The Amphoteric Behavior of Bovine Plasma Albumin. Evidence for Masked Carboxylate Groups in the Native Protein*

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ABSTRACT: The hydrogen ion titration behavior of bovine plasma albumin (BPA) in the pH range acid to the isoionic pH has been reexamined. Infrared difference spectra conducted in D_2O at various values of pD demonstrate that, practically speaking, only carboxyl groups are titrated over this range. The number of protonated carboxyl groups existing at the isoionic pH is estimated by this approach to be 2.5 ± 0.5. Electrostatic corrections were made by measuring the electrophoretic mobility in each system as a function of pH and utilizing the Henry equation to evaluate the ζ potential, which is assumed very nearly equal to the effective surface potential on the protein. Results on BPA in 0.1 M chloride are in accord with the thesis that in the native (N) form of the protein only about 60 of the approximately 100 carboxylate groups are available for protonation. In the case of the AD_{12} complex (D signifying detergent dodecyl sulfate ions) the N form of the protein is stabilized and the results strongly reinforce the above interpretation.

In the presence of 0.05 M perchlorate, most of the masked carboxylate groups are normalized even at the isoionic pH.

It is well established that the hydrogen ion titration curves of both bovine and human plasma albumins are abnormally steep near pH 4. Tanford (1952), who first pointed out this anomaly, suggested that it may result from an expansion of the globular protein molecule with a consequent reduction in the effective electrostatic interaction at a given net charge. Following discovery of the N–F transformation, which occurs in this pH region, it was argued by one of us (Foster and Aoki, 1957) that most of the anomaly is probably associated with this highly cooperative transformation rather than with expansion *per se*, which takes place at somewhat lower pH. It was suggested that the mean pK_{int} of carboxyl groups in the N or native form of the protein is abnormally low while the value becomes essentially normal in the isomerized or F form. Loeb and Scheraga (1956) also presented evidence that the anomaly cannot be explained in terms of expansion but proposed that it results from generation of COOH–COOH hydrogen bonds at low pH, the pK_{int} of the acid form or forms thus being abnormally high.

Foster and Clark (1962) attempted to resolve the situation, in the case of human mercaptalbumin, by utilizing electrophoretic mobility to establish experimentally the values of the electrostatic interaction parameter. They attempted to assess the number of titratable carboxylate groups together with the mean pK_{int} of these groups in both N and F forms by utilizing the well-known Scatchard plot of the data. Such plots clearly exhibited an inflection in the pH range of the N–F transition and demonstrated rather clearly that all carboxyl groups in the F form must titrate relatively normally. Unfortunately, the binding curve in the region above the transition was not well enough defined to permit an unambiguous conclusion as to the precise nature of the abnormality existing in the N form. This uncertainty arose because (1) the precise state of protonation of carboxyl and imidazole groups at the isoionic pH was not known and (2) the transformation to the F form was initiated before a sufficient number of carboxyl groups was titrated to accurately define the curve. In spite of these limitations it was suggested that the most probable explanation of the anomaly is that a large number, perhaps nearly one-half, of the carboxylate groups in the N form are so highly masked that they are effectively nontitratable so long as the protein remains in its native conformation.

The present paper reports results of a similar analysis of the titration behavior of bovine plasma albumin. In order to better define the interpretation of the results, two additional experimental devices have been employed. In the first place, infrared difference spectra have been utilized to define with better precision the number of carboxyl groups already protonated at the isoionic pH and to verify that, practically speaking, only carboxyl groups are being titrated throughout the

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region under consideration. This procedure was used earlier by Susi *et al.* (1959) with considerable success to demonstrate the presence of abnormal carboxyl groups in β-lactoglobulin. In addition, an anionic detergent has been employed to stabilize the N form of the protein to lower pH, thereby extending the range of protonation over which this form can be studied. Evidence has been presented earlier (Foster and Aoki, 1958) that addition of up to 10–12 equiv of such detergent anions/mole of protein shifts the N–F equilibrium at a given pH toward the N form. In addition to these experimental innovations, advantage has been taken of a recent detailed analysis of the alkaline limb of the titration curve of this protein (Decker and Foster, 1967) which leads to a more precise estimate of the state of protonation of histidyl residues at the isoionic pH. The results lead, but with greater conviction, to the same conclusions reached earlier in the case of human mercaptalbumin (Foster and Clark, 1962).

Experimental Section

Materials. Bovine plasma albumin was obtained from Armour Pharmaceutical Co. Three different preparations were employed, each containing approximately 5% of dimer as judged by sedimentation velocity in the Spinco Model E ultracentrifuge. The protein was defatted by acid treatment (Williams and Foster, 1959) followed by centrifugation at 10,000 rpm and filtration through a Millipore filter. The clarified acid solution was then deionized by the procedure of Dintzis (1952).

Reagent grade sodium chloride, hydrochloric acid, potassium chloride, and sodium perchlorate were employed. Sodium dodecyl sulfate was recrystallized twice from methanol. Deionized water with a specific resistance of approximately 10^6 ohms was obtained by passage of distilled water through a Barnstead Bantam Model BD-1 demineralizing column. D_2O which was 99.7% pure was purchased from Columbia Chemicals. Ion-exchange resins employed for deionization of protein solutions were Amberlite-120 and Amberlite-400 (obtained from Mallinckrodt Chemical Works).

Preparation of Deuterated BPA,[1] *DCl, and NaOD.* The defatted, deionized, and lyophilized protein (approximately 8 g) was dissolved in approximately 50 ml of D_2O and left in the cold room at pD 8.9 to permit complete exchange of potentially exchangeable hydrogen atoms of the BPA. After 2 days, the solution was neutralized to pD 6.7 and lyophilized. DCl was prepared by the method of Brown and Groot (1942). Benzoyl chloride and D_2O were mixed in molar proportion, gently heated, and the ensuing DCl gas was dissolved in D_2O. NaOD was prepared by dissolving clean sodium in D_2O. For adjusting the pD of the protein solutions, 0.50 N DCl and 1.0 N NaOD were employed.

Potentiometric Titrations. Titrations were conducted at 22.3 ± 0.01° in a double-walled glass cell through which water was pumped at a constant rate from a constant-temperature water bath. An initial volume of 25.0 ml of BPA solution was titrated with 0.10 N HCl using mechanical stirring. A 0.5-in. Teflon-coated magnetic stirring bar was employed. A Beckman research pH meter which can measure with a precision of ±0.001 pH unit was used in conjunction with a Beckman general-purpose glass electrode and calomel reference electrode with frit junction. Saturated KCl was employed in the liquid junction. The pH meter was calibrated against standard buffer (Sargent) of pH 4.01. The same stock solution was employed for each set of titration studies on the three systems to minimize variations due to errors in protein concentration. Concentration of BPA solutions was determined with a Hitachi Perkin-Elmer 139 UV-VIS spectrometer assuming $E^{1\%}_{1\,cm}$ to be 6.67 at 279.0 mμ. A precalibrated micropipet (L. S. Starret Co. No. 263 MR), readable to 1×10^{-4} ml, was used. Acid was standardized against an NaOH solution which in turn was standardized against potassium hydrogen phthalate. Blank corrections were made by the equation

$$V = V_i - v_i\left(\frac{25 + V_i}{25 + v_i}\right)$$

Here V is the corrected volume, V_i is the volume of acid added to the BPA solution, and v_i is the volume of acid added to a blank solution of the same ionic

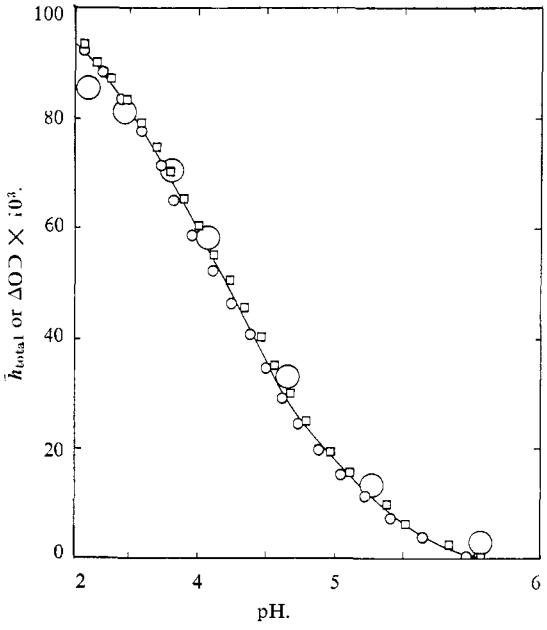

FIGURE 1: Potentiometric titration curves for BPA in 0.10 M NaCl at two protein concentrations, namely, 1.06 (small circles) and 2.53% (squares). For comparison the observed values of ΔOD measured at 1710 cm^{-1}. are shown (large circles) as a function of the "indicated pH" of the D_2O solutions.

[1] Abbreviations used: BPA, bovine plasma albumin; SDS, sodium dodecyl sulfate.

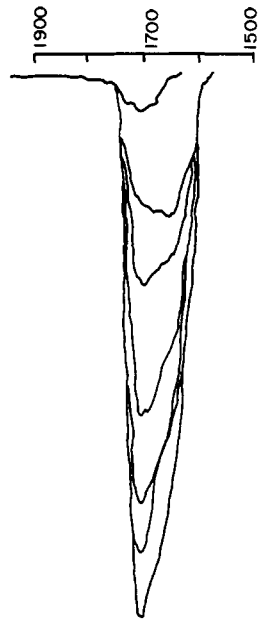

FIGURE 2: Tracings of representative infrared difference spectra over the range 1600–1900 cm^{-1}. Reading from top to bottom the curves correspond to solutions of "indicated pH" (see Discussion) of 5.58, 4.77, 4.16, 3.58, 3.32, 2.97, and 2.34. In all cases the reference solution was at an indicated pH of 8.50.

strength as the protein solution, to bring the pH of the blank solution to the same pH as that of the protein solution. In all studies the molecular weight of BPA was taken equal to 6.6×10^4. All three systems were titrated at two protein concentrations, 1.06 and 2.53%. Figure 1 summarizes the titration data for one system only, namely, BPA in 0.10 M NaCl. In each case approximately 40 data points were collected over the pH range approximately 5.5–2.5. The data employed, as indicated below, were taken from the best smoothed curve through all of the points.

Infrared Titrations. A 10% stock solution of deuterated BPA was prepared by dissolving a weighed sample of deuterated BPA in D_2O. To assure the same concentration in all samples, the following method was chosen. To 3.50 ml of stock solution were added 0.50 ml of 0.5 N KCl (in D_2O) + x ml of DCl + $(1 - x)$ ml of D_2O, where x was varied from 0 to 1 for changing the pD. Matched fixed-thickness cells (0.05 mm, Connecticut Instrument Co.) with CaF_2 windows were used. For the purpose at hand it was absolutely essential that these cells be well matched. The criterion of matching employed was the generation of a flat base line, over the wavelength range 2100–1400 cm^{-1}, in a difference spectrum obtained with the same protein solution in both cells under conditions where the total absorption was approximately 50%. Matching was attained by trial and error substitution of polyethylene spacers and by variation of the compression on the spacers by slight adjustment of the screws.

The reference cell contained a solution of protein at pD 8.9 where all carboxyl groups are assumed to be ionized. Against this reference, samples differing in pD were scanned over the 1900–1600-cm^{-1} region of the infrared spectrum. The sample cell was rinsed three or four times with fresh solution and finally filled with the fresh solution to ensure that the pD of the solution in the cell was the same as in the flask. After scanning of one sample was finished, the cell was thoroughly flushed with H_2O and then dried with dry nitrogen gas.

The following settings were used in the Perkin-Elmer 221 Infrared spectrometer equipped with grating: slit program, 1000 × 2; gain, 5; attenuator speed, 6; scan time, 32 min; suppression, 3; scale, ×5; source current, 0.4 amp.

Tracings of the observed spectra are given in Figure 2. Optical density at the 1710-cm^{-1} absorption maximum was calculated by the peak-height method. In this case, the peak-height method has a distinct advantage over the peak-area method because near the carboxyl peak there would be a negative difference spectrum, due to carboxylate ions, at 1550 cm^{-1}, and the area of the peak could be complicated by this factor. The scanning was done three or four times. Computed optical densities usually did not vary more than ±0.002 from run to run on the same solution. The computed ΔOD values are presented in Figure 1 as a function of the "indicated" solution pH (see below).

Electrophoresis. All electrophoresis experiments were conducted at 22° with a Beckman Model H electrophoresis and diffusion instrument equipped with schlieren cylindrical lens and Rayleigh interference optical systems. Experiments were carried out in a microcell with a capacity of 2.0 ml using a potential gradient of approximately 2.7 v cm^{-1}. Ascending and descending boundaries were generally enantiographic for nearly 4000 sec. After this time, ascending boundaries were generally sharper than descending. Conductivity at the temperature of the electrophoretic experiments was measured by a conductivity bridge which can be read with a precision of $\pm 10^{-8}$ mhos (Industrial Instrument Inc., Cedar Grove, N. J.). Cellulose casing (Visking) was boiled in 2% sodium bicarbonate and thoroughly washed for 3 or 4 days. BPA (15.0 ml) (final concentration of 0.70%) was dialyzed against 1000 ml of the electrolyte for 14–18 hr. The dialysate was stirred with a 1.5-in. magnetic stirring bar. The possibility of concentration dependence of mobility was checked by doing three electrophoretic runs at constant pH with three different concentrations of BPA. It was found that concentration has no appreciable effect on the mobility. An arithmetic average of ascending and descending mobilities was used in all the mobility calculations.

In case of AD_{12} complex, 16.0 ml of 0.5% BPA was dialyzed against 1000 ml of 0.10 M NaCl. After dialyzing for 16 hr the weighed amount of SDS which would give a composition of AD_{12} was dissolved in 4.0 ml of outer solution (0.10 M NaCl) and added to the dialyzed BPA. One hour after addition of SDS

TABLE I: Smoothed Titration and Mobility Data for BPA in 0.10 M Chloride.

pH	$\bar{h}_{total}$	$\mu \times 10^5$	$\epsilon\psi/kT$	$\bar{h}$
5.58	0	−5.3	−0.39	2.5
5.50	0.8	−5.0	−0.36	3.2
5.40	1.9	−4.8	−0.35	4.1
5.20	4.4	−4.0	−0.29	6.5
5.00	7.5	−3.0	−0.22	9.5
4.80	11.4	−2.0	−0.15	13.3
4.60	16.0	−1.0	−0.07	17.8
4.40	21.8	0	0	23.5
4.20	29.3	+1.5	0.11	31.0
4.00	38.7	4.0	0.29	40.4
3.80	47.9	7.4	0.54	49.6
3.60	56.6	9.2	0.67	58.3
3.40	65.7	10.8	0.79	67.4
3.20	75.2	12.0	0.87	76.9
3.00	83.0	13.0	0.95	84.7
2.80	89.1	14.0	1.02	90.8
2.60	94.2	14.6	1.06	95.9

TABLE II: Smoothed Titration and Mobility Data for the AD_m Complex in 0.10 M Chloride.

pH	$\bar{h}_{total}$	$\mu \times 10^5$	$\epsilon\psi/kT$	$\bar{h}$
5.70	0			2.5
5.50	2.2	−5.3	−0.39	4.5
5.30	5.0	−4.7	−0.35	7.1
5.10	8.1	−3.8	−0.28	10.0
4.90	11.9	−2.6	−0.18	13.7
4.70	16.4	−1.7	−0.12	18.2
4.50	21.5	−0.6	−0.05	23.2
4.30	27.3	+0.8	+0.07	28.9
4.10	33.6	2.1	0.16	35.2
3.90	40.7	4.8	0.35	42.4
3.70	48.6	7.2	0.53	50.3
3.50	58.8	10.0	0.74	60.5
3.30	68.9	12.0	0.88	70.6
3.10	77.6	13.6	0.99	79.3
2.90	85.4	15.0	1.08	87.0
2.70	91.5	15.9	1.15	93.1

TABLE III: Smoothed Titration and Mobility Data for BPA in 0.05 M Perchlorate.

pH	$\bar{h}_{total}$	$\mu \times 10^5$	$\epsilon\psi/kT$	$\bar{h}$
5.79	0	−9.5	−0.69	2.5
5.50	3.2	−8.9	−0.65	5.1
5.30	6.1	−8.1	−0.60	7.9
5.10	9.5	−7.0	−0.51	11.2
4.90	13.5	−5.3	−0.39	15.1
4.70	18.0	−3.6	−0.23	19.9
4.50	23.6	−1.7	−0.12	25.0
4.30	29.8	−0.6	0.05	31.2
4.10	37.2	+2.1	0.16	38.6
3.90	44.5	4.8	0.35	46.9
3.70	54.7	7.2	0.53	56.4
3.50	63.3	10.0	0.74	64.7
3.30	71.1	12.0	0.88	72.5
3.10	78.4	13.6	0.99	80.1
2.90	85.0	15.0	1.08	86.9
2.70	91.1	15.9	1.15	93.0

the electrophoretic experiments were started.

To determine the exact location of peaks and distance traveled, the patterns were projected with a Nikon optical comparator (Model 6) equipped with a micrometer stage readable to ±0.002 mm. Usually the graphs relating time and distance were linear.

Results and Discussion

For the calculations that follow values of the total number of protons bound ($\bar{h}_{total}$) and of the electrophoretic mobility (μ) were read for discrete pH values from the titration and pH–mobility plots. These smoothed data are summarized in Table I–III for the three systems investigated. Table I is for BPA in 0.1 M chloride, Table II for the AD_m complex in 0.1 M chloride, and Table III for BPA in presence of 0.05 M perchlorate. In these tables the total uncorrected values for $\bar{h}$ and μ are given in the second and third columns, the derived value of $\epsilon\psi/kT$ in the fourth column, and the corrected value of $\bar{h}$, that is the number of hydrogen ions presumed to be combined with carboxyl groups, in the fifth column. Some explanation is required as to the assumptions involved in the computation of the latter two quantities.

The values for the electrostatic interaction parameter $\epsilon\psi/kT$ (ϵ is the unit charge on the proton, ψ the surface potential on the protein, and k is Boltzmann's constant) were derived from the well-known Henry equation as was done previously (Foster and Clark, 1962; Decker and Foster, 1967), namely, assuming

$$\psi \cong \zeta = \frac{6\pi\eta}{Df(\kappa a)} \qquad (1)$$

In this equation D is the dielectric constant and η the viscosity (in poise) of the solvent. The parameter $f(\kappa a)$ is a slowly varying function of the product of the Debye–Hückel parameter and the radius of the protein, a. As has been discussed previously, this function is not highly sensitive to the assumed value of a. In the present calculations a was assumed to be 30 A for both BPA and the AD_m complex and the corresponding constant relating $\epsilon\psi/kT$ to μ was taken as 7.29×10^3 v sec cm^{-2}.

To compute the corrected value of $\bar{h}$, it is obviously

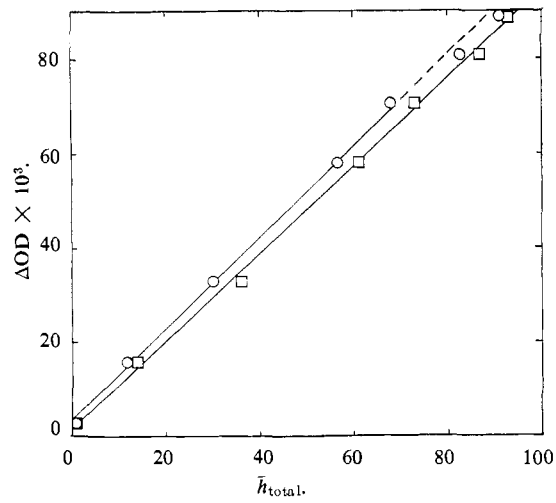

FIGURE 3: Difference in optical density at 1710 cm^{-1} plotted as a function of the total number of hydrogen ions bound, measured from the isoionic pH. The upper curve (circles) corresponds to the assumption that a given degree of protonation in D_2O is the same as that in water at the same indicated value of pH; the lower curve (squares) is the case in which the equivalent pH in water is assumed to be equal to the indicated pH in D_2O minus 0.10.

necessary to add to $\bar{h}_{total}$ the number of carboxyl groups protonated at the isoionic pH and subtract the number of histidyl residues titrated from the isoionic pH to the pH in question. For the histidyl correction it was assumed that the 17 imidazole groups are divided into two classes, 10 of pK = 6.5 and 7 of pK = 7.5, as concluded in another study (Decker and Foster, 1967). These parameters lead to an assumed value of 0.8 unprotonated imidazole residues at the mean isoionic pH. In line with the results of infrared titration, discussed later, it was assumed that 2.5 carboxyl groups are protonated at the isoionic pH in all cases. Obviously, since infrared titrations were carried out only for the one system BPA in 0.1 M chloride, this value is not known with certainty for the other cases. Comparative titration studies for BPA and AD_m in 0.1 M chloride carried out in the alkaline pH range suggest that the titration behavior of the protein in the two cases must be almost exactly the same for the two systems in the region of histidyl and carboxyl titrations down to the isoionic pH (Decker and Foster, 1967). It, therefore, seemed reasonable to make the corrections in the same manner for AD_m as for BPA. The justifiability for invoking the same assumptions in the case of BPA in 0.05 M perchlorate is less certain.

As indicated in the introduction, Foster and Clark (1962) assumed, without proof, that only carboxyl groups are titrated in the pH region below the isoionic pH. This supposition deserves further support in view of known cases of extreme abnormality of prototropic groups in some proteins. Perhaps the case which is most relevant is that of hemoglobin where there is an unmasking of basic groups associated with a reversible conformational change occurring in nearly the same pH range as the N–F transformation. In that case, Steinhardt and co-workers (Steinhardt and Beychok, 1964) have presented strong evidence that the groups involved are imidazoles, approximately 20 in number, which are so strongly masked by the native protein structure that they protonate in a pH range commonly associated with the titration of carboxyl groups alone, and then only as a result of, or in concert with, the disruption of the native protein structure. Such a possibility in plasma albumins should not be dismissed on an *a priori* basis.

To ascertain whether any groups other than carboxyl make an important contribution to the buffering capacity of BPA in the region acid to the isoionic pH, difference infrared spectra were measured in D_2O as a function of pD of the solvent as outlined under Experimental Section. Ehrlich and Sutherland (1954) first demonstrated a small shoulder at 5.87 μ (1710 cm^{-1}) on the main peptide carbonyl absorption which they attributed to unionized carboxyl groups. Susi *et al.* (1959) made use of this band to demonstrate the presence in β-lactoglobulin of two carboxyl groups of abnormally high pK. Figure 2 shows tracings of difference spectra obtained, Figure 1 presents ΔOD as a function of the "indicated pH," and Figure 3 summarizes these results in the form of plots of the difference in optical density, at the band in question, *vs.* the total number of hydrogen ions bound as determined by potentiometric titration. In all cases, the reference solution was at pD 8.9, at which pD it is assumed that all carboxyl groups are ionized.

In deriving Figure 3 a fundamental problem arises due to the fact that the potentiometric titration curves were conducted in H_2O rather than in D_2O. The "pH" values of the D_2O solutions employed in the infrared experiments were read with the same (aqueous) electrode system employed in the titration experiments in water. Several workers (Mikkelsen and Nielsen, 1960; Lumry *et al.*, 1951; Gary *et al.*, 1964) have concluded that a proper pD scale in D_2O is established by adding 0.40–0.44 unit to the indicated pH when measured in this manner. The pD values mentioned previously were obtained by adding 0.4 unit to the indicated pH value. On the other hand, it is also known that the effective pK values of various weak acids in D_2O are elevated by about this same amount or slightly more when compared with the values in H_2O. For example, Rule and La Mer (1938) found the pK values of acetic acid and chloroacetic acid in D_2O to be, respectively, 0.52 and 0.44 unit higher than in H_2O. For guanosine, Bunville and Schwalbe (1966) find a difference of 0.42 unit. Since the objective of these experiments is to compare the results in H_2O and D_2O at the same degree of protonation it would appear that results of the infrared experiments should be compared with $\bar{h}$ measured in water at the same indicated pH or possibly at the pH indicated in D_2O minus 0.1 unit. Some slight

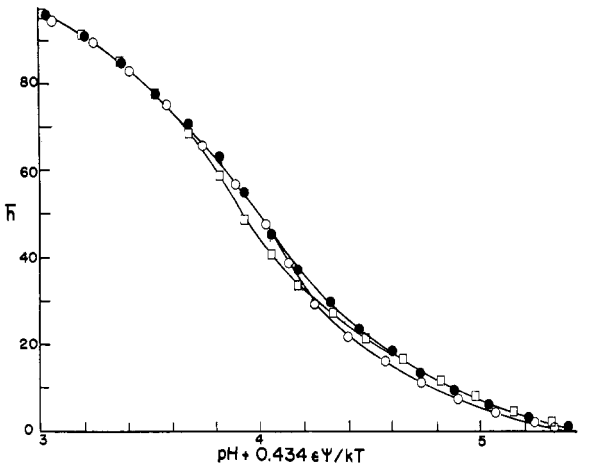

FIGURE 4: Hydrogen ion titration curves with electrostatic corrections applied. The three systems studied are BPA in 0.10 M NaCl (open circles), BPA in 0.05 M perchlorate (solid circles), and AD_{12} complex in 0.10 M NaCl (squares).

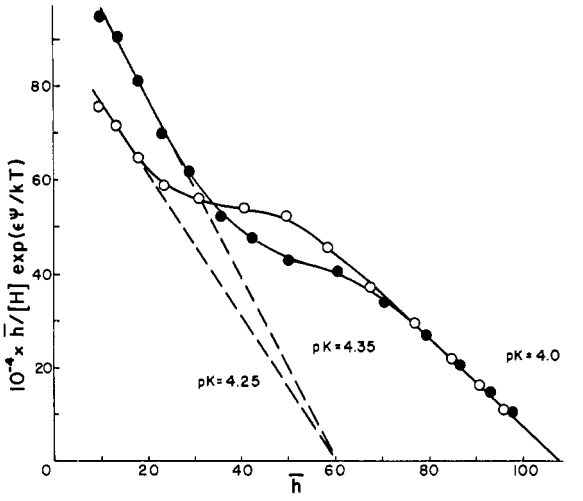

FIGURE 5: Scatchard plots with electrostatic corrections included for BPA (open circles) and for AD_{12} (filled circles), in both cases in 0.10 M chloride.

support for the latter assumption was obtained by measuring the indicated pH of a deionized BPA preparation in H_2O and in D_2O under otherwise similar conditions. The resulting values were: pH (in H_2O) 5.41, indicated pH (in D_2O) 5.52.

In Figure 3 data are plotted according to both assumptions. In either case, ΔOD is seen to be a virtually linear function of $\bar{h}_{total}$. The simpler assumption gives somewhat better fit of the data to the linear function at degrees of protonation less than 70 and a systematic deviation at higher degrees of protonation. This deviation might be due to inadequacies of the binding data at pH values below 3.0, owing to the increasing importance of the blank correction at extreme pH values. As will be seen below, the potentiometric data lead to an extrapolated binding limit (n) of about 108, somewhat greater than the expected value of 99–100. If one makes the simple assumption, corresponding to the upper line in Figure 3, and corrects the potentiometric binding data below pH 3.0 accordingly, the limit obtained from a Scatchard plot is indeed 100 ± 1. Therefore, this upper curve is tentatively considered to be the more correct although the conclusions would not be altered materially if the other curve were used. In either case it must be concluded that only carboxyl groups are making a significant contribution to $\bar{h}_{total}$. The number of protonated carboxyl groups at the isoionic pH, obtained from the ratio of the intercept to the slope, is 3.0 and 2.2 for the two cases considered.

Figure 4 shows the titration data on the three systems based on the smoothed data given in Table I and incorporating the electrostatic corrections and the corrections of $\bar{h}$ computed as outlined earlier. Clearly the major differences between the three systems are in the region of the N–F transition, *i.e.*, near pH 4.0, as was anticipated. In particular, protonation of the AD_m complex is depressed in this region as compared to BPA in either chloride or perchlorate. This is in accord with the fact that the detergent exerts a stabilizing influence on the native protein conformation.

In their analysis of the titration behavior of human mercaptalbumin Foster and Clark (1962) utilized the well-known Scatchard plot with the electrostatic correction incorporated according to the equation

$$\frac{\bar{h}}{[H^+]}\exp(\xi\psi/kT) = 1/K_{int}(n - \bar{h}) \qquad (2)$$

where n is the number of sites of intrinsic dissociation constant K_{int}. Plots according to this equation are shown in Figure 5 for BPA in 0.1 M chloride and for the AD_m complex in the same medium. As in the earlier case (Foster and Clark, 1962), these curves show a definite inflection associated with the N–F transition. The portion of the titration curve corresponding to the N form is not very well defined in the case of BPA, as was true in the earlier work, because of the onset of the N–F transition which takes place at about $\bar{h} = 20$. Unfortunately, data corresponding to $\bar{h} < 10$ are of almost no value in such a plot since $\bar{h}$ enters as a multiplier in the abscissa and since there is an estimated uncertainty of at least ±0.5 in these values. As expected, however, addition of detergent anions suppresses the N–F transformation so that the initial, virtually linear, portion of the plot for AD_{12} is extended to approximately $\bar{h} = 30$. In this case the extrapolation can be made with enhanced confidence to about the same n value of approximately 60 as is obtained with BPA.

Clearly, it cannot be stated with certainty that the actual plot, if the N–F transition could be completely prevented, would be linear. Indeed, it is entirely possible that the plot would curve at higher $\bar{h}$ and approach $n \simeq 100$. This would only imply that the remaining carboxylate groups have a finite basicity and would

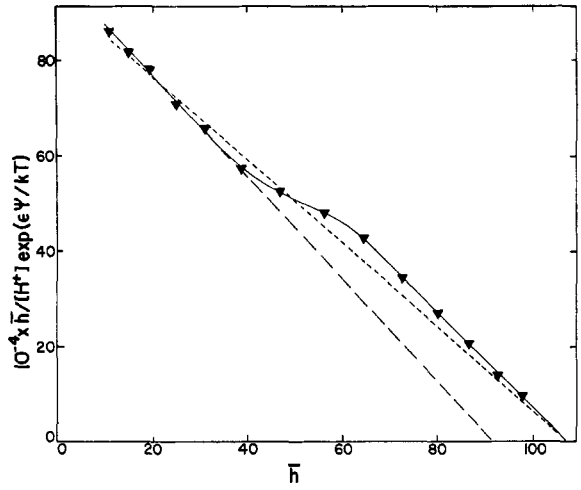

FIGURE 6: Scatchard plot with electrostatic corrections for BPA in 0.050 M perchlorate. See text for a discussion of the significance of the various curves drawn.

not alter the conclusion that only about 60 or so of the carboxylate groups in the native protein have a reasonably normal tendency to protonate and that the others must be highly masked or otherwise stabilized in the anionic state.

The slopes of the initial linear portions of the curves for BPA and for the AD_m complex correspond to pK_{int} values of, respectively, 4.25 and 4.35. These are to be compared with the value 4.3 estimated for the exposed carboxyl groups in the case of human mercaptalbumin (Foster and Clark, 1962). The difference between the values for BPA and AD_m appears to be significant. This may imply that the added detergent ions do not make an electrostatic contribution to the local environment of the carboxylate groups in full proportion to their effect on the potential (electrophoretic mobility) of the protein as a whole. In other words, it is very probable that this is a consequence of the employment of a smeared-charge model which in fact does not apply rigorously.

At pH values below the range of the N–F transition, the curves in the two cases obviously approach coincidence and can be extrapolated to yield a common n value. Unfortunately, this value of $n = 108$ appears to be significantly higher than the value of 98–100 expected on the basis of the best available amino acid composition data, that of Spahr and Edsall (1964). The latter expected value is somewhat uncertain because of the difficulty of determining precisely the amide ammonia content; nevertheless, it seems unlikely that the figure could be off by as much as ten residues per molecule. As suggested earlier, the discrepancy may arise from inaccuracies in the correction, at low pH, for the titration blank of the solvent. It seems surprising that this error could be so large. On the other hand, it is interesting that a Scatchard plot prepared by correcting the binding data at pH below 3.0 based on the linear relation between infrared absorption and $\bar{h}$ discussed above, yielded a limiting value (n) of 100. In that case, also, the limiting slope was somewhat higher, corresponding to $pK_{int} = 4.1$ rather than 4.0. In either case, the limiting slope (for the F form) appears to be significantly less than the initial slope (for the N form).

This later result is not surprising. In a molecule such as BPA, containing 100 carboxyl groups, it could not be anticipated that all would have the same pK_{int}, even in the F form. Further evidence for some inherent heterogeneity of the carboxylate groups in disorganized BPA was obtained by conducting titration studies in the presence of 4.0 M guanidine hydrochloride. In that case, electrophoretic experiments were not performed so electrostatic corrections could not be applied and the results are not presented. However, it might be presumed that electrostatic effects would be minor in the presence of so concentrated an electrolyte and particularly since the protein must be drastically expanded. The Scatchard plot in that case showed no evidence of any inflection but consisted of a smooth curve of decreasing slope (concave upwards) typical of binding to heterogeneous sites. The extrapolated limit (n) was again 108.

To summarize the above conclusions from Figure 5 it is suggested that the N form of BPA (and also AD_m) possesses approximately 60 "normal" carboxylate groups which protonate freely, the conjugate acid groups having pK_{int} values of approximately 4.3 ± 0.05. The remaining carboxylate groups are highly masked or for other reasons resist protonation, the corresponding acid groups having pK_{int} much less than 4.0 (and possible $-\infty$ if indeed they are fully masked). Conversion to the F form exposes all carboxylate groups.

Results of the titration of BPA in presence of 0.05 M perchlorate are similarly summarized in the form of a Scatchard plot in Figure 6. Evidence has been presented earlier (Aoki and Foster, 1957) that in 0.02 M perchlorate the N–F transition takes place in two pH-dependent steps. The present results indicate that in 0.05 M perchlorate many or most of the masked carboxylate groups are essentially normalized, even at isoionic pH. It is in fact debatable whether the inflection shown in Figure 6 is real. A straight line can be drawn through all of the points (dotted line) in such a way that the deviations are scarcely outside the experimental error. However, it is to be noted that the deviations are then systematic. Furthermore, the slope of that straight line corresponds to an anomalously low pK_{int} which would be difficult to understand. It seems probable that the solid line is more realistic and that a cooperative transition also takes place in this case. However, the protein prior to this transition must have most (*ca.* 90%) of its masked carboxylate groups exposed so that they protonate with a reasonably normal pK_{int} of *ca.* 4.0. The extrapolated limit is again 108 as in the other cases.

The results thus support the earlier conclusion of Foster and Clark (1962). As they pointed out, it is difficult to visualize such strong masking of so many

anionic groups in a protein without also assuming participation of cationic partners. They suggested the possible participation of amino groups which, since they are known to titrate reasonably normally (Tanford *et al.*, 1955; Decker and Foster, 1967), might be normalized in the transition which takes place between pH 7 and 9 (Leonard *et al.*, 1963). It is of interest that recent studies by Goldfarb (1966) of the reaction of trinitrobenzenesulfonic acid with human serum albumin lead to the conclusion that only about 19 of the ϵ-amino groups react. Since this protein contains a total of 62 lysine residues (Spahr and Edsall, 1964) the clear implication is that over 40 of these groups must be masked, in excellent accord with the number of masked carboxylate groups inferred in the present study. Barré and van Huot (1965) have presented some evidence for the masking of the guanidinium groups of arginine in human serum albumin but the number of such groups in either human or bovine albumins (22–24) is scarcely great enough to neutralize the large number of carboxylate groups which appear to be masked.

There is considerable evidence to suggest that the N–F transition consists essentially of the separation of two globular folded units of the peptide chain which, in the native protein, must be closely associated (Foster, 1960). Recently evidence has been presented by Laskowski (1966) that the interface between the globular units behaves as a crevice which is permeable to ethylene glycol, methanol, and dimethyl sulfoxide but not to polyethylene glycol, glycerol, or sucrose. As expected, this crevice is destroyed in the N–F transformation (Laskowski, 1966). These results are of great significance, not only in lending further support to the basic nature of the N–F transition, but in that they provide an estimate of the width of the mouth of the crevice and show that it must be essentially hydrophilic in character. It seems reasonable to postulate that approximately 40 of the carboxylate groups of native BPA reside in this narrow, hydrophilic crevice and are paired with a similar number of cationic partners on the opposite surface. The fact that the N–F transition is favored, at a given pH, by increasing ionic strength is in accord with this model, which would attribute much of the stabilization of the N form to electrostatic attraction between the paired anionic and cationic groups. Most importantly, for this model the cooperative normalization of the interfacial carboxylate groups, shown in the present paper, would be an expected consequence of the N–F transition.

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