# Structural Studies of Ribonuclease. XXIV. The Application of Nuclear Magnetic Resonance Spectroscopy to Distinguish between the Histidine Residues of Ribonuclease1

**ARTICLE** *in* JOURNAL OF THE AMERICAN CHEMICAL SOCIETY · SEPTEMBER 1966

Impact Factor: 12.11 · DOI: 10.1021/ja00970a025

18

[Reprinted from the Journal of the American Chemical Society, 88, 4240 (1966).]

# Structural Studies of Ribonuclease. XXIV. The Application of Nuclear Magnetic Resonance Spectroscopy to Distinguish between the Histidine Residues of Ribonuclease

J. H. Bradbury and Harold A. Scheraga

[Reprinted from the Journal of the American Chemical Society, **88**, 4240 (1966).]


# Structural Studies of Ribonuclease. XXIV. The Application of Nuclear Magnetic Resonance Spectroscopy to Distinguish between the Histidine Residues of Ribonuclease[1]

**J. H. Bradbury[2] and Harold A. Scheraga**

*Contribution from the Department of Chemistry, Cornell University, Ithaca, New York.* *Received May 2, 1966*

**Abstract:** Proton magnetic resonance studies of L-histidine, glycyl-L-histidine, L-histidyl-glycine, and poly-L-histidine showed that deuteration of the imidazole group is accompanied by a downfield chemical shift of about 0.9 ppm of the C-2 proton and about 0.4 ppm of the C-4 proton. The former chemical shift is studied as a function of p*D* in $D_2O$ for DL-histidyl-DL-histidine, and approximate values of the microscopic dissociation constants for the two imidazole groups are determined. With ribonuclease, the C-2 proton resonance falls downfield from the aromatic resonance for all values of p*D*. Using a computer of average transients it is found that the resonance splits into three peaks at values of p*D* between 5.4 and 8. The two groups with average microscopic dissociation constants p*k* of approximately 5.4 and 5.8 have been *tentatively* assigned to the two histidines involved in the active site of the enzyme and the two groups with identical p*k* values of approximately 6.6 to the other two histidine residues. In oxidized ribonuclease the four histidine residues are normalized with $pK' \sim 7.4$.

Ribonuclease was the first protein for which a nuclear magnetic resonance (nmr) spectrum was determined by Saunders, Wishnia, and Kirkwood in 1957.[3] This spectrum was interpreted by Jardetzky and Jardetzky[4] on the basis of measurements of the spectra of the amino acids and peptides in water, $D_2O$, and concentrated sulfuric acid.[5,6] The spectra of amino acids and some polymers and proteins have also been determined in trifluoroacetic acid.[7,8] More recent studies comprise several on the interpretation of the nmr spectra of various proteins,[9-11] including the sharpening of the spectra and the appearance of new peaks which is produced by opening up the native structure by denaturants and oxidation of disulfide bonds. Other more specialized applications include (i) a study of the slowly exchanging protons attached to nitrogen atoms in ribonuclease,[12] (ii) contact resonances at very high and low field in cytochrome *c*,[9,13] and (iii) measurement of spin lattice relaxation times of water molecules in protein solutions.[14]

From the foregoing, it is apparent that the nmr technique has been of limited use in the solution of problems of protein chemistry. This is largely due to the fact that the spectra, in general, consist of a number of broad bands, owing to a multiplicity of similar protons, and it is usually impossible to separate the contribution from each kind. The exceptions to this pattern occur with protons attached to nitrogen which, however, form very broad bands,[12] the aromatic protons due to tryptophan, tyrosine, phenylalanine, and the C-4 proton of histidine which can be partially separated in favorable cases, and finally the C-2 protons of histidine.[9,10] The last named show promise because they form a fairly sharp resonance on the low-field side of the aromatic resonance, and previous work[15] with L-histidine indicates

(1) This work was supported by a research grant (AI-01473) 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 (GB-4766) from the National Science Foundation. Presented at the May 1966 meeting of the Australian Biochemical Society.

(2) On leave from the Chemistry Department, Australian National University, Canberra, Australia, 1965. The award of a Fulbright Travel Grant to J. H. B. is gratefully acknowledged.

(3) M. Saunders, A. Wishnia, and J. G. Kirkwood, *J. Am. Chem. Soc.*, **79**, 3289 (1957).

(4) O. Jardetzky and C. D. Jardetzky, *ibid.*, **79**, 5322 (1957).

(5) M. Takeda and O. Jardetzky, *J. Chem. Phys.*, **26**, 1346 (1957).

(6) O. Jardetzky and C. D. Jardetzky, *J. Biol. Chem.*, **233**, 383 (1958).

(7) F. A. Bovey and G. V. D. Tiers, *J. Am. Chem. Soc.*, **81**, 2870 (1959).

(8) F. A. Bovey, G. V. D. Tiers, and G. Filipovich, *J. Polymer Sci.*, **38**, 73 (1959).

(9) A. Kowalsky, *J. Biol. Chem.*, **237**, 1807 (1962).

(10) M. Mandel, *ibid.*, **240**, 1586 (1965).

(11) M. Mandel, *Proc. Natl. Acad. Sci. U. S.*, **52**, 736 (1964).

(12) A. Wishnia and M. Saunders, *J. Am. Chem. Soc.*, **84**, 4235 (1962).

(13) A. Kowalsky, *Biochemistry*, **4**, 2382 (1965).

(14) D. J. Blears and S. S. Danyluk, *Biopolymers*, **3**, 585 (1965).

(15) C. C. McDonald and W. D. Phillips, *J. Am. Chem. Soc.*, **85**, 3736 (1963).

that there is a considerable chemical shift to lower field on protonation of the imidazole ring.

The present paper describes the application of the nmr technique to the C-2 and C-4 protons of the imidazole group in a number of histidine derivatives including ribonuclease. In the latter, two of the four histidines are known to be implicated in the active site of the enzyme.[16-20]

## Experimental Section

**Materials.** Samples of L-histidine, glycyl-L-histidine, L-histidylglycine (Mann Research Labs.), DL-histidyl-DL-histidine (Nutritional Biochemical Corp.), imidazole (City Chemical Co.), and poly-L-histidine (Lot No. HS17, mol wt 8000 estimated from end group analysis, Yeda Chemicals) were used without further purification. Ribonuclease was a five-times-recrystallized product from Sigma Chemical Co. (Type 1A). It was used to prepare ribonuclease A by the method of Hirs, Moore, and Stein[21] as subsequently modified.[22] The latter was used in most cases, although there was no apparent difference between the over-all nmr spectra of the two products.[9] Oxidized ribonuclease was prepared by the method of Hirs.[23] $D_2O$ (99.7%) was obtained from the U. S. Atomic Energy Commission, Chemical Processes Branch, Savannah River Branch, Aiken, S.C., and bottled by the Cornell University Department of Chemistry. A concentrated solution of NaOD was prepared by dissolving sodium in $D_2O$ and concentrated AR–HCl was diluted with $D_2O$ to produce approximately 6 *N* acid, containing about 35% $H_2O$.

**Methods.** Solutions with concentrations ranging from 5 to 60% were prepared by dissolving the material in $D_2O$, with addition of NaOD, or HCl in $D_2O$, if necessary to facilitate dissolution. The pH readings were measured by a Radiometer pH meter, Type PHM4C, No. 42357, fitted with microelectrodes and standardized with Fisher standard buffers at three pH values between 2 and 10. The pH meter reading (error ±0.04) was converted to p*D* by the equation p*D* = pH meter reading + 0.40, which has been verified in the range p*D* 2–12.[24,25] The p*D* was adjusted as required by the addition of a concentrated solution of NaOD, or HCl in $D_2O$, by means of a micrometer syringe, to the stirred solution contained in a small, covered, plastic cup. Approximately 0.4-ml aliquots of the solution were transferred at appropriate p*D* values to nmr tubes.

The solutions were examined, using a Varian Associates high-resolution Model A-60 spectrometer, fitted with a variable temperature probe and a Technical Measurements Corp. C-1024 computer of average transients (CAT). The spectra were all obtained at 32 ± 2°; chemical shifts are recorded in cycles per second (cps) *vs.* the narrow HDO resonance (1 cps = 0.0167 ppm). The position of the latter moves to higher field with increasing temperature[11] but is essentially independent of p*D* between 1 and 13.[5,15] In using the CAT to scan repeatedly over the region of the histidine and the broad aromatic resonances, it was necessary to introduce a narrow resonance to trigger the scan. A large number of liquids were tested and finally paraldehyde was chosen as the narrow resonance because, although the C–H proton produces a quartet rather than a single resonance, its position about 40 cps downfield from HDO is suitable, and it does not appear to affect the nmr spectra of the protein. The relative intensities of various resonances, which are proportional to the number of nuclei producing them, were determined by cutting out and weighing the appropriate peak areas.

(16) H. A. Scheraga and J. A. Rupley, *Advan. Enzymol.*, **24**, 161 (1962).
(17) A. M. Crestfield, W. H. Stein, and S. Moore, *J. Biol. Chem.*, **238**, 2413, 2421 (1963).
(18) R. Heinrickson, W. H. Stein, A. M. Crestfield, and S. Moore, *ibid.*, **240**, 2921 (1965).
(19) T. C. French and G. G. Hammes, *J. Am. Chem. Soc.*, **87**, 4669 (1965).
(20) R. E. Cathou and G. G. Hammes, *ibid.*, **87**, 4674 (1965).
(21) C. H. W. Hirs, S. Moore, and W. H. Stein, *J. Biol. Chem.*, **200**, 493 (1953).
(22) A. M. Crestfield, W. H. Stein, and S. Moore, *ibid.*, **238**, 618 (1963).
(23) C. H. W. Hirs, *ibid.*, **219**, 611 (1956).
(24) P. K. Glasoe and F. A. Long, *J. Phys. Chem.*, **64**, 188 (1960).
(25) N. C. Li, P. Tang, and R. Mathur, *ibid.*, **65**, 1074, (1961).

## Results

Initially, the chemical shifts of the protons attached to the carbon atoms designated α, β, C-2, and C-4 of L-histidine (see Figure 1) were measured in a 5% solu-

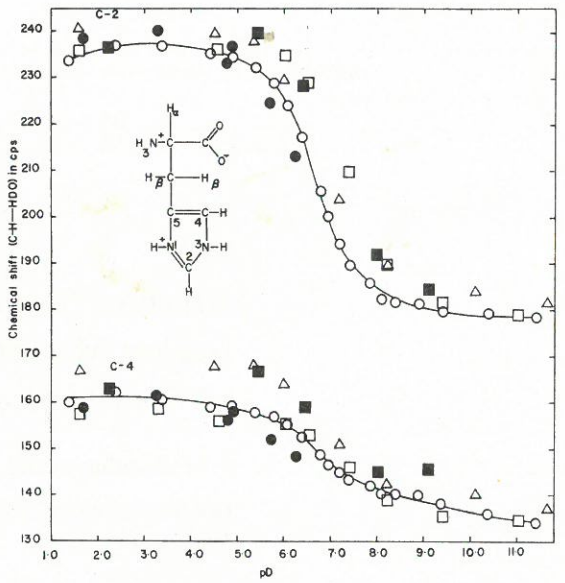


Figure 1. The chemical shifts of the C-2 and C-4 protons as a function of p*D* of L-histidine (○), glycyl-L-histidine (□), L-histidylglycine (△), poly-L-histidine (●), and imidazole (■). For the sake of clarity the lines for only L-histidine have been drawn.

tion at 20 different p*D* values between 1 and 11. The results are the same, within experimental error, as those obtained by McDonald and Phillips,[15] and confirm their interesting observations on the chemical shifts of the various protons due to the proximity of charged groups. The results for the p*D* dependence of the chemical shifts of the C-2 and C-4 protons of L-histidine and four histidine derivatives are given in Figure 1. There is no difficulty in the interpretation of these spectra since the C-2 and C-4 proton resonances are readily identified both by their positions and intensities relative to other resonances. The imidazole ring protons attached to nitrogen exchange rapidly with $D_2O$ and hence are not observed. The C-2 and C-4 resonances for poly-L-histidine were singlets but unfortunately the pH range open to study is limited due to insolubility in $D_2O$ above p*D* 6.4.

In most cases it was possible to calculate an apparent dissociation constant from the S-shaped curve obtained. These results are collected in Table I and compared, where possible, with literature values. The ionic strength could not be maintained constant because of the high concentration needed for nmr measurements and varied from 0.2 to 0.6. This has a direct effect on the chemical shift of the C-2 and C-4 protons[6] and also on the calculation of p*K'* from the data, since the latter is dependent on ionic strength. It is clear that the p*K'* values are only approximate and are therefore reported in Table I to two significant figures.

**DL-Histidyl-DL-histidine.** The nmr spectrum of DL-histidyl-DL-histidine was examined as a function of p*D*, and it was found that the C-2 protons of the two imidazole groups gave a single resonance at low and high

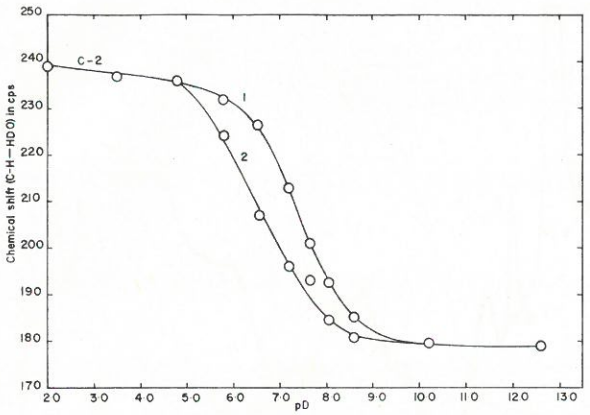


Figure 2. The chemical shifts of the C-2 protons in DL-histidyl-DL-hisitidine as a function of p$D$.

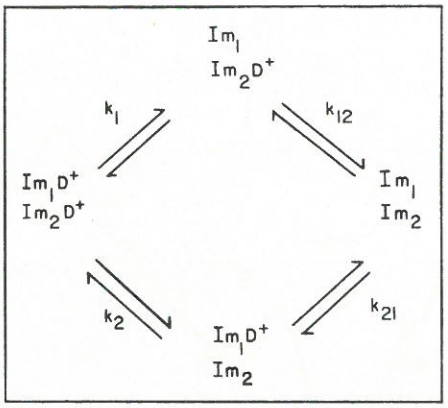


Figure 3. The ionization of the imadazole groups of DL-histidyl-DL-histidine, the carboxyl and amino groups being already ionized. $Im_1D^+$ = deuterated imidazole group 1, $Im_2$ = imidazole group 2 of the dipeptide, etc.

values of p$D$, but in the intermediate region there were two and in some cases three peaks. Two peaks of equal intensity would be expected in the intermediate region due to the different magnetic environments of the C-2 protons, but in fact there was an additional splitting of one of the peaks into a doublet with a separation which varied from about 0 to 15 cps depending on the p$D$. This splitting is almost certainly due to the fact that the sample consists of a mixture of two different racemates which have slightly different spectra. However, because of the smallness of the spectral difference, the position of the center of this doublet was recorded in the data of branch 2 of the graph in Figure 2. Branch 1 remained as a singlet throughout. Splitting of the signal due to the C-4 protons occurred but its complexity precluded any reasonable interpretation of the results.

**Table I.** Apparent Dissociation Constants of Imidazole Groups in Histidine Derivatives

| Samples | Present work, $D_2O$, 32° p$K_2'$ | p$K_3'$ | Lit. values, $D_2O$, 25° p$K_2'$ | p$K_3'$ | Lit. values, $H_2O$, 25° p$K_2'$ | p$K_3'$ |
|---|---|---|---|---|---|---|
| L-Histidine | 6.7 | | 6.65[25] | | 6.05[a] | |
| Glycyl-L-histidine | 7.4 | | | | 6.79[a] | |
| L-Histidyl-glycine | 6.9 | | | | 5.80[b] | |
| DL-Histidyl-DL-histidine | 6.2 | 7.7 | 6.05[25] | 7.30[25] | 5.51[c] | 6.80[c] |
| Oxidized ribonuclease | 7.4 | | | | | |

[a] D. D. Perrin, "Dissociation Constants of Organic Bases in Aqueous Solution," Butterworth and Co. (Publishers) Ltd., London, 1965. Mean of 10 values for L-histidine. [b] J. P. Greenstein, *J. Biol. Chem.*, **101**, 603 (1933). [c] Mean of 3 values reported by Perrin.[a]

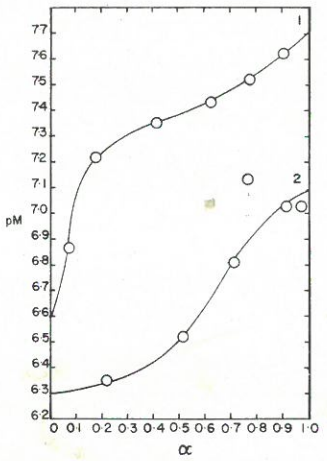


Figure 4. Graph of p$M$ *vs.* $\alpha$ for imidazole groups 1 and 2 of DL-histidyl-DL-histidine.

The results given in Figure 2 can be used to calculate the individual or microscopic ionization constants $k_1$, $k_{12}$, etc., for the complex ionization of the two imidazole groups as shown in Figure 3.[26] The microscopic ionization constants are defined by equations of the form

$$k_1 = (D^+)(Im_1Im_2D^+)/(Im_1D^+Im_2D^+) \quad (1)$$

where $(D^+)$ is the activity of deuterium ion present and the other two terms represent the concentrations of the doubly positively charged acid and the positively charged conjugate base. They are related to the macroscopic apparent dissociation constants $K_2'$ and $K_3'$ by the equations[26,27]

$$K_2' = k_1 + k_2 \text{ and } 1/K_3' = 1/k_{12} + 1/k_{21} \quad (2)$$

The apparent dissociation constants are readily determined from titration data but one requires additional experimental information about the composition of the solution as a function of p$D$, in order to be able to evaluate the microscopic constants. Such information is available from an nmr study which gives, at any particular p$D$, the fraction ($\alpha_1$) of molecules in which the imidazole group 1 is unprotonated and also the fraction ($\alpha_2$) of molecules in which the imidazole group 2 is unprotonated. Using the elegant method of Edsall, Martin, and Hollingworth[27] it is possible to construct graphs of

$$pM = pD - \log[\alpha/(1 - \alpha)] \quad (3)$$

*vs.* $\alpha$ (see Figure 4). Extrapolation of curve 1 to $\alpha = 0$ gives p$k_1$, and extrapolation to $\alpha = 1$ yields p$k_{21}$. The other two constants are then readily calculated.[30] Similar treatment of curve 2 produces, in all, two sets of

(26) J. T. Edsall and J. Wyman, "Biophysical Chemistry," Vol. 1, Academic Press Inc., New York, N. Y., 1958, Chapter 9.
(27) J. T. Edsall, R. B. Martin, and B. R. Hollingworth, *Proc. Natl. Acad. Sci. U. S.*, **44**, 505 (1958).

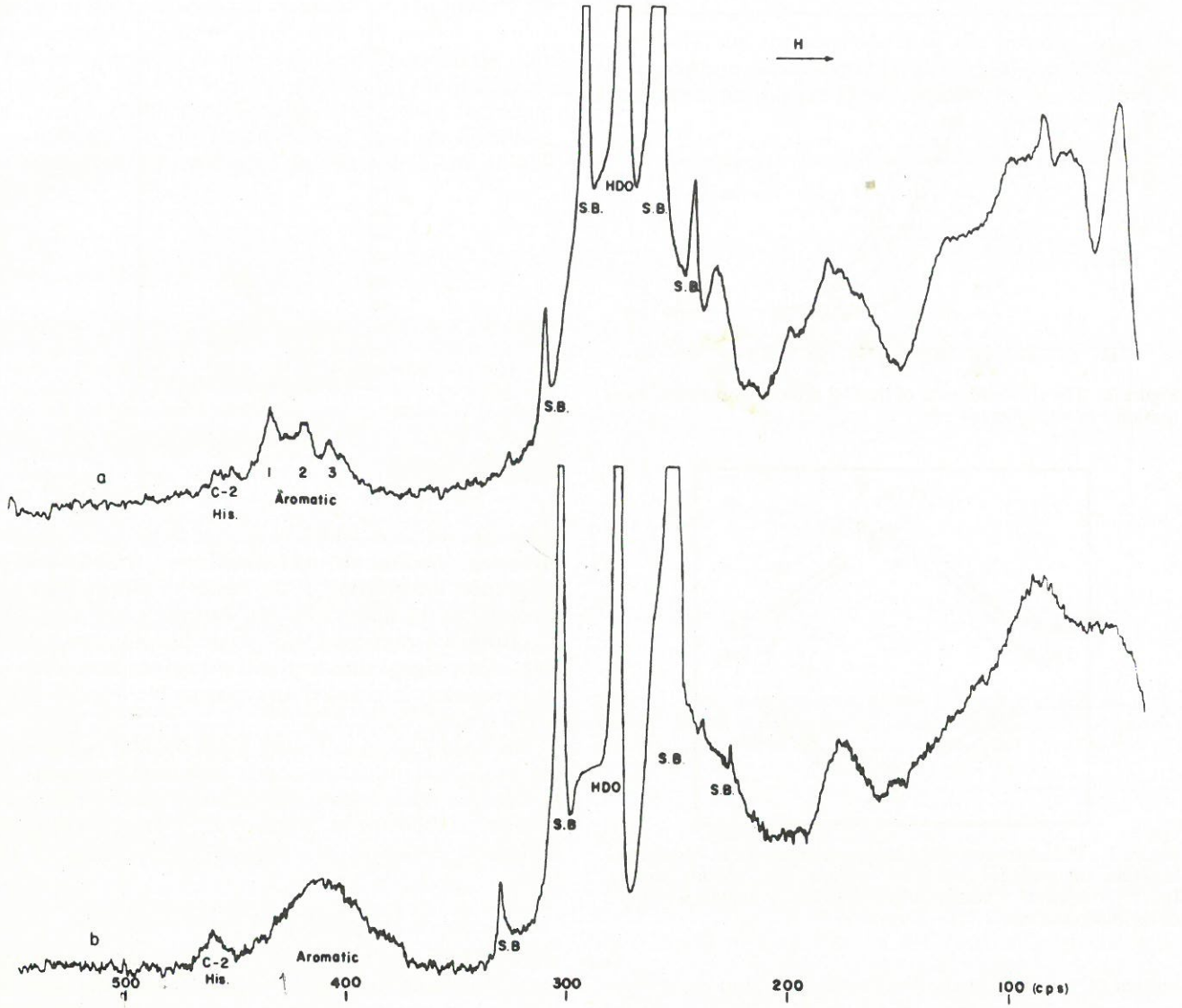


Figure 5. (a) Spectrum of a 25% solution of oxidized ribonuclease in $D_2O$ at p$D$ = 10.15; (b) spectrum of 20% solution of ribonuclease in $D_2O$ at p$D$ = 8.65. SB = Spinning side band.

results for the four microscopic dissociation constants. The latter are given in Table II, and the values of $K'_2$ and $K'_3$ calculated therefrom are recorded in Table I. It is seen that there are inaccuracies in extrapolation of curves 1 and 2, to $\alpha = 0$ and $\alpha = 1$, and this is reflected in the differences in $pk_{12}$ and $pk_{21}$ in Table II. There are also additional difficulties in that the ionic strength is not constant and that the results apply to a mixture of two different racemates. A detailed investigation of these factors is now in progress.

**Table II.** Microscopic Dissociation Constants of DL-Histidyl-DL-histidine[a]

| | Curve 1 | Curve 2 | Mean |
|---|---|---|---|
| $pk_1$ | 6.6 | 6.6 | 6.6 |
| $pk_2$ | 6.4 | 6.3 | 6.4 |
| $pk_{12}$ | 7.5 | 7.1 | 7.3 |
| $pk_{21}$ | 7.7 | 7.4 | 7.6 |

[a] Values calculated from Figure 4.

**Oxidized Ribonuclease.** The spectrum of oxidized ribonuclease was measured in $D_2O$ solution as a function of p$D$. A representative spectrum at p$D$ 10.15 is shown in Figure 5a and has the same characteristic peaks which have been reported by previous workers.[9-11] The only peaks which alter their position appreciably with alteration of p$D$ are the histidine resonances and those designated aromatic 2 and 3.

Confining our attention to this region of the spectrum it is noted in Figure 6 that the upfield displacement of the aromatic peaks 2 and 3 at high p$D$ is about 10–20 cps. Solutions of L-tyrosine in $D_2O$ show resonances in about the same position as aromatic peaks 2 and 3, and the former also give a similar upfield shift at high p$D$ due to ionization of the phenolic group.[28] On the other hand, aromatic peak 1 has the same chemical shift as the aromatic resonance of L-phenylalanine in $D_2O$ and both resonance positions are essentially independent of p$D$. Furthermore, six sets of area measurements were made for the four peaks shown in Figure 6b and relative areas of 4.3:17.5:16.1:9.2 were obtained, whereas the number of C-2, C-4, Phe, and Tyr protons in ribonuclease is 4, 4, 15, and 24, respectively. One can therefore conclude that at high p$D$

(28) We are indebted to Dr. H. Rüterjans for these measurements.

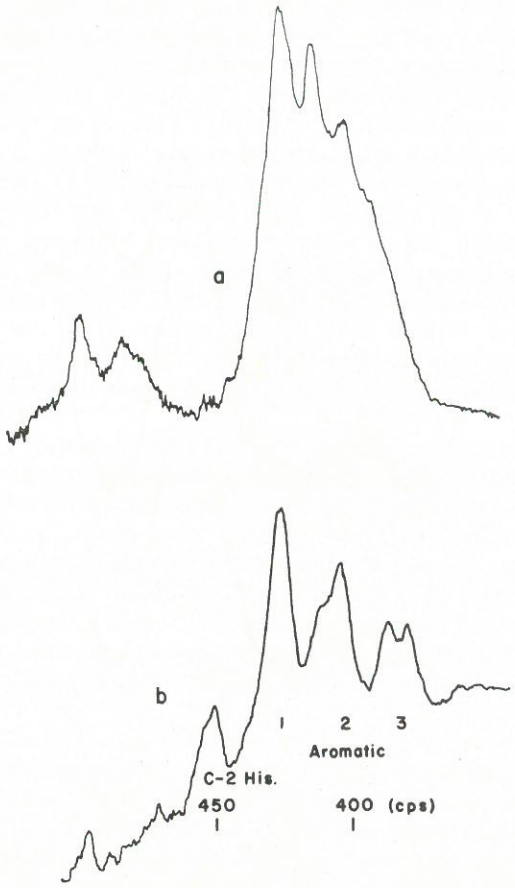


Figure 6. The spectrum of the aromatic and histidine protons of oxidized ribonuclease in $D_2O$ (a) at p$D$ 6.0, (b) p$D$ 12.4.

the resonance at lowest field is due to the C-2 histidine protons; the resonance labeled aromatic 1 represents the aromatic protons of phenylalanine, and aromatics 2 and 3 are due to the aromatic protons of tyrosine. The C-4 histidine protons are included in aromatics 1 and 2 as shown by the area measurements and also by their chemical shift in L-histidine and its derivatives.

As the p$D$ is progressively reduced below 9 there is a downfield shift of the C-2 histidine resonance, and at about p$D$ 6.5 the appearance of a second resonance. This is shown in Figure 6a, and the chemical shift data in Figure 7. The assignment of the C-2 histidine resonance is based on (i) its position with respect to HDO at both high and low pD values (compare Figures 1 and 2 with Figure 7), and (ii) the intensity of the resonance relative to the aromatic resonance (the mean of 8 measurements indicates 4.3 $\pm$0.5 protons). It is therefore clear that the four imidazole groups in oxidized ribonuclease are equivalent, and one can obtain from Figure 7 an approximate value of p$K'$ of 7.4.

The assignment of the additional resonance in Figure 6a is not possible with certainty at the present time. The resonance contains 4.1 $\pm$ 0.5 protons, on a basis of four area measurements compared with the aromatic resonance, and might therefore be attributed to the C-4 imidazole protons. However, the resonance position between p$D$ 1 and 6 is very different from that of the C-4 protons in a wide range of model compounds including poly-L-histidine (see Figure 1). Protons attached to nitrogen atoms are known to have a chemical shift in this region,[12] but it is known that all such

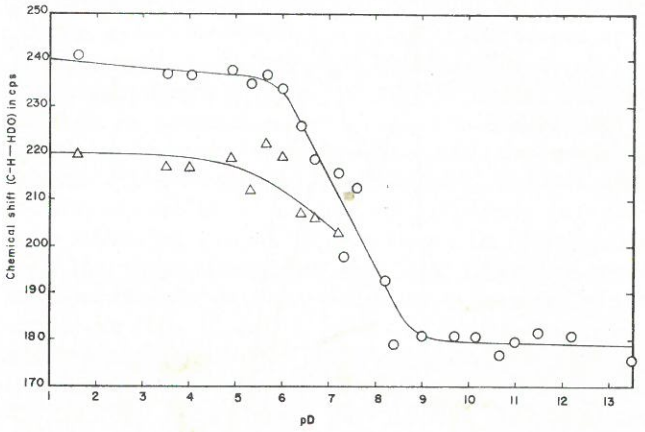


Figure 7. The effect of p$D$ on the chemical shift of the C-2 histidine protons (○) and a second resonance (△) of oxidized ribonuclease.

protons in oxidized ribonuclease exchange rapidly with $D_2O$,[29,30] and hence would not give an observable resonance. In view of the peak size (equivalent to four protons) and the alteration of its chemical shift as a function of p$D$ (which indicates a group with p$K'$ *ca.* 6 to 7), the most likely explanation is that the resonance is due to the C-4 imidazole protons.

**Ribonuclease.** The spectrum of ribonuclease at p$D$ 8.65 is given in Figure 5b and one notices (as previously observed)[9-11] the sharpening of the spectrum which occurs when the protein structure is opened up by oxidation. The aromatic peak is of particular interest because of the assignment of resonance 1 in Figure 5a to phenylalanine and resonances 2 and 3 to tyrosine (resonances 1 and 2 also contain four C-4 imidazole protons). In Figure 8 is shown the way in which the native aromatic resonance (trace a) is rapidly altered at p$D$ 2.02 and 32° to trace b. After cooling to 2°, the spectrum was measured again at 32° and trace a was obtained, showing that the unfolding is reversible. The new resonance in trace b is that of phenylalanine. It appears that reversible acid denaturation allows greatly increased freedom of movement of the phenylalanine as well as the tyrosine residues.[31] Clearly, by suitable choice of reaction conditions, the kinetics of this process could be investigated.

The spectrum of the C-2 protons of the four imidazole groups in ribonuclease are shown in Figure 9. Below p$D$ 4.85, the resonance is essentially single but at higher values of p$D$ there is splitting into three peaks, which move upfield (Figures 9b, c, d) and form one resonance again at p$D \geqslant$ 8.2 (Figure 5b). The chemical shift behavior is given in Figure 10. The assignment of the single resonance at low and high p$D$ to the C-2 protons of the imidazole rings is based on the good agreement between the chemical shift data and those for the various model compounds given in Figures 1 and 2. Also the mean value of the number of protons in the single C-2 resonance, taken over six measurements *vs.* the aromatic resonance, is 3.9 $\pm$ 1.0. Similar area measurements made on CAT traces for five sets of triplet resonances *vs.* the aromatic peak showed that the number of protons represented by branches 1, 2, and

(29) A. Hvidt, *Biochim. Biophys. Acta*, **18**, 306 (1955).
(30) A. Stracher, *Compt. Rend. Trav. Lab. Carlsberg*, **31**, 468 (1960).
(31) R. A. Scott and H. A. Scheraga, *J. Am. Chem. Soc.*, **85**, 3866 (1963).


Figure 8. Spectra of the aromatic resonance of a 25% solution of ribonuclease at p$D$ 2.02, (a) obtained immediately after heating from 2 to 32°, (b) obtained after maintaining the solution at 32° for 10 min and then measuring the spectrum with exactly the same instrumental conditions.

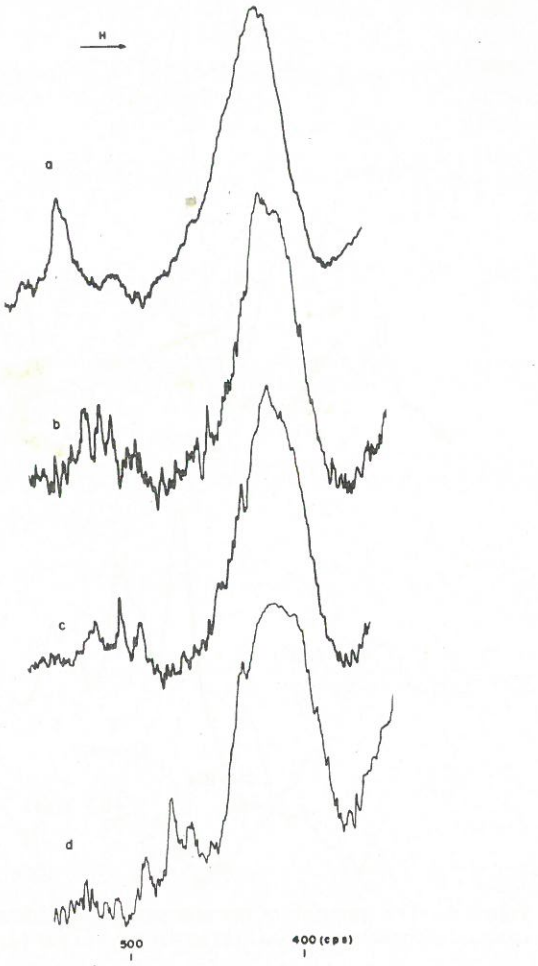


Figure 9. Spectra of a 25% solution of ribonuclease in $D_2O$ at (a) p$D$ 4.5; (b) p$D$ 5.37, 15 spectra combined by CAT; (c) p$D$ 5.89, 16 spectra combined by CAT; (d) p$D$ 6.92, 15 spectra combined by CAT.

3 in Figure 10 is: 1.1 ± 0.2, 1.3 ± 0.3, 1.6 ± 0.3, respectively. It therefore seems likely that branches 1 and 2 each represent one histidine residue, and branch 3 represents two histidine residues.

## Discussion

There is good agreement between the results for the apparent dissociation constant of L-histidine in Table I and the literature values. If one assumes that the increase in p$K'$ in going from $H_2O$ to $D_2O$ is about 0.55 (the average of the values for L-histidine and DL-histidyl-DL-histidine) for these histidine derivatives, the agreement for glycyl-L-histidine is also excellent. However, the values for L-histidyl-glycine and the p$K_3'$ of DL-histidyl-DL-histidine are both 0.4–0.6 p$K$ unit higher than the literature values.

In glycyl-L-histidine, the charged amino group is further away from the imidazole group than in histidine, and hence there is an increase in p$K'$ (in the complete absence of the amino group in imidazole itself p$K'$ = 7.08, in water).[32] The opposite effect, owing to the electrostatic attractive force of the carboxyl group for the deuteron, is not nearly so marked in comparing histidine and L-histidyl-glycine (in fact our results indicate a trend opposite to that expected). In oxidized ribonuclease, in which the four imidazole groups have

(32) J. P. Greenstein and M. Winitz, "Chemistry of the Amino Acids," Vol. 1, John Wiley and Sons, Inc., New York, N. Y., 1961, p 500.

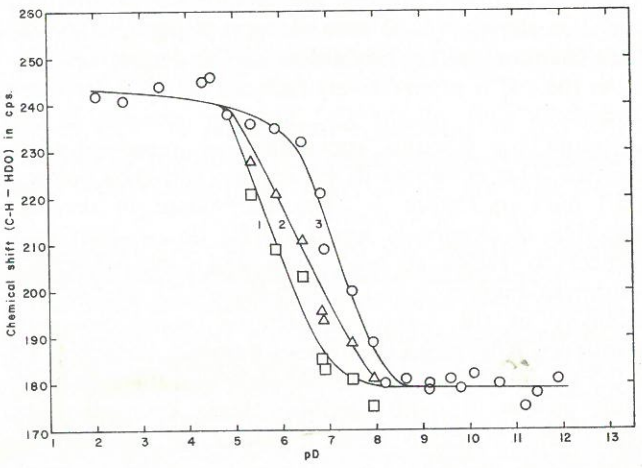


Figure 10. The chemical shifts of the C-2 protons of ribonuclease as a function of p$D$. Different symbols are used for the three branches in order to avoid confusion.

been normalized, it is likely that the high p$K'$ value o 7.4 is due to the fact that the imidazole rings are unlikely to be in the vicinity of positively charged groups but they may be near negatively charged carboxyl or sulfonic acid groups.

In considering the results obtained with DL-histidyl-DL-histidine, it is reasonable to suppose that the imid-

azole group nearest the amino group has a lower p$k$ than the imidazole group nearest the carboxyl group and hence they have subscripts 2 and 1, respectively (see Table II and Figure 3). However, the effect of proximity of an imidazole group to an amino group rather than a carboxyl group is surprisingly small (0.2–0.3 p$k$ unit). On the other hand, the placing of a positive charge on one imidazole group displaces the p$k$ of the other imidazole group downwards by 0.9–1.0 ($pk_{21} - pk_1$ or $pk_{12} - pk_2$). It is also worth noting that $pK_2'$ (6.2) and $pK_3'$ (7.7) in Table I cannot be considered to represent the ionization of the first and second imidazole groups, respectively; a precise description of the total situation can be given only in terms of the microscopic constants.[27]

The formulation of the problem when one has three ionizing groups of approximately the same p$K$ involves 12 different microscopic dissociation constants.[26] The situation is even more complex with the histidines of ribonuclease because, as shown in Figures 9 and 10, we have four histidines, two of which have apparently the same p$K$. Thus there can be no question of attempting to calculate the microscopic dissociation constants from the results given in Figure 10.

It is seen from the treatment of DL-histidyl-DL-histidine that the chemical shift data allow one to calculate $\alpha_1$ and $\alpha_2$, *i.e.*, the fraction of molecules in which the imidazole groups of types 1 and 2, respectively, are unprotonated. According to eq 3, when $\alpha = 0.50$, the function p$M$ = p$D$. It is seen from Figure 4 that the value of, say, p$M_1$ at $\alpha = 0.5$ on curve 1 is very roughly an average of p$k_1$ (p$M_1$ at $\alpha = 0$) and p$k_{21}$ (p$M_1$ at $\alpha = 1$), where p$k_1$ represents dissociation from the doubly protonated and p$k_{21}$ from the singly protonated imidazole species, respectively (see Figure 3).[33] Similarly, for ribonuclease the value of p$M$ at $\alpha = 0.5$, which is actually the value of p$D$ at the midpoint of graphs 1, 2, and 3 in Figure 10, represents a very approximate average of the p$k$ values for the ionization of one particular imidazole group. The values at $\alpha = 0.5$ are p$M_1$ = 5.9, p$M_2$ = 6.3, p$M_3$ = 7.1.

The assignment of these groups to particular histidine residues in ribonuclease is largely speculative and is based on intensity measurements and the work of others. Thus, intensity measurements indicate that the nmr resonance 3 contains approximately two protons indicating two histidine residues in ribonuclease with the same average p$k$ (*ca.* 7.1). There is some evidence that the two histidine residues involved in the active site (His 12 and 119) have differing reactivities and low p$k$ values.[17,18,34] It is therefore likely that the two histidine residues with average p$k$ $\sim$ 7.1 are His 48 and 105. The average p$k$ values in $D_2O$ of about 5.9 and 6.3 are then allotted to the two histidine residues involved in the active site. An unequivocal assignment of nmr resonances should be possible by examination of the spectra of ribonuclease in which His 12 or 119, respectively, is modified.[18] This work is in progress.

After making allowance for the fact that the average p$k$ values should be reduced by about 0.50 to convert from $D_2O$ to $H_2O$ this leaves His 12 or 119 p$k$ $\sim$ 5.4, His 12 or 119 p$k$ $\sim$ 5.8, His 48 and 105 p$k$ $\sim$ 6.6 in water. In view of the errors involved in the measurements, the results of Donovan[35] obtained by ultraviolet-difference spectroscopy (either four histidines of p$K$ = 5.9 or two His p$K$ = 5.6 and two His p$K$ = 6.2) are not in disagreement. Recent kinetic studies[19,20] implicate three ionizable groups of p$K$ = 5, 6, and 6.7, and binding studies[36] implicate two groups of p$K$ = 5.1 and 6.1 at the active site of ribonuclease. Our average p$k$ values fit reasonably well but the two groups at an average p$k$ of 6.6 are considered not to be involved in the active site. Obviously, more experimental information is necessary in order to settle this question.

(33) It should be noted that this approximation becomes worse the greater the deviation of the ratio $k_1/k_2$ from unity. In this case, $k_1/k_2 \sim 2$ but for values of 5 or 0.2 the errors would be much greater.[27]

(34) A. P. Mathias, A. Deavin, and B. R. Robin in "Structure and Activity of Enzymes," T. W. Goodwin, J. I. Harris, and B. S. Hartley, Ed., Academic Press Inc., New York, N. Y., 1964, p 19.
(35) J. W. Donovan, *Biochemistry*, **4**, 823 (1965).
(36) J. P. Hummel and H. Witzel, *J. Biol. Chem.*, **241**, 1023 (1966).