Excited State Chemistry of Aromatic Amino Acids and Related Peptides. I. Tyrosine

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Abstract: Optical excitation of phenolic compounds and tyrosine and tyrosyl peptides in water was carried out using a pulsed (~15 nsec duration) frequency quadrupled neodymium laser emitting at 265 nm. The compounds studied include phenol, anisole, *p*-cresol, tyrosine, *p*-hydroxyphenylpropionic acid, tyramine, tyrosylglycine, *N*-acetyltyrosylglycine, glycyltyrosylglycine, and cystinylbistyrosine. The lifetimes and triplet-triplet absorption spectra of the triplet states of these compounds were determined. The triplets have $\tau \sim 3$-10 μsec and absorb mainly in the uv region. These triplet states are effectively quenched by oxygen ($k_q \sim 5 \times 10^9$ M^{-1} sec^{-1}) and by disulfides RSSR ($k_q \sim 2$-4×10^9 M^{-1} sec^{-1}). The quenching mechanism was established to occur via an electron transfer with the formation of the superoxide radical $\cdot O_2^-$ and the RSSR$\cdot^-$ radical anion. A correlation is indicated between the quenching rate constants of ^{3}Tyr by organosulfur compounds and the reactivity of these compounds toward e_{aq}^-. The photoionization of these phenolic compounds and tyrosyl peptides is shown to occur primarily from the triplet state via a biphotonic process. These laser experiments confirm earlier flash photolysis studies from this laboratory. The pH and temperature dependences of the yields of triplets and photoionization products (e_{aq}^- and phenoxy radicals) have been examined. The implications of these results to the excited state chemistry of tyrosyl containing proteins are discussed.

The macromolecular luminescence of natural proteins is represented largely by the contribution of the luminescence properties of phenylalanine, tyrosine, and tryptophan as perturbed by their presence in a polypeptide chain and the structural influence of the protein. From the studies (see ref 1 and 2 for review) of the fluorescence properties of the aromatic amino acids and proteins at room temperature, and their phosphorescence in organic glasses at low temperature (usually 77°K), various conclusions were reached with regard to energy transfer processes from the singlet excited states and the triplet states of these emitting molecules. The prime interest in these studies was the use of luminescence as a probe for the study of structural properties of proteins. Relatively little work has been carried out on the basic photochemical mechanisms of these systems.

Detailed studies have now been carried out on the chemistry of the excited states of aromatic amino acids, related model compounds, and peptides, with the object of establishing the nature of the precursors leading to the photoionization and photodissociation processes which these molecules undergo. In part I of this series, the particular role of the excited states of phenolic compounds, tyrosine, and tyrosyl peptides will be presented.

The fluorescence quantum yield of phenol and of the zwitterion of tyrosine in aqueous solutions is $\phi_F \sim 0.21$ at room temperature.[1,2] It is strongly dependent upon temperature,[3] decreasing with increasing T. Formation of an amide or a peptide link leads to a reduction in ϕ_F. Fluorescence lifetimes of 3.6 nsec[4] and 5.1 nsec[5] have been reported for tyrosine in water.

The photoionization of phenolic compounds and of tyrosine in water at 25° has been shown[6,7] to occur primarily from the triplet state via a biphotonic process, i.e., the absorption of a second photon by ^{3}Tyr-OH (triplet Tyr-OH)

$$^3\text{Tyr-OH} \xrightarrow{h\nu} \text{Tyr-O}\cdot + e_{aq}^- + H^+ \qquad (1)$$

The flash photolysis technique (time resolution ~10 μsec) was used,[6,7] but the triplet state was not observed. It was also not possible to exclude the possibility that (a) ≤30% of the phenoxy radicals may be produced by rupture of the OH bond, reaction 2, and (b) that a small percentage of the

$$^*\text{Tyr-OH} \longrightarrow \text{Tyr-O}\cdot + H \qquad (2)$$

photoionization process may be occurring by a different mechanism, e.g., from a vibrationally excited singlet state.[8]

Reported below are the results obtained using a frequency quadrupled neodymium laser emitting at 265 nm with pulses of ~15 nsec duration. Some preliminary results have been reported elsewhere.[9]

Experimental Section

A neodymium laser, supplied by Holobeam Inc. (Paramus, N.J.), was frequency quadrupled using temperature-controlled CDA and ADP crystals. The emission at 265 nm could be varied up to ~25 mJ/pulse, as read using a Quantronix 504 power meter and Model 500 head. Singles pulses of ~15 nsec duration were monitored using an ITT biplanar photodiode 4001 which sampled light at 90° to the laser axis via a "Spectrosil" quartz window beam splitter. Narrow laser pulses of ~3.5 nsec duration were obtained using a beam slicer (supplied by Holobeam Inc.) and the intensity was raised back to ~20-25 mJ by employing an amplifier (Holobeam) to increase the 1.06 μ laser output. Unless otherwise stated experiments were performed using pulses of 15 nsec duration. The 265 nm light output typically varied ±7% within the same day or from day to day. It was routinely monitored for every pulse using the ITT photodiode, and the results were normalized.

The jacketted spectrosil quartz optical cell was 10 × 10 × 10 mm, and the diameter of the laser beam was ~8 mm. A Neslab Model RTE-4 was employed as a temperature-controlled circulator.

The 265 nm light intensity was varied by using calibrated Schott filters (No. UG-5). Reproducible results were obtained in this way and this method was used to establish the light intensity dependence of the photophysical and photochemical processes.

Kinetic absorption spectrophotometry of the transients species formed was carried out using either (a) two back-to-back high-intensity Bausch and Lomb monochromators, an EMI 9558QB photomultiplier and associated circuitry[10] (the overall rise time of this circuitry was ≤100 nsec) or (b) a single high-intensity Bausch and Lomb monochromator, with an RCA 1P28 photomultiplier and associated circuitry[11] (the overall rise time of this circuitry was ≤2 nsec). The signal from the IP28 photomultiplier circuitry was either displayed on a Tektronix oscilloscope, Model No. 7904, with plug-ins 7A19, 7A18, and 7B92, or directly processed using an online Biomation 8100 waveform digitizer interfaced to a Hewlett Packard 9830A calculator, programmed for first-order and second-order kinetics.

The monitoring light source was as Osram 250 W Xenon lamp connected to a Kepco KS36-30M power supply. The light output was increased for ~1 msec using a booster circuitry,[11] resulting in an increase of ~400 times at $\lambda \leq 280$ nm.

Solutions were prepared just previous to use, and fresh samples were employed for each laser pulse. Highest purity research grade

Table I. Lifetimes of Triplet States of Phenolic Compounds, Tyrosine, and Related Peptides in Water at 25°

Compd[a]	pK_a	pH	Wavelength monitored, nm	k, sec^{-1}	τ
Phenol (3.4×10^{-4} M)	10.0	7.5	260	$3.0 \pm 0.2 \times 10^5$	3.3 μsec
Anisole (5.6×10^{-4} M)		8.5	250	$3.0 \pm 0.2 \times 10^5$	3.3 μsec
p-Cresol (3.5×10^{-4} M)	10.2	7.5	260	$2.9 \pm 0.2 \times 10^5$	3.4 μsec
p-OH phenylpropionic acid (4.4×10^{-4} M)	4.6, 10.1	7.5	250	$9.9 \pm 0.3 \times 10^4$ [c]	10.1 μsec
Tyrosine	2.2, 9.1, 10.1	6.0	250	$1.8 \pm 0.05 \times 10^5$ [b]	5.6 μsec
Tyramine (3.4×10^{-4} M)	9.5, 10.8	7.5	250	$1.0 \pm 0.2 \times 10^5$ [c]	10.0 μsec
L-Tyrosylglycine (5.0×10^{-4} M)		6.0	250	$2.9 \pm 0.2 \times 10^5$	3.4 μsec
N-Acetyltyrosine (6.0×10^{-4} M)		7.5	350	$3.1 \pm 0.4 \times 10^5$	3.2 μsec
L-Tyrosylglycylglycine (3.0×10^{-4} M)		6.0	250	$3.2 \pm 0.4 \times 10^5$	3.1 μsec
Glycyltyrosylglycine (6.1×10^{-4} M)	2.9, 8.5, 10.5	6.0	250	$2.7 \pm 0.2 \times 10^5$	3.7 μsec
Cystinylbistyrosine (5×10^{-4} M)		5.8	250	$2.6 \pm 0.4 \times 10^7$	38.5 nsec

[a] Numbers in parentheses are the concentration of the substrates at which the lifetimes were determined. [b] Value extrapolated to "zero" tyrosine concentration is $1.0 \pm 0.05 \times 10^5$ sec^{-1}. [c] Small second-order component observed.

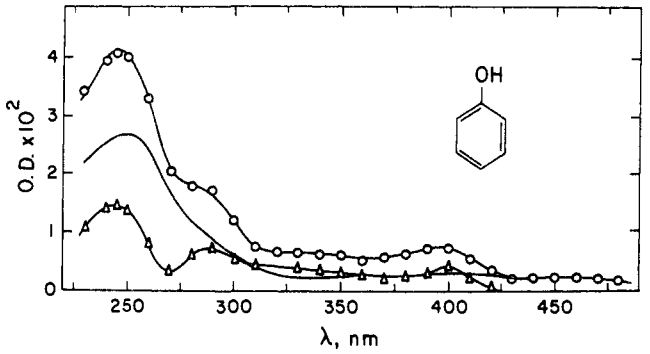


Figure 1. Absorption spectra of the transient species produced on optical excitation at 265 nm of phenol (6.0×10^{-4} M, pH 7.7, 25°) in water. OD read at 20 nsec (○) and at 15 μsec (△) after the 15 nsec laser pulse. The difference spectrum shown by a full line is the T-T absorption spectrum of phenol. At $\lambda > 320$ nm, the transient spectra were obtained in the presence of N_2O (1 atm) and 0.5 M *tert*-butyl alcohol to scavenge the e_{aq}^- produced by photoionization (see text).

chemicals were obtained from Calbiochem, Cyclochemicals, Sigma Chemicals, Baker and Adamson, J. T. Baker, and Aldrich Chemicals. Solutions were buffered using perchloric acid, potassium hydroxide, and ~1.0 mM borate and boric acid or 0.2 mM phosphates.

Actinometry was based on anthracene in cyclohexane. The T-T spectrum of anthracene was monitored at 428 nm (using narrow slits), taking[12] $\epsilon^{428} = 6.47 \times 10^4$ M^{-1} cm^{-1} and $\phi_{ISC} = 0.75$ (from ref 13).

The absorption spectrum of e_{aq}^- produced from the photoionization of the compounds studied is not shown in this work. Its presence was eliminated by saturation of the solution with N_2O (1 atm). The OH radicals produced from the reaction $e_{aq}^- + N_2O \rightarrow OH + N_2 + OH^-$, were scavenged by ~0.1-1.0 M *t*-BuOH. It was established in all cases that under the conditions of these experiments the N_2O, *t*-BuOH, and the β-hydroxy radical[14] produced from the alcohol did not interfere with the transient spectra or with the triplet lifetimes and quenching constants reported below.

The rate constants for quenching of the triplet states were derived from linear plots using three to five different concentrations of the quencher in each experiment.

Results and Discussion

Phenolic Compounds

Phenol. The fluorescence quantum yield of phenol in water is 0.21 and $\tau_F \sim 2.1$ nsec (see ref 1, 2, and 15). The triplet-triplet absorption spectrum and lifetime of ^{3}PhOH, like those of tyrosine and other phenolic compounds, have not been measured. The triplet energy level of phenol is reported[15] to be ~75-80 kcal/mol. The flash photolysis of phenol in water showed[7] the characteristic absorption spectrum of the hydrated electron[16] and the phenoxy radical.[17] The photoionization was found[7] to occur from the triplet state via a biphotonic mechanism.

Table II. Rate Constants for Quenching of Triplet State of Tyrosine and Related Peptides in Water at 25°

Tyrosine/peptides	Quencher	pH	k_q, M^{-1} sec^{-1} [a]
Tyrosine (6.1×10^{-4} M)	O_2	7.5	$4.8 \pm 0.2 \times 10^9$
	OH^-	7–10.3	$2.0 \pm 0.6 \times 10^{10}$
	H^+	2–7	$\leq 1.0 \times 10^5$
	Tyrosine	7.5	$2.5 \pm 0.1 \times 10^8$
	N-Acetyldiglycine	6.5	$1.0 \pm 0.4 \times 10^6$
	Histidine	3.8	$1.6 \pm 0.1 \times 10^8$
	Histidine	7.7	$2.2 \pm 0.2 \times 10^8$
	Lipoic acid (RSSR)	7.5	$4.0 \pm 0.2 \times 10^9$
	Cystamine (RSSR)	5.2	$1.7 \pm 0.2 \times 10^9$
	Cystamine (RSSR)	7.9	$1.5 \pm 0.2 \times 10^9$
	Dithiodipropionic acid	6.6	$1.4 \pm 0.1 \times 10^9$
Tyrosine (1.2×10^{-3} M)	Tryptophan	7.3	$6.0 \pm 1.5 \times 10^9$
Phenol (3.4×10^{-4} M)	O_2	7.1	$6.1 \pm 0.4 \times 10^9$
	Mn^{2+}	7.5	$1.1 \pm 0.1 \times 10^8$
p-Cresol (3.5×10^{-4} M)	O_2	7.5	$5.3 \pm 0.5 \times 10^9$
p-Cresol (3.0×10^{-2} M)	Tryptophan	7.3	$6.0 \pm 2.0 \times 10^9$
Anisole (5.6×10^{-4} M)	O_2	8.5	$6.3 \pm 1.0 \times 10^9$
L-Tyrosylglycine (5.0×10^{-4} M)	O_2	6.0	$3.6 \pm 0.6 \times 10^9$
Glycyltyrosylglycine (6.1×10^{-4} M)	O_2	6.0	$3.9 \pm 0.3 \times 10^9$
	Lipoic acid (RSSR)	6.0	$3.2 \pm 0.4 \times 10^9$

[a] Derived from k (sec^{-1}) vs. quencher concentration plots.

On optical excitation at 265 nm of phenol in oxygen-free water (6.0×10^{-4} M, pH 7.7, 25°) using a 15 nsec laser pulse, a transient absorption is observed immediately after the pulse, see Figure 1 (the spectrum of e_{aq}^- is not shown). At 15 μsec after the pulse, a different transient spectrum is observed. The bands with maxima at ~400, ~290, and ~245 nm correspond mainly to the characteristic spectrum of the phenoxy radical.[17,18] The difference spectrum (Figure 1) with a relatively strong maximum at ~250 nm, and an absorption extending into the visible region, is suggested to be the T-T absorption spectrum of phenol. Throughout this work in order to obtain the T-T spectra, it has been necessary to subtract an absorption measured after the decay of the triplet from an initial value. This method gives a correct spectrum provided that no new longer lived absorbances are produced during the decay of the triplet. Unless otherwise stated, this is assumed to be the case.

The lifetime of triplet phenol in water (3.4×10^{-4} M, pH 7.5) is 3.3 μsec; see Table I. This value is not corrected for the quenching of ^{3}PhOH by ground state phenol (see below). It is quenched by oxygen with $k_q = 6.1 \pm 0.4 \times 10^9$ M^{-1} sec^{-1} (Table II), obtained using three concentrations

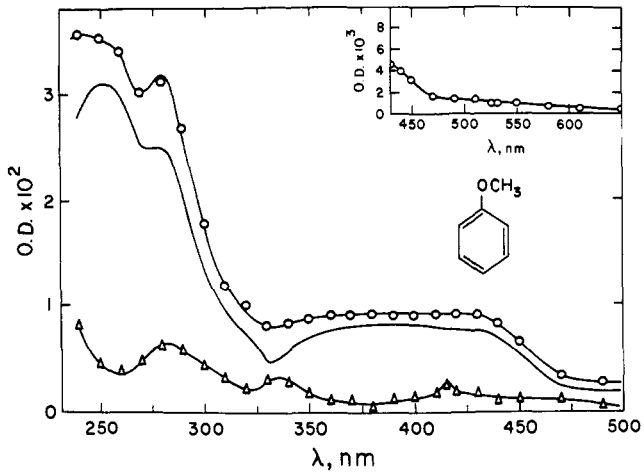


Figure 2. Absorption spectra of the transient species produced on optical excitation at 265 nm of anisole (5.6×10^{-4} *M*, pH 8.5, 25°) in water. OD read at 20 nsec (○) and at 15 μsec (△) after the pulse. The difference spectrum shown by a full line is the T-T absorption spectrum of anisole. At $\lambda > 320$ nm, the spectra were obtained in N_2O (1 atm) and 0.5 *M* *t*-BuOH.

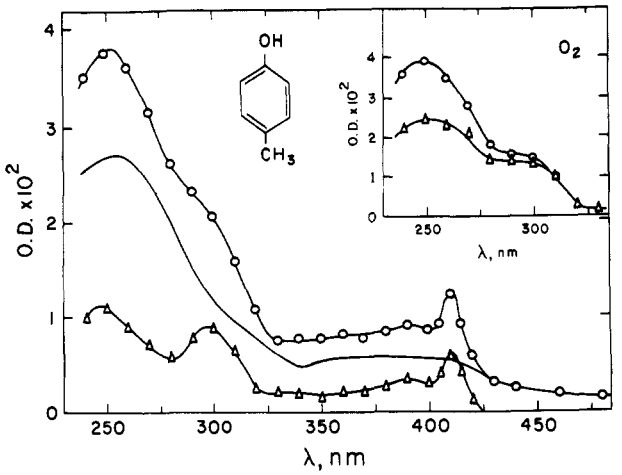


Figure 3. Absorption spectra of the transient species produced on optical excitation at 265 nm of *p*-cresol (6.0×10^{-4} *M*, pH 7.7, 25°) in water. OD read at 20 nsec (○) and at 15 μsec (△) after the laser pulse. The difference spectrum shown by a full line is the T-T absorption spectrum of *p*-cresol. At $\lambda > 320$ nm, the spectra were obtained in N_2O (1 atm) and 0.5 *M* *tert*-butyl alcohol (see text). Insert: transient spectra observed on excitation of *p*-cresol in the presence of oxygen (1 atm); OD read at 20 nsec (○) and 1 μsec (△) after the pulse.

of oxygen. The phenoxy radicals react much more slowly with oxygen,[7] and hence the "base line" on the oscilloscope trace is not affected by the overlapping absorption.

Phenol has a $pK_a = 9.95$. On optical excitation of phenolate ions at pH 11-12, both e_{aq}^- and PhO· radicals are produced *during* the 15 nsec laser pulse with a higher quantum yield compared to pH 7.7. No transient spectrum due to $^3PhO^-$ could, however, be seen (see more below).

Mn^{2+} ions present as $Mn(ClO_4)_2$ were found to quench triplet phenol with $k_q = 1.1 \pm 0.1 \times 10^8\ M^{-1}\ sec^{-1}$.

Anisole. The $\phi_F = 0.29$ and $\tau_F = 8.3$ nsec has been reported[19] for anisole in organic solvents. It has a slightly lower triplet energy level than phenol. Figure 2 shows the transient spectra observed at 20 nsec and at 15 μsec after the laser pulse. The latter spectrum is presumably due to the PhO· radical, together with the $PhOCH_2$· radical and other species.

The difference spectrum shown in Figure 2 is suggested to be the T-T absorption of anisole. It is found to decay (in 5.6×10^{-4} *M* solutions at pH 8.5) with $k = 3.0 \pm 0.2 \times 10^5\ sec^{-1}$. It is rapidly quenched by oxygen with $k_q = 6.3 \pm 1.0 \times 10^9\ M^{-1}\ sec^{-1}$. No changes were observed on increasing the pH from 8.5 to 12.0.

***p*-Cresol.** In cyclohexane solution,[19] $\phi_F = 0.09$ and $\tau_F = 2.3$ nsec. Figure 3 shows the transient spectra observed on laser photolysis of *p*-cresol in water at 20 nsec and 15 μsec after the pulse. The latter spectrum is probably due mainly to the PhO· radical, though one cannot exclude the formation of the *p*-OH benzyl radical. The triplet of *p*-cresol, represented by a full line in Figure 3, decays with $k = 2.9 \pm 0.2 \times 10^5\ sec^{-1}$ (in 3.5×10^{-4} *M* *p*-cresol at pH 7.5).

The ^3p-cresol is quenched by oxygen with $k_q = 5.3 \pm 0.5 \times 10^9\ M^{-1}\ sec^{-1}$. The insert in Figure 3 shows the transient spectra observed on photolysis in the presence of O_2. At 20 nsec after the pulse, the spectrum is essentially the same as that observed in the absence of O_2. At 1 μsec after the pulse (when all the ^3p-cresol are quenched), the spectrum observed is suggested to be due mainly to the phenoxy radical and $\cdot O_2^-$.

$$^3ROH + O_2 \longrightarrow RO\cdot + \cdot O_2^- + H^+ \qquad (3)$$

The $\cdot O_2^-$ radical is known[20] to absorb in the uv region with $\lambda_{max} \sim 245$ nm and $\epsilon_{245} = 2 \times 10^3\ M^{-1}\ cm^{-1}$. As will be discussed below in detail for tyrosine, oxygen can quench the triplet states of phenolic compounds by an electron transfer mechanism with the formation of phenoxy and superoxide radicals. This process is monophotonic whereas in the absence of oxygen a second quantum of light is needed to photoionize ^3ROH molecules.

In the presence of tryptophan, triplet-triplet energy transfer was observed from ^3p-cresol. On monitoring the *formation* kinetics of ^{3}Trp at 440 nm[21], a $k = 6.0 \pm 2.0 \times 10^9\ M^{-1}\ sec^{-1}$ was found (see Table II) for reaction 4. At this wavelength, the absorption of ^3p-cresol is relatively very weak.

$$^3p\text{-cresol} + Trp \longrightarrow {}^3Trp + p\text{-cresol} \qquad (4)$$

***p*-Hydroxyphenylpropionic Acid.** The $\phi_F = 0.20$ and the τ_F of this compound in water are the same as that of tyrosine in neutral solution.[5] The triplet state lifetime in water is 10.0 μsec (Table I), which is longer than that of the tyrosine zwitterion ($\tau = 5.6$ μsec). The protonated α-amino group would seem to shorten the triplet state lifetime in tyrosine.

Tyrosine

Excited States. A considerable amount of work has been carried out on the fluorescence of tyrosine in water. The $\phi_F = 0.21$ and $\tau_F = 3.6$ nsec for the zwitterion.

On optical excitation at 265 nm of tyrosine (pK_a's 2.2, 9.1, 10.1) in oxygen-free aqueous solutions (6.0×10^{-4} *M*, pH 7.5, 25°), somewhat different transient optical spectra are observed when measured at 20 nsec and at 15 μsec after the 15 nsec laser pulse, see Figure 4a. The "15 μsec spectrum" is suggested to represent primarily the characteristic absorption of the tyrosine phenoxy radical Tyr-O·. It may, in addition, include a weak absorption due to other species, e.g., the *p*-hydroxybenzyl radical. Reaction 5 is suggested on the basis of a similar photodissociation reaction observed on optical excitation of phenylalanine;[22,23] but no evidence for this reaction is presently available.

$$HOPhCH_2CH(NH_3^+)COO^- \xrightarrow{h\nu} HOPhCH_2\cdot + \dot{C}H(NH_3^+)COO^- \qquad (5)$$

The difference spectrum shown in Figure 4a is suggested to be the T-T absorption spectrum of tyrosine. Its maxima are at 250, 295, and ~575 nm. This spectrum is much broader than the T-T spectrum of phenol or *p*-cresol. The ^{3}Tyr decays (in 3.4×10^{-4} *M* solutions at pH 6.0) with *k*

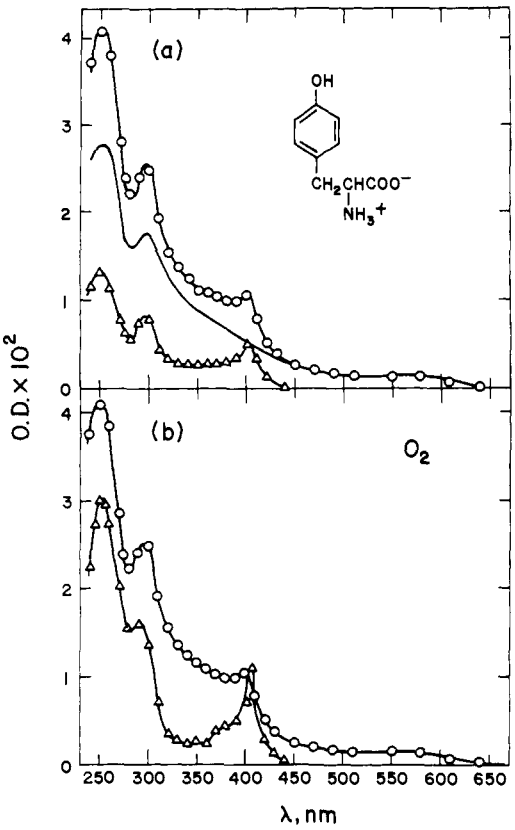


Figure 4. Absorption spectra of the transient species produced on optical excitation at 265 nm of tyrosine (6.0×10^{-4} *M*, pH 7.5, 25°) in water. (a) In argon (1 atm), OD read at 20 nsec (○) and at 15 μsec (△) after the pulse. The difference spectrum shown by a full line is the T-T spectrum of tyrosine. At $\lambda > 320$ nm, N_2O (1 atm) and 0.5 *M* *t*-BuOH were present in the solution. (b) In oxygen (1 atm), OD read at 20 nsec (○) and at 1 μsec (△) after the pulse.

$= 1.8 \pm 0.5 \times 10^5$ sec^{-1}, see Table I. It is quenched by ground state tyrosine with $k_q = 2.5 \times 10^8$ M^{-1} sec^{-1} (Table II). At "zero" tyrosine concentration, the triplet state of tyrosine decays with $k = 1.0 \pm 0.05 \times 10^5$ sec^{-1}.

The triplet state of tyramine (3.4×10^{-4} *M*, pH 7.5) in water, $k = 1.0 \pm 0.2 \times 10^5$ sec^{-1}, decays more slowly than ^{3}Tyr. Its lifetime is similar to that of *p*-hydroxyphenylpropionic acid (see above).

In the presence of oxygen, ^{3}Tyr is rapidly quenched with $k_q = 4.8 \pm 0.2 \times 10^9$ M^{-1} sec^{-1} (Table II). On optical excitation of 6.0×10^{-4} *M* tyrosine in the presence of oxygen (1 atm, $[O_2] = 1.2 \times 10^{-3}$ *M*), the transient spectrum observed at 20 nsec after the pulse is identical to that produced in the absence of oxygen (compare Figures 4a and 4b). At 1.0 μsec after the laser pulse, all the tyrosine triplets have decayed and the observed spectrum is essentially similar to the "15 μsec spectrum" in the absence of oxygen, but more intense by a factor of ~2.2, see Figure 4b. This increase in the yield of phenoxy radicals is suggested to occur from the quenching of triplet tyrosine by O_2.

$$^3\text{Tyr-OH} + O_2 \longrightarrow \text{Tyr-O}\cdot + \cdot O_2^- + H^+ \qquad (6)$$

The superoxide radical has[20] a $\lambda_{max} \sim 245$ nm and $\epsilon_{245} = 2 \times 10^3$ M^{-1} cm^{-1}, and its contribution is mainly to the 255 nm band in Figure 4b. The Tyr-O· radical was found[6] to have an $\epsilon_{405} = 2.8 \times 10^3$ M^{-1} cm^{-1}.

The Tyr-O· radicals in the absence of oxygen decay by second-order kinetics[6] with $2k = 1.2 \times 10^9$ M^{-1} sec^{-1}. In the presence of oxygen, they decay by reaction with O_2 with[7] $k \leq 10^6$ M^{-1} sec^{-1}.

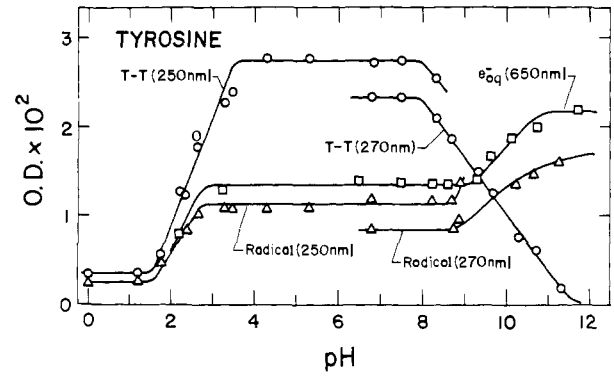


Figure 5. Titration curves of the transient species observed on optical excitation of tyrosine (6×10^{-4} *M*, 25°) in water. The e_{aq}^- were read at 20 nsec, T-T at 20 nsec, and the radical at 15 μsec after the laser pulse.

Dependence upon pH. Figure 5 shows the dependence upon pH of the yields of triplet, e_{aq}^-, and radical (mainly phenoxy radicals) produced from the 265 nm excitation of tyrosine (6×10^{-4} *M*, 25°) in oxygen-free aqueous solutions. The wavelengths monitored in alkaline solutions were changed (as indicated) due to the red shift in the absorption of tyrosinate ions as compared to tyrosine. However, it should be noted that OD_{265} remained constant throughout the pH range studied.

From these titration curves, one can derive in the usual manner approximate dissociation constants. The triplet shows dissociations with $pK_a \sim 2.5 \pm 0.2$ and 9.7 ± 0.2. The e_{aq}^- show pK_a of ≤ 2.2 and $\sim 10.1 \pm 0.2$ and the radicals 2.2 ± 0.2 and 10.1 ± 0.2. These values are to be compared with the ground state pK_a values of 2.2 (COOH), 9.1 (NH_3^+), and 10.1 (OH) for tyrosine. It is clear that ionization of the phenolic proton and of the carboxyl group play an important role in the formation of triplets, e_{aq}^-, and (it follows) phenoxy radicals.

It is important to note the following points: (a) an almost quantitative correlation between the triplet yields and the fluorescence yields[4,5] with pH (both show the same apparent pK_a values and their yields decrease to zero in alkaline solutions and decrease to ~15% in acidic solutions); (b) while the yield of ^{3}Tyr decreases in alkaline solution that of e_{aq}^- (and Tyr-O·) increases following the ionization of Tyr-OH; (c) the lifetime of the triplet at pH >8 is dependent upon $[OH^-]$, with $k = 2.0 \pm 0.6 \times 10^{10}$ M^{-1} sec^{-1} (see Table II), presumably according to reaction 7 (since no

$$^3\text{Tyr-OH} + OH^- \longrightarrow {}^3\text{Tyr-O}^- + H_2O \qquad (7)$$

T-T spectrum of ^{3}Tyr-O$^-$ could be seen, its lifetime is probably very short, $\tau < 10^{-9}$ sec; the decrease in the yield of triplets shown in Figure 5 is *not* due to reaction 7 since the absorbances were read at ~20 nsec); (d) in view of the apparent very short lifetime of ^{3}Tyr-O$^-$, one cannot exclude the possibility of populating the triplet state from ^{1}Tyr-O$^-$; however, the fluorescence of ^{1}Tyr-O$^-$ has been observed only in low temperature glasses indicating that its lifetimes at 20° is probably $\leq 10^{10}$ sec^{-1}; (e) at pH below ~3.5, the lifetime of ^{3}Tyr is not affected by $[H^+]$, Table II.

Dependence upon Light Intensity. Flash photolysis work[7,6] has indicated that the photoionization of tyrosine occurs in neutral solutions from the triplet state via a biphotonic process. Laser photolysis work, Figure 6, supports these conclusions. In alkaline solutions the formation of e_{aq}^- from tyrosinate was monophotonic and was suggested[24] to occur from the triplet state. However, since the T-T absorption of ^{3}Tyr-O$^-$ was not observed the excited state precursor could be either ^{3}Tyr-O$^-$ or ^{1}Tyr-O$^-$. It is not possible at present to establish this point.

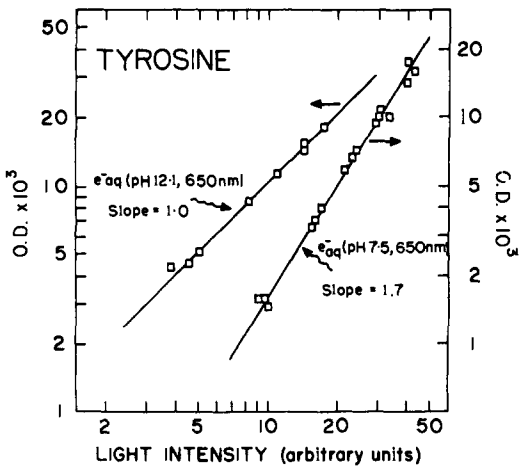


Figure 6. Dependence upon 265 nm light intensity of the yields of e_{aq}^- produced on optical excitation of tyrosine (6×10^{-4} *M*) at pH 7.5 and 12.1, 25°.

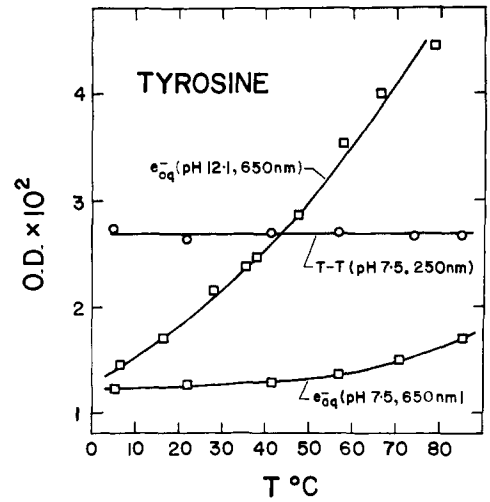


Figure 7. Dependence upon temperature of the yields of transient species produced from the optical excitation of tyrosine (6×10^{-4} *M*) at pH 7.5 and 12.1.

The slope = 1.7 observed in neutral solution (i.e., less than 2.0, as expected for a process αI^2; see Figure 6) may be due to a number of factors, e.g., the formation of e_{aq}^- via a mechanism other than the absorption of a second quantum of light by ^{3}Tyr-OH, as discussed above.

Dependence upon Temperature. Over the temperature range 25–95°, the shape of the fluorescence band of tyrosine in water remains unaltered and ϕ_F decreases with increasing temperature.[3] A similar dependence of ϕ_F upon pH was observed[3] at 25 and 95°.

Figure 7 shows the results observed. The ϕ_T was found to be independent of temperature from 5 to 85° at pH 7.5. The $\phi_{e_{aq}^-}$ from tyrosine (pH 7.5) is somewhat dependent upon temperature, while at pH 12.1 $\phi_{e_{aq}^-}$ is very strongly temperature dependent. It should be pointed out that the absorption maximum of e_{aq}^- is red shifted[16] with increase in temperature, and the $\epsilon(e_{aq}^-)$ at 650 nm decreases with increase in *T*. These effects are expected to lead to an even more pronounced effect of $\phi(e_{aq}^-)$ with *T*, Figure 7. The following additional points can be made. (a) At pH 7.5, one would not expect[7] a strong dependence of $\phi_{e_{aq}^-}$ upon temperature for a biphotonic process. The small temperature dependence observed, while the ϕ_T remains unchanged, may be due to either a more efficient biphotonic process or a temperature dependence of $\phi_{e_{aq}^-}$ produced from another mechanism, e.g., vibrationally excited singlet tyrosine. (b) At pH 12.1, the photoionization reaction is monophotonic. The e_{aq}^- is produced within the 15 nsec (or 3.5 nsec) laser pulse. The strong dependence of $\phi_{e_{aq}^-}$ on *T* is not inconsistent with a vibrationally excited singlet state as the precursor. (c) With phenylalanine,[23] however, photoionization at pH 7.0 and 12.0 occurs from the triplet state via a biphotonic process, and a strong temperature dependence was observed: $\phi_{e_{aq}^-}$ *decreases* with increasing temperature.

Quenching Reactions of Triplet Tyrosine. The quenching of ^{3}Tyr by oxygen and its electron transfer mechanism have been discussed above (see reaction 6). The quenchings by ground state tyrosine, OH^-, and H^+ have also been discussed.

Carbonyl, amide, and peptide groups have been suggested to quench the fluorescence of aromatic amino acids. The lifetimes of the triplet states of the simple peptides of tyrosine (see below), phenylalanine,[23] and tryptophan[21] are all shorter than that of the zwitterions of the corresponding aromatic amino acids. Using *N*-acetylglycylglycine as a model for the peptide linkage, it was found to quench ^{3}Tyr with $k_q = 1.0 \pm 0.4 \times 10^6$ M^{-1} sec^{-1} (see Table II).

Histidine (pK_a = 1.8, 6.0, 9.2) was found to quench ^{3}Tyr with $k_q = 1.6 \pm 0.1 \times 10^8$ M^{-1} sec^{-1} and $2.2 \pm 0.2 \times 10^8$ M^{-1} sec^{-1} at pH 3.8 and 7.7, respectively.

The reaction of tryptophan with ^{3}Tyr is, of course, an important one in proteins since Trp has a lower triplet energy level. We were able to observe energy transfer from ^{3}Tyr to Trp. The rate constant $k = 6.0 \pm 1.5 \times 10^9$ M^{-1} sec^{-1} for reaction 8 was determined by monitoring the rate of forma-

$$^3\text{Tyr} + \text{Trp} \longrightarrow \text{Tyr} + {}^3\text{Trp} \qquad (8)$$

tion of ^{3}Trp at 440 nm.[21] At this wavelength the ^{3}Tyr absorption is relatively weak compared to ^{3}Trp.

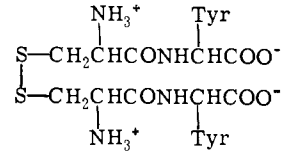
Quenching by RSSR, RSH, and RSR Substrates. The quenching of the fluorescence of tyrosine and tyrosyl residues by disulfides and thiols is well documented,[1,2,25-27] but the mechanism is not established.

The quenching of the triplet state of tyrosine by disulfides (RSSR) was recently[24] demonstrated and the mechanism was established to be an electron transfer to form the $RSSR\cdot^-$ radical anion

$$^3\text{Tyr} + \text{RSSR} \longrightarrow \text{RSSR}\cdot^- + \text{P}\cdot \qquad (9)$$

Using the cyclic disulfide lipoic acid, the characteristic[28] transient absorption spectrum of the lipoate$\cdot^-$ radical anion with $\lambda_{max} \sim 420$ nm was observed.[24] The rate constants of reaction 9 for various disulfides were determined, see Table II. Values ranging from 4.0×10^9 to 1.4×10^9 M^{-1} sec^{-1} for lipoate ions and dithiodipropionic acid, respectively, were derived. The formation of $RSSR\cdot^-$ radicals was confirmed.

The tyrosine in L-cystinylbis-L-tyrosine has a ϕ_F = 0.016[25] in water compared to 0.21 for tyrosine. This loss of



fluorescence is ascribed to "internal" quenching by the -SS- group. On optical excitation of this compound at 265 nm, a very short lived weakly absorbing intermediate was observed which decayed with $k = 2.6 \pm 0.4 \times 10^7$ sec^{-1} (Table II). This is ascribed to the triplet state of cystinylbistyrosine. Subsequent to the decay of this triplet, an absorption with $\lambda_{max} \sim 420$ nm characteristic of the $RSSR\cdot^-$ radical was observed. The lifetime of this triplet state is ~100 times shorter than that of ^{3}Tyr. It is clear that quenching by the -CONH- linkages does not account for the $\tau \sim 38$ nsec (see tyrosine peptides below), and therefore

Table III. Quenching of the Triplet State of Tyrosine by Organosulfur Compounds in Water at 25°[a]

Quencher[b]	pH	Ionic form	k_q, M^{-1} sec^{-1}[c]	$k(e_{aq}^- + Q)$, M^{-1} sec^{-1}[d]
Cysteamine (8.6, 10.7)	5.2	$HSCH_2CH_2NH_3^+$	$5.8 \pm 0.7 \times 10^8$	3.0×10^{10}
Cysteine (1.8, 8.3, 10.8)	7.0	$HSCH_2CH(NH_3^+)COO^-$	$5.2 \pm 0.7 \times 10^8$	1.3×10^{10}
N-Acetylcysteine (~2, 9.5)	5.3	$HSCH_2CH_2(NHCOCH_3)COO^-$	$4.0 \pm 0.4 \times 10^8$	5.6×10^9
S-Methylcysteine (~2, 8.8)	5.5	$CH_3SCH_2CH(NH_3^+)COO^-$	$3.2 \pm 0.2 \times 10^7$	7.2×10^8
Djenkolic acid	5.4	$CH_2[SCH_2CH(NH_3^+)COO^-]_2$	$\leq 2.0 \times 10^7$	1.0×10^{8e}
Thiodiacetic acid (3.3, 4.5)	6.3	$S(CH_2COO^-)_2$	$6.0 \pm 3.0 \times 10^6$	8.3×10^7
β-Thiodipropionic acid (~4)	7.2	$S(CH_2CH_2COO^-)_2$	$6.5 \pm 1.0 \times 10^6$	5.8×10^7
Methionine (2.3, 9.2)	5.7	$CH_3SCH_2CH_2CH(NH_3^+)COO^-$	$5.0 \pm 2.0 \times 10^5$	4.5×10^7

[a] 1.0×10^{-3} *M* concentration of tyrosine used. [b] Numbers in parentheses are the pK_a values of the quencher. [c] Derived from k (sec^{-1}) vs. quencher concentration plots. [d] From ref 29. [e] From ref 16.

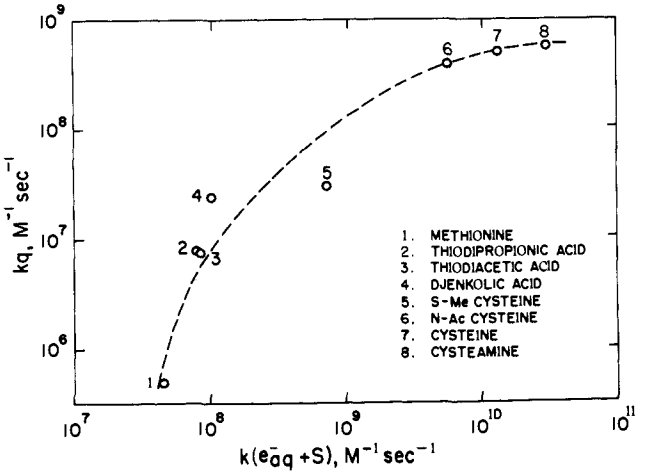


Figure 8. Correlation between the quenching rate constants of the triplet state of tyrosine (~1.0×10^{-3} *M*, 25°) by RSH and RSR compounds and the rate constants of e_{aq}^- with the same organosulfur compounds (see Table III and text).

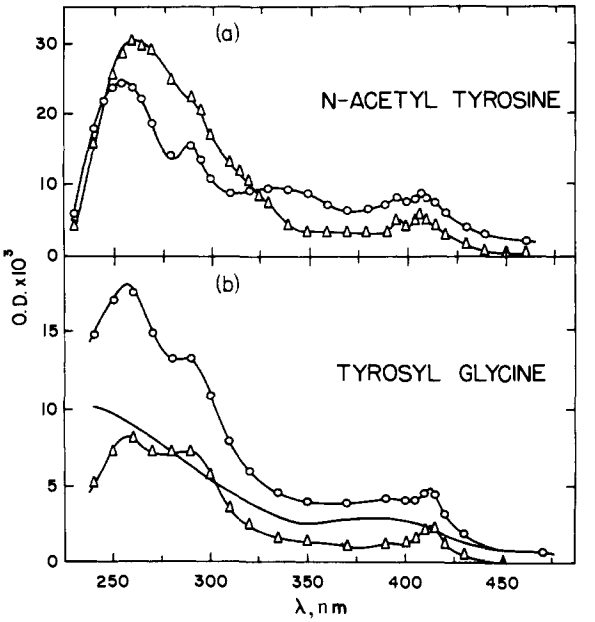


Figure 9. Absorption spectra of the transient species produced on optical excitation at 265 nm in water at 25° of (a) *N*-acetyltyrosine (6×10^{-4} *M*, pH 7.5), and (b) tyrosylglycine (6×10^{-4} *M*, pH 6.0). OD read at 20 nsec (○) and at 15 μsec (△) after the pulse. The difference spectrum in (b) is the T-T spectrum of Tyr-Gly. At $\lambda > 320$ nm, N_2O (1 atm) and 0.5 *M* *t*-BuOH were present in the solution.

the short lifetime is due to internal quenching by the vicinal -SS- group. This finding is considered to be important with respect to the photochemistry of proteins since it is known[28] that the formation of $RSSR^{\cdot -}$ leads to rupture of the disulfide bridge, and therefore may lead to inactivation of the enzyme.

$$RSSR^{\cdot -} \longrightarrow RS^{\cdot} + RS^- \qquad (10)$$

Sulfhydryl groups and thioethers have also been found to quench ^{3}Tyr, but these quenching rate constants are well below the diffusion-controlled limits, see Table III. There appears to be a correlation between the quenching rate constants k_q and the reactivity of the same quenchers toward e_{aq}^-, as determined[29] by pulse radiolysis, see Figure 8.

It is probably the first such correlation found, and underlines the suggested electron transfer mechanism for the quenching of triplet tyrosine. Reactions 12 and 13 have

$$^3Tyr + RSH \longrightarrow (RSH)^{\cdot -} + P^{\cdot} \qquad (11)$$
$$(RSH)^{\cdot -} \longrightarrow R^{\cdot} + SH^-$$
$$R^{\cdot} + RSH \text{ (or } RS^-) \longrightarrow RS^{\cdot} + RH \qquad (12)$$
$$RS^{\cdot} + RS^- \rightleftharpoons RSSR^{\cdot -} \qquad (13)$$

been suggested[29] previously. Small yields of $RSSR^-$ have been found.

The reason for the apparent leveling of k_q at ~10^9 M^{-1} sec^{-1} (Figure 8) is not clear at present.

Tyrosine Peptides. The photoionization of tyrosyl peptides as indicated by the formation of e_{aq}^- and phenoxy radicals has been observed in all cases examined.

***N*-Acetyltyrosine.** The ϕ_F of this compound[30] is 0.18 at pH 7.0. Optical excitation at 265 nm of *N*-Ac-Tyr (6×10^{-4} *M*, pH 7.5) produces, at 20 nsec after the laser pulse, a transient spectrum showing the features of the triplet state, phenoxy radical, and at least one other radical absorbing below ~370 nm; see Figure 9a. At ~15 μsec after the pulse, an increase in absorbance is observed at $\lambda < 320$ nm and a decrease at $\lambda > 320$ nm. The decrease in absorbance is due to the decay of the triplet state, and a $k = 3.1 \pm 0.4 \times 10^5$ sec^{-1} was determined (Table I).

The increase in absorbance around 270 nm was also found to have the same rate constant, indicating that it is produced from the decay of ^{3}*N*-Ac-Tyr. The nature of the species formed, which is presumably a radical(s) produced by a dissociative reaction, is not known. One of the photodissociative reactions produced from ^{3}Phe was shown[22,23] to lead to the formation of $PhCH_2^{\cdot}$ and $CH_3CONHCHCOO^-$ radicals. Comparable species may also be formed from *N*-Ac-Tyr and would be consistent with the observed transient spectrum. Similar photodissociative reactions from the triplet state via a monophotonic mechanism are observed whenever the α-amino group of Tyr is substituted by an acetyl or peptide group; see, e.g., Gly-Tyr-Gly below.

Tyrosylglycine. At pH 6.0, the $\phi_F = 0.07$ for this compound.[30] Laser photolysis of Tyr-Gly (6×10^{-4} *M*) at pH 6.0 shows a transient spectrum whose absorbance decreases between 20 nsec and 15 μsec after the laser pulse, see Figure 9b. The difference spectrum is assigned to the T-T absorption of Tyr-Gly. It decays with $k = 2.9 \pm 0.2 \times 10^5$ sec^{-1} and is quenched by oxygen with $k_q = 3.6 \pm 0.6 \times 10^9$ M^{-1} sec^{-1} (see Tables I and II).

The spectrum observed at 15 μsec after the pulse shows

Table IV. Relative Yields of Intermediate Species Produced from the Optical Excitation at 265 nm of Tyrosine and Related Peptides in Water at 25°

Substrate[a]	pH	e_{aq}^- $\phi_{e_{aq}^-} \times 10^2$	e_{aq}^- Ratio[b]	Initial $OD_{250} \times 10^2$	Radical $OD_{250} \times 10^2$
Tyrosine	7.5	9.5	1.0	3.8	1.1
	11.5	15.1	1.6		
Tyrosylglycine	6.0	5.1	0.5	1.7	0.8
Glycyltyrosyl-glycine	6.0	3.5	0.4	1.0	0.7

[a] Determined from solutions whose absorbance at 265 nm was 0.8. [b] Ratio based on Tyr at pH 7.5, $\phi = 1$.

the presence of another radical(s) in addition to the characteristic absorption of the phenoxy radical.

With tyrosylglycine, as with the other tyrosyl peptides examined, the e_{aq}^- and phenoxy radicals are formed within the 15 nsec laser pulse. As discussed above for tyrosine and other phenolic compounds, the photoionization of tyrosyl peptides is mainly a biphotonic process with the triplet state as the precursor. The photodissociative reactions observed (other reactions are probably occurring but have not been observed by this technique) occur from the decay of the triplet state via a monophotonic mechanism. Hence the latter reactions can occur, e.g., in proteins, even when low intensity light sources are used.

The triplet state of tyrosylglycylglycine was found to decay with $k = 3.2 \pm 0.4 \times 10^5$ sec^{-1} (Table I).

Glycyltyrosylglycine. The quantum yield for photoionization of this peptide is ~40% that of tyrosine (see Table IV). The transient spectra observed at 20 nsec and 15 μsec after the laser pulse are shown in Figure 10. Here again, relatively strong transient absorptions (other than the phenoxy radical) remain after the decay of the triplet, indicative of dissociative reactions. A comparison of the initial and later spectra indicates that these dissociation reactions occur, at least partially, from the triplet state.

The ^{3}Gly-Tyr-Gly decays with $k = 2.7 \pm 0.2 \times 10^5$ sec^{-1} and is quenched by oxygen with $k_q = 3.9 \pm 0.3 \times 10^9$ M^{-1} sec^{-1} and by lipoate ions with $k_q = 3.2 \pm 0.4 \times 10^9$ M^{-1} sec^{-1} (see Tables I and II).

On optical excitation of Gly-Tyr-Gly in the presence of oxygen (1 atm), the same transient spectrum as found in O_2-free solutions is observed at ~20 nsec after the pulse. At ~1 μsec later, all the triplets have been quenched by O_2 and a good fraction of the free radicals have also reacted with O_2 to produce the corresponding peroxy free radicals. The transient spectrum observed (Figure 10) is mainly due to phenoxy radicals (these react slowly with O_2, see above), O_2^-,[20] and peroxy radicals.[15,31] Peroxy radicals and O_2^- usually have $\lambda_{max} \leq 250$ nm, and low extinction coefficients compared to phenoxy radicals. As we concluded above for tyrosine, the quenching of ^{3}Gly-Tyr-Gly by oxygen and by disulfides also occurs via an electron transfer mechanism.

$$^3\text{Gly-Tyr-Gly} + O_2 \longrightarrow \cdot O_2^- + \text{RO}\cdot + H^+ \quad (14)$$

$$^3\text{Gly-Tyr-Gly} + \text{lipoate} \longrightarrow \text{lipoate}^{\cdot -} + R\cdot^+ \quad (15)$$

Conclusions

The lifetimes, absorption spectra, and the chemistry of the triplet states of phenolic compounds, tyrosine, and tyrosine peptides have been studied and determined for the first time. These triplets have lifetimes of ~3-10 μsec in water. They are rapidly quenched by oxygen with $k_q \sim 5 \times 10^9$ M^{-1} sec^{-1} and by disulfides with $k_q \sim 2\text{-}4 \times 10^9$ M^{-1} sec^{-1}.

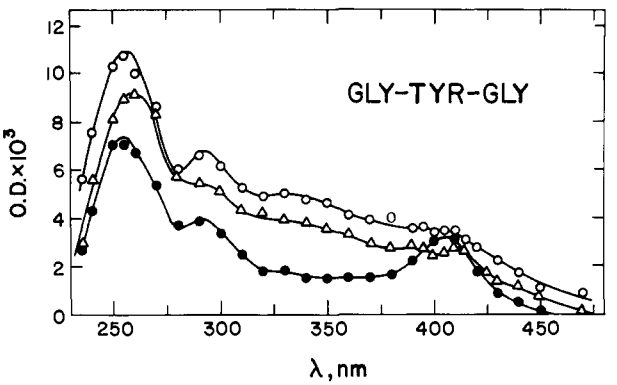


Figure 10. Absorption spectra of the transient species produced on optical excitation at 265 nm of glycyltyrosylglycine (6×10^{-4} M, pH 4.5) in water at 25°. (a) In oxygen-free solutions, OD read at 20 nsec (○) and at 15 μsec (△) after the pulse; at $\lambda > 320$ nm, N_2O (1 atm) and 0.5 M t-BuOH were present in the solution. (b) In oxygen (1 atm), OD read at 1 μsec after the pulse (●).

The quenching of the triplet states of tyrosine and of tyrosyl peptides by O_2 and RSSR is shown to occur via an electron transfer mechanism with the formation of the superoxide $\cdot O_2^-$ radical and the $RSSR\cdot^-$ radical anion. The quenching mechanism which affects tyrosyl residues in proteins associated with vicinal disulfide linkages may be explained on the basis of electron transfer processes to the RSSR groups.

The photoionization of tyrosine and tyrosyl peptides in water has been shown to occur mainly from the triplet state via a biphotonic process. The quantum yield of this reaction is, therefore, dependent on the lifetime of the triplet state, the intensity of the exciting light at the appropriate wavelength, and the quantum yield of the biphotonic process. The ionization of the triplet states by O_2 and RSSR is a monophotonic process, and hence could be of great importance in the photochemistry of tyrosyl containing proteins exposed to low intensities of exciting light.

Finally, the e_{aq}^- produced in the photoionization of tyrosyl proteins can react with aromatic[6,32] and aliphatic[33] amino acids, the peptide linkage,[34] disulfides,[28] and sulfhydryl amino acids[29] and lead to deamination reactions, electron transfer reactions, and ruptures of linkages which may affect the conformation and activity of proteins.

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Excited State Chemistry of Aromatic Amino Acids and Related Peptides. II. Phenylalanine

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Abstract: A pulsed frequency quadrupled neodymium laser emitting at 265 nm (~15 nsec duration) was used to optically excite phenylalanine and derivatives in water at 25°. The following systems were studied: benzene, toluene, phenylpropionic acid, phenylalanine, *N*-acetylphenylalanine amide, phenylalanine amide, and glycylphenylalanylglycine. The triplet-triplet absorption spectra of most of these compounds were observed and their lifetimes determined. These lifetimes are ~1–3 μsec and are shorter lived than those observed for the corresponding tyrosine derivatives. The triplet states are quenched by oxygen with $k_q \sim 3\text{-}5 \times 10^9\ M^{-1}\ \text{sec}^{-1}$. The photoionization and photodissociation of phenylalanine were studied as a function of pH and temperature. The photoionization of phenylalanine and derivatives has the triplet state as the main precursor, and electron ejection occurs in all cases via a biphotonic process. The $\phi_{e_{aq}^-}$ is dependent on the state of protonation of the NH_3^+ group, and decreases when the α-amino group is present as NH_2. The photodissociation reactions occur, at all pH values, primarily via the triplet state as precursor. The dissociation processes can be biphotonic or monophotonic, depending on certain conditions which have been defined. In proteins, phenylalanine is expected to dissociate via a monophotonic process. The photoionization of benzene in water is reported. These and other results are discussed.

On the basis of the singlet excited state and the triplet state energies of phenylalanine, it has generally been considered that the sequence of electronic energy transfer from phenylalanine → tyrosine → tryptophan was feasible and probable in protein macromolecules containing these aromatic amino acids.[1,2] However, the destruction of phenylalanine (Phe) occurs on uv irradiation of proteins.[3] Based on the low extinction coefficient of Phe and its blue-shifted absorption spectrum compared to tyrosine (Tyr) and tryptophan (Trp), there is, however, a low probability for direct optical excitation of Phe in proteins which contain a number of Tyr and Trp molecules.

The fluorescence lifetime and quantum yield of Phe (~10^{-3} *M*, 20°) in aqueous solution[4-6] are $\phi_F = 0.025$ and $\tau_F = 6.8$ nsec. A marked temperature dependence was found,[5] with ϕ_F and τ_F decreasing with increasing temperature in the range 2–68°. This deactivation process was indicated[3] to be due mainly to internal conversion and only to a small extent due to intersystem crossing. The ϕ_F decreases[4] by ~30% on ionization of the COOH group and ~15% on ionization of the NH_3^+ group. The phosphorescence of Phe has been observed[1] only in glasses at 77°K and has a band maximum at 385 nm and $\tau_P \sim 5.5$ sec.

Flash photolysis studies[7] of Phe in water at 20° showed that: (a) the photodissociation process leading to the formation of the benzyl radical and the photoionization process leading to the hydrated electron e_{aq}^- were both strongly dependent on the state of ionization of the free end groups COOH and NH_3^+, and in particular to the amino group (these processes followed the pK_a of the ground state molecule); (b) the excited state precursors of both processes were long lived, and probably the triplet states; (c) the photoionization process was biphotonic in nature; (d) in neutral and acid solutions, the photodissociation processes from Phe, Phe-NH_2, and *N*-Ac-Phe were biphotonic, whereas in alkaline solutions (above the pK_a of NH_3^+) only one quantum was required to bring about the same photodissociation reaction; (e) the triplet state was also involved in alkaline solutions.

$$\begin{array}{l} \text{NH}_3^+\text{CHCOO}^- \\ \quad\quad\vert \\ \quad\text{CH}_2\text{Ph} \end{array} \xrightarrow{h\nu} \begin{cases} \text{PhCH}_2\cdot + \text{NH}_3^+\dot{\text{C}}\text{HCOO}^- & (1) \\ e_{aq}^- + \text{R}\cdot & (2) \\ \text{Ph}\cdot + \dot{\text{C}}\text{H}_2\text{CH}(\text{NH}_3^+)\text{COO}^- & (3) \end{cases}$$

The triplet state of Phe and related compounds was not observed,[7] presumably due to its relatively short lifetime. Reported below is a laser photolysis study of Phe and phenylalanine peptides in water using a quadrupled neodymium laser emitting at 265 nm with single pulses of ~15 nsec duration. The triplet-triplet absorption spectra of β-phenylpropionic acid, toluene, Phe, *N*-Ac-Phe, *N*-Ac-Phe-NH_2, Phe-NH_2, and Gly-Phe-Gly were observed and their lifetimes were determined. The pH and temperature dependen-