The Thermal Perturbation Method for the Estimation of Exposed Tyrosines of Proteins. I. Ribonuclease in Aqueous Glycol, Glycerol, and Denaturants*

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ABSTRACT: A new spectral method is presented for the estimation of exposed chromophores of proteins, and has been applied to the tyrosines of bovine pancreatic ribonuclease. The method is that of thermal perturbation, and is based on the change in spectrum arising from the change in solvation of the chromophore accompanying a change in temperature. Difference spectra are taken from identical solutions at two temperatures, one usually 25°, the other usually 4°, but the temperature range used must be outside the range of a thermal conformational transition. For the tyrosine chromophore the difference absorption band at around 290 mμ is used, and $\Delta\epsilon_{290}$ for RNase is divided by $\Delta\epsilon_{290}$ for the model compound to give the number of exposed tyrosines. For ribonuclease in the denaturants guanidinium chloride and LiBr, the numbers of exposed tyrosines are in good agreement with estimates made by other methods. For ribonuclease in H_2O 3.6 exposed tyrosines were found, in agreement with other spectral data and with X-ray data. For ribonuclease in 75–97% glycerol or ethylene glycol the number of exposed tyrosines is greater than indicated by other methods.

Herskovits and Laskowski (1962) introduced the method of solvent perturbation difference spectra to estimate the number of exposed and buried chromophoric side chains of proteins, such as the phenolic side chain of tyrosyl residues. The method makes use of the change of spectrum when a second solvent component, such as glycol[1] or sucrose, is added. The change in solvent changes the spectrum of accessible chromophores. This change is evaluated with reference to model compounds such as *N*-AcTyr-NH_2. This has been a valuable technique for the study of proteins. However, a difficulty arises in the case in which the transition temperature, T_m, is close to the temperature at which the solvent perturbation experiment is carried out, normally 25°. In such a condition, there will be comparable proportions of native and denatured states, and the addition of perturbing solvent may shift the equilibrium toward one state or the other giving rise to invalid results (Herskovits and Laskowski, 1968). A case in point appears to be that of bovine pancreatic RNase in neat glycol for which T_m is near 25° (Bello, 1969). Herskovits (1964) and Herskovits and Laskowski (1968) studied this system using 20% Me_2SO as the perturbant, and found that about 3.4 tyrosyl residues are exposed, a value similar to that in water. Since T_m in neat glycol is near room temperature, 80% glycol–20% Me_2SO may not be similar to neat glycol. Also, we have presented spectral, rotatory, and circular dichroic data that suggest that in 97% glycol the tyrosine side chains of RNase are largely but not entirely exposed (Bello, 1969).

Since the solvent perturbation method of Herskovits and Laskowski may not be valid when the major solvent component is not water, we sought for another spectral method of finding exposed tyrosines. It occurred to us that a variation of this method might be to perturb the solvent, not by adding another solvent, but by changing the solvent character and the solvation of the chromophore by changing the *temperature*. We found, indeed, that changing the temperature produces substantial difference spectra, which are not merely spectra arising from the difference in concentration accompanying the volume change induced by cooling.

Results

It is to be understood in all of the following that all solutions of RNase and *N*-AcTyr-NH_2 in H_2O, glycol, glycerol, Gu·HCl, and Me_2SO contain a pH 7.0 buffer of 0.075 M ionic strength (see Materials and Methods). In Figure 1 are shown some difference spectra at 4° measured against 25°, in 6 M Gu·HCl, with identical solutions in the two beams of the spectrophotometer. Since the λ values vary with solvent composition, the peaks and troughs will be more conveniently identified by the letters, A, B, and C instead of by wavelength. The principle is to find $\Delta\epsilon_A$ for the protein and for the model compound, *N*-AcTyr-NH_2, and calculate the ratio. This ratio should equal the number of exposed tyrosine side chains. We call this method the thermal perturbation method. The thermal perturbation method can be used only if there is no conformation change in the temperature range studied. A linear plot of $\Delta\epsilon$ *vs.* temperature is considered indicative of no conformation change. The linearity of $\Delta\epsilon_A$ of *N*-AcTyr-NH_2 and RNase with temperature for some solvent compositions containing glycol and glycerol is shown in Figure 2. In the T_m region $\Delta\epsilon_A$ was not linear with temperature.

* From the Department of Biophysics, Roswell Park Memorial Institute, Buffalo, New York 14203. *Received April 7, 1969.* This work was supported by U. S. Public Health Service (Institute of General Medical Sciences) Grant GM 13485 and National Science Foundation Grants GB 7523 and GB 7340.

[1] Abbreviations used are: glycol, ethylene glycol; Ac_3RNase, Ac_4-RNase, Ac_6RNase, RNase having three, four, or six tyrosines O-acetylated; RCM-RNase, RNase with disulfides reduced and carboxymethylated; NMA, *N*-methylacetamide; DMA, *N*,*N*-dimethylacetamide; Gu, guanidine.

TABLE I: Thermal Perturbation Spectral Data in 6 M Gu · HCl.[a]

Solute	λ_A (mμ)	$\Delta\epsilon_A$	$\Delta\epsilon_A'$[b]	$\Delta\epsilon_A'/\Delta\epsilon_A(N\text{-AcTyr-NH}_2)$ Found	Theory
N,*O*-Ac_2Tyr	287	−12			
N-AcTyr-NH_2	287	−47			
RNase	288	−299	−281	6.0	6.0
Ac_3RNase	287	−212	−158	3.3	3.0[c]
Ac_3RNase[d]	287	−184	−138	2.9	2.8
Ac_4RNase[e]	288	−165	−99	2.1	1.9
Ac_6RNase	287	−86	+4	−0.3[f]	0.0

[a] 5° *vs.* 25°. [b] Where $\Delta\epsilon_A' = \Delta\epsilon_A - n\Delta\epsilon_A(N,O\text{-}Ac_2Tyr)$, n being the number of acetyl groups per RNase molecule. Includes correction for disulfides. [c] For this sample the direct spectrum taken before and after thermal perturbation spectrum showed the loss of 0.25 acetyl group/molecule of RNase. The other samples showed no significant loss. The loss of 0.25 acetyl group would result in approximately 0.2 exposed tyrosine too much. [d] This preparation contained 3.2 *O*-AcTyr/molecule of RNase. [e] This preparation contained 4.1 *O*-AcTyr/molecule of RNase. [f] Calculated as *N*,*O*-Ac_2Tyr; −0.1 calculated as *N*-AcTyr-NH_2.

The thermal perturbation method was tested on RNase in 6 M Gu · HCl, a solvent which is an effective unfolder of proteins, although even in this solvent three of the tyrosines of RNase show a slightly higher p*K* than the other three (Nozaki and Tanford, 1967). Thermal perturbation difference spectra were obtained for RNase and *N*-AcTyr-NH_2 in 6 M Gu · HCl. The results are shown in the upper part of Table I, and show that 6.0 (theory 6.0) tyrosines are exposed. This result encouraged us to continue.

In using the thermal perturbation method in a denaturing solvent such as Gu · HCl, it would be desirable to be able to see only those tyrosines which are "buried" when RNase is dissolved in H_2O. Chemical modification of the exposed tyrosines to produce modified tyrosines of low ϵ would be suitable. Of the several methods of modifying tyrosine, the

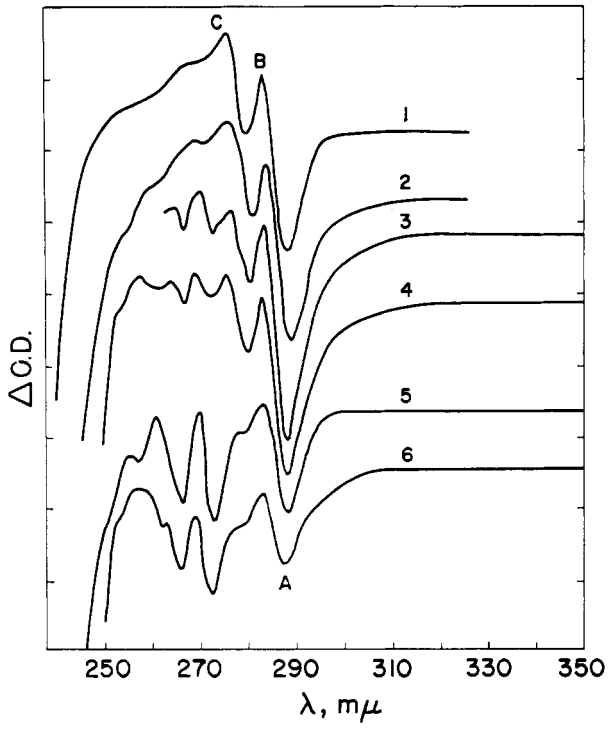

FIGURE 1: Thermal perturbation difference spectra in 6 M Gu · HCl. Spectrum 1, *N*-AcTyr-NH_2, 0.1 mg/ml; 2, RNase, 1 mg/ml; 3, Ac_4RNase, 2 mg/ml; 4, Ac_3RNase, 3 mg/ml; 5, *N*,*O*-Ac_2Tyr, 0.3 mg/ml; 6, Ac_6RNase, 3 mg/ml. Concentrations are rounded. Each division on the Δ_{OD} axis represents 0.01 Δ_{OD}.

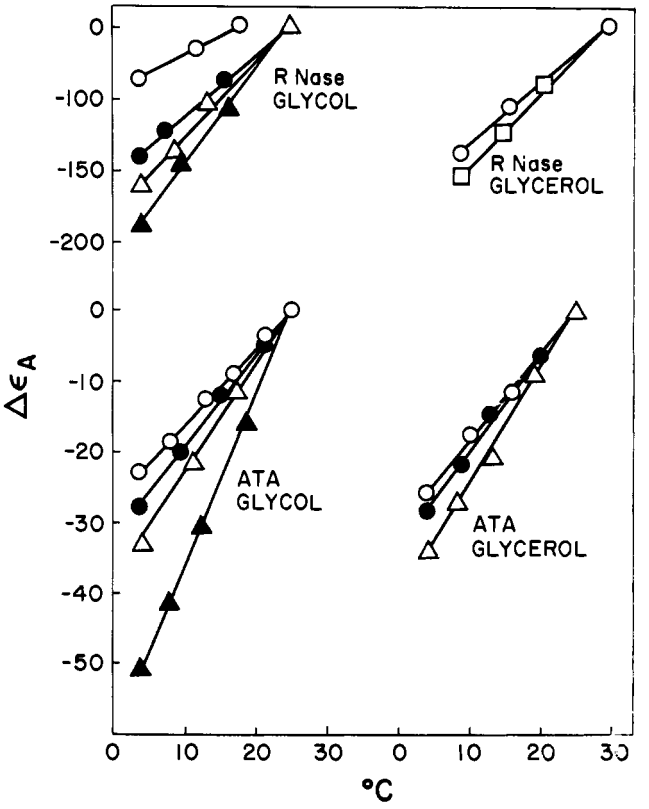

FIGURE 2: $\Delta\epsilon_A$ of *N*-Actyr-NH_2 and RNase as a function of glycol and glycerol concentration. (▲) H_2O, (□) 50% glycerol; (△) 75% glycol or glycerol; (●) 90% glycol or glycerol; (○) 97% glycol or glycerol. All difference spectra are of 4° *vs.* 25° solutions, except for RNase in 97% glycol which is for 4° *vs.* 18°.

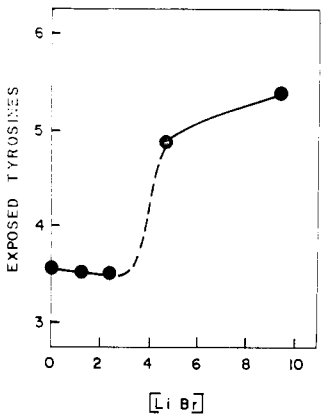

FIGURE 3: Exposed tyrosine of RNase in LiBr.

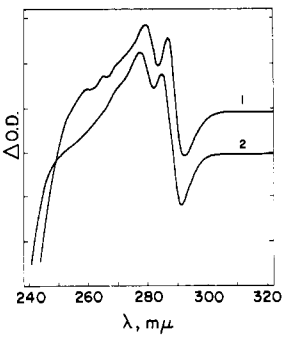

FIGURE 5: Thermal perturbation spectra of *N*-AcTyr-NH_2 and RNase in 97% glycerol. Spectrum 1, RNase, 1 mg/ml; 2, *N*-AcTyr-NH_2, 0.1 mg/ml. Concentrations rounded. Each division on the Δ_{OD} axis represents 0.01 Δ_{OD}.

most suitable appeared to be O-acetylation by *N*-AcIm (Simpson *et al.*, 1963). The ϵ of *N,O*-Ac_2Tyr is about one-tenth that of tyrosine, and the thermal perturbation difference spectrum gave $\Delta\epsilon_A = -12$ in water. Acetylation of RNase in water normally modified three of the six tyrosines (and most of the lysine amino groups) although four can be acetylated with a larger amount of *N*-AcIm. We applied the thermal perturbation method to Ac_6RNase, Ac_4RNase, and Ac_3RNase in 6 M Gu·HCl, with the gratifying results shown in Table I. The thermal perturbation difference spectra for these systems are shown in Figure 1. (The spectra shown in the several figures are direct tracings of the spectrophotometer records, and since the concentrations are not all the same, the $\Delta\epsilon$ values are not shown, but are given in the tables or in the text.) The spectra for *N*-AcTyr-NH_2 and RNase show considerable similarity, as do those of *N,O*-Ac_2Tyr and Ac_6RNase. The spectra for Ac_3RNase and Ac_4-RNase are intermediate in shape, as expected. $\Delta\epsilon_A$ for all solutes in 6 M Gu·HCl was linear with temperature. $\Delta\epsilon_A'$ also includes a correction for the contribution of the disulfides, using oxidized glutathione as the model. Four disulfides give $\Delta\epsilon_A = -18$. The negative value of -0.4 *N,O*-Ac_2Tyr for Ac_6RNase in 6 M Gu·HCl is equivalent to -0.1 tyrosine, within the experimental error.

Thermal perturbation difference spectra were also obtained for RNase and *N*-AcTyr-NH_2 in LiBr at concentrations from 1.2 to 9.4 M, with the results shown in Figure 3. These results are in substantial agreement with those of Sarfare and Bigelow (1967), showing no denaturation up to 2.4 M LiBr, and five exposed tyrosines at 5 M LiBr. An increase in exposed tyrosines is seen between 4.7 and 9.4 M LiBr. Sarfare and Bigelow found an increase in apparent exposure between 5 and 10 M LiBr, but attributed this to the effect of LiBr on exposed tyrosines of RNase being somewhat different from the effect on the model compound. However, it is possible that additional exposure of tyrosines does take place. Thermal perturbation difference spectra in the dashed region of Figure 3 showed clear evidence of renaturation at 4° in the large positive extrema at 280 and 287 mμ. This also agrees with the finding by Sarfare and Bigelow of a temperature dependence in this concentration range. There may also be some contribution to the thermal perturbation spectra from exposure

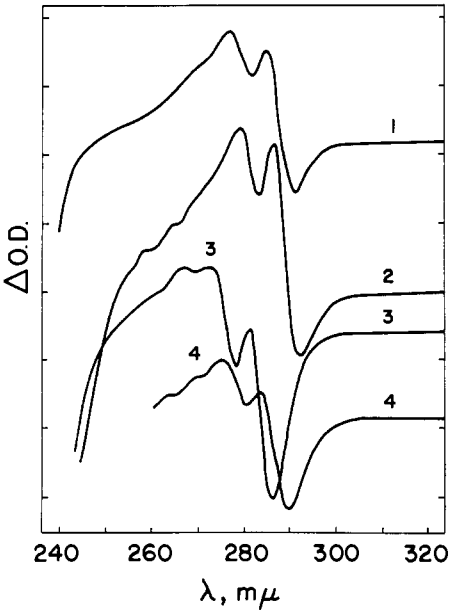

FIGURE 4: Thermal perturbation spectra in water and 97% glycol. Spectrum 1, *N*-AcTyr-NH_2 in glycol, 0.05 mg/ml; 2, RNase in glycol, 1 mg/ml; 3, *N*-AcTyr-NH_2 in H_2O, 0.1 mg/ml.; 4, RNase in H_2O, 1 mg/ml. Concentrations are rounded. Each division on the $\Delta_{O.D.}$ axis represents 0.01 Δ_{OD}.

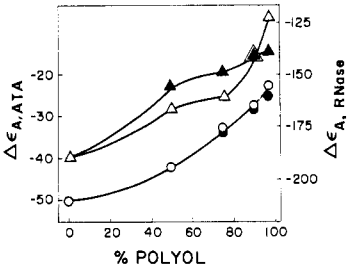

FIGURE 6: $\Delta\epsilon_A$ of *N*-AcTyr-NH_2 and RNase as a function of glycol and glycerol concentration. (○) *N*-AcTyr-NH_2 in glycol; (●) *N*-AcTyr-NH_2 in glycerol; (△) RNase in glycol; (▲) RNase in glycerol. The datum for 97% glycol obtained at 4° *vs.* 18° has been normalized to 4° *vs.* 25° by direct proportion; $\Delta\epsilon_A$, 4° *vs.* 18°, was −82.

TABLE II: Exposed Tyrosines of RNase in Glycol and Glycerol by Thermal Perturbation Difference Spectra.

Solute	Solvent	λ_A(RNase)	$\Delta\epsilon_A$(*N*-AcTyr-NH_2)	$\Delta\epsilon_A$(RNase)	$\Delta\epsilon_A$(RNase)/$\Delta\epsilon_A$(ATA)
RNase	H_2O	288	−50	−190	3.8
RNase	50% glycol	291	−42	−167	4.0
RNase	75% glycol	291.5	−34	−161	4.7
RNase	90% glycol	292	−28	−141	5.0
RNase	97% glycol	292	−23	−123	5.4
RNase	50% glycerol	291	−42	−156	3.7
RNase	75% glycerol	291	−34	−151	4.4
RNase	90% glycerol	292	−28	−140	5.0
RNase	97% glycerol	292	−26	−137	5.3
RCM-RNase	H_2O	287	−50	−290	5.8
RCM-RNase	97% glycol	292	−23	−127	5.5
RCM-RNase	97% glycerol	292	−26	−129	5.0

of disulfides. Exposure of all four disulfides would contribute about 0.4 to the tyrosine count.

The method was then applied to RNase in aqueous mixtures of glycol and glycerol. Thermal perturbation difference spectra for H_2O, 97% glycol, and 97% glycerol are shown in Figures 4 and 5. While λ_A for *N*-AcTyr-NH_2 and RNase are nearly the same in glycol or glycerol, there is a significant difference in H_2O, 4 mμ. At all glycol or glycerol compositions from 50 to 97% all $\Delta\epsilon_A$'s were linear with temperature (Figure 2). (Compositions between 0 and 50% were not investigated.) All of the data of Figure 2 were obtained from difference spectra with the reference solution at 25°, except for RNase at 97% glycol concentration for which 18° was used. In the latter case 25° is near T_m of RNase and some curvature was observed with the reference at 25°. *N*-AcTyr-NH_2 was also measured at both 18 and 25° *vs.* 4° with no significant difference in the final result. In Figure 6 is shown $\Delta\epsilon_A$ as a function of solvent composition. The thermal perturbation data for RNase in glycol and glycerol are summarized in Table II. The estimated number of tyrosines in water is 3.8, compared with 3.4 found by Herskovits and Laskowski (1968) by the solvent perturbation method using sucrose as perturbant. Other perturbants gave smaller values. (With lot 8CA of Worthington type RAF, 3.6 tyrosines are exposed.) With increasing glycol or glycerol content the number increases (Figure 7). There is only a small change of exposed tyrosines until about 75% polyol is reached.

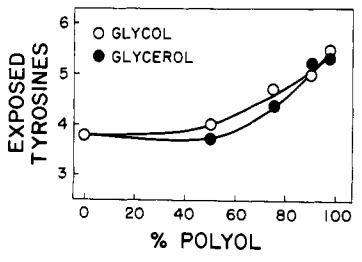

FIGURE 7: Number of exposed tyrosines of RNase as a function of glycol and glycerol concentration, as indicated by the thermal perturbation difference spectral method.

Thermal perturbation difference spectra were also obtained for RCM-RNase with the results shown in Table II. In H_2O, 5.8 tyrosines are shown to be exposed, but in 97% glycol and glycerol smaller values were found.

Turning next to the acetylated RNase in H_2O, glycol, and glycerol, the results of thermal perturbation are shown in Figure 8 and Table III. These results are limited to 75% glycol and glycerol. At 88% glycol solvolysis of *O*-Ac groups was so rapid that useful results could not be obtained. At 50 and 75% glycol there appears to be some additional exposure of tyrosines over that for RNase. The same appears, but to a lesser extent, for 75% glycerol. For Ac_6RNase large negative values of exposed *O*-AcTyr were found in H_2O and 50% glycol, whereas in 6 M Gu·HCl a much smaller negative value was found. Ac_6RNase is less easily soluble in H_2O than is RNase, but is easily soluble in 6 M Gu·HCl.

The thermal perturbation method was then applied to RNase and Ac_3RNase in 90% Me_2SO–10% H_2O (Figure 9 and Table IV), with the indication of complete exposure of

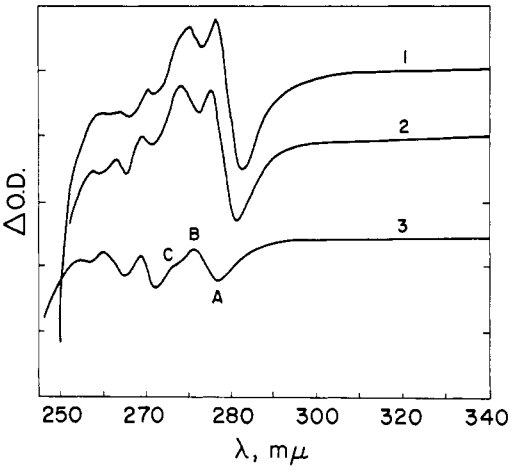

FIGURE 8: Thermal perturbation difference spectra of acetylated RNase in H_2O. Spectrum 1, RNase, 1 mg/ml; 2, Ac_3RNase, 3 mg/ml; 3, *N*,*O*-Ac_2Tyr, 0.15 mg/ml.

TABLE III: Thermal Perturbation Difference Spectral Data for Acetylated RNase in Ethylene Glycol and Glycerol Solutions.[a]

Solute	Solvent	λ_A (mμ)	$\Delta\epsilon_A$	$\Delta\epsilon_A'$	$\Delta\epsilon_A'/\Delta\epsilon_A$(*N*-AcTyr-$NH_2$)
N,*O*-Ac_2Tyr	H_2O	287	−12		
Ac_3RNase	H_2O	291	−90	−54	1.1
Ac_4RNase	H_2O	291	−67	−21	0.4
Ac_6RNase	H_2O	291	−27	+45	−3.8[b]
N,*O*-Ac_2Tyr	50% glycol	288	−7		
Ac_3RNase	50% glycol	292	−73	−53	1.26
Ac_6RNase	50% glycol	292	−12	+30	−2.5[b]
N,*O*-Ac_2Tyr	75% glycol	292	−6		
Ac_3RNase	75% glycol	292	−69	−51	1.5
N,*O*-Ac_2Tyr	75% glycerol	292	−7		
Ac_3RNase	75% glycerol	292	−61	−40	1.2

[a] Temperature difference, 21°. [b] Calculated as *N*,*O*-Ac_2Tyr.

tyrosines. This was supported by the finding that no T_m could be observed spectrophotometrically at temperatures up to 77°, 14° above T_m in H_2O. $\Delta\epsilon_A$, $\Delta\epsilon_B$, and $\Delta\epsilon_C$ were all linear with temperature and nearly parallel to those of *N*-AcTyr-NH_2 in the same solvent. The difference spectra of RNase and *N*-AcTyr-NH_2 were closely similar at all temperatures, with no indication of the sharp change in $\Delta\epsilon_B$ and $\Delta\epsilon_C$ that distinguishes RNase from *N*-AcTyr-NH_2 in the transition region of RNase. It is unlikely that RNase in 90% Me_2SO is exceedingly stable. This is also supported by circular dichroism. In 90% Me_2SO, $\epsilon_l - \epsilon_r$ at 278 mμ is 2.5 for RNase, while it is 7 for RNase in water. Above T_m, $\epsilon_l - \epsilon_r$ is 1 in water for RNase. The circular dichroism data show considerable, but not necessarily complete, unfolding of RNase in 90% Me_2SO.

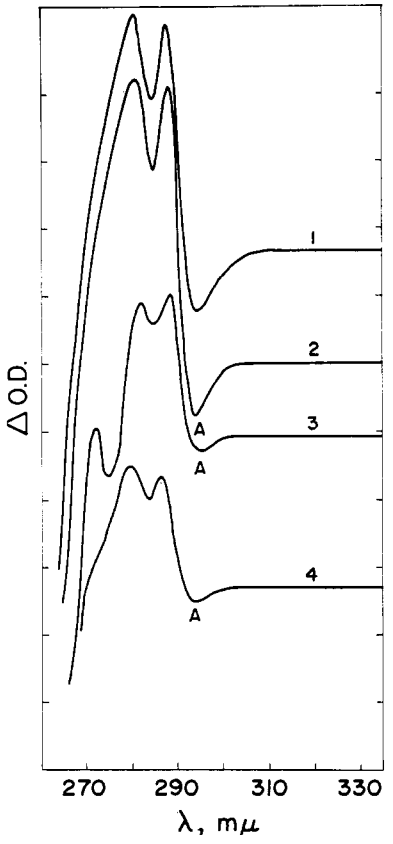

FIGURE 9: Thermal perturbation spectra in 90% Me_2SO. Spectrum 1, RNase, 1 mg/ml; 2, *N*-AcTyr-NH_2, 0.1 mg/ml; 3, *N*,*O*-Ac_2Tyr, 0.3 mg/ml; 4, Ac_3RNase, 1 mg/ml. Concentrations are rounded. Each division on the Δ_{OD} axis represents 0.01 Δ_{OD}.

Since Herskovits and Laskowski (1968) found 3.4 exposed tyrosines by taking the spectral difference between RNase in neat glycol and RNase in 80% glycol–20% Me_2SO, we applied the thermal perturbation method to RNase and *N*-AcTyr-NH_2 in a mixture of 2.5% H_2O, 77.5% glycol, and 20% Me_2SO and found $\Delta\epsilon_A$(*N*-AcTyr-NH_2) and $\Delta\epsilon_A$(RNase) to be −28 and −152, respectively. The ratio gives 5.4 exposed tyrosines. λ_A was 292.5 mμ for both *N*-AcTyr-NH_2 and RNase. $\Delta\epsilon_A$ was linear with temperature, but on rewarming the cooled sample to 25° a small negative absorbance was observed at 275–280 mμ, with $\Delta\epsilon = -50$, which decreased with time. By inserting the sample cuvette into the precooled cuvette holder and measuring the difference spectrum at intervals from 5 min to 1 hr no time dependence was observed. λ_{max} of the direct spectrum of *N*-AcTyr-NH_2 in this solvent was 278.5 mμ.

Thermal Perturbation Spectra of RNase in D_2O. We had

TABLE IV: Thermal Perturbation Spectral Data in 90% Me_2SO.

Solute	λ_A (mμ)	$\Delta\epsilon_A$	$\Delta\epsilon_A'/\Delta\epsilon_A$-(*N*-AcTyr-$NH_2$)
N-AcTyr-NH_2	295	−18	
N,*O*-Ac_2Tyr	295	2	
RNase	294	−113	6.0
Ac_3RNase	294	−59	2.9

thought that information on the state of the tyrosines of RNase in glycol and glycerol might be obtained by taking isothermal difference spectra of RNase in deuterated solvent *vs.* protiated solvent, and similarly of *N*-AcTyr-NH_2 in these solvents. Isothermal difference spectra in D_2O *vs.* H_2O gave the result of six to seven exposed tyrosines. This seemed quite unlikely. Circular dichroism spectra of RNase at 350–250 mμ were nearly identical in D_2O and H_2O. Absorption spectra gave: for RNase in H_2O, λ_{max} 277.5 mμ, ϵ_{max} 9800 ($\epsilon/6$ = 1630); for RNase in D_2O, λ_{max} 277 mμ, ϵ_{max} 10,300 ($\epsilon/6$ = 1720); for *N*-AcTyr-NH_2 in H_2O, λ_{max} 274 mμ, ϵ_{max} 1410; for *N*-AcTyr-NH_2 in D_2O, λ_{max} 274 mμ, ϵ_{max} 1490. If RNase in D_2O were denatured, λ and $\epsilon/6$ would have been closer to those of *N*-AcTyr-NH_2. Spectrophotometric titrations in both D_2O and H_2O showed three tyrosines titrated at pH 12. Thermal perturbation spectra showed RNase in 98% D_2O to be native. λ_A was 286 mμ for *N*-AcTyr-NH_2 and 285 for RNase. $\Delta\epsilon_A$(*N*-AcTyr-NH_2) was −62 and $\Delta\epsilon_A$(RNase) was −235, giving a ratio of 3.8, uncorrected for volume contraction and internal tyrosines. This is the same as the result in H_2O, and is in agreement with the direct spectra, circular dichroism, and titration data in indicating that RNase is essentially native in 98% D_2O.

The number of exposed tyrosines shown in the various tables is reproducible to ±0.2 when the RNase concentration is about 1.5 mg/ml. At lower concentrations the poorer signal to noise ratio makes the reproducibility worse, *e.g.*, about ±0.5 at 0.5 mg/ml. For Ac_6RNase, a concentration of about 3–4 mg/ml is desirable.

The $\Delta\epsilon_A$ data in the tables have not been corrected for concentration changes arising from the volume changes accompanying cooling. The correction to be applied is for the contribution of buried groups to $\Delta\epsilon_A$, that for exposed groups is automatically made by taking the ratio $\Delta\epsilon_A$(RNase)/$\Delta\epsilon_A$(*N*-AcTyr-NH_2). ϵ_{291} is one-fourth of ϵ_{278} (λ_{max} for the direct spectrum); the volume change is 0.3% for H_2O between 25 and 4°, and the buried groups contribute about 60% of the total ϵ. The last is computed from ϵ_{278}(RNase) = 9700, ϵ_{274}(*N*-AcTyr-NH_2) = 1400, $3\epsilon_{274}$(*N*-AcTyr-NH_2) = 4200, giving a difference of ϵ_{278} = 5500 for buried tyrosines, or 56%. We use 60% for a round number. Thus the volume error for H_2O would be $\Delta\epsilon_A$ = 4, making the calculated $\Delta\epsilon_A$ more negative by 4. This is only 2%, but since some of the buried groups are partially exposed the correction is less. In 97% glycol or glycerol the volume change would be four times as great, 1.2% (International Critical Tables, 1928, using 97% polyol as equivalent to neat polyol), but with most of the tyrosines exposed, the correction is smaller, probably one-sixth as much since only 0.5 tyrosine is buried. Thus in 97% glycol the correction to $\Delta\epsilon_A$ is about 2–3, or again about 2%. The intermediate solvent compositions have corrections of the same order. Since a 2% correction would not be significant, it has not been included in the tables. This correction changes the number of exposed tyrosines in H_2O from 3.8 to 3.7 (and from 3.6 to 3.5 for lot 8CA).

$\Delta\epsilon_B$ and $\Delta\epsilon_C$ could not be used in the same manner as $\Delta\epsilon_A$; they gave too large numbers of exposed tyrosines. $\Delta\epsilon_C$ in H_2O for RNase and *N*-AcTyr-NH_2 are 123 and 23, respectively, yielding 5.4 for the number of exposed groups. At λ_C the correction arising from the volume change is much larger than at λ_A because λ_C is near λ_{max} of the direct spectrum. This correction is $\Delta\epsilon$ = 17 (0.3% of 5500), making $\Delta\epsilon_C$ = 106, and the number of exposed tyrosines 4.6. But a further correction can be made.

After this paper was submitted for publication Cane (1969) presented a preliminary account of an independent development of the thermal perturbation method (*Fed. Proc.* April 1969, Abstract No. 1183). Cane took into account the thermal expansion of the protein and obtained 2.8 exposed tyrosines (not included in the abstract.)

Thermal perturbation spectra of *N*-AcTyr-NH_2 were also run in 72% NMA–8% DMA–20% H_2O, in 90% NMA–10% DMA, in neat glycol, and in neat dioxane. The values of $\Delta\epsilon_C$ in these solvents were 90, 130, 40, and 60. The corresponding value in water was 18. For phenol in 80% hexane–20% ethanol $\Delta\epsilon_C$ was 130. We shall consider the interior of the protein to be intermediate in nature between glycol and hexane–ethanol, corresponding to $\Delta\epsilon_C$ = 70, being within a factor of 2 of the extremes. $\Delta\epsilon_A$ in such a medium would be −15, intermediate between −20 in neat glycol and −10 in hexane–ethanol. The X-ray structure of RNase shows that the buried tyrosines are not surrounded largely by hydrocarbon groups, but by a mixture of polar and some nonpolar groups.

The problem here is to find a suitable factor for this effect. To assume that the thermal perturbation spectral effect of the buried tyrosines would be similar to that of exposed tyrosines in solvent equivalent to the interior of the protein would give unacceptable values of exposed tyrosines. Thus, at λ_A, the correction would reduce our computed value of exposed tyrosines, from 3.8 to 2.7; at λ_C the computed value of exposed tyrosines would be negative, −4. Thus, the effective environmental change for buried tyrosines must be much less than for exposed tyrosines in a similar environment.

Whereas the surrounding molecules in a liquid are free to move and reorient around a chromophore (with a change in the number and types of atoms around the chromophore), the protein groups around a buried chromophore are highly restricted in their movement. If there is no conformational change, there will be only a change in distances; the number and types of surrounding atoms will not change. For the temperature range 4–25°, the linear expansion of the solvents used is not more than 1%. Thus, even if we accept a linear coefficient of expansion of this order, the effect on the chromophore must be much smaller than for a model compound.

Using values of 10% of −15 and 70, the correction at λ_A is $\Delta\epsilon$ = 5 (for 3 buried tyrosines) or 1.5%, giving (with the 2% correction for the volume change of the solution), 3.6 exposed tyrosines, and the correction at λ_C is $\Delta\epsilon$ = 21 or 20%. Combined with the correction due to the concentration change arising from volume change of the solution at 4°, the number of exposed tyrosines calculated at λ_C is 3.8, in good agreement with that at λ_A.

Examination of the X-ray structure of RNase indicates that no correction need be made for phenylalanine residues, as these are well buried. The correction for cystine is negligible as there are mostly buried and $\Delta\epsilon$ is small for exposed cystine (see below).

In an effective denaturant, we should find that $\Delta\epsilon_C$ and $\Delta\epsilon_A$ give similar results, without correction for buried groups. In 6 M Gu·HCl, $\Delta\epsilon_C$ for RNase is 165. Corrected for phenylalanine ($\Delta\epsilon_C$ = 9 for three phenylalanines) and disulfides ($\Delta\epsilon_C$ = −20 for four disulfides, using oxidized glutathione as the model), $\Delta\epsilon_C$ is 176. These corrections are needed be-

cause we expect that these chromophores are exposed in 6 M Gu·HCl. Since $\Delta\epsilon_C$ for *N*-AcTyr-NH_2 in 6 M Gu·HCl is 28, the ratio is 6.3 in good agreement with that at λ_A (6.0).

In view of the much smaller spectral contribution of buried tyrosines at λ_A, it appears preferable to use $\Delta\epsilon_A$ rather than $\Delta\epsilon_C$. We have not used $\Delta\epsilon_B$ as it is strongly influenced by both $\Delta\epsilon_A$ and $\Delta\epsilon_C$, the latter two having opposite signs.

Discussion

The thermal perturbation results for RNase and acetylated RNase in 6 M Gu·HCl which show complete exposure of tyrosines strongly indicate that the thermal perturbation method is usable in principle. This is supported further by the good results in LiBr, which are in good agreement with denaturation blue shift data.

The number of exposed tyrosines of RNase in H_2O as indicated by thermal perturbation is 3.5–3.7 compared with 3.4 by solvent perturbation using sucrose as perturbant (Herskovits and Laskowski, 1968). This is good agreement, although some perturbants gave smaller values. Examination of the structure of RNase as obtained by X-ray diffraction (D. Harker, G. Kartha, and J. Bello, unpublished data) shows that the buried tyrosines are not completely buried. Tyr-92 is about 0.7–0.8 exposed and Tyr-25 is about 0.3–0.4 exposed. Tyr-97 is almost completely buried. On the other hand, the 3 exposed tyrosines are not completely exposed, but taken together are equivalent to about 2.5 exposed tyrosines. Thus the total of exposed tyrosines is about 3.6 exposed tyrosines, with 3.2 and 3.8 as extreme values. Both solvent perturbation and thermal perturbation data are consistent with the X-ray data. Of course, the conclusions from X-ray data must be taken with some reservation because of the possible differences between the structure in the crystal and in solution.

A difficulty inherent in both the solvent perturbation and thermal perturbation methods is the question of the suitability of the model compound as an accurate model of the chromophore in the protein. While the model compound is completely accessible to all solvent components, the same is not necessarily true for the chromophore in the protein. Part of the chromophore may be inaccessible to all solvent components, and part may be inaccessible to one of the components. Or part of a chromophore may be accessible to only one part of one of the component molecules, *e.g.*, to a methyl or oxygen of Me_2SO, but not to the whole molecule. Another difficulty is that a free tyrosine may respond differently, in regard to its spectral change, from one in which the hydroxyl group is hydrogen bonded to another group of the protein, changing not only the nature of the solvation but also the electronic transitions. A manifestation of this may be the 4-mμ difference in λ_A between *N*-AcTyr-NH_2 and RNase in H_2O. In the case of a solvent that effectively unfolds the protein, such as 6 M Gu·HCl, the chromophores are well exposed and the above considerations are not applicable. Also, $\Delta\epsilon_A$ for *N*-AcTyr-NH_2 is nearly the same in H_2O and in 6 M Gu·HCl, so that some preferential solvation would not be manifested. From the good results of the solvent perturbation and thermal perturbation methods applied to RNase in H_2O, it appears that there is no cause for concern in this case as well.

The results for Ac_3RNase and Ac_4RNase in H_2O also appear to be reasonable. We assume that in Ac_3RNase only the tyrosines that are normal (Irie and Sawada, 1967) are acetylated, and that the conformation is native. Then, the partial exposure of Tyr-92 and Tyr-25 totaling about 1.0–1.2 tyrosines described above is in good agreement with the thermal perturbation result of 1.1 exposed tyrosines. In the case of Ac_4RNase, we assume that the fourth tyrosine acetylated is Tyr-92, because in our X-ray structure it is the most exposed, and because it can be iodinated at pH 9.5 (Cha and Scheraga, 1963) while the other buried tyrosines, Tyr-97 and Tyr-25, cannot be iodinated. If the conformation of Ac_4RNase is also native, then only Tyr-25 should be exposed to about 0.4, in excellent agreement with the thermal perturbation result. But for Ac_6RNase, having no unmodified tyrosines, the thermal perturbation method indicates −3.8 exposed, or 3.8 buried *O*-AcTyr. This is equivalent to 0.9 buried tyrosine, well beyond the experimental error. Although it is possible that the method gives an incorrect result in this case, it is also possible that some AcTyr are buried. While the *O*-AcTyr is less polar than tyrosine and might tend to be buried in hydrophobic regions, it lacks the hydrogen-bonding capability of tyrosine, a factor partly responsible for the burial of tyrosine in RNase (D. Harker, G. Kartha, and J. Bello, crystallographic results to be published).

Turning to RNase in glycol and glycerol, we see that up to 50% glycol or glycerol there is little, if any, change in the number of exposed tyrosines. Beyond 50%, the number of exposed tyrosines increases, rising to about 5.4 in 97% polyol. If some of the disulfides are exposed the result would be less, about 5 exposed tyrosines (or 1 buried) for 2 exposed disulfides. This result is in agreement with the 0.7–1.4 tyrosines that become exposed on heating, as indicated by $\Delta\epsilon_{287}$ (Bello, 1969), although we are not sure that the latter value represents the total number of buried tyrosines. The results of 75–97% glycerol are higher than is suggested by circular dichroism, optical rotation, and $\Delta\epsilon$ of thermal denaturation (Bello, 1969). The last type of measurement indicates that heating RNase in 97% glycerol results in the uncovering of about 1.3–2.1 tyrosines. Circular dichroism, optical rotation, and $\Delta\epsilon$ of thermal denaturation of RNase change little until beyond 90% glycol or glycerol, and in glycerol, optical rotation does not change even up to 97% glycerol. Nitration of tyrosines of RNase does not exceed that in H_2O till beyond 86% glycol, and not even in 93% glycerol. Thus, beyond 50% glycol or glycerol the thermal perturbation difference spectral method indicates a greater degree of exposure of tyrosines than do other methods. The reason for the disagreement is not clear, but a possible explanation of this is preferential access of H_2O to the chromophore giving too large a value of $\Delta\epsilon_A$.

For RCM-RNase, with disulfides broken, the thermal perturbation method indicates nearly complete exposure of tyrosines in H_2O as expected. The smaller number of exposed tyrosines in 97% glycerol is unexpected. The reason is not apparent to us.

With regard to Ac_3RNase, between H_2O and 75% glycol the increase in exposed tyrosines is 0.4, compared with 0.9 for RNase. The latter may include some further exposure of the exposed tyrosines that are somewhat shielded in aqueous RNase. The increase for Ac_3RNase between H_2O and 75% glycerol is within the experimental error.

The thermal perturbation result of 5.4 exposed tyrosines

for RNase in 97% glycol is considerably greater than the 3.4 proposed by Herskovits and Laskowski (1968) for RNase in neat glycol by the solvent perturbation method using 20% Me_2SO as perturbant. But the solvent perturbation method compares RNase in neat glycol with RNase in 80% glycol–20% Me_2SO, which may be a solvent of different character. The thermal perturbation method applied to RNase in a solvent approaching 80% glycol–20% Me_2SO (2.5% H_2O–77.5% glycol–20% Me_2SO) gave 5.4 exposed tyrosines, the same as the thermal perturbation result in 97% glycol. The small difference in solvent composition is probably not important. The thermal perturbation result might arise from renaturation. But this seems unlikely because Herskovits and Laskowski suggested that Me_2SO destabilized RNase in urea, and we have shown here that RNase is largely unfolded in 90% Me_2SO–10% H_2O. Since both glycol and Me_2SO destabilize RNase, mixing them would be unlikely to restore the native state of the tyrosines. With both methods there is the possibility of some conformation change induced by addition of perturbant in the solvent perturbation method or change of temperature in the thermal perturbation method. If renaturation takes place on cooling there would be a red shift in the direct spectrum with increase in ϵ at 292 mμ, resulting in a diminution of $\Delta\epsilon_A$ in the thermal perturbation difference spectrum leading to a diminution in the calculated number of exposed tyrosines. Only if cooling caused denaturation would the calculated number of exposed tyrosines be too large.

In the solvent perturbation method RNase in glycol is studied by comparison with RNase in glycol–Me_2SO; in the thermal perturbation method RNase is studied only in the solvent of interest, modified by a temperature change. By using the thermal perturbation method below T_m and verifying the linearity of $\Delta\epsilon_A$ with temperature, the probability of a significant conformational change is small. (Of course with a solvent such as 6 M Gu·HCl we work above T_m. The important point is that measurements not be made in the T_m range.)

The results in 90% Me_2SO–10% H_2O indicating complete unfolding are supported by the absence of a thermal transition as measured spectrophotometrically. However, this last may not be absolute support, because there may be a buried tyrosine that does not become exposed on heating. In H_2O, thermal denaturation is not complete; one tyrosine remains buried (Foss, 1960; Bigelow, 1961).

A possible explanation of the result found by Herskovits and Laskowski is preferential binding of glycol to the tyrosines of RNase. Since 20% Me_2SO as a perturbant gives a larger $\Delta\epsilon$ than does 20% glycol (where H_2O is the major solvent in both cases), greater preferential binding of glycol (in 80% glycol–20% Me_2SO) to RNase than to the model compound might give too small a value of $\Delta\epsilon$ and, therefore, too small a value of exposed tyrosines.

An advantage of the thermal perturbation method over the solvent perturbation method is that careful matching of solvent compositions is not required because the same solution is used in both beams of the spectrophotometer. In their solvent perturbation work Herskovits and Laskowski express their data as $\Delta\epsilon_{287}/\epsilon_{277}$, where ϵ_{277} is the molar absorptivity at λ_{max}. This ratio is constant for *N*-AcTyr-NH_2 in several solvents. However, in taking the ratio for RNase to that for *N*-AcTyr-NH_2, an error is introduced, arising from the fact that the buried tyrosines, which contribute to ϵ_{277}, have a higher ϵ_{277} than do exposed tyrosines in the same solvents. This difference is about 30% in H_2O. For *N*-AcTyr-NH_2 this does not arise. Thus, in water $\Delta\epsilon_{287}/\epsilon_{277}$ for the whole protein (with one-half of the tyrosines buried) is approximately 15% smaller than it would be if only ϵ_{277} of exposed tyrosines were being used. This error does not arise in an effective denaturant or in solvents (such as neat glycerol or ethylene glycol) in which ϵ_{277} is the same for buried and exposed groups.

Materials and Methods

Most of the work reported here was done with Worthington Enzyme Corp. (Freehold, N. J.) phosphate-free RNase A type RAF, lot 8BB. Preliminary work was done with type RAF, lot 6509.

Ethylene glycol and glycerol were purified as described earlier (Bello, 1969). Gu·HCl was recrystallized from methanol containing Na_4EDTA. Me_2SO was redistilled *in vacuo*. Double-distilled H_2O was used.

Portions of a single solution were placed in both beams of a Cary Model 15 spectrophotometer. Cuvets were held in close-fitting brass blocks through which tempered water flowed, 25° for the reference beam and another temperature for the sample beam. The sample cuvet holder was inserted in a Cary thermostated cell holder. The tempered water from the brass cuvet holder then passed through the Cary cell holder. Nitrogen gas from liquid nitrogen was used to prevent condensation of moisture. The dynode and sensitivity were usually set at 2, but spectra were also taken at other settings to find the optimum slit and noise conditions. A concentration of RNase of about 1.5 mg/ml was optimum. For Ac_3RNase and Ac_6RNase concentrations of 3 and 4 mg per ml could be used because of their low ϵ values, and were needed because of their low $\Delta\epsilon_A$ values. Base lines generally were adjusted to ± 0.0005, with both sample and reference at the same temperature. This requires temperature agreement within about 0.5°. The temperatures were read with a Yellow Springs thermistor thermometer, using the same probe for both water baths. A second probe was inserted in the water line to the sample cuvet about 2 in. from the cuvet holder. The temperature was not necessarily that in the cuvet, but since the thermal perturbation method requires the ratio of $\Delta\epsilon$ for RNase to $\Delta\epsilon$ for *N*-AcTyr-NH_2, the exact temperature is not required, only that the temperatures for both RNase and *N*-AcTyr-NH_2 be the same. Even the latter is not strictly required since $\Delta\epsilon$ is linear with temperature and $\Delta\epsilon$/deg can be used for both RNase and *N*-AcTyr-NH_2.

RNase and *N*-AcTyr-NH_2 were made up as stock solutions of 60 and 4 mg per ml, respectively, in a buffer containing 2.5 M TMACl and 0.5 M NaAc (pH 7.0), which were diluted to 1.5 and 0.1 mg per ml (40-fold dilution), respectively, with H_2O and organic solvent to obtain the desired concentration. The protein concentration was calculated from the weight of RNase used, corrected for the water content obtained by drying *in vacuo* over phosphorus pentoxide. $\epsilon_{277.5}$ for solutions prepared on this basis was 9700 ± 100, in good agreement with published values (Sela and Anfinsen, 1957). For Ac_3RNase, Ac_4RNase, Ac_6RNase, and *N*,*O*-Ac_2Tyr the required weight (corrected for water content) was weighed out for each solution, because stock solutions are subject to hydrolysis of acetate groups.

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.

References

Bello, J. (1969), *Biochemistry 8*, 4535.
Bigelow, C. C. (1961), *J. Biol. Chem. 236*, 1706.
Cane, W. (1969), *Fed. Proc. 28*, 469.
Cha, C. Y., and Scheraga, H. A. (1963), *J. Biol. Chem. 238*, 2958.
Foss, J. G. (1960), *Biochem. Biophys. Res. Commun. 2*, 193.
Herskovits, T. T. (1964), *J. Biol. Chem. 240*, 628.
Herskovits, T. T., and Laskowski, M., Jr. (1962), *J. Biol. Chem. 237*, 2481.
Herskovits, T. T., and Laskowski, M., Jr. (1968), *J. Biol. Chem. 243*, 2123.
International Critical Tables (1928), Vol. III, McGraw-Hill, New York, N. Y., p 28.
Irie, M., and Sawada, F. (1967), *J. Biochem. (Tokyo) 62*, 282.
Nozaki, Y., and Tanford, C. (1967), *J. Am. Chem. Soc. 89*, 742.
Sarfare, P. S., and Bigelow, C. C. (1967), *Can. J. Biochem. 45*, 651.
Sela, M., and Anfinsen, C. B. (1957), *Biochim. Biophys. Acta 24*, 229.
Simpson, R. T., Riordan, J. F., and Vallee, B. L. (1963), *Biochemistry 2*, 616.

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

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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-

* From the Department of Biophysics, Roswell Park Memorial Institute, Buffalo, New York 14203. *Received April 7, 1969.* This work was supported by U. S. Public Health Service Grant (Institute of General Medical Sciences) GM-13485, and National Science Foundation Grants GB 7523 and GB 7340.

[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.