

AN ABSTRACT OF THE THESIS OF

THOMAS OLIVER TIFFANY for the DOCTOR OF PHILOSOPHY
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COMPONENT PARTS, S-PROTEIN AND S-PEPTIDE

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Dr. Robert R. Becker

Hydrophobic bonding has been suggested to be accompanied by a volume increase. Certain magnitudes have been established in the literature for the volume change resulting from the interaction of non-polar aliphatic and aromatic side chain residues. That there have been certain magnitudes established for the volume change accompanying these interactions has led to the investigation of this phenomenon.

The interaction of S-peptide with S-protein has been reported to involve mainly hydrophobic residues as determined from the X-ray data of ribonuclease-S at 3.5 Å resolution. Therefore ribonuclease-S and its component parts, S-peptide and S-protein, were selected as a model system for this investigation.

The study of the pressure effect on the reversible denaturation of ribonuclease-S and S-protein by difference spectroscopy has led to

the indirect determination of the volume change accompanying the denaturation by pressure of ribonuclease-S and S-protein. The values determined were -80 ± 2 cc/mole for ribonuclease-S and -45 ± 2 cc/mole for S-protein. The difference in these values is assumed to reflect the volume change due to dissociation of S-peptide from S-protein.

Dilatometric measurements were also made to determine the volume change of association and/or dissociation of S-protein and S-peptide. These studies were conducted in the dilatometer through the addition of S-peptide to S-protein, and through the addition of small amounts of acid to ribonuclease-S. A volume change of $+34 \pm 6$ cc/mole S-protein accompanied the addition of S-peptide to S-protein in the dilatometer. A volume decrease of 44 cc/mole to 56 cc/mole was obtained when small amounts of acid were added to ribonuclease-S in the dilatometer and the data were extrapolated to complete carboxylate titration at 25°. Correspondingly a volume decrease of 17 cc/mole to 29 cc/mole was obtained when a similar experiment was conducted at 15°. The difference in these two sets of values is assumed to reflect the volume change accompanying the conformational change of S-protein between 15° and 25°, while the values obtained for the volume change at the lower temperature are thought to reflect the dissociation of S-peptide from S-protein at acid pH.

Circular dichroism studies were conducted on S-protein and the

stepwise addition of S-peptide to S-protein as a function of temperature. The results of these studies indicate that the conformation of S-protein is extremely sensitive to temperature. The reduction of temperature from 26° to 21° was sufficient to cause the appearance of a maximum in the CD band at 237 m μ of S-protein which was similar in magnitude to the 240 m μ CD band of ribonuclease-S. Solutions for these studies were generally made up in 0.01 M sodium acetate buffer (pH 5.01) containing 0.1 M sodium chloride. The stepwise addition of S-peptide to S-protein resulted in a CD spectrum for ribonuclease-S' that did exhibit a 240 m μ CD band similar in magnitude to that of ribonuclease-S.

A plot of the change in the molar ellipticity at 275 m μ vs. S-peptide concentration to an excess S-peptide concentration (approximately seven moles S-peptide to one mole S-protein) allowed the determination of the molar ellipticity of ribonuclease-S'. A dissociation constant of 5×10^{-6} M was calculated for ribonuclease-S' in 0.1 M sodium acetate (pH 5.01) containing 0.1 M sodium chloride.

Some Physical Studies on Ribonuclease-S and its
Component Parts, S-Protein and S-Peptide

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Redacted for Privacy

Professor of Biophysics
substituting for Dr. Robert R. Becker

in charge of major

Redacted for Privacy

Head of Department of Biochemistry

Redacted for Privacy

Dean of Graduate School

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Typed by Clover Redfern for Thomas Oliver Tiffany

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SOME PHYSICAL STUDIES ON RIBONUCLEASE-S AND ITS COMPONENT PARTS, S-PROTEIN AND S-PEPTIDE

PART I. THE DETERMINATION OF THE VOLUME CHANGE ACCOMPANYING THE ASSOCIATION AND/OR DISSOCIATION OF RIBONUCLEASE-S USING HIGH PRESSURE AND DILATOMETRIC STUDIES

INTRODUCTION

The suggestion that "hydrophobic bonding" is accompanied by a volume increase has prompted this laboratory to investigate this phenomenon. Kauzmann (1959), in reference to the earlier work of Masterton (1954) on the transfer of small hydrocarbons into an aqueous environment, states that the formation of hydrophobic bonds should be accompanied by an expansion of 20 cc/mole of aliphatic side chains, and a lesser value for phenylalanine residues. Némethy and Scheraga (1962a, b, c), in a series of papers concerning the thermodynamics of hydrophobic bonding, indicate that the formation of "hydrophobic bonds" can be regarded as a partial reversal of the solvation process with a resulting calculated increase in volume of 3-8 cc/mole depending upon the nature of the side chain group and the extent of interaction. Since certain limits have been established for the magnitude and direction of the volume change resulting from "hydrophobic bonding" this laboratory decided to find a protein system that might allow experimental determination of these parameters.

The recently published X-ray structure of ribonuclease-S at 3.5 Å resolution by Wyckoff et al. (1967) indicates that the S-peptide portion is largely held in place by hydrophobic interactions of Ala 4, Phe 8, His 12, and Meth 13. For this reason Ribonuclease-S and its component parts, S-protein and S-peptide, have been selected as a model system in an attempt to obtain some gross indication as to the magnitude and direction of the volume change accompanying "hydrophobic bonding." This study acknowledges the precarious nature of attempting to attribute too much to hydrophobic bonding as the stabilizing factor in the S-peptide-S-protein interaction, as there clearly are other factors involved. A good example is the demonstration by Hoffman et al. (1966) of the importance of the Asp 14 residue to the overall binding of S-peptide to S-protein. They showed that S-peptide containing residues 1-13 required a molar ratio of approximately 30:1 to fully activate S-protein, whereas S-peptides composed of residues 1-15 and residues 1-16 activated S-protein fully at a molar ratio of 1:1. The X-ray data of ribonuclease-S at 3.5 Å resolution indicates that there is an electrostatic interaction between Asp 14 and Arg 33. However, it must be emphasized that the majority of the interaction as indicated by the data appears to be hydrophobic (Wyckoff et al. 1967). From the knowledge of the interactions involved as indicated by the X-ray data, one can make a gross estimation of the magnitude of volume change that should accompany the formation of

ribonuclease-S'.¹ If one assumes a volume increase of 8 cc per mole of aliphatic side chain interaction and 3 cc per mole of aromatic side chain interaction as suggested by Nemethy and Scheraga (1962b), and estimating a volume increase of 10-20 cc per mole of side chain electrostatic interaction, a total volume increase of approximately 30-40 cc per mole S-protein-S-peptide interaction should accompany the formation of ribonuclease-S'. Correspondingly, if one assumes a volume increase of 20 cc per mole aliphatic side chain interaction as suggested by Kauzmann (1959), a lesser value of 10 cc per mole aromatic side chain interaction, and again estimating 10-20 cc per mole increase for the electrostatic interaction, a total volume increase of approximately 70-80 cc per mole S-protein-S-peptide interaction should accompany the formation of ribonuclease-S'. It is the expressed purpose of this thesis then to determine the direction and magnitude of the volume change accompanying the association and/or dissociation of the S-peptide and S-protein, and to compare the experimental data with the above estimated magnitudes of volume change.

¹Ribonuclease-S' is the commonly used notation indicating the active enzyme formed from the interaction of S-protein and S-peptide. Ribonuclease-S is the commonly used notation indicating the limited subtilisin digested bovine pancreatic ribonuclease-A. Ribonuclease-S is fully active and differs from ribonuclease-A only in the respect that the peptide linkage between residues 20 and 21 or 21 and 22 has been cleaved. (Doscher and Hirs, 1967).

The Indirect Determination of Volume Change From Pressure Studies

Both direct and indirect volume change measurements have been made in this laboratory in the form of dilatometric measurements and indirect determinations of volume change through the study of pressure effects on the equilibrium constant of the reversible thermal denaturation of ribonuclease-S and S-protein. The relationship of the pressure studies to the indirect determination of volume change will be introduced in this section.

The reversible acid and thermal denaturation of ribonuclease-A has been studied in considerable detail (Hermans and Scheraga, 1961; Holcomb and Van Holde, 1962). Richards and Logue (1962) have studied in some detail the S-peptide-S-protein interaction by UV difference spectroscopy. More recently Sherwood and Potts (1965a, b), using UV difference spectral techniques, have studied the effect of subtilisin digestion, the effect of urea denaturation at constant temperature, and the effect of urea on the thermal denaturation of ribonuclease-S, and S-protein. However, studies had not been conducted on the thermal transition of ribonuclease-S and S-protein over a range of acid pH as has been done for ribonuclease-A by Hermans and Scheraga (1961). These studies were subsequently conducted in this laboratory because of the importance to the pressure studies of knowing the state of denaturation of ribonuclease-S and S-protein

with respect to temperature and pH. Utilizing these data an equilibrium expression for the reversible thermal denaturation was set up in a manner used by previous workers (Hermans and Scheraga, 1961; Holcomb and Van Holde, 1962; Brandts, 1964; Gill and Glogovsky, 1965). From this equilibrium expression the enthalpy of denaturation was determined as a function of pH for ribonuclease-S, and the effect of pressure on the equilibrium constant for the reversible denaturation was followed for ribonuclease-S and S-protein at a specified temperature and pH. The change in the equilibrium constant with pressure was related to a change in the partial molar volume of the system through the use of an expression first derived by Planck (1887). The difference in the effect of pressure on the denaturation state of ribonuclease-S and S-protein under similar conditions is assumed to reflect the dissociation of the S-peptide portion of ribonuclease-S. Furthermore, the difference in the change in partial molar volume of the two systems is interpreted to give a direct indication of the magnitude and direction of volume change occurring when the S-peptide dissociates from ribonuclease-S.

The Direct Determination of the Volume Change From Dilatometric Studies

The volume change accompanying reactions as well as phenomena associated with conformational changes in proteins can be followed

by dilatometry. Linderstrøm-Lang and Jacobsen (1941) used the technique of dilatometry to monitor the hydrolysis of β -lactoglobulin by trypsin or chymotrypsin. Their studies indicated the volume change associated with the hydrolysis of the peptide, which was previously found to be accompanied by a contraction of 20 cc per mole of peptide linkage hydrolyzed, could not account for the total contraction observed in β -lactoglobulin. This additional contraction ranged from 100 cc per mole to 700 cc per mole protein at 0°. Their results indicated the initial hydrolysis of β -lactoglobulin must involve a considerable rearrangement in the protein structure. More important it demonstrated that dilatometric studies can contribute information concerning the magnitude and volume changes accompanying protein conformational changes. Other dilatometric studies have been made to determine such phenomena as the volume change resulting from ionization reactions in proteins (Rasper and Kauzman, 1962; Rasper, Bodansky and Kauzmann, 1962), the volume change accompanying the helix-coil transition (Noguchi and Yang, 1963), and the volume change associated with the thermal denaturation of ribonuclease-A (Holcomb and Van Holde, 1962).

Dilatometric studies related to the determination of the volume change due to hydrophobic bonding have been conducted by Lauffer (1964) and by Gerber and Noguchi (1967). These studies were performed with aggregating protein systems in which the exact nature and

number of binding sites involved in the aggregation were not known. Both studies were involved with entropy driven, endothermic processes resulting in an increase in volume with aggregation. Since other non-covalent interactions can likewise exhibit these phenomena, the lack of a defined system has hindered the interpretation of these studies (Lauffer, 1964). As previously stated the X-ray data indicate a considerable portion of the S-peptide-S-protein interaction is hydrophobic (Wyckoff et al., 1967). Therefore, this laboratory has performed dilatometric studies on ribonuclease-S and its component parts, S-protein and S-peptide, in an attempt to obtain the magnitude and direction of the volume change resulting from the association and/or dissociation of S-peptide and S-protein.

An attempt is then made to correlate the dilatometric data and the value determined from the indirect volume change measurements with the estimated magnitudes and direction of the volume change given in the first section of this introduction.

MATERIALS AND METHODS

Materials

Bovine pancreatic ribonuclease-A (Type I-A, Type II-A, and Type XI-A) were purchased from the Sigma Chemical Company, St. Louis, Missouri. Trypsin, IX crystallized, dialyzed salt free, lyophilized, was purchased from the Worthington Biochemical Corporation, Freehold, New Jersey. Subtilisin BPN' (prepared by Nagase and Co., Ltd., Osaka, Japan, and sold under the trade name of Nagarse) was purchased from the Biddle Sawyer Company, 64 Wall Street New York. Tortulla ribonucleic acid was purchased from the Sigma Chemical Company, St. Louis, Missouri. Yeast ribonucleic acid was purchased from P. L. Biochemicals, Milwaukee, Wisconsin. Uranyl acetate and other inorganic reagents used for the preparation of ribonuclease-S were reagent grade chemicals purchased from Baker-Adamson, Allied Chemical Company, Morristown, New Jersey.

Bio-Rex 70 (weakly acidic cation exchange resin, minus 400 mesh), AG 11A8 ion retardation resin, and P-4 (molecular sieve gel, 150 mesh) were purchased from Bio-Rad Laboratories, Richmond, California.

Sephadex G-25 (medium grade) was purchased from the Pharmacia Company, Uppsala, Sweden. Amberlite IR 120 (strongly acidic, sulfonated polystyrene type, cation exchange resin, medium porosity,

hydrogen cycle) and IRA-400 (strongly basic, quaternary ammonium polystyrene type, anion exchange resin, medium porosity, chloride cycle) were purchased from Mallinckrodt Chemical Works, St. Louis, Missouri. Columns were purchased from either Kontes Glass Company, Vineland, New Jersey, or the Fisher Porter Company, Hatboro, Pennsylvania.

Standard hydrochloric acid solutions were prepared from Hellige certified hydrochloric acid reagent concentrate 1/10 N purchased from Hellige Inc., Garden City, New York. Undecane and dodecane were purchased from the J. T. Baker Chemical Co., Phillipsburg, New Jersey. Purified kerosene (odorless) was purchased from the Fischer Scientific Company, Fair Lawn, New Jersey. All other reagents used for the dilatometer experiments were purchased commercially and used without further purification.

Millipore filters, 24 mm and 47 mm in diameter, with a pore size of 0.45μ were purchased from the Millipore Corporation, Bedford, Massachusetts.

Dialysis tubing (18/32) was obtained from the Union Carbide Company, Food Products Division, Chicago, Illinois. An Omnivector continuous counter current dialyzer (Model CD-16) for the dialysis of large volumes of solution, was obtained from Omnivector Instruments, Richmond, California.

Dilatometers were Carlsberg-Type, having a $10 \mu\text{l}$ full scale

precision bore capillary calibrated in 0.05 μ l divisions, and were purchased from F. C. Jacob Glasteknik, Copenhagen, Denmark.

Methods

Ribonuclease Assay

Ribonuclease activity was determined by the method described by Kalnitsky, Hummel, and Kierks (1959), wherein the hydrolysis of yeast ribonucleic acid at pH 5.0 and 0.1 M sodium acetate buffer is determined by measuring the amount of acid-soluble oligonucleotides liberated during a four minute incubation at 37°. Yeast ribonucleic acid was found to have a high blank value when measured at 260 m μ after trichloroacetic acid (TCA) precipitation. The high blank value indicates the presence of free oligonucleotides which can interfere with the assay. Therefore solutions of yeast ribonucleic acid were dialyzed against several changes of 0.1 M sodium acetate buffer (pH 5.0) to remove this contaminant.

Preparation of Ribonuclease-S

Ribonuclease-S was prepared from bovine pancreatic ribonuclease-A type I-A or type II-A according to the method of Richards and Vithayathil (1959), Gordillo, Vithayathil, and Richards (1962), and as reviewed by Marilyn Doscher (1968). Optimum

digestion time of the ribonuclease-A used was determined to be 18 hours by preliminary subtilisin digestion of a 20 mg sample of the protein using a weight ratio of 1:1000 subtilisin BPN' to ribonuclease-A.

The final procedure used in the preparation of ribonuclease-S was a combination of methods so used to obtain better yields with less handling time. A brief outline of the final procedure used is as follows. One to three grams of ribonuclease-A (type I-A or type II-A) was subjected to limited subtilisin BPN' digestion using a weight ratio of 1:1000 subtilisin to ribonuclease-A. After proper handling of the digestion mixture to destroy subtilisin activity, ribonuclease-S was separated from undigested ribonuclease-A and other protein fractions using a Bio-Rex 70 (325 mesh) column having the dimensions of 4.0 cm by 40 cm. A 0.2 M sodium phosphate buffer (pH 6.35) was used as the eluent buffer. Successful separation of ribonuclease-S from ribonuclease-A depends upon proper column pH and therefore required good equilibration of the column before use. For the size column used in these preparations, this often required four or five days of pumping buffer through the column. The volume of buffer used for the equilibration was approximately 10 liters. The ribonuclease-S fraction was dialyzed at 5° against glass distilled water using an Omnivector counter current dialyzer to remove sodium phosphate. Solutions were passed three times through the dialyzer at a

flow rate of three ml/min and with a flow rate for the counter flowing glass distilled water of 60 ml/min. Solutions were then passed through a Millipore vacuum filter apparatus, employing a 47 mm filter having a $0.45\ \mu$ pore size, to remove possible contaminants. Last traces of salt were removed by passing the dialyzed, filtered ribonuclease-S solution over a multi-layered ion exchange column (2.4 cm by 50 cm) consisting of a 7 cm layer of IR-120 (hydrogen cycle), and 18 cm IRA-400 (hydroxide cycle) layer, and an 18 cm layer of IR-120 (hydrogen cycle). This mixed ion exchange column was previously described by Gorodillo et al. (1962), and differs only from the one described by these authors in that one column was employed using sintered glass disks to separate the ion exchange layers. The solutions were finally lyophilized, and the dried, salt free, material was stored desiccated at -20° . The yield, based on the weight of the starting material, was 45 percent. This compared favorably with the reported 30-50 percent recovery reported by Gorodillo et al. (1962).

Preparation of S-Protein and S-Peptide

S-protein and S-peptide were prepared according to the methods reviewed by Marilyn Doscher (1968). S-protein was characterized by its lack of activity towards yeast ribonucleic acid substrate, and the ability to regain activity upon addition of an equimolar quantity of

S-peptide. Amino acid analysis of S-protein and S-peptide were performed on a Beckman Amino Acid analyzer Model 120B. The results of the amino acid analysis were in close agreement with the reported amino acid composition of S-protein and S-peptide given by Richards and Vithayathil (1959).

pH Measurement

All pH measurements reported in these experiments were made on a Corning pH meter Model 12 with an expanded scale for greater accuracy. A Corning semi-micro combination electrode (No. 476050) was employed for these measurements. Adjustments of pH were made with concentrated hydrochloric acid and the pH meter was frequently checked with pH 4.0 and pH 7.0 standard buffers to eliminate errors due to drift.

Thermal Transitions

Thermal transitions of ribonuclease-S and ribonuclease S-protein were followed in a Cary 14 recording spectrophotometer. A 0-0.1 slide wire was employed to increase the sensitivity. A set of matched semi-micro cuvettes from Helma Cell Inc., having a 1.0 cm path length, and fitted with teflon stoppers, were employed for these experiments. The reference compartment was maintained at 9° -11° throughout the experiments. The sample cell was

maintained at temperature by a water bath. Temperature change was regulated with a Neslab Temperature Programmer, which allowed the temperature to change at a rate of 0.5° per minute. The temperature of the sample cell was continuously monitored using a thermistor temperature sensing device produced by the Yellow Spring Instrument Company (Model 423). The thermistor probe was inserted through a specially prepared sample compartment cover into the cuvette. A small rubber stopper surrounding the wire of the probe was used to seal the cuvette. Temperature and optical density change were recorded simultaneously on a duo channel Esterline Angus recorder (Model E 1102S). Reference and sample solutions were in all cases the same. The final plateau region of the thermal transitions were taken as the final reference line. The reasonableness of this assumption is referenced to the fact that concentrated denaturants in conjunction with high temperature are required to normalize the third tyrosine in both ribonuclease-S and S-protein (Sherwood and Potts, 1965b). All experiments were performed on solutions of ribonuclease-S and S-protein that were approximately 0.87 mg per ml in concentration. The protein solutions were made up in 0.01 M sodium acetate buffer (pH 5.0) containing 0.1 M sodium chloride. Ribonuclease-S and S-protein concentrations were determined from their absorbance at 280 m μ using the extinction coefficients listed by Sherwood and Potts (1965a).

Circular Dichroism

Circular dichroism spectra were obtained using a Durrum-Jasco circular dichrograph model CD-SP. The experimental procedures and equipment used is discussed in detail in the Materials and Methods section in the second part of this thesis.

Dilatometric Studies

The dilatometers used in these experiments were Carlsberg type dilatometers manufactured by F. C. Jacob Glasteknik Copenhagen, Denmark, and have been described by Linderstrøm-Lang and Lanz (1938). The dilatometers consist of a precision bore capillary having a full scale range of 10 μ l, and are calibrated in 0.05 μ l divisions. The capillaries are fitted with a standard 7/25 male tapered joint which fits into the dilatometer vessel. The vessel is in the shape of an inverted "V" and allows one to place a sample solution in one leg of the vessel, and a perturbant solution in the other. The volume of the vessel is approximately 16 ml and each leg holds about 6 ml of solution. A mercury regulator (Precision 62541), with a sensitivity of $\pm 0.003^\circ$ in conjunction with a Lapine Control Box was employed to control temperature. Heaters consisted of 60 watt or 100 watt light bulbs, and were used to eliminate the lag time in heating response characteristics of copper heating coils. The heaters

were balanced against a cooling coil. Tap water was used as a coolant. The bath was an insulated 15 gallon aquarium. Temperature control was better than $\pm 0.003^\circ$ over extended periods of time as monitored by a Beckman thermometer. Volume fluctuations of the liquid in the dilatometer capillaries, due to thermal fluctuations of the bath, were observed to be less than $\pm .005 \mu\text{l}$ over the temperature ranges employed for these experiments.

Determination of the volume change of ribonuclease-S and ribonuclease-A upon addition of acid was accomplished in the following manner. A sample of ribonuclease-S or ribonuclease-A, usually 100 mg, was dissolved in 5.0 ml of a 0.1 M sodium chloride solution. This was placed in one leg of the dilatometer. The sample was allowed to equilibrate to the temperature of the bath, and then was degassed carefully to eliminate the possibility of bubbles being formed after the dilatometer was sealed. A 0.100 ml aliquot of 0.200 N (± 0.001 N) hydrochloric acid containing 0.1 M sodium chloride was added to the other leg of the dilatometer using a 100 μl precision Hamilton syringe. An immiscible hydrocarbon, either purified kerosene, undecane, or dodecane, was then layered over the aqueous solutions to a point of filling the dilatometer vessel. The bottom two-thirds of the capillary male tapered joint was then greased with a glycerol-soap mixture (Johansen, 1948) and the upper portion with vaseline. The use of the double grease provided a seal that was inert

both to the hydrocarbon and to water. The capillary was then ground into the dilatometer vessel and the dilatometer was partly submerged into the bath. After the dilatometer was allowed to equilibrate to the temperature of the bath, approximately 15 minutes, the liquid level was adjusted in the capillary to a point below the water level of the bath. This level was then determined accurately with respect to the nearest $0.05 \mu\text{l}$ division through the use of a cathetometer which could be read to $\pm 0.005 \text{ cm}$. After accurately determining the liquid level in the dilatometer, the dilatometer was removed from its holder and the solutions mixed by carefully tipping the dilatometer back and forth in the bath. The dilatometer was then replaced in its holder. The liquid level was then allowed to reach its new level in the capillary and the meniscus height was determined again with respect to the nearest $0.05 \mu\text{l}$ division. At the end of each run the dilatometer was raised out of the bath, water was dried from the outside of the capillary, and then the dilatometer was opened. A 0.3 ml portion of the sample was removed and the pH of the solution was determined. The sample was then replaced in the dilatometer and the whole process repeated. The pH of the solution was needed to determine the number of protons bound to the protein. This was done by subtracting out the amount of free acid of the solution from the amount of acid added. The pH of solutions containing known concentrations of hydrochloric acid in 0.1 M sodium chloride were used to calibrate the pH meter (Rasper

and Kauzmann, 1962). These experiments were performed either at 25° or 15°.

The determination of the volume change accompanying the addition of equimolar quantities of S-peptide to S-protein was made using techniques similar to that described in the previous paragraph. Solutions were prepared using either 0.01 M sodium acetate buffer (pH 5.0) containing 0.1 M sodium chloride or with 0.01 M sodium acetate buffer (pH 5.0) without sodium chloride. Solutions of S-protein were made up in concentrations ranging from two mg/ml to five mg/ml. From 10 mg to 30 mg of S-protein were placed into the dilatometer. A solution of S-peptide that was 20 percent of the S-protein concentration was placed in the other leg of the dilatometer. The volume change of interaction of S-peptide and S-protein was obtained by mixing the two solutions together.

Pressure Studies

The change in the spectral transitions of ribonuclease-S and S-protein with pressure was studied using a stainless steel bomb equipped with sapphire windows. The equipment used was previously described by Morita (1957). The bomb was held in place by a jacketed sample holder, and temperature control was maintained throughout the experiments using a regulated water bath. A Cary recording spectrophotometer model 11 was used to obtain the spectra.

Samples of ribonuclease-S and S-protein were adjusted to a pH at which they were in the initial stage of their thermal transition at the temperature employed in the experiment. A temperature of 25° was used for these experiments. The pH to which the samples were adjusted was determined from the plot of the thermal transitions as a function of pH. The samples were then placed in the bomb, and the spectra were run from 320 m μ to 250 m μ at pressures of 1 atm to 1000 atm. Increments of 200 atm or 300 atm were employed in these studies. Spectra of ribonuclease-S and S-protein were obtained at pH 5.0 and at pH 7.0 respectively and as a function of pressure utilizing the same pressure increments that were employed for the low pH pressure studies. All spectra were obtained using glass distilled water as a reference.

Solutions of the proteins were made up in 0.01 M sodium acetate buffer (pH 5.0) containing 0.1 M sodium chloride. The pH of these solutions was adjusted using concentrated hydrochloric acid or 50 percent sodium hydroxide. The concentration of the protein solutions was determined from their absorbance at 280 m μ according to the procedure of Sherwood and Potts (1965a).

EXPERIMENTAL AND RESULTS

Thermal Transitions

The thermal transitions of ribonuclease-S and S-protein, as a function of acid pH, are shown in Figure 1 and Figure 2. The set of curves obtained for ribonuclease-S are similar to those obtained by Hermans and Scheraga (1961) for ribonuclease-A. The main difference is the increased thermal sensitivity of ribonuclease-S as has been previously shown by Richards and Vithayathil (1960). The change in molar absorbance at 287 m μ for the thermal denaturation of ribonuclease-S at pH 5.0 was calculated to be -1700 and is in close agreement with a value of -1730 obtained by Sherwood and Potts (1965b). A change in molar absorbance at 286 m μ for S-protein of -1100 in the absence of phosphate is to be compared with -800 for S-protein obtained by the same authors in the presence of phosphate. The difference in the two values might reflect additional instability of our system towards thermal denaturation due to the lack of phosphate. The thermal denaturation of ribonuclease-S at pH 3.6 was followed also by circular dichroism (CD) in the region of the side chain contributions (Beychok, 1968) from 320 m μ to 235 m μ . Curves representing three stages of the thermal transition, native ribonuclease-S (13°), near the transition temperature (27°), and at the top of the transition (37°) are shown in Figure 3. Also a partial CD spectrum

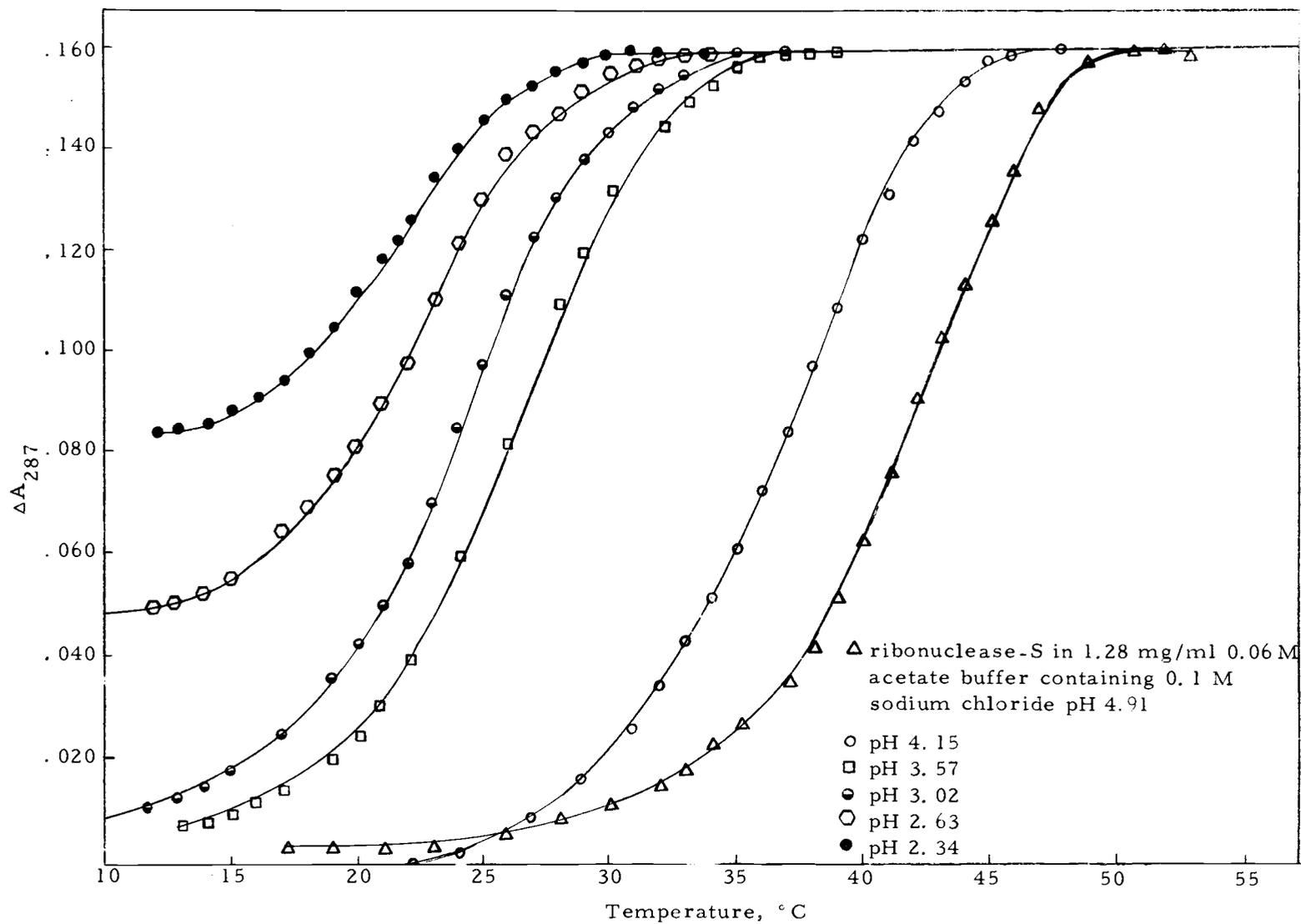


Figure 1. The temperature transition of ribonuclease-S as a function of pH.

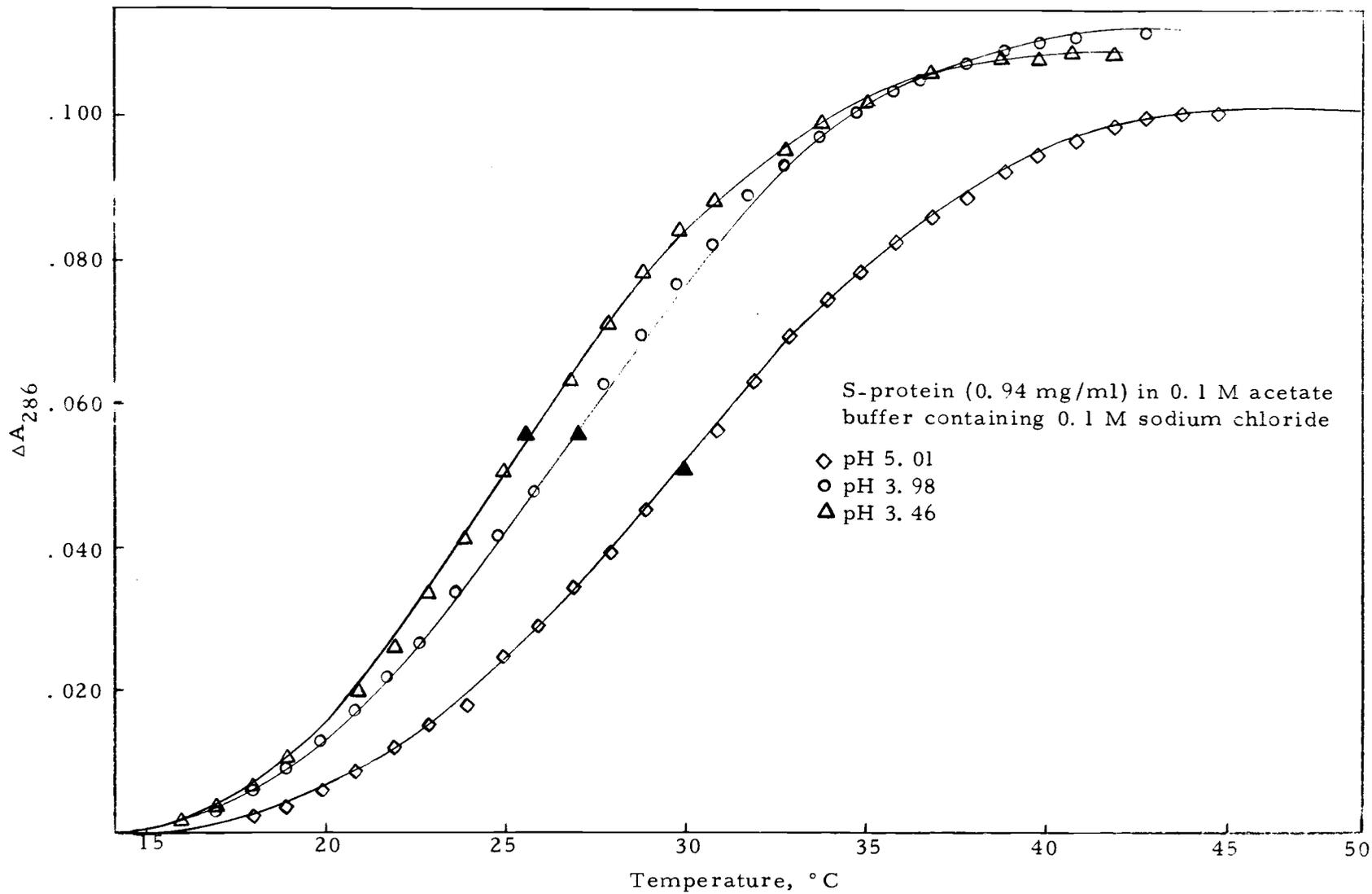


Figure 2. The temperature transition of S-protein as a function of pH.

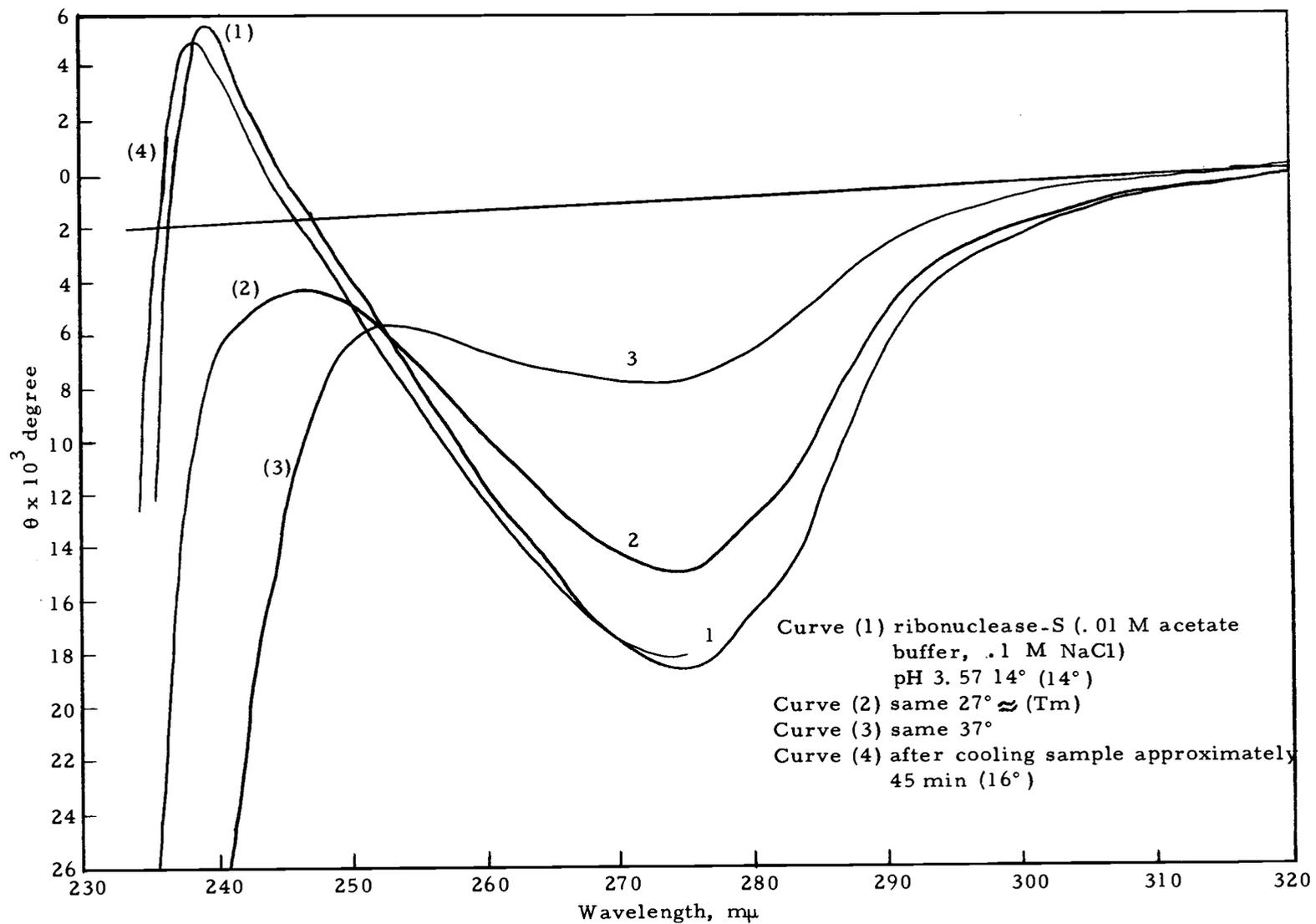


Figure 3. Circular dichroism of ribonuclease-S as a function of temperature at pH 3.57.

of ribonuclease-S at pH 3.6 upon recooling is shown in the same figure. This curve shows the thermal denaturation is nearly, but not completely reversible. A plot of the change in molar ellipticity at 275 m μ as a function of temperature is shown in Figure 4. This experiment was conducted in an effort to obtain additional information concerning the process occurring during the thermal transition. The plot in Figure 4 shows that the change in molar ellipticity at 275 m μ from the CD measurements correlates well with the change in molar absorbance at 287 m μ obtained from difference spectral measurements, with the exception that the transition temperature is higher (31° as opposed to 27°) and the transition appears to occur over a larger temperature range. The difference spectra or change in molar absorbance at 287 m μ is due to the shift in the tyrosine spectra to shorter wavelengths upon denaturation. That subtle differences occur in the CD data and difference spectra data obtained for the thermal denaturation of ribonuclease-S at pH 3.6 might suggest that the 275 m μ CD band results from contributions in addition to changes in tyrosine environment. From the data obtained from the studies of the thermal transitions of ribonuclease-S and S-protein, it is possible to select conditions where the protein is in equilibrium with its denatured state and are suitable for conducting experiments under mild conditions.

Studies conducted by Richards and Logue (1962) and Sherwood

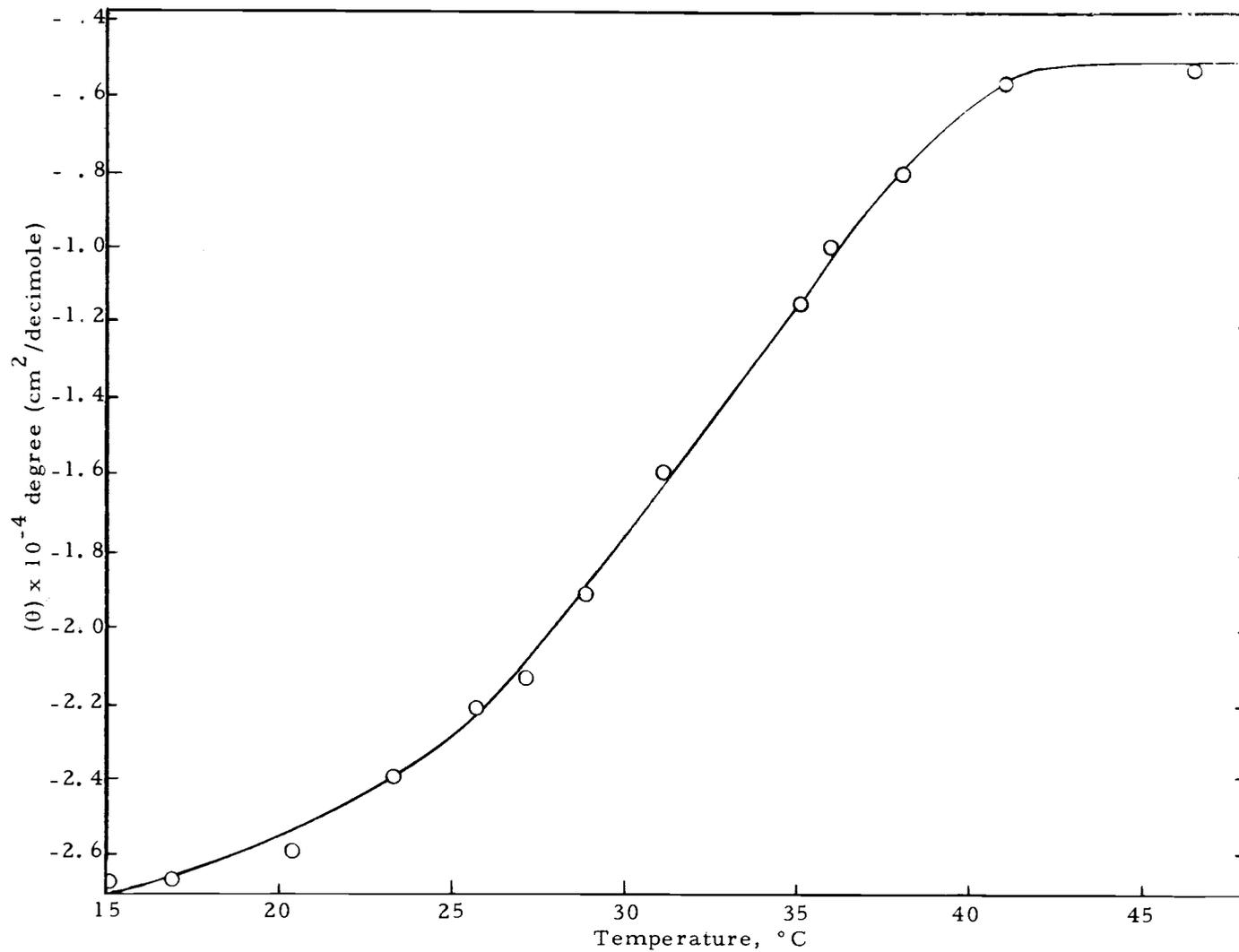


Figure 4. Change in molar ellipticity of ribonuclease-S as a function of temperature (pH 3.61 .01 M acetate 0.1 M NaCl).

and Potts (1965a, b) have attempted to determine some of the conformational changes occurring in S-protein and ribonuclease-S during acid and thermal denaturation. Sherwood and Potts (1965b), using the method of Bigelow (1961) for distinguishing abnormal tyrosines in ribonuclease-A, have studied the abnormal tyrosines of ribonuclease-S and S-protein. Ribonuclease-S is similar to ribonuclease-A in that it has three abnormal tyrosines. These have been labeled simply A, B, and C due to the difficulty of correlating these with the suspected buried tyrosine residues 25, 92, and 97 as determined by the X-ray data (Wyckoff et al., 1967). The addition of S-peptide to S-protein results in an increase in the molar absorbance at 287 m μ and the magnitude of the molar absorbance change indicates that one tyrosine is effected. From this it is concluded that S-protein has two abnormal tyrosine residues and they are suggested to be residues B and C (Sherwood and Potts, 1965b). Simple thermal denaturation exposes residues A and B in ribonuclease-A and ribonuclease-S, and residue B in S-protein. Residue C in all three proteins is exposed only upon subtilisin digestion or denaturation under extreme conditions. Acid denaturation of ribonuclease-A and S-protein are suggested to involve only residue B. However experiments conducted on ribonuclease-S by Richards and Logue (1962) indicate that two residues are exposed at pH 1.75 and 22°. They further demonstrate that the molar absorbance at 287 m μ of a mixture of S-peptide and

S-protein at pH 1.75 is equal to the sum of the individual contributions of the S-peptide and S-protein absorbance at 287 m μ and pH 1.75.

This is indicative of the fact that no interaction occurs between S-peptide and S-protein at this pH and therefore leads Richards and Logue (1962) to conclude that S-peptide is probably dissociated from S-protein at low pH. The usual separation of S-peptide from S-protein involves either TCA precipitation or chromatography on G-75 using 50 percent acetic acid. Although both types of separation are conducted at acid pH, it can be argued that other effects are responsible for the separation of S-peptide and S-protein using these methods. In an attempt to establish the separation of S-protein from S-peptide under milder acidic conditions, ribonuclease-S was adjusted to pH 2.12 in 0.1 M citric acid. This solution was then chromatographed at 5° on a Bio-Rad P-4 column (1.8 cm x 45 cm) which had been previously equilibrated with 0.1 M citric acid (pH 2.12). It was assumed that if dissociation did occur at acid pH and at 5°, that the S-peptide, having a molecular weight of 2200, would be partially included resulting in some separation of the two component parts of ribonuclease-S. Figure 5 shows the results of the column chromatography of ribonuclease-S at pH 2.12 on P-4 at 5°. The results indicate that the separation was not complete, but about 80 percent or more of the peak had no enzyme activity towards yeast RNA. The addition of inactive fractions following the peak, to the peak fraction,

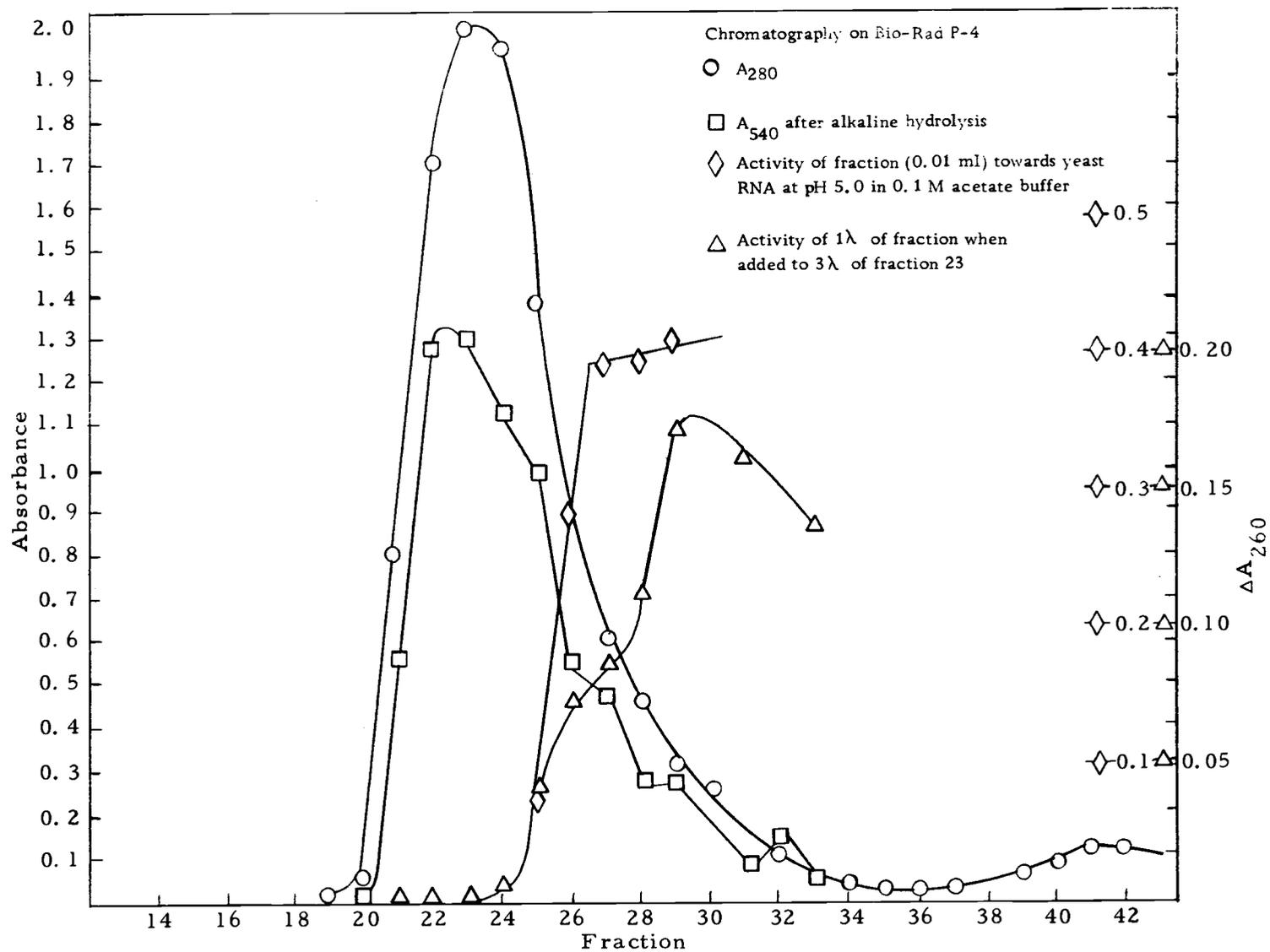


Figure 5. The chromatography of ribonuclease-S at pH 2.12 (5°) on Bio-Rad P-4.

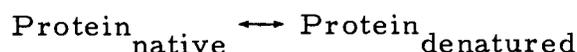
which was inactive, resulted in the restoration of activity. Addition of aliquot samples of fractions directly following the peak to the inactive peak fraction allowed the identification of the S-peptide peak by monitoring the amount of enzyme activity restored. The column volume was approximately 100 ml. The S-protein peak came off at 32 ml and the S-peptide peak at 48 ml. Using a void volume of 36 ml for this column, an E_v/E_{vo} ratio of 1.33 was obtained. E_v represents the elution volume in which the S-peptide came off and E_{vo} is the void volume of the column. The value of 1.33 is very close to the E_v/E_{vo} ratio suggested for a molecule having a molecular weight of 2200 by the Bio-Rad Company.² Therefore under the conditions employed this separation was as good as could be expected. These data support the suggestion of Richards and Logue (1962) that S-peptide is dissociated from ribonuclease-S under acid conditions. It is further suggested from the data of Sherwood and Potts (1965b) that the thermal denaturation of ribonuclease-S must involve the dissociation of the S-peptide portion of the molecule. This would appear to be in direct conflict with the assumption that the S-peptide portion of the molecule is involved in hydrophobic bonding. However, it is suggested that the thermal denaturation of the S-protein portion of the molecule could weaken or disrupt the hydrophobic core with which the

²This information was supplied in the Bio-Rad Laboratories price list July 1, 1968, page 42.

S-peptide is associated.

Establishment of an Equilibrium Expression

It is possible to assume that the reversible denaturation of ribonuclease-S and S-protein can be represented by the following expression (Hermans and Scheraga, 1962; Holcomb and Van Holde, 1962; Brandts, 1964; Herskovits, 1968):



The equilibrium expression for this simple two state system is,

$$K = f_d / (1 - f_d) \quad \text{or} \quad K = \frac{\Delta\epsilon - \Delta\epsilon_n}{\Delta\epsilon_d - \Delta\epsilon}$$

These expressions are explained in detail in a recent review by Herskovits (1968). Having an expression for the equilibrium constant of the denaturation it is possible to obtain the enthalpy of denaturation from the van't Hoff plot of $\ln K$ vs. $1/T$. A plot of $\ln K$ vs. $1/T$ as a function of pH for ribonuclease-S and S-protein is given in Figure 6. Figure 7 shows a similar van't Hoff plot for ribonuclease-S at pH 3.6 from data obtained from the CD study. The enthalpy for ribonuclease-S in the range pH 4.0 to pH 2.6 was calculated to be 56 ± 6 Kcal. The calculated enthalpy for ribonuclease-S at pH 5.0 was 41 Kcal while the enthalpy at pH 2.3 was calculated to be 80 Kcal.

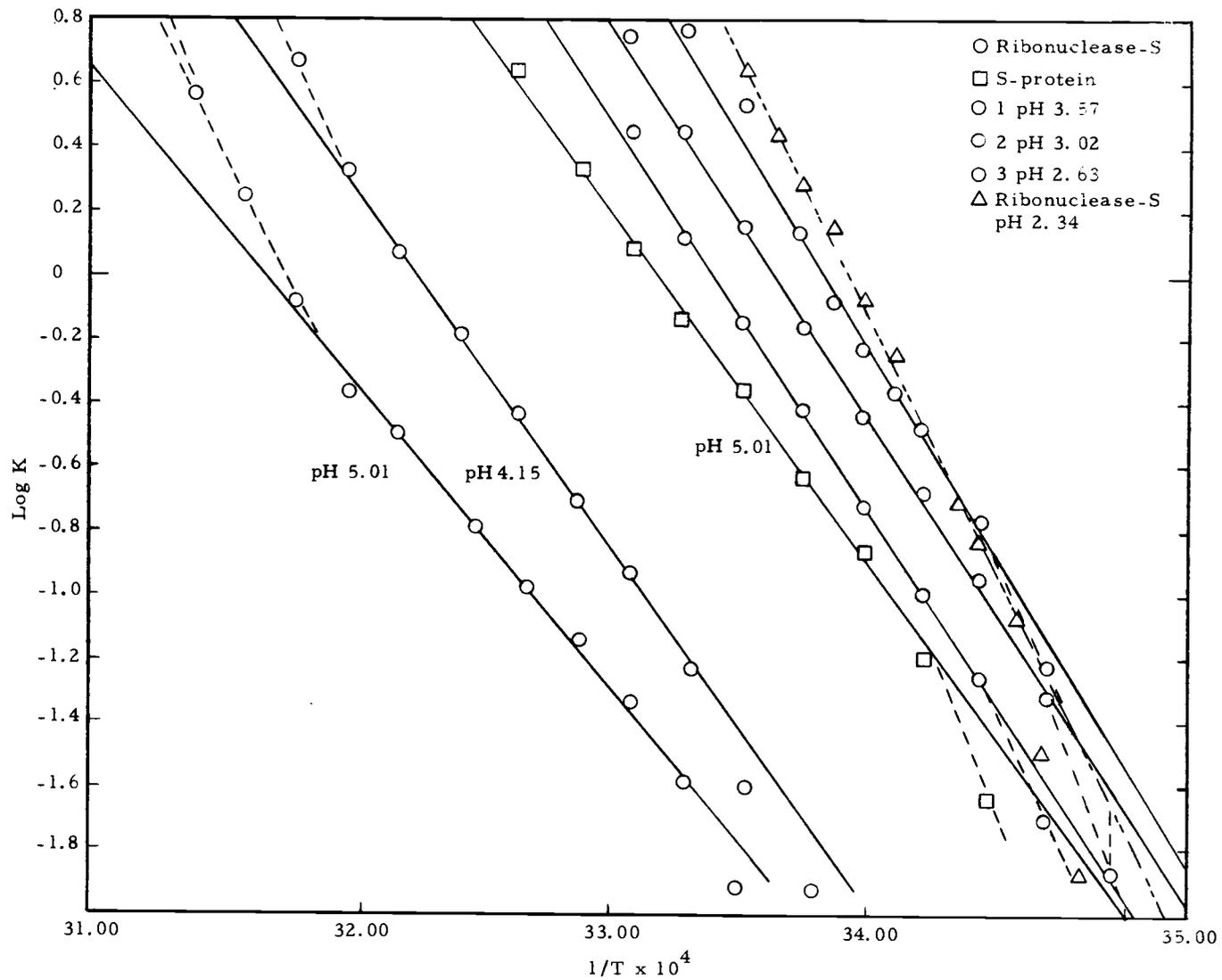


Figure 6. van't Hoff plot for ribonuclease-S as a function of pH.

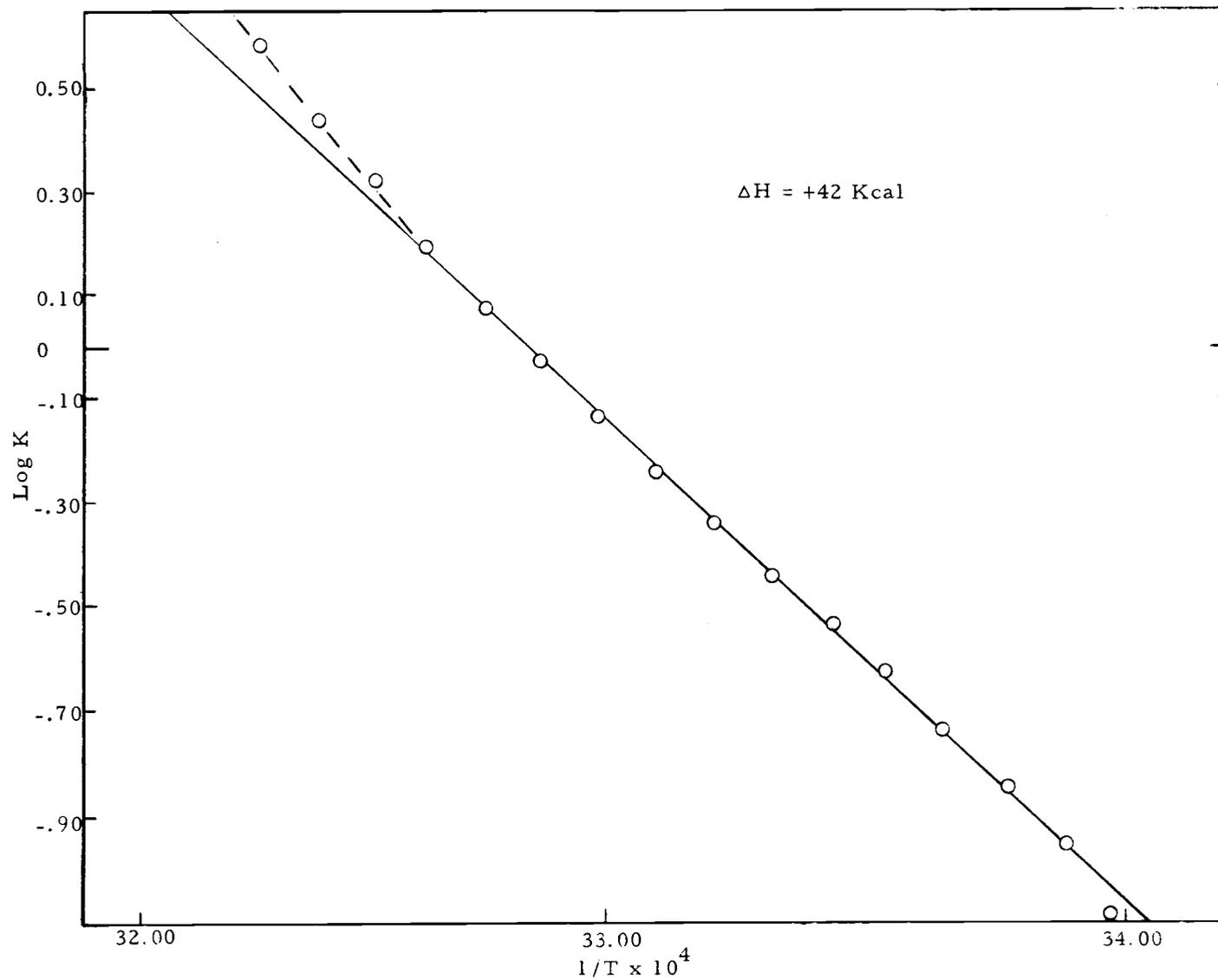


Figure 7. van't Hoff plot for ribonuclease-S (pH 3.61) as determined by CD data.

The value determined from the CD data for the thermal denaturation of ribonuclease-S at pH 3.6 is 42 Kcal. The van't Hoff plot for ribonuclease-S at pH 5.0 is non-linear above the transition temperature. The curves in the pH range of 4.1 to 2.6 are linear over a larger temperature range, but do exhibit some non-linearity in the low and high temperature regions of the curves. The van't Hoff plot for ribonuclease-S at pH 3.6 followed by CD is quite similar to that obtained for ribonuclease-S at pH 5.0 with difference spectra. The non-linearity in the curves, the difference in the enthalpy values, and the difference in the CD and difference spectral data obtained at pH. 3.6 indicate the simple one step denaturation process may not be valid, as suggested previously by Hermans and Sherağa (1961) and Holcomb and Van Holde (1962). However, the use of the one step denaturation process can allow the calculation of thermodynamic parameters with the evaluation of such parameters leading to useful information.

Pressure Studies

It is assumed that the standard molar volume change for denaturation can be obtained from the isothermal pressure dependence of the molar equilibrium constant from a relationship first derived by Planck (1887).

$$\left(\frac{\partial \ln K}{\partial P} \right)_T = \frac{-D\bar{V}^\circ}{RT}$$

The use of this expression will be guided by the following assumptions. The expression holds when experimentation is conducted in dilute solutions where the activity coefficient approaches unity. The use of the equilibrium constant for the one step denaturation process has meaning in the limits that this study is attempting to determine the direction of the volume change and an indication of its order of magnitude. A detailed discussion of the above relationship in terms of K_x , K_m , and K_c for concentrated and dilute solutions has been given by Hamann (1963) with the resulting expression for K_c in dilute solutions:

$$\left(\frac{\partial RT \ln K}{\partial P} \right)_T = -\Delta \bar{V}_{av}^{\infty} + \underbrace{(\sum n_{\text{products}} - \sum n_{\text{reactants}})}_{\Delta n} K_s$$

where $-\Delta \bar{V}_{av}^{\infty}$ is the standard molar volume change in infinite dilution, and K_s is the compressibility of the solvent. For non zero values of Δn , the compressibility term must be considered. However, for the simple one state denaturation process under consideration, Δn is zero. For equilibria expressed in K_x , K_m , and K_c when Δn is zero, an expression for the average volume change can be defined over finite pressure intervals as,

$$\Delta \bar{V}_{av}^{\infty} = -2.303 RT \log \frac{K_2}{K_1} / P_2 - P_1.$$

It is not assumed that $\Delta\bar{V}_{av}^{\infty}$ is constant with pressure (Hamman, 1963), but that this expression, in the limits assumed for the equilibrium expression, relates $\Delta\bar{V}_{av}^{\infty}$ in a gross manner with magnitude and direction, i. e., whether positive or negative, with the volume change resulting from the reversible pressure denaturation. The use of Planck's expression to determine volume change in protein systems is not new and has been extensively reviewed by Johnson, Eyring, and Polissar (1954). A recent paper by Gill and Glogovsky (1965) uses this expression to obtain the volume change of denaturation of ribonuclease-A through the use of optical rotation in conjunction with the pressure studies. Gill et al. (1965) obtained a value for $\Delta\bar{V}_{av}^{\infty}$ of -30 ± 10 cc/mole for the denaturation of rebonuclease-A at 42°C as followed by optical rotation methods, and found that $\Delta\bar{V}_{av}^{\infty}$ varied depending upon which state of thermal denaturation that the pressure studies were initiated from.

The first attempt at following the pressure effect on the reversible denaturation of ribonuclease-S and S-protein, was performed as a direct difference spectra experiment using ribonuclease-S at pH 1.75 as a reference (Richards and Logue, 1962) and the same sample at pH 5.0. The temperature was controlled at 30° . A second set of samples were pressurized with the reference sample being prepared as the first, but ribonuclease-S in the sample cell was adjusted to pH 3.6 and the temperature was set at 25° . The results of

these two experiments are shown in Table 1. It can be seen that pressure had little effect on the change in absorbance at 287 m μ for ribonuclease-S at pH 5.0 and 30°. However when the pH was lowered to 3.6, where native ribonuclease-S is in equilibrium with its denatured state at 25°, the pressure had a direct and marked effect on the difference spectra as evidenced by the decrease in the absorbance at 287 m μ . A distortion was seen in the difference spectra which either was due to a solvent mis-match or distortion in the cell windows. This resulted in an apparent cancellation of the pressure effect on ribonuclease-S at pH 3.6 above 600 atm. For this reason direct spectra of ribonuclease-S at pH 5.0 and a similar sample at the same concentration but at pH 3.6 were run separately as a function of pressure from 1-900 atm and the difference spectra were computed by hand using the sample at pH 5.0 as a reference. This procedure was repeated for S-protein except that the reference sample was adjusted to pH 7.0. S-protein is thermally stable at pH 7.0, but exhibits some thermal instability at pH 5.0 and 25°. The difference spectra are shown in Figures 8 and 9. In all cases the pressure was relaxed after 900 atm and their spectra were again obtained at 1 atm to see if the process had been reversible. In most cases the system returned to its original state. Figure 10 and 11 shows the CD spectra of S-protein at pH 3.4 and pH 7.0 respectively, before and after pressurizing to 900 atm. Little change resulting from irreversible

pressure effects appears to have taken place as detected by CD. From the change in molar absorbance at 287 m μ and 286 m μ respectively, equilibrium constants as a function of pressure were computed for ribonuclease-S and S-protein. A plot of $\log K$ vs. pressure at 25° is shown in Figure 12. From these data a volume change of -80 ± 2 cc/mole was obtained in the pressure range of 300 atm - 900 atm for ribonuclease-S, while S-protein undergoes an apparent volume change of -45 ± 2 cc/mole under similar conditions.

Table 1. The change in A_{287} as a function of pressure.

Ribonuclease-S pH 5.0 <u>vs.</u> pH 1.8	
Pressure	A_{287} *
1 atm	0.139
300 atm	0.135
600 atm	0.139
Ribonuclease-S pH 3.6 <u>vs.</u> pH 1.8	
Pressure	A_{287} *
1 atm	0.116
300 atm	0.092
600 atm	0.068

* Measurements were made with a Cary 11 spectrophotometer equipped with a 0-0.1 slide wire.

Dilatometric Studies

The initial attempts to determine the volume change accompanying the interaction of S-peptide and S-protein by direct dilatometric measurement were not as successful as one would like. A

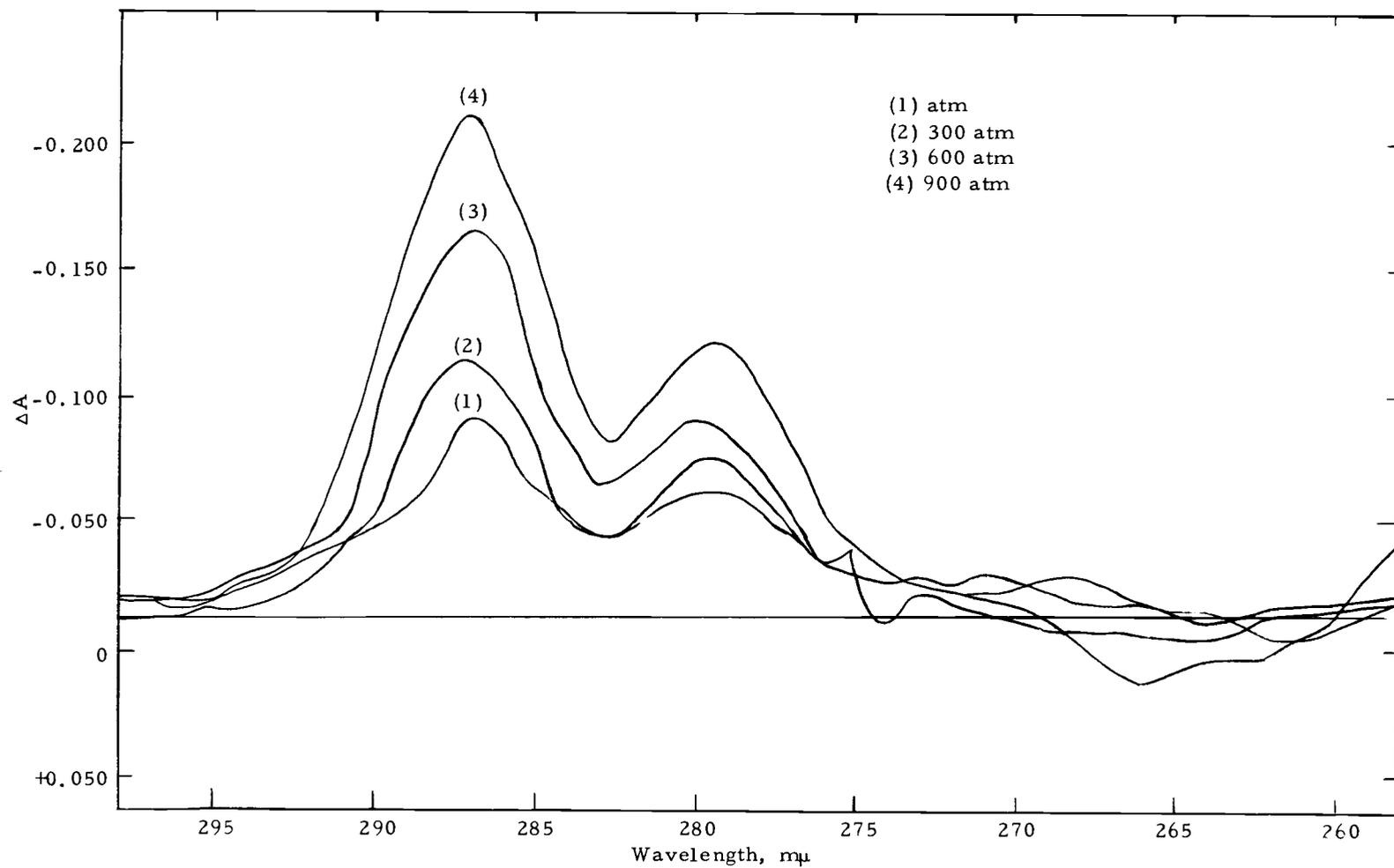


Figure 8. High pressure difference spectra of ribonuclease-S at 25°.

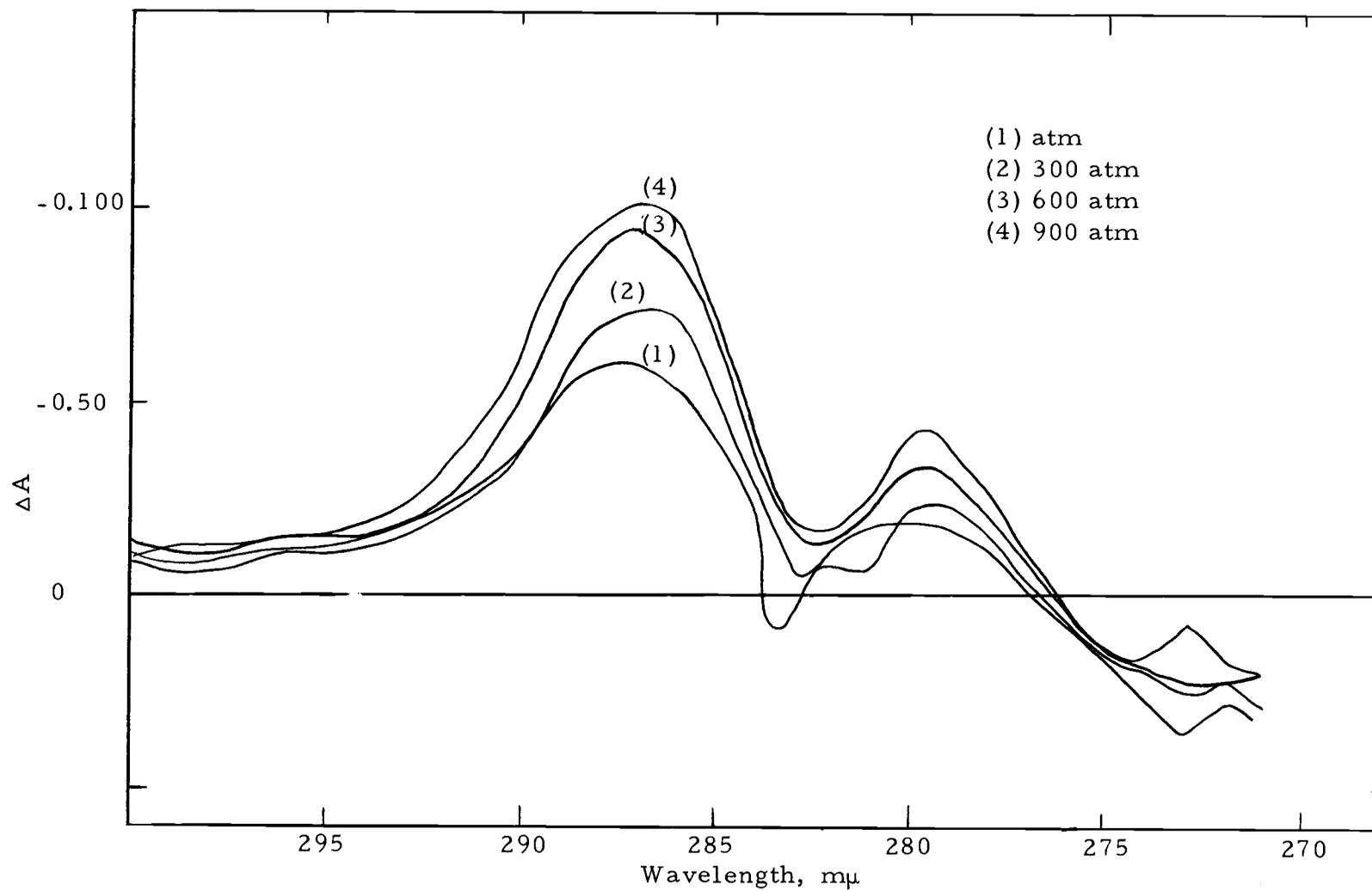


Figure 9. High pressure difference spectra of ribonuclease S-protein at 25°.

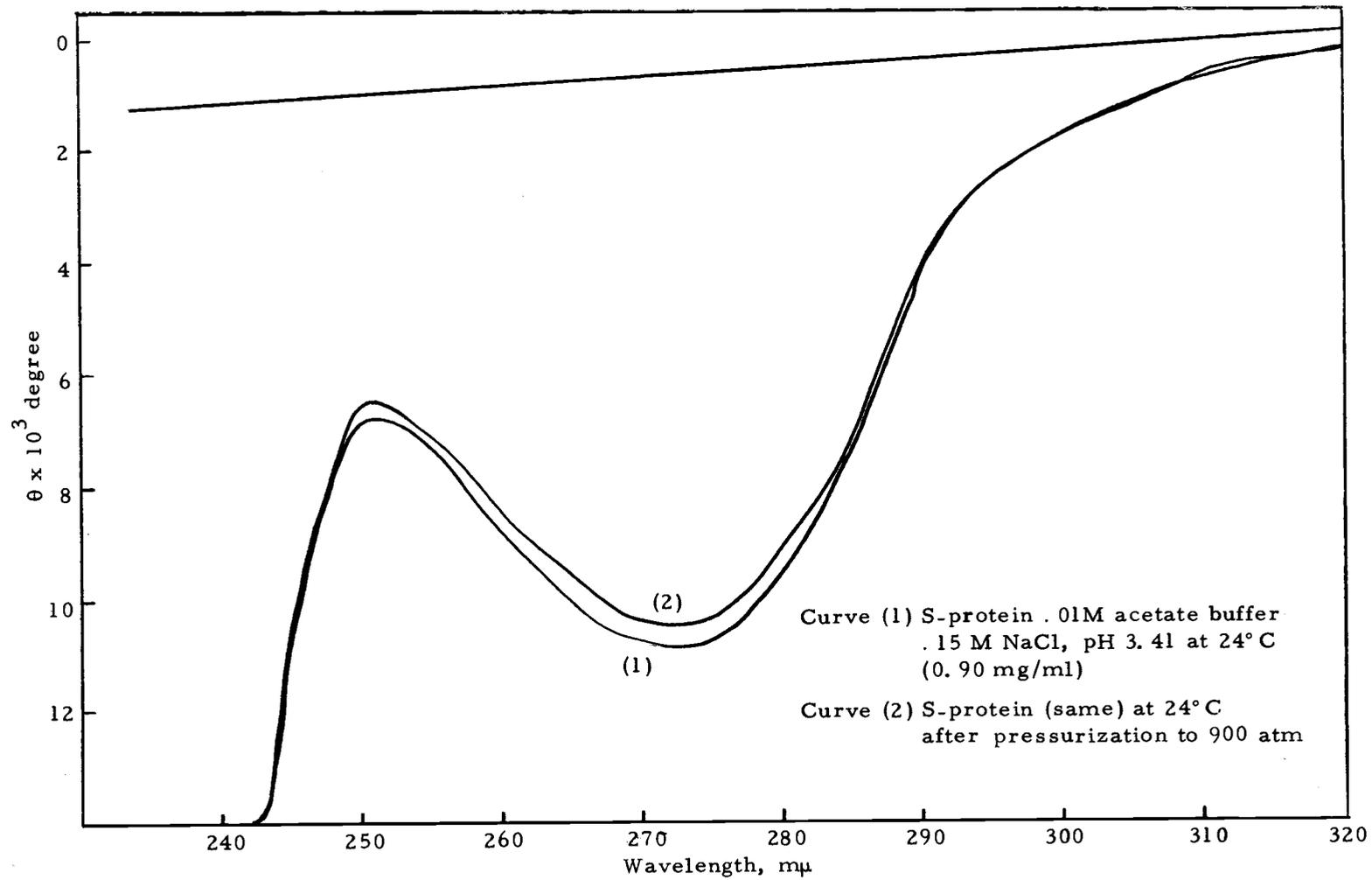


Figure 10. Pressure effects on S-protein at pH 3. 41 and 24°.

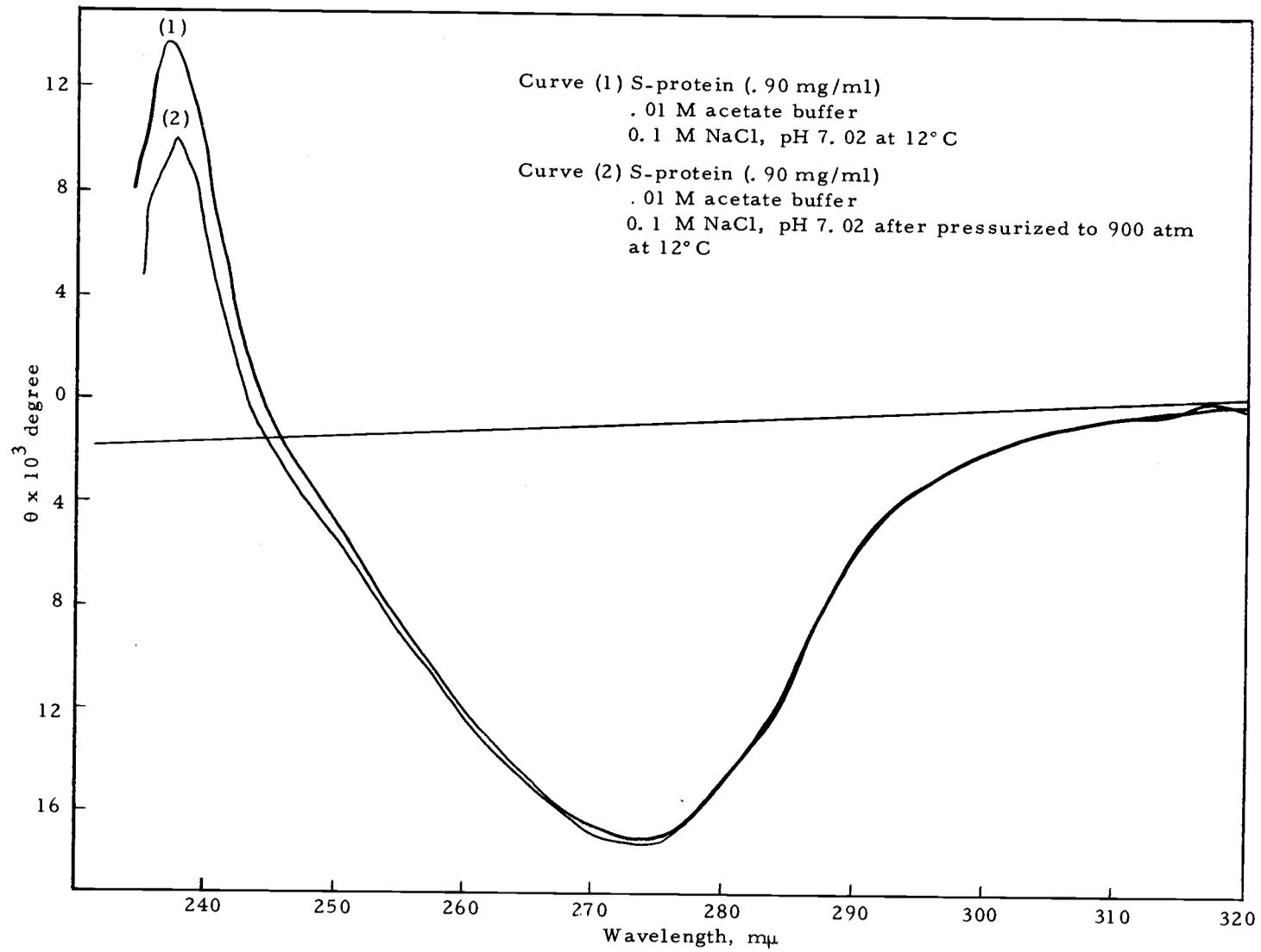


Figure 11. Pressure effects on S-protein at pH 7.0.

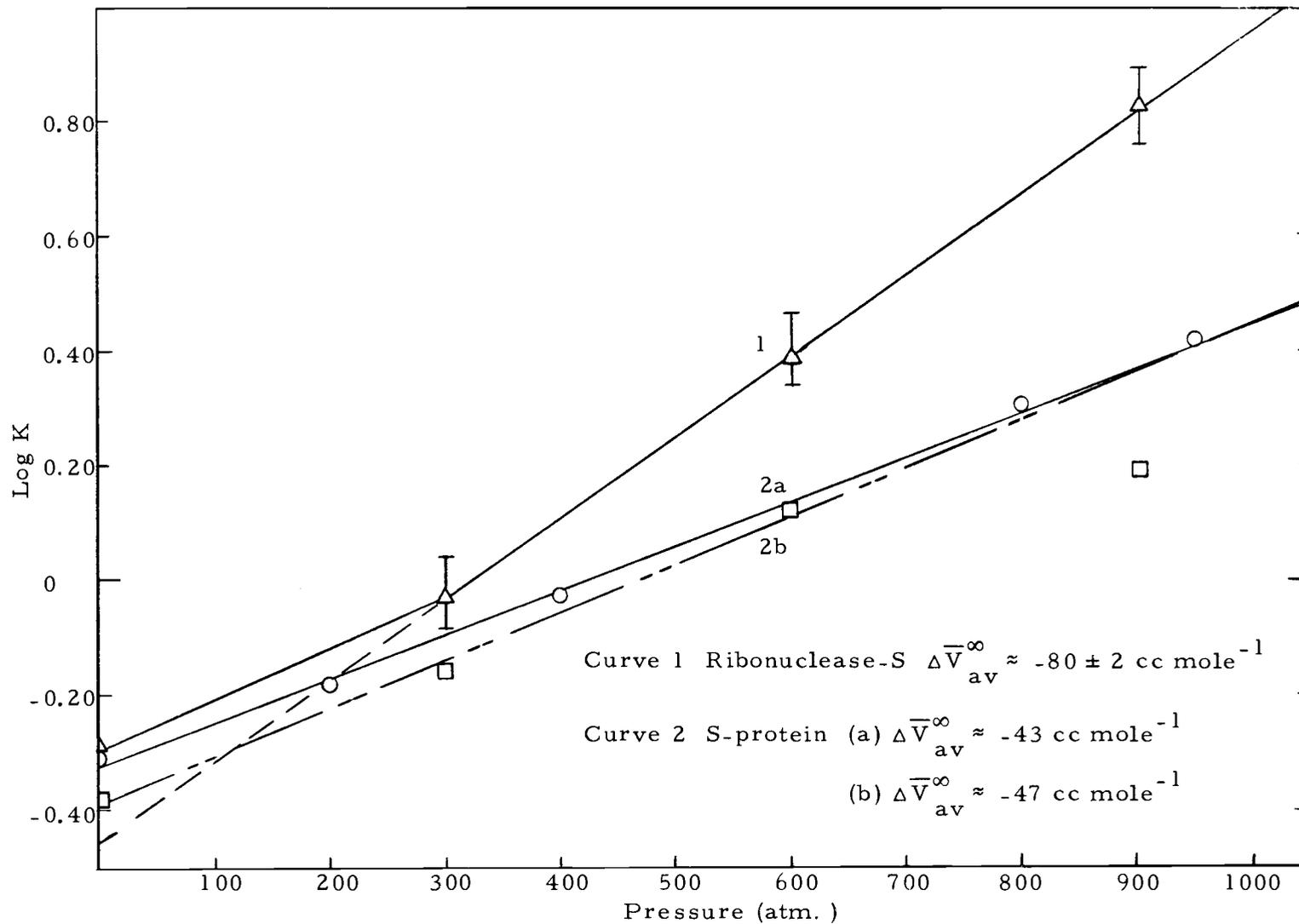


Figure 12. Log K vs. pressure for ribonuclease-S and S-protein at 25°.

preliminary calculation was made to estimate the expected volume change for the interaction. This calculation was based on the value of 20 cc/mole of aliphatic side chain interactions (Kauzmann, 1959) and it was estimated that a volume change of approximately + 100 cc/mole of S-peptide-S-protein interaction should result when the two solutions were mixed together in the dilatometer. Commercial S-protein and S-peptide are quite expensive and therefore it was decided that, with an expected volume change of +100 cc/mole interaction, a five mg sample of S-protein per experiment should be sufficient. This would produce an actual volume change of +0.045 μ l, and with proper temperature control, would be readily obtainable with the precision dilatometers employed in these experiments. Initial measurements showed considerable scatter and also gave a pattern of a gradual volume increase. It was evident from these results that it would be necessary to prepare our own S-protein and S-peptide so that larger quantities of material could be used. The addition of ribonuclease S-protein to S-peptide at higher quantities of material resulted in a volume change that was lower than the predicted value but in the same direction. In Table 2 are listed the quantity of S-protein employed and the resulting volume change. The volume change resulting from the interaction of S-peptide and S-protein from these experiments is an apparent increase of 32 ± 6 cc/mole S-protein.

Table 2. The volume change resulting from S-peptide S-protein interaction.

Moles S-protein	Volume change cc/mole
1.40×10^{-6} M	+38 cc/mole S-protein
1.30×10^{-6} M*	+36 cc/mole S-protein
2.40×10^{-6} M	+25 cc/mole S-protein
0.69×10^{-6} M	+34 cc/mole S-protein
0.38×10^{-6} M	+26 cc/mole S-protein
2.60×10^{-6} M*	+ 9 cc/mole S-protein

* These samples, like the rest, were made up in 0.01 M sodium acetate buffer pH 5.0, but they did not contain 0.1 M sodium chloride.

Evidence was given in the section on Thermal Transitions indicating S-peptide and S-protein are dissociated at acid pH. The evidence cited was the separation of ribonuclease-S into its component parts, S-protein and S-peptide, on a Bio-Rad P-4 column at pH 2.12, and the work of Richards and Logue (1962) on the spectrophotometric properties of ribonuclease-S and its component parts at pH 1.75. Additional evidence was obtained from the CD spectra of ribonuclease-S and S-protein at 12°. The effect of acid pH on the CD spectrum of ribonuclease-S at 12° is shown in Figure 13. The CD spectrum of S-protein at pH 2.34 (12°), and the CD spectrum of S-peptide at pH 2.28 (12°), and the CD spectrum of ribonuclease-S at pH 2.34 and 12° are shown in Figure 14. The dotted line in Figure 14, in the lower wavelength region, is the result of the summation of the CD spectra of

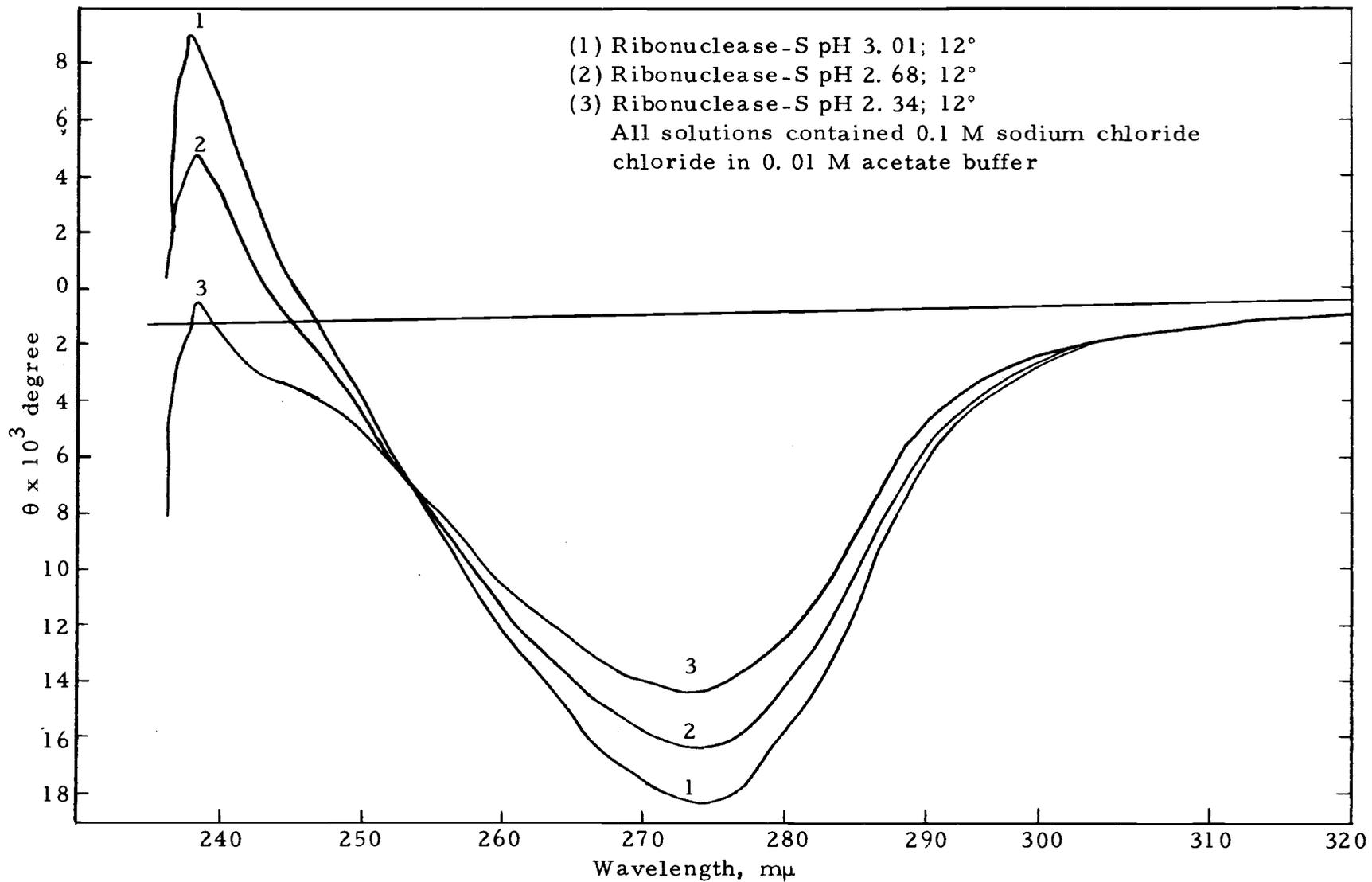


Figure 13. The effect of acid pH on ribonuclease-S at 12° C.

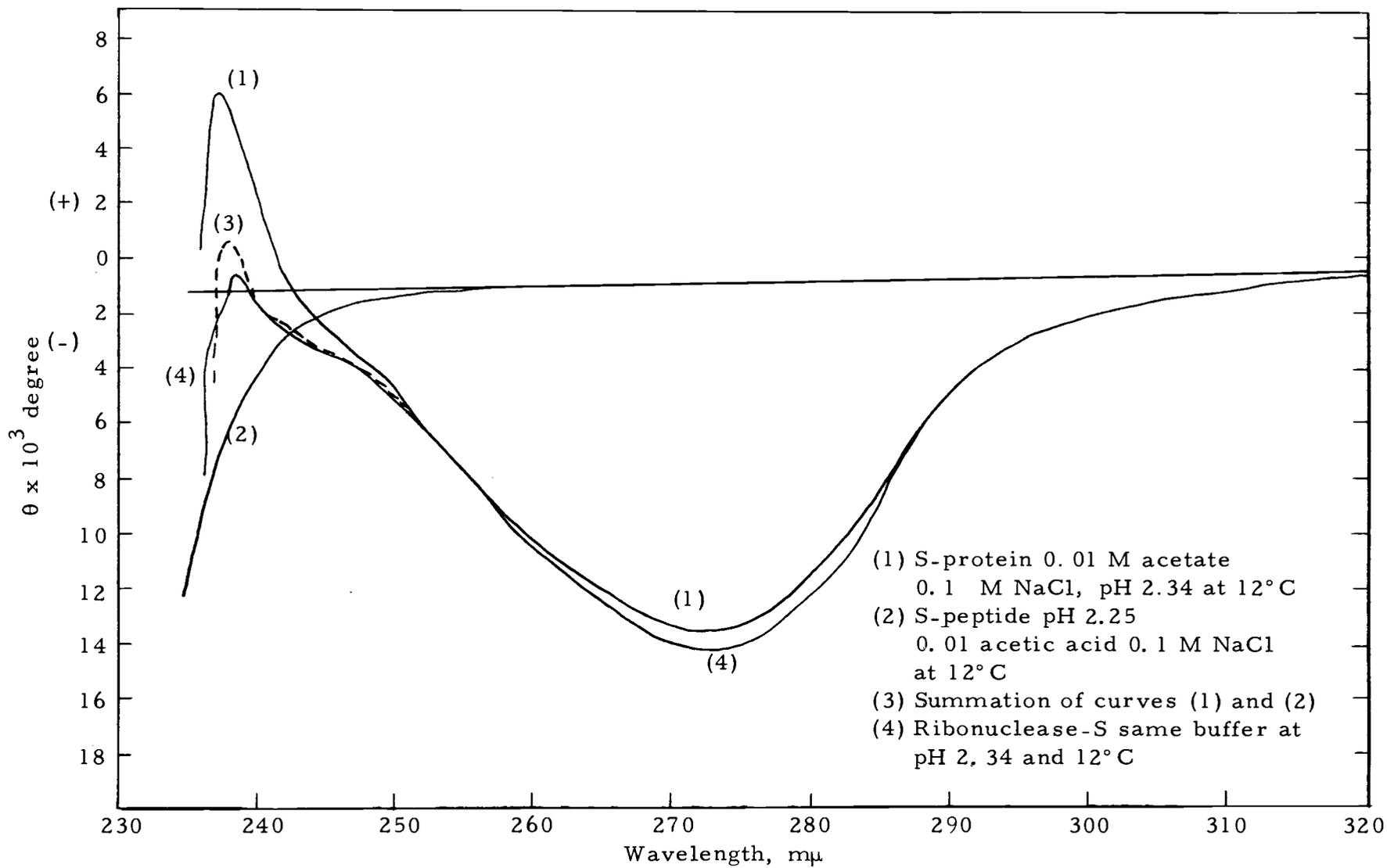


Figure 14. Circular dichroism of S-protein at pH 2. 34 and 12°.

S-protein and S-peptide. The resulting curve is quite similar to the CD spectrum of ribonuclease-S in this region. The additivity of the S-protein and S-peptide CD spectrum resulting in a curve similar to that of ribonuclease-S indicates S-peptide is dissociated from S-protein at this temperature and pH. The apparent dissociation of S-protein and S-peptide at acid pH suggests an alternative method of obtaining the desired volume change through the addition of small amounts of acid to ribonuclease-S in the dilatometer. The assumptions involved in this set of experiments are based on the following information from the literature. The addition of small amounts of acid to ribonuclease-A in the dilatometer resulted in a linear volume change of 11 cc/mole protons reacting between pH 5.5 and pH 2.0 (Rasper and Kauzmann, 1962). An earlier paper by Kauzmann (1958) indicated that the addition of small amounts of acid to ovalbumin resulted in a similar increase of 11 cc/mole proton reacting between pH 5.0 and pH 2.0. However, the addition of small amounts of acid to bovine serum albumin (BSA) did not give similar results. A plot of the volume change per 10^5 grams of BSA vs. moles of protons bound, showed a break in the curve at lower pH corresponding to a decrease in volume of 200 cc/mole BSA to 400 cc/mole BSA depending upon whether or not the experiments were conducted in 0.15 M potassium chloride. The larger value corresponds to addition of acid to BSA in the absence of potassium chloride. The dilatometric data

correlated well with the data obtained by Tanford et al. (1955) which indicate the intrinsic viscosity of BSA increases between pH 4.3 and pH 2.0. This suggests BSA is undergoing a conformational change and the dilatometer is sensitive enough to follow such a change. It is therefore the opinion of this laboratory that the addition of acid to ribonuclease-S in the dilatometer will give results similar to BSA, and will allow the determination of the volume change accompanying the dissociation of S-peptide and S-protein.

The addition of acid to ribonuclease-S in the dilatometer resulted in a non-linear increase in volume characteristic of the results obtained for BSA. Three such experiments were performed on ribonuclease-S at 25°. Also, small amounts of acid were added to ribonuclease-S in the dilatometer at 15°. Finally, small amounts of acid were added to ribonuclease-A in the dilatometer at 25° as a reference and a check of the procedure. A plot of the volume change per 10^5 grams of protein vs. moles of protons bound for these experiments is shown in Figure 15. The titration of ribonuclease-A resulted in a linear curve having a slope corresponding to a volume increase of 10 cc/mole protons reacting. The value obtained by Rasper and Kauzmann (1962) was 11 cc/mole protons reacting. The plot of the data for ribonuclease-S at 25° resulted in a non-linear curve, with the break in the curve occurring below pH 3.6. These data correlate well with the pH dependence of the spectral transition at 287 m μ and

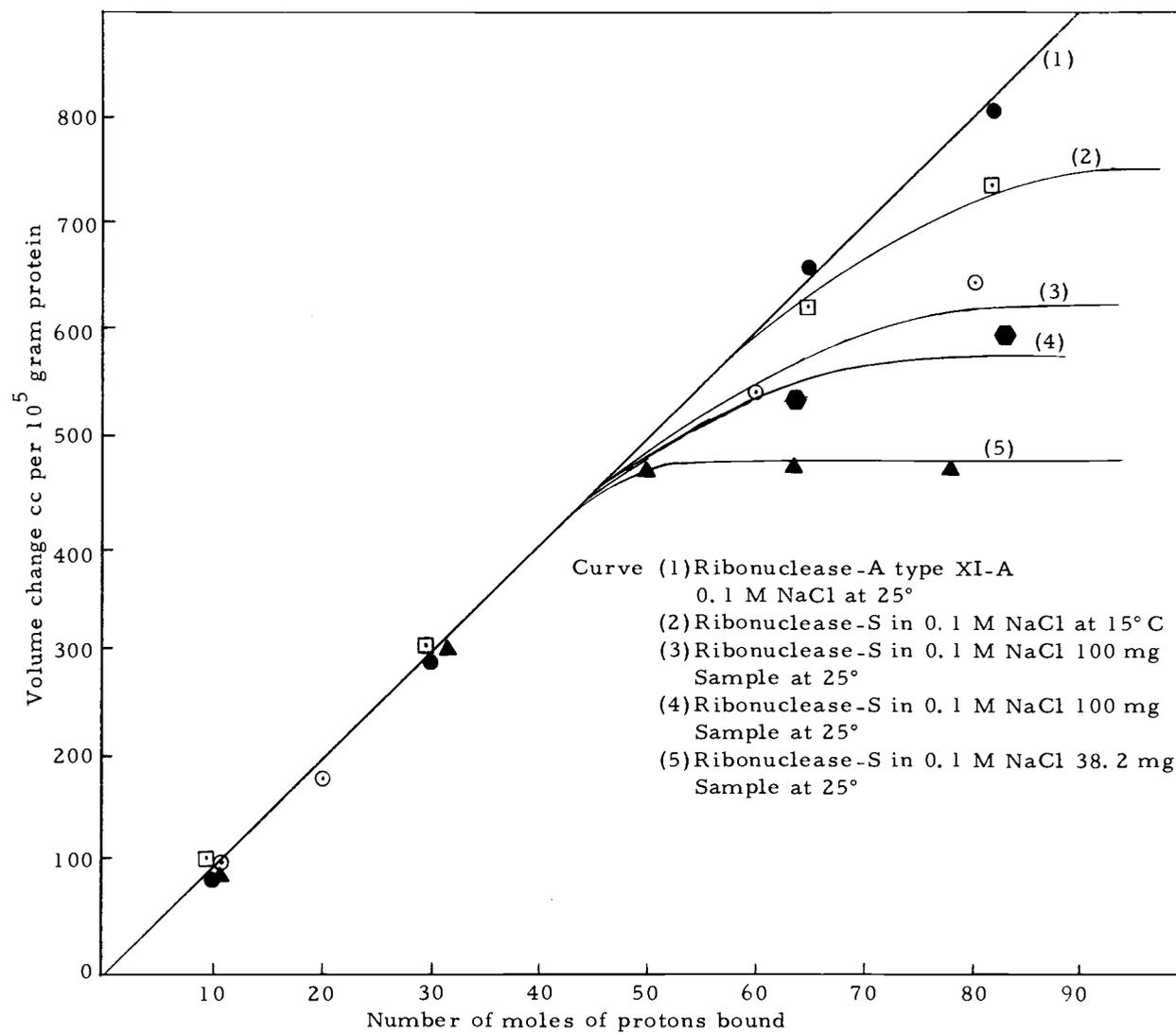


Figure 15. The volume change accompanying titration of ribonuclease-S and ribonuclease-A with hydrochloric acid.

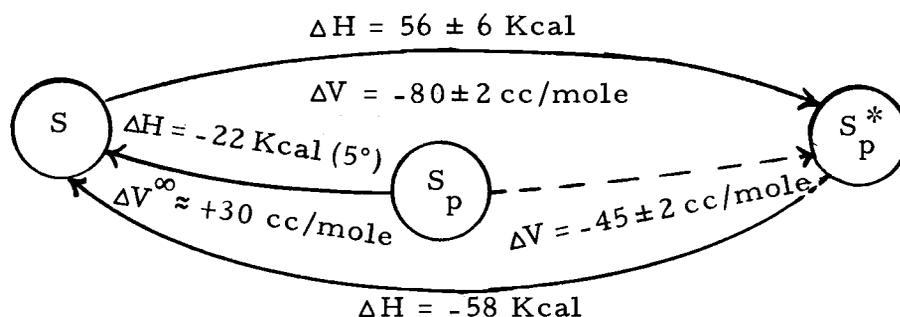
22° shown by Richards and Logue (1962). The spectral transition of ribonuclease-S at 22° begins below pH 3.6 and increases through the region pH 3.5 to pH 2.5. The addition of acid to ribonuclease-S in the dilatometer at 15° resulted in a similar non-linear curve, but the break in the curve occurred at a lower pH and was of lesser magnitude compared to the break in the curve for ribonuclease-S at 25°. The calculation of the volume change from this data was made in a manner similar to Kauzmann (1958). The titration of ribonuclease-A to pH 2.0 and 0.15 M salt results in the protonation of 11 carboxylate groups (Tanford and Hauenstein, 1956). This corresponds to 80.3 groups titrated per 10^5 grams of ribonuclease-A. (The molecular weight of ribonuclease-A is 13,683.) The volume change resulting from the titration of carboxylate ions in proteins is 11 cc/mole proton reacting (Rasper and Kauzmann, 1962). Therefore, a volume change of +883 cc should result when 10^5 grams of ribonuclease-A are titrated from pH 5.5 to pH 2.0 in the dilatometer. Ribonuclease-S possesses one additional carboxylate group due to the limited subtilisin BPN' digestion of peptide linkage 20-21 or 21-22 (Doscher and Hirs, 1967). Therefore, a volume change of +964 cc should result when 10^5 grams of ribonuclease-S are titrated from pH 5.5 to pH 2.0. The addition of acid to ribonuclease-A in the dilatometer performed in this laboratory resulted in a volume increase of 10 cc/mole proton reacting. Using this value the expected volume change for 10^5 grams

of ribonuclease-A and ribonuclease-S should be +800 cc and +876 cc respectively. Extrapolating the data obtained for ribonuclease-S at 25° (Figure 15) to approximately 87 moles protons bound, results in an actual volume increase per 10^5 grams protein of +413 cc to +650 cc. Depending upon whether one chooses the reference volume change of +876 cc or +964 cc as discussed above, an apparent volume decrease of 45 ± 13 cc/mole or 56 ± 14 cc/mole results when ribonuclease-S is titrated from pH 5.5 to pH 2.0 at 25°. Correspondingly, an apparent volume decrease of 17 cc/mole or 29 cc/mole results when ribonuclease-S is titrated from pH 5.5 to pH 2.0 at 15°.

DISCUSSION

A severe limitation of the pressure studies is the equilibrium expression used to describe the process occurring during denaturation. The simple approach is to assume a one step or two state system in which the molecules are in equilibrium with a fraction in the native state and the remaining fraction in the denatured state. There is a question of validity in using this simple model due to the non-linearity seen at higher temperatures in the van't Hoff plot, and also due to differences in enthalpy obtained by various methods. The latter is a question, however, of how accurate the enthalpy values are, i. e., is the value of +42 Kcal obtained from CD significantly different from the value of +56 Kcal obtained by difference spectra?

If one is considering a state process in which many routes occur in obtaining the final denaturation state, it is possible to look at the various enthalpies obtained under different conditions and to see how these compare. Richards et al. (1968) have reported microcalorimetry data for the association of the S-peptide and S-protein as a function of temperature from 5° -40° at pH 5.0 in 0.3 M NaCl. The values obtained were -22 Kcal/mole at 5° and -58 Kcal/mole at 40°. The variations in the enthalpy are largely due to the conformational change in the S-protein. It is possible to assume the following set of processes;



where S is the native ribonuclease-S, S_p represents native S-protein going to ribonuclease-S through addition of S-peptide, and S_p^* represents a reversibly denatured S-protein. The microcalorimetry experiments of Richards are indicative of the lower paths, where one can postulate many states between S_p and S_p^* depending upon the extent of reversible thermal denaturation of S_p as reflected by the variation of enthalpy with temperature. The upper path is postulated in this thesis to be occurring when ribonuclease-S has undergone reversible thermal denaturation or pressure denaturation. Supporting evidence for this is taken to be the apparent involvement of abnormal tyrosine A with the binding of S-peptide and S-protein, one of the two abnormal tyrosine residues is exposed in S-protein, (Sherwood and Potts (1965b) suggest abnormal tyrosine residue A to be normalized), and the exposure of both abnormal tyrosine residues A and B occurs during thermal denaturation. Thus, it would appear that the enthalpy of the upper path obtained by indirect methods and

depending upon the one step denaturation hypothesis agrees somewhat in magnitude and certainly in direction with the value of the lower path obtained through microcalorimetry. This is not intended to validate the one step process, but to suggest that its use in this case has meaning under the limits set for it.

The values obtained for the $\Delta \bar{V}_{av}^{\infty}$ of ribonuclease-S and S-protein calculated from the effect of pressure on the denaturation equilibrium expression were -80 ± 2 cc/mole and -45 cc/mole ± 2 cc/moles respectively, under similar conditions of pH, temperature, pressure, and protein concentration. This is taken to indicate that an approximate volume decrease of -35 cc/mole occurs upon dissociation of the S-peptide, and the concomitant reversible denaturation of the S-protein portion involves a volume change of approximately -45 cc/mole.

The results of the dilatometer studies tend to agree in a gross manner with the results of the pressure studies. The determination of the volume change due to the interaction of S-peptide and S-protein was found to be $+32 \pm 6$ cc/mole S-protein and would appear on the surface to agree quite well with the value of -35 ± 2 cc/mole ribonuclease-S obtained from the pressure studies for the assumed dissociation of S-peptide and S-protein. Information obtained by Allende and Richards (1962) seems to cast some doubt on the meaningfulness of the dilatometer value. Their study gave evidence that S-protein

aggregates above pH 3.0 and at concentrations greater than 3.0 mg/ml. There is no evidence to indicate that the aggregation involves the same region of the molecule vacated by the S-peptide. The aggregates readily dissociate upon addition of S-peptide. The spectral studies of Richards and Logue (1962) are not concentration dependent, suggesting the aggregation does not involve the same sites that bind the S-peptide. Also, tryptic digest intermediates of S-protein show no tendency to polymerize but they do interact with S-peptide to give complexes with at least some enzymatic activity (Allende and Richards, 1962), which would also indicate that polymerization must involve a site separate from the one vacated by S-peptide. There is also a question as to what extent the S-protein population is aggregated at a concentration of 3 mg/ml at pH 5.0 since the concentration dependence of S-protein determined by Allende and Richards (1962) was obtained on samples at pH 8.0. Furthermore, the pH dependence of the aggregation was determined on samples that were 10 mg/ml in concentration and may not be representative of S-protein at a concentration of 3 mg/ml.

Titration of a mixture of S-peptide and S-protein in equimolar concentrations at pH 1.75 and at 22° with concentrated base results in an increase in the molar absorbance at 287 m μ between pH 1.75 and pH 7.0, with the steep portion of the transition occurring in the region pH 2.5-pH 3.5 (Richards and Logue, 1962). The magnitude of this change in molar absorbance at 287 m μ is +1500 indicating two tyrosine residues are affected. Data obtained by Richards and Logue

(1962) further indicate that the binding of S-peptide to S-protein results in an immediate change in the molar absorbance at 287 m μ followed by a time dependent change in the molar absorbance. All this data, in conjunction with our experiment on the separation of S-peptide from S-protein, indicate S-peptide is separated from S-protein in acid pH, and the binding of S-peptide at a pH where the interaction will occur involves a conformational change in S-protein with the extent of this conformational change being dependent upon the temperature at which the interaction takes place. The circular dichroism data presented in the second section of this thesis, as well as the microcalorimeter experiments conducted by Richards et al. (1968), support the conclusions that the binding of S-peptide to S-protein produces a change in the conformation of S-protein. From this it is suggested that the addition of acid to ribonuclease-S in the dilatometer at 25° results in both a dissociation of S-peptide from S-protein and a corresponding change in S-protein conformation. The volume decrease obtained of -45 ± 13 cc/mole or 56 ± 14 cc/mole, depending upon which reference value is chosen, must represent both the dissociation of ribonuclease-S and the change in S-protein conformation. The value obtained for the addition of acid to ribonuclease-S in the dilatometer at 15° was a decrease of 17 cc/mole S-protein or 29 cc/mole again depending upon which reference was chosen. The extent of conformational change of S-protein is greatly reduced at this temperature and it is suggested that these values must reflect the

dissociation of S-peptide from S-protein. Figures 10 and 14 show the effect of temperature on CD spectra of S-protein at low pH, and indicate that S-protein exhibits a considerably native spectrum at pH 2.34 and at 12°. The difference in the value obtained at 25° and 15° is -27 cc/mole protein. This value is suggested to represent the volume change due to the conformational change of S-protein and is to be compared with the value of -45 cc/mole protein obtained for the pressure induced conformational change of S-protein.

From these studies it is concluded that a volume increase of 17 cc/mole to 35 cc/mole accompanies the interaction of S-peptide with S-protein. Furthermore it is suggested that a volume change of -27 cc/mole protein to -45 cc/mole protein must accompany the reversible denaturation of S-protein. From the data obtained it is difficult if not impossible to speculate on the individual contributions to the volume change from the various hydrophobic groups. In a gross manner, it is felt that the suggested value of +20 cc/mole aliphatic interaction (Kauzmann, 1959) is probably too high and that the value must be closer to a value of +8 cc/mole as suggested by Némethy and Scheraga (1962c).

PART II. CIRCULAR DICHROISM STUDIES OF S-PROTEIN
AND THE STEPWISE TITRATION OF S-PROTEIN WITH
S-PEPTIDE AS A FUNCTION OF TEMPERATURE
INCLUDING THE DETERMINATION OF THE DISSOCIATION
CONSTANT OF RIBONUCLEASE -S'

INTRODUCTION

Ribonuclease S-protein, in 0.01 M sodium phosphate buffer (pH 6.8) containing 0.9% sodium chloride, has been shown to undergo a spectral transition between 16° and 42° as determined by difference spectroscopy (Sherwood and Potts, 1965b). That the thermal denaturation is occurring at room temperature (22° to 26°) and under conditions that stabilize ribonuclease-S, makes it imperative that the temperature be adequately defined when conducting physical studies on S-protein.

Simons and Blout (1968), in a recent communication concerning the circular dichroism (CD) of S-protein, cited experimental evidence which demonstrated that ribonuclease-A and ribonuclease-S exhibit a maximum in their respective CD spectra at 240 m μ , but that this maximum is absent in the CD spectra of S-protein and ribonuclease-S. The presence of a maximum in the CD spectrum of ribonuclease-S at 240 m μ and the corresponding absence of this maximum in the CD spectrum of S-protein, in conjunction with other evidence, leads to the conclusion that the dissociation of S-peptide from S-protein disrupts an interaction between a S-peptide residue and one on the

S-protein, and that it is this interaction (tyrosine carboxylate interaction) which is responsible for the CD maximum at 240 m μ . The temperature at which these experiments were conducted was not defined. Therefore an alternative conclusion can be offered as to why the 240 m μ maximum is absent in the S-protein CD spectrum.

A study of the effect of temperature on the CD spectrum of S-protein was conducted in this laboratory. These studies demonstrated that the CD spectrum of S-protein is very sensitive to thermal change, particularly in the region of 237 m μ to 250 m μ . Furthermore it was shown that a maximum exists in the 240 m μ region of the S-protein CD spectrum in the temperature range of 13° to 24°, and that this maximum increases in magnitude with decreasing temperature. The maximum is greater in magnitude for S-protein than that of ribonuclease-A or ribonuclease-S at 13°, and it is shifted from 240 m μ to 237 m μ at all temperatures investigated below 24°. The magnitude of the maximum diminishes below the base line above 24° and the maximum is absent at 26°. Because a maximum in the CD spectrum of ribonuclease S-protein in the 240 m μ region was shown to exist below 24°, we decided to add S-peptide to see what effect this would have on this maximum. Stepwise addition of S-peptide to S-protein, to a slight molar excess of S-peptide, resulted in a shift of the maximum from 237 m μ to 240 m μ at temperatures below 24°. The maximum decreases or increases in magnitude depending upon the temperature

at which the S-peptide is added. This phenomenon appears to be poised at approximately 21°. Addition of S-peptide to S-protein at 26° resulted in the appearance and subsequent increase in magnitude of the 240 m μ maximum.

The negative CD maximum for S-protein at 275 m μ increases in magnitude upon addition of S-peptide. A plot of molar ellipticity vs. S-protein concentration resulted in a titration curve similar to that obtained by Woodfin and Massey (1968) for the titration of S-protein with S-peptide as determined by difference spectroscopy. A dissociation constant for ribonuclease-S' was calculated for such a titration curve, and the value obtained was approximately 5×10^{-6} M at pH 5.0 in 0.01 M acetate buffer containing 0.1 M sodium chloride.

EXPERIMENTAL PROCEDURES

Ribonuclease-S and its component parts S-protein and S-peptide were prepared from Sigma type II-A ribonuclease-A according to the methods of Richards and Vithayathil (1959) and as reviewed by Marilyn Doscher (1968). Subtilisin was Nagarse Subtilisin BPN¹ purchased from the Enzyme Development Corporation. Ribonuclease-S and S-protein were desalted according to the methods described by Gordillo, Vithayathil, and Richards (1962). Amino acid analysis and the lack of activity towards yeast RNA substrate were used to characterize S-protein.

Preparation of Solutions

Solutions of S-protein and S-peptide were prepared from the dry salt-free powders, and made up in 0.01 M acetate buffer pH 5.0 containing 0.1 M NaCl. The concentrations of the S-protein solutions were 0.84 to 0.87 mg/ml as determined by their absorbance of a 1.0 mg/ml solution of S-protein of 0.784 at 280 m μ (Sherwood and Potts, 1965). The concentrations of the S-peptide solutions were 2.2 mg/ml and 10.0 mg/ml respectively.

Circular Dichroism

Circular dichroism spectra were obtained using a Durrum-Jasco circular dichrograph model CD-SP. A rectangular cuvette of

1 cm path length, which was jacketed for temperature control, was used. A Lauda/Brinkmann Circulator Model K-2/R, providing specified temperature control to $\pm .02^{\circ}\text{C}$, was used for temperature regulation. Temperature measurements were made before and after each CD run using a thermistor temperature sensing device manufactured by the Yellow Springs Instrument Company. Inaccessibility to the sample compartment while the circular dichrograph was in operation made it unfeasible to monitor the temperature during the CD runs. Aliquots of S-peptide were added to the S-protein solution using 10 μl and 50 μl Hamilton syringes. Each addition was followed by stirring.

The CD spectra in these experiments were run in the region of side chain optical activity as defined by Beychok (1968). Therefore it was decided to express the CD data in terms of molar ellipticity rather than mean residue ellipticity which has been customary. The molar ellipticity is defined as follows. $[\theta] = \theta/100 c'd$, where θ is the measured ellipticity obtained from the dichrograph in millidegrees/cm, c' is defined as gram-moles/cc, and d is the path length in decimeters. $[\theta]$ has the dimensions of $\text{deg}(\text{cm}^2/\text{decimole})$ (Velluz, Legrand and Grosjean, 1965).

RESULTS AND DISCUSSION

The Circular Dichroism of S-Protein and the Stepwise Titration of S-Protein with S-Peptide as a Function of Temperature

The CD spectra as a function of temperature for S-protein in 0.01 M acetate buffer (pH 5.01) containing 0.1 M sodium chloride, is shown in Figure 16. This family of curves demonstrates clearly that a maximum does exist in the CD spectrum of S-protein in the 240 m μ region, that it is shifted from 240 m μ to 237 m μ , and that the 237 m μ CD band increases in magnitude as the temperature decreases. The CD spectrum at 14° of ribonuclease-S in 0.01 M acetate buffer (pH 3.57) containing 0.1 M sodium chloride is given in curve (5) of Figure 16. The magnitude of the CD band of S-protein at 237 m μ and 13° is approximately three times greater than that for the ribonuclease-S at 240 m μ . We wanted to know whether or not the maximum at 237 m μ of the S-protein spectrum at 21° would be lost upon addition of S-peptide to S-protein. The addition of S-peptide to S-protein did not result in the loss of the 237 m μ band as can be seen in Figure 17. However, the maximum at 237 m μ was shifted to 240 m μ upon addition of an equimolar amounts of S-peptide. Addition of excess peptide does not result in further shift of the 240 m μ maximum. It can also be seen that the negative 275 m μ CD band for the S-protein increases in magnitude upon addition of S-peptide. Titration of S-protein with

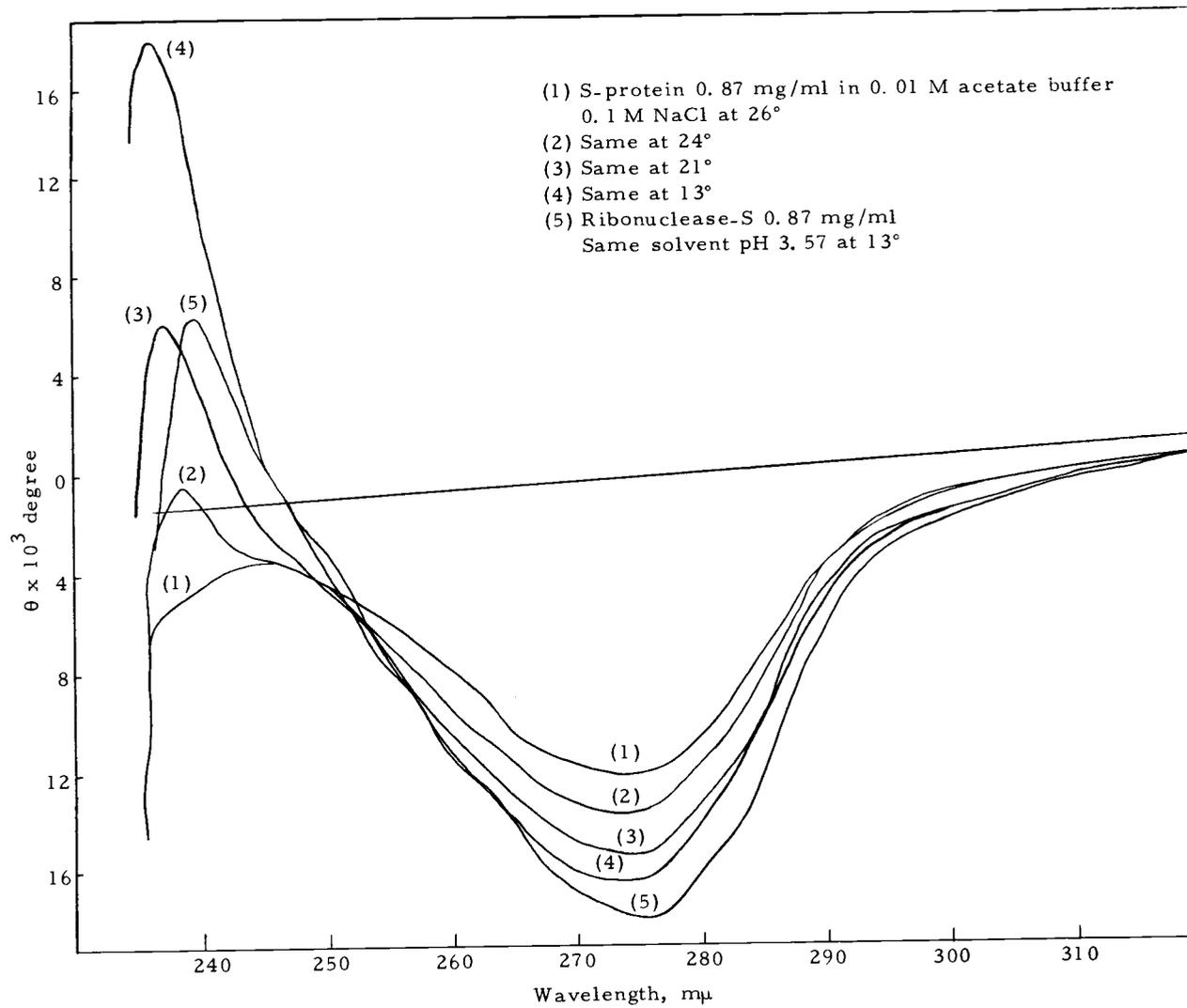


Figure 16. Ribonuclease S-protein circular dichroism as a function of temperature.

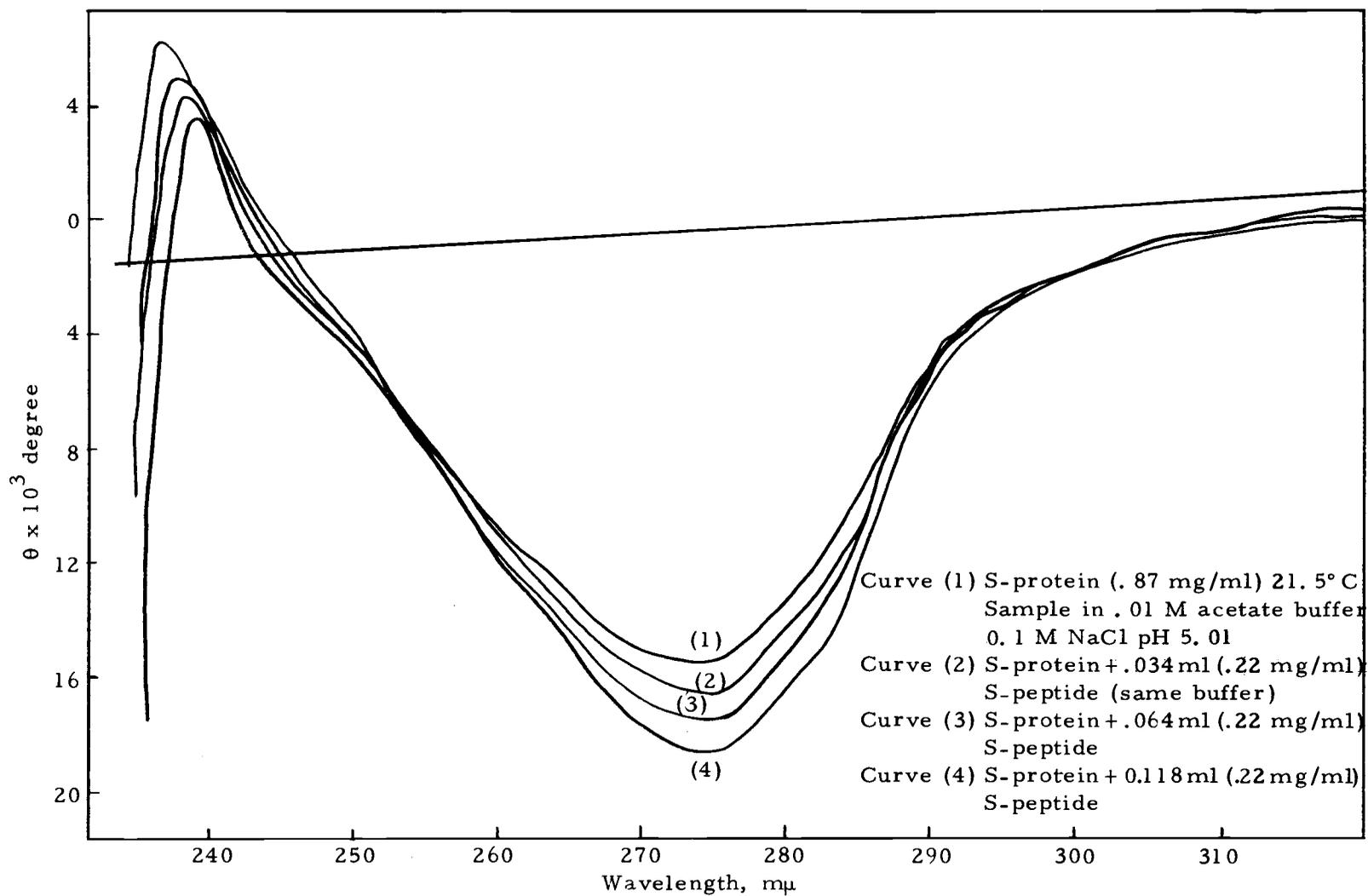


Figure 17. The stepwise formation of ribonuclease-S' by titration of S-protein with S-peptide at 21. 5° C.

S-peptide at 13° and 26° also produces marked change in the CD spectra in the region 320 m μ to 235 m μ as shown in Figure 18 and Figure 19. The stepwise addition of S-peptide to S-protein at 26° can be seen to result in the formation of the 240 m μ maximum and a subsequent increase in its magnitude. We then raised the question, could these results be explained by the additive contributions of both CD spectra in the region 320 m μ to 235 m μ ? The CD spectra of S-peptide, corresponding to approximate S-peptide to S-protein ratios of 0.25, 0.75, and 7.0 are shown in Figure 20. S-peptide contributions to the CD in the region 320 m μ to 270 m μ are negligible. However, the S-peptide contribution becomes apparent in the 250 m μ to 235 m μ region. The decrease in magnitude and shift of the 237 m μ CD band of the S-protein upon addition of S-peptide at 13° might be explained by the additive contribution of the S-peptide CD spectrum between 237 m μ and 240 m μ . The dotted lines in Figure 20 indicate how this contribution of the S-peptide spectrum could cause a decrease in the magnitude and apparent shift in the 237 m μ maximum upon the stepwise addition of the S-peptide to S-protein. Mere additive contributions of the S-peptide CD can not explain the increase in magnitude of the negative 275 m μ band, nor can it explain the increase in magnitude of the 240 m μ CD band which occurs with the stepwise addition of S-peptide to S-protein at 26°. It is evident that the titrations of S-protein with S-peptide have resulted in the formation of ribonuclease-

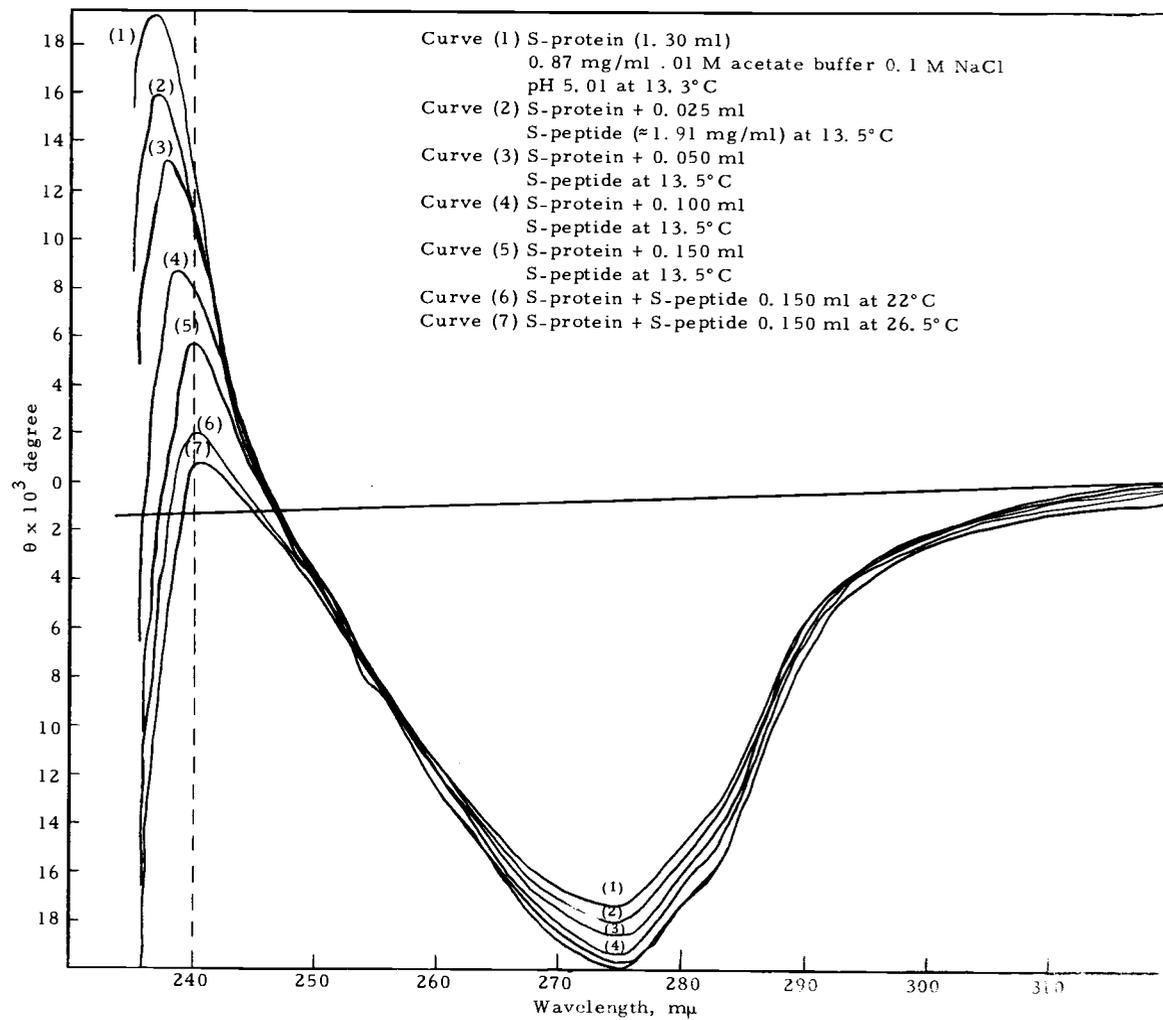


Figure 18. Stepwise formation of ribonuclease-S at 13°C.

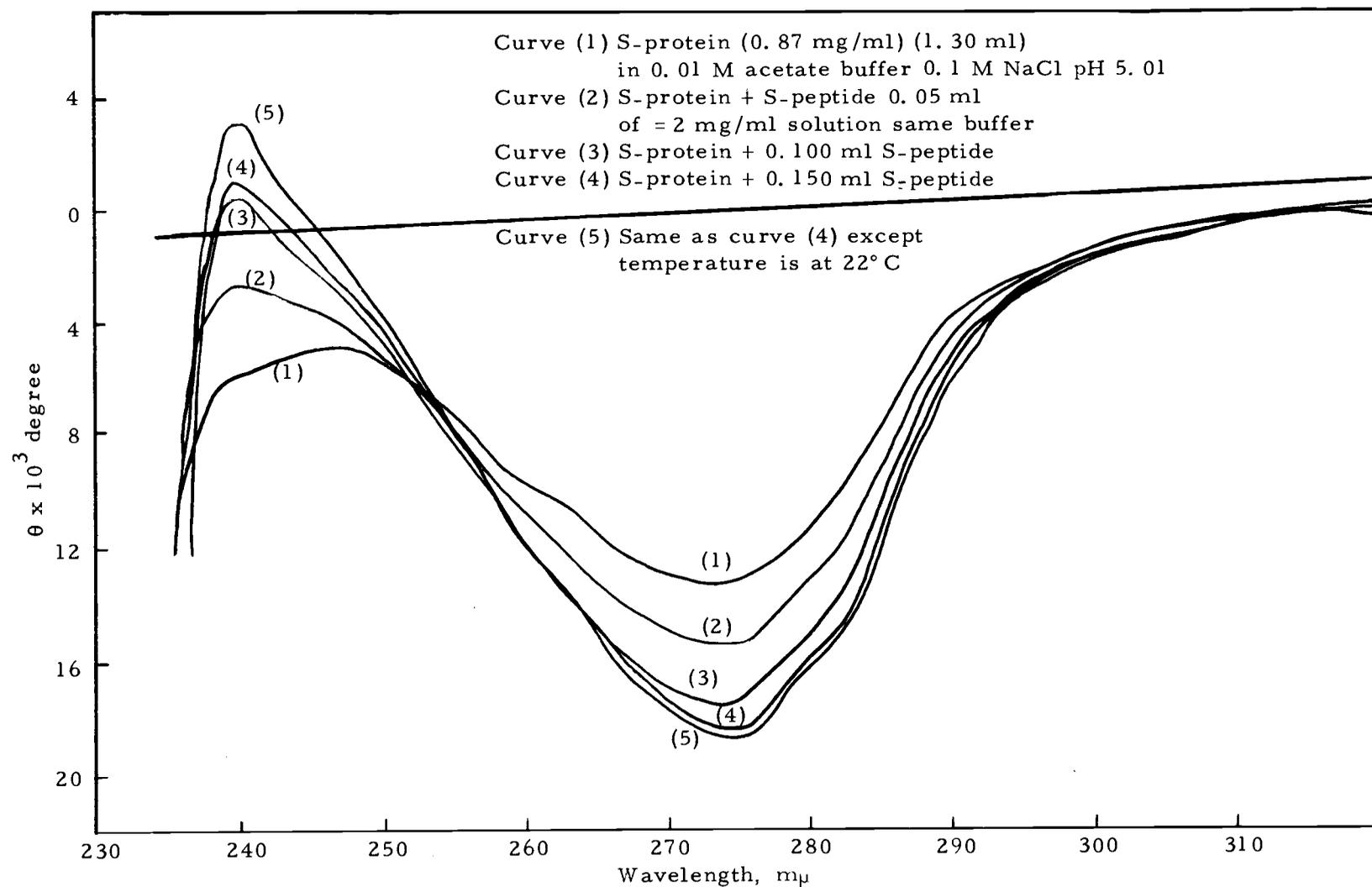


Figure 19. The stepwise formation of ribonuclease-S' by titration of S-protein with S-peptide at 26.5°C.

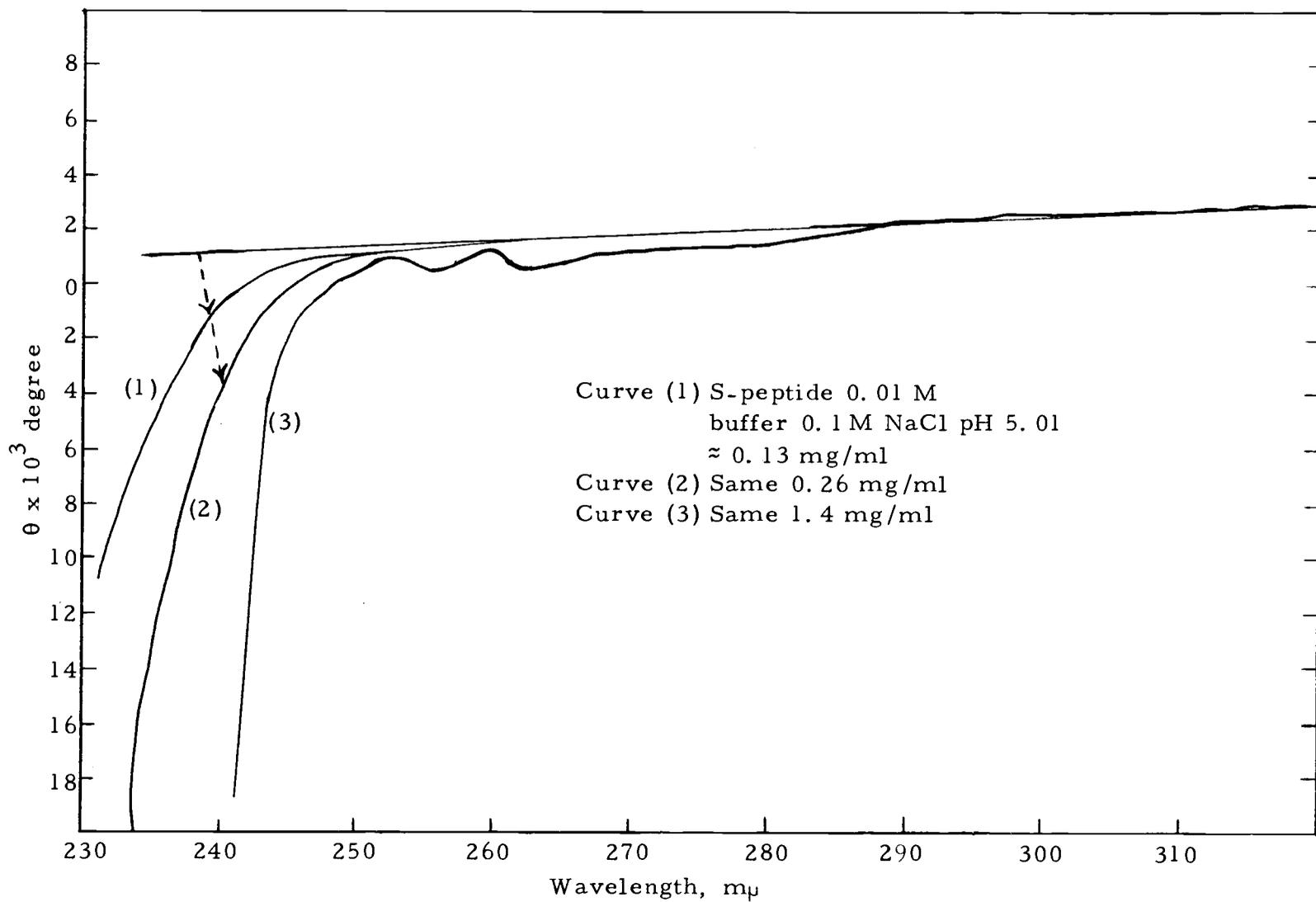


Figure 20. Circular dichroism of S-peptide as a function of concentration at 16°.

S', which exhibits a 240 m μ maximum similar to native ribonuclease-S. It is also evident that the addition of S-peptide to S-protein at 13° results in small incremental increases in the magnitude of the 275 m μ CD band. This also occurs at 21° and 26°, but the size of the incremental change becomes larger in this region. This suggests that the interaction of S-peptide and S-protein to form ribonuclease-S' involves at least two phenomena. The small incremental changes in the 275 m μ maximum at 13° indicates a change is occurring in a tyrosine environment, possibly Tyr 25. The increase in the magnitude of this incremental change in the 21° to 26° region suggests that the S-protein portion of ribonuclease-S' undergoes a conformational change on interaction with S-peptide. The 275 m μ CD band of the S-protein decreases more in magnitude in this temperature range than it does in the 13° to 21° temperature range, and hence a restoration of the S-protein conformation on interaction with S-peptide in the 21° to 26° range would produce a greater incremental change in the 275 band. This corresponds also to earlier findings of Richards and Logue (1962) in which they have shown that a time dependent change in the molar absorbance at 287 m μ occurs on addition of S-peptide to S-protein in 0.1 M acetate buffer (pH 4.50) at 22°. They suggest that this time dependent change is due to an intramolecular change in S-protein conformation.

From our results it is difficult to say which particular residue

is responsible for the positive 237 m μ -240 m μ band seen in the CD spectra of ribonuclease-S, ribonuclease-A, ribonuclease-S' and S-protein. The dissociation of S-peptide from S-protein has been shown to result in the exposure of one abnormal tyrosine (Sherwood and Potts, 1965). If the loss of the 240 m μ maximum in the CD spectrum of S-protein is the result of a disruption of a carboxylate (Asp 14) X or Y interaction, with the exposure of one abnormal tyrosine (Tyr 25) to the solvent, then it becomes difficult to believe that this residue is "buried" again when S-protein is cooled from 26° to 21°. Cooling the S-protein in this temperature range results in the protein exhibiting a positive 237 m μ band at 21°. The loss of the 240 m μ CD band in S-protein may also be ascribed to a thermally induced conformational change in the protein. Further cooling of S-protein from 21° to 13° results in a three fold increase in the molar ellipticity of the positive 237 m μ band of S-protein as compared to ribonuclease-S at 14°. A similar change can not be seen in the molar absorbance of S-protein at 286 m μ when the temperature is raised from 13° to 21° (Sherwood and Potts, 1965b). This suggests that the positive CD band between 237 m μ and 240 m μ reflects a conformational change that does not involve an abnormal tyrosine. It can not be denied that the titration of S-protein with S-peptide results in a shift of the 237 m μ CD maximum to 240 m μ . However, whether S-peptide causes this shift by a direct interaction of one of its residues with a residue of the S-protein, or

by the combination of the contribution of its peptide CD spectrum with a corresponding change in the S-protein conformation remains to be proved. The CD spectrum of S-peptide in solution is not necessarily the same as its CD contribution when interacted with S-protein (Klee, 1968). This would be particularly true in the peptide region of the CD spectrum, for the X-ray data indicates that residues 2-12 of the interacted S-peptide are in an helical configuration (Wyckoff et al., 1967).

The Determination of the Dissociation Constant of
Ribonuclease-S'

A plot of the molar ellipticity vs. S-peptide concentration (Figure 21) resulted in a titration curve similar to that obtained by Woodfin and Massey (1968) for the titration of S-protein with S-peptide as determined from difference spectroscopy. It is our belief that with certain assumptions, the CD data obtained from the titration of S-protein with S-peptide could be used to calculate a dissociation constant for ribonuclease-S'. These assumptions are: (1) The S-peptide can not contribute appreciably in the region where the measurements are being made. (2) The contribution made by the CD spectrum in the region of measurement can be subtracted out. (3) The titration of S-protein with S-peptide to a large molar excess of S-peptide will allow calculation of the molar ellipticity of ribonuclease-S'. (Woodfin and Massey, 1968; NaKamura, Yoshimura and Ogura, 1965). (4) The

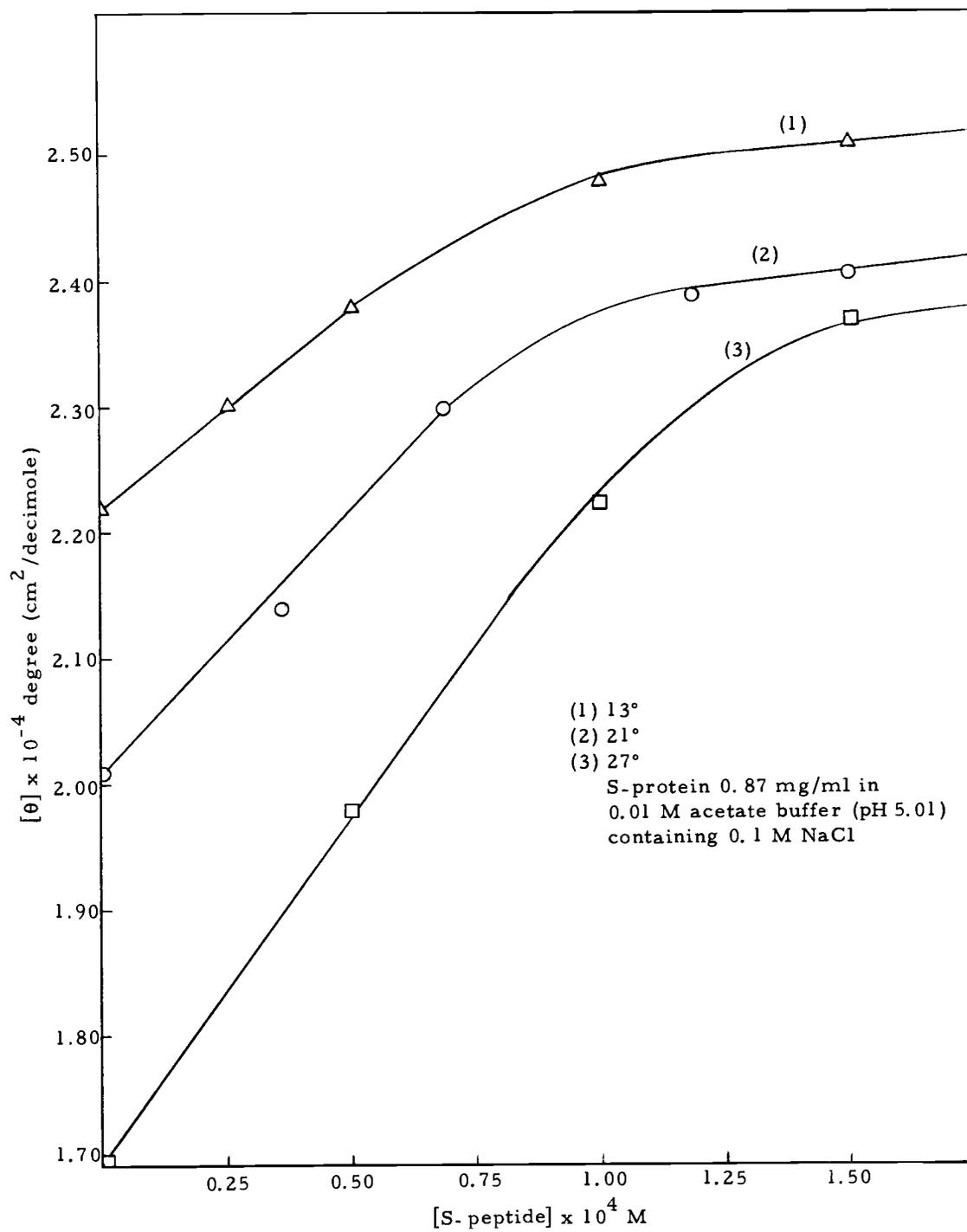


Figure 21. Change in molar ellipticity with the addition of S-peptide to S-protein.

ratio of the molar ellipticity at any point in the titration curve to the molar ellipticity of the ribonuclease-S' as determined from (3) will allow the calculation of the S-protein concentration involved in the complex and therefore enable the calculation of the dissociation constant from the following expression;

$$k_d = (\text{S-peptide})(\text{S-protein})/(\text{S-protein})$$

where k_d represents the dissociation constant of ribonuclease-S'.

The plot of molar ellipticity vs. S-peptide for the addition of an excess molar ratio of S-peptide to S-protein at 21.5° is shown in Figure 22. From this plot the molar ellipticity of the ribonuclease-S' complex was taken to be 2.628×10^4 degree $\text{cm}^2/\text{decimole}$. The measurement of molar ellipticity was made at the negative 275 $\text{m}\mu$ band for the following reasons. This region did not show an appreciable shift in maximum upon addition of S-peptide. The S-peptide does not contribute appreciably to the CD spectrum in this region (Figure 18), and that this, being the region of the tyrosine UV absorption maximum, is probably seeing a similar phenomenon as the UV difference spectrum at 287 $\text{m}\mu$. The same can not be said for the 240 $\text{m}\mu$ region of the CD spectrum. A dissociation constant of 5×10^{-6} M was obtained from the data. The value obtained by Woodfin and Massey (1968) was 7×10^{-5} M. Besides the difference in methods used to obtain this value, the solutions that we employed contained

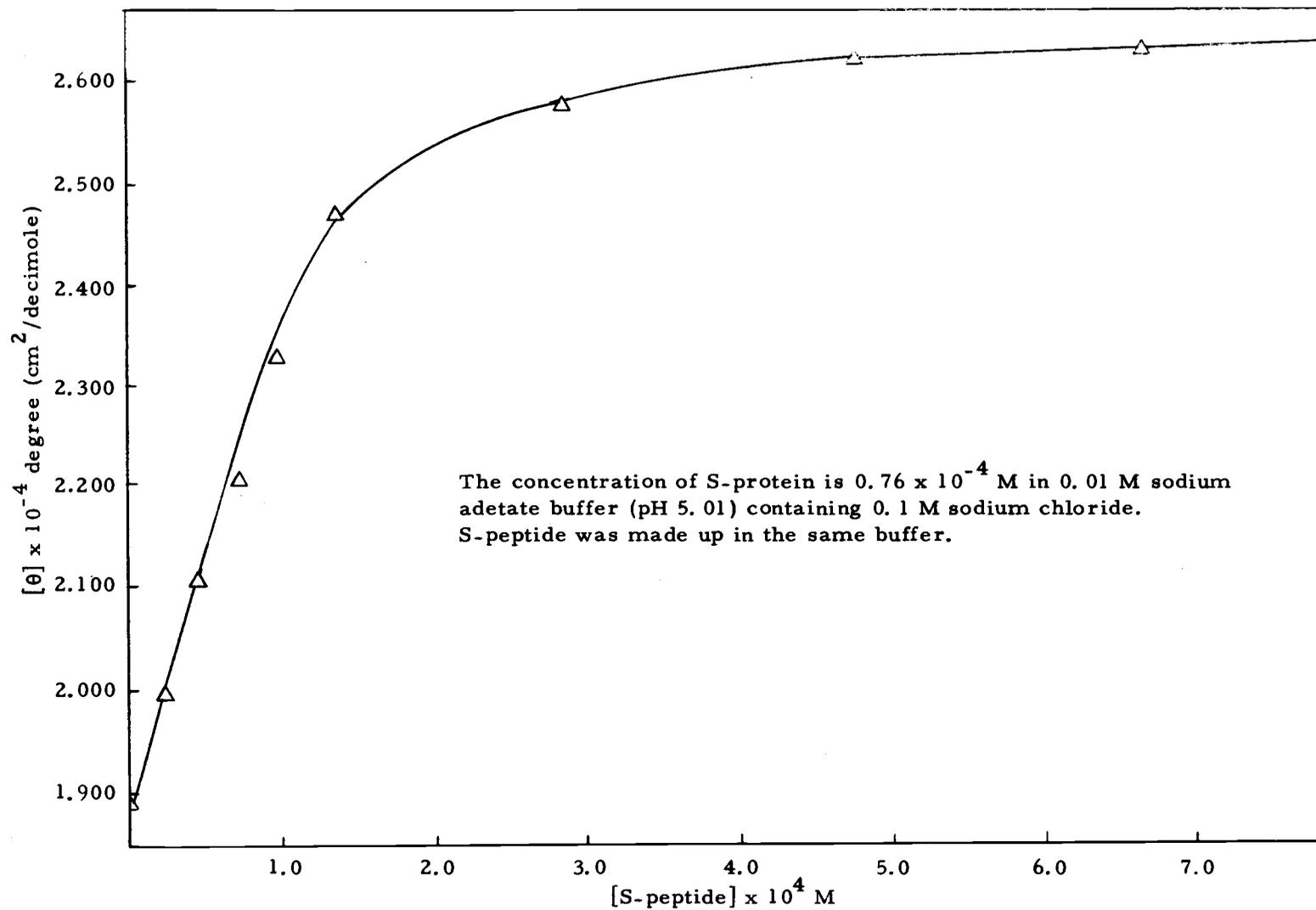


Figure 22. The change in molar ellipticity with addition of S-peptide to an excess.

0.1 M sodium chloride in 0.01 M acetate buffer (pH 5.01), whereas Woodfin and Massey employed 10^{-3} M phosphate buffer at pH 5.45. This 10 fold decrease in the dissociation constant may reflect a stabilizing of ribonuclease-S' by the increased ionic strength of the solution, or a stabilizing effect of the chloride ion.

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