Effects of Hydrogen Bonding and Temperature Upon the Near Ultraviolet Circular Dichroism and Absorption Spectra of Tyrosine and O-Methyl Tyrosine Derivatives

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SUMMARY

To gain information about the properties of tyrosyl residues buried within proteins, the circular dichroism (CD) and absorption spectra of tyrosine derivatives have been investigated in nonpolar solvents. N-Stearyl-L-tyrosine n-hexyl ester dissolved in methylcyclohexane (O-O band at 283 nm) was used to measure the effects of hydrogen-bonding agents. Adding low concentrations of dioxane, N,N-dimethylacetamide, 1-butanol, or methanol causes a 1- to 4-nm red shift in the absorption spectrum and a 10 to 25% increase in the dipole strength. The results with N,N-dimethylacetamide suggest that a hydrogen bond between a tyrosyl hydroxy group and a carbonyl oxygen of the peptide backbone may be one mechanism for producing a large red shift in proteins.

CD spectra were recorded after the hydroxy group of N-stearyl-L-tyrosine n-hexyl ester had been hydrogen bonded. Dioxane and N,N-dimethylacetamide cause the CD spectra to red shift and intensify to the same extent as do the absorption spectra. Evidently hydrogen bonding to these compounds does not alter the conformation of this tyrosine derivative. In contrast, hydrogen bonding of N-stearyl-L-tyrosine n-hexyl ester to butanol or methanol causes a 50% loss of rotatory strength, suggesting an altered conformation. The dependence upon alcohol concentration is the same for both the CD and absorption alterations (half-maximal effect at 30 mM alcohol). Evidence is presented that a polymeric form of the alcohol may simultaneously hydrogen bond to both the hydroxy group and the amide oxygen atom of N-stearyl-L-tyrosine n-hexyl ester. At concentrations greater than 15 μM, N-stearyl-L-tyrosine n-hexyl ester is partially aggregated in methylcyclohexane, causing changes in the CD and absorption spectra. The aggregate is proposed to be mainly a dimer in which the hydroxy group of each tyrosine residue is hydrogen bonded to the amide oxygen of the other residue (K_M ≈ 1000 M⁻¹).

To minimize aggregation, N-acetyl-O-methyl-L-tyrosine ethyl ester (N-Ac-O-Me-L-Tyr ethyl ester) and N-stearyl-O-methyl-L-tyrosine n-hexyl ester were studied. O-Methyl tyrosine derivatives have the same vibronic structure as does tyrosine, but the wave length position is not much shifted by polar organic solvents (O-O band at 283.5 ± 0.5 nm). The rotatory strengths of O-methyl-L-tyrosine derivatives are nearly identical with those of L-tyrosine derivatives in corresponding solvents. For N-Ac-O-Me-L-Tyr ethyl ester dissolved in methylcyclohexane at 297 K, the rotatory strength is especially large (1.3 X 10⁴° c.g.s. with Δε = 0.75 M⁻¹ cm⁻¹ at 277 nm).

Upon cooling N-Ac-O-Me-L-Tyr ethyl ester (dissolved in ether-isopentane-ethanol solvent) from 297 to 140 K, the negative rotational strength is intensified 10-fold. These results are interpreted in terms of temperature shifting the equilibrium distribution of the various conformers of N-Ac-O-Me-L-Tyr ethyl ester. The variable temperature CD technique may provide a way to detect motility of the tyrosyl side chains in proteins.
Information concerning the circular dichroism spectra of model compounds is a prerequisite to understanding the near ultraviolet CD bands of proteins. Recently the CD spectra of several tyrosine derivatives have been investigated both experimentally (1–4) and theoretically (5, 6). The theoretical studies clearly showed that the near ultraviolet tyrosyl CD bands are strongly conformation dependent (5, 6). Unfortunately, predicting the conformation from the observed CD bands is usually difficult because of the many conformations possible for nonrigid molecules. In addition, the relative populations of these conformers may be influenced by solvent-solute interactions, such as hydrogen bonding, and by temperature.

This communication describes the CD spectra of several tyrosine derivatives which were synthesized so that the effects of temperature, solvents, and hydrogen bonding could be investigated in detail. N-Stearyl-L-tyrosine n-hexyl ester is soluble in both nonpolar and polar solvents, but is prone to aggregate. N-Stearyl-o-methyl-L-tyrosine n-hexyl ester and N-acetyl-o-methyl-L-tyrosine ethyl ester do not aggregate as readily and were, therefore, more satisfactory for examining CD spectra at low temperatures. The effects of hydrogen bonding upon the CD spectra were studied with the use of both N-Stearyl-L-tyrosine n-hexyl ester and its corresponding o-methyl derivatives.

MATERIALS AND METHODS

Instrumentation—CD spectra were recorded with an instrument that has been described in detail elsewhere (7, 8). Reliable, low noise CD records were obtained by averaging four to 32 scans at 0.3 nm per sec with a Fabri-Tek model 1072 signal averager (8, 9). The CD intensity was calibrated with an aqueous solution of d-10-campborsulfonic acid ($\Delta \varepsilon = 2.2 \times 10^3$ cm$^{-1}$ at 200 nm (10), where $\Delta \varepsilon$ is the molar extinction coefficient for left circularly polarized light minus that for right circularly polarized light). The techniques for low temperature CD measurements have been described previously (2). In calculating the $\Delta \varepsilon$ values at each temperature, the concentration increase upon cooling was taken into account by using the volume-concentration data for EPA (11) and for methylcyclohexane (12). Unless otherwise indicated, the concentration dependence of these CD spectra was in accordance with Beer’s law over at least a 5-fold concentration range. These findings imply that aggregation is not responsible for the changes in CD spectra observed for N-Ac-o-Me-L-Tyr ethyl ester and N-Ac-l-Tyr ethyl ester upon cooling.

Most absorption spectra were recorded on a Cary model 15 spectrophotometer. In some cases, however, a direct comparison was made between the wave length positions and shapes of the CD and absorption bands by recording absorption spectra on the CD spectrophotometer. This procedure was necessary because the spectral resolution of sharp bands is somewhat poorer on the CD instrument. To record these absorption spectra, a portion of the photomultiplier dynode voltage was fed through a log amplifier (Philbrick-Nexus No. 4351) and then subtracting the log of the solvent voltage. $A = \log (I_0/I) = k \log V - k \log V_0$, where $k$ is a calibration constant, $V$ is the dynode voltage for the sample, and $V_0$ is the voltage for the solvent (13).

Materials—O-Methyl-L-tyrosine (p-methoxyphenylalanine) was obtained from Sigma Chemical Co. (St. Louis, Mo.). p-Cresol and p-methylanisole were obtained from Eastman Organic Chemicals. Perfluorinated hexane was kindly supplied by Dr. J. D. LaZerte (3 M Co., St. Paul, Minn.). All other solvents were spectroquality. The methylcyclohexane did not have any near ultraviolet absorbing impurities (5 cm path length).

N-Ac-O-Me-L-Tyr ethyl ester was synthesized by the acetylation of O-methyl-L-tyrosine ethyl ester hydrochloride with 3-fold excess of acetic anhydride in 1 M sodium bicarbonate. The precipitate was collected and crystallized from ethyl acetate (m.p. 93°).

$\text{C}_9\text{H}_{16}\text{NO}_4$

Calculated: C 74.39, H 11.82, N 2.58
Found: C 74.51, H 11.78, N 2.51

The $\xi$ values for N-Ac-o-Me-L-Tyr ethyl ester at the absorption maxima were found to be 1400 (274 nm) in water, 1750 (277 nm) in EPA, and 1850 (277 nm) in methylcyclohexane.

N-Stearyl-o-methyl-L-tyrosine n-hexyl ester was prepared from O-methyl-L-tyrosine n-hexyl ester hydrochloride (m.p. 149°) by the same pathway described for the synthesis of N-Stearyl-L-tryptophan n-hexyl ester (14) and was recrystallized from petroleum ether (m.p. 41°–49°).

$\text{C}_9\text{H}_{16}\text{NO}_4$

Calculated: C 74.86, H 10.82, N 2.56
Found: C 75.01, H 10.58, N 2.53

The $\xi$ values were assumed to be the same as those of N-Ac-o-Me-L-Tyr ethyl ester.

N-Stearyl-L-tyrosine n-hexyl ester was prepared similarly from L-tyrosine n-hexyl ester hydrochloride (m.p. 165°) and stearic acid (14). The product was recrystallized from ether-petroleum ether (m.p. 71°–73°).

$\text{C}_{15}\text{H}_{26}\text{NO}_4$

Calculated: C 74.57, H 10.73, N 2.63
Found: C 74.45, H 10.55, N 2.48

The $\xi$ value in methylcyclohexane was taken to be the same as that of N-Ac-O-Me-L-Tyr ethyl ester.

N-Stearyl-L-phenylalanine n-hexyl ester was prepared from stearic acid and L-phenylalanine n-hexyl ester hydrochloride (m.p. 127°). The product was semicrystalline.

$\text{C}_{17}\text{H}_{32}\text{NO}_4$

Calculated: C 76.89, H 11.06, N 2.71
Found: C 76.67, H 11.18, N 2.58

RESULTS

Comparison of p-Cresol and p-Methylanisole Spectra—As a preliminary step, we compare the near ultraviolet chromophores of O-methyl-L-tyrosine and of L-tyrosine. The most appropriate model compounds for these chromophores are p-methylanisole and p-cresol, respectively. The vibronic structure of their absorption bands can be clearly seen by using perfluorinated hexane as the solvent. As shown in Fig. 1, the wave length positions and spacings of the vibronic bands are essentially identical in p-methylanisole and in p-cresol when dissolved in this relatively inert solvent. These high resolution solution spectra reveal the same major vibronic bands as have been reported in the
FIG. 1. Absorption spectra of p-cresol and p-methylanisole dissolved in perfluorinated hexane. The spectrum of p-cresol has been offset to separate the records. One centimeter path length.

FIG. 2. CD (top) and absorption (bottom) spectra of N-Ac-O-Me-L-Tyr ethyl ester dissolved in EPA (left), dioxane (middle), and methanol (right). The rotatory strength of N-Ac-O-Me-L-Tyr ethyl ester (27 mM) in EPA at 77 K is approximately the same as that observed at 140 K. The dipole strength of N-Ac-O-Me-L-Tyr ethyl ester is $1.3 \times 10^{-6}$ c.g.s. in all three solvents.

FIG. 3. CD and absorption spectra of N-Ac-O-Me-L-Tyr ethyl ester dissolved in methylecyclohexane at 297 and 225 K. The concentration had to be kept less than 0.4 mM to prevent aggregation from occurring at 225 K. N-Ac-O-Me-L-Tyr ethyl ester did not show any tendency to aggregate in this solvent at 297 K. The spectral half-intensity band widths were 1 nm for the CD measurements and 0.2 nm for the absorption measurements. Trimer after cooling N-Ac-O-Me-L-Tyr ethyl ester to 140 K (Fig. 2, bottom). This band sharpening occurs without any measurable change in the dipole strength of the near ultraviolet absorption band (less than 3% intensification).

Since EPA is a mixed solvent containing both an alcoholic and ether component, the CD spectrum of N-Ac-O-Me-L-Tyr ethyl ester was also examined in dioxane and methanol at room temperature. The CD spectrum of this compound is positive in dioxane and negative in methanol (Fig. 2).

Additional evidence relating to the variability of CD intensity was obtained from N-Ac-O-Me-L-Tyr ethyl ester dissolved in methylcyclohexane. In this solvent, N-Ac-O-Me-L-Tyr ethyl ester possesses an intense, positive CD spectrum with pronounced vibronic structure even at 297 K (Fig. 3). The sharpness of these bands is further enhanced by cooling to 225 K, and the rotatory strength is increased an additional 35% (Fig. 3).

The sharpness and intensity of these bands at 225 K provide an exceptional opportunity to examine the correspondence between CD and absorption bands. To make an accurate comparison, the absorption spectrum of N-Ac-O-Me-L-Tyr ethyl ester in methylecyclohexane at 225 K was recorded using the monochromator in the CD instrument so that differences in spectral band widths would not affect the results. The individual vibronic bands occur at the same wave lengths, and their relative intensities are nearly identical in both the CD and absorption spectrum (less than 10% variation).

N-Stearol-O-Methyl-L-Tyrosine n-Hexyl Ester—The spectra of N-stearol-O-methyl-L-tyrosine n-hexyl ester were also examined dissolved in methylecyclohexane at 297 and 225 K. These CD and absorption spectra are nearly identical with those shown for N-Ac-O-Me-L-Tyr ethyl ester in methylecyclohexane in Fig. 3 (see also Table I).

N-Acetyl-L-tyrosine Ethyl Ester—Variable temperature CD spectra of p-cresol (15) and p-methylanisole (16). The 800 cm⁻¹ spacing (Fig. 1) results from the ring-breathing mode of vibration (1).
N-Ac-L-Tyr ethyl ester solutions and correcting for a change of 250 nm for positive CD bands; for negative bands, only the negative portion was measured.

Cooling brought out a much more intense, negative CD in the region above 265 nm. At 297 K, the CD spectrum is weakly positive (I). Cooling brought out the major vibronic absorption bands (1). The shapes of the 215 and 140 K CD spectra are similar to those of N-Ac-O-Me-L-Tyr ethyl ester dissolved in EPA (Fig. 2), but the CD spectrum has a much more intense, negative CD in the region above 265 nm. At 297 K, the CD spectrum is weakly positive (I).

Circular dichroism intensities of O-methyl tyrosine derivatives compared to those of tyrosine derivatives

| Temperature | Solvent          | Derivative                        | $\Delta = \Delta \epsilon \times \lambda 
^2$ | $\Delta \epsilon \times \lambda 
^2$ |
|-------------|------------------|-----------------------------------|-------------------------|-------------------------|
| 297         | Water-glycerol,  | L-Tyrosine*                       | -0.66                   | 0.33                     |
|             | pH 1.0 (1:1, v/v)| O-Methyl-L-tyrosine               | -0.49                   | 0.27                     |
| 77          | Water-glycerol   | L-Tyrosine*                       | -0.91                   | 0.51                     |
|             | (1:1, v/v)       | O-Methyl-L-tyrosine               | -0.75                   | 0.46                     |
| 297         | Dioxane          | N-Ac-O-Me-L-Tyr ethyl ester*      | -0.40                   | 0.20                     |
|             |                  | N-Ac-O-Me-L-Tyr ethyl ester       | -0.36                   | 0.18                     |
| 297         | Methanol         | N-Ac-L-Tyr ethyl ester            | -0.20                   | -0.13                    |
|             |                  | N-Ac-O-Me-L-Tyr ethyl ester       | -0.22                   | -0.15                    |
| 140         | EPA              | N-Ac-L-Tyr ethyl ester            | -0.17                   | -0.15                    |
|             |                  | N-Ac-O-Me-L-Tyr ethyl ester       | -0.34                   | -0.29                    |
| 297         | Methylocyclohexane| N-Ac-O-Me-L-Tyr ethyl ester       | 1.3                     | 0.75                     |
| 225         | Methylocyclohexane| N-Ac-O-Me-L-Tyr ethyl ester       | 1.8                     | 1.3                      |
| 297         | Methylocyclohexane| N-Stearyl-L-Tyr n-hexyl ester     | 1.1                     | 0.63                     |
|             |                  | N-Stearyl-O-Me-L-Tyr n-hexyl ester| 1.1                     | 0.63                     |
| 225         | Methylocyclohexane| N-Stearyl-O-Me-L-Tyr n-hexyl ester| 1.5                     | 1.0                      |

* $\epsilon$, rotatory strength of the near ultraviolet CD band above 250 nm for positive CD bands; for negative bands, only the negative portion was measured.

* Calculated at the position of the CD maximum between 275 to 280 nm.

* Value calculated from the data in Reference 1, using the $\epsilon$ values given in Reference 17 to obtain the concentrations of the N-Ac-L-Tyr ethyl ester solutions and correcting for a change of the calibration standard (10).

The approximate association of N-Stearyl-L-tyrosine n-hexyl ester was estimated from the increase in absorbance at 287 nm in the more concentrated solutions. This band was assumed to result from the formation of dimers having their O-O hydrogen bond formation in phenol and p-cresol (18-22). The approximate association of N-Stearyl-L-tyrosine n-hexyl ester was estimated from the increase in absorbance at 287 nm in the more concentrated states above 15 $\mu$m. Apparently the CD and absorption spectra at 15 $\mu$m and below represent the monomeric N-Stearyl-L-tyrosine n-hexyl ester dissolved in methylcyclohexane.

The approximate association of N-Stearyl-L-tyrosine n-hexyl ester was estimated from the increase in absorbance at 287 nm in the more concentrated solutions. This band was assumed to result from the formation of dimers having their O-O hydrogen bond formation in phenol and $\mu$-cresol (18-22).

**Effects of Hydrogen Bonding** By adding small amounts of hydrogen-bonding agents to methylocyclohexane solutions, it is possible to obtain hydrogen-bonded tyrosine derivatives dissolved in an essentially nonpolar solvent. Thus the ensuing spectral changes reflect predominantly interactions with the hydrogen-bonding agent, not changes in the bulk properties of the solvent. This technique has been extensively used to measure hydrogen bond formation in phenol and $\mu$-cresol (18-22).

The first experiments were carried out with dioxane and $N,N$-dimethylacetamide-compounds which are able to act only as proton acceptors. The extent of interaction with the phenolic ring of N-Stearyl-L-tyrosine n-hexyl ester was determined from the red shift in the absorption spectrum after adding the proton acceptor (Fig. 5). For example, adding 4 $\mu$m $N,N$-dimethylacetamide causes a shoulder to appear at 286.6 nm.
Fig. 5. Effects of 1-butanol (left), p-dioxane (center), and N,N-dimethylacetamide (right) upon the absorption spectra of N-stearyl-L-tyrosine n-hexyl ester (9 to 10 μM) dissolved in methylcyclohexane. Solid lines were traced from instrument record to reveal the noise level. Path length was 5 cm. The reference cuvet had the same solvent composition as did the sample cuvet. Solvent versus solvent base-lines were flat. The spectral half-intensity band width was 0.4 nm.

Table II

| Acceptor                  | ΔR2 | ΔD2 | ΔR2/ΔD2 | Δλ |
|---------------------------|-----|-----|---------|----|
| N,N-Dimethylacetamide     | 25  | 1.0 | 0.1     | 3.5| 0.2|
| p-Dioxane                 | 13  | 1.0 | 0.1     | 1.9| 0.2|

* Change in rotation strength of near ultraviolet band.

* Change in dipole strength.

* Shift in position of the O-O vibronic band.

Additions of N,N-dimethylacetamide intensify the 286.6 nm band and obliterate the band observed at 283 nm in pure methylcyclohexane. After the N,N-dimethylacetamide concentration reaches 30 mM, the phenolic hydroxy group of essentially all the N-stearyl-L-tyrosine n-hexyl ester molecules are hydrogen bonded to carbonyl groups of N,N-dimethylacetamide, since further additions do not alter the absorption spectrum. In the hydrogen-bonded complex, the maximal ε value (280 nm) is about 10% larger than that of the unhydrogen-bonded N-stearyl-L-tyrosine n-hexyl ester (276.7 nm) even though the latter band is much sharper. Apparently the dipole strength of the near ultraviolet band is about 25% larger when this tyrosine derivative is hydrogen bonded to N,N-dimethylacetamide.
**Table III**

Summary of equilibrium constants for hydrogen bond formation in relatively inert solvents

| Hydrogen bond           | Proton donor          | Acceptor                  | Ka a          | Temperature |
|-------------------------|-----------------------|---------------------------|---------------|-------------|
| N-Stearyl-L-tyrosine    | Dioxane               |                           |               |             |
| n-hexyl ester b         | 16                    | 24 b                      |               |             |
| p-Cresol (22) c         | Dioxane               |                           | 12.4          | 25          |
| Amide, >NH4             | Dioxane               |                           | ~2            | 25          |
| N-Stearyl-L-tyrosine    | N,N-Dimethylacetamide |                           | 400           | 24          |
| n-hexyl ester b         | 1-Butanol             |                           | 33            | 24          |
| Phenol (21)             | N,N-Dimethylacetamide | 205                       | 28            |             |
| Amide, >NH4             | N,N-Dimethylacetamide | ~5                        | 25            |             |
| N-Stearyl-L-tyrosine    | N-Stearyl-L-tyrosine  | N,N-Dimethylacetamide     | 1000          | 24          |
| n-hexyl ester b         | 1-Butanol             | Ethyl acetate             | 4.5           |             |
| Phenol (21)             | 1-Butanol             | Ethyl acetate             | 9.8           | 20          |
| Phenol (20)             | Ethyl acetate         | 9.35                      | 29            |             |
| Phenol (23)             | Ethyl benzene         | 0.35                      | 29            |             |

* Equilibrium constant or association constant.

b This work.

c The number in parentheses is a reference number.

d Estimate based on the report that the amide, >NH, donates its proton to about the same extent as alcohols (23).

e 1-Butanol forms hydrogen bonds having approximately the same strength as ethanol and methanol (26).

![Figure 6](image6.png)

**Figure 6.** Effects of N,N-dimethylacetamide (top) and 1-butanol (bottom) upon the CD spectra of N-stearyl-L-tyrosine n-hexyl ester (9.3 µM) dissolved in methylecyclohexane. BL designates methylecyclohexane base-line. Path length, 5 cm; time constant, 1 sec. Each trace represents the average of 16 scans. The solid lines are actual instrument traces. Peak-to-peak noise in these records is approximately 1 to 2 × 10⁻⁴ ΔA, where ΔA represents absorbance for left circularly polarized light minus that for right circularly polarized light. Spectral half-intensity band width was 1.5 nm. 297 K.

![Figure 7](image7.png)

**Figure 7.** Effect of butanol concentration upon the rotatory strength (K_r) and absorbance (287 nm) of the N-stearyl n-hexyl esters of tyrosine and O-methyltyrosine dissolved in methylecyclohexane at 297 K. Rotatory strength was measured in the region above 270 nm.

butanol, however, is the loss of rotatory strength (Fig. 6). When the butanol concentration reaches 0.08 M, about half of the initial rotatory strength remains. The decline in rotatory strength possesses the same dependence upon butanol concentration as do the alterations in the absorption spectra within our experimental error (Fig. 7). Similar changes were observed after adding small amounts of methanol to N-stearyl-L-tyrosine n-hexyl ester dissolved in methylecyclohexane. In pure butanol the CD spectrum of N-stearyl-L-tyrosine n-hexyl ester is weakly negative from 275 to 290 nm.

To further examine the sites where butanol interacts, CD and absorption spectra were recorded following the addition of butanol to both N-stearyl-O-methyl-L-tyrosine n-hexyl ester and N-stearyl-L-phenylalanine n-hexyl ester. Up to 0.5 M butanol does not blur the vibronic absorption bands of N-stearyl-O-methyl-L-tyrosine n-hexyl ester, although the bands may have been shifted to a slightly shorter wave length (less than 0.2 nm shift). The dipole strength is essentially unchanged. At low concentrations of butanol the loss of rotatory strength is appreciably less than that observed for the tyrosine compound (Fig. 7). At 0.5 M butanol, the rotatory strength of N-stearyl-O-methyl-L-tyrosine n-hexyl ester declines by 43%, which is about the same as observed with the tyrosine derivative.

The possible interactions of butanol with the amide and ester groups were examined with the use of N-stearyl-L-phenylalanine n-hexyl ester dissolved in methylecyclohexane. Measurable changes in the near ultraviolet CD spectrum of this compound began to occur at 0.08 M butanol. As the butanol concentration was increased further, the CD intensity of the O-O band gradually changed from weakly positive to weakly negative. Even at 1.5 M butanol there was no evidence that the binding sites on N-stearyl-L-phenylalanine n-hexyl ester had been saturated. Evidently butanol interacts at numerous sites on N-stearyl-L-phenylalanine n-hexyl ester.

**Discussion**

Combined studies of tyrosine and O-methyl tyrosine derivatives aid in understanding the spectral properties of the ionized tyrosyl side chain. The high resolution solution spectra of model compounds (p-methylanisole and p-cresol, Fig. 1) indicate that the substitution of a p-methoxy group for a p-hydroxy group does not alter the vibronic absorption bands char-
characteristic of the tyrosyl side chain (1). This conclusion is further substantiated by the well resolved vibronic absorption bands of tyrosine and O-methyl tyrosine derivatives dissolved in methylcyclohexane (Figs. 2 and 4) and in EPA at low temperatures (Fig. 2 and References 1 and 2). Furthermore, the ε values are nearly identical for both tyrosine and O-methyl tyrosine derivatives (see under “Materials and Methods” and References 17 and 30). Although the vibronic bands of O-methyl tyrosine derivatives are sharper than those of tyrosine derivatives in polar organic solvents, the dipole strengths are slightly greater for tyrosine derivatives. This difference in behavior results from the phenolic hydroxy group acting as a proton donor in hydrogen bond formation (18). The hydrogen-bonding experiments with N-stearyl-L-tyrosine n-hexyl ester suggest that the stronger hydrogen bonds (measured by the equilibrium constants) may produce greater enhancement of dipole strength. The mechanism involved in this effect has been discussed by other workers (17, 19, 22).

There is only one major spectral difference between O-methyl tyrosine and the un-ionized tyrosyl side chain; the wave length position of the O—O band of O-methyl tyrosine is much less shifted by hydrogen-bonding solvents than is the case for tyrosine. In all organic solvents used, the O—O band of O-methyl tyrosine derivatives occurs at 283.5 ± 0.5 nm (Figs. 2 and 3). By contrast, the O—O band of N-stearyl-L-tyrosine n-hexyl ester is red shifted 1 to 4 nm by hydrogen-bonding agents added to methylcyclohexane (Fig. 5). The red shift observed under these conditions probably depends largely upon the strength of the hydrogen bond (31). Interestingly, N,N-dimethylacetamide, a model compound for the peptide bond, is strongly bound by N-stearyl-L-tyrosine n-hexyl ester and causes a 3.5-nm red shift (Table II). Evidently hydrogen bonding of the tyrosyl hydroxy group to the carbonyl oxygen of a peptide bond should be thermodynamically favorable in proteins and may partially account for the large red shift of the O—O band of some tyrosine residues in proteins (1). For example, in ribonuclease-S one of the 3 tyrosyl residues having its O—O band at 296 nm (32) does have this type of hydrogen bond (see Tyr 92 in Fig. 4 of Reference 33). In addition to hydrogen bonding, other physical properties of the tyrosyl side chain may also influence the position of the O—O band in proteins (22, 34).

Next we shall examine several factors that influence the near ultraviolet rotatory strength of the N-acyl esters of tyrosine and O-methyl tyrosine. The largest rotatory strength for the monomeric forms occurs in a completely nonpolar solvent, methylcyclohexane (Table I). This solvent permits the strongest interactions between the polar groups of the amino acid portion and the aromatic ring. Perhaps these interactions are sufficiently strong to stabilize mainly a single conformation for these moieties. The small increase in rotatory strength upon cooling N-stearyl-O-methyl-L-tyrosine n-hexyl ester and N-Ac-O-Me-L-Tyr ethyl ester in methylcyclohexane (Fig. 3) is consistent with this interpretation (see below). Close contact of the aromatic ring with the amide and ester groups would tend to facilitate coupling with their electronic transitions and may thereby produce a larger rotatory strength (5).

The effects of hydrogen bonding upon rotatory strength were examined by using the red shift of the absorption band to identify when the tyrosyl hydroxy group is hydrogen bonded. These studies, however, are complicated by the occurrence of several other binding sites. To aid in determining which sites are occupied on N-stearyl-L-tyrosine n-hexyl ester, a number of equilibrium constants for hydrogen bonding to model compounds (23-28) have been compared with those of the tyrosine derivative (Table III). Apparently low concentrations of hydrogen-bonding agents should interact preferentially with the phenolic hydroxy group, though the margin over other binding sites is not always great.

After adding small amounts of dioxane or N,N-dimethylacetamide, the CD spectrum of N-stearyl-L-tyrosine n-hexyl ester is red shifted and blurred to the same extent as the absorption spectrum (Figs. 5 and 6). A small increase of rotatory strength occurred in proportion to the increase in dipole strength after hydrogen bonding (Table II). These findings suggest that the conformation of N-stearyl-L-tyrosine n-hexyl ester is not altered when the phenolic hydroxy group is hydrogen bonded to dioxane or N,N-dimethylacetamide. The relatively minor change in the CD spectrum results because the absorption spectrum is altered.

Contrary to the results with dioxane and N,N-dimethylacetamide, adding even small concentrations of butanol or methanol causes the rotatory strength of N-stearyl-L-tyrosine n-hexyl ester to decrease by half. Both the CD and absorption alterations have approximately the same concentration dependence (half-maximal effect at 30 mM butanol, Fig. 7). The apparent equilibrium constant for this interaction agrees well with the values published for alcohols hydrogen bonding to the phenolic hydroxy group (Table III). If each hydrogen-bonding site acts independently, butanol should bind less well to the other sites on N-stearyl-L-tyrosine n-hexyl ester. The second strongest binding site for butanol is expected to be the amine oxygen atom. The equilibrium constants predict that about 35% of the amide oxygens should be hydrogen bonded when the phenolic hydroxy group is essentially fully hydrogen bonded (0.1 M butanol). At 0.5 M butanol, about 75% of the amide oxygens should be hydrogen bonded. Surprisingly, the rotatory strength of N-stearyl-L-tyrosine n-hexyl ester does not decrease any further when the butanol concentration is raised from 0.1 to 0.5 M (Fig. 7).

In contrast, the N-stearyl n-hexyl esters of phenylalanine and O-methyl tyrosine do not have a plateau region. For these compounds, the rotatory strength is less altered at low concentrations of butanol than is the case with the tyrosine compound (Fig. 7). As the butanol concentration is increased to 0.5 M and above, the rotatory strengths of the O-methyl tyrosine and phenylalanine derivatives continue to change. Apparently at these higher concentrations butanol interacts extensively with the amide and ester groups of these compounds lacking a phenolic hydroxy group.

For N-stearyl-L-tyrosine n-hexyl ester, the large initial loss of rotatory strength seems in some way to involve the interaction of butanol with the phenolic hydroxy group. The structures of the alcohol-phenol complexes are not well understood, in part because their hydroxy groups can function as both proton donors and acceptors. When phenol donates a proton to the oxygen atom of an alcohol, the alcohol becomes a stronger proton donor and the phenolic oxygen becomes a better proton acceptor (29). Thus the initial hydrogen bond potentiates additional hydrogen bonds with other alcohol molecules, leading to phenol-polymeric alcohol complexes (29). Undoubtedly a number of different tyrosyl-alcohol complexes are formed, which account for alcohols blurring the absorption spectra (Fig. 5).
An examination of molecular models revealed a group of complexes that is especially interesting. As few as 2 or 3 alcohol molecules joined by hydrogen bonds can simultaneously hydrogen bond to both the hydroxy group and the amide oxygen atom of N-stearyl-L-tyrosine n-hexyl ester. A complex of this type would explain the large initial decrease in rotatory strength at low butanol concentrations and the absence of any further effects between 0.1 and 0.5 m butanol. In any case, it seems highly probable that the binding of even low concentrations of alcohols leads to an altered conformation for N-stearyl-L-tyrosine n-hexyl ester. Binding an alcohol per se does not necessarily alter the rotatory strength, since the rotatory strengths of rigid tyrosyl derivatives are nearly identical in both methanol and dioxane.

When tyrosine and O-methyl tyrosine derivatives are dissolved in polar organic solvents, the rotatory strengths are greatly altered from those in nonpolar solvents (Table I). Apparently the conformations of the N-acetyl esters of tyrosine and O-methyl tyrosine are greatly influenced by certain interactions with the solvent. Interestingly the rotatory strengths of the O-methyl tyrosine derivatives are practically identical with those of the tyrosine derivatives dissolved in corresponding solvents (Table I). Even with pure alcoholic solvents, the conformations of these tyrosine derivatives do not seem to be much affected by the phenolic hydroxy group donating a proton to form a hydrogen bond with the solvent.

Major differences between the CD spectra of tyrosine and O-methyl tyrosine compounds were observed only when the tyrosine derivatives aggregated (Fig. 4). The phenolic hydroxy group in tyrosine derivatives greatly decreases the tendency to self-associate in organic solvents. The aggregation of N-stearyl-L-tyrosine n-hexyl ester in methylethylcemohexane apparently reveals the interacting groups. In contrast to p-cresol aggregation, an interaction between the tyrosyl hydroxy groups does not seem to occur. First of all, the tyrosine interaction is much stronger than that of p-cresol (Table III). Secondly, the aggregation of N-stearyl-L-tyrosine n-hexyl ester in methylethylcemohexane causes a 4-nm red shift (Fig. 4) instead of the short wave length shift observed in p-cresol aggregates (26). Perusal of the hydrogen bond equilibrium constants (Table III) reveals that the strongly possible interactions occur between phenolic hydroxy groups and amide oxygen atoms. Examining models of N-stearyl-L-tyrosine n-hexyl ester showed that a dimer can be formed having the hydroxy group of each tyrosine residue hydrogen bonded to the amide oxygen atom of the other residue. In this dimer most of the two aromatic rings are stacked in van der Waal contact. Evidently this conformation has sufficient interactions to account for the large equilibrium constant and the altered CD spectrum of the aggregate. At high concentrations, long chain polymers of N-stearyl-L-tyrosine n-hexyl ester may be formed by having the hydroxy group of one residue hydrogen bonded to the amide oxygen atom of the following residue.

As our final point, we consider the effect of temperature upon the CD spectrum of N-acetyl-L-Tyr ethyl ester and N-acetyl-O-methyl-Tyr ethyl ester dissolved in EPA. The measured CD spectrum is the population-weighted average resulting from the various conformers existing in equilibria (35). At room temperature, the distribution of the various conformers is such that their population-weighted average gives a very small rotatory strength. Probably this results because the number of conformers with positive CD spectra is approximately equal to the number with negative CD spectra, giving nearly complete cancellation. Cooling EPA solutions of N-Ac-O-Me-L-Tyr ethyl ester and N-Ac-O-Me-L-Tyr ethyl ester from 297 K to 140 K causes the rotatory strength to become strongly negative, especially for N-Ac-O-Me-L-Tyr ethyl ester. Evidently cooling shifts the equilibria in favor of conformers having negative CD spectra, i.e. conformational motility (36) exists.

The large enhancement of rotatory strength upon cooling N-Ac-O-Me-L-Tyr ethyl ester (Fig. 2) is similar to that observed previously with nonrigid tryptophan and phenylalanine derivatives (7, 37). Apparently the noncyclic derivatives of all these aromatic amino acids may have extensive motility in EPA. Low temperature CD measurements are also possible for proteins that dissolve in a water-glycerol solvent without having their native conformation altered (32, 38). Thus variable temperature CD spectra provide a means to detect motility of the aromatic amino acid side chains of proteins. In this technique a residue has already revealed some motility in the tryptophan side chains of carbocyclicprotease A (38).

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