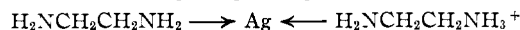
Again there is a parallelism with exchange equilibria of quaternary ammonium cations.[14]

(d) **The Silver–Ethylene Diamine Complex.**—Here it appears that the complex is much more stable in the resin, the effect being far greater than one would expect from van der Waals attraction (by comparison with *n*-butylamine). As a possible explanation we suggest that in the resin the ion $H_2NCH_2CH_2NH_3^+$ ($enH^+$) coördinates with the silver ion to form a doubly charged complex. In dilute solution such a complex would be very unstable on account of electrostatic repulsions; in the resin, however, there will be a considerable screening effect due to the high concentration of negative charges. (For a 5 *M* solution of a uni-univalent salt, the Debye–Hückel parameter, $1/\kappa$, is 1.36 Å.; this gives us an order of magnitude for the screening effects.) The concentration of $enH^+$ in the resin, while small, is about ten thousand times as great as the concentration of uncharged en. It is not unreasonable to suppose, therefore, that most of the silver ions in the resin are coördinated with $enH^+$, and some of them with en as well, giving complexes



$$H_2NCH_2CH_2NH_2 \longrightarrow Ag \longleftarrow H_2NCH_2CH_2NH_3^+$$

If this is so, there will be more $enH^+$, free and bound, in the resin than we thought there was, and therefore more millimoles of ionic ethylene diamine; the neutral en bound to Ag will then be less than we calculated, and the binding of neutral en to silver may be little, if any, stronger in the resin than in the solution. Unpublished data on coördination of silver with ethanolamine support this interpretation.

That a steric repulsion of en from the resin exists is seen from the effects of increasing en:Ag ratio and of increasing the silver concentration in the resin (Fig. 4).

**Acknowledgments.**—We thank the Permutit Company and the Rohm and Haas Company for providing the resins used. Thanks are also due to the University of Colorado Council on Research and Creative Work for assistance in starting this research, and to Leone Cockerell and Kaye L. Motz for help with the experimental work.

BOULDER, COLORADO

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

# Hydrogen Ion Equilibria of Lysozyme[1,2]

BY CHARLES TANFORD AND MYRON L. WAGNER[3]

RECEIVED FEBRUARY 15, 1954

Hydrogen ion titration curves of crystalline lysozyme have been obtained at two temperatures and three ionic strengths. There are found present 10.5 carboxyl groups, 1 imidazole group, 1 $\alpha$-amino group, 5 or 6 $\epsilon$-amino groups, 3 phenolic groups and 12 or 11 guanidine groups, per molecule of assumed molecular weight 14,200. The intrinsic $pK$'s of all basic nitrogen groups appear to be normal, the intrinsic $pK$ of the phenolic groups is about 10.8 instead of the expected 9.6, and the titration of the carboxyl groups appears to be complicated and no $pK$ values for these groups could be obtained. The empirical values of the electrostatic factor $w$ obtained in the isoelectric region suggest that the lysozyme molecule is compact and sparingly hydrated.

## Introduction

It was first shown in the pioneering study of Cannan, Kibrick and Palmer on ovalbumin[4] that the dissociation of hydrogen ions from proteins can often be explained on the basis of the intrinsic dissociation tendencies of the various types of dissociating groups, coupled with the electrostatic effect due to the charges present on protein molecules at any $p$H other than the isoelectric $p$H. The present paper represents a similar study on another protein crystallized from egg white, lysozyme.

The results of such a study serve to reveal abnormalities of any of the dissociating groups, and such are shown to exist in the phenolic and carboxyl groups of lysozyme. The study of hydrogen ion equilibria serves another purpose in that it is an indispensable prerequisite to an understanding of the $p$H-dependence of all properties of a protein molecule, including, in the case of lysozyme, its physiological activity. The experimental and computational methods used have been reviewed in another paper[5] and will receive only brief discussion here.

## Experimental

The lysozyme used in this work was lot 003L1, obtained from Armour and Co., Chicago, Ill. According to the manufacturer's assay the moisture content was 4.12%, ash (sulfated) 1.52%, nitrogen 18.3%. Electrophoretic measurements performed by Armour and Co., in phosphate buffer at $p$H 7.7 and ionic strength 0.2, showed two components with mobilities 1.82 (95%) and 0.60 (5%).[6]

Stock solutions were prepared as needed by dissolving the protein in water. To lower the amount of inorganic impurity indicated by the 1.52% ash content each stock solution was dialyzed twice against conductivity water in bags made from 36/32″ Visking dialysis tubing. There was probably some loss of protein in this procedure. The ash

(1) Presented at the 125th National Meeting of The American Chemical Society, Kansas City, Mo., March, 1954.

(2) This investigation was supported by research grants RG-2350 and H-1619 from the National Institutes of Health, Public Health Service and by a grant from The National Science Foundation.

(3) Abstracted from the Ph.D. Thesis of Myron L. Wagner, State University of Iowa, February, 1954.

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

(5) C. Tanford, "Symposium on Electrochemistry in Biology and Medicine," in press.

(6) The occurrence of about 5% electrophoretic impurity in lysozyme is usual.[7] Tallan and Stein[7] suggest that it may be due to hydrolysis of some of the amide groups of lysozyme, in analogy with the two components of insulin, observed by Harfenist and Craig.[8] The mobilities here given are lower than those found at close to the same $p$H by Tallan and Stein, who, however, used barbiturate buffers instead of phosphate. Abnormally low mobilities in phosphate have been observed by Alderton, Ward and Fevold.[9]

(7) H. H. Tallan and W. H. Stein, *J. Biol. Chem.*, **200**, 507 (1953).

(8) E. J. Harfenist and L. C. Craig, THIS JOURNAL, **74**, 3083 (1952).

(9) G. Alderton, W. H. Ward and H. L. Fevold, *J. Biol. Chem.*, **157**, 43 (1945).

content of the lysozyme in the first three stock solutions prepared in this way was found to have been reduced to 0.15% or less. Subsequent solutions were not ashed.

Six such stock solutions were prepared, and their lysozyme content determined by heating to constant weight at 105–107°. When diluted with KCl solution (final ionic strength 0.15) all of the stock solutions had a *p*H close to 6.0. Slightly different values were obtained with each stock solution.

Six independent determinations of the molecular weight of lysozyme are reported in the literature.[10–15] Five of these lie between 13,400 and 14,900. In all calculations reported in this paper 14,500 g. of the protein in the stock solutions was taken to correspond to one mole. From the titration curve obtained it can be seen that a *p*H of 6.0 in 0.15 *M* KCl corresponds to 8.3 bound protons per mole, so that the formula of the lysozyme in our stock solutions must have been [lysozyme, 8.3 HX], where X is presumably chloride ion, since NaCl is present in large excess during recrystallization. The contribution of 8.3 HX to the weight of one mole is therefore about 300, and our assumption therefore corresponds to a molecular weight of about 14,200 for isoelectric lysozyme, which is a good compromise between the various experimental values.

Standard HCl was prepared by dilution of constant boiling HCl, potassium hydroxide was prepared $CO_2$-free by the method of Kolthoff[16] and standardized against potassium acid phthalate. Reagent grade KCl was used without further purification.

Potentiometric measurements were made in the water-jacketed apparatus previously described,[5] using a Beckman glass electrode (type 1190-80) and a Beckman model G *p*H meter. Measurements were made at 25° and at 4°. Measurements at high *p*H were made in a nitrogen atmosphere.

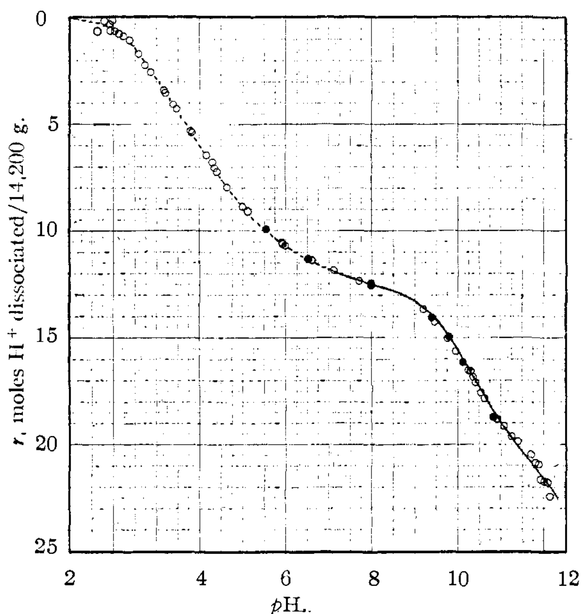


Fig. 1.—Titration curve at 25° and $\mu$ = 0.15. Solid circles represent points reversed from *p*H 11. The solid line between *p*H 7 and 12 is a curve calculated by equation 1 for 1 imidazole group ($pK_{int}$ 6.70), 1 $\alpha$-$NH_2$ group ($pK_{int}$ 7.55), 5 $\epsilon$-$NH_2$ groups ($pK_{int}$ 10.27), 3 phenolic groups ($pK_{int}$ 10.70), 12 guanidine groups ($pK_{int}$ 12.65) and a constant value of 0.080 for *w*.

Spectrophotometric titration of the phenolic hydroxyl groups was carried out by the method originated by Crammer and Neuberger[17] as modified in this Laboratory.[18] Measurements were made at 15° and at 35° in a Beckman model DU spectrophotometer equipped with thermospacers. A special cover was constructed for the cell housing which made it possible to carry out measurements in a nitrogen atmosphere.

**Construction of Titration Curves.**—The titration curves of Figs. 1, 2, 3 and 5 were constructed from *p*H measurements in the manner previously described.[5] The number of hydrogen ions bound or dissociated is first referred to the original state of the protein in the stock solution. Since the six stock solutions employed in the present study differed slightly in *p*H, the original state of one of these was arbitrarily selected as a common reference point, and the values for bound or dissociated hydrogen ions for the other stock solutions were corrected to this reference point by the number of hydrogen ions which would be required to change the original *p*H to the arbitrary reference *p*H. The original data and all calculations leading to the construction of Figs. 1, 2, 3 and 5 are available on microfilm from the American Documentation Institute.[19]

## Results

The experimental data at 25° and $\mu$ = 0.15 are shown in Fig. 1. This figure shows several points obtained by first exposing the lysozyme (initially near *p*H 6, as previously explained) to *p*H 11, followed by addition of acid to lower the *p*H to the desired final values. These points fall on the same curve as those obtained without previous exposure to high *p*H, thus showing that the titration curve is completely reversible between *p*H 6 and 11.

Figure 2 shows that the titration curve is also

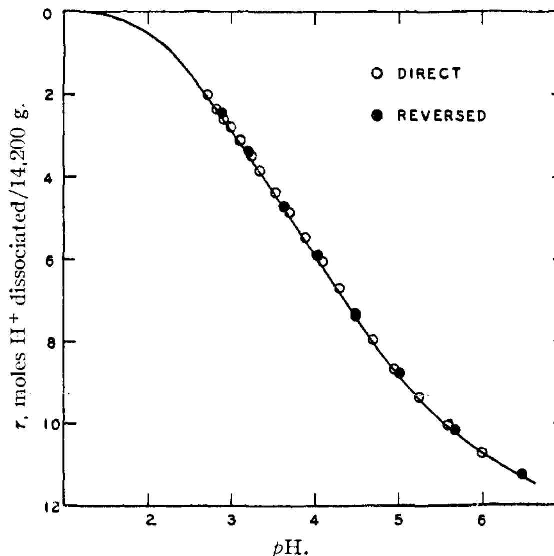


Fig. 2.—Acid reversibility at 25° and $\mu$ = 0.15. These data were obtained with an undialyzed sample of lysozyme. The curve drawn is the experimental curve of Fig. 1, representing data obtained with a dialyzed sample.

(10) K. J. Palmer, M. Ballantyne and J. A. Galvin, This Journal, **70**, 906 (1948).

(11) C. Fromageot and M. P. de Garilhe, *Biochim. et Biophys. Acta*, **4**, 509 (1950).

(12) J. C. Lewis, N. S. Snell, D. J. Hirschmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **186**, 23 (1950).

(13) M. Halwer, G. C. Nutting and B. A. Brice, This Journal, **73**, 2786 (1951).

(14) L. R. Wetter and H. F. Deutsch, *J. Biol. Chem.*, **192**, 237 (1951).

(15) R. C. Rhees and J. F. Foster, *Iowa State College J. Sci.*, **27**, 1 (1952).

(16) I. M. Kolthoff, *Z. anal. Chem.*, **61**, 48 (1923).

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

(18) C. Tanford and G. L. Roberts, Jr., This Journal, **74**, 2509 (1952).

(19) Material supplementary to this article has been deposited as Document number 4216 with the ADI Auxiliary Publications Project, Photoduplication Service, Library of Congress, Washington 25, D. C. A copy may be secured by citing the Document number and by remitting in advance $2.50 for photoprints, or $1.75 for 35 mm. microfilm, payable to Chief, Photoduplication Service, Library of Congress.

reversible between $p$H 2.7 and 6. The data in this figure were obtained from a sample of lysozyme which had not been dialyzed to remove inorganic salts.

These data indicate that lysozyme undergoes no irreversible changes between $p$H 2.7 and $p$H 11. Alderton, Ward and Fevold[9] have reported that there is no rapid loss of biological activity at $p$H 2.8 or at $p$H 11.8.

The effect of ionic strength on the titration curve is shown in Fig. 3. As expected, an increase in ionic strength leads to steepening of the curves. It

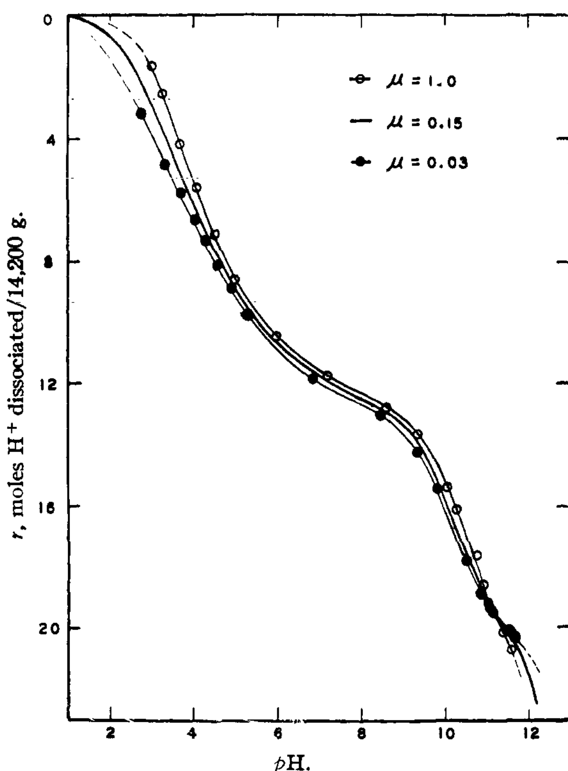


Fig. 3.—Titration curves at 25° and $\mu$ = 0.03 and 1.0. The central curve is an experimental curve through the data of Fig. 1, at $\mu$ = 0.15.

is of interest to note that the curves at the three ionic strengths employed intersect at a single point, within the limits of experimental error, at $r$ = 19 and $p$H 11.1 ± 0.1. This indicates that there is not much difference between the isoionic and isoelectric points of lysozyme, and that these points are not very dependent on the concentration of KCl, a result which is in accord with expectation since chloride ion is not bound by lysozyme at its isoelectric point,[20] and binding of potassium ion under such conditions is unlikely. If one takes the point of intersection to be the isoelectric point, therefore, the $p$H obtained is identical with that found from electrophoretic measurement. Anderson and Alberty[21] found values of 11.0 and 11.2, respectively, at ionic strengths 0.01 and 0.05 in sodium glycinate buffers. Alderton, Ward and Fevold[9] also observe a value close to $p$H 11.[22] Mobility data by both groups of workers are plotted in Fig. 4, together with values of the charge per lysozyme molecule obtained in the present study. It would appear that the mobilities observed near the isoelectric point closely follow the relation $\mu \times 10^5 = 1.0\,Z$.

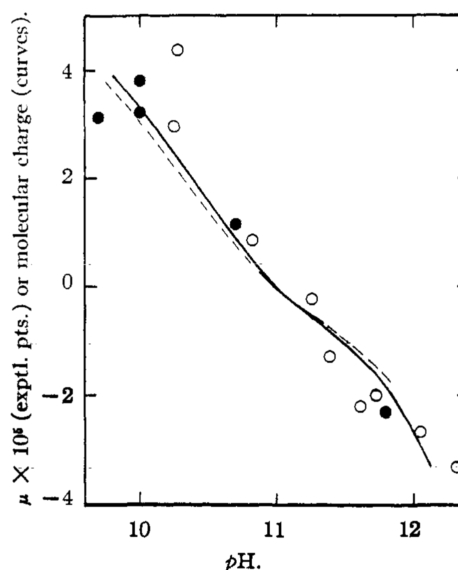


Fig. 4.—Comparison of titration curves at $\mu$ = 0.03 (dashed curve) and $\mu$ = 0.15 (solid curve) with electrophoretic mobility data of Anderson and Alberty[21] (open circles) and of Alderton, Ward and Fevold[9] (solid circles). The electrophoretic mobilities were obtained at ionic strengths varying from 0.01 to 0.10.

Figure 5 shows the effect of temperature on the titration curve and from these data the apparent heats of ionization, $\Delta H = 2.303\,R\,[\Delta p\mathrm{H}/\Delta(1/T)]_r$, were calculated. These are plotted as a function of $r$ in Fig. 6.

Figure 7 shows the spectrophotometric titration of the phenolic groups. The data are seen to be lacking in self-consistency in that the results at different wave lengths are in disagreement. The curves are also flatter than would be expected (see below). An attempt to obtain heats of ionization of the phenolic groups by comparison of spectrophotometric data at 15 and 35° proved unsuccessful because, again, the results at different wave lengths were in disagreement.

The reason for these inconsistencies may lie in part in the high tryptophan content of lysozyme, as a result of which the change in absorption due to phenolic ionization at any of the wave lengths used is only a small fraction of the total absorption at that wave length. It should be pointed out, however, that a similar study (in glycine buffers of about the same ionic strength as here used) made by Fromageot and Schnek[23] shows no similar difficulty. While these workers do not report their

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

(21) E. A. Anderson and R. A. Alberty, *J. Phys. and Colloid Chem.*, **52**, 1345 (1948).

(22) The isoelectric point reported by Alderton, Ward and Fevold is actually 10.5. They arrive at this figure, however, by ignoring their experimental data between $p$H 10 and 11, for reasons shown to be invalid by Anderson and Alberty.[21] That their data are entirely compatible with an isoelectric point at $p$H 11 is shown by Fig. 4.

(23) C. Fromageot and G. Schnek, *Biochim. et Biophys. Acta*, **6**, 113 (1950).

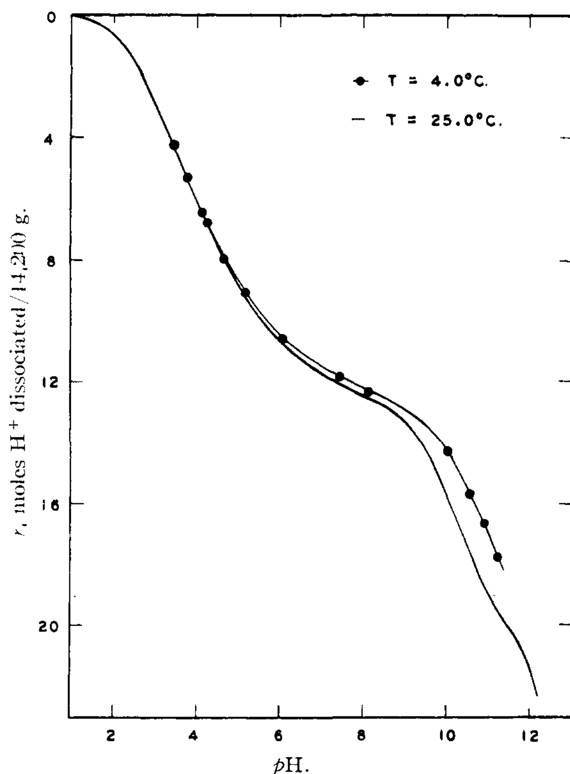


Fig. 5.—The effect of temperature at $\mu$ = 0.15. The curve at 25° is the experimental curve of Fig. 1.

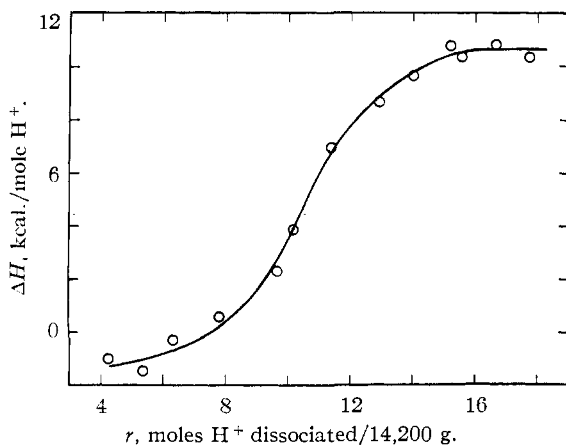


Fig. 6.—Apparent heat of ionization as a function of $r$, at $\mu$ = 0.15.

data at different wave lengths, they give a titration curve averaged from the results at several wave lengths. This curve is considerably steeper than ours, and, in fact, comes close to coinciding with the calculated curve of Fig. 7.

**Stoichiometry.**—From the fact that the isoionic point of lysozyme occurs at a value of $r$ = 19, one can conclude that this protein must contain 19 basic nitrogen groups. This figure agrees with the amino acid analyses of Fromageot and de Garilhe[11] and of Lewis, Snell, Hirschmann and Fraenkel-Conrat[12] who report, respectively, 18.8 and 16.9 basic nitrogen groups per 14,200 g. To these must be added the single terminal amino group (lysine)

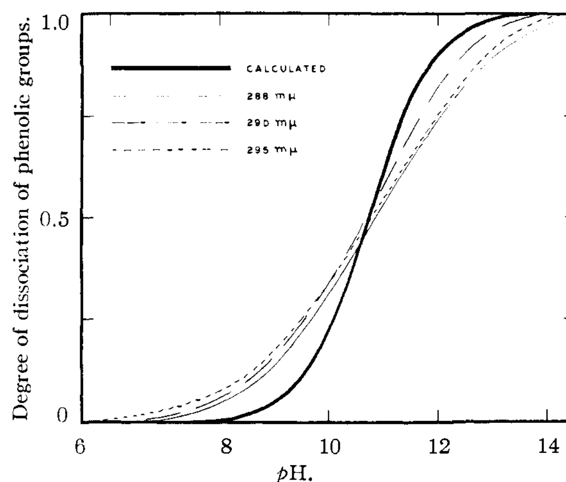


Fig. 7.—Spectrophotometric titration of phenolic groups, at 25° and $\mu$ = 0.15. The experimental curves were interpolated from actual data at 15 and 35°. The theoretical curve corresponds to $pK_{int}$ 10.8 and $w$ = 0.092. An experimental curve by Fromageot and Schnek[23] lies close to this calculated curve.

suggested by data from four laboratories.[24-27] The flat portion of the titration curve in the region of $p$H 6 to 9 corresponds to the titration of two groups with well-separated $pK$'s, which are presumably the terminal amino group, and the single imidazole group arising from the single histidine residue of lysozyme.[11,12] The steep region between $p$H 9 and 12 might correspond to the titration of either 8 or 9 groups. Of these three are phenolic groups, as indicated by the total change in absorption with $p$H at 295 m$\mu$,[28] as well as by amino acid analysis.[11,12] The other groups titrated in this region are $\epsilon$-amino groups, so that the presence of 5 or 6 of these is indicated. Fromageot and de Garilhe[11] have reported the presence of 5.8 such groups per 14,200 g., while the figure given by Lewis, *et al.*,[12] is 5.5.[29] There remain, therefore, 11 or 12 basic nitrogen groups, which must be guanidine groups. Amino acid analysis indicates the presence of 12.0[11] or 10.4[12] of these groups per molecule.

From Fig. 6 it is seen that the apparent heat of ionization rises sharply between the values of $r$ = 9 and $r$ = 12. Since the characteristic heat of ionization of carboxyl groups is close to zero, the presence of 10 to 11 of these groups is indicated. More precise values of the number of these groups may be obtained from the observed value of $r$ at $p$H 7.00, where the titration of carboxyl groups must be about 99.9% complete, and that of the imidazole group about 90% complete, while the more basic groups can contribute less than 0.6 to the value of $r$; and from the observed value of $r$ at $p$H 8.00,

(24) A. R. Thompson, *Nature*, **168**, 390 (1951).
(25) M. Jutisz and L. Penasse, *Bull. soc. chim. biol.*, **34**, 480 (1952).
(26) W. A. Schroeder, THIS JOURNAL, **74**, 5118 (1952).
(27) W. A. Landmann, M. P. Drake and J. Dillaha, *ibid.*, **75**, 3638 (1953).
(28) A total change in extinction of about 7500 per mole of lysozyme was observed. Three phenolic groups would be expected to contribute about 6900.[18]
(29) These figures point to 6 rather than 5 $\epsilon$-amino groups. The evidence of Tallan and Stein[7] concerning ninhydrin color suggests, however, that the lower figure should not be discarded.

where the titration of carboxyl and imidazole groups is essentially complete, the contribution of the $\alpha$-amino groups is about 0.9 and that of more basic groups is less than 0.2. The values obtained in this way for the number of carboxyl groups are, respectively, 10.45 ± 0.15 and 10.54 ± 0.15. It is therefore concluded that our preparation of lysozyme contained 10.5 carboxyl groups per molecule.[30] One of these groups is presumably the single terminal carboxyl group,[31-33] so that one would conclude that 9.5 free aspartyl and glutamyl carboxyl groups must be present per lysozyme molecule. This figure differs considerably from the values reported by Fromegeot and de Garilhe[11] and by Lewis, *et al.*,[12] which are, respectively, 1.3 and 6.5. These values, however, differ so greatly from one another and depend so much on the analysis for amide groups, which is often known to be high, that the figure obtained by us is most likely to be correct. An alternative possibility is that our sample of lysozyme contained more free carboxyl groups, because of amide hydrolysis, than samples studied by previous workers. If this were so, however, we should have found an abnormally low isoelectric point, which, as shown by Fig. 4, is not the case.

Another possibility is that some of the anions associated with the lysozyme in the stock solutions (*cf.* Experimental section) are acetate or carbonate ions. This possibility would seem to be remote because dialysis had virtually no effect on the acid titration curve (Fig. 2), and because the presence of extraneous titratable ions would lead to a high value of $r$ at the isoionic point, whereas a reasonable value was obtained.

**Electrostatic Factor and Intrinsic $pK$'s.**—The equation governing the dissociation of hydrogen ions from any set of $n_i$ identical groups is[5]

$$\log \frac{r_i}{n_i - r_i} = pH - (pK_{int})_i + 0.868Zw \qquad (1)$$

where $r_i$ is the number of these groups dissociated at any $p$H, $Z$ is the net charge per molecule at that $p$H, $w$ is an electrostatic factor (which may or may not be a constant independent of $Z$) such that $2ZwRT$ is the work required, in addition to the intrinsic free energy of dissociation, to remove a proton from the molecule; and $(pK_{int})_i$ is the intrinsic dissociation constant. The value of $r$ at any $p$H, as experimentally observed, is the sum of the values of $r_i$ for all the different kinds of dissociable groups.

By the methods given in detail in two preceding papers,[5,34] involving in part appropriate graphical procedures and in part trial and error methods, values of $pK_{int}$ for the various types of groups, and values of $w$ as a function of $Z$, can be evaluated from the experimental data. The results of such calculations are given in Tables I and II. Table II also contains values of $w$, computed from the Debye–Hückel theory, for spherical ions of radius 17.0 Å. This is the radius of a sphere of molecular weight 14,200 with a specific volume of 0.75, with a hydration layer of specific volume 1.0, the amount of hydration being assumed to be 0.1 g. per gram of protein.

As Table I shows, unequivocal values of the intrinsic $pK$'s could not be obtained from our data, and for the carboxyl groups no values could be obtained at all, for reasons explained in the discussion below. Values for the parameter $w$ (Table II) could be computed only for the isoelectric region ($p$H 9.5 to 11.5), and again only a range of possible values was obtained. The lower limit of this range, at each ionic strength, agrees very well with the values computed by means of the Debye–Hückel theory. The experimental data, however, do not exclude values somewhat higher than those computed in this way.

TABLE I

DISSOCIATING GROUPS OF LYSOZYME

| Group | Number of groups/14,200 g. Analytical data | Obsd. | Intrinsic $pK$ Expected value[a] | Obs. possible range Lower limit | Upper limit |
|---|---|---|---|---|---|
| $\alpha$-Carboxyl | 1[b] | 10.5 | 3.8 | ?[a] | |
| $\beta,\gamma$-Carboxyl | 1.3,[c] 6.5[d] | | 4.6 | ?[a] | |
| Imidazole | 0.96,[c] 0.95[d] | 1 | 6.5 | 6.5 | 7.0 |
| $\alpha$-Amino | 1[e] | 1 | 7.8 | 7.5 | 7.9 |
| Phenolic | 2.9,[c] 2.8[d] | 3 | 9.6 | 10.42 | 10.9 |
| $\epsilon$-Amino | 5.8,[c] 5.5[d] | 5 or 6 | 10.1–10.6 | 10.27 | 10.6 |
| Guanidine | 12.0[c], 10.4[d] | 12 or 11 | >13 | 12.65 | 13.3 |

[a] See text. [b] Ref. 24–27. [c] Fromageot and de Garilhe.[11] [d] Lewis, *et al.*[12] [e] Ref. 31–33.

TABLE II

ELECTROSTATIC FACTOR IN THE ISOELECTRIC REGION

| Ionic strength | Value of $w$ calcd. for sphere | Obsd. possible range of $w$ Lower limit | Upper limit |
|---|---|---|---|
| 0.03 | 0.124 | 0.12 | 0.15 |
| 0.15 | .080 | .08 | .12 |
| 1.00 | .052 | .045 | .07 |

This type of calculation is normally expected to yield fairly definite results, at least in the isoelectric region. The uncertainties in the present calculations arise from the uncertainty in the spectrophotometric data of Fig. 7. The disagreement between the data at different wave lengths has already been pointed out. In addition, the curves of Fig. 7 are considerably flatter than would be expected. This could be explained if the three phenolic groups of lysozyme had appreciably different $pK$'s, but this result turns out to be incompatible with the potentiometric titration curves. One is therefore forced back to the conclusion already reached from the wave length effect, that the observed changes in light absorption are not a true representation of the ionization of the phenolic groups. This conclusion is strengthened by the steeper spectrophotometric curve obtained by Fromageot and Schnek (*cf.* Fig. 7).

Accordingly, the phenolic groups have been assumed identical and subject to equation 1 with the same value of $w$ as applies to the amino groups titrated in the same $p$H region. The potentiometric

(30) As previously stated,[6] the preparation of lysozyme here used contains two electrophoretic components, which may differ in the number of free carboxyl groups because of amide hydrolysis. A non-integral number of carboxyl groups is therefore quite reasonable. However, the uncertainty in the molecular weight of lysozyme assumed in this paper is also sufficiently great to account for a non-integral number of these groups.

(31) A. R. Thompson, *Nature*, **169**, 495 (1952).

(32) J. I. Harris, THIS JOURNAL, **74**, 2944 (1952).

(33) J. T. Edward and S. Nielsen, *Chemistry and Industry*, 197 (1953).

(34) C. Tanford and J. Epstein, THIS JOURNAL, **76**, 2163 (1954).

titration curves can then be fitted with various possible $pK$'s and values of $w$, as shown by Tables I and II. The fact that the number of $\epsilon$-amino groups may be 5 or 6 contributes to the uncertainty in these parameters. A calculated curve for the spectrophotometric titration, shown in Fig. 7, is based on a $pK$ of 10.8 for the phenolic groups and a value of $w$ close to the lower limit given in Table II. It falls very close to the experimental curve of Fromageot and Schnek.

Extension of the calculations to the region of $p$H 7 to 9 is possible with any values of $w$ within the limits observed for the isoelectric region (Table II), the lower $pK$'s for the imidazole and $\alpha$-amino groups (Table I) being those corresponding to the lowest values of $w$ and *vice versa*.

The greatest difficulty in calculation is encountered in the acid region, where the carboxyl groups are titrated. The first reason for this is that the binding of chloride ion by lysozyme becomes important in this region, so much so that the variation of $Z$ with $p$H becomes quite small. It is thus essential to have very accurate information on the extent of chloride binding. Unfortunately, the chloride binding studies by Carr,[35] while no more inaccurate than corresponding studies with other proteins, are not sufficiently precise. In fact, the limits of accuracy of Carr's data are such that they do not exclude the possibility that chloride binding increases more rapidly with decreasing $p$H than the binding of hydrogen ion, a possibility which would lead to decreasing values of $Z$ with decreasing $p$H.

The second and more fundamental difficulty with the acid region of the titration curve is that it is much flatter than would be predicted on the basis of equation 1 for any reasonable value of $w$. This curve is too flat (empirical $w = 0.2$ at $\mu = 0.15$) even if no chloride binding were to occur at all, and, since binding of chloride ion should steepen the titration curve, the difficulty is greatly increased by Carr's finding that chloride binding is important. One possible explanation is that the carboxyl groups are not identical, but differ widely in intrinsic $pK$; an alternative possibility is that all the carboxyl groups are located in close proximity at one end of the lysozyme molecule, instead of being evenly distributed over its surface. To attempt precise calculations based on either of these possibilities is, of course, not worthwhile until the charge at any $p$H can be estimated more accurately than is now possible.

## Discussion

From the data presented above and the calculations based upon them, and despite the uncertainty of some of the calculated parameters, it is possible to draw several conclusions of interest concerning the structure of lysozyme.

One of these is that the phenolic groups must be hydrogen-bonded or prevented in some other way from extending from the protein molecule into the solvent. For the "normal" intrinsic $pK$ of phenolic groups, as estimated from the $pK$ values of appropriate small molecules[18] is $9.6 \pm 0.2$, and values in this range have been calculated by the methods used in this paper from titration data on two macromolecules, poly-L-tyrosine ($pK_{int}$ 9.5)[36] and insulin ($pK_{int}$ 9.6).[34] Table I shows that the corresponding $pK$ in lysozyme must be much higher, as has already been pointed out by Fromageot and Schnek.[23]

As has already been mentioned, the carboxyl groups must also be abnormal.

The basic nitrogen groups, on the other hand, appear to be normal. The intrinsic $pK$ values for these groups, given in Table I, are in good agreement with values to be expected from comparison with appropriate small molecules. Ellenbogen,[37] for example, has deduced that $pK$ values of 7.8 and 10.6 should be expected, respectively, for $\alpha$ and $\epsilon$-amino groups. An alternative reasonable value for the latter is the $pK$ of $ROOC-(CH_2)_4-NH_3^+$, which is 10.1.[38] Some of the variations in $pK$ of imidazole groups of small molecules are not yet understood,[38] but the intrinsic $pK$ observed for the imidazole group of lysozyme lies in the range observed for other proteins; *e.g.*, ovalbumin 6.7,[4] insulin 6.4,[34] serum albumin 6.3,[39] and $\beta$-lactoglobulin 6.8.[40] The intrinsic $pK$ of the guanidine groups is very high, as is to be expected, since values above 13 have been observed for guanidine itself and for substituted guanidines.[38,41]

Finally, the observed values of $w$ (Table II) suggest that the lysozyme molecule, near its isoelectric point, is compact and sparingly hydrated. Empirical values of $w$ for other proteins, such as serum albumin,[5] ovalbumin[4] and $\beta$-lactoglobulin[5,40] have always been smaller than the values which would be computed by the Debye–Hückel theory for a spherical molecule containing up to 0.2 g. of water of hydration per gram of protein. This has been explained on the basis of expansion or asymmetry. In lysozyme, however, the empirical values of $w$ are certainly not less than the calculated values, and may actually be a little larger.

As demonstration of the validity of the calculated $pK$'s and electrostatic factor a titration curve has been computed for $p$H 7 for 25° and ionic strength 0.15. This curve, with the actual $pK$'s and the value of $w$ used, is shown in Fig. 1, and is seen to agree with the experimental data.

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