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Reversible Boundary Spreading as a Criterion of the Microheterogeneity of Plasma Albumins*

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ABSTRACT: Several samples of bovine plasma albumin of known microheterogeneity, as judged by the criterion of the solubility-pH profile method, have been examined by the reversible boundary spreading technique. For charcoal-defatted samples a linear relationship exists between h , the standard deviation of the mobility distribution, and $\Delta\text{pH}_{10}^{90}$, the pH range required to alter the solubility of the protein in 3 M KCl from 10 to 90%. The straight line extrapolates to $\Delta\text{pH}_{10}^{90}$ approximately 0.1 when $h = 0$, i.e., for a

hypothetically homogeneous protein exhibiting no electrophoretic heterogeneity. This limiting value of $\Delta\text{pH}_{10}^{90}$ is shown to be consistent with published evidence that precipitation is associated with the N-F transition together with evidence on the difference in protonation behavior of N and F forms. Nondefatted and acid-defatted samples deviate from this simple linear relationship in such a way as to indicate that extrinsic contaminants make a greater contribution to $\Delta\text{pH}_{10}^{90}$ than to the electrophoretic heterogeneity, h .

The technique of electrophoresis is well known for detecting gross electrical heterogeneity or contamination in solutions of proteins (Alberty, 1948a; Hess, 1951; Taylor, 1953). A protein which is multimodally heterogeneous will exhibit multiple boundaries in a moving-boundary experiment (Longworth, 1959). But the fact that a protein fails to develop more than one moving boundary in a given buffer solvent cannot be considered sufficient evidence that all protein molecules have the same electrophoretic mobility. As pointed out by Alberty (1948b) "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 electric field than expected for diffusion alone but will become sharper upon reversal

of this field." This is the basic idea behind the criterion of reversible boundary spreading as a test of purity of proteins. Working with hemocyanins, Tiselius and Horsfall (1939) and Horsfall (1939) were the first to observe reversible boundary spreading. Later, Sharp *et al.* (1942) developed a theory which was modified by Alberty and associates (Alberty *et al.*, 1948; Anderson and Alberty, 1948) and Brown and Cann (1950). Alberty and coworkers applied this method to fourteen purified proteins and concluded (Anderson and Alberty, 1948) that none of the preparations satisfied this criterion of homogeneity. The method has received further elaboration and support by Longworth (1959) who pointed out that fluctuations in charge and shape, to be expected in any isoelectric protein, should be rapidly reversible and should not contribute to the heterogeneity in mobility observed by this method.

Colvin *et al.* (1954) published a stimulating review on the microheterogeneity of proteins. They suggested that the data then available "provide a wide base for the conclusion that all protein preparations examined to date are microheterogeneous at best." They concluded much of this microheterogeneity to be inherent. Following Haurowitz (1950), they suggested that a native protein should be considered as a population of closely related but not identical molecules.

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Sogami and Foster (1963) invoked this concept to explain the fact that the N and F forms of plasma albumin can be resolved electrophoretically even though the rate of the interconversion of the isomeric forms appears to be very fast. They visualized that the N-F equilibrium is dependent on hydrogen ions to a high order (>12) so that for any homogeneous set of molecules the pH dependence of the reaction would be very sharp. They proposed that a given plasma albumin sample must consist of a spectrum of molecules which differ in the critical pH at which they undergo the isomerization reaction. Thus at a particular pH some of the molecules of the population would exist virtually 100% of the time in the N state, others in the F state. Hence resolution of N and F forms actually involves a subfractionation of the population.

This concept has been verified and amplified in the various papers of this series. Particularly germane to the present work is the demonstration of Petersen and Foster (1965a) that the insolubility of the F form in presence of high concentrations of salt (typically 3 N KCl has been employed) permits subfractionation of albumin samples by control of pH in the range of the N-F transition, approximately 4.5–3.8. Fractions precipitating at different pH values within this range differ from each other in their solubility-pH profiles and in other properties. The symbol $\Delta\text{pH}_{10}^{90}$ was employed to describe the degree of microheterogeneity of a given albumin sample. This corresponds to the pH range required to alter the percentage of the protein which is soluble in a given medium, usually 3 M KCl, from 10 to 90%.

The reversible boundary spreading technique as developed by Alberty and coworkers seems to have been ignored by most workers in the field, probably on the ground of possible anomalies. The availability of a known group of microheterogeneous proteins, the plasma albumins, has made it possible to reexamine the method. In this study several preparations of bovine plasma albumin and human mercaptalbumin of known microheterogeneity have been examined. The results clearly indicate that h , the standard deviation of the mobility distribution, is related to the relative degree of microheterogeneity as determined by the solubility-pH profile method.

Experimental Section

Materials. Crystallized bovine plasma albumin used in the investigations as such or in preparing acid-defatted or charcoal-defatted samples was obtained from Armour Pharmaceutical Co. The acid-defatted samples were prepared by the method of Williams and Foster (1959). The charcoal-defatted samples were prepared by Chen's method (Chen, 1967) as modified by Sogami and Foster (1968). Noncrystallized bovine plasma albumin (Fraction V), lot 46, was obtained from Pentex. Lot numbers in all the cases are given in Table I, which also shows the results of the reversible boundary spreading experiments.

The sample of human mercaptalbumin used was the same as employed by Petersen and Foster (1965a,b).

For removing the dimer and polymer contaminants from acid-defatted bovine plasma albumin, Sephadex G-150 (Pharmacia, Uppsala, Sweden) was used in a laboratory column K 25–100. The eluent used was 0.1 N KCl. About

10 ml of 10% bovine plasma albumin was applied at a time. A flow rate of 25–30 ml/hr was maintained with a pressure head of 10–15 cm. A Gilson fraction collector was used and 4 ml was collected per tube. The monitoring was done by measuring optical densities in the Hitachi Perkin-Elmer Model 139 spectrophotometer. The monomer obtained yielded a single band on disc gel electrophoresis.

For preparing monomer from the charcoal-defatted bovine plasma albumin (from crystallized bovine plasma albumin, Armour, lot D 71309) 40 ml of 10% protein was applied to Sephadex G-150 in a laboratory column K 50–100. The eluent was 0.1 N $(\text{NH}_4)_2\text{SO}_4$. A flow rate of 70–80 ml/hr was maintained with a pressure head of about 15 cm. A Gilson fraction collector was used and the amount collected per tube was 12 ml. The monitoring was done in the Hitachi Perkin-Elmer Model 139 spectrophotometer. A single band was obtained when the monomer was tested for its purity by disc gel electrophoresis. The pooled monomer was concentrated to about 8% in a concentration cell (Amicon, Model 400) using a Dialflow Membrane UM-2 (Amicon Corp.). It was diluted with deionized water and again concentrated to about 8% to remove as much of the ammonium sulfate as possible. The protein was then lyophilized and stored at 2°. The lyophilized material showed a pH of 4.4 when dissolved in deionized water. The pH was readjusted to 5.5 before storing the solution or carrying out any experiment with it.

The charcoal-defatted monomeric bovine plasma albumin which was aged for 17 days at pH 7.85 was prepared from crystallized bovine plasma albumin (Armour lot D 71309). Another sample of charcoal-defatted monomer which was aged for 81 hr at pH 8.40 was prepared from crystallized bovine plasma albumin (Armour, lot D 71209).

Methods. The subfractionation of bovine plasma albumin in 3.0 N KCl was conducted according to the procedure described by Sogami and Foster (1968). The subfractionation based on solubility in $(\text{NH}_4)_2\text{SO}_4$ was performed by the method of Wong and Foster (1969). The pH-solubility profiles in 3.0 N KCl were obtained by the method described by Sogami and Foster (1968).

Protein concentrations were routinely determined by measuring absorbance in a Hitachi-Perkin Elmer 139 spectrophotometer. The values of $\epsilon_{1\text{cm}}^{1\%}$ used were 6.67 at 279 $m\mu$ for bovine plasma albumin and 5.30 at 280 $m\mu$ for human mercaptalbumin. A Radiometer Model 25 pH meter, equipped with expanded scale and a combined electrode GK 2026C and calibrated with Sargent pH 4.01 and 7.00 buffers, was employed for pH measurements.

Reversible boundary spreading experiments were carried out in a Beckman Model H electrophoresis-diffusion instrument. The temperature of the bath was maintained at $22 \pm 0.01^\circ$. In all experiments the 11-ml cell was used. The buffer used in the case of bovine plasma albumin and human mercaptalbumin was 0.01 N sodium acetate (pH 4.88 ± 0.02). The protein solutions were dialyzed for 12–16 hr at room temperature before filling the electrophoresis cell. No significant difference was observed between the pH values of buffer and protein solutions after dialysis. Current densities were limited to 1 V/cm which corresponds to a heat input in the cell of less than 10^{-3} W/cc. This is lower than the heat input in the experiments of Alberty by a factor of nearly 20 and should preclude thermal convection in spite of the

TABLE I: Data on Reversible Boundary Spreading of Various Samples of Bovine Plasma Albumin and Human Mercaptalbumin.

Sample	Protein Sample	pH of 0.01 N NaOAc Buffer	<i>J</i>	Voltage Gradient (V/cm)	Heterogeneity Constant <i>h</i> × 10 ⁵			ΔpH_{10}^{90}
					Left	Right	<i>Av</i>	
1	Bovine plasma albumin, crystallized, nontreated	{ 4.86 4.87 4.87	{ 38.0 37.0 37.5	{ 0.67 0.83 1.0	{ 0.70 0.68 0.69	{ 0.70 0.68 0.72	0.70	0.31
2	Bovine plasma albumin, noncrystallized, non- treated	4.86	33.0	0.83	1.33	1.25	1.29	0.31
3	Bovine plasma albumin, crystallized, acid defatted	{ 4.87 4.86	{ 38.0 40.0	{ 0.67 1.0	{ 1.86 1.65	{ 1.86 1.65	1.75	0.48
4	Bovine plasma albumin, crystallized, C defatted	{ 4.87 4.86	{ 43.0 44.0	{ 0.83 1.0	{ 0.62 0.66	{ 0.62 0.65	0.64	0.18
5	Bovine plasma albumin, noncrystallized, C defatted	4.86	49.0	0.83	1.23	1.13	1.18	0.24
	Bovine plasma albumin, noncrystallized, C defatted							
6a	Subfraction I ^a	4.86	46.0	0.9	0.99	0.97	0.98	0.16
6b	Subfraction II ^a	4.86	24.0	1.0	0.75	0.92	0.84	0.16
	Bovine plasma albumin, acid-defatted monomer							
7a	Subfraction I	4.88	27.4	0.83	0.95	1.36	1.16	0.34
7b	Subfraction III	4.88	{ 30.2 30.4	{ 0.83 0.83	{ 0.90 0.78	{ 0.90 0.78	0.84	0.28
	Bovine plasma albumin, C defatted, crystallized							
8a	Subfraction I ^a	4.85	49.2	0.83	0.88	0.88	0.88	0.15
8b	Subfraction III ^a	4.85	42.0	0.83	0.62	0.62	0.62	0.14
9	Bovine plasma albumin, C-defatted monomer, aged 81, hr pH 7.40	4.87	28.0	0.67	1.53	1.86	1.70	0.26
10	Bovine plasma albumin, C-defatted monomer, aged 17 days pH 7.85	4.87	33.4	0.83	1.90	2.20	2.05	0.28
11	Human mercaptalbumin, acid-defatted monomer	{ 4.90 4.87	{ 33.4 29.3	{ 0.50 0.83	{ 1.91 1.56	{ 1.91 1.93	1.91	0.29
12	Human mercaptalbumin, C-defatted monomer	4.86	29.2	0.80	1.25	1.25	1.25	0.18

^a Subfractions prepared by (NH₄)₂SO₄ fractionation as described by Wong and Foster (1969). The other subfractions (7a and 7b) were prepared by the low pH 3 M KCl method described by Petersen and Foster (1965a).

use of a temperature well away from the temperature of maximum density.

Before carrying out an electrophoresis experiment the boundaries were compensated at 4 mA until both left- and right-hand boundaries could be seen in the middle of the cell. After stopping the compensation, the boundaries were allowed to diffuse for a period of 12–16 hr before starting electrophoresis in order to minimize any contribution from initial boundary imperfections. The reversible boundary spreading experiments were carried out at voltage gradients ranging from 0.5 to 1.0 V per cm. Table I records all the

details. Usually the current was reversed after 4–5-hr electrophoresis in one direction. Combined photographs of schlieren patterns and Rayleigh fringes were taken at intervals of 50–70 min during the initial electrophoresis. After reversing the current, only one or two photographs were taken. Photographs were always taken when starting electrophoresis, reversing the current and stopping the current.

For reading Rayleigh fringes a Nikon Shadowgraph Model 6C was used. The magnification factor was unity. The fringes were counted and their positions were recorded according to the procedure described by Schachman (1957).

Details regarding the determination of the standard deviation, σ , and the diffusion coefficient, D , are given in a later section.

Theory of Reversible Boundary Spreading. In the electrophoresis of a heterogeneous protein with a mobility distribution, $g(\mu)$, carried out under ideal conditions the refractive index gradient at a time, t_D , after the formation of the boundary and time, t_E , after the application of the electric field is given (Alberty, 1948b) by eq 1, where D is the diffusion

$$\frac{\partial n}{\partial x} = \frac{n_1 - n_2}{2\sqrt{\pi Dt_D}} \int_{-\infty}^{\infty} g(\mu) \exp\left\{-\frac{(x - \mu Et_E)^2}{4Dt_D}\right\} d\mu \quad (1)$$

coefficient, assumed to be the same for all protein molecules, μ is the electrophoretic mobility, E is the electric field strength, and $n_1 - n_2$ is the difference in refractive index of the protein solution and the buffer.

For a protein at its isoelectric point the mean mobility is zero. If it is assumed further that the distribution of mobilities is a Gaussian function, we may write

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

For the mobility distribution which has been normalized to unity, h has the dimensions of mobility and is the standard deviation. Substitution of this form of $g(\mu)$ in eq 1 and subsequent integration gives eq 3.

$$\frac{\partial n}{\partial x} = \frac{n_1 - n_2}{\sqrt{2\pi(E^2 h^2 t_E^2 + 2Dt_D)}} \times \exp\left\{-\frac{x^2}{2(E^2 h^2 t_E^2 + 2Dt_D)}\right\} \quad (3)$$

Hence if there is a Gaussian distribution of mobilities, the electrophoresis gradient curves should be Gaussian.

If electrophoresis is initiated after the boundary has been diffusing Δt seconds, then σ , the standard deviation, is given by

$$\sigma = \sqrt{E^2 h^2 t_E^2 + 2Dt_E + 2D\Delta t} \quad (4)$$

Here $2D\Delta t$ is the square of the standard deviation of the gradient curve, σ_0 , at the moment the electric field was applied. Rearranging, we have

$$D^* = \frac{\sigma^2 - \sigma_0^2}{2t_E} = D + \frac{E^2 h^2}{2} t_E \quad (5)$$

D^* is the "apparent coefficient."

Hence if the protein is heterogeneous and has a Gaussian distribution of mobilities, a plot of D^* vs. t_E should yield a straight line of slope $E^2 h^2/2$ from which the heterogeneity constant h may be calculated.¹

¹ This is a special case. In the general case, eq 5 still applies with h replaced by β , the standard deviation of a mobility distribution also representable as a Gram-Charlier series. Since our gradient curves were closely Gaussian in shape, the mobility distribution must also be essentially Gaussian so we are concerned only with the special case.

The variation of the apparent diffusion coefficient with time following reversal of the direction of the current, if t_E is the total time the current has been flowing in both directions and t_1 is the time after which the current was reversed, is given by

$$D^* = D + \frac{E^2 h^2}{2} \frac{(2t_1 - t_E)^2}{t_E} \quad (6)$$

Thus the apparent diffusion coefficient decreases with time and becomes equal to the diffusion coefficient at $t_E = 2t_1$, provided that irreversible effects such as convection are negligible.

Determination of σ . The same cell and optical system used in moving-boundary electrophoresis may be employed for the determination of the diffusion coefficient and for the reversible boundary spreading experiments. The details of the method using schlieren optics are given by Alberty (1948a) and Alberty *et al.* (1948). We used the Rayleigh interferometric method as described by Gosting (1956). The total number of Rayleigh fringes, J , is given by eq 7,

$$J = \frac{a(n_1 - n_2)}{\lambda} \quad (7)$$

where $n_1 - n_2$ is the refractive index difference across the boundary, λ the wavelength of the monochromatic light, and a the cell thickness along the light path. For calculations, basically two kinds of measurements of the Rayleigh fringes are required. First is determination of the total number of fringes and second, measurement of fringe positions. An early picture, before the fringes are completely resolved is used to determine the fractional part of J , and a later photograph, after fringes are distinctly separated, to determine the integral part (Schachman, 1957).

The photographs were first placed on the stage of the shadowgraph and aligned so that the straight portions of the pattern were parallel to the motion of the stage. Starting at one end of the boundary with the stage carefully aligned, the screw of the shadowgraph was turned and the number of "white" fringes crossed in traversing the boundary was counted and recorded. The first fringe minimum when all the fringes were parallel to each other is denoted by 0. Comparator readings X_j were then taken as the cross-hair passed over successive fringes $j = 1, 2, 3, \dots$, etc. On reaching the $J/2$ position, the cross-hair was readjusted by an amount equivalent to the fractional value of J . The further readings of minima then take into account the fractional value of J and correspond to X_{J-j} . For example, if $J = 40.4$ a sequence of reading was made on fringes $j = 1, 2, 3, \dots, 20$. At 20 the cross-hair was readjusted and the screw gauge moved further horizontally to successive minima. These readings correspond to 20.4, 21.4, 22.4, \dots , etc.

Combination of eq 3 and 4 gives

$$\frac{\partial n}{\partial x} = \frac{n_1 - n_2}{\sqrt{2\pi}\sigma} e^{-x^2/2\sigma^2} \quad (8)$$

Integrating between $-\infty$ and X_j , and recognizing the propor-

tionality between j and n , we have

$$\frac{j}{J} = \frac{1}{\sqrt{2\pi}\sigma} \int_{-\infty}^{X_j} e^{-x^2/2\sigma^2} dx \quad (9)$$

This is of the form of the standard probability integral

$$\frac{j}{J} = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^z e^{-\alpha^2/2} d\alpha \quad (10)$$

where $\alpha = x/\sigma$. CRC Standard Mathematical Tables (Selby and Girling, 1965) were used to obtain values of z corresponding to values of j .

The following procedure was employed for the calculation of diffusion coefficients from the Rayleigh fringe data. If the concentration of the protein was such that there were less than about 30 fringes in the boundary, then the position and number of all the fringes except the first three and the last three was recorded. The reason for not taking into account the first few and the last few fringes is that the accuracy in locating these fringes is low (Schachman, 1957) owing to the great width and small curvature of these fringes at the ends of the boundary. If there were as many as 30 fringes, then the position and number of every other fringe was recorded, omitting once again the first few (5 or 6) and the last few (5 or 6) fringes. If we denote X_j as the reading on the shadowgraph for the position of fringe j , then a plot of X_j vs. z will give a straight line for a Gaussian distribution. The slope of this line gives the reciprocal of σ , i.e.,

$$\frac{\Delta z}{\Delta X_j} = \frac{1}{\sigma} \quad (11)$$

The value of σ obtained in this way was used to calculate D^* (eq 5). A plot of D^* vs. time of electrophoresis gave h , the heterogeneity constant.

Results

The results obtained are given in Table I. The value of J is given in each case to give an idea of the concentration of the protein. A value of J of 50 corresponds to a protein concentration of about 0.25%. The voltage gradient used was in the range 0.5–1.0 V/cm. For more homogeneous samples a higher value of E (about 1 V/cm) is recommended for the simple reason that the changes in D^* are then more pronounced and a calculation of h is easier. A typical plot of z vs. X_j is shown in Figure 1. A typical plot of D^* vs. t_E is shown in Figure 2. In Figure 3 the results are presented in the form of a plot of h vs. ΔpH_{10}^{90} . All the points shown in Figure 3 have been numbered corresponding to the sample numbers given in Table I.

Discussion

At first glance, the results summarized in Figure 3 appear to suggest that no simple relation holds between the heterogeneity constant, h , and the degree of microheterogeneity estimated from low pH-solubility profiles, ΔpH_{10}^{90} . However, on closer examination meaningful relations do emerge.

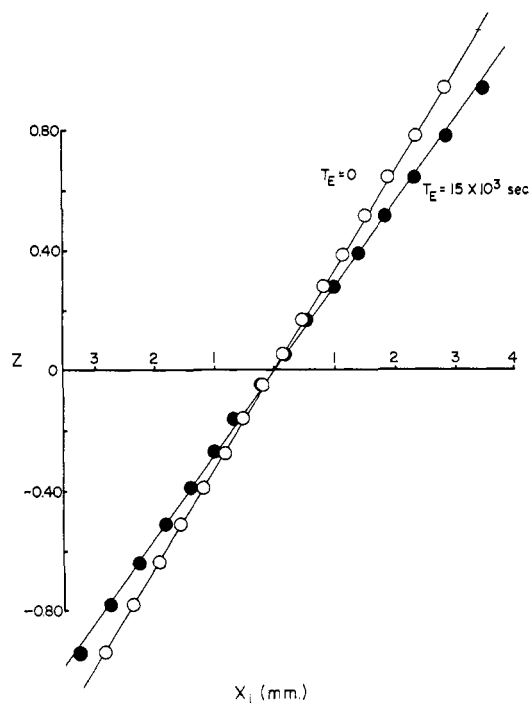


FIGURE 1. A typical plot of z vs. X_j , normalized to the center of the boundary. This run corresponds to sample 6a (Table I). The excellent linear dependence of z on X_j demonstrates that boundaries were Gaussian to high degree of precision.

Both McMenemy and Lee (1967) and Sogami and Foster (1968) have concluded that bound contaminants make a substantial contribution to microheterogeneity and the former authors refer to this as "extrinsic" microheterogeneity. Sogami and Foster (1968) concluded that the acid-defatting procedure does not completely remove these extrinsic contributors to microheterogeneity and concluded further that the acid-defatting process results in an additional broadening of the population due to some unknown irreversible alterations of the protein. If attention is focused for the moment only on charcoal-defatted bovine plasma albumin samples, the circles which are shaded in Figure 3, it is seen that a reasonably good linear relation exists between h and ΔpH_{10}^{90} . The lower points on this line consist of charcoal-defatted, crystallized bovine plasma albumin and subfractions prepared therefrom by partial precipitation at low pH (Petersen and Foster, 1965a). The noncrystallized sample (point 5) is somewhat more heterogeneous, as judged by both criteria. Two samples (points 9 and 10) which are artificially broadened by permitting intramolecular disulfide interchange to take place lie toward the top of the line showing that the electrophoretic heterogeneity is also increased by this process.

It is suggested, then, that this line represents the relationship between intrinsic microheterogeneity and electrophoretic heterogeneity. In these terms, it is interesting that the line appears to extrapolate to a value of ΔpH_{10}^{90} of approximately 0.1. This is entirely reasonable, since an ideal homogeneous bovine plasma albumin sample of $h = 0$ would still be expected to exhibit a precipitation curve of finite breadth. Assuming that precipitation is associated with the N-F transition, as we have contended (Rachinsky and Foster, 1957), the

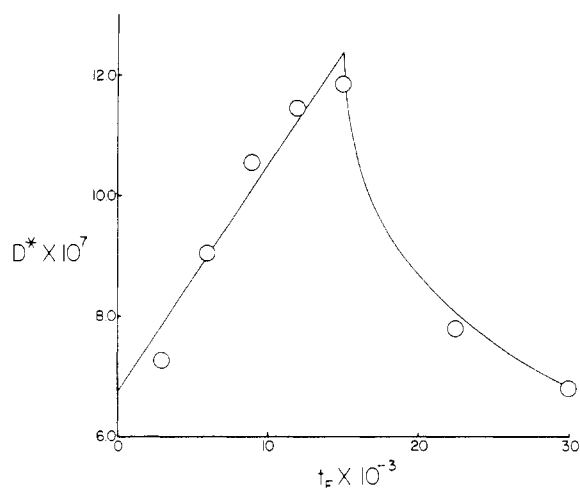
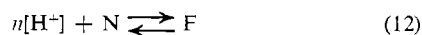


FIGURE 2: A typical plot of D^* vs. t_E . D^* is in units of $\text{cm}^2 \text{sec}^{-1}$, t_E in sec. This run corresponds to sample 6a (Table I). The curved line representing the change in D^* during reversal of the current was calculated from the value of h corresponding to the initial straight line. The excellent fit of this line to the experimental points indicates that the boundary spreading was entirely reversible.

limiting value of $\Delta\text{pH}_{10}^{90}$ should be calculable from the degree of cooperativity, n , in eq 12. It is very difficult to



determine the precise value of n because of the microheterogeneity of the samples plus the fact that the rate of interconversion of N and F is much too rapid to permit independent titration curves to be measured on the N and F forms. We have previously estimated (Foster and Aoki, 1957) that n is approximately 12, but this is almost certainly a lower limit. Vijai and Foster (1967) have presented evidence that the difference in hydrogen ion titration properties of the N and F forms results from the fact that N has only approximately 60 carboxylate groups available for protonation while in F all of the approximately 100 such groups are free and titratable. They further estimated the pK of the exposed groups in the N form to be 4.25. Assuming that the same intrinsic pK is applicable to the F form, and assuming further that electrostatic factors can be neglected under the high ionic strength conditions (3 M KCl) employed in determining the solubility-pH profiles, the mean degree of protonation of carboxyl groups in the N and F forms at the pH of the midpoint of precipitation, 4.33, can be calculated to be 27.3 and 45.5 equiv per mole, respectively. Thus the value of n in eq 12 should be the difference between these numbers or approximately 18. The limiting value of $\Delta\text{pH}_{10}^{90}$ can then be calculated as

$$\Delta\text{pH}_{10}^{90} = \frac{\log 9 - \log (1/9)}{18} = 0.10 \quad (13)$$

Thus the extrapolated limit of the line drawn in Figure 3 is entirely reasonable and consistent with earlier interpretations of the titration anomaly, the N-F transition and their relationship to the solubility-pH behavior.

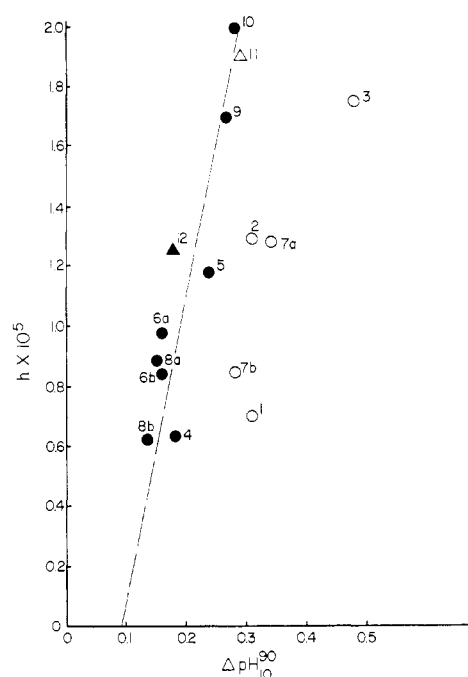


FIGURE 3: Summary of results expressed as h vs. $\Delta\text{pH}_{10}^{90}$. Circles represent bovine plasma albumin and triangles human mercaptalbumin samples. Shaded symbols represent samples which were charcoal defatted. All symbols are labeled with sample numbers corresponding to Table I.

The nontreated samples (points 1 and 2) clearly lie well to the right of the line representing the effect of intrinsic microheterogeneity. This indicates that the contaminants present in the nontreated protein, presumably chiefly fatty acids, affect the solubility-pH profile much more than they affect the electrophoretic heterogeneity. In fact, comparison of points 2 and 5 and points 1 and 4 indicate that the effect of contaminants on h is negligible. Similarly, the acid-defatted samples (points 3, 7a, and 7b) exhibit an inordinate breadth in the solubility-pH profiles as compared with their electrophoretic heterogeneities. Two samples of the human protein, human mercaptalbumin, were also examined and the results are shown as triangles in Figure 3. Little can be concluded from these two points except that the acid-defatted sample is more heterogeneous by both criteria than is the charcoal-defatted sample.

The relationships observed between h and $\Delta\text{pH}_{10}^{90}$ are consistent with the observation of Petersen and Foster (1965b) that different subfractions of bovine plasma albumin have differing values of isoionic pH. In fact it was that observation which suggested the present study of reversible boundary spreading.

Finally, it should be pointed out that the values of the heterogeneity constant h found in this study are somewhat greater than that reported by Alberty *et al.* (1948). Their value at 0.01 ionic strength, 0.16×10^{-5} , must be corrected for the difference in temperature employed, which results in an approximate doubling. Even so, the value lies below any of the values found in this study. Unfortunately, they did not provide any details on their albumin preparation other than to note that it contained "a small amount of α -

globulin." They also noted that about 15% of a separate "rather heterogeneous component" with a more positive mobility appeared in their patterns, and the relatively low h value they report evidently applies to the remainder of the protein. Such a separate boundary was not observed in our experiments. Perhaps the most significant difference between their experiments and ours lies in the fact we employed a much higher temperature and lower current density. Conceivably, charge (mobility) differences could be accentuated or altered at the higher temperature due to differences in the enthalpy of ionization of different groups in the various species of the population.²

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² Electrophoresis of albumin in dilute acetate buffers similar to those used in these experiments and those of Alberty *et al.* (1948) can give rise to multiple boundaries which have been attributed to the interaction of the protein with unionized acetic acid (Cann, 1958). Possibly the temperature dependence of this reaction is such as to account for the differences observed in the two investigations.