DETERMINATION OF THE RATE CONSTANT OF THE SOLVENT CAGE REORIENTATION AROUND A PHOTOEXCITED MOLECULE USING PHASE MODULATION FLUOROMETRY

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Summary

The fluorescence lifetime and quantum yield of ethylamino-9-methoxy-2-chloro-6-acridine in glycerol solution were measured at three temperatures using the phase modulation technique. The results were interpreted in terms of a relaxation process in the excited state that involved two emitting species: one (designated F) directly excited and one (designated R) resulting from the reorientation of the solvent cage around the F state. The rate constant of this process was evaluated; it was greatly increased on raising the temperature, which also enhanced the radiationless deactivation rate constants of both emitting species, although to a minor extent. At 60 °C, this relaxation process is so fast that the whole fluorescence is emitted from R species.

1. Introduction

9-aminoacridinic compounds (9AA) are widely used as fluorescence probes in biological fields. Our aim was to study the change of their fluorescence properties in solution under various experimental conditions (viscosity, temperature, pH, solvent used etc.). A previously published study showed that a solvent relaxation process occurs after the photoexcitation of molecules of 9AA [1]. This process can be investigated by performing phase modulation fluorescence measurements if it occurs at a rate that is of the same order of magnitude as the rates of other deactivation mechanisms from the excited state [2, 3]. The results may then be interpreted by using the two excited state (F and R) model of Lakowicz and Balter [4]. In our case, the directly photoexcited F state would be solvated as in the ground state while the excited state R would result from a different orientation of solvent molecules around the excited state F (Fig. 1).

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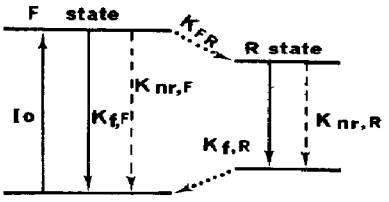

Fig. 1. Energy diagram of F and R states: I_0 is the number of photons absorbed per second; $k_{f,F}$ and $k_{f,R}$ are the rate constants of radiative transitions from the F and R states; k_{FR} is the rate constant of the F → R transition; $k_{nr,F}$ and $k_{nr,R}$ are the rate constants of other radiationless transitions from F and R states.

In this work we have studied 9-ethylamino-2-methoxy-6-chloroacridine (ECMA) in glycerol solutions at various temperatures. Our purpose was to look for any influence of the viscosity of the medium and the temperature on the solvent relaxation rate constant k_{FR}. We determined k_{FR} from phase modulation and quantum yield measurements.

2. Experimental details

ECMA was synthesized at the laboratory of Dr. P. Jacquignon (Institut des Substances Naturelles, Gif sur Yvette, France). Fluorescence spectra were recorded using a Jobin–Yvon 3 D apparatus. Absorbance measurements were performed using a Uvikon 820 spectrophotometer. Fluorescence quantum yields were determined by comparison with a standard of known fluorescence quantum yield [5]. The standard was a solution of atebrin in methanol with a quantum yield of 0.111 [6].

Phase shift and demodulation factor measurements were performed using an SLM 4800 spectrofluorometer. The apparent phase fluorescence lifetime τ_ϕ was determined from the phase difference ϕ between the fluorescence and the exciting light modulated at the angular frequency $\omega = 2\pi\nu$ according to

$$\tau_\phi = \frac{1}{\omega} \tan \phi \tag{1}$$

The apparent modulation fluorescence lifetimes τ_m were determined from the demodulation factor m according to the relation

$$\tau_m = \frac{1}{\omega}\left(\frac{1}{m^2} - 1\right)^{1/2} \tag{2}$$

Each τ_ϕ and τ_m represent the average of a set of ten measurements, each of which, in turn, was the average of a set of 100 separate values obtained at a sampling rate of approximately 5 s^{-1}. The maximum coefficient of variation in an independent measurement of either τ_ϕ or τ_m was 5%. Data were corrected for the wavelength–time response of the photomultiplier tube accord-

ing to the method of Jameson and Weber [7]. Consequently, an error treatment was rather difficult to perform.

When the coefficient of variation between the τ_ϕ and the τ_m reached 20%, we considered that these parameters had different values which were dependent on the experimental conditions. However, when this coefficient was only 6%, τ_ϕ and τ_m were considered to be equal whatever the experimental conditions.

Thus, our interpretation relied not only on the eventual difference between τ_ϕ and τ_m measured under the same experimental conditions but also on the change in both these parameters with angular frequency or observation wavelength. Control measurements were performed according to the technique of Spencer and Weber [8] to confirm that the rotational Brownian motion of the molecules had no influence on the experimental data.

3. Results

3.1. Phase modulation experiment

The data from phase modulation measurements of a 2.5×10^{-5} M ECMA–glycerol solution at 5 °C are listed in Table 1. It can be seen that for short wavelengths $\tau_\phi < \tau_m$, $m/\cos\phi < 1$ and τ_ϕ, τ_m and $m/\cos\phi$ were decreasing functions of ω; for long wavelengths $\tau_\phi > \phi_m$, $m/\cos\phi > 1$ and τ_ϕ, τ_m and $m/\cos\phi$ were increasing functions of ω. At a wavelength close to 530 nm, τ_ϕ and τ_m were equal whatever the modulation frequency. In addition, τ_ϕ and τ_m increased with λ over the whole wavelength range studied. All these results are typical of an excited state process occurring with a rate constant of the same order of magnitude as those of other deactivation processes from this excited state [2 - 4]. This excited state process may be due to the reorientation of solvent molecules around photo-excited ECMA molecules, as is the case for other similar compounds [3].

TABLE 1

Phase modulation fluorescence measurements on a 2.5×10^{-5} M ECMA–glycerol solution at 5 °C

λ_{obs} (nm)	*30 MHz*			*18 MHz*			*6 MHz*		
	τ_ϕ	τ_m	$m/\cos\phi$	τ_ϕ	τ_m	$m/\cos\phi$	τ_ϕ	τ_m	$m/\cos\phi$
460	5.6	8.3	0.78	7.2	10.1	0.85	9.2	12.7	0.95
480	9.5	12.6	0.80	10.8	13.2	0.88	13.8	13.9	1.00
500	10.9	14.0	0.81	13.6	14.6	0.95	14.0	14.4	0.99
520	13.2	14.6	0.92	14.6	15.2	0.97	14.8	15.0	1.00
540	16.7	15.3	1.09	16.7	15.5	1.04	15.6	15.5	1.00
560	18.1	16.1	1.08	17.9	15.7	1.04	15.7	16.2	0.99
575	21.6	16.3	1.30	19.5	16.0	1.12	16.3	16.0	1.01

The fluorescence was then emitted from both F and R states (Fig. 1) and τ_ϕ and τ_m may be expressed as [9]

$$\tau_\phi = \frac{\alpha\tau_F(1 + \omega^2\tau_R^2) + (1 - \alpha)(\tau_F + \tau_R)}{\alpha(1 + \omega^2\tau_R^2) + (1 - \alpha)(1 - \omega^2\tau_F\tau_R)} \quad (3)$$

$$\tau_m = \left(\frac{\tau_F^2\tau_R^2\omega^2 + \tau_F^2 + \tau_R^2 - \alpha^2\tau_R^2}{1 + \alpha^2\tau_R^2\omega^2}\right)^{1/2} \quad (4)$$

In eqns. (3) and (4) τ_F and τ_R are the lifetimes of the excited states F and R and α is the contribution of the F state to the overall fluorescence.

τ_R was determined as 15.0 ± 0.5 ns, *i.e.* the mean value of τ_ϕ and τ_m obtained by interpolation at λ = 530 nm where τ_ϕ is close to τ_m (and $m/\cos\phi$ is close to unity) and τ_ϕ and τ_m are independent of modulation frequency. A theoretical study of the variations in τ_ϕ and τ_m with α showed that in this case $\alpha = \tau_F/\tau_R$ and eqns. (3) and (4) reduce to $\tau_\phi = \tau_m = \tau_R$ [3, 9]. Using τ_R = 15.0 ns, τ_F and α can be calculated from eqns. (3) and (4) for each observation wavelength and modulation frequency. The results are listed in Table 2.

TABLE 2

τ_F and α determined at different observation wavelengths for an ECMA–glycerol solution at θ = 5 °C and θ = 21 °C

		λ (nm)						
		460	*480*	*500*	*520*	*540*	*560*	*575*
θ = 5 °C								
30 MHz	τ_F (ns)	4.0	4.0	3.0	3.0	4.0	4.5	4.5
	α	0.66	0.38	0.34	0.30	0.26	0.23	0.22
18 MHz	τ_F (ns)	4.0	4.5	4.0	—	5.0	4.5	4.5
	α	0.59	0.39	0.31	—	0.24	0.22	0.20
θ = 21 °C								
30 MHz	τ_F (ns)	1.5	1.5	0.8	—	1.5	1.2	1.3
	α	0.33	0.16	0.14	0.10	0.09	0.08	0.07
18 MHz	τ_F (ns)	1.7	2.5	—	2.0	1.5	—	1.0
	α	0.32	0.16	—	0.09	0.08	—	0.06

Experiments were performed at 21 °C and 60 °C to determine the influence of temperature and viscosity on the rate constant k_{FR}.

At 21 °C, the relaxation process in the excited state was still apparent, as shown by the changes in τ_ϕ and τ_m with ω and λ, and their relative values (Table 3). Nevertheless, τ_F was shorter: τ_F = 1.5 ± 0.5 ns at 21 °C compared with τ_F = 4.1 ± 0.6 ns at 5 °C; in addition the α were also smaller (Table 2).

At 60 °C, the excited state process was no longer apparent. τ_ϕ and τ_m could be considered equal within experimental error and were independent of observation wavelength and modulation frequency used (Table 4). Thus,

TABLE 3

Phase modulation fluorescence measurements on a 2.5×10^{-5} M ECMA–glycerol solution at 21 °C

λ_{obs} (nm)	*30 MHz*			*18 MHz*			*6 MHz*		
	τ_ϕ	τ_m	$m/\cos\phi$	τ_ϕ	τ_m	$m/\cos\phi$	τ_ϕ	τ_m	$m/\cos\phi$
460	5.1	10.7	0.62	7.6	12.2	0.77	9.9	13.5	0.95
480	9.9	13.5	0.77	12.1	13.7	0.92	12.5	13.6	0.98
500	11.2	14.2	0.82	13.4	14.4	0.95	12.9	11.9	1.01
520	14.7	14.4	1.01	15.0	14.4	1.02	13.9	13.0	1.01
540	16.5	14.5	1.10	15.4	14.4	1.06	14.5	13.5	1.02
560	17.7	14.5	1.20	15.9	14.2	1.09	14.9	14.2	1.01
575	19.7	14.6	1.31	16.8	14.4	1.13	15.2	14.5	1.01

TABLE 4

Phase modulation fluorescence measurements on a 2.5×10^{-5} M ECMA–glycerol solution at 60 °C

λ_{obs} (nm)	*30 MHz*			*18 MHz*		
	τ_ϕ	τ_m	$m/\cos\phi$	τ_ϕ	τ_m	$m/\cos\phi$
460	9.8	11.5	0.88	10.9	12.6	0.91
480	10.4	10.3	1.01	9.9	11.0	0.94
500	10.4	10.2	1.02	10.1	10.2	1.00
520	10.2	9.9	1.02	10.2	10.9	0.96
540	10.4	9.9	1.04	9.8	11.2	0.92
560	9.7	10.7	0.93	9.8	10.8	0.95
575	10.6	10.6	1.00	10.1	10.8	0.96

the mechanism of reorientation of solvent molecules around the F state was fast enough that fluorescence was emitted from the R state only, and thus the excited state process could not be detected in phase modulation measurements [3, 10]. τ_ϕ and τ_m were equal to 10.5 ± 0.6 ns, and were now a measure of the lifetime of the excited state R.

3.2. Quantum yield measurements and determination of rate constants

The total fluorescence quantum yield Q_T was determined at 5, 21 and 60 °C (Table 5). By definition Q_T can be written (using the notation of Fig. 1) as

$$Q_T = \frac{k_{f,F}[F] + k_{f,R}[R]}{I_0} \tag{5}$$

where [F] and [R] represent the concentration of F states and R states respectively.

TABLE 5

Temperature variations in the fluorescence quantum yields, fluorescence lifetimes and deactivation rate constants of F and R states

θ (°C)	Q_T	Q_F	τ_F (ns)	$k_{f,F}$ ($\times 10^7$ s^{-1})	k_{FR} ($\times 10^7$ s^{-1})	$k_{nr,F}$ ($\times 10^7$ s^{-1})	Q_R	τ_R (ns)	$k_{f,R}$ ($\times 10^7$ s^{-1})	$k_{nr,R}$ ($\times 10^7$ s^{-1})
60	0.177	≈ 0					0.177	10.5	2.0	7.5
21	0.257	0.033	1.5	2.2	52.2	12.3	0.224	14.3	2.0	5.0
5	0.286	0.090	4.1	2.2	15.9	6.3	0.196	15.0	2.0	4.7

Under photostationary conditions we can write

$$\frac{d[F]}{dt} = 0 = I_0 - (k_{f,F} + k_{nr,F} + k_{FR})[F] \quad (6)$$

$$\frac{d[R]}{dt} = 0 = k_{FR}[F] - (k_{f,R} + k_{nr,R})[R] \quad (7)$$

Expressing eqn. (5) in terms of eqns. (6) and (7) it becomes

$$Q_T = k_{f,F}\tau_F + k_{FR}\tau_F k_{f,R}\tau_R \quad (8)$$

where

$$\tau_F = \frac{1}{k_{f,F} + k_{nr,F} + k_{FR}} \quad (9)$$

and

$$\tau_R = \frac{1}{k_{f,R} + k_{nr,R}} \quad (10)$$

The total fluorescence quantum yield can therefore be expressed as a sum of two terms. The first represents the F fluorescence quantum yield Q_F; the second is not exactly the R fluorescence quantum yield since the R state is never directly photoexcited. Nevertheless we shall denote it Q_R since it is related to R emission. In addition, the product $k_{FR}\tau_F$ represents the probability that an F state will be converted into an R state.

Phase modulation fluorescence data allow the resolution of the total fluorescence into its F and R components (Fig. 2) since α was determined for various wavelengths. Consequently it is possible to calculate Q_F and Q_R at 21 °C and at 5 °C (Table 5). However, at 60 °C fluorescence was only emitted from the R state and eqn. (8) can then be written as

$$Q_T = Q_R = k_{FR}\tau_F k_{f,R}\tau_R$$

which implies that $k_{f,F}$ is negligible relative to the product $k_{FR}k_{f,R}\tau_R$.

As τ_F and Q_F were determined at 21 and 5 °C, the rate constant $k_{f,F} = Q_F/\tau_F$ of fluorescence emission could be calculated at both temperatures. It

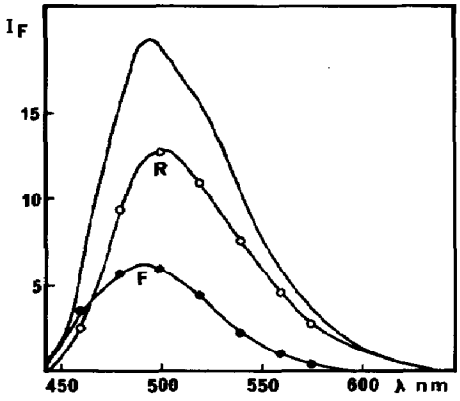

Fig. 2. Resolution of the total fluorescence of an ECMA–glycerol solution at 5 °C into its F and R components.

was equal to 2.2×10^7 s^{-1} in both cases (Table 5). This rate constant changed with neither the temperature nor the viscosity of the medium and this completely agrees with the results of Birks [11]. Likewise, it seemed quite rational to us to assume that the rate constant $k_{f,R}$ of the R state fluorescence emission did not significantly change with temperature [9, 12]. Nevertheless, the radiative rate constants do change with the refractive index n of the solvent used. In a water–ethanol solution (θ = 21 °C) $k_{f,R}$ has been determined as 1.7×10^7 s^{-1} [9]. Consequently, its value in a glycerol solution (θ = 21 °C) can be calculated by using the equation

$$k_{f,R}(2) = \left(\frac{n_2}{n_1}\right)^2 k_{f,R}(1)$$

where 1 and 2 refer to the two different solvents [13]. We obtained $k_{f,R}$ = 2×10^7 s^{-1}. The refractive index can be considered as constant for the glycerol solutions in the temperature range studied; consequently $k_{f,R}$ was also constant. From eqn. (8) the solvent relaxation rate constant k_{FR} was calculated at 21 °C ($k_{FR} = 52.2 \times 10^7$ s^{-1}) and at 5 °C ($k_{FR} = 15.9 \times 10^7$ s^{-1}); $k_{nr,F}$ and $k_{nr,R}$ were deduced from eqns. (9) and (10). All these results are listed in Table 5.

4. Discussion

At 5 °C, the fluorescence emitted by an ECMA–glycerol solution arises from both F and R, F being the directly photoexcited state and R being the state that results from a reorientation of solvent molecules around the F state.

Let P be the deactivation probability of an F or R state, defined by the expression $P = k\tau$ where k is the rate constant of the deactivation process concerned and τ is the lifetime of the state considered. P_{FR}, $P_{nr,F}$, $P_{f,F}$, $P_{nr,R}$ and $P_{f,R}$ can be calculated at 5 °C and at 21 °C (Table 6).

TABLE 6

Temperature variations in F and R state deactivation probabilities

θ (°C)	$P_{FR} = k_{FR}\tau_F$	$P_{f,F} = k_{f,F}\tau_F$	$P_{nr,F} = k_{nr,F}\tau_F$	$P_{f,R} = k_{f,R}\tau_R$	$P_{nr,R} = k_{nr,R}\tau_R$
60	0.84	≈ 0	0.16	0.21	0.79
21	0.78	0.03	0.18	0.29	0.71
5	0.65	0.09	0.26	0.30	0.70

Thus, of 100 F states directly photoexcited at 5 °C, 65 were converted into R states, 26 were deactivated by non-radiative transitions and 9 emitted fluorescence. Of the 65 R states produced, 19 emitted fluorescence and 46 were deactivated by non-radiative transitions.

At 21 °C, the process was qualitatively identical but quantitatively different. The temperature jump appeared in a large increase in k_{FR} and a relatively small increase in the radiationless rate constants $k_{nr,F}$ and $k_{nr,R}$. Consequently, the lifetime of F decreased to a large extent while that of R decreased much less significantly. Therefore, of 100 F states directly photoexcited only 3 emitted fluorescence, 18 were deactivated by non-radiative transitions and 78 were converted into R states. Of the latter, 23 emitted fluorescence and 55 were deactivated by non-radiative transitions.

Raising the temperature to 60 °C results in an increase in $k_{nr,F}$ and also in k_{FR}. Consequently, the lifetime of excited F was so short that the fluorescence probability $P_{f,F}$ of the F state could be considered as negligible: the whole fluorescence emitted was ascribed to the R state. Using $Q_R = 0.177$ and $P_{f,R} = k_{f,R}\tau_R = 0.21$ it can be deduced from eqn. (8) that $P_{FR} = 0.84$. Consequently, $P_{nr,R} = 0.79$ and $P_{nr,F} = 0.16$ since $P_{f,F}$ is assumed to be negligible. Thus, among 100 F states directly photoexcited at 60 °C, 84 were converted into R states of which 18 emitted fluorescence and 66 were deactivated by non-radiative transitions. The rate constant ratio $k_{FR}/k_{nr,F}$ was calculated from the ratio of P_{FR} to $P_{nr,F}$. It was equal to 5.2 at 60 °C compared with 4.2 at 21 °C and 2.5 at 5 °C.

Conclusions

In conclusion, an increase in temperature of an ECMA–glycerol solution induced an increase in the F and R radiationless transition rate constants as expected, and resulted in a more significant increase in the rate constant of the relaxation process. This strongly suggests that the rate determining step of this process is a solvent cage reorientation around photoexcited ECMA molecules.

References

1 A. Marty and P. Viallet, *J. Photochem.*, *20* (1982) 213.
2 J. R. Lakowicz and H. Cherek, *Biochem. Biophys. Res. Commun.*, *99* (1981) 1173.
3 A. Marty, M. Bourdeaux, M. Dell'Amico and P. Viallet, *J. Photochem.*, *28* (1985) 71.
4 J. R. Lakowicz and A. Balter, *Biophys. Chem.*, *16* (1982) 99.
5 J. B. Birks, *J. Res. Natl. Bur. Stand., Sect. A*, *80* (1976) 389.
6 J. L. Cline Love, L. M. Upton and A. W. Ritter, *Anal. Chem.*, *50* (1978) 2059.
7 D. M. Jameson and G. Weber, *J. Phys. Chem.*, *85* (1981) 953.
8 R. D. Spencer and G. Weber, *J. Chem. Phys.*, *52* (1970) 1654.
9 A. Marty, *Thesis "Doctorat d'Etat"*, Perpignan, France, 1984.
10 A. L. Campillo and T. M. Ordonez, *C. R. Acad. Sci. Paris*, *293* (1981) 271.
11 J. B. Birks, *Photophysics of Aromatic Molecules*, Wiley-Interscience, London, 1970, p. 312.
12 K. Kasama, K. Kikuchi, Y. Nishida and H. Kokubun, *J. Phys. Chem.*, *85* (1981) 4148.
13 R. A. Lampert, S. R. Meech, J. Metcalfe, D. Phillips and A. P. Schaap, *Chem. Phys. Lett.*, *94* (1983) 137.