ORGANIC AND BIOLOGICAL CHEMISTRY

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, PURDUE UNIVERSITY]

Electrophoretic Behavior of Bovine Plasma Albumin at Low pH[1]

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Electrophoresis of bovine plasma albumin was carried out in the pH range 2.0 to 4.7 using 0.2% solutions mainly at 0.02 ionic strength chloride and at 0°. Some experiments were conducted under other conditions, that is, changing the ionic strength (0.10) or temperature (25°), or changing the medium (0.02 ionic strength acetate and thiocyanate). In 0.02 ionic strength chloride and at 0°, there were two boundaries (F and N forms) in the pH region between 3.5 and 4.5, and the percentage composition changed continuously with pH. At pH 4.0 N and F forms existed approximately in equal amounts. The results are explained by an equilibrium, $N + 3H^+ \rightleftarrows F$. Reversibility of the isomerization process was demonstrated. Thermodynamic parameters of the reaction were calculated by comparing results at 0 and 25°. Values when the N form is changed to the F form are: $\Delta F^0 = -15.0$ kcal./mole, $\Delta H^0 = +3.0 \pm 1.5$ kcal./mole and $\Delta S^0 = +66 \pm 4$ e.u./mole. The magnitude and the sign of ΔH^0 is similar to that found calorimetrically by Gutfreund and Sturtevant. The half-time of the N–F equilibrium could not be determined in the present study, but it was deduced to be less than two hours. Under other experimental conditions similar results were obtained except for minor shifts in the equilibrium constant and possibly in the number of hydrogen ions involved. Charge–mobility curves were drawn at the two ionic strengths. It was found that the slopes of these curves changed at pH 3.5, indicating that the electrophoretic behavior was different above and below this pH. Radius of bovine plasma albumin was calculated from electrophoretic mobility and Z value using Henry's equation. It was 33 ± 2 Å. above pH 3.5 at the two ionic strengths for both N and F forms, agreeing with previous estimates. With decrease of the pH from 3.5 the molecule expanded. At pH 2.0 the radius was 50 Å. at 0.02 ionic strength and 43 Å. at 0.10 ionic strength. The isoelectric point varied with the medium and its ionic strength.

Introduction

It has been known that serum albumin is not electrophoretically homogeneous in the pH region near 4; that is, two or more boundaries exist in the electrophoretic pattern. Luetscher[2] observed that crystallized human and horse serum albumins showed two electrophoretic boundaries in acetate buffer of pH 4.0 and ionic strength 0.02. Neurath and co-workers[3] stated that, while horse serum albumin migrated with a single boundary in pH regions remote from the isoelectric point, such as 7.6 and 3.6, the boundary became complex as the pH of the solution approached that of the isoelectric point. More recently, Longsworth, *et al.*,[4] made an electrophoretic study of bovine plasma albumin (BPA) associated with anion binding to that protein. They obtained heterogeneous boundaries in pH regions near the isoelectric point. Alberty[5] observed that BPA was resolved into three components in 0.15 M sodium chloride between pH's 4.0 and 4.5. Alberty and co-workers[6,7] made extensive studies of the heterogeneity of proteins by the "reversible boundary spreading" method at their isoelectric points. Furthermore, the heterogeneity of human serum albumin in the acidic region was observed by Miller, *et al.*,[8] and by Saifer, *et al.*[9]

Independently it has been suggested that serum albumin expands at low pH. Both Scatchard[10] and Tanford[11] suggested such expansion to account for the character of the titration curve of this protein. Measuring viscosity and optical rotation of BPA, Yang and Foster[12] interpreted the behavior at low pH in terms of molecular expansion. More recently Tanford and co-workers[13] suggested the existence of an additional intermediate form, the "expandable" form.

The object of the present investigation was to gain more insight into the heterogeneity of BPA as related to the molecular expansion of this protein. The electrophoretic behavior of BPA was investigated over a wider pH region, 2.0 to 4.7. Experiments were carried out mainly in chloride media of 0.02 ionic strength and 0°. Other experiments were carried out in chloride media changing the temperature (25°) or the ionic strength (0.10). Electrophoresis was also conducted in other media, such as acetate and thiocyanate of 0.02 ionic strength.

Experimental

Materials.—Pentex bovine plasma albumin, Lot No. A 1201, was used without further purification. All the reagents used, except thiocyanic acid, were of C.P. grade. Thiocyanic acid was obtained by passing potassium thiocyanate solution through cationic ion exchanger (IR-120) in the hydrogen form.[14]

Procedure.—Electrophoresis was carried out at 0° in a Tiselius type electrophoresis apparatus, Model 35 of the Perkin-Elmer Corporation, equipped with the schlieren scanning system. The pH of the sample was measured with a Beckman Model G pH meter. Concentration of BPA was determined spectrophotometrically using a Beckman Model DU spectrophotometer. Extinction of BPA in distilled water was assumed to be $E^{1\%}_{1\,cm.}$ 6.67 at 279 mμ.[15] Electri-

(1) Supported in part by the National Cancer Institute, National Institutes of Health, Grant C-2248. Presented in part before the Division of Biological Chemistry, American Chemical Society, September, 1956.

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(11) C. Tanford, *Proc. Iowa Acad. Sci.*, **59**, 206 (1952).

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TABLE I

ELECTROPHORETIC DATA AND RADIUS OF BPA AT 0.02 CHLORIDE IONIC STRENGTH AND 0°

pH	Mobility, U_0, cm.²/v. sec. × 10⁵ Slow	Fast	Area, % Slow	Fast	Z Total (N, F)	Z/u_0 × 10⁻⁵ Slow	Fast	Radius, Å. Slow	Fast
2.11		+13.7			+73		5.3		50
2.55		+13.1			+63		4.8		47
2.99		+11.9			+46		3.9		41
3.08		+11.7			+43		3.7		40
3.42		+ 9.7	0	100	+28		2.9		34
3.62	+7.8	+ 8.6	19	81	+21 (+19, +22)	2.4	2.6	31	33
3.75	+6.6	+ 7.4	23	77	+18 +(16, +19)	2.4	2.6	31	33
3.82	+5.2	+ 6.6 (+7.0, +6.2)	23	77	+16 (+14, +17)	2.7	2.6	33	33
3.90	+4.5	+ 5.7 (+6.0, +5.3)	27	73	+13.5 (+12, +15)	2.7	2.6	33	33
4.00	+3.5	+ 5.3	60	40	+11.0 (+9.5, +12.5)	2.7	2.4	33	31
4.11	+2.5	+ 4.1	64	36	+ 8.5 (+7, +10)	2.8	2.4	34	31
4.16	+2.3	+ 3.7	67	33	+ 7.5 (+6.5, +9.5)	2.8	2.6	34	33
4.30	+1.2	+ 2.6	86	14	+ 4.5 (+3.5, +6.5)	2.9	2.5	34	32
4.55	−1.0	+ 0.8	94	6				33 ± 2 Å.	

cal conductivity was measured by a conductivity bridge, Model RC 16 of the Industrial Instruments, Inc. The *p*H values described in this paper were obtained by measuring at room temperature the *p*H of the protein solution after dialysis. The conductivity of the protein solution was measured at 0°.

Three different supporting media were used in the present investigation. They were (1) mixtures of hydrochloric acid and sodium chloride, (2) acetic acid and sodium acetate, and (3) thiocyanic acid and potassium thiocyanate. The total ionic strength of the media was kept constant, 0.02 or 0.10. Dialysis was carried out in a cold room at 1–2° for at least 15 hours with continuous mechanical agitation. A protein concentration of 0.2% was employed when the ionic strength was 0.02 for the reason described in the next section. For analysis, patterns were enlarged approximately eight diameters and traced carefully on graph paper. Resolution of components was carried out in the customary manner by sketching Gaussian peaks in such a manner that they summed to yield the observed gradient curve. Areas were measured using a planimeter and the percentage composition obtained from relative areas on the assumption that each component had the same refractive increment.

Results

Effect of Protein Concentration and Determination of Limiting Mobilities.—Since most of the studies were conducted at relatively low ionic strength (0.02) and in poorly buffered media (chloride) one must be concerned with the possibility of salt and *p*H gradients which would vitiate interpretation of the patterns. The only practical criterion of such effects appears to be the degree of enantiography of the patterns obtained on the rising and descending sides. At 0.5% protein and above, in the low ionic strength media, patterns were far from enantiographic, the rising boundary being very sharp, the descending boundary broad and diffuse. At 0.2% boundaries were reasonably enantiographic, in general showing the same number of boundaries and very nearly the same relative areas under such boundaries. At 0.05% results were qualitatively very similar to those at 0.2% but areas could not be estimated with sufficient precision. Mobilities could, however, be determined with no difficulty.

The relationship between electrophoretic mobilities and protein concentration was studied in a mixture of hydrochloric acid and sodium chloride at ionic strength 0.02, *p*H 2.1 and temperature 0°. As is seen in Fig. 1, the difference in mobility calculated from the ascending side and that from the descending one became greater with increase in the protein concentration. When the protein concentration was 0.5% or greater, a diffuse and asymmetric boundary was obtained on the descending side. In this case the centroidal ordinate[16] was used to calculate the mobility. It was found that the mobility value U_0, mobility corresponding to zero protein concentration, could be given by the following empirical equation at any protein concentration in 0.02 chloride ionic strength at 0°

$$U_0 = U_D + \frac{2}{3}(U_A - U_D) \qquad (1)$$

where U_D and U_A represent the electrophoretic mobility found on the descending side and that found on the ascending side, respectively. This relation was found to hold at *p*H 2.6 and 3.0 as well as at *p*H 2.1. At *p*H 3.5 and above the concentration dependence was less pronounced and U_0 could be calculated equally well by eq. 1 or by simple averaging of U_A and U_D.

***p*H–Mobility Curves.**—Figure 2 shows typical electrophoretic patterns obtained in chloride at 0.02 ionic strength. Below *p*H 3.5 and above 4.6 there was a single boundary; between 3.5 and 4.6 there were, in general, two. Strictly speaking, in the narrow *p*H range between 3.80 and 3.95 there were three boundaries. At *p*H lower than 3, where the mobility calculated from the migration velocity in the ascending limb was appreciably different from that calculated from the descending limb, the empirical eq. 1 was used to give the mobility value U_0 at zero protein concentration. Mobility values are shown in Table I. The *p*H–mobility curve is shown in Fig. 3. In the *p*H region above 3.5 there are two curves. When three components existed, mobilities of the two faster components are shown in parentheses in Table I, and mobility of the slowest component and the mean of the two faster components were plotted.

Electrophoretic data obtained in chloride media of 0.10 ionic strength are shown in Table II. In this case the pattern was single below *p*H 3.5 and was not single between *p*H 3.5 and 4.6. In the latter *p*H region the pattern usually consisted of three components, similar to those obtained by Alberty.[5]

Electrophoretic data at 25° and at 0.02 chloride ionic strength are shown in Table III. Below *p*H

(16) L. G. Longsworth, THIS JOURNAL, **65**, 1755 (1943).

TABLE II

ELECTROPHORETIC DATA AND RADIUS OF BPA AT 0.10 CHLORIDE IONIC STRENGTH AND AT 0°

*p*H	Mobility, U_0, cm.²/v. sec. × 10⁵ Slow	Fast	Area, % Slow	Fast	*Z* Total (N, F)	$Z/u_0 \times 10^{-5}$ Slow	Fast	Radius, Å. Slow	Fast
2.12		+8.1			+59		7.3		43
2.66		+7.8			+49		6.2		40
3.20		+6.4			+35		5.5		37
3.50		+5.4			+26		4.8		34
3.79	+3.4	+4.1	21	79	+17 (+15, +18)	4.4	4.4	32	32
3.95	+2.1	+2.8 (+2.9, +2.5)	30	70	+11 (+9.5, +12.5)	4.5	4.5	33	33
4.01	+1.6	+2.4 (+2.5, +2.2)	52	48	+ 9 (+8.5, +10.5)	4.7	4.4	34	32
4.10	+0.7	+1.5 (+1.6, +1.4)	56	44	+ 4 (+3, +6)	4.3	4.0	32	31
4.13	+0.4	+1.4 (+1.6, +1.2)	70	30				33 ± 2 Å.	
4.30	−0.5	+0.4	82	18					
4.50	−2.1	−0.4	93	7					
4.69	−2.7								

3.5 there was a single boundary, and between *p*H 3.5 and 4.7 there were two or three boundaries in agreement with the results at 0°.

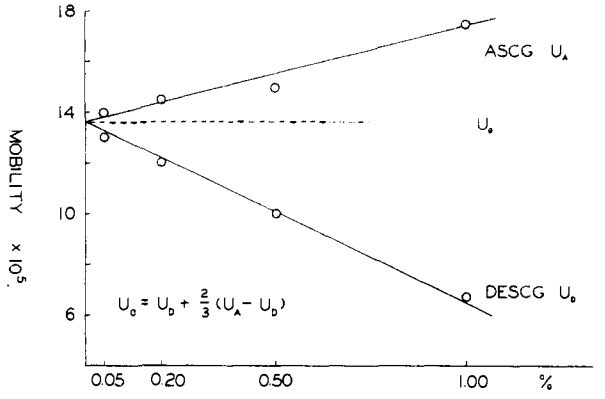

Fig. 1.—Relation between the mobility and the concentration of BPA: *p*H 2.1, 0.02 ionic strength chloride.

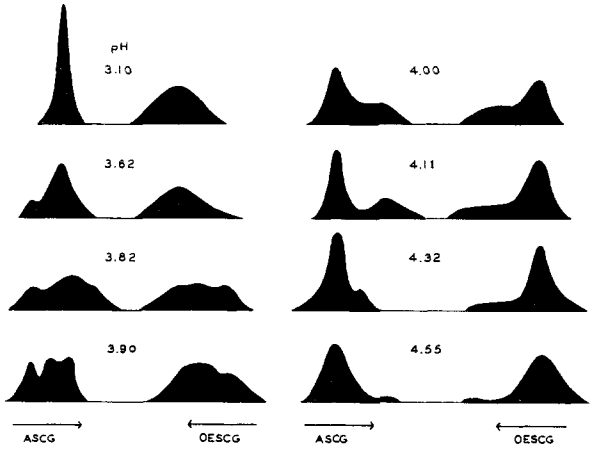

Fig. 2.—Electrophoretic patterns, 0.02 ionic strength chloride, 4800 sec., 7.7 volt cm.$^{-1}$.

Electrophoresis was carried out in acetate media and in thiocyanate media at 0.02 ionic strength. Mobilities obtained in acetate media are shown in Table IV.[17] The degree of resolution of the boundaries in acetate media was almost the same as in

(17) Detailed discussion of the results in thiocyanate media will be given in a later publication.

TABLE III

ELECTROPHORETIC DATA OF BPA AT 0.02 CHLORIDE IONIC STRENGTH AT 25°

*p*H	Mobility, cm.²/v. sec. × 10⁵ Slow	Fast	Area, % Slow	Fast
2.10		+26.6		
2.42		+26.3		
2.97		+23.7		
3.48		+18.6		
3.65		+16.2		
3.71	+13.5	+15.3	30	70
3.91	+10.1	+11.8	32	68
4.01	+ 8.1	+10.3	42	58
4.05	+ 7.0	+ 9.1	53	47
4.12	+ 6.1	+ 8.0	62	38
4.25	+ 4.5	+ 6.0	70	30
4.39	+ 4.2	+ 1.9	86	14

the mixture of hydrochloric acid and sodium chloride. Below *p*H 4.6 there were two boundaries. In a narrow *p*H range between 3.80 and 3.95 (the same region as in the chloride media), there were three boundaries. When the *p*H was around 4.5

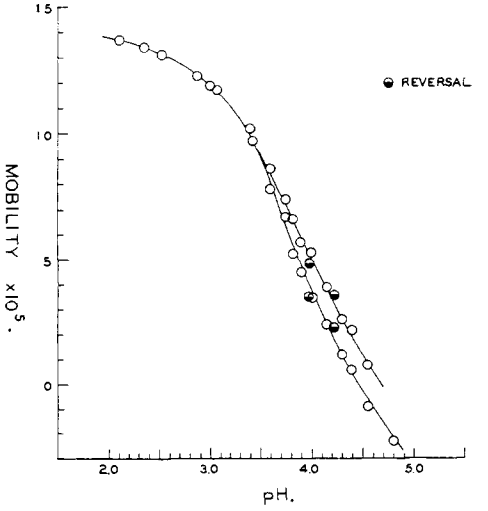

Fig. 3.—*p*H-mobility curve at 0.02 ionic strength chloride and at 0°.

the pattern was better in the acetate media than in chloride, possibly due to the improved buffering

TABLE IV

ELECTROPHORETIC DATA OF BPA AT 0.02 ACETATE IONIC STRENGTH AT 0°

pH	Mobility, U_0, cm.²/v. sec. × 10⁻⁵ Slow	Fast	Area, % Slow	Fast
3.79	+6.5	+7.3		
3.84	+6.2	+6.9	23	77
3.92	+5.8	+6.6	35	65
4.07	+4.5	+5.7	61	39
4.17	+3.7	+5.3	73	27
4.26	+3.0	+4.4	77	23
4.46	+1.6	+3.0	85	15
4.60	+0.7	+2.1	92	8
4.79	−0.3		100	0
5.00	−1.7			

in the acetate media.[18] In thiocyanate the resolution was poor, but two boundaries were separated after prolonged migration. In thiocyanate the migration was slow, therefore the difference in the electrophoretic mobilities of two components was smaller. This is doubtless the reason that a longer time was necessary to attain resolution. Below *p*H 3.2 and above *p*H 4.3 there was a single boundary. In the region between these two *p*H's there were two boundaries.

Percentage of the Two Components.—As was described above, the relative area of each component changed continuously with *p*H within a particular *p*H range. Percentages of the areas of each component were calculated on the ascending pattern. When three boundaries were observed, the ratio of the sum of areas of the faster two boundaries to that of the slowest one was taken. The results are shown in Tables I to IV. The results in chloride at 0.02 ionic strength and at 0°, and those at the same ionic strength at 25°, are plotted in Fig. 4.

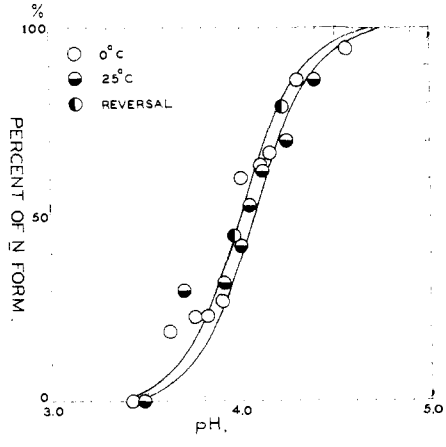

Fig. 4.—Relation between *p*H and the % of the area, 0.02 ionic strength chloride at 0° and 25°.

Discussion

Equilibrium between Two Forms of BPA.—In all of the experiments conducted the percentage composition was found to change continuously with *p*H over the *p*H range in which two or more components existed. This range depended primarily on the medium employed, slightly on temperature and ionic strength. If we term the slow or normal electrophoretic form N and the faster form F, the percentage composition is found to conform closely in all cases to an equilibrium of the form

$$N + nH^+ \rightleftharpoons F$$

Further, the value of n is in all cases between 2 and 3, usually closer to 3. It is inferred that there is an equilibrium of this nature between two discrete forms of the protein which differ in hydrogen-ion binding by approximately three ions. The results in various media are summarized according to this interpretation in Table V which gives the value of n as well as the *p*H of the mid-point of the transition curve (*p*H at which two forms exist in equal amounts). The equilibrium constant corresponding to

$$K = \frac{[F]}{[N][H^+]^n}$$

is also given in each case.

TABLE V

CONSTANTS FOR N-F EQUILIBRIUM

Conditions	n	Mid-point	K
0.02 Cl^-, 0°	3	4.0	$10^{12.0}$
.02 Cl^-, 25°	3	4.07 ± 0.03	$10^{12.2 \pm 0.1}$
.10 Cl^-, 0°	3	4.05	$10^{12.15}$
.02 Ac^-, 0°	2	4.1	$10^{8.2}$
.02 SCN^-, 0°	3	3.75	$10^{11.1}$

Recently, Phelps and Cann[20] observed two electrophoretic components in BPA at *p*H near the isoelectric point and noted further that the relative proportion of faster form increased, at constant *p*H, when part of the chloride ion was replaced by acetate. This phenomenon is explained readily by our results. In chloride media the two components existed in equal amounts at *p*H 4.0 and they followed the three hydrogen ion equilibrium. In the acetate media the two components existed in equal amounts at *p*H 4.1 and followed the two hydrogen equilibrium. Therefore when part of chloride ion is replaced by acetate at any *p*H above 3.8 the area of the faster moving boundary increases.

Reversibility of the Process.—To observe whether or not the transition process is reversible the following experiment was carried out. BPA solution of *p*H 2.11, consisting of hydrochloric acid and sodium chloride, was prepared and stored in a cold room about 24 hours. Then by adding the proper volume of sodium hydroxide, the *p*H of the protein solution was changed to around 4.0. The final solution had 0.02 ionic strength and 0.2% protein concentration. After dialysis

(18) The slower moving boundary in chloride media around *p*H 4.5 separated into two after a longer migration as was described before.[19] This phenomenon was not observed in acetate media at the same *p*H.

(19) K. Aoki and J. F. Foster, THIS JOURNAL, **78**, 3538 (1956).

(20) R. A. Phelps and J. R. Cann, *ibid.*, **78**, 3539 (1956). While there is qualitative agreement between their results and ours, there does appear to be a serious quantitative discrepancy. Thus their patterns at *p*H 4.7 in 0.025 ionic strength chloride are complex whereas we find only one boundary under such conditions. In a private communication Dr. Cann has informed us that the protein concentration employed in their published experiments was 1%. He further states that they have obtained less complex patterns in subsequent experiments at lower concentration.

in a cold room overnight, the protein solution was analyzed electrophoretically. Electrophoretic mobility and relative area of the two components, with the experimental condition, are shown in Table VI. These data are plotted in Figs. 3 and 4. It is observed that the data obtained in this experiment are the same as those obtained on the fresh BPA solution. This means that the transition process is reversible, at least insofar as electrophoretic behavior is concerned.

TABLE VI

DATA OF THE REVERSAL EXPERIMENT

*p*H		Mobility, cm.²/v. sec. × 10⁵		Area, %		
Initial	Final	Slow	Fast	Slow	Fast	Exptl. condition
2.11	→ 3.96	+3.5	+4.8	45	55	Kept at *p*H 2.11 in a cold room for 23 hr. and dialyzed 20 hr.
2.11	→ 4.22	+2.3	+3.6	79	21	Kept at *p*H 2.11 in a cold room for 23 hr. and dialyzed 24 hr.

Thermodynamic Parameters of the N–F Transition.—It is observed in Fig. 4 that the equilibrium was shifted slightly to a higher *p*H with rise of the temperature. The shift of the mid-point resulting from a rise of 25 degrees was 0.07 ± 0.03 *p*H unit. Assuming the three hydrogen equilibrium, the equilibrium constant K was calculated to be $10^{12.0}$ at 0° and $10^{12.2 \pm 0.1}$ at 25° as is shown in Table V. The free energy change is

$$\Delta F^\circ = -RT \ln K = -15 \text{ kcal./mole. (at } 0^\circ)$$

Enthalpy change is

$$\log \frac{K_0}{K_{25}} = -\frac{\Delta H^\circ}{2.303R}\left(\frac{1}{T_0} - \frac{1}{T_{25}}\right)$$

$$\Delta H^\circ = +3.0 \pm 1.5 \text{ kcal./mole}$$

Entropy change is

$$\Delta H^\circ = \Delta F^\circ + T\Delta S^\circ$$

$$\Delta S^\circ = +66 \pm 4 \text{ e.u./mole}$$

The enthalpy of formation of side-chain hydrogen bonds in protein has been estimated as −5 to −10 kcal., the entropy as −20 to −30 e.u.[21] On the basis of the observed third-order dependence on hydrogen ion and the calculated entropy change it is tempting to suggest the transition as involving rupture of three intramolecular hydrogen bonds by protons. The enthalpy of the reaction, while small, is strikingly similar to that found calorimetrically by Gutfreund and Sturtevant.[22] They observed that 3.1 kcal./mole of heat was absorbed when the *p*H of BPA was changed from 4.5 to 3.4. These two *p*H values correspond to the practical extremes of the transition in our experiments. They also stated that the process is reversible, had a half-time of 2.5 to 3 minutes, and is first order with respect to protein concentration.

Half-time of the Transition Reaction.—It was observed that the electrophoretic pattern was not affected by the time of dialysis; that is, when the time of dialysis was changed between 19 and 75 hours in 0.02 ionic strength and at 0°, the pattern was not changed. When the medium of HCl–NaCl was used, prolonged dialysis was necessary because of the poor buffering.

In an attempt to estimate the rate of attainment of the N–F equilibrium electrophoresis of BPA was carried out without dialysis in the acetate buffer. The electrophoresis was conducted directly after the protein was dissolved in the buffer solution at *p*H 4.11 and 0.02 ionic strength. A picture taken two hours after dissolving the protein, however, showed the same resolution as obtained on a sample at the same *p*H and ionic strength and dialyzed 18 hours. It seems that the N–F equilibrium is completed in a shorter time than two hours, hence is too rapid to study by the electrophoretic technique.

It has been realized that no resolution of boundaries will occur in moving boundary transport experiments, electrophoresis or sedimentation, where two or more components are in an equilibrium which is established rapidly compared with the differential rate of migration. A single boundary will be observed which moves with the weight-mean velocity of its components, and which spreads more rapidly than is accounted for by diffusion. Some discussions have been given[23–25] on this problem. Accordingly, the fact that the two forms were resolved electrophoretically tends to indicate the transition reaction to be slower than that of Gutfreund and Sturtevant.[22] As there is no sufficient theory applicable to the present system, the following experiment was conducted[26] to see whether or not the pattern was affected by the re-equilibration of the system. Protein solution of the same *p*H and ionic strength was electrophoretically analyzed changing the current density. BPA solution at *p*H 4.08 and 0.02 ionic strength was analyzed with different currents of 2.2, 1.1 and 0.4 ma. Pictures were taken when the same number of coulombs had passed, that is, at 6,000 sec. in the 2.2-ma. run, at 12,000 sec. in the 1.1-ma. run, and at 33,000 sec. in the 0.4-ma. run. These runs yielded the same mobilities and the same area distribution. Results are shown in Table VII. The picture taken in the 0.4 ma.-run was slightly broader than that in 2.2-ma. run.

TABLE VII

EFFECT OF CURRENT DENSITY ON ELECTROPHORESIS

	Mobility, cm.²/v. sec. × 10⁵		Area, %		Voltage gradient,
*p*H	Slow	Fast	Slow	Fast	v./cm.
4.08	+4.6	+3.1	75	25	5.80
4.08	+4.6	+3.1	73	27	2.90
4.08	+4.5	+3.0	72	28	1.05

Although it is not known whether this broadening is more than expected only by diffusion, the value of the per cent. of area agrees with the other results

(21) M. Laskowski and H. A. Scheraga, THIS JOURNAL, **76**, 6305 (1954).

(22) H. Gutfreund and J. M. Sturtevant, *ibid.*, **75**, 5147 (1953).

(23) R. F. Smith and D. R. Briggs, *J. Phys. Colloid Chem.*, **54**, 33 (1950); R. A. Alberty and H. H. Marvin, *ibid.*, **54**, 47 (1950).

(24) E. O. Field and A. G. Ogston, *Biochem. J.*, **60**, 661 (1955).

(25) G. A. Gilbert and R. C. L. Jenkins, *Nature*, **177**, 853 (1956).

(26) This experiment was suggested by Dr. S. J. Singer, Department of Chemistry, Yale University, and is the same kind of experiment done by him and his co-workers.[27]

(27) S. J. Singer and D. H. Campbell, THIS JOURNAL, **77**, 3499, 4851 (1955).

within experimental error. This indicates the area distribution was not disturbed seriously by the re-equilibration in the present system.[28]

The Third Component.—As was described above, there were three boundaries in the *p*H region between 3.80 and 3.95 at 0.02 chloride or acetate ionic strength and at 0°. There were three boundaries over a wider *p*H region at higher ionic strength or at room temperature. Even when the BPA was dissolved and dialyzed in a cold room, there were three components in this particular *p*H region. This boundary probably was not caused by thermal convection.[30]

It has been observed by the ultracentrifuge[32] and by light scattering[33] that BPA aggregates under some conditions. BPA has more tendency to aggregate at higher ionic strength and at lower *p*H. Aggregation of BPA was demonstrated by the above techniques in media, the ionic strength of which was more than 0.1. Kronman, *et al.*,[33] observed the effect of ionic strength on the aggregation of BPA at *p*H around 3. There is hence some possibility that the third component may be aggregated form. Ultracentrifuge runs were made at 0.02 ionic strength and at *p*H 3.85 both at room temperature and around 6°. There was a single boundary with a minor faster boundary amounting to only a few per cent. This minor boundary in the ultracentrifuge pattern is interpreted as the "dimer impurity" which exists in a wider *p*H region. Therefore the third component is not the aggregated form.

Another possibility is that the third component may be the denatured form. Levy and Warner[34] studied the effect of *p*H on the denaturation of BPA, and found that the denaturation velocity was maximum in the *p*H region between 3.5 and 4.0 at 46.2°. It seems probable that this is not the explanation since the component was present even when care was taken not to expose the protein solution to temperatures above 2°.

It seems probable that the third component is real and may in fact represent an intermediate in the transition.[28]

Calculation of the Net Charge of BPA.—The charge Z at a particular *p*H is governed by the total ion binding, *i.e.*, both cations and anions. The number of hydrogen ions bound may be determined from the titration curves of Tanford, *et al.*,[35] or of Foster and Sterman.[15] Chloride binding data are available from the work of Coleman,[36] Carr[37] and Alberty and Marvin.[38] Since it is known that sodium ion does not combine,[39,40] or at most combines only very slightly,[41] to BPA the net charge below the isoelectric point is given by the difference between hydrogen ion binding and chloride binding. The two sets of data on hydrogen ion binding agree reasonably well; the chloride ion binding data are not so complete and not in such good agreement and hence constitute the main uncertainty in calculation of net charge.

Coleman's studies were conducted with bovine mercaptalbumin and chiefly at the isoionic point. According to his results the chloride binding may be calculated under a given set of experimental conditions through the equation

$$\nu_{x^-} = \sum \frac{k_i n_i C_{x^-} \exp(2Zw)}{1 + k_i C_{x^-} \exp(2Zw)} \qquad (2)$$

where ν_{x^-} represents the number of anion x^- bound to one BPA. C_{x^-} is the molar concentration of the anion. Z is the net charge and n_i is the number of groups with intrinsic constant of k_i. w is a function expressed as

$$w = \frac{e^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + a\kappa}\right) \qquad (3)$$

where e, D, k and T, represent the electronic charge, dielectric constant, Boltzmann's constant and the absolute temperature, respectively. b and a are the radius of the protein and the exclusion radius of the protein. κ is the Debye–Hückel function expressed as

$$\kappa = \sqrt{\frac{8\pi e^2 N}{1000DkT}}\sqrt{\mu} \qquad (4)$$

where N and μ represent Avogadro's number and the ionic strength, respectively. At 25° κ is

(28) While this result would seem to justify the quantitative deductions we make with respect to the equilibrium between, and properties of, the N and F forms, the important question of why re-equilibration is not serious remains unanswered. This is especially true in light of the earlier mentioned experiment which indicates the half-time for the transition to be much shorter than two hours (possibly approximately 30 minutes). The theory of Gilbert and Jenkins[25] would offer a possible explanation if the equilibrium were of the nature A + B ⇄ C, but this seems most improbable. Another explanation which we have considered is that the boundaries are stabilized by a *p*H gradient arising in electrophoretic separation. This seems doubtful on two grounds: (1) in such case the observed enantiography would not be expected; (2) resolution is very similar in the very poorly buffered chloride systems and in the somewhat better buffered acetate systems. Dr. Charles Tanford has suggested to us that resolution might conceivably be explained along the lines of the Gilbert–Jenkins theory if the transition were actually stepwise, involving one or more intermediates. This suggestion is of great interest to us because we had already reached the conclusion that the transition must indeed be stepwise, probably involving three intermediate forms.[29] If this is true, it must be considered that the thermodynamic parameters given herein refer to some composite of the separate steps rather than to any one step.

(29) J. F. Foster and K. Aoki, *J. Phys. Chem.*, in press.

(30) The heat H developed in the cell by the current is expressed by

$$H = i^2/q^2k \text{ watt/cm.}^3$$

where i is the current, q is the cross-sectional area of the cell, and k is the specific conductivity. According to Alberty[31] experience has shown that when the thermostat temperature is about 1°, the maximum power which might be dissipated in the electrophoresis cell without causing convection at the usual protein concentration is about 0.15 watt/cm.³. In the present study the H value was 0.08 at these *p*H's. Therefore it seems probable that there was no convective disturbance.

(31) R. A. Alberty, *J. Chem. Education*, **25**, 426, 619 (1948).

(32) H. A. Saroff, G. J. Loeb and H. A. Scheraga, THIS JOURNAL, **77**, 2908 (1955); P. Bro, S. J. Singer and J. M. Sturtevant, *ibid.*, **77**, 4924 (1955); M. E. Reichman and P. A. Charlwood, *Can. J. Res.*, **32**, 1092 (1954).

(33) M. J. Kronman, M. D. Stern and S. Timasheff, *J. Phys. Chem.*, **60**, 829 (1956).

(34) M. Levy and R. Warner, *ibid.*, **58**, 106 (1954).

(35) C. Tanford, S. A. Swanson and W. S. Shore, THIS JOURNAL, **77**, 6414 (1955).

(36) J. S. Coleman, Ph.D. thesis, Massachusetts Institute of Technology, 1953.

(37) C. W. Carr, *Arch. Biochem. Biophys.*, **40**, 286 (1952); **46**, 417 (1953).

(38) R. A. Alberty and H. H. Marvin, THIS JOURNAL, **73**, 3220 (1951).

(39) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, *ibid.*, **72**, 535, 540 (1950).

(40) I. M. Klotz, "Modern Trends in Physiology and Biochemistry," E. S. G. Barron, Ed., Academic Press, New York, N. Y., 1952, p. 430.

(41) C. W. Carr, *Arch. Biochem. Biophys.*, **62**, 476 (1956).

0.47×10^7 at 0.02 ionic strength, and 1.03×10^7 at 0.10 ionic strength. Using these values of κ and taking a as 30 Å.[42] and b as 32.5 Å., w is calculated at 25° to be 0.053 at 0.02 ionic strength and 0.035 at 0.10 ionic strength. Again in Coleman's equation, constants are

$n_1 = 1$, $n_2 = 8$, $n_3 = 18$, $k_1 = 2400$, $k_2 = 100$ and $k_3 = 3.3$

Using eq. 2, a relation between Z and ν_{Cl^-} is obtained. Since $Z = \nu_{H^+} - \nu_{Cl^-}$, and since ν_{H^+} at a particular pH is given by the titration curve, a curve relating ν_{Cl^-} and pH is obtained. If this chloride binding curve is perfectly correct, the pH at which Z is zero should agree with the isoelectric point found in the present study. Actually there was some discrepancy between the experimental isoelectric points and those calculated, amounting to approximately three charge units and nine units at 0.02 and 0.10 ionic strength, respectively. Accordingly, the entire chloride binding curves were increased by 3 and 9 chloride ions in the two cases. The resulting Z values are given in Tables I and II and are employed in further calculations below.[44]

Charge–Mobility Curve.—Using the pH–mobility curve and the pH–charge curve, the charge–mobility curves were drawn for cases of 0.02 and 0.10 ionic strengths. The charge–mobility curves in the pH region above 3.5, where two components exist, were determined in the following way. In this pH region at 0.02 ionic strength and room temperature, only a single boundary was obtained in the ultracentrifuge pattern.[45] This means that the F and N forms have similar size and different charge. The Z values shown in Tables I and II are the average ones. From these values the net charge of each form, F and N, was found, assuming that the difference in net charge of the two forms is three and that the chloride ion binds in the same way to the two forms, and considering that average Z is calculated using the charge of each form and its percentage in the electrophoretic pattern. The Z values of each form thus calculated are shown in parentheses in Tables I and II. The mobility values of each form were plotted against the Z values of each form. As is seen in Fig. 5 these plots lie on a common straight line, showing that the assumption that the charge difference in the two forms is three is probably right. This is additional evidence that the equilibrium is N + $3H^+$ ⇄ F. Charge–mobility curves over the whole pH region are shown in Fig. 6. It is seen that the relation between charge and mobility is linear above pH 3.5 but not below that pH. This indicates that the radius of BPA is constant above pH 3.5 but increases at lower pH, in accord with previous conclusions.

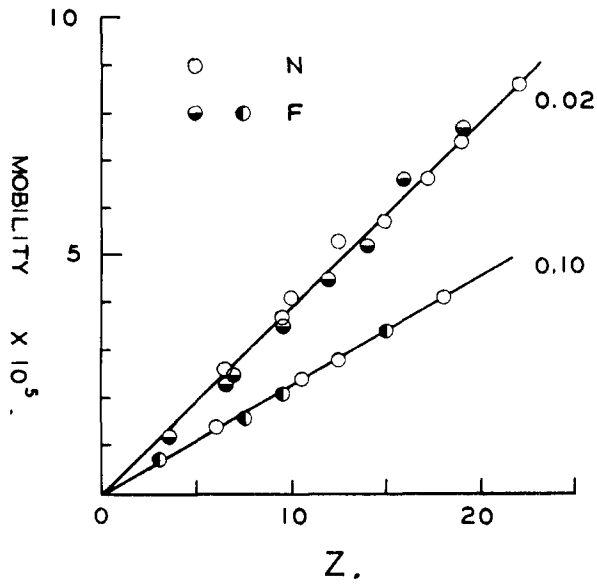

Fig. 5.—Relation between charge (Z) and mobility in the pH region above 3.5.

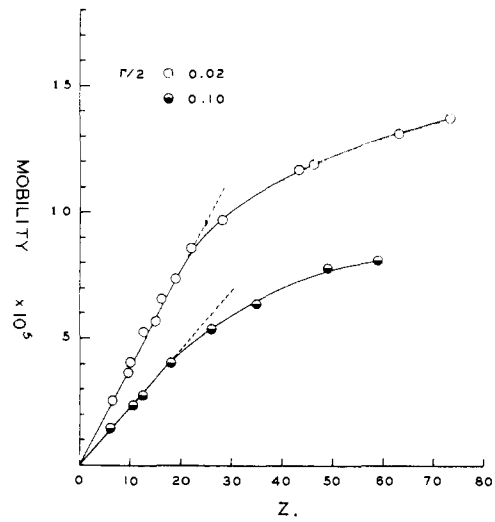

Fig. 6.—Relation between charge (Z) and mobility in the whole pH region.

Calculation of the Radius of BPA.—By use of Henry's equation[46] the radius of BPA was calculated from electrophoretic mobility data and Z values. Henry's equation is

$$Q = \frac{6\pi\eta r(1 + \kappa r + \kappa r_i)}{f(\kappa r)(1 + \kappa r_i)} u \qquad (5)$$

where, Q is the charge (coulomb per molecule), r, the radius of molecule (cm.), r_i, the average radius of electrolyte ion, η, the viscosity coefficient of the solvent, κ, the reciprocal thickness of double

(42) Serum albumin approximates a prolate ellipsoid of 150 Å. major axis and 38 Å. minor axis.[33] Molecular volume of this prolate ellipsoid corresponds to a sphere of 30 Å. radius.

(43) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Colloid Chem.*, **51**, 184 (1947).

(44) This mode of correction of the Z values is of course rather arbitrary. It leads to better agreement with the binding data of Carr at low pH but poorer agreement in the pH range 4 to 5. Carr's data would predict an isoelectric point of approximately 5.0 at ionic strength 0.02, a result which is far outside the experimental error. It appears that either both the binding data of Carr and of Coleman are too low near the isoelectric point, or the titration data are in error. The later possibility appears much less likely. Another conceivable explanation lies in the fact that we have restricted our consideration to the titration of carboxyl groups exclusively. This amounts to the assumption that all of the (16) imidazole groups are in the acid (cationic) form at the isoelectric point. It is conceivable that the discrepancy of 3 charge units observed in 0.02 ionic strength could result from this assumption, but not the discrepancy at 0.1 ionic strength.

(45) M. J. Kronman and J. F. Foster, *Arch. Biochem. Biophys.*, in press.

(46) D. C. Henry, *Proc. Roy. Soc.* (*London*), **A133**, 106 (1931).

tember 1956.

(6) H. M. Diutzis, Ph.D. thesis, Harvard University, 1952.

Mobilities at zero concentration were estimated using equation 1 in the earlier paper.[3]

layer which is expressed by eq. 4, μ, the electrophoretic mobility (cm.^{2}/volt. sec.), and $f(\kappa r)$ is a function of κr given by Henry. The equation is written using Z as

$$\frac{Z}{u} = \frac{300}{4.80 \times 10^{-10}} \frac{6\pi\eta r(1 + \kappa r + \kappa r_i)}{f(\kappa r)(1 + \kappa r_i)} \qquad (6)$$

The value of r_i was taken as 2.5×10^{-8} cm., and η as 0.01792. The value of κ was described above.

In the above equation r is the radius of the spherical molecule in question. BPA is not a sphere but possibly a prolate ellipsoid with the axial ratio of 4/1. Therefore the r value obtained by this equation must be corrected. According to Gorin[47] a cylinder of an axial ratio of 4/1 has a mobility equal to 70% of that of a sphere of the same molecular volume in the case of 0.02 ionic strength, and 67% in the case of 0.10 ionic strength. This means that this cylinder has approximately the same mobility as the sphere of a radius of 100/70 or 100/67 times, depending upon the ionic strength, larger than that of the sphere having the same volume as the cylinder. The effective radius of the BPA molecule is thus taken to be 70/100 or 67/100 that of r obtained from Henry's equation. Results obtained after correction in this manner are shown in Tables I and II, and also shown in Fig. 7. It is

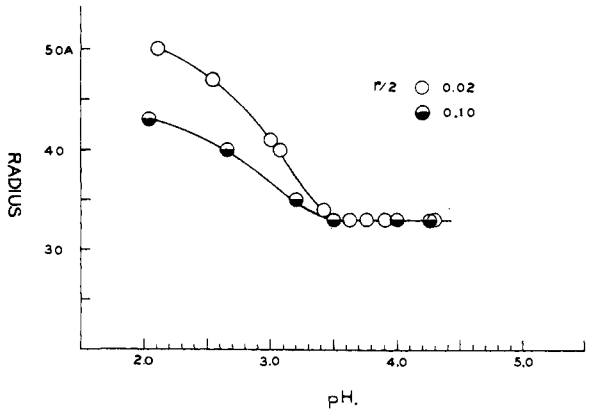

Fig. 7.—Relation between pH and radius of BPA.

found that when the pH is above 3.5 the effective radius of BPA is 33 ± 2 Å., in good agreement with the value deduced by Oncley, *et al.*,[43] and independent of ionic strength. At pH 2.1 the radius is 50 Å. (expansion of 3.4 times) when the ionic strength is 0.02, and 43 Å. (expansion of 2.2 times) when the ionic strength is 0.10.[48] With the increase in the ionic strength, the expansion is suppressed, which is the same conclusion obtained from the measurement of viscosity and optical rotation.[12]

Isoelectric Point.—As is shown in Table VIII, the isoelectric point differs with the media in which the protein is dissolved. Both the isoelectric point and the mid-point were changed with the composition of the media, in the order of higher pH, $SCN^- < Cl^- < Ac^-$. This series is in accord with the result by Longsworth, *et al.*[4] They showed that the isoelectric point of BPA was changed in the order shown above, when a part of the acetate buffer was replaced by other media. So far as the present study, at 0.02 ionic strength, is concerned, the pH at which the F form appears is close to the isoelectric point.

TABLE VIII

MEAN ISOELECTRIC POINT OF BPA IN VARIOUS MEDIA AT 0°

0.02 Cl^-	4.52
.10 Cl^-	4.22
.02 SCN^-	4.25
.02 Ac^-	4.72

A relation between the isoelectric point and ion binding is derived readily. At the isoelectric point $Z = 0$ and $r = \nu_{H^+} = \nu_{x^-}$ so that the titration equation becomes (assuming binding only to carboxyl groups of pK_0)

$$(p\mathrm{H})_I = pK_0 + \log \frac{n - (\nu_{x^-})_I}{(\nu_{x^-})_I} \qquad (7)$$

where x^- is the anion in the medium, $(p\mathrm{H})_I$ represents the isoelectric point, and $(\nu_{x^-})_I$ means the number of anions x^- which bind to the protein at the isoelectric point. It is recognized that the pH at which $r = 1$ is approximately equal to the isoionic point. At this pH, $Z = \nu_{x^-}$ and $\log(n - r)/r$ becomes very nearly $\log n$. Thus eq. 2 is written as

$$(p\mathrm{H})_0 = pK_0 - 0.868(\nu_{x^-})_0 w + \log n \qquad (8)$$

where, $(p\mathrm{H})_0$ represents the isoionic point and $(\nu_{x^-})_0$ the number of the anions bound to the protein at the isoionic point. Combining eq. 7 and 8 equation (9) is obtained

$$(p\mathrm{H})_I = (p\mathrm{H})_0 + \log\left(\frac{1}{(\nu_{x^-})_I} - \frac{1}{n}\right) - 0.868\,(\nu_{x^-})_0 w \qquad (9)$$

It is seen that the difference between the isoionic point and the isoelectric point is a function of the medium and its ionic strength, because w is a function of the ionic strength and ν_{x^-} changes with the media and its ionic strength. In the case of BPA at 0.02 chloride ionic strength the calculated $(p\mathrm{H})_I$ is 4.5 ± 0.1, the experimental value 4.52.

Effect of Temperature.—Comparing the mobility values at 25° and those at 0° at 0.02 ionic strength, it is found that the former are almost exactly double the latter within experimental error. The ratio of viscosity coefficient at 0° to that at 25° is 2.005. Therefore the product of ηu is seen to be constant[49] in this pH region. In Henry's eq. r_i is thought to be constant regardless of temperature. It is found also that κ is nearly independent of temperature.[51] Thus when the ratio of Henry's

(47) H. A. Abramson, L. S. Moyer and M. H. Gorin, "Electrophoresis of Proteins," Reinhold Publ. Corp., New York, N. Y., 1942, p. 131.

(48) Values of the radius found in the present study are smaller than those found at room temperature by Tanford, *et al.*,[18] and by Kronman and Foster.[46] The main reason is that the authors assumed BPA as a prolate ellipsoid, while they assumed it as a sphere.

(49) Watanabe, *et al.*,[50] studied the change of the mobility of horse serum albumin with temperature. It was found that the product ηu increased with temperature at pH 7.7. This may be attributed to the increase of the dissociation of the basic groups with temperature. This is reasonable when we consider that the heat of dissociation of the amino groups is much larger than that of the carboxyl group.

(50) I. Watanabe, N. Ui and M. Nakamura, *J. Phys. Colloid Chem.*, **54**, 1366 (1950).

(51) In eq. 4 variables are T and D. The ratio of κ at 25° (κ_{25}) to that at 0° (κ_0) is almost unity as

$$\frac{\kappa_{25}}{\kappa_0} = \sqrt{\frac{273 D_0}{298 D_{25}}} = 1.013$$

since D_0 (dielectric constant at 0°) is 87.8 and D_{25} (that at 25°) is 78.3.[52]

(52) "International Critical Tables," McGraw-Hill Book Co., New York, N. Y., Vol. VI, p. 78.

eq. at 25° to that at 0° is taken (suffix means the temperature), it becomes

$$\frac{Z_{25}}{Z_0} = \frac{r_{25}g(r_{25})}{r_0 g(r_0)}$$

where $g(r)$ is a new function of r. The hydrogen ion binding of proteins in the carboxyl titration region is known to be nearly temperature independent.[53,54] Evidence also exists that chloride ion binding to human serum albumin has a low temperature coefficient (enthalpy).[39] The change of the isoelectric point with temperature is not great, in this study less than 0.1 pH unit between 0 and 25°. These results suggest Z to be nearly independent of temperature. If we assume that Z is constant at the two temperatures, the radius of BPA is also constant.

(53) C. Tanford, THIS JOURNAL, **72**, 441 (1950).
(54) J. Wyman, *J. Biol. Chem.*, **127**, 1 (1939).

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, PURDUE UNIVERSITY]

Electrophoretic and Hydrogen Ion Binding Behavior of Bovine Plasma Albumin in the Presence of 0.02 *M* Thiocyanate Ion[1,2]

BY KOICHIRO AOKI[2] AND JOSEPH F. FOSTER

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Electrophoretic studies have been conducted on bovine plasma albumin over the pH range 2.0–4.5 in 0.02 *M* thiocyanate. Below pH 3.0 and above 4.3 single boundaries were obtained. Between 3.2 and 4.3 two boundaries were found, the composition varying continuously with pH in accord with the same N–F equilibrium previously reported in presence of chloride. The mid-point of the equilibrium occurs at pH 3.75 in comparison to 4.0 in chloride. Hydrogen ion titration studies were conducted in the region acid to the isoionic point in presence of 0.02 *M* thiocyanate. Molecular radii estimated from electrophoretic mobilities through Henry's equation are in agreement with values in chloride and indicate no appreciable molecular expansion to take place over the pH range of the molecular transition. Assuming a constant value for the electrostatic interaction parameter w, and constant number of binding sites, the apparent intrinsic pK of the carboxyl groups is shown to parallel very closely the electrophoretic composition in both chloride and thiocyanate. These results strongly suggest that the anomaly in the titration behavior of this protein is due essentially to the N–F transition, the carboxyls in the N form having a pK of approximately 3.7 due to stabilization by the native protein structure, while in the F form these stabilizing interactions are largely removed (pK = 4.4).

Introduction

It has been shown in chloride media that bovine plasma albumin (BPA) undergoes an isomerization reaction (N–F equilibrium)[3] in the pH range 4.5 to 3.5, and that the molecule expands below pH 3.5.[3,4] It has been suggested that the anomalous character of the titration curve of this protein above pH 3.5 can be explained on the basis of this equilibrium if it is assumed that the carboxylate groups are stabilized in the N form.[5] To test this idea further, electrophoretic studies have been conducted in the presence of 0.02 *M* thiocyanate ion, which is known to be much more strongly bound to this protein than is chloride. In addition hydrogen ion titration curves were determined in the same medium.

Experimental

Materials.—Pentex bovine plasma albumin, Lot No. A1201, was used. In the electrophoretic study this was used without further purification. In the titration study the protein solution was deionized by passing through the mixed ion-exchange column of Dintzis.[6] Potassium thiocyanate was of C.P. grade. Thiocyanic acid was obtained by passing potassium thiocyanate solution through cationic ion exchanger in the hydrogen form. In the titration study only ion-exchanged water was used.

Procedure.—Electrophoresis was carried out at 0° in a Tiselius type electrophoresis apparatus, Model 35 of the Perkin-Elmer Corporation, equipped with the schlieren scanning system. All the procedures in electrophoresis and the method of calculation were exactly the same as used in the previous work.[3] The total ionic strength of the media was 0.02 and the concentration of protein was 0.2%.

In the titration study the same apparatus as used by Foster and Sterman[7] was used. It consisted of a Beckman Model G pH meter and an external electrode cell assembly. The cell assembly consisted of a Beckman No. 1190-80 General Purpose glass electrode and a Beckman No. 1070-71 sleeve type calomel reference electrode. All temperature sensitive parts of the electrode cell assembly were maintained at a constant temperature, 25 or 0°. The procedure for determining the titration curve at 25° was also the same as was followed by the above workers.[7] In the studies at 0° the electrode system was standardized at each measurement against a standard phthalate buffer of pH 4.00, and the pH reading of the solution, which was prepared in a cold room, was taken after attaining thermal equilibrium (about 15 minutes).[8] In the calculations the molecular weight of BPA was assumed to be 70,000.

Results

Electrophoresis of BPA in the thiocyanate media of 0.02 ionic strength and at 0° was carried out in the pH range of 2.0 to 4.5. Below pH 3.0 there was a single boundary, and between this pH and 4.3 there were two boundaries. The percentage of the area of each form changed continuously with pH in the same way as in chloride media.[9] Here

(1) Supported in part by the National Cancer Institute, National Institutes of Health, Grant C-2248.

(2) Presented in part at the 41st Meeting of the Federation of American Societies for Experimental Biology, Chicago, 1957.

(3) K. Aoki and J. F. Foster, THIS JOURNAL, **78**, 3538 (1956); **79**, 3385 (1957).

(4) J. T. Yang and J. F. Foster, *ibid.*, **76**, 1588 (1954); C. Tanford, *et al.*, *ibid.*, **77**, 6421 (1955); W. F. Harrington, P. Johnson and R. H. Ottewill, *Biochem. J.*, **62**, 569 (1956).

(5) J. F. Foster and K. Aoki, Abstracts 130th ACS Meeting, September 1956.

(6) H. M. Dintzis, Ph.D. thesis, Harvard University, 1952.

(7) J. F. Foster and M. D. Sterman, THIS JOURNAL, **78**, 3656 (1956).

(8) In measurements at 0°, the temperature dial of the pH meter was adjusted to 25° and the pH reading multiplied by 1.09 (= 298/273).

(9) The areas were measured on the ascending pattern. Patterns were reasonably enantiographic in all cases. Resolution of the two components was not quite as good as in either chloride or acetate probably because of the decreased mobility in presence of thiocyanate. Mobilities at zero concentration were estimated using equation 1 in the earlier paper.[3]