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Hydrogen Ion Equilibria of Bovine Serum Albumin[1]

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Hydrogen ion titration curves of de-ionized bovine serum albumin have been obtained at four ionic strengths and two temperatures. They have been analyzed by equations treating the electrostatic effect as an empirical factor. The ionizing groups found present and their intrinsic pK's at 25° are: 1 α-carboxyl (3.75 assumed), 99 side chain carboxyl (3.95), 16 imidazole (6.9), 1 α-amino (7.75 assumed), 57 ϵ-amino (9.8), 19 phenolic (10.35) and 22 guanidyl (>12). The pK values found for carboxyl, amino and phenolic groups are abnormal: they suggest that these groups all participate in some kind of internal bonding, such that the carboxylate ion is favored over the un-ionized COOH group, the neutral NH_2 group over the NH_3^+ ion, and the un-ionized phenolic groups over the phenolate ion. The heats and entropies of ionization are incompatible with carboxylate-phenolic hydrogen bonds previously suggested. Empirical values of the electrostatic interaction factor w are independent of pH between pH 4.3 and 10.5, but fall off sharply outside this region. Thus serum albumin appears to maintain constant shape and size between pH 4.3 and 10.5, but it must expand outside this region. This conclusion is in agreement with data obtained by other methods.

This paper presents an experimental study of the dissociation of hydrogen ions from bovine serum albumin (BSA), and the subsequent analysis of the results in terms of (1) the numbers of the various types of dissociable groups present per molecule, (2) the *intrinsic* dissociation constants of these groups, *i.e.*, the dissociation constants they would have in the absence of electrostatic effects, and (3) the electrostatic interaction factor which causes the effective dissociation constants to vary continuously with charge, *i.e.*, with pH. Similar studies of this kind have been made by Cannan and coworkers, for the proteins ovalbumin[3a] and β-lactoglobulin,[3b] and by one of the present authors and associates for human serum albumin (HSA),[4] insulin[5] and lysozyme.[6]

This type of investigation is intended primarily to provide evidence regarding the state of the acidic and basic side-chain groups in the protein, and, through interpretation of the electrostatic interaction factor, to give some idea of the size and shape of the protein molecules in solution. In the case of BSA the latter becomes a major factor. Whereas the electrostatic interaction factors in ovalbumin[3a] and lysozyme[6] appear to be constants independent of pH, and the same is true at least on the acid side, for β-lactoglobulin,[3b] the electrostatic factor for BSA varies markedly with pH. This leads to the conclusion, discussed in detail in the following paper, that BSA (and HSA) undergoes reversible expansion below pH 4.3 and above pH 10.5.

A minor objective of the present paper is a comparison between hydrogen ion dissociation in BSA and in HSA. It will be shown that differences between the results of this paper and those previously obtained for HSA[4] can be accounted for largely by the fact that an erroneous value for the number of terminal amino groups was used in the HSA study, and by the attempt made in the HSA study to strain the data into fitting equations allowing for no variation in electrostatic interaction. Real differences between HSA and BSA appear to be quite small.

(1) Presented at the 128th National Meeting of the American Chemical Society, Minneapolis, Minnesota, September, 1955.

(2) Abstracted in part from the M.S. Thesis of Sigurd A. Swanson, State University of Iowa, June, 1955.

(3) (a) R. K. Cannan, A. C. Kibrick and A. H. Palmer, *Ann. N. Y. Acad. Sci.*, **41**, 243 (1941), (b) R. K. Cannan, A. H. Palmer and A. C. Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

(4) C. Tanford, THIS JOURNAL, **72**, 441 (1950).

(5) C. Tanford and J. Epstein, *ibid.*, **76**, 2163 (1954).

(6) C. Tanford and M. L. Wagner, *ibid.*, **76**, 3331 (1954).

Experimental

Bovine Serum Albumin.—The results reported here were obtained over a period of three years on four different lots of Armour's crystalline BSA. In chronological order, the lot numbers were 128–175, R 370295 A, M 66909 and N 67009. Concentrated stock solutions of the protein were de-ionized and made isoionic by being passed through an ion exchange column of the type designed by Dintzis.[7] The concentration of each stock solution was determined by drying to constant weight at 105–107°.

Small differences between the four lots used were observed. They are the cause of the deviations between neighboring points in the neutral pH region, seen in Fig. 1. They are of such magnitude as to suggest that different preparations of BSA may differ from each other by not more than one in the number of carboxyl groups per molecule. (The three experimental points at ionic strength 0.15 falling below the curve between pH 9 and 9.5 come from the earliest series of measurements. This series was confined largely to the acid and neutral portion of the curve, and less care was taken in keeping out CO_2 than in the remainder of the measurements. The three points deviating from the remainder thus do not necessarily represent a difference in lot 128–175.)

All calculations are reported in terms of a molecular weight of 65,000 for BSA.[8]

Solutions for measurement were made up by weight from the stock solutions of BSA, and from standard solutions of HCl, CO_2-free KOH, KCl, and conductivity water. Each experimental point in Fig. 1 represents a separate solution. The protein concentration varied between 0.6 and 4.2%.

Measurement of pH.—The apparatus and procedure used for pH measurement have been described elsewhere.[9] Beckman pH meters, Models G or G-S, were used with external general purpose glass electrodes. No difference between different meters or electrodes could be detected. Two determinations near the acid end-point were made with Clark-type hydrogen electrodes, similar to those used with HSA.[4] The results agreed well with those obtained with glass electrodes.

The calculation of moles of H^+ bound to or dissociated from one mole of isoionic BSA has been described in the reference cited above.[9]

Results

The results of 25° at four different ionic strengths are shown in Fig. 1. Detailed numerical data are contained in the thesis of Swanson.[2] The data are reported in terms of the number (r) of hydrogen ions dissociated from the acid end point of the curve, rather than the direct experimental number (h) of hydrogen ions added to or dissociated from

(7) H. M. Dintzis, Ph.D. Thesis, Harvard University, 1952.

(8) J. M. Creeth, *Biochem. J.*, **51**, 10 (1952).

(9) C. Tanford in T. Shedlovsky, ed., "Electrochemistry in Biology and Medicine," John Wiley and Sons, Inc., New York, N. Y., 1955.

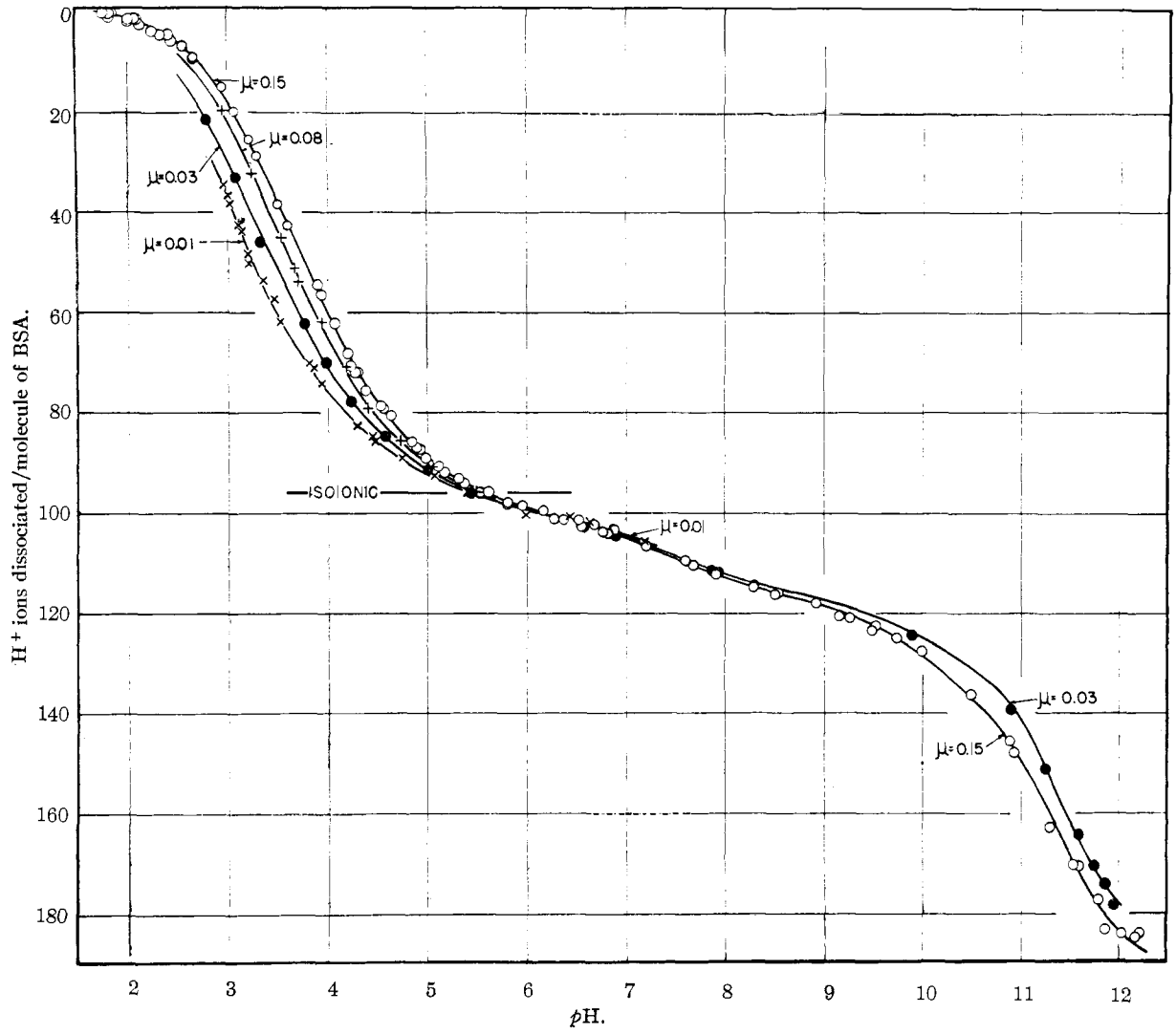

Fig. 1.—Hydrogen ion titration data at 25° at four ionic strengths. The curves drawn are calculated, using equation 1 and the parameters of Tables I and III, and of Fig. 6.

the isoionic point. The two are related through the maximum value of h at the acid end-point (96 ions/molecule), *i.e.*, $h = 96 - r$, negative values of h signifying hydrogen ions dissociated from the isoionic protein.

Reversibility.—Figure 2 shows data (all at ionic strength 0.15) designed to test the reversibility of the titration curve. Protein solutions were made up with sufficient acid or base to bring the *p*H to 2.5, 10.5 or 12. After standing about 10 minutes, base or acid was added to return the final *p*H closer to (and in two solutions beyond) the isoionic point. The value of r for the final solution was calculated from the *net* acid or base added and the final *p*H. The points fall almost, but not quite, on the same curve as the points of Fig. 1. It must be realized, however, that the *net* acid or base added to these solutions is the difference between two relatively large numbers, and the deviations observed for solutions reversed from *p*H 2.5 and 10.5 appear to be essentially within experimental error. Somewhat larger deviations were found for solutions reversed from *p*H 12, and it seems probable that some irreversible change occurs at this highest *p*H.

In any event, the deviations are sufficiently small to permit thermodynamic analysis of the data. The calculated titration curve can be made to fit the reversed points by changes in pK's smaller than the probable uncertainty in these quantities.

That the titration of the phenolic groups of BSA is reversible up to *p*H 12 was shown in a previous paper.[10]

Heats of Ionization.—A titration curve was also obtained at 5°. It was confined to ionic strength 0.15, and to the range of *p*H 4.5 to 10. From this curve the apparent heat of ionization, $\Delta H = 2.303R(\partial\ p\mathrm{H}/\partial(1/T))_r$, at given values of r, was computed. A plot of these data is shown in Fig. 3. It shows the typical low heat of ionization in the region where carboxyl groups ionize, followed by a ΔH of about 6.5 kcal./mole in the imidazole group region, and values of about 12 kcal./mole in the amino group region.

In a similar study made with BSA which had not

(10) C. Tanford and G. L. Roberts, Jr., THIS JOURNAL, **74**, 2509 (1952).

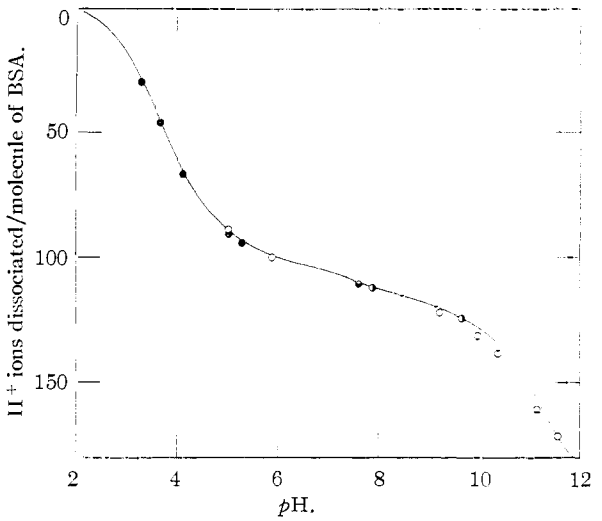

Fig. 2.—Reversibility of the titration curve of BSA at μ = 0.15 and 25°: ●, reversed from *p*H 2.5; ◑, reversed from *p*H 10.5; ○, reversed from *p*H 12. The curve drawn is the direct titration curve at μ = 0.15 from Fig. 1.

been purified by ion exchange, much the same heats of ionization were obtained. In this study the heat of ionization was computed down to *p*H 2: a gradual decrease from 2 kcal./mole in the isoionic region to zero near *p*H 3 was found. Below *p*H 3, the apparent heat of ionization was slightly negative.

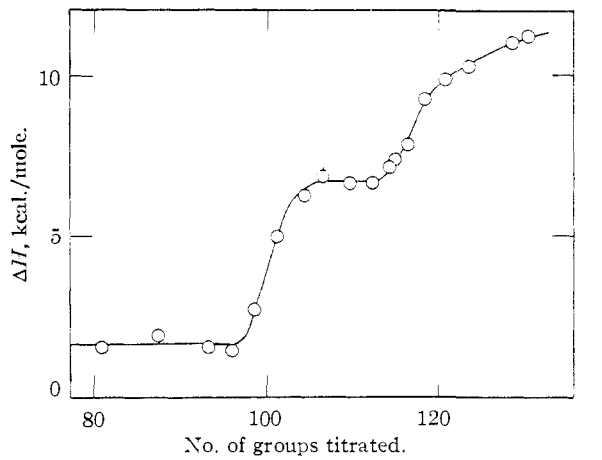

Fig. 3.—The apparent heat of ionization of BSA, as a function of the number of groups titrated.

Calculated Results

The numbers of the various types of groups were calculated by the methods used previously.[5,6,9] The maximum acid binding gives the sum of imidazole, amino and guanidine groups. The value of r at the inflection point of Fig. 1 near *p*H 6 can be used, with the aid of calculations quite insensitive to the numbers and pK's of other groups, to compute the number of carboxyl groups. A check on this figure can be obtained from the corresponding inflection point in Fig. 3. In a similar way the point of inflection at *p*H 8.6 in Fig. 1 and the corresponding inflection point in Fig. 3 are a measure of the number of imidazole groups. The number of phenolic groups has been previously discussed.[10] The sum of the phenolic and ϵ-amino groups and, hence, by difference, the number of ϵ-amino groups, is obtained by extrapolation of the most alkaline portion of Fig. 1 to a reasonable horizontal termination. The guanidine groups are not titrated below *p*H 12 (if they were the alkaline portion of Fig. 1 would not appear to level off as it does). The number of such groups can be computed, however, as the difference between maximum acid binding and the sum of the imidazole and amino groups. All the numbers so calculated are shown, together with comparable figures from amino acid analyses, in Table I. The uncertainty is about one group in each case.

TABLE I

TITRATABLE GROUPS PER 65,000 GRAMS

	Found[a]	Amino acid anal.
α-Carboxyl	(1)[b]	1[c]
β,γ-Carboxyl	99	$\geqslant$89.9[d]
Imidazole	16	16.8[d]
α-Amino	(1)[b]	1[e,f,h]
ϵ-Amino	57	57.0[d]
Phenolic	19	18.1[d]
Guanidine	22	22.0[d]
Sulfhydryl (free)	(0)[b]	0.7[g]

[a] Probable uncertainty about one group in each case. [b] The number of terminal carboxyl and amino groups and the number of free sulfhydryl groups is too small to be distinguished experimentally from other types of groups, and the values given were assumed. Free SH groups were assumed absent because they have about the same pK as the ϵ-amino groups, and because their number (by analysis) is less than the uncertainty in the number of amino groups. Drs. R. and R. E. Benesch have informed us that BSA samples in use for periods of several months, as ours were, are in any event likely to have a reduced sulfhydryl content. [c] J. T. Edward and S. Nielsen, *Chemistry and Industry*, 197 (1953). [d] W. H. Stein and S. Moore, *J. Biol. Chem.*, 178, 79 (1949). [e] H. Van Vunakis and E. Brand, Abstracts, 119th National Meeting of the American Chemical Society, Boston, 1951. [f] B. Keil, V. Tomasek and J. Sedlackova, *Chem. Listy*, 46, 457 (1952). [g] R. Benesch and R. E. Benesch, *Arch. Biochem.*, 19, 35 (1948). [h] E. O. P. Thompson, *J. Biol. Chem.*, 208, 565 (1954).

The agreement of the titration data with analysis is seen to be good, except that more carboxyl groups are titrated than the amino acid analysis would lead one to expect. It is difficult to see how the value obtained by us can be in error by more than one group. The accuracy of the amino acid analysis the present authors are unable to assess. It should be noted, however, that the number of free carboxyl groups given by amino acid analysis is in any event a *minimum* number, owing to the fact that the analysis for amide groups gives a maximum figure.[11]

The method of mathematical analysis of the titration curves has also been described previously.[9] It is assumed tentatively (and in the present case no reason appeared to alter this assumption) that all of the dissociable groups of any one type are intrinsically identical, *i.e.*, that they all have the same intrinsic dissociation constant, $(K_{\text{int}})_i$. If

(11) It should also be noted that one recent microbiological assay[12] gives 99 glutamyl residues per mole compared to 73 given by the chromatographic analysis cited in Table I.

(12) L. E. McClure, L. Schieler and M. S. Dunn, THIS JOURNAL, **75**, 1980 (1953).

there are n_i such groups per BSA molecule, the number, r_i, dissociated at any pH, is then given by the equation

$$p\mathrm{H} - \log \frac{x_i}{1 - x_i} = p\mathrm{H} - \log \frac{r_i}{n_i - r_i} = (pK_{int})_i - 0.868wZ \quad (1)$$

where $x_i = r_i/n_i$ is the fraction dissociated. The total number, r, of hydrogen ions dissociated, as given by the ordinate of Fig. 1, is then the sum of the r_i for all possible groups.

The factor $0.868wZ$ appearing in equation 1 takes into account the electrostatic interaction between the net charge Z of the protein at any pH and the dissociating hydrogen ion, such that $2kTwZ$ is the electrostatic work required to remove a proton from the protein molecule to infinity. In the absence of any knowledge concerning the distribution of charged groups in the molecule, the value of this factor is assumed independent of the type of group from which the proton is dissociated. The factor w is treated as an empirical parameter, which is not necessarily constant over the entire pH range.

The charge Z is primarily due to bound or dissociated protons, but is also influenced by bound salt ions. Scatchard and co-workers[13,14] have shown that in NaCl solutions, sodium ion is not bound at all by isoionic serum albumins. We have assumed that the same is true of potassium ion in KCl solutions, and, also, that no appreciable binding of the ion occurs up to pH 11. Scatchard and co-workers have shown, however, that chloride ion is relatively strongly bound. We have used their data to estimate as well as possible the chloride-binding at any pH and ionic strength. Our estimates reach well outside the region studied by Scatchard and coworkers. The entire chloride-binding curve at ionic strength 0.15, for example, is based on a single measurement at pH 3.2 (confirmed, however, by two other workers)[15,16] and on a calculated value for $\bar{\nu}_{Cl}$ (chloride ions bound per BSA molecule) at the isoionic point, based on constants obtained by Coleman[14] from studies at lower ionic strengths. The curve through these two points was drawn so as to parallel the curves at lower ionic strengths. From these estimates Z was calculated as $96 - r - \bar{\nu}_{Cl}$.

Most regions of the titration curves of Fig. 1 represent regions where more than one kind of group is being titrated, so that the change in r with pH does not represent the change in any individual r_i. In the region of pH 2 to about 5.5, however, virtually all of the groups titrated are carboxyl groups, and after a relatively unimportant correction for imidazole dissociation at the alkaline end of the pH region, may be used as a measure of r_i for carboxyl groups only.[17] Equation 1 may therefore be applied directly, and the plots of Fig. 4 obtained.

Spectroscopic data obtained previously[10] are a direct measure of r_i values for phenolic groups, and lead to the logarithmic plot for these groups shown in Fig. 5. Since the region of pH 9.5 to 12 represents essentially the titration of phenolic and ϵ-amino groups only, and r_i values for the former are known from the independent spectroscopic data, one may obtain r_i for the ϵ-amino groups by difference, and obtain a similar plot, also shown in Fig. 5.[18] The plots of Fig. 5 are at one ionic strength only, since the spectroscopic data are available only at $\mu = 0.15$.

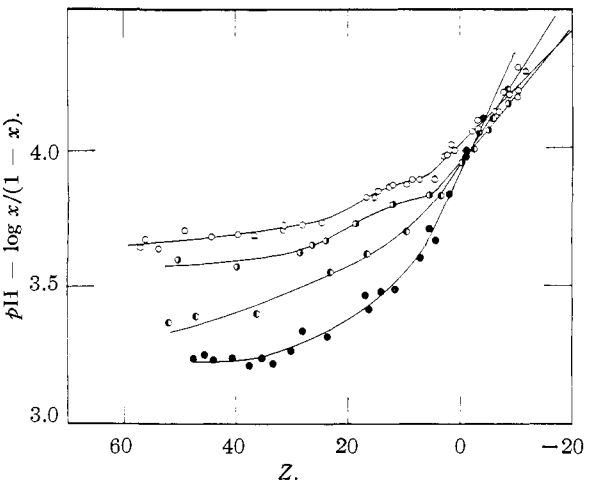

Fig. 4.—Logarithmic plot for the dissociation of carboxyl groups, at: ionic strength 0.01 (●); 0.03 (◐); 0.08 (◑); 0.15 (○).

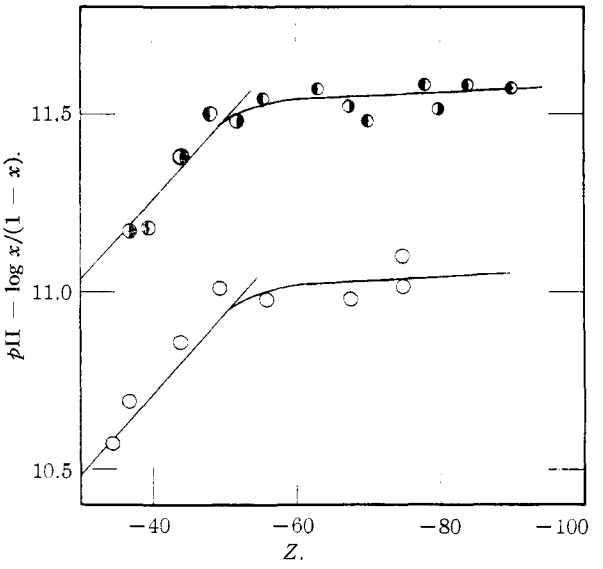

Fig. 5.—Logarithmic plots for the dissociation of the phenolic and ϵ-amino groups. The upper curve represents phenolic groups, using the data of Tanford and Roberts,[10] using buffered solutions (◐) and points taken from a smooth curve through unbuffered solutions (◑). The lower curve represents the ϵ-amino groups, using data from the present paper.

The curves of Figs. 4 and 5 are not straight lines. For Z less than about $+5$, however, the curves of

(13) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., THIS JOURNAL, **72**, 535 (1950).

(14) J. S. Coleman, Ph.D. Thesis, Massachusetts Institute of Technology, 1953.

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

(16) C. W. Carr, *Arch. Biochem. Biophys.*, **40**, 286 (1952).

(17) The method of correcting for imidazole groups is described in our paper on insulin (ref. 5).

(18) Allowance for the single α-amino group is made by assigning it an assumed pK_{int} of 7.75.

Fig. 4 become relatively steep and approximately linear. The same is true for the curves of Fig. 5 with Z more positive than about -55. Accordingly, a least-squares treatment has been applied to these portions of these curves, and to a similar (linear) curve at $\mu = 0.15$ for the imidazole groups. The slopes calculated, together with their standard deviations are shown in Table II.

TABLE II

"LEAST-SQUARES" SLOPES OF PLOTS OF LOG $x_1/(1 - x_1) - p$H *versus* Z

Type of group and ionic strength	Slope and stand. dev.	Calcd. slope[a]
Carboxyl, $\mu = 0.01$	-0.047 ± 0.0033	0.045
$\mu = .03$	$- .031 \pm .001$	.034
$\mu = .08$	$- .024 \pm .001$	.026
$\mu = .15$	$- .020 \pm .0017$	
Imidazole, $\mu = .15$	$- .026 \pm .005$	.022
ϵ-Amino, $\mu = .15$	$- .024 \pm .004$	
Phenolic, $\mu = .15$	$- .022 \pm .0035$	

[a] Using a value of w 0.8 of that calculated by equation 2.

The curves of Fig. 4 may be used to calculate intrinsic dissociation constants of the carboxyl groups, since they include the point $Z = 0$, where, according to equation 1, the ordinate is equal to pK_{int}. A value of pK_{int} for the imidazole groups may be computed by extending the linear logarithmic plot to $Z = 0$. The steep linear portions of the curves of Fig. 5 have, within experimental error, the same slopes as are obtained at the same ionic strength for the imidazole groups, and for the linear portion of the carboxyl group plot, as is required by the simplest electrostatic theory. One may therefore extrapolate these plots to $Z = 0$ to obtain intrinsic dissociation constants for the ϵ-amino and phenolic groups. All pK_{int} values so obtained are shown in Table III, together with comparable values for other proteins and values to be expected from measurements on suitable molecules of low molecular weight.

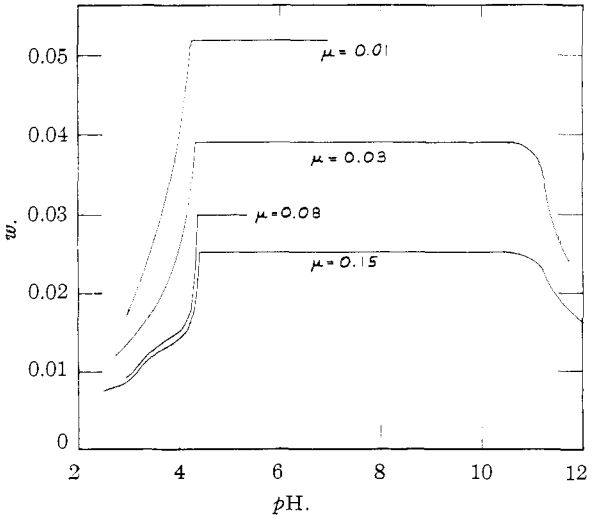

Fig. 6.—The empirical electrostatic interaction factor, w, as a function of pH. Taken from logarithmic plots such as Figs. 4 and 5, except above pH 10.5 at $\mu = 0.03$ where the values are those required to provide a good fit for Fig. 1.

TABLE III

INTRINSIC DISSOCIATION CONSTANTS

Type of group and ionic strength	pK_{int} obsd.[a]	Expected value (appropriate small molecules)	Range of values found in other proteins[m]
α-Carboxyl	(3.75)[b]	3.7[c]	3.6[g]
β,γ-Carboxyl, $\mu = 0.01$	3.92		
.03	3.95	4.6[d]	4.3–4.73[g-i]
.08	3.96		
.15	4.02		
Imidazole, $\mu = 0.15$	6.9	7.0[e]	6.4–7.0[g-i]
α-Amino,	(7.75)[b]	7.8[f]	7.45–7.9[g,i]
ϵ-Amino, $\mu = 0.15$	9.8	10.1–10.6[f]	10.1–10.6[i,j]
Phenolic, $\mu = 0.15$	10.35	9.6[f]	9.5–9.9[g,k,l] 10.4–10.9[j]
Guanidine	>12	>13[f]	11.9–13.3[g,i]

[a] Probable uncertainty about 0.05 for carboxyl groups, about 0.1 for the other groups. [b] Assumed values. [c] E. Ellenbogen, THIS JOURNAL, **74**, 5198 (1952). [d] Ref. 9, Table III. [e] The value for imidazole, reduced by about 0.1 to allow for the effect of the peptide chain. It should be noted, however, that the effect of substituents on this pK is not fully understood (ref. 6). [f] See discussion in ref. 6. [g] Insulin, ref. 5. [h] β-Lactoglobulin, ref. 3b. [i] Ovalbumin, ref. 3a. [j] Lysozyme, ref. 6. [k] Ribonuclease, study in progress in this Laboratory. [l] Polytyrosine, E. Katchalski and M. Sela, THIS JOURNAL, **75**, 5284 (1953). [m] Values for HSA are discussed at the end of this paper.

Since the pK_{int} for imidazole groups is considerably different from the value given in an earlier study of HSA,[4] an independent check of the value given in Table III is desirable. This may be obtained by taking the mid-point of the 6.5 kcal. plateau of the heat of ionization curve (Fig. 3) as the mid-point of the titration of imidazole groups. The pH at this point (7.48 ± 0.15) is by equation 1 equal to $pK_{int} - 0.868wZ$. The value of Z is -22; if one takes for w the value of 0.026 given in Fig. 6, this gives $pK_{int} = 7.0 \pm 0.15$. The calculation is quite insensitive to the value of w. If w is chosen as small as 0.020, say, or as large as 0.032, the resulting change in pK_{int} is only 0.05.

Once values of pK_{int} are established, values of the electrostatic interaction factor w can be calculated directly by use of equation 1. The values so calculated are shown in Fig. 6. It should be noted that the curves for phenolic and amino groups (Fig. 5) are parallel and that the same value of w at any pH is obtained from either curve, as required by the simple electrostatic treatment here employed. That this requirement is fulfilled by actual experimental data has not been previously demonstrated for any protein.

It should be noted that the values of Z of Fig. 5 are calculated with the assumption that no potassium ion is bound. This probably is not true. At the point where the acid expansion of BSA begins, a simultaneous sharp increase in chloride binding occurs, presumably because of the appearance of new sites,[19] and a similar situation may well occur in the break in Fig. 5 with regard to potassium ion binding. If so, the alkaline values of w in Fig. 6 will drop off less steeply.

In order to test the calculated results here given, the entire titration curve at $\mu = 0.15$, and the acid portions of the curves at the other ionic strengths have been calculated, using the constants of Table

(19) G. Scatchard, Richards Medal Address, *The Nucleus*, **31**, 211 (1954).

III and Fig. 6. These are the curves of Fig. 1, and they are seen to fit the experimental data, as they should. The alkaline portion of the curve at $\mu = 0.03$ was also calculated, using the same pK_{int} values as at $\mu = 0.15$ and the w values, chosen for best fit, given by Fig. 6. The titration curve follows the experimental points very closely, and the curve for w lies roughly where one would have expected it to lie in comparison with the independently calculated curve at $\mu = 0.15$.

Discussion

Studies of hydrogen ion dissociation in two proteins, ovalbumin and lysozyme, have shown that experimental curves could be described by equations of the form of equation 1 with values of w independent of pH. The same is true of β-lactoglobulin between pH 2 and 9. This has been interpreted as indicating that these proteins are rigid, maintaining a constant shape and size independent of charge.

If one now assumes that these protein molecules are spheres impenetrable to salt ions, and that their net charge is evenly distributed over their surface, one may calculate w according to the following equation, based on the Debye–Hückel theory[20-22]

$$w_{calc} = \frac{\epsilon^2}{2DkTR}\left(1 - \frac{\kappa R}{1 + \kappa a_i}\right) \qquad (2)$$

where R is the radius of the sphere (usually calculated with the assumption that about 20% of the sphere consists of water bound to polar groups), a_i is the distance of closest approach of salt ions (exceeds R by about 2.5 Å. in KCl), κ is the Debye–Hückel constant proportional to the square root of ionic strength, D is the dielectric constant of water, k Boltzmann's constant, ϵ the protonic charge, and T the absolute temperature. For the proteins mentioned above, w calculated by equation 2 has predicted correctly the ionic strength dependence of the titration curves; empirical values of w have, however, been somewhat smaller than those calculated by equation 2.

The same situation exists for BSA for Z approximately between +5 and −55, roughly between pH 4.3 and 10.5. In this region curves plotted according to equation 1 are linear; their slopes, within reasonable experimental uncertainty,[23] are independent of pH; and, moreover, agree with slopes calculated on the basis of equation 2, if the values of w used are about 0.8 the calculated values. Slopes computed in this way are shown in the last column of Table II.[24]

As shown by Fig. 6, empirical values of w fall well below their neutral region values below pH 4.3 and above pH 10.5. The most straightforward explanation of this is that BSA undergoes reversible expansion outside the region of pH 4.3 to 10.5. This same conclusion already has been put forward by Scatchard (based on the titration curve of HSA)[25] and by the present author[9] (based on preliminary titration data of unpurified BSA). That this conclusion is amply supported by several other experimental methods, and that these methods also support the conclusion that BSA is not deformable in the neutral region, *i.e.*, that they support the use of a constant w between pH 4.3 and 10.5, is shown in detail in the following paper. The significance of the break in the curve at $\mu = 0.15$, near pH 4, is also discussed in that paper.

It remains to consider the intrinsic dissociation constants of Table III. Of the four constants computed, three, those for side-chain carboxyl groups, ϵ-amino groups and phenolic groups, are abnormal. The intrinsic free energy of ionization deviates from normal values by about 1000 cal. per mole of hydrogen ions dissociated, in such a direction as to favor the carboxylate ion, the uncharged amino group, and the un-ionized phenolic group.

Serum albumin is the only protein known where such discrepancies are so prevalent: at least 175 of approximately 200 side-chain groups show abnormal behavior. While some abnormal behavior has been noted in other proteins, especially for phenolic groups, the number of groups involved has been relatively small. It is possible that this is due to the fact that BSA is larger than other proteins studied in this way: there must as a result be fewer locations available on the surface of the molecule, and it is on the surface that a dissociable group must be if it is to show normal thermodynamic behavior with reference to a small molecule possessing the same dissociable group. It is also possible, however, that this extensive mutual interaction between side-chain groups is part of the general perversity of the albumin molecule, its configurational adaptability, as Karush has termed it.[26]

Table IV presents thermodynamic data for the dissociation of the phenolic groups of BSA,[10] as compared with "normal" values. Laskowski and Scheraga[27] have presented convincing arguments to show that simple hydrogen bonding is probably responsible for the difference. One of the present authors[28] has speculated that carboxylate ions may be the electron-donating portion of the phenolic hydrogen bonds, a suggestion also supported by Laskowski and Scheraga. It seems probable, for two reasons, that this idea is invalid. Firstly, BSA contains 99 side chain carboxyl groups, but only 19 phenolic hydroxyl groups. If 19 of the 99 carboxyl groups were appreciably different from the rest, it would most certainly have shown up in the titration curve. Since it did not, we must suppose that BSA contains 80 side chain groups of equal hydrogen bonding power as the phenolic groups. There happen to be approximately 80 aliphatic hydroxyl groups, but it is unlikely that they would act as efficient hydrogen bonding agents. The second and stronger reason for rejecting the idea lies in the entropy (and heat) changes of Table IV. If the deviations for phenolic groups are due to the rupture of phenolic–carboxylate bonds, the deviations for

(20) K. Linderström-Lang, *Compt. rend. trav. lab., Carlsberg*, **15**, No. 7 (1924).

(21) G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949).

(22) C. Tanford, *J. Phys. Chem.*, **59**, 788 (1955).

(23) Because of the possibility of systematic error in correcting for overlap of neighboring groups, the uncertainty may be somewhat greater than the standard deviations given in Table II.

(24) Empirical values of w in ovalbumin (ref. 3a) are also about 0.8 as great as calculated by equation 2; the same is true, in work in progress in this Laboratory, for ribonuclease.

(25) G. Scatchard, *American Scientist*, **40**, 61 (1952).

(26) F. Karush, THIS JOURNAL, **72**, 2705 (1950).

(27) M. Laskowski, Jr., and H. A. Scheraga, *ibid.*, **76**, 6305 (1954).

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

carboxyl groups should be due to the formation of such bonds: they should be equal in magnitude, but opposite in sign. This relationship exists indeed for the ΔF^0 values: it does not exist, however, for the heat and entropy changes. The deviation of the entropy of ionization of the carboxyl groups is a *positive* one, quite incompatible with the *negative* deviation in ΔS^0 of 15 e.u. which should be observed if the bond formed on carboxyl ionization is the same as the bond broken on phenolic ionization. We must conclude, therefore, that the electron-donating portion of the phenolic hydrogen bond is still unidentified.[29]

TABLE IV

INTRINSIC THERMODYNAMIC PROPERTIES FOR HYDROGEN ION DISSOCIATION

	ΔF^0, kcal./mole	ΔH^0, kcal./mole	ΔS^0, kcal./mole/deg.
COOH groups, normal	6.3	0 to 1.0	−18 to −21
in BSA	5.4	1.5 to 2.0	−11 to −13
Phenolic groups, normal	13.1	6.0	−24
in BSA	14.1	11.5	− 9

An alternative explanation lies in the possible existence of salt links between COO^- and cationic nitrogen. One would not normally expect much attraction between oppositely-charged groups in a medium of high dielectric constant[30] and evidence against such bonds has been presented by Jacobsen and Linderström-Lang.[31] Nevertheless, and perhaps especially in view of the attraction of some presumably positively-charged site on the BSA for as inert an anion as chloride, the possibility of such bonds should be kept in mind. If such bonds did exist they would be expected to give rise to a lowered pK and to a more positive ΔS of ionization (due to release of solvent) for the carboxyl groups, as actually observed. The dissociation of hydrogen ions from amino groups, however, would then be correspondingly impeded, contrary to what is observed.

One other piece of experimental evidence of interest in this connection is the ionic strength dependence of the intrinsic pK's for carboxyl groups shown in Table III. The intrinsic pK is defined as the apparent pK, at the pH where Z is zero, at whatever ionic strength is being used. That such a pK should vary slightly with ionic strength is evident if one considers the equilibrium $—COO^- + —ImH^+ \rightleftarrows —COOH + Im$, where Im stands for an un-ionized imidazole group. This equilibrium, change in which involves no dissociation of H^+ and no change in Z, will clearly move to the left with increasing ionic strength and thus produce an apparent *decrease* in pK_{int} for carboxyl groups. The observed change in pK is in the opposite direction, however.

Once again the postulate of salt bridges provides a possible explanation: salt bridges would be most stable at low ionic strength. Increasing ionic strength would therefore decrease any stabilization of the carboxylate ion by salt bridges and bring about an apparent *increase* in pK_{int} for carboxyl groups.

Salt bridges would also provide an explanation for the fact that BSA expands outside the region of pH 4.3 to 10.5, and not within it, as discussed in the following paper.

One other idea, put forward by Schellman,[32] is that the side-chain groups of BSA might be in an environment of low dielectric constant. This would stabilize the *uncharged* form of each group, and could account for the abnormal pK's of the amino and phenolic groups. The pK shift for the carboxy groups, however, is in the wrong direction.

Clearly no one of these explanations can account for all of the deviations observed. It will probably be necessary to wait for the detailed application to proteins of a more direct technique, such as infrared spectroscopy, before the exact nature of the internal bonds of BSA can be determined.

Comparison with Human Serum Albumin.—A number of the calculated results of the present paper differ from the corresponding figures obtained from the titration study of HSA.[4] Especially prominent are the differences in pK_{int} for the imidazole, ϵ-amino and phenolic groups. These differences do not represent differences between HSA and BSA, but are due to the fact that our knowledge of these proteins and our technique in analyzing their titration curves has improved. Thus in 1950 it was believed that HSA had nine terminal α-amino groups, although it is now known that there is only one.[33] We allowed for four such groups in the calculated titration curve of HSA, which had the effect of reducing the observed pK_{int} for imidazole groups. We also attempted to calculate the titration curve of HSA with a value of w independent of pH, which resulted in a very poor fit above pH 11 and too low values for pK_{int} for the groups titrated in this area.

That there is in fact remarkably little difference between HSA and BSA is shown by the fact that titration data from both proteins at $\mu = 0.15$ are close to superimposable. The small differences which exist below pH 10 can be accounted for if HSA has one or two extra carboxyl groups, one or two extra groups titrated near pH 6 (which may be the fatty acid groups removed by the ion-exchange method of purification) and one or two fewer imidazole groups. The HSA data above pH 10 are insufficiently precise to decide whether there is any difference at all between the two proteins above that pH.[34]

It is thus unlikely that the intrinsic pK's for the various groups of HSA are appreciably different

(29) This argument based on thermodynamic data might be invalid if there were carboxyl-carboxyl hydrogen bonds between un-ionized COOH groups. Such bonds have been suggested by Laskowski and Scheraga (ref. 21) to account for the *curvature* of the curves of Fig. 4. However, this curvature is accounted for quantitatively in terms of the expansion of BSA.

(30) *Cf.* our experimental study of ammonium and guanidinium ions, C. Tanford, THIS JOURNAL, **76**, 945 (1954).

(31) C. F. Jacobsen and K. U. Linderström-Lang, *Nature*, **164**, 411 (1949).

(32) J. A. Schellman, *J. Phys. Chem.*, **57**, 472 (1953).

(33) H. van Vunakis and E. Brand, Abstracts, 119 National Meeting of the American Chemical Society, Boston, 1951; B. Keil, V. Tomasek and J. Sedlackova, *Chem. Listy*, **46**, 457 (1952); E. O. P. Thompson, *J. Biol. Chem.*, **208**, 565 (1954).

(34) The titration data for HSA above pH 10 are badly scattered, because hydrogen electrodes, which were used in the HSA study, are much less accurate in alkaline protein solutions than glass electrodes, perhaps because of the greater activity of the sulfide group above pH 10, which would tend to poison the platinum surface of the electrodes.

from those of BSA. The variation of w with pH is also essentially the same, as seen on the acid side by the fact that the curve for HSA is as steep as that for BSA, and as best shown on the alkaline side by the similarity between the titration curves for the phenolic groups.[10]

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[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, STATE UNIVERSITY OF IOWA]

The Reversible Expansion of Bovine Serum Albumin in Acid Solutions[1]

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Bovine serum albumin exists in a compact form between pH 4.3 and 10.5. Below pH 4.3 and above pH 10.5 it undergoes expansion. This paper shows that the compact form is held together by a network of bonds involving the side-chain groups of the protein. Near pH 4.2 or 10.5 the titration of some of these side chains results in transition to an *expandable* form. This form undergoes continuous expansion, increasing with charge and decreasing with ionic strength, so as to reduce the electrostatic free energy. A further slow molecular change occurs on standing. The entire process, including the final slow change, is reversible.

Numerous investigators during the last five years have discovered, independently, that the behavior of serum albumin in acid solutions is considerably different from that of other well-known proteins. The first indication of this difference came from the titration curve of human albumin which, in the acid region, is much steeper than theory predicts.[2] Both Scatchard and one of the present authors have interpreted this as the result of an *expansion* of the protein molecule with increasing acidity.[3,4] About the same time, Macheboeuf, Barbu and co-workers[5] observed an anomalous viscosity increase, which they interpreted as an *association*, and shortly thereafter Weber[6] observed an anomalous increase in the apparent rotational diffusion constant (from studies of the depolarization of fluorescence) which he interpreted as a *dissociation*. Pedersen[7] has stated, however, that unpublished diffusion–sedimentation studies made by him preclude the possibility of a change in molecular weight, and the same conclusion was reached from light scattering by Yang and Foster.[8]

Yang and Foster's is the most detailed study of the problem to date: they studied viscosity, optical rotation and light scattering, showing that all of them point to expansion as the probable cause of the anomalous behavior. Moreover, they found an absence of flow birefringence, so that the expansion appears to be essentially isotropic. Yang and Foster have also pointed out that the decrease in fluorescence polarization observed in acid BSA[6] is compatible with expansion if, as seems reasonable, the expansion is accompanied by increase in freedom for internal rotation.

Jirgensons[9] has also observed the same changes in optical rotation as Yang and Foster, and Gutfreund and Sturtevant[10] have observed an anomalous uptake of heat in acid solutions, which they have also ascribed to expansion.

The present investigation of bovine serum albumin (BSA) was intended primarily to confirm Yang and Foster's work, and to compare the progress of the expansion as reflected by viscosity measurements with its progress as reflected by changes in the empirical electrostatic interaction factor derived from the titration curves of BSA.[11,12] During the course of the investigation it soon appeared, however, that the expansion process is more complicated than was originally supposed. Yang and Foster[8] had visualized it as a single "all-or-none" equilibrium between an expanded and a compact form of BSA; our own initial idea[4,11] had been that it was a continuous change in configuration. It is shown in the present paper that there are actually at least three stages to the reaction: an initial "all-or-none" conversion from a compact to an *expandable* form, followed by continuous expansion of the expandable form, followed by a slow third stage, possibly an aggregation.

As a result of the discovery of this greater complexity of the reaction, this paper actually represents only a preliminary study, for each stage of the reaction now requires more detailed investigation, not only by the methods here given, but by some of the other techniques which have been used to provide evidence for the existence of the expansion.

Experimental

Crystalline BSA was purchased from Armour and Co. (lot P67502). The protein was dissolved in water and the solutions were passed through a mixed bed ion-exchange column as described by Dintzis.[13] The resulting stock

(1) A preliminary report on this work was presented at the 126th National Meeting of the American Chemical Society, New York, N. Y., September, 1954.

(2) C. Tanford, THIS JOURNAL, **72**, 441 (1950).

(3) G. Scatchard, *American Scientist*, **40**, 61 (1952).

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(5) E. Gavrilesco, E. Barbu and M. Macheboeuf, *Bull. soc. chim. biol.*, **32**, 924 (1950); S. Bjornholm, E. Barbu and M. Macheboeuf, *ibid.*, **34**, 1083 (1952).

(6) G. Weber, *Biochem. J.*, **51**, 155 (1952).

(7) K. O. Pedersen, *Disc. Faraday Soc.*, No. **13**, 49 (1953).

(8) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954).

(9) B. Jirgensons, *Arch. Biochem. Biophys.*, **41**, 333 (1952).

(10) H. Gutfreund and J. M. Sturtevant, THIS JOURNAL, **76**, 1595 (1954).

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

(13) H. M. Dintzis, Ph.D. Thesis, Harvard University, 1952.