The Phosphorescence of Oligopeptides Containing Tryptophan and Tyrosine*

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ABSTRACT: In oligopeptides containing both tryptophan and tyrosine, the phosphorescence of tyrosine is largely suppressed in 50% ethylene glycol. The ionization of tyrosine produces a major decrease in the ratio of fluorescence to phosphorescence. The excitation spectrum shifts significantly to longer wavelengths. Possible explanations include some form of energy migration.

The three aromatic amino acids phenylalanine, tyrosine, and tryptophan all exhibit fluorescence and phosphorescence in the free state (Teale and Weber, 1957; Nag-Chaudhuri and Augenstein, 1964; Steiner and Edelhoch, 1963; Longworth and Bovey, 1966; Longworth, 1961, 1966; Vladimirov and Burshtein, 1960; Hercules, 1966; Weber, 1961; Steele and Szent-Györgyi, 1957; Debye and Edwards, 1952). The luminescent properties of all three amino acids are profoundly modified when incorporated into a protein. The luminescence of phenylalanine appears to be entirely suppressed. When tryptophan is present, the fluorescence of tyrosine is largely or entirely quenched and its phosphorescence is suppressed to a variable extent (Longworth, 1961).

A major unsolved problem in this area is the correlation of luminescent properties with specific features of the molecular environment. An important aspect of this problem is the question of internal energy transfer. Although radiationless energy transfer at the singlet level from un-ionized tyrosine to tryptophan is theoretically plausible, definite evidence for its occurrence in proteins is lacking (Weber, 1961). However, there is considerable evidence for transfer from tryptophan to ionized tyrosine (Steiner and Edelhoch, 1963; Edelhoch *et al.*, 1967). Transfer at the triplet level has not generally been believed to be a significant factor and the quenching of tyrosine phosphorescence in proteins has been ascribed to other causes.

One approach to the problem is the systematic investigation of fluorochrome-containing model compounds. In this paper the luminescent properties of a series of tryptophan- and tyrosine-containing oligopeptides have been examined, with particular attention to the dependence of quantum yield, spectral distribution, and excited lifetime upon composition and to the possible occurrence of internal energy transfer.

Experimental Section

Luminescence Spectra. Measurements of luminescence spectra at low temperatures were made with an Aminco-Bowman spectrofluorometer adapted for observations at liquid nitrogen temperatures (Steiner *et al.*, 1967). The cell holder was mounted reproducibly by means of a brass clip upon a vertical rod in the cell compartment, which was in thermal contact with a liquid nitrogen reservoir. A stream of dry nitrogen gas continuously flushed out the cell compartment and prevented frosting. At equilibrium the temperature of the sample was close to 85°K.

The cells were sections of quartz electron spin resonance tubing of 3-mm diameter (obtained from Varian), which were sealed at one end. The cell was positioned in a V-shaped groove in the cell holder.

The photomultiplier response was registered either by a galvanometer or by a Tetronix storage oscilloscope. Spectral selectivity was controlled by sets of interchangeable slits of varying widths, which intercepted the activating and emitted beams.

A rotating shutter was used when it was desired to observe phosphorescence spectra alone and was removed for measurements of total emission. The luminescent spectra reported here have not been corrected for the variation with wavelength of lamp intensity and photomultiplier response.

Lifetime Measurements. For determinations of phosphorescence decay curves the electronic power supply for the photomultiplier was replaced by a battery power supply. By means of a manually operated rotating shutter, a slit placed initially so as to admit the exciting beam was rotated by 90° at zero time, so as to intercept the luminescent beam. The decay of phosphorescence was observed on the oscilloscope screen and photographed.

Values of relative intensity as a function of time were obtained from the enlarged photographs of the oscilloscope traces. If the decay is strictly exponential, as in the case of a single phosphorescent component, then the intensity (I) varies with time (t), $I/I_0 = \exp(-t/\tau)$, where I_0 = initial intensity and τ = excited lifetime. In this case a plot of log I *vs.* t yields a straight line of

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TABLE I: Values of Excited Lifetime of Phosphorescence.

Material	pH	$\lambda_{excitation}$	λ_p	τ (sec) (±10%)
	Systems Showing Exponential Decay, with a Single Decay Time			
Tyr	7.0	260	400	2.2
	11.4	290	400	1.3
Try	5.1	285	430	6.3
	5.1	285	400	6.3
Try-Gly-Gly	5.25	285	430	6.3
Try-Tyr	6.1	285	430	5.7
	6.5	285	430	6.2
	12.1	285	430	6.0
	12.1	300	430	6.1
	12.1	310	430	5.8
Try-Gly-Tyr	6.4	285	430	6.7
	12.0	285	430	6.6
Try-Gly-Gly-Tyr	5.1	285	430	5.7
	5.1	285	400	5.7
	12.2	300	430	5.5

Material	pH	$\lambda_{excitation}$	λ_p	τ_1 (±10%)	τ_2 (±10%)	% Contributed by Component 1
	Systems Showing Two Decay Times					
Try + Tyr (1:1)	5.3	285	400	2.5	5.2	26
Try + Tyr (1:1)	12.2	300	400	1.5	~5	>90
Try + Tyr (1:1)	12.2	285	430	1.6	5.1	49
Try-Tyr	12.1	300	400	2.4	5.1	33
Try-Gly-Tyr	12.2	300	400	2.1	5.0	35
Try-Gly-Gly-Tyr	12.2	300	400	1.6	4.4	46

slope $1/\tau$. Values of τ were computed in this way.

Under some conditions, the observed decay curves were biphasic. The plots of log I *vs.* t showed curvature, approaching linearity at long times. The lifetime of the more slowly decaying component was obtained from the limiting linear slope at long times. By linear extrapolation to shorter times the contribution of the long-lived component to the total intensity was estimated and subtracted. The lifetime of the short-lived component was obtained from a logarithmic plot *vs.* time of the residual intensity. The ratio of residual intensity at zero time to the total intensity provided an index of the magnitude of the contribution of the short-lived component.

Absorption Spectra. Measurements of absorption spectra were made with a Cary recording spectrophotometer.

Solvents. All measurements reported in this paper were made upon solutions in 50% ethylene glycol–0.05 M PO_4^{2-}, which were titrated to the indicated pH. All pH values were measured at room temperature, using a Radiometer pH meter with a glass electrode.

Materials. Tryptophan and tyrosine were purchased from Sigma. Tryptophyltyrosine (Trp-Tyr) and glycyltyrosine (Gly-Tyr) were purchased from Cyclo. Tryptophylglycylglycine (Trp-Gly-Gly), Trp-Gly-Tyr, and Trp-Gly-Gly-Tyr were prepared by Dr. M. Wilchek, Weizmann Institute, Rehovoth, Israel, and were obtained through the courtesy of Dr. H. Edelhoch, National Institutes of Health, Bethesda, Md.

Results

Tryptophan and Tyrosine. The luminescent properties of tryptophan and tyrosine have been described in other publications (Teale and Weber, 1957; Longworth, 1966; Bishai *et al.*, 1967). The present results are in general accord with the earlier data. At neutral pH and 85°K in 50% ethylene glycol, tryptophan shows a single fluorescence peak at 320 mμ and a structured phosphorescence spectrum with maxima at 406, 433, and 455 mμ.

Under these conditions the intensities of tryptophan fluorescence and phosphorescence show no important variation with pH between pH 5 and 12 (as measured at 25°), in agreement with the results of Bishai *et al.* (1967). This contrasts with the well-known quenching of tryptophan fluorescence above pH 11 in water at room temperature (Weber, 1961) and with the reported enhancement of phosphorescence in 0.5% glucose in the same pH range (Truong *et al.*, 1967). Apparently, for the temperature and solvent composition utilized

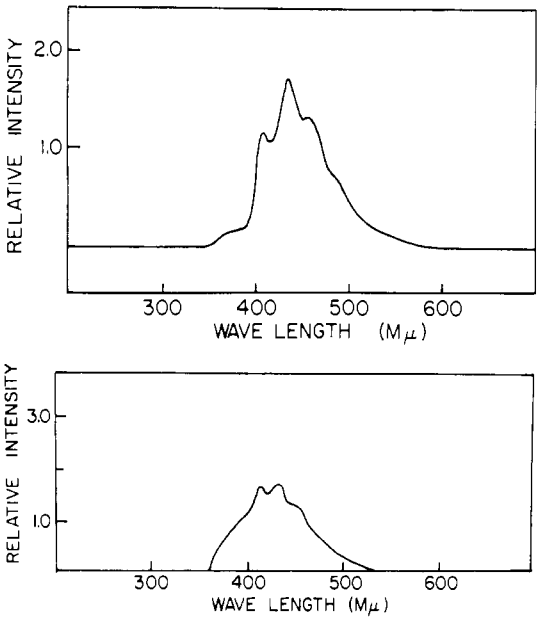

FIGURE 1: Phosphorescence spectra. Upper: a 1:1 mixture of tryptophan and tyrosine (2.4×10^{-4} M) at pH 5.1 ($\lambda_{excitation}$ 285 mμ). Lower: same, at pH 12.18 ($\lambda_{excitation}$ 285 mμ).

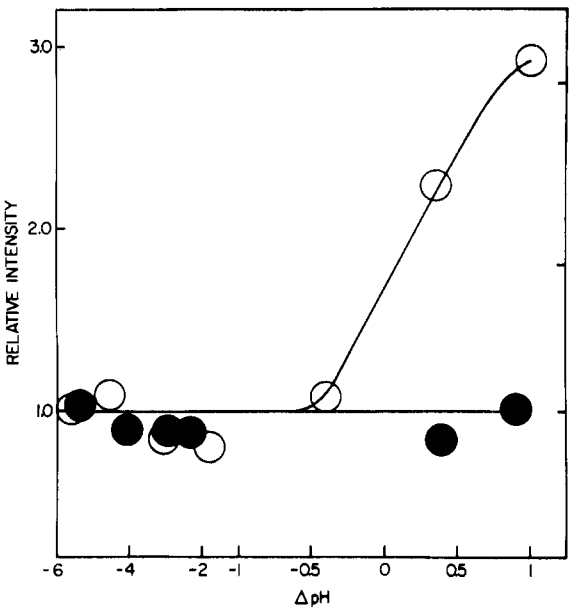

FIGURE 2: Relative intensities of phosphorescence ($\lambda_{excitation}$ 285 mμ; λ_p 430 mμ) for Trp-Gly-Gly (1.6×10^{-4} M) and for Trp-Gly-Tyr (7.0×10^{-5} M) as a function of pH in 50% ethylene glycol–0.05 M PO_4. The abscissa is the difference between the ambient pH and the pH corresponding to the midpoint of the ionization of Gly-Tyr, as determined by the quenching of fluorescence at 300 mμ (see text). (○) Try-Gly-Tyr and (●) Trp-Gly-Gly.

here, these effects occur only for more alkaline conditions than were examined here.

At neutral pH tyrosine gives a single fluorescence band at 298 mμ and a single phosphorescence peak at 387 mμ (Nag-Chaudhuri and Augenstein, 1964). A pronounced change in the luminescent properties of tyrosine occurs upon ionization. The fluorescence intensity at 85°K decreases by 80%, while a tenfold increase occurs of intensity of phosphorescence excited at 290 mμ. The position of the phosphorescence maximum shifts to 408 mμ and that of the fluorescence maximum to 320 mμ. The lifetime decreases from 2.2 to 1.3 sec (Table I). A shift in excitation spectrum for phosphorescence also occurs, the position of the maximum (uncorrected) changing from 273 mμ at neutral pH to 290 mμ at pH 12, in accord with expectations if the change in luminescent properties arises from the ionization of tyrosine. A 1:1 mixture of tyrosine and tryptophan shows a phosphorescence spectrum which represents the summation of the spectra of the individual components (Figure 1), the tyrosine band being prominent below 400 mμ.

Oligopeptides Containing Tryptophan. The oligopeptide Trp-Gly-Gly has luminescence properties characteristic of tryptophan, with similar positions of the fluorescence and phosphorescence maxima. The excited lifetime for phosphorescence (6.3 sec) is close to the value for free tryptophan. The intensities of fluorescence and phosphorescence were essentially constant between pH 5 and 12 (Figure 2) as was the excited lifetime (Table I).

Oligopeptides Containing Tryptophan and Tyrosine. At neutral pH in 50% ethylene glycol, the decay curves of phosphorescence excited at 285 mμ for oligopeptides of the series Trp-$(Gly)_x$-Tyr (x = 0, 1, 2) are strictly exponential at 400 and at 430 mμ, with lifetimes characteristic of tryptophan (Table I). There is no evidence of any significant component of shorter lifetime arising from tyrosine. Moreover the phosphorescence spectrum is closely similar to that of free tryptophan, with no apparent shoulder below 400 mμ which would reflect the contribution of tyrosine (Figure 1). It may be concluded that the direct contribution of un-ionized tyrosine to the phosphorescence of this series of peptides is largely suppressed.

The intensity of phosphorescence ($\lambda_{excitation}$ 285 mμ) of all three peptides shows a two- to threefold increase in the pH region in which tyrosine ionizes (Figure 2). The spectral distribution of phosphorescence is not significantly altered, corresponding to that of tryptophan. The enhancement of phosphorescence is accompanied by a major quenching of fluorescence and by a pronounced decrease in the F/P ratio (Figure 3). In contrast, the F/P ratio of Trp-Gly-Gly is independent of pH in this range (Figure 3).[1]

The theoretically expected attenuation of the alkaline enhancement with increasing numbers of glycines between Trp and Tyr was not observed, possibly being

[1] The data of Figures 2 and 3 are plotted *vs.* the displacement of the measured pH from that of the midpoint of the ionization of the tyrosine group of Gly-Tyr, as determined from the quenching of fluorescence at 300 mμ at 85°. The data are presented in this way because of uncertainties in the relation of the pH measured at room temperature to that of the glass at 85°K and because of the unknown effect of the mixed solvent upon the glass electrode. The observed apparent midpoint of Gly-Tyr ionization was 11.0 ± 0.15 but because of the above no quantitative significance should be attached to this figure.

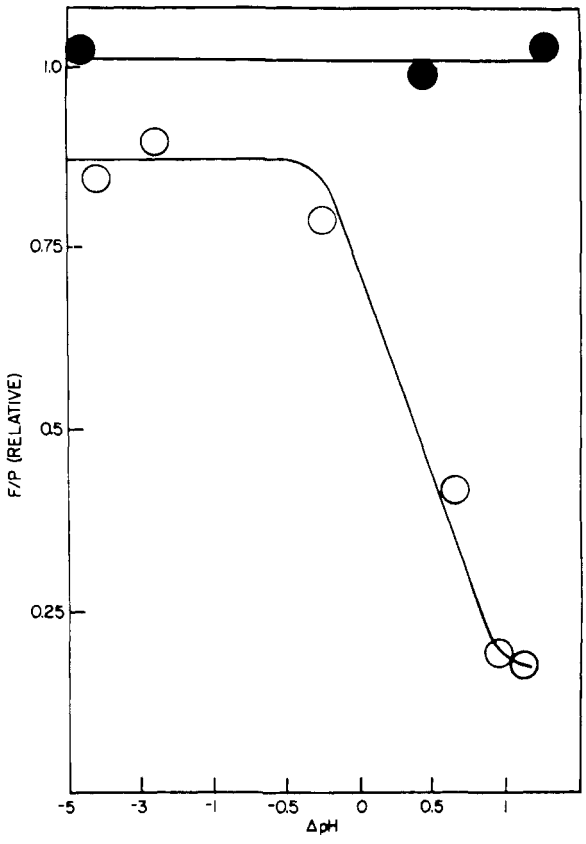

FIGURE 3: pH dependence of fluorescence/phosphorescence (F/P) ratio (uncorrected) for Trp-Tyr (4.3×10^{-4} M) ($\lambda_{excitation}$ 270 mμ) (○) and for Trp-Gly-Gly (●). The abscissa has the same significance as for Figure 2.

obscured by experimental scatter. The uncorrected excitation spectrum at pH 12, as compared with pH 5, shows a significant shift by 5–7 mμ to longer wavelengths (Figure 4). Only a minor change ($\sim$1 mμ) occurs in the case of Trp-Gly-Gly, paralleling a slight shift in absorption spectrum, arising from ionization of the α-amino group.

Excitation of Trp-Gly-Tyr at 308 mμ at pH 12 results in a detectable phosphorescence spectrum characteristic of tryptophan (Figure 5). A solution of Trp-Gly-Gly of equivalent molar concentration shows no phosphorescence in excess of background.

For excitation at 285, 300, or 310 mμ and emission at 430 mμ, the phosphorescence decay curves of Trp-Tyr, Trp-Gly-Tyr, and Trp-Gly-Gly-Tyr at pH 12 are exponential with no significant tyrosine component. However, for excitation at 300 mμ, where most of the absorption is due to ionized tyrosine, and emission at 400 mμ, a component of lifetime $\sim$1.5 sec appears for all three peptides corresponding to ionized tyrosine (Table I). There is thus a detectable contribution of ionized tyrosine at these wavelengths, although this accounts for only a very minor fraction of the *total* phosphorescence.

In summary, for the above set of oligopeptides containing tryptophan and tyrosine, the phosphorescence characteristics are largely those of tryptophan, irrespective of the state of ionization of tyrosine. The ionization

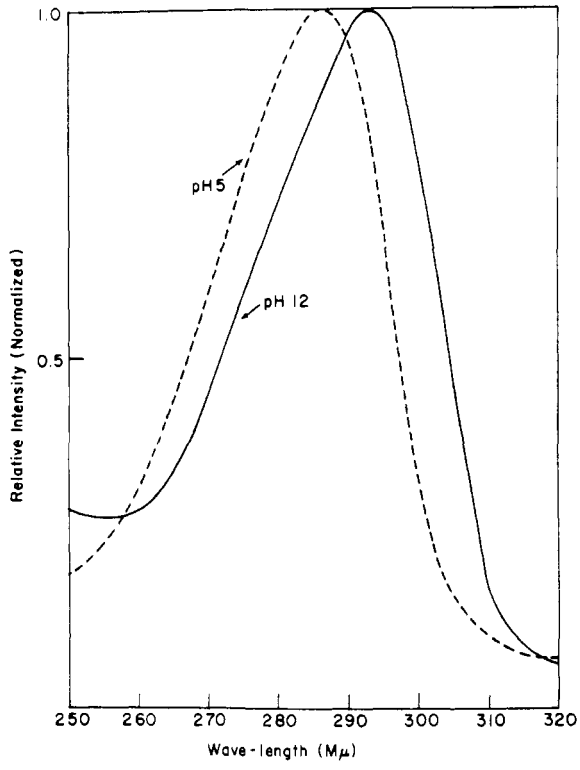

FIGURE 4: Relative excitation spectra (uncorrected) of Trp-Gly-Tyr (1.4×10^{-4} M) at neutral and alkaline pH.

of tyrosine results in a fall in the F/P ratio and in an increase in tryptophan phosphorescence.

Discussion

From the preceding it is clear that, in an oligopeptide containing both tryptophan and tyrosine: (1) the phosphorescence of tyrosine, whether ionized or un-ionized, is almost entirely suppressed; (2) the ionization of tyrosine results in the partial suppression of tryptophan fluorescence, while its phosphorescence is enhanced; and (3) the excitation spectrum for tryptophan phosphorescence is shifted to longer wavelengths upon ionization of tyrosine.

The suppression of tyrosine phosphorescence in oligopeptides which also contain tryptophan indicates that some form of interaction between the two residues results in a depletion of the triplet state of tyrosine, whether

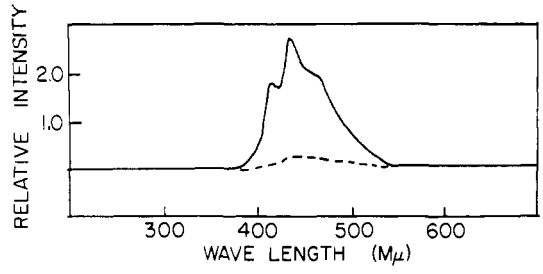

FIGURE 5: Relative phosphorescence spectra for Trp-Gly-Tyr (———) and Trp-Gly-Gly (– – –) at pH 12.1 ($\lambda_{excitation}$ 308 mμ). The molar concentration is 2.5×10^{-4} in each case.

ionized or un-ionized. It is possible that one of the factors involved in the quenching in the latter case is radiationless energy transfer at the singlet level between tyrosine and tryptophan (Förster, 1959).

The quenching of tryptophan fluorescence upon ionization of tyrosine could of course be explained in terms of radiationless energy exchange between tryptophan and tyrosine according to the Förster mechanism (Förster, 1959). This model is theoretically plausible in view of the finite overlap between the emission band of tryptophan and the absorption band of ionized tyrosine (Edelhoch *et al.*, 1967).

The exaltation of tryptophan phosphorescence accompanying the ionization of tyrosine, which is accompanied by a quenching of tryptophan fluorescence, indicates that the proximity of ionized tyrosine serves to populate the triplet state of tryptophan. It appears unlikely that this reflects solely the enhancement of singlet → triplet conversion in tryptophan, in view of the shift in excitation spectrum, although this cannot be excluded as a factor. A further possibility is triplet–triplet energy transfer between ionized tyrosine and tryptophan (Roy and El-Sayed, 1964; Helene *et al.*, 1966; Dexter, 1953).

Energy transfer at the triplet level has been interpreted in terms of exchange–resonance interactions between a donor in the triplet state and an acceptor in the ground state (Helene *et al.*, 1966; Dexter, 1953). Interactions of this kind become possible if the peripheries of the electron shells of the donor and acceptor overlap. The probability of energy transfer by this mechanism is dependent upon the extent of the overlap of the phosphorescence (triplet → singlet) spectrum of the donor and the singlet → triplet absorption band of the acceptor. The latter is not ordinarily accessible to direct measurement. If it shows approximate mirror symmetry with the phosphorescence emission band (triplet → singlet) of the acceptor, overlap will be greater if the phosphorescence spectrum of the donor is at shorter wavelengths than that of the acceptor; *i.e.*, the triplet state of the donor is of higher energy than that of the acceptor.

The above requirements are met by the present system. The phosphorescence emission band of tyrosine is at lower wavelengths than that of tryptophan. Triplet–triplet transfer remains a possibility for this system.

However, it cannot be claimed that these results provide a rigorous proof of the existence of triplet–triplet transfer. This will require measurements of the polarization of phosphorescence as a function of excitation wavelength for the ionized and un-ionized forms of the oligopeptides.

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