RECENT ADVANCES IN THE STUDY OF PROTEINS BY ELECTROPHORESIS[1]

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Recent improvements in the experimental procedure for electrophoretic analysis by the moving-boundary method are described. These include the new Tiselius cell and the schlieren methods now available for recording the complete electrophoretic pattern. With the aid of the new procedures a study has been made of the conditions under which satisfactory patterns of the proteins of human plasma may be obtained. Of the many buffer solutions tested as solvents for the plasma proteins, a 0.1 *N* sodium diethylbarbiturate solution having a pH of 8.6 at 25°C. gave the best patterns for electrophoretic analysis. Using a typical pattern obtained in this solvent as an example, the current methods of computing protein concentrations from the patterns have been critically examined and the assumptions that are necessary in making these computations have been stated. Moreover, from a consideration of the necessity of conserving matter in the transport of materials by electrophoresis it has been possible to derive relations that are of value in checking the consistency of the analyses and in testing the validity of one of the assumptions.

I. INTRODUCTION

Four years have elapsed since Tiselius (21) described his modification of the moving-boundary method for the electrophoresis of proteins and related materials. It is one of the purposes of this report to describe the method in its present form, including recent improvements in the experimental procedure. Moreover, the author will take this opportunity to include details of manipulation and procedure which have hitherto escaped publication.

Since its introduction in 1937 the method has been used for the study of a variety of problems, particularly for the electrophoretic analysis of naturally occurring protein mixtures. Human plasma is an important example of such a mixture, and the analysis of this material has recently been improved by a study, the results of which are also reported in this paper, of the conditions under which satisfactory patterns may be obtained. Moreover, an interpretation of the patterns of protein mixtures is suggested, and the current methods of computing concentrations from the patterns are critically examined.

II. THE NEW TISELIUS ELECTROPHORESIS CELL

In the moving-boundary method for the electrophoresis of proteins a sharp boundary between the protein and buffer solutions is formed initially in each of the two sides of a U-shaped channel, with the denser protein solution underneath.

[1] Presented at the Symposium on Physicochemical Methods in Protein Chemistry, which was held under the joint auspices of the Division of Physical and Inorganic Chemistry and the Division of Biological Chemistry at the 102nd meeting of the American Chemical Society, Atlantic City, New Jersey, September 8-12, 1941.

The latest cell devised by Tiselius (5, 20) for this purpose is shown in cross section in figure 1 and consists of the three sections I to III. These may be slid over one another along the planes *a-a'* and *b-b'*. Through the cell runs a U-shaped channel having a rectangular cross section of 3 x 25 mm. With the cell in place in its support, which will be shown below, the boundaries are formed as follows: The bottom section III is filled with the protein solution, the filling extending above *b-b'*, and the contents of this section are then isolated by displacement of this section to the left, as shown at A in figure 1. One side of section II is filled with protein solution and the other side rinsed (with the aid of a long stainless-steel needle attached to a syringe), and filled with buffer solution, both sides being filled to a level above *a-a'*. After connecting to the electrode vessels and partially filling the latter with buffer solution, the assembly is placed in a thermostat regulating at 0.5°C. When thermal equilibrium has been established in the channel, section II is displaced to the right, after

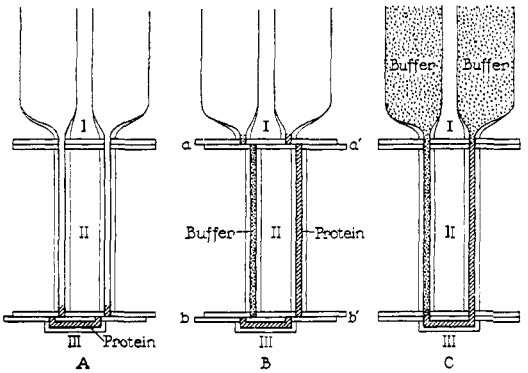

FIG. 1. Diagrams illustrating the initial formation of the boundaries in the Tiselius electrophoresis cell with the tall center section.

which the bottom section is returned to the center, as shown in B of figure 1. The excess protein solution is then rinsed out of the top section and both sides of this section, together with the attached electrode vessels, are filled with buffer solution, as will be described below. Now by returning section II to the center position, figure 1 C, boundaries between the protein and buffer solutions are formed at the junction of sections I and II in one side of the channel and between sections II and III in the other side.

The support for the cell and the electrode vessels, together with the mechanism (5) for moving the sections of the cell in relation to each other, are shown in figure 2. With the electrophoresis cell filled as described, but before the boundaries have been formed, buffer solution is introduced into the electrode vessels to the levels *l-l'*. The silver–silver chloride electrodes E and E' (6) are next inserted, care being taken not to trap air bubbles as the ground glass stopper f is seated. Concentrated potassium chloride is then introduced around each electrode by allowing 25 ml. of 1 *N* potassium chloride to flow slowly down each of the silver tubes t and t'. As the buffer solution around the electrode E, for

example, is displaced, the liquid level rises in the hollow stopper p and the excess buffer solution overflows through c. The tube t is closed while still filled with solution and the capillary rubber tube c, also filled with solution, is then connected, as shown in figure 2, to the syringe d, the latter having been previously filled with buffer solution and clamped in position in the "compensator." The latter device, not shown in the figure, imparts, with the aid of a small synchronous motor, a uniform movement, at the desired rate, to the piston of the syringe. By turning the stopcock m clockwise through 90° from

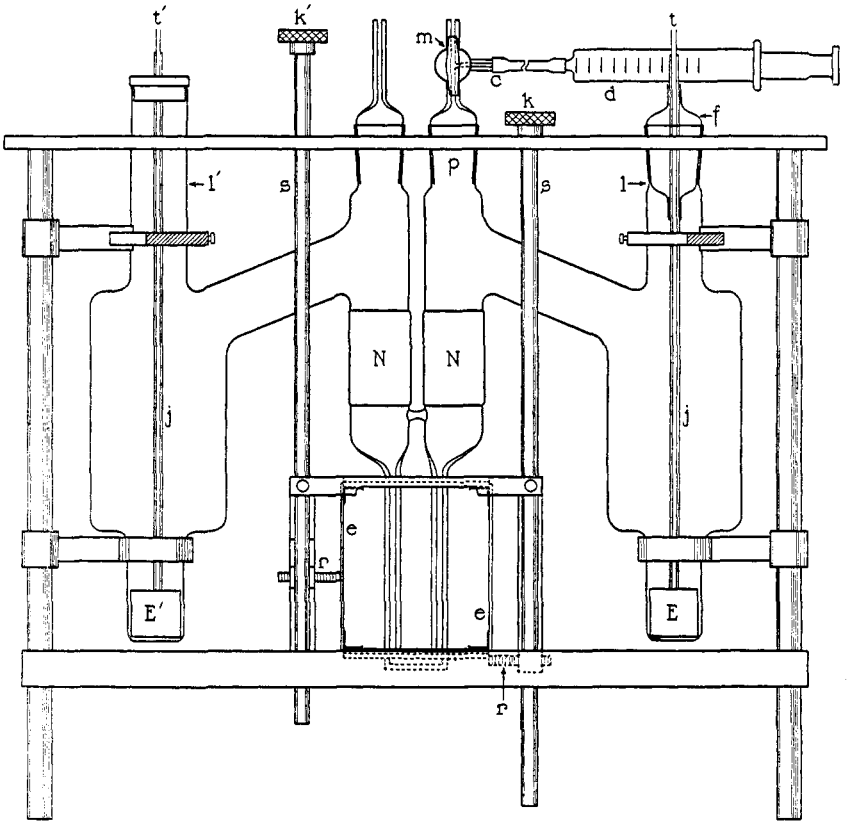

FIG. 2. Electrophoresis cell, electrode vessels, and support

the position shown in figure 2, the right-hand side of the apparatus is closed, connection with the compensator being retained. It is essential that air bubbles be excluded from the closed side, and the manipulation outlined here achieves that purpose. The boundaries are then formed as has been described and are brought out from behind the opaque horizontal plates of the cell by slow injection of buffer from the syringe, after which the latter is isolated by turning the stopcock m through 45°. Application of a potential in the appropriate direction then causes, in general, the boundary in one side of the center section to rise and that in the other side to descend. If the protein solution contains several

components the mobilities of which differ sufficiently, the initially single boundary in each side of the channel will separate, on electrophoresis, into a number of separate boundaries exclusive of the δ and ϵ (7) effects, equal to the number of components in the mixture. In the electrophoresis of plasma at pH values above 7, all of the components are negatively charged. In this case the pattern of the rising boundaries may also be termed the anode or positive pattern, while that of the descending boundaries is the cathode or negative pattern.

The tall center section II of figure 1 is interchangeable with, and replaces, the two identical short center sections of the original Tiselius cell, thereby eliminating the horizontal glass plates obscuring the middle of the field. With the new section it is possible to spread the boundaries over more than twice the usual distance, with the possibility of disclosing more detail in the electrophoretic pattern. This is illustrated later in this paper, the patterns of a human plasma, as obtained in the short section, being shown in figure 5 and those of the same material in the new tall section in figure 6. Although some of the increased resolution apparent in figure 6 is due to the use of a different buffer solvent and a higher concentration of protein, the improvements of the patterns have, in part, been made possible by the use of the new type of center section. This new section is to be recommended for electrophoretic analyses and also for electrophoretic separations except in the case of a mixture of two components. In this latter case it is usually possible to fill a short compartment with a separated component, and isolation of this section then permits easy recovery of the material.

With mixtures of more than two components, however, it is usually not possible to fill a short compartment with a separated component before one of the boundaries has been drawn into the bottom section. Under these conditions the layer of solution containing the separated component may be recovered with the aid of the "convection-proof" pipet of Tiselius (22) or, as carried out in this laboratory, with a fine glass capillary attached to the compensator syringe. The tip of the capillary is lowered, with the aid of a rack and pinion, to the proper level in the channel and the desired solution withdrawn. The introduction of the capillary does not disturb the boundaries appreciably, and the entire procedure is subject to schlieren observation and control. The presence of the capillary in the channel does not interfere with the electrophoresis and it is thus possible to begin the withdrawal of material from between two boundaries soon after their separation has occurred. The two processes may then proceed simultaneously, thereby saving considerable time.

III. RECORDING THE COMPLETE ELECTROPHORETIC PATTERN

Probably the most important advance in electrophoresis procedure has been in the development of methods by means of which the complete electrophoretic patterns are obtained. The optical devices for this purpose are based upon an effect employed by Foucault and Toepler for testing lenses and called by the latter the "schlieren method," since it rendered small differences of refractive index visible as schlieren or shadows. As originally adapted by Tiselius, a

boundary appeared in the focus of the schlieren camera as a dark band. This method, however, did not furnish information as to the variation of the refractive index in the boundary and was soon replaced by methods which yielded the complete electrophoretic pattern. The latter is a plot of the refractive-index gradient in a thin layer of solution in the channel as ordinate against the height of the layer as abscissa. One of the methods, the schlieren-scanning method, was developed by the author (2), while the other, the cylindrical-lens method, is due to Philpot (12) and has been modified by Svensson (18, 19). A pattern obtained with the aid of the latter method is shown in figure 3b, which also includes, for comparison, a pattern of the same material as given by the scanning procedure (figure 3a). Both the simple schlieren and the schlieren-scanning methods have been adequately described elsewhere (7) and will not be reviewed here.

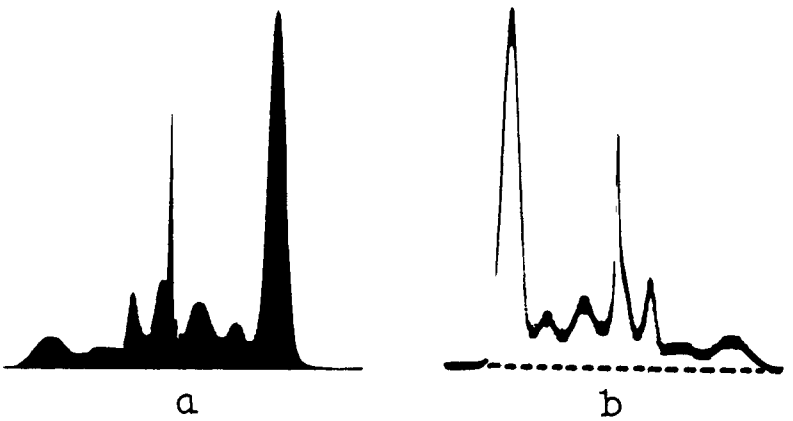

FIG. 3. Electrophoretic patterns of a human plasma obtained with the aid of (*a*) the schlieren-scanning method and (*b*) the cylindrical-lens method.

The cylindrical-lens method, however, affords an inexpensive and useful addition to the electrophoresis apparatus, and since the existing descriptions of its operation are not readily available to American chemists, a brief outline of this method will be presented here, with the aid of figure 4. An illuminated horizontal slit, present on the left of S but not shown in the figure, is focussed by means of the schlieren lens, S, in the plane of the schlieren diaphragm, D. The latter contains a diagonal slit, kk, as shown in the front view, D′. The camera objective, C, is focussed on the electrophoresis cell, E, and forms, in the absence of the lens H, a normal image of the cell on the ground-glass or photographic plate at G. The cylindrical lens, H,[2] with its axis vertical, is

[2] The objective C is a carefully corrected achromat of 2″ diameter and 36″ focal length. It is thus used at an aperture of *F*/18. The cylindrical lens H, on the other hand, is a single element lens of the same diameter but has a focal length of 16″ and hence works at the much higher aperture of *F*/8. Since this exceeds the maximum aperture of about *F*/16 usually considered safe (11) for photography with a simple lens, the use of the uncorrected cylindrical lens at an aperture of *F*/8 doubtless introduces aberrations in the patterns.

focussed on the schlieren diaphragm and also on the plate at G. Viewed from the side (figure 4a), the cylindrical lens has no effect on the pencils of light forming the cell image. Thus the vertical coördinate of each point in the image is conjugate to the corresponding level in the channel E and, owing to the focussing action of the camera lens, C, this also remains true for pencils that may be deflected by gradients in the channel. Viewed from above, however (figure 4b), the cylindrical lens, in conjunction with the diagonal slit, causes, as will be shown below, a lateral deviation of a pencil of light that is proportional to the vertical deflection the pencil has suffered in a boundary. The curve to the right of G in figure 4a represents the pattern of the boundary, B, as it would appear on the screen if the latter were hinged at the side and turned toward the reader, whereas if it were hinged at the top and turned, the pattern would appear as in figure 4b.

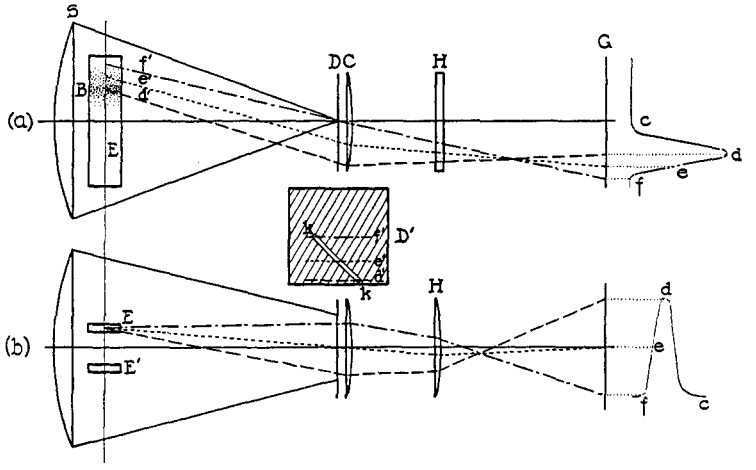

FIG. 4. Diagram of the cylindrical-lens method for the observation of the electrophoretic patterns.

If the fluid in the electrophoresis cell is homogeneous, all of the light through the channel is concentrated in an image of the illuminated slit at the upper or normal level on the diaphragm, i.e., f′ of D′, or D. As can be seen from the figure, only the extreme left-hand portion of the light in this image passes through the diaphragm to form a straight vertical line, i.e., the base line, on the screen at the position *c-f*. The width of this line varies with the width of the diagonal slit and, since a wide line is undesirable, Svensson has made the practical suggestion of tapering the end of the slit kk to a point.

If, on the other hand, a boundary B is present in the cell, a pencil through the layer of solution in the boundary having the maximum gradient, for example, is deflected downward as indicated by the line *d′d* and forms an image of the slit at the lower level *d′* on the diaphragm. Owing to the angle the diaphragm slit makes with the vertical, the portion of the light in the lower image *d′* that enters the slit is shifted laterally from the position at which the normal pencil enters by an amount proportional to the vertical deflection in the boundary

gradient. The cylindrical lens consequently imparts to this pencil a corresponding lateral shift in the opposite direction to the position *d*, figure 4b, without affecting its vertical position, i.e., *d* of figure 4a.

The path of a pencil through another portion of the boundary is indicated by the line *e'*-*e* and forms the corresponding element in the pattern. All other elements in the complete pattern are formed similarly.

Our camera has been modified recently so that either the scanning or the cylindrical-lens procedures can be used interchangeably. The cylindrical lens has been mounted in the aluminum tube that serves as the camera "bellows" in such a manner that it may be easily placed in, or removed from, the path of the light forming the cell image. With this lens in the optical path, a mask with a diagonal slit is inserted at the schlieren diaphragm and the camera then yields patterns of the type shown in figure 3b. When, however, the scanning procedure is to be used, the diagonal slit is removed and the cylindrical lens moved out of the optical path. This arrangement is proving to be very satisfactory.

Although a slit as diaphragm has been described here in conjunction with the cylindrical lens, one may also use a diagonal straight edge, as was done by Philpot. Moreover, it is also possible to use either a slit (16) or a straight edge, in this case in a horizontal position, with the scanning method. With either method a slit as diaphragm gives the pattern as an illuminated line on a dark background, as is indicated in figure 3b, whereas in the case of a straight edge the pattern is obtained as the contour between a light and a dark field, figure 3a. The latter type of diaphragm has been criticized in that the position of the contour shifts slightly with the exposure and development of the photographic plate, whereas the center of the line obtained with a slit is more nearly independent of these factors. While this criticism of the straight edge is justified theoretically, no difficulty has been experienced in keeping the error from this source well within the limits of accidental error from other sources.

The cylindrical-lens method is convenient for visual observation during an experiment and for the control of electrophoretic separations, since the pattern may be viewed directly on the screen of the camera. For visual observation a slit diaphragm is preferable to a straight edge, since the greater quantity of light reaching the screen in the latter case causes a decrease in the apparent contrast between the pattern and the background. For photographic work, however, the straight edge is to be preferred, owing, in part, to the superior resolving power and simpler diffraction phenomena (3) characteristic of this diaphragm. Consequently the permanent photographic records of an experiment, on which the analyses are based, are obtained with a straight edge as diaphragm and with the scanning procedure. With the latter method the optical errors inherent in the uncorrected cylindrical lens are eliminated.

IV. THE ELECTROPHORESIS OF PROTEIN MIXTURES AND CHOICE OF THE BUFFER SOLVENT

Although the electrophoretic method may be used for precise measurements of the mobilities of pure proteins as functions of the pH and ionic strength of the buffer solution in which they are dissolved, the method is finding its widest

application in the electrophoretic analysis of naturally occurring protein mixtures. Of the many mixtures that are available for study, the plasma proteins are among the most important. Consequently it is of interest to consider the sources of error in the electrophoretic analysis of plasma and to establish the experimental conditions, particularly the buffer solution used as solvent for the plasma proteins, that yield satisfactory patterns.

In an ideal electrophoresis of a protein mixture the volumes swept through by the rising and descending boundaries due to each component are identical and are proportional to the mobilities of the separate components. Moreover, the area under each "peak" in the electrophoretic pattern is proportional to the concentration of the component in the mixture to which it is due. In the ideal or limiting case the patterns for the two sides of the channel are mirror images of each other. The actual patterns, however, never exactly meet this condition

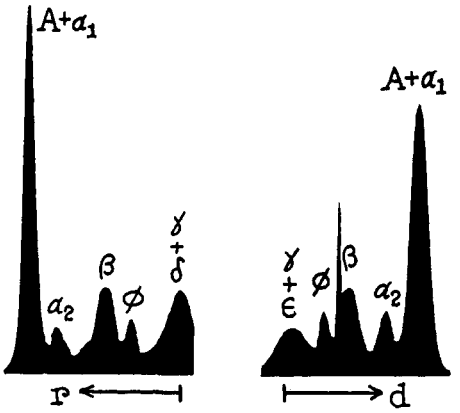

FIG. 5. Electrophoretic patterns of (r) the rising and (d) the descending boundaries of a human plasma, diluted 1:4, in a 0.025 N LiCl–0.025 N LiV–0.025 N HV (V = diethylbarbiturate) buffer at pH 7.9 after electrolysis, at 0.5°C., for 5750 sec. at 7.76 volts per centimeter. The initial boundary positions are indicated by the ends of the arrows.

and only approach it as the protein concentration is decreased. At such low protein concentrations the areas under the peaks are, however, not of sufficient magnitude to be determined accurately, and the vertical density gradients in the boundaries are frequently insufficient to stabilize the latter against the disturbing effects of convection.[3] The asymmetries between the patterns that appear as the protein concentration is increased are due to the contribution of the protein ions to the conductance and viscosity of the buffer solution in which they are dissolved and also to the influence of these ions upon the pH of the solution and upon the transference numbers of the buffer electrolytes. Typical

[3] Thus it has been found necessary to use a smaller current for the electrophoresis of, say, a 0.1 per cent solution of a protein in a given buffer than for a 1 per cent solution in the same solvent. The importance of the vertical density gradient in reducing disturbances due to convection and electroösmosis is also illustrated by the work of Shedlovsky and Smadel (14) on the elementary bodies of vaccinia.

asymmetries, i.e., "boundary anomalies," are illustrated in the patterns of figures 5 to 7. Thus the rising boundary due to albumin is sharper than the corresponding descending boundary. Boundaries characterized by large gradients, such as the rising albumin spike of figure 7, are not desirable for the practical reason that they cannot be recorded completely and, theoretically, because their areas are subject to the convergence and curvature errors discussed by Svensson (19). Moreover, all of the rising boundaries in the figures have swept through larger volumes, but have smaller areas, than the correspond-

TABLE 1

Buffer solutions used as solvents for the electrophoretic analysis of human plasmas and sera

(1) BUFFER	(2) μ	(3) pH AT 25°C.	(4) $\kappa_B \times 10^8$	(5) SERUM OR PLASMA	(6) DILUTION*	(7) NUMBER OF PEAKS	(8) SYMMETRY	(9) γ SEPARATION
0.1 *N* NaCac†–0.02 *N* HCac	0.1	6.8	3.10	P	1:2	‡	0.65	Yes
0.008 *M* NaH_2PO_4–0.064 *M* Na_2HPO_4	0.2	7.7	4.81	P	1:2	6§	0.39	Yes
0.004 *M* NaH_2PO_4–0.032 *M* Na_2HPO_4	0.1	7.7	2.62	S	1:4	5	0.52	Yes¶
0.025 *N* LiV‖–0.025 *N* HV–0.025 *N* LiCl	0.05	7.9	1.96	P	1:4	5	0.75	No
0.025 *N* LiV–0.025 *N* HV–0.075 *N* LiCl	0.1	7.9	3.97	S	1:4	4	0.75	No
0.025 *N* LiV–0.025 *N* HV–0.0675 *N* LiCl–0.0025 *M* $CaCl_2$	0.1	7.9	4.00	S	1:4	4	0.70	No
0.02 *N* NaV–0.02 *N* HV–0.08 *N* NaCl	0.1	7.9	5.13	P	1:2	5	0.54	No
0.04 *N* NaV–0.02 *N* HV–0.06 *N* NaCl	0.1	8.2	4.58	P	1:2	7	0.56	Yes¶
0.025 *N* $NaHCO_3$–0.1 *N* NaCl	0.125	8.2	6.64	S	1:4	4§	0.54	No
0.1 *N* NaV–0.02 *N* HV	0.1	8.6	3.03	P	1:2	7	0.81	Yes
0.1 *N* LiV–0.02 *N* HV	0.1	8.6	2.41	P	1:2	7	0.81	Yes¶
0.1 *N* NaOH–0.6 *N* glycine	0.1	9.0	3.48	P	1:2	6§	0.44	Yes¶

* With a dilution of 1:2 the new tall center section was used, otherwise the old short section.

† Cac = cacodylate.

‡ Partial precipitation of the proteins occurred in this buffer solvent.

§ Incomplete separation of α_1 from albumin.

¶ Separation was less complete than in 0.1 *N* NaV at pH 8.6.

‖ V = diethylbarbiturate.

ing descending boundaries. Finally, there are the δ and ϵ peaks, of quite different areas, that do not correspond to any component of plasma. The ϵ boundary represents a gradient of buffer electrolyte concentration that remains, on electrophoresis, near the initial boundary position. The δ boundary, on the other hand, is due to concentration gradients of both buffer salts and proteins. Moreover, it was suggested in an earlier paper (7) that the salt gradients in the δ and ϵ boundaries are balanced, partially at least, by small changes of salt concentration across each of the protein boundaries. These inverted salt gradients in the protein boundaries decrease the area of the latter slightly and, as will be shown below, should be considered in interpreting the patterns.

The abnormally sharp spike usually observed in the descending β-globulin boundary is a different type of asymmetry from those mentioned above and appears to be characteristic of this component.

The author has obtained patterns of human plasma, or serum, in a series of buffer solutions the compositions, ionic strengths, pH values, and specific conductances of which are given in the first four columns, respectively, of table 1. Three of these patterns, all of the same sample of plasma, are reproduced in figures 5 to 7 and the corresponding experimental conditions are recorded in the legends for the figures. The patterns of figure 5 were obtained under the same conditions as in previous work (9) from this laboratory on plasma and serum. A comparison of the patterns of this figure with those of figure 6 indicates that

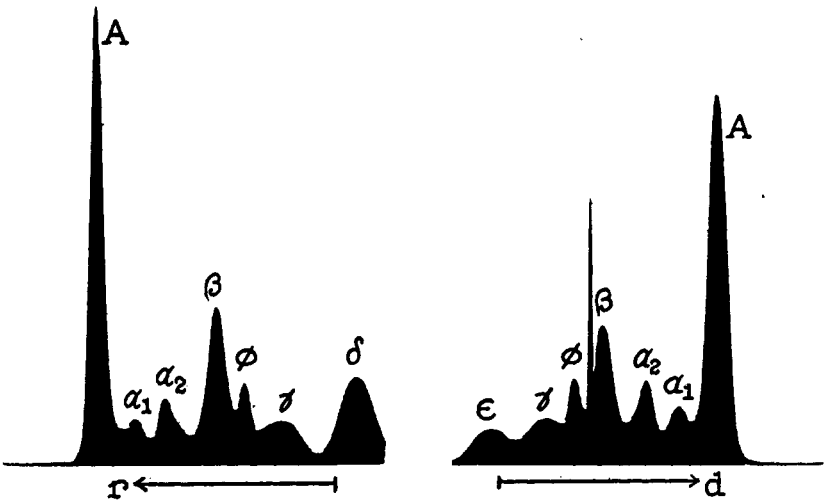

FIG. 6. Electrophoretic patterns of the same plasma as that shown in figure 5 but diluted 1:2 and dissolved in a 0.1 N NaV–0.02 N HV buffer at pH 8.6. Patterns were obtained after electrolysis for 14,000 sec. at 5.38 volts per centimeter.

in the 0.1 N sodium diethylbarbiturate buffer at pH 8.6, a previously unrecognized component, designated as α_1, has separated from the albumin. Also the δ and ϵ boundaries have separated from the peak due to γ-globulin, thereby permitting a more accurate determination of the concentration of this important component.

The patterns of figure 7 were obtained in the buffer solvent used by Svensson (20) in his work on animal sera. In this solvent the α_1-globulin appears only as a shoulder on the albumin peak, fibrinogen, ϕ, is not adequately separated from the β-globulin, and the rising albumin boundary is too sharp to be recorded completely. Horse plasma was also examined in the same solvents as those used in obtaining the patterns of figures 6 and 7. In contrast with human plasma this material gave a more satisfactory pattern in the phosphate than in the diethylbarbiturate buffer, thereby suggesting that the proper solvent for the analysis of the plasma of a given type of animal varies with the species and should be determined experimentally.

Sufficient data pertaining to the patterns obtained in the other solvents listed in table 1 are included in that table to show that, on the basis of symmetry and number of peaks clearly resolved, none is superior to the 0.1 N sodium diethylbarbiturate solution at pH 8.6 used in obtaining the patterns of figure 6. The letter in the fifth column indicates whether serum, S, or plasma, P, was the material studied. The dilution factor is given in column 6 of the table and in the next column the number of clearly resolved peaks in the pattern. One more peak, due to fibrinogen, is expected in a plasma pattern than in a serum pattern. In column 8 is given the ratio of the maximum refractive index gradient in the descending albumin boundary to that in the rising albumin boundary. While this ratio depends upon a number of factors, it nevertheless furnishes an index

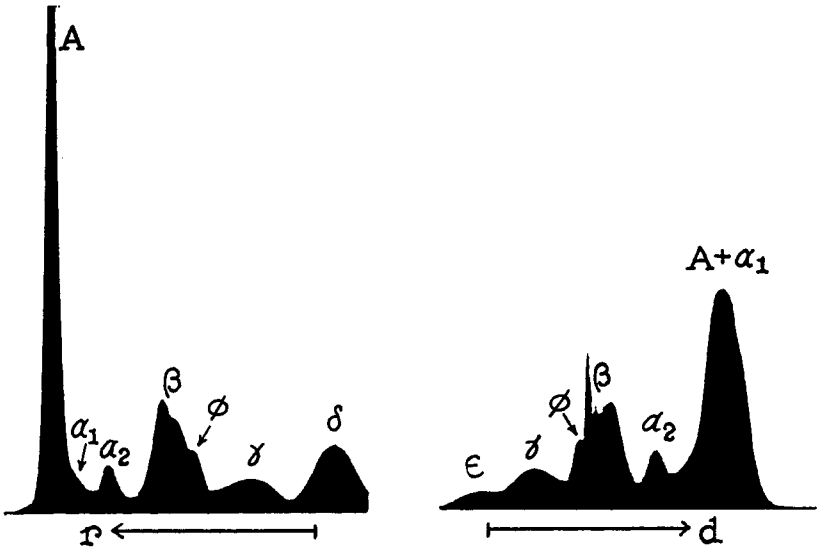

FIG. 7. Electrophoretic patterns of the same plasma as that shown in figure 5 but diluted 1:2 and dissolved in a 0.008 M NaH_2PO_4–0.064 M Na_2HPO_4 buffer at pH 7.7. Patterns were obtained after electrolysis for 20,500 sec. at 4.67 volts per centimeter.

of the symmetry of the patterns, the value approaching unity in the ideal case. As Svensson has suggested (20), a rapid spreading of the descending albumin boundary is undesirable, since the adjacent globulin peak is apt to be masked, as is the case with the α_1 component in figure 7d. Separation of the δ and ϵ boundaries from the γ-globulin peak is indicated in the last column of the table.

The same sample of material was not used in all of the experiments reported in table 1 and it is not possible, therefore, to make a quantitative study of the effect of the buffer solvent upon the relative concentrations of the components. There is some evidence (4, 15) that the buffer ions do have specific effects on protein mobilities. Consequently the redistribution of mobilities that occurs when one buffer solvent is substituted for another may cause appreciable variations in the apparent composition of a plasma. The following observation was,

however, made in connection with the patterns represented by table 1: Of the solvents listed in that table, the 0.025 N sodium bicarbonate–0.1 N sodium chloride solution at pH 8.2 is nearest in ionic composition to that of blood, and the analysis of a serum in this solvent was in good agreement with the analysis of the same serum in the diethylbarbiturate solvent used in our early work on human plasmas and sera. Moreover, the patterns of figures 5 and 6 are of the same sample of plasma and yield closely agreeing results if account is taken of the fact that, in figure 5, α_1 migrated with the albumin and the γ-globulin did not separate from the δ and ϵ boundaries. These observations appear to justify the use of the diethylbarbiturate buffer as solvent for the plasma proteins, in spite of the fact that this buffer ion is not a normal component of blood.

Some additional observations in connection with table 1 are worthy of note. Although pattern asymmetries are reduced by increased ionic strengths, the improvement is generally more marked if the increase is due to buffer salt, which simultaneously raises the buffer capacity, than if it is due to neutral salt.

A pattern obtained in a 0.1 N lithium diethylbarbiturate solution at pH 8.6 was quite indistinguishable from a pattern of the same material in a solution of the corresponding sodium salt, except that in the latter solvent greater separation between the δ, or ϵ, boundary and the γ-globulin boundary occurred. This observation has been of value in the interpretation of the δ and ϵ effects. In figure 6d, in which the end of the arrow indicates the initial boundary position, it can be seen that the separation between the γ and ϵ boundaries is due, in part, to a slight cathodic displacement of the latter boundary. This displacement is known to be proportional to the change of the transference number, T_+, with the concentration (10). From the conductance data of table 1 and the mobility of the sodium ion, one can estimate a value of $T_+ = 0.73$ for 0.1 N sodium diethylbarbiturate and 0.66 for 0.1 N lithium diethylbarbiturate. Since the change of transference number with concentration is roughly proportional to the deviation of T_+ from 0.5 (1), this change is less for the lithium salt than for the sodium salt and, in agreement with theory, the observed cathodic displacement of the ϵ boundary is less in the 0.1 N lithium diethylbarbiturate buffer than in the 0.1 N sodium diethylbarbiturate buffer solution.

V. INTERPRETATION OF THE ELECTROPHORETIC PATTERNS OF PLASMA

A. Determination of relative concentrations

Since the specific refractive increments of the electrophoretically separable plasma proteins have not yet been measured it is only possible, at present, to determine their concentrations as differences in refractive index and not in terms of protein nitrogen or dry weight. The available evidence indicates that the refractive index is proportional to the protein concentration as determined by other methods, although the proportionality factor doubtless varies with the nature of the protein. While it would be of considerable interest to compare concentrations, as determined from the areas of the electrophoretic patterns, with values given by other methods, the present lack of knowledge of the specific

refractive increments cannot be considered a limitation of the electrophoretic method, since there is no *a priori* reason for selecting one method of measuring protein concentrations in preference to others.

In determining the area, A_i, due to a given component, i, in the pattern it has been customary to make a more or less arbitrary separation, owing to the fact that the gradients of the different boundaries overlap. Following the procedure of Tiselius and Kabat (23), this has been done by drawing an ordinate

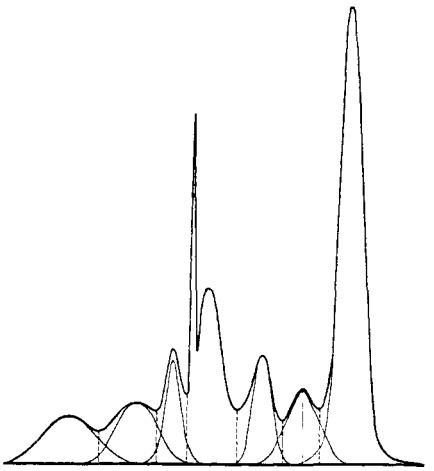

FIG. 8. Tracing of the electrophoretic pattern of figure 6d. In this diagram two methods of distributing the areas among the plasma components are illustrated.

TABLE 2

Concentrations and electrophoretic volume displacements of the components of a human plasma

	COMPONENT i	A	α_1	α_2	β	ϕ	γ	δ	ϵ
1	A_{id}	356	54	85	181	61	77		67
		(343)	(72)	(64)	(199)	(50)	(76)		(77)
2	A_{ir}	325	45	75	163	56	77	141	
3	A_{id}/A_{Ad}		0.152	0.239	0.508	0.171	0.216		
4	A_{ir}/A_{Ar}		0.138	0.231	0.502	0.172	0.237		
5	V_{id} (ml.)	3.297	2.699	2.208	1.568	1.144	0.659		0.128
6	V_{ir} (ml.)	3.597	3.037	2.520	1.815	1.376	0.811	0.305	
7	ρ_i (eq. 5)	0.923	0.899	0.890	0.884	0.862	0.864		

from the lowest point between two adjacent peaks. These ordinates are shown as dotted lines in figure 8, which is a tracing of the cathode pattern of figure 6. If it is divided in this manner, the areas under the various peaks have the values in arbitrary planimeter units, given in the first line of table 2. With the exception of the peak due to β-globulin, it is also possible to resolve, as described by Pedersen (17), the pattern into a series of symmetrical curves. These curves are indicated by the light lines of figure 8 and the corresponding areas are recorded in parentheses in table 2. The two methods yield values for the areas

that differ by as much as 33 per cent in the case of α_2-globulin and exhibit an average deviation of 15 per cent. The plasma globulins, with the exception of fibrinogen, are so far differentiated entirely on the basis of their electrophoretic mobilities. The differences in areas noted above are due, partially at least, to the fact that the two methods of dividing the pattern define the globulins by assigning to each a somewhat different range of mobilities. The method of Tiselius and Kabat enjoys the advantage of simplicity. In using the latter method the author has observed that the distribution of areas among the peaks shifts slightly as the distances between them increase and appears to approach that given by the Pedersen method. The arbitrary nature of the methods of separation must be recognized. The comparison made above may serve to indicate the uncertainties that arise from this source.

B. Assumptions

As previously noted, there are small salt gradients in each of the protein boundaries and the observed area, A_i, is thus the sum of an area, P_i, due to gradients of the i^{th} protein alone and an area, $-S_i$, due to salt, the negative sign indicating the inverted nature of this gradient. Since the magnitudes of these salt gradients are not known, except that they are relatively small, it has been found convenient to make certain assumptions regarding them in order to compute, from data obtained from the electrophoretic patterns, relative concentrations of the proteins. Thus the concentration of one protein component relative to that of another, say albumin, may be computed from values of A_i from the cathode pattern, designated as A_{id} since they refer to the descending boundaries, if the assumptions are made (I) that S_i is proportional to P_i and (II) that the proportionality factor is the same for all components. With these assumptions $P_{id}/P_{Ad} = A_{id}/A_{Ad}$. This relation was used in computing the concentrations of the plasma globulins, relative to albumin, from the pattern of figure 6; the results are given in the third line of table 2.

Values of A_i from the anode pattern, i.e., A_{ir}, since they refer to the rising boundaries, may also be used to compute relative concentrations if the additional assumption (III) is made that all of the protein components are held in the same proportion through the δ boundary. Values of A_{ir}/A_{Ar} are given in the fourth line of table 2 and are in reasonably good agreement with corresponding values of A_{id}/A_{Ad}.

The available evidence indicates that the first of the assumptions made in these computations, namely, that the salt gradients in a protein boundary are proportional to the gradients of protein, is valid.[4] The second assumption that the proportionality constant is the same for all proteins probably introduces, on the other hand, a small error, since it is unlikely that all proteins influence the transference of the buffer electrolytes to the same extent. The third assumption that all components are held in the same proportion through the δ boundary places restrictions, as will be shown below, upon the volume displacements of

[4] The author has observed (see 13), for example, that the transference number of potassium chloride in aqueous solution varies linearly with the concentration of added glycine.

the various components as well as upon the areas. Since the displacements can be measured with somewhat more precision than the areas, it is thus possible to test the validity of the third assumption. In order to obtain the relations required for this test it is necessary to consider the transference of material from one side of the channel to the other during electrophoresis and to apply the principle of the conservation of matter to these processes.

C. Electrophoretic transference of proteins and buffer electrolytes

Since the passage of an electric current does not produce changes of composition in the body of a homogeneous solution, the composition of the protein solution in the bottom section, and also that of the buffer solution in the two sides of the top section, remains constant during electrophoresis. The *total* area of an electrophoretic pattern is thus proportional to the integral

$$\int_B^P (\mathrm{d}n/\mathrm{d}h)\mathrm{d}h = n_P - n_B$$

in which n is the refractive index at the level h in the channel. The integration is from the original protein solution P to the buffer solution B and, since these limits are the same for both anode and cathode patterns, the total areas of the two patterns are the same and are independent of the time during electrophoresis at which the patterns are recorded. This relation may be written as

$$A_\epsilon + \Sigma A_{id} = A_\delta + \Sigma A_{ir}$$

or as its equivalent,

$$A_\epsilon + \Sigma(P_{id} - S_{id}) = P_\delta + S_\delta + \Sigma(P_{ir} - S_{ir}) \qquad (1)$$

in which the summation is for all of the protein components and P_δ and S_δ are the portions of the area of the δ peak due to protein and salt gradients, respectively.

During electrophoresis the plasma proteins neither enter nor leave the cell. Consequently, if the cathode pattern indicates a loss of one of these components, the anode pattern should indicate a corresponding gain in that component. If V_{id} is the volume through which the i^{th} component descends, the loss of this component from the cathode channel is proportional to $V_{id}P_{id}$ and this must equal the corresponding gain, $P_{ir}V_{ir} - (P_{id} - P_{ir})V_\delta$, in the anode channel. Thus for each of the i components

$$V_{id}P_{id} = V_{ir}P_{ir} - (P_{id} - P_{ir})V_\delta \qquad (2)$$

Considerable quantities of buffer electrolytes, on the other hand, move through the cell during an experiment. The total amounts of these materials in the cell at any instant remain essentially constant,[5] however, and from considerations

[5] Negligibly small quantities of buffer electrolytes may be gained, or lost, by the cell, owing to small volume changes resulting from electrophoretic separation if the specific volumes of the various components are not additive.

similar to those above, i.e., conservation of matter, the following relation may be obtained:

$$A_\epsilon V_\epsilon + \Sigma S_{id} V_{id} = S_\delta V_\delta + \Sigma S_{ir} V_{ir} \tag{3}$$

Since the net movement of buffer salts is from the anode to the cathode side of the channel and that of protein is in the opposite direction, the total transport of material is given by the difference between equation 2, summed over all protein components, and equation 3, i.e.,

$$\Sigma V_{id}(P_{id} - S_{id}) - A_\epsilon V_\epsilon = \Sigma V_{ir}(P_{ir} - S_{ir}) - V_\delta[S_\delta + \Sigma(P_{id} - P_{ir})]$$

but

$$P_i - S_i = A_i, \qquad \Sigma(P_{id} - P_{ir}) = P_\delta, \quad \text{and} \quad P_\delta + S_\delta = A_\delta$$

Therefore,

$$\Sigma V_{id} A_{id} - V_\epsilon A_\epsilon = \Sigma V_{ir} A_{ir} - V_\delta A_\delta \tag{4}$$

The relation 4 states that, if the area of each peak in the pattern be multiplied by the volume displacement of the peak, account being taken of the direction of migration, the sum of these products for the anode pattern is equal to the corresponding sum for the cathode pattern. Although equations 1 and 4 have been derived with reference to the electrophoretic analysis of plasma, they are valid for protein mixtures generally and represent two relations that may be used to advantage in checking these analyses. The relation 4 will be recognized as analogous to the well-known test for a successful transference experiment by the Hittorf or gravimetric method,—namely, that the loss of material at one electrode should equal the gain at the other electrode. From the data of table 2 a value of 1903 may be computed for the left-hand members of equation 4. This differs by 0.8 per cent from the value, 1887, obtained for the right-hand terms.

D. The δ boundary and a test of assumption III

Since P_{ir} and P_{id} are measures of the concentration of the i^{th} component on the two sides of the δ boundary, the ratio P_{ir}/P_{id}, which may be designated ρ_i, is the dilution that a component undergoes in this boundary. Solution of equation 2 for this ratio gives

$$P_{ir}/P_{id} = \rho_i = (V_{id} + V_\delta)/(V_{ir} + V_\delta) \tag{5}$$

an expression for ρ_i involving only boundary displacements. Values of the latter, as obtained from the pattern of figure 6d, are recorded in lines 5 and 6 of table 2, and in the last line of this table are reported values of ρ_i for each component as given by equation 5. It may be noted that the values of ρ_i are not strictly constant, as required by the assumption III that all components are held in the same proportion through the δ boundary, but show a drift that is outside the limit of error in the volume determinations.

It is also possible to compute values of ρ_i from determinations of area. Thus

with the aid of assumption I, $\rho_i = A_{ir}/A_{id}$; i.e., ρ_i is the ratio of the area of the i^{th} peak in the anode pattern to that of the corresponding peak in the cathode pattern. In the case of plasma only the areas of the albumin peaks are of sufficient magnitude to give a reasonably accurate value, i.e., 0.913, for ρ_A. This value, it will be noted, is in satisfactory agreement with the corresponding value, 0.923, from the displacements of the albumin boundaries.

An average value, $\bar{\rho}$, for all components may be computed with the aid of equation 1, i.e.,

$$\bar{\rho} = \Sigma A_{ir}/\Sigma A_{id} = (A_t - A_\delta)/A_t - A_\epsilon)$$

in which A_t is the total area of either the anode or the cathode pattern. The data of table 2 yield the value of 0.909 for $\bar{\rho}$. This is also in approximate agreement with an average value of ρ_i from table 2. Thus in all cases in which the areas can be determined with sufficient precision they lead to values of ρ in essential agreement with those computed from the boundary displacements.

The small variations among the values of ρ for the different plasma proteins, as recorded in the last line of table 2, would appear to indicate that assumption III is not strictly valid but represents a close approximation. In the preceding discussion of the electrophoretic analysis of plasma, it has been tacitly assumed that there is no interaction between the protein components, i.e., that one protein has no influence upon the transference of another protein. From a comparison with systems in which interaction is known to occur (8), the plasma patterns appear to be relatively free from this effect, although it should be recognized that some of the observed asymmetries may possibly be due to such interaction.

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