# Studies of the Location of Tyrosyl and Tryptophyl Residues in Proteins. I. Solvent Perturbation Data of Model Compounds*

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ABSTRACT: For the study of the location of tyrosyl and tryptophyl groups in proteins by the solvent perturbation technique, and the simultaneous assessment of the number or fraction of both of these chromophores exposed in proteins, carefully measured molar absorptivity difference values of the *N*-acetyl ethyl esters of tyrosine and tryptophan have been obtained with nine of the most commonly employed perturbants ranging in diameter from 2.0 to 9.4 Å. Molar absorptivity differences in the wavelength region from 350 to 240 mμ have been collected and tabulated at convenient wavelength intervals, using aqueous solutions of 90% deuterium oxide and 20% methanol, dimethyl sulfoxide, ethylene glycol, glycerol, erythritol, glucose, hexaethylene glycol (Carbowax 300), and sucrose as perturbants. For the study of urea-denatured proteins, model data have also been obtained in 8 M urea, with 20% dimethyl sulfoxide, ethylene glycol, glycerol, and sucrose as perturbants. The additivity of solvent perturbation difference spectra has been checked and found valid for both tyrosyl and tryptophyl model compounds and proteins, and the range of applicability in studies of proteins, rich in both tyrosine and tryptophan, has been defined and tested.

Studies of the location of tyrosyl and tryptophyl residues and their involvement in protein and enzymic reactions and transformations have gained considerable attention in recent years (for the more recent literature of the subject see Wetlaufer, 1962; Scheraga and Rupley, 1962; Edsall, 1963; Hayashi *et al.*, 1964; Herskovits, 1967; Cuatrecases *et al.*, 1967). The solvent perturbation method of difference spectroscopy (Herskovits and Laskowski, 1960, 1962a), which employs mild nonaqueous additives as spectral perturbants or probes for locating the chromophoric groups in proteins, is one of the methods which has greatly facilitated such studies. Thus the location of tyrosyls in ribonuclease (Herskovits and Laskowski, 1960, 1968), the serum albumins (Herskovits and Laskowski, 1962a), ovomucoid (Herskovits and Laskowski, 1962b; Donovan, 1967), insulin (Weil *et al.*, 1965; Herskovits, 1965), the dehydrogenases (Libor *et al.*, 1965; Cross and Fisher, 1966), the heptalbumins (Waks and Alfsen, 1966), paramyosin (Riddiford, 1966), thyroglobin (van Zyl and Edelhoch, 1967), streptococcal nuclease (Cuatrecases *et al.*, 1967), taka-amylase (Friedman and Epstein, 1967), and bovine fibrinogen (Mihalyi, 1968) have been examined. Protein studies involving the tryptophans in carboxypeptidase (Fujioka and Imahori, 1963), lysozyme (Hayashi *et al.*, 1964; Williams *et al.*, 1965), α-chymotrypsin and chymotrypsinogen (Williams *et al.*, 1965), α-lactalbumin (Kronman and Holmes, 1965; Herskovits, 1965), β-lactoglobulin (Herskovits, 1965), trypsin (Edelhoch and Steiner, 1965), L-glutamate dehydrogenase (Cross and Fisher, 1966), myoglobin (Williams, 1966), cytochrome *c* (Stellwagen and Van Rooyan, 1967), hemoglobin and hemocyanin (Herskovits and Greenblatt, 1967), and bovine fibrinogen (Mihalyi, 1968) have also been reported.

Most of these studies, on the one hand dealing with only tyrosine-containing or tyrosine-rich proteins and on the other with tryptophan-rich proteins, have justifiably ignored the explicit consideration of the different spectral contribution of the minor chromophoric components. This was possible because the tyrosyl perturbation at the first 291–294-mμ peak is small, and correspondingly the tryptophan difference spectrum at the major 286–288-mμ tyrosyl peak is relatively small, although not as small as has been assumed in the past. For example, the contributions of the three and four tyrosyl residues to the perturbation difference spectra of the six and eight tryptophyls in lysozyme and α-chymotrypsinogen, respectively, are at most 5%. On the other hand, in serum albumin the contribution of the two tryptophyls to the difference spectra at the 286–288-mμ maximum, due to the perturbations of some of the 21 tyrosyls, may be as much as 30%.[1]

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[1] This is readily apparent from the tyrosine and tryptophan difference spectra of Figure 1B. The estimates given are based on the data of Tables I and II of this paper employing the relation: $\Delta\epsilon_\lambda(\text{protein}) = a\Delta\epsilon_\lambda(\text{Trp}) + b\Delta\epsilon_\lambda(\text{Tyr})$ assuming that the tyrosyl and tryptophyl residues in lysozyme and α-chymotrypsinogen are about equally exposed. Here the subscript λ refers to the wavelength of interest, *i.e.*, 286–288 or 292–94 mμ, *a* refers to the number of tryptophyls, and *b* is the number of tyrosyls in the protein in question. The exposure of tryptophyl residues in bovine serum albumin and their difference spectral contributions are discussed in the accompanying paper (Herskovits and Sorensen, 1968).

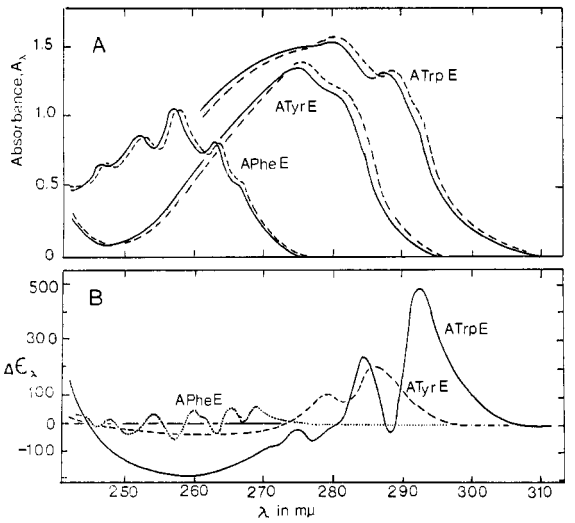


FIGURE 1: Relation between spectral shifts and solvent perturbation difference spectra of *N*-acetyl ethyl esters of tryptophan, tyrosine, and phenylalanine. (A) Solid lines indicate spectra in water; broken lines, spectra in 20% dimethyl sulfoxide. (B) Solvent perturbation difference spectra obtained with 20% dimethyl sulfoxide solutions plus water blanks in the sample beam and aqueous solution in the reference beam of the spectrophotometer. Concentrations: $3 \times 10^{-4}$ M *N*-acetyl-L-tryptophan ethyl ester, $1 \times 10^{-3}$ M *N*-acetyl-L-tyrosine ethyl ester, and $5 \times 10^{-3}$ M *N*-acetyl-L-phenylalanine ethyl ester. For purposes of representation in Figure B, the otherwise negligible absorptivity difference of *N*-acetyl-L-phenylalanine ethyl ester is multiplied by a factor of four. Buffer: $\Gamma/2 = 0.01$, pH 6.8 phosphate.

In proteins where both tyrosyl and tryptophyl residues contribute materially to the absorption and solvent perturbation difference spectra, that is, where the tyrosine to tryptophan molar ratios approach about 3:1,[2] in principle one should be able to obtain reasonable estimates of the exposure of both tyrosyl and tryptophyl groups. Fujioka and Imahori (1963) were the first to attempt to obtain such information on carboxypeptidase. Owing to instrumental limitations, however, they were unable to obtain reliable information on other than the exposure of tryptophyl residues. Donovan (1964) was able to resolve both the tyrosyl and tryptophyl contributions to the perturbation difference spectra of native and acid-denatured aldolase, employing a Cary Model 11 double-beam spectrophotometer, and obtain estimates of the fraction of exposed tyrosyl and tryptophyl residues perturbed by ethylene glycol. In a recent study, Mihalyi (1968) examined the location of tyrosyls and tryptophyls in bovine fibrinogen, employing essentially the same methods of analyses. In these studies the additivity of tyrosyl and tryptophyl contributions to the solvent perturbation difference spectra were assumed, as this has been logically assumed in the first published papers on the subject (Herskovits and Laskowski, 1960, 1962a,b), and the protein difference spectra in the 320–260-mμ region were then fitted visually with the best combination of molar tyrosyl and tryptophyl absorptivity differences obtained with the same perturbant.

The need for tabulated values of the molar absorptivity differences of model compounds and the desire to define and to test the instrumental reliability and the additivity of solvent perturbation difference spectra of tyrosyl and tryptophyl chromophores in model compound mixtures and proteins have prompted the present study. In addition to the latter tests, carefully measured molar absorptivity differences of *N*-acetyl ethyl esters of tyrosine and tryptophan in aqueous solutions in the wavelength region ranging from 350 to 240 mμ employing the nine most commonly used perturbants ranging in diameter from 2.0 to 9.4 Å are reported in this paper, together with selected model data necessary for protein denaturation studies in 8 M urea. In the accompanying paper some of the model data is applied to the study of the location of tyrosyls and tryptophyls in rabbit muscle aldolase, pepsin, and bovine serum albumin, with the main purpose of defining the methods and procedures to be employed and the discovery of the best way of handling solvent perturbation data for the study of this class of tyrosine- and tryptophan-rich proteins.

## Experimental Section

*Materials.* *N*-Acetyl ethyl esters of tyrosine (Ac-Tyr-OEt), tryptophan (Ac-Trp-OEt), and phenylalanine (Ac-Phe-OEt) were Mann Research Laboratories products. Polyethylene glycol (Carbowax 300; degree of polymerization, 6) was a Union Carbide product, while deuterium oxide (99.89%) was a Bio-Rad Laboratories product. All other solvents and reagents were spectroscopic quality of the best commercially available grade. Bovine pancreatic ribonuclease was purchased from Sigma Chemical Corp. Lysozyme was a Worthington Biochemical Corp. product. The water employed was prepared in an all-glass still.

*Methods.* Difference spectral measurements were made as already described (Herskovits and Laskowski, 1962a; Herskovits, 1967) employing a Cary 14 recording spectrophotometer, equipped by the manufacturer with a scale multiplier which permits one to expand the optical density scale by a factor of ten. Matched cylindrical 1.0-cm path-length tandem cells were employed (Herskovits and Laskowski, 1962a). Before each set of experiments the base line or zero line was adjusted with solvent and perturbant blanks as close to zero as the instrumental multipots would permit (Herskovits and Laskowski 1962a; Herskovits, 1967). With the use of the scale expander small shifts in base line due to cell repositioning or even small temperature fluctuations were noted. We found that more reproducible $\Delta\epsilon_M$ values could be obtained by a linear extrapolation of the base line in each run, using the nonabsorbing 350–315-mμ region as a guide. We also found that with our instrument a small deviation in the 275–260-mμ region with a minimum of about −0.002 to −0.004 $\Delta A$ unit (around 265–68 mμ) could not be eliminated with the

[2] Examination of the data of Tables I and II indicates that the molar absorbances of tryptophan at 292–294 mμ obtained with the most commonly employed perturbants are about three times the average values obtained with tyrosine at the 286–288-mμ peak. The molar extinction coefficient of tryptophan in water is correspondingly higher: 5550 at 282 mμ, as compared to 1340 for tyrosine at 274.5 mμ.

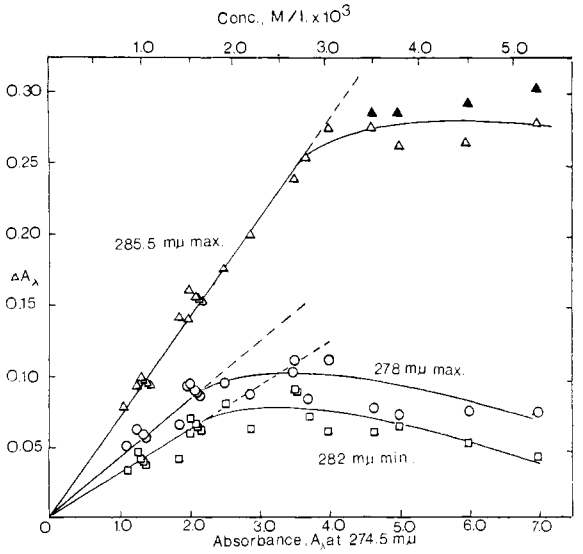


FIGURE 2: Test of Beer's law for solvent perturbation difference spectral measurements, $\Delta A_\lambda$ *vs.* $A_{274.5\ m\mu}$ and Ac-Tyr-OEt concentration, obtained with 20% ethylene glycol as perturbant. Measurements at 285.5 mμ (Δ) represent dynode voltage setting 3; at 285.5 mμ at dynode voltage setting 4 (▲); at 278 mμ (○), and at 282 mμ (□), dynode voltage settings 3. Ac-Tyr-OEt concentrations were $9 \times 10^{-4}$–$5.2 \times 10^{-3}$ M; 0.1 M KCl, 0.01 M pH 6.8 phosphate.

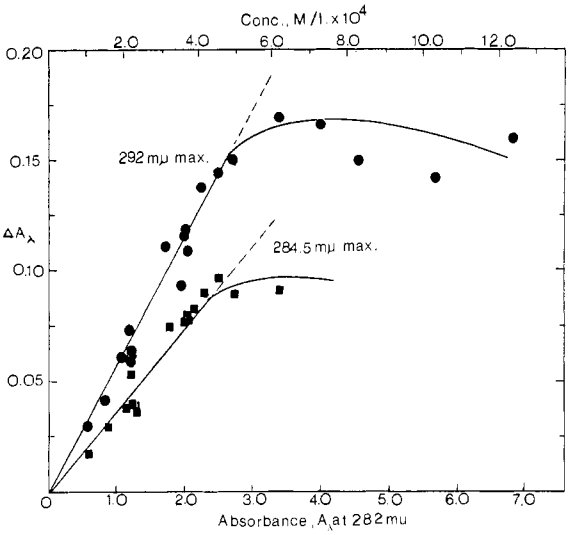


FIGURE 3: Test of Beer's law for solvent perturbation difference spectral measurements, $\Delta A_\lambda$ *vs.* $A_{282\ m\mu}$ and Ac-Tyr-OEt concentrations, obtained with 20% ethylene glycol as perturbant. The $\Delta A\lambda$ values at 292 and 284.5 mμ were obtained at dynode voltage settings of 3. Ac-Tyr-OEt concentrations were $1 \times 10^{-4}$–$1.2 \times 10^{-3}$ M; 0.1 M KCl; 0.01 M pH 6.8 phosphate.

neighboring 275- and 260-mμ multipots (such a base line is shown in the bottom part of Figure 4). Therefore, in addition to the above-mentioned base line extrapolation, this small nonzero contribution to the difference spectra was subtracted in each experiment. In working with 90% deuterium oxide, solutions were prepared at concentrations ten times the desired final values. Employing a 500-μl pipet, 0.50 ml of model or protein solution was delivered twice to two 5.0-ml flasks. To one solution 4.5 ml of 99.8% $D_2O$ was added, while to the other, 4.5 ml of water was added, employing the same pipets. After the $D_2O$ was delivered, pipets were rinsed once or twice with water and the water deliveries were made. Blowout pipets were utilized. The content of the tip of the pipets used was blown out as previously described (Herskovits and Laskowski, 1962a; Herskovits, 1967).

The concentrations of all liquid perturbants are expressed in volume per cent (v/v). The concentrations of sugars which are solids are 21.6 g of sucrose, 21.36 g of erythritol, and 21.52 g of glucose per 100 ml of final solution. In *water*, this corresponds to 20% (w/w).

Concentrations were determined spectrophotometrically using the following molar extinction coefficients: 195 for Ac-Phe-OEt at 258.5 mμ, 1340 for Ac-Tyr-OEt at 274.5 mμ, 5550 for Ac-Trp-OEt at 282 mμ (Beaven and Holiday, 1952; Wetlaufer, 1962), 37,750 for lysozyme at 282 mμ (Sophianopoulos *et al.*, 1962), and 9800 for ribonuclease at 277.5 mμ (Wetlaufer, 1962). Extinction coefficients for Ac-Tyr-OEt and Ac-Trp-OEt in 8 M urea are 1420 and 5870, respectively. These values were determined by measurements on aqueous and 8 M urea solutions and using the above aqueous value from the literature as a reference. A set of three to five measurements was made by delivering from a common aqueous stock solution 1.0 ml each to 4 ml of water or 4 ml of 10 M urea. The same pipet was used for the deliveries and dilutions in 5-ml volumetric flasks.

Difference spectral measurements were made at least in triplicate at two different optical densities for both Ac-Tyr-OEt and Ac-Trp-OEt. Solutions were measured at optical densities of about one and about two with eight of the nine perturbants. Because of solubility limitations measurements with $D_2O$ could be made only at the lower absorbance. All measurements were performed at 25 ± 1°. Instrument reliability tests for difference spectral measurements were made with Ac-Tyr-OEt and Ac-Trp-OEt using 20% ethylene glycol as perturbant, employing a range of absorbances up to about 7, and making instrumental adjustments with the dynode voltage setting and the slit-width control. The accuracy of the scale-expander slide wire was tested with model compound solutions. Within experimental error the same absorbance differences were obtained with and without the scale expander (*i.e.*, to better than ±0.002 absorbancy unit). Measurements were made at a scanning speed of 5 Å/sec.

## Results and Discussion

*Instrument Reliability.* It has been the experience of workers using difference spectral techniques in the past that the manufacturer's specifications concerning the optimum noise to resolution ratio and absorbance ceiling are not applicable to difference spectroscopy, having been determined for direct spectral work, and relying on them could lead to erroneous results (Herskovits, 1967).

For difference spectral studies involving the use of model data the linear or Beer's law range of solution

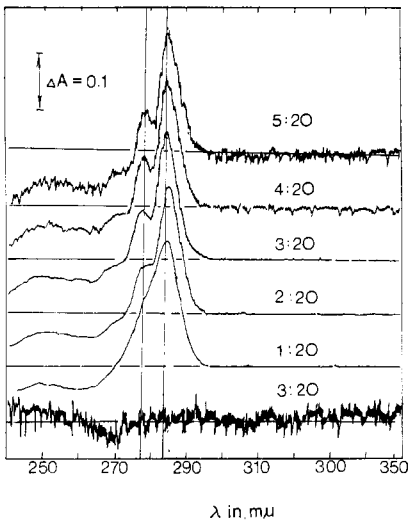


FIGURE 4: Difference spectra tracings of Ac-Tyr-OEt in 20% ethylene glycol at various dynode voltage and slit-width control adjustments. The first number indicated is the dynode voltage setting and the number following the colon is the slit-width control adjustment. The spectral band widths at the 285.5-mμ maximum were 5.5, 12, 22, 38, and 70 Å for the Ac-Tyr-OEt curves from the uppermost (5:20) to the lowest Ac-Tyr-OEt curve (1:20). Ac-Tyr-OEt concentration = $2.63 \times 10^{-3}$ M.

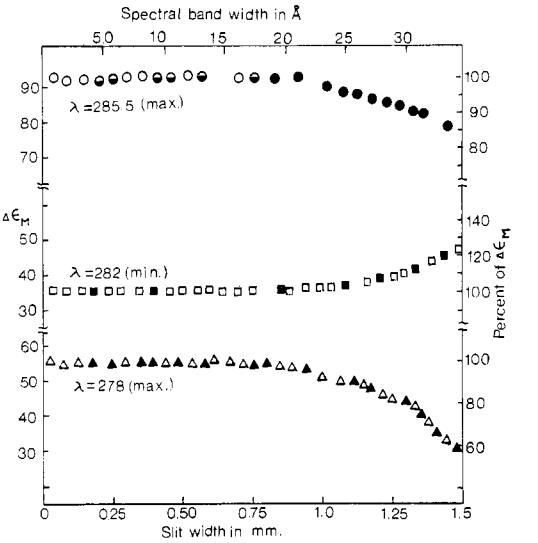


FIGURE 5: Molar absorptivity differences for Ac-Tyr-OEt due to 20% ethylene glycol are shown as a function of slit width and spectral band width. Measurements at 285.5 mμ (○ absorbance of approximately 1; ◒ absorbance, 3; ● absorbance, 5); at 282 mμ (□ absorbance, 1; ■ absorbance, 3); and at 278 mμ (△ absorbance, 1; ▲ absorbance, 3).

concentrations needs to be defined and tested for each individual instrument employed, since only in this solution range will the reported difference spectral parameters have meaning in terms of reproducibility and additivity required for the use of such data.

Adherence to Beer's law was tested in the present study on a Cary 14 double-beam instrument. Absorbance differences at the first tyrosine and tryptophan difference spectral maxima (274.5 and 282 mμ; see Figure 1) showed a linear relationship up to about 2.5 absorbance units (Figures 2 and 3) when plotted as a function of concentration and/or optical density. These measurements were obtained using 20% ethylene glycol as perturbant. Measurements were made with the dynode voltage adjustment at 3 and the slit-width control at 25. Wherever possible use was made of the scale expander (*i.e.*, with most of the solutions in the Beer's law range having $\Delta A_\lambda$ values of less than 0.1 at their respective difference spectral maxima). In order to try to extend the linear range, an attempt was made to use a dynode voltage adjustment of 4, but this instrumental adjustment provided an inconvenient amount of noise without appreciable improvement in resolution. Therefore, the dynode voltage adjustment of 3 was used throughout this work. Figure 4 is an example of some of the difference spectral tracings at various dynode settings. The data of this figure emphasizes an important fact, namely that a difference spectral peak (at 278 mμ) of a sufficiently concentrated and optically dense solution (absorbance at 274.5 mμ of 3.52) can visually disappear at very high slit widths or spectral band widths, *i.e.*, at low noise level.

Because most proteins absorb maximally around 280 mμ, and the tyrosyl and tryptophyl difference spectral peaks center respectively around 286 and 292 mμ, it is important to note that the actual absorbance values measured are considerable less at these longer wavelengths than at 280 mμ. The actual absorbance values of the solutions employed in our Beer's law study were only 54 and 55% of the absorbance at the 274.5- and 282-mμ tyrosine and tryptophan maxima. The apparent discrepancies suggested by the lower concentrations or optical densities at which the second tyrosine and tryptophan difference spectral maxima and minima deviate from Beer's law are largely due to the fact that at 278, 284.5, and 282 mμ the respective absorbance values are 94, 89, and 83% of the absorbance values at the maxima. We recommend that this limit of 2.2–2.5 absorbance units above which deviations from Beer's law are apparent be not exceeded, unless one is only trying to obtain a more accurate determination of absorbance difference at the first tyrosyl or tryptophyl difference spectral maxima rather than the whole difference spectral profile.

The slit width and spectral band width limitation for reliable measurements have also been studied (Figure 5). Manual operations employing the instrumental dynode voltage adjustment and the slit-width control, as well as varying the solution absorbance, effected changes in slit width in this study. Reproducible values for the molar absorptivity differences ($\Delta\epsilon_M$) were obtained with slit and spectral band widths up to 0.75 mm and about 17 Å, respectively, at which point good resolution of both maxima and minima was lost. Since the spectral band width did not exceed 10 Å in the measurements reported below, this limitation has not been a problem in the present study. Yet it is noteworthy and apparent from the data of Figure 5 that errors as large as 40% would result at slit widths of 1.5 mm or spectral band widths of about 35 Å.

*Model Compound Data.* Figure 1 also illustrates the relation between solvent-induced shifts or solvent per-

TABLE I: Molar Absorptivity Difference Values for *N*-Acetyl-L-tyrosine Ethyl Ester due to Several Perturbants in Aqueous Solutions.[a]

| λ (mμ) | 90% $D_2O$ | 20% Methanol | 20% DMSO[b] | 20% Ethylene Glycol | 20% Glycerol | 20% Eryth-ritol | 20% Glucose | 20% Carbowax 300 | 20% Sucrose |
|---|---|---|---|---|---|---|---|---|---|
| 350.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 315.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 310.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 305.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 300.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 297.5 | −1.0 | 0.2 | 1.5 | 0.6 | 0.0 | 0.0 | 0.2 | 1.4 | 0.0 |
| 295.0 | −2.8 | 1.3 | 12.7 | 4.1 | 2.5 | 0.9 | 1.5 | 8.6 | 1.0 |
| 294.0 | −5.6 | 1.9 | 21.7 | 5.8 | 3.4 | 2.0 | 3.0 | 15.3 | 2.2 |
| 293.0 | −7.8 | 4.0 | 32.6 | 9.8 | 7.6 | 4.2 | 5.0 | 24.7 | 3.8 |
| 292.5 | −9.8 | 8.1 | 35.5 | 13.4 | 10.4 | 7.3 | 6.2 | 32.9 | 5.6 |
| 292.0 | −12.2 | 12.3 | 48.6 | 16.1 | 12.9 | 8.6 | 9.6 | 40.7 | 7.0 |
| 291.5 | −14.0 | 16.8 | 62.7 | 20.1 | 16.7 | 10.4 | 11.7 | 49.8 | 8.9 |
| 291.0 | −16.8 | 19.4 | 73.2 | 24.6 | 21.5 | 13.8 | 12.8 | 57.1 | 11.3 |
| 290.0 | −27.1 | 26.4 | 110.5 | 38.2 | 33.3 | 23.1 | 18.0 | 88.0 | 18.8 |
| 289.0 | −37.5 | 39.7 | 145.6 | 53.5 | 50.1 | 31.8 | 24.2 | 125.8 | 25.7 |
| 288.5 | −41.9 | 46.7 | 164.5 | 64.1 | 57.3 | 37.1 | 28.2 | 140.6 | 29.8 |
| 288.0 | −48.1 | 54.2 | 183.7 | 71.6 | 64.9 | 42.5 | 32.0 | 156.0 | 33.7 |
| 287.0 | −58.7 | 68.7 | 207.2 | 84.3 | 75.8 | 52.2 | 38.1 | 179.0 | 42.2 |
| 286.0 | −65.6 | 74.8 | 213.7 | 91.9 | 84.1 | 60.3 | 41.5 | 187.0 | 45.7 |
| 285.5 | −67.1 | 75.5 | 203.9 | 92.1 | 83.4 | 60.3 | 40.7 | 181.0 | 46.1 |
| 285.0 | −66.4 | 72.8 | 186.6 | 88.2 | 79.8 | 58.3 | 38.6 | 163.0 | 43.8 |
| 284.0 | −59.9 | 58.8 | 141.7 | 73.8 | 64.7 | 50.9 | 32.5 | 117.0 | 40.0 |
| 283.0 | −42.9 | 34.0 | 98.9 | 51.1 | 47.0 | 39.2 | 24.3 | 81.7 | 29.3 |
| 282.0 | −30.6 | 27.2 | 77.2 | 32.5 | 34.9 | 32.4 | 19.4 | 63.9 | 24.0 |
| 281.5 | −25.4 | 21.7 | 75.4 | 34.5 | 32.0 | 30.1 | 19.1 | 64.7 | 23.7 |
| 281.0 | −25.0 | 21.6 | 85.5 | 34.9 | 32.4 | 30.4 | 19.5 | 64.9 | 24.5 |
| 280.0 | −27.2 | 29.6 | 101.4 | 43.2 | 39.1 | 32.4 | 23.9 | 81.2 | 27.6 |
| 279.0 | −34.9 | 38.7 | 107.2 | 53.2 | 47.5 | 39.6 | 26.2 | 95.3 | 32.2 |
| 278.5 | −37.5 | 41.4 | 105.1 | 54.9 | 48.5 | 42.0 | 25.9 | 94.1 | 32.6 |
| 278.0 | −38.2 | 40.1 | 92.4 | 54.5 | 47.1 | 41.5 | 24.8 | 87.1 | 31.4 |
| 277.0 | −35.5 | 31.1 | 67.0 | 43.9 | 39.1 | 38.4 | 17.9 | 60.8 | 28.4 |
| 276.0 | −24.9 | 19.8 | 33.7 | 30.0 | 25.8 | 30.0 | 14.6 | 31.0 | 25.0 |
| 275.0 | −15.1 | 10.0 | 14.3 | 17.4 | 14.0 | 24.8 | 7.9 | 8.8 | 15.3 |
| 274.0 | −10.2 | 2.5 | 0.0 | 10.3 | 6.4 | 20.1 | 3.8 | −1.0 | 12.0 |
| 272.5 | −6.8 | −1.0 | −15.9 | 5.5 | 3.0 | 14.1 | 3.0 | −9.6 | 8.9 |
| 270.0 | −5.9 | −6.5 | −22.8 | 1.4 | −1.2 | 10.6 | −1.2 | −17.6 | 4.5 |
| 267.5 | −3.2 | −10.5 | −37.3 | −7.0 | −10.0 | 6.4 | −4.1 | −30.4 | 1.4 |
| 266.0 | −0.8 | −13.0 | −41.6 | −9.1 | −12.2 | 2.3 | −6.6 | −36.1 | −0.5 |
| 265.0 | −0.7 | −12.4 | −39.8 | −9.7 | −13.6 | 1.0 | −7.4 | −32.9 | −1.4 |
| 262.5 | −1.8 | −12.3 | −40.2 | −8.0 | −14.0 | −3.0 | −8.2 | −41.8 | −2.4 |
| 260.0 | −2.2 | −12.1 | −34.8 | −8.0 | −13.2 | −5.1 | −8.8 | −39.8 | −3.7 |
| 255.0 | −7.1 | −10.3 | −25.4 | −6.6 | −10.4 | −6.4 | −9.1 | −23.7 | −4.7 |
| 250.0 | −14.8 | −7.9 | −18.8 | −2.9 | −8.9 | −6.2 | −10.4 | −18.8 | −4.4 |
| 245.0 | −39.3 | −6.6 | −11.2 | −6.9 | −10.7 | −8.2 | −13.0 | −16.1 | −4.4 |
| 242.5 | | −5.6 | −9.1 | −5.4 | −11.4 | −8.7 | −14.0 | −12.5 | −1.5 |
| 240.0 | | −3.2 | −4.6 | −3.0 | −5.8 | −4.4 | −11.4 | −8.2 | 6.2 |

[a] Measurements made at absorbances of about 1.3 and 2 at 276 mμ; 0.1 M KCl; 0.01 M pH 6.8 phosphate; 25°.
[b] DMSO, dimethyl sulfoxide.

TABLE II: Molar Absorptivity Difference Values for *N*-Acetyl-L-tryptophan Ethyl Ester due to Several Perturbants in Aqueous Solutions.[a]

| λ (mμ) | 90% $D_2O$ | 20% Methanol | 20% DMSO | 20% Ethylene Glycol | 20% Glycerol | 20% Erythritol | 20% Glucose | 20% Carbowax 300 | 20% Sucrose |
|---|---|---|---|---|---|---|---|---|---|
| 350.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 315.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 310.0 | −10.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.9 | 0.0 | 0.0 | 0.9 |
| 305.0 | −35.2 | 2.4 | 24.9 | 2.5 | 13.6 | 14.3 | 12.6 | 0.0 | 15.5 |
| 300.0 | −90.8 | 8.7 | 116.7 | 43.9 | 66.5 | 54.4 | 51.5 | 33.0 | 48.0 |
| 297.5 | −124.5 | 33.5 | 190.4 | 89.4 | 114.1 | 81.2 | 90.3 | 103.7 | 86.4 |
| 295.0 | −160.2 | 95.2 | 340.7 | 168.7 | 198.1 | 128.0 | 142.2 | 209.9 | 134.9 |
| 294.0 | −176.0 | 137.9 | 405.7 | 218.7 | 243.7 | 157.0 | 168.9 | 273.5 | 166.9 |
| 293.0 | −192.9 | 185.0 | 478.5 | 271.7 | 284.5 | 200.0 | 190.3 | 372.6 | 184.0 |
| 292.5 | −201.5 | 207.3 | 489.9 | 291.9 | 300.5 | 189.1 | 192.2 | 434.9 | 192.2 |
| 292.0 | −203.6 | 220.9 | 489.0 | 305.1 | 304.4 | 186.1 | 156.3 | 462.2 | 191.3 |
| 291.5 | −200.5 | 235.4 | 468.9 | 293.9 | 295.6 | 176.7 | 138.8 | 495.2 | 183.5 |
| 291.0 | −186.2 | 231.1 | 366.5 | 261.8 | 270.8 | 144.0 | 120.9 | 518.7 | 164.6 |
| 290.0 | −133.7 | 163.6 | 149.3 | 187.4 | 166.0 | 77.6 | 85.9 | 448.1 | 91.8 |
| 289.0 | −68.9 | 68.5 | −21.1 | 62.1 | 67.5 | 14.3 | 33.5 | 273.5 | 40.8 |
| 288.5 | −52.0 | 45.2 | −28.6 | 29.8 | 48.1 | 5.6 | 26.2 | 155.6 | 34.4 |
| 288.0 | −34.7 | 35.9 | −34.5 | 31.3 | 51.5 | −4.0 | 33.0 | 42.4 | 40.8 |
| 287.0 | −58.7 | 59.2 | 1.9 | 69.7 | 95.6 | 3.5 | 67.5 | 0.0 | 70.9 |
| 286.0 | −104.1 | 111.7 | 168.4 | 138.4 | 152.9 | 66.3 | 100.0 | 94.3 | 107.8 |
| 285.5 | −120.0 | 135.9 | 204.8 | 172.2 | 178.2 | 78.2 | 111.7 | 127.3 | 118.0 |
| 285.0 | −131.1 | 156.3 | 228.7 | 189.4 | 195.6 | 91.1 | 115.5 | 193.3 | 123.8 |
| 284.0 | −151.0 | 174.3 | 240.2 | 189.4 | 190.3 | 79.2 | 104.4 | 278.3 | 116.0 |
| 283.0 | −112.8 | 152.4 | 147.4 | 166.2 | 157.8 | 55.9 | 82.0 | 292.4 | 95.1 |
| 282.0 | −92.3 | 129.1 | 60.3 | 130.3 | 132.0 | 32.2 | 57.8 | 235.8 | 77.2 |
| 281.5 | −76.0 | 117.5 | 19.1 | 108.6 | 118.0 | 22.7 | 53.4 | 183.9 | 65.1 |
| 281.0 | −74.0 | 113.1 | −9.6 | 105.1 | 101.9 | 23.0 | 49.0 | 165.0 | 63.1 |
| 280.0 | −64.5 | 102.9 | −16.3 | 89.4 | 85.0 | 13.6 | 39.3 | 122.6 | 50.5 |
| 279.0 | −58.4 | 90.8 | −34.5 | 72.2 | 67.5 | 6.9 | 29.6 | 84.9 | 39.8 |
| 278.5 | −55.7 | 83.0 | −49.8 | 71.2 | 60.2 | 6.0 | 28.2 | 61.3 | 35.9 |
| 278.0 | −53.1 | 79.6 | −71.8 | 56.1 | 53.9 | 5.5 | 22.3 | 56.6 | 33.5 |
| 277.0 | −55.1 | 78.2 | −57.4 | 55.6 | 53.4 | −2.5 | 20.9 | 37.7 | 37.4 |
| 276.0 | −63.8 | 86.4 | −42.1 | 71.7 | 63.1 | −2.0 | 29.1 | 28.3 | 42.2 |
| 275.0 | −73.0 | 94.7 | −16.3 | 82.3 | 71.8 | 5.5 | 31.0 | 42.4 | 47.1 |
| 274.0 | −76.0 | 94.7 | −20.1 | 76.3 | 61.7 | −7.9 | 22.3 | 70.7 | 41.8 |
| 272.5 | −60.2 | 78.2 | −72.7 | 50.3 | 37.4 | −12.4 | 1.0 | 51.8 | 30.6 |
| 270.0 | −45.9 | 55.3 | −93.8 | 25.8 | 4.4 | −40.6 | −15.1 | 0.0 | 11.2 |
| 267.5 | −42.4 | 34.5 | −128.2 | 2.0 | −18.5 | −50.5 | −31.6 | −47.1 | −1.5 |
| 266.0 | −47.5 | 16.5 | −147.4 | −13.6 | −35.0 | −58.4 | −41.8 | −61.3 | −9.2 |
| 265.0 | −49.5 | 11.2 | −154.1 | −24.7 | −51.0 | −66.3 | −48.5 | −70.7 | −15.1 |
| 262.5 | −59.2 | −10.2 | −230.4 | −46.5 | −73.8 | −73.0 | −58.3 | −94.3 | −27.7 |
| 260.0 | −76.5 | −28.6 | −290.0 | −63.1 | −89.3 | −76.7 | −67.0 | −127.3 | −34.0 |
| 255.0 | −105.7 | −67.0 | −180.9 | −102.5 | −121.4 | −94.1 | −100.0 | −179.2 | −46.1 |
| 250.0 | −137.8 | −88.4 | −124.4 | −123.7 | −133.0 | −97.5 | −86.0 | −231.1 | −42.2 |
| 245.0 | −165.8 | −75.2 | 2.9 | −102.4 | −101.0 | −63.4 | −64.1 | −177.5 | −16.0 |
| 242.5 | −201.5 | −48.5 | 120.0 | −82.8 | −61.2 | −45.1 | −44.7 | −131.1 | 7.3 |
| 240.0 | | 0.2 | | −19.7 | −4.9 | −24.8 | −24.3 | −75.0 | 50.5 |

[a] Measurements made at absorbances of about 1 and 2 at 282 mμ; 0.1 M KCl; 0.01 M pH 6.8 phosphate; 25°.

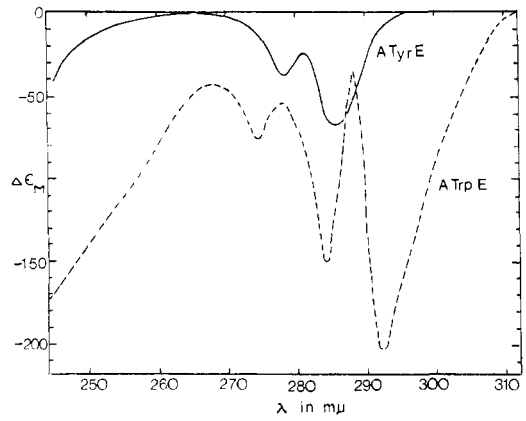


FIGURE 6: Solvent perturbation difference spectra of Ac-Trp-OEt and Ac-Tyr-OEt due to the perturbation of 90% deuterium oxide in pH 6.8 phosphate; $\Gamma/2 = 0.1$; absorbances range from 1.0 to 1.3 absorbance units at the tyrosine and tryptophan absorbance maxima at 274.5 and 282 mμ.

turbation difference spectra[3] (Herskovits and Laskowski, 1960, 1962a) and direct spectra of tyrosine, tryptophan, and phenylalanine model compounds. It is fairly apparent from this figure that the phenylalanine contribution to the solvent perturbation of most proteins will be close to zero above 275 mμ and can be safely neglected. Thus only the tyrosyl and tryptophyl contributions to protein difference spectra need to be considered explicitly above 275 mμ, and even from 245 to 275 mμ the contribution of phenylalanine to the difference spectra of most proteins will be negligible.

For the study of the location of tyrosyl and tryptophyl groups in proteins by the solvent perturbation technique, and the simultaneous assessment of the number or fraction of both of these chromophores exposed in proteins, carefully measured molar absorptivity difference values of tyrosine and tryptophan model compounds are necessary. Tables I and II present a summary of the data collected and tabulated at convenient wavelength intervals for the *N*-acetyl ethyl esters of tyrosine and tryptophan with nine of the most commonly employed perturbants ranging in diameter from 2.0 to 9.4 Å. The data presented represent averages of a minimum of six measurements having standard deviations of ±3% at the 286–288-mμ tyrosyl and the 291–293-mμ tryptophyl peaks. For the tyrosine model compound, each three of these measurements were made at absorbances of approximately 1.3 and 2.0 absorbance units. In making measurements on the tryptophan model compound, absorbances were approximately 1.0 and 2.0. The measurements using 90% deuterium oxide were made at the lower absorbance only ($A_{max}$ 1.3 and 1.0, respectively), due to solubility limitations. To rule out

[3] Difference spectra of tyrosyl and tryptophyl model compounds produced by various additives, salts, and denaturants have been reported in a number of earlier studies (Wetlaufer *et al.*, 1958; Bigelow, 1960; Bigelow and Geschwind, 1960; Herskovits and Laskowski, 1960, 1962a; Hamaguchi and coworkers, 1963, 1964). Further references on the subject can be found in two of the more recent reviews dealing with the subject (Laskowski, 1966; Herskovits, 1967).

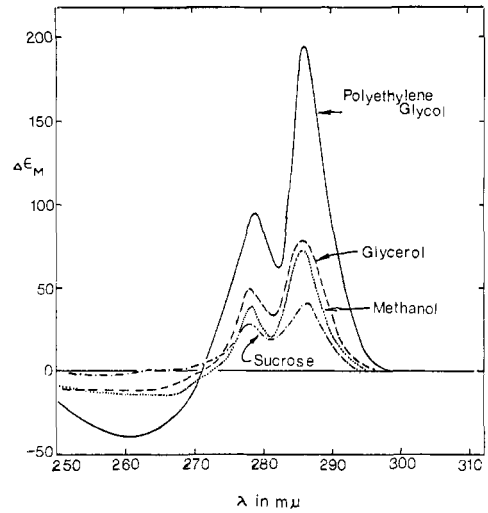


FIGURE 7: Typical solvent perturbation difference spectra of Ac-Tyr-OEt in aqueous solutions due to the perturbing influence of 20% polyethylene glycol, glycerol, methanol, and sucrose. $\Delta\epsilon_\lambda$ represents the molar absorptivity differences due to the various perturbants employed. Absorbances range from 1.3 to 2.1 absorbance units at 274.5 mμ; 0.1 M KCl; 0.01 M phosphate.

the possibility of pH effects on absorbance, the chromophores perturbed by 20% dimethyl sulfoxide were measured at neutral and acid pH. The molar absorptivity difference values were not found to be pH dependent. Results of these measurements are tabulated in Tables I and II, and are illustrated in Figures 6–9.[3]

Since 8 M urea has an effect on both absorbance and molar absorptivity differences of Ac-Tyr-OEt and Ac-Trp-OEt (Herskovits, 1965; Williams *et al.*, 1965) which is of the order of ±3–30% depending on the perturbant employed (see also Table I of Herskovits and Sorensen, 1968) additional data have been obtained for four of the most commonly employed perturbants, 20% dimethyl

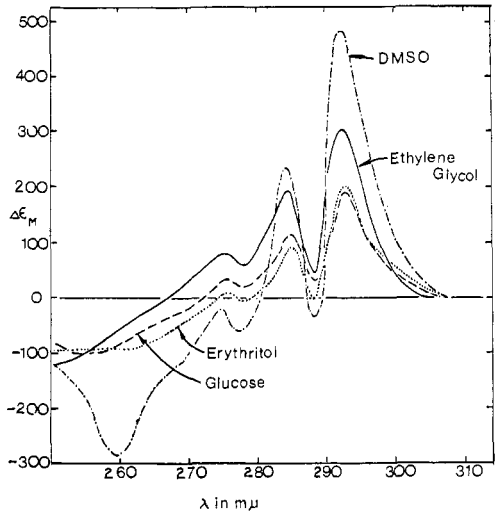


FIGURE 8: Solvent perturbation difference spectra of Ac-Trp-OEt due to the perturbation by 20% dimethyl sulfoxide, ethylene glycol, erythritol, and glucose. $\Delta\epsilon_\lambda$ represents the molar absorptivity differences due to these perturbants. Absorbances range from 1.0 to 2.1 absorbance units at 282 mμ; 0.1 M KCl, 0.01 M pH 6.8 phosphate.

TABLE III: Molar Absorptivity Difference Values for *N*-Acetyl-L-tyrosine and *N*-Acetyl-L-tryptophan Ethyl Esters in 8 M Urea in Some Perturbants.[a]

| | Ac-Tyr-OEt | | | | Ac-Trp-OEt | | | |
|---|---|---|---|---|---|---|---|---|
| λ (mμ) | 20% DMSO | 20% Ethylene Glycol | 20% Glycerol | 20% Sucrose | 20% DMSO | 20% Ethylene Glycol | 20% Glycerol | 20% Sucrose |
| 350.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 315.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 310.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 6.5 | 4.5 |
| 305.0 | 0.0 | 0.0 | 0.0 | 0.0 | 12.4 | 7.3 | 19.0 | 22.6 |
| 300.0 | 0.0 | 0.0 | 0.0 | 0.6 | 92.0 | 44.9 | 59.8 | 54.3 |
| 295.0 | 10.2 | 6.1 | 0.5 | 5.7 | 266.2 | 148.2 | 166.3 | 141.0 |
| 294.0 | 16.3 | 6.6 | 0.8 | 7.0 | 328.4 | 181.5 | 184.8 | 162.9 |
| 293.0 | 30.6 | 10.8 | 2.4 | 8.1 | 403.0 | 225.3 | 227.7 | 176.5 |
| 292.5 | 39.1 | 14.2 | 5.9 | 8.7 | 427.9 | 246.7 | 259.9 | 190.0 |
| 292.0 | 48.0 | 21.2 | 6.3 | 9.4 | 435.3 | 270.8 | 285.9 | 194.6 |
| 291.5 | 65.2 | 28.8 | 9.5 | 9.8 | 427.9 | 286.7 | 288.0 | 199.0 |
| 291.0 | 81.7 | 33.0 | 14.2 | 11.7 | 415.4 | 298.9 | 289.1 | 185.5 |
| 290.0 | 102.1 | 51.9 | 22.2 | 18.1 | 303.5 | 285.2 | 285.3 | 162.9 |
| 289.0 | 138.9 | 54.7 | 34.8 | 23.6 | 179.1 | 219.2 | 217.4 | 117.6 |
| 288.5 | 159.4 | 81.1 | 42.7 | 26.4 | 124.4 | 152.2 | 173.9 | 95.0 |
| 288.0 | 179.8 | 108.5 | 50.6 | 29.7 | 82.1 | 127.1 | 130.4 | 76.9 |
| 286.0 | 230.8 | 116.0 | 75.9 | 48.1 | 159.2 | 129.8 | 135.9 | 104.1 |
| 285.5 | 219.7 | 119.8 | 79.1 | 48.5 | 186.6 | 165.0 | 173.9 | 126.7 |
| 285.0 | 210.4 | 117.0 | 77.5 | 44.7 | 211.4 | 198.2 | 190.2 | 149.3 |
| 284.0 | 178.8 | 103.8 | 72.8 | 40.1 | 236.3 | 201.2 | 217.4 | 158.4 |
| 283.0 | 128.7 | 88.7 | 66.5 | 35.3 | 213.9 | 246.1 | 228.3 | 158.4 |
| 282.0 | 102.0 | 61.3 | 46.4 | 28.4 | 164.0 | 216.4 | 195.6 | 126.7 |
| 281.5 | 102.3 | 54.7 | 45.3 | 26.3 | 99.5 | 205.6 | 179.3 | 122.2 |
| 281.0 | 102.0 | 47.2 | 44.3 | 25.4 | 92.0 | 191.4 | 163.0 | 117.6 |
| 280.0 | 117.5 | 53.8 | 31.6 | 27.2 | 74.6 | 168.5 | 146.7 | 108.6 |
| 279.0 | 132.8 | 63.2 | 41.1 | 31.4 | 49.7 | 147.4 | 135.9 | 99.5 |
| 278.5 | 136.9 | 67.0 | 44.3 | 32.5 | 32.3 | 137.7 | 130.4 | 95.0 |
| 278.0 | 132.8 | 71.7 | 47.5 | 32.1 | 24.8 | 127.7 | 127.7 | 90.5 |
| 277.0 | 112.3 | 73.6 | 44.3 | 29.0 | 19.9 | 122.2 | 125.0 | 81.4 |
| 276.0 | 76.6 | 61.3 | 41.1 | 24.1 | 24.8 | 123.3 | 103.3 | 76.9 |
| 275.0 | 48.0 | 40.6 | 25.3 | 20.3 | 37.3 | 131.4 | 126.1 | 81.4 |
| 274.0 | 35.6 | 34.9 | 19.0 | 17.4 | 42.3 | 140.6 | 125.0 | 81.4 |
| 272.0 | 18.4 | 16.0 | 3.2 | 13.3 | 0.0 | 123.0 | 108.7 | 63.3 |
| 270.0 | 5.1 | 11.3 | 0.0 | 9.2 | −32.3 | 105.4 | 97.8 | 54.3 |
| 267.5 | −10.2 | 0.0 | −12.7 | 4.6 | −64.8 | 71.7 | 59.8 | 49.8 |
| 266.0 | −28.6 | −6.0 | −17.4 | 1.7 | −92.0 | 64.9 | 54.3 | 36.2 |
| 265.0 | −30.6 | −6.0 | −17.4 | 0.5 | −99.5 | 62.1 | 48.9 | 20.7 |
| 262.5 | −35.8 | −6.0 | −14.2 | −1.4 | −114.4 | 46.6 | 38.0 | 9.0 |
| 260.0 | −30.6 | −6.0 | −6.3 | −3.1 | −131.8 | 28.6 | 32.6 | 0.0 |
| 255.0 | −10.2 | −2.8 | −4.7 | −3.0 | −181.2 | 4.0 | 5.4 | −18.1 |
| 250.0 | +10.2 | 0.0 | 0.0 | −2.2 | −124.4 | −12.2 | −16.3 | −49.8 |

[a] Measurements made at absorbances of 1.4–1.8; pH 6.8–7.1; $\Gamma/2 = 0.1$; 25°.

sulfoxide, 20% glycerol, 20% ethylene glycol, and 20% sucrose. Results of these measurements required for the analysis of protein denaturation data in 8 M urea are summarized in Table III. The standard deviation of the first difference spectral maxima at 286–288 and 291–293 mμ are found to be somewhat larger than for aqueous solution, *i.e.*, about ±5%, reflecting to some extent the experimental difficulties encountered in dealing with

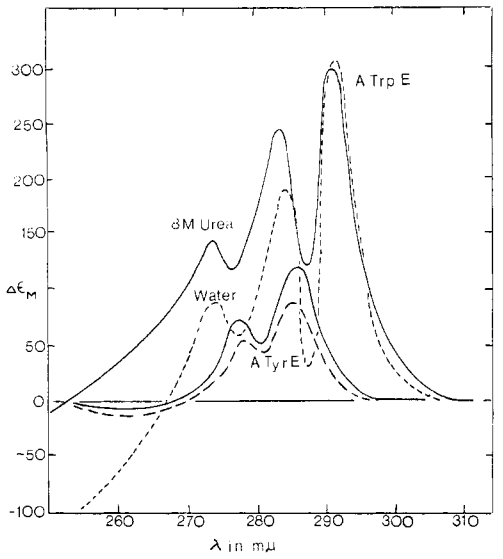


FIGURE 9: Comparison of the molar absorptivity differences of Ac-Tyr-OEt and Ac-Trp-OEt in 8 M urea and water due to 20% ethylene glycol. Absorbances range from 1.0 to 2.1 absorbance units at $\lambda_{max}$; 0.1 M KCl; 0.01 M pH 6.8 phosphate.

concentrated urea solutions. With sucrose, having the smallest molar absorptivity difference, especially with the Ac-Tyr-OEt model compound, the standard deviation is ±7%. Figure 9 shows a comparison of Ac-Tyr-OEt and Ac-Trp-OEt solvent perturbation difference spectra in water and 8 M urea.

*Additivity of Solvent Perturbation Difference Spectral Data.* A basic assumption of this method is that chromophore absorbances are additive. This assumption has been checked in the present study for both model compounds and proteins. For the model experiments a 5.02 × $10^{-4}$ M Ac-Tyr-OEt and 1.03 × $10^{-4}$ M Ac-Trp-OEt mixture was used to obtain the difference spectra with 20% glycerol as perturbant and compared with calculated curves based on the data of Tables I and II. This curve was calculated by use of the relation, $\Delta A_\lambda$ = 5.02 × $10^{-4}\Delta\epsilon_\lambda$ (Ac-Tyr-OEt) + 1.03 × $10^{-4}\Delta\epsilon_\lambda$ (Ac-Trp-OEt). The resulting calculated curve and the experimental curve are compared in Figure 10. Theoretical values for lysozyme and ribonuclease were also calculated and absorptivity differences of a mixture of the enzymes measured and illustrated in Figure 11. For these experiments 1.31 × $10^{-4}$ M ribonuclease and 2.83 × $10^{-5}$ M lysozyme solutions were measured separately and then measured as a 6.55 × $10^{-5}$ M ribonuclease and 2.83 × $10^{-5}$ M lysozyme mixture with 20% ethylene glycol as perturbant. The theoretical points represented by open circles were calculated from the former runs using the separate enzymes. Close agreement between the experimental and calculated curves in both cases indicates that the additivity assumptions are valid within the experimental uncertainties of the method. It is worth noting, for example, that the slight positive and negative deviation of the third difference spectral maximum in Figures 10 and 11 centering around 276 mμ represents only a ±0.002–0.004 measured optical density difference.

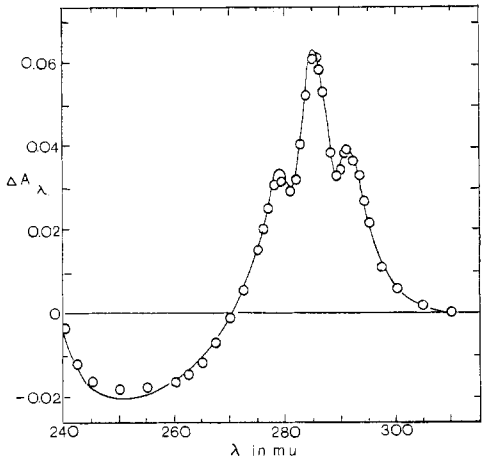


FIGURE 10: Test of additivity of solvent perturbation of model mixtures. Solvent perturbation difference spectrum of 5.02 × $10^{-4}$ M Ac-Tyr-OEt and 1.03 × $10^{-4}$ M Ac-Trp-OEt due to 20% glycerol (solid curve), and the calculated theoretical curve based on the data of Tables I and II (open circles); 0.1 M MCl; 0.01 M pH 6.8 phosphate; dynode setting = 3.

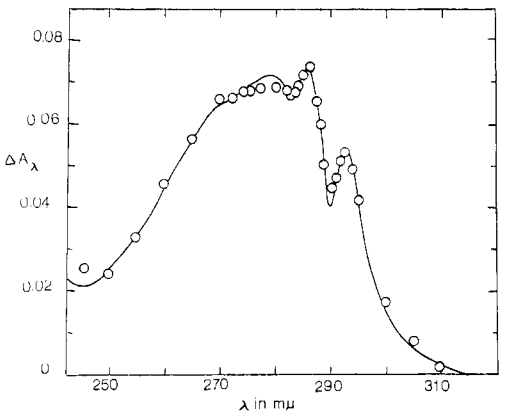


FIGURE 11: Test of chromophore additivity of proteins. Measured absorptivity differences for a mixture of 6.55 × $10^{-5}$ M ribonuclease and 2.83 × $10^{-5}$ M lysozyme due to 20% ethylene glycol are plotted (solid line) and compared to calculated values (open circles) based on pure ribonuclease and lysozyme data obtained in separate experiments; 0.1 M KCl; 0.01 M pH 6.8 phosphate; dynode setting = 3.

Further work on protein solvent perturbation difference spectra, exploring the limits of applicability in relation to tryptophan to tyrosine content, are reported in the accompanying paper (Herskovits and Sorensen, 1968). The applicability of the method to be employed is illustrated and tested by studying the location of tyrosyls and tryptophyls in rabbit muscle aldolase, pepsin, and bovine serum albumin.

## References

Beaven, G. H., and Holiday, E. R. (1952), *Advan. Protein Chem. 7*, 319.

Bigelow, C. C. (1960), *Compt. Trav. Lab. Carlsberg 31*, 305.

Bigelow, C. C., and Geschwind, I. I. (1960), *Compt. Rend. Trav. Lab. Carlsberg 31*, 283.

Cross, D., and Fisher, H. (1966), *Biochemistry 5*, 880.
Cuatrecases, P., Fuchs, S., and Anfinsen, C. B. (1967), *J. Biol. Chem. 242*, 4759.
Donovan, J. W. (1964), *Biochemistry 3*, 67.
Donovan, J. W. (1967), *Biochemistry 6*, 3918.
Edelhoch, H., and Steiner, R. F. (1965), *J. Biol. Chem. 240*, 2877.
Edsall, J. T. (1963), *in* Aspects of Protein Structure, Ramachandran, G. N., Ed., New York, N. Y., Academic, p 179.
Friedman, T., and Epstein, C. J. (1967), *J. Biol. Chem. 242*, 5131.
Fujioka, H., and Imahori, R. (1963), *J. Biochem. (Tokyo) 53*, 244.
Hamaguchi, K., and Kurano, A. (1963), *J. Biochem. (Tokyo) 54*, 497.
Hamaguchi, K., Kurano, A., and Gato, S. (1963), *J. Biochem. (Tokyo) 54*, 259.
Hamaguchi, K., Hayashi, K., Imoto, T., and Funatsu, M. (1964), *J. Biochem. (Tokyo) 55*, 24.
Hayashi, K., Imoto, T., and Funatus, M. (1964), *J. Biochem. (Tokyo) 55*, 516.
Herskovits, T. T. (1965), *J. Biol. Chem. 240*, 628.
Herskovits, T. T. (1967), *Methods Enzymol. 11*, 748.
Herskovits, T. T., and Greenblatt, J. (1967), Southestern Regional Meeting of the American Chemical Society, Atlanta, Ga., Nov 1, Abstract 55.
Herskovits, T. T., and Laskowski, M., Jr. (1960), *J. Biol. Chem. 235*, PC56.
Herskovits, T. T., and Laskowski, M., Jr. (1962a), *J. Biol. Chem. 237*, 2481.
Herskovits, T. T., and Laskowski, M., Jr. (1962b), *J. Biol. Chem. 237*, 3418.
Herskovits, T. T., and Laskowski, M., Jr. (1968), *J. Biol. Chem. 243*, 2123.
Herskovits, T. T., and Sorensen, M. (1968), *Biochemistry 7*, 2533 (this issue; following paper).
Kronman, M. J., and Holmes, L. G. (1965), *Biochemistry 4*, 562.
Laskowski, M., Jr. (1966), *Federation Proc. 25*, 20.
Libor, S., Elodi, E., and Nagy, Z. (1965), *Biochim. Biophys. Acta 110*, 484.
Mihalyi, E. (1968), *Biochemistry 7*, 208.
Riddiford, L. M. (1966), *J. Biol. Chem. 241*, 2792.
Scheraga, H. A., and Rupley, J. A. (1962), *Advan. Enzymol. 24*, 161.
Sophianopoulos, A. J., Rhodes, C. K., Holcomb, D. N., and Van Holde, K. E. (1962), *J. Biol. Chem. 237*, 1107.
Stellwagen, E., and Van Rooyan, S. (1967), *J. Biol. Chem. 242*, 4801.
van Zyl, A., and Edelhoch, H. (1967), *J. Biol. Chem. 242*, 2423.
Waks, M., and Alfsen, A. (1966), *Arch. Biochem. Biophys. 113*, 304.
Weil, L., Seibes, T. S., and Herskovits, T. T. (1965), *Arch. Biochem. Biophys. 111*, 308.
Wetlaufer, D. B. (1962), *Advan. Protein Chem. 17*, 303.
Wetlaufer, D. B., Edsall, J. T., and Hollingworth, B. R. (1958), *J. Biol. Chem. 233*, 1431.
Williams, E. J. (1966), *Arch. Biochem. Biophys. 115*, 21.
Williams, E. J., Herskovits, T. T., and Laskowski, M., Jr. (1965), *J. Biol. Chem. 240*, 3574.