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TITLE: THE DESIGN OF CONDUCTIVITY AND PERMITTIVITY DETECTION DEVICES FOR ELECTROPHORESIS

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ABSTRACT

THE DESIGN OF CONDUCTIVITY AND PERMITTIVITY DETECTION DEVICES FOR ELECTROPHORESIS

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Electrophoresis has been a major method for research in the life sciences for almost sixty years. The importance of this method is that it is one of the most powerful methods available for the separation of charged biomolecules.

Since Tiselius introduced electrophoresis as an analytical tool in 1930, there have been many variations in the development of this technique. Other techniques are presently being studied to furnish more suitable methodology for analyzing biopolymers. For example, the capability of electrophoresis in the analysis of biopolymers has recently been compared with High Performance Liquid Chromatography. Through the use of laboratory computers and commercialized instruments, automated versions of electrophoresis, therefore, seem more desirable.

Several traditional electrophoretic separation schemes have involved the use of support media such as gels. These techniques are slow, prone to poor reproducibility, and are not competitive with state-of-the-art methods found in some commercial methods. Modern

detectors offer features such as sample preservation, selectivity for the analyte, dynamic range, low cost and convenience. Since a great deal of research has been done in the area of detectors for liquid chromatography, technology is available to provide very sensitive devices in the area of capillary zone electrophoresis.

Since zone electrophoresis is the form most commonly used in clinical labs, interest was focused on developing an automated form in which a conductivity detector is placed directly over the cellulose acetate support medium. This procedure involved the analysis of serum hemoglobin. The result was that the hemoglobin fractions were affected by the detector and discrete zones did not form. A high frequency method was also attempted with similar results. There have been recent developments in capillary zone electrophoresis/conductivity detection. However, there are still questions concerning the use of these methods as universal detectors applicable to biomolecules. Permittivity detectors are also being investigated to research the use of these devices in capillary zone electrophoresis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS

SYMBOL	DEFINITION
a. c.	Alternating Current
C.Z.E.	Capillary Zone Electrophoresis
cm	Centimeter
Q	Charge
C	Conductance
i	Current
^{o}C	Degrees Celsius
DNA	Deoxyribonucleic Acid
d. c.	Direct Current
μ	Electrophoretic mobility
EDTA	Ethylenediaminetetra-acetic acid
Hb	Hemoglobin
HPLC	High Performance Liquid Chromatography
i. d.	Inside diameter
IR	Infrared
I	Ionic strength
pI	Isoelectric point
pH	Log (negative) of Hydrogen Ion Concentration
MHz	Megahertz
mL	Milliliter

mm	Millimeter
mV	MilliVolts
min	Minute
v	Migration velocity
ng	Nanogram
o. d.	Outside diameter
pF	PicoFarad
E	Potential Difference (or Electric field)
AA_2	Serachem Hemo Control-Normal
$AFSA_2$	Serachem Hemo Control-Sickle Cell Anemia
AFSC	Serachem Hemo Control-Sickle Cell Disease
ASA_2	Serachem Hemo Control-Sickle Cell Trait
UV	Ultraviolet
η	Viscosity
V	Volts
VCM	Voltage-Controlled Multivibrator

Chapter 1

Introduction

The Concept of Electrophoresis

The separation of charged particles under the influence of an applied electric field is the basis for electrophoresis (Figure 1). In electrophoresis, the species being studied are suspended in a liquid medium which is usually supported by an inert stationary solid. This can be paper, cellulose acetate, polyacrylamide gel or a similar substance. The liquid is the conductor for the electric current created by applying an external voltage to the system. A molecule moves towards the anode or cathode during electrophoresis with a certain velocity which is called the migration velocity of the molecule, usually referred to as v.[1] This value is characteristic of both the molecule itself and of the strength of the applied electric field. When a molecule migrates in a specific liquid medium, the migration velocity of this molecule increases with increasing field strength, E. This can be written as

$$v = \mu \times E \qquad (1)$$

where μ is a constant.[1] The constant μ is the electrophoretic mobility of the molecule. It is the ratio of migration velocity, v, to field strength E, and is the migration velocity of a particle under the influence of an applied electric field of strength 1 V/cm.[1]

Under the influence of the force of the electric field, a discrete molecule will start to move towards the electrode of opposite charge. It will accelerate rapidly from the point of application, but this acceleration will be opposed by a frictional force as the molecule starts to move through the medium. This frictional force is usually referred to as viscous drag. The magnitude of the force of viscous drag depends on the viscosity, η, of the medium. Media which are very viscous will cause greater resistance of movement for a molecule through them. Size and shape of the molecule are also factors affecting the movement. A small molecule which is compact will encounter less resistance to its movement than one that is large and irregular in shape. Figure 2 illustrates the various forces acting in the electric field.

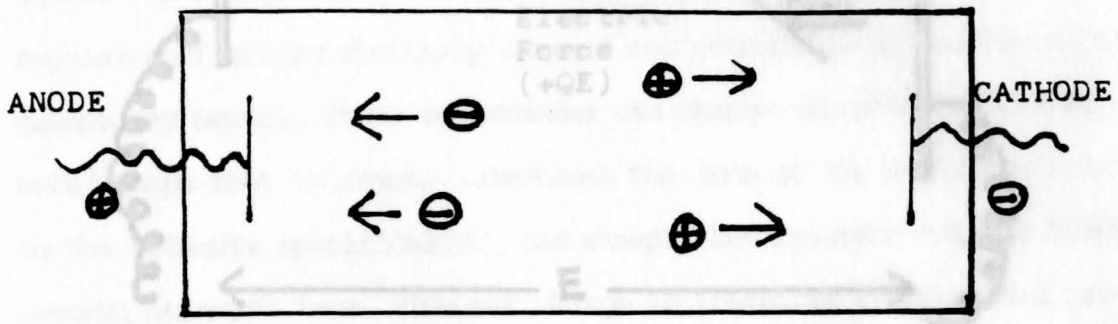


FIGURE 1

Movement of Charged Molecules
Under the Influence of an
Applied Electric Field

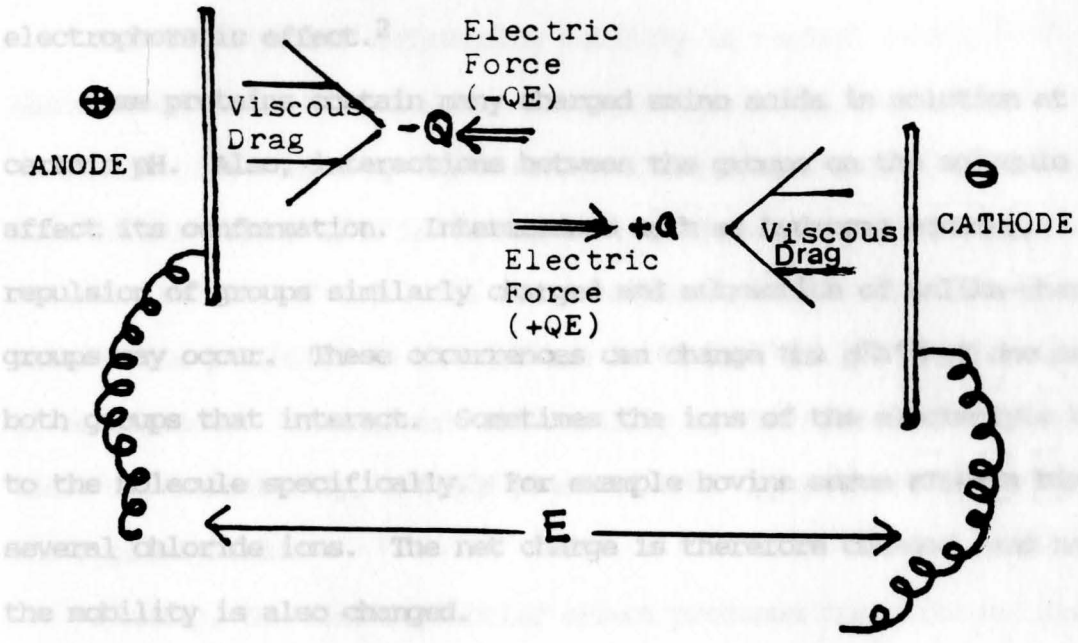


FIGURE 2

Forces Acting On Charged Molecules
In An Electric Field

Mobility-Influencing Factors of Biopolymers

Specifically, there are various parameters which affect the mobility of a substance through an electrolyte. Some of these are the charge and conformation, zeta potential, thermal energy and the electrophoretic effect.[2]

Some proteins contain many charged amino acids in solution at a certain pH. Also, interactions between the groups on the molecule affect its conformation. Interactions such as hydrogen bonding, repulsion of groups similarly charged and attraction of unlike-charged groups may occur. These occurrences can change the pKa's of one or both groups that interact. Sometimes the ions of the electrolyte bind to the molecule specifically. For example bovine serum albumin binds several chloride ions. The net charge is therefore changed, and hence the mobility is also changed.

The zeta potential is another factor influencing the mobility. Zeta potential is the average effective electrical field strength ("potential") produced by charges on macromolecules and any particles embedded in the solvent carried along ("water of hydration") with the macromolecule.[2] Under normal conditions, with binary electrolytes, the solid surface has an excess of anionic charge resulting from ionization of surface functional groups. Counterions to these anions are in a stagnant double layer. The zeta potential is created; it is dependent upon the dielectric constant of the medium. Ionic strength describes electrophoretic solutions. The effect of the interactions of small ions of the electrolyte with macromolecules is proportional to the square of the charge on the small ions. For a solution, the ionic strength, I, is written:

$$I = 1/2C_1Q_1^2 + 1/2\ C_2Q_2^2 + \ldots = 1/2\ \Sigma C_nQ_n2 \quad (2)$$

For electrolyte solutions that are univalent, like NaCl, the ionic strengths and concentrations are the same.

The third factor influencing mobility is thermal energy.[2] Macromolecules do not move in a straight line because of random thermal motion caused by the electric field. Macromolecules do not move in a straight line in an electric field because of random thermal motion. Because of this, the ion atmosphere around the molecule that causes zeta potential is changed and it takes time for the counterions to be replaced. This lowers the mobility of the macromolecule because there is, momentarily, a field produced that opposes the direction of the applied field.

Finally, the electrophoretic effect produces the aforementioned "viscous drag". The motion of the macromolecule is against a "flow" of solvent and the mobility is reduced further. Since equal charges are moving in opposite directions, there is little "net" flow of solvent in either direction.

Electrophoretic Methods

The Swedish chemist Tiselius introduced electrophoresis as an analytical tool in 1930. His original work was with "moving boundary electrophoresis", and from this technique the additional methods have been derived. Since 1950 several "zone" electrophoretic techniques have been developed. Newer techniques are in isoelectric focusing and isotachophoresis. There are a variety of formats in which electrophoresis may be done. Two major categories are "free

solution", in which no stabilizers are used, or using anticonvection stabilizers such as papers or gels.[3]

Support Media

During electrophoresis, narrow sample zones are very favorable because less migration distance is necessary to achieve separation, resulting in less time for analysis. However, there is a technical difficulty in using thin zones with a rather high concentration of analyte: convection. Convection is the bulk flow which occurs because the zones actually "fall" through the solvent faster than electrophoresis occurs.[2] Separations such as this are thus best carried out on a support medium to counteract the effects of convection and diffusion and to facilitate immobilization of the separated proteins. The basis for the support medium, then, is to allow free penetration of the material to be separated and yet to cut off convection. This is achieved by restricting the pore size for movement of the macromolecules. Capillary tubes have the same effect. Support media such as paper, cellulose acetate, starch gels and polyacrylamide gels are all common.

Gels are jellylike solids; they are three-dimensional polymeric networks with random structures. The average pore size of a gel depends on the concentration of the polymer. One example is polyacrylamide gels formed by the copolymerization of acrylamide monomers with a crosslinking agent to form a three-dimensional network.[3] The most commonly used crosslinker is N,N'-methylene

bisacrylamide, but others are available to impart special properties. Molecular sieving effects are apparent when microporous gels permit separations based on molecular size through a sieving action.[3] For example, by changing the gel concentrations of the starch or agar, one can vary pore size. If the pore size is near the diameter of the molecules being electrophoresed, molecular sieving occurs. The most effective application of gel-sieving to proteins involves denaturing ionic detergents such as sodium dodecyl sulfate.[1] With sodium dodecyl sulfate, complexes have similar charge-to-size ratios. Therefore, the migration velocity is dependent only upon the size of the complex.

Papers or sheets of plastic coated with cellulose acetate are sometimes used as solid supports because of the mechanical strength that they have, and because no preparation time is needed. Some of the problems with paper electrophoresis have been overcome by the introduction of the use of cellulose acetate. In cellulose acetate most of the hydroxyl groups of cellulose have been esterified by acetylation, so there are few hydroxyl groups available to interact with sample molecules. Because of this, adsorption of samples onto the cellulose and the subsequent distortion of the separation are avoided. The separation of analyte components would be quicker. Moreover, cellulose acetate supports are more homogeneous in structure. For scanning by densitometry, cellulose acetate can be made transparent. A disadvantage of cellulose acetate is that it may contain sulfate and carboxylic acid groups which produce an electro-osmotic anode-to-cathode flow of water during electrophoresis.

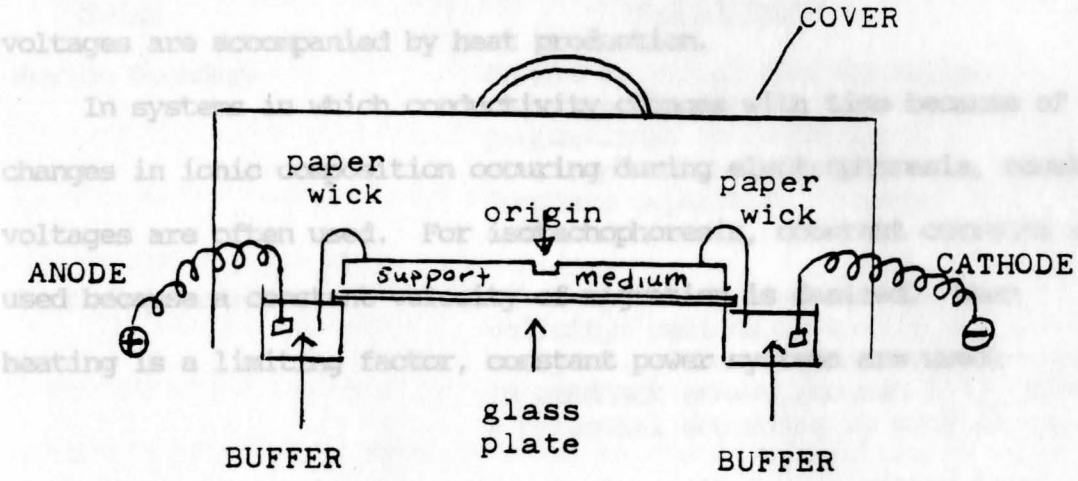
Free solution methods of electrophoresis are a convenient alternative to the use of support media, principally because the procedures involved are shorter in time and less tedious.

Conditions for Choosing a Method

Table 1 highlights the different modes of electrophoresis and various parameters for their use.[4] The selection of the method and conditions used is based upon what type of knowledge of the analyte is available. Knowing the molecular weight of the molecule and the isoelectric point can help determine the type of electrolyte to use, the pH of the electrolyte, the acidity, the voltage and the current. Also, the end result of the electrophoresis is a factor in choosing a method. Dyes are sometimes desirable when visualization is necessary. Dying a plate gives the analyst a visual aid at the end of the actual separation. This can be kept as a record of the separation or used for comparison with other separations.

There is no theoretical reason for choosing vertical or horizontal electrophoresis.[2] However, horizontal electrophoresis places less mechanical stress on the support. Neither horizontal nor vertical methods should ever be exposed to the atmosphere due to potential evaporation of liquid during electrophoresis. Figure 3 illustrates a system for horizontal electrophoresis.

FIGURE 3



Apparatus for Horizontal Electrophoresis

The power supply used in electrophoresis is a very important factor in choosing a method. Electrophoresis may be carried out at constant voltage, constant power, or constant current.[2] This depends on the power supply. Maximum voltage is desirable to complete the electrophoresis as quickly as possible. However, it is necessary to keep the electrophoresis chamber as cool as possible because high voltages are accompanied by heat production.

In systems in which conductivity changes with time because of the changes in ionic composition occuring during electrophoresis, constant voltages are often used. For isotachophoresis, constant currents are used because a constant velocity of migration is desired. When heating is a limiting factor, constant power systems are used.

Table I

Modes of Electrophoresis

Modes	Description
Moving Boundary	Sample is placed between buffer solutions in a tube; migration is proportional to component's electrophoretic mobility; Complete separation is never effected.
Isotachophoresis	All samples migrate with the same velocity; cations or anions are the analytes. Substances are concentrated in gradient zones produced by applying a potential according to mobilities.
Zone Electrophoresis	Sample is applied as a narrow band surrounded by buffer; as an electric field is applied, each zone migrates according to its own mobility. Analogous to elution chromatography.
Isoelectric Focusing	Separation of amphoteric samples in a pH gradient; a low anodic pH to a high cathodic pH is applied; samples cease to migrate when it reaches the pH at its isoelectric point.
Two-dimensional	One mode followed by another mode. The second mode perpendicular to the first; very powerful separation method.

Techniques for Detection and Quantitation

Staining

There are a wide variety of dyes that achieve the goals of high analyte resolution and specificity. For example, stains commonly used in the detection of proteins are Ponceau S, Coomassie blue and Silver stain. For nucleic acids, the silver stain and Ethidium bromide give fluorescent bands with DNA.[3] Other stains are specific for lipoproteins, glycoproteins and enzymes (Table 2).[2] These depend upon the support medium used.

The staining procedure can be quite cumbersome. For example, the procedure utilizing dyes such as Ponceau S and Coomassie blue usually involve a "fixative", such as trichloroacetic acid, which precipitates proteins and prevents their diffusion out of the gel.[3] The electrophoresis plate must be soaked in the stain to permit uniform staining. Unbound dye must be removed using a destaining rinse. With thicker solids and gels used as the stationary phase, the length of time for these combined procedures (staining/destaining/densitometry) can be quite long. Staining and destaining takes up quite a large portion of the time to complete an analysis. The silver stain procedure developed by Merril et al.[3] requires one day to complete. This procedure involves procedures of fixing, rinsing and soaking the plates in several solutions: acidic dichromate, silver nitrate, formaldehyde and dilute acetic acid.

Table 2

Compounds Detected by Certain Stains

Class of Compound	Stain	Support Medium
Amino Acid	Ninhydrin	Paper, Cellulose acetate
Serum protein	Ponceau S Coomassie blue 250	Cellulose acetate Polyacrylamide
Lipoproteins	Oil Red O	Agarose
Glycoproteins	PAS(periodic acid-Schiff)	Agarose
Nucleic Acids	Ethidium bromide(fluorescent)	Agarose
Hemoglobins	Ponceau S Silver stain o-Dianisidine Ferricyanide Peroxide	Cellulose acetate Agar
Isoenzymes Lactate dehydrogenase	Fluorescent NADH or tetrazolium	Agarose
Creatine kinase	Fluorescent NADH or tetrazolium	Agarose
Alkaline phosphatase	1-Naphthylphosphate + fast blue B or 5-Bromo-4-chloro-indolylphosphate	Polyacrylamide Cellulose acetate
Immunoglobulins	Coomassie blue 250	Agarose
Specific antigens by immunological electrophoresis	Amido black 10B	Agarose

After the staining and destaining procedure, the plates are placed in solutions to dehydrate and clear the plates. This is necessary for visualization and scanning by densitometry.

Alternative Detection Methods

Detection is not limited to staining followed by densitometry. Other methods used for detection include autoradiography, use of immunoreagents and on-line devices.[3]

In autoradiography, an image is formed by the decay of a suitable radioisotope, such as tritium. The sample molecules may contain the radioactive atoms within their structures, or they can be tagged with them. The emitted beta particles affect the silver grains on a photographic emulsion. Black spots appear at these positions when the film is developed. Exposure times of one day to a week are common with this technique.

Detection using immunoreagents is also a common method. Immunoelectrophoresis is a combination of electrophoresis and an antigen-antibody interaction.[3] Several methods of immuno-electrophoresis are available. The methods offer accurate identification of various proteins by the use of specific antibodies, but at the cost of increased analysis time. However, the resolution of overlapping bands made possible with this procedure make this detection method highly desirable. Certain proteins, such as monoclonal antibodies, are routinely analyzed by this technique.

On-line detection[3] is desirable for such reasons as ease of use, low cost, convenience, and ease of quantitation. Moreover, automation is ideal because the detection mechanisms eliminate the time consuming

and cumbersome staining/destaining/densitometry procedure used routinely in clinical labs. Many detection methods have been used for on-line[3] detection. Among these are optical, electrical and thermal methods. The advantage of choosing on-line devices is that the methods are performed in free-solution, in tubes. Gels and solid supports may actually interfere with many forms of on-line detection.

Electrical detection is one type of on-line detection method commonly employed. As an example, this detector is utilized in isotachophoresis. In isotachophoresis, zones travel in order of decreasing mobility. The electric field increases with decreasing mobility of zones. In this way, all zones travel with the same velocity. The electric field can be monitored by placing tiny electrodes within the capillary. The resulting electropherogram looks like a series of steps in which the steps correspond to successive zones in the capillary. Conductivity can be monitored in the zones. Conductivity will decrease in each successive zone due to decreased mobilities in the zones.

Thermal methods are available which take advantage of the fact that constant currents are present throughout the capillary. The electric field is increased with each zone, however. Thermocouples may be placed on the capillary to monitor the temperature.

Optical methods are fairly obvious to the practicing scientist. Some examples of optical detection routinely employed in electrophoresis are absorption, fluorescence, refractive index, and light scattering. These methods are useful in isotachophoresis, moving boundary electrophoresis and in capillary zone electrophoresis.

In comparing different methods, one may come to realize that some of the simpler electrophoretic methods may require less than one hour to complete; some of the more difficult procedures may require days to complete. Instrumental versions seem more desirable.

There have been many variations on techniques employed in discovering better detection methods. Many of these have been in the area of electrophoresis as well as other areas such as chromatography. Electrophoresis itself is a technique that has been in use for sixty years. It is the premier method in protein separations. There are now several known commercial manufacturers of instrumentation in this area. Still, however, there is a need to develop more detection methods. For example, chromatography has developed over the years and provides analytical labs with quick and efficient procedures. Several samples can be analyzed simultaneously, and require only minutes of work by the analyst.

Statement of the Problem

Quantitation of analyte zones is important in carrying out an electrophoretic analysis. Staining with subsequent densitometry has historically been the premier method to quantify proteins; it still remains common. There are certain problems associated with staining, however. One problem appears to be that of comparing protein patterns from gels and solids stained by different techniques.[2] Certain proteins stain to a different degree depending upon which procedure for staining is used. Also, the electropherogram of the separation of blood or serum may contain several protein constituents. There may be present lipoproteins, haptoglobins, hemoglobins, among other proteins.

The type of staining procedure depends upon the analyte. There are always problems with contamination in analytical techniques, also. These can be observed with staining techniques in the sample preparation procedure. Another problem observed is the lack of uniformity in the surface of the solid phase, which creates erratic separations. Sometimes, also, there is a lack of uniformity in the stated molecular weights of proteins in purchased standards.[2] Among these problems is the fact that the staining of plates can be tedious and time-consuming. Many densitometers don't even have integrators to help quantify the protein separations. This is especially important because of the fact that state-of-the-art instruments ensure good reproducibility of data. With non-integrating instruments, this would not be achieved.

In electrophoresis, good reproducibility depends upon close adherence to quality control protocols. It is important to pursue new detection techniques or improve well-known detection principles for quality analyses. In light of this, the development of two types of detectors was studied; conductivity and permittivity detectors. These are two types of electrical detectors. In zone electrophoresis, electrical field detectors work because the ions travel in order of increasing mobility. When electrodes are placed side by side in the capillary channel, the electric field in a zone is measured as a voltage between the electrodes. These detectors can be viewed as equivalent circuits. Therefore, the ideas of capacitance (permittivity detectors), potentiometry, amperometry, and coulometry

may be employed in detection devices. Electrical conductivity can also be measured because the mobility of different ions are different in the electric field.

Conductance may be measured by measuring current across the conductor at some applied voltage difference. Equation 3

$$C = i / E \qquad (3)$$

relates conductance, C, with the current, i (amperes), and the potential difference, E, (volts).[5] Conductance is related to the chemical properties of the sample.

It was thought that since it is the basis of charge that separates components of a sample in electrophoresis, it might be beneficial to make a conductivity detector for zone electrophoresis. A detector such as this has been reported, where a conductivity detector was placed directly over the agarose gel plate.[6] A conductivity meter was connected to an X-Y recorder. It was reported that a separation of the components of a serum sample with discrete zones formed. A similar method was attempted with cellulose acetate to attempt confirmation of these results. There has not been a report of this type of detector in the chemical literature, but there have been numerous conductivity detectors applied to free-solution techniques.

The theory for permittivity detection has been given a detailed treatment in liquid chromatography.[5] There are many ways to measure permittivity. The permittivity detector measures permittivity which is related to a molecule's polarizability.[5]

Chapter 2

Literature Review

A literature search was conducted using Chemical Abstracts. From this search, it was found that many detection methods in several fields based upon conductivity have been reported in the literature.[7-15] Only a few in the past few years have been reported for electrophoresis. This thesis deals with zone electrophoresis on a support medium of cellulose acetate. Recently, however, many detectors for capillary zone electrophoresis have been developed. The theory concerning both of these modes (and other modes) is similar. Therefore, new detection methods deserve mentioning.

With capillary zone electrophoresis, an on-column conductivity detector was described by Huang, et al.[7] Previously, it was not thought to be beneficial to use electrical detection modes with capillary zone electrophoresis because with CZE, there is a relatively large background of supporting electrolyte (buffer) upon which a low concentration of sample ion is superimposed.[3] The detector by Huang is one which holds promise as a universal detector. The detector was constructed by fixing platinum wires through diametrically opposite holes in fused-silica capillary tubing. These holes were made with a CO_2 laser. This can be used to detect and quantify any species causing a conductivity change with respect to the background electrolyte. It is easy to couple it to a data acquisition system. The total cost of its construction was only about $200. The power required was only a few watts. Low molecular weight carboxylic acids were analyzed with this same detector by Huang.[8]

Modifications to a high-frequency contactless conductivity detector were described by Vacik, et al.[9] Its properties (reproducibility, stability and dependence on the sensitivity of the concentration or conductivity and relative permittivity of the measured solutions) were demonstrated. Its applicability to capillary isotachophoresis was illustrated by a test separation. The apparatus has a four-electrode probe. The apparatus, including the electronics, was placed in a thermostated box to ensure reproducibility.

Electronic circuits for the measurement of conductivity in isotachophoresis have been described by Mulder, et al.[11] A conductimeter was used. It was found that by coating the electrodes, the conductometric method of detection can have better sensitivity. The reaction at the electrodes was suppressed in order to diminish the changes in capacity between the electrode surface and the electrolyte. The concept of cell impedance was utilized. The impedance can be measured by analyzing the potential difference between the electrodes or by applying an alternating signal to measure impedance.

A high-frequency bridge (500 kHz) was constructed for application in isotachophoresis by Stankoviansky, et al.[12] Platinum electrodes were used in the measuring cell because graphite electrodes had lower sensitivity. In the two trials, PTFE capillaries of dimensions 0.4mm I.D. and 0.7 mm O.D., then of dimensions 0.4 mm I.D. and 1.0 mm O.D. were used. The current range was 5-100 microamperes and the voltage range 2.5-12kV.

Conductivity and permittivity detectors for liquid chromatography have been reported in the literature.[13-18] A review of detectors for liquid chromatography was made by Yeung.[5] The first review of these

specific detectors was reported in 1974 by Haderka.[14] The theory was extensively reviewed with a mathematical treatment deriving from the first Maxwell equation. It was derived that the magnetic field in the space between the capacitor electrodes is parallel to the electrodes, and its magnitude is just equal to the magnitude of the surface current density on the plates.[14] The concept of the flow-through capacitor as a sensor was reviewed especially with regard to the design of the detectors. Two kinds of capacitor designs were recommended: a coaxial design for lumped-parameter circuits and a flat circular design, with coaxial and cavity resonators, for frequencies of the order of 10^8 Hz.

Another description by Haderka was of a simultaneous detector for both conductivity and permittivity in a single cell.[14] The combination of the two detectors complements the analysis, giving the system wide applicability. For example, the effect of band broadening can be reduced since it eliminates the need to use two cells connected in series and it eliminates the time lag between signals. Both qualitative data with regard to polarization/ionization of the eluent and quantitative information can be provided.

A permittivity detector for high-performance liquid chromatography (HPLC) and flowstream analysis employing 3 oscillators was described by Alder, et al.[15] The cell circuit was of the Franklin type, and the third oscillator allowed operation over the complete range of permittivities and prevented locking. This detector was applied to the HPLC of amino acids and alcohols.

The study by Alder, et al. of a microcomputer-based temperature corrected permittivity detector for liquid chromatography was made to

digitize the oscillator frequency data for subsequent manipulation.[16] A combination of these data and those obtained from a simultaneous measurement of the eluent temperature within the cell allowed an algorithmic correction of the base line for long-term drift. This allowed the analysis of three trialkyl phosphates to be detected at injected levels of 200-450 ng in 5% methanol in hexane.

The use of a liquid chromatographic mobile phase of higher permittivity than the solute lowers the sensitivity and improves the response linearity of a capacitance detector.[17] Relations between the detector response and solute concentrations were derived for both the interference and bridge methods of detection by Haderka.

The basic characteristics of the liquid chromatograph permittivity detector were analyzed theoretically by Haderka using the principle of resonance.[18] Equations were derived for the response of the detector to either the real or imaginary component of complex permittivity of the carrier liquid-analyzed mixture and the response of either of the components was a linear function of concentration. A nonconductive substance with minimal dielectric losses was the most suitable carrier liquid when using the resonance principle.

The generation and implementation of the aforementioned detectors from the literature can be a difficult task. Materials available, power supplies, and electronics are specific to the project, and hence, affect the results. Detection limits, therefore, vary. Even various parameters such as buffer used, concentrations, current ranges and voltage ranges are specific to the method. Attempts to improve qualitative and quantitative analysis, however, have much in common.

Chapter 3

Experimental

List of Materials

Samples used: Helena Laboratories Hemo controls AFAC, ASA_2, $AFSA_2$ and AA_2

Buffer: Helena Labs Supre-Heme Buffer Cat. no. 5802 for hemoglobin electrophoresis; contains Tris-EDTA-boric acid

Applicator Kit: Super Z Cat. No. 4088 (Helena Labs)

Microdispenser Tubes: Helena Laboratories Cat. no. 6010

Plates: Helena Labs Cat. no 3023 Titan III Zipzone Cellulose Acetate Plates for serum protein electrophoresis (25-60 x 76 mm)

Chamber: Helena Labs

Infrared to mV converter: OS-500 Omega Engineering, Inc. Model OS-500C
Calibration 1 mV/deg C. for monitoring temperature.

Keithley 169 Multimeter

Power Supply: Bio-Rad Labs model 1420B/150

Stain: Ponceau S stain (cat no. 5005)

Wicks: Zipzone Chamber wicks (cat. no. 5081)

Microdispensor: Helena Labs cat. no. 6008.

Clear Aid: Helena Labs cat. no. 5005

Densitometer: Quick Scan TLC (Helena Labs)

Oscilloscope/counter/timer/multimeter: Techtronix 2236 100 MHz

Universal Counter: Hewlett Packard 5314A

Breadboard: Heathkit laboratory breadboard ET 3300

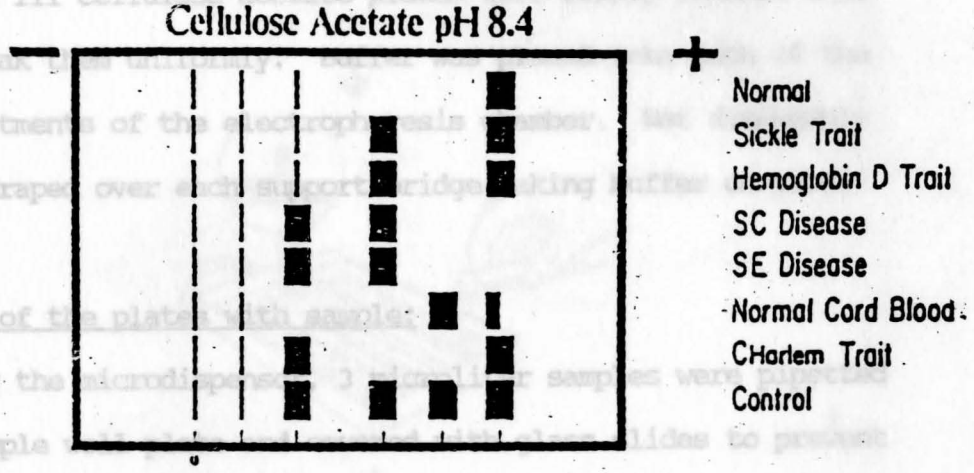
Resistors: brown/black/orange/gold; 10,000 Ohms or 10 kiloOhms
brown/black/red/gold ; 1000 Ohms or 1 kiloOhm

Capacitor: 100 pF, 25 V

Chip: CS2917/8333

The Helena Labs serum hemoglobin electrophoresis procedure[10] served as a basis for most of the experimental work. This was chosen because it is used routinely in clinical labs. This procedure was a type of zone electrophoretic procedure in which the mixture of components were separated completely from one another during electrophoresis, forming discrete zones. Cellulose acetate was used as a support medium because it yielded rapid separation of Hb A, F, S, and C and many other hemoglobin mutants with minimal preparation time. Figure 4 is a diagram of a stained electropherogram using cellulose acetate as a support medium. It shows the mobilities of possible Hemoglobin mutants. A buffer composed of Tris-EDTA and boric acid, pH 8.2-8.6 was used as a mobile phase. A detailed description of the procedure was provided by Helena Laboratories[10] (see Appendix). The procedure involved preparing the cellulose acetate plates, preparing the electrophoresis chamber, sample preparation and application, electrophoresis of the sample plate, staining the plates containing the hemoglobin bands, and evaluation of the hemoglobin bands.

FIGURE 4



Electrophoretic Mobilities

Of Hemoglobin Showing

Possible Mutants

Initial Studies

Initial separation of hemoglobin components was carried out by the traditional Helena Labs procedure. Figure 5 illustrates the step-by-step method of the procedure. The procedure is provided in detail in the appendix.

Typical Experimental Procedure

Preparation of the chamber:

Titan III cellulose acetate plates were slowly lowered into buffer to soak them uniformly. Buffer was placed into each of the outer compartments of the electrophoresis chamber. Wet disposable wicks were draped over each support bridge making buffer contact.

Preparation of the plates with sample:

Using the microdispensor, 3 microliter samples were pipetted into the sample well plate and covered with glass slides to prevent drying. The plates were taken out of the buffer. As quickly as possible, each plate was blotted and spotted with sample. The plates were placed in the chamber (cellulose acetate side down) and were run for 25 min. at constant voltage (350 V).

Staining Procedure:

After the electrophoresis time, the plates were removed from the chamber and placed in Ponceau S stain for 5-6 minutes. The plates were removed from the stain and then destained in 3 successive washes of 5% acetic acid (allowing the plates to stay in each wash 2 minutes).

FIGURE 5

PROCEDURE

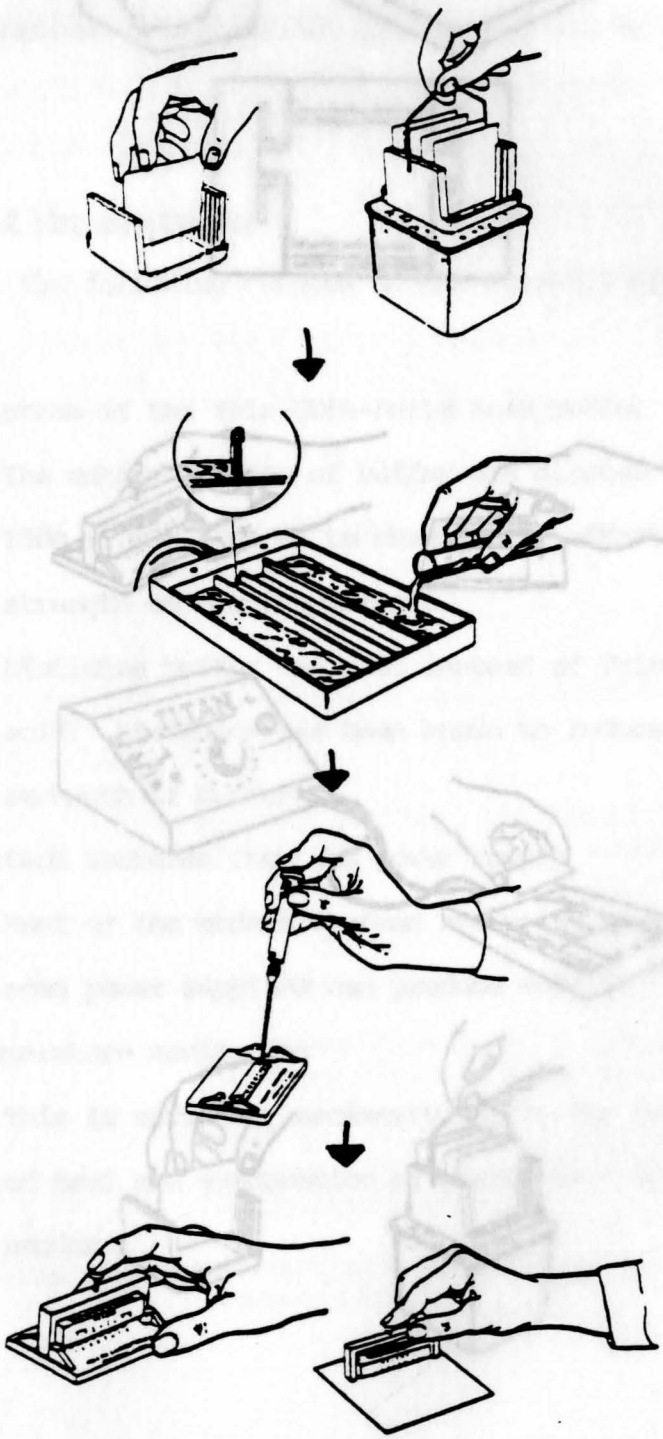
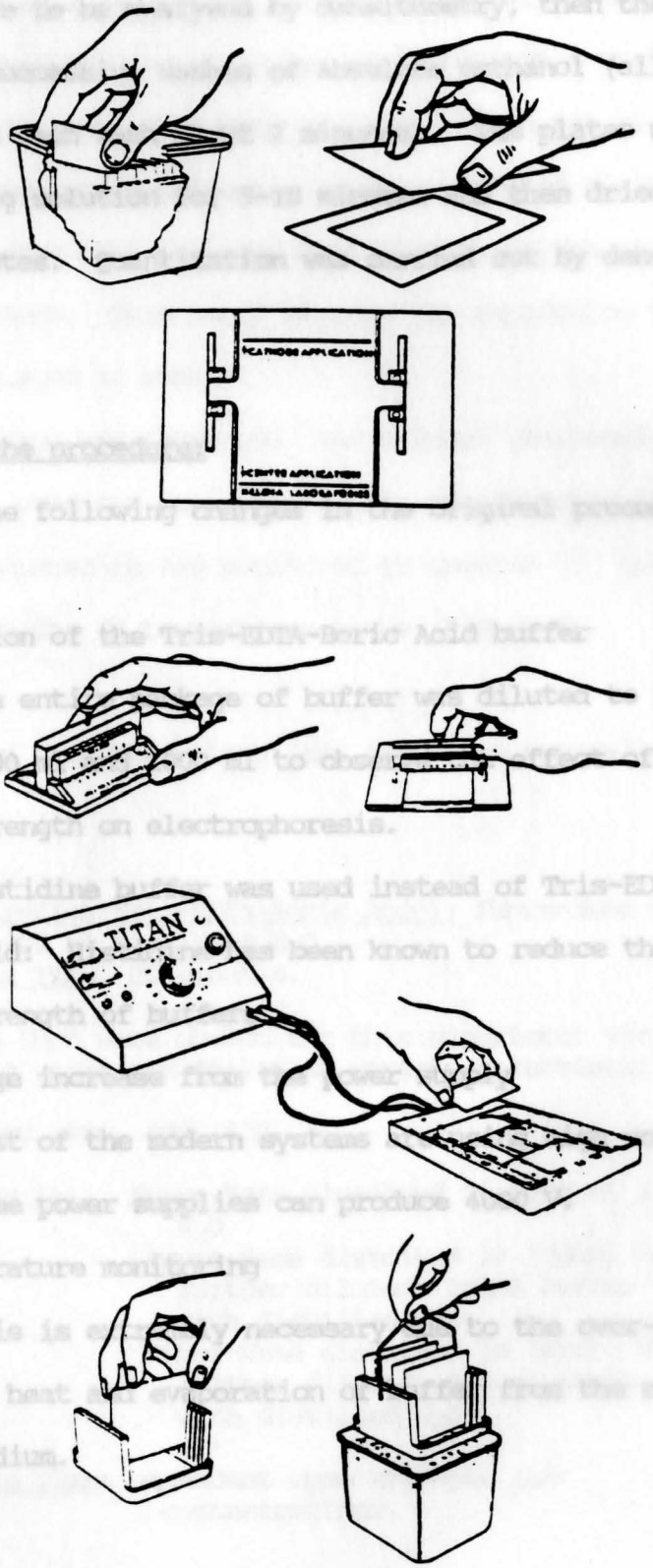


FIGURE 5, CONTINUED



If the plates were to be analyzed by densitometry, then the plates were dehydrated in 2 successive washes of absolute methanol (allowing the plates to stay in each wash about 2 minutes). The plates were then placed in clearing solution for 5-10 minutes and then dried in an oven for about 10 minutes. Quantitation was carried out by densitometry after drying.

Modification of the procedure:

In brief, the following changes in the original procedure were carried out:

A. Dilution of the Tris-EDTA-Boric Acid buffer

The entire package of buffer was diluted to 2000 ml, 1500 ml and 1000 ml to observe the effect of ionic strength on electrophoresis.

B. 1% Histidine buffer was used instead of Tris-EDTA-Boric acid: Histidine has been known to reduce the ionic strength of buffers.

C. Voltage increase from the power supply

Most of the modern systems are using high voltages; some power supplies can produce 4000 V.

D. Temperature monitoring

This is extremely necessary due to the over-production of heat and evaporation of buffer from the support medium.

It was suspected that if the buffer concentration was decreased, the concentration of analyte molecules would exhibit a much larger sensitivity of detection. A buffer of 1% Histidine was also thought to be a buffer which would produce a lower supporting electrolyte conductivity. It was also believed that increasing the voltage would benefit the procedure. This would shorten the separation time and decrease the diffusion of zones.

When the voltage was increased, the current increased. The increased current produced Joule heating if the voltage was too high. Therefore, the temperature was monitored to observe the consistency in the signal from the IR temperature probe (Figure 6).

The parameters that the Helena Labs procedure typically employed were:

Voltage: 350 V
Buffer concentration(Tris-EDTA-Boric Acid): Supre-Heme dissolved in 980 ml distilled H_2O.
Electrophoresis Time: 25 minutes.

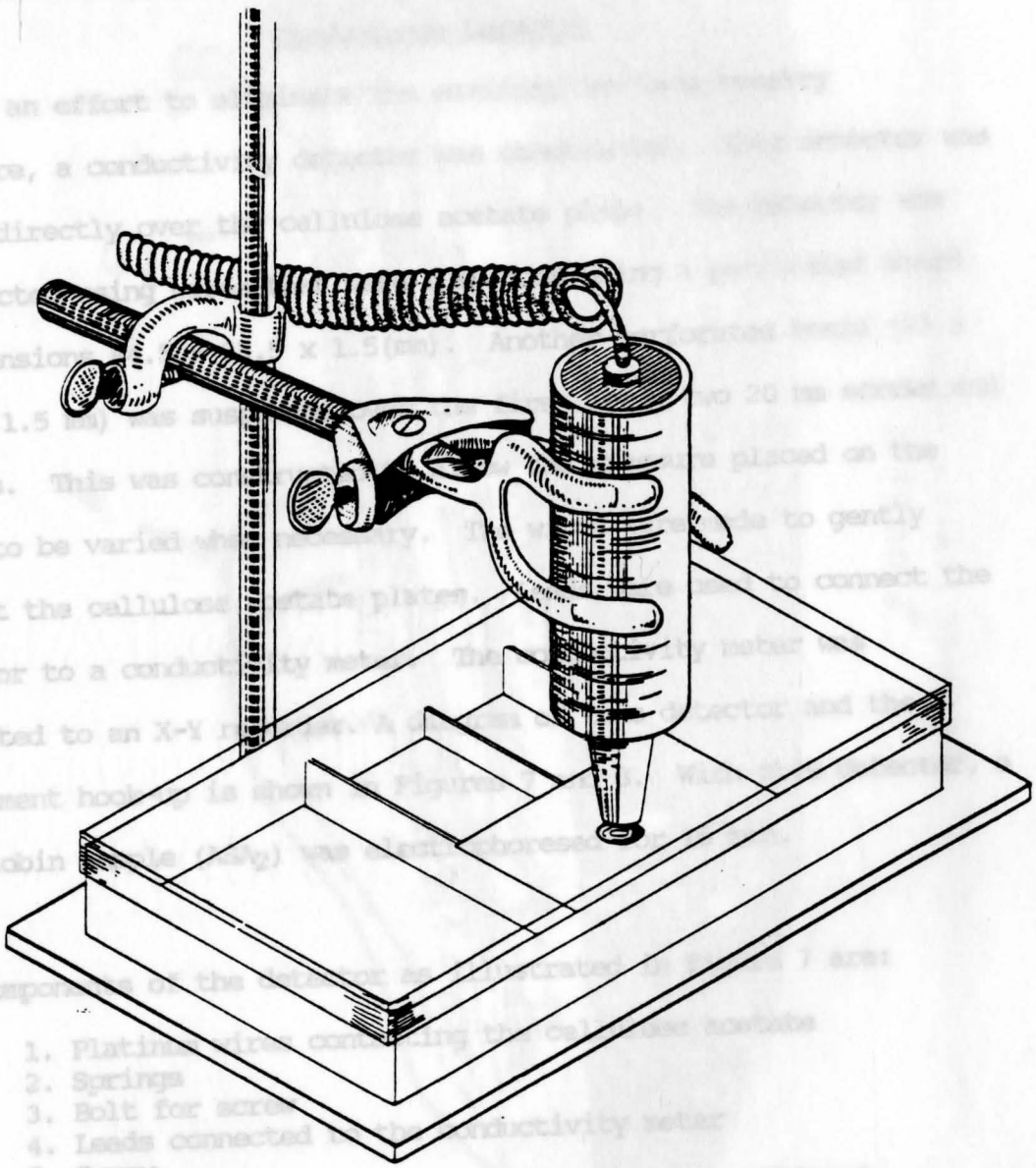
The parameters that were chosen for this experiment were based on electrophoresis using the following various parameters:

Voltages(V): 300, 350, 400, 450

Buffer conc.(Tris): Supre-Heme dissolved in 1200 ml distilled H_2O
Supre-Heme dissolved in 1000ml H_2O then further diluted: 250ml buffer to 1000 ml with distilled H_2O
Super-Heme dissolved in 1000ml H_2O and further diluted: 500 ml buffer to 1000 ml with distilled H_2O

Electrophoresis Time: dependent upon voltages and concentrations.

FIGURE 6



IR PROBE

Optimum parameters of voltage and concentration were chosen before continuing. These were 0.0250 ionic strength and 350 volts. Qualitative observation of the electropherogram led to these parameters. Figures 11-14 illustrate the densitometry scans of the four control samples using these parameters.

Conductance Detector

In an effort to eliminate the staining and densitometry procedure, a conductivity detector was constructed. This detector was placed directly over the cellulose acetate plate. The detector was constructed using wires that were suspended using a perforated board of dimensions 84.5 x 83.5 x 1.5(mm). Another perforated board (23 x 84.5 x 1.5 mm) was suspended over the first using two 20 mm screws and springs. This was constructed to allow the pressure placed on the plate to be varied when necessary. The wires were made to gently contact the cellulose acetate plates. Leads were used to connect the detector to a conductivity meter. The conductivity meter was connected to an X-Y recorder. A diagram of this detector and the instrument hook-up is shown in Figures 7 and 8. With this detector, a hemoglobin sample (ASA_2) was electrophoresed for 15 min.

The components of the detector as illustrated in Figure 7 are:

1. Platinum wires contacting the cellulose acetate
2. Springs
3. Bolt for screw
4. Leads connected to the conductivity meter
5. Screw
6. Perforated board onto which the wires are soldered
7. Perforated board upon which the cellulose acetate plate rests
8. Cellulose acetate plate.

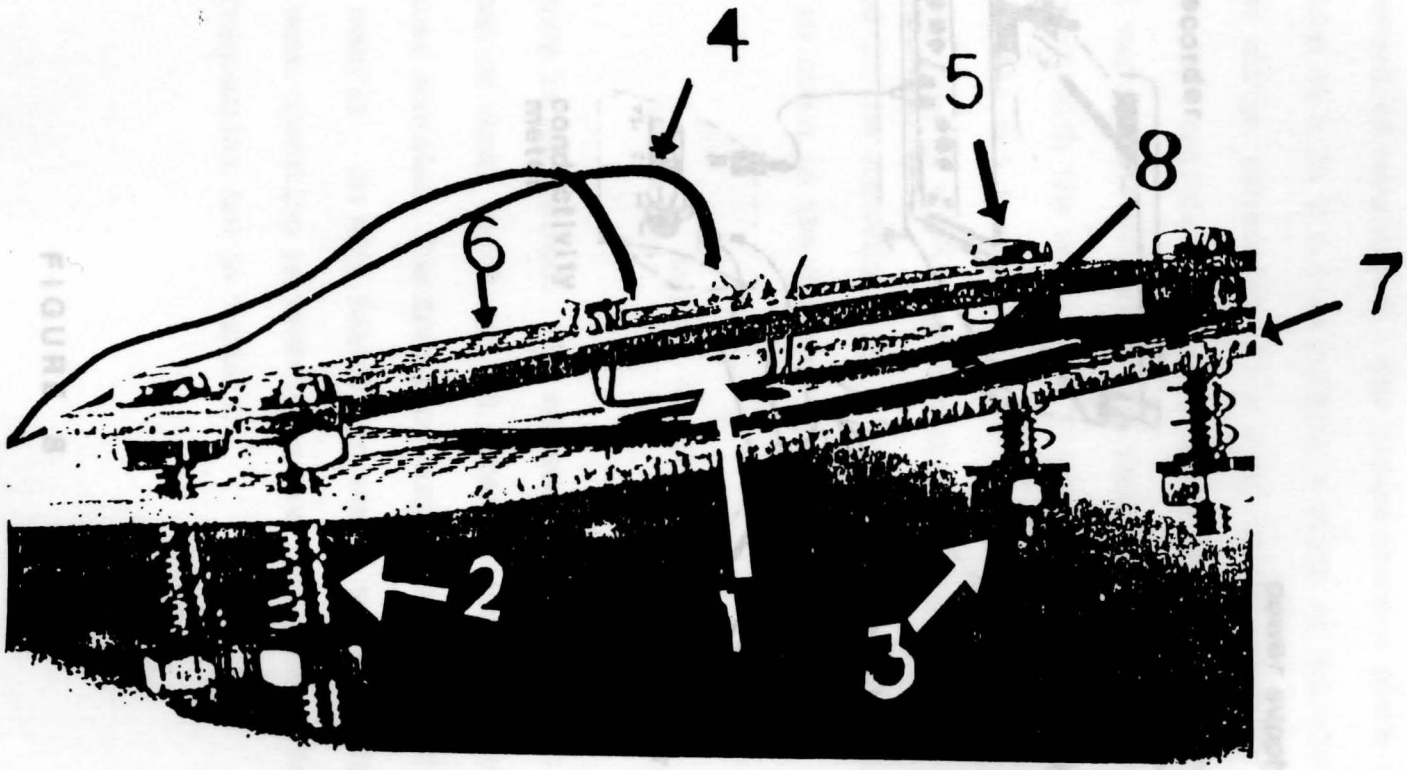


FIGURE 7
CONDUCTIVITY DETECTOR

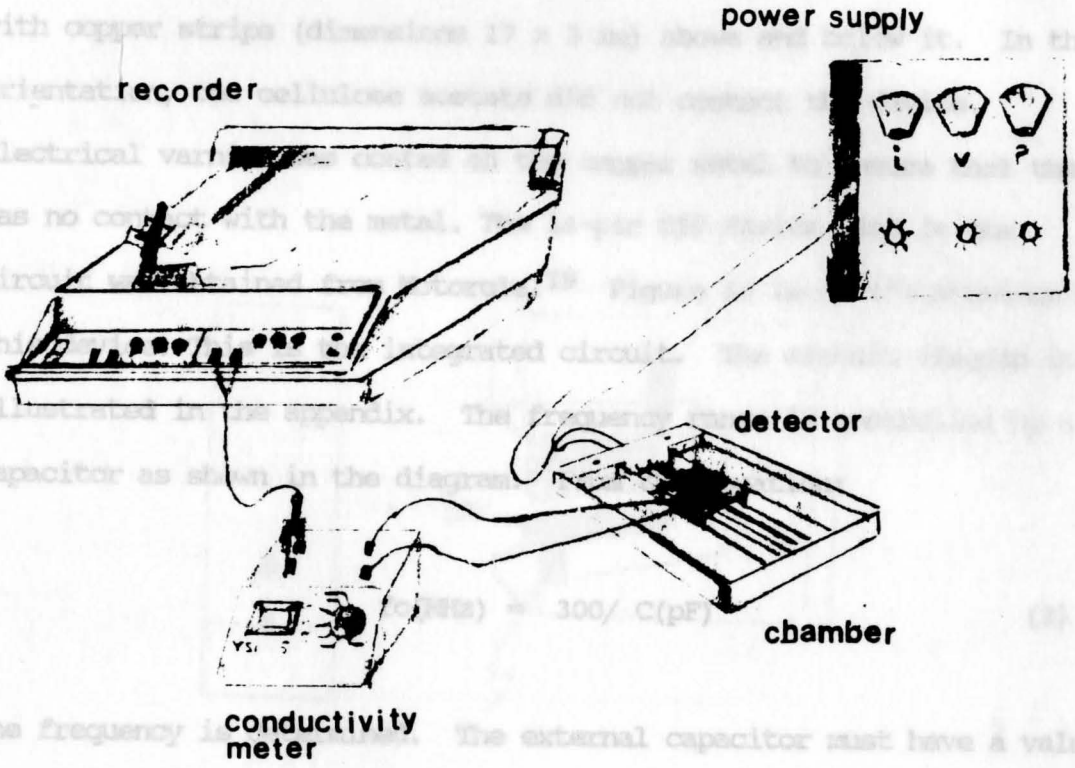

power supply
i
v
P
recorder
detector
chamber
conductivity
meter

FIGURE 8

Permittivity Detector

A permittivity detector (Figure 9) was constructed using a voltage controlled oscillator. The figure shows a piece of plexiglass of dimensions 45 x 31 x 6.5 mm holding a strip of cellulose acetate with copper strips (dimensions 17 x 3 mm) above and below it. In this orientation, the cellulose acetate did not contact the device. Electrical varnish was coated on the copper metal to ensure that there was no contact with the metal. The 14-pin DIP device used in the circuit was obtained from Motorola.[19] Figure 10 is an illustration of this device. This is the integrated circuit. The circuit diagram is illustrated in the appendix. The frequency range is controlled by a capacitor as shown in the diagram. From the equation:

$$fo(MHz) = 300/\ C(pF) \qquad (3)$$

the frequency is determined. The external capacitor must have a value greater than or equal to 100 pF. A detector was placed directly over the cellulose acetate. The frequency output was observed on the frequency counter. An ASA_2 hemoglobin sample was electrophoresed under the same operating parameters as previously described. The observed frequencies are in Table 3.

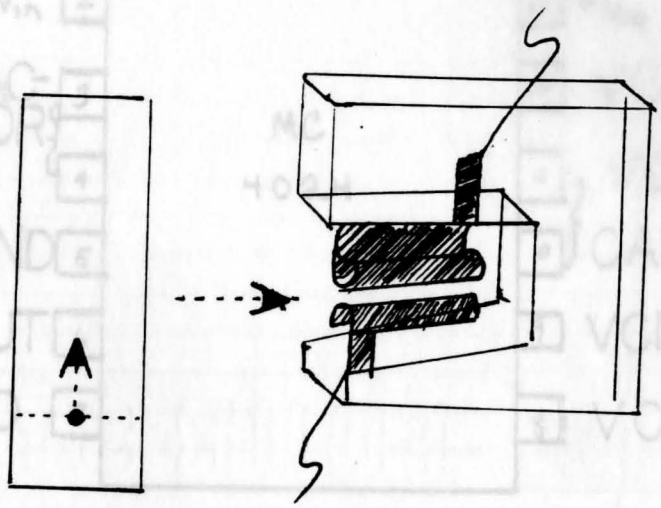
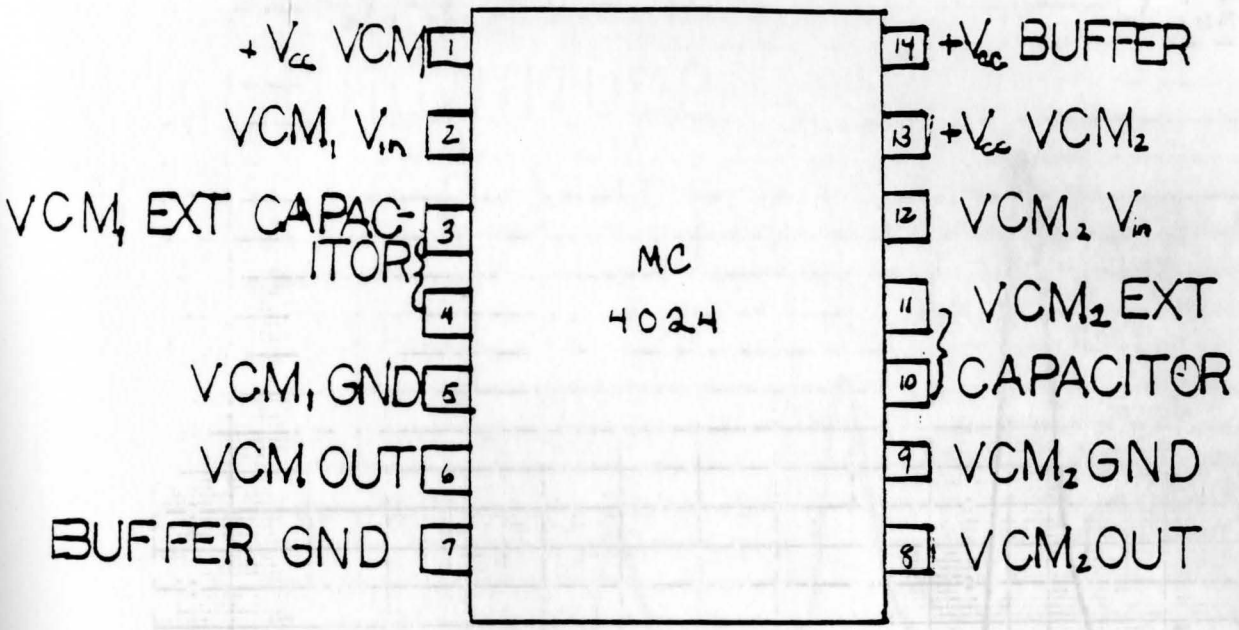


FIGURE 9

Permittivity Detector

FIGURE 10



Pin Diagram of the MC 4024

Voltage-Controlled Multivibrator

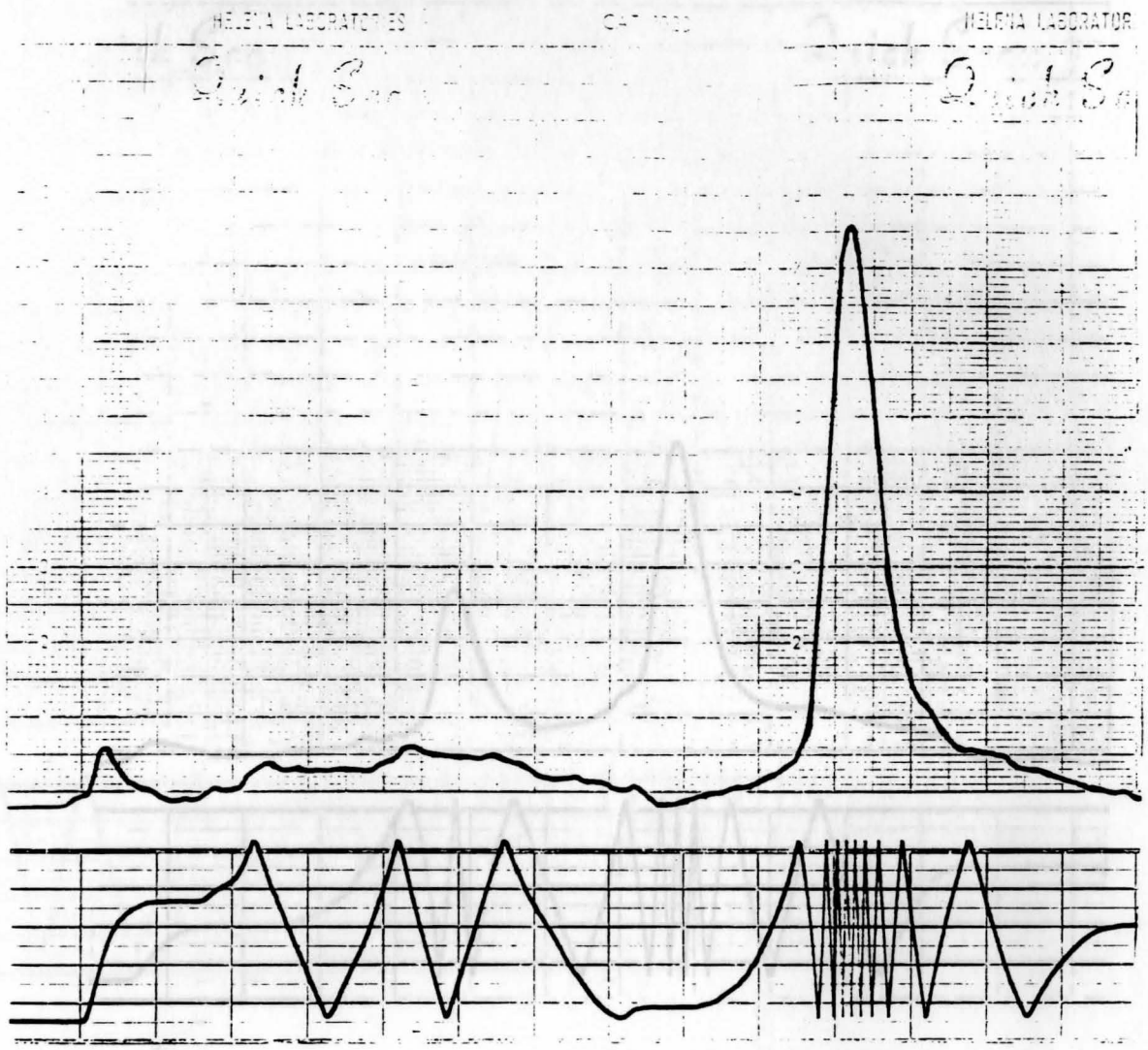


FIGURE 11

Scan of AA_2 Hemoglobin Control

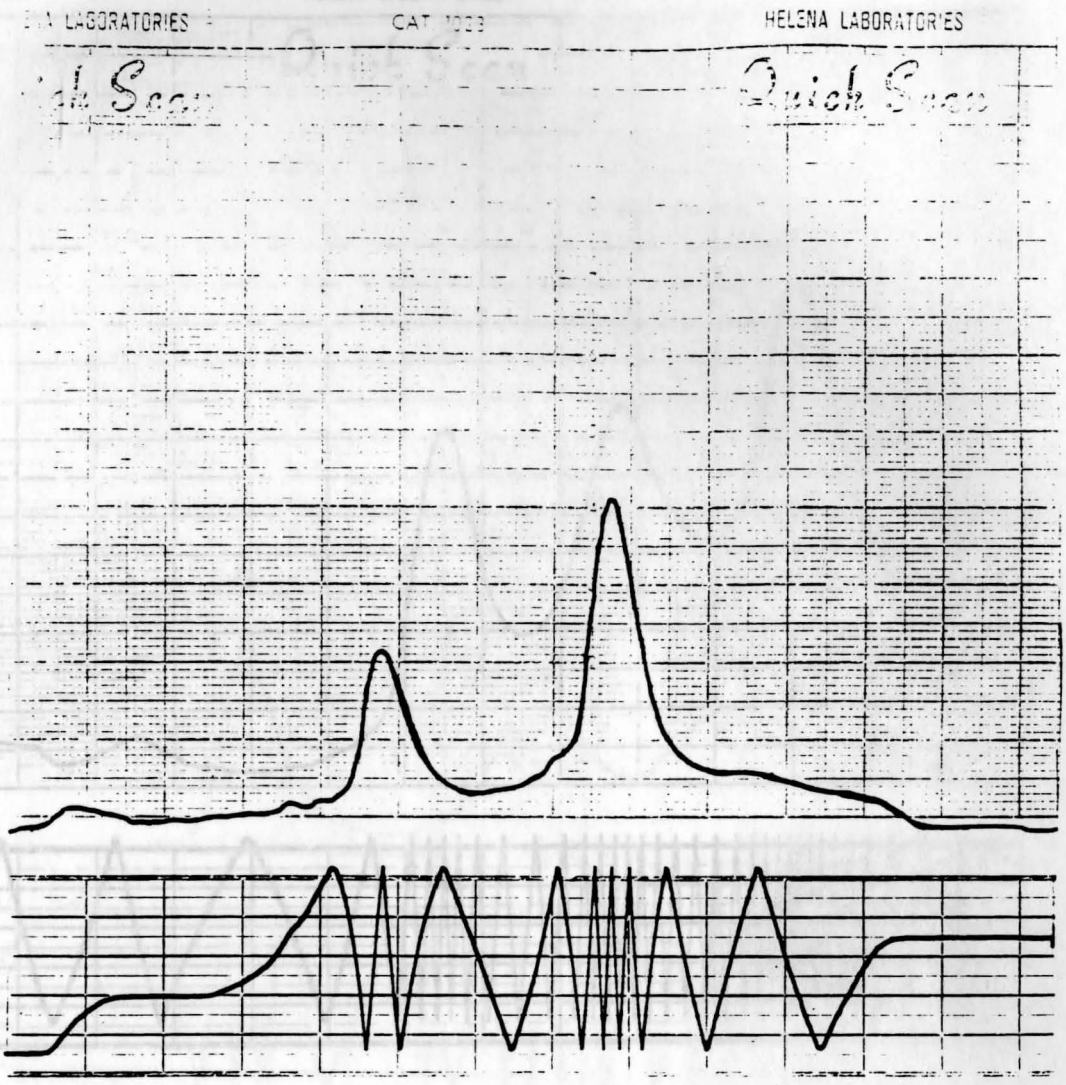


FIGURE 12

Scan of ASA_2 Hemoglobin Control

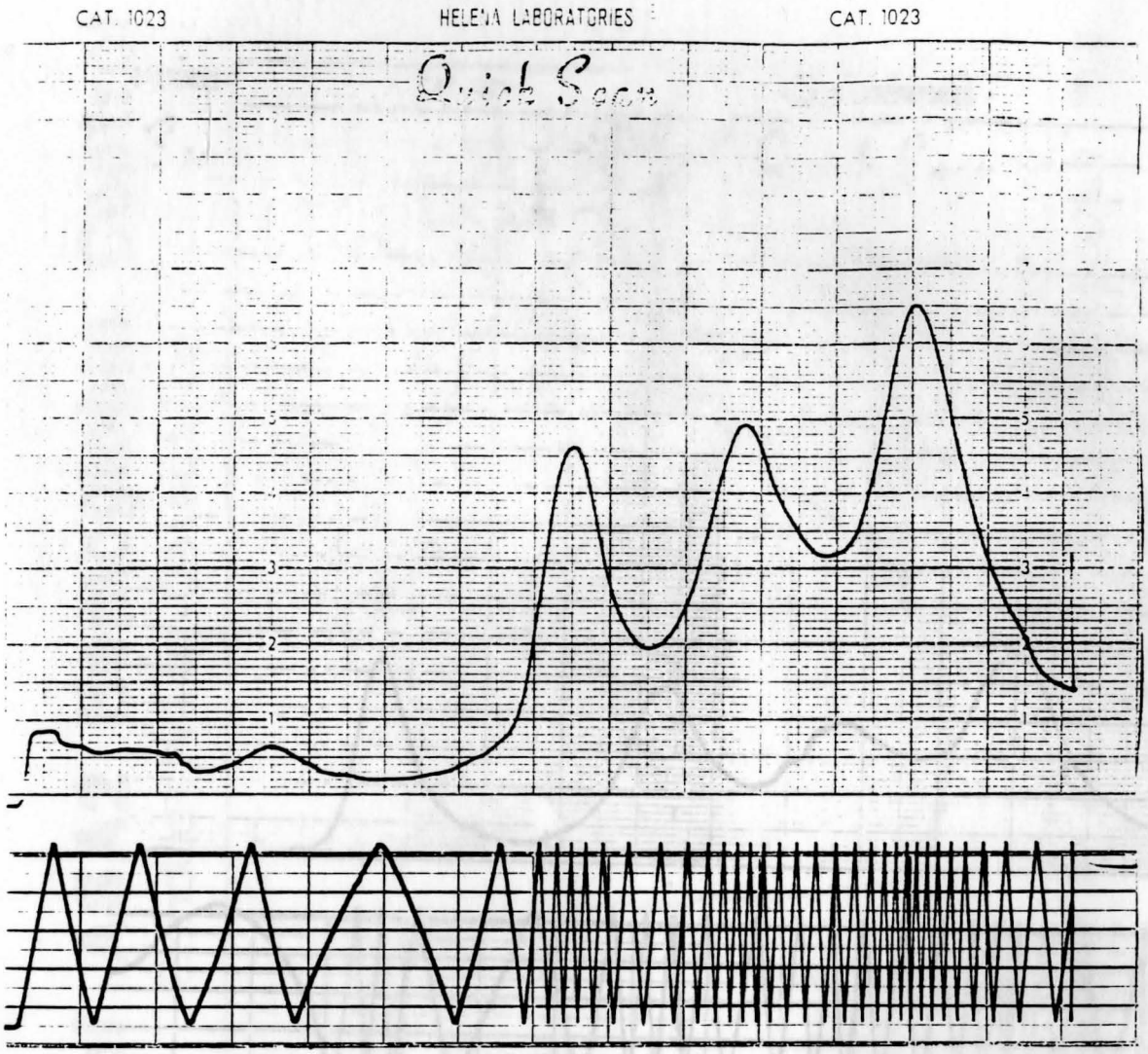


FIGURE 13

Scan of $AFSA_2$ Hemoglobin Control

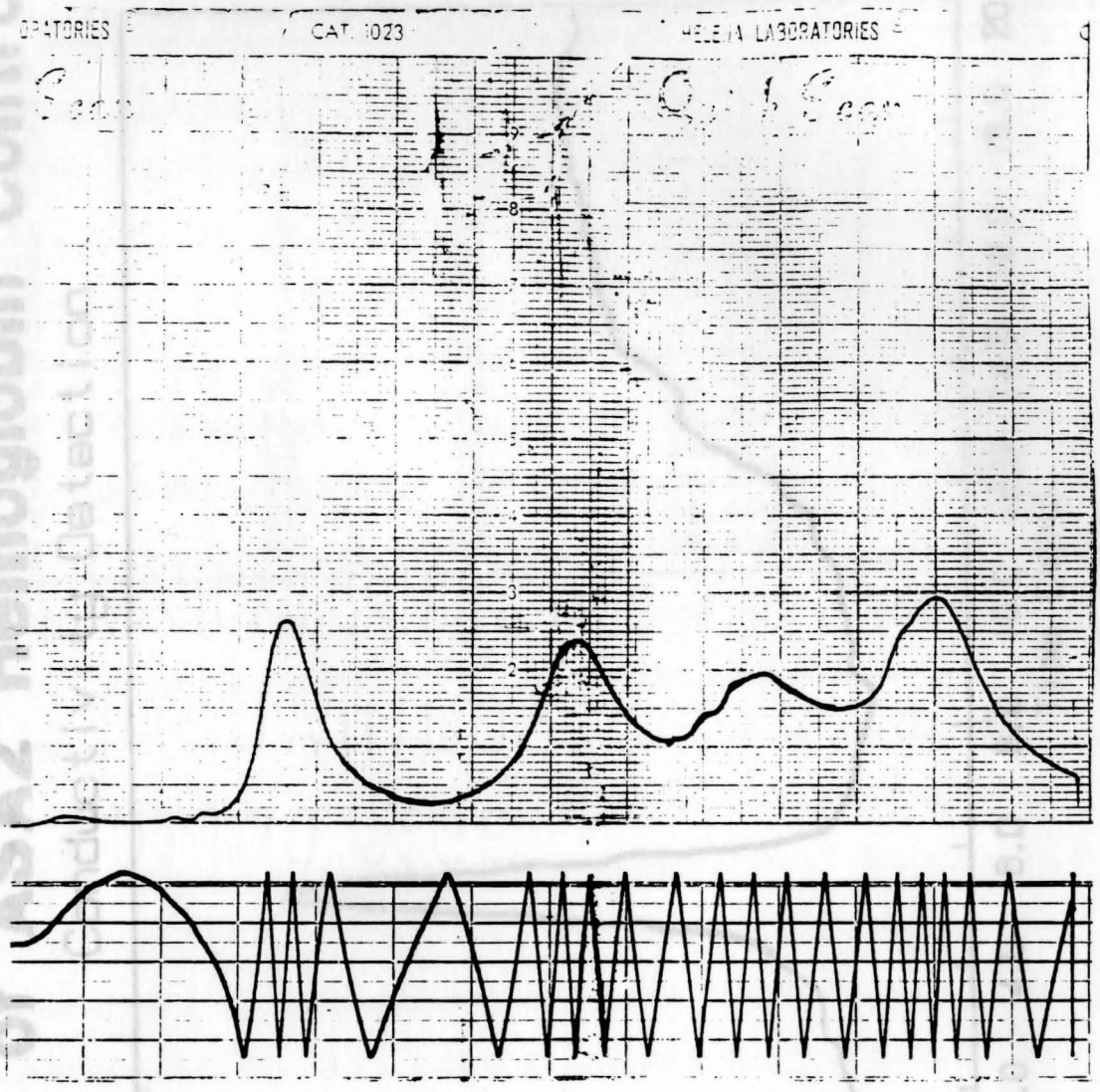


FIGURE 14

Scan of AFSC Hemoglobin Control

Scan of ASA2 Hemoglobin Control

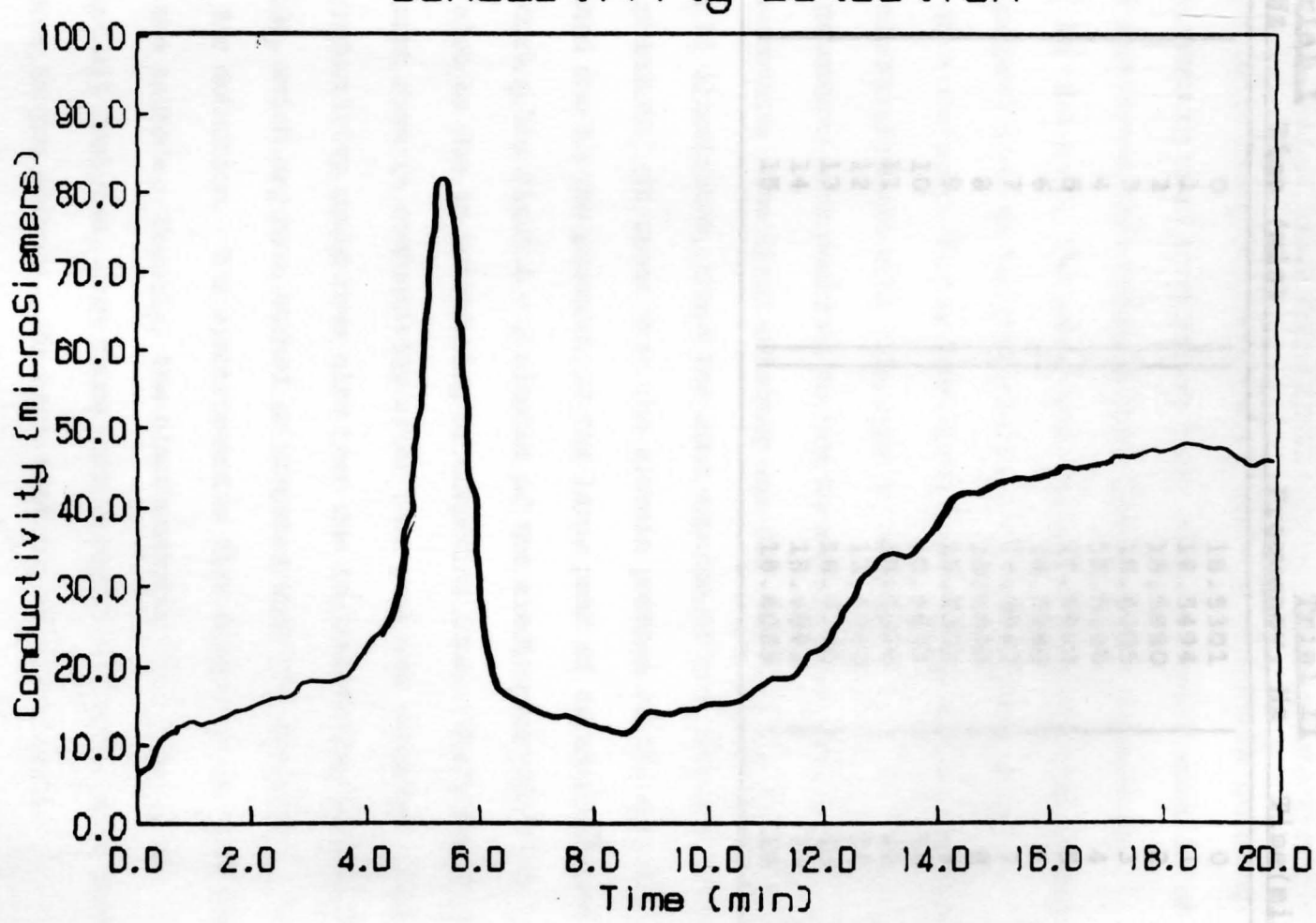


FIGURE 15

Table 3

Frequency (Hz) Observed During Electrophoresis of ASA_2 Permittivity Detection

Trial I		Trial II	
Frequency, Hz	Time (min)	Frequency, Hz	Time(min)
18.8410	0	18.5301	0
18.8412	1	18.5494	1
18.8605	2	18.5880	2
18.8533	3	18.6005	3
18.8490	4	18.5400	4
18.8441	5	18.5992	5
18.8298	6	18.5990	6
18.8347	7	18.5927	7
18.8270	8	18.5630	8
18.8301	9	18.5981	9
18.8214	10	18.5624	10
18.8408	11	18.5524	11
18.8195	12	18.4760	12
18.8167	13	18.5760	13
18.8163	14	18.6591	14
18.8169	15	18.6089	15

Chapter 4

Results and Discussion

The objective of these experiments was to design a detection system to analyze hemoglobin samples without the cumbersome staining procedure commonly used in clinical labs. The end result would be an automated instrument that requires much less time and expense to operate. In this case, the instrument furnished an electrical signal that was proportional to the concentration of a substance being analyzed in a mixture. It has been shown that the agarose gel medium supports electrophoresis with this type of detection.

Two detectors were designed to try to achieve this goal on cellulose acetate. The first detector was the conductivity detector. As Figure 15 illustrates, there was some reponse of the detector from the ASA_2 standard. It seems that the albumin portion of the sample was detected due to the presence of the large peak of 84 microSiemens. This was during the first 4 - 8 minutes of the electrophoresis time. This may also be due to interfering or competing ions. There was also a significant rise in conductivity after this peak was observed. This rise in conductivity could have also been due to interfering ions such as chloride, which may have masked or competed with the desired analytes for detection. The electroosmotic flow direction is from the anode to the cathode. Usually, the electroosmotic flow rate is so strong that all analytes, even those with a negative charge, move past the detector to the cathode. However, when the electrophoretic mobilities of some anions are higher than the electroosmotic flow mobility of the bulk electrolyte, these anions will escape detection.

The configuration of the conductivity detector was as shown in Figure 7. Several configurations were attempted, and this was the best. The others suffered from disadvantages such as the application of too much pressure upon the support medium, and interference of the electric field vector which caused the flow of sample around the platinum wires instead of underneath them.

The circuit for the permittivity detector was an a. c. circuit. The biggest advantage of alternating current is that it can readily be transformed to high voltages. It usually is desirable to use high voltage and low current for power transmission. The frequency of the alternating current was being measured in this circuit. The result on the electropherogram was that the hemoglobin components were not travelling underneath the detector wires; they were going around them. As a result, the measured frequencies were not due to hemoglobin components being detected. These frequencies were probably due to the buffer ions. Stray fields (electric or magnetic) may have been produced to make the pattern of mobility change. Frequency was observed on the Techtronix frequency counter. An added problem with this circuit was the need to convert frequency to a d. c. electrical signal to make a "hard copy" by plotting the signal. In this way, a comparison could be made to the densitometry scans for the hemoglobin samples.

Free solution methods may prove to be more beneficial in producing automated instruments since there would not be any interference from solid or gel support media. Some problems associated with solid supports are differing pore sizes and ionic composition. Also, there could possibly be chemical reactions

occurring on the surface of the electrodes inhibiting the passage of the hemoglobin components. A problem with both free solution methods and traditional zone methods on solid supports is that of extracting a small signal from a large background. Impedance is always a function of the composition of liquid between the two measuring electrodes. This problem would be reduced by decreasing the ionic composition of the electrolyte. In this experiment, 0.025 ionic strength buffer produced the best results. Histidine was also used since some literature sources have had success with this buffer; however, Tris-EDTA-Boric Acid proved better for this method.

In conclusion, detectors with great sensitivity for the hemoglobin components need to be produced to eliminate the problems associated with this method. Interference of bulk electrolyte seems to be the major difficulty. Already, new methods have been achieved with capillary zone electrophoresis for free solution methods. However, a method suitable for zone electrophoresis on solid support media must be considered because of the common use of this method in clinical labs.

APPENDIX

HEMOGLOGIN ELECTROPHORESIS PROCEDURE: USING CELLULOSE ACETATE PLATE IN ALKALINE BUFFER
HELENA LABORATORIES

STEP-BY-STEP METHOD

A. Preparation of the Titan III-H Plate
   1. Dissolve one package Supre-Heme Buffer in 980 ml purified water.
   2. Properly code the required number of Titan III-H Plates by marking on the glossy hard side with a Helena Marker.
   3. Soak the required number of plates in Supre-Heme Buffer for 5 minutes. The plates should be soaked in the bufferizer according to the instructions provided. Alternately the plates may be wetted by slowly and uniformly lowering a rack of plates into the buffer. The same soaking buffer may be used for soaking up to 12 plates or for approximately one week if stored tightly closed. If used for a prolonged period, residual solvents from the plates may build up in the buffer and cause poor separation of the proteins or evaporation may cause greater buffer concentration.

B. Preparation of Zip-Zone Chamber
   1. Pour approximately 100 ml of Supre-Heme Buffer into each of the outer sections of the Zip-Zone Chamber.
   2. Wet two disposable wicks in the buffer and drape one over each support bridge being sure it makes contact with the buffer and that there are no air bubbles under the wicks.
   3. Cover the chamber to prevent buffer evaporation. Discard the buffer after use.

C. Sample Preparation and Application
   1. Prepare a hemolysate of the patient samples as follows:
      a. Using whole blood: Add 1 part whole blood to 3 parts Hemolysate Reagent. Mix well and allow to stand for 5 min.
      b. Using packed cells: Mix 1 part packed red blood cells to 6 parts Hemolysate Reagent. Mix well and allow to stand for 5 minutes.
   2. Place 5 microliters of the prepared patient hemolysates or 5 microliters of at least one of the Helena HemoControls into the wells of the Sample Well Plates using the Microdispenser. NOTE: The Helena Hemo Controls are used directly from the vial. Do not prepare a hemolysate.
   3. To prevent evaporation, cover the Sample Well Plate with a glass slide if the samples are not used within 2 min.
   4. Prime the applicator by depressing the tips into the sample wells 3 or 4 times. Apply this loading to a

piece of blotter paper. Priming the applicator makes the second loading much more uniform. Do not load applicator again at this point, but proceed quickly to the next step.

5. Remove the wetted Titan III Plate from the buffer with the fingertips and blot once firmly between two blotters. Place the plate in the aligning base cellulose acetate side up, aligning the bottom edge of the plate with the black scribe line marked "CATHODE APPLICATION". The identification mark should be aligned with sample No. 1. Before placing the plate in the aligning base place a drop of water or buffer on the center of the aligning base. This prevents the plate from shifting during the sample application.
6. Apply the sample to the plate by depressing the applicator tips into the sample well 3 or 4 times and promptly transferring the applicator to the aligning base. Press the button down and hold it for 5 seconds.

D. Electrophoresis of Sample Plate

1. Quickly place the plate in the electrophoresis chamber, cellulose acetate side down. Place a weight (glass slide, coin, etc.) on the plate to ensure contact with the wicks.
2. Electrophorese the plate for 25 minutes at 350 volts.

E. Staining the Hemoglobin Bands

1. Remove the plates from the electrophoresis chamber and stain in Ponceau S for 5 minutes.
2. Destain in 3 successive washes of 5 % acetic acid. Allow the plates to stay in each wash about 2 minutes.
3. The plates may be dried and stored for a permanent record at this point. If a transparent background is desired (i.e., for densitometry) proceed to the next step.
4. Dehydrate the plates in 2 successive washes of absolute methanol. Allow the plates to stay in each wash about 2 minutes.
5. Place the plate in clearing solution for 5-10 minutes. Prepare the clearing solution as follows: 30 parts glacial acetic acid, 70 parts methanol, 4 parts Clear Aid.
6. Remove the plate from the clearing solution and drain off the excess solution.
7. Place the plate, cellulose acetate side up, on a blotter pad under the Micro-Hood, or in the I.O.D. or other laboratory drying oven, at 56 ^{o}C for 10 minutes or until dry.

F. Evaluation of the Hemoglobin Bands

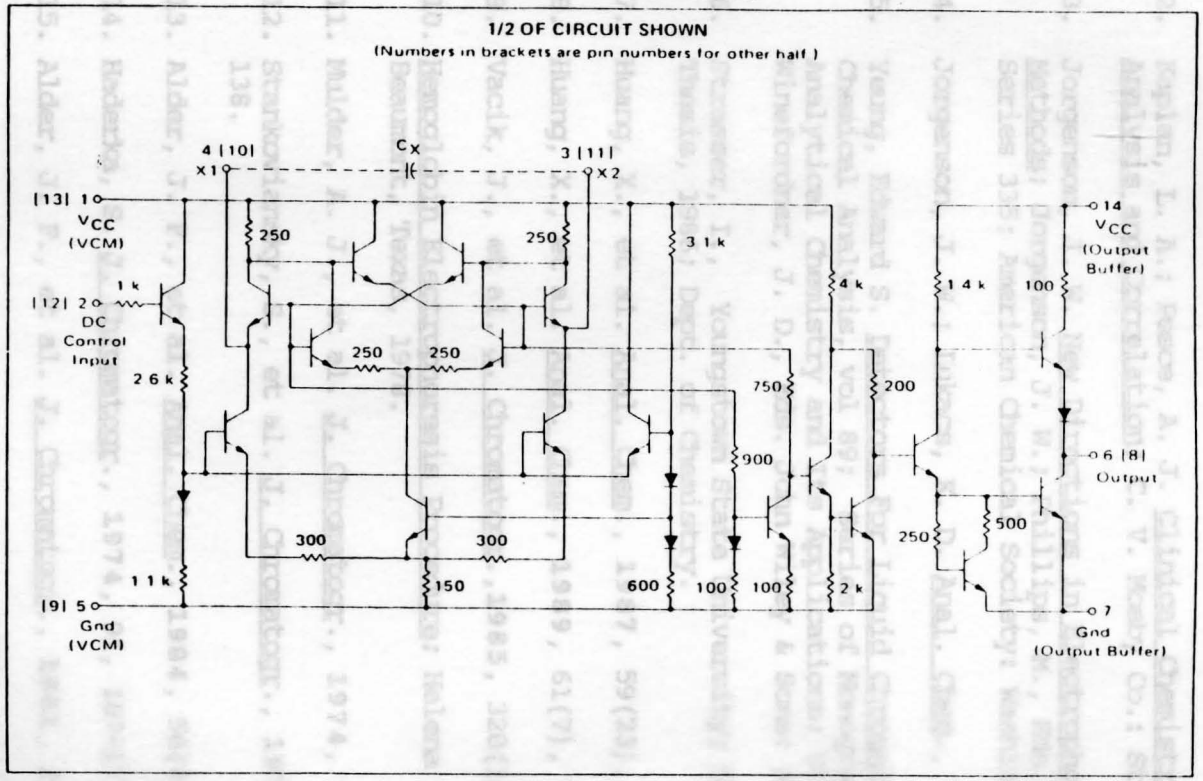
1. Qualitative evaluation: The hemoglobin plates may be inspected visually for the presence of abnormal hemoglobin bands. The Helena Hemo Controls provide a marker for band identification.
2. Quantitative evaluation: Determine the relative percent of each hemoglobin band by scanning the cleared and dried plates in the densitometer using a 525 nm filter.

HARDWARE	CAT NO.
Super Z-12 Applicator Kit (12 samples)	4093
Super Z Applicator Kit (8 samples)	4088
Zip Zone Chamber	1283
Microdispenser and Tubes	6008
700 Staining Set	5114
Micropreparation Dish	8003
Bufferizer	5093
Micro-Hood	8009
I.O.D.	5116
EWS Digital Power Supply	1520

Consumables:

Titan III Cellulose Acetate (94 mm x 76 mm)	3021
Titan III Cellulose Acetate (76 mm x 60 mm)	3022
Supre Heme Buffer	5082
Hemo $AFSA_2$	5330
Hemo AA_2	5328
Hemo AFSC	5331
Hemo ASA_2	5329
Hemolysate Reagent	5125
Ponceau S	5525
Clear Aid	5005
Titan Blotter Pads	5034
Hemoglobin Report Forms	5212
Zip Zone Prep	5090
Titan Plastic Envelopes	5052
Helena Plastic Marker	5000
I.D. Labels	5006
Zip Zone Chamber Wicks	5081
Glue Stick	5002

FIGURE 6 – CIRCUIT SCHEMATIC



Courtesy Motorola Semiconductor Products, Inc.

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