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9-Amino-2-methoxy-6-chloroacridine monocation fluorescence analysis by phase-modulation fluorometry

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Abstract. The fluorescence of the 9-amino-2-methoxy-6-chloroacridinic monocation (ACMA) in various alcohol-water solutions was studied by phase-modulation fluorometry. Apparent phase and modulation lifetimes were determined at different observation wavelengths for three modulation frequencies. The results are explained by an orientational relaxation of the solvent cage subsequent to ACMA photoexcitation.

Key words: 9-amino-2-methoxy-6-chloroacridine monocation, fluorescence lifetimes, phase-modulation fluorometry, solvent cage reorientation

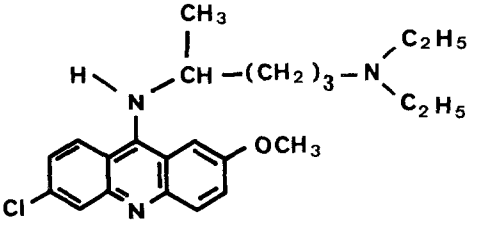
1. Introduction

9-aminoacridine derivatives have been widely used as fluorescent probes to monitor properties of biological samples. In particular, intracellular proton concentrations have been studied by a fluorescence quenching method using 9-(N,N-4′-diethylamino-1′-methyl-n-butylamino)-2-methoxy-6-chloroacridine (atebrin) and 9-amino-2-methoxy-6-chloroacridine (ACMA) (Dufour et al. 1982; Takio and Takaoki 1981). Structures of atebrin and ACMA are presented in Fig. 1.

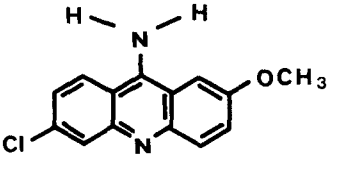
As yet, no data concerning the fluorescence decay of the ACMA monocation has been published. This is quite surprising because the pK_a value (8.6) which characterizes the monocation $\rightleftharpoons$ neutral molecule equilibrium indicates that the charged form is the predominant one in physiological solutions (Albert 1965; Capomacchia and Schulman 1975).

Another reason for our interest in the monocation fluorescence is that complex fluorescence decays are observed for atebrin and atebrin mustard (Andreoni et al. 1980; Arndt-Jovin et al. 1979; De Silvestri et al. 1981). The observation that these decays are not mono-exponential has been interpreted in terms of a multi-proton transfer mechanism in the excited state, involving atebrin mono-, di- and trications. Such an explanation conflicts with the results of Capomacchia and Schulman (1975): it seems quite unlikely that these three charged species could exist in equilibrium because of the large differences between the pK_a values corresponding to each atebrin protonation site.

Phase-modulation fluorometry was used to study the fluorescence decay of the ACMA monocation. The pH value was carefully controlled in order that this species was the only one detectable. Nevertheless, the fluorescence decay observed was not mono-exponential. Moreover the variations of both apparent phase and modulation lifetimes with modulation frequencies and observation wavelengths suggested that some relaxation process occurred in



ATEBRINE



ACMA

Fig. 1. Atebrin and ACMA structures

the excited state. The influence of temperature and solution viscosity were studied. These results suggest that orientational relaxation of the solvent cage could be involved in the excited state process.

2. Experimental

ACMA was synthesized and purified in the laboratory of Dr. P. Jacquignon (Institut des Substances Naturelles, Gif-sur-Yvette, France). Because of its poor water solubility, this probe was dissolved in ethanol-water (20/80, V/V). The viscosity of the medium was changed using glycerol-water (12/88, V/V). A few drops of 0.1 N HCl were added to obtain the ACMA monocation fluorescence spectrum. All solution concentrations were approximately $2\times10^{-5}\text{mol}\cdot\text{l}^{-1}$.

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

$$\tau_\varphi = \frac{1}{\omega}\tan\varphi\,. \tag{1}$$

The apparent modulation lifetime, τ_m, was determined from the demodulation factor, m, according to the relation:

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

The data were corrected for wavelength-dependent time responses of the photomultiplier tube according to the method of Jameson and Weber (1981).

Absorption spectra were recorded on a SAFAS 1800 spectrophotometer and fluorescence spectra on a JOBIN-YVON 3D spectrofluorometer. Quantum yields were calculated according to the comparison method of Parker (1968). pH measurements were performed on a Tacussel PH N 81 apparatus with a TB/HA electrode.

The equilibrium between the singly and doubly charged cations derived from ACMA was studied in sulfuric acid solutions. The correct Hammett acidity scale (H_0) of Jorgenson and Hartter (1963) was employed to calibrate these solutions.

To determine the equilibrium constant between the neutral molecule and anion derived from ACMA, a solution of sodium methoxide (0.025 M) in various dimethyl sulfoxide-methanol mixtures was used as the solvent. These solutions were calibrated by the H^- acidity function (Stewart et al. 1962; More O'Ferral and Ridd 1963).

The ground state dissociation constants (pK_a) were determined by absorptiometric pH titration. The excited state dissociation constants (pK_a^*) were determined by fluorometric pH titration. The fluorescence was excited at an isobestic point of the absorption spectra. The inflection point in the titration curve of ACMA fluorescence with H_0 or H^- was assigned to the dissociation constant for the excited state equilibrium (Capomacchia and Schulman 1975).

3. Results

ACMA can exist as a dication (D) protonated at the acridinic nitrogen and the amino group, a monocation (M) protonated at the acridinic nitrogen, a neutral molecule (N) or an anion (A) when ionization of the amino group occurs. The absorption and fluorescence spectra of all these species are presented in Figs. 2 and 3.

The corresponding acidity constants were determined in the ground state (pK_a) and in the first singlet excited state (pK_a^*). These values are the following (Marty 1984):

	$D \rightleftharpoons M$	$M \rightleftharpoons N$	$N \rightleftharpoons A$
pK_a	− 8.6	8.6	18.0
pK_a^*	− 4.7		13.3

Previous studies by Capomacchia and Schulman (1975) have led to similar results. Throughout the acidity range −3 to 7 the spectra recorded were identical to spectrum (2) of Figs. 2 and 3. In the acidity range 10 to 11.5 the spectra recorded were identical to spectrum (3) of Figs. 2 and 3. In the acidity range 7 to 10, spectrum (2) was seen to change to spectrum (3). The fluorescence spectra of M and N changed within the same pH region as the absorption spectra. Capomacchia and Schulman (1975) have postulated that excited state proton exchange does not occur between M and N.

The τ_φ, τ_m values and the $(m/\cos\varphi)$ ratios are listed in Table 1 for ACMA in ethanol-water solution at pH 4 and $\theta = 21\,^\circ\text{C}$. Under these conditions, the ACMA monocation is protonated on the acridinic nitrogen atom in the ground state and in the first excited singlet state (Albert 1965; Capomacchia and Schulman 1975). Experimental results can be summarized as follows: On the short wavelength side of the spectrum $\tau_\varphi < \tau_m$, $(m/\cos\varphi) < 1$; τ_φ, τ_m, $(m/\cos\varphi)$ decrease as the modulation frequency increases. The opposite is observed for the long wave-

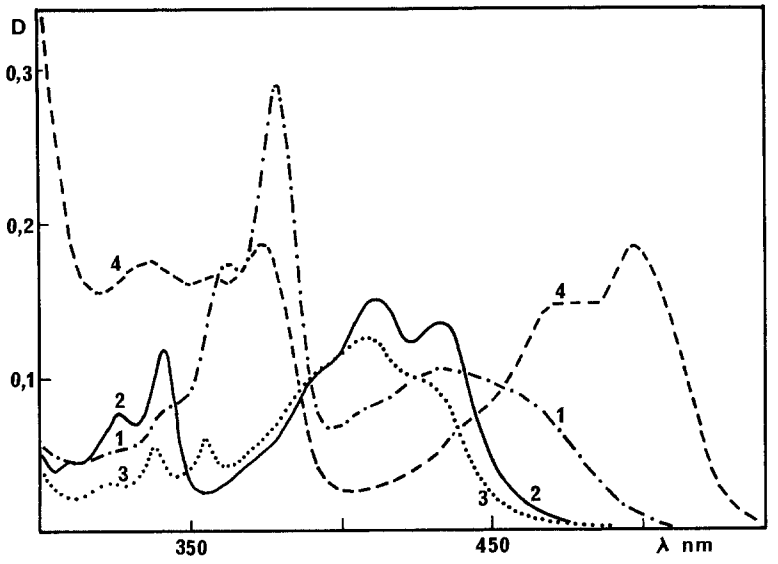


Fig. 2. Absorption spectra of ACMA (2.1×10^{-5} mol · l^{-1}): (1) dication in H_2SO_4 18 mol · l^{-1} ($H_0 = -10$); (2) monocation in 40% ethanol – 60% water (pH = 6.5); (3) neutral molecule in 40% ethanol – 60% water (pH = 11); (4) anion in 2% methanol – 98% dimethylsulfoxide containing 0.025 mol · l^{-1} sodium methoxide ($H^- = 20$). At pH 4 the absorption spectrum is identical to spectrum (2)

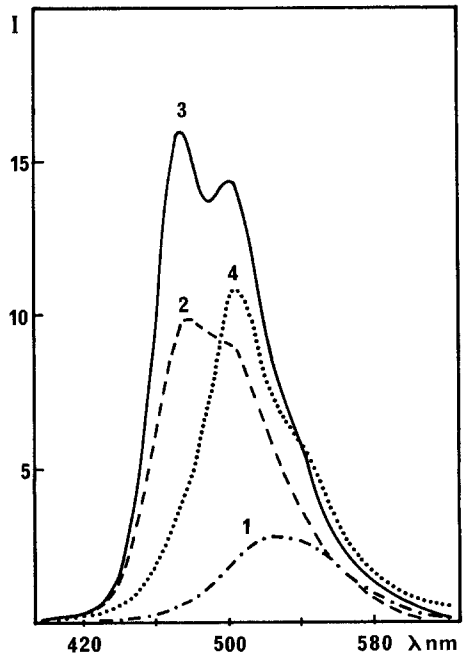


Fig. 3. Fluorescence spectra of ACMA (2.1×10^{-5} mol · l^{-1}): (1) dication in sulfuric acid 12.6 mol · l^{-1} ($H_0 = -7$, λ_{ex} = 440 nm); (2) monocation in 40% ethanol – 60% water (pH = 6.5, λ_{ex} = 395 nm); (3) neutral molecule in 40% ethanol – 60% water (pH = 11, λ_{ex} = 395 nm); (4) anion in methanol containing 2 mol · l^{-1} sodium methoxide ($H^- = 15$, λ_{ex} = 440 nm). At pH = 4 the fluorescence spectrum is identical to spectrum (2)

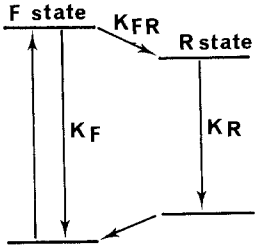


Fig. 4. Energy diagram of F and R states

length side of the spectrum. At a wavelength close to 500 nm, τ_φ and τ_m values are nearly equal and $(m/\cos\varphi)$ is close to 1. In addition, τ_φ and τ_m increase with increasing λ for the entire wavelength range studied.

These results are characteristic of an excited state process occurring within the same timescale as other processes leading to deactivation of the first singlet excited state (Lakowicz and Balter 1982a; Marty 1984). It can be deduced that fluorescence is simultaneously emitted from two excited states which we call F and R. Because the F state is the only one directly photoexcited, the R state has to be populated through the F one as shown in Fig. 4 (Lakowicz and Cherek 1981). Consequently, the expressions for τ_φ, τ_m and $(m/\cos\varphi)$ can be written (Marty 1984; Marty et al. 1985):

$$\tau_\varphi = \frac{\alpha\,\tau_F(1+\omega^2\tau_R^2) + (1-\alpha)\,(\tau_F+\tau_R)}{\alpha\,(1+\omega^2\tau_R^2) + (1-\alpha)\,(1-\omega^2\tau_F\tau_R)} \tag{3}$$

$$\tau_m = \left[\frac{\tau_F^2\tau_R^2\omega^2+\tau_F^2+\tau_R^2-\alpha^2\tau_R^2}{\tau_R^2\alpha^2\omega^2+1}\right]^{1/2} \tag{4}$$

$$\frac{m}{\cos\varphi} = \frac{1+\alpha^2\omega^2\tau_R^2}{\alpha\,\omega^2\tau_R\,(\tau_F+\tau_R) + (1-\omega^2\tau_F\tau_R)}\,. \tag{5}$$

In these expressions, ω is the angular modulation frequency; α measures the fractional contribution of the F excited state to the overall fluorescence emission and its numerical value is dependent on the observation wavelength; τ_F and τ_R are the fluo-

Table 1. Phase-modulation fluorescence data (τ_φ and τ_m in ns) for an ACMA ethanol-water solution at pH 4 and $\theta = 21\,^\circ C$

λ_{obs} [nm]	30 MHz			18 MHz			6 MHz		
	τ_φ	τ_m	$\frac{m}{\cos\varphi}$	τ_φ	τ_m	$\frac{m}{\cos\varphi}$	τ_φ	τ_m	$\frac{m}{\cos\varphi}$
440	3.3	8.8	0.61	5.1	12.0	0.69	9.8	15.0	0.93
460	8.3	16.1	0.58	11.9	17.7	0.75	16.0	17.5	0.97
480	15.7	18.2	0.87	17.5	18.9	0.94	18.8	18.7	1.00
500	20.2	18.7	1.07	19.7	19.1	1.03	19.5	18.6	1.02
520	24.4	18.6	1.29	21.2	19.3	1.08	19.2	18.1	1.02
540	27.6	19.3	1.40	22.6	19.4	1.14	19.5	18.1	1.02
560	29.3	19.3	1.49	22.9	19.6	1.14	19.3	18.6	1.01

Table 2. τ_F (in ns) and α values for an ACMA ethanol-water solution at pH 4 and $\theta = 21\,^\circ C$

λ [nm]		440	460	480	500	520	540	560
30 MHz	τ_F	1.5	2.0	3.5	–	–	1.5	1.5
	α	0.60	0.23	0.13	0.11	0.10	0.09	0.09
18 MHz	τ_F	1.5	1.5	1.0	–	3.0	2.5	3.5
	α	0.54	0.22	0.13	0.11	0.10	0.08	0.08

Table 4. τ_F and τ_R values (in ns) for an ACMA ethanol-water solution at pH 4 and $\theta = 55\,^\circ C$

λ [nm]		520	540	560
30 MHz	τ_F	0.2	0.2	0.2
	τ_R	18.5	18.4	18.9
18 MHz	τ_F	0.2	0.3	0.3
	τ_R	18.5	18.1	18.3

Table 3. Phase-modulation fluorescence data (τ_φ and τ_m in ns) for an ACMA ethanol-water solution at pH 4 and $\theta = 55\,^\circ C$

λ_{obs} [nm]	30 MHz			18 MHz			6 MHz		
	τ_φ	τ_m	$\frac{m}{\cos\varphi}$	τ_φ	τ_m	$\frac{m}{\cos\varphi}$	τ_φ	τ_m	$\frac{m}{\cos\varphi}$
440	5.9	14.8	0.50	9.5	17.0	0.68	14.6	18.0	0.94
460	13.4	18.1	0.76	15.5	18.3	0.88	17.4	18.0	0.99
480	18.6	18.9	0.99	18.1	18.5	0.98	18.1	18.5	0.99
500	19.5	18.9	1.03	18.7	18.5	1.01	18.1	18.4	0.99
520	21.4	18.5	1.15	19.8	18.5	1.06	18.2	19.0	0.99
540	21.9	18.4	1.18	19.9	18.1	1.08	18.6	18.1	1.01
560	22.0	18.9	1.15	19.8	18.3	1.07	18.4	18.9	0.99

rescence lifetimes of the F and R states and could be written as $\tau_F = \frac{1}{k_F + k_{FR}}$, $\tau_R = \frac{1}{k_R}$ where k_F and k_R represent the sum of all deactivation rate constants and k_{FR} represents the rate constant for the process leading to population of the R excited state (see Fig. 4).

The τ_R value was determined to be 19.0 ± 0.5 ns, i.e. the mean of the τ_φ and τ_m values obtained at 500 nm. A theoretical study of the variations of τ_φ and τ_m with α showed that in this case, $\alpha = \frac{\tau_F}{\tau_R}$ (Marty 1984; Marty et al. 1985). Equations (3) and (4) are then reduced to $\tau_\varphi = \tau_m = \tau_R$.

Using $\tau_R = 19.0$ ns, the τ_F value could be calculated from Eqs. (3) and (4); τ_F was determined to be 2.1 ± 0.9 ns (Table 2). Lastly, α values were calculated for each observation wavelength (Table 2). It is obvious that F and R emissions overlap throughout the entire fluorescence spectrum.

The influence of temperature on phase-modulation fluorescence data was studied. The results obtained in a water-ethanol solution at pH 4 and $\theta = 55\,^\circ C$ are listed in Table 3. Results obtained under these conditions could still be explained by an excited state process. However, at long wavelengths (520, 540 and 560 nm) the τ_φ, τ_m and $(m/\cos\varphi)$ values change in relation to ω but are independent

of the observation wavelength. Therefore, the fluorescence is only emitted by the R excited state ($\alpha = 0$) and the τ_φ, τ_m expressions can be written (Lakowicz and Balter 1982a; Marty 1984; Marty et al. 1985):

$$\tau_\varphi = \frac{\tau_F + \tau_R}{1 - \omega^2 \tau_F \tau_R} \tag{6}$$

$$\tau_m = (\tau_F^2 + \tau_R^2 + \omega^2 \tau_R^2 \tau_F^2)^{1/2} . \tag{7}$$

Consequently, τ_F and τ_R values could be calculated from these two equations. The results obtained are listed in Table 4.

4. Discussion

Phase-modulation measurements have shown that an excited state process is involved in the fluorescence decay of ACMA solutions at pH 4. Various mechanisms may be suggested, a priori, to explain this result.

First, because of the poor solubility of ACMA in water, the possibility of aggregate formation has to be considered. These aggregates do not exist in the ground state as proved by the linear plot of absorbance against ACMA concentration in the range $10^{-6} - 2 \times 10^{-4}$ mol · l^{-1} (Fig. 5). Moreover, absorbance did not change with ethanol concentration. Finally, the equilibrium constant for the monomer-dimer equilibrium of 9-aminoacridine, the parent compound of ACMA, has been found to be 10^3 mol · l^{-1} (Gangola et al. 1981). Therefore, at the concentration used in our experiments, it seems likely that ground state aggregates need not be taken into account. Consequently, the excited state process cannot be explained by monomer formation in the excited state occurring subsequent to aggregate formation.

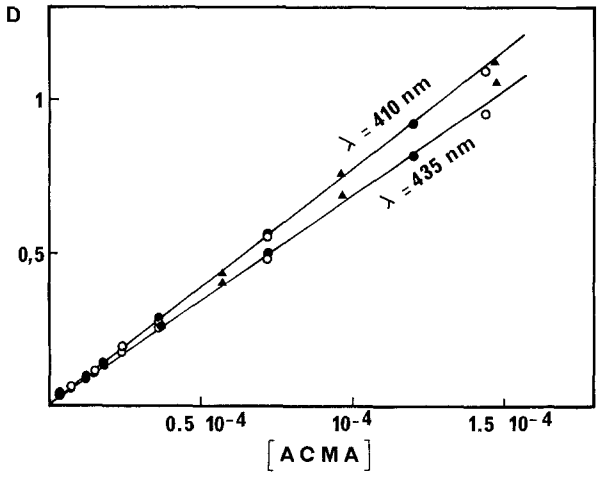


Fig. 5. Absorbance plotted against ACMA monocation concentration in 10% ethanol – 90% water (●); 20% ethanol – 80% water (○); 40% ethanol – 60% water (▲)

Further, the fluorescence excitation and emission spectral features were compared at different ACMA concentrations and for solutions of different ethanol content. The ratio of relative fluorescence emitted at two different wavelengths did not change. This observation precludes the possibility of excimer formation because this would result in increasing intensity on the low energy side of the spectrum.

A second mechanism that could be involved would be a proton transfer process in the excited state. In particular, this process could be the neutralization of M^* to N^*. Our titrimetric results exclude this possibility. In fact, the inflection point in the fluorometric titration curve corresponds approximately to a pH equal to the pK_a of the ground state $M \rightleftharpoons N$ process. This result means either that no proton transfer occurs in the excited state (from M^* to N^*) and then fluorescence measurements reflect what happens in the ground state, or this transfer does occur, but does not change the $M^* \rightleftharpoons N^*$ equilibrium, i.e. $pK_a^* = pK_a$. Then, as our measurements were performed at pH = 4, the excited state process revealed by phase fluorometry is not a proton transfer from M^* to N^*.

An excited state ionization could also take place at one of the other groups of the ACMA monocation, with the exception of chlorine. However this would occur between two states that emit exactly the same fluorescence intensity; that is possible, though rather unlikely. However our results can actually be explained in this way.

Nevertheless, this mechanism necessarily implies changes of charge distribution in the excited state; moreover, solvent is necessarily concerned with this process because of its proton donor character. With no data to show unambiguously what kind of mechanism is actually concerned in the excited state, this process will be considered as a "solvent cage reorientation", which has a broad meaning including all kinds of interaction between solvent and ACMA monocation in the excited state.

A reorientation of solvent molecules around the photoexcited ACMA monocations must be rigorously considered as a continuous relaxation mechanism (Bakhshiev et al. 1966; De Toma 1983). Nevertheless, experimental data cannot be analysed according to such a complex process. The theoretical study of Lakowicz and Balter (1982a) showed that these data can be analysed with a simplified model valuable for phase-modulation fluorometry experiments where only two excited states have to be considered (Lakowicz et al. 1980; Marty et al. 1985). The first excited state, called F, is in the same solvation condition as the ground state. It is directly photoexcited and emits fluorescence before any solvent shell relaxation occurs. For the second excited state called

Table 5. τ_F (in ns) and α values for an ACMA glycerol-water solution at $\theta = 21\,^{\circ}C$ and $\theta = -3\,^{\circ}C$

		440 nm	460 nm	480 nm	500 nm	520 nm	540 nm	560 nm
$\nu = 30$ MHz								
$\theta = 21\,^{\circ}C$	τ_F	3.0	4.5	5.0	4.0	5.0	5.5	–
	α	0.83	0.49	0.30	0.28	0.22	0.20	–
$\theta = -3\,^{\circ}C$	τ_F	5.5	8.5	–	7.5	9.0	10.0	10.0
	α	~1.00	0.74	0.50	0.49	0.45	0.42	0.40
$\nu = 18$ MHz								
$\theta = 21\,^{\circ}C$	τ_F	4.0	4.0	–	5.0	4.5	5.5	5.0
	α	0.80	0.50	0.32	0.28	0.21	0.20	0.18
$\theta = -3\,^{\circ}C$	τ_F	6.0	10.0	–	5.5	8.0	10.0	9.5
	α	1.00	0.69	0.50	0.48	0.45	0.41	0.38

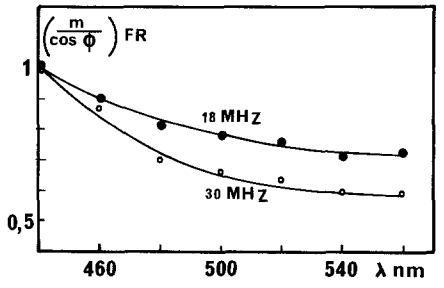


Fig. 6. $(m/\cos\varphi)_{FR}$ plotted against observation wavelength for the ACMA monocation in glycerol-water solution at $\theta = -3\,^{\circ}C$

R, relaxation is complete when fluorescence is emitted. Moreover, the R excited state is populated through F with a rate constant k_{FR}. Therefore, when the k_{FR} value is within the same timescale as other F excited state deactivation rate constants, F and R states can simultaneously emit fluorescence. This is what is observed for an ACMA monocation water-ethanol solution (Table 2). In more viscous media, the solvent shell relaxation rate constant is likely to decrease. In fact, α values are always higher (Table 5), i.e. the F contribution to the overall fluorescence increases. Also, the τ_F value increases from 4.5 ns at $\theta = 21\,^{\circ}C$ to 8.3 ns at $\theta = -3\,^{\circ}C$.

In contrast, when the temperature of the solution is raised to $55\,^{\circ}C$, k_{FR} is likely to increase. Then, α values are actually lower and become equal to 0 on the low wavenumber side of the spectrum, and the τ_F value is only 0.2 ns (Table 4). To confirm that a k_{FR} change was actually involved, fluorescence quantum yields were measured at $21\,^{\circ}C$ and $55\,^{\circ}C$ in water-ethanol solutions. The quantum yield values were $Q(21) = 0.325$ and $Q(55) = 0.355$. Now, fluorescence quantum yield can be written as:

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

where $k_{f,F}$ and $k_{f,R}$ are the fluorescence rate constants of the F and R excited states, independent of temperature (Birks 1970). So, we obtain:

$$\frac{Q(21)}{Q(55)}\,\frac{\tau_F(55)}{\tau_F(21)} = \frac{k_{f,F} + k_{f,R}\, k_{FR}(21)\, \tau_R(21)}{k_{f,F} + k_{f,R}\, k_{FR}(55)\, \tau_R(55)}\,. \tag{8}$$

When substituting the known values $Q(21) = 0.325$, $Q(55) = 0.355$, $\tau_F(21) = 2.1$ ns and $\tau_F(55) = 0.2$ ns it is obvious that expression (8) is less than unity. Moreover, $\tau_R(21) = 19$ ns and $\tau_R(55) = 18.5$ ns do not significantly differ. So it can be concluded that $k_{FR}(21) < k_{FR}(55)$: the effect of temperature is to increase the R state population rate constant, i.e. the solvent shell relaxation rate.

Our purpose here is to establish whether the excited state process is a single step or a continuum mechanism. In fact, since our experiments performed in a glycerol medium at $-3\,^{\circ}C$ showed a blue side spectral region where only the F state emitted light ($\alpha = 1$), it was possible to calculate the $(m/\cos\varphi)_{FR}$ ratio relative to the initially excited state. According to Lakowicz and Balter (1982a), if this ratio exceeds unity on the low wavenumber side of the spectrum, then the spectral relaxation must be the result of more than a single step process. The $(m/\cos\varphi)_{FR}$ ratios obtained in glycerol at $-3\,^{\circ}C$ were plotted against λ. As shown in Fig. 6, nowhere does $(m/\cos\varphi)_{FR}$ exceed unity. Lakowicz and Balter (1982b) have obtained similar results for 2-p-toluidinyl-6-naphthalene-6-sulfonic acid in propylene glycol at $-25\,^{\circ}C$, where solvent relaxation is very likely to occur. They suggest that this observation is a result of large spectral overlap of the emission from the excited states. The same situation may exist for the ACMA monocation. Finally, our measurements were used to resolve the individual spectra of the F and R states. Under all experimental conditions used, the R emission spectrum was about $600\ cm^{-1}$ red shifted compared to the F one; that corresponds to an energy difference around $1.7\ kcal \cdot mol^{-1}$ between these two states, i.e. of the order of magnitude of van der Waals bond energy. Similar results have been obtained previously for

the neutral ACMA molecule, though solvent relaxation was then complete in water-ethanol at 21 °C (Marty et al. 1985). So the additional proton of the ACMA monocation seems to reduce the rate of solvent cage reorientation around photoexcited states.

In conclusion, the analysis of ACMA monocation fluorescence by the phase modulation method reveals an excited state process; our results strongly suggest that the rate determining step of this process is a solvent cage reorientation.

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