THE CONDUCTOMETRIC DETERMINATION OF ELECTROPHORETIC PATTERNS

by

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ABSTRACT

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Electrophoresis, an analytical method in the field of separation science, is widely used today in biochemical and clinical fields for the analysis of biological macromolecules such as proteins, polysaccharides, and nucleic acids.

Electrophoresis most often involves the separation of a sample on a stabilized supporting medium. This is accomplished by application of an electric field to the sample resulting in migration of separated components at different rates towards one of the electrodes. This migration is possible provided the biological macromolecule bears a net charge. The supporting medium functions to allow the investigator a means of fixing the separated components after each has traveled a certain distance.

After electrophoretic separation on the supporting medium, detection and chemical characterization of the separated components are usually achieved by the utilization of colored dyes. This staining procedure, however, suffers several drawbacks:

1. Dyes are not universal; there exists a specificity of protein stains, carbohydrate stains, and so forth, and it is the ultimate task of the experienced clinician or scientist to determine the appropriate dye for a specific class of macromolecules so as to ensure selectivity of detection.

2. The staining and destaining procedures are very time consuming and the utilization of dyes often introduces many complications (variation in staining densities/ intensities, qualities of dye differing considerably with time and different batches, and cracking of dyed gels during drying) resulting in irreproducibility of the band patterns.

3. The supporting medium is rendered inactive following staining and therefore may not be reused for multiple analyses.

With this in mind, an alternate detection method is developed and studied, where conductivity of the analytes, proteins, will serve as the physical property for characterization of separated components. To allow for measurement of this property, a conductivity cell is constructed and a conductance meter attached. This is the first known report of the successful characterization of protein electrophoresis employing this conductivity detection system.

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LIST OF SYMBOLS

SYMBOL	DEFINITION
A	Area
AC	Alternating Current
A/D	Analog-to-Digital Conversion
c	Concentration
C	Celsius
cm	Centimeter
d	Distance
D/A	Digital-to-Analog Conversion
DC	Direct Current
E	Potential Gradient; Electric Potential; Voltage
F	Force of Electrostatic Attraction
Fs	Frictional Resistance
g	Gram
G	Conductance
hr	Hours
Hb	Hemoglobin
I	Current
I'	Ionic Strength of Buffer
k	Specific Conductance; Conductivity
k'	Constant
K	Cell Constant
mg	Milligram

SYMBOL	DEFINITION
min	Minute
mL	Milliliter
M	Molar
n	Viscosity
N	Normal
Q	Charge of Particle
r	Radius of Particle
R	Resistance
t	Time
TCA	Trichloroacetic Acid
u	Electrophoretic Mobility
v	Velocity of Particle; Distance Per Unit Time
w/v	Weight Per Unit Volume
z	Ionic Charge; Valence
α_1	Alpha One Protein Fraction
α_2	Alpha Two Protein Fraction
β	Beta Protein Fraction
γ	Gamma Protein Fraction
µL	Microliter

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CHAPTER I

INTRODUCTION

Principles of Electrophoresis

Electrophoresis is defined as the movement of charged particles in an electric field. Specifically, biological macromolecules, if they bear a net charge, will migrate at different rates either to the cathode or to the anode as an electric potential is applied. The rate of migration is a function of charge density (the ratio of charge to mass) and electric field strength. Hence, it is the analysis of differential migration of particles of the sample that forms the fundamental basis for electrophoretic separation.

The migration of the ions is caused by two specific forces, a driving force (force of electrostatic attraction) and a retarding force (frictional resistance of the medium).[1] The driving force may be expressed as follows:

$$F = QE \qquad (1)$$

where F is the force of electrostatic attraction, Q is the charge of the particles in the sample, and E is the potential gradient or the electric potential applied to the cell. The retarding force, due to the viscosity of the medium through which the particles move, can be defined in the following equation:

$$Fs = 6\pi rvn \qquad (2)$$

where Fs is the frictional resistance of the medium, r is the particle's radius, v is the particle's velocity, and n is the viscosity of the medium.

The two forces may then be equated (since there exists a certain velocity at which the two forces balance each other) to give the equation:

$$EQ = 6\pi rvn \tag{3}$$

Electrophoretic mobility, defined as the velocity of migration of a particle under the influence of an electric potential gradient, serves as a useful property in the characterization of a sample and may be expressed as follows:

$$U = d/tE \quad \text{or} \quad U = v/E \tag{4}$$

where U is the electrophoretic mobility, d is the distance traveled by the particle, t is the time required to travel a certain distance, E is the potential gradient or the electric potential applied to the cell, and v is the velocity, the distance per unit time.' Since electrophoretic mobility refers to the ability of a particle to move, which is dependent upon the forces discussed above, equation 3 may now be substituted into equation 4 to give the following:

$$U = \frac{Q}{6\pi rn} \tag{5}$$

This is the basic equation of electrophoresis.[2] With $6\pi n$ serving as a constant in a laboratory experiment, electrophoretic mobility may be reduced to the following equation:

$$U = \frac{Q}{k'r} \tag{6}$$

Hence, mobility is a function of the ratio of charge to radius of the particles in a sample. This explains how two samples of equal charge but varying sizes may be separated using the principle of electrophoretic mobility.

The Charged Species

To illustrate the electrophoretic process and the requirement that the molecule be charged, let us now consider the separation of a specific class of macromolecules, namely proteins.

Proteins are composed of amino acids linked together by **peptide** bonds. Amino acids, monomeric building blocks of all proteins, consist of amino ($-NH_2$), carboxyl (-COOH), and hydrogen (-H) groups attached to a central carbon atom. It is the R group which varies and distinguishes one amino acid from another. The general structure of an amino acid is shown in Figure 1.

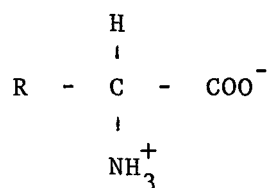
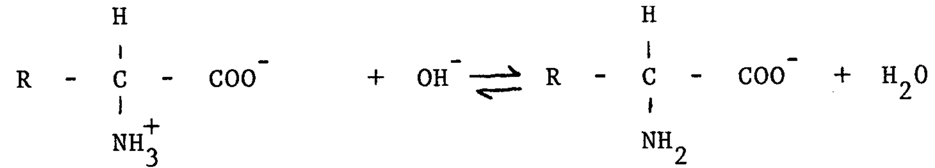


Figure 1 - General Structure of an Amino Acid

Proteins carry ionic charges since they contain amino acids having carboxylate as well as ammonium, guanidinium and imidazolium groups. The charge shall depend, however, on the pH of the buffer or medium in which the protein is found.[3] For example, the protein shall exhibit a negative charge (more COO- groups) in alkaline medium and alternatively a positive charge (more NH_3^+ groups) in an acid medium. The direction of movement of the charged species shall be toward the electrode bearing opposite charge. Equations which illustrate the protein's (amino acid's) charge dependency on the pH of the buffer solution are shown in Figure 2.[4] When the protein carries no net charge at a certain pH (same number of NH_3^+ as COO- groups), the protein is electrically neutral and defined to be at its isoelectric point. In this state of neutrality,

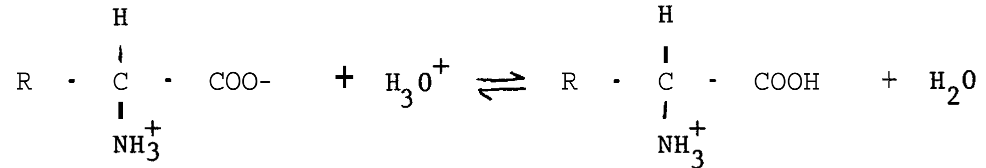
the protein will not migrate to either anode (positive electrode) or cathode (negative electrode); there is no migration at the isoelectric point.

In alkaline medium:



(migrates toward positive electrode - ANODE)

In acid medium:



(migrates toward negative electrode - CATHODE)

Figure 2 - Protein's (amino acid's)[4] charge dependency on the pH of the buffer solution.

The Supporting Medium

Electrophoresis may be carried out in free solution (Tiselius's moving boundary/free electrophoresis) or on a supporting medium (zone electrophoresis). The advantages of the latter type of electrophoretic separation are twofold. The supportive medium minimizes deleterious effects of convection and diffusion. Convective disturbances are

caused by heating effects during electrophoresis which can ultimately interfere with separation of bands or zones; diffusion results in constant broadening of bands or zones even after electrophoresis has been stopped.[5] Hence, zone electrophoresis allows for more complete and sharper resolution of a mixture into its respective components, as shown in Figure 3.[6]

Various requirements are necessary when selecting the supporting medium. Choice of support is largely related to the analytical task at hand. The supporting medium in general, however, should possess the following characteristics:[6]

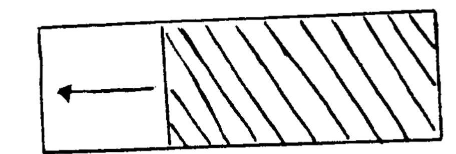
1. It should be stable over a wide range of pH, ionic strength, and temperature.
2. It should be transparent so as to allow for visual evaluation.
3. It should have good tensile strength so as to assure ease in handling and in application of sample.
4. It should be non-toxic.
5. Its cost should be low.

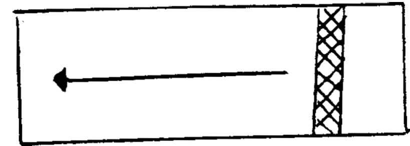
Many supporting media are used today for zone electrophoresis. These include paper, cellulose acetate, and other thin-layer materials as well as gels of agar, agarose, starch and polyacrylamide. The former class of materials serves mainly for support and to decrease convection. Here, separation takes mainly into account the charge density of the migrating species at a particular pH. The latter class, the gels, not only minimizes convection and diffusion but may also interact with the migrating species in a process known as molecular sieving. Molecular sieving refers to the interaction of sample (pore size) as it passes through the porous supporting

Frontal/Moving Boundary Type

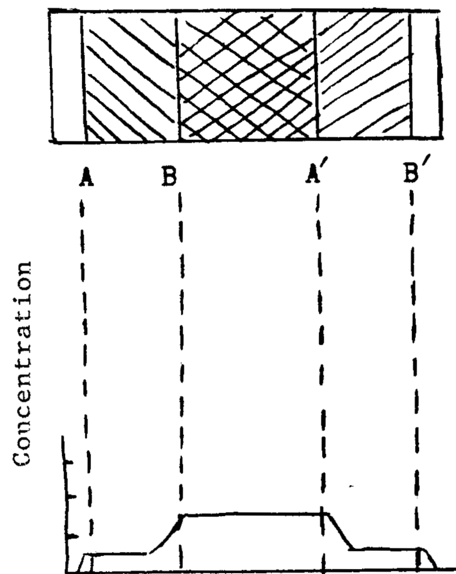
Zonal Type

Before Fractionation





After Fractionation



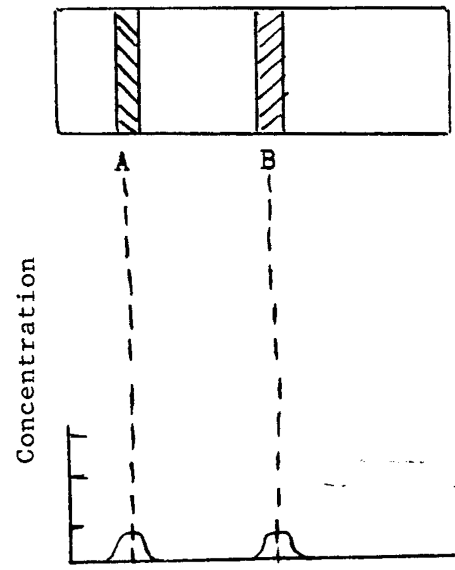


Figure 3 - Concentration distribution before and after the run in frontal and zonal electrophoresis of a mixture containing two components.[6]

medium. This sieving-effect contributes significantly to the electrophoretic separation when the supporting medium's pore size approximates or is of the same magnitude as that of the migrating species. Hence, separation is influenced by both charge density and size. It is possible then to effectively separate two proteins of identical charge densities but different sizes. The migration rate of the larger protein will be impeded more strongly in comparison to that of the smaller protein because of the molecular sieving effect. Gel systems, therefore, are far superior to paper or cellulose acetate medium, since molecular size allows for further possibilities of separation. As a result, gel systems afford sharper and more numerous bands or zones.[5]

Agar gel is a polysaccharide obtained from the cell walls of red algae and used as an electrophoretic medium. It consists of two fractions, agarose and agaropectin. Although agar is relatively inexpensive and can be preserved for long periods of time, agarose, the purified fraction of agar, is preferred. The undesired fraction, agaropectin, gives considerable electroendosmosis and background color due to the presence of its sulphate and carboxylic acid groups.[7] Besides neutrality (which results in considerable reduction of electroendosmosis), agarose has other advantages over agar, such as its ability to form stronger gels, its utilization with a wider variety of buffer solutions, and its ability not to inactivate enzymes.[3]

The Buffer System

Currently, no universal buffer system exists for the electrophoretic separation of biological macromolecules such as proteins.

Rather, the choice of—,buffer for a particular separation is done by trial and error. Two properties which assist the investigator in the appropriate selection of buffer are pH and ionic strength.

As discussed in the previous section, large molecules possess both anionic and cationic groups. The net charge on such a molecule' depends upon pH of the environment - the buffer system with which the molecule comes in contact. The buffer system influences electrophoretic separation by varying the charge on different molecules. It is therefore inherently responsible for differential mobilities, the fundamental principle in electrophoresis.

The selection of the pH of the buffer is relative to that of the isoelectric points of the proteins to be separated. With the pH of the buffer very close to the average isoelectric point of proteins, one is able to effectively increase separation since greater charge differences between the proteins are attained. Alternatively, reduced times for electrophoretic separation as well as decreased diffusion (decreased band spreading) is apparent when the pH of the buffer is very far from the isoelectric points of proteins to be separated. This is due to a higher charge on the proteins and greater mobility. Ultimately, a pH range must first be chosen over which the proteins under study are stable.[5]

Ionic strength of the buffer is another parameter which affects electrophoretic mobility and hence separation.[4] Ionic strength is defined in the following manner:

$$I' = \tfrac{1}{2} \sum cz^2 \tag{7}$$

where I' is the ionic strength of the buffer, c is the molar concentration of a particular ion, and z is the ionic charge or valence of that ion.

Mobility is **inversely** proportional to the square root of the ionic **strength.**[1] Therefore, low ionic strength buffers allow for faster migration rates, where separation is achieved in a minimal amount of time with little production of heat. High ionic strength buffers, on the other hand, promote sharper resolution due to decreased diffusion coefficients. The migration rates using high ionic strength buffers are however longer and there is a greater degree of heat production. Longer migration rates are due to the greater number of buffer ions which are available to carry the current. Greater heat production is the result of lower electrical resistance (greater conductivity) afforded by the higher concentration of buffer. This lower electrical resistance, according to Ohm's law, results in a greater degree of current carried at a given applied voltage generating a greater amount of heat output.[1,8] (The effects of heat on separation during electrophoresis are numerous. They are discussed in the section entitled "The Electric **Field".)**

A compromise must be reached to allow for rapid migration rates with sharp resolution of bands or zones without the abundant production of heat, which would have deleterious effects in the separation process. It has been found that buffer ionic strengths within the range 0.05 - 0.1 M accomplish this goal.

To complete the discussion of buffer systems, it is essential to introduce the following terms: continuous buffer systems and discontinuous (multiphasic) buffer systems. Continuous buffer systems refer to the presence of the same buffer throughout the sample, supporting medium such as gels, and buffer reservoirs (electrode

vessels) at a constant pH. Here the sample is loaded directly onto a small-pore resolving gel, which represents the medium in which the separation will occur. Contrast this to the discontinuous (multiphasic) buffer system where different buffer solutions are used in the supporting medium and the buffer reservoirs (electrode vessels). The sample is now loaded directly onto a large-pore stacking gel which is polymerized on top of a small-pore resolving gel. Discontinuities in both buffer composition and pH may occur. Hence the latter type of buffer system offers high resolution compared to continuous buffer systems, since the sample effectively becomes concentrated into narrower zones or bands as it migrates from stacking gel to resolving gel. The use of continuous and discontinuous buffer systems in rod and slab gels is shown in Figure 4.[5]

The Electric Field

To allow for migration of charged particles within the supporting medium a direct current (DC) is used as a driving force. Specifically, an alternating current (AC) from a wall plug is converted into a pulsating direct current by a rectified power supply. This signal is then further filtered and conducted to the negative electrode of the electrophoretic apparatus. Free electrons are generated to the supporting medium, buffer solution, positive electrode, and finally back to the power supply to complete the circuit. As the electrons flow through the supporting medium, they come in contact with the charged particles from the sample which result in their movement toward the oppositely charged pole.[9]

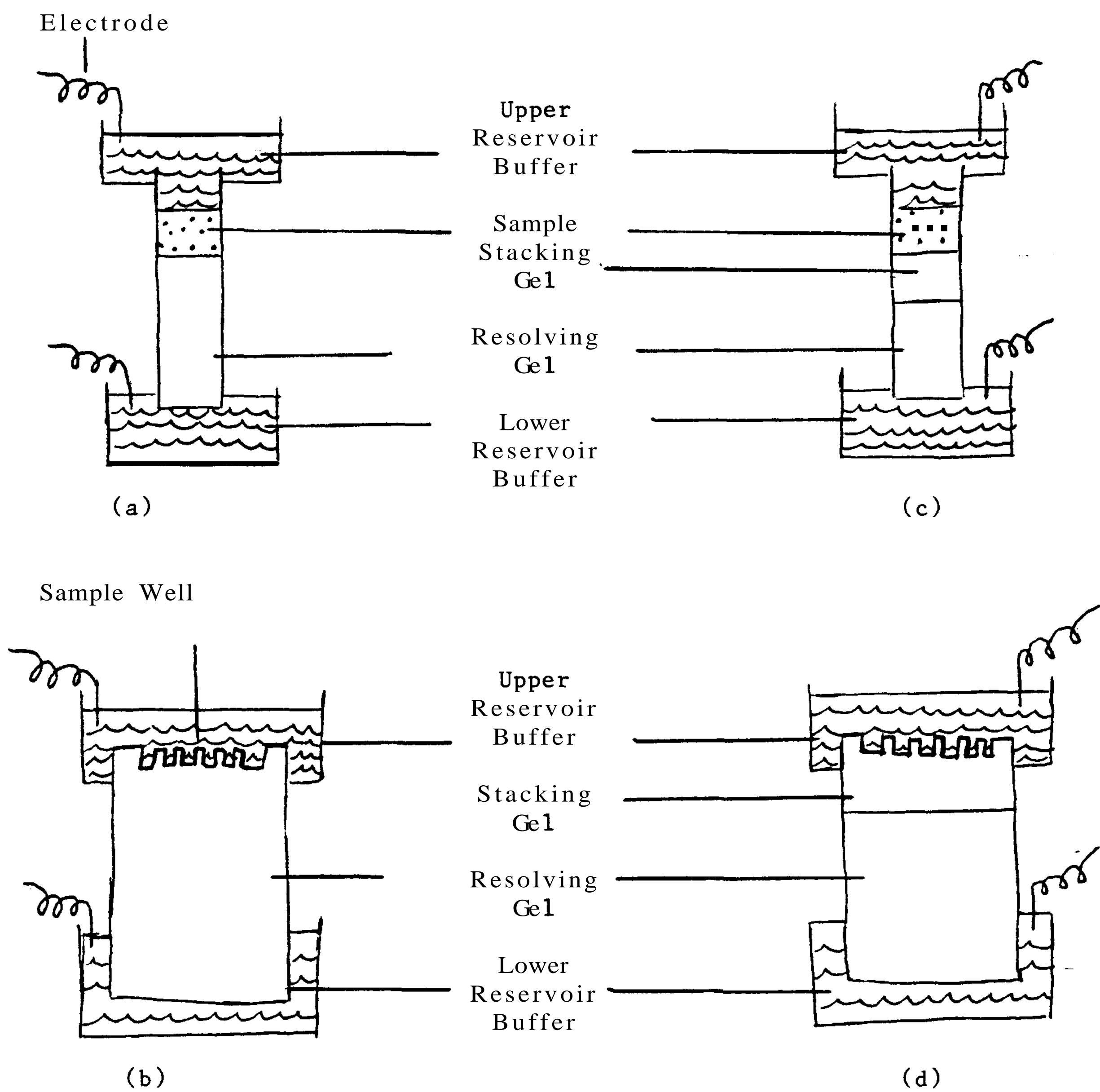


Figure 4 - Use of continuous and discontinuous buffer systems with rod and slab gels. (a) continuous buffer system used in conjunction with a rod gel; the sample is loaded directly onto the resolving gel. (b) continuous buffer system used in conjunction with a slab gel; samples are loaded into wells formed directly in the resolving gel. (c) and (d), discontinuous buffer system used in conjunction with rod and slab gels, respectively; samples are loaded directly onto the stacking gel or into wells formed in the stacking gel, respectively.[5]

The electricfield is determined by power supplies which operate at either constant current or constant voltage. At constant voltage, there is a concomitant and gradual increase in current which results in the evolution of heat:[7]

$$\text{Heat} = (E)\,(I)\,(t) \tag{8}$$

where E is the voltage, I is the current, and t is the time. This increase in current, manifesting itself as elevations in temperature, (Joule heat) affects the electrophoretic separation in many ways:[1,6]

1. Decrease in viscosity. Viscosity may be defined as an exponential function of $1/T$ where T is the absolute temperature. Therefore, it has been found that for an increase in temperature from $0°$ to $25°$ in free aqueous medium, viscosity is halved whereas electrophoretic mobility is doubled. This decrease in viscosity results in an increase in diffusion which reduces resolving power.

2. Increase in diffusion and ionic mobility (about 2.4 percent per degree Celsius rise in temperature). This is the result of an increase in thermal agitation of the molecules and a decrease in viscosity or retarding force of the supporting medium.

3. Increase in conductivity. An increase in the evaporation of water from the supporting medium causes an increase in ion concentration (conductance). This leads to a more pronounced Joule heating.

4. Modifications in ionic strength and pH of buffer. The evaporation process alters these parameters and in so doing interrupts the homogeneity of the electric field.

5. Denaturation of proteins or loss of enzymatic activity.

6. Incineration of the supporting medium.

The above deleterious effects are cumulative and inherent in electrophoresis with the passage of the electric current. Although these effects may never completely be abolished, they can be minimized. For example, built-in refrigeration units may be incorporated into the electrophoretic apparatus to offset the heating effects. Alternatively,

lower ionic strength buffers may be utilized, since they provide lower conductivity (and hence increased resistance). At constant voltage the effect is ultimately a decrease in current which overcomes any heating problem, since Joule heating is defined as follows[4]:

$$\text{Wattage} = (E)(I) = (R)(I)^2 \qquad (9)$$

where E is the voltage, I is the current, and R is the resistance. Nevertheless, constant voltage is used for short-term electrophoresis (one-half hour or less) where heat production is kept to a minimum as current gradually increases. For long-term electrophoresis, power supplies which provide constant current are used, since temperature will not increase appreciably during the run.[8] For example, when the resistance of the system decreases as is present in the evaporation process, the voltage will also decrease. This keeps the heating effect to a minimum as is expressed in Equation 8, allowing migration rates to remain relatively constant. It must be stressed, however, that constant-current power supplies do not eliminate heat production entirely.

To summarize, optimum electrophoresis consists of obtaining ". . . the highest degree of resolution, and the fastest migration rate with no degradation due to overheating."[10] Neither constant-voltage nor constant-current power supplies meets this requirement. Electrical resistance of the electrophoretic system may change with time, affecting heat output. In order to help resolve this dilemma, another type of power supply known as constant-power power supply is entering the scientific arena. This type of power supply, commercially available today for analytical work of the highest accuracy, ensures optimum conditions for electrophoretic separation. It allows

the investigator to regulate power independent of electrical resistance changes. Therefore, although heating effects are not eliminated, they are kept nearly constant during a run. Power can now be regulated in such a manner so as to achieve maximal electric field strength (allowing for more rapid electrophoretic separation) without the generation of excess heat, which the system could not tolerate.[10]

Electroendosmosis

Electrophoresis consists of sample migration of charged particles with time in an electric field. Another process which operates concurrently involves the movement of buffer in a supporting medium subjected to the electric current. This buffer flow is known as electroendosmosis (or endosmosis). Although it does not prevent separation of the charged particles, electroendosmosis can modify the migration distance of these particles affecting their final position on the supporting medium. Also, electroendosmosis is responsible for "hydrostatic pressure heads" which affect the homogeneity of the electric field by causing a build-up of water at one end and the diminution and drying out at the other end.[6]

Specifically, electroendosmosis occurs as the supporting medium becomes negatively charged when placed in alkaline buffer or water due to the adsorption of hydroxyl ions. These negatively-charged ions cannot migrate because of their fixation onto the rigid electrophoretic supporting medium. Positively-charged ions from the buffer, however, become attracted to these negative sites in the supporting medium and, as a result of current applied to the system, will flow toward the cathode (negative electrode).

Movement of water in this direction will also result, since these positively-charged ions are highly hydrated. Furthermore, this movement of solvent (water) will be accompanied by migration of its solutes, any weakly-charged molecule(s) which tend(s) to migrate toward the anode because of negative charge. The net effect is that buffer flow is counter to current flow of proteins migrating to the anode, as is illustrated in Figure 5.[8] As a result, all protein molecules will be slowed in their migration or ultimately moved with the buffer in the opposite direction to that which is expected. Rate of buffer flow can be determined by utilizing uncharged molecules such as urea, dextran, or deoxyribose and monitoring the distance that these are passively carried by the buffer.[1]

Serum proteins may be fractionated on a supporting medium such as cellulose acetate. Five bands will be obtained at the conclusion of an electrophoretic run, namely albumin, alpha one (α_1), alpha two (α_2), beta (β), and gamma (γ) globulins, respectively, beginning with the species which exhibits greatest negative charge (or greatest migration toward the anode). The more mobile fractions will move toward the anode whereas the protein fraction exhibiting the lowest electrophoretic mobility, the gamma band, is displaced toward the cathode due to electroendosmosis.

Staining and Destaining Methods

Detection of the separated components of a sample is achieved utilizing a variety of methods as is shown in Table 1.[6] Chemical characterization involving staining reactions, however, is probably the most frequently used method of detection.

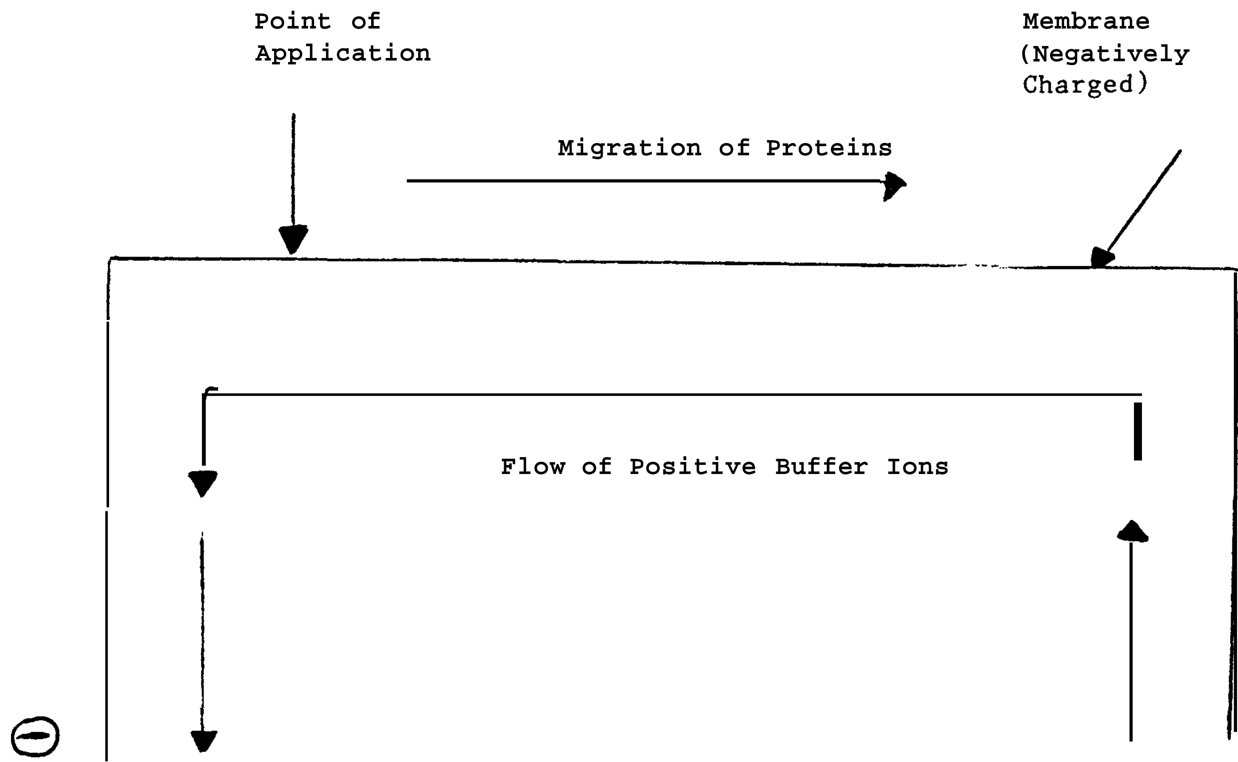


Figure 5 - Illustration of electroendosmosis. Buffer movement is counter to the current flow and migration of the serum proteins.

TABLE 1

PROPERTIES SUGGESTED FOR ADDING A CHARACTERIZING DIMENSION DURING OR AFTER ELECTROPHORETIC SEPARATION[6]

Properties	Methods
physical	- light absorption or dispersion - radioactivity - precipitability - interaction with complexing substances - molecular dimensions
chemical	- staining characteristics
enzymic	- determined after elution - determined by enzymoelectrophoresis - determined by conventional histiochernical procedures
immunochemical	- interaction with antibodies
biological	- colicines, haemolysins, lysozyme

There are numerous fundamental requirements which are necessary in order to ensure successful detection of electrophoretic separations. They are as follows:[11]

1. The colored dye must be able to react and form a complex with the macromolecule under analysis to subsequently yield colored insoluble bands.

2. The dye should not bind to the supporting medium, so that high background colorization will be avoided and will therefore not interfere with the interpretation of electrophoretic results.

3. The dye should ultimately be specific for a particular class of macromolecules. If this is not possible (i.e. the dye interacts and complexes with several types of substances) the complexes formed should exhibit different distinguishable colors.

Ths staining procedure is very simple. It consists of immersing the supporting medium into a dye bath for a specific amount of time. Often, a fixing step is introduced simultaneously or prior to staining in order to avoid diffusion or losses during staining and destaining.[1] A fixing agent such as trichloroacetic acid (TCA) is employed. This fixing step is performed only when the retention of enzymic or biological activity is unimportant, since inactivation of sample is often apparent.

There are currently a number of dyes commercially available for staining biological macromolecules. A summary of staining-. procedures for proteins is shown in Table 2.[1] Each staining procedure differs in staining mixture composition, staining time, destaining mixture composition, staining reactions, and sensitivity. A high sensitivity silver staining method for proteins is described in Table 3.[1,12]

TABLE 2

SUMMARY OF STAINING PROCEDURES FOR PROTEINS (adapted from 1)

Staining mixture	Staining time (hr)	Destaining mixture	Comments	Staining reactions
Amido Black 10B 1-10 g dissolved in 250 mL methanol; then add 100 ml acetic acid + 650 mL H_2O)	0.5-2.0	10% acetic acid	Very widely used, simple; prefixing in 12.5% TCA may be advantageous	
Procion Brilliant Blue RS 2-10 g dissolved in up to 500 mL methanol; then add 100mL acetic acid + H_2O to 1 liter	1.0-2.0	500 mL methanol + 100 mL acetic acid + 400 mL H_2O	Very suitable for densitometry; use fresh dye each time. 2x as sensitive as Amido Black 10B	Forms covalent bonds with -OH, $-NH_3^-$, -NH, and peptide groupings of proteins
Coomassie Blue R250 1 g dissolved in 500 mL methanol; then add 100 mL acetic acid + 400 mL H_2O or	1.0	500 mL methanol + 100 mL acetic acid + 400 mL H_2O	Very sensitive. 3x as sensitive as Amido Black 10B	Forms electrostatic bonds with NH_3^+ groups and non-covalent bonds with non-polar regions in the proteins
0.5 g in 50 mL H_2O mixed with 950 mL 12.5% TCA	0.5-1.0	10% TCA for 20-30 min (store in 7% acetic acid)	Before staining, gels can be prefixed for 0.5 hr in 12.5% TCA (or overnight if desired)	
Anilinonaphthalene sulphonate 30 mg in 1 liter of 0.1 N sodium phosphate pH 6.8	0.05		Fluorescent labeling; less sensitive than staining methods but faster and preserves biological/ enzymic activity	

TABLE 3

HIGH-SENSITIVITY SILVER STAINING METHOD FOR PROTEINS * (1)

	Staining procedure (successive steps)	Time
(1)	Soak gel(s) in 10% aqueous glutaraldehyde	30 min
(2)	Rinse three times in 500-1000 mL distilled water	10 min
(3)	Soak in water	>2 hr or over-night
(4)	Drain off water, add fresh ammoniacal silver solution. Stir continuously to prevent deposition of silver on the gel surface. (Ammo-niacal silver solution prepared by adding 1.4 mL of conc. NH_4OH to 21 mL of 0.36% NaOH. Stir vigorously and slowly add 4 mL of 19.4% $AgNo_3$. When the transient brown precipitate has cleared make up to 100 mL with water)	≤ 15 min
(5)	After no more than 15 min wash gel in water	2 min
(6)	Transfer into a fresh solution of 0.005% citric acid and 0.019% formaldehyde. Protein zones become visible at this stage. Remove gel from solution when background begins to darken.	5-30 min
(7)	Wash gels thoroughly in distilled water (at least 3 changes) .	> 1 hr

* Claimed to be 100 times more sensitive than Coomassie Blue R250 extending staining methods down to the sensitivity levels of radioactive procedures.

Following staining is destaining, the removal of excess dye which colors the whole supporting medium and not just the zones which contain the bands of separated components to be analyzed. Two methods are available to destain gels, namely, soaking the gels in a destaining solution (diffusion destaining) or further electrophoresis (electrophoretic destaining).

In the former method, diffusion destaining, leaching out of excess dye is achieved by immersing the supporting medium in a destaining solution (12.5% isopropanol, 10% acetic acid), usually for 24 hours or more. This solution consists of solvents which dissolve free dyes but leave the complexes formed between macromolecules and the dye intact. The procedure is time consuming. It can be accelerated, however, by changing the destaining solution often, by stirring, or by increasing the temperature of the system. This causes a high concentration gradient of dye between the gel and the destaining solution. Another method by which to accelerate destaining is to use a dialysis bag filled with ion exchange resin (Dowex 50 for basic dyes or Dowex 1 for acidic ones) or activated charcoal in a destaining bath. Adsorption of dye molecules from the gels onto the suspended bag is then possible.[1,11]

In the latter method, electrophoretic destaining, excess dye is removed as an electric potential is applied to the gels. The gels are positioned between two electrodes in a 7% acetic acid solution. Electrophoretic destaining may be carried out longitudinally (where unbound dye moves in a similar direction as the macromolecule during separation) or transversely (where electrophoresis at right angles to the direction of macromolecule separation achieves the

removal of unbound dye). Transverse electrophoretic methods are preferred as a more rapid means of removal of unbound dye because of the characteristic shorter electrophoretic distances involved. Destaining times of 15 to 30 minutes have been recorded.[1,11]

When comparing diffusion destaining with electrophoretic destaining, it is evident that the latter allows for a short destaining period. However, it is important to note that the investigator must execute greater care in electrophoretic destaining. Specifically, excessive electrophoretic destaining is avoided, since weakly stained bands or faint bands may be destained completely. Loss of color intensity of separated protein bands is also possible. For these reasons diffusion destaining is more often employed.

CHAPTER II

STATEMENT OF PROBLEM

Traditional methods of separation of serum proteins in a sample rely upon an electrophoretic procedure involving interactions of the proteins with buffer and a supporting medium. This is usually followed by a detection method-visualization of protein bands by the utilization of specific staining solutions or dyes. This type of detection method, however, suffers several drawbacks, the first being the time factor involved in staining and destaining procedures, as well as the selection of specific dyes for a particular class of macromolecules. Another drawback is that the supporting medium cannot be reused for multiple analyses.

The primary goal and scope of this research entails the use of an electrophoretic apparatus and a conductance meter to successfully measure conductivity (conductance), which serves as the physical property for detection of separated components. The application of an electric potential upon an electrolyte solution results in the migration of charged particles to the respective oppositely-charged electrode. Conductance is a measure of the current produced as a potential is applied. It is defined as the reciprocal of electrical resistance and may be expressed as follows:[13]

$$G = 1/R \tag{10}$$

where G is the conductance of the solution and R is the resistance.

Substituting the definition of resistance from Ohm's law ($E = IR$ where $R = E/I$) into the above equation gives the following:

$$G = I/E \quad (11)$$

where I is the current and E is the electric potential or voltage.

Conductance is therefore the ratio of current to voltage. Current is carried by any given charged species in a solution, so that conductance is dependent upon the concentration of a particular species as well as its mobility in a supporting medium.[13] Mobility, furthermore, is limited by frictional forces present in the system.

To determine conductivity (specific conductance), a versatile conductivity cell was designed. The conductance of the conductivity cell immersed in the solution to be analyzed (observed conductance) is measured. Observed conductance (1/R) of a solution is directly proportional to electrode area and inversely proportional to the distance between the electrodes and can be expressed in the following manner:[14]

$$1/R = k\ (A/d) \quad (12)$$

where 1/R is the observed conductance, k is the specific conductance or conductivity, A is the electrode area, and d is the distance between the electrodes. Conductivity is then calculated by relating the conductance reading measured at the cell terminals to the cell constant. The cell constant (K) is defined as the ratio d/A. Therefore, equation 12 may be solved for conductivity to give the following:[14]

$$k = (1/R)\ K \quad (13)$$

Conductivity is the product of observed conductance and the cell constant. The conductance value of the solution is displayed on the conductance meter. Cell constant values vary with the physical configuration of the cell and its electrodes.[14]

The development of this alternate detection method has the advantage of eliminating some of the major problems encountered in the chemical detection of separated protein utilizing dyes. Also, such an analytical method provides for more rapid, sensitive, and possibly more dependable results than previous available detection of separated methods.

CHAPTER III

MATERIALS AND APPARATUS

Materials

The Supporting Medium (TITAN GEL Serum Protein System)

TITAN GEL Agarose Systems kits were obtained from Helena Laboratories (Helena Laboratories, 1530 Lindbergh Drive, P.O. Box 752; Beaumont, Texas 77704). The TITAN GEL Serum Protein System (Cat. No. 3041) used in this research included the following:

1. TITAN GEL Serum Protein Plates (10) containing 1.0% agarose in 5.0% sorbitol with 0.4% arabic acid and 0.02% thimerosal added as preservatives.

2. TITAN GEL Serum Protein Buffer (1 package), a Barbital-Sodium Barbital Buffer with sodium azide and thimerosal added as preservatives, pH 8.4-8.8.

3. TITAN GEL Serum Protein Stain (1 vial), a 0.25% (w/v) Amido Black Stain made by dissolving the dry stain in 1000 mL of Fixative/Destain Solution, consisting of 1000 mL methanol, 1000 mL purified water, and 200 mL glacial acetic acid.

4. TITAN GEL Blotters (20).

5. TITAN GEL SPE Templates/Application Slits (10).

Serum Control

The serum control used was Kemtrol Serum Control Normal (Cat. No. 7024) from Helena Laboratories. This product is a lyophilized pooled human serum control which is reconstituted with 2.0 mL purified water and stored at 2° to 6°C. Stability period is five days when reconstituted or one year unreconstituted. The serum

control may be used as a qualitative **and/or** quantitative control for serum protein electrophoresis.

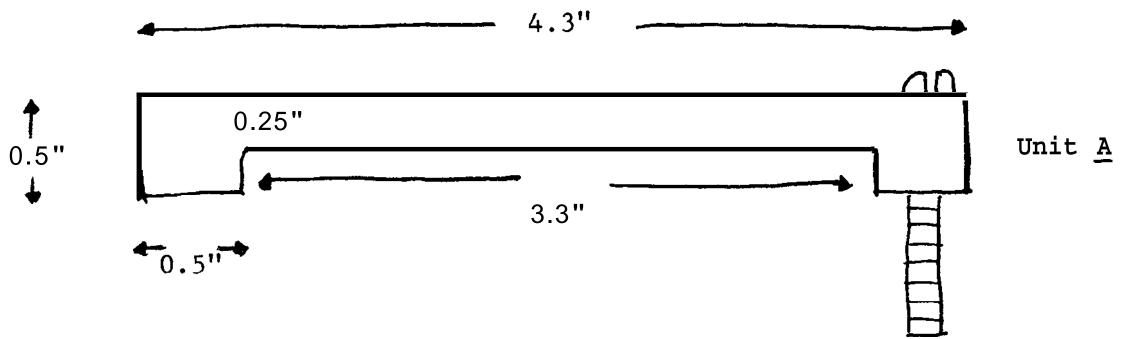
Apparatus

Zip Zone® Chamber

A Zip Zone@ Chamber(Cat. No. 1283) from Helena Laboratories served as the electrophoretic apparatus consisting of two outer and two inner compartments. Sponges were used as wicks and supports for the agarose gel. Buffer and sponges were then placed into each outer section of the Zip Zone® Chamber. The gel could then be placed agarose-side down so as to make good contact with the top surface of the sponges which were saturated with buffer. The positioning of the gel in this manner allowed for electrical connection. Elimination of vapor condensation on the gel using this approach was achieved resulting in minimum air movement and maximum vapor saturation.

Conductivity(Conductance) Cell

The conductivity cell shown in Figure 6 was constructed of plexiglass consisting of two units, A and B. Unit A was cut. to a length of 4.3 inches(10.9 cm). An inner width of 0.25 inches (0.64 cm) extended for 3.3 inches(8.4 cm) of the total length, where the remainder of the length was divided equally as outer joints, 1 and 2 (0.5 inch length, 0.5 inch width). A 0.19-inch (0.48 cm) diameter hole was then made through each of the centers of these joints. Similar dimensions were utilized in the construction of Unit B. Hence, the joints formed between the units could be



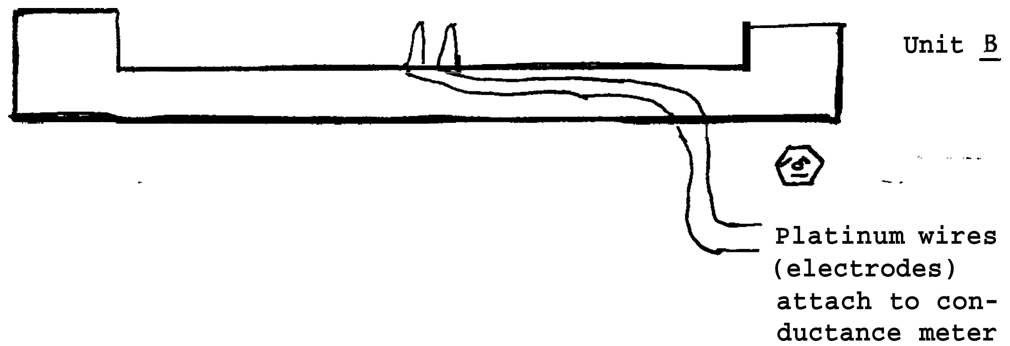


Figure 6 - Conductivity Cell Design

attached by machine screws and nuts. The machine screws were extended upward from Unit B to Unit A. Two additional holes were drilled through the center of Unit B to accommodate two 24-gauge platinum wires (electrodes) for attachment to a conductance meter.

Power Supply, Conductance Meter, XY Recorder

A Bio-Rad Laboratories Model 1420B/150 Power Supply (Bio-Rad, Chemical Division, 1414 Harbour Way South; Richmond, California 94804) was used to supply the appropriate voltage necessary to electrophorese samples on the gel plates. Conductance measurements were monitored using a YSI Model 35 Conductance Meter (Yellow Springs Instruments Company, P.O. Box 279; Yellow Springs, Ohio 45387) whose output was recorded on a Houston Instrument Model 200 XY Recorder (Houston Instrument, One Houston Square; Austin, Texas 78753).

CHAPTER IV

EXPERIMENTAL

Preliminary Studies

Introduction

The initial experiment involved measuring and observing the conductance of the supporting medium alone, agarose gel, on the display unit of a YSI Model 35 Conductance Meter. Protein samples were then placed onto the gel where it was found that the initial conductance reading(s) varied as compared to gel alone by a factor of about 1.5.

Computer-Assisted/Controlled Electrophoresis

With the advent of the computer as an integral component of analytical instrumentation, instrument-computer interfacing was the next avenue of research explored. It was initially thought that conductance measurements and electrophoresis could not be made simultaneously. A computer was required to stop the application of electrophoresis voltage while making conductance measurements. This was accomplished employing a computer controlled relay box. A computer which would assist in monitoring and controlling the operation of the power supply and the conductance meter by interfacing with digital-to-analog (D/A) converters and analog-to-digital (A/D) converters was necessary. A D/A converter sends control voltages to the instrument (power supply) whereas A/D converters read voltages from the instrument.

A program written in BASIC language was made to accompany an Apple IIe computer. The program made use of the following variables:

1. Power Supply Voltage
2. Time of Electrophoresis Period in Seconds
3. Conductivity Meter Deactivation Time in Seconds
4. Conductivity Meter Activation Time in Seconds
5. Detection Time in Seconds

For further elucidation of the program, refer to Appendix A.

The experimental set-up was next constructed as shown in Figure 7. This involved connection of the Zip Zone® Chamber, power supply, conductivity cell, conductance meter, XY recorder, computer, and relay box.

Experiments were then conducted over a period of five months using the same conductivity cell, cleaning it with dilute nitric acid and rinsing with distilled water prior to each experiment. Various Hb samples were run employing a Helena Titan IV® Citrate Hemoglobin Electrophoresis procedure (Cat. No. 2400) from Helena Laboratories.[15,16,17] Electrophoresis was performed for 45 minutes at 50 volts.

Serum Protein Electrophoresis

Research continued as a new sample, serum protein control, was selected. Materials and apparatus used, as well as the specific procedure(s) employed, are outlined in this and previous chapters. A pre-conditioned step was undertaken consisting of electrophoresis of gel alone at 50 volts for 20 minutes so as to obtain stabilized conductance readings.

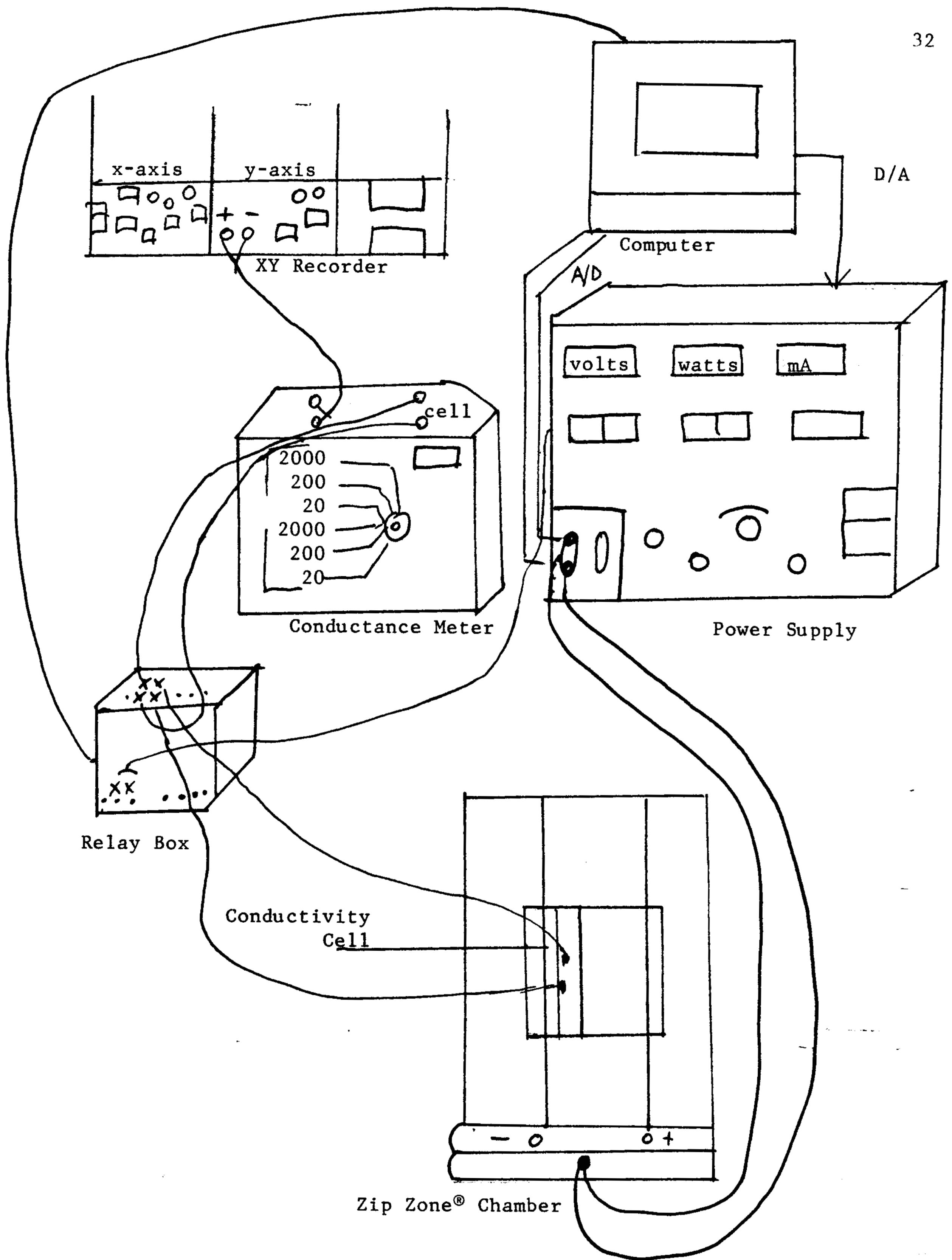


Figure 7 - Experimental Set Up (Computer-Assisted/Controlled Electrophoresis)

Procedure

Part A: Preparation of Reagents and Control

The first part of this research involved preparation of the Zip Zone® Chamber by making up the TITAN GEL Serum Protein Buffer. This was done by diluting the entire package of barbital-sodium barbital buffer to 1500 mL with deionized water. The pH of the buffer was 8.6.

The Kemtrol Serum Control Normal vial was next reconstituted with 2.0 mL of deionized water. The serum control was gently swirled and allowed to stand for 20 minutes so that all protein particles would dissolve. Refrigeration at 2° to 6°C was then necessary to preserve the solution until application of the sample as described in Part C.

Finally, the TITAN GEL Serum Protein Stain and fixative/destain solution were prepared as previously discussed (See MATERIALS AND APPARATUS, "The Supporting Medium").

Part B: Experimental Set Up

The experimental set up was next constructed as shown in Figure 8. This involved connection of all apparatus which included Zip Zone® Chamber, power supply, conductivity cell, conductance meter, and XY recorder.

Part C: Positioning of the Gel and the Conductivity Cell

A rectangular plate (which served as the protective covering for the TITAN IV Citrate Agar Plates) measuring 3.8 inches (9.7

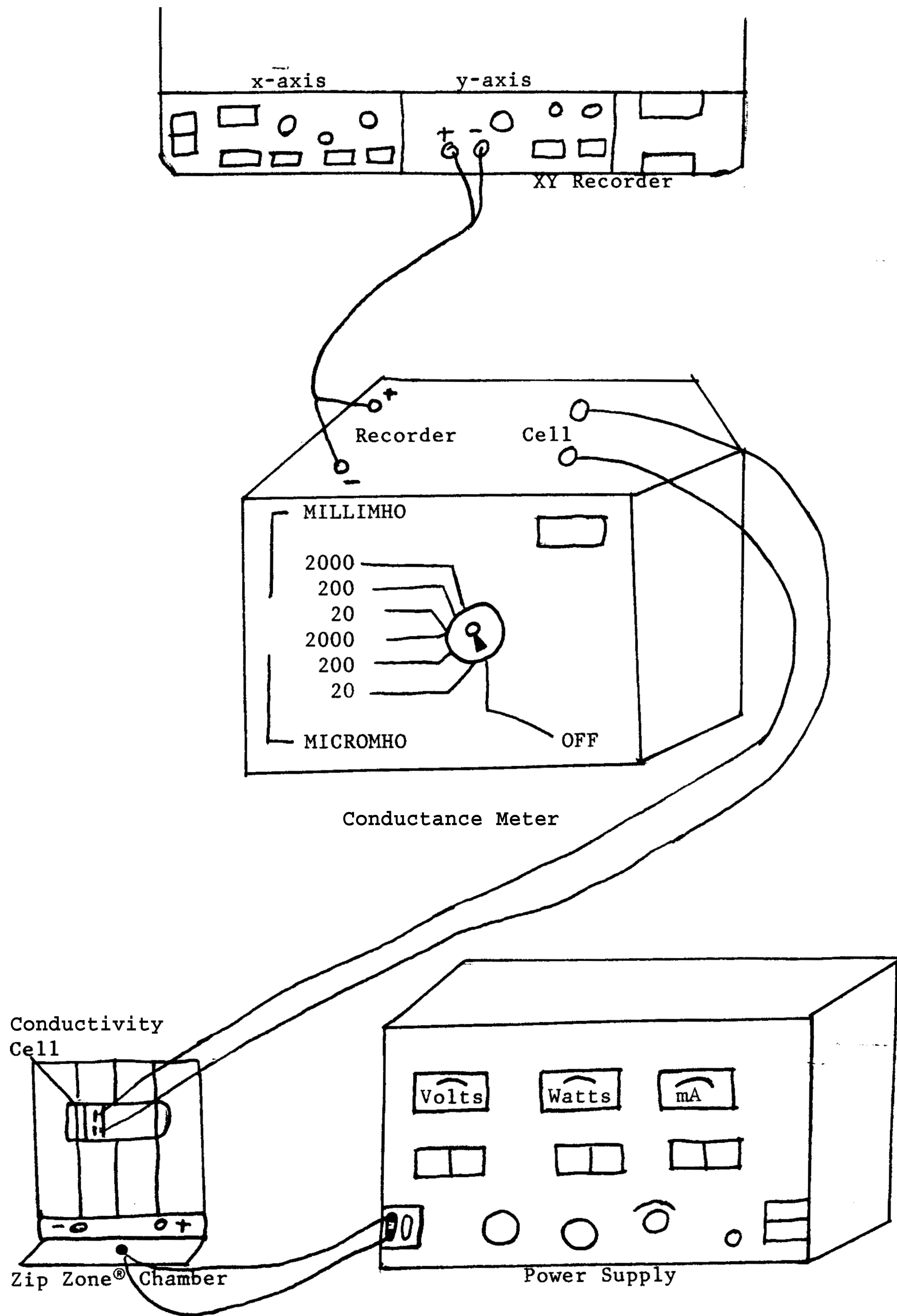


Figure 8 - Experimental Set Up

cm) in length and 3.1 inches (7.9 cm) in width was secured along the edges of conductivity cell Unit A with epoxy resin. Unit A was positioned down from the end about 1/3 of the entire length of the plate itself.

The TITAN GEL Serum Protein plate was subsequently removed from the protective packaging and the gel and its support glued onto the rectangular plate.

Finally, Unit A was lowered down onto Unit B so that the platinum electrodes would make contact with the gel. Unit A was then raised slowly as an imprint was left on the gel. This would serve as a marker for the sample application point.

Part D: Electrophoresis on the Gel

A TITALGEL SPE Template was initially placed slightly above the imprint on the gel (on the upper 1/3 of the plate) as shown in Figure 9. Kemtrol Serum Control Normal was applied next placing 3.0 μL volume of sample on every other template slit, spreading the sample across the entire slit. Care was taken in positioning one of the samples to align directly between the platinum electrodes, so as to assure optimum detection of separated components of serum protein as they migrated between the electrodes. A serum absorption time of 4 minutes allowed the samples to diffuse into the agarose. The template was then carefully removed from the gel surface.

Conductivity Cell Unit A was then lowered down onto Unit B and tightly fastened. The plate was quickly placed agarose-side down in the Zip Zone@ Chamber so that the agarose layer made contact with the top surfaces of the sponges saturated with buffer. The

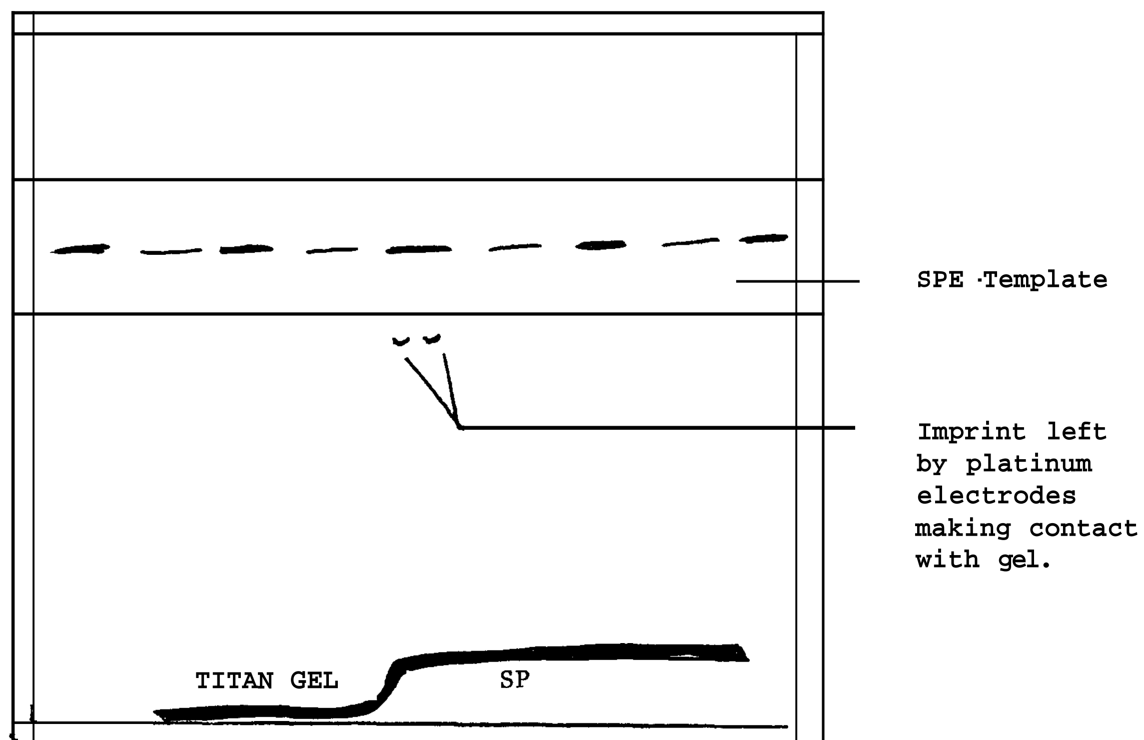


Figure 9 - Placement of TITAN GEL SPE Template on the gel plate

plate was positioned in such a manner that the application point was on the cathodic (-) side.

The sample was electrophoresed at 120 volts for the amount of time which corresponded to the complete separation of serum protein into its components. The results of the separation were displayed on the recorder as peaks which indicated the migration of various bands as they passed the detector. The experiment was conducted at ambient temperature.

Part E: Visualization of the Protein Bands

Upon completion of electrophoresis, the plate was removed from the chamber and conductivity cell. It was placed in methanol for destaining for 5 minutes. Next, the plate was transported to a laboratory drying oven and dried at 60° to 70°C for 5 minutes. It was then immersed in the staining solution for 10 minutes followed by two consecutive washes in destaining solution for 1 minute each. The destained plate was then dried at 60° to 70°C.

CHAPTER V

EXPERIMENTAL RESULTS AND DISCUSSION

Conductivity (Conductance) Cell

A conductivity (conductance) cell which monitored electrolytic conductance changes in the supporting medium against time was successfully constructed.

Preliminary Studies

Introduction

From the data generated by the initial experiment involving measurement of conductance of the gel alone versus protein samples placed onto the gel, it was apparent that conductance changes were being detected by the conductivity cell. Further work could hence be directed at developing a physical detection method which would ultimately provide a simple, dependable, and rapid technique for detection of separated components.

Computer-Assisted/Controlled Electrophoresis

The recordings produced in the electrophoresis of various Hb samples revealed lack of reproducibility of the conductance measurements during the experiments. Separation of hemoglobins was not accomplished. This lack of migration could have been the result of the computer program which controlled electrophoresis time for a set amount of time (seconds) followed by the shut-down

of power supply, activation and stabilization of the conductance meter, and finally deactivation of the meter and subsequent turn-on of the power supply. The time lag between successive electrophoretic runs could have been such that there was inadequate time for continual mobilization of bands and hence detection of any conductance reading changes would be difficult.

As mentioned previously, it was initially thought that the operation of the conductance meter would be affected by the electrophoresis power supply voltage. This was later found to be not true.

The remainder of the research consisted of performing electrophoresis without the aid of a computer. Initially, Hb samples were run, but once again neither a clear separation nor significant variations in conductance readings were apparent. Signs of deterioration of the agarose plates (cloudiness) could be considered a possible explanation of unsuccessful separation. Furthermore, the inability to detect changes in conductance may have been due to the fact that hemoglobin's conductance may be very near to that of the buffer contained in the agarose plates.

Serum Protein Electrophoresis

The conductance of the supporting medium, the gel, is shown in Figure 10. Results obtained for the electrophoresis of Kemtrol Serum Control are shown in Figure 11. This recording represents conductance changes in the supporting medium against time. As can be seen, the conductance of the supporting medium is altered significantly by the sample's components as they migrate past the

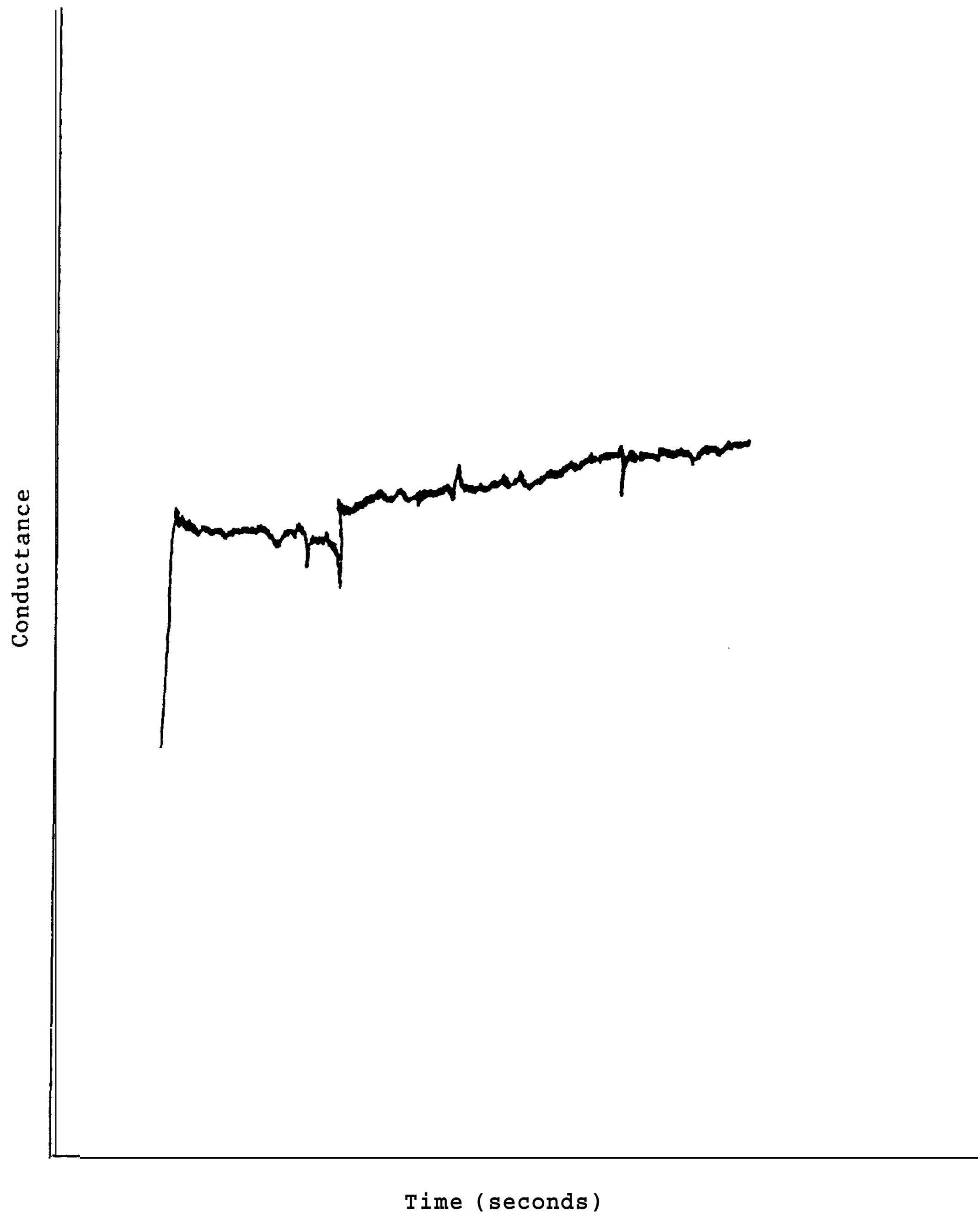


Figure 10 - Conductance of the supporting medium

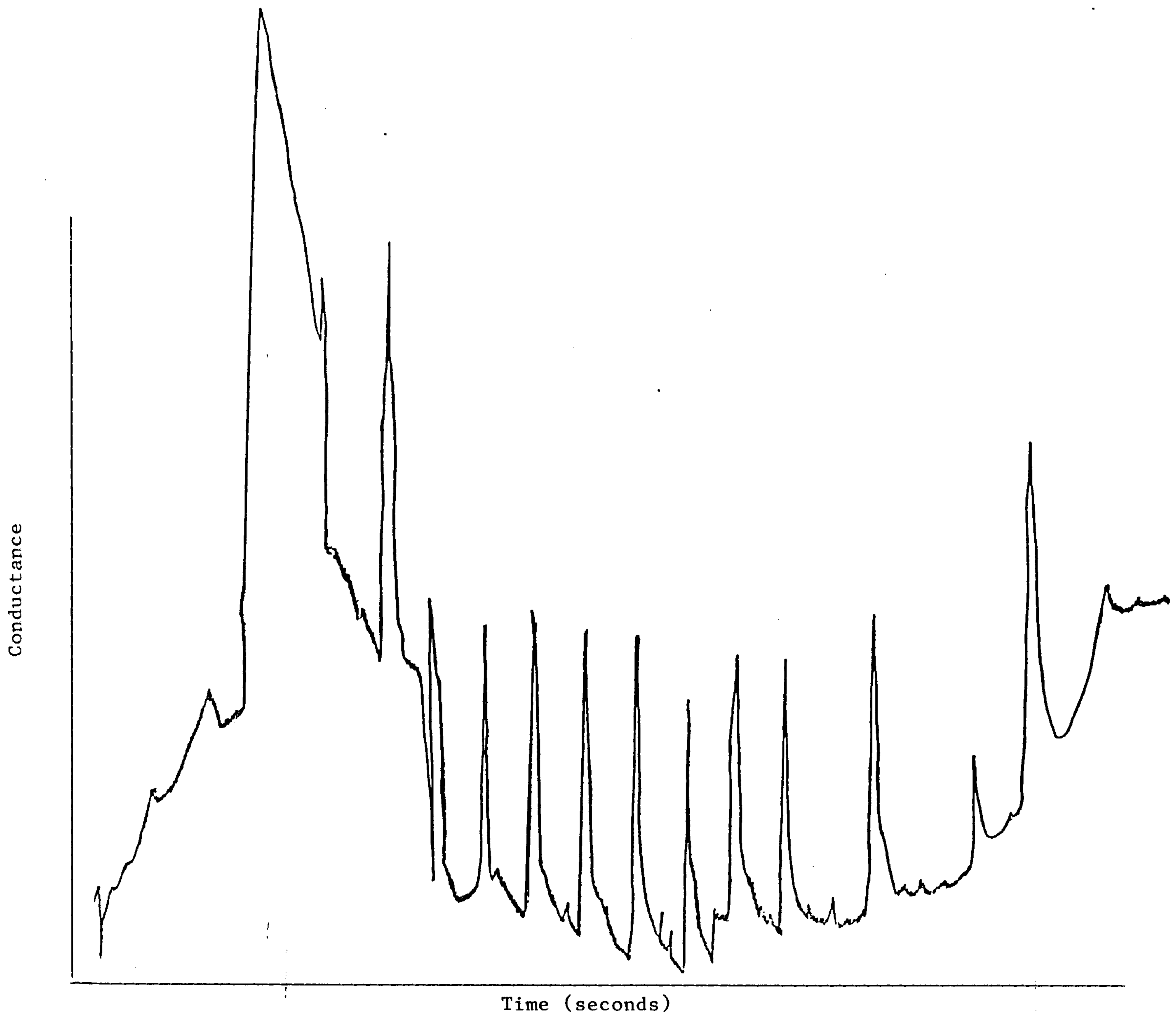


Figure 11 - Electrophoresis and the condoctometric determination of serum proteins

electrodes. Conductivity of a solution depends on the number of charge carriers (ions) present and their mobility. This is indicated by peaks shown on the recorder scan. Each peak represents a separated fraction of serum protein. The albumin fraction exhibits greatest migration toward the anode. It represents the first major fraction to pass the platinum electrode detectors. The peaks that follow are believe to designate a greater degree of detection of protein separation than is possible with a conventional electrophoretic system, a staining method, which yields five to ten bands or zones. (See "Visualization of the Protein Bands" as described below). Conductometric detection systems afford more numerous bands or zones.

Visualization of the Protein Bands

Visualization of the protein bands by a staining procedure served a dual purpose, identification of complete separation as well as determination of electrophoretic mobilities of the albumin, alpha one, alpha two, beta and gamma protein bands on the TITAN GEL Serum Protein Plate. A representation of the TITAN GEL Serum Protein Plate is illustrated in Figure 12.

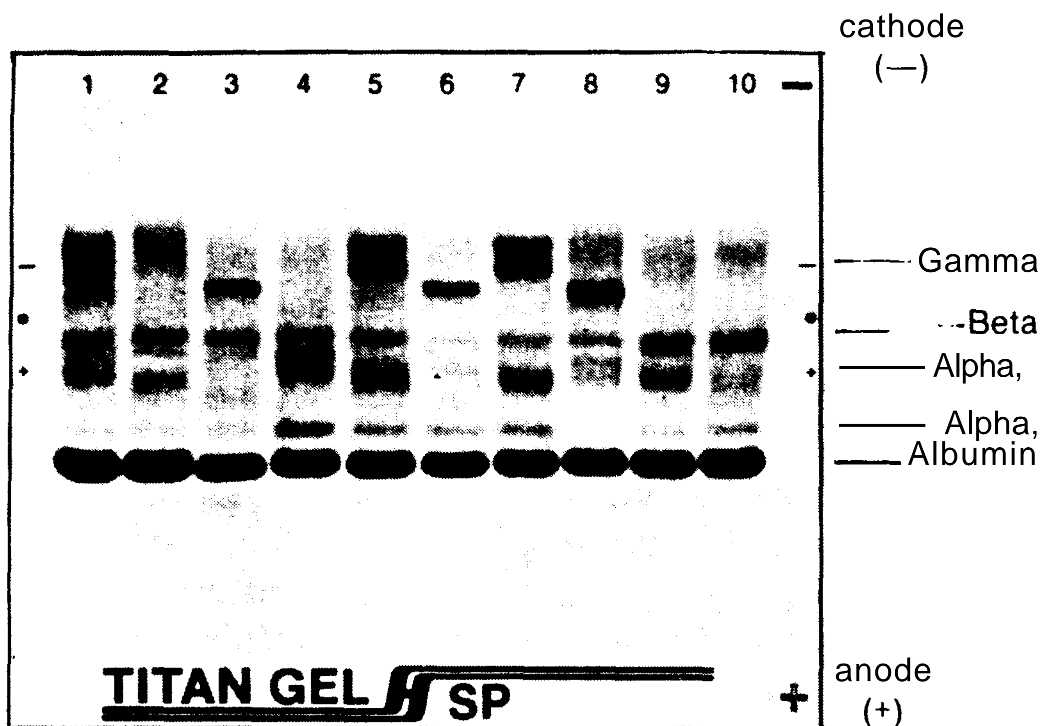


Figure 12 - A representation of a TITAN GEL Serum Protein Plate illustrating the electrophoretic mobilities of albumin and alpha one, alpha two, beta and gamma globulins.

CHAPTER VI

CONCLUSION

The work done in this research is believed to be the first documented report of the successful conductometric determination of serum proteins. The design of the conductivity cell is satisfactory in that successive results are reproducible over a period of time. This alternate detection method rivals traditional chemical detection methods utilizing dyes, in both time (one-half hour or less for complete analysis compared to several hours to a day) and from an economic standpoint (reusability of gels for multiple analyses versus an inactive supporting medium following staining from a single analysis). Furthermore, the greater degree of detection of protein separation using this method labels conductometric determination as a high resolution system.

Therefore, the use of conductance measurements as an alternate detection method in monitoring separated components of a sample yields a new dimension to electrophoresis by providing a rapid and highly sensitive technique for qualitative information.

Further work should involve preparation of one's own supporting medium and therefore the construction of an alternate conductivity cell. Also worthy of further study would be the qualitative and/or quantitative analysis of the separated components of proteins achieved in this project.

APPENDIX A

COMPUTER PROGRAM FOR COMPUTER-ASSISTED/CONTROLLED ELECTROPHORESIS

```
1  HIMEM:  36095:D% = 0:  DIM C%(5), Q%(5), D%(10):  PRINT CHR$ (4)
     "BRUN QUICK I/O"

20  INPUT "POWER SUPPLY VOLTAGE?"; V

21  IF V<800 THEN GOTO 29

22  IF V>800 THEN PRINT

23  PRINT "YOUR POWER SUPPLY VOLTAGE IS TOO HIGH FOR THIS SYSTEM
      PRESENTLY!"

24  PRINT "WOULD YOU LIKE TO CHOOSE ANOTHER VOLTAGE?  (Y OR N)":
      INPUT YN$

25  IF YN$ = "Y" THEN GOTO 20

26  IF YN$ = "N" THEN GOTO 260

29  PRINT

30  INPUT "TIME OF ELECTROPHORESIS PERIOD IN SECONDS'?";D1

31  PRINT

33  INPUT "CONDUCTIVITY METER DEACTIVATION TIME?"; D4

35  IF D4>D1 THEN GOTO 40

37  IF D4<D1 THEN PRINT

38  PRINT "WARNING THE DEACTIVATION TIME PERIOD SHOULD EXCEED THE
      ELECTROPHORESIS TIME PERIOD BY A SUFFICIENT MARGIN TO ALLOW
      THE POWER SUPPLY TO ACHIEVE ZERO OUTPUT VOLTAGE."

39  PRINT

40  INPUT "CONDUCTIVITY METER ACTIVATION TIME IN SECONDS?";D2

41  IF D2>D4 THEN GOTO 49

42  IF D2<D4 THEN PRINT

43  PRINT
```

```
44  PRINT "WARNING!!!  A SUFFICIENT TIME PERIOD SHOULD EXIST TO
      PERMIT STABILIZATION OF THE CONDUCTIVITY METER BEFORE A
      READING IS TAKEN."

45  PRINT

46  PRINT "WOULD YOU LIKE TO CHOOSE A NEW ACTIVATION TIME?":
      INPUT YN$

47  IF YN$ = "Y" THEN GOTO 40

48  IF YN$ = "N" THEN GOTO 260

49  PRINT

50  INPUT "DETECTION TIME IN SECONDS?"; D3

51  IF D3>D2 THEN GOTO 100

52  IF D3<D2 THEN PRINT

53  PRINT

54  PRINT "DETECTION TIME SHOULD BE A TIME FOLLOWING THE ACTIVATION
      TIME!"

55  PRINT

56  PRINT "WOULD YOU LIKE TO CHOOSE A NEW DETECTION TIME? (Y
      OR N)"  INPUT YN$

57  IF YN$ = "Y" THEN GOTO 50

58  IF YN$ = "N" THEN GOTO 260

100 D% = 32767: & TO 1

120 & TI1:  T = D%

145 D% = 0:  & DOO:  & DO1

150 T1 = T - (D1 * 10)

160 D% = 4.00 * V:  & AOO

170 & TI1:  IF D%>T1 GOTO 170

180 D% = 0:  & AOO

185 T4 = T - (D4 * 10)

190 & TI1:  IF D%>T4 GOTO 190

205 D% = 1:  & DOO:  & DO1
```

```
206  T2 = T - (D2 * 10)
210  & TI1:  IF D%>T2 GOTO 210
220  T3 = T - (D3 * 10)
230  & TI1:  IF D%>T3 GOTO 230
240  & AIO:  FOR I = 1 to 50:  NEXT I:  &AIO
245  PRINT "ANALOG INPUT = "D% / 2000" VOLTS"
250  GOTO 100
260  PRINT "THANK YOU":  END
```

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