Fluorescence Decay of Tryptophan Conformers in Aqueous Solution[1]

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Abstract: The fluorescence decay parameters of aqueous solutions of tryptophan and several tryptophan and indole derivatives are reported. The fluorescence decays of tryptophan, tryptophan ethyl ester, and 5-methyl- and 6-methyltryptophan are all described by double exponential kinetics. The relative proportions of the two components vary with emission wavelength. The fluoresence of all other derivatives obeys single exponential decay kinetics. In the case of tryptophan the two components, τ_1 = 3.1 ns and τ_2 = 0.51 ns, had fluorescence maxima at 350 and 335 nm, respectively. The origin of this behavior is discussed in terms of several models but is most consistent with the assignment of the emission to different rotamers or conformers of the alanyl side chain of tryptophan.

The fluorescence of the aromatic amino acid tryptophan has been extensively investigated.[2] Several workers have studied the fluorescence properties of the parent indole molecule and its derivatives,[3-13] tryptophan derivatives, tryptophan-containing oligopeptides,[4,14-16] and tryptophan-containing proteins.[17-22] This is largely due to the utility of tryptophan fluorescence as an intrinsic probe in the structure and function of proteins and enzymes. In addition the photodecomposition of tryptophan has been implicated in the photoinactivation of proteins. Besides this biophysical interest the spectroscopic properties of indoles, particularly their fluorescence behavior, have stimulated many photophysical studies.[23-29] Yet despite this extensive effort there remain controversial differing viewpoints of the origin of the special fluorescence properties of indole, tryptophan, and proteins containing tryptophan.

The origins of the large Stokes shift in the fluorescence spectra of indoles in polar solvents has received the most attention. Studies of the effect of solvents of varying polarity on the fluorescence of indole derivatives indicated that the Stokes shift of the fluorescence maxima was linearly correlated with the the solvent dielectric properties and refractive indices.[7,8,11,30,31] These studies indicated that there was a large change in the dipole moment of the excited ^1L_a state[32] compared to the ground state. It was concluded that the origin of the Stokes shift resulted from a rapid reorientation of the solvent shell surrounding the excited solute indole forming a new solvent equilibrated excited ^1L_a state which was of lower energy than the initially excited ^1L_a state. The energy of this solvent equilibrated state falls below that of a close lying ^1L_b excited state. But it is pertinent to note that these correlations between Stokes shift and dielectric constants of solvents all showed severe deviation from linearity when hydrogen-bonding solvents were studied.

This concept of two close-lying excited states was supported by the resolution of the absorption spectra, using solvent perturbation techniques by Strickland and co-workers.[34-36] Polarized fluorescence and excitation spectra confirmed this resolution of the absorption band[6,37,38] and suggested that the fluorescence of indoles and tryptophan may be composed of two emission bands.

On the other hand, because of the lack of correlation of the Stokes shift with dielectric constant in polar hydrogen bonding solvents, Lumry and co-workers[10] proposed that the large Stokes shift in polar solvent was due to the formation of a solvent–indole exciplex. By varying the concentration of the polar solvent, butanol, in a nonpolar solvent containing indole they were able to observe a progressive shift of the emission maximum to lower energy. The concentration of butanol was sufficiently low that the solvent dielectric properties were not affected. More recently Lasser, Feitelson, and Lumry[9] studied the interaction between 3-methylindole in cyclohexane and ethyl acetate or dimethylacetamide. They presented compelling evidence for the formation of an excited-state complex in the case of 3-methylindole and ethyl acetate. They also indicated that a ground-state complex was formed in the case of 3-methylindole and dimethylacetamide.

Recently Lami[39] suggested a third alternative that the Stokes shift may be due to emission from a solvated Rydberg state of the indoles.

As Tatischeff and co-workers have aptly proposed,[12] the solvent can interact in at least three different ways with the indole chromophore depending on the nature of the solvent. One can have effects due to the dielectric properties of the solvent, or exciplexes of varying stability may form depending on the solvent. Finally, in hydrogen-bonding solvents hydrogen-bond complexes with the indole N–H may be formed. A combination of these effects may satisfactorily explain the Stokes shift of indole fluorescence in polar solvents.

The effect of other variable parameters in the fluorescence of indoles and tryptophan has also been extensively investigated. Different salts have been reported to have a variety of effects depending on the indole derivative studied.[4,40-42] In some cases the quantum yields (ϕ_f) increased slightly while in others slight decreases were observed. There was virtually no effect on the emission maxima.

The dependence of ϕ_f on excitation wavelength has been reported.[23,24] ϕ_f is constant down to 260 nm and then decreases with further increases in excitation energy. It was suggested that this was due to an increase in the yield of a photoionization process when the indole derivatives were excited into higher singlet states.

Several workers have reported temperature studies on a number of derivatives in different solvents.[3,7,25,43-48] They have shown that, in polar solvents at least, there are two nonradiative deactivation processes, one of which is temperature dependent and the other not. The frequency factors were dependent on the solvent system.

These differing rationalizations are important in biological fluorescence studies of proteins. The fluorescence of tryptophan in proteins is heterogeneous[19,20,22,49,50] having differing fluorescence maxima and quantum yields depending on the protein and its state of naturation. It has been suggested that these properties are dependent on differing solvent exposure of the tryptophans or on the polarity of their environment. Until recently it was generally stated that the fluorescence decay of aqueous tryptophan and tryptophan derivatives obeyed single exponential kinetics. A lifetime of ~3 ns at 20 °C was reported for tryptophan. Using improved time correlated single photon counting fluorescence techniques which accounted for critical

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instrumental artifacts[51,52] we demonstrated that the fluorescent decay of aqueous solutions of tryptophan could be resolved into two exponentially decaying components.[53]

The one with an apparent emission maximum at 335 nm had a lifetime of 0.5 ns while the other with a maximum at 350 nm had a lifetime of 3.1 ns (pH 7, 20 °C). In our preliminary report we suggested a tentative rationalization that this dual exponential behavior may originate from the two ^1L_a and ^1L_b solvent equilibrated excited states. The observed kinetic parameters were inconsistent with the formation of a water-tryptophan exciplex

While the work reported herein was in progress Fleming and co-workers[54] reported that they had observed dual exponential kinetics in the fluorescence decay of tryptophan with lifetimes of 2.1 and 5.4 ns. Using mode locked frequency quadrupled neodymium laser pulses with streak camera detection they measured the total sample emission above 380 nm. They interpreted their results as originating from two conformers which did not interconvert on a nanosecond time scale.

Fluorescence decay measurements of several tryptophan-containing proteins have been shown to display multiexponential kinetics and yet contain only a single tryptophan residue.[19,55] In proteins containing more than one tryptophan, multiexponential kinetics were also observed and the ratio of the two components was wavelength dependent. The fluorescence of the lac repressor protein was resolved into two spectral components with lifetimes of 3.8 and 9.8 ns and emission maxima at 322 and 344 nm, respectively.[56]

In this work we report fluorescence decay experiments which further characterize the dual exponential decay behavior of tryptophan fluorescence. We have measured the fluorescence decay parameters of several tryptophan and indole derivatives elaborating the influence of the side-chain substituents, solvent structure, and other relevant factors on these parameters. The results are discussed in terms of the several models proposed to account for the fluorescence properties of tryptophan. We now consider that they are most consistent with the concept that the fluorescence of tryptophan originates from at least two conformers.

Materials and Methods

All compounds were purchased from commercial sources except for 3-indolylpropionic acid ethyl ester and 3-indolylpropionamide, which were synthesized. 1,2-Dimethylindole, indole, and 3-methylindole were purified by vacuum sublimation. Most other compounds were recrystallized several times from different solvent systems: 3-indolylbutyric acid (ethanol-water); 3-indolylacetic acid (water); 5-methoxyindole (*n*-pentane); 5-methyltryptophan (water); 6-methyltryptophan (ethanol-water); *N*-acetyltryptophanamide (methanol-ether); *N*-acetyltryptophan ethyl ester (ethyl acetate); *N*-acetyltryptophan (methanol-water); tryptophanamide (methanol-ethyl acetate); tryptophan (water or ethanol-water). Tryptamine (Sigma), 3-indolylpropionic acid (Polysciences), and tryptophan ethyl ester (Sigma) were used without further purification. 3-Indolylpropionamide was synthesized from 3-indolylpropionic acid according to the literature[57] and recrystallized from ethanol-water, mp 131.5–133.5 °C. 3-Indolylpropionic acid ethyl ester was obtained by esterification of the acid and recrystallized from pentane, mp 40–41 °C.

Solutions were freshly prepared for each decay measurement and degassed by bubbling with high-purity nitrogen prior to use. The concentrations were approximately 5×10^{-5} M. The buffers were 0.01 M sodium cacodylate at either pH 7 or 5. Cyclohexane was MCB spectroquality grade. Deuterium oxide (99.7%) was obtained from Merck Sharp and Dohme.

Absorption spectra were performed on a Cary 118CX spectrophotometer. Fluorescence spectra were measured on a Perkin-Elmer MPF 44A spectrofluorimeter and corrected according to the methods outlined by Melhuish.[58]

The fluorescence decay measurements were made at 20 °C at different emission wavelengths with 8-nm band-pass on the instrument described previously.[52] The multichannel analyzer was a Tennecomp TP5/11 system using a Digital Equipment Corp. (DEC) 11/04 computer. Data was transferred via a direct link to a DEC 11/34 computer for data processing.

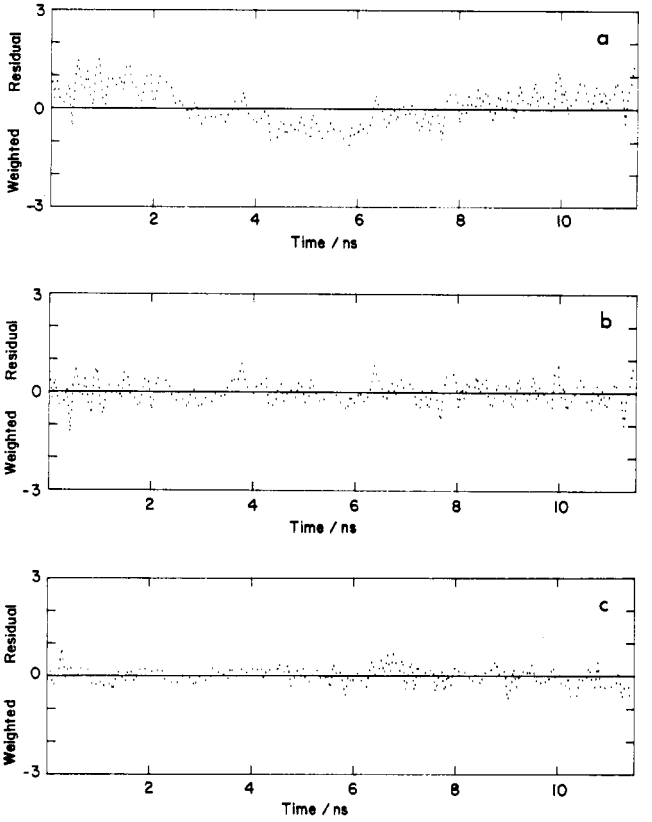

Figure 1. Weighted residual plots for calculated "best fit" emission decay profile after deconvolution of the corrected emission decay profile, λ_{ex} 280 nm, λ_{em} 340 nm: (a) for tryptophan and only one exponential decay component; (b) for tryptophan and two exponential decay components; (c) for PPD in ethanol and one exponential decay component.

Prior to deconvolution the data was corrected by previously described procedures[52] for the instrumental time response variation with wavelength. Deconvolution was performed by a nonlinear least-squares convolution process.[59] Inspection of the weighted residual plots was used as the criterion for adequacy of the curve fitting.

Fluorescence quantum yields were measured relative to tryptophan itself ($\phi = 0.14$[90]) by comparison of the areas under the respective corrected fluoresence spectra. The excitation wavelength was 280 nm, at which the optical densities of the solutions did not exceed 0.05. The excitation and emission band-passes were 3 nm.

Results

In our earlier work on fluorescence decay measurements we described criteria by which one could have a high degree of confidence in the physical model and kinetic parameters obtained after deconvolution of fluorescence decay data.[52,53,59] The most satisfactory criterion for the acceptance of the results is the random distribution of the weighted residuals[60] obtained after deconvolution. When we measured the fluorescence decay of an aqueous buffered solution of tryptophan (pH 7, 20 °C) with $\lambda_{ex} = 280$ nm and $\lambda_{em} = 340$ nm, the residuals obtained after deconvolution for a single exponential decay function were clearly not randomly distributed (Figure 1a). When a double exponential decay function of the form

$$F(t) = Ae^{-k_1 t} + Be^{-k_2 t} \quad (1)$$

was fitted, the weighted residuals appear to be randomly distributed (Figure 1b), indicating that the measured fluorescence decay was adequately described by this function. The lifetimes

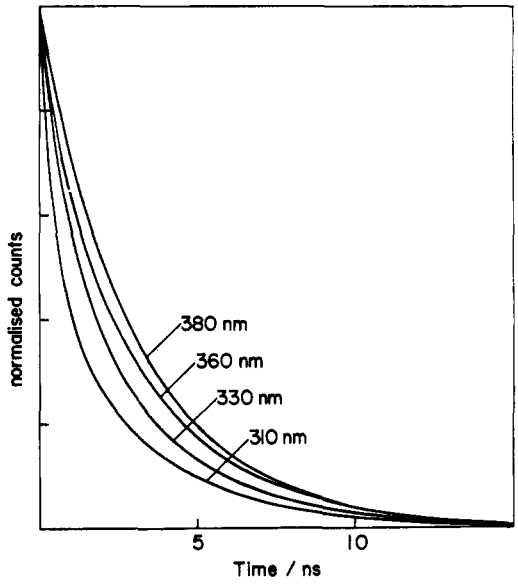

Figure 2. Normalized calculated "best fit" emission decay profiles after deconvolution of the corrected emission decay profiles of aqueous tryptophan, λ_{ex} 280 nm, λ_{em} (a) 310, (b) 330, (c) 360, (d) 380 nm. Curves (a), (b), and (c) are the calculated decay profiles for two exponential decay components. Curve (d) is for one exponential decay component.

Table I. Fluorescence Decay Times and Ratio of Preexponentials for Aqueous Tryptophan at pH 7 and 20 °C[a] at Different Emission Wavelengths

$\lambda_{em} \times nm^{-1}$	$\tau_1 \times ns^{-1}$	$\tau_2 \times ns^{-1}$	R^b
310	3.08 ± 0.15	0.46 ± 0.16	0.96 ± 0.15
315	3.18 ± 0.08	0.54 ± 0.16	1.2 ± 0.1
320	3.17 ± 0.12	0.67 ± 0.15	1.5 ± 0.2
325	3.21 ± 0.05	0.44 ± 0.07	1.7 ± 0.2
330	3.13 ± 0.04	0.53 ± 0.10	2.1 ± 0.2
335	3.22 ± 0.03	0.54 ± 0.12	2.7 ± 0.3
340	3.12 ± 0.04	0.62 ± 0.12	2.9 ± 0.2
350	3.09 ± 0.03	0.65 ± 0.20	4.1 ± 0.5
360	3.25 ± 0.03	0.65 ± 0.14	4.3 ± 0.3
370	3.18 ± 0.05	0.66 ± 0.25	7.3 ± 1.2
380	3.10 ± 0.01		
mean value: τ_1 = 3.13 ± 0.03 ns, τ_2 = 0.51 ± 0.04 ns			

[a] Values reported are the means of several measurements made on degassed buffered solutions with λ_{ex} 280 nm. [b] R is the ratio of preexponential terms corresponding to τ_1 and τ_2, respectively.

of the two components were 3.12 ± 0.04 and 0.62 ± 0.12 ns with a ratio of preexponentials, A/B, being 2.88 ± 0.18.

At all wavelengths below 380 nm the fluorescence decay of aqueous tryptophan was best described by a double exponential function (Table I) with the same two lifetimes, within experimental error, but different relative values of A and B. The weighted mean lifetimes[61] were 3.13 ± 0.02 and 0.51 ± 0.04 ns. The preexponentials A and B were always positive and their ratio increased with increasing wavelength until 380 nm, where the fluorescence decay obeyed single exponential kinetics with a lifetime of 3.10 ± 0.01 ns, a value equal to that of the longer component at the shorter wavelengths.

The lack of any instrumental artifact causing these results was shown when we measured the fluorescence decay of an ethanol solution of the scintillator PPD using identical conditions and computational procedures. The weighted residual plot (Figure 1c) for the expected single exponential decay process (τ = 1.35 ± 0.04 ns) appeared random. Computer-simulation studies of double exponential decaying systems showed that our computational procedures could resolve the kinetic parameters of fluorescence decays such as observed in the case of tryptophan.

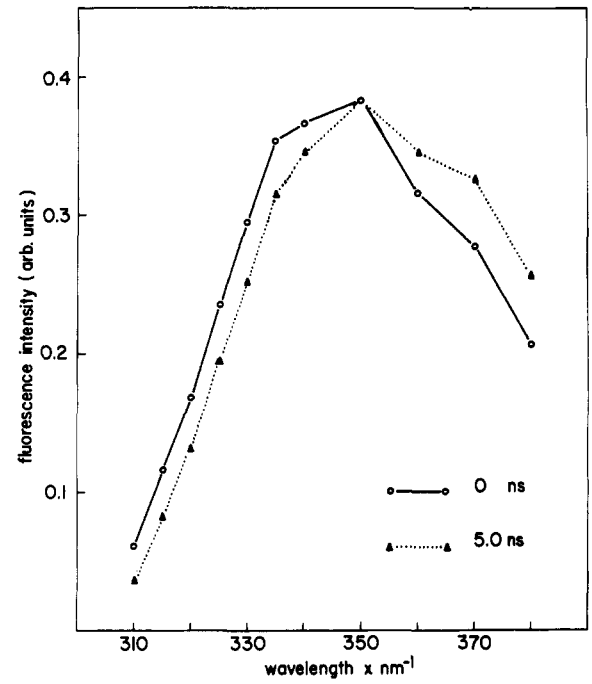

Figure 3. Fluorescence spectra of aqueous tryptophan (pH 7, 20 °C) at t = 0 and 5 ns with peak heights normalized at 350 nm.

The deconvolved fluorescence decay curves at several wavelengths are shown in Figure 2. It can be seen that the decay curve measured at 310 nm has significantly less of a long-lifetime component than that measured at 380 nm. From these curves and the steady-state fluorescence spectrum of tryptophan it was possible to generate time-resolved spectra of tryptophan fluorescence. The fluorescence intensity at time t at a wavelength of λ is $F(\lambda,t)$ and may be expressed in terms of the steady-state fluorescence intensity of tryptophan at wavelength λ, $F_{ss}(\lambda)$, according to the expression

$$F(\lambda,t) = F_{ss}(\lambda)(R_\lambda e^{-t/\tau_1} + e^{-t/\tau_2})/(R_\lambda \tau_1 + \tau_2) \quad (2)$$

where $R_\lambda = A_\lambda/B_\lambda$.

If the reasonable assumption is made based on these experimental measurements that the emission at 380 nm is due entirely to that of the 3.1-ns component and that the spectrum at 5 ns consists only of this same component, it is possible to calculate an approximate fluorescence spectrum of the short-lived component at t = 0 ns. The spectrum at t = 5 ns (Figure 3) is normalized so that the intensity value at 380 nm is equal to the value at this wavelength at t = 0 ns. Subtracting these values from the total t = 0 ns spectrum gives the zero-time spectrum of the short-lived component (Figure 4). This component has a maximum at ~335 nm while that of the 3.1-ns component is at 350 nm, the usual steady-state fluorescence maximum recorded for aqueous tryptophan.

The contribution of the 0.5-ns component to the steady-state fluorescence spectrum of tryptophan may be estimated in the following manner as outlined by Wahl and co-workers.[56,62] The total fluorescence at wavelength λ for the 3.1-ns component, $F(\lambda)$, is given by

$$F_1(\lambda) = \frac{R\tau_1}{R\tau_1 + \tau_2} F_{ss}(\lambda) \quad (3)$$

and for the 0.5-ns component, $F_2(\lambda)$, by

$$F_2(\lambda) = \frac{\tau_2}{R\tau_1 + \tau_2} F_{ss}(\lambda) \quad (4)$$

The resolution of the steady-state spectrum into these components is shown in Figure 5. Clearly the 0.5-ns component

Table II. Kinetic Parameters of the Fluorescence Decay of Tryptophan Derivatives[a]

compd	λ_{em} × nm^{-1}	τ_1 × ns^{-1}	τ_2 × ns^{-1}	τ_{lit} × ns^{-1} [c]	R[b]	ϕ[c]
tryptophan	330	3.13 ± 0.03	0.53 ± 0.05	(3.0)[63]	2.1 ± 0.2	0.14 (0.14)[90]
	350	3.09 ± 0.03	0.65 ± 0.12		4.1 ± 0.5	
pH 10.5	330	10.1 ± 0.4	4.55 ± 0.4	(8.9)[63]	1.2 ± 0.3	0.31 (0.36)[67]
N-acetyltryptophanamide	330	3.00 ± 0.01		(2.9)[19]		0.14 (0.15)[3]
N-acetyltryptophan	330	4.80 ± 0.01		(4.8)[14]		0.21 (0.23)[3]
tryptophanamide pH 5	330	1.61 ± 0.01		(1.7)[67]		0.11 (0.09)[4]
pH 9	330	6.86 ± 0.03				
tryptophan ethyl pH 5 ester	330	1.53 ± 0.09	0.47 ± 0.03		0.22 ± 0.04	0.029 (0.024)[3]
	350	1.87 ± 0.09	0.51 ± 0.02		0.13 ± 0.03	
N-acetyltryptophan ethyl ester	330	1.87 ± 0.07	0.81 ± 0.16		1.8 ± 0.3	0.066 (0.067)[4]
	350	1.84 ± 0.02	0.50 ± 0.1		2.5 ± 0.3	
5-methyltryptophan	330	3.14 ± 0.13	1.40 ± 0.14		1.2 ± 0.2	0.13
	350	2.74 ± 0.02	0.72 ± 0.12		4.7 ± 1	
6-methyltryptophan	330	3.02 ± 0.16	1.50 ± 0.3		1.7 ± 0.3	0.11
	350	2.98 ± 0.19	1.53 ± 0.3		1.8 ± 0.4	

[a] Values reported are those for measurements made on degassed buffered solutions at 20 °C, pH 7 (unless otherwise noted), with λ_{ex} 280 nm. [b] R is the ratio of the preexponential terms corresponding to τ_1 and τ_2, respectively. [c] The values in parentheses are literature values which are representative of several determinations by various authors. The literature values of the fluorescence lifetimes were all measured using filters in the emission beam.

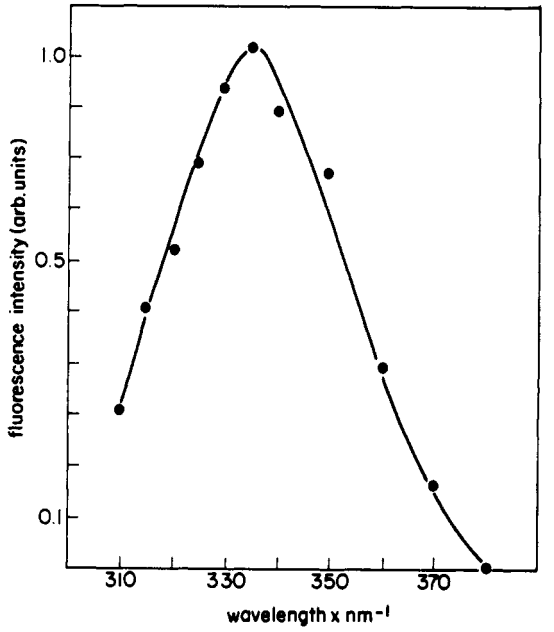

Figure 4. Calculated zero time emission spectrum of the 0.5-ns decay component.

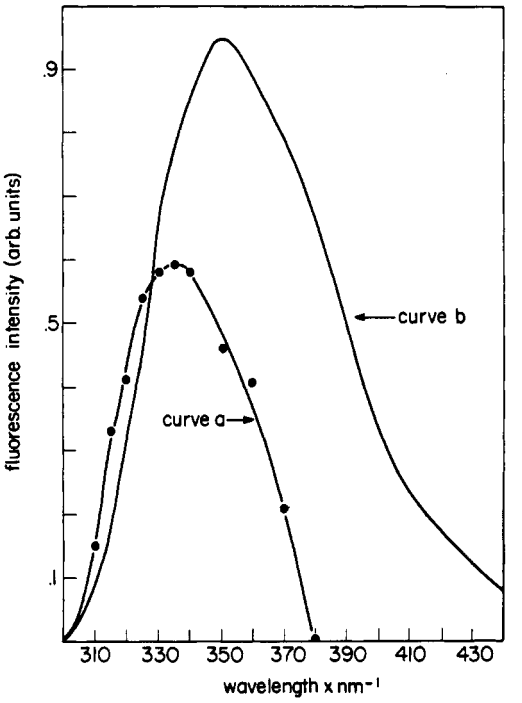

Figure 5. Resolution of the steady-state fluorescence spectrum of aqueous tryptophan (pH 7, 20 °C) into the two components from the fluorescence decay data; curve (a), 0.5-ns component multiplied by a factor of 10; curve (b), 3.1-ns component.

makes a very small contribution to the total steady-state fluorescence intensity at any wavelength.

The generality of the observation of dual exponential kinetics in other tryptophan derivatives was examined. The results are summarized in Table II. For those derivatives exhibiting single exponential fluorescence decay behavior our values are in reasonable agreement with the literature. In the case of tryptophan the longer of the two lifetimes is similar to literature values. The lifetimes of the remaining derivatives have to the best of our knowledge not been reported earlier. The fluorescence of the derivatives *N*-acetyltryptophanamide, *N*-acetyltryptophan, and tryptophanamide all decayed with single exponential kinetics. By contrast the fluorescence decays of tryptophan ethyl ester, *N*-acetyltryptophan ethyl ester, 5-methyltryptophan, and 6-methyltryptophan were described by double exponential functions.

Since the pK_a of the α-amino group in tryptophan ethyl ester and tryptophanamide is close to 7, their fluorescence decay was measured at pH 5. The fluorescence decay parameters of tryptophan at pH 5 were identical with those at pH 7.

The fluorescence decay of tryptophanamide at pH 9, where the α-amino group is not protonated, is significantly longer than at pH 5, where it is protonated. In the case of tryptophan at pH 10.5, where the α-amino group (pK_a = 9.3) is unprotonated (93% NH_2, 7% $(NH_3)^+$), again a double exponential decay is observed with lifetimes of 10.1 ± 0.3 and 4.8 ± 3 ns with a ratio of preexponentials of 1.27. This result is different from that observed by Wahl (8.9 ns).[63]

The lifetimes of tryptophanamide and the longer lifetime of tryptophan ethyl ester and *N*-acetyltryptophan ethyl ester are all shorter than the corresponding lifetimes in the other derivatives. These results show that the dual exponential be-

Table III. Fluorescence Parameters of Indole Derivatives[a]

compd	solvent	$\tau \times ns^{-1}$	$\tau_{lit} \times ns^{-1}$ [b]	ϕ[b]	$\tau_f \times ns^{-1}$ [c]	$k_0 \times 10^{-7}, s$[d]
indole	H_2O	4.82 ± 0.02	4.8[13]	0.25 (0.23)[45]	19.3	15.6
	C_6H_{12}	7.93 ± 0.03	9.0[13]	0.48 (0.37)[13]	16.5	6.5
1,2-dimethylindole	H_2O	5.51 ± 0.03		0.36	15.3	11.6
	C_6H_{12}	3.37 ± 0.01		0.48	7.0	15.4
3-methylindole	H_2O	9.37 ± 0.06	9.4[14]	0.37 (0.34)[67]	25.3	6.7
	C_6H_{12}	3.70 ± 0.01	3.4[9]	0.31	11.9	18.6
5-methoxyindole	H_2O	4.48 ± 0.01	4.0 ± 0.1[65]	0.27 (0.29)[65]	16.6	16.3
2-(3′-indolyl)acetic acid	H_2O	9.19 ± 0.07	8.7[14]	0.39 (0.33)	23.6	6.6
3-(3′-indolyl)propionic acid	H_2O	10.76 ± 0.08		0.44 (0.45)[48]	24.5	5.2
4-(3′-indolyl)butyric acid	H_2O	10.33 ± 0.07		0.42 (0.48)[48]	24.6	5.6
3-(3′-indolyl)propionamide	H_2O	8.67 ± 0.05		0.39 (0.42)[4]	22.2	7.0
3-(3′-indolyl)propionic acid ethyl ester	H_2O	3.36 ± 0.04		0.15	24.0	25.6
3-(3′-indolyl)ethylamine[e]	H_2O	6.14 ± 0.05	6.0[14]	0.34 (0.30)[3]	19.8	11.2

[a] See footnote *a*, Table II. λ_{em} 330 nm. [b] See footnote *c*, Table II. [c] τ_f is the radiative lifetime ($1/k_f$) calculated from the relationship $\phi\tau_f = \tau$. [d] k_0 is the rate constant for the nonradiative processes calculated from the relationship $\tau = 1/k_f + k_0$ (see text). [e] Tryptamine.

Table IV. Kinetic Parameters of the Fluorescence Decay of Tryptophan and Indole Derivatives in D_2O Solution[a]

compd	$\tau_1 \times ns^{-1}$	$\tau_2 \times ns^{-2}$	R[a]
tryptophan	7.05 ± 0.35	2.76 ± 0.2	1.34 ± 0.4
N-acetyltryptophan ethyl ester	2.23 ± 0.14	1.14 ± 0.19	1.28 ± 0.6
N-acetyltryptophanamide	3.58 ± 0.01		
tryptophanamide[b]	2.22 ± 0.01		
tryptamine	8.57 ± 0.06		
3-methylindole	13.0 ± 0.1		
indole	6.0 ± 0.03		

[a] See footnotes in Table II. [b] pH 5.

Table V. Fluorescence Decay Parameters in 2 M $NaClO_4$ Solution, pH 7

compd	$\tau_1 \times ns^{-1}$	$\tau_2 \times ns^{-1}$
tryptophan	2.85 ± 0.02	0.47 ± 0.04
N-acetyltryptophanamide	3.46 ± 0.01	
3-methylindole	10.8 ± 0.06	

havior of tryptophan derivatives is dependent on the α-carbon substituents.

The fluorescence decay of several indole derivatives related to tryptophan was measured. These are summarized in Table III. In all these compounds *only* a single exponential fluorescence decay was observed. As in tryptophan the lifetimes were dependent on the nature of the substituent. Our values are in reasonable agreement with those measured by other workers previously.

To the best of our knowledge the lifetimes of 3-indolylpropionic acid and its derivatives have not been reported earlier. The lifetime of the ethyl ester derivative is the shortest followed by the amide and then the carboxylate group. This parallels the quantum-yield values and indicates that the ester group is the most effective quencher of the indole ring if the radiative rate constants are equivalent (vide infra).

The values of the quantum yield and calculated radiative lifetimes, listed in Table III, are in most cases in good agreement with the literature. While these values may be inaccurate owing to continuing controversy over the quantum yield of tryptophan, their relative order and their lack of constancy are observations which are important to our discussion.

A comparison of the effect of changing the solvent from water to cyclohexane on the fluorescence of indole and 3-methylindole is interesting. In the case of indole the lifetime is longer in cyclohexane (7.9 ns) solution than in water (4.8 ns), while in the 3-methylindole the reverse is true; cyclohexane (3.7 ns), water (9.4 ns).

The fluorescence decays of some derivatives were measured in D_2O in order to determine the effect of deuterium substitution of the amino hydrogens on the decay kinetics. Several workers have earlier reported an enhancement of the fluorescence of tryptophan and tryptamine in D_2O solution. Our results are presented in Table IV. In all cases the same multiplicity of decay as in water is found but the lifetimes are longer. The lifetimes of the two components in the tryptophan decay are both markedly longer in D_2O. The ratios of the fluorescence lifetime in D_2O to that in H_2O of the compounds with monoexponential decay are similar, ranging from 1.2 to 1.4, in agreement with quantum-yield measurements.[3,5,67]

To test the role of solvent structure on the double exponential decay behavior of tryptophan fluorescence we investigated the effect of 2 M sodium perchlorate solutions of tryptophan, *N*-acetyltryptophanamide, and 3-methylindole. The results in Table V show that there is no effect on the order of the decay processes in high ionic strength solutions. There was a small increase in the lifetime of *N*-acetyltryptophanamide and 3-methylindole and a smaller decrease of the long-lifetime component of tryptophan fluorescence.

We verified that the proportion of the two components in the fluorescence decay of tryptophan derivatives also varied with wavelength. Decay measurements at 330 and 350 nm showed that the relative amount of the longer lifetime component was greater at 350 nm. The results for tryptophan ethyl ester are an interesting exception since in this case the relative amount of shorter lifetime component is greater than the long-lived component at the two wavelengths and there is even more of the former component at 350 nm.

Discussion

Prior to considering the rationalization of the results presented herein, comments on the validity of our observation of double exponential fluorescence decay kinetics of aqueous tryptophan are appropriate. This is important in view of the fact that earlier workers using instruments of varying degrees of sophistication[14,45,63,64,67] who measured the fluorescence decay of tryptophan reported only single exponential kinetics. During the preparation of this manuscript Fleming and coworkers[54] reported the observation of dual exponential kinetics of tryptophan fluorescence decay above 380 nm. There has been one preliminary report of multiexponential fluorescence decay behavior in indole by Mataga.[68] However, this result has not been confirmed in any subsequent publication by that group. Balcavage and Alväger[69] reported dual exponential decay behavior of tryptophan fluorescence that was dependent on excitation wavelength. The lifetimes which they reported

were markedly different from any previously reported or found by ourselves.

We consider that we have been able to observe the dual exponential decay behavior because of instrumental refinements and improvements in the criteria for deciding on the adequacy of the curve-fitting or deconvolution calculations. In our instrument we eliminate scattered exciting light and achieve wavelength resolution of the emission by using monochromators rather than filters in both the excitation and emission beams. The use of filters in the emission beam makes the detection of a second component in the decay very difficult or unlikely because of the large contribution of the long-lived component to the total emission intensity. We also correct for the variation of the instrumental time-response function with wavelength which we have shown[51,52] is essential for accurate measurement of fluorescence decay parameters. Finally we base our acceptance of kinetic parameters obtained after deconvolution on the inspection of the weighted residual plots. Our criteria of acceptance is that the weighted residuals must appear random for the physical model used in the deconvolution; otherwise alternate models describing the fluorescence decay must be considered. Several other workers[19,56,70] measuring fluorescence decays using time-correlated single photon counting techniques also recommend similar criteria.

As we indicated earlier we eliminated artifactual explanations of the observed dual emission. The recurrence, within experimental error, of the same two lifetimes in the case of tryptophan with changing emission wavelength allows us to state that the double exponential decay represents a physical model for the decay processes and is not simply mathematical curve fitting. The small variation in the lifetime of the short-lived component illustrates the difficulties that are encountered when making these types of measurements where deconvolution involves the estimation of four highly correlated unknown parameters. With improved time resolution one may expect to reduce the standard deviation in the lifetimes of the shorter component. The observation of single exponential kinetics in the fluorescence decay of *N*-acetyltryptophanamide and *N*-acetyltryptophan, which have similar excitation and emission spectra as tryptophan and lifetimes as the long-lived component in tryptophan, is a verification that the dual exponential behavior in the latter case is not due to any instrumental or data-processing artifact.

The discrepancy between our results above 380 nm and those of Fleming[54] may be due to the different techniques of excitation and emission detection. We found that at all wavelengths above 380 nm only a single component with a lifetime of 3.1 ns was observed. This value of 3.1 ns agrees with the results of earlier workers who would have detected only the most predominant component. Measurements with tryptophan obtained from the same source as Fleming in our hands gave a single exponential decay with a lifetime of 3.1 ns at 390 nm. Perhaps the high photon density of their pulsed laser beam resulted in photochemical alteration of the sample. We found that, if we irradiated a degassed solution of tryptophan for a short time (<5 min) with a pen ray lamp and subsequently measured the fluorescence decay at 380 nm (λ_{ex} 280 nm), then we observed double exponential decay kinetics with lifetimes of 6.9 and 1.8 ns in a ratio of 2:1. These results are in agreement with those obtained by Fleming and co-workers. Their lack of observation of a 0.5-ns component in their study is consistent with our time-resolved spectra which show that there is a negligible amount of this component above 370 nm, the lower wavelength cut off of the streak camera used by them.

In our preliminary communication[53] we suggested that the double exponential fluorescence decay kinetics of aqueous tryptophan was due to the emission from two solvent equilibrated $^1L_a^e$ and $^1L_b^e$ states which were not interconvertible. One requirement of this rationalization is that the initially excited ^1L_a and ^1L_b states, prior to solvent equilibration, should be nearly degenerate. This suggestion was an extension of the concepts advanced by other workers[11,30,45,71] to explain the large Stokes shift in tryptophan and indole fluorescence in polar solvents. They proposed that the shift is due to a reorganization of the solvent shell surrounding the solute caused by the large dipole change of the ^1L_a state relative to the ground state. The solvent reorganization occurs rapidly (<10 ps[72]) and results in a lowering of the energy of the ^1L_a state below that of an initially close-lying ^1L_b state. Andrews and Forster[8] discussed the possibility of simultaneous emission of thermally equilibrated ^1L_a and ^1L_b levels in indole derivatives with one state or other dominating the emission depending on the substituent on the indole nucleus. Furthermore, recent polarization studies of Valeur and Weber[6] suggest that the emission of tryptophan is comprised of two bands.

If indeed our original explanation was correct, then "a priori" one would have expected that the fluorescence of *N*-acetyltryptophanamide and *N*-acetyltryptophan should also have exhibited dual exponential decay kinetics. These derivatives are different from tryptophan only in the substituents on the α carbon and this is not expected to markedly affect the relative order of the two nearly degenerate ^1L_a and ^1L_b states. We conclusively confirmed that the fluorescence decay of these compounds obeyed single exponential kinetics at all wavelengths.

On the other hand it has been shown that substituents on the aromatic ring such as in 5-methylindole and 3-methylindole cause a change in the relative positions and/or separation of the ^1L_a and ^1L_b states[8,34] when compared with indole. Thus an effect on the relative position of these two states in 5-methyltryptophan and 6-methyltryptophan when compared with tryptophan is a reasonable possibility and a difference in the fluorescence decay kinetics of these molecules may have been expected. Actually the fluorescence decay of these derivatives again clearly obeyed double exponential decay kinetics and the ratio of the two components varied with emission wavelength. Further, for all the indole derivatives examined lacking an alanyl type side chain, especially those for which an assignment of the positions of the ^1L_a and ^1L_b states has been made (vide supra), only single exponential decay kinetics were observed in both polar and nonpolar solvents at all wavelengths studied.

These considerations indicate that we must reexamine our earlier rationalization and suggest alternative explanations which are more consistent with the results.

Several alternatives were considered. As stated earlier the formation of an exciplex and concomitant exciplex kinetics should be discounted since at all wavelengths both preexponentials were always positive and above 380 nm a single exponential decay was observed. If the fluorescence was due to exciplex emission only, then either the preexponentials should be equal in magnitude but opposite in sign or, if the rate constant for exciplex formation is rapid, then the fluorescence decay would be described by a single exponential decaying function. If the spectrum included both exciplex and monomer emission, then at one wavelength a discontinuity would be observed in the preexponentials and in one wavelength range the preexponentials would be positive while in another range negative preexponential terms would be observed.

An alternate explanation could involve excited-state p*K* changes or a similar equilibrium process. However, arguments similar to those discussed above can rule this explanation out.

A fourth rationalization which we now favor is that the dual emission originates from different conformers or rotamers of the alanyl side chain of tryptophan. This concept was indeed suggested by Fleming and co-workers in their report. A similar explanation has been invoked recently by Wahl and co-work-

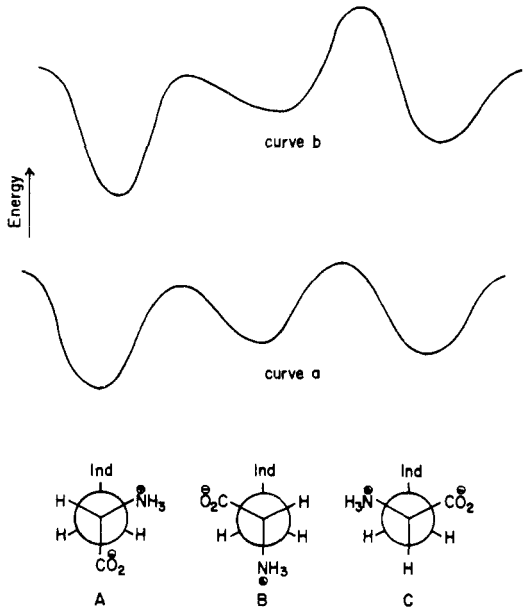

Figure 6. Newman projections of three rotamers along the C_α-C_β bond of L-tryptophan and depiction of potential energy surfaces of three rotamers in (a) the ground state; (b) the excited singlet state as per discussion in the text.

ers[62,73] in their fluorescence decay study on tyrosine derivatives and diketopiperazines. This interpretation of the observation of dual exponential decay kinetics of tryptophan and the temporal resolution of the fluorescence spectrum has appealing relevancy to biochemical systems.

We propose that the dual exponential decay of fluorescence of tryptophan originates from two or more different configurations of the alanyl side chain in reference to the indole nucleus.

The existence of preferred populations of C_α-C_β bond rotamers in amino acids has been amply demonstrated by analysis of the coupling constants of their NMR spectra. Cavanaugh[74] found that the relative populations of three rotamers of the carboxylate anion form of tryptophan (alkaline pH) was similar to that of phenylalanine. Unfortunately the limited solubility of the zwitterion form of tryptophan has prevented its NMR spectral resolution. Thus, by analogy with amino acids, particularly phenylalanine, and by inspection of space-filling molecular models we can discuss the relative populations of three α-β rotamers of tryptophan shown as A, B, and C in Figure 6.

For each of the above rotamers further conformational variation is possible by rotation of the indole ring around the C_β-C_γ bond. The ease of rotation of the indole ring around this bond depends on the particular C_α-C_β rotamer since the steric interactions of the ring with the substituents differ in each rotamer. Based on examination of space-filling models an assessment of the effect of rotation around the C_β-C_γ bond on these interactions can be made. In rotamers A and B the rotation of the indole ring is relatively free, perhaps slightly more so in rotamer A, unless certain conformations are favored because of specific intramolecular interactions. Because of this rotational freedom about the C_β-C_γ bond any effect of this rotation on the fluorescence decay of these rotamers will be averaged. The rotation of the indole ring around the C_β-C_γ bond in rotamer C is restricted and one can propose an energy barrier between two conformations. However, we feel that the C_β-C_γ rotamers do not affect our rationalization of the spectral assignment in terms of rotamers A, B, and C. Therefore we have restricted our discussion to rotamers about the C_α-C_β bond.

It has been shown that rotamer A is the favored rotamer in phenylalanine and tyrosine (approximately 50%) with approximately equal populations of B and C.[75-78] In the case of tryptophan the electrostatic repulsion of the negatively charged carboxylate and the Π-electron cloud of the indole nucleus would reduce the populations of rotamer B relative to A. Inspection of models indicates that there is considerable steric crowding between the C_α substituents and the indole nucleus in rotamer C. Owing to the increased size and the Π-electron density of the indole nucleus compared to a phenyl ring one may speculate that if anything the population of rotamer A is even higher than that in phenylalanine. Thus the predominant rotamer in tryptophan would be A where the positively charged ammonium substituent is close to the indole nucleus. Rotamer B with the carboxylate substituent near the indole ring would be the least favorable while the C rotamer with the indole nucleus and the two α substituents eclipsed may have a slightly larger population than B, i.e., [A] ≫ [C] > [B].

With this conformer model we initially attempted to interpret the dual exponential behavior in terms of the relative quenching efficiencies of the substituents and their relationships to the indole nucleus. The observation of only two decay components rather than three may be due to very similar lifetimes of two of the rotamers and our inability to resolve them or perhaps the fluorescence yield of one of the rotamers is negligible.

It has previously been shown[4,66] that the relative quenching efficiency of indole fluorescence by substituents on the 3 position decreases in the following order:

$$-CO_2H \geq -CO_2C_2H_5 > CONH_2 \gtrsim -CO_2^-$$

The quenching efficiency of the ammonium group in tryptophan or tyrosine has been discussed in terms of an electrostatic effect on the quenching efficiency of the carboxyl group and not attributed to a direct quenching process.[4,54,73,79]

However, the data of Feitelson[4] shows that tryptamine fluorescence is quenched by 15% at acidic pH compared to the fluorescence at alkaline pH where the amino group is not protonated. Moreover, Lehrer[82] states that the α-ammonium group quenches the fluorescence of tryptophan as evidenced by studies of the deuterium isotope effects. Eisinger and Navon[45] showed that there was a large deuterium isotope effect on the fluorescence of 1-methyltryptophan and we have recently found[83] that the fluorescence quantum yield of 1-methyltryptamine increases by 30% in D_2O compared to H_2O. Nakanishi and Tsuboi[80] in their stop-flow fluorescence measurements on tryptophan have demonstrated that at least 83% of the enhancement of the fluorescence of tryptophan in D_2O solution relative to H_2O is due to the exchange of the α-ammonium hydrogens by deuterium. These reports indicate that the α-ammonium substituent does quench the fluorescence of the indole nucleus. There are at least two mechanisms by which the ammonium group could act as a fluorescence quencher. It may either be by a proton-transfer mechanism as suggested by some workers[41,82] or an electron-transfer mechanism as suggested by other reports.[29,84] At present we are unable to offer any definitive choice between these two mechanisms except that the work of VanderDonckt[26] shows that the excited-state pK_a of indoles is >2, a limiting value which does not rule out the proton-transfer mechanism.

In earlier work[67] the radiation lifetimes of the indole derivatives were assumed to be similar. From our lifetime results and our measured quantum yields (Table III) we can calculate a radiative lifetime for each derivative in aqueous solution. It is apparent that the radiative lifetimes are not constant, though they have values of the same order ranging from 15 to 25 ns for the different derivatives. It is noteworthy that for the 3-substituted indole series the radiative lifetimes are similar

(τ_f(mean) = 24.2). From these radiative lifetimes ($1/k_f$) and the measured singlet state lifetimes ($1/k_0 + k_f$) we can calculate the nonradiative rate constant k_0. These values, listed in Table III, are a preferred comparison of the relative fluorescence quenching efficiency of the indole ring by a substituent on the 3 position. This order is

$$CH_2CH_2CO_2C_2H_5 > -CH_2CH_2NH_3^+$$
$$> -CH_2CH_2CONH_2 = CH_3 > -CH_2CH_2CO_2^-$$

Based on this series we were tempted to assign rotamer A with the ammonium group closest to the indole ring (and perhaps rotamer C as well) as the short-lived (0.5 ns) component and rotamer B with the carboxylate group closest to the indole ring as the long-lived (3.1 ns) component. However, this assignment of the rotamers breaks down on considering the spectral results. If we assume that the radiative lifetimes of the rotamers are similar (vide supra), the ratio of the quantum yields of the two components may be estimated. Then the ratio of the initial concentrations of the two components may be estimated from the expression

$$\frac{[335]}{[350]} = \frac{S_{335}\,\phi_{350}}{S_{350}\,\phi_{335}} \qquad (5)$$

by comparing spectral areas of the two components (Figure 5). In the expression [335] and [350] represent the initial concentrations of the 335- and 350-nm components and S_λ represents the areas of their time-resolved fluorescence spectra. From these relationships we found that the ratio of the initial concentrations of the 350-nm component to that of the 335-nm component was 3.7. Despite the approximations and assumptions used in arriving at this number, this ratio is reasonably close to the ratio of the ground-state populations of rotamers A and B or C to the populations of rotamers C or B, respectively, as estimated from the NMR measurements discussed above. Therefore our initial assignment based on the quenching efficiency of the ammonium group is not consistent with the rotamer populations.

We propose that the spectral features, lifetimes, and rotamer populations may be resolved by one of the following similar schemes. One can estimate the general form of a potential energy surface for the interconversion of the rotamers A, B, and C in the ground state where A is the most stable, followed by C and then B. In the excited state changes in the potential surface will result owing to the following interactions. In the excited state of rotamer A the large dipole of the excited indole nucleus which has additional negative charge on atomic positions remote from the indole nitrogen[33,81] will be stabilized by interaction with the positively charged ammonium group. On the other hand, the interaction between the excited indole nucleus and the carboxylate substituent will be less favorable owing to electrostatic repulsion and will destabilize rotamer B in the excited state For rotamer C it is not intuitively clear as to what effect the electrostatic interactions with the C_α substituents will be but one might consider that any stabilization may be small. This discussion is summarized in Figure 6.

This scheme indicates that rotamer A will have a fluorescence spectrum at lower energy than the other rotamers. The ratio of concentrations of the 350- to 335-nm components is consistent with the 350-nm component being the most populous ground-state rotamer. Further, we suggest that, because of the electrostatic interaction between the carboxylate substituent and the excited indole nucleus in rotamer B, the carboxylate group may be "*pushed*" away from the indole ring and by a rapid one-third of a rotation form rotamer A. The formation of rotamer C from B by a rotation in the opposite direction would be much less favorable owing to the steric and electrostatic interactions occurring when the carboxylate group passes the indole nucleus. By analogy it is reasonable that, during the lifetime of the excited state, rotamer C does not form either rotamer A or B. Therefore we suggest that the 335 nm may be assigned as being due to rotamer C. This explanation accounts for the ratio of the two components being 3.7, as the ratio of populations of rotamers A and B to rotamer C is close to 3.5 in the ground state.

A rationalization of the observed lifetimes of the two species in terms of this scheme is possible. One might consider that in rotamer C there are two quenching groups, with the carboxylate and the ammonium substituents close to the indole ring, whereas in rotamer A there is only the ammonium group in this position. Recalling that the carboxylate group is the least effective quencher in the indolepropionic acid derivative series this explanation may be inappropriate. However, one could imagine that the ammonium group is closer to the indole nucleus in rotamer C owing to the unfavorable electrostatic interaction between the carboxylate group and the indole.

An alternative explanation based on similar considerations would be that rotamers A and C have similar emission spectra and similar lifetimes of 3 ns and would be assigned as the 350-nm component. Then rotamer B would be assigned as the 335-nm component. The short lifetime of this component could then be due to the efficient nonradiative process of rotation to rotamer A. At the present time we are not able to distinguish between these two schemes which rationalize the experimental observations according to different rotamer populations. While the assignment of rotamer A as the rotamer with a fluorescence lifetime of 3.1 ns and a spectral maximum of 350 nm is consistent with our data and the ground-state rotamer populations, the assumption of the similarity of radiative rate constants for the components is crucial.

In the above discussion we have neglected any reference to the electronic nature of the fluorescent singlet state of tryptophan and how it may vary with the rotamers. It is conceivable that in one rotamer the ^1L_a state may be the lowest state and in another rotamer the ^1L_b state is the more stable. The local electrostatic interactions between the α-carbon substituents and the different atomic positions on the excited indole nucleus will vary according to the state designation owing to the different electron densities on the indole carbon atoms in the two states.[81]

Also the role of the solvent on the stability of the rotamers and in the interactions with the excited state is difficult to evaluate. The large Stokes shift of both components is reasonably due to a very rapid solvent reorganization after excitation of the solute molecule. The ground-state solvent structure and reorganization in the excited state will depend on the charge on the α-carbon substituents. That the bulk solvent properties do not influence the fluorescence kinetics is shown by the results obtained in concentrated sodium perchlorate solution (Table V). Sodium perchlorate is considered to be an effective water structure breaker.[85] Yet we found that the fluorescence decay kinetics of tryptophan in 2 M $NaClO_4$ were similar to those in low buffer concentrations. Hence the intimate solvation of tryptophan is not affected by the high salt concentration; otherwise a change in the proportion of the two components would be anticipated.

The results which we obtained in D_2O solution offer some clarification of the assignment and rationalization of the results in H_2O. The significant increase in the lifetime of the short-wavelength component (τ_{H_2O} = 0.51, τ_{D_2O} = 2.76 ns) is inconsistent with assignment of the 335-nm component as rotamer B as in the second scheme discussed above. In that scheme we suggested that the lifetime was governed by an efficient nonradiative rotational process. The exchange of H_2O by D_2O should not affect the rate of rotation to any significant extent, yet the lifetime of this component increases by a factor of 5. As stated earlier the exchange of the α-ammonium hy-

drogens for deuterium has a large effect on the fluorescence intensity of tryptophan. In rotamer B the α-ammonium group is farthest away from the indole ring. In rotamer A and C this group is close to the ring. Since in our previous rationalization rotamer A is assigned as the 350-nm component, these D_2O results support the assignment of rotamer C as the 335-nm component.

The much larger increase in the fluorescence lifetime of tryptophan compared to the increase in the fluorescence decay time of *N*-acetyltryptophanamide in D_2O confirms that the α-ammonium group, whose hydrogen atoms have been exchanged for deuterium, is a very effective quencher of the fluorescence of tryptophan.

The results obtained for the tryptophan derivatives with different α-carbon substituents can be discussed in terms of the rotamer scheme and a variation of the quenching efficiencies of these substituents. But these explanations are subject to qualifying considerations. Firstly the solvation and hydrogen bonding of the derivatives will vary depending on their nature and ionic charge. Secondly the ground-state rotamer populations and relative stabilities are not necessarily similar to those of the zwitterion tryptophan. Finally the observation of only one exponential decay in some derivatives may be the result of similar unresolvable lifetimes of rotamers or by rapid rotational motion the lifetimes average to a single exponential.

In *N*-acetyltryptophanamide both α substituents are amide functions having similar quenching efficiencies. Hence, if there were preferred rotamer populations, they might be expected to have the same lifetimes.

However, the double exponential behavior of *N*-acetyltryptophan ethyl ester suggests that there are preferential populations of rotamers and their lifetimes may depend on the different quenching ability of the ester and amide substituent. It is relevant that Newmark and Miller[75] found that in the analogous phenylalanine derivative a rotamer with the ester group close to the aromatic ring was the favored rotamer.

The difference in the fluorescence decay behavior of tryptophan ethyl ester (two exponentials) and tryptophanamide (one exponential) is more difficult to understand. Both have the same positively charged ammonium substituent (pH 5) and shorter mean lifetime than tryptophan. Perhaps this different behavior reflects a difference in the hydrogen-bonding interactions between the α substituents. These results with derivatives of tryptophan are important in another sense because they demonstrate that the fluorescence decay behavior of tryptophan residues in proteins will depend on the interactions of the indole ring with other amino acid residues.

As stated earlier our rationalization of the tryptophan results in terms of different conformers is similar to that of Wahl and co-workers in their work on tryptophan diketopiperazine derivatives[62] and in dipeptides containing tyrosine.[73] In the former case they have temporally resolved the fluorescence spectra of *cyclo*-(glycyltryptophyl) and *cyclo*-(alanyltryptophyl) in Me_2SO solution into two components, one with a λ_{max} near 335 nm and the other main component with a λ_{max} at 350 nm. They attributed the dual behavior to two different conformers which differ in the interaction of the indole ring with the diketopiperazine ring. In their dipeptide work the dual exponential fluorescence decay behavior was observed in some but not all derivatives. It was assigned to the emission from different rotamers and the differing interactions of the tyrosyl residue with the carboxylate or ammonium terminal groups.

In another small peptide, lysyltryptophyllysine, we have observed dual exponential decay behavior with lifetimes of 2.3 and 0.8 ns. Again this must be due to different interactions of the indole ring in at least two conformers.[86]

From these fluorescence decay studies on small amino acid constituents a consistent picture is presented which confirms Grinvald and Steinberg's work on proteins,[19] Wahl and co-workers' work on the lac repressor protein[56] and human serum albumin,[18] and Conti and Forster's report on the fluorescence decay of glucagon.[87] The first authors have found that dual exponential fluorescence decay kinetics are observed in many proteins which contain only a single tryptophan. Moreover, the porportion of the two components varied with emission wavelength. In the lac repressor protein the emission of the two tryptophans could be temporally resolved into two components with emission maxima at 320 and 345 nm. Either this is due to a different environment for each of the two tryptophans in the protein or the fluorescence reflects the emission from two different protein conformations. In the case of glucagon Conti and Forster found double exponential fluorescence decay kinetics at pH 10 and suggested that it originated from different tryptophan environments in different conformers of the peptide.

The continual structural fluctuations of proteins in solution implies that the interactions of the amino acid residues vary. While this manuscript was in preparation, Munro and co-workers,[88] using synchrotron light pulse techniques, showed that in some proteins the internal mobility of chromophoric residues such as tryptophan may be quite rapid, having rotational correlation times less than 1 ns, while in other proteins the rotational times of these residues are very long (>30 ns). In these latter examples, because of the high rotational energy barriers[89] of the aromatic residues, it is clear that they may exist in quite different interactive environments depending on the protein conformation.

Earlier Burstein and co-workers[22] reported that the fluorescence maxima of proteins changed from ~330 to ~350 nm when the proteins were denatured by urea. They attributed this variation to a change in the solvent accessibility and environment of the tryptophan residues in the proteins with the "buried" residues having a fluorescence maxima close to 330 nm and the "exposed" residues a maximum near 350 nm. Considering our results the variation in spectra observed by these authors may also be the result of changes in the interactions of the indole ring within the protein as a result of conformational changes induced by urea. Solvent effects will still be operative but may not be the most significant factor.

Our results on the fluorescence decay of tryptophan and its derivatives taken together with these other reports of multiexponential decay kinetics in proteins provide a basis for interpreting the steady-state fluorescence spectra and fluorescence decay properties of proteins. Since time-resolved fluorescence spectra of aqueous solutions of tryptophan may be attributed to emission from at least two rotamers in which the interactions of the indole ring are different, the interactions of this moiety in proteins may be probed by this technique. Investigations aimed at further evaluation of our rationalization are continuing.

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(60) The weighted residuals are defined according to

$$r(k) = w(k)^{1/2}[h_e(k) - h_c(k)]$$

where $h_e(k) - h_c(k)$ is the difference between the experimentally observed number of counts in the kth channel ($h_e(k)$) and the value obtained after convolution in the same channel ($h_c(k)$). The weight for the kth channel $w(k)$ is defined as $1/h_e(k)$. The weighted residual plot is a plot of $r(k)$ vs. k.
(61) The weighted mean was calculated according to the formula $\bar{x} = \sum w_i \cdot x_i / \sum w_i$ where w_i is the weight assigned to each value of x_i according to the relationship $w_i = 1/(SE_i)^2$ where SE_i is the standard error of each value of x_i.
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