AN ELECTROPHORETIC STUDY OF NORMAL CEREBROSPINAL FLUID PROTEIN

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A B S T R A C T

Part I.

The theoretical factors in electrophoretic investigation are considered and a brief review of the literature of electrophoretic methods is given. The problem of proper preparation of electrophoretic samples is considered. The normal values of protein components in serum are listed and the values obtained by separate methods are compared. A review of the electrophoretic investigations of cerebrospinal fluid protein is given up to December 1954. Cerebrospinal fluid probably contains albumin, alpha-2 globulin, beta globulin, and gamma globulin, whereas other components designated X-1, X and T, alpha-1 globulin and fibrinogen have not been consistently demonstrated. A simple method for electrophoresis of cerebrospinal fluid proteins is required, in which reproducible results may be obtained from small samples of fluid; and in which no component is denatured.

Part II.

After ruling out other methods of concentration as unsatisfactory, fifteen samples of cerebrospinal fluid from clinically normal patients were subjected to concentration by dialysis and negative pressure exerted through collodion bags. On an electrophoresis apparatus, samples of serum, of diluted and reconcentrated serum, of diluted and reconcentrated plasma as well as concentrated cerebrospinal fluid were run. The method was shown to be simple, did not denature the protein components, and was capable of reproducible results. A component designated as X was consistently seen preceding the albumin fraction, and the beta fraction was consistently increased. The other components consistently present were albumin, alpha-2 globulin, and gamma globulin. The method is considered to be suitable for clinical and research studies.

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REVIEW OF ELECTROPHORETIC TECHNIQUE AS APPLIED TO CEREBROSPINAL PROTEINS

Introduction

The cerebrospinal fluid proteins have been subjected to fractionation by chemical and electrophoretic methods. The chemical[18,33] and immuno-chemical methods[25,40,41] have resulted in the separation of cerebro-spinal fluid albumin and globulin, with the globulin being further fractionated to gamma globulin by immuno-chemical means.[42,43] The usual chemical test for determining the albumin-globulin ratio is not useful or dependable.[18,57] The colloidal gold tests of Lange[56,57,58,59,60,61,62] and others are not helpful in differentiating the type of globulins present[86] except in the case of gamma globulin, which shows a "D" (paretic, or first zone) curve in spite of the mixture of other globulins or of salts. The complicated chemical fractionation methods of Cohn[7,8] and others,[9,72,87] which have resulted in the separation of over thirty serum protein components, have not been applied to cerebro-spinal fluid proteins.

The development of a dependable, simple electrophoresis technique in the last fifteen years has made available an opportunity to study the cerebrospinal fluid protein components. To be useful as a clinical and experimental adjunct, these techniques require the concentrated product of small amounts of cerebrospinal fluid. This is now possible and it is the purpose of this paper to discuss the theory of microelectrophoretic technique and the application of electrophoresis to the study of cerebrospinal fluid proteins.

I. Theory of Electrophoresis and the Development of the Original Method

The quantitative and qualitative estimation of cerebrospinal fluid proteins by electrophoresis was the direct consequence of the development of a satisfactory device by Tiselius[92,93] for the measurement of the movement of protein particles in an electrical field.

This property of colloidal particles of moving in an electrical field had been known for well over a hundred years, and had been specifically applied by Hardy[36] as early as 1899 to protein particles. (He showed at this time that the migration velocity of the particles depended on the acidity of the medium).

Earlier methods of direct observation involved the use of a microscope but the observation of many of these small particles is impossible by this method. Therefore, it is necessary to study a layer of proteins rather than a single particle. If this layer moves into an adjacent medium of the same electrolyte concentration, two methods of analysis are open.[94] (a) The first is to take out the layer and analyse it for its biological and chemical properties. (b) The second method is to measure the distance the layer, or more precisely, the boundary of the layer, moves. These two methods can be combined.

At first the measurement of the distance the boundary moved could only be applied to colored substances, but gradually other methods came into use. Thus in 1926, Svedberg and Tiselius made use of the property of protein of absorbing ultra-violet light and this allowed them to measure colorless substances. The method illustrated some of the

difficulties which might be encountered. It was found that there were unavoidable disturbances at the electrodes, so that these had to be separated from the protein solution by a calculated amount of buffer; and that the ultra-violet method was not sensitive enough to pick up many of the protein boundaries thought to be present. However, these early methods did illustrate where the isoelectric point of certain proteins might be and what the characteristic mobility might be.

Although these workers had been using electrophoresis for more gross separation of protein fractions, it was not until 1937 that Tiselius[42] overcame the most serious difficulties that stood in the way of isolating separate protein fractions from the serum.

Tiselius early pointed out[94] the limitations of his method, namely that the only property of protein being measured by electrophoresis is the mobility of the protein component, that many large particles (i.e. polysaccharides) carry no charge at any pH, that many protein components (i.e. the albumins) have nearly or almost nearly the same isoelectric points (although they move at quite different mobilities at another pH). Tiselius felt that "serological reactions and solubilities of crystalline material are more specific properties than the electrophoretic mobility even if the separation by these methods may not always seem so clear cut as that obtained by electrophoresis". He noted the spreading of the boundaries which occurred when the substances were run for a long time, which he did not attribute to diffusion but to separation, and which could be reversed. This indicated in his

opinion (now confirmed) that the protein components were not homogeneous, or that there was an unknown source of disturbance in his apparatus.

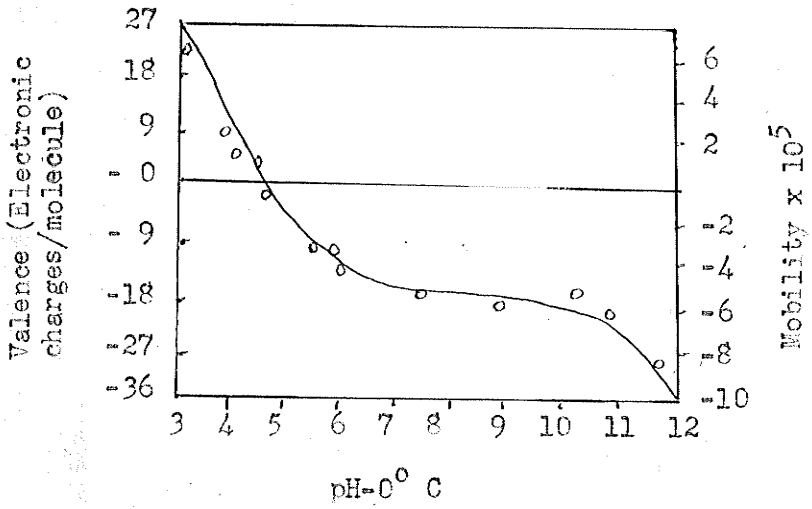
II. Preliminary Considerations in Electrophoretic Technique

(a) Selection of a Buffer

The action of variations in the pH, ionic strength and type of electrolyte present on the electrophoretic behaviour of the protein ion is important.

(i) The action of changes in pH--Protein molecules of different nature each have an isoelectric point, a pH at which the negative and positive changes seem to balance each other, and at which, among other things, the solubility of the protein is at its minimal, and at which it does not move in an electrical field.[37] If the protein solution is made strongly alkaline, the protein molecule which is in the "non-migrating state" changes; the amino groups become completely dissociated, and the carboxy groups are unchanged so that the molecule has a net negative charge and it will migrate to the positively charged pole. On the other hand, if from the isoelectric point the solution be made strongly acid, the amino groups are unchanged while the carboxy groups are combined with H $^{+}$ ions so that the molecule has a net positive charge. It can be shown that the number of these "free" charges, (either $^{+}$ or -) on the protein molecule is directly related to the mobility of the protein molecule. Thus, if the ionic strength is kept constant, and the pH varied from 3 to 12; and if with each change of pH (a) the number of free negative and positive charges are determined by titration, and

(b) the mobility of the protein solution is determined, then it will be seen that the electrophoretic mobility and "valence" of the protein solution at a given ionic strength will be a function of the pH.[37] These points are illustrated in Diagram I below.



Diag. I - Electrophoretic Mobility and Valence of Ovalbumin at 0.10 Ionic Strength.

The solid line represents valence, and the circles represent electrophoretic mobilities. (from Longsworth).

From a consideration of Diagram I one might deduce the pH at which electrophoresis should be carried out.

(ii) <u>Effect of ionic concentration</u>

It can be shown that if the ionic strength is decreased, there will be a more precipitous change in the mobility of the protein solution with each change of pH. This phenomenon is thought to be due to a "shielding" effect which the absorbed ions from the buffer exert upon the protein molecule.

(iii) Effect of type of electrolyte present

The effect of the ions in the supporting medium is reflected not only in their amount, but also in their type, i.e. charge. Thus, a polyvalent ion in the buffer, is more likely to cause a variation in the mobility of the protein molecule, even if the pH and ionic strength are the same, than a univalent ion might.

In addition to these factors, the ionic atmosphere must provide a uniform pH, and a uniform electric field throughout the whole electrophoretic apparatus. In this regard, such a field and pH can never be perfectly constant throughout because of the effect on the buffering ions of the protein ions themselves as they move and separate. Such a field can be sought for by using a relatively high electrolyte concentration with a relatively dilute protein solution.

These factors allow us to select a "buffer" for any particular mixture of protein to be subjected to electrophoresis. Such a buffer would have to have its maximal buffering pH well above or below the isoelectric point of most of the components (allowing free charges to occur), and to consist mostly of univalent ions, to be in a relatively high electrolyte concentration, yet not combining with the protein to form insoluble precipitates. In the case of the Tiselius apparatus diethylbarbiturate (veronal) buffer of 0.10 ionic strength at pH 8.6 gives the best resolution.

(d) Preparation of the substance to be examined

Most investigations have been done with serum.[34,64,65,71,85]

(i) Hemolysis:

If hemoglobin is added to serum in sodium-phosphate buffer of pH 7.4, an increase in the optical density is seen, and the gamma globulin is increased in proportion to the amount of hemoglobin added.

If hemoglobin is added to serum in veronal buffers at pH 8.6, the area of beta globulin is increased. In the presence of hemolysed biological substances, such as blood serum or plasma, the contribution of hemoglobin to the pattern can be estimated by subtracting the beta globulin obtained in phosphate buffer from that obtained in barbiturate buffer provided that the samples are not dialyzed more than 24 hours.

(ii) Dialysis:

If serum is diluted with buffer, then subjected to dialysis, no change not within the experimental method is seen. There may be some general decrease in pattern area with veronal buffer after 24-48 hours of dialysis.

However, mobilities may be affected in the case of the Tiselius apparatus, because in the ascending limb of the apparatus Cl^- ions migrating from the serum ahead of the albumin are thought to cause a slowing of the components of this limb.

(iii) Storage:

If the serum is refrigerated at ice box temperature only minor changes appear in the electrophoretic pattern. Some workers believe the optimal temperature to be -8°C. If however, serum is stored for days

(48 hours) at room temperature, changes appear, especially in the alpha and beta portions.

Similarly if plasma is allowed to remain in contact with red blood cells even if refrigerated, changes also appear in the alpha and beta globulins.

(iv) Lipide removal:

(a) Ether extraction. Most of the changes appear after the first extraction. These consist of a marked diminishing of the areas associated with the alpha and beta components. If left for four or five days no further change is seen in the electrophoretic pattern.

(b) Ultracentrifuge. If the fatty layer is removed by ultracentrifuge changes in the height of the alpha and beta components are again noted.

(c) Storage at room temperature followed by ether extraction causes a further marked reduction in the alpha and beta components.

(d) Fasting does not appear to change the electrophoretic components.

(v) Freezing in Dry Ice-Alcohol Bath and Lypholization

These procedures caused an increase in the opalescence associated with the alpha globulin, a decrease in the area representing albumin and a larger more diffuse gamma globulin area. These changes

probably represent albuminous degeneration.

(vi) Heating

If the serum is heated at a high enough temperature, for long enough (i.e. 58°C. for 20 minutes) a component designated as the C component appears in the area formerly occupied by the alpha and beta components. Even if the serum is ether extracted, before and after heating, the component still appears.

According to Moore, Roberts, Costello and Schonberger[71] the changes associated with simple storage at room temperatures or by the usual manipulation of the serum are due to changes in the lipide content of the serum "whereas changes produced by more drastic changes in temperature are due to denaturation of the proteins independent of the presence of lipides".

These findings suggest that specimens for electrophoresis should be centrifuged, the supernatant separated immediately, the protein solution dialyzed not more than 24 hours, kept in an ice box, and used as soon as possible.

III. Types of Electrophoretic Apparatus

Only a brief description of these will be given here except in the case of apparatus concerned with paper electrophoresis. Innumerable modifications of Tiselius' original model make detailed description impossible.

(1) "Free" Electrophoresis apparatus

(a) Macro-Cell Type. Modified types of apparatus are based on

Tiselius' original model.[92]

The cell consists of a U-shaped cell, with rectangular limbs (3 x 25 mm. and 25 mm. long) built in three sections. Each section may be slid relative to another. The bottom section is filled with protein solution, and separated and isolated from the top and middle sections. One limb is then filled with protein solution, and the other with buffer. The top section of the protein solution filled limb is then separated from the middle compartment, and the top section filled with buffer. All compartments are then put in continuity giving sharp "boundaries". After the apparatus has been cooled sufficiently, the electrode vessels are added and these are capacious enough to prevent the products formed from reaching the protein solution. The electrodes themselves are usually silver wire coated with silver chloride. Electrophoresis takes place at 1°C. This reduces convection, caused by the heating of the current. Several hundred volts of electrical potential are then applied, and this causes the boundary on one side of the center section to rise and the other to descend. Gradually each component forms a separate boundary, the small or highly charged ones moving fastest. Besides the boundaries that move, there are boundaries in the ascending and descending limb which do not move, and are probably not related to protein components but to changes in salt concentration. By complex optical and photographic methods the boundaries are measured and presented as curves, and their mobilities calculated.[66,67,74,75,88,95]

(b) Micro-Cell Type.

Antweiler,[1] Lotmar,[68,69] and others[53] devised a micro cell apparatus for use on occasions where only small amounts of fluid ($\frac{1}{4}$ cc.) were available. By a complex system of lenses, the use of an interoferometer plate, and a small electrophoresis cell, the back and front of which are used as mirrors, these authors could allow the rays of light to traverse the channels twice and to double the sensitivity of the apparatus. The process could be watched through an eye piece or recorded on a photographic plate.

Booij[2] devised a type of micro-cell apparatus by inserting glass rodes in the limbs of the macro cell apparatus.

(2) Zone Electrophoresis

Because of the expense and complexity of the "free" electrophoresis methods, workers in Germany (Weiland et al),[98] United States (Durrum)[13,14] and Sweden (Tiselius et al)[10] began independently the development of electrophoresis by simpler methods.

(i) Theory of Zone Electrophoresis[52,94,99]

Zone electrophoresis is based on the same properties of colloids which allows their measurement in the moving boundary technique of electrophoresis, namely that an electrical field causes the different components of a colloidal mixture to migrate at a rate proportional to the surface charge per unit area of the particles or molecules, and in a direction determined by the sign of this charge.

These rates are characteristic of certain proteins.

A simple mathematical explanation of the events which occur is described below:

Ohm's Low - Volts = $\frac{i}{R}$ (1)

where i is the current and R the resistance.

But the field strength is measured in units of distance (i.e. cm.).

In the case of the electrophoretic cell, the field strength E or potential gradient per cm. is equal to

$$E = \frac{V}{l} \quad (2)$$

where V is the voltage across the cell and l is the length of the channel.

This can also be expressed as $E = \frac{i}{qk}$ (3)

where i is the current, q the cross section of the tube, and k the specific conductivity of the solution.

Thus E is expressed as Volts/cm.

According to hypothesis, if the force E is exerted on a molecule it will move x cms. in time 't'.

Thus E will cause a velocity of $\frac{x}{t}$ (i.e. cm./sec.).

The velocity which one unit electric field will cause is designated as u.

$$\therefore \quad u = \frac{x/t}{E} \quad \text{or} \quad u = \frac{x/t}{v/l} = \frac{xl}{Vt} \quad (4)$$

and since $E = i/qk$

$$u = xqk/it \quad (5)$$

Furthermore, if a current i passed time t causes the protein boundary to move x cms., in a tube whose cross sectional area is q cms.^{2}, and if P equals the grams of protein per ml., then in time t, x q P grams of protein have moved the distance x.

If we presume that the average protein molecule moves the distance of u (its velocity per one unit electric field) times E_p (the toal electric field) in a time t, then the amount of protein (cm./sec.) that has moved can also be expressed as u Ept q P.

$$\therefore \quad x\,q\,P = u\,E_p t\,q\,P \tag{6}$$

$$\text{and } u = \frac{x}{Ept} \tag{7}$$

$$\text{since } Ep = \frac{i}{q\,K_p}$$

$$\therefore \quad u = \frac{x}{t} \div \frac{i}{q\,K_p} = \frac{xqk}{i\,t} \quad \text{(see 5 above)} \tag{8}$$

$$\text{or } u = \frac{x}{E\,t} \tag{9}$$

The equation (8) therefore gives the volume swept through a certain distance when a force of i t coulombs of electricity are passed through the cell.

The value for K is determined in the case of free electrophoresis by measuring the resistance of the protein solution beforehand in a conductivity cell.

Also in the case of free electrophoresis, convection currents are set up if the electric fields are too high. Heat develops at the rate of $\frac{i^2}{Q^2K}$ WATT/cm.^{3} In order that no convection currents be set

up, at 1° C. this value must not exceed 0.15 watts/cm.^{3}

According to Kunkel and Tiselius[51,52] however, these equations for free electrophoresis do not hold true for the paper apparatus which is really "liquid in a highly porous medium". They showed that $\frac{V}{l}$ did not equal $\frac{i}{qk}$, i.e. the electric field strength arrived at by measuring the length of the paper, and the field strength arrived at by measuring the cross-section of the paper did not correlate. This, Kunkel and Tiselius felt, was because the voltage was not being exerted through a straight column of liquid, such as the channels of an electrophoretic cell but through a tortuous channel in the paper. Thus the distance over which the voltage might drop was not l (the actual length of the strip) but "l_1", (the theoretical length of the paper). They also presumed that the protein molecule itself does not travel the distance d (or x) but the distance "x_1", i.e. that it follows the path of the fluid in the paper and instead of being a fraction of l is a fraction of l_1 (see appendix A).

As a result of experimentation, Kunkel and Tiselius arrived at the conclusion that the length l_1 could be correlated with the resistance of the paper strip.

$\therefore\ l_1 = Rq_aK$ (15) Where R is the measured resistance over the paper strip and q_a is the cross section of the paper.

By use of this formula, substituted in the above equation, it was possible to determine mobilities on paper strips and compare them with free electrophoresis. When the correction factor due to the resistance of the paper was applied, the mobilities by the two methods correlated fairly well. (Without this correction factor, the mobilities

on paper seemed to be slower). This correction factor was shown to actually depend on the paper, not on the buffer used, and was found to be the same in the same paper for any of the buffers Kunkel and Tiselius used. It was seen however, that some of the fractions were affected more than others. Thus alpha component migrated relatively more slowly than beta with the result that the two migrated closer together on the paper than in free electrophoresis.

There is another factor which comes into consideration when theoretical deductions are made regarding the behaviour of protein particles on paper strips. This is the problem of electrosmosis. The pores in the filter paper act like small test tubes and their walls are thought to have a negative charge relative to the buffer medium. Because the pores cannot move, and buffer can, there is a movement of the buffer to the cathode because of its relatively positive charge. This movement causes a shift of gamma globulin to the cathode side in many cases, even though its charge is negative and it should theoretically move to the anode. Kunkel and Tiselius, to measure this influence of electrosmosis searched for a substance which had no inherent electrophoretic mobility (no charge) but which would move with the electrosmotic fluid. Such a substance should not diffuse, and should stain easily. They selected dextran, and they found that up to a point the lower the concentration and smaller the spot, the greater was the "back migration" of the spot.

Thus by knowing how far back the dextran went, and how far forward the albumen went, the actual distance migrated by the albumen

could be calculated. (See appendix "B").

If the current was changed, or run for varying lengths of time, no difference in the ratio $\frac{x\ dex}{x\ alb / x\ dex}$ occurred. The ratio did change with the type of paper used. The relationship $\frac{x\ dex}{x\ alb}$ was therefore also constant for any type of paper and so they determined what these would be for a certain type of paper at a certain pH and temperature, and then used this ratio without further measurement in individual cases of the distance moved by dextran, as "x dex" can be determined from the formula. (At lower ph's dextran has some positive electrophoretic mobility).

Gronwall[32] points out that the electrolytes in the buffer must be kept constant, (i.e. their concentration and composition) because of the known effects these variations have on mobility (as shown in free electrophoresis). These can be altered by evaporation and by disturbances in reaction which occur (at the electrode). These precautions are described later. He points out that heat is generated which causes evaporation thereby affecting concentration.

Kunkel and Tiselius did not attempt to estimate the effect of varying ionic concentrations of salt in the buffer, in order to determine the effects on the mobilities on filter paper, but they did show the effect of changing the type of buffer, changing pH and the value of paper electrophoresis in determining the isoelectric point. They mixed two protein solutions together, and showed that they moved approximately the same distance when mixed as they did

separately. They showed also that 1:1 mixtures of albumin and gamma globulin did not give these proportions by dye elutions because of the lesser affinity of gamma globulin for bromphenol blue. This problem is illustrated in free electrophoresis where mixtures of albumin and globulin mixed in a 1:1 ratio exist in ratio of 55:45 in the free electrophoresis machine because of gradients in the barbiturate and protein ion concentration.

Woods and Gillespie[99] also considered the theoretical problems behind the estimation of mobilities by electrophoresis. They claim that albumin moves only in approximately a straight line relationship with the length of time the current is applied, and that there is only an approximate ratio between the rate of movement and the potential gradient when the protein is applied in the center of the strip.

The protein moves faster nearing the anode side. They observed a straight line relationship however, when the protein was applied 6 cm. from the cathode end of the glass strip and allowed to run for 18 cm. until it was 6 cm. from the anode side. These effects are thought to be due to the effects of liquid flow of buffer in the reverse direction in the paper, i.e. of a combination of evaporation resulting in a movement of fluid from both ends toward the center, and of endosmosis which causes a flow of buffer from anode to cathode as mentioned above. This non-uniform liquid flow causes variations in the potential gradient at various times during the process of electrophoresis.

Thus the voltage across the paper gradually rises, then falls toward the end of electrophoresis, while the current shows a constant rise. These workers use therefore the average potential gradient in their calculation of mobilities, but point out that the liquid flow itself may be influenced by time, potential gradient, position of the spot of protein on the paper, method of wetting paper, the extent of evaporation, pH and the ionic strength of the buffer. Nevertheless their results using potential gradient correction, rather than the correction for the resistance of the paper as suggested by Kunkel and Tiselius, agree with the results obtained by Kunkel and Tiselius, which in turn agree with those of the free electrophoresis. Such agreement would suggest that not all the factors are explained by theoretical calculations as yet made.

From all this investigation it becomes apparent that the measurement of mobilities by paper electrophoresis is at best a complicated and not too well understood method, which does not appear totally reliable unless the results are checked by similar experiments with free electrophoresis. This appears to be because the behaviour of the protein molecule in the paper strip is not well understood. However these considerations would suggest that unless the same type of paper, of the same degree of wetness, with the same buffer used of the same ionic strength and pH, at the same temperature, for the same length of time, at exactly the same potential gradient, with the same amount of protein applied in exactly the same spot so that the forces of evaporation,

endosmosis and mobility caused by the potential gradient, are all of the same degree in separate experiments, experiments which depend on the identification of new components by their position on one paper strip, as compared with another, even if done at the same time, will to a widely variable degree, be unreliable. Whether this unreliability applies to protein solutions applied on the same strip of paper is less doubtful. Kunkel and Tiselius have shown at least that such proximity does not alter the properties of each protein to any marked degree.

(ii) Types of Zone Electrophoresis Apparatus

Methods prior to 1948 using glass wool, gels and other materials for electrophoresis were not applicable to situations requiring small amounts of fluid.[32,35] Haugaard and Kroner,[38] using paper chromatography noted than an electrical field helped to separate components among amino acid. Weilland and Fischer [98] separated amino acids and peptides. Svensson and Brattsten[90] used a lucite box filled with glass wool, applied an electrical potential at right angles to the path of the protein solution as it passed downwards. Durrum[13,14] modified this technique by using filter paper, and was able to collect the components. Durrum[14] tried several other techniques and devised a method whereby a glass support is used to suspend the narrow filter paper between the poles of the electrodes in buffer. Later, in order to get two dimensional effects he suspended his paper from a wire running lengthwise in the box a few inches below the lid. This type of apparatus was developed further by Flynn and de Mayo[21] and separate compartments

for the electrodes were built in the bottom of the box. At the time of Durrum's first work on this subject, Cremer and Tiselius [10] devised a method of electrophoresis between glass plates, under a chlorobenzene bath. Kunkel and Tiselius[52] clamped the edges of the plates, did electrophoresis in air. They did important experimental work, pointed out the necessity of large volume electrode vessels to prevent changes at the electrodes from reaching the strips, measured mobility, tested the effects of various types of buffers, compared chemical analysis of the strips with dye elutiation estimates of the protein content. Kõiw, Wallenius and Gronwall[50] further modified this method, adding labyrinthine systems around platinum electrodes, meters, signal lamp, fuse and other apparatus all compactly arranged. Goa[26] simplified the process, used a glass plate, over which was suspended another glass plate a few millimeters above the strip, forming a channel for the strip. These are but a few of the many varieties of zone electrophoresis apparatus in use.

(iii) Methods of Protein Estimation after Zone Electrophoresis

(a) Staining and Elutriating Methods

Usually the strip is dried in an oven (60°), the protein fixed by coagulation, and stained under standardized conditions. The requirements for good staining are constant composition of the solutions, same quality of paper, stain with specificity and high affinity for protein without staining the background. Typical dyes used are bromphenol blue,[10] azocarmine S,[96] amidoblack 10B.[29] A

typical staining program[52] is 5 minutes in 1% bromphenol blue in saturated alcoholic $HgCl_2$ solution, 15 minutes in 1% $HgCl_2$ in methanol, 15 minutes in ethanol, 5 minutes in methanol. The latter are wash solutions. The paper usually dries in a few minutes, is cut in strips 5 mm. wide elutriated in Na_2CO_3 or NaOH and read on the photoelectric colorimeter.

(b) Staining, Photometry, and Planimetry Methods.

The strip is dyed as above, usually dipped in paraffin oil to render it transparent, then passed through a light beam, with a curve being obtained of the interference of the protein spot.[29,73] The readings are then plotted, with the maximal reading (designated as 100, or merely the actual reading) along the ordinate, and the distance along the abscissa. The points are joined by a continuous line, and the areas beneath the peaks calculated by planimetry.

(c) Actual Nitrogen Determination Method

Kunkel and Tiselius[52] modified a quantitative protein test of Folin so that they could compare actual estimates of the nitrogen content of the strips at various spots, with the results obtained by dying and elutriating.

Levin and Oberholzer[63] use two strips. They stain the first, cut the second in sections corresponding to maximal protein concentration of various components. These pieces are subjected to micro-Kjeldahl analysis. This gives actual protein amount, not the protein-lipid-

carbohydrate complex so that correspondence between it and the dye method is not exact in some cases of disease.

Kunkel and Tiselius showed by their method that albumin may bind more bromphenol blue than globulin. Whatever the factor, results with zone electrophoresis do not agree with those of free electrophoresis exactly. For this reason, Cremer and Tiselius multiplied their results for globulin by a factor (1.6). Other workers have pointed out that this factor is not the same for all globulins, especially abnormal ones. Köiw, Gronwall and Wallenius[50] do not believe a correction should be made to make the results of paper electrophoresis agree with those of free electrophoresis. Abnormal results should be plotted against normal results obtained by the same method.

(iv) Results of Electrophoresis

(a) Serum Proteins

Because certain dyes have lesser affinity for globulin than albumin,[52] and because some lipoproteins do not stain,[63] values for albumin are higher, and globulins lower in zone electrophoresis than free electrophoresis, and sometimes peaks appear in free electrophoresis which are not seen in zone electrophoresis (i.e. hyper-betaglobulinemia). For this reason, free electrophoresis is considered more sensitive. According to Köiw,[49] however, they are equally valuable in diagnosis. Table I compares the serum components calculated by the two methods.

Protein Component	"Free" Electrophoresis		"Zone" Electrophoresis		Modified Folin
	Koiw	Tiselius	Koiw	Tiselius	
Albumin	62.1	53.3	72.9	65.9	52.9
Alpha-1	3.4	6.2	1.4	3.9	6.3
Alpha-2	5.2	10.8	3.5	8.0	12.2
Beta	11.8	13.7	8.6	9.0	10.1
Gamma	17.0	15.6	13.6	13.1	18.5

TABLE I. Normal Values (%) of Serum Protein Components by Different Methods of Estimation (see text).

This table illustrates also that differences occur even among workers using different methods of zone electrophoresis. Only an approximate identification can be given to the bands relative to one another and normal values vary with the methods used, if not with the operator.

Beirring and Neilssen,[5] working with the Antveiler type of micro-electrophoresis also identify these components with the exception of alpha-1 globulin. Their apparatus gives higher values for the albumin plus alpha-1, areas, and lower beta areas than the Tiselius apparatus.

Actual mobilities obtained by paper electrophoresis can be correlated with those of free electrophoresis but the basis for this

correlation based on the resistance of the paper, or of the "mean potential" throughout electrophoresis, is not completely understood. (see above).

Lipids, [91] polysaccharides, [49] iron, [32] and radioactive iodine labelled thyroxin, [27] as well as radioactive phosphorous bound to protein can be measured by zone electrophoresis.

(b) Cerebrospinal Fluid Proteins

(i) Methods used. Kabat et al [45,46] (1942) concentrated large volumes of cerebrospinal fluid to 2 cc. in a cellophane dialysing membrane, under nitrogen pressure, over 2-3 days. Tiselius free electrophoresis was used. Mobilities were measured. They isolated albumin, beta globulin and gamma globulin. Sometimes "x" component with a mobility faster than albumin; alpha globulin; and "fibrinogen" were seen. Scheid and Scheid [77,78] (1944) performed similar experiments. Booij [2,3] (1949 and 1952) concentrated 15 cc. to 4 cc. by in-vacuo evaporation, and modified the Tiselius cell by inserting glass tubes in the channels. He obtained albumin only in 21 of 48 cases, and albumin and beta globulin in the other 27. Fish, Chanutin and Klingman [20] used large amounts of cerebrospinal fluid, concentrated by dialysis through collodion sacs. Using free electrophoresis, they obtained the component designated as "x" in 66 cases and a further component with a still faster mobility (x-1) in 9 cases. Serum subjected to the same procedures did not show these components.

Schneider and Wallenius [84] (1951) and Wallenius [97] (1952) were successful in using small amounts of fluid (4-9 cc.) concentrating

the material to .1-.2 cc. in a cellophane dialysis tube. Paper electrophoresis under a chlorobenzene bath was performed and albumin, alpha globulin, beta globulin, and gamma globulin were seen in repeated examinations of 5 specimens. Labhart, Schweizer and Staub[54,55] (1951) using a concentration method of Ewerbeck,[19] and a vacuum apparatus, concentrated to 1 Gm.% from 5-15 cc. in 5-7 days. Antweiler-Lotmar[1,69] micro-electrophoresis was performed. Their normal values are not clear. Albumin, beta and gamma globulin were seen in some pathological cases, but no differentiation was made between alpha-1 and alpha-2 globulin. Serum similarly prepared was unchanged by the procedure.

Bücher, Matzelt and Pette[4] (1952) used 10 cc. of fluid and precipitated protein with acetone in a cold room. This was redissolved in cooled buffer, and electrophoresis was carried out in a moist chamber. Staining was with amidoblack 10B and photometry was used. Slight reduction in the albumin and alpha-1 peak occurred, but they could demonstrate a component migrating in front of albumin, and another appearing immediately after the beta globulin, as well as alpha, beta, and gamma globulin. They did not think the new peak described was related to fibrinogen. Hoch and Chanutin[39] (1952) pressure dialysed large quantities of centrifuged fluid to 3-5 cc. and used the Klett-Tiselius microcell electrophoresis. When phosphate buffer of pH 7.85 and ionic strength 0.05 was used, boundary anomalies could be used to identify rapidly migrating components appearing in front of the albumen. Thus "x" component was identified in all samples. Since gamma globulin does not migrate from the salt boundary, the area encompassed by this

component must be calculated, and with this in mind the upper and lower limits of normal values for the other components can be calculated. Since the value of the salt boundary increases with protein concentration, and "x" components seem to increase relatively, these authors show that the actual values for "x" may be 2/3 its apparent value, and that of gamma globulin 1-2 times its apparent value. In 9 of 49 cases, x-1 appeared. In 21 of 49 cases with protein under 40 mgm. the component migrating after beta globulin was seen. In one case, alpha-1 was seen.

Mies[70] (1953) concentrated 5 cc. of fluid in a specially shaped collodion bag, dialysed against Ringer' solution, with ex vacuo concentration. The concentrate was redissolved in buffer, electrophoresis being done in a wet chamber. The "x" component, albumin, alpha, beta, gamma globulin as well as the component migrating after beta globulin, were seen.

Cumming,[11] Easton, and Gardner[17] could not identify the "x" component of spinal fluid, or the component migrating after beta globulin.

In reviewing these methods, it is seen that some methods of concentration are known to affect serum proteins. These are dialysis over 24 hours, freezing, ether extraction. Some require large amounts of fluid and the use of free electrophoresis. Other simpler methods do not show all the components which likely exist. The careful selection of a buffer with pH below 7.9 is likely important. The method of Mies seems likely to combine the qualities of simplicity and accuracy best.

Author	X	Albumin	Alpha	Beta	T	Gamma	No. of Cases
Kabat et al (a)	6.4	64.2	5.1	23.8	- (1)	8.0	4
(b)	---	62.5	6.7	19.9	-	15.1	4
Hoch et al							
(c)	I 11.7	58.9	8.5	12.1	3-8	8.9	36 (2)
	II 11.0	55.4	7.7	11.2	-	14.7	
(d)	I 11.2	61.8	8.5	13.8	-	4.7	13
	II 10.1	56.6	7.8	12.8	-	12.7	
Bucher et al	4.4	49.7	15.4	11.5	8.0	11.0	10
Wallenius	---	56.5	9.8	15.8	-	18.0	5 (3)
Mies	6.4	53.9	13.5	8.0	6.2	12.0	5
Roboz et al	---	57.3	11.0 (4)	9.2	-	16.5	-

TABLE 2. Normal Values (%) of C.S.F., Protein Components

Protein Levels (mgms.) (a) 27-38, (b) 30-38, (c) 0-29, (d) 30-39

I and II signify upper and lower levels given salt boundary

(1) Fibrinogen (1 case) 5.4%
(2) X-1 appeared in 9 cases, Alpha -1 appeared in 1 case
(3) 9 determinations of 5 cases
(4) Alpha -1 6.1%; Alpha -2 11.0%

From a consideration of Table 2, in which is summarized the work of authors using both free and zone electrophoresis, it would seem that normal cerebrospinal fluid protein consists of at least five components. These are designated "x", albumin, alpha globulin, beta globulin, and gamma globulin since these components have been consistently seen by different workers using separate methods. A sixth component designated as "T" may be present, but its status is uncertain because it is so close to the position of fibrinogen, which therefore is also uncertain. Another component, x-1, has been seen by one group of workers using free electrophoresis. Alpha-1 and alpha-2 are generally not separated if they are present in normal spinal fluid.

(c) Some Properties of CSF Protein Components

(a) Mobilities: Kabat et al[46] reports the mobilities of spinal fluid protein as compared to serum as follows: (Mobility expressed as u x 10^5)

	CSF	Serum
Albumin	5.8	5.0
Alpha globulin (one case)	4.1	3.4
Beta globulin	3.0	2.6
Gamma globulin	.9	.78

They suggest that these mobilities are so close to being similar that they probably represent the same components in serum and spinal fluid.

Koch and Chanutin[39] believe that the concentration of albumin influences the mobility of the "x" component. It moves further at higher concentrations of albumin. "x" component moves 1.3 as fast as

albumin.[20] If the faster moving component is separated it moves in a pattern as below:

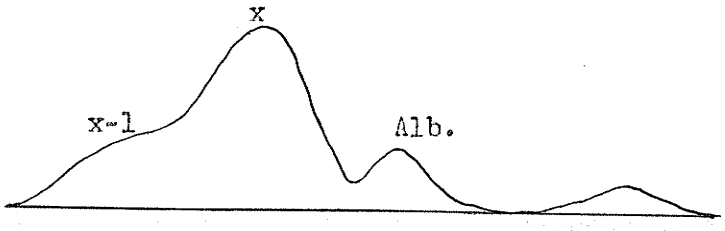


Fig. 2 - Electrophoretic Pattern of Rapidly Migrating Component of Cerebrospinal Fluid (from Hoch and Chanutin).

65% of this component is "x", presumably the leading boundary is x-1. The remainder is probably albumin. The motility of "x" is 11.8 x 10^{-5} cms.^{2} volt^{-1} sec.$^{-1}$, while x - 1 is 14.0 x 10^{-5} cm.^{2} volts^{-1} sec.$^{-1}$, with the buffers used (phosphate buffer pH 7.5 ionic strength .05)

Hoch and Chanutin present the mobilities with albumin expressed as 1, and presuming there is the same concentration of albumin in spinal fluid and serum.

	CSF		Serum
Alpha-2	0.79		.84
Beta	0.57		0.55
"T"	0.42	Fibrinogen	.30
Gamma	did not move		.24

Mobilities of the CSF protein components have not yet been calculated on filter paper. The mobilities of the components therefore are similar, but not the same.

(b) Sedimentation rate in Ultracentrifuge: As far as can be determined only the fast moving component of spinal fluid has been subjected to this test.[39] 95% of it sediments as a homogenous component with the

rate of albumin, the remainder sediments 2.2 times as fast.

(c) Combination with Colloidal Gold: Gamma globulin obtained from the spinal fluid in two cases of multiple sclerosis, and four cases of neurosyphilis by Kabat et al,[45,46] showed markedly positive "first zone" curves on colloidal gold examination. Gamma globulin obtained from spinal fluid in other cases not syphilitic and not having multiple sclerosis, did not possess this reactivity to such a marked degree, and in some cases not at all. It is not clear from their paper however, whether the concentration of gamma globulin in the amount used to test the colloidal gold was the same in both instances, but presuming it was, then special properties would have to be attributed to the gamma globulin of multiple sclerosis and neurosyphilis. Normal serum gamma globulin[30,44] will produce this reaction in the colloidal gold.

Spinal fluid albumin has some function in the protection of colloidal gold curves, just as serum albumin has. By itself, it does not produce any coagulation in the colloidal gold curve, even when obtained from syphilitic spinal fluid.

There is no report on the action of spinal fluid protein components alpha and beta globulins, although Scheid and Scheid[78,79] report some coagulation with beta globulin.

SUMMARY

1. Cerebrospinal fluid specimens for electrophoresis should be centrifuged, the supernatent removed immediately, not dialyzed over

24 hours, kept or stored in an ice box (but not frozen) and used as soon as possible.

2. The buffer used should have its maximal buffering pH well above or below the isoelectric points of the components, consist mostly of univalent ions, to have an ionic content higher than that of the protein solution, but not too high to cause precipitation.

3. The position of a protein spot on an electrophoresis strip does not identify it exactly with another spot on another electrophoresis strip unless extreme precaution is taken in technique. This is especially true if different protein solutions are used.

4. The results of electrophoresis of cerebrospinal fluid protein by one method cannot be compared exactly with results obtained by any other method. However, the ideal method should probably consistently show at least five easily recognizable components in normal cases.

5. No electrophoretic experiment on cerebrospinal fluid protein has unequivocally identified any component to be the same components as that found in serum.[47]

PART II - Electrophoretic Patterns of Normal Cerebrospinal Fluid Protein

The electrophoretic analysis of normal cerebrospinal fluid protein is complicated by two problems; the concentration of small amounts of fluid to relatively high concentrations of protein without denaturation, and the use of an electrophoretic method which requires only a minute amount of fluid. Probably because of these difficulties workers do not agree on the normal cerebrospinal fluid protein constituents. Most workers[1-14] have demonstrated fractions comparable to albumin, alpha globulin, beta globulin, and gamma globulin in the blood. There is evidence, however, that some components seen in spinal fluid are not present in blood. Thus separate workers have shown components designated as x-1[10,15] and x[1,3,9,10,11,15] migrating faster than albumin. These components have not been seen regularly by others.[5,6,7,8,12,13] Another component has been shown to migrate slightly slower than beta globulin.[9,10,11] Conversely, some components seen regularly in serum or plasma are not seen consistently in spinal fluid. Thus, alpha component is only rarely separated into alpha-1 and alpha-2 component[10,14] and fibrinogen is only seen occasionally in spinal fluid.[1,2,10] With the description of a simple method of concentration of spinal fluid by Mies,[11] and the development by many workers[16,17,18,19] of a reliable paper electrophoresis technique, it is now possible to separate the spinal fluid proteins and compare them directly with serum and plasma.

The method described below is designed to provide an

accurate simple means of comparing cerebrospinal fluid to serum in a way that will be clinically valuable, and possibly applicable to pathological states. The concentration procedure is much simpler and apparently as accurate as precipitation techniques and does not need to be carried out in a cold room.

MATERIALS AND METHODS:

(1) Collection of Material:

Five to ten ml. of normal cerebrospinal fluid was obtained at the time of lumbar puncture for spinal anaesthesia, from the first 10 ml. of fluid obtained at air encephalograms which later proved normal, and occasionally from lumbar puncture done for diagnostic purposes. An aliquot of each sample was tested for cells, protein and colloidal gold reaction. In each case, clinical and laboratory findings were normal. The remainder was centrifuged and the supernatent stored in a refrigerator or used immediately.

Samples of blood were obtained as closely as possible to the time of cerebrospinal fluid collection and always within 24 hours.

(2) Method of Concentration:

Concentration under positive nitrogen pressure, by chemical precipitation,[9,14] and by pressure dialysis[1,2,7] was attempted, and for various reasons each was discarded as unsatisfactory. The method of Mies[11] using collodion sacs was adopted. Concentration by this method requires from one to four hours. Bags can be obtained cheaply from the manufacturer.* The Congo red must be washed thoroughly from

the bag, and they may be reused several times providing that they are kept moist. The concentrate may be stored overnight in the refrigerator with no change in the pattern produced.

Samples of serum and of plasma were diluted to the protein levels of normal spinal fluid, then subjected to this concentration method. No disturbance in the pattern of normal serum was seen (Figure 1, Panel C) indicating that the method does not affect the normal serum protein pattern. In the case of plasma, the fibrinogen component was not as clearly defined in the reconcentrated fluid as in plasma not subjected to this concentration method, but was still clearly present.

(3) Electrophoresis:

The method of Goa[18] was used employing several modifications. Only three channels were used and the size of the electrode beakers was increased to 100 ml. The air spots in the paper were pressed firmly out with a moistened pad of cheese-cloth. Three strips were run simultaneously. These strips used were Whatman #1, 4 cm. wide and 37 cm. long.

Preliminary experience has shown that the positions of the spots representing the different protein components could not be correlated when the serum was run on separate strips of paper. (Figure 1, Panel A) Differences of 15 mm. occurred in some cases, no matter whether the albumin, the starting points, or the albumin-starting point distance was used as a constant. When two drops of

serum were run on the same strip however, the correlation is within 3 mm. (Figure 1, Panel B).

For this reason, 0.01 ml. of plasma, and 0.01 ml. of cerebrospinal fluid concentrate were applied 6 cms. from the cathode end of the same strip of paper.

Electrophoresis was for 9 hours and at 1.8 milli-amperes. Veronal buffer of pH 7.8 and ionic strength 0.05 was used.

(4) Staining and Photometry

The paper was dried in an oven, then stained 5 minutes in 1% bromphenol blue, 15 minutes in 1% alcoholic $HgCl_2$ solution, 15 minutes in ethanol, and 5 minutes in methanol. Strip was dried and inserted into a simple reading device.[20] Readings were taken at 2 mm. intervals on a photo-electric colorimeter at 575 mu. The readings were plotted in such a way that the highest reading could be expressed as 100, and the rest proportionately. The readings of cerebrospinal fluid concentrate were generally 3/4 those of undiluted serum or plasma.

RESULTS

In five separate cases, 0.01 ml. of serum was applied side by side with 0.01 ml. of cerebrospinal fluid concentrate on the same strip of paper. Typical results are shown in Figure 1, Panels D, E, and F. Albumin, alpha, beta and gamma globulin peaks appeared in both patterns. The peaks of corresponding fractions between cerebrospinal

fluid and serum did not vary over 3 mm. from one another after electrophoresis. A fraction appeared in front of the albumin fraction of cerebrospinal fluid, which was not seen in serum. The height of the cerebrospinal fluid "beta" peak was consistently higher than that of cerebrospinal fluid gamma globulin, a pattern not seen in these sera. Alpha-1 fraction did not separate from albumin in cerebrospinal fluid, if indeed it is present in cerebrospinal fluid, or in serum, using this method.

Figure 1, Panels G, H and I illustrate the results obtained when plasma and cerebrospinal fluid are run on the same strip. A clearly defined fibrinogen band is seen in plasma but no corresponding curve is demonstrated in cerebrospinal fluid.

In ten other cases cerebrospinal fluid was run alone on a strip of paper. A typical cerebrospinal fluid pattern was observed in all cases. The readings at 11 points representing the peaks and valleys of the pattern (Figure 2) expressed as a proportion of 100 are summarized in Table I (The five CSF patterns mentioned above are also included). Figure 2 represents the cerebrospinal fluid pattern, a composite of the results listed in Table I.

DISCUSSION:

With this method, the peaks of the patterns of the same serum when run on separate strips of paper cannot be correlated exactly by simple methods of superimposition. Kunkel and Tiselius[17]

have shown that the actual distance migrated by albumin can be calculated if a constant based on the "backflow" due to osmosis is calculated. The use of this constant, dependent on the type of paper used, at a certain pH and temperature, would probably allow closer correlation between separate strips, but a very close correlation of the peaks (within 3 mm.) can be made without using involved calculations if the drops are simply applied on the same strip. Furthermore, it is known that in some pathological conditions changes in the cerebrospinal fluid pattern are merely a reflection of abnormalities in the serum,[1,2] so that the serum should be examined in every case.

Besides the constant albumin, and alpha, beta, and gamma globulin peaks, two consistent features appear in the cerebrospinal fluid pattern, one of which is never seen in serum. The first is a component appearing in front of albumin, designed "x" by previous workers. It has been demonstrated by many separate investigators, and the failure to demonstrate it by others may be due to faults in concentration procedure which result in denaturation.[21] It was never seen in serum or plasma subjected to dilution, and reconcentrated. An "x-1" component[10,15] described in free electrophoresis was not seen. The second consistent feature of the cerebrospinal fluid pattern was an increased height of the "beta peak" relative to the gamma peak. This is occasionally seen in serum, but was a constant feature of cerebrospinal fluid. In the latter instance, it may be a

reflection of the band in cerebrospinal fluid described by various workers[9,10,11] as occurring after the beta component and separate from both beta and gamma component. Our method may not be sensitive enough to clearly define this band. It should be noted[21] that hemoglobin in hemolyzed blood or plasma, will contribute to an increase in the area of beta globulin in veronal buffers.

When the cerebrospinal fluid and serum are run side by side on the same strip, we have noted that the peaks of albumin, and alpha, beta, and gamma globulin serum correspond (within 3 mm.) in position with peaks appearing in the cerebrospinal fluid concentrate, so that the cerebrospinal fluid albumin and alpha, beta, and gamma globulins have electrophoretic patterns very similar to serum constituents, and may be identical with them. Alpha-1 and alpha-2 do not separate clearly enough even in serum with this method to allow any conclusions to be drawn about their presence in cerebrospinal fluid. Fibrinogen was not seen in normal cerebrospinal fluid as a definite band. Precipitation at the point of application occurred in several cases, but if the buffer and concentrate were at approximately the same temperature when mixed before being applied to the strip, and if the concentrate was clear, no precipitation occurred. Fibrinogen has however, been reported in normal cerebrospinal fluid using free electrophoresis.[1,2]

Because we are not certain that the peaks demonstrate only one main component no attempt has been made to fractionate the

various components by planimetric methods for quantitative analysis. Inspection of Table I reveals the definite pattern which exists, and shows the variation in the readings which may be expected. The variations probably depend on the degree of concentration which cannot be controlled exactly, and of course on the degree of separation which takes place.

SUMMARY

1. A method of concentrating normal cerebrospinal fluids has been tested and found to be valuable.

2. With the electrophoretic method used the pattern of cerebrospinal fluid protein is characterized by a component in front of the albumin and an increased height of the beta globulin peak.

* Membranfiltergesellschaft
Sartorius-Werke Aktiengesellschaft & Co.
(20b) Gottingen (Han),
Germany.

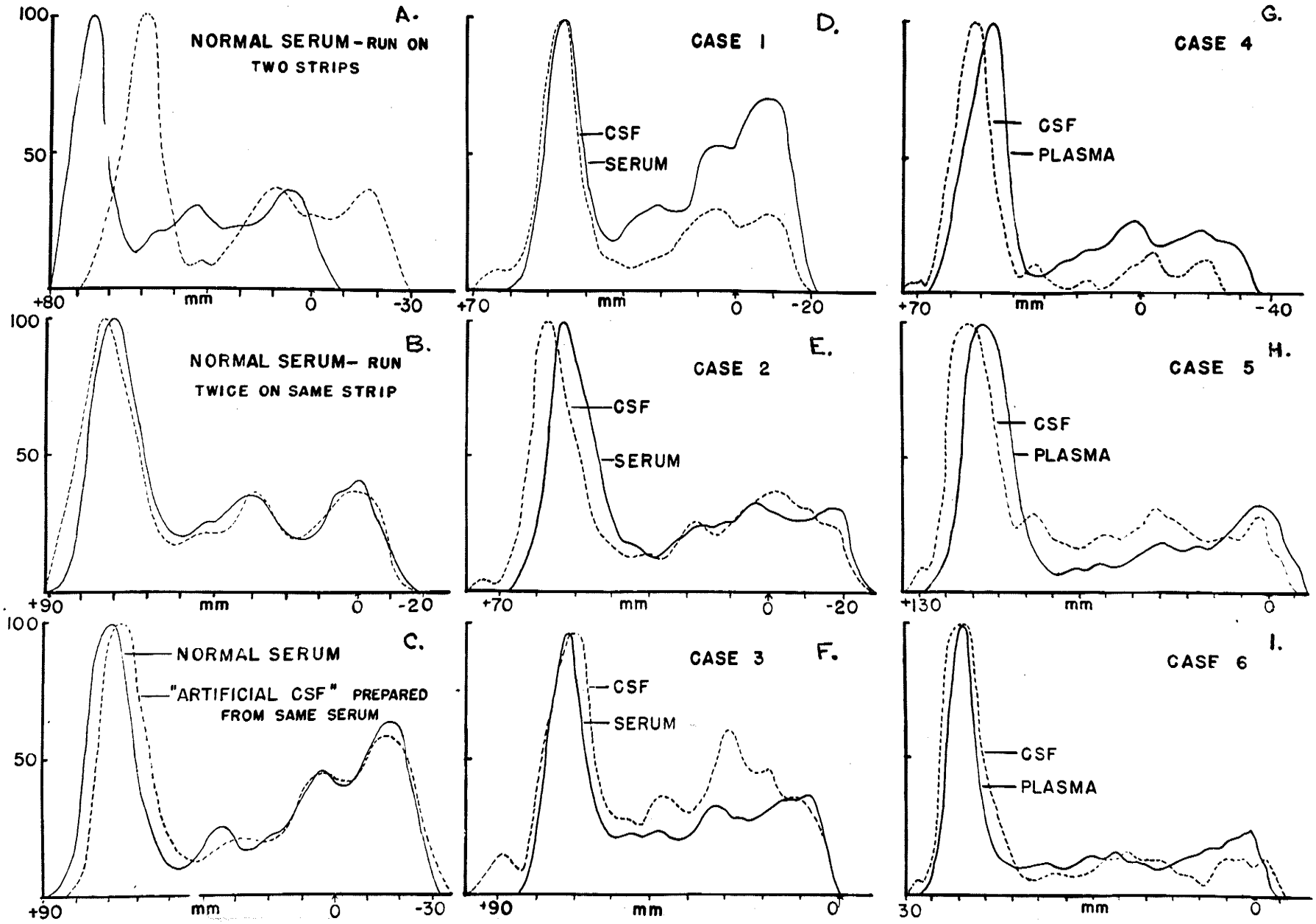


FIGURE I. ELECTROPHORETIC PATTERNS OF C.S.F. SERUM AND PLASMA

TABLE I

THE RELATIVE HEIGHTS OF SELECTED POINTS IN THE ELECTROPHORETIC PATTERN OF NORMAL CEREBROSPINAL FLUID.

Case No.	POINT ON FIGURE 2.										
	A	B	C	D	E	F	G	H	I	J	K
1	0	14	12	100	22	32	30	45	28	38	0
4	0	14	12	100	15	18	16	50	25	35	0
6	0	13	13	100	23	25	23	48	18	20	0
7	0	12	13	100	16	20	18	37	14	15	0
11	0	20	18	100	5	14	10	28	12	22	0
17	0	13	12	100	6	12	11	19	6	14	0
25	0	15	15	100	12	14	8	18	10	12	0
36	0	25	20	100	25	28	22	58	30	45	0
37	0	4	3	100	12	22	20	36	24	24	0
38	0	18	17	100	6	14	10	28	14	20	0
45	0	14	8	100	26	34	30	55	36	38	0
54	0	8	8	100	9	10	10	30	24	28	0
70	0	9	8	100	23	30	15	33	18	28	0
73	0	4	3	100	6	10	9	18	4	17	0
77	0	6	4	100	2	6	2	18	8	14	0

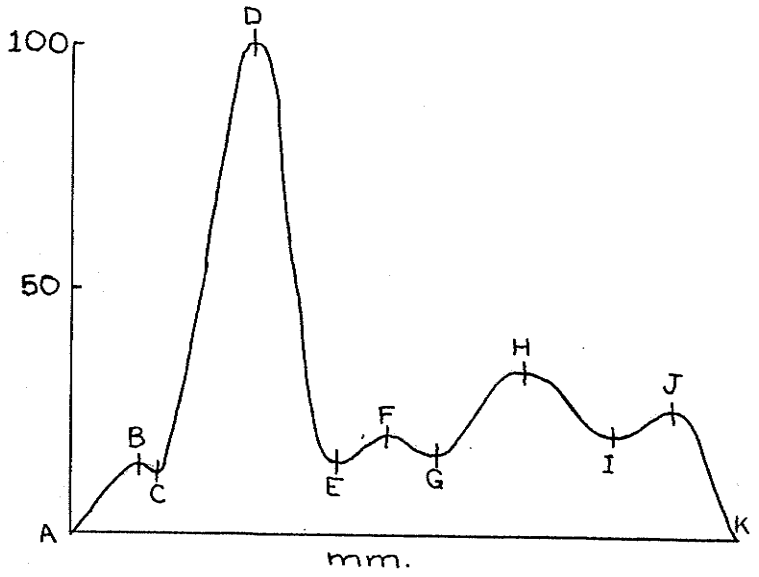


FIGURE 2

THE ELECTROPHORETIC PATTERN OF CEREBRO SPINAL FLUID PROTEIN.

A composite of the findings in 25 separate cerebrospinal fluids.

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APPENDIX

1. Item A [52].

The distance on the paper strip which the voltage drops is not the actual distance "l" but the theoretical distance "l_1", the tortuous course of fluid through the paper.

The distance that the protein molecule travels is not "x" the measurable distance, but "x_1" a theoretical distance travelled as it follows the path of the fluid in the paper.

$$\frac{x_1}{l_1} \text{ as } \frac{x}{l} \quad \text{and} \quad \frac{x_1}{l_1} = \frac{x}{l}$$

$$x_1 = \frac{x\,(l_1)}{(l)} \tag{10}$$

In a free solution, the protein particles travel the distance "x", i.e.

$X = uEt$ or (See 9)
$x = \frac{utV}{l}$ (See 4)

Substituting $X_1 = \frac{utV}{l_1}$ (11)

$$\frac{x\,(l_1)}{(l)} = \frac{utV}{\frac{l(l_1)}{(l)}}$$

$$x = \frac{utV}{l}\,\frac{(l)^2}{(l_1)} \tag{12}$$

but $x = \frac{uti}{qk}$ (See 5)

In zone electrophoresis, "q" is determined by the cross

section of the paper, and is designated "q_a".

$$X_1 = \frac{uti}{q_a k} \qquad (13)$$

Substituting $\underset{(1)}{x(l_1)} = \frac{uti}{q_a k}$

$$x = \frac{uti(1)}{q_a k(l_1)} \qquad (14)$$

As a result of experimentation, Kunkel and Tiselius found that the length l_1, could be correlated with the resistance of the paper strip. Thus

$$l_1 = Rq_a k \qquad (15)$$

where R is the resistance of the paper strip.

2. Item B.

Let x_{dex} = distance travelled by dextran.

x_{alb} = distance apparently travelled by albumin,

therefore actual distance travelled by albumin = $x_{dex} \diagup x_{alb}$

since $u = \frac{x}{Et}$ (see 7)

then $u_{alb} = \frac{x_{dex} \diagup x_{alb}}{Et} \qquad (16)$

similarly, the mobility of the electrosmatic flow (u_{el}) is

$$u_{el} = \frac{x_{dex}}{Et}$$

therefore $\frac{x_{dex}}{x_{alb} \diagup x_{dex}} = \frac{u_{el}}{-u_{alb}} \qquad (17)$

since $\frac{u_{el}}{-u_{alb}}$ is a constant, no matter what distance, time or field strength are used, $\frac{x_{dex}}{x_{alb} \diagup x_{dex}}$ should also be a constant.