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Dr. Robert R. Becker

The spectrophotometric titration of the tyrosine residues, their reaction with N-acetylimidazole, and the effect of pH on the thermally induced conformational transition of ribonuclease T_1 have been investigated. The results of spectrophotometric titration in 0.20 M KCl/0.001 M sodium phosphate suggest that titration of the tyrosine residues occurs in two steps, the first with an apparent pK of 10.7, and the second with an apparent pK greater than 12. About 8 of the 9 tyrosine residues titrate in the first step, which is characterized by a sharp dependence of the degree of ionization on pH. This behavior is suggestive of a cooperative titration. In 8 M urea-0.20 M KCl/0.001 M sodium phosphate the tyrosine residues titrate in one step with an apparent pK of 10.9. Acetylation of ribonuclease T_1 in 0.02 M Veronal, pH 7.5, indicates that about 2 tyrosine residues are readily acetylated with a 60-180 fold molar

excess of acetylimidazole, while other tyrosine residues react only at much higher concentrations of acetylating reagent. In 8 M urea-0.02 M Veronal, pH 7.5, about 8 of the 9 tyrosine residues are readily acetylated. Studies on the thermal transition of ribonuclease T_1 showed that, above about pH 4, the transition temperature decreases with increasing pH. Thermal transition occurred with the exposure of tryptophan and tyrosine residues to solvent. The above properties of ribonuclease T_1 have been contrasted with those of bovine pancreatic ribonuclease A.

The homogeneity of ribonuclease T_1 was investigated by high-speed sedimentation equilibrium measurements, and the protein was found to be homogeneous under the conditions used in the experiments. Molecular weights of ribonuclease T_1 calculated from the sedimentation equilibrium experiments agreed, within experimental error, with the molecular weight based on the known amino acid sequence.

Physical and Chemical Properties of Ribonuclease T_1

by

Terry Lee Miller

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TO CAROLYN AND MY PARENTS

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PHYSICAL AND CHEMICAL PROPERTIES OF RIBONUCLEASE T_1

PART I. SPECTROPHOTOMETRIC TITRATION OF THE TYROSINE RESIDUES AND THEIR REACTION WITH N-ACETYLIMIDAZOLE, AND THE THERMALLY INDUCED CONFORMATIONAL TRANSITION

INTRODUCTION

The isolation of ribonuclease T_1 (RNase T_1) by Sato and Egami (1957) has prompted many studies of its enzymatic and chemical properties. The results of most of these studies have been reviewed by Egami, Takahashi and Uchida (1964) and by Egami (1966). These authors have compared the properties of RNase T_1 to those of the more thoroughly investigated protein, bovine pancreatic ribonuclease A (RNase A). Many differences, and a few similarities, in the enzymatic and chemical properties of these nucleases have been noted. Most of the studies on RNase T_1, however, have been directed toward an understanding of those properties directly associated with enzymatic activity, and not toward an understanding of those properties directly associated with protein structure. Studies of the latter type are of utmost importance, for they have as their end the understanding of the relationship of the structure of a protein to its specific function.

One step in the direction of determining the structural properties of RNase T_1 was the elucidation of its complete covalent

structure by Takahashi (1965). Figure 1 shows schematically the complete sequence of the amino acid residues, and the arrangement of the disulfide bonds, of this protein. The protein consists of a single polypeptide chain of 104 amino acid residues cross-linked by 2 disulfide bridges between the 4 half-cystine residues. The presence of 1 tryptophan residue, the low content of basic amino acids (1 lysine, 1 arginine, and 3 histidine residues), and the absence of methionine, point to marked differences in the amino acid composition of RNase T_1 from that of RNase A. Ribonuclease A has 4 methionine, 4 histidine, 4 arginine, and 10 lysine residues (Hirs, Moore and Stein, 1960; Smyth, Stein and Moore, 1963).

The complete structural characterization of RNase T_1 requires, in addition to its covalent structure, knowledge of the chemical and physical properties related to its three-dimensional structure, as well as the three-dimensional structure itself. Many chemical and physical methods are available to the protein chemist for the study of such properties, and application of these methods to the study of RNase T_1 should yield valuable information. Ultimately, such studies will lead to knowledge of the relationship of the structure of RNase T_1 to its function. The work to be described in this thesis, while only a start, is directed toward this goal. The chemical and physical methods used in this work will now be briefly discussed.

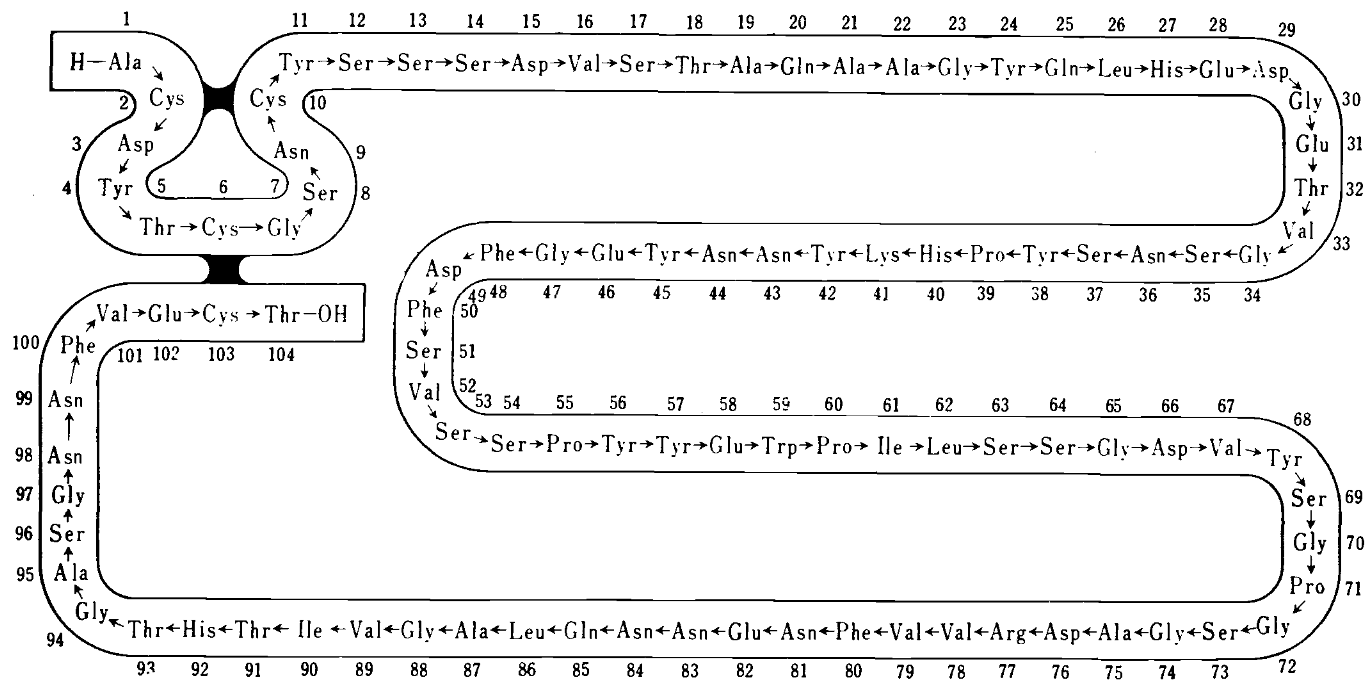

Figure 1. The amino acid sequence of ribonuclease T_1. (After Takahashi, 1965)

Spectrophotometric titration of the tyrosine residues of a protein can yield information on the gross environment of these chromophores. Thus, if the residues titrate normally (*i.e.*, with a pK near to that of tyrosine and closely following a theoretical titration curve) they are considered to be exposed to solvent, as opposed to being "buried" in the interior of the protein molecule. On the other hand, abnormal titration behavior (*i.e.*, an abnormally high pK and marked departure from a theoretical titration curve) indicates that the tyrosine residues are "buried" or "partially buried" in the interior of the molecule, or that they are strongly interacting with other residues in the protein. It is usually found that part of the tyrosine residues of a given protein will titrate normally, whereas others will titrate abnormally or "partially" abnormally (Tanford, 1962). RNase A has been titrated by the spectrophotometric method, as have many other proteins (Tanford, 1962), resulting in the conclusions that 3 of its 6 tyrosine residues are exposed to solvent, while the others are buried in the interior of the molecule (Tanford, Hauenstein and Rands, 1955). It is interesting to note that the above conclusions can be verified by the recent determination of the three-dimensional structures of RNase A (Kartha, Bello and Harker, 1967) and RNase S (Wyckoff *et al.*, 1967).

Reaction of the tyrosine residues of a protein with N-acetylimidazole provides another method for determining the number

of these residues which are exposed to solvent. It has been shown that, in most instances, the exposed tyrosine residues react readily with the acetylating reagent while the buried residues are unreactive (Riordan, Wacker and Vallee, 1965). For example, 3 of the 6 tyrosine residues of RNase A are readily acetylated, whereas the remaining residues are acetylated only after disruption of the native conformation of the protein (Riordan, Wacker and Vallee, 1965).

Studies on the thermal transition of proteins have given information on the conformational stability of these macromolecules, as reflected by the "melting" temperature and enthalpy of transition (Brandts, 1964, 1967). One can also, through difference spectral measurements, determine the types of chromophores that are exposed to solvent during the thermal transition, thus allowing the inference that these residues are buried in the interior of the native molecule.

The work described in this thesis, then, is directed toward a more complete understanding of the physical and chemical properties of RNase T_1 as related to its structure. To this end, the spectrophotometric titration of the tyrosine residues, their reaction with N-acetylimidazole, and the effect of pH on the thermally induced conformational transition of this macromolecule have been investigated.

Part II of this thesis is a study of the homogeneity and

molecular weight of RNase T_1 (prepared by the method of Uchida (1965)) by sedimentation equilibrium measurements.

MATERIALS AND METHODS

Materials

Sanzyme R (ribonuclease T_1 source) was purchased from Calbiochem.

Ribonucleic acid (Torula yeast; Type IV) was purchased from Sigma Chemical Co. It was exhaustively dialyzed against glass distilled water and lyophilized before use. The desicated powder and stock solutions were stored frozen.

Ribonuclease T_1 was prepared from Sanzyme R according to the procedure of Uchida (1965). The specific activity (calculated by the method of Takahashi (1961)) of the purified protein was 720 when assayed against Torula yeast ribonucleic acid, in good agreement with the reported value of 500-700 (Takahashi, 1961; Uchida, 1965). Spectral constants, calculated from a spectrum of the purified protein, are as follows: $OD_{278}/OD_{251} = 3.40$ and $OD_{278}/OD_{260} = 2.28$. These are in agreement with the reported values of $OD_{278}/OD_{251} = 3.01$-3.66 and $OD_{278}/OD_{260} = 2.22$-2.36 (Takahashi, 1962; Minato *et al.*, 1966). Results of amino acid analysis were in agreement with the known amino acid composition (Takahashi, 1965).

Isopropenyl acetate and N-Acetylimidazole were purchased from Eastman Chemical Co. The N-acetylimidazole was recrystallized from isopropenyl acetate and stored *in vacuo* over P_2O_5.

The recrystallized material had a melting point of 101.8-103.2°.

Urea (ultra pure grade), purchased from Mann Chemical, was used without further purification.

Veronal was a product of Merck Chemical.

Dialysis tubing, size 18/32, made by the Viscose process, was purchased from the Food Products Division of the Union Carbide Corporation. The tubing was rinsed thoroughly with distilled water before use.

Methods

Spectrophotometric Titration

Measurements of the optical density at 295 mμ (OD_{295}) and/or 245 mμ (OD_{245}) were made with a Beckman DU spectrophotometer equipped with thermospacers. The temperature in the cell was maintained at 25 ± 1° by circulating water from a constant temperature bath through the thermospacers. Difference spectra were obtained with a Cary Model 11 recording spectrophotometer. Measurements of pH were made with a Corning Model 12 pH meter equipped with a Corning semi-micro combination electrode (no. 476050). Between pH measurements, the electrode was returned to pH 7 standard buffer to check for instrument and electrode drift. Little restandardization was necessary throughout the titration. The

electrode was rinsed with distilled water and dried with a Kimwipe before each pH measurement.

The titrations were carried out at the concentrations and in the solvents indicated in Figures 2, 3, and 4. All changes in optical density were measured relative to a solution of the protein at the same concentration and in the same solvent, but at neutral pH, as that being titrated. The solutions to be titrated were prepared as follows: an appropriate amount of ribonuclease T_1 (from a stock solution of 10 mg/ml of distilled water) was added to about 8 ml of solvent. The concentration of the solution was determined from the optical density at 278 mμ, taking the extinction coefficient of RNase T_1 as $E_{278}^{0.1\%} = 1.91$ (Takahashi, 1962). Approximately 3 ml aliquots were then placed in each of two cuvets and the solution titrated as indicated below. A very small amount of NaOH, delivered from an Agla precision syringe, was placed on the end of a small glass stirring rod and then added to the solution cuvet. The solution was continuously stirred during the addition by use of a micro magnetic stirring bar. The concentration of NaOH used depended on the pH range of the titration as follows: 1) pH 7-11, 1M NaOH, 2) pH 11-12, 4M NaOH, and 3) pH 12-13, saturated NaOH. The total amount of base added (50-100 μl) contributed negligibly to the total volume and, thus, the complete titration could be performed on a single sample. After addition of base the pH of the solution was determined and the

OD_{295} and/or OD_{245} recorded. Immediately after determination of the optical density, the pH of the solution was measured again and these final pH values are reported in the figures. Reverse titrations were carried out in the same manner, but with the addition of HCl. Values of the molar difference extinction coefficients, $\Delta\epsilon_{295}$ and $\Delta\epsilon_{245}$, were computed from the known protein concentration and optical density changes.

Acetylation with N-Acetylimidazole

The procedure employed in the acetylation reactions was analogous to that of Riordan and Vallee (1967). The pH of the reaction was maintained with a Radiometer titrator (Type TTT1) equipped with automatic titration equipment (Type SBR2/SBU1/TTA31). Microelectrodes (Radiometer type G2222B and K4112) were used in the micro titration assembly. A stock solution (10 mg/ml) of acetylimidazole in isopropenyl acetate was freshly prepared before each series of experiments and stored over anhydrous Na_2SO_4. An appropriate aliquot of this solution was placed in the titration vessel and isopropenyl acetate removed by evaporation with a stream of air, which was dried by first flowing it through a tube of Drierite and then through a tube of P_2O_5. The acetylimidazole was dissolved in 0.9 ml of 0.025 M Veronal, pH 7.5, and then 0.1 ml of RNase T_1 solution (10 mg/ml) was added. The molar ratios of acetylimidazole to

protein are indicated in Figure 8. The reaction was maintained at pH 7.5 by addition of 0.2 M NaOH for reaction with 100 fold molar excess of acetylimidazole and 1 M NaOH for all other concentrations of acetylimidazole. After reaction for one hour at 25.0 ± 0.1^{o}, the reaction mixture was transferred to dialysis tubing. The reaction vessel was rinsed twice with 0.2 ml portions of Veronal, the rinses added to the dialysis tubing, and the mixture dialyzed at 4° against three changes (at least eight hours each) of a 100 fold excess of 0.1 M sodium phosphate, pH 7.5. For experiments in urea, a 10.5 M solution of urea in 0.025 M Veronal was prepared. The acetylimidazole was dissolved in 0.8 ml of this solution and 0.1 ml of 0.025 M Veronal was added. The pH of the solution was brought to 7.5 by addition of a small amount (about 5 μl) of 1 M HCl, and then 0.1 ml of RNase T_1 solution (10 mg/ml) was added. The final concentration of urea was 8.4 M. A control sample for each series of experiments was carried through the same procedure, but without addition of acetylimidazole.

Determination of the Number of O-Acetyltyrosine Residues

The number of tyrosine residues acetylated, N, was determined from the increase in absorbance at 278 mμ upon addition of hydroxylamine to a solution of the acetylated protein, as suggested by Riordan and Vallee (1967). A solution of hydroxylamine was

freshly prepared before each series of experiments as follows. Distilled water (3 ml) was added to 5 ml of 7.2 M $H_2NOH \cdot HCl$ and the solution was cooled to below 15°. The stirred solution, maintained at 15° or less, was adjusted to pH 7.6 by the slow addition of saturated NaOH. The solution was diluted to 10 ml with distilled water and placed on ice.

The dialysate from the above dialysis (filtered through a Gelman Metrical filter, GA-8, which had been previously washed with dialysate) was used as a reference solvent in the spectrophotometric measurements. The dialyzed acetylated RNase T_1 was filtered through the same filter and diluted to about 2.5 ml with reference solvent. A portion of this solution (2.00 ml) was placed in the sample cuvet and the OD_{278}, relative to 3.00 ml of reference solvent, was determined from its absorption spectrum obtained with a Cary 11 recording spectrophotometer. The Cary 11 was equipped with a thermostatted cell holder, and the temperature of the solution cuvet was maintained at 25.0 ± 0.1° during the course of the experiments. Hydroxylamine was then added to the reference (0.30 ml) and solution (0.20 ml) cuvets and the solutions were thoroughly mixed. The reaction was allowed to proceed until no further change in OD_{278} was observed (always less than 45 minutes). The equation,

$$N = \frac{\Delta OD_{278} \times M}{\Delta \epsilon'_{278} \times C},$$

where ΔOD_{278} is the increase in absorbance upon deacetylation after correction for dilution due to H_2NOH addition, C the concentration of RNase T_1 (mg/ml), M its molecular weight, and $\Delta\epsilon'_{278}$ the change in molar extinction coefficient upon acetylation of N-acetyl-tyrosine, was used to calculate N. C was determined from the optical density at 278 mμ after deacetylation. The value of 1160 for $\Delta\epsilon'_{278}$ was used in the calculations (Riordan and Vallee, 1967). The control samples were treated in the same manner.

Studies of the Thermal Transition

Changes in optical density with temperature were obtained with a Cary Model 14 recording spectrophotometer equipped with a thermostatted cell holder in the solution compartment. The temperature of the solution cuvet was regulated by circulating water from a heating bath whose temperature was regulated by a programmer (Neslab Instruments). The temperature of the reference cuvet was maintained at 17-19°. The Cary 14 was also equipped with a retransmitting potentiometer attached to a voltage bucking circuit, the signal of which was passed through a simple lag circuit[1] (with a 1 second time constant) and into a dual channel recorder (Esterline

[1]Simple Lag Circuit No. II-15, described in "Applications Manual for Computing Amplifiers", Copyright 1966 by George A Philbrick. Researches, Inc., Nimrod Press Inc., Boston, Mass., p. 46.

Angus Model E 1102S). For most of the experiments, the recorder was adjusted so that full-scale deflection corresponded to a 0.25 optical density change. A thermistor probe (Yellow Springs Instrument Co., Model 423), inserted through a rubber stopper fitted to a semi-micro cuvet and placed in direct contact with the solution, was used to measure the temperature in the solution cuvet. The temperature ($\pm$ 0.2^{o}) was recorded on the second channel of the recorder. In this way, changes of OD with temperature were continuously monitored.

The experiments were carried out in the buffers (all 0.01 M and adjusted to an ionic strength of 0.14 with KCl) indicated in Table 1. Protein concentrations were of the order of 0.5 mg/ml. All changes in OD are relative to a reference solution at the same concentration as the sample solution, but with a pH of 7.4 (0.01 M sodium phosphate, ionic strength = 0.14).

RESULTS

Titration of the Tyrosine Residues of RNase T_1

Difference spectra obtained during spectrophotometric titration of RNase T_1 are shown in Figure 2 as a plot of $\Delta\epsilon$ against wavelength. The optical density was initially set to zero at 360 mμ. These spectra, with maxima at 295 and 245 mμ, are very similar to tyrosine ionization difference spectra (Wetlaufer, 1962; Hermans, 1962). Furthermore, the ratio of $\Delta\epsilon_{245}$ to $\Delta\epsilon_{295}$ of 4.7 is close to that of 5.2, 4.2, 4.5, and 4.9 reported for tyrosine, ribonuclease A, myoglobin (Hermans, 1962), and pepsinogen (Perlmann, 1964), respectively. Thus one can conclude that the spectral changes observed during titration of RNase T_1 can be attributed to tyrosine ionization.

Results of the spectrophotometric titration of RNase T_1 in 0.20 M KCl/0.001 M sodium phosphate are shown in Figure 3 as a plot of $\Delta\epsilon_{295}$, and in Figure 4 as a plot of $\Delta\epsilon_{245}$, against pH. The different sets of points on the forward titration curve of Figure 3 represent data obtained at different protein concentrations. Within experimental error, all of the points fall on the same smooth curve. This indicates that the results of the titration are not effected by protein concentration and, also, that Beer's law holds under the conditions used in these experiments.

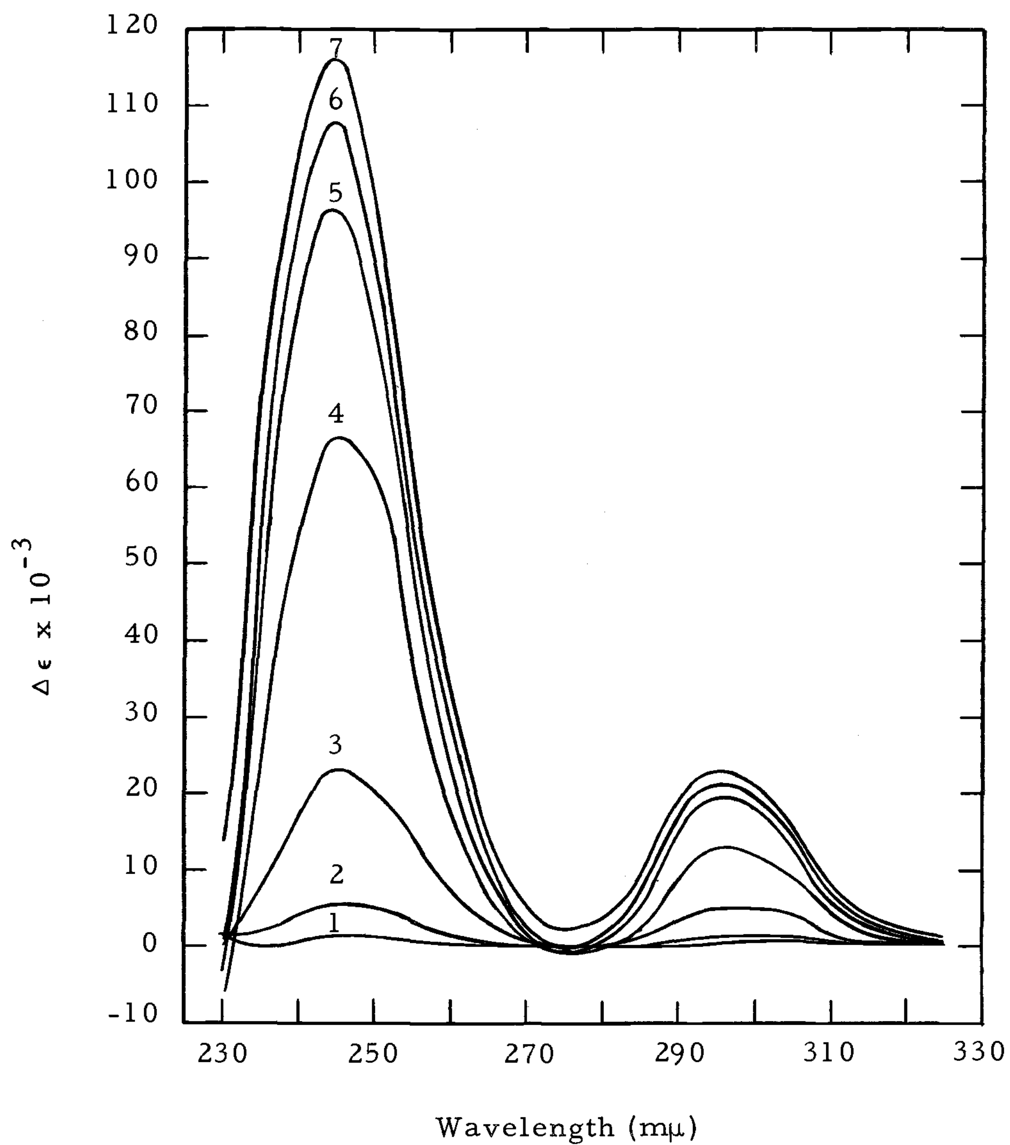

Figure 2. Difference spectra of ribonuclease T_1 as a function of pH. pH 8.60, (1); pH 9.65, (2); pH 10.48, (3); pH 10.78, (4); pH 11.35, (5); pH 11.95, (6); pH 12.66, (7).

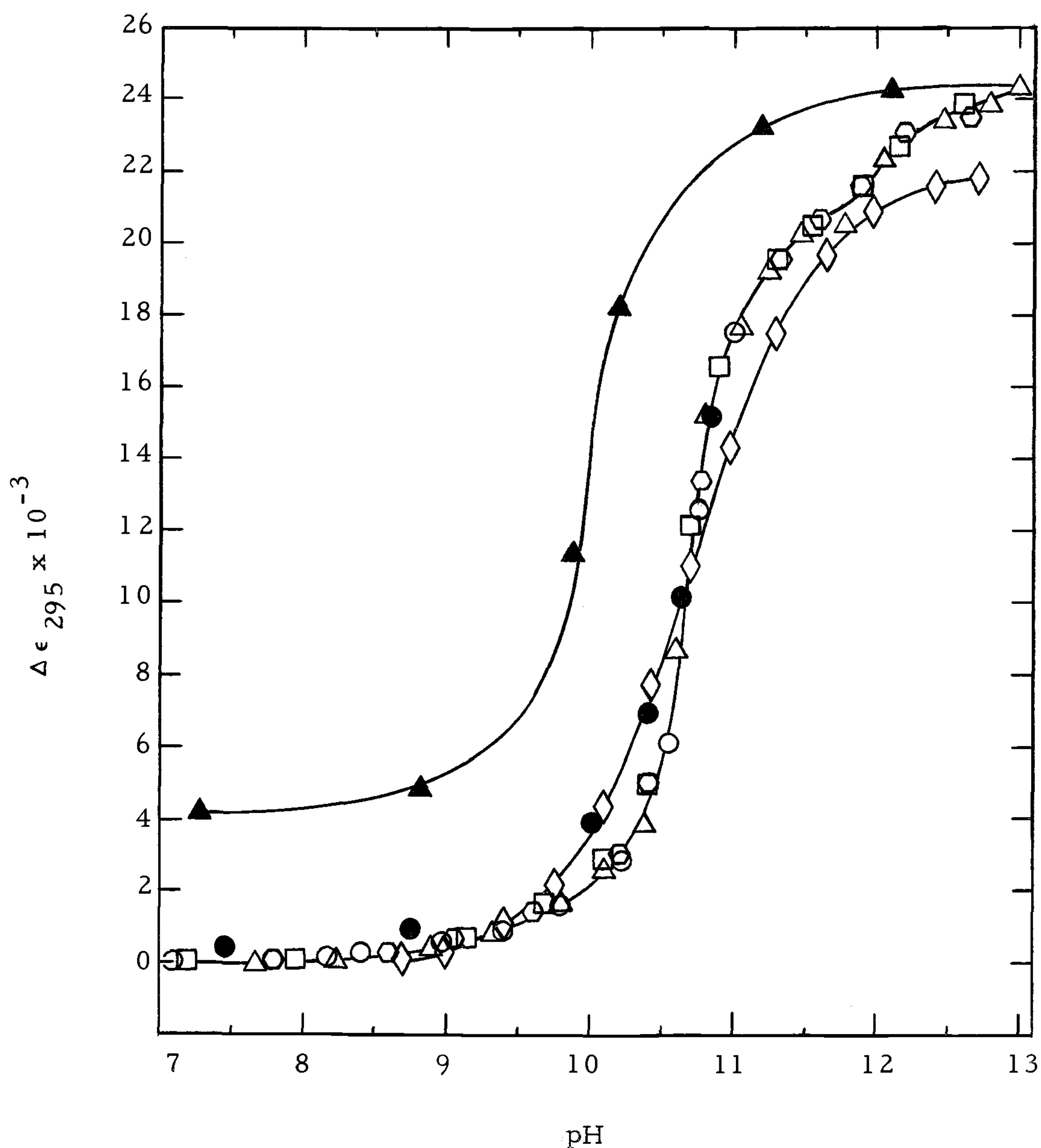

Figure 3. Spectrophotometric titration at 295 mμ of the tyrosine residues of RNase T_1 in 0.20 M KCl/0.001 M sodium phosphate at 25°. Forward titration at the indicated protein concentration: (□), 0.70 x 10^{-5} M; (⬡), 1.38 x 10^{-5} M; (○, △), 2.31 x 10^{-5} M. Reverse titration at a protein concentration of 2.31 x 10^{-5}M: (●), from pH 11; (▲), from pH 13. Calculated curve for the titration of tyrosine with an apparent pK of 10.7: (◊).

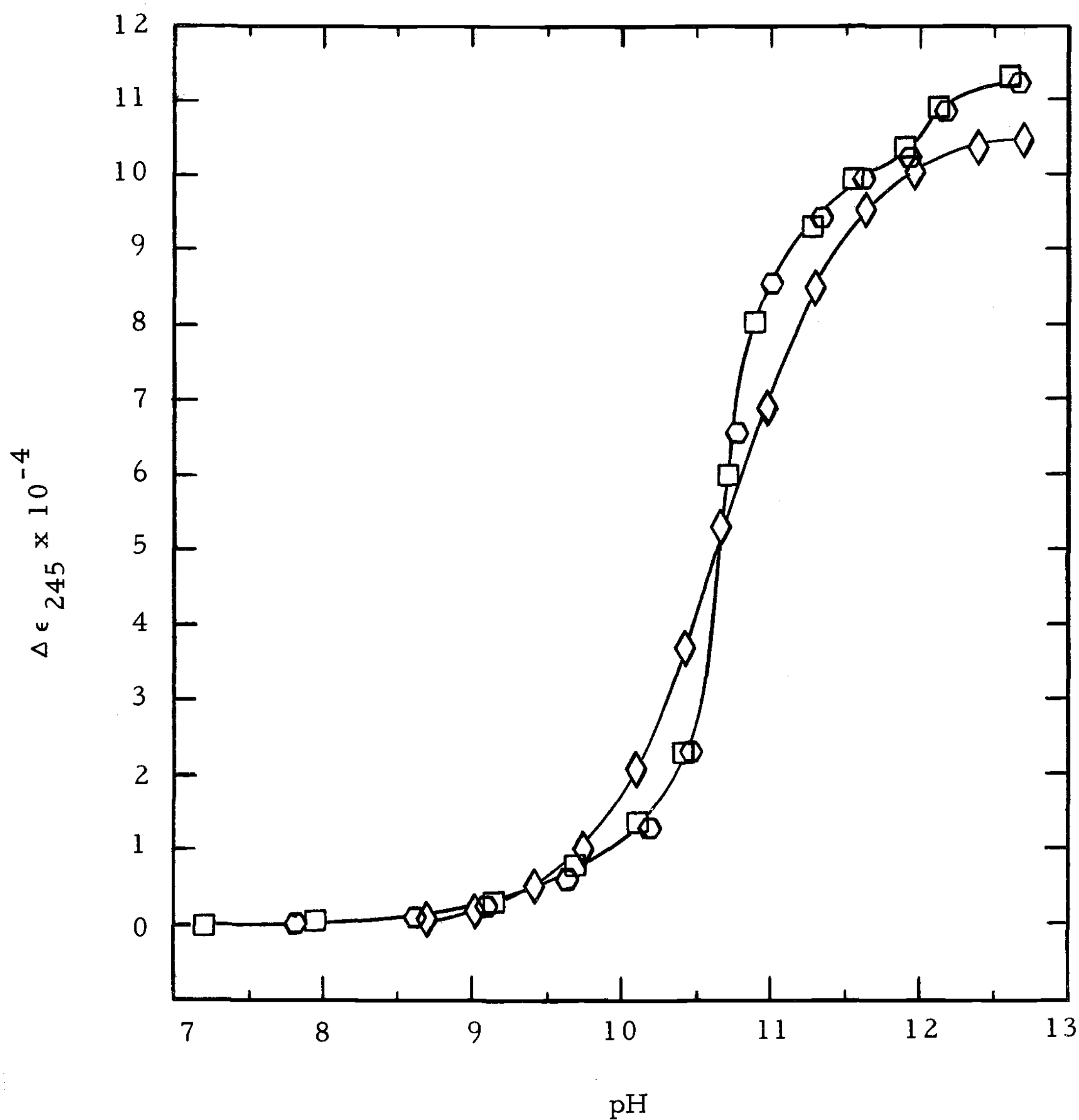

Figure 4. Spectrophotometric titration at 245 mμ of the tyrosine residues of RNase T_1 in 0.20 M KCl/0.001 M sodium phosphate at 25°. Forward titration at the indicated protein concentration: (□), 0.70 x 10^{-5} M; (△), 1.38 x 10^{-5} M. Calculated curve for the titration of tyrosine with an apparent pK of 10.7: (◊).

Figures 3 and 4 also show that titration occurs in two steps, the first occurring between pH's 7-12 and the second above pH 12. Two models attempting to account for the break in the titration curve, taking into consideration that there are nine tyrosine residues in RNase T_1 (Takahashi, 1965), will be presented below.

The first model to be considered (Model A) is one in which eight tyrosine residues titrate in the first step with an apparent pK of 10.7, while the remaining residue titrates with an apparent pK > 12. For this model, the contribution of each tyrosine residue to the total change in molar extinction coefficient at 295 mμ is readily calculated as $\Delta\epsilon_{295} = (24.3 \times 10^3)/9 = 2.70 \times 10^3$. Using this value, the total $\Delta\epsilon_{295}$ of 21.8×10^3 for the first step corresponds to the titration of about 8.1 residues, whereas the value of 2.50×10^3 for the second step indicates the titration of 0.9 residues. Similar considerations at 245 mμ yield a $\Delta\epsilon_{245} = (112.0 \times 10^3)/9 = 12.4 \times 10^3$ per tyrosine residue. The total $\Delta\epsilon_{245}$ of 104.0×10^3 in the first step suggests titration of about 8.4 residues, whereas the value of 8.0×10^3 suggests titration of about 0.6 residues in the second step.

The second model to be considered, Model B, is one in which all nine tyrosine residues titrate in the first step with an apparent pK of 10.7, while the absorbance increase in the second step is attributed to light scattering contributions to the optical density.

In this case, the contribution of each tyrosine residue to the total $\Delta\epsilon_{295}$ is $(21.8 \times 10^3)/9 = 2.4 \times 10^3$, while $\Delta\epsilon_{245} = (104.0 \times 10^3)/9 = 11.5 \times 10^3$.

The above values of $\Delta\epsilon_{295}$ and $\Delta\epsilon_{245}$ for RNase T_1 may be compared to the corresponding values obtained for tyrosine and for other proteins. These are as follows: $\Delta\epsilon_{295}$ of 2.33×10^3 for tyrosine (Wetlaufer, 1962; Beaven and Holiday, 1952), 2.63×10^3 for ribonuclease A (Tanford _et al._, 1955), 2.43×10^3 for bovine serum albumin (Tanford and Roberts, 1952), and 2.54×10^3 for pepsinogen (Perlmann, 1964); and $\Delta\epsilon_{245}$ of 10.0×10^3 for tyrosine (Hermans, 1962), 10.1×10^3 for myoglobin (Hermans, 1962), and 12.5×10^3 for pepsinogen (Perlmann, 1964). It is readily seen that the values of $\Delta\epsilon_{295}$ and $\Delta\epsilon_{245}$ vary considerably from one protein to another. Thus, simply comparing these values with those obtained for RNase T_1 does not allow one to decide whether the titration of the tyrosine residues in this molecule is described by Model A or Model B. The following experiment was performed to help in this decision. A difference spectrum between RNase T_1 at pH 12.7 and pH 11.5 was obtained (Figure 5). If Model A were correct, one would expect the difference spectrum to have maxima at 295 and 245 mμ, and the value of $\Delta\epsilon_{295}$ should correspond to the difference between $\Delta\epsilon_{295}$ at pH 12.7 and 11.5 of Figure 3. Similar considerations apply to the value of $\Delta\epsilon_{245}$. As shown in Figure 5, there are

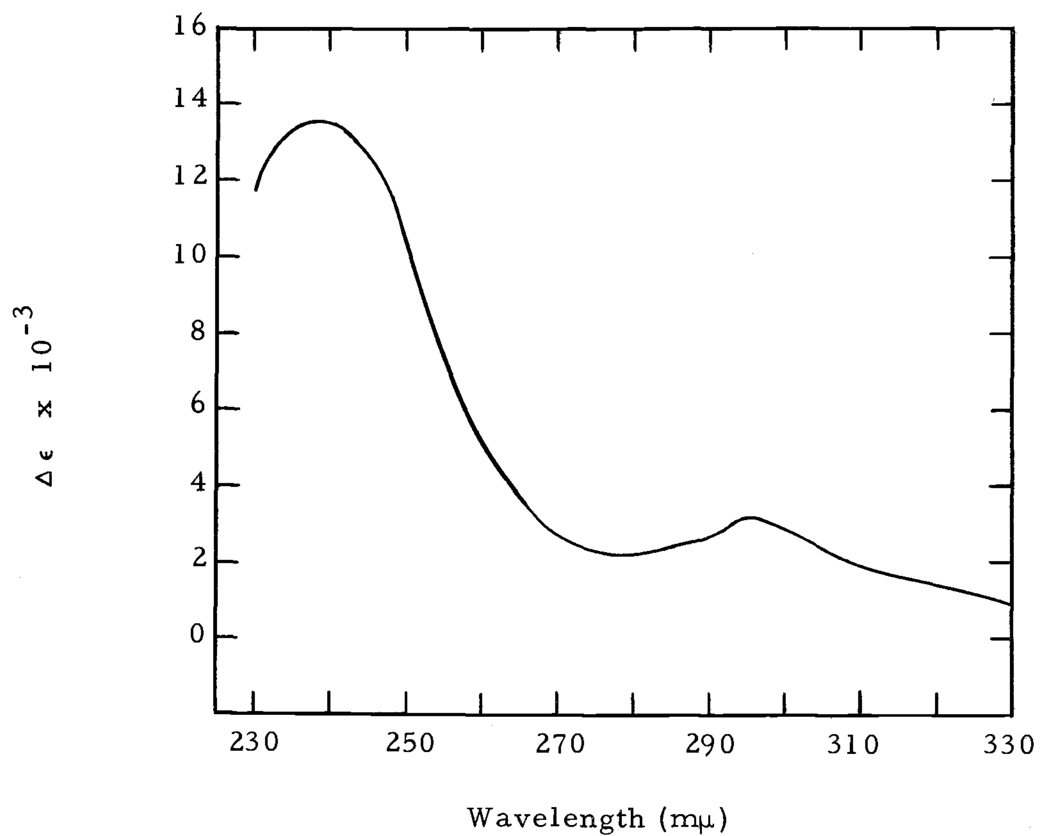

Figure 5. Difference spectrum of RNase T_1 at pH 12.6 (relative to pH 11.5). Protein concentration = 1.38×10^{-5} M.

maxima at 295 and 238 mμ, and the values of $\Delta\epsilon_{295}$ and $\Delta\epsilon_{245}$ are 3.1×10^3 and 13.0×10^3, respectively. However there is apparently no maximum at 245 mμ, and there is an unusually large difference in extinction coefficient in the region above 310 mμ. Similar absorbance above 310 mμ has been observed in the spectrophotometric titration of acid-denatured RNase A (Bigelow, 1961), and has been attributed to light scattering contributions. An interpretation of the difference spectrum of Figure 5, and whether or not it allows one to choose between Model A and Model B, will be presented in the Discussion section.

Above pH 10.2 the degree of ionization is time dependent. The values of $\Delta\epsilon_{295}$ and $\Delta\epsilon_{245}$ shown in the figures are those obtained after the optical density showed no further change with time (usually about 25 minutes).

Data on the reversibility of the titration are included in Figure 3. It was found that titration to pH 13 is irreversible. This is indicated by the fact that the $\Delta\epsilon_{295}$ does not return to zero upon back titration to neutral pH. The irreversibility of the titration was further reflected in a difference spectrum (relative to the reference solution used during titration) of this sample at neutral pH (curve 1 of Figure 6). This spectrum shows a large non-specific increase in difference extinction coefficient with decreasing wavelength (down to 305 mμ), presumably caused by light scattering from RNase T_1 molecules

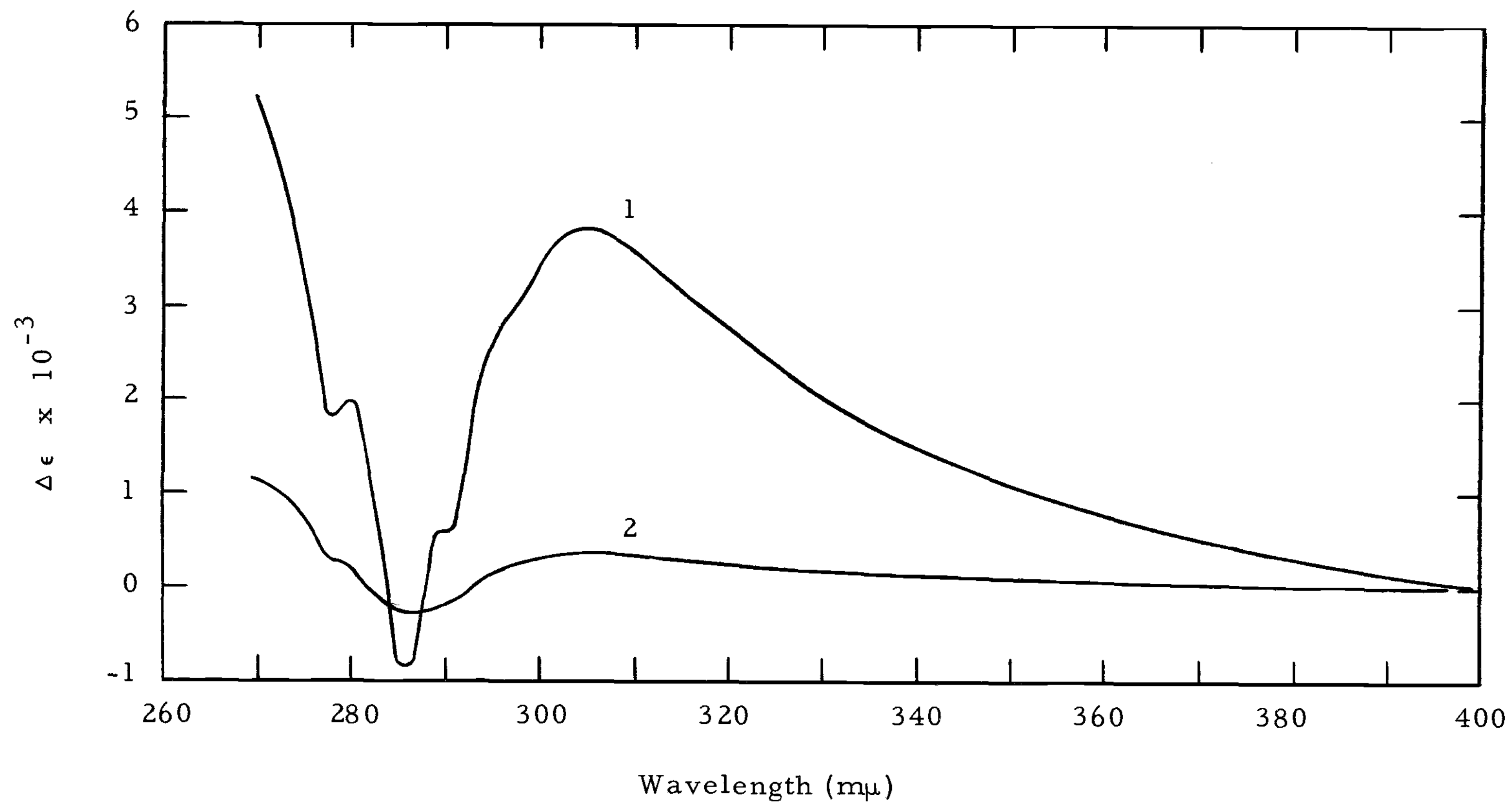

Figure 6. Difference spectra of RNase T_1 obtained after back titration to neutral pH. Curve 1: back titration from pH 13. Curve 2: back titration from pH 11.

which have aggregated at high pH. The spectrum also shows minima at 290, 286, and 279 mμ, which can be explained in the following way. It has been shown that protein denaturation produced by changes in temperature, pH, solvent composition, etc., is reflected as changes in spectra and difference spectra (Herskovits, 1967). In particular, the transfer of tyrosine and tryptophan residues from the non-polar environment in the "inside" of a protein to the more polar environment of the solvent results in generation of a difference spectrum characteristic of such transfer. The difference spectrum for the transfer of tyrosine to a more polar environment shows minima at 286-288 mμ and 278-281 mμ, while that for the transfer of tryptophan shows minima at 292-294 mμ and 281-284 mμ (Herskovits, 1967). The presence of minima at 290, 286, and 279 mμ in curve 1 of Figure 6 thus indicates that there are tyrosine and tryptophan residues exposed to solvent which are not so exposed in native RNase T_1. Similar effects have been observed in a study of alkali-denatured RNase A (Bigelow, 1961). Titration to pH 11, on the other hand, is more nearly reversible. Curve 2 of Figure 6 indicates the presence of only a very small amount of turbidity after back titration and only small differences in extinction coefficient.

The first stage of the forward titration is characterized by a very sharp dependence of the degree of ionization on pH. This is particularly obvious when the titration curves of Figures 3 and 4

are compared to the calculated curves for the titration of tyrosine with an apparent pK of 10.7.

Results of the spectrophotometric titration of RNase T_1 in 8 M urea-0.20 M KCl are shown in Figure 7. The tyrosine residues titrate in one group with an apparent pK of 10.9. This behavior is similar to that of RNase A in 8 M urea (ionic strength = 0.1), where all tyrosine residues titrate in one class with an apparent pK of 10.9, as indicated in Figure 1 of the paper of Blumenfeld and Levy (1958). The total $\Delta\epsilon_{295}$ of 23.6×10^3 represents a value of 2.62 $\times 10^3$ per tyrosine residue, contrasted to 2.55×10^3 for the tyrosine residues of RNase A in 8 M urea (Blumenfeld and Levy, 1958). Figure 7 also shows, for purposes of comparison, a calculated curve for the ionization of nine tyrosine residues with a pK of 10.9. The curve obtained in 8 M urea is very similar to the calculated curve; therefore, the tyrosine residues appear to be normalized in this solvent.

Acetylation of the Tyrosine Residues of RNase T_1

Results of the reaction of N-acetylimidazole with the tyrosine residues of RNase T_1 are shown in Figure 8. The number of tyrosine residues acetylated is an increasing function of acetylimidazole concentration, at least in the range of concentrations studied here. Acetylation of other proteins has indicated that a 60-180 fold molar

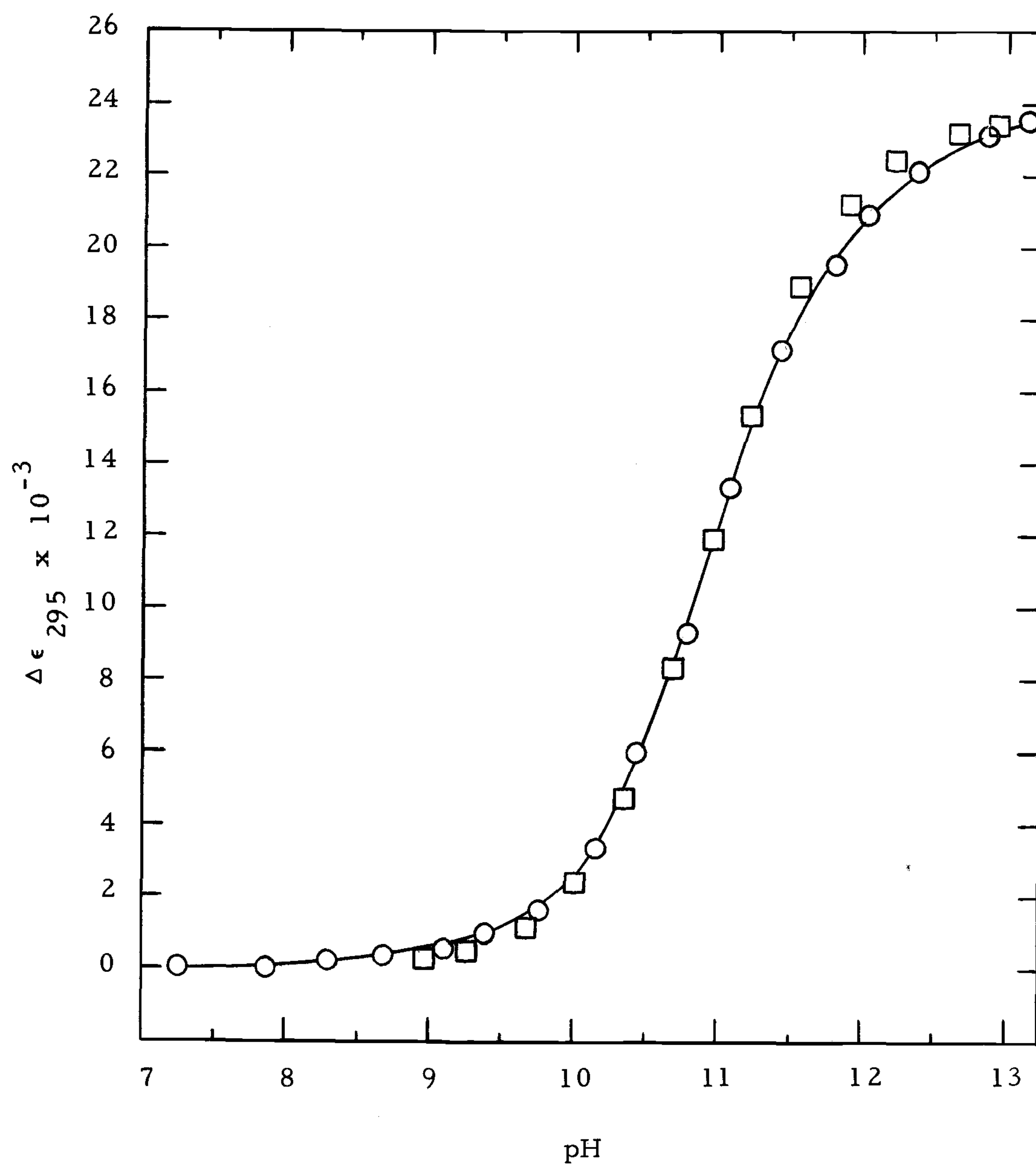

Figure 7. Spectrophotometric titration of the tyrosine residues of RNase T_1 in 8 M urea-0.20 M KCl/0.001 M sodium phosphate at 25°. Forward titration at a protein concentration of 1.88×10^{-5} M: (○). Calculated curve for the titration of 9 tyrosine residues with an apparent pK of 10.9: (□).

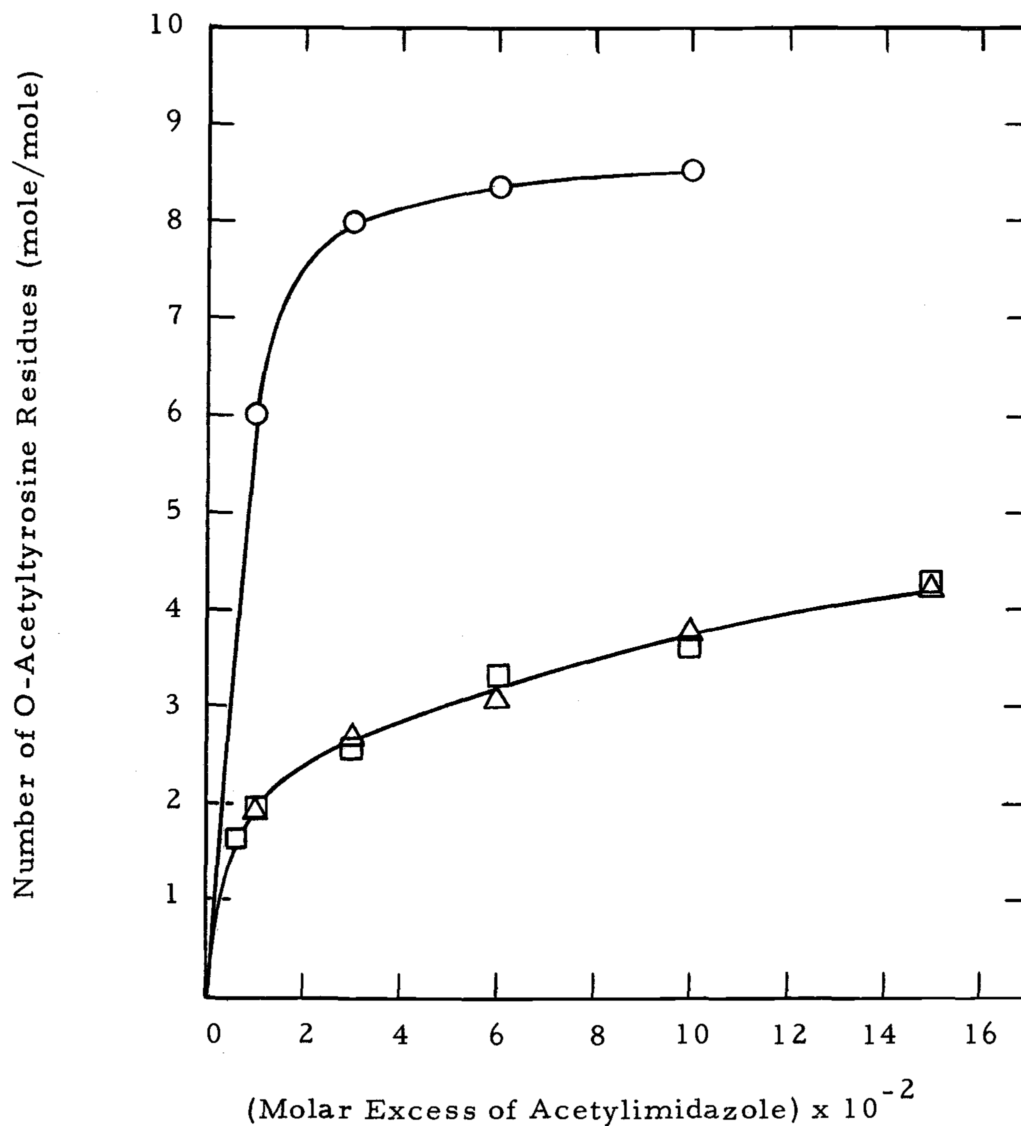

Figure 8. Reaction of the tyrosine residues of RNase T_1 with N-acetylimidazole at 25.0°. Acetylation in 0.02 M Veronal, pH 7.5: (△, □). Acetylation in 8 M urea-0.02 M Veronal, pH 7.5: (○).

excess of acetylimidazole to protein is sufficient to acetylate all of the completely exposed tyrosine residues and to produce maximal acetylation (Riordan, Wacker and Vallee, 1965). In the present study, only 1 to 2 tyrosine residues are acetylated in the range of 60-180 fold molar excess, but other tyrosine residues are acetylated at much higher concentrations of acetylating reagent. These results indicate that 1 to 2 of the tyrosine residues of RNase T_1 are completely exposed to solvent and that some react only under "forcing" conditions.

Also shown in Figure 8 are the results of acetylation in 8 M urea-0.02 M Veronal, pH 7.5. About eight of the nine tyrosine residues appear to be acetylated readily under these conditions.

Thermal Transition of RNase T_1

The difference spectrum of RNase T_1 at 64° and pH 7.41, relative to a solution of the same concentration and pH but at 19°, is shown in Figure 9. The minima at 278 and 285.5 mμ are, as mentioned above, characteristic of the exposure of tyrosine residues to a more polar environment, while the minimum of 290 mμ is characteristic of tryptophanyl exposure. The number of each of these residues exposed during thermal transition may be estimated by consideration of perturbation difference spectra of tyrosine and tryptophan. In 20% ethylene glycol, approximately 87% of the $\Delta\epsilon_{290}$ for a

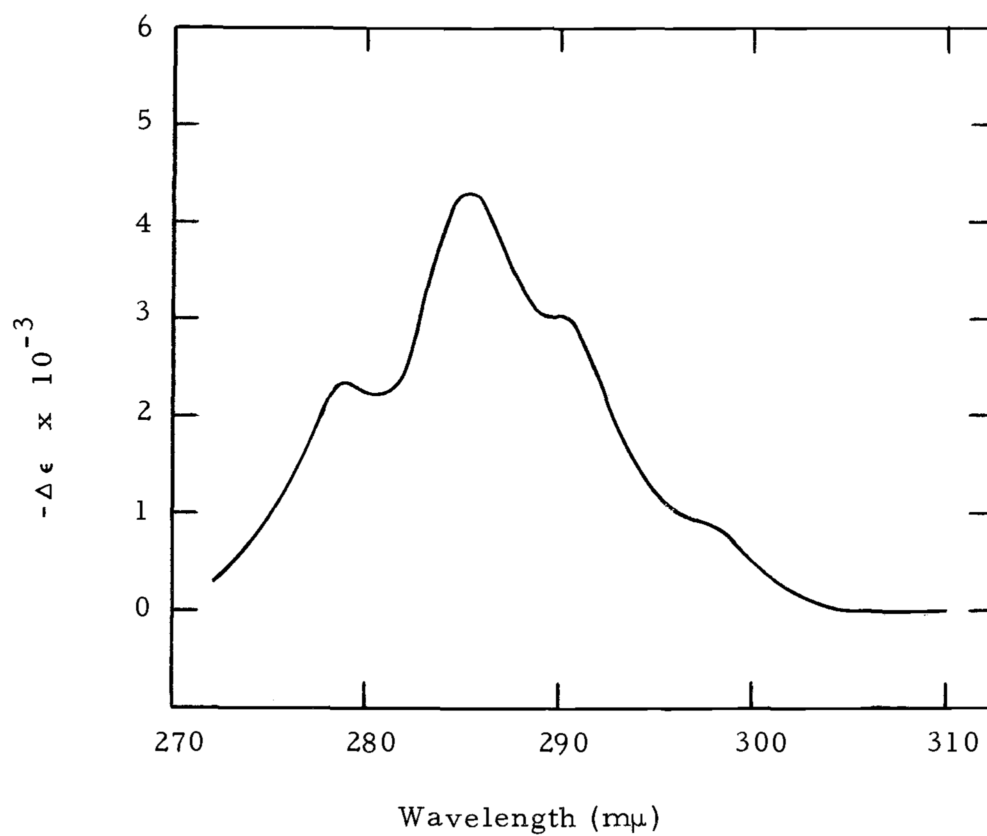

Figure 9. Difference spectrum of RNase T_1 at pH 7.41 and 64°. The reference solution was at pH 7.41 and 19°.

tyrosine-tryptophan mixture is due to tryptophan, while the ratio of $\Delta\epsilon_{290}$ to $\Delta\epsilon_{285.5}$ for tryptophan is 2.45, both estimated from Figure 3 of the paper by Donovan (1964). Assuming the applicability of the above to the temperature difference spectrum of RNase T_1, one can calculate that the contribution of tryptophan to the $\Delta\epsilon_{290}$ of Figure 9 is -3000 x 0.87 = -2610, and the contribution of tyrosine to the $\Delta\epsilon_{285.5}$ is -4290 - (-2610/2.45) = -3200. This value of $\Delta\epsilon_{290}$ for tryptophanyl exposure can be compared to the value of -2350, estimated from Figure 1 of Donovan (1964), for the exposure of the tryptophan residues of aldolase at low pH. On this basis, it can be concluded that the single tryptophan residue of RNase T_1 is exposed during thermal transition. Other evidence which indicates that the tryptophan residue in native RNase T_1 is not exposed to solvent is the lack of reactivity of this residue with N-bromosuccinimide (Takahashi, 1962). The number of tyrosine residues exposed during thermal transition is not readily determined since the contribution of each to the $\Delta\epsilon_{285.5}$ is not known. If one assumes that the change in molar extinction coefficient of about -1000 for the exposure of the tyrosine residues in RNase A (Bigelow, 1961) is a maximum, then at least three tyrosine residues are exposed during the thermal transition of RNase T_1.

Since the greatest change in OD in the difference spectrum is at 285.5 mμ, this wavelength was used to monitor the progress of

the thermal transition. The effect of pH on the midpoint of the thermal transition (T_m) of RNase T_1 is summarized in Table 1. Thermal transition at pH 1.56 and 2.95 was accompanied by extensive aggregation, and it was thus necessary to estimate the T_m at these pH's. This was done by assuming that the total $\Delta\epsilon_{285.5}$ due to chromophore exposure was the same under these conditions as at pH 4.53.

The transition at pH's 8.14 and 7.41 is completely reversible. Super-imposability of the forward and reverse thermal transition curves was the criterion for reversibility used in this study.

Shown in Figure 10 is the transition curve obtained at pH 8.14, and the effect of temperature on $\Delta\epsilon_N$, the extinction coefficient of the native state (Brandts, 1964). The limited data obtained above the transition indicate that $\Delta\epsilon_D$, the extinction coefficient of the denatured state, is independent of temperature, and this was assumed to be true for the calculations that follow.

The data of Figure 10 were used in the determination of ΔH^o for the thermal transition. The equilibrium constant was determined using the equation

$$K = \frac{\Delta\epsilon - \Delta\epsilon_N}{\Delta\epsilon_D - \Delta\epsilon} ,$$

Table 1. Effect of pH on the T_m[a] of RNase T_1.

pH	Buffer[b]	Tm[a]	Reversibility
1.56	HCl	46[c]	No
2.95	0.01 M glycine hydrochloride	52[c]	No
4.53	0.01 M sodium acetate	59.7	Partial
5.89	0.01 M sodium phosphate	56.3	Partial
7.41	0.01 M sodium phosphate	49.0	Complete
8.14	0.01 M sodium phosphate	46.6	Complete
9.52	0.01 M sodium bicarbonate	43.0	Partial

[a] T_m defined as the midpoint of the thermal transition.

[b] All buffers were adjusted to an ionic strength of 0.14 with KCl.

[c] Estimated as described in text.

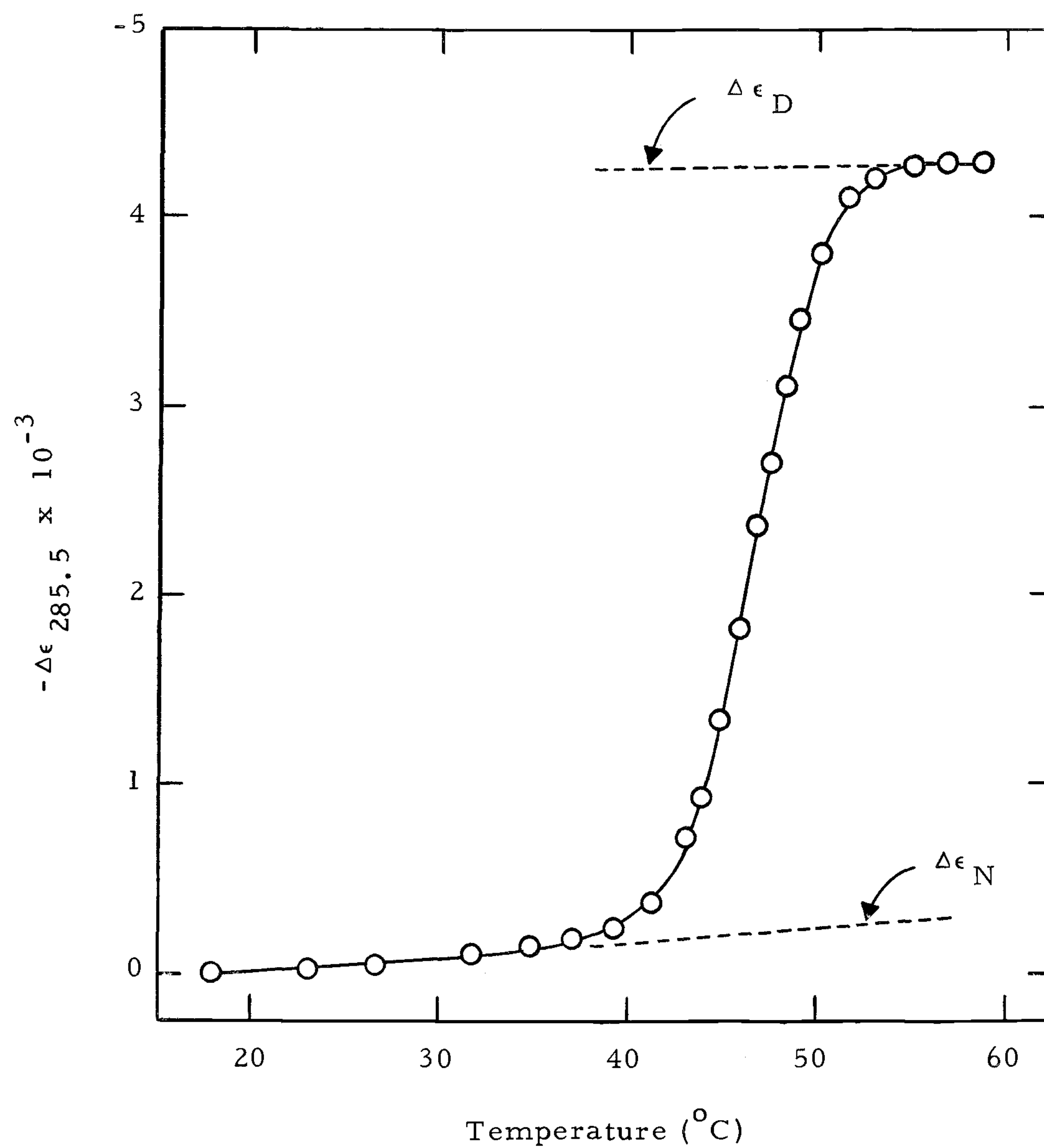

Figure 10. Thermal transition curve for RNase T_1 in 0.01 M sodium phosphate, pH 8.14 (ionic strength = 0.14).

as given by Brandts (1964). A plot of log K against the reciprocal of the absolute temperature is shown in Figure 11. The ΔH^o computed from the linear portion of the plot is +110 kcal/mole. This large positive value is indicative of a highly cooperative transition and is in the range of values of ΔH^o found for the thermal transition of other proteins (Brandts, 1967).

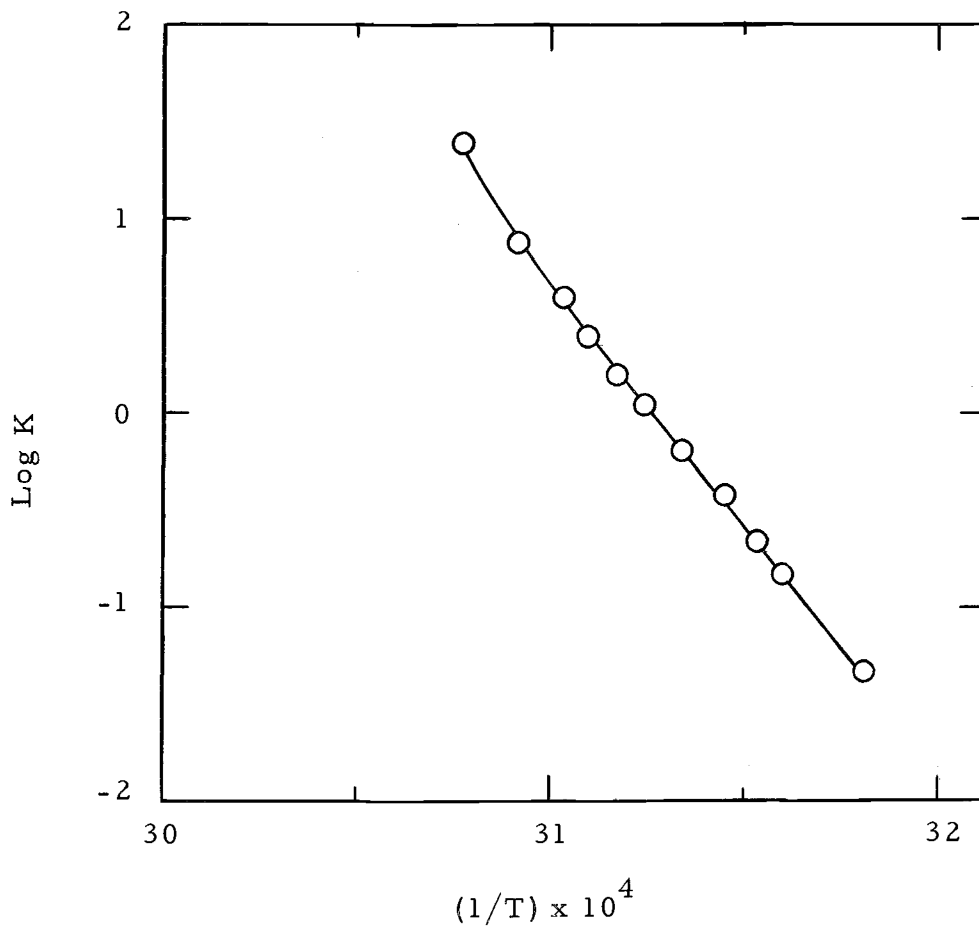

Figure 11. Plot of the logarithm of the equilibrium constant, calculated from the data of Figure 10, against the reciprocal of the absolute temperature.

DISCUSSION

The chemical and physical properties of RNase T_1 previously studied (reviewed by Egami et al. (1964) and Egami (1966)) are generally quite different from those of RNase A. The present work points to further differences in the properties of these proteins, and the following discussion will center on a comparison of these differences.

Spectrophotometric Titration

The spectrophotometric titration of RNase A reveals that three of the tyrosine residues titrate normally, whereas the others titrate cooperatively and irreversibly above pH 12 (Tanford et al., 1955). The residues titrating normally are considered to be completely exposed to solvent, while the others are referred to as being "buried" (or "partially buried") in the interior of the molecule. By contrast, a cooperative region in the spectrophotometric titration of RNase T_1 is reached at a much lower pH and is at least partially reversible. The cooperative titration of RNase T_1 may best be explained by the occurrence of a cooperative conformational change in the molecule after, or coincident with, the tiration of one to two tyrosine residues at pH's < 10.5 (Figures 3 and 4), thus exposing the other tyrosine residues to solvent. A study of the titration by circular dichroism

or optical rotatory dispersion would be useful in determining if such a conformational change occurs (Beychok, 1966). Unfortunately, a lack of the necessary equipment precluded such a study. The low pH, compared to that for RNase A, at which the cooperative titration occurs can be rationalized through consideration of the isoelectric points of RNase A and RNase T_1. RNase A is isoelectric above pH 9 (Tanford, 1962) and RNase T_1 at pH 2.9 (Takahashi, 1962). Thus, near pH 10, RNase T_1 would possess a highly negative charge whereas RNase A would be considerably less negatively charged. The introduction of more negative charges, by the ionization of the one to two tyrosine residues, could be imagined to decrease the conformational stability of the protein, thus inducing a conformational change. Of course, the possibility of the ionization of a specific tyrosine residue (perhaps directly involved in maintaining conformational stability) or of another amino acid residue, resulting in a conformational change cannot be eliminated. The partial reversibility of the cooperative titration is probably the result of the pH at which it is reached, that is, a pH low enough that irreversible structural modifications do not occur. This explanation gains support from the fact that a difference spectrum obtained after back titration from pH 11 showed little tyrosyl or tryptophanyl exposure to solvent, whereas the difference spectrum obtained after back titration from pH 13 showed such exposure (see Figure 6).

The values of $\Delta\epsilon_{295}$ and $\Delta\epsilon_{245}$ obtained from the difference spectrum of Figure 5 suggest the ionization of one tyrosine residue and, thus, that the spectrophotometric titration of the tyrosine residues of RNase T_1 is correctly described by Model A. Indeed, a similar difference spectrum (but limited to the region between 285 and 317 mμ) obtained in the spectrophotometric titration of acid-denatured RNase A has been interpreted (Bigelow, 1961) as showing, unequivocally, the titration of one tyrosine residue. However, because of the light scattering contributions to the difference spectra of both RNase T_1 and RNase A, such interpretations should be made only with caution. The difficulties involved in interpreting difference spectra which are complicated by light scattering effects have recently been emphasized by Olins, Olins and von Hippel (1967). In the vincinity of an absorption band an anomalous dispersion of the refractive index, and thus also of light scattering, is expected. For an isolated absorption band, anomalous scattering in the peak region would result in a positive contribution to the apparent absorbance on the long wavelength side of the peak and in a negative contribution on the short wavelength side. However, such anomalous scattering apparently does not contribute to the difference spectrum of Figure 5. From the above considerations, such a contribution would require that the peak of the strong absorption band at 245 mμ be displaced to longer wavelengths, rather than to shorter wavelengths as

observed. This observation tends to support Model A. It also suggests that scattering and absorbance are additive in this system. It should be emphasized that, in the event of anomalous scattering, it would not be possible, because of the various absorption bands involved in the difference spectra of proteins, to sort out the relative contributions of scattering and "true" absorbance to the observed optical densities. It is conceivable that anomalous light scattering effects can be large enough to partially or completely mask the absorbance due to the chromophore itself.

If, as suggested above, Model A is correct, it is interesting to speculate on the location of the tyrosine residue which titrates above pH 12. The speculation is based on the amino acid sequence of RNase T_1 (Takahashi, 1965) and a knowledge of the location of the abnormally ionizing tyrosine residues of RNase A. In this regard, the three abnormally ionizing tyrosine residues of RNase A are located next to, or one or two residues removed from, disulfide bridges (Li, Riehm and Scheraga, 1966). In RNase T_1 (see Figure 1), one tyrosine residue, amino acid number 4, is located in a loop provided by the disulfide bridge of cysteines number 2 and 10, with another tyrosine located at position 11. The tyrosine residue at position number 4 is also close to a disulfide bridge between cysteines 6 and 103. The remainder of the tyrosine residues are removed from the region of the smaller loop. It is possible that this

region is less accessible to solvent, and the ionization of the tyrosine residues located in this region could be influenced accordingly. The titration of the tyrosine residue at a pH $>$ 12 might be preceded by a β-elimination reaction of one or both of the disulfide bonds, thus destroying the structure imposed by them. This type of reaction has been shown to occur in RNase A at high pH (Bohak, 1964).

The apparent pK (10.9) for the titration of the tyrosine residues of RNase T_1 in 8 M urea-0.20 M KCl is high compared to the pK for normal tyrosyl ionization. However, because of the large negative charge that RNase T_1 possesses at high pH, an apparent pK of this order for tyrosyl ionization is not unexpected. That is, the net negative charge on the molecule would tend to suppress the ionization of the tyrosine residues. An alternative explanation has been offered by Blumenfeld and Levy (1958) for the high apparent pK (10.9) for tyrosyl ionization in RNase A in 8 M urea. These authors suggest that the phenolic hydroxyls are hydrogen bonded to urea, having the net result of raising the pK. Perhaps this effect also contributes to the high pK found for the tyrosine residues of RNase T_1. The intrinsic pK of the tyrosine residues of RNase T_1 in 8 M urea might be of use in assessing the contribution of each of the above possibilities to the apparent pK of these residues. Unfortunately, the limited availability of RNase T_1 precludes the determination of the intrinsic pK since large amounts of protein (of the order of a 0.5-3%

solution) are required for an electrometric titration (Nozaki and Tanford, 1967).

Acetylation of the Tyrosine Residues

The observations that two tyrosine residues of RNase T_1 are acetylated at low concentrations of acetylimidazole and titrate below pH 10.5 indicate that these residues are completely exposed to solvent. The other tyrosine residues are not as available, becoming reactive only at higher concentrations of acetylimidazole. In view of their titration behavior, the reason that other tyrosine residues become available is not clear. Perhaps the acetylation of two tyrosine residues leads to conformational instability, thus making others available. In the presence of 8 M urea, it appears that all but one of the tyrosine residues in RNase T_1 are readily acetylated.

It has been suggested that acetylimidazole is a suitable reagent for distinguishing between "free" and "buried" tyrosine residues, where the tyrosine residues are operationally defined as "free" if their pK is between 9.5 and 10.5 and "buried" if their pK is above 10.5 (Riordan *et al.*, 1965). The results of the present work suggest that tyrosine residues with pK's higher than those associated with "free" tyrosine residues may also be acetylated, albeit at rather high acetylimidazole concentrations. Another example of acetylation of tyrosine residues with pK's > 10.5 is found in ovomucoid. All of

the tyrosine residues of this protein titrate with an apparent pK of 12 (Donovan, 1967), but they are also acetylated with a 60 fold molar excess of acetylimidazole (Riordan *et al.*, 1965). These examples show that knowledge of the reactivity of the tyrosine residues toward acetylimidazole does not allow prediction of their titration behavior, and *vice versa*. Thus they serve to endorse the suggestion of Riordan *et al.* (1965) that more direct evidence is required in classifying tyrosine residues as "free" or "buried".

Thermal Transition Studies

The data on the thermal transition of RNase T_1 shows that, above about pH 4, the T_m is lowered as the pH is increased. This result can be contrasted with those for RNase A (Hermans and Scheraga, 1961) and chymotrypsinogen (Brandts, 1964), where the T_m for both of these proteins is lowered as the pH is decreased. A simple explanation for the effect of pH on the T_m of these proteins is the following. RNase A and chymotrypsinogen are isoelectric at a pH $>$ 9 (Tanford, 1962) and thus acquire a large positive charge at low pH. The conformational stability of these proteins at low pH would be reduced due to electrostatic repulsions between the positively charged groups, and, as a result, the T_m would be lowered. On the other hand, RNase T_1 is isoelectric at pH 2.9 (Takahashi, 1962) and the conformational stability of this protein would be

decreased by electrostatic repulsions between negative charges at high pH. The effect of pH on the T_m of RNase T_1 is thus opposite to that for the proteins with high isoelectric points listed above. Whether or not this explanation for the dependence of T_m on pH will hold for other proteins will depend in part on the extent to which the protein is negatively or positively charged at a given pH. Admittedly the above explanation is oversimplified, but it seems to satisfactorily explain the behavior of the above proteins.

PART II. SEDIMENTATION EQUILIBRIUM STUDIES

INTRODUCTION

Several criteria have been used to establish the homogeneity of ribonuclease T_1 (RNase T_1) prepared by the methods of Takahashi (1961, 1962), and Uchida (1965). Thus, the protein has been found to be homogeneous by (a) column chromatography, (b) paper and moving boundary electrophoresis, and (c) N-terminal amino acid analysis (Takahashi, 1961, 1962; Uchida, 1965). Homogeneity has been further indicated by the symmetry of the schlieren refractive index gradient curves obtained during the determination of the diffusion and sedimentation coefficients (Ui and Tarutani, 1961).

The above criteria, while sensitive to certain types of heterogeneity, are relatively insensitive to heterogeneity in molecular weight. By contrast, "high-speed" sedimentation equilibrium measurements can provide a great deal of information about such heterogeneity (Yphantis, 1964; Van Holde, 1967). Thus, measurements of this type would contribute further information concerning the homogeneity of RNase T_1 preparations. In addition, a comparison of the molecular weight of RNase T_1 obtained by sedimentation equilibrium measurements with that determined from the sedimentation and diffusion coefficients (Ui and Tarutani, 1961) and from the

known amino acid composition (Takahashi, 1965) would be of interest. Consequently, a sedimentation equilibrium study of ribonuclease T_1, prepared by the method of Uchida (1965), was undertaken.

Theory

The differential equation for sedimentation equilibrium of a homogeneous, ideal solute can be written (Van Holde and Baldwin, 1958) as

$$\frac{d \ln c(r)}{dr^2} = AM, \qquad (1)$$

where r is the distance (cm) from the axis of rotation, c(r) the concentration of solute at a point r in the cell, and M the molecular weight of the solute. The constant A is given by the equation

$$A = \frac{(1 - \bar{v}\rho)\,\omega^2}{2\,RT}, \qquad (2)$$

where $\bar{v}$ is the partial specific volume of the solute, ρ the density of the solution, ω the angular velocity of the rotor (radians/second), R the gas constant, and T the absolute temperature. Equation (1) can be used directly for the determination of M by plotting $\ln c(r)$ vs. r^2. The slope of this plot, at any point r in the cell, gives the weight average molecular weight at that point. Therefore, for a

homogeneous, thermodynamically ideal solute, the $\ell n\ c(r)$ vs. r^2 plot will be linear; and any departure from linearity is an indication of heterogeneity and/or thermodynamic non-ideality. Integration of equation (1) from the meniscus r_a to point r yields

$$\ell n \frac{c(r)}{c(a)} = AM(r^2 - r_a^2) \quad (3)$$

or

$$c(r) = c(a)e^{AM(r^2 - r_a^2)}, \quad (4)$$

where c(a) is the concentration of solute at the meniscus. Use of the above equations requires that c(r) or some quantity proportional to it be known at each point in the cell at equilibrium. One of the optical systems of the ultracentrifuge is used for this purpose, as described below.

At the present time, the Rayleigh interference optical system offers higher accuracy than either the absorption or schlieren optical systems (Richards and Schachman, 1959) and is therefore the system of choice. This optical system yields directly the difference in concentration between any point r and the meniscus. Thus, the absolute value of c(r) is readily calculated from interference data if c(a) is known, and a number of methods for determining the latter quantity have been proposed (Van Holde and Baldwin, 1958; Richards

and Schachman, 1963; LaBar, 1965).

Yphantis (1964) has avoided the determination of c(a) by operating the centrifuge at such a high speed that the solute concentration at the meniscus becomes essentially zero. This technique is variously referred to as the "meniscus depletion method," the "high-speed method," or the "Yphantis method". The high-speed method has become very popular for the following reasons: 1) because c(a) is essentially zero, the interference optical system gives c(r) directly, greatly simplifying both the calculations and experimental procedure, 2) measurements on dilute solutions are feasible, 3) analysis of paucidisperse solutions which, in many cases, permits determination of the smallest macromolecular component present, is possible, and 4) the time to equilibrium is relatively short. The parameter σ, given by the equation (Yphantis, 1964)

$$\sigma = \frac{\omega^2 M (1 - \bar{v}\rho)}{RT} , \qquad (5)$$

is useful in defining the experimental conditions to be used in the high-speed equilibrium experiment. Yphantis has shown that for a 3-mm solution column a σ of about 5 cm^{-2} is required to assure meniscus depletion. Also, with this σ and solution column height, moderate resolution and convenient equilibrium times are obtained.

The molecular weight range which one can study by the

high-speed method using a 3 mm solution column is limited primarily by the speed limitations of the cell components and by the requirement that σ be about 5 cm^{-2}. The rotor speed should be no greater than is required to maintain a negligible c(a) so that (a) buffer redistribution, leading to an extensive density gradient, is minimized and (b) the maximum range of solute concentration can be observed. Furthermore, a practical limit on the rotor speed is 45,000-50,000 rpm for the following reasons: 1) window distortion at high rotor speeds results in poor fringe quality, 2) extensive centerpiece distortion can result in permanent damage, and 3) distortion of the double-sector centerpiece increases the danger of interchannel leakage. Under the conditions of a rotor speed limited to 50,000 rpm and a 3 mm solution column, one can calculate from equation (5) that the lowest molecular weight which can be studied is 15,000 (assuming that $\bar{v}$ = 0.700 ml/g, ρ = 1.00 g/ml, and T = 298°K). The conditions to be used in the study of lower molecular weights by the high-speed method can be established by use of the equation

$$\frac{c(b)}{c(a)} = e^{AM(r_b^2 - r_a^2)} \qquad (6)$$

relating the concentration of solute, c(b), at the base of the solution column, r_b, to that at the meniscus. By choosing a c(a) which is

negligible compared to c(b), one can calculate for a given AM the length of the solution column needed to maintain the required ratio of c(b) to c(a). Then the equation

$$c_o = c(a) \left[\frac{e^{AM(r_b^2 - r_a^2)} - 1}{AM\,(r_b^2 - r_a^2)} \right], \qquad (7)$$

which is equation (4) integrated over the entire solution column and expresses the relationship of c(a) to the initial solute concentration c_o, is used to compute the maximum c_o which can be used and still maintain a negligible c(a).

MATERIALS AND METHODS

Materials

The materials used in these experiments have been described in Part I.

Methods

Partial Specific Volume

The partial specific volume ($\bar{v}$) of RNase T_1 was calculated by the method of Cohn and Edsall (1943) from the amino acid composition as given by Takahashi (1965). The calculations, shown in Table 2, yield a $\bar{v}$ of 0.699 ml/g, and this value was used in the following calculations.

Determination of the Conditions Required for a High-Speed Experiment

The molecular weight of 11,085 for RNase T_1 (Takahashi, 1965) is below the limit of molecular weights which can be studied by the high-speed method when using a 3 mm solution column and restricting the rotor speed to less than 50,000 rpm. Indeed, to be able to study RNase T_1 by this method with a σ of 5 cm^{-2} would require a rotor speed of about 58,000 rpm. Therefore, equations (6) and (7) were used to find a set of conditions which would permit

Table 2. Calculation of the Partial Specific Volume of RNase T_1.

Amino acid residue	No. of residues/ molecule[a]	W_i (% by weight of residue)	V_i (specific volume of residue)[b]	V_iW_i (% by volume of residue)
Aspartic acid	6	6.23	0.60	3.74
Threonine	6	5.47	0.70	3.83
Serine	15	11.78	0.63	7.42
Glutamic acid	6	6.98	0.66	4.61
Proline	4	3.50	0.76	2.66
Glycine	12	6.18	0.64	3.96
Alanine	7	4.48	0.74	3.32
Half cystine	4	3.68	0.63	2.32
Valine	8	7.15	0.86	6.15
Isoleucine	2	2.04	0.90	1.84
Leucine	3	3.06	0.90	2.75
Tyrosine	9	13.25	0.71	9.41
Phenylalanine	4	5.31	0.77	4.09
Lysine	1	1.15	0.82	0.94
Histidine	3	3.71	0.67	2.49
Arginine	1	1.40	0.70	0.98
Tryptophan	1	1.67	0.74	1.24
Asparagine	9	9.26	0.62	5.74
Glutamine	3	3.46	0.67	2.32
Total	104	$\Sigma W_i =$ 99.76		$\Sigma W_iV_i =$ 69.81

$$\Sigma W_iV_i/\Sigma W_i = \bar{v} = 0.699$$

[a] From Takahashi (1965)

[b] Data of Cohn and Edsall (1943)

lower rotor speeds to be used. The rotor speed was arbitrarily limited to 40,000 rpm, and a solute concentration of 0.01 fringes at the meniscus, when c(b) is 10 fringes, was required. The calculations, shown in Table 3, indicate that at least a 4.4 mm solution column is required to obtain a ratio of c(b) to c(a) of 1000. And to be able to realize a meniscus concentration of 0.01 fringes requires an initial solute concentration of less than 1.44 fringes (about 0.36 mg/ml). It should be emphasized that the calculated solution column length represents the minimum, and c_o the maximum, value that can be tolerated. To assure that the condition of a high-speed sedimentation equilibrium experiment is fulfilled (namely, that c(a) be essentially zero), the following experimental conditions were used in the present work: 1) a rotor speed of 44,000 rpm, 2) a solution column of approximately 6 mm, and 3) an initial solute concentration of about 0.30 mg/ml.

The time to attain sedimentation equilibrium (t_{eq}) was calculated by the method of Van Holde and Baldwin (1958) using the equation

$$t_{eq} = \frac{F(\alpha)(r_b - r_a)^2}{D} , \qquad (8)$$

which states that the time to attain equilibrium depends directly on the square of the length of the solution column, $(r_b - r_a)$, and

Table 3. Calculation of the Solution Column Length and Initial Solute Concentration.

$\bar{v} = 0.699$ ml/g [a]	$T = 298^{o}K$
$\rho = 1.0042$ g/ml [b]	$r_b = 7.10$ cm
$M = 11,085$ [c]	$c(b)/c(a) = 1000$ [d]
$R = 8.314 \times 10^{7} \frac{erg}{deg\text{-}mole}$	$c(a) = 0.01$ fringes [d]

$$A = \frac{(1 - \bar{v}\rho)\omega^2}{2RT}$$

$2RT = 49.55 \times 10^9$ $\qquad$ $(1 - \bar{v}\rho) = 0.3001$

$\omega = (2\pi/60)\,(rpm)$

$\omega = (2\pi/60)\,(44,000) = 4.189 \times 10^3$ $\qquad$ $\omega^2 = 17.55 \times 10^6$

$(1 - \bar{v}\rho)\omega^2 = 5.271 \times 10^6$

$A = (5.271 \times 10^6)/(49.55 \times 10^9)$

$A = 1.063 \times 10^{-4}$

$$r_a^2 = r_b^2 - \frac{1}{AM} \ln \frac{c(b)}{c(a)}$$

$r_b^2 = 50.41$

$AM = (1.1085 \times 10^4)\,(1.063 \times 10^{-4}) = 1.169$

$\ln\,[c(b)/c(a)] = 6.909$

$r_a^2 = 50.41 - (6.909/1.169) = 44.70$

$r_a = 6.68$

Continued on next page

Table 3 Continued.

$$\text{solution column length} = r_b - r_a$$
$$= 0.44 \text{ cm}$$
$$= (0.44)(10) = 4.4 \text{ mm}$$

$$c_o = c(a)\left[\frac{e^{AM(r_b^2 - r_a^2)} - 1}{AM(r_b^2 - r_a^2)}\right]$$

$AM(r_b^2 - r_a^2) = 6.909$

$[e^{AM(r_b^2 - r_a^2)} - 1]/[AM(r_b^2 - r_a^2)] = 144$

$c_o = (0.01)(144) = 1.44$ fringes

concentration (mg/ml) $\cong$ (no. of fringes) (1 mg/ml/4 fringes)

$c_o \cong (1.44)(1/4) \cong 0.36$ mg/ml

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[a] Calculated from the amino acid composition as described above.

[b] Obtained from the Handbook of Chemistry and Physics (Chemical Rubber Co.).

[c] From Takahashi (1965).

[d] As described in the text.

inversely on the diffusion coefficient, D. The quantity $F(\alpha)$ is defined as

$$F(\alpha) = -\frac{1}{\pi^2 U(\alpha)} \ln \left[\frac{\pi^2 U^2(\alpha)\epsilon}{4[1+\cosh(1/2\alpha)]} \right] \quad (9)$$

where

$$\alpha = \frac{2RT}{M(1-\bar{v}\rho)\omega^2(r_b^2 - r_a^2)} \quad (10)$$

$$U(\alpha) = 1 + (1/4\pi^2\alpha^2) \quad (11)$$

$$\epsilon = (\Delta C_{eq} - \Delta C_t)/\Delta C_{eq} \quad (12)$$

$$\Delta C = C_b - C_a. \quad (13)$$

Figure 1 of Van Holde and Baldwin (1958) is a plot of $F(\alpha)$ vs. α for $\epsilon = 0.001$, from which the value of $F(\alpha)$ used in the present work was obtained. A sample calculation of t_{eq} is shown in Table 4.

Sedimentation Equilibrium Experiments

Sedimentation equilibrium experiments were performed with a Spinco Model E ultracentrifuge equipped with an electronic speed control and the RTIC temperature control unit. The Rayleigh

Table 4. Calculation of the Time to Attain Sedimentation Equilibrium.

$\bar{v} = 0.699$ ml/g[a]	$R = 8.314 \times 10^{7}$ erg/deg-mole
$\rho = 1.0042$ g/ml[b]	$T = 298^{\circ}K$
$M = 11,085$[c]	$r_b = 7.10$ cm
$D = 12.0 \times 10^{-7}$ cm^2/sec[d]	$r_a = 6.50$ cm

$$a = \frac{2RT}{M(1-\bar{v}\rho)\omega^2 (r_b^2 - r_a^2)}$$

$2RT = 49.55 \times 10^{9}$	$(1 - \bar{v}\rho) = 0.3001$
$\omega = (2\pi/60)\,(rpm)$	$M(1 - \bar{v}\rho) = 3.327 \times 10^{3}$
$\omega = (2\pi/60)\,(48,000)$	$r_b^2 - r_a^2 = 8.16$

$\omega = 21.22 \times 10^{6}$

$M(1-\bar{v}\rho)\omega^2 (r_b^2 - r_a^2) = 5.76 \times 10^{11}$

$a = (49.55 \times 10^{9})/(5.76 \times 10^{11})$

$a = 0.086$

$F(a) \cong 0.21$[e]

$$t_{eq} = \frac{F(a)\,(r_b - r_a)^2}{D} = \frac{(0.21)\,(0.60)^2}{12.0 \times 10^{-7}}$$

$$= 6.29 \times 10^{4} \text{ seconds}$$

Continued on next page

Table 4 Continued.

$$t_{eq} = (6.29 \times 10^4)/(3.6 \times 10^3) = 17.5 \text{ hours}$$

[a]Calculated from the amino acid composition as described above.

[b]Obtained from the Handbook of Chemistry and Physics (Chemical Rubber Co.).

[c]From Takahashi (1965).

[d]From Ui and Tarutani (1961).

[e]Obtained from Figure 1 of Van Holde and Baldwin (1958).

interference optical system, focused by the method of Gropper (1964), was used in all experiments. A standard 12-mm-thick aluminum-filled epoxy double-sector centerpiece, sapphire windows, and the An-D rotor, were used throughout.

Kodak II G spectroscopic plates were used to record the Rayleigh interference patterns. Exposure times, using a Wratten 77A filter to isolate the 546 mμ Hg line, were of the order of 60-90 seconds. Development of the plates in Kodak D-19 developer for five minutes at 20° was followed by fixing in Kodak General Purpose Hardening Fixer.

Solutions of RNase T_1 were prepared by dialyzing an aqueous solution of the protein against a large volume of 0.10 M sodium acetate, pH 5.63. The retentate was diluted to the desired protein concentration with dialysate, and the dialysate was further used as the reference solvent. Precision syringes of 0.25 ml capacity were used to measure 0.22 ml (corresponding to about a 6 mm solution column) of solution and dialysate into the solution and reference sides, respectively. When the cell was viewed from the screw-ring end with the filling holes up, the solution side was the one on the right. Matched column heights were used to minimize the effects of buffer ion redistribution.

It has been shown that the time required to attain sedimentation equilibrium can be shortened by making appropriate speed changes

during the early hours of an experiment (Hexner, Radford, and Beams, 1961). This technique was used in the present work, and the following changes in rotor speed were made. The rotor was first accelerated to the operating speed of 44, 000 rpm and initial photographs taken (see below). Then the rotor speed was (a) increased to 52, 000 rpm for about 45 minutes, (b) decreased to 48, 000 rpm for about 165 minutes, (c) decreased to 40, 000 rpm for 25 minutes, and (d) finally increased to 44, 000 rpm for the remainder of the experiment. With this "overspeeding-underspeeding" routine, sedimentation equilibrium was attained in about 12 hours. A similar routine was used for experiments at 48, 000 rpm.

Photographs, with both monochromatic and achromatic light, were taken at the initial attainment of operating speed (i.e., before overspeeding) as a check on the base line. About 12 hours after the initial photographs, periodic exposures were taken, at about 2-4 hour intervals, until no further changes in net fringe displacement could be measured. Then a series of monochromatic photographs of different exposure times were taken and the best exposure was selected for measurement. Also, achromatic photographs were taken on the same plate adjacent to the monochromatic photographs.

The plates were measured with a Nikon Model 6C microcomparator. Alignment of the radial direction along the x-coordinate of the comparator was initially performed on an achromatic

photograph, making use of the zero-order fringe located in each of the reference holes. The plate was then shifted to an adjacent monochromatic photograph, and any adjustment in radial alignment was made. Then the y-coordinates of five black fringes were determined as a function of the x-coordinate. Measurements were made at 500-1000 μ intervals until a fringe displacement of about 30 μ, relative to the meniscus region, was observed. After this point, readings were taken at 200 μ intervals until the bottom of the solution column was reached or the fringes could no longer be resolved.

Analysis of the Data

For each of the five fringes read, the y-coordinates of the region up to about 30 μ displacement were plotted as a function of the x-coordinate. The zero-concentration reference level for each of the fringes, y_o, chosen from this graph, was subtracted from the fringe displacement, $y(r)$, of the corresponding fringe. The resulting values, $y(r) - y_o$, for each of the five fringes were averaged to yield the net fringe displacement $c(r)$. The x-coordinates were converted to distances in centimeters r from the axis of rotation by the equation

$$r = (x - x_{mid})/FMAG + 6.5000 \quad , \qquad (14)$$

where FMAG is the camera magnification factor and x_{mid} is the center of the measured frame in relative micrometer readings.

RESULTS

The Rayleigh interference patterns obtained at equilibrium showed no detectable net fringe displacement in the upper half of the solution column, indicating that the solute concentration at the meniscus is essentially zero. Furthermore, calculations similar to those of Table 3 show that for a 6 mm solution column and a rotor speed of 44,000 rpm, the ratio of c(b) to c(a) should be 1.1×10^5. If it is assumed that RNase T_1 is the smallest solute species present, measured net displacements of 8-10 fringes at the base of the solution column show that the maximum meniscus concentration is $10/(1 \times 10^5) = 9 \times 10^{-5}$ fringes. These results show that the conditions for the high-speed sedimentation equilibrium experiment have been fulfilled.

Three independent sedimentation equilibrium experiments were performed, two at 44,270 rpm and one at 48,240. Figure 12 shows a determination of zero-concentration levels from an experiment at 44,270 rpm. Using these zero-concentration levels, the reduced data shown in Table 5 were obtained. Because net fringe displacements of less than 100 μ are less reliable (Yphantis, 1964), only larger displacements were used. Figure 13 shows plots of the reduced data of Table 5 and of the experiment at 48,240 rpm. These plots show no detectable departures from linearity.

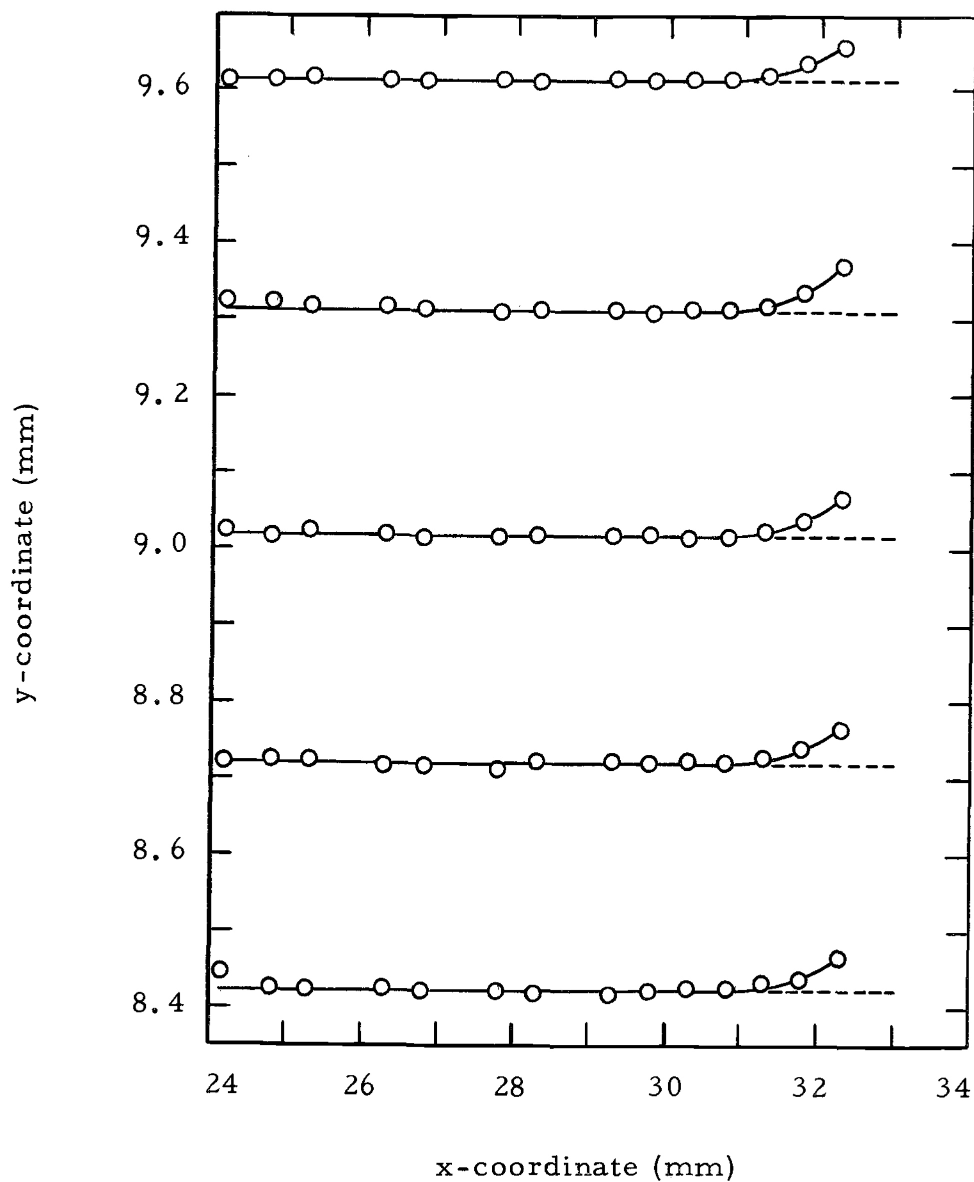

Figure 12. Plot of the y-coordinate against the x-coordinate, and the resulting zero-concentration levels, for an experiment at 44, 270 rpm.

Table 5. Reduced Data: Concentration, Natural Logarithm of the Concentration, and Square of the Radial Distance.[a]

r^2 (cm^2)	c(r) (x 10^1)	ℓn c(r)
49.2734	0.1308	-4.3367
49.4070	0.1638	-4.1117
49.5408	0.2054	-3.8854
49.6748	0.2444	-3.7115
49.8090	0.2970	-3.5166
49.9433	0.3640	-3.2985
50.0778	0.4406	-3.1222
50.2126	0.5326	-2.9326
50.3475	0.6604	-2.7175
50.4825	0.8062	-2.5180
50.6178	0.9824	-2.3203
50.7532	1.2044	-2.1166
50.8888	1.4746	-1.9142
51.0246	1.8058	-1.7116
51.1606	2.2008	-1.5138
51.2968	2.7296	-1.2984
51.4331	3.3402	-1.0966

X_{mid} = 2.2331

FMAG = 2.1026

[a] Calculated as described in the text.

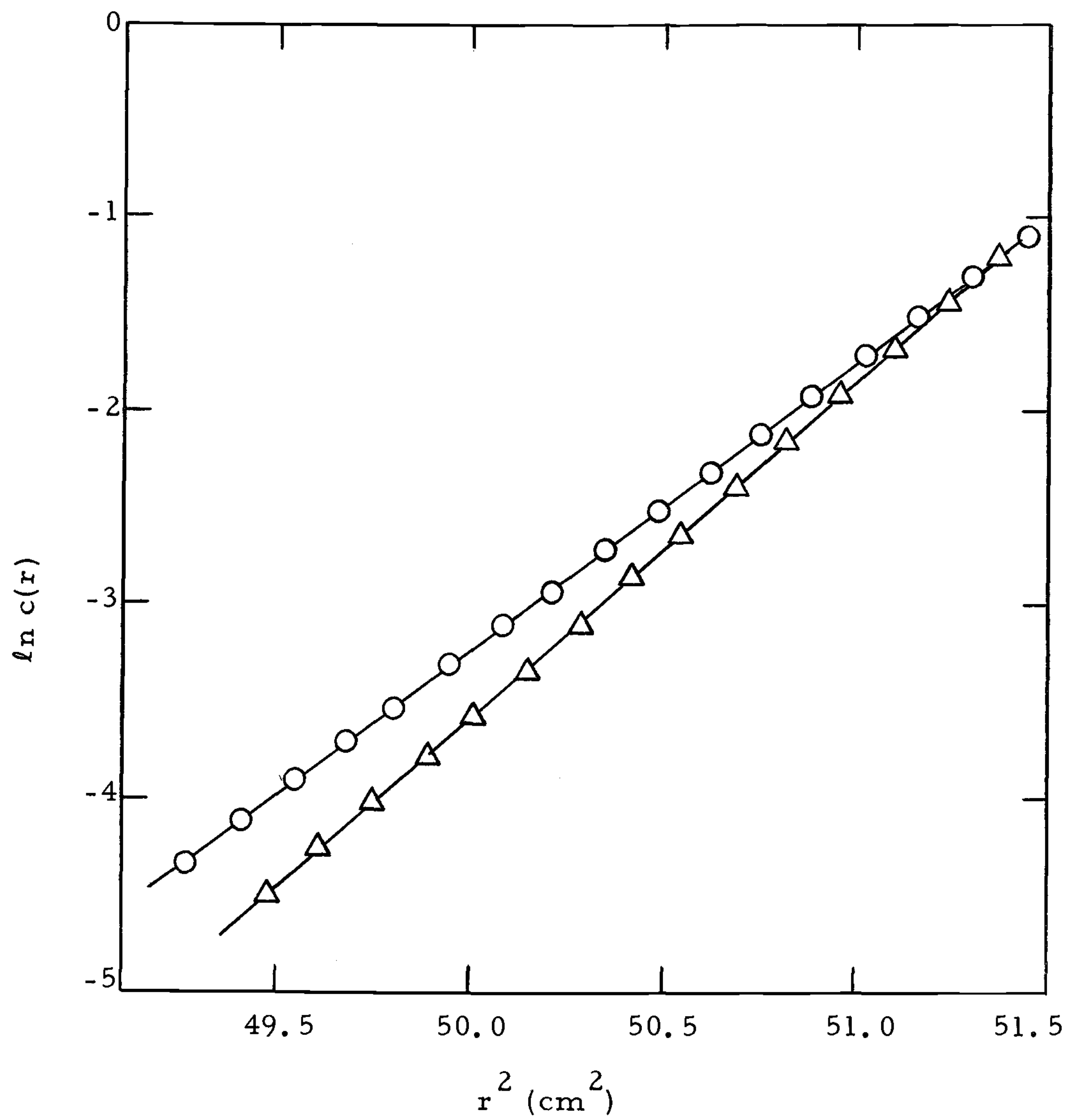

Figure 13. Plot of $\ell n\ c(r)$ against r^2. Experiment at : 44, 270 rpm, (○); 48, 240 rpm, (△).

The weight average molecular weight of RNase T_1 was calculated from the equation

$$\bar{M}_w = (\text{slope}) \frac{2RT}{(1 - \bar{v}\rho)\,\omega^2} . \tag{15}$$

The slope was determined from an unweighted linear least squares treatment of the reduced data (i.e., $\ell n\, c(r)$ and r^2). Molecular weights of 11, 271 and 11, 316 for the experiments at 44, 270 rpm, and 11, 200 for the experiment at 48, 240 rpm, were obtained.

DISCUSSION

Under the conditions of these experiments, this sample of RNase T_1 appears homogeneous and thermodynamically ideal. This conclusion is based on the fact that the $\ell n\ c(r)$ vs. r^2 plots are linear and, therefore, do not depart from equation (1) for a homogeneous, ideal solute. Thus, RNase T_1 does not undergo irreversible aggregation or self-association under these conditions. It should be emphasized that, for the experiments at 44, 270 rpm, measurements were obtained over the entire solution column. Therefore, the presence of dimer or higher aggregates would have been indicated by curvature of the $\ell n\ c(r)$ vs. r^2 plot near the base of the solution column. These results provide another demonstration of the homogeneity of RNase T_1 prepared by the method of Uchida (1965).

The above results should not be taken as evidence that RNase T_1 is monodisperse under other conditions. The reason for this is that the extent of irreversible aggregation and self-association of proteins has been shown, in some cases, to be markedly dependent on the pH and solute concentration (Yphantis, 1964; Adams, 1967).

The values of the molecular weight of RNase T_1 calculated from the sedimentation equilibrium experiments agree, within experimental error, with the theoretical value of 11, 085 based on the

known amino acid composition (Takahashi, 1965). However, they are all slightly higher than the theoretical value. There are at least two explanations that can account for this behavior. One is that the protein may bind buffer ions and thus increase its effective molecular weight. The other is that the partial specific volume calculated by the method of Cohn and Edsall (1943) may be incorrect. Since a 1% error in the partial specific volume results in about a 3% error in the calculated molecular weight, a very accurate value for this parameter is needed before one can draw conclusions regarding the small differences in molecular weight indicated above. The accuracy of calculated partial specific volumes is indicated by the work of McMeekin and Marshall (1952). These authors found that, for a number of proteins, the calculated values agree to within 1% of the values determined by conventional methods. Clearly, this level of accuracy is not sufficient to allow assessment of the above molecular weight differences. The large amounts of protein required for a conventional determination of the partial specific volume precluded such a study with RNase T_1. Therefore, in the absence of further information, nothing more can be said about buffer ion binding to RNase T_1.

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