Acetylations of tyrosines of RNase were carried out with *N*-AcIm (Simpson *et al.*, 1963). Ac_4RNase was made as described previously (Bello, 1969).

Acknowledgment

The experimental work was performed by Mrs. H. R. Bello.

Added in Proof

The uncertainty in the correction for buried tyrosines would be significant in the case of a protein with numerous buried tyrosines and few exposed tyrosines.

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The State of the Tyrosines of Bovine Pancreatic Ribonuclease in Urea–Sulfate Solutions*

Jake Bello

ABSTRACT: Spectrophotometric titration of the tyrosines of ribonuclease in 8 M urea containing 0.5 M K_2SO_4, Na_2SO_4, or $(TMA)_2SO_4$ shows a forward time dependence at pH 11–12, attributed to unfolding of the protein. In 8 M urea–0.5 M Cs_2SO_4 a *reverse* time dependence is observed at pH 10.5–12.5 indicating refolding of the protein and covering of nearly two tyrosine residues. Thermal perturbation difference spectra show that RNase and Ac_3RNase (three tyrosines O-acetylated) in 8 M urea–0.25 M K_2SO_4 are substantially renatured as compared with 8 M urea. Quick cooling of RNase in 8 M urea or in 8 M urea–0.25 M K_2SO_4 appears to freeze-in the 25° conformation, which slowly changes to a partially renatured conformation.

The greater the concentration of sulfate in 8 M urea, the smaller the extent of acetylation, iodination, and nitration.

Bovine pancreatic ribonuclease RNase[1] (polyribonucleotide 2-oligonucleotidotransferase, cyclizing) is denatured in 8 M urea, as evidenced by the normalization of the titration of tyrosine residues (Blumenfeld and Levy, 1958), increase of viscosity (Sela *et al.*, 1957), alteration of optical rotatory properties (Sela and Anfinsen, 1957), and decrease of optical density at 287 mμ (Bigelow, 1961). In the presence of phosphate or sulfate the properties of RNase partially revert to those of the native state. Thus, at 287 mμ, ε for RNase in 8 M urea is about 2300 less than in water, corrected for the effect of urea on hypothetical fully folded RNase (Bigelow and Geschwind, 1960). In the presence of 0.08 M alkali phosphate $\Delta\epsilon$ of refolding is 1580 (70%) at 25° (Barnard, 1964). There is also a partial return to the original viscosity (Sela *et al.*, 1957). It would be of interest to have more detailed information about the state of RNase in urea solutions containing anions that refold the conformation. We here report the behavior of the phenolic side chains of RNase in 8 M urea containing alkali sulfates, toward titration, acetylation, iodination, and nitration.

Materials and Methods

Most of the work was done with Worthington type RAF phosphate-free RNase, lots 6509, and 8BB, and Mann five-times-crystallized RNase, lot T3709, and with two-times-

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[1] Abbreviations used are: RNase, polyribonucleotide 2-oligonucleotide transferase, cyclizing; TNM, tetranitromethane; I_6-RNase, RNase containing three I_2Tyr residues per molecule; I-RNase, iodinated RNase; RCM-RNase, RNase with its disulfides reduced and carboxymethylated; $(TMA)_2SO_4$, tetramethylammonium sulfate.

crystallized Worthington RNase, lot R6KA. No significant differences were observed among the several batches. *N*-AcIm was purchased from Pierce Chemical Co., recrystallized from benzene–hexane, and stored in a desiccator over phosphorus pentoxide. Tetranitromethane was purchased from Aldrich Chemical Co. (Milwaukee, Wis.) and kept at 4° as a solution 0.84 M in 90% ethanol.

Urea solutions were deionized with MB-3 mixed-bed ion-exchange resin shortly before use; other reagents were reagent grade.

Iodinations described in Table II were carried out on solutions of 5 mg of RNase in 5 ml of solvent at room temperature for 20 min, using either 0.17 or 0.25 ml (9 or 14 moles, respectively, of I_2 per mole of RNase) of 0.21 M I_2 in 0.1 M KI–0.1 M (pH 6.7) ammonium acetate. Some iodinations were carried out by the method of Woody *et al.* (1966) using the above I_2 solutions. Some iodinations were carried out at other temperatures and pH values as noted under Results. Spectra of iodinated RNase were measured on the reaction mixtures after destruction of excess iodine with thiosulfate, except for the spectral titrations, for which the RNase was filtered through Sephadex G-25 and freeze dried.

Nitrations were done with TNM (Riordan *et al.*, 1966). To 15 mg of RNase in 15 ml of solvent (0.2 M in Tris-HCl, pH 8.0) was added 0.4 ml of TNM (0.84 M in 90% ethanol), added below the surface of the vigorously stirred solution. The vessel was wrapped in dark paper and the contents were stirred for 2 hr with a TRI-R magnetic stirrer (Tri-R Instruments, Jamaica, N. Y.) in which the driving magnet enclosure does not become warm. The reaction mixture was filtered through Sephadex G-25 and freeze dried.

Acetylation was carried out with *N*-AcIm by the method of Simpson *et al.* (1963), except that no buffer was used, the imidazolium acetate formed on hydrolysis of *N*-AcIm acting as buffer; the initial pH was adjusted to 8.0 ± 0.3, the final pH being about 6.3. RNase (25 mg) was dissolved in 10 ml of solvent, to which 45 mg of *N*-AcIm was added; after reaction the solution was gel filtered through Sephadex G-25 and freeze dried.

The extent of acetylation was estimated from the optical density at 277 mμ, and the extent of nitration from the optical density of the nitrophenolate peak at pH 9.2, and at the isosbestic point.

Spectrophotometric titrations were carried out on a Cary 15 spectrophotometer; pH adjustments were made with potassium hydroxide. Cuvets were stoppered to prevent uptake of carbon dioxide, and were thermostated at 25°. The concentration of RNase was about 0.4–0.5 mg/ml.

Difference spectra were obtained with identical solutions in both beams; the base line was adjusted to flatness (±0.0005) at 25°. Then the sample cuvet in the sample beam was adjusted to the desired temperature. Nitrogen was passed through the sample compartment to prevent condensation of moisture. The temperature was measured with a thermistor thermometer (Yellow Springs Instruments Co.) with the probe inserted 2 in. from the cuvet holder.

Results

The work reported here has been done with sulfate salts rather than with phosphate, because the latter has a strong buffering action which would have required large quantities of alkali for pH adjustment and because phosphate causes rapid decomposition of *N*-AcIm (half-life of 3 min in 0.13 M phosphate, pH 7, compared with 60 min in water). Herskovits and Laskowski (1968) showed that 0.2 M sulfate is slightly more effective than 0.25 M phosphate in restoring the number of buried tyrosines, as indicated by the solvent perturbation method.

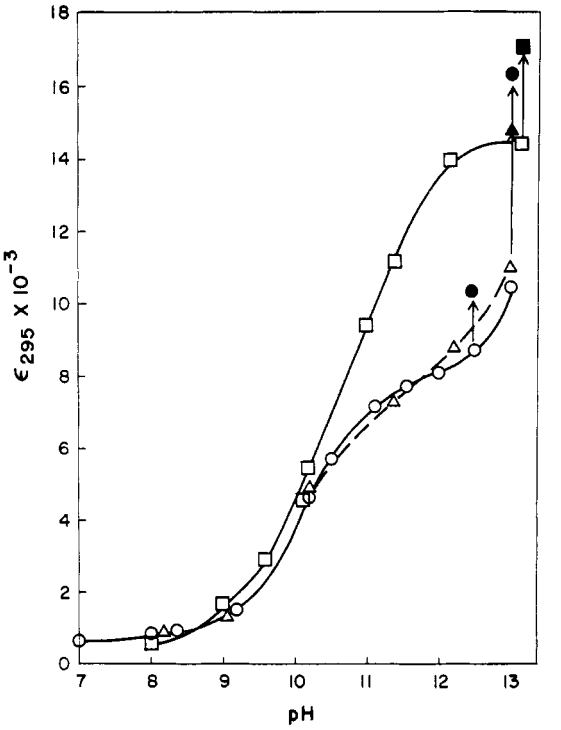

FIGURE 1: Spectrophotometric titration of RNase. (○) in H_2O, (△) in 0.25 M K_2SO_4, and (□) in 8 M urea. Filled symbols and arrows indicate time-dependent data after 30 min; unfilled symbols are for t = 0. Unfilled symbols with no corresponding filled symbols indicate the absence of time dependence.

Spectrophotometric Titration. Shugar (1952) and Tanford *et al.* (1955) found that the tyrosines of RNase are titrated in two steps, the first being the titration of the three normal tyrosines with a pK near 10. The second step was found to be time dependent above pH 12.2. Such a titration curve is shown in Figure 1, similar to numerous such curves reported by others. ϵ_{295} for the anion of *N*-AcTyr-NH_2 at pH 12 is 2550 in our hands; for the titration of six tyrosyl residues ϵ_{295} should be about 15.3×10^3. It has been considered that on reaching a value of ϵ 16×10^3 at pH 13, the titration is complete. Tanford *et al.* reported ϵ 16×10^3 at pH 13, when extrapolated to zero time (t = 0); Shugar's ϵ extrapolated to t = 0 at pH 13 was 12,500 and our Figure 1 gives ϵ 10,500. Our value corresponds to the instantaneous titration of about 4 tyrosines and that of Shugar to about 5. Blumenfeld and Levy found ϵ 11,000 at pH 13 but did not indicate a time dependence; probably, their ϵ_{295} was at t = 0. Figure 1 also shows the titration of RNase in 8 M urea. ϵ at t = 0 at pH 13 is 14,500. Since ϵ_{295} for *N*-AcTyr-NH_2 in 8 M urea is 2425, 14,500 is equivalent to 5.9 tyrosines. Blumenfeld and Levy found ϵ_{295} 14,500 for RNase in 8 M urea at pH 12.

The time dependence at high pH in water appears to arise from a chemical reaction involving the disulfides (Brown

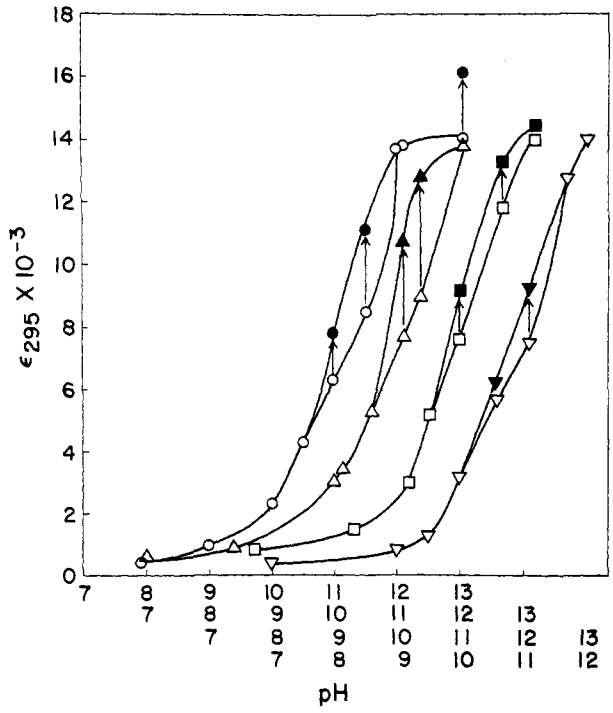

FIGURE 2: Spectrophotometric titration of RNase in urea–sulfates. (○) 0.25 M K_2SO_4; (△) 0.5 M Na_2SO_4, corrected for sodium ion error; (□) 0.5 M $(TMA)_2SO_4$; and (▽) 0.25 M Cs_2SO_4. Significance of filled symbols as in Figure 1.

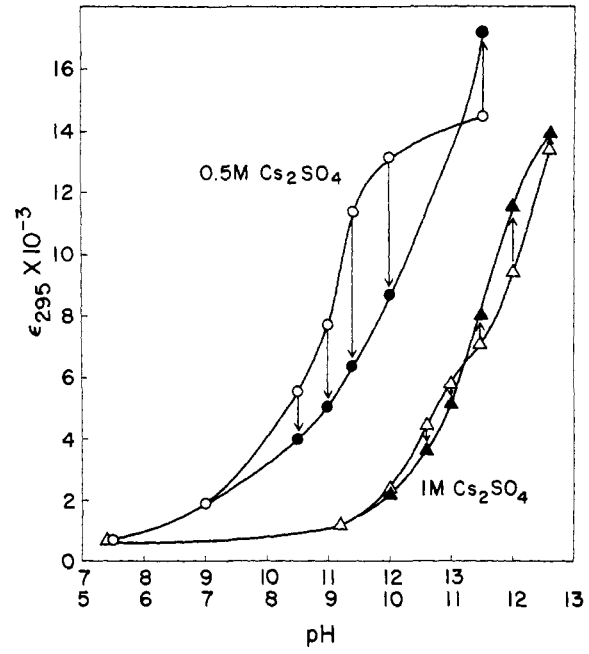

FIGURE 3: Spectrophotometric titration of RNase in 8 M urea–Cs_2SO_4. Significance of filled symbols as in Figure 1.

et al., 1959; Clarke and Inouye, 1931; Donovan, 1967). While Donovan found that cystine gives no spectral change, we have found that oxidized glutathione does, with an increase in ϵ over the range 350–250 mμ. Titration of RNase in which the disulfides were broken by reduction and protected by carboxymethylation (RCM-RNase) showed time dependence at pH 13. On acidification of RCM-RNase held at pH 13 for 0.5 hr, the transient odor of a mercaptan was noted. Tramer and Shugar (1959) showed that there was no time dependence in the titration of oxidized RNase in which the disulfides had been broken by oxidation with performic acid. Since oxidized and reduced RNase are unfolded in water (Harrington and Sela, 1959) and since these as well as RNase in 8 M urea show nearly complete titration of the tyrosines at pH 12 without time dependence, the time dependence at pH 13 for RNase in 8 M urea and of RCM-RNase in water must be the result of a reaction of the disulfides in the former and, probably, β elimination of mercaptoacetic acid in the latter.

Figure 1 also shows the titration of RNase in 0.25 M K_2SO_4, which is seen to be similar to that in water. Titration in 8 M urea (Figure 1) shows a simple curve with one step and complete titration. Titration in 8 M urea–0.25 M K_2SO_4 (Figure 2) shows time dependence at pH 11 and 11.5, but no other indication of two classes of tyrosines. (A solution of RNase in 8 M urea–0.25 M K_2SO_4 was observed spectrophotometrically at pH 11.5 for 30 min and was then neutralized to pH 5.6. The optical density returned to the original value at pH 5.6 to within 1%, corrected for the volume change. Thus no irreversible spectral change took place.) There was no time dependence at pH 12. Similar results were obtained in 8 M urea containing 0.5 M K_2SO_4 (not shown), 0.5 M Na_2SO_4, 0.5 M $(TMA)_2SO_4$, or 0.25 M Cs_2SO_4 (Figure 2), except for a small time dependence at pH 12 for $(TMA)_2SO_4$. The largest time-dependent $\Delta\epsilon$, exclusive of pH 13, was 3800 at pH 11.4 in 8 M urea–0.5 M Na_2SO_4; this is equivalent to 1.5 tyrosines.

However, in 8 M urea containing 0.5 M Cs_2SO_4 a remarkably different result was obtained (Figure 3). In this case a reverse time dependence was observed at pH 10.5–12, the optical density decreasing with time. At pH 11.4 the magnitude of the decrease was $\Delta\epsilon = 5000$, equivalent to the protonation of about 2 tyrosines. At pH 13 the usual forward time dependence was observed. In 8 M urea–1 M Cs_2SO_4 (Figure 3) only a small reverse time dependence was observed up to pH 11.2, and a forward effect above pH 11.2. A pH 11.5 solution of RNase in 8 M urea–0.5 M Cs_2SO_4 showed no change in pH during 30 min; a pH decrease would have indicated an increase of cesium binding. A similar absence of pH change was observed for 8 M urea–0.25 M K_2SO_4, or in 8 M urea–0.5 M K_2SO_4.

Thermal Perturbation Spectra. It has been shown (Bello, 1969; Cane, 1969) that perturbing a protein solution by changing the temperature results in difference spectra that are useful in estimating the number of exposed tyrosyl side chains. Encouraging results were obtained for RNase in H_2O, in LiBr, and in 6 M guanidinium chloride (Bello, 1969). Such difference spectra were obtained by comparing identical solutions at two temperatures, such as 25 and 4°. Some examples are shown in Figure 4. (In all the thermal perturbation difference spectra shown here, the $\Delta\epsilon$ 0 base lines coincide with the horizontal parts of the spectra at long wavelength.) All spectra were flat to 350 mμ, but are shown only to 325 mμ. The spectra have been shifted on the Δ OD axis for clarity. The thermal perturbation data are for unbuffered solutions at pH 6.7.

The method is based on taking the ratio of $\Delta\epsilon$ at trough A

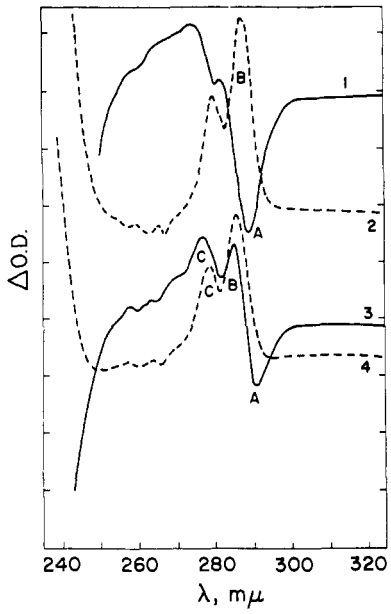

FIGURE 4: Thermal perturbation difference spectra of RNase in 8 M urea and 8 M urea–0.25 M K_2SO_4 at 4° and 18°. Spectrum 1, 8 M urea, 4°; 2, 8 M urea, 18°; 3, 8 M urea–0.25 M K_2SO_4, 4°; 4, 8 M urea–0.25 M K_2SO_4, 18°. Each division on optical density axis is 0.01 optical unit. Concentrations of RNase are about 1 mg/ml for spectra 2, 3, and 4 and 1.5 mg/ml for spectrum 1.

(Figure 4) for RNase to that for *N*-AcTyr-NH_2, and assumes that in the absence of a conformation change $\Delta\epsilon$ is the result only of a change in solvation of the chromophore (except for the concentration change arising from the volume change accompanying cooling, which is negligible). At extremum A, the spectral change due to buried tyrosines is small (Bello, 1969). Trough A for *N*-AcTyr-NH_2 and RNase is at 287–292 mμ, the wavelength varying with the solvent. To use this method there must be no conformational change over the temperature interval used. In dilute aqueous salt solutions the magnitude of $\Delta\epsilon_A$ is linear with temperature for both RNase and *N*-AcTyr-NH_2 (Bello, 1969). Departure from linearity is taken as evidence for conformational change.

Before we attempt to estimate the number of exposed tyrosines we must examine the characteristics of the spectra. All of the difference spectra at 4° *vs.* 25° display a negative absorption at around 290 mμ, trough A, and two positive peaks at 285–275 mμ, peaks B and C.

On raising the temperature to an intermediate value, *e.g.*, 18°, the difference spectra of RNase in 8 M urea and 8 M urea–0.25 M K_2SO_4, 18° *vs.* 25°, change in a manner different from those of *N*-AcTyr-NH_2 in these solvents or from that of RNase in H_2O. In the latter cases all peaks and troughs diminish linearly with temperature. But in the case of RNase in 8 M urea trough A vanishes, and peaks B and C increase, B more than C. The increase in B and C is indicative of renaturation. The strong negative absorption at 240 mμ becomes positive, also indicative of renaturation (C. C. Bigelow and E. P. A'Zari, unpublished data). For RNase in 8 M urea–0.25 M K_2SO_4, on raising the temperature to 18°, trough A also vanishes, peak B increases, and the negative absorption at 240 mμ becomes positive. Thus, raising the temperature from 4° appears to promote renaturation, in apparent contradiction to the results of Foss (1961) and of Barnard (1964). However, if the temperature program is reversed and the sample cuvet is cooled from 25° to lower temperature, peaks B and C also increase, in agreement with Foss and with Barnard. From this we infer that their temperature programs were from 25° down. These results are shown in Figure 5. (Both temperature programs must give $\Delta\epsilon$ 0 at 25° *vs.* 25°.) Also, maintaining a quickly cooled solution at 4° for 3 days increases peaks B and C (Figures 5 and 6). The 3-day thermal perturbation difference spectra (Figure 6) of RNase in 8 M urea and 8 M urea–0.25 M K_2SO_4 show several differences: larger $\Delta\epsilon_B$ and $\Delta\epsilon_C$ values for the former, a distinct negative absorption trough A for the latter, and some differences in the 240–260-mμ regions. Both show evidence of renaturation in the B and C peaks and the positive absorption at 240 mμ. $\Delta\epsilon_B$ for RNase in 8 M urea is 960 and in 8 M urea–0.25 M K_2SO_4 is 530, corresponding to covering of about 1 and 0.5 tyrosine, respectively (Bigelow, 1961). The response of peak B to temperature suggests that on quick cooling the 25° conformation is frozen-in. On slow cooling from 25°, or on warming from 4°, there is opportunity for conformational

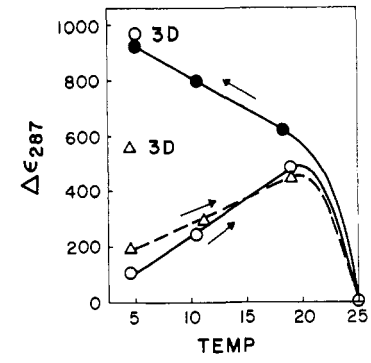

FIGURE 5: Effect of temperature on $\Delta\epsilon_{287}$ (peak B) of thermal-solvent perturbation spectra of RNase in 8 M urea and 8 M urea–0.25 M K_2SO_4. Circles are for 8 M urea and triangles for 8 M urea–0.25 M K_2SO_4. Arrows indicate direction of temperature program. Symbols labeled 3D are for samples cooled at 4° for 3 days.

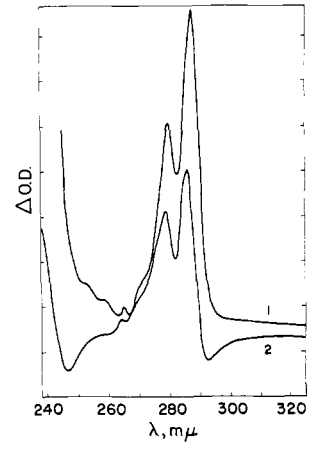

FIGURE 6: Thermal difference spectra of RNase in 8 M urea (spectrum 1) and 8 M urea–0.25 M K_2SO_4 (spectrum 2), after 3 days at 4°. Each division on optical density axis is 0.01 optical density unit. Concentrations are 1 mg/ml.

TABLE I: Estimated Number of Exposed Tyrosines in Urea by Thermal Perturbation Difference Spectra.[a]

Solute	Solvent	$\Delta\epsilon_A$	$\Delta\epsilon'_A$[b]	Exposed Tyrosines $\Delta\epsilon'_A/\Delta\epsilon_A$-(*N*-AcTyr-$NH_2$)
Ac_2Tyr	8 M urea	−10		
Ac_2Tyr	8 M urea–0.25 M K_2SO_4	−9		
N-AcTyr-NH_2	8 M urea	−49		
N-AcTyr-NH_2	8 M urea–0.25 M K_2SO_4	−51		
N-AcTyr-NH_2	0.25 M K_2SO_4	−50		
RNase	8 M urea	−244		5.0
RNase	0.25 M K_2SO_4	−157	−157	3.1
RNase	8 M urea–0.25 M K_2SO_4	−161	−161	3.5
Ac_3RNase	8 M urea	−177	−147	3.0[c]
Ac_3RNase	8 M urea	−153	−123	2.5[d]
Ac_3RNase	8 M urea–0.25 M K_2SO_4	−107	−80	1.4[e]
Ac_6RNase	8 M urea	−77	−7	0.7[e]

[a] Temperatures: 25 and 4°. [b] $\Delta\epsilon'_A = \Delta\epsilon_A$ (RNase) − $n\Delta\epsilon_A(Ac_2Tyr)$ where n is the number of *O*-acetyltyrosine residues per RNase molecule. [c] This sample contained 3.0 *O*-acetyltyrosines/molecule. [d] This sample contained 3.3 *O*-acetyltyrosines, or 2.7 tyrosines per molecule. [e] Calculated as Ac_2Tyr. 0.14 calculated as *N*-AcTyr-NH_2.

change. The thermal perturbation difference spectra were taken after a 20-min equilibration at 4°. Spectra were also taken 5, 10, 15, 20, and 30 min after inserting the sample cuvet into the precooled holder. There was no time dependence for RNase in 8 M urea–0.25 M K_2SO_4 during this time interval, none in 8 M urea during the first 15 min, and only a small change after 30 min. This suggests that the 25° conformation is frozen-in at 4°, and changes only slowly. The data used in Table I were taken after 10 min.

Turning to the question of attempting to estimate the number of exposed tyrosines from thermal perturbation difference spectra we assume that on quickly cooling to 4°, the 25° conformation is frozen-in and that $\Delta\epsilon_A$ is a true measure of the 25° conformation. The reasons for using $\Delta\epsilon$ at λ_A and not at other wavelengths have been discussed (Bello, 1969). The results are shown in Figures 7 and 8, and in Table I. The last column, $\Delta\epsilon'_A/\Delta\epsilon_A$(*N*-AcTyr-$NH_2$), gives the calculated number of exposed tyrosyl side chains. For RNase in 0.25 M K_2SO_4 the value of 3.1 is significantly smaller than the value of 3.6 found for RNase in H_2O (Bello, 1969). For RNase in 8 M urea 5.0 tyrosines are exposed. For RNase in 8 M urea–0.25 M K_2SO_4 the number, 3.5, is, within the experimental error, the same as for H_2O. For Ac_3RNase in 8 M urea all nonacetylated tyrosines are exposed. The value of 1.4 for Ac_3RNase in 8 M urea–0.25 M K_2SO_4 shows a significant refolding and is slightly higher than the 1.1 value for Ac_3RNase in H_2O (Bello, 1969). It should be noted that after the difference spectra were taken the 4° sample was warmed to 25° and the difference spectrum was retaken; the result was flat to within the same limits as the original base line. Thus, no appreciable differential hydrolysis of acetyl groups took place, and probably no significant hydrolysis at all. Also a difference spectrum of phenylalanine showed no significant effect at around 290 mμ. No correction has been made for exposed disulfides, which, based on oxidized glutathione, would reduce the number of exposed tyrosines by about 0.4 for completely unfolded RNase. For essentially native RNase with 3.5 exposed tyrosines, the disulfide correction should be small.

TABLE II: O-Acetylation in Urea–Sulfate at 25°.[a]

Solvent	Moles of *O*-AcTyr Residues/Mole of RNase
H_2O (9800)[b]	3.0–3.3
8 M urea (8600)[b]	6.0
8 M urea + 0.25 M Na_2SO_4 (9800)[b]	5.9
8 M urea + 0.5 M Na_2SO_4 (9900)[b]	5.1
8 M urea + 1 M Na_2SO_4	3.2
8 M urea + 1 M Na_2SO_4 (twice-concentrated *N*-AcIm)	4.0
8 M urea + 0.5 M Cs_2SO_4	4.8

[a] Reaction time was 45 min. [b] Figure in parentheses is ϵ_{277} for unmodified RNase in the solvent indicated.

Chemical Modifications. An alternate method of studying the state of the tyrosines is by chemical modifications. Whereas spectral methods "see" the time average state, chemical reactions, being slower, can also "see" tyrosines which are buried much of the time but exposed during a small percentage

TABLE III: Spectral Properties of Iodinated RNase (I-RNase) and of ITyr and I_2Tyr.

Solute	Iodination Solvent[a]	Moles of I_2/ Mole of RNase	λ_{max} pH 9.5 (mμ)	ϵ_{max} pH 9.5	$\epsilon_{max}/\epsilon_{350}$ at pH 9.5
I-RNase	H_2O	9	309	6,000	14
I-RNase	8 M urea	9	312.5	22,000	3.4
I-RNase	8 M urea–0.25 M K_2SO_4	9	313	17,000	3.3
I-RNase	8 M urea–1 M Cs_2SO_4	9	310	9,800	5.1
ITyr	8 M urea[b]		307	3,000	58
I_2Tyr	8 M urea		312	5,100	14
I_2Tyr	H_2O[b]		310	5,400	15
I-RNase	H_2O	14	309	6,800	12
I-RNase	8 M urea–0.25 M K_2SO_4	14	313	23,000	2.3
I-RNase	8 M urea	14	314	27,000	2.4

[a] 0.05 M ammonium acetate in all solvents, 20-min reaction time. Spectra were obtained on iodination reaction mixtures, but changed by the addition of 0.05–0.1 ml of 0.1 N $Na_2S_2O_3$ to 10 ml and by the addition of NaOH to adjust the pH. ε values were corrected for the volume change. [b] Solvent for spectral data.

of the time, and also tyrosines which are largely buried but in which the reactive site, *e.g.*, the hydroxyl group of the phenolic group, is exposed.

In Table II are shown the results of acetylation of tyrosines in 8 M urea containing sulfate. In 8 M urea–0.25 M K_2SO_4 acetylation of tyrosines is substantially complete although the restoration of ε is 70% complete (Barnard, 1964). In 8 M urea–1.0 M Na_2SO_4 the number of acetylated tyrosines is reduced to about three, but this result appears to arise in part from the effect of sulfate on the stability of *N*-AcIm, reducing the half-time for decomposition from 60 to 25 min. By using twice as much *N*-AcIm the number of acetylated tyrosines was raised to 4.0 in 8 M urea–1 M Na_2SO_4. However, even in water, the use of four times the usual amount of *N*-AcIm results in acetylation of 4 tyrosines.

Acetylation at 4° in precooled (20 min or 4 days) 8 M urea for 3 hr (four times the reaction time at 25°) gave 6 acetyltyrosines, and acetylation in 8 M urea–0.25 M K_2SO_4 precooled 4 days gave 4.7 AcTyr.

The second method of modification was iodination.

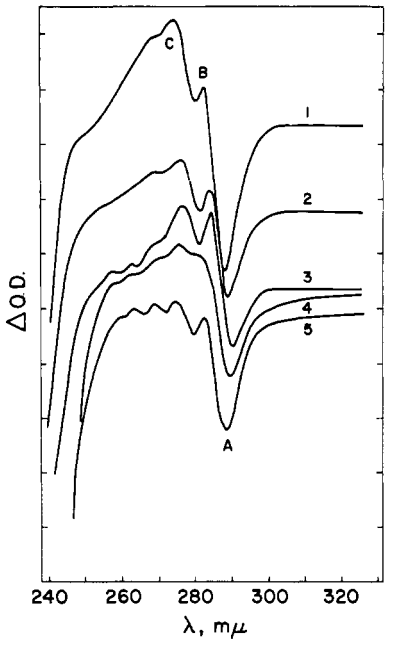

FIGURE 7: Thermal perturbation difference spectra of RNase, Ac_3RNase, and *N*-AcTyr-NH_2. Curve 1, *N*-AceTyr-NH_2 in 8 M urea–0.25 M K_2SO_4, 0.1 mg/ml; 2, *N*-AcyTyr-NH_2 in 8 M urea, 0.07 mg/ml; 3, RNase in 8 M urea–0.25 M K_2SO_4, 1 mg/ml; 4, Ac_3RNase in 8 M urea–0.25 M K_2SO_4 2 mg/ml; 5, Ac_3RNase in 8 M urea, 2 mg/ml. Each division on the optical density axis is 0.01 optical density unit.

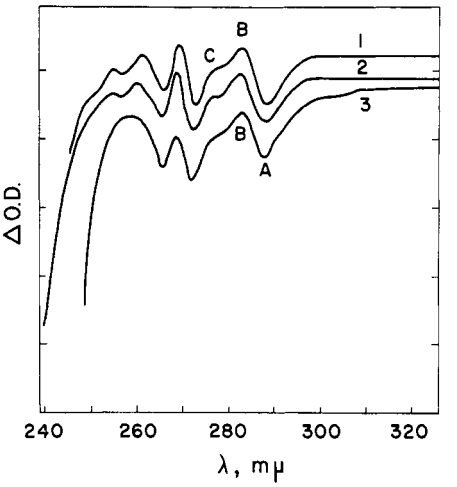

FIGURE 8: Thermal perturbation difference spectra of Ac_2Tyr and Ac_6RNase. Spectrum 1, Ac_2Tyr in 8 M urea; 2, Ac_2Tyr in 8 M urea–0.25 M K_2SO_4; 3, Ac_6RNase in 8 M urea–0.25 M K_2SO_4. Each division on optical density axis is 0.01 optical density unit. Concentrations are 0.3 mg/ml for Ac_2Tyr and 3 mg/ml for Ac_6RNase.

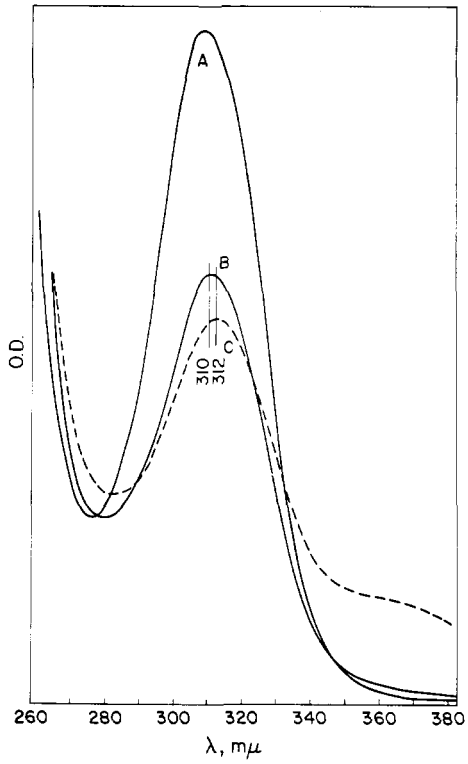

FIGURE 9: Spectra of I_2Tyr and iodinated RNase. Spectrum A, I_2Tyr; B, RNase iodinated in 8 M urea, pH 11.2; C, RNase iodinated in 8 M urea, pH 6.7.

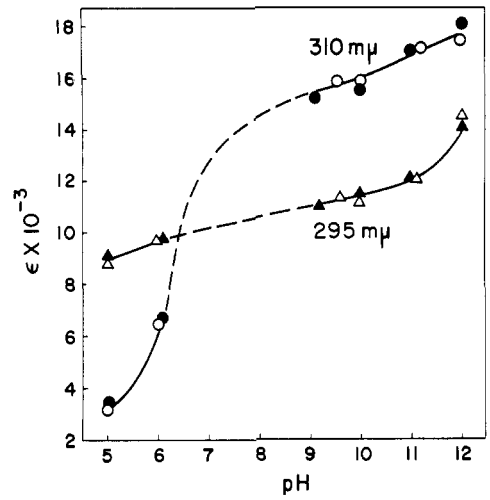

FIGURE 10: Spectrophotometric titration of RNase iodinated in 8 M urea. Filled symbols for heated samples; unfilled for unheated samples.

Iodinations were done for short times, 20 min; complete iodination of the normal tyrosines of native RNase required 3 days at 4° and gave ϵ 16.5 × 10^3 (Woody *et al.*, 1966). The results are shown in Table III. Since the p*K* values of I_2Tyr and ITyr are 6.4 (Gemmill, 1955) and 8.2 (Herriott, 1947), respectively, ϵ at pH 9.5 is sufficient to measure the ionized ITyr and I_2Tyr, while the contribution at 310–313 mμ from the 295-mμ peak of ionized tyrosine is only ϵ 550 in 8 M urea for completely uniodinated RNase and less for partially iodinated RNase. That reactions other than iodination of tyrosine took place is indicated by the shoulder at 375–350 mμ (Figure 9) in some of the samples, which is not shown by ITyr or I_2Tyr. The ratio of $\epsilon_{max}/\epsilon_{350}$ (Table III) indicates the extent of this side reaction. Hughes and Straessle (1950) found a lesser degree of side reactions at pH 9–10.2 than at pH 7.1, but at lower pH much more iodine was used. In their most heavily iodinated preparation, the spectrum of Hughes and Straessle at pH 10.8 showed λ_{max} 315, and $\epsilon_{315}/\epsilon_{350}$ 5.5, but there was no distinct shoulder in the 350–375-mμ regions.

λ_{max} for ITyr was 307 in urea compared with 303 mμ in H_2O, and ϵ_{max} was 3000 compared with 3600 in H_2O (Woody *et al.*, 1966). The corresponding values for I_2Tyr in water were 310 and 5400, compared with 310 and 5500 found by Woody *et al.* (1966). From λ_{max} and ϵ_{max} an estimate may be made of the number of ITyr and I_2Tyr residues produced by iodination. But in those cases in which $\epsilon_{max}/\epsilon_{350}$ is low, λ_{max} is probably at longer wavelength and ϵ_{max} is greater than would be given by ITyr and I_2Tyr residues in the absence of side products.

Using 9 moles of I_2/mole of RNase, the data of Table III show that there is much more iodination in 8 M urea than in water, and about 25% less in 8 M urea–0.25 M K_2SO_4 than in 8 M urea. Iodination in 8 M urea was incomplete at 9 moles of I_2/mole (12 moles would be required, not including iodine consumed in side reactions); all of the iodine was consumed. Reaction of the free amino acid histidine with I_2 in 8 M urea was about one-fourth as fast as that of *N*-AcTyr-NH_2, and reaction of free methionine in 8 M urea with I_2 was as fast as that of *N*-AcTyr-NH_2, with a half-time of about 1–2 min, as measured by the disappearance of iodine color at 440 mμ. However, in RNase in urea the reaction of methionine appears to be slower than iodination of tyrosine; otherwise we would not have obtained so nearly complete iodination of the latter. For RNase iodinated in water the values λ_{max} 309 mμ and ϵ_{max} 6000 suggest that about 1 I_2Tyr residue and a small amount of ITyr were formed; in 8 M urea 4 I_2Tyr were formed, and in 8 M urea–0.25 M K_2SO_4 3 I_2Tyr. In 8 M urea–1 M Cs_2SO_4 iodination is markedly less than in 8 M urea. A combination of about 1.3 I_2Tyr and 0.9 ITyr would give the observed λ and ϵ.

Increasing the I_2/RNase ratio from 9 to 14 caused an increase in the extent of iodination of tyrosine (and of the side reaction). Again, there was less iodination in 8 M urea–0.25 M K_2SO_4 than in 8 M urea. During 20-min reaction times the loss of I_2 in the reaction media used, without RNase, was less than 5%. Thus, the lesser extent of iodination in urea–sulfate compared with urea is not the result of insufficient I_2.

Woody *et al.* published a spectrophotometric titration curve for I_6RNase (*i.e.*, 3 I_2Tyr/molecule), which is difficult to understand. Between pH 12 and 13 the increment of ϵ_{295} was 8000–9000, which they explained as resulting from the titration of 3 abnormal tyrosines (based on ϵ 2800 for 1 tyrosine). But between pH 8.5 and 12, $\Delta\epsilon_{295}$ was 6000, equivalent to about 2.4 tyrosines based on our ϵ_{295} 2550. Since the p*K* of I_2Tyr is 6.4 (Gemmill, 1955), 99% of these should have been titrated below pH 8.5; therefore the tyrosines titrated at pH 8.5–12 (with over-all p*K* of about 10.3) would have

TABLE IV: Iodination of RNase at pH 11.2.[a]

Solvent	ϵ_{max}	λ_{max} (mμ)	$\epsilon_{max}/\epsilon_{350}$
H_2O	24,300	311	13
8 M urea	27,500	311	12
0.5 M Cs_2SO_4–8 M urea	27,900	312	12

[a] Spectral data at pH 9.5.

TABLE V: Iodination of RNase,[a] Precooled 5 Days at 4°.

Solvent	Reaction Time (min)	λ_{max} 9.5 (mμ)	ϵ_{max} pH 9.5	$\epsilon_{max}/\epsilon_{350}$
8 M urea–	40	312	16,300	5.0
0.25 M K_2SO_4	90	312	18,500	4.3
8 M urea	40	312	23,700	4.7
	90	312.5	27,900	4.5

[a] In 0.05 M ammonium acetate, pH 6.7, 14 moles of I_2/mole of RNase.

to be the uniodinated tyrosines which are perhaps easily uncovered in I_6RNase. If this is correct $\Delta\epsilon_{295}$ above pH 12 is probably due to reactions at the disulfides. Also the titration curve of Woody *et al.* shows no experimental points between pH 6 and 9. We iodinated three batches of RNase under the conditions of Woody *et al.*, but we did not fractionate to obtain homogeneous I_6RNase. These batches were a Worthington phosphate-free RAF, lot 8BB, a Worthington two-times-crystallized lot R6KA, and a Mann Laboratories five-times-crystallized lot T3079. The first and second lots were iodinated as received and all three lots were also iodinated after heating for 10 min at 66–68° at pH 3.5, to dissociate aggregates. Heating resulted in no significant differences.

The titration curves of all 5 iodinated RNases gave ϵ_{310} 15.8–17.5 × 10^3 at pH 9.5 equivalent to about 3 I_2Tyr residues, in agreement with the 16.5 × 10^3 of Woody *et al.*, although our products were not fractionated as was theirs. All showed time-dependent turbidity at pH values from 6.5 to 8.5 (to pH 9 for Mann). At 295 mμ the changes in ϵ from pH 8.5 to 11.5 were about 1000, compared with 5000 shown by Woody *et al.* Titration of the uniodinated tyrosines (shown by the 295-mμ curve) of iodinated RNase began at about pH 11–11.5, compared with pH 12 for the second group of tyrosines of native RNase. The optical densities of Figure 10 were for t = 10 min, there being a time dependence, largely completed at t = 10 min,of a bout $\Delta\epsilon$ = 1000 at pH 11.5–12. Only data for lot 8BB are shown; the others are so nearly similar that their exhibition would be redundant. The titration curve from 6 to 9 was drawn on the assumption that only I_2Tyr is being titrated with a pK of 6.4. Titration of I_2Tyr between pH 5 and 7.5 cannot easily be observed at 295 mμ as this is very close to the isosbestic point.

If our titration of buried tyrosines at pH 12 corresponds to that of Woody *et al.*, we do not understand what they were titrating at pH 8.5–11.5.

Iodination in 8 M urea or in water at pH 11.2 resulted in extensive iodination (Table IV). RNase in the solvent being used was adjusted to pH 11.2, allowed to stand for 30 min to allow for the change of conformation shown by the optical density (Figure 2), and then iodinated with 14 moles of I_2/mole of RNase for 20 min. The extent of iodination ranged from nearly 5 to nearly 6 I_2Tyr for all 3 solutions of Table IV.

It was noted above (Figure 9) that a shoulder at 350 mμ was observed in the spectrum of iodinated RNase that was not present in I_2Tyr and ITyr. This shoulder was greater, the greater the extent of iodination, except in the case of iodination at pH 11.2 in which it was nearly absent. With regard to the product absorbing at 350–375 mμ, reaction at pH 11.2 appears to be cleaner than at 6.7, in accord with the suggestion of Hughes and Straessle.

Iodinations were also carried out on solutions precooled at 4° for 5 days (Table V). Reaction times of 40 and 90 min were chosen, the latter on the assumption of a twofold change in reaction rate per 10° change in temperature. In 8 M urea there is a difference of about 2200 in ϵ between 40 and 90 min, while in 8 M urea–0.25 M K_2SO_4 the difference is 4200. The difference between urea and urea–sulfate at 4° is about twice as large as at 25°, suggesting that the latter refolds more than does the former at low temperature. The results of acetylation also show this effect.

The third chemical modification used was nitration by TNM with results shown in Table VI. For 3-nitrotyrosine λ_{max} at pH 9.2 and the isosbestic point were found to be 425 and 379 mμ, respectively, and ϵ_{425} and ϵ_{379} were found to be 4200 and 2200, respectively, in agreement with those of Riordan *et al.* (1966), except that the latter found the two wavelengths to be 428 and 381 mμ.

However, in nitrated RNase λ_{max} at pH 9.2 and the isosbestic point were not the same as for 3-nitrotyrosine. The reason is not clear but may be related to the findings of Bruice *et al.* (1968) who showed that TNM acting on phenols yields other products in addition to simple nitrophenols, including bimolecular addition products of phenols. Intermolecular cross-

TABLE VI: Nitration of Ribonuclease.

Solvent	Nitrotyrosines/RNase at 428 mμ[a]	at 381 mμ[a]
H_2O	3.1 (423)	3.8 (378)
8 M urea	4.2 (426)	5.2 (380)
8 M urea–0.25 M K_2SO_4	3.6 (427)	4.6 (382)
8 M urea–1 M K_2SO_4	2.5 (427)	3.1 (382)
8 M urea–0.5 M Cs_2SO_4	3.3 (427)	4.0 (380)
6 M Gu·HCl	4.9 (429)	4.7 (382)

[a] Nominal wavelength. Actual wavelength is shown in parentheses. ϵ_{428} taken from spectrum at pH 9.2 (0.025 M Tris-HCl); ϵ_{381} taken from intersection of pH 9.2 spectrum and spectrum in water, pH not measured.

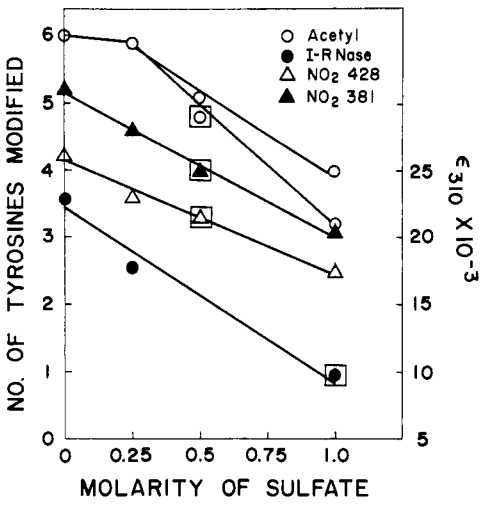

FIGURE 11: Extent of modification of tyrosines of RNase in 8 M urea as a function of sulfate concentration. All data are for K_2SO_4 except those symbols in squares which are for Cs_2SO_4. The two symbols for acetylation are for two different quantities of *N*-AcIm (see Table II). Iodination data are for 9 moles of I_2/mole of RNase. Left axis is for acetylation and nitration, right axis for iodination. The two sets of data for nitrated RNase are calculated at the wavelengths indicated.

linking of proteins, probably through phenolic groups, has been shown (Doyle *et al.*, 1968). Intramolecular cross-linking may also occur. In denatured RNase normally separated tyrosines may approach each other. Also in native RNase Tyr-73 and Tyr-115 are close together as shown by X-ray crystallography in this laboratory. We found that paper chromatography of the nitration reaction mixture of tyrosine in water showed a ninhydrin-positive spot at the origin, which was not nitrotyrosine and was not present in the control. The number of calculated nitrotyrosines in RNase differs at the two wavelengths. The best agreement was obtained in 6 M guanidinium chloride, namely, 4.7 at 382 mμ and 4.9 at 429 mμ. The difficulty in the analytical values for nitrotyrosine may mean that nitrotyrosines and nonnitrated tyrosines reacted to yield products with absorption in the region of that of nitrotyrosine. Tyrosines that react in some manner other than the formation of nitrotyrosines must also be exposed. In 8 M urea the maximum number is 5.2 instead of the 6 expected.

In Figure 11 are shown the effects of 0.25 M K_2SO_4 and 0.5 or 1 M Cs_2SO_4 on the chemical modification in urea. The approximate linearity of the relations suggest that in the neutral pH region Cs_2SO_4 acts similarly to K_2SO_4. Also, although ϵ_{277} is almost invariable from 0.25 M K_2SO_4 to 1 M K_2SO_4 (Table II) in 8 M urea, the amount of chemical modification is affected significantly.

Discussion

The forward time dependence at pH 10.5–11.5 in 8 M urea–sulfate (except 0.5–1 M Cs_2SO_4) must arise from a time-dependent exposure of buried tyrosines. Since titration in water or in 8 M urea shows no time dependence at pH 10.5–11.5, there is no reaction of disulfides in this pH range. The absence of time dependence at pH 12 in urea–sulfate, the similarity of ϵ at pH 12 to that at pH 13 at $t = 0$, and the good agreement at pH 12 between ϵ_{295} for RNase and that expected for 6 *N*-AcTyr-NH_2 suggest that the titration is nearly complete at pH 12. The time dependence at pH 10.5–11.5 shows that in 8 M urea–0.25 M K_2SO_4 (and in 0.5 M Na_2SO_4, 0.25 M Cs_2SO_4, and 0.5 M $(TMA)_2SO_4$) the tyrosines of RNase are in a state intermediate between that of RNase in water and in 8 M urea; *i.e.*, all of the tyrosines can be titrated with a p*K* near that of RNase in 8 M urea, but some are buried, although less tightly than in RNase in water.

The number of tyrosines titratable in 8 M urea–0.25 M K_2SO_4 up to the beginning of the time-dependent range is about 2.3. This probably does not mean that nearly four tyrosines are buried. It more probably means that buried tyrosines are more easily uncovered than during titration in water, and become reactive before the initially exposed tyrosines have been completely titrated. In 8 M urea–0.25 M K_2SO_4 the number of tyrosines that become exposed with time is about 1.5.

Extrapolation of ϵ at pH 13 to zero time in aqueous medium gives ϵ 10,500, equivalent to 4.2 tyrosines. This cannot be taken to mean that only 4 tyrosines can be titrated at pH 13, as the extrapolation probably overcorrects. The time dependence at pH 13 is probably the sum of reaction of disulfides and slow exposure of tyrosine. It does suggest that on reaching pH 13, the instantaneous value of ionized tyrosines is 4.

The fourth tyrosine that is immediately titrated at pH 13 may be residue 92, because this is the most exposed of the three abnormal tyrosines (crystal structure to be published, G. Kartha, D. Harker, and J. Bello), and because this tyrosine can be iodinated at pH 9.5 (Cha and Scheraga, 1963), while tyrosine residues 97 and 25 are resistant to iodination.

The difference in the number of tyrosines titrated at pH 13, $t = 0$ (6 by Tanford *et al.*, 5 by Shugar, and 4 in this work), may arise from variations in the conformations of different batches. (We have noted variations in reduction of disulfides, spectra, crystallizability, and crystal diffraction spectra.)

The reverse time dependence of RNase in 8 M urea–0.5 M Cs_2SO_4 suggests that cesium binds to RNase more strongly than do sodium or potassium, but there was no time-dependent pH change in support. A pH change caused by cesium binding may be balanced by a change caused by sulfate binding.

The $t = 0$ titration curve for RNase in 8 M urea–1 M Cs_2SO_4 shows some indication of a step near pH 11, at the crossover of the $t = 0$ and $t = 30$ curves, suggesting that about two tyrosines are easier to titrate than the rest.

Herskovits and Laskowski (1968), by the solvent perturbation method, showed that 84–88% of the tyrosines of RNase in 8 M urea are accessible to glycerol or sucrose. This is equivalent to one inaccessible tyrosine residue; this was attributed to partial shielding of tyrosine near disulfide bonds. Although titration in 8 M urea shows no abnormal tyrosine, titration in 8 M urea–0.25 M sulfate shows up to 1.4 such. In 8 M urea–0.25 M sulfate (cation not specified) Herskovits and Laskowski showed by the solvent perturbation method that 3.6 tyrosines are accessible, at pH 2.8, or 2.4 tyrosines are inaccessible, compared with 3.4 accessible in water. The smaller number of inaccessible tyrosines indicated by titration (Figure 2) may arise from several causes: (1) exposure during pH adjustment (*i.e.*, before $t = 0$); (2) exposure below pH 10.5, as a result of greater instability of part of the conforma-

tion to pH and urea than to either alone, or (3) to partial burial of tyrosine, with the hydroxyl group more exposed than the benzene ring.

The thermal perturbation difference spectrum of RNase in 8 M urea showed 5 exposed tyrosines at pH 6.7, in agreement with the result of Herskovits and Laskowski (1968) at pH 5.8. However, at pH 5.5–5.7, we found 6.1 exposed tyrosines by thermal perturbation. But Ac_3RNase in 8 M urea at pH 6.7 showed complete exposure of nonacetylated tyrosines, instead of one buried tyrosine, as expected from the results with RNase. This may indicate that Ac_3RNase unfolds more easily than does RNase. The thermal difference spectrum of RNase in 8 M urea–0.25 M K_2SO_4 indicated about 3.5 exposed tyrosines, or 2.5 buried. This is in good agreement with the results of Herskovits and Laskowski, but as our pH was about 6.7 compared with their 2.8, we might have expected a less denatured state. The value of 1.4 exposed tyrosine, or 1.6 buried, in Ac_3RNase in 8 M urea–0.25 M K_2SO_4 is in agreement with the 1.5 abnormal tyrosines shown by titration data of unmodified RNase. The thermal perturbation difference spectrum of Ac_3RNase in water (Bello, 1969) indicated 1.1 exposed tyrosines.

The value of 3.5 exposed tyrosines for RNase in 8 M urea–0.25 M K_2SO_4 is similar to the value of 3.6 in H_2O, but higher than the value of 3.1 in 0.25 M K_2SO_4. The last two figures indicate that 0.25 M K_2SO_4 tightens the conformation of RNase in both water and urea. From the chemical evidence, and from the only partial restoration of viscosity and optical properties effected by K_2SO_4 in urea, we should have expected to find greater exposure of tyrosines in urea–sulfate than in H_2O.

The acetylation experiments in 8 M urea–0.25 M K_2SO_4 show that tyrosines buried by physical criteria are exposed for chemical reaction. But greater concentrations of sulfate cause more extensive reburial. Iodination and nitration were more sensitive to sulfate, suggesting that *ortho* carbons of the phenolic groups may be more reburied than are the hydroxyl groups.

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