[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY]

# Structural Studies of Ribonuclease. I. Hydrogen Ion Equilibria in a Denaturing Solvent[1,2]

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RECEIVED JUNE 9, 1959

A denaturing solvent (5 *M* in guanidine hydrochloride and 1.2 *M* in urea, designated "GU") has been used to disrupt internal interactions in the ribonuclease molecule. As a result the carboxyl and three of the tyrosyl groups, which show abnormal ionization behavior in the native protein, titrate normally in GU, the $pK$'s in GU being similar to those of acetic acid and phenol in this same solvent. Also, the electrostatic interaction parameter $w$, for the titration of ribonuclease in GU, is the same at both low and high $p$H, reflecting not only the normal behavior of the carboxyl and tyrosyl groups but also the absence of any significant $p$H-dependent configurational changes in this solvent.

## Introduction

Recent progress[3,4] on the elucidation of the amino acid sequence and positions of the disulfide bonds of ribonuclease has stimulated many investigations of the tertiary structure of this protein. Such work on the internal structure of both native and denatured ribonuclease is also in progress in this Laboratory and, in this paper, we report the results of titration studies of ribonuclease in a denaturing solvent.

Previous studies of hydrogen ion titration curves[5] and of the phenolic hydroxyl ionization[6-8] of ribonuclease have indicated that an indeterminate number of the carboxyl groups and 3 of the 6 tyrosyl groups behave abnormally. The abnormality of the carboxyl groups has variously been attributed to local electrostatic effects[5] and to hydrogen bonding[9] and that of the tyrosyl groups to hydrophobic bonding[6] and to hydrogen bonding.[9,10] A possible structure for ribonuclease involving 3 tyrosyl-carboxylate ion hydrogen bonds has been suggested by Linderstrøm-Lang and Schellman.[11] Anfinsen[4] has suggested that one or more hydrogen-bonded tyrosine residues may be intimately concerned with the active center of ribonuclease and that such a linkage presumably exists only in the native configuration. In order to be able ultimately to identify the nature of the internal interactions which manifest themselves in these abnormalities, this initial investigation was concerned with the hydrogen ion equilibria of the carboxyl and tyrosyl groups in a solvent which would denature the protein and render these groups normal.

(1) This investigation was supported by a research grant (E-1473) from the National Institute of Allergy and Infectious Diseases, of the National Institutes of Health, U. S. Public Health Service, and by a Research Grant (G-6461) from the National Science Foundation.

(2) Presented before the Division of Biological Chemistry at the 136th meeting of the American Chemical Society, Atlantic City, New Jersey, September, 1959.

(3) C. H. W. Hirs, W. H. Stein and S. Moore, "Symposium on Protein Structure," Ed. A. Neuberger, John Wiley and Sons, Inc., New York, N. Y., 1958, p. 211.

(4) C. B. Anfinsen, ref. 3, p. 223.

(5) C. Tanford and J. D. Hauenstein, THIS JOURNAL, **78**, 5287 (1956).

(6) C. Tanford, J. D. Hauenstein and D. G. Rands, *ibid.*, **77**, 6409 (1955).

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

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

(9) H. A. Scheraga, *Biochim. Biophys. Acta*, **23**, 196 (1957).

(10) W. F. Harrington and J. A. Schellman, *Compt. rend. trav. Lab. Carlsberg, Ser. chim.*, **30**, 21 (1956).

(11) K. U. Linderstrøm-Lang and J. A. Schellman, "The Enzymes," Vol. I, Ed. P. D. Boyer, H. Lardy and K. Myrback, Academic Press, Inc., New York, N. Y., 1959, p. 443.

Guanidine hydrochloride at moderate concentrations denatures ribonuclease.[12,13] However, the intrinsic $pK$'s of the various ionizable groups are also modified by this solvent. In order to avoid large solvent effects on the intrinsic $pK$'s, a mixed solvent was used (5 *M* in guanidine hydrochloride and 1.2 *M* in urea, designated "GU"). The $pK$ of acetic acid is the same in GU as it is in 0.15 *M* KCl, and the $pK$'s of imidazole, phenol and *n*-butylamine in GU differ very slightly from the corresponding values in 0.15 *M* KCl.[14] A similar use has been made of GU in structural studies of lysozyme.[14] Complete titration curves, as well as spectrophotometric ones in the alkaline $p$H range, were therefore obtained for ribonuclease in GU in order to determine whether the carboxyl and tyrosyl groups behave normally in this denaturing solvent.

## Experimental

**Material.**—Armour ribonuclease (lot 381-059) was used in this study. The moisture content was determined by drying to constant weight at 110–120° and found to be 10.2%. Solutions were prepared by direct weighing with a correction for the moisture content. The concentrations were also checked by spectrophotometric measurement at 278 mμ and found to agree with the values determined by direct weighing.

All other chemicals were reagent grade unless otherwise indicated. Eastman guanidine hydrochloride was purified according to the method of Kolthoff, *et al.*,[15] and dried at 50° for 5–6 hr. in a vacuum oven. Mallinckrodt urea (U.S.P.) was recrystallized from ethanol. The urea was air-dried at room temperature and then *in vacuo* for 4–5 hours. The GU solution was prepared by direct weighing. Baker Chemical Co. HCl and Mallinckrodt KCl and KOH were also used, the base being prepared $CO_2$-free according to the method of Kolthoff.[16] The HCl was diluted and standardized against Mallinckrodt borax, the latter having been purified as described by Vogel.[17] The $CO_2$-free KOH was then standardized against the HCl.

The solutions used to standardize the $p$H meter at 25° were Beckman $p$H 4 and $p$H 7 buffers, and 0.01 *M* borax ($p$H 9.18) prepared as described by Bates.[18]

All solutions were prepared with conductivity water.

Weighed amounts of ribonuclease were dissolved in known volumes of GU.

(12) C. B. Anfinsen, W. F. Harrington, A. Hvidt, K. Linderstrøm-Lang, M. Ottesen and J. Schellman, *Biochim. Biophys. Acta*, **17**, 141 (1955).

(13) C. B. Anfinsen, *Federation Proc.*, **16**, 783 (1957).

(14) J. W. Donovan, M. Laskowski, Jr., and H. A. Scheraga, *J. Molecular Biol.*, in press.

(15) I. M. Kolthoff, A. Anastasi, W. Stricks, B. H. Tan and G. S. Deshmukh, THIS JOURNAL, **79**, 5102 (1957).

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

(17) A. I. Vogel, "Quantitative Inorganic Analysis," 2nd Ed., Longmans, Green and Co., Inc., New York, N. Y., 1951, pp. 231–232.

(18) R. G. Bates, "Electrometric $p$H Determinations," John Wiley and Sons, Inc., New York, N. Y., 1954, pp. 118–121.

**Titration Apparatus.**—An automatic titration assembly (Radiometer, type TTT1; recorder SBR 2a from Welwyn International Inc.) was used to obtain the titration curves. The buret was a syringe of 0.5-ml. capacity with a micrometer graduated in 1250 divisions. The buret assembly was calibrated by titrating HCl against KOH. Radiometer glass electrodes type G202B and calomel electrodes K 100 were used for the *p*H measurements. The titration cell was a 30-ml. lipless beaker enclosed in a water jacket through which constant-temperature water was circulated. The temperature of the water-bath was kept constant at 25° within 0.1° or better. A thin piece of iron wire enclosed in a glass tube was used in conjunction with a magnetic stirrer to stir the solutions. Nitrogen was passed slowly over the solution being titrated in order to avoid the absorption of carbon dioxide. The electrodes, buret and nitrogen inlet were inserted into the titration cell through a rubber stopper.

**Titration Procedure.**—The TTT1 titrator was standardized with about 10 ml. of standard buffers *p*H 4, *p*H 7 and *p*H 9.18 in the titration cell. Ten ml. of the sample solution to be titrated then was placed in the cell and allowed to stand about ten minutes with stirring and bubbling of nitrogen gas for temperature equilibration at 25°.

The protein solution (0.35–0.50%) was brought to *p*H 1.8 by addition of HCl and titrated with automatic recording using 3.300 *N* KOH. In order to examine the reversibility of the titration, the solution was brought to *p*H 11.8 by addition of KOH and titrated with 3.013 *N* HCl using the same procedure as above. The duration of the titration runs was about 25 minutes. After the titration, the TTT1 *p*H meter was checked against standard buffers to make sure that the standardization was not off by more than 0.01 *p*H unit.

Similar titrations were carried out on the solvent, the titration curve being obtained by subtraction of the solvent curve from that of the protein solution. A correction for the small volume change due to the addition of titrant has not been made. However, this introduces only a negligible error in the ionic strength and none in the protein concentration since the calculations were based on the actual amount of protein present in the solution. As a result, no differences could be detected between the data obtained by this continuous titration procedure and the point-by-point method.

**Spectrophotometric Titration.**—Ultraviolet absorption measurements were made on a Beckman model DU spectrophotometer using 1-cm. silica cells covered with glass-stoppers to avoid absorption of $CO_2$. Temperature in the cell compartment was maintained at 25° by thermostatting. The protein concentration was 0.04 g./100 cc. An appropriate amount of KOH was added to the GU solutions to bring them to the desired *p*H. Since the optical density of the GU solutions is very high (approximately 2), GU rather than water was used in the reference cell. Since the optical density of GU is *p*H-dependent, the reference GU solution was kept at a constant *p*H of 5. Therefore, the ultraviolet absorption spectrum of the tyrosyl groups was obtained by subtracting the optical density of the blank GU at various *p*H's from that of the ribonuclease solutions in GU at the corresponding *p*H's. Measurements were made at three different wave lengths, 295, 290 and 288 mμ. The data for relative absorption at all three wave lengths agreed within the experimental error. The values at 295 mμ are reported in this paper. *p*H measurements for the spectrophotometric titration were made on a Beckman model GS *p*H meter using the A scale. The solutions were placed in a small cell through which constant-temperature water was circulated during the *p*H measurement. Borax buffer (*p*H 9.18 at 25°) and Beckman *p*H 7 buffer were used for calibration.

## Results

**Direct Titration.**—Titrations of ribonuclease in 0.15 *M* KCl at 25°, and of the 0.15 *M* KCl solvent, were carried out with the autotitrator. The titration curve (*i.e.*, the number of protons dissociated from a molecule of ribonuclease at any given *p*H) was obtained by subtracting the titration curve of the solvent from that of the protein solution at various *p*H's. A molecular weight of 13,683[19] was used in the computations. The resulting curve agreed with that of Tanford and Hauenstein[5] within 0.1–0.5 group.[20] As has been discussed by these workers, the carboxyl groups have an abnormally low intrinsic $pK$, about 4.1. Also, 3 of the 6 phenolic groups have abnormally high $pK$'s as previously reported[6-8] on the basis of spectrophotometric titrations. As pointed out by Tanford and Hauenstein,[5] the titration curve may be computed on the assumption that no chloride ions are bound to the protein. This is based[5] on the calculation, by the method of Scatchard and Black,[21] that no bound chloride ion is indicated by the ionic strength dependence of the titration curve.

The titration curve for ribonuclease in GU at 25° was obtained in a similar manner and is shown in Fig. 1. The two sets of points represent titrations performed in opposite directions and demonstrate the reversibility of the hydrogen ion equilibria involved. The small difference in the experimental data for the forward and reverse titrations in the *p*H range 10–11.5 is not considered significant as far as the question of reversibility is concerned. The curve of Fig. 1 is a theoretical one computed, as discussed below, with the parameters given in Table I. At low *p*H (*p*H 2) the curve is observed to flatten, *i.e.*, the proton uptake by ribonuclease remains unchanged below *p*H 2. Thus, the titration curve could be extrapolated to $r = 0$,

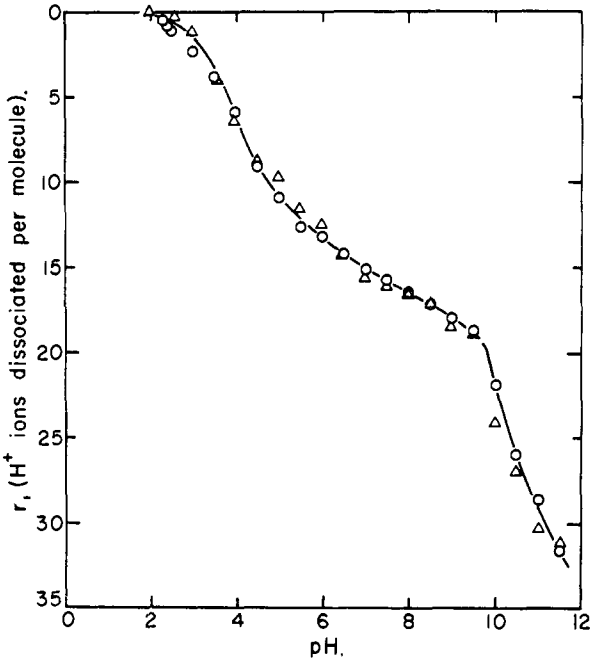


Fig. 1.—Titration curve of ribonuclease in GU at 25°. The circles represent the titration from *p*H 2 to 12 and the triangles from *p*H 12 to 2. The curve is a theoretical one calculated from the parameters in Table I.

(19) C. H. W. Hirs, S. Moore and W. H. Stein, *J. Biol. Chem.*, **219**, 623 (1956).

(20) The discrepancy becomes as large as 0.5 group in the *p*H region 9.6–10 where our points fall on the theoretical curve, whereas those of Tanford and Hauenstein deviate from the curve by 0.5 group.

(21) G. Scatchard and E. S. Black, *J. Phys. Colloid Chem.*, **53**, 88 (1949).

TABLE I

PARAMETERS[a] USED TO CALCULATE TITRATION CURVE OF FIG. 1 ($w$ = 0.056)

| Group | $pK'_{int}$ | |
|---|---|---|
| Carboxyl | 4.6 | |
| Imidazole | 6.5 | |
| $\alpha$-Amino | 7.8 | |
| Tyrosyl | 10.0 | |
| $\epsilon$-Amino | 10.2 | |
| Guanidine | >12 | (does not influence curve in the $p$H range shown in Fig. 1) |

[a] The values for carboxyl and tyrosyl groups were determined from Figs. 3 and 4, respectively. The values for the other groups, being the same in GU as in 0.15 $M$ KCl, were taken from Tanford and Hauenstein.[5]

where $r$ is the number of protons dissociated per molecule, in order to obtain the maximum positive net charge $Z_{max}$. The determination of $Z_{max}$ also involves a knowledge of the isoionic point. If one assumes no chloride ion binding, as assumed by Tanford and Hauenstein for the 0.15 $M$ KCl solvent,[5] and uses the figure of 19 for the number of basic groups,[19] the isoionic point, computed from the experimental curve in GU by adding 19 to the value $r$ = 0 at the flat portion of the curve, is $p$H 9.6, in agreement with that reported[5] for 0.15 $M$ KCl. The value of $Z_{max}$ is thus +19. In addition to the constant value of $r$ below $p$H 2, the curves in GU and in 0.15 $M$ KCl were observed to coincide in the $p$H range of 6–8.5, the region of ionization of imidazole and $\alpha$-amino groups.

**Spectrophotometric Titration.**—The spectrophotometric titration curve for the tyrosyl groups in GU at 25° is shown in Fig. 2. Whereas only 3 of the 6 tyrosyl groups titrate reversibly in 0.15 $M$ KCl[6–8] all 6 of them behave normally in GU, as they do in 8 $M$ urea,[8] as is indicated by the single-stage titration curve of Fig. 2. The reversibility of the hydrogen ion equilibria involved was already demonstrated in the direct titration experiments on which Fig. 1 was based. However, whereas the reversibility test in Fig. 1 was carried only to $p$H 11.8, and the data of Fig. 2 extend to $p$H 12.8, we rely on the consistency of the data (in the absence of a direct test) to indicate that the region between $p$H 11.8 and 12.8 also involves a reversible reaction and that the thermodynamic treatment in the Discussion section may be applied to the data. There was no difference (in the spectrophotometric or in the direct titration) in titration curves between solutions aged for 30 min. and 1 hr., respectively. Therefore, the denaturation of ribonuclease in GU at 25° has been assumed to be complete within 30 min.

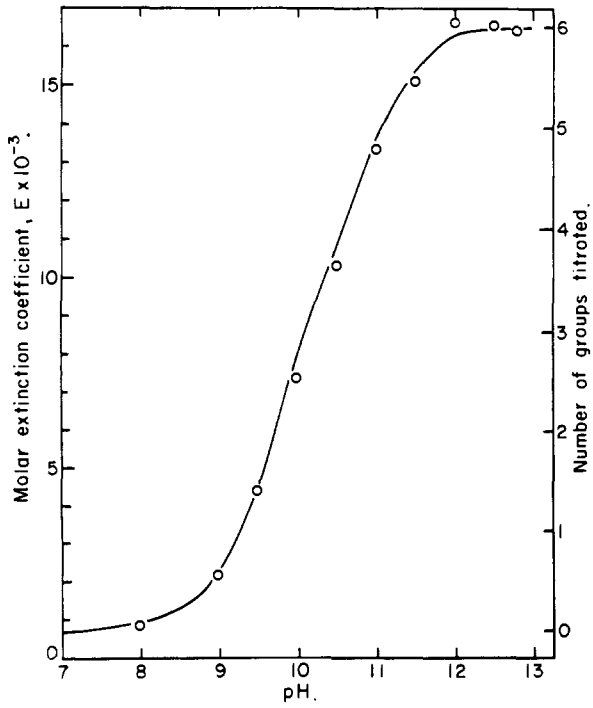


Fig. 2.—Spectrophotometric titration curve of ribonuclease in GU at 25° at a wave length of 295 m$\mu$. See text for a discussion of the reversibility. The curve is a theoretical one for $pK'_{int}$ = 10.0 and $w$ = 0.056.

The change in molar extinction coefficient $E$ at 295 m$\mu$ in GU is 15,800 for 6 tyrosyl groups or 2630 per single group. This may be compared with the values of 2430 for bovine serum albumin[22] and 2300 for tyrosine[22,23] at the same wave length in 0.15 $M$ KCl and a value of about 2550 for ribonuclease in 8 $M$ urea without buffer (estimated from Fig. 1 of the paper by Blumenfeld and Levy).[8]

## Discussion

Regarding the hydrogen ion equilibria in GU as reversible, the titration data may be interpreted in terms of equations of the following form for each type of ionizable group[24]

$$pH - \log \frac{x}{1-x} = pK'_{int} - 0.868wZ \qquad (1)$$

where $x$ is the fraction of the groups of the given kind which have dissociated protons at the given $p$H, and the $pK'_{int}$ is the negative logarithm of the intrinsic dissociation constant for the corresponding groups. The quantity $pK'_{int}$ refers to the solvent used (*e.g.*, GU in this case, and does not represent the value at zero ionic strength). The ordinate of the titration curve is given by the expression

$$r = \Sigma n_i x_i \qquad (2)$$

where $n_i$ is the number of groups in each set and a subscript i has been placed on $x$ to specify the type of group ionizing. The term 0.868$wZ$ arises from the electrostatic interaction between the proton and protein molecule which bears the net charge $Z$. The quantity $w$ may be regarded as an experimental parameter and would be expected to be constant at a given temperature and ionic strength over a $p$H range in which the size, shape and permeability of the molecule do not vary.

The value of $w$ may also be computed from theory, *e.g.*, that of Linderstrøm-Lang,[25] in which the protein is regarded as a spherical ion of radius $b$ with the charge distributed uniformly over the surface. The theoretical expression for $w$ then

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

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

(24) See, *e.g.*, C. Tanford in T. Shedlovsky, ed., "Electrochemistry in Biology and Medicine," John Wiley and Sons, Inc., New York, N. Y., 1955, p. 248.

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

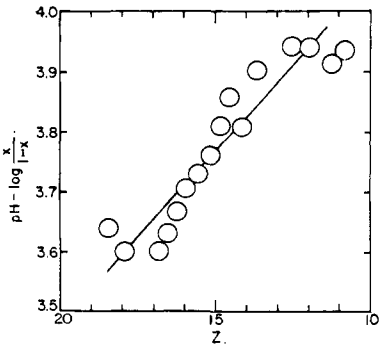


Fig. 3.—Plot of data for carboxyl groups according to equation 1. The line was computed by the method of least squares.

becomes[22]

$$w = \frac{\epsilon^2}{2DkT}\left[\frac{1}{b} - \frac{\kappa}{1 + \kappa a}\right] \qquad (3)$$

where $a = b + 2.5$ in Å. and is the radius of exclusion, $\epsilon$ is the electronic charge, $D$ is the dielectric constant, $k$ is Boltzmann's constant, $T$ is absolute temperature and $\kappa$ is the Debye–Hückel parameter depending on ionic strength. According to eq. 3, the value of $w$ should be independent of $p$H if the model applies and if the size and shape of the molecule are not $p$H-dependent. It is thus of interest to see whether $w$ for ribonuclease is the same in the regions of both carboxyl and tyrosyl ionizations in GU.

The data for the carboxyl and tyrosyl groups[26] were plotted according to equation 1 and are shown in Figs. 3 and 4, respectively. The values of $Z$ were obtained from Fig. 1, taking the isoionic point as 9.6 and assuming no binding of chloride ions. Within the experimental error, the curves of Figs. 3 and 4 are seen to be straight lines from which $w$ and $pK'_{int}$ were determined from the slope and intercept at $Z = 0$. The values are listed in Table II together with similarly obtained values for

TABLE II

EXPERIMENTAL PARAMETERS FOR IONIZATION OF CARBOXYL AND TYROSYL GROUPS IN RIBONUCLEASE[a]

| | $pK'_{int}$ (in 0.15 $M$ KCl) | $pK'_{int}$ (in GU) | $w$ (in 0.15 $M$ KCl) | $w$ (in GU) |
|---|---|---|---|---|
| Carboxyl—11 groups | 4.1 | 4.6 | 0.061[b] | 0.057 |
| Tyrosyl—3 groups | 9.95 | 10.0 | 0.061 | .056 |
| 3 groups | Inaccessible | 10.0 | | .056 |

[a] For the carboxyl groups of lysozyme in GU, the values of $pK'_{int}$ and $w$ are 4.6 and 0.052, respectively.[14] [b] The value 4.1 was obtained[5] by a procedure involving the use of $w = 0.061$ originally obtained at high $p$H in the region of tyrosyl ionization.

TABLE III

EXPERIMENTAL PARAMETERS FOR THE IONIZATION OF MODEL COMPOUNDS[14]

| | $pK$ (in 0.15 $M$ KCl) | $pK$ (in GU) |
|---|---|---|
| Acetic acid | 4.62 | 4.62 |
| Imidazole | 7.07 | 7.20 |
| Phenol | 9.92 | 10.03 |
| $n$-Butylamine | 10.79 | 10.74 |

(26) The value of $x$ for the tyrosyl groups was obtained from the equation $x = (E - 700)/16{,}500$ (see Fig. 2).

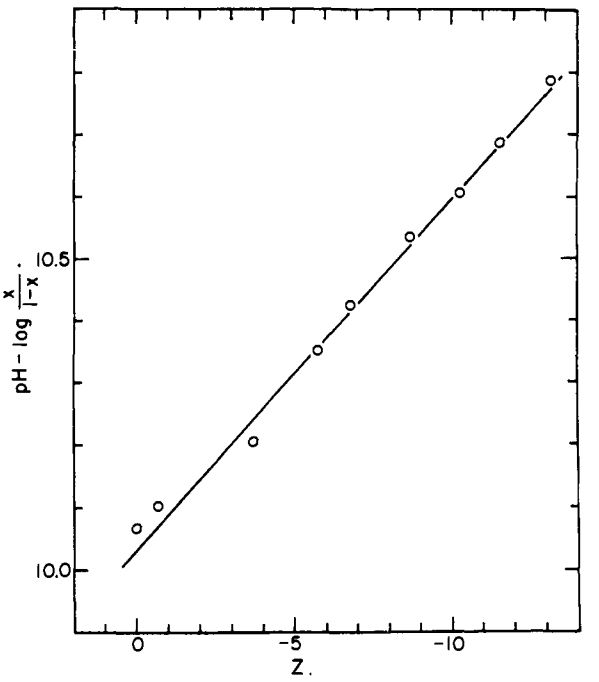


Fig. 4.—Plot of data for tyrosyl groups according to equation 1. The line was computed by the method of least squares.

lysozyme.[14] For a proper interpretation, the $pK'_{int}$ values should be compared to those for model compounds in the same solvent, which are listed in Table III. A comparison of the data of Tables II and III indicates that the 11 carboxyl and 6 tyrosyl groups of ribonuclease titrate normally in GU. This is in contrast to the low $pK$ for the 11 carboxyl groups and inaccessibility of 3 tyrosyl groups of the native molecule in 0.15 $M$ KCl. Although no details are presented here, it was found that the titration of the imidazole, $\alpha$-amino and $\epsilon$-amino groups is the same in both 0.15 $M$ KCl and in GU. As a further indication of the normality of the carboxyl and tyrosyl groups in GU, and of the absence of significant $p$H-dependent configurational changes of ribonuclease in this solvent, it is seen that the $w$-values obtained from Figs. 3 and 4 are identical. The only indication of the lack of applicability of the Linderstrøm-Lang model of a rigid, impermeable sphere is that the experimental $w$-value of 0.056 does not agree with a value of 0.04, obtained from equation 3 using parameters of Tanford and Hauenstein.[5] However, as pointed out by numerous workers, *e.g.*, Tanford,[24] $w$ should better be regarded as an empirical parameter; therefore, this lack of agreement should not be taken too seriously. It thus seems valid to conclude that internal interactions, which render the carboxyl and half of the tyrosyl groups of native ribonuclease in 0.15 $M$ KCl abnormal, are removed in the denaturing solvent GU. A few words about the possible nature of these internal interactions are, therefore, in order.

It seems likely that the denaturing solvent GU causes the rupture of internal hydrogen bonds. These hydrogen bonds could involve the tyrosyl and carboxyl groups directly,[9,10] *e.g.*, tyrosyl-

carboxylate ion bonds, rendering the observed $pK$ of carboxyl groups lower than normal and that of tyrosyl groups higher than normal in the native protein. On the other hand, the abnormality could be due to specific local electrostatic effects[5] or hydrophobic bonding.[6] All three types of interaction might require the specific configuration of the native protein. Since GU disrupts the native configuration,[12] such interactions could be eliminated. There is not yet sufficient experimental evidence to decide between these alternatives. If tyrosyl–carboxylate ion hydrogen bonds do exist in the native protein, they may play a role in the stabilization of the configuration, and their rupture (by binding hydrogen ions to carboxylate ion groups) at low $p$H might lead to an unfolding of the molecule. While deuterium–hydrogen exchange studies[27] at $p$H 1 indicate that exposure to low $p$H leads to instability, this instability arises only after some irreversible change has taken place in the molecule. Similarly, the titration of the 3 inaccessible tyrosyl groups is possible only after an irreversible process has taken place.[6,7] Thus, while some rupture of hydrogen bonding in GU is indicated by the data reported here, the identification of the specific bonds broken will have to await the results of further investigation.

(27) C. L. Schildkraut and H. A. Scheraga, THIS JOURNAL, **82**, 58 (1960).

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# Structural Studies of Ribonuclease. II. Deuterium Exchange Studies of Two Crystalline Forms of Ribonuclease[1,2]

BY CARL L. SCHILDKRAUT AND HAROLD A. SCHERAGA

RECEIVED JUNE 9, 1959

Deuterium exchange studies were carried out on Armour ribonuclease 381-059 and two samples (ribonuclease II and III) prepared by King and associates. Complete deuteration cannot be achieved unless the molecule is above the transition temperature. A significant fraction of the hydrogens exchange slowly, in agreement with the results of Hvidt and Haggis. Below the transition temperature, *e.g.*, at 38°, about 20 hydrogens are sufficiently shielded to prevent exchange. Two groups of 25 hydrogens each exchange more rapidly at 0° but still slowly enough to suggest that they are involved in some folding of the molecule. The remaining 175 exchange essentially instantaneously at 0°, suggesting that they are in random-coil-like parts of the molecule. Ribonuclease II and Armour ribonuclease 381-059 are indistinguishable by the method of deuterium–hydrogen exchange. By this criterion, ribonuclease III appears to have a configuration in solution in which a greater fraction of its hydrogens are shielded.

## Introduction

King and associates recently have prepared several crystalline forms of ribonuclease and have characterized them by X-ray diffraction[3] and by their melting points in contact with their mother liquor.[4] In accounting for the properties of these crystals King has suggested the possibility that different molecular configurations of ribonuclease might exist in solution. The possibility that such differences might exist prompted us to look for them by the deuterium–hydrogen exchange method.[5–7] Differences in configuration can be detected by this method only if the various configurations are stable under the conditions of the experiment and if the different types of molecules exhibit a significant difference in the rate or degree of exchange. Two crystalline forms, designated ribonuclease II and III, have been selected for study. Form III has a lower melting point in the mother liquor[4] and form II appears to be the stable form over a wide range of conditions.[3] In conjunction with these experiments, some observations also were made on the relation between the maximum number of exchangeable hydrogens and the temperature of deuteration. These observations are relevant to the question of the transition in ribonuclease observed by Harrington and Schellman.[8]

## Experimental

**Materials.**—Two different crystalline forms were prepared by King[9] from Armour ribonuclease Lot 381-059 by essentially the same procedure outlined by King, *et al.*[3] Modification II was grown from a solution of 50 volume % 2-methyl-2,4-pentanediol and 5 volume % methanol. Modification III was grown from a solution of 50 volume % *n*-propyl alcohol. For purification the crystals were dissolved in water, centrifuged to remove traces of insoluble material and lyophilized.

All other determinations were performed on Armour Ribonuclease Lot 381-059, used without further purification.

Deuterium Oxide, 99.5%, batch No. 7, from the Stuart Oxygen Company, San Francisco, California, was used to prepare the standards for density determinations and for deuteration of the samples.

**Procedure.**—The procedure was essentially the same as method 2 developed by Linderstrøm-Lang.[6,10] A 150-$\mu$l. sample was placed in a tulip-shaped vessel and the water removed *in vacuo* by cryosublimation into a cold trap at −78°. When the substance was almost dry, it was heated for 4 hr. at 60° while still in the cryosublimation tube. Deuterium oxide (99.5%) then was added to the dried protein

(1) This investigation was supported by a research grant (E-1473) from the National Institute of Allergy and Infectious Diseases, of the National Institutes of Health, U. S. Public Health Service, and by a Research Grant (G-6461) from the National Science Foundation.

(2) Presented before the Division of Biological Chemistry at the 136th meeting of the American Chemical Society, Atlantic City, New Jersey, September, 1959.

(3) M. V. King, B. S. Magdoff, M. B. Adelman and D. Harker, *Acta Cryst.*, **9**, 460 (1956).

(4) M. V. King, *Biochim. Biophys. Acta*, **27**, 503 (1958).

(5) A. Hvidt, G. Johansen, K. Linderstrøm-Lang and F. Vaslow, *Compt. rend. trav. Lab. Carlsberg, Sér. chim.*, **29**, 129 (1954).

(6) I. M. Krause and K. Linderstrøm-Lang, *ibid.*, **29**, 367 (1955).

(7) A. Hvidt and K. Linderstrøm-Lang, *ibid.*, **29**, 385 (1955).

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