Some Specific Ion Effects on the Conformation and Thermal Stability of Ribonuclease*

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ABSTRACT: Ribonuclease at pH 2.1 and low ionic strength shows variations in conformation and in thermal stability that are functions of the anions present. Measurements of optical rotation, optical rotatory dispersion, ultraviolet absorption, and viscosity at low temperature indicate a more ordered structure in the sulfate than in the chloride case. In addition, sulfate stabilizes the molecule against thermal denaturation, raising the transition temperature from 29 (chloride) to 43° (sulfate). After the transitions, the two salt forms are still not identical, the sulfate form retaining more ordered regions, and both forms being less disorganized than ribonuclease in 5 M guanidine hydrochloride. Nevertheless, in all cases heat produces practically identical changes in the properties measured. The results suggest that ribonuclease contains a thermolabile region that is reversibly disrupted by heat to an *extent* that is largely independent of the anion present, but that the lability is controlled by specific ion binding in a thermostable region of the molecule. The *sharpness* of the thermal transition also is dependent on the anion present which, together with other observations, can be related qualitatively to the existence of equilibria between various conformations at low temperatures. At low pH sulfate ion stabilizes a more ordered and compact configuration (without a corresponding increase in apparent net helical content) than does the monovalent chloride ion. The effects of phosphate are intermediate between those of chloride and sulfate, and can be related to an incomplete saturation of the stabilizing binding site(s) by divalent phosphate ion.

The concept that proteins in solution readily undergo conformational changes, with a particular configuration stabilized or destabilized through the binding of a specific low molecular weight substance, was introduced by Koshland (1958) and Linderstrøm-Lang and Schellman (1959). Various properties of proteins, including particularly those characteristic of enzymic catalysis, could be interpreted on this basis. Monod *et al.* (1963) have proposed further that such induced conformational changes play an essential role in the regulation of enzyme activity. Bovine pancreatic ribonuclease, which shows a varying specificity toward simple inorganic anions, was used in the study reported here to test the influence of chloride, phosphate, and sulfate ions at pH 2.1 on the conformation and thermal stability of this protein.

There have been numerous reports of phosphate and sulfate anion effects, which simulated the action of known substrates or substrate competitive inhibitors of ribonuclease when tested. These effects have been observed either in the protection from chemical modification of the amino acid residues known to be involved in the active site (Barnard and Stein, 1959; Crestfield *et al.*, 1963; Hirs, 1962; Cooke *et al.*, 1963) or in the stabilization of the molecule (Sela *et al.*, 1957; Sela and Anfinsen, 1957; Neumann *et al.*, 1962; Craig *et al.*, 1963; Nelson *et al.*, 1962). Crestfield *et al.* (1962) have reported also that phosphate ion stabilizes active aggregates of ribonuclease. In addition, Mathias *et al.* (1960) demonstrated complex formation between ribonuclease and the 2′- or 3′-phosphates of cytidylic acid, which are competitive inhibitors of this enzyme. Specific anion binding in the case of inorganic ions has been indicated by the work of Crestfield and Allen (1954), Rosemeyer and Shooter (1958, 1961), Saroff and Carroll (1962), and Loeb and Saroff (1964). These specific anion effects, in contrast to the general neutral salt effects studied by von Hippel and Wong (1963, 1964), are exerted at very low concentrations of the anion in question (0.0001–0.01 M if the binding affinity constant $\leq$ 1000) and can be differentiated from general salt effects on this basis.

Configurational changes in the ribonuclease molecule were thermally induced (Harrington and Schellman, 1956) in the following studies of the different anion

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effects.[1] Preliminary experiments indicated that the thermal denaturation of ribonuclease in the presence of low concentrations of divalent anions at low ionic strength is more completely reversible at acid pH, where this protein has a decreased thermal stability (Hermans and Scheraga, 1961), than at neutral pH. Accordingly, only the results obtained at pH 2.1 are reported. At low pH and temperature, in contrast to neutral pH conditions (Hermans and Scheraga, 1961), specific anion effects are enhanced due to the disordering of the protein effected by acid pH with only chloride ion present and the capability of the divalent sulfate ion to induce a form which approximates that observed at neutral pH. The relatively large changes produced by heat in the optical rotation, ultraviolet absorption, and viscosity as a function of the anion present provide information on the potential type and magnitude of structural changes which can be induced in a protein macromolecule through combination with a specific small molecule.

Materials and Methods

Bovine pancreatic ribonuclease, Type XIIA (Lot No. 13B-755), obtained from the Sigma Chemical Co.[2] and analyzed at 0.04–0.1 mole % phosphate, was used directly without further purification since chromatographic analysis of this lot by the method of Hirs *et al.* (1953) showed the presence of only ribonuclease fraction A. A complete deionization of this material, as needed, was achieved by cycling a 2–4% ribonuclease solution in water three times through a deionizing column of the type described by Dintzis (1952). The deionized stock solution was collected in polypropylene tubes, quickly passed through a 1.2-μ Millipore filter with a Swinney-type hypodermic adaptor, and immediately stored in a closed vessel containing Ascarite (Arthur H. Thomas Co.) at 2–4°. Such stock solutions of deionized ribonuclease initially and after as long as 3 months storage over Ascarite in the cold had pH 9.60 ± 0.01 and a conductivity of about 3×10^{-3} ohm^{-1}. However, in practice, the isoionic ribonuclease was used soon after its preparation. Solutions throughout were prepared by titrating a weighed aliquot of isoionic ribonuclease with the acid of the specific anion desired in the final solution. When necessary, a salt of the same anion was added to adjust the ionic strength of the medium to the desired value.

The concentrations of the deionized ribonuclease solutions were determined by measuring the optical density of a diluted aliquot (by weight) in 0.2 M sodium phosphate buffer at pH 6.46 (density at 20° = 1.0240) at 277.5 mμ using a partial specific volume of 0.695 ml/g (Harrington and Schellman, 1956), ϵ 9800 (Sela *et al.*, 1957), and a molecular weight of 13,683 (Hirs *et al.*, 1956). For ribonuclease solutions at pH $\leq$ 6, dilutions (by weight) with water were made for the concentration determinations.

Deionized water with a conductivity $\leq 1.7 \times 10^{-6}$ ohm^{-1} was obtained from a water deionizing unit (Model DJ-128) of the Crystal Research Laboratory, Inc., and this water was used exclusively. Corrections for the amount of ultraviolet absorbing material in the water arising from the resin were applied in the spectral work.

The barium salt of *cytidine 2',3'-cyclic phosphate* (Lot No. 6301) was obtained from Schwarz BioResearch, Inc., and it assayed at 97.3% pure spectrophotometrically using ribonuclease at pH 7 to effect the conversion to cytidine 3'-phosphate (ϵ 8970 at 272 mμ). The *RNA* used was prepared according to Crestfield *et al.* (1955) and was a gift from Dr. C. B. Anfinsen. *Pepsin* (Lot PM687), crystallized two times, was obtained from Worthington Biochemical Corp., as was the *hemoglobin* substrate (Lot No. 584) used in the pepsin assay. The XE-64, or IRC-50, resin was obtained from Fisher Scientific Co. under the trade name of Rexyn-CG51(H) (Lot No. 706080), and it was of 200–400 mesh size in the H^+ form. It was cycled through the Na^+ and H^+ forms before its equilibration with 0.2 M sodium phosphate buffer at pH 6.47 (Hirs *et al.*, 1953). The pH standards used were obtained from the National Bureau of Standards. All other materials were reagent grade.

Ribonuclease assays were performed at room temperature, usually by both the spectrophotometric methods of Kunitz (1946) and Crook *et al.* (1960).

Viscosity measurements were made in an Ostwald viscometer with an average shear gradient of 153 sec^{-1} and an outflow time of 113.04 ± 0.05 seconds for 1 ml of water at 25.0°. The average temperature fluctuation at 25.0° was ±0.003°, but varied as much as 0.1° at the very high temperatures, and about 0.03° at the very low temperatures. Because of evaporation during the runs at high temperatures, it was necessary to redetermine the concentration at the end of each series and to make small corrections (1–5%) in the actual concentration at each temperature. All solutions were filtered through a 1.2-μ Millipore filter with a Swinny-type hypodermic adapter before being pipetted into the viscometer.

A 10-ml pycnometer calibrated to 0.002 ml in the capillary stem was used for the determination of solvent densities at 25.00 ± 0.003°. Solvent densities at other temperatures were calculated from the relative water densities for those temperatures. Solution densities were calculated from the weight fraction of the protein and the partial specific volume at 20°, neglecting the temperature dependence of $\bar{v}$ (Holcomb and Van Holde, 1962), since this is such a small correction in the concen-

[1] The reversible thermal transition of ribonuclease has been examined by numerous investigators (Harrington and Schellman, 1956; Kalnitsky and Resnick, 1959; Foss and Schellman, 1959; Hermans and Scheraga, 1961; Foss, 1961; Holcomb and Van Holde, 1962; Scott and Scheraga, 1963). The specific optical rotation, the specific activity, the absorption spectrum, the intrinsic viscosity, and the sedimentation rate of ribonuclease have been used as indicators of the configurational changes in the molecule produced by heat.

[2] We are grateful to Mr. Louis Berger of the Sigma Chemical Co. for supplying details on the procedures used in preparing the XIIA ribonuclease.

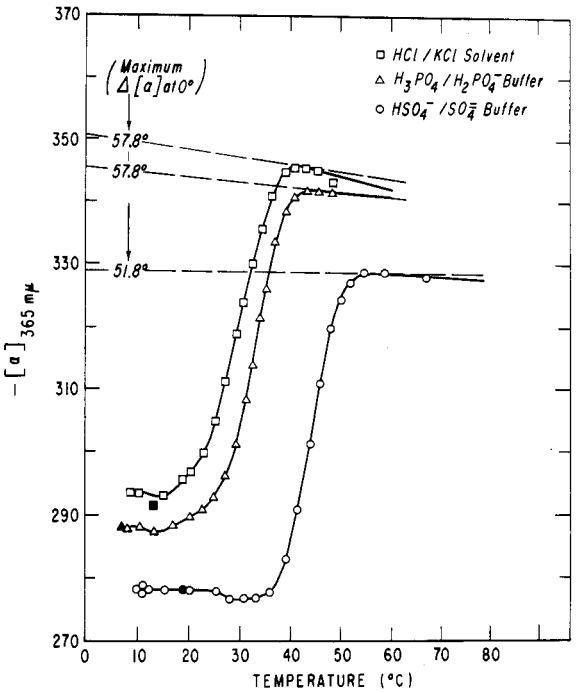

FIGURE 1: The specific optical rotation at 365 mμ as a function of temperature of 1.3% ribonuclease solutions at pH 2.1 and 0.019 ionic strength. Measurements after cooling from the high temperatures are shown by the solid symbols.

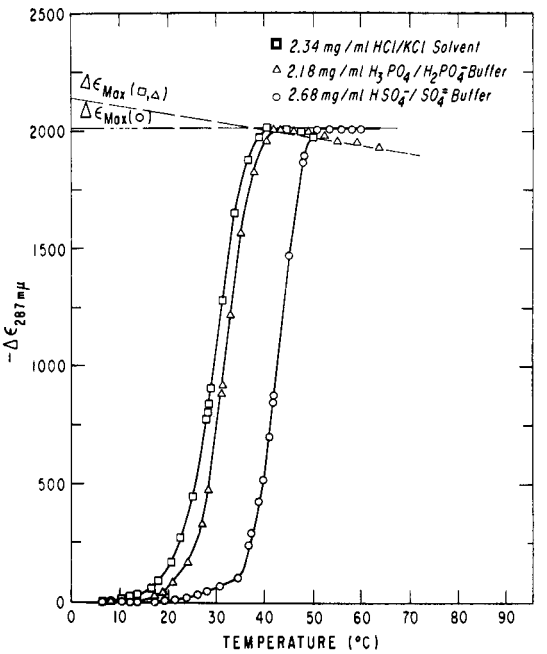

FIGURE 2: The change in the molar absorption at 287 mμ as a function of temperature of ribonuclease solutions at pH 2.1 and 0.019 ionic strength.

tration ranges used. The viscosities of the ribonuclease solutions at low ionic strength and pH 2.1 were not corrected for an electroviscous effect since this was found to be small by Buzzell and Tanford (1956), as can be anticipated from the anion binding (Saroff and Carroll, 1962; Loeb and Saroff, 1964) which lowers the net charge on the molecule.

pH Measurements and Titrations. Either the Beckman Model G pH meter or the Radiometer TTT-1 equipped with a type PHA-630 scale expander and a GK-2641C electrode was used for routine pH determinations. The Leeds and Northrup No. 124138 miniature pH electrode assembly, adapted to the Radiometer TTT-1 unit, was used for the titrations and at all other times when it was desirable to keep the ionic strengths of the solutions constant.[3] Before each pH determination, and both before and after each titration, linear calibration curves at the temperature to be used were constructed with the standard pH buffers and a commercial pH 2 buffer over the appropriate pH range. The accuracy in the titration experiments was ±0.01 pH unit. During titrations, the standard acids were added to a weighed aliquot of isoionic ribonuclease in water by means of a micrometer syringe previously calibrated at 0.0198 ml/division and reading to 0.01 division. The activity coefficient of the hydrogen ion at the different pH values and ionic strengths of the titration experiments with ribonuclease was estimated by titrations of dilute KCl or NaCl solutions without protein present.

Ultraviolet spectra and changes in the absorption at 287.0 mμ of ribonuclease solutions were measured with a Cary Model 14 recording spectrophotometer equipped with a thermostatable cell compartment. A copper block (designed by Dr. George Loeb), holding four 1-cm cells and through which water was pumped from an external thermostated bath, was used in the studies in which the temperature was varied. Temperature equilibrium between the solutions in the cells fitted into the copper block and the external bath was accomplished in about 5 minutes, at which time no further change in absorption was observed (Scott and Scheraga, 1963). The solution temperatures inside the copper cell holder were monitored with a Teflon-coated thermistor probe (Yellow Springs Instrument Co. No. 44103) which was bonded into a ⅌ 9 Teflon stopper by the use of a Teflon bonding kit supplied by the B & W Plastics Co., Inc. A cell containing the thermistor probe in water was used throughout the experiments as a reference cell to detect changes in the instrument balance or adventitious changes in absorption due to temperature. The reference spectrophotometer compartment was kept ≤25°. Ultraviolet spectra were recorded from the solutions at the lowest and highest temperatures, but throughout the heating cycle, the wavelength setting was kept at 287.0 mμ. All 3-ml cuvets, containing about

[3] The reference electrode of this assembly has an extremely low leakage of KCl. For example, 1 ml of deionized water into which these electrodes were immersed for 24 hours at 24° had a conductivity of 2.4×10^{-4} ohm^{-1} which was equivalent to a leakage in this period of only 4×10^{-6} mole of KCl.

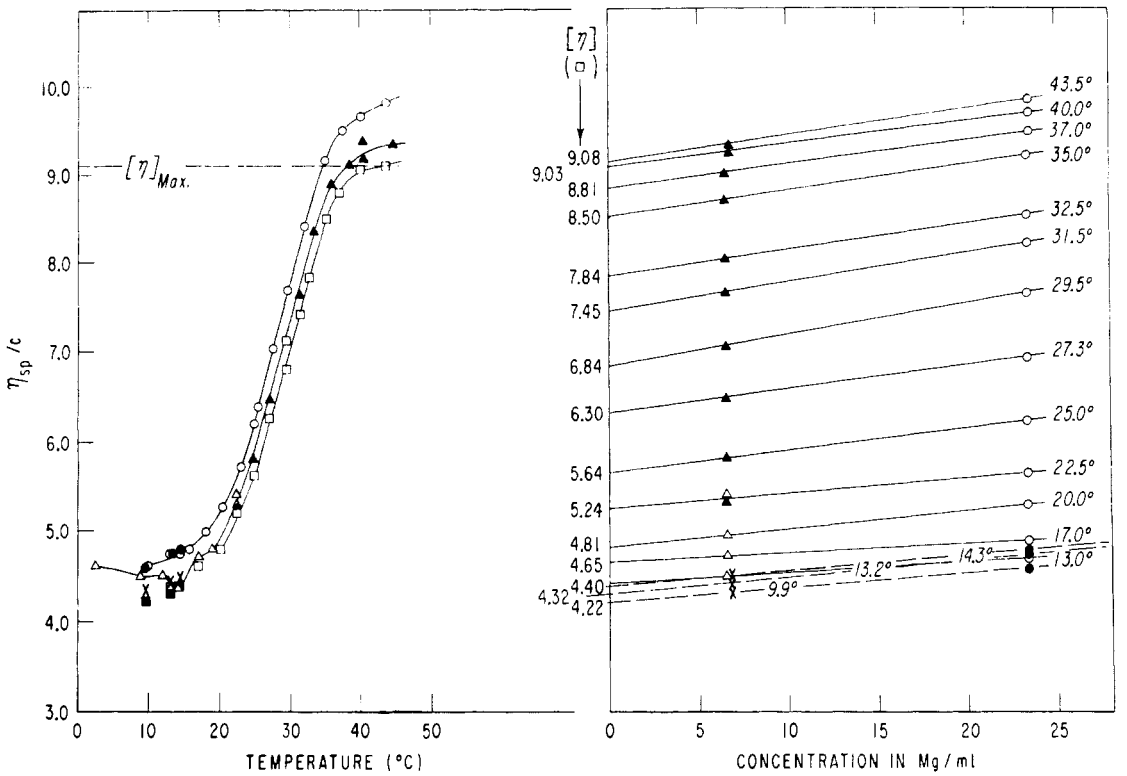

FIGURE 3: The reduced and intrinsic viscosities (in ml/g) of ribonuclease in a HCl–KCl solvent at pH 2.1 and 0.019 ionic strength as a function of temperature (left). The temperature dependence of the η_{sp}/c *vs.* c slopes (right). At 6.6-mg/ml concentration, the symbols (△) and (▲) are first and second heating cycles (after overnight cooling at 4°), respectively. A single heating cycle at 23.4-mg/ml concentration is shown as (○). After cooling from 44 to 4° overnight, final reduced viscosity measurements are indicated by (×) and (●) for the two concentrations indicated at right. The intrinsic viscosities (□) corresponding to (×, ●) are shown as the solid symbol ■.

2.1 ml each, were tightly stoppered with standard taper ⅌ 9 Teflon stoppers which effectively prevent evaporation. When temperatures below 25° were used, a slow stream of dry air was passed through the spectrophotometer cell compartments to prevent condensation.

Optical rotation measurements were made with a Rudolph Model 80 precision polarimeter equipped with the photoelectric attachment and oscillating polarizer. A zirconium light source was used for the wavelength range 750–330 mμ for optical rotatory dispersion measurements. An Hanovia quartz mercury burner with a Type 98B Hg bulb was used for the readings at the 365 mμ Hg line for greater sensitivity in the studies of optical rotation change with temperature. A ±5° oscillation was used for all measurements. Solutions were placed in a jacketed 1-dm capillary cell (about 1-ml volume) equipped with silica end plates. The end screws were marked so that the screw distortion of the end plates could be approximately reproduced for the cell corrections. For temperature control (±0.03°) water was pumped from an external thermostated bath through the cell jacket, a small condenser holding a thermometer, and the thermostating jacket of the Rudolph Model 80 compartment. Temperature equilibrium and stability of the observed optical rotation were attained within 10–20 minutes. All solutions were filtered through a 1.2-μ Millipore filter directly into the polarimeter cell and were kept at 2–4° overnight prior to the low-temperature measurements. A continuous stream of dry air was put through the sample compartment of the polarimeter when low temperatures were employed which kept all windows free of condensate. The optical rotatory dispersion data are expressed in the terms suggested by Moffit and Yang (1956) with λ_0 assigned the value of 212 mμ. The parameter b_0 was used to estimate the per cent helical content of the molecules (Urnes and Doty, 1961). The dispersion in refractive index of the aqueous solvents employed was approximated from the dispersion of the refractive index of water at 25°. To convert the values from the indices of refraction of water to indices of refraction for the protein solutions (concentrations = c_2 in g/ml), the experimental value of Armstrong *et al.* (1947) for $dn/dc_2 = 1.86 \times 10^{-4}$ was used. The temperature dependence of the refractive index of water at the sodium line was used to estimate the refractive index at temperatures different from 25°.

Pepsin digestion of ribonuclease was initiated by adding an aliquot of pepsin solution prepared according to Edelhoch (1957) to the ribonuclease solution. Pepsin

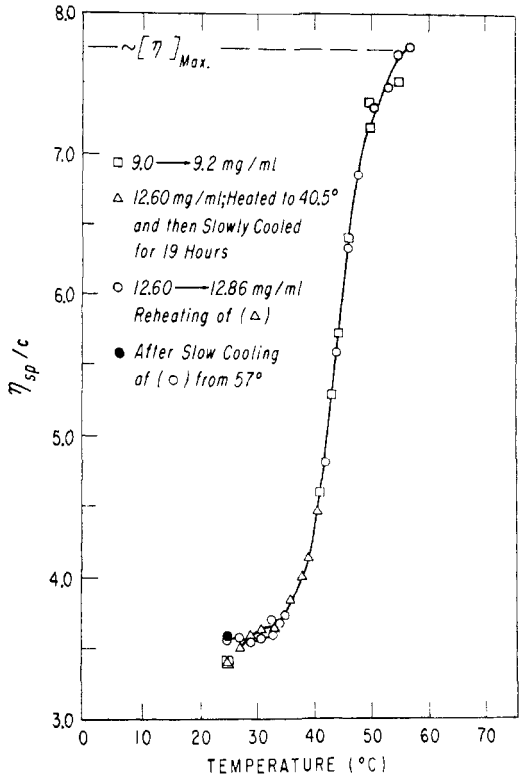

FIGURE 4: The reduced viscosity of ribonuclease in a SO_4^{2-}–HSO_4^- buffer at pH 2.1 and 0.019 ionic strength as a function of temperature.

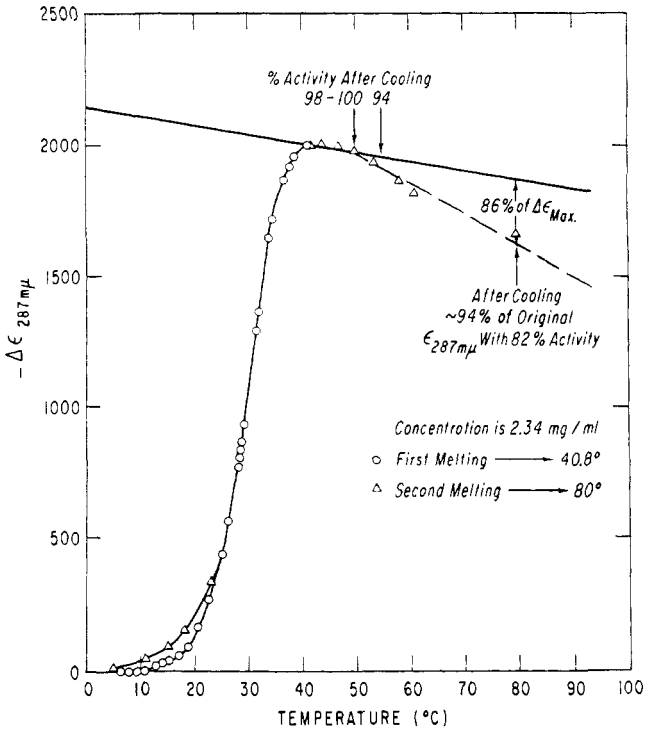

FIGURE 5: The effect of temperature on the reversibility of the molar absorption changes at 287 mμ and the recoverable activity of ribonuclease in the HCl–KCl solvent at pH 2.1 and 0.019 ionic strength.

action was terminated by the addition of alkali to pH 7.2. Amino nitrogen values, determined by the method of Moore and Stein (1948) as modified by Rosen (1957), provided a measure of peptide bonds broken by the action of pepsin. The possible inhibition of pepsin by sulfate ions was tested by using the assay procedure of Anson (1938) at pH 1.8, 0.06 ionic strength, and 25° with either HCl–KCl or H_2SO_4 added to the hemoglobin substrate.

Results

Temperature Dependence of the Optical Rotation, Tyrosyl Absorption, and Viscosity. The specific rotations at 365 mμ of ribonuclease at pH 2.1 with Cl^-, $H_2PO_4^-$, or SO_4^{2-} present are shown in Figure 1 as a function of temperature. Figure 1 illustrates the main features of the effect of the various anions on the conformation and thermal stability of the protein. Corresponding plots of the changes in absorption at 287 mμ (Figure 2) and the reduced viscosity (Figures 3 and 4) show the parallelism of these property changes with increasing temperature.

As the temperature is raised, the specific rotation (Figure 1) becomes more negative as ordered regions within the molecule become disrupted (Harrington and Schellman, 1956). Correspondingly, Figure 2 confirms the observation that of the three abnormal tyrosyl residues present in native ribonuclease (Shugar, 1952; Tanford *et al.*, 1955) only two are normalized by heat treatment (Hermans and Scheraga, 1961). The viscosity changes induced by heat (Figures 3 and 4) indicate that an unfolding which leads to rather gross configurational changes is the main response to increasing temperatures.

In contrast to the optical rotatory and viscosity properties (Figures 1, 3, and 4), the different salt forms had the same initial molar absorptions at 287 mμ and low temperatures. However, ribonuclease in the presence of sulfate ions routinely showed a slight red shift at low temperatures with a maximum absorption at 277.8 mμ rather than the usual absorption maximum at 277.5 mμ. Under the conditions of these experiments, this red shift could not be detected in the 287-mμ readings. Also, the maximum absorption for all thermally denatured anion forms was at 276.0 mμ, which is the same as that of ribonuclease in 8 M urea (Sela *et al.*, 1957).

The $\Delta\epsilon_{287}$ for the main transition of about −2000 (Figure 2) for the normalization of two tyrosyl residues is slightly higher than that previously reported by Hermans and Scheraga (1961), Foss (1961), and Scott and Scheraga (1963). This could be due to the low ionic strengths and/or pH employed in these studies since a change from acid to neutral pH conditions causes an apparent decrease in $-\Delta\epsilon_{287}$. In addition, this value is sensitive to the wavelength and molar extinction value at 277.5 mμ used.

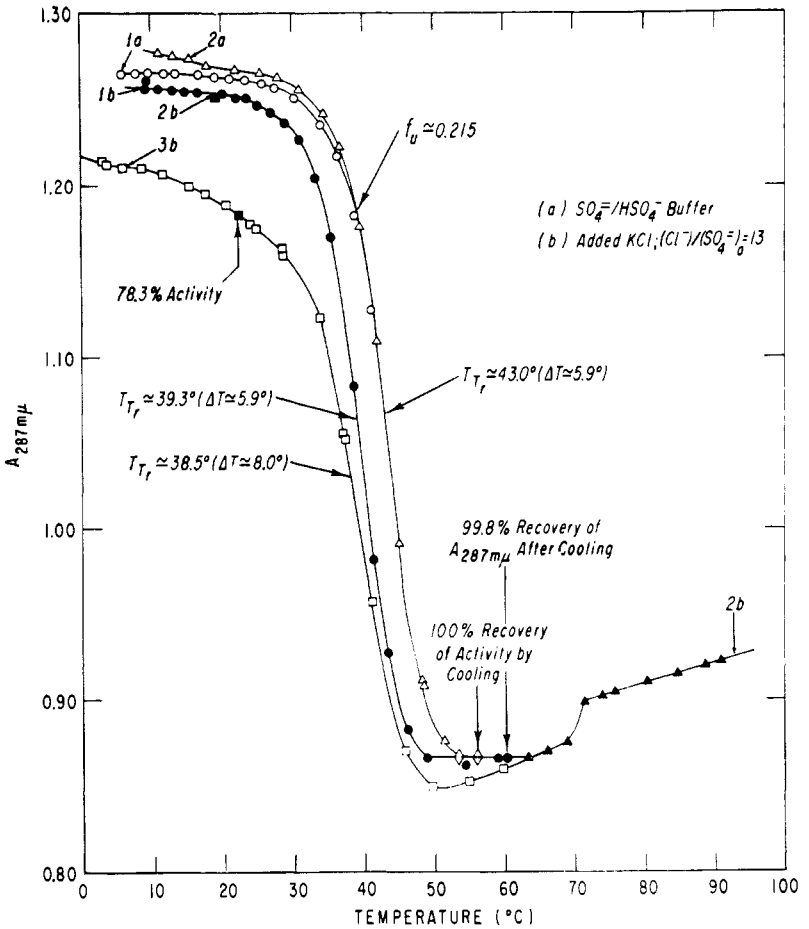

FIGURE 6: Anion competition during the melting out of ribonuclease structures. The absorbance at 287 mμ is shown as a function of temperature. Curves 1a and 2a: 1.958×10^{-4} M ribonuclease in a SO_4^{2-}–HSO_4^- buffer at pH 2.1 and 0.014 ionic strength with moles of free sulfate ion to ribonuclease about equal to 27; 1a (○), first heating to 41.2°; 2a (△), after fast cooling of (○) from 41.2 to 2° and equilibration at 2° overnight; second heating to 56°. Curves 1b, 2b, and 3b: KCl added to an unheated portion of the above solution at 23.5°; ribonuclease = 1.944×10^{-4} M, ionic strength = 0.083, free chloride to sulfate ions = 13, moles of chloride ion to ribonuclease $\cong$ 358; 1b (●), first heating to 60.2° after overnight equilibration at 9.5°; 2b (▲), second heating to 91° after slow overnight cooling of (●) to 10.6°; 3b (□), third heating to 60° after slow overnight cooling to 20° followed by equilibration of (▲) at 2° for 17 hours; (■), single measurement after slow overnight cooling of (□) from 60 to 22.5°.

The specific rotations of the high-temperature forms of ribonuclease (Figure 1) with either Cl^- or $H_2PO_4^-$ anions present show a temperature dependence similar to that observed for unfolded ribonuclease (Harrington and Schellman, 1956). The extrapolation of the observed specific rotations at high temperatures to 0° is made according to the procedure of these workers who assumed that the high temperature forms show the intrinsic temperature dependence of a random-coil type of configuration. It is seen that the net height of the heat-induced transition of ribonuclease with either of the monovalent anions present is about the same. However, the specific rotation of the sulfate form shows no temperature dependence from about 52 to 60°. As shown in Figure 2, the more accurately obtained absorption data for the sulfate form are constant also from 51 to 60°, whereas there is an absorption increase at high temperatures in the monovalent anion cases. Thus, the extrapolations of ϵ_{287} for the monovalent forms from high to low temperatures parallel the corresponding extrapolations of $[\alpha]_{385}$ shown in Figure 1. An intimate relationship between these different properties could exist if the temperature dependence of ϵ_{287} at high temperatures due to the intrinsic temperature dependence of tyrosine itself (Foss, 1961) is manifested only where tyrosyl residues have the configurational freedom provided by a random polypeptide chain arrangement.

In Figure 3, the value of the intrinsic viscosity at the completion of the main transition at about 40° appears to remain roughly constant. The intrinsic viscosity, which indicates gross macromolecular changes, would be expected to be relatively insensitive to the type of changes involved in the rotation and absorption parameters at high temperatures. However, if the changes in the

specific rotation and 287-mμ absorption at high temperatures (Figures 1 and 2) were not due to rotational and solvent–solute interactions of the unfolded polypeptide regions, but rather due to intermolecular interactions, such as aggregation, the viscosity measurements would readily reveal them.

The measured intrinsic viscosity of ribonuclease at pH 2.1 in the HCl–KCl solvent (Figure 3) is 4.40 ml/g at low temperature. This value is significantly higher than either the 3.30 or 3.4 ml/g measured, respectively, for native ribonuclease at neutral pH (Harrington and Schellman, 1956) or at pH 2.1 in the presence of sulfate (Figure 4). This difference might be attributed partially to an electroviscous effect. However, the difference is quite consistent also with the results shown in Figures 1 and 4 if a decreased compactness and corresponding increased effective volume of the chloride form at pH 2.1 is related to the more negative specific rotation of this form. Since the height of the thermal transition as measured by viscosity is about the same for both the chloride and sulfate forms, the presence of sulfate also lowers the viscosity of the unfolded form at high temperatures.

The increase in the concentration dependence of the chloride form with increasing temperatures shown in Figure 3 indicates that an increased particle interaction, presumably from a corresponding increase in the asymmetry of the molecular dimensions, develops as the temperature is raised.

Just prior to the main transitions shown in Figures 1, 3, and 4 there are observable small 'inverted' transitions which are similar to the one observed at the sodium line by Harrington and Schellman (1956) for ribonuclease at neutral pH. Foss and Schellman (1959) have observed greatly magnified inverted transitions in their studies on the thermal transition of ribonuclease in urea solutions, which were interpreted in terms of a preferential adsorption of urea from the mixed solvent to sites of the protein made available by the thermal unfolding process.[4] It is evident that the inverted transitions of Figure 1 with the monovalent anions present are barely perceptible (within experimental error), whereas that with the divalent sulfate anion present is of a greater magnitude. Although these inverted transitions are small in magnitude, they were quite reproducible. The inverted transitions observed at low temperatures in the optical rotatory properties do not appear in the tyrosyl perturbations. Instead, a small degree of melting out occurs at the low temperatures which is in agreement with the report of Scott and Scheraga (1963). Figures 3–6 illustrate further that these transitions observed at low temperatures are dependent upon prior sample treatment.

In Figures 1, 3, and 4, the points recorded after the solutions were cooled overnight show that the specific rotation and viscosity changes are reversible. Also, not shown, the cooled solutions assayed at 97–100% activity indicating that the changes in this property also are reversible.

An interesting phenomenon arose during the cooling of these solutions which appears to be related to specific ion effects. If the solutions containing sulfate or phosphate were cooled rapidly from above the transition temperatures, a visible precipitation took place, whereas solutions of ribonuclease with chloride ion remained clear after the same treatment. The precipitates which formed in the sulfate and phosphate solutions could not be dissolved by prolonged heating. Once this observation had been made, all solutions containing sulfate or phosphate routinely were allowed to cool slowly from above the transition temperature to room temperature, under which conditions these solutions remain clear. Further cooling from room temperature then could be performed rapidly.

Table I shows the optical rotatory dispersion parameters of some different ribonuclease solutions at low, intermediate, and high temperatures. The average specific rotations at the sodium line are indicated also for direct comparisons with the results of other investigators. Although the lack of theoretical justification is recognized for the calculation of helical contents from the different b_0 values, these are shown also for qualitative comparisons. Ribonuclease at neutral pH and moderate temperatures is known to be in a compact, folded configuration. However, at pH 2.1 and 27° with only chloride ion present, both the rotation and viscosity measurements (Figure 3) reflect an unfolding or disordering of the molecule as compared to the neutral pH form, which is in accord with the results of Hermans and Scheraga (1961) for ribonuclease at this pH and temperature. Sulfate ion inhibits the disordering process at low pH, as concluded by Neumann *et al.* (1962), and the rotation and viscosity values (Figure 4) at pH 2.1 and 27° are nearly identical with those for this protein at neutral pH.

Since Jirgensons (1963) has found that the b_0 parameter is zero for ribonuclease in 8 M urea, the high-temperature b_0 values of Table I indicate that residual helical or ordered structures remain after thermal denaturation. That is corroborated by a comparison of the specific rotations at the sodium line (Table I) and the viscosity values (Figures 3 and 4) at high temperatures to the corresponding values (Sela *et al.*, 1957), −108° rotation and 10 ml/g, respectively, for ribonuclease in 6 M guanidine hydrochloride. Since the latter denaturant at high concentrations is not affected by the presence of moderate amounts of divalent anions (Sela *et al.*, 1957), the optical rotation and viscosity values obtained for ribonuclease in 6 M guanidine hydrochloride can be

[4] A preferential adsorption (binding) of anions as a function of temperature has not been directly measured. However, the following results were obtained. No difference in the number of sulfate ions bound per molecule of ribonuclease was found between a sample kept at 15° and one which had been heated to 40° at pH 2.1 in the sulfate–bisulfate buffer. Anion binding measurements by the method of Loeb and Saroff (1964) were performed on both samples after a back-titration at 15° to pH 4 with NaOH (adjustment to pH 4 would cause the loss of about two anions), and in both the heated and unheated samples 3.2 sulfate ions were bound per molecule. This result also indicates that at least 3.2 sulfate ions are not melted off irreversibly as the temperature is raised to 40° (unpublished data of G. I. Loeb and A. Ginsburg).

TABLE I: Temperature Dependence of Optical Rotatory Dispersion Parameters.

Solvent Buffer	Ribonuclease Concn (mg/ml)	pH	Ionic Strength	Temperature (°C)	$-[\alpha]_D$ (deg)	$-a_0$ (deg)	b_0 (deg)	Apparent Helical Content (%)
HPO_4^{2-}–$H_2PO_4^-$	8.52	6.8	0.001	27	74.9	420	−96	15
Cl^-	8.73	6.8	0.001	27	74.8	425	−102	16
SO_4^{2-}–HSO_4^-	8.77	2.1	0.016	27	75.4	425	−102	16
HCl–KCl	8.60	2.1	0.019	27	85.6	490	−77	12
HCl–KCl	12.55	2.1	0.019	13–14	79.6	448	−99	16
				48.1	96.2	557	−55	9
				13[a]	79.2[a]	448	−92[a]	15
$H_2PO_4^-$–H_3PO_4	13.36	2.1	0.019	13–14	78.1	444	−92	15
				48.1	95.8	558	−50	8
				13–14[a]	78.2[a]	445	−86[a]	14
SO_4^{2-}–HSO_4^-	12.60	2.1	0.019	11	76.1	432	−83	13
				60.2	93.9	549	−38	6
				20[a]	74.1[a]	427	−93[a]	15

[a] Reversibility after overnight cooling.

used for the unfolded form of the enzyme. From these considerations it would appear that roughly one-third of the native structure is thermally stable. There is some nonparallelism between the low temperature specific rotations and the b_0 values of the different anion forms which could be significant since the decrease in the b_0 parameter between the chloride and sulfate forms is reflected at both the low and high temperatures.

Figure 5 is supplementary to Figure 2 and illustrates the effect of heating considerably above the transition temperature. Ribonuclease in a HCl–KCl solvent at pH 2.1 was heated just to the end of the transition, cooled, and then reheated to 80°. On the second heating, the points of the main transition duplicated those obtained in the first heating cycle except that the low-temperature melting out was increased. As the temperature is increased beyond the completion of the main transition, irreversible inactivation occurs. After cooling from 80°, the activity loss is greater than the decrease in the absorption at 287 mμ. Also, there is a high-temperature slope change in the absorption properties of the denatured forms at the point at which activity is irreversibly lost. The marked decrease in $-\Delta\epsilon_{287}$ at temperatures greater than 10° above the end of the main transition suggests that the solvent interactions of the tyrosyl residues normalized by heat treatment approach those of the free amino acid, and that the configuration of this denatured form has a decreased ability to re-form the native structure upon cooling.

Anion Competition during the Thermal Denaturation Process. The experiments shown in Figure 6 provide additional information on the low-temperature form and the heat-induced transition of ribonuclease with sulfate bound. Curve 1a shows the usual behavior of the sulfate form. The deviation of curve 2a from 1a is an effect brought about by rapid cooling from just below the transition temperature (41°). Freezing from 25° or slow cooling from 41 to 25° with subsequent equilibration at 2° did not produce this effect.

The addition of a 13-fold excess of chloride to sulfate ion with subsequent cooling and heating produced the 1b curve. The amount of chloride added was governed by the affinity constants determined by Saroff and Carroll (1962) for sulfate and chloride ion binding to ribonuclease at pH 4.4 (see also Loeb and Saroff, 1964). On that basis, a tenfold excess of chloride ion should convert about half of the molecules to the chloride form, with the other half being in the sulfate form, by anion competition at each binding site. If the thermal denaturation of each form is an independent process, the melting curve for this mixture should approximate an average of the chloride and sulfate curves shown in Figure 2. It will be recalled that the denatured sulfate and chloride forms have nearly indistinguishable ultraviolet absorptions, as do the native sulfate and chloride forms, so that the fraction of unfolded molecules calculated at any temperature (see below) accounts for both denatured anion forms. Accordingly, a calculation at 39.3° where, as shown in Figure 6–1b, there is an apparent 50% conversion to the denatured form (f_u = 0.5) as compared to 21.5% in the case of the sulfate form, yields an apparent 57% conversion of the sulfate to the chloride form with the 13-fold excess chloride ion present. However, an inspection of the 1b curve at lower temperatures reveals that the sulfate to chloride form appears to predominate in about a 9:1 ratio. The temperature independence of the absorption (curves 1b and 2b) between 49 and 60° is characteristic also of the sulfate case (curve 2a). Finally, it is apparent that instead of a true mixed anion curve (with no interconversion between the chloride and sulfate forms, a broad transition, and a 6–7° shift in the average melting tem-

perature), there is a sharp transition with less than a 4° shift in the transition temperature. This means that there must be a temperature-dependent interconversion of the different forms. The apparent increase in the conversion to the chloride form between the temperature extremes can be explained best by taking into account differences in stabilities of the various anion forms present, the equilibria of which are temperature dependent.

From the results shown in Figure 6, the stabilities of the various anion forms in approximate temperature ranges can be summarized as follows: (15–33°) $N \cdot SO_4^{2-} > D \cdot SO_4^{2-} > D \cdot Cl^- > N \cdot Cl^-$, (33–36°) $D \cdot Cl^- \approx N \cdot SO_4^{2-} > N \cdot Cl^- > D \cdot SO_4^{2-}$, (36–44°) $D \cdot Cl^- \approx D \cdot SO_4^{2-} > N \cdot SO_4^{2-} > N \cdot Cl^-$, and (52–61°) $D \cdot SO_4^{2-} > D \cdot Cl^- > N \cdot SO_4^{2-} > N \cdot Cl^-$, where N and D refer to the native and denatured enzyme forms, respectively. The quantity (df_u/dT) of curve 1b becomes identical with that of curve set a at temperatures 36–44°. Since it is at these temperatures that the denatured sulfate form becomes stabilized with respect to the native sulfate form (Figure 2), the sharpness of curve 1b in terms of ΔT[5] in this temperature range reflects primarily the equilibrium between these two forms as it does throughout the main transition of ribonuclease in the HSO_4^-–SO_4^{2-} buffer. At slightly lower temperatures, the equilibria among the chloride and sulfate native and denatured forms must all be taken into account. A two-state mechanism for the thermal denaturation process (Schellman, 1955) is implicitly assumed.

The temperature independence of the denatured sulfate form as compared to the chloride form (Figure 2) has indicated that perhaps secondary changes in the denatured chloride form occur at high temperatures. Indeed, a similar transition can be induced in the sulfate form by heating to temperatures greater than 63°. The reproducibility of curve 1b after cooling from 60° (as evidenced by curve 2b) indicates that the original equilibria between the different forms at low temperature is re-established after cooling from 60°. If the bound divalent anion does prevent the secondary structural changes from occurring (53–63°), as reflected in a temperature independence of A_{287}, then the bound sulfate ion also is necessary for the complete reversibility of the thermally induced changes. Curve 3b of Figure 6 shows that after the high-temperature transition only partial enzyme activity and original absorption at 287 mμ are restored by the cooling process. These results are similar to those shown in Figure 5 where heating the chloride form above 50° caused a loss in recoverable activity and 287-mμ absorption. Curve 3b shows the behavior of the mixed anion system with irreversibly denatured forms present. In this case, where an interconversion of native and irreversibly denatured forms is blocked, the width of the transition (ΔT) is significantly increased (much as would be anticipated in the case of curve 1b had the chloride and sulfate forms denatured independently). However, the transition temperature (T_{tr}) for curve 3b only decreased by 1°, which illustrates the insensitivity of this parameter in the case where the initial absorption is considered to be representative of a single state. After the completion of the transition (curve 3b), there is observed a temperature dependence of A_{287}. After cooling from 60°, a single determination fell on the 3b curve which indicates that structures retaining activity behave as those in curve 1b with the deviations from curve 1b due to the fraction of irreversibly denatured enzyme forms present.

It may be assumed that the chloride and sulfate native forms at pH 2.1 do not have equivalent conformations energetically, as they are approximately at neutral pH, so that there is a shift in the apparent affinity constant ratio favoring the sulfate form at low temperatures in this system.[4] Actually, it can be predicted that binding constants will vary according to the configuration of the native protein. Since the sulfate form has the approximate optical rotatory and viscosity properties of ribonuclease at neutral pH, whereas the chloride form by the same criteria is in a more disordered state, it can be proposed that a 10-fold increase in the apparent affinity constant ratio reflects a corresponding decrease in the affinity constant for chloride ion binding to the pH 2.1 form. Alternatively, under these conditions, the effectiveness of the free chloride ion in converting the stabilized native sulfate form to the more disordered native chloride forms cannot be expressed simply in terms of a competitive displacement according to relative affinity constants. It is possible that some disorganization of the protein must be effected first either by increasing considerably the concentration of the competing anion (with interacting binding sites involved) or by sufficiently increasing the temperature.

In particular, a consideration of the phosphate case involves such an alternative interpretation. That is, the deviations of the behavior of the phosphate forms from that of the chloride forms (Figures 1 and 2) at low pH appear to be due to a partial saturation of one or both of the two neutral pH anion binding sites of the protein with divalent phosphate ion. This was indicated by titrations of isoionic ribonuclease with phosphoric acid at 15° in which the apparent proton uptake per molecule at pH 5 and below was significantly lower than that found in titrations of ribonuclease with sulfuric acid under the same conditions of ionic strength and temperature (where sulfate binding should give a comparable shielding effect). The apparent discrepancy in the proton uptake with the different acids as titrants can be resolved in terms of a persistent binding of the divalent phosphate ion at acid pH, in which case one unaccounted-for proton is provided to the medium by each divalent anion bound. (At pH 2.1 and 15°, the molar ratio of solvent buffer $H_2PO_4^-$ to ribonuclease is only about 14 with 2.08×10^{-3} M ribonuclease.) Once the divalent ion has been displaced from the binding site by monovalent phosphate, it forms a part

[5] ΔT is defined according to von Hippel and Wong (1963) as the temperature differences between $f_u = 0.75$ and $f_u = 0.25$ (see also Flory, 1961).

TABLE II: Parameters from the Reversible Thermal Denaturation of Ribonuclease at pH 2.1 and 0.019 Ionic Strength.

Solvent Mixture	HCl–KCl			$H_2PO_4^-$–H_3PO_4		SO_4^{2-}–HSO_4^-			
Measurement	$\Delta\epsilon_{287}$	$\Delta[\alpha]_{365}$	$\Delta[\eta]$	$\Delta\epsilon_{287}$	$\Delta[\alpha]_{365}$	$\Delta\epsilon_{287}$	$\Delta[\alpha]_{365}$	$\Delta(\eta_{sp}/c)$	
Transition height (→ 0°)	2140 M^{-1} cm^{-1}	57.8°	4.68 ml/g	2140 M^{-1} cm^{-1}	57.8°	2010 M^{-1} cm^{-1}	51.8°	4.38 ml/g	
Concn (mg/ml)	2.34	12.55	→ 0	2.18	13.36	2.03, 2.68	12.60	8.66	12.60
Transition temp, T_{tr} (°C)	29.9	29.9	28.8	31.9	32.8	42.7	44.1	43.4	44.1
$\Delta H°_{obsd}$ (kcal)	47	51	46	56	59	69	76	69	76
$\Delta S°_{obsd}$ (eu)	157	168	160	182	192	220	239	218	239
ΔT (°C)	7.5	7.9	8.2	6.1	6.3	5.9	6.1	6.0	6.1

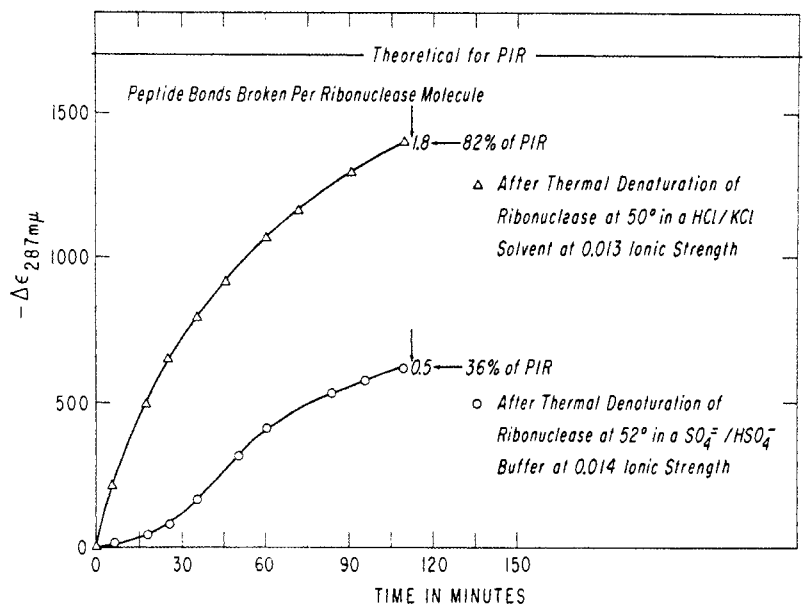

FIGURE 7: The susceptibility of "refolded" ribonuclease to pepsin digestion after thermal denaturations at pH 2.2 in the presence of different anions. The change in absorbance at 287 mμ (Sela and Anfinsen, 1957) as a function of the digestion time; conditions: pH 2.2, 25°, pepsin : ribonuclease = 1 : 4000 by weight.

of the $H_2PO_4^-$–H_3PO_4 buffer. Accordingly, divalent phosphate binding would not be expected on a strictly competitive basis in which each binding site is considered independently.

The intermediate behavior of ribonuclease in the phosphate buffer at pH 2.1 in Figures 1 and 2 (between the chloride and sulfate cases) can be analyzed as a mixed mono- and divalent anion system, once it is attributed to an incomplete saturation of the neutral pH binding sites with divalent phosphate anions. The optical rotation of ribonuclease at low pH and temperature in Figure 1 could give an estimate of the fractions of the divalent phosphate form present, if it is assumed that the divalent and monovalent phosphate forms have the specific rotations of the sulfate and chloride forms, respectively. On this basis, 31% of the molecules at pH 2.1 in the $H_2PO_4^-$–H_3PO_4 buffer have the specific rotation of the divalent anion form. The sharpness and transition temperature of the thermal denaturation of ribonuclease in the $H_2PO_4^-$–H_3PO_4 buffer (Table II) is consistent also with this system being representative of a mixed-anion system resembling the sulfate–chloride case shown in Figure 6. The slight difference in the high temperature behavior of the chloride and phosphate forms observable in Figures 1 and 2 and Table I does suggest that a complete conversion of the di- to the monovalent phosphate forms does not occur up to 48°.

It is appropriate to point out here that, in the sulfate–bisulfate buffer at pH 2.1 and 0.019 ionic strength (free SO_4^{2-}:HSO_4^- is ~2.8 at 15°), the bisulfate ion concentration was such that the ribonuclease could have at most 3% of any one binding site occupied by the HSO_4^- ion, with the SO_4^{2-} ion filling the site to the extent of 97%, on the basis of an affinity constant ratio of ~11 for the affinity of the divalent to that of the monovalent

species. Then, ribonuclease in the HSO_4^-–SO_4^{2-} buffer at pH 2.1 would be representative at most of only 3% of the mixed-anion case in Figure 6. In addition, when the experiments shown in Figures 1 and 2 were conducted at pH 2.2, under which conditions the bisulfate concentration is lower, no differences from the results shown were observed. Thus, the effect, if any, of the bisulfate ions on the behavior of ribonuclease in the HSO_4^-–SO_4^{2-} buffer, is a very small one.

The Susceptibility of Ribonuclease at pH 2.2 to Attack by Pepsin with Either Chloride or Sulfate Ions Present. The results from a study of the peptic digestion of ribonuclease with the different anions present are shown in Figure 7. The change in absorbancy at 287 mμ indicates inactivation of ribonuclease during the digestion (Sela and Anfinsen, 1957), and the reported value of $-\Delta\epsilon_{287}$ for ribonuclease which has been completely inactivated by pepsin (PIR)i s shown. In the experiment of Figure 7, ribonuclease solutions from such thermal denaturation studies as illustrated in Figure 2 were used. Similar results were obtained with unheated ribonuclease, which demonstrates again the reversibility of the denaturation process. Pepsin, when assayed by the method of Anson (1938) in a SO_4^{2-}–HSO_4^- buffer, had the same activity as in a HCl–KCl solvent. Therefore, the inhibition of pepsin in the digestion of ribonuclease in the SO_4^{2-}–HSO_4^- buffer is due to the action of the anion on ribonuclease rather than to an effect on pepsin.

The excess cleavages over that necessary to produce inactivation of the molecule with chloride bound undoubtedly are related to a preferential attack by pepsin on those chloride forms which are unfolded at this temperature (Figures 1 and 2). This conclusion is supported by the fact that chromatography of such a digest (pepsin action terminated at 17% inactivation) by the method of Hirs *et al.* (1953) showed that almost all the activity resided in material which chromatographed as the native protein.

Treatment of the Data Obtained during the Thermal Denaturation of Ribonuclease. The two-state approximation of Schellman (1955), which is an assumption that the protein occurs as either the native or denatured form, has been applied to the reversible denaturation reactions illustrated in Figures 1–4: native protein (folded) $\rightleftharpoons$ denatured protein (unfolded). At any temperature, t, the fraction of molecules unfolded or in the denatured state, f_u, is calculated (Schellman, 1955; Harrington and Schellman, 1956; Hermans and Scheraga, 1961) from the different measurements by

$$f_u = 1 - f_t = \frac{\Delta[\alpha]_t}{\Delta[\alpha]_{max}} = \frac{\Delta(\epsilon_{287})_t}{\Delta(\epsilon_{287})_{max}} = \frac{\Delta[\eta]_t}{\Delta[\eta]_{max}}$$

where $\Delta[\alpha]_t$, $\Delta(\epsilon_{287})_t$, and $\Delta[\eta]_t$ are the differences between the specific rotation, the molar absorption at 287 mμ, or the intrinsic viscosity at a temperature t and the minimum $-[\alpha]$, the maximum ϵ_{287}, or the minimum intrinsic viscosity observed at the low temperatures, respectively. Using this procedure, the occurrence of inverted transitions is reflected in the quantity f_u. The maximum change in the different properties is computed from the difference between the high and low temperature values, using the same low temperature value as is used in the numerator calculation and the high temperature values indicated in Figures 1–4. Where the properties at the high temperatures appear to be temperature dependent, linear extrapolations along the appropriate dashed lines in these figures to the temperature t are used in the calculations of the denominator quantities (Harrington and Schellman, 1956).

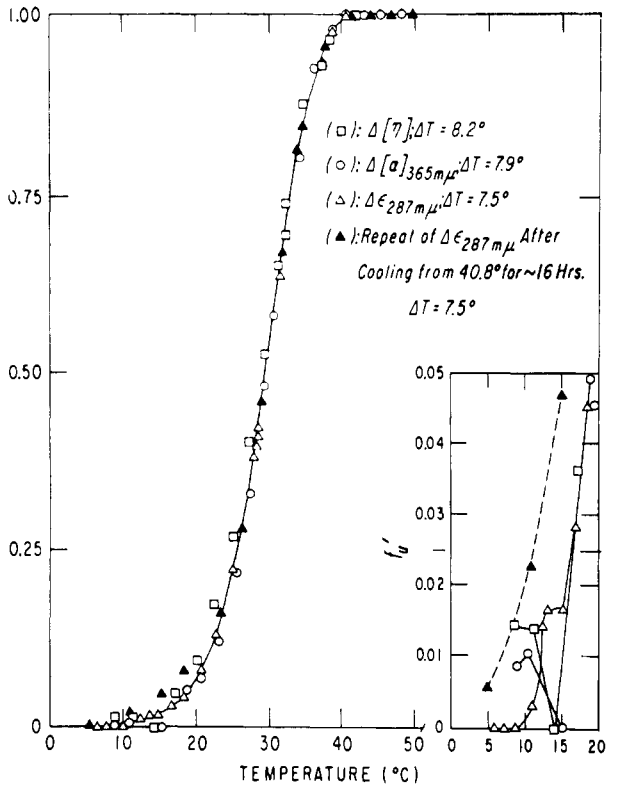

FIGURE 8: The heat-induced transition of ribonuclease in a HCl–KCl solvent at pH 2.1 and 0.019 ionic strength. Only the points (△) are connected. The apparent fractions of unfolded molecules (f_u'), calculated from the changes in the different properties at various low temperatures (see text), are shown as an insert in the main figure.

The results of these calculations for ribonuclease in the HCl–KCl solvent and in the SO_4^{2-}–HSO_4^- buffer are shown in Figures 8 and 9, respectively. In addition, the sharpness or "degree of cooperativeness" for each transition is shown in the form of ΔT.[5] It is seen that the heat-induced transition of the sulfate form is at least 1.5° sharper than that of the chloride form from the ΔT values (see also Table II). In addition, the heat-induced changes in the different measurements parallel or overlap one another throughout most of the respective transitions. The variations noted at the low temperatures in Figures 1–3 between the trends of the temperature dependence of the different properties are illustrated in the inserted plot of Figure 8. The reduced viscosity values of Figure 4, which showed little con-

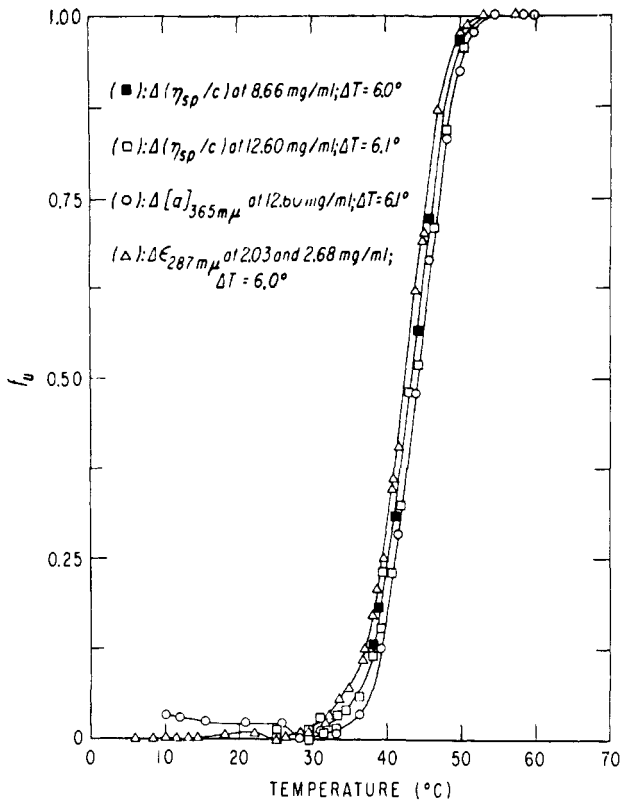

FIGURE 9: The heat-induced transition of ribonuclease in a SO_4^{2-}–HSO_4^- buffer at pH 2.1 and 0.019 ionic strength. The fractions of unfolded molecules (f_u), which are calculated from the observed changes in the different properties with temperature (see text) are plotted as a function of temperature.

centration dependence,[6] as illustrated in Figure 9, indicate that there is a concentration dependence in the thermal denaturation process for ribonuclease. At 12.60-mg/ml concentration, the f_u *vs.* temperature curves (from f_u = 0.2 to 1.0) from the reduced viscosity and specific rotation data exactly correspond. However, the same plot from (η_{sp}/c) values at the 8.66-mg/ml concentration falls exactly between the curve constructed at the 12.60-mg/ml concentration and the curves from ϵ_{287} at 2.03- and 2.68-mg/ml concentrations. This concentration dependence is reflected in all but one of the transition temperatures recorded in Table II.

If the only differences between the main transitions in the different properties of ribonuclease in the presence of a particular anion are due to a concentration dependence as indicated, these transitions then exactly correspond. This suggests that the changes in the different properties during the heat-induced transition are intimately related, and that the increase in tyrosyl perturbation, the decrease in ordered structures, and the increase in the frictional properties all concern the same region or regions in the ribonuclease molecule.

An equilibrium constant for the thermal denaturation process can be defined by $K_{obsd} = f_u/(1 - f_u)$, using the assumptions implicit in the definition of f_u and a van't Hoff plot constructed by plotting log K_{obsd} against the reciprocal of the absolute temperature (Figure 10). The standard-state enthalpy change, $\Delta H°_{obsd}$, in the process is obtained then from the slope (assuming a constant enthalpy over the temperature range of the transition). The transition temperature is defined as that temperature at which log K_{obsd} = 0, and the standard-state entropy change, $\Delta S°_{obsd}$, for the denaturation reaction is estimated from the enthalpy change divided by the transition temperature since the standard-state free-energy change at this temperature is zero by definition. The data of Figures 8 and 9 along with similar data from the transitions in the presence of the phosphate buffer at pH 2.1 were used directly to construct the van't Hoff plots shown in Figure 10. The linear portions of the curves in Figure 10 were used to compute the thermodynamic parameters given in Table II.

It is seen that the different anions have an apparently large effect on the enthalpy and entropy changes related to the heat-induced transition of ribonuclease, and this could be predicted from the inverse proportionality of ΔH to ΔT (Flory, 1961). This then indicates that the thermal transitions of the different anion forms are not identical in spite of their similarities, and that the effects of the anions are more complicated than simple in-

[6] A concentration dependence of the reduced viscosities of the chloride form (Figure 3) reflects an apparent increase in the transition temperature with *decreasing* concentrations.

fluences on the initiation of the melting out process. Another complication is obvious from the van't Hoff plots in Figure 10: the curvature at high temperatures and the scattering of points in the low temperature region are not compatible with the assumption of a simple two-state transition and confirms the multiplicity of forms existing at both ends of the transition that was deduced from other information.

The increasing ΔH with increasing temperature at high temperatures has been reported previously (Harrington and Schellman, 1956; Hermans and Scheraga, 1961; Holcomb and Van Holde, 1962), and various explanations for the skewed heat-induced transition curves observed for ribonuclease have been proposed (Schellman, 1958; Hermans and Scheraga, 1961; Scott and Scheraga, 1963). The fluctuation treatment of Schellman (1958) and the recent more comprehensive statistical mechanical treatment of Poland and Scheraga (1965) of the helix-coil equilibrium of a polypeptide of a given length could be applicable to the observations of Figures 1–4. In such a treatment, the recognition of fluctuating native structures is a natural consequence (Schellman, 1958), and a stabilization of intermediates under certain conditions is quite possible. The complexities of the Schellman and Poland and Scheraga treatments make it quite impossible to apply them quantitatively to the experimental observations. Accordingly, the relatively simple scheme which assumes a single-step process in the denaturation of different native forms, and upon which basis the observations can be interpreted qualitatively, is adopted here (see below). Poland and Scheraga (1965) have shown that the enthalpy and entropy changes which are calculated by the assumption of a two-state theory when in reality a mechanism of denaturation involving gradual "unwinding" is applicable can be in considerable error. Thus, the absolute values listed in Table II for $\Delta H°_{obsd}$ and $\Delta S°_{obsd}$ are quite uncertain. However, Poland and Scheraga found the transition temperatures to be unaffected by the same considerations so that the values of T_{tr} in Table II can be accepted without reservations as to the applicability of the two-state approximation.

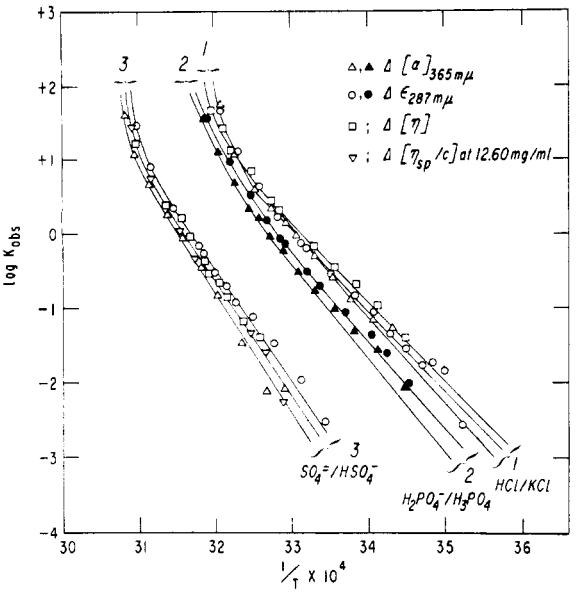

FIGURE 10: van't Hoff plots of the thermal denaturation of ribonuclease at pH 2.1. The logarithm of the equilibrium constant for the thermal denaturation reaction in various solvent buffers is plotted against the reciprocal of the absolute temperature. Straight lines have been drawn to fit the results obtained near the thermal transition where log $K_{obsd} \cong -1$ to $+0.2$. At high temperatures, the observed curvature only is known.

Discussion

Accepting low negative rotation and low intrinsic viscosity as indicators of increased specific structure, it is apparent that the sulfate ion encourages a more ordered, compact configuration at every temperature than does the chloride ion. In fact, from the differences between the optical rotations and viscosities of the chloride and sulfate forms over a wide temperature range, it is tempting to conclude that sulfate introduces a unique organization to a region that is stable to heat over the whole range studied. Except for a slight red shift observed in the absorption maximum of the sulfate form at low temperatures, the different anions do not perceptibly affect the low and high temperature ultraviolet spectra. The red shift could mean that sulfate ion produces an enhancement in the folding of structures involving tyrosyl residue(s). In addition to the apparent thermostable effects on the conformation of ribonuclease at low temperatures, the divalent sulfate ion markedly stabilizes the thermolabile regions of this molecule at acid pH. That is, there is a 14° increase in the temperature required to induce the thermal transition of the sulfate form over that of the chloride form. Sulfate also decreases the width of the main transition indicating an increased "degree of cooperativeness" in the denaturation process of this form. However, the heights of the main heat-induced transitions, whether measured by changes in optical rotation, in tyrosyl absorption, or in intrinsic viscosity, are essentially the same with either chloride or sulfate ions present.

The observations made in the low-temperature ranges of the transition curves can be interpreted in terms of the existence of multiple conformations (represented simply as N_1 and N_2 below) of ribonuclease in solutions at those temperatures. Such forms are visualized as being in equilibrium with each other, with each conformation having a different thermal stability and a characteristic specific rotation and viscosity. The binding of anions would stabilize particular configurations (Linderstrøm-Lang and Schellman, 1959), and it is the differences between the capabilities of chloride and sulfate ion binding (Saroff and Carroll, 1962; Loeb and Saroff, 1964) to induce a preferred configuration which

is compared here. Chloride ions do not provide a stabilization which narrows the range of probable configurations as much as does the divalent sulfate ion. In contrast, the net specific rotation and viscosity of the sulfate forms suggest that a relatively large proportion of ordered and compact forms are stabilized at low temperature. The magnitude of the inverted transition observed in the specific rotation could represent the displacement of the low-temperature equilibria with increasing temperatures to a more ordered form, with the configuration with the lowest $-[\alpha]$ and intrinsic viscosity being more stabilized by the specific divalent sulfate anion. Then, the inverted transition appears to be due to a shift in the relative concentrations of the different native and denatured forms, with the stability of each form being temperature dependent.

Since the tyrosyl perturbations at low temperatures are in the same direction as the main transition and since prior sample treatment influences the magnitude of this change, it is impossible to quantitate this effect. The native chloride and sulfate forms are not distinguishable by the tyrosyl absorption at 287 mμ which could explain the absence of an inverted transition of this property. However, the tyrosyl perturbation at low temperatures can be the counterpart of the inverted transitions observed in the specific rotations at low temperatures if it is caused by a modification of structure in a region remote from the tyrosyl residues located in the thermolabile region(s).

In summary, the different effects of chloride and sulfate ions on the conformation and thermal stability of ribonuclease at pH 2.1 can be qualitatively represented by Scheme I. Consider that N_1 represents a

SCHEME I

$$\begin{array}{ccc} N_1 & \rightleftharpoons & N_2 \\ \downarrow\uparrow & & \downarrow\uparrow \\ D_1 & \rightarrow D' \leftarrow & D_2 \end{array}$$

native form of low thermal stability with a specific rotation and intrinsic viscosity similar to that measured as an average for the chloride forms; the presence of sulfate ion mostly stabilizes a native form N_2, and the sulfate forms have a net specific rotation and intrinsic viscosity which are fairly characteristic of this form. N_1 and N_2 are meant to represent averages of about two stability extremes since fluctuations about and between these conformations undoubtedly exist in solution. The denatured anion forms, D_1 and D_2, result from the unfolding of the thermolabile region(s) in the molecule which is(are) the same in either the N_1 or N_2 case. The differences between the D_1 and D_2 forms are assumed to reside in a thermostable region of the molecule. Irreversibly denatured molecules, if formed, are shown as D'.

It is useful for purposes of discussion to divide the ribonuclease molecule into two regions, I and II. Region I includes the thermolabile structures which unfold as $N_{1,2} \rightarrow D_{1,2}$. All of the molecule excluded by region I is included in region II so that II includes all thermostable structures, which are estimated to be about one-third of the low temperature secondary and tertiary structures from viscosity and optical rotation results. Region II, which determines the thermal stability of the N forms and the specific rotation and viscosity properties of both the N and D forms, is more ordered and compact in the presence of sulfate than chloride ion. Whatever structure is encouraged by sulfate, it is not a right-handed α-helix by the usual criterion (by a comparison of b_0 parameters). Also, the one tyrosyl residue, out of the three abnormal tyrosine groups in native ribonuclease (Shugar, 1952; Tanford *et al.*, 1955; Cha and Scheraga, 1963), which is not normalized by heat treatment is located in region II. The unfolding of region I which occurs during the thermal denaturation process is responsible for the gross changes observable in optical rotation, viscosity, and ultraviolet absorption. The changes in these properties show that region I contains two abnormal tyrosyl residues in the folded states, that it is a more ordered region at low temperature than at high temperature, and that the unfolding of this region produces gross configurational changes. Although it is possible that no large segment of α-helix is in region I, some helix must be included in this region. The optical rotatory dispersion data do appear to minimize the "helical content" of region I, as do these data for native ribonuclease (see Urnes and Doty, 1961) when compared to the results of Blout *et al.* (1961) and Englander (1963) who applied quite different methods for estimating the quantity of helix in the native enzyme. Also, the sharpness of the heat-induced transition indicates a large degree of cooperativeness in the unfolding process which in turn suggests that some kind of regular ordered structure is involved. Sulfate ion markedly increases the thermal stability of region I without influencing the extent of unfolding induced by heat treatment. An intimate relationship between regions I and II is suggested by these observations which show that sulfate ion markedly affects structures present in region II while simultaneously increasing the stability of region I. From the thermodynamic parameters estimated for the thermal unfolding process of the different anion forms (assuming a two-state mechanism and considering a particular anion form fairly representative of either N_1 or N_2), it appears that regions I and II are coupled throughout the denaturation process. The values of $\Delta H^\circ_{obsd} = 69$ and 46 kcal with $T_{tr} = 43$ and 29° were calculated for the denaturation of the sulfate and chloride forms, respectively.

After the completion of the main transition, dissimilarities between the behavior of the chloride and sulfate forms at high temperatures (represented as D_1, D_2, and D') are observed. That is, the specific rotation and the tyrosyl absorption of the chloride form exhibit a slight temperature dependence which to a small degree approximates that of a random-coil configuration. It is possible that some secondary changes occur in the configuration of the chloride form at high temperatures, which would result in different denatured forms (all represented as D') of the enzyme being present, possibly even in the temperature range of the main transi-

tion. By this criterion, sulfate ion may stabilize only reversibly denatured configurations at the high temperatures.

It can be predicted from the above scheme that a concentration dependence for the denaturation process could be evident and that prior sample treatment could be an important factor in the observations at low temperatures, both of which are observed.

The above scheme is consistent with the report of Scott and Scheraga (1963) that the equilibrium transition (as measured by the temperature dependence of the tyrosyl absorption changes where the different native and different denatured forms each have one characteristic absorption throughout the temperature range of the transition) of ribonuclease as the chloride form(s) at acid pH can be resolved into a two-component transition, each giving a linear van't Hoff plot. The thermodynamic values calculated by Scott and Scheraga for the two independent transitions at different acid pH values were similar at pH 2 to the chloride and sulfate cases, which were used here to estimate these parameters for $N_1 \rightarrow D_1$ and $N_2 \rightarrow D_2$, respectively. Quantitative agreement cannot be expected because the experimental techniques are not comparable. The pH dependence of the thermodynamic parameters for each transition (Scott and Scheraga, 1963) could represent simply a stabilization of native forms with increasing pH with the low-temperature melting being increasingly associated with a secondary effect in the temperature range of the inverted [α] transition. In addition, Scott and Scheraga studied the kinetics for the thermal denaturation of ribonuclease and, although the rate data were found to be complex, empirically they could be resolved into a slow and a fast first-order reaction. Qualitatively relating the above scheme to these results of Scott and Scheraga, the fast first-order reaction could be the rate of the denaturation of either N_1 or N_2, and the slow rate could reflect both the reversal of the denaturation reaction and the interconversion of native forms, assuming the latter to be relatively temperature independent.

The titration of isoionic ribonuclease to pH 2.1 with phosphoric acid and the properties of the protein in this buffer system suggest that one or two of the anion binding sites present at pH 5 (Saroff and Carroll, 1962) is involved in the stabilization against thermal denaturation and therefore in the ordering of thermostable structures in the molecule. One such anion binding site involves a cluster of histidines 12 and 119 and lysine 41 which composes at least a part of the active site of the enzyme (Richards, 1958; Stark *et al.*, 1961; Vithayathil and Richards, 1961; Hirs, 1962; Crestfield *et al.*, 1963). Similarly, Nelson *et al.* (1962) observed that the binding of a single anion is involved in the stabilization of the ribonuclease molecule against urea denaturation (Sela *et al.*, 1957).

Sulfate ion inhibits the rate of the inactivation of ribonuclease by pepsin just as increasing the pH does (Ginsburg and Schachman, 1960; Hermans and Scheraga, 1961). Since the inactivation by pepsin is caused by the hydrolysis of the 120–121 peptide bond (Anfinsen, 1956), the inhibition of this bond cleavage by the presence of sulfate ion suggests that a binding site involves the C-terminal end of the molecule. Also, the inactivation of ribonuclease by pepsin produces a normalization of two tyrosyl residues at room temperature (Sela and Anfinsen, 1957) which can be attributed to an uncoupling of the thermostable and thermolabile regions since the state of region II is shown here to influence the stability of region I. The results of Rupley and Scheraga (1963) and Ooi *et al.* (1963) already have indicated that N- and C-terminal regions of ribonuclease do not unfold during the thermal transition. The slight red shift observed in the sulfate case could involve an effect on the tyrosyl 115 residue which is located near the active site. Thus, it is not unreasonable to propose that sulfate ion exerts its effect on the conformation and thermal stability of ribonuclease at acid pH by a specific binding at the active site of this enzyme. In this regard, the results of Kalnitsky and Resnick (1959) indicate that RNA, in contrast to the small specific anions and perhaps the pyrimidine 2′,3′-cyclic phosphates, destabilizes the ribonuclease molecule, and this could arise from an interaction of RNA with other side-chain groups of ribonuclease.

Doscher and Richards (1963) found that crystalline suspensions of ribonuclease S in concentrated ammonium sulfate solutions are catalytically active toward the pyrimidine nucleoside 2′,3′-cyclic phosphates. This may be due to the ability of sulfate, which stabilizes configurations more easily crystallizable (Kunitz, 1940), to maintain the correct atomic distances at the active site for catalysis to occur. The fact that ribonuclease is active in the crystalline state does not decrease the probability that fluctuant configurations can and do occur in solution (Koshland, 1958; Linderstrøm-Lang and Schellman, 1959). Indeed, a configurational adaptability of the ribonuclease molecule in solution is suggested by the results presented here.

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