−6 kcal. and −20 e.u. Schellman's[24] analysis of the properties of urea as modified by Kauzmann[25] leads to $\Delta H = -1.5$ kcal. and ΔS (unitary) = −3.3 e.u. Values of $\Delta H = -4$ kcal. and $\Delta S = -10$ e.u. for similar hydrogen bonds in synthetic polynucleotides were obtained by Warner and Breslow.[26] The transitions A → B and B → C would thus correspond to the *formation* of one to two and disruption of two or three such bonds respectively.

In a molecule the size of conalbumin the observed phenomena may be the resultant of many interactions. In particular, Kauzmann[25] has suggested that the transfer of a non-polar residue from the interior of a protein molecule to the aqueous interface would have a ΔH of −2.5 kcal. and ΔS of −18 e.u. A denatured species, probably C, is known to expand[4,5] with hydration, increasing from 75 to 200%. Transfers and bond-breaking could proceed together, with the net change in ΔH and ΔS remaining small.

The kinetic data do not permit further refinement. It has been concluded that the changes in the molecule are of a limited nature. This seems reasonable in a general sense from the fact that the reactions are reversible, with substantial amounts of all species present at equilibrium, and because the solubility discrimination into three species rationalizes the kinetics.

Comparison of the data on rates and equilibrium positions of the reactions discussed here with the conditions under which the two-component electrophoretic patterns were observed has permitted the identification of the slower component as A + B and the faster as C.[5] The increase in net positive charge and expansion of the protein are thus associated with B → C.

An altogether different, non-specific electrostatic theory for the *p*H dependence of the rate of denaturation has been suggested by Kauzmann.[27] This treatment may explain, qualitatively, the greater stability of proteins near their isoelectric points. However, the behavior of conalbumin, particularly that illustrated by Figs. 2 and 3, like that of lysozyme[12] and bovine plasma albumin,[9,10] shows that the detailed variation in rate of denaturation with *p*H cannot be accounted for by simple functions of the net charge.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, NEW YORK UNIVERSITY SCHOOL OF MEDICINE, NEW YORK, N. Y.]

The Hydrogen-ion Equilibria of Conalbumin[1]

BY ARNOLD WISHNIA,[2] IONE WEBER AND ROBERT C. WARNER

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Titration curves of conalbumin have been obtained at 5, 15 and 25° at ionic strengths of 0.01, 0.03 and 0.1. Time dependent proton equilibria near *p*H 4 have been observed with the rapid flow titration technique. A *p*H dependent transition between two species of conalbumin in this *p*H range has been investigated by electrophoresis. These have been correlated with the species indicated by kinetic studies to be formed during denaturation. The mobilities of these species and the electrostatic interaction factors obtained in the forward and reverse titrations indicate that the native and denatured forms are initially compact but expandable. The behavior of the tyrosyl residues indicates that 7 are buried (*i.e.*, not in hydrogen ion equilibrium) and that a large portion of the rest, possibly the 6 involved in chelating Fe^{+++} to form the iron complex, are hydrogen-bonded. Specific local electrostatic interactions are indicated in the entire *p*H range. The implications of the data for a model of the conalbumin molecule are discussed.

Hydrogen ion equilibria, electrophoretic mobility and denaturation kinetics depend, in different but related ways, on the charge, the charge distribution and the underlying structure of the protein molecule. These properties of conalbumin were the object of the present studies.[3]

The Linderstrøm-Lang model,[4] derived from Debye–Hückel theory applied to a sphere the net charge of which is smeared over its surface, has long been used as the point of departure for the discussion of protein titration curves[5–7] although deviations already were observed in its first application.[5] Scatchard[8] showed that the combinatorial terms used by Cannan[5] could be neglected when the number of identical groups exceeded four. His result, generalized to several types of groups, has been used in the extensive studies from Tanford's laboratory.[9–16] The final equations are

(1) Taken in part from a thesis submitted by Arnold Wishnia in partial fulfillment of the requirements for the degree of Doctor of Philosophy at New York University, June, 1957. An account of this work was presented at the Biocolloid Symposium, "Transformations of Proteins in Acidic Media," 132nd Meeting, American Chemical Society, New York, N. Y., September, 1957. A. Wishnia and R. Warner, *Abstr.*, p. 18-I. This work was supported by a research grant, H-1642, from the National Heart Institute, Public Health Service.

(2) Department of Biochemistry, Dartmouth Medical School, Hanover, N. H.

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$$pH - \log \frac{r_i}{n_i - r_i} = (pK_{int.})_i - 0.868\, Z\, w \quad (1)$$

$$w = \frac{\epsilon^2}{2DkT}\left(1/b - \frac{\kappa}{1 + \kappa a}\right) \quad (2)$$

where n_i is the number of ionizable groups characterized by the intrinsic dissociation constant $(pK_{int.})_i$, r_i the number ionized, Z the mean charge of the protein. D, ϵ, k, T and κ have their usual meaning. The radius of the spherical protein molecule is b and a is the radius of exclusion. Low experimental values of w were immediately found and attributed to hydration or non-spherical form[5-11] or to non-uniform charge distributions.[11]

The problem of discrete charge distributions has been investigated using Kirkwood–Westheimer theory.[17-18] Scatchard[8] remarked that tetrahedra and cubes, with identical groups at the vertices, would show behavior consistent with equations 1 and 2, although b might lose its exact physical meaning. Linderstrøm–Lang,[19] Hill[20] and particularly Kirkwood and Tanford[21,22] have treated more general systems of discrete charges in greater quantitative detail. Tanford[22] has shown that the most common effect will be on $pK_{int.}$. Only markedly unbalanced nearest neighbor charge distributions will also show strongly anomalous values of w. The carboxyl groups of lysozyme may fall in this class, but the phenolic groups conform to no current theory.

While such considerations account for some of the observed deviations from equations 1 and 2, there remain some rather large effects which require special explanation. For example, the steep acid branch of the titration curve of serum albumin[9,14] occurs in a region where viscosity studies[23-25] indicate expansion. Tanford's theory,[26] postulating penetration by solvent and small ions into a swollen spherical protein molecule, correlates the electrostatic and hydrodynamic data quite well. On the other hand, the more complex behavior of ferrihemoglobin[27,28] requires the association of protons to groups not previously in hydrogen-ion equilibrium in the native molecule.

Laskowski and Scheraga[29] have developed a theory of specific hydrogen bonding to account for anomalous behavior. Quantitative application to specific proteins has been difficult. However, such interactions may occur when sterically favored. A strong carboxyl–carboxylate interaction (a case not specifically treated by these workers) is an important factor in the equilibria of maleic acid[30] and diethylmalonic acid[31] among others.

Experimental

Conalbumin and iron-conalbumin, prepared by the method of Warner and Weber,[32] were deionized by passage through an ion-exchange column.[33] Carbonate-free KOH solutions[34] were standardized against potassium acid phthalate (NBS acidimetric standard). HCl was standardized against Na_2CO_3 and checked against the KOH. Glass-redistilled CO_2-free water was used to make up all solutions. Aliquots of stock conalbumin and acid or alkali were added to 5 ml. volumetric flasks using Carlsberg pipets[35] of 0.1 to 1.0 ml. capacity calibrated to 0.0002 ml. by weight. The usual protein concentration was 0.4%. A continuous titration showed no obvious deviations and the back titration was done in this manner.

Measurements of e.m.f. below pH 9 were made with a Cambridge model R pH meter and condenser-type electrode or with a Beckman model G pH meter and standard electrodes, suitably thermostated. At higher pH's only the model G was used, as the Beckman all-purpose glass electrodes showed remarkably little alkaline error in the potassium salt solutions.

Hydrogen-ion concentrations and pH were computed by assuming that the liquid-junction potentials and mean activity coefficients of the experimental solutions were the same as those of reference solutions of the same ionic strength and similar composition. Primary reference solutions for the pH extremes were 0.01 M HCl,[36] 0.01 M HCl–0.02 M KCl,[37] 0.01 M HCl–0.09 M KCl[37] and 0.01 M KOH–0.09 M KCl.[38] Secondary standards, 0.05 M potassium acid phthalate, 0.025 M KH_2PO_4–0.025 M Na_2HPO_4 and 0.01 M borax, were also used to check for drifts.[43] Computation of free (H^+) and (OH^-) and of h, the number of protons bound per mole of 76,600, was then done in a straightforward manner.

The flowing titrations were made on the apparatus described by Steinhardt and Zaiser.[44] Solutions of conalbumin in 0.1 M KCl and varying concentrations of HCl in 0.1 M KCl were mixed and the pH was determined during flow (3 seconds after mixing) and 10 minutes after stopping the flow. The average room temperature during these experiments was 28.7°.

Hydrogen-ion equilibria of the phenolic groups were determined by the technique introduced by Crammer and Neuberger.[45] In one set of experiments, done at about 22° in an air-conditioned room, conalbumin and iron conalbumin, diluted to low concentration in glycine–KOH–KCl and

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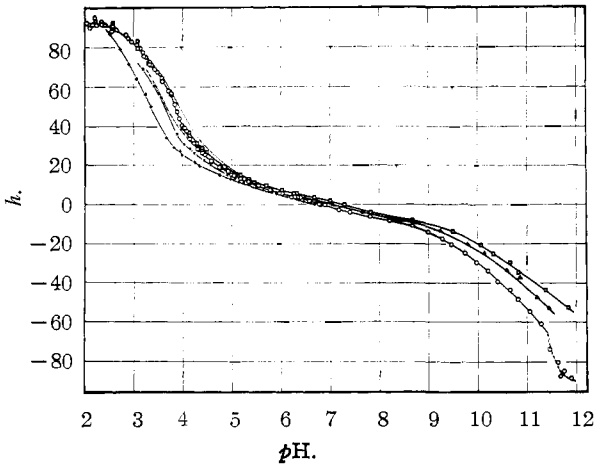

Fig. 1.—The titration curve of conalbumin: □, 5°, 0.1 ionic strength; △, 15°, 0.1 ionic strength; ○, 25°. 0.1 ionic strength; ×, 25°, 0.03 ionic strength; +, 25°, 0.01 ionic strength. Most of the points for temperature variation in the acid range have been omitted in order to simplify the graph. The back titration is indicated by ..... and the rapid flow titration by - · · · -.

KOH–KCl buffers at 0.1 ionic strength, were compared, in 1 cm. cells, with the respective proteins at neutral *p*H, over the spectral range 270–305 mμ, in a Beckman recording spectrophotometer. The spectra of many samples were also recorded against blanks. Although an initial shoulder at 290–291 mμ becomes a peak as *p*H increases, the maximum differential rise always occurs at 295 mμ. Two isosbestic points are readily observed in the difference spectra and departure from these points at high *p*H is another indication that the changes found in this region are not equilibrium phenomena.

The second set of experiments was performed on the unbuffered KOH–KCl solutions of the over-all titration, using a Beckman DU spectrophotometer at 295 mμ and a light path of 0.100 cm. Temperature was regulated to ±0.03° at 25 and 15° and 0.1° at 5° by the use of four Beckman thermospacers. The experimental data were converted to molar quantities using 76,600 as the molecular weight and 11.1 as the absorbancy at 278 mμ in a 1 cm. cell of a 1% solution of conalbumin in 0.02 *N* HCl.[46]

Electrophoresis was conducted at 0° in buffers of 0.1 ionic strength as previously described.[47] The *p*H of each buffer was measured at 25° and corrected to 0° using the equation of Harned.[42b] Determinations on cacodylate and barbital buffers at 10, 25 and 40° could be accommodated to the Harned equation (with $p = 5 \times 10^{-5}$) by using values of θ of 30 and 175°, respectively. All of the patterns obtained were closely enantiographic and showed only the characteristic differences in sharpness and mobility between the ascending and descending boundaries.

Results

The hydrogen-ion equilibria of conalbumin determined at 5, 15 and 25° and at 0.01, 0.03 and 0.10 ionic strength are shown in Fig. 1. Phenolic equilibria are shown in Fig. 2.[48]

Stoichiometry.—The anchor of the whole analysis, the acid end-point, gives 92 as the number of cationic groups. 93 is almost equally probable.

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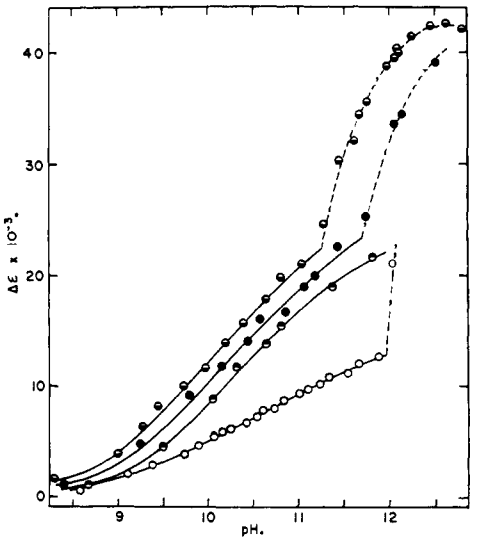

Fig. 2.—Phenolic equilibria; $\Delta\epsilon$ was measured at 295 mμ and refers to the increment over the molar extinction at neutral *p*H: ◒, 25°; ●, 15°; ◓, 5°; ○, iron conalbumin, 22°; all are at 0.1 ionic strength.

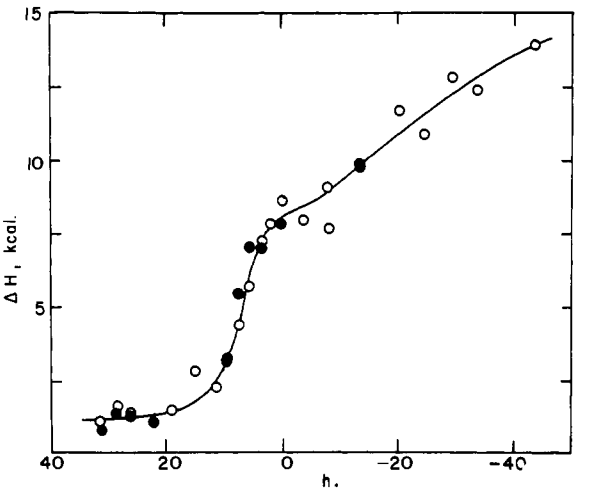

Fig. 3.—Apparent heats of ionization as a function of *h:* temperature interval for calculation, ○, 5–25°; ●, 15–25°.

From inflection points with overlap corrections, from the ΔH curve (Fig. 3), from self-consistent reconstruction of the titration curves, there are 85 carboxyl groups of which one is assumed to be a C-terminal α-carboxyl to match the single N-terminal residue[49] and 13 imidazole groups. These data agree moderately well with the amino acid analysis[50] (*cf.* Table I).

There was no separate determination of amino groups by formol titration. However, 52 ε-amino groups gives a consistent picture. Moreover, from considerations below, this cannot be an overestimate, and the total of cationic groups would make this figure quite uncomfortable as an underestimate. The figure of 26–27 guanidino groups cannot be stretched to 33 as given by amino acid analysis, and

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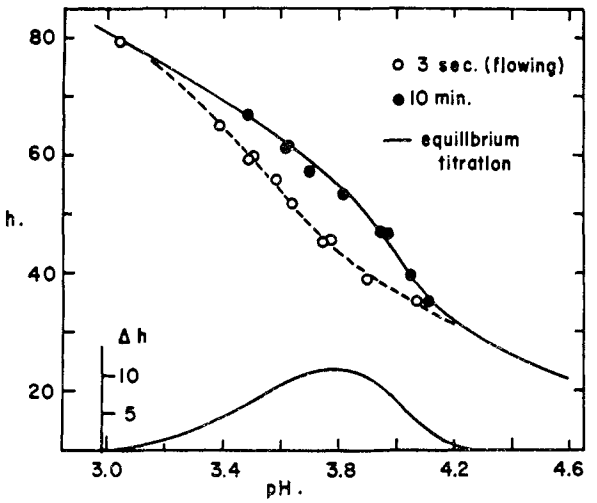

Fig. 4.—Rapid flow titration; the lower inset shows the difference between the equilibrium and rapid flow curves.

this discrepancy is unresolved. The Van Slyke amino nitrogen estimate, 61,[50] cannot possibly be correct.

TABLE I

TITRATABLE GROUPS

	Found	Amino acid analysis[a]
α-Carboxyl	(1)	..
β,γ-Carboxyl	84	82
Imidazole	13	13
α-Amino	1	1[b]
ϵ-Amino	52	52
Phenolic	18	19
Guanidino	26	33
Total cationic	92	99

[a] Ref. 50, [b] Ref. 49.

From the spectrophotometric titration at 295 mμ, 18 tyrosyl residues are found, with a residue absorbancy of 2350, compared to 2300 for tyrosine,[45] 2430 for BSA,[10] an anomalous 2500 for lysozyme[12] and 2630 for ribonuclease.[13] It must be emphasized, however, that the molar absorbancy of 18 × 2350 is determined on alkaline denatured material, whose properties are considerably different from the native. It is not yet excluded that native conalbumin and iron-conalbumin have higher residue absorbancies.

There are no sulfhydryl groups.[50]

Heat of Ionization.—In Fig. 3 is given the apparent heat of ionization as a function of the number of protons associated, calculated from the curves at 5, 15 and 25° at 0.1 ionic strength according to Wyman.[51] The inflection at $h = 7$ is obvious. The doubtful presence of an expected inflection at $h = -6$ is a reflection of the rather high heat of ionization of imidazoles which is also observed in the intrinsic pK. The increase of ΔH with $-h$ is determined by a difference in electrostatic interaction factors at 5 and 25°. On the carboxyl side this leads to a decrease in ΔH as h increases. However, in the accessible range before denaturation ΔH does not assume negative values.

Rapid Flow Titration.—The results of the rapid flow measurements are plotted in Fig. 4. The

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

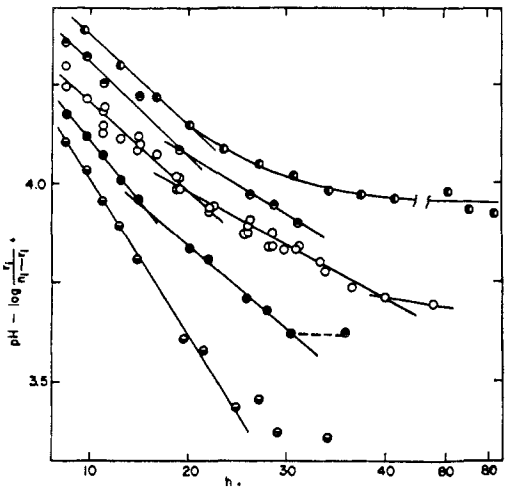

Fig. 5.—Plot for determining w in the carboxyl region of the titration curve: native, 25°, 0.1 ionic strength, O; 0.03 ionic strength, ●; 0.01 ionic strength, ◒; 5°, 0.1 ionic strength, ◓; back titration, 25°, 0.1 ionic strength, ◐. The compressed abscissa at high values of h has been used only for the uppermost curve.

similarity of conalbumin to hemoglobin is obvious.[27,44] Both proteins exhibit a time dependent denaturation in the same pH range accompanied by an increase in proton binding and changes in the solubility and other properties. The three-second points in Fig. 4 begin to diverge from the static curve just below pH 4.2. The difference between the two curves (lower part of Fig. 4) rapidly rises to a maximum of 11 equivalents per mole. In this pH range the instantaneous proton equilibria with the native conalbumin are more rapid than the denaturation. As the pH is lowered, the latter becomes increasingly rapid until at pH 3 the proton equilibria are established instantaneously (<< 3 seconds) with the denatured form. The data reported here on electrophoresis show a transition from a slow to a fast component in this pH range with the appearance of two-component patterns at intermediate pH's. A more quantitative comparison between the two sets of data is not possible because of differences in the temperature and ionic composition of the media.

Electrostatic Interaction and Intrinsic pK.—If the Linderstrøm-Lang model for the protein is adequate, a plot of the quantity (pH − log $r_i/n_i - r_i$) against charge, Z, will be linear (equation 1). It usually has been the practice to use the more readily measured quantity, h, the number of protons bound, in place of Z. The data, treated in this way, are presented in Figs. 5 and 6 and in Table II. Correction of possible errors introduced by neglecting ion binding will be considered later.

The electrostatic interaction factor, w, computed from equation 2 with $b = 30$ Å. (20% hydration; $[V = M/N\bar{v} + 0.2]$) and $a - b = 2$ Å. for KCl,[42] is 0.033, 0.046 and 0.061 at 0.10, 0.03 and 0.01 ionic strength. Somewhat smaller values are expected if there is greater hydration or non-spherical form,[5,11] the probable situation in conalbumin.

In the interval $20 > h > -37$ at 25°, and as far as -52 at 5°, the anticipated linear relations of

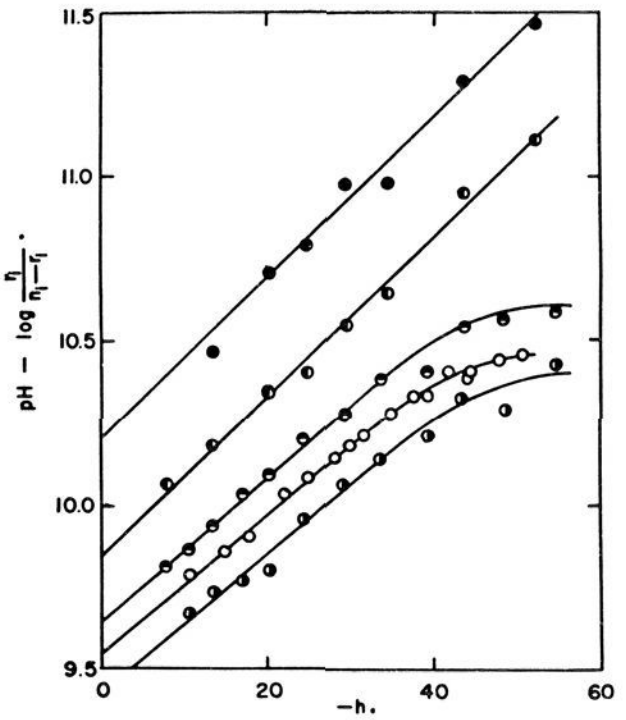

Fig. 6.—Plot for determining w in the alkaline pH range: ϵ-amino groups, 5°, ●; 25°, ◓; phenolic groups, 5°, ◐; 25°, ◑; phenolic groups iron conalbumin, 22°, O. Ionic strength, was 0.1.

equation 1 are observed and reasonable values of w obtained. The titration is, however, still reversible outside these limits—at 0.1 ionic strength from + 32 to − 55. The rapid flow technique extends the equilibrium data for native protein to $h = 45$. At lower pH's there is extensive denaturation in three seconds.[3] The observed departures from linearity in Figs. 5 and 6 reflect properties of native conalbumin.

TABLE II

ELECTROSTATIC FACTORS AND INTRINSIC pK

Group	Temp., °C.	w	pK
ϵ-Amino, 0.10 ionic strength	25	0.0248	9.64
	5	.0279	10.20
Phenolic, 0.10 ionic strength	25	.0252	9.41
	15	.027	9.63
	5	.0283	9.85
Phenolic, 0.10 ionic strength, Fe complex	22	.025	9.55
Imidazole	25	.026	6.75
	5	.030	7.20
β,γ-Carboxyl, 0.10 ionic strength	25	.025	4.43
.03		.035	4.41
.01		.046	4.41
.10	5	.0274	4.54
β,γ-Carboxyl, 0.10 (back titration)	25	.025	4.60

Electrophoresis.—When conalbumin not previously exposed to acid was examined electrophoretically at pH's above 4.4 or below 3.9, it migrated as a single species. The data are presented in Fig. 8 as plots of *u versus* pH and *u versus h*. In the intermediate range in formate buffers two distinct components were evident in the patterns. The relative amount of the faster component increased as the pH was lowered. A representative pattern is shown in Fig. 7. The mobilities of the slower component fall on the mobility-change relation prevailing above pH 4.4 (straight line on *u vs. h* plot and lower curve on *u vs.* pH in Fig. 8). The

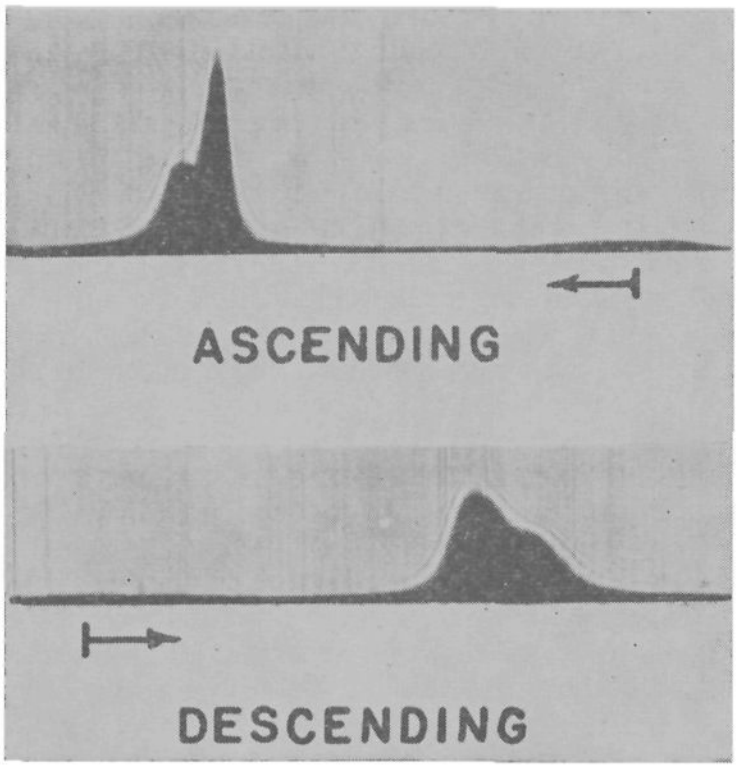

Fig. 7.—Electrophoretic pattern of conalbumin at pH 4.21, 0.1 ionic strength, in a formate buffer. The fast component comprises 43% of the area. The same distribution and mobilities were found in an experiment in which conalbumin was adjusted to pH 3.8 in formate buffer and then dialyzed 3 days against pH 4.21 buffer. In other experiments the following areas for the fast component were found: pH 4.32, 26%; pH 4.07, 56%; pH 3.99, 74%.

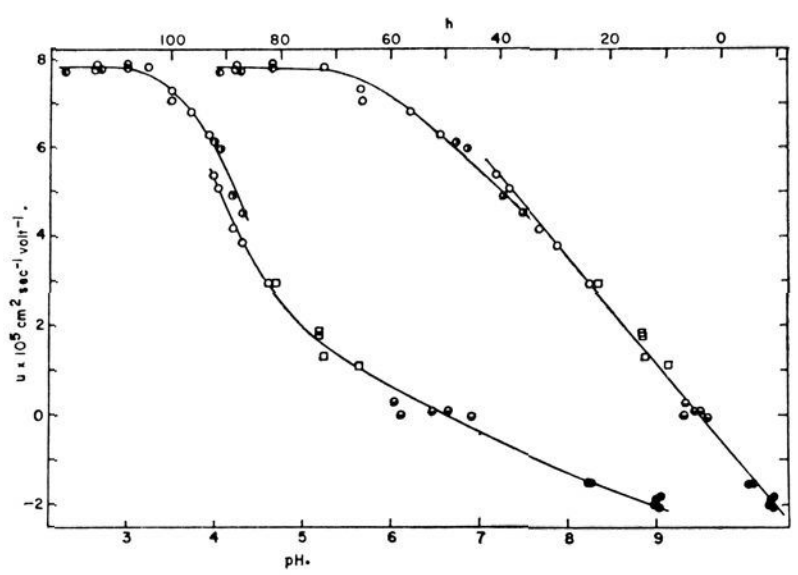

Fig. 8.—Electrophoretic mobility of conalbumin as a function of pH (left hand curve and lower abscissa) and of h (right hand curve and upper abscissa) at 0° and 0.1 ionic strength in the following buffers: ◑, phosphate; O, ◑, formate; □, acetate; ◒, cacodylate; ●, barbital. The faster component of the two-component patterns in formate is indicated by ◑. For the calculation of h for these patterns the rapid flow titration was used for the slow component and the back titration was used for the fast component. Points are included in the acetate, cacodylate and barbital regions for conalbumin exposed to formate buffer at pH 3.7 and then adjusted to the pH of electrophoresis by dialysis over a period of 2 days. Good agreement with the straight line is given by points calculated from the data in ref. 53.

mobilities of the faster component fall on a curve which is continuous with the mobilities of the single boundary below pH 4. It is thus indicated that the slow species is converted to the fast species as the pH is lowered through this interval and that this

transition corresponds to the one observed by rapid flow titration and by change in solubility.[3]

This conversion is reversible. The same pattern was obtained in this *p*H range regardless of whether the *p*H of electrophoresis was achieved by direct adjustment of a neutral stock solution or whether the stock was first adjusted to *p*H 3.3–3.8 with formic acid and subsequently dialyzed against the electrophoresis buffer, provided a dialysis period of about 70 hr. was used in each case. This reversal experiment was performed at three different *p*H's within the range 4 to 4.4. The two components can be identified with the species postulated from kinetic measurements. Consideration of the relative amounts and rates of formation indicates that the slow component consists of the species identified as A and B in our investigation of the kinetics of denaturation of conalbumin.[3] The fast component is the same as C[3]. The rate of the transition B to C is such that they should behave as independent components according to the theory of Cann and Kirkwood[52] and produce enantiographic patterns as they indeed do. After long periods of time particularly at low *p*H, species C undergoes further changes which appear to be irreversible and which may involve aggregation.

The sedimentation properties of conalbumin also were investigated. However, since our results confirm those of Cann and Phelps,[53] who have established that there is no change in molecular weight at low *p*H, they will not be reported in detail.

When the *p*H is returned to a value of 4.6 or above after a brief exposure to acid, two components may also be found, but the relative amounts depend on the time of standing over very long periods. This can be eliminated at least in formate buffers by a much shorter period of standing with gradual *p*H adjustment through the range 4 to 4.4 before going to a higher *p*H. This is consistent with the *p*H dependence of the rate of A → B and B → C both of which fall off extremely rapidly above *p*H 4.[3] It is not clear whether the apparent reversal of much higher *p*H's (*ca.* 8) leads to the same products. These observations explain the time dependence of pattern noted by Longsworth, Cannan and MacInnes[54] who first identified the electrophoretic complexity of conalbumin.

In the presence of anions other than formate, multicomponent patterns were obtained in the same *p*H range (4 to 4.4) with phosphate and sulfate. In the presence of these anions the rate of denaturation[3] was similar to or slower than in formate. Those anions which increase the rate, trichloroacetate, nitrate and chloride, shifted the range of two-component patterns to higher *p*H's. These effects have not been examined in detail and are complicated by the high degree of binding of divalent anions as indicated by electrophoresis (sulfate, pyrophosphate and selenate).

It is difficult to compare our results with some of those of Cann and Phelps[53] because of the difference in the stock conalbumin solutions. They have prepared conalbumin by precipitation at *p*H 3 in the presence of sulfate and subsequent dialysis at *p*H 7, while we have used conalbumin not previously exposed to acid.[32] The two components, C_1 and C_2, of Cann and Phelps in reversible equilibrium between *p*H 4.5 and 7.5 have no counterpart in our patterns, although we have observed them in conalbumin treated with sulfate at low *p*H. This equilibrium could not be reproduced in Cann and Phelps' experiments if their conalbumin was again exposed to *p*H 3, this time in chloride at low ionic strength. We conclude that the components observed by Cann and Phelps are a result of changes in conalbumin subsequent to the equilibrium that we have studied. They may also be due in part to the use of sulfate which is a strongly bound anion and one having a marked effect on the rate of the reaction[3] and on aggregation.[53]

(52) J. R. Cann, J. G. Kirkwood and R. A. Brown, *Arch. Biochem. Biophys.*, **72**, 37 (1957).

(53) J. R. Cann and R. A. Phelps, *ibid.*, **52**, 48 (1954); R. A. Phelps and J. R. Cann, *ibid.*, **61**, 51 (1956).

(54) L. G. Longsworth, R. K. Cannan and D. A. MacInnes, *J. Am. Chem. Soc.*, **62**, 2580 (1940).

Discussion

The Non-Carboxyl Region.—Although the phenol ionization is observed spectrophotometrically, a source of ambiguity resides in the fact that denaturation occurs long before all the groups are titrated. Since it is clear that the groups are not equivalent, their distribution must be inferred.

The number of phenolic groups in hydrogen-ion equilibrium in the native protein lies between 10 (from the absorbancy at the *p*H of discontinuity) and 18. If the absorbancy change per group on ionization is 2350 at 295 mμ, then a value of 11 gives the best parallelism with the ε-amino group data, reproducing the slope and curvature of the 15 and 25° data and the slope and linearity of the 5° data (Fig. 4). A somewhat larger value of $\Delta\epsilon_{TYR}$ (perhaps 2500) or 12 groups at 2350 would begin to stretch the data out of coincidence. Still larger values of $\Delta\epsilon$ or n would cause distinct upward curvature in the 5° w-plot (an increase of w with *p*H for which there is no precedent) and marked divergence from the ε-amino group curves.

In this connection it is important to consider the spectrophotometric titration of the ferric complex of conalbumin. Earlier work[46] had suggested that each of the two ferric ions is bound to three phenolate residues. The shift in the spectrum at neutral *p*H is consistent with this hypothesis.

Iron-conalbumin is more stable than conalbumin itself. At 22° the titration may be extended to *p*H 11.9, where the phenolic ionization is virtually complete, having clearly begun by *p*H 8.5. Since the iron association enhances the entire spectrum, caution regarding the use of 2350 as the group absorbancy is indicated. However, if it is assumed that five tyrosyl residues are normal, parallelism with the conalbumin data is achieved. Using six groups or a larger $\Delta\epsilon_{295}$ does not shift the pK_0 very much but increases w and the length of the linear portion of the w-plot to values comparable with the 5° data of conalbumin. This result, implying a more stable, compact structure for iron conalbumin than for conalbumin at the same temperature, is attractive, but hardly established.

Any consistent choice shows a difference of six titratable phenol groups between conalbumin and

its iron complex, establishing the nature and number of the chelating groups beyond question as three phenolate residues per ferric ion. Furthermore, the most reasonable numbers for normal phenols are eleven and five. Seven phenolic groups, then, are not included in the hydrogen-ion equilibria of the native proteins. Since this is a large fraction of the total, since the pK_0 of the other groups is low and the titration followed to fairly high pH and since these groups appear only at discontinuities where denaturation and expansion demonstrably occur, the possibility that the seven groups merely have a higher pK, because of hydrogen bonding, is slight. The best explanation is that these groups are buried in the inaccessible hydrophobic regions of the protein and do not participate in the metastable equilibria of native conalbumin (*cf.* ref. 13).

It has been shown that the conalbumin titration is still reversible for some distance on either side of the interval $20 > h > -37$ in which the w-plots are linear. The curvature of the w-plots at 25 and 15° for the ϵ-amino- and phenolic groups may not be ascribed to a fixed distribution of pK's or to cation binding, since either would affect the 5° data as well. It must be concluded that some structural change occurs. Whether all the molecules are modified or whether there is an equilibrium between native and altered proteins is not known. The evidence on the acid side suggests the former. As with bovine serum albumin,[14] the phenomenon occurs at much higher net charge on the alkaline than on the acid side.

The extrapolated values of the intrinsic pK of carboxyl, phenolic and ϵ-amino groups are low, that for ϵ-amino groups especially so. If salt bridges or hydrogen bonds were responsible, one would expect ΔF^0, ΔH^0 and ΔS^0 to shift but in opposite directions for the groups of the pair. This condition is not met (compare discussion in ref. 14).

The low pK of the ϵ-amino groups is reflected in ΔH^0, which is about 1 kcal. less than usual.[51] This suggests that most of the amino groups are segregated in their own relatively positive region. The high ΔH^0 and ΔS^0 of the tyrosyl groups imply interaction with some group, possibly with each other (hydrogen-bonding is at least sterically favored for six of the residues); the data do not invite the conclusion that carboxylate groups are ligands for the phenolic groups.

The heat of ionization of the imidazole residues is about 2 kcal. greater than in hemoglobin.[51] However, the ΔH^0 for model imidazole compounds is also higher (imidazole, 7.7 kcal.; 4-methylimidazole, 8.6 kcal.; 2,4-dimethylimidazole, 9.2 kcal.),[55] and it is quite possible that hemoglobin is the abnormal type. ΔS^0 is, however, less negative than expected.

The Carboxyl Region.—It is instructive to begin the discussion of the carboxylic equilibria with the back titration. At low pH, conalbumin is denatured. As with BSA,[16] Tanford's inflated sphere model gives good agreement with the hydrodynamic data: at pH 3.0, 0.1 ionic strength, the radius of conalbumin is 45 Å. (viscosity[53]), 44 Å. (sedimentation[53]) and 44 A. (electrostatic factor, eq. 17 of ref. 26.) As protons are removed, the protein collapses, increasing w. By $h = 25$ the molecule is refolded to its original compactness, equation 2 is valid and the electrostatic radius, 35 Å., compares with the viscosity (36 Å.) and sedimentation (35 Å.) radii.[53] The intrinsic pK for the carboxyl groups is a "normal"[22] 4.6. The reneutralized protein is in many ways similar to, but not identical with, native conalbumin.[53,54]

The carboxyl titration of native conalbumin, with an intrinsic pK of 4.42 (4.34 with chloride binding) and, initially, the same value of w found in the alkaline region, begins to exhibit anomalous behavior at $h = 20$, when only 13 of the 84 β,γ-carboxylate groups have been protonated. Beyond this point the apparent value of w falls continuously. At $h = 45$, the last point at which even the three-second rapid flow data may be treated with any confidence, w has decreased to three-fourths of its initial value. At $h = 30$, the fractional decrease is about the same at all three ionic strengths.

TABLE III

THERMODYNAMIC QUANTITIES[a]

	pK_0[b]	ΔF^0,[b] kcal.	ΔH^0, kcal.	ΔS^0, e.u.
β,γ-COOH, conalbumin	4.42	6.0	2.1	−13.2
typical	4.5	6.2	0–1	−18 to −21
Phenolic, conalbumin	9.41	12.8	8.3	−15.2
typical	9.6	12.9	6.0	−24
ϵ-Amino, conalbumin	9.64	13.2	10.7	− 8.2
typical	10.2	13.7	11.5	− 7.5

[a] Typical values taken from ref. 14 and references therein. [b] At 25°.

If it is assumed that a constant w applies to each class, no distribution of carboxyl groups between two classes with different pK_0 will fit the data. More elaborate schemes within this framework cannot be expected to change this picture. The apparent decrease in w might be attributed to extensive non-linear chloride binding, of which the electrophoretic data give no indication, to some rearrangement in or expansion of all the molecules (about 2 Å. at $h = 40$; ionic strength 0.10), or to an equilibrium between native and reversibly modified protein. The last possibility, which is of significance in the electrophoretic work, is unlikely, since the observed denaturation equilibria[3] are reached too slowly in this pH region.

A modification similar to the "expandable" form of BSA[14] probably occurs at both pH extremes prior to denaturation. The sedimentation data of Phelps and Cann[53] are drawn to show a change of the proper magnitude starting at pH 4.5 ($h = 24$), but the scatter of the data leaves the matter somewhat uncertain.

Below pH 4 the rapid-flow and static titrations diverge. The steep pH 4.0 to 3.8 portion of the latter represents the pseudoequilibrium time-dependent transition from native conalbumin to an acid-denatured form. In this respect conalbumin resembles hemoglobin[27,28] rather than BSA.

However, no "masked" (*i.e.*, non-equilibrium) groups appear to be involved. All the cationic residues have been accounted for in the ordinary

(55) Y. Nozaki, F. R. N. Gurd, R. F. Chen and J. T. Edsall, *J. Am. Chem. Soc.*, **79**, 2123 (1957).

way. The difference between the forward and reverse titrations, at most 11 protons per mole, arises principally from the difference in the intrinsic pK of the carboxyl groups, and secondarily from the somewhat surprisingly small difference in electrostatic factor, between the species involved.

The pK_0 of either species is not far from normal. The question is whether one is low or the other high. Tyrosyl-carboxylate bonding, and the purely electrostatic effect of positive near-neighbors, which would lower pK_0 in the native state, are manifested mainly in a decreased ΔH^0 of ionization, which is not observed. The formation of salt bridges, which is primarily an entropic effect, is plausible as far as the carboxylates are concerned. The cationic ligands would have to be guanidinium groups, since the data preclude participation by the lysyl and histidyl residues. It is possible that the foregoing relations are unchanged in the acid-modified protein and that during refolding of the denatured molecule a rearrangement occurs permitting a certain amount of carboxyl–carboxylate (COOH. . . .COO^-) interaction, which at this state of ionization would increase the apparent pK.[29] Carboxyl–carboxyl (COOH. . . .COOH) interactions would be important only at the acid end of the titration. Moreover, the data for small molecules suggest that when steric factors are favorable, the carboxyl–carboxylate interaction is the stronger.[30,31]

Binding of Ions Other Than Protons.—Ion-binding is rather a difficult point in most discussions of hydrogen-ion equilibria, because the available data are inadequate. For example, although the problem has long been recognized, only in two papers from Tanford's laboratory[14,16] has an effort been made to include ion-binding, and this has been on an admittedly arbitrary or insecure basis. Ion-binding equilibria are determined with more difficulty and less precision than hydrogen-ion equilibria. Moreover, theoretical interpretation of the results is hampered because the nature of the sites is unknown and even their uniqueness is not readily established, whereas even a crude study of proton-binding allows one to count and distinguish specific groups (carboxyl, phenolic, etc.). Studies usually have been made in the isoionic region and at a few isolated pH values. Carr, in his informative papers,[56] has been satisfied to report the data and to demonstrate the inadequacy of current knowledge to explain them.

Anion binding to conalbumin may be estimated in two ways. The electrophoretic mobility at 0.1 ionic strength is a linear function of h from $+40$ to -10 and probably to -37, using five different monovalent buffer anions. While theoretical problems (see below) preclude calculation of the absolute mean charge, it may be stated that the mean charge is also a linear function of h. Consequently, anion binding is either constant or linear in this range. At acid pH replacing acetate by chloride results in decreases in mobility corresponding to the additional binding of about two anions per mole of protein. At the isoelectric point four moles of cacodylate are bound per mole of protein. If it is assumed that the association constants for glycinate, barbituate, acetate and chloride are in the same ratio for conalbumin as for serum albumin[57] and the same order of magnitude, it may be estimated that the maximum binding of chloride at 0.1 M before acid denaturation occurs is not more than 10 ions per mole.

As Lowey[58] has shown, the isoionic pH of conalbumin increases with KCl concentration. According to the theory of Scatchard and Black[59] the purely electrostatic effect is small, and the increase in pH at 0.01, 0.10 and 1.0 M KCl may be attributed to the binding of 1, 7 and 14 moles of chloride, respectively.[58] From these data we would compute that there are 17 equivalent sites with an association constant of 10. Using the Debye–Hückel activities for chloride and for 20% hydrated spherical conalbumin an estimate of chloride binding at any pH may be obtained. The charge on the acid side of the isoionic point is then indeed a linear function of h: $Z = 0.80\ h - 7.4$, $0.74\ h - 3.3$, $0.71\ h - 1.0$ at 0.1, 0.03 and 0.01 M KCl, respectively. In the interval $0 < h < 20$ the experimental w would then agree with the theoretical values for the compact (30 Å.) molecule. These results, however, should be viewed with reservation since this assignment leaves some questions unanswered and creates new problems. *Ad hoc* explanations for the breaks at $h = 20$ (Fig. 5) and for the increase of w at 5° still are required. The computed degree of isoionic chloride binding also would require that the intrinsic pK of carboxyl groups decrease with increasing ionic strength (from 4.36 to 4.31 to 4.27 on going from 0.01 to 0.03 to 0.10 M KCl), while any interactions which lead to the already low value of pK_0 would be minimized by the increased salt concentration. Finally, the calculated association constant for chloride binding is rather low.[57,60]

The small electrostatic effect of ionic strength on isoionic pH predicted by Scatchard and Black[59] arises from small fluctuations in the mean charge at the isoionic pH, spread out over the whole protein sphere. Actually, nearest-neighbor interactions may be as great at this as at any other pH, and an increase of 0.14 in pK_0 between 0.01 and 0.10 ionic strength is not unusual.[22] Support for this conjecture is found in the recent work on β-lactoglobulin. Nozaki, *et al.*,[16] computed the binding of Ca^{++} and K^+ from the decrease in isoionic pH using the theory of Scatchard and Black[59] and compared these data to the membrane-electrode values of Carr[37] adjusted to this pH. The agreement for Ca^{++} is fair, falling off at higher concentration; the K^+ data are underestimated by half. It is as if an increase in pH, dependent on ionic strength, subtracts significantly from the smaller effects observed for K^+, while changing the larger Ca^{++} effects by a smaller fraction. If the carboxyl data are extrapolated [by equation 1] to the appro-

(56) C. W. Carr, *Arch. Biochem. Biophys.*, **40**, 286 (1952); *ibid.*, **46**, 417 (1953); *ibid.*, **62**, 476 (1956).

(57) I. M. Klotz and J. M. Urquhart, *J. Phys. and Colloid Chem.*, **53**, 100 (1949).

(58) S. Lowey, *Arch. Biochem. Biophys.*, **64**, 111 (1956).

(59) G. Scatchard and E. S. Black, *J. Phys. and Colloid Chem.*, **53**, 88 (1949).

(60) G. Scatchard, J. S. Coleman and A. L. Chen, *J. Am. Chem. Soc.*, **79**, 12 (1957).

priate zero charge computed from the actual K^+ binding, self-consistency (identical binding from the net ΔpH and from the length of the carboxyl extrapolation) is achieved when, at 0.01 and 0.135 M KCl, pK_0 COOH is 4.82 and 4.88, and binding of K^+ is 1.2 and 5.8 ions per mole. The new values lie on a curve which parallels the Ca^{++} data and passes through the experimental value for K^+. Nozaki,[16] *et al.*, observed the change in the intrinsic pK of the carboxyl groups without realizing the implications for K^+ binding. The intrinsic pK of the imidazole residues is constant at 7.45 and the quantitative influence of these groups at pH 5.2 is small compared to the carboxyl groups.

We conclude that an important part of the increase in isoionic pH with ionic strength found with conalbumin is an electrostatic effect. The binding of four cacodylate ions to conalbumin at the isoelectric point is determined in a relatively unambiguous way. If the association constant is between 10 and 20 the number of sites is between eight and six and the increase in chloride binding between $h = 0$ and $h = 40$ is probably not more than 2 to 4 ions per mole of protein.

Potassium ion binding does not occur in many proteins (*e.g.*, hemoglobin,[56] lysozyme[56]) even at higher than isoionic pH (ovalbumin,[56] serum albumin[56,60,61]). On the other hand, the association of K^+ to β-lactoglobulin[56] and to myosin[61] is well known. In the region $-10 > h > -50$ where the lysyl and tyrosyl residues titrate, chloride binding is, by any scheme, slight. If it is supposed that the low experimental values of w in this region are attributable entirely to potassium binding, then it follows that K^+ binding is a linear function of h, and greater at 25° than at 5° at least in the ratio $\frac{(0.033 - 0.025)}{(0.033 - 0.028)} = 1.6$. Qualitatively it may already be said that the heat of binding is positive. To get a more quantitative estimate of ΔH^0 we assume a simple model containing n sites of K^+ binding of a single type. It may be shown that, at constant h

$$\frac{\Delta H}{2.3R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right) = \frac{2w}{2.3}(\nu_2 - \nu_1) + \log\frac{\nu_2}{\nu_1}\frac{(n - \nu_1)}{(n - \nu_2)}$$

ν is the number of potassium ions bound.

Assuming that n is large and neglecting the electrostatic term, we obtain the minimal value, $\Delta H^0 = +4$ kcal. However, if n is finite or if the electrostatic term is included, ΔH^0 will increase with increasing pH. A larger heat will be computed from more complex models. If allowance is made for residual chloride binding, the minimal ΔH^0 may approach 8 kcal.

These results are contrary to experience. The average heat of association of potassium to myosin[61] is negative (less K^+ bound at 27° than at 5°); and for the proposed model[61] ΔH is zero for one set of sites and -10 kcal. for the other set. In the better defined systems of small molecules negative heats are encountered: For ethylenediaminetetraacetate ΔH^0 is 0, -1.4, and -4 to -14 for Li^+, Na^+ and various M^{++}[62] (K^+ is not bound; in the Z^2/r correlation K^+ would fall between Na^+ and the M^{++} series); for divalent cations and various carboxylates[63] and imidazoles[55] the heat of association is negative at -3 to -6 kcal.

We conclude that K^+ binding may be neglected. A reasonable description of the charge at 0.1 ionic strength, 25° from all the foregoing, is $Z = 0.92\ h - 4.5$, $20 > h > 0$. (Unless no chloride binding sites disappear when imidazole and amino groups titrate, the slope for $h < 0$ is closer to unity.)

The values of w at 25° and 5° still differ from the predicted value and from each other. Radii computed from (2) are 34 Å. (carboxyl) and 35 Å. (ϵ-amino, phenolic) at 25° and 0.1 ionic strength. The 5° values are about 1.5 Å. smaller. Other modifications might, of course, produce the same effect.

The Size and Shape of Conalbumin.—The molecular weight of conalbumin, determined in three different laboratories by four methods, is 76,600. Because of the inherent precision of the method, the sharpness of the end-points and the clear-cut absence of non-specific binding, the most reliable estimate of the molecular weight, 76,600 ± 400, is obtained from the stoichiometry of the ferric and cupric complexes of conalbumin.[46] The light-scattering value, 76,200 ± 1,700, reported by Timasheff and Tinoco,[64] and the value determined from osmotic pressure by Phelps and Cann,[53] 77,300 ± 2,500, are in good agreement. The sedimentation-diffusion molecular weight,[53] when properly evaluated, is 76,900. Higher values have been obtained by others[65,66] but the weight of evidence favors the lower molecular weight.[64]

To investigate the size and shape of conalbumin we evaluate the quantities

$$\beta_D = D[\eta]^{1/3}M^{1/3}\eta/kT \text{ and } \beta_s = N_s[\eta]^{1/3}\eta/M^{2/3}(1 - \bar{v}\rho)$$

introduced by Scheraga and Mandelkern.[67] The available hydrodynamic data are: $S_{20,w} \times 10^{13} = 5.10$,[53] 5.05,[66] 5.26 (Fe Con),[66] 5.4[65]; $D_{20,w} \times 10^7 = 6.01$,[53] 5.30,[66] 5.72 (Fe Con),[66] 5.66[65]; $[\eta] = 0.040$.[53] Phelps and Cann[53] report $\bar{v} = 0.756$ for native and 0.741 for reneutralized conalbumin; Fuller and Briggs[66] give $\bar{v} = 0.732$. The specific volume calculated from the amino acid analysis[50] using the residue volumes in Cohn and Edsall[68] is 0.733. McMeekin and Marshall[69] have compiled an impressive list (to which Kay[70] has made the most recent additions) showing that the partial specific volumes of proteins may be predicted extremely well from their composition. We therefore accept the value $\bar{v} = 0.732$. In this case, β_s[53] = β_D[53] = 2.17×10^6 (and incidentally, $M = 76{,}900$) while β_s[66] = 2.13×10^6 and β_D[66] = 1.95×10^6 (reflecting the low $D_{20,w}$ that gives an unacceptably high molecular weight).

(61) M. S. Lewis and H. A. Saroff, *J. Am. Chem. Soc.*, **79**, 2112 (1957).

(62) R. G. Charles, *ibid.*, **76**, 5854 (1956).

(63) E. L. King, *J. Chem. Ed.*, **30**, 71 (1953).

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(65) J. A. Bain and H. F. Deutsch, *J. Biol. Chem.*, **172**, 547 (1948).

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(67) H. A. Scheraga and L. Mandelkern, *ibid.*, **75**, 179 (1953).

(68) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 372.

(69) T. L. McMeekin and K. Marshall, *Science*, **116**, 142 (1952).

(70) C. M. Kay, *Biochim. Biophys. Acta*, **38**, 420 (1960).

The theoretical value of β for a sphere, 2.12×10^6, is within the experimental error of this determination, and it is not improbable that conalbumin is a spherical molecule. From the relations $r_S = M(1 - \bar{v}\rho)/(6\pi\eta Ns)$, $r_D = kT/(6\pi\eta D)$, $r_V = (30 M[\eta]/\pi N)^{1/3}$ we obtain, using Phelps and Cann's[53] data at *p*H 5, 0.10 ionic strength, $r_S = 35.3$ Å., $r_D = 35.6$ Å., $r_V = 36.5$ Å. For reneutralized denatured conalbumin, $r_S = 35.2$ Å., $r_V = 35.9$ Å. We note that the equivalent electrostatic radius is 34–35 Å.

At *p*H 3.0, 0.1 ionic strength, if the same $\bar{v}$ is applicable, then from $S_{20,w} = 4.07 \times 10^{-13}$, $[\eta] = 0.074$,[48] we obtain $\beta_S = 2.13 \times 10^6$, $r_S = 44.2$ Å., $r_V = 44.8$ Å. For an inflated sphere, the experimental electrostatic term, w, gives $r = 44$ Å. A sphere of volume $\bar{v}M/N$ has a radius of 28.1 Å., so that the hydration in this state is about 200%. A spherically symmetrical model is certainly expected, but in view of the arguments regarding flexibility[71] and the anomalies in BSA[24,25] it is curious that a *rigid* sphere seems indicated. The situation for BSA at acid *p*H[24,25] is quite analogous. Similarly, if the native molecule is a sphere, the hydration is about 75%.

Problems in Electrophoretic Theory.—At the present time, no general theory of the electrophoretic mobility of globular proteins exists. Henry's[72] extension of Debye–Hückel theory has limited applicability. For a sphere, the mobility, u, is related to the electrokinetic potential, ψ, by[73]

$$u = \psi D\phi(\kappa b)/6\pi n \qquad (3)$$

If we may identify the surface of shear with the surface of the molecule and use the spherical smeared-charge estimate of potential

$$\psi = (\epsilon Z/D)[1/b - \kappa/(1 - \kappa a)] \qquad (4)$$

we obtain, after rearranging

$$u = \frac{\epsilon Z}{6\pi n b} \frac{[(1 + \kappa)(a - b)]}{1 + \kappa a} \phi(\kappa b) \qquad (5)$$

From equations 2 and 4 the intimate relation of ψ and w is obvious. One would expect that whatever precise theory or parameters apply to one would apply to the other, so that both could be evaluated from the experiments appropriate to either, as long as the protein is not radically different from the model. In the cases to be discussed, the potential is small enough to make relaxation efforts negligible.[74]

Beychok and Warner[75] computed the charge on lysozyme from the mobility, using (5). Comparison with the hydrogen-ion binding data[12] then reproduced Carr's chloride binding[56] fairly well. In the neighborhood of the isoelectric point, $u = 1.0 \times 10^{-5}Z$,[12] approximately, for ionic strengths from 0.01 to 0.15. The theory [equation 5] predicts $u = 1.0 \times 10^{-5}Z$ at 0.15 ionic strength, $1.3 \times 10^{-5}Z$ at 0.03 ionic strength. These results, however, are not typical. Longsworth and Jacobsen[76] report that u/h for β-lactoglobulin and BSA is two-thirds of the theoretical value. The behavior of aldolase is similar.[77]

In two cases the data are more complete and the contradictions sharper. In the interval $20 > h > -20$, in 0.01 M chloride, the computed mobility of BSA from Schlessinger's data[78] is $u = (0.318\ h - 1.11) \times 10^{-5}$ cm.^{2} volt^{-1} sec.$^{-1}$. The degree of chloride binding, $\nu = 3.5$ at $h = 3.5$, agrees well with $\gamma = 4.0$, $h = 0$, found by Scatchard, Coleman and Shen.[60] From the latter work, chloride binding at 0.01 M is essentially linear in the interval $-20 < h < 20$, increasing from 0 to 8 ions per mole. The corrected expression for mobility is then $u = 0.398 \times 10^{-5}Z$. Using, in (5), the radius 35 Å. appropriate to the *experimental* value of w,[14] we find that $u = 0.680 \times 10^{-5}Z$. The value computed for a 30 Å. radius is $u = 0.849 \times 10^{-5}Z$.

In the case of conalbumin the experimental mobility in the linear region is $u = (0.149\ h - 0.60) \times 10^{-5}$ cm.^{2} sec.$^{-1}$ volt^{-1}, at 0° and 0.1 ionic strength. The experimental value of w, computed from the hydrogen-ion equilibria, includes in itself the possible ion-binding and shape effects, within the general framework of the theory. These do not therefore have to be taken explicitly into account. From the 5° data ($w = 0.028$), $u = 0.424 \times 10^{-5}\ h$ cm.^{2} sec.$^{-1}$ volt^{-1} at 0°. The 25° data give $u = 0.379 \times 10^{-5}\ h$. This is the largest discrepancy yet reported. It would seem that some fundamental concept has been overlooked in current electrophoretic theory.

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