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Electrophoretic analysis and its application to the study of the serum protein fractions in man and their alteration in various pathologic processes

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ELECTROPHORETIC ANALYSIS AND ITS APPLICATION TO THE STUDY OF THE SERUM PROTEIN FRACTIONS IN MAN AND THEIR ALTERATION IN VARIOUS PATHOLOGIC PROCESSES

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INTRODUCTION

Although the electrophoretic phenomenon and its associated measurement has been noted and studied to some extent for the past 150 years, it has only been within the last decade that their study and use has been of any practical value. In 1937 Tiselius[157] introduced his apparatus for electrophoretic analysis, and since then its application has found use in many and widely varied fields of scientific research. Continued improvement of apparatus and technique and study of the basic principles involved is ever widening its scope of applicability.

The first work in this field was in 1808 when Reuss, a Russian physicist, observed the flow of water through clay (electroosmosis) under the influence of an impressed electric field and the migration of the clay particles in the opposite direction (electrophoresis). In 1861 Quincke made the first actual measurements on the migration of particles in fluids due to an impressed voltage.

Besides the early important theoretical studies by Helmholtz, on the origin of the electric charge on colloidal particles, our knowledge in this field was further advanced with the discovery of the relationship between current and the electric field by Ohm in 1827, and the laws relating electricity and chemical change set forth by Faraday in 1834. Out of these basic investigations and the more recent work of Longsworth[86, 89] and Dole[46], we have evolved the moving boundary theory upon which the determinations and calculations of electrophoretic analyses are based.

BASIC PRINCIPLES AND THEORY OF ELECTROPHORETIC ANALYSIS

Colloidal Particles.

Colloidal particles, in general, are electrically charged with respect to the dispersion medium. This was stated in Coehn's Law that if two heterogeneous substances are in contact, the one with the higher dialectric constant is positively charged with respect to the other. Since water has a high dialectric constant ($\varepsilon = 81$), it is usually positive. Coehn's Law, however, is not absolute in certain cases[18].

In the protein molecule there are a large number of amino (NH_3^+) and carboxyl (COO^-) groups which act as if they were on the surface. These determine the net charge on the molecule, and since it is constantly taking on and giving up H^+, the instantaneous charge is a small integral number. However, the statistical nature of the charging process allows the time average of the resultant charge to be fractional, and it is this fractional nature of this net charge that makes possible the wide variety of electrophoretic identifications at a given pH. At a very low pH, the protein is charged predominately positive by the protons attached to the substituted ammonium groups and therefore, migrates to the negative pole; at a high pH the protein is predominately negative by a loss of protons from substituted ammonium groups and carboxyl groups and therefore, goes to the positive pole. At the isoelectric point there is no net charge and therefore, no migration.

Origin of the Electric Charge on Colloidal Particles.

The nature and structure of this electric charge existing

on and about colloidal particles has been and still is a subject of much discussion. For a critical analysis of these, the reader is referred to a discussion by Reimer[18]. The early work in this field was done by Helmholtz and Lamb[55]. In their hypothesis they consider that the two phases become charged in a manner like electrification of frictional electricity. They also assumed that the outer oppositely charged ions were concentrated in a single layer at a definite distance from the surface, the so called "double layer."

The Freundlich-Bancroft[36] hypothesis, however, states that the charge is due to the selective adsorption of ions and depends on the relative adsorption of cations and anions. Briggs[18] stated that every solid has a specific adsorbing power for a given ion which depends upon the specific surface of the solid, temperature, concentration of the ion, and other ions present or previously adsorbed on the solid. The possible sources of these ions would be the dissociation of H_2O, solution of the solid, and extra ions added to the solution.

More recent work[54] has tended to show that the charge is due to an orientation of the molecules of this liquid at the surface and within the pores of the solid.

Structure and Properties of the Electric Double Layer.

Since the electrokenetic phenomenom cannot be accounted for on the basis of a rigid double layer, various hypotheses have arisen to explain its structure and properties.

Since there must be a "slip" or "give" between the layers, it was thought[80] that in electrophoresis, for example, there must first

be a poliarization of the colloid medium system (solid-liquid phases), followed by a transfer of the charge on the layer made up of the molecules of the medium (liquid) to other contiguous molecules. In this way the particle is "handed on" by the molecules of this medium.

The Gouy-Deby theory[54] postulates that the outer layer, instead of being sharp, consists of an ionic atmosphere with the charge density decreasing to zero from a large valve at the surface, and although the effect of diffusion tendency is appreciable in the outer part of the double layer, its influence on the rigidity of the inner part is small owing to the very large adsorptive forces. The thickness of this diffuse layer is defined as that distance of separation of an equivalent Helmholtz layer of charge, Q, which would produce the same potential drop as on the diffuse layer. The potential difference[115] between the particles of radius, R_1 and the assumed Helmholtz layer of radius R_2 is:

$$V_1 - V_2 = \int_{R_2}^{R_1} \frac{Q}{Dr^2}\, dr = \frac{Q(R_2 - R_1)}{D R_1 R_2}$$

Q = charge on the particle.
D = dialectric constant.

This drop in potential in the diffuse layer is termed the zeta (ζ) potential. As expressed by Stern[54],

$$\zeta = \frac{4\pi e \delta}{\varepsilon}$$

e = charge on unit surface.
δ = distance between the sides of this double layer.
ε = dialectric constant.

Stern considered the double layer to be a condensor, one side of which is the solid surface having attached to it a rigid layer of approximately molecular thickness and beyond this a diffuse layer extending into this liquid. Part of the electric charge is concentrated on this surface and the fall in potential in this fixed layer is sharp, while the density of the charge in this fluid diminishes assymptotically to zero. The total potential fall in the two layers is the epsilon potential, (ϵ), while the fall in the diffuse layer is the ζ potential.

Calculation of Electrophoretic Mobility.

The determination of electrophoretic mobility is of great importance in interpretation of electrophoretic patterns and in the identification of proteins. This can be done directly or indirectly depending on what measurements are available. In general, the quantities needed are the distances moved by this boundary and this electric field strength.

Beginning with Stokes' Law[115] the particles will tend to migr-ate with a velocity of

$$V = \frac{EQ}{6\pi\eta}$$

E = impressed field in volts/cm.
η = viscosity.
Q = charge on particles.

However, due to the backward drag of the ionic double layer, the velocity will be less.

$$V = \frac{DE\zeta}{6\pi\eta}$$

D = dialectric constant for a particle so small that the distortion of the electric field by the particle is negligible.

For larger particles:

$$V = \frac{DE\zeta}{4\pi\eta}$$

By using the development of equations for the electro-osmotic flow, another equation for velocity can be arrived at[36].

u = velocity of moving liquid.
ζ = cross section of the capillary tube.
E = EMF of the electric field.

An electric double layer is formed at the surface of the contact of tube and liquid. Assume the solid portion of the double layer is not in the solid itself but in the film of liquid adhering immoveably to the solid, then the frictional force, f, resisting the moving liquid is directly proportional to the coefficient of viscosity, η, the velocity and the area of the moving surface of the electric double layer and inversely proportional to the distance, δ, between the two sides of the electric double layer.

Therefore, for a unit surface,

$$f = \frac{\eta u}{\delta} \qquad (1)$$

but since $$u = \frac{v}{\varphi} \qquad (2)$$

where v = volume of fluid passing through a cross section in a unit time.

$$\zeta = \frac{\eta v}{\delta \varphi} \qquad (3)$$

If e is the charge on a unit surface of the moving side of the electric double layer and X equals the potential gradient in the tube, then the electric force acting on a unit surface of the moving layer is eX. Since this is the force necessary to just overcome the frictional force and impart a constant velocity to the moving side of the electric double layer, then

$$eX = \frac{\eta v}{\delta \varphi} \qquad (4)$$

If we regard the electric double layer as a condensor of potential ζ in a medium having a dialectric constant, ε, its capacity per unit area is

$$\frac{e}{\zeta} = \frac{\varepsilon}{4\pi d}$$

$$\zeta = \frac{4\pi d e}{\varepsilon} \qquad (5)$$

Equation (4) combined with equation (5) is

$$\zeta = \frac{4\pi\eta v}{\varepsilon \varphi X}$$

or $$v = \frac{\varphi \varepsilon \zeta X}{4\pi\eta} \qquad (6)$$

Then equation (2) combined with equation (6) and if a particle is

stationary in the tube and the liquid is moving past it,

$$u = \frac{\varepsilon \cdot \zeta \cdot E}{4\pi\eta}$$

u = velocity that the fluid moves past the particle.

Then if the particle is free to move and the fluid is stationary, it must move with the same velocity. This, then, would be the velocity of electrophoresis.

The third method of arriving at electrophoretic mobility is as follows[5]. The force of an electric field on an ion is proportional to the rate with which the potential is changing with distance (potential gradient) at that particular point. The potential gradient is greatest in the electrophoretic cell because here the cross section is smallest and, therefore, the resistance is the highest. (The cross section can be calculated from the weight of Hg necessary to fill a definite height of each limb). If q is the cross sectional area, the potential gradient or electric field strength, E, (in volts/cm) may be calculated from the current flowing, i, (in amperes) and the specific conductivity of the solution, k, (in $Ohm^{-1}\,cm.^{-1}$).

$$E = \frac{i}{qK} \qquad (2)$$

The current is reassured with a sensitive milliameter or a potentiometer and a known resistance.
The specific conductivity is calculated from the resistance, R, of the solution in a conductivity cell of constant, C.

$$K = \frac{C}{R} \qquad (3)$$

If the passage of current, i, for time, t, causes a protein boundary to

descend, Δx cm., then Δx q P grams of protein have been transported into the bottom section of the u-tube (P = grams of protein/ml. of protein solution). The amount of protein transported into the bottom section may also be expressed in terms of mobility and is u·E·p·t·q·P where u·E·p·t is the distance moved by an average protein molecule in the body of the solution. By equating these expressions and solving for u, we get

$$u = \frac{\Delta x}{E_p t} \qquad (6)$$

Since

$$E_p = \frac{i}{q K_p}$$

$$u = \frac{\Delta x q K_p}{i t} \qquad (7)$$

$\Delta x q$ = volume swept through by the boundary when one coulomb passes through the cell.

Equation (7) can be written in terms of the volume swept through by the descending boundary per coulomb V_d.

$$u = V_d K_p \qquad (8)$$

The sign of the mobility is the same as the charge on the ion. If the descending boundary is symetrical, the distance through which the maximum ordinate has moved may be used to calculate V_d. If not symetrical, the centroid ordinate should be used[85].

The Moving Boundary Theory[6].

Moving boundaries are also used for the determination of mobilities and transference numbers of inorganic ions. The basic theory is well worked out for simple mixtures, and this has then been extended to include more complex mixtures such as plasma.

To derive the relationship between ion mobility and transference numbers, we must consider the specific conductivity, K.

$$K = \frac{l}{R q} \quad ohm^{-1} cm^{-1} \qquad (2)$$

R is resistance in ohms of a column of conductor of cross section area, q, and length, l.

Substituting E/i for R by Ohm's Law

$$K = \frac{i l}{E q} \qquad (3)$$

Therefore, K equals the current in amperes carried through a one cm. cube of conductor between two opposite faces differing in potential by one volt.

For solutions of electrolytes, the current is equal to the summation of the rates of transport of electric change by the different ion species. If there are C_j Faraday equivalents of an ion per liter of solution and this ion has a mobility of u_j cm.^{2} volt^{-1} second^{-1}, the electric charge carried by the ion through a square cm. cross sectional area perpendicular to the electric field in one second per unit of potential gradient is $u_j C_j/1000$ equivalents. To get current in amperes (coulombs/second), this must be multiplied by the number of coulombs in an equivalent (Faraday's constant - 96,500).

$$\text{Transference No.} = T_j = \frac{i_j}{i} = \frac{u_j c_j}{u_1 c_1 + u_2 c_2 + \cdots + u_n c_n} \quad (5)$$

From equation (4) we get

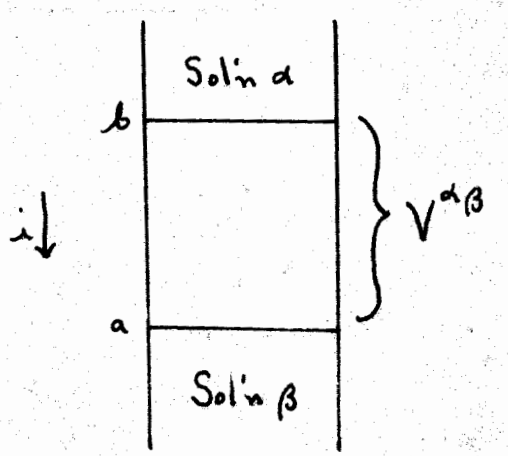
$$T_j = \frac{u_j c_j}{\left(\frac{1000}{96,500}\right) K} \quad (6)$$

These equations give the relationship between mobility and transference numbers.

The fundamental law in the study of moving boundaries is the moving boundary equation which relates the displacement of a separated single boundary and the concentration transference numbers of the ions in the homogeneous solutions on either side of the boundary. In the case of proteins or other weak electrolytes, this equation is applicable only so far as the buffer maintains a constant pH.

As considered by Longsworth[86], a boundary, a, between solutions, α and β, moves against the current, i, through a volume, $V_a^{\alpha\beta}$ (liter/Faraday) corrected for the electrode reaction to the position, b, on passage of one Faraday equivalent of electricity (figure 1).

Figure I.



If C_j^{α} is the equivalent concentration of an anion constituent, j, in solution α and C_j^{β} its concentration in β solution, the number of equivalents present initially in $V^{\alpha\beta}$ is $C_j^{\alpha} V^{\alpha\beta}$ and after the passage of current, $C_j^{\beta} V^{\alpha\beta}$. Also a number of equivalents of j ion equal to its transference number, T_j^{β} , in β solution simultaneously enter this volume through the plane at a, while T_j^{α} equivalents leave through the plane at b. By applying the Law of Conservation of Mass to j ion in $V^{\alpha\beta}$, we get

$$T_j^{\alpha} - T_j^{\beta} = V^{\alpha\beta}\left(C_j^{\alpha} - C_j^{\beta}\right) \qquad (6)$$

$V^{\alpha\beta}$ is positive if it moves with the current. A similar equation can be derived for a cation if C_j is given the sign of the charge on the ion.

If one ion is absent on one side of the boundary; that is, it disappears on the boundary, equation (6) becomes

$$T_j = V_j^{\alpha\beta} C_j \qquad (7)$$

This is the usual case with proteins. By substituting equation (7) for T_j in equation (5) and solving for u,

$$u_j = \frac{V_j^{\alpha\beta} K}{\frac{96500}{1000}} = V_j K \qquad (8)$$

V_j = volume in cm.^{3} moved through by j boundary/coulomb.

This is the same as equation (8) under calculation of electrophoretic mobility.

To discuss the concentration change at this position of the initial boundary which gives boundary anomalies, we must introduce the "regulating function," w, as discovered by Kohlroush[6].

$$w = \frac{c_1}{u_1} + \frac{c_2}{u_2} + \cdots + \frac{c_n}{u_n} \qquad (9)$$

This quantity remains constant for any given level in the electrophoretic cell regardless of the number of boundaries which pass by that level. Thus for a moving boundary, w must have the same value in the two solutions on either side. However, for a stationary boundary, w has different values in the solution on either side. The ratio of the values of w for two solutions separated by a stationary boundary is equal to the concentration across the boundary.

$$\frac{w^\alpha}{w^\beta} = \frac{c_1^\alpha}{c_1^\beta} = \frac{c_2^\alpha}{c_2^\beta} = \cdots = \frac{c_n^\alpha}{c_n^\beta} \qquad (10)$$

In the case of constant relative ion mobilities, as in Dole's development[46], all ionic species are diluted by the same factor at the stationary boundary. Here although relative mobilities are constant, the absolute ion mobilities vary somewhat because of a difference in salt concentration and viscosity. If the protein solution is diluted with the unionized part of the buffer by the factor before electrophoresis, the δ and ϵ boundaries disappear because of the equality of the regulating functions[92].

A complete theoretical description of the boundary displacements and concentration changes through the boundaries could be obtained from the composition of the original solutions, the differential equations of continuity, the electro-neutrality requirements and a specification of ion mobilities as a function of composition. This is only practical in a simple case of three ions; however, because of the interrelation of diffusion and electrical migration in this case, it can only be done if mobilities are regarded as constant. To avoid mathematical complexity, an equation

can be developed in a form independent of the particular path by which ion concentration changes between phases.

Dole's[46] general solution for the moving boundary equation assumes only that the relative ion mobilities are constant. Thus, a system that contains n ions will, in general, form a maximum of n-1 boundaries, one of which is a stationary boundary. If the system contains p anions and q cations, there will generally be p-1 boundaries with a negative velocity and q-1 boundaries with a positive velocity. If, however, we use only relative ion mobilities, $r_1, r_2, \cdots r_n$ as in Dole's theory, we must define a "relative" specific conductivity,

$$\sigma = r_1 C_1 + r_2 C_2 + \cdots r_n C_n \qquad (11)$$

This is analogous to specific conductivity. The $V\sigma$ products (where V = volume moved through by the boundary/Faraday) are obtained a solution of polynomenals of the type.

$$\frac{r_1 - r_n}{r_1 - x} C_1^{\alpha} + \frac{r_2 - r_n}{r_2 - x} C_2^{\alpha} + \cdots + \frac{r_{(n-1)} - r_n}{r_{(n-1)} - x} C_{n-1}^{\alpha} = 0 \qquad (12)$$

If the values of x are ordered from the extreme negative to the most positive, these correspond to the $V\sigma$ products for the boundaries from the one with the most negative velocity to the one with the most positive velocity.

The concentration changes of each ion species across each moving boundary may be obtained from

$$C_j^{\beta} = \left[\frac{(V^{\alpha\beta}\sigma^{\beta})(r_g - r_j)}{(r_g)(V^{\alpha\beta}\sigma^{\beta} - r_g)}\right] C_j^{\alpha} \qquad (13)$$

r_g = relative mobility of the ion species absent in solution .

From the above equation, one can then compute the analysis of an assumed mixture. For a more extensive discussion of the moving boundary theory, the reader is referred to the work of Dole[46], Hoch[62], and Alberty[4].

Sources of Deviation from Ideality.

The main deviation from Dole's theory is caused by variations in protein mobilities from small alterations in pH. Thus, his assumption of constant relative ion mobilities is not strictly valid.

Further errors are introduced because with the above mentioned variations in pH, the charge on the protein molecules will also vary. Also the size and shape of different proteins will effect their movement through any viscus media.

In addition, a diffuse ionic atmosphere surrounds any ion, especially those of a larger size. The electric field will act on this atmosphere as well as on the protein ion, and since the ions of the atmosphere are predominately opposite in charge to that of the protein, they will tend to move in a direction opposite to that of the protein ions. This effect will then be transmitted to the liquid itself.

APPARATUS AND TECHNIQUE

Method of Study.

In general, the methods of studying electrophoresis may be divided into two types: the microscopic method and the moving boundary method. With the first method, the particles under study are neutral particles which are coated with the substance being investigated and are actually observed and their movements measured microscopically. Since this method is not widely applicable to the study of protein mixtures, it is only mentioned here for completeness.

However, a comparative study of macro and micro electrophoretic analysis of human serum[24] showed good agreement by both methods as to mobility and percentage composition of various components.

The moving boundary method, which is the one used to study protein mixtures such as serum and plasma, involves the formation of a series of invisible moving boundaries each corresponding to a different component of the mixture. These boundaries are then visualized and measured by means of various types of optical systems. The moving boundary method was first used as early as 1906; however, it was only after the improvements of Tiselius[157] that its application became widely used.

The moving boundary method had many difficulties which had to be overcome before it could be of any practical use[115]. Proteins must be in a salt solution thus making the current high which would result in mixing+convection from the generation of heat. Also proteins usually have a low electrophoretic mobility and differ very little, one from another in this respect, necessitating a long tube in order to get adequate separation. And finally, proteins are usually colorless and not easily observed.

With the use of the Tiselius apparatus, many of these difficulties are overcome. By using an electrophoretic cell of rectangular cross section to increase the dissipation of heat and a low temperature bath to maintain a temperature near that of constant density for the solution, convention currents are eliminated. Since buffer can be forced in the opposite direction to electrophoretic flow, the analyses can be greatly prolonged and better separation can be obtained. With a large volume of buffer between the boundaries and electrodes, a constant pH is maintained around the protein molecules and by using large Ag/AgCl electrodes, the volume change resulting from gas formation and electrolytes is minimized.

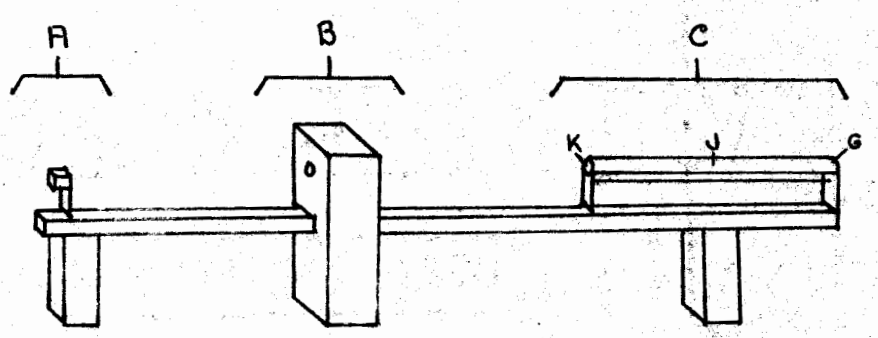
Apparatus.

There are several basic necessities which are necessary in a moving boundary type of electrophoretic apparatus[90] in order that the measurements obtained by its use may be valid. In general, they are as follows: a sharp boundary must be formed between a solution of the material to be studied in a suitable buffer and the buffer itself; the electric field and pH in which these boundaries are formed should be constant; the electrode processes should not involve the evolution of gas or other uncertain volume changes; and finally, the electrode product should not reach the regions where the boundaries are moving. These necessities are fairly adequately accounted for in the Tiselius[157] apparatus.

The apparatus itself consists of three basic units (Figure I.). "A" is a horizontal slit illuminated by an adequate light source, "B" is

made up of the electrophoretic cell itself with its auxiliary equipment and a low temperature bath in which it is suspended, and "C" is the optical system for recording the boundaries and their movements. This is equipped either for direct visual observation or photographic reproduction. These three basic units are so attached to the longitudinal metal tract that they may be optically oriented with respect to each other.

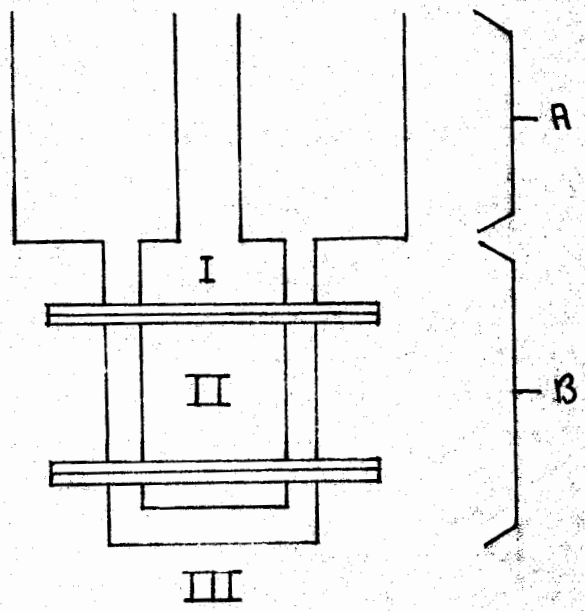
Figure I.



In Figure II. are shown the basic details of the electrophoretic cell itself. It is composed of two portions, the larger dialated section, A, that contains the electrodes and the smaller capillary portion, B, in which the boundaries are formed. The capillary portion is composed of three sections, I, II, and III; the middle section being moveable in a horizontal direction independent of the other two. The movements of these sections are controlled by an auxillary gear mechanism. The technique

will be discussed later.

Figure II.



The original Tiselius electrophoretic cell had two short moveable center sections. This type of cell is still used to some extent for the recovery of an isolated component; however, the new type of cell has a greater distance over which the boundaries can be spread and thus affords better detail of their structure[84]. Most electrophoretic units have a compensator connected to one limb of the cell. This allows the operator to produce small carefully controlled pressure changes in that half of the tube, and thus control the level of the boundaries in the capillary portion of the tube.

The electrodes are of the Ag/AgCl type. They must be capable of carrying 30 milliamperes for long periods with no gas evolution[90]. Sufficient capacitance is obtained by using an Ag strip wound in a tight

spiral for the electrode. On passage of the current, the reactions at the electrodes are as follows:

At the anode (+) $Ag^{+} + Cl^{-} \longrightarrow AgCl + e$
At the cathode (-) $AgCl + e \longrightarrow Ag^{+} + Cl^{-}$

Thus, no gaseous electrode products are formed. The third unit of the apparatus which is composed of the recording apparatus will be discussed in greater detail in the section on optical methods. Basically, it is made up of a Schlieren diaphram or lens, K, at the proximal end of a hollow tube, J. At the other end, G, is the recording photographer plate or ground glass.

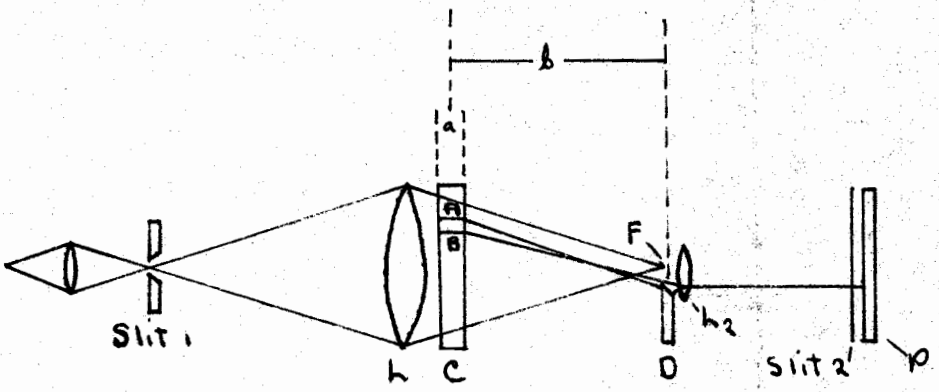
Optical Methods.

Since the boundaries formed by protein solutions are usually colorless, some optical method of their visualization must be used. In general, all these optical devices are based on the effect employed by Foucault and Taepler for testing lenses and is called the Schlieren (shadow) method. These devices are of two types[115]. The Schlieren scanning method of Longsworth [82, 88, 92], and the cylindrical lens Schlieren method of Philpot with the modification of Svensson[154]. Only the basic details of these methods will be discussed here, and for greater detail, the reader is referred to the original article.

The patterns from the above mentioned methods show the refractive index gradient (dn/dx) in a thin layer of solution in the channel on the ordinate as a function of the height, x, in the cell on the abcissa.

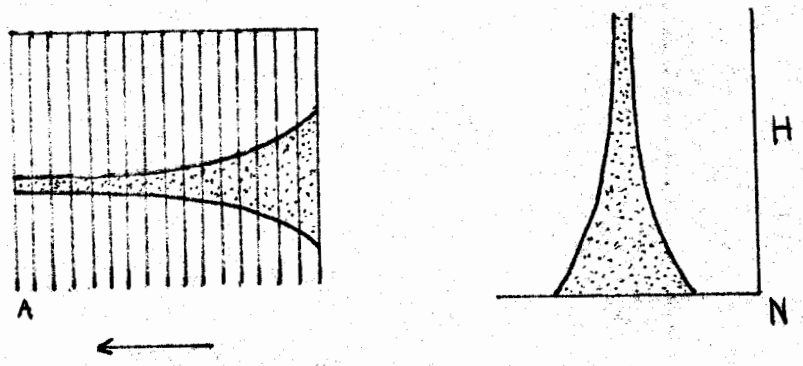
In the Schlieren scanning method[22] (Figure III.) monochromatic light from the horizontal slit 1 is focused by the large lens, L at F in the plane across which diaphragm, D, can be moved.

Figure III.



The camera objective, L_2, focused on electrophoretic cell, C, produces on the photographers plate, P, an image of that part of C in which the protein is migrating. If ray A passes through the solution where the gradient of concentration of protein is highest, it will be bent and cut off by D, since the gradient of refractive index will also be greatest at this point. Then corresponding to the level in the cell through which A passes, there will be a dark spot on a positive of the photograph. This is shown in Figure IVA by a dark spot in section A which is the first of a series of photographs.

Figure IV



(A) (B)

The rest of the photographic plate is obscured by a plate in which there is a vertical slit 2 which admits light the width of 1a. For the second photograph, P is moved in a horizontal direction or with the arrow in Figure IIA, and D is moved up thus cutting off rays bent slightly less than A as well as A. Therefore, the second dark spot is slightly wider than a. This process is continued to build to the pattern in Figure IVA. In practice, P and D are coordinated with gears and move continuously with a motor. For reading, the Schlieren diagram the pattern is rotated 90° as in Figure IVB. H is a mark on cell C. This serves as a base in calculating the level in C at which there is the greatest change of refractive index. N is the base line of the pattern. Ideally, it is straight and represents levels in the cell at which the solution in the cell is homogeneous.

The mathematics of this method[90] are derived from the fact that the angular deviation of a pencil of light in the boundary is proportional, under proper conditons, to the gradient, dn/dx, of the refractive index and the horizontal breadth, a, of the boundary. The displacement, Δ, of the Schlieren diaphragm necessary to intercept the deflected pencil is proportional to the optical lever arm, b, of the apparatus; thus:

$$\Delta = ab\, dn/dx \qquad (1)$$

a + b are constants for the instrument. dn/dx varies vertically through the boundary.

As the Schlieren diaphram is raised, the first pencils of light to be intercepted are those from the steepest gradient of refractive index, ie.

the center of the boundary. With increased displacement of the diaphram, we would get an indication of the refractive index through the boundary. Since the refraction power of a solution is proportional to the concentration of protein, quantative estimates of protein concentration can be derived. If, as was noted above, there is a slow simultaneous horizontal movement of slit 2 and vertical displacement of the Schlieren diaphram, there would be produced on the photographic plate, a transparent area proportional to $\int \Delta dx$. Then from equation (1), we get,

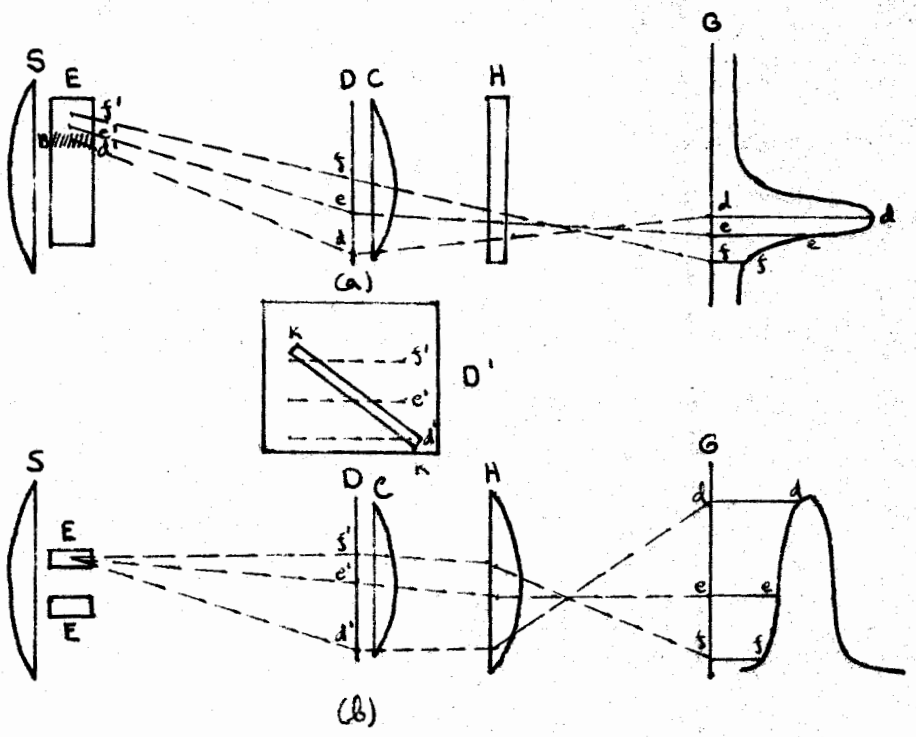
$$\int \Delta dx = \int ab \frac{dn}{dx} dx = ab \Delta n$$

Δn = the refractive index increment due to the protein constituent causing the boundary.

This may be applied to the quantative analysis of protein mixtures[81, 82].

With the cylindrical lens method of Philpot, (Figure V.), the illuminated horizontal slit is focused by the Schlieren lens, S, in the plane of the Schlieren diaphram, D. The latter contains a diagonal slit, kk, as shown in D'. The camera objective, C, is focused on the electrophoretic cell, E, and forms in the absence of lens, H, a normal image of the cell on the ground glass or photographic plate, G.

Figure V.



The cylindrical lens, H, with its axis vertical, is focused on the Schlieren diaphram and also on the plate, G. Viewed from the side (Figure Va.) H has no effect on the light pencils forming the cell image. Thus, the vertical coordinate of each point in the image is conjugate to a corresponding level in the channel of the cell, E, and, owing to the focusing action of lens, C, this also remains true for pencils that may be refracted by gradients in the channel. Viewed from above, however (Figure Vb.), the cylindrical lens, H, in conjunction with the diagonal slit in D, causes a lateral deviation of a pencil of light that is proportional to the vertical deviation the pencil suffered in the boundary. The curves to the right in Figure V. represent the patterns of boundary, B (in Va.) as it would appear on the screen if the latter

were hinged at the side and turned toward the reader. In Vb, the pattern is hinged at the top and turned.

If the fluid in the cell is homogeneous, all the light through the channel is concentrated in an image of the slit at the upper or normal level of the diaphram (L' of D'). Only the extreme left hand portion of the light in this image passes through the diaphram to form a straight vertical line; ie., the base line on the screen (e-f). If there is a boundary in the cell, a pencil through the maximum gradient is deflected downward as d'-d and forms an image of the slit at the lower level, d', of the diaphram. Owing to the angle the diaphram slit makes with the vertical, the portion of the light in the lower image, d', that enters the slit is shifted latterally from the position at which the normal pencil enters by an amount proportional to the vertical deflection in the boundary gradient. The cylindrical lens, H, consequently imparts to the pencil a corresponding lateral shift in the opposite direction to position d in Figure Vb without effecting its vertical position, d, in Figure Va. All intermediate rays are effected to a corresponding extent.

Most types of electrophoretic apparatus are equipped to use the scanning and cylindrical lens procedures interchangeably. Either one of these methods can be used with either a slit or a straight edge. This is quite advantageous since each method has certain advantages in a given situation. The slit shows the pattern as an illuminated lene, and when combined with the cylindrical lens method, is usually preferred for visual observation because of the better contrast. The straight edge is preferred for photographic work due to the superior resolving power and

simpler diffraction phenomenom[81] characteristic of this diaphram. By using the scanning method, we can get away from the inherent optical errors of the uncorrected cylindrical lens (see section on technique errors).

Buffers.

The choice of a buffer is of great importance in electrophoresis because different buffers not only give variation with respect to the configuration of the various peaks of the pattern, but also variations in the number of peaks produced. There are several points to consider in the choice of a buffer. A buffer should have a high capacity to relatively reduce the buffer action of the protein itself. Buffer capacity, β, is defined as the number of equivalents of a strong acid or base taken up by one liter of buffer to change the pH one unit.

$$\beta = \frac{2.3[A^-][HA]}{[A^-]+[HA]}$$

$[A^-]$ = concentration of ionized forms of buffer.
$[HA]$ = concentration of unionized forms of buffer.

Since pK (-log K) is the pH of maximum buffer capacity (K equals dissociation constant), this will aid in determining the pH at which the buffer should be used. A buffer should also have a low specific conductance to decrease disturbances from the heating effect of the current. Since, however, both capacity and conductance increase with concentration of buffer salts, these first two considerations are not compatable, and a compromise must be found.

Since buffer capacity does not depend on ionic mobilities, buffer salts with ions of low mobility should be selected. These ions show less effect on mobility of the proteins at the same pH and ionic strength. Thus Li is better than Na, and Na is better than K. In general, high concentration of weak electrolytes should be avoided unless allowance is made for the effects these have on the temperature of maximum density of the solution.

Many buffers have been tried in an effort to overcome the incompatabilities of some of the above mentioned considerations, and to achieve the best possible resolution of the protein fractions. Probably the most used buffer today and the one that gives the best symetry and resolution of the peaks is the Sodium Diethyl Barbiturate buffer at pH of 8.6 and ionic strength of 0.1. Also used is a phosphate buffer of pH 7.7 and ionic strength of 0.2 or a Lithium veronal buffer of pH 7.9 and ionic strength of 0.05. The last two are mostly used in special situations.

Technique.

Only an outline of the technique of electrophoresis will be given here. For more elaborate discussions, see references 81, 84, 88, 90, and 154.

The first step in electrophoretic analysis is the dialysis of the protein solution against a portion of the buffer which is to be used in the upper portion of the cell. If this is done with a mechanical agitator and cellophane tubing, equilibrium will be reached in about two hours[133].

The electrophoretic cell is then filled as follows: the bottom section is filled with the solution of dialized protein and buffer; one arm of the middle section is filled with this same solution while the other is filled with plain buffer. The middle section is then slid to one side and the two upper arms of the cell are filled with plain buffer. The entire cell is then placed in the thermostat at 0.5° C., and the entire system is allowed to reach equilibrium. When this has been obtained, the electrode vessels are filled with buffer, concentrated KCl is introduced around the electrodes, the middle section of the cell is slid back into place to form the boundaries, and by means of a compensator the boundaries are brought to any desired level in the tube. The current is then allowed to flow for a given length of time, usually two hours, and the results are recorded, either visually or photographically.

A new method of simplified electrophoretic analysis at room temperature has been developed by Abramson[1], but since it has only attained limited acceptance, it will not be discussed here. It has been used mainly for the physical separation of protein constituents.

Possible Sources of Error Associated With Technique and Apparatus.

In general, there are two main possible sources of error; that associated with the optical system, and error due to thermal convection current in the cell.

If a straight edge is used with a photographic plate to record the results, the position of the contour of the pattern shifts slightly with the exposure and development of the plate[84]. This is well within experimental error, however, and does not influence the accuracy of the end result. A source of error which may introduce more severe aberations

in the pattern is seen with the use of the cylindrical lens method[84]. The objective C (Figure V.) is a corrected achromat, usually of two inches in diameter and 36 inches focal length and as such, is used at an aperture of F/18. The cylindrical lens, however, is a single element of the same diameter but has a focal length of 16 inches. It, therefore, works at the higher aperture of F/8. Since this exceeds the maximum aperture of about F/16 usually considered safe[83] for photography with a simple lens, an error in the optical system may be introduced.

The prevention of thermal convection currents is of the utmost importance in the accuracy of electrophoretic analysis. Since heat is generated in each volume element of the solution but flows to the thermostat only through the wall of the tube, the solution along the axis of the tube is hotter than that along the wall where the solution will be heavier and falling, thus setting up convection currents. This source of error is minimized by having the solution at/or near its temperature of maximum density so the temperature difference will not give associated density variations. The temperature, t_s, of a solution in a steady state as a function of distance, r, from the axis is[90]:

$$t_r = t_0 - t_s = \frac{I^2 \epsilon}{4 k K_s} r^2$$

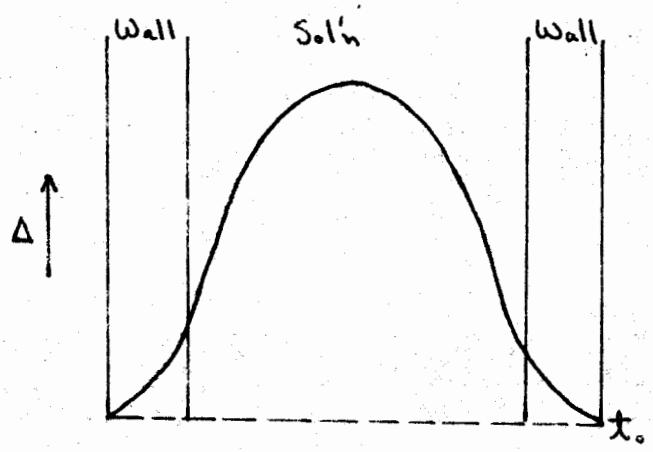
The temperature of the glass, t_g, is:

$$t_g - t_0 = \frac{a^2 I^2 \epsilon}{2 k K_g} \ln \frac{b}{r}$$

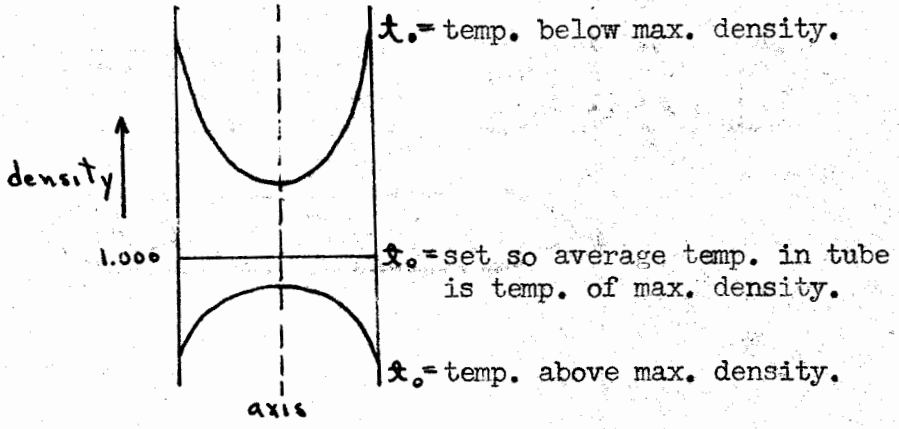
$a + b$ = inside and outside radii.
$K_s + K_g$ = thermal conductivities of solution and glass.
I = current intensity.
$\mathfrak{E}$ = electrical equivalent of heat.
t_o = thermostat temperature.

Using the above equations, the following diagrams were developed to show the association between temperature and density which result in convection currents.

Change of Temperature in the Wall and Solution



Changes in Density at Different Distances from the Axis of the Tube at Various Thermostat Settings



Another consideration must be the greatest amount of current that can be put through a cell without developing heat at a greater rate than it can be dissipated[5]. The current develops heat at the rate of

$$H = \frac{i^2}{g^2 K} \text{ watts/cm}^3 \qquad (4)$$

Experience shows that with the thermostat at 1° C., the maximum power which may be dissipated in the cell without causing convection currents at the usual protein concentration is .15 watts/cm^3. By eliminating i between equation (4) and (2),

$$E = \sqrt{\frac{H}{K}}$$

This shows that the maximum allowable field strength is proportional to 1/K. Higher potential gradients may be used with buffers of low conductivity, but this increases electrophoretic anomalies.

THE NORMAL ELECTROPHORETIC PATTERN

Development.

The first work by Tiselius was done on horse serum[156], and in this he assigned symbols to the various fractions that are still used today. He showed albumin as the fastest boundary, then α, β, γ, globulin in decreasing order of mobility, and a fifth stationary border attributed to a Δ component. In addition, plasma showed a sixth boundary between γ and Δ globulin, and is due to fibrinogen[63, 149]. Later the stationary Δ boundary in the ascending limb and the corresponding ϵ boundary in the descending limb were shown to be boundary anomalies[159] due largely to the transport of buffer ions by the proteins during electrophoresis, but also due to a superimposed protein gradient[84]. Following this, a low peak of high mobility shown in normal plasma and serum[111] was recognized as a false moving boundary by Svensson. It was due to the use of a buffer containing two negative ions. The so called pseudoglobulin and euglobulin were shown to be mixtures of several globulin components[154, 156], which differed chiefly in solubility behavior rather than in composition.

The above findings were extended and confirmed, and more accurate measurements obtained by the use of many refined methods such as the optically integrating refractive index technique[84], the mechanical integrating Schlieren scanning method[82, 88, 92], the light absorption method of Svedberg[153], and the scale refractive index method of Lamm. Thus, through continued work, research, the complexities of the electrophoretic patterns are ever being better understood.

The Ideal Case and Variations[6, 84].

The ideal case of electrophoresis would be a very dilute protein solution in a buffer of high electrolyte concentration so that the conductivity of the solution will be determined almost entirely by the buffer ions because of the low mobility and low equivalent concentration of proteinsions. Thus, there would be a constant pH and uniform electric field for the proteins in which to migrate, and the protein concentration would vary from a constant value below a boundary to zero above. As this ideal case is approached, however, the low protein concentration results in areas under the peaks which are not of sufficient magnitude to be determined accurately and the vertical density gradient in the boundaries are frequently insufficient to stabilize the latter against the disturbing effects of convection.

The pattern resulting from the "ideal case" would have the "ideal" attributes which are strived for in all electrophoretic analyses. The volume swept through by the ascending and descending boundaries due to each component would be identical and proportional to the mobilities of the separate components. In addition, the area under each peak would be proportional to the concentration of the component, and the patterns from the two sides of the channel would be mirror images. In actual practice, consistant variation from the ideal case will be noted. In general, the distance moved by the boundaries will be greater in the ascending limb, the rising albumin boundary will be sharper than the descending, the area of the δ peak is greater than the ϵ peak, and the areas under a given peak are not the same in the ascending and descending boundaries although the total areas including the stationary boundaries are the same.

Also to be considered is the presence of salt gradients in the δ and ϵ boundaries which, although partially balanced by small changes in salt concentration across each of the protein boundaries[92], must be considered in interpreting the pattern because of the corresponding decrease in the area under the protein boundaries.

The assymetry between the patterns is due to the fact that the contribution of the protein ions to the conductivity of the solution is not negligible and this causes small gradients of conductivity, pH, and protein concentration across the boundaries in addition to the gradients of the protein constituent that disappears in the boundary. These effects are of different magnitudes for different proteins and buffer concentration, and thus the analysis varies with both. The true composition of the protein mixture may be obtained by extrapolation of apparent concentration either to zero protein concentration at constant ionic strength or to infinite salt concentration at constant protein content[7].

The volume swept through by the ascending boundary is larger than the corresponding volume for the descending boundary. This is due to the fact that the field strength is greater between the ascending boundary and δ than it is in the original protein solution. The volume swept through by the descending boundary and the conductivity of the protein solution are used to calculate mobility since the calculation is more complex when using the ascending pattern[92].

In contradistinction, the total area of the ascending and descending patterns are equal and proportional to the refractive index differing between protein solution and buffer.

$$\int_{top}^{bottom} \frac{dn}{dx}\, dx = n_p - n_o$$

This is independent of the time of electrophoresis, provided no boundaries leave the cell. However, since $A_\delta > A_\epsilon$, the area of the moving peaks is less in the ascending pattern.

The shape of the peaks is controlled by two general factors; the rate of boundary spreading and the difference in field strength. The rate of boundary spreading is determined partially by the superimposed electric field gradients and partially by diffusion and inhomogeneity of the protein. The descending peaks are broader and shorter than the corresponding ascending peaks. This is because the field strength is greater on the leading edge of the descending peak than on the trailing edge so that the molecules move more rapidly in the leading edge, thus getting broader boundaries than one would expect for diffusion alone (conductivity effect). Thus difference in field strength is partially compensated for by the difference in pH of the solutions on different sides of the boundary. Since the pH is lower on the leading edge of the descending boundary, the molecules have a lower mobility and in some cases, this pH effect may predominate over the difference in the field strength.

The Normal Electrophoretic Pattern.

The normal electrophoretic pattern and proportions and concentration of the various components has been determined by many workers (see references 7, 45, 57, 58, 68, 74, 91, 99, 111, 123, 129, 136, 138, 147, and 155) and although the analyses vary somewhat with different

buffers[110], the results with a given buffer are in very good agreement. The most widely used buffer is Sodium diethyl barbiturate, and the pattern and results obtained with its use are shown in Figure I and Table I.

Figure I.

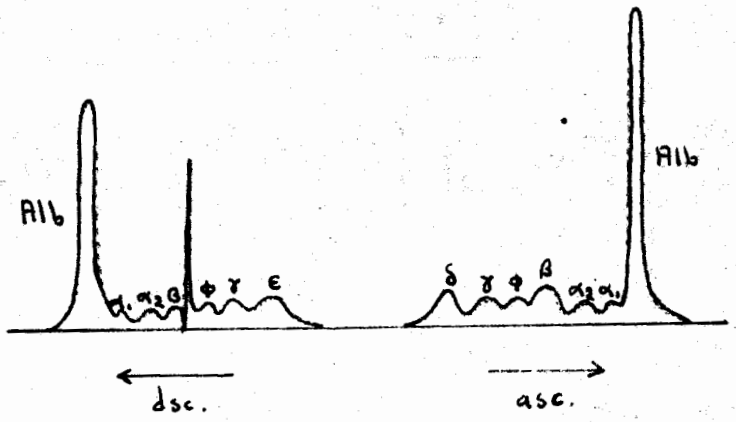


Table I.

		Albumen		α_1		α_2		β		ϕ		γ		A/G
		ratio	gm%	ratio	gm%	ratio	gm%	ratio	gm%	ratio	gm%	ratio	gm%	
Concentrations	Mean	.603	4.04	.046	.31	.072	.48	.121	.81	.051	.34	.110	.74	1.53
	S.D	.028	.27	.007	.051	.013	.083	.019	.126	.006	.059	.025	.151	.181
Mobilities (cm^2/volt sec $\times 10^5$)	Mean	5.94		5.07		4.04		2.83		2.14		1.02		
	S.D.	.267		.236		.256		.241		.252		.282		

All the studies referred to in this paper unless otherwise indicated were carried out with Sodium diethyl barbiturate as the buffer. The excellent agreement between the results of many workers shows the high degree of precision with which electrophoretic analyses can be carried out.

Studies on the plasma proteins of normal children have been carried out by Knapp and Routh[72]. They divided their patients into four age groups: group 1, infants, 6 to 52weeks old; group 2, preschool, 1 to 4 years; group 3, mid-childhood, 5 to 11 years; and group 4, adolescence, 13 to 17 years. In general, there were only minor changes on most fractions. The albumin level was quite normal except in group 3 in which it was slightly decreased. A small but persistant decrease was noted in the alpha-1 globulin of all groups. The most striking changes were noted in the gamma globulin and alpha-2 globulin. The gamma globulin showed a marked drop from the newborn until one year of age. Following this, there was a steady increase until it reached the normal level in midchildhood. Since antibodies are known to be associated with the gamma globulin fraction, the noted changes in this fraction would seem to correlate with the loss of acquired maternal antibodies and the gradual acquisition of active antibodies. There seemed to be a reciprocal relationship between the alpha-2 globulin and gamma globulin, with the alpha-2 globulin being low in the newborn period, increasing to above normal in infancy, and then decreasing to the adult level with age. The percentage of albumin was noted to decrease from group 1 to group 3, and was lowest in group 3. However, since the total serum protein was increased in group 3, the increase in actual concentration

of gamma globulin, fibroinogen, and other globulins takes place without any significant decrease in absolute concentration of albumin. The presence of a recent infection was noted to cause an increase in gamma globulin[97], and observation during the acute febrile stage of an infection showed a rise in alpha globulin followed later by an increase in gamma globulin.

Comparative studies of normal material, infant and fetal sera, have been carried out by several workers[87, 117, 138], as well as detailed work on embryonic sera alone[37, 116, 132, 161]. The study of maternal sera revealed a decrease in the albumin, a slight increase in the alpha globulin, and a marked increase in the beta globulin. There was no change in the gamma globulin. The study of the corresponding fetal sera showed diametrically opposite changes. There was an increased albumin and gamma globulin and a decrease in the alpha and beta globulin. In general, however, the fetal proteins were more nearly normal than the maternal proteins. Thus, although the relative concentration of fetal gamma globulin is above normal, and that of the beta globulin is below normal, the differences are not great while with maternal sera the alpha-1, alpha-2, and especially the beta globulin are markedly above normal as was noted above. Changes in the first few weeks of life have also been determined[117]. In the first few days of post natal life, the alpha and beta globulin increases, the albumin shows a slight increase, and the gamma globulin then begins to decrease. An increase in the A/G which is noted during the first few days is due mainly to the increase of alpha and beta globulin while the noted decrease in the ratio in the next three weeks is due to the decrease in gamma globulin. The

further increase of the A/G up to ten weeks is due to the increase of the albumin and a persistance of the low gamma globulin.

The Individual Components of the Electrophoretic Pattern.

The individual peaks in the normal electrophoretic pattern have been extensively studied and many efforts have been made to determine the elements of their composition. By various methods of chemical and physical characterization, the complex nature of the albumin and the various globulins has been shown[26, 27, 28, 29, 61, 108, 146, 154] as well as the presence of biologically active proteins in amounts too small to recognize in the patterns of the native proteins[28, 121, 128, 139]. In general, the various fractions are considered native complexes of the protein moieties with low molecular non-protein substances.

The albumin peak is the tallest and best defined in both ascending and descending boundaries. It has been shown that it will separate into two components on prolonged electrophoresis[16], especially at a pH just above 4[98, 146] and in the presence of certain ions[7].

The globulin peaks are in general lower and subject to a greater degree of electrophoretic spreading. This progressive spreading or blurring of the boundaries is due only partly to diffusion, and the remainder is caused by a slight electrochemical inhomogeneity of the material. This second component in contrast to diffusion is essentially reversible in nature[2, 143, 147]. This wide reversible spreading is most marked in the gamma globulin boundary and shows its heterogeneity [63, 65, 144, 145]. The beta globulin peak shows an anomaly in the descending peak which is like as total reflection phenomena and is ascribed to the instability of this component after electrophoretic separation from

the other serum proteins[91].

The association of carbohydrates and lipoids with blood proteins is intimate[16, 20, 156, 164], and these substances effect both the refractive increment and mobility of the proteins to which they are bound, especially the alpha ad beta globulin[17, 74, 79, 126, 164]. Tiselius considered the albumin to contain bilirubin in addition to carbohydrate, and the beta globulin to contain the lipids such as cholesterol. However, subsequent work has shown that all the fractions contain some cholesterol and phospholipids in bound form, the alpha and beta globulin being much richer in these components than the albumin and globulin. It has also been shown that all fractions contain some carbohydrate with the alpha and beta globulin being richest in this component also. In spite of the above findings, more than 50% of the total lipids and carbohydrates are contained in the albumin and gamma globulin due to the relatively small concentrations of the alpha and beta globulin[16, 164].

Calculations.

The calculation of the concentration of the various protein fractions present in the electrophoretic pattern is based on the determination of the area, A_i, under that part of the curve which is due to component, i, of the serum studied. Since the specific refractive increments of the electrophoretically separable plasma proteins have not been measured, it is only possible to determine their concentration as differences in refractive indexes by the area determination and not in terms of protein nitrogen or dry weight.

In order to develop the basic calculation necessary for the determinations, several assumptions are necessary. Each protein boundary

is considered to contain a small salt gradient, thus the observed area, A_i, is the sum of an area P_i, due to the gradient of protein, i, and an area, $-S_i$, which is due to the salt; the negative sign indicating the inverted nature of this gradient. Two other assumptions that are made are that S_i is proportional to P_i and that this proportionality factor is the same for all components. Thus, it can be seen that the concentration of one component can be calculated relative to another by the equation:

$$P_{id}/P_{Rd} = A_{id}/A_{Rd}$$

d indicates that the determinations were made from the descending or cathode patterns.
A indicates the total pattern area.

Valves from the anode or rising pattern, A_{ir}, can be used if the additional assumption is made that all the protein components are held in this same proportion through the δ boundary. Experimental data[132] indicates that these assumptions are valid within the experimental error of the calculations.

Due to the incomplete resolution of the protein peaks, the gradients of the different boundaries overlap and various methods have been devised to separate these areas due to the various components into measureable quantities. Tiselius and Kabat[159] drew an ordinate from the lowest point between two adjacent peaks. This method has been superceded, however, by the method of constructing Gaussian-shaped curves[16] for each of the peaks such that their ordinates at every point add up

to give the experimental curve. These curves may not be symetrical due to the gradient of pH and conductivity in the boundary, and because the proteins in one component may not be homogeneous with respect to size, shape, and iso-electric point.

In actual practice, the area of component, i, is measured with a planimeter[87]. This is designated A_{id} if the boundary is descending and A_{ir} if the boundary is rising. The total area, A_t, should be the same for both sides of the pattern since it is proportional to the integral,

$$\int_{\eta_B}^{\eta_P} \left(dn/dh\right) dh \qquad (1)$$

whose limits, the refractive indices of the protein, and buffer, are the same for both channels. The final relationship is:

$$\int_{\eta_B}^{\eta_P} \left(dn/dh\right) dh = \eta_P - \eta_B = A_t/abGE_cE_t\pi \qquad (2)$$

h = height in the channel.
a = cell thickness.
b = optical distance from cell to Schlerein diaphram.
G = ratio of plate to diaphram travel.
E_c = camera enlargement.
E_t = tracing enlargement.
π = planimeter units per $cm.^2$.

The corresponding protein concentration would then be:

$$\rho = (\eta_P - \eta_B)/K \qquad (3)$$

K = specific refraction increment.

If D is the factor by which the plasma or serum is diluted before

electrophoresis, the original concentration in grams per cent of protein is

$$P = D_P \qquad (4)$$

There are, however, various errors inherent in D and K in the above calculation. Errors in D are due to the fact that during dialysis, a small amount of H_2O enters the bag, and also with plasma, the diluting effect of the solution of anticoagulation must be considered. Errors in K result from variations in temperature, wave length, and type of protein.

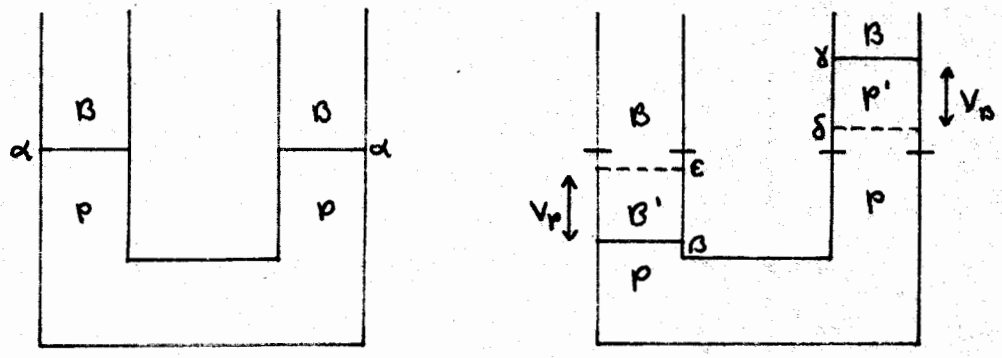
In order to finally compute the concentration, P_i, of the individual components, correction must be made for the δ and ϵ effects. These are boundary anomalies as will be shown later, and are not due to protein fractions. Thus, the relative concentration of the i component is given very closely by:

$A_{id}/A_t - A_\epsilon$ for the descending boundary, and

$A_{ir}/A_t - A_\delta$ for the rising boundary.

Boundary Anomalies.

The general principles of the formation of the boundary anomalies δ and ϵ have been worked out by Longsworth[90] and greatly extended by Hoch[62], and a brief discussion of their formation will be presented here. In Figure 2 is shown the initial and terminal phase of an electrophoretic process.



β, P = concentration of buffer and protein respectively.
α = original boundary.
β, γ = final descending and ascending boundaries.
V_P = volume of buffer solution moving into protein and having a concentration of beta-1.
V_B = volume of protein solution moving into buffer and having a concentration of P-1.
ϵ, δ = the boundary anomalies.

Since the concentration of buffer salt is different beta and P after dialysis due to the Donnan equilibrium, beta-1 has to be "adjusted," in general, to a value different from beta in such a way that its regulating function has the same value as that of the protein solution it has replaced. As was noted before, the regulating function defines a property of the solution which, at any given point, retains a constant value independent of changes of concentration caused by electrolytic migration. If, from such a migration, a different ion species appears at a point, their concentration will be adjusted to values compatable with the constant determined by the initial concentration of the solution. Since a boundary will form where a change in concentration occurs, the boundary will

thus be formed between two solutions of the same salt but at different concentration. A similar boundary will be formed at due to a difference in protein concentration, P and P^1, as the protein replaces buffer solution. This boundary has a greater visability since it involves a gradient of protein concentration.

Minor boundary anomalies may arise from small differences in pH between the protein and buffer solution as required by the Donnan equilibrium. These may be greatly minimized by the proper choice of a buffer and exact technique.

Factors Influencing Electrophoretic Patterns and Mobility.

Factors which have an influence on the electrophoretic pattern and protein mobility may, in general, be divided into four basic categories: physical factors, factors connected with buffer, technique, and protein concentration. These are usually minimized by the use of standard technique; however, they may be a necessary consideration in the interpretation of certain types of electrophoretic analyses.

Studies on the effects of storage[7, 74, 96, 113, 138], freezing, and drying[30, 39, 60, 73, 104, 125, 131, 148, 156, 160] on the electrophoretic pattern have been extensively carried out. In general, there was no effect noted which resulted from any of the above treatments. Irradiation with ultraviolet light[39] gave a new peak from the denatured protein which resulted at the expense of the other components. This same effect was also noted in heat denatured sera[39, 160].

The buffer effects the ionic mobility and electrophoretic pattern in three ways: pH, ionic strength, and types of ions present. It has been shown[2] that the electrophoretic mobility depends on pH, and

that the ionic strength and curves of mobility at various pH's are closely related to the acid-base titration curves of the protein involved. The mobility is likewise effected by the types of ions present in the buffer[7] and in addition, it has been noted that interaction between proteins and other proteins[21, 73, 93, 114, 158, 157] or smaller ions[7, 8, 95, 101, 119, 130] may give minor variations in the electrophoretic pattern.

The variations in technique such as voltage, dialysis, protein concentration and the presence of hemolysis may effect the electrophoretic pattern. While an alteration in voltage shows little variation in the pattern[64], a decrease in protein concentration gives a relative and absolute increase in albumin along with a relative decrease of gamma globulin and a slight increase of the other globulins[64, 123]. When hemalysis resulted in the addition of Hb to the protein, there was an increase in gamma globulin when a phosphate buffer was used and an increase in beta globulin when a barbiturate buffer was used[118]. The effect of dialysis, continued after one to two days, was shown to be minimal[118].

ELECTROPHORETIC PATTERNS IN PATHOLOGIC CONDITIONS

Virus Diseases.

In poliomyelitis[70, 71], the only significant deviation from the normal pattern was a varying abnormality of the beta disturbance. The change, varying all the way from minor alterations to complete absence, was noted in 80% of poliomyelitis cases when a phosphate buffer was used. There was no correlation between the change noted and the duration or severity of the disease. Since the beta anomaly is thought to be due either to convections from decreased stability in the neighborhood of the beta globulin[94, 91] or to a turbidity from the liberation of a lypoprotein complex[91, 20] in this area, it would seem that in poliomyelitis there either was a decreased dissociation tendency of this lypoprotein or else it is absent or decreased in concentration. The findings in encephalomyeloradiculitis (Guillain-Barre's Disease) were similar to those in poliomyelitis[54].

The electrophoretic pattern in infectious hepatitis and homologous serum jaundice[105, 134, 57, 109] showed a decrease in the albumin which was approximately mirrored by an increase of the gamma globulin. The deviation from normal was in general associated with the severity of the disease with the maximum decrease of albumin occurring in the first ten days. This may be correlated with the temporary parenchymatous damage to the liver. There was some variation in the alpha-2 and beta globulin but this change was quite variable. During the course of the disease, the pattern tended toward normal; however, the severe cases showed residual abnormalities even in the absence of symptoms. A check-up two to three years later still showed minor varia-

tion in most patterns, especially if the disease had been quite severe. The alterations in pattern could either be due to the hepatic dysfunction or the infectious process; however, the secondary increase of the gamma globulin is probably due to a different mechanism[105]. It is thought to be due either to a compensatory mechanism in an attempt to maintain osmotic relationships, or to the formation of antibodies or to an alteration in the relative rate of production and utilization of the albumin and globulin.

In studies on lymphogranuloma venerium[51, 91] a marked increase of all globulin fractions was noted.

Bacterial Diseases.

In acute bacterial diseases such as pneumonia[91, 100], peritonitis[91], and tonsillitis[91], an increase of the alpha globulin with an accompanying decrease of albumin was noted. This is typical of the change noted in most acute infectious processes.

Studies on rheumatic fever[48, 11, 91, 99, 136] showed a decrease in albumin with a varying increase in the alpha-1, alpha-2, and gamma globulin. These changes were most marked during the acute stage but they persisted to a lesser degree during inactivity. No definite changes were noted before and after salicylate therapy. Changes in scarlet fever[48] were similar to those noted in rheumatic fever. There was, however, a delay in the return to normal in cases which developed rheumatic fever following the attack of scarlet fever. This was most noticed as a persistant increase of the alpha-2 globulin.

In tuberculosis[100, 140, 141, 142], the electrophoretic pattern could be correlated with the stage of the disease. In early cases there is a decrease in albumin and fibrinogen with an increase

in gamma globulin. As the disease progresses, the above changes become more marked and are associated with an increase in the alpha globulin and the appearance of an "x" component with a mobility slightly greater than albumin. This probably shows sensitization to the tuberculin protein and has been shown to increase in declining clinical courses and to decrease with improvement. Several explanations have been offered for the increase in the alpha globulin. Either it is associated with a specific antibody to a fraction of tuberculin or to sensitization of the host or to tissue distruction[142]. In terminal stages, an increase of beta globulin was noted. Changes noted in leprosy[141] were similar with an increase of alpha and gamma globulin as the most marked variation from normal.

Alterations in the electrophoretic pattern of patients with brucellosis were similar to those noted with infectious hepatitis[134].

Rickettsial Diseases.

Changes in typhus fever[50] were a marked increase in gamma globulin and a slight decrease of alpha-2 globulin. The total serum protein was very low during the acute stage, and was abnormally high during convalescence. The albumin was also decreased during the acute stage. The early increase of gamma globulin is probably not due to antibodies since the Weil-Felix and complement fixation tests were negative at that time.

Spirochetal Diseases.

Studies on syphilitic sera have been carried out by many workers [12, 31, 33, 34, 35, 40, 41, 120]. The most characteristic change was a decrease in the albumin. This was noted in primary, secondary

and tertiary lues, and was evident in both relative and absolute concentration. An increase of alpha-1 globulin was noted only in the secondary stage, while the alpha-2 globulin was increased in both the secondary and tertiary stages. A moderate increase was also noted in the gamma globulin in all stages, but this was not as marked as the changes in the albumin. Studies of sera with false positive serologic reactions or following treatment showed no characteristic changes[31, 12]. Other investigators[34, 35] using electrophoretic and serologic examination of fractions removed from flocculated sera have shown that the beta and gamma globulins are the carriers of the Kahn and Wasserman reagins.

Studies of the sera from patients with Pinta and Yaws[43] have been carried out. With the Pinta sera, the only change noted was a slight increase in gamma globulin and a decrease in albumin, but these were not characteristic. In the sera from Yaws' patients, the globulin peaks were noted to be larger than in normal syphilitic or Pinta sera. It was also noted that the beta anomaly was absent in the sera from both Pinta and Yaws.

Protozoan Diseases.

Investigation of malaria sera[47, 59] show that following a paroxysm, there is a decrease in the A/G from a slight decrease of albumin, but mainly from an increase in fibrinogen and gamma globulin. The total serum protein was normal in all cases. These changes tended toward normal after the paroxysms were stopped by therapy in spite of a persistant infection. Thus it seems that the alterations in the electrophoretic pattern appears to be due to the hosts reaction to the

acute process of the paroxysm rather than to the mere presence of the parasites.

In Kala Azar, there was a marked inversion of the A/G with an increase of gamma globulin and a decrease in albumin. The gamma globulin peak showed a skewing on the slow side and a diffuseness on the fast side, and its mobility was outside normal limits. This is different than the gamma globulin changes seem in most other disease processes for here there is a new component of slower inability present.

Non-infectious Diseases.

Plasma from patients in cardiac failure[100] showed a decrease in albumin and an increase in the beta and gamma globulin. The alpha globulin and fibrinogen were within normal limits or slightly reduced. Effusions differed from the plasma in that there was a relatively higher albumin concentration and a relatively lower beta globulin concentration. The various types of liver disease show characteristic patterns. In portal cirrhosis [57, 75, 99, 100, 134, 150, 151], there is a marked decrease in albumin and an increase in gamma globulin with associated smaller increases of the alpha and beta globulin. These changes become more marked as ascities developed. Similar changes are seen in arsenic poisoning and other heavy metals[57]; however, the beta globulin peak becomes more prominent. Early obstructive jaundice gives little change in the electrophoretic pattern, but later there developes a decrease in albumin and increase in beta globulin. Since it has been shown[57], however, that extra hepatic jaundice can be quite severe with no associated alteration in the electrophoretic pattern, the above noted changes must be evidence of hepatic parenchymatous disorder[57, 91, 162].

Infectious hepatitis gives a decrease in albumin and an increase in gamma globulin with small associated increases in the alpha-2 and beta globulin[57, 105, 109] while infiltration with carcinoma gives more reduction in albumin and less increase in the gamma globulin[57].

The various stages of glomerulonephritis also gives quite characteristic patterns, especially the so called nephrotic stage[13, 14]. In acute glomerulonephritis the changes are minimal; however, the decrease in the albumin is more than is shown by the usual salting out methods. There is also a relative and actual increase in gamma globulin, while the alpha and beta globulin are normal unless an active infection is present. In the nephrotic stage, there is a decrease in albumin and gamma globulin with an associated increase in the alpha and beta globulin[91, 94, 99]. The pattern is almost diagnostic. A considerable part of the increase in the alpha and beta globulin is lipoids associated with the fractions which contribute more to the refractive increment than to the nitrogen content[7, 94]. In the terminal stage of glomerulonephritis, there is seen a moderate decrease of albumin and some increase in the globulin and fibrinogen[99].

In rheumatoid arthritis[49], there was found a relative decrease in albumin and a lowering of the A/G. This was associated with a marked increase in alpha globulin, a slight increase in the gamma globulin and a slight decrease in the beta globulin. All of these changes returned to normal with clinical improvement. There was no diagnostic significance that could be associated with these findings since they couldn't be distinguished from rheumatic fever.

Diseases of Unknown Etiology.

The sera from patients with uncomplicated essential hypertension[79]

showed only slight variations from normal. The most consistant change noted was a low alpha-2 globulin. This is significant since the renin substrate has the same mobility as the alpha-2 globulin. The only other diseases, however, which show this persistant decrease of the alpha-2 globulin are myxedema[76] and hypoadrenal activity of pituitary origin, and these are associated with a low arterial blood pressure. As the disease process enters the malignant phase, this decrease of alpha-2 globulin becomes more marked, and a marked increase in beta globulin is now noted. This may be an important change with vascular diseases since it is also seen in the various stages of glomerulonephritis and in uncontrolled diabetes with diabetic retinopathy[77]. No correlation could be found between fibrinogen and blood pressure which had been advocated by some workers[79].

Studies of the so-called collogen diseases[11, 23, 51, 141, 142], rheumatic fever, lupus erythematosis, scleroderma and sarcoidosis show significant electrophoretic changes which are common to all of them, but also there are various differential points which distinguish the various disease processes. In general, the electrophoretic pattern change common to all were a decrease in albumin and an increase in gamma globulin. However, with acute disseminated lupus erythematosis, there was a fixed increase in the gamma globulin, and an inverse relationship was noted between the albumin concentration and the severity of the disease while in the cases of sarcoidosis there seemed to be an inverse relationship between the albumin and gamma globulin. The reader is referred to the previous write up on rheumatic fever for the electrophoretic changes in that disease process. In several cases of acute ulcerative

colitis which were studied in conjunction with the above series[11], there was noted a varying decrease of the albumin fractions but no associated increase of the gamma globulin was found.

In cases of infectious mononucleosis[25, 59, 134, 152], there was found to be a decrease in albumin and an increase in gamma globulin. The changes in the alpha-1 and beta globulins were equivocal. These are almost identical findings to those in virus hepatitis, and may be indicative of hepatic involvement. The patterns before and after heterophil absorption were practically identical, thus showing that the increase in gamma globulin is not due to the heterophil antibodies.

Diseases of Endocrine Dysfunction.

Sera from patients with hyperthyroidism showed an absolute and relative decrease of the albumin with no consistant change found in the globulin fractions[76]. Following surgery, this pattern returned to normal in several months; however, if progressive exophthalmus or other residual signs of Graves' Disease remained, the decrease in the albumin fraction persisted. It may be that the low albumin fraction is indicative of impaired liver function and liver damage which is found in many hyperthyroid patients. In cases of hypothyroidism, a decrease in the albumin fraction was also noted; however, it was associated with a decrease in alpha globulin and an increase in beta globulin. Here also with proper therapy, the electrophoretic pattern returned to normal.

In cases of diabetes mellitus, the condition of the patient and the severity of the disease are reflected in the electrophoretic pattern[77, 137]. In severe uncontrolled diabetes with acidosis, there

was a decrease in albumin and an increase in beta globulin with a normal total serum protein while in mild diabetic sera with no acidosis, the only abnormality noted was a slight decrease in the total serum protein. Proper treatment in either of these examples resulted in a return to normal of the electrophoretic pattern. However, if complications were present such as diabetic retinopathy, there was a greater decrease in the albumin and an increase in the beta globulin than was noted in the uncomplicated cases. In these cases, it was also necessary to add a high protein diet to the therapeutic regime in order to restore the pattern to normal.

Cases of Addison's Disease[106, 107, 100] showed a total serum protein which was at the upper limit of normal. There was an absolute and relative decrease in albumin and an increase in the total globulin which was due mostly to increases in the beta and gamma fractions. When the patient was adequately maintained on adrenal extract and desoxycorticosterone, the total serum protein returned to normal, but the albumin remained low in many instances. This may indicate an inadequacy in the present therapy of Addison's Disease.

In Cushing's syndrome[78], there was found to be a decrease in the albumin and gamma globulin fractions with a slight increase in the alpha-2 globulin. Since adrenal extract causes a release of gamma globulin into the plasma from the lymphoid tissue, the increase in adrenal activity should give an increased gamma globulin. Therefore, the decrease in gamma globulin in these cases is probably due an exhaustion of the gamma globulin reserves in the lymphoid tissue due to the long duration of the adrenal stimulation. Following therapy

by x-ray to the pituitary and bilateral hemiadrenalectomy, the pattern in most cases tended toward normal.

Diseases of the Blood and Blood Forming Organs.

Many of the diseases of the blood cells and their precursors show pattern changes, but in general they are hard to interprete since the secondary effects may also alter the protein fractions. Fever and malnutrition are frequent complications and must be taken into consideration. Also encountered are alterations in the functions of the hemapoetic system in the formation of the globulins as well as liver damage resulting from anemia or infiltration.

In leukemias and lymphosarcoma, there are no significant alterations in the electrophoretic pattern unless the patient is very debilitated[91, 127]. In this case there is a decrease in the albumin and an increase in the alpha-1, gamma globulin and fibrinogen. Hodgkin's Disease[127, 135, 153], however, seems to produce an earlier and more severe systemic intoxication than do the other neoplastic diseases of lymphatic and hemopoetic organs. It usually gives the pattern of a chronic infection or wasting disease with a decrease in albumin and an increase in alpha and gamma globulin. Therapy of leukemias or Hodgkin's Disease with ACTH, cortisone, x-ray or nitrogen mustard gave no change in the electrophoretic pattern[52, 127].

The sera of multiple myeloma has been widely studied since its electrophoretic pattern is quite characteristic[3, 15, 58, 69, 91, 112]. In general it shows a new protein peak that has a mobility that varies between that of beta and gamma globulin. This new protein component varies from a slight abnormality to a high narrow spike, and many efforts

have been made to correlate its presence with that of the urinary Bence-Jones protein which is found in some cases of this disease. This, however, has not been very satisfactory. It has been shown that when this abnormal component has a mobility like beta globulin, its characteristics in blood and urine are similar, but when it has the mobility of gamma globulin, the urinary protein is different from the new component found in the blood. Much work is being done at present to try and determine the exact origin and fate of this new protein component.

Physical Injury.

Following severe bodily injury such as burns, fractures, hemorrhage, or freezing, it has been shown that there is first a decrease in the albumin and this is followed by an increase in alpha globulin[19, 53, 122, 147]. The decrease in the albumin may indicate the severity of the injury and always occurs earlier than the increase in alpha globulin. The pattern in these cases looks very much like that of a febrile infection, and suggests that this reaction of the proteins to injury is related to adrenal stimulation and the protein catabolic reaction.

Ocular Disturbances.

Bellows and his workers[10] have studied the electrophoretic patterns of sera from patients with various types of ocular pathology; namely, chronic glaucoma, iridocyclitis, and sympathetic ophthalmia. They noted that in all cases the ascending beta globulin boundary was composed of two or more peaks. This was seen only in the ascending boundary and they assumed that it was due to the ocular pathology since it was not present in any of the previous studies of other

diseases. These pattern changes were not characteristic of each type of ocular pathology, but seemed to be present in all cases of chronic intraocular disease. These workers surmised that the alteration in the ascending beta boundary was due either to a breaking up of some complex in the ascending beta boundary, or to the elaboration of a fraction in response to an antigen in the eye such as uveal pigment or lens protein. In this latter instance, the uveal pigment would probably be involved since these pattern changes were seen in pathologic processes not involving the lens.

DISCUSSION

As can be seen from the foregoing section, the only disease in which the electrophoretic patterns are of any diagnostic significance are in nephrosis and some types of myelomatosis. Instead, it seems that more often the pattern is characteristic of the hosts reaction to the disease or injury rather than typical of the type of pathology present, and many times the changes can be correlated with the severity of the physiologic disturbance. This, in turn, can be divided into several contributing factors; the duration or stage of the disease, state of nutrition, and a disturbance in the production or loss of plasma proteins due to the involvement of certain organs such as liver or kidney.

In general, there are two definite types of changes seen in the electrophoretic pattern. Those involving the albumin and those involving the globulins. In nearly every pathologic state, there is seen both a relative and absolute decrease in the albumin. This is probably associated with two factors which are common to many disease processes; a deficiency of protein and a general reaction of the body to injury or infection.

The alterations seen in the globulins can be divided into two distinct patterns and these can be seen electrophoretically in response to any infectious process. During the febrile state of an acute infection, there is first seen an increase of the alpha globulin as well as the above noted decrease in the albumin fraction. There is only one exception to this initial increase of the alpha globulin in a febrile illness, and that is seen in vivax malaria. This increase in

the alpha globulin may well be related to the so-called "C-reactive protein" which is in human sera in acute infections and is found in the alpha globulin fraction. A later change in the globulin fractions which is found in nearly all infection is a secondary rise of the gamma globulin. Similar changes to those noted above are seen in liver disease. This similarity between the patterns of infectious processes and liver disease suggests that liver dysfunction or injury may play a part in the changes seen in infection in addition to the more obvious increment of the gamma globulin by stimulated antibody production.

At present, the practical clinical applications of electrophoretic analysis are greatly limited both by the complex and time consuming technique and the expense and space necessary to set up the apparatus. At the time of this writing, however, there are several new types of apparatus being developed, which overcome many of the above mentioned limitations. Their further development and continued research will undoubtedly greatly extend the use of electrophoretic analysis in clinical medicine as well as broaden present day conceptions of a hosts response to injury and disease.

SUMMARY

1. The history of electrophoretic analysis was briefly discussed.
2. Basic principles, theory, and mathematics were developed as well as a brief summary of the apparatus and technique.
3. The normal electrophoretic pattern was discussed as well as its interpretation and various factors which tend to alter it.
4. The electrophoretic changes seen in various pathologic conditions were presented.
5. The basic changes seen in the serum protein fractions were discussed with their possible diagnostic and clinical applications.

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