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Circular Dichroism Studies on the Conformation and Interaction of T_1 Ribonuclease*

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ABSTRACT: Circular dichroic spectra of T_1 ribonuclease have been measured from pH 1 to 12 at varying temperature (4–70°) and in 8 M urea. Analyses of the circular dichroism bands in the 240–320-nm region have been attempted in terms of contributions from various side-chain chromophores, especially those of the tyrosines. The results indicate that many of the tyrosine groups are buried, but un-ionized; the results also suggest possible exciton interaction between the tyrosine chromophores. The circular dichroism spectra in the 200–230-nm region show that T_1 has a low α-helical content and a low β-structural content (probably less than 20% as compared to model compounds). The sharp temperature transition profile observed in the circular dichroism of the side-chain chromophoric region does suggest, however, that T_1 has a certain organized structure formed by interaction of side-chain groups. The circular dichroism spectrum in the 240–310-nm region of a mixture of T_1 with 2′-GMP (or 3′-GMP, or certain of their analogs) differs significantly from the algebraic sum of the circular dichroism of T_1 and the circular dichroism of the nucleotide measured separately. Strong extrinsic Cotton effects are induced with positive and negative dichroic bands at 250 and 280 nm, respectively. The magnitudes of the circular dichroism maxima (10^4–10^5 deg (cm^2 dmole^{-1})), the symmetry of the transitions about 270-nm wavelength, and the approximately equal rotational strength of the two transitions (the 280-nm band is usually smaller) of the opposite sign suggest an exciton coupling effect. Moreover, the transitions remain invariant in wavelength, under varying pH and temperature and with a variety of inhibitor analogs. This substrate-induced exciton effect in T_1 could arise from electronic interactions between the substrate purine group and the aromatic tyrosines and/or tryptophan located at or near the active site of the enzyme.

The formation of the T_1–nucleotide complex as shown by the induced circular dichroism bands has a qualitative relation with the binding constants obtained by gel filtration techniques. The inhibitors with high constants, $>10^3$ M^{-1}, all show induced dichroic bands. However, the magnitude of the induced bands per mole of complex calculated from the binding constant is not proportional to the binding constants. The relative intensities per mole of complex are 3′-GMP > 2′-GMP > 3′-dGMP > (9-GEtP, 9-GBuP).

Nucleases are suitable enzymes for studying the mechanism of nucleic acid and protein recognition and interaction. These enzymes are usually small, soluble, without subunits, and devoid of complex cofactors. Ribonuclease T_1 is a particularly interesting enzyme (Egami and Nakamura, 1969; Takahashi *et al.*, 1970). Its activity is limited to phosphoryl transfer and hydrolysis of the guanylic acid residues of RNA. It is less active on some analogs of GMP (such as IMP or XMP).[1] In mechanism, its activity is similar to RNase 1-A, for it forms an intermediate 2′,3′-cyclic nucleotide which is finally hydrolyzed to a 3′-nucleotide. Its physical and chemical properties are, however, very different from those of RNase 1-A. It is an acidic protein, with an isoelectric point at pH 2.9. It has 104 amino acids and its sequence has been determined (Takahashi, 1965). It has two disulfide linkages which form a very compact loop at the N-terminal end of the protein, as well as closing almost the entire sequence into a circle. T_1 has nine tyrosines, one tryptophan, and four phenylalanines; it has a large excess of acidic groups (12) and very few basic groups: one arginine, one lysine, the N-terminal amine, and three histidines. In spite of its acidic nature, its natural sub-

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[1] Abbreviations used are: GMP, guanylic acid; IMP, inosinic acid; XMP, xanthosinic acid; 9-GEtP, 9-(2′-hydroxyethyl)guanine 2′-phosphate; 9-GBuP, 9-(4′-hydroxybutyl)guanine 4′-phosphate.

strate is the negatively charged nucleic acid. Thus its recognition step must involve limited and highly specific parts of its sequence. An acidic group (glutamic acid 58) has been implicated in the active site (Takahashi *et al.*, 1967). The role of this group in catalysis is not understood. The histidines, the single arginine, and the tryptophan also appear to be involved in the active site (Takahashi *et al.*, 1970; Pongs, 1970b).

T_1 has also been studied by fluorescence and proton magnetic resonance. Thus the binding of substrate inhibitor produces marked quenching in the fluorescence of both tryptophan and tyrosines (Pongs, 1970a,b). By observing the chemical shifts of the C-2 protons of the three histidine residues, Ruterjans *et al.* (1969) found that two of the histidines titrated abnormally high, while the third behaved so anomalously that its pK could not be determined. The C-2 proton resonances of two of the histidines changed in the presence of 3′-GMP, thus implicating them in the active site.

Qualitative and semiquantitative binding studies of substrate inhibitors to T_1 were reported by Sato and Egami (1965), Egami and Nakamura (1969), and Pongs (1970b). Their studies showed that T_1 has only one binding site. Quantitative binding measurements are now also available using gel filtration techniques (Campbell and Ts'o, 1971).

Takahashi (1966) reported the uv spectrum and optical rotatory dispersion pattern of T_1 in water and 8 M urea. He concluded that the enzyme is reversibly unfolded in urea, and the α-helical content of the active enzyme is low. At the termination of this study, Yamamoto and Tanaka (1970) recently published a study of the ultraviolet absorption, circular dichroism, optical rotatory dispersion, and fluorescent properties of T_1. Their results will be further discussed in the following section.

In this study, the circular dichroism of T_1, particularly in the wavelength regions of the side-chain chromophores, is presented in detail. The circular dichroism spectrum is perturbed by acid, alkali, thermal, and solvent denaturation of T_1. New dichroic bands are induced in the circular dichroism spectrum of T_1 by inhibitors, whose effects are qualitatively correlated with the binding data of Campbell and Ts'o (1971). The asymmetry of the active site is evident in induced dichroic bands with nonasymmetric molecules.

Materials and Methods

T_1 ribonuclease was isolated from the crude extract of *Aspergillus oryzae* by the method of Uchida (1965) with minor modifications, as described below. The crude extract (Takadiastase powder, Sanzyme R. Sankyo) was purchased from Calbiochem (Los Angeles, Calif.). The DEAE-cellulose was Whatman DE23. The Japanese acid clay was purchased from Kukita Yakukin Kogyo Co., Ltd., Tokyo, Japan. Gel filtration was with Sephadex G-25. 2′-GMP and 3′-GMP were kindly supplied by Dr. Mary Campbell, who prepared them by the method of Cohn and Volkin (1951). Some 2′-GMP and 3′-GMP were also purchased from Sigma Chemicals (St. Louis, Mo.).

3′-XMP was prepared by nitrous acid deamination of 3′-GMP by Dr. Olaf Pongs of our laboratory. Samples of 9-(2′-hydroxyethyl)guanine 2′phosphate and 9-(4′hydroxybutyl)-guanine 4′-phosphate were generous gifts from Dr. Akihiro Yamazaki (Central Research Laboratories, Aginomoto Co., Japan). All other chemicals were reagent grade or the best available.

In following the Uchida method, the crude Takadiastase extract was equilibrated with DEAE-cellulose in phosphate buffer and then eluted with batches of increasing ionic strength buffer. The solution was acidified (pH 1.5) and the precipitate discarded. The supernatant was heated to 80° to inactivate other enzymes. After cooling, the enzyme was adsorbed onto the Japanese acid clay. It was eluted in citrate buffer. This step was facilitated by grinding the clay in a Virtis grinder, followed by centrifuging. The supernatant was acidified and the enzyme was precipitated in ammonium sulfate. The precipitate was collected, resuspended in buffer, adjusted to neutral pH, and dialyzed against water. The dialyzed enzyme was concentrated in a rotary evaporator. The solution was then chromatographed in a DEAE-cellulose column. The elution profile was bimodal. The leading major peak was clear and showed high activity. The trailing peak was colored and showed higher activity. The leading peak was concentrated by evaporation, desalted on a Sephadex G-25 column, and evaporated to dryness. A clear crystalline enzyme preparation was obtained. This material provided a spectrum typical of T_1 ribonuclease, with a maximum at 278 nm, a shoulder at 284 nm, and a minimum at 251 nm, and a maximum/minimum ratio of 3.3.

This preparation was compared with a sample of ribonuclease T_1 kindly supplied by Dr. H. Ruterjans of the Institute fur Aerobiologie, Saurland, Germany, and found to be similar. In a second preparation, the two peaks in the DEAE-cellulose elution were not resolved. These preparations were also compared to a Sankyo sample (Sankyo Lot No. 901073, Calbiochem, Cat. No. 556785) provided by Dr. Mary Campbell. This sample gave optical properties identical with the first preparation's in both ultraviolet absorption and circular dichroism spectra. In this study, the two preparations are sometimes indicated, for there appears to be some slight difference.

The concentrations of enzyme and inhibitors were determined by ultraviolet absorption in a Cary 15 spectrophotometer. For T_1, the maximum molar extinction coefficient of 2.1×10^4 was used (Sato and Egami, 1965). The GMP concentrations were determined using the acid molar extinction coefficients of 12×10^3 (pH 1). Deoxyguanylic and riboguanylic acids as well as the guanosines have similar spectra. For this reason, the extinction coefficients of the 9-(2′-hydroxyethyl)guanine 2′-phosphate and 9-(4′-hydroxybutyl)guanine 4′-phosphate were also taken to be 12×10^3 in acid solutions. For IMP the acid extinction coefficient was 11.7×10^3, and for XMP it was 8.4×10^3.

The circular dichroism spectra were measured in a Cary 60 spectropolarimeter adapted with a Model 6001 circular dichroism accessory. The calibration of the Cary 6001 circular dichroism attachment was rechecked against *d*-camphorsulfonic acid and found satisfactory. Most measurements were made at the most sensitive setting (0.02-deg full-range ellipticity). To obtain reliable, reproducible data at this setting, the cell location between repeated readings must not be changed. This was achieved by mounting the thermojacketed cells (Optical Cell Co., Beltsville, Md.) permanently under strain-free conditions on removable cell holders. The cells attached to their removable holders can thus be removed, rinsed, refilled, and relocated exactly. Cells of 10, 2, and 0.5 mm were used. The 10-mm cell was used down to approximately 240–250 nm. At shorter wavelengths, 2- and 0.5-mm cells were used. The sample temperature was controlled by an external circulating bath (Haake–Brinkmann, Model KT 62). The temperature readings were accurate to ±0.5° at temperatures above 20 and ±1° at temperatures of approximately 5°.

The absorption of the solutions at 278-nm maximum varied from 0.5 to 2.5 optical density units. In its most sensitive

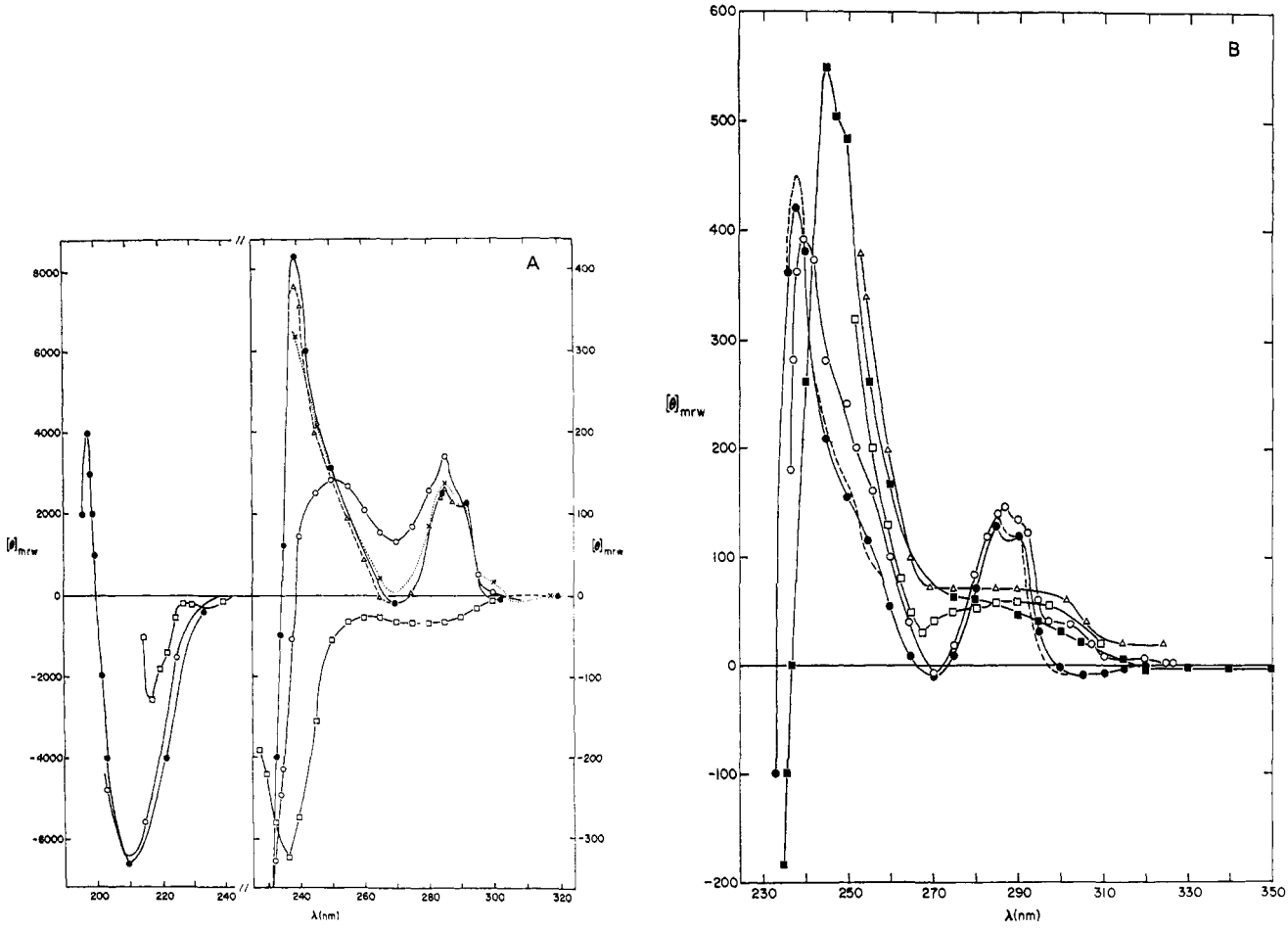

FIGURE 1: The circular dichroism spectra of T_1 ribonuclease. (A) As a function of pH (acid titration) and 8 M urea at 23°: (●) pH 7.5 (preparation II), (Δ) pH 5.0 (preparation I), (×) pH 4.0 (preparation I), and (○) pH 0.9 (preparation II); (□) 8 M urea, neutral pH. The temperature is 23°, T_1 concentration, 5×10^{-5} to 5×10^{-4} M. In the left-hand side inset the scale is changed. The far-ultraviolet regional data at pH 7.5 were obtained in water and the acid data in HCl. (B) As a function of pH (alkaline titration) at 23°: (●) pH 7.5 (preparation II), (– – –) pH 8.6 (preparation I), (○) pH 10.6 (preparation I), (□) pH 11.3 (preparation I), (Δ) pH 12.0 (preparation I), (■) pH 12.5 (preparation II). The T_1 concentration is 5×10^{-5} to 10^{-4} M. The pH adjustments were made with NaOH.

range (0.02-deg ellipticity full range), the signal to noise ratio, particularly with highly absorbing solutions, is small. It was thus necessary to run the instrument in synchronous drive, using an expanded wavelength and a 1.0 time constant setting. In the far-ultraviolet region and in highly absorbing solutions, the decrease in signal-to-noise ratio increased the error in the data. The error in measurements in pure enzyme solutions is estimated to be approximately ±25 deg (cm² dmole⁻¹) at 290 nm, ±50 at 240 nm, and ±1000 at 210 nm. The error is slightly higher in enzyme–inhibitor and acetate buffer solutions.

The ellipticity of T_1 and complexes is expressed as deg (cm² dmole⁻¹) and is normalized to the mean residue weight of the protein (110). In studies on enzyme–inhibitor complexes, the ellipticity is also expressed per decimole of enzyme. The data are not corrected for refractive index.

Results and Discussion

Circular Dichroism of T_1 Ribonuclease. T_1 ribonuclease is reversibly inactivated in acid and irreversibly in alkali (pH 10.6) (Sato and Egami, 1965; Egami *et al.*, 1964). In alkaline titration the ultraviolet absorption of T_1 changes drastically at pH 10.8 (Takahashi, 1966; Iida and Ooi, 1969; Yamamoto and Tanaka, 1970). The absorption maximum shifts from ~278 to ~290 nm with an increase in absorption of approximately 30%. This is due to the ionization of tyrosines. In 8 M urea, T_1 is reversibly inactivated. The ultraviolet absorption spectrum shows a blue shift and there is a large change in the optical rotatory dispersion spectrum indicating unfolding of the protein in urea (Takahashi, 1966).

The circular dichroism of T_1 from 200 to 320 nm at pH 1 to 7.5 and in neutral 8 M urea solution is shown in Figure 1A. At neutral pH, the native enzyme has two prominent positive dichroic bands, a band in the 240-nm region with ellipticity varying from 300 to 400 deg (cm² dmole⁻¹) and a smaller band of ~120 deg at 285 nm. This latter band has a subsidiary peak at 290.5 to 291 nm. In the 240-nm region, the intensity and wavelength of a circular dichroism band would be greatly effected by overlap with the predominantly negative dichroism originating in the peptide chromophores of the shorter wavelengths. Another positive band is suggested at 250 nm as a shoulder of the 240-nm band. Negative bands, partially masked by the adjoining positive bands, are found in the 265–270 and 300–305-nm wavelength regions. These bands are repeatedly observed in many different scans from many samples. The experimental errors in the circular dichroism measurements observed from many scans (see Methods) do not reflect the relative changes in the circular dichroism of individual scans. Thus the peak at 285 nm and the accompanying

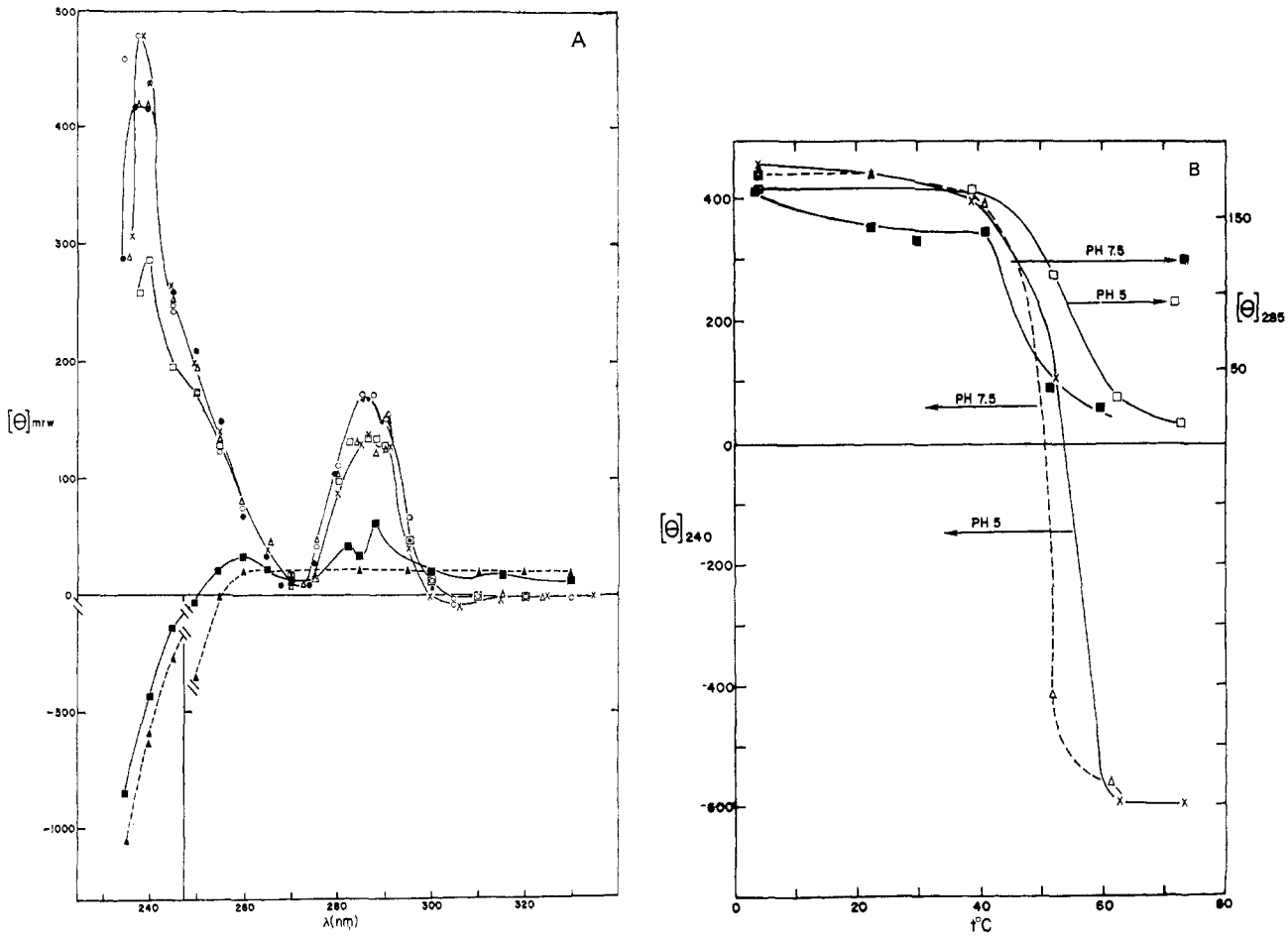

FIGURE 2: The effects of temperature. (A) On the circular dichroism of T_1 ribonuclease at pH 7.5. (○) 3–5°, (×) 23°, (△) 30°, (□) 41°, (■) 51°, (▲) 60°, and (●) 23° after heating. Solvent water, pH adjusted with NaOH. T_1 concentration, 5×10^{-5} M. (B) On two chromophores in the circular dichroism of T_1 ribonuclease at pH 5 and 7.5. (■) Ellipticity at 285 nm at pH 7.5; (□) ellipticity at 285 nm at pH 5; (×) ellipticity at 400 nm at pH 5; (△) ellipticity at 400 nm at pH 7.5. T_1 concentration 5×10^{-5} M.

291 nm subsidiary peak may be in error to ±25 deg (cm^2 $dmole^{-1}$). However their relative values to each other are unchanged. The repeated scans provided the resolution. The same applies to the 265- and the 300–305-nm wavelength regions.

At pH 0.9, the positive band in the 240-nm region and the negative band in the vicinity of 270 nm are both lost due to the acidification. The peak at 290 nm also vanishes. The large change in the circular dichroism at 270 nm suggests that this negative band is apparently not as weak as expected from observations on the native enzyme. However, the 285-nm band is retained in acid solution and the 250-nm shoulder is observed as a fairly strong positive band.

In neutral solution of 8 M urea, the positive bands at 280–290, 250, and 240 nm are all erased.

In alkaline titration (Figure 1B), all the bands except the negative one at ~305 nm persist to pH 10.6. When the pH exceeds 10.6, the 285–290-nm bands usually assigned to aromatic chromophores are reduced, but a new band, difficult to resolve, appears at 300 nm. This latter band is probably due to tyrosyl groups. The negative band in the vicinity of ~270 nm is also reduced. The circular dichroism spectrum at pH 12.5 shows the major changes due to alkaline inactivation and unfolding. The 285- to 290-nm bands are reduced to approximately half their intensity, while the circular dichroism in the 300-nm region increases appreciably. The negative band in the 270-nm region is no longer apparent. The strong positive band observed in the 240-nm region of the native T_1 is shifted to ~245 nm with approximately 25% increase in intensity. The shoulder at 250 nm remains apparent. The absorption of this sample of T_1 (pH 12.5) showed a hyperchromic effect on denaturation (an increase of 34%). The maximum absorption shifted from 278 to 290 nm. These ultraviolet absorption changes are in agreement with those of Takahashi (1966) and of Yamomoto and Tanaka (1970) and indicate that the enzyme is completely denatured.

The signal-to-noise ratio of the circular dichroism spectrum of T_1 at the far-ultraviolet region (Figure 1A) is very high; however, approximate circular dichroism values in this region were obtained. There is a minimum in the vicinity of 210 nm and a weak maximum at about 196–198 nm. Estimated errors are ±1000 deg (cm^2 $dmole^{-1}$) at the 210-nm minimum and 2000 deg (cm^2 $dmole^{-1}$) at wavelengths below 205 nm. Because of large errors, any circular dichroism differences between native and acid denatured T_1 in the minimum at 210 nm could not be resolved. However, the far-ultraviolet spectrum of T_1 in 8 M urea does show a large difference. The minimum is reduced and there appears to be a small trough at 230–233 nm. This trough is also observed as a weak inflection in the circular dichroism of T_1 in H_2O.

The far-ultraviolet spectrum of T_1 is consistent with a very low degree of regular organized structure. The α helix gives

strong negative bands at 206 and 222 nm and a very strong positive band at 190 nm. The molar ellipticities of these bands in a 100% α helix are very large: $-(4 \times 10^4)$ at 206 and 222 nm and 7×10^4 at 190 nm (Holzwarth and Doty, 1965). T_1 ribonuclease apparently is very low in helical content, for the only band comparable to the α helix is the 210-nm band, with a value of approximately $-(6 \times 10^3)$, indicating very little α-helical structure. The β helix has a broad negative band in the vicinity of 218 nm with a molar ellipticity of -2×10^4 and a positive band at 198 nm with a value of 3×10^4 (Sarker and Doty, 1966; Townend *et al.*, 1966). T_1 apparently has little β structure, for the molar ellipticity near 218 nm is very small (4×10^3). The presence of some β structure, however, is suggested by the location of a maximum near 198 nm. At this wavelength, the circular dichroism of a random coil protein is negative (Holzwarth and Doty, 1965; Jirgensons, 1970). The circular dichroism spectrum of T_1 ribonuclease thus indicates a very low helical content, lower than in ribonuclease A (Tamburro *et al.*, 1968; Jirgensons, 1969, 1970). The contributions to the circular dichroism in the far-ultraviolet region by bands arising from the side-chain chromophores is not expected to alter these values significantly. The present evidence indicates that ribonuclease T_1 can be classified among the nonhelical proteins (Jirgensons, 1969, 1970). At such a low level of helical content and β structure, perhaps it is not meaningful to cite an accurate number for T_1; roughly speaking our data indicate that the per cent of helical content and β structure is less than 20%, or approximately 15%. This value is not far off from the value of 22% estimated by Takahashi (1966) from a modified Moffitt plot on optical rotatory dispersion data. More recently, Yamamoto and Tanaka (1970) reported their circular dichroism and optical rotatory dispersion studies on the T_1 enzyme from neutral to alkaline (down to 260 nm only) solution, presumably at room temperature. At neutral pH, the near-ultraviolet circular dichroism spectrum was similar to that of Figure 1, except that the values of Yamamoto and Tanaka are approximately twice as high. In the far-ultraviolet region they obtained minima at ~212 nm and 204 nm. Their extreme minimum at 204 nm is again approximately two times that obtained in this study. Consequently, their estimation of helical content was about 30–40%, twice as high as ours. Since our results on four preparations of T_1 enzyme (two from our own preparation, and two from Sankyo Co.) gave nearly identical results, and the instrument Cary 60 with 6001 circular dichroism attachment was calibrated satisfactorily with *d*-camphorsulfonic acid, the reason for this discrepancy is unknown at present. The enzyme preparation of Yamamoto and Tanaka was from Sankyo and their method for the estimation of protein concentration was not cited in their paper.

The effect of temperature on the side-chain chromophores at pH 7.5 is shown in Figure 2A. From 4 to 40°, the circular dichroism bands in the 280-nm region are essentially unchanged, while the spectral band at 240 nm begins to decrease at 41°. At 50°, all the positive bands are lost. At 60° the spectrum of T_1 begins to resemble that obtained at 8 M urea (Figure 1A) though the σ values remain slightly positive at wavelengths above 250 nm.

The effects of temperature on T_1 at pH 5.0 are essentially the same as that at pH 7.5 and are not reported here. However at pH 5.0, the enzyme appears to be more stable to thermal denaturation and this is shown in Figure 2B. Thus, at pH 5.0 and 52°, the bands in the 280-nm region are reduced to about 70% of their original value (instead of 30% as at pH 7.0) and the band at 250 nm is more prominent ($[\sigma]_{mrw} = 100$, instead of $[\sigma] = 0$ at pH 7.0). It should be noted that the circular dichroism spectrum of this enzyme sample (Figure 2A) returns to the original, native profile upon cooling to 23° after the rather lengthy heating period at 50–60°. The temperature profiles of $[\sigma]_{240\ nm}$ and $[\sigma]_{285\ nm}$ at pH 7.5 and 5.0 are shown in Figure 2B. There is an abrupt change around 50–60°, with the transition at pH 5.0 taking place at higher temperature than that at pH 7.5. This sharp transition is indicative of the cooperativity in the thermal denaturation of T_1.

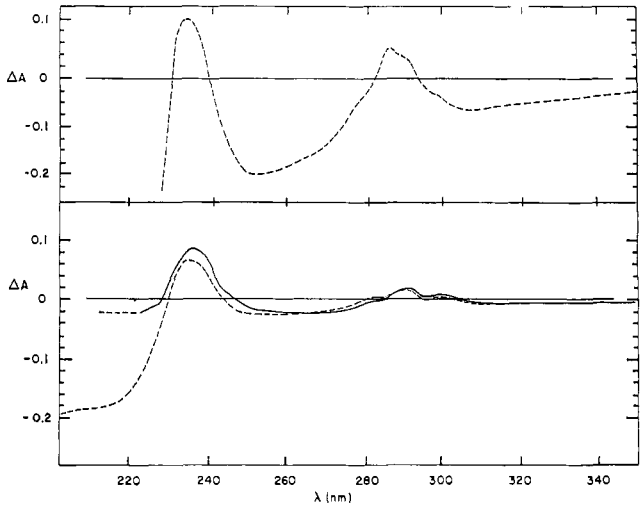

FIGURE 3: The ultraviolet absorption difference spectra of T_1 ribonuclease. Upper part: T_1 in H_2O, pH 7.5/T_1 in 8 M urea, neutral pH. The urea absorbance has been subtracted out. Lower part: T_1 in H_2O, pH 7.5/T_1 in acid, pH 0.9; solid line, freshly acidified, interrupted line, 17 hr after acidification. T_1 concentration, 3.6×10^{-5} M (0.75 optical density unit at 278 nm). The data were traced directly from a spectrophotometer scan, room temperature.

The ultraviolet difference spectra of the denaturation of T_1 by acid and 8 M urea have been studied (Figure 3) to obtain more information for the assignment of circular dichroism bands. A difference spectrum from 300 to 275 nm in the presence and absence of 8 M urea has been previously reported by Takahashi (1966) and was similar to that portion of the profile shown in Figure 3. Both urea and acid denaturation reveal changes in $\Delta\epsilon$ at similar wavelengths, positive $\Delta\epsilon$ at 235 nm and 285–290 nm and a negative $\Delta\epsilon$ at 250–270 nm. The negative $\Delta\epsilon$ at longer wavelengths (>300 nm) due to urea is attributed to light scattering, which would have an effect on the exact magnitude of the position of the urea difference spectrum. The positive $\Delta\epsilon$ at 285 and 290 nm is attributed to changes in the environment of the tyrosines and the trytophan which have high extinction coefficients at these wavelengths (tyrosine, log ϵ_{max} 3.16; tryptophan, log ϵ_{max} 3.70; phenylalanine, log ϵ_{max} 2.35; Donovan, 1969). The positive $\Delta\epsilon$ at 285 nm indicates a red shift in the transfer of the tryosine chromophore from the aqueous solution to the interior of the protein. The $\Delta\epsilon$ at 290 nm is associated with similar changes in the environment of the tryptophan moiety. The contribution to absorption by the phenylalanines is negligible (log ϵ_{max} 2.35). In acid, the $\Delta\epsilon$ at 285–290 nm is smaller than that obtained in 8 M urea. Thus denaturation by acid is less effective than by 8 M urea. This is in qualitative agreement with the circular dichroism data (Figure 1A).

The positive $\Delta\epsilon$ observed at 235 nm is larger than that in the 285–290 nm. Large $\Delta\epsilon$'s at this wavelength have been observed in other proteins (Glazer and Smith, 1960, 1961). Glazer and Rosenheck (1962) associated the positive $\Delta\epsilon$ at 235 nm with

the n–π^* transitions of the peptide backbone. Subsequent work (Donovan, 1968; Mora and Elodi, 1968) showed that the major contribution to the $\Delta\epsilon$ at ~235 nm is due to changes in the transitions of aromatic groups (tyrosine and trytophan). This was done by observing parallel changes in the $\Delta\epsilon$ at both the 235- and 285-nm wavelengths. The latter authors also indicated that any n–π^* peptide contribution was small (<10%) at this wavelength. It thus appears that the $\Delta\epsilon$ of T_1 at 235 nm is also associated with the tyrosines and tryptophan.

The negative $\Delta\epsilon$ at 250–270 nm observed in acid and in 8 M urea are in the absorbing regions of both cystines and phenylalanine chromophores, which have fairly small extinction coefficients (log ϵ_{max} 2.5 for cystine; Donovan, 1969). The phenylalanine, being hydrophobic, would be expected to behave like the other aromatic groups and give positive $\Delta\epsilon$ with denaturation instead of negative $\Delta\epsilon$. The large negative $\Delta\epsilon$ seen at these wavelengths (245–270 nm) due to 8 M urea denaturation could be again largely attributed to light scattering. The possibility that T_1 has enhanced absorption in 8 M urea at 300 nm is unlikely for T_1 has no chromophores in this region. A part of this negative region could possibly be attributed to inadequate subtraction of urea but the 8 M urea used had only a small absorbance at these wavelengths. It should be noted that whatever the origin of the large negative $\Delta\epsilon$ at $\lambda > 300$ nm and in the vicinity of 245–270 nm the qualitative results are not affected, except insofar that the intensities of the positive peaks at 235 and 285–290 nm may even be larger than shown in Figure 3. The studies in the changes of $\Delta\epsilon$ in general support the circular dichroism data. The hydrophobic aromatic groups undergo red shifts on going from the aqueous environment to the hydrophobic interior of the enzyme. The effect of 8 M urea is much larger than that of acid (pH 0.9).

Assignment of the Side-Chain Chromophores. The dichroic bands observed in the near-ultraviolet wavelengths (longer than 240 nm) arise from transitions in the nonpeptide chromophores. The main bands may originate from the π–π^* transitions of aromatic groups (tyrosine, tryptophan, and phenylalanine) and the n–σ^* transition in cystine. T_1 has nine tyrosines, one tryptophan, four phenylalanines, and two cystine residues. The phenylalanine group produces very weak Cotton effects since its magnetic and electric dipole moments are perpendicular (Moscowitz *et al.*, 1965), and no mixing with n–π^* transitions is possible. Tryptophan residues are difficult to assign, particularly in the presence of tyrosines. The indole moiety has two overlapping transitions, which produce strong dichroic bands in the 290- to 305-nm regions of proteins (Strickland *et al.*, 1969). The position and sign of the tryptophan dichroic bands vary considerably with solvent polarity and neighboring charge groups as is evident from studies on model compounds (Edelhoch *et al.*, 1968). They usually overlap with circular dichroism bands from tyrosines and their intensities are comparable. Since there is only one tryptophan to nine tyrosines in T_1, the tryptophan contribution may be small (~20%) in the 285- to 290-nm region. The tyrosine chromophore has strong π–π^* absorption bands at 270 and 222 nm. The corresponding dichroic bands in proteins are observed in the 270- to 290-nm range for the former, while the latter are usually masked by the peptide dichroism. In the ionized form, tyrosines absorb at 235 and 287 nm, while the ionized tyrosines of proteins exhibit circular dichroism bands at slightly longer wavelengths.

T_1 in the above studies has been shown to have positive dichroic bands in the 240-, 250-, 285-, and 290-nm wavelength regions, and negative bands in the vicinity of 265–270 and 305–310 nm. There may also be a band in the vicinity of 230–233 nm. The conspicuous positive bands in the 240- and 285-nm regions of the native T_1 are lost in the unfolded enzyme, when T_1 is denatured by heat or 8 M urea (Figures 1A and 2A). At acid pH (Figure 1A), the change in the circular dichroism is less drastic, for the intensity at 240 nm is reduced, but the peak at 250 nm is retained. Also, the effect in the 285-nm region is slight, for there appears to be only a loss of the 290-nm shoulder. At alkaline pH (12.5), the 240-nm band is shifted to 245 nm and its intensity increases, while the 285- to 290-nm bands are reduced in intensity and are shifted to longer wavelengths. This is consistent with the spectral shift associated with the ionization of tyrosines. Bands very similar to the 240-nm and the 285- to 290-nm bands are observed in poly-L-tyrosine (Beychok and Fasman, 1964; Chen and Woody, 1969; Friedman and Ts'o, 1971). These bands are often shifted, depending upon the environment. The 240-nm band is also found in L-tyrosine but at alkaline pH, above its pK (Beychok and Fasman, 1964). The 240-nm band has been observed in other proteins, *e.g.*, carbonic anhydrase (Beychok *et al.*, 1966) and in lysozyme (Ikeda *et al.*, 1967). In ribonuclease A the band at 240 nm is less conspicuous but becomes obvious upon alkaline titration (Simmons and Glazer, 1967; Simons and Blout, 1968; Pflumm and Beychok, 1969). In all these proteins some tyrosines titrate abnormally (Riddiford *et al.*, 1965; Tanford *et al.*, 1955). In T_1, seven out of nine tyrosines titrate with abnormally high pK's (Iida and Ooi, 1969).

Based upon the above evidence and by analogy with the behavior of other proteins, the band in the 240-nm region can be assigned to buried tyrosines. It was proposed by Pongs (1970a,b) from the quenched fluorescent state of the tyrosines in T_1 and their abnormally high pK (Iida and Ooi, 1967) that the majority of the tyrosines in T_1 may be in a unique environment—some buried but interacting with tryptophan, others buried but interacting with acidic groups. The latter tyrosines would behave as if quenched and would not be titratable, due to their proximity to carboxyl groups. The recent paper of Yamamoto and Tanaka (1970) also concluded that most of the tyrosines and the tryptophan are buried in T_1. The exact nature of the interaction between the buried tyrosine and the carboxylic acids is not yet known, since the quenched fluorescent state of the tyrosines inside a protein does not unequivocally indicate that the tyrosines are in an ionized state, for quenching can occur by energy dissipation to neighboring aromatic groups, as occurs in the tyrosine–tryptophan case observed by Pongs (1970a).

Currently, there are no data to indicate that the buried tyrosines are in deprotonized (or ionized) form. The seven abnormal tyrosines in T_1 all titrate at ~pH 11 and show the usual long-wavelength shift associated with protonation; the ultraviolet spectrum of native T_1 enzyme does not indicate the presence of deprotonated tyrosines. The strong positive circular dichroism band in the 240-nm region is shifted to 245 nm, that is, to longer wavelength at alkaline pH (12.5). A positive circular dichroism band in the vicinity of 245 nm has been observed in poly-L-tyrosine at pH 11.2 above the pK of tyrosine (Beychok and Fasman, 1964; Friedman and Ts'o, 1971). At a lower pH (10.6) and in methanol, the positive band occurs at ~230 nm (Friedman and Ts'o, 1971; Shiraki and Imahori, 1966; Damle, 1970). Thus the peak at 245 nm and not at 240 nm is indicative of ionized tyrosines. The 245-nm peak in T_1 has an ellipticity based upon the mean residue weight of ~550. If only tyrosines contribute to this band at pH 12.5, the molar ellipticity per tyrosine residue is ~6200, which agrees with the value of 6700 obtained by Beychok and Fasman (1964) with poly-L-tyrosine. The

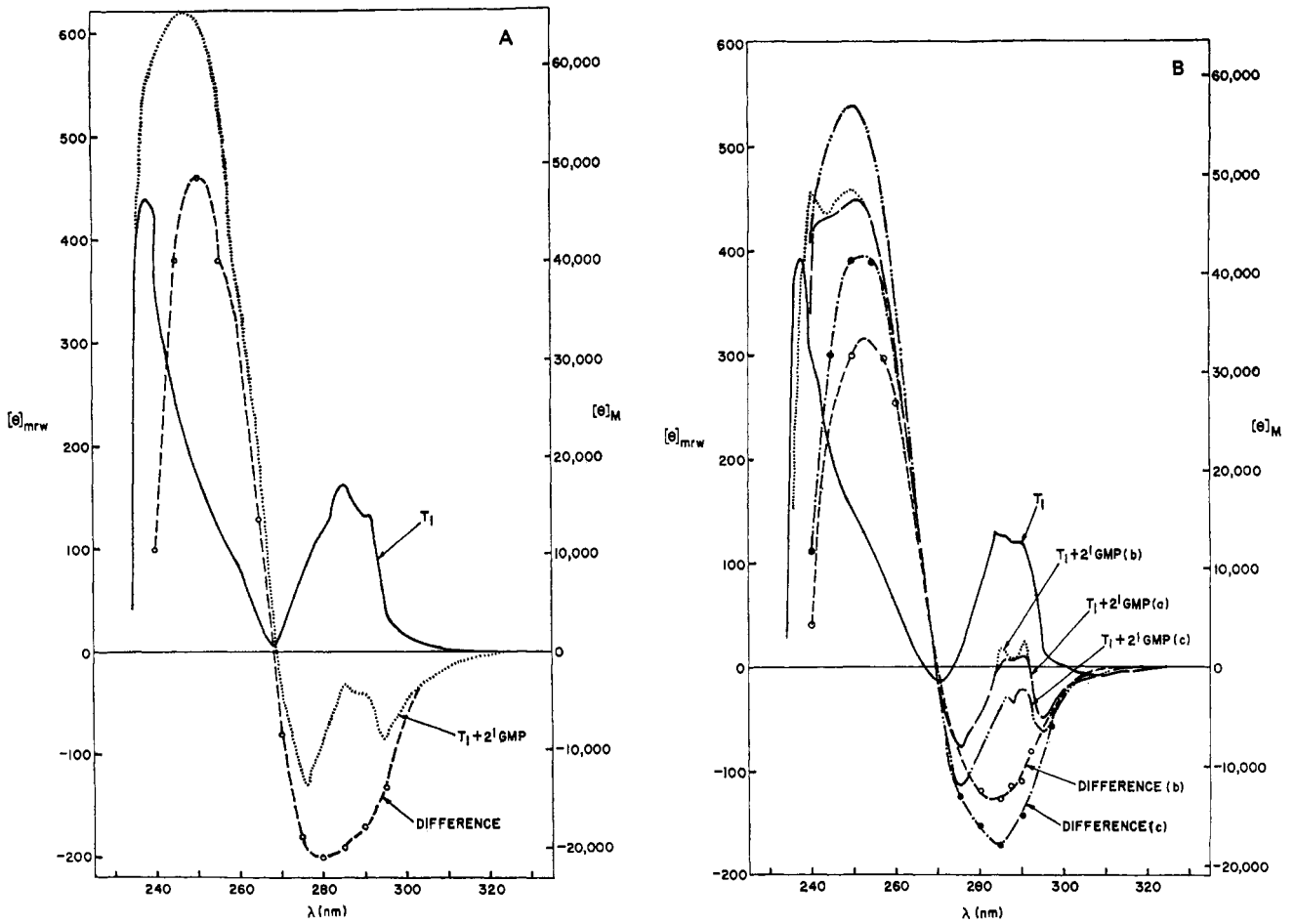

FIGURE 4: Inhibitor studies. (A) The effect of the inhibitor 2′-GMP on the circular dichroism of side-chain chromophores of T_1 ribonuclease at pH 5. Solid line, T_1 in 0.025 M sodium acetate buffer; (. . . .) T_1 4.5×10^{-5} M, + 2′-GMP, 2×10^{-4} M; (O) difference curve [T_1 + 2′-GMP] mixture − T_1; temperature 23°. (B) The concentration dependence on 2′-GMP inhibitor of the circular dichroism of T_1 ribonuclease at pH 5. (——) T_1; expt a: T_1 (10^{-4} M) + 2′-GMP (10^{-4} M); expt b: T_1 (5×10^{-5} M) + 2′-GMP (5×10^{-5} M); expt c: T_1 (4.5×10^{-5} M) + 2′-GMP (2×10^{-4} M); (O) difference curve for expt b: *i.e.*, [T_1 (5×10^{-5} M) + 2′-GMP (5×10^{-5} M)] mixture − T_1; (-●-●-) difference curve for expt c, *i.e.*, [T_1 (4.5×10^{-5} M) + 2′-GMP (2×10^{-4} M)] mixture − T_1. Sodium acetate buffer.

circular dichroism of L-tyrosine shifts from ~230 to ~245 nm on ionization (Beychok and Fasman, 1964). The strong positive band at 240 nm occurs only in the native T_1. Upon denaturation either by heat or by urea, it is completely lost; but on acidification, it is partially lost. If the 240-mμ band is attributed to tyrosine, it must result from wavelength shift of the nonionized tyrosine 230-nm circular dichroism band. Such a shift could occur by exciton coupling between tyrosine residues in close proximity to each other. A positive band would be produced in the ~240-nm wavelength region, while the negative band would be in the vicinity of 220 nm. Unfolding of the enzyme would destroy the coupling mechanism.

The positive band at 285–290 nm and the negative band in the vicinity of 270 nm may also be associated with aromatic groups, particularly tyrosines (Pflumm and Beychok, 1969; Yamamoto and Tanaka, 1970; Horwitz *et al.*, 1970). This band is especially informative for the RNase A, since this enzyme has no tryptophan. The negative band at 305 nm may be due to disulfide chromophores. These chromophores absorb weakly, but have considerable asymmetry (Horwitz *et al.*, 1970). The 230–233-nm band is also likely to be due to disulfide chromophores (Coleman and Blout, 1967).

The 250-nm band is difficult to assign. From the circular dichroism studies, it seems to be more resistant to denaturation than the other positive bands. It is not perturbed in acid but is lost in urea and at high temperatures; *i.e.*, it is lost upon complete unfolding of the protein. The ultraviolet difference spectra reveal that its absorption increases to a large extent in urea, whereas in acid its increase is much less. Titration studies do not support an assignment to tyrosines. From the melting profile, it does seem to be related to the cooperative structure of the enzyme. Circular dichroism bands at this wavelength have been observed in oxytocin (Beychok, 1966; Beychok and Breslow, 1968). By observing the retention of this band in analogs of oxytocin which have their tyrosines replaced by nonaromatic groups, Beychok and Breslow could unequivocally assign the band at this wavelength to cystine residues. Assignments of bands at this wavelength to SS chromophores have also been made by Coleman and Blout (1967). In T_1, the assignment of all these bands (305, 250, and 235 nm) may be assisted by measurements on the reduced enzyme or on fragments of T_1.

Effects of Inhibitors on the Circular Dichroism of T_1 Ribonuclease. The nucleotide 3′-GMP, the final enzymic hydrolysis product from RNA, is an inhibitor (Sato and Egami, 1957; Egami and Nakamura, 1969). Another effective inhibitor is 2′-GMP which at pH 5 binds more strongly to T_1 than 3′-GMP (Campbell and Ts'o, 1971). 5′-GMP shows no inhibition (Sato-Asano and Egami, 1960; Pongs, 1970a; Egami and Nakamura, 1969). Other, less effective, inhibitors and sub-

TABLE I: Molar Ellipticity of the Induced Circular Dichroic Bands from T_1–2′-GMP Complex.[a]

	T_1 (M)	2′-GMP (M)	Complex (M)	% Sites Occupied	[Molar Ellipticity]$_{complex}$ (deg cm^2 (dmole^{-1})) 280 nm	250 nm
(A)	1×10^{-4}	1×10^{-4}	6.1×10^{-5}	61	−23,000	+55,000
(B)	5×10^{-5}	5×10^{-5}	2.5×10^{-5}	50	−28,000	+70,000
(C)[b]	4.5×10^{-5}	2×10^{-4}	3.9×10^{-5}	87	−21,000	+49,000
					−24,000	+56,000

[a] The table is derived from Figure 4A,B; the binding constant used is $k = 4 \times 10^{+4}$ M^{-1} (Campbell and Ts'o, 1971). [b] Two preparations of T_1 enzymes were used, and the results are similar.

strates are 2′- and 3′- IMP and 2′- and 3′- XMP. The binding data of Campbell and Ts'o (1971) show that the order of affinity for T_1 at pH 5 is the following: 2′-GMP > 3′-GMP > 2′-IMP ≈ 2′-XMP ≫ 5′-GMP. The synthetic analogs 9-GEtP and 9-GBuP have very high binding constants—close to that of 2′-GMP. The above compounds, together with 3′-dGMP, 3′-IMP, and 3′-XMP, were studied for their effect on the circular dichroism of the side-chain chromophores of T_1. The concentrations and molar ratios were limited by the allowable ultraviolet absorption of the instrument.

The effect of 2′-GMP on the circular dichroism spectrum of T_1 in the wavelength region of side-chain chromophores is shown in Figure 4A. Minimal acetate buffer was used to maintain the pH at 5. Based on the binding constant of 2′-GMP to T_1, $4 \times 10^{+4}$ M^{-1} (Campbell and Ts'o, 1971), the enzyme should be 87% saturated with inhibitor at this pH and concentration. Figure 4A shows the circular dichroism spectrum for: (a) the unperturbed enzyme, (b) the enzyme plus inhibitor, (c) the algebraic difference spectrum, b − a. The ellipticity values as shown are normalized to the mean residue weight of protein and also per decimole of enzyme. (The left-hand side ordinate of these figures is based on the mean residue weight of protein, while the right-hand side is per decimole of enzyme.) The circular dichroism spectrum shows a marked effect due to the inhibitor, particularly at wavelengths in the vicinity of 250 and 280 nm. At 280 nm there is a new negative band, while at 250 nm there is a large positive band. The fine structure of the circular dichroism spectrum of the T_1 enzyme (*i.e.*, the peaks at 285 and 290 nm) appears to be little affected by the presence of inhibitor. The difference curve is smooth, with a positive maximum at 250 nm and a negative maximum at 280 nm. The two circular dichroism bands, of opposite sign, must arise from two transitions about 30 nm apart, and appear to be symmetrical about the 270-nm wavelength.

The concentration dependence of the effect of 2′-GMP on T_1 ribonuclease at pH 5 is shown in Figure 4B. Three concentrations were used. Since the data are normalized per mean residue weight of protein or mole of enzyme, the two concentrations of complex in expt A and B are not resolved in the figure. Comparison of the results from expt B and C, however, clearly shows the concentration dependence of the effect of 2′-GMP on the induced bands of T_1. These experiments also show that the binding constant of 2′-GMP to T_1 is high, thus supporting the high binding constant of Campbell and Ts'o (1971). Table I summarizes the concentration effect. It shows the concentrations of T_1, 2′-GMP, and complex, based on the binding constant 4×10^4 M^{-1}. The table also shows the mole per cent of T_1 occupied by inhibitor. From these data, the molar ellipticity of the T_1–2′-GMP complex was calculated at 250 nm (+56,000, maximum) and at 280 nm (−24,000 minimum). Experiment B, using low concentrations of both T_1 and inhibitor, shows the widest deviation and provides an approximate estimate for the minimum measurable concentration of 2′-GMP–enzyme complex needed in this experiment ($\sim 5 \times 10^{-6}$ M). In spite of the difficulties in measurements, the data are reproducible.

The influence of temperature on the induced dichroic bands at pH 5.0 is shown in Figure 5A. In each case the algebraic difference was obtained by subtracting the enzyme curve from that of an enzyme–2′-GMP complex obtained at the same temperature. Up to 40° there are no changes in the difference curves within experimental error. At 50° the enzyme–complex mixture showed no change with temperature; however, subtracting the curve of the partially unfolded T_1 would distort the difference curve, thus giving the curve indicated 50° (a). However, if the data from native T_1 at 40° are subtracted from the 50° complex curve, the 50° difference curve (indicated as 50° (b)) superimposes on that of the 40° curve (Figure 5A). The difference curves obtained at higher temperatures are even more distorted (not shown). As presented in Table I, T_1 at these concentrations and 23° is 87% saturated by 2′-GMP. Apparently, the inhibitor stabilizes the native structure of T_1. Thus, the equilibrium between the native and unfolded T_1 is shifted in favor of the active structure due to interaction with 2′-GMP. Since both the circular dichroism spectrum of T_1 and that of the T_1–2′-GMP complex are essentially unchanged over the range of 0–50°, this observation suggests that the enthalpy change of the binding is small from consideration of the Van't Hoff equation. It is likely, therefore, that the free energy change of T_1–2′-GMP complex formation measured (Campbell and Ts'o, 1971) is mainly entropic in origin.

The influence of temperature on the induced T_1–2′-GMP dichroic bands at pH 7.5 is shown in Figure 5B. At this pH, the binding affinity of T_1 for 2′-GMP is low and was not measured accurately before (Campbell and Ts'o, 1971). Data in Figure 5B inidcate that some binding must take place, for the induced dichroic bands at pH 7.5 are nearly half those obtained at pH 5. The reason for presenting Figure 5B is that the effect of temperature on the induced bands can be definitely observed from 5 to 40° in contrast to that shown in Figure 5A. There is a gradual reduction in the rotational strength of the induced circular dichroism bands as the temperature is increased. Since the circular dichroism spectrum of the enzyme at pH 7.5 is stable up to 40° (Figure 2A), indicating that the enzyme conformation is unchanged until the temperature is above 40°, the temperature dependence of the complex bands

from 5 to 40° suggests a contribution of an enthalpic term in the complex formation at pH 7.5. At pH 5.0, the enthalpic contribution is not detected as shown above.

Since 3′-GMP is the final product in the hydrolysis step of the enzyme and is a natural inhibitor, the circular dichroism spectra of the T_1–3′-GMP complex are presented for several pH values at concentrations shown in Figure 6A–C. At pH 5.0, the circular dichroism curves of T_1 (6×10^{-5} M) with 3 concentrations of 3′-GMP (6.2×10^{-5}, 1.9×10^{-5}, and 1.3×10^{-5} M) have been measured; since the results of the latter two concentrations of 3′-GMP were nearly identical, only the curves at 6.2×10^{-5} and 1.3×10^{-5} M are shown in Figure 6A. The circular dichroism of T_1 and 3′-GMP both at 6.2×10^{-5} M has also been measured at pH 5.5 and was found to be nearly the same as that at pH 5.0 (curve a in Figure 6A). The circular dichroism spectra of the T_1–3′-GMP complex measured at pH 6.0 with two concentrations of 3′-GMP and at pH 7.5 with one concentration of 3′-GMP are shown in Figure 6B,C, respectively. The concentration dependence is again apparent in Figure 6A,B, the higher concentration having the larger effect. The binding constant of 3′-GMP to T_1 at pH 6.0 is not available, but comparing Figure 6A,B, the intensity of the induced bands at pH 6.0 is lower by approximately two-thirds than that obtained at pH 5.0. At pH 7.5 the intensity of induced bands measured at the same concentrations of the enzyme and 3′-GMP is even further reduced and the relative error in the measurement becomes high. Comparison among Figure 6A–C clearly indicates the influence of pH on the induced dichroic bands and thus on the complex formation between T_1 and 3′-GMP. As the pH is increased from pH 5.0 to 7.5, the intensity of the induced bands decreases considerably, indicating the binding of 3′-GMP to T_1 has been greatly reduced. This observation may explain why the hydrolysis rates, V_{max}, of guanine-containing dinucleoside monophosphates (GpN) by T_1 are significantly higher at pH 7.5 than at pH 5.0 (Irie, 1968). The reason for the decrease of the interaction between the guanosine 2′- and 3′-phosphate and the T_1 enzyme is uncertain; it could be due to the ionization of the secondary phosphate group of the nucleotides, or the ionization of other groups in the enzyme (such as the imidazole groups). As for the quantitative estimation of the optical properties of the T_1–3′-GMP complexes, at pH 5.0, currently there are two values of binding constants; *i.e.*, the value of 2.9×10^3 M^{-1} measured by gel filtration (Campbell and Ts'o, 1971) and the value of 5.8×10^3 M^{-1} measured by microcalorimetry (H. Ruterjans, G. Schoene, and O. Pongs, personal communication). The K value obtained by gel filtration may have a tendency of being low, especially at low K values. A better fit of the calculated molar ellipticity for the complex (Table II) can be obtained if the higher value of K (5.8×10^{-3} M^{-1}) is used in the calculation of the circular dichroism data from Figure 6A. As shown in Table II, the average value of the calculated molar ellipticity of the complex is about +90,000 deg (cm^2 $dmole^{-1}$) at 250 nm and −45,000 deg (cm^2 dmole) at 280 nm; these values are about two-thirds higher than those obtained for T_1–2′-GMP complex (Table I). It is possible that the binding constant adopted for the T_1–3′-GMP complex is still too low; however, it is more likely that the induced dichroic bands in the T_1–3′-GMP complex are much larger than those from the T_1–2′-GMP complex.

It is of interest to investigate the role of the 2′-hydroxy group in the interaction between the nucleotide and the enzyme, in view of the fact that T_1 is inactive in DNA. In Figure 7, the circular dichroism spectra of T_1 (6×10^{-5} M) with 3′-dGMP at 1.2×10^{-4} and 5×10^{-5} M are shown. The difference curve at the lower concentration of 3′-dGMP is difficult to estimate in the 280-nm region and is not included. The difference curve at T_1 (6×10^{-5} M) and 3′-dGMP (1.2×10^{-4} M) shown in Figure 7 can be compared directly with that of T_1 (6.1×10^{-5} M) and 3′-GMP (1.2×10^{-4} M) shown in curve b (Figure 6A). The difference spectrum from the 3′-dGMP is is very similar to that from the 3′-GMP except that the intensity of the circular dichroism bands of the deoxynucleotide at

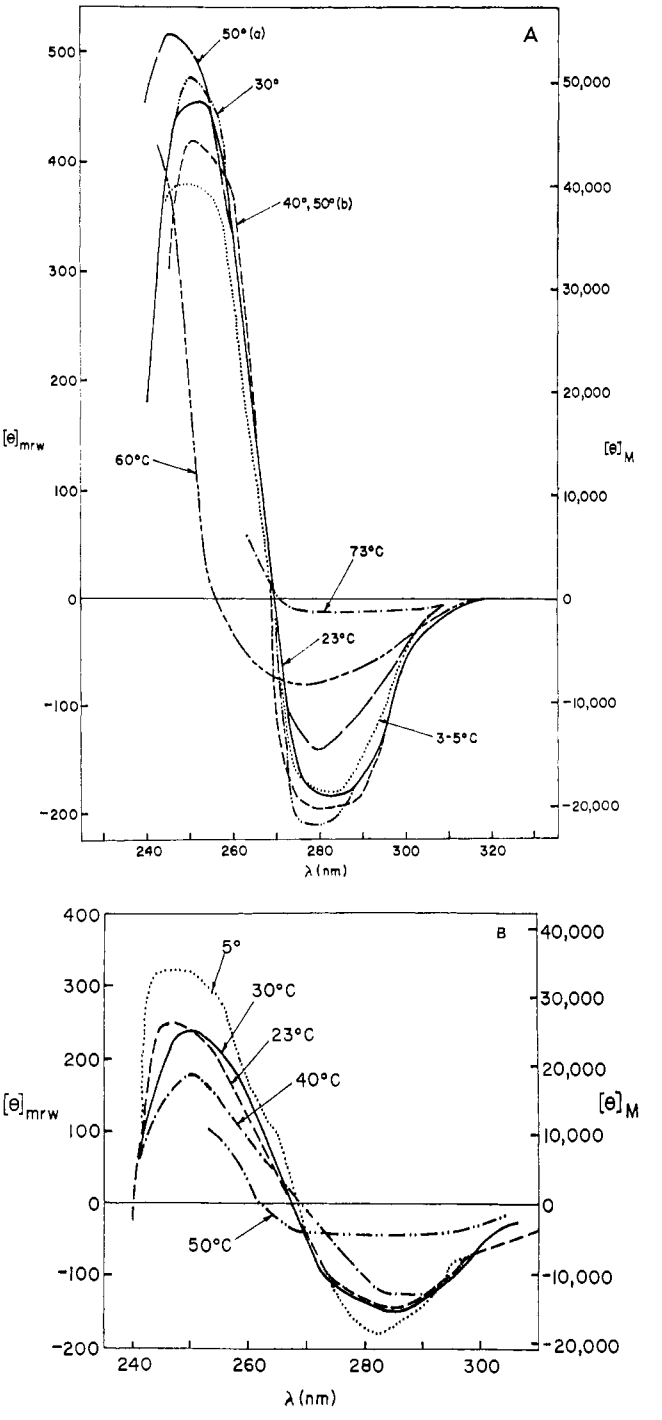

FIGURE 5: The temperature effects. (A) On the difference curves from (T_1 + 2′-GMP) − T_1; T_1, 4.5×10^{-5} M, 2′-GMP, 2×10^{-4} M. (····) 3–5°, (——) 23°, (—·—) 30°, (– – –) 40°, (— —) 50°, (–··–) 60°, and (—·—) 73°; pH 5.0. (B) On difference curves [T_1 + 2′-GMP] mixture − T_1 at pH 7.5; T_1, 4.5×10^{-5} M; 2′-GMP, 2×10^{-4} M. (····) 3–5°, (– – –) 23°, (———) 30°, (—·—) 40°, and (–··–) 50°.

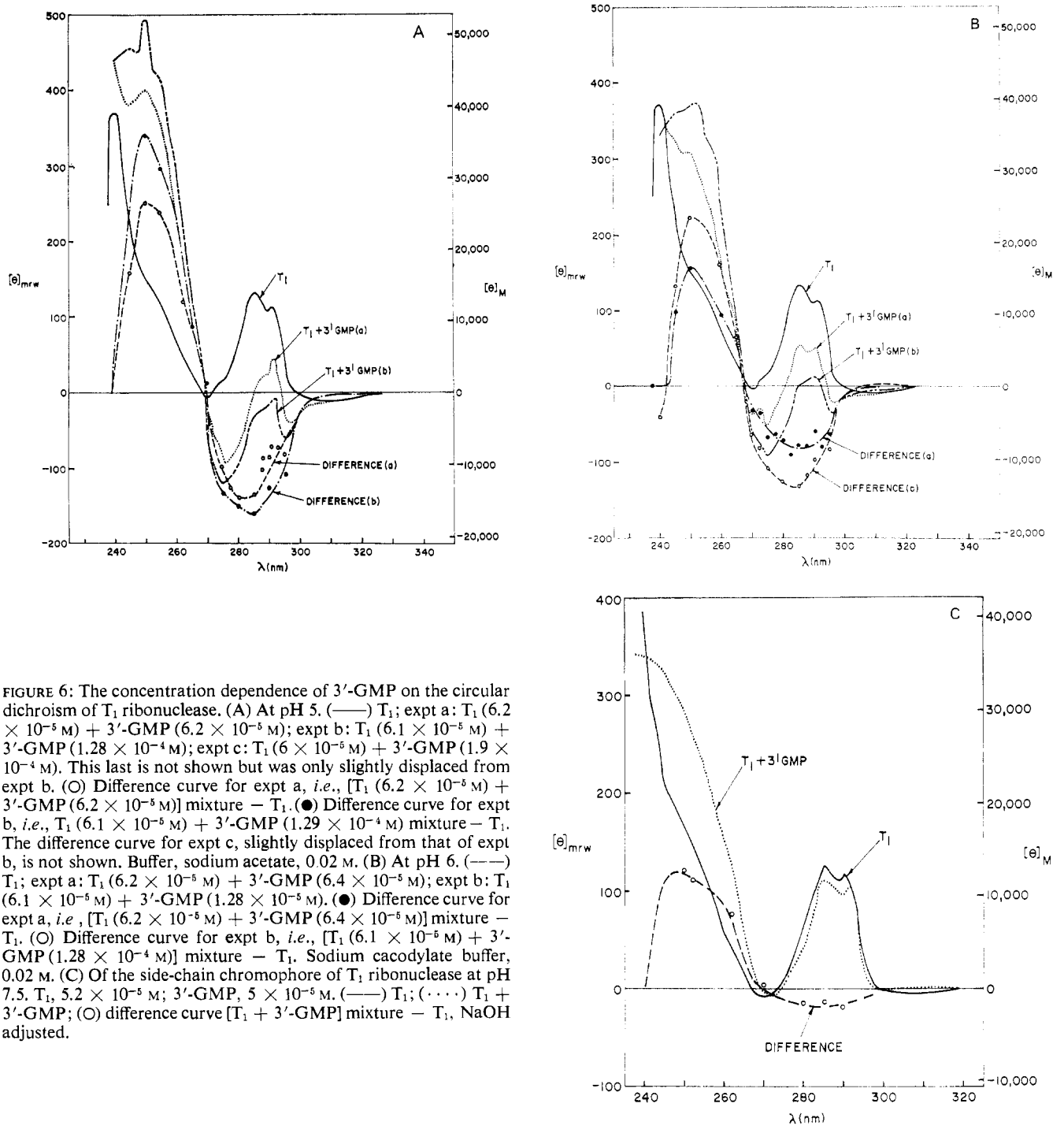

FIGURE 6: The concentration dependence of 3'-GMP on the circular dichroism of T_1 ribonuclease. (A) At pH 5. (——) T_1; expt a: T_1 (6.2×10^{-5} M) + 3'-GMP (6.2×10^{-5} M); expt b: T_1 (6.1×10^{-5} M) + 3'-GMP (1.28×10^{-4} M); expt c: T_1 (6×10^{-5} M) + 3'-GMP (1.9×10^{-4} M). This last is not shown but was only slightly displaced from expt b. (○) Difference curve for expt a, *i.e.*, [T_1 (6.2×10^{-5} M) + 3'-GMP (6.2×10^{-5} M)] mixture − T_1. (●) Difference curve for expt b, *i.e.*, T_1 (6.1×10^{-5} M) + 3'-GMP (1.29×10^{-4} M) mixture − T_1. The difference curve for expt c, slightly displaced from that of expt b, is not shown. Buffer, sodium acetate, 0.02 M. (B) At pH 6. (– – –) T_1; expt a: T_1 (6.2×10^{-5} M) + 3'-GMP (6.4×10^{-5} M); expt b: T_1 (6.1×10^{-5} M) + 3'-GMP (1.28×10^{-5} M). (●) Difference curve for expt a, *i.e* , [T_1 (6.2×10^{-5} M) + 3'-GMP (6.4×10^{-5} M)] mixture − T_1. (○) Difference curve for expt b, *i.e.*, [T_1 (6.1×10^{-5} M) + 3'-GMP (1.28×10^{-4} M)] mixture − T_1. Sodium cacodylate buffer, 0.02 M. (C) Of the side-chain chromophore of T_1 ribonuclease at pH 7.5. T_1, 5.2×10^{-5} M; 3'-GMP, 5×10^{-5} M. (——) T_1; (· · · ·) T_1 + 3'-GMP; (○) difference curve [T_1 + 3'-GMP] mixture − T_1, NaOH adjusted.

TABLE II: Molar Ellipticity of the Induced Circular Dichroic Bands from T_1–3'-GMP Complex.[a]

	T_1 (M)	3'-GMP (M)	Complex (M)	% Sites Occupied	[Molar Ellipticity]$_{complex}$ (deg cm^2 (dmole^{-1})) 280 nm	250 nm
(a)	6.2×10^{-5}	6.2×10^{-5}	1.58×10^{-5}	26	−58,000	+100,000
(b)	6.1×10^{-5}	1.28×10^{-6}	2.34×10^{-4}	39	−35,000	+93,000
(c)	6×10^{-5}	1.9×10^{-4}	2.60×10^{-5}	43	−33,000	+84,000

[a] In these data the binding constant (5.8×10^3 M^{-1}) of H. Ruterjans, G. Schoene, and O. Pongs (personal communication) was used.

280 nm is about 30% and the intensity at 250 nm is about 60% of the circular dichroism bands and of the ribosyl compound, respectively, indicating the interaction of T_1–3′-dGMP is much weaker.

In the study of Campbell and Ts'o (1971), it was shown that the 9-GEtP and 9-GBuP, in which the pentose moiety has been replaced by a more flexible hydrocarbon bridge (9-(2′-hydroxyethyl) and 9-(4′-hydroxybutyl), respectively), have binding constants to T_1 at similar levels as 2′-GMP at pH 5.0. These compounds of high affinity to T_1 contain no asymmetric carbon atom and thus have no optical activity. The circular dichroism spectra of the T_1 (6.2×10^{-5} M)–9-GEtP (5×10^{-5} M) complex at pH 5.0 and the induced dichroic bands (the difference spectrum) are shown in Figure 8A. The induced bands are about one-third of those from the T_1–2′-GMP under similar conditions (curve a, Figure 6A) with the same maximum and minimum spectral positions. The circular dichroism spectra of the T_1 (5.4×10^{-5} M)–9-GBuP (5×10^{-5} M) complex at pH 5.9 and the induced dichroic bands are shown in Figure 8B. The magnitudes of the difference spectra in Figure 8A,B are quite comparable. The studies shown in Figure 8A,B provide four important results. (1) Optical activity can be induced from nonasymmetric compounds in the asymmetric binding sites; (2) the ribose moiety is not critical in the binding site; (3) the ribose moiety is not the source of optical activity; (4) the induced dichroic bands from these nonasymmetric compound–T_1 complexes have two approximately equivalent positive and negative regions, suggesting that the origin of the induced optical activity is from exciton splitting.

As for other nucleotides, 5′-GMP is a very weak inhibitor with a very low binding constant (Campbell and Ts'o, 1971). It showed no effect on the circular dichroism of T_1 at either pH 5 or 7 using concentrations comparable to those of the above experiments. Other nucleotide analogs of 3′-GMP, such as 3′-XMP in which the two amino group is replaced with a keto group, and 3′-IMP in which the 2-amino group has been removed, were also studied. No changes in the circular dichroism spectrum of T_1 ($\sim 5 \times 10^{-5}$ M) were observed with 3′-XMP ($\sim 2 \times 10^{-4}$ M) at either pH 4.7 or 6.0, on either side of the pK_a of the base (5.5). The ultraviolet absorption spectrum of this compound is shifted to the blue as compared to that of GMP, and if a similar shift in the circular dichroism is observed, then the origin of the induced circular dichroism bands could be identified. No effect was observed also with 2×10^{-4} M 3′-IMP and 4×10^{-5} M T_1.

Nature of the Induced Dichroic Bands in T_1–GMP (or Analogs) Complex. Nuclease–nucleotide complexes have been studied previously by optical rotatory dispersion and circular dichroism methods. Cathou *et al.* (1965) reported that the RNase A–2′-CMP (or 3′-CMP) complex at pH 5.5 was slightly more negative at 228 nm than calculated by addition of the rotation of the free enzyme and nucleotide. In addition, a weak positive Cotton effect of the enzyme at 278 nm was lost in the complex. They ascribed the observed changes to a slight alteration in protein structure and/or to interaction of nucleotide–protein chromophores. Samajima *et al.* (1969), in circular dichroism studies on thionucleotides, found that the RNase A–4-thiouridylic acid complex exhibits a different circular dichroism spectrum with a strong negative band around 330 nm and a weak positive band around 385 nm. They can identify the origin of these induced bands, which is from the chromophore of the nucleotide and not from the enzyme since the thionucleotide absorbs at that wavelength region while the enzyme does not. In view of the fact that the

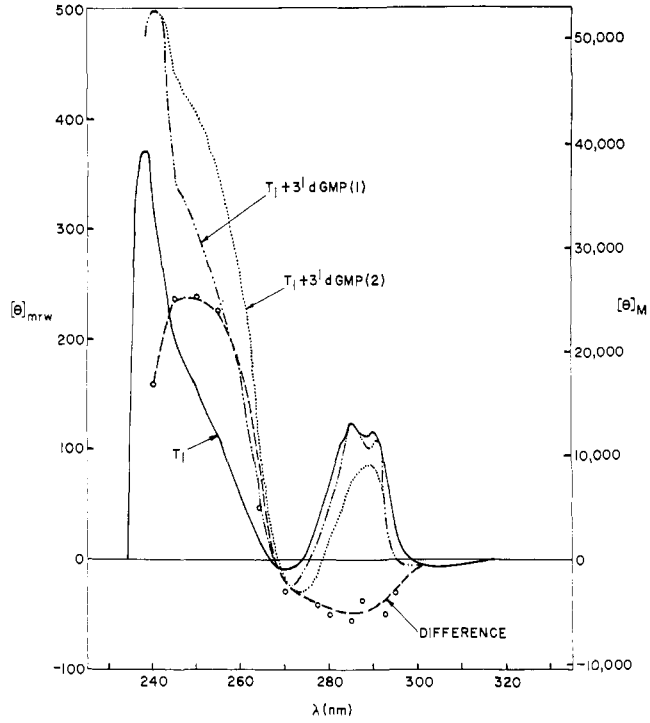

FIGURE 7: The effect of the inhibitor 3′-dGMP on the circular dichroism of side-chain chromophores of T_1 ribonuclease and its concentration dependence at pH 5. T_1, 6×10^{-5} M; 3′-dGMP (1) 5×10^{-5} M, (2) 1.25×10^{-4} M. (———) T_1; (· · · ·) T_1 + 3′-dGMP (2); (O) difference curve [T_1 + 3′-dGMP (2)] mixture − T_1; (—·—·) [T_1 + 3′-dGMP (1)]. The difference curve for the lower concentration (1) of 3′-dGMP is not shown. Sodium acetate 0.025 M, 23°.

circular dichroism spectrum of 4-thiouridylic acid in solution has a positive circular dichroism band at 330 nm (as well as at the 270-nm region), opposite to that of the observed induced negative band, these authors suggest "that the configuration of the nucleotide may alter significantly in the molecule of the complex. This gross change in configuration of the nucleotide may cause the alteration of rotation strength giving the opposite sign in optical activity" (Samajima *et al.*, 1969). The observation of the small induced positive circular dichroism band was left unexplained. Omenn *et al.* (1969) found strong positive dichroic bands induced in staphylococcal nuclease by its substrate dpTp in the 280-nm wavelength region. The enzyme alone has a negative band at this wavelength, while the substrate has a positive band. The difference circular dichroism spectrum could be accounted for by a ~80% decrease in the ellipticity of substrate. Concentration dependence of the induced bands gave a binding constant equivalent to that obtained by other methods. These authors suggest "that perturbation of the tyrosyl residues of nuclease contribute to the observed ellipticity difference, since 2 or 3 of them interact directly with the nucleotide" (Omenn *et al.*, 1969).

Substrate induced dichroic bands have been quantitized in other proteins. Thus, Chignell (1968, 1969a,b, 1970) has obtained binding constants from induced changes in the circular dichroism of serum albumins with aromatic drugs. Foster (1968) measured the binding parameters of proflavin to chymotrypsin from induced circular dichroism measurements. More recently R. L. Biltonen and M. Johnson (personal communication) also studied induced dichroic bands in the proflavin–chymotrypsin system. They obtained thermodynamic parameters, ΔG, ΔH, and ΔS, for the binding process.

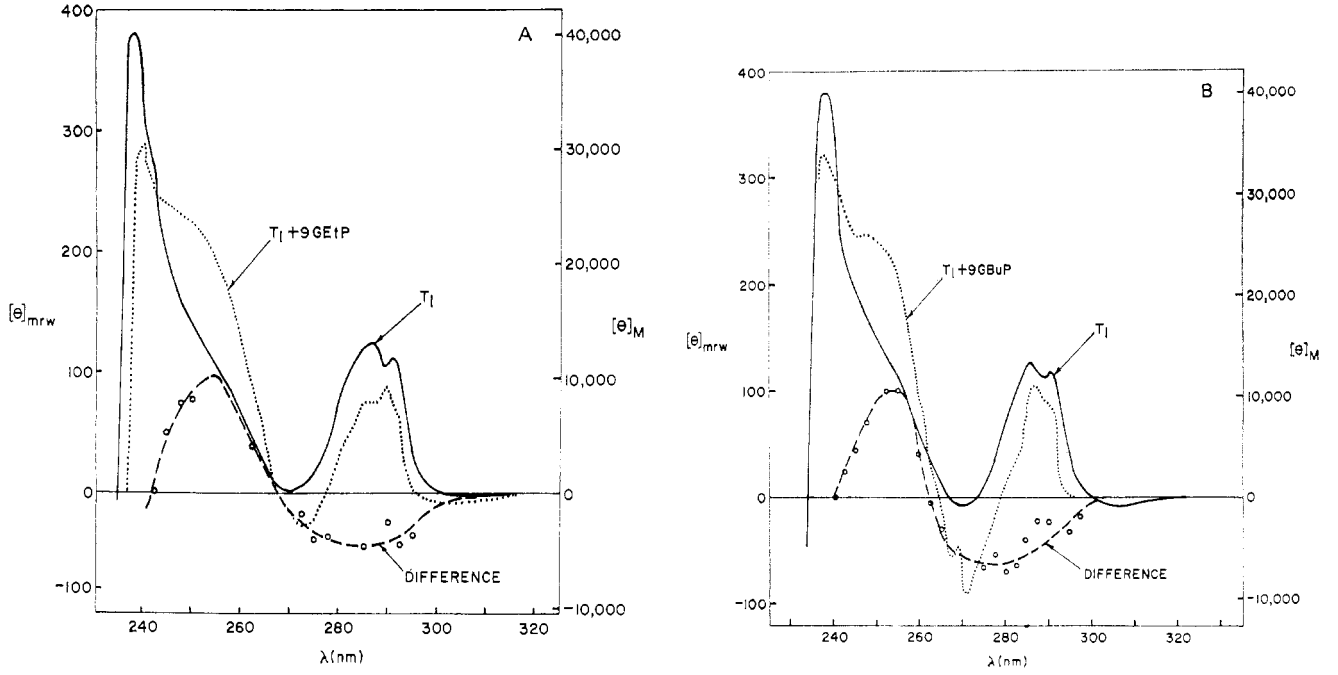

FIGURE 8: Inhibitor studies. (A) The effect of the inhibitor 9-GEtP on the circular dichroism of side-chain chromophores of T_1 ribonuclease at pH 5.1. T_1, 6.2×10^{-5} M; 9-GEtP, 5×10^{-5} M. (——) T_1; (. . . .) T_1 + 9 GEtP mixture; (○) T_1 + 9 GEtP mixture − T_1; 0.025 M sodium acetate buffer, 23°. (B) The effect of the inhibitor 9-GBuP on the circular dichroism side-chain chromophores of T_1 ribonuclease at pH 5.9. T_1, 5.4×10^{-5} M; 9-GBuP, 5×10^{-5} M. (——) T_1; (. . . .) T_1 + 9-GBuP mixture; (○) T_1 + 9-GBuP mixture − T_1; 23°.

Moreover, they correlated the thermodynamic parameters with the structural and rotational strength parameters.

Considerable effort was made to measure carefully the very weak circular dichroism spectrum of 2′-GMP at pH 5.6 and 7.2 as shown in Figure 9. There is a weak positive band between 275 and 280 nm and a negative band at ~250 nm. The weak positive band has been overlooked. However, in the data of Guschlbauer and Courtois (1968), there appears to be a weak inflection in both the circular dichroism and optical rotatory dispersion of guanosine at pH 7.0. Cantor *et al.* (1971) also observed a very small Cotton effect in the circular dichroism spectrum of dG at long wavelengths. The positive band in circular dichroism at 280 nm has its counterpart in the ultraviolet absorption spectrum of GMP, in which a fairly strong transition is observed near this wavelength.

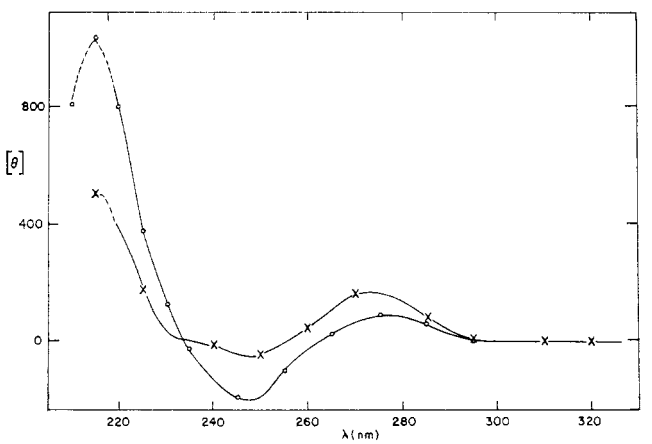

FIGURE 9: The circular dichroism spectrum for 2′-GMP at 20°. (×) 2′-GMP in H_2O at pH 7.2; (○) 2′-GMP in sodium acetate 0.02 M, pH 5.6. Concentration of GMP, 2.0×10^{-4} M.

The information available at present is not yet sufficient to determine the nature of the induced dichroic bands from the complex, since we are not certain whether the induced optical activity originates from the chromophores of the protein, or from the chromophore (guanine) of the nucleotides, or from the interaction of both. Any proposal would have to account for the following observations. (1) The molecular ellipticity of the induced bands from the complex is very high, *i.e.*, about 10^4–10^5 deg (cm^2 dmole^{-1}). (2) The induced spectra consist of one negative band at about 280 nm and one positive band at about 250 nm with the crossing-over at about 270 nm; the rotational strength of these two bands is similar, with the 280-nm band usually smaller. The ratio of the intensities (band area) of these two bands (I250 nm/I280 nm) varies from 1.3 to 4, mostly around 2. (3) The induced circular dichroism bands can be obtained from optically inactive compounds (containing no asymmetric carbon); thus, the presence of the asymmetric pentose moiety is not required.

The proposal we favor at present is that the induced bands arise from exciton coupling interaction between the guanine of the inhibitors and the aromatic chromophores of the protein, especially the tyrosines and trytophan in or near the active site. Exciton coupling by identical chromophores produces a splitting in energy and gives rise to two circular dichroism bands of opposite sign which are centered about the absorption maximum of the individual isolated chromophore. For exciton resonance to be observable, the oscillators must have intense absorption, be noncoplanar, and be sufficiently close together, since the energy separation of the exciton transitions is proportional to the cube of the reciprocal distance between the two dipoles. (For a recent discussion, see Schellman, 1968, and Urry, 1970.) Application of this theory has been successful in the understanding of the dinucleoside

monophosphates' optical rotatory dispersion spectra (Bush and Tinoco, 1967). In the present situation, if these induced bands originate from exciton coupling, the coupling may involve interactions between two dissimilar aromatic groups, a guanine base on one hand and one (or two) protein aromatic side chain on the other. It is unlikely that the induced bands are originated entirely from tyrosine–tyrosine interaction in the enzyme, since the circular dichroism of the helical poly-L-tyrosine has only very weak bands (10^2 deg (cm^2 dmole^{-1}) in the region of 280 and 250 nm, though the band at 280 nm is negative and that at 250 nm is positive, having the same sign as the induced circular dichroism bands (Damle, 1970; Shiraki and Imahori, 1966; Friedman and Ts'o, 1971).

Concluding Remarks

T_1 ribonuclease is an unusual enzyme. Its high tyrosine content ($^9/_{104}$) and the single trytophan make it a good choice for the comparative assignment of circular dichroism bands. Positive circular dichroism bands in the 240- and 285–290-nm regions can be assigned to tyrosine. Denaturation (unfolding) by heat and 8 M urea shows that the tyrosines are buried. Spectral shifts on denaturation by alkali show them to be un-ionized. The location of un-ionized bands at 240 nm suggests the possibility of exciton effects. The bands at ~235 and ~250 nm are less certain and are tentatively assigned to cystine residues. Negative bands at ~270 nm and possibly ~305 nm are even less known.

The far-ultraviolet circular dichroism spectrum shows T_1 to be a nonhelical protein. This does not mean that it is a random coil but that the level of organized conformation is low in terms of the α helical and β structures of model peptides and proteins.

The circular dichroism spectrum in the 240–310-nm region of a mixture of T_1 with 2′-GMP (or 3′-GMP, or certain of their analogs) differs significantly from the algebraic sum of the circular dichroism of T_1 and the circular dichroism of the nucleotide measured separately. Strong extrinsic Cotton effects are induced with positive and negative dichroic bands at 250 and 280 nm, respectively. The magnitudes of the circular dichroism maxima (10^4–10^5 deg (cm^2 dmole^{-1})), the symmetry of the transitions about the 270-nm wavelength, and the approximately equal rotational strength of the two transitions (the 280-nm band is usually smaller) of opposite sign suggest an exciton coupling effect. Moreover, the transitions remain invariant in wavelength, under varying pH and temperature and with a variety of inhibitor analogs.

A part of the active site has the interesting sequence: Pro^{55}-Tyr-Tyr-Glu-Trp-Pro^{60} (Takahashi *et al.*, 1967). Both the Glu^{58} and Trp^{59} have been implicated in the active site (Takahashi *et al.*, 1967; Terao and Ukita, 1969). The neighboring two tyrosines and the trytophan residue all provide a source of π electrons for interaction with the substrate. The effect of the histidines also implicated in the active site would also bring other residues in the vicinity of His^{27}, His^{92}, or His^{40} into close proximity with the 55–60 amino acid sequence. This may bring additional aromatic groups into the active site.

Exciton effects arise in the coupling of oscillators from different aromatic groups. The substrate-induced exciton effect in T_1 could arise from electronic interactions between the substrate purine group and the aromatic tyrosines and trytophan of the enzyme.

The formation of the T_1–nucleotide complex as shown by the induced circular dichroism bands has a qualitative relation with the binding constants obtained by gel filtration techniques. The inhibitors with high binding constants, $>10^3$ M^{-1}, all show induce dichroic bands. However, the magnitude of the induced bands per mole of complex calculated from the binding constant is not proportional to the binding constants. The relative intensities per mole of complex are 3′-GMP > 2′-GMP > 3′-dGMP > (9-GEtP, 9-GBuP).

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References

Beychok, S. (1966), *Science 154*, 1288.
Beychok, S., Armstrong, J. M., Lindblow, C., and Edsall, J. T. (1966), *J. Biol. Chem. 241*, 5150.
Beychok, S., and Breslow, E. (1968), *J. Biol. Chem. 243*, 151.
Beychok, S., and Fasman, G. D. (1964), *Biochemistry 3*, 1675.
Bush, C. A., and Tinoco, I. (1967), *J. Mol. Biol. 23*, 601.
Campbell, M., and Ts'o, P. O. P. (1971), *Biochim. Biophys. Acta* (in press).
Cantor, C. R., Warshaw, M. N., and Shapiro, H. (1971), Oligonucleotide Interactions III Biopolymers (in press).
Cathou, R. E., Hammes, G. G., and Schimmel, P. R. (1965), *Biochemistry 4*, 2687.
Chen, A. K., and Woody, R. W. (1969), 158th National Meeting of the American Chemical Society, New York, N. Y., Sept, Abstract BIOL 275.
Chignell, C. F. (1968), *Life Sci. 7*, 1181.
Chignell, C. F. (1969a), *Mol. Pharmacol. 5*, 244.
Chignell, C. F. (1969b), *Mol. Pharmacol. 5*, 455.
Chignell, C. F. (1970), *Mol. Pharmacol. 6*, 1.
Cohn, W. E., and Volkin, E. (1951), *Nature (London) 167*, 483.
Coleman, D. L., and Blout, L. R. (1967), *in* Conformation of Biopolymers, Vol. 1, Ramachandran, G. N., Ed., New York, N. Y., Academic Press.
Damle, V. N. (1970), *Biopolymers 9*, 937.
Donovan, J. W. (1968), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 27*, 337.
Donovan, J. W. (1969), *in* Physical Principles and Techniques of Protein Chemistry, Leach, S. J., Ed., New York, N. Y., Academic Press, pp 101–170.
Edelhoch, H., Lippoldt, R. E., and Wilchek, M. (1968), *J. Biol. Chem. 243*, 4799.
Egami, F., and Nakamura, K. (1969), *Mol. Biol. Biochem. Biophys. 6*, 19.
Egami, F., Takahashi, K., and Uchida, T. (1964), *Progr. Nucl. Acid Res. 3*, 59.
Foster, R. J. (1968), *Abstr. Fed. Proc. Amer. Soc. Exp. Biol.*, No. 3200.
Friedman, S., and Ts'o, P. O. P. (1971), *Biochem. Biophys. Res. Commun. 42*, 510.
Glazer, A. N., and Rosenheck, K. (1962), *J. Biol. Chem. 237*, 3674.
Glazer, A. N., and Smith, E. L. (1960), *J. Biol. Chem. 235*, PC 43.
Glazer, A. N., and Smith, E. L. (1961), *J. Biol. Chem. 236*, 2942.
Guschlbauer, W., and Courtois, Y. (1968), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 1*, 183.

Holzwarth, G., and Doty, P. (1965), *J. Amer. Chem. Soc. 87*, 218.
Horwitz, J., Strickland, E. H., and Billups, C. (1970), *J. Amer. Chem. Soc. 92*, 2119.
Iida, S., and Ooi, T. (1969), *Biochemistry 8*, 3897.
Ikeda, K., Hamaguchi, K., Imanishi, M., and Amano, T. (1967), *J. Biochem. (Tokyo) 62*, 315.
Irie, H. (1968), *J. Biochem. (Tokyo) 63*, 649.
Jirgensons, B. (1969), *Mol. Biol. Biochem. Biophys. 5*, 82.
Jirgensons, B. (1970), *Biochim. Biophys. Acta 200*, 9.
Mora, S. and Elodi, P. (1968), *Eur. J. Biochem. 5*, 574.
Moscowitz, A., Rosenberg, A., and Hansen, A. E. (1965), *J. Amer. Chem. Soc. 87*, 1814.
Omenn, G., Cuatrecasas, P., and Anfinsen, C. (1969), *Proc. Nat. Acad Sci. U. S. 64*, 923.
Pflumm, M. N., and Beychok, S. (1969), *J. Biol. Chem. 244*, 3982.
Pongs, O. (1970a), *Biochem. Biophys. Res. Commun. 38*, 431.
Pongs, O. (1970b), *Biochemistry 9*, 2316.
Riddiford, L. M., Stellwagen, R. H., Mehta, S., and Edsall, J. T. (1965), *J. Biol. Chem. 240*, 3305.
Ruterjans, H., Witzel, H., and Pongs, O. (1969), *Biochem. Biophys. Res. Commun. 37*, 247.
Samejima, T., Kita, M., Saneyoshi, M., and Sawada, F. (1969), *Biochim. Biophys. Acta 179*, 1.
Sarker, P. K., and Doty, P. (1966), *Biochemistry 5*, 981.
Sato, K., and Egami, F. (1957), *J. Biochem. (Tokyo) 44*, 753.
Sato, S., and Egami, F. (1965), *Biochem. Z. 342*, 437.
Sato-Asano, K., and Egami, F. (1960), *Nature (London) 185*, 462.
Schellman, J. A. (1968), *Accounts Chem. Res. 1*, 144.
Shiraki, M., and Imahori, K. (1966), *Sci. Pap. Coll. Gen. Educ., Univ. Tokyo 16*, 215.
Simmons, N. S., and Glazer, A. N. (1967), *J. Amer. Chem. Soc. 89*, 5040.
Simons, E. R., and Blout, E. R. (1968), *J. Biol. Chem. 253*, 218.
Strickland, H., Horwitz, J., and Billups, C. (1969), *Biochemistry 8*, 3205.
Takahashi, K. (1965), *J. Biol. Chem. 240*, 4117.
Takahashi, K. (1966), *J. Biochem. (Tokyo) 60*, 239.
Takahashi, K., Stein, W. H., and Moore, S. (1967), *J. Biol. Chem. 242*, 4682.
Takahashi, K., Uchida, T., and Egami, F. (1970), *Advan. Biophys. 1*, 53.
Tamburro, A. M., Scatturin, A., and Moroder, L. (1968), *Biochim. Biophys. Acta 154*, 154.
Tanford, C., Hauenstein, J. D., and Runds, D. G. (1955), *J. Amer. Chem. Soc. 77*, 6409.
Terao, T., and Ukita, T. (1969), *Biochim. Biophys. Acta 181*, 347.
Townend, R., Kumosinski, T. F., Timasheff, S. N., Fasman, G. D., and Davidson, B. (1966), *Biochem. Biophys. Res. Commun. 23*, 163.
Uchida, T. J. (1965), *Biochemistry 4*, 547.
Urry, D. W. (1970), *in* Spectroscopic Approaches to Biomolecular Configuration, Chicago, Ill., American Medical Association.
Yamamoto, Y., and Tanaka, J. (1970), *Biochim. Biophys. Acta 207*, 522.

Alterations in Specific Transfer Ribonucleic Acids in a Spectrum of Hepatomas*

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ABSTRACT: Tyrosyl-, histidyl-, asparaginyl-, and phenylalanyl-tRNAs from a spectrum of Morris hepatomas were compared to the same respective aminoacyl-tRNAs from normal rat liver by means of methylated albumin kieselguhr column chromatography. The tyrosyl-, histidyl-, and asparaginyl-tRNAs from hepatoma 3924A had altered elution profiles similar to those found earlier in Novikoff hepatoma. Hepatomas 9121, 9098, 3683F, and 5123C had altered phenylalanyl-tRNA profiles. Codon recognition properties of tyrosyl-tRNAs from Novikoff hepatoma, hepatomas 3924A and 9098, and normal rat liver were also examined. In the case of Novikoff hepatoma, the tyrosyl-tRNA was further fractionated on a DEAE-Sephadex A-50 column. All of the tyrosyl-tRNAs recognized the normal tyrosine codons UAU and UAC; the response to UAU was always greater than the response to UAC. None of the tyrosyl-tRNAs were bound by the chain terminator codons UAG or UAA. The biological and functional significance of these findings are discussed.

A previous study of the tRNA obtained from the Novikoff hepatoma demonstrated pronounced differences in the MAK[1] column elution profiles of tyrosyl-, histidyl-, and asparaginyl-tRNAs, when compared to the corresponding aminoacyl-tRNAs from control rat liver (Baliga *et al.*, 1969). The appearance of new species of isoaccepting tRNAs and of

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[1] Abbreviation used is: MAK, methylated albumin kieselguhr.