

AN ABSTRACT OF THE THESIS OF

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Title PREPARATION AND PROPERTIES OF POLY-L-TYROSYL  
ACETAMIDINATED RIBONUCLEASE

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Polypeptidyl derivatives of bovine pancreatic ribonuclease (RNase) containing two or three tyrosine chains with an average of two to three residues per chain have been prepared. The effects of various perturbants such as neutral salts, ethanol, urea and guanidine hydrochloride on the thermal transition of the derivatives have been studied. The forces responsible for maintaining the native conformation of RNase appear to be unaffected by chemical modification. In the association-dissociation reaction of the polypeptidyl derivatives of RNase, hydrophobic interactions are strongly implicated.

Acetamidinated-RNase I was prepared by treating a RNase solution with excess acetimidate hydrochloride at pH 7.8 in 0.1 N  $\text{NaHCO}_3$  buffer containing  $\text{Na}_2\text{SO}_4$ . After removal of reagents by dialysis, polypeptidylation was carried out under the same conditions by reacting the acetamidinated-RNase derivative with excess N-carboxy-L-tyrosine anhydride. There were then obtained a soluble fraction (IA) and an insoluble fraction (IB) of polytyrosyl-acetamidinated RNase

(PT-Ac-RNase). IB could be dissolved in 0.0025 N HCl (IC). Fractions IA and IC were purified by gel filtration prior to experimental investigation. Another preparation (PT-Ac-RNase II) was carried out in phosphate buffer under comparable conditions as the preparation of PT-Ac-RNase I.

Analysis for added amino acid residues and for the number of sites amidinated or peptidylated indicated that IA contained three peptide chains of average chain length slightly less than three tyrosines. The fraction IC had 17 moles of tyrosine per mole of protein, giving an average chain length of about six tyrosines. PT-Ac-RNase IIA had 4 moles of tyrosine per mole of protein attached to two sites. The enzymatic activities of the PT-Ac-RNase derivatives were shown to be about 5 percent of the unmodified enzyme by two methods of assay.

The melting temperatures ( $T_m$ ) as determined by difference spectra at 287  $m\mu$  and 237  $m\mu$  for RNase, acetamidinated RNase and PT-Ac-RNase in 0.05 M salt at pH 2.1 were very similar and varied between 33° and 35°. But calculations for  $\Delta\epsilon_{287\ m\mu}$  and  $\Delta\epsilon_{237\ m\mu}$  revealed respective values of 1000 and 3000 for the PT-Ac-RNase derivatives.

The  $T_m$  of PT-Ac-RNase IA was shown to be dependent on pH, urea, ethanol and guanidine hydrochloride. Lowering pH or increasing the concentration of perturbant would shift the  $T_m$  to lower temperature. However, the same observation has also been reported for native RNase.

PT-Ac-RNase IC became insoluble between pH 5 and pH 9, but IA was soluble throughout this pH range. Although turbidity formation was enhanced by increasing concentration of KCl, addition of guanidine hydrochloride could prevent turbidity formation. Increasing concentration of KCl in the PT-Ac-RNase II solution at pH 2.1 shifted the  $T_m$  to higher temperatures and could induce turbidity formation at 1.2 M concentration. A similar effect of increasing the  $T_m$  of PT-Ac-RNase II was observed with  $\text{CaCl}_2$  at pH 2.1. With 0.5 M KSCN precipitation of the protein derivative occurred. A higher enrichment of tyrosine as a result of peptidylation must account for these experimental observations.

Spectrophotometric titration of PT-Ac-RNase II revealed seven normal and three abnormal tyrosines, indicating that the tyrosine residues covalently attached to the surface of the protein molecule are titrated normally.

A thermodynamic treatment of the melting profiles of native RNase, Ac-RNase and PT-Ac-RNase derivatives in the presence of various concentrations of  $\text{CaCl}_2$  was carried out. The approximation of two-state transition was employed to estimate the various thermodynamic parameters. The increase in heat capacity due to addition of extra tyrosine residues in the PT-Ac-RNase derivatives could probably account for the striking curvature at both low and high temperatures in the van't Hoff plots. However, the

theory of gradual "unwinding" of the molecule cannot be excluded.

PREPARATION AND PROPERTIES OF POLY-L-TYROSYL  
ACETAMIDINATED RIBONUCLEASE

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To my dear wife and parents.

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# PREPARATION AND PROPERTIES OF POLY-L-TYROSYL ACETAMIDINATED RIBONUCLEASE

## I. INTRODUCTION

When the covalent bonds in polypeptide chains of proteins somewhat fix the interatomic distances and bond angles in the chain, there are numerous spatial arrangements of various atoms of the backbone of the polypeptide. But noncovalent interactions are responsible for the unique conformation that is required of the protein. These noncovalent interactions are composed of interactions of side-chains and side-chains, side-chains and the backbone of the polypeptide and the solvent and solute; they contribute to maintaining the native structure of a protein.

There is a considerable amount of chemical and physicochemical evidence to support the hypothesis that noncovalent interactions exist in proteins. For instance, Tanford and others (1955) reported that three out of six phenolic groups in the ribonuclease molecule could be titrated normally by the spectrophotometric method. It was suggested that two types of  $pK$  corresponding to two types of ionization could explain this observation. Potentiometric titration of chymotrypsinogen and of guanidinated chymotrypsinogen by Wilcox (1961) revealed that two of the phenolic groups were inaccessible to titration. Yanari and Bovey (1960) demonstrated a red shift in the tyrosine

spectra when the refractive index of the media was increased, particularly when tyrosine was transferred from an aqueous medium to a hydrocarbon medium. These experimental observations indicate the presence of noncovalent interactions surrounding perturbable chromophores in the protein tertiary structure. The culminating work on the hypothesis that noncovalent interactions are present in a protein molecule was described by Kendrew (1962). He demonstrated the existence of noncovalent interactions in myoglobin through X-ray diffraction studies.

Since 20 to 40 percent of the amino acid residues in most proteins possess nonpolar side-chains (Kauzmann, 1959), the importance of nonpolar interactions (hydrophobic bonds) may surpass that of other noncovalent interactions in the stabilization of the native conformation of proteins. Valuable information on nonpolar interactions has been obtained using synthetic polypeptides. Models such as homopolymers, copolymers and block polymers possessing nonpolar side-chains have been synthesized. Investigation on the conformational stability of these models by physicochemical methods has been very fruitful.

It has been found that in the homopolymers such as poly-L-lysine and poly-L-leucine, the  $\alpha$ -helical stability depends largely on the hydrophobic side-chain interactions (Fasman, 1962; Gratzner and Doty, 1963; Bixon, et al., 1963). In a copolymer of L-glutamic and L-leucine, the  $\alpha$ -helical

stability seems to withstand a higher temperature than a homopolymer of L-glutamic. This  $\alpha$ -helical stability with increasing temperature has been attributed to the leucyl-leucyl hydrophobic interactions. A most recent work published by Sage and Fasman (1966) deals with a copolymer of L-glutamic and L-phenylalanine. A similar observation of the helical stability with increasing temperature again strongly suggests the importance of hydrophobic interactions between the aromatic rings of phenylalanine.

In most globular proteins, the forces that have been implicated in the stabilization of the secondary-tertiary structure of proteins are charge-charge interactions, hydrogen bonds, and hydrophobic bonds. Among these non-covalent interactions, hydrophobic bonds have been recognized to play a prime role in the stabilization of the native conformation. Even in proteins that are known to have subunit structures, hydrophobic bonds have been attributed to preserving the integrity of the quaternary structure, though other forces may be just as or more important. For instance, human carbon monoxide-hemoglobin can be dissociated into two symmetrical subunits in the presence of 3 M NaCl while ethanol or n-propanol, a good solubilizing agent for the hydrophobic side-chains, does not affect the quaternary structure (Tanford, 1964). The ability of the inorganic salt to dissociate the hemoglobin molecule suggests that hydrophobic groups are not involved. However,

in other proteins that exhibit subunit structures hydrophobic interactions are suggested. The polymerization of protein subunits of tobacco mosaic virus provides a suitable example. The polymerization process was shown to be endothermic and entropy-driven (Laufer, 1964). The suggestion was made that strong hydrophobic or electrostatic interactions caused release of "protein-bound" water molecules and therefore, spontaneous polymerization was resulted. More convincing evidence on hydrophobic interactions can be obtained using polypeptidyl proteins since electrostatic interactions can be eliminated from consideration.

A polypeptidyl protein is prepared by reacting N-carboxy-amino-acid anhydrides with a protein which acts as a multi-functional initiator. The polypeptidylation reaction in which the  $\alpha$ - or  $\epsilon$ -amino groups of proteins serve as initiators, usually takes place under mild conditions and therefore, as a rule, the modified proteins still maintain the structural features of the native macromolecules, though some other properties like the enzymic activity may be affected (Katchalski, et al., 1964). For instance, in the study of activation of polyglycyl chymotrypsin, Konigsberg (1956) found that at a high concentration of trypsin, the rate and extent of activation in the modified enzyme was not different from those of the unmodified enzyme. But at a low concentration of trypsin, the rate of activation

was slower in polyglycyl chymotrypsin. With polyvalyl derivatives of chymotrypsin, the enzymic activity was found to be only 56 percent of the activity of the unmodified enzyme when 50 moles of valine were attached per mole of chymotrypsin (Stracher and Becker, 1959). Two other interesting features were found by Glazer and others (1962) in the polytyrosyl trypsin derivatives. The modified protein was sparingly soluble between pH 5 and pH 9 and was found to be more resistant to autolysis than the unmodified enzyme.

Although apparent changes in some properties have been cited with the polypeptidyl proteins, the structural features have not been greatly affected on the basis of some physicochemical measurements. It is, indeed, of utmost importance that the structural features of the native protein be maintained so that the observable changes in properties can be attributed solely to the attached peptidyl chains and not to the disruption of the integrity of the native protein. Epstein and others (1962) found that no differences could be detected in the poly-DL-alanyl trypsin derivatives and the unmodified trypsin in ultracentrifuge studies. Spectrophotometric and optical rotation measurements on the polyalanyl derivatives of ribonuclease did not indicate any differences in the structural features as a result of polypeptidylation (Anfinsen, Sela and Cooke, 1962). With polyvalyl ribonuclease, Becker (1962) could

not detect any conformational changes on the basis of optical rotatory dispersion studies between the polypeptidyl derivative and the native enzyme. However, he observed a reversible aggregation-dissociation reaction as a function of temperature with the polyvalyl ribonuclease derivatives and with chymotrypsin that was peptidylated with amino acids having hydrophobic side-chains. Upon comparing the physical properties of polyglycyl, polyalanyl, polyvalyl, polyisoleucyl, and poly-ter-leucyl derivatives of chymotrypsin, Becker (1962) concluded that the modified enzyme preparations aggregate probably through formation of intermolecular hydrophobic bonds. Interaction of side-chains that were afforded by the peptidyl chains could conceivably enhance aggregation by releasing the "bound" water molecules around the nonpolar groups, resulting in an observable turbidity.

It is suggested that a specific number of chains with proper chain lengths and in the proper configuration is responsible for the aggregation phenomenon that has just been described. But in the polypeptidyl proteins that have thus far been investigated, the aggregation phenomenon cannot be attributed to a limited and specific peptidyl chains, because there are too many random chains attached to the protein molecule. Much more valuable information could be derived from a protein derivative with only two or three peptidyl chains attached, or even better, just one.

In those cases where the aggregation-dissociation phenomenon occurs, the interpretation of experimental data would be simplified. Such derivatives would provide valuable models in studies of hydrophobic interactions. To this end, attempts to prepare derivatives of ribonuclease containing as few peptidyl chains of hydrophobic nature as possible seemed in order.

There are two approaches to the problem of attaching a limited number of peptidyl chains onto the protein molecule. One of the approaches involves utilization of the rates of acylation of different groups in the polypeptidylation reaction. The kinetics are, however, dependent on conditions of the reaction: pH, salt effects, and concentrations of reactants. It has been found that the nine  $\epsilon$ -amino groups on the periphery of the ribonuclease molecule are very reactive toward the guanidinating reagent, O-methylisourea (Klee and Richards, 1957). Selective acylation therefore appears difficult, and this was not attempted.

A second approach involving first blocking the reactive amino groups of a protein with a reagent and then acylating the less reactive groups appear more feasible. The use of O-methylisourea as a guanidinating agent with chymotrypsinogen (Chervenka and Wilcox, 1956) and later with ribonuclease (Klee and Richards, 1957) suggested it as a possible reagent. But the conditions in which the

reaction was carried out appeared to be too harsh since the integrity of the protein after reaction appeared to be questionable. Hunter and Ludwig (1961), however, reported acetimidate hydrochloride as a very useful reagent. According to their kinetic studies, the amidination reaction appeared to be complete in a short course of time under mild conditions. Further experimentation using reduced ribonuclease demonstrated the usefulness of acetimidate hydrochloride as a reagent to block the reactive  $\epsilon$ - and  $\alpha$ -amino groups on a protein molecule. Wolsky and Singer (1963) also studied this reagent and reacted a bovine serum albumin solution with acetimidate hydrochloride under mild conditions to obtain a derivative useful in immunological studies.

Much information on the physical properties of ribonuclease together with the complete sequence of the primary structure have made the protein molecule a good choice for the limited polypeptidylation work. It has been discovered that in the three-group catalytic center, a lysine residue is involved (Anfinsen, 1962). Further examination of the extent of polypeptidylation revealed that two other  $\epsilon$ -amino groups of lysine may be protected by the buffer anion (Cooke, et al., 1963). These three  $\epsilon$ -amino groups of lysine have been identified to be in positions 7, 37, and 41. But upon extensive reaction with high concentration of N-carboxy amino acid anhydride, the lysine residues in

positions 7 and 37 can be peptidylated.

With this information regarding the reactivity of the lysine residues in the ribonuclease molecule, it appeared reasonable to react ribonuclease with acetimidate hydrochloride under such conditions that an acetamidinated derivative with most of the reactive  $\epsilon$ - and one  $\alpha$ -amino groups being acetamidinated. The acetamidinated derivative of ribonuclease could then be further reacted with N-carboxy-L-tyrosine anhydride to grow tyrosine chains on the somewhat unreactive  $\epsilon$ -amino groups such as those in the positions 7 and 37. The choice to grow tyrosine peptides onto the ribonuclease molecule is clear upon considering the chromophoric moiety of tyrosine. The chromophore permits spectral investigations of the derivative, and above all, permits ready estimation of the extent of peptidylation when the molar absorptivity of tyrosine is known.

There have been many experimental approaches attempted to define the complex pattern of noncovalent interactions in proteins. Among the approaches is the common technique of perturbing the protein environment. Since a particularly stable secondary-tertiary structure of a protein corresponds to one conformational structure with the minimum free energy, its geometrical arrangement of groups is greatly dependent on the solvent environment. Additions of perturbants to the protein solution may offset the balance of intramolecular interactions sufficiently to favor

rearranging the conformational structure. Commonly used perturbants have included temperature change, additions of salts, urea, guanidine hydrochloride and alcohols, or water-miscible organic solvents.

The thermal transition of ribonuclease was first discovered by Harrington and Schellman (1956) and has been intensively studied to shed light on the mechanism of action of some neutral salts on the conformational stability of ribonuclease molecule at neutral pH (von Hippel and Wong, 1965). It is generally accepted that the thermal transition involves a cooperative unfolding of at least a portion of the native ribonuclease molecule in dilute aqueous salt solutions. The use of the temperature of the mid-point of transition ( $T_m$ ) has helped understand the effect of different perturbants on the conformation stability as  $T_m$  can be progressively shifted up or down depending on the type of perturbant used.

Using optical rotation measurements, Foss and Schellman (1959) discovered that the  $T_m$  of ribonuclease could be shifted to lower temperatures with increasing concentrations of urea. Presumably the high concentration of urea disrupted some of the noncovalent bonds in such a way that little heat was further required to completely denature the protein molecule. Hermans and Scheraga (1961) found that the  $T_m$  of ribonuclease was a function of pH. Hypothetical models enabled them to make calculations to

account for the S-shaped curve obtained from plotting  $T_m$  against pH. They proposed that the tyrosyl groups were imbedded in the protein interior, associating or hydrogen bonding with the "abnormal" carboxyls. Alcohols which are dielectric constant depressants have been shown to shift the  $T_m$  of ribonuclease to lower temperatures. These observations imply that hydrophobic interactions may be crucially involved in the conformational stability of the protein.

In an attempt to understand the molecular mechanism of neutral salts and denaturants like urea, guanidine hydrochloride, alcohols and other water-miscible organic solvents on the conformational stability of ribonuclease at neutral pH, von Hippel and Wong (1965) investigated the shifts of  $T_m$  with increasing concentration of perturbants. The experimental data enabled them to propose an indirect mechanism of interaction between the additives and the water molecules around the protein. However, Ginsburg and Carroll (1965), after having studied the thermal transition of ribonuclease at pH 2.1 under very low anion concentration, proposed a specific binding of anion with the protein to account for the stability of ribonuclease in sulfate and phosphate buffers. Due to different conditions employed by the two groups of investigators, there can be no comparison and correlation of the experimental data.

In the work to be described, derivatives of

polytyrosyl acetamidinated RNase with two or three peptide chains have been prepared. Investigation of the thermal denaturation of these derivatives in the presence of neutral salts, urea, ethanol, and guanidine chloride should help understand what short-range forces of interactions are responsible for the conformational stability of RNase at low pH.

The results in this study further support the presence of noncovalent interactions in the globular protein ribonuclease. Among the noncovalent interactions that are implicated in the stabilization of the protein molecule are electrostatic interactions, hydrogen bonds and hydrophobic bonds. However, in the association-dissociation process, hydrophobic bonds are clearly demonstrated.

## II. MATERIALS AND METHODS

Tetrahydrofuran, reagent grade, was purchased from Matheson, Coleman and Bell. It was refluxed with lithium aluminum hydride overnight and distilled just prior to use. The distillate in the range of 65°-66° was found to be satisfactory.

Acetonitrile, reagent grade, was obtained from Matheson, Coleman and Bell. It was refluxed with  $P_2O_5$  for two hours and then distilled at 81°C just before use.

Petroleum ether, B.p. 30°-60°, reagent grade, was obtained from J. T. Baker. It was stored over  $CaCl_2$  for two weeks; and just prior to use, it was filtered over a Buchner funnel.

Ethyl ether, anhydrous, was J. T. Baker analyzed reagent. Previously unopened bottles were used without further purification.

Ethyl acetate, reagent grade, was obtained from Eastman Chemical Co. It was stored over calcium chloride for two weeks and was distilled just prior to use. The distillate obtained in the range of 76°-77° was found to be satisfactory.

Hydrogen chloride gas was generated by adding concentrated sulfuric acid dropwise into solid sodium chloride and was bubbled through concentrated sulfuric acid before passing into the reaction flask.

L-lysine mono hydrochloride, A grade, and L-tyrosine, A grade, were obtained from Calbiochem.

Phosgene gas was purchased from Matheson Co. It was passed through concentrated sulfuric acid before bubbling into the reaction flask.

Lithium aluminum hydride was purchased from Metal Hydrides, Inc.

Urea, reagent grade, was purchased from Sigma Chemical Co. and was used without recrystallization.

Calcium chloride, potassium thiocyanate, lithium chloride, phosphorous pentoxide, potassium chloride, sodium chloride, sodium bicarbonate, all analytically reagent grade, were obtained from J. T. Baker.

Potassium phosphates, mono basic and dibasic, sodium hydroxide were all reagent grades and purchased from Baker and Adamson.

Tris (hydroxymethyl) aminomethane, obtained from Sigma Chemical Co., was used to make necessary buffers.

Cation exchange resin, B10-REX70, minus 400 mesh, was obtained in the sodium form from Bio-Rad Laboratories. It is equivalent to IRC-50 cation exchange resin.

Sephadex G-150 was purchased from Pharmacia Co.

Guanidine hydrochloride (GuCl), reagent grade, was obtained from Matheson, Coleman and Bell Co. It was recrystallized from ethanol-benzene mixture and from methanol (Whitney and Tanford, 1965).

Ribonuclease, lot 124B-2610, recrystallized five times with activity of 75 Kunitz unit per mg, was purchased from Sigma Chemical Co. It was further chromatographed on an IRC-50 column according to the procedure of Crestfield, Stein and Moore (1963) to yield RNase A which was used throughout this work.

Sodium salt of cytidine-2',3'-cyclic phosphate was purchased from Sigma Chemical Co.

Yeast ribonucleic acid was purchased from P-L Biochemicals Inc.

Uranyl acetate was purchased from Mallinckrodt Chemical Co. A solution of 0.75 percent uranyl acetate in 25 percent perchloric acid was prepared.

pH Measurements. Either a Radiometer TTT-1 or a Corning pH Meter Model 12 was used for routine pH determinations. A combination electrode, purchased from Sargent Co. was adapted to the Corning pH Meter and used to determine the pH of solutions of high alkalinity.

Turbidity Measurement. Turbidity was estimated at 320  $m\mu$  in a Beckman DB recording spectrophotometer with 1-cm cells.

Ultraviolet Spectra. Ultraviolet spectra and difference spectra were measured in a Carey Model 14 recording spectrophotometer equipped with a thermostatable cell compartment. In the studies of temperature difference

spectra as used by Foss (1961), the cell compartment was externally connected to a thermostated bath whose temperature was controlled by a temperature programmer at a rate of  $1^{\circ}$  per 3 minutes, either increasing or decreasing. A copper strip to which were bonded in series five thermocouples of copper Constantine was attached to the side of the cuvette. The cuvette was insulated from the cuvette holder by thin cardboard. Another thermopile made of five thermocouples in series was immersed in an ice bath and used as a reference. Two wires, each bonded to one thermopile, were then connected to the Model 2CD Moseley X-Y recorder. A retransmitting potentiometer was attached to the Carey slide wire to permit direct correlation of optical densities on the Y axis of the Moseley recorder. The reference compartment of the spectrophotometer was kept at about  $25^{\circ}\text{C}$ . All 3-ml cuvettes were tightly stoppered with Teflon stoppers which effectively prevent evaporation.

The melting temperature,  $T_m$ , was defined as the temperature of the mid-point of the transition, and the size of the transition as the difference between the high and low temperature ends of the curves.

Ribonuclease Assay. The spectrophotometric method of Kalnitsky, et al. (1959) and the titrimetric method according to Josefsson and Lagerstedt (1962) were used to assay for the enzyme activity at  $25^{\circ}\text{C}$ . The spectrophotometric method involves using yeast ribonucleic acid. After the

ribonuclease has hydrolyzed the ribonucleic acid, the big fragments of ribonucleic acid are precipitated by uranyl acetate. An appropriate dilution of the supernatant is then read at 260  $m\mu$  to determine the amount of soluble nucleotides. The activity is thus computed with a known concentration of ribonuclease. The titrimetric method involves using cytidine-2',3'-cyclic phosphate as a substrate. As the substrate is hydrolyzed by ribonuclease at neutral pH, a proton is released. The amount of base consumed to keep the pH constant with a known concentration of enzyme is a measure of the rate of hydrolysis.

## III. EXPERIMENTAL

1. Preparation of Ethyl Acetimidate Hydrochloride

This was prepared according to the method of McElvain and Nelson (1942) with some modification. A mixture of absolute ethanol (29 ml) anhydrous acetonitrile (25 ml) and anhydrous ether (27 ml) was cooled at 4°C for an hour. Dry hydrogen chloride was bubbled into the solution until 10-15 gm had been added. The reaction flask was stoppered tightly and then kept in the freezer. After 48 hours, a white crystalline compound was filtered from the reaction mixture, washed with anhydrous ether and stored in vacuo over P<sub>2</sub>O<sub>5</sub> and KOH pellets. The compound decomposed at 110°-112° with gas evolution.

2. Preparation of N-Carboxy-L-Tyrosine Anhydride (Tyrosine NCA)

This was prepared according to the method of Berger, et al. (1958) with some improvement. L-tyrosine (5 gm) which had been dried in vacuo at 100° for one day, was suspended in anhydrous tetrahydrofuran (100 ml). Dry phosgene was bubbled into the suspension being stirred at 40°. After one hour, a clear solution was obtained. Dry nitrogen gas was then bubbled into the solution for two hours to remove excess phosgene. A turbid solution resulted. After removal of solvent on a rotatory

evaporator at 25°C, a white material, not very soluble in warm ethyl acetate (20 ml), was left in the flask. The insoluble material was filtered. Into the filtrate was added petroleum ether (100 ml) to precipitate tyrosine NCA. The crystalline compound was filtered and washed with petroleum ether. Both the insoluble material and the crystalline compound decomposed at 190°C; in the literature, 195°C.

### 3. Preparation of Acetamidinated-Ribonuclease (Ac-RNase)-I

Ribonuclease A (450 mg) was dissolved in distilled water (50 ml) containing sodium bicarbonate (550 mg) and sodium sulfate (200 mg). Acetimidate hydrochloride (400 mg) was dissolved in 4 N sodium hydroxide (0.5 ml). The latter was poured into the protein solution being stirred at 4°C. In the initial phase of reaction, drops of 4 N NaOH were added to the solution to maintain the pH at about 8. The pH of the solution, after four hours of reaction, was 7.8. The solution was then poured into Visking tubing No. 18 for exhaustive dialysis against distilled water. After dialysis, the solution from the bag was directly used in the polypeptidylation experiment.

### 4. Preparation of Acetamidinated-Ribonuclease (Ac-RNase)-II

A solution of ribonuclease A (500 mg) in 0.2 M

phosphate buffer (85 ml) was adjusted with 4 N NaOH to pH 8. Into the protein solution was added acetimidate hydrochloride (500 mg) in 1 ml of 4 N sodium hydroxide. The solution was stirred at pH 7.8, kept constant by adding drops of 4 N sodium hydroxide. After two hours of reaction at 4°C, the solution was emptied into a Visking tubing size No. 18 for exhaustive dialysis against distilled water. After dialysis, the solution from the bag was directly used in the polypeptidylation experiment.

5. Preparation of Polytyrosyl-Acetamidated-Ribonuclease (PT-Ac-RNase) -I

Into a solution of Ac-RNase I (300 mg in 40 ml) were introduced sodium bicarbonate (200 mg) and sodium sulfate (100 mg). Solid tyrosine NCA (100 mg) was then added to the protein solution being stirred at 4°C. After one day of reaction, a turbid solution resulted; and it was emptied into a cellophane tubing size No. 18 and dialyzed exhaustively. The suspension was centrifuged at 11,000 xg for one half hour. The supernatant was lyophilized and then stored in the freezer as was the precipitate.

6. Preparation of Polytyrosyl-Acetamidated-RNase (PT-Ac-RNase) -II

Into a solution of Ac-RNase II (360 mg in 80 ml) was added sufficient dipotassium hydrogen phosphate (0.70 gm) to bring the pH to 8.5. Solid tyrosine NCA (100 mg) was

then added into the solution being stirred at 4°C. After one day of reaction, the turbid solution was dialyzed against distilled water. The suspension was centrifuged to yield a supernatant fraction and a pellet. The precipitate was washed with distilled water and then centrifuged and stored in the freezer.

#### 7. Dinitrophenylation of Ac-RNase and of PT-Ac-RNase

About 2.0 ml aliquots containing 4 mg Ac-RNase I from the dialysis bag was directly used to determine the degree of acetamidination. 100 mg of sodium bicarbonate was dissolved in the protein solution, followed by the addition of 3.0 ml of 5% dinitrofluorobenzene in 100% ethanol. After the flask, wrapped with aluminum foil to exclude light, had been shaken mechanically at room temperature for four hours, a yellow precipitate was obtained. 6 N HCl was used to acidify the yellow suspension, which was then centrifuged in a clinical centrifuge to obtain the dinitrophenylated derivative. The yellow precipitate was washed with 95% ethanol and with ether to remove unreacted dinitrofluorobenzene and dinitrophenol.

This same procedure was followed to obtain dinitrophenylated derivatives of PT-Ac-RNase IA, Ac-RNase II and PT-Ac-RNase IIA.

## 8. Purification of PT-Ac-RNase through Sephadex G-150

PT-Ac-RNase I yielded supernatant fraction (IA) and an insoluble pellet IB. IB could be dissolved in 0.0025 N HCl to yield another fraction (IC). Fraction IA was applied to a column of Sephadex G-150 (1.8 x 42 cm) and then eluted with 0.0025 N HCl. A symmetrical peak emerged from the column. After thorough washing of the column with 0.0025 N HCl fraction IC was put on the top of the same Sephadex G-150 column and eluted with 0.0025 N HCl. Two peaks were obtained: a small peak emerged at about the void volume and the major peak followed right after. The major peak was used in the studies as preparation IA.

No filtering over Sephadex G-150 was done for PT-Ac-RNase IIA, IIB was found insoluble in 0.0025 N HCl.

## 9. Hydrolysis of Dinitrophenylated and Polypeptidyl Derivatives

The following procedure was employed for hydrolysis. A derivative in the range of 2 to 3 mg was introduced into a hydrolysis ampule, and it was followed by adding one ml of constant boiling HCl. After the ampule had been exchanged with nitrogen gas, it was sealed under vacuum and then lowered into a toluene bath boiling at 110°. Twenty-four hours of hydrolysis was sufficient for complete hydrolysis of the protein. After hydrolysis, the tip of the ampule was broken, the solvent evaporated, and the

hydrolysate taken up in citrate buffer. The amino acid content in the hydrolysate was analyzed by the Spinco Automated Amino Acid Analyzer (Spackman, Stein and Moore, 1958).

The  $\epsilon$ -DNP lysine was determined in a more precise system devised by Dr. A. H. Nishikawa in our laboratory. The system involves dissolving p-hydroxy-benzoate and benzoate respectively in the citrate buffer at two different pH values, which would cause elution of a symmetrical peak of  $\epsilon$ -DNP lysine.

## IV. RESULTS

1. Extent of Reaction of RNase with Reagents

The extent of reaction between acetimidate hydrochloride and ribonuclease could not be ascertained by determining the  $\epsilon$ -amidino lysine because the latter was partially hydrolyzed by 6 N HCl at 110°C for 24 hours to yield lysine and  $\epsilon$ -amidino lysine (Hunter and Ludwig, 1962; Wolsy and Singer, 1963). Treatment with 2,4-dinitrofluorobenzene, however, on the modified enzyme could help determine the availability of amino groups for further reaction such as polypeptidylation.

TABLE I. DEGREE OF ACETAMIDINATION AND POLYPEPTIDYLATION

	Moles $\epsilon$ -DNP-lysine per mole RNase	Moles tyrosine added per mole RNase
Ac-RNase IA	4.04	----
PT-Ac-RNase IA	0.92	7.48
PT-Ac-RNase IC	----	16.80
Ac-RNase IIA	2.28	----
PT-Ac-RNase IIA	0.64	3.70
(PT-Ac-RNase IIB gave tyrosine only with contamination of protein.)		

According to the results of dinitrophenylation shown in Table I, there were approximately three sites in Ac-RNase I available for tyrosination and two sites in Ac-RNase II. Although there is one  $\alpha$ -amino terminal group in

the native RNase, it is assumed to have been reacted with acetimidate hydrochloride which was present at one hundred fold excess per amino group of the enzyme. A parallel observation was reported by Anfinsen, Sela and Cooke (1962), who demonstrated that the  $\alpha$ -amino terminal group of native ribonuclease was tyrosinated with smallest amount of tyrosine NCA because of the higher intrinsic reactivity of the  $\alpha$ -amino group compared with the  $\epsilon$ -amino group. Hence, the average number of residues per polypeptidyl chain in the PT-Ac-RNase derivatives is between 2 and 2.6 moles of tyrosine per mole of enzyme.

## 2. Enzymic Acitivity

The enzymic activities of the modified proteins may provide some information on whether or not the lysine at position 41 or the native RNase has been reacted as this lysine is necessary for activity. The activities were assayed by two independent methods, and results are shown in Table II.

TABLE II. ENZYMIC ACTIVITY OF MODIFIED RNase

	Spectrophotometric assay at pH 5.0	Titrimetric assay at pH 7.0
Native RNase	100 %	100 %
PT-Ac-RNase IA	8.0%	3.9%
PT-Ac-RNase IC	Inactive	Inactive
Ac-RNase II	6.8%	3.1%
PT-Ac-RNase IIA	2.0%	2.2%

It was evident that Ac-RNase derivatives did not have much activity. Presumably amidination had taken place at the 41 residue of the RNase to cause extensive loss of activity. Although there was further decrease in the enzymic activity for PT-Ac-RNase, tyrosination may have affected the substrate binding site to bring about the further slight drop in activity.

### 3. Difference Spectra at 287 m $\mu$ and 235-7 m $\mu$

It has been established that the peak at 287 m $\mu$  in the difference spectrum is a result of perturbing the environment of tyrosyl chromophores on the protein either by changing the pH of the protein solution (Scheraga, 1957), or by treating the protein solution with urea or guanidine hydrochloride (Cha and Scheraga, 1960), or by denaturing the protein thermally (Schellman, 1955). A large peak in the difference spectrum between 230-240 m $\mu$  has also been observed when a denatured protein is compared to the native protein by one of the above methods. Perturbation of either the tyrosyl chromophores (Eisenberg and Edsall, 1963) or the peptide chromophores (Glazer and Smith, 1961) has been advanced to explain the existence of this peak. However, denaturation may perturb tyrosyl and peptide chromophores in a cooperative manner to give rise to the peak.

An attempt was made using the PT-Ac-RNase derivatives

to investigate the cause of the peak between 230 and 240  $m\mu$ . As for the two soluble PT-Ac-RNase preparations, the  $T_m$  was found the same at both wavelengths. The striking difference appears in the pronounced trough and maximum. In Figures 1(A), (B), and (C) are illustrated these findings. When the native RNase solution was used, the  $T_m$  was also shown to be the same at 287  $m\mu$  and at 237  $m\mu$ . But in the pretransition region (low temperature), the melting profile at 287  $m\mu$  ascends gradually while that at 237  $m\mu$  descends slowly to form a slight trough. As for the post-transition region, the 287  $m\mu$  line levels off but the 237  $m\mu$  shows a slight maximum. The Ac-RNase solution was also shown to have the same  $T_m$  at both wavelengths under identical conditions and also the same characteristics in the pre- and post-transition regions.

At present no conclusive information may be derived from the findings on the cause of the low UV peak. The factors responsible for the production of the 287  $m\mu$  peak, however, are probably the same as those for the 235-7  $m\mu$  peak on the grounds that PT-Ac-RNase was shown to have the same  $T_m$  at both wavelengths. Hence, throughout this work the interchangeable use of both wavelengths to determine the  $T_m$  values is justified.

Although the difference spectra at 235  $m\mu$  have been used in melting experiments by Scott and Scheraga (1963), no illustrated melting curves are available so that a

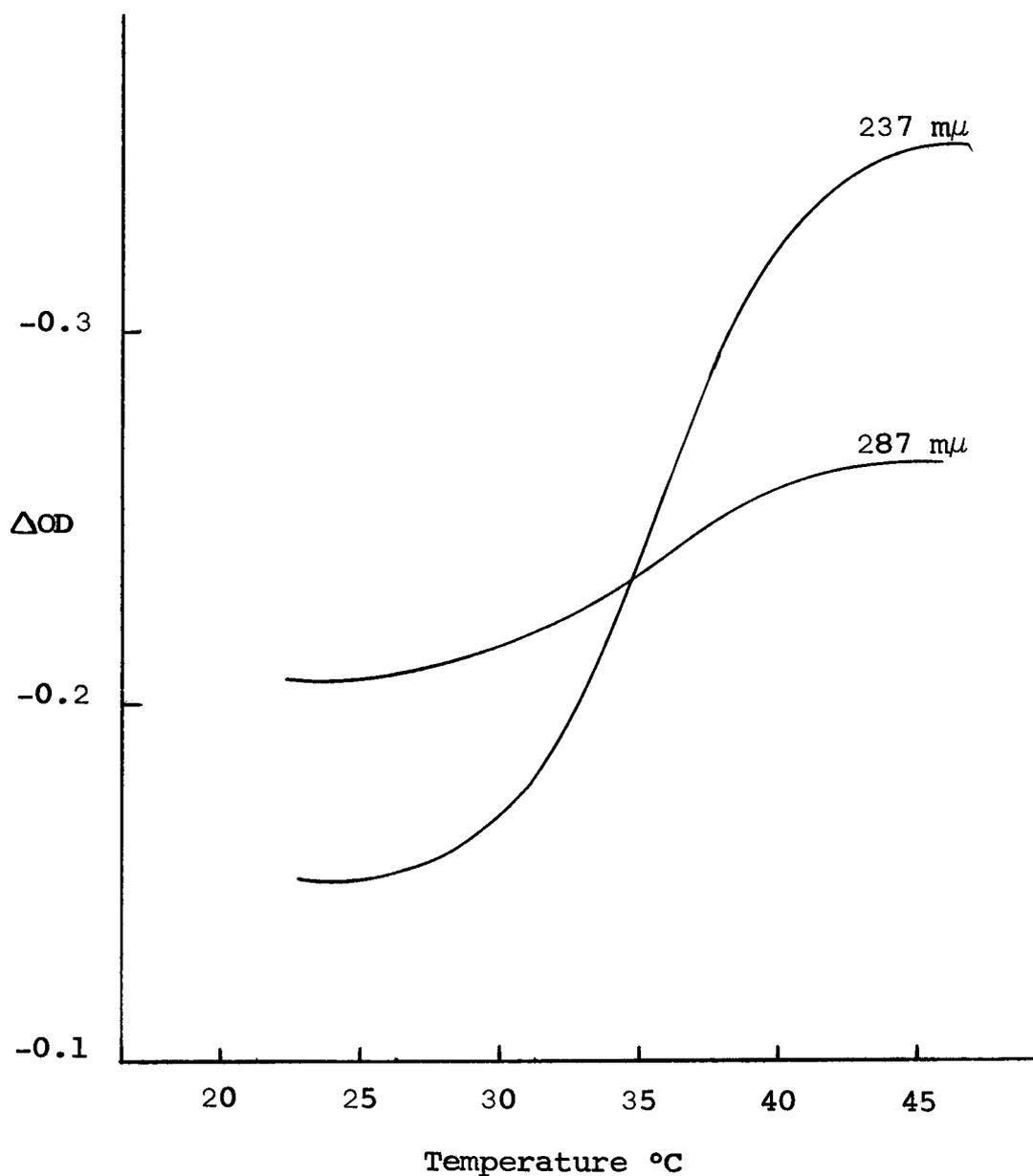


Figure 1(A). The thermally induced transition of ribonuclease (concentration =  $4.6 \times 10^{-5}$  M) in a KCl-HCl buffer at pH 2.1 and 0.05 M ionic strength. Monitoring of the two melting curves was done separately at 287 mμ and 237 mμ as shown in the figure.

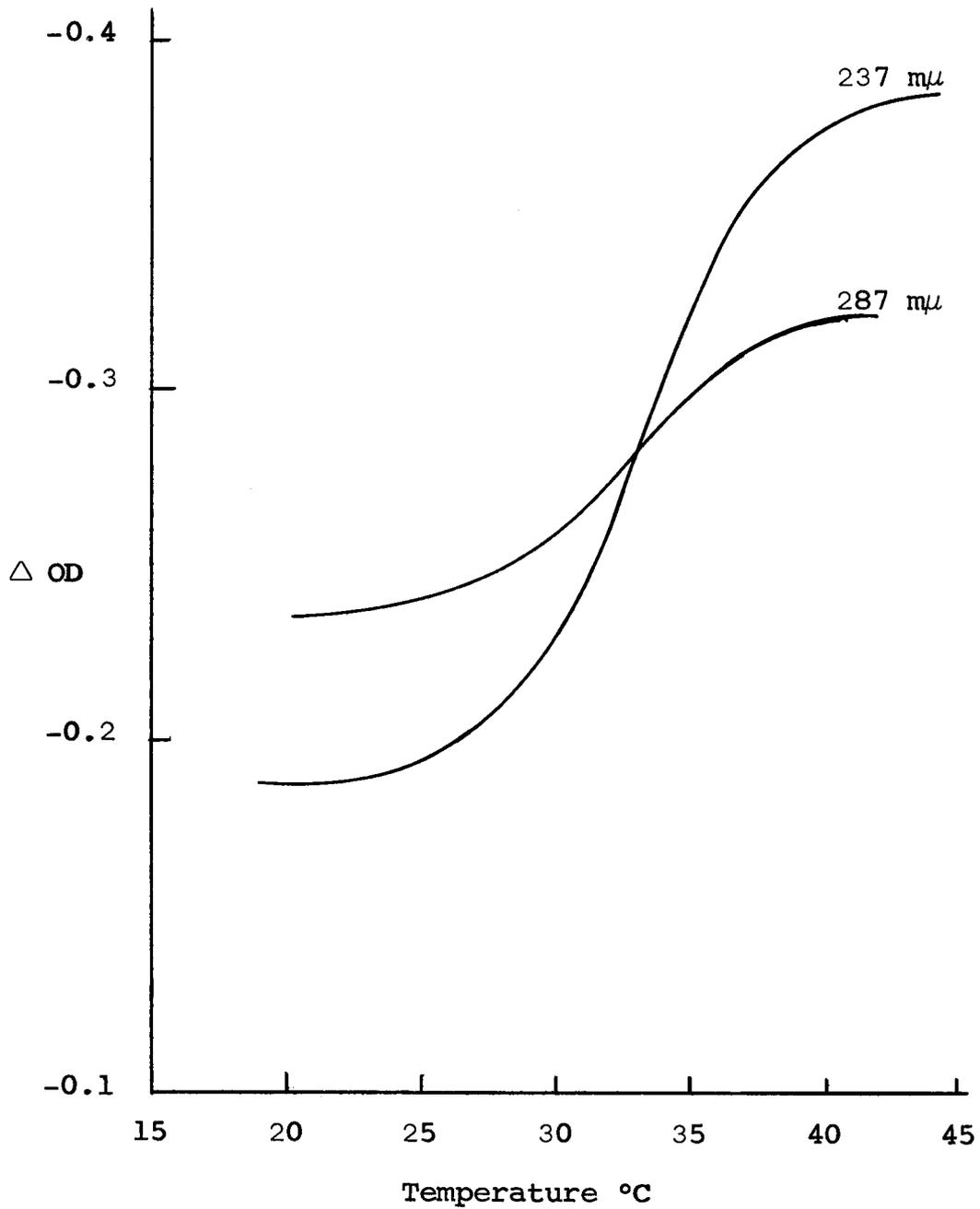


Figure 1(B). The heat-induced transition of acetamidated ribonuclease (concentration =  $4.2 \times 10^{-5}$  M) in a KCl-HCl buffer at 2.1 and 0.05 M ionic strength. Monitoring of the two melting curves was done at 237 mμ and 287 mμ as shown in the figure.

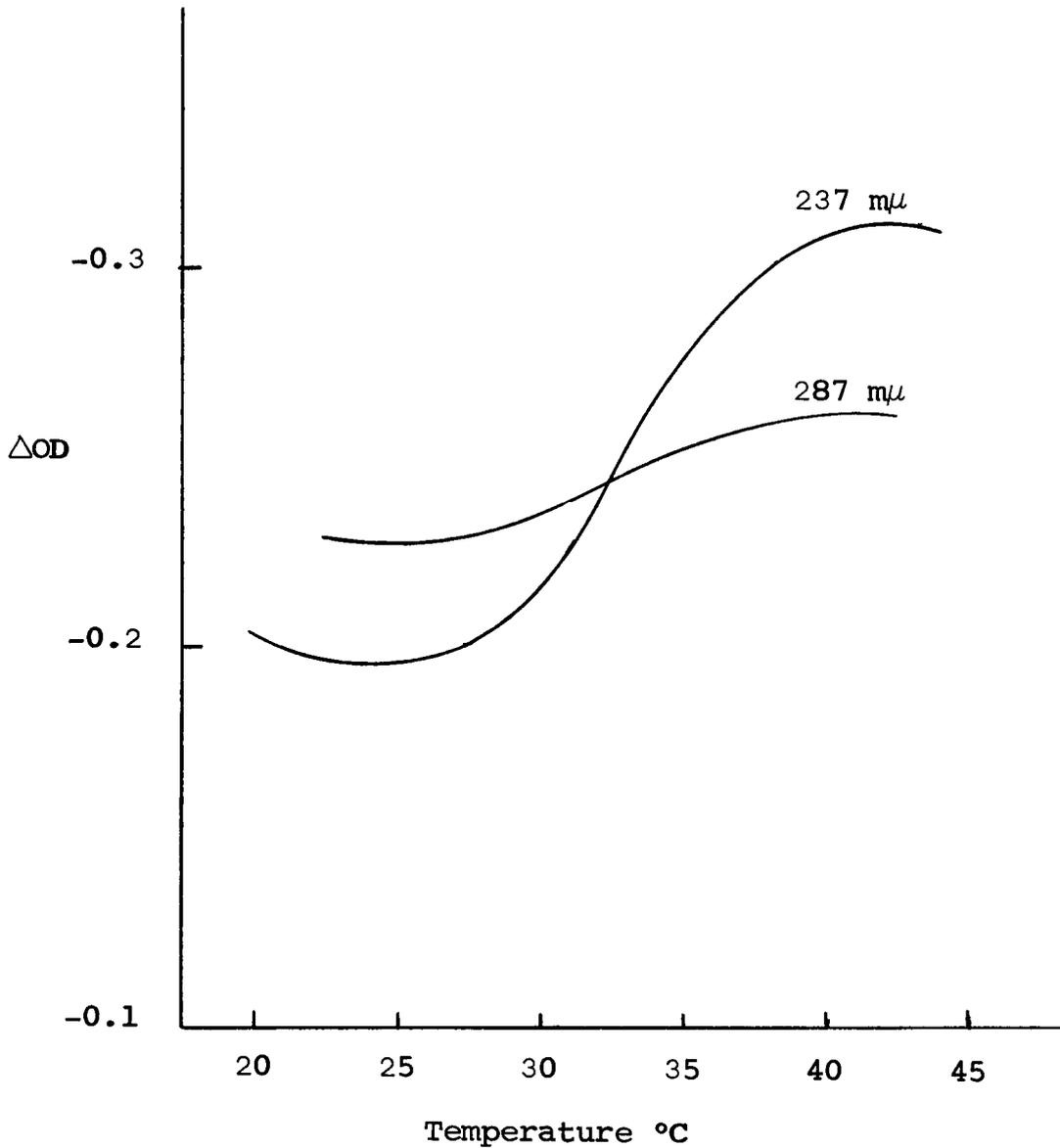


Figure 1(C). The heat-induced transition of polytyrosyl acetamidated ribonuclease IA (concentration =  $3.6 \times 10^{-5}$  M) in a KCl-HCl buffer at pH 2.1 and 0.05 ionic strength. Monitoring of the two melting curves was done separately at 237 m $\mu$  and 287 m $\mu$  as shown in the figure.

comparison may be made with this work. By analyzing the kinetics of the thermally induced transition of RNase, they suggested that the 235  $m\mu$  peak was probably the consequence of perturbing the environment of the peptide chromophores.

#### 4. Environment of Attached Tyrosyl Residues

The solvent perturbation technique of Herskovits and Laskowski (1962) has been used as a probe in detecting the presence of chromophoric groups "buried" in the protein molecule by comparing the influence of perturbants such as sucrose on the spectra of native and unfolded proteins. Unpublished results from this laboratory using this technique have indicated that some tyrosine residues appear to interact with the parent protein molecule in that surface residues are not all perturbed. A logical question is raised with regard to the environment of the added chromophoric residues. Since a limited number of tyrosine residues have been attached to a RNase molecule whose physical parameters and conformation have been characterized, the PT-Ac-RNase derivatives may be a better model for studying the environment of the added chromophores. If some of the added tyrosyl residues in PT-Ac-RNase are "buried" in the RNase molecule, the thermal-induced transition will normalize some of the "buried" groups. The technique of the temperature difference spectra should prove helpful in probing the conformation of these derivatives.

Figure 2 shows the melting profiles of RNase derivatives. Both reference and sample cells contained the same concentration of PT-Ac-RNase IA at pH 2.1 and 0.05 M KCl. As the sample cell was warmed, the S-shaped melting curve resulted. When the experiment was repeated with native RNase in the reference cell with an optical density at 277  $m\mu$ , the same as that of PT-Ac-RNase IA in the sample cell, the melting curve could not be differentiated from the previous melting curve. A control with Ac-RNase in the sample cell and with native RNase in the reference cell was carried out. Only in the control is there a large size of transition. The size of transition is defined as the difference between the lowest and highest optical densities observed.

It is suggestive that these attached residues of tyrosine are not in a different solvent environment than any of the normal tyrosine residues in the native RNase molecule. But upon thermal denaturation of the derivative, the added tyrosine residues could interact with the chromophoric group strongly to effect a decrease in the size of transition as shown in the figure. In short, the added tyrosine residues may mask a chromophoric group and the consequent normalization of the chromophoric group, as detected by the technique of difference spectrum, was not possible.

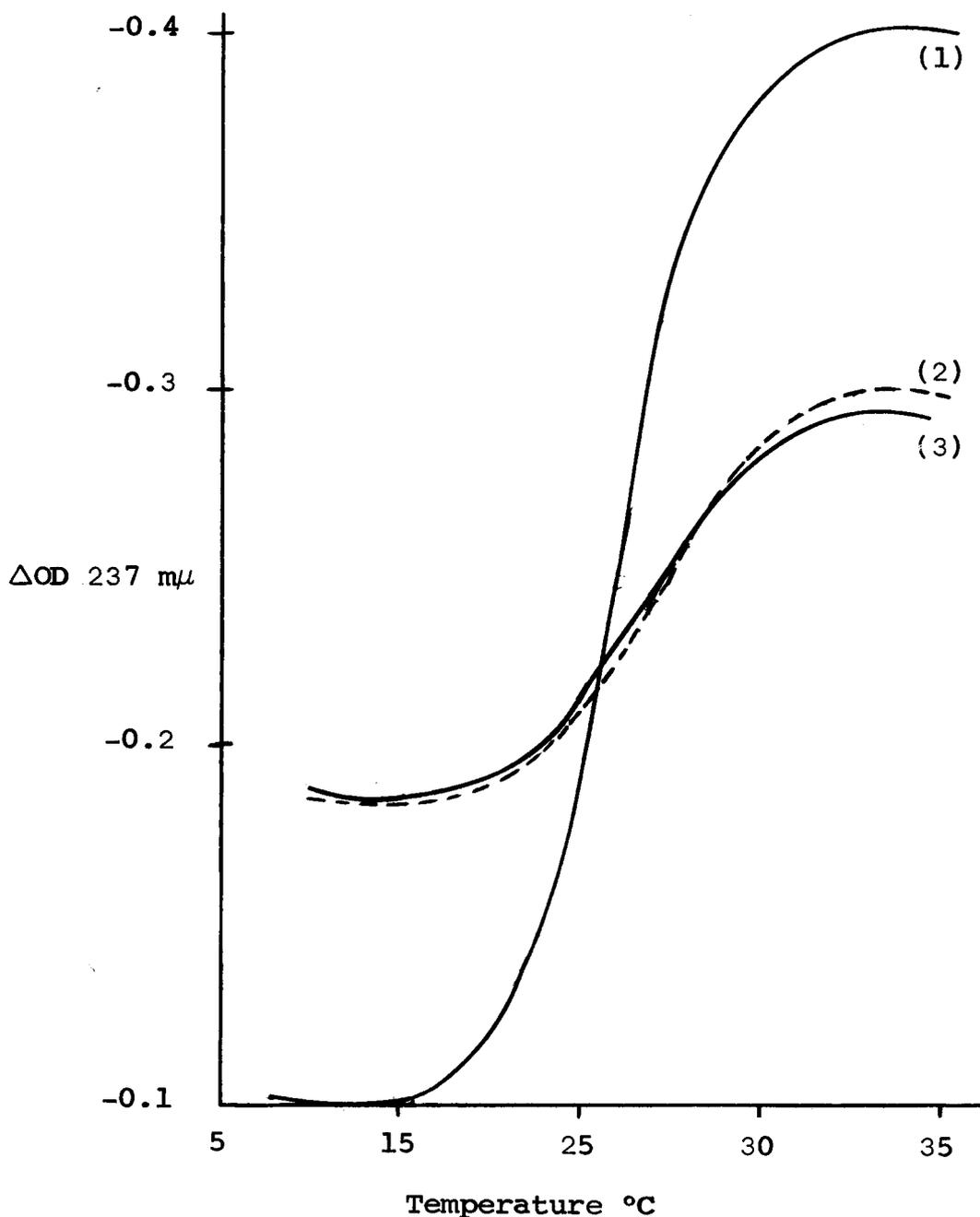


Figure 2. The heat-induced transition of polytyrosyl acetamidated ribonuclease IA and acetamidated ribonuclease in KCl-HCl buffer at pH 2.1 and 0.05 M ionic strength.

- (1) Acetamidated RNase compared with native RNase (OD = 0.7).
- (2) PT-Ac-RNase IA compared with PT-Ac-RNase IA (OD = 0.7).
- (3) PT-Ac-RNase IA compared with native RNase (OD = 0.7).

### 5. Effect of pH on $T_m$ of PT-Ac-RNase

The variation of  $\Delta OD_{235} \text{ m}\mu$  with temperature at a series of pH values at an ionic strength between 0.05 M to 0.1 M is shown in Figure 3. The data were obtained in the following manner: 0.5 ml of PT-Ac-RNase IA was mixed with a 2.0 ml buffer. The reference cell containing PT-Ac-RNase was kept constant at pH 7.1 while the pH in the sample cell containing the derivative was varied. In the pH range covered, it appears that the  $T_m$  is independent of pH below 1 and above 6.

Using a hypothetical model whose structure was assumed to be stabilized by tyrosyl-carboxylate ion and tyrosyl-histidine hydrogen bonds, Scheraga (1960) obtained a S-shaped curve by plotting  $T_m$  versus pH. He concluded that the value of  $T_m$  was the greatest in the pH region where the degree of hydrogen bonding was the greatest, and that the carboxylate-tyrosine hydrogen bond might be imbedded in a nonpolar region. Hermans and Scheraga (1961) observed a similar S-shaped curve when the RNase molecule was thermally denatured at different pH values. Their finding strengthened the hypothesis that the tyrosine-carboxylate hydrogen bond could be, as suggested, imbedded in the nonpolar interior of the protein.

In spite of the uncertainty in such an interpretation of the results, it is clear that the added tyrosine

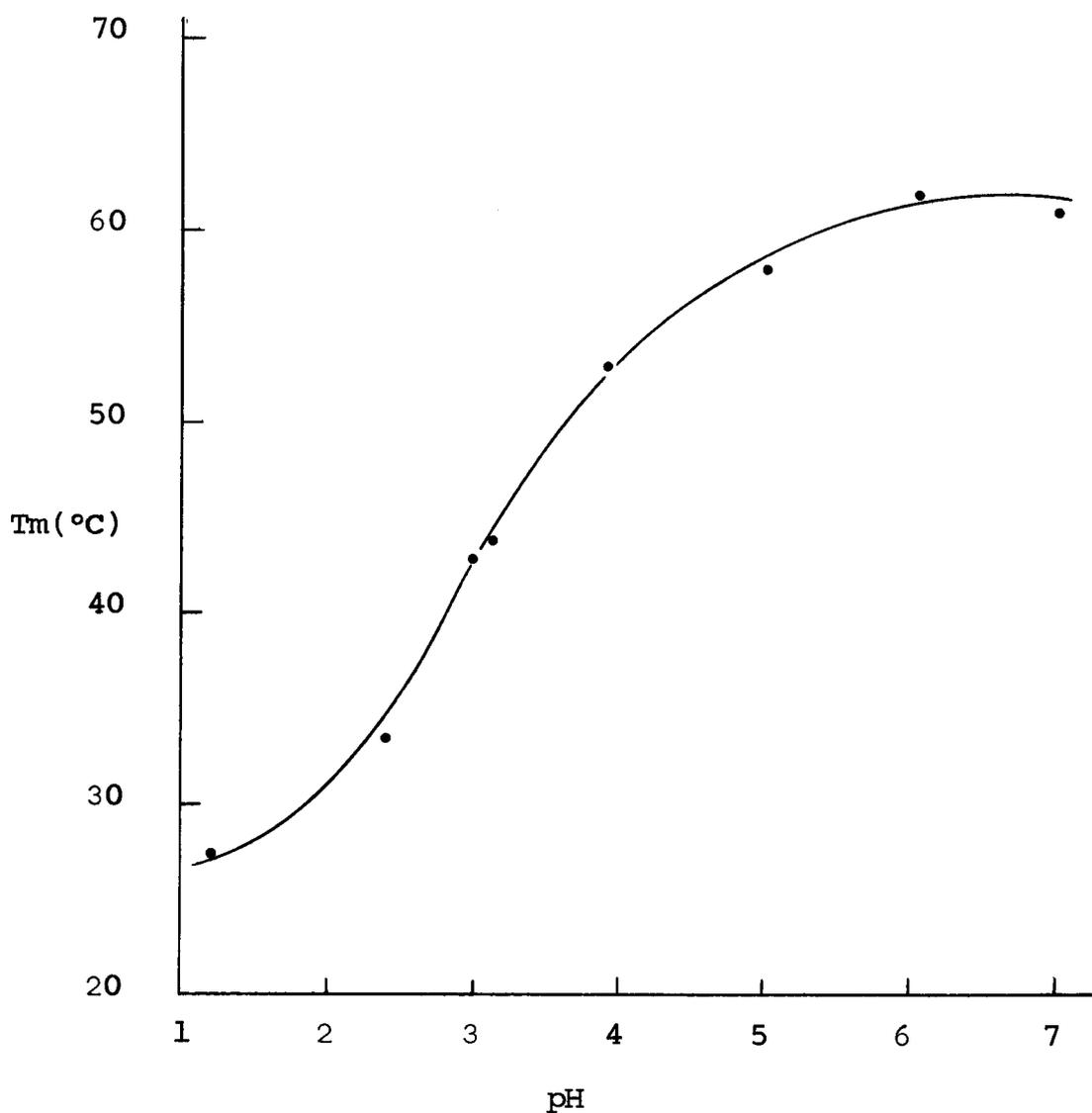


Figure 3. Transition temperature of PT-Ac-RNase IA (concentration =  $2.3 \times 10^{-5}$  M) at different pH values. 0.5 ml of PT-Ac-RNase IA was mixed with a 2.0 ml buffer. The pH was determined after heating.

residues of the PT-Ac-RNase derivative have not changed the magnitude and direction of the transition since the curves for native RNase and the derivative are identical. Whatever other effects may be attributed to the added residues, the groups buried within the protein interior are still sensitive to pH.

#### 6. Guanidine Hydrochloride, Urea and Ethanol

It has been shown that the  $T_m$  can be progressively shifted to lower temperatures by alcohols (Shrier and Scheraga, 1962), by urea (Foss and Schellman, 1959), and by guanidine hydrochloride (von Hippel and Wong, 1965). These denaturants, formerly considered to be hydrogen bond competitors, have recently been recognized as potent breakers of the hydrophobic bond (Nozaki and Tanford, 1963; Wetlaufer, et al, 1964). Therefore, a study of the effects of these denaturants on the  $T_m$  of RNase derivatives to which hydrophobic residues have been added might provide additional information as to possible interaction of the added residues with other groups in the protein.

The results of the effects of GuCl and ethanol on the  $T_m$  are shown in Figure 4. The PT-Ac-RNase solutions were adjusted to pH 7 and then various concentrations of solid GuCl were added to the solutions. The  $T_m$  was determined for each concentration of GuCl. To obtain the various concentrations of ethanol, the solution was first adjusted

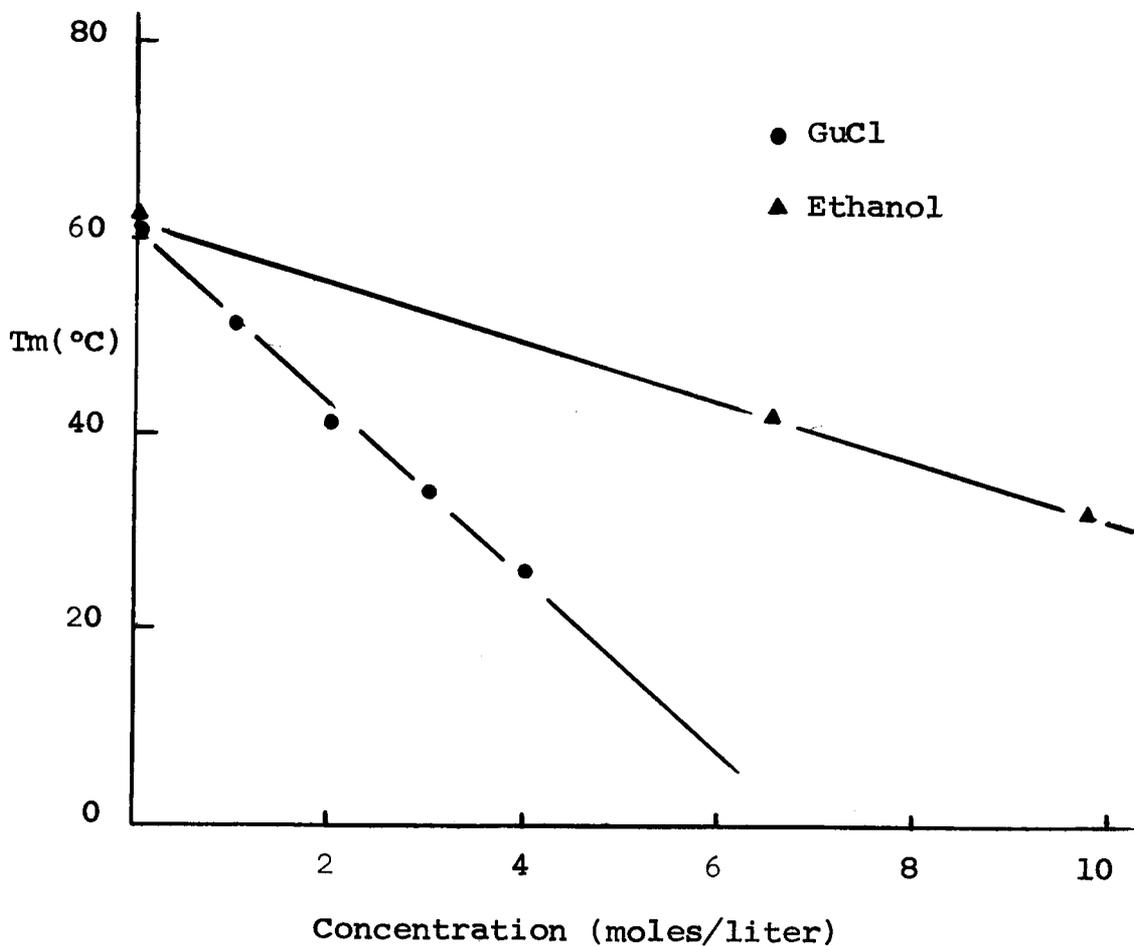


Figure 4. Transition temperature of PT-Ac-RNase IA (concentration =  $1.2 \times 10^{-5}$  M) in various concentrations of guanidine hydrochloride and ethanol. All of the samples were buffered in a KCl-HCl system at pH 2.1 and 0.05 M ionic strength.

to pH 7, followed by addition of 95 percent ethanol to yield the desired concentration. There is no marked difference in the plot of the  $T_m$  versus the concentration of GuCl between the native RNase and PT-Ac-RNase in the plot of  $T_m$  versus the concentration of ethanol. At a concentration of more than 60 percent ethanol, the PT-Ac-RNase precipitates from the solution. From the figure, the observation is made that the two denaturants are potent depressants of  $T_m$ . They further decrease the size of transition with increasing concentration of denaturant. The latter observation is contrary to that made by von Hippel and Wong (1965), who reported an increase in the size of transition using specific rotation as the physical measurement, indicating that the two techniques measure different phenomenon.

The effect of GuCl on the size of transition of PT-Ac-RNase is more striking than that of RNase. At 4 M concentration of GuCl, the size of transition is decreased to one fourth of the maximal size while decrease to only one half of the maximal size is obtained from RNase. Presumably, the effectiveness of depressing  $T_m$  by GuCl may be accompanied by some other effects on the structure of the protein molecule.

Urea was also tested for its potency on depressing the  $T_m$ . It was added to a PT-Ac-RNase solution at pH 2.1 which was compared with the same concentration of protein

at pH 7. The melting curves are illustrated in Figure 5. Again, urea can shift the  $T_m$  to lower temperatures linearly with increasing urea concentration. It also decreases the size of transition: at a concentration of 4 M urea, the size is only one fifth of that at 2 M urea concentration.

#### 7. Insoluble Region of PT-Ac-RNase IC

A unique characteristic of the PT-Ac-RNase IC was that it became insoluble as the pH was adjusted to values between 5.0 and 9.0. This insolubility phenomenon was investigated to determine the pH at which maximal turbidity occurred. 1.0 ml of the protein solution was mixed with 4.0 ml buffer ranging from pH 5.0 and 9.0. After five minutes, the suspension was measured at 320  $m\mu$  for the extent of turbidity. Figure 6 shows the plot of turbidity against pH, and it is clear that the PT-Ac-RNase IC was least soluble at around pH 8.1.

A similar observation was reported by Glazer, Bar-Eli and Katchalski (1962) in a study of a polytyrosyl trypsin derivative which was obtained as a milky suspension right after the polypeptidylation reaction. Upon exhaustive dialysis against 0.0025 N HCl at 4°, the suspension became clear and then was found to be sparingly soluble between pH 5.0 and 9.0.

The high content of tyrosine residues must be responsible for the insolubility phenomenon as the native

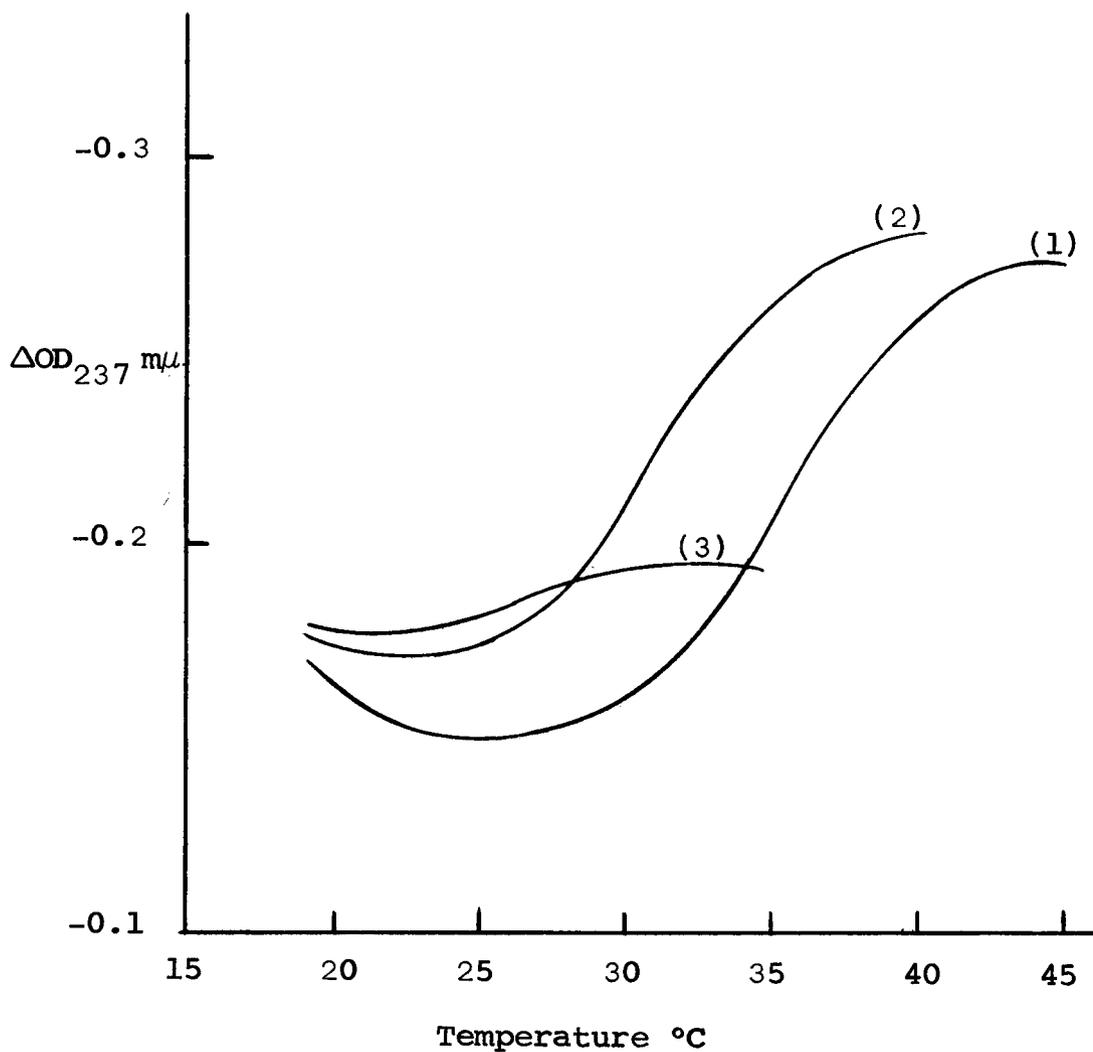


Figure 5. The heat-induced transition of polytyrosyl acetamidated ribonuclease IA (concentration =  $3.6 \times 10^{-5}$  M) in a KCl-HCl buffer at pH 2.1 and 0.05 ionic strength containing no urea (1); 2 M urea (2); 4 M urea (3).

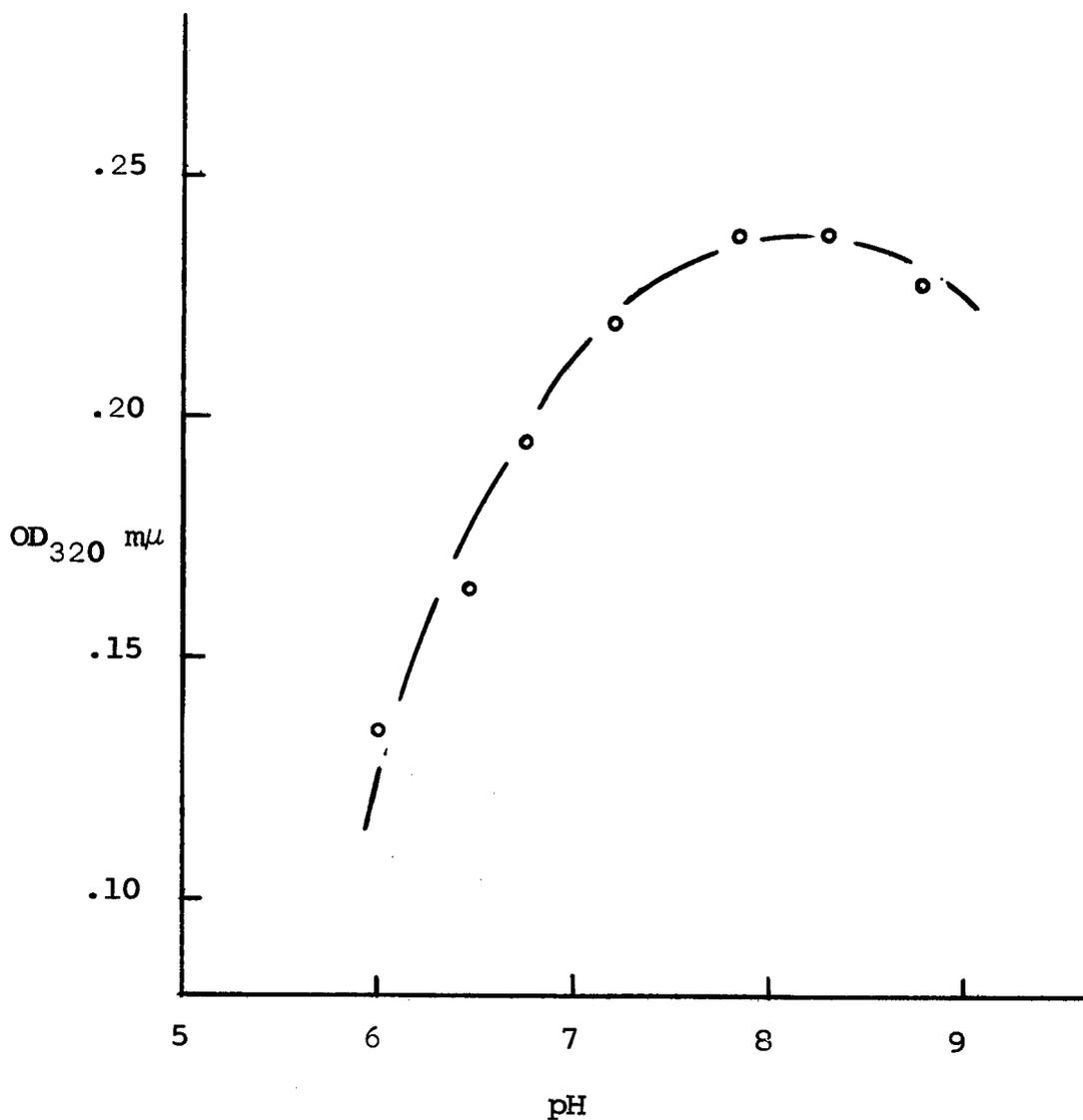


Figure 6. Turbidity formation of polytyrosyl acetamidinated ribonuclease IC as a function of pH. The concentration of PT-Ac-RNase IC was  $4.6 \times 10^{-6}$  M, and phosphate (0.1 M) and tris (0.2 M) buffers were used.

proteins are soluble throughout the pH range. Besides, a lightly modified derivative such as IA is also soluble. A plausible explanation may be that at the isoelectric point hydrophobic interactions more than offset the electrostatic repulsive interactions to cause intermolecular aggregation.

#### 8. KCl and Turbidity Formation

PT-Ac-RNase IC was observed to have certain properties similar to those of polyvalyl RNase. At high concentration of salt, either monovalent or divalent, turbidity arises at low pH and room temperature. The hydrophobic side chains of tyrosine presumably exhibit attractive apolar interactions when the electrostatic repulsive forces are diminished by higher ionic strengths.

Since the rate of turbidity formation was maximum after five minutes as shown in Figure 7(A), the optical density measured at  $320\text{ m}\mu$  was taken to mean that a maximal turbidity was reached and that another known concentration of salt could be added to produce further aggregation. Figure 7(B) shows the plot of optical density measured at  $320\text{ m}\mu$  against the concentrations of potassium chloride at pH 3.5. In the plot are included the effects of the salt on Ac-RNase and native RNase. A sigmoidal curve is shown. The KCl salt restricts the electrostatic interaction by a charge-shielding effect and therefore enhances the

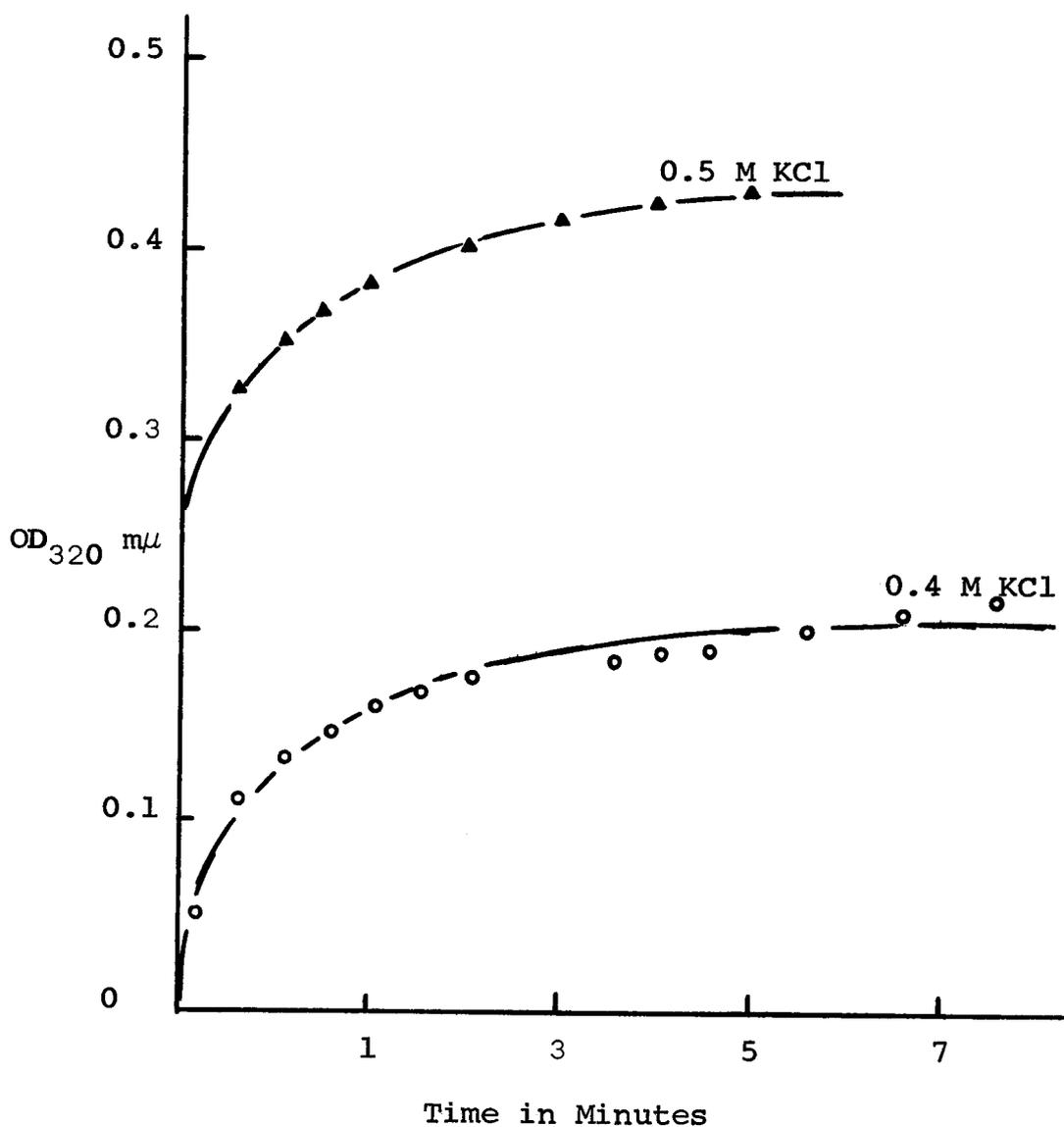


Figure 7(A). Rate of turbidity formation of polytyrosyl acetamidated-RNase IC in the presence of potassium chloride.

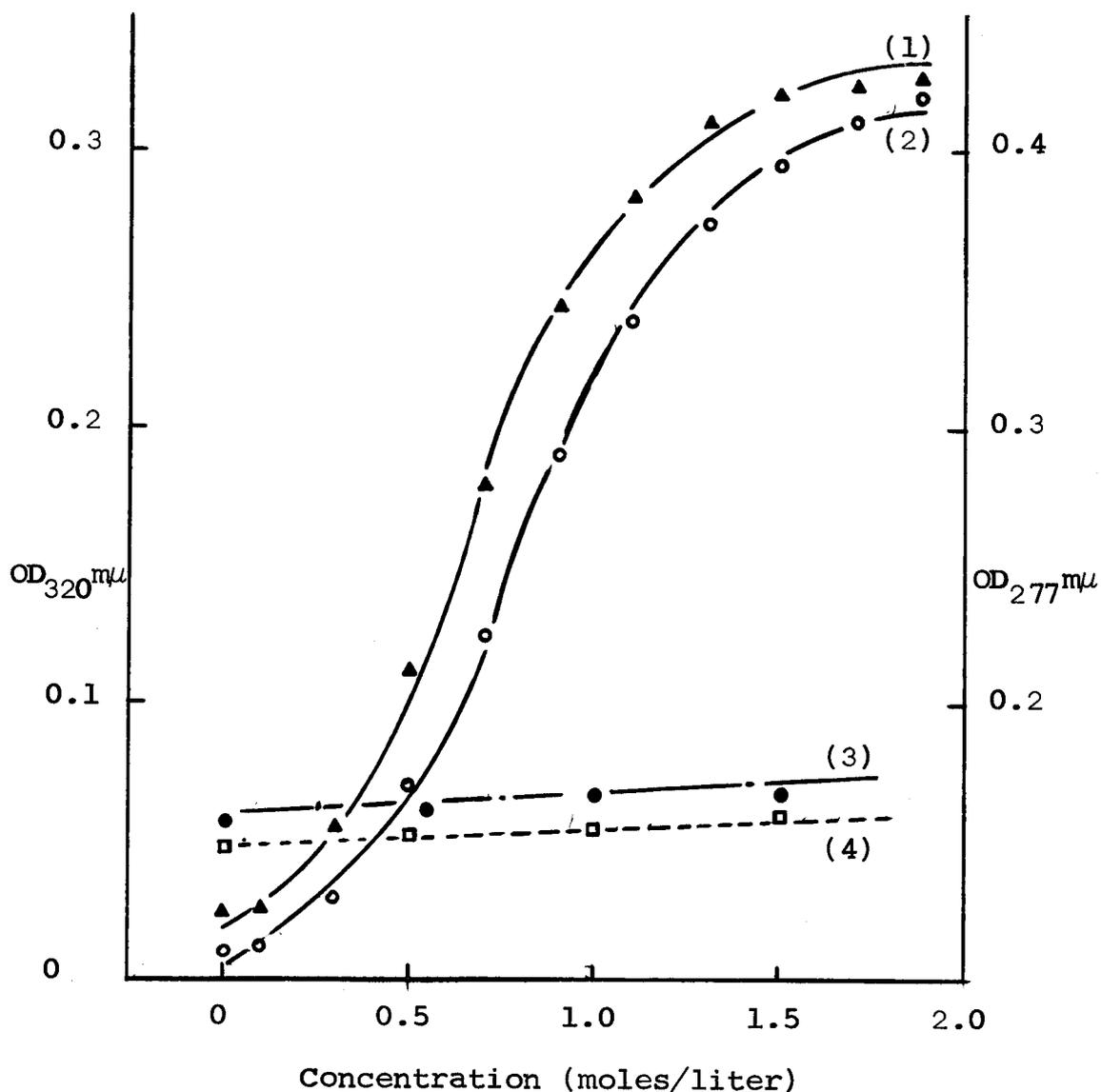


Figure 7(B). Turbidity formation of polytyrosyl aceta-midinated ribonuclease IC in various concentrations of KCl.

- (1) PT-Ac-RNase IC, turbidity monitored at 277 mμ.
- (2) Turbidity monitored at 320 mμ.
- (3) Acetamidated ribonuclease, at 320 mμ.
- (4) Native ribonuclease, at 320 mμ.

hydrophobic interaction of the added tyrosyl residues. As would be expected, additional salt causes increasing turbidity formation until the shielding effect reaches the maximum, and a leveling off of the curve is seen.

It is desirable to ascertain that the effect of salt on turbidity formation is, indeed, caused by the additional tyrosine residues and not due to disruption of the protein entity. In parallel measurements to turbidity formation, the peak at 277  $m\mu$  was followed. The position of the peak is an indication of the molecular integrity. Any denaturation process will cause a "blue shift," namely, the shifting of the peak to lower wavelengths. Since no shift is observed at 277  $m\mu$ , as shown in the figure, turbidity formation results from perturbing the hydrophobic groups of tyrosine.

A recent study of carboxypeptidase A in the presence of high salt concentrations suggests an important role of hydrophobic interactions (Bethune, 1965). The enzyme was shown by ultracentrifuge experiments to form a dimer in the presence of 2.0 M sodium chloride. The author suggests that since an "active" tyrosine residue is found in the catalytic center of carboxypeptidase A, the hydrophobic interaction to form a protein-protein dimer is not unlikely.

#### 9. Dissociation Effects of Hydrochloride

According to the work of Robinson and Jencks (1963)

GuCl possesses the solubilizing effects of urea, but is two to three times as effective per mole (Tanford, 1964). Also, it has been shown that carboxymonoxide-hemoglobin can be dissociated into subunits by 3 M GuCl whereas even 8 M urea does not bring about dissociation (Tanford, 1964). The potency of GuCl as a dissociating agent is further revealed by its effect on the PT-Ac-RNase IC turbid solution. The turbidity produced by adding various concentrations of KCl at pH 3.5 could be diminished by the addition of GuCl as shown in Figure 8.

Although there are no direct data relating the solubilizing effect of GuCl on hydrophobic groups, the available data for urea might be extrapolated to GuCl (Nozaki and Tanford, 1963). Thus, the free energy involved in transferring a tyrosine side-chain from an aqueous environment to a 3 M GuCl is approximately  $-0.7$  kcal. If the observable turbidity is caused by hydrophobic interactions of tyrosine side-chains, the free energy of transfer of these groups from water into the GuCl solution favors the dissociation of aggregates and therefore, a clear solution.

#### 10. Difference Spectrum of PT-Ac-RNase IC in Ethanol

In the interpretation of the melting curves of RNase derivatives, it is assumed that as long as the abnormal tyrosine may be normalized in the same manner as those in native RNase, the native conformation is preserved. Since

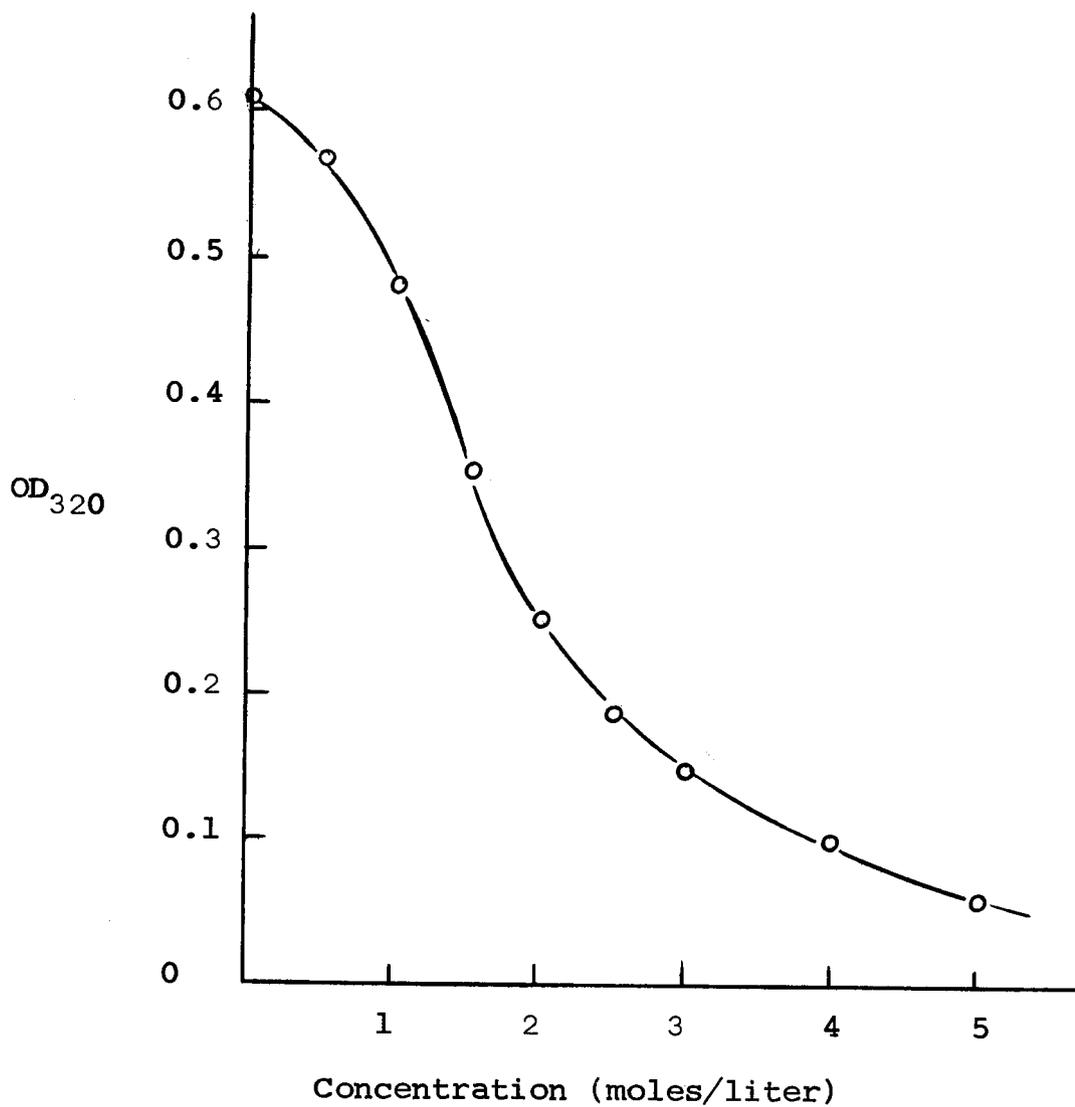


Figure 8. Dissolution of aggregates of polytyrosyl acetylated ribonuclease IC in various concentrations of guanidine hydrochloride at pH 3.5.

PT-Ac-RNase IA has been shown to possess the native conformation by the technique of the temperature difference spectrum, a pertinent question of whether or not PT-Ac-RNase IC which is more highly substituted, has the native conformation appears to be desirable. Unfortunately, no interpretable melting curve could be obtained for this derivative. Presumably either intermolecular or intramolecular interactions of the side-chains were so predominant that the chromophores' environment was not changed substantially over the temperature range studied. However, it was possible to obtain a difference spectrum in the presence of ethanol (Figure 9), since this low dielectric solvent is capable of disrupting the strong interactions of the tyrosine residues.

#### 11. Spectrophotometric Titration of PT-Ac-RNase II

PT-Ac-RNase II prepared in phosphate buffer instead of bicarbonate contains four added tyrosine residues attached to approximately two sites. A similar melting experiment as already described previously for PT-Ac-RNase I was carried out, and a melting curve identical to that for PT-Ac-RNase I was observed (Figure 1). The size of transition not increased by the tyrosine residues, suggests that they reside on the surface of the RNase molecule. This possibility can be confirmed by the spectrophotometric titration. Although the spectrophotometric titration helps

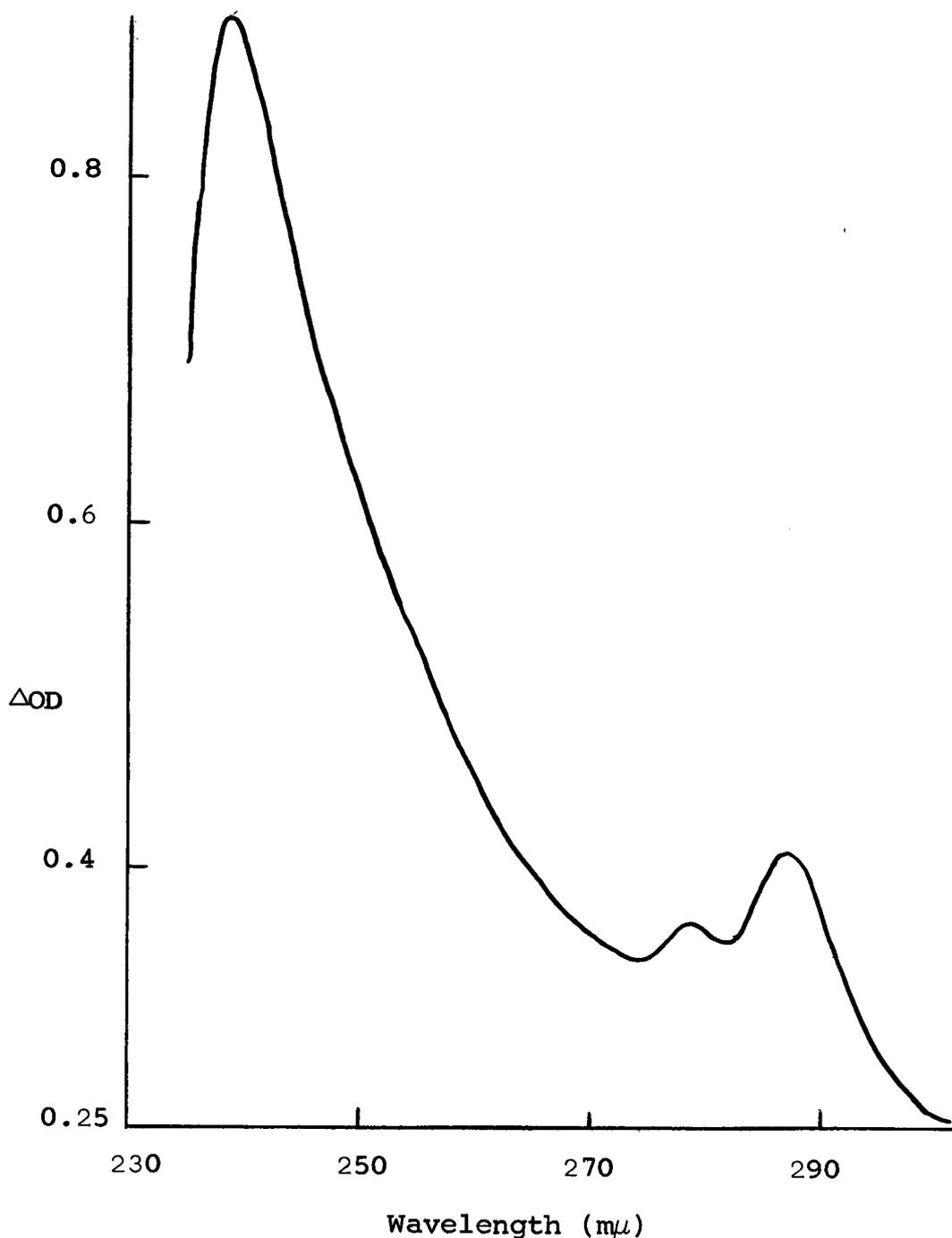
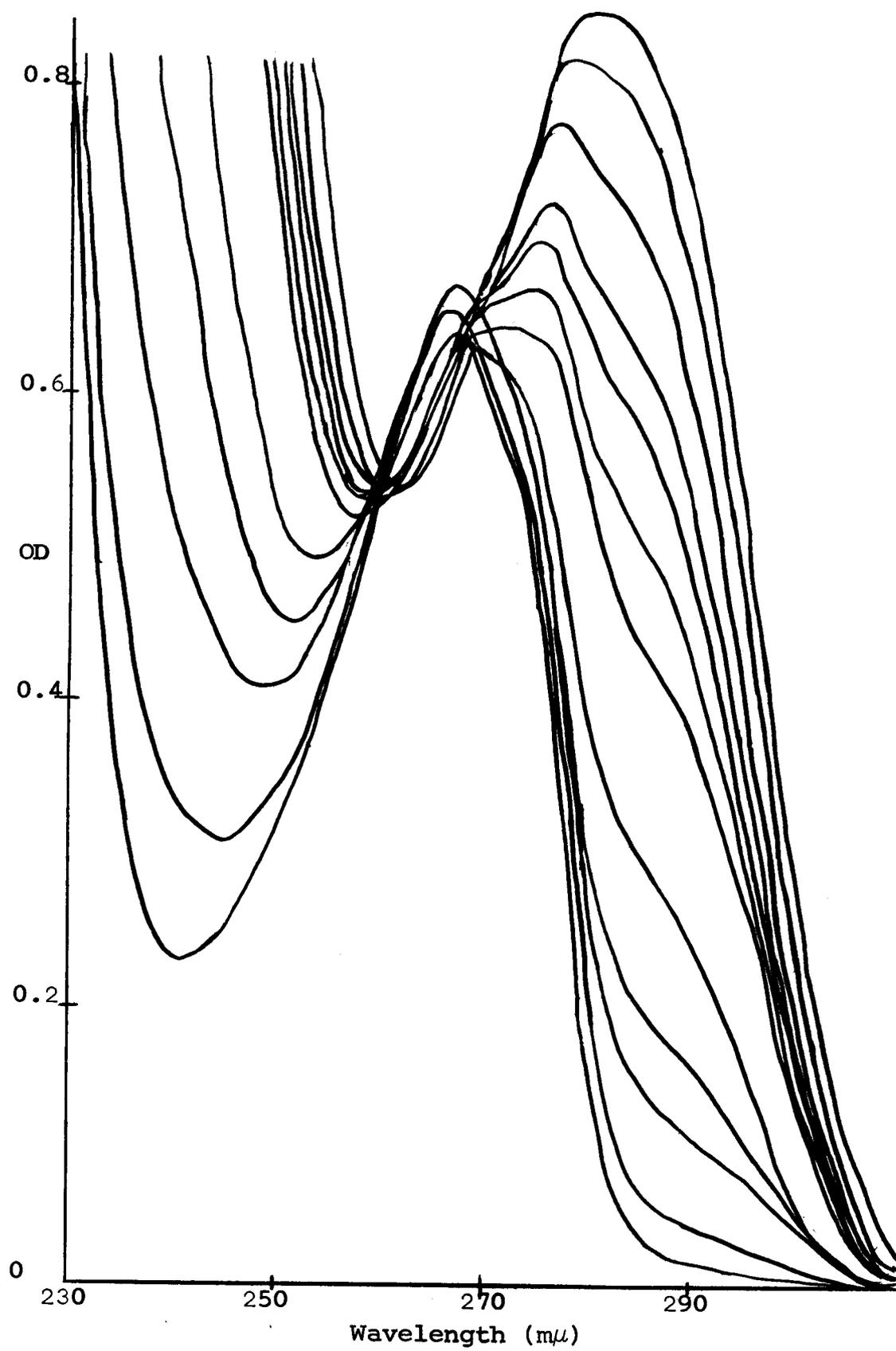


Figure 9. Difference spectrum of polytyrosyl acetamidinated ribonuclease IC ( $2.6 \times 10^{-5}$  M). The reference cell contained the derivative solution in 37% ethanol and phosphate buffer at pH 6.7 while the sample cell contained the same concentration of the derivative in KCl-HCl buffer at pH 2.1.

to ascertain whether or not the added tyrosyl groups are located in an environment different from the normal ones, the question of intermolecular interaction with the side chains remained a possibility.

The UV spectra of PT-Ac-RNase II as a function of pH are shown in Figure 10. Figure 11 shows the plot of the molar extinction coefficient  $\epsilon$  at 295  $m\mu$  against pH. There are two distinct portions of the S-shaped curves, which presumably correspond to the two stages of ionization of tyrosine. The change in  $\epsilon$  at 295  $m\mu$  for each stage can be fixed: for the first stage, there is an increase from 900 to 18,500; and for the second stage, an increase from 18,500 to 26,000. If the change in  $\epsilon$  at 295  $m\mu$  per phenolic group as obtained from the corresponding curve for the native RNase is 2500, a comparable value to that reported (Tanford, Hauenstein and Rands, 1956), then the first stage represents the ionization of seven tyrosine residues and the second stage, the ionization of three abnormal tyrosines. Native RNase contains three residues titrating in the first stage (normal) and three that titrate in the second stage (abnormal). In the PT-Ac-RNase derivative, according to analysis for added tyrosine, there are, indeed, four moles per mole of protein (see Table I). This implies that the four tyrosine residues are located on the surface to account for the first stage of ionization of seven phenolic groups. Moreover, it is likely that the

Figure 10. Ultraviolet spectra of polytyrosyl acetamidinated ribonuclease IIA (concentration =  $4.3 \times 10^{-5}$  M) under the influence of different pH values.



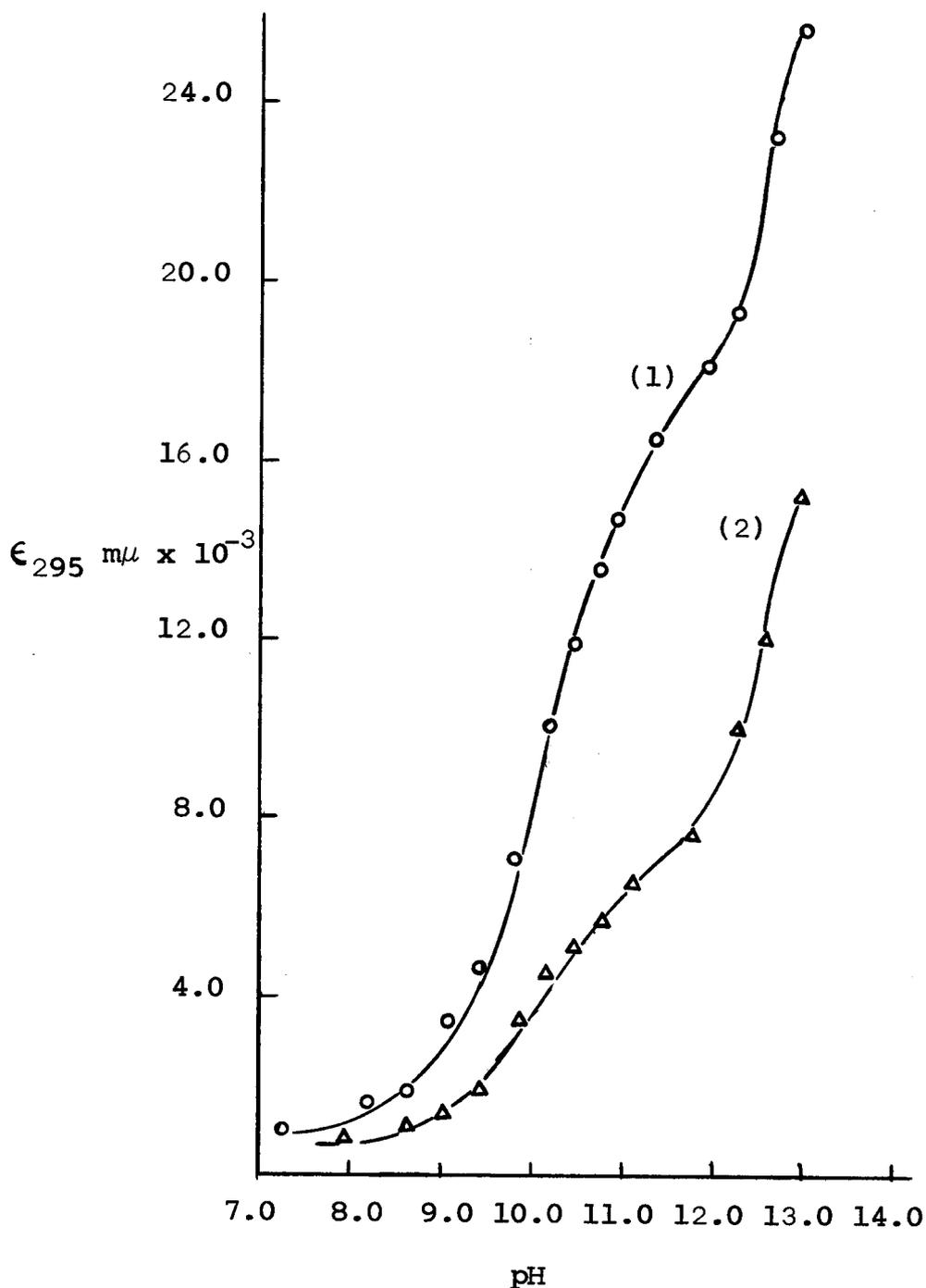


Figure 11. Spectrophotometric titration of polytyrosyl acetamidated ribonuclease IIA (1) and native ribonuclease (2). A plot of molar absorptivity  $\epsilon$  at 295  $m\mu$  versus different pH values showed two stages of ionization of tyrosine residues.

three abnormal tyrosines ionizing at high pH's are the same three abnormal ones in the native RNase molecule.

## 12. Effect of KCl on Tm at pH 2.1

Recent investigation of the effects of neutral salts on the conformational stability of collagen (von Hippel and Wong, 1963) and ribonuclease (von Hippel and Wong, 1965) suggest that salts do not necessarily play a role only in shielding electrostatic interactions. Experimental findings enabled them to propose that the salts have a general effect on the structure of the solvent. However, another recent work on RNase at low pH and low anion concentration has permitted Ginsburg and Carrol (1965) to advance a different mechanism, namely, a specific binding of salt to the protein molecule. The better the salt is bound, the more resistant to thermal denaturation is the protein molecule. Since there is a difference in the proposed mechanisms, investigation with the PT-Ac-RNase derivative at low pH with increasing salt concentrations should yield valuable information bearing on both suggested mechanisms.

The data were obtained in the following manner. PT-Ac-RNase IIA solution with a concentration of  $3.4 \times 10^{-5}$  M, 0.05 M ionic strength and pH 2.1 was present in the sample cell and also the reference cell. As the solution in the sample cell was gradually heated, the temperature

of transition was observed and recorded. After the solution had been thermally denatured, the solution was cooled to room temperature to yield the original absorption. Solid KCl was then added to the solution to make 1 M concentration. This solution was again heated and  $T_m$  recorded. This procedure was repeated for various concentration of KCl. It was shown that the original optical density was obtained upon sufficient cooling, demonstrating reversibility of the system.

At a concentration of 1.2 M KCl, however, aggregation was induced by a temperature around  $27^\circ$  and thus the  $T_m$  could not be determined. The results of this series of melting profiles are shown in Figure 12. Mention must be made about the size of transition. As the concentration of KCl is increased, the size of transition appears to be diminished. Another pertinent observation is that as KCl concentration is increased the  $T_m$  becomes shifted toward higher temperatures. It appears that KCl stabilizes the conformational structure of RNase. When Ac-RNase and native RNase were separately tested to distinguish the effects of various concentrations of KCl on the  $T_m$ , a similar result, as previously described, was observed. Figure 13 shows the plot of  $T_m$  versus concentration of KCl for native RNase, Ac-RNase and PT-Ac-RNase IIA. Differences are only qualitative, and KCl does not markedly affect the conformational structure of RNase even when there are extra

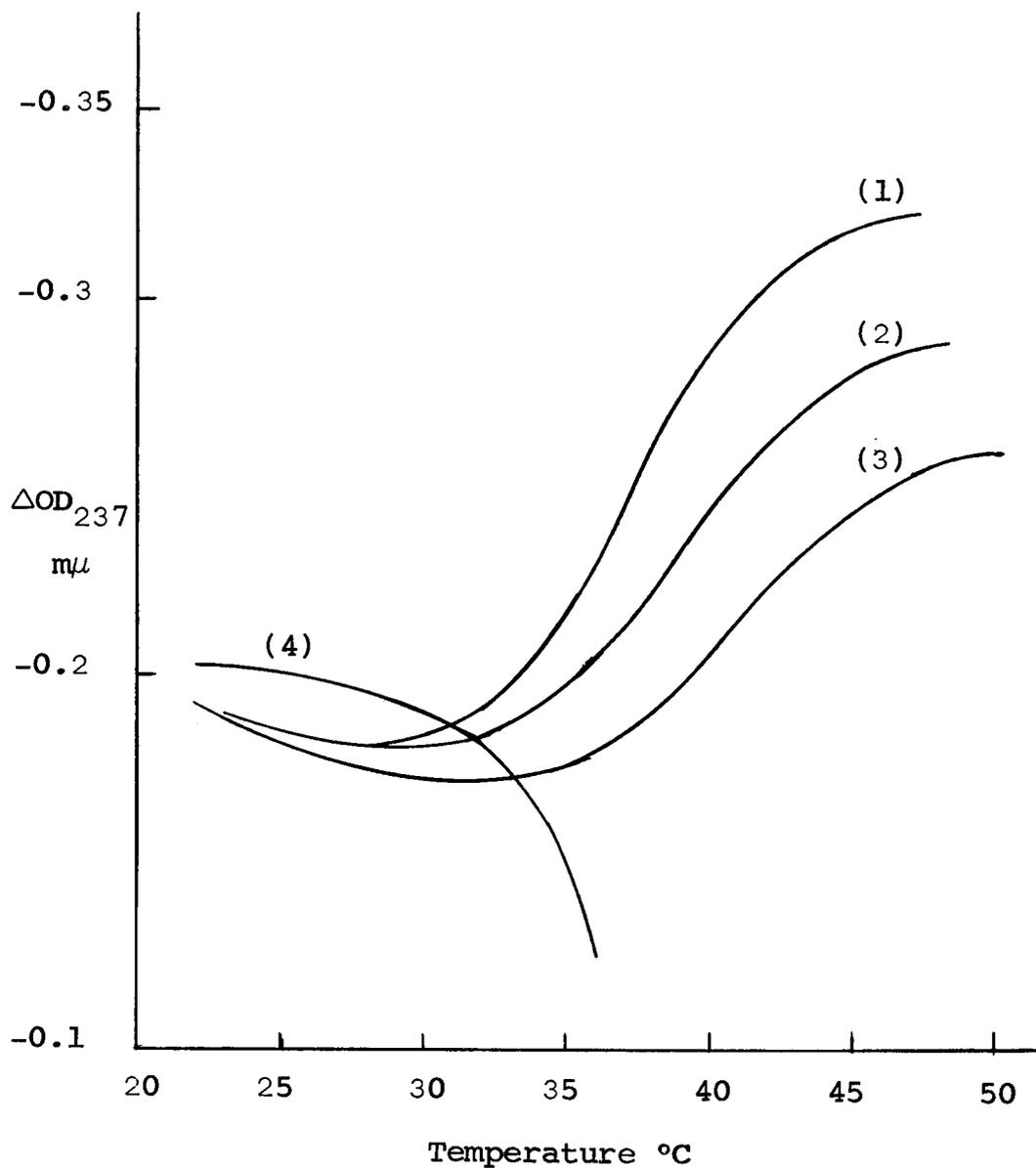


Figure 12. The heat-induced transition of polytyrosyl acetamidated ribonuclease IIA ( $3.4 \times 10^{-5}$  M) in various concentrations of potassium chloride at pH 2.1.

- (1) PT-Ac-RNase IIA in 0.05 M KCl solution.
- (2) PT-Ac-RNase IIA in 0.3 M KCl solution.
- (3) PT-Ac-RNase IIA in 0.6 M KCl solution.
- (4) PT-Ac-RNase IIA in 1.2 M KCl solution.

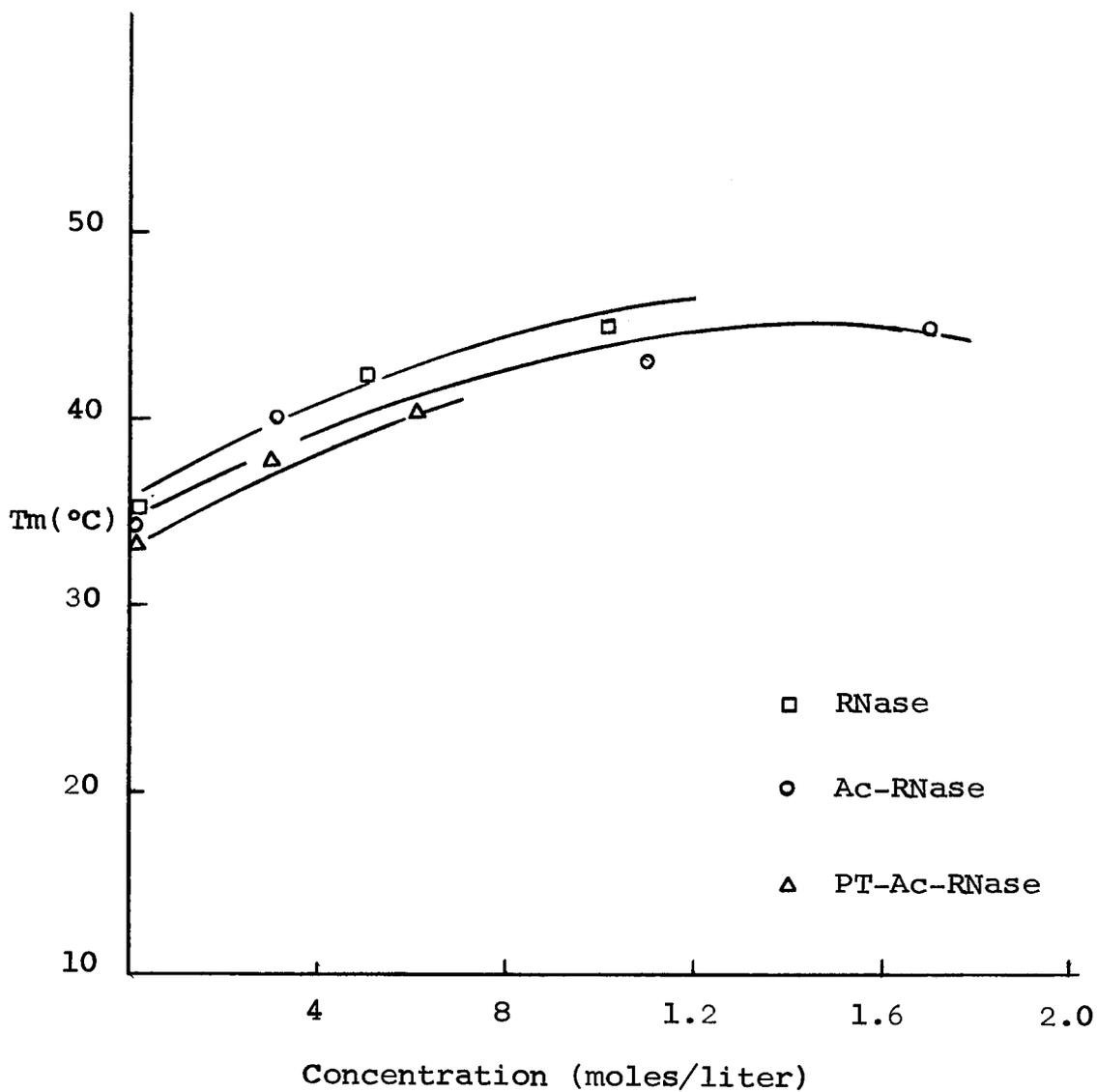


Figure 13. Transition temperature of polytyrosyl acetamidated ribonuclease IIA (concentration =  $4.6 \times 10^{-5}$  M) in various concentrations of KCl. Transition temperatures of Ac-RNase and native RNase in various concentrations of KCl were plotted for comparison.

tyrosine residues in the protein molecule.

### 13. Effect of $\text{CaCl}_2$ on $T_m$ at pH 2.1

Calcium chloride was found to be a potent destabilizer of the conformation of RNase at neutral pH (von Hippel and Wong, 1965). It was also found to diminish the size of transition when  $T_m$  was shifted on increasing salt concentrations. It is of interest to examine the potency of this salt on PT-Ac-RNase at pH 2.1.

PT-Ac-RNase IIA, Ac-RNase II and native RNase were all tested with calcium chloride dihydrate. Identical conditions of pH 2.1 and 0.06 M dilute salt were employed. In all cases, solid  $\text{CaCl}_2$  was introduced into the protein solution to make up the desired concentration of salt. The effect of  $\text{CaCl}_2$  on the  $T_m$  was in contrast to that observed at neutral pH. All  $T_m$ 's were shifted toward higher temperature. In Figure 14 are shown the melting profiles of PT-Ac-RNase IIA in the presence of various amounts of  $\text{CaCl}_2$ . A plot of  $T_m$  versus concentrations of the salt for PT-Ac-RNase IIA, Ac-RNase and native RNase is shown in Figure 15.

It is clear that the size of transition is diminished as the  $T_m$  is shifted toward high temperature, and the slope of each line in the plot of  $T_m$  versus concentration of salt remains approximately the same for all the derivatives in question. Although there is no qualitative difference among the derivatives examined,  $\text{CaCl}_2$ , however, stabilizes

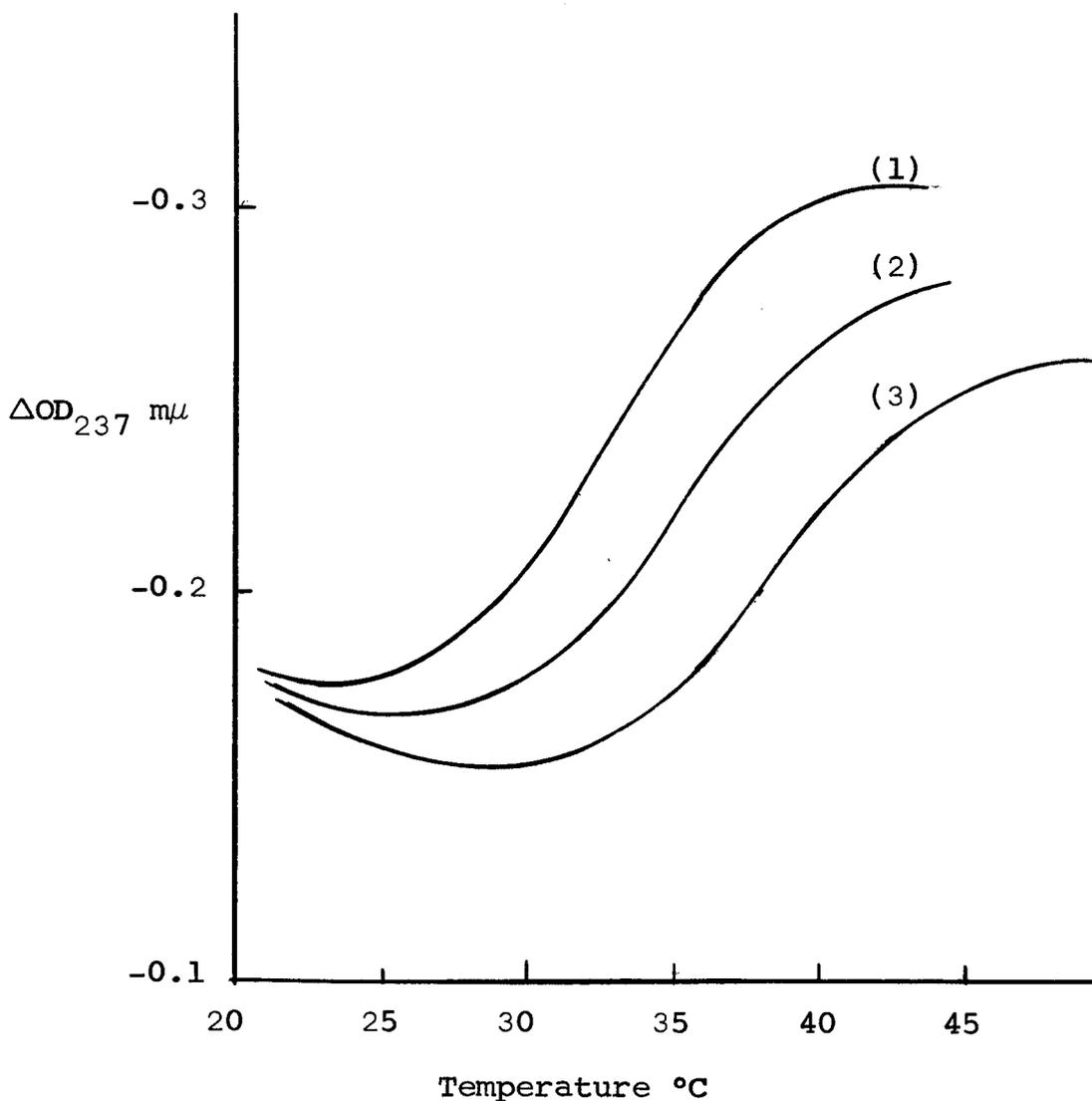


Figure 14. The heat-induced transition of polytyrosyl acetamidated ribonuclease IIA in various concentrations of  $\text{CaCl}_2$ . All samples (concentration equal to  $4.6 \times 10^{-5} \text{ M}$ ) were adjusted to pH 2.1 in KCl-HCl buffer.

- (1) PT-Ac-RNase IIA in 0.05 M KCl.
- (2) PT-Ac-RNase IIA in 0.1 M  $\text{CaCl}_2$ .
- (3) PT-Ac-RNase IIA in 0.2 M  $\text{CaCl}_2$ .

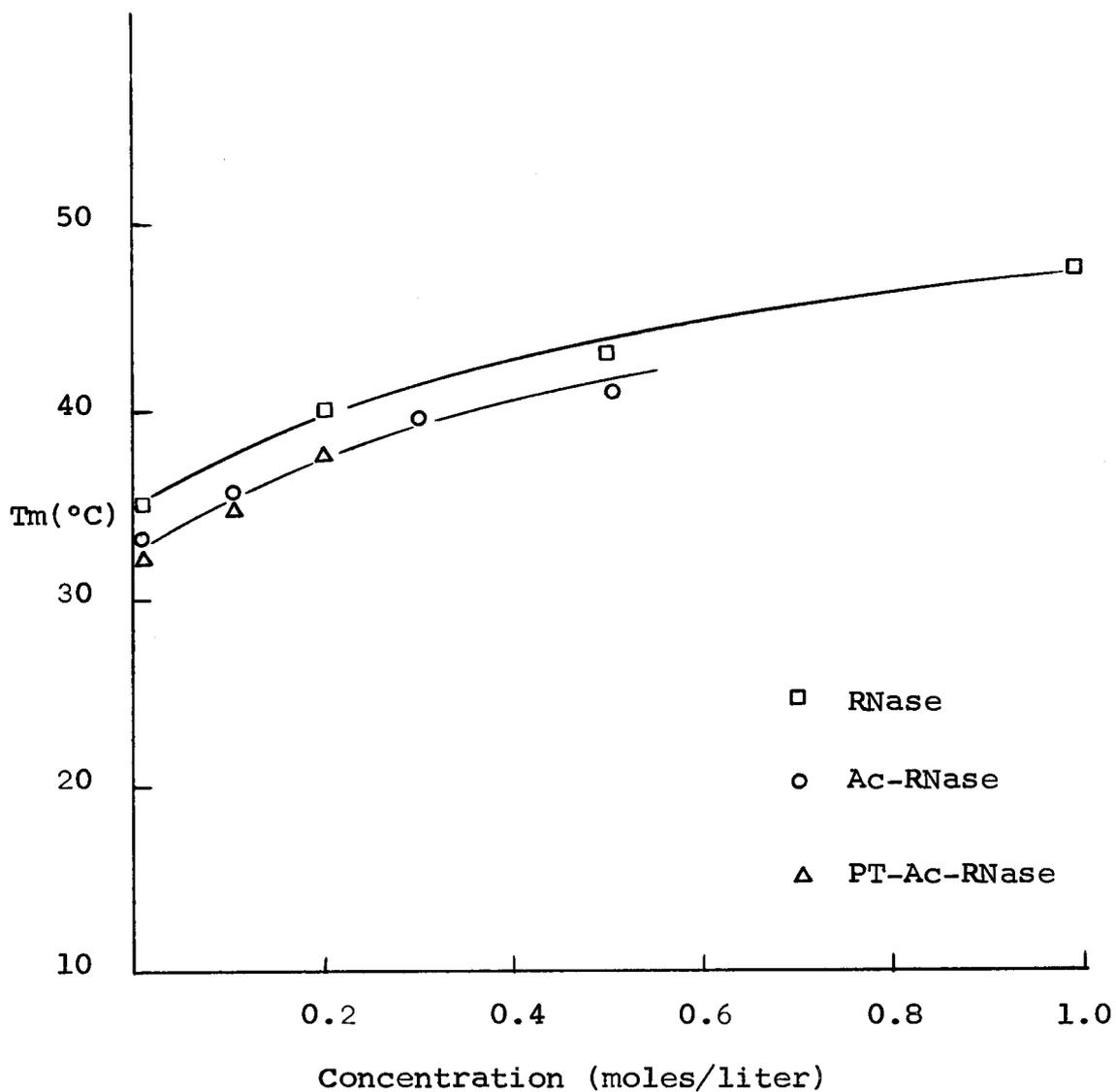


Figure 15. Transition temperature of polytyrosyl acetamidated ribonuclease IIA (concentration =  $4.6 \times 10^{-5}$  M) in various concentrations of  $\text{CaCl}_2$ . Transition temperatures of Ac-RNase and native RNase in various concentrations of  $\text{CaCl}_2$  were plotted for comparison.

the conformation of the RNase molecule at pH 2.1. Von Hippel and Wong (1965) found that at neutral pH, the effect is the reverse.

#### 14. Effect of KSCN on PT-Ac-RNase IIA at pH 2.1

Another potent destabilizer of conformation of RNase at neutral pH is potassium thiocyanate KSCN, reported by von Hippel and Wong (1965). This salt, however, exhibits some unusual phenomena on PT-Ac-RNase. At a concentration of  $4.6 \times 10^{-5}$  M of the derivative solution, 0.05 M KSCN could be dissolved in the protein solution causing a shift of  $T_m$  from 33 to 36. 0.1 M KSCN could also be dissolved further causing a shift of  $T_m$  to 37. But a concentration of 0.2 M KSCN immediately caused appearance of turbidity as a result of intermolecular interactions.

If the turbid solution was allowed to warm up to  $40^\circ$ , the turbidity disappeared. Upon cooling to room temperature turbidity reappeared. This whole process of appearance and disappearance of turbidity was reversible. A control with native RNase and Ac-RNase II at the same concentration as PT-Ac-RNase did not show this turbidity phenomenon. However, with higher concentration of KSCN about five times as that used in PT-Ac-RNase, a reversible turbidity may be observed. As for RNase, a much higher concentration of KSCN could also effect the phenomenon just described. Since it was not possible to determine the  $T_m$

with more than 0.2 M KSCN present in PT-Ac-RNase IIA solution, the change of size of transition could not readily be ascertained. With native RNase, the size of transition was diminished five times as much as at the 0.2 M concentration.

Figure 16 shows the plot of  $T_m$  versus concentration of KSCN for native RNase, Ac-RNase and PT-Ac-RNase II. There is definitely a qualitative difference in all the three lines. KSCN appears to stabilize the conformation of PT-Ac-RNase by its ability to shift the  $T_m$  toward higher temperature. But it is with some reservation to assess the extent of stabilization as high concentration than 0.2 M KSCN cannot be used. While KSCN has a destabilizing effect on the native RNase, it has little effect on the Ac-RNase derivative.

The effect observed in PT-Ac-RNase must be due to the presence of additional tyrosine side-chains. Because  $SCN^-$  is a polarizable ion, its ability of affecting the vicinal water structures around the hydrophobic side chains enhances the attractive apolar interactions. Since the hydrophobic bond becomes more stable at high temperature (Nemethy and Scheraga, 1962), it is not unreasonable to suggest that the  $T_m$  shift toward higher temperature is a result of enhancing the hydrophobic interactions.

It was observed that formation of turbidity was a function of pH. At high pH there was no turbidity

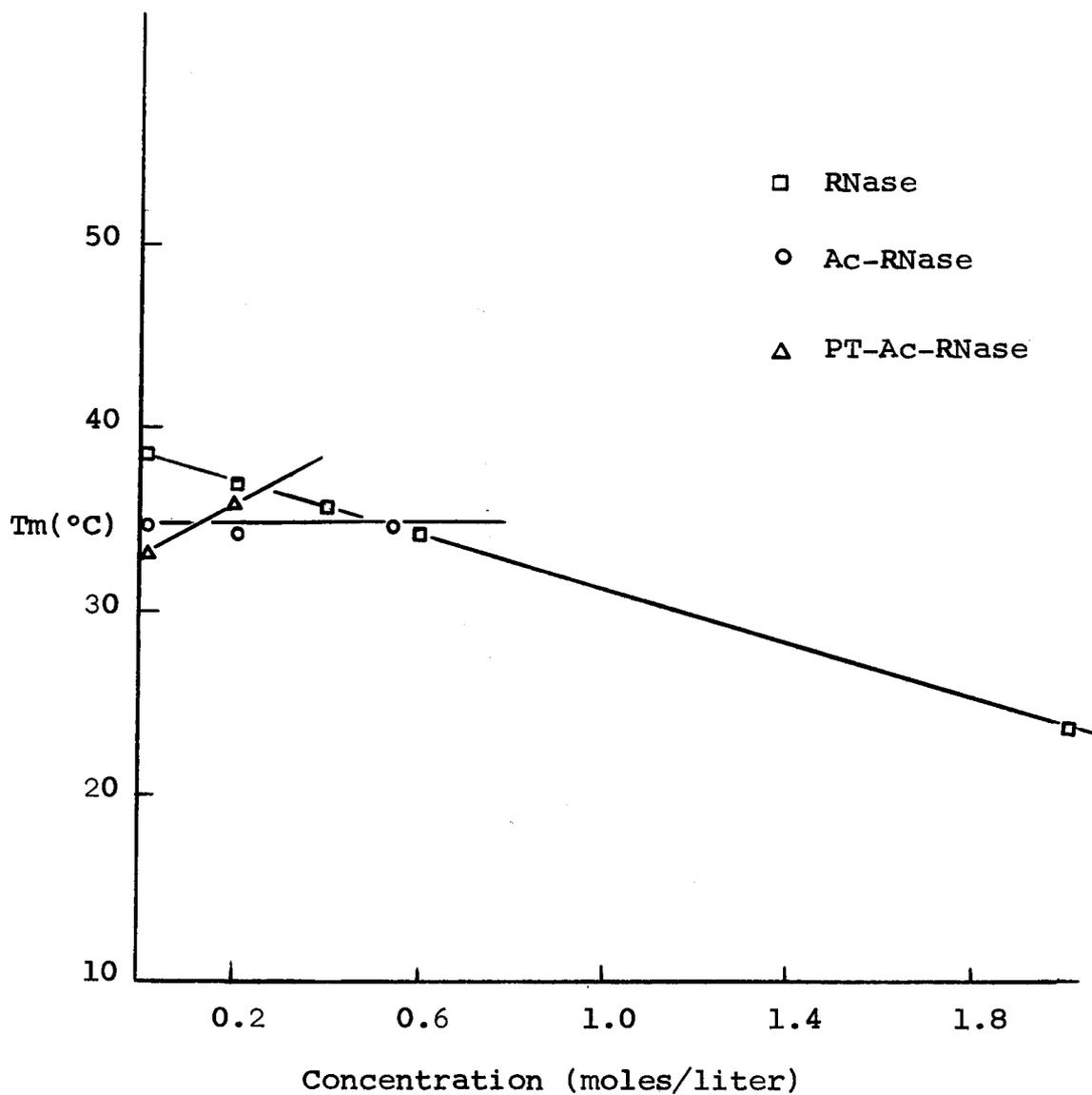


Figure 16. Transition temperature of polytyrosyl acetamidated ribonuclease IIA (concentration =  $4.6 \times 10^{-5}$  M) in various concentrations of KSCN. Transition temperatures of Ac-RNase and native RNase were plotted for comparison.

formation when a constant amount of KSCN was used throughout the pH range. The extent of turbidity was determined by measuring the optical density at 320  $m\mu$ . In Table III are tabulated the optical densities as a function of pH values for native RNase, Ac-RNase II and PT-Ac-RNase IIA. The optical densities were determined after introducing 0.5 M KSCN into the respective protein solutions whose concentrations are shown in the table. The pH and optical density measurements were taken after five minutes to insure formation of maximal turbidity.

TABLE III. TURBIDITY FORMATION AS A FUNCTION OF pH IN 0.5 M KSCN

pH	RNase $2.2 \times 10^{-5}$ M	Ac-RNase II $2.2 \times 10^{-5}$ M	PT-Ac-RNase IIA $1.2 \times 10^{-5}$ M
1.35	0.06	0.16	1.25
2.40	0.05	0.04	1.10
4.05	0.05	0.03	0.03
5.00	0.05	0.03	0.01
5.80	0.05	0.03	0.02
6.80	0.05	0.03	0.03

#### 15. Kinetics Studies of KSCN on PT-Ac-RNase IIA

Kinetic studies involving the KSCN and PT-Ac-RNase IIA system at low pH values may be informative with respect to the mechanism of action of the salt. Into a solution of PT-Ac-RNase of concentration  $1.2 \times 10^{-5}$  M, buffered at a pH with 0.05 M ionic strength was added solid KSCN to make 0.5 M concentration. Mixing was accomplished in less

than 1 second, and the optical density at 320  $m\mu$  was followed with time at about 26°. The rates of turbidity formation at various pH values are shown in Figure 17(A).

If the rates of turbidity formation are plotted against the pH as shown in Figure 17(B), a leveling off at around pH 4.0 is observed. As the formation of turbidity occurred rapidly at pH 2.0 and below, the rates unfortunately could not be precisely determined and consequently it is shown as the dotted line in the figure.

If each of the turbid solutions was heated in the same manner as in the melting experiments with the exception that the monitoring was done at 320  $m\mu$ , a sigmoidal curve was observed for each pH value. But turbidity did not become diminished upon cooling to room temperature and this meant that the system was irreversible. Since the equilibrium at any temperature cannot be established, the S-shaped curves obtained as a result of the thermal-induced aggregation do not have any physical significance. However, the size of the sigmoidal curve became diminished as the pH was decreased. This observation is consistent with Figure 17(B) in which turbidity formation is shown to reach the higher values at lower pH's. At low pH the extent of turbidity formation had reached a maximum and further heating could not cause more aggregation.

Since a concentration of 0.2 M KSCN could precipitate the PT-Ac-RNase derivative and not the native RNase or

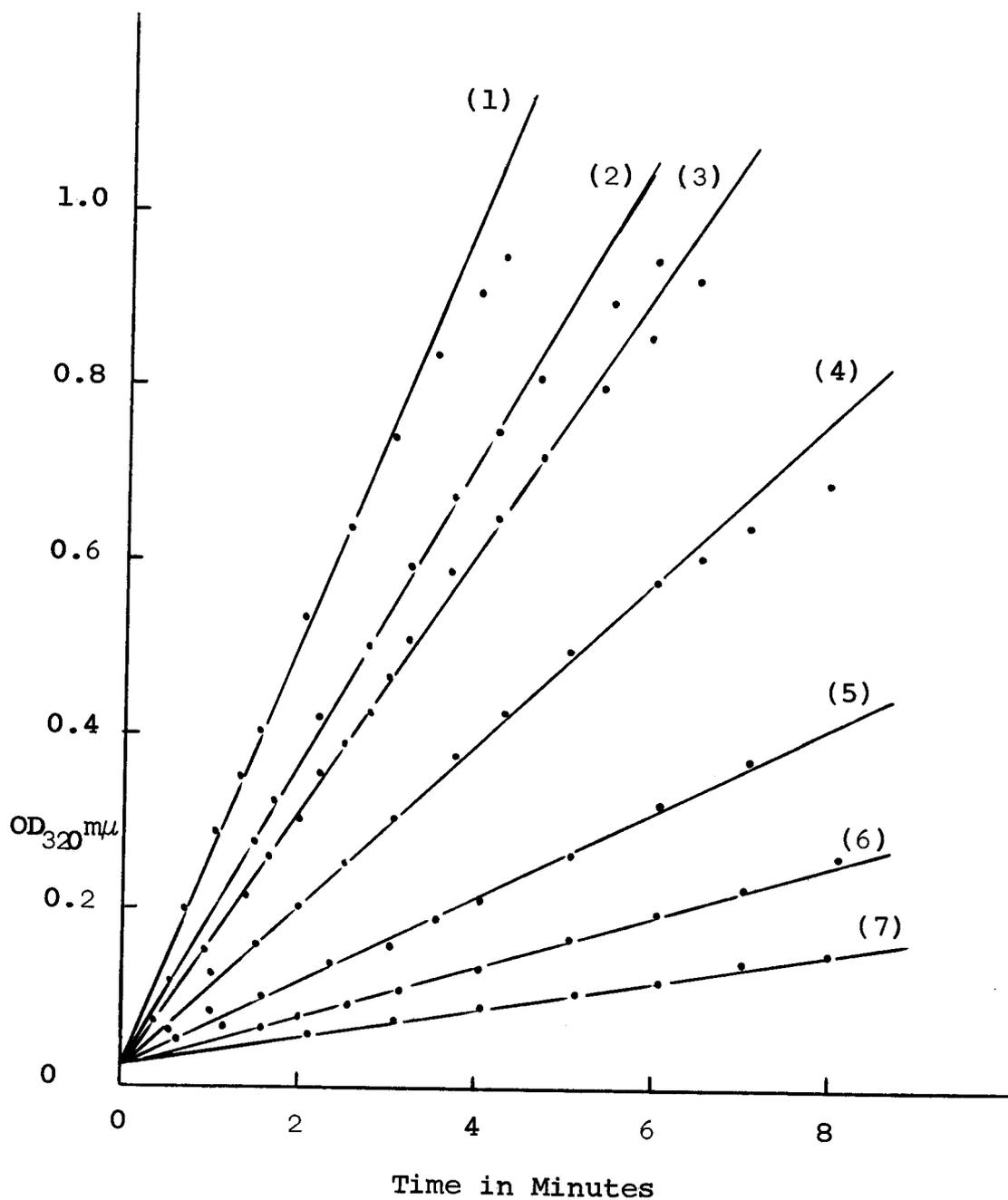


Figure 17(A). Rate of turbidity formation of polytyrosyl acetamidated IIA (concentration  $1.2 \times 10^{-5}$  M) under the influence of various values of pH and in 0.5 M KSCN. (1) pH 2.6; (2) pH 2.8; (3) pH 3.0; (4) pH 3.2; (5) pH 3.4; (6) pH 3.6; (7) pH 3.8.

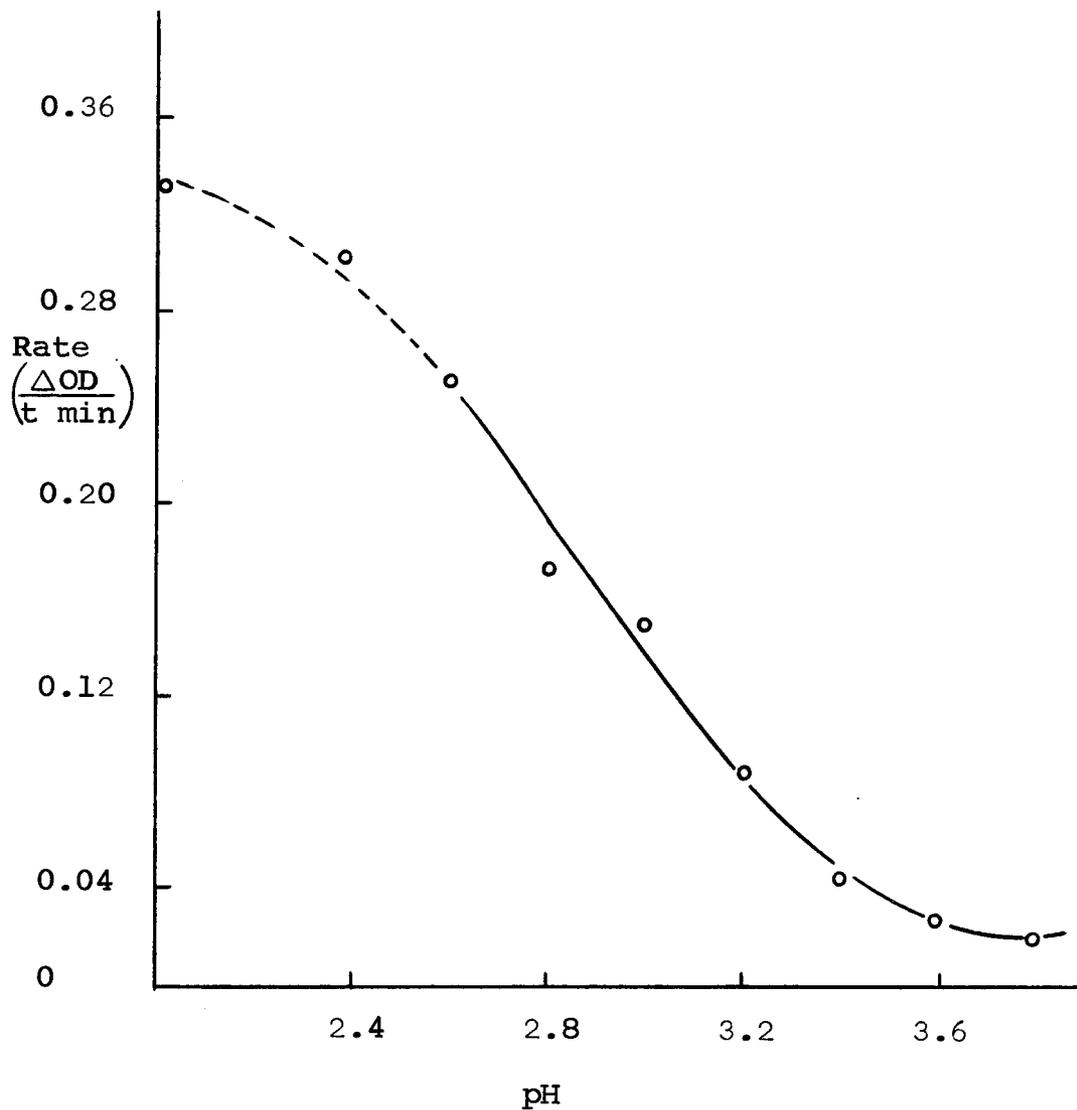


Figure 17(B). A plot of rates of turbidity formation of polytyrosyl acetamidated ribonuclease IIA as a function of pH. All samples had a concentration of  $1.2 \times 10^{-5}$  M PT-Ac-RNase IIA and 0.5 M KSCN.

acetamidinated RNase, the presence of extra tyrosine residues in the PT-Ac-RNase derivative must account for this phenomenon of aggregation. That a low concentration of KSCN could induce turbidity formation better than KCl (See Figure 12) probably argues for the greater polarizability of the thiocyanate ion. Because of the high polarizability of the thiocyanate ion, the effect of KSCN on the structure of the solvent may conceivably be more striking than that of KCl. At 0.2 M KSCN, the rearrangement of water structures around the thiocyanate ion, which behaves as a competitor for solvent molecules, promotes the tyrosine-tyrosine interaction through intermolecular hydrophobic bonding. Consequently, the strong interaction results in turbidity formation or in a "salting out" process.

#### 16. Thermodynamic Treatment of Denaturation Data in $\text{CaCl}_2$

The two-state approximation of Schellman (1955) has been applied to the thermally induced transitions of RNase and the derivatives in this work. This theory states that a protein molecule occurs in either a native or a denatured state, and in a reversible denaturation, the native state is in equilibrium with the denatured state. Hence, at any temperature,  $t$ , the fraction of molecules,  $f_d$ , in the denatured state can be calculated from the expression as shown (Schellman, 1955; Hermans and Scheraga, 1961; Ginsburg and Carroll, 1965)

$$f_d = 1 - f_n = \frac{\Delta(\text{OD}_\lambda)_t}{\Delta(\text{OD}_\lambda)_{\max}} = \frac{\Delta(\epsilon_\lambda)_t}{\Delta(\epsilon_\lambda)_{\max}}$$

where  $\text{OD}_\lambda$  is the optical density at any wavelength and  $\epsilon$  the molar absorptivity at the corresponding wavelength. The value of  $\Delta\text{OD}_{\max}$  is computed from the difference between the high and low temperature values while  $\Delta\text{OD}_t$  is the value at any temperature,  $t$ .

The results of the calculation of  $f_d$  for RNase, Ac-RNase II and PT-Ac-RNase IIA and PT-Ac-RNase IA in a dilute salt medium as well as in various concentrations of  $\text{CaCl}_2$  are shown in Figure 18(A), (B), (C), and (D). It is noticed that the curves are similar to those in Figure 15. As for the degree of cooperativeness which is defined according to von Hippel and Wong (1963) as the temperature difference between  $f_d = 0.25$  and  $f_d = 0.75$ , there is no detectable difference in RNase and in the derivatives.

If the equilibrium constant for the thermal denaturation process is defined by  $K_{\text{obs}} = \frac{f_d}{1-f_d}$  a van't Hoff plot can be constructed by plotting  $\text{Rln}K_{\text{obs}}$  versus the reciprocal of the absolute temperature. The change of standard-state enthalpy  $\Delta H_{\text{obs}}$  is then obtained from the slope of the plot with the assumption that  $\Delta H_{\text{obs}}$  is independent of temperature. The transition temperature,  $T_m$ , is thus defined as that temperature at which the standard-state free energy change is equal to zero. The standard-state entropy change can be estimated from  $\Delta H_{\text{obs}}$  divided by  $T_m$ .

The data as obtained from Figure 18(A), (B), (C), and (D) are used to construct the van't Hoff plots and are shown in Figure 19(A), (B), (C), and (D). Curved portions of the plots are noted in the high temperature regions in all cases examined. In dilute salt solution, however, the figures for the PT-Ac-RNase derivatives exhibit curved portions both at high and low temperatures. But at higher  $\text{CaCl}_2$  salt concentrations, the curved portions are not noticeable in all cases. Only steeper slopes for each protein derivative are detected as the concentration of  $\text{CaCl}_2$  is increased. Computations from the straight portions of the lines also reveal an increase in  $\Delta H_{\text{obs}}$  with increasing  $\text{CaCl}_2$  concentration. In Table IV are shown the values of the thermodynamic parameters for RNase, Ac-RNase II, PT-Ac-RNase IIA and PT-Ac-RNase IA.

TABLE IV. THERMODYNAMIC PARAMETERS FROM THE REVERSIBLE DENATURATION OF RNase AND DERIVATIVES IN DILUTE SALT AND IN  $\text{CaCl}_2$

CaCl <sub>2</sub> conc.	RNase			
	0.05	0.1	0.5	1.0
$\Delta\epsilon_{287}$	1580	--	--	--
$\Delta\epsilon_{237}$	4500	--	--	--
$T_m$ (°C)	35	40	43	47
$\Delta H_{\text{obs}}^\circ$ (kcal)	85 <sup>±4</sup>	100 <sup>±5</sup>	103 <sup>±5</sup>	105 <sup>±5</sup>
$\Delta S^\circ$ (eu)	278 <sup>±14</sup>	319 <sup>±16</sup>	236 <sup>±12</sup>	328 <sup>±16</sup>
$\Delta T$ (°C)	4.5	4.5	4.5	4.5

TABLE IV (Continued)

Ac-RNase				
CaCl <sub>2</sub> conc.	0.05	0.1	0.3	0.5
$\Delta\epsilon_{287}$	1550	--	--	--
$\Delta\epsilon_{237}$	4400	--	--	--
T <sub>m</sub> (°C)	33	36.5	39.5	42
$\Delta H_{obs}^{\circ}$ (kcal)	80 <sup>+4</sup>	85 <sup>+4</sup>	95 <sup>+5</sup>	105 <sup>+5</sup>
$\Delta S^{\circ}$ (eu)	261 <sup>+13</sup>	275 <sup>+14</sup>	304 <sup>+15</sup>	334 <sup>+16</sup>
$\Delta T$ (°C)	5.3	4.5	4.3	4.1
PT-Ac-RNase I				
CaCl <sub>2</sub> conc.	0.05	0.1	0.3	0.5
$\Delta\epsilon_{287}$	1100	--	--	--
$\Delta\epsilon_{237}$	3300	--	--	--
T <sub>m</sub> (°C)	33	36.5	38.5	41
$\Delta H_{obs}^{\circ}$ (kcal)	75 <sup>+4</sup>	90 <sup>+5</sup>	105 <sup>+5</sup>	115 <sup>+6</sup>
$\Delta S^{\circ}$ (eu)	245 <sup>+12</sup>	291 <sup>+15</sup>	337 <sup>+16</sup>	366 <sup>+18</sup>
$\Delta T$ (°C)	5	4.5	4.5	4.5
PT-Ac-RNase II				
CaCl <sub>2</sub> conc.	0.05	0.1	0.3	0.5
$\Delta\epsilon_{287}$	970	--	--	--
$\Delta\epsilon_{237}$	2800	--	--	--
T <sub>m</sub> (°C)	33	36	38.5	41.5
$\Delta H_{obs}^{\circ}$ (kcal)	80 <sup>+4</sup>	90 <sup>+5</sup>	100 <sup>+5</sup>	120 <sup>+6</sup>
$\Delta S^{\circ}$ (eu)	261 <sup>+13</sup>	291 <sup>+15</sup>	321 <sup>+16</sup>	382 <sup>+19</sup>
$\Delta T$ (°C)	5.5	4.7	4.5	4.0

It is noted that CaCl<sub>2</sub> not only stabilizes the conformation of the protein molecule by shifting the T<sub>m</sub> to

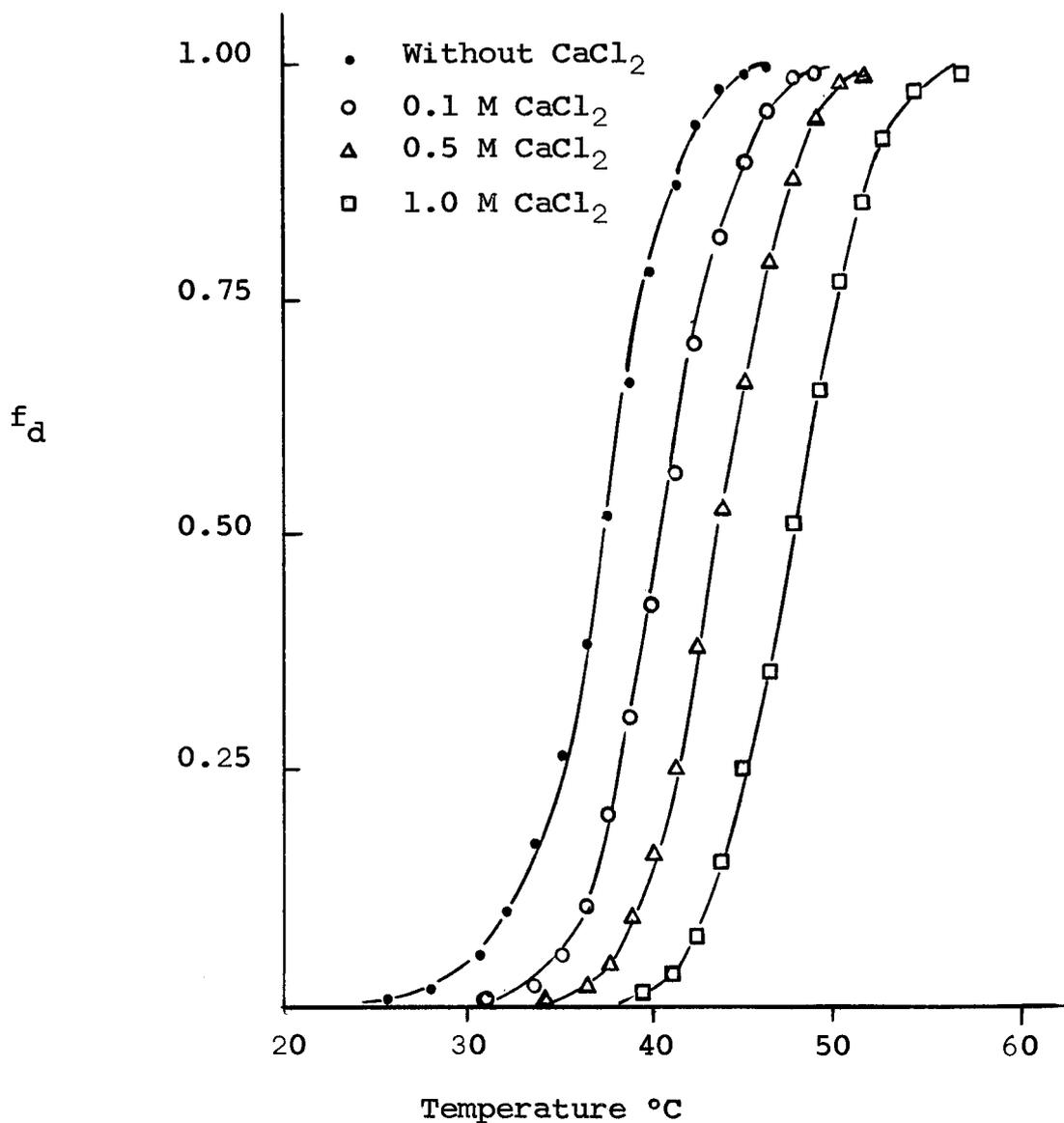


Figure 18(A). The heat-induced transition of ribonuclease in various concentrations of  $\text{CaCl}_2$  at pH 2.1. The fractions of denatured molecules ( $f_d$ ) which are calculated from the temperature difference spectra (see text) are plotted as a function of temperature.

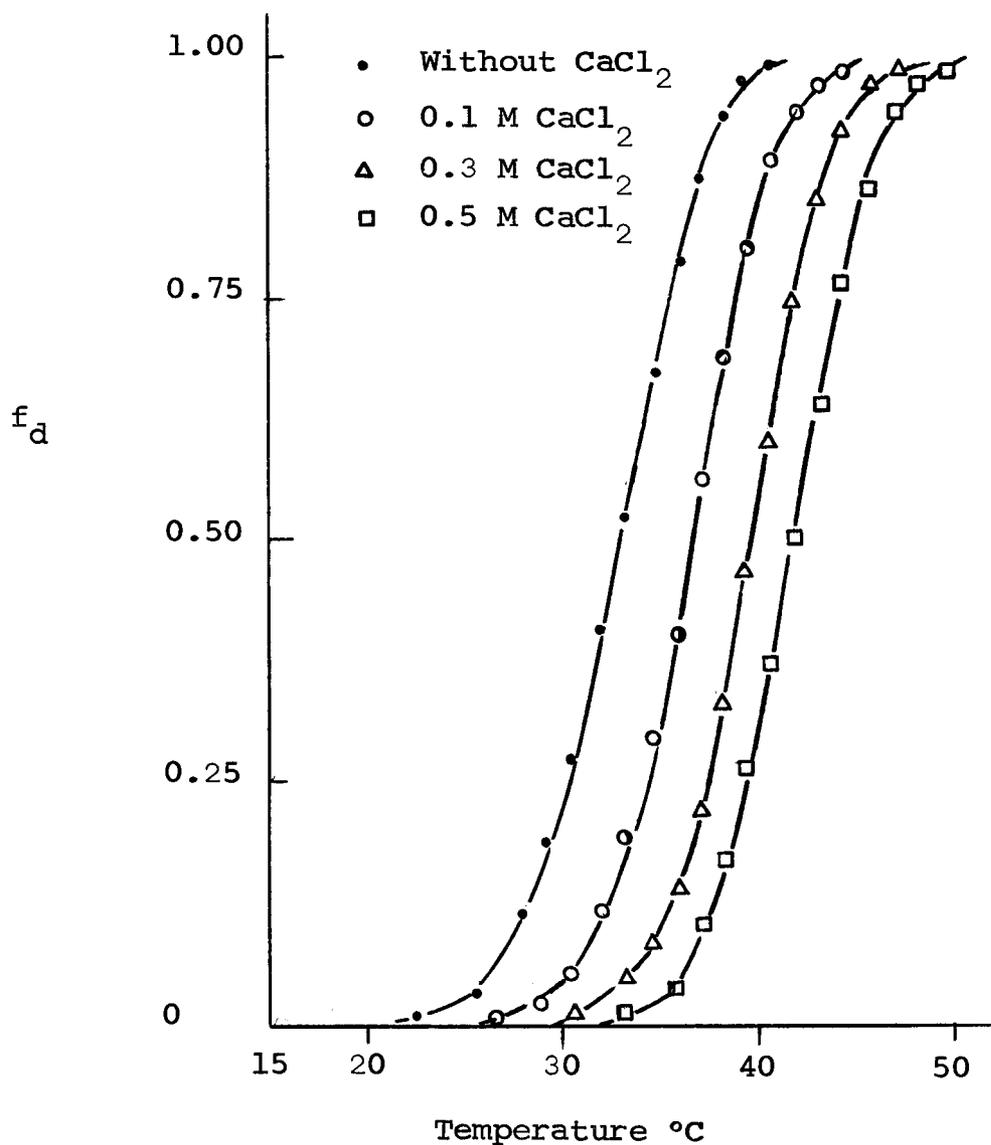


Figure 18(B). The heat-induced transition of acetaminated ribonuclease in various concentrations of  $\text{CaCl}_2$  at pH 2.1. The fractions of denatured molecules ( $f_d$ ) which are calculated from the temperature difference spectra (see text) are plotted as a function of temperature.

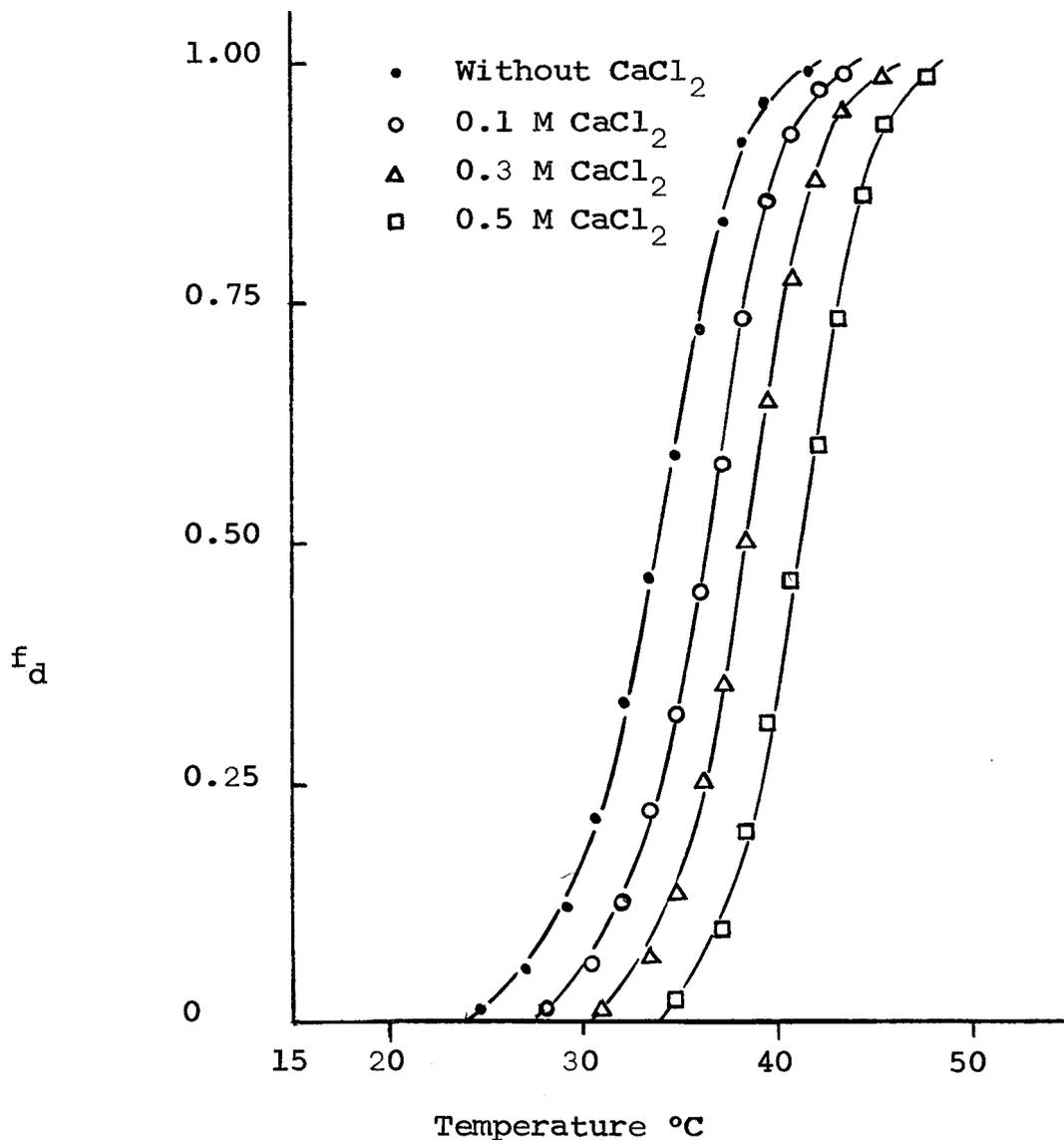


Figure 18(C). The heat-induced transition of polytyrosyl acetamidated ribonuclease IA in various concentrations of  $\text{CaCl}_2$  at pH 2.1. The fractions of denatured molecules ( $f_d$ ) which are calculated from the temperature difference spectra (see text) are plotted as a function of temperature.

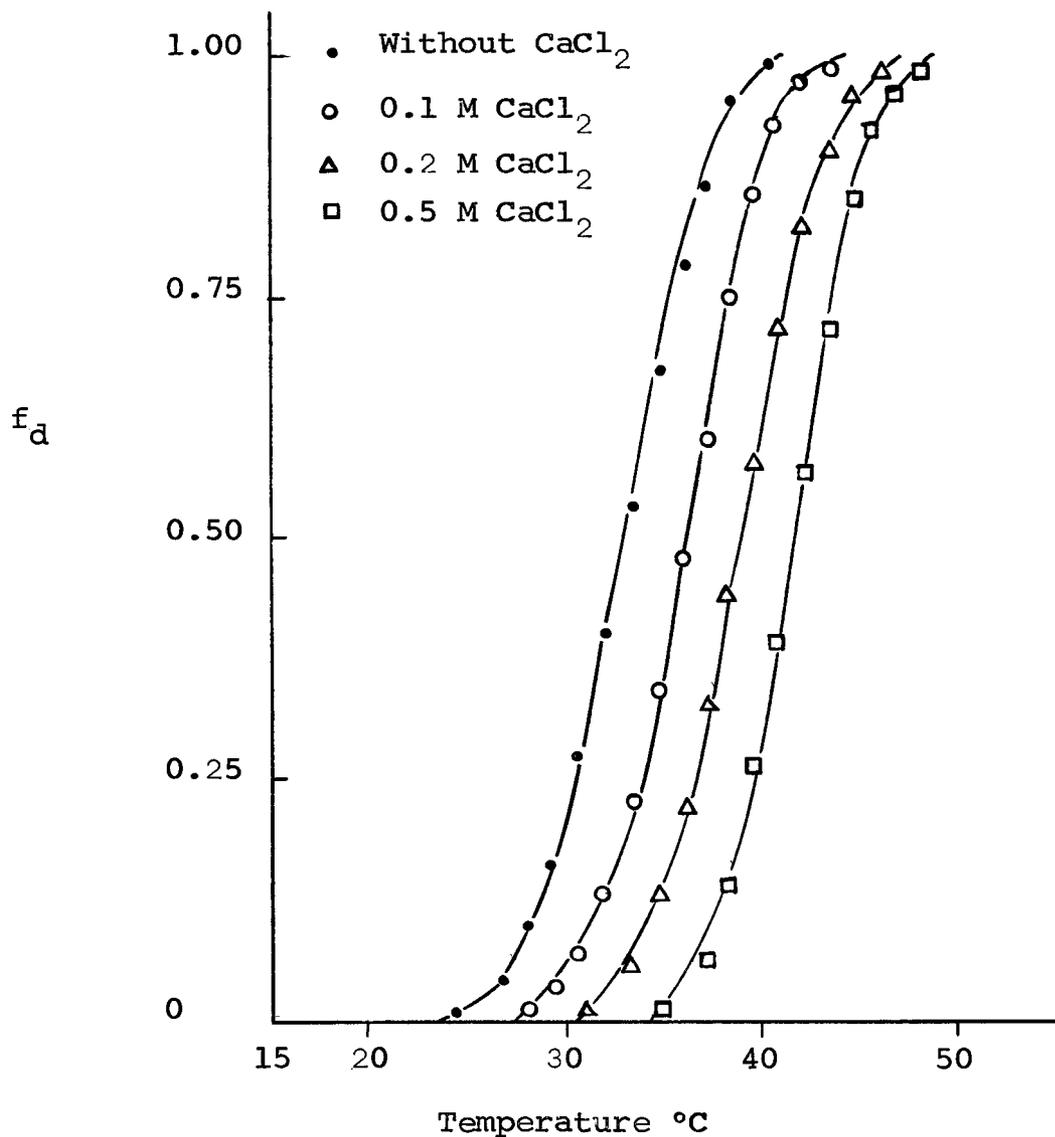


Figure 18(D). The heat-induced transition of polytyrosyl acetamidated ribonuclease IIA in various concentrations of  $\text{CaCl}_2$  at pH 2.1. The fractions of denatured molecules ( $f_d$ ) which are calculated from the temperature difference spectra (see text) are plotted as a function of temperature.

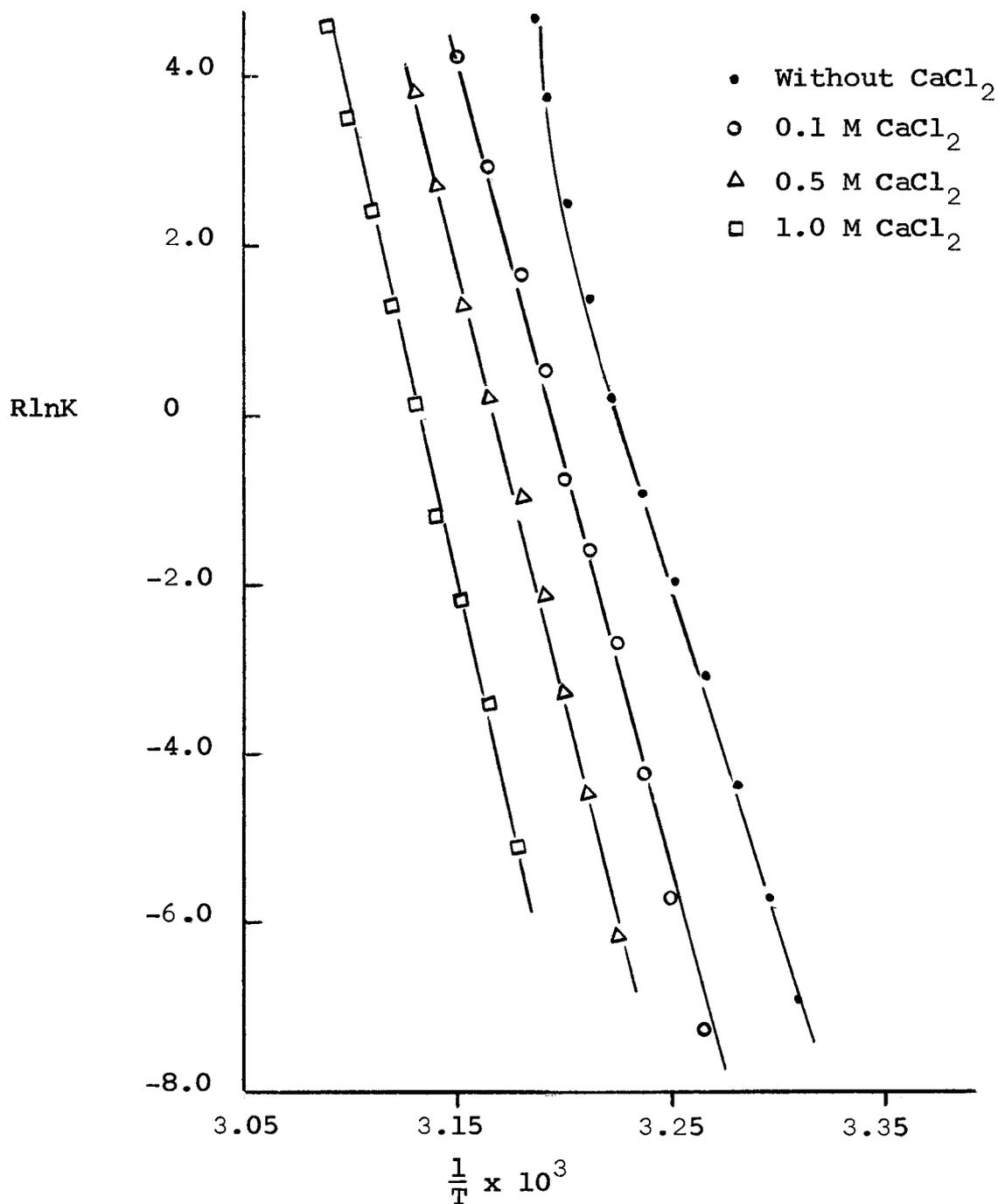


Figure 19(A). Van't Hoff plots of the thermal denaturation of ribonuclease at pH 2.1. The product of the gas constant ( $R$ ) multiplied by the natural logarithm of the equilibrium constant for the thermal denaturation reaction in various concentrations of  $\text{CaCl}_2$  is plotted against the reciprocal of the absolute temperature.

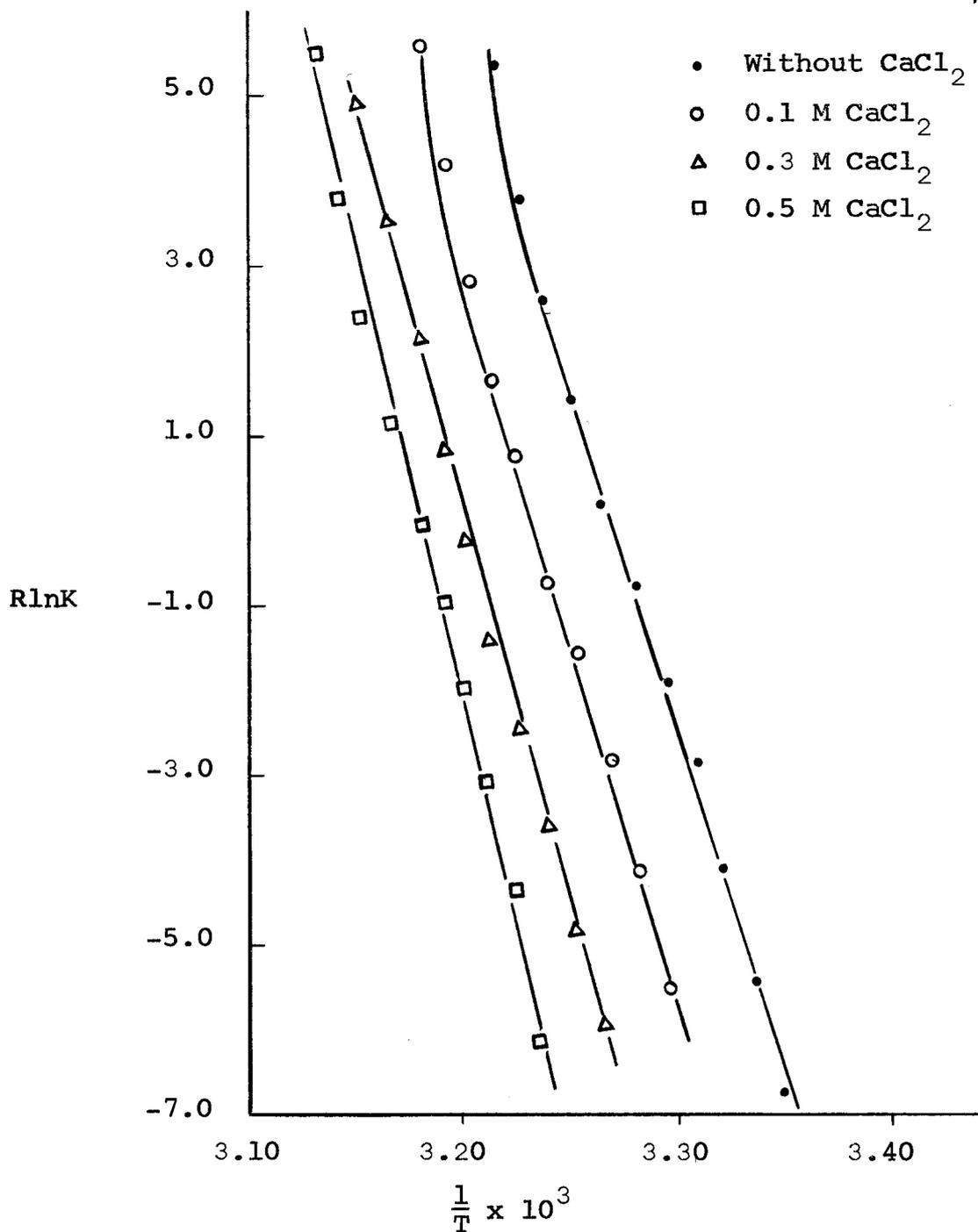


Figure 19(B). Van't Hoff plots of the thermal denaturation of acetamidinated ribonuclease at pH 2.1. The product of the gas constant ( $R$ ) multiplied by the natural logarithm of the equilibrium constant for the thermal denaturation reaction in various concentrations of  $\text{CaCl}_2$  is plotted against the reciprocal of the absolute temperature.

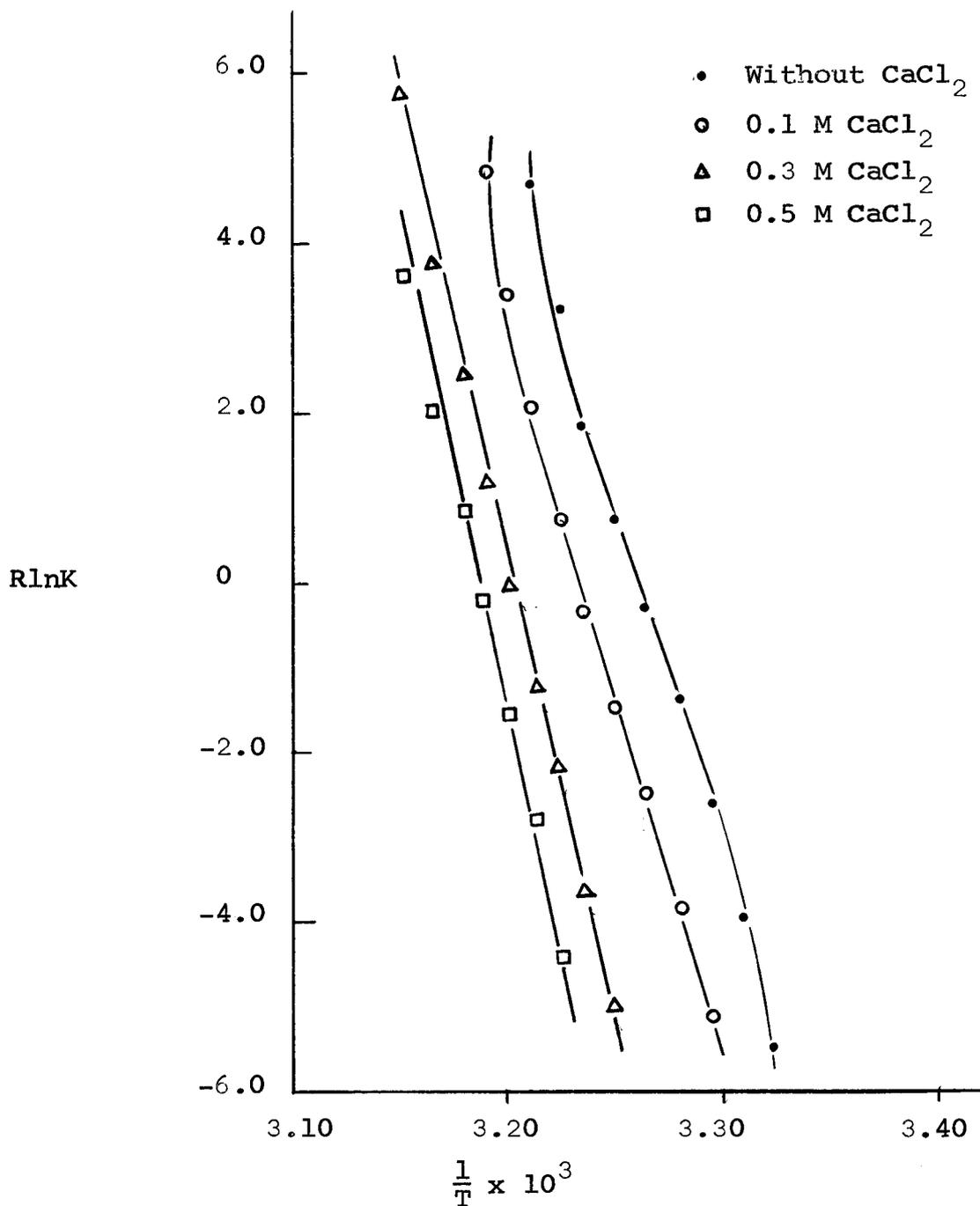


Figure 19(C). Van't Hoff plots of the thermal denaturation of polytyrosyl acetamidated ribonuclease IA at pH 2.1. The product of the gas constant ( $R$ ) multiplied by the natural logarithm of the equilibrium constant for the thermal denaturation reaction in various concentrations of  $\text{CaCl}_2$  is plotted against the reciprocal of the absolute temperature.

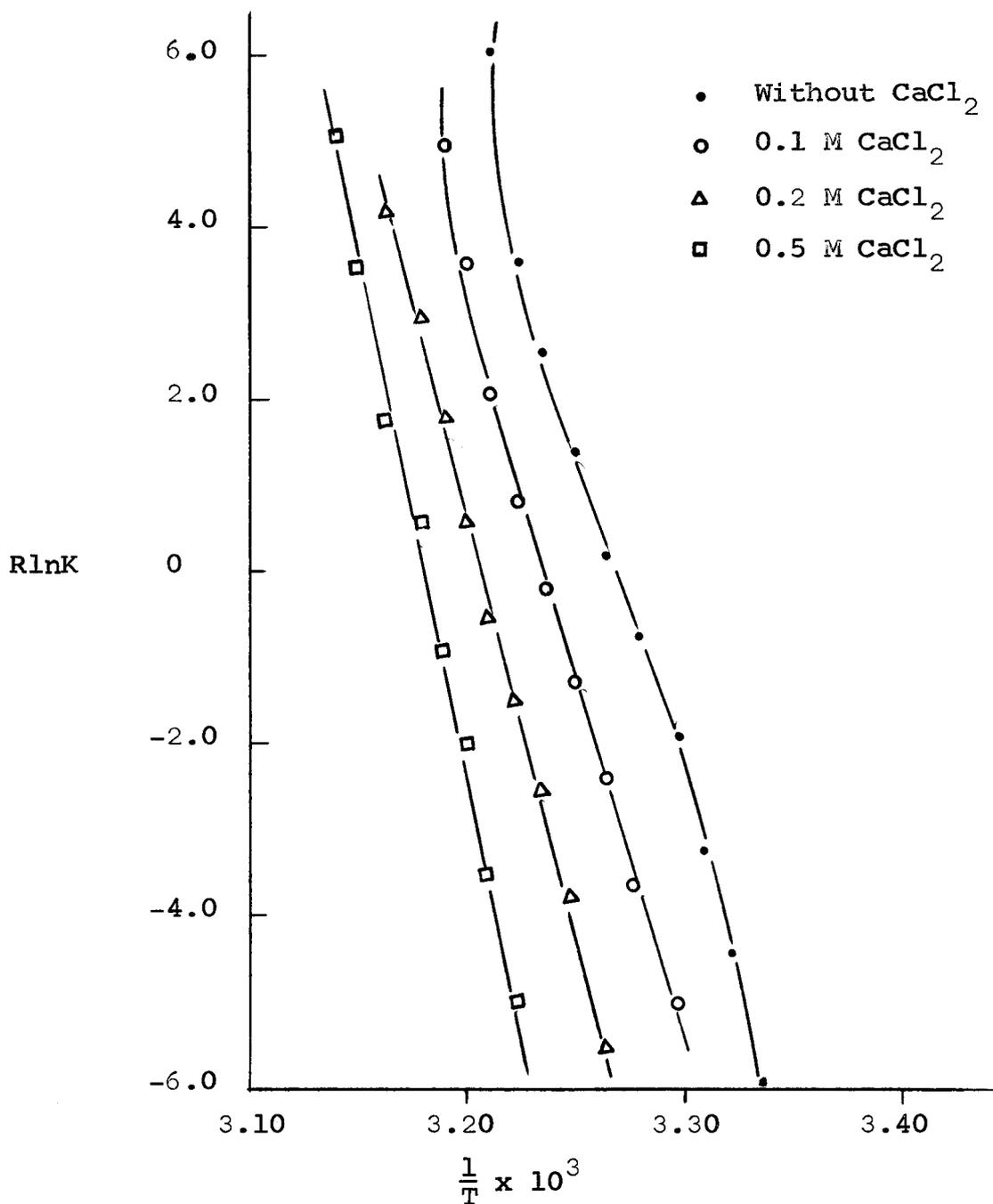


Figure 19(D). Van't Hoff plots of the thermal denaturation of polytyrosyl acetamidated ribonuclease IIA at 2.1. The product of the gas constant ( $R$ ) multiplied by the natural logarithm of the equilibrium constant for the thermal denaturation reaction in various concentrations of  $\text{CaCl}_2$  is plotted against the reciprocal of the absolute temperature.

higher temperature, but also changes the apparent values of  $\Delta H_{\text{obs}}$ . Although the increasing  $\Delta H_{\text{obs}}$  with increasing temperature at high temperatures had been reported (Harrington and Schellman, 1956; Hermans and Scheraga, 1961), the skewed portions at high temperatures are no longer detectable in high  $\text{CaCl}_2$  concentration, but distinguishable at low salt concentration. Various explanations have been advanced to account for the skewed portions. There is the view that intermediates exist in equilibrium with either the native or the completely denatured state. A statistical treatment on the helix-coil transition of a polypeptide of a given length has permitted Poland and Scheraga (1965) to suggest a gradual "unwinding" of the native molecule on thermal denaturation, and the all-or-none process, according to their view, cannot justify the enthalpy and entropy changes.

A second view argues that a two-state approximation is sufficient when the heat capacity of the system is taken into consideration. It was reported that the change in heat capacity for RNase was independent of pH but dependent on temperature (Brandts, 1965). The van't Hoff plot thus obtained, however, still shows the skewed portion at high temperatures.

In the van't Hoff plot for PT-Ac-RNase derivatives, it is noticed that the skewed portions are more apparent at low temperatures than the native RNase and Ac-RNase.

It is possible to conceive that the added tyrosine polypeptides increase the change of heat capacity thus perturbing the equilibrium between the native and denatured state. As a result, the van't Hoff plot is obtained as shown in the figures. Because of the complexity of Poland and Scheraga treatment, it is impossible to apply the theory quantitatively to the experimental data. The thermodynamic data obtained from the van't Hoff plots have, therefore, only qualitative significance.

Inspection of Table IV reveals that the  $\Delta\epsilon_{287}$  and  $\Delta\epsilon_{237}$  values for the PT-Ac-RNase derivatives are about two-thirds of those values for the native RNase and Ac-RNase. In light of such evidence it has been reported that in the melting of RNase, two tyrosyl residues are normalized, that is to say, transferred from a "buried" environment to one that is exposed to solvent (Hermans and Scheraga, 1961; Scott and Scheraga, 1963). An explanation may be acceptable if one assumes that the added tyrosyl groups protect the normalization of one of these two tyrosyl residues of RNase. Instead, through hydrophobic bonding, only one tyrosine is normalized in the PT-Ac-RNase molecule upon thermal denaturation.

## V. SUMMARY AND CONCLUSIONS

Polypeptidyl derivatives of RNase containing a limited number of chains of tyrosine peptides have been prepared. Studies of these derivatives have yielded some valuable information with regard to interactions, either inter- or intramolecular, of the added tyrosine residues with the parent protein molecule.

Dinitrophenylation results show that eight moles of tyrosine are attached to three sites of RNase in PT-Ac-RNase IA, and that four moles of tyrosine are attached to two sites of RNase in PT-Ac-RNase IIA. Enzymatic activities of PT-Ac-RNase and acetamidinated RNase derivatives were shown to be about two to ten percent of RNase by two independent methods of assay. These results indicate that the lysine in the position of 41 has been reacted and that tyrosine peptide chains may have been attached to the relatively unreactive lysine groups such as 7, 37 and 41 (Cooke, Anfinsen and Sela, 1963).

The spectrophotometric titration data reveal that the added tyrosine residues most probably reside on the surface and behave normally in the ionization process. The results of this and other experiments suggest that the conformation of the Ac-RNase and of PT-Ac-RNase derivatives is most probably the same as that of the native RNase molecule. At the sites of attachment of peptidyl chains,

some rearrangement of vicinal amino acid residues to accommodate the added residues is conceivable. However, if such changes exist, they are not apparent in spectrophotometric and optical rotatory measurements of polyalanyl and polyvalyl derivatives (Anfinsen, et al., 1962; Becker, 1962).

Although the added tyrosine residues have been assumed to reside on the surface of the RNase molecule, the results of temperature difference spectra suggest an intramolecular tyrosine-tyrosine interaction among the added tyrosine residues and the tyrosine of the parent molecule in the partially unfolded form. Since a strong intramolecular tyrosine-tyrosine interaction might exist at higher temperatures as hydrophobic bonding persists at temperatures between 50° and 70° (Nemethy and Scheraga, 1962), it may prevent one of the two "buried" tyrosine chromophores from being normalized by thermal denaturation. Such a process could bring about a decrease in  $\Delta\epsilon_{237 \text{ m}\mu}$  and  $\Delta\epsilon_{287 \text{ m}\mu}$  (see Table IV).

The more highly substituted PT-Ac-RNase derivatives were sparingly soluble at pH values between 5 and 9. The maximal insolubility was observed at pH 8.1, which is probably the isoelectric point for the derivative. At this pH the net charge on the protein is zero, and strong intermolecular interactions of the added hydrophobic groups favor the process of aggregation and turbidity ensues.

Different types of salt have been tested on PT-Ac-RNase, Ac-RNase, and native RNase with respect to their stabilizing or destabilizing effect on the protein conformation at low pH. The size of transition (overall  $\Delta OD$ ) is progressively decreased with increasing salt concentrations but the  $T_m$  is progressively shifted toward higher temperatures. It is the ion concentration and not the ion type that appears to stabilize the conformation of the protein molecule. This effect may be attributed to the nonspecific binding or shielding of charge by the added electrolytes, thus diminishing electrostatic interactions, which are responsible partially for the conformation of the protein molecule.

Potential hydrogen bond breakers such as urea and guanidine hydrochloride show a destabilizing effect on the conformation of PT-Ac-RNase by diminishing the size of transition and shifting the  $T_m$  to lower temperatures. These results point to a role for hydrogen bonding in the stabilization of the native conformation. Since the PT-Ac-RNase molecule becomes denatured at high temperatures, the change in stability of the molecule with temperature must involve the enthalpy terms due to interactions among which intramolecular hydrogen bonds may show a dominant temperature dependence (Scheraga, 1963).

The importance of hydrophobic bonding in the stabilization of the native conformation is reinforced when

PT-Ac-RNase is studied in the presence of salts. Presumably aggregation results from formation of intermolecular hydrophobic bonding. The effects of KCl and  $\text{CaCl}_2$  on the PT-Ac-RNase derivatives are similar to that described for the polyvalyl RNase system (Kettman, et al., 1966). At low concentrations of salt, higher temperatures were required to induce aggregation. As the salt concentration was increased, the rate of turbidity formation was faster and at a lower temperature. That only a low concentration of KSCN can induce aggregation when compared with KCl, argues for the greater polarizability of the thiocyanate ion than the chloride ion. But the aggregation of PT-Ac-RNase could be lessened and the aggregates dispersed in the presence of KSCN at a temperature greater than  $40^\circ$ . These inconsistent results pertaining to hydrophobic interaction may be attributed to tyrosine possessing a phenolic moiety. On the basis that tyrosine is found to be much less soluble in water at neutral pH than phenylalanine, it appears that the hydroxyl group of the phenolic moiety of tyrosine may contribute to the interactions in a different way. The solubility properties of polytyrosine derivatives cannot, therefore, be accounted for solely on the basis of hydrophobic interactions.

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