The State of the Tyrosines of Bovine Pancreatic Ribonuclease in Ethylene Glycol and Glycerol*

Jake Bello

ABSTRACT: The state of the tyrosines of bovine pancreatic ribonuclease was investigated in aqueous ethylene glycol and glycerol. By spectrophotometry, circular dichroism, and optical rotation, a conformational transition was found at all concentrations of polyol up to and including 97%. Up to 75% glycol or glycerol, the thermal melting profile by optical rotation is sigmoid, with [α] becoming more negative with rising temperature. At 75% glycol or glycerol no transition is seen; but above 75% the melting profile is bell shaped, with [α] becoming first more positive and then more negative. Light scattering measurements give similar bell-shaped profiles, showing increased and then decreased scattering. The value of T_m extrapolated to neat glycol is close to 25°. All three optical methods show little change in conformation up to 90% glycol or glycerol. Marked changes occur between 90 and 97% polyol. This is confirmed by nitration in glycol, but for glycerol there is little nitration even at 93% glycerol.

Of the six phenolic side chains of RNase three are exposed and normal and three are "buried" or abnormal, when RNase is dissolved in water. The state of the tyrosines when RNase is dissolved in other solvents, especially in ethylene glycol, has been investigated in several laboratories. The published work on glycol[1] solutions is contradictory. Sage and Singer (1962) measured the ϵ_{277} of RNase and of TyrEt in water and in neat glycol. They found that the ratio of ϵ for RNase to that for tyrosine ethyl ester, at their respective λ_{max} values, was 6.9 in water and 6.1 in neat glycol. The excess above 6.0 represents the effect of the tyrosine chromophore not being entirely exposed to solvent. In an effective denaturant the ratio is expected to be close to 6.0. Sage and Singer concluded that the ratio of 6.1 in glycol demonstrated that all of the tyrosine side chains are exposed to solvent. They also found that all of the tyrosine phenolic hydroxyls can be titrated as one class in glycol, while, as is well known, they are titrated as two distinct classes of three each in water (Shugar, 1952). This was also taken as indicating exposure of all the tyrosines in glycol. Neither of these conclusions follows from the data. Since ϵ_{277} for buried tyrosines is close to that of model tyrosine compounds in neat glycol, the ratio of the ϵ values of RNase and model compound must approach 6 whether the tyrosine side chains are exposed or buried. As for the titration data, they show only that all of the tyrosines are similar in the pH range near the pK. This is not necessarily true at neutral pH; in glycol RNase may become denatured at a lower pH than in water thereby exposing tyrosines that were buried at neutral pH.

Herskovits and Laskowski (1968) made a spectral solvent-perturbation study of RNase in a mixture of 80% glycol and 20% Me_2SO (v/v) and concluded that three of the tyrosines are buried, as in water. It is not certain that the state of RNase in this solvent is the same as in neat glycol. We shall present evidence that they may not be the same.

Finally, von Hippel and Wong (1965) studied the T_m of RNase in glycol by optical rotation and found that on heating RNase solutions $\Delta\alpha$ decreases with increasing glycol concentration and vanishes at about 75% (v/v) glycol. This was taken as demonstrating the unfolding of RNase at room temperature at 75% glycol. We shall show that a transition exists even at 97% glycol.

Materials and Methods

RNase was purchased from several sources. Most of the optical work was performed on one batch (lot 8BB) of phosphate-free RNase type RAF from Worthington Biochemical Corp., Freehold, N. J. Some preliminary work was done with type RAF (lot 6509).

Reagent quality glycol and "Spectroquality" glycerol (Matheson, Coleman & Bell, Norwood, Ohio) were treated overnight with about 3 g of $NaBH_4$/l., deionized with dried Amberlite MB-3 mixed-bed resin, and distilled at 2–3-mm pressure through a 70-cm vacuum-jacketed column packed with stainless-steel helices (Scientific Glass Apparatus Co., Bloomfield, N. J.).

Urea was reagent grade; solutions were deionized shortly before use. Gu·HCl was Mann Ultra Pure, or reagent grade recrystallized from methanol. Filtration of the Ultra Pure was required before use. *N*-AcIm was purchased from Pierce Chemical Co. and was recrystallized from hexane–benzene. Tetranitromethane was purchased from Aldrich Chemical Co. and was dissolved in 90% ethanol and stored in the cold.

Spectra were taken on a Cary Model 15 spectrophotometer. The sample was held in a close-fitting brass block through which tempered water was circulated, the water then passing through the Cary thermostated cell holder (part no. 1540750). Water at 25° was circulated through the hollow sample chamber wall to maintain the reference cuvet at 25°. Nitrogen, from

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[1] Abbreviations used are: TMACl, tetramethylammonium chloride; glycol, ethylene glycol; MPD, 2-methylpentane-2,4-diol; RCM-RNase, RNase having its disulfides reduced and carboxymethylated.

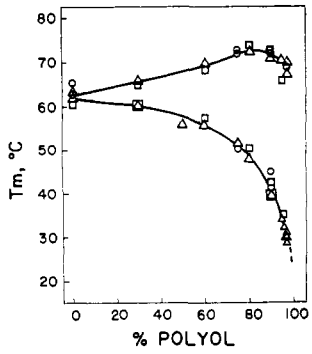

FIGURE 1: T_m of RNase as a function of glycol and glycerol concentration. Upper curve for glycerol; lower curve for glycol. (○) Circular dichroism, (△) difference spectra at 287 mμ, and (□) optical rotation.

liquid nitrogen, was passed through the sample and reference compartments to prevent condensation of water at low temperatures and to sweep warm air out of the reference compartment at elevated temperatures. Silicone rubber tubing was used inside the sample compartment. Other types of rubber and plastic tubing resulted in the condensation of volatile substances from the tubing on the compartment windows. Difference spectra were obtained with the 0–0.1-optical density slidewire for concentrations of 1 mg or less. The base line was adjusted with sample in both compartments, at 25°, and was generally flat to 0.0005. In those cases in which flatness was not worse than 0.001 corrections were made. No spectra were taken with base lines worse than ±0.001. Concentrations of RNase were generally 1 mg/ml or less (optical density less than 1) to maintain small slits at reasonable noise levels. Some experiments were also done at up to 4 mg/ml with poorer resolution to obtain T_m as a function of concentration and to look for evidence of turbidity at concentrations used for optical rotation. During thermal equilibrations the wavelength was set at about 500 mμ to minimize ultraviolet damage. *N*-AcTyr-NH_2 concentrations were 0.05–0.1 mg/ml, to give optical density values similar to those of RNase solutions.

Optical rotation and circular dichroism data were obtained with a Durrum-Jasco ORD-5-CD apparatus. At low temperatures nitrogen was passed through the sample compartment to prevent condensation of moisture. Circular dichroism spectra were taken at RNase concentrations of 1 mg/ml and *N*-AcTyr-NH_2 concentrations of 0.05–0.1 mg/ml in 5-cm cells. Normally two or three slit widths were used, usually 0.5, 1.0, and 1.5 mm. Good agreement was obtained at small and large slits.

RNase solutions were prepared from stock solutions of 40 mg/ml in a buffer containing 2.5 M TMACl and 0.5 M ammonium acetate. Appropriate volumes were diluted with glycol or glycerol and water to give the desired concentration of glycol or glycerol, and an RNase concentration usually of 1 mg/ml. Mixing 1 volume of RNase stock solution with 39 volumes of glycol or glycerol gives a polyol content of 97.5% by volume, referred to in this paper as 97%, because the polyols contained several tenths per cent of H_2O, as indicated by Karl Fischer titration. By this method the maximum polyol content consistent with 4 mg/ml of RNase is 90%, and with 2 mg/ml is 95%. When lower concentrations of RNase were desired, smaller volumes of stock solutions were used, and the difference was made up with buffer to maintain the same final buffer composition as at 1 mg/ml of RNase. Solutions were mixed with a Vortex mixer (Scientific Industries, Inc., Queens Village, N. Y.). The final ionic strength was 0.075 and the pH in the absence of organic solvent was 7.0.

Nitrations were done by a modification of the method of Riordan *et al.* (1966). To 15 mg of RNase in 10 ml of solvent, 0.062 M in TMACl, and 0.013 M in ammonium acetate was added 0.4 ml of 0.84 M TNM in 90% ethanol. The mixture was stirred in the dark on a magnetic stirrer of the type that does not heat the reaction vessel (Tri-R Co., Jamaica, N. Y.) for the required time. Glacial acetic acid (1 ml) was added to lower the pH to quench the reaction and the solution was then filtered through Sephadex G-25 and freeze dried. For analytical calculations spectra were measured in water (pH was not measured) for one of the isosbestic curves, and at pH 9.1 in 0.01 M Tris buffer for the other isosbestic curve and for ϵ_{428}. The analytical data are discussed under Results.

Light-scattering measurements were made with an Aminco-Bowman spectrophotofluorometer with both monochromators set at the same wavelength, either 320 or 400 mμ. Since relative scattering only was required, the instrument was not calibrated. Meter sensitivity was 0.03 or 0.1 and slits were 2 mm in both incident and fluorescent beams. Data were recorded with a Moseley X–Y recorder. Cross-linking of RNase was accomplished with glutaraldehyde (Quiocho and Richards, 1964), with the following modification. Crystals, their weight estimated from their dimensions, solvent composition of 50%, and protein density of 1.4, were soaked for 2 weeks in 75% MPD (25% H_2O) containing approximately 8 moles of glutaraldehyde/mole of RNase. They were then soaked in H_2O for 4–5 days with daily changes, and then for 4–5 days against glycol or glycerol with daily changes.

Results

Importance of Solvent Purity. In the initial stages of this work we used Spectroquality glycerol without further purification. However, we found that on heating RNase in glycerol erratic results and irreversible high optical densities resulted. We attribute this to light scattering resulting from the formation of cross-linked aggregates formed by reaction with impurities (glyceraldehyde?) in the glycerol. After adoption of the purification method described under Methods and Materials, no further difficulties were encountered with freshly prepared solutions. However, solutions of RNase in glycol, and more so in glycerol, that had been kept at 4° for some months showed marked increases in light scattering. All results shown here are for freshly prepared solutions.

Spectrophotometric Results. ϵ_{max} values of *N*-AcTyr-NH_2 in H_2O, 97% glycol, and 97% glycerol are 1400, 1850, and 1860, respectively, and for RNase in the same solvents they are 9,700, 11,220 and 11,260, respectively. The values for *N*-AcTyr-NH_2 and RNase in 97% glycol are in good agreement with the corresponding values of Sage and Singer (1962) for H_2O and neat glycol. With increasing glycol or glycerol content, λ_{max} of *N*-AcTyr-NH_2 changes from 274.5 in H_2O to 278 mμ in 97% polyol, and λ_{max} of RNase changes from 277.7 to 278.5 mμ.

Figure 1 shows the spectrophotometric T_m of RNase as a function of solvent composition, while Figure 2 shows the

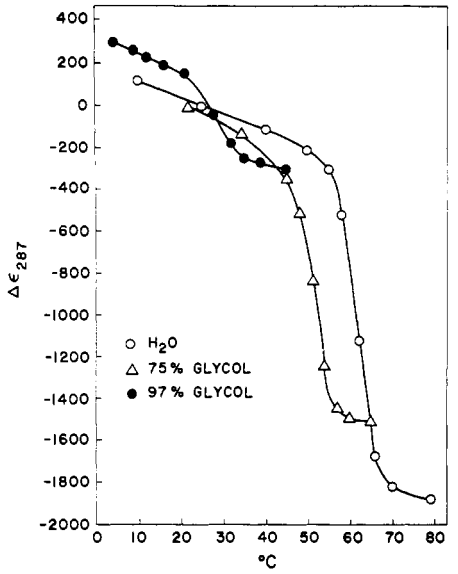

FIGURE 2: Melting profiles of RNase by difference spectra at 287 mμ. Concentration 7.3×10^{-5} M (1.0 mg/ml).

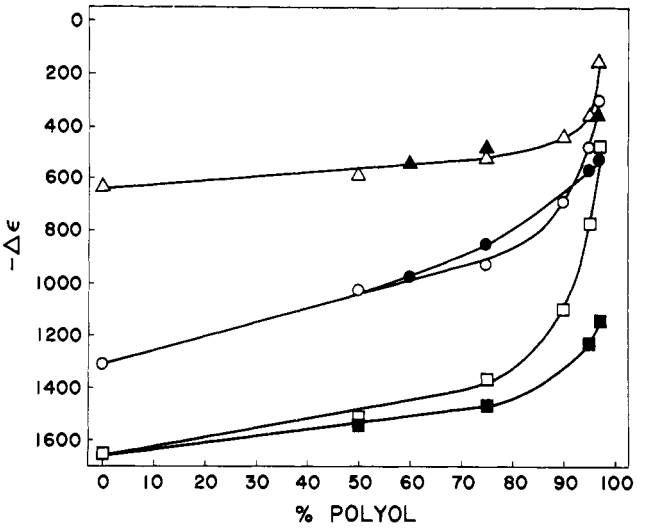

FIGURE 3: $\Delta\epsilon$ accompanying the thermal transition, as a function of polyol content. Filled symbols are for glycerol; unfilled for glycol. See text for explanation of the three methods Method. 1 (□, ■), method 2 (○, ○), and method 3 (△, ▲).

melting profiles at three solvent compositions. T_m was taken from the midpoint of the steep part of the profile. T_m at 97% glycol in Figure 2 is 29°, one of the three values given in Figure 1, 29, 30, and 31°. Similar T_m values were obtained at RNase concentrations of 0.4–4 mg/ml. It is clear that the spectral T_m exists at a glycol concentration of 97%. We have not gone beyond 97% because of the difficulty of making such solutions. Extrapolation of the T_m of Figure 1 suggests that in neat glycol T_m is near room temperature. It is probable that attempts to measure the state of RNase in neat glycol at room temperature would give rise to variable results because of the effects of small differences of water content, ionic strength, specific ion effects, pH, and variations in the various batches of RNase.

In Figure 3 are shown the change in $\Delta\epsilon$ accompanying the transition, calculated three ways: (1) from the total change in $\Delta\epsilon_{287}$ between room temperature (or 5° in the case of 97% glycol) and the bottom of the melting profile; (2) from $\Delta\epsilon_{287}$ between the bottom of the melting profile and the value of $\Delta\epsilon_{287}$ obtained by extrapolation of the linear part of the low-temperature data to above T_m; and (3) from thermal difference spectra, taking the difference between the $\Delta\epsilon$ values at the 287- and 280-mμ peaks. The last method is of value in cases in which turbidity increases with temperature. Corrections for turbidity are uncertain, especially where a long extrapolation from a wavelength above an absorption extremum is required. In using difference spectra to measure T_m, as we have done, the influence of the positive extremum at 290 mμ added to the difficulty of correcting for turbidity. Since $\Delta\epsilon_{287}$ changes more rapidly than $\Delta\epsilon_{280}$ with change in temperature through the T_m range, and since these extrema are close together, thereby reducing the uncertainty in the turbidity correction, we have used $\Delta\epsilon_{287} - \Delta\epsilon_{280}$ to measure T_m. We shall show below that turbidity is a factor at high glycol content. Melting profiles obtained by the $\Delta\epsilon_{287} - \Delta\epsilon_{280}$ method were sigmoid and resembled those of Figure 2. T_m values obtained by this method were very similar to those of Figure 1, but extrapolating to about 22° at neat glycol. While the results of T_m obtained from $\Delta\epsilon_{287} - \Delta\epsilon_{280}$ are similar to those from $\Delta\epsilon_{287}$, the former is more convenient to use and entails less uncertainty. Perhaps results of the $\Delta\epsilon_{287} - \Delta\epsilon_{280}$ method testify that our turbidity corrections were reasonably well done in the $\Delta\epsilon_{287}$ method. With more turbid solution the $\Delta\epsilon_{287} - \Delta\epsilon_{280}$ method may be particularly useful. All three methods of treating $\Delta\epsilon$ of transition show that at 97% glycerol $\Delta\epsilon$ is about twice as great as for 97% glycol.

The thermal difference spectrum of *N*-AcTyr-NH_2 is similar to that of RNase below T_m. $\Delta\epsilon_{285}$ for *N*-AcTyr-NH_2 was found to be linear with temperature in 97% glycol and glycerol to above the T_m of RNase. The change in ϵ_{285} for *N*-AcTyr-NH_2 in 97% glycerol between 25° and 80° is −95. The corresponding value for glycol from 20° to 42° is −53. The significance of these values is considered in the Discussion.

Glycerol, in contrast to glycol, raises T_m, but above 80% glycerol T_m begins to fall. The extrapolation to neat glycol that is temptingly obvious in Figure 1 may not be justified, as we do not know if there is a drastic change in conformational stability above 97% glycerol.

Optical Rotation. In Figure 1 is shown T_m as a function of solvent composition, as measured by optical rotation. von Hippel and Wong (1965) measured T_m of RNase at 0–50% glycol and found results similar to those reported here. But at 13 M glycol, about 75% by volume, they found no transition; *i.e.*, $\Delta[\alpha] = 0$. We have confirmed this. However, on increasing the glycol content above 75%, we have found a transition. But whereas the rotatory melting profiles below 75% glycol are sigmoid, resembling those of Figure 2, but inverted, the melting profiles above 75% glycol are bell shaped as shown in Figure 4. There is first a change toward more positive values, then toward more negative values. In glycerol there is a similar effect. Similar results were obtained at 300, 400 and 450 mμ, except for the magnitudes of the effects. The T_m values of Figure 1 were taken from the descending limbs of melting profiles such as that shown in Figure 4. In Figure 5 are shown the magnitudes for the

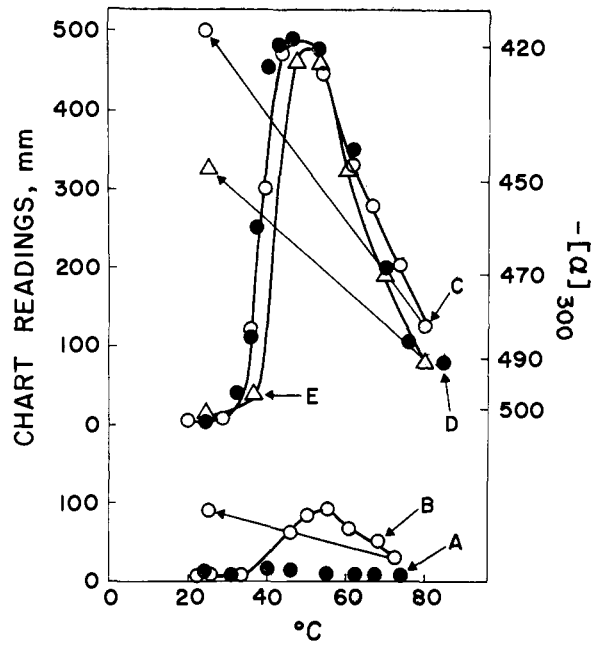

FIGURE 4: Light scattering and [α] of RNase in ethylene glycol with temperature. All except curve D are for light scattering. Curve A: 1 mg/ml, 75% glycol, 400 mμ; B: 1 mg/ml, 90% glycol, 320 mμ; C: 4 mg/ml, 90% glycol, 320 mμ; D: optical rotation, 4 mg/ml, 90% glycol, 300 mμ; E: 2 mg/ml, 90% glycol, 400 mμ. 25° points at ends of arrows are for samples cooled to 25° from highest temperature.

descending limb, starting from a negative value and becoming positive, crossing zero near 75% glycol and glycerol. The value of Δ[α] at 90% glycol was the same whether measured at 4 mg/ml in a 1-cm cuvet or at 0.5 mg/ml in a 5-cm cuvet. However T_m at 0.5 mg/ml was 4° higher then at 3 or 4 mg per ml; the latter was used in Figure 1.

The optical rotation at 25° as a function of polyol content is shown in Figure 6. Whereas in glycerol the rotation increases linearly up to 97% glycerol, in glycol there is a sharp change in rotation between 90 and 97% glycol.

Difference spectra of heated solutions measured against unheated solutions suggested that the heated solutions evidenced a greater degree of light scattering than did unheated solutions. Measurement of light scattering confirmed this (Figure 4). The agreement with the rotatory data is notable. Cooling of the solutions to room temperature for several hours resulted in increased scattering, except for 75% glycol. There was no visible turbidity in any of these experiments during the heating. Figure 4 shows a strong concentration effect between 1 mg/ml and 4 mg/ml, but the curve for 2 mg/ml at 400 mμ is unexpectedly similar to that for 4 mg/ml at 320 mμ. It is not clear whether the optical rotatory transition is entirely an artifact produced by turbidity. A Ba_2SO_4 suspension gave a positive rotation but the visible turbidity of the Ba_2SO_4 was extremely great compared with that of the RNase, which was invisible to the eye. It appears likely that the turbidity is caused by, or accompanies, a conformation change, so that the observed Δ[α] is probably a combination of the two effects. The good agreement of the rotatory T_m with the spectral and circular dichroism T_m values supports this. It is possible that rotatory changes observed for several proteins in mixed solvents are influenced by aggregation.

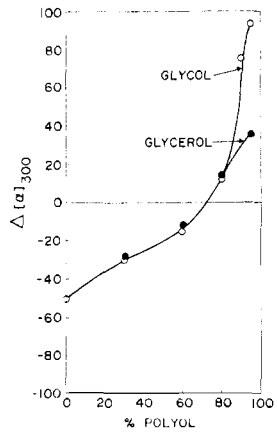

FIGURE 5: [α] of transition of RNase as a function of glycol or glycerol concentration.

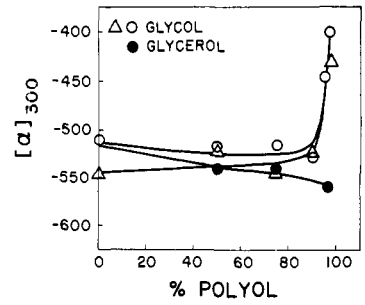

FIGURE 6: Optical rotation of RNase as a function of glycol or glycerol content at 25°. (●) Glycerol and (○,△) glycol, two lots of RNase.

An attempt by optical rotation to measure T_m of RNase in a mixture of 78% glycol, 19.5% Me_2SO, and 2.5% H_2O was not successful as a result of high turbidity which developed on heating. In 97% glycol, turbidity was not visible, although detectable by light scattering, and vanished above T_m. While this solvent is not exactly the glycol–Me_2SO (80:20) mixture of Herskovits and Laskowski (1968), the difference in turbidity suggests that the state of RNase in glycol–Me_2SO is not the same as in glycol.

Circular Dichroism. Here again, a transition is observed at 97% glycol or glycerol (Figure 7). The circular dichroism spectrum of RNase in water was similar to that of Simmons and Glazer (1967), but that of *N*-AcTyr-NH_2 did not show the positive peak at 283 mμ. In Figure 1 are T_m values obtained from circular dichorism measurements. These are in good agreement with those obtained by other methods. In Table I are shown $\epsilon_l - \epsilon_r$ for *N*-AcTyr-NH_2 at 25° and for RNase below and above T_m. RNase in 97% glycol and, more so, in 97% glycerol has $\epsilon_l - \epsilon_r$ greater than expected for 6 free tyrosine residues. Only above T_m does $\epsilon_l - \epsilon_r$ fall to near the value expected for fully exposed side chains of tyrosine. But the value of $\epsilon_l - \epsilon_r$ in 97% glycol above T_m is greater than that in glycerol. It will also be noted that $\epsilon_l - \epsilon_r$ above T_m is lower in 75% and 90% than in 97% glycol. For Ac_6RNase $\epsilon_l - \epsilon_r$ was 1 and for *N,O*-Ac_2Tyr was about 0.01–0.02. The circular dichorism at room temperature as a function of glycol or glycerol concentration is shown in Figure 8.

Crystallographic Results. Crystals soaked only in water

TABLE I: Circular Dichroism of RNase below and above T_m.

Solute	Solvent	Temp (°C)	$(\epsilon_l - \epsilon_r)$/Mole of RNase	$(\epsilon_l - \epsilon_r)$/Mole of Residue of Tyr
RNase	H_2O	25	7.0[a]	1.16
RNase	H_2O	74	1.1	0.18
RNase	97% glycol	10–25	3.4–4.0[b]	0.57–0.67[b]
RNase	97% glycol	42	2.2	0.37
RNase	97% glycol	62	2.1	0.35
RNase	97% glycerol	25	6.4–7.0[b]	1.07–1.16[b]
RNase	97% glycerol	84	1.5–1.7[b]	0.25–0.28
N-AcTyr-NH_2	97% glycol	25		0.16
N-AcTyr-NH_2	97% glycol	42		0.16
N-AcTyr-NH_2	97% glycerol	25		0.20
N-AcTyr-NH_2	97% glycerol	82		0.18
N-AcTyr-NH_2	H_2O	25		0.15[c,d]

[a] Simmons and Glazer's value was 6.4. [b] Values for two lots of RNase. [c] Simmons and Glazer's value was 0.13 as calculated from their spectrum. [d] No temperature dependence within experimental error.

gave good diffraction patterns on the a^*, b^*, c^*, and 101 axes, only slightly changed from those of noncross-linked crystals in 75% MPD, the medium in which RNase crystals are equilibrated for X-ray work. Crystals soaked in 90% glycerol gave diffraction patterns very similar to those in H_2O. But the crystals in 97% glycol and glycerol were entirely disordered. These soaking solutions contained the same buffer as all the solutions for optical studies. (A more complete investigation of cross-linked crystals as a function of solvent composition is in progress.)

Chemical Modification. An alternative approach to the study of the state of the tyrosines is chemical modification. This approach may give a different result from physical methods. The latter report time averages, while the former, because of the long reaction times required, may report as exposed groups that are buried most of the time but are accessible to reagent part of the time. The most commonly used chemical methods for the study of tyrosines in proteins are O-acetylation by *N*-AcIm, iodination, and nitration. The first two could not be used. Acetylation in 50–90% glycol or glycerol resulted in very little reaction, even with *N*-AcTyr-NH_2. It was found that *N*-AcIm is solvolyzed two to three times more rapidly in these solvents than in water. But even the use of threefold quantities of *N*-AcIm, or of addition of increments of *N*-AcIm during the reaction, was not successful in increasing the number of *O*-acetyl groups above 1, whereas three tyrosines are acetylated in water. Longer reaction times were of no avail because of solvolysis of *O*-acetyltyrosine, which is much faster in glycol–H_2O than in H_2O. The possibility that acetylation in glycol does not proceed further because the tyrosines are buried is not in accord with the preponderance of the data. Iodination could not be used because the rate of iodination in these media was extremely slow and was overshadowed by side reactions.

Nitrations, however, could be carried out in aqueous glycol and glycerol. The results of nitration are shown in Table II. Nitrations were carried out in the usual TMA buffer so that results could be compared with the optical data, even though at pH 7 the rate of nitration is slower than at the more usual pH of 8–9. To compensate for the slower rate, a reaction time of 20 hr, and in one case, 68 hr, was used. Because of the addition of TNM, in ethanol the concentrations of glycol, glycerol, urea and Gu·HCl were reduced from 75 to 72%, 90 to 86%, 97 to 93%, 6 to 5.76 M, and 8 to 7.68 M. We have noted some discrepancies between our results on

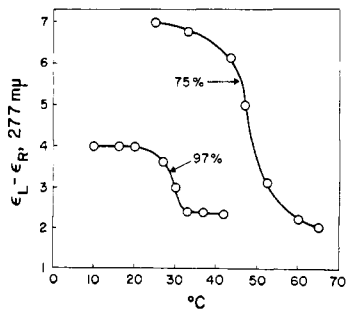

FIGURE 7: Circular dichroism melting profiles of RNase in 75 and 97% glycol.

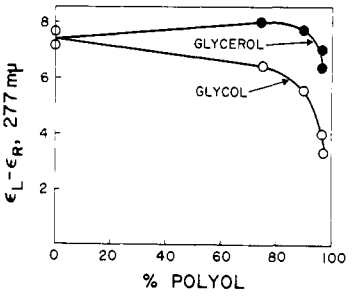

FIGURE 8: Circular dichroism of RNase as a function of glycol and glycerol concentration. The two points each for 97% glycol and glycerol are for two batches for RNase.

TABLE II: Nitration of RNase.[a]

Solvent	λ_{max} (mμ)	*N*-Tyr[b,c]	Isosbestic[d] Point (mμ)	*N*-Tyr[c]
H_2O	423	3.1	378	3.8
72% glycol	424	3.0	379	3.4
86% glycol	427	3.3	381	3.8
93% glycol	431	5.2	382	5.7
72% glycerol	421	2.1	378	2.7
93% glycerol	423	1.9	379	2.2
89% glycerol (three-times concentrated TNM)[e]	425	2.6	379	3.1
93% glycerol (68 hr)	424	2.4	379	3.0
5.76 M Gu·HCl	429	4.9	382	5.2
7.68 8 M urea	426	4.2	380	5.2

[a] 20-hr reaction time, room temperature, Worthington RAF lot 8BB. Spectra were taken on aqueous solutions. [b] pH adjusted to 9.2 with 0.05 ml of 1 M Tris buffer added to 2 ml of aqueous solution. [c] Moles of nitrotyrosine residues per mole of RNase at wavelength in preceding column. [d] pH not adjusted or measured. [e] Threefold quantity of TNM used.

nitration in water and those of Riordan *et al.* (1966). λ_{max} for nitrotyrosinate groups of protein was generally not at 428 mμ and the isosbestic point was not at 381 mμ as reported (Riordan *et al.* 1966). For the model compound L-3-nitrotyrosine λ_{max} was 425 and the isosbestic point was 379 mμ; ε values at the two wavelengths were 4260 and 2200, respectively. Also the number of nitrotyrosines calculated from the peak near 425 mμ and from the isosbestic point did not agree. There may have been side reactions, which raises the possibility of the calculated number of nitrotyrosines being different from the actual number of modified tyrosines. Nevertheless, it is apparent that in 93% glycol most or all of the tyrosines reacted. With increasing glycol and glycerol content the wavelengths increase and in 86 and 93% glycol are longer than for water. This is also true for 5.76 M Gu·HCl, in which incomplete nitration appears to have taken place. However, as the wavelengths are long, side reactions may have taken place, raising the number of modified tyrosines. The same consideration applies to 86 and 93% glycol. On the basis of the wavelengths the reaction in glycerol appears to be the cleanest, even with the threefold quantity of TNM. In 93% glycerol the number of nitrotyrosines is about 2 for a 20-hr reaction and 2.4–3 for a 68-hr reaction. To make sure that the value for glycerol was not the result of faster side reaction at the other tyrosines, this product was renitrated in 8 M urea, with the production of 4.9 nitrotyrosines, with λ_{max} and the isosbestic point at 429 and 382 mμ, respectively, and with the same value of nitrotyrosine calculated at both wavelengths. The low degree of nitration might have arisen from a reduced rate of reaction in glycerol, from rapid decomposition of TNM in this solvent, or from inaccessibility of tyrosines. To test the first two possibilities *N*-AcTyr-NH_2 was nitrated in H_2O and in 93% glycerol, the rate being followed by the increase in optical density at 440 mμ. Decomposition of TNM in 93% glycerol was much faster than in H_2O, but about 10% slower than in 93% glycol. Corrected for decomposition of TNM, the rate of nitration of *N*-AcTyr-NH_2, as indicated by optical density (Riordan *et al.*, 1966), was slightly faster in 93% glycerol than in 93% glycol. These results suggest that the tyrosines of RNase in glycerol are no more accessible than in water, while in glycol they become more accessible with increasing glycol content, and become entirely accessible, or nearly so, in 93% glycol.

Discussion

At the outset of this work we had three sets of experiments on the state of RNase in high concentrations of glycol, two of which contradicted the third, namely, Sage and Singer's (1962) spectral and titration data and von Hippel and Wong's (1965) rotatory data, which were interpreted as indicating denaturation, opposed to Herskovits and Laskowski's (1968) solvent perturbation spectra data, which indicated that the tyrosines were similar to those of native RNase in H_2O. From the demonstrations of transitions at up to 97% glycol and glycerol by spectrophotometry, optical rotation, and circular dichroism, it is clear that RNase is not completely denatured in these solvents.

Since ε(*N*-AcTyr-NH_2) in 97% glycol or glycerol (1850) is very close to that of a buried tyrosine of RNase (1830), direct spectra cannot distinguish between buried and exposed tyrosines. The value of $\epsilon = 1830$ for buried tyrosines of RNase in H_2O is based on ε(RNase) 9700, 3 ε(*N*-AcTyr-NH_2) = 4200, and $\frac{1}{3}$ of the difference of 5500 (1830). The value of ε for *N*-AcTyr-NH_2 is taken at 275 mμ, and for RNase at 278 mμ. $\epsilon(N\text{-AcTyr-NH}_2)_{278}$ is 40 less than at 275 mμ; but the exposed tyrosines of RNase are partly buried, which should raise ε.

Although ϵ_{max} of *N*-AcTyr-NH_2 in 97% glycol or glycerol is very nearly the same as that of the average value of ε of buried tyrosines, the spectra of the two are not identical. λ_{max} of *N*-AcTyr-NH_2 in 97% glycol or glycerol is 278, while that of RNase in the same solvents is 278.5. Therefore, λ_{max} of buried tyrosines must be at a somewhat longer wavelength. Since λ_{max} of *N*-AcTyr-NH_2 in H_2O is 274 and λ_{max} of RNase in H_2O is 277.5, λ_{max} of the three buried tyrosines may be around 280 mμ. Thus the spectrum of the buried tyrosines of RNase may lie about 2 mμ to the red of that of *N*-AcTyr-NH_2 in 97% glycol or glycerol, and 2 mμ to the red of that of exposed tyrosines (perhaps a bit less than 2 mμ if exposed tyrosines are not entirely exposed and therefore not identical with *N*-AcTyr-NH_2). A 2-mμ blue shift of the spectrum of *N*-AcTyr-NH_2 corresponds to $\Delta\epsilon = -330$ near 287 mμ. This is the value expected for unfolding at room temperature. At higher temperature an additional contribution enters. The transition may be considered as equivalent to the transfer of buried tyrosines to glycol or glycerol at room temperature, plus the heating of these tyrosines from room temperature to above T_m. (Although ε of the interior tyrosines must change during heating this change is included in the Δε of the hypothetical exposed groups just mentioned.)

The value of $\Delta\epsilon_{287}$ to be used for heating of a newly exposed

tyrosine from 25° to above T_m is the corresponding value for *N*-AcTyr-NH_2 over the appropriate temperature range: $\Delta\epsilon = -95$ in 97% glycerol and -50 in 97% glycol. Combining these values with $\Delta\epsilon_{287}$ for exposure of a buried tyrosine we obtain $\Delta\epsilon_{287}$ of approximately -430 in 97% glycerol and -380 in 97% glycol for each tyrosine exposed by heat. Figure 3 (middle curves) shows the observed values of $\Delta\epsilon_{287}$ for RNase to be -550 in 97% glycerol and -300 in 97% glycol. (The middle curves of Figure 3 are corrected for $\Delta\epsilon$ of heating tyrosines that are already exposed at 25°.) Thus we estimate that on heating RNase in 97% glycol or glycerol, approximately 0.7 and 1.3 tyrosines, respectively, become exposed. (One of the reviewers suggested that λ_{max} of buried tyrosines is 279 mμ instead of 280 mμ. This would change the over-all calculated $\Delta\epsilon$ values to 215 for glycol and 260 for glycerol, giving 1.4 and 2.1 tyrosines exposed by heating.) Heating in water exposes two of the three buried tyrosines (Bigelow, 1961). Whether or not heating in glycol or glycerol exposes all of the buried groups is not obvious. The circular dichroism data of 97% glycol or glycerol above T_m show a residual effect greater than for *N*-AcTyr-NH_2, by an amount roughly equivalent to one tyrosine with native rotatory power. However, since in H_2O the circular dichroism of denatured RNase (per residue of tyrosine) is very similar to that of *N*-AcTyr-NH_2, we cannot decide from circular dichroism data.

Extrapolation of the T_m curve for glycol to 100% glycol brings T_m to near room temperature. As Herskovits and Laskowski (1968) have pointed out, if measurements are made on a system close to T_m, the proportions of native and denatured forms may be significantly altered by any perturbation, such as another solvent added to measure solvent perturbation spectra. Since T_m of RNase in neat glycol is near room temperature, adding Me_2SO may alter the equilibrium. The $\Delta\epsilon$ data of Figure 3, circular dichroism data of Figure 8, and the optical rotatory data of Figure 7 suggest that the conformation of RNase in 90% glycol at room temperature is not appreciably denatured, although T_m has fallen to 45°. Above 90% glycol the conformation changes rapidly with glycol concentration by all of these physical criteria. The chemical evidence from nitration is also in good agreement with the physical evidence.

Since T_m for RNase in neat glycol is near, or even slightly below (Figure 1), room temperature, the state of RNase in the solvent is probably strongly dependent on small variations in conditions, *i.e.*, purity of solvent, salt concentration, nature of salt, pH, ligands bound to RNase, traces of water, etc. The low value of circular dichroism at 97% glycol even at 4–10°, well below T_m, suggests that RNase is partly unfolded in this solvent at low temperature.

Comparison of all the criteria presented here, except X-ray diffraction, shows that up to 97% glycerol RNase is more stable than in glycol. The difference between the rotatory and circular dichroism data at 90–97% glycerol suggests that a slight change of conformation occurs, too small to affect rotation, which may be insensitive to small local changes, but sufficient to affect circular dichroism which is sensitive to changes in small regions around tyrosine residues. Only X-ray diffraction on cross-linked crystals shows RNase to be denatured in 97% glycerol. Perhaps the chemical modification by glutaraldehyde makes RNase more easily denatured. The X-ray evidence says nothing about the state of the tyrosines, and may reflect chiefly a disorganization of the crystal with relatively little unfolding of individual molecules.

The data presented here demonstrate that in 97% glycol and glycerol RNase is not entirely unfolded, that the greatest change in conformation takes place above 90% polyol, and that RNase is less unfolded in glycerol than in glycol.

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