for other peptides. The product chromatographed as a single spot but differently from the natural peptide. On hydrolysis it gave the expected products.

Comparison of Synthetic and Natural Peptides.—The synthetic and natural peptides were compared separately and as mixtures on paper chromatograms in a two dimensional system using isoamyl alcohol–pyridine–water (35/35/30) in one direction and phenol–water (70/30) in the second. One dimensional chromatograms were also run. Of the three peptides synthesized only L,L-N$^{\epsilon}$-(glycyl-α-glutamyl)-lysine behaved like the natural peptide.

Discussion

Peptide bonds involving the ϵ-amino groups of lysine are not novel in biochemical experience. Biocytin[31] and Bacitracin[32] contain such structures. Schweet[33] describes the incorporation of lysine into a soluble enzyme fraction of guinea pig liver through the epsilon amino group.

The isolation of the ϵ-lysine peptide from an acid hydrolysate of collagen indicates the existence of the structure in the protein. Strong acid hydrolysis at low temperature is not likely to produce artifacts. It seems likely that the α-carboxyl and the α-amino groups of lysine were originally part of a "normal" α-linked peptide chain, but of course this should be demonstrated by isolation of lysine peptides containing such linkages as well as the ϵ-linked amino acids. The amounts of peptide isolated accounts for only a fraction of a per cent. of the total lysine present, but the conditions of hydrolysis would be likely to yield only a little of any particular peptide, although there may be many more ϵ-amino branch points. If the deficit in the appearance of ϵ-dinitrophenyllysine on treatment of gelatin or collagen with dinitrobenzene fluoride[34] is accepted as evidence for protected ϵ-amino groups, about 40% of the lysine must be protected by peptide linkage. Bowes and Kenten[35] suggested the presence of peptide-bound ϵ-amino groups in collagen because of the agreement between titratable amino groups and the Van Slyke amino nitrogen and the deficit in both with respect to the total lysine contents. However, they express some doubts of the validity of the analyses for lysine with which they compare these figures. Revision of the analyses[36] still leaves a deficit of 31–37%. Courts and Stainsby[37] adduce evidence for the existence of multi-chain gelatin molecules and Pouradier[38] submits evidence that some gelatin molecules bear no terminal carboxyl groups. It is consistent with these findings and ours to propose that collagen contains peptide bonds formed between terminal carboxyls of α-linked chains and the ϵ-amino groups of lysine. The peptide we have isolated may be the residue of such a ring. The only direct evidence against the existence of covalently bound ϵ-amino groups is in the work of Bowes and Kenten[39] who found no lysine capable of yielding carbon dioxide with L-lysine decarboxylase after deamination of collagen with nitrous acid.

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NEW YORK, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, NEW YORK UNIVERSITY COLLEGE OF MEDICINE]

Denaturation and Electrophoretic Behavior of Lysozyme[1,2]

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The rate of denaturation of lysozyme has been studied as a function of *p*H at 94.3 and 84.6° at two ionic strengths. The change in rate constant as *p*H varies from 1 to 6.3 is unusually small. Using a set of postulates developed by Levy and Benaglia, two equilibrium constants, which govern the variation of rate constant with *p*H, were evaluated. Mobilities from electrophoretic measurements were employed in calculating the potential at the surface of shear of the protein as a function of *p*H. These potentials are used in a modified form of the usual titration equation for proteins and the electrostatic factor involved in the titration of lysozyme from *p*H 2.0 to 6.0 has been calculated. The construction of a theoretical titration curve requires that about half of the carboxyls have a pK_0 of 3.53, the other half a pK_0 of 5.08. The relation of these values to hydrogen bonds involving carboxyls is discussed. It is concluded that the extreme stability of lysozyme at acid *p*H's does not depend on the hydrogen bonded structure but on some other factor such as disulfide cross-linking. Small modification in the stability of this structure by rupture or formation of the weaker prototropic hydrogen bonds may be responsible for the small variation of rate of denaturation with *p*H.

The variation in the rate of denaturation with *p*H of several proteins has been accounted for by the assumptions that there are a limited number of proton dissociations which are critical for the stability of the molecule and that a different specific rate constant can be assigned to the dissociated and undissociated forms.[4–9] Such a dependence might arise from the existence of cross-linking hydrogen bonds involving these dissociating groups.

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(2) Taken in part from a dissertation presented by Sherman Beychok in partial fulfillment of the requirements for the degree of Doctor of Philosophy at New York University, June 1957.

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(6) M. Levy and R. C. Warner, *J. Phys. Chem.*, **58**, 106 (1954).

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The proteins that have been studied in this way have been those such as ricin[5] or bovine plasma albumin[6,7] which denature more or less rapidly below 70°, or proteins which show unusual instability at room temperature in some particular *p*H range such as pepsin,[4] conalbumin[8] or ferrihemoglobin.[9] This investigation describes an examination of the protein, lysozyme, from the same point of view. Lysozyme was chosen for study because it differs from these other proteins in its relative stability to temperatures as high as 100°. In addition, there are only eleven groups per molecule which titrate in the carboxyl region. Tanford and Wagner have found, however, that this part of the titration curve could not be described as due to a set of eleven groups with the same intrinsic *pK*.[10] It seemed possible that the same groups, modified by involvement in prototropic hydrogen bonds, might be responsible for the aberrant titration curve and might also appear as critical groups in the denaturation kinetics. The electrophoresis of lysozyme has been studied as an adjunct to the interpretation of the titration curve.

Experimental

All of the data reported in this paper were obtained with six times recrystallized lysozyme. The protein was crystallized using a modification of the method of Alderton and Fevold.[11,12] Armour and Co. provided a small amount of lysozyme as the lyophilized powder, lot No. 381-187. This was recrystallized and was indistinguishable from the freshly prepared protein under all conditions reported here.

Lysozyme, when prepared as above, is reported to contain a second component which is approximately 5% of the total protein.[13] Whatever the nature of this minor component, it could not be detected electrophoretically at any *p*H reported here (*p*H 1 to 6.25). Similarly, sedimentation showed only a single, well-defined peak at all *p*H's.

Lysozyme contains a large number of amide groups relative to the total charge.[14] Since the free carboxyl content determined from the amino acid analysis[15,16] does not agree well with that from the reported titration curve,[10] it appeared possible that different preparations might contain different numbers of amide groups. When a 1% solution of lysozyme was passed through an ion-exchange column (hydrogen, hydroxyl mixed bed resin), the solution had a *p*H of 10.72 to 10.75. The isoionic point of lysozyme determined from the titration curve is 11.1. Electroneutrality demands a sufficient net charge on the protein to balance the hydroxyl ion charge, and it is predicted from the titration curve that this will occur at 10.70. This indicates the same number of free carboxyls in our preparations as in those used for the titration.

The criterion for denaturation used in this investigation was loss of solubility in a solution of specified *p*H and salt concentration, the stopping buffer. The stopping buffer establishes conditions in which no further reaction occurs and quantitatively precipitates denatured material while native protein remains in solution. The requirements for a satisfactory stopping buffer have been fully discussed elsewhere.[6] A stopping buffer which meets these requirements for lysozyme was prepared that contained 0.766 *M* potassium trichloroacetate maintained at *p*H 4.45 with 1.7 *M* acetate buffer. Except for the composition of the stopping buffer, the experimental techniques used in measuring the denaturation reaction were the same as those described by Levy and Warner.[6]

Electrophoresis was conducted as previously described.[17] Ionic strength was 0.15, made up entirely of buffer ions. The position of the boundary was determined directly from the photographic plates using a two-coördinate comparator. The centroidal ordinate was located using an averaging process described by Longsworth.[18]

Sedimentation experiments were made in the Spinco Model E ultracentrifuge. The position of the boundary and all reference lines on the plates were measured on the comparator in the same way as for electrophoresis.

Results

A plot of the logarithm of rate constant for denaturation (log *j*) *versus* *p*H at 94.3 and 84.6° is shown in Fig. 1. No quantitative measurements were made above *p*H 6.5 because of the low solubility of the native lysozyme in this range. The rate constants above *p*H 7, however, are always greater than anywhere below. It is therefore evident that in this temperature interval the protein has maximum stability at about *p*H 5.5—five and one half *p*H units acid to the isoelectric point. The rate of denaturation increases steadily below 5.5, but at *p*H 3 begins to fall off somewhat until at *p*H 2 another minimum on the curve appears. Thereafter, the stability decreases rapidly.

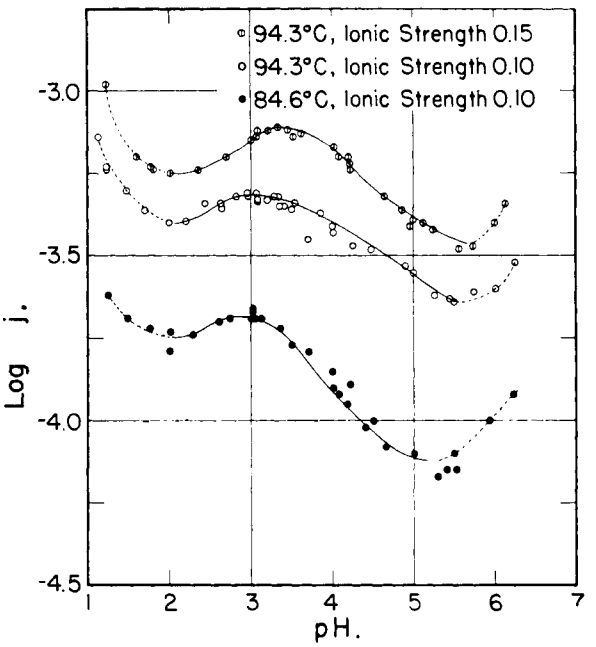

Fig. 1.—The effect of *p*H on the first-order rate constant of denaturation of lysozyme at 94.3 and 84.6°. The solid lines are calculated from equations found in the text. The broken lines are smooth curves drawn through the experimental points.

In view of the very large effects of *p*H usually observed, the most striking aspect of Fig. 1 is the small over-all variation in rate of denaturation. The logarithmic plot was actually unnecessary but was made because it is most suitable for the analysis which follows.

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(18) L. G. Longsworth, THIS JOURNAL, **65** .1755 (1943).

The general equation developed by Levy and Benaglia[5] for the variation in rate of denaturation with pH is

$$j = \frac{k_1h^n + k_2K_1h^{n-1} + \ldots k_n K_1K_2..K_{n-1}h + k_{n+1}K_1K_2\ldots K^n}{h^n + K_1h^{n-1} + K_1K_2h^{n-2} + \ldots K_1K_2..K_{n-1}h + K_1K_2..K_n} \quad (1)$$

where

j = first-order constant for denaturation
h = hydrogen ion activity
k_{n+1} = rate constant for species resulting from the dissociation governed by equilibrium constant, K_n

In previous cases, this equation has been fitted to the data and K's estimated by making use of pH regions in which a linear relation of log j to pH with an integral slope was obtained. It should be noted, however, that in the application of this equation to a single critical ionization, for which

$$j = \frac{k_1h + k_2K}{h + K} \quad (2)$$

a slope of unity is obtained only when one of the specific rate constants is about ten times the other. The pK can be evaluated only if regions exist above or below the integral slope in which the rate is independent of the pH.

The data for lysozyme show two minima and a maximum so that at least a 4th power of hydrogen ion concentration is required for the kinetic equation. Thus four critical dissociations are involved. There are no portions of the curve showing integral slopes or slopes even approaching unity, except at the very acid end. Since there is no leveling off at pH 1 and 6.3, there must be a pK somewhere below 1 (this was observed for ricin and bovine plasma albumin, also) and one somewhere above 5.5. These cannot be evaluated because no estimate can be made of the rate constants of the most acid and least acid species of lysozyme in the region being examined.

Between pH 2.0 and 5.5, at least two dissociations are critical. In order to estimate these, we have assumed that the occurrence of a minimum or maximum indicates dominance of the over-all rate in that region by a single species. Then the value of (j) at a given minimum is equal to the rate constant for the species determining the rate at that minimum. The dissociation constants can then be calculated from eq. 2. If the assumption made above is correct, then the values for K_2 and K_3 permit little variation. Nonetheless, little confidence can be placed in the absolute values derived for the parameters in the case of log j *vs.* pH curves of this kind. The number of parameters is determined by the changes in slope, but in the absence of integral slopes they cannot be uniquely determined. To show their order of magnitude, we have calculated a set of parameters for each of the three curves and these are recorded in Table I. Successive approximation was necessary for close fitting, but an adequate fit could be made by direct application of eq. 2. A curve constructed from these constants is drawn as the solid line between pH 2 and 5.5 in Fig. 1.[19]

(19) The occurrence of more than one minimum on the curve is evidence in favor of the "critical group" theory of protein denaturation. Behavior of this sort was also observed for bovine plasma albumin below pH 4.0.[6] It is known that albumin undergoes an expansion at pH's below 4, and the greater stability of the molecule might be related to this change in configuration. Lysozyme, however, undergoes no such expansion in acid solution. We have determined the intrinsic viscosity of lysozyme as a function of pH and found it to be constant in the entire pH region under consideration.

TABLE I

PARAMETERS FOR DENATURATION

Ionic strength	0.15	0.10	0.10
Temp., °C.	94.3	94.3	84.6
k_2	5.0×10^{-4}	3.8×10^{-4}	1.5×10^{-4}
k_3	9.0×10^{-4}	5.4×10^{-4}	2.6×10^{-4}
k_4	3.3×10^{-4}	2.3×10^{-4}	6.8×10^{-5}
K_2	1.4×10^{-3} (pK = 2.85)	2.5×10^{-3} (pK = 2.60)	3.2×10^{-3} (pK = 2.49)
K_3	6.0×10^{-5} (pK = 4.22)	6.5×10^{-5} (pK = 4.19)	1.4×10^{-4} (pK = 3.85)

In the ricin and bovine albumin cases, a small number of constants was necessary in fitting data which covered many pH units. In both instances, the rate of denaturation varied greatly with small changes in pH and a large number of proton dissociations were known to occur. Lysozyme, on the other hand, requires four constants between pH 1 and 6.3 and none of the dissociations corresponding to these can be said to be critical to the stability of the protein, since each gives rise to a very small change in the over-all rate of denaturation. To emphasize this point, we may note that over the entire pH range examined for lysozyme, the total variation in rate constant for denaturation is just over threefold. For the same pH interval, in bovine plasma albumin and ricin, the variation is several thousand-fold. We may conclude that none of the groups titrating acid to pH 6.5 in lysozyme is involved in a prototropic hydrogen bond the formation or rupture of which affects, except in a minor way, the stability of the molecule to heat.

Analysis of the Titration Curve.—Tanford and Wagner have titrated lysozyme but were unable to assign an intrinsic dissociation constant for the carboxyls.[10] In the first place, the binding of chloride ion is considerable in the acid region and has not been accurately measured. Secondly, the acid region of the curve is too flat even if no binding occurs. Tanford and Wagner suggest that the carboxyl groups are not all alike in titration behavior, but that calculation of the constants was not worthwhile until the charge at any pH could be estimated more accurately.

The usual form of the equation used to describe protein titration curves is[10]

$$\log r_i/(n_i - r_i) = p\mathrm{H} - (pK_0)_i + 0.868Zw \quad (3)$$

in which n_i is the total number of identical groups in a set having an intrinsic constant for dissociation $(K_0)_i$, r_i is the number of these groups dissociated at any pH, Z is the net charge and

$$w = \frac{\epsilon^2}{2Dk\bar{T}}[(1/b - \kappa/(1 + \kappa a)] \quad (4)$$

in which (b) is the radius of the molecule, (a) is the radius of exclusion and (k) is the Boltzmann constant. From the Debye–Hückel theory of strong electrolytes, the potential at the surface of a sphere

of charge Z spread uniformly over the surface is[20]

$$\psi = \frac{\epsilon Z}{D}\,[1/b - \kappa/(1 + \kappa a)] \qquad (5)$$

and from eq. 4

$$w = \psi \frac{\epsilon}{2ZkT} \qquad (6)$$

Substitution of eq. 6 into eq. 3 yields

$$\log r_1/(n_1 - r_1) = p\mathrm{H} - (pK_0)_1 + 0.434\,\frac{\epsilon\psi}{kT} \qquad (7)$$

It is possible, then, to analyze the titration curve without knowledge of the charge Z, provided the potential at the surface of the molecule is known as a function of pH.[21] If it is assumed that this potential is equal to that at the surface of shear (electrokinetic potential), then the required information comes directly from the observed electrophoretic mobilities since[22]

$$u = \frac{\psi D}{6\pi\eta} \times f(\kappa a) \qquad (8)$$

where (u) is the mobility, ψ is the potential at the surface of shear, (D) is the dielectric constant of the solvent, η is the viscosity of the solvent, (a) is the radius of exclusion and $\kappa = \sqrt{(4\pi N\epsilon^2/1000DkTT)}$. The function, $f(\kappa a)$, was evaluated by Henry.[22]

Equation 8 does not take into account the relaxation effect. Overbeek[23] has evaluated this for particles having the dimensions of proteins and shown it to be a function of $\epsilon\psi/kT$ and of the nature of the surrounding electrolyte. For values of $\epsilon\psi/kT$ less than 1.2 (approximately 25 mv. potential) at 0°, overlooking the relaxation effect introduces a maximum error of 3% in a univalent symmetrical electrolyte.

It should be pointed out that the radius of the particle enters into the calculation only in the term, $f(\kappa a)$, which changes logarithmically with the dimensions of the particle. Thus a small error in evaluating the radius will not be important in calculating the potential. The calculation of charge from mobility or potential, however, involves a direct dependence on the dimensions and shape of the particle, which can often only be approximated, so that attempts to evaluate charge from mobility data are considerably less accurate.

Equation 8 can be used to evaluate potential if this potential is no greater than about 25 mv. ($\epsilon\psi/kT$ less than 1.2) in a symmetrical electrolyte and the particle is spherical or nearly so. In Fig. 2, the mobility–pH curve for lysozyme is shown. Columns 3 and 5 of Table II give the values of ψ and $\epsilon\psi/kT$, respectively, calculated from eq. 8 using a radius of 19 Å. in the term $f(\kappa a)$; the radius was evaluated from the diffusion constant.[12] Column 6 gives $0.434\,(\epsilon\psi/kT)$.

With the values listed for the electrostatic interaction term, it is impossible to construct the acid region of the titration curve using a single intrinsic dissociation constant for all the carboxyl groups. The construction can be made for the 10.5 carboxyls present[10] by assuming two sets of groups, one of six carboxyls, the other 4.5 carboxyls and assigning to the first a pK_0 of 3.53 and the second a pK_0 of 5.08. It would appear that there is some flexibility in the choice of constants since there are three degrees of freedom in the fitting, but if a total of 10.5 carboxyls is assumed, then the experimental curve allows little variation. In Fig. 3, Tanford and Wagner's acid titration for lysozyme is shown (upper curve, h). The line through the points was calculated from eq. 7 with $(pK_0)_1$ of 3.53 and $(pK_0)_2$ of 5.08.

TABLE II

pH	$u \times 10^5$, cm.²/sec. v.	ψ, mv.	$\psi \times 10^8$ ergs/e.s.u.	$\epsilon\psi/kT$	$0.434\ \epsilon\psi/kT$
1.5	7.91	25.3	8.43	1.06	0.463
2.0	7.60	24.4	8.11	1.04	.451
2.3	7.56	24.2	8.06	1.03	.447
2.6	7.56	24.2	8.06	1.03	.447
2.9	7.51	24.0	8.01	1.02	.443
3.2	7.41	23.7	7.89	1.01	.439
3.5	7.24	23.2	7.71	0.988	.428
3.8	6.94	22.2	7.41	.948	.412
4.1	6.51	20.9	6.96	.885	.384
4.4	6.35	20.3	6.77	.863	.375
4.7	6.33	20.2	6.75	.862	.374
5.0	6.26	20.0	6.67	.852	.370
5.3	6.13	19.6	6.54	.836	.363
5.6	5.93	19.1	6.32	.806	.350
5.9	5.70	18.2	5.77	.737	.320

If the electrostatic contribution is as indicated in Table II,[24] then it is not permissible to assign four constants evenly distributed between 3.53 and 5.08 since the extreme acid end then becomes too flat. If four are used, two cluster around 3.5 and two around 5.1.

An Estimate of the Charge from pH 2 to pH 6.—The calculation of charge from potential is subject to greater error than the calculation of the potential from mobility for reasons stated above. Nonetheless, interest attaches to an estimate of the charge. For a spherical particle of radius (b) and average radii of the counterions (a_i), the relation between charge and potential is[25]

$$Q = \psi D b\,\frac{1 + \kappa\,(a_i + b)}{1 + \kappa a_i} \qquad (9)$$

The value used for (a_i) was 2.5 Å. The charge Z, where $Z = Q/\epsilon$ is shown as a function of pH in Fig. 3. Using the charge arrived at in this way, the term (w) can then be calculated from eq. 3, and it is equal to 0.065.

The difference between the titration valence (h) and the charge calculated from electrophoresis (Z) may be taken as the number of anions bound at any pH. This difference $(h - Z)$, is plotted *versus* pH in Fig. 3. In the figure also are shown

(20) G. Scatchard, in, "Proteins, Amino Acids and Peptides," Ed. E. J. Cohn and J. T. Edsall, Reinhold Publ. Corp., New York, N. Y., 1943, p. 55.

(21) A. Katchalsky, N. Shavit and H. Eisenberg, *J. Polymer Sci.*, **13**, 69 (1954).

(22) D. C. Henry, *Proc. Roy. Soc.* (*London*), **A133**, 106 (1937).

(23) J. Th. Overbeek, *Adv. in Colloid Sci.*, **3**, 97 (1950).

(24) The calculation of the electrostatic contribution using information from electrophoresis assumes that the binding of buffer ions—formate, acetate and acid phosphate—is approximately equal to the binding of chloride. The justification for this is that mobilities measured in HCl buffers overlapped well with those in phosphate buffer. The same was true for phosphate–formate and formate–acetate.

(25) H. A. Ahramson, L. S. Moyer and M. H. Gorin, "Electrophoresis of Proteins," Reinhold Publ. Corp., New York, N. Y., 1942.

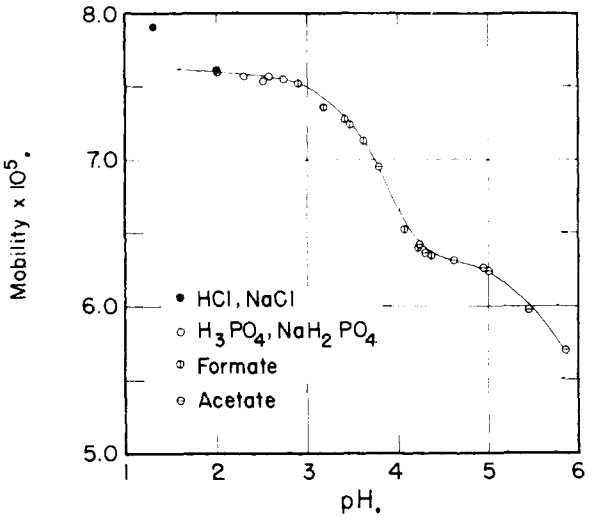

Fig. 2.—The electrophoretic mobility of lysozyme at 0° and ionic strength 0.15, as *p*H is varied. Mobility units are cm.²/sec. v.— Ionic strength made of buffer ions only, as indicated.

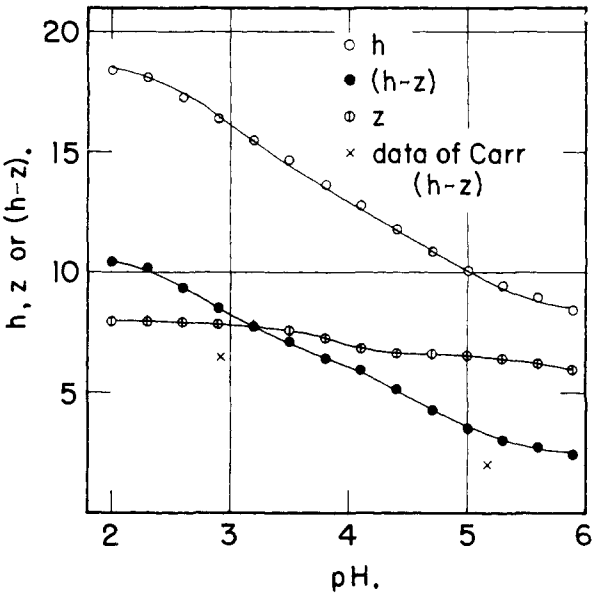

Fig. 3.—The binding of hydrogen ions and other small ions and the charge resulting from these as a function of *p*H. The curve marked (*h*) is a theoretical titration curve calculated as explained in the text. The points on this curve are experimental from Tanford and Wagner.[10] Curve (*Z*) is calculated from electrophoretic data as described in the text. (*h* − *Z*) shows the binding of buffer ions to lysozyme. Two points measured by Carr[26] are for binding of chloride ion.

two points which Carr found for chloride binding.[26] A plot of (*h*) *versus* (*h* − *Z*) is fairly linear and gives a slope of 0.8 from *p*H 2.0 to 6.0. If there is any specific interaction between the carboxyls and the basic binding sites, such interaction appears to be unrelated to the difference in the carboxyls.

Figure 2 shows an unusual variation of mobility with *p*H between *p*H 3 and 5. The mobility begins to level off near *p*H 5, then increases rapidly before becoming constant at *p*H 3. This increase is equivalent to 1.2 charge per molecule and thus is barely evident in the curves of Fig. 3 which are drawn to a different scale. Since the curve for *Z* in Fig. 3 is almost independent of *p*H, it appears that approximately one anion is bound per proton bound. The jump in Fig. 2 may then be associated with a single protonation (*pK* about 3.6) which is unique in not being associated with the binding of an anion.

TABLE III

SEDIMENTATION COEFFICIENTS

*p*H	State of protein	$s_{20,w}$
5.47	Native	1.94
5.47	Denatured	1.97
3.64	Native	1.79
3.65	Denatured	1.83
2.27	Native	1.71

Some Properties of Denatured Lysozyme.—Electrophoretic measurements revealed no difference in the mobilities of native and heat treated lysozyme if the duration of heating was for one half-period of denaturation. When the protein was denatured for more than two half-periods, a second component appeared and increased in amount with increasing time of denaturation. After four half-periods, at *p*H 3.64 in formate buffer, the second component was 12% of the total protein. The major component had a mobility of 7.07×10^{-5}; the minor component had a mobility of 5.82×10^{-5}. Calculation from eq. 8 and 9 indicates a valence difference of 1.2, the minor component being less positive.

It was thought at first that the second component represented a dimer or higher polymer of lysozyme formed through possible disulfide exchange reactions. Lysozyme was sedimented at several *p*H's. The denatured samples used were the same as those which underwent electrophoresis. The results are shown in Table III. No second component was evident.[27]

The second component in electrophoresis thus must be looked upon as a monomer with approximately one less positive charge (net charge) on the molecule. Since this occurred at all *p*H's from 5.9 to 2.3, it did not appear likely that the extra negative charge was a carboxylate resulting from amide hydrolysis.

A possible explanation for this is that at the elevated temperatures and under the conditions employed, the disulfide is hydrolyzed with subsequent formation of ionized sulfenic acid. Cecil[28] has shown that acetate ion catalyzes this hydrolysis at *p*H 4.9 with sulfenic acid the major product. Alternatively, the solutions used at 100° may provide a sufficiently good oxidizing medium for production of a sulfoxide with subsequent formation of the acid.

Discussion

Laskowski and Scheraga[29] have formulated the relation between the dissociation constant K_1 of a

(26) C. W. Carr, *Arch. Biochem. Biophys.*, **46**, 417 (1953).

(27) The slight change in sedimentation constant with *p*H for both native and denatured protein occurs because no correction was made for concentration of protein which varied somewhat.

(28) R. Cecil, *Biochem. J.*, **47**, 572 (1950).

(29) M. Laskowski, Jr., and H. A. Scheraga, THIS JOURNAL, **76**, 6305 (1954).

carboxyl group and the observed constant K_{obsd} when the carboxylate ion (acceptor) is hydrogen bonded to, say, a tyrosyl residue (donor) as

$$K_{obsd} = K_1 (1 + K_{ij}) \quad (10)$$

where K_{ij} is the equilibrium constant for the formation of a hydrogen bond. From theoretical estimates of the entropy and enthalpy of model hydrogen bonds and a single postulated case of a tyrosine–carboxylate bond in a protein, Laskowski and Scheraga calculated a value for K_{ij} of 4.0.

To obtain an intrinsic pK of 3.5 for a carboxyl group, the value of K_{ij} must be about 9.0 if pK_1 is 4.5. The analysis of the alkaline region of lysozyme was successfully made by Tanford and Wagner[10] if they assigned to the tyrosines an intrinsic pK of 10.4 to 10.9. A spectrophotometric titration had earlier given a value of 10.8.[30] The normal pK_0 is about 9.6. Thus the three phenolic groups are displaced by an amount corresponding to the displacement of the carboxyls from the normal value. If carboxylate–tyrosine bonds existed, this would be predicted since K_{obsd} for the donor would be

$$K_{obsd} = \frac{K_2}{1 + K_{ij}} \quad (11)$$

The value of K_{ij} for the tyrosines would also be of the order of 9. The carboxyls with a pK_0 of 5.08 are presumably also modified. The assumption of carboxyl–carboxyl hydrogen bonds would account for the high pK_0.[29]

Recently, Donovan, Laskowski and Scheraga[31] examined the differential ultraviolet spectrum of lysozyme and found no evidence of carboxyl–tyrosine hydrogen bonds. However, the extinction at 295 mμ was dependent on the state of ionization of the carboxyl groups. From their data, they concluded that some carboxyls must be located very near the tryptophan residues and that ionization of these carboxyl groups alters the absorption of the tryptophan groups. They showed that two distinct kinds of carboxyls were involved and, while it was not possible to estimate the number of carboxyls in each set, they were able to calculate apparent pK's of 3.2 and 6.1. These authors suggest that the abnormal pK's are due either to hydrogen bonding or very large electrostatic effects. Since these are apparent constants, the agreement for the low value with ours is quite good. Further comment on the discrepancy in the value for the carboxyls titrating with high pK requires knowledge of the actual number of these affecting the tryptophan absorption.

The evidence from the titration analysis is in favor of at least some hydrogen bonded carboxyls and probably more than half the titratable total. The conclusion was drawn earlier from the denaturation kinetics that no prototropic bonds exist, the formation or rupture of which has a large effect on the stability of the molecule to heat. These observations indicate that the extraordinary over-all stability of lysozyme below pH 6 is not due to hydrogen bonding involving the groups which titrate below pH 6. Confirmation of a sort comes from experiments of Jirgensens[32] in which native lysozyme was treated with varying concentrations of guanidine hydrochloride. The reduced viscosity of native lysozyme was 0.031. In 2.5 M guanidine, the reduced viscosity was 0.029 at pH 4.6. Bovine serum albumin, under the same conditions, showed a threefold increase in reduced viscosity compared to native albumin.

The presence of 5 disulfide bonds in a relatively small molecule may be responsible for the unusual stability of lysozyme below pH 6. The variations in rate shown in Fig. 1 would then be attributed to modifications in the stability of the disulfide bonded structure by titration of critical hydrogen bonded groups, each of which makes only a small contribution to the stability in comparison with a disulfide bond. It is assumed that a disulfide bond must be broken in order to produce insoluble protein but not necessarily that this give rise to a negative charge. The little evidence that we have from electrophoretic experiments indicates that the production of a negative charge is secondary to denaturation. At higher pH's, where the disulfide groups are more subject to hydrolysis, exchange reactions and oxidation, their instability may be a factor in the rate of denaturation.

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