LAB #3: Electorphoretic Separation of Proteins

Objectives:

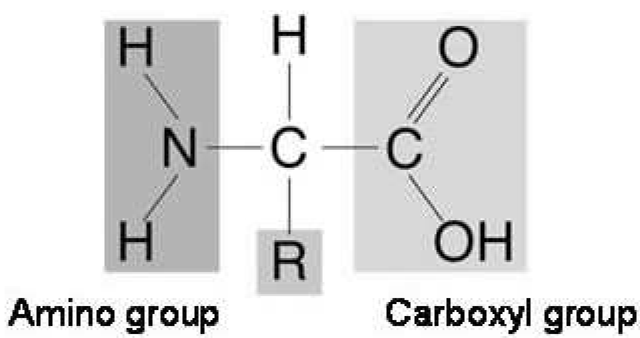
- Understand the isoelectric point of proteins.
- Identify the principles of electrophoresis.
- Given an electrophoretic buffer and the isoelectric point of specific proteins, predict the migration of a protein in an electric field.
- Separate four proteins according to their net charge using electrophoresis.

Introduction:

Proteins occupy a central position in the structure and function of all living organisms. Some proteins serve as structural components while others function in communication, defense, and cell regulation. The enzyme proteins act as biological catalysts which control the pace and nature of essentially all biochemical events. Indeed, although DNA serves as the genetic blueprint of a cell, none of the life processes would be possible without proteins.

The fundamental unit of proteins is the amino acid. The common amino acids have the general structure shown in **Figure 1**. Each amino acid has an amino group (NH_2) and a carboxyl acid group (COOH) attached to a central carbon atom called the alpha carbon (α). Also attached to the alpha carbon are a hydrogen atom and an R-group or side chain.

Figure 1



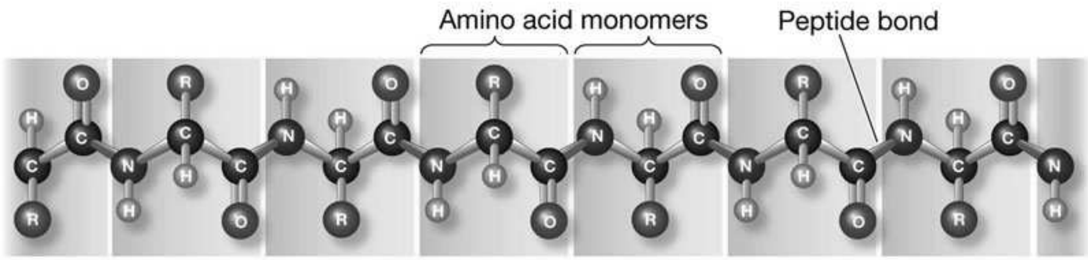
There are twenty amino acids commonly found in proteins and these differ from each other in the nature of the R-groups attached to the alpha carbon. A convenient classification of amino acids depends on the number of acidic and basic groups that are present. Thus, the neutral amino acids contain one amino acid one carboxyl group. **Table 1** lists the major amino acids found in proteins.

Table 1

Acidic Amino Acids	Basic Amino Acids	Neutral Amino Acids		
aspartic acid	arginine	glycine	methionine	leucine
asparagine	lysine	valine	tyrosine	serine
glutamic acid	histidine	isoleucine	proline	cysteine
glutamine		threonine	alanine	phenylalanine
			tryptophan	

Proteins are composed of amino acids linked into chains by peptide bonds as shown in **Figure 2**. Two amino acids joined by a single peptide bonds form a dipeptide; three amino acids form a tripeptide; and a large number of amino acids joined together constitute a polypeptide. A protein is a polypeptide chain that contains more than 50-100 amino acids. The monomer units in the chain are known as amino acid residues. The average protein contains about 350 amino acid residues although proteins with as many 1000 residues and those with as few as 100 are not uncommon.

Figure 2



The sequence of order of amino acids along a polypeptide chain is referred to as the primary structure of the proteins. The primary structure of the protein myoglobin is given in **Figure 3**. This protein serves to bind and store oxygen in muscle. The primary structure of over 500 proteins is now known.

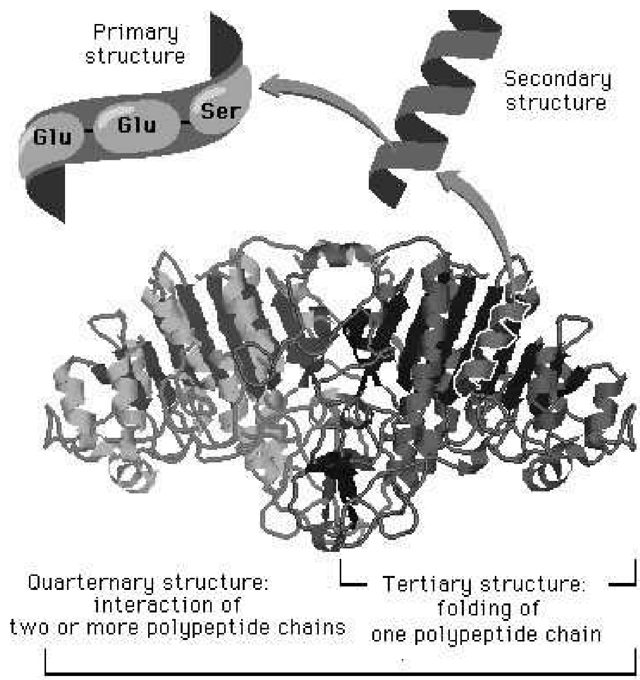
Figure 3

Val-Leu-Ser-Glu-Gly-Glu-Trp-Gln-Leu-Val-Leu-His-Val-Tyr-Ala-Lys-Val-Glu-Ala-Asp-Val-Ala-Gly-His-Gly-Gln-Asp-Ile-Leu-Ile-Arg-Leu-Phe-Lys-Ser-His-Pro-Glu-Thr-Leu-Glu-Lys-Phe-Asp-Arg-Phe-Lys-His-Leu-Lys-Thr-Glu-Ala-Glu-Met-Lys-Ala-Ser-Glu-Asp-Leu-Lys-Gly-His-His-Glu-Ala-Glu-Leu-Thr-Ala-Leu-Gly-Ala-Ile-Leu-Lys-Lys-Lys-Gly-His-Glu-Ala-Glu-Lys-Leu-Lys-Pro-Leu-Ala-Gln-Ser-His-Ala-Thr-Lys-His-Lys-Ile-Pro-Ile-Lys-Tyr-Leu-Glu-Phe-Ile-Ser-Glu-Ala-Ile-Ile-His-Val-Leu-His-Ser-Arg-His-Pro-Gly-Asn-Phe-Gly-Ala-Asp-Ala-Gln-Gly-Ala-Met-Asn-Lys-Ala-Leu-Glu-Leu-Phe-Arg-Lys-Asp-Ile-Ala-Ala-Lys-Tyr-Lys-Glu-Leu-Tyr-Gln-Gly

In the cell, the polypeptide chain is folded into a highly ordered shape or conformation. Most proteins are globular in shape and these proteins are usually soluble in water or in aqueous media containing salts. This group includes the enzymes, antibodies, and a variety of other proteins. Less frequently, proteins are long and fibrous and most of these elongated molecules are insoluble in water and serve a role in the maintenance of cell structure.

The three-dimensional structure of a protein is due to the type and sequence of its constituent amino acids. Since the amino acid sequence of each protein is unique, it follows that different proteins assume different shapes. Thus, there is a remarkable diversity of three-dimensional protein forms. The conformation of a protein is usually of critical importance in the protein's function. For example, a protein can be unfolded into a polypeptide chain that has lost its original shape. In general, proteins such as enzymes are rendered nonfunctional upon unfolding because functional activity is dependent on the protein's native shape. This process is called denaturation. Most proteins can be denatured by heating, by certain detergents, and by extremes of pH. The ionic detergent, sodium dodecyl sulfate (SDS), is often used to denature proteins. The denaturing treatment can frequently be reverse, for example, by removing the detergent of by neutralizing the pH. During this renaturing process, the polypeptide chain spontaneously refolds into its original conformation and the protein regains its biological activity. A similar folding process occurs in the cell for when a polypeptide is constructed on the ribosomes, it folds into a biologically active conformation. Thus, the three-dimensional folding of a protein and its biological properties are directed by the sequence of amino acid residues along the polypeptide chain.

Biochemists have identified three structural levels that define the three-dimensional shape of a protein. These levels of organization are secondary structure, tertiary structure, and quaternary structure. **Figure 4** shows examples of these levels of organization. The major force involved in the formation and maintenance of these structures are various types of weak, noncovalent bonds that are formed between the amino acid residues and between the amino acid residues and water. Although a noncovalent bond typically has less than 1/ 20 the strength of a covalent bond, a large number of noncovalent bonds participate in the folding of a single protein into its native conformation. Figure 4:

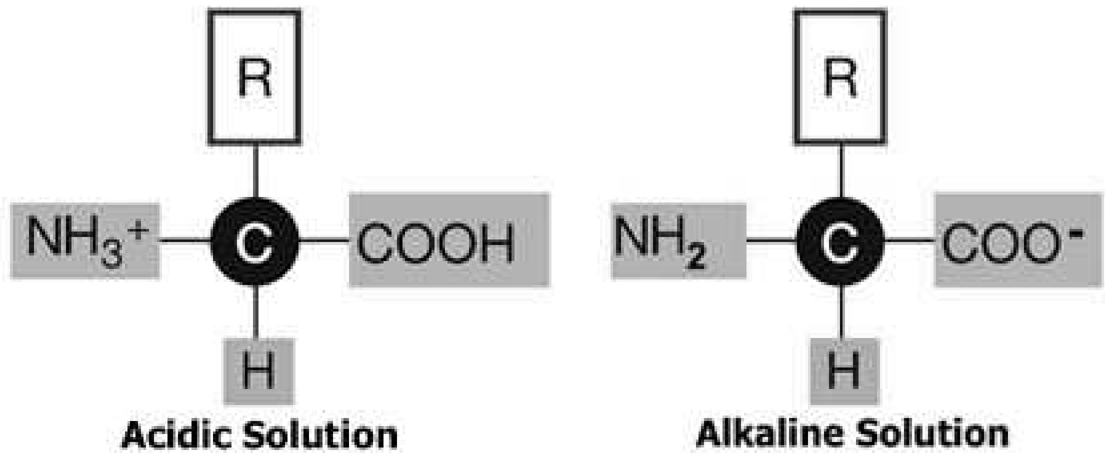


Electrophoresis is the movement of charged molecules under the influence of an electric field. Because amino acids and proteins are charged molecules, they migrate in an electric field at appropriate pH values. In the most common form of electrophoresis, the sample is applied to a stabilizing medium, which serves as a matrix for the buffer in which the ample molecules travel. The agarose gel is frequently used in the electrophoretic separation of non-denatured proteins since low percentage gels (i.e. 1% agarose) form a sponge-like network which serves as a medium for the buffer, but has pores large enough to allow even the largest proteins to pass unimpeded. However, changing the concentration of agarose can affect how molecules move through the matrix.

The agarose gel, containing preformed sample wells, is submerged in a buffer, which is contained within the electrophoretic chamber. Samples to be separated are then loaded into the sample wells. Current from the power supply travels to the negative electrode, supplying electrons to the conductive buffer solution, gel, and positive electrode, thus completing the circuit.

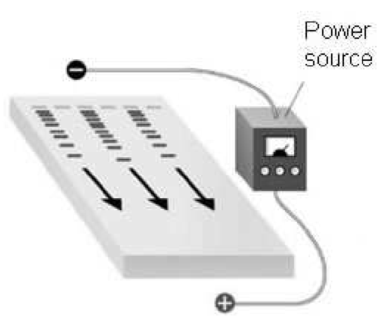
All amino acids contain at least one amino and one carboxyl group. In acid solutions, the amino groups are positively charged while the carboxyls are not ionized (**Figure 5**). Therefore, in strong acid solutions, amino acids are positively charged and migrate in an electric field to the negative electrode. In basic or alkaline solutions, the carboxyls are negatively charged while the amino groups are not ionized. It follows then, that in strong alkaline solutions, amino acids are negatively charged, and migrate to the positive electrode during electrophoresis.

Figure 5



There must be an intermediate pH at which each amino acid bears no net charge and does not migrate in an electric field. The pH at which an amino acid or protein does not migrate in an electric filed is called an **isoelectric point**. Most neutral amino acids have isoelectric points around pH 6.0. The isoelectric points of aspartic acid and glutamic acid, however, are close to pH 3. Therefore, at pH 6 these acidic amino acids carry a negative charge and migrate to the positive electrode during electrophoresis. The isoelectric points of the basic amino acids, lysine and arginine, are ph 9.7 and 10.8 respectively. These amino acids carry a positive charge at pH 6, and, hence migrate to the negative electrode. These differences in charge permit the electrophoretic separation of acidic, neutral, and basic amino acids at pH 6, as illustrated in **Figure 6**.

Figure 6



The positively and negatively charged side chains of proteins cause them to them to migrate like amino acids in an electric field. The electrochemical character of a protein is dependent primarily on the numerous positively charged ammonium groups (-NH_3) of lysine and arginine and the negatively charged carboxyl groups (-COO-) of aspartic acid and glutamic acid. The isoelectric points of most proteins is in the range of pH 5 to 7. Electrophoresis of proteins is usually preformed at a pH above the isoelectric point of most proteins. The pH of the electrophoresis buffer used in the exercise described in this manual is 8.6. Thus, at pH 8.6, most proteins are negatively charged and when applied to sample wells at the negative electrode end of the gel, they travel towards the positive electrode. The rate of migration of a protein species in an electric field depends upon its net charge; the higher the charge the faster the protein will travel. For example, serum albumin, which has an isoelectric point of 4.8, will carry a strong negative charge in a buffer of pH 8.6 as compared to myoglobin, which has an isoelectric point of 7.2. Therefore, at pH 8.6 albumin will migrate toward the positive electrode at a much faster rate than myoglobin. These considerations form the basis for the electrophoretic separation of proteins according to net charge.

The isoelectric point of a protein is defined as the pH at which the protein does not migrate in an electric field. The isoelectric points of the four colored proteins that we will study in this exercise are listed in **Table 2**. A brief description of the functions and properties of these proteins is given below.

Table 2

Protein	Color	Isoelectric Point	Net Charge at pH 8.6
cytochrome C	orange	10.2	positive
myoglobin	brown-red	7.2	negative
hemoglobin	red	6.8	negative
serum albumin*	blue	4.8	negative

Cytochrome C is one of a class of cell protein pigments found in plant and animal tissues. Cytochrome C, which is one of the most well characterized of the cytochromes, is an integral part of the electron transport system in mitochondria and is involved in cell energy production. Cytochrome C consists of a single polypeptide chain which is wound around a central, nonproteinaceous compound called a heme. It is the iron containing heme group which is responsible for the orange-brown color of this protein. The protein is basic in nature primarily because it contains a high concentration of lysine residues. The isoelectric point of horse cytochrome C is 10.2 and at pH 8.6 the protein carries a net positive charge. Thus cytochrome C, unlike most proteins, migrates differently during electrophoresis at ph 8.6.

Myoglobin and hemoglobin also contain an iron containing heme group and the iron is involved in oxygen binding. Myoglobin binds and stores oxygen in muscle and hemoglobin is involved in the transport of oxygen in the blood. The isoelectric point of myoglobin from horse is 7.2.

Hemoglobin is the major protein found in red blood cells. This protein transports oxygen from lungs to tissues. The isoelectric point of hemoglobin from rabbit is 6.8.

Serum albumin, is the major protein found in red blood plasma. This protein binds and transports a large number of smaller molecules in blood. Unlike the other proteins described above, albumin is not naturally colored. However, a tracking dye has been added to your serum albumin sample and some of this dye will bind and remain bound to the albumin during the electrophoretic run, turning the albumin band blue. The remainder of the tracking dye will migrate faster than albumin and when this free dye has migrated to the positive electrode end of the gel, the electrophoretic separation is complete. Serum albumin is a relatively acidic protein and has the lowest isoelectric point of the proteins that will be used in this exercise. Thus, this protein possesses a very negative net charge at pH 8.6 and will migrate faster than the other three proteins described above.

SAFETY

WHEN ATTACHING ELECTRODES TO YOUR GEL BOX, BE SURE THE POWER SUPPLY IS TURNED OFF. CONNECT BLACK TO BLACK AND RED TO RED.

Procedure:

1. Seal the ends of the gel-casting tray with masking tape and insert comb in the middle position.
2. Carefully pour enough agarose solution at approximately 50 degrees C into the casting tray to fill to a depth of about 4 mm. The gel should cover only about 1/3 of the height of the comb teeth (approximately 0.5 cm).
3. Do not move or jostle the casting tray while agarose is solidifying. As it polymerizes—about 10-15 minutes—the agarose will change from clear to opaque.
4. When agarose is set, unseal ends of casting tray. Play tray on platform of the gel box, so that the black and red bands match up with their respective-colored dots.
5. Fill the box with appropriate buffer, to a level that just covers the entire surface of the gel.
6. Gently remove the comb, taking care not to rip the gel.
7. Make certain that well holes left by the comb are completely submerged. If you notice "dimples" around the wells, slowly add buffer until they disappear.
8. Continue to slowly fill the electrophoresis chamber with buffer until the gel is covered with a 4-5 mm layer of buffer.
9. Load 15 μL of each of the four colored proteins into 4 wells on your gel (remember which is which):
    a. CYTOCHROME C
    b. MYOGLOBIN
    c. HEMOGLOBIN
    d. SERUM ALBUMIN
10. Plug your gel boxes into the power source (black to black and red to red). Turn on the power source and apply 100V (check with instructor if this is correct—time is an issue).
11. Electrophorese for 10 minutes, and then turn off and disconnect the power supply.
12. Remove the lid of the electrophoresis unit and note the relative position of the four proteins as compared to their point of application at the sample wells.
13. Resume electrophoresis until the bromophenol blue in the serum albumin sample has migrated to within 1 cm of the positive electrode end of the gel.

14. Turn off power, detach electrodes, and remove gel and place in large staining dish.
15. Label dish with your name and hand to the instructor for staining and destaining using Coomassie blue.
16. Photograph (if you have the ability to) and/or also draw a diagram of your gel.
17. Measure the distance of the proteins (in cm) from the sample origin.

Hints For Your Lab Report:

The Introduction section of your lab report should include an explanation of how gel electrophoresis works (cite sources) as well as how proteins (amino acids) can be ionized (Understanding the Nature of Amino Acids) as well as all other necessary information as described in the lab manual.

The Results section of your lab report will include a data table for your measured results along with a diagram (to scale—or photo) of your gel with each protein lane labeled and distances measured and labeled. You might have to use your Photoshop© skills…

The Discussion section of your lab report will include the answers to the following questions (among lots of other information as described in your lab manual):

1. What is the molecular basis for the differences in the electrophoretic mobilities of the four proteins analyzed in this exercise? Relate your answer to the isolelectric points of these proteins that were given.
2. Explain how your results would have been affected if the electrophoretic separation was carried out in a pH of 10.5. (You can use a labeled diagram to supplement your written answer.)
3. Explain how your results would have been affected if the separation was carried out at pH 6.0. (You can use a labeled diagram to supplement your written answer.)
4. What are two different variables you can alter to affect the speed of migration of molecules through the agarose gel via electrophoresis? Describe each sufficiently.