# Chapter 2

# Polyacrylamide Gel Electrophoresis (PAGE) of Blood Proteins

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## Contents



## Introduction

This laboratory exercise is used at Northwestern for the introductory biology sequence during the quarter that covers the topics of Cell Biology and Physiology. In this exercise, the students learn how to use several techniques common to many research laboratories. These techniques include, differential precipitation, centrifugation, and detergent extraction. The majority of the students take this course during their sophomore year and most are either biology majors or students taking the pre-medicine curriculum. The students are taught in groups of 24 and work in pairs. Each pair of students shares a gel with another pair. This is a convenient arrangement that requires only six gel apparatuses per room.

This lab requires a great deal of preparation and organization. Although the lab can be accomplished by the students in less than 3 hours, it will be necessary for the preparatory staff to spend the following day staining and de-staining gels so that the students can come in on the third day and check their results. A detailed list of materials and protocols can be found in the Appendices.

Depending on the level of the students, this exercise may need to be modified. For example, there is little discussion of disulfide bonds and the role they play in protein structure. The sample buffer used in this exercise contains a reducing agent that creates an environment of excess thiols which reduces all the disulfide bonds present in the proteins. Therefore, the protein will be denatured into individual protein subunits.

The polyacrylamide gels used in this exercise differ from those used in research laboratories in that there is no stacking gel — sometimes referred to as a discontinuous buffer system. A stacking gel is a separate gel that is poured on top of the running gel and has a lower percentage acrylamide and a different pH buffer. Because of these conditions, the proteins will eventually stack into concentrated narrow bands at the bottom of the stacking gel. Stacking gels give better resolution, but involve more preparation time and require that the gels be poured vertically. The gels that we use are poured horizontally, which saves more time and is less prone to leakage. A discussion of stacking gels and the procedures for doing polyacrylamide electrophoresis can be found in two different laboratory manuals: Sambrook et al. (1989) and Ausubel et al. (1990).

## Student Outline

## Introduction

The various types of chromatography represent powerful and widely-utilized techniques for separating mixtures of biological molecules into their individual components. But as you might imagine, those clever and industrious biochemists have developed during the course of the past century many other procedures for separating molecules from mixtures as well. Chief among these are the various electrophoretic procedures, with polyacrylamide gel electrophoresis (PAGE) and agarose electrophoresis being the major types. Polyacrylamide gel electrophoresis, with all of its different modifications is probably the most widely-utilized procedure in contemporary biochemistry and molecular biology. All electrophoretic procedures are based on the fact that charged molecules in aqueous solution will migrate in an electric field. Also important are the various kinds of batch separatory techniques such as differential precipitation, differential denaturation, and differential extraction — all of which are based on the solubility properties of the molecular species of interest. Although not as widely utilized now as they were formerly, batch techniques are still essential for many applications — especially those in which large amounts of material are being purified. In this laboratory exercise, you will utilize differential precipitation and polyacrylamide gel electrophoresis in combination with centrifugation and detergent extraction to fractionate the complex mixtures of proteins found in mammalian red blood cells and mammalian blood plasma and to characterize some of the component proteins.

Of the various classes of organic molecules within cells, it is the proteins that are without doubt the most variable in terms of size, structure, and function. Thus, the separation and isolation of proteins from natural sources has traditionally been a major area of research in biochemistry and continues so today. Because proteins are the "working molecules" of the cell, these large and complex macromolecules are of interest to researchers working in many areas of contemporary biological research. Many proteins are stable only under a rather limited range of experimental conditions; if the limits are exceeded, denaturation as a result of intramolecular alteration and/or fragmentation occurs with the subsequent loss of biological activity. The two fractionation techniques which you will investigate in this laboratory exercise are especially advantageous for purifying proteins because under carefully-controlled conditions, degradation of the molecules is minimal. Differential precipitation may be regarded as a "brute force" method for fractionating a mixture of proteins because it is typically used to divide the mixture into a small number of different fractions, each of which contains many different molecular species. Furthermore, any specific protein might be present in more than one fraction, for example, 80% might occur in the so-called major fraction with 10% each in two minor fractions. Polyacrylamide gel electrophoresis is much more sophisticated in that it can separate individual proteins — even those that are structurally quite similar — from each other.

Mammalian blood has been selected as the source of proteins to be separated in this exercise for several reasons. It is a substance which contains both a fluid component (the plasma) and a cellular component, and the two are easily separated by centrifugation. The plasma contains many proteins in solution. The cells contain both the soluble intracellular cytoplasmic proteins as well as the membrane proteins (both peripheral and integral). Thus, mammalian blood provides membrane proteins, cytoplasmic proteins, and extracellular proteins to work with.

You will begin by separating the cells from the plasma by centrifugation. The cells will then be washed to remove traces of plasma proteins and subsequently lysed in a hypotonic medium. Cell membranes (ghosts) will be separated from cytoplasmic proteins by high-speed centrifugation. Meanwhile, aliquots of plasma will be subjected to two different fractional precipitation procedures with ammonium sulfate and ethanol being used as the precipitating agents. Finally, all fractions will

be treated with dithiothreitol (DTT), a reagent which breaks disulfide bonds, and subjected to polyacrylamide gel electrophoresis in the presence of the detergent, sodium dodecyl sulfate (SDS). Background information about the various aspects of this laboratory exercise is summarized below.

## Objectives

When you have completed this laboratory exercise, you should be able to:

1. Name and briefly describe the major proteins in mammalian plasma.
2. Name and briefly describe the major proteins associated with the mammalian erythrocyte membrane.
3. Explain what is meant by fractional precipitation, solvation, salting out, and dielectric constant; describe the mechanisms by which ammonium sulfate and ethanol cause fractional precipitation of proteins.
4. Explain the principles upon which electrophoresis is based; tell what the equation, $Ez = fv$, means; explain how μ is defined and why it is significant; explain what charge-density means.
5. Tell what SDS-PAGE is and how it differs from PAGE; describe the roles of polyacrylamide and SDS in the electrophoretic procedure.
6. Tell how to prepare protein samples for SDS-PAGE and why they are prepared in this way; include the role of DTT in your discussion.
7. Interpret SDS-PAGE data.

## Background

### Plasma Proteins

Mammalian plasma is a complex fluid — complex in the sense that it contains many different molecular species. This complexity derives from its biological role as the major transport medium in the mammalian body, for as you might imagine, there are many different molecular substances to be distributed. Nutrient molecules are picked up from the digestive system, transported throughout the body, and delivered to all of the cells. Waste molecules are picked up from all cells and delivered to the appropriate excretory sites: primarily kidneys, lungs, and skin. Regulatory molecules are picked up from the various endocrine cells and transported to the various target tissues. Tissue-derived plasma enzymes appear in variable amounts, especially in connection with injury or disease. Then there are the defense molecules: antibodies against invading organisms as well as a group of molecules which have the ability to coalesce and form a clot to prevent hemorrhage when the blood vessels are broken. Many of these substances are themselves proteins or are carried by transport proteins. Approximately 100 different proteins have been purified and characterized from human plasma, but numerous minor proteins remain to be characterized. The major plasma proteins of mammalian blood can be listed as follows:

**Albumin**, a single polypeptide chain containing approximately 580 amino acid residues, is the most abundant protein in mammalian plasma (35–45 mg/ml). Its molecular weight is approximately 66 kD. (You will remember that the Dalton, which is equal to the mass of a hydrogen atom, is the basic unit of molecular mass, and the abbreviation, kD, means kilodalton

or 1,000 Daltons.) Albumin, which is soluble in half-saturated ammonium sulfate, is the most acidic of the major plasma proteins. When electrophoretic techniques were first developed, plasma was one of the first substances to be studied.

The **globulins** are a large family of plasma proteins which were originally defined on the basis of their solubility properties; globulins include all plasma proteins which are insoluble in half-saturated ammonium sulfate. The **α globulins** are a diverse lot with regard to their molecular weights and functions. Prothrombin (72 kD), a major protein of the clotting system, is one of the α globulins.

The **β globulins** comprise a second subgroup of the globulins. The β globulins are also a diverse group of molecules from the standpoints of both size and function. Transferrins, the most abundant β globulins, have a molecular weight range that varies from 76–81 kD.

The **γ globulins**, also termed **immunoglobulins**, include the antibody molecules. Three major classes (designated A, G, and M) and two minor classes (D and E) are typically present. Immunoglobulin G (IgG) is the second most abundant protein in human plasma (8–18 mg/ml). All γ globulin molecules consist of four polypeptide chains held together by disulfide bonds: two heavy chains (45–55 kD) and two light chains (25–35 kD). They differ in the nature of the chains which are involved. Their molecular weights range from 150–190 kD, except for IgM, an aggregate molecule which is much larger at approximately 1,000 kD.

**Fibrinogen** is the most abundant of the coagulation proteins present in plasma (2.0–4.5 mg/ml). Its molecular weight is 340 kD.

## Membrane Proteins

Mammalian blood contains several different kinds of cells. Everyone remembers from high school biology the so-called red corpuscles (erythrocytes) and white corpuscles (leucocytes). Erythrocytes are by far the most abundant ($5.4 \times 10^9$ cells per ml of blood in human males, $4.8 \times 10^9$ cells per ml in human females); the leucocytes, of which there are several types, are much rarer ($7–10 \times 10^6$ cells per ml of blood in humans). Mammalian erythrocytes exist as thin, round, biconcave disk-like cells which lack nuclei and all other intracellular organelles. Because of this comparatively simple structure, and the fact that their proteins can be easily extracted using detergents such as sodium dodecyl sulfate (SDS), it is not surprising that mammalian erythrocytes have become the favorite research object of biochemists and cell biologists who are interested in the structure of the plasma membrane. As a consequence, one can say that the membrane proteins of mammalian erythrocytes are the best known of any cell. You will find them discussed in detail and illustrated in pages 510–516 in *Molecular Cell Biology* (Second Edition) by Darnell et al. and in pages 301–307 in *Biochemistry* (Third Edition) by Stryer.

When the membrane proteins of mammalian erythrocytes are subjected to SDS-PAGE and stained with Coomassie Brilliant Blue R such as you will do in this exercise, approximately 10 major bands can be observed. These major bands have been numbered starting with band 1, the largest protein at the top of the gel (see Figures 13-32 in *Molecular Cell Biology* and 12-35 in *Biochemistry*).

Bands 1 and 2 are the α (molecular weight 240 kD) and β (220 kD) monomers of **spectrin**, a peripheral membrane protein which resides on the cytoplasmic face. Spectrin, a long, filamentous protein which exists as an αβ dimer, serves as a major component of the cytoskeleton.

Bands 2.1 and 2.2 are **ankyrin**, another peripheral membrane protein located on the cytoplasmic side. Ankyrin, a globular protein of approximately 200 kD molecular weight, is also a component of the cytoskeleton. Ankyrin has two binding domains: one specific for spectrin and the other for the transmembrane band 3 protein. Thus, it serves to attach the spectrin cables to the inner side of the erythrocyte membrane.

**Band 3 protein** is the first of two major integral membrane proteins. It exists as a dimer of two identical chains, each with 929 amino acid residues having a molecular weight of approximately 93 kD. Each monomer includes a C-terminal domain (509 residues) which lies embedded in the phospholipid bilayer of the membrane and an N-terminal domain (420 residues) which extends into the cytoplasm of the erythrocyte. Band 3 protein is a "multipass" transmembrane protein; its transmembrane C-terminal domain passes in and out through the erythrocyte membrane twelve times. Band 3 protein serves as an anion channel (a pore through the membrane through which anions such as chloride and bicarbonate can be exchanged). In addition to its transport function, band 3 protein has an ankyrin-binding domain on its cytoplasmic N-terminus. Thus, band 3 molecules serve as the sites to which the cytoskeleton is attached. Band 3 protein is abundantly represented in the erythrocyte membrane; it comprises approximately one-third of total membrane protein. Band 3 protein is also a glycoprotein, carrying on its external domain a small, oligosaccharide residue.

**Band 4.1 protein**, approximately 82 kD molecular weight, is another peripheral membrane protein on the cytoplasmic face of the membrane and another component of the cytoskeleton. It is a globular protein with two binding domains: one for spectrin and a second for actin.

Band 5 protein is **actin**, a globular protein of 43 kD molecular weight. Actin is another peripheral membrane protein on the cytoplasmic side which participates in the cytoskeleton. Actin monomers polymerize to form filaments and these participate in the cytoskeleton at the junctions of spectrin cables to which they are linked by band 4.1 protein.

Band 6 protein is **glyceraldehyde-3-phosphate dehydrogenase**, an enzyme which catalyzes the conversion of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate. This is one of the major enzymes in the glycolytic pathway, a series of biochemical reactions involved in cellular bioenergetics. Glyceraldehyde-3-phosphate dehydrogenase is a tetrameric protein with a molecular weight of 140 kD. Each of its four identical subunits consists of a single polypeptide chain (35 kD) of approximately 330 amino acid residues.

One final membrane protein to be mentioned is **glycophorin**, the second of the two major integral membrane proteins. Glycophorin is not numbered in the sequence because it does not stain with Coomassie blue. It consists of 131 amino acid residues with a molecular weight of approximately 30 kD. It is a "single-pass" transmembrane protein, that is, the polypeptide chain passes through the phospholipid bilayer just once. Its amino terminus (some 70 amino acid residues) projects on the external side of the erythrocyte membrane. This external domain is heavily glycosylated with multiple carbohydrate residues, these sugars making up 64% of the molecular weight of the purified protein.

A careful reading of the above summary will reveal that nothing has been written about the identity of bands 4.2, 4.9, and 7 — proteins which are illustrated in Figure 13-38 in *Molecular Cell Biology*. This is because the functions of these proteins have yet to be discovered. Furthermore, the proteins, adducin and tropomyosin, which are illustrated as important components of the erythrocyte cytoskeleton in Figure 13-38 in *Molecular Cell Biology* were not mentioned either. This is because these proteins do not seem to be abundant enough to be routinely present in SDS-PAGE gels.

## Cytoplasmic Proteins

It has been estimated that what with its enzymes, regulatory proteins, and structural (i.e., cytoskeletal) proteins, the cytoplasm of a typical mammalian cell contains on the order of a few hundred different kinds of protein molecules. Erythrocyte cytoplasm is unique, however, in two respects. First, it has a higher protein content than that of typical somatic cells. Second, approximately 75% of this protein is hemoglobin (15 kD subunits), the respiratory pigment which confers upon erythrocytes their major function (transport of oxygen and carbon dioxide throughout the body). The remaining 25% includes a variety of different enzymes, structural molecules, and regulatory molecules, but many of the proteins normally present in somatic cells are not found in erythrocytes. They are lost, together with the nucleus, in the process of erythrocyte maturation.

## Fractional Precipitation

Fractional precipitation is a technique which depends on the differential solubilities of the different molecules in a solution. By now, you are very familiar with the concepts of hydrophobicity and hydrophilicity as they pertain to the side-chains of amino acids, to fatty acids, to the heads and tails of phospholipids, and to organic molecules in general. You are also familiar with the concept of the general shape and polarity of water molecules, with the hydrogen atoms being relatively positive and the oxygen atom being relatively negative. Thus, you will have no difficulty in recognizing that when molecules are dissolved in water (and don't forget that all living organisms are essentially highly-organized mixtures of organic molecules dissolved in water and surrounded by membrane), each molecule will be surrounded by a shell of water molecules which are oriented toward the solute in such a way that the free energy is maximal. This phenomenon is referred to as solvation. Ionic molecules or groups and polar molecules or groups may be solvated by a shell consisting of several layers of water molecules organized very specifically whereas molecules that are midway on the hydrophobic-hydrophilic scale may be solvated with very thin shells of water molecules which are somewhat randomly oriented and very loosely held.

Having established the concept that molecules in aqueous solution are solvated by shells of water molecules, it follows that these shells serve to keep the dissolved molecules from coming in contact with each other, and further, that any change in the aqueous medium which would disturb the shells by thinning or eliminating them so that the individual solute molecules could contact each other might permit the solutes to stick together more tightly than they hold the water molecules. Giant clusters might form and these would settle out of the solution as a precipitate. This is essentially what happens in fractional precipitation. How might one alter the aqueous medium in a way such that the shells might be disturbed? There are three major ways: change the pH, add a salt, or add a water-miscible organic solvent. It is the second and third of these three techniques that you will utilize in this laboratory exercise.

Fractional precipitation of proteins by adding a salt is known as “salting out”. In theory, any salt whose ionic components do not irreversibly react with and denature proteins might be utilized, but in practice, ammonium sulfate, $(NH_4)_2SO_4$, is the salt of choice. Ammonium sulfate is enormously soluble in water; 767 g must be added per liter of water to produce a saturated solution. Thus, there is a great range over which its concentration can be varied. Also, both ammonium ions and sulfate ions become heavily solvated. Thus, they are very effective in stripping water molecules away from the shells solvating proteins. In this exercise, you will dilute a saturated solution of ammonium sulfate with an equal volume of plasma at room temperature; the resulting mixture will

be at the 50% saturation level. Under these conditions, one expects that globulins will precipitate but albumin will remain in solution.

Fractional precipitation of proteins by adding a water-miscible organic solvent such as ethanol occurs because of a change in the dielectric constant (ε) of the solution. The dielectric constant is a value that describes the strength of the force exerted by one electric charge on another. You will recall that water molecules are polar structures, and their intramolecular charges exert force on other water molecules and on dissolved solutes such as proteins. (It is in fact these intermolecular forces which result in the phenomenon of solvation described in the previous paragraphs.) When relatively non-polar organic solvents such as methanol, ethanol, and propanol are mixed with water, they serve to separate the water molecules from each other and thus reduce the force which they can exert on each other. For pure water, $\varepsilon = 80$ statcoulomb$^2$/dyne-cm$^2$ whereas for pure ethanol, $\varepsilon = 32$ statcoulomb$^2$/dyne-cm$^2$ (both are approximate values). Adding increasing amounts of ethanol to an aqueous solution of proteins progressively reduces the dielectric constant and ultimately interferes with protein solvation to the point that protein-protein interactions become stronger than protein-water interactions, and the proteins precipitate. In this exercise, you will add absolute ethanol to plasma in the ratio of 1:2 (vol:vol) at 0°C; the resulting mixture will be 33% ethanol with a dielectric constant of approximately 60. Under these conditions, one expects that the globulins will precipitate but the albumin will remain in solution.

## SDS-PAGE

SDS-PAGE is the acronym for the electrophoretic procedure in which polyacrylamide gel is utilized as the matrix material and the detergent, sodium dodecyl sulfate, is included in the electrophoresis buffer. How are these two substances related to the movement of charged molecules (in our case, proteins) in an electric field? To begin with, if one simply took a U-shaped glass tube, filled it with a protein solution in an appropriate buffer, placed a cathode in one arm of the tube and an anode in the other, and applied an electric current, the proteins in solution would begin to migrate in the electric field. After a while, the concentration of the various proteins in the different portions of the tube would no longer be uniform: positively charged species would be more concentrated in the cathode arm while negatively charged species would be more concentrated in the anode arm. Any separation so obtained would be very unstable, however, for as soon as the current was shut off, if the apparatus was not moved so that the separated molecules would be mixed physically, they would quickly return to their randomized positions due to diffusion. Thus, in order for electrophoresis to be practical, researchers had to find a way in which to keep the separated molecules apart after they had been separated.

This technical problem was solved when procedures for carrying out the electrophoretic process in a porous matrix were developed. Paper was the first substance which was utilized as matrix material. The paper was saturated with buffer, a protein mixture was applied at an origin much as you did in thin-layer chromatography (except that the origin was typically in the middle of the sheet rather than at one end), and the sheet was draped over a supporting rod with the ends dipped into two buffer baths: one containing the cathode and the other, the anode. The whole system was enclosed in an air-tight chamber to prevent the paper from drying out. A related system, cellulose acetate electrophoresis, made use of a thin layer of buffer-saturated cellulose acetate on a plastic backing enclosed in a similar chamber. Both of these systems worked well, but they were technically tricky to carry out because it was difficult to keep the paper or cellulose acetate layer from drying at least somewhat, and they were soon replaced by gel matrix systems which are much more convenient.

Starch gel was the first of the gel matrices to be utilized for electrophoresis. Later, agarose gels and polyacrylamide gels were found to be preferable. The gels are cast in either a horizontal bed or

a vertical bed (Figure 2.1). Because the gel beds can be enclosed, drying out and the concomitant heating that occurs as an electric current passes through an increasingly dryer medium are never problems. The chemistry of polyacrylamide formation is illustrated in Figure 2.2. Acrylamide is the monomeric molecule which polymerizes, N,N'-methylene-bis-acrylamide (known simply as bis) is the cross-linking molecule which co-polymerizes with the acrylamide, and ammonium persulfate, $(NH_4)_2S_2O_8$, and N,N,N',N'-tetramethylethylenediamine (TEMED) are catalysts for the polymerization. By varying the amount of acrylamide in the polymerizing mixture, one can vary the amount of cross-linking in the polymer. Decreasing the degree of cross-linking results in an increase in the average size of the pores within the polyacrylamide matrix, and this directly affects the rate at which molecules of various sizes will move through the matrix. Thus, one can chose the polyacrylamide concentration which will best resolve the particular protein in which one is interested

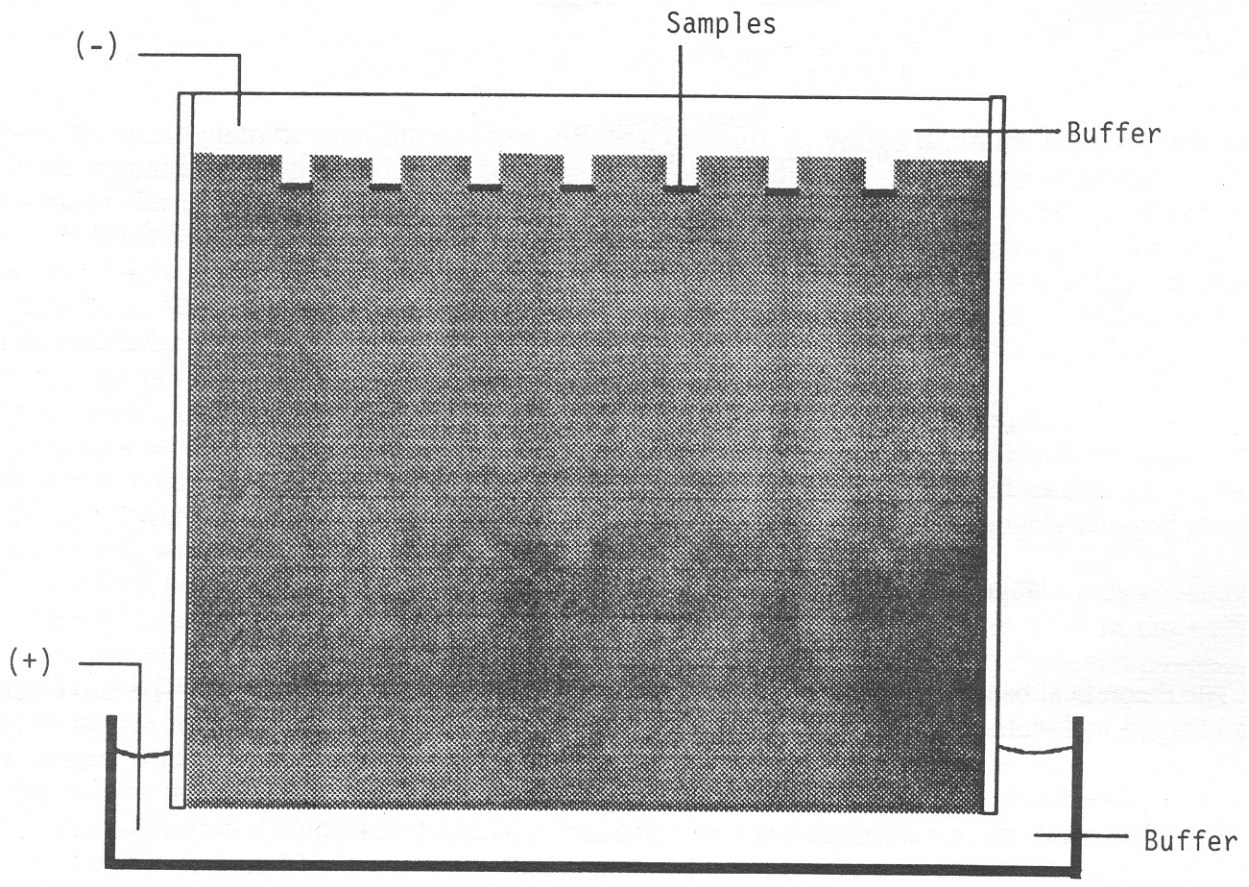



**Figure 2.1.** Diagram of the experimental apparatus for polyacrylamide slab-gel electrophoresis.

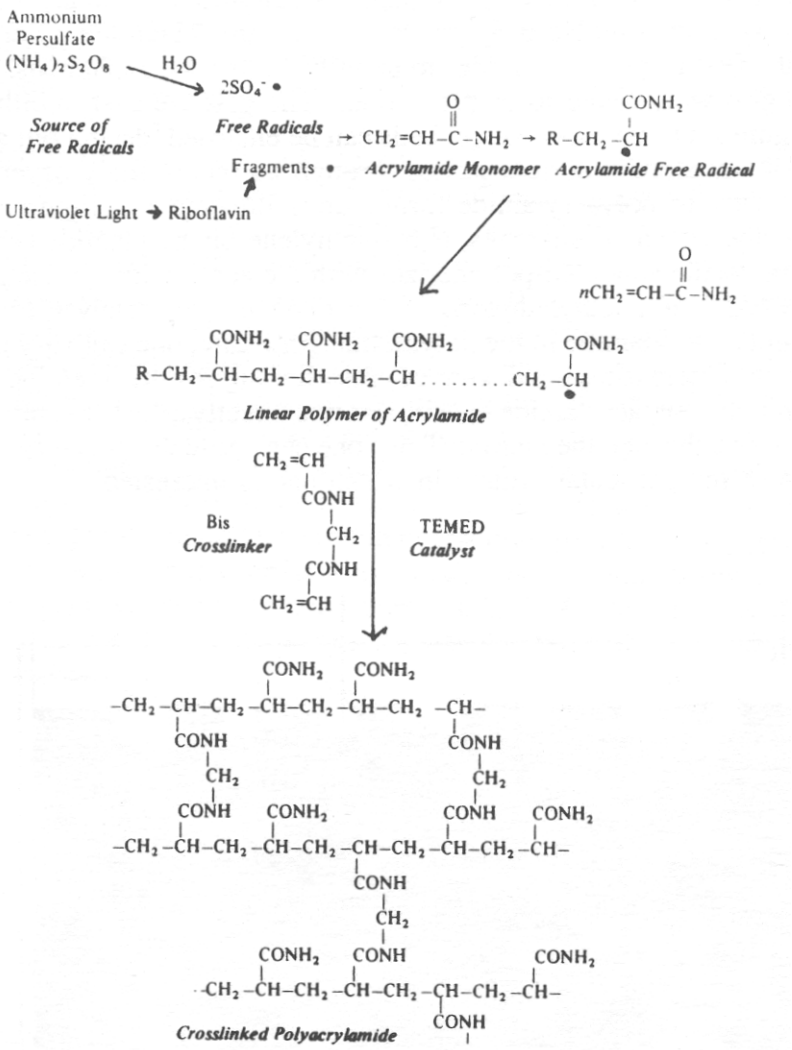



**Figure 2.2.** Chemistry of polymerization of polyacrylamide gels.

The theoretical basis of the electrophoretic process may be described as follows: The movement of a charged molecule in an electric field is described by the equation

$$Ez = fv$$

where $E$ is the force of the electric field (in volts/cm), $z$ is the net charge on the molecule, $v$ is the velocity at which the molecule is moving (in cm/sec), and $f$ is a frictional coefficient which depends on the size and shape of the molecule. Rearranging the equation,

$$v = \frac{E\,z}{f} \quad or \quad v = E\left(\frac{z}{f}\right)$$

emphasizes the point that the velocity, $v$, is proportional to the strength of the electric field, $E$, and the net charge on the molecule, $z$, and inversely proportional to the frictional coefficient, $f$. Thus, the stronger the electric field and the greater the net charge, the faster the molecule will move, while the larger and/or more asymmetrical its shape, the greater will be the frictional force or drag slowing

it down. Because molecular weight is a significant component of the frictional coefficient, *f*, the (*z*/*f*) term is referred to as the charge-mass ratio or the charge density.

This equation also allows us to compare the behavior of two different charged molecules in the same electric field. Since the effect of the electric field on the two different molecular species will be the same, differences in *v* must be due to differences in *z*/*f*, the charge-mass ratios of the two molecules in question. This point can also be illustrated by defining another parameter, electrophoretic mobility (μ), the velocity per unit electric field,

$$\frac{v}{E} = \frac{z}{f} = \mu$$

Thus, the electrophoretic mobilities of two different proteins, $\mu_1$ versus $\mu_2$, in the same electric field will be proportional to the difference between the charge densities or the charge-mass ratios of the two molecular species. Electrophoretic mobility (μ), like molecular weight, is a physical property of each different molecular species. Note, however, that unlike molecular weight, it is not a physical constant, but rather, a variable property which depends on the pH of the solution in which the molecule is dissolved. While the nature and number of ionizable groups in a molecule is a constant property of its molecular structure, whether these groups are ionized or not depends on the pH of the solution. Thus, *z*, the net charge on a molecule, varies as a function of pH. A general rule is that as pH goes down, *z* tends to become more positive, and your general understanding of the process of ionization as it relates to organic molecules should be such that you can explain why this is the case. The direction of migration also depends on the net molecular charge; positively-charged species (i.e., cations) will move toward the cathode (i.e., the negatively-charged electrode), while negatively-charged species (i.e., anions) will move toward the anode.

One final point remains to be discussed: the difference between PAGE (regular polyacrylamide gel electrophoresis) and SDS-PAGE (polyacrylamide gel electrophoresis with SDS in the buffer. You are already familiar with the role of the detergents such as SDS in extracting integral membrane proteins (see pages 504–505 in *Molecular Cell Biology* to refresh your memory). You will utilize this technique to solubilize the erythrocyte membrane proteins in this exercise. When proteins are pre-treated with SDS and subsequently subjected to polyacrylamide gel electrophoresis with SDS in the buffer, their electrophoretic mobilities are markedly different from those of intact proteins. This is because SDS disrupts the secondary structure of the molecules and binds to the polypeptides in the same way that it surrounds integral membrane proteins and wrenches them out of their thermodynamically comfortable positions in the lipid bilayer. The amount of SDS bound per unit weight of protein is constant (1.4 g of SDS per g of protein) and consequently, the charge densities (i.e., the *z*/*f* values) of all proteins become identical, with the charge being contributed by the bound SDS rather than the R-groups of the protein. Under these conditions, electrophoretic mobility becomes a function strictly of molecular weight because of the molecular-sieving property of the polyacrylamide gel. In other words, smaller molecules will move through the gel pores faster than larger molecules. When distance migrated is plotted as a function of log molecular weight, one obtains a straight line such as those illustrated in Figure 2.3. Thus, SDS-PAGE not only separates the different proteins in a mixture, but gives a reasonably accurate estimate of their molecular weights as well.

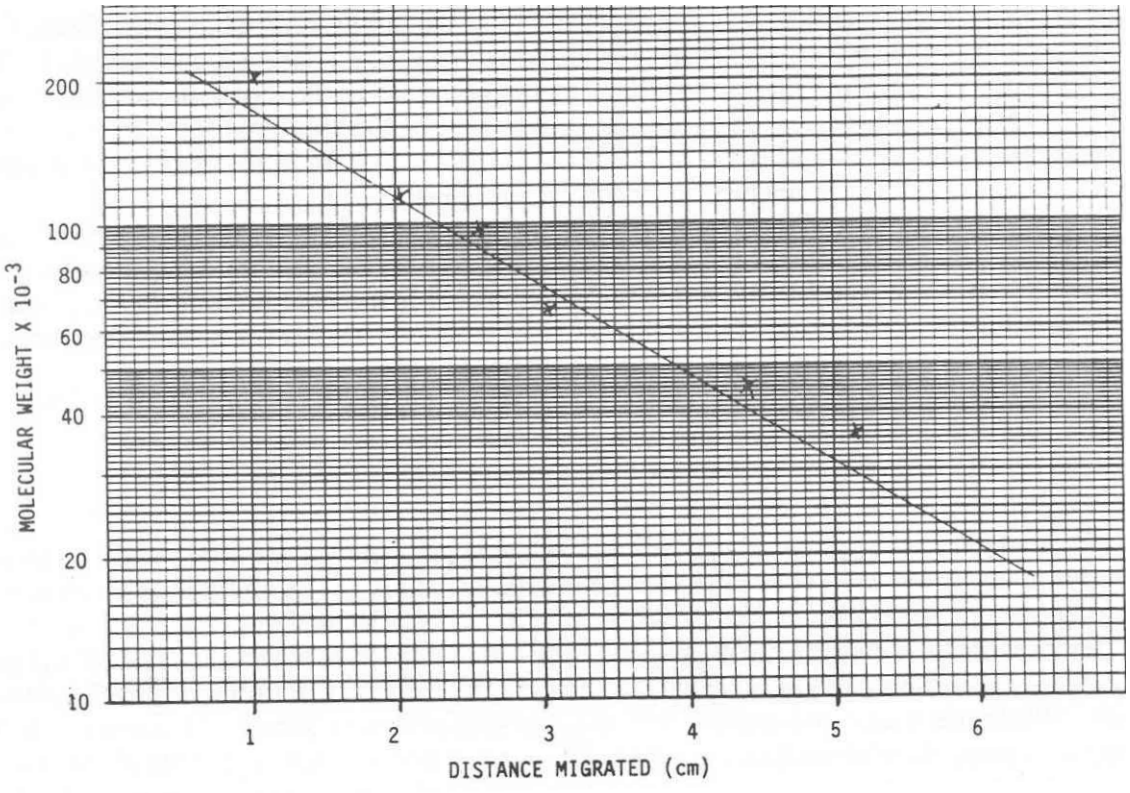



**Figure 2.3.** Molecular weight as a functio n of distance migrated for different proteins that will be available in the standard mixtures for this exercise.

## Laboratory Procedures – Day 1

### Safety Precautions

1. **DANGER!** *Shock hazard!* Although it is equipped with a safety lid, the electrophoresis apparatus should always be operated with caution. Careless handling can result in a painful (but not lethal) electrical shock.
2. Acrylamide and bis are potent neurotoxins which in either pure (i.e., powder) form or dissolved in water are readily absorbed through the lungs as well as through the skin. You will not have to deal with these substances, however, since your gel will be cast for you by the preparatory staff. Polyacrylamide is not toxic, but as some unpolymerized monomers may still be present in the gel, disposable **rubber gloves *must always be worn*** when handling gels or washing glassware that has come in contact with acrylamide.
3. **Take care** to avoid burns when using the boiling water baths.

**Part 1: Separation of blood into cellular and plasma fractions**

1. Remove the 12 ml calibrated centrifuge tube containing 10 ml mammalian blood from your ice bucket. Dry its exterior with a Kimwipe and load it into one of the buckets of a refrigerated clinical centrifuge.

   • If you cannot counterbalance your tube with that belonging to another pair of students, obtain a water-filled balance tube to put in the bucket on the opposite side of the centrifuge rotor.

2. Centrifuge for 5 minutes at top speed.

3. With a plastic pasteur pipet, carefully aspirate one pipet-full of the supernatant plasma into a 15 × 85 test tube. *Be careful* not to stir up the sedimented cells. Label **Pl**. Store the tube in your ice bucket until needed later.

   • Remove the rest of the supernatant with your pasteur pipet and discard it in the plastic waste beaker.

4. Add 6 ml ice cold isotonic washing buffer (150 mM sodium chloride + 5 mM sodium phosphate + 0.1 mM EDTA, pH 7.4) from a repipetter to the pelleted cells. Cover with parafilm and *gently* vortex the cells until they are evenly mixed with the washing buffer.

5. Add enough buffer to balance the tube with another and centrifuge again at top speed for 5 minutes.

6. Decant the supernatant washing buffer into a plastic waste beaker.

7. Repeat step 4.

**Part 2: Isolation of cellular proteins**

1. Fit a large-bore blue tip onto a 1000 µl Eppendorf pipetter. Carefully draw up 1000 µl (i.e., 1 ml) of packed cells into the tip.

2. Eject the cells into 19 ml ice-cold hypotonic hemolysis buffer (5 mM sodium phosphate + 0.1 mM EDTA, pH 8) in a 50 ml polycarbonate centrifuge tube which can be found in your ice bucket. Rinse the tip if necessary by carefully drawing hemolysis buffer into it several times.

3. Cover with parafilm and vortex vigorously.

4. Balance your centrifuge tube with that of another group by adding a few drops of chilled hemolysis buffer to the lighter tube.

   • Remember to *dry* your centrifuge tube on the outside before balancing; then load it immediately into the centrifuge rotor.

5. Spin the hemolyzed cells at 14,000 RPM for 30 minutes to pellet the membranes. During this time, proceed with step Part 3.

6. When the rotor has stopped, *gently* remove your tube. The ghosts will form a *very loose pellet*, therefore you must handle the tube very carefully or you will resuspend them.
   (a) With a plastic pasteur pipet, carefully aspirate one pipet-full of the bright red supernatant from the top of the tube; transfer it to a 15 × 85 test tube. This will be your cytoplasmic protein fraction; label it **Cy** and store in your ice bucket.
   (b) Next, *carefully* decant the rest of the supernatant into the plastic waste beaker. Remember to decant over the pellet so that it will not drop down into and flow out of the tube with the

supernatant. If you do this successfully, your ghosts (**G**) fraction will remain as a loose pellet in the bottom of your centrifuge tube. It may be necessary to remove any residual buffer with a plastic pasteur pipet.

(c) Add 10 ml of ice-cold hemolysis buffer from a repipetter to the pellet and gently mix with a plastic pasteur pipet. Balance and spin at 14,000 RPM for 10 minutes. Decant the supernatant and the pellet is your ghost (**G**) fraction.

**Part 3: Fractionation of plasma proteins by salting out**

1. To 250 µl saturated ammonium sulfate in a 1.5 ml microcentrifuge tube (labeled **A**), add 250 µl plasma with a 250 µl Eppendorf pipetter.
2. Close the cover of the microcentrifuge tube and vortex briefly but thoroughly; then store at room temperature for 5 minutes.
3. Centrifuge 3 minutes at room temperature. (Make sure that your tube is balanced with another one.)

   • When loading your microcentrifuge tube into the rotor, position it with the *hinge toward the center* of the rotor. This will enable you to decant over the pellet, thereby reducing the chance that loose pellets will be dislodged with the supernatant.
4. Carefully decant the supernatant into a 15 × 85 ml test tube; label **As** and store in your ice bucket. Twist a Kimwipe and insert it into the microcentrifuge tube to draw off the last of the supernatant from above the precipitated proteins by capillary action.
5. From a repetitive pipetter, add 1 ml half-saturated ammonium sulfate to the precipitate to wash out the last traces of supernatant. Resuspend by repeated aspiration with a plastic pasteur pipet.
6. Centrifuge again as described above. Decant the wash supernatant into the plastic waste cup. Remove the last drop with a twisted Kimwipe. The precipitate will be your **Ap** fraction; store it in your ice bucket.

**Part 4: Fractionation of plasma proteins by ethanol precipitation**

1. To 250 µl ice-cold absolute ethanol in a 1.5 ml microcentrifuge tube (labeled **E**), add 500 µl plasma with a 500 µl Eppendorf pipetter.
2. Close the lid of the microcentrifuge tube and vortex briefly but thoroughly; then store in your ice bucket for 5 minutes.
3. Centrifuge 3 minutes in a refrigerated microcentrifuge. (Make sure that your tube is balanced with another one.)
4. Carefully decant the supernatant into a 15 × 85 test tube; label **Es** and store in your ice bucket. Twist a Kimwipe and insert it into the microcentrifuge tube to draw off the last of the supernatant from above the precipitated proteins by capillary action.
5. From a repetitive pipetter, add 1 ml chilled 50% ethanol to the precipitate to wash out the last traces of supernatant. Resuspend by repeated aspiration with a plastic pasteur pipet. Try to do this quickly so that you don't heat up your preparation with the warmth of your fingers.

6. Centrifuge again in the refrigerated microcentrifuge as described above. Decant the wash supernatant into the plastic waste cup. Remove the last drop with a twisted Kimwipe. The precipitate will be your **Ep** fraction; store it in your ice bucket.

**Part 5: Preparation of samples for SDS-PAGE**

1. Turn up the heat on your hot plate so that the water will be boiling when your samples are ready.
2. From a repetitive pipetter, add 450 µl sample buffer (80 mM Tris + 100 mM DTT + 2% SDS) to each of four 1.5 ml microcentrifuge tubes.
   (a) With a 50 µl Eppendorf pipetter, add 50 µl of the three liquid fractions (cytoplasmic proteins, ammonium sulfate supernatant, and ethanol supernatant) each to a different microcentrifuge tube. Label **Cy**, **As**, and **Es**. Mix gently by tapping the side of the tube.
   (b) With a 50 µl Eppendorf pipetter and a wide-bore yellow tip, add 50 µl of ghosts to a microcentrifuge tube; label **G**.
3. Add 900 µl sample buffer to the two microcentrifuge tubes containing your ammonium sulfate and ethanol precipitates (**Ap** and **Ep**, respectively).

   • Close the lids and vortex these two tubes to mix the precipitates thoroughly with the sample buffer. If you experience trouble resuspending the pellet, use the 1000 µl Eppendorf pipetter (with tip) to break up the pellet.
4. Put the microcentrifuge tubes into a rack in the boiling water bath and boil for 5 minutes. Only the tips of the tubes need to be in contact with the boiling water. Adjust the heat so that the water does not boil too vigorously.
5. Remove your microcentrifuge tubes from the boiling water bath, dry their exteriors with a Kimwipe, and centrifuge at room temperature for 3 minutes to pellet any insoluble materials that may have precipitated during boiling.

   • Your samples are now ready to be loaded into the appropriate wells of the gel. Take care that you do not stir up the precipitates before loading.

**Part 6: Loading the gel and carrying out the electrophoresis**

1. The gel apparatus which you are using is of the latest design commonly sold to research laboratories. Your gel will have been cast for you and mounted in the gel apparatus by the preparatory staff. You should inspect your set-up, however, to figure out how it is put together.
   (a) Identify the safety cover which opens by lifting back to enable you to work with the apparatus. Locate the binding posts where the electrical leads are attached. Note that you cannot attach the leads while the cover is open, and thus, you will not be able to accidentally dip your fingers into the buffer baths after the current is turned on.
   (b) The upper and lower buffer chambers will be filled with electrophoresis buffer. Lying in the bottom of each chamber will be the platinum wire electrodes which supply the current to the system.
   (c) The gel slab is sandwiched between two glass plates which are clamped in a vertical position in the apparatus. White teflon spacers run vertically on each edge of the slab. The sandwich should be clamped securely into the apparatus so that electrophoresis buffer from

the upper chamber is not leaking out. If your system appears to be leaking, summon your teaching assistant.

(d) Notice that the glass plates are of unequal height; the back plate is shorter than the front one, and this allows the back side of the gel to come in contact with electrophoresis buffer in the upper chamber. In the top of the gel, you will find 20 sample wells. It is into these that you will load your samples. Because the gel is translucent, it is hard to see the wells. Using a marker, make a line at the bottom of the well so that you will be able to easily find the wells while loading.

2. Use a 10 µl Eppendorf pipetter for loading the gel. The technique is to draw each substance to be loaded into the tip of the Eppendorf, introduce the tip beneath the surface of the electrophoresis buffer between the glass plates and above the well into which you wish to put that sample, and then *slowly* depress the button, emptying the sample into the well. Because the sample buffer contains 10% glycerol, it will be more dense that the electrophoresis buffer, and you can watch the sample drop through the electrophoresis buffer to the bottom of the well.

   (a) You will have six samples and three standards to load. **Follow the loading protocol given in the Data section**. Mark the order on the piece of tape right below the wells. Make sure that you do not load two samples in one well. Notice that one empty well is left on the left side of the gel and two on the right so that you will be able to ascertain which side is which in the case that the gel gets turned over during the staining and destaining procedures.

   (b) Load 10 µl of your plasma preparations (As, Ap, Es, and Ep) and the standards. Load 20 µl of your erythrocyte preparations, G and C.

3. Close the cover of the gel box and attach the electrodes. Placement of the red and black leads is indicated on the box.

4. Turn the amperage to full clockwise and the voltage to 200. At these settings, it will take approximately 4 hours to run your gel.

5. Note the code number on your gel box. Record this number in your Data section; it will enable you to identify your gel after it is stained and destained.

**Part 7: Staining and destaining the gel**
*(These procedures will be performed for you by the preparatory personnel)*

1. When the tracking dye reaches approximately 2 cm from the bottom of the gel, turn off the power supply and disconnect the electrodes.

2. **Pull on** a pair of rubber gloves (small, medium, and large sizes are available).

3. Carry your gel box, with its back side toward you, over to the sink. Open the safety cover and empty the buffer from both upper and lower chambers into the sink.

4. Back at your desk, loosen the gel clamps by rotating the black knobs and remove the gel sandwich from the box.

5. Lay the sandwich on absorbent lab paper with the shorter plate uppermost. Slide the spacer along one edge half-way out from between the glass plates; then twist it in such a way that it lifts the top plate from the gel. You will have to apply considerable force, as the plate sticks tightly to the gel. When it springs loose, put both the top plate and the spacer into a clean dishpan filled with soapy water. Then remove the second spacer and put it into the dishpan as well.

6. With your fingers, carefully loosen the gel slab from the bottom plate and transfer it into a plastic gel-staining box that has been pre-filled to a depth of 2 cm with a solution of Coomassie Brilliant Blue R.
7. Cover your gel-staining box and place it on the wobble-table.
8. After 1 hour, decant the stain into a container for used stain (for reuse). Add destaining solution #1 to a depth of 2 cm and return your box to the wobble-table. By removing excess stain from the solution, this process can be greatly shortened. This can be accomplished by wadding up 4 or 5 Kimwipes and putting them in the destaining solution.
   (a) After a 3–4 hour interval, remove your staining box from the wobble table, pour off the destaining solution into the sink with cold running water, replace it with fresh solution, and return it to the wobble table.
   (b) You will need to repeat the above step two or three times for a total of three to four batches of destaining solution. It is usually most convenient to let it destain overnight.
9. The next morning you will transfer the gel to destaining solution #2. Destaining solution #1 has a high percentage of methanol and the gel shrinks a great deal. Destaining solution #2 has a low percentage of methanol and the gel returns to its original size.
10. When your gel has been adequately destained, wrap it in a piece of cellophane so that you can handle it easily for evaluation.

## Laboratory Procedures – Day 2

• If you are not doing the staining and destaining yourself, allow 2 days for the preparatory staff to complete the procedure.

### Examination of the destained gel

1. You will find your gel together with a Xerox copy of the gel in Room 104 Cresap Laboratories. Xerography produces a fairly good copy of the gel, although some of the lighter bands may be missing and closely-spaced dark bands may appear as one.
2. Put your cellophaned, destained gel on the light box. The strip of white tape above the gel will have the identity of the sample in each well.
3. Compare your gel with the Xerox copy, indicating on the latter any minor components which were too light for the copier to pick up and any broad dark bands which are in reality two closely-spaced dark bands.
4. Use a plastic millimeter ruler to measure the distance that each component traveled; record these values in table in the Data section. Measure from the bottom of the well to the middle of each band. Note also the relative density of each band (very dark, dark, light, very light are suggested categories).

5. The identities and molecular weights (in kD) of the components in the standard mixtures are:

S1: bovine serum albumin.....66
egg albumin.....45
glyceraldehyde-3-phosphate dehydrogenase.....36
carbonic anhydrase.....29
trypsinogen.....24
soybean trypsin inhibitor.....20
α-lactalbumin.....14

S2: myosin.....205
β-galactosidase.....116
phosphorylase.....97
bovine serum albumin.....66
egg albumin.....45
carbonic anhydrase.....29

S3: bovine serum albumin.....66

***Note:*** These proteins are used only as standards and do not necessarily represent any of the unknowns in your fractions.

## Laboratory Report

1. Summarize the distances traveled by each component in the standards and each sample in tabular form.
2. Using semi-log graph paper, plot molecular weight (in kD; on the log scale) of the standards versus the distance migrated (cm; on the linear scale). Your graph should resemble Figure 2.3.
   (a) Draw a line of best-fit for the linear portion of the graph. Extend the line by inspection if the extreme values do not fall in line.
   (b) Using this standard curve, estimate the molecular weights of the components in your six samples. Because the large and small components of the standards may not fall on the curve, estimate the molecular weights of the large and small components in your samples by inspection. Record these values in your table.
3. Using these data (i.e.,molecular size, molecular abundance, and the fraction in which a band is found) together with information given in the background section of this exercise and in your textbooks, speculate on the identity of the various components in each of your six samples.
4. Draw conclusions about (a) the comparative efficacy of the two fractional precipitation procedures, (b) the unexpected presence or unexpected absence of various components in each sample, and (c) other points as indicated by your teaching assistant.
5. Your Data section should be included in your report.

## Data

1. Gel code: _____

2. Loading protocol (wells numbered left to right)

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| - | G | Cy | As | Ap | Es | Ep | - | S1 | S2 | S3 | - | G | Cy | As | Ap | Es | Ep | - | - |

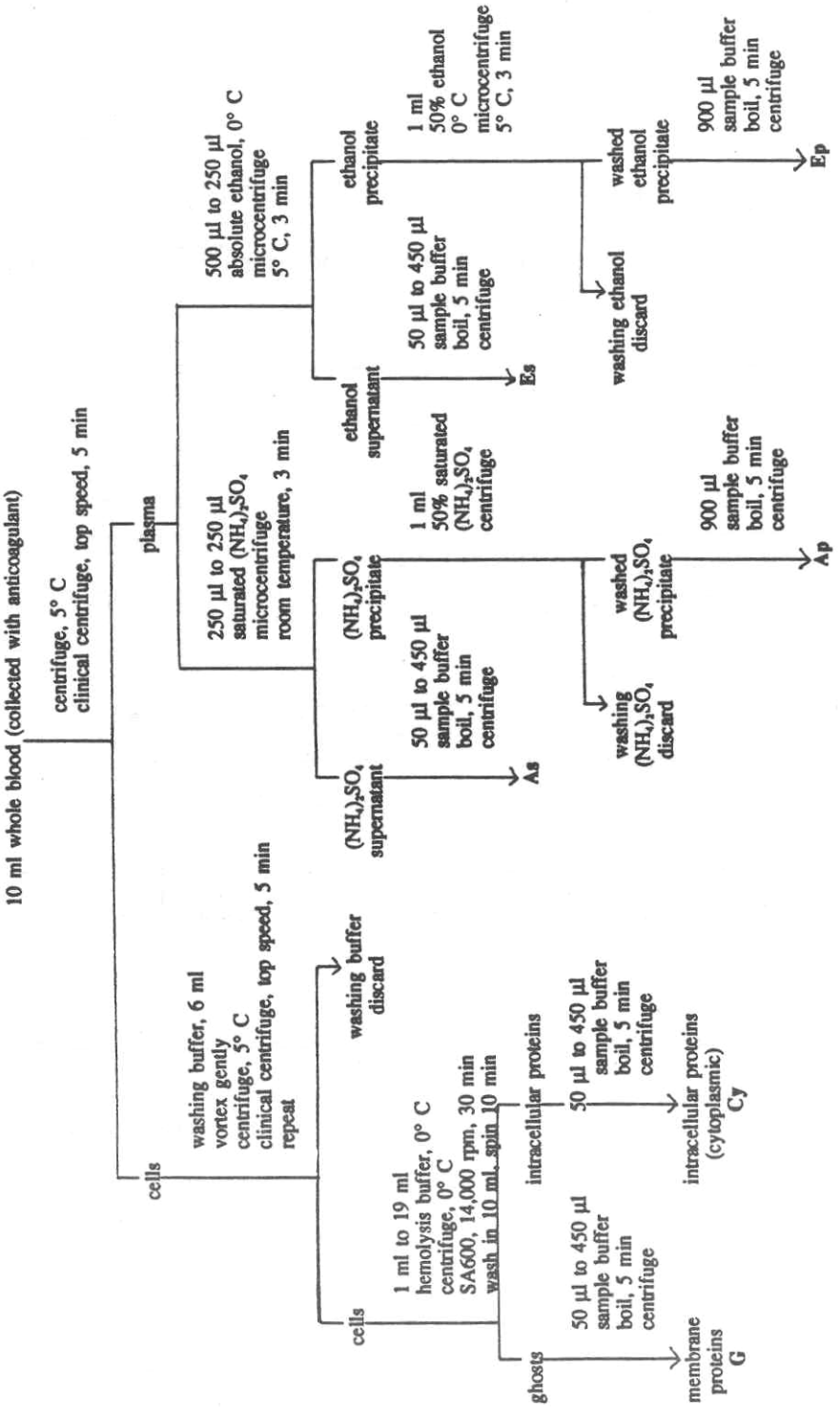
Team 1 will load samples in wells 2–7; team 2 will use wells 13–18.

3. Appearance of gels (migration distance in mm; band densities as VD, D, L, or VL):

| Band | G | Cy | As | Ap | Es | Ep | S1 | S2 | S3 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | | |
| 2 | | | | | | | | | |
| 3 | | | | | | | | | |
| 4 | | | | | | | | | |
| 5 | | | | | | | | | |
| 6 | | | | | | | | | |
| 7 | | | | | | | | | |
| 8 | | | | | | | | | |
| 9 | | | | | | | | | |
| 10 | | | | | | | | | |
| 11 | | | | | | | | | |
| 12 | | | | | | | | | |

4. Comments regarding bands that are streaked, smeared, overloaded, etc.:

## Procedural Flow Chart



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# APPENDIX A
*Experimental Outcome*

The following is a list of proteins that appear in each fraction. This will give you a guideline for grading the students' results.

*Note:* It is probable that not all of these proteins will be present because of abundance or bad technique. Because albumin and hemoglobin are so abundant, it is likely that they will contaminant many of the fractions.

**Ghost Fraction (G)**

spectrin (bands 1 and 2) 240, 220 kD
ankyrin (bands 2.1 and 2.2) 200 kD
band 3 protein 93 kD
band 4.1 protein 82 kD
actin (band 5) 43 kD
glyceraldehyde-3-phosphate dehydrogenase (band 6) 35 kD

**Ammonium Sulfate Fraction**

| Soluble (As) | Precipitate (Ap) |
|---|---|
| albumin 66 kD | α globulins (prothrombin) 72 kD |
| | β globulins (transferrin) 76–81 kD |
| | γ globulins subunits 25–30 kD and 40–50 kD |

**Ethanol Fraction**

| Soluble (Es) | Precipitate (Ep) |
|---|---|
| albumin 66 kD | α globulins (prothrombin) 72 kD |
| | β globulins (transferrin) 76–81 kD |
| | γ globulins 25–30 kD and 40–50 kD |

**Cytoplasmic Fraction (Cy)**

hemoglobin subunits 15 kD

# APPENDIX B
*Preparator's Guide*

## Materials

One section has 24 students who work in pairs. Six gels are needed per section as two pair can load one gel.

**Medium sized tray:** (per pair)

100 ml plastic tripour beaker for waste
Lab pen with fine point suitable for marking microcentrifuge tubes that will be boiled
Plastic pasteur pipets (4)
Microcentrifuge tube holder
Microcentrifuge tubes (4)
Microcentrifuge tube labeled **A** containing 250 µl saturated ammonium sulfate at room temperature (1)
Small wire test tube rack holding four 15 × 85 test tubes, taped
Wooden applicator stick (1)

**Ice bucket** (approximately half-full with ice): (per pair)

12 ml glass calibrated centrifuge tube containing 10 ml blood (1)
(*Note:* If blood is a pretty red and thin-looking, it is probably lysed and cannot be used. Blood must be fresh.)
50 ml polycarbonate centrifuge tube containing 19 ml hemolysis buffer (1)
Microcentrifuge tube containing 250 µl absolute ethanol; labeled **E** (1)

**In lab room on lab bench:** (for 2 pairs of students)

Vortex mixer
Hot plate
400 ml beaker with 2 inches of water
10 cm evaporating dish to serve as lid for beaker
Round plastic holder for microcentrifuge tubes
Forceps, nickel-plated
Tray containing:
- 50 µl Eppendorf pipet
- 1000 µl Eppendorf pipet (1)
- 250 µl Eppendorf pipet (1)
- Kimwipes

**In refrigerator or coldroom:**

Microcentrifuge (1)
Table-top clinical centrifuges (2)

**On front desk or a side bench:**

12-hole microcentrifuges (2)
Microcentrifuge tubes for balance tubes

Repipetter in ice bucket labeled "washing buffer"; set to 6 ml
Repipetter in ice bucket labeled "hemolysis buffer"; set to 10 ml
Small 1 ml repipetter labeled "sample buffer"; set to 0.45 ml
Small 1 ml repipetter labeled "ammonium sulfate"; set to 1 ml
Ice bucket with small 1 ml repipetter labeled "50% EtOH"; set to 1 ml
Container of blue tips
Container of blunt blue tips
Container of yellow tips
Container of blunt yellow tips

**Take into lab later at loading time:**

Medium tray labeled "Standards" and "Loading kit" (for 2 pairs):
- 5 µl Eppendorf (1)
- 10 µl Eppendorf (1)
- Plastic autoclavable tray with yellow tips
- 3 standards in microcentrifuge tubes:
  - S1 (= SDS 7)
  - S2 (= SDS 6H)
  - S3 (= albumin)
- Holder for microcentrifuge tubes

## Solutions

### Washing buffer

150 mM sodium chloride (= 8.766 g/liter)
5 mM sodium phosphate (= 0.71 g/liter, dibasic sodium phosphate = 1.42 g/2 liters)
0.1 mM EDTA (= 0.038 g/liter)
pH 7.4

- Amount required: 6 ml/pair/wash × 2 washes = 12 ml/pair
- Reduce from pH of 9.13 to 7.4 by adding approximately 8 drops of concentrated HCl.
- Dispense from repipetter set to 6 ml and placed in ice bucket on the front desk.

### Hemolysis buffer (hydrolyzing buffer)

5 mM sodium phosphate (use dibasic sodium phosphate) (= 0.71 g/liter)
0.1 mM EDTA (= 0.038 g/liter)
pH 8

- Amount required: 29 ml/pair students
- Reduce from pH of 9 to 8 by adding approximately 1.5 drops of concentrated HCl.
- Store in refrigerator. Dispense into centrifuge tubes in ice bucket from repipetter set to 9.5 ml.

### Ammonium sulfate (saturated)

- Amount required: 250 µl/pair
- Aliquot to labeled microcentrifuge tubes.
- Store at room temperature.

• Make 100 ml by adding ammonium sulfate until it no longer goes into solution. This will take approximately 75–80 grams.

**Tris-glycine stock solution**

50 mM Tris (= 6.0 g/liter)
380 mM glycine (= 28.8 g/liter)

• Amount required: 1 liter/section for electrophoresis buffer. Dilute 10-fold.

**SDS stock solution**

10% SDS (wt:vol)

• Amount required per section (make 150 ml per section): 100 ml for electrophoresis buffer (dilute 100-fold), 10 ml for sample buffer (dilute 5-fold), 6 ml for gel preparation.
• Mix by careful magnetic stirring to avoid foaming.

**Electrophoresis (Tris-glycine-SDS) buffer**

5 mM Tris
38 mM glycine
0.1% SDS wt:vol

• Amount required: approximately 1 liter/two pairs of students
(two pair students/gel).
• Dilute Tris-glycine stock solution 10-fold with SDS stock solution and deionized water to reach final concentration: 100 ml Tris-glycine stock solution, 10 ml SDS stock solution, 890 ml deionized water per liter.
• Suggestion 1: Pour Tris-Glycine Stock into erlenmeyer, fill to 900 ml with deionized water, add SDS, and fill to 1 liter mark with deionized water. This prevents foaming.
• Suggestion 2: Make in 4-liter batches (enough for three gels); add 400 ml Tris-glycine stock solution in a 500 ml graduated cylinder + 40 ml SDS stock solution in a 50 ml graduated cylinder + enough deionized water to make 4 liters in a 4-liter erlenmeyer. It is best to mix the Tris-glycine stock with the deionized water to a level of 3500 ml, then add the SDS and fill to the 4000 ml level. This prevents foaming.

**Sample (Tris-DTT-SDS-glycerol-bromphenol blue) buffer**

80 mM Tris (= 9.6 g/liter = 0.96 g/100 ml))
100 mM DTT (Dithiothreitol) (= 15.42 g/liter = 1.542 g/100 ml)
2% SDS (wt:vol) (= 20 ml stock/100 ml)
10% glycerol (vol:vol) (= 10 ml/100 ml)
0.02% bromphenol blue (= 0.02 g/100 ml)

• Amount required: 5 ml/pair
• For 100 ml add: 70 ml Tris-DTT solution in deionized water, 20 ml SDS stock solution, 10 ml glycerol, 0.02 g bromphenol blue.

**Gel (Tris) buffer**

3.0 M Tris, pH 8.9
MW = 121.4 × 3 = 364.2 g/liter × 3.5 liters = 1274.7 g

• Amount required: 15 ml/pair

• pH will be 10.95 to begin. Make 3.5 liter in a 4-liter beaker. Fill with 2 liter deionized water. Gradually add Tris until dissolved. Adjust pH. Then add water to the 3.5 liter mark. Check and adjust pH again.

### Acrylamide-bis

30% acrylamide (wt:vol)
0.8% bis (wt:vol)

• Amount required: 15–20 ml/pair
• Add 210 g acrylamide + 5.6 g bis to 700 ml of deionized water; stir.

**Ammonium persulfate (APS)**

0.11% (wt:vol) or 0.14% (wt:vol)

• Prepare fresh.
• Amount required: 300 µl portions
• Use 100 mg APS per 1 ml distilled water.
• Suggestion: Make 2 ml at a time; this will provide 1.8 ml for a batch of gel mixture sufficient to cast six gels. Weigh out 200 mg portions of APS in small erlenmeyers. Cover with parafilm and store in desiccator in cold room.

**Gel mixture**

| 10% gel mixture | | | |
|---|---|---|---|
| Ingredients | 4 gels | 6 gels | 8 gels |
| Deionized water | 94 ml | 150 ml | 188 ml |
| Gel buffer | 22.5 ml | 36 ml | 45 ml |
| SDS stock | 1.8 ml | 2.9 ml | 3.6 ml |
| Acrylamide-bis | 59.5 ml | 95 ml | 119 ml |
| APS | 1 ml | 1.5 ml | 2 ml |
| TEMED | 125 µl | 200 µl | 250 µl |
| Total volume = 59.5 ml (the amount needed for casting one gel) | | | |

**Coomassie blue stain**

0.25% Coomassie Brilliant Blue R in methanol:acetic acid:water, 5:1:5 (vol:vol:vol) (= 0.25 g/100 ml)

• Make 200 ml per section: 91 ml methanol, 18.2 ml acetic acid, 91 ml deionized water.

**Destaining solution 1**

• For 1 litre: 450 ml methanol, 100 ml acetic acid, 450 ml distilled water.
• Work under hood to make solution.

**Destaining solution 2** (the rehydrating destain step)

• For 1 litre: 50 ml methanol, 75 ml acetic acid, 875 ml deionized water.
• Work under hood to make solution.

**Half-saturated ammonium sulfate**

• Amount required: 1 ml/pair
• Use the saturated and dilute by half.
• Put into small repipetter set to 1 ml and store at room temperature.

### 50% EtOH

• Put in small repipetter set to 1 ml and store in cold room.

### Absolute EtOH

• Aliquot 250 µl into microcentrifuge tubes labeled "E". Place in microcentrifuge tube racks and into freezer.

### Standards

For SDS 7 and SDS 6H:

- Dissolve in 1.5 ml sample buffer.
- Aliquot 30 µl into labeled microcentrifuge tubes
- Boil 5 minutes.
- Place in microcentrifuge tube racks and put in freezer. Take out when the lab is beginning the third hour.

Label SDS 7 as "S1" (low molecular weight)
Label SDS 6H as "S2" (high molecular weight)
Label albumin as "S3"

Bovine albumin (0.71 mg/ml)

- Make 2 ml in sample buffer: 0.71 mg x 2 ml = 1.42 mg = 0.0014 g
- Follow the same instructions as for S1 and S2.

## Setting Up the Plates

After the glass plates have been thoroughly cleaned, the spacers should be arranged on the larger of the two plates. The glass plate should be on a platform (rubber stopper) so that there is space to attach the clamps. Next, position the smaller plate over the spacers making sure that the foam blocks are tight against the plate. Place two clamps on each side and the bottom. Leave the plates on the platform in a horizontal position.

### Making the gels

1. Add the first five components in order; mix (swirl) after each addition. This should be done in a side arm flask.
2. Attach the flask to a vacuum and de-gas for 1 minute.
3. Add TEMED and mix.
4. While holding the plates at a 30° angle, pour the gel, minimizing the bubbles that get trapped between the plates. If bubbles become trapped between the plates, hold the plates vertically and firmly tap on the plates. This will knock the bubbles loose, and they will rise to the top.
5. Place the gel horizontally and insert the comb all the way. Clamp the top of the plates at the position of the comb; this prevents any acrylamide from polymerizing between the comb and the plate. It will take about 45 minutes to polymerize.
6. After polymerization remove the clamps and the comb. Rinse the plates with water to get rid of any dried acrylamide stuck to the outside of the plates. Clamp the gel to the gel apparatus and fill the reservoirs with buffer. The spacers don't always fit tightly and there may be some leakage. This can usually be stopped by the addition of some vacuum grease to the joints. Place a piece of tape on the glass plate directly under the wells so that the students can clearly mark the loading order.

**Additional set-up**

1. Have view boxes ready.
2. Spread lab paper over benches to set up gel forms.
3. Shakers for staining and destaining boxes.
4. Lab paper covering area to wrap completed gels in cellophane. Place on tray with section number near view boxes.
5. Under hood: set-up for making destaining solution.
6. Turn on ultracentrifuge on the morning of the first lab period.
7. Set up balances for balancing centrifuge tubes. Have two 125 ml erlenmeyers of hemolysis buffer with pasteur pipet in two ice buckets for balancing. Have extra centrifuge tubes for balancing.
8. Hook up two 1 liter side arm flasks to water suction for gel mixture preparation.

**Gel plates, spacers, and combs**

For successful electrophoresis, it is essential that the glass plates be free of residual polyacrylamide, grease spots (i.e., fingerprints) and dust particles. Thus, prompt and proper cleaning of the plates is essential.

*The following procedure is recommended for plates:*

1. Place the glass plates into a clean dishpan containing a small amount of non-abrasive detergent in deionized water as soon as the gel is removed.
2. Rinse each plate individually under the tap with warm (*not hot*) water to remove all residual gel. Rub each plate on each side with a clean cellulose sponge.
3. Transfer each plate to a clean dishpan filled with deionized water and swish several times to rinse off detergent.
4. Rinse each plate on both sides under running deionized water.
5. Stand each plate on a folded paper towel in a wire-basket cart, bracing it between the wires so that it stands vertically without touching another plate to air dry.
6. Prior to use, wipe the gel surface of each plate with a Kimwipe soaked in 70% ethanol to remove all finger prints.
7. Use clean dishpans for this procedure. Also use a new sponge.

*Plastic spacers and combs should be cleaned as follows:*

1. Place these items into a clean dishpan containing a small amount of non-abrasive detergent in deionized water as soon as they are removed from the gel.
2. Rinse in warm running tap water as above; rinse in deionized water as above; air-dry in a wire-basket cart.

**Miscellaneous**

• For blunt blue and yellow tips, slice off the end with a sharp razor blade.

# APPENDIX C
## *Source of Materials*

**Citrated sheep, pig, bovine, or horse blood:** Lampire Biological Laboratories, P.O. Box 170, Pipersville, PA 18947. The cost is $30 US for 0.5 liter and $45 US for 1 liter. *Note:* At Northwestern University, we are required to have a protocol approved by the animal care committee before we may proceed with this laboratory exercise. The reason is that, even though a mammal is not sacrificed for this exercise, mammalian products are used.

All the **electrophoresis grade reagents**, including glycine, Coomassie Brilliant Blue R, TEMED, acrylamide, bis-acrylamide, ammonium persulfate, and Tris: BIORAD, 3300 Regata Blvd., Richmond, CA 94804.

All **other chemicals**, including the **standards**: Sigma Chemical Company, P.O. Box 14508, St. Louis, MO 63178. Standards: High Molecular Weight Standard Mixture (catalog #SDS-6H), $11 US per vial; Dalton Mark VII-L (catalog #SDS-7), $11 US per vial.

**Electrophoresis equipment:** GIBCO BRL Life Technologies, 3175 Staley Rd., Grand Island, NY 14072. Plastic gel box V15-17 (catalog #1080VA), $299 US each; glass plates, 3 pair/set (catalog #1074GC), $20 US per set; spacers, 1.5 mm (catalog #1077GH), $20 US per set of 36; combs, 1.5 mm, 20-tooth, (catalog #1076GH), $30 US each.

**Miscellaneous equipment:** Gel staining trays (catalog #144210X), $30 US each from Research Products International, North Business Center Dr., Mount Prospect, IL 60056; Lab-Line Variable Junior Orbit Shaker (catalog #S1060-1), $940 US each from Baxter Scientific Co., 1210 Waukegan Rd., McGaw Park, IL 60085-6788.