Ultraviolet Difference Spectra of Tyrosine Groups
in Proteins and Amino Acids*†

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The ultraviolet absorption of protein solutions at wave lengths greater than 2500 Å is due chiefly to the aromatic side chains of tyrosine, phenylalanine, and tryptophan (1, 2). When a protein is titrated with alkali, a new, stronger absorption band appears with a maximum at about 2930 Å, because of the ionization of tyrosyl residues. This marked change in absorption has been used to estimate the tyrosine and tryptophan content of proteins (1, 3) and as a direct means for measuring the acid strength of tyrosyl groups in proteins (4–7).

On close comparison of spectra of proteins and their constituent amino acids at neutral pH, they are found to be not quite identical. The observation that in proteins the absorption bands of the aromatic components are displaced slightly to the red as compared to the amino acids was made over 30 years ago by Stenström and Reinhard (8), and has been amply confirmed (4, 5, 9, 10). Similar small spectral shifts occur in proteins under conditions where no phenolic ionization would be expected; when, for example, the protein is titrated with acid (5, 11), or subjected to enzymic hydrolysis (11–13), or denatured with urea or guanidine salts at neutral pH (12–14).

If we consider the molar extinction coefficients, \( \epsilon_t \) and \( \epsilon_a \), of a given molecule in two different states, 1 and 2, then the difference spectrum is obtained by plotting \( \Delta \epsilon = \epsilon_2 - \epsilon_1 \), as a function of wave length. The representation of spectral shifts, such as those discussed above, as difference spectra, was introduced by Laskowski et al. (11). The same authors have proposed the hypothesis that these small displacements of the spectra reflect changes in hydrogen bonding of tyrosyl groups, and this view has been widely accepted (12, 15–18).

We have observed, however, that similar displacements of the spectra of tyrosine and some of its simple derivatives accompany the ionization of other groups adjoining the benzene ring, without ionization of the phenolic group. For steric reasons hydrogen bonding between the phenolic group and the other ionizing group is impossible in the cases we have studied; yet the difference spectra are very similar, both in position and in magnitude, to those found in proteins such as insulin and ribonuclease. The addition of high concentrations of such reagents as urea or acetate ion also gives rise to similar difference spectra.

**EXPERIMENTAL**

**Materials**

**L-Tyrosine**—Chromatographically pure L-tyrosine (Mann Research Laboratories, Inc.) was recrystallized twice from water, and dried over \( \text{P}_2\text{O}_5 \) in a vacuum. Kjeldahl N was 7.74 per cent, theoretical, 7.73 per cent. At pH 6, \( \lambda_{\text{max}} = 2745 \) Å, \( \epsilon_{\text{max}} = 1400 \); at pH 12, \( \lambda_{\text{max}} = 2930 \) Å, \( \epsilon_{\text{max}} = 2400 \).

**O-Methyl-L-tyrosine** (p-methoxyphenylalanine)—This was a gift from Professor H. T. Clarke (19). The product as received was twice recrystallized from water and dried in a vacuum over \( \text{P}_2\text{O}_5 \). Evidence of purity was obtained from the pH titration; on titration with alkali a constant value of \( \text{pK}_a' = 9.27, \nu = -0.018 \) (at 25° and ionic strength 0.16) was obtained over a pH range from 8.4 to 9.7. The product was examined by paper chromatography with a butanol-acetic acid-water solvent system. It showed but a single ninhydrin-positive spot, \( R_f = 0.48-0.62 \). From tyrosine controls (20) it was further demonstrated that as little as 1 per cent tyrosine impurity in the O-methyltyrosine could have been detected.

No increase in absorbancy at 2950 Å was observable in a 0.001 m solution at pH 12 compared to pH 7, by which a tyrosine impurity of 0.3 per cent should be detectable. At pH 6, \( \lambda_{\text{max}} = 2740 \) Å, \( \epsilon_{\text{max}} = 1380 \); at pH 11.6, \( \lambda_{\text{max}} = 2750 \) Å, \( \epsilon_{\text{max}} = 1430 \).

**Chloroacetyl-L-tyrosine**—L-Tyrosine was suspended in ethyl acetate and refluxed with excess chloroacetyl chloride under anhydrous conditions, according to the procedure of Ronwin (20). After filtering off unchanged tyrosine, the solvent was evaporated, yielding a light yellow oil which crystallized on rubbing. After recrystallization from acetone-chloroform, the yield was 50 per cent, and the m.p. 152–155°. In the literature (20) the m.p. is 155–156° corrected.

**Glycyl-O-methyl-L-tyrosine**—Chloroacetyl-L-tyrosine was methylated with dimethyl sulfate in alkaline aqueous solution at room temperature with constant stirring and with addition of sodium hydroxide solution dropwise to maintain pH >10. After two hours the mixture was acidified and allowed to stand overnight. The acidified solution was extracted with ethyl acetate, the organic phase dried over anhydrous sodium sulfate and the ethyl acetate evaporated to yield an oil which crystallized...
in part. This material was aminated by treatment with con-
centrated ammonium hydroxide at room temperature. After
two days a clear solution was obtained, which was evaporated
to dryness in a water bath at 50°. The solid was extracted with
two days a clear solution was obtained, which was evaporated
to dryness in a water bath at 50°. The solid was extracted with

time. The yield, after two recrystallizations, was 32 per cent of theory,
based on the chloroacetyl-L-tyrosine taken.

Analysis
C₇H₁₄NO₆
Calculated: C, 57.1; H, 6.40; N, 11.10
Found. C, 50.7, H, 0.50, N, 11.04

At pH 6, λₘₐₓ = 2745, εₘₐₓ = 1400; at pH 12, λₘₐₓ = 2745,
εₘₐₓ = 1390. By potentiometric titration the value of pKₐ
for the ammonium group (at 25° and ionic strength 0.16) was
found to be constant (8.15, σ = ±0.015) over a pH range from
7.5 to 9.3. This indicates the presence of no more than 0.3 per
cent inert impurity. A very slight increase in the absorbancy
of a 0.001 M solution was observed (Fig. 8) at 2950 A at pH 12,
compared to pH 6. If this were assumed to be due to glycyl-
trypsin, it would correspond to a content of less than 0.3 per
cent of the latter. Such a degree of impurity would affect
neither the experimental results reported here, nor the conclu-
sions drawn therefrom; and the small observed change may in
fact be experimental error.

Urea—Eastman Kodak purified urea was used. It was
twice recrystallized from ethanol-water at -8 to -10° according
to Steinhardt (21).

Sodium acetate—Fisher Certified Reagent was used.

Water—The water used was either redistilled from glass or
dionized in a mixed bed ion exchange column.

All other materials were of reagent grade or better.

Methods
Spectroscopic measurements were made at 25° with a Beckman
model DU spectrophotometer equipped with thermostapsers in
10 mm. cells which had been previously calibrated. The wave
length scale on the spectrophotometer was checked periodically
with a mercury vapor lamp, and the nominal wave lengths are
valid to within ±1 A. The instrument was routinely operated at
a monochromatic slit-width of 0.10 mm. Wave lengths are
specified in this work with an estimated accuracy of ±0.5 A
(maximum deviation). For these measurements recommenda-
tions for precision spectrophotometry were followed (22).

Although we have reported many of our data as difference
spectra, the measurements were not made by the technique of
"differential spectrophotometry" (23). Absorbancies were
measured in the conventional way against a blank cuvette
containing solvent only, and the resulting spectra were used to
compare one solution with another, and thus to compute a
difference spectrum. When we speak of the difference spectrum
of state 2 versus state 1, or of state 2 referred to state 1, we mean:
Δε = ε₂ - ε₁. It is of course essential that in spectral regions
where Δε/Δλ is large, absorbancy readings be made at a single
setting of the wave length scale; otherwise, large errors can result
from the failure to duplicate a previous setting exactly. This is a
more tedious way of obtaining difference spectra than by the
technique of Bastian et al. (23), but it has the advantage of
reducing the effective bandwidth of light obtained from the
monochromator. Since this method also yields complete spectra,
it is easy to detect impurities in samples.

Aqueous solutions for spectrophotometric observation were
prepared in buffers with KCl added to a final ionic strength of
0.10. For the most acid solutions unbuffered HCl was used,
and for the other pH ranges, acetate, tris(hydroxymethyl)-
aminomethane, glycinate, and ε-amino-caproate buffers were
employed at buffer-ion concentrations ranging from 0.02 to
0.10 M. We have found no evidence for specific buffer effects
on the spectra of tyrosine or O-methyltyrosine at these low
concentrations. The concentrated sodium acetate solutions
were adjusted from pH >8 (hydrolysis) to pH 7.0 with glacial
acetic acid.

Measurements of pH were carried out at 25° with a Beckman
model G pH meter, calibrated with standard buffers prepared
according to the specifications of the National Bureau of Stan-
dards.

RESULTS
In Fig. 1, it is seen that tyrosine and O-methyltyrosine have
nearly congruent spectra. Figs. 2, 3, and 4, upper, show the
perturbation of the spectra of both tyrosine and O-methyl-
tryosine associated with the ionization of a carboxyl and an
amino group. Since the amino and phenolic groups in tyrosine
ionize in the same pH range, the small effect of the ammonium
group ionization is largely obscured by the red shift and intensifi-
cation of absorption which occurs when the phenolic group
ionizes (λₘₐₓ shifts from 2745 to 2930 and ε shifts from 1400
to 2400 at the respective maxima). This is seen in Fig. 4, upper,
in the difference spectra of O-methyltyrosine. The difference
spectra due to the ionization of the carboxyl group are seen to
be practically the same for tyrosine (Fig. 4, upper) as for O-
methyltyrosine (Fig. 3).

We have calculated the apparent ionization constant of the
ammonium group of O-methyltyrosine from the difference
spectrum at pH 9.65, assuming that the spectra of the two ex-
treme forms, the isoelectric amino acid and the anion, are
obtained at pH 5.7 and at pH 11.5, respectively. The equation
employed was pK' = pHH - log [α/(1 - α)], where α = (Δε/
FIG. 2. Spectra of aqueous solutions of O-methyl tyrosine at various pH values. At pH 1.08, O; at pH 5.7, Δ; at pH 9.6, □; at pH 11.5, ●.

$\Delta_{\text{max}} \lambda$. The mean result from calculations at nine wave lengths between 2810 A and 2890 A is $pK_2' = 9.31 \pm 0.05$, in good agreement with titration results which yield $pK_2' = 9.27 \pm 0.02$. These results clearly indicate that this difference spectrum is due to the ammonium ionization, and not due to an impurity or an artifact.

Difference spectra of O-methyltyrosine at pH 11.4 versus pH 5.7 were obtained at $10^{-3}$ M and at $4 \times 10^{-4}$ M. The results (Fig. 5) show that this molar difference spectrum is concentration-independent, and therefore not due to intermolecular interactions, nor to instrumental artifacts of the sort described by Fridovich et al. (24). The concentration dependence of difference spectra arising from carboxyl group ionization in tyrosine and glycyl-O-methyltyrosine was also tested in this way; in no case was a concentration dependence found.

Concentrated solutions of urea (Fig. 6) and sodium acetate (Fig. 7) also give rise to difference spectra in tyrosine. The effects are seen to be qualitatively the same for tyrosine as for its O-methyl derivative, but with some quantitative differences. That the result with sodium acetate is not simply a general electrolyte effect is shown by the difference spectra obtained with 3 M NaCl, (Fig. 4, lower). The curve in Fig. 4, lower, showing the difference spectrum of isoelectric tyrosine in 3 M NaCl versus aqueous isoelectric tyrosine is seen to correspond to a small general spectral intensification, and is itself of a very different form from that resulting from addition of sodium acetate or the ionization of adjacent groups. This result is confirmed by the other curve of Fig. 4, lower, which shows that acid tyrosine referred to isoelectric tyrosine, with 3 M NaCl in both tyrosine solutions, gives rise to a difference spectrum very similar to that found at low ionic strength, the peaks being slightly higher at the high salt concentration.

Spectra of glycyl O-methyltyrosine were also determined at various pH values (Fig. 8). Here the increment in $\Delta \varepsilon$ on carboxyl ionization is, within experimental error, the same as that produced by the carboxyl ionization of O-methyltyrosine and tyrosine. On the other hand the ammonium ionization in glycyl O-methyltyrosine gives a negligibly small difference spectrum, quite in contrast to O-methyltyrosine.

**DISCUSSION**

The difference spectra which result from carboxyl ionization in tyrosine and from both carboxyl and ammonium ionizations in O-methyltyrosine may be qualitatively characterized by the appearance of two clearly separated peaks, one at about 2770 A and the other at about 2850 A, the latter almost twice as high as the former. These difference spectra are very similar to those reported by Laskowski et al. (11) for insulin at pH 1 compared to pH 7, and for trypsin-digested insulin compared to native insulin. Scheraga (26) has reported similar difference spectra for ribonuclease at pH 7 compared at pH 1, as have Glazer et al. (16) for acid ovalbumin and acid serum albumin, with reference to these proteins at neutrality. If one starts with the native protein in a neutral solution, and alters the

As is apparent from Fig. 2, difference spectra of tyrosine derivatives will show additional peaks at wave lengths below 2600 A. This has also been observed in the work of Schwert and Takenaka (25). However, most difference spectra of proteins have been determined above 2600 A, and we have therefore accepted this as a practical lower limit for the present work.

The location of the peaks in the difference spectrum of O-methyltyrosine at 2770 A and 2840 A is consistent with the fact that the long wave side of its absorption band is displaced, with respect to that of tyrosine, about 10 A toward shorter wave lengths.
The effect of NaCl on tyrosine difference spectra. Tyrosine at pH 5.4, 3 M NaCl versus pH 0.50, 3 M NaCl, ○. Tyrosine at pH 5.4, 3 M NaCl, versus tyrosine at pH 5.7, $\Gamma/2 = 0.16$, □.

Fig. 4. Upper, difference spectra for tyrosine. Isoelectric tyrosine (pH 5.7) versus tyrosine pH 1.02, △. Tyrosine at pH 8.05 (phenolic group 2.8 per cent ionized) versus 5.7, ○. Lower, the effect of NaCl on tyrosine difference spectra. Tyrosine at pH 5.4, 3 M NaCl versus pH 0.50, 3 M NaCl, ○. Tyrosine at pH 5.4, 3 M NaCl, versus tyrosine at pH 5.7, $\Gamma/2 = 0.16$, □.

environment either by titration with acid, by proteolytic digestion, or by adding large amounts of urea to the solution, the absorbancy decreases ($\Delta\varepsilon < 0$) between 2750 and 2900 $\mu$m. Titrations of the protein with base leads, of course, to absorbancy increases of much greater magnitude because of tyrosyl phenolic ionization—although we are not here concerned with this effect.

Quantitative comparisons of these and similar difference spectra can be made only with difficulty, for, more often than not, difference spectra have not been given in a quantitative form. Where such comparisons are possible, however, spectra observed in proteins are of the same order of magnitude, are of the same general form, and have their peak locations within 10 to 30 $\mu$m of the difference spectra observed in the simple tyrosine derivatives studied in this paper. For example, ribonuclease at pH 7 versus pH 1 yields a difference spectrum (20) whose peak is at 2870 $\mu$m, with ($\Delta\varepsilon$/tyrosine residue) $\approx 100$. When ribonuclease is urea-denatured, the change, involving a decrease in $\varepsilon$, in this range of wave lengths, is about twice this (12), and when ovalbumin is acid-denatured, ($\Delta\varepsilon$/tyrosine residue) is $\approx -150$ at 2870 $\mu$m, urea denaturation leading again to a value about twice as large (16). Of course, if not all of the tyrosyl groups of a protein are involved in the spectral perturbation, the change per residue involved is obviously greater than the values just calculated. Clearly, the magnitude of the spectral perturbations found in our studies with tyrosine and its derivatives ($\Delta\varepsilon$/tyrosine residue) = $-50$ to $-250$ is of the same order as the changes found when proteins are submitted to similar alterations in the solvent medium. It probably will be difficult to give a quantitative treatment of these spectral shifts since there is, for example, no simple correlation between hydrogen-bond strength and ultraviolet spectral shift (27, 28).

For O-methyltyrosine, it seems certain that the difference spectrum may arise from the ionization of either the ammonium or the carboxyl groups, since a reliable ionization constant for the ammonium group can be calculated from the difference spectrum at a known pH (Fig. 3). Tyrosine-tyrosine interactions cannot be the source of these difference spectra, since the values of $\Delta\varepsilon$ (molar) were found to be independent of concentration, over a range of 2.5-fold. Examination of molecular models of tyrosine and its O-methyl derivative show that it is impossible to bring the phenolic —OH or $-OCH_3$ group close enough to either the ammonium or the carboxyl group to form an intramolecular hydrogen bond. It seems therefore necessary to assume that the ionization of either group results in the transmission of a small perturbation to the chromophore, but whether the transmission is mainly through the molecule or through the medium we cannot say at the moment. The closest analogy in proteins to the case at hand would be the perturbation of the aromatic portion of a C-terminal tyrosine by ionization of its own carboxyl residue. Although this residue has seldom been found as C-terminal in proteins or peptides, proteolytic digestion of a protein will often give peptide fragments with C-terminal tyrosine, so that this may not be an entirely trivial case. On the available evidence, however, it appears that charged groups from other amino acid residues in the protein are more likely to be responsible for such ion-chromophore interactions as may occur.

The fact that tyrosine gives a “characteristic” difference spectrum on addition of urea would be expected on the assumption that these difference spectra are due to hydrogen bonding, since urea is well recognized as an effective hydrogen-bonding agent. Indeed, this experiment has been done before by Wetlauffer (29) for phenol, and by Laskowski (30) for acetyl tyrosine ethyl ester. In both these earlier cases, as here with tyrosine itself, a characteristic difference spectrum was shown with strong

4 R. Iavazzo, unpublished studies in this laboratory, 1957.

5 In principle, similar effects should occur in N-terminal tyrosine residues in proteins. However, even though N-terminal amino groups usually ionize with an apparent pH below 8, ionization of the protein tyrosyl residues will already have begun (see Fig. 4, upper) before the N-terminal ionization is complete, with the result that both spectral effects will occur with overlap in this pH range. It is questionable whether the two effects could be unambiguously sorted out.
urea solutions. If the experiments went no further, one could simply formulate this reaction as shown.

\[
\text{Ph-OH} \rightarrow \text{O} + (\text{urea}) \cdot \text{H}_2\text{O} \quad (1)
\]

However, we must also consider that O-methyltyrosine gives the same kind of difference spectrum but clearly cannot be fitted into the same phenolic-hydrogen-donating scheme. Of course, a turnabout scheme in which the phenolic oxygen becomes a hydrogen bond acceptor from a urea H-donor can be suggested, but for two reasons this seems unlikely. First, given a choice between two potential donors, the stronger acid is more likely to be the donor, and phenol is a stronger acid than urea. Conversely, of two bases, the stronger is likely to be the acceptor, and urea is a much stronger base than the ether oxygen of O-methyltyrosine. It is more likely that urea and O-methyltyrosine interact as two dipoles, without actual hydrogen bonding, whereas for tyrosine and urea a hydrogen-bonding interaction is an additional possibility.

The fact that urea causes an absorption increase with simple phenolic groups, but an absorption decrease with proteins (12, 14) is not necessarily an inconsistency. In all probability, urea has the same effect on exposed chromophores of the protein as it has on similar chromophores in simple compounds. Superimposed on this, however, is a larger effect of opposite sign caused by the disappearance of intramolecular interactions between the tyrosyl residues and other ionic or polar groups within the protein, because of the general configurational disruption induced by the urea.

The suggestion that tyrosyl hydroxyl groups may be bonded to carboxylate groups in a protein originated with Crammer and Neuberger (4), and has been given some experimental support by the work of Harrington (31) and of Scheraga (26). In model systems, it has been shown that concentrated alkali acetate induces characteristic displacements of the spectra of phenol (29) and acetyl tyrosine ethyl ester (30). However, it has not yet been possible to establish the nature of this phenolic-carboxylate interaction, nor the equilibrium constant. In our experiments it is seen that O-methyltyrosine as well as tyrosine, gives the usual difference spectrum in concentrated sodium acetate solutions. In the former case hydrogen bonding cannot occur, since neither acetate ion nor the methoxyphenyl residue has a hydrogen which can engage in hydrogen bonding. This spectral perturbation would seem to be due to a less intimate type of ion-dipole interaction.

The foregoing experiments show that ion-dipole and dipole-dipole interactions can give rise to difference spectra in tyrosine, and we may reasonably presume that tyrosyl residues in proteins likewise interact with other charged or dipolar groups of the same molecule. Whether such interactions may serve to stabilize a given protein configuration, is a question of considerable interest, but an answer cannot be inferred from the present experiments.\(^*\)

\(^*\)Theoretical and experimental estimates of the strength of tyrosyl-carboxylate interactions have, however, been made (29, 30, 32).
tyrosine, a.

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(with reference to aqueous solutions). Tyrosine, O; 0-methyl tyrosine, X.

There is the analogous possibility of a tyrosyl residue in a protein arc based on but a single experimental case, and cannot be obtained.

Efficiency transmission routes for such perturbations. Con-spectra will not arise from the ionization of distant groups; that is to say, groups more than \(-10 \text{ A}\) away from the chromophore, assuming, of course, that the protein does not provide high-efficiency transmission routes for such perturbations. Conversely an increase in the difference spectrum should be expected if the perturbing ion approaches the tyrosyl chromophore more closely than is the case in tyrosine. Of course, these conclusions are based on but a single experimental case, and cannot be accepted without reservation until confirmatory evidence is obtained.

For tyrosine difference spectra produced by acetate or urea, there is the analogous possibility of a tyrosyl residue in a protein interacting, for example, with a neighboring glutamate or glutamyl residue. The two groups so interacting in a protein need not be neighbors in a sequence along a polypeptide chain; indeed, they need not be in the same chain. For hydrogen bonding, the interacting groups necessarily have to approach each other about as close as their collision diameters and remain "fixed" with relatively little freedom (32). With the available alternatives of ion-dipole and dipole-dipole interactions, however, transmission of the effect through the medium becomes feasible, and we might expect spectral perturbation to result from interaction over distances somewhat greater than those allowable for hydrogen bonding. Thus the tyrosyl residues of a protein that are spectrally perturbed need not be as rigidly fixed as the hydrogen-bonding model suggests.

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A consideration of the nature of the ultraviolet absorption spectrum of tyrosine suggests a basis for the characteristic location of the peaks in its difference spectra. These peaks fall at about 2780 A and at 2850 A, where the absorption curves are steepest. If the spectral perturbation should result only in a red shift of the spectrum, then the result shown in the continuous curve of Fig. 5 is obtained. This hypothetical difference spectrum is based on the premise of a 10 A red shift of the O-methyl-tyrosine spectrum. The result is seen to agree well with experiment in the location, and fairly well in the relative heights of the peaks, but there is no agreement at all below approximately 2750 A. Disagreement in this last feature means that the case was not as simple as was assumed. However, the form of this hypothetical curve indicates that the location and relative size of the peaks are a consequence of the initial shape of the absorption spectrum and of the experimental fact that it undergoes relatively little deformation when perturbed.

Earlier Interpretations of Protein Spectral Shifts

The red shift in protein tyrosyl-group spectra was attributed by Stenström and Reinhard (8) to partial tyrosyl ionization due to vicinal electrostatic effects. That this is not so is seen immediately in a comparison of the difference spectrum of tyrosine at 2.8 per cent ionization (Fig. 4, upper) with the difference spectra shown here for tyrosine and O-methyltyrosine (Figs. 3, 4, 5, 6, and 7), which are closely similar to those observed in certain proteins. The mechanism suggested by Stenström and Reinhard, namely that the (electrostatic) influence of adjacent positively charged amino acid residues is responsible for the spectral shift, does seem to have some truth in it, in view of our present findings.

Simple peptides of tyrosine and tryptophan have their absorption maxima shifted from 5 to 15 A to the red, compared with the free amino acids in aqueous solution (1, 33, 34). Thus the hydrolysis of a peptide or protein to the constituent amino acids gives a difference spectrum (\(\Delta \varepsilon \) negative) qualitatively similar to those observed in our studies. Beaven et al. (34) suggested that additional long wave absorption shifts in proteins result from fastening the chromophores onto a "semi-rigid framework." The results on which this suggestion was based seem sound; however, they involve observations of the spectra of tryptophan and phenylalanine in gels or dried films. We believe that these interactions reasonably fall into the somewhat broader classes of dipole-ion and dipole-dipole interactions, as illustrated in the present work. Whether the mechanical rigidity of a chromophore can be separated for consideration from the environmental factors which enforce such rigidity is difficult to say.\(^7\)

Hydrogen-bonding hypotheses for explaining spectral shifts seem to have had their origin in the classic work of Crammer and Neuberger on ovalbumin (4). Strong experimental support for tyrosyl-carboxylate hydrogen bonds in ovalbumin has come

\(^7\) In proteins it is probable that chromophoric groups other than tyrosine will undergo spectral perturbation by one or more of the mechanisms suggested for tyrosyl residues. From spectral measurements of acid and alkaline solutions of tryptophan and phenylalanine (1, 34), it is certain that their spectra are perturbed by ionization of one or both of their ionizing groups. Difference spectra derived from phenylalanine due to ionization would probably show several small peaks between 2600 A and 2700 A, whereas tryptophan would probably show a broad trough between 2500 and 2800 A, a small peak at about 2830 A, and a larger peak around 2930 A. (see Beaven and Holiday (1), Figs. 2 and 3).
from the studies of Harrington (31), who showed that, when subjected to denaturation by guanidinium chloride, ovalbumin exposes about eight carboxylate groups and about eight base-binding groups whose acid pK is close to 10. This fits well with Crammer and Neuberger's finding that about eight tyrosyl groups in native ovalbumin are not free to ionize. However, an alternative explanation of Harrington's experiment is possible. As Tanford has recently suggested for the denaturation of hemoglobin (35), acid or base uptake may accompany denaturation due to a decrease in the electrostatic work factor when the protein assumes a more expanded configuration. Ovalbumin does show a characteristic spectral shift when denatured by acid or by urea(16) but in spite of the decisive evidence that tyrosyl groups in ovalbumin are by no means "normal," it is as yet impossible to say with any certainty whether this is because they are hydrogen bonded.

It may be mentioned that if a chromophore of a protein is imbedded in a hydrophobic region of the molecule, as has been occasionally suggested (6, 36), crude qualitative predictions can be made as to the spectral change to be expected. Such a group, being in a medium of lower dielectric constant, would have its absorption peak at slightly shorter wave lengths than in a medium of high dielectric constant such as water (37-39). In the case of a tyrosyl residue, its hypothetical transport from a nonaqueous medium (in the interior of a native protein molecule) to an aqueous medium, as in denaturation, probably would entail the formation of at least one phenol-water hydrogen bond. Changing from a less polar to a more polar medium, whether or not this is accompanied by formation of a discrete hydrogen bond, would induce a shift of the electronic spectrum. This shift might be either to longer or to shorter wave lengths, in view of the data discussed above, and in Footnotes 8 and 9.

However, only shifts to shorter wave lengths result when proteins are denatured, or otherwise subjected to treatment which may be expected to disrupt "hydrophobic bonds" and expose previously unavailable groups to the solvent.

**REFERENCES**


**SUMMARY**

Mechanisms have been investigated which can give rise to a displacement of the spectrum of the tyrosyl residues in proteins when acid and neutral solutions are compared. The difference spectrum is characterized by two peaks: the larger at 2850-2870 Å, and a second peak at 2770-2800 Å, about half the height of the first. In solutions of tyrosine, O-methyltyrosine, and glycy1-O-methyltyrosine, it is shown that the ionization of an adjacent group can give rise to difference spectra similar to those observed in proteins when the pH is displaced from neutrality to acid values near 2. In all these cases, e increases with increasing pH in the wave length region between 2750 and 2900 Å. For the spectral changes on ionization in tyrosine, O-methyltyrosine, and glycy1-O-methyltyrosine, mechanisms of intermolecular hydrogen bonding are ruled out because the effect is concentration-independent, and intramolecular hydrogen bonding is ruled out on steric grounds.

Urea, which at high concentrations produces similar difference spectra with both tyrosine and O-methyltyrosine, acts by a dipole-dipole interaction, which, in the case of tyrosine, may take the form of hydrogen bonding. Concentrated solutions of sodium acetate also produce a characteristic displacement in the spectra of tyrosine and O-methyltyrosine, with a difference spectrum similar to that produced by the ionization of a group within the molecule. This leads to the conclusion that the acetate-tyrosyl interaction may be due to a general ion-dipole interaction or to specific hydrogen bonding. Difference spectra obtained from glycy1-O-methyltyrosine suggest that the ionizing group may be within a few Angstroms of a tyrosyl residue to give rise to a spectral perturbation. The implications of these findings for difference spectra in proteins are discussed.

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27. Tsubomura, H., Nippon Kagaku Zasshi, 73, 811, 920 (1952); also Chem. Abstr., 47, 3096 a, c (1953).
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