tion is involved since the isotope exerts an effect whether it is close to the point of attachment of the amino acid to the resin or at a distance. The only requirement is that it be adjacent to a charged atom.

It therefore appears certain that the isotope exerts its effect on the ionic state of the molecule. The possibility of an inductive effect owing to a shift of electrons away from the heavier isotope as compared to the lighter atom might be considered. This would require that C^{14} be less electronegative than C^{12}. The result would be that in the 2-position of an α-amino acid, the heavier isotope would tend to stabilize the adjacent ammonium group and thereby increase the statistical chances of attachment to the sulfonate groups of the resin. In the 1-position the isotope would tend to increase the chances of the carboxylate group being protonated and thereby decrease the average repulsive force between this group and the resin. In the case of a carboxylate group located further from the ammonium group, such as the 4-position in aspartic acid, the effect would be less since at the *p*H of the eluting buffer this group would be largely protonated and its importance as a repulsive group would be less. In addition, its greater separation from the point of attachment might be expected to decrease its contribution to the repulsive forces.

This inductive effect can be considered more simply, but less mechanistically, as a decrease in acidity of either a carboxyl or ammonium group which is adjacent to the C^{14} atom. The more basic ionic species travel more slowly on a cation-exchange resin.

All of the data fit this suggested explanation. However, there is apparently no independent evidence in support of the necessary requirement that C^{14} be less electronegative than C^{12}. At the same time it must be remembered that a difference too small to be observed by most methods might be sufficient to explain the observed isotope effect. The effect is small and ion-exchange methods provide an extremely sensitive procedure for observing small differences.

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

Hydrogen Ion Equilibria of Ribonuclease[1]

BY CHARLES TANFORD AND JACK D. HAUENSTEIN[2]

RECEIVED APRIL 23, 1956

The dissociation of hydrogen ions from crystalline ribonuclease has been studied at 25° at three ionic strengths. The number of dissociating groups of any one kind, *i.e.*, carboxyl, imidazole, amino, guanidyl, etc., agrees precisely with the known amino acid content of the molecule. The *pK* values suggest that the dissociating groups are located at the surface of the molecule, except that three phenolic groups may be buried in the interior, as previously reported. About half the carboxyl groups appear formally to have an abnormally low *pK*. The possible structural significance of this is discussed in light of the structure of ribonuclease proposed by Hirs, Moore and Stein and by comparison with other proteins.

This paper reports a study of hydrogen ion titration curves of ribonuclease and their interpretation in terms of the intrinsic dissociation tendencies of the various types of dissociating groups and in terms of the electrostatic effect of the protein charges. Previous studies of a similar nature have been made for ovalbumin,[3] β-lactoglobulin,[4] human serum albumin,[5] bovine serum albumin,[6] insulin[7] and lysozyme.[8] The experimental technique and underlying theory have been previously summarized.[9]

Experimental

The ribonuclease used in this study was lot 381-059, purchased from Armour and Co. Chromatographic analysis has shown that this particular lot consists of one principal and two minor components. The minor component present in larger amount appears to differ from the principal component only in having a single additional free carboxyl group.[10]

Isoionic, salt-free stock solutions of the protein were prepared by passing a solution of the protein down an ion exchange column.[11] Solutions for measurement were prepared by weight from such stock solutions by addition of standard HCl, KOH and KCl. Conductivity water was used throughout. The final protein concentration was usually about 0.5%. In some solutions a 2% concentration was used, with no apparent difference.

Most of the *p*H measurements were made using the precision potentiometer on a Beckman Model GS *p*H meter. The scale, rated at 0.200 mv. per division, was calibrated and shown to correspond to 0.1992 mv. per division. The full scale of the potentiometer enables one to measure a potential difference corresponding to about 3.3 *p*H units. By means of this scale we evaluated accurately the *p*H of two commercial buffers (*p*H 7 and *p*H 10), comparing them with Bureau of Standards potassium hydrogen phthalate (*p*H 4.005 at 25°), which was used as primary standard. This provided standard solutions lying within the desired range of 3.3 *p*H units of any *p*H between 1 and 13.

Some of the measurements at ionic strength 0.03 and 0.15, near the isoionic point, were made by continuous titration (under nitrogen) in a beaker, with volume addition of acid or base. Most of these are not shown in Fig. 1, for lack of space. There was no indication that data obtained in this way differed from those evaluated in the usual manner. (One would expect a lack of precision by this method only as one moves far from the isoionic point, and at low ionic

(1) Presented at the 129th National Meeting of the American Chemical Society, Dallas, Texas, April, 1956.

(2) Abstracted in part from the Ph.D. thesis of Jack D. Hauenstein, State University of Iowa, August, 1955.

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

(4) R. K. Cannan, A. H. Palmer and A. C. Kibrick, *J. Biol. Chem.*, **142**, 803 (1942).

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

(6) C. Tanford, S. A. Swanson and W. S. Shore, *ibid.*, **77**, 6414 (1955).

(7) C. Tanford and J. Epstein, *ibid.*, **76**, 2163, 2170 (1954).

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

(9) C. Tanford in T. Shedlovsky, ed., "Electrochemistry in Biology and Medicine," John Wiley and Sons, Inc., New York, N. Y., 1955.

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

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

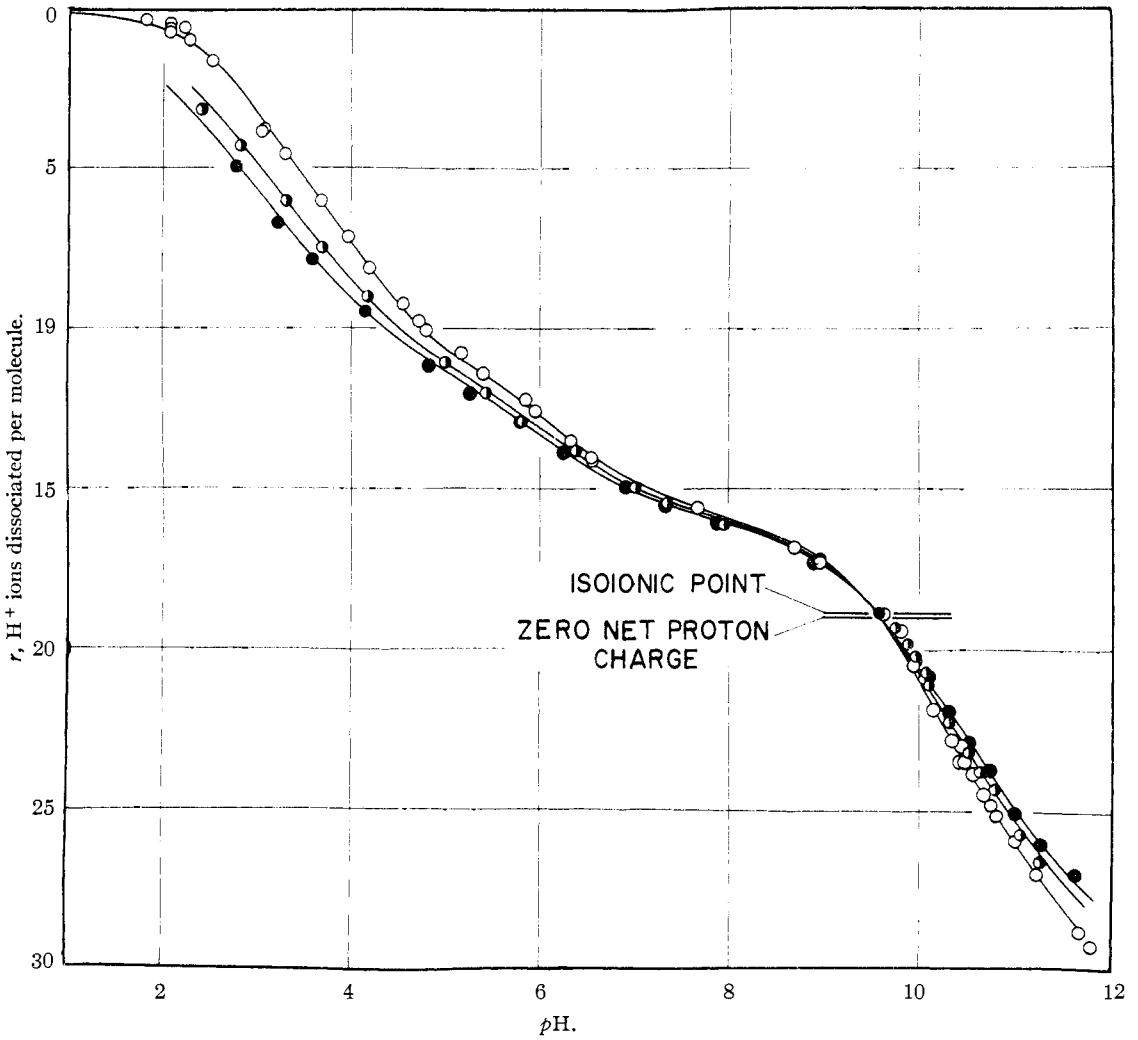

Fig. 1.—Titration data at 25°, at ionic strengths 0.01 (●), 0.03 (◑) and 0.15 (○). The curves are calculated, using the parameters of Table I and others discussed in the text.

strength, where the liquid junction resulting from use of a dipping calomel electrode becomes unsatisfactory.)

Results

Data were obtained at 25° at three ionic strengths, 0.01, 0.03 and 0.15. Detailed tabulation of the results is given in the Ph.D. thesis of J. D. Hauenstein.[2] Plots of the data are shown in Fig. 1. The results are all based on a molecular weight of 13,683, which is calculated from the complete amino acid content determined by Hirs, Moore and Stein.[12,13]

Figure 2 contains data designed to test the reversibility of the titration. Solutions exposed to *p*H 1.8 or to *p*H 10.8 before being adjusted to their final *p*H are seen to fall, within experimental error, on the direct titration curve, showing that between *p*H 1.8 and 10.8, and presumably up to *p*H 11.5, the titration is reversible. Above *p*H 11.5 solutions decrease in *p*H on standing, and, furthermore, exposure to a *p*H above 11.5 causes dissociation of hydrogen ions which, on reversal, recombine with the protein at lower *p*H. There is quantitative agreement between this observation and the irreversible titration of three of the phenolic groups of ribonuclease reported in an earlier paper.[14]

Isoionic Point.—The protein stock solution which is obtained after passage down the ion-exchange column is, by definition,[15] isoionic for it contains no non-colloidal ions other than H^+ or OH^-. The *p*H of a 0.5% solution of isoionic protein in aqueous KCl is 9.604 (μ = 0.001) and it increases with ionic strength to 9.658 (μ = 1.0). This is probably a purely electrostatic effect[16] and not an indication of chloride ion binding (see below). In view of this high isoionic *p*H, and the corresponding excess of

(12) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **219**, 623 (1956). We are grateful to these authors for making the results of this paper, and that cited in ref. 24, available to us in advance of publication.

(13) Our results were originally based on a molecular weight of 13,895, as quoted in earlier work by Hirs, Moore and Stein, *ibid.*, **211**, 941 (1954). If reference is made to the tabulation in Hauenstein's thesis,[2] a correction must be applied.

(14) C. Tanford, J. D. Hauenstein and D. G. Rands, THIS JOURNAL, **77**, 6409 (1955).

(15) For a discussion see R. A. Alberty, in H. Neurath and K. Bailey, ed., "The Proteins," Vol. 1, Academic Press, Inc., New York, N. Y., 1953, p. 477.

(16) Equation of A. Brown, as discussed by G. Scatchard and E. S. Black, *J. Phys. Colloid Chem.*, **53**, 88 (1949).

hydroxyl ions in the solution, there must be a small positive charge on the isoionic protein, *i.e.*, the point of zero net proton charge is at a slightly higher *p*H as indicated in Fig. 1.

In the absence of specific binding of chloride or potassium ion, the point of zero net proton charge should be identical with the isoelectric point (*i.e.*, zero net total charge). Anderson and Alberty[17] have obtained an isoelectric point of 9.45 in good agreement with this prediction: especially so since variation in the proportion of the components of ribonuclease may vary the isoelectric point a few tenths of a *p*H unit.[10]

Chloride Binding.—Some experiments performed in this laboratory by R. W. Monsul, using silver–silver chloride electrodes, showed that no chloride is bound by ribonuclease above *p*H 7, but indicated that some binding occurs acid to *p*H 7, up to about 5 chloride ions per molecule at *p*H 2. The electrodes, however, behaved poorly and we believe that Monsul's result arises from some sort of interaction between the protein and the electrode surface.[18] If one calculates the chloride binding from the ionic strength dependence of the titration curve, using the method of Scatchard and Black,[16] one obtains the result that none is bound, even at the lowest *p*H reached, and we have accepted this result as correct.[19]

Maximum Acid Binding.—The most acid points of the titration curve at $\mu = 0.15$ extrapolate reasonably to a maximum proton uptake, reckoned from the point of zero net proton charge, of 19.1 protons per molecule, in excellent agreement with the analytical figure[12] of 19.0 basic nitrogen groups per molecule. It is of interest that if a molecular weight of 13,895[13] is assumed, the maximum acid binding becomes 19.4 protons per molecule, with a probable error considerably less than 0.4. The present data thus provide confirmation of Hirs, Stein and Moore's revised value of the molecular weight,[12] since a non-integral value for the maximum acid binding is not acceptable.

Calculated Results

Since the titration of ribonuclease is reversible up to *p*H 11.5 the results may be treated thermodynamically, as previously described.[9] It is supposed that the ionizable groups of the protein occur in sets, all members of each set being more or less randomly distributed over the exterior of the protein molecule. The degree of dissociation of the groups in any set at a given *p*H and total charge Z is then given by x_i, where

$$pH - \log \frac{x_i}{1 - x_i} = (pK_{int})_i - 0868wZ \qquad (1)$$

Here $(pK_{int})_i$ is the negative logarithm of the intrinsic dissociation constant of the groups in a given set and $2kTwZ$ is the work required to remove a proton (after dissociation) from the protein molecule to infinity. If there are n_i groups in each set, then the ordinate of the titration curve, the number of hydrogen ions dissociated per protein molecule, is given as

$$r = \sum_i n_i x_i$$

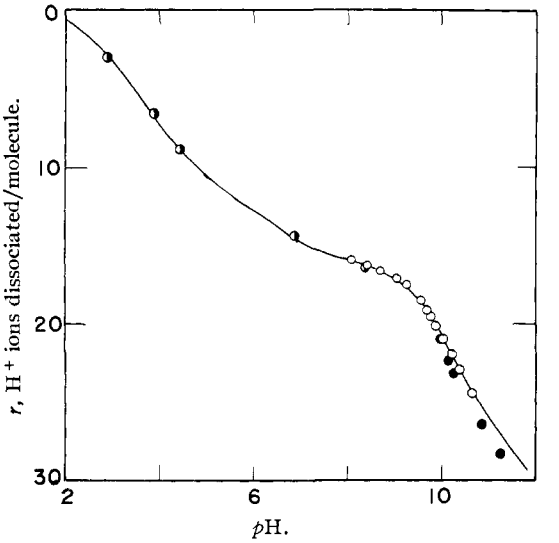

Fig. 2.—Reversibility study at ionic strength 0.15. The curve is the experimental curve through the points of Fig. 1 at the same ionic strength. It differs slightly from the calculated curve of Fig. 1 between *p*H 9.5 and 10.0. The experimental points were obtained after exposure to *p*H 1.8 (◑). *p*H 10.8 (○) and *p*H 12.2 (●).

The value of w depends upon ionic strength and temperature, and upon the solvation, shape and possible aggregation or dissociation of the protein molecule. In the case of ribonuclease, light scattering and viscosity measurements have been performed in this Laboratory and will be published in the near future. They indicate that no change in molecular weight or in shape or solvation occurs between *p*H 2 and 11. The value of w at each ionic strength should then be a constant independent of *p*H, *i.e.*, the values found should correspond closely to those previously reported for the phenolic groups.[14]

In view of this expectation, the most interesting result of the mathematical analysis is that apparently high values of w are obtained when a plot according to equation 1 is made for the carboxyl groups, as shown in Fig. 3, the dashed lines of which show the slopes to be expected on the basis of the values of w obtained from our study of the phenolic groups. Formally, there are two ways to account for this observation, excluding the possibility of a change in shape or solvation and maintaining the form of equation 1.

(1) The value of w in equation 1 may actually be different in the *p*H region where carboxyl groups are titrated. This could happen, for example, if the carboxyl groups were located below the surface of the protein molecule, or if they were all bunched together at one end. Such an arrangement would

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

(18) We have had other indications that ribonuclease may interact with solid surfaces, *e.g.*, viscometers which have been used for ribonuclease are much more difficult to clean than those used for serum albumin.

(19) If the results obtained from e.m.f. measurements are accepted as reflecting chloride binding, then one gets the following curious result: the logarithmic plot for carboxyl groups (Fig. 3) becomes extremely steep, suggesting very strong electrostatic interaction, but it also becomes independent of ionic strength, indicative of no electrostatic interaction at all.

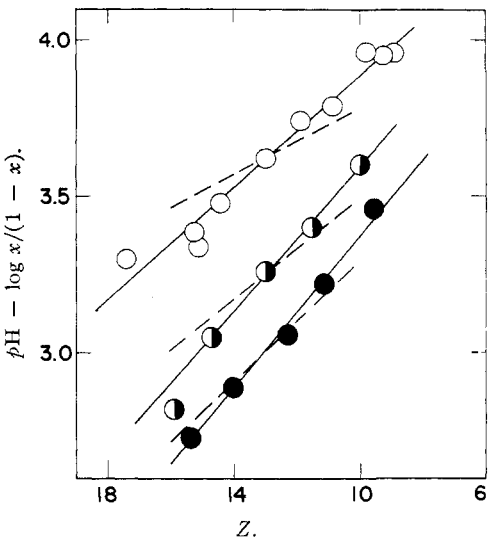

Fig. 3.—Data for the carboxyl groups, plotted according to equation 1. Ionic strength is 0.15 for the top curve, 0.03 for the middle one, and 0.01 for the lowest one. The solid lines are the least squares lines through the points. The dotted lines show the slopes to be expected on the basis of the probably correct value for the factor w. The lowest point at ionic strength 0.03 was not included in the least squares calculation. It appears to deviate from the rest both here and in Fig. 1.

result in a higher change in electrostatic free energy per proton added. However, it would result in a *proportionately* higher value of w at each ionic strength, whereas in fact the difference between observed and expected values is the same at each ionic strength. Also, if the carboxyl groups were located below the surface, an abnormally *high* pK_{int} should be observed,[20] whereas, in fact, a calculation within the present assumption leads to a low pK_{int}.[21]

(2) The intrinsic pK may not be the same for all the carboxyl groups, *i.e.*, the carboxyl groups may fall into two or more sets, rather than a single set. Using the "phenolic" values of w, this assumption was found to lead to complete agreement with experiment if the carboxyl groups were assumed about equally divided between two sets, one with pK_{int} near 4.0, the other with pK_{int} near 4.7. The calculated curves of Fig. 1 were based on the present assumption and it is especially noteworthy that they reproduce accurately the ionic strength dependence of the acid portion of the titration curve.

It should be noted that this difference between carboxyl groups may well be a formal result of the form in which equation 1 is written. For the electrostatic term ($0.868wZ$) of this equation allows the inclusion of only those electrostatic effects which vary with the *total charge*. Electrostatic effects independent of the total charge (in the pH region being considered) must appear formally in pK_{int}.

(20) J. A. Schellman, *J. Phys. Chem.*, **57**, 472 (1953).

(21) A calculation of pK_{int} may be made by extrapolating to zero carboxyl group charge, *i.e.*, to $Z = 19$, using the slopes of Fig. 3, and then correcting for the positive charge by the values of w previously found for the phenolic groups. These or slightly lower values of w are also applicable to the titration of imidazole and ϵ-amino groups. The result of the calculation at each ionic strength is $pK_{int} = 4.1$.

In fact, the most reasonable explanation of the result obtained is that it is an electrostatic effect, *i.e.*, that about half the carboxyl groups are forced into close contact with centers of positive charge and thus subjected to a force increasing dissociation, but independent of Z.

It has not been possible to find other reasonable explanations of the course of titration of the carboxyl groups. For example, models involving reasonable hydrogen bonds will not fit the data.

The remainder of the titration curve does not present any startling problem. Empirical values for w can be obtained for the ϵ-amino groups from the data between pH 10 and 11.5. These values are somewhat smaller than the "phenolic" values of w, possibly because the ϵ-amino groups extend a little further from the protein surface than other groups. The difference is quite small, however. No difficulty was found in fitting the central region of the curve, pH 5 to 9, with the "phenolic" values of w.

A complete summary of all calculated results is shown in Table I. The numbers of the various types of groups are in agreement with amino acid analysis. The intrinsic pK's are within the range expected for groups lying at the surface, in contact with solvent, except in so far as the carboxyl groups are concerned, about half of these having an unusually low pK, if explanation (2) given above for their titration behavior is accepted.

TABLE I

SUMMARY OF NUMERICAL RESULTS

	Number of groups: Found by titration	Number of groups: Predicted by amino acid content	Intrinsic pK: Obsd.	Intrinsic pK: Normal value[d]
α-Carboxyl	(1)[a]	1	(3.75)[a]	3.75
β,γ-Carboxyl	10.2[b]	10.2[b]	See text	4.6
Imidazole	4	4	6.5	6.5–7.0
α-Amino	(1)[a]	1	7.8	7.8
ϵ-Amino	10	10	10.2	10.1–10.6
Phenolic[c]	3[c] + 3[c]	6	9.95[c]; Inaccessible[c]	9.6
Guanidyl	4	4	$\geqslant 12$	>12

[a] The values in parentheses were assumed. [b] The number of free carboxyl groups may be obtained with an accuracy of about 0.3. The "best" value here given was not adjusted to agree with the amino acid analysis. At the time it was chosen, amino acid analysis was believed to lead to 11.2 rather than 10.2 free carboxyl groups. The 0.2 in the predicted value is due to the presence of more than one component in the ribonuclease used. [c] *Cf.* ref. 14. [d] The normal values have been discussed in several of our preceding papers.

The curves drawn through the data of Fig. 1 are curves computed with the constants of Table I, and the "phenolic" values of w, *i.e.*, 0.112, 0.093 and 0.061, respectively, at $\mu = 0.01$, 0.03 and 0.15. There is a slight discrepancy between calculation and observation between pH 9.6 and 10.0, which region includes the isoionic point. No simple way can be found of accounting for this. Mathematically, it could be accounted for if there were a double hydrogen bond[22] between pairs of $-NH_3^+$ groups, but such a bond is clearly impossible. Possibly some reversible surface rearrangement occurs near

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

the isoionic point. If the ϵ-amino groups, like the carboxyl groups, fell into two classes, the discrepancy would be in the opposite direction.

Discussion

This study shows ribonuclease to be a compactly folded protein with no drastic changes in configuration between pH 1.8 and 11.5. Three of the phenolic groups, as previously discussed,[14] appear to lie in the interior of the folded protein molecule. All of the other dissociable groups must lie at the surface, and all, except about half the carboxyl groups, must have an essentially neutral environment. About half the carboxyl groups have an abnormally low pK, an observation most simply explained if they are forced into close contact with centers of positive charge.[23]

With this behavior of the carboxyl groups in mind, it is of interest to examine the partial amino acid sequence of ribonuclease obtained by Moore, Hirs, and Stein.[24] Two of the free carboxyl groups in their proposed structure immediately stand out: the glutamic acid group at position no. 2 in the chain is adjacent to a terminal lysine group, containing, in the range of pH in which the carboxyl groups are titrated, two positive charges; and the aspartic acid group at position no. 38, in peptide O-Tryp 7, lies between a lysine and an arginine residue, both with positive charges. These carboxyl groups would almost certainly be expected to have a lower intrinsic pK than normal carboxyl groups. One other carboxyl group (No. 9) lies adjacent to a single positive charge, and three others may lie adjacent to a single positive charge (the amino acid sequence is not completely determined at their locations); five carboxyl groups definitely cannot be near positive charges, except as a result of folding of the peptide chain. The amino acid sequence thus shows that the explanation offered for the anomalously flat titration curve on the acid side is a reasonable one.

Comparing the titration of the carboxyl groups of other proteins with that in ribonuclease we find in one, lysozyme,[8] even more markedly anomalous behavior. In this protein the plots corresponding to Fig. 3 of the present study are at least three times as steep as for ribonuclease, while the expected slopes are about equal. In lysozyme there is definite evidence for chloride binding, both e.m.f. measurements and calculations by the method of Scatchard and Black giving results of the same order of magnitude. Sufficiently precise numerical values for chloride binding have not been obtained to make possible a meaningful attempt to evaluate pK's of the carboxyl groups.

In no other protein has evidence been found suggesting that the carboxyl groups are not all equivalent. In insulin,[7] for example, a reasonable account of the acid side of the titration curve could be given in terms of the known association–dissociation equilibrium of this protein, assuming all of the β- and γ-carboxyl groups to have an intrinsic pK of 4.7. The amino acid sequence of this protein[25] shows no carboxyl groups conspicuously close to two centers of positive charge, and only one adjacent to even a single positive charge.

In the serum albumins[5,6] all of the carboxyl groups have a low intrinsic pK (about 4.0). Thus one might suppose that all of its carboxyl groups lie adjacent to centers of positive charge. In contrast to ribonuclease, however, the titration of both carboxyl and amino groups (both in the direction of their uncharged forms) is accompanied by expansion of the molecule. We were therefore led to suggest[6] that the alignment of these groups near one another is not the result of the amino acid sequence, but arises instead from the folding of the polypeptide chain, which in turn was thought to be the result of attractive forces between COO^- side chains and side chains carrying positively charged nitrogen atoms. The effect of high ionic strength and of urea[26] is to increase the intrinsic pK, in support of the explanation offered.[27]

The result obtained with ribonuclease may be regarded from another point of view as merely a failure of the equation for the titration curve, which assumes a uniform distribution of all dissociating groups over the molecular surface. If the electrostatic term of this equation could be expressed in terms of the actual locations of the dissociating groups, then the intrinsic pK would presumably have its normal value for all of the carboxyl groups.

Acknowledgment.—This investigation was supported by research grant NSF-G 326 from the National Science Foundation and by research grant H-1619 from the National Heart Institute, of the National Institutes of Health, Public Health Service.

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(23) *Cf.* ref. 9, p. 256. The same effect may be observed in simple peptides. For example, the α-COOH groups of glycylglycine and other dipeptides with uncharged side chains have pK values in the range of 3.0 to 3.2 (tyrosyltyrosine is listed as 3.52). For tyrosylarginine, with a positive charge on the side chain nearest the carboxyl group we have a pK of 2.64, for histidylglycine with a positive charge on a shorter side chain further from the carboxyl group we have a pK of 2.40, for lysyllysine with positive charges on both side chains we have a pK of 1.95. The data here are taken from E. J. Cohn and J. T. Edsall, "Proteins, Amino-Acids and Peptides," Reinhold Publ. Corp., New York, N. Y., 1943.

(24) S. Moore, C. H. W. Hirs and W. H. Stein, *J. Biol. Chem.*, in press; *Federation Proc.*, in press.

(25) F. Sanger, *et al.*, *Biochem. J.*, **49**, 481 (1951); **53**, 353, 366 (1953).

(26) M. D. Sterman and J. F. Foster, paper presented at 128th National Meeting of the American Chemical Society, Minneapolis, Minn., Sept. 1955.

(27) The effect of urea on the titration curve of ribonuclease has not been studied. The effect of ionic strength is not unequivocal, because the titration region of the carboxyl groups does not encompass the point of zero charge. Thus an interpretation in terms of pK's requires an assumption about w. With the assumptions here made no change of intrinsic pK with ionic strength was called for.