Coupled Magneto-Electrophoresis For Improved Hemoglobinopathy Resolution

by

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Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in the Chemistry Program

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ABSTRACT

Coupled Magneto-Electrophoresis For Improved Hemoglobinopathy Resolution

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Misinterpretation of different hemoglobin variants has been the cardinal source of errors in hemoglobinopathies screening prior to genetic counseling, or medical treatment designed to ameliorate a pathological condition.

In this study, a magnetic field is coupled toanelectrophoretic power supply in order to enhance the resolution of separated globin chains over conventional electrophoresis. The diamagnetic susceptibility of hemolysates is utilized to oppose the mobilities due to the charged globin chains, when an applied voltage is on. Normal hemolysate has been investigated, and distinct band separation of HbA and HbA_2 was observed. The resolution was definitely superior to conventional electrophoretic method. The technique is easy to perform and provides time optimization.

It is envisaged that coupled magneto-electrophoresis, though a novel idea, may be useful in the establishment of loci for the various hemoglobinopathies. Because the variants have differing net charges, with the iron in a divalent state, a - repulsion or enhancement will result when placed in a magneto-electrophoretic field. The direction may be valuable to the diagnostic clinician.

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LIST OF ABBREVIATIONS

ABBREVIATIONS	DEFINITIONS
°C	Degree Centigrade
dL	Deciliter
2,3 - DPG	2,3 – Diphosphoglycerate
DEAE – cellulose	Diethylaminoethyl Cellulose
Fe^{2+}	Iron (II)
g	Gram
Hb	Hemoglobin
HbCO	Carboxyhemoglobin
HPLC	High Performance Liquid Chromatography
mmHg	Milliliter of Mercury
mL	Milliliter
O_2	Oxygen
o/o	Percent
p^H	Hydrogen Ion Concentration Index
p^I	Point of Electrical Neutrality
$p^{O}2$	Oxygen Tension
$p^{CO}2$	Carbon Dioxide Tension
μ	Micro
v	Volume
V	Volt

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CHAPTER I

INTRODUCTION

Electrophoresis is a useful tool that a clinician, or investigator could employ to diagnose pathological states or in vitro life processes in the laboratory. Electrophoresis is basically the separation of macromolecules under the influence of an electric field. It is the classical method in use today in the laboratory for the analyses and separations of proteins, lipoprotein, hemoglobin, and other biologic polymers since most biologic polymers are electrically charged. Moreover, it has been accepted as the reference method in all diagnostic procedures of charged species furnishing medical investigators with knowledge which would have otherwise been difficult to achieve. The most widely used method is zone electrophoresis. A spot or thin layer of the sample is applied on an electrically conducting medium; an electric field is applied and molecules migrate on or through the supporting medium. The rate of migration of a molecule in an electric field depends on the support, the magnitude of the net charge on the molecule, and the size and shape of the molecules. Other factors that affect electrophoresis include the following: degree of dissociation, extent of ionization, pH, voltage applied, distance between electrodes, viscosity of supporting medium, concentration and ionic strength of electrolyte, temperature, solubility of sample, and affinity between separating material and the supporting medium.

The work of Tiselius (1920) in his famous U-tube experiment, revolutionized the technique conceived by the Russian scientist Reuss (1809), who performed a study of the kinetic behavior of colloids. Tiselius used sliding baffles to establish sharp boundaries between sample and buffers, since Sample - buffer mixing was an inherent defect. Due to the absence of a solid matrix, his technique was termed free - solution electrophoresis.[1] Here the rate of mobility was found to be dependent on the charge to mass ratio. For this endeavor, he was awarded a Nobel prize in 1948. An illustration of Tiselius' arrangement is shown in Figure 1.

The numerous difficulties associated with the free-solution electrophoresis are alleviated or eliminated when separations are carried out in a stabilizing medium such as paper, a layer of finely granulated solid, or a column packed with suitable solid. Hence electrophoresis in a stabilizing medium has been tagged electrochromatography and this has been employed in various applications in the diagnostic laboratory and biochemistry. Electrochromatography facilitates the separation of proteins and other macromolecules contained in serum, urine, spinal fluid, gastric juices, and other body fluids. Electrochromatography has been employed by biochemists to fractionate alkaloids, antibiotics, nucleic acids, vitamins, natural pigments, steroids, amino acids, carbohydrates, and other organic acids.

Since the property of the medium is pertinent to the separation process, results have been primarily due to a combination of the electrophoretic effect and absorption, ion exchange, or other distribution equilibria. Numerous solid media have been

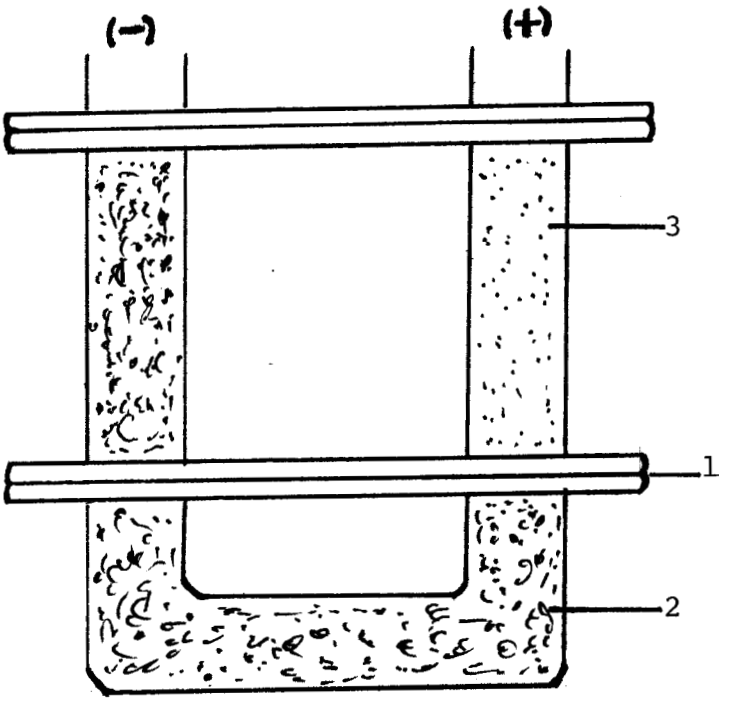

Figure 1. Tiselius[1] **electrophoresis arrangement. (1), sliding baffle to facilitate unique loading of sample and buffer; (2), sample and buffer; (3), buffer only.**

employed and these include: paper, cellulose acetate membranes, cellulose powders, starch gels, ion exchange resins, glass powders, agar gels, and polyacrylamide gels. The most widely used support medium is cellulose acetate; this is a plastic material which can be made into thin strips. The strips contain pores and tracks through which ions can migrate by capillary action. A typical cellulose acetate electrophoresis set up is illustrated in Figure 2.

Kohn introduced cellulose acetate in 1958. The acetate support medium has the advantage of rapid analysis using small sample volumes, great tensile strength when wet, and a pure and relatively uniform structure. Further, sample absorption is almost absent, and it possesses low affinity for dyes, A crystal clear acetate strip facilitates quantitation.[2,3]

Agar Gel Electrophoresis

Agarose gel electrophoresis has proven successful in separations involving aqeous buffers supported within a polymerized gel matrix. Agar gel electrophoresis has the distinct advantage in that it can contain larger samples than cellulose acetate or celiulose nitrate systems; hence, this process can be employed in preparative scale electrophoresis of macromolecules. The property of the gel matrix can be freely manipulated since gels enhance the friction as well as molecular sieving action. Viscosity and pore size have a strong influence on electrophoretic mobility.[2]

Agarose itself is a polygalactose polymer which has proven successful in its applicability to separating large macromole-

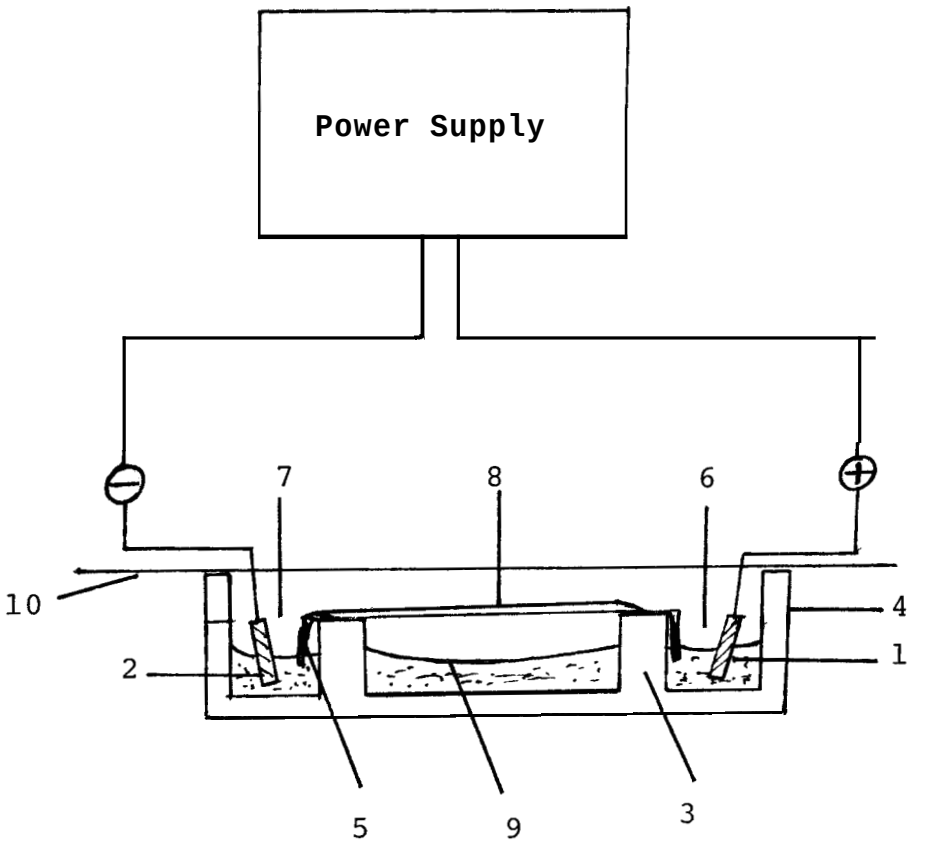

Figure 2. Cellulose acetate electrophoresis. Buffer is in the anode and cathode compartments. The anode (1) and cathode (2) are platinum wires positioned in the proper compartments (6) and (7); (3), strip support; (4), plastic tank; (5), wick; (8), cellulose acetate strip; (9), ice/buffer solution. and (10), is a plastic covering which minimizes evaporation.

cules such as nucleic acids, lipoproteins, lactate dehydrogenases, and bacteriophages.

Agar gel electrophoresis has gained prominence in most clinical laboratories in routine diagnosis of macromolecules. Its ease of management coupled with versatility has increased its acceptability as the gel medium of choice and could possibly displace cellulose acetate.

Structure of Hemoglobin

Hemoglobin is a globular protein mass with a central cavity and having a molecular weight of 67,000 daltons. The Hb molecule is radially segmented into four tetrahedrals, each segment consisting of loops of globin chains and each projects to the exterior surface of the sphere an ion-porphrin complex (heme) which is essentially the site of oxygen binding.

The spheroidal Hb tetramer consists of two groups of identical globin chains and each chain is associated with a heme molecule. In adult hemoglobin (HbA), these chains are commonly designated as alpha and beta. The two alpha and two beta chains are arranged as in Figure 3 and Figure 4. Each adjacent alpha and-beta chain interact via narrow strips between them ($alpha_1$ $beta_2$ contact points and two broad areas of contact, $alpha_1$ $beta_1$). During periods of oxidative interconversion from oxygenated Hb to the deoxygenated state, there is a rotation of the $alpha_1$ $beta_1$ contact point such that the central cavity enlarges enough to accomodate a molecule of 2,-3 - diphosphoglycerate (DPG), a product of glucose metabolism. Conversely, oxygenation causes a rotation of the two segments

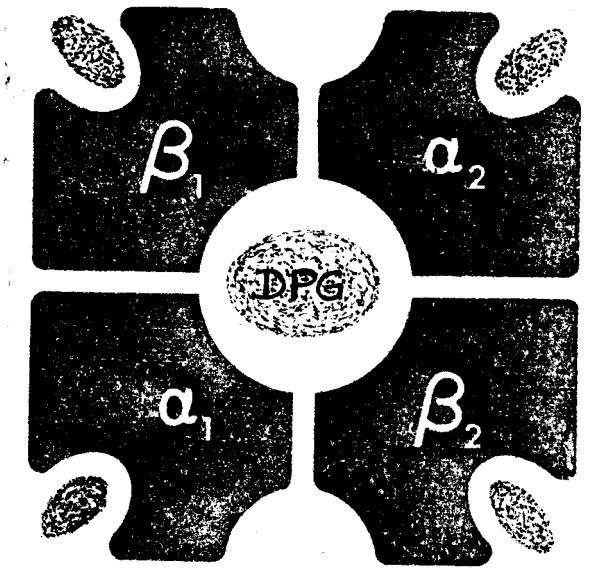

Figure 3. Schematic tetrameric composition of hemoglobin showing two pairs of identical globin chains.[5] For HbA, there are two alpha and two beta chains. Each globin chain has an attached heme group (light gray oval). 2,3-DPG, a product of glucose metabolism, occupies the central cavity in the deoxygenated state.

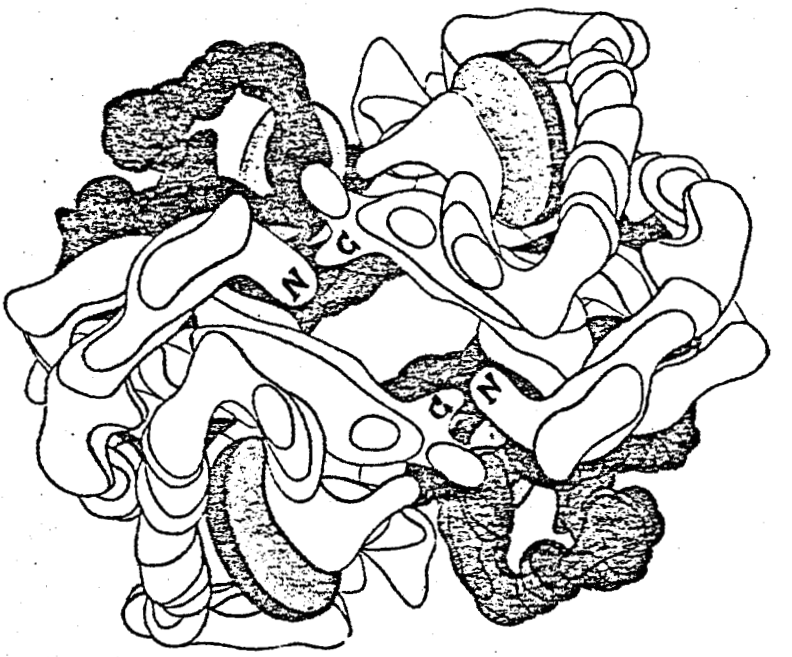

Figure 4. Three dimensional structure of Hb molecule based on x-ray crystallography.[5] The alpha chains (light gray) overlie the beta chains (dark gray). The central cavity (site of DPG binding) is visible. The disc-like objects are heme groups, one in each globin chain. N and C represent the amino and carboxyl terminals of the alpha chains.

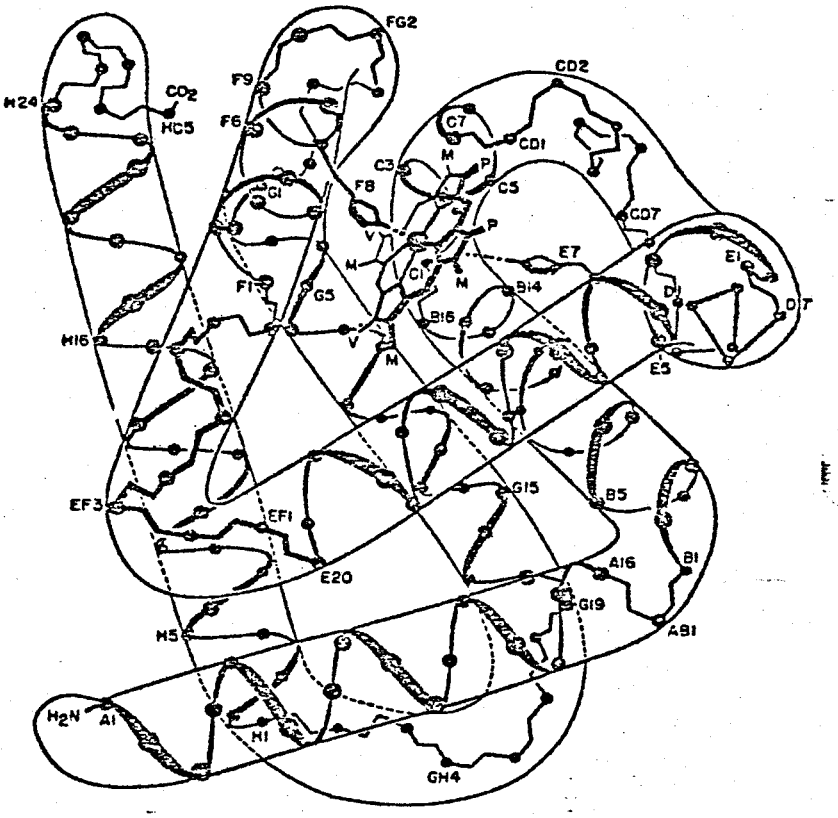

Figure 5. The basic unit of hemoglobin is a long chain of amino acids so looped to provide a cleft that is occupied by the heme group.[5] The globin chain consists of a helical arrangement (A to H) linked by short non-helical chains of amino acids. For the beta chain, A2 is the site of substitution for Hbs S and C; F8 is the proximal histidine position, which interacts with the iron in the heme group.

of Hb molecule resulting in a diminution of the cavity and consequent loss of the DPG.

In addition to $alpa_2$ $beta_2$, normal adult Hb also contains HbA_2, designated as $alpha_2$ $delta_2$, composed of two alpha and two delta chains. This is a minor component of normal red blood cells.[5] There is a fraction in normal red blood cells known as HbA_3 whose proportion is accentuated with aging of the Hb. This component is thought to be linked to glutahione molecule at the beta chain. It has the formula $alpha_2$ $beta_2$. In addition to Hbs-A_1; A_2, and A_3, adult Hb contains other minor components which include one having the formula $alpha_2$ $betabeta^x$ in which $beta^X$ chain has an NH_2 terminal group attached to a hexose moiety. This type of Hb, the glycosylated hemoglobin, is of significance in diabetic patients and the monitoring of the HbA_{1c} fraction has proven to be a valuable diagnostic tool.

A major hemoglobin component of intrauterine life is fetal hemoglobin, HbF, and trace amounts persist in adult life. HbF consists of two alpha and two gamma chains ($alpha_2$ $gamma_2$) and is made up of F_1 and F_2. While F_1 is associated with two gamma chains, F_2 apparently has no gamma chains since the N-terminal end is blocked.

Hemoglobin Gower II is an embryonic type and has no counterpart in adult life. Each globin chain is a long strand of amino acids, 141 in each alpha chain and 146 in each beta, delta, or gamma chain. Each chain contains helical segments, seven in an alpha chain and eight in a beta chain, delineated by short non-coiled segments. The homology of the beta, gamma,

and delta is unique, with the beta and delta chains differing by only 10 amino acids.

Each globin chain is looped about itself so as to form a pocket which embodies the heme group, and this pocket is surrounded by essentially hydrophobic amino acids. The heme moiety is suspended within this cleft by a non-covalent linkage of the iron atom to the imidazole group of the proximal histidine (position 92 of the beta chain or position 87 of the alpha chain). The distal histidine has its imidazole group at position 63 of the beta chain or position 58 of the alpha chain which is continuous with the heme iron, but it is able to swing in and out of this position to permit the binding and release of O_2. Whether Hb is oxygenated or deoxygenated, the four iron atoms of the tetrameric hemoglobin molecule exist in a divalent state. The structure of a single heme group may be represented as a square planar complex with the four nitrogens of the polyphyrin rings at the angles as illustrated in Figure 4.

The central iron atom is in a hexavalant coordination state analogous to the inorganic iron complex, ferrocyanide. Of the two remaining coordination bonds, one is closely associated with an imidazole residue from the specific globin chain to which the heme group is attached. The remaining bond is available for the reversible uptake of oxygen. No ligand is known to occupy this latter site in deoxyhemoglobin.[6]

Function of Hemoglobin

Hemoglobin is uniquely adapted for the reversible uptake of oxygen within a critical O_2 tension. The functional feature

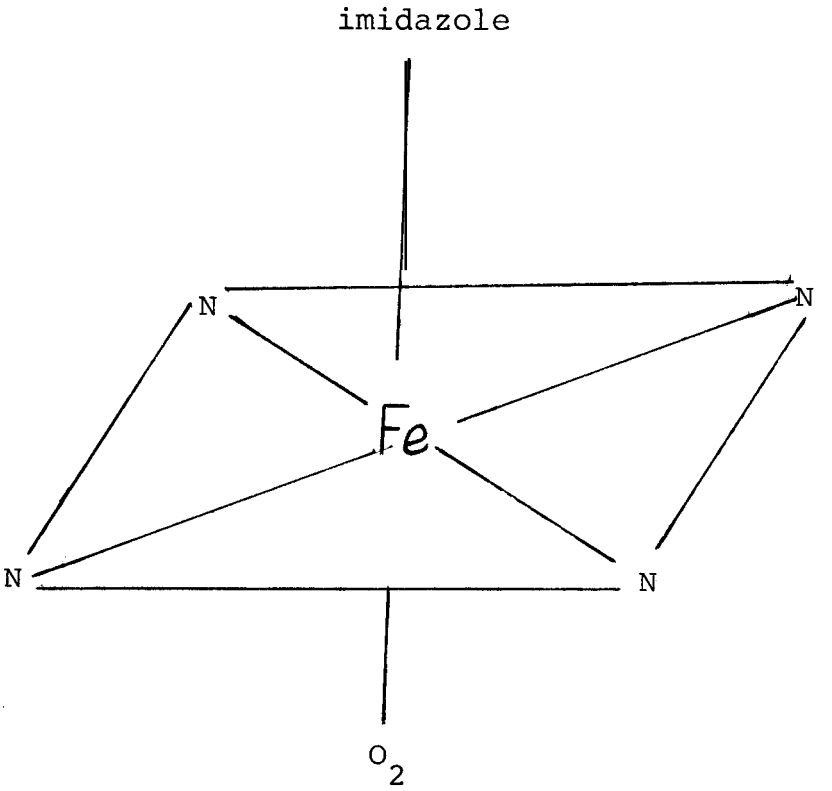

Figure 6. Idealized representation of heme molecule.[6]

is a chain-to-chain interaction whereby an alteration in spatial arrangement of one molecule to another facilitates the uptake or release of 0_2. The dissociation curve for normal blood hemoglobin is sigmoidal. This is a result of interactions between the four subunits comprising a hemoglobin molecule. These interactions are termed cooperativity.

Dissociation of 0_2 from hemoglobin (deoxygenation) occurs in four distinct steps, each having a dissociation constant because of cooperativity changes that accompany each succes-sice release of 0_2.

$$Hb_4 0_8 \longrightarrow Hb_4 0_6 + 0_2 \quad K_1 \qquad (1)$$

$$Hb_4 0_6 \longrightarrow Hb_4 0_4 + 0_2 \quad K_2 \qquad (2)$$

$$Hb_4 0_4 \longrightarrow Hb_4 0_2 + 0_2 \quad K_3 \qquad (3)$$

$$Hb_4 0_2 \longrightarrow Hb_4 + 0_2 \quad K_4 \qquad (4)$$

Values for each K are not established yet. K values are defined as equilibrium constants by

$$K_1 = \frac{(Hb_4 0_6)(0_2)}{(Hb_4 0_8)} \quad \text{units in moles} \qquad (5)$$

The association constant is the reciprocal of K. The smaller the dissociation constant, the more tightly bound is the oxygen and the more stable is the hemoglobin-oxygen complex.[6] The converse is true for the association constant.

In the saturated hemoglobin molecule all of the oxygen are essentially equivalent; a decrease in the ambient oxygen tension may cause any of them to be released first. The release of the fourth 0_2 does not occur at physiologic conditions.

The above sequence of events accounts for the sigmoidal shape of the normal oxygen dissociation curve as illustrated in Figure 7. The properties of the Hb molecule facilitate the loading and unloading of large quantities of oxygen over a physiologically critical range of pO_2. The total oxygen content of normal blood is about 20 mL/100 mL (v/v): the release of 5 mL/100 mL is equivalent to releasing one oxygen molecule from a hemoglobin tetramer which culminates from a pO_2 of about 60 mmHg (point a to V). The release of a second O_2 requires a further decrease of 15 mmHg in the pO_2 from 40 down to 25 mmHg. The release of the third oxygen molecule can then be effected by a decrease in the pO_2 of only 10 mmHg.

Once the hemoglobin molecule has taken up a molecule of O_2 at the heme site, and given up 2,3-diphosphoglycerate, the remaining three sites are oxygenated with a slight increment in pO_2. Conversely, a molecule with all four heme sites oxygenated produces oxygen availability at three other sites. This coordination of oxygen "tension" changes is dependent upon (1) the reversible binding of certain amino acids across the $alpha_1beta_2$ contact point, allowing the alpha beta dimers to flex back and forth as the Hb molecule is oxygenated and deoxygenated, and (2) the movement of DPG in and out of the central cavity-- DPG stabilizes the deoxygenated configuration.

In vivo DPG concentration markedly affects the O_2 dissociation curve of Hb as illustrated in Figure 7. As the concentration of 2,3-DPG increases, the dissociation curve shifts to the right manifesting diminished oxygen affinity for hemoglobin and elevated O_2 delivery to the tissues. On the contrary,

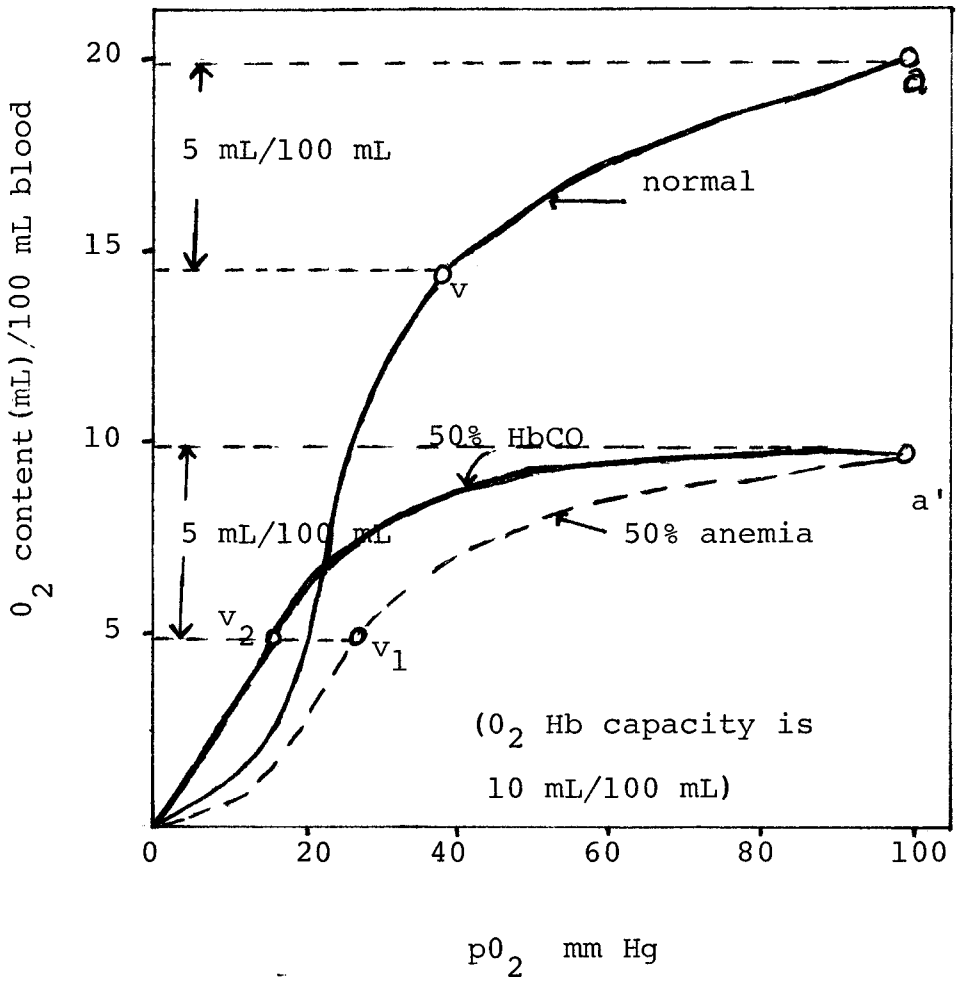

Figure 7. Normal oxyhemoglobin dissociation curve and curve for the case of a 50% anemia.[6] The delivery of 25% of the total oxygen content of fully oxygenated arterial blood (5 mL/100 mL blood requires a drop in pO_2 of about 60 mmHg, from a to v).

a low DPG concentration results in shifting the curve to the left. This indicates an elevated 0_2 affinity for hemoglobin, with a corresponding decrease in delivery to the tissues. This latter situation is problematic in blood banking, wherein blood preserved in acid-citrate dextrose solution shows progressive diminution in DPG concentration.

Changes in blood pH and $p0_2$ influence oxygen affinity for hemoglobin. For a decrease in blood pH or an increase in $pC0_2$, as in acidosis, there is a corresponding shift of the dissociation curve to the right. This phenomenon is a case of the classical acid Bohr effect. Conversely, a decrease in $pC0_2$, or an increase in blood pH, may be due to alkalosis. The resultant shifting of the curve to the left is termed the alkaline Bohr effect.

Clinical Significance of Hemoglobinopathies

Synthesis of hemoglobin is under genetic control and numerous hemoglobin variants, many capable of producing severe hemolytic anemias and even fatality, have been detected. Early detection of certain hemoglobinopathies have proven useful in the avoiding of serious medical problems. Counseling of individuals or marital partners to determine the probability of having offspring with abnormal hemoglobins has resulted in broad-based screening programs.

All inherited abnormal hemoglobinopathies fall into one of three categories: (a) inherited abnormalities of the structure of one or more of the globin chains (b) inherited abnormalities relating to the frequency of synthesis of one or more

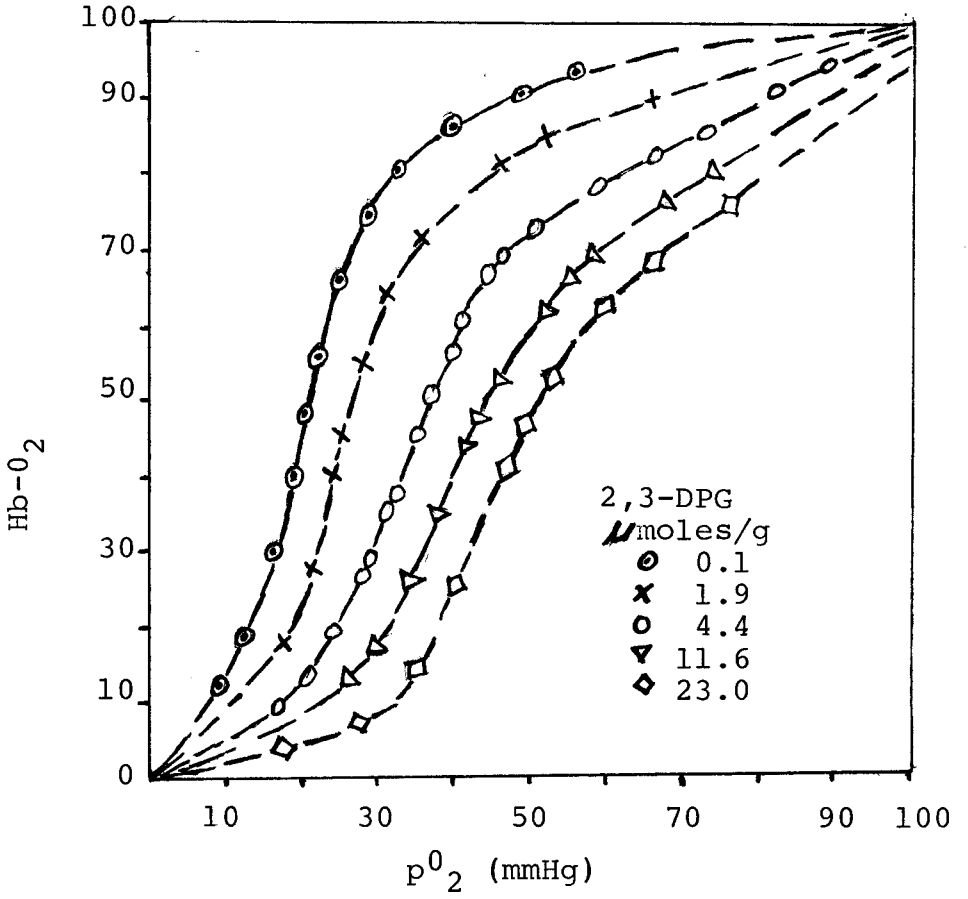

Figure 8. The normal oxygen dissociation curve is sigmoidal. Changes in 2,3-DPG concentration markedly influence the position of the curve to the right, and the converse is true. The action of 2,3-DPG is a pertinent mechanism for controlling the rate of oxygen delivery to the tissues.

of the globin chains, or (c) absence of the usual switch from (F) to adult (A) hemoglobin synthesis.

The first type is a result of the substitution of one or more amino acids in a globin chain. A good example is the difference between hemoglobin S and hemoglobin A, where valine is substituted for glutamic acid in the sixth position. Numerous other hemoglobinopathies belong to this class.

The thalassemias fall into the second group and are the result of a genetic defect in the production rate of adult hemoglobin.[3] This reduction in the amount of hemoglobin A_1 is due to a variety of alternations in the hemoglobin patterns which have shown the genetic significance of the thalassemias.

Some thalassemias interact with beta-chain hemoglobin variants such as HbS, and are also associated with increased amounts of hemoglobin F and A_2 due to the effective beta-chain production; these are designated as beta-thalassemia major. The heterozygous carrier states are termed beta-thalassemia minor. Beta-thalassemia major is usually severe. The Hb pattern for this homozygous thalassemia is indicative of a variable elevation in HbF with low, normal, or elevated level of hemoglobin A_2. Hemoglobin F level may vary from 40 to 90 percent.

Clinical distinction of the heterozygous beta-thalassemia minor varies from moderately severe anemia to normal findings. The severe forms are rare and individuals having them are of mediterranean nations. The sub-clinical forms of beta-thalassemia minor are known to exist in many individuals.

Hemoglobin F is slightly elevated from 2 to 6 percent in 50% of the cases, whereas HbA_2 is elevated 3.5 to 7 percent. The balance is due to HbA_1. The beta-thalassemias are characterized by persistent fetal hemoglobin production, elevation of hemoglobin A_2 levels, and interaction with beta-chain structural hemoglobin variants.

In some patients with a thalassemia blood semblance there is no elevation of hemoglobins A_2 and F levels; however, there are small amounts of hemoglobins H and Bart's in the cells. Hemoglobin H is a tetramer of normal beta chains (B_4), while hemoglobin Bart's is a tetramer of gamma chains (γ_4). Evidence suggests that hemoglobins H and Bart's are probably caused by defective alpha-chain synthesis, and this class of thalassemia does not interact as in B-thalassemia. These interact with alpha-chain variants and are referred to as alpha-thalassemias. Delta thalassemias and beta-delta thalassemias have also been documented.[5]

Interpretation of Hb Electrophoresis

Hemoglobin electrophoresis is indispensible for the detec- of hemoglobinopathies. Hemoglobins are genetically controlled and the presence of abnormal hemoglobins is often associated with functional, physical and morphologic abnormalities in the erythrocyte as well as pathological manifestation such as hemolytic anemia.

The normal pattern is characterized by an intense band of HbA_1 and if the concentration of hemolysate is high enough, a weak HbA_2 band is demonstrated. HbA_1 may have a blurred faint

band of HbA_3 moving in front of it.

Normal values are:

HbA_1 - HbA_3	96.0-98.5%
HbA_2	1.5-4.0%

Sickle Cell Trait

Sickle cell trait is a heterozygous state with a distinct electrophoretic pattern of HbA_1SA_2. Hemoglobin values are HbA, 55-65%, HbS 35-45%, Hb F is less than 2%, Hb A_2 is normal.

This trait confers protection from falciparum malaria and is present in about 10 percent of all blacks in the United States. Blood smears are usually normal, but supravital preparations of fresh blood reveal sickling.

Sickle Cell Anemia

Sickle cell anemia is a homozygous state. The hemolysate consists of mostly HbS, about 95 percent, with variable amounts of HbF--usually less than 15 percent in the adult. No HbA is observed. On electrophoresis at alkaline pH, HbS migrates to the same position--HbS can be distinguished from other variants by citrate agar electrophoresis at pH 6.0-6.5. It can as well be distinguished from HbD and HbG by a positive sickle cell test (Sickledex).

The major clinical manifestations include chronic hemolytic anemia, impairment of growth and development with increased susceptibility to infection, vasoocclusive "crisis" and organ damage due to vasoocclusive events, leg ulcers, and renal dysfunction. This is the most common abnormal hemoglobin.

Testing for Sickle Cell Disease

Sickle cell is usually tested by two methods in the laboratory. The first method, the oxygen deprivation method, demonstrates the presence of HbS in erythrocytes. In this technique, a cover slip is used to cover a drop of blood, and the frequency with which the cells sickle is observed.

The other test is the solubility test for sickle cell screening. Here whole blood is placed in a hypertonic phosphate buffer solution to release the sickle Hb from the red cells when saponin is added. The solution turns cloudy due to the formation of insoluble crystals which are unique to HbS as well as HbC_{Harlem}, $C_{Zingurchor}$, and S_{Travis}. Hemoglobins A, C, D, F, G, I, J, and O_{Arab} yield negative results. Consequently, electrophoresis is a better technique for screening.

Sickle Cell Disease

Sickle cell disease is a heterozygous state with approximately equal amounts of HbS and HbC. This form of sickle cell anemia is the most common genetic variant though clinically milder than sickle cell anemia. It has fewer complications when it occurs in childhood. Sometimes adults are asymptomatic only to be diagnosed when a routine smear exposes target cells, rare irreversibly sickled cells, and a relative measure of reticulo cytosis (3 to 10 percent).

HbC Disease

HbC disease is a homozygous state; the hemolysate contains

more HbC and variable amounts of HbF. HbA_1 is lacking.

On electrophoresis at alkaline pH, HbC migrates slowly to the same position as HbA_2, HbE and HbO. It can be separated from HbA_2 by column chromatography, and from other Hb variants by acid pH electrophoresis on citrate agar.

The homozygous state produces hemolytic anemia that is mild to moderate, with hemoglobin levels from 8 to 12 gram/100 mL. The majority of red cells on peripheral blood smear are target cells. HbC cells form intracellular crystals when suspended in a hypertonic solution.

Sickle Cell HbD_{punjab} Disease

HbD_{punjab} hemoglobinopathy causes a severe hemolytic anemia, though it is milder than HbSS disease. It is inherited as a double abnormal homozygous trait, one for the sickle hemoglobin trait and the other, a gene for HbD_{punjab}. It can be distinguished from HbS by electrophoresis on citrate agar at acid pH. HbD_{punjab} is suspected in heterozygotes when a negative sickle test occurs, yet an Hb component is evident at the S position.

HbE Disease

HbE disease is due to the homozygous inheritance of the gene for HbE. HbE is the third most prevalent abnormal hemoglobin in the world and occurs primarily amongst orientals. HbEE is associated with microcytosis, target cell formation, and a mild hemolytic anemia.

At acid pH in agar electrophoresis, HbE migrates with

HbA and not with HbO or HbC. Whole blood 0_2 dissociation curves demonstrate a slight shift to the right.

The heterozygous state, HbAE or HbE trait, is commonly encountered and has not shown any hematologic abnormality.

Thalassemia Minor

The hemolysate consists of HbA_1 with HbA_2 elevated above 4 percent, often with a slight increase in HbF. The erythrocytes exhibit hypochromia and microcytosis in confirmatory hematologic diagnosis.

HbS-Beta Thalassemia

The typical pattern is that of HbA_1. Clinical and hematologic abnormalities are mild.

HbC-Beta Thalassemia

The hemolysate contains more HbC than HbA_1 with variable amounts of HbF. Typical values are: HbC 65-95 percent, HbA about 20 percent. HbA_2 is completely lacking. This pattern is identical to HbC diseases.

HbC-beta thalassemia is a hematologic disorder with little clinical evidence of disease. The mean corpuscular hemoglobin is reduced, with hypochromic target cells, fragmented red cells, and microspherocytes seen on peripheral blood smears.

Thalassemia Major

The clinical diagnosis is uniquely that of HbF electrophoretic pattern as high as 98 percent with variable amounts

of HbA_1, or HbA_1 may be completely absent. The HbA_2 value is usually normal.

Alpha Thalassemia

The clinical condition is characterized by marked microcytosis and hypochromia of the red cell associated with mild anemia and erythrocytosis; HbA_2 and HbF levels are normal or low. Iron deficiency must be ruled out in alpha thalassemia.

A variant of homozygous beta thalassemia is thalassemia intermedia, a less severe impairment in beta-globin synthesis, with fewer alpha-chains inclusion. Individuals with this disorder maintain a hemoglobin level of 6-10 g/dL. This condition ranges from a very mild disease to a more severe disease state.

The electrophoresis pattern manifests hemoglobin values of 20 to 100 percent HbF, up to 7 percent HbA_2 and 0 to 80 percent HbA, depending on the genotype of the patient. Iron overload is a complication because of the highly accelerated but ineffective erythropoiesis. There is an increase in plasma iron turnover and increased gastrointestinal iron absorption.

Unstable Hemoglobin Disease

Unstable hemoglobin disease is characterized by the presence of unstable hemoglobins. The latter are structural variants of HbA; these undergo denaturation within the red cell which results in irreversible precipitation and the formation of insoluble inclusion (Heinz bodies). Anemias due to unstable hemoglobins are designated as unstablehemoglobin hemolytic anemias.

Unstable hemoglobins are inherited as an autosomal dominant trait. Of the 70 known Hb mutants that are associated with unstable hemoglobin hemolytic anemia, most are of beta-chain origin; only 8 alpha-chain mutants are known. Many of the substitutions associated with this condition are neutral, and their electrophoretic migration are indicative of HbA.

The Heinz bodies are selectively removed in the spleen. Because of the low gene frequency, a homozygous state is infrequently encountered in life. However, Heinz body anemia is variable, it may be quite mild or moderately severe depending on the variant. Most variants exhibit some degree of variability in oxygen binding affinity; however, inclinations to normal binding exists. Variants exhibiting decreased oxygen affinity tend to manifest lower hemoglobin levels.

Hemoglobinopathies with Abnormal Oxygen

Hemoglobinopathies with abnormaloxygen prevail as an erythrocytosis in patients with elevated 0_2 affinity and a reduction in 0_2 unloading, and as an anemia in patients with decreased oxygen affinity (Hb Kansas). However, clinical manifestations are minor; cyanosis might occur in variants with decreased oxygen affinity.[3,15]

Some clinically important hemoglobinopathies are illustrated in Table 1.

TABLE 1. SOME CLINICALLY IMPORTANT HEMOGLOBINOPATHIES

Clinical Manifestation	Hemoglobin Designation	Substitution	Comments
Hemolytic anemia	S	beta^{6} glu→val	Sickling
	C	beta^{6} glu→lys	Target cells
	$E_{}$	beta^{26} glu→lys	Target cells
	O_{Arab}	beta^{121} glu→lys	Target cells
	Köln	beta^{98} val→met	Unstable Hb
	Zurich	beta^{63} his→arg	Unstable Hb
	H	$beta_{4}$	Unstable Hb
Cyanosis	M_{Iwate} (Kankakee)	alpha^{87} his→tyr	This group of rare abnormal hemoglobins is characterized by abnormal absorption spectra.
	M_{Boston} (Osaka)	alpha^{58} his→tyr	
	$M_{Saskatoon}$ (Chicago)	beta^{63} his→tyr	
	$M_{Milwaukee\ 1}$	beta^{67} his→tyr	
	$M_{Hyde\ Park}$	beta^{92} his→tyr	
	Kansas	beta^{102} asn→thr	Rightward displacement of O_2 dissociation curve.

Clinical Manifestation	Hemoglobin Designation	Substitution	Comments
Erythrocytosis	Chesapeake	alpha^{92}arg→leu	This group of abnormal globins is characterized by marked leftward displacement of the O_2 dissociation curve.
	$J_{Capetown}$	alpha^{92}arg→glu	
	Malmo	beta^{97}his-+gin	
	Ypsilanti	beta^{99}asp→tyr	
	Kempsey	beta^{99}asp→asn	
	Yakima	beta^{99}asp→his	
	Rainier	beta^{145}tyr→cys	
	Bethesda	beta^{145}tyr→his	

Statement of Problem

The study of hemoglobin variants and hemoglobinopathies have been employed in clinical diagnostic settings mainly by electrophoretic techniques. Electrophoresis as defined, is the separation of charged macromolecules by means of an applied potential. The technique of electrophoresis has been successfully utilized to fragment biologic macromolecules such as serum proteins, enzymes, and hemoglobin. The prime goals in any electrophoresis procedure is the detection and quantitation of the migrated fractions. In the normal protein or hemoglobin electrophoresis, a stain, usually Ponceau S is used to enhance the visualization of the resolved fragments. The intense red bands produced correspond to the separated components, and these can be quantitated by means of a densitometer. However, the detection is resolution limited. A distinct resolution of the separated bands will obviously yield a more accurate detection result.[5]

A macromolecule such as hemoglobin is associated with numerous variant forms. Each is genetically controlled as well as connected with functional, physical, and morphologic abnormalities in the erythrocyte, resulting in pathological manifestations. Each variant form has a distinct electrophoretic mobility, but numerous diagnostically significant variants have similar electrophoretic mobilities. The various mobilities are mainly due to the quantity of the charge/mass of each at a particular pH.

In the identification of hemoglobinopathies, two basic electrophoretic techniques are usually employed. Electrophoretic

separation of hemoglobins on cellulose acetate at pH 8.6 identifies all pathological variants including hemoglobin S. There is increased mobility towards the anode as illustrated in Figure 9 below. Poor resolution is a problem in separating for HbF, HbA, HbD, HbG, HbL, and HbS which all have about the same mobility.

When agar gel is used to separate hemoglobins under typical electrophoreticconditions HbA and HbF are better resolved, but the cluster of variants A, A_2, E, G and D have the same mobility. However, the concentration of Hb applied to the gel is directly proportional to the mobility. Large concentrations of HbA will result in increased electrophoretic mobility. Care is therefore cardinal in adjusting the Hb concentration of samples. Equal concentration is needed for better interpretation.

The quest for a better resolution technique has been difficult. It is proposed that a simpler, more precise procedure that will combine modern electrophoresis with the magnetic property of hemoglobin should be investigated. The advantage of this technique is believed to be the use of an applied magnetic field to fix the hemoglobin molecule while the applied electric field moves the charged particles in an anodic direction. Unlike a conventional electrophoresis, a better resolution could be achieved in a relatively shorter time. The apparent advantage in this technique is that the need for buffer change is eliminated. Only one buffer type is required, and time optimization will be achieved if the technique proves successful.

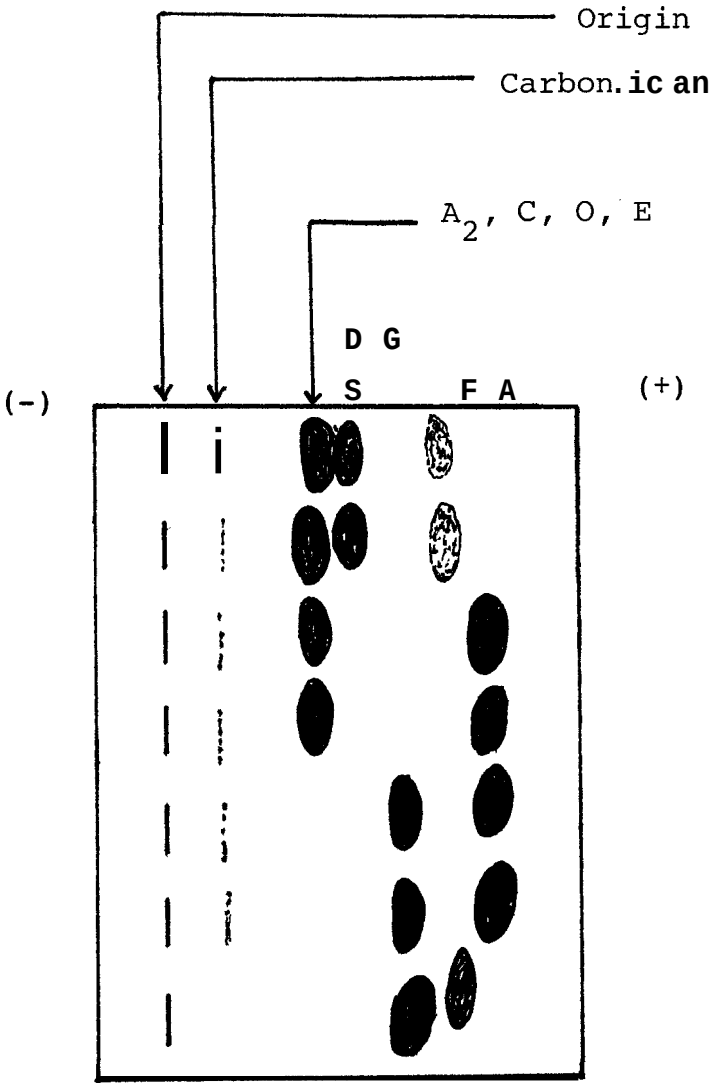

Figure 9. Hemoglobin electrophoresis on cellulose acetate at pH 8.6, showing the relative positions of various hemoglobins.[5] Hemoglobin F is poorly resolved.

Background for the Magneto-electrophoresis System

A magnetic effect study based on the Gouy technique was carried out using a micro-electrophoretic cell. The micro-cell consists of rectangular plates of plexiglass which have been glued together after wells have been milled into the upper half.

A study of the effect of an applied magnetic field alone on a sample of hemolyzed red blood cell containing hemoglobin showed that the attraction of the magnetic field was ineffective in causing the sample to migrate. The illustration of the effect is shown in Figure 10a. Supre Heme buffer was used in this study.

When the same study was performed on a 2 mL blood sample which had been treated with 5 grams of sodium thiosulfate, there was significant movement from the point of application - as illustrated in Figure 10b. Capillary action of the buffer solution had no significant effect on the mobility of the blood sample.

Theoretical Basis for a Coupled Magneto-electrophoresis Enhancement Technique

In this study the induced magnetization and the susceptibility of the substance to magnetization are utilized to attempt to enhance the resolution of separated macromolecules.

Studies have shown that a hemoglobin molecule solution that has been reduced to ferrohemoglobin by means of sodium thiosulfate is paramagnetic, whereas oxyhemoglobin is diamagnetic.[20] These findings are all based on the Gouy technique

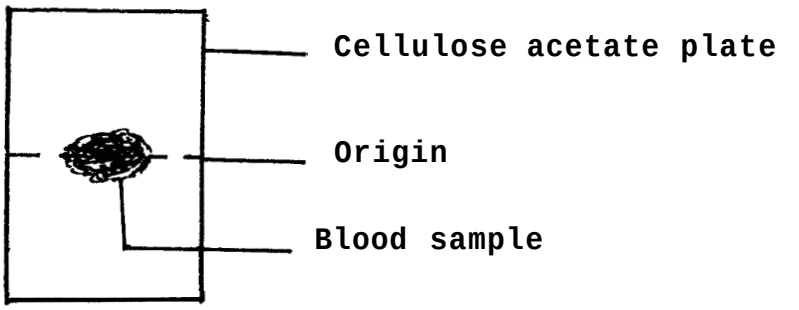

Figure 10a. Effect of applied magnetic field from a 14.7 kilogauss NMR magnet for a two hour duration.

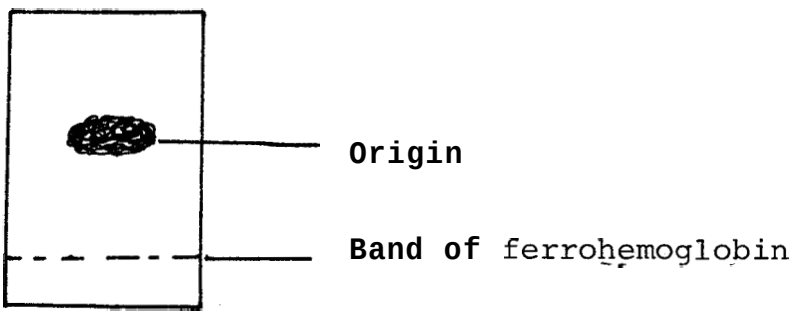

Figure 10b. The effect of the same magnetic field on a sample of ferrohemoglobin in an electrophoretic cell.

which is relied on in this study. Under the influence of a magnetic field, a diamagnetic molecule will have its bonding electrons in a plane perpendicular to the magnetic field. The result of this motion is the development of a secondary field which opposes the primary field. The resultant is smaller since the nucleus has been shielded from the applied field. Illustration of this is in Figure 11. The intensity of magnetization induced thus is less than that produced in vacuum. A paramagnetic substance such as ferrohemoglobin produces the opposite effect.

The result is a repulsion between the substance and the applied field. The susceptibility is therefore negative according to the equation

$$M = -X_m H \tag{6}$$

where M is the magnetization, H is the magnetic field strength, and X_m is the magnetic susceptibility.

Paramagnetic intensity due to the induced magnetization is greater in the substance than that of the applied field in vacuum. Attraction of the material towards the applied field is characteristic of paramagnetics, and the magnetic susceptibility X_m is always positive. Though all paramagnetic compounds have a built-in diamagnetism as a universal property, the magnitude of paramagnetism usually overwhelms the feeble, opposing diamagnetism.

Paramagnetic susceptibility is independent of the applied field but is usually inversely related to temperature according to the equation

$$X_m = Nu_o \quad (\mathcal{E} + u_m^2/3KT) \tag{7}$$

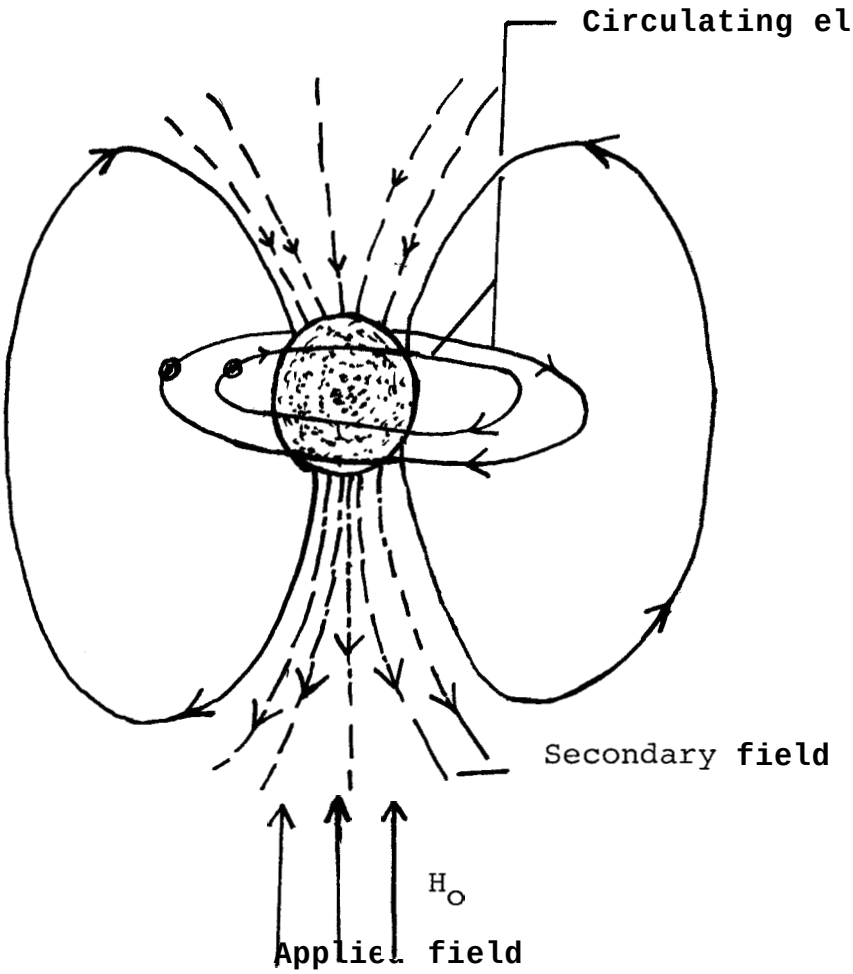

Figure 11. Diamagnetic shielding of a nucleus.[2]

where u_m is the magnetic dipole moment, $\mathcal{E}$ is the molecular magnetizability. From (7) above magnetism has a contribution proportional to the term $u_m^2/3KT$. Magnetic susceptibility is thus related to the magnetizability, the dipole moment, and temperature.[21] To reiterate, this is usually determined by the Gouy balance.

It is apparent that a permanent magnetic moment of a macromolecule that is paramagnetic can be employed to position the induced moments at a more or less stationary locus. An applied potential from an electrophoretic power source can be used to separate different hemoglobin types in an anodic direction. The differing intensities of magnetic susceptibilities would generate well defined rates of mobilities unique to the variants. In essence, the induced magnetization will retard the migration of mutant Hb, which could result in a better resolution of these variants.

CHAPTER II

LITERATURE REVIEW

Methods

Hemoglobin variants have been detected, studied and evaluated by several techniques in the literature, but the technique of diagnostic important in the laboratory is electrophoresis. It is the technique of choice in most hemoglobinopathy screening programs. Currently the novel technique of isoelectrofocusing has been employed. These valuable techniques are discussed below.

Electrophoretic Techniques

The identification of various hemoglobin variants is carried out by electrophoresis at various pH levels using a variety of support media.

The most commonly used diagnotic procedures for hemoglobinopathy evaluation are electrophoresis on cellulose acetate at pH 8.6 and electrophoresis in agar gel at pH 6.2. Other support media occasionally used to identify abnormal hemoglobin variants include potato starch gel, paper, agarose, and polyacrylamide gel. Because cellulose acetate provides sharp resolution of hemoglobin bands in a relatively short time, it is readily available, it permits staining and clearing, and it allows densitometric quantitation; it is the support medium of choice.

Cellulose acetate was first discovered and employed in protein fractionation by Kohn in 1957. He established that

cellulose acetate, as a support medium, was amongst all three media in use then, paper (Durum, 1950), agar gel (Gordon, 1949), most similar to Tiselius' moving boundary method. Since then cellulose acetate has proven to be a valuable diagnostic tool for routine investigation superseding paper medium. Commercial kits of cellulose acetate have further enhanced its availability, and kits commonly used include Microzone (Beckman Instruments, Inc., Fullerton, California 92634), Sepratek (Gelman Instrument Co., Ann Arbor, Michigan 48106), and Zip Zone (Helena Laboratories Corp., Beaumont, Texas 77704). These have been evaluated and proven satisfactory with the Zip Zone kit providing the best overall performance in the separation of hemoglobin variants.[7,8]

Routine protein fractionation by agar gel was first reported by Gordon.[9] It has gained use, parallel to cellulose acetate, in the separation of some hemoglobin variants. Agar gel has superseded paper as a support medium for zone electrophoresis. It has a more homogenous structure and provides faster and sharper resolution, and may be dried to a transparent thin film. This technique is however, concentration limited as far as the mobility of hemoglobin is concerned.[9]

Ion-Exchange Chromatography

Since the inception of ion exchange chromatography, numerous protein oligomers have been fractionated using various ion-exchange materials. Of importance in hemoglobin screening programs is diethylaminoethyl cellulose (DEAE cellulose), whose backbone is a polysaccharide; DEAE-cellulose is a weak anion-

exchanger which may be bound to protein. Eventual elution of the protein is accomplished by varying the pH or salt concentration.

This technique has been employed in evaluating individuals with Beta-Thalassemia. Efremov et al had reported a simplified protocol utilizing ultragranular DEAE-cellulose and Pasteur pipets as columns.[10,11] Several alternative methods have been discussed in the literature, but these suffer from considerable imprecision. Because ion-exchange column chromatography is primarily employed for the diagnosis of beta-thalassemia, it is restrictive in scope and is less valuable a tool for routine investigation of hemoglobinopathies.

A recent advance in hemoglobin analysis has been put forward by Hannash et al. This technique incorporates anionic exchange material in the use of DEAE-cellulose with high performance liquid chromatography. The procedure has been used to separate Hbs A_2, S, F. Details of this technique, anion-exchange HPLC, are discussed in the literature.[12]

Isoelectrofocusing Technique

Iso-electrofocusing in polyacrylamide gel is gradually gaining acceptance in clinical investigations especially in the fractionation and characterization of proteins including hemoglobin variants. Iso-electrofocusing was first employed for hemoglobin variants by Righetti, P. et al (1971).[13] Since then several reports have been documented involving iso-electrofocusing in protein separation. It is a simple technique which basically employs the same apparatus as used for electro-

phoresis but posesses a greater resolving ability than electrophoresis. Other advantages include optimal resolution, and it does not require application of the sample in a narrow zone as in conventional electrophoretic procedure. The concentrating property of isolectrofocusing provides a much lower detection limit than conventional electrophoresis, and proteins differing slightly in pI values can be resolved.

Also the presence of polyacrylamide gel as a support medium reduces the effect of electroendosmosis to a minimum for the gel itself is devoid of bound charged groups. Moreover, polyacrylamide gel is a high capacity stabilizer, which is able to withstand the high voltage applied in iso-electrofocusing.[14]

Basis of Electrofocusing

Every electrophoretic separation of proteins is due to the fact that proteins have a net charge that is dependent on the pH of its surroundings, and this net charge equals the sum of the positive and the negative charges on the surface of the protein. A state of continuous change in the net charge exists as the protein traverses a gradient. At a certain well defined pH, the net charge on the protein becomes zero. This point is termed the iso-electric point (pI).

Electrofocusing employs a stationary and stable pH gradient that is incremental in a cathodic direction. If a protein is placed at a pH higher than its pI, the net charge will be negative and such a protein will be anodic in its migration in an applied electric field. Gradual increases in pH result along the gradient until it reaches its pI where the net charge

will be zero and movement will cease. On the other hand, plating such a protein at a pH lower than its pI results in its acquisition of a net positive charge and migration will be cathodic. Likewise, the protein will be static at its pI position. Thus, regardless of where a protein sample is introduced into the pH gradient, each one will end up as a narrow zone at a specific pI position unique to the protein. Diffusion effects are eliminated by the applied electric field.

Separation and Determination

As mentioned above, isoelectrofocusing is a special type of electrophoresis. A pH gradient is established along the support medium (polyacrylamide gel), and each protein in the sample migrates to the point where the pH equals the pI. The pH gradient is caused by ampholytes added to the medium, which migrate in the electrophoretic current. Ampholytes are mixtures of molecules carrying positive and negative charges. Commercial ampholytes consist of a mixture of arginine, aspartic acid, and glumatic acid with synthetic polyamino-polycarboxylic acids. One end of the gel is immersed in acid and the other in base. Hemoglobin moves within the gel until it becomes concentrated at its pI. The relative order of hemoglobins seen after isoelectrofocusing, beginning at the acidic anodal end is HbA, HbS, and HbC.[5,15]

Isoelectrofocusing can be done in columns or thin-layer polyacrylamide gels. Thin-layer plates are available commercially as ampholine PAG plate kit, pH 3.5 to 9.5. The LKB 2117 Multiphor concept, a recent advance, has proven successful in hemoglobinopathy screening. Hemoglobin variants with small

charge difference, such as Hb_{Malmo} (beta 97 his--→ gln), not detectable by conventional electrophoresis, have been separated from HbA_1. The more recent application, high voltage electrofocusing, which optimizes time schedule and provides sharper zones, and visual examination of the concentrate, could be used.[16]

Electrophoretic Technique

Separation and Determination

In cellulose acetate electrophoresis, separation and identification of hemoglobinopathies are carried out under alkaline condition. Usually Tris-EDTA-boric acid buffer, pH 8.6 at 25 degrees Centigrade is used and electrophoresis is performed at 450V for 30 minutes. Under these conditions, hemoglobins migrate according to their net charge in an anodic direction. The relative orders are HbC, (A_2, E and O_A); HbN, HbS (D and G); HbF, HbA and HbK; and HbH, HbI and HbJ. The problem of misinterpretation exists for corresponding mobilities of different variants.

Dr. Rose Schneider has introduced a simplified technique for separating globin chains and this can be used to confirm other hemoglobins. In this procedure, erythrocyte hemolysate is electrophoresed on cellulose acetate in urea-2-mercaptoethanol buffers in the presence of additional 2-mercaptoethanol. The latter severs heme from globin, whereas the urea cleaves the alpha and beta chains; these migrate on cellulose acetate according to their net charges. The mobilities are dependent upon the pH and buffer composition. This technique is very simple and provides excellent resolution of the globin chains

in alkaline as well as acidic buffers, and it requires a small amount of hemolysate.

The Helena Laboratories electrophoresis procedure has now become the world's model for screening programs. It utilizes Supre Heme alkaline buffer and is capable of detecting hemoglobins S and C and estimating elevated amounts of hemoglobins A_2 and F. Other hemoglobins such as D move in the same area as S and hemoglobins such as O and E have the same mobility as C under alkaline conditions. Nevertheless, the Helena protocol has proven to be the definitive determination for all hemoglobinopathies. It is fast, easy and has the added advantage of cost effectiveness.

Helena Laboratories has also developed an acidic buffer procedure which distinguishes hemoglobins S and C using citrate agar as the support medium.

In the Helena electrophoresis protocol, a drop of packed red blood cells is hemolyzed with hemolyzing reagent, applied to a Titan III cellulose acetate plate, and electrophoresed in Supre Heme buffer for 25 minutes at 350V. The Supre Heme buffer is reconstituted by dilution to 980 mL using deionized water. This method alleviates the problem of artifacts production on cellulose acetate. The patterns may be interpreted immediately after electrophoresis, or carried through an intensifying stain, usually Ponceau S, for permanent storage or enhanced readability or quantitation. Quantitation may be carried out by means of a densitometer and is necessary for the identification of thalassemia minor. Controls are usually run on the same plate as each group of samples for screening.

CHAPTER III

Materials and Apparatus

Materials needed for the construction of the mini electrophoresis cell include a 1/4 plexiglass sheet, manufactured by Baker Plastics, Inc., Duro Epoxie Resin and Hardware, platinum wire strips of about 55 micrometer gauge size and Ersin Multicore Econo real solders (0.040" diameter, 60/40V and ER 220). Other materials are 8/32 inch tap for milling threads for screws, a Drill Press Motor tool model 210, Panavise soldering pen #338G, and alpha colored wires from Alpha Wire Corporation #22-7/30 PVC wire of type B Mil-w-16878D. All materials were obtained from the Chemistry Department of Youngstown State University.

Other materials needed for the experiment are a Fisher Scientific Kodak Timer by Eastman Kodak Company, disposable pipets, Whatman Filter papers (Ashless type #42), permanent-broad tip marker, Sargent magnetic stirrer for proper stirring of the buffer solution during preparation, the Helena Laboratories micro-dispenser, Zip Zone applicator, and a pair of tongs for plate handling.

In carrying out this study, Titan IIIH 1" by 3" cellulose acetate plates from Helena Laboratories were used. The blood specimens used were obtained from Saint Elizabeth Hospital Medical Center. The blood was hemolyzed by adding hemolysate reagent from Helena Laboratories and preserved in the refrigerator until needed for use.

The buffer used in this study (Tris-EDTA-Boric Acid, pH

8.2 to 8.6) was obtained from Helena Laboratories of Beaumont, Texas. The buffer was reconstituted to specification using the required amount of deionized water as outlined in Chapter IV. The staining dye, Ponceau S, used for the affinity coloration of the hemoglobin was prepared from capsules. Ponceau S has a very long shelf life. It was properly capped when not in use.

All water used in this study, either for buffer reconstruction or solution preparation, was deionized water.

Apparatus

A Heath Schlumberger Regulated Power Supply model #SP-17A capable of producing 400V of Direct Current electricity was available for this study. A magnet from the Chemistry Department's DA60 Nuclear Magnetic Resonance Spectrometer, capable of producing 14.7 kilogauss field strength, was used. The radio frequency components of the NMR were not involved in this study.

The micro-electrophoresis cells were constructed in the machine shop at Youngstown State University. The metal sander, drill press, band saw and vertical mill located in B-17 were useful in putting the cells together.

CHAPTER IV

Experimental

Cell Design and Construction

The electrophoresis cell was drawn to scale and cut into dimension by means of a band saw. The cut specimen of plexiglass was properly smoothed out by use of a sander. A vertical mill was used to engrave the desired wells according to the design in Figure 12 and 13.

Once the main body of the cell was completed, the components including the lid were soaked overnight in tap water to remove the glued paper label, which indicates the plexiglass manufacturer. Then the cells were dried by means of a lintless cloth.

A drill press was used to make holes for the insertion of the platinum electrodes. The platinum wires were cut and put in place by means of Epoxy glue and allowed to dry. Threads for screws were made by means of a 8/32 inch Tap. Thereafter, the wires were soldered to the colored alpha wires for positive (red) and negative (black) leads; a soldering iron was used for this purpose. The cell was tested for proper functioning.

A vertical span electrophoretic cell as well as a horizontal one was constructed for the study. Illustrations are in Figure 12 and 13.

Preparation of Solutions

The Supre Heme buffer (Tris-EDTA-Boric Acid, pH 8.2 - 8.6) from Helena Laboratories, Beaumont, Texas was reconstituted

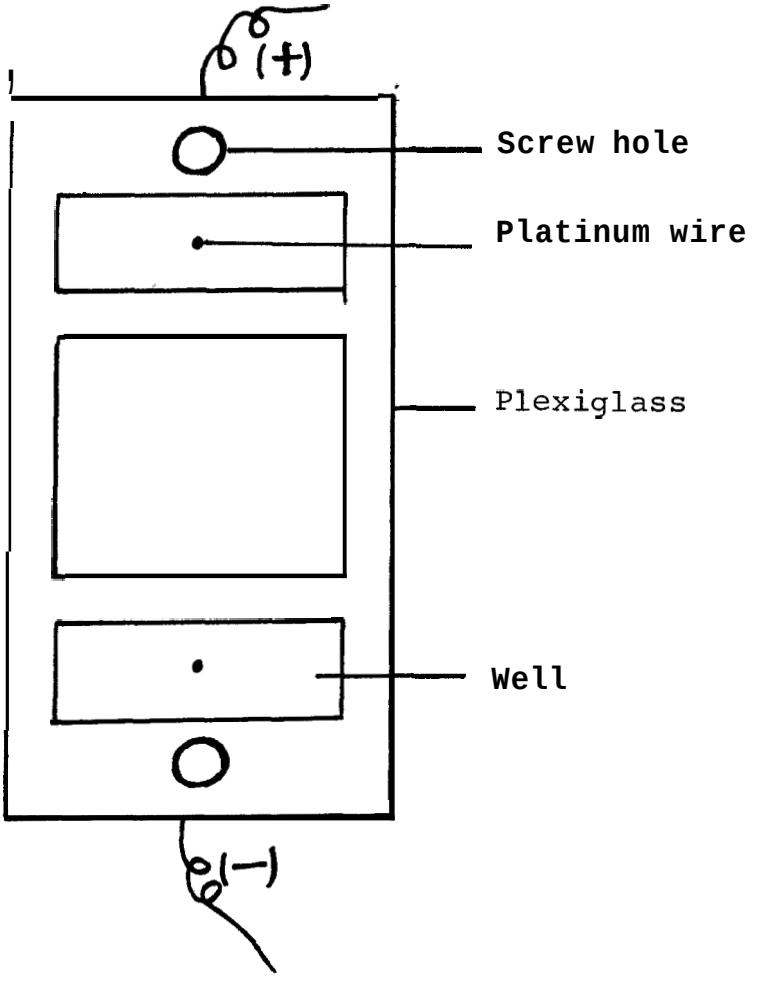

Figure 12. Mini electrophoretic cell for coupled magneto-electrophoresis (vertical span).

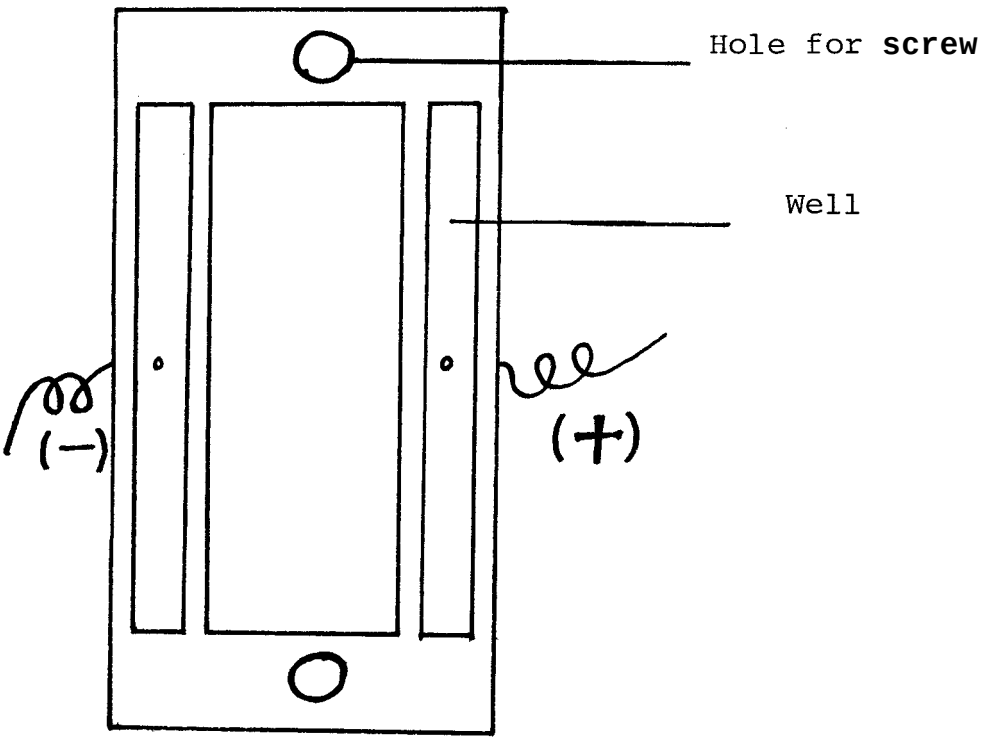

Figure 13. Mini electrophoretic cell for coupled magneto-electrophoresis (horizontal span).

according to manufacturers instructions. Each packet was reconstituted in 980mL of deionized water. The buffer solution is stable for a month at temperature of 2 to 80°C. Consequently, when not in use the buffer solution was stored in the refrigerator.

The 5 percent acetic acid solution for destaining was freshly prepared by mixing 5mL of glacial acetic acid with 95 mL of deionized water.

Ponceau S stain was prepared by dissolving 20g Ponceau S dye in 100mL of dilute aqeous trichloroacetic acid (30g/100mL). Deionized water was used for the rinsing of destained cellulose acetate plates before visual examination.

Method

The protocol used in this study is that of the Helena Laboratories. The steps are slightly modified to adapt to the micro-cells. The basic directions are outlined below:

1. The cellulose acetate plate was cut into strips for the minielectrophoretic cells.
2. Some of the reconstituted buffer was poured into a plastic boat from which some was retrieved to--soak the cotton in each of the outer compartments. A disposable micro-pipet was used to drain the cotton swabs.
3. Two cut strips of Zip Zone Chamber wicks were soaked in the buffer and then draped over each support bridge so that contact was made with the buffer. The covered chamberwas now ready for electrophoresis.
4. The leads of the cell are connected to the Heath Power supply. By means of a switch then regulationwas kept

at standby. At the same time the marked acetate strip was soaked in the buffer for 5 minutes prior to use.

5. The hemolysate containing globin chains under study was properly shaken; it was allowed to stand for a while and swirled just prior to use.
6. Using the microdispenser 5 microliter of the hemolysate was fed into the well plate. The maximum number of well plates filled was three, but initially one Sample well plate was used. The hemolysate was added as a drop into the well plate.
7. The sample applicator tips are cleaned in deionized water and dry blotted prior to use. The applicator was loaded by depressing the tips into sample wells several times. The first loading was used to prime the applicator and hence was applied to a blotter pad. The second and more uniform loading was used.
8. The wetted cellulose acetate plate was removed from the buffer and firm blotted once. With the cellulose acetate side up, the applicator was depressed about 10 millimeters from the cathodic base. Additional superimposed application may be necessary for the enhancement of minor fractions.
9. The strip was quickly placed over the cell with the acetate side down. The application site was nearest the cathode. The lid of the mini-cell was put in place and the voltage was turned on to 350V. The stop-clock was set for 2 minutes.
10. At the end of the time, power was cut off and the strip was removed from staining.

11. Thereafter, the strip was stained for 3 minutes in Ponceau S.

12. The stained strip was destained in three successive washes of 5 percent acetic acid for 2 minutes each or until the background turned white. The strip was now dried and inspected, the strip can be stored away for permanent record.

The electrophoresis study was carried out in two phases, one in the form of a conventional electrophoresis as described above. The other was the coupling of the applied field strength of the NMR magnet. Once step #9 was reached, the prepared Sample and cell are placed within the two faces of the magnets as illustrated in Figure 14, prior to turning on the power supply. All other steps remained the same. By comparison of the two runs, the effect of the magnetic susceptibility on the resolution of the hemolysate was determined. Electrophoresis under the influence of the magnetic field was carried out in the X,Y, and Z directions. The polarity of the mini-cell was reversed to investigate which direction, in relation to the polarity of the cell, was more favorable and reproducible. Several determinations were performed on each of these parameters mentioned above.

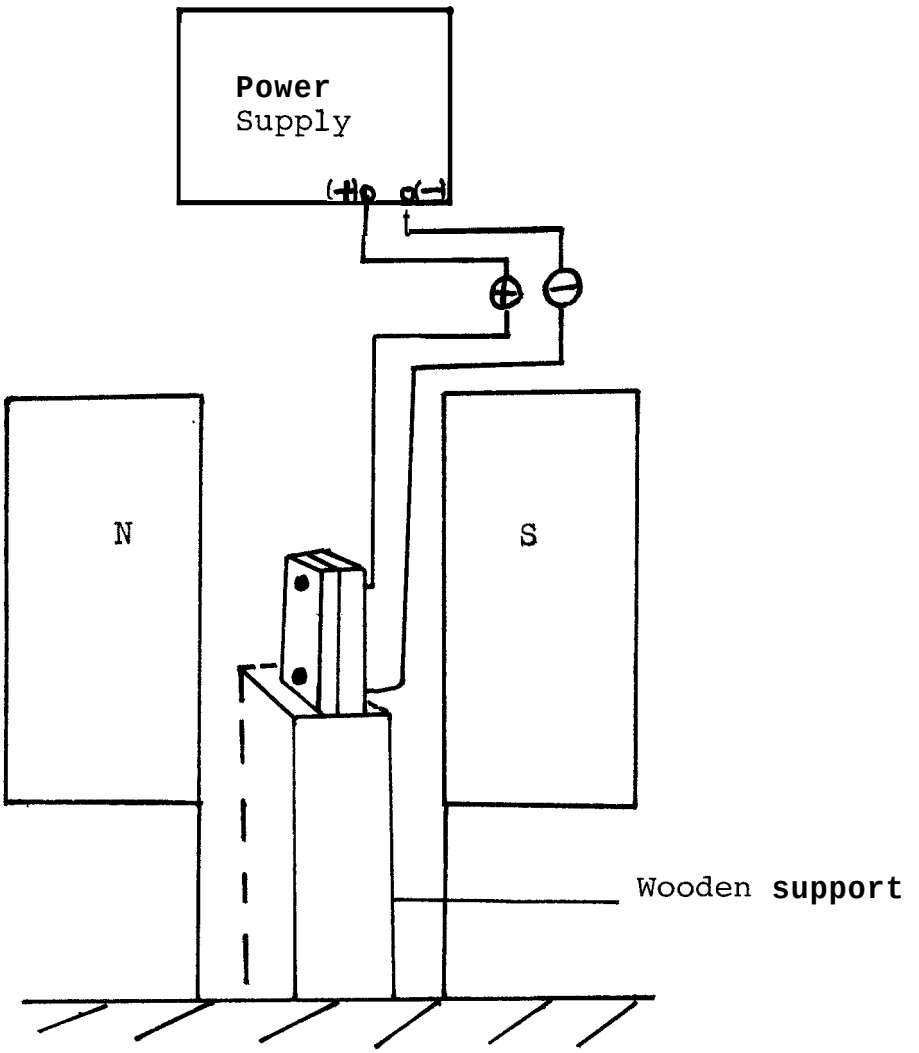

Figure 14. The set-up for coupled magneto-electrophoresis.

CHAPTER V

Results and Discussion

The results of this study are based on time-scheduled determinations for 2 minutes. The hemolysate migrated properly at 25°C and 350V without the presence or appearance of any artifacts.

When the hemolysate was electrophoresed within the poles of a magnetic field in the X, Y, Z directional axes, the outcome was distinctly different for each run. In the Z-axis, the resolution was poor due to the clustering of the bands along an aligned angle. In the X-axis, the resolution produced was only slightly improved when compared to the conventional runs. Comparatively, the migration path length for both the X-axis and the conventional runs were approximately the same. However, electrophoresis performed in the Y-axis produced some distinct and noticeable differences when compared to the conventional electrophoresis technique.

First, there was a splitting of the separated bands into two distinct peaks with intense coloration on staining with Ponceau S. The shades of color intensity of the two peaks correspond to HbA_1 for the more intense band while the less intense one corresponded the HbA_2 fraction. Next, on comparing the migration distances of the conventional and magneto-electrophoresis runs, a striking result was that mobility distances for magneto-electrophoresis diminished by about half the length for the conventional runs. Equal distances were obtained when magneto-electrophoresis was performed at a time schedule nearly

4 minutes instead of 2. The set up for the Y-axis runs is illustrated in Figure 14. When the polarity of the applied electric field was reversed, the results were similar to the conventional electrophoresis run.

Magneto-electrophoresis runs had results (for Y-directional axis) which are due to the coupled effect of the magnetic susceptibility of the hemolysate and the applied voltage. Diamagnetic effect of the heme or globin chains produced a retarding force in the applied magnetic field. Thus, the combined effect was a greater separation of hemoglobin variant due to the magnetic field. This technique results in an enhanced separation of hemoglobin A_2 from HbA. This is shown in Figure 15. Further efforts will employ other variant specimens.

The clustering effect produced in the Z-axis may be due to the multi-nucleate nature of the hemoglobin molecule. Paramagnetic susceptibility is well pronounced only in mononucleated molecules when subjected to a magnetic field in the Z-direction.[20,21]

In the X-axis, the applied magnetic field is ineffective since the electrophoresed sample appeared to be enveloped in the centre of the field. The circulating secondary field either hampered, or barely improved the resolution. Many runs in the X-axis produced diffused bands as well.

Conclusions

Based on this study, it is evident that the combination of electrophoresis with an applied magnetic field is a useful technique for the separation and evaluation of hemoglobinopathies.

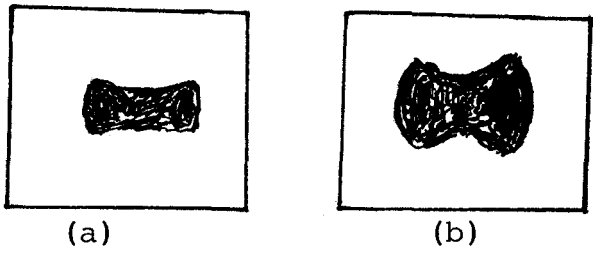

Figure 15. Illustration of the effect of magneto-electrophoresis on a normal Hb hemolysate. (a) conventional electrophoresis of hemolysate, (b) **the same hemolysate after magneto-electrophoretic separation. The time schedules for the same migration distance as shown above: 2 minutes for a,** 4 minutes **for b.**

This technique produced more distinct band resolution over the conventional electrophoresis; it is relatively easy to perform and may provide time optimization for multiple samples, if a larger cell is employed.

The effective net charge on mutant globin chains can be very prominent in establishing the various magneto-electrophoresis loci, since the migration path length happens to be shorter than the conventional electrophoresis. The net charge will either enhance or retard the magneto-electrophoretic migration, which will be related to the effective globin chain's magnetic susceptibility.

One problem is that of the magnetic field strength; the magnetic field used in this preliminary study is small (14.7 kilogauss). It is not high enough to penetrate each globin mass and demonstrate its effect on the iron (II) molecule. Each globin mass has one iron (II) molecule. The massive arrangement of peptides partially shields the iron atom from the applied magnetic field. For better effects, a magnetic field strength of about 25.3 kilogauss has been recommended in the literature.

In addition to the above assertions, it was conclusively proven that paramagnetic materials such as ferrohemoglobin do migrate substantially when placed in an applied magnetic field. In this study, hematin (ferrohemoglobin) was shown to migrate substantially, about 2.0 cm, in a cellulose acetate electrophoresis cell placed in a magnetic field. No electric field voltage was involved. On the other hand, normal Hb that has been treated with sodium thiosulfate migrated only slightly against gravity (about 0.40 cm) confirming that normal Hb is diamagnetic.

It is hoped that in the future other graduate students will undertake the evaluation of the magneto-electrophoretic effect on some of the known globin **mutants or variants; this will eventually culminate into the establishment of the different loci for these hemoglobinopathies.**

APPENDIX A

The Gouy Technique

The Gouy technique is often used to measure magnetic susceptibilities, and the instrument consists of a sensitive balance from which hangs the sample in the form of a narrow cylindrical tube as in Figure 15.

The sample hangs between the poles of a magnet. If the sample is paramagnetic, its energy is apparently less within the magnetic field; hence, there is a drawing force into the field. Contrariwise, a diamagnetic substance has a lower energy outside the field, and so there is a repelling force out of the field. This force is proportional to the susceptibility. Determining the balance point allows X_m to be determined. The instrument is usually calibrated against a sample of known susceptibility.[20]

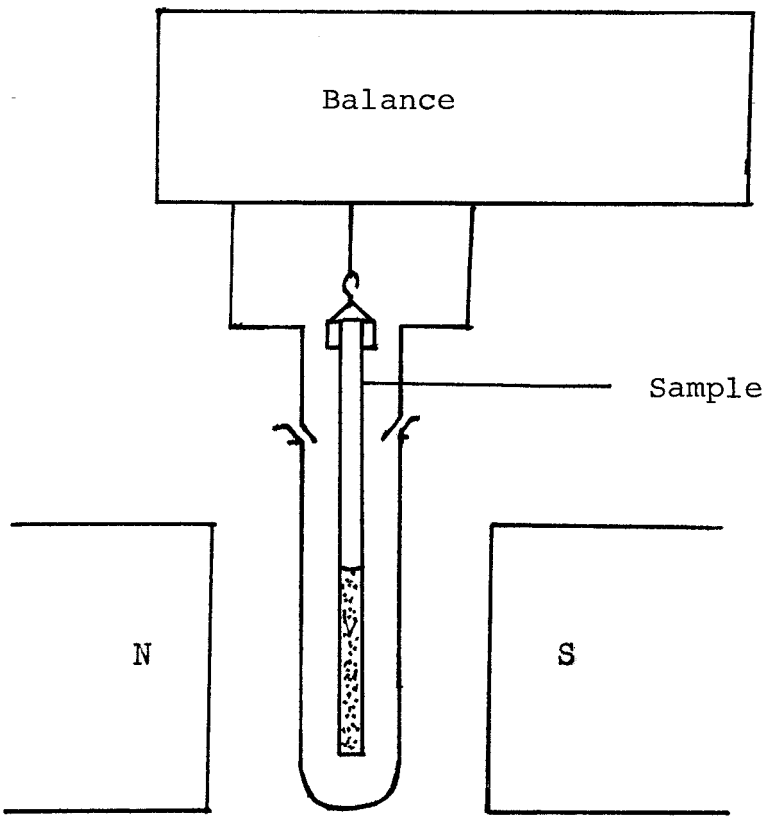

Figure 16. Gouy balance arrangement for measuring magnetic susceptibility: [21]

APPENDIX B

The Modified Gouy Technique

The modified Gouy technique by Quinke is mainly applicable to liquids and solutions. In this version, the magnetic force acting on the capillary sample is measured in terms of the hydrostatic pressure. By experimentation, the change in capillary column, Δh, with the field on and off is determined by means of a cathetometer.

Paramagnetic liquids are known to show the greatest increase in height; diamagnetic liquids show a decrease. Applied field of 25×10^3 Gauss are recommended for use, and results are comparable to the Gouy method.

Since solutions exposed to air consist of a dynamic equilibrium mixture of air and its vapor, the relationship could be expressed by equation (8)

$$\frac{2g(\rho - \rho_o)\Delta h}{H^2} = k - k_o \qquad (8)$$

where ρ and ρ_o are densities of the liquid and the gas above the liquid respectively; k is the volume susceptibility of the liquid and k_o is the volume susceptibility of the gas above the meniscus; H is the applied field, and g is the acceleration due to gravity.

The hydrostatic pressure, $g(\rho - \rho_o)\Delta h$ is counterbalanced by the field strength, $1/2H^2(k - k_o)$ to yield equation (8) above.

By rearrangement, the susceptibility per gram, X, is defined by equation (9)

$$X = \frac{k}{\rho} = \frac{2g\ h}{H^2} + x_o \rho_o/\rho \qquad (9)$$

where x_o and $_o$ are the gram susceptibility and density of gas above the liquid. However, the term $x_o \rho_o/\rho$ is negligible when compared to the larger diameter reservior to the capillary in addition to the small gas susceptibility, x_o. Thus, a simpler definition is given by

$$X = \frac{2g\Delta h}{H^2} \qquad (10)$$

measures the liquid or solution susceptibility. Thus, susceptibility measurements are independent of density and temperature.

Under identical conditions, a reference sample is treated in like manner as the sample investigated. Then the relation

$$\frac{x_s}{x_r} = \frac{\Delta h_s}{\Delta h_r} \qquad (11)$$

holds; x_s and x_r are the susceptibilities of sample and reference respectively.

Illustration of the Quinke balance is shown in Figure 17.

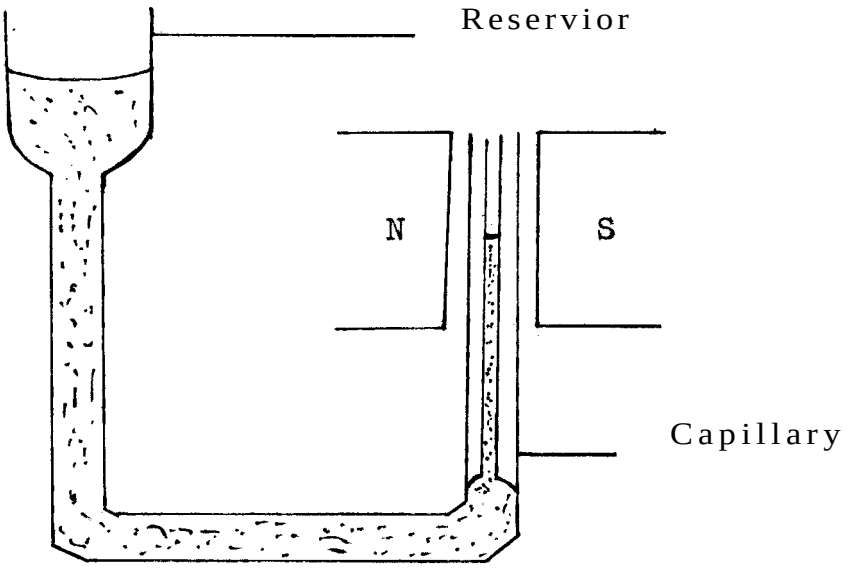

Figure 17. Principle of the Quinke balance based on Gouy technique.[20]

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