has recently been derived independently by Strehlov and Stackelberg.[11] This derivation, which we understand will soon be published, follows the general pattern of Ilkovic's original derivation[1] and that of MacGillavry and Rideal[2] but retains in the intermediate mathematical operations those terms, neglected in the original derivations, which represent the curvature of the electrode surface.

Summary

The following new equation has been derived for the diffusion current observed with the dropping mercury electrode at 25°

$$i_d = 607nD^{1/2}Cm^{2/3}t^{1/6}\left(1 + \frac{39D^{1/2}t^{1/6}}{m^{1/3}}\right)$$

This equation differs from the familiar Ilkovic equation by the term in parentheses, which has a value in the neighborhood of 1.1 with capillaries of the usual characteristics. This term represents the curvature of the electrode surface which the Ilkovic equation neglects. The new equation accounts quantitatively for the experimentally observed linear increase in the diffusion current "constant" $i_d/Cm^{2/3}t^{1/6}$ with increasing values of the ratio $t^{1/6}/m^{1/3}$. From the new equation the true diffusion current constant is $i_d/Cm^{2/3}t^{1/6}(1 + 39D^{1/2}t^{1/6}m^{-1/3})$.

(11) Private communication from Dr. H. Strehlov and Prof. M. v. Stackelberg received after the conclusions in this paper were formulated.

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[CONTRIBUTION FROM THE HARVARD MEDICAL SCHOOL]

Preparation and Properties of Serum and Plasma Proteins. XXIII. Hydrogen Ion Equilibria in Native and Modified Human Serum Albumins[1a,1b]

BY CHARLES TANFORD[1c,1d]

Introduction

The use of electromotive force measurements to obtain information about the acid- and base-binding groups of proteins dates back to 1898,[2] and the first measurements on bovine serum albumin were made in 1913.[3,4] The study on human serum albumin reported in this paper was undertaken because it is now possible to interpret such electromotive force measurements with far greater certainty than ever before, for the following reasons: (1) Human serum albumin preparations of high purity and consistent properties are now available. (2) The work of Linderstrøm-Lang and Scatchard has provided a theoretical basis for an understanding of the binding of small ions by proteins in terms of fundamental equilibrium constants. (3) Interaction studies on ions other than hydrogen or hydroxyl ions have been made, and their effect on the hydrogen ion equilibria can be evaluated. (4) Well-defined chemical modifications of human serum albumin have been prepared in this Laboratory, the hydrogen ion titration curves of which should differ in a predictable manner from native albumin.

(1a) This paper is Number 80 in the series "Studies on the Plasma Proteins" from the Harvard Medical School, Boston, Massachusetts, on products developed by the Department of Physical Chemistry from blood collected by the American Red Cross.

(1b) This work was originally supported by grants from the Rockefeller Foundation and from funds of Harvard University. It was aided early in 1941 by grants from the Committee on Medicine of the National Research Council which included a grant from the American College of Physicians. From August, 1941, to July, 1946, it was carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. Since then it has been aided by a grant recommended by the Panel on Hematology of the National Institutes of Health.

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(1d) Some of the initial experiments leading up to this work were performed while the author held a Lalor Foundation Post-doctoral Fellowship (1947-1948) at Harvard Medical School.

(2) S. Bugarszky and L. Liebermann, *Pflügers Arch.*, **72**, 51 (1898).

(3) K. Manabe and J. Matula, *Biochem. Z.*, **52**, 369 (1913).

(4) A complete review of early work in this field is given by E. J. Cohn, *Physiol. Rev.*, **5**, 349 (1925); see also ref. 17.

Materials and Methods

Human Serum Albumin.—Two preparations of human serum albumin were used, one crystallized from decanol (preparation decanol-10),[5] the other first crystallized as the mercury dimer, from which mercury has been subsequently removed.[6] The latter preparation, which has one free sulfhydryl group per molecule, has been given the name mercaptalbumin. Stock solutions of the protein were prepared by dissolving crystals in water. The solutions were exhaustively electrodialyzed, and the protein concentrations (in grams per kg. of water) were measured by heating at 110° to constant weight. The molecular weight of human serum albumin (of both preparations) is known to be 69,000.[7]

Guanidinated Albumin.—Preparation U of Hughes, Saroff and Carney, made from decanol-crystallized albumin[8] has been used. The maximum number of free amino groups are guanidinated in this preparation. Stock solutions of this protein could not be electrodialyzed because it is insoluble at its isoionic point. They were instead dialyzed against 0.15 *M* sodium chloride, and protein concentrations were measured as the difference between the dry weights of the protein solution and the dialyzing solution. The molecular weight of the guanidinated albumin, calculated from the extent of guanidination, is 71,500.

Iodinated Albumin.—A preparation of iodinated albumin prepared from decanol crystals, and containing 17 atoms of iodine per mole, was obtained from Drs.

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

(6) W. L. Hughes, Jr., *ibid.*, **69**, 1836 (1947).

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

(8) W. L. Hughes, Jr., H. A. Saroff and A. L. Carney, THIS JOURNAL, **71**, 2476 (1949).

Hughes and Straessle.[9] Stock solutions were made up and electrodialyzed, as for native albumin. The calculated molecular weight of this protein is 71,000.

Acid, Base and Salt.—Stock solutions of hydrochloric acid, sodium hydroxide and sodium chloride were made up from C. P. reagents. The composition of the solutions was determined on a weight basis.

Hydrogen.—Tank hydrogen was used, and purified by passage through a "Deoxo" hydrogen purifier.[10]

E. m. f. Measurement.—The system employed for electromotive force measurements consisted of a hydrogen half-cell, a saturated potassium chloride bridge, and a 0.1 *N* calomel half-cell. The latter was maintained at 25°. The hydrogen electrode vessels were of the rocking type developed by Clark,[11] but were modified in that the main body of the vessels, between the two stopcocks, was jacketed. Water from large reservoirs, maintained at 0, 5, 25 and 37.8°, could be pumped through the jackets. The liquid junction between the protein solution and the potassium chloride bridge was made in the stopcock at the time of the measurement. Thirty minutes of rocking was allowed for attainment of equilibrium.

Ultraviolet Light Absorption.—Light absorption measurements were made in a Beckman spectrophotometer, model DU, using a light path of 1 mm. The quartz cells and inserts were calibrated before use.

Hydrogen Electrode Potentials and Their Interpretation

The determination of a single ion activity from an electromotive force requires a non-thermodynamic assumption. We have chosen to assume that the liquid junction potentials are independent of the solution whose *p*H is being measured, and therefore constant at any temperature. The equation relating *p*H to electromotive force then becomes

$$p\mathrm{H} = -\log a_{\mathrm{H}^+} = (E - E_0')/2.303\,(RT/F) \quad (1)$$

where E_0' is the sum of all the constant potentials in the cell, including the liquid junction potentials.[12] E_0' has been evaluated at each temperature by use of solutions of known *p*H, *viz.*, Bureau of Standards phthalate and borate buffers,[13] and 0.1 *N* hydrochloric acid.[14] At 25° identical values for E_0' were obtained with all of the solutions used.[15]

The *p*H is related to the molality of free hydrogen or hydroxyl ion by the equation

$$p\mathrm{H} = -\log m_{\mathrm{H}^+}\,\gamma'_{\mathrm{H}^+} = \log (m_{\mathrm{OH}^-}\,\gamma'_{\mathrm{OH}^-}/K_{\mathrm{W}}) \quad (2)$$

where the values of K_{W} obtained by Harned and co-workers have been used.[16] The activity coefficients have been designated γ' because they are different from activity coefficients determined by any other method, or with other assumptions about liquid junction potentials and about K_{W}.

To obtain the molality of free hydrogen or hydroxyl ion in a solution containing protein, it is assumed that the activity coefficients depend only upon the non-protein components of the system.[17] In our solutions, in other words, where sodium chloride and hydrochloric acid or sodium hydroxide are the only non-protein solutes present, the activity coefficients are taken as a function only of the amounts of these components, independent of whether any protein is present, and of whether any of the added ions are bound to the protein. This assumption, although certainly inexact, leads to remarkably consistent results, especially in the presence of added salt and and at reasonably moderate protein concentrations.[18]

Activity coefficients in the absence of protein have been determined by measuring the *p*H of solutions of known molalities of hydrochloric acid or sodium hydroxide at a total ionic strength of 0.150. These coefficients for rounded values of the activities are listed in Tables I and II, and compared with values previously determined with a similar experimental arrangement by Green.[19]

The computation of the number of moles of acid or base bound per mole or gram of protein at any *p*H is now obvious since it is simply the difference between the acid or base originally added to a solution, and that found present in the free state.

It should be noted that the amount of free H^+ or OH^- is significantly large only at relatively low or relatively high *p*H values, so that the fact that no activity coefficients can be obtained where *p*H or *p*OH > 3 (because of the fact that such solutions in the absence of protein would be very much unbuffered) does not matter much. We have as-

TABLE I

HYDROGEN ION ACTIVITY COEFFICIENTS AT 25° (Γ/2 0.150)

*p*H	$-\log \gamma'_{\mathrm{H}^+}$	Values of A. A. Green
1.5	0.065	0.052
2.0	.055	.046
2.5	.047	...
3.0	.047	...

TABLE II

HYDROXYL ION ACTIVITY COEFFICIENTS (Γ/2 0.150)

*p*OH	$-\log \gamma'_{\mathrm{OH}^-}$ 0°	$-\log \gamma'_{\mathrm{OH}^-}$ 37.8°	$-\log \gamma'_{\mathrm{OH}^-}$ 25°	Values of Green, 25°
1.5	...	...	...	0.210
2.0	0.238	0.204	0.205	.210
2.5	.252	.217	.220	.220
2.8	.309	.240	.248	...

(9) W. L. Hughes, Jr., and R. Straessle, THIS JOURNAL, **72**, 452 (1950).

(10) From Baker and Co., Inc., 113 Astor St., Newark 5, N. J.

(11) W. M. Clark, "The Determination of Hydrogen Ions," Williams and Wilkins Co., Baltimore, Md., 1920, p. 128.

(12) See D. A. MacInnes, "The Principles of Electrochemistry," Reinhold Publishing Corp., New York, N. Y., 1939, Chapter 15.

(13) W. J. Hamer, G. D. Pinching and S. F. Acree, *J. Res. Nat. Bur. Stand.*, **36**, 47 (1946); G. G. Manov, N. J. De Lollis, P. W. Lindvall and S. F. Acree, *ibid.*, **36**, 543 (1946).

(14) G. Scatchard, THIS JOURNAL, **47**, 696 (1925).

(15) Detailed experimental data on this matter, and complete calculations of the titration curves to be discussed subsequently, can be obtained from the American Documentation Institute, 1719 N Street, N.W., Washington 6, D. C., by ordering Document 2690, remitting 50¢ for microfilms or $1.40 for photoprints.

(16) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold Publishing Corp., New York, N. Y., 1943, p. 485.

(17) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 454.

(18) R. K. Cannan, A. Kibrick and A. H. Palmer, *Ann. N. Y. Acad. Sci.*, **41**, 243 (1941).

(19) A. A. Green, THIS JOURNAL, **60**, 1108 (1938). These values refer to the molarity rather than the molality scale, but the difference should be very small.

sumed that the activity coefficients remain constant from *p*H or *p*OH = 3 to neutrality.

Probable Error

Electromotive force measurements on the acid side at 25° are usually reproducible to 0.2 mv., *i. e.*, to 0.003 *p*H unit. This means that there will be an error of 0.003 in the logarithm of the molality of free acid, *i. e.*, an error of somewhat less than 1%. In a 0.5% protein solution near *p*H 3 the amount of free acid will be only about 20% of the total added to achieve that *p*H. Hence the error in the amount of bound acid will be 0.2% or less. At *p*H 2 in a 0.5% protein solution, however, one-half or somewhat more of the added acid is free, so that the error in computing the bound acid now becomes 1% or a little more. In a 1% protein solution more acid is bound in proportion and the error is reduced to less than 1%. The accuracy is slightly less good at temperatures other than 25°, as well as on the alkaline side. Here, however, there is in any case a greater error due to instability (see below). The error in computing bound base should be less than 2% below *p*H 12, however.

Between *p*H 5.5 and about *p*H 9, serum albumin is a relatively poor buffer, and *p*H measurements are less reproducible than elsewhere. Deviations of several hundredths of a *p*H unit have been observed.

Stability and Reversibility

All solutions acid to the isoionic point were found to be unchanged in *p*H after twenty-four hours of standing. This was observed even for solutions with *p*H below 2. Furthermore, the titration curves on that side appear to be perfectly reversible.

On the basic side of the isoionic point we have, first of all, the weakly buffered region previously referred to, in which a certain amount of *p*H drift is always present, such that an observed *p*H may be in error by several hundredths of a *p*H unit. At or above *p*H 11 this drift virtually disappears. Whenever observed, however, the *p*H drift has *always* been toward the isoionic point. Furthermore, whenever solutions have given points falling off the best curve through the majority of the points the displacement has always been in this same direction. It would appear then that an irreversible process consuming hydroxyl ions must be occurring. Attempts to investigate this process further have so far been bafflingly fruitless.

Within the 0.02 *p*H unit or so which must be considered the probable error over most of the basic side of the titration curves, these curves have been found to be reversible.

It is interesting to observe, in particular, that the tyrosine ionization, as followed by the ultraviolet absorption spectrum of the protein solution, is perfectly reversible up to *p*H 12, over a period comparable to that required for a measurement. Some pertinent absorption curves are shown in Fig. 1. This observation is quite contrary to that found for egg albumin by Crammer and Neuberger,[20] but corresponds to their findings for insulin.

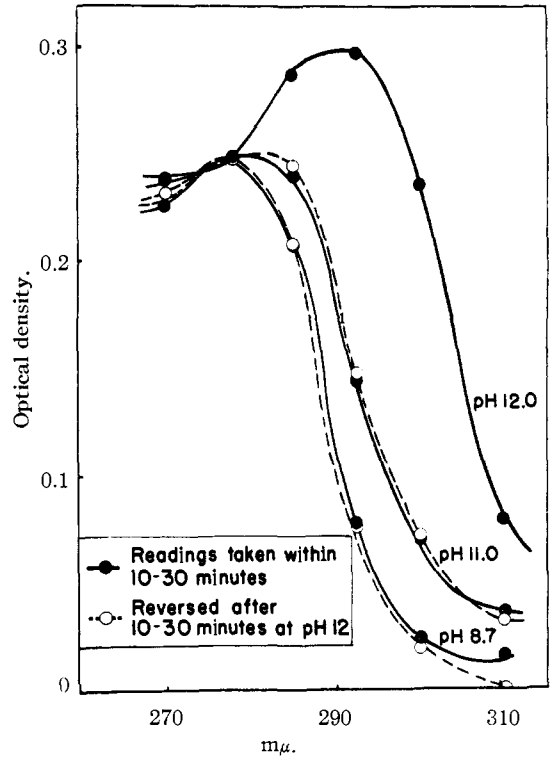

Fig. 1.—Reversibility of tyrosine ionization (decanol crystals) at 25°.

Determinations on Human Serum Albumin

Experimental results for human serum albumin are given in Tables III to VI. The starting materials used were electrodialyzed preparations (*i. e.*, isoionic). The tables give the number of moles of hydrogen or hydroxyl ion bound per mole (69,000 grams) of protein as a function of *p*H. Most of the points are averages of duplicate determinations. Detailed data and calculations can be obtained elsewhere.[21] It will be shown subsequently that all isoionic preparations of human serum albumin studied are capable of binding just 100 hydrogen ions per mole. By the simple numerical addition of 100 we can accordingly refer the state of the protein to the *p*H of maximum binding rather than to the isoionic point. These figures are the most convenient for subsequent computation and are given in the last columns of Table III to VI. It should be noted that data at temperatures other than 25° are not of interest below *p*H 4, and none have therefore been obtained.

Figure 2 is a plot of all points at 25° obtained with decanol-crystallized serum albumin, as well

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

(21) See Ref. 15.

TABLE III

HUMAN SERUM ALBUMIN (DECANOL-10), 25°, Γ/2 0.150

Protein, g./kg. H_2O	H^+ added, moles/kg. H_2O $\times 10^3$	pH	Free H^+, moles/kg. H_2O $\times 10^3$	Bound H^+, moles/mole protein	Moles H^+ dissociated per mole protein from pH of max. acid binding
5.14	18.20	2.018	10.87	98.5	1.5
5.15	13.74	2.248	6.34	99.0	1.0
5.14	9.06	2.686	2.28	91.1	8.9
5.14	5.09	3.491	0.35	63.6	36.4
5.16	2.54	4.057	.10	32.6	67.4
5.14	0.00	5.270	.00	0.0	100.0
	OH^- added, moles/kg. H_2O $\times 10^3$		Free OH^-, moles/kg. H_2O $\times 10^3$	Bound OH^-, moles/mole protein	
5.14	1.83	8.411	0.00	24.5	124.5
5.15	2.56	9.531	.06	33.5	133.5
5.11	3.78	10.294	.36	46.2	146.2
5.13	5.13	10.730	.96	56.0	156.0
5.15	6.39	11.022	1.88	60.3	160.3
5.15	7.68	11.180	2.72	68.1	168.1
5.15	10.30	11.472	4.98	71.1	171.1
5.14	12.82	11.629	7.01	77.8	177.8

TABLE IV

HUMAN SERUM ALBUMIN (MERCAPTALBUMIN), 25°, Γ/2 0.150

Protein, g./kg. H_2O	H^+ added, moles/kg. H_2O $\times 10^3$	pH	Free H^+, moles/kg. H_2O $\times 10^3$	Bound H^+, moles/mole protein	Moles H^+ dissociated per mole protein from pH of max. acid binding
10.33	37.12	1.712	22.27	99.3	0.7
7.81	20.24	2.096	9.07	98.7	1.3
5.15	9.17	2.668	2.35	91.4	8.6
4.92	2.53	4.066	0.10	34.2	65.8
4.89	1.53	4.358	.05	20.9	79.1
4.91	1.02	4.586	.03	13.9	86.1
4.92	0.50	4.844	.02	6.8	93.2
5.17	0.00	5.169	.00	0.0	100.0
	OH^- added, moles/kg. H_2O $\times 10^3$		Free OH^-, moles/kg. H_2O $\times 10^3$	Bound OH^-, moles/mole protein	
4.96	0.52	5.704	0.00	7.3	107.3
5.16	0.51	5.790	.00	6.8	106.8
5.15	1.01	6.766	.00	13.5	113.5
5.16	1.02	6.838	.00	13.6	113.6
5.18	1.52	7.580	.00	20.3	120.3
5.15	2.03	8.404	.01	27.0	127.0
5.27	2.09	8.408	.01	27.3	127.3
5.18	2.52	9.228	.03	33.1	133.1
5.12	3.52	9.942	.16	45.3	145.3
5.18	3.57	9.951	.16	45.4	145.4
5.18	5.13	10.660	.81	57.5	157.5
5.18	7.67	11.096	2.23	72.5	172.5
7.87	19.18	11.688	7.98	98.2	198.2

as with mercaptalbumin. In addition the determinations made by Mrs. Blanchard in this Laboratory some years ago,[22] on decanol-crystallized albumin are included. The curve drawn through these points is a theoretical curve which will be discussed later.

(22) M. H. Blanchard, unpublished results.

TABLE V

HUMAN SERUM ALBUMIN (MERCAPTALBUMIN), 0°, Γ/2 0.150

Protein, g./kg. H_2O	H^+ added, moles/kg. H_2O $\times 10^3$	pH	Free H^+, moles/kg. H_2O $\times 10^3$	Bound H^+, moles/mole protein	Moles H^+ dissociated per mole protein from pH of max. acid binding
4.92[a]	2.53	4.113	0.09	34.4	65.6
4.89[a]	1.53	4.400	.05	21.0	79.0
4.91[a]	1.02	4.645	.03	14.0	86.0
4.92[a]	0.50	4.891	.02	6.9	93.1
5.17	0.00	5.303	.00	0.0	100.0
	OH^- added, moles/kg. H_2O $\times 10^3$		Free OH^-, moles/kg. H_2O $\times 10^3$	Bound OH^-, moles/mole protein	
4.96[a]	0.52	5.819	0.00	7.3	107.3
5.15	1.01	7.055	.00	13.5	113.5
5.18	1.52	7.852	.00	20.3	120.3
5.15	2.03	8.920	.00	27.1	127.1
5.27	2.09	8.897	.00	27.3	127.3
5.12	3.52	10.659	.10	46.0	146.0
5.18	5.13	11.400	.57	60.6	160.6
5.18	7.67	11.866	1.05	88.3	188.3
7.87	19.18	12.449	5.66	118.5	218.5

[a] Run at 5°.

TABLE VI

HUMAN SERUM ALBUMIN (MERCAPTALBUMIN), 37.8°, Γ/2 0.150

Protein, g./kg. H_2O	H^+ added, moles/kg. H_2O $\times 10^3$	pH	Free H^+, moles/kg. H_2O $\times 10^3$	Bound H^+, moles/mole protein	Moles H^+ dissociated per mole protein from pH of max. acid binding
4.92	2.53	4.123	0.08	34.4	65.6
4.89	1.53	4.383	.05	21.0	79.0
4.91	1.02	4.592	.03	13.9	86.1
4.92	0.50	4.830	.02	6.9	93.1
5.17	0.00	5.158	.00	0.0	100.0
	OH^- added, moles/kg. H_2O $\times 10^3$		Free OH^-, moles/kg. H_2O $\times 10^3$	Bound OH^-, moles/mole protein	
4.96	0.52	5.671	0.00	7.3	107.3
5.16	0.51	5.716	.00	6.8	106.8
5.15	1.01	6.660	.00	13.5	113.5
5.18	1.52	7.367	.00	20.3	120.3
5.15	2.03	8.050	.01	27.0	127.0
5.27	2.09	8.157	.01	27.3	127.3
5.18	2.52	8.802	.03	33.2	133.2
5.12	3.52	9.507	.14	45.4	145.4
5.18	3.57	9.593	.17	45.2	145.2
5.18	5.13	10.255	.50	61.7	161.7
5.18	7.67	10.706	2.24	72.4	172.4
7.87	19.18	11.286	6.30	113.0	213.0

It is apparent that the author's points for decanol crystallized albumin coincide almost exactly with those obtained by Mrs. Blanchard. The

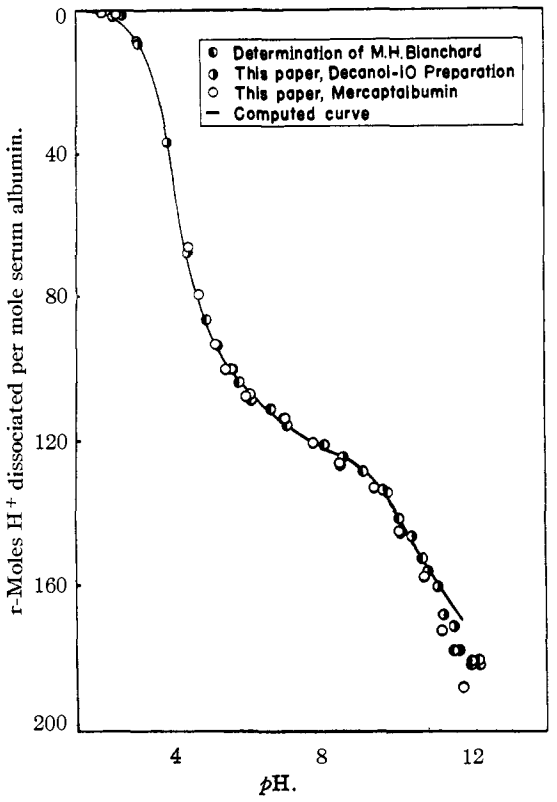

Fig. 2.—Titration curve of human serum albumin at 25°, Γ/2 0.150.

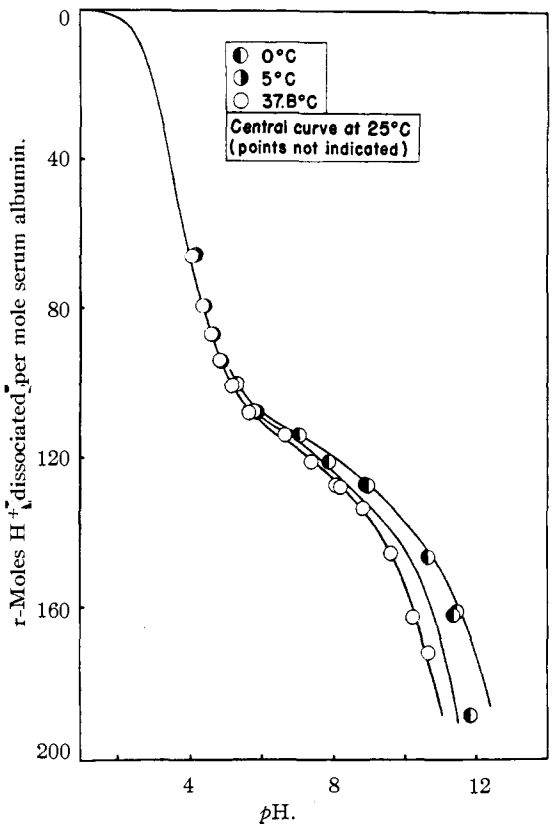

Fig. 3.—Titration curves of human serum albumin (mercaptalbumin) at different temperatures, Γ/2 0.150.

points for mercaptalbumin fall a little below those for decanol-crystallized albumin on the basic side.

Figure 3 is the best experimental plot of the data for mercaptalbumin at 0, 25 and 37.8°. (A few of the points are at 5°.) Figure 4 is a plot of the apparent heat of ionization as a function of the state of ionization of the albumin. The apparent heat of ionization is defined as $\Delta H_{ion} = \left(\partial p\mathrm{H}/\partial\frac{1}{T}\right)_r$ *i. e.*, the values plotted are $\Delta p\mathrm{H}/\Delta\frac{1}{T}$ at fixed values of r, the number of moles of hydrogen ion dissociated from the reference point of maximum acid binding. Near the isoionic point no change in r takes place as the temperature of a given solution changes. The values of ΔpH are therefore those obtained from single solutions whose pH was measured very accurately at different temperatures. On the basic side, however, there is encountered first the region of low buffering capacity and pH instability already referred to. Moreover, a point is soon reached where r changes with temperature for any one solution. For $r > 110$, therefore, the ΔpH values have been taken from the smooth curves of Fig. 3. Between $r = 120$ and $r = 140$, say, the ΔH values must be considered very unreliable, because of the pronounced pH drift in that region, which would tend to introduce an error into the curves of Fig. 3, particularly those at 0 and 37.8°.

The observed heat of ionization for the range 0 or 5–25° differs from that for the range 25–37.8°. This is to be expected, as will be discussed below, because of the difference in heat capacity between the ionized and un-ionized forms of the ionizing groups.

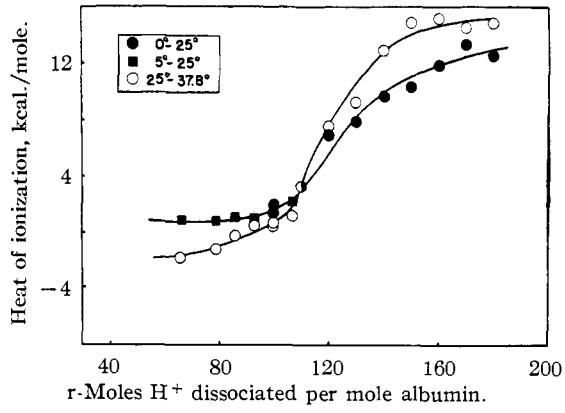

Fig. 4.—Apparent heat of ionization of human serum albumin (mercaptalbumin).

Figure 5 shows the change in the ultraviolet absorption spectrum of decanol-crystallized serum albumin. This represents *in part* the effect of the ionization of the phenolic hydroxyl groups of tyrosine. There is a complicating factor, however,

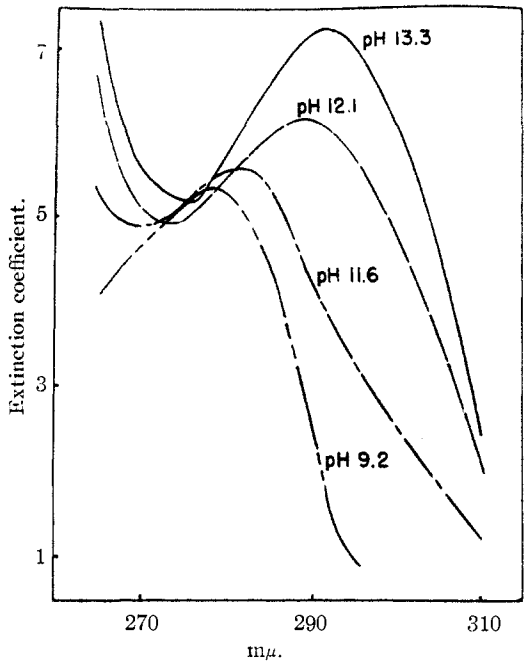

Fig. 5.—Ultraviolet absorption spectrum of human serum albumin (decanol crystals).

in that an appreciable fraction of the absorption is due to tryptophan. In addition, though we are primarily concerned with the peak at 279 mμ, some contribution certainly arises from peaks at lower wave length. If the simplifying assumption is made that the coefficient of absorption at 293 mμ (the peak at the highest *p*H) is a measure of the ionization, the "titration curve" for the phenolic OH of albumin given in Fig. 6 is obtained. The point of inflection of this curve, *i. e.*, the *pK* of the group, is at *p*H 11.7, a very reasonable value. The curve is steeper, however, than one would expect on the basis of theory, taking electrostatic effects into account. In view of the naive manner in which the curve has been constructed, no significance should be attached to this fact at this time.

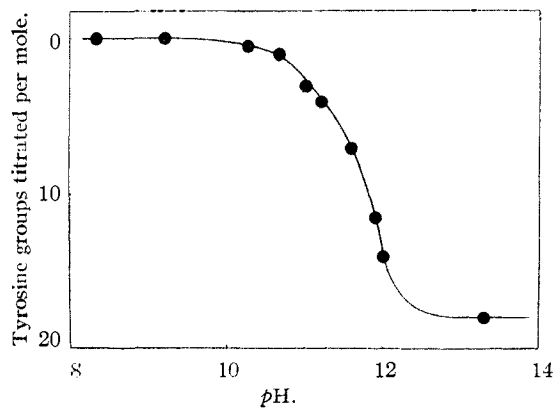

Fig. 6.—Tyrosine titration in human serum albumin (decanol crystals) at 25°.

Determinations on Modified Albumins

Table VII and Fig. 7 give data on guanidinated serum albumin, at 25°. These data were obtained with slightly less precision than others discussed in this paper in that solutions were made up by volume instead of by weight. The difference between the curves for guanidinated and or-

TABLE VII

GUANIDINATED ALBUMIN, 25°, Γ/2 0.150

Protein, g./kg. H_2O	H^+ added, moles/kg. H_2O × 10^3	*p*H	Free H^+, moles/kg. H_2O × 10^3	Bound H^+, moles/mole protein	Moles H^+ dissociated per mole protein from *p*H of max. acid binding
11.37	41.74	1.691	23.44	115.2	1.8
11.37	32.55	1.894	14.58	113.0	4.0
9.50	22.97	2.153	7.92	113.2	3.8
4.74	6.69	3.202	0.69	90.4	26.6
4.74	3.34	4.15	.08	49.2	67.8
4.73	0.42	6.598	.00	6.3	110.7
4.73	0.00	7.400	.00	0.0	117.0
	OH^- added, moles/kg. H_2O × 10^3		Free OH^-, moles/kg. H_2O × 10^3	Bound OH^-, moles/mole protein	
4.73	0.41	8.566	0.01	6.1	123.1
4.73	0.82	9.652	.08	11.2	128.2
4.73	1.63	10.438	.49	17.3	134.3
4.73	3.27	11.009	1.83	21.8	138.8
4.74	5.73	11.398	4.26	22.2	139.2
4.74	8.20	11.544	5.82	35.8	152.8
9.54	25.33	12.104	20.28	38.2	155.2

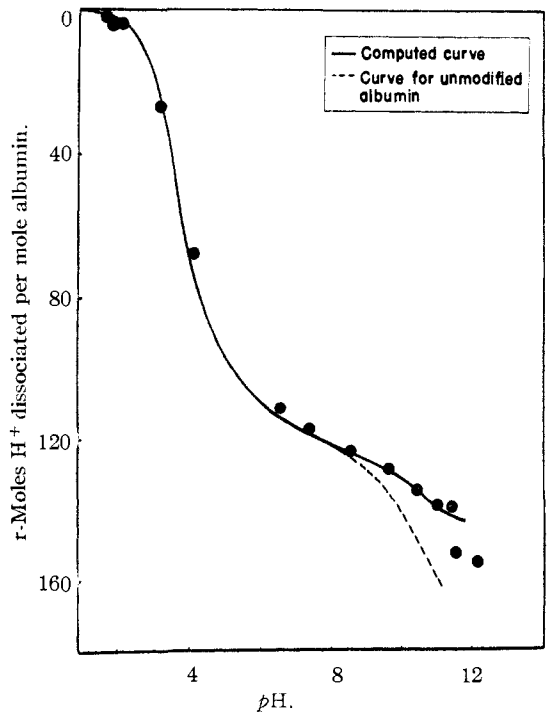

Fig. 7.—Titration curve of guanidinated serum albumin at 25°, Γ/2 0.150.

dinary albumin, as shown by Fig. 7, is so striking, however, that the decrease in precision is not important. Since this particular albumin is quite insoluble at its isoionic point, the starting material was not electrodialyzed. A solution made up to ionic strength of 0.150 without addition of acid or base had a *p*H of 7.400. For the unmodified decanol crystals 117 hydrogen ions must be bound to attain maximum acid binding from *p*H 7.40: it has been assumed that the same figure will apply to the guanidinated albumin. This figure certainly cannot be in error by more than 2 groups.

The guanidinated albumin is insoluble in 0.15 molar salt from *p*H 3.5 to 5.5. There is one point in this region on the curve, where the *p*H was obtained from a glass electrode measurement on the protein suspension. The curve in Fig. 7 is again a theoretical curve (see discussion below). The dotted line is the curve for unmodified serum albumin (decanol crystals).

Table VIII and Fig. 8 give data on an iodinated serum albumin containing 17 atoms of iodine per mole of albumin. The curve in Fig. 8 is that for unmodified decanol crystals, and it is seen that the points for the iodinated albumin fall remarkably close to it. This result was not anticipated, and will be discussed below.

TABLE VIII

IODINATED SERUM ALBUMIN, 25°, Γ/2 0.150

Protein, g./kg. H_2O	H^+ added, moles/kg. H_2O $\times 10^3$	*p*H	Free H^+, moles/kg. H_2O $\times 10^3$	Bound H^+, moles/mole protein	Moles H^+ dissociated per mole protein from *p*H of max. acid binding
10.46	36.97	1.711	22.34	99.5	0.5
7.73	20.16	2.084	9.38	99.2	0.8
5.12	9.21	2.629	2.57	92.1	7.9
4.32	2.57	4.018	0.11	40.5	59.5
4.33	0.00	5.298	.00	0.0	100.0
5.16	0.00	5.305	.00	0.0	100.0
	OH^- added, moles/kg. H_2O $\times 10^3$		Free OH^-, moles/kg. H_2O $\times 10^3$	Bound OH^-, moles/mole protein	
4.22	0.46	5.926	0.00	7.8	107.8
5.15	1.04	6.619	.00	14.4	114.4
5.15	1.54	7.776	.00	21.3	121.3
5.14	2.07	8.873	.01	28.5	128.5
5.15	2.58	9.419	.05	35.0	135.0
5.14	3.36	9.804	.11	44.9	144.9
5.17	3.84	10.073	.21	49.8	149.8
5.15	3.87	10.120	.23	50.3	150.3
5.10	3.92	10.244	.32	50.2	150.2
4.36	3.85	10.524	.60	53.4	153.4
4.35	5.25	10.906	1.44	62.2	162.2
5.09	6.37	10.958	1.62	66.4	166.4
4.33	7.63	11.239	3.01	75.9	175.9
3.86	11.32	11.598	6.56	87.8	187.8
7.79	19.14	11.733	8.81	94.2	194.2

Ultraviolet absorption measurements were also made, both on guanidinated and iodinated albumins. The whole curve for the guanidinated albumin is similar to that of Fig. 6, but shifted toward neutrality. The shift, however, is not nearly as great as would be expected from the fact that the guanidinated serum albumin molecule in the region of tyrosine ionization bears a much smaller charge than the unmodified serum albumin in a corresponding region. The iodinated serum albumin curve, which might have been expected to show considerable ionization of phenolic hydroxyl groups near *p*H 6, since the *pK* of diiodotyrosine is 6.5,[23] actually showed a gradual change from *p*H 5 up. Brief reference to this fact will be made again below, but in view of the difficulty (already mentioned) in interpreting the ultraviolet absorption data, no quantitative inferences can be made at this time.

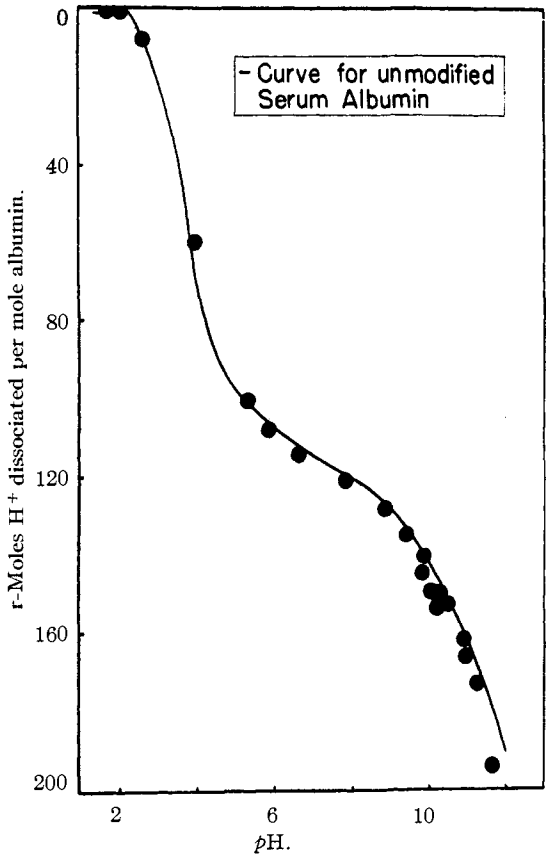

Fig. 8.—Titration curve of iodinated serum albumin at 25°, Γ/2 0.150.

Stoichiometry. Comparison with Amino-Acid Analyses

Table IX gives a list of the ionizable groups in serum albumin, together with analytical data, and reasonable ionization constants and heats. The analytical data reported are from the compilation of Brand[24] corrected for a molecular weight of

(23) P. S. Winnek and C. L. A. Schmidt, *J. Gen. Physiol.*, **18**, 889 (1935).

(24) E. Brand, *Ann. N. Y. Acad. Sci.*, **47**, 210 (1946).

TABLE IX

IONIZABLE GROUPS IN SERUM ALBUMIN (DECANOL-10)

Group	Number present per mole (analysis)[a]	Reasonable pK_0	Ionizing range anticipated (pH)	Heat of ionization,[j] kcal.	Observed this paper no. of groups	Observed this paper pK_0
Carboxyl	106	4.3,[c] 4.3,[d] 4.6[e]	2–5.5	±1.5	106	4.00[k]
Imidazole	16	6.0,[f] 6.7,[d] 6.8[e]	6–8	6.9–7.5	16	6.10
α-Amino	9	7.8[g]	8–9.5	10–13	4	8.00
ε-Amino	58	9.7,[g] 10.1[d]	9–12.5	10–12	56	9.40
Phenolic OH	18	9.6[h]	9–12.5	6.0	18	9.60
Sulfhydryl	0.7[b]	.....	~11[i]	.....	...	...
Guanidinium	24	.....	>11[i]	.....	24	...
Total basic nitrogen[l]	107	.....	.....	.....	100	...

[a] With two slight modifications these data are from Brand, *Ann. N. Y. Acad. Sci.*, **47**, 210 (1946). See full discussion in text. [b] W. L. Hughes, Jr., private communication; mercaptalbumin contains one free SH group per mole. [c] The pK of a normal fatty acid, corrected for the presence of the peptide chain at the β- or γ-position. [d] Value observed for egg albumin; R. K. Cannan, A C. Kibrick and A. H. Palmer, *Ann. N. Y. Acad. Sci.*, **41**, 243 (1941). [e] Value observed for β-lactoglobulin; R. K. Cannan, A. H. Palmer and A. C. Kibrick, *J. Biol. Chem.*, **142**, 803 (1942). [f] Value observed for histidine: E. J. Cohn and J. T. Edsall, ref. 17, p. 85. [g] Value observed for $^+NH_3COOR$ or $^+NH_3(CH_2)_3COOR$: E. J. Cohn and J. T. Edsall, ref. 17, p. 122. [h] Value observed for tyrosylarginine, E. J. Cohn and J. T. Edsall, ref. 17, p. 85. [i] These groups will make no appreciable contribution in the region studied by us. [j] E. J. Cohn and J. T. Edsall, ref. 17, p. 445. [k] See text. [l] Imidazole + amino + guanidinium.

69,000. The figure for the number of free carboxyl groups is obtained by addition of the analytical values for aspartic and glutamic acids (136) plus 1 for the average free fatty acid content[5] plus 9 α-carboxyl groups (equal to the 9 α-amino groups found by Brand) minus the analytical value for the number of amide groups. The latter is given by Brand as 44. His corresponding figure for bovine albumin is 43, whereas Stein and Moore[25] have recently given a maximum value of 39. Assuming a similar error in Brand's figure for human albumin, and reducing the number of amide groups by 5, leaves 106 free carboxyl groups. The values of pK_0 listed in Table IX are reasonable values for the groups concerned, appropriately attached to a peptide chain, which, however, has a zero net charge. The anticipated ionization range given in the table takes into account the fact that the serum albumin molecule is in actuality highly charged over most of the titration curve. The last two columns of Table IX refer to conclusions which will be reached in the discussion presented in the paragraphs immediately following. They are included in Table IX so that a convenient comparison of these conclusions with analytical data can be made.

Because of the differences in anticipated ionizing ranges and heats of ionization considerable information on the stoichiometry can be obtained directly from an examination of Figs. 2 and 4. Breaks in the titration curve should occur between pH 5.5 and 6, and about pH 8, which should give the number of carboxyl and imidazole groups present. Such breaks do actually occur and correspond to 106 or one or two more carboxyl groups and very close to 16 imidazole groups. In addition the heat of ionization begins to rise sharply from the low value appropriate to carboxyl groups where r is somewhere between 105 and 110. If there are 16 imidazole groups present the heat of ionization should level off near its maximum value where r is somewhere close to 122. That this is not obvious from Fig. 4 is not unexpected, however, since the greatest inaccuracy in heat of ionization determination occurs precisely in that region. An additional clue from Fig. 4 is the point at which the curve for the lower temperature range crosses that for the upper range. The change in heat of ionization with temperature is simply the change in heat capacity on ionization

$$\partial \Delta H_i / \partial T = \Delta Cp_i$$

For the carboxyl group ΔCp_i is generally between -30 and -40 cal./degree/mole[26] so that ΔH_i for the 25–37.8° range should be smaller by about 1 kcal. than ΔH_i for the lower temperature range. For substituted ammonium groups, on the other hand, ΔCp_i is generally zero or positive[27] so that the heat of ionization for such groups should be higher in the higher temperature range. The cross-over point is near $r = 108$, and again supports the contention that we have 106 carboxyl groups or one or two more.

Since at the pH of maximum acid binding the basic nitrogen groups all bear a positive charge, while all other groups are uncharged, we can obtain the number of such nitrogen groups from the number of hydrogen ions which can be bound per mole by an isoionic preparation of serum albumin. This number has been found by us to be between 99 and 100 for both decanol-crystallized serum albumin and mercaptalbumin, as well as for the iodinated albumin we have studied. In addition, a curve obtained for decanol crystals at zero added salt[28] gives a maximum acid binding of 99. The maximum number of basic nitrogen groups found

(25) W. H. Stein and S. Moore, *J. Biol. Chem.*, **178**, 79 (1949).

(26) H. S. Harned and B. B. Owen, "The Physical Chemistry of Electrolytic Solutions," Reinhold Publishing Corp., New York, N. Y., 1943, p. 514.

(27) D. H. Everett and W. F. K. Wynne-Jones, *Trans. Faraday Soc.*, **35**, 1380 (1939).

(28) M. H. Blanchard, unpublished data.

by titration is therefore 100, compared with the analytical figure of 107. It should be noted that the agreement with analysis cannot be improved by assuming that our titration is incomplete at the acid end, for any groups not titrated must be carboxyl groups, and we already have as many carboxyl groups (or even one or two more) as the analysis requires.

Because of the fact that *p*H measurements become unreliable and that peptide linkages may be split above *p*H 12 the titration curve is incomplete at the basic end. Virtually all the guanidinium groups and a third or more of the amino and tyrosine groups have not been titrated at *p*H 12, so that no analysis of the stoichiometry of these groups is possible. (The single sulfhydryl group, of course, would not be noticed anyway.) However, further information can be obtained from the curve for guanidinated albumin (Fig. 7). A Van Slyke nitrogen analysis on the preparation used has indicated that up to 10 free amino groups are present. This figure is certa nly high.[29,30] Actually only four amino groups are required to fit the curve, and, furthermore, these must be assigned a low *pK* appropriate for α-amino groups. It therefore appears that all ϵ-amino groups are guanidinated in our preparation of guanidinated albumin, and we have assumed that the remaining four groups represent all of the α-amino groups initially present in the native albumin. Assuming that there are 24 arginine residues per mole (analytical figure), this leaves 56 ϵ-amino groups, compared with Brand's value of 58. This is not serious disagreement.

That Brand's value for α-amino groups might be too high is not unreasonable, since it is obtained as the difference between a van Slyke analysis (which tends to be high) and the lysine figure. One complication is that a reduction in the assumed number of α-amino groups automatically reduces the number of α-carboxyl groups which one would expect to be present on the assumption that both end groups of all peptide chains are free. However, the number of terminal amide groups used in computing the figure for the free carboxyl groups might still be too high, and therefore compensate for the possible deviation in the α-carboxyl groups. In addition it might be mentioned that Chibnall has suggested the possibility that the number of α-carboxyl groups in a protein may not be equal to the number of α-amino groups.[31]

It should be emphasized that these remarks are not intended to throw doubt upon the analytical data for amino-acid content. While it has been shown that slight revision of two of the least certain of the analytical figures (those for amide and α-amino groups) will produce complete accord with the stoichiometry of our titration curves, such complete accord is not necessary, for it is easy to visualize cross-linkages, etc., which might make certain groups inaccessible to hydrogen ion titration. Finally it might be pointed out that the incompleteness of the titration curve at the basic end permits some variation in the number of amino, tyrosyl and guanidinium groups required to fit the curve. However, no appreciable adjustment is possible in the number of carboxyl and imidazole groups, nor in the total basic nitrogen, *i. e.*, the sum of amino and guanidinium groups is effectively fixed.

(29) W. L. Hughes, Jr., private communication.

(30) NOTE ADDED IN PROOF: Since this paper was submitted for publication, I have seen the paper by S. R. Hoover, E. L. Kokes and R. F. Peterson, *Textile Res. Journal*, **18**, 423 (1948), who report four terminal α-amino groups in bovine serum albumin. They do not give any data for human albumin, but the correspondence with the figures in this paper is worth noting.

(31) A. C. Chibnall, Bakerian Lecture, *Proc. Roy. Soc.* (*London*), **B131**, 136 (1942).

Computed Titration Curves. Discussion[32]

If all ionizable groups of a protein were completely independent of one another, then each one would obey an ionization equation of the form

$$(\text{conjugate base})\ \gamma_{\text{base}}\ a_{H^+}/(\text{acid})\ \gamma_{\text{acid}} = K$$

where ( ) represent concentration and the γ's are activity coefficients. At a given ionic strength and for any one type of group the γ's can be incorporated in the equilibrium constant K, so that we get

$$(\text{conjugate base})a_{H^+}/(\text{acid}) = K'$$

or

$$\log\ [(\text{conjugate base})/(\text{acid}] - p\text{H} = -pK' \quad (3)$$

where $p\text{H} = -\log a_{H^+}$ and $pK' = -\log K'$

The equilibrium constant K' is related to the free energy of ionization

$$\Delta F^\circ_{\text{ion}} = -RT \ln K' = 2.303RTpK'$$

Now, part of this free energy is electrostatic in nature. It is the negative of the work which must be done to increase the charge of the conjugate base molecule by one.

Consider now that the ionizable group is on the surface of a protein molecule, which shall be taken to be a sphere, and which is to have a charge Z evenly distributed about its surface. The electrostatic free energy required to increase the charge to $(Z + 1)$ is then

$$\frac{(Z+1)^2Ne^2}{2D}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right) - \frac{Z^2Ne^2}{2D}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right) = \frac{(2Z+1)Ne^2}{2D}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right) = -(\Delta F^\circ_{\text{ion}})_{\text{elec.}}$$

where b is the radius of the sphere, a its radius of exclusion, κ has the meaning customary in the Debye–Hückel theory, D is the dielectric constant of the medium and ϵ is the protonic charge.

Let us now use the subscript zero to denote that the protein sphere has zero net charge when the

(32) The treatment given here is a simplified version of that orlginally developed by Linderstrøm-Lang, *Compt. rend. trav. lab., Carlsberg*, **15**, No. 7 (1924), and recently put into more convenient form by G. Scatchard, *Ann. N. Y. Acad. Sci.*, **51**, 660 (1949). A rigorous derivation of an equation essentially similar to equation 6 can be found in Scatchard's paper.

ionizing group in question is in the form of its conjugate base. Then

$$-[(\Delta F^\circ_{\text{ion}})_{\text{elec.}}]_0 = \frac{N\epsilon^2}{2D}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right)$$

If we assume that the difference in ionization constant of a particular group on the protein sphere is the same, whether neighboring groups are ionized or not, except insofar as the electrostatic free energy changes with the charge Z, then clearly

$$[\Delta F^\circ_{\text{ion}}]_z - [\Delta F^\circ_{\text{ion}}]_0 = 2.303\,RT\,(pK'_z - pK_0)$$
$$= \frac{N\epsilon^2}{2D}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right) - \frac{(2Z+1)N\epsilon^2}{2D}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right)$$
$$= -\frac{2ZN\epsilon^2}{2D}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right)$$

or

$$pK'_z - pK_0 = -\frac{2Z}{2.303}\frac{N\epsilon^2}{2DRT}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right)$$

It is customary to place

$$\frac{N\epsilon^2}{2DRT}\left(\frac{1}{b} - \frac{\kappa}{1+\kappa a}\right) = w \qquad (4)$$

so that at any charge Z

$$pK = pK_0 - \frac{2Zw}{2.303}$$

Equation (3) then becomes

$$\log \text{(conjugate base)/(acid)} = p\text{H} - pK_0 + 2Zw/2.303 \qquad (5)$$

The value of w depends upon the dimensions of the protein sphere and the valence and size of the reacting group. It is not affected by the chemical nature of the group under consideration.

Let the protein we are considering have a limited number of group types (those of Table IX, for example). Let there be n_i of each per protein molecule, and at any pH let r_i of these be in the form of the conjugate base, and $n_i - r_i$ therefore in the acid form. Equation (5) then becomes

$$\log \frac{r_i}{n_i - r_i} = p\text{H} - (pK_0)_i + \frac{2Zw}{2.303} \qquad (6)$$

If the charge on the protein sphere at any pH is known we can calculate r_i for each type of group from equation (6). Each r_i is equal to zero at the pH of maximum binding, and, clearly, the number of moles of hydrogen ion dissociated at any pH, which we have designated by r in all the curves given earlier in the paper, is just the sum of the r_i; *i. e.*, at any pH

$$r = \sum_i r_i \qquad (7)$$

To obtain the value of the charge Z at any pH we first read off from the experimental titration curve the number of hydrogen ions dissociated or bound per mole from the isoionic point. Next we must find what other ions are bound by the proteins. For human serum albumin (decanol crystals) in sodium chloride Scatchard and co-workers[33] have shown that only chloride ion is bound, and, at 25°, have evaluated the number of ions bound over a pH range near the isoionic point as a function of chloride concentration. In 0.150 molar chloride at the isoionic point seven chloride ions are bound per mole of albumin. This number increases toward lower pH values, and decreases toward higher pH values: in our case where increase in pH (at constant total sodium chloride + sodium hydroxide in the system) is accompanied by a decrease in chloride concentration the number must gradually fall to zero.

To calculate w (equation 4) for serum albumin we have assumed that the serum albumin molecule is equivalent to a sphere of radius 30 Å., with a radius of exclusion of 32.5 Å.[33] At ionic strength 0.150 this leads to a value for w of 0.0303.

It now becomes possible, from equations 6 and 7 to compute the titration curve for human serum albumin (decanol −10) at 25°.[34] Using the figures in the last two columns of Table IX for the numbers per mole of the various group types and their pK_0's, excellent agreement is obtained from the isoionic point to beyond pH 11, taking into consideration all groups except arginine. Near pH 12 base is known to be used up in splitting off ammonia, and it has therefore not been thought worthwhile to fit the tail end of the curve by taking arginine ionzation into account. On the acid side, however, the experimental curve is far steeper than any curve computed from equation 6 on the assumption that the chloride binding remains small.[35] It is possible to fit the experimental points only if it is assumed that the chloride binding increases sharply as the protein acquires a positive charge, so that this positive charge always remains small. The computed curve of Fig. 2 is obtained if a pK_0 of 4.00 is used for all the carboxyl groups, and if the number of chloride ions bound per mole of albumin is taken to be 24 at pH 4, 71 at pH 3 and 89 at pH 2.

It seemed desirable to discover whether such a marked increase in the chloride binding could actually be observed. Accordingly, a solution of serum albumin in 0.15 M chloride and at a pH of 3.2 was prepared, and the chloride binding was very kindly determined by Dr. I. H. Scheinberg by an electromotive force method.[36] The average of a number of determinations gave 31 chloride

(33) G. Scatchard, A. C. Batchelder and A. Brown, THIS JOURNAL, **68**, 2320 (1946); G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., THIS JOURNAL, **72**, 535 (1950).

(34) Both the analytical data and chloride binding figures are available only for the decanol crystals. Figure 2 indicates that mercaptalbumin differs slightly from decanol crystals on the alkaline side.

(35) The steepness of the curve is not a new observation. Cohn (ref. 4), for example, was able to fit experimental data on serum albumin with a single pK for the acid groups, with no electrostatic interaction terms. He was unable to fit the data for egg albumin in this manner, and Cannan and co-workers (ref. 18) have found that this protein, as well as β-lactoglobulin, behaves normally if a theoretical treatment essentially similar to that discussed in this paper is used.

(36) G. Scatchard, I. H. Scheinberg and S. H. Armstrong, Jr., THIS JOURNAL, **72**, 540 (1950).

ions bound per mole. While this figure is only about half as great as that which has been used in constructing the curve of Fig. 2, it is much higher than the number bound at the isoionic point, and explains in part the steepness of the titration curve. It is perhaps unreasonable to expect better agreement between experiment and theory on the acid side. Serum albumin is known to consist of more than one component below *p*H 4,[37] and the value of w in that region may well differ from that at the isoionic point.

A computed curve has also been obtained for the guanidinated albumin (see Fig. 7), using precisely the same number of groups of each type and the same pK_0 values, except that all ϵ-amino groups are now taken to be guanidinium groups, and also making the same assumptions about chloride binding. Only the four α-amino groups are assumed to be intact, and, as has already been mentioned, this is a reasonable number. The agreement between the computed curve and the experimental points is very encouraging.

It would also have been desirable to obtain a computed curve for the iodinated albumin used in our experiments, using a pK appropriate for diiodotyrosine for half the tyrosine groups originally present. It is, however, apparent at a glance at Fig. 8 that such a computed curve would not fit the data. Both from the titration curve, and from the change in ultraviolet absorption spectrum, it is clear that no appreciable ionization (over and above that in unmodified albumin) takes place acid to *p*H 10, whereas, if the 17 atoms of iodine had gone to convert about half the tyrosine content into diiodotyrosine, then a difference of up to 9 groups would have been expected. The need for more experimental study of iodinated albumins is indicated.

Acknowledgments.—The author wishes to express his gratitude to Dr. E. J. Cohn for suggesting this problem, and to Drs. J. T. Edsall, J. L. Oncley, George Scatchard and W. L. Hughes, Jr., for many invaluable discussions. He is especially indebted to Dr. Hughes for advice regarding the choice of protein preparations, and for making some of these available to him.

(37) J. A. Luetscher, Jr., *ibid.*, **61**, 2888 (1939).

Summary

1. Acid–base titration curves have been obtained for human serum albumin, as well as for two of its chemical modifications, guanidinated and iodinated albumin, by means of hydrogen electrode measurements.

2. Hydrogen ion uptake was found to be reversible, or very nearly so, from *p*H 2 to *p*H 12. This is true even of the tyrosyl groups, as shown independently by ultraviolet light absorption.

3. Two preparations of albumin were used, mercaptalbumin and a decanol preparation. The titration curves differ slightly on the basic side.

4. The titration curve for the native albumin, as well as observed heats of ionization, are adequately explained on the basis of amino-acid analyses of Brand, with the exception that the titration curve shows only 100 basic nitrogen groups per mole, compared with the analytical figure of 107.

5. A computed titration curve has been constructed which fits the experimental points for unmodified serum albumin. The electrostatic interaction between hydrogen ions and the charged protein molecule has been taken into account in the computation. The steepness of the curve on the acid side has been interpreted as indicating that the protein molecule never acquires a high positive charge, presumably because a large number of chloride ions can be bound on the acid side. A chloride binding study partially confirms this interpretation.

6. The dissociation constants used in the computed curve have been corrected for electrostatic interaction. The pK_0 values for carboxyl, imidazole, α-amino, ϵ-amino and phenolic hydroxyl are 4.00, 6.10, 8.00, 9.40 and 9.60, respectively.

7. A computed curve for guanidinated albumin can be constructed with the same constants, taking into account the fact that all ϵ-amino groups have been converted into guanidinium groups. This curve also fits the experimental data.

8. Only a small difference was observed between the titration curves for iodinated and unmodified albumin. This is contrary to expectations.

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