are available at ionic strengths of 0.004 and 0.16, a substantial reduction in $\Delta pK'_2$ may be anticipated if it is based on a lysine pK'_2 at higher ionic strength. (The directly comparable pK for tyrosine is pk_{123}, which drops from 9.95 to 9.70, as the ionic strength is raised from zero to 0.16. An *increase* of similar magnitude in pK'_2 for lysine may be anticipated.) As experimental data for lysine are not available at higher ionic strength, we can only guess what the value of $\Delta pK'_2$ would be at the higher ionic strength. A reasonable guess is 0.40. The $\Delta pK'_2$ in 6 *M* GuHCl, which is only 0.26, thus may represent an appreciable further decrease. It should be noted, however, that the interaction between the amino groups is by no means abolished entirely. The $\Delta pK'_2$ of 0.26 in 6 *M* GuHCl is still close to half the $\Delta pK'_2$ value at ionic strength 0.01.

Both the ineffectiveness of GuHCl in suppressing the interactions between charges of oppositive sign in glycine and tyrosine, and its somewhat greater effectiveness in suppressing the interaction between charges of like sign in lysine, may be simple consequences of the fact that the salt ions which provide the source of the ionic strength are large in size, having dimensions comparable to the distance between charges on the amino acid molecules. If the ionic strength of concentrated GuHCl is to provide an effective shield between charges of opposite sign, it is necessary that both GuH^+ and Cl^- ions have a high probability of being simultaneously in close proximity to the interacting charges. Steric considerations may well make this probability vanishingly small. For GuHCl to decrease the interaction between the positive charges of lysine, on the other hand, it is required only that a single Cl^- ion have a high probability of being in close proximity to the charges. Steric repulsions will clearly be less effective in this case.

Conclusions. It can be concluded from the results of this paper that concentrated GuHCl is a suitable medium for acid–base titrations, which does not differ significantly from concentrated solutions of alkali metal chlorides, such as KCl or CsCl. It is however usable only up to a pH of about 11 or 11.5. Prohibitive amounts of base are required to attain higher pH's because of the dissociation of the GuH^+ ion to uncharged Gu, and further difficulties arise from the apparent instability of Gu under these conditions. The fragmentary data given earlier suggest that the product formed from Gu may be biguanide. If so, its formation would result from the condensation of Gu with GuH^+, and loss of NH_3. The NH_3 would presumably be removed in any titrimetric experiment by the nitrogen gas normally employed to keep alkaline solutions free from CO_2. The formation of biguanide would thus be accompanied by some irreversibility in the uptake of base, and evidence for such irreversibility has in fact been found in titration studies carried above pH 11.

Concentrated solutions of GuHCl have remarkably little effect on the pK's of amino acids. In particular, intramolecular electrostatic interactions, which one might expect to be substantially reduced in a solvent medium of ionic strength 6.0, are in fact almost as strong as in dilute salt solutions, especially when the interacting charges are of opposite sign. This finding suggests that GuHCl may not be able to suppress electrostatic interactions in proteins entirely, even when the protein molecules are completely unfolded to a randomly coiled state.

Proteins as Random Coils. II. Hydrogen Ion Titration Curve of Ribonuclease in 6 *M* Guanidine Hydrochloride[1]

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Abstract: The hydrogen ion titration curve of ribonuclease in 6 *M* guanidine hydrochloride has been determined. The results differ significantly from titration data in a similar medium reported by Cha and Scheraga. All 32 known titratable groups of the protein (except for perhaps three phenolic groups) appear to be titrated with the pK values to be expected for fully exposed groups, subject to no interactions of any kind. Even electrostatic interactions appear to be reduced to less than experimentally significant magnitude. The three phenolic groups excepted from this generalization are subject to very weak interactions, leading to pK values about 0.4 higher than expected. These results are compatible with the conclusion, reached on the basis of other data, that ribonuclease and other proteins, in 6 *M* guanidine hydrochloride, behave as randomly coiled polypeptide chains even when their disulfide bonds are intact. An anomalous uptake of about one hydroxyl ion per protein molecule was observed at high pH and shown to be due to the occurrence of a β-elimination reaction at cystine residues.

Evidence presented in two earlier papers[2,3] suggests that proteins dissolved in concentrated guanidine hydrochloride (GuHCl) lose essentially all elements of their native structure, and exist as randomly coiled chains in which no important noncovalent interactions remain. Although this conclusion was based primarily on data obtained under conditions where disulfide bonds of the proteins were broken, viscosity and optical

(1) Supported by research grants from the National Science Foundation and from the National Institutes of Health, U. S. Public Health Service.

(2) C. Tanford, K. Kawahara, and S. Lapanje, *J. Biol. Chem.*, **241**, 1921 (1966).

(3) C. Tanford, K. Kawahara, and S. Lapanje, *J. Am. Chem. Soc.*, **89**, 729 (1967).

rotatory dispersion studies suggest that the conclusion is also true when disulfide bonds are intact. In this situation, a protein polypeptide chain (containing disulfide bonds) cannot be a "random coil" in the sense that a linear chain without cross links can be. Our results have indicated however that such a chain may well be random except as restricted by the disulfide bonds, and that the existence of structured beads, held together by noncovalent bonds, is unlikely.[3]

If the foregoing conclusion is correct, then the titration curves of proteins should become very simple in concentrated GuHCl solutions. Effects of hydrogen bonds and hydrophobic bonds should virtually disappear. Moreover, since GuHCl is a strong electrolyte, electrostatic interactions should also be greatly reduced. As indicated in the preceding paper,[4] interactions between closely spaced charges, separated by distances comparable to the dimensions of the GuH^+ and Cl^- ions, may not be entirely abolished, but the residual interactions should certainly be small.

To express this prediction mathematically,[5] we would say that all chemically identical groups have identical intrinsic dissociation constants ($pK_{int}^{(i)}$), and that the equation for the degree of dissociation (x_i) of each group at any pH must approach the simple relation

$$\log \frac{x_i}{1 - x_i} = \mathrm{pH} - pK_{int}^{(i)} \qquad (1)$$

in which no allowance is made for any electrostatic or other kind of interaction between the titratable groups. The symbol i in this equation refers to the various kinds of chemically different groups. Only nine classes should ordinarily exist, these being the terminal carboxyl and amino groups at the ends of polypeptide chains and the seven chemically distinct kinds of titratable side chains: β-carboxyl (aspartic acid), γ-carboxyl (glutamic acid), imidazole (histidine), sulfhydryl (cysteine), phenolic (tyrosine), ϵ-amino (lysine), and guanidyl (arginine). The number n_i of groups in each class should correspond exactly to the number of residues of the corresponding amino acid present in each protein molecule.

Previous work on titration of proteins in GuHCl or similar denaturing media does not support the expectations set forth above, but indicates that some of the features of the titration curves of native proteins may be retained even after the proteins are fully denatured.

Cha and Scheraga,[6] for example, have reported a titration curve of ribonuclease in a mixture of 5 *M* GuHCl and 1.2 *M* urea, which can be fitted with the expected n_i values, but which requires the use of the conventional semiempirical equation of Linderstrøm-Lang[5,7]

$$\log \frac{x_i}{1 - x_i} = \mathrm{pH} - pK_{int}^{(i)} + 0.868w\bar{Z}_H \qquad (2)$$

to describe the course of titration of each set of n_i groups of type i. In this equation, $\bar{Z}_H$ is the average charge per molecule, owing to proton binding or dissociation, and w is a semiempirical parameter. In the original derivation[7] of eq 2, w was assumed to reflect electrostatic interactions only, but it is now recognized[5,8] that all kinds of interactions, including those which in fact affect pK_{int}, can lead to a high value for the parameter w as it is normally determined from experimental data.

That eq 2 had to be used, rather than eq 1, is in itself not surprising, since, as we have pointed out, electrostatic interactions are not expected to be entirely eliminated under the conditions of the experiment. However, the value of w obtained from the data of Cha and Scheraga[6] is surprisingly large. They obtained, for titration of both carboxyl and phenolic groups, $w = 0.056$, which is almost as large as the value of 0.061 which is required to fit titration data of *native* ribonuclease[9] in 0.15 *M* KCl, implying therefore that noncovalent interactions continue to be almost as large in the denatured protein as in the native protein. Although Blumenfeld and Levy[10] have reported a much lower and more reasonable value of w for the titration of the phenolic groups of ribonuclease in 8 *M* urea, at an ionic strength of 0.1, a repetition of the titration is important, because a confirmation of the result of Cha and Scheraga would raise serious doubt about our own earlier conclusion[2,3] that proteins such as ribonuclease behave as random coils in solvents containing high concentrations of GuHCl or urea.

Data obtained for proteins other than ribonuclease raise doubts about the stoichiometry of hydrogen ion dissociation, especially in the acid branch of the titration curve. It has been found for a number of proteins, including some studied in this laboratory (to be reported at a later date), that more groups are titrated in the acid branch in concentrated GuHCl than in dilute salt solutions. This in itself is not alarming, for it could simply reflect abnormality of the titration behavior of the native proteins in dilute salt solutions. In two proteins, however, the data cannot be explained in this way. In lysozyme,[11] the number of carboxyl groups titrated in 8 *M* GuHCl (or 5 *M* GuHCl plus 1.2 *M* urea) exceeds the number of free carboxyl groups which this protein is known to possess on the basis of its amino acid sequence.[12] In ovalbumin,[13] denaturation by guanidine hydrochloride at low pH has been reported as being accompanied by the titration of eight carboxyl groups, which exist in the native protein, to very low pH, as carboxylate ions. However, all free carboxyl groups known to be present on the basis of the best available amino acid analyses are already titrated in the native protein.[14] While the analytical data could be in error, it is unlikely that they are in error by as much as eight groups per molecule.[15]

These experiments concerning the number of groups titrated may therefore reflect the existence of some un-

(4) Y. Nozaki and C. Tanford, *J. Am. Chem. Soc.*, 89, 736 (1967).
(5) For a general discussion of protein titration curves see C. Tanford, *Advan. Protein Chem.*, 17, 69 (1962).
(6) C.-Y. Cha and H. A. Scheraga, *J. Am. Chem. Soc.*, 82, 54 (1960).
(7) K. Linderstrøm-Lang, *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, 15, No. 7 (1924).
(8) F. Karush and J. Sonenberg, *J. Am. Chem. Soc.*, 71, 1369 (1949).
(9) C. Tanford and J. D. Hauenstein, *ibid.*, 78, 5287 (1956).
(10) O. O. Blumenfeld and M. Levy, *Arch. Biochem. Biophys.*, 76, 97 (1958).
(11) J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, *J. Am. Chem. Soc.*, 82, 2154 (1960).
(12) R. E. Canfield and A. K. Liu, *J. Biol. Chem.*, 240, 1997 (1965).
(13) W. F. Harrington, *Biochim. Biophys. Acta*, 18, 450 (1955).
(14) R. K. Cannan, A. Kibrick, and A. H. Palmer, *Ann. N. Y. Acad. Sci.*, 41, 247 (1941).
(15) Furthermore, if eight carboxylate ions remain untitrated in the native protein, then the experimental maximum positive charge, $(\bar{Z}_H)_{max}$, as measured in the native protein, should be less than the total number of basic groups by the same number, whereas there is in fact agreement between $(\bar{Z}_H)_{max}$ and the analytical figures for basic groups.[14]

expected artifact. They suggest the possibility that GuHCl may react with proteins, in acidic solution, so as to produce new titratable groups not normally present on native proteins.

The study reported here was undertaken with these questions in mind. Ribonuclease is a protein of known amino acid content and sequence.[16] It can therefore provide an exact test of the stoichiometry of hydrogen ion dissociation, to determine whether new titratable groups are generated by interaction with guanidine hydrochloride. Furthermore, it will be possible to analyze deviations from the limiting ideal behavior in the titration of individual groups (ideal behavior meaning complete absence of interactions between groups) in terms of the amino acid sequence, *i.e.*, to see whether such deviations can be explained in terms of reasonable residual weak interactions between groups which are closely spaced by virtue of the amino acid sequence alone.

Experimental Section

Ribonuclease. The sample used was Lot 114B-1510 from the Sigma Chemical Corp. It was designated Type I-A, phosphate free. Stock solutions were prepared by dissolving the protein in water, dialyzing the solution against water, and finally deionizing it by passage through a mixed-bed, ion-exchange resin column as described by Dintzis.[17] The protein concentration of each stock solution was determined by the residual weight of an aliquot, after drying to constant weight at 107°.

Two stock solutions were prepared without the deionization step, a longer period of dialysis against several changes of distilled water being substituted. It was found that the dialysis procedure did not bring the protein even close to its isoionic pH. The pH measured (in 0.15 *M* KCl) after dialysis was found to be 5.60, corresponding to a molecular charge of about +7. Since anions must have been present to neutralize this charge, the dry-weight method used to determine protein concentration must, for these stock solutions, have measured the residual weight of protein plus the neutralizing anions. On the other hand, the anions should not interfere with the titration of the protein, unless they contain acidic or basic moieties. Though the anions are presumably tightly held to the native protein, they would become dissociated when the protein is unfolded in the GuHCl solution and make a negligible contribution to the total ionic strength. Electrometric titration data obtained with these stock solutions indicated that the neutralizing anions in fact did not contain acidic or basic groups. The data were found to be virtually superimposable on the curve obtained with deionized protein, if the contribution of the anions to the dry weight was taken as 4%, *i.e.*, 550 g per mole of protein. This is a reasonable figure. Four sulfate ions, for example, would add 380 g to the dry weight per mole of protein.

Other Reagents. The purification of GuHCl and the preparation of standard acid and base solutions were described in the preceding paper.[4]

Electrometric Titration. The procedure followed is the same as described in the previous paper.[4] The number of H^+ ions bound to or removed from the protein at any pH was calculated with the aid of the apparent activity coefficients given in that paper. Because GuH^+ ions themselves dissociate in alkaline solution (leading to very small values for γ'_{OH}), the precision with which $\bar{Z}_H$ values can be calculated becomes poor at pH's much above 11. Some chemical deterioration of guanidine also sets in between pH 11 and 12. Because of these features of the behavior of GuHCl, we have obtained no experimental data above pH 11.3. All measurements were made at 25.0°.

Spectrophotometric Titration. The procedure followed is the same as that described for the titration of tyrosine in the preceding paper.[4] Measurements were made at 25.0°.

Results

The pH of Zero Net Proton Charge.[5] The pH of deionized solutions of ribonuclease was found to agree quite closely with values previously reported. At zero ionic strength the pH is 9.60, in 0.15 *M* KCl it is 9.71. (Tanford and Hauenstein[18] reported a value of 9.65 for ribonuclease A in 0.15 *M* KCl.) Addition of GuHCl to high concentrations reduces the pH. Two separate samples in 6 *M* GuHCl gave values close to pH 9.45. At the protein concentrations at which these measurements were made, the amount of base required to produce a pH of 9.45 corresponds to about 0.3 mole/mole of protein. This relatively high figure is a result of the low values of γ'_{OH}, which in turn reflect the fact that a significant conversion of GuH^+ to uncharged Gu (1 mole per 5×10^5) occurs when GuHCl is exposed to pH 9.45.[4] The required base must be derived from the protein itself, so that the protein molecules at the isoionic point must bear an average charge of about +0.3. The pH at which the protein molecules have zero net proton charge is, therefore, higher than 9.45 by the change in pH required to titrate 0.3 group. This leads to a pH of 9.54 for the point of zero net proton charge.

Electrometric Titration. Several independent titrations were performed, using protein from two deionized stock solutions and from two dialyzed stock solutions. Only the results obtained for the deionized stock solutions are reported as primary data, although, as pointed out above, essentially identical results were obtained with the dialyzed stock solutions, after a reasonable correction of the protein content as determined by dry weight analysis.

The deionized stock solutions were stored (at pH 9.6) under CO_2-free air. Nevertheless, small amounts of CO_2 were absorbed, either during storage or during the initial handling which was required for the introduction of the protein into the titration vessels. The initial pH in 6 *M* GuHCl was generally somewhat below the pH of isoionic protein as measured on the freshly deionized stock solutions. Initial titration to below pH 5 generally required a few tenths of a mole of acid more (per mole of protein) than was required for back titration after the solution had been allowed to stand in the titration vessel for a short period below pH 5. Such standing would be expected to lead to removal of any CO_2 in the solution, as the titration vessels were always kept under a stream of nitrogen. The data reported here are only those obtained after removal of CO_2 by initial titration to below pH 5. We do not believe that there was any possibility for contamination by CO_2 after the titration itself was begun.[19]

The results obtained are shown in Figure 1. The primary data were measured as $\bar{Z}_H$ values relative to the initial pH of titration, and they were adjusted to absolute $\bar{Z}_H$ values by taking $\bar{Z}_H$ to be zero at pH 9.54. The over-all reproducibility of different titrations was about 0.1 group per mole over most of the titration range. The maximum deviation observed below pH 10 was near pH 5, where a difference of about 0.2 group per mole was observed between two of the titration experiments. The titration between the acid end point and pH 10.0 was found to be perfectly reversible. (The data above pH 10 will be discussed below.)

(16) D. G. Smyth, W. H. Stein, and S. Moore, *J. Biol. Chem.*, 238, 327 (1963).

(17) H. Dintzis, Ph.D. Thesis, Harvard University, 1952.

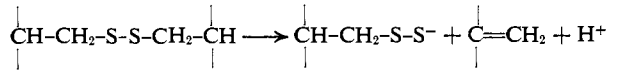
(18) C. Tanford and J. D. Hauenstein, *Biochim. Biophys. Acta*, 19, 535 (1956).

(19) No contamination by CO_2 was detected at any time in the titration data obtained with dialyzed stock solutions, which were stored and introduced into the titration vessels at pH 5.6.

Figure 1 shows that the acid end point of the titration occurs at $\bar{Z}_H = +19.2$. The theoretical figure is 19.0, corresponding to the content of basic groups of the protein:[16] four histidines, ten lysines, four arginines, plus the terminal amino group. The agreement between experiment and theory is probably within the over-all experimental error.

Visual examination shows that the curve may be divided into three distinct titration regions: an acid branch from $\bar{Z}_H = 19$ to $\bar{Z}_H = 8$, indicating the presence of 11 carboxyl groups; a neutral branch from $\bar{Z}_H = 8$ to $\bar{Z}_H = 3$, indicating the presence of five groups in the class of imidazole and α-amino groups; and an alkaline branch which is incomplete at the highest pH to which the titration was carried, but which can be extrapolated to an end point at $\bar{Z}_H = -14$, indicating the presence of 17 groups in the class of tyrosyl and ϵ-amino groups. The number of groups titrated in the first two branches again agrees with the known amino acid content of the protein[16] (five aspartic acids, five glutamic acids, four histidines, and the terminal carboxyl and amino groups). The number of groups titrated in the alkaline region, on the other hand, exceeds the number expected on the basis of amino acid content, which is 16 groups (six tyrosines, ten lysines). This anomaly cannot be the result of faulty extrapolation of the data of Figure 1. An extrapolation to an end point of $\bar{Z}_H = -13$ is clearly unreasonable, requiring much too sharp a break in the curve, as can be seen by comparison with the calculated curve also shown in the figure.[20,21]

The most likely explanation for the anomaly in the alkaline branch of the curve is that we are observing the β-elimination reaction of disulfide bonds,[23] which was observed to take place in native ribonuclease at higher pH or higher temperature by Bohak.[24] This reaction leads to dissociation of a proton from cystine and creation of a residue of dehydroalanine.



Bohak found that this reaction occurs in aqueous solutions of native ribonuclease, at room temperature, only above pH 12. At a temperature of 100°, however, the reaction could be observed already at pH 8. Bohak noted that the conditions required for occurrence of the reaction were similar to the conditions required for denaturation of ribonuclease. It is not unreasonable that we observe the reaction under milder conditions, since the ribonuclease in our experiments is already denatured by the presence of GuHCl, and the disulfide bridges are presumably freely accessible to hydroxyl ions at all times. It may be noted that a time of about 2–3 hr was required for each alkaline titration, and that the results require that only one of the four disulfide bonds have reacted during that time.[25]

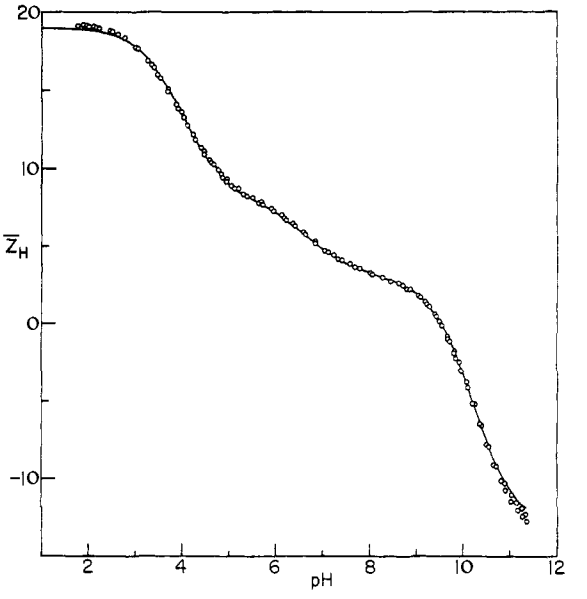


Figure 1. Electrometric titration of ribonuclease in 6 *M* guanidine hydrochloride, at 25°. The curve is a calculated one, based on the parameters of Table I.

It has been shown that the dehydroalanine residue formed in the β-elimination reaction is unstable, and that it reacts with lysine residues to form a new amino acid, lysinoalanine.[24] The presence of this amino acid can thus serve as a confirmation of the occurrence of β-elimination. We accordingly hydrolyzed a sample of the protein which had been titrated to pH 11.3 and subjected it to amino acid analysis.[26] A peak in the elution pattern was observed, which emerged ahead of lysine, exactly in the position shown by Bohak for lysinoalanine.[24] No exact quantitation was attempted, but comparison with the data of Bohak suggested that about 0.6 mole of lysinoalanine had been formed in the solution subjected to analysis.

The proton dissociated by the β-elimination reaction should be regained by back titration to lower pH. If the thiocysteine residue loses sulfur and becomes converted to cysteine, as has been suggested,[23] the new pK would be about 9.1.[5] While no detailed studies of reversibility were carried out, the few data we have obtained are compatible with a pK of that order of magnitude, *i.e.*, back titration from pH 11.3 showed a hysteresis of about 0.5 group which disappears at pH 7 or below.[27]

Spectrophotometric Titration. The results of spectrophotometric titration of the phenolic groups are shown in Figure 2. This study was carried out with dialyzed protein. The total change in molar extinction

(20) The theoretical curve which is drawn in Figure 1 indicates that 1.35 groups (not counting guanidyl groups) remain to be titrated above pH 11.3. If this figure is added to the experimental $\bar{Z}_H$ values at that pH, an end point of -13.85 ± 0.25 is obtained.

(21) We are assuming that the guanidyl groups of arginine residues do not make a significant contribution to the titration curve at a pH as low as 11.3. The pK of GuH^+ itself is 13.74.[4] A substantially lower value (12.5) has been reported for the guanidyl groups of arginine,[22] but even this lower value corresponds to the titration of only 0.24 of the 4.0 guanidyl groups of ribonuclease at pH 11.3. The extrapolated end point would still be too high by 0.6 group if allowance for 0.24 titrated guanidyl group is made.

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

(23) R. Cecil and J. R. McPhee, *Advan. Protein Chem.*, **14**, 255 (1959).

(24) Z. Bohak, *J. Biol. Chem.*, **239**, 2878 (1964).

(25) The data of Figure 1 above pH 10 represent two independent titrations, one of which yielded the upper set of points (corresponding to the dissociation of 0.6 anomalous proton), the other the lower set of points (1.1 anomalous protons). We do not know what caused the difference. The data obtained with dialyzed stock solutions agree almost exactly with the upper points.

(26) We are indebted to Dr. R. E. Bradshaw for carrying out this analysis for us.

(27) Titration studies of bovine serum albumin in 6 *M* GuHCl show the same anomaly as we have reported here for ribonuclease. More detailed reversibility studies were performed with this protein, and the midpoint of back titration of the anomalous proton uptake could be definitely fixed at pH 9.0.

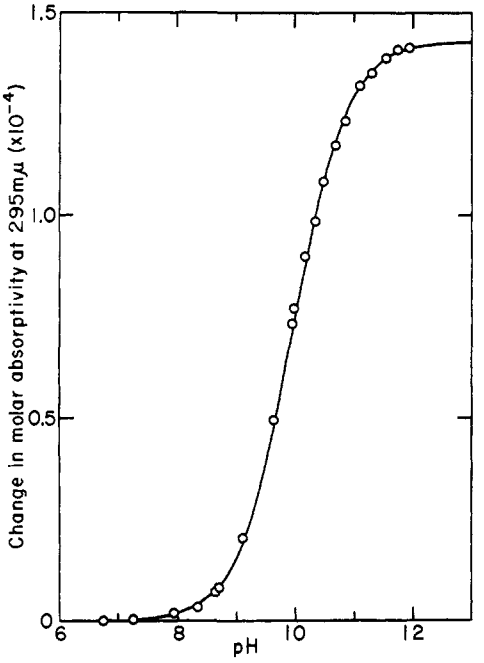


Figure 2. Spectrophotometric titration of ribonuclease in 6 *M* guanidine hydrochloride, at 25°. The curve is a calculated one, based on the parameters of Table I.

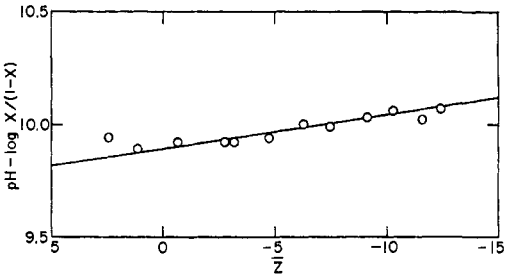


Figure 3. Logarithmic plot of the data of Figure 2.

at 295 mμ, after correction for the probable contribution of anions to the dry weight of the sample, is 14,300. This change is in the expected range for dissociation of six phenolic groups. The value of $\Delta\epsilon$ per phenolic group in 6 *M* GuHCl was found to be 2450 in the titration of tyrosine,[4] corresponding to 14,700 for six groups.

Mathematical Analysis. In Figure 3, the titration data for the phenolic groups are plotted in the conventional way,[5] according to eq 2, with $\bar{Z}_H$ values taken from the electrometric titration data. All data between $x_i = 0.05$ and $x_i = 0.95$ are included. It is seen that the data yield a linear plot. The intercept at $\bar{Z}_H = 0$ gives $pK_{int} = 9.90$, and the value of w calculated from the slope is 0.017. This is much smaller than the value of 0.056 reported by Cha and Scheraga[6] for similar data, but agrees well with the value of 0.018 obtained by Blumenfeld and Levy[10] for titration of the phenolic groups in 8 *M* urea.

A similar analysis was made for the titration of the side chain carboxyl groups, *i.e.*, for the data of the acid branch of Figure 1 after correction for titration of the single α-carboxyl group, and for overlap with the titration of imidazole groups. Making the usual assumption that aspartyl and glutamyl groups can be treated as identical, we obtain $pK_{int} = 4.3$ and $w = 0.020$. This value of w is essentially the same as that determined for the phenolic groups and again substantially smaller than the value of $w = 0.057$ obtained for the carboxyl groups by Cha and Scheraga.[6]

Although the titration of the carboxyl and phenolic groups can be described formally in terms of eq 2, with the assignment of a quite small value of w, this approach cannot be used to obtain a formal fit for the electrometric titration curve as a whole. The alkaline branch of the titration curve, for example, is sufficiently steep to require that w for the lysyl amino groups be essentially zero. The parameter w of eq 2 thus represents in this case aspects of titration behavior which are specifically applicable to the titration of carboxyl and phenolic groups, rather than being a measure of the derivative of the electrostatic free energy of the whole molecule with respect to charge, which is the formal meaning of w in the original derivation of eq 2.[7,28] The fact that approximately equal values of w apply to the titration of both carboxyl and phenolic groups must be accidental. (This will be borne out in the Discussion. It will be seen that the value of w for carboxyl groups becomes essentially zero when proper account is taken of the expected difference in pK between aspartyl and glutamyl carboxyl groups.)

If w is to be considered as an empirical parameter,[5] it may reflect any of a number of phenomena. Its nonzero value could result from local electrostatic interactions (with charges on adjacent residues, for example), which affect some or all carboxyl groups, and some or all phenolic groups, but which do not influence lysyl amino groups. On the other hand, it could equally well result from causes which should properly be considered as affecting pK_{int} values, *i.e.*, arise from factors other than interactions between titratable groups.[5,8]

It thus becomes of interest to analyze the titration data in a different way. We shall assume for this analysis that $w = 0$ for all groups and ask what is the minimum range in $pK_{int}^{(i)}$ which must be assigned to groups commonly considered as identical, in order to account for the experimental data. The result of such an analysis is shown in Table I. It is found necessary, in order to describe the data of Figure 2, to consider the phenolic groups as belonging to two different classes. To describe the acidic branch of the titration curve, it is necessary to consider the side chain carboxyl group as belonging to two different classes. No other diversity need be introduced, however. (The anomalous dissociation of a proton as a result of the β-elimination reaction could be *formally* described in terms of the presence of a single group with $pK_{int} = 11.05$, but this would have no physical significance.)

The theoretical curves based on the parameters of Table I are the curves through the experimental data of Figures 1 and 2. The maximum deviation between these curves and the experimental data is the deviation of 0.2 group which necessarily occurs at the acid end point, no matter what theoretical curve is used, because the experimental points extrapolate to a maximum value of 19.2 for $\bar{Z}_H$, rather than the theoretical value of 19.0. Elsewhere (*i.e.*, above pH 2.75) the maximum deviation between calculated and average experimental $\bar{Z}_H$ values is 0.1 group, compared to a maximum deviation of 0.2 group between individual experimental runs.

(28) C. Tanford, "Physical Chemistry of Macromolecules," John Wiley and Sons, Inc., New York, N. Y., 1961, Chapters 7 and 8.

Table I. Formal Description of the Titration Curve without Interactions between Groups

Class of group	No. per molecule, anal	Titration curve — No. per molecule	Titration curve — pK_{int}
α-Carboxyl	1	1	3.4
β,γ-Carboxyl	10	5	3.8
		5	4.3
Imidazole	4	4	6.5
α-Amino	1	1	7.6
Phenolic	6	3	9.75
		3	10.15
ε-Amino	10	10	10.35
Guanidyl	4	4	>12.5
Anomalous	...	~1	*a*

[a] It is possible to fit the entire titration curve formally by assigning a pK_{int} of 11.05 to the anomalous group.

Discussion

It is convenient to consider the titration curve of ribonuclease in terms of the equation

$$\bar{Z}_H = (\bar{Z}_H)_{max} - \sum_i n_i x_i \qquad (3)$$

in which $\bar{Z}_H$ is the protonic charge at any pH, $(\bar{Z}_H)_{max}$ is the maximum value which $\bar{Z}_H$ attains (*i.e.*, +19), n_i represents the number of titratable groups which can be assigned a given $pK_{int}^{(i)}$, and x_i is the degree of dissociation of each group of that type at any pH. The number of classes of groups of type i which must be used to describe the curve is one of the adjustable parameters.

In an ideal random coil, without interactions between the constituent acidic and basic groups, the classes of groups would correspond to the kinds of chemically distinct titratable groups, and each n_i would correspond to the number of such groups as given by amino acid analysis. The values of x_i could be determined by eq 1 (*i.e.*, eq 2 with $w = 0$), and the $pK_{int}^{(i)}$ values would be those expected on the basis of model compound studies.

We have seen that this ideal behavior is not quite attained by ribonuclease in 6 *M* GuHCl solution. It is thus necessary to consider the deviations from limiting ideal behavior in some detail, to see whether they can be accounted for quantitatively in terms of properties of a random coil, or whether the deviations are too large for such an explanation and require abandonment or modification of the conclusion reached on the basis of hydrodynamic studies[3] that proteins in 6 *M* GuHCl, even with disulfide bonds intact, retain no elements of ordered structure.

The first question to be considered concerns the side chain carboxyl groups. Rigorously, they fall into two classes, aspartyl and glutamyl groups, but these two classes are customarily treated as a single class in the interpretation of titration curves of native proteins,[5] because the difference in intrinsic p*K* between the two kinds of groups has been believed to be small, so that it would be overshadowed by the effects of interactions of the groups. Since even small differences in pK_{int} are important in the interpretation of the present curve, we have reexamined the question of the difference in p*K* between aspartyl and glutamyl carboxyl groups. This work will be described in another paper.[29] The result obtained was quite surprising. It indicates that aspartyl and glutamyl groups should differ quite significantly, and, moreover, that the intrinsic p*K*'s are probably slightly smaller than the values normally assigned. The best values in water are probably $pK_{int} = 4.05 \pm 0.1$ for aspartyl groups and $pK_{int} = 4.5 \pm 0.1$ for glutamyl groups, at 25°. As p*K*'s for carboxyl groups in 6 *M* GuHCl are lower by about 0.1 to 0.2 than p*K*'s in water,[4] the expected pK_{int} values in 6 *M* GuHCl become about 3.9 and 4.35, respectively.

These estimates (which cannot be considered as reliable to better than 0.1) are in remarkable agreement with the $pK_{int}^{(i)}$ values which were obtained empirically (see Table I) and used in drawing the calculated curve of Figure 1. Since ribonuclease in fact possesses five aspartyl and five glutamyl side chains, it is evident that the expected difference in $pK_{int}^{(i)}$ between these two kinds of carboxyl groups accounts quantitatively for the deviations from limiting ideal behavior which appear to be present when the two kinds of groups are assumed to have the same p*K*. (Although the difference between the pK_{int} values of Table I, 3.8 and 4.3, respectively, and the foregoing estimates, 3.9 and 4.35, respectively, is within the uncertainty of the estimates, differences of this order of magnitude are in fact to be expected on the basis of electrostatic interactions, as will be explained below.)

The other p*K*'s listed in Table I are also essentially identical with the expected values. The best values for the intrinsic p*K*'s of imidazole, α-amino, and ε-amino groups, in dilute salt solution, are 6.4, 7.7–7.8, and 10.4, respectively.[5] To judge from the p*K* values of imidazole and *n*-butylamine in 6 *M* GuHCl (Table IV of the preceding paper[4]), the difference in p*K* between basic groups in 6 *M* GuHCl and in dilute aqueous salt solution should be negligibly small.

Thus the only real discrepancy between the experimental data of Figures 1 and 2 and the result expected on the basis of the limiting ideal behavior of a random coil without any interactions lies in the higher than expected p*K* which must be assigned to three of the six phenolic groups. (The expected pK_{int} is close to 9.8.) It is possible that this result may be due to the expected thermodynamic nonideality of randomly coiled polypeptide chains. As was mentioned in paper I of this series,[3] aqueous GuHCl solutions are likely to be poor solvents for hydrophobic groups. Hydrophobic groups (especially on adjacent residues) are therefore likely to prefer contacts with each other to contacts with solvent molecules, and the average environment of titratable groups on side chains which are predominantly hydrophobic may thus be significantly more nonpolar than the environment of the same group on an amino acid molecule in 6 *M* GuHCl solution. Since tyrosyl side chains are among the most hydrophobic of protein side chains,[30] they may be particularly prone to be affected in this way. The result of the effect would be to increase pK_{int}.

Examination of the amino acid sequence of ribonuclease[16] shows that two of the tyrosyl groups (no. 25 and 73) are adjacent to disulfide bonds, and a third (no. 115) occurs in the sequence Pro-Tyr-Val-Pro-Val. These three groups could well have a slightly higher than normal p*K* as a result of their environment.[31]

(29) C. Tanford and Y. Nozaki, paper in preparation.

(30) C. Tanford, *J. Am. Chem. Soc.*, 84, 4240 (1962).

Electrostatic Interactions. Electrostatic interactions between charged acidic and basic groups can be important even on randomly coiled polypeptide chains if the interacting charges are close together in the primary sequence of amino acids.[28,32] A high concentration of an electrolyte should greatly diminish these interactions. We have shown[4] however that 6 *M* GuHCl does not entirely abolish the interactions between side chain charges and the charges of α-amino and α-carboxyl groups of amino acid molecules. The removal of the positive charge from the α-amino group of tyrosine, for example, was shown to alter the pK of the phenolic group by 0.4. Similarly, the interaction between the α-amino group and the side chain ϵ-amino group of lysine was estimated as leading to a change in pK of 0.26. Electrostatic effects can therefore not be dismissed entirely in the analysis of the titration curve of ribonuclease in 6 *M* GuHCl. Interaction between charges on adjacent residues at least should be considered, though those with charges on more distant residues can probably be safely neglected.

It is not possible to estimate quantitatively the ΔpK which such interactions can produce, because this will depend on how effectively the ions of GuHCl can shield the charges from each other. Certainly, the effect of a charge on a neighboring residue will be less than the effect of a charge on an α-amino or α-carboxyl group in the parent amino acid. However, effects leading to changes in pK of the order of 0.1 would not be surprising.

If we examine the locations of the aspartyl and glutamyl side chains in the primary structure of ribonuclease,[16] we see that five of them (residues 2, 9, 38, 49, and 86) are adjacent to residues which bear charges. In each case the charge is positive. In each case the pK of the charged group is well outside the titration range of carboxyl group, *i.e.*, the charges are fixed and will exert the same electrostatic effect at all stages of titration of the pertinent carboxyl groups. Mathematically, therefore, the electrostatic interaction can be taken into account by using eq 1 for the titration with an effective pK_{int} which differs from the true intrinsic pK. In other words, for half the carboxyl groups, the pK_{int} values required to fit the experimental data can be expected to be lower than the true intrinsic pK's by about 0.1. This difference is clearly within the uncertainty with which true intrinsic pK's can be estimated. It should be noted, however, that the pK_{int} values given for aspartyl and glutamyl residues in Table I are 0.1 and 0.05, respectively, below the initially estimated values, and this difference may well be a reflection of the weak electrostatic effects here discussed. Part of the pK spread observed for phenolic groups may also be due to electrostatic effects, since two tyrosine residues (no. 92 and 97) are adjacent to lysine residues.

We have considered the influence of such interactions in some detail, to assure ourselves that they lead to no inconsistencies with the experimental data. For example, it should be noted that only one of the carboxyl groups affected is an aspartyl residue, and it (residue 38) is adjacent to two positive charges. Four of the glutamyl residues are affected, one of them (residue 2) being adjacent to two positive charges, and three (residues 9, 49, and 86) to single charges. It might therefore be expected that one of the five aspartyl groups should have an *effective* pK_{int} which is about 0.2 less than the true pK_{int} for aspartyl groups. One of the glutamyl groups should have an effective pK_{int} about 0.2 less than the true pK_{int} for glutamyl groups, and three should have an effective pK_{int} about 0.1 less than that figure. A best fit of the data is obtained if the true intrinsic pK's are set equal to 3.87 and 4.35, respectively (initial estimates were 3.9 and 4.35), leading to one group with pK_{int} = 3.67, four with pK_{int} = 3.87, one with pK_{int} = 4.15, three with pK_{int} = 4.25, and one with pK_{int} = 4.35. The calculated curve based on these pK values turns out to be indistinguishable from the calculated curve based on five groups with pK_{int} = 3.8 and five with pK_{int} = 4.3. A similar result was obtained for the entire titration curve. The expected electrostatic effects are no larger than the uncertainties in the true intrinsic pK values, and the experimental data can be fitted with an elaborate theoretical curve which takes such interactions into account, as well as (but not significantly better than) they can be fitted with the simpler theoretical curve based on the parameters of Table I.

Appearance of Anomalous Titratable Groups. It was pointed out in the introduction that existing titration data for lysozyme and ovalbumin suggest the possibility that GuHCl may react with proteins in acid solutions so as to produce new titratable groups other than the usual acidic and basic groups of protein molecules. No indication of such a phenomenon could be observed in the present study with ribonuclease. Since proteins in concentrated GuHCl behave essentially as random coils, which means that individual amino acid residues behave essentially as independent entities, reactions specific to individual proteins are not expected to occur. The titration data of lysozyme and ovalbumin which suggest the presence of anomalous groups may thus be artifactual. Such artifacts could arise from impurities known to be present in GuHCl which has not been carefully purified. However, artifacts of this kind should not contribute to the titration curves of proteins if proper blank titrations of the solvent are carried out. An alternative possibility is that the anomalies observed in lysozyme and ovalbumin arise from an effect of GuHCl on tryptophan residues, as this amino acid is not present in ribonuclease. However, no anomalies were observed in the titration of tryptophan itself, as reported in the preceding paper.[4] We plan to reinvestigate the titration of lysozyme in the near future to resolve the difficulty raised by these apparently conflicting results.

Although we did not observe the appearance of anomalous titratable groups in acid solutions, we did observe the anomalous dissociation of a proton in alkaline solution. It was shown to arise from the β-elimination reaction of disulfide bonds. Such a reaction is expected to occur in a randomly coiled polypeptide chain in which the disulfide bonds are readily accessible to OH^- ions.

Conclusion. It is evident that the titration data presented in this paper are compatible with the conclusion reached earlier, on the basis of viscosity and sedimenta-

(31) The parameters given in Table I do not, of course, constitute a unique description of the course of titration of the phenolic groups. For example, two groups each, with pK_{int} = 9.75, 9.95, and 10.15, would give essentially the same calculated curve as the one drawn in Figure 2.

(32) S. A. Rice and M. Nagasawa, "Polyelectrolyte Solutions," Academic Press Inc., New York, N. Y., 1961, Chapter 7.

tion studies.[3] Ribonuclease dissolved in 6 *M* GuHCl has been shown to possess the titration behavior expected of a randomly coiled polypeptide chain, without important noncovalent interactions. Twenty-nine of the 32 titratable groups are titrated exactly in accord with the limiting ideal curve, based on expected p*K* values and an absence of any interactions between titratable groups. Some phenolic groups have a somewhat higher p*K* than the others, but the difference is no greater than 0.4 p*K* unit, and there is no difficulty in visualizing weak interactions, which could remain in a random structure, to account for it. One anomalous group was titrated per molecule, but was shown to represent a chemical reaction which is probably common to most unfolded proteins which retain intact disulfide bonds.

A Nuclear Magnetic Resonance Study of the Reversible Hydration of Aliphatic Aldehydes and Ketones. I. Oxygen-17 and Proton Spectra and Equilibrium Constants

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Abstract: Oxygen-17 nuclear magnetic resonance spectra of aqueous solutions of a number of aliphatic carbonyl compounds enriched in ^{17}O are reported. It is shown that the resonances of the unhydrated carbonyl species and of the hydrated *gem*-diol species fall in two different regions. The carbonyl oxygen resonance falls in the range -520 to -560 ppm (relative to $H_2{}^{17}O$), and that of the *gem*-diol falls close to the water line, often being concealed by it. Proton magnetic resonance spectra have also been studied and used to determine equilibrium constants, K_d, for the hydration–dehydration reaction of the carbonyl group. It is shown that a good correlation between K_d values, which span a range of seven orders of magnitude, and $\Sigma\sigma^*$ is obtained provided account is taken of the number of aldehydic protons in the molecule. The following free-energy relationship between K_d and $\Sigma\sigma^*$ is proposed, $-\log K_d = \rho^*\Sigma\sigma^* + B\Delta + C$, where ρ^*, B, and C are constants, and Δ is the number of aldehydic hydrogens in the molecule. A best-fit analysis gave $\rho^* = 1.70 \pm 0.07$, $B = 2.03 \pm 0.10$, and $C = -2.81 \pm 0.13$. These results are discussed in terms of adjacent bond interaction.

The reversible hydration–dehydration (reaction 1) of carbonyl compounds in aqueous solutions has, because of its great chemical importance, been the subject of a great number of scientific publications.[1]

$$R_1R_2C(OH)_2 \rightleftharpoons R_1R_2C{=}O + H_2O \qquad (1)$$

This reaction represents one of the simplest addition reactions to the carbonyl group and is of great importance in understanding many organic reactions. Previous studies have shown that the extent of hydration varies over a wide range and depends strongly on the nature of the substituents directly bonded to the carbonyl group. Equilibrium constants[1]

$$K_d = [R_1R_2C{=}O]/[R_1R_2C(OH)_2] \qquad (2)$$

ranging from 10^{-4} to 10^{3} for simple aliphatic carbonyl compounds have been reported. The rates of the hydration–dehydration reaction are usually too fast to allow the determination of K_d by isolating one of the compounds, and it is therefore necessary to apply methods which do not perturb the equilibrium. Moreover, in many cases the equilibrium concentration of one of the components may be so small as to be detectable only by very sensitive spectroscopic methods. In ref 1, the various methods of studying reaction 1, the results obtained, and their significance are thoroughly reviewed. The most common methods to determine K_d are ultraviolet spectroscopy and proton magnetic resonance (pmr), although in a few cases other methods have been employed.

The main features of the pmr spectra of aqueous solutions containing a mixture of free and hydrated carbonyl compounds have been described by several authors.[2-5] When the equilibrium constant, K_d, is between $\sim 10^{-2}$ to $\sim 10^{+2}$, it is usually possible to observe two sets of lines corresponding to the hydrated and unhydrated species. Regularities in the chemical shifts of the aldehydic and α-protons of these two species have been observed. It is found that the resonance due to the protons bonded to the *gem*-diol carbon atom in the hydrated species is shifted 4.6 to 5.0 ppm upfield relative to the aldehydic hydrogen in the unhydrated form. Similarly, α-protons in both hydrated aldehydes and ketones are found to be shifted $+0.7$ to $+0.9$ ppm relative to the corresponding unhydrated compounds. β-Protons are also found to shift upon

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