CHARGE DIFFERENCE SPECTRA OF MODEL COMPOUNDS FOR TYROSYL CHROMOPHORES

by

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ABSTRACT

Charge difference spectra were determined for tyrosine and seven model compounds. A perturbing charge may cause either an intensity decrease or an intensity increase depending on its sign and orientation with respect to the hydroxyl group. The magnitude of a spectrum diminishes as the distance between the charge and the chromophore is increased. The spectra observed must be largely due to intensity changes; an equation for the change in dipole strength of a charge-perturbed transition is derived and applied to spectra of phenols and indoles. Only transition dipoles are important in the intensification of phenol spectra but both permanent and transition dipoles contribute to intensity changes in indoles. The magnitude of phenol difference spectra is generally smaller than that of indole spectra. It appears that inductive effects cannot be the principal cause of the difference spectra observed. Applications of these results to protein spectra are considered.

CHAPTER 1

INTRODUCTION

Ultraviolet spectroscopy is among the physical methods that have been most extensively applied in the study of protein structure. Much information about the environments of individual protein chromophores can be gained using spectrophotometric techniques in the ultraviolet region of the spectrum.

Proteins contain several types of chromophores that absorb ultraviolet light; these include the amino acids cysteine, phenylalanine, tyrosine, tryptophan and histidine, the disulfide bond of cystine and the peptide bond itself. All of these chromophores have absorption bands between 200 and 250 nm. It is the region above 250 nm, however, that is most characteristic of protein structure (Donovan 1969) since at lower wavelengths there is much overlapping of absorptions due to different chromophores. While cystine shows an absorption maximum at 250 nm, only the aromatic amino acids phenylalanine, tyrosine and tryptophan have absorption maxima above 250 nm. They contain the phenyl, phenolic and indolyl chromophores, respectively, and in each case absorption is due to a $\pi \rightarrow \pi^*$ transition. Phenylalanine can ordinarily be neglected since its absorption is $\sim$10 times weaker than that of either tyrosine or tryptophan (Donovan 1969).

Understanding the ultraviolet spectra of proteins is a formidable problem. A chromophore in solution is affected by interaction with its environment; with respect to the vapor phase spectrum, absorption bands may be shifted and vibrational structure may diminish or disappear altogether. For a chromophore in a protein molecule, the environment includes not only solvent molecules but also the rest of the macromolecule; thus protein spectra may depend markedly on conformation. Local environments may differ even for chromophores of the same type. The respective contributions of the different chromophores must also be determined.

Two approaches have been taken to make the interpretation of protein spectra a tractable problem. The first is the use of model compounds; the second is difference spectroscopy.

Much effort has been devoted to the study of small model compounds containing only a single chromophore (see below). Such spectra show no overlapping of absorptions and there are no conformation-dependent effects. Well-characterized synthetic polypeptides have been used as models for conformational changes (Glazer and Smith 1961, Glazer and Rosenheck 1962).

Difference spectroscopy has proved especially useful in determining the effect of environment on chromophore absorption. In this technique, the absorbance of a compound in a particular test state is measured relative to the absorbance of the same compound at the same concentration in a reference state; the difference spectrum thus obtained shows the difference in absorbance of the two states. The reference state may be defined arbitrarily.

If a molecule containing acidic or basic groups has different charges in the two states, it is subject to a charge perturbation; the molecule's charge can be changed by adjusting the pH of the solution. Solvent perturbations may be brought about by putting the compound in solvent systems of differing composition. (It is important to note that a small amount of perturbant may produce spectral changes without altering the protein conformation while a large amount of the same perturbant may cause denaturation.)

Four possible causes of pH difference spectra have been suggested (Leach and Scheraga 1960, Herskovits 1967, Donovan 1969). These are:

1. Vicinal charge effects, i.e., effects of charges near chromophores acting along the covalent bonds of the molecule or directly through space.
2. Hydrogen bonding.
3. Changes in polarity and polarizability of chromophore environment, perhaps involving solvent molecules.
4. Ionization of tyrosyl residues in alkaline solution.

All of these factors may be important in difference spectra due to tyrosyl residues.

Ionization of tyrosyl residues can be readily detected by spectrophotometric titration. As the phenolic chromophore is ionized, there is a pronounced shift in wavelength (from 270 to 287 nm) and a large intensification (from $\log\mathcal{E} = 3.16$ to $\log\mathcal{E} = 3.41$) (Donovan 1969).

Much work has also been done on hydrogen bonding of tyrosine and related model compounds (Grinspan, Birnbaum and Feitelson 1966; Chignell

and Gratzer 1968; Bailey et al. 1968; Strickland et al. 1972). It has been reported that hydrogen bonding of tyrosyl residues is probably responsible for the pH difference spectrum of bovine plasma albumin (Williams and Foster 1959).

Less attention, however, has been directed toward vicinal charge effects or polarity and polarizability changes. The acid versus neutral charge difference spectrum of tyrosine itself has been reported (Wetlaufer, Edsall and Hollingworth 1958; Mihalyi 1968; A'zary and Bigelow 1970), as have those for a few model compounds (Wetlaufer et al. 1958, Yanari and Bovey 1960).

Furthermore, there is little understanding of how such effects might operate. Yanari and Bovey (1960) have claimed that inductive vicinal charge effects are not sufficient to account for pH difference spectra in proteins, but they also maintain that vicinal charges may be important in specific cases. It is apparent, however, that a charge must be quite close to a chromophore if it is to affect the absorption (Wetlaufer et al. 1958; Donovan, Laskowski and Scheraga 1961). It is not known whether such effects are transmitted to the chromophore primarily by an inductive route along covalent bonds within the molecule or whether they are mediated by the solvent.

Such questions are especially important in view of the fact that Donovan et al. (1961) have reported that pH difference spectra of lysozyme are due to charges near indolyl chromophores. Andrews and Forster (1972) have studied charge difference spectra of a variety of indole derivatives. It is desirable to determine if analogous effects

are associated with the tyrosyl residues of other proteins and to make a study of model compounds containing the phenolic group.

This work is an attempt to determine charge difference spectra for model compounds containing the phenolic chromophore, to determine if possible how such effects occur and to offer generalizations that would be applicable to protein difference spectra.

CHAPTER 2

METHODS AND MATERIALS

Techniques of difference spectrophotometry are well-established. Possible sources of error are of two types: errors in preparation of solutions and instrumental errors.

Stock solutions of the model compounds used in this study were prepared by dissolving dry samples in distilled water in 500 or 1000 ml flasks; these stock solutions had concentrations around 2×10^{-3} M. Absorption spectra were taken of solutions 10-20 times more dilute; the solutions used for difference spectra were about 10^{-3} M. In all cases, the spectra were obtained the same day the solutions were made up; several of the stock solutions showed decreases in peak heights after two or more days.

A Cary 14 double beam spectrophotometer was used in all experiments. The scans were begun at 350 nm, well above the region of absorption. Difference spectra were taken with the dynode voltage setting at 2 and the slit control at 25; slit widths were below 0.8 mm until about 235 nm, when the slit began to open rapidly. The absorbances of the solutions used were around 1.5 and in no case greater than 2. Direct absorption spectra were obtained with a dynode setting of 1.

The absorbances obtained in this study are well within the limits suggested by Herskovits and Sorensen (1968) after their study of solvent

perturbations of model compounds. Using a Cary 14 instrument, they found that a plot of absorbance versus concentration will be linear below 2.2-2.5 absorbance units, i.e., ε will be independent of concentration. Photometric errors therefore are presumed to be absent. Also, Herskovits and Sorensen (1968) found that $\Delta\varepsilon$ is independent of slit width as long as the width is below 0.75 mm. Spectral bandwidth is not as critical for the accurate determination of a difference spectrum as it would be for a direct absorption spectrum (Donovan 1969); the height of a difference spectrum is determined by the slope of the absorption spectrum and at a resolution insufficient to determine the height of an absorption spectrum accurately, the absorption at the sides of the peak, and thus the difference spectrum, will be determined somewhat more accurately. Donovan (1969) estimated that slit widths three to five times greater than optimal can be used without loss of resolution of a difference spectrum.

The stock solution of each compound investigated was diluted in 0.1 M HCl and pH 7 buffer. A 5 ml pipet and 10 ml volumetric flasks were used for the preparation of the diluted solutions. Both the buffer and the acid solutions used in making the dilutions were non-absorbing over the wavelength region of interest. Reference-reference baselines were run before each difference spectrum. These were nearly flat (corrections 0.005 or less) down to 230-240 nm, indicating that the cells were well-matched. The buffer solution was used for the reference and the acid solution (about pH 1.3) was used for the sample for all compounds except p-hydroxyphenylacetic acid, for which the reverse

is true. The spectrum of this compound is shown in Figure 1 as if the neutral solution had been used as the reference, however. After determination of the baseline, the sample cell was emptied, rinsed, dried and filled with the sample solution. The difference spectrum was then obtained.

The pH 7 buffer used was pHydrion buffer manufactured by Micro Essential Laboratory, Inc. Of the model compounds studied, tyramine hydrochloride, o-hydroxyphenylacetic acid, m-hydroxyphenylacetic acid, p-hydroxyphenylacetic acid and 3-(p-hydroxyphenyl)-propionic acid were obtained from Aldrich Chemical Company. L-tyrosine ethyl ester and N-acetyl-DL-tyrosine were from Schwartz/Mann while L-tyrosine was gotten from Calbiochem. The compounds were dried before use but not further purified.

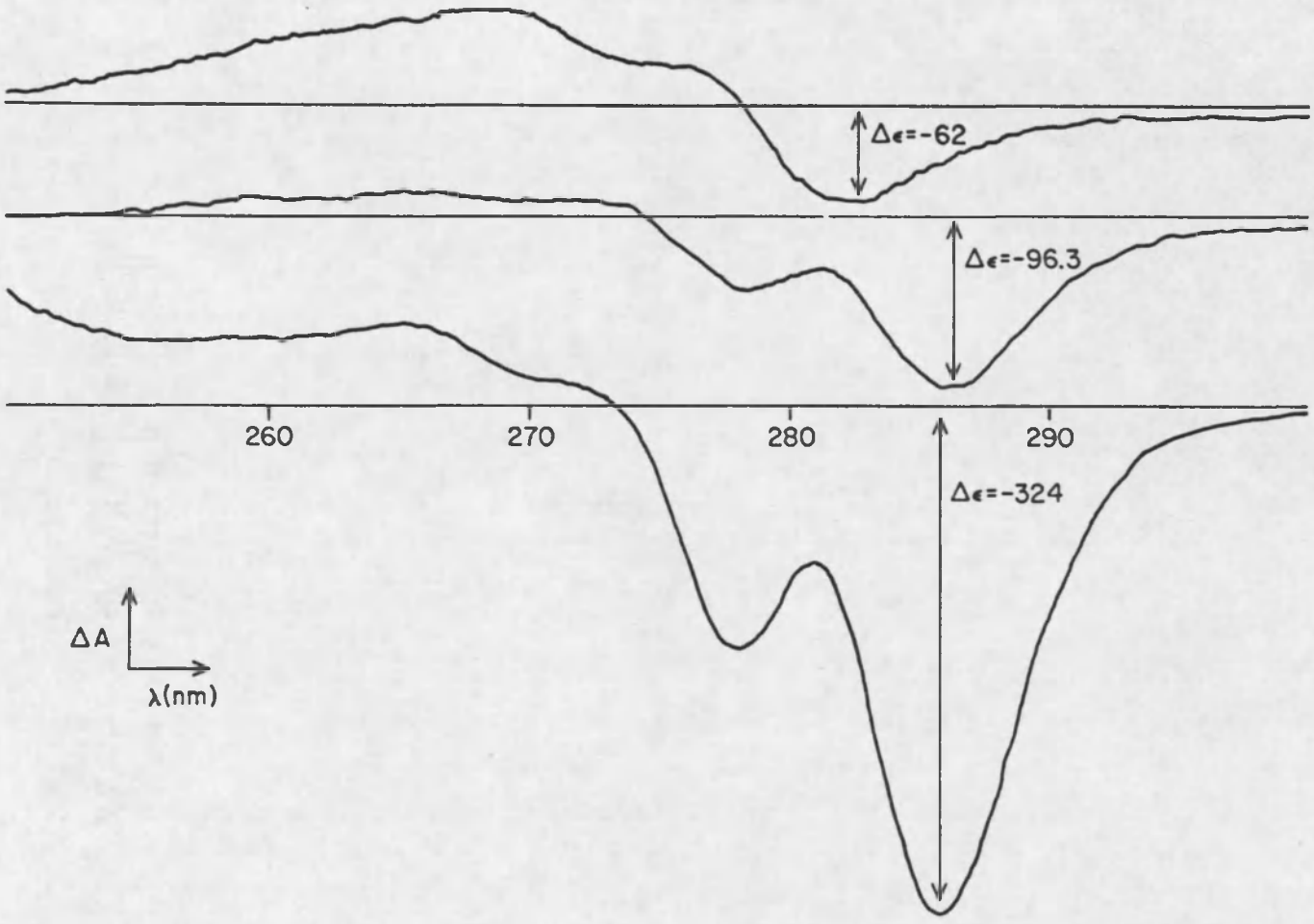

Figure 1. Acid versus Neutral Difference Spectra of (Top to Bottom) o-Hydroxyphenylacetic Acid, 3-(p-Hydroxyphenyl)-Propionic Acid and p-Hydroxyphenylacetic Acid.

CHAPTER 3

THEORY

There are three possible causes of difference spectra: spectral shifts, intensity changes and broadening or sharpening of vibrational structure.

Although changes in structure may be important in solvent-induced difference spectra (Coggeshall and Lang 1948), they will not be considered further here. Water was the only solvent used in these experiments so individual vibrational transitions were not resolved in the absorption spectra. Furthermore, it is difficult to see how a perturbing charge could affect vibrational bands differently to produce a difference spectrum.

As a first approximation, a difference spectrum is considered to result from a shift of the entire absorption spectrum. As discussed by Donovan et al. (1961), if an absorption spectrum described by $\varepsilon(\lambda)$ is shifted to a longer wavelength by an amount $\Delta\lambda$, the resulting spectrum is given by $\varepsilon(\lambda - \Delta\lambda)$ and may be expanded in a Taylor series about a wavelength λ_k

$$\varepsilon(\lambda_k - \Delta\lambda) = \varepsilon(\lambda_k) + \sum_{n=1}^{\infty} \frac{(-\Delta\lambda)^n}{n!} \left(\frac{d^n\varepsilon}{d\lambda^n}\right)_{\lambda_k} \qquad (1)$$

If only the first derivative is retained, the difference spectrum is given by

$$\Delta\varepsilon(\lambda) = \varepsilon(\lambda - \Delta\lambda) - \varepsilon(\lambda) = -\Delta\lambda\frac{d\varepsilon}{d\lambda} \qquad (2)$$

Thus the difference in extinction coefficient is directly proportional to the wavelength shift, the proportionality constant being the negative of the slope. Equation (2) will be most accurate at extrema of the difference spectrum; at such wavelengths, $\frac{d^2\varepsilon}{d\lambda^2}$ will be zero.

A bathochromic or red shift occurs when the spectrum is shifted toward longer wavelengths. If the shift is toward shorter wavelengths, it is termed a hypsochromic or blue shift.

It is of interest to note that if a symmetric absorption peak showing no structure is shifted without intensification, a difference spectrum with positive and negative lobes of the same size and shape will result.

From Tinoco and Bush (1964), the frequency shift in an electronic transition due to a static electric field is

$$\Delta\nu = \frac{1}{h}(\vec{E} \cdot \vec{\Delta\mu}) \qquad (3)$$

where $\Delta\nu$ is the frequency shift, h is Planck's constant, $\vec{E}$ is the electric field vector of the perturbing charge and $\vec{\Delta\mu}$ is the difference in the dipole moment vectors of the ground and excited states. Converting this expression to one in terms of wavelength, one obtains

$$\Delta\lambda = \frac{\lambda^2(\vec{E} \cdot \vec{\Delta\mu})}{hc} \qquad (4)$$

Thus the wavelength shift is seen to depend on the region of the spectrum where the shift occurs, the magnitude of the electric field due to the charge, the magnitude of the difference in dipole moment between the initial and final states and the relative orientation of the two vectors.

The intensity of an electronic transition may be defined in several ways, but the quantity usually discussed is the oscillator strength, which can be related directly to the experimental spectral data as well as to the computed transition dipole moment (Suzuki 1967).

The integrated intensity of an absorption band is the area under the band and may be determined from the spectrum. If the extinction coefficient is given as a function of wave number, the integrated intensity is the integral

$$A = \int_{\tilde{\nu}_1}^{\tilde{\nu}_2} \varepsilon(\tilde{\nu})\, d\tilde{\nu} \qquad (5)$$

The limits of the integration may be ambiguous if different absorption bands are not clearly separated from one another.

The oscillator strength f_{if} is proportional to the integrated intensity. In gaussian units,

$$f_{if} = \frac{10^3 (1n10) mc^2}{\pi e^2 N} \int_{\tilde{\nu}_1}^{\tilde{\nu}_2} \varepsilon(\tilde{\nu})\, d\tilde{\nu} \qquad (6)$$

Here m and e are the mass and charge of an electron, respectively, c is the speed of light and N is Avogadro's number. The transition is from state i to state f.

For a sufficiently narrow absorption band, f_{if} will be proportional to the square of the transition moment integral

$$f_{if} = \frac{8\pi^2 mc}{3h} \tilde{\nu}_{if} M_{if}^2 \tag{7}$$

M_{if}^2 is called the dipole strength and is given by

$$M_{if}^2 = \left[\int \psi_i^* \left(\sum_i \vec{r}_i - \sum_I Z_I \vec{r}_I \right) \psi_f d\tau \right]^2 \tag{8}$$

ψ_i and ψ_f are the vibronic wave functions of the initial and final states, $\vec{r}_i$ is the position vector of the ith electron and Z_I and $\vec{r}_I$ are the atomic number and position vector of the Ith nucleus.

No treatment of intensity changes due to intramolecular charge perturbations has been published. A derivation of a general expression for the dipole strength of a charge-perturbed transition is given here along with the expression for the special case of a three level system.

The potential energy of a system consisting of a charge and an electric dipole is

$$U = -\vec{p} \cdot \vec{E} \tag{9}$$

where $\vec{p}$ is the dipole moment and $\vec{E}$ the electric field due to the charge. The distance from the charge to the dipole is assumed to be much greater than the length of the dipole itself.

The perturbation Hamiltonian will be taken to be

$$H' = -\vec{p} \cdot \vec{E} = e \sum_k \vec{r}_k \cdot \frac{\hat{R}q}{R^2} \tag{10}$$

R is the distance between the charge and the dipole, $\hat{R}$ a unit vector, q the perturbing charge and e the charge of an electron. For a system of axes fixed in the chromophoric portion of the molecule, the position of the kth electron is given by $\vec{r}_k$; the sum is over all electrons of the chromophore. It is assumed that the perturbing charge is not part of the chromophore itself. In choosing this form for the Hamiltonian, the additional ssumption is made that the perturbing charge acts directly through space and not inductively along bonds.

Using first order perturbation theory for non-degenerate energy levels, the perturbation matrix elements can be written

$$H'_{ij} = (\psi_i^0|H'|\psi_j^0) = (\psi_i^0|e\sum_k \vec{r}_k|\psi_j^0) \cdot \frac{\hat{R}q}{R^2} \tag{11}$$

The wave functions ψ_i^0 and ψ_j^0 are assumed to be real and normalized. If i = j,

$$(\psi_i^0|e\sum_k \vec{r}_k|\psi_i^0) = \vec{p}_{ii} \tag{12}$$

while if $i \neq j$,

$$(\psi_i^0|e\sum_k \vec{r}_k|\psi_j^0) = \vec{M}_{ij} \tag{13}$$

The vectors $\vec{p}_{ii}$ and $\vec{M}_{ij}$ correspond to the real dipole of the molecule in state i and the transition dipole for the $j \rightarrow i$ transition, respectively.

The transition dipole for the $0 \rightarrow 1$ absorption is, in general,

$$(\psi_1^0 | e \sum_k \vec{r}_k | \psi_0^0) = \int \left[\psi_1^0 + \sum_{m \neq 1} \frac{H'_{m1}}{E_1^0 - E_m^0} \psi_m^0 \right] e \sum_k \vec{r}_k$$

$$\left[\psi_0^0 + \sum_{n \neq 0} \frac{H'_{n0}}{E_0^0 - E_n^0} \psi_n^0 \right] d\tau$$

$$= \vec{M}_{10} + \sum_{n \neq 0} \frac{H'_{n0}}{E_0^0 - E_n^0} \vec{M}_{1n} + \sum_{m \neq 1} \frac{H'_{m1}}{E_1^0 - E_m^0} \vec{M}_{m0}$$

$$+ \sum_{m \neq 1} \sum_{n \neq 0} \left(\frac{H'_{m1}}{E_1^0 - E_m^0} \right) \left(\frac{H'_{no}}{E_0^0 - E_n^0} \right) \vec{M}_{mn} \qquad (14)$$

The energy $E_i^0 (i = 0,1,m,n)$ is that of an unperturbed molecule in the ith state and the transition dipoles $\vec{M}_{10}$, etc., are those of unperturbed transitions.

Squaring this expression and keeping only the terms in q/R^2, there results

$$M^2 = M_{10}^2 + \frac{2\vec{M}_{10} \cdot \hat{Rq}}{(E_1^0 - E_0^0) R^2} \vec{M}_{10} \cdot (\vec{P}_{00} - \vec{P}_{11})$$

$$-2 \sum_{n \geq 2} \frac{\vec{M}_{n0} \cdot \hat{Rq}}{(E_n^0 - E_0^0) R^2} \vec{M}_{10} \cdot \vec{M}_{1n} - 2 \sum_{m \geq 2} \frac{\vec{M}_{m1} \cdot \hat{Rq}}{(E_m^0 - E_1^0) R^2} \vec{M}_{10} \cdot \vec{M}_{m0} \qquad (15)$$

The Hermitian property of the dipole moment operator has been used in collecting terms.

If only three energy levels are considered, the summations give simply

$$M^2 = M_{10}^2 + \frac{2\vec{M}_{10} \cdot \hat{Rq}}{(E_1^0 - E_0^0) R^2} \vec{M}_{10} \cdot (\vec{P}_{00} - \vec{P}_{11})$$

$$- \frac{2\vec{M}_{20} \cdot \hat{Rq}}{(E_2^0 - E_0^0) R^2} \vec{M}_{10} \cdot \vec{M}_{12} - \frac{2\vec{M}_{21} \cdot \hat{Rq}}{(E_2^0 - E_1^0) R^2} \vec{M}_{10} \cdot \vec{M}_{20} \qquad (16)$$

The intensity change produced by a charge is therefore seen to depend on geometrical factors, various actual and transition dipoles, the spacing of the energy levels and the sign of the charge.

CHAPTER 4

RESULTS AND DISCUSSION

The results obtained in this study are shown in Figures 1 and 2, and Tables 1, 2, and 3. The figures show the difference spectra obtained for tyrosine and for seven model compounds. The spectra have been offset for clarity and the absorbance scale is not the same for different spectra. Numerical data from the difference spectra are given in Table 3. Tables 1 and 2 give data from absorption spectra which are not reproduced here; the absorption spectrum of tyrosine closely resembles those of all the model compounds except o- and m-hydroxyphenylacetic acid.

The absorption spectra of o- and m-hydroxyphenylacetic acid are qualitatively quite different from those of the other compounds. All the other compounds have an absorption maximum at 275-276 nm and another at 221-224 nm. While the ortho- and meta-substituted compounds have maxima at 272-273 nm, they have no other maxima above 200 nm. There are shoulders at about 215 nm, but they are not prominent. These results are in accord with those of Dearden and Forbes (1959), who studied many substituted phenols and found that ortho- and meta-substituted phenols had low wavelength absorption spectra quite different from those of para isomers. In addition, the absorption of o-hydroxyphenylacetic acid at

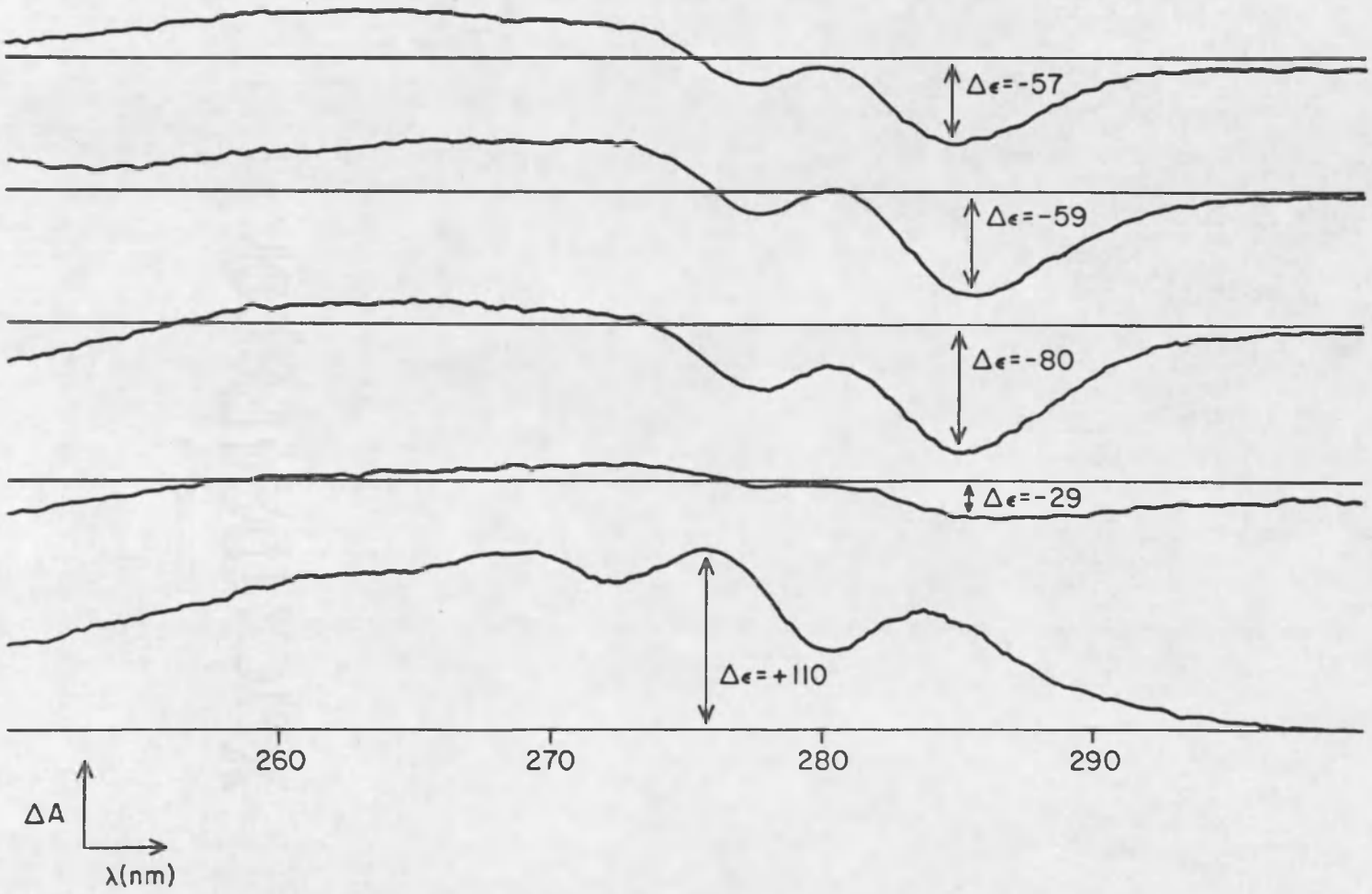

Figure 2. Acid versus Neutral Difference Spectra of (Top to Bottom) Tyrosine, N-Acetyl Tyrosine, Tyrosine Ethyl Ester, Tyramine and m-Hydroxyphenylacetic Acid.

Table 1. Absorption Maxima 200-300 nm.

Compound	λ_{max} (nm)	ε_{max} (1/mole-cm) $\times 10^{-3}$
tyrosine	275.0	1.42
	223.0	8.06
o-hydroxyphenylacetic acid	272.2	1.98
m-hydroxyphenylacetic acid	272.8	1.42
p-hydroxyphenylacetic acid	276.0	1.37
	223.6	6.76
3-(p-hydroxyphenyl)-propionic acid	275.7	1.50
	222.0	7.31
N-acetyl tyrosine	275.5	1.24
	223.0	7.76
tyramine·HCl	275.0	1.40
	221.3	7.09
tyrosine ethyl ester	275.5	1.33
	223.0	7.83

Table 2. Extrema in Difference Spectra 250-300 nm.

Compound	λ(nm)	$\Delta\varepsilon$ ($M^{-1}cm^{-1}$)	$\lvert\Delta\varepsilon/\varepsilon_{max}\rvert$
tyrosine	285.1	- 57	0.040
	277.8	- 19	0.013
tyramine·HCl	286.7	- 29*	0.021
tyrosine ethyl ester	285.1	- 80	0.060
	277.9	- 42	0.032
N-acetyl tyrosine	285.8	- 59	0.048
	277.9	- 13	0.010
o-hydroxyphenylacetic acid	282.2	- 62	0.031
	268.1	+ 58	0.024
m-hydroxyphenylacetic acid	283.8	+ 73	0.051
	275.7	+ 110	0.0775
	268.3	+ 107	0.0754
p-hydroxyphenylacetic acid	285.5	- 324	0.236
	277.7	- 161	0.118
	264.6	+ 53	0.039
3-(p-hydroxyphenyl)-propionic acid	286.2	- 96.3	0.0642
	278.3	- 41	0.027

reference at pH 7, sample at pH 1.3

* lower limit

Table 3. Absorption of Tyrosine in H_2O.

λ(nm)	ε(1/mole-cm)	λ(nm)	ε(1/mole-cm)
204	6370	275	1420
206	5300	276	1390
208	5000	278	1240
210	5060	280	1200
212	5310	282	1130
214	5690	284	910
216	6210	286	548
218	6850	288	260
220	7520	290	121
222	7970	292	19
223	8060	294	1
224	7970	296	1
226	7440	298	0
228	6370	300	0
230	4880		
232	3300		
234	1940		
236	1120		
238	631		
240	390		
242	288		
244	241		
246	251		
248	260		
250	269		
252	288		
254	306		
256	362		
258	455		
260	557		
262	659		
264	752		
266	910		
268	1020		
270	1180		
272	1320		
274	1400		

272.2 nm is far more intense than the long wavelength absorption of any of the other compounds. The extinction coefficient for the meta isomer, however, is the same as that of tyrosine and comparable to those of the other para-substituted compounds.

The difference spectra of all the para-substituted phenols (pH 1.3 versus pH 7) are quite similar in shape, showing two negative peaks to the red of the absorption maximum and a broad structureless positive peak at shorter wavelengths. The positive peaks are of much smaller area than the negative peaks. Difference spectra of o- and m-hydroxyphenylacetic acid differ in appearance from those of the para compounds and from each other. The difference spectrum of m-hydroxyphenylacetic acid shows three peaks and does not cross the baseline; o-hydroxyphenylacetic acid has both a negative and a positive peak but the positive peak has a greater area.

Difference spectra of all eight compounds have their longest wavelength extrema about 10 nm to the red of the absorption maxima. For the para compounds, these extrema occur at 285-287 nm; o- and m-hydroxyphenylacetic acid have extrema at slightly shorter wavelengths.

There are wide differences in the magnitudes of the difference spectra as measured by $|\Delta\varepsilon/\varepsilon_{max}|$. Values of $|\Delta\varepsilon/\varepsilon_{max}|$ vary from 0.236 for p-hydroxyphenylacetic acid to less than $\frac{1}{7}$ this number. The value for p-hydroxyphenylacetic acid is much higher than that for any of the other compounds studied; the next largest value (for m-hydroxyphenylacetic acid) is only about one-third that of the para isomer.

In consideration of pH difference spectra, it has often been agreed that a perturbing charge must be quite close to a chromophore--within only a few bond lengths--to produce a measureable difference spectrum.

The effect of the separation of the perturbing charge and the tyrosyl chromophore has been considered previously by Wetlaufer et al. (1958). They found that the difference spectrum due to the ionization of the amino group in glycyl-O-methyltyrosine (in which the amino group is three atoms removed from the α carbon)is barely detectable, while for the comparable difference spectrum of O-methyltyrosine $\Delta\varepsilon \sim 230$ 1/mole-cm. These workers predicted that a charge will have no effect on a chromophore unless they are closer than ~ 10 Å apart.

In this study the effect of distance is shown in the difference spectra of p-hydroxyphenylacetic acid and 3-(p-hydroxyphenyl)-propionic acid. Although the spectra are very similar in shape (see Figure 1), the $\Delta\varepsilon$ for p-hydroxyphenylacetic acid is more than three times greater than that for 3-(p-hydroxyphenyl)-propionic acid. The increase in charge-chromophore distance with chain length can be seen by comparing the space-filling molecular models shown in Figures 3 and 4. While 3-(p-hydroxyphenyl)-propionic acid does have certain conformations in which the charge is closer to the chromophore than in the model shown in Figure 4, these conformations are sterically somewhat crowded.

The amino group of tyramine is very close to the phenolic ring--no more than one van der Waals radius away as illustrated in Figure 5. The difference spectrum of tyramine is very small but this can be

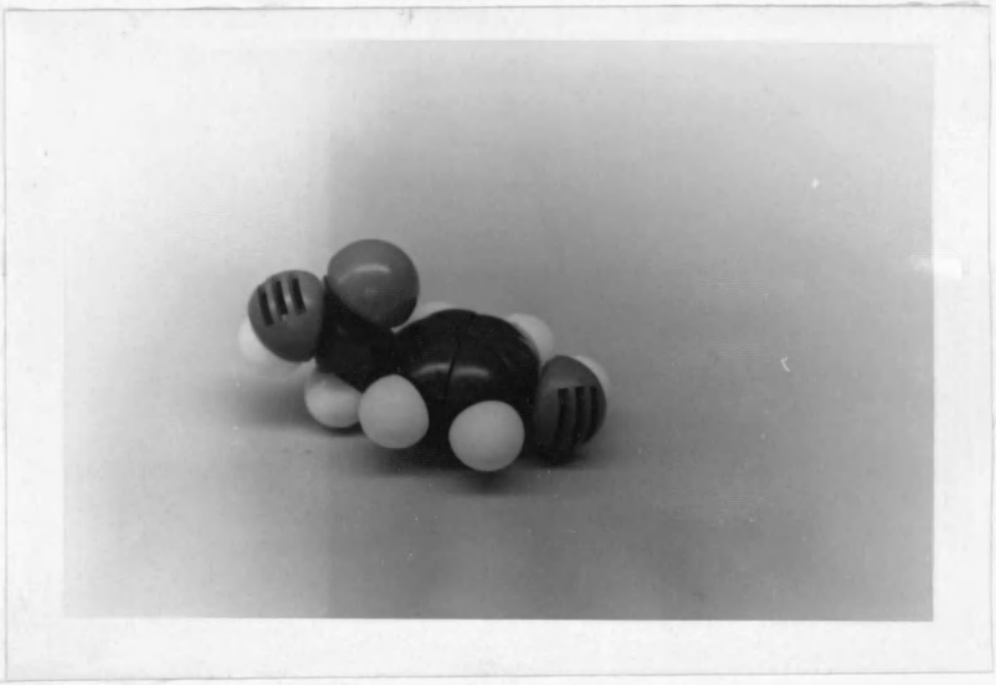

Figure 3. Molecular Model of p-Hydroxyphenylacetic Acid Showing Typical Conformation.

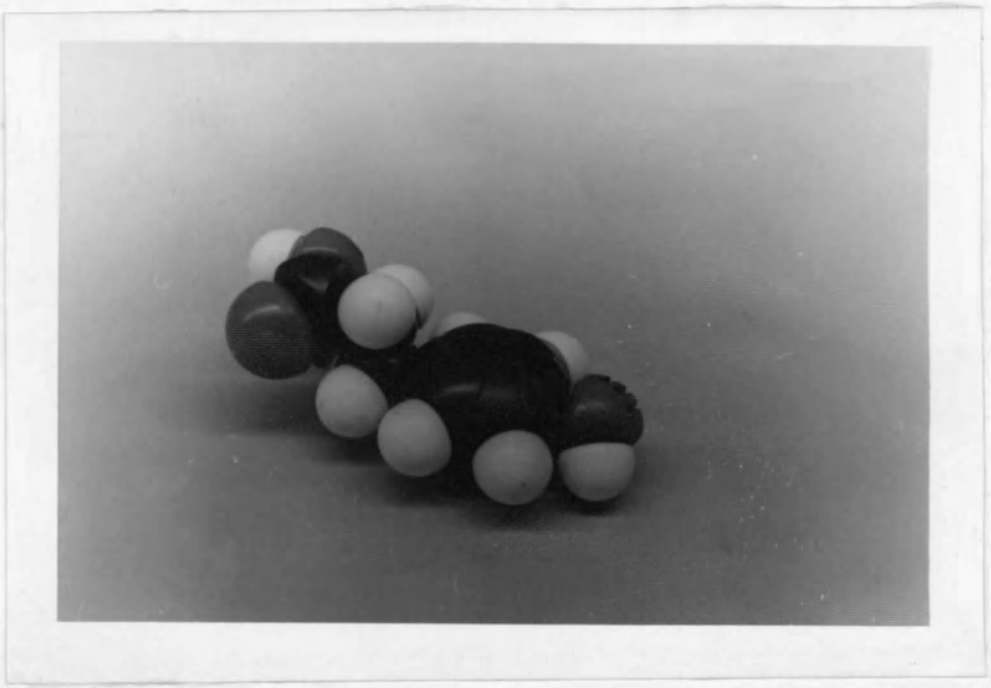

Figure 4. Molecular Model of 3-(p-Hydroxyphenyl)-Propionic Acid Showing Unstrained Conformation.

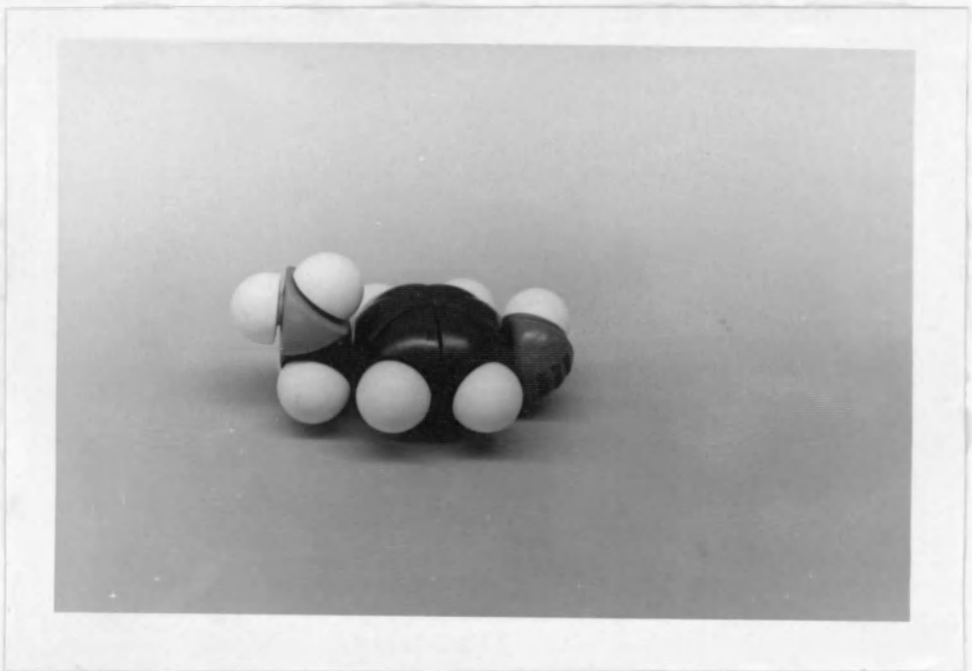

Figure 5. Molecular Model of Tyramine Showing Typical Conformation.

attributed to the fact that at pH 7 nearly all the tyramine molecules will already have been protonated; the $\Delta\varepsilon$ value determined for this compound is therefore a lower limit. The actual value cannot be determined since at pH values sufficiently high for the amino group to be uncharged, the phenolate ion will be formed and its spectrum will obscure that of the amino group (Wetlaufer et al. 1958). The small observed spectrum may be due to differences in ionic strength between the sample and the reference. Such an effect presumably would be the same for all of the difference spectra obtained so the result for tyramine gives an upper limit to the possible error in the spectra due to the presence of salts.

It has been noted several times that inorganic salts can produce difference spectra when added to solutions of phenolic compounds (Wetlaufer et al. 1958, Bigelow and Geschwind 1960, A'zary and Bigelow 1970). In particular, A'zary and Bigelow (1970) found that concentrated HCl causes an increase in $\Delta\varepsilon$ for the ^1L_b band of tyrosine after protonation of the carboxylate group is complete; the ^1L_a band is unaffected. Data of these workers suggests that such an effect occurs below about pH 2.

In N-acetyl tyrosine and 3-(p-hydroxyphenyl)-propionic acid, there are three carbon atoms between the aromatic ring and the oxygen atoms of the carboxyl group. Steric strain is important in many conformations of N-acetyl tyrosine; in the less strained conformations, the carboxyl group is quite far from the chromophore (see Figure 6). Crowding is less serious for the simpler, less bulky substituent of 3-(p-hydroxyphenyl)-propionic acid, and the carboxyl group of this

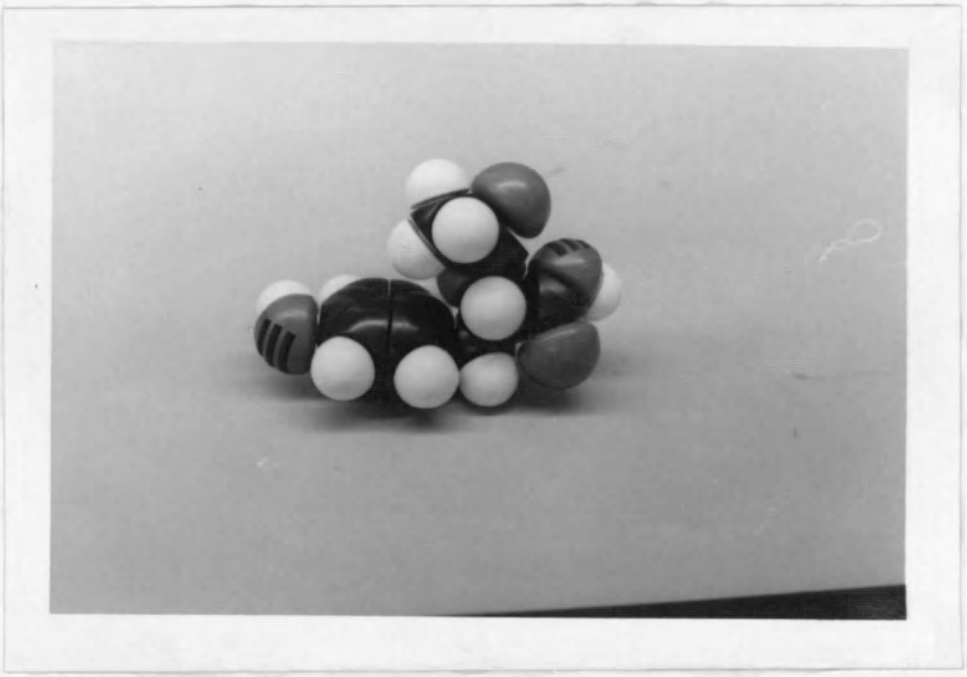

Figure 6. Molecular Model of N-Acetyl Tyrosine Showing Unstrained Conformation.

molecule would be expected, on the average, to be somewhat closer to the phenolic chromophore than the carboxyl group of N-acetyl tyrosine. This interpretation is in agreement with the difference spectra; $\Delta\varepsilon$ is -59 for N-acetyl tyrosine and -96.3 for 3-(p-hydroxyphenyl)-propionic acid.

For tyrosine, tyrosine ethyl ester and p-hydroxyphenylacetic acid, there are two carbon atoms between the aromatic ring and the charged atoms. When the three molecules are in conformations such that the charge-chromophore distance is a maximum, the amino groups are somewhat further from the chromophore than is the carboxyl group of p-hydroxyphenylacetic acid (see Figures 3, 7 and 8). On the other hand, the charge of a carboxylate group will be divided between the two oxygen atoms (accordingly, the charge may not behave as a point charge, especially close to the chromophore, so Equation (16) may not be adequate), and the oxygen atoms of p-hydroxyphenylacetate cannot simultaneously be brought as close to the chromophore as the nitrogen atom of tyrosine or tyrosine ethyl ester.

Data for the three compounds indicates that an inductive effect cannot be the principal cause of these difference spectra. An inductive effect would depend only on the number of carbon atoms between the charge and the chromophore, so if the perturbation acted inductively, $|\Delta\varepsilon|$ for the three compounds should be the same. For p-hydroxyphenylacetic acid, however, it is more than four times greater than that for either tyrosine or tyrosine ethyl ester. Charge difference spectra of phenols therefore are probably primarily due to interactions directly through space.

These results for phenols may be compared with those of Donovan et al. (1961) and Andrews and Forster (1972) for indole derivatives.

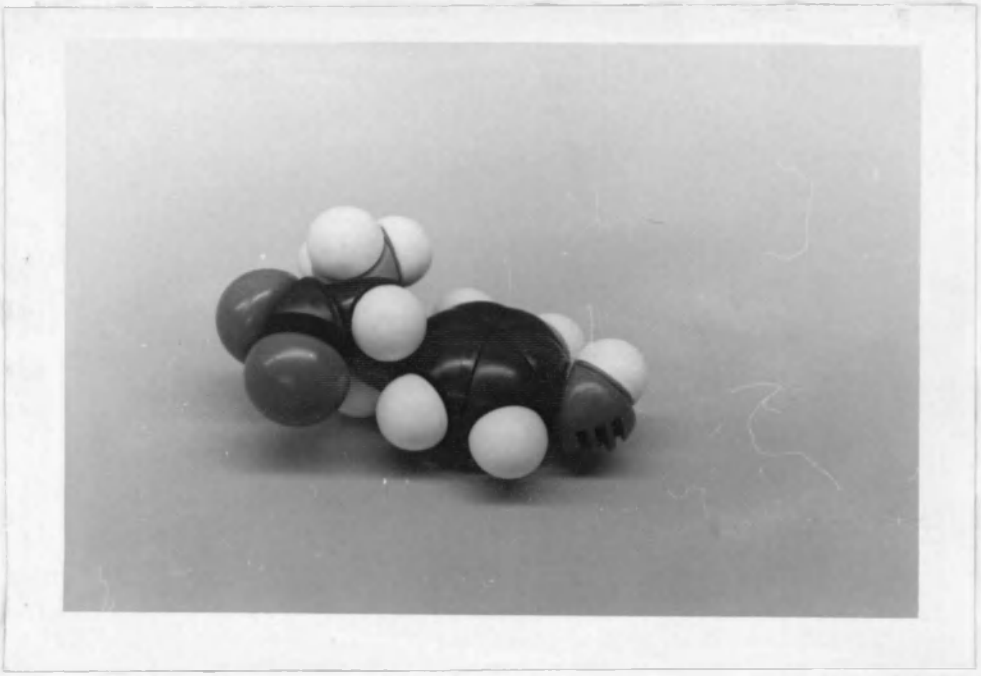

Figure 7. Molecular Model of Tyrosine Showing Maximum Charge-Chromophore Distance.

Figure 8. Molecular Model of Tyrosine Ethyl Ester Showing Maximum Charge-Chromophore Distance.

Donovan et al. (1961) compared the amino group ionizations of glycyl-tryptophan and tryptophan. In tryptophan there are two atoms separating the amino group from the indolyl chromophore; in glycyltryptophan there are five. These workers found that $\Delta\varepsilon$ is ten times less in glycyltryptophan than in tryptophan. Andrews and Forster (1972) studied 3-indolebutyric acid, 3-indolepropionic acid and 3-indoleacetic acid. The $\Delta\varepsilon$ values for these compounds were approximately in the ratio 1:3:8.

It is clear that both for phenol and indole derivatives there is a pronounced decrease in the magnitude of charge difference spectra as the distance between the charge and the chromophore is increased. The actual size of the decrease, however, varies widely among different model compounds.

As mentioned above, the absorption spectra of o- and m-hydroxyphenylacetic acid have a close resemblance but both differ from the spectrum of the para isomer. The difference spectra of the three isomers, however, are all different. As would be expected, the substituents of the ortho isomer are in close proximity (see Figure 9). Specific interactions such as intramolecular hydrogen bonding are therefore possible if the substituents are ortho. Space-filling molecular models reveal, however, that in the meta isomer the substituents cannot approach closer than 2-3 van der Waals radii (see Figure 10); interactions between substituent groups are unlikely at such distances.

All the para compounds investigated have difference spectra (acid versus neutral) in which the negative lobe is substantially larger than the positive lobe. The spectrum of o-hydroxyphenylacetic acid has a

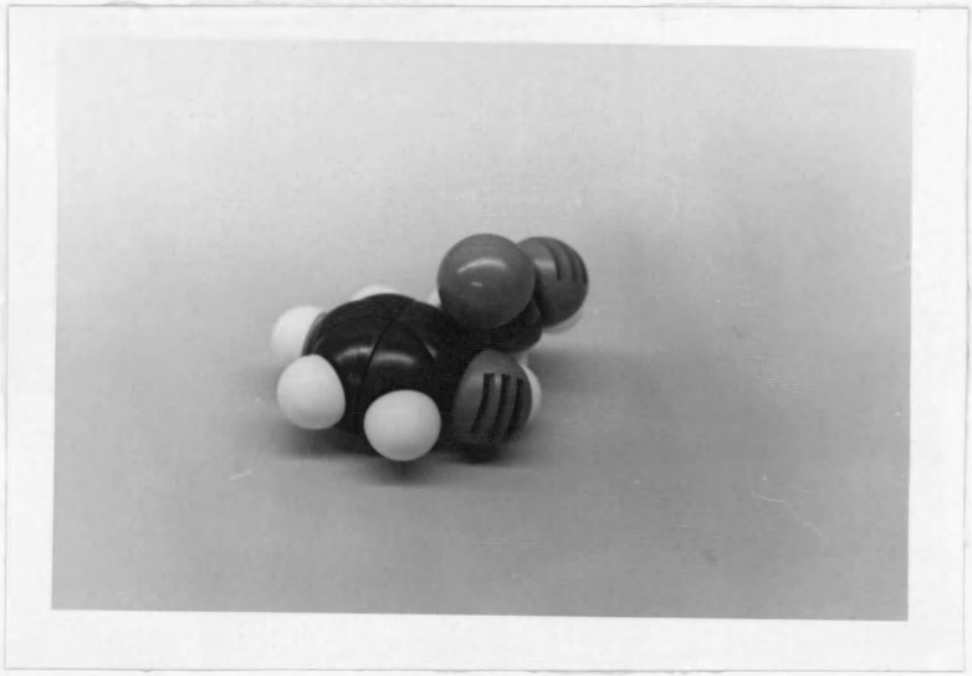

Figure 9. Molecular Model of o-Hydroxyphenylacetic Acid Showing Typical Conformation.

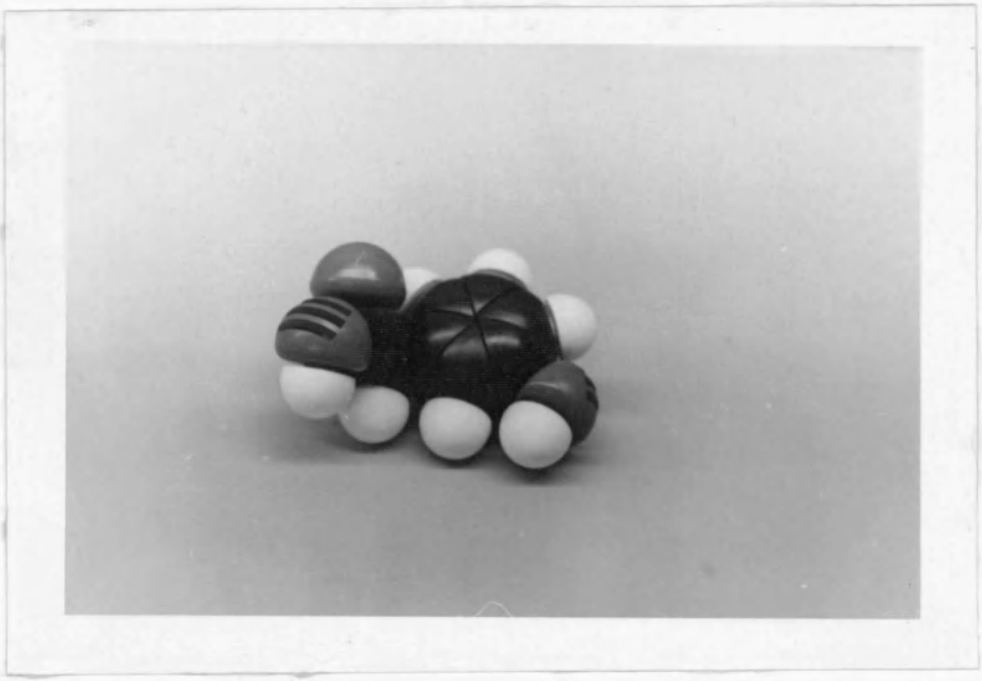

Figure 10. Molecular Model of m-Hydroxyphenylacetic Acid Showing Closest Approach of Substituents.

positive lobe that is much larger than the negative lobe, and the spectrum of m-hydroxyphenylacetic acid does not cross the baseline at all.

If the difference spectra were due solely to a wavelength shift of the longer wavelength $^1A \rightarrow {}^1L_b$ band, they would show positive and negative lobes of approximately equal size. (The compounds used have a slight shoulder on the long wavelength side of the absorption maximum and quite a long tail on the short wavelength side, so they would not be expected to have symmetric difference spectra.)

For indole derivatives, it is possible to explain difference spectra with one lobe much larger than the other with the observations (Andrews and Forster 1972) that the ^1L_a and ^1L_b states are close together and that charge perturbations can shift the two transitions in opposite directions; the transitions may overlap in such a way that one of the lobes is obscured. Such an explanation will not apply to phenols, however, since the ^1L_a and ^1L_b bands are separated by about 50 nm; the $^1A \rightarrow {}^1L_a$ band can have only a negligible effect on the longer wavelength $^1A \rightarrow {}^1L_b$ transition.

Thus the difference spectra of the compounds studied must be due in large part to intensity changes. Shifts also occur, but they cannot account for all the features of the spectra. (See Appendix for a discussion of attempts to perform a gaussian analysis on the spectra.)

It has been shown (see Theory) that if the correction terms with a $1/R^2$ dependence are large enough, there is a substantial change in the dipole strength. Intensity changes are therefore a plausible cause of difference spectra.

As the data for the model compounds shows, the effect of a perturbing charge depends on the orientation of the substituents. For para isomers, a negative charge causes an intensity increase and a positive charge causes an intensity decrease. For ortho isomers, it appears that perturbation by a negative charge leads to intensification while for meta isomers a negative charge seems to lead to an intensity decrease.

Both tyrosyl and tryptophyl residues can give rise to protein difference spectra in the near ultraviolet. It is of interest, therefore, to compare the general features of the spectra of appropriate phenols and indoles.

Absorption spectra of indole and phenol derivatives differ in an important way. While for phenols the two transitions seen in the ultraviolet spectrum are always well separated, they overlap in certain indoles. Furthermore, the superimposed indole transitions respond differently to environmental influences (Andrews and Forster 1972). Solvent perturbations appear always to shift the ^1L_a and ^1L_b bands in the same direction. In certain model compounds, however, charge perturbations shift the two bands in opposite directions. Intensity changes play only a minor role in the difference spectra of most indoles. Charge difference spectra of phenols and indoles, therefore, are due to different causes.

In most of the model compounds that have been studied so far, $\Delta\varepsilon$ values for phenols are substantially less than those for indoles. It therefore appears probable that tryptophyl residues will make considerably more contribution to charge difference spectra in proteins than will tyrosyl residues.

The relative importance of the correction terms in Equation (16) can be partially assessed using data available in the literature. Berndt and Kwiatkowski (1970) have given the angles of polarization for the electronic transitions of phenol. The actual symmetry of phenol is only C_1; the effective symmetry, however, is usually considered to be C_{2v}. Only if the bonds to the oxygen atom made a 180° angle could the molecule have a C_2 axis and two symmetry planes. Since the hydroxyl group is freely rotating, the phenol molecule is regarded as belonging to the C_{2v} point group. This assumption has also been made for tyrosine and p-cresol (Horwitz, Strickland and Billups 1970; Sponer 1942) and is consistent with the vibronic spectra of these compounds. Considering the symmetry to be C_{2v} and defining the z axis as the axis through the oxygen atom and C_1 and C_4, the y axis as the other axis in the plane of the aromatic ring and the x axis as the axis perpendicular to the plane, the $0 \rightarrow 1$ transition is y-polarized and the $0 \rightarrow 2$ transition is z-polarized. The transition moments $\vec{M}_{10}$ and $\vec{M}_{20}$ therefore are perpendicular and the final correction term vanishes. Assuming that the ground state wave function ψ_0 transforms as the totally symmetric A_1 representation and considering the direct product representations of the integrands of the transition moments, ψ_1 is found to transform as B_2 and ψ_2 to transform as A_1; $\vec{M}_{12}$ will be y-polarized. The second correction term will then be expected to be large since $\vec{M}_{10}$ and $\vec{M}_{12}$ will be parallel. Information in the literature about permanent dipole moments for excited states of phenols is scanty, but Lombardi (1969) has determined that the dipole moment in the first excited state of phenol differs only slightly from that of the

the ground state but is probably increased. In the $0 \rightarrow 1$ transition, the phenol molecule contracts along the z axis; the excited state apparently has appreciable quinoid character. For a charge para to the hydroxy group, $\hat{R}$ will be along the y axis and $\vec{M}_{10} \cdot \hat{R}$ will be zero. Only the second correction term will be important for charge-perturbed para-substituted phenols; the difference in permanent dipole moment of the ground and excited states has no effect.

This analysis will apply only to para-substituted phenols; those with substituents ortho or meta to the hydroxyl group will have only C_s symmetry. Intensification does not appear to be very important in the difference spectrum of o-hydroxyphenylacetic acid so phenols with ortho substituents will not be discussed further. The spectrum of m-hydroxyphenylacetic acid, however, does not cross the baseline at all. A change in intensity must be the primary cause of this difference spectrum. Assuming that the transition dipole moments have the same orientations regardless of substitution on the phenolic ring, group theoretical arguments analogous to those above show that ψ_0, ψ_1 and ψ_2 all transform as A' for phenols of C_s symmetry. The last correction term in Equation (16) will vanish since $\vec{M}_{10}$ and $\vec{M}_{20}$ will be perpendicular. The vectors $\hat{R}$ and $\vec{M}_{12}$ will each have components along both axes in the plane of the aromatic ring, so the second correction term may be large. The importance of the first correction term cannot be determined without information about permanent dipole moments of substituted phenols, which is unavailable.

For indole, the transition moments of the $^1A \rightarrow {}^1L_b$ and $^1A \rightarrow {}^1L_a$ transitions make a 92^o angle (Yamamoto and Tanaka 1972). The final correction term in Equation (16) will then be very small. Indole is of low symmetry and neither the $\vec{M}_{10}$ nor the $\vec{M}_{20}$ transition moment is along any of the axes of the molecule so the second correction term may be presumed to be quite important. For indoles, in contrast to the situation for phenols, the first correction term is likely to be important. Although information on the orientation of indole permanent dipole moments is lacking, the magnitude of the difference in dipole moment between the excited and ground states has been reported to be ~5 D (Mataga, Torihashi and Ezumi 1964); unless the dot product is small, this term is likely to make a large contribution. Permanent dipole moments appear, therefore, to be more important in the intensification of charge-perturbed spectra of indoles than of phenols.

CHAPTER 5

SUMMARY

Charge difference spectra of tyrosine and seven model compounds were studied. All the para isomers investigated have difference spectra that are very similar in shape although they differ greatly in magnitude. The difference spectra of o-, m- and p-hydroxyphenylacetic acid are qualitatively very different, however. In all the difference spectra, one lobe is larger than the other; for m-hydroxyphenylacetic acid the spectrum does not cross the baseline at all. Such effects can only be due to changes in the intensity of absorption. Evidence was obtained which suggests that interactions through space, rather than inductive interactions, are the principal cause of these difference spectra. An expression for the change in dipole strength when a transition is perturbed by a charge was derived using perturbation theory. Examination of this equation shows that the change in permanent dipole moment upon excitation appears to be of minor importance in the intensification of spectra of para-substituted phenol derivatives; such changes are likely to be much more significant for indoles. Transition dipole terms will be important in the intensification of spectra of both types of compound. Charge difference spectra are generally smaller for phenol than for indole derivatives so it is likely that tryptophan will be more important than tyrosine in protein pH difference spectra.

APPENDIX

ATTEMPTED GAUSSIAN ANALYSIS OF THE SPECTRA

It is desirable to perform a gaussian analysis on a difference spectrum to determine the wavelength shift and intensification as quantitatively as possible. In this study, analysis of the difference spectra was attempted both by means of a digital computer program and a Dupont 310 curve resolver.

The computer program will fit up to ten gaussian peaks to a spectrum. It requires as input parameters the estimated positions, heights and widths at half-height of the number of gaussian peaks to be used in the fitting as well as the observed heights at regular wavelength intervals. The program then adjusts the estimated gaussian curves so that their sum fits the observed spectrum to a specified precision.

The program successfully fit an absorption spectrum with three gaussian peaks but not with four or five. With more than three peaks, the program shifted intensity away from the center of the spectrum to the wings; when too much intensity built up near the extremes of the absorption band, the program inverted peaks of its own accord.

In view of the progression of vibronic peaks in the low temperature spectra of tyrosine and other para-substituted phenols (Horwitz et al. 1970), fitting an absorption spectrum with only three gaussian curves is not physically realistic.

In principle, once the absorption spectrum of a compound has been fitted, the resulting gaussian peaks can all be shifted in wavelength, inverted and combined with the unshifted corrected peaks to give input parameters for the fitting of a difference spectrum.

When the program fitted a difference spectrum with three inverted and three normal peaks, it shifted two of the inverted curves to longer wavelengths and the other to shorter wavelengths. The program evidently lacks sufficient constraints to ensure that curve fitting of a difference spectrum gives reasonable results. Use of a computer was abandoned.

Use of a curve resolver also proved unfeasable. The difference spectra to be fitted were of small magnitude, and absorption spectra at the same concentrations had absorbances so large that the instrument's system of mirrors could not display the entire absorption spectrum at one time. The instrument also had a serious instability; even over a period of a few minutes, curves often did not retain their original gaussian shape. Furthermore, it was impossible to get a flat baseline with the large peaks required; the deviations from flatness in the baselines were nearly as large as the difference spectra themselves and the spectra could not be accurately fitted.

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