NANOSECOND RELAXATION PROCESSES IN LIPOSOMES

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INTRODUCTION

The fluorescence characteristics of aromatic molecules are often strongly influenced by their immediate environment. For this reason, fluorescence probes have found wide application for monitoring changes occurring in biological or model membrane systems (Azzi, 1975; Radda, 1975; Badley, 1976). The majority of the reported studies have made use of steady-state fluorescence measurements. These reflect a time average of any excited-state interactions that occur on the nanosecond time scale. Fluorescence decay measurements, on the other hand, provide direct kinetic information regarding these interactions. Direct measurements are possible in a time interval limited on the short side by the time resolution of the instrumental technique and on the long side by the decay time of the fluorophore. As a consequence, the time-dependent characteristics of the fluorescent probe environment are better defined.

The parameters characterizing the excited state behavior of 2-anilinonaphthalene (2-AN) in fluid solutions such as cyclohexane, ethanol, and mixtures thereof, and in a viscous solvent (glycerol) have previously been reported (DeToma and Brand, 1977). They were found to be strongly influenced by the presence of relatively small amounts of polar molecules in a nonpolar solvent. This particular property makes 2-AN a valuable fluorescent probe for a heterogeneous polar-apolar liposomic environment. In addition, the information obtained in the model solvent systems mentioned above constitutes a firm basis for interpreting the results obtained with liposomes.

In the present communication we describe the results obtained by measuring the fluorescence decay, time-resolved emission spectra (TRES), and the decay of the emission anisotropy (DEA) of 2-AN adsorbed to dimyristoyllecithin (DML) single bilayer liposomes. It will be shown that the rotational motion of 2-AN as well as its excited-state interactions occur on the nanosecond time scale both above and below the gel-liquid transition temperature of these vesicles. The nanosecond relaxation processes found to occur with the liposomes will be compared to those observed with 2-AN in homogeneous solvents.

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METHODS

Experimental

2-AN was obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.) and purified as previously described (DeToma and Brand, 1977). The DML single bilayer liposomes were prepared as described (Chen et al., 1977), and the fluorescence measurements were carried out in 0.01 Tris-HCl buffer, pH 8.5, containing 0.1 M NaCl. 4 μl of a 1-mM solution of 2-AN in ethanol was added to 4 ml of liposomes in the Tris buffer followed by rapid vortexing for about 1 min. The mixture was left to equilibrate for 2-3 h before the optical measurements. The lipid to dye ratio was about 700. The glycerol was spectral grade for fluorescence microscopy and was obtained from AG Merck (Darmstadt, W. Germany).

The instrumental and computational techniques for obtaining deconvolved nanosecond time-resolved emission spectra and the decay of the emission anisotropy have been described by Easter et al. (1976) and Chen et al. (1977), respectively. The monophoton counting technique was used to obtain the fluorescence decay data. Each curve and its corresponding excitation profile were collected semi-simultaneously to minimize the errors associated with long-term timing variations in the excitation source. The excitation was performed with an air, nanosecond flash lamp through a Baird Atomic 3,150-Å filter (Baird Atomic, Inc., Bedford, Mass.).

Steady-state fluorescence spectra were obtained both with the monophoton counting instrument and with a Perkin-Elmer MPF4 spectrofluorometer Perker-Elmer Corp., Instrument Div., Norwalk, Conn.). They were measured at the start and end of each series of decay measurements for every sample and found to be identical. This indicated the absence of intervening photodecomposition. To avoid errors due to time-dependent changes in the emission anisotropy, all the decay measurements were carried out under "magic angle" conditions (Spencer and Weber, 1970). Excitation was with vertically polarized light, and the emission was observed through a polarizer whose transmission axis was at 54.7° to the vertical direction.

RESULTS

The interaction of 2-AN with the DML vesicles results in enhanced fluorescence intensity and a blue shift in the fluorescence emission spectrum. The normalized, corrected emission spectra shown in Fig. 1 indicate that 2-AN has a structured spectrum in cyclohexane with a maximum at 372 nm and that the emission maximum when adsorbed to DML vesicles is intermediate between that in cyclohexane and the red-shifted emission observed in water.

The nanosecond time-dependent behavior of 2-AN in DML vesicles was examined both below (1°C) and above (37°C) the crystalline-liquid crystalline phase transition temperature (22°C). The procedure of Easter et al. (1976) was used to obtain the nanosecond time-resolved fluorescence profile at each temperature. Fluorescence decay curves, together with the corresponding lamp profiles, were collected at selected wavelengths spanning the emission band. Impulse response functions for each decay curve were obtained by the method of nonlinear least squares. A sum of exponential terms was used as the fitting function. The results of a typical fit to experimental data are shown in Fig. 2. The good visual agreement between the experimental and theoretical decay, as well as the residuals and autocorrelation of the residuals, all indicate that a sum of three exponentials represents an adequate function to represent the impulse

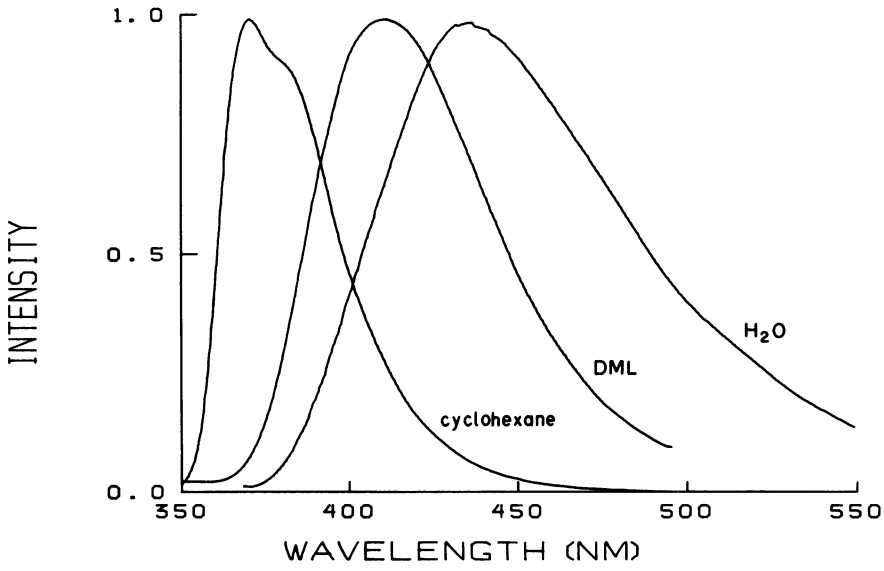

FIGURE 1 Steady-state fluorescence emission spectra of 2-AN in cyclohexane, water, and DML liposomes. The spectra have been normalized at the peak emission and are corrected for non-linear transmission of the monochromator and sensitivity of the detector. The fluorescence spectra were obtained with the Perkin-Elmer MPF-4 spectrophotofluorometer with an excitation and emission bandwidth of 10 nm. The single bilayer DML vesicles (~0.5 mM in lipid) were suspended in 0.01 M Tris-HCl, 0.1 M NaCl at pH 8.5 at 20°C.

response. It can be seen that a fitting function of only two exponential terms is not sufficient to describe the decay in this wavelength region.

The impulse response (deconvolved decay) obtained at four representative wavelengths is shown in Fig. 3. Two salient features are immediately apparent. First, the decay at the red portion of the emission (500 nm) is characterized by an initial rise. This shows up as a negative preexponential term in the decay analysis (see Table I) and indicates that at least a portion of the emission at this wavelength region has its origin in species created during the lifetime of the excited state. The pattern of decay as a function of emission wavelength is similar to that observed with 2-AN dissolved in glycerol. A second feature of the impulse response curves is that the mean decay time $\langle\tau\rangle = \sum_j \alpha_j \tau_j^2 / \sum_j \alpha_j \tau_j$ increases with increasing wavelength. This is presented more explicitly in Fig. 4, which shows the variation in mean lifetime with emission wavelength at temperatures above and below the phase transition of the liposomes. It is of interest that in the long wavelength region the mean decay time decreases with increased temperature whereas a small enhancement of the mean decay time is found at short wavelengths. The impulse response curves (deconvolved fluorescence decay) at 380 and 460 nm at 1° and 37°C are shown in Fig. 5. The increase in mean decay time at 37°C is evident at 380 nm.

The results of an analysis of the decay data at five representative wavelengths are presented in Table I. More than two exponential terms are required to obtain a good

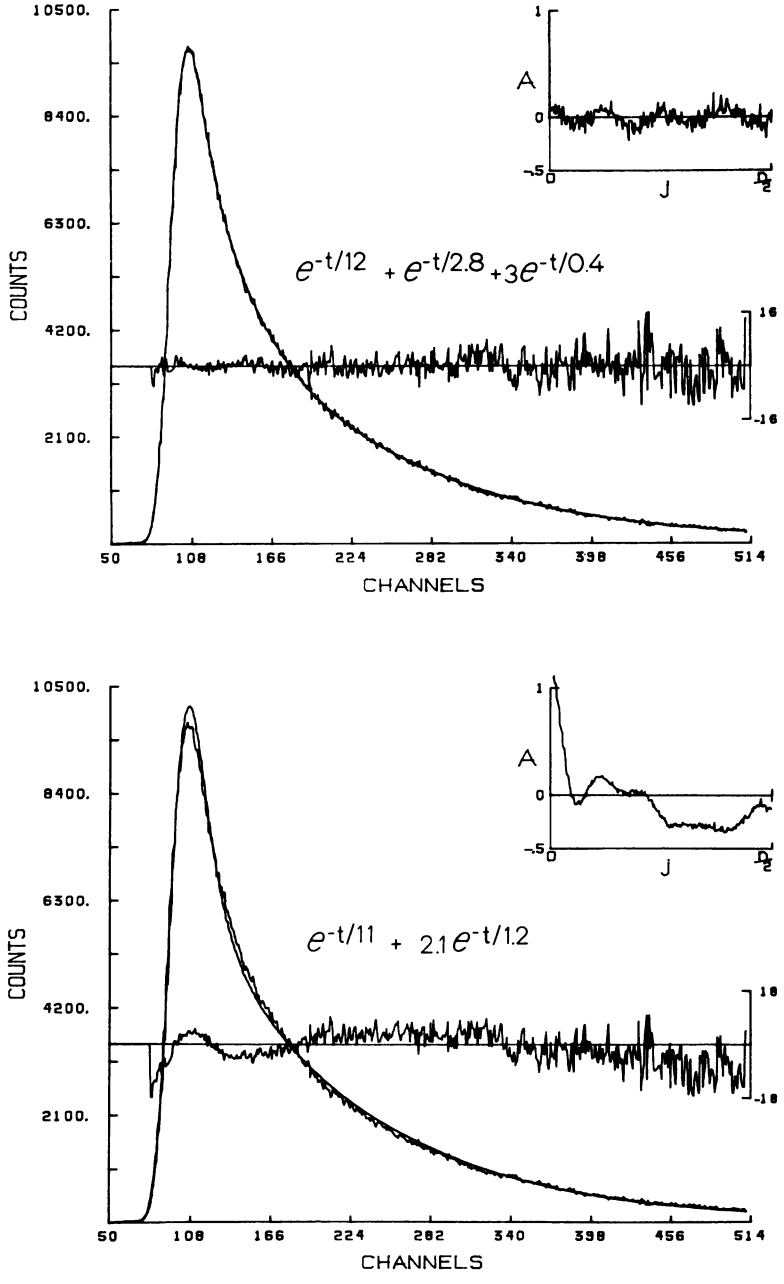

FIGURE 2 Double and triple exponential analysis of a typical decay curve (375 nm) of 2-AN in DML single bilayer liposomes. The smooth curve representing the impulse response convoluted with the lamp flash is superposed on the raw decay data. The weighted residuals of the fit are plotted along the horizontal line. The autocorrelation function of the residuals is shown in the upper right inset. (Above) three exponential fit. (Below) two exponential fit. The timing calibration was 0.101 ns/channel and the bandpass was 13 nm (4-mm slits). Conditions of 2-AN/DML mixture are as in Fig. 1. Temperature was 37°C.

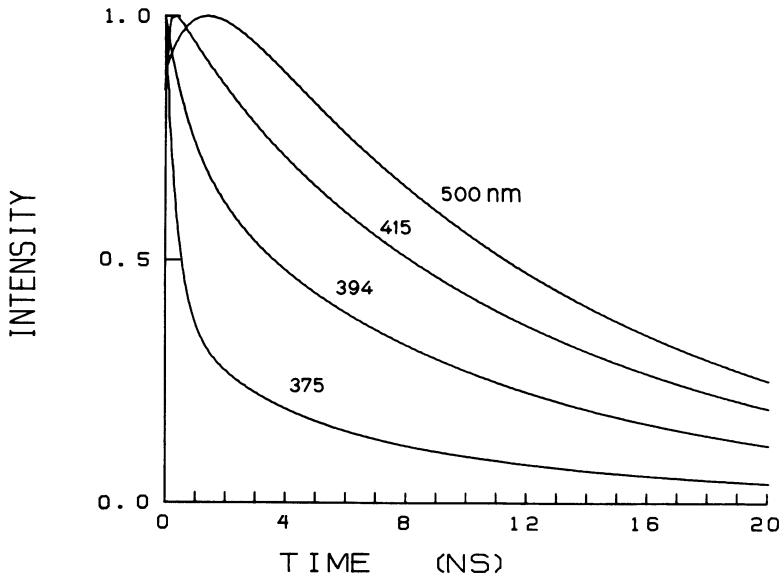

FIGURE 3 Normalized impulse response functions of 2-AN in DML single bilayer liposomes at 375, 394, 415, and 500 nm. Conditions are as in Fig. 2.

fit in the blue region of the emission band. Another representation of the decay surface is shown in Fig. 6 which shows the nanosecond time-resolved emission spectra obtained at three representative times, 0.4, 1.4, and 12 ns. There is a clear shift of the emission maxima to lower energy with time. The time-course of these spectral shifts is represented in Fig. 7, which shows the wave number of maximum emission intensity as a function of time. It is seen that after photoexcitation, 2-AN undergoes relaxation

TABLE I

EMPIRICAL DECAY PARAMETERS OF THE 2-AN/DML COMPLEX AT 1° AND 37°C

Temperature	Wavelength	α_1	τ_1	α_2	τ_2	α_3	τ_3	$\langle\tau\rangle$
			ns		*ns*		*ns*	
1°C	375	0.74	0.23	0.16	3.34	0.10	11.87	8.35
	394	0.30	0.68	0.33	4.23	0.37	13.11	10.78
	418	0.32	3.96	0.68	13.71	—	—	12.51
	460	−0.12	1.12	0.23	7.44	0.65	15.37	14.37
	500	−0.23	3.39	0.28	4.78	0.49	15.50	14.95
37°C	375	0.60	0.38	0.20	2.84	0.20	12.27	9.81
	394	0.26	0.90	0.19	4.31	0.55	12.77	11.56
	418	0.13	5.70	0.87	12.86	—	—	12.42
	460	−0.23	1.04	0.77	12.63	—	—	12.92
	500	−0.22	1.14	0.78	12.45	—	—	12.75

Empirical decay parameters (nanoseconds) obtained by a nonlinear least-square fit of a multiexponential model function to the fluorescence decay of the 2-AN/DML complex at 1° and 37°C. At each wavelength the sum of pre-exponential terms is normalized to 1.

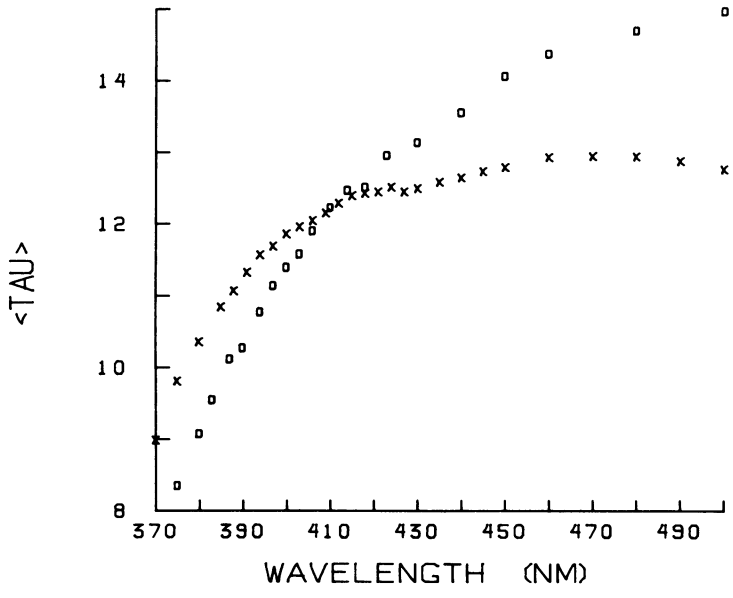

FIGURE 4 Mean lifetime of fluorescence emission decay of 2-AN in DML single bilayer liposomes vs. emission wavelength. □, 1°C; x, 37°C. Conditions are as in Fig. 2.

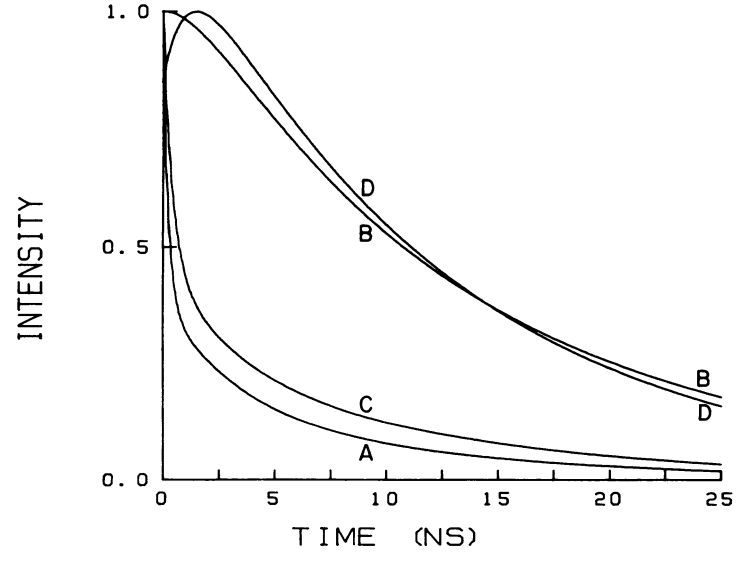

FIGURE 5 Normalized impulse response functions of 2-AN in DML single-bilayer liposomes at 1°C: (A) 380 nm, (B) 460 nm; and at 37°C: (C) 380 nm, (D) 460 nm. Conditions are as in Fig. 2.

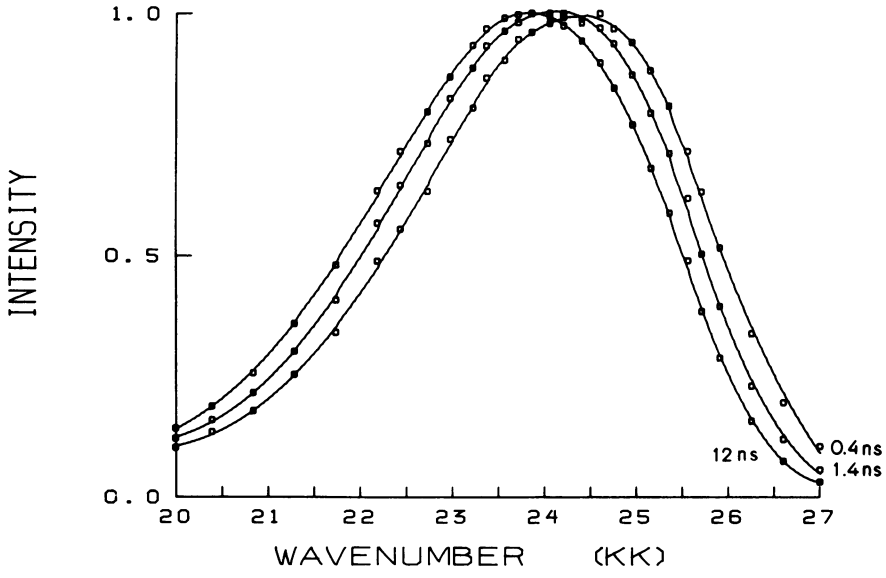

FIGURE 6 Peak normalized time-resolved emission spectra at 0.4, 1.4, and 12 ns. Conditions are as in Fig. 2.

processes both on the subnanosecond and the nanosecond time scales. The subnanosecond processes occur before the nanosecond time window of our experimental technique and are inferred from the apparent zero-time emission maxima observed on the nanosecond time scale. In the liposomes, for instance, 2-AN starts its nanosecond spectral relaxation from an apparent zero-time position close to that in glycerol and

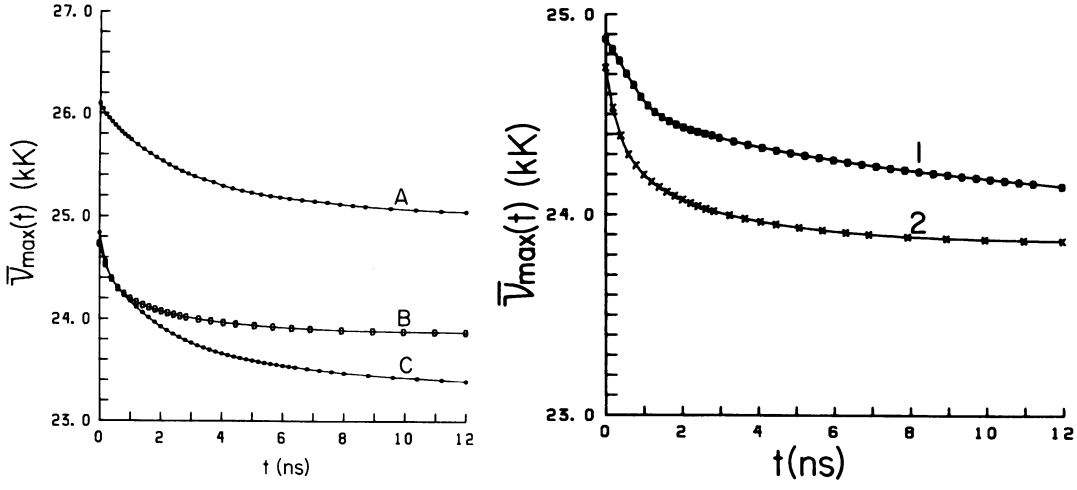

FIGURE 7 Right: wave number of maximum intensity vs. time of 2-AN in: (A) cyclohexane + 0.1 M ethanol mixture at 20°C; (B) DML single bilayer liposomes at 37°C; and (C) glycerol at 10°C. Left: variation of curve B with temperature: (*1*) at 1°C, (*2*) at 37°C.

more than 1 kK lower than the corresponding situation in cyclohexane containing 0.1 M EtOH. On the other hand, as shown in Fig. 7 (right), the zero-time spectral position of 2-AN in liposomes is practically independent of their physical state although the nanosecond relaxation shows a different behavior above and below the phase transition. To quantitate this difference, the curves representing the kinetics of the nanosecond spectral shift were analyzed in terms of an empirical multiexponential model, and the results are shown in Table II.

The best fit was obtained in each case with two exponentials and a constant. The constant represents the fully relaxed spectral position, which, as shown in Table II, is independent of temperature. In contrast, this position is reached approximately three times faster above the phase transition than below it. The additional, fast subnanosecond decay constant could be interpreted as the tail end of the inferred subnanosecond interaction mentioned above.

The data presented thus far indicate that 2-AN adsorbed to bilayer vesicles undergoes interactions with polar residues both on the subnanosecond and the nanosecond time scale. These interactions might involve mutual reorientational motion of the interactive species. It is, thus, of interest to follow the relaxation characteristics of the rotational motion of the fluorophore by measuring the decay of the emission anistropy both above and below the phase transition. These measurements can in principle be used to ascertain whether 2-AN undergoes rotational motion on the nanosecond time scale and whether this motion is related to the excited-state solvation reactions described above.

The fluorescence emission anisotropy is defined as follows:

$$r(t) = \frac{D(t)}{S(t)} = \frac{I_{VV}(t)G - I_{HV}(t)}{I_{VV}(t)G + 2I_{HV}(t)},$$

where $I_{VV}(t)$ is the observed decay with a vertical polarizer in both the excitation and emission beam. $I_{HV}(t)$ is the decay obtained with horizontally polarized excitation and

TABLE II

EMPIRICAL DECAY PARAMETERS ABOVE AND BELOW T_m

	Below T_m	Above T_m
Spectral relaxation	$\bar{\nu}_{max}(t) = 0.44e^{-t/0.51} + 0.61e^{-t/8.74} + 23.84$	$\bar{\nu}_{max}(t) = 0.44e^{-t/0.40} + 0.42e^{-t/2.81} + 23.86$
Anisotropy relaxation	$r(t) = 0.09\,e^{-t/6.5} + 0.07$	$r(t) = 0.13e^{-t/1.5} + 0.03$
Anisotropy in glycerol	$r(t) = 0.19\,e^{-t/397.0}$	$r(t) = 0.20e^{-t/28.8}$

Empirical decay parameters (nanoseconds) for spectral and anisotropy relaxation of 2-AN in DML bilayer liposomes above and below the crystalline-liquid crystalline phase transition (T_m = 22°C). The spectral relaxation experiments were carried out at 1° and 37°C and the anisotropy relaxation experiments at 2° and 40°C. Also shown is the decay of the emission anisotropy of 2-AN in glycerol at 0° and 28°C.

vertically polarized emission. $G = I_{HH}/I_{VH}$ and represents a small correction factor (Chen and Bowman, 1965).

The instrumental methods used to obtain the emission anisotropy data and the computational procedures utilized to extract the parameters characterizing $r(t)$ are similar to those described by Chen et al. (1977). Briefly the procedure was as follows. $I_{VV}(t)$ and $I_{HV}(t)$, together with a lamp profile, were collected during the same time period using the instrument in the sample alternation mode. The sum curve, $S(t)$, was analyzed in terms of a multiexponential decay law using the method of nonlinear least squares. A model function consisting of a sum of exponentials plus a constant was chosen for $r(t)$: $r(t) = \sum_i \beta_i e^{-t/\phi i} + c$. The values of the variable parameters β_i, ϕ_i, and c were obtained by convolving the product $r(t) \cdot s(t)$ with the lamp profile and searching for the best fit to the experimental difference curve, $D(t)$, by the nonlinear least-squares method. The best parameters obtained for the fluorescence emission anisotropy of the 2-AN vesicle system below (2°C) and above (40°C) the melting transition are given in Table II. The impulse response curves (deconvolved anisotropy) are shown in Fig. 8. Also shown in Table II are the parameters for the decay of the emission anisotropy for 2-AN in glycerol at 0° and 28°C. At both temperatures the decay of the anisotropy gave excellent fits to a single exponential. In contrast, 2-AN in lipo-

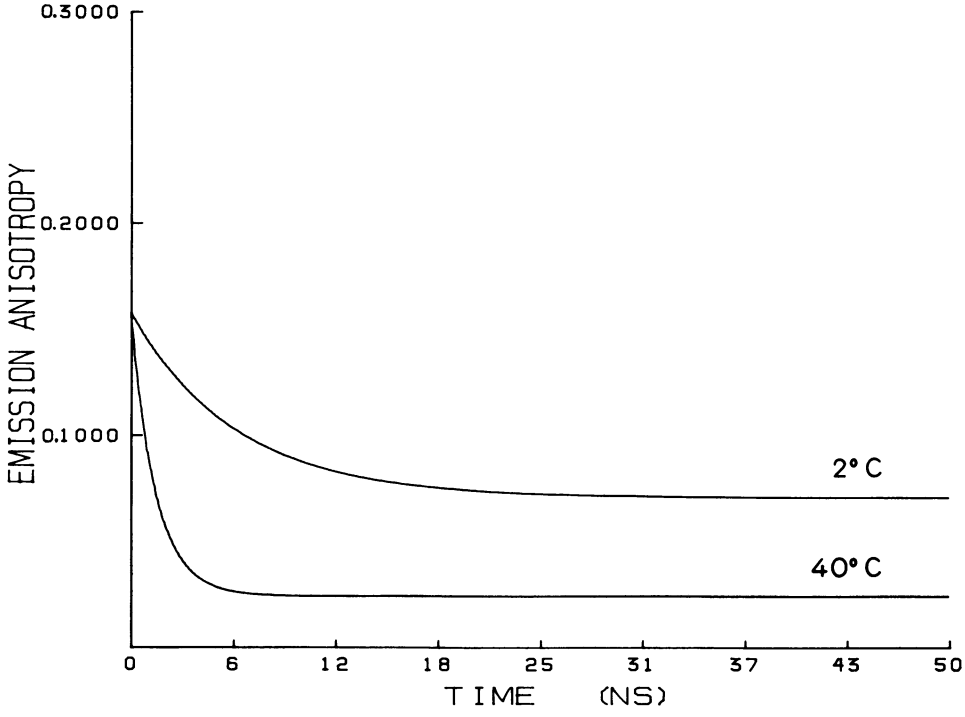

FIGURE 8 The impulse response curves for the decay of the emission anisotropy at 2° and 40°C. The parameters of these decays are given in Table II.

somes gave a decay of the emission anisotropy made up of a single exponential term plus a constant. This is consistent with the notion that the rotation of the dye in the liposomes is restricted. The restrictions are more stringent below the phase transition of the liposomes than above it. This is indicated by the higher value of the constant term at 2°C relative to that at 40°C. The rate at which these constant anisotropy levels are attained is also temperature dependent. Thus, anisotropy relaxation occurs about four times faster above the phase transition temperature than below it. As mentioned above, spectral relaxation had a similar temperature variation pattern. The potential implications of this similarity will be discussed below.

DISCUSSION

The results presented above show that 2-AN adsorbed to DML single-bilayer liposomes undergoes excited-state reactions on the nanosecond time scale. The decay kinetics across the emission band suggest a continuous type of relaxation rather than a simple two-state reaction. Thus the decay times are not wavelength independent, and more than two exponentials are required to fit the fluorescence decay data. Concomitant with this spectral relaxation, the molecule also rotates on the nanosecond time scale. Its emission anisotropy relaxation, initially exponential, was found to level off at a nonzero value at longer times. Thus, the rotation is restricted both above and below the phase transition of the liposomes. In each of the two physical states of the liposome, the initial rate of anisotropy relaxation is comparable to the nanosecond rate of spectral relaxation (Table II).

The interpretation of these data should be considered in relation to the location of 2-AN in liposomes. It is generally accepted that hydrophobic molecules not carrying a net charge such as 2-AN will be located in the apolar hydrocarbon region of the bilayer (Azzi, 1975; Badley, 1976). Thus the nuclear magnetic resonance study of Colley and Metcalfe (1972) located the related molecule 1 anilinonaphthalene (1-AN) deep inside the egg lecithin lipid bilayer, whereas the charged probe 1-anilinonaphthalene-8-sulfonate (1, 8 ANS) was found to be at the surface. Radda and Vanderkooi (1972) used the D_20 fluorescence enhancement effect exhibited by several fluorescence probes to estimate their location in different lipid bilayers. Again 1-AN was found to be better protected than 1, 8 ANS, suggesting its deeper location. Overath and Trauble (1973) have also concluded that 1-AN is a probe of the interior of the bilayer in contrast with charged molecules, such as ANS, which probe mainly the surface of the bilayer.

It seems reasonable to assume that 2-AN is also located in the interior of the bilayer. The characteristics of the relaxation of 2-AN emission anisotropy in glycerol and the liposomes are in agreement with this location. Whereas in glycerol the molecule rotates isotropically (monoexponential decay of the emission anisotropy), in the DML liposomes its rotation was found to be restricted both above and below the phase transition temperature. Similar results have been found with 1, 6-diphenyl-1,3,5-hexatriene (DPH) in DML liposomes (Chen et al., 1977; Kawato et al., 1977). This molecule has also been assumed in a variety of studies (see Chen et al., 1977, for a re-

view) to probe the interior of the bilayer. The initial relaxation times of DPH emission anisotropy are 5.2 ns below the phase transition (14.8°C) and 1.5 ns above it (37.2°C). As shown in Table II, the corresponding parameters of 2-AN anisotropy relaxation are similar (1.5 ns at 40°C, 6.5 at 2°C). Thus the temperature-induced change in the local microenvironment of the probe is reported in a parallel manner by these two molecules. In contrast the results of 2,6 toluidinonaphthalene sulfonate (2,6 TNS) emission anisotropy decay (Chen et al., 1977) adsorbed to the same liposomes present a different picture. The initial anisotropy relaxation time varies from 1.5 ns at 37°C above the phase transition to only 2.9 ns below it (practically independent of temperature in the range 1°–14°C). The molecule 2,6 TNS is similar in structure to 2-AN except for the negatively charged sulfonate group at the 6 position of the naphthalene ring. Like 1,8 ANS its location in the liposomes is considered to be in the polar head region.

The anisotropy relaxation results can be summarized as follows. A tighter packing of the lipids in the crystalline phase affects more markedly the initial rate of the anisotropy decay for probes of the interior than for those of the liposome surface. This temperature differential effect could be used as an initial indication of a potential location. However, the fluorescence anisotropy measurements by themselves could not assign a definite location to a molecular probe in the liposome.

If, based on all the evidence presented above, it is assumed that 2-AN is located in the interior of the bilayer, the origin of the nanosecond time-dependent spectral shifts remains to be explained. DeToma and Brand (1977) found that 2-AN dissolved in cyclohexane solution containing 0.1 M ethanol undergoes a classic two-state excited-state reaction. Its spectral relaxation time profile is represented as curve A in Fig. 7. In contrast tht decay kinetics of 2-AN in glycerol are not consistent with a two-state exiplex reaction and were interpreted in terms of a continuous multistate, solvation scheme. As shown in Fig. 7 (curve C), the initial spectral position of 2-AN in glycerol is more than 1 kK lower than the corresponding position in the cyclohexane—0.1 M ethanol system. It is clearly seen that the spectral relaxation profile of 2-AN in liposomes (curve B, Fig. 7) follows more closely the glycerol model system than the two-state exiplex system. As mentioned above, the empirical decay parameters presented in Table I also support this conclusion. This behavior indicates that 2-AN in liposomes undergoes continuous relaxation during which many polar residues are simultaneously interacting with the probe. Thus, either the probe is located close to the polar head region or small polar molecules (water, for instance) have access to it. This latter possibility is in contradiction with the D_20 experiments of Radda and Vanderkooi (1972). It could, however, be argued that the considerable permeability to water of phospholipid bilayer liposomes (5–100 $\times$ 10^{-4} cm/s; Papahadjopoulos and Kimelberg, 1973) is sufficient to induce a positive time-resolved emission spectroscopy effect and insufficient for an isotope fluorescence enhancement.

Our data support the possibility that the probe is interacting with polar moieties more rigidly held in place than with highly mobile, small polar molecules. This tentative conclusion is based on the comparable relaxation rates of spectral position and

anisotropy both above and below the phase transition (see Table II). Thus, the nanosecond spectral relaxation is characterized by an empirical time constant of 2.81 ns above the phase transition and 8.74 ns below it. The corresponding values for the initial anisotropy relaxation are 1.5 and 6.5 ns (Table II). In glycerol such a correlation is not found. The nanosecond part of the interaction between the probe and the polar moieties proceeds mainly through mutual reorientational motion of the interactive species (Bakhshiev et al., 1966). If this mutual reorientation motion is performed mainly by the probe, then both spectral and anisotropy relaxation would monitor it. It becomes the source of the common time scale for both effects. On the other hand, in a more mobile environment, such as glycerol, the dipolar reorientational motion is more likely to be performed mainly by the solvent molecules than by the excited chromophore. This would explain why in this case, when the relaxation processes are not monitoring a common motion, uncorrelated temperature variation patterns would be found.

Thus, the combination of time-resolved emission spectroscopy with the decay of the emission anisotropy provides a detailed kinetic picture of the interactions that small molecules undergo in biological systems. The parameters obtainable by steady-state measurements lump together many hidden variables each with its own physical state and temperature dependence. As shown by DeToma et al. (1976), the Bakhshiev theoretical approach (Bakhshiev et al., 1966) to solvent relaxation can be used to interpret in a self-consistent way the time-resolved emission spectroscopy data. In this analysis the time and energy dependence of the fluorescence intensity $F(\bar{\nu}, t)$ is described as a product of two terms, namely: $F(\bar{\nu}, t) = i(t)\rho(\bar{\nu}, t) = i(t)\rho(\bar{\nu} - \bar{\nu}_{max}(t))$, where $F(\bar{\nu}, t)$ is the observed fluorescence emission behavior, $i(t)$ is an electronic damping term that would characterize the decay law in the absence of spectral shifts, and $\rho(\bar{\nu} - \bar{\nu}_{max}(t))$ represents the normalized elementary fluorescence spectral contour shifted in energy by the amount $\bar{\nu}_{max}(t)$ at time t. This latter function, $\bar{\nu}_{max}(t)$ is obtained independently of the Bakhshiev theoretical model. In the present case its course in time has been empirically characterized in terms of two exponentials and a constant (Table II). The nanosecond decay constants are strongly temperature dependent. In contrast, the two decay constants characterizing the electronic damping term $i(t)$ are both temperature and wavelength independent (around 12.5 and 2.4 ns, respectively). Similar experiments carried out with 2,6 TNS absorbed to egg lecithin vesicles (Easter et al., 1977) showed that with this more polar probe the corresponding decay constants for $i(t)$ were temperature dependent. Their values decrease from 1.9 to 1.0 ns and from 9.7 to 4.8 ns as the temperature is increased from $-1°$ to 32°C. It is proposed that this difference between 2,6 TNS and 2-AN also reflects the different location of the two probes in the liposome. The latter molecule being more deeply buried is better protected against temperature-dependent quenching processes.

Comparative studies at the level of these hidden variables are potentially much more meaningful. For instance, as shown in Fig. 4, 2-AN in DML liposomes exhibits a negative temperature coefficient of the mean decay time in the blue region, whereas a

positive one is found in the red region. The tempting hypothesis of different locations of the probe in the liquid-crystalline and crystalline phases is, however, not warranted in this case. The decay constants for $i(t)$ being phase independent, the likelihood of such a situation is greatly diminished. Additional studies of this nature are needed to determine whether the fast relaxation processes discussed in this work are related to the ability of membranes to carry out their function of selective transport.

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