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

Phenolic Hydroxyl Ionization in Proteins. II. Ribonuclease[1a]

BY CHARLES TANFORD, JACK D. HAUENSTEIN[1b] AND DAVID G. RANDS

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The six phenolic groups of ribonuclease have been shown to be of two types. Three of the groups ionize instantaneously and reversibly between *p*H 9 and 11.5, the other three ionize slowly and irreversibly above *p*H 11.5. At 25° and ionic strength 0.15 the first three groups have an intrinsic pK of 9.9, an intrinsic standard heat of ionization of 7 kcal./mole, and an intrinsic standard entropy of ionization of −22 cal./deg./mole. These figures are essentially normal for the ionization of phenolic groups and thus indicate that the reversibly ionizing phenolic groups lie at the surface of the molecule. It is suggested that the irreversibly ionizing groups are embedded in non-polar portions of the ribonuclease molecule, and that they may be part of a "hydrophobic" bond which helps to maintain the native structure of the molecule.

The spectrophotometric method for following the dissociation of hydrogen ions from the phenolic groups of proteins was first used by Crammer and Neuberger in 1943.[2] It was adapted to modern spectrophotometers, and to measurements at different temperatures, in the preceding paper of this series.[3]

The method has been applied to several proteins and to poly-L-tyrosine, and marked differences have been observed. In poly-L-tyrosine[4] and in insulin[5] the ionization proceeds reversibly and with a normal intrinsic dissociation constant. In serum albumin[3] and in lysozyme[6] the ionization is reversible, but the intrinsic pK (and, in serum albumin at least, also the heat and entropy of ionization) is abnormally high. This suggests that the phenolic groups are hydrogen-bonded in these proteins.[7] Finally, in ovalbumin,[2] the phenolic groups cannot be titrated at all without destruction of the native structure of the protein. This suggests that they are embedded in the interior of the molecule.

The present paper reports a similar study on the low molecular weight protein, ribonuclease. In this protein the phenolic groups are shown to be of two types: half of them are essentially normal, the other half are inaccessible, like those of ovalbumin.

Experimental

Ribonuclease was purchased from Armour and Co. Two lots were used, no. 381-059 and 381-062. Chromatographic analysis by the method of Hirs, Moore and Stein[8] showed that lot 381-059 consisted largely of the component designated by these investigators as component A, whereas lot 381-062 contained components A and B in about equal amounts. A separate study[9] has shown that these two components of ribonuclease differ only in the number of free carboxyl groups, component B having one more than component A, with no other detectable difference. Thus the only difference between the two lots used, as far as the present study is concerned, is a slight difference in average molecular charge at any *p*H. The actual charges could be determined from titration curves of both lots which have been obtained in this Laboratory.[9]

All solutions for measurement were prepared from isoionic, salt-free stock solutions, obtained by dissolving the crystalline protein in water and passing it down a column of ion exchange resins of the type described by Dintzis.[10] Measurements were made on solutions containing 0.18 to 0.5% ribonuclease. The *p*H was adjusted by addition of standard KOH or HCl, the ionic strength by further addition of KCl. Conductivity water was used throughout.

Measurements of *p*H were made on a Model G Beckman *p*H meter, using the technique and apparatus previously described.[11]

(1) (a) Presented at the 128th National Meeting of the American Chemical Society, Minneapolis, Minnesota, September, 1955. (b) The major portion of this paper is abstracted from the Ph.D. thesis of Jack D. Hauenstein, State University of Iowa, August, 1955.

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

(3) C. Tanford and G. L. Roberts, Jr., THIS JOURNAL, **74**, 2509 (1952).

(4) E. Katchalski and M. Sela, *ibid.*, **75**, 5284 (1953).

(5) G. L. Roberts, Jr., unpublished data; C. Tanford and J. Epstein, *ibid.*, **76**, 2163 (1954).

(6) C. Fromageot and G. Schnek, *Biochim. Biophys. Acta*, **6**, 113 (1950); C. Tanford and M. L. Wagner, THIS JOURNAL, **76**, 3331 (1954).

(7) *Cf.* M. Laskowski, Jr., and H. A. Scheraga, *ibid.*, **76**, 6305 (1954).

(8) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **200**, 493 (1953).

(9) C. Tanford and J. D. Hauenstein, *Biochim. Biophys. Acta*, in press.

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

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

Preliminary measurements of ultraviolet light absorption were made on a Cary recording spectrophotometer. A few of these measurements are shown in Fig. 1.

All of the ultraviolet light absorption data for quantitative evaluation were measured on a Beckman model DU spectrophotometer, with thermostated cell compartment. A stream of nitrogen passed slowly through the cell compartment avoided absorption of carbon dioxide and prevented the *p*H drifts observed with serum albumin[3]; 1-cm. quartz cells were used, usually containing inserts to reduce the light path to 3 mm.

Optical density readings were taken at three wave lengths, 292, 295 and 298 mμ. The readings were corrected for protein concentration and light path, and are reported as molar extinction coefficients, ϵ, *i.e.*, as log I_0/I for a 1 *M* solution with 1-cm. light path. No effect of protein concentration was observed in the concentration range used.

The results reported are all based on the data at 295 mμ. However, for a portion of the data, plots similar to Fig. 3 were also obtained for the other wave lengths. The results were essentially identical to those obtained at 295 mμ.

Throughout this paper, the molecular weight of ribonuclease is taken as 13,895.[12] The number of phenolic groups per molecule, as determined by Hirs, Moore and Stein,[12] is six.

Results

The over-all absorption spectrum in the region of 250 to 330 mμ, and its dependence on *p*H, is shown in Fig. 1. The typical change due to progressive ionization of the phenolic groups is seen. When the absorption at any wave length from 285 to 310 mμ is plotted *versus* *p*H, however, as is done for the wave length of 295 mμ in Figs. 2 and 3, it is seen that the ionization proceeds in two distinct stages,

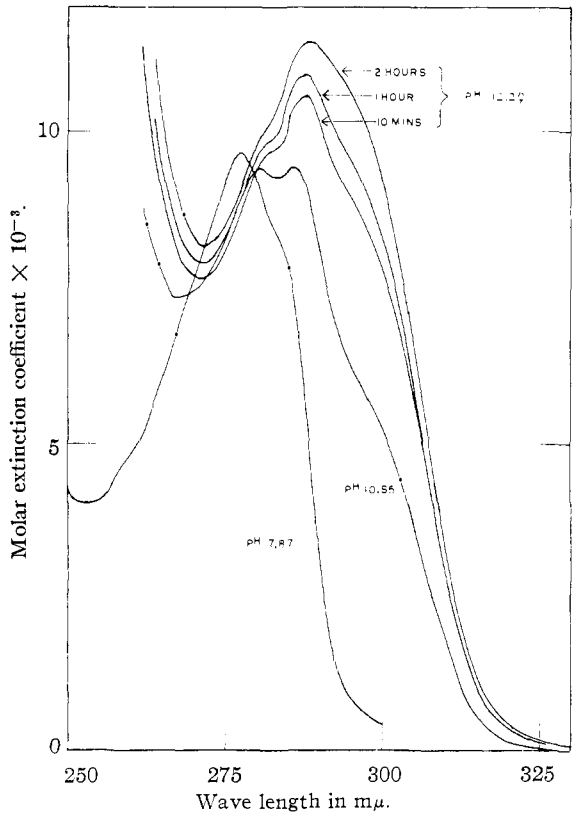

Fig. 1.—Ultraviolet absorption spectrum of ribonuclease as a function of *p*H and (at *p*H 12.2) as a function of time. There is no time dependence at the lower *p*H values.

(12) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **211**, 941 (1954).

one occurring roughly between *p*H 9 and 11.5, the second above *p*H 11.5.

By assuming that the curves of Figs. 2 and 3 (and some others not shown) all represent the superposition of two S-shaped curves, each corresponding to one of the stages of the ionization, the change in ϵ during each stage could be fixed: for the first stage there is an increase from 700 to 8600, for the second stage an increase from 8600 to about 17,000, all of these figures being for 295 mμ. Thus the total increase in each stage is almost the same, so that each stage may be taken to represent the ionization of one-half the phenolic groups of ribonuclease, *i.e.*, of three such groups. The change in ϵ at 295 mμ per phenolic group is thus 2630, compared to values of 2430 found for serum albumin[3] and about 2300 for tyrosine[2,3] at the same wave length.

The two stages of the ionization are very different. The first stage is time-independent and completely reversible, as shown by Fig. 2. The second stage, on the other hand, is time-dependent and irreversible, the phenolic groups titrated in this region recombining with hydrogen ions at a much lower *p*H. The situation is quite similar to that observed in the titration of the phenolic groups of ovalbumin[2] and in the acid titration of hemoglobin.[13]

The main objective of the present study was to obtain thermodynamic data for the reversibly ionizing phenolic groups. The most careful and numerous observations were therefore made in the range of *p*H 9 to 11.5. The data, at two temperatures and three ionic strengths, are shown in Figs. 2 and 3. A detailed thermodynamic analysis of these data is presented in the following section.

A few qualitative observations may be noted concerning the time-dependent second stage of the reaction. At 25°, this process appears to occur very rapidly, the experimental points shown in Fig. 2 being extrapolations to zero time from measurements made at intervals, beginning about 5 minutes after mixing of individual solutions.[14] The initial ionization is seen by Fig. 2 to occur much more slowly at lower temperature.

The change in ϵ with time after the initial measurements still appears to be largely a consequence of further slow ionization, as shown by the wave length dependence of the increase given by the upper three curves of Fig. 1. The nature of the rate process is therefore quite different from that which occurs in serum albumin,[3] and which was ascribed to aggregation.

Thermodynamic Calculations

The ionization of the first three phenolic groups of ribonuclease represents reversible equilibrium and thus obeys the equation[11]

$$p\mathrm{H} - \log \frac{x}{1-x} = pK_{\mathrm{int}} - 0.868Zw \qquad (1)$$

where x is the degree of ionization at any *p*H, measurable in the present instance as $x = (\epsilon - 700)/$

(13) J. Steinhardt and E. M. Zaiser, *ibid.*, **190**, 197 (1951).

(14) It is not suggested that the points correspond to the experimental values which would be obtained in a "3-second titration" of the type used by Steinhardt and Zaiser.[13] It is more likely that, as in the case of hemoglobin, the appearance of the new titratable groups is most rapid in the initial few seconds.

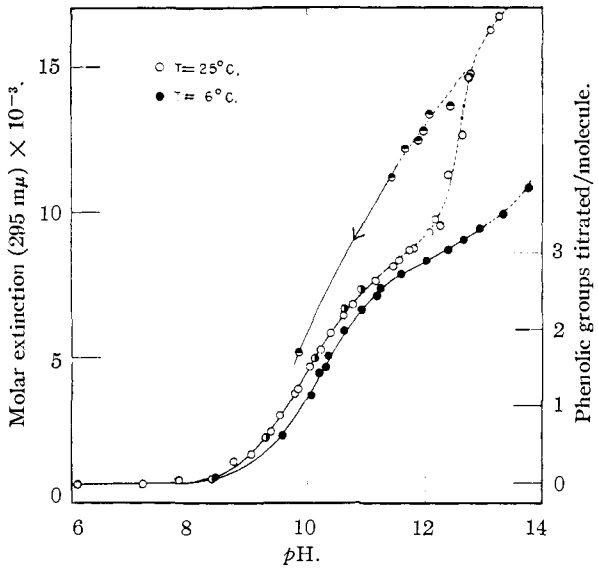

Fig. 2.—Ionization of the phenolic groups at ionic strength 0.15. The ionization is independent of time where solid lines have been drawn, it increases with time where dashed lines have been drawn: ◑, solutions at 25° reversed after exposure to *p*H 11.5; ◒, solutions at 25° reversed after exposure to *p*H 12.7.

tation of w requires that the three phenolic groups are intrinsically identical. If they are not, however, this is likely to be reflected in abnormally large values of w, or in pronounced curvature of plots of the type made in Fig. 4, neither of which is observed for ribonuclease.

Figure 4 shows plots of $p\mathrm{H} - \log x/(1-x)$ *versus* Z, the values of Z being obtained from the titration curve,[9] on the assumption that no binding of salt ions occurs.[15] It can be seen from equation 1 that plots of this kind can be used to calculate pK_{int}, as the value of the ordinate where $Z = 0$. They can then be used to obtain values for the empirical interaction factor w. The plots of Fig. 4 do not differ significantly from being straight lines, so that w appears to be a constant in the *p*H region here under consideration. To obtain the best straight lines through the data one can employ a least squares technique weighting all points equally. Alternatively, one can give greatest weight to the points in the center of the curve, since these are subject to the least error. The results do not differ appreciably. The probable uncertainty in the intercepts (*i.e.*, pK_{int}) is less than 0.05, the uncertainty in w is less than 0.010.

The values obtained for pK_{int} are shown in Table I. It is seen that slightly different values are ob-

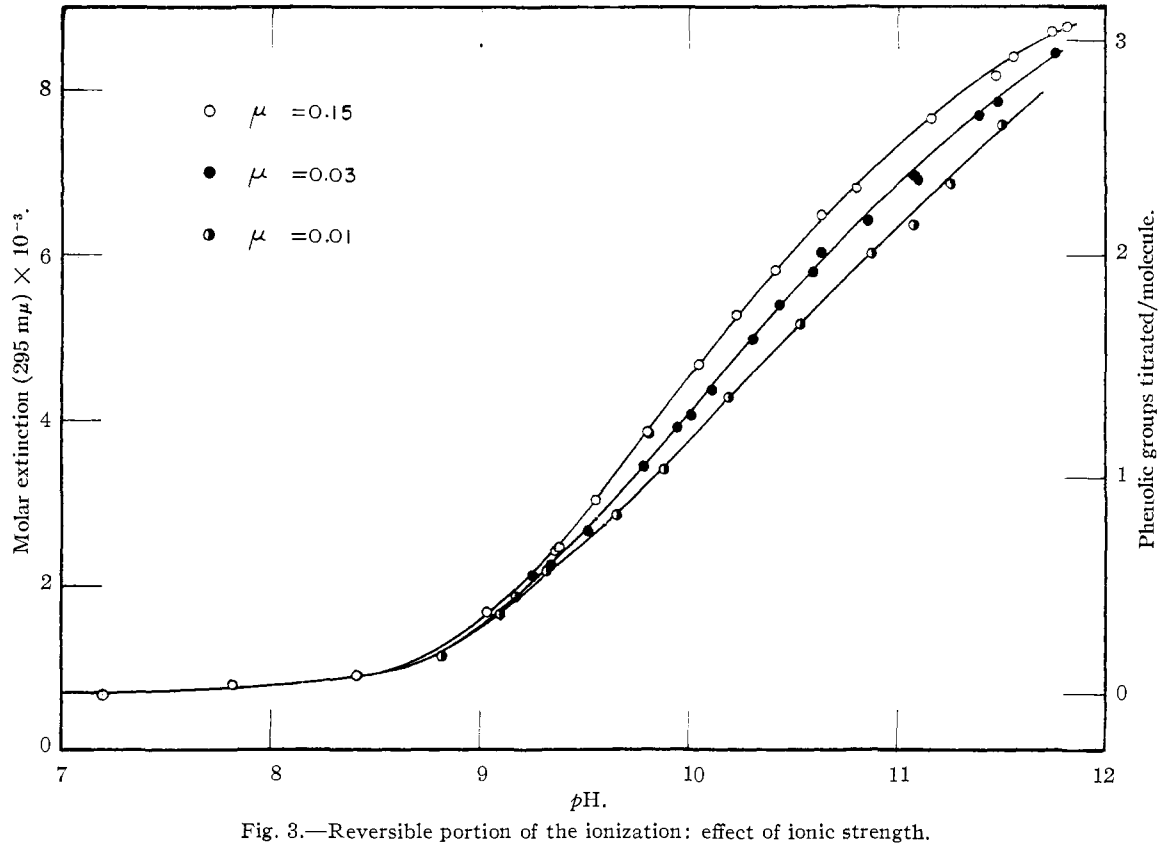

Fig. 3.—Reversible portion of the ionization: effect of ionic strength.

7900, Z represents the charge at any *p*H, pK_{int} is the intrinsic dissociation constant of the three phenolic groups, and w is an empirical electrostatic interaction factor such that $2kTwZ$ is the work required to remove a proton from the surface of the protein ion of charge Z to infinity. This interpre-

(15) Potentiometric studies with Ag–AgCl electrodes have shown that no chloride ions are bound above *p*H 7. When KCl is added to the isoionic protein in conductivity water, the *p*H does not fall, as it would if potassium ions were bound. In fact, there is a small increase in *p*H, comparable in magnitude to that which would be expected on the basis of the equation derived by A. Brown and quoted by G. Scatchard and E. S. Black, *J. Phys. Colloid Chem.*, **53**, 88 (1949).

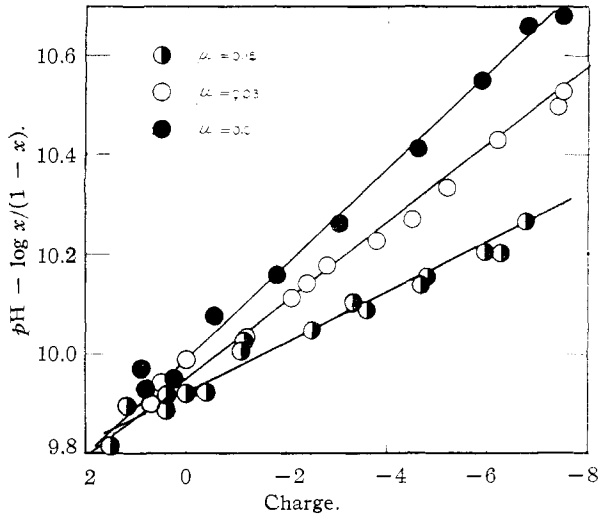

Fig. 4.—Plot of data of Fig. 3 according to equation 1.

tained at 25° at the three ionic strengths used. This is theoretically not unexpected, since the reference state used here, to which the intrinsic pK refers, varies with ionic strength.[11,16]

TABLE I

THERMODYNAMIC CONSTANTS FOR PHENOLIC IONIZATION AT 25°

	pK_{int}	$\Delta H°_{int}$, kcal./mole	ΔH_{app}, kcal./mole	$\Delta S°_{int}$, cal./deg. mole
Ribonuclease, μ = 0.01	9.99	..	..	..
Ribonuclease, μ = 0.03	9.95	..	..	..
Ribonuclease, μ = 0.15	9.92	7.0	5.5	−22
Ribonuclease, μ = 0.15, 6°	10.27			
Phenol[a]	9.78	6.1	..	−24
Tyrosine[b]	9.66	6.0	..	−24
Tyrosylarginine[a]	9.6	6.0	..	−24
Polytyrosine[c]	9.5	..	..	..
Insulin[d]	9.7	..	7.5	−19
Pepsin[e]	(9.5)	..	6.0	−23
Serum albumin[f]	10.35	..	11.5	− 9
Lysozyme[g]	10.8	..	..	..
Ovalbumin[h]	Not accessible in native protein			
Ribonuclease (3 groups)				

[a] As compiled in E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publ. Corp., New York, N. Y. [b] Ref. 3. The observed pK of 10.05 has been corrected by means of the Kirkwood-Westheimer theory for the presence of the negatively charged carboxyl group on the molecule. [c] Ref. 4. [d] Ref. 5. [e] G. L. Roberts, Jr., unpublished data. A personal communication from Dr. Harold Edelhoch informs us that in the alkaline pH range pepsin is dissociated into half-molecules. The thermodynamic constants given therefore do not refer to native pepsin. [f] Ref. 3. [g] Ref. 6. [h] Ref. 2.

From the pK's at 25 and 6° one can calculate the standard heat and entropy of ionization, $\Delta H°_{int} = 2.303R\ d\ pK_{int}/d(1/T)$ and $\Delta S°_{int} = \Delta H°_{int}/T - 2.303RpK_{int}$. Values for these are also listed in Table I, and all of the thermodynamic data are compared with similar data for the phenolic groups of other proteins and of suitable small molecules.

In the case of serum albumin the heat of ionization, and the entropy of ionization calculated from it, were used as the principal argument in support of the existence of hydrogen bonds involving phenolic groups.[3,7] It was not, however, $\Delta H°$ which was used for this purpose, since the titration region of the phenolic groups in serum albumin is far from the point where $Z = 0$ and the pK_{int} values cannot therefore be evaluated with any accuracy. The heat of ionization obtained for serum albumin was the apparent heat of ionization, $\Delta H_{app} = 2.303R(\partial pH/\partial(1/T))_x$, the derivative being taken at constant degree of ionization (x), *i.e.*, at constant ϵ. It was assumed that $\Delta H_{app} \backsimeq \Delta H°$.

The present study provides an opportunity for estimating the accuracy of this procedure. By equation 1, ignoring the small contribution (which is much less than 1 kcal./mole) due to change in w with temperature, $\Delta H_{app} = 2.303R[d pK_{int}/d(1/T) - 0.868w(\partial Z/\partial(1/T))_x] = \Delta H° - 2Rw\ (\partial Z/\partial(1/T))_x$. Thus ΔH_{app} would be expected to be the same as $\Delta H°$ only if protein solutions at different temperatures with the same value of x(or ϵ) also have the same value of molecular charge. This condition is not satisfied with ribonuclease. Ten amino groups are titrated in the same pH region as the phenolic groups, their heat of ionization is very different, and, hence, protein solutions at different temperatures with the same degree of phenolic ionization will not have the same degree of amino ionization, and will thus correspond to different values of Z. In fact one can estimate the change in Z from the titration curve and obtains $[\partial Z/\partial(1/T)]_\epsilon \sim 4400$ at ionic strength 0.15. Using the appropriate value of w, this leads to the relation $\Delta H_{app} = \Delta H° - 1100$. Thus one would expect ΔH_{app} to be smaller than $\Delta H°$ by about 1100 cal. Actually we observe ΔH_{app} ~5.5 kcal./mole, which is about 1500 cal. lower than $\Delta H°$.

In serum albumin the heat of ionization of phenolic and amino groups is about the same and one therefore expects little change in Z with temperature at constant ϵ. (An examination of the titration curve confirms this.) Thus there seems no reason to change the estimate previously given, that ΔH_{app} should lie within about 1 kcal./mole of $\Delta H°$. For insulin and pepsin the values of ΔH_{app} listed in Table I should also be reasonably close to $\Delta H°$, since in these molecules the phenolic groups greatly outnumber the amino groups.

The values of w obtained from the slopes of the plots of Fig. 4 are 0.112, 0.093 and 0.061, respectively, at ionic strengths 0.01, 0.03 and 0.15. They are to be compared with values calculated from the relation

$$w = \frac{\epsilon^2}{2DkT}\left(\frac{1}{b} - \frac{\kappa}{1 + \kappa a_i}\right) \qquad (2)$$

This equation gives the values of w to be expected for a spherical protein ion of radius b with the net charge distributed evenly over the surface. Salt ions are excluded to a distance a_i from the center of the protein ion, a_i being about 2.5 Å. greater than b. In equation 2 ϵ is the protonic charge, D the dielectric constant, k Boltzmann's constant, T the absolute temperature and κ the Debye–Hückel constant proportional to the square root of the ionic strength. A value of 17.1 Å. has been used for b. This is based on a molecular weight of 13,895,[12] a partial specific volume of 0.71[17] and the inclusion of 0.2 g. of tightly-bound water of hydration per gram of dry protein.

Values of w thus calculated by equation 2 are 0.137, 0.113 and 0.079, respectively, at ionic strengths 0.01, 0.03 and 0.15. They represent correctly the observed ionic strength dependence, but the observed values of w are seen to be uniformly smaller (by about 20%) than those calculated.

(16) The observed change is in the direction to be expected. The intrinsic ionization constant is defined as the apparent ionization constant where $Z = 0$, so that changes in pK_{int} with ionic strength may be conveniently interpreted in terms of equilibria which do not involve changes in Z. Applicable to the present situation is the equilibrium $-C_6H_5O^- + -NH_3^+ \rightleftarrows -C_6H_5OH + -NH_2$, which will clearly move to the *left* with increasing ionic strength. This will decrease the apparent pK of the phenolic groups (as observed) and increase the apparent pK of the ϵ-amino groups.

(17) A. Rothen, *J. Gen. Physiol.*, **24**, 203 (1940).

A discrepancy of this order of magnitude has been observed for many proteins, and might be due to non-spherical shape of the protein ion or to the fact that the charge distribution on the ion is discrete rather than continuous.[18]

Discussion

The data of Table I show clearly that three of the phenolic groups of ribonuclease have values of intrinsic pK and of intrinsic heat and entropy of ionization very close to the values obtained for phenolic groups of model compounds dissolved in water. The same fact is true of *all* of the phenolic groups of polytyrosine, of insulin and of pepsin.

The other three phenolic groups of ribonuclease appear inaccessible to hydrogen ions, and behave in a fashion similar to all (or nearly all) of the phenolic groups of ovalbumin. It would not seem likely that the abnormal behavior of these phenolic groups could be explained on the basis of hydrogen bonding alone. A single hydrogen bond would more likely lead to an increase of about 1000 cal. in the free energy of ionization (*i.e.*, an increase of about 0.7 in pK_{int}) and changes in ΔH° and ΔS° of about 6000 cal. and about 15 e.u., respectively. Theoretical justification for figures of this order of magnitude has been given by Laskowski and Scheraga.[7] Thus the data given for serum albumin and for lysozyme *do* suggest hydrogen bonding as an explanation.

In the case of the three abnormal groups of ribonuclease and for those of ovalbumin the change in environment of the phenolic groups must be greater than could be provided by simple hydrogen bonding. Possibly, there are phenolic hydrogen bonds which form part of a network of bonds maintaining the native configuration of the molecule, so that ionization of a phenolic group cannot occur without simultaneous and irreversible rupture of other bonds. Alternatively there may be present in ovalbumin and ribonuclease strong "hydrophobic" bonds of the type recently discussed by Kauzmann.[19] These bonds arise because non-polar side chains of a protein molecule extending into an aqueous medium interfere with the hydrogen bonding between water molecules, *i.e.*, they make "holes" in the structure of water, with a resulting free energy increase. These non-polar groups therefore tend to aggregate together in the interior of the molecule and can be brought to the surface and in contact with water only by rupture of the native structure. Phenol is somewhat less soluble in aliphatic hydrocarbons than in water, but the difference is much less than a factor of 10. In benzene, phenol is twice as soluble as in water.[20] The transfer of an un-ionized phenolic side-chain from an aqueous medium into one of these hydrophobic regions would thus be expected to occur without any large increase in free energy. In proteins containing a large number of phenylalanine side chains (providing possibility of a benzene environment) there might even be a free energy decrease. Thus it is likely that some phenolic side-chains of some proteins will be found part of such hydrophobic regions, and, if they are, they would be expected to show precisely the kind of ionization behavior observed for the abnormal phenolic groups of ribonuclease and for those of ovalbumin.

This raises a question concerning certain speculations about the structure of ribonuclease made in a recent paper by Anfinsen and co-workers.[21] These authors suggested that the four disulfide bonds of ribonuclease are the principal cross-links in the molecule, and thus responsible for maintenance of its compact, globular structure. The present paper would seem to indicate that other strong bonds exist and that these might be just as important as the disulfide bridges.

Germane to this question are the studies on urea denaturation by Kauzmann and co-workers.[22] They have shown that bovine serum albumin, despite having 15 disulfide bonds, has an easily deformable structure, while ovalbumin, which has only one disulfide bond, is deformed only slowly at high urea concentrations. These two proteins serve to indicate that the presence of disulfide bonds does not necessarily lead to a rigid structure, and, conversely, that a rigid structure may be achieved where insufficient disulfide bonds exist to account for it.

Ribonuclease has a sufficient number of disulfide bonds so that these *could* be the vital links in the native structure. But ribonuclease also resembles ovalbumin (and, so far, no other protein) in having some of its phenolic groups involved in internal bonding of considerable strength. There is no reason to believe that either kind of bond is necessarily the most important. Indeed, the possibility exists that they are essentially the same bond, *i.e.*, that the disulfide bridges, like the phenolic groups, may be dissolved in the hydrophobic portions of the molecule.

NOTE ADDED IN PROOF.—It has just come to our attention that the principal qualitative conclusion of this paper, that there are two very different kinds of phenolic groups in ribonuclease, was already fully established in 1952 by D. Shugar,[23] using the same method as in the present study. Shugar, however, did not study the effect of ionic strength or temperature, nor did he calculate intrinsic thermodynamic constants.

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(18) For a fuller discussion see C. Tanford, *J. Phys. Chem.*, **59**, 788 (1955).

(19) W. Kauzmann in W. D. McElroy and B. Glass, ed., "The Mechanism of Enzyme Action," Johns Hopkins Press, Baltimore, Md., 1954.

(20) A. Seidell, "Solubilities of Organic Compounds," 3rd ed., D. Van Nostrand Co., New York, N. Y., 1941.

(21) C. B. Anfinsen, R. R. Redfield, W. L. Choate, J. Page and W. R. Carroll, *J. Biol. Chem.*, **207**, 201 (1954).

(22) W. Kauzmann, *et al.*, THIS JOURNAL, **75**, 5139, 5152, 5154, 5157, 5167 (1953).

(23) D. Shugar, *Biochem. J.*, **52**, 142 (1952).