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A Quantitative Study of Reversible Boundary Spreading in the Electrophoresis of Proteins[1]

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Introduction

The usual criterion for the electrophoretic homogeneity of a protein is that it migrates as a single boundary in an electric field in buffers of various hydrogen ion concentrations and ionic strengths. However, this alone is not sufficient evidence that all the protein molecules have the same electrophoretic mobility, and further evidence regarding the electrophoretic homogeneity may be obtained by studying the rate with which the protein gradient spreads in the electrical field. If the molecules in a protein "family" vary with respect to electrophoretic mobility because of differences in net charge or size or shape, the protein gradient will spread faster in the electrical field than expected for diffusion alone but will become sharper upon reversal of the field.[2] While in the case of a heterogeneous protein spreading is observed at both boundaries in the U-tube, in the case of spreading caused by conductivity[3] or *p*H[4] gradients in the moving protein gradient, spreading at one boundary is accompanied by sharpening at the other. Spreading and sharpening caused by these superimposed gradients may be minimized by performing the electrophoresis experiment at the average isoelectric point of the protein.[5] Convection caused by the temperature gradient set up in the electrophoresis cell by electrical heating or caused by electroosmosis along the cell wall may also spread the protein boundary. Such convection effects would not be reversed by reversing the electric field because they are a result of turbulence, and so it is possible to test electrophoresis experiments for their presence.

Reversible electrophoresis spreading has been cited as evidence for the electrophoretic heterogeneity of *Helix pomatia* and *Helix nemoralis* hemocyanins,[6,7] ovomucoid,[5] alfalfa mosaic virus,[8] horse pseudoglobulin GI,[9,10] pectin[11] and bovine γ_1- and γ_2-globulins.[12]

Two quantitative methods for representing boundary spreading have been proposed,[6,13] but neither of these has been used to calculate the actual distribution in mobility among the protein molecules. The purpose of this paper is to present a quantitative method for the determination of the mobility distribution in certain cases.

Theory

If the electrophoresis of a heterogeneous protein with a mobility distribution $g(u)$ is carried out under conditions such that no convection is caused by temperature gradients or electroosmosis in the electrophoresis cell and there are no conductivity or *p*H gradients through the boundary between protein solution and buffer, the refractive index gradient, $\partial n/\partial x$, as a function of height in the electrophoresis cell, x, at time t_D after the formation of the boundary and time t_E after application of the electric field, is given by equation

(1) Presented before the Division of Physical and Inorganic Chemistry, Atlantic City, April 18, 1947.

(2) Tiselius, *Nova Acta Reg. Soc. Scient. Upsala*, (IV) **7**, No. 4 (1930).

(3) Longsworth and MacInnes, THIS JOURNAL, **62**, 705 (1940).

(4) Longsworth, *J. Phys. Coll. Chem.*, **51**, 171 (1947).

(5) Longsworth, Cannan and MacInnes, THIS JOURNAL, **62**, 2580 (1940).

(6) Tiselius and Horsfall, *Ark. Kem. Min. Geol.*, **13A**, No. 18 (1939).

(7) Horsfall, *Ann. N. Y. Acad. Sci.*, **39**, 203 (1939).

(8) Lauffer and Ross, THIS JOURNAL, **62**, 3296 (1940).

(9) Sharp, Cooper and Neurath, *J. Biol. Chem.*, **142**, 203 (1942).

(10) Sharp, Hebb, Taylor and Beard, *ibid.*, **142**, 217 (1942).

(11) Speiser, Copley and Nutting, *J. Phys. Coll. Chem.*, **51**, 117 (1947).

(12) Hess and Deutsch, THIS JOURNAL, **70**, 84 (1948).

(13) Sharp, Taylor, Beard and Beard, *J. Biol. Chem.*, **142**, 193 (1942).

(1).[14] D is the diffusion constant which is assumed to be the same for all the protein molecules, u is electrophoretic mobility, E is the electric field strength, and $(n_1 - n_2)$ is the difference in refractive index of the protein solution and buffer. The boundary will spread faster than expected for diffusion alone because of the difference in rates of migration of the protein ions.

$$\frac{\partial n}{\partial x} = \frac{(n_1 - n_2)}{2\sqrt{\pi D t_D}} \int_{-\infty}^{+\infty} g(u) e^{\frac{-(x-uEt_E)^2}{4Dt_D}} du \quad (1)$$

If diffusion is negligible during the electrophoresis experiment, Sharp, *et al.*,[10] point out that equation (1) assumes a simple form, and a heterogeneity constant, H, may be defined by

$$H = \Delta\sigma/\Delta t E \quad (2)$$

where $\Delta\sigma/\Delta t$ is the time rate of change of the standard deviation, σ, of the refractive index gradient. For this case, H characterizes the mobility heterogeneity of the protein and the mobility distribution may be determined directly from the gradient curves.

If diffusion is not negligible during the electrophoresis experiment, it is theoretically possible to obtain the mobility distribution $g(u)$ from the experimental refractive index gradient curves by using equation (1), but this is not a practical method for studying the distribution in mobilities because of the difficulties in computation. However, a suitable function for $g(u)$ with parameters to be evaluated from the experimental curves may be introduced in equation (1). In the case of a protein at its "average" isoelectric point so that the most frequent molecule has a mobility of zero, it is convenient to try a Gaussian distribution function with h, the heterogeneity constant, the parameter to be evaluated.

$$g(u) = \frac{1}{h\sqrt{2\pi}} e^{-u^2/2h^2} \quad (3)$$

h has the dimensions of mobility and is the standard deviation for the mobility distribution which has been normalized to unity. Substituting this form of $g(u)$ in (1) and integrating, we obtain the equation giving the refractive index gradient as a function of height in the cell for such a protein during electrophoresis.

$$\frac{\partial n}{\partial x} = \frac{(n_1 - n_2)}{\sqrt{2\pi}\sqrt{E^2h^2t_E^2 + 2Dt_D}} e^{-x^2/2(E^2h^2t_E^2 + 2Dt_D)} \quad (4)$$

This equation shows that if there is a Gaussian distribution of mobilities, the electrophoresis gradient curves should be Gaussian, as is closely realized for the systems studied.

The heterogeneity constant, h, may be evaluated by noting that the standard deviation, σ, of the experimental curve should be

$$\sigma = \sqrt{E^2h^2t_E^2 + 2Dt_E + 2D\Delta t} \quad (5)$$

if the electrophoresis is started after the boundary has been diffusing Δt seconds. $2D\Delta t$ is the square of the standard deviation of the gradient curve at the moment the electric field was applied, σ_0^2. Rearranging

$$D^* = \frac{\sigma^2 - \sigma^2}{2t_E} = D + \frac{E^2h^2}{2} t_E \quad (6)$$

D^* is the "apparent diffusion constant" calculated from the experimental gradient curves during the electrophoresis.[15] According to this equation, the apparent diffusion constant should plot as a straight line against time of electrophoresis and extrapolate back to the normal diffusion constant at zero time. If the protein is heterogeneous and has a Gaussian distribution of mobilities, the straight line will have a slope $E^2h^2/2$ from which the heterogeneity constant h may be calculated. Since the heterogeneity constant is the standard deviation for the mobility distribution, the actual mobility distribution curves may be constructed using a table of values for the Gaussian probability function.

Equation (5) for the standard deviation of the electrophoresis curves in terms of the heterogeneity constant h may be used to show the relationship between the heterogeneity constant, H, introduced by Sharp, Taylor, Beard and Beard[13] and h. For the case in which electrophoresis and diffusion start simultaneously

$$H = \frac{\sigma}{tE} = \sqrt{\left(\frac{2D}{E^2}\right)\frac{1}{t} + h^2} \quad (7)$$

This shows that if diffusion is negligible, $H = h$. If diffusion is not negligible, H will decrease with time approaching h asymptotically.

As a check on irreversible spreading during electrophoresis, the direction of the electric field may be reversed for an equal period of time. The manner in which the apparent diffusion constant varies with time during the reversal period may be shown by using equation (5). If t_E is the total time the current has been flowing in both direc-

(14) This equation has been but slightly modified from that given by Sharp, Hebb, Taylor and Beard.[10] The times in the equation have been given subscripts to indicate whether they are the time of diffusion or electrophoresis so that the more general case in which these are not equal may be treated. Sharp, *et al.*, prefer to use a modified diffusion constant D_1 which may not be the same as D since they state variations in mobility of individual particles with time may affect the rate of diffusion under the influence of an electric field. If, however, as assumed by Tiselius (ref. 2, p. 26) diffusion is simply superimposed on the electrophoretic migration, it should not be necessary to distinguish between two diffusion constants provided convection can be eliminated, and no distinction is made in this paper.

(15) The apparent diffusion constant may be calculated from the gradient curves by any of the standard methods which will all give the same result provided the refractive index gradient is Gaussian in form. In this paper the apparent diffusion constants have been calculated from enlarged tracings of the photographs by using the half width x, of the gradient curves at the inflection point $\frac{Y}{\sqrt{e}}$, or from the area, A, and maximum height, Y,

$$D_\sigma^* = \frac{x^2 - x_0^2}{2t_E G^2} \qquad D_A^* = \frac{\left(\frac{A}{Y}\right)^2 - \left(\frac{A}{Y}\right)_0^2}{4\pi t_E G^2}$$

If x, A and Y are measured in centimeters on a tracing of the photograph, G is the number of cm. on the tracing paper corresponding to one cm. in the electrophoresis cell.

tion, and t_1 is the time after which the current was reversed, for $t_E > t_1$

$$D^* = D + \frac{E^2h^2}{2}\frac{(2t_1 - t_E)^2}{t_E} \qquad (8)$$

Thus the apparent diffusion constant decreases with time and becomes equal to the diffusion constant at $t_E = 2t_1$. The slope of the D^* *vs.* t_E plot immediately after reversing the current ($t_E = t_1$) is $-\frac{3}{2}E^2h^2$, and when $t_E = 2t_1$ the slope is zero showing that an error of a few minutes in the time of reversing the current will generally not cause D^* to differ significantly from D.

More general distribution functions with as many constants as justified by the precision of the experimental data may be used in place of the error function.[16] In cases in which the gradient curves are symmmetrical but not Gaussian, the sum of two or more Gaussian distribution functions may be used to represent the mobility distribution $g(u)$. Such a distribution function may be integrated conveniently when substituted in equation (1), and the parameters may be evaluated from the successive moments of a single gradient curve by the same method used in determining the diffusion constants and relative amounts of two or more independently diffusing molecules in a polydisperse system.[17]

Experimental

The optical system used in this work was the cylindrical lens schlieren optical system with a schlieren lens on each side of the thermostat arranged so that light from the horizontal slit passed through the cell in a parallel beam.[18] A diagonal slit (0.50 mm. wide) was used in the optical system, and the photographs were taken on Eastman Kodak Co. CTC plates and enlarged and traced. The gradient curves were obtained by averaging the ordinates of the two edges of the band of light and constructing the corresponding mean curve. It was found that the diffusion constants determined by this method did not vary with the exposure time as did those determined by the diagonal knife edge method.

It was found that when diffusion boundaries were formed in the standard Tiselius electrophoresis cell by the usual method and compensated into the optical system at the rate of 2–3 cm. per hour using a mechanically driven syringe, thirty to ninety minutes had to be added to the diffusion times in order to obtain a constant diffusion constant for short time diffusions (less than ten hours).[19] The quality of the initial boundary is very important in an electrophoresis spreading experiment since it must be carried out in a short period compared to the usual 3–4 day diffusion experiment. As shown by equation (6), the apparent diffusion constant may be calculated even when the initial gradient is diffuse, provided it is Gaussian, by using the method of differences, and this is satisfactory if the electrophoresis spreading is large. However, in the case of a homogeneous protein the spreading of the gradient during the experiment is not large and D^* calculated by the method of differences is subject to rather large experimental errors. In order to avoid the correction for the width of the initial boundary, the sharpening technique of Kahn and Polson[20] may be applied. In forming these so-called "sharpened" boundaries for some of the electrophoresis spreading experiments, a capillary was lowered into the protein boundary after it had been compensated into the cell, which was set up with both sides open to the atmosphere, and the diffuse portion of the boundary was drawn off by suction at a rate of about 0.4 cc. per minute. The sharpening of the boundary was followed with the schlieren optical system, and when the boundary became no sharper, the suction was practically stopped while the capillary was carefully withdrawn, and this time was taken as the starting time for the diffusion. Figure 1 shows the results of a short diffusion of crystallized bovine albumin[23] using a sharpened boundary. The zero time correction was negligible after one hour, and the agreement between the diffusion constants calculated by two methods indicates the precision obtained with the schlieren optical system. The average diffusion constant, 3.1×10^{-7} cm.^{2} sec.$^{-1}$ at 1.5°, is in agreement with earlier values.[21]

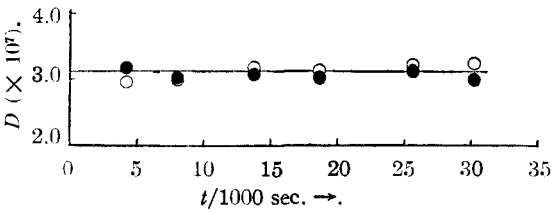

Fig. 1.—Diffusion constant for crystallized bovine albumin, $\Gamma/2 = 0.1$, *p*H 4.6, 0.6% protein, at 1.5°: ●, calculated from the half width at the inflection point; ○, calculated from the height and area.

The disadvantage of applying the sharpening technique in electrophoresis spreading experiments is the difficulty of sharpening both boundaries simultaneously. When only one of the boundaries is sharpened and conclusions are drawn from its behavior alone, sharpening or broadening of the peak caused by the field and *p*H gradients may go undetected, and an erroneous conslusion as to the homogeneity of the protein may be drawn.

More than the usual care to avoid thermal convection must be taken in electrophoresis spreading experiments. In electrophoresis load tests with alternating current Tiselius[22] found that power dissipations of 0.5 to 1 watt/cc. could be used in flattened electrophoresis cells near the temperature of maximum density of water. Loads of about 0.15 watt/cc. are used routinely in this Laboratory with the standard 11-cc. cells, but for spreading experiments the loads were kept below 0.015 watt/cc. It has been found that loads which do not cause convection in short experiments (two hours) may cause convection in prolonged spreading experiments as the density gradient becomes progressively less.

In order to reduce the difference in buffer salt concentration in the protein solution and equilibrium buffer caused by the Donnan effect and to reduce optical errors inherent in measuring high refractive index gradients, protein concentrations of 0.5 to 0.8% were used in all experiments. The electrophoresis samples were dialyzed two days in the cold before electrophoresis. The conductivity of the equilibrium buffer measured at the temperature of the thermostat (1°) was used in calculating the potential gradient, and the *p*H of the buffers was measured at 25° using a glass electrode.

(16) For examples of such functions see Rinde, "The Distribution of the Sizes of Particles in Gold Sols," Inaugural Dissertation, Upsala, 1928; Lansing and Kraemer, THIS JOURNAL, **57**, 1369 (1935); Jullander, *Ark. Kem. Min. Geol.*, **21A**, No. 8, 14 (1945).

(17) Neurath, *Chem. Rev.*, **30**, 357 (1942).

(18) Svensson, *Kolloid Z.*, **87**, 181 (1939); **90**, 141 (1940).

(19) This is similar to the observation by Longsworth that diffusion boundaries compensated into the electrophoresis cell are imperfect. THIS JOURNAL, **69**, 2510 (1947).

(20) Kahn and Polson, *J. Phys. Coll. Chem.*, **51**, 816 (1947).

(21) Cohn, Hughes and Weare, THIS JOURNAL, **69**, 1753 (1947); Stern, Singer and Davis, *J. Biol. Chem.*, **167**, 321 (1947).

(22) Tiselius, *Trans. Faraday Soc.*, **33**, 524 (1937).

Results

Bovine Albumin.—The isoelectric point of crystallized bovine albumin was found to be *p*H 4.6 at 0.10 ionic strength in acetate buffers. A number of spreading experiments carried out at this *p*H showed that although there was partial resolution of about 5% of another protein constituent, the same rate of boundary spreading was observed in both limbs of the U-tube. The additional protein component would not appear to be α- or β-globulin.[23] In these experiments the boundaries were compensated into the electrophoresis cell at a rate of about 1 cm. per hour, and after they had diffused until the maximum gradient was recorded by the optical system, a photograph was taken to determine σ_0 and the electric field applied. In the experiment shown in Fig. 2 one boundary was sharpened and the electric field applied immediately. The vertical arrows indicate the reversal time and the end of the experiment. The fact that the apparent diffusion constant at the end of the experiment is in agreement with the diffusion constant obtained from free diffusion (3.1×10^{-7} cm.^{2} sec.$^{-1}$, Fig. 1) is evidence that thermal and electroosmotic effects of the current did not disturb the boundaries appreciably. This is in agreement with the observation of Longsworth[24] that the correct diffusion constant for a raffinose boundary in 0.1 *N* lithium chloride was obtained in an electric field provided the power dissipation was not too great and is contrary to the conclusion of Janssen[25] that boundaries of non-electrolytes spread more rapidly in an electric field than expected from diffusion alone. A small amount of reversible spreading is evident in the case of bovine albumin, and although this amount of electrical spreading is not much greater than the experimental error, the electrical heterogeneity of bovine serum albumin at its isoelectric point has been confirmed by spreading experiments at 0.01 ionic strength where higher field strengths may be used.[26]

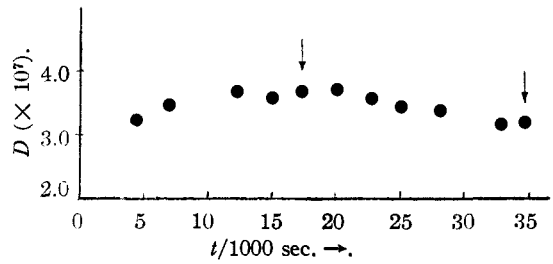

Fig. 2.—Electrophoretic spreading experiment with crystallized bovine albumin at $\Gamma/2 = 0.10$, *p*H 4.61 at 1.59 volts/cm. and 0.0135 watt/cc. using a sharpened boundary. The apparent diffusion constants were calculated by the inflection point method.

Human γ_2-Globulin.—Human γ_2-globulin[27] was studied by electrophoresis spreading experiments at several ionic strengths. The sample of protein used contained less than 2% of γ_1- and β-globulins and albumin as judged by electrophoresis at *p*H 8.6, $\Gamma/2 = 0.10$. Some of the refractive index gradient curves obtained during the electrophoresis of human γ_2-globulin at its average isoelectric point at 0.10 ionic strength are given in Fig. 3. The experimental refractive index gradient curves in solid lines are plotted with their bisecting ordinates located at the time at which the photograph was taken. The superimposed dashed curves give the patterns which would have been obtained if the spreading had been caused by diffusion alone (calculated using $D_O^0 = 2.0 \times 10^{-7}$). Note that after the direction of the electric field was reversed at 300 minutes, the experimental gradient curves became sharper and that after the electric field had been applied for equal times in the two directions, the experimental gradient was just that expected from diffusion alone. Figure 4 shows a comparison of a refractive index gradient curve obtained in the electrophoresis of human γ_2-globulin with the Gaussian probability curve in normal coördinates.[28] This gradient is nearly enough Gaussian

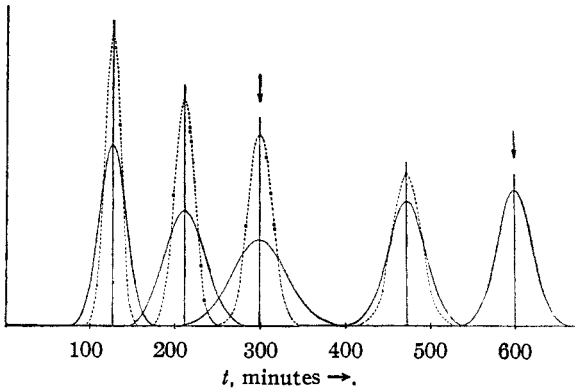

Fig. 3.—Refractive index gradient curves (solid lines) from the electrophoresis of human γ_2-globulin at $\Gamma/2 = 0.1$, *p*H 7.27, at 1.70 volts/cm. and 0.0131 watt/cc. using a sharpened boundary. The superimposed dashed curves give the patterns which would have been obtained if the spreading had been caused by diffusion alone. The field was reversed after 300 minutes.

(23) The crystallized bovine albumin was from Lot 46 prepared by Armour Laboratories, Chicago, Illinois. Electrophoresis experiments in *p*H 8.6, 0.1 ionic strength diethyl barbiturate buffer indicate no appreciable content of globulin impurities, while immunological tests carried out as described by Cohn, Hughes and Weare, THIS JOURNAL, **69**, 1755 (1947), indicate that less than 0.01% α-globulin is present. Thus the immunological tests would indicate that any impurity could not be the same protein as that which constitutes most of the alpha globulin component of plasma. They do not eliminate the possibility that another protein component similar to albumin in its antigenic behavior may be present. Private communication from Dr. J. B. Lesh.

(24) Longsworth, THIS JOURNAL, **69**, 1288 (1947).

(25) Janssen, *Rec. trav. chim.*, **65**, 564 (1946).

(26) Alberty, Anderson and Williams, Colloid Symposium, Stanford University, June, 1947, *J. Phys. Coll. Chem.* **52**, 217 (1948).

(27) Deutsch, Alberty and Gosting, *J. Biol. Chem.*, **165**, 21 (1946).

(28) Lamm, *Nova Acta Reg. Soc. Scient. Upsala*, (IV), **10**, No. 6 (1937).

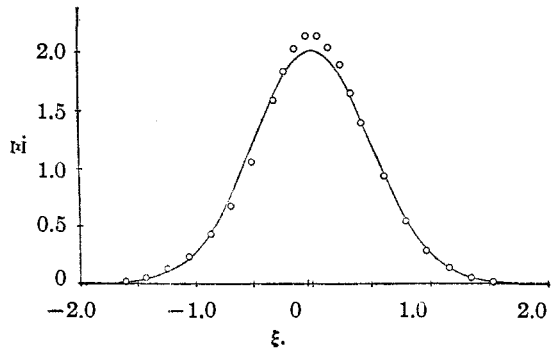

Fig. 4.—Comparison of the third experimental refractive index gradient curve given in Fig. 3 (circles) with the Gaussian probability function (solid curve) in normal coördinates.

that the mobility distribution may be represented by the error function. Figure 5, which shows the variation in apparent diffusion constant during the sharpened electrophoresis experiments represented in Fig. 3, indicates that D^* plots as a straight line *vs.* t_E during the period before the current was reversed as predicted by equation (6) for a Gaussian distribution of mobilities. The apparent diffusion constants calculated by the height and area method were somewhat higher than those calculated by the inflection point method in the case of the gradients spread both by diffusion and electrical heterogeneity, as would be expected because of the deviation of the gradients from perfect Gaussian form (Fig. 4). Since the apparent diffusion constant calculated from the half width at the inflection point is less affected by the resolution of small amounts of γ_1- and β-globulin from the main peak, the heterogeneity constant was calculated from the slope of the straight line through these points. The initial straight line extrapolates back to the diffusion constant for γ_2-globulin at zero time, and the heterogeneity constant, h, calculated from the slope is 0.52×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$. When the direction of the current was reversed for an equal period of time, the correct diffusion constant was obtained, and this indicates that irreversible spreading caused by thermal convection and electroosmosis were negligible in this experiment. The values expected for the apparent diffusion constant during the reversal period were calculated using equation (8) and $h = 0.52 \times 10^{-5}$. The calculated values are represented by the solid curve in Fig. 5, and the agreement with the experimental points is further evidence that the electrical heterogeneity of human γ_2-globulin may be represented by a Gaussian mobility distribution.

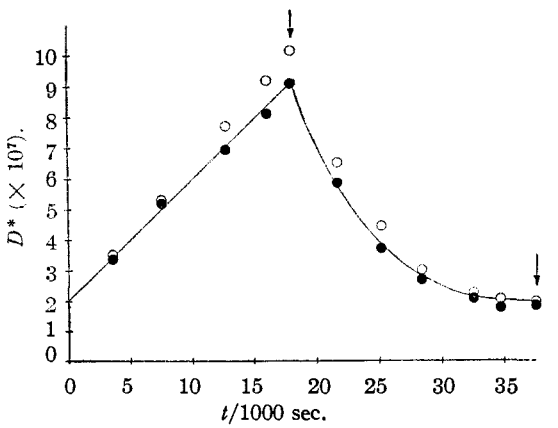

Fig. 5.—Plot of apparent diffusion constant *vs.* time of electrophoresis for the electrophoresis experiment with human γ_2-globulin given in Fig. 3: ●, calculated from half width at inflection point; ○, calculated from height and area.

The heterogeneity constant, H, of Sharp, *et al.*,[13] calculated from the data of this experiment using equation (2) has been plotted against time in Fig. 6. As expected H drifts downward because diffusion is not negligible, and H approaches the value of the heterogeneity constant, h, calculated from Fig. 5, asymptotically. In Fig. 6 the solid curve through the experimental points has been calculated from equation (7) using $h = 0.52 \times 10^{-5}$, $D = 2.0 \times 10^{-7}$, and adequately represents the experimental points.

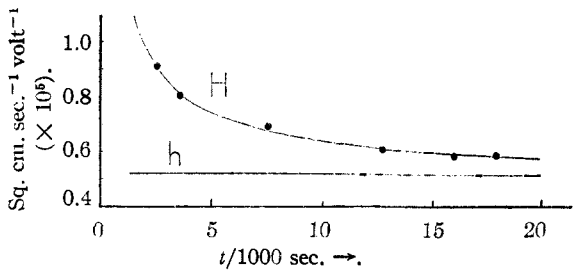

Fig. 6.—Plot of H *vs.* time of electrophoresis for human γ_2-globulin during the initial period of the experiment given in Fig. 3.

In order to study the variation of h for human γ_2-globulin with ionic strength, electrophoresis spreading experiments were also performed at 0.15 and 0.010 ionic strengths close to the isoelectric points under these conditions. The data on these experiments are given in Table I, and the

TABLE I

ELECTROPHORESIS SPREADING EXPERIMENTS WITH HUMAN γ_2-GLOBULIN

$\Gamma/2$	pH	Buffer[a]	Heating watt/cc.	E volt/cm.	h[b]
0.010	8.12	0.01 *N* NaV	0.0145	6.27	0.88×10^{-5}
					$.98 \times 10^{-5}$
.10	7.27	.04 *N* NaCac	.0131	1.70	$.52 \times 10^{-5}$
		.06 *N* NaCl			
.15	6.70	.02 *N* NaCac	.0155	1.40	$.40 \times 10^{-5}$
		.13 *N* NaCl			$.38 \times 10^{-5}$
.16	8.01	.01 *N* NaV	.0140	1.67	$.40 \times 10^{-5}$
		.05 *M* $CaCl_2$			$.43 \times 10^{-5}$

[a] V = diethyl barbiturate, Cac = cacodylate. [b] The heterogeneity constant (in cm.^{2} sec.$^{-1}$ volt^{-1}) obtained on the ascending side is placed below that obtained on the descending side.

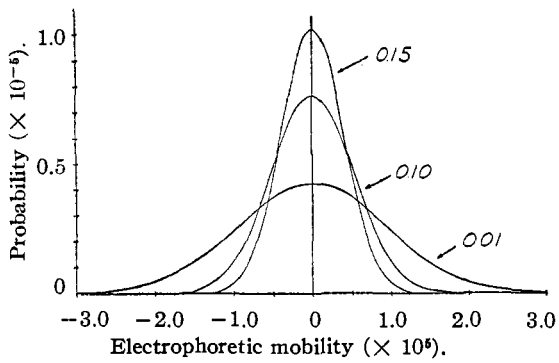

Fig. 7.—Mobility distributions for human γ_2-globulin at several ionic strengths.

mobility distributions are plotted in Fig. 7. The agreement between the heterogeneity constants obtained on the ascending and descending sides is evidence that electrical sharpening and spreading effects are of negligible importance. However, it should be pointed out that agreement between the heterogeneity constants determined from ascending and descending boundaries is not sufficient evidence that sharpening and spreading caused by conductivity and *p*H gradients is negligible in the case of a heterogeneous protein at a *p*H away from the isoelectric point. For example, in the case of human γ_2-globulin at *p*H 8.6, $\Gamma/2 = 0.10$, veronal buffer, the ascending peak has very nearly the same shape as the descending peak,[29] in spite of the tendency to sharpen on the ascending side because of the conductivity effect. This is probably not a result of the *p*H effect or a reversal of the conductivity effect because $(1/u)(du/dp\mathrm{H})$ and u are quite small.[4] The ascending boundary is more diffuse than expected from a superposition of the conductivity effect and diffusion in this case probably because the protein boundary velocity is greater on the ascending side, and this causes the actual separation of two molecules of different mobility in the heterogeneous protein to be greater on the ascending side after a given time than on the descending. Consequently too great a value for the heterogeneity constant would be obtained from both boundaries. In the experiments described here, the mobilities of the center of the boundaries were very low (less than 0.3×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1} uncorrected for electrode volume changes), and the difference in mobility on the ascending and descending sides was no larger than the usual experimental error.

Discussion

Variation of Heterogeneity with Ionic Strength.—The variation in mobility heterogeneity with ionic strength is a combination of a number of factors. In the case of human γ_2-globulin some euglobulin is not soluble at the average isoelectric point at 0.01 ionic strength so that the protein studied at this ionic strength is not identical with that studied at 0.10 and 0.15 ionic strength. The valence of a protein molecule a given number of *p*H units away from the isoelectric point generally decreases with decreasing ionic strength.[30] However, in spite of this decrease in valence, the electrophoretic mobility a given number of *p*H units from the isoelectric point generally increases with decreasing ionic strength[31] because of the decrease in the screening effect of the ionic atmosphere[32] and the modification of the viscous flow of solvent past the moving particle.[33] Assuming to a first approximation that the γ_2-globulin molecule may be represented by a sphere of 55 Å. radius (calculated from $D_0^0 = 2.0 \times 10^{-7}$), the effect of ionic strength on mobility may be estimated from electrophoretic theory.[31,33,34] The mobility resulting from a given net charge should be proportional to $\phi(\kappa a)/(1 + \kappa a)$ where κ is the reciprocal of the "thickness" of the ion atmosphere, a is the radius of the protein molecule, and $\phi(\kappa a)$ is Henry's function. Assuming that the net charge distribution in γ_2-globulin at the isoelectric point is independent of ionic strength, the standard deviation of the mobility heterogeneity should be proportional to $\phi(\kappa a)/(1 + \kappa a)$. Since the values of this function at 0.01, 0.10, and 0.15 ionic strengths at 0° are 0.379, 0.178, and 0.153, the heterogeneity constants expected at 0.01, 0.10, and 0.15 ionic strength are 1.11×10^{-5}, (0.52×10^{-5} assumed) and 0.45×10^{-5}. Although this is not an exact calculation it is seen that the direction and magnitude of the variation in h observed experimentally (Table I) is in agreement with that expected from electrophoretic theory.

Northrop[35] and Rothen[36] have concluded that the reversible electrophoresis spreading shown by crystalline diphtheria antitoxin of constant solubility can not be attributed to a heterogeneous preparation but is connected with electroösmosis because the spreading was diminished by the addition of calcium chloride to their buffer to a concentration of 0.05 *M*. In order to test for this possibility with human γ_2-globulin, a similar experiment was performed. It was not found possible to prepare the 0.067 *M*, *p*H 7.2, veronal buffer mentioned by Rothen because of the insolubility of the diethylbarbituric acid at this *p*H and 1°, and so the experiment was performed by using the 0.01 ionic strength veronal buffer indicated in Table I. Some of the buffer used for the spreading experiment at 0.01 ionic strength was made 0.05 *M* in calcium chloride by the addition of solid calcium chloride. Although a smaller potential gra-

(29) Cohn, Oncley, Strong, Hughes and Armstrong, *J. Clin. Invest.*, **23**, 417 (1944). Fig. 2.

(30) Cannan, Kibrick and Palmer, *Ann. N. Y. Acad. Sci.*, **41**, 247 (1941); Cannan, Palmer and Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

(31) Tiselius and Svensson, *Trans. Faraday Soc.*, **36**, 16 (1940).

(32) Debye and Hückel, *Physik Z.*, **24**, 305 (1923).

(33) Henry, *Proc. Roy. Soc.* (*London*), **A133**, 106 (1931).

(34) Longsworth, *Ann. N. Y. Acad. Sci.*, **41**, 267 (1941).

(35) Northrop, *J. Gen. Physiol.*, **25**, 465 (1942).

(36) Rothen, *ibid.*, **25**, 487 (1942).

dient had to be used in the case of the buffer containing calcium chloride, reversible spreading was observed, and the slope of the graph of D^* *vs.* time of electrophoresis yielded a heterogeneity constant of 0.42×10^{-5} (average of two limbs). Although this value for the heterogeneity constant is smaller than that obtained in the 0.01 ionic strength buffer ($h = 0.93 \times 10^{-5}$) before the addition of calcium chloride, it is about what should be expected at the higher ionic strength of the calcium chloride buffer ($\Gamma/2 = 0.16$) for reasons outlined in the preceding paragraph. The heterogeneity constant obtained with the calcium chloride buffer is in good agreement with that obtained at 0.15 ionic strength (0.02 *N* NaCac, 0.13 *N* NaCl) and does not indicate that the reversible spreading of human γ_2-globulin is caused by electroosmosis.

Sensitivity.—Spreading experiments at lower ionic strengths are a more sensitive test of electrophoretic homogeneity because higher electric field strengths may be employed without causing thermal convection and the standard deviation of the mobility distribution of a heterogeneous protein is greater at lower ionic strengths as illustrated in Fig. 7. As electrophoresis spreading experiments are carried out at present, the smallest heterogeneity constant which may be determined is limited by the conductivity of the buffer and the sensitivity of the schlieren optical system. Assuming an increase of 1×10^{-7} in the apparent diffusion constant during five hours electrophoresis is the minimum increase which may be measured and 0.015 watt/cc. is the maximum permissible heat dissipation in an electrophoresis cell of 0.77 cm.^{2} cross section, the smallest heterogeneity constant which could be determined in a buffer of 50×10^{-4} ohm^{-1} cm.$^{-1}$ conductivity at 0° is 0.2×10^{-5} while in a buffer of 1×10^{-4} ohm^{-1} cm.$^{-1}$ conductivity, 0.03×10^{-5} would be detected.

Interpretation.—Several of the possible interpretations of the reversible electrophoresis spreading of γ_2-globulin may be eliminated. Since this protein is apparently homogeneous with respect to sedimentation and diffusion, the variation in mobility cannot be attributed to a difference in the frictional coefficients of the molecules. Also the spreading cannot be attributed to the existence of different charged forms of identical protein molecules which are in equilibrium in a solution of given *p*H and ionic strength because any individual molecule in the system is constantly giving up and taking on protons, so that the time average of its net charge, considered over an appreciable time interval in which many proton exchanges take place, is identical with the mean net charge of all the molecules in the system.[37] Therefore, on the basis of electrophoresis spreading experiments it may be concluded that human γ_2-globulin consists of a mixture of molecules differing with respect to their average net charge.

The actual variation in net charge among the molecules in human γ_2-globulin as indicated by reversible electrophoresis spreading is not large. Calculation of the proportionality constant between valence of the protein ion and electrophoretic mobility by the method of Abramson, Moyer and Gorin[38] at 0.10 ionic strength (assuming a cylindrical molecule with an axial ratio of 7 and a molecular weight of 160,000) indicates that a molecule with a mobility equal to the standard deviation of the mobility distribution (0.52×10^{-5}cm.^{2} sec.$^{-1}$ volt^{-1}) has a net charge of approximately 3 electrons. This variation in net charge among the molecules would indicate a small variation in their contents of the ionizable amino acids, a variation in arrangement resulting in different end groups or perhaps only a variation in steric effects of groups neighboring ionizable amino acids residues which affects the dissociation constants. Human γ_2-globulin prepared from plasma pools contains antibodies to many different antigens,[39] and therefore according to present theories of antibody structure the molecules have a variety of different configurations. It might be expected therefore, that the molecules would not be identical with respect to net charge at a given *p*H and ionic strength even if they were identical with respect to amino acid composition.

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Summary

1. A method for the determination of the electrophoretic mobility distribution in a heterogeneous protein having a Gaussian distribution of mobilities has been developed for the case in which diffusion during the electrophoresis experiment is not negligible. In this case the apparent diffusion constant is a linear function of time of electrophoresis and the heterogeneity constant, *h*, which is the standard deviation of the mobility distribution, may be calculated from the slope.

2. It has been shown that electrophoresis spreading experiments may be carried out under conditions such that spreading caused by the conductivity and *p*H effects, thermal convection, and electroösmosis are negligible.

3. Crystallized bovine serum albumin has been found to show a small amount of reversible spreading at its isoelectric point at 0.10 ionic

(37) Cohn and Edsall, "Proteins, Amino Acids, and Peptides as Ions and Dipolar Ions," Reinhold Publishing Corp., New York, N. Y., 1943, *cf.* p. 468.

(38) Abramson, Moyer and Gorin, "The Electrophoresis of Proteins," Reinhold Publishing Corp., New York, N. Y., 1942.

(39) Enders, *J. Clin. Invest.*, **23**, 510 (1944); Deutsch, Alberty, Gosting and Williams, *J. Immun.*, **56**, 183 (1947).

strength. Human γ_2-globulin shows a large amount of reversible spreading and the standard deviation for the mobility distribution has been found to vary with ionic strength in the direction expected from electrolytic solution theory.

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Photochemistry of Tryptophan, *p*-Dimethylaminobenzaldehyde and Reaction Products in Sulfuric Acid Solution[1]

BY JOSEPH R. SPIES AND DORRIS C. CHAMBERS

During a previously reported study[2] of the color-forming reactions between tryptophan, *p*-dimethylaminobenzaldehyde (DAB) and sodium nitrite in sulfuric acid solution, it became necessary to study the effects of light on the reactants and reactions involved. This paper describes a method for studying the effects of illumination (the term "illumination" refers to exposure to light by a procedure described below), the effect of light on the stability of tryptophan, the effects of light on reactions I and II,[3] and a photochemical "after effect" produced by illumination of DAB in acid solution. The reactions were carried out in 19 *N* sulfuric acid because this concentration was found suitable for the determination of tryptophan in proteins by a procedure to be described in later papers.

Photochemical development of color from the colorless condensation product of tryptophan and DAB was first reported by Boyd[4] who attributed this effect to the ultraviolet rays of sunlight.[5]

The effect of illumination on the stability of tryptophan in 19 *N* acid at 25° is shown in Table I. Light accelerates the decomposition of tryptophan as losses of 3, 11 and 34% occurred on illumination for one-half, one and three hours, respectively, compared with a loss of only 8% on standing in the dark for forty-eight hours.

The effects of illumination during reaction I under conditions such that reactions I and II were proceeding simultaneously are shown in Table II. Illumination for the first ten seconds of reaction I caused no increase in pre-nitrite color[6] nor any loss of tryptophan. But illumination for thirty seconds caused an increase in pre-nitrite color and

TABLE I

EFFECT OF LIGHT ON THE STABILITY OF FREE TRYPTOPHAN IN 19 *N* SULFURIC ACID AT 25°[a]

Time illuminated or dark, hours	Loss of tryptophan, % Illuminated	Dark[b]
0	0	..
0.1	0	0
.25	0	..
.50	3	0
1	11	0
2	..	0
3	34	..
4	..	3
24	..	6
48	..	8

[a] Procedure: nine ml. of 21.4 *N* acid at 25° was placed in tube A, Fig. 2, and 100 γ of tryptophan in 1.0 ml. of water was added. The solution was mixed and tube A was placed at once in holder B through which water at 25 ± 0.1° was circulating. Illumination was started twenty seconds after adding the tryptophan to the acid solution. After the desired interval of illumination the solution was poured onto 30 mg. of DAB in a 25-ml. glass-stoppered Erlenmeyer flask. Tryptophan was then determined by procedure C, Paper I.[2] [b] Results taken from Table VII, Paper I[2].

destruction of 6% of the tryptophan. The pre-nitrite color increased with time of illumination until after 180 minutes it amounted to 76% of the total. The loss of tryptophan, caused by illumination, increased to 25% of the total during the first five minutes of reaction I and then remained constant. Reaction I is 87% completed in five minutes (Table VI).[2] Therefore the destructive effects of illumination occur chiefly during the condensation of tryptophan and DAB.

Rate of reaction II caused photochemically is rapid at first and then becomes quite slow as 70, 88 and 93% of the color was developed by five, twenty, and 120 minutes illumination, respectively.[7] After five minutes of illumination only 94% of the potentially available color could be obtained by subsequent oxidation with sodium nitrite. This destructive effect, however, was not progres-

(1) Paper II in a series entitled, "Chemical Determination of Tryptophan." Presented at the 111th meeting of the American Chemical Society held at Atlantic City, New Jersey, April, 1947. Not subject to copyright.

(2) Spies and Chambers, *Anal. Chem.*, **20**, 30 (1948).

(3) The condensation of tryptophan and DAB to form the leuco base is called reaction I and the oxidative development of the blue color, either photochemically or with sodium nitrite, is designated reaction II.

(4) Boyd, *Biochem. J.*, **23**, 78 (1929).

(5) Ruemele, *Z. Untersuch. Lebensm.*, **79**, 453 (1940), observed that light effected the formaldehyde–tryptophan colorimetric reaction but no detailed study was made.

(6) The term pre-nitrite color will refer to that color which develops in a test solution either spontaneously or as a result of exposure to light. The term post-nitrite color refers to that color which develops in a test solution after addition of sodium nitrite.

(7) Procedure: To 1.607 mg. of tryptophan and 482 mg. of DAB in a glass-stoppered Erlenmeyer flask was added 160.7 ml. of 19 *N* acid. The solution was kept in the dark at 25° for twenty-two hours. After illumination of 10 ml. aliquots of this solution pre-nitrite transmittancies were read and expressed as per cent. of the maximum color obtainable under ideal conditions of the test (procedure E, Paper I[2]).