Ultraviolet Irradiation Effects in Poly-L-tyrosine and Model Compounds. Identification of Bityrosine as a Photoproduct*

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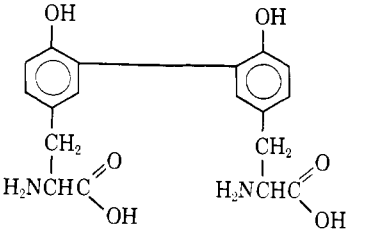
ABSTRACT: The blue fluorescence observed in solutions of poly-L-tyrosine, copolymers of tyrosine, and L-tyrosine monomer is due to the production of bityrosine by ultraviolet irradiation. Analogous products are formed by irradiation of other model phenolic compounds. The identification was made by a spectral comparison of the photoproduct with bityrosine and *o,o'*-bicresol and by a chromatographic and electrophoretic comparison of irradiated polymer hydrolysates and controls.

In addition, some spectral characteristics and quantum yields of the bicresol chromophore were obtained in different solvents. It is also shown that the phenomenon of excited state ionization which occurs in aqueous solution does not appreciably occur in solutions of dimethyl sulfoxide.

Previous fluorescence studies of poly-L-tyrosine (PLT)[1] had shown that under certain conditions a second emission band was present with a peak in the 410–430-mμ region in addition to the usual tyrosine band at 305 mμ (Lehrer and Fasman, 1964). This new band was also observed in solutions of copolymers of tyrosine (Pesce *et al.*, 1964) depending upon the treatment and history of the polymer sample. Changes in the fluorescence intensity were produced with little change in the absorption spectrum. In addition, the wavelength of maximum excitation was the same for both the new blue fluorescence and the usual ultraviolet tyrosine fluorescence in PLT. The new band was, therefore, tentatively interpreted as the result of an excited-state interaction (excimer) between two neighboring tyrosine residues in the helical polymer.

A more recent study was reported that characterized excimer emission in tyrosine model systems (Lehrer and Fasman, 1965). This study showed that excimer emission occurred in the 350-mμ region for phenolic chromophores, not in the 420-mμ region, and suggested that the latter band was either owing to a spectral interaction of tyrosine residues, different from the excimer as characterized, or that some impurity was present. The study to be reported herein will show that the new band observed in PLT is the result of a new chemical species produced by ultraviolet irradiation and that similar photoproducts can be obtained in monomer model systems of tyrosine.

It will be shown that the new species is bityrosine (BT),[1] which is the *o,o*-biphenol analog of tyrosine.



BT

This is demonstrated by comparison of the fluorescence spectra of the photoproduct with bityrosine (BT) and by chromatographic and electrophoretic separation of the photoproduct from L-tyrosine after hydrolysis of the polymer. In addition, corrected fluorescence spectra and quantum yields of BT and other model phenolic compounds in different solvents will be presented.

The biochemical importance of BT is indicated by recent studies which show that it can be produced enzymatically from tyrosine (Gross and Sizer, 1959) and that it is found in a structural protein (Andersen, 1963, 1964, 1966). In these latter studies of Andersen, BT was isolated from resilin and its spectral characteristics were studied. The similarity of these spectral characteristics and the characteristics of the photoproduct observed in PLT suggested possible identification.

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[1] Abbreviations used: BT, bityrosine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; OBC, *o,o'*-bicresol; OBP, *o,o'*-biphenol; PEP, *p*-ethylphenol; PLT, poly-L-tyrosine.

Materials and Methods

Calibration of Fluorometer. A Zeiss XFM 4C prism spectrofluorometer equipped with a 450-w Osram xenon lamp was used to obtain emission and excitation spectra. All the spectra reported here were corrected for instrumental variations of sensitivity and of variations of exciting light output with wavelength by a method similar to that employed by Melhuish (1962). To obtain correction factors for excitation spectra, a Rhodamine B solution in ethylene glycol (3 g/l.) was employed as a quantum counter to measure the combined effects of the xenon lamp output and excitation monochrometer dispersion as a function of wavelength. This was done by recording the fluorescence at 650 mμ as a function of exciting wavelength at constant slit width. The geometry was kept constant by using the tilted-cell position in the fluorometer so that fluorescence was viewed from the same face as the excitation. Stray light was reduced by using the >620-mμ filter on the emission monochrometer. To obtain emission correction factors, the radiation from the now calibrated source was reflected from the face of an ultraviolet spectral grade quartz plate rather than an aluminum mirror (Melhuish, 1962), and both monochrometers were varied for maximum output, in unit steps of about 5 mμ at constant slit width.

Quantum Yield Determinations. Quantum yields were determined by comparing the area under the corrected fluorescence spectrum expressed in frequency units with the corresponding area of an L-tyrosine standard in solution whose known absorbance at the exciting wavelength was <0.1 in a 1-cm cuvet. The quantum yield, Q, was then calculated with the equation $Q = 0.2(FA_T/F_TA)K$, where F and F_T are the integrated fluorescence intensities, and A and A_T are the corresponding absorbances of the unknown and the L-tyrosine standard, respectively, at the exciting wavelength; 0.2 is the assumed quantum yield of L-tyrosine (Teale and Weber, 1957); and K is a constant which corrects for the difference in intensity of radiation if the unknown is excited at a different wavelength than the L-tyrosine standard. Calculations of quantum yield were for the processes indicated in the tables. That is, in the cases where more than one species absorbed in the region of excitation, only that fraction of the absorbance which contributed to the fluorescence was considered. Measurements were made at room temperature (25°) which remained constant within a few degrees. The error in quantum yield is estimated to ≃10% and was mainly due to fluctuation in exciting light intensity caused by the wandering of the arc of the xenon lamp.

Irradiation Methods. Most of the solutions were irradiated in the fluorometer by exciting at 295 or 280 mμ, with a band width of ≃21 mμ. This enabled the increase in fluorescence in the 410-mμ region to be conveniently monitored during the irradiation. The solutions were stirred either by bubbling N_2 gas directly into the cuvet or by interrupting the irradiation and periodically shaking. Some experiments with *p*-ethylphenol (PEP)[1] and L-tyrosine (Tyr) and L-tyrosyl-L-tyrosine (TyrTyr) were performed in cells that had been degassed on a vacuum pump (0.001 mm) by alternate freezing and thawing in liquid N_2.

Absorption Spectra. A Cary 14 recording spectrophotometer was used to obtain spectra often utilizing the high-sensitive slide wire (0–0.1-OD full scale). These values were used in the quantum yield calculations.

Cells. The fluorescence and absorption cells used were of ultrasil quality and had path lengths of 1 and 5 cm. They were obtained from Hellma and Pyrocell.

Hydrolysis Methods. After irradiation of PLT in alkaline solution to maximum blue fluorescence, the polymer was precipitated by adding an excess of 1 N HCl and the precipitate was washed with 0.1 N HCl to remove excess salts. The polymer was then transferred to thick-walled hydrolysis tubes (≃1 mg in each tube) and dried in a vacuum desiccator over KOH. Concentrated HCl (12 N, 2 ml) was added to each tube and the tubes were sealed off after degassing, by alternate freezing, evacuating with a vacuum pump, and thawing. The samples were heated for 8 days at 118–120°. The long hydrolysis time was found to be necessary because initial studies indicated oligomeric products were present when shorter times were used. The insoluble polymer slowly dissolved as it hydrolyzed over this period of time. After 7 days, all of the polymer was found to be dissolved. The tubes were opened and the excess HCl was removed in a vacuum desiccator over KOH overnight. A small amount of H_2O was added and the solutions were passed through a Millipore (HAWP 0.45 μ) filter. Control samples of Tyr, TyrTyr, and BT were subjected to the same hydrolysis treatment.

Thin Layer Chromatography. Thin layer chromatography was done on Eastman silica gel sheets type K301R in two dimensions. The solvent system used in the first dimension was 1-butanol–acetic acid–water (4:1:1) prepared the same day. The sheet was then dried and run in the second dimension using 2-propanol–concentrated ammonium hydroxide–water (8:1:1). In the second solvent system, BT barely migrates, as found by Andersen (1966), if precautions are taken to first expose the sheet to ammonia vapor for a few minutes before starting the run. The solutions were applied with 1-μl pipets. The quantity of hydrolysate applied was estimated from the absorption spectra to contain ≃10^{-6} mmole of BT. The BT spots were easily located by the bluish fluorescence produced by excitation with a portable mercury ultraviolet lamp which increased in intensity when exposed momentarily to ammonia fumes. Ninhydrin and 1-butanol were also used to locate the spots of the various amino acids.

High-Voltage Electrophoresis. High-voltage electrophoresis at pH 1.9 in an acetic acid–formic acid buffer system at 80 v/cm was run on the polymer hydrolysate and controls after application to Whatman No. 1 paper. Location and development were done as de-

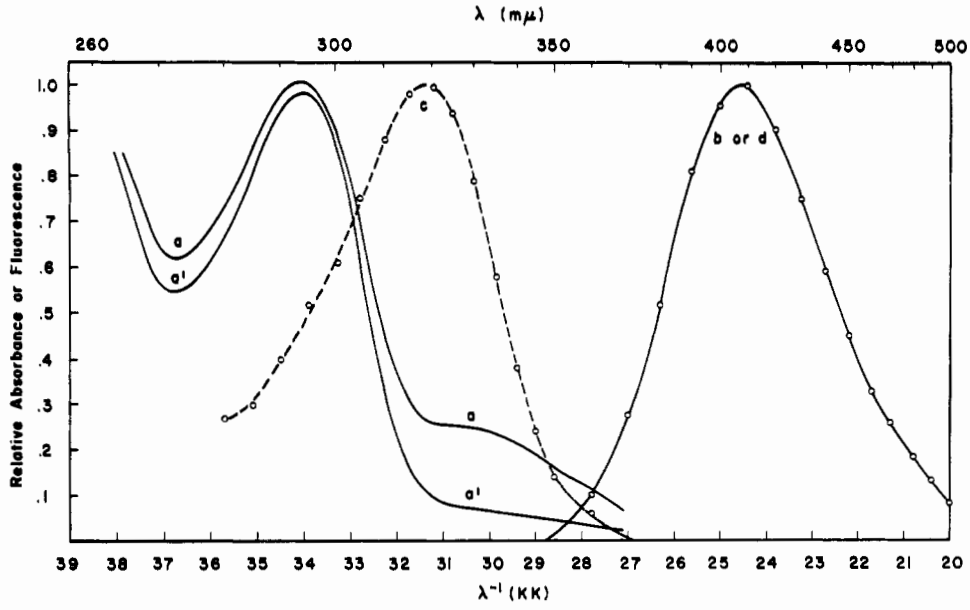

FIGURE 1: Absorption and fluorescence spectra of poly-L-tyrosine in 0.1 N NaOH before and after irradiation. (a) Normalized absorption spectrum before irradiation; (a′) absorption spectrum after irradiation relative to a; (b) normalized emission spectrum of photoproduct, $\lambda_{excitation}$ 315 mμ; (c) normalized excitation spectrum of photoproduct; and (d) normalized emission spectrum of the photoproduct of *p*-ethylphenol.

scribed previously for the thin layer sheets.

Materials. The following compounds were used as purchased: L-tyrosine (CP grade), Mann Research Laboratories; L-tyrosyl-L-tyrosine, New England Nuclear; dimethyl sulfoxide (spectroquality), Matheson Coleman and Bell; dimethylformamide (fluorometric grade), American Instrument Co. The following compounds were purified by sublimation before use: *p*-ethylphenol, Gallard Schlesinger; phenol, Baker and Adams; *o,o′*-biphenol, Alldrich; *o,o′*-bicresol, a gift from Dr. S. O. Andersen.

BT was prepared by Mr. J. Kappler using the enzymatic method of Gross and Sizer (1959). The reaction mixture was acidified and separated from unreacted L-tyrosine and impurities on a phosphocellulose column (45 × 1 cm), equilibrated, and eluted with 0.2 M acetic acid. Final purification and desalting was accomplished on a P-2 Bio-Gel column (1 × 30 cm) by eluting with water. In these purifications, only the fraction which exhibited visible fluorescence was collected. The final fraction was recrystallized from ethanol and stored under ether.

PLT was synthesized as previously described (Fasman *et al.*, 1964) using the *N*-carboxyanhydride of *O*-carbobenzoxy-L-tyrosine with sodium methoxide as initiator (Blout and Karlson, 1956) at a ratio of anhydride:initiator of 200. After polymerization, the blocking group was removed by the HCl–HBr treatment (Fasman *et al.*, 1961), dissolved in NaOH to pH 12, extracted with ether, and dialyzed *vs.* 0.01 N HCl for 3 days. The polymer precipitate was dried under high vacuum at 80°. The resulting intrinsic viscosity, $[\eta]$ = 1.12 at pH 12 in 0.2 M NaCl, indicated a high molecular weight.

Results

Spectral Characterization of the Photoproduct in PLT and Model Compounds

PLT. The fluorescence and absorption spectra of PLT in aqueous solution was only studied in the basic pH range due to the lack of solubility of the un-ionized polymer. The absorption spectrum in 0.1 N NaOH shows a maximum at 295 mμ characteristic of ionized tyrosine residues. Ionized tyrosine fluoresces very inefficiently in the 350-mμ region ($Q \simeq 0.01$) (Cornog and Adams, 1963), and this emission was observed for PLT solutions. During the measurement of fluorescence, an irradiation effect was observed, when exciting in the 295-mμ region, which resulted in a large increase in fluorescence in the 400–410-mμ region. This blue fluorescence reached a maximum value after a short time of irradiation, the maximum intensity depending upon the wavelength of irradiation.

The fluorescence emission and excitation spectra of the photoproduct in 0.1 N NaOH are shown as curves b and c, respectively, in Figure 1 and the absorption spectra before and after 280-mμ irradiation to maximum 410-mμ fluorescence are seen in curves a and a′. It is seen that an increase in "tail" absorption occurred (≃330 mμ) after irradiation, showing the presence of a longer wavelength absorbing species. The excitation spectrum of the high-wavelength band, which is uncomplicated by any ionized tyrosine contribution, shows that the new species has an ab-

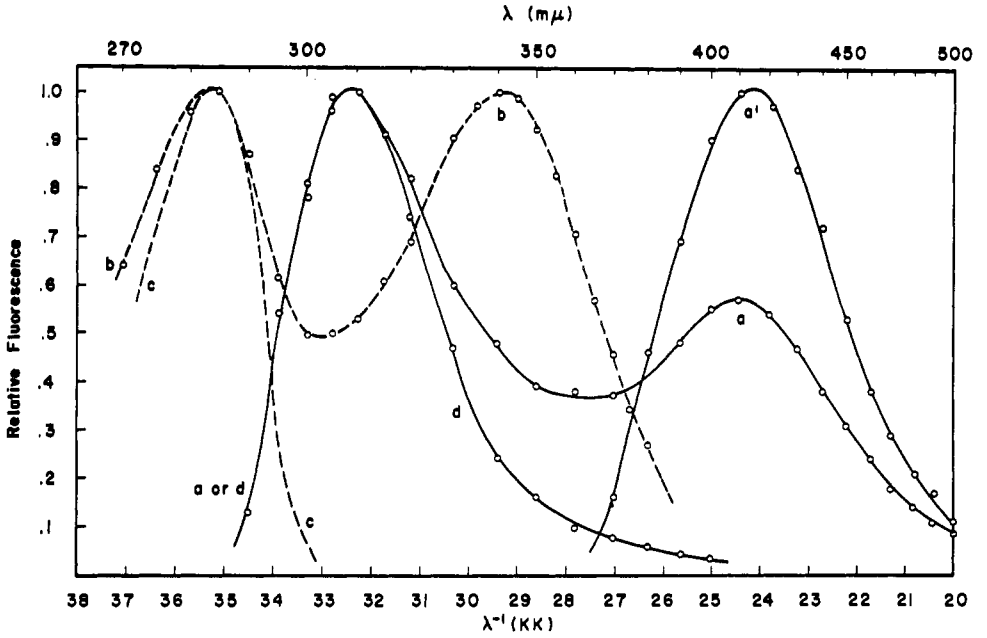

FIGURE 2: Normalized fluorescence emission and excitation spectra of poly-L-tyrosine in dimethyl sulfoxide. Fluorescence spectra, –O–O–. Excitation spectra, - -O- -O- -. (a) Poly-L-tyrosine previously irradiated in base, $\lambda_{excitation}$ 282 mμ; (a′) poly-L-tyrosine previously irradiated in base, $\lambda_{excitation}$ 330 mμ; (b) excitation spectrum of 415-mμ band; (c) excitation spectrum of 308-mμ band; and (d) emission of sample never exposed to base, $\lambda_{excitation}$ 280 mμ.

sorption maximum near 320 mμ. From these results, it could be estimated that the quantum yield of the photoproduct was at least an order of magnitude greater than that of ionized tyrosine. This partly explains why a large change in fluorescence could be observed without much change in absorption spectrum.

This irradiation effect did not depend upon the presence of oxygen. When oxygen was present, however, a competing irradiation effect occurred which caused the loss of both monomer and photoproduct fluorescence, indicating probable photooxidation effects. Since the oxidation products did not appear to be fluorescent and hence did not complicate the fluorescence results, their identification was not pursued.

A correlation between the previous history of the polymer samples with the initial amount of the 410-mμ fluorescence and also the amount of "tail" absorption was made for PLT samples and for block copolymer samples of glutamic acid and tyrosine, *e.g.*, (L-Glu)(L-Tyr)(L-Glu) (Fasman *et al.*, 1964). After polymerization, the polymers were routinely dissolved in base and dialyzed *vs.* acid for many hours. It was found that the largest 410-mμ fluorescence band and most prominent "tail" absorption correlated with those polymers which were left longest in base. Perhaps the intensity of the long-wavelength ultraviolet radiation from the room fluorescent lights was sufficient to produce some photoproduct in these basic systems.

The PLT polymers were also studied in dimethyl sulfoxide (DMSO).[1] In curves a and a′ of Figure 2 the emission spectra of PLT solutions which were previously irradiated in base, precipitated by acid, and redissolved in DMSO are shown for excitation at 330 and 282 mμ, respectively. The main contribution of the fluorescence is from the tyrosine band at 306 mμ, for 282-mμ excitation, but the blue photoproduct fluorescence is also quite prominent (curve a) while excitation at 330 mμ yields only the 412-mμ emission (curve a′). The photoproduct fluorescence in DMSO is shifted several millimicrons to longer wavelengths compared with the aqueous solution results. Also shown in Figure 2 (curve d) is the fluorescence spectrum of a sample which was never dissolved in base during its preparation history. It is clear that little photoproduct is present in this case. The excitation spectra of both fluorescence bands are presented in Figure 2 (curves b and c), where it is seen that the excitation spectrum of the tyrosine fluorescence band has a maximum at 282 mμ. The excitation spectrum of the 410-mμ emission consists of two bands, a long wavelength band at 342 mμ and one near 282 mμ. Only a slight increase in "tail" absorption was observed in the ultraviolet absorption spectra before and after irradiation, again indicating the relatively high quantum yield of fluorescence. The coincidence of the excitation spectra for both fluorescence bands in the region of 280 mμ indicates the presence of energy transfer. That is, energy was transferred from the tyrosine to the photoproduct probably *via* a long-range intramolecular mechanism (Förster, 1959).

These polymers were also studied in dimethylformamide (DMF)[1] and similar results were observed. In this case, the quantum yield of fluorescence of

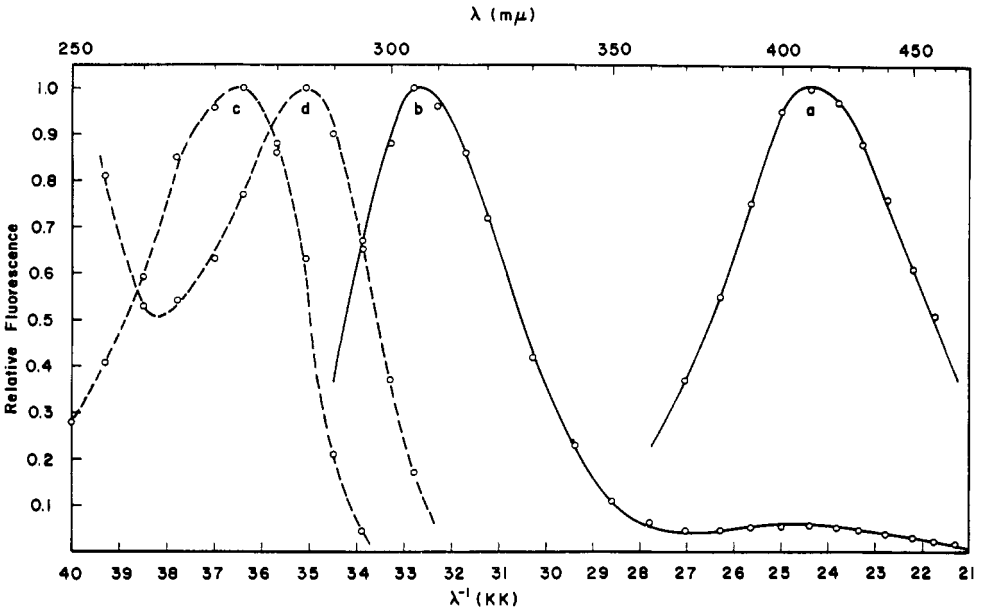

FIGURE 3: Normalized fluorescence emission and excitation spectra of irradiated *p*-ethylphenol at pH 4. Fluorescence spectra, –O–O–. Excitation spectra, - -O- -O- -. (a) *p*-Ethylphenol previously irradiated in base, $\lambda_{excitation}$ 295 mμ; (b) *p*-ethylphenol previously irradiated in base, $\lambda_{excitation}$ 280 mμ; (c) excitation spectrum of 305-mμ band; and (d) excitation spectrum of 410-mμ band.

the 305-mμ band was approximately one-tenth that of PLT in DMSO (Q_{DMSO} = 0.09). Since the 420-mμ band appeared more intense relative to the 305-mμ band, the quantum yield of the photoproduct in DMF is still high. It was also observed that heating at 100° for several minutes in both DMSO and DMF in the absence of oxygen increased the 420-mμ band but that heating in the presence of oxygen caused some loss of total fluorescence.

Model Compounds. Studies on model compounds were undertaken to investigate whether nonpolymeric phenolic compounds show similar ultraviolet irradiation effects and also to be able to study the phenomena in a pH range inaccessible to the polymer system. Irradiation studies on *p*-ethylphenol, phenol, L-tyrosine, and L-tyrosyl-L-tyrosine showed that the new fluorescence band was produced for all these compounds by irradiation in basic or neutral solutions. The excitation and emission spectra of the new band for the *para*-substituted phenols were essentially the same as that found with PLT. This is illustrated in Figure 1 for irradiated PEP. The photoproduct was not as efficiently produced in phenol and the spectrum of the phenol photoproduct was shifted $\simeq$15 mμ to lower wavelengths compared to PEP.

The excitation spectrum of the photoproduct of *p*-ethylphenol in acid solution was different from that found in basic solution. This is illustrated in Figure 3, where it is seen that although the same emission near 410 mμ is produced, the excitation spectrum for this emission is completely different at these two pH values. The excitation spectrum for the usual 305-mμ band coincided with the absorption spectrum and had a maximum at 275 mμ. These results show that the absorption spectrum of the photoproduct is different in acid and base, but that the emission remains the same.

The fluorescence of PEP in DMSO was also studied and the results were qualitatively similar to the PLT system. The quantum yield of the main band at 310 mμ was found to be 0.09, approximately the same as PLT. In the absence of oxygen, heating at 100° for several minutes caused an increase in the long-wavelength band fluorescence which had a maximum at 423 mμ. In the presence of oxygen, heating caused a loss in the total fluorescence indicating loss of fluorescent species by oxidation.

Kinetics. Studies of the kinetics of the photoreaction in the systems PEP, Tyr, and TyrTyr in base indicated that saturation of the new fluorescence band occurred after a short irradiaion time as with PLT. In one experiment, the initial rate and final value of photoproduct fluorescence was compared for Tyr and TyrTyr, both solutions having the same absorbance at the irradiation wavelength. The initial rate and final value was fourfold higher for the dipeptide. Irradiation experiments performed in the same manner as a function of PEP concentration showed that the initial rate was a function of the concentration of the absorbing species. These observations indicate that more than one phenolic group is involved in the photoreaction.

An attempt was made to see if the saturation effect was due to a photostationary state set up by a reverse photochemical process. A sample of PEP in base previously irradiated to produce the new fluorescence

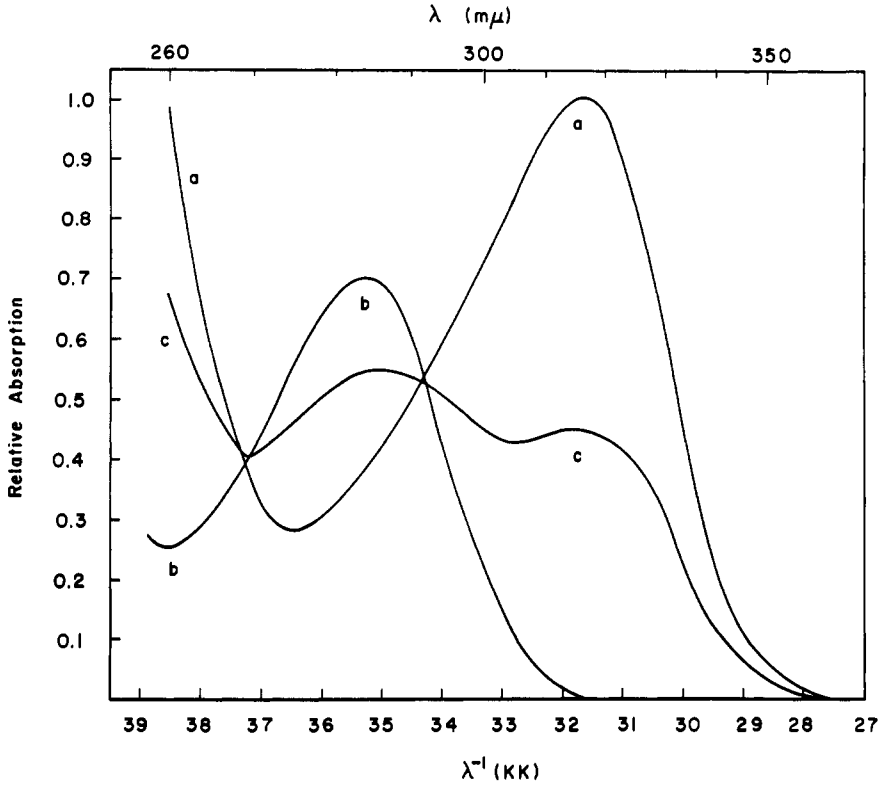

FIGURE 4: Absorption spectra of bityrosine at different pH values: (a) pH 11; (b) pH 3; and (c) pH 7.

band was further irradiated in the new absorption band at 325 mμ. A loss of the 410-mμ fluorescence without a corresponding increase in the 350-mμ ionized tyrosine band was observed. From this result it appears that the saturation phenomenon is probably due to a further photoreaction of 410-mμ photoproduct to nonfluorescent species.

Spectral Characteristics of Bityrosine and Related Compounds

Bityrosine (BT), o,o'-Biphenol (OBP),[1] *and o,o'-Bicresol (OBC)*[1] *in Aqueous Solution.* It has previously been noted that the ionization constant of the first hydrogen in OBP is shifted three orders of magnitude to p$K \simeq 7$ compared to phenol (Musso and Matthies, 1961), and that the fluorescence and absorption spectra of the ionized species is different from the un-ionized species (Bridges *et al.*, 1961). The ultraviolet absorption of BT and the fluorescence emission and excitation characteristics of OBC of the un-ionized and singly ionized species of each are shown in Figures 4 and 5. These spectra are in agreement with those published by Andersen (1966). It is clear from these spectra that the emission is from the excited, singly ionized

TABLE I: Spectral Characteristics and Quantum Yields of Fluorescence of Bityrosine (BT), *o,o'*-Bicresol (OBC), and *o,o'*-Biphenol (OBP).

Sample	pH	Absorption Max, λ (mμ)	Excitation Max, λ (mμ)	Absorbing[a] Species	Emitting[a] Species	Emission[b] Max, λ (mμ)	*Q* for[c] Process
BT	3.6	284	283	^{0}S	^{0}S*	410	0.42
BT	10.7	316	315	$^-$S	$^-$S*	410	0.64
OBC	3.8	286	285	^{0}S	$^-$S*	412	0.55
OBC	10.8	317	317	$^-$S	$^-$S*	412	0.68
OBP	3.7	278	278	^{0}S	$^-$S*	396	0.60
OBP	10.9	308	308	$^-$S	$^-$S*	396	0.65

[a] ^{0}S and $^-$S = ground-state species, un-ionized and ionized, respectively. ^{0}S* and $^-$S* = excited-state species, un-ionized and ionized, respectively. [b] ±2 mμ. [c] ±10%.

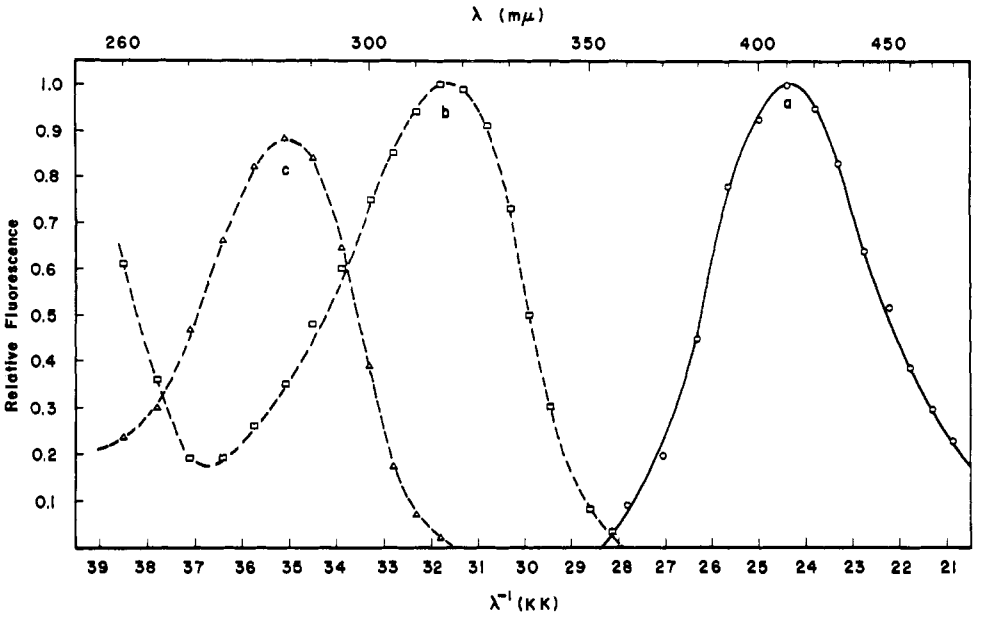

FIGURE 5: Fluorescence and excitation spectra of *o,o'*-bicresol in H_2O. Fluorescence spectra, –O–O–. Excitation spectra --O--O--. (a) Fluorescence spectrum, pH 3.8 and 10.8; (b) excitation spectrum, pH 10.8; and (c) excitation spectrum, pH 3.8. The excitation spectra were normalized to equal peak absorbance so that they represent relative quantum yield.

species from the observed mirror image symmetry of curves a and b. This excitation spectrum is due to the ionized species. However, the same fluorescence spectrum is obtained from excitation of the un-ionized species (curve c). Therefore, the phenomenon of excited-state ionization occurs. This phenomenon was first investigated by Förster (1950) and has also been shown to occur for OBP (Bridges *et al.*, 1961). The same phenomena occur for BT as indicated in Table I. The slightly lower quantum yield for BT at pH 3.6 compared to OBC is probably due to some quenching by carboxylate ion as is observed for tyrosine (White, 1959; Pesce *et al.*, 1964).

o,o'-Bicresol (OBC) in DMSO. The spectral characteristics of OBC were studied in DMSO for comparison with the PLT study. The absorption spectrum of OBC dissolved in DMSO is shown in curve a of Figure 6. It should be noted that the ionized absorption peak in DMSO is shifted 35 mμ to longer wavelength compared to the aqueous solutions discussed above. This indicates a strong interaction between DMSO and singly ionized species. Also, the extent of ionization of OBC in DMSO is quite high as evidenced by the magnitude of the high-wavelength-ionized absorption band. This ionized absorption band was also seen for OBC dissolved in DMF, but only the un-ionized absorption band was observed in methanol. The spectrum of OBC in DMSO containing approximately 5% of aqueous 0.1 N NaOH or 0.1 N HCl (v/v) are shown in curves c and d of Figure 6. It is to be noted that the ratio of absorbance of the un-ionized form to ionized form is higher in DMSO than in aqueous solution at pH 7 by a comparison with Figure 4. The purity of the DMSO determines the extent of ionization.

The fluorescence spectra of OBC in DMSO are seen in Figure 7 for two excitation wavelengths. When the excitation is absorbed only by the ionized species, the fluorescence (curve b) can only be from the excited ionized species and appears with a maximum at 427 mμ. When the excitation is partially absorbed by un-ionized species, some fluorescence is observed in the 365–70-mμ region (curve a). The excitation spectra for each emission band (Figure 7c,d,d') shows that the 350-mμ band is associated with absorption by un-ionized species, but that the un-ionized species does not contribute appreciably to the 427-mμ band; *i.e.*, little excited-state ionization occurs. This is different from the aqueous solution. In the DMSO–H_2O (1:1, v/v) system, however, some excited-state ionization of OBC occurs in addition to un-ionized emission. This was indicated by a large contribution of the un-ionized excitation band to the ionized emission. Only a small quantity of H_2O is necessary to produce some excited-state ionization. When 0.1 N HCl, approximately 5% by volume, is added to DMSO to ensure that only un-ionized OBC is present, some ionized fluorescence is still produced. This is seen in Figure 8 (curve a) by the high-wavelength tail present in the main fluorescence band. The resolution of the composite

TABLE II: Spectral Characteristics and Quantum Yields of Fluorescence of *o,o'*-Bicresol in DMSO, Water, and Mixtures.

Solvent	pH	Absorption Max, λ (mμ)	Excitation Max, λ (mμ)	Absorbing[a] Species	Emitting[a] Species	Emission[g] Max, λ (mμ)	*Q* for[h] Process
H_2O	3.8	286	286	^0S	$^0S^*$	*b*	*b*
H_2O	10.8	286	286	^0S	$^-S^*$	412	0.55
H_2O	10.8	317	317	^-S	$^-S^*$	412	0.63
H_2O–DMSO (1:1, v/v)		*Ca.* 292[d]	291	^0S	$^0S^*$	*Ca.* 370[d]	0.07
		Ca. 292[d]	292	^0S	$^-S^*$	415	0.47
		325 (sh)[c]	325	^-S	$^-S^*$	415	0.56
DMSO–5% 0.1 N HCl[e]		290	292	^0S	$^0S^*$	360	0.14
DMSO–5% H_2O		290	292	^0S	$^-S^*$	425	0.02
DMSO–5% 0.1 N NaOH[e]		345	345	^-S	$^-S^*$	425	0.70
DMSO		294	294	^0S	$^0S^*$	370[i]	0.18
		294	294	^0S	$^-S^*$	427	0[f]
		350	350	^-S	$^-S^*$	427	1.06

[a] Symbols as in Table I. [b] No emission observed. [c] Shoulder on main peak. [d] Peak value estimated because of distortion due to overlap of more than one band. [e] The estimation of the aqueous volume is only approximate and the quantum yields are quoted to indicate trends in the various solvent systems. [f] Estimated from lack of contribution of un-ionized species to excitation spectrum of 425-mμ band. [g] ±2 mμ. [h] ±10 %. [i] Broad peak.

band into the two contributions, which enabled the respective quantum yields to be calculated, is indicated by curve c. A summary of the spectral characteristics and quantum yields for the processes indicated is given in Table II.

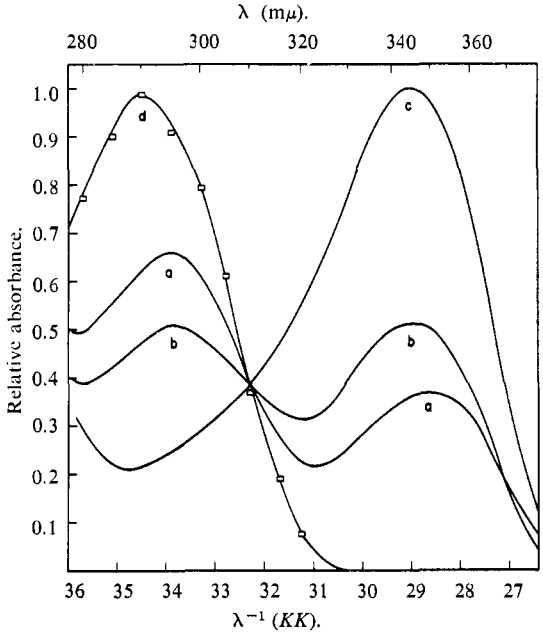

FIGURE 6: Absorption spectra of *o,o'*-bicresol in dimethyl sulfoxide. (a) Dimethyl sulfoxide; (b) dimethyl sulfoxide with ≃ 5% H_2O by volume; (c) dimethyl sulfoxide with ≃ 5% 0.1 N NaOH by volume; and (d) dimethyl sulfoxide with ≃ 5% 0.1 N HCl by volume.

Thin Layer Chromatography

Chromatographic separation and identification of BT was performed in hydrolysates of irradiated PLT even though, as discussed above, the relative quantity of BT produced by irradiation is small. A sample of PLT was dissolved in 0.1 N NaOH and irradiated at 280 mμ (band width ≃ 12 mμ) after saturating the solution with N_2 gas. This irradiation wavelength was chosen to minimize loss of dimer by further photoreaction, because the ratio of absorbance of monomer to dimer was estimated to be high from the extinction coefficients reported by Andersen (1966). A spectral study of the hydrolysate indicated the presence of BT and Tyr, as observed in the unhydrolyzed polymer, with some relative loss of BT. In addition, certain impurities were observed in all the samples, including the unirradiated controls, which indicated that additional products were formed by the hydrolysis procedure. One impurity found in the polymer and the tyrosine control (T_h) fluoresced at ≃450 mμ, excited maximally by radiation at 320 mμ in acid and 355 mμ in base. Another impurity found in the bityrosine control, $(BT)_h$, was observed to fluoresce near 325 mμ, excited maximally by 300-mμ radiation. Further identification of these impurities was not attempted. The results of the two-dimensional thin layer chromatography of the hydrolysate are shown in Figure 9. In this figure all samples subjected to the hydrolysis conditions are labeled with the subscript h. The spots identified in the chromatogram are indicated and were assigned by running the controls in the same manner as the polymer. The small amount of

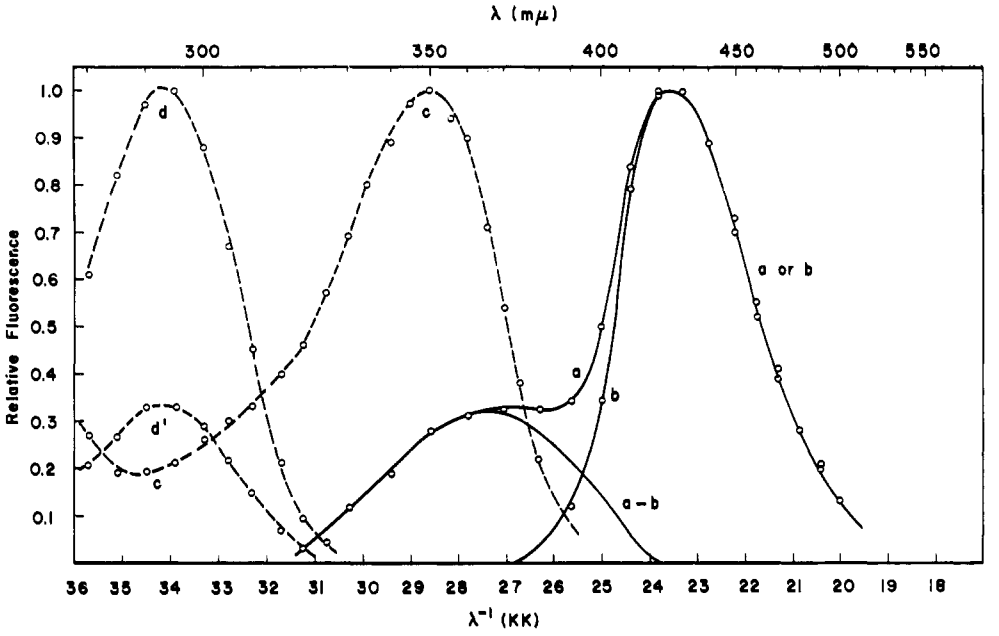

FIGURE 7: Normalized fluorescence of *o,o'*-bicresol in dimethyl sulfoxide. Fluorescence spectra, –O–O–. Excitation spectra, - -O- -O- -. (a) Emission spectrum, $\lambda_{excitation}$ 280 mμ; (b) emission spectrum, $\lambda_{excitation}$ 345 mμ, normalized at peak to a; (a–b) difference spectrum; (c) excitation spectrum, $\lambda_{emission}$ 440 mμ; (d) excitation spectrum, $\lambda_{emission}$ 370 mμ; and (d') curve d normalized to curves a–b.

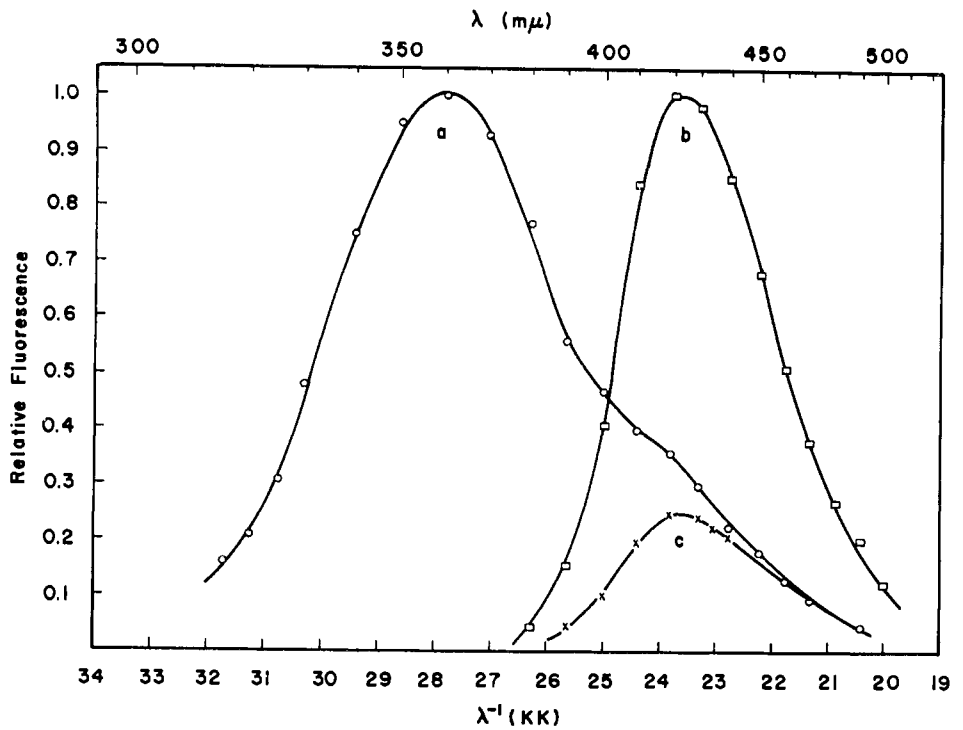

FIGURE 8: Normalized fluorescence of *o,o'*-bicresol in dimethyl sulfoxide solution. (a) Dimethyl sulfoxide with ≃ 5% 0.1 N HCl, $\lambda_{excitation}$ 280 mμ; (b) dimethyl sulfoxide with 5% 0.1 N NaOH, $\lambda_{excitation}$ 280 mμ; and (c) contribution of 420-mμ band in curve a.

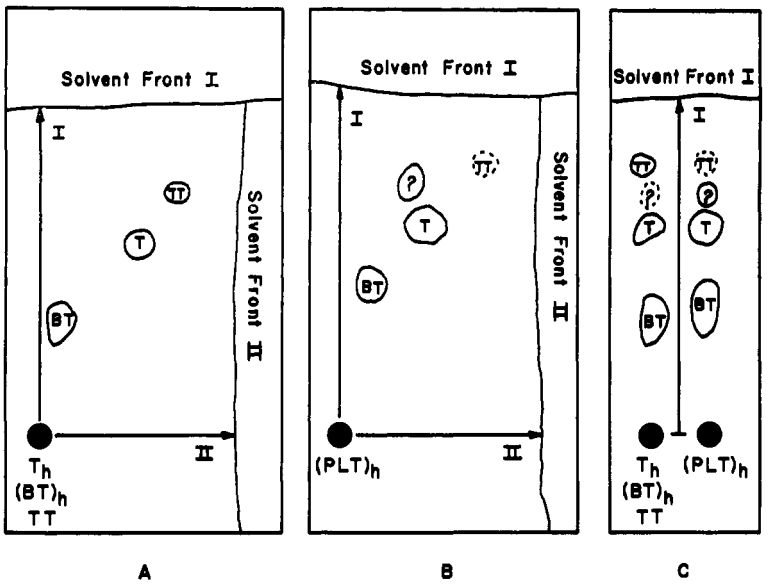

FIGURE 9: Two-dimensional thin layer chromatograph of irradiated poly-L-tyrosine hydrolysate and controls. The control hydrolysate consisted of bityrosine, $(BT)_h$, and tyrosine, $(T)_h$. A sample of unhydrolyzed L-tyrosyl-L-tyrosine (TT) was also applied to the control spot. Chromatograms A and B were first run in direction I using the first solvent and then in direction II using the second solvent. Chromatogram C was run only in solvent I. The spots were located by their fluorescence and/or by ninhydrin.

TyrTyr shown in section C was seen when very large quantities of hydrolysate were applied. This indicated that the hydrolysis was not quite complete. The spots, labeled ?, which also gave a bluish fluorescence, are probably the impurities identified spectrally and produced during the hydrolysis. This is evident because a slight bluish fluorescence was also observed in chromatogram C for the control near the same R_F value as the polymer hydrolysate. A greater quantity of this impurity is to be expected in the polymer hydrolysate since a larger quantity of material was applied for this system.

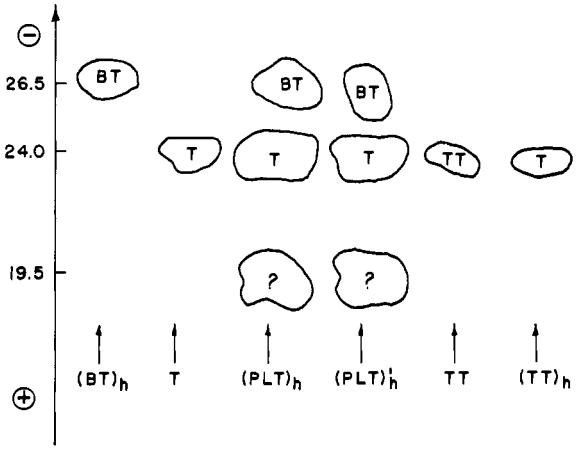

FIGURE 10: High-voltage electrophoresis of an irradiated poly-L-tyrosine hydrolysate and controls at pH 1.9 at 80 v/cm. Symbols as in Figure 9. Two hydrolysis samples were used here. The numbers on the left side indicate the distance in centimeters from the origin.

High-Voltage Electrophoresis

High-voltage electrophoresis was also used to separate and identify the bityrosine produced by the polymer irradiation, and subsequent hydrolysis. The result of this separation is shown in Figure 10. The BT spots were identified by the inherent blue fluorescence and by the subsequent ninhydrin stain.

The BT spot was observed to migrate faster than the other principal products and hence was identified quite easily. The spot labeled ? which also produced a bluish fluorescence was probably the same impurity observed in the thin layer chromatogram and observed by its different fluorescence characteristics in the hydrolysate mixture. Under sufficiently long hydrolysis conditions, TyrTyr can be completely hydrolyzed.

Discussion

It is clear from the work reported here that the characteristic blue fluorescence observed in phenolic systems is due to an irradiation effect which causes dimerization of the phenolic group to the corresponding *o,o'*-biphenol. This was shown to occur for various phenolic compounds as well as for PLT. The observation of this photoproduct was aided by the larger quantum yields and higher extinction coefficients of the dimer compared to the monomer. This is true both for alkaline solutions where $Q(\text{monomer})_{base} \simeq 0.01$ and $Q(\text{dimer})_{base} \simeq 0.64$. In DMSO the principal fluorescing species are the nonionized monomer and

the singly ionized dimer. For DMSO solutions Q-(monomer)$_{DMSO} \simeq 0.09$ and Q(dimer)$_{DMSO} \simeq 1.00$. Hence, only a small amount of dimerization affects the fluorescence spectrum markedly without a corresponding effect on the absorption spectrum. For example, it is estimated that a molar concentration of less than 0.5% dimer is necessary to produce equal monomer and dimer fluorescence for excitation at the respective absorption maxima, in an alkaline solution of tyrosine. At this concentration the absorbance of the dimer at 320 mμ would be less than 3% of the monomer peak absorbance and the irradiated absorption spectrum would not be easily distinguishable from the unirradiated spectrum.

The saturation of the 410-mμ fluorescence and corresponding absorption is to be interpreted as further reaction of the biphenol to higher oxidation products which do not fluoresce in the blue region. Andersen has reported that the biphenolic compounds were unstable to ultraviolet radiation, resulting in loss of the characteristic blue fluorescence (Andersen, 1966). This phenomenon was also observed here. The mechanism of the photooxidation of phenolic compounds has recently been extensively studied. It has been found that dimeric products are produced *via* the combination of transient phenoxy radicals formed by photoelectron ejection from the phenol (Land and Porter, 1963; Dobson and Grossweiner, 1965; Joschek and Miller, 1966). The primary product of ultraviolet irradiation of *p*-cresol has been found to be OBC and further irradiation of OBC yielded higher oxidation products (Joschek and Miller, 1966).

The observation that photodimerization of tyrosine occurs efficiently in PLT, copolymers, and the dipeptide, TyrTyr, indicates that this phenomenon may possibly occur in certain protein systems if the steric arrangement of Tyr residues is such as to allow close approach. Thus, the report that ultraviolet irradiation of collagen results in the production of blue fluorescence in the region of 450 mμ which is activated maximally by radiation in the 350-mμ region (Fujimori, 1965, 1966) indicates the strong probability of the formation of biphenolic products. The irradiated collagen was found to differ from the native collagen by its increased viscosity, in its inability to form native fibrils, and in its thermal transition profile as measured by viscosity, fluorescence, and optical rotation. These observations are consistent with the formation of intermolecular cross-links *via* tyrosine dimers.

Another report in the literature indicated unexplained blue fluorescence, with a peak near 410 mμ, in aqueous solutions of Tyr (Longin, 1959). This again is most probably due to inadvertent photochemical formation of bityrosine.

In general, it should be noted that photochemical alteration in proteins containing tyrosine, phenylalanine, and tryptophan can occur *via* the transient phenoxy, benzyl, and indolyl radicals formed by the ultraviolet irradiation (Grossweiner *et al.*, 1963). The secondary products formed will depend on the immediate environment of these chromophoric groups. The variety of spectral and other changes reported for ultraviolet irradiation (McLaren and Shugar, 1964) of various proteins is in part due to this multitude of possible further reactions of the transient species.

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