Fluorescence Lifetimes of Carbocyanine Lipid Analogues in Phospholipid Bilayers†

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Received November 2, 1984

ABSTRACT: The fluorescence lifetimes for the 1,1′-dialkyl-3,3,3′,3′-tetramethylindocarbocyanine ($C_N$diI) dyes (N = 12, 18, and 22) in a variety of lipid bilayer membranes were measured. Effects of bilayer physical state, probe chain length, probe concentration, charge, lipid head group, and cholesterol concentration were examined. Even in single-phase membranes these probes did not exhibit single-exponential decays. Rather, the data were fit by biexponential decays with lifetimes of ~0.3–0.4 and ~0.9–1.3 ns with no significant improvement in χ^2 convergence with the addition of a third component. Average lifetimes were dependent upon lipid phase and to a lesser degree surface charge and the phospholipid head group. In dipalmitoylphosphatidylcholine (DPPC)–cholesterol membranes, the C_{18}diI lifetime was sensitive to membrane reorganizations at both 20 and ~33 mol % cholesterol. In egg phosphatidylcholine (EPC) bilayers, the C_{18}diI lifetime was essentially independent of its concentration below 1:10³.

The $C_N$diI's[1] and related carbocyanine dyes have been extensively used as lipid analogues in biophysical studies of membranes (Derzko & Jacobson, 1980; Axelrod, 1979; Dragsten & Webb, 1978; Ethier et al., 1983; Fahey & Webb, 1978; Fahey et al., 1977; Klausner & Wolf, 1980; Peters, 1981; Peters & Richter, 1981; Schlegel et al., 1980; Schlessinger, 1977; Sims et al., 1974; Waggoner et al., 1977; Wolf & Voglmayr, 1984; Wolf et al., 1981a,b; Wu et al., 1977) and lipoproteins (Barak & Webb, 1981). They have been particularly useful as lipid analogues in fluorescence recovery after photobleaching studies of lipid lateral diffusion in both model and native membranes [for review, see Peters (1981)]. Results from recent experiments indicate that these probes select between coexistent lipid phases or domains by virtue of their alkyl chain length (Klausner & Wolf, 1980; Ethier et al., 1983). Data with the $C_N$diI's on cell membranes suggest that they may be selecting for specific domains within the membrane (Wolf et al., 1981a,b). The availability of structural variants of these probes with overlapping spectral properties (Sims et al., 1974) indicates their potential usefulness in a variety of donor–acceptor combinations in nonradiative resonance energy-transfer studies (Förster, 1948) of lateral membrane organization. A critical first step in such an approach is a consideration of the factors that govern the fluorescence lifetimes of these probes in lipid bilayer membranes.

In this paper we report studies of $C_N$diI lifetimes in a variety of single- and mixed-phase lipid bilayer environments. Specifically, we consider the effects of lipid phase, surface charge, lipid head group, and cholesterol and probe concentrations. These results are consistent with previous studies indicating selectivity of these probes between coexistent lipid phases and enable us to estimate partition coefficients for this selection. Our results also indicate that nonradiative resonance energy-transfer studies of membrane lateral organization with these probes should be feasible provided the probe concentration is ≤1:10³.

Materials and Methods

All phospholipids were purchased from Sigma (St. Louis, MO) and stored at −20 °C; previous batches from these lots had been verified to be of 99+% purity by thin-layer chromatography and quantitation with an Iatroscan TH-10 analyzer and differential scanning calorimetry. The C_NDiI's were prepared by Molecular Probes (Junction City, OR) as perchlorates. Their structure is shown in Figure 1. Purity of the dyes was confirmed by thin-layer chromatography.

For sample preparation, the dyes were dissolved in chloroform–methanol (2:1), and their concentrations were determined spectrophotometrically (Sims et al., 1974). For the preparation of liposomes, appropriate concentrations of lipids were premixed in $CHCl_3$–methanol (2:1). A solution of dye and lipids was shaken on a vortex genie, dried at room temperature under vacuum for at least 8 h, and stored under nitrogen.

Liposome Preparations. All liposomes were made by the following procedure: phosphate-buffered saline (PBS) at 80–82 °C was added to test tubes containing samples (also equilibrated to 80–82 °C) dried on the tube walls. Final lipid to buffer concentrations were 0.2–1.0 mg of lipid/mL of buffer. The tubes were immediately agitated rigorously on a vortex genie for a total of 60 s, returning them to the 80–82 °C water bath every 20 s for 2–5 s. After equilibration to room temperature, the contents were transferred to quartz cuvettes, and fluorescence lifetime measurements were made.

Liposomes in the gel, fluid, and 1:1 (gel:fluid) states were obtained by using distearoylphosphatidylcholine (DSPC), dioleylphosphatidylcholine (DOPC), and a 1:1 (DSPC:DOPC) mixture. (Phase transitions occur for DSPC and DOPC at 55 and −12 °C, respectively). In order to determine partition

† This work was supported by DOE Contact DE-Ac03-76SF00098 (Melvin P. Klein), NIH Training Grant T32AM07349 (B.S.P.), NIH Grant HD 17377 (D.E.W.), and a private grant from the A. W. Mellon Foundation to the Worcester Foundation.
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[1] Abbreviations: $C_N$diI, 1,1′-dialkyl-3,3,3′,3′-tetramethylindocarbocyanine (alkyl chain length = N); DOPC, dioleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; EPC, egg phosphatidylcholine; PA, phosphatidic acid; PG, phosphatidylglycerol; PS, bovine brain phosphatidylserine.

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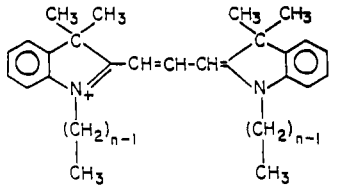

FIGURE 1: Structure of the $C_N$diI's where N is the alkyl chain length.

coefficients, fluorescence lifetime measurements were made in liposomes of each composition for $C_N$diI's of chain lengths 12, 18, and 22 at a molar dye to lipid ratio of 1:10^4.

To examine the effect of $C_N$diI concentration on fluorescence lifetimes, a series of egg phosphatidylcholine (EPC) liposomes was made with the concentration of $C_N$diI (N = 18) increasing from 9.9 × 10^{-4} to 9.1 mol %.

The effects of head groups and surface charge were examined by comparing the measured parameters of diI in the following phospholipidic environments: phosphatidylcholine (DOPC and DSPC), dipalmitoylphosphatidylcholine–dipalmitoylphosphatidylethanolamine (DPPC–DPPE) (1:1), stearylamine (SA), phosphatidic acid (PA), bovine brain phosphatidylserine (PS), sphingomyelin, and phosphatidylglycerol (PG). The dye:phospholipid ratio was 1:10^4 as above.

Lifetime Measurements. Fluorescence lifetimes were measured by a reverse single photon timing system in a previously described apparatus (Nairn, 1981; Turko et al., 1983) that contains a Spectra Physics synchronously pumped mode-locked laser (SP 171 argon ion laser and SP 362 mode locker) operating with a 12-ns pulse separation and a pulse full-width at half-maximum of ~200 ps (Figure 2). The limit of resolution of the instrument is ~80 ps. Excitation of samples was at 514.5 nm, and emission was detected at 565 nm with a cooled RCA C31034A photomultiplier. Samples were counted long enough to acquire 1 × 10^4 photons in the peak channel of a 1024-channel Northern NS636 multichannel analyzer. The response function (see Figure 2) of the apparatus was determined by measuring the scattering of the pulsed laser light at the excitation wavelength (514.5 nm) from an unlabeled liposome sample or a colloidal suspension of Ludox particles. Background scattering by the Ludox particles at the emission wavelength rarely exceeded the dark count rate. The true decay was obtained by iterative reconvolution with an assumed decay law approximated by a sum of exponentials:

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i)$$

where a_i is the fraction, normalized to unity, of molecules with lifetime τ_i. Minimizations of the deviations from this sum were guided by determining the least number of terms for convergence (Leskovar et al., 1976). Deviations from attempted fits are indicated by the pattern and magnitude of ΔI.

Data Analysis. The average lifetime was defined as

$$\langle\tau\rangle = \frac{\sum_{i=1}^{N} \int_0^\infty t\alpha_i \exp(-t/\tau_i)\, dt}{\sum_{i=1}^{N} \int_0^\infty \alpha_i \exp(-t/\tau_i)\, dt}$$

which, upon integration, is

$$\langle\tau\rangle = \sum_{i=1}^{N} \alpha_i\tau_i^2 / \sum_{i=1}^{N} \alpha_i\tau_i$$

As indicated below, data could always be fit by a biexponential decay with $\chi^2 \sim 1$–2 with no improvement in χ^2 convergence with the addition of a third component. This corresponds to uncertainties of ~80 ps in the τ's and 0.1 in the α_i's.

Partition Coefficients. Assuming that in mixed-phase vesicles the signal is composed of α_g, the fraction of the signal from the biexponential decay of $C_N$diI in the gel state, and $\alpha_f = 1 - \alpha_g$, the fraction of the biexponential decay of $C_N$diI in the fluid state, then the average lifetime $\langle\tau\rangle$ is given by

$$\langle\tau\rangle = \frac{\alpha_g \sum_{i=1}^{2} \alpha_{ig}\tau_{ig}^2 + (1-\alpha_g) \sum_{i=1}^{2} \alpha_{if}\tau_{if}^2}{\alpha_g \sum_{i=1}^{2} \alpha_{ig}\tau_{ig} + (1-\alpha_g) \sum_{i=1}^{2} \alpha_{if}\tau_{if}}$$

By rearranging and making the substitutions

$$TF_i = \tau_{if}\langle\tau\rangle - \tau_{if}^2$$

$$TG_i = \tau_{ig}\langle\tau\rangle - \tau_{ig}^2$$

α_g, the fraction of diI in the gel phase, is equivalent to

$$\alpha_g = \frac{\sum_{i=1}^{2} \alpha_{if} TF_i}{\sum_{i=1}^{2} (-1\alpha_{ig}) TG_i + \sum_{i=1}^{2} \alpha_{if} TF_i}$$

and α_f, the fraction in the fluid phase, is equivalent to $1 - \alpha_g$.

RESULTS

In all cases $C_N$diI's in bilayer environments did not exhibit monoexponential decays; empirically, convergence was achieved when curves were fit by a sum of two exponential terms. An example of this is illustrated in Figure 2, which shows the experimental decay and deviations' plots of (a) one- and (b) two-component fits for C_{22}diI in DOPC. Data from all experiments are summarized in Tables I and II.

Physical States. To determine the effect of phospholipid physical state within a single lipid class, the phosphatidylcholines, we performed two sets of experiments: (1) the C_{18}diI lifetime was measured at room temperature in EPC, DOPC, DPPC, and DSPC (see Table I). (2) C_{12}diI, C_{18}diI, and C_{22}diI lifetimes at room temperature were compared in pure gel DSPC, pure fluid DOPC, and mixed-phase bilayers of 1:1 DSPC–DOPC (see Table II). These data are summarized in Tables I and II.

For C_{18}diI we found an average lifetime of 0.80 ns in the pure fluid-state lipid DOPC and longer lifetimes of 1.20 and 1.32 ns in the gel-state lipids DPPC and DSPC, respectively. Similar, though somewhat reduced, effects of state were observed for C_{12}diI and C_{22}diI. In a 1:1 binary mixture of DOPC and DSPC, membranes known to have coexistent gel and fluid domains (Ladbrooke & Chapman, 1969), each probe exhibited an average lifetime intermediate to that measured in the pure lipid phases. Similarly for C_{18}diI, we observed an intermediate average lifetime of 0.96 ns in EPC bilayers that contain a heterogeneous mixture of chains. Qualitatively, one can assign a fluid-phase preference to C_{12}diI and C_{22}diI and a gel-phase preference to C_{18}diI by considering whether the average lifetime in the 1:1 membranes is closer to that observed in the pure fluid or the pure gel. A quantitative estimate of the partition can be calculated as described above. This equation gives the fraction of probe in the gel state, α_g, as 0.01 for C_{12}diI, 0.93 for C_{18}diI, and 0.22 for C_{22}diI. The validity of assumptions implicit in this calculation is discussed below.

Concentration Series. In order to determine the effect of $C_N$diI concentration, and more specifically at what concentrations significant probe–probe complexing occurs, we prepared vesicles of EPC with increasing mole percents of C_{18}diI from 9.9 × 10^{-4} to 9.1. The results of these experiments are shown in Figure 3 and summarized in Table I. We observed that the average lifetime was essentially constant at ~0.8 ns

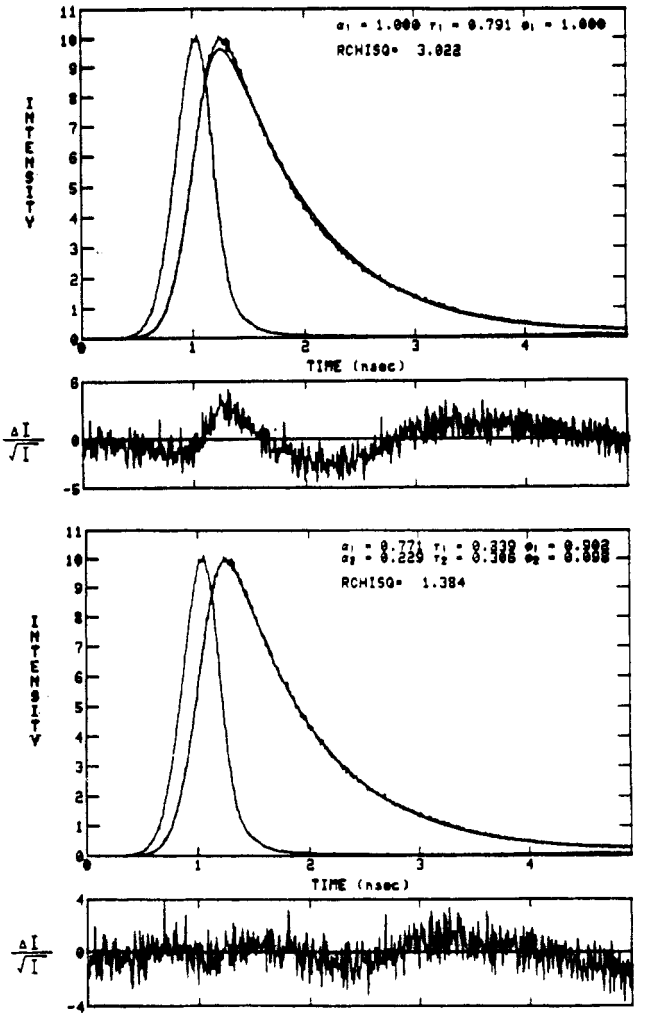

FIGURE 2: Fluorescence decay curves for C_{12}diI in DOPC bilayers 1:10^4 at room temperature. Top shows data fitted to a monoexponential decay. Leftmost curve is the incident light profile. Middle curve is the experiment fluorescence data. Rightmost curve is the best fit to the data. Bottom shows same data fitted to a biexponential decay. The experimental and best fit are barely distinguishable. Below each intensity plot are the statistically weighted least-squares residuals. $\Delta I/I^{1/2}$ is shown, where ΔI is the intensity difference experimental minus fit and I is the experimental intensity.

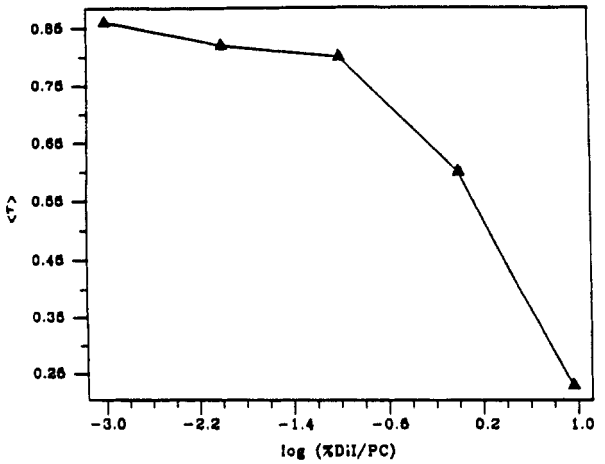

FIGURE 3: Average fluorescence lifetime as a function of C_{18}diI concentration in EPC membranes.

from 9.9 × 10^{-4}% to 9.9 × 10^{-2}% and then fell off rapidly to ~0.46 ns at 0.99% and 0.22 ns at 9.1%.

Head-Group Series. Since the fluorescent domain of the $C_N$diI's is in the polar head region of the bilayer (Axelrod,

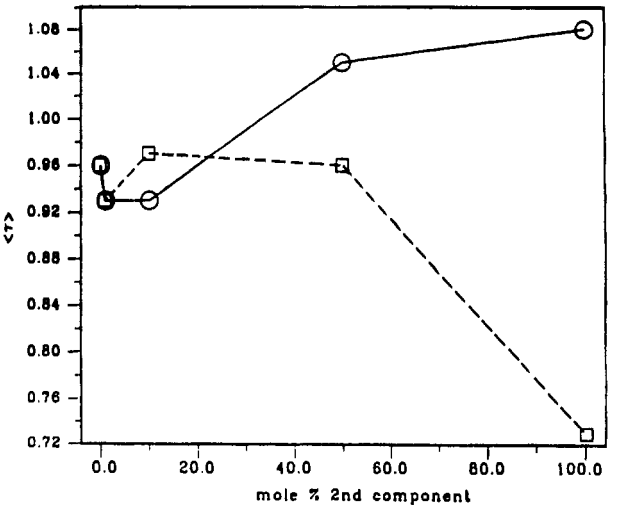

FIGURE 4: Average fluorescence lifetime for C_{18}diI in EPC membranes 1:10^4 doped with phosphatidic acid and stearylamine.

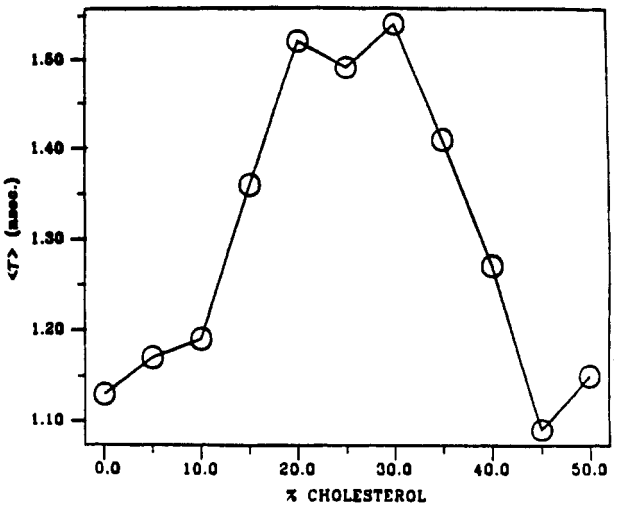

FIGURE 5: Average fluorescence lifetime for C_{18}diI in DPPC + cholesterol membranes (1:10^4 probe) at room temperature as a function of mole percent of cholesterol.

1979) and since this region is cationic, one might expect the fluorescence lifetimes of these probes to be sensitive to the nature of the polar head region of the lipid bilayer. To examine this possibility, two series of measurements were performed:

In the first series, the lifetime of C_{18}diI was measured in EPC bilayers with increasing amounts of phosphatidic acid (minus charge) or stearylamine (plus charge). The results of these experiments are shown in Figure 4 and summarized in Table I. Phosphatidic acid has only a slight increasing effect on C_{18}diI lifetime with variation of only about 10% going from pure EPC to pure PA. SA has no effect on C_{18}diI lifetime below 50%, but average lifetime decreases from 0.96 ns at 50% SA to 0.73 ns at 100% SA. This latter value is the lowest lifetime that we obtained for C_{18}diI in lipidic environments.

In the second series of experiments, membranes containing C_{18}diI were made with lipids of a variety of polar head groups: DPPC, PS, DPPC–DPPE (1:1), PG, and sphingomyelin. These data are summarized in Table I. Again, only small differences in average lifetimes were observed. The longest average lifetime observed for C_{18}diI, 1.44 ns, was obtained in DPPC–DPPE (1:1).

Cholesterol in DPPC Series. To measure the effect of cholesterol on C_{18}diI lifetime, we prepared DPPC membranes with 1:10^4 C_{18}diI and cholesterol in increments of 5% up to

Table I: Fluorescence Lifetimes for C_{18}diI in Lipid Bilayers

lipid	mol % C_{18}diI	one-component fit		two-component fit			
		τ	χ^2	α_1, α_2	τ_1, τ_2	χ^2	$\langle\tau\rangle$
Phosphatidylcholines Gel vs. Fluid							
EPC	1×10^{-2}	0.90	3.58	0.60, 0.40	1.10, 0.47	1.21	0.96
DOPC	1×10^{-2}	0.74	6.16	0.76, 0.24	0.86, 0.33	1.29	0.80
DPPC	1×10^{-2}	1.10	2.37	0.80, 0.20	1.19, 0.34	1.40	1.13
DSPC	1×10^{-2}	1.34	3.08	0.83, 0.17	1.37, 0.31	1.58	1.32
DOPC–DSPC (1:1)	1×10^{-2}	1.19	4.93	0.67, 0.33	1.42, 0.40	1.58	1.30
Concentration Series EPC + C_{18}diI							
EPC	9.9×10^{-4}	0.82	2.04	0.49, 0.51	1.05, 0.42	1.23	0.86
EPC	9.9×10^{-3}	0.82	1.32	0.67, 0.33	0.91, 0.34	0.99	0.82
EPC	9.9×10^{-2}	0.80	2.02	0.75, 0.25	0.86, 0.29	1.32	0.80
EPC	9.9×10^{-1}	0.61	1.75	0.77, 0.23	0.64, 0.19	1.33	0.60
EPC	9.1	0.22	2.49	0.24, 0.76	0.32, 0.17	1.95	0.23
Addition of Surface Charge							
100% EPC	1×10^{-2}	0.90	3.58	0.60, 0.40	1.10, 0.47	1.21	0.96
99% EPC + 1% SA	1×10^{-2}	0.87	3.86	0.57, 0.43	1.08, 0.45	1.20	0.93
90% EPC + 10% SA	1×10^{-2}	0.82	7.92	0.36, 0.64	1.29, 0.47	1.10	0.97
50% EPC + 50% SA	1×10^{-2}	0.85	5.69	0.42, 0.58	1.22, 0.49	1.15	0.96
100% SA	1×10^{-2}	0.45	14.3	0.01, 0.99	0.73, 0.00	5.96	0.73
100% EPC	1×10^{-2}	0.90	3.58	0.60, 0.40	1.10, 0.47	1.21	0.96
99% EPC + 1% PA	1×10^{-2}	0.84	6.60	0.55, 0.45	1.08, 0.35	1.04	0.93
90% EPC + 10% PA	1×10^{-2}	0.90	5.66	0.52, 0.48	1.02, 0.12	2.84	0.93
50% EPC + 50% PA	1×10^{-2}	0.94	6.69	0.57, 0.43	1.20, 0.34	1.15	1.05
100% PA	1×10^{-2}	0.96	6.82	0.55, 0.45	1.26, 0.42	1.22	1.08
PS	1×10^{-2}	0.98	3.59	0.60, 0.40	1.25, 0.40	1.20	1.10
sphingomyelin	1×10^{-2}	1.05	3.83	0.77, 0.24	1.22, 0.35	1.15	1.15
PG	1×10^{-2}	0.80	4.18	0.76, 0.24	0.94, 0.28	1.39	0.88
DPPC–DPPE (1:1)	1×10^{-2}	1.29	7.16	0.70, 0.30	1.54, 0.34	1.54	1.44
Cholesterol Series DPPC + Cholesterol							
100% DPPC	1×10^{-2}	1.10	2.37	0.80, 0.20	1.19, 0.34	1.40	1.13
5% cholesterol	1×10^{-2}	1.14	2.34	0.80, 0.20	1.23, 0.34	1.38	1.17
10% cholesterol	1×10^{-2}	1.16	2.48	0.80, 0.20	1.25, 0.32	1.48	1.19
15% cholesterol	1×10^{-2}	1.31	2.58	0.80, 0.20	1.42, 0.32	1.53	1.36
20% cholesterol	1×10^{-2}	1.47	2.56	0.82, 0.18	1.58, 0.34	1.71	1.52
25% cholesterol	1×10^{-2}	1.38	4.67	0.63, 0.37	1.61, 0.28	1.63	1.49
30% cholesterol	1×10^{-2}	1.46	3.88	0.69, 0.31	1.62, 0.23	1.59	1.54
35% cholesterol	1×10^{-2}	1.29	6.16	0.53, 0.47	1.57, 0.25	1.57	1.41
40% cholesterol	1×10^{-2}	1.16	7.29	0.45, 0.55	1.48, 0.24	1.46	1.27
45% cholesterol	1×10^{-2}	0.97	6.28	0.41, 0.59	1.38, 0.39	1.44	1.09
50% cholesterol	1×10^{-2}	1.00	6.75	0.37, 0.63	1.50, 0.41	1.67	1.15

Table II: Fluorescence Lifetimes for $C_N$diI's in Homogeneous and Mixed-Phase PC Bilayers

bilayer		C_{12}diI	C_{18}diI	C_{22}diI
DSPC	χ^2	1.33	1.58	1.40
	α_1, τ_1	0.68, 0.98	0.83, 1.37	0.73, 1.16
	α_2, τ_2	0.32, 0.35	0.17, 0.31	0.27, 0.42
	$\langle\tau\rangle$	*0.89*	*1.32*	*1.07*
DOPC	χ^2	1.26	1.29	1.16
	α_1, τ_1	0.77, 0.84	0.76, 0.86	0.74, 0.88
	α_2, τ_2	0.23, 0.31	0.24, 0.33	0.26, 0.36
	$\langle\tau\rangle$	*0.78*	*0.80*	*0.81*
1:1	χ^2	2.09	1.58	1.72
	α_1, τ_1	0.83, 0.84	0.67, 1.42	0.69, 0.96
	α_2, τ_2	0.17, 0.30	0.33, 0.40	0.31, 0.38
	$\langle\tau\rangle$	*0.80*	*1.30*	*0.87*

50%. These data are shown in Figure 5 and are summarized in Table I. As the concentration of cholesterol is increased, the average lifetime rises slightly between 0% and 10%; this is followed by a sharp rise (from ca. 1.2 to 1.5 ns) between 10% and 20%. The value remains at ca. 1.5 ns from 20% to 30%. Above 30%, the average lifetime drops and at 45% begins to level off at ca. 1.1 ns.

Discussion

We have found that the $C_N$diI's exhibit nonmonoexponential decay even in single-component lipid bilayers. Similar multiple-component decays have been observed for other fluorescent dyes in lipid bilayers as well as for related dyes in organic solvents. Such behavior does not necessarily imply a chemically heterogeneous or racemic population of probe but can also result from energy-level heterogeneities and excited-state reactions (Badea & Brand, 1979; Chakrabarti & Ware, 1971; DeToma & Brand, 1976; DeToma et al., 1976; Matayoshi & Kleinfeld, 1981). As such, it may not be appropriate to assign any physical significance to the two individual exponential components but rather to treat them as empirical parameters. Thus, for the present discussion we will confine our discussions of physical significance to the parameter average lifetime, which will be independent of the specific model chosen to fit the decay. This is so because the same average lifetime should be obtained from any function that equally (has the same χ^2) fits the decay data.

The $C_N$diI's have been shown to selectively partition between different lipid domains within a membrane (Ethier et al., 1983; Klausner & Wolf, 1980). This selectivity, which is dependent upon the $C_N$diI's alkyl chain length, makes them useful tools for probing the lateral organization of both model and natural lipid bilayers. It was the purpose of the measurements presented in this paper, by determining the effect of lipid environment and probe concentration on $C_N$diI fluorescence lifetimes, to facilitate such studies of lateral membrane organization.

The most straightforward environmental difference to consider is that between the gel and fluid states of a single

phospholipid class. Such a comparison may be made by comparing our data for C_{18}diI in DOPC, fluid at 23 °C, with those for C_{18}diI in DPPC and DSPC, both gels at 23 °C. Comparison may also be made for C_{12}diI and C_{22}diI in DOPC vs. DSPC. In all cases, the average fluorescence lifetime is longer in the gel state than in the fluid state. Comparable effects have been observed for other membrane-soluble fluorophores (Klausner et al., 1980; Sklar et al., 1977).

If we compare pure phase data to those of membranes that are made from a 1:1 mixture of the two lipids and contain both fluid and gel domains, we see that the lifetimes for C_{12}diI and C_{22}diI in the 1:1 mixture are much closer to those in the pure fluid than the pure gel phase, while C_{18}diI's lifetime is closer to that of the gel. We can thus qualitatively assign a fluid preference to C_{12}diI and C_{22}diI and a gel preference to C_{18}diI. This is consistent with both thermodynamic (Ethier et al., 1983) and photobleaching studies (Klausner & Wolf, 1980) with these probes. As discussed above, we can model these data to obtain fractions of probe in the gel state: $\alpha_g = 0.93$ for C_{18}diI, 0.01 for C_{12}diI, and 0.22 for C_{22}diI. It must be pointed out that since the environmental data can always empirically be fit by biexponential decays, treating the signals from the 1:1 samples as being composed of α_g times the biexponential decay of a pure gel and $1 - \alpha_g$ times the biexponential decay of the pure fluid is somewhat arbitrary and thus does not necessarily provide a unique solution to the partition.

Our calculation of the partition was based upon the conventional [see, for instance, Demas (1983) or Lakowicz (1983)] definition of the average lifetime as the mean dwell time of the molecule in its excited state given by the ratio of the first- and zero-order moments. The less conventional definition of $\langle\tau\rangle \equiv \int_0^\infty I(\tau)\, dt$ has the advantage that the partition takes the simple interpolation form:

$$\alpha_g = \frac{\langle\tau\rangle_f - \langle\tau\rangle}{\langle\tau\rangle - \langle\tau\rangle_g}$$

where $\langle\tau\rangle_f$ and $\langle\tau\rangle_g$ are the average lifetimes obtained in pure fluid and gel phases, respectively. This form is true, independent of the empirical parameters that fit the data. Of course the empirical parameters must be used to apply this formula. Use of this formula gives values for α_g of 0.50, 0.76, and 0.16 for C_{12}diI, C_{18}diI, and C_{22}diI, which exhibit the same trend as the values that we have calculated.

Since the $C_N$diI's are cationic and since the fluorescent group is at the bilayer water interface (Axelrod, 1979), one might expect it to be sensitive to either surface charge or the characteristics of the phospholipid head-group region.

If one confines oneself to considering bilayers doped with 10% PA or SA or less, where one should not need to be concerned with lateral segregations or the formation of nonbilayer phases, one finds that the C_{18}diI lifetime is not particularly sensitive to the addition of either positive, SA, or negative, PA, surface charge.

Data for the effect of lipid head group upon C_{18}diI lifetime are complicated by the fact that some of the lipids are fluid and some are gel at 23 °C. However, if one compares data for the pure fluid-state lipid, DOPC, we see that C_{18}diI has an average lifetime of 0.80 ns, while in each of the solid-state lipids, DPPC, DSPC, DPPC–DPPE (1:1), and sphingomyelin, the average lifetime is $\geq$1.2 ns. Similarly, if we compare the data for EPC, PS, and PG (the PS and PG are commercially derived from the same EPC by the action of phospholipases and thus should contain roughly the same chain heterogeneity), we see that the C_{18}diI lifetime is always $\leq$1.1 ns. While one cannot make a definitive statement without comparing vesicles of many more compositions, the present data suggest effects on fluorescence lifetime of physical state dominate over those of lipid head groups.

Data from the cholesterol series are in accord with its observed effects on phase transitions in lipid bilayers (Melchior et al., 1980). The peak in average lifetime that is a plateau between 20 and 30 mol % cholesterol (Figure 5) suggests that in this range C_{18}diI molecules are probing an environment of relatively increased order. Presti et al. (1982) have suggested that below 20 mol % pure phospholipid domains coexist with 2:1 phospholipid–cholesterol complexes, between 20 and 33 mol % boundary lipids are disappearing leaving the 2:1 complexes in media of increasing cholesterol concentration, and between 33 and 50 mol % cholesterol-rich domains coexist with the remaining phospholipids in 1:1 hydrogen-bonded complexes with cholesterol. Our data show that the C_{18}diI fluorescence lifetime is sensitive to both the putative change in lateral membrane organization at 20 mol % and the change at ~33 mol %.

As discussed above by virtue of their alkyl chain length dependent phase partition, the $C_N$diI's should be useful as probes of lateral membrane organization. A particularly promising approach to the problem is to use the $C_N$diI's in nonradiative resonance energy-transfer experiments. Critical to such studies is a knowledge of the concentration range in a membrane in which these probes exist as monomers. As has been shown previously (Sims et al., 1974), as these probes dimerize and form larger size aggregates, the spectra become altered, and the fluorescence is significantly quenched. The effect of C_{18}diI concentration in EPC membranes in fluorescence lifetime was measured to determine in which range monomeric fluorescence was obtained. As seen in Figure 3, there is little change in fluorescence lifetime from a C_{18}diI:lipid ratio of $1:10^5$ to $1:10^3$. Above this concentration, lifetime shortens dramatically, presumably indicating dimerization and larger scale aggregation.

Conclusions

In conclusion, we have found the following: (1) The $C_N$diI's exhibit nonmonoexponential decay even in homogeneous lipid phases. Empirically, the data are best fit by a biexponential decay with one lifetime of ~1–1.3 ns and the other ~0.3–0.4 ns. (2) $C_N$diI average fluorescence lifetime is sensitive to lipid environment, lipid phase, and to a lesser extent lipid head group. In some instances, average lifetime can be used to estimate the partition of probe between coexistent lipid phases. (3) C_{18}diI fluorescence lifetime is sensitive to membrane cholesterol. In DPPC–cholesterol membranes, average fluorescence lifetime for C_{18}diI is sensitive to changes in membrane organization at both 20 and ~33 mol % cholesterol. (4) The Effect of C_{18}diI concentration in EPC membranes indicated that the probe primarily exists in its monomeric form for concentrations $\leq 1:10^3$.

Acknowledgments

We are grateful to Drs. Melvin P. Klein, Kerry K. Karukstis, Alan Kleinfeld, Ludwig Brand, Jay Knutson, David A. Pearlman, and Dana Walbridge for their many constructive discussions and to Stephanie Hagopian for her help in preparing samples. We are also grateful to one of our reviewers for the suggestion of an alternative definition of the average lifetime.

Registry No. C_{12}diI, 84109-08-0; C_{18}diI, 41085-99-8; C_{22}diI, 84109-15-9; DOPC, 4235-95-4; DPPC, 2644-64-6; DSPC, 4539-70-2;

SA, 124-30-1; DPPE, 3026-45-7; cholesterol, 57-88-5.

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