Unusual Difference Spectra of Proteins Containing Tryptophan. I. Studies with Model Compounds*

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ABSTRACT: The difference spectra of many proteins containing tryptophan are found to exhibit an atypical extremum around 300 mμ under certain conditions. This paper reports on a systematic study of some model compounds, namely, tryptophan and some of its derivatives, which was designed to improve our understanding of this unusual effect. Our study leads to the following conclusions. (1) The extinction difference observed at 300 mμ when the solvent is changed behaves quantitatively and qualitatively quite differently from the differences observed at shorter wavelengths; for example, changing the aqueous solvent by the addition of an organic solvent caused a characteristic positive difference between about 275 and 295 mμ, but a sometimes negative difference at 300 mμ. (2) The value of the extinction observed at 300 mμ is not changed very much by changes in the nonpolar nature of the medium. It is much more sensitive to changes in the electrostatic environment of the chromophore. (3) There is strong, but not conclusive, evidence that the unusual spectral behavior of indole chromophores at 300 mμ is due to the existence of a minor absorption band, buried under the long-wavelength side of the main π–π^* bands. The model compound studies reported in this paper are used in the following paper to interpret, in a self consistent way, the hitherto puzzling spectral behavior of some tryptophyl-containing proteins.

Difference spectra of proteins have received much attention over the past 10 years (Wetlaufer, 1962). When used to study the effect, say, of an organic solvent on a protein, difference spectroscopy typically gives the following results. At concentrations below denaturing the difference spectrum shows a series of positive peaks above 270 mμ due to a red shift of the spectrum of the protein. When the concentration of the organic solvent reaches the point where the protein begins to unfold, the well-known "denaturation blue shift" (Bigelow and Geschwind, 1960) occurs which usually overcomes the solvent effect red shift and results in a blue shift difference spectrum showing a series of negative troughs. As the concentration of the organic solvent is increased further, after the denaturation process is completed, the difference troughs experience the red shift again; at sufficiently high concentration of the solvent, these may appear as positive peaks, if the total red shift is greater than the denaturation blue shift. (The behavior just described is not *always* observed, though, as stated it is typical behavior when the organic additives are relatively small. Ray *et al.* (1966) showed that octanol causes a blue shift with bovine serum albumin even at the lowest concentrations, and some ionic compounds do too; Bigelow, 1960; Polet and Steinhard, 1968.)

In the course of a study of the denaturation of β-lactoglobulin A by various denaturing agents, we have repeatedly observed difference spectra which do not behave in this simple fashion, and which have not hitherto been satisfactorily explained (*e.g.*, Figure 1). When a difference spectrum is measured by comparing β-lactoglobulin A in denaturing concentrations of an organic solvent with the protein in water it exhibits an atypical trough around 300 mμ in addition to the usual peaks in the 280–295-mμ region which are mainly due to changes in the environment of tryptophyl residues. In the experiments reported below we have attempted to find out what causes the 300-mμ anomaly, and whether it may have some value in protein studies.

A survey of the literature reveals that such atypical difference spectra have been observed with surprising frequency (see Ananthanarayanan and Bigelow, 1969, for references), but in none of these examples was any serious attempt made to account for the effect. In one paper, however (Donovan *et al.*, 1961), there were presented difference spectra for tryptophan similar to the ones we are discussing here. These were caused by changes of pH, and not by changes in other solvent conditions. However, as we shall show, this work has been very important to us in facilitating our interpretation of the unusual difference spectra we observe under other conditions.

This paper reports the results of a systematic study of the effect of the environment on model compounds (indole, tryptophan, and related compounds). The following paper (Ananthanarayanan and Bigelow, 1969) employs the results obtained in this paper to interpret the unusual difference spectra of tryptophyl-containing proteins.

Materials and Methods

β-Lactoglobulin A was prepared from the milk of individual homozygous cows by the method of Aschaffenburg and Drewry (1955). Tryptophan was from Nutritional Biochemicals Corp. *N*-Ac-Trp-ethyl ester, *N*-Ac-Trp, and Trp-ethyl ester were Mann Assayed samples. Indole was a Fisher Certified sample. Salts like LiCl, LiBr, and KCl were reagent grade. Urea was Mann Ultra-Pure grade. Dioxane was distilled and stored under nitrogen at 0°. 2-Chloroethanol was from Eastman Organic Chemicals and was distilled before use. Ethylene glycol and its monomethyl ether were Fisher Certified reagents. Isooctane, methanol, and dimethyl sulfoxide were

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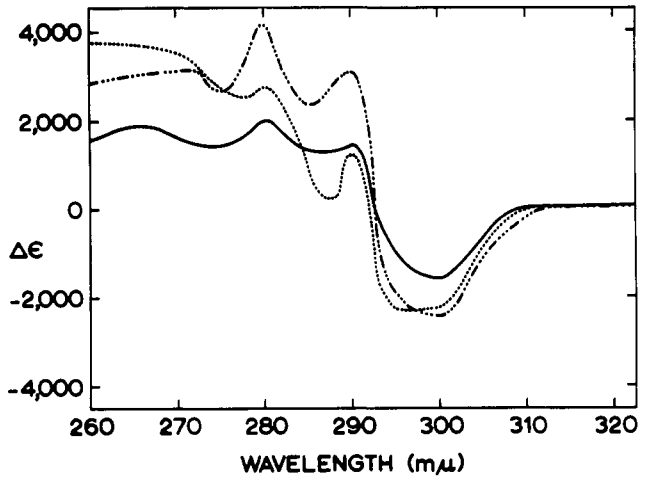

FIGURE 1: Difference spectra of β-lactoglobulin A in aqueous organic solvents. (— —) 82% ethylene glycol, pH 3.5; (—· · ·) 73% dioxane, pH 3.0; (· · ·) 83% 2-chloroethanol (pH 1.0). The reference solution is the protein in water at pH 6.0.

Fisher Spectranalyzed solvents; 95% ethanol and absolute alcohol were obtained from Commercial Alcohols Ltd., Quebec. The water used was distilled in a Corning all-glass distillation unit. All solutions were routinely filtered through appropriate Millipore filters (0.45 μ diameter).

Difference spectral measurements were carried out with a Cary Model 15 recording spectrophotometer using matched silica cells. Correction was made for absorption by the solvent, whenever necessary. pH values, measured with a Radiometer pH meter, were determined for the aqueous stock solutions before mixing with organic additives.

For low-temperature spectral measurements, the amino acid along with pure dry KBr was made into a transparent pellet and cooled by using liquid nitrogen to 80°K in the modified sample compartment of a Cary Model 14 spectrophotometer. The spectrum was recorded using as reference a KBr pellet of equal size at the same temperature. The optical density of the sample was around 3.

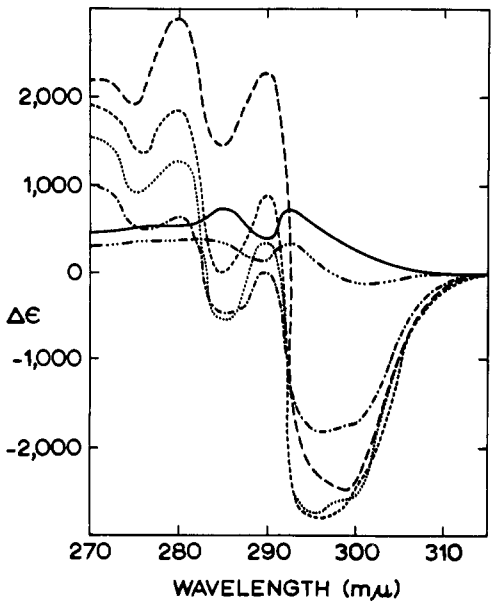

FIGURE 2: Difference spectra of β-lactoglobulin A in aqueous dioane. Dioxane concentration: (—) 20%, (—· · ·) 25%, (—·) 30%, (· · · ·) 35%, (- - -) 40%, and (— —) 60%. The reference solution is the protein in water. Both sample and reference solutions are at pH 3.0.

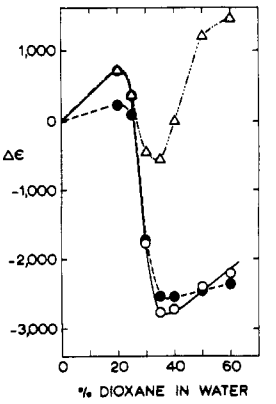

FIGURE 3: Plot of Δε *vs.* dioxane concentration, using data from Figure 2. △, 285 mμ; ○, 293 mμ; and ●, 300 mμ.

Results

Before actually presenting the results obtained with model compounds, it is necessary and useful to summarize briefly a few of the results obtained with proteins, which acted as the incentive for the model compound studies. Figure 1 shows the difference spectra of β-lactoglobulin A in aqueous dioxane, 2-chloroethanol, and ethylene glycol solutions with the protein in water as the reference. The concentrations of the organic solvents used were over 70% by volume in water and were much higher than necessary for denaturation. At such high concentrations, the solvent effect red shifts at 284 and 292 mμ were large enough to overcome the denaturation blue shift, with the result that we find positive differences in extinction at these wavelengths. However, there is a large, atypical, negative trough around 300 mμ in all of these difference spectra. At nondenaturing concentrations of the organic solvents, the difference spectra were normal and did not reveal any significant peak or trough near 300 mμ. It was therefore necessary to see what connection exists between the appearance of the anomaly and the denaturation of the protein. This was done by carrying out experiments at close intervals of the denaturant concentration.

Results obtained in this way using dioxane are shown in Figures 2 and 3. The major extrema at 284 and 292 mμ in these difference spectra showed the expected behavior, namely, red shift before denaturation (up to 20% dioxane), denaturation blue shift (from 25 to 35% dioxane), and red shift after denaturation (at above 40% dioxane). The behavior of the 300-mμ extremum was quite different. There was no significant peak or trough near this wavelength before denaturation whereas during denaturation, a large negative difference absorbance value was obtained, a distinct negative shoulder being discernible at 35% dioxane concentration. After completion of denaturation, there was very little effect of the solvent on the magnitude of the absorbance difference at 300 mμ. This is more clearly seen from Figure 3, where values of Δε

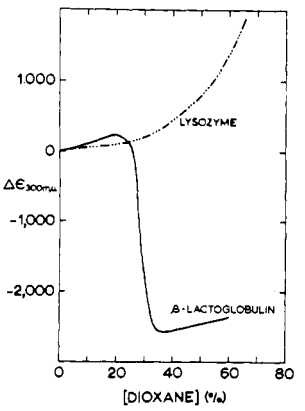

FIGURE 4: Plot of $\Delta\epsilon_{300}$ mμ *vs.* dioxane concentration for β-lactoglobulin A and lysozyme. The data for lysozyme are those of Hamaguchi and Kurono (1963).

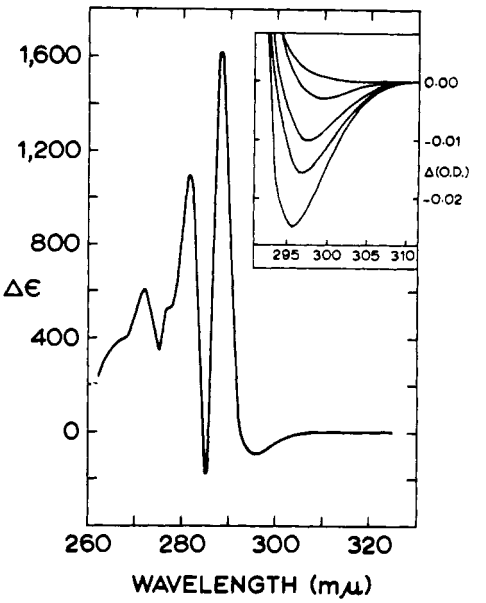

FIGURE 5: Difference spectrum of indole in 90% ethanol in water. Reference solution: indole in water. The inset shows the spectrum in the 290-300-mμ range recorded on the expanded scale of the Cary 15 instrument. The curves, from top to bottom, represent ethanol concentrations of 18, 36, 54, 72, and 90%.

at 284, 292, 294, and 300 mμ, obtained from data in Figure 2, are plotted against the solvent concentration. While the curves for the first two wavelengths showed a large response to both changes in the solvent and denaturation, the 300-mμ curve showed little response to the solvent changes, but a large response to denaturation.

It was of interest to inspect the behavior of the extremum at 300 mμ during the denaturation of other tryptophyl-containing proteins. A survey of the literature showed not only that denaturation did not always give a negative value of $\Delta\epsilon_{300}$, but also that sometimes a *positive* value of $\Delta\epsilon_{300}$ could be observed. That is, a "denaturation red shift" seemed to take place at 300 mμ. An example is given in Figure 4, where values of $\Delta\epsilon_{300}$ are plotted against the dioxane concentration for two proteins, β-lactoglobulin and lysozyme (Hamaguchi and Kurono, 1963). A detailed examination of the protein data is deferred to the following paper. The results in Figure 4, and similar results, show clearly that the spectral behavior at about 300 mμ in tryptophyl-containing proteins is different from that at about 292 mμ. At the latter wavelength, denaturation always brings about a blue shift which, as is well known, is caused by the transfer of tryptophyl residues from the nonpolar interior of the protein molecule to the relatively polar solvent medium.

It thus became desirable to carry out a systematic study of the influence of different variables like the solvent composition, pH, ionic strength, and temperature, on ϵ_{300}, using model compounds. In the experiments to be discussed, we have found occasion to use indole, tryptophan, *N*-Ac-Trp-ethyl ester, *N*-Ac-Trp, and Trp-ethyl ester.

Solvent Effects. The effect of a change in the solvent on the spectrum of indole and tryptophan was studied by difference spectroscopy.[1] For example, Figure 5 shows some of the difference spectra obtained when ethanol was added to aqueous indole. A *negative* trough whose magnitude increased with ethanol concentration was observed in these difference spectra at 297.5 mμ. This is in marked contrast to the positive peaks obtained at 282 and 290 mμ. The same negative trough around 298 mμ can be seen in data from a similar experiment by Foss (1961; Figure 4, curve D); however, he did not further investigate the phenomenon.

Similar experiments with systems like tryptophan and aqueous methanol or ethanol, and *N*-Ac-Trp-ethyl ester, *N*-Ac-Trp, or Trp-ethyl ester and aqueous dioxane revealed qualitatively similar negative troughs around 298 mμ and positive maxima at 282 and 290 mμ. A positive peak at about 300 mμ can be noticed in the difference spectrum, obtained by Polet and Steinhardt (1968), of tryptophan in water compared with tryptophan in aqueous methanol.

Difference spectra were also collected for indole and tryptophan in a variety of organic additives besides those mentioned above. The results are summarized in Table I. The sign of the difference extrema at 282 and 290 mμ were positive in all the cases studied. The sign and value of $\Delta\epsilon$ around 298 mμ, on the other hand, did not follow this trend; it can be seen from the table that it was sometimes positive, sometimes negative. It can also be seen that a particular solvent does not necessarily give the same sort of effect with both indole and tryptophan. We are unable to explain the rather varied response of these chromophores to the solvents; however, as we will show, these *solvent* effects are quantitatively quite small, and they are not likely to be very significant in proteins.

It should be added here that while a negative *extremum* can be observed at 298 mμ in these experiments, we do not observe a positive *extremum* at this wavelength. Rather there is a long-wavelength tail on the difference spectrum, because the difference peak here is so small and the 290-mμ peak so large that the two are not resolved in the difference spectrum. One

[1] Direct measurements on the shifts in the positions of the absorption bands are useful for a rigorous quantitative evaluation of the spectral changes produced, especially when complications like broadening and intensification of the absorption bands arise by the addition of the organic solvent which would make the difference method less useful for such purposes. However, in the experiments described here, the latter method was found more useful for an easier, though qualitative, understanding of the spectral changes. The former method was used in each case as a check on the conclusions drawn from the latter.

TABLE I: Effect of Organic Solvents on the Difference Spectra of Indole and Tryptophan.[a]

Solvent	Ref Index[b]	Indole			Tryptophan		
		$\Delta\epsilon_{297.5}$	$\Delta\epsilon_{288}$	$\Delta\epsilon_{282}$	$\Delta\epsilon_{300}$	$\Delta\epsilon_{290}$	$\Delta\epsilon_{282.5}$
Methanol	1.3288	−125	+1320	+840	−93	+1140	+808
Ethanol	1.3621	−105	+1660	+1120	−34	+1245	+675
2-Chloroethanol	1.3950	+25	+1195	+885	+38	+742	+627
Ethylene glycol monomethyl ether	1.4015	−40	+2035	+1305	+190	+1616	+837
p-Dioxane	1.4224	−105	+1835	+1205	+238	+1540	+741
Ethylene glycol	1.4274	+15	+1885	+1030	+143	+1426	+684
Dimethyl sulfoxide	1.4790	+353	+3028	+2170	+1211	+2540	+821
Sodium dodecyl sulfate		−45	+905	+553	−13	+475	+209

[a] Reference: solution in each case was the compound in water. Concentration of organic solvents used was 90% (by volume) and that of sodium dodecyl sulfate was 5% (by volume). [b] Refractive indices are for the pure solvents.

can now detect similar long-wavelength tails in some earlier difference spectra published on model compounds. For example, those published by Bigelow and Geschwind (1960) show that concentrated urea and LiBr caused sizable positive values of $\Delta\epsilon_{300}$ in the difference spectra of aqueous tryptophan solutions.

pH, Ionic Strength, and Temperature. The effect of changes in the electrostatic environment of the chromophore on ϵ_{300} could be understood from pH difference spectra measured for tryptophan. As mentioned above, these have earlier been studied by Donovan *et al.* (1961) and Yanari and Bovey (1960). These authors have brought out clearly the fact that electrostatic effects do affect the absorption characteristics of the indole chromophore. A red shift is observed for the major absorption band when a titratable molecule containing this chromophore becomes more negatively charged by dissociation of protons. The data of Donovan *et al.* (1961) and those from our own experiments reveal significant broadening of the extremum at 292 mμ in the pH difference spectra, extending to about 310 mμ; that is, $\Delta\epsilon_{292}$ and $\Delta\epsilon_{300}$ have the same sign. As is to be expected, experiments with *N*-Ac-Trp showed no significant changes in the chromophore's absorption in the alkaline pH range, those with Trp-ethyl ester showed negligible changes at acid pH values and those with indole and *N*-Ac-Trp-ethyl ester showed no marked effect in any accessible pH range.

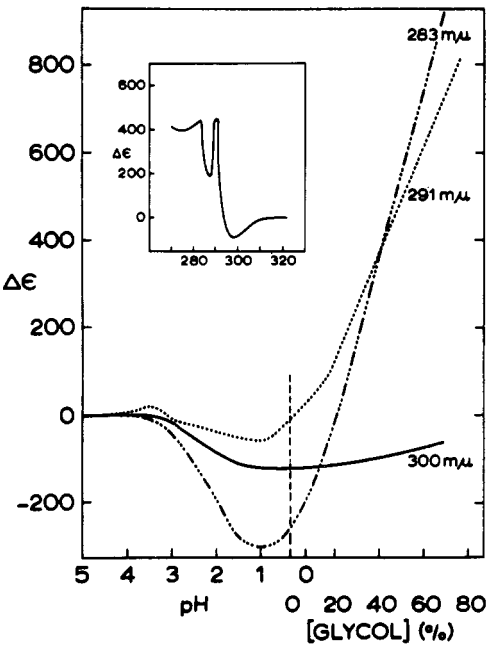

FIGURE 6: Plot of Δε *vs.* pH and ethylene glycol concentration for tryptophan. Reference solution is tryptophan in water at pH 6.0. See text for details. The inset shows the difference spectrum produced at pH 0.35 and 60% glycol.

The combined effect of an organic solvent and pH on $\Delta\epsilon_{300}$ in tryptophan provides an interesting study which proves useful in understanding the unusual difference spectra of proteins (see Ananthanarayanan and Bigelow, 1969). Figure 6 shows the results of experiments in which the pH of an aqueous solution of tryptophan is changed from neutral to low values following which ethylene glycol is added in increasing concentrations. Tryptophan in water at a pH of about 6 was the reference solution in these experiments. The values of Δε at 284, 291, and 300 mμ are plotted against both the pH and the glycol concentration on the abscissa. It is important to note that the positive Δε values observed at 283 and 291 mμ on the addition of the organic solvent are very much more pronounced than the negative values caused by the pH change. On the other hand, there is a sizable negative Δε value at 300 mμ caused by change of pH but practically no solvent effect; the small positive values actually observed at 300 mμ on adding the organic solvent may be mainly due to the red shift of the major bands. These data clearly show that the change in absorption at 300 mμ has a different origin from the changes in absorption at lower wavelengths. The inset in Figure 6 shows a very "unusual" difference spectrum which resulted from the combined effects of pH and solvent even in this simple compound.

Increasing the temperature or ionic strength of a solution of indole or tryptophan was found to lead to a positive $\Delta\epsilon_{300}$ as evidenced by a significant "tailing" of the major difference peak. Addition of solutes like urea or guanidine hydrochloride also had the same effect. Because we believed that in order to interpret the results already presented we would have to pos-

tulate the existence of an absorption band distinct from, but buried under, the main π–π^* band of these chromophores, we also carried out some studies of the spectrum of tryptophan and indole in KBr pellets at 80°K. The existence of such a band has been alluded to in a review article by Beaven (1961), but he did not present any data in that review. We therefore recorded a few spectra of tryptophan and N-Ac-Trp-ethyl ester in KBr pellets at room temperature and 80°K. A clearly visible shoulder in the spectrum around 298–299 mμ is found even at room temperature, while at the low temperature a separate peak is partially resolved. However, we are not very sure whether this peak observed at 300 mμ is a new one we have been looking for or is just the one at 290 mμ observed in aqueous tryptophan solutions which has moved up by about 10 mμ on the wavelength scale in the presence of KBr. A similar shoulder at 300 mμ was also observed when sucrose was used in the place of KBr.[2]

Discussion

The results described in this paper show that the spectral behavior of indole chromophores is somewhat more complex than has hitherto been appreciated. The difference spectra of earlier workers show that the value of $\Delta\epsilon_{290}$ varies in quite predictable fashion with the polarity of the solvent, but our new data show that the value of $\Delta\epsilon$ near 300 mμ cannot be accounted for so simply. In fact it is quite insensitive to solvent polarity but, as Donovan *et al.* (1961) showed, and as our data also show, it is especially sensitive to charged groups in the vicinity of the chromophore.

As mentioned in the introduction two different explanations seem possible. One is that there exists a separate electronic band which would be small compared with the main bands, and which would be buried under the long-wavelength side of the major bands. Such a minor band would presumably be due to a different electronic transition, and if so, that could account for its different response to changes in the environment. The second possibility is that there is a change in the shape of the absorption spectrum as well as a spectral shift when the environment is changed. There is, of course, no question that a change in the spectral shape is *observed* (this is just another way of saying that the difference spectra are unusual and it can be seen directly from the spectra). The question, rather, is whether a new electronic transition is the underlying cause of the shape change.

None of our own data is conclusive on this question. Nevertheless we believe that a separate band exists, and in what follows we will summarize the evidence for this belief. Subsequently we will interpret the results in terms of such a band.

While a detailed analysis of the unusual difference spectra of proteins will be presented in the following paper, it should be pointed out here that whether we accept the probable existence of a minor absorption band or not, the fact that we observe both denaturation blue shifts and red shifts at 300 mμ in proteins can be readily explained merely by supposing that in the former case, indole chromophores are separated from negative charges and in the latter, from positive charges. This conclusion is justified by a study of the effects of pH on the spectra of the model compounds studied by Donovan *et al.* (1961) and by us.

It is worthwhile considering, at this point, what references can be made from the earlier literature as to the existence of the putative minor band. As mentioned earlier Beaven (1961) alluded to its existence. On the other hand, Bigelow and Geschwind (1960), Yanari and Bovey (1960), and Mataga *et al.* (1964) did not report any evidence for such a band, though of course they were not looking for any.

It is worthwhile considering the spectra of naphthalene and thionaphthene in conjunction with the spectrum of indole (Badger and Christie, 1956). The arguments for expecting these compounds to have similar spectra have been summarized by Jaffe and Orchin (1962). Badger and Christie (1956) showed that both thionaphthene and indole absorb with medium intensity around 288 mμ, as does naphthalene. Naphthalene and thionaphthene were both shown to have smaller bands at longer wavelengths: 312 mμ for naphthalene, and 300 mμ for thionaphthene.

Thus our belief in the existence of a minor band in the indole spectrum is not unreasonable.

While studying the fluorescence polarization spectra of indole, tryptophan, and related compounds and of proteins containing tryptophan, Weber (1960) observed a maximum at 300–305 mμ in these spectra along with other extrema at lower wavelengths. He found that this maximum did not have a corresponding feature in the absorption spectrum of indole measured in propylene glycol at −70°. The observed polarization spectra were interpreted by him on the assumption that the long-wavelength absorption band of tryptophan consists of two independent electronic transitions, one from the ground state to a first singlet excited state and another from the ground state to a second singlet excited state. The maximum at 305 mμ was considered to arise from the excitation of the first singlet excited state alone. The two electronic transitions mentioned above (both being π–π^* transitions) were considered responsible for the major absorption bands in indole by other workers also (Mataga *et al.*, 1964; Wetlaufer, 1962). Yeargers (1968) has recently carried out a self-consistent field molecular orbital calculation on indole and tryptophan and his results are consistent with Weber's interpretation of the fluorescence polarization spectrum. A maximum near 300 mμ in the fluorescence polarization spectra of indole, tryptophan, and tryptophan-containing peptides has also been observed in a recent study by Lynn and Fasman (1968).

In a very recent study of the ultraviolet optical rotatory properties of poly-L-Trp, Cosani *et al.* (1968) have made circular dichroism measurements on this polymer in trifluoroethanol solution. The circular dichroism spectrum reported by these authors exhibits a *negative* trough at about 295 mμ and positive bands at 290 and 272 mμ with shoulders at 280 and 286 mμ. Since the circular dichroism spectra of optically active chromophores normally reflect the characteristics of their absorption spectra, the presence of a circular dichroism band near 295 mμ is consistent with the existence of an absorption band at the same wavelength in the spectrum of tryptophan. A similar conclusion can be drawn from the work of Lederer (1968) on acetoacetate decarboxylase which shows a

[2] While this paper was in preparation, Keyes *et al.* (1969) presented an interpretation of the 300-mμ difference which was based on spectral broadening rather than on the existence of a separate absorption band. More work will undoubtedly be required to decide absolutely conclusively between the two interpretations.

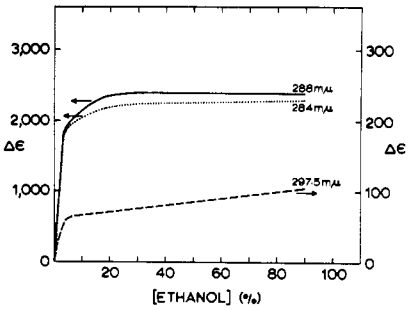

FIGURE 7: Plot of $\Delta\epsilon$ at 284, 288, and 297.5 mμ *vs.* ethanol concentration, for indole–isooctane–ethanol system. Indole in pure isooctane is the reference solution.

weak positive circular dichroic band above 305 mμ, besides the negative bands at 290, 283, and 270 mμ.

The possibility that an absorption band near 300 mμ may arise from a n–π^* transition seems to be ruled out from our observation that addition of ethanol to an isooctane solution of indole causes a red shift at about 290 and 298 mμ (Table I). This is illustrated in Figure 7 where the $\Delta\epsilon$ values at 284, 288, and 297.5 mμ are plotted against ethanol concentration. A blue shift would be expected for the new band if it were due to a n–π^* transition and if hydrogen-bond formation in the hydroxylic solvent caused a spectral shift (Kasha, 1960; Glazer and Rosenheck, 1962). The leveling off of the curves for 284 and 288 mμ in Figure 6 at as low as 5% ethanol concentration, confirms the involvement of hydrogen bonding in the observed red shift of the major bands. However, there is a continuous increase in the $\Delta\epsilon$ values at 297.5 mμ even up to 80% ethanol concentration. (The initial steepness of the curve at this wavelength is most probably due to the overlapping effect of the adjacent major band.) This would mean that hydrogen-bond formation is not the reason for the red shift of the new band.

Some insight into the nature of the new band could be obtained by noting the nature of the shift of this band in indole in different solvents, as given in Table I. An examination of the type of solvents which produce blue shifts and of those which cause red shifts of this band, leads us to believe that the behavior of the minor band in indole–organic solvent systems is closely related to the hydrophobic character of the solvent—an increase in the hydrophobic nature of the solvent as compared with water causes a blue shift of this band whereas an increase in the "polarity" of the solvent produces a red shift. However, this generalization fails to hold good when applied to tryptophan–solvent systems as could be seen from Table I. We believe that this is due to the electrostatic charges carried by the tryptophan molecule and their interaction with the solvent—the actual nature and extent of such an interaction would depend upon the type of solvent employed.

It can be mentioned that Hamaguchi and Kurono (1963) found a negative trough near 300 mμ when tryptophan in aqueous 2-chloroethanol was compared with tryptophan in water. The apparent contradiction between their result and ours (Table I) arises from the fact that the 2-chloroethanol solutions used by Hamaguchi and Kurono were at pH values between 2.6 and 3.6 while the reference solutions were at neutral pH. Their difference spectra therefore resemble the "anomalous" one we found by changing both the pH and the concentration of organic solvent (Figure 6, inset).

TABLE II: Values of $\Delta\epsilon_{298-300}/\Delta\epsilon_{288}$ in the Difference Spectra of Indole and Tryptophan.

Compound	Reference	Sample	$\Delta\epsilon_{298-300}/\Delta\epsilon_{288}$
Indole	Water	90% 2-chloro-ethanol	0.02
	Water	90% ethylene glycol	0.008
	Water	90% dimethyl sulfoxide	0.12
	Isooctane	90% ethanol	0.044
	Water	7.4 M LiCl	0.21
	Water	7.4 M urea	0.11
Tryptophan	Water pH 13.0	Water, pH 0.4	0.58
	Water pH 6.0	Water, pH 0.4	0.33

It is useful, at this point, to calculate the value of the ratio $\Delta\epsilon_{297.5}/\Delta\epsilon_{290}$ and use this in comparing the magnitudes of the effect of organic solvents, on the one hand, and the effect of pH, on the other hand, on the new, minor band. Table II contains values of this ratio obtained for indole. It is to be noted that in all these cases, the shift of the minor band is bound to be influenced by those of the major bands so that we can assume that the comparison of the ratio values has been made under more or less similar conditions. The important fact that arises from such a comparison is that the effect of changing the polarity of the solvent on the minor band is very much smaller (lower value for the extinction ratio) than that arising from changes in the electrostatic environment. As will be discussed in the following paper (Ananthanarayanan and Bigelow 1969), this fact is useful in understanding the behavior of the minor band in proteins.

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Unusual Difference Spectra of Proteins Containing Tryptophan. II. Studies with Proteins*

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ABSTRACT: An attempt is made to interpret unusual difference spectra observed with various tryptophyl-containing proteins in a consistent fashion working from studies made with model compounds. It is found that an analysis of the behavior of the hitherto ignored extremum around 300 mμ in these difference spectra can yield information about the electrostatic environment of tryptophyl residues in the native form of the proteins and about small local conformational changes around these residues which take place under certain conditions in some of these proteins.

Proteins containing tryptophyl residues exhibit, under certain conditions, an atypical extremum or shoulder around 300 mμ in their difference spectra. Figures 1 and 2 of the preceding paper and Figure 1 of this paper give examples of such unusual difference spectra obtained by us with β-lactoglobulin A. From the literature one can cite observations made on the proteins listed in Table I.

Studies made with model compounds (see Ananthanarayanan and Bigelow, 1969) have revealed that the unusual nature of these difference spectra probably arises from the presence of a weak long-wavelength band in the absorption spectrum of the indole chromophore and the characteristic behavior of this minor band toward changes in the chromophore's environment. Use is made of these results in this paper to interpret the unusual difference spectra of various tryptophyl-containing proteins.

Materials and Methods

β-Lactoglobulin A was prepared as outlined in the preceding paper. Lysozyme was a three-times-recrystallized sample from Worthington. Chymotrypsinogen was a Worthington sample. Other reagents were the same as described in the preceding paper (Ananthanarayanan and Bigelow, 1969).

Difference spectral measurements were carried out with a Cary Model 15 spectrophotometer using matched silica cells. pH measurements were made on a Radiometer pH meter.

Results and Discussion

The reader is referred to Figures 1 and 2 of the preceding paper (Ananthanarayanan and Bigelow, 1969) which show some of the unusual difference spectra obtained with β-lactoglobulin A when it undergoes denaturation by organic solvents. In Figure 1 of this paper are shown the difference spectra of β-lactoglobulin A denatured thermally and by urea. In both cases a distinct negative shoulder at about 300 mμ is found to be present along with the minima at 284 and 292 mμ.

Figure 2 shows the difference spectra of chymotrypsinogen, pepsin, and bovine albumin after they have been denatured by urea or Gu·HCl. A faintly discernible negative shoulder around 298 mμ was obtained in the case of chymotrypsinogen in 6 M urea whereas a broad positive maximum centered around 300 mμ was found in the case of pepsin in 7.2 M Gu·HCl. In both cases minima were found at 292.5 and 283–285 mμ. With bovine albumin there was practically zero difference absorp-

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