of the variation they found but is of a much smaller magnitude. There may be some question as to whether the variation is real in light of the precision involved. However, the trend cannot be denied.

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

Phenolic Hydroxyl Ionization in Proteins. I. Bovine Serum Albumin[1-3]

BY CHARLES TANFORD AND GEORGE L. ROBERTS, JR.

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The ionization of the phenolic hydroxyl groups of bovine serum albumin has been studied by measuring the ultraviolet light absorption at 295 mμ as a function of *p*H at three temperatures. The ionization curves are steeper than theory would predict, and can be fitted with a computed curve only if the effective negative charge on the albumin molecule is assumed not to rise much above −50. The intrinsic *pK* for ionization at 25° lies between 10.0 and 10.3; the heat of ionization is 11.5 kcal./mole; the intrinsic entropy of ionization is −8 e.u. The heat of ionization of di-iodo phenolic groups in iodinated albumin is 7.0 kcal./mole. These figures all suggest that the phenolic OH group in native albumin may be hydrogen-bonded; however, the hydrogen bonds are certainly not involved in maintaining the native structure of the molecule, for the ionization proceeds instantaneously and reversibly.

While numerous potentiometric studies have been made of hydrogen ion dissociation in proteins, such studies are always complicated, particularly in the alkaline range, by the fact that the ionization ranges of different groups overlap. Crammer and Neuberger,[4] however, have shown that one of the important groups contributing to hydrogen ion dissociation in the alkaline range, the phenolic hydroxyl group, can be studied independently of other groups by utilization of the shift in the ultraviolet absorption spectrum which accompanies the dissociation of a hydrogen ion from this group. Crammer and Neuberger in this way made a study of the ionization of the phenolic hydroxyl groups of tyrosine, insulin and egg albumin. The present paper attempts a considerably more detailed investigation for bovine serum albumin. The results obtained are of rather special interest for two reasons: (1) because they throw light on the problem of hydrogen bonding in proteins, in which phenolic hydroxyl groups may be expected to play a prominent role; and (2) because the work described constitutes a precise thermodynamic study of a protein reaction at a *p*H very far removed from the iso-ionic point, and, hence, a very exacting test of the theory underlying the effect of electrostatic charge upon protein reactions.

Experimental

Reagents.—Armour crystalline bovine serum albumin was used in this study. Its concentration in stock solution was determined from the light absorption at 280 mμ wave length in an appropriately diluted solution. The method was standardized by measurements on a solution whose concentration was determined independently by drying at 105°. The value of $E^{1cm}_{1\%}$ was found to be 6.60, in agreement with a value reported by Cohn, Hughes and Weare.[5] The molecular weight has been taken to be 69,000.[6] Stock solutions of piperidine buffers, hydrochloric acid and potassium chloride were prepared from reagent grade materials; stock solutions of potassium hydroxide were prepared carbonate-free by the method of Kolthoff.[7] Carbon dioxide-free water was used throughout.

Solutions for measurement contained between 0.2 and 0.3% serum albumin and their *p*H was adjusted by the careful addition of appropriate amounts of acid or base, or by means of the piperidine buffers. Potassium chloride was used to bring the total ionic strength of each solution to 0.15.

Measurement of *p*H.—Measurements of *p*H were made on a Model G Beckman *p*H meter, using external electrodes and a small cell which could be lowered into any one of three constant temperature baths, maintained at 15.0, 25.0 and 35.0°. Bureau of Standards borax *p*H standard (*p*H 9.18 at 25°) was used for calibration. The instrument was checked at high *p*H values by means of potassium hydroxide solutions of known molality, containing sufficient potassium chloride to bring the ionic strength to 0.15. The *p*H of such solutions could be computed from the activity coefficients obtained from hydrogen electrode measurements by Tanford[8] or Green.[9] (The substitution of potassium for sodium should not alter these activity coefficients appreciably.) It was found that individual measurements with the glass electrode showed random deviations from the computed values of a few hundredths of a *p*H unit; average values were not significantly in error, however. It is concluded that *p*H measurements were identical with hydrogen electrode measurements, with an accuracy of about 0.02 *p*H unit, even in the neighborhood of *p*H 13.0.

Ultraviolet Light Absorption.—Measurements were made on a Beckman model DU spectrophotometer. 10-mm. quartz cells with 7 mm. inserts were used to give a 3 mm. light path. Each cell was always used with the same insert, and calibrated with both water and tyrosine solution. The temperature in the cell compartment was maintained at 15.0, 25.0 or 35.0° by use of four Beckman thermospacers,[10] through which water from the appropriate constant temperature bath was circulated.

Except in Fig. 2, light absorption measurements are re-

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(1) This work was supported by a grant from the Research Corporation.

(2) Presented at the XIIth International Congress of Pure and Applied Chemistry, New York, September, 1951.

(3) Abstracted from the thesis submitted by George L. Roberts, Jr., in partial fulfillment of the requirements for the Ph.D. degree, State University of Iowa, August, 1951.

(4) J. L. Crammer and A. Neuberger, *Biochem. J.*, **37**, 302 (1943).

(5) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

(6) G. Scatchard, A. C. Batchelder and A. Brown, *ibid.*, **68**, 2320 (1946).

(7) I. M. Kolthoff, *Z. anal. Chem.*, **61**, 48 (1922).

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

(9) A. A. Green, *ibid.*, **60**, 1108 (1938).

(10) Arrangement "D," Bulletin 227, National Technical Laboratories, South Pasadena 3, California.

ported as molar extinction coefficients ($\log_{10} I_0/I$ for a 1 M solution with 1 cm. light path).

Ultraviolet Absorption by Serum Albumin

The ultraviolet absorption spectrum of bovine serum albumin in neutral or acid solution and that in alkaline solution (pH 13) are shown in Fig. 1. The alkaline spectrum is merely approximate, since it changes rapidly with time (see below), but is not likely to be far wrong above 280 mμ. Below 280 mμ the molar extinction figures are probably somewhat too high.

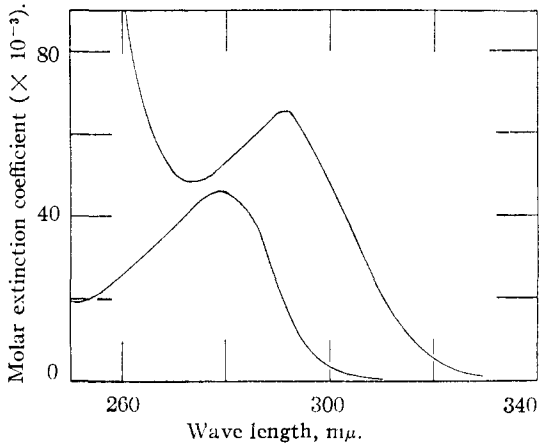

Fig. 1.—The ultraviolet absorption spectrum of bovine serum albumin in neutral or acid solution (lower curve) and above pH 13 (upper curve).

Qualitatively, the two curves are very similar to the absorption spectra of tyrosine before and after ionization of the phenolic hydroxy group. Both the absorption band shown in the figure, and that which gives rise to the absorption increase at lower wave length (the peak of which is not shown) are similar to those seen in tyrosine.[4,11] It would appear then that a measure of the amount of phenolic hydroxyl ionization which has occurred in an albumin solution at any pH can be obtained from the molar extinction coefficient either near 250 or near 295 mμ.

The latter wave length was chosen since the total absorption is less there, allowing the use of more concentrated protein solutions than would be possible at the low wave length. This choice turned out to be a fortunate one, as the instability of the spectrum is less at 295 mμ than in the lower wave length region.

Time Dependence.—It was observed early in this study that the optical density of an alkaline albumin solution changes with time. The change is negligibly slow over a portion of the pH range which it was desired to investigate, but becomes very pronounced above pH 12, especially at 35°, the highest temperature used. At pH 13, to give rough figures, the molar extinction at 295 mμ rises instantaneously from about 9000 to 60,000 (even by taking readings less than one minute after solutions were prepared, no suggestion could ever be found that the rate of this initial rise is measurable by ordinary means). After this instantaneous rise, however, a further slow increase occurs, over a period of two or more hours, until the final extinction is perhaps 70,000.

It is important to decide whether this is due to the existence of a few special phenolic hydroxyl groups, the ionization of which proceeds slowly, or whether it is due to some extraneous process (perhaps one of the many which fall under the heading of protein "denaturation").

To decide this point, a study of this change was made at three wave lengths, 265, 278 and 295 mμ and the results are shown in Fig. 2. It is at once apparent that the optical density change occurring is not due to the slow ionization of some of the phenolic hydroxyl groups. This ionization, both in tyrosine[4,11] and in serum albumin (Fig. 1), is accompanied by a relatively large increase in extinction at 295 mμ and a considerably smaller increase at 265 mμ. At 278 mμ, on the other hand, no change in absorption (or at most a very small change) occurs. The process observed in Fig. 2, however, shows the largest rise at 265 mμ, the smallest at 295 mμ, with 278 mμ intermediate. It appears, therefore, to be due to some denaturation process.[12] An increase in Rayleigh scattering due to aggregation would appear to be a possible cause; however, the change with wave length indicated by Fig. 2 is greater than that of an inverse fourth-power relation, which would be required by light scattering.[13,14]

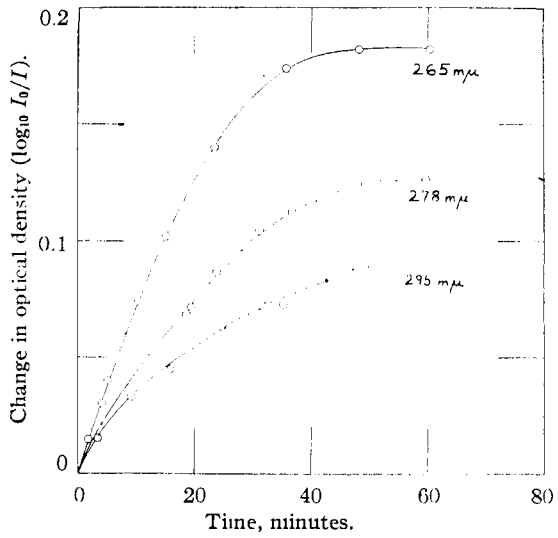

Fig. 2.—Increase in absorption with time at three wave lengths for a roughly 0.2% albumin solution near pH 13, using a light path of 3 mm.

It would appear then that the ionization of all the phenolic groups is instantaneous, the subsequent change being due to denaturation. It should therefore be possible to obtain equilibrium data for the native protein if optical density readings are extrapolated to zero time. The methods used to do this are discussed below, in the presentation of the data for serum albumin.

(11) N. Kretchmer and R. Taylor, THIS JOURNAL, **72**, 3291 (1950).

(12) It is interesting to compare these results with a similar study on egg albumin. Crammer and Neuberger (Ref. 4) have shown that most of the phenolic groups of this protein cannot be ionized without denaturation, and that the ionization is then irreversible. We have found that the ionization proceeds slowly (over a period of hours), and that the spectral change accompanying it is in accord with this, *i.e.*, there is no change at 278 mμ, a large change at 295 mμ, and a relatively small change at 265 mμ.

(13) P. Doty and J. T. Edsall, *Adv. in Protein Chemistry*, **6**, 35 (1951).

(14) Provided no increase in molecular size takes place, no appreciable change in scattering is to be expected on going from an iso-ionic protein to even a highly charged molecule, as long as the protein concentration is low and the ionic strength fairly high, as is true in our experiments.

To prove the validity of the method it must be shown (1) that the ionization curves are reversible, (2) that heats of ionization determined for the 15–25° range are the same as those for the 25–35° range (the difference to be expected because of the large value of ΔC_p in this reaction lies within the experimental error of our results), (3) that the change in extinction in going from an albumin molecule in which none of the phenolic groups are ionized to one in which ionization is complete shall be independent of temperature, and (4) that this change can be accounted for quantitatively on the basis of the number of tyrosyl residues in the protein molecule. The first two of these points are dealt with in a later section of this paper; the last two are discussed in the following paragraphs.

Change in Molar Extinction.—It is found that the molar extinction at 295 mμ of all solutions between *p*H 4 and 8 is 8800. This is therefore taken to the molar extinction of serum albumin before ionization of the phenolic hydroxyl groups.

The molar extinction corresponding to complete ionization of these groups is less easily established, since complete ionization is never quite reached, even at *p*H 13. However, the extinction *vs.* *p*H curves at all three temperatures (Figs. 3 and 5) appear to approach the same value, and, while there is some margin for error in the extrapolation, a final extinction of 59,800 is not unreasonable. Using this value, we thus obtain a total change in extinction of 51,000.

This change is easily shown to be of the right order of magnitude for the ionization of the 21 phenolic hydroxyl groups present in the albumin molecule.[15] The difference in the molar extinction of tyrosine before and after ionization of the phenolic group has been measured in this Laboratory and found to be 2300 (the same figure was obtained by Crammer and Neuberger[4]). For 21 tyrosyl residues, therefore, a change of 48,300 would be calculated if the absorption of tyrosine itself were identical with that of tyrosyl residues in the albumin molecule. Since it cannot be expected that the absorption in the two cases will be exactly identical, the agreement between this calculated figure and that actually observed is considered satisfactory.[16]

It remains to consider the possibility that the tryptophan and cystine residues of serum albumin may contribute to the absorption increase. Both tryptophan and cystine absorb light at 295 mμ, and both show a small increase with *p*H. However, in the case of cystine, the change is negligible; at 295 mμ the molar extinction coefficient is 25 in acid solution and 50 in alkaline solution.[17] For the 16 cystine residues present in a serum albumin molecule[15] the expected change would therefore be only 400, *i.e.*, less than 1% of the total change.[18] The corresponding change for tryptophan, as observed in this Laboratory, is from 1400 to 2400 (Crammer and Neuberger's figures are 1400 and 2300[4]), *i.e.*, the two tryptophan residues of the serum albumin molecule[15] would contribute about 2000, or somewhat less than 4%, to the total observed change of 51,800.

Actually, it appears highly probable that the tryptophan in the protein molecule will not make even this small contribution. Our studies have shown that the change in tryptophan itself is a small general increase rather than a pronounced shift, suggesting that it is not caused by an important alteration in the indole ring. Furthermore, the change occurs between *p*H 9 and 11, with virtually no increase above that *p*H. This strongly indicates that the change is due to the dissociation of a hydrogen ion from the amino group of the tryptophan molecule, which of course, cannot occur when the tryptophan is incorporated in a protein molecule.

Ionization Curves from Spectra.—It is concluded from the preceding discussion that the instantaneous increase in absorption at 295 mμ is due entirely to the ionization of the phenolic hydroxyl groups. The degree of ionization, x, at any *p*H, can therefore be calculated from the observed molar extinction coefficient at that *p*H by means of the relation

$$x = (\epsilon - 8800)/51{,}000 \qquad (1)$$

Results with Tyrosine

As a test of the experimental method, the ionization constants at 15, 25 and 35°, and the heat of ionization, were determined for tyrosine itself. In this case, of course, there is no change in the spectrum with time. The *pK* values obtained are 10.19, 10.05 and 9.90, respectively, at 15, 25 and 35°. The heat of ionization is obtained from the relation

$$\Delta H = 2.303R \left[\frac{\Delta p\mathrm{H}}{\Delta(1/T)}\right]_{x=\text{const}} \qquad (2)$$

which holds true provided the *p*H difference is taken at a given value of the degree of ionization. In order to test the consistency of the data, ΔH values were computed at three different values of the degree of ionization. The values are given in Table I. The average value is seen to be 6.0 kcal./mole. It should be emphasized that the probable error in this determination is close to 1 kcal./mole. The measured *p*H differences are accurate only to 0.02 *p*H unit, which, for a 10 degree temperature range, corresponds to 0.8 kcal./mole in the heat of ionization.

TABLE I

HEAT OF IONIZATION OF TYROSINE

Degree of ionization	*p*H 15°	*p*H 25°	*p*H 35°	ΔH, kcal./mole 15–25°	ΔH, kcal./mole 25–35°
0.25	9.59	9.45	9.31	5.5	5.9
.50	10.19	10.05	9.90	5.5	6.3
.75	10.76	10.60	10.45	6.3	6.3
			Average	6.0 ± 1.0	

The entropy of ionization (ionic strength 0.15), $\Delta S^0 = (\Delta H + RT \ln K_0)/T$, has the value -26 ± 4 e.u. at 25°.

The values obtained for the ionization constants agree reasonably well with previous determinations. Corrected to zero ionic strength by means of the Debye–Hückel equation (with an a_i value of 6 Å.[19]), our value at 25° becomes 10.14. The value obtained by Crammer and Neuberger, corrected to 25° and zero ionic strength, is 10.03.[4] Values obtained from solubility measurements, corrected in the same way, lie somewhere between 10.10 and 10.15.[20,21] The heat of ionization of the phenolic

(15) E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 187 (1946).

(16) J. S. Fruton and G. I. Lavin (*J. Biol. Chem.*, **130**, 375 (1939)) have shown that the effect of the presence of a peptide bond on the absorption spectrum of tyrosine in *acid* solution is small. However, changes of the order of 5% (as required here) would not appear to be excluded by their data.

(17) H. Ley and B. Arends, *Z. physik. Chem.*, **17B**, 177 (1932).

(18) In relatively strong alkaline solution, in the presence of oxygen, cystine undergoes slow oxidation (A. P. Matthews and S. Walker, *J. Biol. Chem.*, **6**, 289 (1909); J. C. Andrews, *ibid.*, **65**, 161 (1925)). We have found that this reaction leads to some changes in the ultraviolet absorption, but these are very small, of the order of 10% of the molar extinction of cystine.

(19) The empirical value for benzoate and salicylate ions (J. Kielland, THIS JOURNAL, **59**, 1675 (1937)). A value of 7 or 8 Å. would give about the same result.

(20) D. I. Hitchcock, *J. Gen. Physiol.*, **6**, 747 (1924).

(21) P. S. Winnek and C. L. A. Schmidt, *ibid.*, **18**, 889 (1935).

hydroxyl group in tyrosine has not been previously determined. Our value of 6 kcal./mole, however, is in very good agreement with the heats of ionization of phenol and peptides containing tyrosine (see Table IV).

Results with Serum Albumin

Ionization Curves.—As has been shown above, the ionization of the phenolic groups of serum albumin proceeds instantaneously, but denaturation sets in almost at once, leading to an increase in light absorption. In order to obtain significant measurements, it was therefore necessary to determine the values for a given solution as a function of time and extrapolate back to zero time. Even this procedure was unsatisfactory in the region of *p*H 10 to 12, in which albumin solutions were found to exhibit a pronounced downward drift in *p*H (similar to that observed with human serum albumin[22]). In this region, therefore, buffered solutions containing piperidine and piperidinium chloride at ionic strength 0.15 were used. (The pK of piperidinium ion is about 11.3 at 25°.)

The experimental results are shown in Figs. 3 to 5. Open circles represent extrapolated optical density and *p*H readings of unbuffered solutions, and solid circles those of buffered solutions. The substitution of piperidinium for potassium ion appears not to shift the ionization curves, indicating that both ions are either not bound by the protein, or bound to the same extent.

The crosses in Figs. 3 to 5 represent unextrapolated readings, obtained 10 to 15 minutes after the preparation of solutions. At high *p*H, especially at 35°, these points deviate much from the extrapolated ones. They have not been used in drawing the ionization curves. The curves of Fig. 4 are computed curves, discussed below.

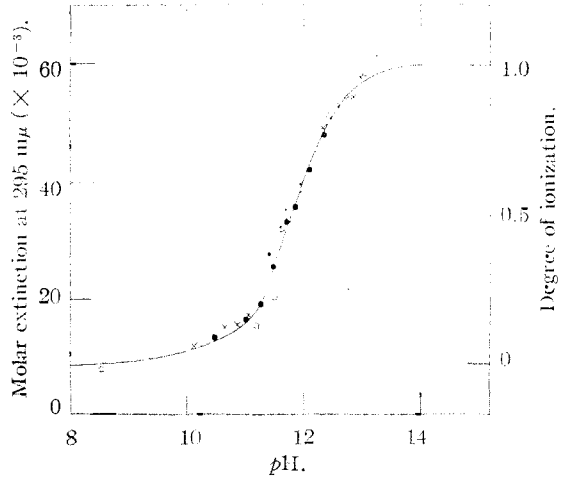

Fig. 3.—Ionization curve for serum albumin at 15°.

Reversibility.—As was mentioned earlier, it is necessary, to justify the extrapolation method used, to show that the ionization curves obtained in this way are reversible. To show this, a few solutions were made (at 25°) containing sufficient potassium hydroxide to bring the *p*H above 12.0. They were maintained there for exactly one minute,

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

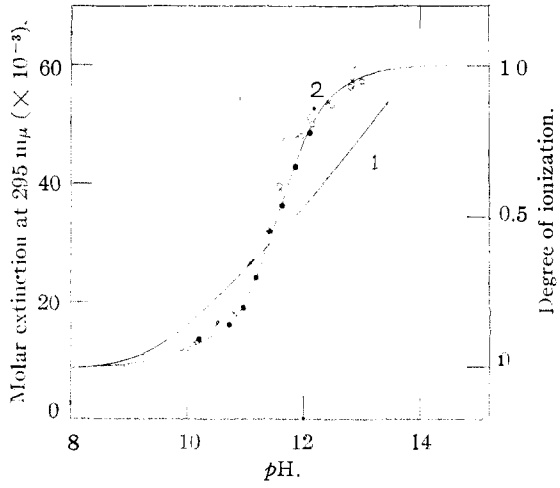

Fig. 4.—Ionization curve for serum albumin at 25°. Curves 1 and 2 are theoretical curves and are discussed in the text.

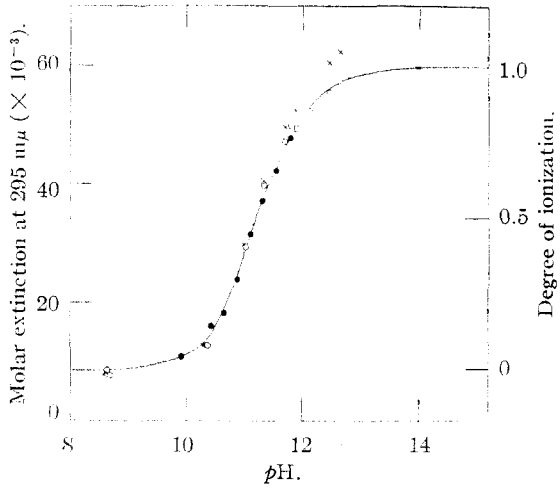

Fig. 5.—Ionization curve for serum albumin at 35°.

which, as has already been indicated, is more than sufficient time for the attainment of ionization equilibrium. Hydrochloric acid was then added to lower the *p*H, and optical density and *p*H readings were obtained by extrapolation. The results are shown by the black circles in Fig. 6. It can be seen that these coincide almost exactly with the open circles of Fig. 6, which represent a regular

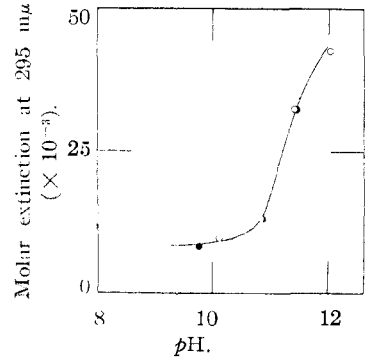

Fig. 6.—Reversibility of phenolic hydroxyl ionization in serum albumin at 25°.

titration with KOH carried out at the same time. It is therefore concluded that the ionization is reversible. It is of special interest that the sharp upswing in the ionization curves near *p*H 11 appears to be reversible.

Computed Ionization Curve.—The method for computing titration curves, taking into account the intrinsic ionization constants of the groups involved, as well as the electrostatic effect due to protein charge, has been described in a previous paper.[22] If a given protein contains n groups of intrinsic ionization constant K_0, the average number of those groups which have lost their proton at a given *p*H, designated by r, is given by the equation

$$\log r/(n - r) = p\text{H} - pK_0 + 2Zw/2.303 \quad (3)$$

where Z is the total charge on the protein molecule at the given *p*H, and w is the usual electrostatic interaction factor which is easily computed if the dimensions of the protein molecule are known. In terms of the degree of ionization, x, which is equal to r/n, this equation becomes

$$\log x/(1 - x) = p\text{H} - pK_0 + 2Zw/2.303 \quad (4)$$

This equation has been used to compute an ionization curve for the phenolic hydroxyl groups of bovine serum albumin at 25°. At ionic strength 0.15 at 25° the value of w is 0.0303.[23] The charge, Z, depends largely on the total number of hydrogen ions dissociated at a given *p*H. This value, for bovine serum albumin, has been assumed the same as for human serum albumin,[22] an assumption which cannot be in error by more than 2 or 3, since the amino acid compositions of the two proteins are almost identical. Binding of chloride ion will also affect the charge. Estimates of the number of chloride ions bound at any *p*H can be made from the data on human and bovine albumin obtained by Scatchard and co-workers.[24,25] It remains only to estimate a "reasonable" value for pK_0. The value for tyrosine (at ionic strength 0.15), 10.05, is unquestionably too high, since the tyrosine molecule, in the *p*H region in which phenolic hydroxyl ionization occurs, bears a negative charge. The value for tyrosylarginine would appear to be a much more reasonable choice. The average of two recorded determinations for this peptide[26] gives pK_0 9.60. The pK for phenol at ionic strength 0.15 is 9.78.[27] Since the peptide links would be expected to lower this, a value of 9.60 for the phenolic groups in albumin therefore appears in accord with this, too.

The curve computed using these figures is shown as curve 1 in Fig. 4. It is quite clear that this curve does not fit the experimental points. A higher value of pK_0 appears to be required to fit the low *p*H portion of the curve. In addition the theoretical curve is much too flat above *p*H 11. It is possible to produce a steeper curve in only one way, namely, by reducing the effect of the electrostatic factor. This is equivalent to saying that the effective charge on the albumin molecule cannot increase beyond *p*H 11 by nearly as much as the hydrogen ion titration curve would lead one to suppose. Accordingly, curve 2 of Fig. 4 has been computed using a pK_0 value of 10.00, and the effective charge values given in Table II. The curve provides an excellent fit of the data. It is seen that the charge values required are just about the same as estimated from the titration curve up to a charge of −59, but no further increase occurs beyond that. It should be emphasized that while these figures may indicate that the charge actually fails to attain its expected value (presumably because of cation binding), it is probable that the low effective charge is caused at least in part by an unfolding or expansion of the molecule, which would result in a failure of the charge actually on the molecule to exert its full electrostatic effect, since the latter is calculated on the basis of a spherical molecule. It should be mentioned that a pK_0 lower than 10.00 could not be used to fit the experimental data. A somewhat higher value could be used, however, in conjunction with charge deviations beginning at a somewhat lower *p*H. A curve computed with a pK_0 value of 10.30, and the effective charge values shown in the last column of Table II would provide about as good a fit of the data as is given by curve 2 of Fig. 5.

TABLE II

EFFECTIVE CHARGE FOR COMPUTED CURVES

*p*H	Charge from titration curve	Effective charge pK_0 10.00	Effective charge pK_0 10.30
8.0	−33	−33	−30
8.5	−35	−35	−31
9.0	−37	−37	−32
9.5	−39	−39	−33
10.0	−44	−44	−34
10.5	−53	−53	−41
11.0	−64	−59	−47
11.5	−77	−59	−47
12.0	−91	−59	−47
12.5	(−101)	−59	−47
13.0	(−107)	−59	−47

These computations are of special interest in that they mark an alkaline limit to the applicability of the Debye–Hückel type of electrostatic correction, approximately where the charge reaches a value of −50. Whatever change in the protein molecule occurs at that point, whether it is an unfolding or cation binding,[28] appears to be a reversible one, as shown by the reversibility experiments described above.

It is of interest in this connection that to fit the hydrogen ion titration curve of human serum albumin on the acid side[22] it is necessary to restrict the effective positive charge to a value of +10. In this case about half of the observed deficiency in charge could be ascribed to chloride binding, the rest being presumably due to deformation.[29]

Klotz and Urquhart[30] have found that the effective charge exerted by 100 and 50 bound dodecyl sulfate anions on iodinated albumins is only 20 and 12, respectively.

An assumption inherent in the preceding discussion is that pK_0 and the absorption characteristics are identical for all the phenolic groups. This assumption is the simplest one which can be made, and there is some evidence for it. If there were to be a range of pK_0 values, one would obtain an ionization curve flatter than theoretical, rather than steeper, as actually observed. If different phenolic groups had different spectra, this would be expected to be accompanied by a difference in thermodynamic properties, *e.g.*, by a gradual change in ΔH from low to high *p*H. Behavior of this type has been observed in studies on insulin, currently in progress in this Laboratory.

Heat and Entropy of Ionization.—The calculation of the apparent heat of ionization is made in

(23) The same value as for human serum albumin, the dimensions of which are the same (ref. 22).

(24) G. Scatchard, A. C. Batchelder and A. Brown, THIS JOURNAL, **68**, 2320 (1946).

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

(26) J. P. Greenstein, *J. Biol. Chem.*, **101**, 603 (1933); J. Melville and G. M. Richardson, *Biochem. J.*, **29**, 187 (1935).

(27) J. W. Murray and N. E. Gordon, THIS JOURNAL, **57**, 110 (1935).

(28) A sharp increase of gegenion binding at some sufficiently high charge would be predicted by the ion association theory of N. Bjerrum (*Kgl. Danske Videnskab. Selskab., Math.-fys. Medd.*, **7**, No. 9 (1926)).

(29) A rough titration curve of the phenolic hydroxyl groups of human serum albumin (ref. 22) shows the same characteristic steepness as that for bovine albumin, *i.e.*, the effective negative charge of human serum albumin is probably limited to about the same value as that of bovine albumin.

(30) I. M. Klotz and J. M. Urquhart, THIS JOURNAL, **73**, 3182 (1951).

the manner already described for tyrosine, and the results are shown in Table III. The apparent heat of ionization obtained from the 15–25° range is the same as that for the 25–35° range, which is evidence for the validity of our method of extrapolation.

TABLE III

HEAT OF IONIZATION OF SERUM ALBUMIN

Degree of ionization	pH 15°	pH 25°	pH 35°	ΔH, kcal./mole 15–25°	ΔH, kcal./mole 25–35°
0.25	11.38	11.09	10.76	11.4	11.4
.50	11.77	11.50	11.20	10.6	12.6
.75	12.29	11.99	11.72	11.8	11.4
				Av.	11.5 ± 1.0

The intrinsic entropy of ionization, $\Delta S^0 = (\Delta H + RT \ln K_0)/T$, is -8 ± 4 e.u. at 25°, the large error being due to the error in the determination of ΔH, rather than the uncertainty in pK_0.

Results with Iodinated Albumin

One possible way to avoid the difficulties encountered with native albumin with respect to the time-dependence of the optical density and pH is to study an iodinated albumin instead, *i.e.*, one in which the tyrosyl residues have been converted into di-iodo tyrosyl residues. The pK of the phenolic hydroxyl group of diiodotyrosine has the low value of 6.48,[21] so that the ionization of the phenolic groups of such a protein should take place in the neutral region where denaturation processes do not occur.

This supposition was confirmed by studies made on a sample of iodinated human serum albumin, kindly supplied to us by Dr. W. L. Hughes, Jr., of Harvard University.[31] Using a wave length of 315 mμ to measure the appearance of the diiodophenoxide ion, we found that buffered solutions showed no change at all in optical density for several hours. Unfortunately, the sample obtained by us, which contained 40.5 atoms of iodine per molecule, appeared to have only 25 to 30 of these incorporated in the tyrosyl residues.[32] In view of the uncertainty of the location of the remaining iodine a detailed analysis of our data was not attempted. However, the heat of ionization of the diiodophenolic groups was determined from values of $\Delta p\text{H}/\Delta(1/T)$ at constant optical density in the region of pH 6 to 8. The value was found to be 7.0 kcal./mole (again with a maximum error of 1 kcal.)

Discussion

One of the most interesting aspects of this problem concerns the possibility that the phenolic hydroxyl groups of serum albumin may participate in hydrogen bonding, and, possibly, help to maintain the native structure of the protein. The suggestion that such hydrogen bonding may be an important factor in protein structure was made originally by Mirsky and Pauling[33] and has recently again been emphasized by Pauling and co-workers[34]

(31) W. L. Hughes, Jr., and R. Straessle, *ibid.*, **72**, 452 (1950).

(32) A similar observation on a similar sample of iodinated albumin has been made by Klotz (Ref. 30).

(33) A. E. Mirsky and L. Pauling, *Proc. Natl. Acad. Sci.*, **22**, 439 (1936).

(34) L. Pauling, R. B. Corey and H. R. Branson, *ibid.*, **37**, 205 (1951); L. Pauling and R. B. Corey, *ibid.*, **37**, 235 *et seq.*, (1951).

That the phenolic hydroxyl groups of egg albumin are involved in such hydrogen bonding was indicated by the work of Crammer and Neuberger previously referred to,[4] who showed that ionization of these groups and irreversible denaturation of egg albumin occur simultaneously. In a few studies made on egg albumin in this Laboratory the findings of Crammer and Neuberger have been qualitatively confirmed.

In serum albumin, the situation is quite different from egg albumin, in that the ionization of the phenolic groups is reversible. However, the data obtained in this paper would seem to indicate that the phenolic groups are nonetheless strongly hydrogen-bonded to some other groups as yet unknown.

The principal factor pointing in this direction is the high heat of ionization observed by us. In Table IV are summarized the heats of ionization for phenolic hydroxyl groups in a number of compounds. Whereas the normal value appears to be near 6 kcal./mole, that for serum albumin is 11.5 kcal./mole.

TABLE IV

HEATS OF IONIZATION OF PHENOLIC GROUPS, KCAL./MOLE

Phenol[a]	6.1
Tyrosine[b]	6.0
Aspartyltyrosine[c]	6.2
Tyrosylarginine[c]	6.0
Serum albumin[b]	11.5
Pepsin[d]	6.0
Diiodotyrosine[e]	0.8
In iodinated albumin[b]	7.0

[a] Landolt–Börnstein "Tabellen." [b] This paper. [c] Reference 26. [d] Preliminary value obtained in this Laboratory. [e] Reference 21.

It can easily be shown both theoretically and from related experimental data that this high value of the heat of ionization cannot be due to the effect of electrostatic charge. The difference between the apparent and intrinsic ionization constants of any group is merely the contribution of the electrostatic term in equation (3)

$$pK - pK_0 = -2Zw/2.303 \qquad (5)$$

Differentiation with respect to reciprocal temperature will give the difference between the apparent and intrinsic heats of ionization

$$\Delta H - \Delta H_0 = 2.303R \frac{\partial}{\partial(1/T)}(pK - pK_0) = -2R[\partial(Zw)/\partial(1/T)] \qquad (6)$$

Noting that ΔH values are always obtained at constant degree of ionization, *i.e.*, constant charge,[35] we can compute the value of the right-hand side of equation (6) with the aid of Wyman's empirical equation for the temperature dependence of the dielectric constant of water.[36] For bovine serum albumin at ionic strength 0.15, $\Delta H - \Delta H_0$ turns out to have a value of less than 1 kcal./mole even if the effective negative charge reaches 100, which, we have shown, is never true.

(35) In view of the fact that the amino groups of albumin have about the same heat of ionization as the phenolic groups, the derivative at constant degree of ionization is probably also a derivative at constant total charge. However, the possibility of error on this point undoubtedly exists.

(36) J. Wyman, Jr., *Phys. Rev.*, **35**, 623 (1930).

This theoretical conclusion is easily confirmed experimentally. Apparent heats of ionization of carboxyl, imidazole and amino groups have been obtained for human serum albumin[22] and oxyhemoglobin.[37] The values in each case were found to agree, within experimental error, with the normal heats of ionization of the groups involved, even though the protein charge undoubtedly reached high values.[38]

Since electrostatic forces appear thus unable to account for the high heat of ionization, it is necessary to seek a chemical explanation, and hydrogen bonding is the logical choice. The difference observed, about 6 kcal., is very close to the average value of the heat required to break virtually all known O—H · · · O and O—H · · · N bonds.[39]

It is noteworthy that the heat of ionization of iodinated albumin is also higher than the normal heat of ionization of a di-iodophenolic group (Table IV), and again by about 6 kcal./mole. It is also of great interest that preliminary data with pepsin indicate that its phenolic groups have a "normal" heat of ionization.

The entropy values would also seem to support the existence of hydrogen bonding. The value in serum albumin (−8 e.u. at 25°) is much more positive than that in tyrosine (−26 e.u.) or in phenol (−24 e.u.), indicating the destruction of an element of order in the protein which is not present in tyrosine or phenol. (The negative value for the over-all process merely reflects the organization of water molecules around the newly-formed phenoxide ion.)

Hydrogen bonding is perhaps also indicated by the fact that the intrinsic pK_0 for ionization is 10.00 or greater, whereas a reasonable "normal" value is 9.60. Even more suggestive is the fact that the absorption peak for serum albumin in acid or neutral solution lies at 279–280 mμ, whereas that for tyrosine itself (and that for phenol) lies near 275 mμ. The peak in alkaline solution, on the other hand, occurs at 292.5 mμ both for serum albumin itself and for tyrosine.

Finally, however, it should be pointed out that even though the tentative conclusion has been reached here, that the phenolic groups of serum albumin are strongly hydrogen bonded to some other groups in the molecule, the evidence does *not* indicate that these bonds are concerned with maintaining the native structure of the molecule, for the ionization process occurs both instantaneously and reversibly. The difference between egg albumin and serum albumin in this respect has already been pointed out above. The kinetic studies on the alkaline denaturation of pepsin and ricin, made by Steinhardt[40] and Levy and Benaglia[41] are of interest in this connection, for they suggest that only a very small number of specific hydrogen bonds are involved in maintaining the native structure of the protein molecules. It would appear that in egg albumin some of the hydrogen bonds involving the phenolic groups are among these vital few; in serum albumin, however, they are not, and apparently can be broken without causing denaturation.

(37) J. Wyman, Jr., *J. Biol. Chem.*, **127**, 1 (1939).

(38) The observation that electrostatic charge has only a minor effect on the heat of ionization appears also to hold true for small molecules. The ionization constant of a carboxyl group (and therefore the free energy of ionization) can be changed considerably by electrostatic charge. In histidine, glycylglycine, acetic acid and hydrogen succinate, for example, we have, respectively, charges of +2, +1, 0 and −1 on the molecule prior to ionization of the carboxyl groups. The corresponding pK values are accordingly very different: being 1.82, 3.08, 4.76 and 5.55, respectively. The heats of ionization, however, have, within 1 kcal., the same value in all four cases (*cf.* E. J. Cohn and J. T. Edsall, "Proteins, Amino-Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, chapters 4 and 5).

(39) See, for example, M. L. Huggins, *J. Org. Chem.*, **1**, 487 (1937).

(40) J. Steinhardt, *Kgl. Danske Videnskab. Selskab, Math.-fys. Medd.*, **14**, No. 11 (1937).

(41) M. Levy and A. E. Benaglia, *J. Biol. Chem.*, **186**, 829 (1950).

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[CONTRIBUTION FROM THE LABORATORIES OF THE ROCKEFELLER INSTITUTE FOR MEDICAL RESEARCH]

The Partial Specific Volumes, in Aqueous Solution, of Three Proteins

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With the aid of the magnetic float method the densities at 25° of aqueous solutions of crystalline ovalbumin, crystalline bovine serum albumin and bovine γ-globulin have been determined. From these data the apparent specific volumes have been computed. Since these values are constant, in the concentration range studied, they are also the partial specific volumes of these proteins in aqueous solution.

In the sedimentation velocity method for the determination of the molecular weights, M, of proteins, with the ultracentrifuge, the familiar equation

$$M = \frac{sRT}{D(1 - \bar{V}\rho)}$$

is employed. Here s is the sedimentation constant, D, the diffusion coefficient, $\bar{V}$, the partial specific volume and ρ, the density of the solution. An inspection of this equation indicates that the accuracy of the determinations of M are particularly sensitive to errors in the product $\bar{V}\rho$. Since that product has the order of magnitude of 0.8, such errors are multiplied by four in their effects on M. Molecular weights obtained by the sedimentation equilibrium method are equally affected by these errors. In spite of this fact, comparatively little attention has been paid to improvement of the methods of determining the densities of protein solutions, from which the partial specific volumes are obtained. In many cases in which molecular weights have been reported, average or assumed values of $\bar{V}$ have been employed in the computations.