

# Determination of Tyrosine Exposure in Proteins by Second-Derivative Spectroscopy<sup>†</sup>

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**ABSTRACT:** The mutual interference between the second-derivative bands of tyrosine and tryptophan in proteins has been evaluated in terms of the ratio  $r$  between two peak to peak distances. The  $r$  values have been found to be not only related to the tyrosine/tryptophan ratio but also dependent on the

polarity of the medium in which tyrosyl residues are embedded. The results obtained on purified proteins have been found consistent with the available X-ray information and with the existing solvent perturbation data.

**S**econd-derivative spectroscopy has proved to be an effective analytical tool because of its ability to resolve overlapping bands in the normal spectrum (O'Haver & Green, 1975; Butler, 1979). This technique has been utilized to resolve the complex protein absorption spectrum into the individual contributions of the three aromatic amino acids (Ichikawa & Terada, 1977; Balestrieri et al., 1978a, 1980; Irace et al., 1979) and to carry out relatively simple methods for their quantitative estimation (Balestrieri et al., 1978a, 1980; Ichikawa & Terada, 1979; Servillo et al., 1982). More recently, second-derivative spectroscopy has been employed for detecting conformational changes involving the microenvironments of aromatic amino acids (Servillo et al., 1980; Ichikawa & Terada, 1981; Karelampi & Hynninen, 1981; Yamagishi et al., 1981; Ruckpaul et al., 1980).

The present report describes the possibility of examining the state of tyrosyl residues in proteins. It is well-known that the absorption of tyrosyl residues is largely masked by the stronger absorption of tryptophanyl residues. Attempts to improve the resolution between the spectral bands of these two chromophores by using second-derivative spectroscopy were also unsuccessful (Balestrieri et al., 1978a, 1980). The mutual interference between the second-derivative bands of tyrosine and tryptophan, evaluated in terms of the ratio between two peak to peak distances, has been recently utilized for a simultaneous determination of these two residues at neutral pH (Servillo et al., 1982). We now show that the same ratio may be used in detecting the degree of exposure of tyrosyl residues even in the presence of a relatively high content of tryptophanyl residues.

## Materials and Methods

**Proteins and Model Compounds.** Bovine  $\alpha$ -chymotrypsinogen was purchased from Mann Research Laboratories; sperm whale myoglobin, pepsin, egg lysozyme, and horse heart cytochrome *c* were obtained from Sigma Chemical Co.; mitochondrial bovine aspartate aminotransferase from beef heart was a gift of Dr. G. Marino (Department of Organic and Biochemistry, University of Naples). Tuna ferrimyoglobin was prepared according to the methods previously described (Balestrieri et al., 1973, 1978b). Apomyoglobins were prepared by the butanone method of Teale (1959); the contamination of apoprotein by myoglobin was assessed spectrophoto-

metrically. In all cases, no significant absorption was observed in the Soret region.

*N*-Acetyl-L-tryptophanamide (*N*-AcTrpNH<sub>2</sub>), *N*-acetyl-L-tyrosinamide (*N*-AcTyrNH<sub>2</sub>), and *N*-acetyl-L-phenylalanine ethyl ester (*N*-AcPheOEt) were purchased from Sigma Chemical Co. Chemicals not mentioned above were reagent grade.

**Protein Concentration.** The protein concentrations were determined spectrophotometrically by using the following absorption coefficients:  $\alpha$ -chymotrypsinogen,  $A^{1\%} = 21$  (Desnuelle & Rovey, 1961) and the molecular weight ( $M_r$ ) is 25 600 (Brown & Hartley, 1966); cytochrome *c*,  $\epsilon_{550} = 29.5 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  (Van Gelder & Slater, 1962); sperm whale myoglobin,  $\epsilon_{408} = 179 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  (Harrison & Blout, 1965); lysozyme,  $\epsilon_{280} = 37.8 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$  (Canfield, 1963). The molar absorption coefficients at 280 nm of tuna apomyoglobin, pepsin, and aspartate aminotransferase were calculated from their tryptophan and tyrosine contents according to Wetlaufer (1962). In all the experiments, the protein absorbances at 280 nm were around 0.2. Experiments were also performed using lower protein absorbance values and ordinate scale expansion, without any appreciable difference in accuracy.

**Model Compound Concentrations.** Model compound concentrations were estimated by absorption measurements using  $\epsilon_{280.8} = 5690 \text{ M}^{-1} \text{ cm}^{-1}$  for *N*-AcTrpNH<sub>2</sub> (Wetlaufer, 1963),  $\epsilon_{275.5} = 1490 \text{ M}^{-1} \text{ cm}^{-1}$  for *N*-AcTyrNH<sub>2</sub> (Edelhoch, 1967), and  $\epsilon_{257.7} = 195 \text{ M}^{-1} \text{ cm}^{-1}$  for *N*-AcPheOEt (Mihalyi, 1976).

**Instruments.** All the normal and second-derivative spectra were recorded with a Perkin-Elmer Model 575 spectrophotometer equipped with an electronic derivative accessory (Hitachi 200-0507 derivative spectrum unit) using the instrumental conditions described elsewhere (Balestrieri et al., 1980).

## Results

**Model Compounds.** The second-derivative spectrum of *N*-AcTrpNH<sub>2</sub> (Figure 1) in the spectral region between 280 and 300 nm shows two maxima centered around 287 and 295 nm and two minima at 283 and 290.5 nm, the position of which is only marginally affected by changing the polarity of the solvent.

We have focused our attention on the ratio between two peak to peak distances, i.e., the peak to peak distance between the maximum at 287 nm and the minimum at 283 nm, which will be indicated as *a*, and the peak to peak distance between the maximum at 295 nm and the minimum at 290.5 nm, indicated as *b*. In spite of small variations observed in the

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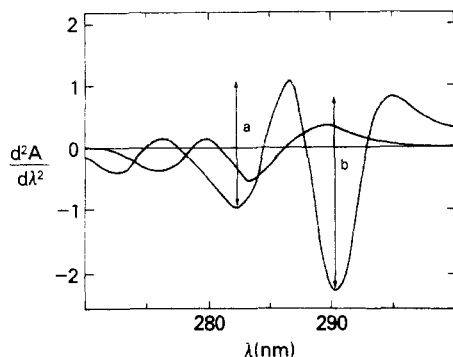


FIGURE 1: Second-derivative spectra of equimolar solutions of *N*-AcTrpNH<sub>2</sub> and *N*-AcTyrNH<sub>2</sub> dissolved in 6.0 M Gdn·HCl-0.05 M phosphate, pH 6.5. The spectrum of *N*-AcTrpNH<sub>2</sub> is identified by the two arrows a and b, which indicate the peak to peak distances between the maximum at 287 nm and the minimum at 283 nm and the maximum at 295 nm and the minimum at 290.5 nm, respectively.

Table I: Effect of Various Solvents on the Ratio  $r_p$  between the Peak to Peak Distances 287–283 and 295–290.5 nm of the Second-Derivative Spectrum of *N*-AcTrpNH<sub>2</sub>

solvent	$r_p$	solvent	$r_p$
<i>p</i> -dioxane	0.68	CH <sub>3</sub> OH	0.70
ethyl acetate	0.71	ethylene glycol	0.67
CHCl <sub>3</sub>	0.68	Gdn·HCl, 6.0 M	0.67
<i>n</i> -C <sub>4</sub> H <sub>9</sub> OH	0.67	formamide	0.68
<i>n</i> -C <sub>3</sub> H <sub>7</sub> OH	0.69	H <sub>2</sub> O	0.68
C <sub>2</sub> H <sub>5</sub> OH	0.70		

position of maxima and minima, it is noteworthy to observe that the value of the ratio  $a/b$  (which will be referred as  $r_p$ ) is almost the same over a wide range of solvent polarities. Table I shows the  $r_p$  values determined for *N*-AcTrpNH<sub>2</sub> in various solvents. The mean value of  $r_p$  for *N*-AcTrpNH<sub>2</sub> has been found to be  $0.68 \pm 0.02$ .

The second-derivative spectrum of mixtures containing *N*-AcTrpNH<sub>2</sub> and *N*-AcTyrNH<sub>2</sub> shows the same general features observed for tryptophan, i.e., two minima at 283 and 290.5 nm and two maxima at 287 and 295 nm, even when the molar ratio between tyrosine and tryptophan is very high, i.e., 5 or more. The dependence of  $r_p$  on the molar ratio between *N*-AcTyrNH<sub>2</sub> and *N*-AcTrpNH<sub>2</sub> (which will be indicated as  $x$ ) can be predicted on the basis of the Lambert's law. In fact, the absorbance at any given wavelength of a mixture of the two chromophores is

$$A = \epsilon_{\text{Trp}} C_{\text{Trp}} + \epsilon_{\text{Tyr}} C_{\text{Tyr}}$$

The difference between the absorbances at two distinct pairs of wavelengths, i.e.,  $\Delta A_1 = A_{287} - A_{283}$  and  $\Delta A_2 = A_{295} - A_{290.5}$ , will be

$$\Delta A_1 = \Delta \epsilon_{1,\text{Trp}} C_{\text{Trp}} + \Delta \epsilon_{1,\text{Tyr}} C_{\text{Tyr}} \quad (1)$$

$$\Delta A_2 = \Delta \epsilon_{2,\text{Trp}} C_{\text{Trp}} + \Delta \epsilon_{2,\text{Tyr}} C_{\text{Tyr}} \quad (2)$$

Dividing  $\Delta A_1$  by  $\Delta A_2$  and rearranging the terms, we obtain the following expression:

$$\frac{\Delta A_1}{\Delta A_2} = \frac{\Delta \epsilon_{1,\text{Trp}}/\Delta \epsilon_{2,\text{Trp}} + (\Delta \epsilon_{1,\text{Tyr}}/\Delta \epsilon_{2,\text{Tyr}})(C_{\text{Tyr}}/C_{\text{Trp}})}{1 + (\Delta \epsilon_{2,\text{Tyr}}/\Delta \epsilon_{2,\text{Trp}})(C_{\text{Tyr}}/C_{\text{Trp}})} \quad (3)$$

Since Beer-Lambert's law is not affected by differentiating 2 times, eq 3 can also be referred to the second-derivative differences  $\Delta A_1''$  and  $\Delta A_2''$ ; therefore, we can write

$$\frac{\Delta A_1''}{\Delta A_2''} = \frac{Ax + B}{Cx + 1} \quad (4)$$

where  $x$  is the molar ratio between tyrosine and tryptophan, the subscripts 1 and 2 refer to the two distinct pairs of

Table II: Numerical Values of the Coefficients  $A$ ,  $B$ , and  $C$  Which Appear in the Equation Relating  $r_d$  to the Molar Ratio  $N$ -AcTyrNH<sub>2</sub>/ $N$ -AcTrpNH<sub>2</sub><sup>a</sup>

solvent	condition I			condition II		
	$A$	$B$	$C$	$A$	$B$	$C$
<i>p</i> -dioxane	-0.41	0.68	-0.04	-0.45	0.66	-0.05
CHCl <sub>3</sub>	-0.03	0.68	-0.05	-0.03	0.66	-0.05
<i>n</i> -C <sub>3</sub> H <sub>7</sub> OH	-0.12	0.69	-0.03	-0.17	0.66	-0.05
ethylene glycol	-0.18	0.64	-0.04	-0.19	0.66	-0.04
Gdn·HCl, 6.0 M	0.20	0.66	-0.09	0.20	0.66	-0.09
H <sub>2</sub> O	0.21	0.66	-0.06	0.18	0.66	-0.05

<sup>a</sup> The data reported under condition I were obtained by dissolving the two chromophores in the indicated solvent; the data under condition II were obtained by using a tandem cell which allowed us to dissolve tryptophan in a fixed solvent, i.e., 6.0 M Gdn·HCl, and tyrosine in the indicated solvent.

wavelengths, i.e., 287–283 nm and 295–290.5 nm, and  $A$ ,  $B$ , and  $C$  are three constants which correspond to

$$A = \frac{\Delta \epsilon_{1,\text{Tyr}}''}{\Delta \epsilon_{2,\text{Trp}}''} \quad B = \frac{\Delta \epsilon_{1,\text{Trp}}''}{\Delta \epsilon_{2,\text{Trp}}''} \quad C = \frac{\Delta \epsilon_{2,\text{Tyr}}''}{\Delta \epsilon_{2,\text{Trp}}''}$$

$\Delta \epsilon''$  being the difference between the second derivatives of the molar extinctions at the two fixed pairs of wavelengths. The ratio  $\Delta A_1''/\Delta A_2''$  corresponds to  $r_p$  if the positions of the peaks coincide with the pairs of wavelengths which appear in eq 4. We will indicate the ratio  $\Delta A_1''/\Delta A_2''$  as  $r_d$  in order to distinguish the two parameters. However, the two values are often coincident, the wavelength shifts usually being small ( $\pm 1$  nm).

A close inspection of eq 4 reveals that  $r_d$  depends not only on the molar ratio  $x$  between tyrosine and tryptophan but also on the solvent composition. In fact, the three coefficients which appear in eq 4 are expected to change because of the spectral shifts which occur in various solvents. The numerical values of the three coefficients in different solvents have been calculated from the second-derivative differences at the two above-mentioned pairs of wavelengths. The data presented under condition I of Table II show the values of  $A$ ,  $B$ , and  $C$  determined on samples prepared by dissolving both chromophores in the same solvent, whereas the data under condition II were obtained by using a tandem cell in order to keep *N*-AcTrpNH<sub>2</sub> in a fixed solvent [6.0 M guanidine hydrochloride (Gdn·HCl)] and to change the *N*-AcTyrNH<sub>2</sub> solvent. No dependence on solvent composition has been observed for coefficient  $B$ ; this result was to be expected since  $B$  is the value of  $\Delta A_1''/\Delta A_2''$  determined in the absence of tyrosine, and, therefore, it would correspond rather closely to the  $r_p$  values shown in Table I. The numerical values of coefficients  $A$  and  $C$  have been found to be strongly dependent on the properties of the solvent; however, the variations of  $C$  are much more limited than those relative to  $A$  and, therefore, do not affect  $\Delta A_1''/\Delta A_2''$  much for a given Tyr/Trp ratio. The converse holds for  $A$ , the numerical value of which is positive or negative depending on the solvent used.

The good correspondence between the numerical values of  $A$  in the two sets of experiments shown in Table II suggests that the variations observed for this coefficient, i.e., from the negative values observed in nonpolar solvents to the positive values obtained in water and in 6.0 M Gdn·HCl, are mostly due to the solvent in which *N*-AcTyrNH<sub>2</sub> is dissolved. This conclusion is consistent with the data reported in Table I showing that the ratio  $a/b$  for *N*-AcTrpNH<sub>2</sub> is not influenced by the solvent composition.

Figure 2 shows the dependence of  $r_d$  on the molar ratio between *N*-AcTyrNH<sub>2</sub> and *N*-AcTrpNH<sub>2</sub> in different solvents.

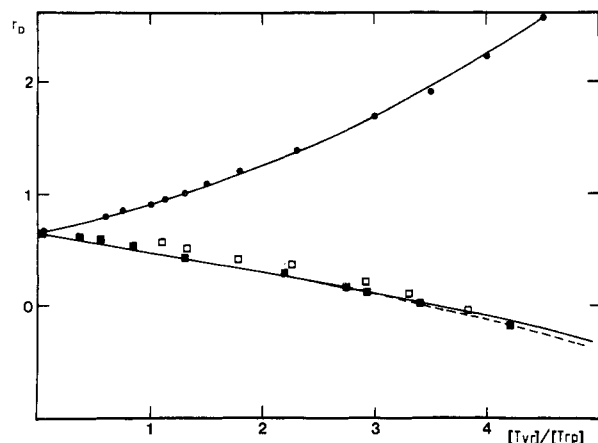


FIGURE 2: Dependence of  $r_d$  on the molar ratio  $x$  between  $N$ -AcTyrNH<sub>2</sub> and  $N$ -AcTrpNH<sub>2</sub> in different solvents: (●) 6.0 M Gdn-HCl; (□) ethylene glycol; (■) ethylene glycol for  $N$ -AcTyrNH<sub>2</sub> and 6.0 M Gdn-HCl for  $N$ -AcTrpNH<sub>2</sub> (a tandem cell was used in this experiment). The continuous lines are theoretical and based on solution of eq 4 by using the constants reported in Table II.

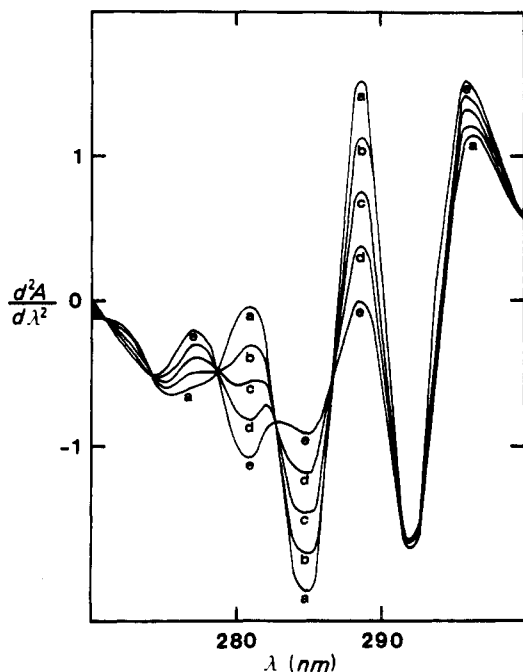


FIGURE 3: Computed second-derivative spectra of a mixture containing four  $N$ -AcTyrNH<sub>2</sub> and two  $N$ -AcTrpNH<sub>2</sub> residues obtained by combining the spectra of  $N$ -AcTrpNH<sub>2</sub> in 6.0 M Gdn-HCl with (a) four  $N$ -AcTyrNH<sub>2</sub> residues in 6.0 M Gdn-HCl, (b) three  $N$ -AcTyrNH<sub>2</sub> residues in 6.0 M Gdn-HCl and one  $N$ -AcTyrNH<sub>2</sub> residue in ethylene glycol, (c) two  $N$ -AcTyrNH<sub>2</sub> residues in 6.0 M Gdn-HCl and two  $N$ -AcTyrNH<sub>2</sub> residues in ethylene glycol, (d) one  $N$ -AcTyrNH<sub>2</sub> residue in 6.0 M Gdn-HCl and three  $N$ -AcTyrNH<sub>2</sub> residues in ethylene glycol, and (e) four  $N$ -AcTyrNH<sub>2</sub> residues in ethylene glycol.

The continuous lines are theoretical and based on solution of eq 4 by using the constants reported in Table II. Since the curve obtained by dissolving the two chromophores in ethylene glycol and that obtained by dissolving  $N$ -AcTyrNH<sub>2</sub> in ethylene glycol and  $N$ -AcTrpNH<sub>2</sub> in 6.0 M Gdn-HCl were practically coincident and within experimental error over a wide range of molar ratios, it is evident that the numerical value of  $r_d$  for a given ratio Tyr/Trp is determined by the solvent in which tyrosine is dissolved.

A possible explanation is the greater sensitivity of the  $N$ -AcTyrNH<sub>2</sub> absorption to the environment; in fact, a careful inspection of Figure 1 shows that a blue shift of the tyrosine

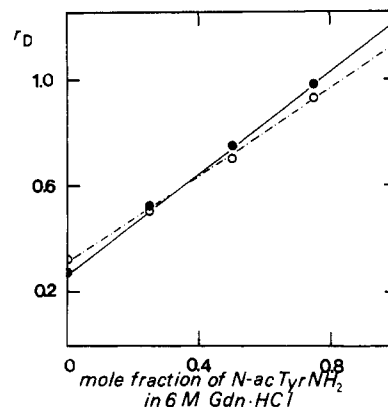


FIGURE 4: Dependence of  $r_d$  on the distribution of  $N$ -AcTyrNH<sub>2</sub> between ethylene glycol and 6 M Gdn-HCl. The data were taken from the spectra reported in Figure 3.

Table III: Effect of Protein Unfolding on the Ratio between the Peak to Peak Distances 287–283 and 295–290.5 nm and Its Correlation with the Fraction  $\alpha$  of Tyrosyl Residues Exposed to Solvent in the Native Structure

protein	$r_n^a$	$r_u^b$	Tyr/Trp <sup>c</sup>	$r_a^d$	$\alpha^e$
lysozyme	0.74	0.79	0.5	0.56	0.78
$\alpha$ -chymotrypsinogen	0.76	0.79	0.5	0.56	0.87
horse apomyoglobin	0.58	0.94	1.0	0.48	0.22
sperm whale apomyoglobin	0.64	1.09	1.5	0.39	0.36
sperm whale metmyoglobin	0.62	1.09	1.5	0.39	0.33
bovine aspartate aminotransferase	0.93	1.15	1.67	0.36	0.72
tuna apomyoglobin	1.10	1.30	2.0	0.30	0.80
pepsin	1.43	1.79	3.2	0.07	0.79
cytochrome c	0.88	2.22	4.0	-0.10	0.42

<sup>a</sup> Determined in 0.05 M phosphate–0.15 M KCl, pH 7.0.

<sup>b</sup> Determined in 6.0 M Gdn-HCl containing 0.05 M phosphate, pH 6.5. <sup>c</sup> Calculated from the amino acid composition. <sup>d</sup> Calculated from eq 4 by using the coefficients relative to ethylene glycol (Table II, condition I). <sup>e</sup> Calculated from eq 5.

spectrum would increase  $a$  and decrease  $b$ , thus producing an enhancement of the ratio  $a/b$ . Figure 3 shows the computed second-derivative spectra of a mixture of four  $N$ -AcTyrNH<sub>2</sub> and two  $N$ -AcTrpNH<sub>2</sub> residues obtained by combining the spectra of the model compounds in 6.0 M Gdn-HCl and in ethylene glycol. The lowest  $r_d$  value was found when four  $N$ -AcTyrNH<sub>2</sub> residues in ethylene glycol were combined with two  $N$ -AcTrpNH<sub>2</sub> residues either in 6.0 M Gdn-HCl or in ethylene glycol. Increasing the amount of  $N$ -AcTyrNH<sub>2</sub> in Gdn-HCl produces an enhancement of the peak to peak distance  $a$  without a large variation of  $b$ , thus determining an increase of the ratio  $a/b$ . Figure 4 shows the linear dependence of  $r_d$  on the distribution of  $N$ -AcTyrNH<sub>2</sub> between the two solvents. For each  $N$ -AcTyrNH<sub>2</sub> distribution, the  $r_d$  is quite insensitive to the solvent chosen for  $N$ -AcTrpNH<sub>2</sub>.

**Proteins.** The second-derivative spectrum of proteins containing both tyrosyl and tryptophanyl residues shows the same general features observed for mixtures of  $N$ -AcTyrNH<sub>2</sub> and  $N$ -AcTrpNH<sub>2</sub>, i.e., two minima centered around 283 and 290.5 nm and two maxima around 287 and 295 nm. Since the position of the peaks does not change much after exposure to perturbing agents, we have analyzed the second-derivative spectra of proteins in terms of the ratio between the peak to peak distances  $a$  and  $b$  (see Figure 1).

Table III shows the values of the ratio  $a/b$  of several native proteins and their corresponding randomly coiled forms reached in 6.0 M Gdn-HCl. The ratios determined for the

native proteins ( $r_n$ ) are in all cases lower than those observed for the unfolded proteins ( $r_u$ ). Moreover, the values determined for denatured proteins correspond rather closely to those determined for mixtures of *N*-AcTyrNH<sub>2</sub> and *N*-AcTrpNH<sub>2</sub> having the same molar ratios. This observation has been recently utilized to carry out a relatively simple method for the simultaneous determination of tyrosine and tryptophan in proteins (Servillo et al., 1982).

The data obtained on low molecular weight model compounds suggest that the increase of the ratio  $a/b$  between the native and unfolded forms of proteins is related to the changes occurring in the tyrosyl microenvironments, which become more polar following protein denaturation. Therefore, we thought that an appropriate analysis of the data reported in Table III could provide the absolute exposure values of tyrosyl residues in native proteins. The degree of exposure has been calculated by the following equation:

$$\alpha = (r_n - r_a)/(r_u - r_a) \quad (5)$$

where  $r_n$  and  $r_u$  are the numerical values of the ratio  $a/b$  determined for the native and unfolded protein, respectively;  $r_a$  is the  $a/b$  value of a mixture, containing the same molar ratio of aromatic amino acids dissolved in a solvent possessing the same characteristics of the interior of the protein matrix. In this respect, ethylene glycol appears to be the most appropriate reference solvent because of its ability to give and to accept protons. Moreover, comparison of the perturbation produced by the protein with the perturbation produced by 20% ethylene glycol indicates that the interior of a protein is equivalent to 120% ethylene glycol (Donovan, 1969).

Table III shows the values of the fraction of tyrosyl residues which are exposed to solvent in the native structure of several proteins as determined by second-derivative measurements. A good correspondence between the data reported in Table III and those available in the literature appears to exist. The X-ray analysis of lysozyme has revealed that the three tyrosyl residues are on the surface of the molecule (Canfield & Liu, 1965; Jollès et al., 1964), one of the residues (Tyr-54) being hydrogen bonded and the other two (Tyr-20 and -23) freely accessible and not sufficiently close to potential proton donors or acceptors. Reactivity with respect to cyanuric fluoride (Kurihara et al., 1963) and acetylimidazole (Kronman & Robbins, 1970) showed one unreactive group which was supposed to be the one involved in hydrogen bonding. In addition, one of the three tyrosyl groups appears to be less free to titrate than the other two (Kronman & Robbins, 1970). These observations are quite in agreement with our result relative to lysozyme indicating that two of the three residues are exposed to solvent and the remaining one only partially buried. As far as sperm whale myoglobin is concerned, the fraction of tyrosyl residues accessible to solvent determined by the second derivative, i.e., 0.33, is consistent with the mean static accessibility calculated from the data of Lee & Richards (1971), i.e., 0.24, the difference being due to the fact that the latter value is a static measurement which does not take into account the protein flexibility. The lower accessibility value found for horse myoglobin, i.e., 0.22, is probably due to the lack of Tyr-HC3 (Edmundson, 1965), which has been reported to be on the surface of the molecule with its phenolic group projecting into the medium (Kronman & Robbins, 1970). The converse holds for tuna apomyoglobin, the tyrosyl fraction of which exposed to solvent is very high. This may be related to the low  $\alpha$ -helical content found for the protein and to the more open conformation compared to that of sperm whale (Fosmire & Brown, 1976).

Herskovits & Sorensen (1968) have found that 10–12 of

the 17 tyrosyls are exposed in the native pepsin; this corresponds to a fraction of exposed residues (60–70%) which is only slightly lower than that found in our calculations (79%). On the other hand, we have found a lower fraction of tyrosyl residues exposed in cytochrome *c*, i.e., 0.42 compared with the existing data, i.e., 0.50, thus indicating that two groups are exposed in the native state (Kronman & Robbins, 1970). No data are available at present to assist in a comparison, as far as aspartate aminotransferase is concerned.

## Discussion

The local environment of the aromatic chromophores of tyrosine and tryptophan residues in proteins has been largely investigated by ultraviolet spectroscopy. A particularly useful way to probe chromophore accessibility is to record the difference between the spectra of a perturbed and an unperturbed protein molecule (Donovan, 1969, 1973). Perturbations are commonly produced by changing the temperature or the solvent composition (Donovan, 1969) using the tandem cell arrangement described by Herskovits & Laskowski (1962) and Holmes & Kronman (1964).

A rather serious limitation of the solvent perturbation technique is its applicability in determining tyrosine exposure for proteins having tyrosine/tryptophan ratios lower than 2. In such cases, an uncertainty of about  $\pm 50\%$  has been reported to exist. Better estimates of tyrosine exposure can be obtained comparing the complete perturbation spectrum of the protein with those calculated for varying degrees of tyrosine and tryptophan exposure using the perturbation spectra of the low molecular weight model compounds.

The data reported in this paper show that second-derivative spectroscopy provides a useful tool for examining the state of tyrosyl residues in proteins containing tryptophan. The method is based on the greater sensitivity of tyrosine to the solvent, the spectral shift being larger than that observed for tryptophan (Donovan, 1969). The results have been found consistent with the available X-ray information and with the existing perturbation data. Herskovits and Sorensen (1968) have critically examined the optimal spectrophotometer parameters for carrying out perturbation measurements. Unfortunately, the high protein concentrations required to record difference spectra severely affect this method because of stray light effects which may result in distortion of the spectra or in deviation from Beer–Lambert's law.

In this respect, the derivative method proposed in this paper offers some advantages since it does not require high protein concentrations, reproducible spectra being obtained at absorbance values as low as 0.2 or less.

This allows the possibility of working at concentrations where the occurrence of other phenomena such as protein–protein interactions and distortion due to turbidity etc. can be reasonably excluded.

**Registry No.** Lysozyme, 9001-63-2;  $\alpha$ -chymotrypsinogen, 9035-75-0; aspartate aminotransferase, 9000-97-9; pepsin, 9001-75-6; cytochrome *c*, 9007-43-6; tyrosine, 60-18-4; tryptophan, 73-22-3.

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## Light-Scattering Investigation of the Subunit Structure and Sequential Dissociation of *Homarus americanus* Hemocyanin<sup>†</sup>

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**ABSTRACT:** The hemocyanin of the North American lobster *Homarus americanus* has a dodecameric subunit structure organized from two basic hexameric units characteristic of arthropod species. Various neutral salts and ureas have been found to dissociate this hemocyanin. The molecular weight profiles studied as a function of reagent concentration and constant hemocyanin concentration, using light-scattering intensity methods, suggest a two-step reaction of dissociation and assembly of the functional dodecameric protein. The dissociation data obtained with urea, NaBr, and some of the other salts of this study show an initial decrease in the molecular weights to values close to  $4.7 \times 10^5$ , the molecular weight of the basic hexameric unit. This intermediate plateau region of the molecular weight transitions is followed by further dissociation of the hexamers to monomers with a corresponding

decrease in molecular weights approaching  $0.8 \times 10^5$ . The equations developed for analyzing the light-scattering dissociation data in previous studies were applied to the sequential steps of dodecamer to hexamer and hexamer to monomer dissociation-association reactions. The apparent number ( $N_{app}$ ) of amino acid groups at the contact areas of the hexamers in the functional dodecamers and the contact areas of the monomers in the half-molecules or hexamers affected by the dissociating reagent were found to be about the same. Similar  $N_{app}$  estimates were obtained previously from the dissociation behavior of the *Homarus* hemocyanin dodecamers and the isolated hexamers, modified by acetylation. These findings suggest that the contact areas of the hexamers and the monomers forming the dodecamers of arthropod hemocyanins must be comparable in size.

**T**he hemocyanin of the North American lobster *Homarus americanus* is a dodecameric subunit protein built from two basic hexameric units held together by noncovalent interactions

in solution (Morimoto & Kegeles, 1971; Herskovits et al., 1981b). The hemocyanins of the lobsters *Panulirus interruptus* (Van Schaick et al., 1981) and *Homarus* (the subject of the present investigation) consist of one or two basic hexameric units, whereas the hemocyanins of some of the other arthropods, represented by the spider *Eurypelma californicum* (Markl et al., 1981) or the scorpion *Androctonus australis* (Lamy et al., 1981) and the horseshoe crab *Limulus poly-*

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