The Properties of Bovine Pancreatic Ribonuclease in Ethylene Glycol Solution*

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The ultraviolet absorption spectrum, the spectrophotometric titration behavior, and the optical rotatory dispersion of bovine pancreatic ribonuclease in ethylene glycol solution have been determined and compared with the same parameters in aqueous solution. Aqueous solutions of ribonuclease are known to exhibit anomalously large absorption at wave lengths near 280 mμ. This hyperchromicity is absent in ethylene glycol solutions of the protein. Furthermore, it is known that the titration of three of the six tyrosine hydroxyl groups of ribonuclease in aqueous solution is anomalous and irreversible. By contrast, all six hydroxyl groups titrate normally and reversibly in ethylene glycol. These results suggest that the hydrophobic regions of the ribonuclease molecule which are present in aqueous solution are disrupted in ethylene glycol. Since the enzymatic activity of ribonuclease dissolved in ethylene glycol is recovered into aqueous solution, this disruption is reversible. On the other hand, the optical rotatory dispersion data are interpreted to indicate that the helical content of ribonuclease undergoes little net change in ethylene glycol compared to water.

In recent years it has become increasingly clear that water plays a vital role in determining the secondary and tertiary structure which proteins display in an aqueous environment. Part of the evidence for this conclusion has come from studies of proteins dissolved in nonaqueous solvents. For example, Rees and Singer (1956) showed that gross configurational changes, measured by changes in sedimentation constants and viscosity increments, were produced in proteins dissolved in hydrazine and ethylene diamine. Yang and Doty (1957) demonstrated that the internal configuration of protein molecules could be radically altered in nonaqueous media, as reflected in changes in optical rotation and optical rotatory dispersion.

In pursuing our studies of nonaqueous solutions of proteins (Rees and Singer, 1955, 1956), we have investigated in some detail the system bovine pancreatic ribonuclease (RNase) in ethylene glycol solution. This system was chosen because it allowed several independent and sensitive criteria to be employed simultaneously in determining the effect of solvent on the structure of the protein.

First of all, the recovery of the enzymatic activity of RNase into aqueous solution, from solution in ethylene glycol under various conditions, was used to discriminate between reversible and irreversible changes in structure that had been produced in glycol.

Secondly, the potentiometric and spectrophotometric titration behavior of the tyrosine residues of RNase was determined in ethylene glycol solution. It is known that, in aqueous solution, three of the six tyrosine phenolic hydroxyl groups of RNase titrate normally but the other three do not titrate until an abnormally high *p*H is reached (Shugar, 1952; Tanford *et al.*, 1955); their titration is irreversible, and is accompanied by a parallel loss of enzymatic activity (Sela and Anfinsen, 1957). These findings have therefore suggested, first, that three of the six tyrosines are present, probably hydrogen-bonded, in hydrophobic regions of the RNase molecule (Tanford *et al.*, 1955), where they are relatively inaccessible to hydroxyl ions, and second that one or more of the six tyrosines is part of a structure whose integrity is vital to RNase enzymatic activity (Sela *et al.*, 1957).

Accompanying this anomalous titration behavior in aqueous solution is an anomalously large ultraviolet absorption. The molar extinction coefficient of RNase in neutral aqueous solution at close to 280 mμ is considerably larger than six times that of tyrosine itself, presumably because of the enhanced absorption of the three anomalous tyrosines. Therefore, a third method of investigating structural changes in ethylene glycol solutions of RNase was through ultraviolet absorption measurements.

A fourth method was through optical rotatory dispersion measurements, which have been interpreted to reflect changes primarily in the helical structure of proteins (Yang and Doty, 1957; Moffitt and Yang, 1956).

On the other hand, studies of possible gross conformational changes by hydrodynamic methods were precluded by the observation that RNase is extensively aggregated in ethylene glycol solution.

During the course of this investigation, an extensive study of viscosity and optical rotatory properties of ribonuclease in chloroethanol-water mixtures

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was published (Weber and Tanford, 1959). The results of this study will be discussed below.

Experimental

Materials.—A preparation of bovine pancreatic RNase, Armour and Co. lot #381-059, was the principal one employed in these studies and was used without further chemical treatment. As obtained, it contained 9–10% water, and before use it was dried overnight in a vacuum oven at 50°. Several other RNase samples were examined in a preliminary fashion, as described below.

The compounds L-tyrosine ethyl ester hydrochloride (TEE), Mann Research Laboratories, and L-tyrosine, Delta Chemical Works, were similarly dried before use. C.P. or equivalent-grade KOH from freshly opened bottles was used without further treatment. C.P. KCl was vacuum dried at 50°.

Ethylene glycol was Fisher Certified Reagent Grade (99.9%, 0.06% H_2O) for most of these studies. As completely rigorous exclusion of water in these experiments was not attempted, this reagent was used without further treatment. Formamide was obtained as a 99% pure grade from the Matheson Co. and was further purified by treatment with anhydrous Na_2SO_4 followed by three vacuum distillations. Other organic solvents were procured in the highest purity commercially available and were not further purified.

Yeast ribonucleic acid (RNA) (Schwarz Laboratories) was used to prepare a substrate for the enzymatic assay of RNase, according to the method of Klee and Richards (1957).

Solubility Studies.—Direct solution of RNase in a number of nonaqueous solutions was attempted by preparing a sealed glass tube containing 1 mg RNase and 1 ml of solvent and rotating it gently overnight at room temperature (Rees and Singer, 1956). In those cases where it appeared that the protein had dissolved, the solution was diluted to 0.1 mg RNase/ml in acetate buffer, *p*H 5.0, $\Gamma/2$ 0.1, and assayed for enzymatic activity as described below. While RNase was not directly soluble from the dry state in *N,N*-dimethylformamide, a stable solution in the pure solvent could be prepared by dialyzing an aqueous RNase solution at 4° against water–*N,N*-dimethylformamide mixtures successively richer in the latter component, and finally against several portions of the pure solvent (Geiduschek and Gray, 1956).

Potentiometric Measurements and Titration Apparatus.—Potentiometric titrations were made with a Beckman Model GS *p*H meter at 25°, with a no. 1190-80 externally shielded glass electrode used in conjunction with a Ag-AgCl reference electrode and a salt bridge consisting of a saturated solution of LiCl in ethylene glycol. Before use, the glass electrode was wiped off carefully, dipped in methyl alcohol, and dried with a stream of dry N_2 gas. After use, the electrode was allowed to stand in distilled water. The reference electrode used in most of these experiments was prepared as follows: The saturated KCl solution and calomel electrode assembly were removed from a Beckman #1170 calomel electrode. A piece of 18-gauge pure silver wire, which had been coated with AgCl by electrolysis in a solution of NaCl, was silver-soldered to the cap of the electrode and the whole assembly was dried under vacuum. A saturated solution of LiCl in ethylene glycol was used for the liquid junction in place of the saturated aqueous KCl. Periodic replacement of the LiCl solution and storage of the electrode in a vacuum dessicator when not in use were the only precautions found necessary.

For the experiments reproduced in Figures 4 and 6, the equivalent reference electrode was prepared by modifying a Beckman No. 41236 Ag-AgCl electrode, the aqueous salt bridge solution of which was replaced with a saturated solution of LiCl in ethylene glycol.

Two different glass electrodes, designated A and C, were employed in most of the experiments reported in this paper. Variation in the properties of different glass electrodes is discussed below.

The continuous titration apparatus consisted of an electrically grounded flat-bottomed vessel with two side-arms (to facilitate a continuous flow of nitrogen through the vessel) and a four-holed rubber stopper in which were snugly fitted the glass electrode, the reference electrode, and two pieces of capillary tubing. Through one of the capillary tubes the KOH in ethylene glycol was added by means of a calibrated syringe-type microburet fitted with a 6-in. 20-gauge stainless steel needle. Through the other capillary tube samples were removed by syringe for optical density measurements. Mixing of the solutions inside the vessel was done with a magnetic stirring apparatus. The vessel was immersed in a thermostat at 25.0 ± 0.05°.

For the titration experiments, solutions of L-tyrosine ethyl ester hydrochloride (0.10 mg/ml) and RNase (1.0 mg/ml) were accurately and freshly prepared in 0.2 M KCl in ethylene glycol and were titrated with 0.2 M KOH in ethylene glycol under N_2 atmosphere. Samples were removed periodically during the titration, the optical density at 296 mμ (see below) was measured in ground-glass-stoppered cells, and the samples were then returned to the vessel. With 0.2 M KOH in ethylene glycol, a maximum of only 5.3.-5.4 of the six tyrosines of RNase could be titrated, and to complete the titration 0.5 M KOH was required. To examine the reversibility of the titration curve, solutions of RNase prepared in 0.2 M KOH in ethylene glycol were back-titrated with 0.2 M HCl in ethylene glycol (prepared by dissolving anhydrous HCl in the glycol).

Absorption Measurements.—Ultraviolet spectra and difference spectra were obtained with a Cary Model 11S Recording Spectrophotometer. Absorption measurements during the continuous titration of L-tryosine ethyl ester hydrochloride and RNase, and for the enzymatic activity determinations of RNase samples as described below,

were made with a Beckman Model DU Spectrophotometer.

Concentration Determinations.—For ultraviolet absorption and spectrophotometric titration measurements, solutions of L-tyrosine ethyl ester hydrochloride and RNase were prepared by weight. Aqueous solutions of these compounds were then carefully analyzed by Nessler N determinations to correct for the residual water content of the compounds. (Direct treatment of ethylene glycol solutions with the Nessler reagent was not possible because of the extensive carbonization that occurred on acid digestion.) For the optical rotatory dispersion measurements, the concentration of a solution of RNase in ethylene glycol was obtained by optical density measurements on a carefully prepared dilution at 278 mμ with the use of ϵ_{278} = 11,400, which was determined as indicated above.

Enzymatic Activity Measurements.—These were carried out essentially as described by Klee and Richards (1957). Solutions of RNase in nonaqueous solvents were adjusted, if necessary, to a concentration of 1 mg/ml; 1.0 ml of this solution was diluted tenfold with acetate buffer, *p*H 5.0, Γ/2 0.1; 0.1 ml of this diluted solution was then added to 1.0 ml of the substrate RNA solution containing 5.0 mg RNA/ml in the acetate buffer. After 15 minutes at 25°, the reaction was stopped by adding 1.0 ml of precipitating reagent (0.5% uranyl acetate, 2.5% trichloroacetic acid in the acetate buffer). The sample was centrifuged, the supernatant was diluted tenfold with water, and the optical density was measured at 260 mμ. A blank containing all the reagents used in the assay was prepared by adding the precipitating agent to the substrate, followed by the RNase. For each nonaqueous solvent studied, a separate standard of enzymatic activity was prepared by the addition, with thorough mixing, of 9.0 ml of a solution containing 0.11 mg RNase/ml in the acetate buffer to 1.0 ml of the nonaqueous solvent. This solution was then carried through the analysis as described. The presence in the assay mixture of 1.0% of any of the nonaqueous solvents employed, however, did not have any noticeable effect on the activity measurements.

Optical Rotation Studies.—Optical rotatory dispersion measurements were kindly carried out for us by Dr. Susan Lowey of the Children's Cancer Memorial Hospital of Boston, on a Rudolph Spectropolarimeter, Model 80 Q3/200AS/650, with a Xenon-Mercury arc as the light source. The measurements were made at 23°, in the range from 589 mμ to 365 mμ. A symmetrical angle of 2° was used throughout. A freshly prepared solution of RNase at a concentration of 0.92 g/dl in 0.2 M KCl in ethylene glycol was examined in a 2-dm polarimeter tube.

In addition, less accurate measurements were made in our laboratory with a Rudolph Model 200 Photoelectric Polarimeter at the Na *D* line on a solution of RNase in 0.2 M KOH in ethylene glycol as a function of time.

Results

Solubility and Recoverable Activity of RNase in Nonaqueous Solvents.—Only a preliminary survey of several likely solvents (Rees and Singer, 1956) was made. Armour and Co. RNase was directly and rapidly soluble to the extent of at least 1 mg/ml in formamide, ethylene glycol, and dimethylsulfoxide. From the first two solvents, the RNase recovered into aqueous buffer retained all of its enzymatic activity within 5%. The RNase recovered from dimethylsulfoxide had a residual activity of 50–60%. No attempt was made to improve this figure. In addition, solutions of RNase in *N*,*N*-dimethylformanide were prepared by dialysis from an aqueous solution, and the RNase was found to have retained 90% of its activity. Other liquids tested which did not dissolve RNase directly were ethanol, methanol, dioxane, acetone, pyridine, piperidine, acetonitrile, and propylene carbonate. No attempt was made to prepare RNase solutions in these solvents by the dialysis procedure.

For the purpose of performing a spectrophotometric titration, several properties are required of a nonaqueous solvent other than its capacity to dissolve RNase. It must dissolve sufficient quantities of acid (HCl), base (KOH), and salt (KCl); it must be stable to acid and base; it must be essentially transparent in the spectral region around 260–300 mμ. Formamide and *N*,*N*-dimethylformamide were found to hydrolyze at appreciable KOH concentrations. Dimethylsulfoxide was ruled out since KOH is very poorly soluble in it, although a quaternary ammonium hydroxide might be sufficiently soluble. In all respects, ethylene glycol was satisfactory, and our further work was confined to it as a solvent for RNase. One per cent solutions of RNase in this solvent could be prepared readily.

At a fairly late stage in these studies, when the supply of the sample of Armour and Co. RNase was dwindling, the solubility of several other RNase samples in ethylene glycol was investigated. Mixtures containing 7.5 mg RNase/ml were prepared. Armour lot #381-062 was equally readily soluble as our original lot #381-059. Worthington RNase lot #R535 was soluble but dissolved slowly and formed a cloudy solution. Sigma lot #R 99-80, L. Light and Co. "1st lot," and Pentex lot #26-6, were found to be almost insoluble, although the latter showed some solubility overnight. These results may have been connected with the state of subdivision of the solid RNase preparations, since the soluble Armour samples were light and fluffy, while the others were particulate. This was not further investigated, however, and the studies reported in this paper were confined to Armour RNase lot #381-059.

Potentiometry in Ethylene Glycol Solutions.—Only a rudimentary study of this part of the problem was undertaken, since our objectives were met by measurements of potential on a relative rather than an absolute scale and since the quanti-

tative measurement and interpretation of acidity in nonaqueous systems is not yet well defined.

Our first concern was to determine whether the usual glass electrode can function as a hydrogen electrode in ethylene glycol solutions. The use of the glass electrode in nonaqueous media has received some attention (Bates, 1954), but mostly in acid-base titrations. The role of residual water on the glass membrane in facilitating the passage of hydrogen ions across the solution-glass boundary has been emphasized. However, we made no studies of this effect; the procedure described above for treating the glass electrode was found to give satisfactory and reproducible results.

A continuous spectrophotometric titration of L-tyrosine ethyl ester HCl in 0.2 M KCl in ethylene glycol was carried out as described, and the results can be compared with the corresponding titration in 0.2 M KCl in water (Fig. 1). A very close correspondence exists between the two titration curves. This indicates that the measured voltage of ethylene glycol solutions of L-tyrosine ethyl ester HCl is closely proportional to the logarithm of the activity of H^+ *in water*, in the *p*H range covered by the titrations.

Furthermore, if the glass electrode functions as a hydrogen electrode in ethylene glycol solutions and is not influenced by the variable activity of water, the measured potential should be given by the relation (Bates, 1954)

$$E = \text{const} - \frac{RT}{F} \ln a_{H^+} \qquad (1)$$

where the constant is the sum of the standard half-cell potentials and the (assumed constant) liquid junction potential, F is the Faraday, and a_{H^+} is the activity of hydrogen ion in ethylene glycol solution, with the standard state taken as, say, the infinitely dilute solution of H^+ in glycol. For the ionization equilibrium of the phenolic OH group of L-tyrosine ethyl ester HCl we may write

$$a_{H^+} = K_A \frac{a_{ROH}}{a_{RO^-}} \qquad (2)$$

where K_A is the acid dissociation constant of L-tyrosine ethyl ester HCl in ethylene glycol, and a_{ROH} and a_{RO^-} the activities of the phenolic and phenoxide ion forms of L-tyrosine ethyl ester HCl respectively. As is shown below, the increment in molar extinction coefficient, $\Delta\epsilon$, of a partially titrated solution of L-tyrosine ethyl ester HCl in ethylene glycol at 296 mμ is essentially proportional to the mole fraction of the phenoxide ion species. Assuming that activity coefficients effectively cancel out, it follows that

$$E = \text{const} - \frac{RT}{F} \ln K_A - \frac{RT}{F} \ln \frac{\Delta\epsilon_T - \Delta\epsilon}{\Delta\epsilon} \qquad (3)$$

where $\Delta\epsilon_T$ is the molar extinction coefficient at 296 mμ of the phenoxide ion species. Therefore, under these circumstances, E should vary linearly with log $[(\Delta\epsilon_T-\Delta\epsilon)/\Delta\epsilon]$ with a slope of -0.0591 at 25°. In Figure 2, this expectation is shown to be realized, using the value $\Delta\epsilon_T = 2400$ determined at the titration extreme shown in Figure 1.

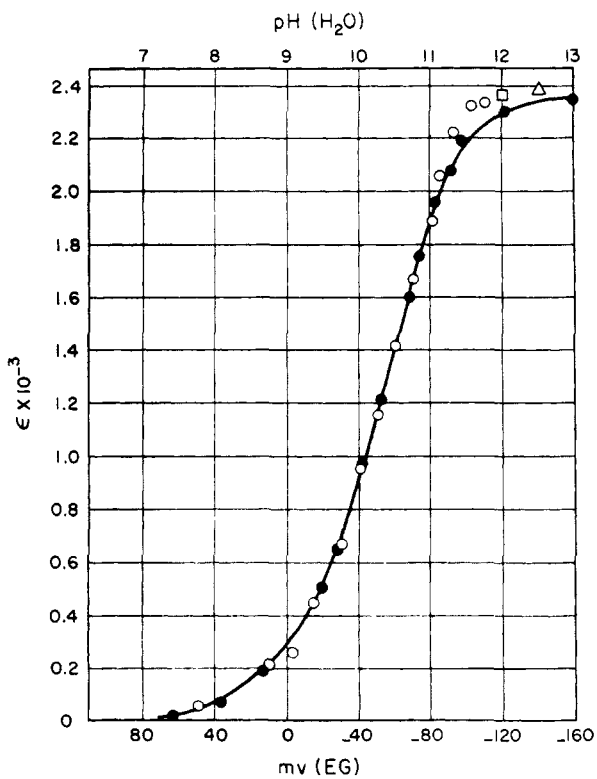

FIG. 1.—Continuous titration of L-tyrosine ethyl ester HCl in 0.2 M KCl–ethylene glycol (EG) (open circles, lower scale) and in 0.2 M KCl-H_2O (filled circles, upper scale), as described in text. □, separate solution of L-tyrosine ethyl ester HCl in 0.1 M KOH–0.1 M KCl–ethylene glycol; △, separate solution of L-tyrosine ethyl ester HCl in 0.2 M KOH in ethylene glycol. Measurements of ϵ made at 296 mμ in ethylene glycol and 295 mμ in H_2O. The curve is drawn through the H_2O data only.

In a single experiment carried out simultaneously with three different glass electrodes but the same reference electrode, titration curves parallel to that of Figure 1 for L-tyrosine ethyl ester HCl in ethylene glycol were obtained, but with different voltages characterizing their midpoints, over a range from about +0.050 to −0.230 v. The results in independent experiments with any one glass electrode were, however, quite reproducible, if the electrodes were treated as indicated in the previous section. Two glass electrodes, designated A and C, were used in the experiments reported herein; these electrodes correspond to two separate periods during which these studies were pursued. Electrode A was employed in the continuous titration experiments, and C in conjunction with studies of the difference spectra, recorded below. Voltage values between these two sets of experiments are, therefore, not directly comparable, while those within a set are.

Spectral Properties of Tyrosine, L-Tyrosine Ethyl Ester HCl, and RNase in Water and Ethylene Glycol.—A comparison of the ultraviolet spectra of L-tyrosine ethyl ester HCl and RNase in neutral

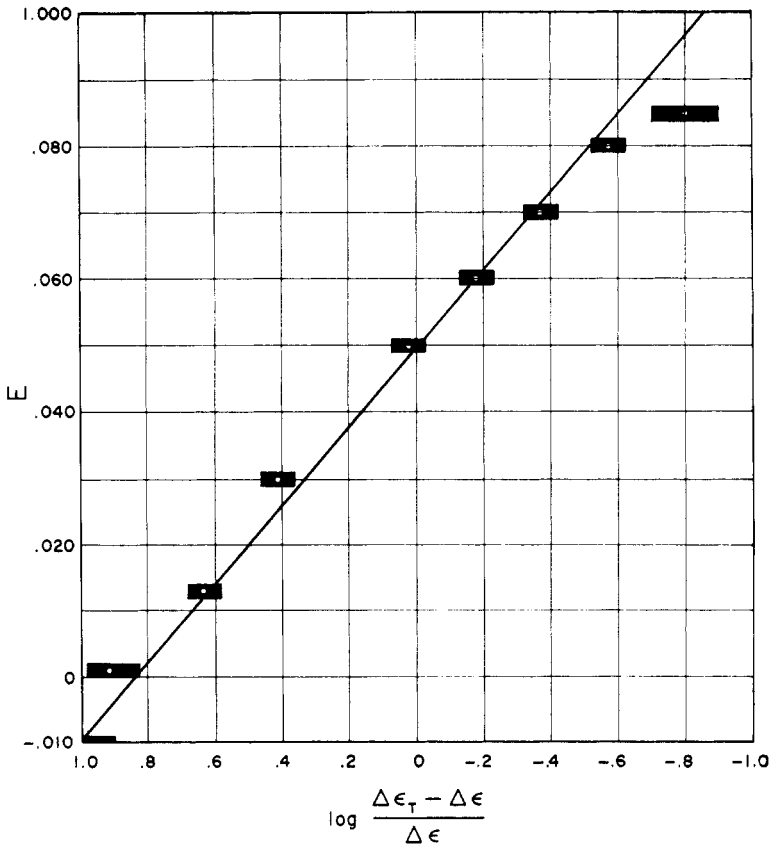

FIG. 2.—Plot of titration data of L-tyrosine ethyl ester HCl in 0.2 M KCl–ethylene glycol according to equation (3) of text. Line drawn through the data has slope of 0.0591 v.

water and ethylene glycol solutions containing 0.2 M KCl is given in Figure 3, and the absorption maxima and molar extinction coefficients are recorded in Table I. While the shapes of all these spectral curves are similar in water and in ethylene glycol, it may be seen that there is a small shift of absorption maxima to the red in the nonaqueous solvent both with L-tyrosine ethyl ester HCl and with RNase, accompanied by an appreciable increase in absorption. L-Tyrosine ethyl ester HCl and tyrosine in either of these two solvents have very similar absorption spectra, the molar extinction coefficients at the maximum being indistinguishable. It is of considerable interest that, while the maximum molar extinction coefficient of RNase in water is considerably more than six times as large as that of L-tyrosine ethyl ester HCl in water (Shugar, 1952), in ethylene glycol it is close to six times that of L-tyrosine ethyl ester HCl in glycol (Table I).

In order to define the spectral changes that occur in the ionization of the phenolic hydroxyl group of tyrosine in ethylene glycol solution, difference spectra were obtained as follows. Solutions of tyrosine were prepared by weight in mixtures of various proportions of 0.2 M KCl in ethylene glycol and of 0.2 M KOH in ethylene glycol, and their

TABLE I

SPECTRAL CHARACTERISTICS OF L-TYROSINE ETHYL ESTER HCL AND RNASE

Compound	Solvent	Absorption Maximum (mμ)	Molar Extinction Coefficient
L-Tyrosine ethyl ester HCl	0.2 M KCl–H_2O	275	1400
	0.2 M KCl–ethylene glycol	277	1850
RNase	0.2 M KCl–H_2O	277	9700
	0.2 M KCl–ethylene glycol	278	11,400

ultraviolet spectra were obtained against blanks containing the same concentration of tyrosine in 0.2 M KCl in ethylene glycol. The potentials characterizing these solutions were also measured with the potentiometric apparatus described above with glass electrode C. Some of the difference spectra are shown in Figure 4. These are generally similar to the corresponding difference spectra obtained in the titration of 0.2 M KCl–water solutions of tyrosine as measured against a blank at *p*H 6.20 (Fig. 5). The maximum extinction coefficient of the phenoxide ion form of tyrosine in ethylene glycol (2400) is similar to the value in water (2350), as is the wave length of maximum absorption (296 in the former case, compared to 295 mμ).

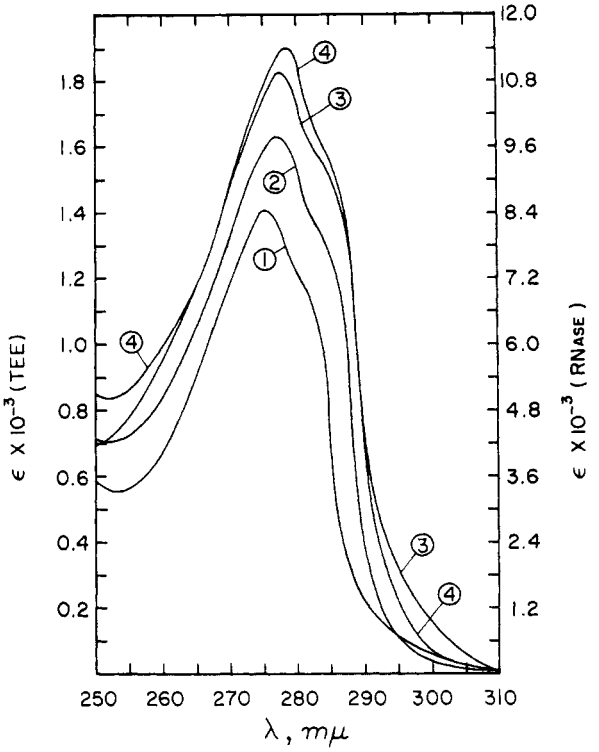

FIG. 3.—Ultraviolet absorption spectra of L-tyrosine ethyl ester HCl (TEE) in, ①, 0.2 M KCl-H_2O and, ③, 0.2 M KCl-ethylene glycol; and of RNase in, ②, 0.2 M KCl-H_2O, and, ④, 0.2 M KCl–ethylene glycol.

Closer examination of the spectra, however, reveals several interesting features. All the curves obtained with tyrosine in aqueous media (Fig. 5) intersect at two values of λ, 266.5 and 278 mμ. The former intersection is an apparent isosbestic point, with $\Delta\epsilon = 0$. The latter intersection, however, occurs at an apparent $\Delta\epsilon = 80$. On the other hand, the spectra obtained with tyrosine in ethylene glycol media (Fig. 4) are more complicated in appearance. They do not intersect at two points, but exhibit rather two clusters of intersections around 263 and 283 mμ. Furthermore, at low degrees of titration the curves are markedly asymmetric. Analogous difference spectra for L-tyrosine ethyl ester HCl and tyrosine in ethylene glycol are very similar, so that these features are not connected with the ionization of the carboxyl group of tyrosine.

The primary object in presenting the tyrosine difference spectra of Figures 4 and 5 is for comparison with the corresponding difference spectra obtained with RNase. A detailed discussion of possible explanations for the complexities of the tyrosine difference spectra is not appropriate here. Suffice it to say that these complexities probably arise from the perturbing effect of the vicinal α-NH_3^+ group on the spectral properties of the phenolic OH group of tyrosine (Wetlaufer *et al.*, 1958).

In a similar manner, difference spectra were obtained for the titration of the tyrosine phenolic

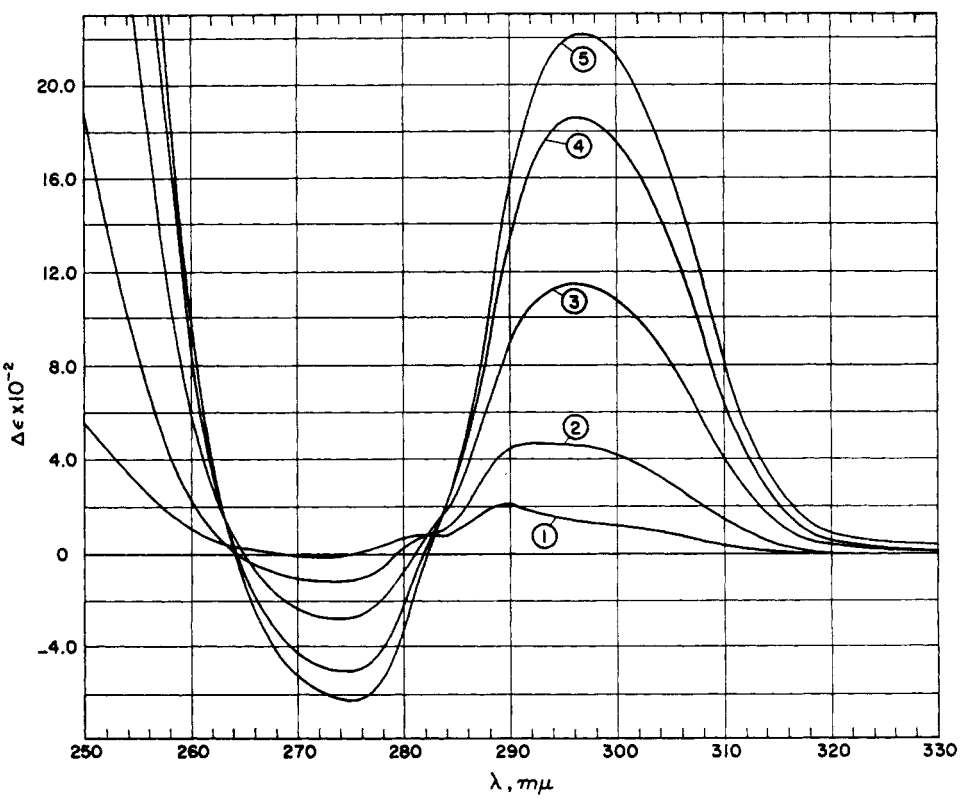

Fig. 4.—Difference spectra of tyrosine in 0.2 M KCl-ethylene glycol, as a function of the degree of titration of the phenolic OH group, against a blank at +191 mv. ① +42 mv; ② −13 mv; ③ −53 mv; ④ −84 mv; ⑤ −120 mv.

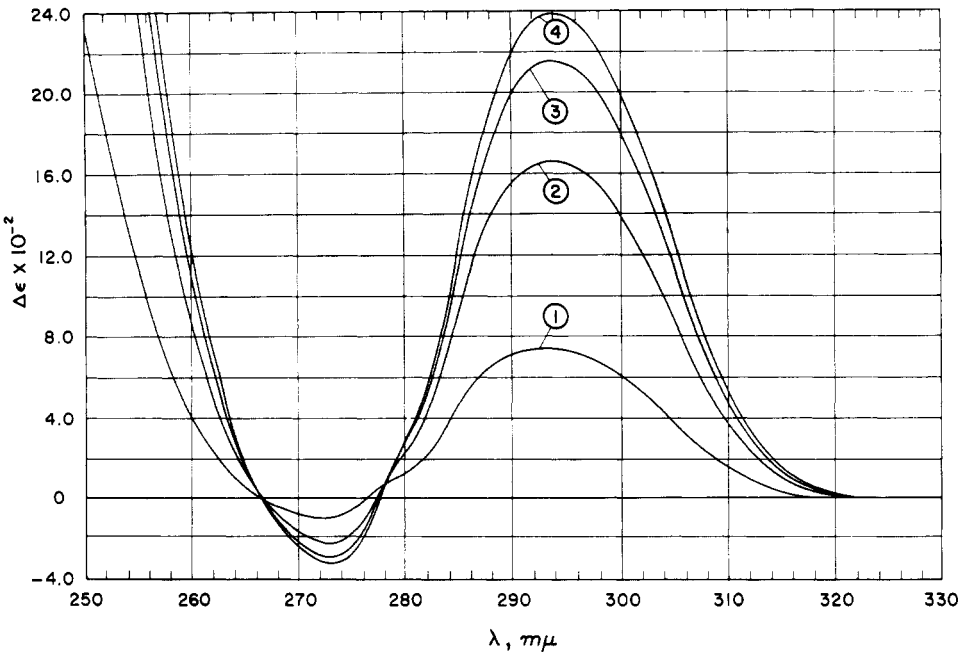

FIG. 5.—Difference spectra of tyrosine in 0.2 M KCl-H_2O, as a function of the degree of titration of the phenolic OH group, against a blank at *p*H 6.2. ① *p*H 9.23; ② *p*H 10.12; ③ *p*H 10.78; ④ *p*H 12.75.

groups of RNase in ethylene glycol (Fig. 6) and in water (Fig. 7) media containing 0.2 M KCl. The spectra in water media are consistent with earlier published findings (Shugar, 1952; Tanford *et al.*, 1955). The titration of approximately the first three tyrosine residues (curve 3 in Figure 7 corresponds to an average of 2.7 residues titrated) occurs more or less normally; the curves exhibit a maximum at 295 mμ, an apparent isosbestic point at 280 mμ, and a cluster of intersections around 271 mμ. At higher degrees of titration, however, absorption at all wave lengths increases in parallel fashion with time, and the curves no longer intersect.

The difference spectra for RNase in ethylene glycol media (Fig. 6) are different from those in water in several notable respects. There are no apparent isosbestic points, but rather two clusters of intersections of slightly positive $\Delta\epsilon$ at about 267 and 282 mμ. The blurring of isosbestic points may be attributable, as in the case of the tyrosine titration in ethylene glycol mentioned previously, to accompanying changes in vicinal charges on nearby amino acid residues, such as lysine, in the RNase molecule (Wetlaufer *et al.*, 1958). More striking, however, is the fact that even at much higher degrees of titration (curve 5 in Figure 6 corresponds to an average of 4.3 tyrosine residues titrated), the curves have normal intersections and are much less time-dependent compared to the corresponding water spectra. These observations are extended by the results of the continuous titration experiments which are discussed next.

Spectrophotometric and Potentiometric Titration of RNase in Ethylene Glycol.—These experiments were carried out as indicated in the Experimental section, with the same glass electrode A employed in the titration of L-tyrosine ethyl ester HCl in ethylene glycol shown in Figure 1. Optical density measurements were made at 296 mμ, corresponding to the maximum in the difference spectra described in the previous section. The results are plotted in Figure 8 (Sage and Singer, 1958), along with the corresponding curve obtained in aqueous solution. With a suitable translation of abscissa, the titration curves in the two solvents are almost superimposable up to the point that corresponds to about two tyrosine residues titrated. Beyond this point, however, the titration curve in water shows the inflection attributed to the abnormally titrating tyrosines whereas the curve in ethylene glycol continues smoothly until essentially all six tyrosines are titrated. Furthermore, the titration curve in ethylene glycol is reversible, even after maintenance of the RNase with over five of its tyrosines titrated at 25° for 1 hour. This is in contrast to the irreversible nature of the higher *p*H regions of the RNase titration curve in water (Tanford *et al.*, 1955).

The value of ϵ_{296} for RNase in ethylene glycol at the asymptote in Figure 8 corresponding to essentially complete titration of all six tyrosine residues is 15,800±200, or 2630±30 per phenoxide ion. This is the same value as that obtained by Tanford *et al.* (1955) for the titration of the normal three tyrosine residues of RNase in water, and by Cha and Scheraga (1960) for the titration of all six tyrosines in 5 M guanidine, 1.2 M urea, in H_2O.

It is also of interest that the observed voltage at the midpoint of the titration curve of RNase in

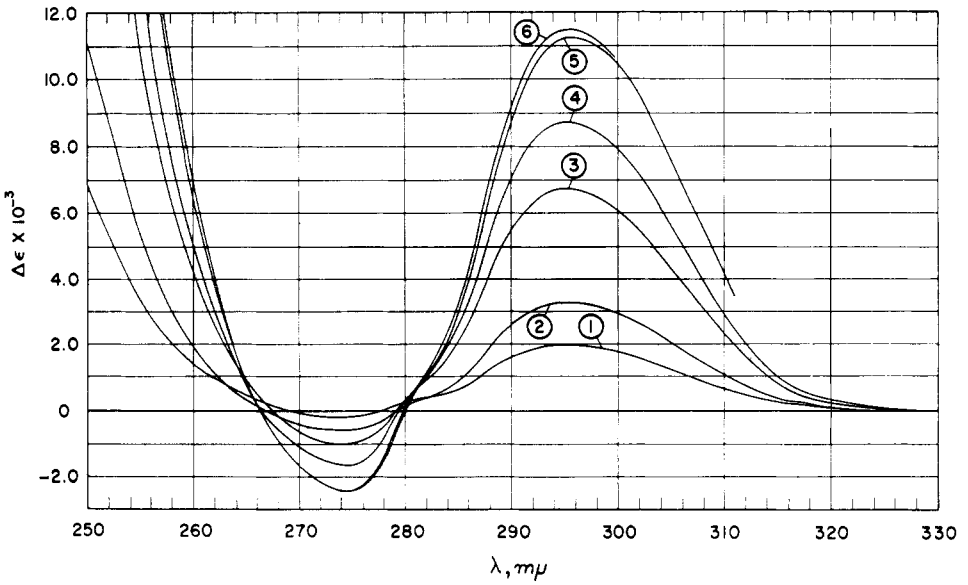

FIG. 6.—Difference spectra of RNase in 0.2 M KCl–ethylene glycol, as a function of the degree of tiration of the phenolic OH groups of the tyrosine residues, against a blank at +150 mv. ① +8 mv; ② − 3 mv; ③ − 50 mv; ④ − 59 mv; ⑤ − 105 mv; ⑥ − 105 mv after 40 minutes at 25°.

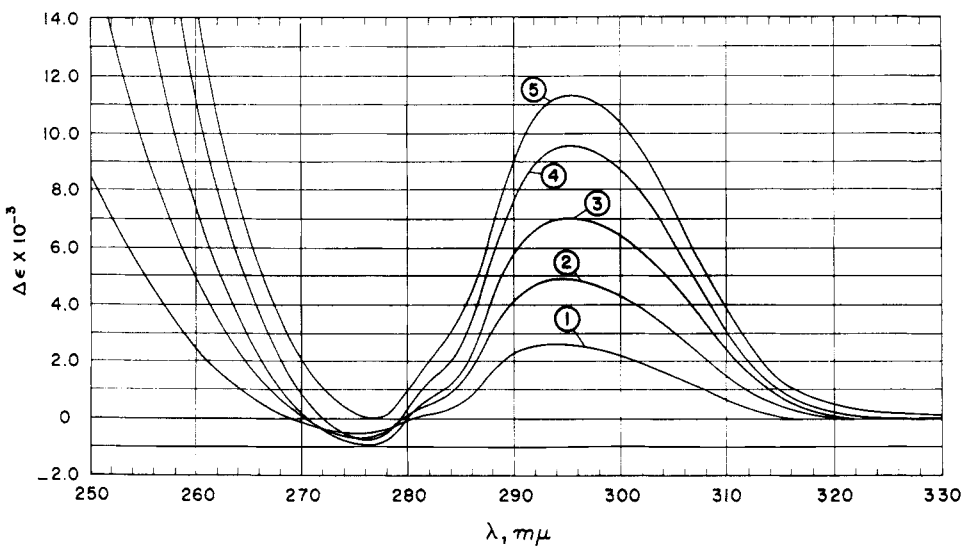

FIG. 7.—Difference spectra of RNase in 0.2 M KCl-H_2O, as a function of the degree of titration of the phenolic OH groups of the tyrosine residues, against a blank at *p*H 5.2. ① *p*H 9.52; ② *p*H 10.41; ③ *p*H 11.38; ④ *p*H 12.32; ⑤ *p*H 12.32 after 40 minutes at 25°.

ethylene glycol, −0.083 v, is considerably more negative than the corresponding voltage for L-tyrosine ethyl ester HCl in ethylene glycol, −0.050 v, the same electrode assembly having been used in both titrations.

Recoverable Enzymatic Activity of RNase Titrated in Ethylene Glycol.—Solutions of RNase (1.14 mg/ml) were prepared in (*a*) 0.5 M KOH in ethylene glycol, (*b*) 0.2 M KOH in ethylene glycol, and (*c*) 0.2 M KCl in ethylene glycol at room temperature. Optical density measurements at 296 mμ were quickly made, and within 1 minute after the RNase was dissolved a portion of each of the solutions was diluted into the acetate buffer used for enzymatic activity determinations. These are referred to as zero-time measurements. At regular intervals thereafter further portions of the three samples were removed, their optical densities were measured, and their enzymatic activities after recovery into aqueous media were determined. The relative enzymatic activity is referred to the activity recovered from solution (*c*) above at zero time, and the results are given in Table II. It may be seen that there is no instantaneous loss of recoverable

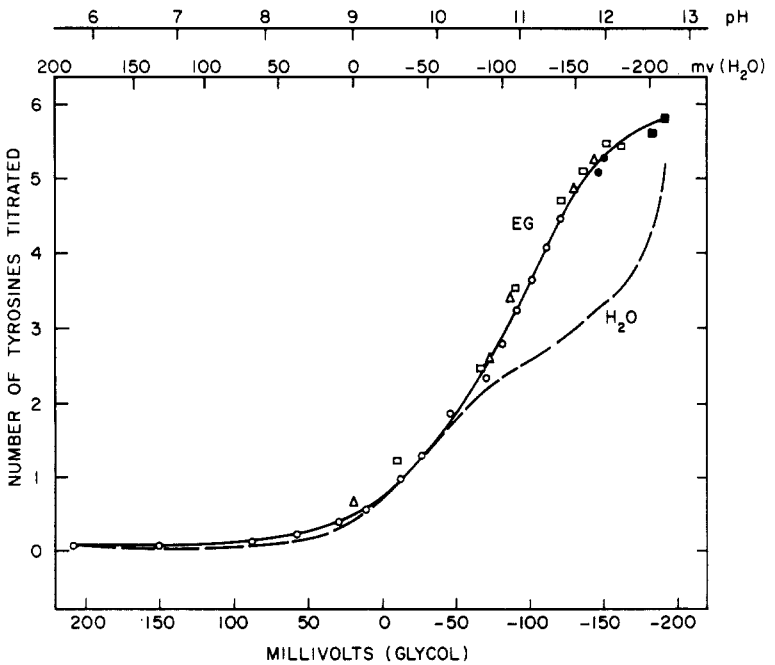

FIG. 8.—Continuous titration curve of RNase in 0.2 M KCl–ethylene glycol (EG) and in 0.2 M KCl-H_2O. Absorption data at 296 mμ converted to the number of tyrosine OH groups titrated using $\epsilon = 2630$ per phenoxide ion group in both solvents. The upper mv and *p*H scales refer to the H_2O titration, the lower scale to ethylene glycol. The two mv scales are the same, and have been translated with respect to each other to superimpose the initial parts of the two titration curves. The points represent data in ethylene glycol solutions only: ○, forward titration (continuous); ●, separately prepared samples in KCl-KOH solutions at 0.20 M total ionic concentration; ■, separately prepared samples in KCl-KOH solutions at 0.35 M total ionic concentration; □, back titration (continuous), starting in 0.1 M KOH–0.1 M KCl solution at −150 mv; △, back-titration (continuous) after 1 hour of incubation at 25° in 0.1 M KOH–0.1 M KCl. Data for the H_2O titration are omitted for convenience.

RNase activity on solution in KOH in ethylene glycol, even though all six tyrosine groups are in the phenoxide ion form; appreciable loss occurs, however, over a period of hours, more rapidly in more alkaline solutions.

TABLE II
RECOVERABLE ACTIVITY OF RNASE

Solvent	No. of Tyrosines Titrated	Time (min.)	Relative Activity
0.2 M KCl–ethylene glycol	0	0	1.00
		30	0.96
		180	0.98
		360	0.95
0.2 M KOH–ethylene glycol	5.5	0	0.98
		120	0.61
0.5 M KOH–ethylene glycol	6.0	0	1.04
		30	0.71
		180	0.31
		360	0.14

Optical Rotation Studies.—The optical rotation of RNase in 0.2 M KCl in ethylene glycol at 25° was found to be independent of time over a period of several hours. The data given in Table III reveal that RNase is considerably more dextrorotatory, at all wave lengths examined, in ethylene glycol than in aqueous solution. For example, $[\alpha]_D$ is −37° compared to −71.5° in 0.2 M NaCl in water (Yang and Doty, 1957). The Lorentz factor, $(n^2 + 2)/3$, however, differs by only 3% for the two solvents.

TABLE III
OPTICAL ROTATORY DISPERSION OF RNASE IN ETHYLENE GLYCOL

λ, mμ	589	578	546	436	405	365
$-[\alpha]$	36.6	39.8	46.0	83.5	106	149

The optical rotatory properties of proteins have been interpreted through the use of relation (4) (Moffitt and Yang, 1956)

$$[\alpha] = \frac{100}{M_0}\frac{n^2+2}{3}\left\{\frac{a_0\lambda_0^2}{\lambda^2-\lambda_0^2} + \frac{b_0\lambda_0^4}{(\lambda^2-\lambda_0^2)^2}\right\} \quad (4)$$

where M_0 is the average residue molecular weight in the protein, taken here as 110, and $\lambda_0 = 212$ mμ. With polypeptide model systems in essentially completely right-handed α-helical form, $b_0 = -630°$ practically independent of solvent effects (Moffitt and Yang, 1956). On the assumptions that only right-handed α-helices are present in RNase, and only these contribute to the value of b_0, an estimate of the fractional helical content can be made. From the plot of $[\alpha]$ $(\lambda^2 - \lambda_0^2)$ against

$(\lambda^2 - \lambda_0^2)^{-1}$ shown in Figure 9, the value $b_0 = -92°$ is obtained, which is close to the value $b_0 = -105°$ found in neutral aqueous solutions of RNase (Weber and Tanford, 1959).

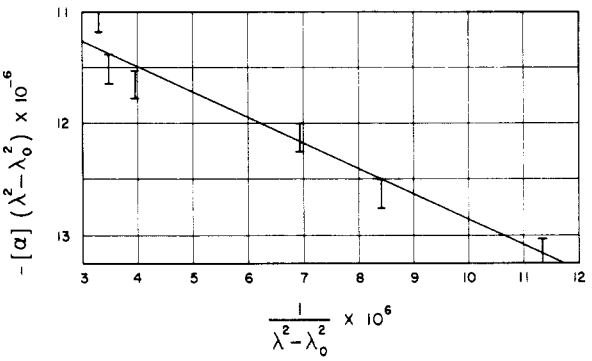

Fig. 9.—The Moffitt plot of the optical rotatory dispersion of RNase in 0.2 M KCl–ethylene glycol.

Measurements were also made at the Na *D* line with a solution of RNase in 0.2 M KOH in ethylene glycol, with about 5.5 of the 6 tyrosines of RNase in the phenoxide ion form, according to the optical density of the solution at 296 mμ. It was found that $[\alpha]_D$ was a function of time (Table IV), starting out at a value which, in view of our experimental error, was not significantly different from the value obtained in 0.2 M KCl in ethylene glycol with all six tyrosines un-ionized. Over a period of several hours, the solution became still more dextrorotatory.

TABLE IV
TIME-DEPENDENCE OF OPTICAL ROTATION OF RNASE IN ALKALINE ETHYLENE GLYCOL

Time (hr.)	0.5	1.5	3.0	7.0	20
$-[\alpha]_D$	41	38	30	23	21

Molecular Weight of RNase in Ethylene Glycol.—A preliminary ultracentrifugal molecular weight determination was made for RNase in 0.2 M KCl in ethylene glycol. The modification by Ehrenberg (1957) of the procedure of Archibald (1947) was utilized, in conjunction with a synthetic boundary cell (Pickels *et al.*, 1952). At a concentration of 6.5 mg RNase/ml, taking the partial specific volume of RNase to be the same as in aqueous media (0.709 [Rothen, 1940–1]), the molecular weight calculated was approximately 100,000. It is apparent that under these conditions RNase is extensively aggregated, and further hydrodynamic studies in ethylene glycol therefore were not pursued.

DISCUSSION

Potentiometry in Ethylene Glycol.—Before discussing the properties of RNase in ethylene glycol solution, let us consider briefly the significance of our few results on potentiometric measurements of hydrogen ion activity in ethylene glycol solutions by means of a glass electrode. Although a considerable amount of work has been done on potentiometric titrations with the glass electrode in nonaqueous solvents (*cf.* Harlow *et al.*, 1956; Cundiff and Markunas, 1956), this has generally not required the demonstration that the glass electrode functioned precisely as a hydrogen electrode in these solvents. The plot of Figure 2 suggests that the glass electrode can function as a hydrogen electrode in very dilute solutions of H^+ in ethylene glycol even in the presence of 0.2 M KCl. Before this conclusion can be stated firmly, however, further studies should be made of the effects of a number of variables, including variable water content of the glycol solutions, on the measured potentials. Should this conclusion be confirmed, it might be feasible to investigate experimentally the acid-base behavior of simple substances and of those proteins which are soluble in ethylene glycol with the glass electrode in a manner analogous to the extensive studies that have been carried out in aqueous solution. A precise definition of a "*p*H" scale in a nonaqueous solvent is elusive (Gutbezahl and Grunwald, 1953), but an operational "*p*H" scale can be defined and measured potentiometrically with the use of suitable standard buffers (Bates, 1954) as is done in aqueous media. Such studies are likely to be of considerable interest in many areas of protein chemistry, and particularly in the application of electrolyte theory to protein solutions.

It is noteworthy, for example, that the voltage at the midpoint of the phenolic titration of RNase in ethylene glycol is shifted to a considerably more negative value than the corresponding voltage for the titration of L-tyrosine ethyl ester HCl or of tyrosine itself in the ethylene glycol (Fig. 8 and 1). In other words, the apparent "*pK*" of the phenolic hydroxyl group is larger in ethylene glycol solutions of RNase than of L-tyrosine ethyl ester HCl or tyrosine. Similar behavior is observed in aqueous solutions of these compounds (Tanford *et al.*, 1955) and of many related systems, and is attributed to the effect of the net charge of the protein molecule on the ionization of specific acidic groups. This is generally investigated quantitatively with the aid of the Linderström-Lang theory (1924), which accounts surprisingly well for the phenomenon. In ethylene glycol, with dielectric constant 37.7 (Weissberger *et al.*, 1955) at 25°, compared to the value 78.5 for H_2O, these electrostatic effects might be expected to be pronounced, and the influence of ionic strength on the titration curves should be particularly marked. This problem appears worthy of further investigation.

Romberg and Cruse (1959) have shown that the glass electrode can also be used for exact potentiometric measurements in acetonitrile solutions of various acids and bases. The glass electrode may therefore be found to be useful as a precise hydrogen electrode in a variety of nonaqueous solvents.

Solubility of RNase in Nonaqueous Solvents.—The finding that RNase is significantly soluble in

at least four relatively aprotic solvents, formamide, *N,N*-dimethylformanide, ethylene glycol, and dimethylsulfoxide, is in itself of interest. In the studies of Yang and Doty (1956) with RNase in nonaqueous solvents, strongly acidic solvents and solvent mixtures, including dichloroacetic and formic acids, were employed and no investigation of recoverable enzymatic activity was made. In such protic solvents, the possibility of chemical reactions with proteins exists (particularly if they contain traces of water), in addition to the fact that ionization equilibria of groups on the protein molecule are likely to be radically different from those in water. More useful information probably can be obtained ultimately in relatively aprotic solvents.

RNase is also soluble in 2-chloroethanol (Weber and Tanford, 1959; Doty *et al.*, 1958), and some of its properties in this solvent have been investigated.

The Conformation of RNase in Ethylene Glycol.— Several different kinds of evidence indicate that the structure of RNase in ethylene glycol is indeed different from that in water. The hyperchromic effect which characterizes the three anomalous tyrosine residues of RNase in aqueous media is absent in ethylene glycol. The molar extinction coefficient of RNase at the peak of tyrosine absorption is now close to six times that of L-tyrosine ethyl ester HCl or tyrosine itself (Table I). Furthermore, all six phenolic groups can be titrated with a normal sigmoid titration curve and with complete reversibility in ethylene glycol, in contrast to the situation in aqueous solution (Fig. 8). If the anomalous tyrosines of RNase in aqueous solution are buried in a hydrophobic region of the molecule (Tanford *et al.*, 1955; Kauzmann, 1959), this region must be largely disrupted in ethylene glycol solution to account for the now normal behavior of all six tyrosine residues.

RNase in the visible and near ultraviolet is considerably more dextrorotatory than in water. Such a change is often attributable to an increase in helical content of proteins (Yang and Doty, 1957). However, in this case, this change appears to be largely a solvent effect, since the parameter b_0 in equation (4), which apparently reflects primarily the helical content of proteins (Moffitt and Yang, 1956), is essentially unchanged in ethylene glycol as compared to aqueous solutions. Similar effects have been observed with other globular proteins in mixtures of H_2O and nonaqueous solvents (Tanford *et al.*, 1960). It appears therefore that no significant net change in the helical content of the RNase molecule accompanies the disruption of its hydrophobic regions in ethylene glycol. This, however, may be the result of at least two extreme alternative situations. Either (*a*) the helical regions present in water solution simply persist in ethylene glycol, in which case the hydrophobic regions of the molecule present in water solution are in nonhelical regions; or (*b*) there is a balance between extensive disruption of those helical regions normally present in water and the formation of new helical regions in ethylene glycol, in which case the hydrophobic regions of the molecule which exist in water solution may correspond, at least in part, to the helical regions of the molecule. Our data do not permit a choice between these alternatives.

In this connection it should be emphasized that the helical regions and hydrophobic regions of a protein molecule need not be mutually exclusive; in fact, there may be considerable coincidence of the two. This is demonstrated directly in the structure of myoglobin as revealed by x-ray methods (Kendrew *et al.*, 1961). In this protein, all the phenylalanine and methionine side-chains are directed inward in what are clearly hydrophobic regions of the molecule, and of these residues about half are located in helical regions and half in nonhelical regions.

That lyophobic interactions (generalizing the term hydrophobic interactions [Kauzmann, 1959] to include other solvents than water) are weaker in ethylene glycol than in water solutions is reasonable in view of the properties of simple compounds in these solvents. Thus, relatively nonpolar substances are generally considerably more soluble in ethylene glycol than in water at 25° (Curme and Johnston, 1952). For example, saturated solutions of benzene in ethylene glycol and in water contain 0.046 and 0.00035 mole fraction hydrocarbon respectively. Thus, the lyophobic interaction of benzene molecules for one another appears to be weaker when benzene molecules are in contact with ethylene glycol than with water. More precisely, the transfer of a mole of benzene from an infinite amount of a solution at mole fraction x in water to an infinite amount of a solution at mole fraction x in ethylene glycol at 25° involves a decrease in free energy of 2880 cal.

It is more difficult to assess the effect of a change in solvent on solute-solute hydrogen bonding, such as occurs intramolecularly in polypeptide helix formation. The heats of formation, the stoichiometry, and the detailed stereochemistry of solvent-solvent and solvent-solute hydrogen bonds must all be involved, but no quantitative evaluation of these factors is possible at present. From a qualitative point of view, however, water and ethylene glycol have the same numbers of hydrogen bond donor and acceptor atoms per mole, and similar heats of formation per mole of hydrogen bond (Pimentel and McLellan, 1960). It is, therefore, not unreasonable that solute-solute hydrogen bonding in these two solvents might occur to about equal extents. Unfortunately, no pertinent experimental data on small molecule hydrogen-bonding solutes in these two solvents are available, to our knowledge.

In a recent study of RNase in chloroethanol-water mixtures, Weber and Tanford (1959) found that in the solvent composition range around 79 mole % H_2O the intrinsic viscosity of RNase was larger than that in either solvent alone, while the value of b_0 of equation 4 was $-175°$ in the solvent mixture compared to $-95°$ in acidic aqueous solu-

tion and −380° in chloroethanol itself. From the fact that the intrinsic viscosity was dependent on ionic strength in 79 mole % H_2O and was practically independent of ionic strength in either water or chloroethanol alone, they concluded that the increased intrinsic viscosity in this solvent mixture was due to an unfolding of part of the RNase molecule into a random-coil form. Since the helical content had simultaneously increased, if anything, they argued that the unfolding could have originated only from hydrophobic regions of the RNase molecule present in aqueous solution. While this chain of argument is plausible, the implication of hydrophobic regions in the conformational change is unfortunately indirect, as no specific property of the intact hydrophobic regions was shown to undergo change in this solvent mixture. However, if it is concluded that the anomalous tyrosines form part of the hydrophobic regions present in aqueous media, the evidence that the hydrophobic regions are disrupted in ethylene glycol is direct. Furthermore, since all three anomalous tyrosines of RNase exhibit normal behavior in ethylene glycol, the extent of the disruption of the hydrophobic regions must be nearly complete in that solvent, whereas the extent of disruption in the chloroethanol-water mixture is indeterminate.

Our preliminary measurements suggest that little further change in $[\alpha]_D$ accompanies the titration of all six tyrosine phenolic residues in ethylene glycol, suggesting that only slight, if any, molecular conformational changes result from the titration. This observation again is consistent with the view that the phenolic groups of the protein in ethylene glycol are exposed and accessible to H^+. Furthermore, $[\alpha]_D$ of the fully tyrosine-titrated molecule in ethylene glycol slowly becomes more positive on standing. Whether this is the result of further helix formation, or of some primary valence bond scission, or both, or other causes, requires further investigation.

It is also noteworthy that RNase in 0.2 M KCl in ethylene glycol is highly aggregated, whereas it is molecularly dispersed and highly soluble at its isoelectric point in water. This behavior is apparently quite different from that of insulin, which is insoluble at its isoelectric point in water and is aggregated even in acidic and basic aqueous media, but which is predominantly molecularly dispersed in neutral *N,N*-dimethylformamide and dimethylacetamide solutions (Rees and Singer, 1956). Clearly quite different interactions are of primary importance in the solution and aggregation behavior of these two proteins.

Recovery of RNase Activity from Nonaqueous Solvents.—The fact that the enzymatic activity of RNase is almost completely recoverable from neutral ethylene glycol solution indicates that the conformational changes in RNase accompanying the transition from water to ethylene glycol solution are rapidly and essentially completely reversible. Similarly, those conformational changes, if any, which occur in the RNase molecule upon solution in formamide and *N,N*-dimethylformamide must be completely, and in dimethylsulfoxide, substantially, reversible. In our opinion, it is in general essential to establish whether such reversibility exists, in order to reduce the possibility that any observed conformational changes are primarily the result of the scission or formation of primary valence bonds in the protein in nonaqueous media.

In aqueous solutions of RNase at *p*H values higher than 12.7, the enzymatic activity is irreversibly lost (Sela and Anfinsen, 1957). This observation, along with others, has raised the possibility that at least one of the anomalously titratable tyrosines is intimately associated with the active site of the enzyme (Sela *et al.*, 1957) in a structure which is irreversibly destroyed upon ionization of the specific tyrosine phenolic residue(s). In ethylene glycol solution, however, the titration of all six tyrosine phenolic groups of the enzyme, and the loss of activity, are clearly dissociable phenomena in time (Sage and Singer, 1958). The loss of activity is a relatively slow process, whereas the titration is instantaneous. This suggests that a process independent of the ionization of the six tyrosine phenolic residues is responsible for the inactivation in both highly alkaline aqueous and ethylene glycol media; perhaps this involves the scission or rearrangement of some disulfide or other primary valence bonds (Ryle and Sanger, 1955; Zahn and Osterloh, 1955) in the RNase molecule. In any event it is therefore unnecessary to implicate a tyrosine residue in the active site of the enzyme. The same conclusion is forthcoming from the observations that in 8 M urea solutions in water (Blumenfeld and Levy, 1958) and in 5 M guanidine–1.2 M urea solutions in water (Cha and Scheraga, 1960), all six tyrosine phenolic residues titrate normally, and enzymatic activity is recoverable from these media.

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Incorporation of Dicarboxylic Amino Acids into Soluble Ribonucleic Acid

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Although it is known that many amino acids are incorporated into soluble ribonucleic acid to yield amino acyl RNA derivatives, little information is available concerning the dicarboxylic amino acids. In the present work, enzymatic formation of glutamyl RNA and aspartyl RNA was observed, and experiments were carried out to determine which of the two carboxyl groups of these amino acids were linked to RNA, or whether both were involved. Although an α-glutamyl-RNA linkage would be consistent with results obtained with other amino acids, the finding of γ-glutamyl RNA might be of significance in relation to the function of widely distributed enzymes that catalyze γ-glutamyl transfer. Enzyme preparations from baker's yeast and rat liver were incubated with soluble RNA, ATP, Mg^{++}, and C^{14}-glutamic or aspartic acids. The C^{14}-amino acyl RNA derivatives obtained were treated with ammonia under several sets of conditions and the radioactive products found after ammonolysis were examined. Aspartyl RNA gave mainly isoasparagine and small amounts of aspartic acid, but no asparagine. Ammonolysis of glutamyl RNA gave isoglutamine and small amounts of glutamic acid; neither glutamine nor pyrrolidone carboxylic acid was formed. Similar results were obtained on ammonolysis of ribonuclease-treated amino acyl RNA preparations. The evidence indicates that α-aspartyl RNA and α-glutamyl RNA were formed by the enzymes employed here. The rates of hydrolysis of aspartyl RNA and glutamyl RNA were determined at 37° at *p*H values between 6.65 and 8.65. At *p*H 7.25, these derivatives, glycyl RNA, and arginyl RNA were hydrolyzed at similar rates, while valyl RNA exhibited significantly greater stability.

It is now generally believed that amino acyl RNA derivatives formed by amino acid–activating enzymes in the presence of soluble RNA, ATP, and amino acids are involved in the incorporation of amino acids into proteins and probably in the synthesis of proteins. Amino acyl RNA formation has been observed in systems containing activating enzymes and soluble RNA (Holley, 1957; Berg and Ofengand, 1958; Hoagland *et al.*, 1958; Weiss *et al.*, 1958; Schweet *et al.*, 1958), and studies in this laboratory have provided evidence that the amino acyl moiety of synthetic tryptophanyl adenylate is specifically transferred by an activating enzyme to RNA (Wong *et al.*, 1959; Wong and Moldave, 1960). The available data are consistent with the belief that the amino acyl moieties are attached to terminal adenosine residues of soluble RNA, and that they are probably linked to a 2′ or 3′ hydroxyl group; isolation of amino acyl adenosine after treatment of amino acyl RNA with ribonuclease has been reported in studies with leucyl RNA and valyl RNA (Zachau *et al.*, 1958, Preiss *et al.*, 1958).

Although a number of reports on the formation and reactions of amino acyl RNA compounds have appeared, relatively little information is available