EMISSION WAVELENGTH-DEPENDENT DECAY OF THE 9-ANTHROYLOXY-FATTY ACID MEMBRANE PROBES

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ABSTRACT Using the phase-modulation technique, we have measured the fluorescence decay of 2- and 12-(9-anthroyloxy)-stearic acid (2- and 12-AS) and 16-(9-anthroyloxy)-palmitic acid (16-AP) bound to egg phosphatidylcholine vesicles or dissolved in nonpolar solvents. Heterogeneity analysis demonstrates that the decay is generally not monoexponential and exhibits large component variations across its emission spectrum. The mean decay time increases (and in parallel, the steady-state polarization decreases) monotonically with increasing wavelength from values at the blue end. The decay at the red side of the emission spectrum contains an exponential term with a negative amplitude, indicating that emission occurs from intermediates created in the excited-state. This behavior is interpreted as arising from intramolecular fluorophore relaxation occuring on the time scale of the fluorescence lifetime. We believe this to be the first study of wavelength-dependent fluorescent emission which is dominated by an intramolecular relaxation process. Although the three probes exhibit qualitatively similar effects, the emission band variations are greatest for 2-AS and smallest for 16-AP. The differences among the probes are not entirely due to environmental factors as demonstrated, for example, by the emission polarization differences observed in the isotropic solvent paraffin oil. In summary, while these findings point out some of the complexities in the 9-anthroyloxy-fatty acids as membrane probes, they also indicate how these complexities might be used as a sensitive measure of lipid-probe interaction.

INTRODUCTION

In recent years, fluorescent probes have been widely used in studying bilayer structure in both model and biological membranes (Radda and Vanderkooi, 1972; Radda, 1975; Azzi, 1975; Badley, 1976; Shinitzky and Barenholz, 1978). The anthroyloxy-fatty acid derivatives (Waggoner and Stryer, 1970; Bashford et al., 1976; Cadenhead et al., 1977; Shaklai et al., 1977; Thulborn et al., 1978*a*, *b*) offer a number of advantages as membrane probes. In particular, they appear to partition into model membranes with the probe fatty acid chain aligned parallel to the phospholipid acyl chains and the anthroate group positioned at a defined depth in the bilayer (Podo and Blasie, 1977). A series of derivatives with the anthracene group covalently attached at different points along the chain appears to offer the possibility of studying the "fluidity gradient" in model and biological membranes (Thulborn et al., 1978*a*, *b*). As a result of their well-defined location at various depths in the bilayer, it has also been suggested that they might be used as acceptors or quenchers in determining the location of tryptophan residues in membrane proteins (Kleinfeld et al., 1979; Haigh et al., 1979).

In the present study,[1] we show that these anthroyloxy-fatty acid derivatives are characterized by fluorescence decay kinetics that vary as a function of wavelength across the emission band. Unlike the case for fluorophores dissolved in viscous polar solvents or located at the polar membrane-water interface, such as 1-anilinonaphthalene-8-sulfonate (ANS)[2] and 2-(*p*-toluidino) naphthalene-6-sulfonate (TNS) (Chakrabarti and Ware, 1971; DeToma et al., 1976; Easter et al., 1976, 1978) or *N*-phenyl-2-naphthylamine (2-NPN) (Badea et al., 1978) and *N*-phenyl-1-naphthylamine (1-NPN) (Matayoshi, 1979), this effect appears to be due to intramolecular relaxation of the fluorophore during its excited-state lifetime. These results demonstrate the importance of fluorophore molecular structure in interpreting the fluorescence in terms of environmental influences, and in particular, have implications for the use of these compounds as membrane probes.

MATERIALS AND METHODS

The fluorescent probes 2 and 12-(9-anthroyloxy) stearic acid (2-AS and 12-AS), and 16-(9-anthroyloxy) stearic acid (16-AP) were obtained from Molecular Probes (Plano, Texas). Absorption and fluorescence spectral measurements and thin layer chromatography demonstrated the absence of any fluorescent impurities. Paraffin oil (Uvasol grade) from E. Merck (Darmstadt, West Germany), and triolein from Nu-Chek Prep, Inc. (Elysian, Minn.) were used without further purification. Egg phosphatidylcholine (PC) was obtained from two sources, both of which yielded identical results. It was initially prepared from hen eggs by the method of Litman (1973), and shown to be > 99% pure by thin layer chromatography. PC was subsequently obtained from Makor Chemicals (Jerusalem, Israel), and its purity was also found to be > 99% by the same method. Cyclohexane (spectro grade) and decane (Gold Label) were products of Aldrich Chemical Co., Inc. (Milwaukee, Wis.), and glycerol (spectro grade) and methanol (spectro grade) were obtained from Fisher Scientific Co. (Pittsburgh, Pa). All other solvents and chemicals were of the highest purity commercially available.

Small unilamellar PC vesicles were prepared by sonication of lipid dispersions using the method of Huang and Thompson (1974). Lipid that had been stored at -20°C in chloroform or ethanol was lyophilized and suspended in buffer (20 mM Tris-HCl, 50 mM NaCl, pH 7.4) at a concentration of ~ 30 mM phospholipid. This suspension was sonicated for 1 h at 4°C under nitrogen using a Branson sonicator set at ~ 70 W (Branson Sonic Power Co., Danbury, Conn.). Titanium particles were removed by centrifugation in a Sorvall SS-34 rotor at 17,000 rpm for 45 min. (DuPont Co., Instrument Products Div., Sorvall Biomedical Div., Wilmington, Del.), and the vesicles were stored under nitrogen at 0°–4°C. The vesicles were not further chromatographed (e.g., on Sepharose 4B) and are therefore a mixture of small (~250Å Diam) vesicles and a small amount (probably < 5%) of larger multilamellar liposomes. Lipid concentration was determined as inorganic phosphate by the method of Gomori (1942).

To incorporate the fluorescent probes into lipid vesicles, concentrated stock solutions of probe were added while vortexing a 1–3 mM (phospholipid) suspension of vesicles. Stock solutions of the anthroyloxy-fatty acids were prepared in either ethanol or *N,N*-dimethylformamide at concentrations ranging from 0.01 to 0.1 M. The final concentration of solvent in the labeled vesicle suspension was kept

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[1]These results were reported in preliminary form at the Biophysical Society meeting in Atlanta, Ga. 1979. *Biophys. J.* 25:168*a*.

[2]Abbreviations used in this paper: ANS, 1-anilinonaphthalene-8-sulfonate; TNS, 2-(*p*-toluidino) naphthalene-6-sulfonate; 2-NPN, *N*-phenyl-2-naphthylamine; 1-NPN, *N*-phenyl-1-naphthylamine; 12-AS, 2-AS, n-AS are, respectively, 12-, 2-, and n-(9-anthroyloxy)-stearic acid; 16-AP, 16-(9-anthroyloxy)-palmitic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; PC, egg phosphatidylcholine; S_0, ground state; S_1^{FC}, first excited singlet state, Franck-Condon geometry; S_1^{EQ}, first excited singlet state, equilibrium geometry; 11-AU, 11-(9-anthroyloxy)-undecanoic acid.

< 0.2% (vol/vol), and the solvent used, at least at these concentrations, had no effect on the results. Since the probes exhibit very low quantum efficiencies in water, the time-course of their uptake into vesicles can be followed by steady-state fluorescence; equilibrium was reached within seconds. At the lipid-to-probe ratios at which these measurements were performed (>500:1) essentially all of the probe is bound to the vesicles.

Fluorescence Measurements

Fluorescence lifetimes were determined by the phase-modulation technique, in an instrument built by SLM Instruments (Urbana, Ill.). Steady-state polarizations were measured in the same apparatus, using Glan-Thompson polarizers in the excitation and emission channels. Monochromators were used for both excitation and emission in all steady-state and lifetime measurements. Most of the lifetimes in the present work were initially obtained with modulation frequencies of 10 and 30 MHz, but some were re-measured at 30, 18, and 6 MHz after the instrument was rebuilt. An excitation polarizer set at 35° from the vertical was used in all lifetime measurements in order to eliminate the effects of Brownian rotations (Spencer and Weber, 1970). Measurements were also made using a blank containing all components except the probe, and when necessary, appropriate corrections for scattering contributions were applied to lifetimes and polarizations.[3]

The theory of phase and modulation fluorometry has been discussed in detail before (Spencer and Weber, 1969; Birks, 1970; Spencer, 1970). For a homogeneous system with single exponential decay, the lifetimes determined by either phase delay (τ_p) or relative modulation (τ_m) are equal at all frequencies. When the emission is given by a sum of exponentials, the observed τ_p and τ_m differ and are given by:

$$\tau_p = w^{-1} \tan \Phi \tag{1}$$

$$\tau_m = w^{-1}(M_r^{-2} - 1)^{1/2} \tag{2}$$

$$\tan \Phi = \frac{\sum_{i-1}^{n} \alpha_i \sin \phi_i \cos \phi_i}{\sum_{i-1}^{n} \alpha_i \cos^2 \phi_i} \tag{3}$$

$$M_r^2 = \left(\sum_{i-1}^{n} \alpha_i \sin \phi_i \cos \phi_i\right)^2 + \left(\sum_{i-1}^{n} \alpha_i \cos^2 \phi_i\right)^2 \tag{4}$$

in which $w = 2\pi f$ (f = modulation frequency), Φ and M_r are the observed phase angle and relative modulation, ϕ_i is the phase angle of the ith component with fluorescence lifetime τ_i ($\tau_i = w^{-1} \tan \phi_i$), and α_i is the fractional steady-state intensity ($0 \leq \alpha_i \leq \alpha_i \leq 1$) due to the ith component ($\Sigma_{\alpha_i} = 1$). From these Eqs., it follows that $\tau_m > \tau_p$ at all frequencies (Spencer and Weber, 1969; Spencer, 1970). Under optimal conditions, if a system containing n exponential components is measured at n modulation frequencies, the complete set (α_i, τ_i) will be obtainable: Eqs. 3 and 4 represent $2n$ Eqs. in ($2n - 1$) unknowns.

When an excited-state reaction or relaxation process is present and new species or intermediates are created that emit at red-shifted wavelengths, the decay kinetics will vary across the emission spectrum, with the mean decay time increasing with increasing wavelength. If observed directly by the nanosecond pulse technique, the fluorescence intensity at the red side of the emission spectrum is seen to build up before it decays. Such behavior is indicated, in the multiexponential expression for intensity [$I(t)$], by the presence of a term with a negative amplitude (Birks, 1970; Loken et al., 1972; Grinvald and

[3]The corrections for scattering and the color effect will be detailed in a subsequent publication on the analysis of phase modulation-derived lifetimes (A. M. Kleinfeld and E. D. Matayoshi, manuscript in preparation). These two effects, which tend to cancel one another, are not very large in the present case and do not affect the conclusions.

Steinberg, 1974; DeToma et al., 1976):

$$I(t) = \sum_{i=1}^{n} A_i \exp(-t/\tau_i) \tag{5}$$

where the A_i are not limited to positive values. The fractional intensities (α_i) in Eqs. 3 and 4 are related to these pre-exponential amplitudes (A_i) by the expression $\alpha_i = A_i \tau_i / \Sigma A_i \tau_i$. When a system with response function given by Eq. 5 is observed (in which some $A_i < 0$) using phase-modulation fluorometry, the expression for the observed phase and modulation remain as given in Eqs. 1–4, but some of the α_i may be negative with $\Sigma\alpha_i = 1$ (Matayoshi, 1979) and hence the α_i no longer represent fractional intensities. In this case τ_m and τ_p can satisfy less restrictive conditions than when the decay corresponds to ordinary heterogeneity ($A_i > 0$ in Eq. 5): (*a*) τ_p can exceed τ_m; (*b*) τ_p can assume negative values; (*c*) τ_p or τ_m may increase with increasing frequency. Since any of these conditions is contrary to the behavior of τ_p and τ_m for ordinary heterogeneity, their observation alone is sufficient (but not necessary) to demonstrate the presence of an excited-state reaction. For two-state systems in which a spectral red shift accompanies the excited-state reaction, it is predicted that (*a*) the condition $\tau_p > \tau_m$ may occur at the longer emission wavelengths, (*b*) $\tau_p > \tau_m$ will characterize the decay at the shorter emission wavelengths, and (*c*) $\tau_p = \tau_m$ (apparent homogeneity) will be observed at some intermediate wavelength (Matayoshi, 1979; Lakowicz et al., 1980).

In this context, it is important to point out that the phase lifetimes determined with the SLM fluorometer (as equipped with EMI 9813 and 9814 photomultiplier tubes; EMI Gencom Inc., Plainview, N.Y.) must in general be corrected because the phase response of this instrument is a function of the wavelength of the detected light. This problem is in part a consequence of the wavelength dependence of the photo-electron transit time (Müller et al., 1965), and the magnitude of this dependence is in turn a function of photomultiplier tube voltage and modulating frequency. We have found that, in contrast, the modulation lifetimes determined in this instrument are not wavelength dependent, and hence can serve under certain conditions as a check on the calibration of the phase measurements. All τ_p data presented here have been corrected for the transit-time effect (Matayoshi, 1979).[3] Employing these correction factors we have found that the membrane probes 1-NPN (in methanol, ethanol, cyclohexane, paraffin oil) and DPH (in all solvents examined) exhibit constant and homogeneous lifetimes as a function of emission wavelength (Matayoshi, 1979). The emission bands of these probes cover a spectral range (390–550 nm) that encompasses the emission band of the 9-anthroyloxy derivatives, and thus demonstrate that these correction factors are appropriate for the AS derivatives.

The τ_p and τ_m data, obtained at two or three modulation frequencies, were analyzed for two or three exponential components by a procedure in which χ^2, defined by

$$\chi^2 = \sum_{w} \left(\frac{\tau_p' - \tau_p}{\sigma_p}\right)^2 + \left(\frac{\tau_m' - \tau_m}{\sigma_m}\right)^2 \tag{6}$$

is minimized by varying the α_i and τ_i. τ_p' and τ_m' are the theoretical phase and modulation lifetimes calculated according to Eqs. 1–4. σ_p and σ_m are the estimated standard deviation of the measured lifetimes and the sum is performed over all modulation frequencies at which data were obtained.[3]

The steady-state polarization, p, was measured according to the definition:

$$p = \frac{(I_\parallel - I_\parallel^B) - (I_\perp - I_\perp^B)}{(I_\parallel - I_\parallel^B) + (I_\perp - I_\perp^B)} \tag{7}$$

in which $I_\parallel$ and $I_\parallel^B$ are the parallel polarized intensities corresponding to sample and blank, respectively, and $I_\perp$ and $I_\perp^B$ the analogous perpendicular polarized intensities. The polarized intensities were measured by fixing the emission polarizer in the vertical orientation while moving the excitation polarizer between the vertical and horizontal orientations to obtain the parallel and perpendicular components, respectively. The intensities in Eq. 7 were corrected for the difference in the intensity of the vertically and horizontally polarized exciting light.

RESULTS

Solvent Studies

The emissive properties of 12-AS were investigated in two viscous nonpolar solvents, triolein and paraffin oil. These solvents were chosen as isotropic analogues to the hydrophobic region of egg lecithin bilayers. As shown in Fig. 1 *A*, the steady-state polarization of 12-AS dissolved in triolein varies as a function of wavelength across its emission spectrum. At O°C, p decreases monotonically from a value of 0.279 at 400 nm, to 0.169 at 550 nm. Similar decreases in p of $\sim$ 40% were observed at temperatures of 20° and 40°C. Fig. 1 *B*, which shows that the values of τ_m determined at 30 MHz increase monotonically with increasing wavelength, demonstrates that polarization and lifetime are inversely related. The values of τ_p at 30 MHz and τ_m and τ_p at 10 MHz (not shown) also increase across the emission spectrum, and their large differences (up to 3-ns splitting between τ_m and τ_p at each frequency) indicate considerable decay heterogeneity. At 30 MHz, it was observed that $\tau_m > \tau_p$ on the blue side, but $\tau_p > \tau_m$ on the red side ($\lambda \geq 450$ nm) which, as discussed under Materials and Methods, is indicative of an excited-state reaction.

Previous studies of emission wavelength-dependent fluorescence decay were performed in viscous polar solvents (such as glycerol) with fluorophores that undergo a change in dipole moment in the excited-state (Chakrabarti and Ware, 1971; DeToma et al., 1976; Easter et al., 1976, 1978; Badea et al., 1978; Matayoshi, 1979). The origin of the emission wavelength variations in these studies was attributed to dielectric relaxation or solvent-probe complexing. We have found that in triolein the fluorophore l-NPN also exhibits an increase in mean decay time across the emission spectrum (Matayoshi and Kleinfeld, manuscript in preparation), but that the decay of DPH, which probably remains nonpolar in its excited-state, is monoexponential and constant across its emission spectrum (Matayoshi, 1979). Since 9-anthroic acid esters undergo an increase in dipole moment in the first excited-state (Werner and Hoffman, 1973), it is unclear whether interactions at the polar ester group of triolein contribute to the effects observed with 12-AS in this solvent. Indeed, when l-NPN is dissolved in paraffin oil, its decay is homogeneous and wavelength independent.

If this were also the case with 12-AS it would strongly suggest dielectric relaxation for the mechanism leading to the observed emission wavelength lifetime variations. However, as shown in Fig. 2, the lifetime and polarization of 12-AS in paraffin oil at 10°C are wavelength dependent. The probe was dissolved initially in cyclohexane, and diluted 1,000-fold into paraffin oil. A complete set of τ_m and τ_p data at three modulating frequencies, taken at 0°C, is presented in Table I. The qualitative behavior of the data is similar to that for triolein: the mean decay time increases with wavelength, $\tau_m > \tau_p$ at the blue end, and at the red side $\tau_p > \tau_m$. These results in paraffin oil demonstrate that the excited-state spectral shift occurs in the absence of dielectric relaxation effects, and imply that a relaxation process intrinsic to the molecule itself is involved.

It should be pointed out that the lifetimes measured in all systems discussed in this paper are independent of excitation wavelength, but the polarization values are not. As shown in Fig. 3, exciting at wavelengths < 380 nm results in a considerable reduction of p. This rapid change in p is due to mixing of the "submerged" higher energy transition (Jaffee and Orchin, 1962). (All 9-anthroic acid ester derivatives retain the absorption spectral properties of the parent anthracene chromophore; see Discussion.) The high energy transition is polarized

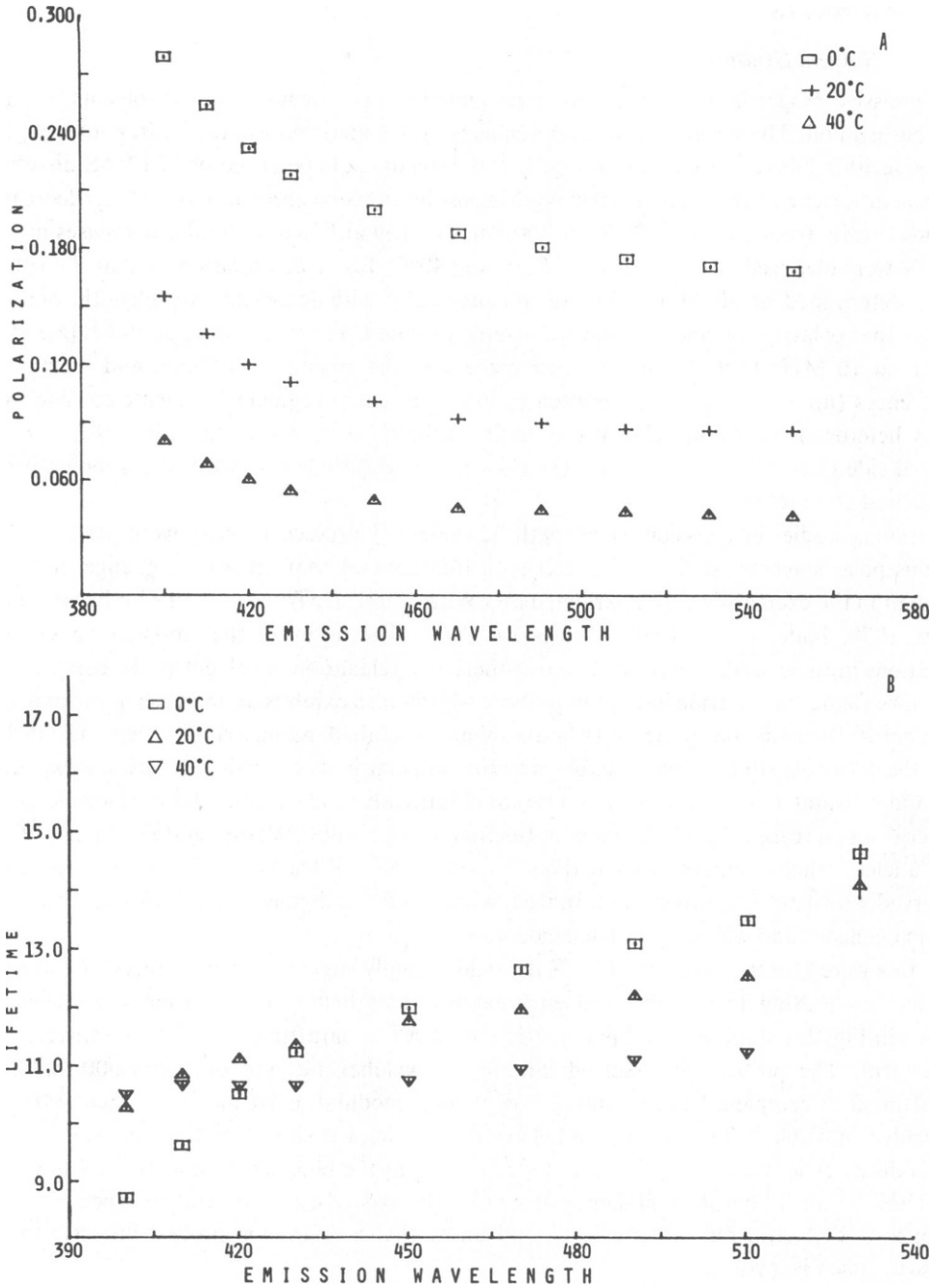

FIGURE 1 Wavelength dependence of the fluorescence of 12-AS dissolved in triolein, at 0°C, 20°C, and 40°C. Excitation = 385 nm; monochromator bandpasses were 1 nm (excitation) and 8 nm (emission). Sample contained 10 μM probe; 12-AS was initially dissolved in ethanol and then diluted 1,000-fold into triolein. (Identical results were obtained if cyclohexane was substituted for ethanol.) The standard deviation is indicated by the vertical bars within the symbols. (*A*) Steady-state polarization as a function of emission wavelength (nm) (error in p is ± 0.001). (*B*) Modulation lifetimes (ns) (30 MHz) as function of emission wavelength (nm).

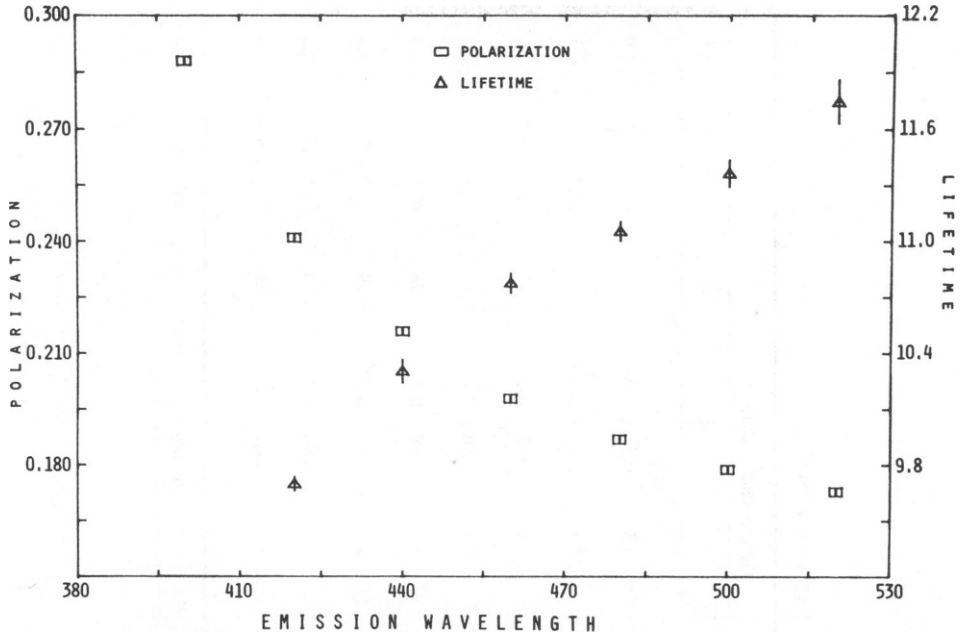

FIGURE 2 Emission wavelength dependence (nm) of modulation lifetimes (ns) at 18 MHz (Δ) and steady-state polarization (□) at 10°C for 12-AS dissolved in paraffin oil. 12-AS was initially dissolved in cyclohexane, followed by 1,000-fold dilution into paraffin oil to a final concentration of 20 μM 12-AS. Excitation = 385 nm (1 nm bandpass), emission bandpass was 8 nm for polarizations, 16 nm for lifetimes.

parallel to the long axis of the anthracene ring and the low energy transition is short-axis polarized. Fig. 3 also shows that the excitation-polarization data obtained by observing emission at either the blue side or red side of the spectrum parallel one another, indicating that the relaxation mechanism is unrelated to the extent to which the higher vibrational or electronic levels are excited. All polarization data on 9-anthroyloxy fatty acid derivatives presented in this paper were obtained using 385-nm excitation.[4]

Several interesting effects are observed in nonviscous solvents at room temperature (Fig. 4). In ethanol, the fluorescence of 12-AS is highly quenched and the short lifetime (3.7 ns) is constant across the emission band to within the ± 0.1 ns SD of the measurement. The decay appears to be monoexponential, except possibly for a slight degree of heterogeneity on the blue side where τ_p is ~ 0.3 ns shorter than τ_m. The significance of this splitting is not clear, since such small differences approach the absolute precision of the measurements, and lifetimes in this case were obtainable at only one modulating frequency. In cyclohexane, where the quantum yield is high, the modulation lifetime shows a small but experimentally significant increase across the emission band. In comparison to the behavior seen in the viscous systems discussed above, the τ_m–τ_p splittings at the blue end are much smaller (1.5 ns at 30 and 18 MHz), and τ_p is approximately equal to τ_m at the emission red edge. When 12-AS is dissolved

[4]The low value of p_0 = 0.29 for the limiting polarization, recently reported by Thulborn et al. (1978*a*), was evidently obtained using an excitation wavelength of only 337 nm, and a broad band 420-nm cut-off filter in the emission.

TABLE I
EMISSION HETEROGENEITY OF 12-AS IN PARAFFIN OIL

λ-emission	Measured values			Heterogeneity analysis						
	30 MHz	18 MHz	6 MHz	α_1	A_1	τ_1	α_2	A_2	τ_2	χ^2
(nm)		*(ns)*					*(ns)*			
405	7.5 ± 0.2	8.2 ± 0.1	9.3 ± 0.4	0.48 ± 0.08	0.75	3.7 ± 0.3	0.52 ± 0.08	0.25	11.8 ± 0.2	1.0
	5.5 ± 0.3	6.2 ± 0.1	7.8 ± 0.2							
415	8.3 ± 0.1	9.1 ± 0.1	9.9 ± 0.4	0.35 ± 0.08	0.61	3.9 ± 0.3	0.65 ± 0.08	0.39	11.4 ± 0.2	0.1
	6.4 ± 0.3	7.3 ± 0.2	8.7 ± 0.3							
430	9.4 ± 0.2	10.0 ± 0.1	10.6 ± 0.2	0.3 ± 0.1	0.47	5.2 ± 0.4	0.7 ± 0.1	0.53	11.8 ± 0.3	0.6
	8.0 ± 0.5	8.9 ± 0.2	10.2 ± 0.4							
445	10.1 ± 0.1	10.6 ± 0.2	11.0 ± 0.3	0.4 ± 0.1	0.51	7.1 ± 0.5	0.6 ± 0.1	0.49	12.8 ± 0.3	2.9
	9.3 ± 0.4	9.9 ± 0.2	11.0 ± 0.2							
460	10.8 ± 0.2	11.0 ± 0.2	11.4 ± 0.3	0.1 ± 0.1	0.06	17 ± 3	0.9 ± 0.3	0.94	10.5 ± 0.2	1.5
	10.8 ± 0.5	11.0 ± 0.2	11.6 ± 0.3							
475	11.1 ± 0.1	11.5 ± 0.2	11.5 ± 0.3	0.03 ± 0.08	0.03	11 ± 9	0.97 ± 0.08	0.99	11.3 ± 0.2	2.3
	11.5 ± 0.9	11.4 ± 0.3	12.0 ± 0.3							
490	11.5 ± 0.2	11.7 ± 0.1	11.4 ± 0.4	0.04 ± 0.2	0.04	11.2 ± 7	0.96 ± 0.2	0.96	11.6 ± 0.2	2.2
	12.0 ± 1	12.0 ± 0.4	12.6 ± 0.5							
505	11.8 ± 0.2	11.8 ± 0.2	11.8 ± 0.3	−0.01 ± 0.02	−0.07	2 ± 3	1.01 ± 0.02	0.93	12.0 ± 0.2	2.4
	14.0 ± 2	12.6 ± 0.4	12.8 ± 0.4							

Analysis of modulation and phase lifetimes at 30, 18, and 6 MHz, as a function of emission wavelength in paraffin oil, 0°C. At each frequency, τ_m is given first, with τ_p directly under it. The sample contained 20 μM 12-AS and 0.1% (vol/vol) cyclohexane. Excitation = 380 nm, monochromator bandpasses were 1 nm (excitation) and 16 nm (emission).

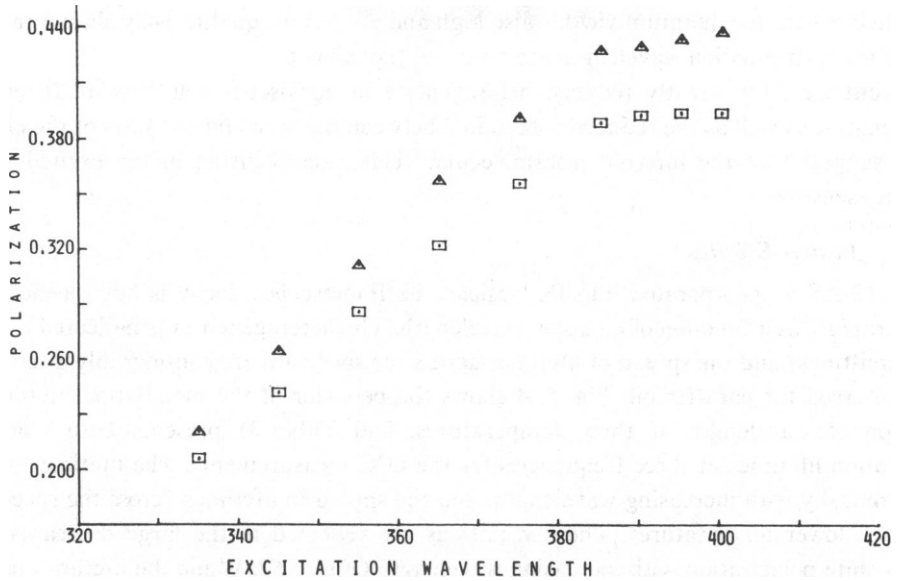

FIGURE 3 Polarization of 12-AS across its last absorption band. Sample contained 10 μM 12-AS in glycerol at 10°C. Excitation bandpass was 4 nm, emission bandpass (at 420 [Δ] or 480 nm [□]) was 8 nm. For purposes of demonstrating the mixed polarization across the absorption band, glycerol was chosen because the high polarization of 12-AS in this solvent maximized the changes in p value. The excitation wavelength is expressed in nanometers.

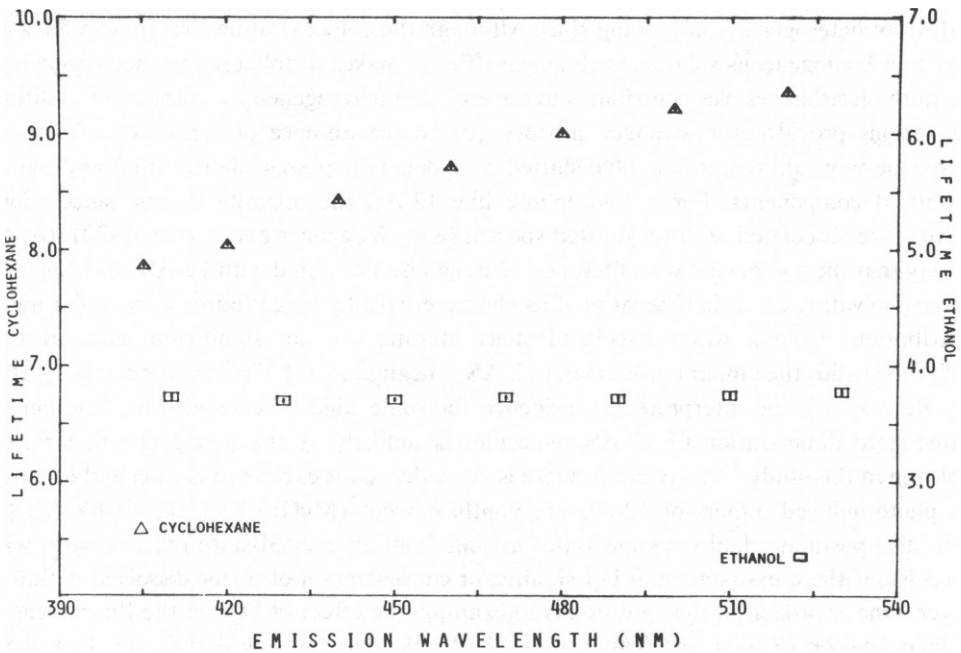

FIGURE 4 Modulation lifetimes (ns) (30 MHz) of 12-AS as a function of emission wavelength (nm) in ethanol (□) and cyclohexane (Δ). Samples were flushed with argon before measurement; temperature = 20°C.

in decane, where the quantum yield is also high and $\tau \sim 9.5$ ns, qualitatively similar increases in lifetime with emission wavelength are observed (not shown).

In summary, the greatly reduced heterogeneity in nonviscous solutions at all emission wavelengths, as well as the reduced spread in τ between the blue and red sides of the emission band, suggest that the inferred intramolecular relaxation occuring in the excited state is viscosity sensitive.

Vesicle Studies

When 12-AS is incorporated into PC vesicles, its fluorescence decay is heterogeneous and varies rapidly as a function of emission wavelength. The heterogeneity (as indicated by the $\tau_m - \tau_p$ splittings) and the spread of lifetimes across the spectrum are considerably greater than that observed for paraffin oil. Fig. 5 *A* shows the behavior of the modulation lifetime as a function of wavelength at three temperatures, and Table II presents both phase and modulation lifetimes at three frequencies for the O°C measurements. The lifetimes increase monotonically with increasing wavelength, and the spread in lifetimes across the spectrum is larger at lower temperatures. These variations are reflected in the large decreases in the steady-state polarization with increasing wavelength (Fig. 5 *B*). While the lifetimes in Fig. 5 *A* represent only τ_m at 30 MHz, it can be seen in Table II that τ_p at 30, and τ_m and τ_p at 18 and 6 MHz all increase monotonically across the emission spectrum. At the blue end, $\tau_m < \tau_p$, but at the red side, τ_p is longer than τ_m. As discussed above, the observation of an increase in decay time with wavelength and the conditions $\tau_p > \tau_m$ and $\tau(30) > \tau(6)$ or $\tau(18)$ at some wavelengths, indicate that an excited-state reaction is occurring.

In general, an increase in the observed mean lifetime across the emission spectrum could result from heterogeneity of binding sites. Although the demonstration that these effects also occur in a homogeneous solution such as paraffin oil makes it unlikely that heterogeneity of sites in the lecithin vesicles contributes to the emission heterogeneity, a number of additional observations provide even stronger support: (*a*) In the absence of relaxation effects, site heterogeneity would require the blue-shifted components to possess shorter lifetimes than the red-shifted components. For a fluorophore like 12-AS the opposite is true since shorter lifetimes are associated with red-shifted spectra (e.g., Waggoner and Stryer, 1970). (*b*) It is unlikely that the component with lifetime < 2 ns can be associated with 12-AS emission under ordinary solvating conditions because of its characteristically long lifetime in nonpolar media. Contributions from a water-associated short lifetime are not significant since at these lipid:probe ratios the amount of unbound 12-AS is negligible. (*c*) The condition $\tau_p > \tau_m$ at the red side can only be interpreted as evidence for some kind of excited-state reaction. An excited-state dimerization of 12-AS molecules is unlikely at the lipid:probe molar ratios employed in this study.[5] In any event, there is no evidence for excimer emission and it appears that photo-induced dimers of 12-AS are nonfluorescent (McGrath et al., 1976). (*d*) One might also postulate dual emission states arising from an excited-state reaction with water molecules, if there exists a small but significant concentration of water dissolved within the bilayer. One approach to this question is to examine the effect of D_2O on the fluorescence of the bound probe (Radda and Vanderkooi, 1972). We have observed that the fluorescence

[5]Kleinfeld and Solomon, submitted for publication.

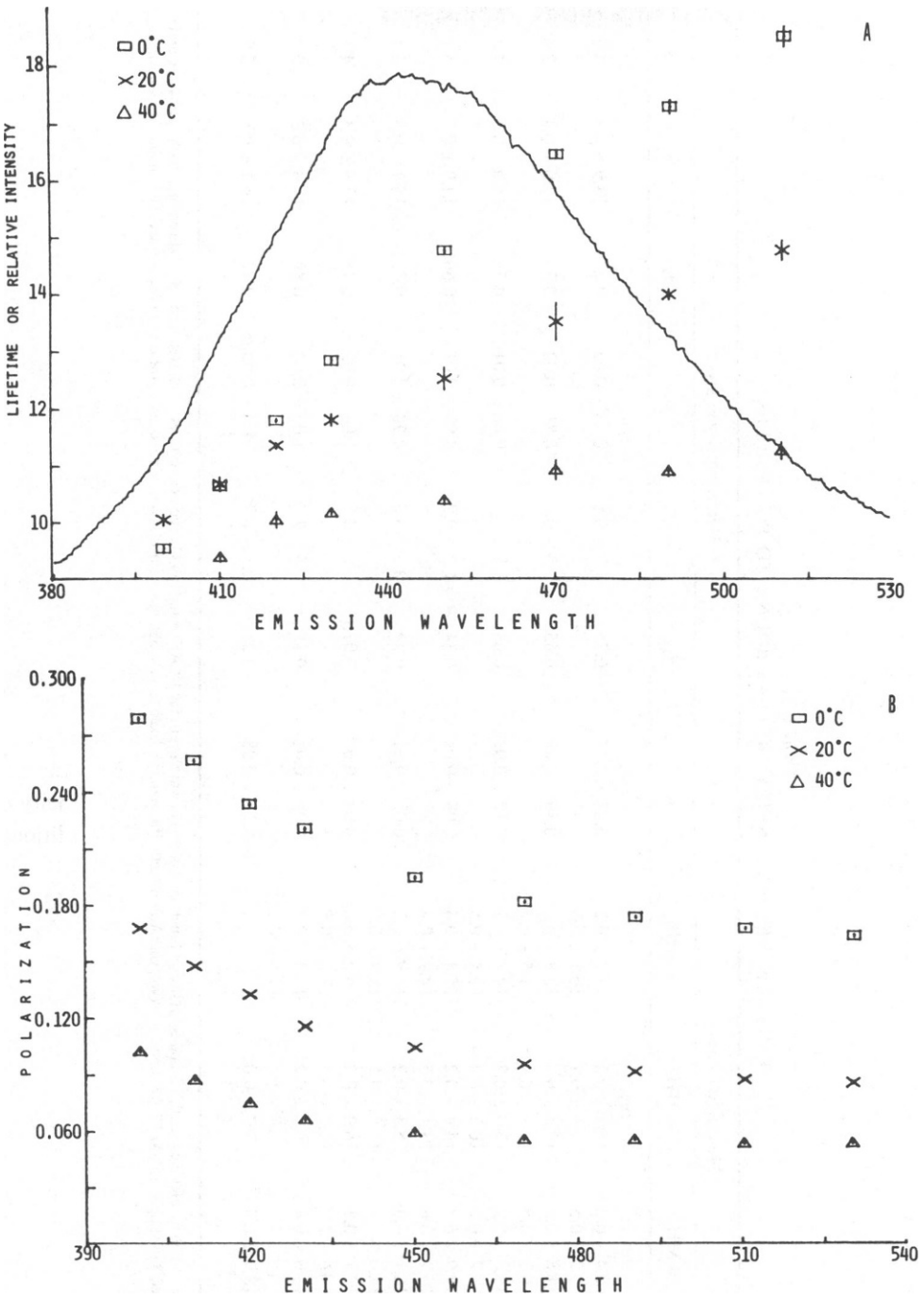

FIGURE 5 Wavelength dependence of the fluorescence of 12-AS bound to PC vesicles, at 0°C, 20°C, and 40°C. Excitation = 385 nm, monochromator bandpasses at 1 nm (excitation) and 8 nm (emission). Sample contains 2.5 mM lipid, 12 μM 12-AS, and 0.1% (vol/vol) ethanol. (*A*) Modulation lifetimes (ns) (30 MHz) vs. emission wavelength (nm). The technical emission spectrum is also shown (solid curve). (*B*) Steady-state polarization vs. emission wavelength (nm).

TABLE II
EMISSION HETEROGENEITY OF 12-AS BOUND TO PC VESICLES

λ-emission	Measured values			Heterogeneity analysis						
	30 MHz	18 MHz	6 MHz	α_1	A_1	τ_1	α_2	A_2	τ_2	χ^2
(nm)		*(ns)*					*(ns)*			
405	10.4 ± 0.2	11.2 ± 0.2	13.3 ± 0.3	0.27 ± 0.02	0.75	1.7 ± 0.5	0.73 ± 0.02	0.25	13.9 ± 0.4	3.2
	5.3 ± 0.5	7.1 ± 0.1	10.1 ± 0.6							
415	11.6 ± 0.2	12.1 ± 0.2	13.8 ± 0.3	0.19 ± 0.02	0.65	1.8 ± 0.6	0.81 ± 0.02	0.35	14.0 ± 0.4	2.8
	6.3 ± 0.5	8.7 ± 0.4	11.5 ± 0.5							
430	13.3 ± 0.2	13.3 ± 0.2	14.8 ± 0.2	0.12 ± 0.01	0.48	2 ± 1	0.88 ± 0.01	0.52	15.0 ± 0.5	4.8
	8.2 ± 0.6	10.5 ± 0.4	13.4 ± 0.5							
445	14.9 ± 0.2	14.4 ± 0.2	15.4 ± 0.4	0.04 ± 0.01	0.34	1.2 ± 0.9	0.96 ± 0.01	0.66	14.9 ± 0.3	5.2
	10.2 ± 0.6	13.1 ± 0.5	14.9 ± 0.4							
460	16.1 ± 0.3	15.3 ± 0.2	15.9 ± 0.2	0.02 ± 0.01	0.39	0.5 ± 0.5	0.98 ± 0.01	0.61	15.7 ± 0.3	3.1
	12.0 ± 1	14.7 ± 0.5	15.9 ± 0.5							
475	17.1 ± 0.3	16.0 ± 0.2	16.2 ± 0.6	−0.03 ± 0.02	−0.07	6 ± 3	1.03 ± 0.02	0.93	15.9 ± 0.3	4.1
	16 ± 2	16.7 ± 0.5	17.3 ± 0.5							
490	18.0 ± 0.4	16.5 ± 0.2	16.4 ± 0.3	−0.05 ± 0.02	−0.11	6 ± 2	1.05 ± 0.02	0.89	16.3 ± 0.3	2.9
	18 ± 3	18 ± 1	17.6 ± 0.5							
505	18.9 ± 0.5	17.0 ± 0.03	16.7 ± 0.4	−0.12 ± 0.08	−0.21	6.1 ± 0.9	1.2 ± 0.08	0.79	16.1 ± 0.8	2.0
	21 ± 6	20 ± 1	18.5 ± 0.6							

Analysis of modulation and phase lifetimes at 30, 18, and 6 MHz as a function of emission wavelength for 12-AS bound to PC vesicles at 0°C. At each frequency, τ_m is given first, with τ_p directly under it. The sample contained 3 mM lipid, 15 μM 12-AS, and 0.1% (vol/vol) *N,N*-dimethylformamide. Excitation = 380 nm, monochromator bandpasses were 1 nm (excitation) and 16 nm (emission).

intensity and spectrum of 12-AS bound to PC vesicles is unaltered by the addition of 95% D_20 to the aqueous medium, thus suggesting that reactions with bilayer water are insignificant. But the negative result might be considered ambiguous because the mechanism of the solvent isotope effect is not well understood, and with unknown concentrations of bilayer water present in a hydrophobic environment, it is unclear how the emission of 12-AS should be perturbed when external H_20 is replaced by D_20. However, the following result supports the same conclusion. As will be discussed below, the spread in the decay time and polarization across the emission band is much smaller for 16-AP than 12-AS; we have attributed this difference to the fact that the anthracene is attached at the end of the chain in 16-AP. If the large wavelength-dependent variations witnessed for 12-AS bound to PC vesicles were instead due to excited-state reactions with water, then a probe such as 11-(9-anthroyloxy)-undecanoic acid (11-AU), located at nearly the same depth in the bilayer, should exhibit similarly large variations across its emission spectrum. We find, in fact, that these variations with wavelength for 11-AU are small and of similar magnitude to that observed with 16-AP. Hence, the presence of bilayer water is highly unlikely to constitute a major source of the nanosecond spectral shift observed in the vesicle system.

Behavior of 2-AS and 16-AP

Figs. 6 *A* and 6 *B* show that in comparison with 12-AS (Fig. 5 *B*), 2-AS exhibits a slightly greater spread in p, whereas the spread for 16-AP is considerably smaller. In view of the observed effects of viscosity on 12-AS emission, the difference in the spread of p across the emission band between the three probes might be predicted as a consequence of the "fluidity gradient" in the bilayer. 16-AP, located in the most fluid region of the bilayer, should be the least hindered of the three probes. In addition, differences in the absolute magnitude of p between the three probes, when bound to vesicles, are caused in part by differences in the lifetime: for emission averaged over the center and red side of the spectrum, τ = 9.5 ns (2-AS), 13.4 ns (12-AS), and 15 ns (16-AP) (detailed data to be presented in a subsequent paper). However, even in paraffin oil (Fig. 7 and Table III), 16-AP yields low p values, and likewise (as in vesicles) shows the smallest changes in p and across the emission band. Both 2-AS and 12-AS exhibit an increase in average decay times of ~2–2.5 ns between 410 and 490 nm, but higher polarization values are obtained for 2-AS despite its slightly higher range of mean lifetimes. These data imply that even in an isotropic solvent, the mobility of the anthracene ring in these three fatty acid derivatives differs, such that the polarization increases in the order 16-AP < 12-AS < 2-AS.

DISCUSSION

Wavelength-dependent nanosecond decay kinetics, such as those observed here, have been studied particularly in systems where solvent dielectric relaxation or solvent-solute complexing is important (Ware et al., 1971; Chakrabarti and Ware, 1971; Egawa et al., 1971; Easter et al., 1976; DeToma et al., 1976; DeToma and Brand, 1977; Badea et al., 1978). In the 12-AS/PC vesicle system, the anthroate group of 12-AS should be located well within the hydrophobic region of the bilayer (Podo and Blasie, 1977), so it seems unlikely that solvent-dielectric relaxation is involved. We have already discussed (see Results) the evidence that argues against the possibility of an excited-state reaction with bilayer water. Furthermore

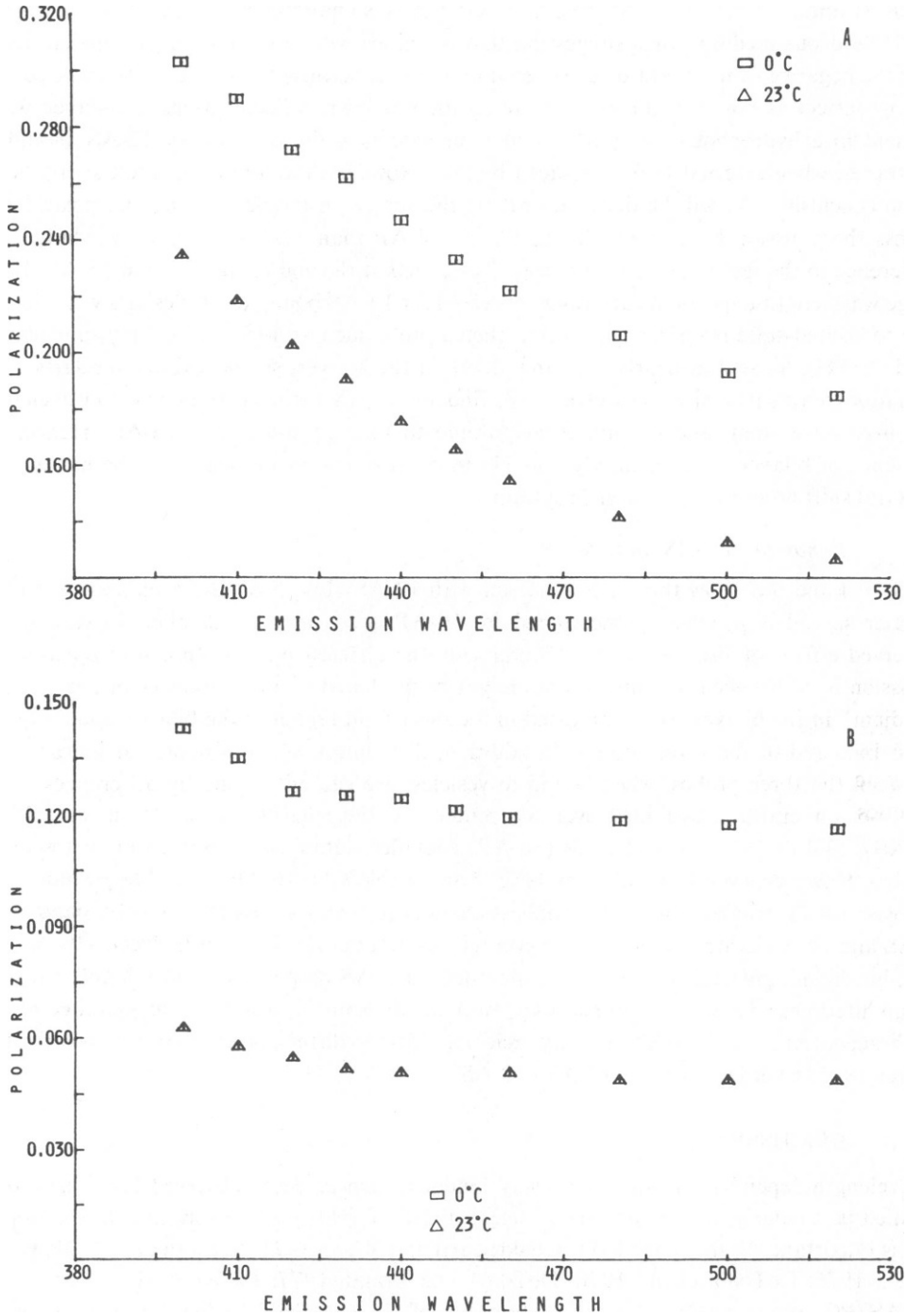

FIGURE 6 Steady-state polarization vs. emission wavelength (nm) of 2-AS and 16-AP bound to PC vesicles. Excitation = 385 nm, and monochromator bandpasses were 8 nm (excitation) and 8 nm (emission). Samples contained 1 mM lipid, 2 μM probe, and 0.04% (vol/vol) ethanol. (*A*) 2-AS; (*B*) 16-AP.

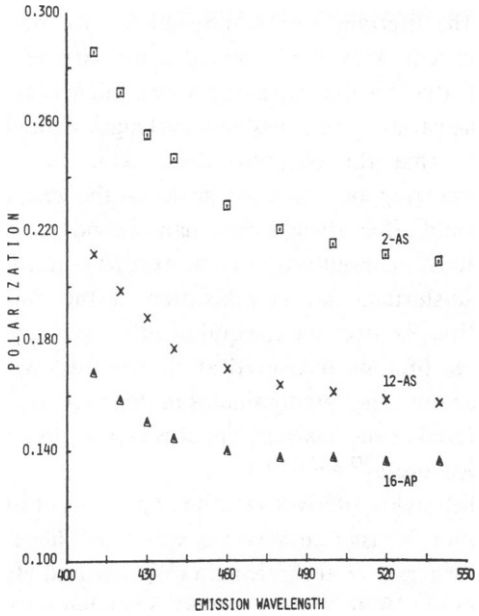

FIGURE 7 Steady-state polarization vs. emission wavelength (nm) of 2-AS, 12-AS, and 16-AP in paraffin oil. The samples contained 5 μM probe dissolved in a paraffin oil – 5% decane mixture; temperature = 3°C. Excitation = 385 nm, monochromator bandpasses were 2 nm (excitation) and 16 nm (emission).

our evidence suggests that water of hydration, which might be associated with the anthroyl fatty acid crystals, from which our solutions are prepared, cannot cause the observed decay kinetics. If this water were loosely associated with 12-AS, then we would expect the decay to differ if the probes are dissolved in cyclohexane as compared with ethanol. However as explained in the caption to Fig. 1, no such differences were observed. Two observations argue against tightly bound water. First, if 12-AS in solution has a shell of water tightly associated with it, we would expect the fluorescence to be highly quenched and generally insensitive to the dielectric constant of the solvent. Fig. 4 demonstrates that this is not the case in ethanol

TABLE III
LIFETIMES AT THREE EMISSION WAVELENGTHS FOR 2-AS, 12-AS, and 16-AP DISSOLVED IN PARAFFIN OIL

	Wavelength		
	410 nm	450 nm	490 nm
2-AS	11.6	12.9	13.3
12-AS	10.1	11.8	12.5
16-AP	12.6	12.8	12.9

Modulation lifetimes (ns) at 18 MHz at three emission wavelengths; the standard deviation was ± 0.1 ns. The samples and conditions were identical to those described under Fig. 7.

and cyclohexane where the lifetimes are ~ 3.8 and 8.5 ns, respectively. Second, a tight hydration shell is inconsistent with the observed sensitivity to the viscosity of nonpolar solvents, as was demonstrated by the wavelength dependence of the decay in paraffin oil versus cyclohexane and the variation in emission wavelength with viscosity (see below).

We therefore conclude that the observed decay kinetics are due to intramolecular fluorophore relaxation occurring on the time scale of the excited-state lifetime. Such a conformational change would affect the observed nanosecond decay in the following manner. The fluorescence from the S_1^{FC} configuration (first excited state, Franck-Condon geometry) would be maximally blue-shifted, and as relaxation of the nuclear framework proceeds towards the S_1^{EQ} state (first excited state, equilibrium geometry) longer wavelengths are emitted. Thus, the average lifetime measured at the shortest wavelengths will be shorter, because emission at the earliest times predominates in this region of the spectrum. Conversely, the average lifetime measured at successively longer wavelengths will be longer, reflecting the rate of formation of one or more S_1^{EQ} geometries.

For the case of the 9-anthroyloxy derivatives, the hypothesis of intramolecular relaxation is supported by the results of Werner and co-workers, who have studied the fluorescence properties of several alkyl esters of 9-anthroic acid (Werner and Hercules, 1969: Werner and Hoffman, 1973; Werner et al., 1976; Werner, 1976). They have proposed that the $S_1^{FC} \rightarrow S_1^{EQ}$ transition involves a change in molecular configuration in which the carbonyl rotates from a perpendicular orientation (relative to the plane of the anthracene ring), towards a more coplanar position. The evidence implicating such a process in the excited state of 9-anthroyloxy fatty acid probes includes the following: (*a*) All of the 9-anthroyloxy-fatty acid derivatives exhibit anthracenelike absorption spectra, implying a lack of resonance interaction between the carbonyl and the anthracene ring in S_0 and S_1^{FC}. (*b*) In contrast to anthracene, the emission spectra of 9-anthroyloxy-fatty acids in nonviscous solvents are broadened and display a Stokes shift and quantum yield which are solvent dependent. A ring-to-carbonyl geometry which has acquired increased coplanarity in the excited state would create an S_1^{EQ} state with a significant amount of charge transfer character. (*c*) The temperature dependence of the emission spectrum of the AS probes in nonpolar solvents is consistent with a viscosity or temperature-dependent change in nuclear geometry in the excited state. We have observed that the emission spectrum of 12-AS in paraffin oil exhibits changes in structure and a blue shift of several nanometers when the temperature is lowered from 50° to 0°C. In addition, the wavelength of maximum emission increases in the following solvents (as does 1/viscosity): paraffin oil < decane < heptane; the detailed spectral shape also differs in these solvents. In this regard, it should be noted that the fluorescence spectrum of 12-AS in ethanol at 77°K (McGrath et al., 1976) acquires a highly structured anthracenelike appearance. The most likely explanation of this effect is that the solvent glass has strongly inhibited the extent to which the proposed excited-state rotation can take place, and emission occurs from a state with geometry similar to S_1^{FC}.

In order to discuss the wavelength-dependent lifetimes in terms of the proposed conformational change, it is necessary to deconvolute the observed decay in terms of the decay from the component states S_1^{FC} and S_1^{EQ}. Although the decay may actually occur from a continuum of intermediates during the relaxation from S_1^{FC} to S_1^{EQ}, we will perform the analysis in terms of two discrete states. We do this since we can at most resolve two components (if measurements

are carried out at three modulation frequencies) and because two components provide a reasonable fit to the data.

We therefore assume that the fluorescence can be described by

$$I(\lambda,t) = A_1(\lambda)\, e^{-t/\tau_1} + A_2(\lambda)e^{-t/\tau_2} \tag{8}$$

in which τ_1^{-1} and τ_2^{-1} correspond to the decay rates from the S_1^{FC} and S_1^{EQ} states, respectively.

The results of a two-component heterogeneity analysis for 12-AS in egg PC and paraffin oil are shown in Tables I and II. These results demonstrate that for egg PC, at least on the blue side of the spectrum, the data are reasonably fit by a long (~ 16-ns) component and a short (~ 2-ns) component. Furthermore, although the lifetimes are fairly constant across the band, the component fractional intensities exhibit appreciable variation. We associate the short component with S_1^{FC} and the longer component with S_1^{EQ}. The association of a 2-ns component with the decay of S_1^{FC} is not unreasonable because the lifetime of anthracene is relatively short (~ 5 ns in deoxygenated cyclohexane), and as noted above, there is evidence that implicates the anthracenelike character of S_1^{FC}. A longer lifetime for S_1^{EQ} is also expected because, as discussed by Werner (1976), the relaxation process moves the singlet state below the second excited triplet state, which decreases the crossover rate and thereby lengthens the lifetime.

At the very blue side of the spectrum (405 nm) there are three times ($A_1 = 0.75$) as many short-lived emitting species as long-lived ($A_2 = 0.25$). This is expected since, as discussed above, there appears to be no resonance interaction between the carbonyl and the anthracene ring, and therefore the S_1^{FC} state should be blue shifted with respect to S_1^{EQ}. The ratio of A_1 to A_2 decreases with increasing emission wavelength until, at the middle of the spectrum, a value of about zero is obtained. This sort of behavior is also characteristic of a two-state system in which the initially formed state (S_1^{FC}) decays with a rate k_{ex} to a state which subsequently fluoresces (Birks, 1970; Loken et al., 1972; Grinvald and Steinberg, 1974; Matayoshi, 1979). In this case

$$I(t,\lambda) = S_{1,0}^{FC}\left\{\left[I_{FC}(\lambda) + \frac{k_{ex}}{\left(\frac{1}{\tau_2} - \frac{1}{\tau_1}\right)} I_{EQ}(\lambda)\right] e^{-t/\tau_1} - \frac{k_{ex}\, I_{EQ}(\lambda)}{\left(\frac{1}{\tau_2} - \frac{1}{\tau_1}\right)} e^{-t/\tau_2}\right\}, \tag{9}$$

in which $I_{FC}(\lambda)$ and $I_{EQ}(\lambda)$ are the normalized emission spectra of S_1^{FC} and S_1^{EQ}, respectively and $S_{1_0}^{FC}$ is the concentration of S_1^{FC} states at $t=0$. Since $\tau_1 < \tau_2$ the second term is negative, and with appropriate values of $I_{FC}(\lambda)$ and $I_{EQ}(\lambda)$ the amplitude factor for $\exp(-t/\tau_1)$ will vanish. An interesting aspect of this phenomenon is that the finding of an intermediate wavelength at which the decay is monoexponential argues against probe "site" heterogeneity. If the latter were significant, wavelength-dependent emission characteristic of each site would contribute to the observed decay, and it is unlikely that monoexponential decay from each site would simultaneously occur at the same wavelength and at the same rate.

At the red end of the spectrum we find that $A_1 < 0$, which manifests itself in the phase-modulation observables as $\tau_p > \tau_m$. A negative pre-exponent is typical for a system in which a fluorescent species is formed from a precursor that decays earlier (Birks, 1970). This effect becomes apparent at the red side of the spectrum simply because the relaxed state dominates the intensity in this region.

Although the results in paraffin oil are qualitatively similar, there are some differences, especially in the values of the lifetimes and the behavior at the red side of the spectrum. First of all, the long lifetime component is considerably shorter ($\sim$ 12 ns compared with $\sim$ 16 ns) than in PC and does not exhibit the small increase in τ_2 with wavelength. On the other hand τ_1 appears to be somewhat longer than in the PC case; the values at the center-red side are not reliable since the data are well fit by a single exponential and therefore the error in α_2 easily encompasses a value of 0. In paraffin oil only the red-most wavelength (505 nm) yielded a negative A_2 value.

There are probably two independent explanations for these differences. First, in the experiments with paraffin oil no attempt was made to deoxygenate the solution. As a result, we would expect the long lifetimes to be preferentially quenched, which also leads to a blue shift because the longer lifetimes correspond to the red-shifted part of the spectrum. Indeed, the emission spectrum of 12-AS in paraffin oil is about 15 nm blue-shifted, and also exhibits some structure (a shoulder to the red side of the spectral maximum) not evident in the more symmetrically broadened spectrum observed in egg PC. The second factor contributing to a difference may be an increased degree of rotational constraint of the anthracene in paraffin oil as opposed to PC. This would account for the smaller lifetime spread and blue shift in paraffin oil.

Although a two-component analysis provides a reasonable description of the results, the fit is not uniformly excellent. In the present case there are three degrees of freedom in the fit: six measured values (τ_p and τ_m at each frequency) and three independent fit parameters (τ_1, τ_2 and α_2 [$\alpha_1 = 1 - \alpha_2$]). A 5% confidence limit therefore corresponds to a normalized chi-square value (absolute chi-square value/ number of degrees of freedom) of 2.6. As can be seen in Table II most of the chi-square values obtained with egg PC are > 2.6, although most of the values in paraffin oil (Table I) are smaller. In addition, the value of τ_1 in egg PC, while constant below 475 nm, undergoes an abrupt increase to 6 ns above 475 nm, and its value in paraffin oil is anything but constant. No explanation for these difficulties is offered; however, as noted above, a pure two-component fit was not expected to be perfect.

Whether or not the emission wavelength variation of the decay can be completely described by a two-component system, the existence of these intramolecular effects certainly complicates the interpretation of the fluorescence of the 9-anthroyloxy probes in terms of membrane structure. The results in Fig. 7 and Table III emphasize the difficulties in attempting to apply the 2-,12- and 16- derivatives as probes of relative "fluidity" along the bilayer vertical ("fluidity" gradient). Apart from the non-exponentiality and wavelength dependence of their decay, the rotational motion of the anthracene moiety is clearly affected by its point of attachment along the fatty acid chain. An examination of molecular models indicates that this may be caused by differences in steric hindrance to ring rotation posed by the fatty acid chain itself. Thus, 2-AS and 16-AP, because of their position, may represent anomalies to the series of *n*-(9-anthroyloxy) fatty acids. If this is the case, the properties of other derivatives, in which the anthracene ring is not located at the end positions, should closely mimic 12-AS. Preliminary studies with 3-AS, 6-AS, 7-AS, 9-AS and 11-(9-anthroyloxy)-undecanoic acid (to be detailed in a subsequent paper) are consistent with this hypothesis. When the 3, 6, 7, and 9 derivatives are dissolved in paraffin oil, their polarization profiles as a function of emission wavelength are quantitatively similar to that obtained for 12-AS. In contrast,

11-(9-anthroyloxy)-undeconoic acid, in which the anthracene is attached at the end of the fatty acid chain, displays a wavelength dependence when dissolved in paraffin oil that is quantitatively similar to 16-AP. In a recent report by Tilley et al. (1979), similar conclusions regarding the anomalous behavior of 2-AS and 16-AP were reached.

Another complication is related to the fact that the point of attachment of the anthroyloxy moiety also determines the shape of the emission spectrum and the variation in lifetime across the spectrum. The shape of the emission spectrum is important since measurements (either p or τ) performed using wide-band (cutoff) filters are necessarily an average over the emission spectrum, and therefore each probe will be subjected to a different averaging. The importance of this effect in determining a "fluidity" gradient or even changes in membrane structure can be gauged from the influence of viscosity change on the emission spectrum and lifetimes. As was noted above, the probes undergo a blue shift in more viscous solvents such as paraffin oil and exhibit a reduced spread and shorter lifetime. Hence, although paraffin oil appears to restrain the relaxation of the anthroyloxy more than PC, the polarization in paraffin oil (exciting above 380 nm) is actually lower. This implies that the rotations around the axis formed by the ester linkage and the short axis of the anthracene ring are more restrained in paraffin oil than in PC, while rotations about perpendicular axes are less restrained, possibly reflecting the anisotropy of the membrane.

Although in this discussion we have emphasized the complexities of the fluorescence response of AS, the deeper understanding of the mechanism that this work provides may allow for a more sensitive probe of membrane structure. It is clear from the comparison of the PC and paraffin oil data that the emission-decay properties reflect one type of rotational restraint while polarization (and more properly the time-dependent polarization) reflects another. Ideally, a quantitative measure of the constraint would be obtained from the S_1^{FC} to S_1^{EQ} decay rate: k_{ex}. Even better we should like to dispense with the two-state model and determine the functional form of the decay mechanism. This will only be possible once the range and number of frequencies is increased in the phase-modulation technique or the pulse width shortened in the pulse method.

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