Fine Structure in the Near-Ultraviolet Circular Dichroism and Absorption Spectra of Tryptophan Derivatives and Chymotrypsinogen A at 77°K*

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ABSTRACT: To aid in identifying tryptophanyl circular dichroism bands in proteins, the circular dichroism spectra of model compounds have been characterized, using low temperature to enhance circular dichroism fine structure. The circular dichroism spectra of *N*-acetyl-L-tryptophanamide and seven other low molecular weight compounds may be grouped into four classes: (1) ^1L_b bands intense, (2) ^1L_a bands intense, (3) both ^1L_a and ^1L_b bands intense, and (4) fine structure whose origin was not readily identified. Both the 0–0 and 0 + 850 cm^{-1} ^1L_b transitions occur together and have the same circular dichroism sign. A number of ^1L_a transitions could be identified, but their relative intensities varied greatly. In chymotrypsinogen A, the tryptophanyl circular dichroism bands arise mainly from ^1L_a transitions. The results of these studies indicate that in proteins the circular dichroism fine structure occurring between 290 and 305 mμ is most readily used to identify tryptophanyl circular dichroism bands. The effect of temperature upon the rotatory strength provides information about the interconversions among different conformations. In *N*-acetyl-L-tryptophanamide the rotatory strength at 77°K is 18 times greater than that at 298°K, which appears to indicate a high degree of conformational mobility at 298°K. In contrast, chymotrypsinogen shows only a 25% intensification upon cooling. Apparently the tryptophan residues in chymotrypsinogen have relatively rigid positions at 298°K, but a minor degree of mobility may exist.

Identifying the tryptophanyl circular dichroism bands in proteins has been difficult, because several other amino acid residues also have bands in the near-ultraviolet region (Beychok, 1968). Vibronic structure, however, may offer a way to distinguish among the various near-ultraviolet circular dichroism bands. In some proteins, tyrosyl and phenylalanyl circular dichroism bands can be identified from their characteristic vibronic structure (Horwitz *et al.*, 1969a,b). It may also be possible to identify the tryptophanyl circular dichroism bands of proteins on the basis of their vibronic structure, since some fine structure has been reported in the circular dichrosim spectra of L-tryptophan (Myer and MacDonald, 1967), tryptophanyl diketopiperazines (Edelhoch *et al.*, 1968), and poly-L-tryptophan (Stevens *et al.*, 1968; Cosani *et al.*, 1968; Peggion *et al.*, 1968).

This communication describes the near-ultraviolet circular dichroism spectra of tryptophan, seven of its derivatives, and chymotrypsinogen A. Fine structure patterns are examined in both the circular dichroism and absorption spectra by using high-resolution spectra recorded at 77°K. Guide lines are suggested for identifying the tryptophanyl circular dichroism bands in proteins. In addition, the extent of conformational mobility in chymotrypsinogen and NAc-L-Trp-amide is investigated by comparing the intensities of circular dichroism spectra recorded at 77 and 298°K.

Experimental Section

Instrumentation. To facilitate measurement of small circular dichroism signals at 77°K, we have modified the circular dichroism spectrophotometer used in our previous investigation (Horwitz *et al.*, 1969a). The modulation frequency for the electrooptical crystal was increased from 94 to 2000 Hz and mechanical chopping of the light beam was eliminated. These changes reduced the inherent instrument noise by fivefold.

Reliable resolution of fine structure in the circular dichroism spectra was obtained by using a computer of average transients (Horwitz *et al.*, 1968). Spectra were scanned repetitively at 0.3 mμ/sec using a 1-sec time constant. Spectral intensity half-band widths were less than 2 mμ. In view of the complexity of low-temperature circular dichroism spectra, all data are presented as photographs of the tracings obtained from the computer. The circular dichroism intensity was calibrated using an aqueous solution of *d*-10-camphorsulfonic acid ($\Delta\epsilon^1$ = 2.1 at 290 mμ).

Absorption spectra were measured on a Cary Model 15 spectrophotometer (Horwitz *et al.*, 1969a).

The wavelength settings of both instruments were calibrated at 253.7 and 296.8 mμ by using a mercury lamp. The wavelength positions of well-resolved tryptophan bands are accurate to within ±0.5 mμ.

Mathematical resolution of spectra was carried out using the Du Pont 310 curve resolver. Each component band was represented by a Gaussian curve, in accordance with the work of previous investigators (Jørgensen, 1962; Urry, 1968). The correctness of the Gaussian shape for vibrational fine structure was verified by curve-fitting isolated vibronic transitions which were exceptionally well resolved in the circular dichroism and

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[1] Abbreviations used are: $\Delta\epsilon$, ϵ for the left circularly polarized light minus that for right circularly polarized light; ΔA, absorbance for left circularly polarized light minus that for right circularly polarized light.

absorption spectra of phenylalanine and tyrosine derivatives at 77°K. Gaussian bands gave a good fit, whereas Lorentzian bands did not. The spectra of tryptophan derivatives were well fit using Gaussian bands.

Low-Temperature Circular Dichroism Technique. The procedure previously described (Horwitz *et al.*, 1969a) has been modified so that low-temperature circular dichroism spectra can be recorded more quickly and conveniently. The samples in the spectroscopic dewar were always kept completely immersed in liquid nitrogen to ensure a temperature of 77°K throughout the circular dichroism measurements. Using this procedure, the light beam passed through liquid nitrogen both before and after traversing the sample. Usually a stream of nitrogen gas bubbled through the liquid and thus passed through the light beam. Nevertheless, no significant noise was introduced into the circular dichroism signal, because the detector system, being tuned to 2000 Hz, filtered out the low-frequency noise caused by bubbling. Control experiments showed that having the liquid nitrogen in the light path did not cause any depolarization of the light.

Four solvent systems were used for low-temperature circular dichroism spectra: water–glycerol (1:1, v/v), tetrahydrofuran–diglyme (4:1, v/v), methanol–glycerol (9:1, v/v), and ethyl ether–isopentance–ethanol (5:5:2, v/v). The procedures used to freeze methanol–glycerol and ethyl ether–isopentane–ethanol were described previously (Horwitz *et al.*, 1969a). Tetrahydrofuran–diglyme had to be frozen rapidly to avoid obtaining an opaque solid. Plunging the cuvet into liquid nitrogen gave clear glasses for path lengths of 0.1 mm. Water–glycerol was frozen slowly over a period of several minutes. Water–glycerol glasses had cracks, but no depolarization occurred for paths up to 0.2 mm in length.[2] Longer path lengths were not used because depolarization of the light occurred under these conditions. On some occasions both water–glycerol and methanol–glycerol glasses gave a sloping base line,[3] *i.e.*, the leading edge of the circular dichroism curve sloped even though no real circular dichroism existed at these wavelengths (330–315 mμ). When this artifact occurred, the sample was discarded and a new glass was prepared.

The necessity of using short path lengths at 77°K dictated having a high tryptophan concentration, since the circular dichroism signals were small. The possibility exists that in some cases the solute aggregated during cooling to 77°K. In related studies on phenylalanine derivatives at 77°K, an example of aggregation has been identified from circular dichrosim spectra (Horwitz *et al.*, 1969a). With tryptophan derivatives, however, no evidence of aggregation was observed. Unfortunately, equipment limitations prevented a systematic investigation of the aggregation question, because the tryptophan concentration could not be varied sufficiently.

The measurement of small circular dichroism signals at 77°K can be vitiated by optical artifacts unless attention is paid to several critical details. First the dewar had to be aligned so that its windows were perpendicular to the light beam. Second the windows had to be masked so that light which was scattered by the frozen sample would not reflect from the dewar walls to the photomultiplier. Third, the angle and position of the photomultiplier face plate (end-on type) had to be adjusted to eliminate artifacts arising from internal reflections. After completing these alignments, no artifacts were observed for samples having absorbances as great as 1. (No experiments were performed at absorbances greater than 1.) If proper optical alignment was not obtained, the optical artifact could be observed by recording the apparent circular dichroism signal from nonoptically active compounds. For a given optical misalignment, the sign of the apparent circular dichroism signal was always the same; but the magnitude increased with the absorbance of the sample. *No artifacts were observed under the conditions used to record the circular dichroism spectra presented here.* As a precaution, however, the base lines for all small circular dichroism signals were recorded using a nonoptically active sample having absorbance identical with that of the optically active sample. These base lines give a direct measure of the instrument noise for each wavelength range (photomultiplier voltage).

Materials. L-Trp, D-Trp, DL-Trp, and NAc-L-Trp were obtained from Calbiochem (Los Angeles, Calif.). L-Arg-L-Trp, L-His-L-Trp, L-Trp-L-Phe, Gly-L-Trp, NAc-L-Trp-ethyl ester, NAc-D-Trp-amide, and NAc-DL-Trp-amide were from Cyclo Chemical Co. (Los Angeles). Bovine pancreatic chymotrypsinogen A (five-times crystallized) was obtained from Worthington Biochemical (Freehold, N. J.). NAc-L-Trp-amide was from Miles Laboratory (Elkhart, Ind.). All solvents were Spectroquality except tetrahydrofuran, which was chromatoquality from Matheson Coleman and Bell.

All concentrations were determined spectrophotometrically at 282 mμ: for Trp, ϵ 5500; for chymotrypsinogen, ϵ 50,200 (Wetlaufer, 1962); for tryptophan derivatives in organic solvents, ϵ was assumed to be approximately 6000. The latter value is probably accurate to within ±10%.

Results

Absorption Spectra of Tryptophan Derivatives. The best resolution was obtained using NAc-L-Trp-amide and NAc-L-Trp-ethyl ester, since these derivatives dissolved readily in solvents which gave the greatest band sharpening at 77°K. Figure 1 shows the absorption spectrum of NAc-L-Trp-amide in methanol–glycerol before and after freezing with liquid nitrogen. At 77°K, the 290-mμ band is sharpened; a shoulder appears on the long-wavelength edge; and the broad absorption band near 281 mμ is resolved into two bands located at 280 and 283 mμ. Other shoulders are evident at 274, 270, and 265 mμ. Measurements of the areas under the 77 and 298°K spectra indicate that there is no change in the absorption intensity due to cooling.

In an attempt to obtain better resolution, the absorption spectrum of NAc-L-Trp-ethyl ester was recorded in ethyl ether–isopentane–ethanol at 77°K (Figure 2). Even in this solvent not all of the individual vibronic transitions are resolved, although the long-wavelength shoulder does become more prominent.

By using circular dichroism spectra in conjunction with the absorption spectra, the positions of certain additional bands

[2] The absence of depolarization was demonstrated by measuring the circular dichroism intensity of a camphorsulfonic acid standard which was placed in the light beam after emerging from the spectroscopic dewar. Any depolarization by the glasses would have caused a decrease in the apparent circular dichroism intensity of the standard. Since no change occurred, our tests showed that the circular dichroism calibration at 77°K was identical with that at 298°K.

[3] This probably resulted when the surfaces of the cracks were not exactly parallel to the direction of the light beam (Horwitz *et al.*, 1969a).

can be identified tentatively. Our operational hypothesis is that each circular dichroism band must have a counterpart in the absorption spectrum. Thus both types of spectra have been simultaneously fit using Gaussian bands positioned at the same wavelengths and having the same band widths in circular dichroism and absorption. However, the ratio of circular dichroism to absorption intensity for a given band has been permitted to differ among the vibronic transitions (see Discussion). Applying this constraint to NAc-L-Trp-ethyl ester in ethyl ether–isopentane–ethanol permits identifying three absorption bands not previously resolved (Figure 2; see also circular dichroism sections). At least two overlapping bands are present in the leading shoulder (297.5 and 294 mμ); and a third band is needed to fill in the shallow trough near 286 mμ. At shorter wavelengths no unique resolution of the absorption spectrum is possible.

The absorption spectra of other tryptophan derivatives contain essentially the same component Gaussian bands as found for NAc-L-Trp-ethyl ester in ethyl ether–isopentane–ethanol at 77°K. However, the intensity of the 297.5-mμ band varied considerably. For NAc-L-Trp-amide at 77°K this band is enhanced by certain solvents (ethyl ether–isopentane–ethanol > methanol–glycerol > water–glycerol). Furthermore, NAc-L-Trp-ethyl ester gives more intense absorption at 297.5 mμ than NAc-L-Trp-amide.

Circular Dichroism Spectra of Tryptophan and Its Derivatives. Since these spectra are either unusually weak or have little fine structure at 298°K, the low-temperature circular dichroism technique was applied (Wellman *et al.*, 1963; Horwitz *et al.*, 1969a). Figure 3 shows the effect of temperature upon the circular dichroism spectrum of NAc-L-Trp-amide dissolved in methanol–glycerol. The 298°K circular dichrosim spectrum reveals a weak positive band at 290 mμ and a broad negative band centered near 265 mμ.[4] After cooling the NAc-L-Trp-amide, the shape of the circular dichroism spectrum changes and the total intensity increases. At 77°K the circular dichroism around 265 mμ becomes positive, and two prominent circular dichroism bands occur at 290.2 and 283.2 mμ. These results illustrate, first of all, that the shape of the circular dichroism spectrum is not necessarily an intrinsic property of each compound and, secondly, that circular dichroism fine structure is better resolved at 77°K. In the case of NAc-L-Trp-amide in methanol–glycerol, the increased resolution results mainly because the total rotatory strength is 18 times greater at 77 than at 298°K, as measured by the increase in total area of the circular dichroism spectrum.

In the remainder of this section we describe four types of circular dichroism spectra which have been observed for tryptophan and its derivatives. In view of the great circular dichroism intensification at low temperature, most of our search was done at 77°K.

TYPE I has prominent ^1L_b bands superimposed on weak ^1L_a bands. This circular dichroism spectrum is illustrated by NAc-L-Trp-ethyl ester in ethyl ether–isopentane–ethanol at 77°K (Figure 4). Sharp vibrational bands are seen at 283.5 and 290.5 mμ. The wavelengths of these circular dichroism bands coincide with the positions of two bands resolved in the absorption spectrum (Figure 2). A weak vibrational circular

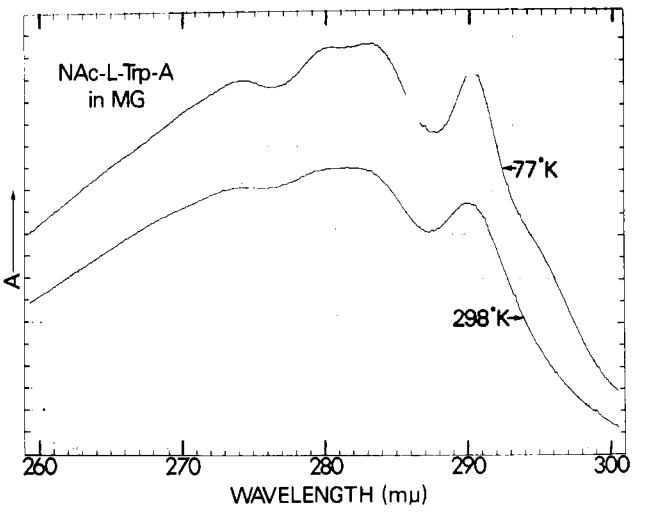

FIGURE 1: Instrument trace of absorption spectra of 12 mM *N*-acetyl-L-tryptophanamide in methanol–glycerol at 298 and 77°K. Path length, 0.1 mm. Base line for 77°K spectrum was offset 0.11 unit to separate the spectra. Both solvent base lines were flat. The areas under the two curves were the same, within our experimental error for methanol–glycerol. (Some evaporation of methanol occurred during the freezing.)

dichroism band is resolved at 278.5 mμ. All three of these circular dichroism bands appear to be ^1L_b transitions (see Discussion).

The ^1L_a bands are not well resolved in the type I circular dichroism spectrum. However, a comparison of the circular dichroism and absorption spectra indicates that weak ^1L_a circular dichroism bands do occur between 294 and 300 mμ. Since the ratio of circular dichroism to absorption increases in going from 300 to 294 mμ, at least two bands are required to fit simultaneously both spectra in this region (Figure 5). Another ^1L_a circular dichroism band is needed at 286.5 mμ to partially fill the trough between the ^1L_b bands at 290.5 and 283.5 mμ.

A number of other tryptophan derivatives also give the type I circular dichroism spectrum at 77°K. These include: NAc-L-Trp in ethyl ether–isopentane–ethanol; NAc-L-Trp-ethyl ester in methanol–glycerol and tetrahydrofuran–diglyme; NAc-L-Trp-amide in water–glycerol, methanol–glycerol, and ethyl ether–isopentane–ethanol; L-His-L-Trp in methanol–glycerol; Gly-L-Trp in methanol–glycerol; and L-Arg-L-Trp

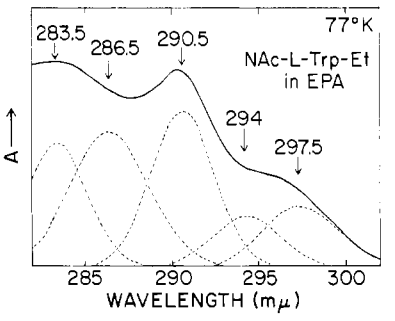

FIGURE 2: Resolved absorption spectrum of *N*-acetyl-L-tryptophan ethyl ester in ethyl ether–isopentane–ethanol at 77°K.

[4] By averaging more scans, additional fine structure can be resolved even at 298°K (see Figure 8).

TABLE I: Effect of Solvent on the Position of ^1L_b Transitions at 77°K.

Glass	Compound	Absorption 0–0[a]	Circular Dichroism 0–0[a]	Circular Dichroism 0 + 850 cm^{-1}
Water–glycerol	NAc-L-Trp-amide	289.0	289.0	282
Methanol–glycerol	NAc-L-Trp-amide	290.2	290.2	283.2
Methanol–glycerol	NAc-L-Trp-ethyl ester	290.3	290.2	283.2
Ethyl ether–isopentane–ethanol	NAc-L-Trp-ethyl ester	290.5	290.5	283.5
Tetrahydrofuran–diglyme	NAc-L-Trp-ethyl ester	290.4	291	284

[a] See footnote 5 for a description of this designation.

in methanol–glycerol. In all these cases, the ^1L_b bands have positive signs.

The wavelengths of the ^1L_b circular dichroism bands are relatively unaffected by the solvent in which the sample is frozen, provided that the compound retains the ^1L_b circular dichroism bands. Table I shows that the range of positions for each ^1L_b circular dichroism band was only 2 mμ in our four different glasses. Furthermore, the movement of these circular dichroism bands coincided with that of the corresponding absorption bands.

TYPE II appears to result mainly from ^1L_a bands. Tryptophan dissolved in neutral water gives this circular dichroism spectrum (Figure 6). In contrast to the tryptophan circular dichroism spectrum reported by Myer and MacDonald (1967), our circular dichroism spectrum does not possess any fine structure. This lack of fine structure could not be due to poor resolution by our circular dichroism instrument, since it can resolve the complex fine structure in phenylalanine (Horwitz *et al.*, 1969a). We have further verified our results by showing that D-Trp and L-Trp give mirror image circular dichroism spectra (Figure 6). The justification for associating the tryptophanyl circular dichroism spectrum with the ^1L_a transitions is given in the discussion.

The circular dichroism spectrum of tryptophan in methanol–glycerol at 77°K also appears to be mainly the ^1L_a type, although the weak fine structure evident at 290 mμ probably is a ^1L_b band (Figure 7).

TYPE III spectra have prominent circular dichroism bands belonging to both the ^1L_a and ^1L_b transitions. NAc-L-Trp-amide in methanol at 298°K illustrates one form of this spectrum (Figure 8). The sharp positive bands at 290 and 283 mμ correspond to the major ^1L_b transitions. Broader negative bands are centered near 297 and 269 mμ, regions of ^1L_a transitions (see Discussion).

A type III circular dichroism spectrum was also obtained at 77°K, provided that an appropriate solvent was used. Even though the circular dichroism spectrum of NAc-L-Trp-amide is identical in both methanol and methanol–glycerol at 298°K, the latter solvent was unsatisfactory because these solutions, converted from type III into I upon freezing (Figure 3). In tetrahydrofuran–diglyme, NAc-L-Trp-amide does give a type III circular dichroism spectrum at 77°K (Figure 9), although it is not identical with the one shown in Figure 8. The most obvious difference between the two type III spectra is that the

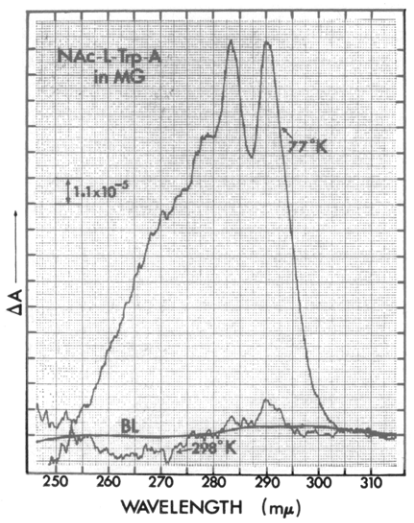

FIGURE 3: Circular dichroism records of 12 mM *N*-acetyl-L-tryptophanamide in methanol–glycerol at 298 and 77°K. Path length, 0.1 mm; 15 scans. BL indicates base line. The area under the 77°K spectrum was 18 times greater than that under the 298°K spectrum (from 310 to 253 mμ). The absorption spectra of these samples are shown in Figure 1.

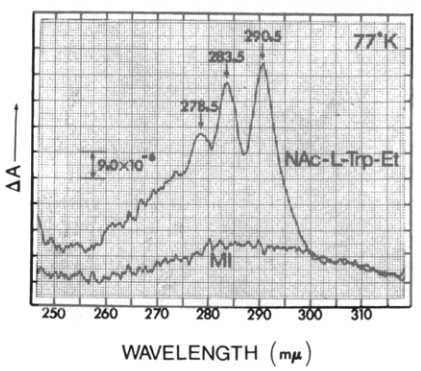

FIGURE 4: Circular dichroism records of 3.8 mM *N*-acetyl-L-tryptophan ethyl ester in ethyl ether–isopentane–ethanol at 77°K. The base line (MI) is 3-methylindole having the same absorbance as NAc-L-Trp-ethyl ester. Path length, 0.2 mm; 40 scans; sensitivity, 9.0×10^{-6} ΔA per large division.

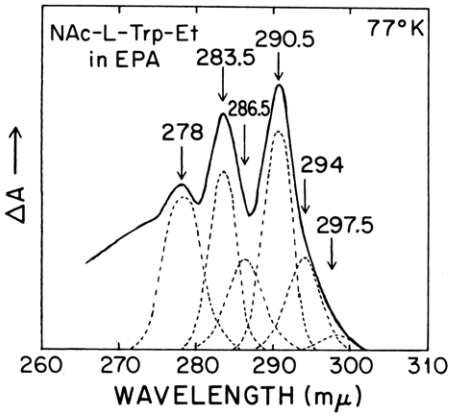

FIGURE 5: Resolved circular dichroism spectrum of *N*-acetyl-L-tryptophan ethyl ester in ethyl ether–isopentane–ethanol at 77°K. The resolved absorption spectrum of this sample is shown in Figure 2.

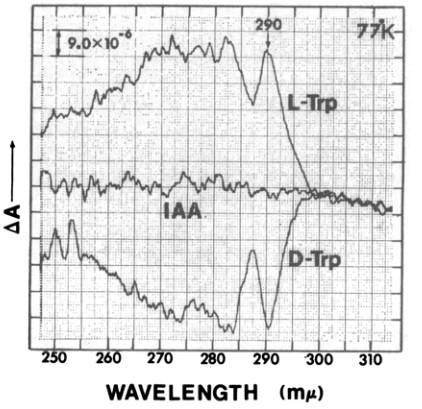

FIGURE 7: Circular dichroism record of 12 mM L-Trp and D-Trp in methanol–glycerol at 77°K. The base line (IAA) is indole-3-acetic acid having the same absorbance as Trp. Path length, 0.1 mm; 40 scans.

short-wavelength ^1L_a band near 269 mμ is positive in tetrahydrofuran–diglyme at 77°K instead of negative as in methanol at 298°K. A number of other circular dichroism bands are especially well resolved in tetrahydrofuran–diglyme at 77°K. The strong ^1L_b circular dichroism bands appear at 291.3 and 284 mμ. In addition, a weak band is evident at 278 mμ. A long-wavelength, negative ^1L_a band is at 298 mμ. By applying our hypothesis that each vibronic transition must occur in both circular dichroism and absorption, the circular dichroism spectrum in Figure 9 can be analyzed in terms of its Gaussian components (Figure 10). In addition to the bands described above, a positive circular dichroism band appears to be present near 294 mμ, a region of ^1L_a absorption. This band causes a plus 0.5-mμ shift in the apparent position of the neighboring ^1L_b band and distorts its shape. The data fit is further improved by including a positive circular dichroism contribution from the 286-mμ absorption band. The sign pattern of the ^1L_a circular dichroism bands in Figure 10 is unusual in that the longest wavelength ^1L_a band has the opposite sign from the other ^1L_a bands.

TYPE IV spectra have circular dichroism fine structure which does not coincide with the positions which have been identified as either ^1L_b or ^1L_a transitions. Both L-Trp-L-Phe in methanol–glycerol at 77°K and NAc-L-Trp in tetrahydrofuran–diglyme (1:1, v/v) at 77°K give type IV spectra. Positive circular dichroism bands are present at 291 and 284 mμ in L-Trp-L-Phe and at 292 and 285 mμ in NAc-L-Trp. These bands are about 1.5 mμ to the red from the apparent positions of the major ^1L_b bands in the absorption spectra.

CHYMOTRYPSINOGEN A. The circular dichroism spectra of this protein in water–glycerol are shown in Figure 11. The 298°K spectrum has strong positive bands at 297 and 288 mμ and weak negative bands at 305 and 291.5 mμ. The exact positions of the weak bands are difficult to determine from the 298°K spectrum due to the extensive overlapping of neighboring bands. After cooling chymotrypsinogen to 77°K, the circular dichroism bands are sharpened and their heights are doubled (Figure 11). Thus the 77°K circular dichroism spectrum gives a more reliable indication of the wavelengths of these bands because there is much less overlap by neighboring circular dichroism bands. Incidentally the sharpening of the negative circular dichroism bands at 77°K seems to preclude their being pseudo-bands resulting from the tails of the strong negative circular dichroism bands located below 280 mμ. Resolution of the 77°K circular dichroism spectrum into Gaussian components reveals that the positive circular di-

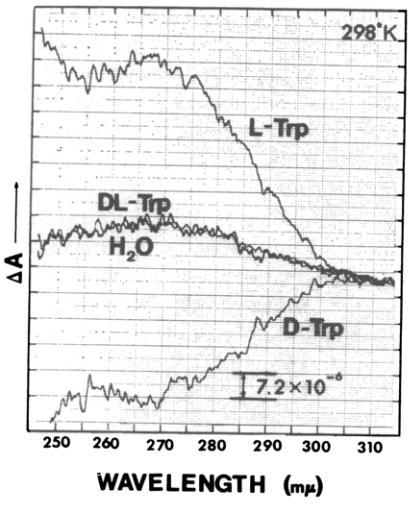

FIGURE 6: Circular dichroism, records of 0.14 mM L-Trp, D-Trp, and DL-Trp in neutral water at 298°K. DL-Trp (dark trace) gave the same base line as water (faint trace). Path length, 10 mm; 40 scans.

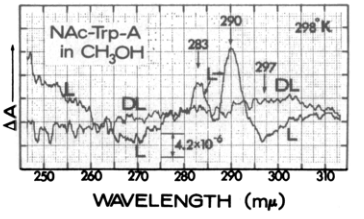

FIGURE 8: Circular dichroism record of 0.13 mM *N*-acetyl-L-tryptophanamide and *N*-acetyl-DL-tryptophanamide in methanol at 298°K. Path length, 10 mm; 80 scans; 0.13 mM NAc-D-Trp-amide gave the mirror image of the circular dichroism spectrum shown here.

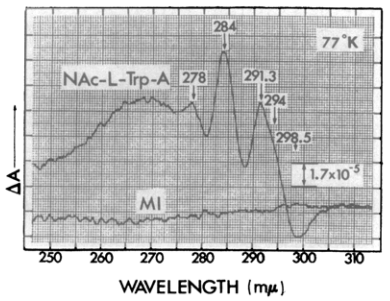

FIGURE 9: Circular dichroism record of 11 mM *N*-acetyl-L-tryptophanamide in tetrahydrofuran–diglyme at 77°K. The base line (MI) is 3-methylindole having the same absorbance as NAc-L-Trp-amide. Path length, 0.1-mm path; 40 scans; 11 mM NAc-D-Trp-amide gave the mirror image of the circular dichroism spectrum shown here.

chroism bands are at 297.5 and 290.5 mμ and the negative bands are centered at 301 and 293 mμ. The 297.5- and 301-mμ bands have an especially high ratio of circular dichroism to absorption intensity.

Lowering the temperature from 298 to 77°K increases the apparent area of the chymotrypsinogen circular dichroism curve by 60% in the region above 285 mμ. The measured areas, however, are not proportional to the total rotatory strength, because the circular dichroism intensity is partially cancelled by overlapping of positive and negative bands. More cancellation occurs in the 298°K spectrum than in the 77°K spectrum due to low-temperature sharpening of each band. The true circular dichroism intensity can be obtained by adding the areas of the individual Gaussian bands obtained from the curve resolver. These measurements showed only a 25% increase in the total rotatory strength of these circular dichroism bands upon cooling chymotrypsinogen from 298 to 77°K.

The absorption spectrum of chymotrypsinogen does not have much structure even at 77°K. However, cooling brings out a prominent shoulder near 298 mμ. The area under the

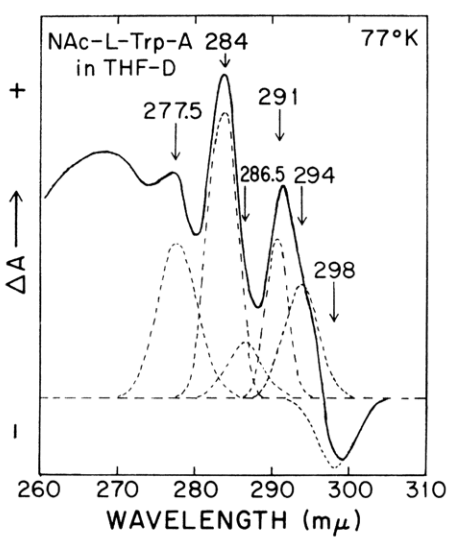

FIGURE 10: Resolved circular dichroism spectrum of *N*-acetyl-L-tryptophanamide in tetrahydrofuran–diglyme at 77°K.

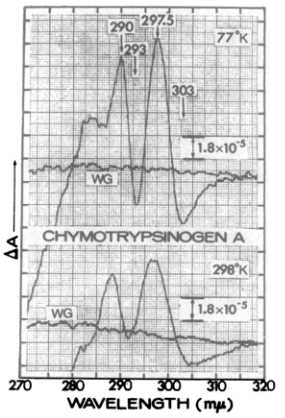

FIGURE 11: Circular dichroism records of 2.0 mM chymotrypsinogen A in water–glycerol with 25 mM sodium phosphate (pH 6.4) at 298 and 77°K. Path length, 0.1 mm; 20 scans. Base lines are water–glycerol.

absorption spectrum at 77°K differs by less than 10% from that at 298°K.

Discussion

The near-ultraviolet absorption spectrum of tryptophan and its derivatives appears to result from two overlapping electronic transitions in the indole moiety (Weber, 1960a). Konev (1967) has investigated the characteristics of these transitions by using both indole and carbazole, a related model in which the ^1L_a and ^1L_b bands are well separated. Both bands contain a number of vibronic transitions, *i.e.*, the absorption process may lead to excitation of vibrational modes in addition to the electronic excitation. In polar solvents the ^1L_b band has sharper vibrational fine structure than the ^1L_a band. In addition, the ^1L_a transitions are markedly red-shifted by hydrogen bonding, whereas the ^1L_b transitions are only slightly shifted. When indole is dissolved in hydrogen-bonding solvents, the 0–0 transition[5] of the ^1L_a absorption band occurs at a slightly longer wavelength than the 0–0 ^1L_b band (Zimmermann and Joop, 1961; Konev, 1967).

For tryptophan derivatives in polar solvents, the ^1L_a and ^1L_b transitions are not well resolved at 298°K. However, cooling to 77°K gives increased resolution of several bands. The reasons for this sharpening have been discussed by Sinsheimer *et al.* (1950). The distinction between ^1L_a and ^1L_b transitions can be made from both circular dichroism and absorption polarization spectra of tryptophan fluorescence. The latter technique indicates that ^1L_b transitions are located at about 290 and 283 mμ, while the ^1L_a transitions are responsible for most of the rest of the tryptophan absorption (Lynn and Fasman, 1968; Konev, 1967). Both these ^1L_b transitions can be resolved in the absorption spectra at 77°K; the remaining absorption bands (297.5, 294.5, 286, 280, 274, 270,

[5] In spectroscopic terminology, the vibrational states involved in excitation are designated by numbers preceding the transition; *e.g.*, 0–0 refers to a transition from the 0 vibrational state in the ground electronic level to the 0 vibrational state in the excited electronic level; 0 + 850 cm^{-1} indicates a transition from the 0 vibrational state in the ground electronic level to the 850-cm^{-1} vibrational state in the excited electronic level (Suzuki, 1967).

and 265 mμ) appear to be ^1L_a transitions. The poor resolution of the bands occurring at the short wavelengths indicates that the ^1L_a transitions are densely packed.

In the chymotrypsinogen absorption spectrum the tryptophanyl transitions are not well resolved even at 77°K. This probably results from two causes. First, below 289 mμ the tryptophanyl bands may be overlapped by tyrosine absorption bands (unpublished experiments). Secondly, this absorption spectrum represents the average of eight tryptophan residues, which probably have slightly different environments. In chymotrypsinogen, the shoulder near 290 mμ is too poorly resolved to use in identifying the 0–0 ^1L_b transitions of its tryptophan residues. However, the absorption polarization spectrum of chymotrypsinogen fluorescence suggests that the ^1L_b tryptophan transitions occur around 293 and 286 mμ (Figure 11 in Weber, 1960b). Thus the ^1L_b tryptophan bands in chymotrypsinogen seem to be located about 2 mμ further to the red than was observed with tryptophan derivatives.

The 77°K absorption spectrum of chymotrypsinogen does reveal a prominent shoulder near 298 mμ. Similar bands have been reported for other proteins containing tryptophan (Beaven, 1961). Apparently these bands are ^1L_a transitions of tryptophan, because none of the other amino acids has comparable absorption in this wavelength region at 77°K. In chymotrypsinogen these ^1L_a bands are red shifted relative to the positions observed for tryptophan derivatives dissolved in water–glycerol. This result suggests that many of the tryptophan residues in chymotrypsinogen are not exposed to the solvent. Williams *et al.* (1965) reached a similar conclusion using the solvent perturbation technique.

The information gained from the 77°K absorption spectra facilitates interpreting the circular dichroism spectra. Each vibronic transition in an optically active molecule possesses both an absorption intensity and a circular dichroism intensity. When spectra are displayed in terms of the underlying vibronic transitions, each circular dichroism transition must coincide with an absorption transition located at the same wavelength. The positions of vibronic transitions are revealed by the vibrational fine structure superimposed on each electronic transition. Thus each fine-structure absorption band gives the position of a possible circular dichroism band, and conversely each fine structure circular dichroism band indicates the location of an absorption band. The ratio of circular dichroism to absorption for each vibronic transition may vary from practically zero to a large positive or negative value (Weigang, 1965; Weigang and Dodson, 1968). These principles are clearly illustrated in the near-ultraviolet circular dichroism and absorption spectra of phenylalanine derivatives (Horwitz *et al.*, 1969a).

The correspondence between circular dichroism 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 circular dichroism and absorption bands. This is illustrated by the $n-\pi^*$ transition of 3(+)-methylcyclopentanone. In cyclohexane there is a correspondence between the positions of the vibrational fine structure in both circular dichroism and absorption; whereas in water the fine structure is blurred, and the circular dichroism maximum is red shifted relative to the absorption maximum (see Figures 4 and 5 in Urry, 1968). Frequently spectra having many overlapping transitions will exhibit an apparent wavelength mismatch between the observed circular dichroism and absorption bands. In many of these cases, it may not be obvious how to resolve the circular dichroism and absorption spectra into their individual vibronic transitions. This situation is common in circular dichroism spectra having (1) degenerate dipole–dipole coupling (Schellman, 1968), (2) accidental closeness of bands, or (3) solvation or conformational effects producing two or more solute species having appreciably different absorption spectra (Moscowitz *et al.*, 1963b). Resolution of these overlapping bands is facilitated by using low temperature to sharpen the circular dichroism and absorption bands. With these factors in mind, let us examine the four types of circular dichroism spectra obtained with tryptophan derivatives.

Type I spectrum has three fine structure circular dichroism bands occurring about 290, 283, and 278 mμ. The first two fine structure circular dichroism bands can be identified as the 0–0- and 0 + 850-cm^{-1} ^1L_b transitions, since they coincide with the ^1L_b bands in the absorption spectrum. These transitions are sufficiently well resolved in the type I circular dichroism spectrum that no complications would be expected from solvation effects. The third fine structure circular dichroism band (278 mμ) may be a weak ^1L_b transition, for in a circular dichroism spectrum having two strong ^1L_b transitions other ^1L_b bands would be expected to occur weakly at shorter wavelengths (Horwitz *et al.*, 1969a). The type I circular dichroism spectrum must also contain weak ^1L_a bands, because some circular dichroism exists even in regions where ^1L_b transitions do not occur, *e.g.*, 298 mμ.

Type II spectrum can be identified as a ^1L_a type, since these broad circular dichroism bands extend over wavelengths where the ^1L_a absorption bands occur. In our two examples of type II spectra, the circular dichroism intensity was weak in the long-wavelength ^1L_a region.

Type III circular dichroism spectra may be viewed as a mixture of types I and II in that both ^1L_a and ^1L_b bands contribute strongly to the circular dichroism. The circular dichroism spectrum of NAc-L-Trp-amide in tetrahydrofuran–diglyme at 77°K (Figure 9) is especially useful, since a number of transitions appear to be resolved. The fine structure circular dichroism bands at 291 and 284 mμ have the same characteristics as the 0–0- and 0 + 850-cm^{-1} ^1L_b bands identified in the type I spectrum. The circular dichroism band at 298 mμ occurs in a region of ^1L_a absorption that is far from the ^1L_b bands. This implies that the 298-mμ circular dichroism band involves a ^1L_a transition, even if solvation effects, aggregation, or other complications exist. Thus all major aspects of this type III circular dichroism spectrum are explained in terms of the vibronic transitions observed in the absorption spectrum.

Additional information may be obtained from a mathematical resolution of the 77°K type III circular dichroism spectrum using the basic concept that circular dichroism and absorption transitions coincide. Even though this analysis may fail to detect some transitions, the positions of the resolved bands should be approximately correct. This procedure will lead to incorrect results only if the broadness of the ^1L_a bands is caused by the presence of two or more solute species having greatly different absorption transitions. Table II summarizes the ^1L_a transitions resolved mathematically. The lowest energy ^1L_a band resolved at 77°K is assumed to be the 0–0 transition. It is particularly noteworthy that the same spacings between ^1L_a transitions were obtained from several model compounds having different circular dichroism

TABLE II: Wavelength Positions for Tryptophanyl Circular Dichroism Bands in Model Compounds at 77°K.[a]

Compound	λ (mμ)				
	^1L_a			^1L_b	
	0–0	0 + 400	0 + 1400	0–0	0 + 850
N-Ac-L-Trp-ethyl ester in ethyl ether–isopentane–ethanol	297.5	294	286.5	290.5	283.5
N-Ac-L-Trp-amide in methanol–glycerol	297.5	294	285.5	290	283
N-Ac-L-Trp-amide in tetrahydrofuran–diglyme	298	294	286.5	291	284
Chymotrypsinogen	301	297.5	290.5	293	286

[a] These data are based on the positions of the individual Gaussian components, not the apparent band locations. Identification of the ^1L_a transitions is tentative (see text).

spectra (Table II). Even though these assignments of ^1L_a transitions must be considered tentative, they do provide a unified description of the circular dichroism and absorption spectra of many tryptophan derivatives.

Type IV circular dichroism bands may indicate the locations of additional ^1L_a transitions that are obscured in type I, II, and III spectra. On the other hand, type IV bands may be the summation of nearby, overlapping bands. Another possibility is that these circular dichroism bands are moved away from the observed absorption transitions by solvation effects (Moscowitz *et al.*, 1963b).

The sign pattern of the ^1L_b circular dichroism bands differs from that of the ^1L_a bands. In our circular dichroism spectra of tryptophan derivatives only positive ^1L_b bands were observed. However, both ^1L_b circular dichroism bands of L-Trp-L-Trp-diketopiperazine are negative (Edelhoch *et al.*, 1968). Apparently the ^1L_b circular dichroism bands may have either positive or negative signs, but not mixed signs. In contrast, the ^1L_a circular dichroism bands appear to sometimes have mixed signs. It is difficult to determine whether the mixed signs result from vibronic coupling (Weigang, 1965) or from solvation effects (Moscowitz *et al.*, 1963b).

Even though the circular dichroism bands of the tryptophanyl moiety have been characterized, the identification of these bands in a protein is not necessarily simple, because other moieties may also contribute circular dichroism between 270 and 310 mμ. Conjugated proteins, *e.g.*, heme proteins, are especially difficult because the tryptophanyl circular dichroism bands may be completely overlapped by the prosthetic group circular dichroism bands. Even in nonconjugated proteins the tryptophanyl circular dichroism bands may be partially overlapped by tyrosine and disulfide bands. These difficulties can be minimized by cooling the protein to sharpen the circular dichroism fine structure.

At 77°K the tyrosyl circular dichroism bands may overlap the tryptophanyl circular dichroism bands located below 289 mμ. The tyrosyl moiety has two major circular dichroism bands (0–0 and 0 + 800 cm^{-1}), which probably always have the same sign (Horwitz *et al.*, 1969b; unpublished experiments). When a tyrosine residue is exposed to the water–glycerol medium, the 0–0 transition occurs near 282 mμ. For buried tyrosines, however, the 0–0 transition may shift as far as 289 mμ. Thus in a protein containing several different tyrosine sites and several tryptophan sites, the circular dichroism spectrum from 270 to 290 mμ may be too complex to interpret.

The tryptophanyl circular dichroism bands of proteins also may be overlapped by disulfide circular dichroism bands (Beychok and Breslow, 1968). Even at 77°K the disulfide circular dichroism bands can extend up to 310 mμ (unpublished experiments). Disulfide bands, however, do not have fine structure, even when frozen in nonpolar solvents at 77°K. Thus in nonconjugated proteins the tryptophanyl circular dichroism bands can be identified on the basis of fine structure located between 290 and 305 mμ.

This background information permits analyzing the circular dichroism spectra of chymotrypsinogen. The four fine structure circular dichroism bands located above 289 mμ must arise from tryptophan residues. Interestingly, the spacings of these bands in chymotrypsinogen are the same as those observed in the tryptophan derivatives (Table II), which suggests that these bands may result mainly from residues having their 0–0 transitions at the same wavelength. If so, the great red shift of the 0–0 ^1L_a band implies that these tryptophan residues are buried within chymotrypsinogen.

In the other proteins the tryptophanyl circular dichroism bands will not necessarily be as simple as those in chymotrypsinogen. Although the tryptophanyl moiety should retain its vibronic structure even in a protein, a variety of circular dichroism spectra are possible, because the protein environment will influence the signs and intensities of the tryptophanyl circular dichroism bands (Schellman, 1966). The circular dichroism spectra of tryptophan derivatives, however, suggest that only certain sign combinations are possible for the prominent ^1L_b bands; they seem to be either both positive or both negative. In proteins containing more than one tryptophan, very complex circular dichroism spectra may occur if each tryptophan has its 0–0 ^1L_a transition at a different wavelength.

As our final point, we note that low-temperature circular dichroism can provide information about conformational mobility. In NAc-L-Trp-amide the rotatory strength at 77°K is 18 times larger than that at 298°K, even though the total

absorption intensity does not increase. The increased rotatory strength appears to indicate the existence of conformational mobility at 298°K (Moscowitz *et al.*, 1963a). NAc-L-Trp-amide may have a number of conformations which are interconvertible by rotations about single bonds in the amino acid moiety. The 298°K circular dichroism spectrum appears to represent the weighted average of all these conformations. For some, the circular dichroism bands of a given vibronic transition may be negative; for others, the same circular dichroism bands may be positive. Thus at 298°K the circular dichroism strength for some conformations would cancel that of others. When the temperature is lowered, the lower energy conformations would become more heavily populated in accordance with the Boltzmann factor. The average circular dichroism strength would increase because a much larger fraction of the tryptophan molecules would have the same circular dichroism spectrum. Since tryptophan has so many overlapping vibronic transitions, the multiple conformations existing at room temperature would tend to cause an especially low circular dichroism strength.

In contrast to NAc-L-Trp-amide, chymotrypsinogen shows relatively little circular dichroism intensification after cooling from 298 to 77°K. The enhanced fine structure in the chymotrypsinogen circular dichroism spectrum at 77°K resulted mainly from the extensive sharpening of absorption bands. The rotatory strength of its tryptophanyl circular dichroism bands increased by only 25%. This finding suggests that only small changes occur in the orientation of the tryptophan residues during cooling. Apparently these residues have a relatively rigid position even at 298°K. The small circular dichroism enhancement in chymotrypsinogen at 77°K does, however, imply that a minor degree of mobility may exist in the tryptophan residues at 298°K.

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