Spectrophotometric Titration of the Imidazole Groups of Bovine Pancreatic Ribonuclease*

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ABSTRACT: The dissociation of protons from the imidazole groups of ribonuclease can be followed as a function of *p*H by measurements of ultraviolet difference spectra in the wavelength region near 235 mμ. The dissociation is reversible and, in agreement with earlier potentiometric studies, an average intrinsic *pK* of 6.5 has been calculated for the four imidazole groups. The *p*H dependence of difference spectra in the 250- to 300-mμ wavelength region shows charge perturbation by imidazole and α-amino groups of one or more of the abnormal tyrosine residues, and charge perturbation by an imidazole group of one or more of the phenylalanines in this molecule. Spatial proximity of these ionizable groups and chromophores is highly probable.

The spectrophotometric titration of ionizable chromophores in proteins has provided a remarkable amount of information, not only about the ionization of these chromophores, but also about the conformation of proteins. The most widely studied chromophore in this respect has been the phenolic chromophore of the tyrosine residue. The change in absorbancy resulting from its ionization in proteins was first studied by Stenström and Reinhard (1925). Further studies by Crammer and Neuberger (1943), Shugar (1952), and Tanford *et al.* (1955), to mention only a few examples, have demonstrated how the abnormalities in the ionization of this chromophore can be interpreted in terms of the tertiary structure of particular proteins.

More recently, it has proved possible to follow the ionization of the sulfhydryl group of the cysteine residue in proteins by the spectrophotometric method (Benesch and Benesch, 1955, 1959; Donovan, 1964). Just as for the phenolic chromophore, information can be obtained not only about the ionization of the sulfhydryl chromophore, but also about the *p*H dependence of the conformation of the protein.

The present study was undertaken in an effort to extend the spectrophotometric method to the ionization of the imidazole chromophore of the histidine residue in proteins. Since this chromophore has often been implicated in enzymatic activity, and presumably is part of the active site of some enzymes (including ribonuclease, the protein studied here), its ionization and binding properties are of particular interest.

The remarks made previously in connection with sulfhydryl groups (Donovan, 1964) about the difficulties of spectrophotometric titrations in the 235-mμ region are applicable in the present case. In addition, the change in absorption of the imidazole chromophore on dissociation of a proton is approximately ten times smaller than the corresponding change for the sulfhydryl group. The accuracy and precision of the present measurements therefore suffer in comparison with corresponding measurements for sulfhydryl groups. Fortunately, ribonuclease does not appear to undergo a conformational change in the *p*H region in which the imidazole groups ionize. Such a conformational change, together with the very small change in absorbance observed here, would probably have completely prevented the spectrophotometric observation of imidazole ionization.

Experimental

Spectrophotometric measurements were obtained using a Cary Model 15 recording spectrophotometer equipped with PM 49 photomultipliers. Usual conditions of operation were: high photomultiplier dynode voltage, 0.8-second time constant for pen damping, and scan rate of 1 A/sec. The 0.1 OD scale was used for difference measurements, readings being taken from the chart to 0.0001 OD unit. Reproducibility averaged 0.0002 OD unit, determined from duplicate baseline (reference solution–reference solution) scans on different days.

Since only a small change in absorption is produced by the ionization of the imidazole chromophores in ribonuclease, in these measurements using the difference technique it was desirable to use as concentrated a protein solution as experimentally feasible. Because unabsorbed stray light from the monochromator gives incorrect values for absorption, and because large slit widths give poor resolution, a maximum absorbance for the protein solutions of 2 OD units at the working wavelengths (near 235 mμ) was chosen. Thus, 1% of the incident light passed through the sample and reference solutions (of approximately equal absorbance). Since the stray light from the monochromator is less

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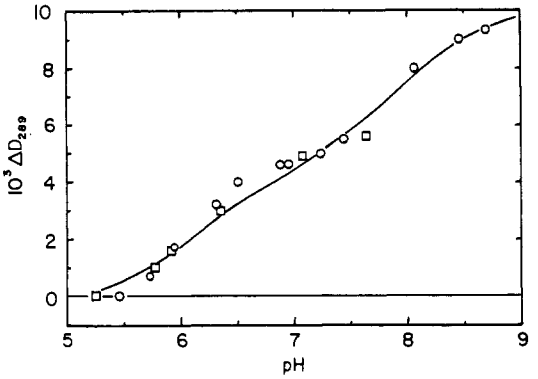

FIGURE 1: The *p*H dependence of the tyrosine difference spectrum observed for 8.23×10^{-5} M solutions of ribonuclease, reference *p*H 5.4, no salt added. Circles, forward titration to higher *p*H; squares, back titration from *p*H 9. The curve drawn through the points is a calculated titration curve for two ionizable groups with apparent *pK* of 6.1 and 7.9, with the height of the first step, 0.0046 OD unit; the second step, 0.0060 OD unit.

than 0.001% in this wavelength range (Applied Physics Corp., 1963), the ratio of transmitted light at the nominal wavelength to stray light (assumed unabsorbed) is 1000. At this concentration of protein, the change in absorbance for complete ionization of the imidazole groups was about 0.02 OD unit at the 237-mμ peak in the difference spectrum. Thus, the intensity of light transmitted through the sample solution was reduced by only 5% by the change in absorbance of the imidazole chromophores, and the ratio of intensities of light at the nominal wavelength to stray light was not significantly altered. Even if the stray light level were *one hundred times* that specified, essentially the same difference spectra would have been recorded, only about a 10% reduction in their magnitude being noted. For the difference spectra presented for ribonuclease, the spectral slit width (half-intensity band width) at the 237-mμ difference peak was less than 10 A. It increased with decreasing wavelength, and was 25 A at 212 mμ, the shortest wavelength for which data are presented for protein solutions.

Two different samples of bovine pancreatic ribonuclease were used in these experiments, Armour lot 381-059 and Sigma Chromatographed Grade Type II, lot R101B-67. Most of the data presented are for the Sigma material. Solutions were prepared by dissolving the dry protein in water, then adding KCl solution (when used) to give the desired ionic strength. The resulting solutions were used directly, without dialysis. Solutions were not prepared using pipets, as in earlier work (Donovan *et al.*, 1961), since errors from pipetting are too large for the present experiments. Care was taken to prevent evaporation, the largest source of error. In some cases, corrections for evaporation were made upon the observed spectra. Except for the *p*H, the sample and reference solutions were identical. One-cm matched cells were used. The *p*H of the sample was adjusted with concentrated HCl (12 N) or KOH (10 N). Dilution corrections were negligible, since very small amounts of acid and base were required in this limited *p*H range near neutrality. Readings of *p*H were made with a Beckman Model G *p*H meter with probe electrodes, calibrated against buffers prepared according to National Bureau of Standards specifications. All measurements of *p*H and absorbance were at 22°. Compounds used were: histidine methyl ester, CalBiochem lot 120607; imidazole, cat. 8826 of Matheson Coleman and Bell; acetyltyrosine ethyl ester, Mann chromatographically pure lot D3678.

Results

At *p*H between 4 and 7, one of the prominent changes in the absorption spectrum of ribonuclease occurs near 290 mμ. Two peaks, characteristic of the perturbation of the spectrum of phenolic groups (Laskowski *et al.*, 1956), were observed in the difference spectrum at 281 and 289 mμ, the first about half the height of the other, as is usual. This difference spectrum observed between *p*H 4 and 7 has not been reported previously. It is an order of magnitude less intense than the phenolic difference spectra which have been observed when ribonuclease is denatured by heat or acid.

Figure 1 shows the *p*H dependence of the 289-mμ difference peak. Above *p*H about 7.5, the observed values have been corrected for the superimposed difference spectrum for the ionization of the phenolic groups of the normal tyrosine residues (peak maximum at 295 mμ). Above *p*H about 9, the magnitude of the difference spectrum for tyrosine ionization makes it difficult to determine the value of ΔD_{289} with much accuracy. The observed value of ΔD_{289} (*p*H 7–4) was 0.0016 at 3.0×10^{-5} M and 0.0046 at 8.2×10^{-5} M. Beer's law is obeyed within the limits of experimental error, and thus the observed $\Delta\epsilon_{289}$ is 56 ± 3. This is the same order of magnitude as the spectral change produced on the phenolic chromophore of the amino acid tyrosine by ionization of its carboxyl group (Wetlaufer *et al.*, 1958; Malik, 1962, cited by Wetlaufer, 1962). This perturbation of the phenolic chromophores in ribonuclease is reversible from *p*H 9, and does not appear to be time dependent.

Difference spectra for the ionization of the imino groups in imidazole, histidine methyl ester, and ribonuclease are shown in Figure 2. Values of $|\Delta\epsilon|$ at the longest wavelength difference peak agree quite well with earlier published values (Donovan *et al.*, 1961), but the present difference spectrum for imidazole appears to occur at somewhat shorter wavelengths than reported previously. The direction of this change in wavelength indicates that the stray light level of the previous experiments was probably much greater than in the present experiments, and increased markedly with decreasing wavelength. The sign of $\Delta\epsilon$ for the ionization of the imino group of histidine methyl ester is the same as that for imidazole, in contrast to the

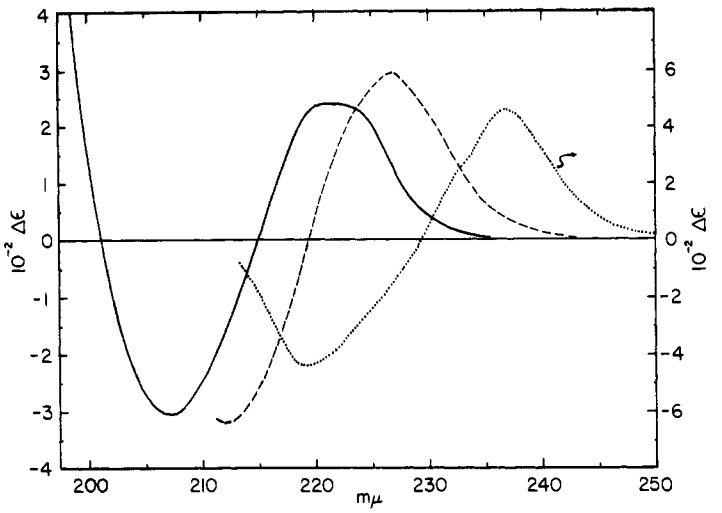

FIGURE 2: Difference spectra for the dissociation of a proton from the imidazole chromophore. Solid curve, imidazole, *p*H 9.5 versus *p*H 3.3, determined at a concentration of 4.4×10^{-4} M; dashed curve, histidine methyl ester, *p*H 6.6 versus *p*H 4.1, determined at a concentration of 6.9×10^{-4} M; dotted curve, ribonuclease, *p*H 6.5 versus *p*H 5.0, determined at a concentration of 3.0×10^{-5} M, no salt present, and uncorrected for superimposed phenolic perturbation.

sign of $\Delta\epsilon$ for the ionization of the imino group in histidine.

The *p*H dependence of the difference peak at 237 mμ for ribonuclease, uncorrected for superposed phenolic perturbations, is given in Figure 3. Beer's law appears to be obeyed. No time dependence of the difference spectrum was observed, and the titration curve appeared to be reversible (after a few minutes from adjustment of *p*H). The apparent *pK* of the imidazole groups calculated from this curve is 5.9.

Apart from a tendency to give inconvenient amounts of turbidity when dissolved in salt solutions, the Armour lot of ribonuclease listed gave results essentially identical to those obtained with the Sigma lot in all the experiments in which comparisons were made. In particular, the difference spectra of 3×10^{-5} M solutions of the Armour and Sigma materials, between *p*H 7 and 4, did not differ by more than 0.001 OD unit at all wavelengths between 225 and 260 mμ. At this concentration of protein, 0.001 OD unit is 5% of the observed difference peak height at 237 mμ.

Between *p*H 5 and 7 the difference spectra of ribonuclease exhibit very small but recognizable peaks at 249, 254, 259, and 266 mμ; $\Delta\epsilon_{259}$ (*p*H 7–5) $\simeq$ 8. These are interpreted as a difference spectrum of one or more of the three phenylalanine chromophores of ribonuclease. A difference spectrum of the amino acid phenylalanine, produced by the ionization of its carboxyl group, has been published previously (Donovan *et al.*, 1961).

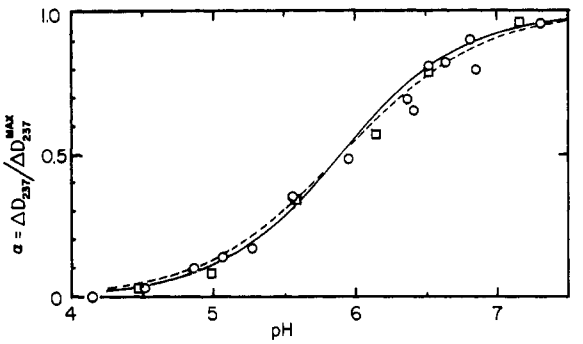

FIGURE 3: Spectrophotometric titration curve of the imidazole groups of ribonuclease, reference *p*H 4.1. Circles, forward titration to higher *p*H in 0.01 M KCl at 4.53×10^{-5} M; squares, reverse titration from *p*H 6.5, no salt present, at 3.05×10^{-5} M. The solid curve drawn through the points is a calculated titration curve for four groups of identical apparent *pK* of 5.9, the dashed curve for two sets of two groups each with apparent *pK* of 5.6 and 6.2.

Discussion

Tyrosine Perturbation Difference Spectrum. Since the imidazole difference spectrum requires correction for the change in absorption of phenolic chromophores in the same wavelength region, the change in absorption of the phenolic chromophores will be treated first.

The variation with *p*H of the perturbation of the phenolic chromophores of the tyrosine residues, presented in Figure 1, is probably not due to conformational changes in the RNAase molecule in which the chromophores become exposed to the solvent. The abnormal tyrosine residues do not release hydrogen ion to the solvent until above *p*H 11, so that solvent penetration to these residues at lower *p*H values is un-

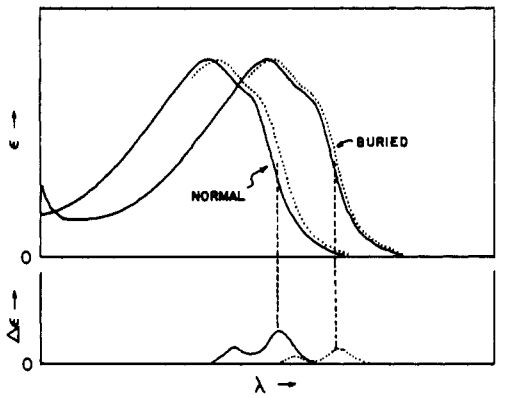

FIGURE 4: Schematic illustration of the relation between the spectra and the difference spectra of "normal" and "buried" chromophores. At top, the solid lines are intended to represent the spectra of "normal" (left) and "buried" (right) chromophores. The dotted lines are these spectra shifted to the red upon application of a perturbation to these chromophores. The differences between the shifted and the unshifted spectra are given at bottom. The difference spectrum for the perturbation of the buried chromophore appears at longer wavelength than that for the normal chromophore. The heights of the difference spectra are proportional to the shifts in each case. Not to scale. Red shift exaggerated.

likely. Furthermore, the sign of the spectral change is opposite to that resulting from exposure of the chromophore to water solvent. If there is a conformational change, it is one resulting in a net increase in refractive index (polarizability) of that portion of the RNAase molecule surrounding tyrosine residues.

It appears more likely that the perturbation results from the release of protons from one or more imidazole groups, and from the α-amino group. The same phenolic chromophore or more than one phenolic chromophore may be affected by these imidazole and α-amino groups. However, it appears likely that only one phenolic chromophore is close to the α-amino group and only one is close to a histidine residue. The release of protons from the perturbing groups may produce a direct electrostatic perturbation ("charge effect") of a phenolic chromophore, or induce a small conformational change in its neighborhood. No effect of ionic strength was observed in the range 0–0.01 M KCl.

It is necessary to account for the position of the peaks in the observed tyrosine perturbation difference spectrum. If the perturbation of a chromophore results simply in a shift of the spectrum of the chromophore, then the difference spectrum will have positive and negative peaks approximately at points of maximum slope in the original spectrum. The magnitude of the difference spectrum will be directly determined by the amount of the shift. For the wavelengths of the peaks in the difference spectrum, the following relation is approximately true (Chervenka, 1959; Donovan *et al.*, 1961): $\Delta\epsilon = -(d\epsilon/d\lambda)\Delta\lambda$, where $\Delta\lambda$ is taken as positive for a red shift.

Only the larger of the two peaks in the tyrosine difference spectrum will be considered, for simplicity. An examination of Figure 4 will be helpful in understanding the argument following. In acetyltyrosine ethyl ester (a model for the tyrosine residue in a protein) the maximum slope at the long-wavelength end of the ultraviolet spectrum is -200 ± 10 $\epsilon/m\mu$, at 285.0 ± 0.3 mμ. The point of maximum slope of the spectrum of the phenolic chromophores of the normal tyrosine residues in ribonuclease can be determined as follows: Perturbation of these chromophores, which are in contact with the solvent, by addition of ethylene glycol to the amount of 20%, gives difference peaks at 278.5 and 286.0 mμ, with $\Delta\epsilon_{286} = 120$ for each of three residues (Herskovits and Laskowski, 1960; experiments repeated by author). Thus, this solvent causes a shift of 0.6 mμ. Since the wavelength of the peak in the difference spectrum is halfway between the points of maximum slope of the shifted and unshifted spectra, the point of maximum slope of the spectra of the normal phenolic chromophores in ribonuclease is at $286.0 - 0.3$, or 285.7 mμ, about 0.7 mμ to the red of that for acetyltyrosine ethyl ester.

The point of maximum slope for the phenolic chromophore(s) perturbed by the *p*H change in the neutral *p*H region can be determined as follows: Perturbation of these chromophores produces the difference spectrum peaks at 281.5 and 289.0 mμ, with $\Delta\epsilon$ at the latter wavelength equal to 56 (at *p*H 5, see Figure 1). This shift must be about 0.3 mμ (assuming one phenolic chromophore), so the point of maximum slope of the charge-perturbed chromophore, before charge perturbation, must be at $289.0 - 0.15$, or 288.9 mμ at *p*H 5 (289.1 mμ at *p*H 7). This is based on the assumption of one chromophore; for more than one chromophore, the wavelength calculated would be closer to 289.0 mμ. Thus (assuming only one chromophore) the spectrum of the chromophore must be shifted an amount: $288.9 - 285.7 = 3.2$ mμ to the red. This residue must be one of the three abnormal tyrosine residues of ribonuclease. Using the foregoing relation, the expected value of $\Delta\epsilon$ for complete normalization of this chromophore should be: $-200 \times 3.2 = -640$. The wavelength at which this $\Delta\epsilon$ of normalization would be observed (the peak wavelength of the denaturation difference spectrum) is $288.9 - 1.6$, or 287.3 mμ. This wavelength of 287 mμ, approximately, is characteristic of the normalization of the abnormal tyrosine residues of ribonuclease (Scheraga, 1957; Sela and Anfinsen, 1957; Bigelow, 1960, 1961; Hermans and Scheraga, 1961).

It is of interest to determine, if possible, which of the abnormal tyrosine residues (assuming only one) is being perturbed by one or more of the histidine residues. A recalculation of the $\Delta\epsilon$ of normalization of the three abnormal tyrosines, using the general method of Bigelow (1960) upon the data available in the literature (Scheraga, 1957; Sela and Anfinsen, 1957; Bigelow, 1960, 1961; Hermans and Scheraga, 1961; Scott and

Scheraga, 1963) gave the following values of $-\Delta\epsilon$ at *p*H 7: tyrosine A, 920; tyrosine B, 860 (360 at acid *p*H); tyrosine C, 720. Using the calculated values of the points of maximum slope of the spectrum of the charge-perturbed phenolic chromophore from the present experiments, values of $-\Delta\epsilon$ for normalization of this residue at *p*H 5 and 7, respectively, are 640 and 715. This would identify the abnormal tyrosine (again, assuming only one) as tyrosine C. It has been suggested that tyrosine C is residue number 97 (Scott and Scheraga, 1963). However, the accuracy of the present calculations, as well as the identification of tyrosine A, B, and C, are subject to a considerable amount of uncertainty, and for the time being it is best to conclude from these experiments only that the tyrosine residue perturbed by histidine is an abnormal tyrosine.

The phenolic perturbation by the α-amino group is of particular interest. First, it lends support to suggestions (Vithayathil and Richards, 1960; Potts *et al.*, 1963) that the residues close to the N-terminal end of the molecule are attached to the body of the molecule, rather than "wagging" freely in solution. Although the N-terminal lysine residue is not essential for activity (Eaker, 1961), it must remain in a fixed position on the molecule in order for the α-amino group to produce the strong charge perturbation on a phenolic chromophore observed in these experiments. Second, the phenolic chromophore which the α-amino group perturbs has characteristics which closely resemble those attributable to tyrosine C, which may be tyrosine 97. The spatial association of histidine residues 12 and 119 (Crestfield *et al.*, 1963) allows conformations of the ribonuclease molecule in which the α-amino group is close to tyrosine 97.

Imidazole Ionization Difference Spectra. The difference spectra for the ionization of the model compounds containing the imidazole chromophore (Figure 2) show, in order of decreasing wavelength, a positive peak, a negative peak of approximately equal height, then a positive peak (not shown) deeper in the ultraviolet (194 and 198 mμ for imidazole and histidine methyl ester, respectively). Only the positive peak of longest wavelength will be considered here, since the wavelength range imposed by the strong absorption of other chromophores present in proteins precludes the accurate observation of the negative peak. Values of $\Delta\epsilon$ are: imidazole, $\Delta\epsilon_{222} = 245$; histidine methyl ester, $\Delta\epsilon_{227} = 295$. Areas under these peaks are: imidazole, 2500 $\Delta\epsilon \cdot$mμ; histidine methyl ester, 3300 $\Delta\epsilon \cdot$mμ.

The imidazole ionization difference spectrum for ribonuclease, also shown in Figure 2, closely resembles the difference spectra of the imidazole model compounds, even without correction for the tyrosine perturbation difference spectrum. However, it is considerably shifted to the red compared with the model compounds. The observed difference spectrum requires correction, since the tyrosine perturbation difference spectrum, with positive peaks at 281 and 289 mμ, must also have a negative peak in the 235-mμ region (Malik, 1962). Assuming an equal frequency shift for the negative tyrosine difference peak at 232.5 mμ,

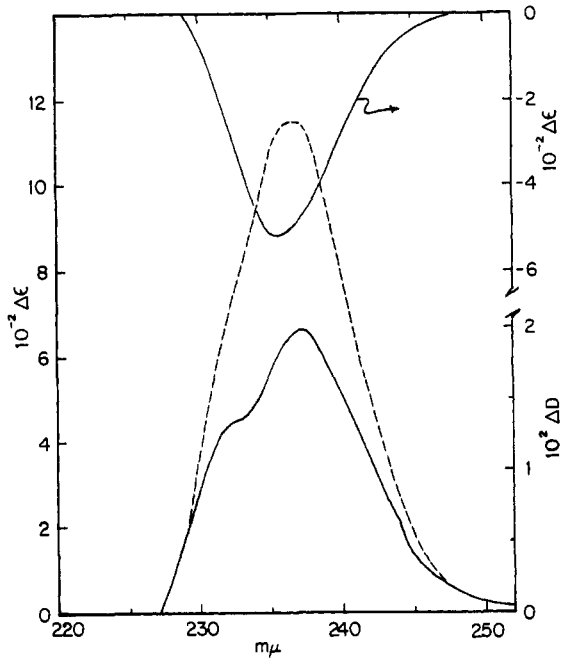

FIGURE 5: Calculation of the corrected difference spectrum for the dissociation of protons from the four imidazole groups of ribonuclease. Lower solid curve, observed difference spectrum for a *p*H 7.0 solution against a *p*H 4.1 reference solution, concentration 3.05×10^{-5} M; upper solid curve, calculated difference spectrum for perturbed tyrosine chromophores (see text); dashed curve, imidazole difference spectrum corrected for superimposed tyrosine difference spectrum.

this peak would be shifted 3 mμ to 235.5 mμ. The correction curve, calculated from the magnitude of the 289-mμ peak, is shown as the solid line at the top of Figure 5. When this (negative) correction curve is subtracted from the observed difference spectrum for the imidazole ionization, the corrected imidazole difference spectrum (dashed line) shown in Figure 5 is obtained. The height of the peak of the corrected curve, its half-width, and area are approximately what would be expected from four imidazole groups on the basis of the difference spectrum for histidine methyl ester. The peak $\Delta\epsilon$ of 1150 $\pm$ 120 at 237 mμ is 3.9 $\pm$ 0.4 times that of histidine methyl ester and 4.7 $\pm$ 0.5 times that of imidazole. The width at half peak-height is 10.6 mμ for the corrected imidazole difference peak, 10.6 mμ for histidine methyl ester, and 10.2 mμ for imidazole. The area under the difference peak for the ribonuclease imidazole ionization is 12,000 $\Delta\epsilon \cdot$mμ, 3.5 $\pm$ 0.4 times that under the histidine methyl ester difference peak, and 4.8 $\pm$ 0.5 times that under the imidazole difference peak.

The position of the peak wavelength of the difference spectrum of the imidazoles in RNAase deserves comment. The positive difference peak is at 237 mμ. The corresponding wavelengths for imidazole and histidine methyl ester are 222 and 227 mμ. It appears that the

spectra of the imidazoles in RNAase are shifted anomalously far to the red for normal imidazoles. The maximum slope of the absorption curve of imidazole is -270 $\epsilon/m\mu$. Since the difference extinction coefficient for imidazole is 245, only a 1-mμ shift in the spectrum occurs when dissociation of a proton takes place. Thus this small shift cannot explain the large red shift observed, and indicates that the spectra of both the undissociated and dissociated forms are shifted to the red by 10 mμ compared with the model compound histidine methyl ester. This shift may be a result of a partial hydrocarbonlike environment of these chromophores, or a strong charge perturbation, such as may occur in the "clusters" of charge proposed by Carroll and Saroff (1962) and Loeb and Saroff (1964).

Titration Curve of the Imidazole Groups. The titration curve of the imidazole groups given in Figure 3, determined from measurements of the peak in the difference spectrum, appears to correspond closely with the potentiometric titration curve of Tanford and Hauenstein (1956). Although the spectrophotometric data can be fitted slightly better by a plot of degree of ionization versus *p*H for two groups of two imidazoles each, with apparent *pK* of 5.6 and 6.2, than by a similar plot for four imidazoles of apparent *pK* 5.9, the precision of the data is not sufficient to distinguish between these alternatives. Intrinsic *pK* values for the cases above are 6.2 and 6.8 for the first case and 6.5 for the second case. In this calculation, Tanford and Hauenstein's (1956) electrostatic factor of 0.112 for 0.01 ionic strength was used. Tanford and Hauenstein report an intrinsic *pK* for the imidazoles (considered identical) of 6.5.

The correction given in Figure 5 was not applied to the titration curve of the imidazoles (Figure 3) because of the limited accuracy of the first portion of the curve in Figure 1, which would form the basis for these calculations. Since the perturbation given in Figure 1 has the same *p*H dependence as one of the imidazole groups, it was considered better not to make the correction. If the *pK* of 6.1 quoted above for the first part of the curve in Figure 1 is correct, then if the correction were made it would raise the pK_{int} given here by 0.1 *pK* unit.

Phenylalanine Perturbation Difference Spectrum. A comparison of the magnitude of the difference spectrum of the benzene chromophore of phenylalanine to the magnitude of its absorption spectrum shows that the perturbation of one or more of the benzene chromophores of ribonuclease is as strong as the observed perturbation of the abnormal phenolic groups. Thus: $\Delta\epsilon_{259}/\epsilon_{max} \simeq 8/200 = 0.04$ for the benzene chromophore of phenylalanine, and $\Delta\epsilon_{289}/\epsilon_{max} \simeq 55/1400 = 0.04$ for the (assumed one) abnormal phenolic group at *p*H 7. Values of ϵ_{max} are taken from Fromageot and Schnek (1950).

Since phenylalanine residue 120 is immediately adjacent to histidine 119 in the amino acid sequence of ribonuclease, and no other phenylalanine residues are adjacent to histidine (Hirs *et al.*, 1960; Smyth *et al.*, 1963), the difference spectrum of phenylalanine observed here is probably due to the charge effect of histidine 119 on phenylalanine 120. If this conclusion is correct (it can be checked by observing whether the perturbation of the phenylalanine residue disappears in singly carboxymethylated ribonuclease), it is in principle possible to study the ionization of histidine 119, one of the histidine residues in the active site (Stein and Barnard, 1959), independently of the ionization of the other three histidines in the molecule, by observation of the *p*H dependence of this perturbation. Since the magnitude of the spectral change is so small, these experiments would be difficult but not impossible to carry out with presently available equipment.

Spectrophotometric Measurements of Imidazole in Proteins. From these results it is apparent that in favorable instances the titration of imidazole in proteins can be carried out spectrophotometrically. Ideally, no conformational changes should occur in the neutral *p*H region in which the imidazole groups are being titrated, and the ratio of imidazole to other chromophores such as phenolic and indole should be relatively high. However, even in instances for which the requirements on stability of conformation are not met, it still may be possible to carry out such a titration, since the wavelength region near 290 mμ allows a separate determination of conformational and other effects on the phenolic and indole chromophores. In general, greater precision and accuracy will be obtainable from the potentiometric hydrogen ion titration method. However, the spectrophotometric method should prove useful when the amount of protein available is limited, or solubility in the neutral *p*H range is very low.

The spectrophotometric method does not offer much promise in determining the number of imidazole groups in a protein from the observed spectral change. This is partly owing to lack of a suitable model compound, and partly to environmental effects upon the imidazole chromophore which can perturb the spectrum of both the undissociated and dissociated forms. Instances of this latter effect are quite common with the phenolic chromophores (Beaven and Holiday, 1952; Wetlaufer, 1962). They are by now easily recognized, and suitable methods have been devised to correct for the perturbing effects.

It is possible that the spectrophotometric method for the titration of the imidazole groups is applicable to the study of other processes in which charge changes in the vicinity of these groups occur. Binding of metal ions, or perhaps anions, to the histidine residue may cause changes in the spectrum of the imidazole chromophore of sufficient magnitude that the binding can be more conveniently studied spectrophotometrically than by such laborious methods as equilibrium dialysis. This is already the case with metal ions having absorption in the visible or near-ultraviolet (d–d transitions), or in cases where charge transfer bands occur (Williams, 1964). When neither of these possibilities occurs, there may still be observable a perturbation of the spectrum of the chromophore in the protein to which binding takes place.

Finally, it should be pointed out that the present

results on the perturbations of the phenolic and benzene chromophores by the imidazole and α-amino groups provide criteria by which proposed models of ribonuclease conformation in solution may be judged. In the absence of knowledge about the particular residues perturbed, these criteria are not so stringent as they might be. With further experiments it may be possible to define these interactions more exactly.

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