Tryptophan Phosphorescence in Fluid Solution

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Received April 4, 1995[⊗]

Abstract: The weak delayed luminescence of some indole derivatives was monitored for the first time in aqueous solutions at room temperature by means of an apparatus capable of detecting emissions with quantum yields as low as 10^{-7} and lifetimes as short as 10 μs. In liquid solution the total luminescence spectrum is composed of normal phosphorescence and of delayed fluorescence that originates from triplet−triplet anhilation. In water at 20 °C, the lifetime (τ) of the triplet state of indole, tryptophan, and *N*-acetyltryptophanamide was found to be 1.2 ms while for 1-methyltryptophan it was 0.6 ms. Compared with published data, obtained by triplet−triplet absorption measurements, these values of τ are almost two orders of magnitude larger. Discrepancies with absorption data were also found for the second-order rate constant that describes the quenching by molecules in the ground state and by H_3O^+. The viscosity (η) dependence of τ in propylene glycol/water mixtures was determined between 10^9 and 10^{-2} P. At high viscosity ($\eta > 3 \times 10^3$ P) the log $1/\tau$ vs log T/η plot had a slope of 0.29 while at lower viscosity the slope increases to 0.7 and in either case the slope is smaller than 1, the value expected for diffusion-controlled quenching reactions. By comparison phosphorescence quenching experiments conducted in the same viscosity range with O_2, Cu^{2+}, and NO_3^- yielded a log−log plot with a gradient of 1. The difference in slope together with a distinct lifetime for 1-methyltryptophan suggest that τ obtained by phosphorescence is little affected by diffusional quenching and therefore represents the truly intrinsic lifetime.

Introduction

Since the initial report of tryptophan phosphorescence from proteins in fluid solutions[1] the potential of the triplet state as an intrinsic probe of protein structure has been expanding rapidly. As with fluorescence, structural and dynamical information on these macromolecules has been derived from the rate of energy/electron transfer[2−6] between reacting centers, the polarization of the emission,[7−10] as well as the rate of permeation of small quenching molecules.[11,12] Due to the several orders of magnitude difference in lifetime, the triplet state permits the monitoring of much slower processes extending the observation time scale from the nanosecond range of fluorescence up to millisecond-to-second range of phosphorescence. Additional potential of the triplet state as a structural probe comes from the drastic dependence of the lifetime (τ) upon the solvent viscosity (η),[13] a correlation that in the last ten years has been greatly exploited as a measure of protein flexibility on a local scale.[14−16]

The empirical correlation established between τ and solvent viscosity emphasizes the enhancement of radiationless transitions in fluid media, an effect that is largely independent of the polarity/polarizability of the solvent. The large reduction in τ that accompanies the transition from rigid to fluid media leads to an equally large decrease in phosphorescence yield. Limited by the very poor yield in liquid solution, phosphorescence measurements of free indole compounds were until now carried out only in viscous supercooled fluids ($\eta > 10^4$ P). For this reason, our present knowledge of the triplet lifetime in liquid solutions at room temperature is derived entirely from triplet−triplet absorption studies employing the technique of flash photolysis. For all the indole derivatives studied, the triplet lifetime ranged between 11 and 29 μs,[17−21] again with minor differences between organic and aqueous solvents. Relative to the 6−6.5 s observed in rigid glasses, τ in fluid solutions is over 5 orders of magnitude smaller. However, such an unprecedented reduction in τ contrasts with the much smaller sensitivity to solvent viscosity displayed by other aromatic hydrocarbons[19] and raises therefore some questions on the validity of τ determination. With long-lived excited states it is generally recognized that at low viscosity quenching reactions are so efficient that permanent or transient impurities at the trace level may be sufficient to preclude the determination of the truly unimolecular decay rate. In this regard, for naphtalene it was found that[22] τ obtained with the absorption method was considerably shorter than that obtained with phosphorescence

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[⊗] Abstract published in *Advance ACS Abstracts*, July 1, 1995.

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and that the discrepancy could be attributed to the large production of quenching photoproducts by flash photolysis.

The aim of the present investigation was to develop an apparatus sufficiently sensitive to monitor the phosphorescence of indole compounds in aqueous solutions at ambient temperature and then reinvestigate the photophysics of the triplet state under conditions of low excitation intensity. Experiments were also carried out over a wide range of solvent viscosity in order to extend the empirical correlation between τ and η from the previous lower limit of 10^4 P down to the centipoise range of liquid water.

The choice of indole derivatives was dictated by two considerations: (1) the need to use high-purity compounds and (2) the need to employ variations in structure of the chromophore that might point out specific intramolecular quenching processes. In particular we wished to examine the ability to exchange protons at the ring nitrogen and the proximity of ionized amino, carboxy, and peptide bond moieties, as these might play a specific role in determining the intrinsic lifetime of free tryptophan (Trp) or Trp in peptides. Among the selected compounds, indole, Trp, 1-methyltryptophan, and *N*-acetyltryptophanamide (NATA), the latter is the closest analogue of tryptophan in proteins. For this reason most experiments were carried out only with NATA. Among glycerol, ethylene glycol, and propylene glycol, the common viscogenic organic cosolvents that with water form supercooled fluids and glasses, only the latter could be obtained to a satisfactory level of purity. Therefore, the viscosity dependence of τ was determined solely for propylene glycol/water mixtures.

Materials and Methods

High-purity indole, L-tryptophan, 1-methyltryptophan, and *N*-acetyltryptophanamide were purchased from Sigma Chemical Co. (St. Louis, MO) and prior to use were recrystallized three times from ethanol/water. Spectroscopic grade propylene glycol (Merck, Darmstadt) was treated with a reducing agent ($NaBH_4$) and distilled under vacuum twice prior to use. Water, doubly distilled over quartz, was further purified by the Milli-Q Plus system (Millipore Corporation, Bedford, MA). Polypropylene containers and glassware used for sample preparation were conditioned in advance by being left standing for 24 h in 10% HCl suprapur (Merck, Darmstadt). Phosphorescence measurements were carried out on freshly prepared samples thoroughly deoxygenated by a method described elsewhere.[23]

The viscosity of propylene glycol/water mixtures (50/50 and 90/10, w/w) was determined at various temperatures and spanned the range from 10^6 to 10^{-2} P. In the low-viscosity range Ostwald viscosimeters were employed while for the high-viscosity range the "falling ball" method was adopted. Both apparatuses were calibrated at each temperature change with a glycerol/water (50/50, w/w) solution of known viscosity.[24] The standard deviation of these measurements was better than 5% with Ostwald viscosimeters and 10−15% with the "falling ball" method. Each viscosity value is the average of three independent determinations.

Apparatus and Procedure for Phosphorescence Measurements. The layout of the home-made apparatus employed for phosphorescence decay measurements is given in Figure 1. Pulsed excitation is provided by a frequency-doubled flash-pumped dye laser (UV500M—Candela) tuned at 292 nm. The pulse duration is 1 μs and the light energy per pulse was varied between 0.03 and 30 mJ. Blocking direct light from the flash lamp is paramount because otherwise the long (~0.3 ms) tail interferes with the detection of very weak phosphorescence emission during the first millisecond after excitation. Thus, before frequency doubling, the fundamental at 584 nm is filtered through a 570 nm cutoff filter (Schott OG 570) to remove traces of visible and UV light that

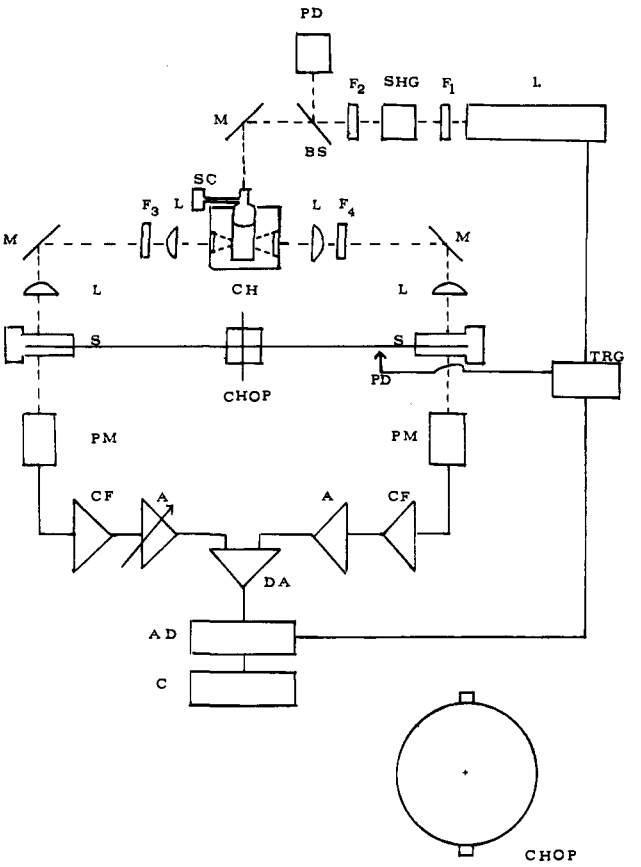

Figure 1. Block diagram of the apparatus for monitoring time-resolved delayed luminescence. All optical components are in a vertical plane. L = dye laser; F1 = 570 nm cutoff filter (Schott OG570); F2 = UV transmitting filter (Schott UG5); F3 = 420 nm cutoff plus DT-blau filters; F4 = 550 nm cutoff + 650 nm short pass filters; SHG = second harmonic generator; BS = quartz plate beam splitter; M = mirror; SC = sample cuvette; CH = cuvette holder; L = quartz lens; S = slit; CHOP = chopper blade; PD = photodiode; PM = EMI 9635B photomultiplier; CF = cathode follower; A = voltage amplifier; DA = differential amplifier; AD = digital board averager (ISC-16, RC Electronics); C = computer; TRG = trigger generator.

propagates directly from the flash lamp. After frequency doubling, a UV transmitting filter (Schott UG5) removes both the fundamental frequency of the dye laser and any remaining light from the flash lamp.

The sample is placed in an especially designed vacuum proof quartz (spectrosil) cuvette that allows excitation of the solution from above—a configuration that was found to minimize the collection of both plasma and impurity emission from the cuvette wall. The sample cuvette is placed in a thermostated copper block which is provided with 4 quartz windows in order to hold a solution acting as a cutoff filter. By changing the solution the cutoff wavelength is changed from 400 nm for phosphorescence measurements to 310 nm for delayed fluorescence measurements. The function of this filter is to block any scattered excitation light and prevent long-lived secondary emission from the lenses in the emission collection path. The advantage of a solution filter is that, unlike colored glasses, its own emission can be completely quenched.

In spite of all the efforts to abate spurious light from the apparatus its intensity in the visible spectrum, when compared to the typical phosphorescence intensity of Trp, is not negligible during the first 100−150 μs from the excitation pulse. Thus, to improve the time resolution of phosphorescence measurements, spurious emission was subtracted from the overall signal utilizing a double beam configuration in a T format. In one arm of the T a filter combination with a trasmission window between 420 and 480 nm (420 nm long pass plus interference filter DT-Blau, Balzers) selects for Trp phosphorescence while in the opposite arm a filter combination with a window between 550 and 650 nm (550 nm long pass plus 650 nm short pass, Oriel) detects mostly spurious emission. The photomultiplier outputs are amplified and fed

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to a differential amplifier for subtraction. The output is digitized and stored and consecutive decays are averaged by a computerscope system (ISC-16, RC Electronics). To correctly subtract spurious emission the sample is first run in the presence of O_2 in sufficient quantity to quench any phosphorescence. Under these conditions, both photomultipliers detect only spurious emission and the difference signal is set to zero by adjusting their relative amplification. The subtraction procedure turned out to be sarisfactory because the decaying characteristics of spurious light are very similar in the two spectral windows. When the measurement is repeated on the deoxygenated sample the difference signal represents the net phosphorescence since deoxygenated blanks mantained a balanced output. In order to have the same scattering power of protein samples the blank consisted of a protein such as ribonuclease A, which lacks Trp residues. In general, different sample cuvettes or scattering powers of the sample required new readjustment of the relative amplification. Delayed fluorescence is monitored in the same manner except for a change in trasmission window of the filter combination from 420−480 to 320−400 nm. In buffer, and under the prevailing conditions of excitation intensity and sample concentration, the delayed fluorescence of small indole derivatives is several folds more intense than the corresponding phosphorescence signal, and in general, there is no need of substracting spurious emission.

The photomultipliers are protected from the intense fluorescence light pulse by a chopper blade that closes the slits during laser excitation. (Photomultiplier gating circuits were also tried but they turned out inadequate because the photocathode remains energized by the fluorescence pulse for as long as 1 ms). A photodiode reveals the position of the chopper blade to a triggering circuit which, with suitable delays, synchronizes the closing of the slits with triggering of the laser and data aquisition. The time resolution of the apparatus is set by the speed of the chopper and the slit width. At the highest speed (167 Hz) the linear velocity of the blade is 0.3 mm/μs. Considering a 2 mm wide slit and 1 mm overlap of the blade at the time of excitation the minimum dead time of the apparatus is about 10 μs. In terms of sensitivity, Trp phosphorescence ($\phi_P \sim 10^{-7}$) can be detected above background emission down to the micromolar concentration.

The spectrum of delayed emission was obtained by removing the filter set and inserting a 0.25 m grating monochromator (H 25, Jobin-Ivon) after the chopper in one of the two emission paths. In this case spurious emission was not subtracted but was eliminated by discarding the first 60 μs of the decay curve. Intensity points at various wavelenghts represent the area under the emission decay curve obtained with extensive averaging of multiple sweeps. Due to the poor collection efficiency of monochromators relative to filters, the slits were kept wide open (2 mm) and spectral resolution was thus limited by the 15 nm band-pass.

All luminescence decays were analyzed in terms of a sum of exponential components by a nonlinear least-squares fitting algorithm (Global Unlimited, LFD, University of Illinois).

Results

Phosphorescence Lifetime of Indole Derivatives in H_2O at 20 °C. Initially, phosphorescence decay measurements were conducted in 10^{-4} M solutions of the chromophore, the concentration employed in triplet−triplet absorption studies ever since the first report of Bent and Hayon.[17] With a laser light energy of 3 mJ per pulse providing an excitation intensity through the sample of 2.3×10^{17} photons/cm^2, the phosphorescence emission, which was sufficiently intense to require no averaging of multiple sweeps, decayed in a distinctly nonexponential fashion. The average lifetime of indole, tryptophan, and *N*-acetyltryptophanamide (NATA) ranged between 50 and 60 μs, a value considerably larger than the 12−20 μs reported by Bent and Hayon,[17] 23 μs reported by Volkert et al.[19] for some of these derivatives, and 29 μs reported by Pepmiller et al.[20] for 1-methylindole. These findings pointed out immediately that the intrinsic lifetime, τ_0, is certainly larger than presumed from absorption studies and that all past determinations of τ must have been dominated by quenching reactions.

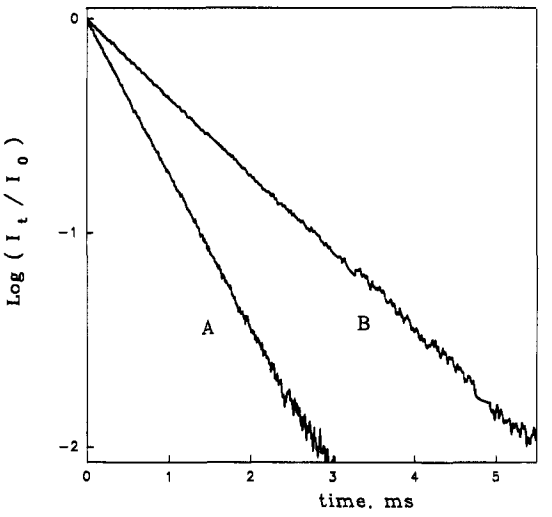

Figure 2. Example of a decay of delayed emission from a 3×10^{-6} M solution of NATA in H_2O, at 20 °C, collected in the spectral windows of 320−400 nm for fluorescence (A) and 420−480 nm for phosphorescence (B). Each decay is the average of 100 sweeps.

The rate of decay of the excited triplet state, T, can be influenced by a number of quenching processes. A general expression is given in eq 1:

$$1/\tau = k_0 + k_1 + k_T[T] + k_{Ph}[Ph] \qquad (1)$$

where $k_0 = 1/\tau_0$ is the sum of radiative and radiationless transition probabilities, and $k_1 = k_{So}[So] + k_q[Q]$ represents the rate of pseudo-first-order quenching by ground state molecules (So) and impurities (Q). k_T and k_{Ph} are second-order quenching rate constants for respectively triplet−triplet anhilation and the interaction with reactive photoproducts (Ph). Among the latter are the cation and neutral radicals, the solvated electron, and products of their recombination. As for [T], the concentration of photoproducts is time dependent. Thus, significant contributions from terms 3 and 4 in the right-hand side of eq 1 will cause the decay to deviate from an exponential law. Since the weight of these terms depends on the amount of excitation absorbed by the sample (OD $\times$ I_0) their contribution can be reduced and made negligible by lowering either the pulse energy (I_0) or the optical density of the sample (OD). With a chromophore concentration of 3×10^{-6} M, monoexponential decays were obtained with pulse energies $I_0 \leq 0.05$ mJ ($\leq 5 \times 10^{15}$ photons/cm^2 pulse). Under these experimental conditions, where time dependent terms 3 and 4 did not contribute significantly, τ increased from 5- to 10-fold. Its value, however, varied substantially from one sample preparation to another—an indication that probably pseudo-first-order terms (k_1 of eq 1) still made a nonegligible contribution to the decay. To obtain stable decays it required thorough and meticulous purification of water and solutes and extensive conditioning of all glassware employed. Under such rigorous controls, the phosphorescence of NATA, collected in the spectral window 420−480 nm, decays exponentially in intensity for at least two decades (Figure 2). The value of τ measured from the slope of first-order plots was 1.2 ms with a reproducibility of ±5%. This value is at least 40-fold larger than old and recent determinations by the method of flash photolysis. Whether the value of 1.2 ms is truly the intrinsic lifetime we cannot know, although indirect evidence such as the dependence of τ on solvent viscosity (see below) suggests that this value must be close to the truly unimolecular rate.

The long (millisecond) triplet lifetime observed with NATA was found also with indole, tryptophan, and 1-methyltryptophan.

Table 1. Phosphorescence Lifetime and Second-Order Quenching Rate Constants by Molecules in the Ground State Obtained for Indole Derivatives in H_2O at 20 °C

sample	τ (ms)	k_{So} (M^{-1} s^{-1})
indole	1.2 ± 0.05	1.1×10^7
Trp	1.16 ± 0.04	1.2×10^7
1-Me-Trp	0.6 ± 0.03	9.7×10^6
NATA	1.2 ± 0.06	1.7×10^7

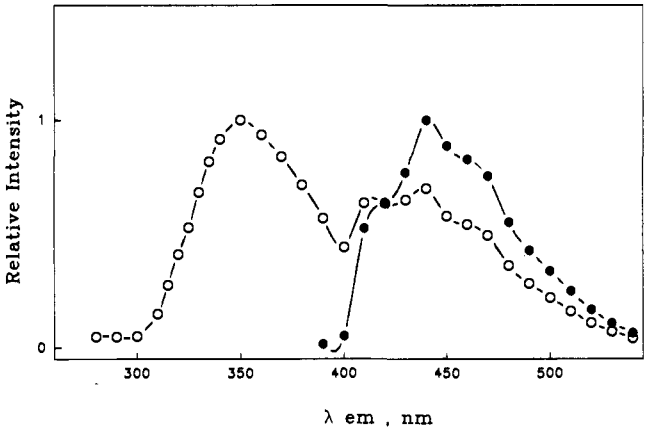

Figure 3. Spectrum of the delayed emission from a 10^{-5} M solution of NATA in H_2O, at 20 °C (○). Subtraction of the fluorescence component, assuming the fluorescence spectrum to be identical to that of prompt fluorescence, yields the net phosphorescence spectrum (●) which is the same as that of the total delayed emission obtained in viscous PG/H_2O (90/10, w/w) where delayed fluorescence is absent.

The values of τ are collected in Table 1. Except for 1-methyltryptophan for which the magnitude is roughly half that of NATA, the other indole derivatives have very similar lifetimes.

Quenching by complex formation with molecules in the ground state occurs with all four derivatives. Values of k_{So}, the second-order quenching rate constant, as obtained from the slope of linear, lifetime Stern–Volmer plots (not shown) are collected in Table 1. Once again there is a substantial difference with the data derived from absorption experiments, the values of k_{So} being roughly 10-fold larger in the latter.[17] More striking is the difference with indole for which no quenching was reported by the absorption technique.

Spectrum of Delayed Emission from NATA. The spectrum of the weak delayed emission from a 3×10^{-6} M aqueous solution of NATA at 20 °C is shown in Figure 3. In the wavelength range between 400 and 550 nm the spectrum displays the characteristic vibronic band structure of NATA phosphorescence. Relative to low-temperature glass matrices, the band maxima are shifted to longer wavelengths by 7–8 nm and the intensity in the red part of the spectrum (460–470 nm shoulder) is enhanced in comparison to that of the 0–0 vibronic band (416–420 nm)—spectral differences that are in part accounted for by solvent relaxation about the triplet state. At wavelengths shorter than 400 nm the spectrum is practically superimposable to that of prompt fluorescence and, naturally, it is assigned to delayed fluorescence. This is the first report of delayed fluorescence from the indole nucleus in solution and the following experimental evidence clearly establishes that it originates from triplet–triplet anhilation:

$$T_1 + T_1 \rightarrow S_1 + S_0 \rightarrow h\nu_f + 2S_0$$

(1) Its intensity, right after the excitation pulse, is proportional to the square of the amount of light absorbed by the sample, $(\Delta I)^2$, and therefore to the square of the initial triplet state population, $[T_1]_0$.

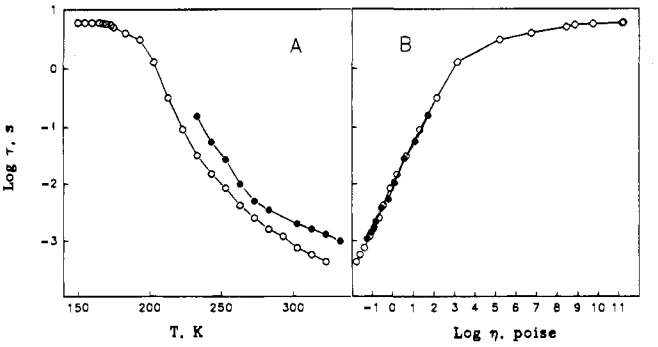

Figure 4. (A) Temperature dependence of the phosphorescence lifetime of NATA (10^{-5} M) in 50/50 (○) and 90/10 (●) PG/H_2O solvent mixtures. (B) Plot of the phosphorescence lifetime as a function of solvent viscosity. Viscosities above 10^6 P were taken from Strambini and Gonnelli.[13]

(2) For small ΔI, or after a sufficiently long period of time such that the rate of decay of phosphorescence by triplet–triplet anhilation is negligible, the decay of this emission is exponential in time and as expected for P-type delayed fluorescence[25] its lifetime is one-half that of phosphorescence (see Figure 2).

(3) When diffusion of the chromophore is drastically reduced by employing either viscous solvents (e.g. propylene glycol) or large molecular weight derivatives (e.g. tryptophan-containing peptides) the delayed fluorescence is no longer detected.

The only other possibility is E-type delayed fluorescence. This is ruled out on energetic grounds ($E_{S1} - E_{T1} \gg kT$) and was in fact not observed up to 80 °C.[26]

Dependence of τ on Solvent Viscosity (η). Much interest on the triplet state of tryptophan stems from the realization that its lifetime may serve as a direct probe of the local flexibility of protein sites harboring the aromatic amino acid. A previous report[13] showed that the ~6 s lifetime in viscous supercooled fluids is reduced dramatically in fluid media and that the enhancement of radiationless transitions is governed principally by the viscosity of the medium. Due to instrumental limitations in measuring rapid decay kinetics, that study investigated only viscosities above 10^4 P. These measurements were now repeated with 10^{-5} M solutions of NATA in 50/50 and 90/10 (w/w) propylene glycol/H_2O and the viscosity extended down to the centipoise range.

The phosphorescence lifetime of NATA in PG/H_2O mixtures was measured over a large temperature range and the results are presented in Figure 4 as a function of solvent viscosity. Since τ is little affected by temperature[27,28] the results confirm a strong dependence of τ on solvent viscosity. They also point out that solvents of different composition, namely 50 and 90% PG, yield lifetimes that fall on the same τ vs η curve.

The decrease in solvent viscosity favors molecular diffusion, and quenching reactions by impurities in the solvent may in part be responsible for the decrease in τ. According to Debye equation, in the regime of diffusion control

$$k_q = 8RT/3000\eta$$

and eq 1 without time-dependent terms becomes

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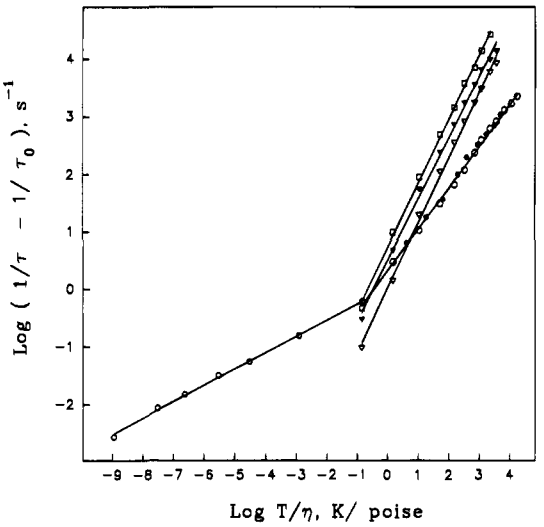

Figure 5. Log–log plot obtained from the data of Figure 4 (○, ●), using $\tau_0 = 6$ s. Inserted in the same diagram is the log–log plot obtained from the decrease in τ when 2.5 μM O_2 (□), 18 μM $CuCl_2$ (▽), and 18 μM $NaNO_3$ (▼) are added to a 10^{-5} M solution of NATA in 50/50 PG/H_2O.

$$1/\tau - k'_0 = k'_0(\eta) + (8R[Q]/3000)T/\eta$$

where $k_0 = k'_0 + k'_0(\eta)$ and $k'_0 = 1/6.5$ s^{-1} is the inverse of the lifetime at infinite viscosity (6.5 s is the lifetime in glasses). Thus, should quenching dominate the decrease in τ a $\log(1/\tau - k'_0)$ vs $\log(T/\eta)$ plot would have unit gradient. Figure 5 shows that such a plot consists of two linear sections with slopes of 0.29 at high viscosity ($\eta > 25$ P) and 0.70 at low viscosity, both magnitudes substantially smaller than unity. Although this finding suggests that the decrease in τ is not due to diffusion mediated quenching by impurities, deviations from Debye's law may occur if the quenching efficiency changes with η or if the diffusion coefficient of Q does not follows the Stokes–Einstein law ($D = kT/6\pi\eta r$) (see Discussion).

To test whether the difference in slope between 0.7 and 1 is significant the quenching behavior of small, neutral and charged, molecules such as O_2, Cu^{2+}, and NO_3^- was investigated in the low-viscosity range. The variation in τ of 10^{-5} M NATA in 50/50, PG/H_2O containing fixed amounts of O_2 ($[O_2] = 2.5$ μM), $CuCl_2$ ($[CuCl_2] = 18$ μM), or $NaNO_3$ ($[NaNO_3] = 18$ μM) is shown in Figure 5. Concentrations were corrected at each temperature for the change in volume. Given the very slow rate of equilibration,[29] oxygen exchange with the gaseous phase is negligible and the amount of O_2 in solution was considered to remain constant. The results yield linear log–log plots and do confirm that in this solvent mixture the slope is for each quencher close to 1 (1.05 for O_2, 0.97 for Cu^{2+}, and 0.99 for NO_3^-) as expected from the Debye relation.

Dependence of τ on pH. The pH of the solution was varied with the addition of ultrapure HCl and NH_3. To verify that no quenching impurities were introduced with these solutes the lifetime of NATA was measured at neutral pH before and after an acid solution (10^{-3} M HCl) was neutralized with NH_3. As τ was practically the same in the two cases both acid and base were deemed sufficiently free of quenching impurities. At 20 °C, the lifetime of NATA (3×10^{-6} M) between pH 3 and 10.5 is given in Table 2. τ decreases in acid solution but remains unaffected at basic pH. Analyzing the decrease in τ in terms of a second-order quenching reaction with the proton one obtains $k_H^+ = 1.2 \times 10^7$ M^{-1} s^{-1}—again a value that is 10-fold less than that reported by Bent and Hayon[17] by the absorption method.

Table 2. pH Dependence of the Phosphorescence Lifetime of NATA at 20 °C

pH	3	4	5	6	7	8	9	10.5
τ (ms)[a]	0.10	0.51	1.01	1.20	1.21	1.18	1.20	1.16

[a] The standard error in lifetime determinations is about 5%.

Discussion

Thanks to the development of sensitive instrumentation capable of detecting delayed emissions with yields as low as 10^{-7} and lifetimes down to 10 μs, it was possible to investigate the phsophorescence characteristics of indole derivatives even in aqueous solution at room temperature. The results show that the spectrum of the delayed emission is composite. At wavelengths greater than 400 nm it exhibits the typical vibronic structure of indole phosphorescence whereas at shorter wavelengths it is superimposable with prompt fluorescence. This is the first observation of delayed fluorescence from indole compounds in solution and by all criteria the emission was shown to derive from triplet–triplet anhilation. Long-lived emission with spectral properties of fluorescence was also detected with the tobacco mosaic virus protein.[30] Its lifetime, however, has not been reported and the origin of this emission has not been determined. Clearly in proteins with isolated, fixed chromophores triplet–triplet anhilation cannot occur. Further, thermal repopulation of the excited singlet state from the triplet state does not take place even at much higher temperatures.[26] Consequently, it is likely that any delayed fluorescence from Trp in proteins results from electron recombination with Trp radical ions subsequent to photochemistry.

The lifetime of the excited triplet state was found to be 1.2 ms, a value which is almost two orders of magnitude larger than reported by the numerous studies employing the triplet–triplet absorption technique. Other discrepancies with previous reports concern the magnitude of the self-quenching second-order rate constant, k_{So}, found by us to be similar among the four derivatives studied and roughly an order of magnitude smaller than previously reported. The contrast with absorption measurements is even larger with indole for which no quenching by ground state molecules was found by flash photolysis. Clearly the method of flash photolysis, as it has been applied until now, is unsuitable for studying the photophysics of the triplet state of indole chromophores. Given the relatively high excitation intensity and concentration employed a main cause of the discrepancies in τ and k_{So} is the underestimation of triplet–triplet anhilation as a deactivation route. Other quenching pathways are afforded by the large production of photoproducts: species such as the cation radical, the neutral radical, the hydrated electron, and products of their recombination. Some of these moieties are quite reactive and are likely to quench the triplet state in diffusional encounters as pointed out by Chen Tsai and Robinson[22] for napthalene in fluid media. Another problem associated with the presence of these transients is that their absorption spectrum in part overlaps with that of the triplet state thereby making spectral resolution more difficult to achieve. The ambiguity can be serious because the type and distribution of photoproducts will depend on the nature of the solvent or of the molecules surrounding the chromophore. For example, Trp residues in proteins will behave according to the side chains in their proximity and consequently their photochemistry is bound to be diverse and largely unpredictable. The

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limitations imposed by the photochemistry of indole should in part be alleviated by selecting an excitation wavelength close to 300 nm, instead of the usual 260 nm, because at the long wavelength the photoproduct yield is substantially smaller.[31,32]

The triplet lifetime of aromatic hydrocarbons, particularly when this is large ($\tau > 1$ s) in rigid media, is generally sensitive to the fluidity of the solvent. The extent to which the medium viscosity affects radiationless transitions, however, has long been debated because the occurrence of diffusion-mediated quenching reactions in fluid media makes it difficult to ascribe changes in τ exclusively to intrinsic molecular parameters. In fact, with an intrinsic lifetime $\tau_0 = 10^{-3}$ s and a quenching rate constant $k_q = 10^{10}$ M^{-1} s^{-1} a quencher concentration $[Q] = (1/\tau_0)k_q = 10^{-7}$ M, which is near the limit of detection for many substances, is sufficient to double the decay rate. For indole derivatives τ was found to decrease from 6.5 s in glasses to about 10^{-3} s in fluid solutions of 10^{-2} P. The following indirect evidence indicates that the millisecond phosphorescence lifetime measured for indole in water at room temperature must be essentially τ_0: (1) The dependence of τ on solvent viscosity is less steep than expected for diffusion-controlled reactions. In PG/H_2O mixtures the slope of log $1/\tau$ vs log T/η is, depending on the η range, either 0.29 or 0.7 against the unit gradient found whenever the lifetime was dominated by diffusional quenching. Gradients smaller than unity can occur also for quenching reactions if the diffusion coefficient of Q is not proportional to $1/\eta$ as predicted by the Stokes–Einstein equation ($D = k_T/6\pi r\eta$, D = the diffusion coefficient). Indeed, the above expression was derived for the diffusion of particles whose size is appreciably larger than that of the molecules of the surrounding medium and deviations are plausible when the size of the solute approaches or is smaller than that of the solvent. The quenchers employed in this study (O_2, Cu^{2+}, and NO_3^-) are among the smallest molecules available. Because they do obey the Stokes–Einstein equation, we anticipate that any other quenching impurity would do the same and its presence would be manifested by a slope close to one. (2) If impurity quenching did dominate the triplet decay rate then there would be no distinction in lifetime among indole derivatives. Certainly it does not dominate τ of 1-methyltryptophan which is one-half that of other derivatives nor, likewise, τ of NATA at acid pH. The difference cannot certainly be imputable to a greater yield of quenching photoproducts or to larger quenching impurities in the commercial source of this compound as, below a given threshold, τ is independent of both excitation intensity and concentration. Instead, it may be worth noting that the difference in τ for this derivative is also associated with a red-shifted phosphorescence spectrum, an indication that methylation of the indole nitrogen does modify molecular orbitals and energies, and concievably also the intrinsic decaying rate.

The high sensitivity displayed by the phosphorescence lifetime of the indole chromophore toward the viscosity of aqueous solutions raises the question as to the underlying mechanism. Chen Tsai and Robinson[22] reported a relatively small effect of η on the radiationless transition of naphthalene in 3-methylpentane. This is in agreement with a theoretical model that treats the solvent merely as a thermal bath, a model probably adequate when the chromophore–solvent interactions are weak. Indole, however, is a polar molecule and is even more so in the excited singlet state. Besides dipolar interactions with the solvent it can form H-bonds or exchange the proton at the ring nitrogen. Thus, for indole, solute–solvent interactions are considerably stronger and probably a new treatment of radiationless transitions is needed to take this into account. One possibility is that the increase of radiationless transitions is due to out-of-plane distortions of the aromatic ring and vibronic coupling to higher energy molecular orbitals of singlet character thereby removing in part the spin forbiddeness of the transition to the ground state. The change in slope in the log($1/\tau$) vs log(T/η) plot (Figure 5) suggests that the mechanism might differ between low- and high-viscosity regimes. At present what we can exclude is that H-exchange at the ring nitrogen is responsible for enhancing radiationless transition in fluid media since substitution of the proton with a methyl group, if anything, increases the rate.

For tryptophan phosphorescence to become an important probe of the local flexibility of protein structures it is necessary to know which other factors, besides the viscosity of the medium, can affect the intrinsic lifetime. Temperature is known to have a weak influence on τ since tryptophan residues in rigid protein sites have lifetimes of a few seconds even at 300 K.[26,28,33] Aspecific solvent properties such as polarity/polarizability also appear to be relatively unimportant. Indeed, a previous study limited to viscosities above 10^4 P showed that large changes in solvent polarity did not alter τ,[13] as did variations from 30 to 90% in PG content in this investigation. On the other hand, specific interactions with the solvent, through the formation of weak exciplexes or protonation of the ring nitrogen, may provide additional routes for the relaxation of the triplet state particularly at low viscosity. These might explain, for example, the modest decrease in τ found in going from water to propylene glycol solutions and the dependence on pH.

Acknowledgment. The authors are grateful to Ms. Claudia Neri for typing the manuscript and to Mr. A. Puntoni, L. Favati, G. Chiti, and T. Piaggesi for technical assistance.

JA9510931

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