Biophysical Chemistry 9 (1979) 223–233

RATES OF DEACTIVATION PROCESSES OF INDOLE DERIVATIVES IN WATER-ORGANIC SOLVENT MIXTURES – APPLICATION TO TRYPTOPHYL FLUORESCENCE OF PROTEINS

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Received 21 July 1978

The fluorescence quantum yield and the fluorescence decay of indole, 3-methylindole, 1-methylindole and *N*-acetyltryptophanamide have been measured in different water-dioxane mixtures. For the first three derivatives, the fluorescence decays were found independent of the emission wavelength and were analyzed as single exponential functions. In the case of *N*-methylindole the rate of the non radiative deactivation processes k_{nr} increased linearly with the molar fraction of dioxane whereas for indole and scatole the variation of k_{nr} was biphasic. This behaviour can be explained by two excited state deactivations of these non *N*-methylated compounds in water and high water content mixtures; one of these deactivation processes occuring through an hydrogen bond between the N-H group and a water molecule. The rate of non radiative deactivation of *N*-acetyltryptophanamide was dominated by the internal quenching involving the intramolecular carbonyl. The rate of the radiative deactivation process k_F of these four compounds increased linearly with the wavenumber of the fluorescence spectrum maximum. Data relative to the three non *N*-methylated derivatives fell practically on the same straight line. Data from other works have been gathered in order to check if a similar relation between the intrinsic k_F and $\bar{\nu}_F$ values can exist for the tryptophyl fluorescence emission of proteins.

1. Introduction

It is well known that tryptophan dominates the fluorescence of class B proteins. The wavenumber ($\bar{\nu}_F$) of the fluorescence spectrum maximum arising from tryptophan residues varies from protein to protein [1]. This can be explained by the fact that a tryptophan residue fluorescence depends on the local environment of that residue in the protein. Therefore, a protein molecule containing several tryptophan residues is expected to have a composite spectrum [2]. A direct evidence for such an heterogeneity came from luminescence measurements at low temperature [2,3] and room temperature fluorescence excitation spectra taken at different emission wavelengths [4,5]. Other evidences have been obtained by observation of shifts of emission spectra induced by external perturbants [6,7,8]. More recently, protein spectra heterogeneity has been detected by fluorescence decay measurements [9,10]. By analysing the fluorescence decay at several emission wavelengths it has been possible to reconstitute the spectrum of the two individual tryptophan residues of the lac repressor protein of *E. coli* [10]. Quantum yield heterogeneity of individual tryptophan residues is also shown up by decay measurements. For example, it should be possible to determine the fraction of tryptophans which is non fluorescent in a protein [11,12]. But, for a more complete interpretation of these measurements, the knowledge of the rate of radiative deactivation k_F is needed [11,12].

It has been shown that, for indole and indole derivatives solutions, k_F depends on the solvent properties [13,14]. It is then expected that k_F will depend on the environment of each protein tryptophan residue. This is confirmed by some observations. For example when the *lac* repressor protein of *E. coli* binds the inducer isopropyl-*β*-*D*-thiogalactoside (IPTG), the protein average decay time decreases whereas its quantum yield remains constant [10,15]. In the same way, the quantum yield and average lifetime ratios of the wheat germ agglutinin (WGA) are not equal in presence and in absence of tri-*N*-acetylchitotriose [11].

The present work was undertaken in the hope of finding a relation between the value of k_F and the wavelength maximum of tryptophan emission spectra in proteins. In many cases, the emission heterogeneity complicates the direct determination of k_F in proteins. So, we decided to study several model compounds in solvents of different polarities. The compounds chosen were indole, scatole, *N*-methylindole and *N*-acetyltryptophanamide. The solvents were dioxane–water mixtures in which the maximum fluorescence spectrum of these compounds varies in the same wavelengths range as the maximum fluorescence spectrum of proteins in aqueous medium. We determined also the rate of non radiative deactivation process of these compounds in the mixtures and obtained interesting informations concerning the fluorescence quenching mechanism by the solvents. Finally, the variation of k_F with the fluorescence maximum wavenumber $\bar{\nu}_F$ of these compounds was compared with data gathered from proteins containing one or two emitting tryptophan residues.

2. Materials and methods

2.1. Materials

Indole, 1-methylindole and 3-methylindole were obtained from Fluka. Indole and 3-methylindole were sublimated under vacuum and stored at 4° in the dark. 1-methylindole was twice-distilled under reduced pressure. This product was stored under vacuum and dilutions were made in a glove-box filled with nitrogen. *N*-acetyltryptophanamide was obtained from Sigma and used without further purification. Water and dioxane were freshly twice-distilled. These solvents were found spectroscopically pure.

Table 1

	x_2	Q	τ $\times 10^9$ s	k_F $\times 10^{-7}$ s^{-1}	k_{nr} $\times 10^{-7}$ s^{-1}	$\bar{\nu}_F$ $\times 10^{-4}$ cm^{-1}	$\lvert M\rvert^2$ (debye)
Indole	0	0.28	4.4	6.3	16.3	2.899	3.8
	0.05	0.39	5.5	7.1	11.1	2.920	3.9
	0.12	0.45	5.9	7.65	9.4	2.990	3.9
	0.24	0.49	5.5	8.9	9.3	3.040	4.2
	0.45	0.48	4.95	9.7	10.5	3.082	4.1
	1.00	0.45	4.85	9.3	11.3	3.205	3.4
3-methylindole	0	0.36	9.2	3.9	7.0	2.736	2.9
	0.05	0.42	11.6	3.6	5.0	2.774	2.4
	0.12	0.48	11.2	4.3	4.7	2.809	2.6
	0.24	0.52	10.0	5.2	4.8	2.863	2.9
	0.45	0.57	8.7	6.6	4.9	2.920	3.3
	1.00	0.66	5.5	11.9	6.15	3.032	5.15
N-methylindole	0	0.42	8.5	4.9	6.8	2.874	3.1
	0.05	0.44	8.2	5.4	6.8	2.899	3.1
	0.12	0.44	7.7	5.7	7.3	2.950	3.0
	0.24	0.40	6.5	6.15	9.2	2.994	3.0
	0.45	0.38	5.2	7.3	11.9	3.067	3.2
	1.00	0.34	4.1	8.3	16.1	3.155	3.2
N-acetyltrypto-phanamide	0	0.14	3.1	4.6	28.1	2.809	3.5
	0.05	0.17	3.5	4.9	24.0	2.833	3.5
	0.12	0.26	3.7	6.8	20.3	2.890	4.35
	0.24	0.32	4.1	7.7	16.4	2.894	4.4
	0.45	0.40	5.1	8.0	11.6	2.959	4.25
	1.00	0.46	5.2	9.0	10.2	3.012	4.15

Fluorescence parameters of indole derivatives in water–dioxane mixed solvents. (Temperature: 20°C).

Absorption spectra and optical densities were measured with a Beckman Acta III spectrophotometer.

In order to avoid fluorescence quenching by oxygen, solutions were contained in 10 × 10 mm quartz cells (Hellma) with a special glass attachment for bubbling of gas. Gentle bubbling of argon (type N 55, purity >99.995%, Air Liquide) was performed during two hours. This was found comparable to the technique of degassing by successive "freeze-thaw" cycles [14] and evaporation of solvents was prevented.

Unless otherwise stated, in the steady-state or transient fluorescence measurements, the excitation wavelength was chosen at 276.5 nm near the absorption maximum of the ^1L_a electronic transition of indole chromophore. Cells were thermostated at 20°C.

2.2. Steady-state fluorescence measurements

Fluorescence measurements were performed with a Jobin-Yvon spectrofluorimeter modified in our laboratory. The apparatus was equipped with a XBO-150 W xenon lamp, two prism monochromators and a 6256 B EMI photomultiplier. Fluorescence quantum yields of indole derivatives in mixed solvents were measured relatively to that of the derivatives in pure water. Their determinations were made according to the method described by Demas and Crosby [16]. Solution absorbances at the excitation wavelength were generally below 0.1 but all results were corrected for the "inner filter effect". Emission spectra were corrected for photomultiplier sensitivity and prism dispersion. The quantum yields (ϕ) were corrected for the refractive index by using the following equation:

$$\phi_u = \phi_s \times \frac{A_u}{A_s} \times 10^{(\mathrm{OD}_u - \mathrm{OD}_s)/2} \times \frac{\mathrm{OD}_s}{\mathrm{OD}_u} \times \left(\frac{n_u}{n_s}\right)^2 \qquad (1)$$

where s and u refer to the standard and unknown solution respectively and where the symbols n, OD, A designate the refractive indexes, the optical densities and the area under the fluorescence spectra of the samples. These quantum yields have been determined with tryptophan as standard. We assumed that the tryptophan quantum yield in 0.14 in water. The validity of this choice has been extensively discussed by Tatischeff and Klein [52] and Lumry and Hershberger [53]. Any correction of this value would lead to a corresponding correction of k_f and k_{nr}. But the relative variation of these parameters will not be affected.

2.3. Fluorescence decay measurements

The fluorescence decay experiments were carried out using a single photoelectron counting apparatus supplied with a peak stabilizer described earlier [17]. The excitation light pulse was generated by a free running flash lamp operating in hydrogen/neon mixtures (Auchet and Wahl, unpublished work) with a frequency at 10 kHz. The excitation wavelength was selected by a Bausch and Lomb 250 mm monochromator with a bandwidth of 13 nm. At 90° of the exciting beam, the fluorescence emission wavelength was selected through a 500 mm Bausch and Lomb monochromator with a bandwidth smaller or equal to 6 nm. It was verified that under these optical conditions the straylight can be neglected. Photons were collected on a Radiotechnique XP 2 020 photomultiplier. Counts were accumulated with a counting rate of 1–2% of the flash frequency in order to stay in the single photoelectron condition. This condition was realized by the mean of neutral MTO filters placed at the output of the excitation monochromator in order to reduce the excitation light. Experiments were stopped after 1–2 hours when the total counts in the fluorescence curve was about 1×10^6. This time could be appreciably reduced to 15–20 min. by using as detector a RCA 8 850 photomultiplier selecting at its outpout the single photoelectron pulses. In this last case, the counting rate was set up to 15 or 20% of the flash frequency.

2.4. Fluorescence decay analysis

The fluorescence decay $I(t)$ is related to the observed fluorescence pulse $i(t)$ by the convolution product:

$$i(t) = \int_0^t G(T)I(t-T)\,\mathrm{d}T, \qquad (2)$$

where $G(T)$ is the apparatus response function to the excitation pulse. $G(T)$ is dependent on experimental excitation and emission wavelengths [18]. Instead of determining $G(T)$, our deconvolution computer programs use the apparatus response function obtained with a solution of para-terphenyl in deaerated cyclohexane ($\tau = 0.96$ ns) according to a method already described [19,20].

The values of the decay parameters obtained by computation were appreciated by examining the deviation functions DV^k and the residual R defined by the following expressions:

$$DV^k = (i_c^k - i_{ex}^k)/(i_{ex}^k)^{1/2} \qquad (3)$$

$$R = \frac{1}{n}\sum_{i=1}^{n} (i_c^k - i_{ex}^k)^2/(i_{ex}^k) \qquad (4)$$

where i_{ex}^k and i_c^k are the number of counts in the k^{ith} channel of the experimental and the calculated curves respectively. Good fits are found when the deviation curves oscillate randomly along the time axis.

Determination of the rates of radiative and non radiative deactivation are obtained by the following expressions:

$$k_F = \phi/\tau, \qquad k_{nr} = (1 - \phi)/\tau . \qquad (5,6)$$

3. Results

We have studied the fluorescence decays and the quantum yields of scatole, indole, *N*-methylindole and *N*-acetyltryptophanamide in water-dioxane mixtures. All measurements have been performed at 20°C ± 0.1°.

3.1. Decay measurements

The fluorescence decays of scatole solutions of different solvent composition are shown in fig. 1. The excitation wavelength was 276.5 nm. These curves were analyzed as previously described. They could be fitted with single exponential functions as shown in fig. 1 where the continuous curves represent the computed convolutions. The average weighted residue (R) which gives the degree of agreement between experimental and computed values is 1.80. In fig. 2 are represented the corresponding deviation functions which can be considered as satisfactory. This was confirmed by checking the fluorescence decay of pure 2,5-diphenyloxazole which is expected to exhibit a monoexponential decay. We obtained a decay of 1.35 ns which compares well with data available in the literature ($\tau = 1.36$ ns). The corresponding deviation function was randomly distributed and fluctuations where of the same order of magnitude than that obtained with scatole ($R = 1.81$). However, in order to determine if more than one contribution could participate to the fluorescence, emission wavelength were varied systematically and fluorescence decay curves were analyzed for three solvent mixtures. This is illustrated by fig. 3. At 50 and 100 percent dioxane, excitation wavelength used was 276.5 nm. At 20% dioxane, results reported are obtained with ex-

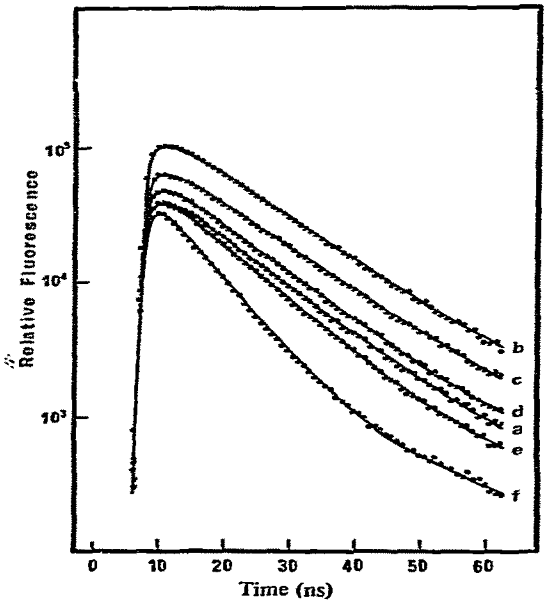

Fig. 1. Effect of solvent composition on fluorescence decays of 3-methylindole. (a) 3-methylindole in pure water; (b,c,d, e) 3-methylindole in 20, 40, 60 and 80 percent dioxane–water mixture (v/v) respectively; (f) 3-methylindole in pure dioxane. Excitation wavelength: 276.5 nm. Emission wavelength: 356 nm. Temperature: 20°C. Solute concentration: 5×10^{-5} M. The solid lines are calculated convolution products assuming a single lifetime. The points correspond to experimental values. Relative fluorescence intensities have been normalized.

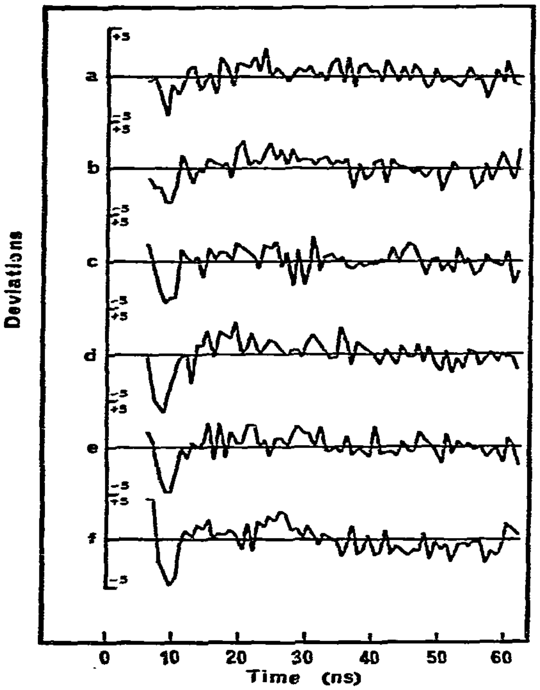

Fig. 2. Effect of solvent composition on fluorescence decays of 3-methylindole. Deviation curves (defined in section 2) between convolution products and experimental values of fluorescence decays illustrated by fig. 1. A single lifetime is assumed. Same symbols and experimental conditions as in fig. 1.

citation wavelength 295 nm and compared with a value obtained at excitation wavelength 276.5 nm. It can be seen that no significant variation of the fluorescence lifetime with emission wavelength was detectable.

Lifetimes of scatole were also measured in two other binary solvent mixtures: water-DMSO and water-terbutanol. In fig. 4, are represented the variations of scatole lifetime with the solvent composition. These curves exhibit a marqued biphasic profile in the three solvent systems. The scatole decay times were found nearly identical in the water—DMSO and water—terbutanol mixtures at all solvent compositions. As molar fractions and dielectric constants are not identical for these solvents, this equivalence should be considered as accidental. As already pointed out [14], oxygen is a very effective fluorescence quench-

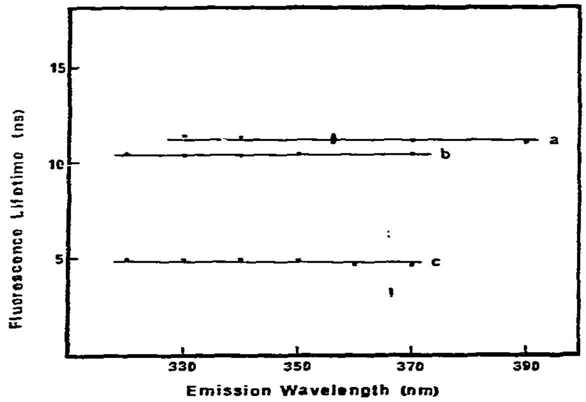

Fig. 3. Fluorescence lifetimes of 3-methylindole. Analysis at different excitation and emission wavelengths in various solvent mixtures. (a) ● 20 percent dioxane/water (v/v). Excitation wavelength: 295 nm. ▲ excitation wavelength: 276.5 nm. (b) 50 percent dioxane/water (v/v). Excitation wavelength: 276.5 nm. (c) pure dioxane. Excitation wavelength: 276.5 nm.

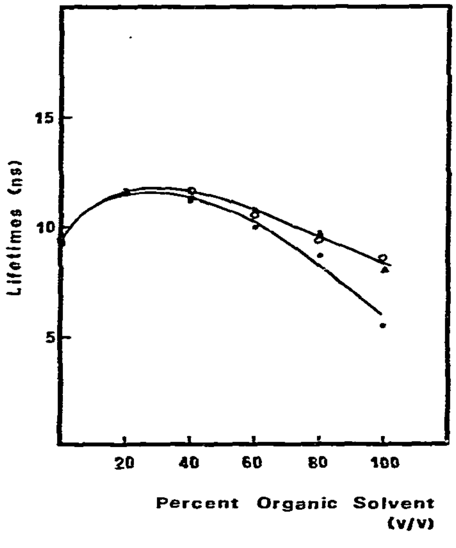

Fig. 4. Effect of the addition of organic solvents on the fluorescence lifetimes of 3-methylindole: ● dioxane, ○ dimethylsulfoxide, ▲ ter-butanol. Excitation wavelength: 276.5 nm. Emission wavelength: 330 nm.

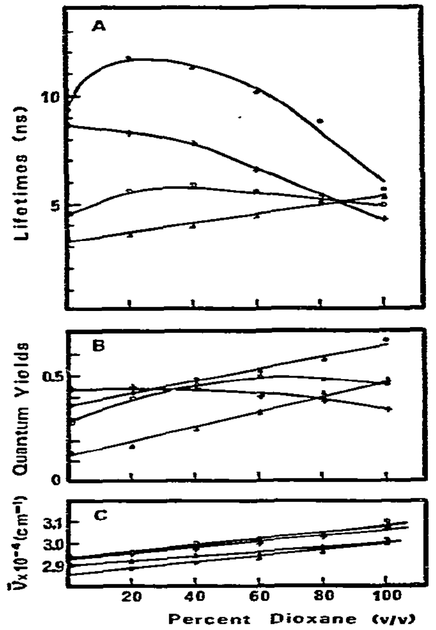

Fig. 5. Fluorescence properties for aqueous solutions of indole derivatives upon addition of dioxane: o indole, + 1-methylindole, • 3-methylindole, ▲ *N*-acetyltryptophanamide. A: lifetimes. B: quantum yields. C: maximum wavenumber of fluorescence spectra.

er in organic solvents. Since oxygen was removed by bubbling argon through the solutions during two hours (see section 2) the decrease of the lifetime with increasing proportions of organic solvent was not attributable to oxygen quenching.

The curves representing fluorescence lifetimes of indole and *N*-methylindole as a function of solvent composition (fig. 5), also display a biphasic profile, but to a lesser extent. In contrast, fluorescence lifetimes of *N*-acetyltryptophanamide rise linearly with increasing amounts of organic solvent. It can be pointed out that the lifetimes measured in water solutions are spread over a large range of values while in pure dioxane all compounds show a value close to 5 ns.

3.2. Determination of quantum yields

The quantum yields of indole and indole derivatives solutions of binary solvents have been determined as explained in the method section. The fluorescence spectra were corrected (fig. 6). The variation of their maximum wavenumber with solvent composition are shown on fig. 5. The absolute quantum yields of these components in water solution were taken from the literature. The variations of the quantum yields with solvent composition are also shown on fig. 5. Finally the rates of radiative (k_F) and non radiative (k_{nr}) deactivation process were calculated according to relation (5) and (6).

For the four compounds studied, k_{nr} has been represented as a function of the dioxane molar fraction "x_2" in the mixture (fig. 7). In this figure one can distinguish three types of variation of k_{nr} with x_2:

1) k_{nr} increases linearly. This is the case of *N*-methylindole.

2) k_{nr} decreases monotonously as in the case of *N*-acetyltryptophanamide.

3) k_{nr} has a biphasic variation with x_2. This is the case of indole and scatole. First it decreases rapidly

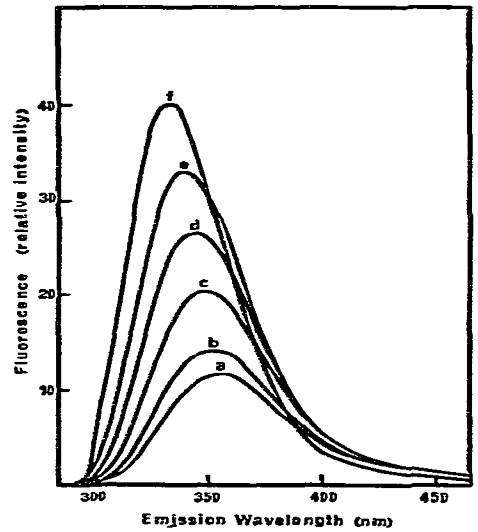

Fig. 6. Effect of solvent composition on fluorescence emission spectra of *N*-acetyltryptophanamide. Spectra are corrected as described in Methods. a) *N*-acetyltryptophanamide in pure water, (b,c,d,e) *N*-acetyltryptophanamide in 20, 40, 60 and 80 percent dioxane-water mixtures (v/v) respectively, (f) *N*-acetyltryptophanamide in pure dioxane. Excitation wavelength: 276.5 nm. Temperature: 20°C. Solute concentration: 2 × 10^{-5} M.

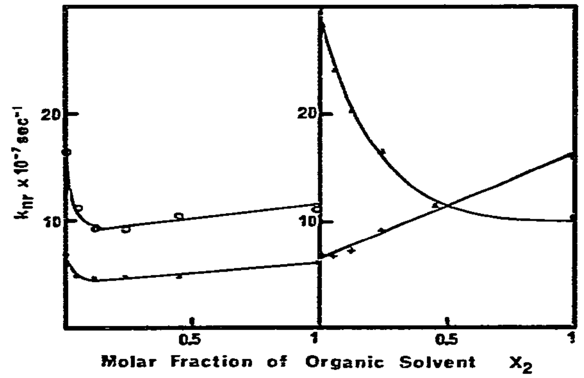

Fig. 7. Rates of non radiative deactivation processes of indole derivatives in water-dioxane mixtures: o indole, + *N*-methylindole, ● 3-methylindole, ▲ *N*-acetyltryptophanamide.

until a minimum reached for x_2 = 0.1—0.15; then it increases linearly. By comparing this curve with the curve relative to *N*-methylindole, one is led to write the rate of non radiative deactivation process of indole and scatole in aqueous solution:

$$k_{nr} = k_1 + k_2, \qquad (7)$$

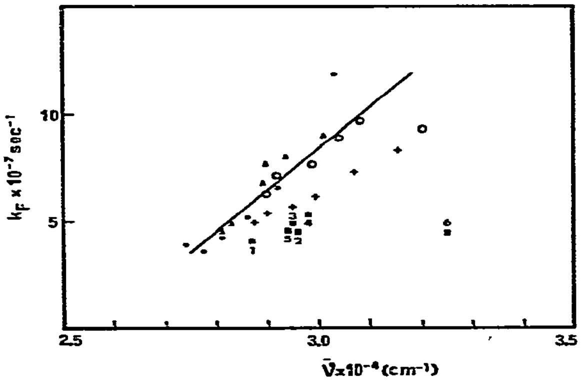

Fig. 8. Plots of k_F versus $\bar{\nu}_F$ the wavenumber of the maximum fluorescence spectra obtained in various water-dioxane mixtures: o indole, + *N*-methylindole, ● 3-methylindole, ▲ *N*-acetyltryptophanamide. Numbers correspond to protein in aqueous medium: 1) wheat germ agglutinin, 2) wheat germ agglutinin-tri-*N*-acetylchitotriose complex, 3) S_4 *E. coli* ribosomal protein, 4) *Staphylococcus aureus* endonuclease, 5) yeast phosphoglycerate kinase, 6) azurin from *Pseudomonas aeruginosa*.

with the following values: $k_1 = k_2 = 8 \times 10^7$ s^{-1} for indole; $k_1 = 4 \times 10^7$ s^{-1} and $k_2 = 3 \times 10^7$ s^{-1} for scatole. These rate constants are obtained by extrapolating the linear part of the curves until it cuts the k_{nr} axis. Then k_1 is the ordinate of this intersection.

In fig. 8 k_F is represented as a function of $\bar{\nu}_F$ for all compounds studied in the dioxane-water mixture; it appears from that figure that (at the experimental accuracy) the points representing the solutions of indole, scatole and *N*-acetyltryptophanamide fall on the same straight line. It seems not to be the case for *N*-methylindole and proteins.

4. Discussion

At the resolution provided by our apparatus, the fluorescence decay of scatole dissolved in water and water dioxane mixtures appeared to be monoexponential. Furthermore we found that the fluorescence decay time was independent of the emission wavelength. Recently Rayner and Szabo [21] using an apparatus having a similar resolution as ours, found that the fluorescence decay of water solution at neutral pH showed two decay times with values 0.51 ns and 3.14 ns. These derivatives of tryptophan had distinct spectra with a maximum at 335 and 350 nm respectively. Two decay times for aqueous solutions of tryptophan had also been found by Balcavage and Alväger, but with quite different values [22]. Whatever may be the difference between these results on tryptophan, the authors concluded that the emission occurs from two non interconvertible excited states, the solvent equilibrated ^1L_a and ^1L_b states of indole. If this conclusion was correct, it would indeed apply to scatole. As we did not find two lifetimes for scatole, results on the heterogeneity of tryptophan emission could more probably be due to internal quenching. Such an explanation has already been proposed in order to account for the fluorescence of cycloglycyl and cycloalanyltryptophan [23]. Fluorescence decays of indole, *N*-methylindole and *N*-acetyltryptophanamide also fitted with a single exponential decay. In agreement with our results, Donzel et al. [23] reported that the decay of scatole and *N*-acetyltryptophanamide solutions in water and DMSO were single exponential functions with time constants independent of emission wavelength. Our

Table 2
Quantum yields and lifetimes of indole derivatives in water and dioxane. Comparison with literature data, a) values obtained in this work, b) best value taken from literature and used in this work.

	Water		Dioxane	
	Q	τ (ns)	Q	τ (ns)
Indole	$0.28^{b)}$	$4.4^{a)}$	$0.45^{a)}$	$4.8^{a)}$
	0.28 [36]	5 [39], 4.9 [37],	0.42 [36]	4.1 [13], 3.6 [14]
		4.1 [13]		5.4 [44]
	0.28 [45]	4.3 [41], 4.8 [14]		
		4.3 [42], 4.5 [44]		
3-methylindole	$0.36^{b)}$	$9.2^{a)}$	$0.66^{a)}$	$5.5^{a)}$
	0.34 [37],	7.6 [37], 6.1 [39]		
	0.34 [36]	9.0 [40], 9.1 [38]		
	0.36 [46]			
N-methylindole	$0.42^{b)}$	$8.5^{a)}$	$0.34^{a)}$	$4.1^{a)}$
	0.43 [31]	10.3 [37], 8.5 [38]		
		8.8 [41]		
N-acetyltryptophanamide	$0.14^{b)}$	$3.1^{a)}$	$0.46^{a)}$	$5.2^{a)}$
	0.14 [44]	2.9 [12], 2.8 [42]	0.30 [44]	4.5 [42], 4.6 [44]
		3.0 [41]		
	0.15 [36]	2.7 [43]		

decay time values of indole derivatives in water and dioxane generally agree with values published in the literature and compare well with that of Walker et al. [13,38] as it can be seen on table 2. The values relative to dioxane are generally more dispersed than in water. This is probably due to incomplete removing of oxygen in some of the works reported.

It has been found in this work that the rate of the non radiative deactivation process k_{nr} of *N*-methylindole dissolved in water-dioxane mixtures increases linearly with the molar reaction of dioxane x_2. In contrast with that simple case the biphasic variation of k_{nr} with x_2 suggests that there is at least two deactivation processes in indole and scatole solutions of high water content. From the comparison of this behaviour with that of *N*-methylindole solutions, one concludes that the nitrogen proton of indole and scatole is involved in one of these deactivation processes with rate constant k_1 probably through an hydrogen bond with a water molecule. The disappearance of this process for values of x_2 greater than 15% suggests that it is linked to the structure properties of water. It has been shown by a number of physical properties that the structure of water changes by adding a small amount of organic solvents [24]. For example, NMR relaxation measurements show that addition of dioxane up to a molar fraction of 20% increases the water molecule correlation time and decreases its self diffusion coefficient [25,26].

In addition to external quenching by the solvent, internal quenching by the molecular carbonyls should be considered in order to interprete the k_{nr} values of *N*-acetyltryptophanamide in water-dioxane mixtures [23,27,28]. Additions of dioxane produce a decrease of k_{nr} which can be explained by the progressive decrease of this internal quenching process which practically disappears in pure dioxane; in this solvent, the value of k_{nr} relative to *N*-acetyltryptophanamide is close to that relative to indole.

The study of the variation with temperature of the fluorescence quantum yield of aqueous solutions of indole, scatole and *N*-methylindole led to consider k_{nr} as the sum of a temperature independent term k_0 and a temperature dependent term $k(T)$ having a high activation energy [13,29,31]. In addition the fluorescence intensity ratio at 20 and 60°C indicates that the high activation process exists only in organic solvent-water mixtures of high water content [29]. More precisely it has been found [13] for indole in aqueous solutions that the activation energy and entropy increased with ethanol concentration up to a molar concentration of 0.05 and then decreased. This biphasic behaviour with a maximum at low organic solvent concentration recalls the variation of k_{nr} observed in the present work in the case of indole and 3-methylindole dissolved in water-dioxane mixtures. This led to the conclusion that the quenching process characterized by $k(T)$ is linked to the water structure. Comparison of k_0 and $k(T)$ values [29–31] reported in the literature with the results obtained for the water solutions studied in the present work, suggests that the two quenching processes characterized by k_1 and k_2 are both temperature dependent. More precise conclusions about this dependence might be obtained by accurate measurements of quantum yield and decay time of the organic solvent-water solutions as a function of temperature.

For the indole derivatives solutions in the dioxane-water mixtures studied in this work, the rate of the radiative deactivation process k_F increased with x_2. Furthermore it has been found that k_F values of indole, scatole and *N*-acetyltryptophanamide (the three derivatives with a non methylated ring nitrogen) were linked by the same relation to the wavenumber ($\bar{\nu}_F$) of the maximum fluorescence spectrum. The electronic transition moments obtained from these values of k_F still vary with the solvent composition. This result might be explained by considering that the fluorescence is emitted from a thermally equilibrated population of the two excited states ^1L_a and ^1L_b [32,33]. Recent measurements of the electronic transition moments as a function of temperature of indole derivatives in water and hydrocarbon solutions disagree with such a possibility [34]. But both type. of solvents may represent extreme cases where emission comes from a unique excited state, namely ^1L_b in hydrocarbon and ^1L_a in water. Dioxane-water mixtures are intermediate cases in which dual emission might occur.

The empirical relation found between k_F and $\bar{\nu}_F$ by studying the model compounds in water–dioxane mixtures incited us to determine whether a similar relation might be found in the case of the proteins tryptophyl emission. In order to establish such a relation it would be very useful to dispose of a series of proteins containing one tryptophyl residue in a definite environment. For such proteins the radiation rate constant would be:

$$k_F = \phi/\tau \tag{8}$$

Experiments show that the conditions are rarely fulfilled. If one admit, however, that a relation exists between k_F and $\bar{\nu}_F$, it suffices to select proteins in which the number of emitting tryptophans is small and in which the fluorescence decay does not depend on emission wavelength. In such a case, one can write:

$$k_F = \frac{\langle\phi\rangle}{\langle\tau\rangle} \tag{8'}$$

Table 3

Fluorescence parameters of several proteins in aqueous medium and room temperature

	$\langle Q\rangle$	$\langle\tau\rangle$ ns	k_F $\times 10^{-7}$ s^{-1}	$\bar{\nu}_F$ $\times 10^{-4}$ cm^{-1}
WGA [11]	0.12 × 3/2	4.32	4.1	2.87
WGA [11] + chitotriose	0.15 × 3/2	5	4.5	2.96
S_4	0.16 [47]	3.25 [48]	4.9	2.95
Nuclease	0.29 [49]	5.5 [50]	5.3	2.98
P.G.K. [51] (pH 3.9)	0.08	1.7	4.6	2.94
Azurin [12]	0.21	4.7	4.5	3.25

WGA: wheat germ agglutinin.
S_4: S_4 *E. coli* ribosomal protein.
Nuclease: *Staphylococcus aureus* endonuclease.
P.G.K.: yeast phosphoglycerate kinase.
Azurin: azurin from *Pseudomonas aeruginosa*.

⟨ϕ⟩ and ⟨τ⟩ must be obtained by excitation at a wavelength in which only the tryptophyl residues absorb (295 nm). Furthermore, one has to assume that the molar absorptivity (ϵ) of each residue is the same at that wavelength. Finally, ⟨τ⟩ must be obtained from a detailed analysis of accurate fluorescence decay measurements, after checking that this decay is independent of the emission wavelength. Most of the published decay times of proteins have not been obtained under the conditions required; then they are not suitable for our purpose. Fig. 8 represents the approximated value of k_F as a function of $\bar{\nu}_F$ for a few proteins containing one or two tryptophyl residues per protomer. Their quantum yield and decay times have been carefully measured. In the case of wheat germ agglutinin (WGA) we took into account that, out of three tryptophan residues contained in the protomer, only two are fluorescent [35]. It can be seen from fig. 8 that the value of k_F found for proteins is sensibly smaller than in the case of non *N*-methylated indole compounds dissolved in the dioxane-water mixtures. In addition, the variation of k_F with $\bar{\nu}_F$ is less pronounced than in the case of the solutions of model compounds. These results are important in view of the quantitative determination of the number of non fluorescent tryptophan residues [11,12] and of the determination of the transfer rate between tryptophan residues in a protein.

In future works, additional measurements on proteins containing one or two emitting tryptophan residues must be made in order to ascertain if a relation between k_F and $\bar{\nu}_F$ can be established. Then the power of the analysis of protein fluorescence heterogeneity by decay measurements would be sensibly increased.

Acknowledgement

We are greatly indebted to Dr. R.H. Pain for his gift of a sample of yeast phosphoglycerate kinase.

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