Far-Ultraviolet Absorption and Circular Dichroism Spectra of L-Tryptophan and Some Derivatives

Henry E. Auer*

Contribution from the Department of Biochemistry and the Department of Radiation Biology and Biophysics, University of Rochester School of Medicine, Rochester, New York 14642. *Received July 24, 1972*

Abstract: The far-ultraviolet band structure of tryptophan residues has been investigated in order to characterize their contributions to the spectral properties of proteins. Absorption spectra of 3-methylindole and indole-3-acetic acid derivatives and the absorption and circular dichroism (CD) spectra of L-tryptophan and some derivatives were determined in various solvents at room temperature. Four types of CD spectra were found. All have intense positive ellipticity centered at about 223 nm, but have differing patterns of zero or negative ellipticity, in one or more bands, below about 215 nm. Simultaneous resolution of absorption and CD spectra into Gaussian components yields a consistent set of four bands in the ^1B_b region which is sufficient to describe every spectrum save one, while only one broad band is needed in the ^1B_a region. Solvent effects and vicinal charge effects have been characterized. From our results, we are able to discount the possibility that parts of the observed CD spectra arise from coupling with the $n-\pi^*$ and $\pi-\pi^*$ transitions of carboxyl, amide, or ester moieties.

The far-ultraviolet spectroscopic properties of the aromatic residues in proteins are potentially of great interest, for they are sensitive to subtle changes in their local environments. Investigations in this spectral region are limited, however, because phenylalanine, tyrosine, and tryptophan all have intense transitions which are nearly coincident with each other. In order to characterize the band structure of tryptophan residues, we have obtained far-ultraviolet absorption spectra of some indole derivatives and absorption and circular dichroism (CD) spectra of L-tryptophan and some of its derivatives. Simultaneous resolution of the absorption and CD spectra into Gaussian components has been carried out, yielding a self-consistent pattern of bands. Most of the compounds investigated contain the carboxyl group or a carboxyl derivative, which are themselves chromophores in this spectral region. In order to evaluate their contributions to the adsorption and CD spectra, we have examined the effects of different solvents and of changes in the state of charge of ionizable groups on the recorded spectra. Finally, the spectra of L-tryptophyl-L-tryptophan have been studied in order to determine whether chromophore interaction occurs.

Experimental Section

Materials. All chromophoric solutes were used as provided. L-Tryptophan (Trp) was obtained from Schwarz BioResearch, Inc., Orangeburg, N. Y.; L-tryptophan methyl ester hydrochloride (TME) and L-tryptophyl-L-tryptophan (Trp-Trp) were from Mann Research Laboratories, Inc., New York, N. Y.; and 3-methylindole (skatole, MI), ethyl indole-3-acetate (IAEE), indole-3-acetic acid (IA), indole-3-acetamide (IAM), L-tryptophan ethyl ester hydrochloride (TEE), L-tryptophanamide hydrochloride (TA), and *N*-acetyl-L-tryptophan (NAT) were purchased from Sigma Chemical Co., St. Louis, Mo. Water was glass redistilled. 2,2,2-Trifluoroethanol (TFE) from Eastman Organic Chemicals, Rochester, N. Y., was twice redistilled to attain essentially zero absorbance to 185 nm. Spectroquality cyclopentane (CP) from Matheson Coleman and Bell, East Rutherford, N. J., was used as supplied. Trimethyl phosphate (TMP) from Aldrich Chemical Co., Milwaukee, Wis., was distilled three times at reduced pressure for far-ultraviolet transmission.

Spectra. Absorption spectra were recorded at room temperature on a Cary 14 spectrophotometer purged with nitrogen. A path length of 1.00 mm was used in all cases. An arbitrary amount of solute was dissolved in the solvent, and the spectrum of the solution recorded directly. The maximum absorbance was always between 0.4 and 1.4. Spectra are reported as relative absorbance, normalized to a value of 1.00 at the wavelength of maximum absorbance. They represent the averaged results of duplicate samples. The maximum extinction coefficient for indole has been reported as ϵ_{215} 30,600,[1] for MI in TFE it has been given as ϵ_{220} 28,700,[2] and for Trp in water ϵ_{218} 37,000.[3] A few of the spectral results reported here were obtained on a Unicam SP800 spectrophotometer, which has a practical wavelength limit of 200 nm. The spectral bandwidth on both the Cary and Unicam instruments was 0.5 nm or less.

Circular dichroism spectra were obtained on a Cary 60CD spectropolarimeter equipped with a 6002 CD accessory. Spectra were recorded at the ambient temperature of the instrument, 27°. The spectral bandwidth was programmed at 1.5 nm throughout the wavelength range of interest. Results are presented as molar ellipticities and are based on dry weight concentrations. The spectra represent average values from at least four runs in each case. Error bars indicate standard deviations. Path lengths ranged from 10 to 0.5 mm with concomitant adjustment of concentrations. No deviations from Beer–Lambert behavior were observed. Aggregation of tryptophan and some derivatives was found to be absent at much higher concentrations than those used here.[4] Therefore it is unlikely that any exists in our solutions.

The spectra for aqueous medium which are illustrated in the figures were recorded on unbuffered solutions. NAT (pK = *ca.* 3.7) is almost completely dissociated under these conditions. Trp is present as the zwitterionic (isoionic) species, and TME (pK = *ca.* 7.7) is essentially undissociated. In examining the effect of changing the state of ionization on the wavelength positions of spectral extrema, buffered solutions having pH values equal to the pK ±1.5 units, or more, were used, so that the desired species was present to the extent of 95% or better. Many other experiments were done by adding solid potassium carbonate, or dilute sulfuric acid, to unbuffered solutions of the chromophore in the cuvette. The results were the same as with buffering. In TFE, no acidification experiments were done; but the removal of an acidic proton was apparently readily accomplished by adding a single particle of potassium carbonate.

Curve Resolution. Components of the spectral bands were obtained using a Du Pont 310 Curve Resolver.

(1) H. B. Klevens and J. R. Platt in J. R. Platt and coworkers, "Systematics of the Electronic Spectra of Conjugated Molecules," Wiley, New York, N. Y., 1964, p 145.

(2) A. Cosani, E. Peggion, A. S. Verdini, and M. Terbojevich, *Biopolymers*, 6, 963 (1968).

(3) R. McDiarmid, Ph.D. Thesis, Harvard University, Cambridge, Mass., 1965.

(4) E. H. Strickland, J. Horwitz, and C. Billups, *Biochemistry*, 8, 3205 (1969).

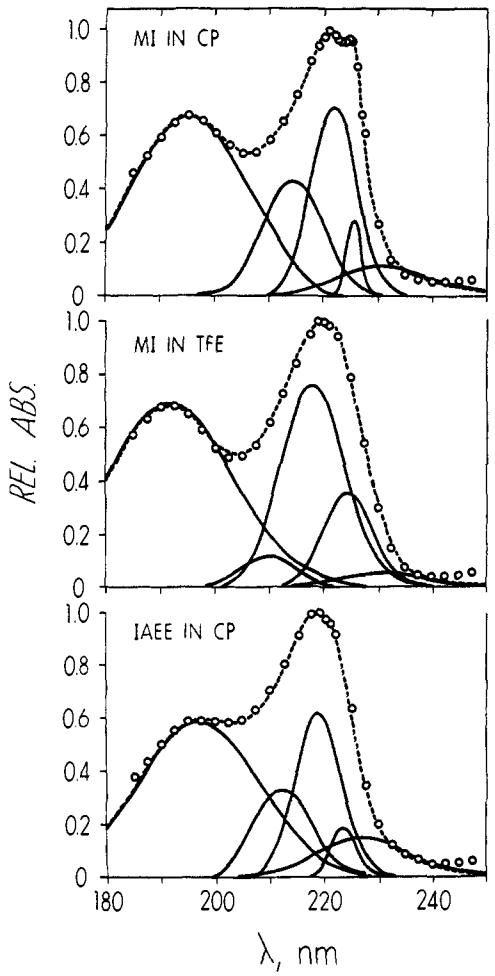

Figure 1. Absorption spectra and resolved Gaussian components of, from top to bottom, 3-methylindole in cyclopentane, 3-methylindole in trifluoroethanol, and indole-3-acetic acid in cyclopentane, plotted as absorbance relative to the absorption maximum: (O-O-O) experimental points, (—) Gaussian components, and (- - -) resultant of Gaussians.

Results

Absorption Spectra of Indole Derivatives. The absorption spectra of MI in CP and TFE and of IAEE in CP are shown in Figure 1 as the experimental points, which were read off the recorded spectra. The resolved spectral components and the resultant fitted curve are also shown. The wavelength positions of the resolved components found for all the absorption and CD spectra reported in this work are presented in Table I. Two bands are evident in the spectra of Figure 1, the ^1B_b at about 221 nm and the ^1B_a at about 195 nm. The relative band locations are consistent with those recently calculated for indole and indole-3-acetic acid.[5] Both bands have been observed in indole,[1] and vibronic fine structure was found in the ^1B_b band of indole in ethanol medium at 93°K.[6] It is seen here that MI in CP exhibits structure in the ^1B_b band at room temperature. This structure is lost in TFE (Figure 1) and in water, evidently because of hydrogen bond formation between these solvents and the indole N–H group. Distinguishable fine structure is also lost upon substitution of a group larger than methyl in the 3 position.

(5) P.-S. Song and W. E. Kurtin, *J. Amer. Chem. Soc.*, **91**, 4892 (1969).
(6) H. Zimmermann and N. Joop, *Ber. Bunsenges. Phys. Chem.*, **65**, 61 (1961).

Table I. Wavelength Positions of Resolved Components in the Spectra of Figures 1–6

Compd	Solvent	Wavelength, nm Abs	CD	Compd	Solvent	Wavelength, nm Abs	CD
MI	CP	195.3		NATEE	TFE	190.0	189.9
		214.1				206.4	
		221.8				215.8	216.3
		225.5				222.2	222.4
		229.3				231.3	230.8
MI	TFE	191.5		TME	Water	191.9	
		209.2				207.2	207.0
		217.8				218.0	217.9
		224.1				225.0	224.6
		229.2				232.3	231.6
IAEE	CP	197.9		TME	TFE	197.2	193.0
		212.0				212.7	213.0
		219.2				218.0	
		223.3				221.0	221.2
		226.4				226.0	225.3
Trp	Water	192.0	193.5	NATEE	CP	191.8	
		206.5	206.8			203.6	203.7
		216.4				209.8	
		222.3	222.6			217.6	
		230.4	231.0			224.2	224.3
NAT	Water	192.6	195.0			226.7	226.2
		208.2	208.1	Trp-Trp	Water	195.5	200.1
		216.6				210.4	210.8
		222.8	222.8			219.2	219.6
		226.2	226.7			224.8	225.3
NAT	TFE	193.0	193.8			233.8	234.4
		210.8					
		216.3	216.1				
		220.8	221.1				
		226.2	226.5				

This may be seen in Figure 1, for example, for IAEE in CP. Absence of structure is also observed with IA, IAEE, and IAM in TFE and in water. In all the spectra studied in this work, the ^1B_b band has somewhat greater intensity and more discernible vibrational fine structure (MI in CP) or more resolvable components than the ^1B_a band, as expected.

Absorption and CD Spectra of Tryptophan Derivatives. The simultaneous analysis of absorption and CD spectra permits the component bands to be resolved, since a given absorption component can contribute positive, negative, or zero optical activity. In this section, therefore, we present the absorption and CD spectra of L-tryptophan and some of its derivatives, as well as the Gaussian components obtained upon curve resolution. A number of different classes of CD spectrum were observed.

Type I. The absorption and CD spectra of Trp and NAT in distilled water are shown in Figure 2. The CD spectra in this class are characterized by a strong positive extremum at 224 nm, a crossover at about 215 nm, a negative shoulder at about 205 nm, and an intense negative extremum at about 195 nm. In the resolved spectra, there are two components contributing to the negative ellipticity, first, a negative contribution from the lowest wavelength component of the ^1B_b band at about 207 nm, and second, a broad, intense contribution from the ^1B_a transition placed near 194 nm. This class is also characterized by the failure of the component having the greatest absorbance in the ^1B_b band, located at 216 nm, to exhibit ellipticity.

The CD spectrum of Trp in water shown here does not exhibit the shoulder at 218 nm nor the negative extremum at 201 nm reported by Myer and Mac-

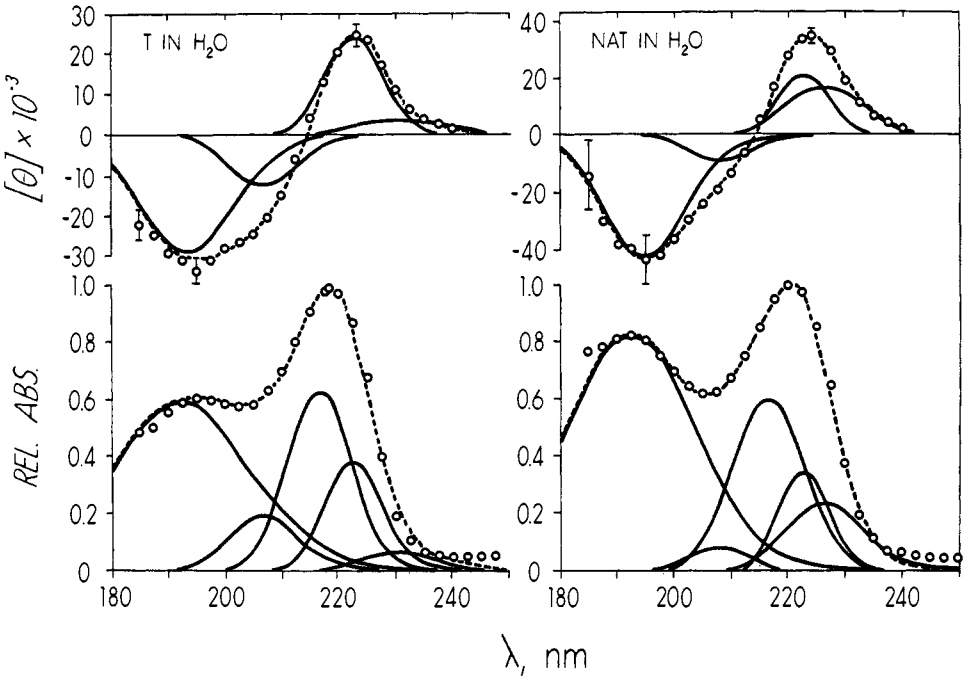

Figure 2. Absorption spectra, CD spectra, and resolved Gaussian components of L-tryptophan in water (left), and *N*-acetyl-L-tryptophan in water (right). Absorption is shown as relative absorbance and circular dichroism as molar ellipticity. Symbols as in Figure 1. Vertical bars indicate standard deviations.

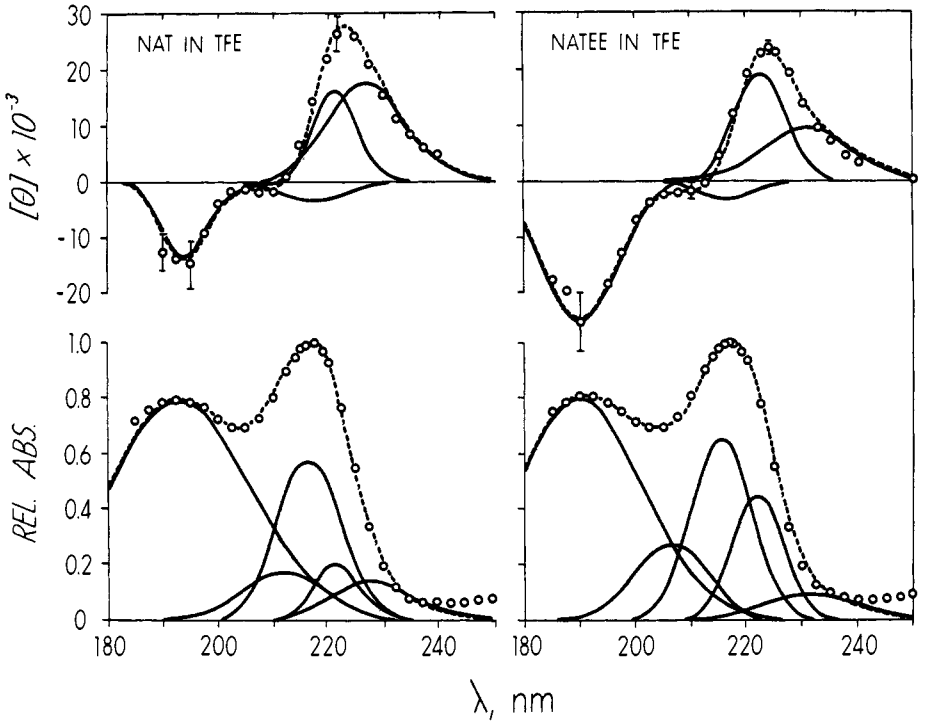

Figure 3. Absorption spectra, CD spectra, and resolved components of *N*-acetyl-L-tryptophan (left) and *N*-acetyl-L-tryptophan ethyl ester (right) in trifluoroethanol. Symbols as in Figure 2.

Donald.[7] Our instrument can readily detect structure when it is present, as can be seen from the figures in this paper. During the course of this work, the CD spectrum of Trp in water was measured on three different instruments, namely, two Cary 60 CD's and one Durrum-Jasco J-20. Virtually identical results were obtained on all the instruments, and the same results were obtained with different sample absorbances. In no case was the spectral structure reported by Myer and MacDonald ever observed.

NAT is almost completely ionized in the dilute solutions used in this work. It was found that the intensities of the CD extrema are diminished by 30–40% upon acidification to the neutral form.

(7) Y. P. Myer and L. H. MacDonald, *J. Amer. Chem. Soc.*, 89, 7142 (1967).

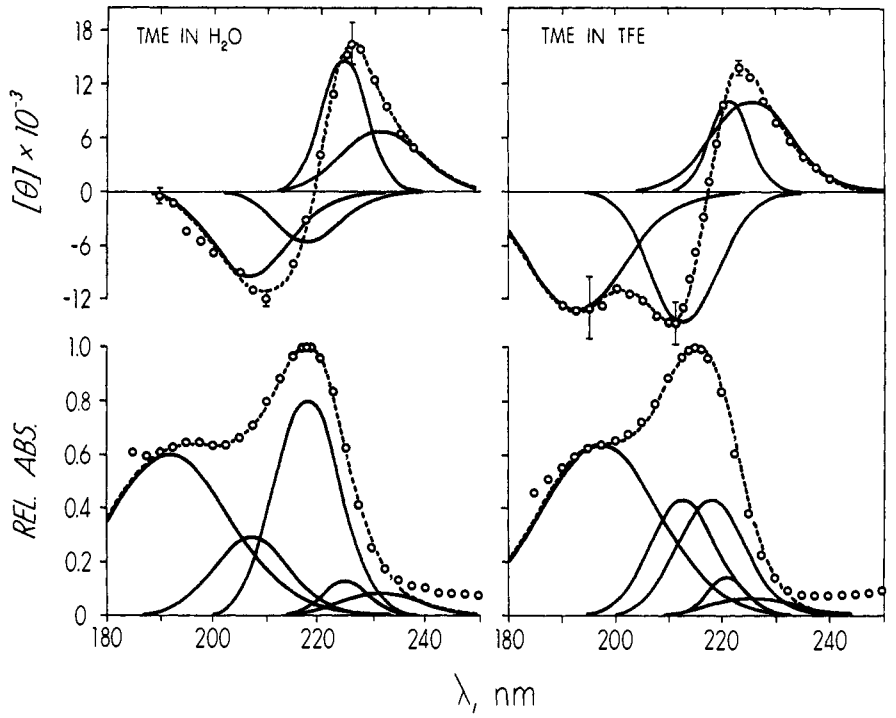

Figure 4. Absorption spectra, CD spectra, and resolved components of L-tryptophan methyl ester hydrochloride in water (left) and in trifluoroethanol (right). Symbols as in Figure 2.

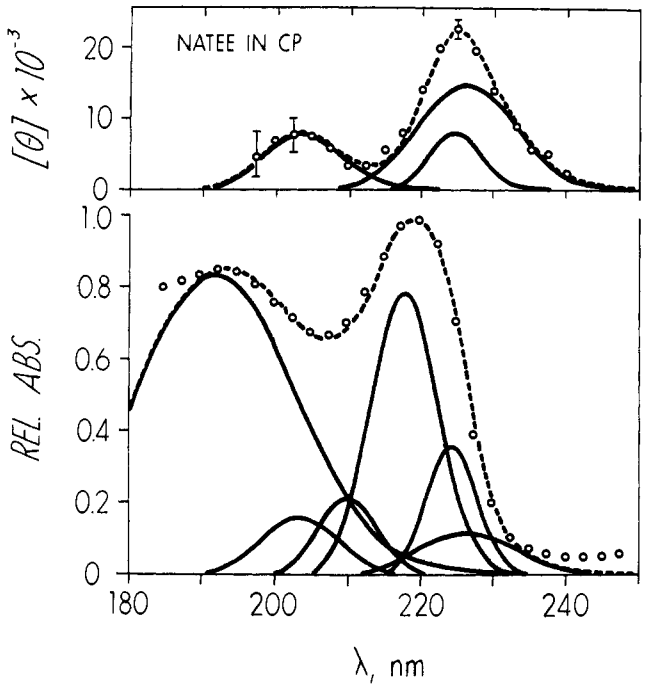

Figure 5. Absorption spectrum, CD spectrum, and resolved components of *N*-acetyl-L-tryptophan ethyl ester in cyclopentane. Symbols as in Figure 2.

The CD spectrum of Trp in TFE is also of type I.

Type II. The absorption and CD spectra of NAT and NATEE in TFE are presented in Figure 3. In this class, there is strong positive ellipticity at about 223 nm, a negative band at 192–195 nm, and negligible ellipticity in the region from 210 to 202 nm. The latter feature arises, in the resolved spectra, from the absence of pronounced optical activity in the components of the ^1B_b transition appearing at 216 nm and at 206–211 nm. This spectral form is also found with NATEE in water, except that the intensities are considerably lower.

Type III. This class of spectrum arises from TME in water and TME in TFE, and is illustrated in Figure 4. In comparison to spectra of the other types, type III CD spectra are characterized by slightly lower amplitudes in the positive extremum, a crossover to negative ellipticity which is at a higher wavelength, and a negative extremum at about 210 nm. The origin of the last two features can be ascribed to the presence of one or two negative components in the range 207 to 218 nm. TME in TFE has an additional negative extremum arising from the ^1B_a transition. This band is completely optically inactive for TME in water.

The CD spectrum of TEE in TFE is similar to that of TME in the same solvent, and the CD spectra of TA and *N*-acetyl-L-tryptophanamide in TFE resemble the spectrum of TME in water.

Type IV. This spectral class, which arises only with NATEE in CP, is characterized by the absence of negative ellipticity, and by the failure of the ^1B_a transition to exhibit any ellipticity (Figure 5). There is the usual positive band, at 225 nm, and a second smaller positive band at 204 nm. Because of the latter band, a total of five components is needed to fit the spectra in the range 230 to 200 nm. Combinations of only four components, including the one derived from the 204-nm CD maximum, could not be found which fitted the observed curves satisfactorily.

CD Spectra for Other Derivatives and Solvents. In addition to the results given above, CD spectra of TME, NAT, and NATEE were examined in TMP. It was found that all the absorption and CD spectra in this solvent are red-shifted by about 5 nm. TME exhibits a spectrum of type III and has no optical activity

Table II. Charge Effects on Wavelengths of Extrema in the Absorption and CD Spectra of L-Tryptophan Derivatives in Water and in 2,2,2-Trifluoroethanol

Compd	Ionization process	pK in water[a]	Solvent	—Δλ (nm) of ^1B_a— Abs	CD	—Δλ (nm) of ^1B_b— Abs	CD
MI	None[b]		H_2O	0.0		−0.3	
			TFE	0.0		+0.1	
NATEE	None[b]		H_2O	0.0		+0.2	
IA	$RCOOH \rightarrow RCOO^-$	4	H_2O	−0.5		+1.5	
			TFE	−0.5		+2.6	
NAT	$RCOOH \rightarrow RCOO^-$	3.67	H_2O	−1.5	−2.0	+1.0	+0.5
			TFE	−2.0		+1.4	
TME	$RNH_3^+ \rightarrow RNH_2$	7.73	H_2O	−2.5		+1.8	
			TFE	−1.6[c]		+1.8	
TEE	$RNH_3^+ \rightarrow RNH_2$	7.83	TFE	−1[c]	*d*	+2.8	+1.5
TA	$RNH_3^+ \rightarrow RNH_2$	7.5	TFE	−1[c]	*d*	+2.6	+3 (−)[e] +2.5 (+)[e]
Trp	$(RNH_3^+)COOH \rightarrow (RNH_3^+)COO^-$	2.38	H_2O	−2	−2	+1.2	+1
Trp	$(RCOO^-)NH_3^+ \rightarrow (RCOO^-)NH_2$	9.39	H_2O	−2.2		+2.2	+2.5

[a] Data for Trp or appropriate analog taken from "Handbook of Biochemistry," H. A. Sober, Ed., Chemical Rubber Co., Cleveland, Ohio, 1968. [b] Control experiment comparing spectra after adding solid K_2CO_3 *vs.* those without K_2CO_3. [c] The absorption band is an unresolved shoulder in the RNH_3^+ species. [d] $[\theta] = 0$. [e] (−), negative extremum, (+), positive extremum.

arising from the ^1B_a transition. The spectrum therefore resembles that of TME in water. The CD spectra of NAT and NATEE in TMP are examples of type II, which was originally defined as arising from the same two compounds in TFE as solvent. Since the appearance of the CD spectra in TMP is not novel, resolution of the observed curves into components was not carried out. A survey of the CD spectra for some other combinations of L-tryptophan derivatives and solvents likewise revealed no new features of interest, as noted in the preceding paragraphs. It seems, then, that the ellipticity patterns of L-tryptophan at room temperature may be completely represented by the set of four spectral types presented above. The central features are a region of positive ellipticity with a maximum at about 223 nm and one or more prominent negative ellipticity bands below 215 nm.

Trp-Trp in Water. The spectra for this case are shown in Figure 6. The molar ellipticity is calculated per mole of tryptophan residues. The main differences with respect to the spectra of Trp in water are that, first, the CD intensity is much lower, second, the ^1B_a band has a relatively greater absorption intensity in the case of Trp-Trp, and, finally, there, is a negative extremum in the CD spectrum at about 201 nm. When subjected to curve resolution, this extremum appears to arise largely from a negative CD component at 198 nm, which has no obvious counterpart in the absorption spectrum.

Vicinal Charge Effects. The effect of changing the state of charge of the indole and tryptophan derivatives on the spectra was assessed, and the results are presented in Table II. In the absence of ionizable groups, no changes in spectral form should be observed upon adding an acid or a base. It was found that the addition of a small solid particle of potassium carbonate to solutions of MI in water, MI in TFE, and NATEE in water had a minimal effect on the positions of the absorption maxima. On the other hand, spectral shifts may occur when an ionizable group undergoes a change in its state of charge. As seen in Table II, it was found that in the absorption spectra the ^1B_a band is shifted between 0.5 and 2.5 nm to the blue, and the ^1B_b band

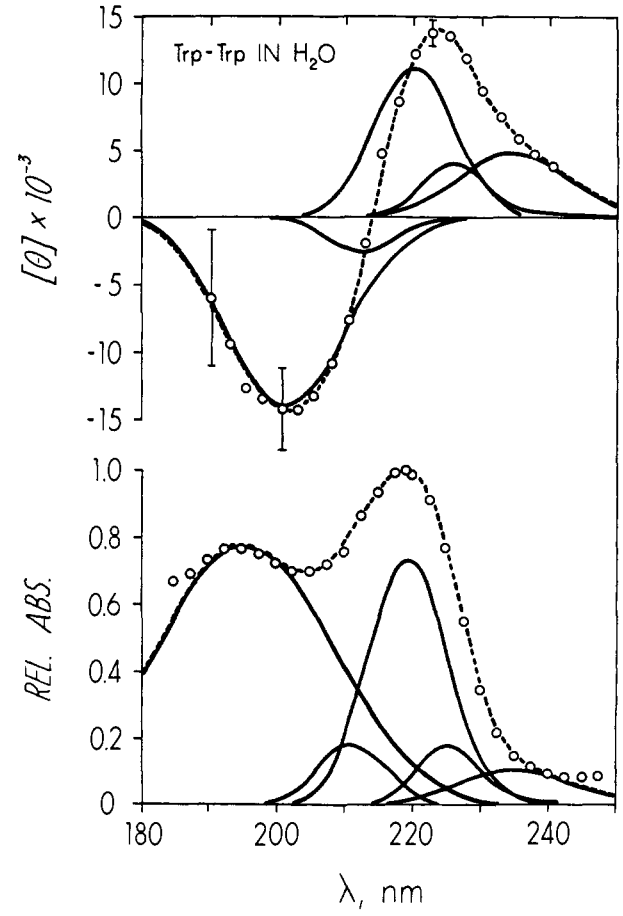

Figure 6. Absorption spectrum, CD spectrum, and resolved components of L-tryptophyl-L-tryptophan in water. The molar ellipticity is calculated per mole of tryptophan residues. Symbols as in Figure 2.

is shifted between 1.0 and 2.8 nm to the red, when the charge on the molecule is made less positive or more negative. In every case where the CD spectral shifts were determined concurrently, the sense and the extent of the shifts are the same as in the corresponding absorption spectra. In no case was the CD band shape

Table III. Solvent Effects on Wavelengths of Extrema in the Absorption and CD Spectra of L-Tryptophan Derivatives

Compd	Band	Wavelength (nm) of absorption maxima in solvent[a] TFE	H_2O	CP	TMP	Wavelength (nm) of (+) or (−) CD extremum in solvent[a] TFE	H_2O	CP	TMP
MI	^1B_a	191.5	192.5	195.0					
	^1B_b	219.5	221.2	222.5[b]					
IAEE	^1B_a	194.5	194.8	197.0					
	^1B_b	216.0	217.2	218.5					
IAM	^1B_a	191.5	194						
	^1B_b	215.0	218.2						
Trp[c]	^1B_a	197[d]	196			193.5 (−)	195.0 (−)		
	^1B_b	216.2	219.5			220.5 (+)	223.0 (+)		
NAT[e]	^1B_a	193.4	194.2			192.5 (−)	197 (−)		198 (−)
	^1B_b	217.5	220.8			222.0 (+)	224 (+)		228.5 (+)
TME[e]	^1B_a	197.2[d]	196.2			192.5 (−)	[f]		[f]
	^1B_b	215.0	217.5			210 (−)	210.0 (−)		212 (−)
						226.0 (+)	226.0 (+)		228.0 (+)
NATEE	^1B_a	191.2	193.0	193.2	195	190 (−)	193 (−)	[f]	198 (−)
	^1B_b	216.8	219.0	219.5	223.4	223.5 (+)	223.0 (+)	224.8 (+)	228.0 (+)

[a] TFE = 2,2,2-trifluoroethanol ($n^{20}D$ 1.28); CP = cyclopentane ($n^{20}D$ 1.405); TMP = trimethyl phosphate ($n^{20}D$ 1.397). [b] Approximate center of gravity of band. [c] Present as the zwitterion. [d] Shoulder. [e] Present as the acidic form. [f] $[\theta] = 0$.

altered upon changing the state of ionization. It should be noted that the charge effects found here are the same regardless of whether a positively charged molecule is transformed into a neutral species, or a neutral species acquires a negative charge.

General Solvent Effects. The wavelength shifts of the absorption and CD extrema of the chromophoric derivatives incurred upon changing solvents were determined in order to characterize the nature of the transitions involved. The results are presented in Table III. Trp is most likely present as the zwitterion in TFE, since in a similar medium, 90% ethanol, this is the predominant species for every amino acid considered.[8] For each of the two transitions of the indole chromophore examined, the absorption and CD spectral extrema suffer concurrent red shifts as the refractive index of the solvent increases. The only exceptions to this observation are for the ^1B_a absorption bands of Trp in TFE and TME in TFE. In both cases these bands are unresolved shoulders under the envelope of the ^1B_b band, so that it is difficult to determine their positions accurately. They acquire their own identity as the solvent is changed to water, with an apparent blue shift. We attribute this to the imprecision of the band position in TFE. Note that for Trp the corresponding CD band undergoes a red shift. In no other case was it found that the sense of the solvent shift differed between the absorption spectra and the CD spectra. The solvent effect caused by TMP is particularly striking, amounting in many cases to 5 nm or more over TFE.

Discussion

Resolution of Spectra into Gaussian Components. The simultaneous resolution of absorption and CD spectra is conveniently achieved with an analog device such as the Du Pont Curve Resolver. Since operator bias can affect the results obtained, it is appropriate to summarize the principles by which the resolved components presented here were obtained. First, the chromophore in question, the 3-substituted indole ring, is common to all the solutes studied. We should therefore expect to be able to resolve the spectra of all of them using the same set of components, and to maintain approximately the same relative weights of the components in all the absorption spectra. Second, if a resolved component is derived from a true vibronic level, the absorption band and the possible circular dichroic band arising from it should have the same wavelength and band width. Accordingly, the absorption and CD spectra of a given solute were resolved simultaneously, using the same set of components with no changes in position or width. Only the amplitudes were adjusted to produce the best possible fit to the observed curves. With these restrictions the freedom with which components can be generated to fit the curves is considerably diminished.

Gaussian components, rather than Lorentzians, were used exclusively in the curve resolution presented here. In a study of the low-temperature absorption and CD spectra of the ^1L_b and ^1L_a bands of L-tryptophan derivatives, the vibronic components, which were resolved experimentally, were excellently simulated using Gaussians, while Lorentzians were completely unsatisfactory.[4] The spectra in that work were plotted on a wavelength scale. Other examples of the simultaneous resolution of absorption and CD spectra, presented on a wavelength abscissa, include the Soret bands of some heme proteins[9] and the peptide bands in synthetic polypeptides.[10] In the latter case as well as in our spectra, the bands are skewed toward short wavelength, and this skew would be enhanced if plotted *vs.* frequency. Although distribution curves exist which are capable of fitting the absorption envelopes of skewed bands, it is not our intention merely to fit the overall shape of the bands. Rather, we seek to fit conjugate absorption and CD spectra for a number of related derivatives and then to examine the similarities between them. Accordingly, the most suitable approach has been to achieve a resolution into the minimum number of Gaussian components consistent with the observed spectra. It has been observed that carrying out such curve resolutions using components which are Gaussian in wavelength introduces a certain degree of skewness into the components, with respect to a frequency plot,

(8) E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold, New York, N. Y., 1943, p 107.

(9) G. E. Willick, G. R. Schonbaum, and C. M. Kay, *Biochemistry*, 8, 3729 (1969).

(10) D. W. Urry, *Annu. Rev. Phys. Chem.*, **19**, 477 (1968).

which may assist in getting satisfactory results.[10] It would be attractive to ascribe physical significance to all the resolved components depicted in this work. While the observation of fine structure in the absorption spectrum of MI in CP and the occurrence of closely spaced resolved components in the CD spectra suggest that the bands represent actual vibronic components, we make no assertion that this is universally the case at present. The experimental resolution attainable under the conditions of these spectra is not great enough to warrant any such identification.

It was found that, in all, four components were required to fit the spectra in the region of the ^1B_b band. From the outset, it was necessary to employ three bands of considerable amplitude to fit the absorption spectrum of MI in CP. These were the bands at 225.5, 221.8, and 214.1 nm. The envelope of the ^1B_b band in many of the other absorption spectra, in which no fine structure is observable, could have been adequately fitted with only two significant components. All three were retained, however, since none of the observed CD patterns could be synthesized using fewer components, and the fitting of the absorption spectrum of MI in CP required them. This was done in conformance with the principle stated above. The fourth band in this scheme was introduced to account for the considerable ellipticity occurring in most of the spectra in the region above 225 nm, even though the corresponding absorption intensity is rather modest. In general, the relative positions, bandwidths, and absorption intensities of these four components were similar from compound to compound.

The ^1B_a absorption band has a broad unstructured appearance. It could be universally fitted with a single broad Gaussian component. Negative deviations from the experimental points on the short-wave side of this band have been tolerated in the case of the derivatives, since there are other chromophores present in these molecules. In fitting the CD spectra in this region, greater liberty was permitted than in the case of the ^1B_b spectra, since there may be unresolved optically active vibronic bands under the observed absorption envelope. Changes in position and decreased bandwidths from those of the absorption band were allowed. In no case was more than one component required to fit the CD spectra in this region. The assignment of these ellipticity bands to the ^1B_a transition of the side-chain chromophore cannot be made unambiguously, however, since negative ellipticity can also arise from the amide $\pi-\pi^*$ transition, as in the 193-nm band of *N*-acetyl-L-alanine-*N'*-methylamide.[11] In our CD spectra, there is neither positive nor negative correlation between the existence of negative ellipticity below 200 nm and the presence of an amide substituent; furthermore, negative CD bands in this region were observed for Trp and TME. Therefore, this optical activity is at least partially ascribable to the ^1B_a transition of the indole chromophore.

The case of NATEE in CP represents an apparent exception to these observations. An additional component situated at 204 nm was needed to fit the CD curve. This is in the region of strong overlap between the ^1B_b and ^1B_a transitions. It is likely that the new component is a member of the set of ^1B_b bands, since it is this transition which is expected to have observable vibronic structure, and the solvent, CP, is one which enhances vibronic structure. On the other hand, since NATEE was studied at saturation in CP, aggregation cannot be ruled out as a possible cause for the observation of the new component. No other spectral evidence of aggregation was observed. None of the other tryptophan derivatives is soluble in CP.

A ^1C Transition in Indole? Klevens and Platt found that the symmetry-forbidden ^1C transitions can be observed in certain fused ring compounds.[12] Zimmermann and Joop[6] cited the presence of extrema in the excitation polarization spectra of isoquinoline and isoquinolinium ions as evidence supporting the existence of a weak band, identified as one of the ^1C transitions, at 240–250 nm. A less prominent feature was found at 235 nm in the corresponding spectrum of indole, but they make no mention of it in the text. In our resolved spectra, there is a broad component having low absorption intensity but pronounced ellipticity in every case examined. Its position ranges from 225 to 232 nm. This is almost coincident in wavelength with the feature observed in indole by Zimmermann and Joop. The breadth of this band is maintained even in CP in which the other resolved components of the ^1B_b transition are somewhat narrower. It occurs at a longer wavelength than the first of the fine structure peaks observed for MI in CP, which is probably the 0–0 band of the ^1B_b transition. Conceivably, then, this component is not part of the ^1B_b system. We wish to raise the possibility that it arises from the same ^1C transition as observed in isoquinoline and its cation.

The Optical Activity of Tryptophan Residues. Four CD spectral types have been found to arise with tryptophan derivatives upon changes in solvent and substitution of the amino and carboxyl groups. In addition, the wavelength extrema of the spectra have been shown to be susceptible to charge effects and solvent effects. In proteins, therefore, the far ultraviolet CD spectra of tryptophan residues may be expected to be quite sensitive to the detailed properties of the microenvironment.

Every tryptophan derivative examined exhibits intense positive ellipticity in the long-wave region of the ^1B_b transition, with amplitudes ranging from 15,000 to 30,000 deg cm^2 dmol^{-1}. The wavelength of maximum ellipticity is almost coincident with the wavelength of the negative extremum in the CD spectrum of α-helical polypeptides, and the CD spectrum of the β structure has considerable negative ellipticity in this region as well. Consequently CD spectral investigations of the polypeptide conformation of proteins with high contents of tryptophan will be required to take this contribution into account. Below about 215 nm, the CD spectral forms exhibited by tryptophan derivatives are quite variable, depending both upon the nature of the derivative and upon the solvent. Some of the spectra have intense negative extrema in the region between 200 and 190 nm. Although it is not possible to predict the nature of the ellipticity behavior of tryptophan residues in proteins from the work presented here, the possibility of intense negative contributions should be considered, based on our results.

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The marked variations of the CD spectral patterns of the tryptophan derivatives illustrated in Figures 2–5 arise upon changes in solvent, and upon substitution of the α-amino and α-carboxyl groups. Changes in the state of charge of ionizable groups had no effect on the spectral type. Among possible origins for these effects, we wish to make note of two. First, changes in solvation may profoundly affect the ellipticity. For example, upon changing solvents, a striking inversion of the CD spectrum has been observed in the case of an optically active cobalt coordination complex which is sufficiently rigid to be incapable of conformational alteration.[13] Second, suppose there are two or more conformers of moderate stability, each having its own characteristic rotatory power. Then changes in substituent, for example, can affect the equilibration between the conformers and so influence the observed CD spectrum. Conformational effects on CD spectra have been characterized by Moscowitz, *et al.*, for some steroid and bridged ring systems.[14] Conformational equilibrium may be important in the case of the aromatic amino acids and their derivatives, for the bulkiness of their side chains restricts the orientation of the aromatic group with respect to the amino and carboxyl groups.[15] On the other hand, we have found instances in which the CD spectrum of a given derivative is the same in different solvents. These include TME in water and in TMP, NAT in TFE and TMP, and NATEE in TFE and TMP. It appears safe to conclude that the conformation is unaffected by the change in solvent in these cases.

Trp-Trp in Water. The absorption spectrum of this derivative may be treated in the simplest approximation as being composed of contributions from Trp plus NAT, or alternatively from L-tryptophanamide plus Trp. The spectrum, which resembles that of many of the monomeric derivatives, is not inconsistent with such a simplification. Even the absorption spectrum of poly-L-tryptophan in TFE[2] is similar to those presented here. The CD spectrum of Trp-Trp, however, suggests that dipole–dipole interaction between the side chains is occurring, since diminished amplitude and the appearance of a new Gaussian component at 198 nm which is uncharacteristic of the monomer spectra were observed. If the latter feature arises from splitting in the ^1B_a band, a positive CD component is to be expected at lower wavelengths. We have an unusually large standard deviation for the CD spectrum of Trp-Trp in water at low wavelengths, and we were unable to obtain readings below 190 nm. With these limitations, no positive component was observed. In this connection, the CD spectrum of α-helical poly-L-tryptophan[16] in TFE has paired positive and negative extrema at 192 and 210 nm, respectively, and another positive extremum at 225 nm,[2] with intensities that are an order of magnitude higher than for any of the monomeric derivatives examined in this work.

Optical Activity of the Carboxyl Chromophore. The derivatives examined in this work contain various forms of the carboxyl chromophore, to wit, carboxylic acids, carboxylate anions, and carboxylic esters and amides. The $n-\pi^*$ transitions in these derivatives occur in the range 200–230 nm,[17] and the corresponding $\pi-\pi^*$ transitions occur in the far and vacuum ultraviolet regions.[3,18] Absorption from these bands contributes to the spectra in the wavelength region under examination here. It is therefore conceivable that some of the variability in optical activity found in the tryptophan derivatives is ascribable to these transitions.[11] Three aspects of our studies bear on this question. First, if the $n-\pi^*$ transitions are optically active, there should be resolved components in the CD spectrum where there are none in the absorption spectrum. This is because $n-\pi^*$ transitions can be strongly magnetically allowed but are electrically forbidden. With an extinction coefficient on the order of 1% that of the indole chromophore, an $n-\pi^*$ transition would be undetectable in our system. In addition, it should be found that the resolved components arising from carboxyl transitions vary as the nature of the carboxyl derivative changes. In this work, carboxylic acid, ester, and amide groups have been systematically varied. In no case, except for NATEE in CP, was there a CD component without a preexisting absorption component, and in all cases, the absorption and CD spectra could be fitted using similar sets of components, although our conditions for obtaining good fit in the ^1B_a region were somewhat relaxed. Second, in studies of charge effects on the spectra, it was found that changes in ionization caused characteristic shifts in the wavelengths of maximum absorption which were matched by identical peak shifts in the CD spectra in all cases measured. The same effects were obtained upon inducing carboxyl–carboxylate transformations as with the ionization of amino groups, which have no transitions at all in this spectral region. Also, the CD spectral form was never altered by these ionization processes. These observations are more characteristic of the effects of vicinal charges on the indole chromophore than on the carboxyl group, since the absorption intensities of the former are much greater than those of the latter. Finally, the absorption and CD spectra of the tryptophan derivatives examined here appear to suffer general solvent shifts. In TFE, a solvent of relatively low polarizability, the bands are at shorter wavelengths than in water, CP, and TMP, whose polarizabilities are considerably higher. This behavior is most pronounced in the latter solvent, where shifts to the red of about 5 nm were observed. It is significant that the same trend occurs in CP, whose dipole moment is zero, and in TMP, which has a high dipole moment. The effects are the same on the absorption maxima and the CD extrema, and the behavior is independent of the nature of the tryptophan derivative. These characteristics are most readily rationalized as arising from the solvent shifts of $\pi-\pi^*$ transitions, in which the excited states are more polar than the ground states and therefore suffer red shifts in more polarizable solvents. In summary, the observation of common spectral components, the occurrence of vicinal charge effects, and the general solvent effects

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are inconsistent with the hypothesis that carboxyl $n-\pi^*$ and/or $\pi-\pi^*$ transitions give rise to portions of the optical activity observed here. Rather, it is more likely that these spectral properties arise directly from the $\pi-\pi^*$ transitions of the chromophore common to all the derivatives studied, the indole residue of the tryptophan side chain. These conclusions apply with greater strength to the region of the ^1B_b band, due to the greater ability to resolve spectral components in this region. In the ^1B_a region contributions to the observed optical activity from amide $\pi-\pi^*$ transitions, as in the case of *N*-acetyl-L-alanine-*N'*-methylamide,[11] cannot be excluded on the basis of our present observations. Finally, it appears that in proteins the peptide chromophore will not couple with the tryptophan side-chain chromophore to generate new absorption or ellipticity bands.

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Nuclear Magnetic Resonance Study of the Interaction of Neodymium(III) with Amino Acids and Carboxylic Acids. An Aqueous Shift Reagent

A. Dean Sherry,[1] Calvin Yoshida, Edward R. Birnbaum,* and Dennis W. Darnall[2]

Contribution from the Department of Chemistry, New Mexico State University, Las Cruces, New Mexico 88003. *Received August 8, 1972*

Abstract: Complexes of neodymium(III) with alanine, histidine, threonine, serine, acetate, butyrate, and 4-aminobutyrate have been studied in aqueous solution by proton magnetic resonance spectroscopy and potentiometric titrations. Stability constants for the various neodymium(III) complexes have been determined using an nmr shift method. The stability constants determined by the nmr method agree well with those determined by potentiometric titration.

Over the past few years there has been a great deal of interest in the use of various β-diketone complexes of lanthanide ions as shift reagents for use in the assignment of nmr spectra of complex organic molecules containing a donor atom.[3] These shift reagents have been used in noncoordinating organic solvents, and the size of the shifts produced generally obeys the $(3\cos^2\theta - 1)r^{-3}$ relation derived by McConnell and Robertson.[4] Armitage, *et al.*,[5] have recently discussed a procedure for evaluating the binding constants of complexes of amines and alcohols with one of the shift reagents, tris(dipivalomethanato)europium(III), whereas Kelsey[6] has carried out a similar study of complexes of organic acetates with tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato)europium(III). Measurement of the induced shifts (δ) as a function of the Eu^{3+} complex concentration [EuL] at high substrate concentrations yields a plot of δ^{-1} *vs.* [EuL] which is linear. The binding constant is determined from the intercept and the chemical shift (ΔH_0) of the "bound" substrate is obtained from the slope. This method assumes only one substrate molecule binds one molecule of the Eu^{3+} shift reagent.

The shifts induced by the addition of lanthanide salts to aqueous solutions of amino acids have only recently been shown to obey the $(3\cos^2\theta - 1)r^{-3}$ relation.[7,8]

We have been interested for some time in using the lanthanide ions as spectroscopic probes of calcium ion binding sites in proteins.[7,9-11] Before an understanding of the aqueous protein systems can be realized, however, it is necessary to investigate model aqueous systems. We report here an nmr method of determining stability constants of amino acids and carboxylic acids in aqueous solution. We have compared the stability constants obtained with the nmr tech-

(1) Postdoctoral Fellow of the National Institute of Neurological Diseases and Stroke (NS-02557), U. S. Public Health Service.
(2) Career Development Awardee (K4-GM-32014) from the National Institutes of Health, U. S. Public Health Service.
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