Protein Difference Spectra. Effect of Solvent and Charge on Tryptophan†

Leonard J. Andrews and Leslie S. Forster*

ABSTRACT: Solvent-and charge-induced difference spectra have been obtained with model compounds containing the indole chromophore. The models which include yohimbine (free base and protonated), dihydroergotamine, and a series of 3-substituted indoles were chosen to encompass a variation of ^1L_a–^1L_b energy separations. The contributions of ^1L_a and ^1L_b shifts to the difference spectra have been differentiated. Whereas a perturbing solvent always shifts ^1L_a and ^1L_b in the same direction, appropriately placed charges can shift the two transitions in opposite directions. In addition, solvents can affect the width of the broad ^1L_a absorption band. Criteria for distinguishing charge from solvent effects in the difference spectra of proteins are presented.

Ultraviolet absorption spectroscopy has been widely used as a tool for the assessment of chromophoric environments in proteins. In particular, difference spectroscopy has been a method of choice for obtaining information about conformational changes associated with denaturation and/or solvent perturbation (for reviews see Donovan, 1969; Kronman and Robbins, 1970). For example, the intensity near 300 nm in the difference spectrum of lysozyme, produced by denaturation, has been ascribed to the separation of charge from the vicinity of an indole moiety (Ananthanarayanan and Bigelow, 1969a). It has been difficult, however, to separate difference spectra induced by a charge from those that are due to solvent perturbation.

The difference spectra of proteins generally consist of the superimposed spectra due to more than one chromophore. Model compounds have been studied in an effort to understand the origin of the perturbation. Early reports on the effect of charge and solvent (Donovan *et al.*, 1961; Yanari and Bovey, 1960) have been followed by extensive work on solvent effects, but only a few studies dealing explicitly with charge effects.

It is fair to say that our understanding of chromophoric difference spectra, especially those associated with indole (tryptophan) in the 250- to 310-nm region, is still unsatisfactory. There are two reasons for this situation. First, the long-wavelength (280 nm) band of indole results from two overlapping transitions, designated ^1L_a and ^1L_b, whose energies are differentially affected by perturbations (Strickland *et al.*, 1970). Secondly, the bandwidth of the broad ^1L_a transition is solvent dependent (Keyes *et al.*, 1969). The interplay of these factors leads to difference spectra which are varied in appearance. In order to display this variety, we have used model compounds in which the separation of ^1L_a and ^1L_b varies. We have then introduced charge and solvent perturbations independently.

It is our aim to present a unified picture that will serve as a general basis for interpreting difference spectra associated with tryptophan residues, with the particular aim of using difference spectroscopy to distinguish charge- from solvent-induced perturbations.

Experimental Section

Difference and circular dichroism (CD) spectra were measured using 1-cm quartz cells with the Cary 14 spectrophotometer and the Cary 60 spectropolarimeter, respectively. The fluorescence apparatus was of conventional design employing 0.25-m excitation and emission monochromators. Right angle emission was monitored. Phase-sensitive detection was used, and a ratio recording circuit was constructed to compensate for variations in the intensity of the 150-W PEK Xenon lamp. The polarization degree, P, is defined as $(I_E - I_B)/(I_E + I_B)$ where E and B refer to vertical and horizontal orientations of the analyzer. Corrections were made for instrumental polarization by measuring I_E and I_B with B polarized exciting light (Azumi and McGlynn, 1962). A DuPont 310 curve resolver (6 channels) was used for the gaussian analysis.

Charge-induced difference spectra were produced by suitably adjusting pH with KOH or HCl. Samples for solvent-induced difference spectra were prepared by diluting 5 ml of stock solution to 25 ml with the appropriate solvent in a volumetric flask. These are referred to as 80% organic solvent–water perturbing solutions.

The following derivatives were used as received: yohimbine·HCl and 2,3-dimethylindole, Aldrich Chemical Co.; 3-indoleethylamine·HCl and 3-indolepropionic acid, Eastman Organic Chemicals; 3-indolebutyric acid and 3-indoleacetic acid, Fisher Scientific Co.; dihydroergotamine methylsulfonate was a gift from Sandoz Pharmaceuticals. The following solvents were used without further purification: Me_2SO, A. R., Mallinckrodt; dimethylformamide, spectroquality reagent, MC/B; CH_3CN, reagent, Baker and Adamson; CH_3OH, A. R., MC/B; and glycerol, 99.5%, MC/B. n-C_3H_7OH, MC/B, was distilled from bromine before use.

Results and Discussion

Spectra of Indole Derivatives. Central to the analysis of indolyl difference spectra is an understanding of the spectra of the unperturbed model compounds. In the parent molecule, indole, ^1L_b is below ^1L_a in the vapor phase (Strickland *et al.*, 1970). ^1L_a is the more sensitive to environment and when indole is dissolved in nonpolar solvents, ^1L_a moves to lower energies. Upon the addition of a small amount of a polar solvent (1-butanol), the ^1L_a red shift is so large that

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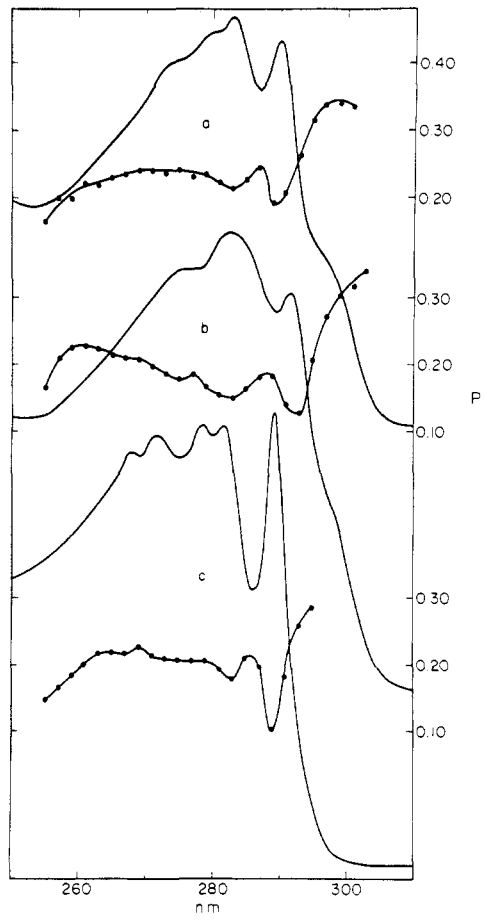
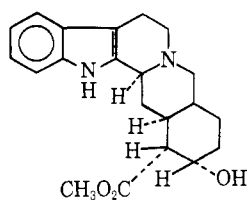


FIGURE 1: Absorption and polarized fluorescence excitation spectra in 1-propanol at $-125°$ of (a) yohimbine (λ_{em} = 321 nm), (b) 3-indolebutyric acid (λ_{em} = 333 nm), and (c) yohimbine·HCl (λ_{me} = 322 nm). Band pass of excitation monochrometer was <2 nm. The spectra of 2,3-dimethylindole are very similar to the yohimbine spectra.

^1L_b becomes the higher state (some broadening of ^1L_a is also evident). A similar situation obtains for 3-methylindole, with an important difference. The methyl substituent lowers ^1L_a more than ^1L_b, and in methylcyclohexane the two states are nearly coincident. Consequently, the separation of ^1L_a and ^1L_b is not only sensitive to external environment, but is dependent upon the intramolecular environment of the indolyl group.

Graphic evidence of the intramolecular perturbation can be seen in the spectra of yohimbine (I) and 2,3-dimethylindole where ^1L_a is markedly red shifted and a pronounced shoulder is developed (Figure 1). The assignment of this shoulder as ^1L_a is made by comparison of the excitation polarization spectra of these molecules and indole (Weber, 1960). Fluorescence in alcoholic solvents arises predominantly in ^1L_a. If excitation is also in ^1L_a, a high degree of polarization (P = 0.30 − 0.50) is expected, as observed in the 300-nm region. The transition moments of ^1L_a and ^1L_b are approximately perpendicular (Konev, 1967). Excitation in ^1L_b should lead to a negative value for P. The minima at 290 and 283 nm in the excitation polarization spectrum correspond to ^1L_b peaks in the absorption spectrum. Overlapping of ^1L_a and ^1L_b is responsible for the positive P at these wavelengths, but the troughs can be assigned to ^1L_b. Thus, the absorption spectrum consists of sharp ^1L_b peaks superimposed on a broad structureless ^1L_a band.



I

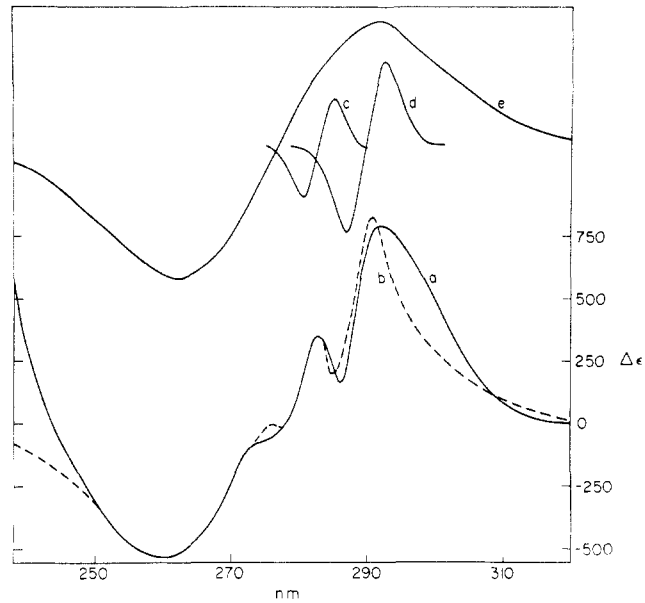


FIGURE 2: Difference spectrum of yohimbine *vs.* yohimbine·HCl in water and its component resolution; (a) experimental spectrum, (b) synthesized spectrum of (a) using six gaussian components, (c and d) contribution of ^1L_b shifts to (b), and (e) contribution of ^1L_a shift to (b).

Charge Perturbations. A static electric field can alter the energy of an electronic transition (Tinoco and Bush, 1964). To first order

$$\Delta\nu_{a\leftarrow 0} = \frac{1}{h}(\mathbf{E}\cdot\Delta\boldsymbol{\mu}(0,a))$$

where $\Delta\nu_{a\leftarrow 0}$ is the frequency shift, $\mathbf{E}$ the electric field vector, and $\Delta\boldsymbol{\mu}(0,a)$ the difference in the ground and excited state dipole moment vectors. According to this equation, a particular transition can be red or blue shifted, depending upon the sign and orientation of the perturbing charge.

When the amine nitrogen in yohimbine (I) is protonated, ^1L_a is strongly blue shifted (the shoulder disappears) but the ^1L_b peaks are only slightly altered (Figure 1). The difference spectrum (I *vs.* I·H$^+$) is shown in Figure 2. Also exhibited in Figure 2 is a gaussian analysis that clearly indicates the origin of the difference spectrum. The difference spectral maxima at 294 and 285 nm are produced by a 1-nm red shift in ^1L_b (0–0) and ^1L_b (0–0 + 900 cm^{-1}), respectively. The effect of the large ^1L_a red shift is to increase the height of the 294 peak relative to the 285-nm peak and to develop the broad negative band at 260 nm.

The difference spectra of 3-indoleethylamine and dihydroergotamine (II) are shown in Figure 3. The results are summarized in Table I. The ^1L_b shifts are reliable, since the

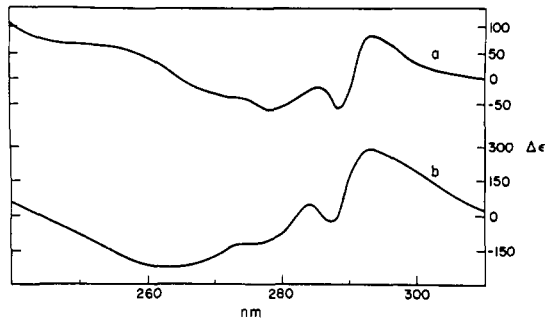
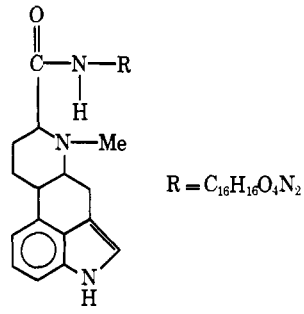


FIGURE 3: Difference spectra in water of (a) dihydroergotamine *vs.* dihydroergotamine·HCl and (b) 3-indoleethylamine *vs.* 3-indoleethylamine·HCl.



$R = C_{16}H_{16}O_4N_2$

II

sharp ^1L_b peaks are resolved in the corresponding absorption spectra. However, ^1L_a shifts should be viewed with caution. In a gaussian analysis, both the amplitude (intensity) and separation of the component curves can be varied and the resolution of a given spectrum may not be unique. We have chosen the amplitudes to reproduce the long-wavelength portions of the corresponding absorption spectra and retained these intensities in the fitting process, *i.e.*, only the separation was varied. Nonetheless, by using more components, different quantitative results might be obtained.

In spite of any uncertainties about the magnitudes of the ^1L_a shifts, one point is clear, *viz.*, the ^1L_a shift in II is to the blue. Only in this manner can the crossover in the difference spectrum at 265 nm be explained. By using space-filling molecular models the positions of the charges in the three molecules can be approximately determined. If we assume that the ^1L_a and ^1L_b dipole moments are the same in all three derivatives, the $\Delta\mu$ vectors for the two transitions can be roughly located (Figure 4).

In locating the positions of the charges by means of space-filling models, we have assumed that the charge perturbation operates through space, whereas it is possible that an inductive mechanism is involved (Donovan *et al.*, 1961). However, the same qualitative conclusions about $\Delta\mu$ would result if an inductive mechanism is assumed. The inductive pathway in yohimbine is through the 2 and 3 positions on the indole ring. Two carbons separate the protonated nitrogen from the 3 position, while only one carbon intervenes in the inductive path to the 2 position. This would lead us to place the "effective" charge at roughly the same angle to the indole framework as indicated in Figure 4c. Similarly, for II, the inductive pathway operates through the 3 and 4 positions, leading to placement of the charge as indicated in Figure 4a.

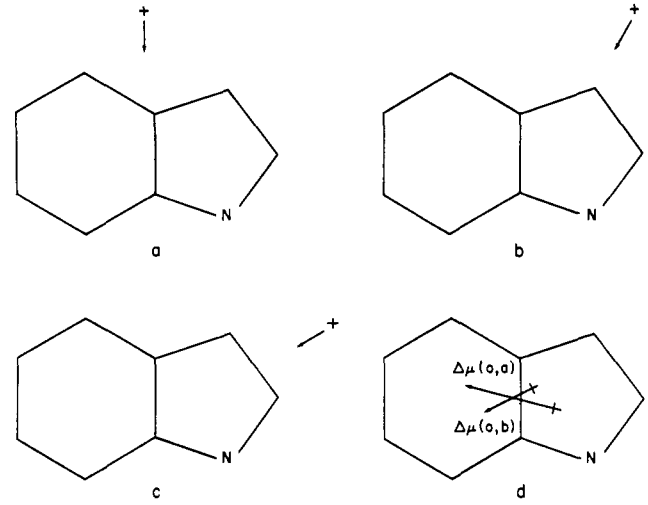


FIGURE 4: Approximate positions of protonated nitrogens in (a) dihydroergotamine·HCl, (b) 3-indoleethylamine·HCl, and (c) yohimbine·HCl and (d) approximate positions of $\Delta\mu(0,a)$ and $\Delta\mu(0,b)$ vectors in indole.

The charge difference spectra of a series of 3-substituted indoles have been recorded. As we have demonstrated, $\Delta\epsilon_{292}$ results both from ^1L_a and ^1L_b shifts. $\Delta\epsilon_{292}$ decreases monatonically with chain length (Table II). We have confirmed, in

TABLE I: ^1L_a and ^1L_b Frequency Shifts from Gaussian Resolved Charge Difference Spectra.

Derivative	Shift (nm)		
	^1L_b (0,0)	^1L_b (0,0 + 900)	^1L_a
Yohimbine	1	1	7
3-Indoleethylamine	≤1	≤1	4
Dihydroergotamine	≤0.5	≤0.5	−1

TABLE II: Δε Values for Charge-Induced Difference Spectra.

Derivative[a]	ϵ_{max} (× 10^{-3})[b]	λ (nm)	Δε	$\Delta\epsilon_2/\Delta\epsilon_1$[c]
Yohimbine	2.81	294	767	0.45
		284	346	
Dihydroergotamine	2.11	294	80	−0.55
		286	−44	
3-Indoleethylamine	1.93	294	293	0.12
		284	35	
3-(3-Dimethylpropylamino)-indole	2.16	295	145	0
		285	0	
3-Indoleacetic acid	2.05	293	285	0.16
		284	45	
3-Indolepropionic acid	2.12	295	98	0
		285	0	
3-Indolebutyric acid	1.97	298	37	−0.16
		285	−6	

[a] The reference compound is either the protonated amine or undissociated acid. [b] ≃280 nm. [c] $\Delta\epsilon_1$ and $\Delta\epsilon_2$ refer to the maxima near 294 and 285 nm, respectively.

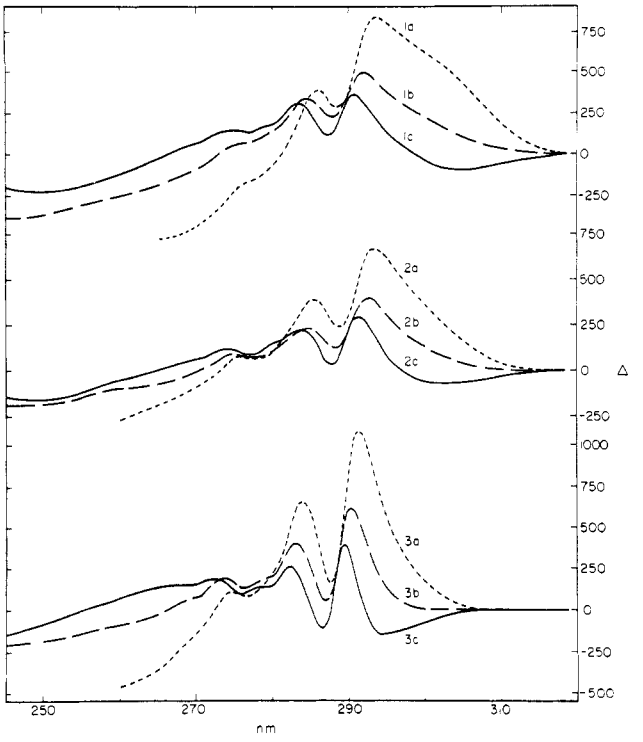


FIGURE 5: Difference spectra induced by 80% organic solvent–water solutions: (1) yohimbine, (2) 3-indolebutyric acid, (3) yohimbine·HCl; (a) Me_2SO ------, (b) glycerol ————, (c) methanol

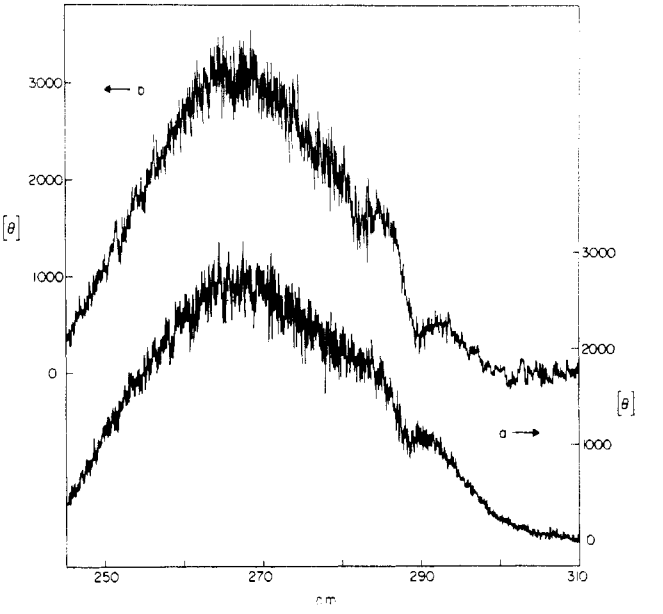


FIGURE 7: CD spectra of yohimbine·HCl in (a) water, (b) 80% methanol–water.

the case of 3-indolebutyric acid, the insensitivity of $\Delta\epsilon_{292}$ to ionic strength (Donovan *et al.*, 1961), which appears to support an inductive mechanism for charge perturbation in 3-substituted indoles. There is, however, one bit of evidence that seems to negate the inductive mechanism. As the chain length increases, not only does $\Delta\epsilon_{292}$ decrease, but the spectra broaden appreciably. This would be expected from a through space interaction, since the range of charge positions in the several conformers increases with chain length, while the site of an inductive perturbation would be invariant to chain length.

Solvent-Induced Difference Spectra. The difference spectra of *N*-acetyltryptophan ethyl ester induced by a variety of perturbing solvents have been recorded (Herskovits and Sorensen, 1968). Although these might be regarded as "typical," from the point of yiew of protein difference spectroscopy, they are no more typical than is the "anomalous" indole difference spectrum (Ananthanarayanan and Bigelow, 1969b). In our view, the varied appearance of solvent difference spectra rises from the initial separation of the ^1L_a and ^1L_b as well as from the nature of the reference and perturbing solvents.

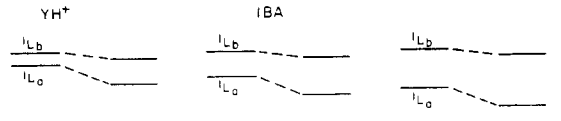


FIGURE 6: Qualitative representation of ^1L_a and ^1L_b shifts in yohimbine·HCl, 3-indolebutyric acid, and yohimbine on going from an aqueous solution to an 80% strongly perturbing solvent–water solution. The ^1L_b energies in water vary from one molecule to another.

Solvent perturbation spectra of three model compounds, chosen to span a range of ^1L_a–^1L_b separations, have been collected. These molecules are: I, IH^+, and 3-indolebutyric acid (Figure 5). The solvents (80% solvent–water) can be classified as strongly perturbing, Me_2SO and dimethylformamide, and moderately perturbing, CH_3OH, CH_3CN and glycerol. Both ^1L_a and ^1L_b are red shifted by the perturbing solvents; the energy levels in water and strongly perturbing solvents are indicated schematically in Figure 6. At one extreme is yohimbine; 3-indolebutyric acid is intermediate while $I \cdot H^+$ represents the other extreme. The combination of a large ^1L_a–^1L_b separation and strongly perturbing solvent is exemplified by the prominent shoulder near 303 nm in the Me_2SO perturbed difference spectrum of yohimbine. As the perturbant strength and/or ^1L_a–^1L_b separation decreases, the difference spectra become more "typical."

Of especial interest are the difference spectra which exhibit a trough in 305-nm region. The CH_3OH-perturbed $I \cdot H^+$ spectrum is exemplary of this class. The trough is correlated with a narrowing of the ^1L_a band in CH_3OH compared to water. This narrowing is seen in the CD spectra (Figure 7). The main CD intensity is associated with ^1L_a; the trough at 288 nm is due to a negative ^1L_b contribution. The small ^1L_b solvent red shifts can also be detected in the CD spectra. A corresponding ^1L_a narrowing obtains in the CD spectrum of $I \cdot H^+$ in CH_3CN. The ^1L_a narrowing is accompanied by a relatively small ^1L_a shift in these two solvents. Within the group of solvents employed here, only ^1L_a and ^1L_b red shifts (relative to water) have been observed.

The shapes of the difference spectra are independent of perturbant concentration in the range 20–80%. Only the $\Delta\epsilon$ magnitudes vary with the concentration of any given perturbant.

These results are summarized in Table III.

Comparison of Charge- and Solvent-Induced Difference Spectra. We have, in addition to the spectra described above, collected a large number of charge- and solvent-induced difference spectra. Based upon an examination of these, and a survey of the literature, we propose several generalizations

TABLE III: $\Delta\epsilon$ Values for Solvent-Induced Difference Spectra.

Solvent[a]	Yohimbine			3-Indolebutyric Acid			Yohimbine·HCl		
	λ (nm)	$\Delta\epsilon$	$\Delta\epsilon_2/\Delta\epsilon_1$[b]	λ (nm)	$\Delta\epsilon$	$\Delta\epsilon_2/\Delta\epsilon_1$	λ (nm)	$\Delta\epsilon$	$\Delta\epsilon_2/\Delta\epsilon_1$
Me_2SO	293.5	840		293.5	678		292	1076	
			0.47			0.59			0.61
	286.5	396		286	398		284	654	
Dimethylformamide	292.5	674		293	518		291	804	
			0.57			0.60			0.56
	285	382		285	509		283	451	
CH_3CN	292	287		292	240		290	270	
			0.67			0.63			0.57
	284	191		283.5	150		282.5	155	
CH_3OH	291	368		291.5	297		289.5	396	
			0.84			0.75			0.65
	283	309		283.5	223		282.5	258	
Glycerol	292	503		292.5	404		290.5	620	
			0.68			0.57			0.64
	285	342		284	230		283	399	

[a] Reference solvent is water, sample solvent is 80% organic solvent–water (see Experimental Section). [b] $\Delta\epsilon_1$ and $\Delta\epsilon_2$ refer to the maxima near 294 and 285 nm, respectively.

which may be useful in distinguishing solvent from charge effects in difference spectra. The two peaks at 293 and 284 nm in the yohimbine charge difference spectra are labeled 1 and 2, respectively. These are always identifiable in difference spectra, albeit at slightly different wavelengths. The guiding principles are: (1) when the $\Delta\epsilon_2$ is negative, a charge effect is indicated; (2) a trough to the red of 300 nm is solvent induced; (3) when $\Delta\epsilon_2/\Delta\epsilon_1 > 0.5$, the perturbation is due to solvent. When $\Delta\epsilon_2/\Delta\epsilon_1 < 0.4$, a charge is implicated. In the range 0.4–0.5, no clear-cut decision is possible.

Although these generalizations are essentially empirical, they can be rationalized. Peak 2 is due to an ^1L_b red shift which would yield a positive $\Delta\epsilon$. When a blue-shifted ^1L_a band is superimposed upon the red-shifted ^1L_b, the 2 peak is moved below the base line. Only an appropriately oriented charge can be responsible for ^1L_a and ^1L_b shifts in opposite directions. Any perturbing solvents will shift ^1L_a and ^1L_b in the same direction. When ^1L_a is narrowed, the superposition of the ^1L_a and ^1L_b shift spectra produce a minimum in the 305-nm region. The explanation of the $\Delta\epsilon_2/\Delta\epsilon_1$ ratios is less obvious, but one possibility can be suggested, *viz.*, a minor intensity increase accompanies the decrease in bandwidth in organic solvents. This would lead to asymmetric ^1L_b difference spectra in which the long-wavelength maxima are more intense than the short-wavelength troughs. Since the $\Delta\epsilon_2$ height depends critically on a partial cancellation of two gaussian curves, the cancellation is less effective in solvent-induced difference spectra resulting in a higher $\Delta\epsilon_2/\Delta\epsilon_1$ ratio. A change in the frequency of the 900-cm^{-1} vibration with solvent might also contribute to the decrease in mutual cancellation.

The utility of these rules for interpreting the more complex protein difference spectra is yet to be demonstrated. It would be desirable to test model compounds with charges in other positions, *e.g.*, 5-substituted indoles. Nonetheless, it does appear likely that charge- and solvent-induced perturbations can be differentiated by the use of difference spectroscopy.

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