STATIC AND TIME-RESOLVED FLUORESCENCE OF AN AMPHIPHILIC FLAVIN IN AEROSOL OT REVERSED MICELLES

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Abstract—Spectral and time-resolved fluorescence studies have been carried out on N(3)-undecyllumi-flavin dispersed in reversed micelles composed of the surfactant sodium di(2-ethylhexyl) sulfosuccinate (Aerosol OT, AOT), various amounts of water and *n*-heptane as continuous phase. The fluorescence spectral properties (spectral distribution, quantum efficiencies and lifetimes) as function of the water to AOT molar ratio suggest that the flavin occupies a position within the surfactant boundary layer in close contact with water. The fluorescence anisotropy exhibits biexponential decay with a short (0.3 ns) and a longer (1.6–2.4 ns) correlation time. The contribution of the short component increases with the growth of the droplet providing evidence for enhanced flexibility of the flavin in the interfacial layer.

INTRODUCTION

Optically transparent, water containing, reversed micelles are very attractive media for the encapsulation of biopolymers (Fendler, 1982; Luisi, 1985; Martinek et al., 1986; Vos et al., 1987a). The size of the water droplet, coated by surfactant, can be such that, for instance, proteins can be encased. A variety of spectroscopic techniques have been applied to reversed micelles in order to establish and quantitate hydrodynamic properties when certain parameters are varied, e.g. the water to surfactant molar ratio (for a survey see Luisi and Straub, 1984). With fluorescence spectroscopy preferential use has been made with probes which are only soluble in the water droplet [Eicke and Zinsli, 1978; Keh and Valeur, 1981; Visser et al., 1984; Luisi and Straub, 1984 (loc cit.)].

Amphiphilic, fluorescent probes have been widely employed in normal, micellar systems and membrane bilayers. Their use in reversed micelles has been rather limited thus far (Rodgers, 1984). In this communication the steady-state and time-resolved fluorescence behaviour of an amphiphilic flavin (N(3)-undecyl-lumiflavin, Fig. 1), solubilized in reversed micelles of AOT (Aerosol OT, sodium di(2-ethylhexyl) sulfosuccinate), is described. This molecule is unique since it contains a polar part and a long alifatic, strictly nonpolar portion. Amphiphilic flavins have been proven very appropriate compounds for probing membrane microenvironments (Schmidt, 1981 and references cited therein),

in Shpolskii spectroscopy (Platenkamp et al., 1980 and 1982) and in dynamic studies of membrane bilayers (Visser, 1982).

The work described in this paper is an extension of a previous study on the fluorescence properties of FMN encapsulated in reversed micelles (Visser et al., 1984). Evidence will be presented that the amphiphilic flavin is flexibly located in the boundary layer between the water droplet and the organic solvent.

MATERIALS AND METHODS

N(3)-undecyllumiflavin has been synthesized according to Michel and Hemmerich (1981). Aerosol OT was purchased from Fluka (Buchs, Switzerland) and purified according to Menger and Yamada (1979). n-Heptane was analytical grade (Merck, Darmstadt, W. Germany). Reversed micelles were prepared as described previously (Visser et al., 1984). The final concentration of AOT was always 67 mM and the final flavin concentration 1.0 μ M. The water content, defined as $w_0 = [H_2O]/[AOT]$, was varied between 0.8 and 33.

Fluorescence spectra were recorded on an Aminco SPF-500 (SLM-Aminco, Urbana, IL, USA) fluorimeter. Timeresolved fluorescence was measured with the mode-locked argon ion laser and single-photon counting set-up as described previously (van Hoek and Visser, 1985). The 457.9 nm laser line was used for excitation and the emission was selected with a KV 500 Schott (Mainz, W. Germany) filter. Data analysis was performed as described previously (Visser et al., 1984) and with a reference method published elsewhere (Vos et al., 1987b). In the fluorescence decay analysis use was made of FMN in water as a reference compound having a single fluorescence lifetime ($\tau = 4.7$ ns, Spencer and Weber, 1972; Visser et al., 1984). The fluorescence decay of FMN was accumulated under the same conditions as for the amphiphilic flavin. The method works very well provided that the decay parameters of the sample to be measured do not contain the 4.7 ns component of the reference compound. The latter condition is fulfilled as will be shown below. All experiments were carried out at 20°C.

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Figure 1. Structure of N(3)-undecyllumiflavin.

RESULTS AND DISCUSSION

Stationary fluorescence properties

Fluorescence spectra of N(3)-undecyllumiflavin in n-heptane and AOT reversed micelles (w_0 is 0.82 and 16.5) are shown in Fig. 2A. At low water content it is evident that the fluorescence maximum is located near 20 500 cm⁻¹, which is close to the

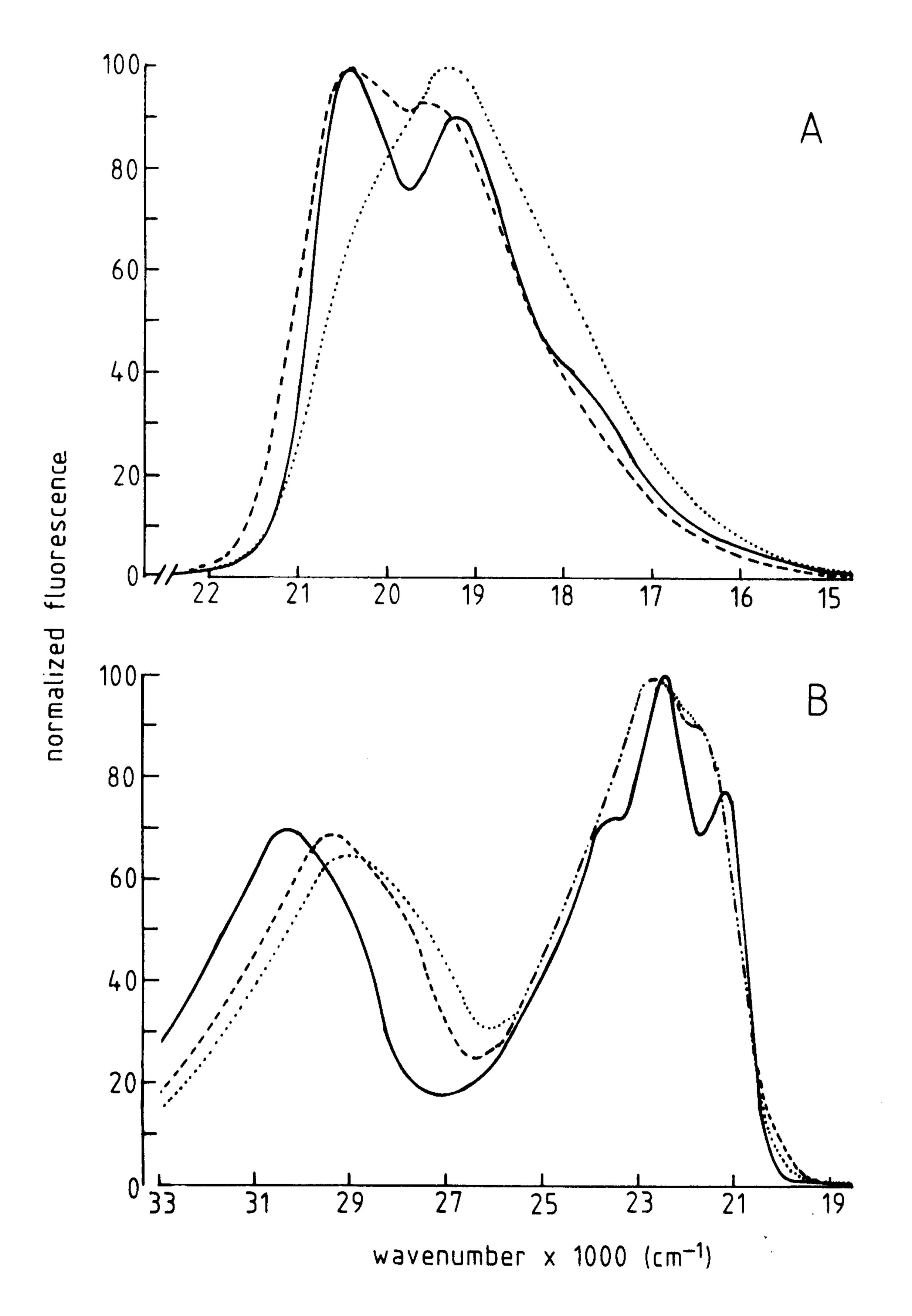


Figure 2. Peak normalized fluorescence (A) and excitation (B) spectra of N(3)-undecyllumiflavin in *n*-heptane and AOT entrapped water pools. (A) Excitation wavelength 430 nm, slits 4 nm. (B) Emission wavelength 550 nm, slits 4 nm. — *n*-heptane, $---w_0 = 0.82$, $w_0 = 16.5$.

0, 0-vibronic transition (0, 0 refers to the electronic transition from the zeroth vibrational level in the ground state to the zeroth vibrational level in the first excited singlet state). Previous studies (Eweg et al., 1979) have shown that flavin compounds dissolved in apolar solvents have most of their fluorescence intensity in the 0,0 transition. A change into a more polar solvent enhances the 1,0 transition relative to the 0,0 transition. The latter effect is also observed in the reversed micelles when the water content increases. The change is quite sudden. At $w_0 > 4$ the emission maximum remains constant at about 19 400 cm⁻¹ (data collected in Table 1). Such an observation implies that the flavin comes into contact with mobile water molecules.

Similar changes are observed in the excitation spectra of N(3)-undecyllumiflavin in n-heptane and in AOT reversed micelles of increasing water content (Fig. 2B). At low w_0 the first absorption band exhibits the characteristic fine structure, which is apparent in apolar flavin solutions (Koziol, 1969; Eweg et al., 1979). Between $w_0 = 2$ and $w_0 = 4$ the shape of the excitation spectrum alters. Above $w_0 = 4$ the excitation spectrum does not change. The location of the second absorption band at 28 570 cm⁻¹ ($w_0 > 2$) is clearly different from the position at 27 830 cm⁻¹ for a comparable flavin compound dissolved in water.

Time-resolved fluorescence

The fluorescence decay of N(3)-undecyllumiflavin in AOT reversed micelles is nonexponential and a biexponential fit to two lifetimes is a minimum hypothesis (Fig. 3A). The long component (8–9 ns) with a relatively small weight can be observed up to $w_0 = 4$ (see Table 1). At $w_0 > 4$ the long lifetime component gets shorter, but its relative weight increases. Therefore the average lifetime increases slightly. Model flavins dissolved in apolar solvents give relatively long lifetimes in agreement with enhanced quantum yields (Visser and Müller, 1979; Eweg et al., 1979). Shorter fluorescence lifetimes are associated with flavins in more polar solvents. They are also observed when FMN is encapsulated in micellar water pools of different surfactants (Visser et al., 1984). The abrupt change in lifetime values and relative weights at $w_0 > 4$ indicates a change in flavin surrounding medium of similar nature as found for the spectral results. The complex fluorescence decay kinetics must be due to a heterogeneous microenvironment.

In Table 1 we have also collected time-resolved fluorescence data on the flavin compound in n-heptane. A two-component analysis yielded a very short picosecond lifetime component and a long lifetime of 8.6 ns. The short lifetime can be ascribed to self-quenching of flavin aggregates, present even at 1.0 μM . The presence of stacked aggregates or

w_0	α_1	$\tau_1 \ (\pm \ 0.1)$ (ns)	$lpha_2$	$\tau_2 \ (\pm \ 0.1)$ (ns)	τ* (ns)	q _{rei} †	F_{max} ‡ (cm $^{-1}$)
0.82	0.81	4.1	0.19	8.6	4.9	1.0	20 470
2.1	0.76	3.9	0.24	8.1	4.9	1.1	20 380
4.1	0.83	4.4	0.17	9.2	5.2	1.2	19 380
8.2	0.49	2.9	0.51	7.2	5.1	1.2	19 380
16.5	0.38	3.2	0.62	6.8	5.4	1.2	19 380
33.0	0.32	3.4	0.68	6.5	5.5	1.2	19 380
8	0.93	<50 ps	0.07	8.6	0.6	0.9	20 470

Table 1. Fluorescence parameters of N(3)-undecyllumiflavin in AOT reversed micelles in n-heptane and in n-heptane at 20° C

^{*}Average lifetime $\bar{\tau} = \alpha_1 \tau_1 + \alpha_1 \tau_2$. †Relative quantum efficiency.

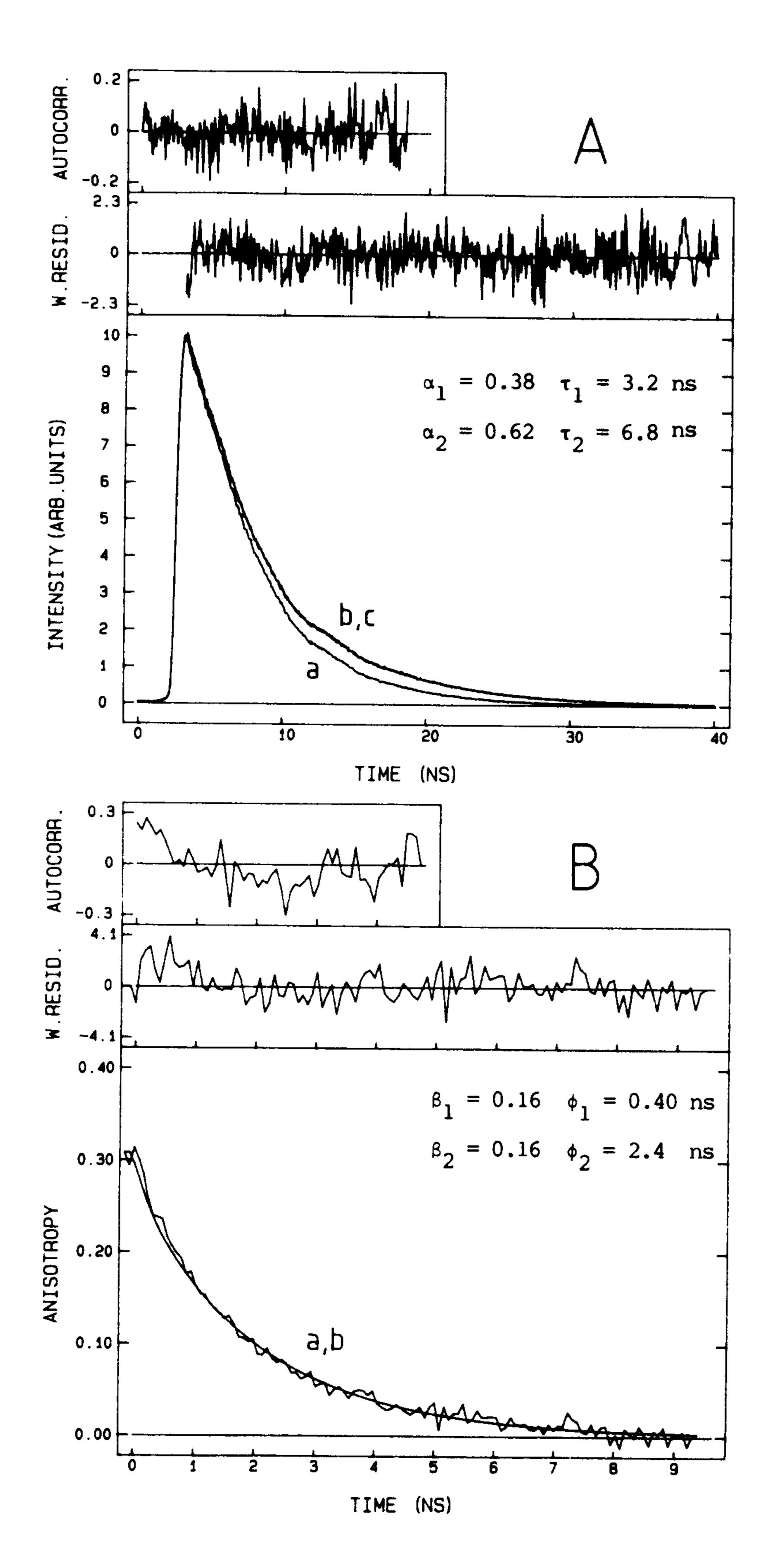


Figure 3. Fluorescence (A) and fluorescence anisotropy (B) decay of N(3)-undecyllumiflavin in AOT reversed micelle entrapped water $(w_0 = 16.5)$. (A) Shown are three curves: the fluorescence decay of FMN (a) and the experimental (b) and calculated (c) fluorescence response curves of N(3)-undecyllumiflavin. The decay parameters (τ) and relative amplitudes (α) of the double exponential decay model are listed. The quality of the fit is good as judged from the plots of the weighted residuals and autocorrelation function of the residuals (top part), which fluctuate around the baseline. (B) Shown are two curves: the experimental (a) and calculated (b) fluorescence anisotropy decay. The correlation times (φ) and relative amplitudes (B) associated with a biexponential decay model are listed. The decay analysis was extended over the whole fluorescence decay curve (see A), but only the initial anisotropy is presented. Weighted residuals and autocorrelation function of the residuals are shown on top.

‡Fluorescence emission maximum.

microclusters in amphiphilic flavins has been demonstrated by Eweg et al. (1979) and Shinkai et al. (1981). As demonstrated by Shinkai and coworkers deaggregation is induced by aqueous solutions of surfactants above the critical micelle concentration. The pronounced shoulder at $21\ 000\ \text{cm}^{-1}$ in the excitation spectrum of the flavin in n-heptane (Fig. 2b) may be in part due to aggregated flavin.

The initial fluorescence anisotropy decay pattern exhibits characteristic changes when the water content varies. The anisotropy decays as a double exponential function with a shorter (0.2-0.4 ns) and a longer (1.7-2.4 ns) characteristic time constant (Fig. 3B). At increasing w_0 the contribution of the short component increases. The short component in the anisotropy decay must be ascribed to a rapid restricted motion of the flavin. The correlation times obtained from analysis of the anisotropy decay are collected in Table 2. The integrated anisotropy (Table 2) also decreases at $w_0 > 4$ indicating the presence of some rapid depolarizing rotation.

In Table 2 we also incorporated the cone angle θ_c that can be derived from a wobbling-in-cone model (Lipari and Szabo, 1980). In the latter model it is assumed that the probe has an unique axis with either the absorption or the emission transition moment pointing along it. This symmetry axis "wobbles" in a cone of semiangle θ_c . Following Lipari and Szabo the cone angle θ_c can be obtained from biexponential anisotropy decay $r(t) = \beta_1$ $\exp(-t/\phi_1) + \beta_2 \exp(-t/\phi_2), \text{ with } \beta_1 + \beta_2 = r(0);$ φ₂ is the characteristic time constant for overall micellar rotation and ϕ_1 is a composite correlation time, $1/\phi_1 = 1/\phi_2 + 1/\phi_i$, where $1/\phi_i$ is related to the wobbling diffusion coefficient, r(0) is the anisotropy at zero time. From the amplitude of the micellar rotation, β_2 , the cone angle can be obtained as explained in the legend of Table 2. The angles are rather high and are indicative of a large amplitude motion. The direction of the first transition moment in the plane of the flavin molecule has been determined (Johansson et al., 1979). It makes an angle of 58° measured counterclockwise with respect to the axis connecting the two nitrogens in the central

 $^{^{\$}}N(3)$ -Undecyllumiflavin in *n*-heptane.

w_0	$\beta_1 \ (\pm \ 0.01)$	$\phi_1 \ (\pm \ 0.05)$ (ns)	$\beta_2 (\pm 0.01)$	$\phi_2 \ (\pm \ 0.1)$ (ns)	*	θ_c †	ф _т ‡ (ns)
0.82	0.08	0.39	0.26	1.7	0.074	24	1.7
2.1	0.14	0.23	0.20	2.1	0.080	33	2.5
4.1	0.14	0.20	0.20	2.1	0.078	33	4.2
8.2	0.16	0.34	0.16	2.3	0.070	38	9.7
16.5	0.16	0.40	0.16	2.4	0.067	38	32.4
33.0	0.18	0.34	0.14	2.4	0.067	41	148.0
§	0.28	<20 ps	0.08	2.5	0.070		

Table 2. Fluorescence anisotropy parameters of N(3)-undecyllumiflavin in AOT reversed micelles in n-heptane and in *n*-heptane at 20°C

ring (see Fig. 1).

The longer correlation times are distinctly shorter than can be calculated from the hydrated radius of the micellar particles (ϕ_m , Table 2). The latter observation is in our opinion an illustration of the fact that the motion of the flavin in the micellar boundary is complex and must arise from several contributions. A pictorial model, in which both flavin and side chain motions are involved, has been proposed earlier (Visser, 1982). A schematic view of the localization of the amphiphilic flavin within the boundary layer of AOT is presented in Fig. 4.

From the results in Table 2 it is clear that the anisotropy decay of the amphiphilic flavin in n-

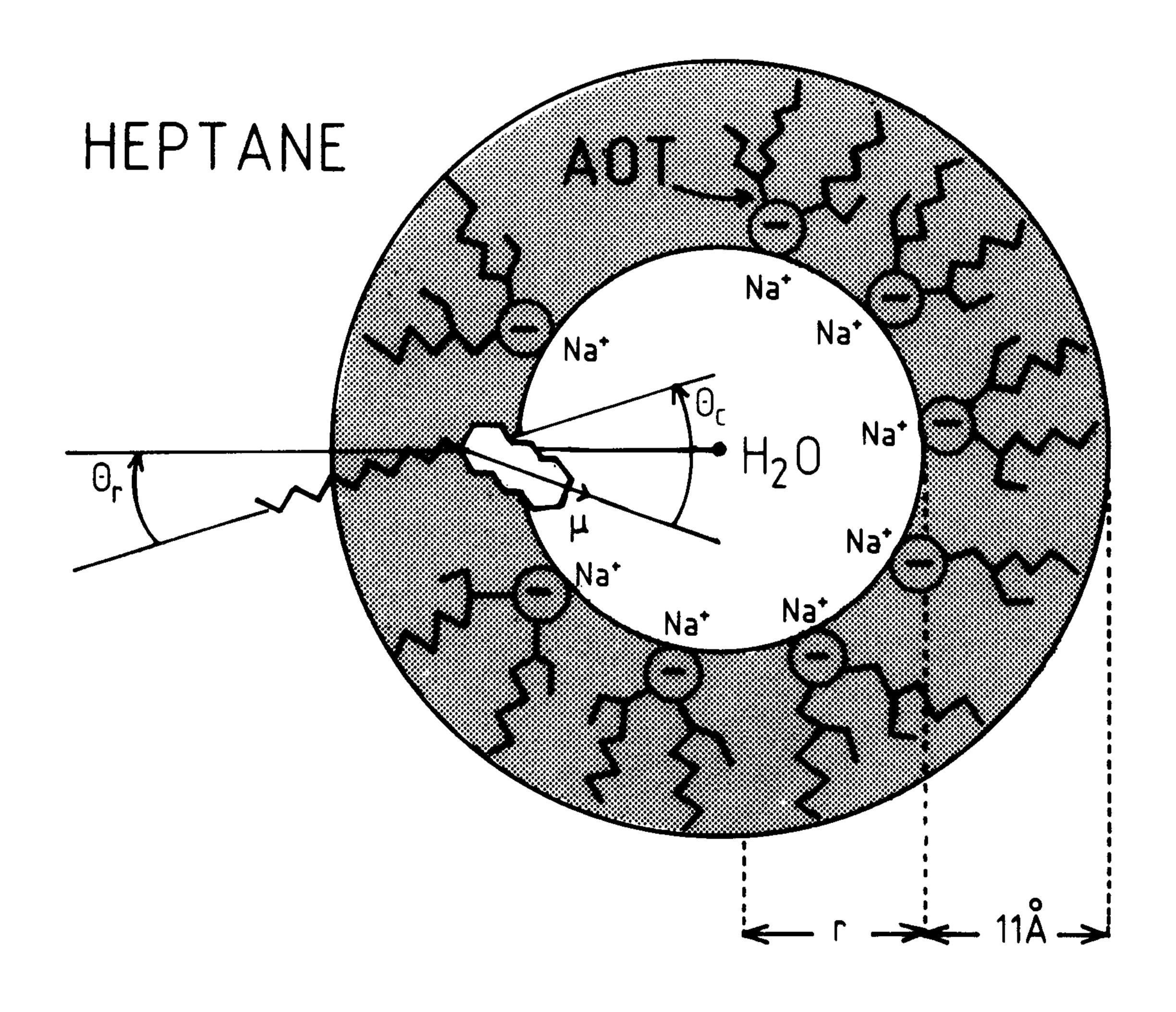


Figure 4. Schematic representation of N(3)-undecyllumiflavin in AOT reversed micelles. The flavin is depicted as a triple hexagon. The transition moment μ of the flavin is the vector, which rotates conically with semiangle θ_c around the long alifatic chain, which in turn performs a restricted rotation around the micellar radius (semiangle

picosecond correlation time must be originated from a rapid depolarization process caused by energy transfer among flavins in the microcluster and is of similar nature as found for biflavinyl compounds (Visser et al., 1983). The longer correlation time can be associated with rotation of the whole microcluster of flavin molecules.

Comparing the correlation times of FMN inside the AOT wter droplet (Visser et al., 1984) with those of the amphiphilic flavin, it is evident that the correlation time of FMN shortens at increasing w_0 , while the longer correlation time of the amphiphilic flavin tends to lengthen slightly. The latter tendency is also observed for FMN in dodecyl ammonium heptane is biexponential as well. The very short propionate (DAP) reversed micelles (Visser et al., 1984). FMN is located near the interface region in DAP reversed micelles.

> Summarizing, probes in the aqueous micellar core rotate differently as compared to probes located in the interface of reversed micelles.

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^{*}Integrated anisotropy $\bar{r} = \int_0^\infty r(t)F(t)dt/\int_0^\infty F(t)dt$

F(t) = fluorescence intensity.

[†]From $\beta_2/(\beta_1 + \beta_2) = \left[\frac{1}{2} \cos \theta_c (1 + \cos \theta_c)\right]^2$

[‡]From $\phi_m = 4\pi R_h^3 \eta/(3kT)$; hydrodynamic radii R_h are obtained from the relation R_h (nm) = 0.175 $w_0 + 1.5$ (Nicholson and Clarke, 1984); η (n-heptane) = 40×10^{-4} kg m⁻¹ s⁻¹ at T = 293 K (Weissberger et al., 1955). $^{8}N(3)$ -Undecyllumiflavin in *n*-heptane.

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