamide chains, cross-links are formed between the polyacrylamide chains leading to a gel (Figure 7.2). In the course of electrophoresis, ions (proteins or nucleic acids) are separated in this gel.

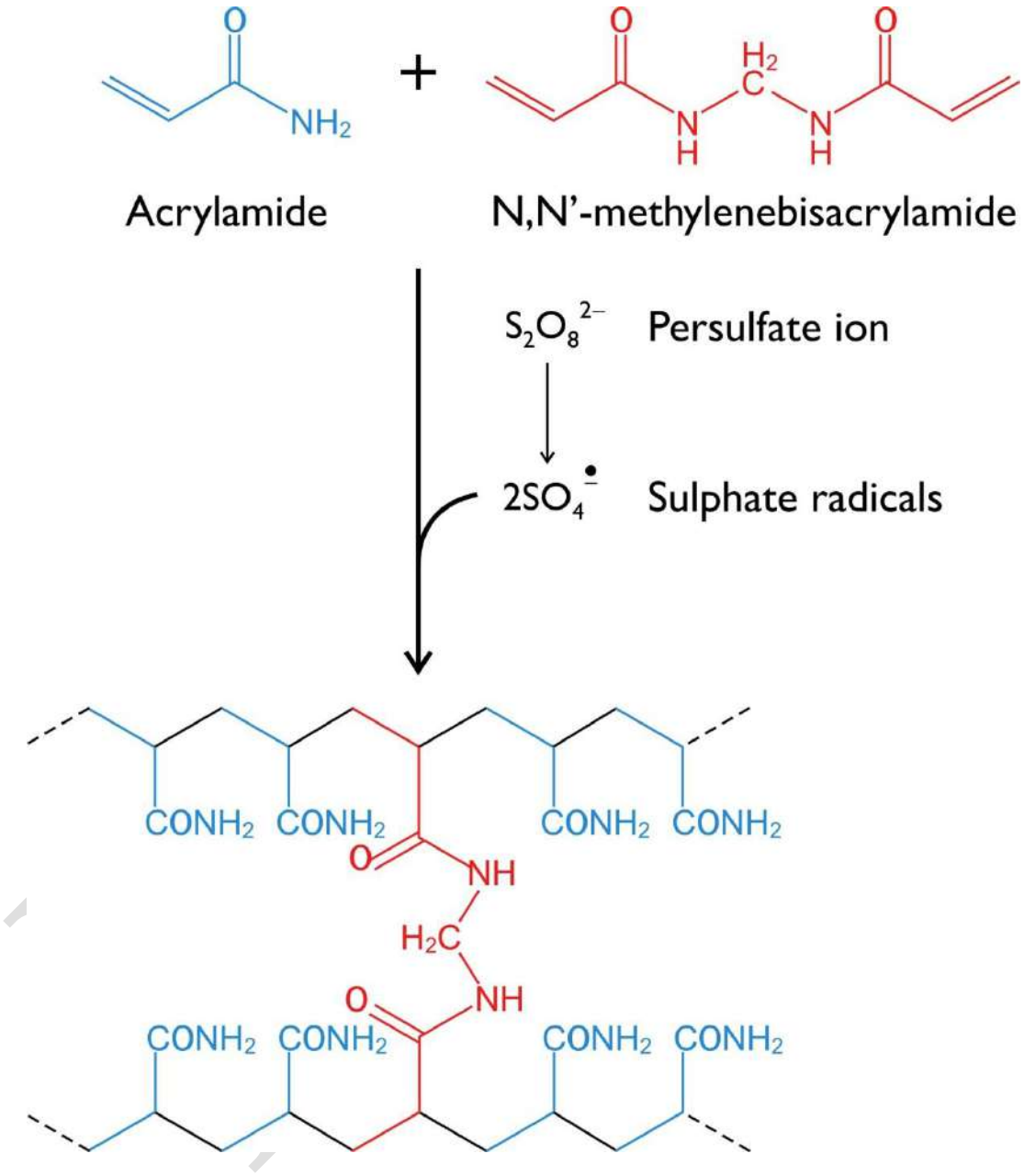


Figure 7.2. Molecular structure of the polyacrylamide gel. The three-dimensional molecular network comes into being by a radical polymerisation of acrylamide monomers and cross-linking *N*,*N'*-methylenebisacrylamide components.

Without any modification, polyacrylamide electrophoresis separates macromolecular ions based on a combination of charge, size and shape. Size (and shape) separation is due to the molecular sieving property of the gel. The size range in which molecules can be separated is dictated by the average pore size of the gel. In the case of polyacrylamide gels, this can be controlled through the concentration of the acrylamide monomer and the proportion of the cross-linking *N*,*N*′-methylenebisacrylamide. The acrylamide concentration can be set in the range of about 4-20 % as this is the range in which the mechanical properties of the gel are appropriate. Below this range the gel will be too soft and it will not keep its shape, while above this range it will be too rigid and prone to break. The optimal proportion of the *N*,*N*′-methylenebisacrylamide component is 1-3 % relative to the acrylamide component. The polyacrylamide gel possesses all advantageous properties necessary for a good electrophoresis medium, i.e. it is hydrophilic, free of electric charges and chemically stable. A further very important property of the polyacrylamide gel is that it does not participate in any non-specific or specific binding interaction with proteins. Furthermore, the polyacrylamide gel does not interfere with common protein staining reactions.

When electrophoresis is performed under native (non-denaturing) conditions, such as near neutral pH and ambient or lower temperature, many enzymes retain their native conformation and, in turn, their enzymatic activity. This way, many enzymes can be separated and specifically detected in the gel after electrophoretic separation.

In the course of creating the gel, a buffer with a properly chosen pH is mixed into the acrylamide/*N*,*N*′-methylenebisacrylamide solution. Radical polymerisation is subsequently triggered by suitable catalyst and initiator compounds. The catalyst is usually ammonium persulfate, which spontaneously decomposes in aqueous media, thereby generating free radicals. These free radicals in themselves cannot efficiently cleave the double bonds of the acrylamide molecule, but are able to excite the electrons of the initiator molecules. This leads to the

generation of free radicals, originating from the initiator molecules, that are able to trigger radical polymerisation of acrylamide monomers. The most frequently used initiator is tetramethylethylenediamine (TEMED).

There are two types of gels according to their geometry. In early gel electrophoretic applications, gel tubes were used that allowed only a single sample to be run. Gel slabs were later introduced, allowing for many samples to be run at the same time in the same gel in parallel. Gel slabs became much more common than gel tubes. Gel slabs are created by pouring the gel-forming solution between two parallel glass sheets prior to polymerisation (Figure 7.3). Besides its higher throughput, this gel geometry provides another important advantage over gel tubes: samples are loaded side by side on such slabs and are run in the same gel at the same time. This allows for a more reliable comparison of the samples, facilitating the interpretation of experimental results.

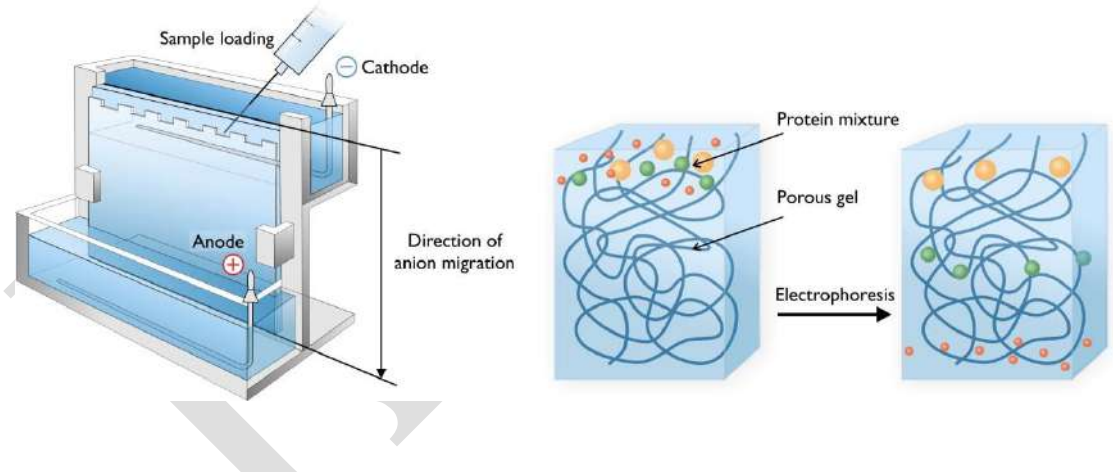


Figure 7.3. Separation of proteins in a polyacrylamide gel. As illustrated in the left panel, several samples can be run in parallel in a slab gel. Ions can move between the two electrodes only through the gel interconnecting the two chambers. The gel acts as a molecular sieve. The larger the molecule, the larger the drag force exerted on it by the gel.

Proper selection of pH and acrylamide concentration is instrumental for successful electrophoresis. For protein electrophoresis, the pH is set usually higher than the pI value of the proteins in the sample. At such a pH, all proteins will be negatively charged and will move towards the anode. The buffer in the medium serves two purposes. One is to set and maintain the proper pH during electrophoresis. The other function of the buffer is to establish the electric current in the medium.

The majority of the electric current is carried by the ions of the buffer. Normally, the protein-ions that are separated by electrophoresis have only a negligible contribution to the current. In other words, proteins have a low ion transport number. However, if the buffer concentration is set too low, the contribution of proteins to carrying the current will increase, and the protein molecules will migrate rapidly. This usually leads to smearing of the bands of migrating proteins. On the other hand, if the buffer concentration is set too high, the mobility of the proteins will be too low. In this case the electrophoresis process would take a very long time. Unnecessary lengthening of the process provides excess time for diffusion, which lowers the resolution of separation.

According to the applied buffer system, gel electrophoretic methods can be classified into two types: continuous and discontinuous. Continuous methods apply the same buffer in the gel and in the two buffer chambers containing the electrodes. The only advantage of this method lies in its simplicity. More complex discontinuous methods were introduced to provide higher resolution. SDS polyacrylamide gel electrophoresis (see later) is usually associated with such a discontinuous system.

The discontinuous system applies two gels of different pore size and three different buffers. One of the gels, the resolving gel, is polymerised at a higher acrylamide concentration. The pore size of this gel is set according to the size range of the proteins to be separated. Another gel, the stacking gel is created on top of the resolving gel. (The gels are mounted in a vertical format.)

The stacking gel is polymerised from a more dilute acrylamide solution to provide larger pores. This pore size does not provide a molecular sieving effect.

As mentioned above, there are three buffers: different ones in each of the two gels and a third one, the so-called 'running buffer' in the buffer chambers containing the electrodes. In the gel buffers, the anion originates from a strong acid; it is usually chloride ion. Dissociation of strong acids does not depend on the pH: these acids always fully dissociate. Consequently, chloride ion is never protonated in the solution: its ionisation state is independent of the pH. On the other hand, the anion component of the running buffer is the conjugate base of a weak acid. Consequently, the ionisation state of this ion depends on the pH of the buffer. Glycinate ion is one of the most frequently used compounds for this purpose. The pH in the running buffer is set to 8.3.

The protein sample is layered on the top of the stacking gel. When an electric field is generated by the power supply, the protein ions and the ions of the running buffer enter the stacking gel. The pH in the stacking gel is set to 6.8. This value is only slightly higher than the pI value of glycine (6.5). At this pH, most glycine molecules are in a neutral zwitterionic state, and only a small portion of the molecules carry a net negative charge. In this state, glycine has a low electrophoretic mobility and a corresponding low transport number. The local sparsity of ions elevates the local electric resistance of the medium. As the electric current must be of the same magnitude at any segments of the electric circuit (there is no macroscopic charge separation), the voltage will increase according to Ohm's law. Due to this effect, the migration speed of the proteins will be relatively high and the protein front will reach the chloride front in the stacking gel. The ion concentration in the chloride front is high and, therefore, here the electric resistance and the voltage are low. This slows down the protein front. This effect results in a very sharp protein front, with the protein molecules being crowded right behind the chloride ion front.

The protein sample will thus enter the resolving gel in a sharp band. The pH in the resolving gel is set to about 8.8. At this pH, almost all glycinate molecules are in the anionic state. Thus, the electric mobility of glycinate increases, and the concentrating effect applied by the stacking gel ends in the resolving gel. Different proteins will be separated in the resolving gel according to their charge, size and shape.

In most electrophoretic methods, a tracking dye is mixed in the sample. Usually, this dye is chosen to have a higher electrophoretic mobility than any of the components of interest (proteins or nucleic acids) in the sample. The function of the tracking dye is to visualise the running front and, in turn, the completeness of the run. The most popular tracking dye is bromophenol blue.

The following sections review the various PAGE methods listed from the simplest to the most complex one.

7.3.2. Native PAGE

Native PAGE is an electrophoresis method to separate native proteins. The conditions are set such that the migrating proteins are kept in their native state. The buffers provide a non-denaturing, native-like milieu, and the electrophoresis is performed at low temperature in order to dissipate heat. Many enzymes retain their native conformation and their enzymatic activities while running in the gel. If certain conditions apply, these enzymes can be highly selectively detected within the gel through a specific ‘staining’ reaction even in the presence of a large excess of ‘contaminating’ proteins. After completion of electrophoresis, the gel is soaked in a solution containing the substrate of the enzyme. As the substrate is usually a small molecule, it quickly diffuses into the gel while the large enzyme molecules do not diffuse out. In an optimal case, the natural product of the enzymatic reaction is a coloured and insoluble compound that precipitates inside the gel and marks the exact location of the enzyme. Of course, most enzymes do not have such natural substrates. However, once the molecular mechanism of catalysis is

revealed, synthetic substrates can be designed that, on the one hand, mimic natural substrates and, on the other hand, lead to colourful insoluble products.

Native PAGE is also a useful method for checking the uniformity of the isolated protein. Even if the purified protein sample contains only a single type of protein, the sample might not be uniform. Some of the molecules might be unfolded or have undergone chemical modifications. Unfolding changes the overall shape of the molecule, while most chemical modifications change the electric charge of native molecules. These alterations can be detected after traditional staining of the purified sample. If no such side products are present, protein molecules will run in a single sharp band. Otherwise, multiple bands or smearing of the band is expected.

In addition, native PAGE can also be used to detect complex formation between proteins. If two (or more) proteins (or proteins and non-proteinous ligands) form a complex, the complex can be detected as an extra band in the gel. This is because in native-like conditions, many non-covalent (subunit-subunit, receptor-ligand, enzyme-inhibitor) interactions are maintained and the complex migrates apparently as a single molecule.

In the course of native PAGE, it is highly important to pay attention to the relationship of the pI values of the proteins or protein complexes and the pH of the gel buffer, as this will determine where individual proteins will migrate in the gel.

7.3.3. SDS-PAGE

SDS-PAGE is an electrophoresis method to separate proteins. However, unlike in the case of native PAGE, here the proteins migrate in their denatured state. As it was mentioned in the general introduction to traditional (native) PAGE, the migration velocity of proteins is a function of their size, shape and the number of electric charges they carry. As the velocity is a complex function of these properties, native PAGE cannot be used to estimate the molecular mass of

proteins. The traditional native PAGE method is similarly unable to assess whether a purified protein is composed of a single subunit or multiple subunits. Even a multi-subunit protein may migrate in a single sharp band.

SDS-PAGE (Figure 7.4) was introduced to analyse such cases and to allow the estimation of the molecular mass of single-subunit proteins or those of individual subunits of multi-subunit proteins. SDS-PAGE is the most prevalent PAGE method currently in use.

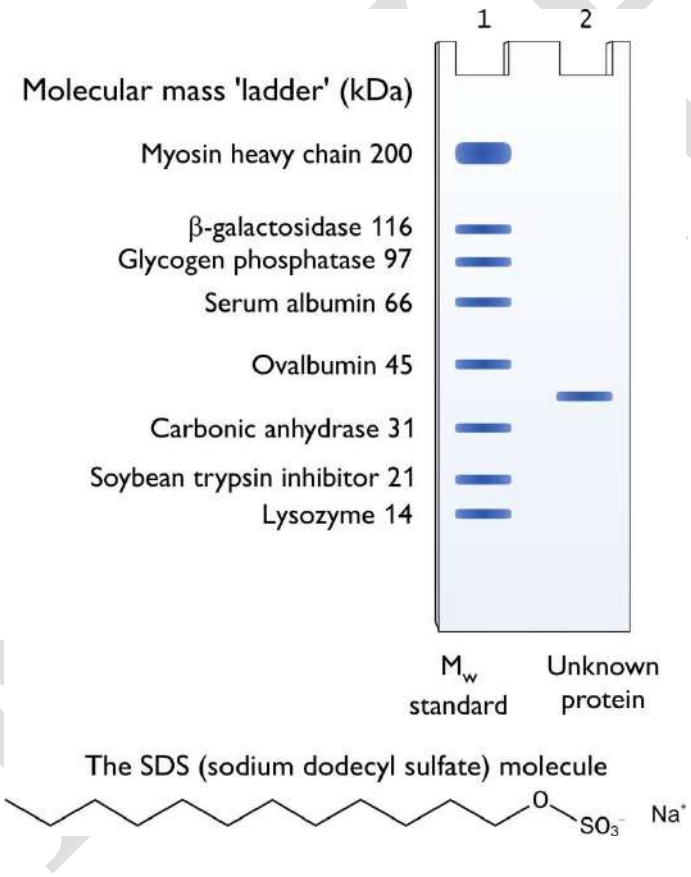


Figure 7.4. SDS polyacrylamide gel electrophoresis. SDS (sodium dodecyl sulphate) is an anionic detergent that unfolds proteins and provides them with extra negative charges. The amount of the associated SDS molecules—and therefore the number of charges—is proportional

to the length of the polypeptide chain. The SDS gel separates individual polypeptide chains (monomeric proteins and subunits of multimeric proteins) according to their size. The velocity of the proteins is an inverse linear function of the logarithm of their molecular mass. Proteins of known molecular mass can be used to establish a calibration curve (a descending line) along which the unknown molecular mass of other proteins can be estimated.

SDS (sodium dodecyl sulphate) is an anionic detergent. When proteins are treated with SDS at high temperature, radical conformational changes occur. The treatment breaks all native non-covalent intermolecular (inter-subunit) and intramolecular interactions. The subunit structure of multi-subunit proteins disintegrates and the proteins unfold. If the native structure is stabilised by disulfide bridges, reducing agents are also added to open up these connections. SDS molecules bind to unfolded proteins in large excess, providing extra negative charges to the molecules.

The amount of the bound SDS molecules is largely independent of the amino acid sequence of the polypeptide chain and it is roughly a linear function of polypeptide length—i.e. the molecular mass of the protein. Therefore, upon SDS-treatment, the specific charge (the charge-to-mass ratio) of different proteins will become roughly identical. Another result of the treatment is that the shape of the different proteins becomes similar. The negatively charged SDS molecules repel each other, which lends a (presumably) rod-like shape to the SDS-treated proteins. These factors together result in a situation analogous to the one already discussed in this chapter for the PAGE separation of linear single-stranded (denatured) DNA molecules. Instead of being separated simultaneously by charge, shape and size, SDS-treated proteins—just like denatured linear DNA molecules—will be separated solely based on their size. As size is a linear function of mass, SDS-PAGE ultimately separates proteins based on their molecular mass.

SDS-PAGE is the most popular cost-effective method to estimate the molecular mass of protein subunits with considerable accuracy. The relative mobility (i.e. the running distance of the protein divided by the running distance of the tracking dye) of the SDS-treated protein is in

inverse linear proportion to the logarithm of the molecular mass of the protein. By running several proteins of known molecular mass simultaneously alongside the protein of interest, a log molecular mass – relative mobility calibration curve (a descending linear graph) can be created. Based on the calibration curve, the estimated molecular mass of the protein in question can be easily calculated.

Table 7.I below shows the useful separating range of polyacrylamide gels as a function of acrylamide concentration. In the useful range, the log molecular mass – relative mobility relationship is linear.

Acrylamide concentration (%)	Linear range of separation (kDa)
15	12-43
10	16-68
7.5	36-94
5.0	57-212

Table 7.I. Relation between acrylamide concentration and the molecular mass of optimally separated molecules

SDS-PAGE is a standard method for assessing whether the sample of an isolated protein is homogeneous. Besides that, SDS-PAGE is a robust method for the analysis of large supramolecular complexes such as multi-enzyme complexes or the myofibril, as discussed below. SDS-PAGE separates and denatures individual subunits of these complexes. Thus, all polypeptide chains will migrate separately in the gel. Via various staining procedures, all subunits can be visualised and the relative amounts of these proteins (subunits) can also be

determined. This allows for the identification of each subunit of a complex and provides a good estimate of the stoichiometry of subunits, too.

7.3.4. Isoelectric focusing

In the course of isoelectric focusing, the conditions are set in a way that proteins will be separated exclusively based on their isoelectric point (Figure 7.5). The two termini and many side chains of proteins contain dissociable groups (weak acids or bases). The dissociation state of these groups is a function of the pH of the environment (as described quantitatively by the Henderson-Hasselbalch equation, see Chapter 3). Isoelectric focusing is based on the pH-dependent dissociation of these groups. Due to this pH-dependent phenomenon, the net electric charge of a protein molecule will be a function of the pH of the medium. If, in a given protein, the number of acidic residues (Asp, Glu) exceeds that of the basic ones (Arg, Lys, His), the protein will have a net negative charge at neutral pH. The isoelectric point (pI) of the protein—i.e. the pH at which the net charge of the protein is zero—will be in the acidic pH range. Such proteins are often denoted as acidic proteins. If the number of basic residues exceeds that of the acidic ones, the protein will be positively charged at neutral pH, and its pI value will be in the basic pH range. These proteins are often called basic proteins.

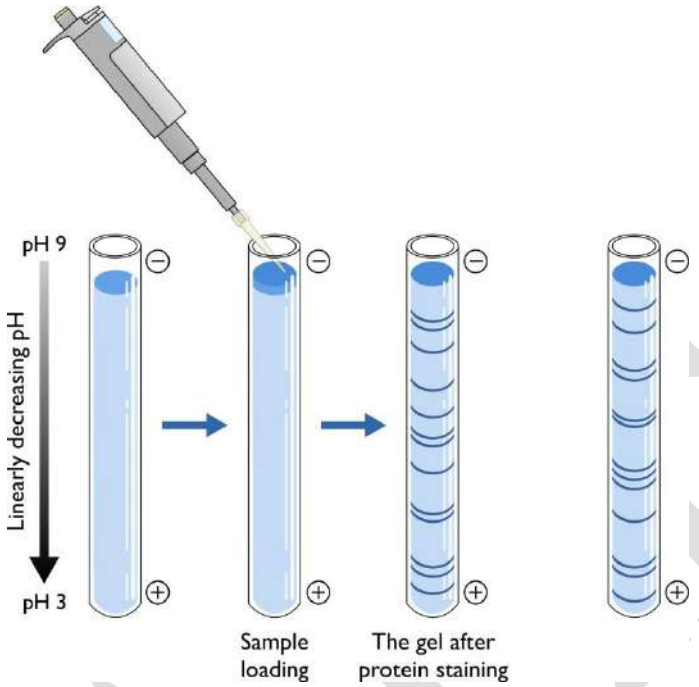


Figure 7.5. Isoelectric focusing. In the course of isoelectric focusing, a pH gradient is created in the gel (usually made of polyacrylamide, less frequently agarose). Upon electrophoresis, various proteins will accumulate in different narrow regions of the gel where the pH equals their individual pI value. At this pH, the number of positive charges equals that of the negative charges on the protein—the net charge will thus be zero. Consequently, no resultant electric force is exerted on the protein.

Isoelectric focusing is an efficient high-resolution method because the pI values of various proteins are spread across a broad range. If the pH is lower than the pI of the protein, the protein will be positively charged and will move towards the cathode during electrophoresis. If the pH is higher than the pH of the protein, the protein will be negatively charged and will migrate towards the anode. If the pH equals the pI value, the net charge of the protein will be zero and the protein will not migrate in the gel any further.

In the course of isoelectric focusing, proteins are placed in a gel representing a special medium in which the pH gradually decreases by going from the negative cathode towards the positive anode. As the protein migrates, it encounters a gradually changing pH and its net charge will also change accordingly. If it has a net negative charge and therefore moves towards the cathode, it will encounter a gradually decreasing pH, i.e. a more and more acidic environment. Consequently, the protein will take on more and more protons—up to a level where its net charge will be zero. This state is reached when the protein reaches a location where the pH equals its pI value. At this point, the protein will stop moving because no electric force will be exerted on it. If it spontaneously diffused further towards the anode, it would take on more protons, would become positively charged and would turn back to migrate towards the cathode. Following the same line of thinking, if a positively charged protein moves towards the cathode, it will encounter increasing pH and lose more and more protons. It will migrate to the place where the pH equals its pI value and will thus stop. If it diffused further towards the cathode, it would become negatively charged and would turn back towards the anode. As one can see, by performing electrophoresis in a medium in which the pH decreases from the cathode towards the anode, each protein will "find its place" according to its pI value and will become sharply focused at that location. In addition, it does not matter where exactly the proteins were introduced in the medium between the cathode and the anode.

A decisive component of this method is the usually linear pH gradient created inside the gel. There are two methods to create such a gradient. One of them applies carrier ampholytes (ampholyte is an acronym from the words amphoteric and electrolyte). Ampholytes or zwitterions are molecules that contain both weakly acidic and weakly basic groups. Just like in the case of proteins, the net charge of ampholytes is a function of the pH. In the course of isoelectric focusing, a mixture of various ampholytes is used such that the pI of the various ampholyte components will cover a range in which the pI values of the "neighbouring" ampholytes differ only slightly. This ampholyte mixture is soaked in the gel and an appropriate

electric field is generated by a power supply. This leads to a process analogous to the one already explained for proteins. Each ampholyte will migrate to the location where its net charge becomes zero. As soon as this steady-state is achieved, ampholytes will function as buffers and keep the pH of their immediate environment constant. This establishes the pH gradient in which the proteins can be separated.

The other, more sophisticated method applies special ampholytes that can be covalently polymerised into the polyacrylamide gel. The appropriate ampholyte gradient is created before the gel is polymerised. This way, the gradient will be covalently fixed in the gel, providing an immobilised pH gradient. The appropriate pH range provided by the ampholyte mixture should be selected based on the pI values of the proteins to be separated.

Regardless of how the pH gradient was created, once the proteins reach the location in the gel where the pH equals their pI, they finally stop moving and the system reaches a steady-state.

One of the potential technical difficulties encountered during isoelectric focusing originates from the fact that the solubility of proteins is lowest at their pI value (see Chapter 5). This can lead to the precipitation of some proteins in the gel. To prevent this unwanted process, urea is most often applied in the gel as an additive. Urea denatures proteins and keeps denatured proteins in solution. As the pI value of proteins is largely independent of their conformational state, this modification does not compromise the method. The solubility of membrane proteins can be further promoted by the addition of non-ionic detergents.

Isoelectric focusing is aimed at separating proteins based exclusively on their pI value—thus, independently of their size. Therefore, the molecular sieving property of the gel in this method should be avoided. The only function of the gel is to prevent free convectional flows in the medium. Accordingly, for isoelectric focusing, polyacrylamide gels are made at very low acrylamide concentrations, and sometimes even agarose gels are applied when very large pores

are needed. Isoelectric focusing is usually performed in a horizontally-mounted electrophoresis apparatus and by applying intense cooling.

7.3.5. Two-dimensional (2D) electrophoresis

The various separation methods are all aimed at separating complex systems to individual components. Separation is always based on at least one physicochemical property that shows diversity among the components. The general problem encountered in the case of complex mixtures is that not all components differ significantly from all other components when only one property is considered. Accordingly, separation based on a single property rarely results in single-component fractions. Some components will be efficiently separated from all others, while some other components will remain in the mixture.

The remaining mixtures can be further fractionated by another separation technique that relies on a different physicochemical property. The most effective separation can be achieved if the combined consecutive separation steps rely on absolutely independent physicochemical properties. A good example of this is the very high-resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) that combines two already discussed electrophoresis methods, isoelectric focusing and SDS-PAGE (Figure 7.6).

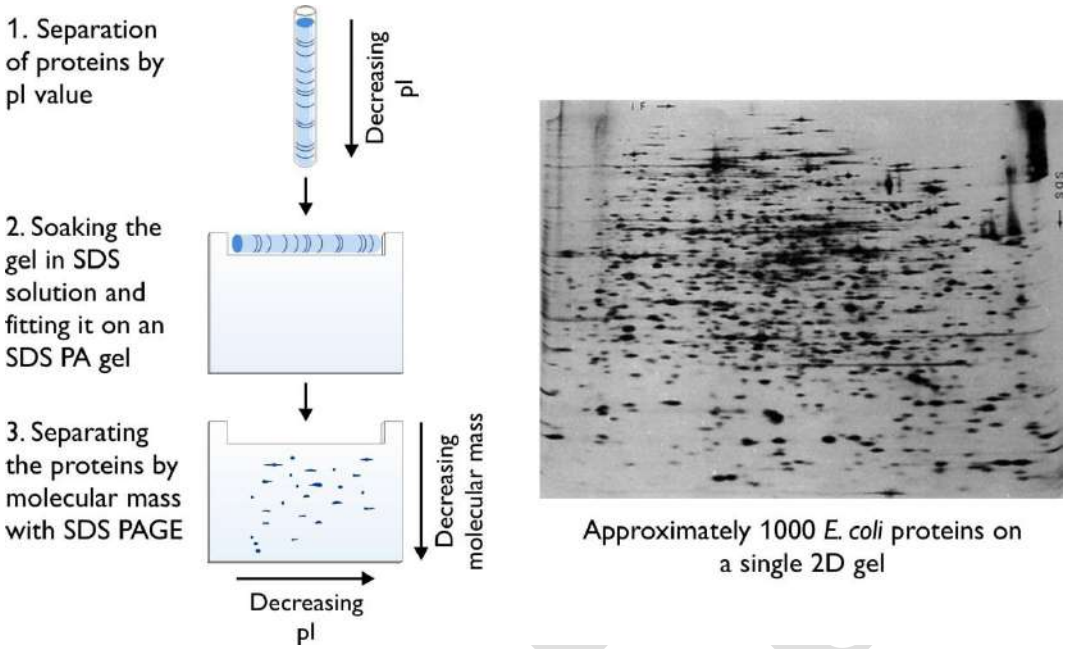


Figure 7.6. Two-dimensional (2D) electrophoresis. 2D electrophoresis is the combination of isoelectric focusing and SDS-PAGE. Proteins are first separated based on their pI values and then based on their molecular mass. As these properties are completely independent, the combination of the two separation methods provides much higher resolution than either of the two methods alone.

As the first step of 2D gel electrophoresis, isoelectric focusing is performed to separate proteins based on their pI values. Only a single sample is loaded on a gel strip in this step. The sample is separated in one dimension both in a primary and in a figurative sense. In a primary sense because the components are separated along a single line, and in a figurative sense as the separation is based on a single well-defined property, the pI value.

After the first separation step has been completed in the first dimension, the gel strip is soaked in an SDS solution and is fitted tightly to one side of a "classical" SDS polyacrylamide gel. The

second separation step is traditional SDS-PAGE, which separates proteins based on their molecular mass. This second step represents a second dimension in both a primary and a figurative sense. The second separation is performed in a second dimension in a direction rectangular to that of the first separation, and the property utilised in the second step (molecular mass) is completely independent of the one utilised in the first step (pI).

If, after the first step, some gel regions contain different proteins that coincidentally have identical pI values, these proteins will be separated from each other in the second step if their molecular mass is different. Note that every aspect discussed for SDS-PAGE also applies to the second separation step of 2D-PAGE. Van der Waals interactions that might have held protein subunits together in the course of isoelectric focusing will break and individual subunits will become separated. If disulfide bridges need to be opened up, some kind of reducing agent needs to be added. Accordingly, in the second separation step, single polypeptide chains will migrate in the gel. If isoelectric focusing collects a multimeric protein at a certain gel location, the second electrophoresis step will dissect it into individual chains. If the multimer contains subunits of different sizes, these subunits will be separated from each other in the second separation step.

UNIT-III

SYLLABUS

Structural Organization and Analysis of proteins : Organization of protein structure into primary, secondary, tertiary and quaternary structures. N-terminal and C-terminal amino acid analysis. Sequencing techniques - Edman degradation. Generation of overlap peptides using different enzymes and chemical reagents. Disulfide bonds and their location. Mass spectrometric analysis, tandem MS. Solid phase peptide synthesis

Proteomics/Protein Primary Structure/Sequencing Methods

Protein sequencing denotes the process of finding the amino acid sequence, or primary structure of a protein. Sequencing plays a very vital role in Proteomics as the information obtained can be used to deduce function, structure, and location which in turn aids in identifying new or novel proteins as well as understanding of cellular processes. Better understanding of these processes allows for creation of drugs that target specific metabolic pathways among other things. Though several methods exist to sequence proteins the two dominant methods are Mass Spectrometry and Edman Degradation. Other methods that are not as frequently used still can serve very specific roles, such as overcoming inadequacies or acting as a preliminary, that compliment the two predominant methods.

History

The advent of protein sequencing can be traced to two almost parallel discoveries by Frederick Sanger and Pehr Edman. Pehr Edman began his work in the Northrop-Kunitz laboratory at the

Princeton branch of the Rockefeller Institute of Medical Research in 1947 where he attempted to find a method to decode the amino acid sequence of a protein using chemicals; specifically he had early success with fluorodinitrobenzene (FDNB) and phenylisothiocyanate (PITC). Throughout his year at Princeton, Edman was able to conduct enough experiments to understand that it was feasible to use reagents like FDNB and PITC to determine amino acid sequence. Edman returned to Sweden in 1947 and after two more years of work he was able to publish his paper that would describe the first successful method to sequence proteins. This groundbreaking paper described a method to determine the amino acid sequence of a protein and would come to be known as the Edman Degradation. Five years earlier, Frederick Sanger had demonstrated a method to determine the amino acid residue located on the N-terminal end of a polypeptide chain by using the reagent fluorodinitrobenzene. While it was thought, that at most,

this method could only provide the sequences found on the N-terminal, Sanger was able to take the method one step further. By using several proteolytic enzymes, partial hydrolysis and early version of chromatography, Sanger was able to cleave the protein into fragments and piece together the residues like a jigsaw puzzle. It wasn't until 1955 that Sanger was able to present the complete sequence of insulin which led to him being awarded a Nobel Prize in Chemistry in 1958. Mass Spectrometry, as a tool for the analysis of individual molecules, had been available many years before either Sanger or Edman began their work on protein sequencing. From its humble beginnings in the late 1800's it has undergone many changes in its hardware and its software and has proven to be so critically important to the field of sequencing that several more Nobel Prizes were awarded to those that were able to improve upon this technology. Despite its importance today, it wasn't until 1966 when K. Biemann, C. Cone, B.R. Webster, and G.P. Arsenault sequenced several oligopeptides containing glycine, alanine, serine, proline, and several other amino acids that the importance of mass spectrometry was fully realized. Further developments were to come in the late 80's as the Mass Spectrometer became a more robust piece

of instrumentation in the laboratory. 1989 saw the first demonstration of fast atom bombardment ionization with Tandem Mass Spectroscopy as applied towards the identification of protein sequences. The work here was the early foundation for the Protein Mass Fingerprinting procedure that would come to use in the early 90's. With the arrival of MALDI and electrospray ionization as two new ionization methods the dynamic range of mass spectroscopy was greatly improved and paved the way for the mass spectrometer to be a dominant tool in the use of protein sequencing. The advent of Proteomics in 1996 saw many rapid developments such as increasing computational power, the growth of the world wide web and protein databases, and the advances in mass spectroscopy multi-quadrupole systems that enabled MALDI-MS/MS and other tandem mass spectrometer methods.

Sequencing Methods

N-terminal Residue Identification - N-terminal residue identification encompasses a technique which chemically determines which amino acid forms the N-terminus of a peptide chain. This information can be used to aid in ordering of individual peptide sequences that were generated using other sequencing techniques that fragment the peptide chain. Frequently, the first round of Edman Degradation will also contain impurities that may make identification of the N-terminus residue difficult. The general process of N-terminal residue identification is described below:

1. The free unprotonated α-amino groups are labeled using a reagent that will selectively label the terminal amino acid. Reagents that can accomplish this include 2,4-dinitrofluorobenzene (DFNB - Sanger's reagent), dansyl chloride, and phenylisothiocyanate (Edman's reagent).

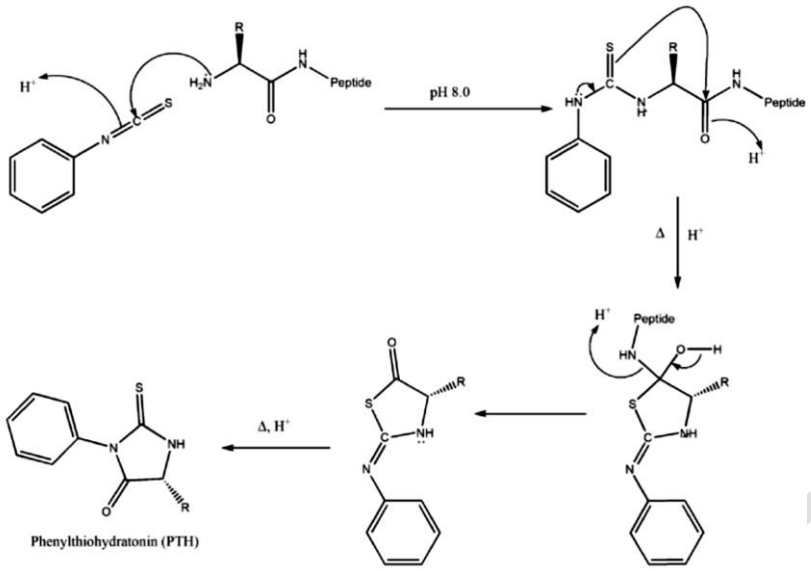
2. The labeled peptide is hydrolyzed with acid which yields the N-terminal residue and other free amino acids.

3. Each of these derivative N-terminal residues can be separated and identified using chromatography. These methods can be used to identify the N-terminal residue of the peptide. This is time consuming process which has decreased in usefulness now that more efficient

sequencing techniques are now available. Further complicating the issue: certain reagents used in the process can also degrade amino acid residues to the point where they are unrecognizable. Of the reagents available to label the N-terminal residue dansyl chloride is about 100 times more sensitive than FDNB due to its highly fluorescent nature which makes it easily detectable in minute amounts. The use of Edman's reagent is also advantageous as it leaves the remaining residues in the peptide chain untouched as described in the next section.

Edman Degradation

Edman Degradation with generic amino acid peptide chain. The Edman Degradation method is based on the principal that single amino acid residues can be modified chemically such that they can be cleaved from the chain without disrupting the bonds between any other residues. The procedure can be achieved with very minute amounts of peptide, usually amounts on the order of 10-100 picomoles will allow for successful completion. Samples must contain only one protein component and should be free of any reagents that interfere with the degradation process such as glycine, glycerol, sucrose, guanidine, ethanolamine, ammonium sulfate, and ammonium salts. The general method is described below:



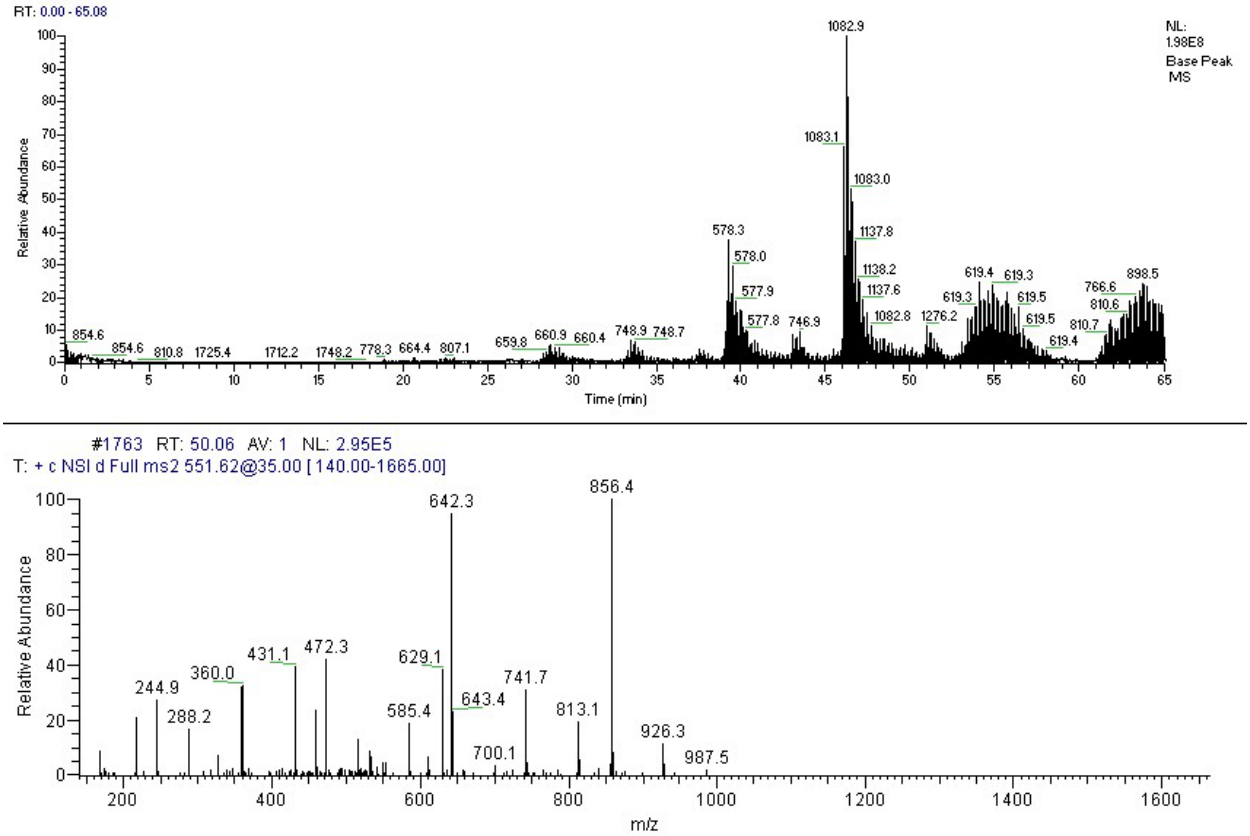
Edman Degradation with generic amino acid peptide chain.

1. The peptides to be sequenced must first be immobilized by being absorbed onto a chemically modified glass or by electroblotting onto a porous polyvinylidene fluoride (PVDF) membrane.
2. Under mildly alkaline conditions phenylisothiocyanate (PITC) is reacted with an uncharged terminal group on the amino acid chain to form a phenylthiocarbamoyl derivative.
3. This phenylthiocarbamoyl derivative is then cleaved using Trifluoroacetic acid producing its anilinothiazolinone derivative (ATZ-amino acid). The next terminal amino acid is now exposed and ready for the same reactions to occur.
4. A wash is performed to remove excess buffers and reagents and the ATZ amino acid is selectively extracted with ethyl acetate and converted to a more stable phenylthiohydantoin (PTH)- amino acid derivative.
5. Identification of the PTH amino acid derivative is accomplished using chromatography or electrophoresis.

6. The process can now be repeated for the remaining residues of the chain. Automation of the Edman Degradation procedure was initiated in 1967[8] and continues to be a favorable sequencing method due to its sensitivity and rapid completion. Sequencers that can automate the Edman Degradation procedure include many models of the Applied Biosystems Procise or Protein Sequencer families. The major drawback of the procedure remains the length of the peptide chain. If the chain exceeds a length of 50-60 residues (30 residues in practice) the procedure tends to fail due to the incompletion of the cyclical derivitization. This can be solved by taking the larger peptide chain and cleaving it into smaller fragments using cyanogen bromide, trypsin, chemotrypsin or any enzyme/chemical which can break peptide chains.

Mass Spectrometry

Full MS and MS2 spectra of a peptide. Mass spectrometry is quickly becoming the gold standard by which to identify protein sequences due to its ease of automation and extreme accuracy. The use of mass spectroscopy now dominates the process of sequencing proteins because prior problems of delivery were solved by John B. Fenn and Koichi Tanaka with their Nobel Prize winning electrospray ionization procedure. The two most popular methods to identify protein sequences using Mass Spectrometry are Peptide Mass Fingerprinting and Tandem Mass Spectrometry.



Full MS and MS2 spectra of a peptide.

Peptide Mass Fingerprinting

This method, also known as Protein fingerprinting, was developed in 1993 by several groups and functions by cleaving an unknown protein into smaller fragments so that these smaller fragments can then be accurately measured with a mass spectrometer. A generalized procedure is shown below:

1. Protein samples are broken up into several smaller peptide fragments by proteolytic enzymes.

2. The resulting fragments are extracted using acetonitrile and dried by vacuum. The peptides are then dissolved in distilled water and ready for analysis.

3. The peptides are then inserted into the vacuum chamber of a mass spectrometer such as ESI-TOF or MALDI-TOF.

The mass spectrometer produces a peak list (i.e. a list of molecular weights), which is then compared against databases such as SwissProt or GeneBank to find close matches. Software is used to translate the retrieved genomic data into proteins which then undergo simulated cleavage by the same enzyme used to cleave the unknown protein. The mass is calculated of these fragments and then compared to that of the unknown protein.

Tandem Mass Spectrometry

Schematic of tandem mass spectrometry Tandem Mass Spectrometry describes the partitioning of mass spectroscopy into separate steps where fragmentation occurs in between these steps. These separations can occur either physically in space, by separate chambers called quadrupoles, using either multiple mass spectrometers or in a single mass spectrometer by time. The generalized procedure is described below:

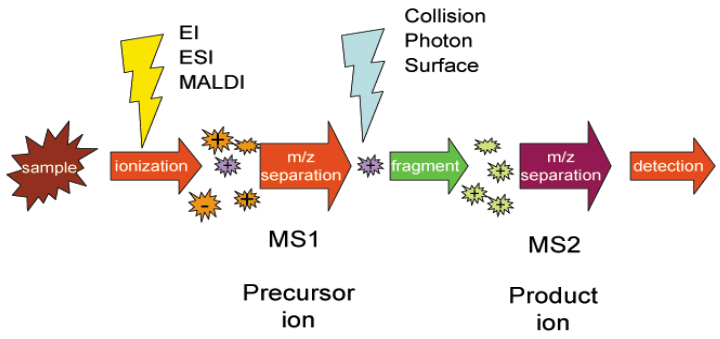
1. Enzymatic or chemical degradation of target protein to produce peptides.

2. Fractionation of peptides by high-performance liquid chromatography.

3. Resulting fragments fed into mass spectrometer for analysis. Analysis of fragments by Tandem Mass Spectrometry occurs in two or more quadrupole systems with the first quadrupole filtering select ions that will undergo further analysis. These filtered ions are transferred to the second quadrupole which acts as a collision center to induce further fragmentation at amide linkages. A third quadrupole is then used to separate these fragments by mass. Tandem Mass Spectrometry mainly generates peptides of the N- and C- terminal types, which are represented in 2D via mass/charge vs. intensity graphs. The spectra produced by a mass spectrometer containing all the molecular weights of the fragments is called a peptide map and can serve as a

means of identifying proteins analyzed by a mass spectrometer. Other approaches to identifying protein sequences include Protein Sequence tags and *de novo* methods. Peptide sequence tags, proposed by Matthias Wilm and Matthias Mann at the EMBL, function by sampling of masses at random points during a Tandem Mass Spectrometry experiment. These handfuls of masses are then used as unique tags to identify specific peptides following further fragmentation by the mass spectrometer. This aids not only in identification but also during the process of attempting to stitch back together the peptides into a full sequence. *Denovo* approaches to protein sequencing identification are also employed along side similarity searches. These de novo methods do not take into account any prior knowledge of the amino acid sequence being analyzed and approach the identification of peptide sequences in novel ways. Examples include Hidden Markov Models, which takes a statistical approach to protein sequence identification, and graph searches of the problem space that helps minimize the search space as to speed up the time needed to identify sequences via a database.

Schematic of tandem mass spectrometry



Solid-Phase Peptide Synthesis (SPPS) and Secondary Structure Determination by FTIR

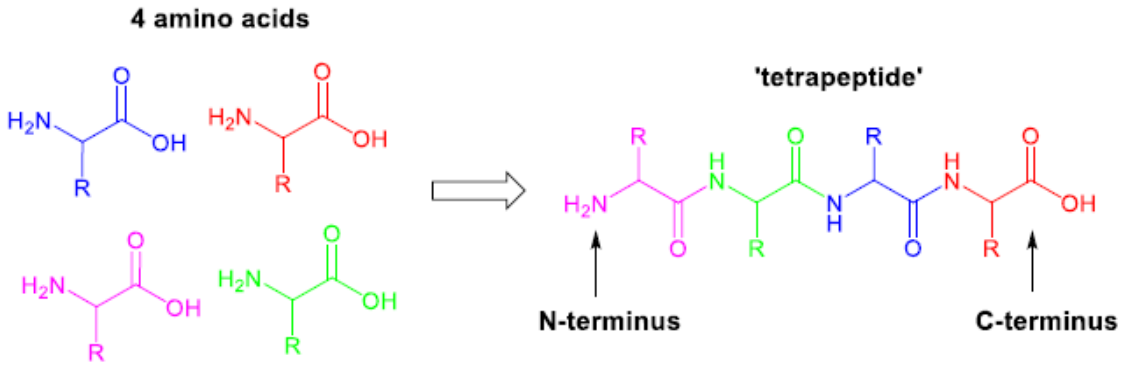
Introduction - Proteins are ubiquitous in living organisms and cells, and can serve a variety of functions. Proteins can act as enzymes, hormones, antibiotics, receptors, or serve as structural supports in tissues such as muscle, hair, and skin. Due to the high molecular

weight and the difficulty in isolating significant quantities of many proteins, scientists have been working for decades to develop methods to synthesize naturally occurring peptides (short proteins) or protein fragments in the laboratory in order to study or mimic the structure and biological activity of full length proteins. Another motivation to develop efficient peptide synthesis techniques is the potential of these molecules to serve as therapeutic agents.

More recently, the natural ability of peptides/proteins to self-assemble into defined structures has also become a target for exploitation in a variety of materials science and biomedical applications. Fibrilliar aggregates and hydrogels formed from peptides and peptide conjugates have been successfully used as biomimetic cell culture scaffolds,drug delivery vehicles, and stimuli responsive biomaterials. Peptides have also been used to control the morphology of larger polymers,[6,7] and direct the assembly of inorganic nanoparticles to form peptide based wires and sensors. As an introduction to this rapidly expanding field, this experiment will cover methods used to synthesize and characterize peptides, as well as evaluate the secondary structure of a peptide following self-assembly.

Basic Peptide Structure - Peptides are formed by sequential addition of specific amino acids. The amino acids all have similar structures that contain an amine on one end and a carboxylic acid on the other (hence the name 'amino acids'), but they vary in the R□group attached to the alpha carbon. To form a peptide, amino acids are joined 'head to tail' by coupling the amine of one amino acid with the carboxylic acid of another amino acid to form an am*ide* bond. The general structure of a peptide containing four amino acids (a 'tetrapeptide') is shown. The end of the peptide containing the amine is called the 'N-terminus' and the end containing the carboxylic acid is called the 'C terminus'. Proteins are naturally synthesized starting at the N terminus, so by convention, the amino acid sequence of a peptide is typically listed from the N to C-terminus. For example, if your peptide contains

arginine, glycine and aspartic acid, the peptide would be referred to as ArgDGlyDAsp or RGD if using the 1Dletter abbreviation for each residue. *Note:* a peptide with the sequence ArgDGlyDAsp is NOT the same as AspDGlyDArg.



General structure of a peptide containing four amino acids

Solid-Phase Peptide Synthesis (SPPS)

In order to efficiently synthesize peptides, a technique known as 'solidDphase peptide synthesis' (SPPS) was first developed in the 1960's. The key feature of SPPS is the sequential attachment of amino acids to a macroscopic solid support matrix (commonly referred to as resins or beads). While a wide variety of solid supports are available, some of the most common are made from small beads (~70D400 microns insize) of polystyrene plastic that have been chemically modified to attach a 'linker' molecule to the surface of the bead.[11] Each bead has multiple linker molecules on its surface. The number of linker molecules on the surface of a particular batch of beads is usually designated by giving the millimoles of linker per gram of beads (mmol/g). The chemical structure of the particular resin that we will use in this lab is shown in Figure 2 (called Wang resin[12]). The hydroxyl group highlighted in blue is the point of attachment (via an ester linkage) to the ***C'terminal*** amino

acid in the peptide chain. The rest of the peptide is then synthesized in a stepDwise fashion by adding one amino acid at a time (see Scheme 1 below). ***Note:*** As mentioned above, proteins are naturally synthesized starting from the NDterminus, but SPPS techniques synthesize peptides starting from the CDterminus for ease of synthesis and to minimize racemization of the amino acids. Therefore, to synthesize the peptide GlyDArgDAsp, you would first add Asp, then Arg, then Gly to the resin.

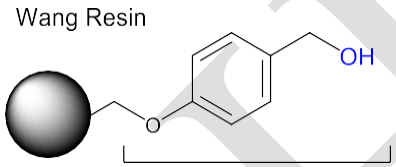


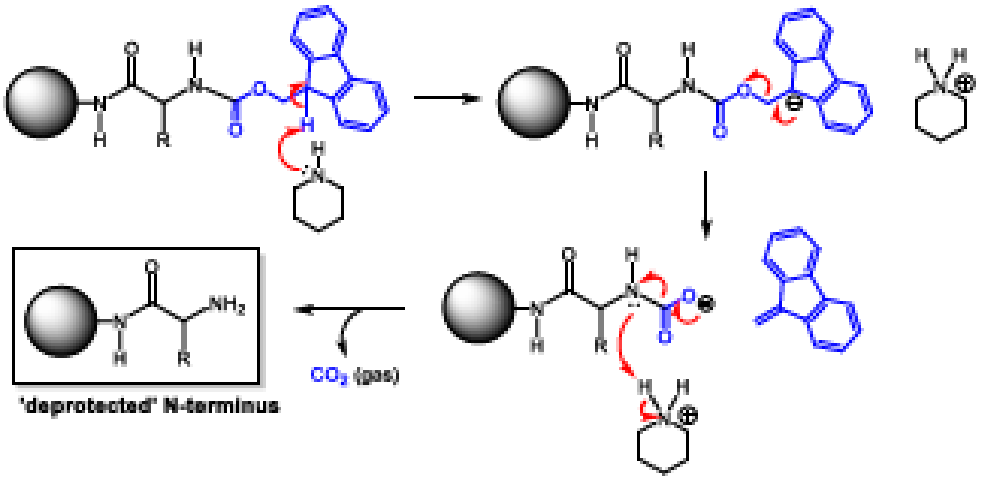
Figure 2. Wang resin linker.

Fmoc Strategy in SPPS

Since each amino acid contains both an amine and carboxylic acid functional group, it has the potential to react with itself. Therefore, in order to synthesize peptides containing a precise sequence of different amino acids, we must use careful protecting group strategies so that we can control which end of the amino acid can participate in the coupling reaction. One of the most commonly used protection strategies is called the 'Fmoc Strategy', in which the amineDend of the amino acids used are first 'protected' with a fluorenylmethoxycarbonyl (Fmoc) group (Scheme 1).[13,14] These derivatives are now commercially available from a variety of vendors. The Fmoc group prevents the amineDend of the amino acid from reacting, so that the coupling is selective between the terminal amine group on the solid phase resin, and the carboxylic acid group on the amino acid to be added. To continue the growth of the peptide chain, the Fmoc group can be removed by reaction with a strong base, such as piperidine, as shown in Scheme 2

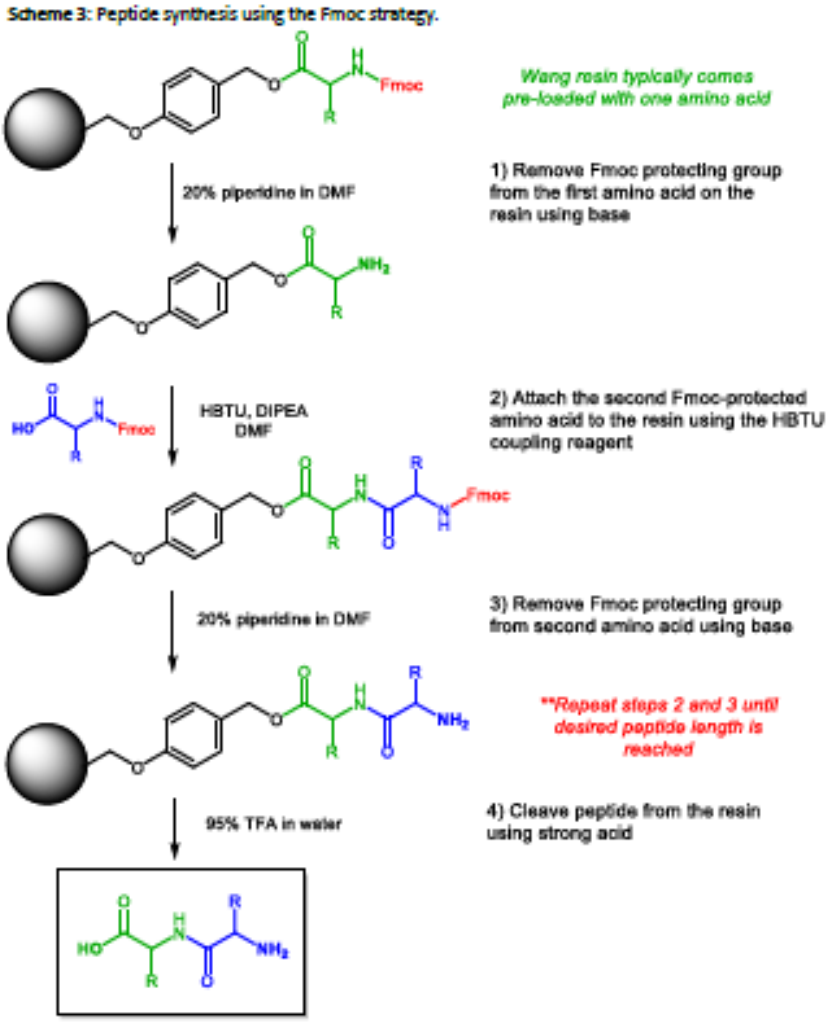
Scheme 1. Synthesis of Fmoc-protected amino acids.

Scheme 2. Mechanism of Fmoc removal from the growing peptide.



The general steps carried out in solid‐phase peptide synthesis using the Fmoc strategy are outlined in Scheme 3. Wang resin is commonly sold with one amino acid already attached. Therefore, the resin must first be 'deprotected' by removing the Fmoc group on the first amino acid (C‐terminal amino acid) using a base such as piperidine. The second Fmoc‐protected amino acid is then attached using a coupling reagent to facilitate the reaction (see further discussion of coupling reagents below). The second

amino acid is then deprotected by treatment with piperidine, and then a third Fmoc amino acid can be coupled. After the desired peptide length is reached, the peptide undergoes a final deprotection step and can be detached from the solid support using trifluoroacetic acid (TFA). When the peptide is cleaved from the Wang resin linker, the carboxylic acid terminus will be regenerated.



Protection of Reactive Side Chains

Several amino acids contain reactive side chains (–OH, –NH, –SH, –COOH) that must also be protected to prevent side–reactions from occurring. The protecting groups for these amino acids must be chosen carefully so that they are compatible with the Fmoc removal conditions.[13,14] While a wide variety of options are available for all of the different reactive amino acids,[15] select examples of common protecting groups are given in Figure 3. As discussed above, the Fmoc groups that block the end of the growing peptide chain are removed using a base. Therefore, to prevent degradation during synthesis, side–chain protecting groups such as *tert*–butyl (*t*–Bu) or *tert*–butyloxycarbonyl (Boc) can be employed due to their stability in basic conditions. These particular protecting groups are also convenient when used in conjunction with Wang resin beads as they are unstable in acid, and can be removed during the final cleavage step of the peptide from the resin beads.

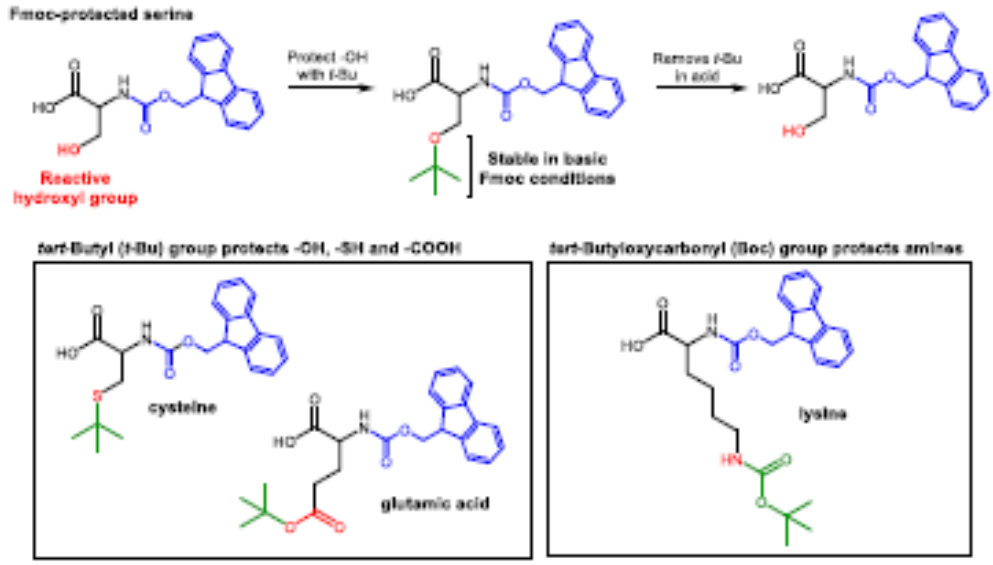
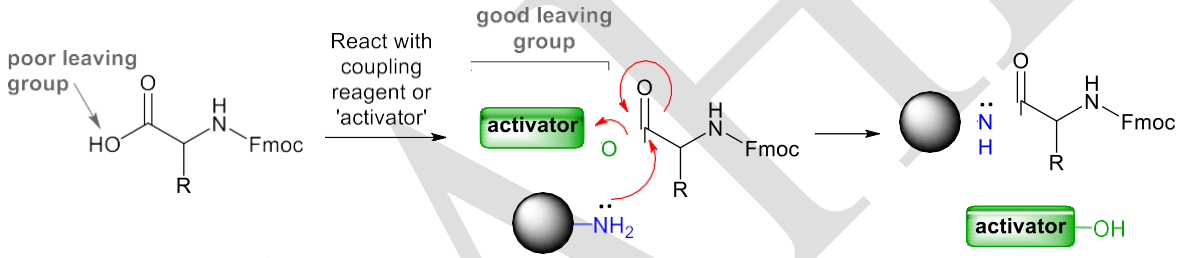


Figure 3. Select examples of protecting groups for some of the reactive amino acids.

Coupling Reagents

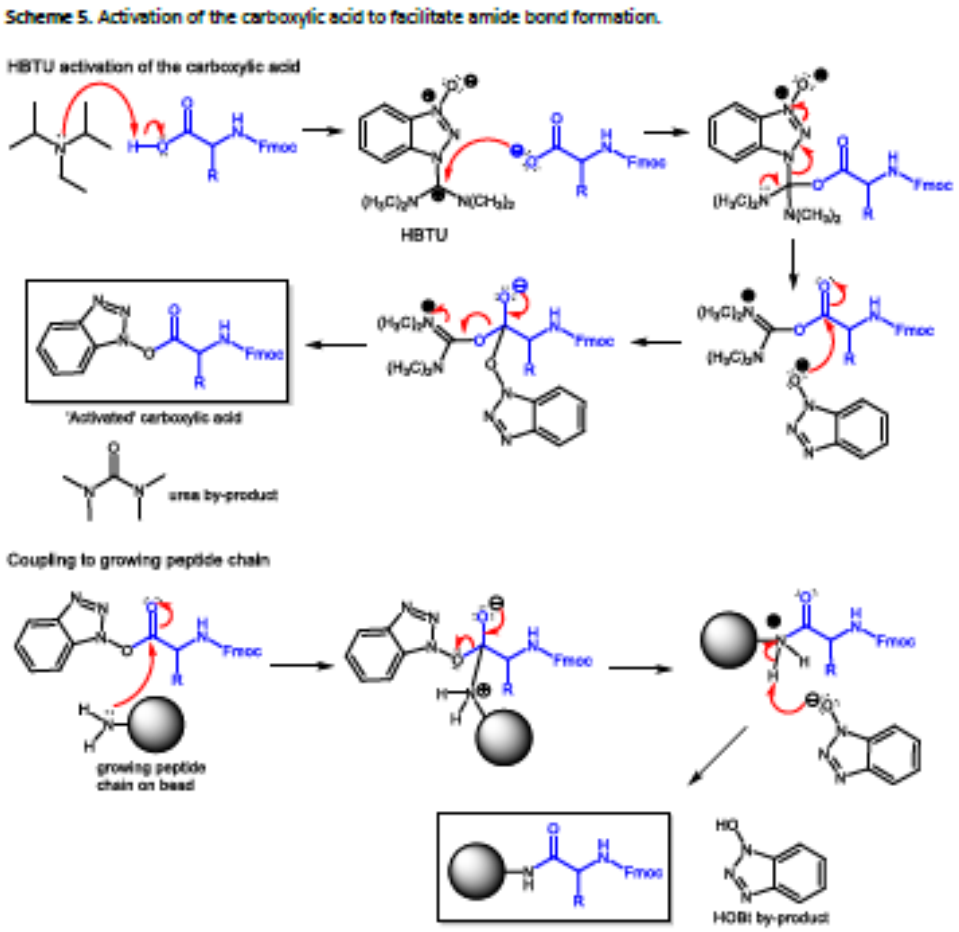
In order to get an efficient reaction between an amine and a carboxylic acid to form an amide bond, a 'coupling reagent' or 'activator' must be used, as illustrated in Scheme 4. The –OH of a carboxylic acid is a poor leaving group, making it difficult to directly displace. Therefore, carboxylic acids are typically converted into an 'activated ester' prior to reaction in order to facilitate displacement of the –OH by the $–NH_2$ on the end of the growing peptide.

Scheme 4. Activation of the carboxylic acid facilitates amide bond formation.



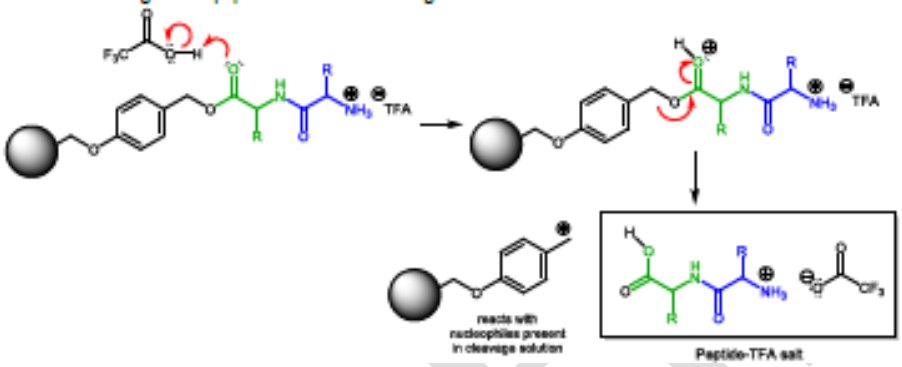
There are many different coupling reagents that have been developed for this purpose.[16] We will use *O*D (benzotriazolD1Dyl)DN,N,N',N'Dtetramethyluronium hexafluorophosphate (HBTU),which reacts as shown in the mechanism given in Scheme 5. While this compound is sold as a 'uronium' salt, it actually has the guanidinium structure shown below.[17] Briefly, an FmocDprotected amino acid is first mixed with HBTU in the presence of base (N,NDdiisopropylethylamine, DIPEA) to convert the carboxylic acid to an ester that is 'activated' toward nucleophilic attack. The free amine on the end of the growing peptide chain can then attack the carbonyl and displace the activator group (here hydroxybenzotriazole, HOBt), forming an amide bond. Over the course of this reaction two byDproducts are generated, 1,1,3,3Dtetramethylurea and HOBt,

which are subsequently washed out.



Cleavage and Isolation of the Peptide - The final step of the synthesis is to cleave the peptides from the resin beads. Before cleavage, any remaining Fmoc groups are removed. As detailed in Scheme 6, peptides are typically detached from Wang resin using

Scheme 6. Cleavage of the peptide from the resin using TFA.



trifluoroacetic acid (TFA), which regenerates the carboxylic acid on the C‐terminus of the peptide. Nucleophilic scavengers are often added to the reaction mixture to prevent further reaction of the benzyl cation produced on the resin. If the peptide has a free N‐terminus, it will become protonated under these acidic conditions, and form a salt with TFA. *Note:* The peptide we will synthesize is N‐acylated, thus will not form a salt.

Advantages and Disadvantages of SPPS

Solid phase reactions have advantages and disadvantages.[13] Since the peptide is anchored to

a solid support and only has one reactive end, a large excess of reagents at high concentrations can be used to drive coupling reactions to completion. Excess reagents and side products can easily be removed by filtration and washing steps after each coupling step. Disadvantages to this approach are the cost of the solid support, the limited number of 'linker' groups on the surface of the beads, and tedious nature of repetitive step□wise synthesis (However, there are commercially available instruments called 'peptide synthesizers' that can do the work for you!). Typically, only peptides containing less than 30 amino acids are synthesized using this method. Even though the reaction conditions have been highly optimized and are quite efficient, if you get 98% of the coupled product at each step, after the addition of 30 amino acids only ~55% of your product will have the correct sequence. Therefore, longer sequences are more commonly obtained through expression by bacterial cells such as *E. coli*.

UNIT-IV

SYLLABUS

Three dimensional structures of protein and Protein Structure Database : Nature of stabilizing bonds - covalent and non covalent. Importance of primary structure in folding. The peptide bond - bond lengths and configuration. Dihedral angles psi and phi. Helices, sheets and turns. Ramachandran map. Techniques used in studying 3-D structures - X-ray diffraction and NMR. Motifs and domains. Tertiary and quaternary structures. Structures of myoglobin and haemoglobin. Denaturation and renaturation of Ribonuclease A. Introduction to thermodynamics of folding and molten globule. Assisted folding by molecular chaperones, chaperonins and PDI. Defects in protein folding. Diseases –Alzheimer's and Prion based. Protein sequence and structure databases (PDB). Use of sequence and domain information. Viewing protein structures using *in silico* tools.

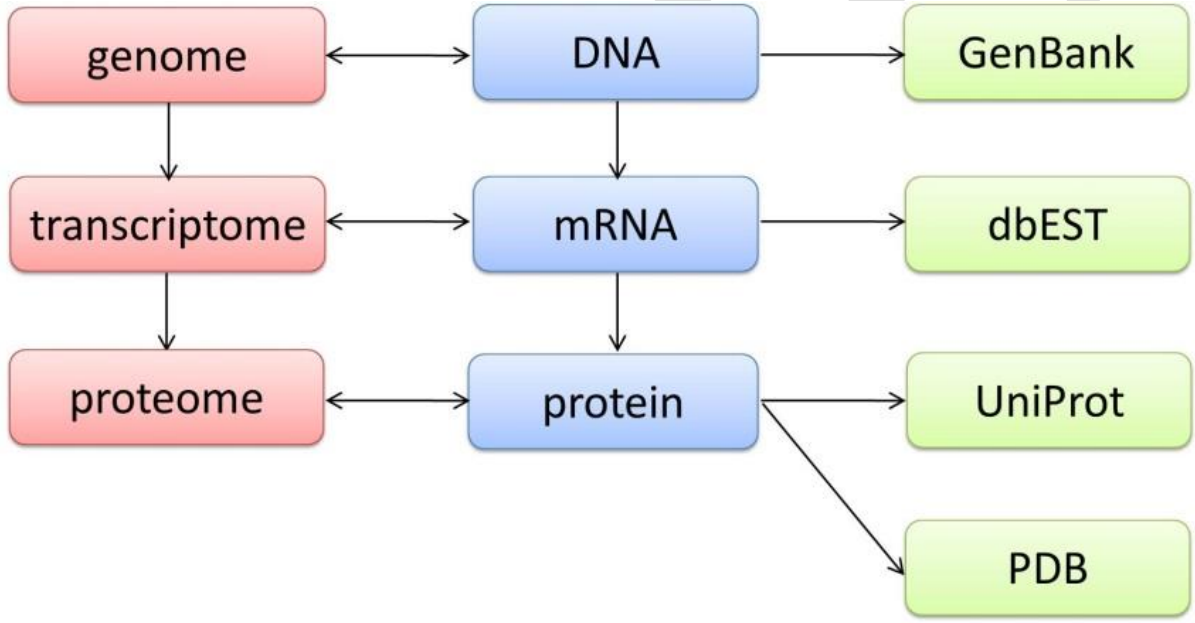
Primary sequence and three-dimensional structure databases

The most common use of bioinformatics for a biologist (and for a student of biology) is a search within primary molecular biological databases. These mostly include sequence and three-dimensional structure databases, and also experimental datasets provided by „omics" HTP methods (protein-protein interactions, large-scale mass spectrometry analysis and identification of lipids, sugars or small-molecule metabolites). The best known nucleotide sequence database is called **GenBank**, which is part of the **Entrez** bioinformatics web portal.

The most familiar protein sequence database is called **UniProt**, which is part of the **ExPASy** portal. The vast majority of amino acid sequences of polypeptides has been determined as nucleotide sequence and subsequently translated *in silico* (by bioinformatics tools) using the genetic code table. (Note that protein sequences determined on amino acid level are more relevant, since quite a few different functional proteins may originate from a single gene,

due to e.g. alternative splicing and/or post-translational modifications. Actually there are many more proteins than genes!) Experimentally-determined three-dimensional structures of macromolecules (proteins, nucleic acids as well as protein-protein and protein-nucleic acid complexes) are stored in the **Protein Data Bank** (PDB). Secondary databases contain data from analysis of sequences and structures, and will be mentioned briefly later in the text.

The relationship between the informational macromolecules and the primary bioinformatics databases is summarised in Figure 11.1.

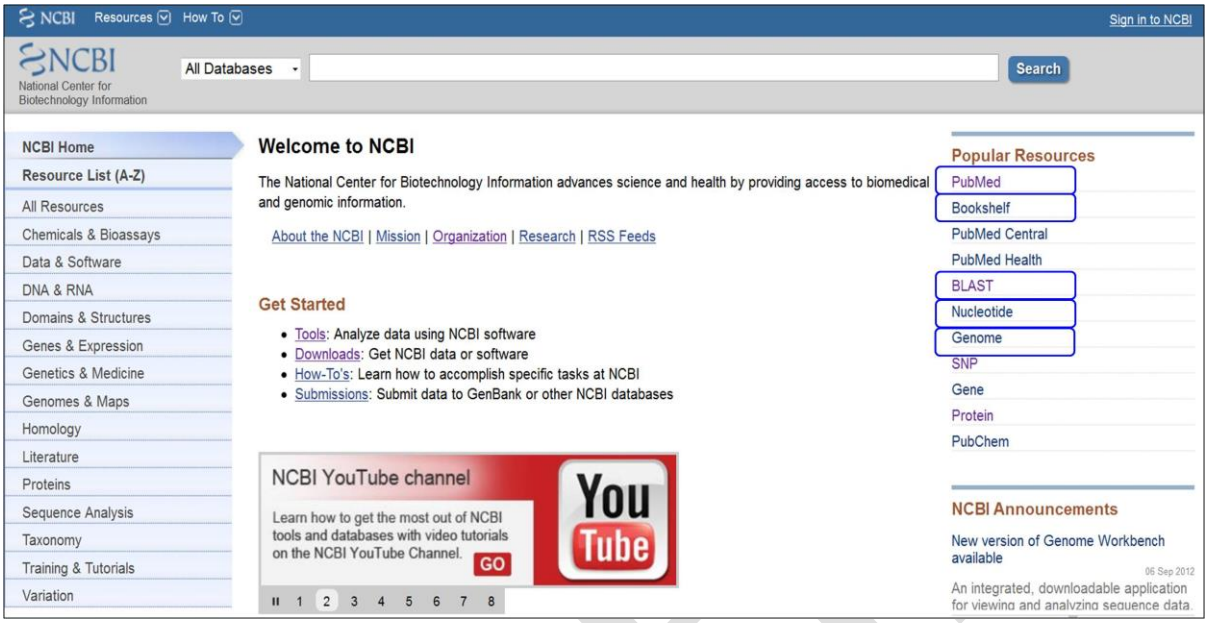


Relationship between informational macromolecules and primary bioinformatics databases

GenBank - **GenBank** (ncbi.nlm.nih.gov/genbank) is a DNA (nucleotide) sequence database maintained by the NCBI (National Center for Biotechnology Information), a US-government sponsored resource for bioinformatics information, which is part NIH (National Institutes of Health).

GenBank currently (late 2012) contains ~150 Gbp (150 billion bp) of information in 160 million sequence files. Only original, experimentally-derived sequences can be submitted to GenBank. It is a redundant database, meaning that a particular sequence can be determined by independent research projects (cloning of a single gene or by genome sequencing projects). GenBank continues to grow at an exponential rate, doubling every 18 months. Presently, the major sources of submitted sequences are **genome projects** (complete sequencing of the full genetic material of an organism). Up to now, more than a thousand genomes have been sequenced, including our own genome. The **Human Genome Project** (www.ornl.gov/Human_Genome), i.e. the sequencing of the 3.2-Gbp human haploid genome (the 23 chromosomes) was finished in 2003. More precisely, only the gene-rich euchromatin region of the chromosomes (~90%) were sequenced because the highly repetitive so-called constitutive heterochromatin (around the centromere and the telomeres of the chromosomes) cannot be cloned. The human genome sequence, and in fact most of the genome sequences, are freely available in GenBank and in other databases (e.g. Ensemble, GenCard). GenBank is an **annotated database**, i.e. the sequences are supplemented with explanations or commentaries on its information content (including the coding region, the source of the sequence, and related publications). Nucleic acid sequences and any analysis derived from those sequences can be published only after they have been deposited in a freely accessible database. The main page of NCBI is shown in Figure, while a sequence entry is shown in Figure. An online example of a sequence record (that of the human hemoglobin beta chain) is accessible here.

Newly determined nucleotide sequences can be identified by and compared using the GenBank database (using the **BLAST** program), and the results of this analysis are GenBank files identified by an accession code (e.g. D32013 in Figure).



The NCBI homepage (http://www.ncbi.nlm.nih.gov/). A few databases that are mentioned in the text are marked.

Thermus aquaticus gene for DNA polymerase, complete cds

GenBank: D32013.1

```
LOCUS       TTHDNAP                 3026 bp    DNA     linear   BCT 26-JAN-2008
DEFINITION  Thermus aquaticus gene for DNA polymerase, complete cds.
ACCESSION   D32013
VERSION     D32013.1  GI:507890
KEYWORDS    .
SOURCE      Thermus aquaticus
  ORGANISM  Thermus aquaticus
            Bacteria; Deinococcus-Thermus; Deinococci; Thermales; Thermaceae;
            Thermus.
REFERENCE   1
  AUTHORS   Ishino,Y., Ueno,T., Miyagi,M., Uemori,T., Imamura,M., Tsunasawa,S.
            and Kato,I.
  TITLE     Overproduction of Thermus aquaticus DNA polymerase and its
            structural analysis by ion-spray mass spectrometry
  JOURNAL   J. Biochem. 116 (5), 1019-1024 (1994)
   PUBMED   7896728
     CDS             1..2499
                     /EC_number="2.7.7.7"
                     /db_xref="GI:507891"
                     /translation="MRGMLPLFEPKGRVLLVDGHHLAYRTFHALKGLTTSRGEPVQAV
                     YGFAKSLLKALKEDGDAVIVVFDAKAPSFRHEAYGGYKAGRAPTPEDFPRQLALIKEL
                     VDLLGLARLEVPGYEADDVLASLAKKAEKEGYEVRILTADKDLYQLLSDRIHALHPEG
                     YLITPAWLWEKYGLRPDQWADYRALTGDESDNLPGVKGIGEKTARKLLEEWGSLEALL
                     KNLDRLKPAIREKILAHMDDLKLSWDLAKVRTDLPLEVDFAKRREPDRERLRAFLERL
                     EFGSLLHEFGLLESPKALEEAPWPPPEGAFVGFVLSRKEPMWADLLALAAARGGRVHR
                     APEPYKALRDLKEARGLLAKDLSVLALREGLGLPPGDDPMLLAYLLDPSNTTPEGVAR
                     RYGGEWTEEAGERAALSERLFANLWGRLEGEERLLWLYREVERPLSAVLAHMEATGVR
                     LDVAYLRALSLEVAEEIARLEAEVFRLAGHPFNLNSRDQLERVLFDELGLPAIGKTEK
                     TGKRSTSAAVLEALREAHPIVEKILQYRELTKLKSTYIDPLPDLIHPRTGRLHTRFNQ
                     TATATGRLSSSDPNLQNIPVRTPLGQRIRRAFIAEEGWLLVALDYSQIELRVLAHLSG
                     DENLIRVFQEGRDIHTETASWMFGVPREAVDPLMRRAAKTINFGVLYGMSAHRLSQEL
                     AIPYEEAQAFIERYFQSFPKVRAWIEKTLEEGRRRGYVETLFGRRRYVPDLEARVKSV
                     REAAERMAFNMPVQGTAADLMKLAMVKLFPRLEEMGARMLLQVHDELVLEAPKERAEA
                     VARLAKEVMEGVYPLAVPLEVEVGIGEDWLSAKE"
ORIGIN
        1 atgaggggga tgctgcccct ctttgagccc aagggccggg tcctcctggt ggacggccac
       61 cacctggcct accgcacctt ccacgccctg aagggcctca ccaccagccg gggggagccg
      121 gtgcaggcgg tctacggctt cgccaagagc ctcctcaagg ccctcaagga ggacggggac
      181 gcggtgatcg tggtctttga cgccaaggcc ccctccttcc gccacgaggc ctacgggggg
      241 tacaaggcgg gccgggcccc cacgccggag gactttcccc ggcaactcgc cctcatcaag
      301 gagctggtgg acctcctggg gctggcgcgc ctcgaggtcc cgggctacga ggcggacgac
```

An example GenBank file (DNA polymerase from *Thermus aquaticus*; accession code D32013)

GenBank is part of the **Entrez** web portal (www.ncbi.nlm.nih.gov/sites/gquery), which is a powerful web tool to search for a large number of bioinformatics databases maintained by NCBI. **PubMed** (ncbi.nlm.nih.gov/pubmed) is a bibliography database of life sciences and biomedical topics (covering practically all scientific journals in biochemistry and molecular biology). It contains more than 20 million bibliographical records of biomedical publications including free abstracts. More and more open access articles are freely available on the original journal websites in html format or downloadable as pdf file (directly accessed via PubMed). The **Bookshelf** online library contains many university textbooks in a fully searchable format (e.g. Stryer: Biochemistry, Lodish et al.: Molecular Cell Biology, Alberts et al.: Molecular Biology of the Cell). Part of the search page of the Entrez portal is shown in Figure.

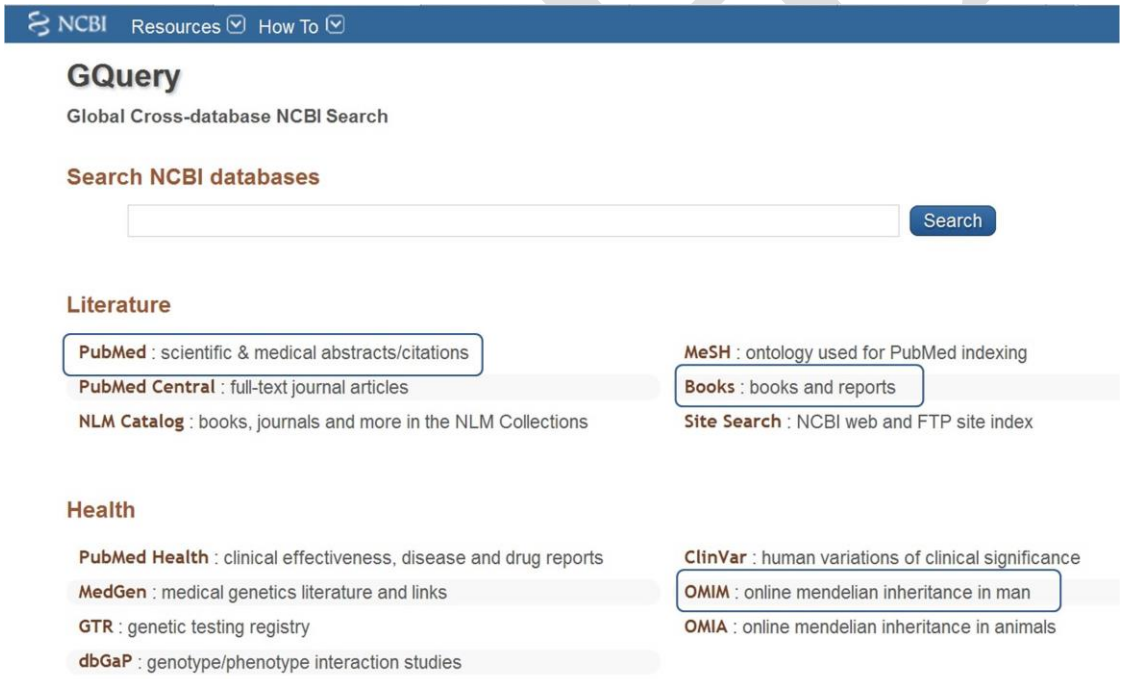


Figure 11.4. Search page of the Entrez portal (databases mentioned in the text are highlighted) (www.ncbi.nlm.nih.gov/sites/gquery)

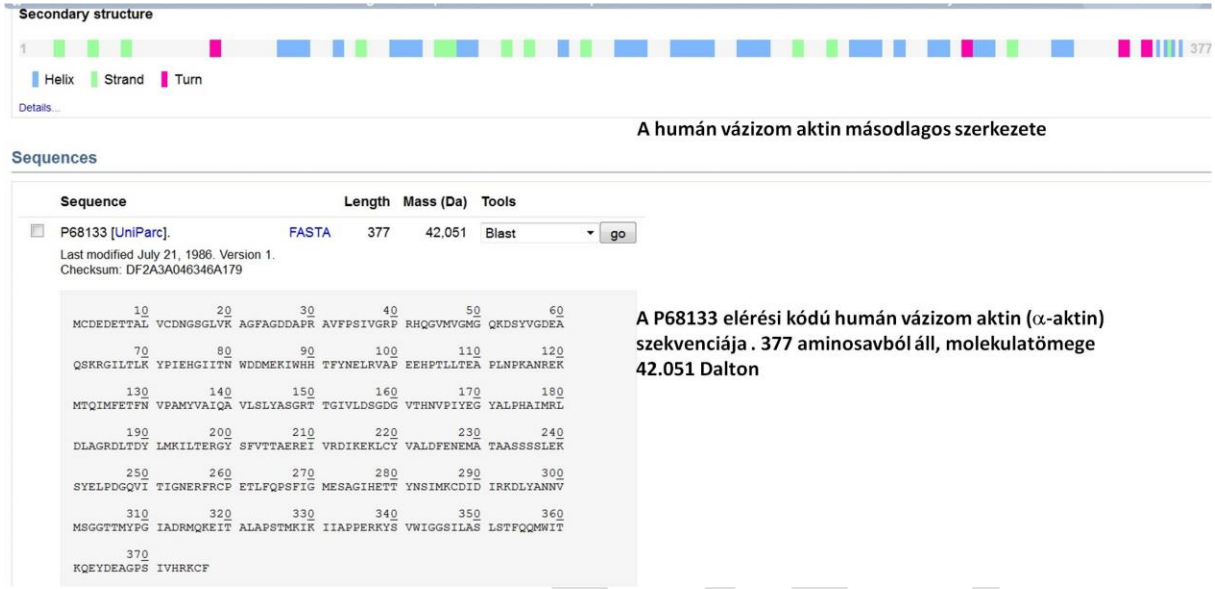
Among the databases accessible via the Entrez search engine, only the most important ones are mentioned here. **Genome** provides views for a variety of *genomes*, complete chromosomes, *sequence* maps from many organisms whose genome has been fully sequenced; **dbEST** (**E**xpressed **S**equence **T**ag) contains **cDNA (complementary DNA) sequences** that were reverse-transcribed from mRNA sequences (transcripts); **OMIM** (**O**nline **M**endelian **I**nheritance in **M**an) contains detailed, full-text, referenced overviews of all human Mendelian disorders (> 12,000 genes). The **Ensembl** (http://www.ensembl.org)database also contains genome sequences. It is maintained as a joint project by the European Bioinformatics Institute (EBI, http://www.ebi.ac.uk/), the European Molecular Biology Laboratories (EMBL) and the British non-profit Wellcome Trust Sanger Institute (named after the double Nobel prize laureate British scientist Frederick Sanger who developed protein as well as DNA sequencing methods). EMBL also maintains a nucleotide database (ENA: European Nucleotide Archive), which contains the same information as GenBank.

UniProt

UniProt (uniprot.org) is an annotated, non-redundant amino acid sequence database that actually consists of two sub-databases. The **Swissprot** division contains only experimentally validated and manually curated (annotated) protein sequences together with references to scientific publications (currently it contains more than 200 million amino acid residues in more than 500,000 annotated sequence files), while the **TrEMBL** division contains automatically translated sequences (currently more than 8 billion amino acid residues in approximately 24 million sequence files) from the EMBL nucleic acid database. Annotations of UniProt files include alternative versions of the particular sequence (alternatively spliced isoforms), other sequence variations (polymorphisms, mutations, sequence conflicts), information on the protein family to which the sequence belongs, structural and functional elements (motifs) of the polypeptide

sequence, posttranslational modifications, cross-references to other databases (nucleotide sequence, structural and secondary databases) and, finally, literature references. An important part of the annotation is the so-called Gene Ontology (GO), a standardised vocabulary of the gene product across species and databases. It covers three attributes of the protein: the **cellular component** is the biological localisation of the protein (the parts of a cell or extracellular environment); the **molecular function**describes the elementary activities at the molecular level, e.g. binding or catalysis; and finally the **biological process**, functions in integrated living units: cells, tissues, organs, and organisms. An example UniProt file is shown in Figure 11.5 and Figure 11.6 (human skeletal muscle α-actin with accession code P68133). Protein sequences are referred to using their accession number (six alphanumeric characters) in research publications. The reader is encouraged to read the following short tutorial about the use of the UniProt database.

Example of a UniProt record (name, general annotation) (http://www.uniprot.org/uniprot/P68133)

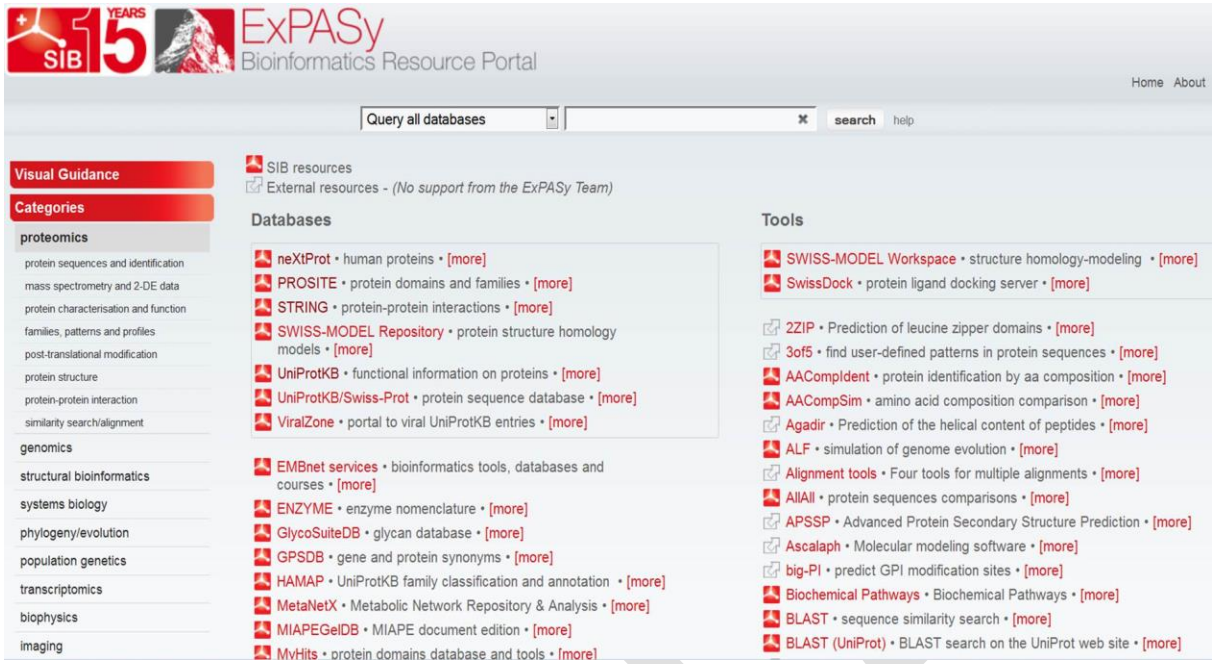


A humán vázizom aktin másodlagos szerkezete

A P68133 elérési kódú humán vázizom aktin (α-aktin) szekvenciája . 377 aminosavból áll, molekulatömege 42.051 Dalton

Example of a UniProt record (secondary structure and amino acid sequence) (http://www.uniprot.org/uniprot/P68133)

The UniProt database is part of the **ExPASy** (**Ex**pert **P**rotein **A**nalysis **Sy**stem; expasy.org/) bioinformatics resource portal, which provides access to scientific databases and software tools to different areas of life sciences including proteomics, genomics, transcriptomics and systems biology. Moreover, it is an entry point to many other secondary databases. For instance, proteomics tools include online programs of DNA-to-protein translation, calculation of the molecular mass and isoelectric point of proteins, prediction of structural and functional motifs, posttranslational modifications and three-dimensional structure. A screenshot of the portal is shown in Figure 11.7 (highlighted are databases and tools described in the text).



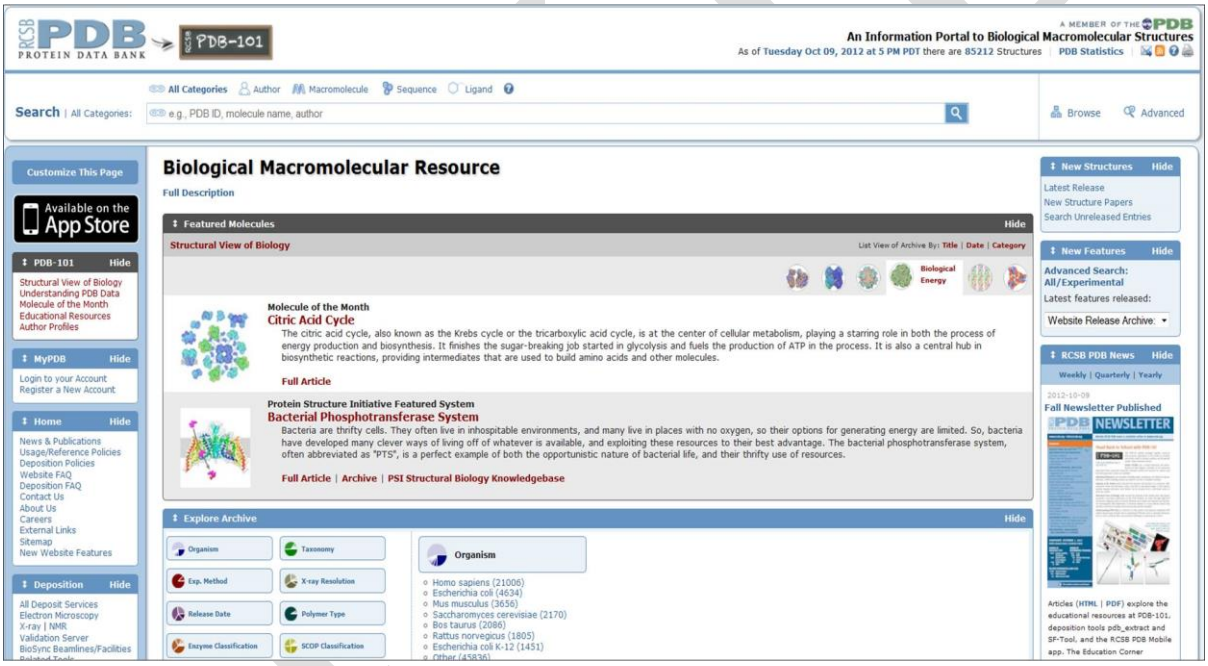
Screenshot of the ExPASy bioinformatics resource portal (www.expasy.org)

Protein Data Bank (PDB)

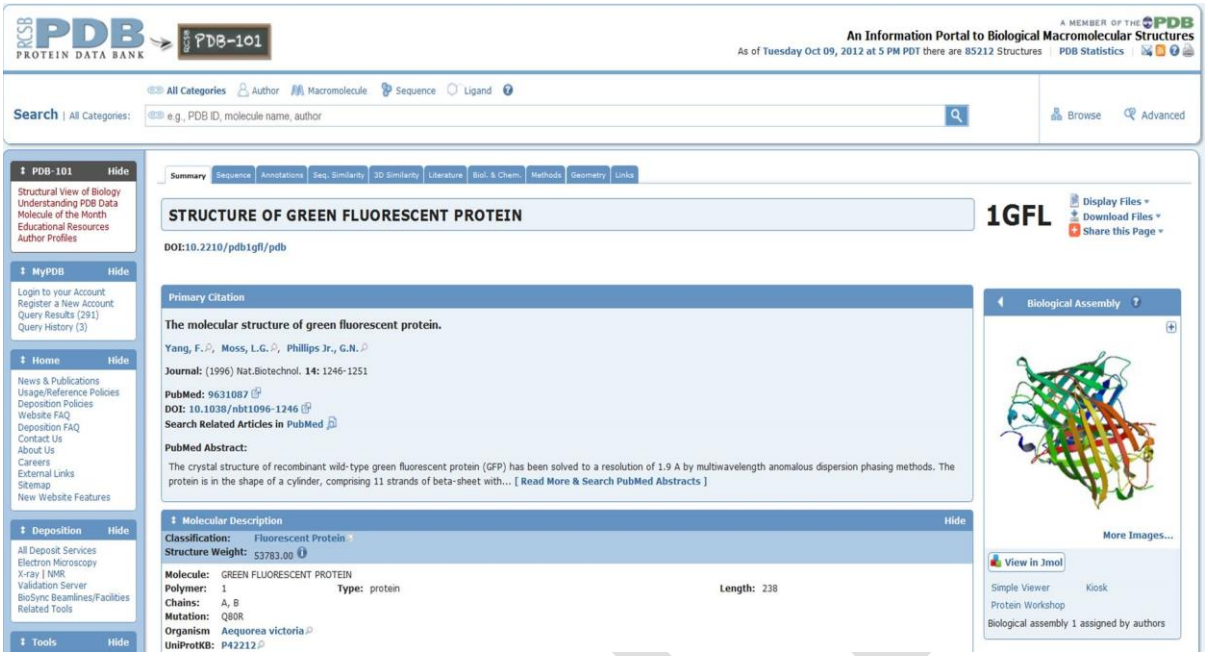
PDB (www.rcsb.org/pdb) is a database of experimentally-determined three-dimensional structures of proteins, nucleic acids and their complexes. Currently it stores nearly 80,000 structures determined by X-ray diffraction and approximately 10,000 structures determined by nuclear magnetic resonance (NMR) spectroscopy. (These two methods can be used to determine atomic-resolution structures of biological macromolecules.) The annotated PDB files contain additional useful information beyond the Cartesian atomic coordinates of the three-dimensional structures. The main page of the PDB website and details of an entry are shown in Figure 11.8 and Figure 11.9. PDB entries have a unique identification code consisting of a number and three letters (e.g. 1GFL is the PDB code of a Green Fluorescent Protein structure shown in Figure 11.9). Three-dimensional structures can be visualised online by using the **Jmol** applet (integrated

into web browsers; see in Chapter 11.4.3). Alternatively, the PDB file can be downloaded and utilised by any of the freely available (open-source) molecular graphics programs.

Molecule of the Month regularly describes the structure and function of an interesting or important molecule. It is part of the PDB-101 interface, an educational resource for exploring a structural view of biology. It is highly recommended to download and study the poster "Molecular Machinery: Tour of the Protein Data Bank" that illustrates 80 PDB entries (enzymes, membrane proteins, motor proteins, DNA-binding proteins, protein complexes such as ribosomes) alongside water and ATP at a scale of one to three million.



Web page of PDB (http://www.rcsb.org), a repository for three-dimensional structural data of large biological molecules such as proteins and nucleic acids



Details of a PDB entry (Green Fluorescent Protein) (http://www.rcsb.org)

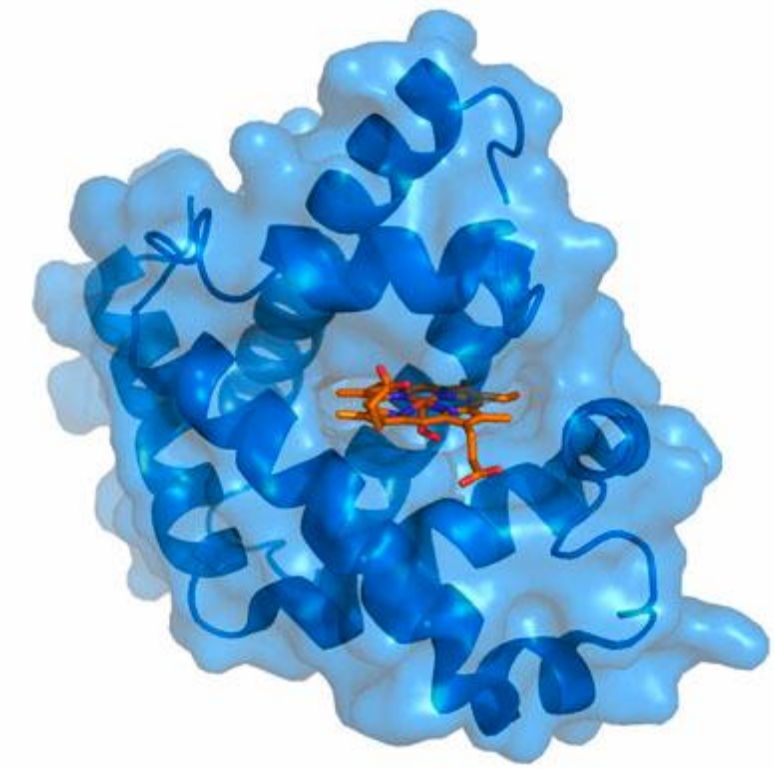
UNIT-V

SYLLABUS

Specialized Proteins and its Applications : Myoglobin and haemoglobin - Oxygen binding curves, influence of 2,3-BPG, CO2 and Cl^- Hill plot. Cooperativity between subunits and models to explain the phenomena –concerted and sequential models. Haemoglobin disorders. Antibodies - Antibody structure and binding to antigens. Actin-myosin motors - ATP activated actin - myosin contractions. Membrane Proteins - Integral and membrane associated proteins. Hydropathy plots to predict transmembrane domains. Significance of membrane proteins - bacteriorhodopsin.

Myoglobin

Myoglobin and hemoglobin are hemeproteins whose physiological importance is principally related to their ability to bind molecular oxygen. Myoglobin is a monomeric heme protein found mainly in muscle tissue where it serves as an intracellular storage site for oxygen. During periods of oxygen deprivation **oxymyoglobin** releases its bound oxygen which is then used for metabolic purposes. The tertiary structure of myoglobin is that of a typical water soluble globular protein. Its secondary structure is unusual in that it contains a very high proportion (75%) of α-helical secondary structure. A myoglobin polypeptide is comprised of 8 separate right handed α-helices, designated A through H, that are connected by short non helical regions. Amino acid R-groups packed into the interior of the molecule are predominantly hydrophobic in character while those exposed on the surface of the molecule are generally hydrophilic, thus making the molecule relatively water soluble.



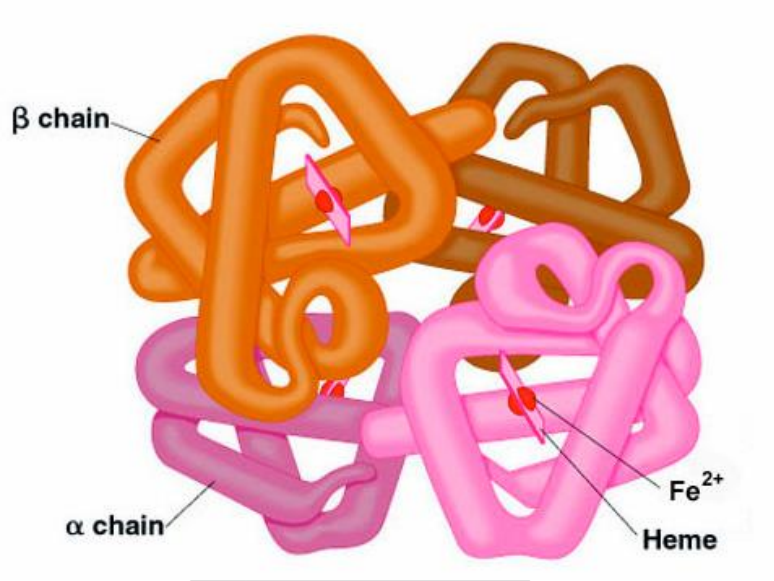
Structure of Myoglobin with Heme

Each myoglobin molecule contains one heme prosthetic group inserted into a hydrophobic cleft in the protein. Each heme residue contains one central coordinately bound iron atom that is normally in the Fe2+, or ferrous, oxidation state. The oxygen carried by hemeproteins is bound directly to the ferrous iron atom of the heme prosthetic group. Oxidation of the iron to the Fe3+, ferric, oxidation state renders the molecule incapable of normal oxygen binding. Hydrophobic interactions between the tetrapyrrole ring and hydrophobic amino acid R groups on the interior of the cleft in the protein strongly stabilize the heme protein conjugate. In addition a nitrogen atom from a histidine R group located above the plane of the heme ring is coordinated with the iron atom further stabilizing the interaction between the heme and the protein. In oxymyoglobin the remaining bonding site on the iron atom (the 6th coordinate position) is occupied by the oxygen, whose binding is stabilized by a second histidine residue. Carbon monoxide also binds

coordinately to heme iron atoms in a manner similar to that of oxygen, but the binding of carbon monoxide to heme is much stronger than that of oxygen. The preferential binding of carbon-monoxide to heme iron is largely responsible for the asphyxiation that results from carbon monoxide poisoning.

Hemoglobin

Adult hemoglobin is a [α(2):β(2)] tetrameric hemeprotein found in erythrocytes where it is responsible for binding oxygen in the lung and transporting the bound oxygen throughout the body where it is used in aerobic metabolic pathways.



Structure of Hemoglobin

For a description of the different types of hemoglobin tetramers see the section below on Hemoglobin Genes. Each subunit of a hemoglobin tetramer has a heme prosthetic group identical to that described for myoglobin. The common peptide subunits are designated α, β, γ and δ which are arranged into the most commonly occurring functional hemoglobins. Although the secondary and tertiary structure of various hemoglobin subunits are similar, reflecting extensive

homology in amino acid composition, the variations in amino acid composition that do exist impart marked differences in hemoglobin's oxygen carrying properties. In addition, the quaternary structure of hemoglobin leads to physiologically important allosteric interactions between the subunits, a property lacking in monomeric myoglobin which is otherwise very similar to the α-subunit of hemoglobin. Comparison of the oxygen binding properties of myoglobin and hemoglobin illustrate the allosteric properties of hemoglobin that results from its quaternary structure and differentiate hemoglobin's oxygen binding properties from that of myoglobin. The curve of oxygen binding to hemoglobin is sigmoidal typical of allosteric proteins in which the substrate, in this case oxygen, is a positive homotropic effector. When oxygen binds to the first subunit of deoxyhemoglobin it increases the affinity of the remaining subunits for oxygen. As additional oxygen is bound to the second and third subunits oxygen binding is further, incrementally, strengthened, so that at the oxygen tension in lung alveoli, hemoglobin is fully saturated with oxygen. As oxyhemoglobin circulates to deoxygenated tissue, oxygen is incrementally unloaded and the affinity of hemoglobin for oxygen is reduced. Thus at the lowest oxygen tensions found in very active tissues the binding affinity of hemoglobin for oxygen is very low allowing maximal delivery of oxygen to the tissue. In contrast the oxygen binding curve for myoglobin is hyperbolic in character indicating the absence of allosteric interactions in this process. The allosteric oxygen binding properties of hemoglobin arise directly from the interaction of oxygen with the iron atom of the heme prosthetic groups and the resultant effects of these interactions on the quaternary structure of the protein. When oxygen binds to an iron atom of deoxyhemoglobin it pulls the iron atom into the plane of the heme. Since the iron is also bound to histidine F8, this residue is also pulled toward the plane of the heme ring. The conformational change at histidine F8 is transmitted throughout the peptide backbone resulting in a significant change in tertiary structure of the entire subunit. Conformational changes at the subunit surface lead to a new set of binding interactions between adjacent subunits. The latter

changes include disruption of salt bridges and formation of new hydrogen bonds and new hydrophobic interactions, all of which contribute to the new quaternary structure.

The latter changes in subunit interaction are transmitted, from the surface, to the heme binding pocket of a second deoxy subunit and result in easier access of oxygen to the iron atom of the second heme and thus a greater affinity of the hemoglobin molecule for a second oxygen molecule. The tertiary configuration of low affinity, deoxygenated hemoglobin (Hb) is known as the taut (T) state. Conversely, the quaternary structure of the fully oxygenated high affinity form of hemoglobin (HbO2) is known as the relaxed (R) state. In the context of the affinity of hemoglobin for oxygen there are four primary regulators, each of which has a negative impact. These are CO2, hydrogen ion (H+), chloride ion (Cl–), and 2,3-bisphosphoglycerate (2,3BPG, or also just BPG). Some older texts abbreviate 2,3BPG as DPB. Although they can influence O2 binding independent of each other, CO2, H+ and Cl– primarily function as a consequence of each other on the affinity of hemoglobin for O2. We shall consider the transport of O2 from the lungs to the tissues first. In the high O2 environment (high pO2) of the lungs there is sufficient O2 to overcome the inhibitory nature of the T state. During the O2 binding-induced alteration from the T form to the R form several amino acid side groups on the surface of hemoglobin subunits will dissociate protons as depicted in the equation below. This proton dissociation plays an important role in the expiration of the CO2 that arrives from the tissues (see below). However, because of the high pO2, the pH of the blood in the lungs (≈7.4 – 7.5) is not sufficiently low enough to exert a negative influence on hemoglobin binding O2. When the oxyhemoglobin reaches the tissues the pO2 is sufficiently low, as well as the pH (≈7.2), that the T state is favored and the O2 released.

4O2 + Hb <——> nH+ + Hb(O2)4

If we now consider what happens in the tissues, it is possible to see how CO2, H+, and Cl– exert their negative effects on hemoglobin binding O2. Metabolizing cells produce CO2 which diffuses into the blood and enters the circulating red blood cells (RBCs). Within RBCs the CO2

is rapidly converted to carbonic acid through the action of carbonic anhydrase as shown in the equation below:

$CO_2 + H_2O$ ——> H_2CO_3 ——> $H^+ + HCO_3^-$

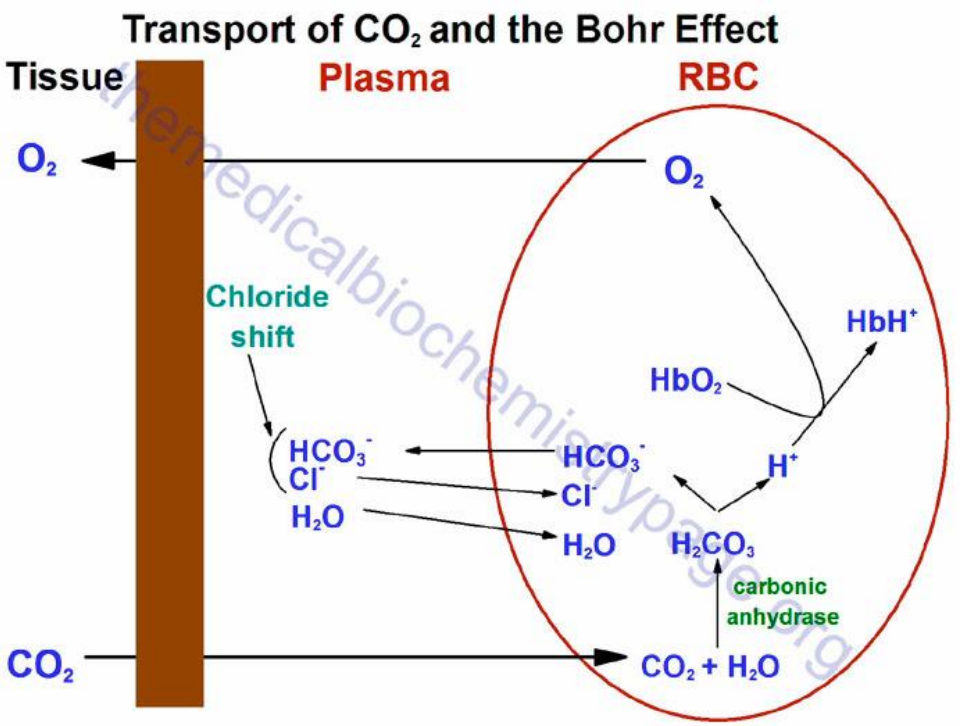
The bicarbonate ion produced in this dissociation reaction diffuses out of the RBC and is carried in the blood to the lungs. This effective CO_2 transport process is referred to as isohydric transport. Approximately 80% of the CO_2 produced in metabolizing cells is transported to the lungs in this way. A small percentage of CO_2 is transported in the blood as a dissolved gas. In the tissues, the H^+ dissociated from carbonic acid is buffered by hemoglobin which exerts a negative influence on O_2 binding forcing release to the tissues. As indicated above, within the lungs the high pO_2 allows for effective O_2 binding by hemoglobin leading to the T to R state transition and the release of protons. The protons combine with the bicarbonate that arrived from the tissues forming carbonic acid which then enters the RBCs. Through a reversal of the carbonic anhydrase reaction, CO_2 and H_2O are produced. The CO_2 diffuses out of the blood, into the lung alveoli and is released on expiration.

In addition to isohydric transport, as much as 15% of CO_2 is transported to the lungs bound to N-terminal amino groups of the T form of hemoglobin. This reaction, depicted below, forms what is called **carbaminohemoglobin**. As indicated this reaction also produces H^+, thereby lowering the pH in tissues where the CO_2 concentration is high. The formation of H^+ leads to release of the bound O_2 to the surrounding tissues. Within the lungs, the high O_2 content results in O_2 binding to hemoglobin with the concomitant release of H^+. The released protons then promote the dissociation of the carbamino to form CO_2 which is then released with expiration.

CO_2 + Hb-NH_2 <——> H^+ + Hb-NH-COO^-

As the above discussion demonstrates, the conformation of hemoglobin and its oxygen binding are sensitive to hydrogen ion concentration. These effects of hydrogen ion concentration are responsible for the well known **Bohr effect** in which increases in hydrogen ion concentration

decrease the amount of oxygen bound byhemoglobin at any oxygen concentration (partial pressure). Coupled to the diffusion of bicarbonate out of RBCs in the tissues there must be ion movement into the RBCs to maintain electrical neutrality. This is the role of Cland is referred to as the **chloride shift**. In this way, Cl– plays an important role in bicarbonate production and diffusion and thus also negatively influences O2 binding to hemoglobin.

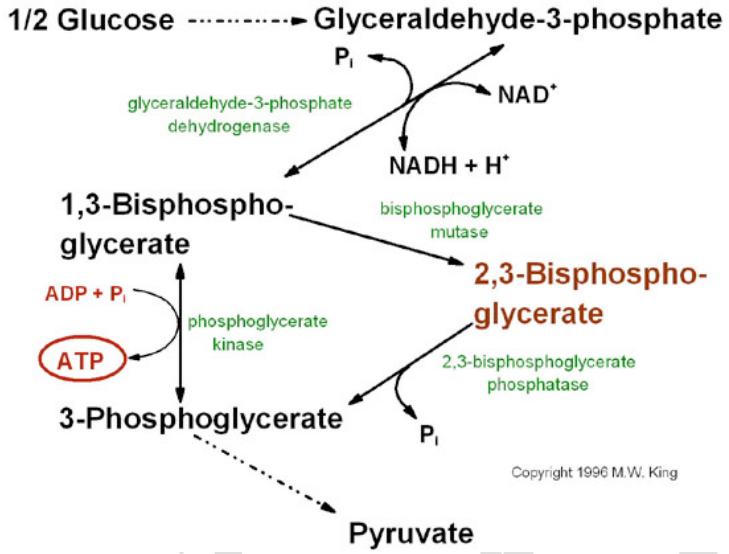


Representation of the transport of CO2 from the tissues to the blood with delivery of O2 to the tissues. The opposite process occurs when O2 is taken up from the alveoli of the lungs and the CO2 is expelled. All of the processes of the transport of CO2 and O2 are not shown such as the formation and ionization of carbonic acid in the plasma. The latter is a major mechanism for the transport of CO2 to the lungs, i.e. in the plasma as HCO3 –. The H+ produced in the plasma by the ionization of carbonic acid is buffered by phosphate (HPO42–) and by proteins.

Additionally, some 15% of the CO2 is transported from the tissues to the lungs as hemoglobin carbamate.

Role of 2,3-bisphosphoglycerate (2,3-BPG)

The compound 2,3-bisphosphoglycerate (2,3-BPG), derived from the glycolytic intermediate 1,3-bisphosphoglycerate, is a potent allosteric effector on the oxygen binding properties of hemoglobin. The pathway to 2,3BPG synthesis is diagrammed in the figure below.



The pathway for 2,3-bisphosphoglycerate (2,3-BPG) synthesis within erythrocytes. Synthesis of 2,3-BPG represents a major reaction pathway for the consumption of glucose in erythrocytes. The synthesis of 2,3-BPG in erythrocytes is critical for controlling hemoglobin affinity for oxygen. Note that when glucose is oxidized by this pathway the erythrocyte loses the ability to gain 2 moles of ATP from glycolytic oxidation of 1,3-BPG to 3-phosphoglycerate via the phosphoglycerate kinase reaction.In the deoxygenated T conformer, a cavity capable of binding 2,3-BPG forms in the center of the molecule.

2,3-BPG can occupy this cavity stabilizing the T state. Conversely, when 2,3-BPG is not available, or not bound in the central cavity, Hb can be converted to HbO2 more readily. Thus,

like increased hydrogen ion concentration, increased 2,3-BPG concentration favors conversion of R form Hb to T form Hb and decreases the amount of oxygen bound by Hb at any oxygen concentration. Hemoglobin molecules differing in subunit composition are known to have different 2,3-BPG binding properties with correspondingly different allosteric responses to 2,3-BPG. For example, HbF (the fetal form of hemoglobin) binds 2,3-BPG much less avidly than HbA (the adult form of hemoglobin) with the result that HbF in fetuses of pregnant women binds oxygen with greater affinity than the mothers HbA, thus giving the fetus preferential access to oxygen carried by the mothers circulatory system.

The Hemoglobin Genes

The α- and β-globin proteins contained in functional hemoglobin tetramers are derived from gene clusters. The α-globin genes are on chromosome 16 and the β-globin genes are on chromosome 11. Both gene clusters contain not only the major adult genes, α and β, but other expressed sequences that are utilized at different stages of development. The orientation of the genes in both clusters is in the same 5' to 3' direction with the earliest expressed genes at the 5' end of both clusters. In addition to functional genes, both clusters contain non-functional pseudogenes.

Hemoglobin synthesis begins in the first few weeks of embryonic development within the yolk sac. The major hemoglobin at this stage of development is a tetramer composed of 2 zeta (ζ) chains encoded within the α cluster and 2 epsilon (ε) chains from the β cluster. By 6-8 weeks of gestation the expression of this version of hemoglobin declines dramatically coinciding with the change in hemoglobin synthesis from the yolk sac to the liver. Expression from the α cluster consists of identical proteins from the α1 and α2 genes. Expression from these genes in the α cluster remains on throughout life. Within the β-globin cluster there is an additional set of genes, the fetal β-globin genes identified as the gamma (γ) genes. The 2 fetal genes called Gγ and Aγ, the derivation of which stems from the single amino acid difference between the 2 fetal genes: glycine in Gγ and alanine in Aγ at position 136. These fetal γ genes are expressed as the embryonic genes are turned off. Shortly before birth there is a smooth switch from fetal γ-globin

gene expression to adult β-globin gene expression. The switch from fetal γ- to adult β-globin does not directly coincide with the switch from hepatic synthesis to bone marrow synthesis since at birth it can be shown that both γ and β synthesis is occurring in the marrow. Given the pattern of globin gene activity throughout fetal development and in the adult the composition of the hemoglobin tetramers is of course distinct. Fetal hemoglobin is identified as HbF and includes both α2Gγ2 and α2Aγ2. Fetal hemoglobin has a slightly higher affinity for oxygen than does adult hemoglobin. This allows the fetus to extract oxygen more efficiently from the maternal circulation. In adults the major hemoglobin is identified as HbA (more commonly HbA1) and is a tetramer of 2 α and 2 β chains as indicated earlier. A minor adult hemoglobin, identified as HbA2, is a tetramer of 2 α chains and 2 δ chains. The δ gene is expressed with a timing similar to the β gene but because the promoter has acquired a number of mutations its' efficiency of transcription is reduced.The overall hemoglobin composition in a normal adult is approximately 97.5% HbA1, 2% HbA2 and 0.5% HbF.

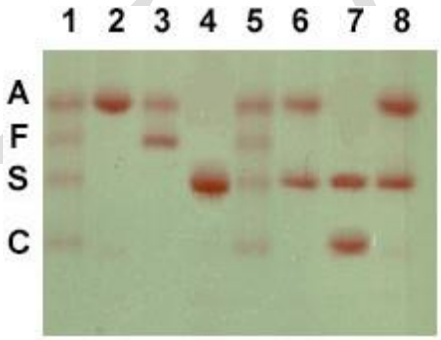
Hemoglobinopathies

A large number of mutations have been described in the globin genes. These mutations can be divided into two distinct types: those that cause **qualitative** abnormalities (e.g. sickle cell anemia) and those that cause **quantitative** abnormalities (the thalassemias). Taken together these disorders are referred to as the **hemoglobinopathies**. A third group of hemoglobin disorders include those diseases in which there is a persistence of fetal hemoglobin expression. These latter diseases are known collectively as hereditary persistence of fetal hemoglobin (HPFH).

Of the mutations leading to qualitative alterations in hemoglobin, the missense mutation in the β-globin gene that causes sickle cell anemia is the most common. The mutation causing sickle cell anemia is a single nucleotide substitution (A to T) in the codon for amino acid 6. The change converts a glutamic acid codon (GAG) to a valine codon (GTG). The form of hemoglobin in persons with sickle cell anemia is referred to as HbS.

The underlying problem in sickle cell anemia is that the valine for glutamic acid substitution results in hemoglobin tetramers that aggregate into arrays upon deoxygenation in the tissues. This aggregation leads to deformation of the red blood cell making it relatively inflexible and unable to traverse the capillary beds. Repeated cycles of oxygenation and deoxygenation lead to irreversible sickling. The end result is clogging of the fine capillaries. Because bones are particularly affected by the reduced blood flow, frequent and severe bone pain results. This is the typical symptom during a sickle cell "crisis". Long term the recurrent clogging of the capillary beds leads to damage to the internal organs, in particular the kidneys, heart and lungs. The continual destruction of the sickled red blood cells leads to chronic anemia and episodes of hyperbilirubinemia. An additional relatively common mutation at codon 6 is the conversion to a lysine codon (AAG) which results in the generation of HbC.

Electrophoresis of hemoglobin proteins from individuals suspected of having sickle cell anemia (or several other types of hemoglobin disorders) is an effective diagnostic tool because the variant hemoglobins have different charges. An example of this technique is shown in the Figure below.



Pattern of hemoglobin electrophoresis from several different individuals. Lanes 1 and 5 are hemoglobin standards. Lane 2 is a normal adult. Lane 3 is a normal neonate. Lane 4 is a homozygous HbS individual. Lanes 6 and 8 are heterozygous sickle individuals. Lane 7 is a SC disease individual.

Another effective tool to identify the genotype of individuals suspected of having sickle cell disease as well as for prenatal diagnosis is to either carry out RFLP mapping or to use PCR. An example of the use of these tools can be seen in the Molecular Tools of Medicine page.

In addition to the missense mutations that lead to HbS and HbC, a number of frameshift mutations leading to qualitative abnormalities in hemoglobin have been identified. A 2-nucleotide insertion between codons 144 and 145 in the β-globin gene results in the generation of **hemoglobin Cranston**. The insertion, which is near the C-terminus of the β-globin protein, results in the normal stop codon being out of frame and synthesis proceeding into the 3'-untranslated region to a fortuitous stop codon. The result is a β-globin protein of 157 amino acids. In the **hemoglobin Constant Spring** variant, a mutation in the α-globin gene converts the stop codon (UAA) to a glutamine codon (CAA) so that the protein ends up being 31 amino acids longer than normal. The resultant α-globin protein in hemoglobin Constant Spring is not only qualitatively altered but because it is unstable it is a quantitative abnormality as well.

Because the globin gene loci contain clusters of similar genes there is the potential for unequal cross-over between the sister chromatids during meiosis. The generation of **hemoglobin Gun Hill** and **Lepore hemoglobins** are both the result of unequal cross over events. Hemoglobin Gun Hill is the result of a deletion of 15 nucleotides caused by unequal cross over between codons 91–94 of one β-globin gene and codons 96–98 of the other. Generation of Lepore hemoglobins results from unequal cross over between the δ-globin and β-globin genes. The resultant hybrid δβ gene is called Lepore and the βδ hybrid gene is called anti-Lepore. As indicated earlier, the promoter of the δ-globin gene is inefficient so the consequences of this unequal cross over event are both qualitative and quantitative.

The **thalassemias** are the result of abnormalities in hemoglobin synthesis and affect both clusters. Deficiencies in β-globin synthesis result in the β-thalassemias and deficiencies in α-globin synthesis result in the α-thalassemias. The term thalassemia is derived from the Greek

thalassa meaning "sea" and was applied to these disorders because of the high frequency of their occurrence in individuals living around the Mediterranean Sea.

In normal individuals an equal amount of both α- and β-globin proteins are made allowing them to combine stoichiometrically to form the correct hemoglobin tetramers. In the α-thalassemias normal amounts of β-globin are made. The β-globin proteins are capable of forming homotetramers (β4) and these tetramers are called **hemoglobin H**, (HbH). An excess of HbH in red blood cells leads to the formation if inclusion bodies commonly seen in patients with α-thalassemia. In addition, the HbH tetramers have a markedly reduced oxygen carrying capacity. In β-thalassemia, where the β-globins are deficient, the α-globins are in excess and will form α-globin homotetramers. The α-globin homotetramers are extremely insoluble which leads to premature red cell destruction in the bone marrow and spleen.

With the α-thalassemias the level of α-globin production can range from none to very nearly normal levels. This is due in part to the fact that there are 2 identical α-globin genes on chromosome 16. Thus, the α-thalassemias involve inactivation of 1 to all 4 α-globin genes. If 3 of the 4 α-globin genes are functional, individuals are completely asymptomatic. This situation is identified as the "silent carrier" state or sometimes as α-thalassemia 2. Genotypically this situation is designated αα/α– (where the dash indicates a non-functional gene) or α–/αα. If 2 of the 4 genes are inactivated individuals are designated as **α-thalassemia trait** or as α-thalassemia 1. Genotypically this situation is designated αα/– –. In individuals of African descent with α-thalassemia 1, the disorder usually results from the inactivation of 1 α-globin gene on each chromosome and is designated α–/α–. This means that these individuals are homozygous for the α-thalassemia 2 chromosome. The phenotype of α-thalassemia 1 is relatively benign. The mean red cell volume (designated MCV in clinical tests) is reduced in α-thalassemia 1 but individuals are generally asymptomatic. The clinical situation becomes more severe if only 1 of the 4 α-globin genes is functional. Because of the dramatic reduction in α-globin chain production in this latter situation, a high level of β4 tetramer is present. clinically this is referred to as **hemoglobin**

H disease. Afflicted individuals have moderate to marked anemia and their MCV is quite low, but the disease is not fatal. The most severe situation results when no α-globin chains are made (genotypically designated – –/– –). This leads to prenatal lethality or early neonatal death. The predominant fetal hemoglobin in afflicted individuals is a tetramer of γ-chains and is referred to as **hemoglobin Bart's**. This hemoglobin has essentially no oxygen carrying capacity resulting in oxygen starvation in the fetal tissues. Heart failure results as the heart tries to pump the unoxygenated blood to oxygen starved tissues leading to marked edema. This latter situation is called **hydrops fetalis**.

A large number of mutations have been identified leading to decreased or absent production of β-globin chains resulting in the β-thalassemias. In the most severe situation mutations in both the maternal and paternal β-globin genes leads to loss of normal amounts of β-globin protein. A complete lack of HbA is denoted as β0-thalassemia. If one or the other mutations allows production of a small amount of functional β-globin then the disorder is denoted as β+-thalassemia. Both β0- and β+-thalassemias are referred to as **thalassemia major**, also called **Cooley's anemia** after Dr. Thomas Cooley who first described the disorder. Afflicted individuals suffer from severe anemia beginning in the first year of life leading to the need for blood transfusions. As a consequence of the anemia the bone marrow dramatically increases its' effort at blood production. The cortex of the bone becomes thinned leading to pathologic fracturing and distortion of the bones in the face and skull. In addition, there is marked hepatosplenomegaly as the liver and spleen act as additional sites of blood production. Without intervention these individuals will die within the decade of life. As indicated, β-thalassemia major patient require blood transfusions, however, in the long term these transfusions lead to the accumulation of iron in the organs, particularly the heart, liver and pancreas. Organ failure ensues with death in the teens to early twenties. Iron chelation therapies appear to improve the outlook for β-thalassemia major patients but this requires continuous infusion of the chelating agent.

Individuals heterozygous for β-thalassemia have what is termed **thalassemia minor**. Afflicted individuals harbor one normal β-globin gene and one that harbors a mutation leading to production of reduced or no β-globin. Individuals that do not make any functional β-globin protein from 1 gene are termed βo heterozygotes. If β-globin production is reduced at one locus the individuals are termed β+ heterozygotes. Thalassemia minor individuals are generally asymptomatic. The term **thalassemia intermedia** is used to designate individuals with significant anemia and who are symptomatic but unlike thalassemia major do not require transfusions. This syndrome results in individuals where both β-globin genes express reduced amounts of protein or where one gene makes none and the other makes a mildly reduced amount. A person who is a compound heterozygote with α-thalassemia and β+-thalassemia will also manifest as thalassemia intermedia. The primary cause of α-thalassemias is deletion, whereas, for β-thalassemias the mutations are more subtle. In β-thalassemias, point mutations in the promoter, mutations in the translational initiation codon, a point mutation in the polyadenylation signal and an array of mutations leading to splicing abnormalities have been characterized.

An interesting and common (up to 30% of persons from Southeast Asia) hemoglobinopathy that has both quantitative and qualitative characteristics is caused by the synthesis of hemoglobin E. Hemoglobin E arises due to a point mutation in codon 26 that changes glutamic acid (GAG) to lysine (AAG). Individulas with this mutation make only around 60% of the normal amount of β-globin protein. The reason for this is that the mutation creates a cryptic splice site such that 40% of the hemoglobin E mRNA is shorter by 16 nucleotides and does not give rise to detectable β-globin protein. There are some individuals in whom the developmental timing of globin production is altered as a consequence of mutation. Persons with hereditary persistence of fetal hemoglobin, HPFH continue to make HbF as adults. Because the syndrome is benign most individuals do not even know they carry a hemoglobin abnormality. Many HPFH individuals harbor large deletions of the δ- and β-coding region of the cluster.

There is no deletion of the fetal globin genes and by an as yet uncharacterized mechanism expression of these genes persists in adulthood.

As discussed above functional hemoglobin is a heterotetramer. Mutations in either the α-globin or the β-globin genes lead to quantitative and qualitative abnormalities in hemoglobin. Therefore, it should not be surprising that complex compound heterozygosities can result in offspring of individuals harboring different mutations.