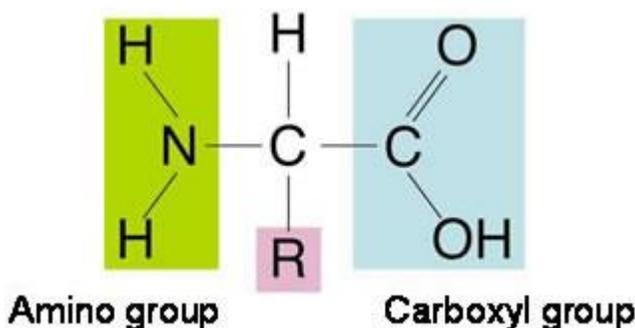


## MOLEBIO LAB #3: Electrophoretic Separation of Proteins

### 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 ( $\text{NH}_2$ ) and a carboxyl acid group ( $\text{COOH}$ ) attached to a central carbon atom called the alpha carbon ( $\alpha$ ). Also attached to the alpha carbon are a hydrogen atom and an R-group or side chain.



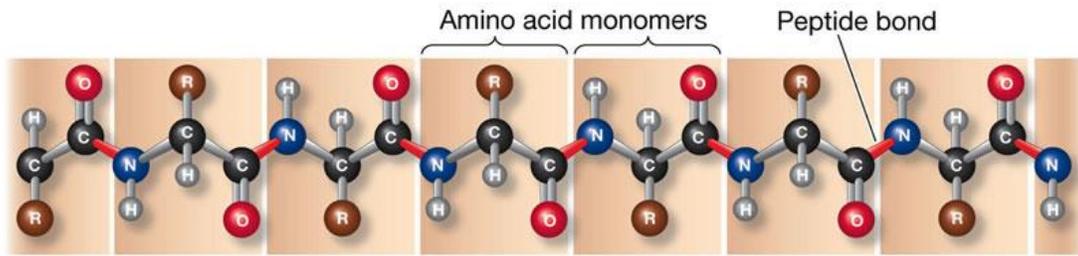
**Figure 1: General structure of an amino acid.**

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: Amino acids classified by their charge at a physiological pH.**

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 usually 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: A brief sequence of amino acids monomers linked by peptide bonds.**

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 cells. The primary structure of thousands of proteins is now known.

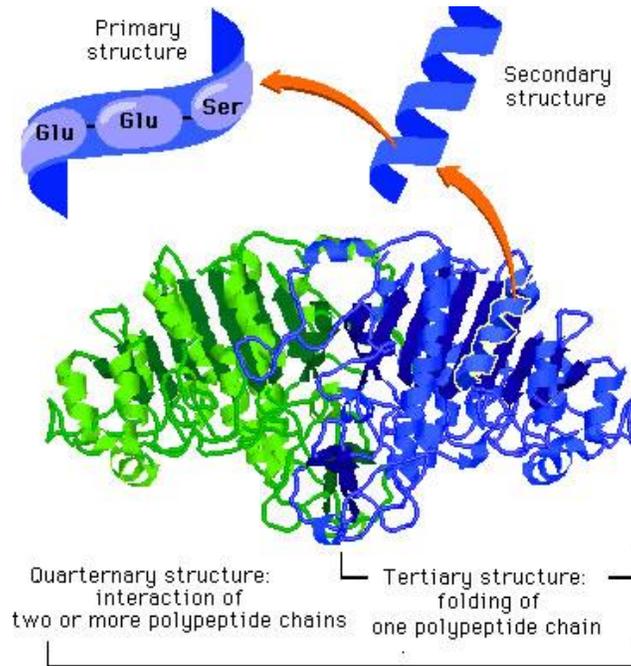
**Val-Leu-Ser-Glu-Gly-Glu-Trp-Gln-Leu-Val-Leu-His-Val-Tyr-Ala-Lys-Val-Glu-Ala-Asp-Val-Ala-Gly-His-Gly-Gln-Asp-Ile-Leu-Ile-Arg-Leu-Phe-Lys-Ser-His-Pro-Glu-Thr-Leu-Glu-Lys-Phe-Asp-Arg-Phe-Lys-His-Leu-Lys-Thr-Glu-Ala-Glu-Met-Lys-Ala-Ser-Glu-Asp-Leu-Lys-Gly-His-His-Glu-Ala-Glu-Leu-Thr-Ala-Leu-Gly-Ala-Ile-Leu-Lys-Lys-Lys-Gly-His-Glu-Ala-Glu-Lys-Leu-Lys-Pro-Leu-Ala-Gl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

**Figure 3: Primary structure of myoglobin.**

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.

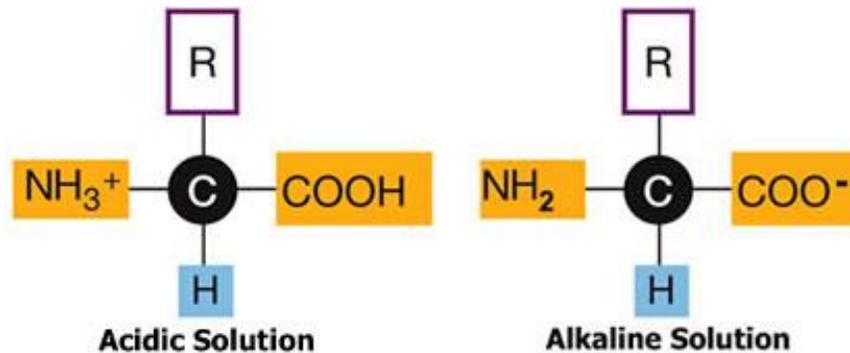
The three-dimensional structure of a protein is due to the type and sequence of its constituent amino acids. Since the amino acid sequence of each protein is unique, it follows that different proteins assume different shapes. Thus, there is a remarkable diversity of three-dimensional protein forms. The 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 reversed, for example, by removing the detergent or 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: Levels of structure in proteins**

All amino acids contain at least one amino and one carboxyl group. In acidic 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: Protonation/deprotonation of amino and carboxyl groups of an amino acid.**

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 field 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.

The positively and negatively charged side chains of proteins cause 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 ( $-\text{NH}_3^+$ ) of lysine and arginine and the negatively charged carboxyl groups ( $-\text{COO}^-$ ) 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 performed 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: Isoelectric points of select proteins.**

Protein	Color	Isoelectric Point	Net Charge at pH 8.6
cytochrome C	orange	10.2	_____
myoglobin	brown-red	7.2	_____
hemoglobin	red	6.8	_____
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 best 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. Prepare 25 mL of 3.5% agarose in 1X Tris-glycine buffer. Follow the steps below for preparing a 5X Tris-glycine stock solution and then dilution to a 1X concentration:
  - a. Add 8.0 g Tris - tris(hydroxymethyl)aminomethane and 3.6 g glycine to 0.1 L of distilled water for a 5X stock solution.
  - b. To dilute, add 4 parts distilled water to one part 5X buffer. ( $V_1C_1 = V_2C_2$ ) Correct for desired pH (8.6) with concentrated HCl. Slowly add HCl with partially diluted stock. Monitor with a pH meter that has been properly calibrated.
  - c. Keep your 5X stock and 1X running buffer in labeled and covered beakers.
3. Also prepare 0.1 L of 0.5 M sodium acetate buffer. (Sodium Acetate = 82.03 g/mol) This will be your 5X stock. Listen up to directions to adjust pH for the 1X running buffer.
4. 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  $\frac{1}{3}$  of the height of the comb teeth (approximately 0.5 cm).
5. Do not move or jostle the casting tray while agarose is solidifying. As it polymerizes—about 5-10 minutes—the agarose will change from clear to opaque.
6. When agarose is set, unseal ends of casting tray. Place tray on platform of the gel box, so that the black and red bands match up with their respective-colored dots.
7. Fill the box with appropriate buffer, to a level that just covers the entire surface of the gel.
8. Gently remove the comb, taking care not to rip the gel.
9. 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.
10. Continue to slowly fill the electrophoresis chamber with buffer until the gel is just covered.
11. Load 10  $\mu$ L of each of the four colored proteins into the first 4 wells on your gel.
  - a. CYTOCHROME C – lane 1
  - b. MYOGLOBIN – lane 2
  - c. HEMOGLOBIN – lane 3
  - d. SERUM ALBUMIN – lane 4

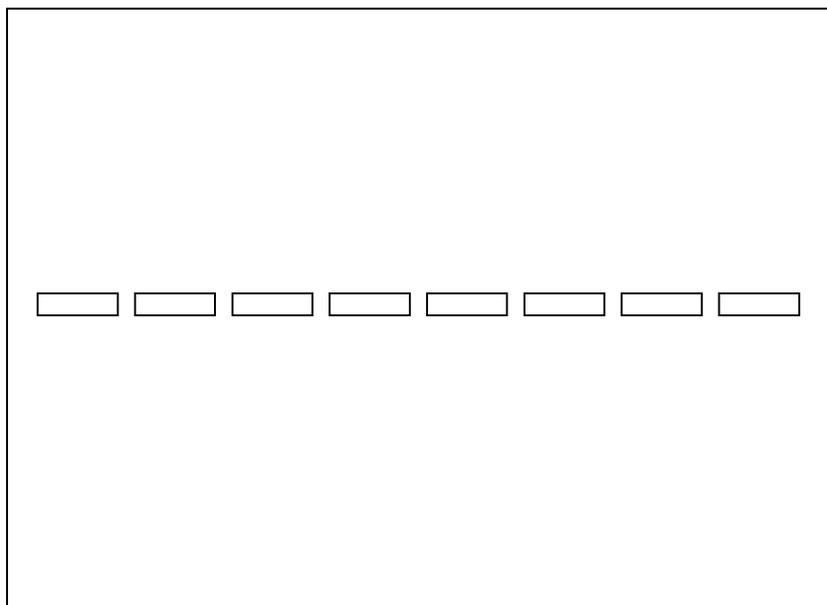
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12. Transfer 10  $\mu\text{L}$  of each of the four proteins into a single clean microtube – label this tube A. Dispense each new protein sample onto the side wall of the microtube. Add 60  $\mu\text{L}$  of distilled water to the microtube. This will bring the concentration down to  $1/10$  of the original stock solutions. Pulse in a balanced centrifuge to bring samples to the bottom of the tube.
13. Transfer 10  $\mu\text{L}$  of the contents of tube A into a single clean microtube – label this tube B. Add 90  $\mu\text{L}$  of distilled water to the microtube. This will bring the concentration down to  $1/100$  of the original stock solutions. Pulse in a balanced centrifuge to bring the protein mixture to the bottom of the tube.
14. Leave the next well (lane 5) over from the previous loading (step 10) empty. Load 10  $\mu\text{L}$  of the mixed protein sample from tube A into the next well over – lane 6. The load 10  $\mu\text{L}$  of the mixed protein sample from tube B into the next well – lane 7.
15. 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). Answer question 1 in the Lab Questions Section.
16. Electrophorese for  $\sim 20$  minutes, and then turn off and disconnect the power supply.
17. Turn off power, detach electrodes, and remove gel and place in the small staining tray.
18. USE GLOVES! Pour enough Coomassie Blue stain to cover the gel. Let sit for  $\sim 2$  minutes.
19. Discard stain into 'recycling container' and flood tray with destaining solution. Gently agitate for  $\sim 2$  minutes.
20. Carefully pour off the destaining solution down the sink and add more destaining to cover the gel. Gently agitate every so often for 3 minutes.
21. Again, carefully pour off the destaining solution down the sink and add more destaining solution to cover the gel.
22. Pour of the destaining solution down the sink.
23. Photograph (if you have the ability to) and/or also draw a diagram of your gel.
24. Measure the distance of the proteins (in cm) from the sample origin. Then, secure plastic wrap over the tray to keep the gel from drying out overnight. Answer questions 2-3 in the Lab Questions Section.

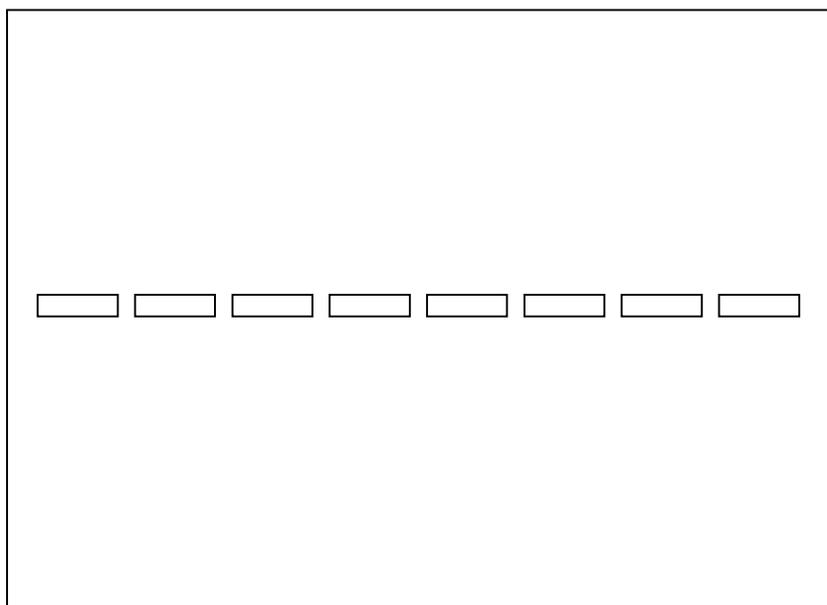
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1. Sketch what you think the results will look like. Label positive and negative 'ends' of the gel.



2. Sketch the final gel below. Include positive and negative 'ends'. Include measurements taken from the actual gel.



Name: \_\_\_\_\_

3. Give a detailed explanation of why the proteins migrated the way they did in the Tris-glycine buffer.

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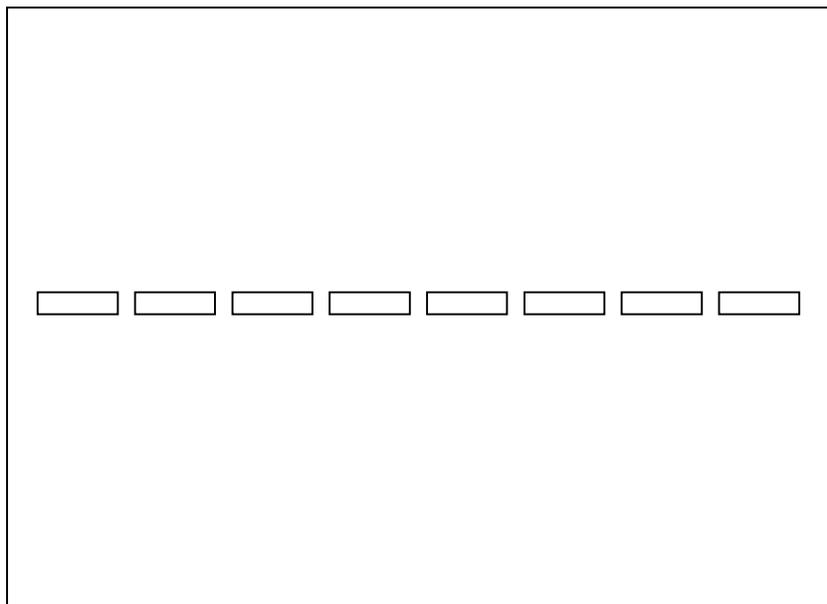
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4. Indicate what buffer you used for your second electrophoresis run: \_\_\_\_\_ @ pH \_\_\_\_\_

5. Sketch your prediction of your second electrophoresis run with a different buffer.



6. Did the proteins move as predicted? Give a detailed explanation of why the proteins migrated the way they did in the second buffer.

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