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Abnormal Ionizable Groups in Lysozyme[1,2]

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Titration curves of lysozyme indicate that all the carboxyl groups in this protein ionize normally in solutions of guanidine hydrochloride but that there are three more acid groups titratable in this solvent than there are in KCl solutions. These three "extra" groups appear in each of two lots of lysozyme which differ in the number of carboxyl groups titratable in KCl but no "extra" groups appear when a methylated lysozyme is titrated in guanidine hydrochloride. The supposition that these "extra" groups are carboxyl groups is supported by the results of amide and methoxyl analyses of the native and methylated lysozymes. These carboxyl groups, present as carboxylate ions in the native molecule, are probably "masked" because of large local electrostatic effects of the positive charges of the ε-amino groups. An acetylated derivative shows three more titratable carboxyl groups in KCl than the native lysozyme but no increase in the number of the carboxyl groups in guanidine hydrochloride. A deaminated lysozyme shows the same number of carboxyl groups in guanidine hydrochloride as in KCl. A guanidinated derivative shows the same number of carboxyl groups in KCl as the native lysozyme and an increase of three carboxyl groups in guanidine hydrochloride. These "extra" groups do not appear to be interacting with the phenolic groups of lysozyme, since the phenolic groups of the acetylated lysozyme in KCl appear to be as abnormal as those in the native lysozyme, while the "extra" groups are "unmasked" in this derivative. However, the phenolic groups appear to ionize normally in guanidine hydrochloride solutions. The kinetics of the uptake and release of hydrogen ion by the protein when guanidine hydrochloride is added to lysozyme solutions indicate that the "extra" carboxylate ions take up protons at the same rate as the increase in levorotation of lysozyme. Thus, a structural alteration of the molecule (fifth order with respect to guanidine hydrochloride) is presumably necessary to "unmask" the three carboxylate ions. The kinetic data also suggest that some of the carboxyl groups which are titratable in KCl solutions have abnormally low pK values and some have abnormally high pK values. This is in agreement with the flatness of the titration curve of lysozyme in KCl, which indicates the presence of both low and high pK carboxyl groups in the native molecule.

Introduction

The location of interactions between specific side-chain groups in a protein can help in distinguishing between several possible spatial configurations of the molecule. This approach to determining protein structures has been applied to insulin[4,5] and ribonuclease,[6,7] and the present paper considers some of the internal interactions of the lysozyme molecule.

The titration curve of lysozyme from egg white has been shown to be abnormal in the region of ionization of the carboxyl groups.[8] It will be shown here that these carboxyl groups appear to titrate normally in solutions containing high concentrations of guanidine hydrochloride and that the rates at which these groups become normal in these solvents give some indications of the nature of the interactions giving rise to these abnormalities. In addition to these carboxyl groups, three "extra" groups appear on the acid side of the titration curve in guanidine hydrochloride. These groups are not titrated in KCl solutions and appear to be "masked" carboxylate ions. They do not seem to be interacting with the tyrosyl groups in lysozyme, which have been reported to have abnormally high[8] pK's, since the tyrosyl groups remain abnormal in an acetylated lysozyme in KCl solution even though the three "extra" groups are titratable in this derivative.

The kinetic data presented here are of the nature of a preliminary study and are included because of their obvious relation to the equilibrium data for the titration of lysozyme in guanidine hydrochloride. The kinetic experiments were patterned after those of Harrington on ovalbumin.[9]

Experimental

Materials.—The lysozyme was obtained from Armour and Co. Two lots were used as received: 003L1, the same lot used by Tanford and Wagner to determine the titration curve of lysozyme[8] and lot 381-187. A comparison of the titration curves for these two lots showed a difference of about 1.5 titratable carboxyl groups (Fig. 1). In KCl solution, 10.5 carboxyl groups are titratable in 003L1[8] and 9 groups in 381-187. Data supplied by the manufacturer for lot 381-187 are: 3.7% moisture, 0.1% ash, 17.1% nitrogen; 95% of the material moves with a mobility of 1.85 in phosphate buffer at pH 7.7 and ionic strength 0.2. Corresponding manufacturer's data for lot 003L1 have been presented.[8] The rotatory dispersion of a 1.9% water solution of 381-187 at pH 5.5 was determined using a Rudolph Model 80 polarimeter, with sodium and mercury lamps. The wave lengths used were 589.3, 578.0, 546.1 and 435.8 mμ, isolated by suitable filters. A straight line plot of $[\alpha]$ *vs.* $\lambda^2[\alpha]$ gave the following constants: $\lambda_c = 251 \pm 3$ mμ, $k = -13.0$ (compare ref. 10 and 11). At 20°, $[\alpha]_D$ was -45.9 ± 0.1. Solutions for the experiments to be described usually were prepared by dissolving a weighed amount of protein in a known volume of solvent. The moisture content of the protein was determined by drying a weighed sample to constant weight in air at 105°. The molecular weight of the isoelectric material was assumed to be 14,200.[8]

The urea (designated U) was reagent grade, Baker and Adamson lot No. J131, used without further purification. The guanidine hydrochloride (designated G) was Eastman Kodak 749. This material contained small amounts of titratable impurities. A large portion of these could be removed by making up a saturated solution and filtering. This appeared to be about as effective as two or three recrystallizations from hot water. The acetic acid and phenol were reagent grade. The imidazole was Eastman No. 4733. The butylamine was obtained from Carbide and Carbon Chemical Company. All other inorganic chemicals were reagent grade. Carbonate free KOH, prepared from reagent KOH by the method of Kolthoff,[12] was standardized against

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(2) Presented before the Division of Biological Chemistry at the 131st and 136th meetings of the American Chemical Society, at Miami, Florida, April, 1957, and at Atlantic City, New Jersey, September, 1959.

(3) Summer Research Fellowships from the Procter and Gamble Company and the Dow Chemical Company are gratefully acknowledged.

(4) L. Gruen, M. Laskowski, Jr., and H. A. Scheraga, THIS JOURNAL, **81**, 3891 (1959).

(5) M. Laskowski, Jr., S. J. Leach and H. A. Scheraga, *ibid.*, **82**, 571 (1960).

(6) C. Y. Cha and H. A. Scheraga, *ibid.*, **82**, 54 (1960).

(7) C. L. Schildkraut and H. A. Scheraga, *ibid.*, **82**, 58 (1960).

(8) C. Tanford and M. L. Wagner, *ibid.*, **76**, 3331 (1954).

(9) W. F. Harrington, *Biochim. et Biophys. Acta*, **18**, 450 (1955).

(10) J. T. Yang and P. Doty, THIS JOURNAL, **79**, 761 (1957).

(11) B. Jirgensons and L. Straumanis, *Arch. Biochem. and Biophys.*, **68**, 319 (1957).

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

potassium acid phthalate. The HCl solutions used were standardized against the KOH, or the chloride concentration determined gravimetrically by precipitation with silver.

Lysozyme Derivatives.—A deaminated lysozyme was prepared according to the procedure of Philpot and Small.[13,14] Three hundred mg. of lot 381-187 was added to 25 ml. of a solution which was 0.5 *M* in sodium acetate and 1 *M* in sodium nitrite at 0°. The solution now was adjusted to *p*H 3.8 with HCl and allowed to stand at 0° for 30 min., after which it was neutralized to stop further reaction and dialyzed extensively against cold distilled water; 250 mg. of cream-colored material was obtained upon lyophilization. The spectrum of this derivative showed an absorption at wave lengths longer than those characteristic of lysozyme. This absorption was essentially constant between 310 and 350 mμ. At 330 mμ, 10^{-4} ϵ_{mol}[15] was 0.7 in 0.15 *M* HCl, 1.0 in 0.16 *M* KOH. Values of ϵ_{mol} at 280, 290 and 295 mμ were within a few per cent. of those for native lysozyme but at 275 mμ were about 10% higher.

An acetylated lysozyme was prepared using the procedure of Hughes, as given by Fraenkel-Conrat.[16] Acetylation is rather specific for amino groups.[14] One gram of lot 381-187 was added to 20 ml. of saturated (at 0°) sodium acetate and the mixture placed in an ice-water bath. A total of 1.2 ml. of acetic anhydride was added in 0.3 ml. increments over a 1.5 hr. period, with constant shaking. Two and a half hours additional was allowed for the reaction to come to completion. The solution was dialyzed against cold distilled water and lyophilized. The ultraviolet absorption spectrum of this material was the same as that of lysozyme, within experimental error.

A guanidinated lysozyme was prepared using the procedure of Hughes, Saroff and Carney.[17] Five hundred mg. of lysozyme, lot 381-187, was added to 2 g. of O-methylisourea[18] in 10 ml. of distilled water at room temperature. When 4 ml. of 1 *M* KOH was added, the *p*H (measured at room temperature) was 9.6. The solution was cooled in ice water and stored in a refrigerator at 4° for two days. At the end of that time, a small amount of precipitate had formed. This was removed by centrifugation, leaving a clear solution of *p*H 9.2. To this solution 1.8 ml. of 1 *M* HCl was added to bring the *p*H to approximately 5. The solution then was dialyzed against cold distilled water and lyophilized. A comparison of the *p*H dependence of the spectrum of this derivative with the alkaline portion of its titration curve showed that less than one lysyl residue per molecule remained unmodified. This observation is in agreement with previous guanidinations.[19,20]

A methyl ester of lysozyme was prepared using the procedure of Fraenkel-Conrat and Olcott,[21] which appears to specifically methylate carboxyl groups.[22] Two g. of lot 381-187 was added to 200 ml. of absolute methanol which contained 2 ml. of 12 *N* HCl. The mixture was shaken at room temperature for 2 hr., then allowed to stand at 23° with occasional shaking. At the end of two days, the supernatant liquid was poured off, and the gel-like solid remaining was dissolved in cold distilled water, dialyzed against cold distilled water and then lyophilized.

Apparatus.—A Beckman Model DU Spectrophotometer with photomultiplier attachment was used to determine ultraviolet absorption spectra. A Beckman Model G *p*H meter (or Model GS, used on the "A" scale) was used for titrations and *p*H determinations. Both *p*H meters were calibrated by the method described by Bates.[23] Standard buffer solutions used were Beckman *p*H 4, 7 and 10, together with 0.05 *M* potassium acid phthalate, prepared according to the directions given by Bates.[24] A Beckman glass electrode (1190-80) and calomel electrodes with asbestos fiber junction, containing either saturated or 3.15 *N* KCl, were used.

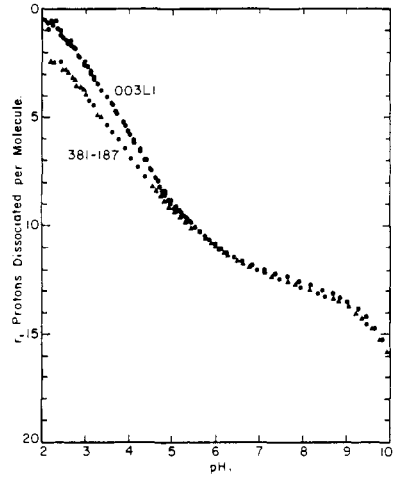

Fig. 1.—Titration curves of two lots of Armour lysozyme in 0.15 *M* KCl at 25°. The titration curve shown here for lot 003L1 checks closely (within 0.1 group) with the curve reported by Tanford and Wagner for the same lot.[8] The *r* values for lot 003L1 are given as ordinate.

Titrations.—Titrations of lysozyme and its derivatives (1–2% solutions) in various solvents were carried out in a water-jacketed cell at 25 ± 0.1°, with a nitrogen flushing system for titrations on the alkaline side. The volume of solution used was generally 5 ml. and was stirred with a small glass-enclosed stirring bar using a magnetic stirrer. Successive increments of titrant were added from a 1 ml. Gilmont ultramicroburet, inserted through the rubber stopper holding the electrodes and the nitrogen inlet. The smallest dial division on the buret was 0.001 ml. and the volumes added were estimated to 0.0001 ml. Titrations of model compounds (0.02 *M* solutions) were usually carried out in small open beakers at room temperature (24 ± 1°). The buret, electrodes, *p*H meter and standardization procedure were the same as for the titrations of protein, so that the results may be compared readily. The titrations of lysozyme and derivatives in KCl were performed by adding 0.15 *M* HCl or KOH to the solution of the protein in 0.15 *M* KCl. In these cases, the calculations were made using the "activity coefficient" of hydrogen or hydroxyl ion calculated from the blank titration. For the titrations of lysozyme and derivatives in solvents other than KCl and for the titrations of model compounds, the titrant used was of 1 *M* concentration and the calculations were performed by subtracting the blank titration graphically. No significant difference between the results of these two methods of calculation was observed when a titration of lysozyme in KCl with 1 *M* HCl was calculated in both ways.

Spectrophotometric Titrations.—These titrations were carried out by adding very small amounts of concentrated KOH from a microburet to the lysozyme solution ($\sim 2 \times 10^{-5}$ *M*). Since the total change in volume of the solution was only 1% up to *p*H 12, it was neglected in the calculations. The optical density of the lysozyme solution at each *p*H was measured against a suitable reference (usually solvent at neutral *p*H). The solvent was similarly treated to obtain the proper corrections to the optical density of the lysozyme solution.

Amide Analysis.—The amide analysis was carried out by a modification of the method of Gordon, Martin and

(13) J. St. L. Philpot and P. A. Small, *Biochem. J.*, **32**, 542 (1938).

(14) F. W. Putnam, in "The Proteins," H. Neurath and K. Bailey, editors, Academic Press, Inc., New York, N. Y., 1953, Ch. 10.

(15) ϵ_{mol} is defined by the equation: $D = \epsilon_{mol} Cl$, where D is the optical density of the solution at a given wave length, C the concentration of protein in moles/l. and l the cell length in cm.

(16) H. Fraenkel-Conrat, in "Amino Acids and Proteins," D. M. Greenberg, editor, C. C. Thomas, Springfield, Ill., 1951, Ch. IX.

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

(18) Kindly supplied by H. A. Saroff.

(19) J. Roche, M. Mourgue and R. Baret, *Bull. soc. chim. biol.*, **36**, 85 (1954).

(20) I. I. Geschwind and C. H. Li, *Biochim. et Biophys. Acta*, **25**, 171 (1957).

(21) H. Fraenkel-Conrat and H. S. Olcott, *J. Biol. Chem.*, **161**, 259 (1945).

(22) H. A. Saroff, N. R. Rosenthal, E. R. Adamik, N. Hages and H. A. Scheraga, *ibid.*, **205**, 255 (1953).

(23) R. G. Bates, "Electrometric *p*H Determinations," John Wiley and Sons, Inc., New York, N. Y., 1954, p. 287.

(24) Ref. 23, p. 118.

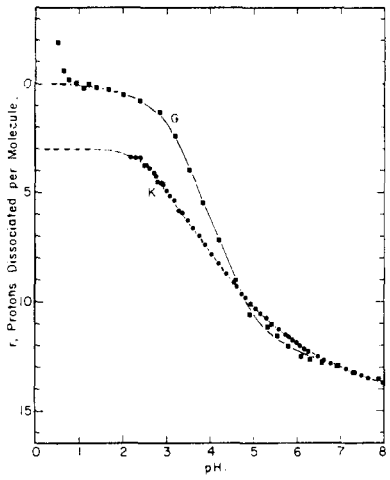

Fig. 2.—The titration curves of Armour lysozyme lot 381-187 in 8 *M* G (approximately 1.5 hr. after solution of the protein) and in 0.15 *M* KCl (K), at 25°. The values of *r* shown as ordinate have been chosen to correspond to the titration in G. The curve K has been moved vertically to coincide with the other at the higher *p*H values.

Synge.[25,26] Samples were hydrolyzed for nine days in 12 *N* HCl at 37°. Aliquots taken were neutralized with NaOH and sodium tetraborate was added to bring the *p*H to 9.5. The ammonia (approximately 0.1 m*M*/aliquot) was distilled into standard 0.1 *N* HCl, and the excess acid back-titrated with standard KOH using Tashiro's indicator. Controls for ammonia were used for the distillations and a blank containing all reagents but the protein was carried through the entire hydrolysis and distillation procedure.

Procedure for Kinetic Experiments.—About 0.2 g. of lysozyme, Armour lot 381-187, in 3–5 ml. of water was adjusted to a *p*H slightly more acid than the *p*H at which the kinetic experiment was to be determined. The *p*H of 15 to 25 ml. of a solution of guanidine hydrochloride (G) was similarly adjusted. (The *p*H rises slightly when the solutions are mixed. This seems to be a combination of dilution and salt effects.) The solutions were mixed, stirred and electrodes placed in the solution. A stop-watch was started at the time of mixing. The first *p*H reading (mv. on the Duodial of the Beckman GS *p*H Meter) usually could be made 20–40 sec. after mixing. At 25°, one division of this dial (a ten-turn 0.1% helipot) represents 0.0034 *p*H unit, and readings could be estimated to two tenths of a dial division. The solution was stirred with a magnetic stirrer between readings. Some kinetic experiments were thermostatted at 25° but this appeared not to affect the results obtained. When the reaction was considered complete (sometimes after 2 hr. or more) the solution was back-titrated through the starting *p*H with standard acid or base, to calibrate the small section of the Duodial used. Since a rather small volume of titrant (about 0.2 ml. of 0.15 *M*) was necessary to accomplish this, the volume of the solution was changed by a negligible amount. The G concentration of the solution was determined from its density. Experiments in which the mixing procedure was carried out without lysozyme showed an initial change in *p*H occurring over 45 sec. to one minute, which was presumed to be the equilibration of the electrodes with the solvent, then no further change in *p*H was observed. Reactions that are nearly complete in the first minute will not be observed by this method. The dilution and salt effects thus are not observed kinetically.

The calculations were made in the following manner: The Duodial readings, taken at various times throughout the reaction, were converted into moles of hydrogen ion, *h*, liberated from, or taken up by, the protein, using the back-titration data. A value of h_∞ was determined from the Duodial readings at completion of the reaction or the Guggenheim method of calculation[27] was employed. The observed reactions appeared to be either a single reaction or two simultaneous reactions. The absorption or release of hydrogen ion in the individual reactions was always observed to be first order. Although some of the reactions are *p*H dependent and their kinetics properly should be determined at constant *p*H, the small changes in *p*H which occur during the reactions do not seem to affect their rates very much.

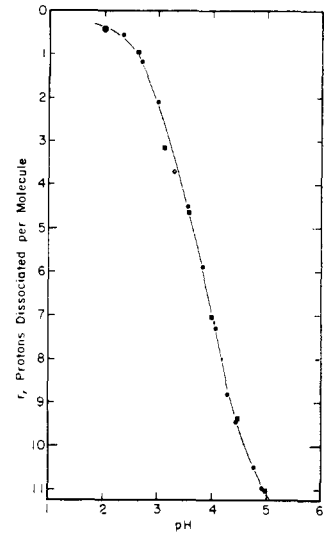

Fig. 3.—The reversibility of the titration curve of lot 381-187 in 5 *M* G at 25°, after fifteen minutes at *p*H 2.08: ●, the points of the titration from *p*H 5 to *p*H 2; ■, the points of the reverse titration. The values of the ordinate have been chosen to correspond with those of Fig. 2.

Results and Discussion

Titrations of Lysozyme.—Figure 2 shows the titration curve of lot 381-187 of lysozyme in 8 *M* G, compared with its titration curve in KCl. In order that the titration curves obtained would be characteristic of a completely "denatured"[28] protein, the titrations were carried out about 1–2 hr. after solution of the lysozyme in G.[31,32] *There appear to be three more titratable acid groups in G than in KCl (i.e., 12, as compared with 9).* Three "extra" groups were also observed for lot 003L1 in 8 *M* G. Because of the higher activity coefficient of hydrogen ion in 8 *M* G, titrations in this solvent provide binding data at lower *p*H's than in KCl. The reversibility of the titration curve in 5 *M* G is shown in Fig. 3. The titration curve in KCl appeared to be reversible when the lysozyme was allowed to stand at *p*H 0.8 for 3 hr. at 25°. Tanford and Wagner[8] have shown reversibility between *p*H's 2.7 and 11. The interpretation of the titration curves presented here is not complicated by changes in the state of aggregation of the lysozyme molecule in solution, since lysozyme does not appear to associate, at least in the acid *p*H range. The specific rotation and reduced viscosity

(25) A. H. Gordon, A. J. P. Martin and R. L. M. Synge, *Biochem. J.*, **35**, 1369 (1941).

(26) See also, S. J. Leach and E. M. J. Parkhill, Proc. Intern. Wool Textile Res. Conf., Vol. C, Australia, 1955, pp. C92–C101. We are indebted to Dr. S. J. Leach for much helpful advice concerning amide analyses.

(27) E. A. Guggenheim, *Phil. Mag.*, **2**, 538 (1926).

(28) Lysozyme recovers its ability to lyse *M. lysodeikticus* after it has been removed from urea[29] or G[30] solutions.

(29) J. Léonis, *Arch. Biochem. and Biophys.*, **65**, 182 (1956).

(30) J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, *J. Molecular Biology*, **1**, 293 (1959).

(31) B. Jirgensons, *Arch. Biochem. and Biophys.*, **89**, 261 (1952).

(32) B. Jirgensons, *ibid.*, **41**, 333 (1952).

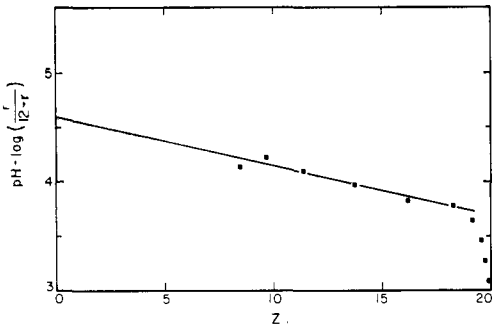

Fig. 4.—A plot of the titration curve of lysozyme lot 381-187 in GU, according to equation 1. The α-carboxyl group has been included and probably causes some of the discrepancy at high Z. The maximum value of Z is assumed to be 20 and no chloride binding is assumed. The value of w, calculated from the slope of the line, is 0.052.

of lysozyme solutions do not appear to be a function of pH or concentration,[33,34] and the variation in the sedimentation and diffusion properties of this protein indicate that there is no aggregation at pH 6.8.[35]

In order to determine whether the pK's of the carboxyls are "normal" in G and to check on a possible large reduction in pK of basic groups (which would make them appear as the "extra" acid groups), the pK's of some model compounds were determined as a function of G, KCl and urea (U) concentration. The results are reported elsewhere[30] and show that there are no changes in pK large enough to shift the ionization of the basic groups into the acid pH region. Acetic acid in 8 M G apparently has a pK which is only about 0.05 unit less than its pK in 0.15 M KCl. The pK of acetic acid is raised by U and lowered by G, and these effects appear to be approximately additive. By selection of the G/U ratio, solvents in which acetic acid has the same apparent pK as in 0.15 M KCl can be prepared. One such is a solution 5.0 M in G and 1.2 M in U, hereafter called "GU."

The apparent pK's of these model compounds in KCl and GU are compared in Table I.

TABLE I

COMPARISON OF THE pK OF MODEL COMPOUNDS IN GU WITH THE pK IN 0.15 M KCl AT 25°

Compound	Obsd. pK in 0.15 M KCl	Obsd. pK in GU	(pK_{GU} − $pK_{0.15M\ KCl}$)[36]
Acetic acid	4.62	4.62	0.0
Imidazole	7.07	7.20	+ .13
Phenol	9.92	10.03	+ .11
n-Butylamine	10.79	10.74	− .05

(33) B. Jirgensons, *Arch. Biochem. and Biophys.*, **74**, 70 (1958).

(34) J. T. Yang and J. F. Foster, THIS JOURNAL, **77**, 2374 (1955).

(35) J. R. Colvin, *Can. J. Chem.*, **30**, 831 (1952).

(36) The apparent changes in pK of these model compounds may not be actual changes in pK, since the liquid junction potential between the calomel electrode salt bridge and the standard buffer solutions may be quite different from the liquid junction potential between the same salt bridge and the solvent used for titration. This difference in liquid junction potentials will be interpreted as a difference in pH, since the assumption made in the calculations is that the liquid junction potentials are equal. The comparison of the protein titration curves in these solvents with the titrations of the model compounds in the same solvents, however, should be unaffected by this error, since the liquid junction potentials will be the same in each case.

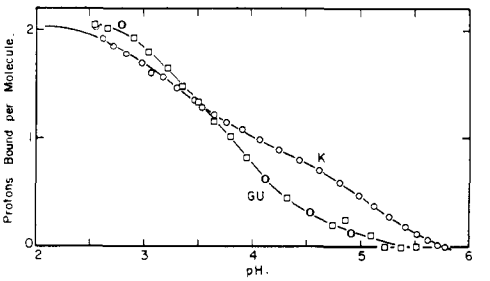

Fig. 5.—Titration curves of the (water soluble) methylated lysozyme at 25° in 0.15 M KCl (K) and in GU. The zero for the ordinate has been chosen arbitrarily.

The titration curve of lot 381-187 in GU is closely the same as its titration curve in 8M G, shown in Fig. 2, and has been reported elsewhere.[30] A plot of the equation[39,40]

$$p\mathrm{H} - \log \frac{r_i}{n_i - r_i} = (pK_i)_{int} - 0.868\, wZ \qquad (1)$$

for the titration curve in GU, with n_i equal to 12, is shown in Fig. 4. Here, n_i is the number of the i^{th} type group having the intrinsic pK value $(pK_i)_{int}$ at the ionic strength at which titration is carried out, and r_i is the number of these groups in the form of the conjugate base at the given pH; Z is the net charge on the molecule, and w is the electrostatic factor.[40] All the groups ionizing below pH 6 on the acid side of the titration curve appear to be carboxyls with normal values of pK_{int} (4.6). This plot, together with reversibility of the titration curve in G, strongly suggests that the "extra" groups are carboxyl groups. Since protons are taken up by the lysozyme molecule when G is added at pH's near 4, as shown by a rise in pH of the solution (over and above that due to the dilution and salt effects), the "extra" carboxyls must be in the carboxylate ion form in KCl solution.

Titrations of Lysozyme Derivatives.—Figure 5 shows the titration curves of the methylated derivative, which was prepared to verify the assumption that the "extra" groups were carboxyl groups. The failure to observe an increase in the number of titratable groups in the methylated lysozyme in G is consistent with the assumption that the "extra" groups are carboxyl groups, since they could have been esterified in the preparation of this derivative.

Another source of error which occurs when solvents containing high solute concentration are used is the reduction in the activity of the water, which appears to take part in the half cell reaction at the glass electrode (see, for example, the discussion given by Bates[37]). Calculations made with data available for the activity of water in concentrated urea and KCl solutions at 25°[38] show that the error is about 0.12 pH unit in 10 M urea solutions, the true pH being lower than the observed pH. The error in saturated KCl solution is appproximately 0.07 pH unit. Literature data are not available for G solutions, but the error should be somewhat greater than for KCl, if only because more concentrated solutions of G are used here. Again, since this error appears both in the determination of the pK's of the model compounds and in the titration of the protein, comparison of the results should lead to no significant error in fitting the titration curve of the protein but the absolute value given to pK_{int} by these calculations may be in error by the amount shown above.

(37) P. 241 of ref. 23.

(38) G. Scatchard, W. J. Hamer and S. E. Wood, THIS JOURNAL, **60**, 3061 (1938).

(39) K. Linderstrøm-Lang, *Compt. rend. trav. lab. Carlsberg*, **15**, No. 7 (1924).

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

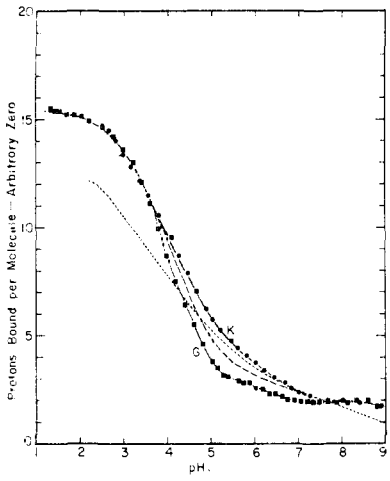

Fig. 6.—Titration curves of the acetylated lysozyme at 25°.

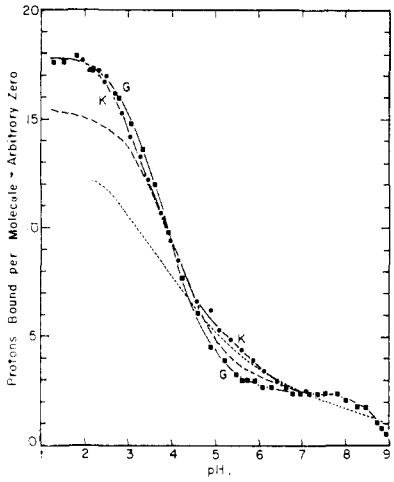

Fig. 7.—Titration curves of the deaminated lysozyme at 25°.

The two groups which are titrated below *p*H 6 in 0.15 *M* KCl have apparent *pK*'s of 3.3 and 4.9. If the net charge of this derivative is assumed to be 20 and w is assumed to be 0.080, as found for the alkaline portion of the titration curve of native lysozyme,[8] then the pK_{int} values for these two groups are approximately 4.7 and 6.3. These groups are thus very likely to be one normal (and unmethylated) carboxyl group[41] and the (single) imidazole group. The large change in *pK* of the imidazole group in GU (see Fig. 5) is rather unexpected and at present unexplained. Analytical data for the number of amide and methoxyl groups in this derivative are presented below.

Since the lowering of the *pK*'s of these three "extra" carboxyl groups in the native lysozyme is of a different magnitude from the raising of the *pK*'s of the three phenolic groups,[8] it seemed possible that these sets of groups were not interacting with one another. Accordingly, since the groups next most likely to be interacting with the "extra" carboxyl groups are the amino groups, acetylated, deaminated and guanidinated lysozymes were prepared. The titration curves at 25° of these derivatives of lot 381–187 are shown in Figs. 6–8. The solvents are 0.15 *M* KCl (K) and 8*M* G. The dashed curve in each figure is the titration curve of the native lot 381–187 in G, the dotted curve, the native in K (Fig 2). All curves are on the same scale and have been moved vertically to coincide at the higher *p*H values. The ordinates for all three figures, the number of protons bound per molecule from some arbitrary reference point, are the same for the titration curves of the *native* lysozyme.

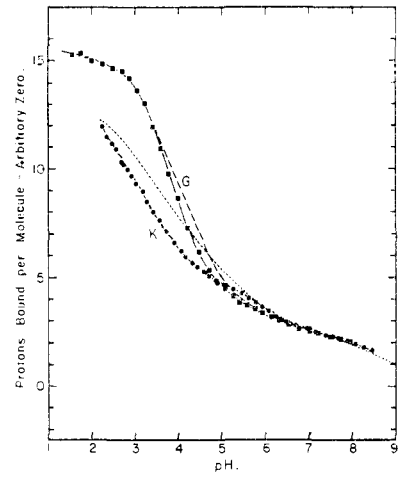

Fig. 8.—Titration curves of the guanidinated lysozyme at 25°.

In the acetylated derivative, the three "extra" acid groups are observed to be titratable in both KCl and G (Fig. 6). Although the three carboxyl groups no longer have their very low values of *pK*, a spectrophotometric titration of the tyrosyl groups of this derivative showed that their apparent *pK*'s were about one-half *pK* unit *higher* than in lysozyme. An increase in *pK* of these groups would be expected because of the increased negative charge on the molecule in this *p*H region. These results indicate that the abnormality of the three carboxyl groups is not caused by the (abnormal) phenolic groups in the native molecule.

The titration curve of the deaminated derivative in KCl (Fig. 7) shows approximately five more titratable acid groups than the native lysozyme but there is no increase in the number of these groups in G. Since nitrous acid has many side reactions,[13,14,16,42] the large number of acid groups in this derivative is not too surprising.

Although the titration curve of the guanidinated derivative in KCl (Fig. 8) does not show the three "extra" carboxyl groups, these groups appear when the titration is carried out in G. The titration curve of this derivative in KCl is almost identical, on the acid side, with the KCl titration curve of a

(41) It should not be inferred that this normal carboxyl group, if it is such, was a normal carboxyl group in the native molecule. It is possible that when a sufficient number of both normal and "masked" carboxyl groups are methylated, the new configuration of the molecule allows a group which was not methylated to become a group of normal *pK* when the molecule is dissolved in KCl solution. Also, this "group" need not be the same in all molecules, nor need all molecules have one group, since the titration curve gives an average for the molecules. It should be pointed out that the assumptions made here about net charge and w probably are unrealistic.

(42) H. Lund, *Acta Chem. Scand.*, 12, 1444 (1958).

"G-treated" lysozyme, prepared by allowing native lysozyme to stand a few hours in 8 *M* G and then removing the G by dialysis.[43] These titration curves presumably differ from the titration curve of the native material because of the different manner in which the molecule has refolded, after it was unfolded either by G or the conditions under which this derivative was prepared.

The Number of Acid Residues in Lysozyme.—Amide and methoxyl analyses of the native lot 381-187 and its methylated derivative support the hypothesis that these "extra" groups are carboxyl groups. A summary of the analytical data is given in Table II. Considering the large number of groups determined, the difference between the number of groups in the native and methylated lysozymes, 3.6 ± 1.0, is in fairly good accord with the observed difference, 3.0 ± 0.2, between the titrations of the native lysozyme in KCl and in GU

TABLE II

ANALYSES OF LYSOZYME, ARMOUR LOT 381-187, AND ITS METHYLATED DERIVATIVE

Group	Native	Methylated
Carboxyl[a]	9.0 ± 0.2	1.0 ± 0.1
Amide[b]	16.6 ± .3	16.0 ± .3
Methoxyl[c]	.....	12.2 ± .1
Total	25.6 ± .5	29.2 ± .5

[a] Estimated from the titration curves in 0.15 *M* KCl. [b] See Experimental section for method. A loss of amide upon methylation, similar to this decrease of 0.6, has been observed by Chibnall, *et al.*,[44] who found 18 amides in Armour lot 20793 (mol. wt. 14,700); Jollès[45] found 18 in his Armour lot 381-187 (mol. wt. 14,800). [c] Analysis performed by Microchemical Specialties Co., Berkeley 3, Calif.

If these groups are carboxyl groups, then they should be found among the total acid residues when the amino acid analyses of the protein are carried out. Up to the present time, most analyses seemed to agree upon 5 glutamic acid and 20 aspartic acid residues[44,46,47] which, together with the C-terminal residue, would give 26 residues total. The amino acids liberated on acid hydrolysis are in many cases partially destroyed by the acid and low values are often found. Jollès,[45] back-extrapolating aspartic acid values after various times of hydrolysis, obtains 21.4 aspartic acid residues per mole (mol. wt. 14,800) and estimates the total number of aspartic acid residues to be 22 ± 1. He has found 22 different aspartic acid residues in peptides obtained by enzymatic hydrolysis of lysozyme. His analyses[45] show 5 glutamic acid residues, in agreement with others. Taking 22 as the aspartic acid value, the total number of acid residues will be 28, including the terminal carboxyl. This does not differ too much from the 29 groups found in the methylated derivative and seems to be consistent with the hypothesis that these "extra" groups in lysozyme are actually masked carboxylate ions.

(43) See curve D in Fig. 2 of ref. 30.

(44) A. C. Chibnall, C. Haselback, J. L. Mangan and M. W. Rees, *Biochem. J.*, **68**, 122 (1958).

(45) P. Jollès, private communication. See also J. Jollès and P. Jollès, 1° Symp. sul lisozima di Fleming, Milan, 1959, in press.

(46) A. R. Thompson, *Biochem. J.*, **60**, 507 (1955).

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

The "Masking" of the Carboxylate Ions.—If these three carboxylate ions are able to accept protons in KCl solution, they must do so below *p*H 2, since no indication of this is observed in the titration curve of lysozyme. If the assumptions made by Laskowski and Scheraga in their treatment of hydrogen bonding in proteins[48] are correct, then either hydrogen bonding fails to explain this extreme lowering of the *pK* of these carboxyl groups, or rather unusual, and *a priori* unlikely, situations of coöperative hydrogen bonding exist in the lysozyme molecule.

It seems reasonable to postulate that these carboxylate ions are in a region of the lysozyme molecule which is quite positively charged and contains several lysyl residues. If it is assumed that the configurations of the derivatives of lysozyme closely resemble that of the native molecule, the titrations of these derivatives support this hypothesis, since the carboxylate ions are titrated when the charges on the lysine residues are removed, but not otherwise. If an electrostatic effect is the explanation of this phenomenon, then: (1) The carboxylate ions may be concealed by the direct effect of the lysyls or (2) the lysyls may interact with some other portion of the molecule, incidentally masking the carboxylate ions. Two results favor the first explanation: (1) The *pK*'s of the lysyl groups have been observed to be normal.[8] This would appear to eliminate other than general electrostatic effects of these groups.[49] (2) An attempt to convert the carboxylate ions into masked un-ionized carboxyl groups (by removal of 8 *M* G from a *p*H 2 solution of lysozyme by dialysis against an HCl solution of *p*H 2) failed, suggesting that these groups can be masked only as carboxylate ions.

The Abnormality of the Tyrosyl Residues in Lysozyme.—It has been mentioned above that the three "extra" carboxyl groups are not interacting with the phenolic groups in the molecule. Thus, the abnormality of the phenolic groups, shown by their high *pK*'s,[8] remains to be explained. Although the phenolic groups in other proteins appear to have normal *pK*'s in urea solution,[50,51] these phenolic groups in lysozyme remain abnormal in urea, even with heating (Fig. 9). The spectrophotometric titration curves of these groups in urea, with and without heating, closely resemble the corresponding curve obtained for the native lysozyme.[8] However, titration curves obtained in 8 *M* G at 25° are shifted about one-half *pK* unit toward acid *p*H's in the region of ionization

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

(49) Assuming a uniform dielectric constant, if one carboxylate ion and two ε-amino groups are placed at the corners of an equilateral triangle, the electrostatic potential at the position of the negatively charged carboxylate ion will be $2e^2/Dr$, where e is the charge on the electron, D the dielectric constant and r the edge length of the triangle. The electrostatic potential at the positions of both positively charged ε-amino groups will be zero, however. This type of spatial arrangement could produce a lowering of the *pK* of a carboxyl group but leave the *pK*'s of ε-amino groups unchanged. There is a sufficient number of lysyl residues in lysozyme[46] to mask three carboxyl groups in this manner.

(50) O. O. Blumenfeld and M. Levy, *Arch. Biochem. and Biophys.*, **76**, 97 (1958).

(51) A. N. Glazer, H. A. McKenzie and R. G. Wake, *Nature*, **180**, 1286 (1957).

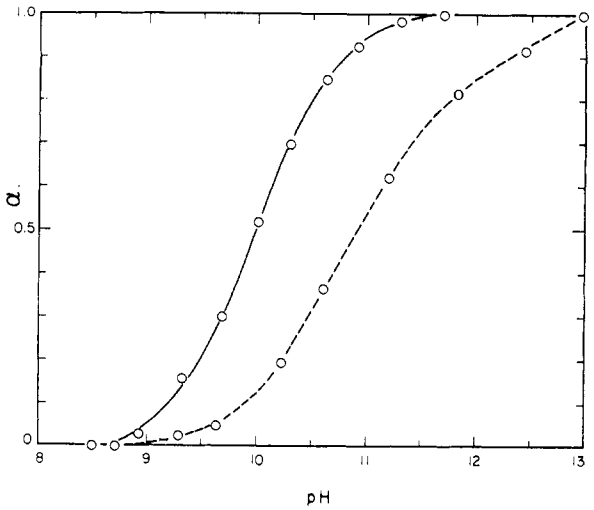

Fig. 9.—Spectrophotometric titration curves of the 3 phenolic groups in lysozyme, Armour lot 381-187. The points are average values of the data obtained at three wave lengths: 290, 295 and 300 mμ. All measurements were made at room temperature (25°): ·····, the ionization of the phenolic groups after the protein was heated in 9 *M* urea at 50° for 24 hr. At *p*H 13.0, a value of 1.0 was assumed for the degree of ionization α. This curve is similar to that reported previously for the ionization of the phenolic groups in KCl solution,[8] and to curves obtained in urea without heating (not shown). ———, GU at 25°: points on this curve were determined after the protein had been in solution for about 2 hr. This sample was not heated. Values of $\Delta\epsilon_{295}$ for 1 mole of lysozyme[15] (3 tyrosyl groups) of 7100 and 7200 in urea and GU, respectively, were obtained.

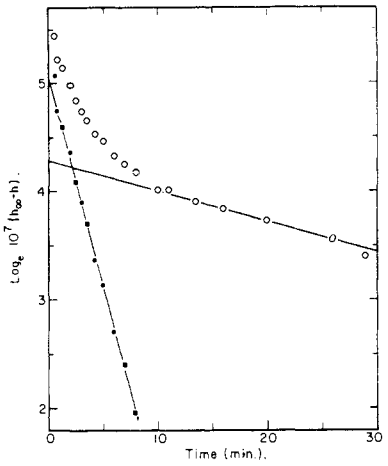

Fig. 10.—The uptake of protons by 181 mg. of lysozyme in 23.0 ml., Armour lot 381-187, at 25° in 3.2 *M* guanidine hydrochloride at *p*H 2.5, as a function of time. The overall reaction has been resolved into two simultaneous first order reactions: O, a slower reaction with rate constant k_2 = 0.028 min.$^{-1}$ (total uptake of hydrogen ion: 0.72 mole/mole protein); ●, a faster reaction with rate constant k_1 = 0.39 min.$^{-1}$ (total uptake of hydrogen ion: 1.25 moles/mole protein).

of the tyrosyl residues, suggesting that the phenolic groups are normal in this solvent. This is not unexpected, since G is much more effective in changing the configuration of lysozyme than is urea.[29,31,52,53] A spectrophotometric titration of the phenolic groups in GU at 25° indicates that these groups are normal in this solvent (Fig. 9). The net charge on the protein in this *p*H range is quite small, and the apparent *pK* in GU may be compared directly with the *pK* of phenol in this solvent (Table I). The nature of the interactions which make the phenolic groups in this molecule abnormal is as yet unknown but some suggestions have been proposed by Tanford and Wagner.[8] A plot of the type shown in Fig. 4, using values of *Z* obtained from titration curves in GU (assuming no chloride binding) gave a negative value of *w* for these phenolic groups. Presumably, the net charge on the molecule in this *p*H range is not the same as the charge affecting the phenolic groups.

The Kinetics of the "Normalization" of the Carboxyl Groups.—The kinetics of the uptake or release of hydrogen ion when G is added to lysozyme solutions allows the classification of some of the carboxyl groups into sets. This classification is possible because different types of abnormality are removed at different rates, as the carboxyl groups are "normalized" by this reagent. The characteristic parameters of these sets are: the amount of hydrogen ion liberated or absorbed and the *p*H dependence thereof. These two quantities are independent of the rate of the reaction and can be treated thermodynamically. Thus, thermodynamic parameters for each set of groups can be obtained separately from those of other sets. This amount of information cannot be obtained from a titration curve alone, where the groups cannot be differentiated in this way. Since the three "extra" carboxyl groups appear in the titration curve in G, if the rate at which these groups are "unmasked" in G is slow enough, the reaction(s) in which these groups are normalized will be observed in addition to the reactions in which some of the remaining nine carboxyl groups are normalized.

A plot of the data from one of the kinetic experiments in which two reactions have been resolved is given in Fig. 10. The rate constant of the faster reaction 1 is designated k_1, that of the slower reaction 2, k_2. Although under the conditions used, at most two reactions were resolved in any one kinetic experiment, a total of four different reaction types were distinguished in the *p*H range of these experiments. Table III contains the observed values of k_1 and k_2, together with the assignments of reaction-type. These assignments were obtained in the following way. A plot of the number of protons taken up per molecule (Δr) for reaction 1 as a function of *p*H gave the curve through the squares in Fig. 11, together with the dashed curve at lower *p*H. The positive portion of the curve drawn through the squares appears to arise from the absorption of protons by carboxyl groups which have abnormally low *pK*'s in the native (before G is added) molecule (reaction type L). Similarly, the negative, higher *p*H, portion of this curve is presumed to be due to carboxyl groups of abnormally high *pK*'s (reaction type H). The

(52) K. Hamaguchi, *J. Biochem.* (*Tokyo*), **45**, 79 (1958).
(53) K. Hamaguchi, *Bull. Chem. Soc. Japan*, **31**, 123 (1958).

TABLE III

KINETIC DATA FOR UPTAKE AND RELEASE OF PROTONS BY LYSOZYME AT 25° IN G[a]

Initial *p*H[b]	Final *p*H	G, *M*	Apparent no. of react.	Reaction 1: k_1, min.$^{-1}$	Reaction 1: $(\Delta r)_1$	Reaction 1: React. type	Reaction 2: k_2, min.$^{-1}$	Reaction 2: $(\Delta r)_2$	Reaction 2: React. type
1.65	1.67	4.5	1				0.030	1.0	R
2.06	2.12	4.75	2	0.48	0.27	S	.030	0.20?	R
2.46[e]	2.60	3.2	2	.39	1.25	S	.028	.72	R
2.70	2.78	5.0	1?	.56	1.10	S			
2.93	3.10	4.5	2	.39	1.84	S	.030	.15	R
2.96	3.07	6.35	1?	.41	1.20?	S			
3.21	3.37	6.2	1?	.17	1.14	S			
3.34	3.38	3.0	2	.24	0.25[d]	L	.058?	.36[d]	S
3.28	3.48	3.62	2	.2	.24	L	.037	1.55	S
3.28	3.41	4.20	2?	~ .3?	.23?	L	.071	1.70	S
3.30	3.46	4.62	1				.121	1.59	S
3.27	3.42	5.20	1				.184	1.66	S
3.33	3.44	6.11	1				.457	1.52	S
3.60	3.74	4.4	2	.16	.74	L	.054	1.10	S
3.97	4.00	3.4	2	.47	.12[d]	L	.031	0.16[d]	S
3.97[c]	4.11	3.8	2	.37	.27	L	.020	.81	S
4.19	4.26	4.6	2	1.4	~ .6	L	.031	.80	S
4.76	4.76	~4.5	2?	?	~ 0	?	?	~ 0	?
5.05	5.02	5.3	2	~ .45	~−0.4	H	~ .05	~−0.1	S?
5.80	5.80	4.6	2	~ .40	~−0.3	H	~ .03	~ 0.2	S?
6.73	6.75	4.0	1	.25	0.05	H			
7.44	7.33	4.8	1				.062	−0.27	?

[a] ? usually means that the best estimate has been made from insufficient data. When applied to the apparent number of reactions, it means that the number given here might have been different at another concentration of G. [b] The initial *p*H was determined by back-extrapolation and may be in error when fast reactions occur. [c] Experiment performed by adding more G to the solution from the previous experiment at the same *p*H. [d] At least the slower reaction has not attained a maximum value at this *p*H, probably because of low G concentration. [e] This experiment is plotted in Fig. 10.

dashed curve appears to join one of the curves (reaction types S) obtained by plotting the Δr values of the slower reactions (with rate constant k_2) as a function of *p*H, and is considered to be an extension of this curve, for reasons which will be explained below. The reactions (with rate constant k_2) at lowest *p*H's appear to be of a distinct type (reaction type R).

Reaction Types R and S.—Curve S appears to result from a structural change of the molecule in G. Jirgenson's data[31] for the increase in levorotation of lysozyme in 4 *M* G at *p*H 4.8 give a first order rate constant of 0.027 min.$^{-1}$. The rate of uptake of protons by lysozyme at *p*H 4.2 in reaction S, at a concentration of 4.6 *M* G, is 0.031 min.$^{-1}$. This uptake is presumably proton binding to molecules which are structurally altered in G. The values of the rate constant for curve S increase markedly with decreasing *p*H, at approximately constant G concentration. The net charge on the molecule increases with decreasing *p*H and could produce a more rapid structural change in G at the lower *p*H's.

The value of Δr for reaction type S reaches a maximum of approximately 2 at *p*H 3 and then appears to decrease with decreasing *p*H (dashed curve). This dashed curve is probably incorrect, for the following reasons. (1) If the maximum in Δr at *p*H 3 were real, this would mean that three carboxyl groups have apparent *pK*'s of approximately 2 in 0.15 *M* KCl solution (see below). However, the observed titration curve gives no indication of the presence of these groups. (2) The titration curve of lysozyme in G differs from the titration curve in KCl by three protons per molecule at low *p*H (Fig. 2), since three carboxyl groups are "unmasked" in G. A total Δr of 3 should then be observed in these kinetic experiments at lower *p*H's if the rate at which these three groups are "unmasked" is slow enough. It would

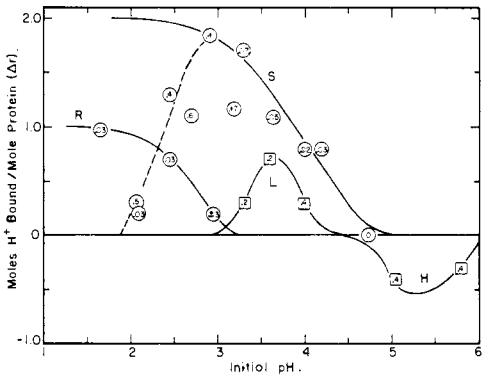

Fig. 11.—The total uptake or release of protons at 25° in G by lysozyme, Armour lot 381-187, as a function of *p*H, for the different reaction types discussed in the text: O, reaction types R and S; □, reaction types L and H. Approximate values of the rate constants (min.$^{-1}$) are given within the symbols. The concentration of G is not indicated but for most points it is between 4 and 5 molar. The data are taken from Table III. The curves drawn through the points are intended to represent the variation of Δr with *p*H for the different reaction types discussed in the text.

be reasonable to expect "masked" groups to become "unmasked" when the molecule undergoes a structural alteration, as postulated for this reaction. (3) The data show that the rate con-

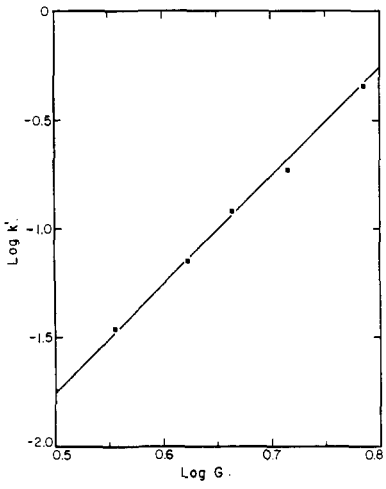

Fig. 12.—The determination of the order of reaction type S with respect to G at 25° and *p*H 3.3. The units of k' and G are min.$^{-1}$ and mole/l., respectively. The line drawn through the points has a slope of 5.00.

stants of reaction type S become quite large at low *p*H, making it difficult to obtain precise values[54] of k and Δr; *i.e.*, for such fast reactions, the values of Δr and k would be expected to be significantly in error. For these reasons, the curve S is extrapolated to $\Delta r = 2.0$ at low *p*H. This extrapolation is consistent with the presumption that curve R represents the slow, *p*H-independent liberation of the third "masked" group ($\Delta r_{max} = 1.0$).

Since the titration curve of lysozyme in 5 *M* G has been shown to be reversible in this *p*H range (Fig. 3), and since, at *p*H 3.3, an increase in G concentration above 3.6 *M* seems to cause no change in the total number of protons taken up by the molecule from solution, it appears valid to interpret curve S as a titration curve of these two carboxyl groups.[55] These two groups appear normal in G, within experimental error. Assuming: (1) no chloride binding, (2) $Z_{max} = 20$ and (3) that w (from Fig. 4) is 0.052, the intrinsic pK of these two groups is calculated to be 4.6. The normality of these groups in G is consistent with the analysis made above of the titration curve in G, which showed that, except for some discrepancy at the low *p*H end of the titration curve, all the ionizable groups fit a straight line plot of equation 1, with intercept $pK_{int} = 4.6$, the pK of a normal carboxyl group (Fig. 4).

(54) Reaction type S has a half-time of approximately 1.5 min. at low *p*H. But, as noted above, the electrodes do not appear to come to equilibrium until after about one minute from the time they are placed in the solution. The back-extrapolation necessary to determine Δr must be done on very few points if a slow reaction (Type R) occurs along with the fast one (Type S) and is thus subject to errors which become larger as the rate constant of reaction type S becomes larger.

(55) The assumptions made here (both of which are substantiated by the data) are that at the end-point of the kinetic experiments, the G has converted the molecule into the form which is titrated in 5 *M* G solution and that the masked groups were completely in the carboxylate ion form in the lysozyme molecule in KCl solution, at least above *p*H 3. Thus, at the end-points of the kinetic experiments, the two carboxylate ions are free to equilibrate with the hydrogen ion in the solution conditioned only by the pK which these groups have, given their position on a structurally altered molecule with the charge configuration peculiar to that molecule. Presumably, electrostatic effects are acting to determine these pK's in G but not intramolecular hydrogen bonds, since it appears likely that these hydrogen bonds would be unstable in G.

The order of reaction type S with respect to G was determined at *p*H 3.3. The data are given in Table III. For an apparent rate constant given by $k' = kG^n$, a plot of log k' against log G will be a straight line with slope n (Fig. 12) if n is constant over this range of G concentration. This experiment would appear to mean that five molecules of G are necessary for the reaction in which two protons are taken up by the lysozyme molecule. This result differs from that of Harrington,[9] who found a total of eight protons taken up per molecule of ovalbumin at acid *p*H's, and an order of eight with respect to G. He observed that an equal number of masked phenolic groups were liberated at the same time by G and concluded that a hydrogen bonding interaction between these groups existed in the native molecule. In the case of lysozyme, the order of the reaction with respect to G is not the same as the maximum number of protons taken up per molecule in the reaction. These interactions are presumably either hydrogen bonds between groups which are non-ionizable in this *p*H range or are not hydrogen bonds at all. Since the "extra" groups are unmasked when these interactions are disrupted, the disruption must result in the spatial separation of the "extra" carboxyl groups and the lysyl groups, which are presumed to be producing the "masking" effect.

Reaction type R has been interpreted as the release of the third "masked" carboxylate ion. It is difficult to reconcile its apparently abnormal pK in G (approximately 2.8 according to Fig. 11) with the plot of Fig. 4. Perhaps this is because the titration curve for Fig. 4 was obtained in GU rather than in G alone. However, these kinetic results near *p*H 2 might be spurious, and reaction type S actually due to three carboxylate ions, the increase in the rate of the reaction with hydrogen ion concentration concealing the fact that this reaction has a maximum Δr of 3 at lower *p*H's, instead of 2 as now postulated.[54]

Thermodynamic Treatment of Reaction Types L and H.—An ionizable group which has an abnormal pK due to some interaction with another portion of the molecule will take up or liberate hydrogen ion in the *p*H range in which it ionizes if this interaction is eliminated (for example, by adding G to the solution of the protein). It is easily shown that, ignoring ordinary electrostatic effects (of the type included in eq. 1): (1) For groups of either abnormally high or low pK, the maximum $|\Delta r|$ will occur at a *p*H halfway between the "normal" and the "abnormal" pK's. (2) The number of protons taken up per molecule will be given up by[56]

$$\Delta r_{max} = m\left(\sqrt{\frac{K'}{K}} - 1\right)\left(\sqrt{\frac{K'}{K}} + 1\right)^{-1} \qquad (2)$$

Here, the equilibrium constant for the dissociation of the normal group is given by K and m is the number of groups having the abnormal equilibrium constant K'.

(56) In using kinetic data of this type, it must be remembered that the disruption of the interaction by G must be complete in order for this analysis to be correct. This can be verified by observing whether $|\Delta r|$ increases when the G concentration is increased. The sign of Δr will be positive when $K' > K$ and negative when $K' < K$.

Given a normal pK value, the first of these relations can be used to determine K' of the abnormally ionizing group from the pK at which $|\Delta r|$ is a maximum. When K' is substituted into equation 2, together with Δr_{max}, the number, m, of abnormal groups of the given type will be obtained.[57]

Reaction Types L and H.—Reaction types L and H of Fig. 11 must be due to the uptake and release, respectively, of protons by carboxyl groups[58] when intramolecular interactions of these groups are removed by G. It is assumed here that normal ionizable groups in the lysozyme molecule will adjust to the new ionic strength rapidly enough so that they will not be observed kinetically. Other abnormal ionizable groups might "normalize" rapidly enough so that they would not be observed either.[59]

For reaction type L, the data in Table III indicate that completeness of bond rupture has been attained at pH 3.3. Completeness will be assumed here throughout the pH range of reaction types L and H. With this assumption, the observed value for Δr_{max} of 0.8 at a pH of approximately 3.6 can be interpreted to mean that two carboxyl groups in the native lysozyme have apparent pK's of 3.2.[60]

Reaction type H appears to be due to the release of hydrogen ion from a carboxyl group of abnormally high pK. With assumptions similar to those made for reaction type L, the apparent pK of this group is estimated to be 6.3, and m, unity.

This interpretation of the reaction types L and H has avoided the use of the hydrogen bonding treatment which has been published previously.[62] If the interactions are treated as hydrogen bonds, the apparent pK's and numbers of groups will be unchanged, but the additional quantity, K_{ij}, the equilibrium constant for the formation of the hydrogen bond, will be obtained in the course of the calculations. For the reaction type L, K_{ij} is found to be about 10, and for reaction type H, about 100. Since the value of K_{ij} for a single hydrogen bond has been estimated to be about unity,[48] then if these interactions are hydrogen bonds, they must be of the coöperative type.[48] Since there appears to be no evidence that these interactions *are* hydrogen bonds, it seems preferable not to treat them as if they *were* hydrogen bonds.

These kinetic results, which indicate the presence of carboxyl groups with both high and low pK's, appear to be in reasonable accord with the equilibrium results obtained from titration curves. Wagner[63] states that if the electrostatic factor for lysozyme were normal, then the observed titration curve[8] in KCl can be accounted for by assuming that half the carboxyl groups have high pK's and half low pK's. Beychok and Warner[61] have recently concluded that the electrostatic factor *is* normal and that (of the 10.5 carboxyl groups in lot 003L1) six carboxyl groups have a pK_{int} of 3.5 and the remainder a pK_{int} of 5.1. Values of pK similar to these have been obtained from difference spectra.[64]

This kinetic study indicates that only about three of the carboxyl groups in lysozyme which are titratable in KCl solution have abnormal pK's. The discrepancy in the number of groups between this kinetic study and the calculations of Tanford and Wagner[8] and Beychok and Warner is presumably due to a very rapid normalization of most of the abnormal carboxyl groups in G.[59] At present the amount of data available is insufficient to permit a detailed description (beyond that already postulated) of the interactions which render the nine carboxyl groups abnormal and mask the three additional groups. It is hoped that a thorough kinetic study will produce sufficient data to permit the determination of the cause of the abnormalities of all of the carboxyl groups.

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(57) Harrington[8] assumed that the interactions masking carboxyl groups in ovalbumin were hydrogen bonds and observed that the order of the unmasking reaction with respect to G was the same as the number of groups unmasked. It has not been shown that one G molecule is required to disrupt one hydrogen bond in simpler systems. If this assumption about hydrogen bonds is true, then the order n of the "normalizing" reaction with respect to G should equal the number of groups m obtained from equation 2, provided that the groups are abnormal because of hydrogen bonding interactions. Presumably, other types of abnormality might not require the equivalence of these two quantities. The orders of reaction types L and H with respect to G have not been determined.

(58) These carboxyl groups, in contrast to those producing reaction types R and S, are presumably titrable in the native protein in KCl solution.

(59) Since the electrodes used did not come to equilibrium for about one minute after G was added to the lysozyme solution, the normal groups (and perhaps some abnormal ones) had this much time to equilibrate with hydrogen ion in the new, higher ionic strength, solvent.

(60) In making this calculation, the charge on the molecule in this pH region for this lot of lysozyme has been estimated from the data of Beychok and Warner.[61] A net charge of eight, independent of pH, has been assumed, together with an electrostatic factor, w, of 0.080.[8] With these assumptions, a normal carboxyl group with pK_{int} of 4.6 would have an apparent pK of 4.0.

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