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pH Dependence of the Fluorescence Decay of Tryptophan*

W. B. De Lauder and Ph. Wahl

ABSTRACT: The fluorescence decay of tryptophan was measured as a function of pH using a nanosecond flash apparatus. Within the pH range 2–10.6 variations in the lifetimes could be understood in terms of the mixtures of different ionic species present in solution. Each species has a characteristic lifetime or contributes to the total decay in proportion to their molar fractions. Within the pH range 10.6–11.5, the fluorescence lifetimes decreased with increased pH due to quenching by hydroxyl ions. It is shown that the average fluorescence lifetime as a function of pH follows the relative quantum yield–pH curve as expected. The results of fluorescence lifetime and quantum yield measurements of some tryptophan derivatives are also reported and quantum yields are proportional to the lifetimes.

The effect of pH on the fluorescence of tryptophan and its derivatives has been the subject of numerous investigations (White, 1959; Cowgill, 1963; Weber, 1961; Konev, 1967). White (1959) was the first to report on the variations in the quantum yield of tryptophan as a function of pH. The quantum yield of tryptophan was found to be constant in the pH range 4–8, to decrease when the pH was lowered below pH 4, to progressively increase as the pH is raised above 8, to reach a maximum and then decrease as the alkalinity is still further increased. Subsequent investigations by several other workers confirmed those results (Cowgill, 1963; Weber, 1961; Konev, 1967). Bridges and Williams (1968) have reported pH–fluorescence curves which showed evidence for five distinct molecular species of tryptophan which were fluorescent.

Previous studies on the pH dependence of tryptophan fluorescence have been confined to measurements of fluorescence emission spectra and quantum yield. Several workers have reported the results of decay-time measurements of tryptophan in polar solvents (Chen *et al.*, 1967; Badley and Teale, 1969; Weinryb, 1969; Eisinger and Navon, 1969), but no literature is available on measurements of the pH dependence of the fluorescence decay of tryptophan. The present study consisted of measurements of fluorescence decay times in order to determine the effect of pH on that parameter. Since tryptophan is an ampholyte, it is capable of dissociation in aqueous solution as either an acid or base

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depending on the solution pH. The dissociation reactions may be written as (Cohn and Edsall, 1965)

$$^{+}H_3NRCOO^{-} + H^{+} \overset{k_1}{\rightleftharpoons} H_3{}^{+}NRCOOH$$

and

$$^{+}H_3NRCOO^{-} \overset{k_2}{\rightleftharpoons} H_2NRCOO^{-} + H^{+}$$

where k_1 and k_2 are the dissociation constants for the respective reactions. Since all three molecular species of tryptophan involved in these reactions are fluorescent (Cowgill, 1963), it was possible to use decay-time measurements in order to accurately determine the pH dependence of the fluorescence of tryptophan and to calculate the relative contributions of the various species as a function of pH.

Experimental Section

DL-Tryptophan and glycyl-L-tryptophan used in these studies were of Puriss grade and were purchased from Fluka AG. Buchs SG. (Switzerland). L-Tryptophyl-L-leucine and L-tryptophylglycine of high quality were obtained from Sigma Chemical Co., St. Louis, Mo. These compounds were used without further purification. Changes in pH were brought about by the addition of appropriate amounts of HCl or NaOH to aqueous solutions of tryptophan. Measurements of pH were made using a Tacussel Electronique pH meter. The pH meter was standardized using standard buffer solutions obtained from E. Merck (Darmstadt, Germany). The accuracy of the measured pH is estimated as ±0.05 pH unit. Freshly prepared solutions of tryptophan were used in all decay-time measurements.

It was found during preliminary experiments that the fluorescence lifetime of tryptophan was not appreciably affected by deaeration of the solutions, and as a consequence oxygen removal from the solutions was not deemed necessary. Preliminary experiments also showed that the lifetime of tryptophan in neutral aqueous solution was constant within the concentration range 10^{-4}–10^{-5} M. Normally solutions with concentrations of 2.5×10^{-5} M in tryptophan or tryptophan derivatives were used in order to ensure that no concentration quenching occurred.

Measurements of optical density were made using a Cary-14 recording spectrometer and fluorescence emission spectra were measured using a Jobin Yvon spectrofluorimeter. The method of Parker and Rees (1960) was used in order to calculate relative quantum yields. Areas under the fluorescence emission spectra curves were measured by planimetry and quantum yields were calculated using tryptophan at neutral pH as the reference.

The nanosecond flash apparatus used in decay-time measurements has been previously described (Wahl, 1969). The flash in an atmosphere of N_2 had a width at half-height of approximately 2.0–2.4 nsec. A shot-glass filter which absorbed light of wavelengths less than 290 nm was placed between the sample, contained in a quartz cell, and the photomultiplier in order to eliminate any scattered light from the measured fluorescence. The wavelength of the exciting light was 280 nm in each case. Solutions were maintained at a constant temperature of 20° during decay-time measurements. Since no buffers were used, the pH of each solution was measured just before and immediately following a measurement and it was found that no change in pH had occurred during the duration of an experiment. The accuracy of decay times greater than 2.0 nsec is ±0.2 nsec, and for decay times less than 2.0 nsec the estimated accuracy is ±0.3 nsec.

The fluorescence response of a solution to an exciting flash may be treated as a product of convolution of the form (Birks and Munro, 1967)

$$n(t) = K \int_0^t g(T)p(t - T)\, dT$$

where K is a constant which depends on the experimental conditions, $g(t)$ is the response function of the flash which is measured by placing a quartz glass reflector in the cell compartment of the apparatus; $p(t)\, dt$ is the probability of the emission of a fluorescent quantum at the time t after an excitation, by a flash of infinitly short duration. In a dilute solution containing only one absorbing solute, one expects for $p(t)$ a single exponential function of the form (Birks and Munro, 1967; Förster, 1951)

$$p(t) = \frac{1}{\tau_0} e^{-t/\tau} \tag{1}$$

where τ is the decay time of the fluorescence; τ_0 is the natural lifetime which would be observed if no quenching occurs, and is related to the absorption spectra (Förster, 1951).

This formula can be easily extended to the case where several absorbing species are present and one obtains

$$p(t) = \sum_i \frac{A_i}{\tau_{0,i}} e^{-t/\tau_i} \tag{2}$$

where τ_i are the decay times, and $\tau_{0,i}$ the natural lifetimes of species i. A_i is the fractional absorption. If the emission spectra are different for the different species, one must take account of the variation of the detector sensibility with wavelength, in evaluating the coefficient A_i (Chen, 1968).

For tryptophan, in the range of pH examined, there is very little change in emission spectra and one can neglect this effect. Very little changes also occur in the absorption spectra. One can reasonably assume therefore that all species present have the same natural lifetime. Furthermore the coefficients A_i are in this case simply the molar fractions of the species. Equation 2 may therefore be written in the form

$$p(t) = \frac{1}{\tau_0} \sum_i A_i e^{-t/\tau_i} \tag{3}$$

In the present study it was not necessary to consider more than two emitting species of tryptophan at any pH. $p(t)$ is related to the quantum yield by the following integration (Förster, 1951)

$$Q = \int_0^\infty p(t)\, dt \tag{4}$$

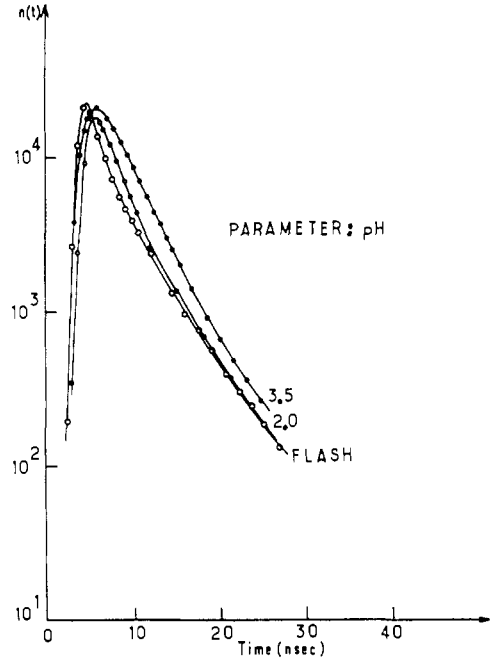

FIGURE 1: Effect of pH on the fluorescence response curves of DL-tryptophan at 20° (low pH). Excitation was at 280 nm.

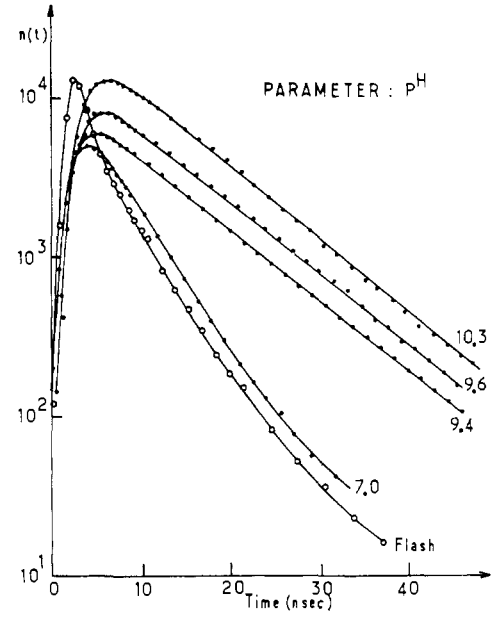

FIGURE 2: Effect of pH on the fluorescence response curves of DL-tryptophan at 20° (high pH). Excitation was at 280 nm.

Taking the expression 1 for p(t) gives the classical result

$$Q = \frac{\tau}{\tau_0} \tag{5}$$

Formula 3 yields

$$Q = \frac{\langle\tau\rangle}{\tau_0} \tag{6}$$

where $\langle\tau\rangle$ is the number-average lifetime

$$\langle\tau\rangle = \Sigma_i A_i\tau_i \tag{7}$$

Therefore one expects that the number-average lifetime should be proportional to the quantum yield.

Convolutions of the decay-time curves were calculated using for this purpose the Olivetti programmer 101. The parameters A_i, τ_i for a given system were chosen as those values which provided the best fit of the convolution function to the experimental curve.

Results and Discussion

The effect of pH on the fluorescence of tryptophan was determined by measuring the fluorescence decay of tryptophan as a function of systematically varied pH. Some representative data obtained from these measurements are plotted in Figures 1–3. The curves plotted in Figure 1 show that the slopes of the decay curves increased as the pH was lowered below pH 4. On the other hand, Figure 2 shows that for pH greater than 9, the slopes of the decay curves decreased. This decrease continued until it reached a minimum at pH approximately 10.6 and then increased at higher pH as can be seen in Figure 3. In addition no change occurred in the fluorescence decay curves of tryptophan in solutions of pH in the range 4–8.

Convolutions of the decay curves were made using the Olivetti programmer 101 as described earlier. The results of that analysis are summarized in Table I. Several interesting results can be seen in the data given in Table I. Firstly the fluorescence decay curves of tryptophan in the pH range 4–8 contained only one component with a lifetime of 3.1 ± 0.2 nsec. This was expected since in this pH range the zwitterion $^+H_3NRCOO^-$ was the predominate species. Secondly in acid solution (pH less than 4) the fluorescence lifetime of tryptophan decreased from that found in neutral solution. Moreover, one is able to identify two distinct lifetimes of tryptophan which are due to the presence of two ionic forms of that amino acid in acid solution. The long component in this case has a lifetime of 3.0 ± 0.2 nsec and may be assigned to the form of tryptophan which predominates at neutral pH (the zwitterion $^+H_3NRCOO^-$), while the short component with a lifetime of 1.2 ± 0.3 nsec is apparently due to the cationic form $^+H_3NRCOOH$. The shorter lifetime obtained for this later species is consistent with the fact that the COOH group quenches the fluorescence of tryptophan (Cowgill, 1963).

Thirdly, two components of quite different lifetimes were observed for decay curves obtained from measurements of tryptophan in the pH range 8 to approximately 10.6. This is in accordance with the fact that two different ionic species of tryptophan are present in this pH range. The short component with a lifetime of 3.1 ± 0.2 nsec corresponds to the zwitterion and the long component with a lifetime of 8.8 ± 0.2 nsec was due to the anionic form of tryptophan (H_2NRCOO^-). The longer lifetime obtained for the later species was to be expected since the anionic form of tryptophan has been reported to have a high quantum efficiency (Cowgill, 1963).

TABLE I: Effect of pH on Fluorescence Lifetime of DL-Tryptophan.[a]

pH	A_1	τ_1 (nsec)	A_2	τ_2 (nsec)	A_3	τ_3 (nsec)	$\langle\tau\rangle$ (nsec)	$\log\frac{A_3}{A_2}$ − pH
2.00	0.78	1.4	0.22	3.0			1.8	
3.50	0.06	1.2	0.94	3.0			2.9	
4.20				3.2			3.2	
7.10				3.0			3.0	
7.60				3.2			3.2	
8.10				3.0			3.0	
8.70			0.83	3.1	0.17	9.0	4.1	9.39
9.40			0.50	3.0	0.50	8.9	6.0	9.40
9.60			0.40	3.0	0.60	8.7	6.4	9.42
9.90			0.24	3.0	0.76	9.0	7.6	9.40
10.10			0.15	3.2	0.85	8.6	7.8	9.45
10.30			0.006	3.0	0.994	8.7	8.7	
10.55						8.9	8.9	
10.90						7.6	7.6	
11.50						4.7	4.7	

[a] The temperature of all measurements was 20°, excitation was at 280 nm.

The data given in column 9 of Table I show that a plot of log A_3/A_2 as a function of pH for the pH range 8–10.1 yields a straight line of slope 1 as is expected for an ordinary titration. The value of the pH, for which log A_3/A_2 is zero, determines the pK. The pK at this point is 9.4. This value corresponds well to the pK_2 of the tryptophan which is 9.38 (Cohn and Edsall, 1965).

Fourthly, at pH greater than 10.6, a single exponential function was found in the convolution and the lifetime of tryptophan was found to decrease as the alkalinity increased. Within the pH range of high alkalinity investigated in this study (10.55–11.5), the anionic form of tryptophan was the predominate species (Cowgill, 1963).

White (1959) has explained the decrease in fluorescence yield which occurred at high alkalinity as due to quenching, by hydroxyl ions. The results reported here for tryptophan in solutions of pH greater than 10.6 are consistent with that view and show that a corresponding decrease in the fluorescence lifetime accompanies the decrease in quantum yield.

Our results are in agreement with the interpretation given by several authors to explain the variation of the quantum yield of tryptophan with pH (White, 1959; Cowgill, 1963; Weber, 1961).

A further confirmation of this agreement is given by the number average lifetime $\langle\tau\rangle$ determined from the data of Table I. Plotted in Figure 4 is the value of $\langle\tau\rangle$ as a function of pH.

Relative quantum yields of tryptophan were also determined as a function of pH using tryptophan in neutral aqueous solution as reference, and it was found that the ratios of the quantum yields were, within experimental error, equal to the ratios of the corresponding average fluorescence lifetimes, as can also be seen in Figure 4.

In view of the above results, it was of interest to conduct similar measurements on some derivatives of tryptophan. Fluorescence decay measurements were therefore made on a few tryptophan derivatives in neutral aqueous solutions. The results of those measurements together with the relative quantum yields determined for those compounds are summarized in Table II. The relative quantum yields given in Table II for Try-Gly and Gly-Try agreed with those reported earlier by Cowgill (1963). It can be seen in Table II that the ratios of the fluorescence lifetimes were also equal to the ratios of the quantum yields for those compounds. A similar result was previously reported by Weinryb and Steiner

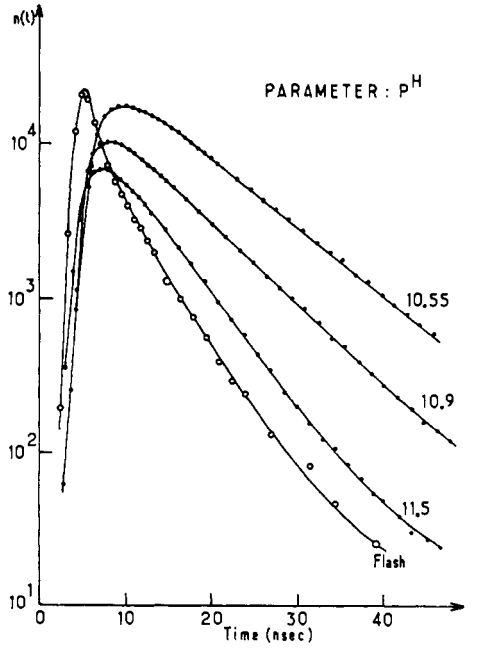

FIGURE 3: Effect of pH on the fluorescence response curves of DL-tryptophan at 20° (high alkalinity). Excitation was at 280 nm.

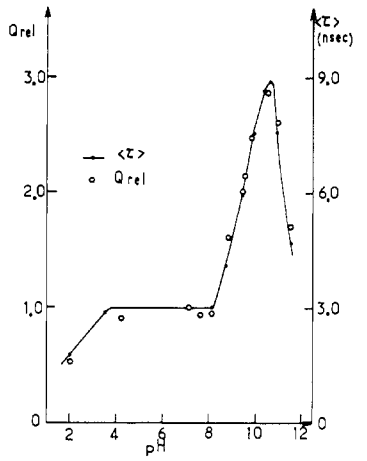

FIGURE 4: Comparison between the variation as a function of pH of $\langle\tau\rangle$ and the relative quantum yield. The relative quantum yield is taken relative to that of tryptophan at pH 7.

(1968) who measured the lifetimes and relative quantum yields of tryptophan and several tryptophan derivatives in 50% ethylene glycol–50% 0.05 M KH_2PO_4 solutions.

In conclusion, in this study, we determined the decay of three ionic species of tryptophan and of three peptides containing tryptophan. All these decays are single exponential, and the time constants are proportional to the quantum yield.

TABLE II: Fluorescence Lifetimes (τ) and Relative Quantum Yields (Q_{rel}) of Some Tryptophan Derivatives.

Compound	τ (nsec)	Q_{rel}
DL-Try	3.0	1.00
L-Try-Gly	2.0	0.68
L-Try-L-Leu	2.3	0.72
Gly-L-Try	1.04	0.30

In the range of transition where two ionic species are present, the decay can be decomposed into two exponential decays, with time constants characteristics of the species, and coefficients equal to the molar ratios of these species.

The proportionality between quantum yield and lifetime indicates that the quenching factors which causes the difference in quantum yield of the different species studied compete with the fluorescence emission.

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