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Effects of Hydrogen Bonding and Solvents upon the Tryptophanyl ^1L_a Absorption Band. Studies Using 2,3-Dimethylindole†

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ABSTRACT: To gain information about the spectral properties of tryptophanyl side chains buried within proteins, the absorption spectra of 2,3-dimethylindole were examined in a variety of solvent systems. Comparing the spectra of 2,3-dimethylindole and 3-methylindole indicates that methylation at the 2 position causes the 0–0 ^1L_a band to red shift away from the 0–0 ^1L_b band even in the nonpolar solvent methylcyclohexane. Thus 2,3-dimethylindole can be used to measure the red shift of the ^1L_a band due to hydrogen bonding the indolyl >NH group in a nonpolar solvent. At low concentrations of hydrogen acceptor molecules, the ^1L_a red shifts are: 3–5 nm for 1-butanol or ethyl acetate, 6–8 nm for *N*,*N*-dimethylacetamide, and 7–9 nm for 1,2-dimethylimidazole. Hydrogen bonding either indole or 2,3-dimethylindole causes only a 0.5–1.5-nm red shift of the 0–0 ^1L_b band. With 1-methylindole, which cannot form a hydrogen bond, no significant spectral shifts occur at low acceptor concentrations. Altering the bulk solvent (water and perfluorocarbons), however, shifts the absorption bands of both >NCH_3 derivatives (1-methylindole, 1,2-dimethylindole) and >NH derivatives (indole, 3-methylindole, 2,3-dimethylindole). Interestingly, water shifts the ^1L_a band to longer wavelengths and the ^1L_b band to shorter wavelengths, whereas perfluorohexane shifts both bands to shorter wavelengths relative to their positions in methylcyclohexane. The results from studying model compounds suggest that in proteins the ^1L_a band may be red shifted by about 3–10 nm due to hydrogen bonding the indolyl >NH to other protein moieties. The largest red shifts are expected for the following hydrogen acceptors: carboxylate ions, —N̈= of histidyl side chain, and the carbonyl oxygens of the peptide backbone and of side-chain amides. Even when an indolyl ring is not hydrogen bonded, the wavelength of the ^1L_a band may be shifted due to the local polarizability and due to nearby polar groups of either the protein or the solvent.

The near-ultraviolet absorption spectrum of tryptophan consists of two overlapping electronic transitions (the ^1L_a and ^1L_b bands). Both of these π–π^* bands are polarized in the plane of the indolyl ring with their transition directions oriented nearly perpendicular to each other (Bernardin, 1970; Konev, 1967). The ^1L_b transition has well-resolved vibronic

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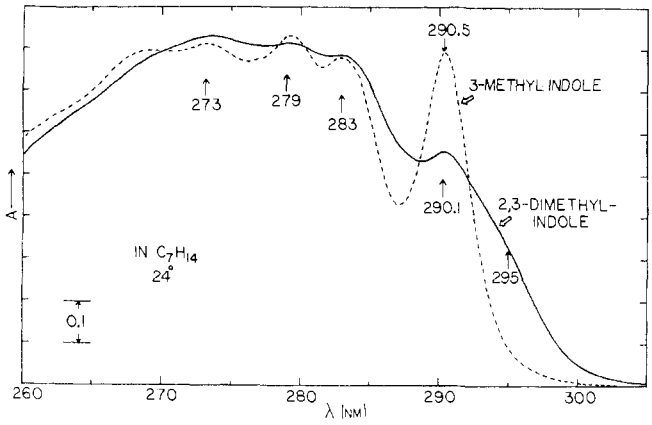

FIGURE 1: Comparison of the absorption spectrum of 1.20×10^{-4} M 2,3-dimethylindole (—) with that of 1.18×10^{-4} M 3-methylindole (- - -) dissolved in methylcyclohexane at 24°; 1.0-cm path length.

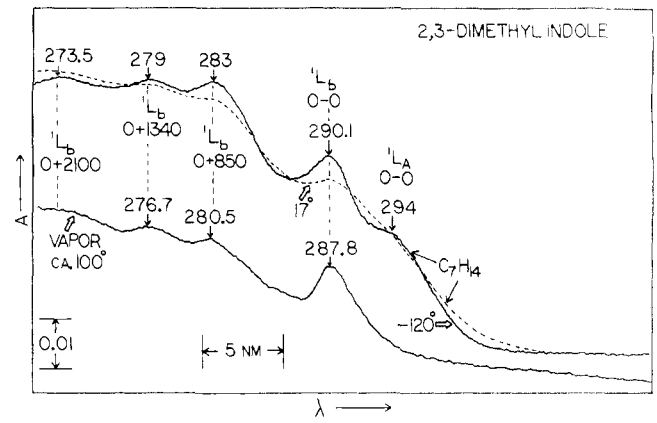

FIGURE 2: Absorption spectra of 2,3-dimethylindole in the vapor state (—, bottom trace) and dissolved in methylcyclohexane at 17° (- - -) and −120° (—, top trace). Path lengths were 10.0 cm for vapor and 1.0 cm for solutions (8×10^{-6} M). The solution spectra were offset 0.006 unit above the vapor trace. Base lines were approximately flat. The 0 + 850 cm^{-1} ^1L_b band may be a composite of 2 unresolved transitions (Strickland *et al.*, 1970).

bands, whereas the ^1L_a transition has relatively broad vibronic bands (Bernardin, 1970; Strickland *et al.*, 1970). When *N*-stearyl-L-tryptophan *n*-hexyl ester is dissolved in nonpolar solvents such as methylcyclohexane, the 0–0 ^1L_a and 0–0 ^1L_b bands occur at about the same wavelength (289.5 nm). Polar organic solvents shift the 0–0 ^1L_a band to the red side of the 0–0 ^1L_b band (Strickland *et al.*, 1969, 1970, 1971). For tryptophan derivatives dissolved in polar solvents, the exact amount of red shift has been difficult to measure, because the ^1L_a bands are broad and not sufficiently separated from the ^1L_b bands. In the absorption spectra of some proteins, however, the wavelength positions of both the 0–0 ^1L_a and 0–0 ^1L_b bands are clearly resolved after cooling to −196° (Strickland *et al.*, 1971). For example, the 0–0 ^1L_a band of the single tryptophan residue in horseradish peroxidase isoenzymes is shifted 12 nm to the red side of the 0–0 ^1L_b band.

Interactions causing the large red shift of the ^1L_a band are examined in this communication. Specifically the effects of hydrogen bonding and solvents are examined by using 2,3-dimethylindole as a model compound for tryptophan, which is a derivative of 3-methylindole. The additional substitution at the 2 position shifts the 0–0 ^1L_a band further to the red side of the 0–0 ^1L_b. This permits measuring the approximate wavelength position of the 0–0 ^1L_a in a variety of solvent systems.

Materials and Methods

Absorption spectra were recorded on a Cary Model 15 spectrophotometer. For the low temperature spectra, the concentration of 2,3-dimethylindole was kept below 10^{-5} M to avoid aggregation upon cooling in a nonpolar solvent.

2,3-Dimethylindole and 1,2-dimethylimidazole were obtained from Aldrich Chemical Co. 1-Methylindole was purchased from K and K Laboratories. 1,2-Dimethylindole was kindly supplied by Professor Rufus Lumry (University of Minnesota). Perfluoromethylcyclohexane was obtained from Pierce Chemical Co. *N*,*N*-Dimethylacetamide and other solvents were spectral quality. 1,2-Dimethylimidazole was distilled under vacuum (20 mm, bp 102°) and stored over KOH pellets in a desiccator to remove any traces of water which may have been present.

The concentrations of methylcyclohexane solutions were calculated using the ϵ value (6900) determined for both 2,3-dimethylindole (273.5 nm) and 3-methylindole (273 nm) by Pappalardo and Vitali (1958).

Results

Identification of the 0–0 ^1L_a and ^1L_b Vibronic Bands. The two overlapping electronic transitions can be distinguished by their differential response to perturbations such as methylation and solvation (Strickland *et al.*, 1970). First of all, the absorption spectra of 2,3-dimethylindole and 3-methylindole may be compared in methylcyclohexane (Figure 1). Within the resolution possible for solution spectra, the sharper vibronic bands occur at the same wavelengths (290, 283, 279, and 273 nm) for both compounds. These are the ^1L_b vibronic bands previously identified in 3-methylindole (Strickland *et al.*, 1970).

The spectra differ mainly in three respects: (A) 2,3-dimethylindole has a broad shoulder (*ca.* 295 nm) that is absent in 3-methylindole; (B) the intensity of the 290-nm band is about 30% less in 2,3-dimethylindole than is the case with 3-methylindole; and (C) at the short-wavelength edge of the near-ultraviolet band, the 2,3-dimethylindole spectrum is shifted slightly to the red of the 3-methylindole spectrum. These differences are readily understood in terms of the previous assignment of bands in 3-methylindole (Strickland *et al.*, 1970). The ^1L_b vibronic bands are superimposed upon numerous ^1L_a bands, which are relatively broad even in methcylcyclohexane. The 0–0 ^1L_a band, which overlaps the 0–0 ^1L_b band in 3-methylindole, is red shifted to about 295 nm in 2,3-dimethylindole. The remaining ^1L_a vibronic bands, although too broad to be individually resolved, are also shifted by the same amount (in wave numbers) as the 0–0 ^1L_a band. Thus methylation at the 2 position red shifts the ^1L_a bands of 3-methylindole, but does not much affect the ^1L_b bands.

Our identification of the 0–0 ^1L_a band is substantiated by comparing the spectra of 2,3-dimethylindole in the vapor phase and in solution (Figure 2). To facilitate observing the differential shift of the ^1L_a band caused by methylcyclohexane perturbation, the 0–0 ^1L_b band of the vapor spectrum has been arbitrarily aligned with the 0–0 ^1L_b band in the solution spectrum. Thus the remaining ^1L_b vibronic transitions should coincide also. Bands with an altered spacing from the 0–0 ^1L_b band may be identified as ^1L_a transitions. This procedure reveals the same ^1L_b vibronic bands as did the com-

TABLE I: Effects of Hydrogen-Bond Acceptors upon the Absorption Bands of Indole Derivatives Dissolved in Methylcyclohexane at 24°.

Proton Acceptor[a]	2,3-Dimethylindole, Δλ (nm)		Indole		1-Methylindole, Δλ (nm)	
			Δλ (nm)	K[c]		
	0–0 ^1L_a	0–0 ^1L_b[b]	0–0 ^1L_b	(M^{-1})	^1L_a[d]	0–0 ^1L_b[e]
1-Butanol	3–5[f]	0.5	0.8	~2	<1.0	0
Ethyl acetate	3–5[f]	0.6	0.6	~5	<1.0	0
N,N-Dimethylacetamide	6–8	1.3	1.3	~50	<1.0	0
1,2-Dimethylimidazole	7–9	1.5	1.5	~50	<1.0	0

[a] Experiments were carried out similar to those shown in Figure 3. The acceptor concentration was increased until subsequent additions caused only minor changes in the spectra of indole and 2,3-dimethylindole. The maximal concentration was 5–10 times larger than that required for half-maximal effect. [b] Measured shift of 0–0 ^1L_b band may be influenced somewhat by shift of underlying ^1L_a bands (see Figure 3). [c] K, approximate equilibrium constant for hydrogen-bond formation, estimated from the acceptor concentration required for half-maximal change in 0–0 ^1L_b band. [d] Spectral shift at the highest acceptor concentration, determined from the short-wavelength edge and from the 0–0 ^1L_a band (shoulder at 290 nm). [e] Band remains sharp after acceptors are added. [f] Since 0–0 ^1L_a band is poorly resolved after adding this acceptor, the ^1L_a shift was also calculated for the short-wavelength edge of the spectrum. Both methods gave approximately the same shift.

parison between the spectra of 2,3-dimethylindole and 3-methylindole dissolved in methylcyclohexane.

Comparing the vapor and solution spectra of 2,3-dimethylindole confirms that a ^1L_a band occurs around 295 nm in methylcyclohexane. This band (0–0 ^1L_a) is clearly resolved at about 294 nm after cooling the methylcyclohexane solution to −120° (Figure 2). In the vapor spectrum of 2,3-dimethylindole, the 0–0 ^1L_a band lies at a shorter wavelength and is overlapped by the ^1L_b bands. Evidently, dissolving 2,3-dimethylindole in methylcyclohexane causes an appreciable red shift of the ^1L_a band, as was observed previously with other indolyl compounds (Strickland *et al.*, 1970).

Effects of Hydrogen Bonding. By adding small amounts of hydrogen-bonding agents to methylcyclohexane solutions, it is possible to obtain hydrogen-bonded 2,3-dimethylindole derivatives dissolved in an essentially nonpolar solvent. When the equilibrium constant for hydrogen-bond formation is large, the ensuing spectral changes reflect predominantly interaction with the hydrogen-bonding agent rather than changes in the bulk properties of the solvent. This technique has been used previously to measure hydrogen-bond formation with the phenolic hydroxy group and with the indolyl >NH group (Baba and Suzuki, 1961; Chignell and Gratzer, 1968; Strickland *et al.*, 1972).

The top of Figure 3 illustrates the family of curves obtained by adding *N,N*-dimethylacetamide to 2,3-dimethylindole dissolved in methylcyclohexane. Even small concentrations of this hydrogen-bond acceptor cause a marked red shift on both the leading and trailing side of the absorption band. The broad shoulder, which appears at about 302 nm, results from the 0–0 ^1L_a band of the hydrogen-bonded complex. The vibronic band at 290 nm (0–0 ^1L_b region) undergoes only a small red shift in the complex (about 1.3 nm).

Table I summarizes the red shifts resulting from hydrogen bonding 2,3-dimethylindole to several other compounds. The ^1L_a band is shifted by 3–9 nm, whereas the ^1L_b band is shifted by only 0.5–1.5 nm. The largest ^1L_a red shift (7–9 nm) occurs for hydrogen bonding to 1,2-dimethylimidazole (Figure 3). The red shift of the 0–0 ^1L_b band in 2,3-dimethylindole is similar to that observed for indole, which has a much better resolved 0–0 ^1L_b band (Table I).

To prove that the observed red shifts actually result from

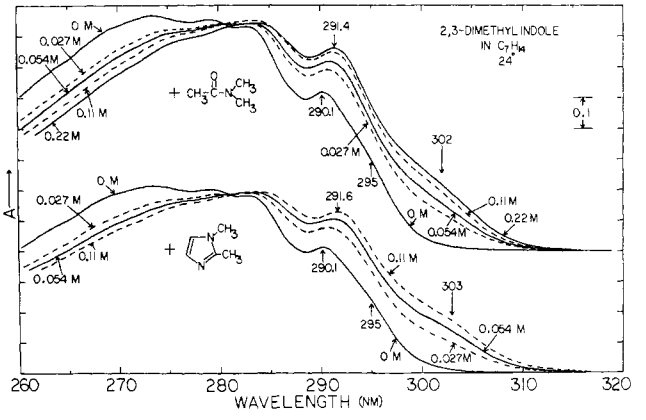

FIGURE 3: Top. Effect of *N,N*-dimethylacetamide (0, 5, 10, 20, 40 μl added) upon the absorption spectrum of 2,3-dimethylindole (initially 2.0 ml of 1.1×10^{-4} M) dissolved in methylcyclohexane. After correcting for dilution, an isosbestic point can be identified at 280.5 nm. Bottom. Effect of 1,2-dimethylimidazole (0, 5, 10, 20 μl added) upon the spectrum of 2,3-dimethylindole (initially 2.0 ml of 0.88×10^{-4} M) in methylcyclohexane. For convenience, 1,2-dimethylimidazole was liquified by heating to about 45° and transferred to the solution in a warm microsyringe (under nitrogen atmosphere to exclude water vapor). An isosbestic point occurs at 281 nm. Labels indicate approximate concentration of added acceptor. Reference cuvet contained the same acceptor concentration as the sample cuvet; 1.0-cm path length, 24°.

hydrogen bonding of the indolyl >NH group, control experiments were carried out with 1-methylindole, a compound that cannot function as a hydrogen donor. At low concentrations of acceptors, the 0–0 ^1L_b band of 1-methylindole is not shifted; and the ^1L_a band is shifted[1] by less than 1 nm (Table I). Evidently the spectral shifts observed for 2,3-dimethylindole result predominantly from the >NH group forming a hydrogen bond with the acceptor molecules added to the nonpolar solvent.

The oscillator strength of the near-ultraviolet band of

[1] Presumably this small shift of the ^1L_a band results because the added acceptor molecules are somewhat concentrated around the 1-methylindole molecules by electrostatic interactions, even though no hydrogen bond is formed; *e.g.*, see Midwinter and Suppan (1969).

TABLE II: Effects of Water and Perfluorohexane upon the Wavelength Positions of the ^1L_a and ^1L_b Absorption Bands of Several Indole Derivatives.

Compound	0–0 ^1L_b Band[a]			0–0 ^1L_a Band[a]		
	λ (nm) in C_7H_{14}	Δλ[b] (nm)		λ in C_7H_{14}	Δλ[b] (nm)	
		In H_2O	In C_6F_{14}		In H_2O	In C_6F_{14}
Indole	287.0	−1.4[c]	−2.5	~279	+[d]	−5
2,3-Dimethylindole	290.1	−2 ± 1[e]		~295	~+4	
3-Methylindole	290.5	−2 ± 1[e]	−2.5	~290.5	+[d]	−5
1,2-Dimethylindole	292.2	−2 ± 1[e]	−2.7	~292	+[d]	−5
1-Methylindole	294.0	−2 ± 1[e]	−3.0	~290	+[d]	~−5

[a] Positions of bands were identified using the procedure described previously (Strickland *et al.*, 1970). [b] Δλ, shift from position in C_7H_{14}. n_D^{20} values for these solvents are: C_7H_{14}, 1.4235; H_2O, 1.333; C_6F_{14}, 1.2509. [c] Band is sufficiently well resolved to measure the shift without much distortion from the ^1L_a band. [d] Measured from the short-wavelength edge of the spectrum. [e] Appears as shoulder, *e.g.*, see Figure 4.

2,3-dimethylindole is not significantly altered by hydrogen bonding (less than 10% change, which is within the experimental error). This result agrees with similar findings for indole (Chignell and Gratzer, 1968).

Effects of Solvents. The ^1L_a band of 2,3-dimethylindole also shifts appreciably when the bulk properties of the solvent are altered. Figure 4 compares the spectra obtained in perfluoromethylcyclohexane, 2-methylheptane, benzene, and water. After examining both the long- and short-wavelength edges of these spectra, one can see that the ^1L_a band occurs at the shortest wavelength in perfluoromethylcyclohexane, the least interacting solvent. Apparently in this solvent the 0–0 ^1L_a band occurs at about the same wavelength as the 0–0 ^1L_b band (*ca.* 289 nm). In 2-methylheptane the spectrum of 2,3-dimethylindole is practically identical with that described for methylcyclohexane as solvent (0–0 ^1L_a at about 295 nm, Figure 1). When benzene is the solvent, the 0–0 ^1L_a band is further red shifted and remains broad (center at about 300 nm, Figure 4). Using water as the solvent also red shifts the ^1L_a band relative to its position in saturated hydrocarbon solvents. The exact shift in water is difficult to measure, because polar solvents cause a great broadening of the absorption bands; *e.g.*, the leading edge extends beyond 320 nm (Figure 4). Comparing the spectrum in water to that in 2-methylheptane for the region from 265 to 275 nm suggests an average ^1L_a red shift of at least 3 nm in water. Examining the long-wavelength edge of the spectrum in water suggests that most species of 2,3-dimethylindole have their 0–0 ^1L_a band between 295 and 305 nm, although some appear at even longer wavelengths (Figure 4).

In the case of the ^1L_b band, the solvent-induced shifts

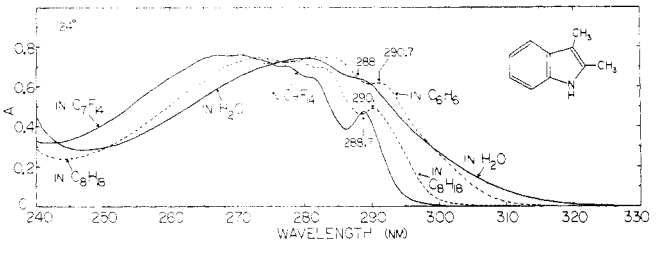

FIGURE 4: Absorption spectra of 2,3-dimethylindole (*ca.* 1.1 × 10^{-4} M) dissolved in perfluoromethylcyclohexane, in 2-methylheptane, in benzene, and in water; 1.0-cm path length, 24°. Refractive indices (n_D^{20}) of these solvents are: C_7F_{14}, 1.2781; H_2O, 1.333; C_8H_{18}, 1.3935; C_6H_6, 1.5011.

(up to 3 nm) are much smaller than those of the ^1L_a band. For the solvent series perfluoromethylcyclohexane, 2-methylheptane, and benzene, both the ^1L_b and ^1L_a bands red shift as the index of refraction increases (Figure 4). In contrast, water causes the ^1L_b and ^1L_a bands of 2,3-dimethylindole to shift in opposite directions from their positions in methylcyclohexane or in 2-methylheptane. Bernardin (1970) has reported similar results for indole.

The spectral shifts caused by water were further evaluated by using several other indole compounds. The directions of the wavelengths shifts for indole, 1-methylindole, 3-methylindole, and 1,2-dimethylindole are the same as described for 2,3-dimethylindole (Table II). Unfortunately the sizes of these shifts cannot be measured accurately, because the bands in water are too broad and are obscured by the ^1L_b bands.

With a perfluorocarbon solvent, however, the absorption shifts can be measured for indole and 1-methyl-, 3-methyl-, and 1,2-dimethylindole. In perfluorohexane both the ^1L_a and ^1L_b bands are shifted to shorter wavelengths relative to their positions in methylcylcohexane (Table II). The extents of these shifts are similar for the various indole compounds, although small differences seem to exist for the well-resolved 0–0 ^1L_b bands.

Discussion

Methylation at the 2 position of 3-methylindole shifts the 0–0 ^1L_a band to the red side of the 0–0 ^1L_b band even for nonpolar solvents, *e.g.*, methylcyclohexane (Figures 1 and 2). In other ways, the characteristic properties of the ^1L_a and ^1L_b bands are essentially identical in both 2,3-dimethylindole and 3-methylindole, which is the model chromophore for tryptophan. The spectra of 2,3-dimethylindole do, however, permit examining certain ^1L_a characteristics that cannot be observed directly in the spectra of tryptophan, 3-methylindole, or indole. For example, Andrews and Forster (1972) have recently used yohimbine, a derivative of 2,3-dimethylindole, to examine difference spectra induced by combinations of polar solvent mixtures and by changes in electric charge. Our investigation has focused upon spectral characteristics of the ^1L_a band revealed by studies using nonpolar solvents; *i.e.*, effects of hydrogen bonding and comparison of nonpolar and aqueous environments.

First, the 0–0 ^1L_a band is inherently broader than the 0–0 ^1L_b band even in methylcyclohexane cooled to −120° (Fig-

ure 2). At room temperature with 2,3-dimethylindole dissolved in a nonpolar solvent, the 0–0 ^1L_a band has considerable width. In polar solvents the width increases further, frequently to the point that the 0–0 ^1L_a band cannot be resolved (Figure 4). The blurring results from the multitude of different interactions between the solvent and the solute (Galley and Purkey, 1970). Each type of solvated indolyl ring may have its 0–0 ^1L_a band at a somewhat different wavelength. The measured spectrum represents the population-weighted average of all chromophoric species. Since the ^1L_a excited state is much more polar than the ^1L_b excited state (Mataga *et al.*, 1964; Song and Kurtin, 1969) the 0–0 ^1L_a band can be spread over a larger wavelength range by perturbing groups in the solvent. Suzuki (1967) has summarized the mechanisms involved in the spectral shifts caused by various solute–solvent interactions.

The 0–0 ^1L_a band of 2,3-dimethylindole provides a means to measure the red shift due to hydrogen bonding. Its size increases as follows: 1-butanol $\approx$ ethyl acetate $<$ *N*,*N*-dimethylacetamide $\approx$ 1,2-dimethylimidazole (Table I). This order corresponds to the strengths of hydrogen bonds measured for indole interacting with related proton acceptors; *e.g.*, $-\Delta H$ varies in the following way: *p*-dioxane $<$ dimethylformamide $\approx$ pyridine (Dunken and Fritzsche, 1962). In addition, the size of the ^1L_a red shift increases in approximately the same order as the equilibrium constant for hydrogen bonding of the indolyl >NH group (Table I; see also Mitsky *et al.*, 1972; Pullin and Werner, 1965). Apparently the strongest hydrogen bonds give the largest red shift of the ^1L_a band. The shift to lower energy results because the indolyl >NH forms an appreciably stronger hydrogen bond in the ^1L_a excited state than in the ground state (Pimentel, 1957; Mataga *et al.*, 1964). Experiments using 1-methylindole confirmed that in the absence of an >NH group no significant shift occurs upon adding small amounts of hydrogen acceptor molecules to methylcyclohexane solutions (Table I).

In contrast, altering the bulk solvent properties causes spectral shifts for indole derivatives having either the >NH or $>NCH_3$ group (Table II). Apparently the shifts caused by nonpolar solvents result mainly from the altered polarizability of the medium (Suzuki, 1967; Bernardin, 1970; Mataga *et al.*, 1964; Yanari and Bovey, 1960). For the fluorocarbon and hydrocarbon solvents both the ^1L_a and ^1L_b bands shift to the red with an increase in the refractive index, a quantity related to the polarizability (Table II and Figure 4; Yanari and Bovey, 1960).

With water as the solvent, the absorption shifts cannot be explained solely in terms of an altered polarizability, because the ^1L_b band shifts to shorter wavelengths and the ^1L_a band shifts to longer wavelengths (Table II). The ^1L_a fluorescence of both >NH and $>NCH_3$ indole derivatives is also red shifted by water and other polar solvents (Van Duuren, 1961). Conflicting explanations have been given for the ^1L_a fluorescence shift (Mataga *et al.*, 1964; Walker *et al.*, 1967; Eisinger and Navon, 1969; Chopin and Wharton, 1969; Vander Donckt, 1969). Nevertheless, regardless of which mechanisms contribute to the fluorescence shift, it is clear that the ^1L_a excited state is much more polar than the ground state (Song and Kurtin, 1969) and thus is able to have stronger electrostatic interactions with other polar molecules[2] even in the absence of a hydrogen bond.

[2] Since the dimensions of the indolyl ring are large compared to the separation between the interacting species, the strength of the electrostatic interaction involves higher order terms than just the dipole–dipole term, which is widely used to evaluate interaction energies.

Unfortunately, the broadness of the absorption bands in water precludes a precise comparison of the absorption ^1L_a red shift for the >NH and $>NCH_3$ indole derivatives. Probably their interactions with water differ in some way, because the >NH derivatives interact more strongly with other polar molecules at lower concentrations than do the $>NCH_3$ derivatives in both the ground state (Table I) and the ^1L_a excited state (see Figures 2 and 3 and Table II in Walker *et al.*, 1967). Most likely the indolyl >NH group is hydrogen bonded in water, and this partially contributes to the ^1L_a red shift. With 1-methyl- and 1,2-dimethylindole, perhaps water molecules oriented around the polar $>NCH_3$ group may cause a red shift by electrostatic interactions even though no hydrogen bond is formed. The size of the ^1L_a shift in both >NH and $>NCH_3$ derivatives probably is also influenced by the water structure around the entire indolyl ring (electrostatic interactions) and by the polarizability of water (Suzuki, 1967; Yanari and Bovey, 1960).

How do the results with model compounds apply to predicting the wavelength positions of the tryptophanyl absorption bands of proteins? The ^1L_a wavelength may be affected by hydrogen bonding, by the polarizability, and by polar groups (either formal charges or dipolar groups) surrounding the tryptophanyl ring. Other aromatic rings (Phe, Tyr, His, Trp), which are more polarizable than aliphatic side chains, may cause red shifts similar to that observed for benzene (*ca.* 5 nm, Figure 4). With indolyl–aromatic interactions, the ^1L_a band may also be shifted due to the >NH group being weakly hydrogen bonded to the π orbitals or due to electrostatic interactions with the neighboring ring (Reinecke *et al.*, 1969). If the tryptophanyl ring is near a polar group of either the protein or solvent, the wavelength of the ^1L_a band may be affected. In principle, a polar group may shift the band to either longer or shorter wavelengths, depending upon the difference in its interaction energies[2] with the ^1L_a excited state and the ground state. Generally, though, a red shift is more likely if the polar group has an attractive interaction with the ground state of the tryptophanyl ring. In this circumstance, the more polar ^1L_a excited state will tend to attract the polar group more strongly than does the ground state, thereby causing a red shift (Suzuki, 1967).

The hydrogen-bonding experiments with 2,3-dimethylindole provide a model for tryptophanyl side chains buried among aliphatic side chains of proteins. Furthermore, since the red shifts are so large for certain hydrogen bonds, they may have a major influence upon the position of the ^1L_a band even for indolyl rings partially exposed on the surface or buried in slightly polar regions of proteins. Table III summarizes the shifts of the ^1L_a band expected to result when the tryptophanyl side chain >NH is hydrogen bonded to the various proton acceptor groups found in proteins.[3]

Hydrogen bonding to butanol was used as a model for binding to the hydroxy oxygens found in amino acid side chains (Ser, Thr, Tyr, Asp, Glu in Table III). The ^1L_a shift due to binding at either the phenolic oxygen or the hydroxy oxygen of a carboxyl group may be slightly less than that observed with butanol, since these oxygen atoms are less electronegative than the alcoholic oxygen atom (Pauling, 1960). Interestingly, the position of the 0–0 ^1L_a band of the tryptophanyl residue in cytochrome *c* (Strickland *et al.*, 1971) is in agreement with the shifts given in Table III. In this protein, the indolyl >NH is hydrogen bonded to the hydroxy oxygen

[3] The hydrogen bonding groups in simple proteins have been summarized by Richards *et al.* (1970).

TABLE III: Expected Position of 0–0 ^1L_a Band When Tryptophanyl Side-Chain >NH Is Hydrogen Bonded to Other Groups within a Nonpolar Region of a Protein.

Proton Acceptor	0–0 ^1L_a (nm)	Model Acceptor
None[a]	289–290	
Hydroxy oxygen of carboxy group (Asp, Glu) Phenolic oxygen (Tyr) Hydroxy oxygen (Ser, Thr)	292–295	1-Butanol
Carbonyl oxygen of carboxy group (Asp, Glu)	292–295	Ethyl acetate
Carbonyl oxygen of peptide bond or of side-chain amide (Gln, Asn)	295–298	*N,N*-Dimethylacetamide
⩾N: (His)	296–299	1,2-Dimethylimidazole
Carboxylate ion (Asp,Glu)	297–302?	

[a] Based upon *N*-stearyl-L-tryptophan *n*-hexyl ester in methylcyclohexane (Strickland *et al.*, 1971).

of a buried propionic acid side chain of the heme (Dickerson *et al.*, 1971).

The red shift resulting from binding the indolyl >NH to the carbonyl oxygen of a carboxyl group was estimated using ethyl acetate as a proton acceptor (Table III). Hydrogen bonding is known to occur at the carbonyl oxygen of ethyl acetate (Grunwald and Coburn, 1958). Carboxylic acids could not be used for these studies, because they interact strongly to form dimers in nonpolar solvents (Murthy and Rao, 1968).

The studies with *N,N*-dimethylacetamide suggest that a 6- to 8-nm ^1L_a red shift may occur when the indolyl >NH binds to a carbonyl oxygens of either a peptide bond or a side-chain amide (Table III). Judging from the experiments with 1,2-dimethylimidazole, a major ^1L_a red shift may also occur, if the indolyl >NH is hydrogen bonded to a histidyl side chain (Table III).

Finally we note that the carboxylate ion is an excellent acceptor for hydrogen bonds, as was pointed out many years ago (Crammer and Neuberger, 1943). Unfortunately, the insolubility of carboxylate ions in methylcyclohexane precluded measuring the shift caused by this acceptor. From a theoretical viewpoint, the carboxylate ion is expected to form a stronger hydrogen bond with the indolyl >NH than do any of the uncharged acceptors, because the strengths of hydrogen bonds depend mainly upon electrostatic interactions (Donohue, 1968). This stronger hydrogen bond may be expected to cause a larger ^1L_a red shift than do those acceptors that dissolved in nonpolar solvents (Table III).

For all the indolyl hydrogen bonds in proteins, the amount of red shift may depend somewhat upon the precise geometry of the hydrogen bond. The increased broadness of the 0–0 ^1L_a band of hydrogen-bonded 2,3-dimethylindole (Figure 3) results from the acceptor molecules having numerous orientations relative to the >NH group (see above). Apparently certain orientations of the acceptor group cause a larger shift than others. In the case of proteins an acceptor group may be constrained to have a single orientation for hydrogen bonding to an indolyl >NH group, thereby accounting for the well-resolved 0–0 ^1L_a bands observed in the spectra of some proteins cooled to −196° (Strickland *et al.*, 1971).

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