Analysis of the Vibrational Structure in the Near-Ultraviolet Circular Dichroism and Absorption Spectra of Tyrosine Derivatives and Ribonuclease-A at 77°K[1]

Joseph Horwitz, E. Hardin Strickland, and Carolyn Billups

Contribution from the Laboratory of Nuclear Medicine and Radiation Biology, University of California, Los Angeles, California 90024.
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Abstract: To identify the tyrosyl circular dichroism (CD) bands in ribonuclease and to estimate their contribution to the rotatory strength, the near-ultraviolet CD and absorption spectra of model compounds have been analyzed. L-Tyrosine, L-tyrosine ethyl ester, N-acetyl-L-tyrosine ethyl ester, and N-acetyl-L-tyrosine amide were studied at 298 and 77°K. When these compounds are cooled to 77°K, a number of vibronic transitions become well resolved. The 0–0 transition starts an intense progression with 800-cm^{-1} spacing to shorter wavelengths. A much weaker progression involves a 1250-cm^{-1} spacing. These vibronic transitions were identified by using *p*-cresol as a model for the phenolic ring of tyrosine and assuming an effective local symmetry of C_{2v}. Only totally symmetrical vibrations are observed in the tyrosine CD and absorption spectra. The vibronic CD bands have the same sign and occur at the same wavelengths as the corresponding absorption bands. The position of the 0–0 transition varies between 282 and 289 nm according to the solvent, but the vibrational spacing remains fixed. This sensitivity to solvent may lead to an apparent mismatch between the wavelength positions of CD and absorption bands for tyrosine dissolved in certain mixed solvents. When ribonuclease-A is cooled to 77°K, the near-ultraviolet CD and absorption spectra reveal much fine structure belonging to the tyrosine residues. Three types of tyrosyl side chains can be identified in the 77°K absorption spectrum of ribonuclease-A: (a) two tyrosine residues have their 0–0 transitions at 286 nm, (b) one tyrosine residue has its 0–0 transition at 289 nm, and (c) three tyrosine residues have their 0–0 transitions at 283 nm. The spectral position of the latter band indicates that only these three residues are exposed to the water–glycerol solvent. Ribonuclease-A has strong CD bands arising from both tyrosine and cystine residues. The existence of a disulfide band is revealed by the gradually increasing CD between 310 and 295 nm in the 77°K spectrum. The CD contributions of tyrosine and cystine residues can be estimated by applying the vibrational analysis worked out for tyrosine model compounds. Between 275 and 310 nm about 40–50% of the CD strength is attributable to disulfide, 45–35% to the three tyrosine residues in a water–glycerol environment (exposed), and 20–15% to the tyrosine residues in a nonaqueous environment (buried). These studies indicate that the tyrosyl CD bands of many proteins may be identified on the basis of the fine structure occurring between 275 and 290 nm.

In principle, the circular dichroism (CD) transitions of tyrosine in the near ultraviolet should provide information about the tertiary structure of proteins in solution.[2] Identifying the tyrosyl CD bands of proteins, however, has been difficult, because tryptophan, phenylalanine, and cystine residues also have bands in the same wavelength range. In some proteins, the tryptophanyl[3] and phenylalanyl[4] CD bands may be identified by their characteristic fine structure, which is especially evident at 77°K. A similar approach may be possible with tyrosine, since some fine structure has been reported in the CD spectra of tyrosyl diketopiperazines at 298°K.[5]

Even in proteins lacking tryptophan, the tyrosyl CD intensities have not been accurately assessed owing to the ambiguity in the disulfide contribution.[6] For example, the 275-nm CD band in ribonuclease-A (RNase-A) has been attributed mostly to the tyrosine residues, although the amount of CD from the disulfide transitions has not been determined.[7] Furthermore, chemical modification, pH, and solvent studies of RNase-A have given conflicting results as to whether the buried or exposed tyrosyl side chains generate the observed CD band.[7–10]

This article describes the near-ultraviolet CD and absorption spectra of tyrosine, three of its derivatives, and RNase-A. High-resolution CD and absorption spectra were obtained by working at 77°K. The fine structure revealed by tyrosine and its derivatives at 77°K is analyzed in terms of the vibronic transitions of *p*-cresol. By using this information, the CD and absorption spectra of RNase-A can be analyzed to determine the relative contributions from the various types of tyrosine residues. In addition, this analysis permits assessing the disulfide CD intensity in RNase-A between 270 and 310 nm.

Experimental Section

Instrumentation. CD spectra were recorded on a highly modified Beckman CD spectrophotometer.[8] Reliable resolution of CD fine structure was obtained by using a computer of average transients.[11] Spectra were scanned repetitively at 0.3 nm/sec using

(1) This work was supported by Contract AT (04-1) GEN-12 between the Atomic Energy Commission and the University of California, and was taken in part from the Ph.D. thesis of J. H, U.C.L.A., 1970.
(2) S. Beychok in "Poly-L-Amino Acids," G. D. Fasman, Ed., Marcel Dekker, New York, N. Y., 1967, p 293.
(3) E. H. Strickland, J. Horwitz, and C. Billups, *Biochemistry*, **8**, 3205 (1969).
(4) J. Horwitz, E. H. Strickland, and C. Billups, *J. Amer. Chem. Soc.*, **91**, 184 (1969).
(5) H. Edelhoch, R. E. Lippoldt, and M. Wilchek, *J. Biol. Chem.*, **243**, 4799 (1968).
(6) S. Beychock, *Ann. Rev. Biochem.*, **37**, 437 (1968).
(7) M. N. Pflumm and S. Beychok, *J. Biol. Chem.*, **244**, 3973 (1969).
(8) R. T. Simpson and B. L. Vallee, *Biochemistry*, **5**, 2531 (1966).
(9) G. H. Beaven and W. B. Gratzer, *Biochim. Biophys. Acta*, **168**, 456 (1968).
(10) N. S. Simmons and A. N. Glazer, *J. Amer. Chem. Soc.*, **89**, 5040 (1967).
(11) J. Horwitz, E. H. Strickland, and E. Kay, *Anal. Biochem.*, **23**, 363 (1968).

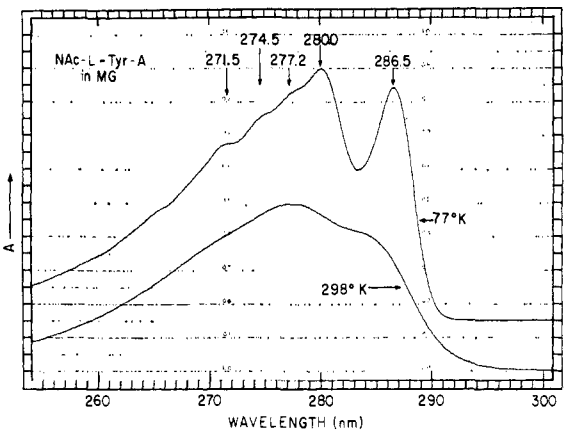

Figure 1. Instrument trace of the absorption spectra of 35 mM NAc-L-Tyr A in methanol–glycerol (9:1, v:v) at 298 and 77°K; path length, 0.10 mm. Base line for 77°K spectrum was offset 0.15 unit to separate the spectra. Both solvent base lines were flat.

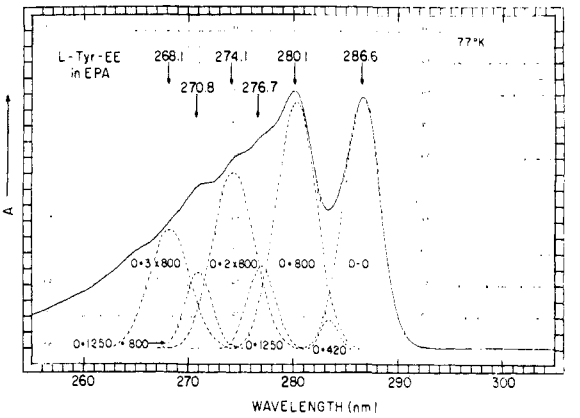

Figure 2. Instrument trace of the absorption spectrum of 24 mM L-Tyr EE in EPA at 77°K; path length, 0.20 mm. The dashed lines under the spectrum indicate the Gaussian bands obtained from the curve resolver. Arrows indicate the positions of the Gaussian bands.

either a 0.3- or a 1.0-sec time constant. For the very sharp bands at low temperature a 1.0-sec time constant was found to be too long, introducing an error of approximately 0.5 nm in the locations of the bands. With a 0.3-sec time constant no tracking error was introduced. Where accurate location of sharp bands was necessary, a 0.3-sec time constant was used. Spectral half-intensity band widths were less than 1.8 nm.

As a matter of convenience, some CD and absorption spectra were represented by a number of Gaussian bands placed in accordance with the vibronic transitions which can be reliably identified by independent spectroscopic experiments (see Appendix). The band widths and intensities of these bands were obtained using the du Pont 310 curve resolver. The correctness of the Gaussian shape for vibrational fine structure of tyrosine was tested by curve fitting the well-resolved 0–0 transitions in the absorption and CD spectra of tyrosine derivatives at 77°K. Gaussian bands gave a good fit, whereas Lorentzian bands did not.

The techniques for low-temperature CD and absorption measurements have been described previously.[3,4] Four solvent systems were used in the present study: EPA (ethyl ether–isopentane–ethanol, 5:5:2, v:v:v), THF–D (tetrahydrofuran–diglyme, 4:1, v:v), M–G (methanol–glycerol, 9:1, v:v), and W–G (water–glycerol, 1:1, v:v). As a demonstration that our low-temperature CD fine structure bands are not artifacts, the base lines for small CD signals were recorded using a nonoptically active sample having absorbance identical with that of the optically active sample.[3] *No artifacts were observed under the conditions used to record the CD spectra presented here.* In addition these base lines give a direct measure of the instrument noise for each wavelength range.

Materials. L-Tyrosine (L-Tyr) was obtained from Sigma Chemical Co., St. Louis, Mo. N-Acetyl-L-tyrosine ethyl ester (NAc-L-Tyr EE), N-acetyl-D-tyrosine ethyl ester (NAc-D-Tyr EE), and N-acetyl-D,L-tyrosine ethyl ester (NAc-D,L-Tyr EE) were from Cyclo Chemical Co., Los Angeles, Calif. N-Acetyl-L-tyrosine amide (NAc-L-Tyr A) was obtained from both Sigma and Cyclo. L-Tyrosine ethyl ester HCl (L-Tyr EE) was obtained from Mann Research Lab., Inc., New York, N. Y. All solvents were spectro quality except tetrahydrofuran, which was chromato quality from Matheson Coleman and Bell.

Bovine pancreatic RNase-A was obtained from Worthington Biochemical Corp., Freehold, N. J. Two different types were used: RAF, which according to the manufacturer may contain some aggregates, and RASE, which is reported to be free of aggregates. The latter preparation was exhaustively dialyzed against 50 mM sodium phosphate (pH 7) to remove the phenol preservative. Both types of RNase-A gave identical CD and absorption spectra. For experiments at 77°K, RNase-A was dissolved in W–G with 25 mM sodium phosphate, pH 7. Within our experimental error, the RNase-A CD spectra obeyed Beer's law over the concentration range which could be examined at 77°K (0.6–3.6 mM). These measurements were made using a short path length (0.2 mm) to avoid depolarization artifacts.[3] The CD and absorption spectra of RNase-A dissolved in either W–G or an aqueous solution at pH 7 were identical at 298°K. Thus the presence of 50% glycerol did not appear to alter the CD contributions of the residues giving rise to the near-ultraviolet bands of RNase-A.

The concentrations of tyrosine derivatives were determined spectrophotometrically using the following values: at 298°K, ϵ_{max} 1430 M^{-1} cm^{-1}; at 77°K for L-Tyr EE and NAc-L-Tyr EE in EPA, ϵ_{280} 1700 M^{-1} cm^{-1}. The latter value was obtained by assuming a constant oscillator strength for all solvents. The calculated concentrations of tyrosine derivatives in the organic solvents may be up to 30% off, since the oscillator strength may not be constant in all solvents.[12] The concentrations of RNase-A were determined at 278 nm (ϵ 9800 M^{-1} cm^{-1}).[13]

Results

Absorption Spectra of Tyrosine Derivatives. The increased resolution achieved at low temperature can be seen in Figure 1, which shows the absorption spectra of NAc-L-Tyr A in M–G at 298 and 77°K. The 298°K spectrum has a shoulder near 286 nm and a broad peak at 278 nm. The vibronic transitions underlying the 298°K spectrum were brought out by cooling. At 77°K well-resolved bands appear at 286.5 and 280.0 nm, and shoulders can be seen at 277.2, 274.5, and 271.5 nm.

The absorption spectra of NAc-L-Tyr EE and L-Tyr EE in nonaqueous solvents at 77°K are identical with the curve of NAc-L-Tyr A in M–G at 77°K, except for a small constant shift in the wavelength positions (see below). The assignments of the vibronic transitions common to all tyrosine derivatives are given for the 77°K spectrum of L-Tyr EE in EPA (Figure 2). Two progressions are seen. The strongest one is obtained by adding multiples of 800 cm^{-1} to the 0–0 band. A much weaker progression involves a 1250-cm^{-1} spacing. In addition to the obvious fine structure, a small contribution from a 0 + 420 cm^{-1} band is needed to fill up the intensity in the region between the 0–0 and 0 + 800 cm^{-1} band. The existence of the 0 + 420 cm^{-1} band is substantiated by its occurrence in the vapor spectrum of *p*-cresol (see Appendix).

The individual vibronic transitions are less well resolved in the spectrum of L-Tyr in W–G at 77°K (Figure 3) than was the case with the derivatives which could be examined in nonaqueous solvents. Nevertheless, the L-Tyr spectrum could be fit using a set of Gaussian bands having the same spacings as those de-

(12) J. E. Bailey, G. H. Beaven, D. A. Chignell, and W. B. Gratzer, *Eur. J. Biochem.*, 7, 6 (1968).
(13) D. B. Wetlaufer, *Advan. Protein Chem.*, 17, 303 (1962).

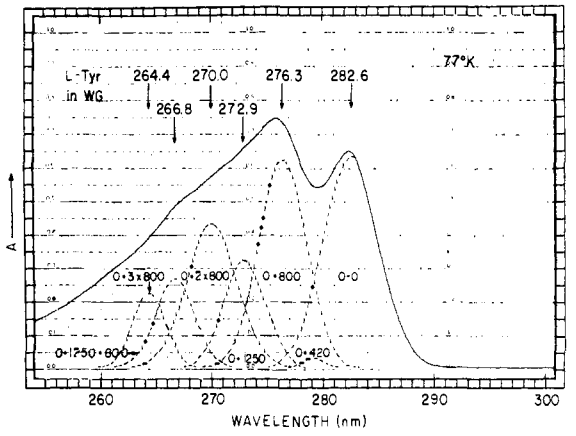

Figure 3. Instrument trace of the absorption spectrum of 32 m*M* L-Tyr in water–glycerol (1:1, v:v) at 77°K; pH 1.0; path length, 0.15 mm. Arrows indicate the positions of the Gaussian bands (dashed lines).

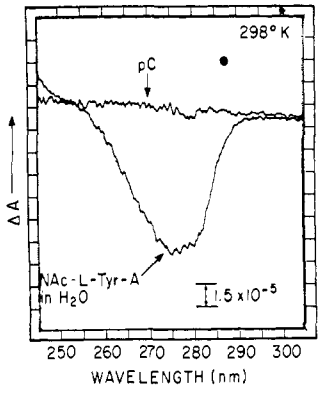

Figure 4. CD record of 0.66 m*M* NAc-L-Tyr A in neutral water at 298°K; path length, 10 mm; 20 scans; time constant, 1.0 sec. Base line (pC) is a *p*-cresol solution having the same absorbance as NAc-L-Tyr A.

scribed above. The blurring in the L-Tyr spectrum results because the band widths of the vibronic transitions are wider in W–G than those observed in nonaqueous solvents.

An addition effect of W–G is to shift the position of the 0–0 transition. For L-Tyr in W–G the 0–0 band occurs at 282.6, whereas for tyrosine derivatives in nonaqueous solvents the 0–0 bands are at higher wavelengths (Table I).

Table I. Effect of Solvent on the Position of the 0–0 Transition of Tyrosine Derivatives at 77°K

Compd	Solvent	0–0 position, nm
NAc-L-Tyr EE	THF–D	287.8
NAc-L-Tyr EE	EPA	287.2
L-Tyr EE	EPA	286.6
NAc-L-Tyr A	M–G	286.5
L-Tyr	W–G	282.6

CD Spectra of Tyrosine Derivatives. NAc-L-Tyr A in water has a CD spectrum whose shape is typical of many tyrosine derivatives at room temperature. This CD spectrum has a negative peak at 275 nm and a strong shoulder at 280 nm (Figure 4). The CD and

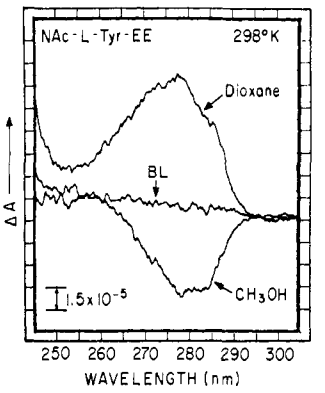

Figure 5. CD records of 0.60 m*M* NAc-L-Tyr EE at 298°K in dioxane (upper curve) and in methanol (lower curve); path length, 10 mm; 20 scans; time constant, 1.0 sec. The base line (BL) was NAc-D,L-Tyr EE having the same absorbance as NAc-L-Tyr EE.

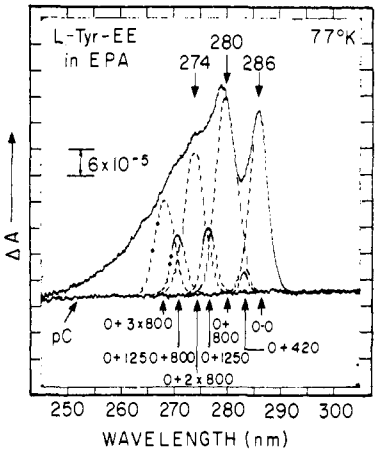

Figure 6. CD record of 24 m*M* L-Tyr EE in EPA at 77°K. Dashed lines indicate the Gaussian bands obtained from the curve resolver. Arrows indicate the positions and assignments of the Gaussian bands; path length, 0.20 mm; 20 scans; time constant, 0.3 sec. The base line (pC) is a *p*-cresol solution having the same absorbance as L-Tyr EE. The absorption spectrum of this sample is shown in Figure 2. The area under the CD spectrum at 77°K is 4 times larger than that at 298°K (298°K spectrum not shown).

absorption spectra of this compound have similar shapes, but are not identical at 298°K. The sign of the CD spectrum differs among the various derivatives and may even be altered by the solvent. For example, NAc-L-Tyr EE has a positive CD band in dioxane and a negative band in methanol (Figure 5).

The increased resolution obtained at 77°K can be seen in the CD spectrum of L-Tyr EE in EPA (Figure 6). It has well-resolved bands at 286 and 280 nm and a shoulder at 274 nm. These bands correspond in wavelength position and relative intensity to the 0–0, 0 + 800 cm^{-1}, and 0 + 2 × 800 cm^{-1} transitions seen in the absorption spectrum of L-Tyr EE (Figure 2). The CD spectrum in Figure 6 can be fit by the same Gaussian bands which fit the absorption spectrum (after a proper scaling factor) without any changes in wavelength positions and with only minor changes (<10%) in the relative intensities of the bands. NAc-L-Tyr EE in THF–D and NAc-L-Tyr A in M–G at 77°K have CD spectra which are approximately the mirror image of the curve shown in Figure 6 for L-Tyr EE.

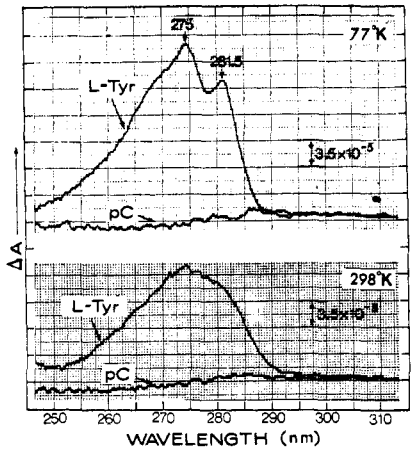

Figure 7. CD records of 32 mM L-Tyr in water–glycerol (1:1, v:v) at 77°K (upper curve) and at 298°K (lower curve); pH 1.0; path length, 0.15 mm; 20 scans; time constant, 1.0 sec. The absorption spectrum of this sample is shown in Figure 3. Labeled arrows indicate the accurate positions of the CD bands obtained using a 0.3-sec time constant. The base line (pC) is *p*-cresol. The area under the 77°K CD spectrum is 33% larger than that under the 298°K spectrum (from 250 to 295 nm). The areas of the corresponding absorption spectra agree to within 10%. Major division indicated by double-headed arrow is 3.5×10^{-5} ΔA.

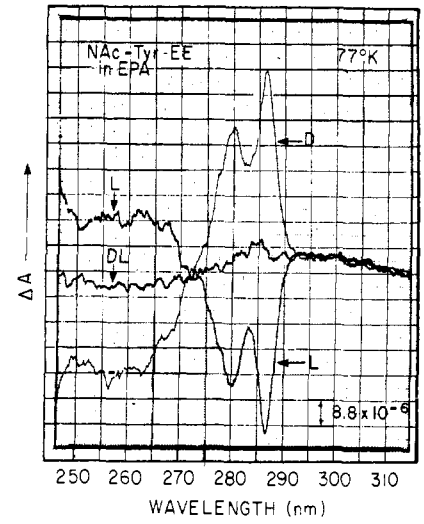

Figure 8. CD records of 32 mM NAc-L-Tyr EE and 32 mM NAc-D-Tyr EE in EPA at 77°K; path length, 0.15 mm; 40 scans; time constant, 1.0 sec. The base line (DL) is 32 mM NAc-D,L-Tyr EE. Major division indicated by double-headed arrow is 8.8 $\times 10^{-6}$ ΔA.

The CD spectrum of L-Tyr in W–G is also sharpened by cooling to 77°K (Figure 7). Just as in the absorption spectra, the individual vibronic transitions are less well resolved in the CD spectrum of L-Tyr in W–G at 77°K than is the case with CD spectra recorded in nonaqueous solvents. In addition, the CD bands of L-Tyr in W–G occur 1.0 nm toward shorter wavelengths than the corresponding absorption bands. The band widths and relative intensities of the resolved bands, however, are approximately the same in both the CD and absorption spectra.

A different type of CD spectrum is obtained for NAc-L-Tyr EE in EPA. At 298°K a low concentration of NAc-L-Tyr EE (0.6 mM) in EPA has a small positive CD band at 270 nm, which is 9 nm below the absorption maximum. This apparent mismatch between CD and absorption seems to result from solvation effects (see Discussion).

A high concentration of NAc-L-Tyr EE (32 mM) in EPA has a negative CD band which coincides with the absorption maximum at 279 nm. This change in the CD spectrum indicates that aggregation occurs at high concentrations. When the 32 mM NAc-L-Tyr EE is cooled to 77°K, another type of CD spectrum results (Figure 8). This spectrum is negative at long wavelengths and positive at short wavelengths, crossing the base line at 272 nm. Another unusual feature of this spectrum is that the 0–0 band is 40% more intense than the 0 + 800-cm^{-1} band. The mirror image CD spectrum was obtained for NAc-D-Tyr EE in EPA at 77°K (Figure 8). The 77°K absorption spectra of these aggregated derivatives are similar to the L-Tyr EE spectrum described previously (Figure 2).

Absorption Spectra of RNase-A. At 298°K the absorption spectrum of RNase-A has a broad maximum at 278 nm and a shoulder at 285 nm. Cooling to 77°K sharpens the spectrum sufficiently to reveal that these bands are actually the summation of several overlapping bands (Figure 9). At 77°K the broad shoulder at 285 nm is resolved into separate bands at 286 and 283.5 nm. The main peak at 278 nm is split into bands at 279.5 and 277 nm. In addition, shoulders can be seen at 274, 271, 268, 264, and 261.5 nm.

The fine structure in the RNase-A spectrum at 77°K can be used to determine which absorption bands arise from each amino acid residue. Only the tyrosine, phenylalanine, and cystine residues contribute to the near-ultraviolet spectrum of RNase-A.[13] The four cystine residues have a broad absorption band which is very weak (about 7% of the RNase-A absorbance at 280 nm).[13] Since disulfide model compounds do not show any fine structure between 250 and 310 nm even at 77°K,[14] the fine structure in the RNase-A spectrum must result from the tyrosine and phenylalanine residues.

From 250 to 268 nm phenylalanine residues have a number of sharp vibronic transitions,[4] whereas tyrosine has only poorly resolved transitions. Presumably the tyrosyl fine structure below 269 nm would not be very evident in a protein containing a number of overlapping bands from different types of tyrosine residues (see below). Apparently the three phenylalanine residues give rise to the shoulders observed at 268, 264, and 261.5 nm in RNase-A. Specifically these bands correspond to the 0–0, 0 + 520, and 0 + 930 cm^{-1} transitions of phenylalanine.[4]

The RNase-A absorption between 270 and 300 nm is dominated by the tyrosyl bands. Consequently, the fine structure in this region can be used to identify the vibronic transitions of the various tyrosine residues in RNase-A. A complete spectral analysis is begun by examining the long wavelength bands to identify the positions of the 0–0 transitions of the buried and exposed tyrosine residues. In addition to the strong bands observed at 286 and 283.5 nm, the mathematical analysis reveals a weaker band at 288.5 nm. When the top half of the first fine structure peak at

(14) No fine structure was found in the near-ultraviolet absorption spectra of the following disulfide compounds at 77 or 298°K: butyl disulfide in isopentane, L-cystine dimethyl ester diHCl in M–G, α-lipoic acid in M–G, and L-cystine in W–G (unpublished experiments).

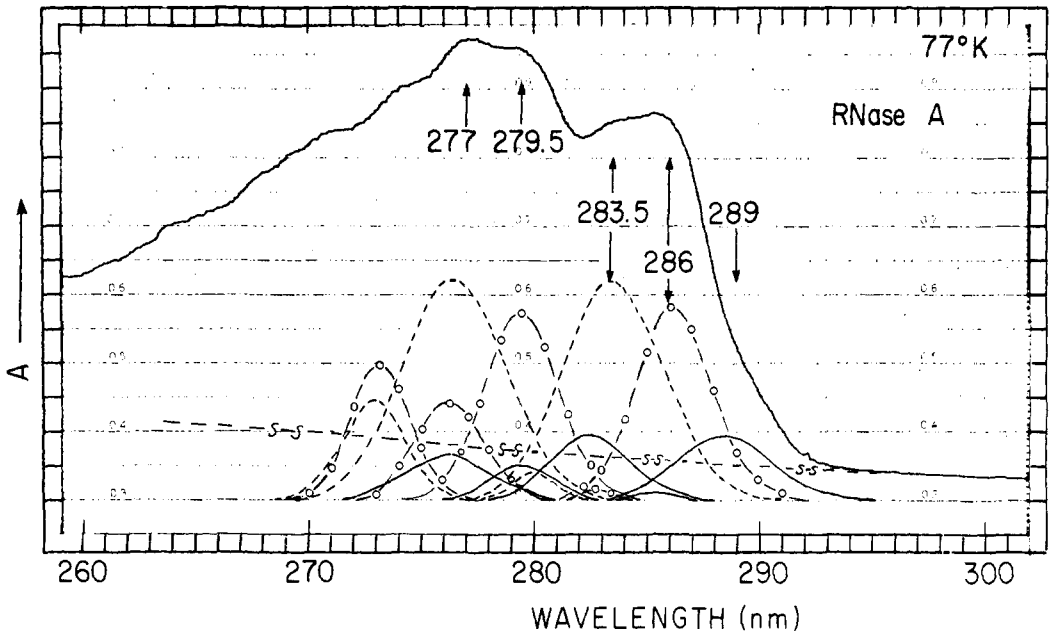

Figure 9. Instrument trace of the absorption spectrum of 2.8 m*M* RNase-A in water–glycerol (1:1, v:v) with 25 m*M* sodium phosphate (pH 7) at 77°K. Individual tyrosine and disulfide bands are presented under the RNase-A spectrum: -S–S-, disulfide, three types of tyrosine residues are identified in terms of the positions of the 0–0 bands; ———, 288.5 nm; —O—O—, 286 nm; and ------, 283.5 nm. The absorption spectrum of each type of tyrosine residue is represented by the 0–0, 0 + 420, 0 + 800, 0 + 1250, and 0 + 2 × 800 cm^{-1} bands, except that the 0 + 2 × 800 cm^{-1} band was omitted from the spectrum having its 0–0 band at 283.5 nm. The remaining short-wavelength transitions were omitted in all cases. The area under the RNase-A spectrum at 77°K was the same as that at 298°K, within our experimental error of ±10%. The path length was 0.20 nm.

286 nm is fit with a single Gaussian band, the leading edge of the RNase-A spectrum between 289 and 293 nm is not fit by the lower part of the Gaussian band. An additional Gaussian band must be placed at 288.5 nm to broaden the leading edge; *e.g.*, compare the sharp rise of the 0–0 transition in tyrosine derivatives (Figures 2 and 3) with the leading edge of the RNase-A spectrum (Figure 9). The existence of the 288.5-nm band is verified by its occurrence in the CD spectrum (see below).

The three longest wavelength bands in RNase-A (288.5, 286, and 283.5 nm) cannot arise from a single type of tyrosine, since tyrosine model compounds do not have strong vibronic transitions closely spaced around the 0–0 transition. Thus these bands must be the 0–0 transitions of three different types of tyrosine, which will be designated types I, II, and III. Types I and II have their 0–0 transitions and band widths corresponding to those found for model compounds in nonaqueous solvents. Type III resembles the absorption spectrum of L-Tyr in water–glycerol (Table I). Having identified the 0–0 transitions of each of the three types, the remaining vibronic transitions of each type of tyrosine can be positioned so as to reconstruct the absorption spectrum found in the tyrosine model compounds. When all the bands are added, the observed RNase-A spectrum is generated (Figure 9).

The area under the curve of each of the three types of tyrosines can be used to estimate the contribution of each to the total intensity. The fractional areas of the three types are approximately: I, 1/6; II, 1/3; III, 1/2. Since RNase-A has six tyrosine residues, this means that one tyrosine residue has its 0–0 transition at 288.5 nm, two residues have their 0–0 bands at 286 nm, and three residues have their 0–0 bands at 283.5 nm.

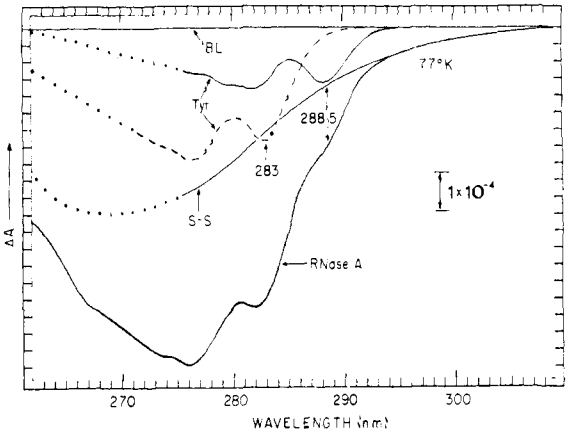

Figure 10. CD spectrum of 3.6 m*M* RNase-A in water–glycerol (1:1, v:v) with 25 m*M* sodium phosphate at 77°K. S–S designates disulfide. Dotted lines represent regions of extrapolation. The method of analysis is described in the text. Distribution of CD intensities is given in Table II; the exposed tyrosyl CD spectrum in this figure has $\Delta A_{277}/\Delta A_{283} = 1.2$; path length, 0.20 mm. Additional shoulders are present at 261 and 255 nm (not shown in figure). Division indicated by the double-headed arrow is $1 \times 10^{-4}\ \Delta A$.

CD Spectra of RNase-A. At 77°K the CD spectrum of RNase-A (Figure 10) has well-resolved bands at 282 and 276 nm and shoulders at 288.5, 267.5, 261, and 255 nm. By using both the data obtained from model compounds and the resolved absorption spectrum of RNase-A, the amino acid residues giving rise to the RNase-A CD bands can be identified. Both CD and absorption spectra of model compounds confirm that tyrosyl and phenylalanyl moieties have fine structure, whereas the disulfide moiety[14,15] does not. The CD

shoulders of RNase-A at 267.5, 261, and 255 nm correspond to phenylalanyl transitions.[4] The CD fine structure seen at 288.5, 282, and 276 nm arises from the tyrosine residues.

The RNase-A CD spectrum is more difficult to analyze than the absorption spectrum, because the disulfide transitions have a strong CD even though their absorption is weak. The disulfide CD band is clearly evident at long wavelengths. Since tyrosine model compounds at 77°K do not have CD at wavelengths longer than 295 nm, the RNase-A CD from 295 to 310 nm (Figure 10) must be the leading edge of a disulfide band. At 77°K disulfide model compounds having a dihedral angle of approximately 90° exhibit CD from about 320 nm.[15] The relatively strong disulfide CD from 295 to 310 nm implies that the disulfide CD contribution cannot be neglected at lower wavelengths. The disulfide CD contribution below 295 nm can be estimated only after the tyrosyl CD bands have been identified in RNase-A. The disulfide CD bands of RNase-A cannot be fit directly from disulfide model compounds, because the ratio of CD to absorption does not follow a consistent pattern throughout the disulfide band.[15] In fact, the disulfide CD band of L-cystine dimethyl ester diHCl in M–G even reverses sign near 280 nm.[15]

It will be shown in the Discussion that each tyrosyl side chain in RNase-A would be expected to have a CD spectrum whose shape corresponds approximately to that observed either for L-Tyr EE in EPA (Figure 6) or for L-Tyr in W–G (Figure 7). Consequently our analysis of the tyrosyl CD bands in RNase involves trying to place a CD band corresponding to each tyrosyl absorption band in RNase-A. In the analysis presented first (Figure 10), the ratio of CD to absorption is kept constant for all bands of a given type of tyrosine residue. The half-band width of each CD band should be the same as its absorption band. The total CD intensity may be different for each type of tyrosine. The shoulder at 288.5 nm fits the position of the 0–0 transition of type I tyrosine found in the RNase-A absorption spectrum. After the height of the 0–0 band is adjusted to fit the CD shoulder at 288.5 nm, the 0 + 800 cm^{-1} and the remaining short-wavelength bands must be placed to correspond with the rest of the type I tyrosine bands in the absorption spectrum (Figure 9). The type II tyrosines with their 0–0 band at 286 nm cannot contribute much to the CD, since no CD band is seen at 286 nm. The band at about 282 nm in the CD spectrum results from the type III tyrosines having their 0–0 transitions at 283 nm. Thus a Gaussian band is put at this position, and its intensity is adjusted to fit the observed spectra at 282 nm. Next the rest of the type III tyrosine bands are put according to the resolved absorption spectrum. When the CD is summed for all tyrosine residues, their total CD intensity is much too weak to fit the observed CD spectra at wavelengths below 282 nm. The RNase-A CD spectrum could be fitted using the tyrosine model compounds and the constant ratio of CD to absorption for each type of tyrosine residue only if the disulfide band is extended as proposed in Figure 10.

Using the procedure described above, the RNase-A CD spectrum at 77°K was analyzed from 310 to 276 nm. Only an approximate curve fitting was done below 276 nm, because too many uncertainties exist in determining the tyrosine and disulfide CD intensities at shorter wavelengths. The relative contributions of tyrosyl and disulfide CD bands were estimated by comparing their areas under the CD spectrum between 275 and 310 nm (Figure 10). About 50% of the RNase-A CD intensity resulted from the disulfides, 35% from the three exposed tyrosine residues, and 15% from the single buried tyrosine residue having its 0–0 band at 288.5 nm. The two tyrosine residues having their 0–0 bands at 286 nm contributed less than 5% and were neglected.

The band shapes used in Figure 10 for the exposed tyrosine residues lead to obtaining the maximal disulfide CD contribution. If the 0 + 800 cm^{-1} CD band of the exposed tyrosine residues was permitted to be more intense than the 0–0 band, then less disulfide CD was required to fill up the 276-nm CD band of RNase-A. Consequently, other analyses were carried out to determine the effects of altering the intensity distribution between the 0–0 and 0 + 800 cm^{-1} CD bands of the exposed tyrosine residues. As shown in Table II, the exact contributions of disulfide and tyrosine changed only slightly, even when the increase in the ratio of CD at the 0 + 800 cm^{-1} band to that at the 0–0 band was made relatively large in comparison to the values observed for tyrosine model compounds.

Table II. Distribution of CD Strength in RNase-A as a Function of the CD Shape Assumed for Exposed Tyrosine Residues

$\Delta A_{277}{}^{a}/\Delta A_{283}$	% of total RNase-A CD strength		
	Disulfide[b]	Exposed[c] Tyr	Buried[c,d] Tyr
1.4	37	47	16
1.3	47	38	15
1.2	50	35	15

[a] In the CD spectrum used to represent the exposed tyrosyl side chains, the ratio of ΔA at 277 nm to ΔA at 283 nm was used to measure the shape. [b] Determined from the area under the disulfide curve from 275 to 310 nm. [c] The total tyrosine contribution was obtained by subtracting the disulfide area from the total area. The distribution between the buried and exposed residues was obtained by comparing the areas obtained by adding the 0–0 and 0 + 800 cm^{-1} bands for each type of tyrosine residue. [d] Tyrosine residue having 0–0 band at 288.5 nm. There was no contribution from the other two buried tyrosine residues.

Attempts were also made to fit the exposed tyrosine contribution using a strong negative CD spectrum and a moderate positive CD spectrum with the 0–0 band offset to slightly longer wavelengths. This combination of bands made the 276-nm CD much more intense than the 282-nm CD. In addition, of course, the bands from the buried tyrosine residue had to be included to obtain the 288.5-nm shoulder. The RNase-A CD spectrum, however, could not be fit using two exposed tyrosyl species with opposite signs unless a large disulfide contribution was added to minimize the CD contribution from the exposed tyrosine residues. When large tyrosine CD bands were used, the resulting

(15) No fine structure was observed in the near-ultraviolet CD spectra of either L-cystine in W–G or L-cystine dimethyl ester diHCl in M–G at 77°K. The long-wavelength edge of these CD spectra appears to begin around 320 nm. These CD spectra at 77°K are similar to the ones recorded at 298°K (unpublished experiments).

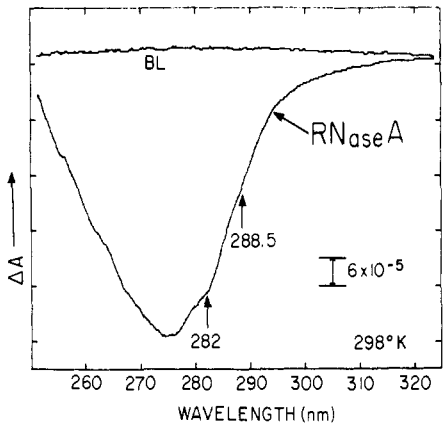

Figure 11. CD record of 72 μM RNase-A with 50 mM sodium phosphate buffer (pH 7) at 298°K; path length, 10 mm; 20 scans; time constant, 1.0 sec; BL indicates the base line. The existence of the band at 288.5 nm was verified in independent experiments.

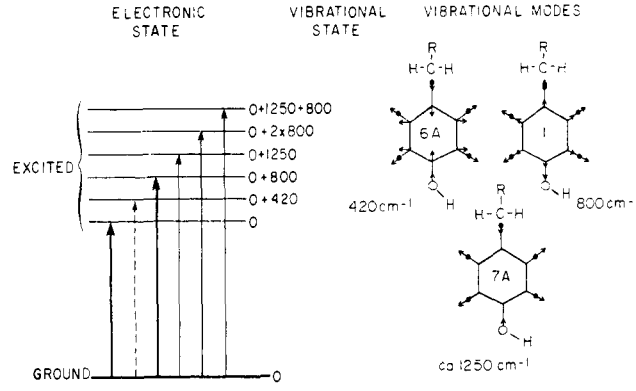

Figure 12. Transitions resolved in the CD and absorption spectra of tyrosine derivatives. The 0–0 and 0 + 800 cm^{-1} transitions are very intense. The 0 + 420 cm^{-1} transition is very weak. The 0 + 1250 and 0 + 1250 + 800 cm^{-1} bands may include several transitions in addition to the one shown (see Appendix).

CD spectrum had much sharper fine structure than was actually observed experimentally. Thus both the relative CD intensities at 276 and 282 nm and the extent of resolution of fine structure in RNase-A preclude a large tyrosine CD contribution.

In light of the above low-temperature analysis, a critical examination of RNase-A at room temperature is necessary. As expected, the CD bands in the 298°K spectrum are not as well resolved as those in the 77°K spectrum. Nevertheless, all bands identified at 77°K can be found in the 298°K CD spectrum of RNase-A. As shown in Figure 11, the 282-, 276-, 268-, 261-, and 255-nm CD bands are easily identified even at 298°K. The existence of the 288.5-nm CD band is indicated by a slight bulge which can be seen by sighting along the trace between 295 and 285 nm. Area measurements demonstrated that total near-ultraviolet CD intensity of RNase-A is approximately the same at both 298 and 77°K (less than 15% intensification upon cooling).

The longer wavelength part of the disulfide contribution is also clearly evident in the room-temperature CD spectrum of RNase-A. Even at 298°K tyrosine does not have measurable CD or absorption at wavelengths above 300 nm; *e.g.*, examine the leading edge of the spectra of tyrosine model compounds (Figures 1, 4, 5, and 7). Thus the long-wavelength tail extending out to 320 nm in the RNase-A CD spectrum (Figure 11) must be a disulfide band.

Discussion

An analysis of the vibrational structure in the absorption spectrum of tyrosine and its derivatives permits understanding the various CD spectra observed for compounds containing tyrosine. Cooling these compounds to 77°K reveals much fine structure. The absorption spectra of tyrosine and all its low molecular weight derivatives possess identical spacings among the fine structure bands. Different solvents, however, shift the 0–0 band between 282 and 288 nm, as has been discussed by other investigators.[16] Over 85% of the total absorption results from the progression obtained by adding multiples of 800 cm^{-1} to the 0–0 band.

(16) D. A. Chignell and W. B. Gratzer, *J. Phys. Chem.*, 72, 2934 (1968), and references cited therein.

The vibronic transitions of tyrosine in the near-ultraviolet region can be identified by using *p*-cresol as a model for the phenolic ring (see Appendix). Figure 12 illustrates the excited state vibrational modes involved in the principal vibronic transitions of tyrosine. All the observed vibrational modes are totally symmetrical. In principle, nontotally symmetrical vibrations may also occur, because the effective local symmetry of tyrosine is C_{2v}. The transitions involving these vibrations, however, are too weak to be identified in the tyrosine absorption spectra (see Appendix).

The vibronic transitions are best resolved for tyrosine derivatives which dissolve in nonaqueous solvents. The water–glycerol solvent widens the band widths of each transition. One major contributor to the broadening of vibronic bands is the random arrangement of solvent molecules around the solute.[17] Unlike a crystal where each absorbing molecule occupies an identical site, glasses and solutions contain multiple sites having different interaction energies between the solute and solvent, thereby broadening the spectrum. In polar solvents the broadening is enhanced by the stronger interaction between solute and solvent.

The information gained from the 77°K absorption spectra facilitates interpreting the CD spectra. Each vibronic transition in an optically active molecule possesses both an absorption intensity and a CD intensity. Thus each fine-structure absorption band gives the position of a possible CD band, and conversely each fine-structure CD band indicates the location of an absorption band. The correspondence between CD and absorption exists only for each vibronic transition. Therefore, if the vibrational fine structure is either not resolved or is ignored, an apparent mismatch may occur between CD and absorption bands.

The 77°K spectra of L-Tyr EE in EPA, NAc-L-Tyr A in M–G, and NAc-L-Tyr EE in THF–D illustrate the ideal behavior expected as to the correspondence in wavelength between CD and absorption. Each vibronic CD transition fits an observed absorption band (within 0.5 nm). Furthermore, the ratio of

(17) (a) R. M. Hochstrasser, *Accounts Chem. Res.*, 1, 266 (1968); (b) H. H. Jaffé and M. Orchin in "Theory and Application of Ultraviolet Spectroscopy," John Wiley & Sons, Inc., New York, N. Y., 1962, p 189.

CD to absorption intensity is nearly constant throughout the spectrum (<10% variation).

These ideal tyrosine CD spectra may be placed in perspective by comparing them with the CD spectra of phenylalanine containing compounds, which also have an effective local symmetry of C_{2v}. In phenylalanine some vibronic transitions containing a single nontotally symmetrical vibration have CD and absorption intensities which are almost as intense as the bands involving only totally symmetrical vibrations.[4] In tyrosine, owing to a large increase in the intensities of the 0–0 band and the transitions involving only totally symmetrical vibrations, the transitions containing a nontotally symmetrical vibration are too weak to be identified.

Next we shall compare the sign properties of the CD transitions of phenylalanine and tyrosine derivatives. The CD spectrum of a phenylalanine compound may have both positive and negative CD bands. In tyrosine compounds, however, all vibronic transitions always have the same sign—either all positive or all negative. The shape of a tyrosine CD spectrum is approximately identical with that of its absorption spectrum, aside from a proportionality factor.

The nontotally symmetrical vibrations are responsible for the mixed signs in the phenylalanine CD spectra.[4] When the transitions involving nontotally symmetrical vibrations are either absent or very weak, as in the case with tyrosine, no mixing of CD signs should occur, and the CD intensity should be approximately proportional to the absorption intensity at all wavelengths. Even very weakly occurring nontotally symmetrical vibrations, however, may cause minor deviations in the proportionality between CD and absorption intensity. This behavior is in accord with Weigang's theoretical description of vibrational structuring in CD spectra.[18]

Finally we shall compare the rotatory strengths of tyrosine and phenylalanine compounds. Some difficulties arise in making this comparison, because solutions of these molecules appear to be equilibrium mixtures of many conformers. The CD intensities of some phenylalanine derivatives increase by as much as 8-fold without any change in absorption intensity, when the temperature is lowered from 298 to 77°K.[4] This suggests that the room-temperature CD spectrum represents a weighted average of different conformations. For some, the CD bands of a given vibronic transition may be positive; for others, the same CD bands may be negative. Thus at 298°K the CD strength for some conformations cancels that of others, resulting in an apparent low intensity. When the temperature is lowered, the minimum energy conformation(s) becomes more heavily populated, in accordance with the Boltzmann factor. The average CD intensity increases because a much larger fraction of the molecules have the same CD spectrum. A similar increase in the CD intensity is observed also with some tyrosine derivatives upon lowering the temperature (see legend of Figure 6). Therefore, to obtain a comparison of the intrinsic rotatory strengths of tyrosine and phenylalanine derivatives, the values obtained at 77°K appear to be the most meaningful.

(18) O. E. Weigang, *J. Chem. Phys.*, 43, 3609 (1965).

Furthermore, since the rotatory strengths vary greatly among different derivatives, it seems most reasonable to compare the tyrosine and phenylalanine compounds having the largest rotatory strengths. This comparison (Table III) suggests that the intrinsic rotatory strength of tyrosine is about 8 times larger than that of phenylalanine in the near ultraviolet. This increase in CD intensity matches the increase in the dipole strength of tyrosine relative to that of phenylalanine (Table III). Thus the recent data on tyrosine and phenylalanine derivatives suggest that the increase in the intrinsic rotatory strength of tyrosine *may* result from the increase in the electric dipole transition moment instead of a change in the magnetic dipole transition moment, as previously suggested by Moscowitz, *et al.*[19]

Table III. Comparison of Dipole and Rotatory Strengths of Phenylalanine and Tyrosine Model Compounds Having Largest Rotatory Strength at 77°K

Compd	D_i[a]	R_i[b] × 10^2	R_i/D_i × 10^2
N-Acetyl-L-phenylalanine amide[c] in M–G	0.15	0.21	1.4
L-Tyrosine ethyl ester in EPA	1.1	1.7	1.5

[a] D_i, dipole strength in Debye squared. [b] R_i, rotatory strength in Debye–Bohr magnetons. [c] Data calculated from ref 4.

The tyrosine CD spectra discussed thus far represent the idealized case in that both the CD and absorption spectra have the same wavelength positions and approximately the same shape. An apparent mismatch between CD and absorption may occur when the solute has two or more species with different absorption spectra.[20] These various solute species may arise from both solute–solvent interactions and multiple solute conformations. Since the wavelength position of the tyrosine absorption spectrum is unusually sensitive to the interactions between the solvent and the solute, it would be expected that some tyrosine CD spectra may not be identical with the corresponding absorption spectra.

The mismatch of 1 nm between CD and absorption of L-Tyr in W–G at 77°K may be one such example. As discussed previously, the solute molecules occupy multiple sites having different interactions with the surrounding glass or solvent. These solute–solvent interactions do not cause major changes in the absorption intensity of each site, but do shift the wavelength positions slightly. On the other hand, the CD intensity is inherently more sensitive to changes in the orientation of the solvent around the solute. Therefore, the glass or solvent may contain a number of solute species with CD bands having different intensities and different wavelength positions.

The manner in which this produces a wavelength mismatch between the positions of the CD and absorption bands is illustrated in Figure 13 using a simplified system with only two sites. The absorption intensities are taken to be the same for both sites, but the CD intensity of the first site is taken to be 6 times

(19) A. Moscowitz, A. Rosenberg, and A. E. Hansen, *J. Amer. Chem. Soc.*, 87, 1913 (1965).

(20) A. Moscowitz, K. M. Wellman, and C. Djerassi, *Proc. Natl. Acad. Sci. U. S.*, 50, 799 (1963).

larger than that of the second site. In the first column, which has the individual bands far enough apart to be resolved, each CD band or shoulder occurs at the same wavelength as one of the absorption bands. In the second column, the CD spectrum does not peak at the same wavelength as the absorption spectrum, because the individual bands are not resolved.

A pronounced mismatch between CD and absorption occurs at a low concentration of NAc-L-Tyr EE in EPA at 298°K. This 9-nm shift of the CD band may be explained by the existence of two solute species having different absorption spectra as a result of interactions with different components in the EPA. For example, NAc-L-Tyr EE gives a positive CD band in dioxane and a negative band in methanol. These spectra were used to simulate two species of NAc-L-Tyr EE in EPA, one interacting with the alcohol and the other with the ether component. The sum of these two spectra duplicated the 9-nm CD shift observed experimentally. This result emphasizes that the mismatch may be large, *when the two species have broad bands and opposite CD signs.*

Minor distortions in band shape and position are possible in many tyrosine spectra. Indeed the small variations observed in the shapes of the room-temperature CD spectra (compare Figures 4, 5, and 7) may be other examples of multiple solute species having slightly different CD and absorption spectra. The extent of these distortions, however, can be minimized by cooling to 77°K to obtain better resolution of each vibronic transition.

Aggregation also influences the appearance of tyrosine CD spectra. When aggregation occurred, the CD spectrum of NAc-L-Tyr EE in EPA at 77°K had sharp negative 0–0 and 0 + 800 cm^{-1} bands and a broad positive band between 270 and 250 nm. Also this spectrum could be generated by using two species, one having a negative CD spectrum and the other having a positive spectrum starting at a slightly shorter wavelength.

In all these cases, each tyrosine species appears to have the same vibronic transitions as observed in the ideal cases. The average CD spectrum, however, may be distorted appreciably when multiple solute species exist with different CD and absorption spectra.

Now that the CD bands of the tyrosyl moiety have been characterized, their identification in proteins may be considered. The major complication in proteins is that other moieties may also contribute CD between 250 and 290 nm. In nonconjugated proteins the tyrosyl CD bands may be overlapped by tryptophanyl, phenylalanyl, and disulfide bands. Conjugated proteins, *e.g.*, heme proteins, are even more complex in that the CD bands of the prosthetic group may also overlap the tyrosyl bands.

An additional difficulty is that proteins usually contain more than one tyrosine residue. When many tyrosine residues are present, the summation of all these individual CD spectra may produce a complicated spectrum. For example, each tyrosine residue in a protein may have its 0–0 transition located at a different wavelength. The exposed side chains would have their 0–0 transitions near 283 nm, whereas the buried side chains would have their 0–0 bands at longer wavelengths (up to 289 nm). These difficulties can be

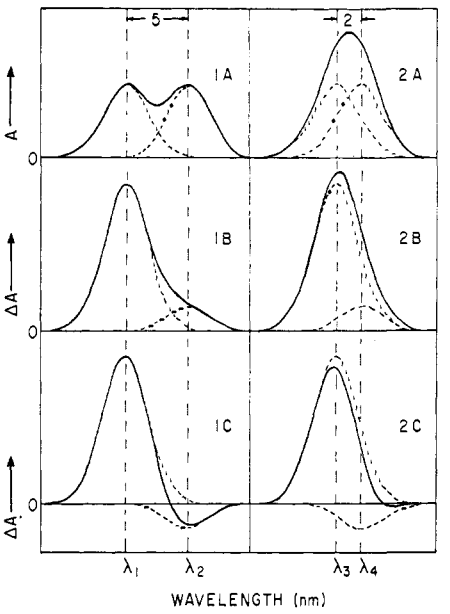

Figure 13. Relationship between the observed CD and absorption bands of an idealized molecule having only a single transition, but distributed equally between two sites: ——, observed spectrum; -----, mathematically resolved spectrum of each site (4-nm half-band width); column 1, sites 5 nm apart; column 2, sites 2 nm apart; row A, absorption spectrum; row B, CD spectrum with both sites having the same sign; row C, CD spectrum with sites having opposite signs. See text for additional details.

minimized by cooling the protein to sharpen the CD and absorption fine structure. For example, even when solvation and aggregation occurred in model compounds, the 0–0 and 0 + 800 cm^{-1} bands were well resolved.

In many cases it should be possible to identify the tyrosyl CD bands in proteins on the basis of their fine structure occurring between 275 and 290 nm. The region from 270 to 250 nm seems unsatisfactory for identifying tyrosyl CD bands. Since these short-wavelength tyrosyl transitions are weak and poorly resolved, the 250–270-nm region is prone to distortion by the presence of multiple species having different CD and absorption spectra, as observed in the model compounds. Furthermore, phenylalanyl CD bands and strong disulfide CD bands also may occur between 250 and 270 nm.

The identification of tyrosyl CD fine structure is most difficult in proteins containing intense tryptophanyl CD bands. Both the ^1L_b and ^1L_a tryptophanyl CD fine structure overlaps the tyrosyl CD bands.[8] In proteins containing several tryptophan sites and several tyrosine sites, the CD spectrum may be too complex to identify the tyrosyl CD bands.

In contrast to the case with tryptophan, the disulfide CD bands do not necessarily hinder identification of the tyrosyl fine structure, because the disulfide bands do not have fine structure. In proteins lacking tryptophan the disulfide CD bands may sometimes be assessed after analyzing the tyrosyl bands.

A complete analysis was carried out on the low-temperature CD and absorption spectra of RNase-A. The absorption spectrum is readily analyzed. Above 270 nm the RNase absorption results principally from the six tyrosine residues, and the structureless disulfide absorption is too small to cause complications. Three

types of tyrosine residues were identified from the long-wavelength fine structure found in the 77°K RNase-A absorption spectrum. Each type possesses the same vibronic transitions as do tyrosine model compounds. The 0–0 transitions, however, occur at different wavelengths, depending upon the local environment. Presumably the three exposed side chains belong to residues 73, 76, and 115, which according to the X-ray studies have their hydroxyl groups exposed.[21] Two buried tyrosine residues have their 0–0 bands at 286 nm, and a single buried residue has its 0–0 transition at 288.5 nm. Other studies have shown that tyrosines 25, 92, and 97 are buried and their hydroxyl groups are involved in hydrogen bonding.[22] This information permits analyzing the 77°K CD spectrum of RNase-A.

Before beginning the CD analysis, however, we must select the proper CD spectrum for each tyrosyl side chain in RNase-A. Several pieces of evidence indicate that the strong, well-resolved CD transitions of each tyrosine residue in RNase-A should have approximately the same relative intensities as observed in the ideal tyrosine model compounds. First the studies with tyrosine model compounds suggest that any major distortion from the ideal CD spectrum must arise from the existence of multiple tyrosine species having different wavelength positions for the 0–0 transition. Even when this distortion exists, the well-resolved 0–0 and 0 + 800 cm^{-1} bands are least affected. Secondly the X-ray studies of RNase-A reveal that each of the six tyrosyl side chains is at least partly constrained in a unique site.[21] This condition minimizes the possibility of having more than one tyrosyl species at each site, especially for the three buried side chains.

Consequently, the single buried residue with 0–0 band at 288.5 nm was represented with an ideal tyrosine CD spectrum. The 15–20% CD contribution from this residue was determined accurately, since its 0–0 band is not overlapped by any other tyrosine bands. The two tyrosine residues with their 0–0 bands at 286 nm do not have detectable CD bands. Possibly one of these tyrosine residues has positive CD bands, whereas the other has negative bands of comparable intensities.

The three exposed tyrosyl side chains give rise to negative CD fine structure at 282 and 276 nm. These bands are a summation of the individual spectra of three different tyrosyl sites. If the 0–0 band at each site is located at a slightly different wavelength, then the total CD spectrum contributed by all three exposed residues may deviate somewhat from that of the tyrosine model compound exposed to the water–glycerol solvent. Consequently, several CD shapes were used to represent the exposed tyrosyl side chains. In L-Tyr in W–G, the ratio of ΔA at the 0–0 band to ΔA at the 0 + 800 cm^{-1} band was 1.3. Analyses were carried out using ratios of 1.4 and 1.2, in addition to the 1.3. These results indicate that the CD contribution of the exposed tyrosine residues remains about 35–45%, even when the ratio is altered appreciably beyond the value observed in the model tyrosine compounds. In all cases, the correct RNase-A band shape can only be obtained by including a broad negative CD band in addition to the tyrosyl bands.

This broad negative band peaking about 270 nm and contributing about 40–50% of the CD intensity above 275 nm is an extension of the disulfide CD band observed at long wavelengths in the RNase-A CD spectrum at 77°K. The shape of the disulfide CD band proposed for RNase-A is similar to that observed in 2-isoleucine-oxytocin,[23] a model compound whose near-ultraviolet CD spectrum arises from a single disulfide bond. Furthermore, just as observed with all disulfide compounds,[15,23,24] the CD intensity of the band in RNase-A is strong even though the absorption intensity is quite weak. The wavelength position of the disulfide band in RNase-A indicates that the dihedral angles of the disulfide bonds are approximately 90°.[25]

The interpretation of the 77°K CD spectrum of RNase-A can be used to estimate the relative CD contributions in the room-temperature CD spectrum. The shapes of both RNase-A CD spectra are identical, except that the tyrosyl CD fine structure is much more evident at 77°K. That is, lowering the temperature sharpened the vibronic transitions, but did not create any new CD bands and did not increase the total rotatory strength. Even the long-wavelength edge of the disulfide CD band can be detected in the room-temperature CD spectrum of RNase-A. Apparently the CD contributions of tyrosine and cystine residues are similar in both the 298 and 77°K RNase-A spectra.

Our interpretation of the near-ultraviolet CD spectrum of RNase-A helps to explain some of the difficulties which arose in the earlier studies of RNase-A. Chemical modification[5,9] and pH effects[10] have not permitted a consistent interpretation of the CD contributions from the buried and exposed tyrosyl side chains,[7] because the large CD contribution from the disulfide bridges has been ignored. The pH studies *per se* are not sufficient to identify the exposed tyrosine residues, since pH changes may also influence the disulfide CD bands. Any ionization in RNase-A may alter the intensity and position of the disulfide CD bands either directly by perturbing the disulfide chromophore or indirectly by altering the protein conformation surrounding the disulfide bond. For example, the disulfide CD bands of 2-isoleucine-oxytocin are sensitive to pH changes.[23] Chemical modification studies also have been difficult to interpret, because the CD bands of the modified residues have not been examined carefully. The modified amino acid residues also have CD bands whose signs and intensities will depend upon the local protein environment. Thus the CD spectrum of any modified protein must be thoroughly analyzed to determine which CD bands remain unaltered and which bands arise from the modified residues.

Appendix

The near-ultraviolet absorption band of tyrosine arises from the π–π^* transitions involving the phenolic ring. The properties of these transitions in tyrosine can be identified by using *p*-cresol as a model compound,

(21) H. W. Wyckoff, K. D. Hardman, K. M. Allewell, T. Inagami, L. N. Johnson, and F. M. Richards, *J. Biol. Chem.*, 242, 3984 (1967).
(22) L. Li, J. P. Riehm, and H. A. Scheraga, *Biochemistry*, 5, 2043 (1966).
(23) S. Beychok and E. Breslow, *J. Biol. Chem.*, 243, 151 (1968).
(24) D. L. Coleman and E. R. Blout, *J. Amer. Chem. Soc.*, 90, 2405 (1968).
(25) G. Bergson, G. Claeson, and L. Schotte, *Acta Chem. Scand.*, 16, 1159 (1962).

Table IV. Spectroscopic Properties of *p*-Cresol and Tyrosine

p-Cresol: Ground state,[a,b] cm^{-1}	*p*-Cresol: Excited state,[a] cm^{-1}	Point group C_{2v} symmetry species[b]	Vibrational mode no.[c]	Tyrosine[d] excited state, cm^{-1}
468	422	A_1	6A	420
642	585	B_2	6B	Not detected
738	694	A_1	12	Not detected[e]
841	808	A_1	1	800
1251[f]	1272	A_1	7A	1250[g]

[a] Reference 30. [b] Reference 27. [c] Notation described in K. S. Pitzer and D. W. Scott, *J. Amer. Chem. Soc.*, **65**, 803 (1943). [d] Taken from Figure 2. [e] The 0 + 694 cm^{-1} transition is weak in *p*-cresol vapor; for *p*-cresol and tyrosine solutions, this band is completely masked by the 0 + 800 cm^{-1} band. [f] In contrast to the rest of the vibrations where the vibrational frequency of the excited state is lower than that of the ground state, vibration 7A has a slightly higher frequency in the excited state (ref 29). [g] In the vapor spectrum of *p*-cresol five bands are closely spaced around 0 + 1250 cm^{-1} (ref 30). These bands cannot be resolved in solution. The 0 + 1272 cm^{-1} transition appears to be the single strongest contributor to the intensity of the 0 + 1250 cm^{-1} band of tyrosine. In addition, the 0 + 420 + 800 cm^{-1} transition also may contribute in this region.

since tyrosine is a derivative of *p*-cresol[26] and has the same near-ultraviolet absorption spectrum as does *p*-cresol. Unfortunately the ultraviolet spectrum of *p*-cresol has not been analyzed completely. Supplementary information, however, is available from studies on phenol and other substituted benzenes.

The effective local symmetry of the phenolic ring in tyrosine appears to be C_{2v}. First an analysis of the infrared and Raman spectra of *p*-cresol indicates an effective symmetry of C_{2v}.[27] Secondly, the near-ultraviolet transitions of phenol have been described in terms of C_{2v} symmetry.[28,29] In view of the effective local symmetry being C_{2v}, the near-ultraviolet band of tyrosine can be identified as an $A_1 \rightarrow B_2$ electronic transition, as is also the case for phenylalanine.[4]

The symmetry species of the excited state vibrational modes were identified from the corresponding ground-state vibrations of *p*-cresol[27,30] (Table IV). The mode numbers for the vibrations were obtained by starting with the assignments for phenol.[28,29] The vibrational frequency of each mode, however, may be somewhat changed in disubstituted benzenes such as *p*-cresol and tyrosine. The magnitude of this change was estimated by calculating the frequency shift for each mode in going from toluene to *p*-xylene (see Tables XIX and XXII in ref 31). When this value was added to the fundamental frequency observed in the excited state of phenol,[28,29] the resulting frequency matches only one of the excited-state vibrational frequencies observed in *p*-cresol (Table III). Thus the identification of the vibrations of *p*-cresol and tyrosine seems reliable.

Consideration must also be given to the absorption intensity of the transitions involving the nontotally symmetrical vibration 6B. In both phenol[28,29] and *p*-cresol (ref 30 and Table III), these transitions are very weak. In the *p*-cresol vapor spectrum, vibration 6B apparently occurs in the 0 + 585 cm^{-1} transition. As would be expected from the weakness of this transition in *p*-cresol vapor,[30] the 0 + 585 cm^{-1} band is too weak to be detected in the tyrosine spectrum even at 77°K. Therefore, the near-ultraviolet absorption spectrum of tyrosine arises almost entirely from the 0–0 band and combinations involving only totally symmetrical vibrations.

Acknowledgments. We wish to thank Professor Albert Moscowitz and Professor Oscar Weigang for helpful discussions concerning the effects of solvation and vibronic coupling.

(26) H. Sponer, *J. Chem. Phys.*, 10, 672 (1942).
(27) R. J. Jakobsen, *Spectrochim. Acta*, 21, 433 (1965).
(28) W. W. Robertson, A. J. Seriff, and F. A. Matsen, *J. Amer. Chem. Soc.*, 72, 1539 (1950).
(29) H. D. Bist, J. C. D. Brand, and D. R. Williams, *J. Mol. Spectrosc.*, 21, 76 (1966).
(30) S. Imanishi, M. Ito, K. Semba, and T. Anno, *J. Chem. Phys.*, 20, 532 (1952).
(31) See Table III, footnote *c*.

Communications to the Editor

Carbon Scrambling upon Electron Impact

Sir:

The occurrence of hydrogen scrambling accompanying fragmentation in the mass spectrometer has been demonstrated in many classes of compounds.[1] In toluene and related compounds scrambling involves ring expansion, some aspects of which have been probed by ^{13}C labeling.[2] For compounds where ring expansion is not possible it has been suggested,[3] by analogy with known photochemical processes, that scrambling might involve valence tautomerism. Some support for this hypothesis has been found[4] in the propensity for H/D scrambling in deuterated thiophenes and the absence of such scrambling in furans, a result which is paralleled by the photoisomerization of arylthiophenes[5] and the absence of such isomerization in arylfurans.[6] Further support for the ring atom scrambling mechanism in thiophenes comes from an electron impact study[7] of

(1) References appear in R. G. Cooks, I. Howe, and D. H. Williams, *Org. Mass Spectrom.*, 2, 137 (1969).
(2) K. L. Rinehardt, *J. Amer. Chem. Soc.*, 90, 2983 (1968). Compare also K. L. Rinehardt, A. C. Bucholtz, and G. E. Van Lear, *ibid.*, 90, 1073 (1968); M. Marx and C. Djerassi, *ibid.*, 90, 678 (1968); A. V. Robertson and C. Djerassi, *ibid.*, 90, 6992 (1968).
(3) K. R. Jennings, *Z. Naturforsch.*, 22a, 454 (1967). Compare K. E. Wilzbach, A. L. Harkness, and L. Kaplan, *J. Amer. Chem. Soc.*, 90, 1116 (1968).
(4) D. H. Williams, R. G. Cooks, J. Ronayne, and S. W. Tam, *Tetrahedron Lett.*, 1777 (1968).
(5) H. Wynberg, R. M. Kellogg, H. van Driel, and G. E. Berkhius, *J. Amer. Chem. Soc.*, 89, 3501 (1967).
(6) A. Padwa and R. Hartman, *ibid.*, 88, 3759 (1966).
(7) T. A. Elwood, P. F. Rogerson, and M. M. Bursey, *J. Org. Chem.*, 34, 1138 (1969).