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Journal

Biophysical Journal, 59(2)

ISSN

0006-3495

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Publication Date

1991-02-01

DOI

10.1016/s0006-3495(91)82240-8

A photophysical model for diphenylhexatriene fluorescence decay in solvents and in phospholipid vesicles

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ABSTRACT The fluorescence decay of 1,6-diphenyl-1,3,5-hexatriene (DPH) in pure solvents and in phospholipid vesicles has been measured using frequency domain fluorometry. Data analysis uses a model with two energetically close excited states. The model explains the high quantum yield and the double exponential decay of DPH observed in some pure solvents and in phospholipid vesicles. This model assumes that after excitation to a first excited state, there is a rapid interconversion to a lower excited state and that most of the emission occurs from this state. The interconversion rates between the two excited states determine the average lifetime. For DPH in solvents, we find that the interconversion rates are solvent and temperature dependent. For DPH in phospholipid vesicles, we find that the back reaction rate from excited state 2 to excited state 1 ($R12$) is what determines the fluorescence properties. The phospholipid phase transition affects only this back reaction rate. The model was analyzed globally for a range of solvents, temperatures and vesicle composition. Of the six parameters of the model, only two, the interconversion rates between the two excited states, varied in all different samples examined. For DPH in phospholipid vesicles, there is an additional feature of the model, which is related to the apparent distribution of the rate $R12$. Significantly better fits were obtained using a continuous lorentzian distribution of interconversion rates. The resulting lifetime distribution was asymmetric and showed a definite narrowing above the phase transition.

INTRODUCTION

DPH (1,6-diphenyl-1,3,5-hexatriene)[1] is one of the most widely used fluorescent probes in studies of biological membranes (Chen et al., 1977; Beddard and West, 1981). Among DPH's favorable spectroscopic properties, such as high quantum yield in hydrophobic environments and negligible fluorescence in water, is a lifetime sensitive to environmental properties.

In natural membranes, different phase states may segregate into domains (Klausner et al., 1980; Barrow and Lentz, 1985; Lentz et al., 1985). Observation and quantitation of coexisting gel and liquid-crystalline phases in a natural bilayer have important implications for understanding the membrane's role in cell life. The partition and activity of membrane-associated proteins and enzymes is believed to be affected by the lipid's phase state (Kimelberg, 1977).

Steady-state fluorescence polarization studies of membranes using DPH as a probe have shown that the transition between the gel and the liquid-crystalline phases can be followed by changes in polarization (Shinitzky et al., 1971). For nonmiscible binary mixtures of phospholipids, DPH polarization detects two distinct phase transitions (Klausner et al., 1980; Barrow and Lentz, 1985). However, steady-state polarization cannot be resolved for the contribution of different emitting species, because fluorescence polarization depends on the contribution of several factors. Consequently, steady-state polarization cannot be used to study phase domain coexistence in natural membranes of unknown composition. A number of time-resolved fluorescence anisotropy studies of membranes utilizing DPH have been made. It is now generally agreed that the anisotropy reports on the order of the system; the r_∞ value is very sensitive to the lipid phase state. In contrast, the rotational diffusion constant that is also obtained from these measurements seems to be quite insensitive to the lipid phase (Jähnig, 1979). The steady-state anisotropy depends both on the fluorescence lifetime and order parameter. For steady-state anisotropy, changes in lifetime and order parameter in different phases are compensatory.

To resolve phase coexistence, it is necessary to analyze the fluorescence decay for at least two emitting components with different spectroscopic properties, such as the lifetime and the limiting anisotropy. The interconversion between phases should correspond to the interconversion between the two sets of spectroscopic properties. Resolution of their properties requires great precision in measuring the fluorescence parameters and

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[1]*Abbreviations used in this paper:* DMOPC, dimyristoleoyl-phosphatidylcholine; DMPC, dimyristoyl-phosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPHpPC, 1-palmitoyl-2[[2-[4-(6-phenyl-*trans*-1,3,5-hexatrienyl)phenyl]ethyl]carboxyl]-3-*sn*-phosphatidylcholine; DPPC, dipalmitoyl-phosphatidylcholine; PBS, phosphate buffered saline.

0006-3495/91/02/466/10 $2.00

a detailed understanding of their relation to the physical state of the membrane.

In liposomes, DPH lifetimes differ in the pure gel and liquid-crystalline phases (Barrow and Lentz, 1985; Dale et al., 1977; Stubbs et al., 1981; Parasassi et al., 1984). Attribution of decay parameters to specific photophysical properties of the DPH molecule, which can then be related to the different phases, is problematic. First, DPH decay in single phospholipid, single-phase liposomes, is not single exponential. Second, lifetime variation does not correlate with specific environmental properties such as temperature.

DPH decay in liposomes has been described by models with discrete exponential components (Barrow and Lentz, 1985; Stubbs et al., 1981; Parasassi et al., 1984) and continuous lifetime distributions (Fiorini et al., 1987). There is good agreement that the main lifetime component in pure gel and pure liquid-crystalline phases is $\sim$10 and 7 ns, respectively. A short-lived component of the DPH decay, with low fractional intensity ($<2\%$), has also been observed in the membrane samples studied (Parasassi et al., 1984, 1987; Fiorini et al., 1987, 1988). The short-lived component was initially attributed to small amounts of photodecomposition product, the concentration depending on preparation of the sample, presence of oxygen in the medium, and unsaturation of the phospholipid acyl chains (Parasassi et al., 1984). We investigated DPH decay in carefully prepared liposomes by successive warming and cooling cycles. The results show that a major fraction of the short component changes reversibly with the temperature cycle (unpublished results). This observation points out that, in "clean" samples, the short-lived component must be primarily attributed to intrinsic features of DPH decay in membranes, although its origin remains unknown. However, even in "clean" samples, a small fraction of this short lifetime component is due to photodegradation product that is commonly observed for DPH in solvents and membranes.

An additional problem complicates the investigation of DPH lifetime changes. The value of the decay rate is not related to any specific properties of the lipid phase. With the aim of establishing a correlation between DPH decay and environmental properties, we measured DPH lifetimes in a series of isotropic solvents. In isotropic solvents at room temperature, the DPH lifetime has been related to the dielectric constant of the medium (Parasassi et al., 1984, 1987; Fiorini et al., 1987, 1988; Zannoni et al., 1983). The lifetime value does not correlate with solvent viscosity. In general, a shorter DPH lifetime is observed for media of higher dielectric constant. Nevertheless, for some solvents, we have observed exceptions to this rule. Moreover, measurements performed in ethanol, in choloroform at low temperatures, and in tetrahydrofuran showed the appearance of a short lifetime component with small fractional intensity similar to the component observed in liposomes. The lifetime of DPH shows an unusual temperature dependence in some solvents. In dodecane, cyclohexane, tetrahydrofuran, and chloroform, the lifetime value shows a small but appreciable increase with increasing temperature. Similar behavior in various solvents has been previously documented (Birks and Birch, 1975; Birch and Imhoff, 1982).

The aim of the present study is to provide evidence supporting a model for the excited state behavior of DPH, which explains (*a*) the origin of the double exponential decay observed in single-phase phospholipid vesicles and in some isotropic solvents, and (*b*) the anomalous behavior of the temperature dependence of lifetime observed in other solvents. The proposed model also reconciles the general observation that DPH lifetimes are much higher than the values calculated from its absorption spectrum (Zannoni et al., 1983; Birks and Birch, 1975; Birch and Imhoff, 1982; Cundall et al., 1979). Our model allows a correlation between certain photophysical parameters of DPH decay and physical properties of the medium.

PHOTOPHYSICAL MODEL FOR DPH FLUORESCENCE DECAY

We propose a model for DPH fluorescence decay based on two separate, interconverting states (Fig. 1). Only state 1 is populated upon absorption; excited state 1 is higher in energy than excited state 2. From state 1, DPH molecules can decay to the ground state with an emission characterized by a fast decay rate of $\sim 0.7\ ns^{-1}$. This value was established by considering the radiative decay rate derived from the absorption coefficient, using the Strichler and Berg approach (Zannoni et al., 1983; Birks and Birch, 1975; Birch and Imhof, 1982; Cundall et al., 1979; Hudson and Kohler, 1973). This predicts that a molecule with a large extinction coefficient must have a short lifetime. Alternatively, DPH molecules can interconvert to state 2 at a high rate, $R21$, which accounts for the high fluorescence intensity. Most of the observed DPH emission originates from state 2, with a decay rate of $\sim 0.06\ ns^{-1}$. This value was fixed in the analysis and was determined on the basis of compatibility with all of the experimental results. This value also corresponds to the radiative decay rate reported by Zannoni et al. (1983). A slower backward interconversion rate, $R12$, from state 2 to state 1 is also a part of the model. Both interconversion rates are sensitive to solvent and temperature effects. The two emitting states can have different spectra. The relative observation of one state with

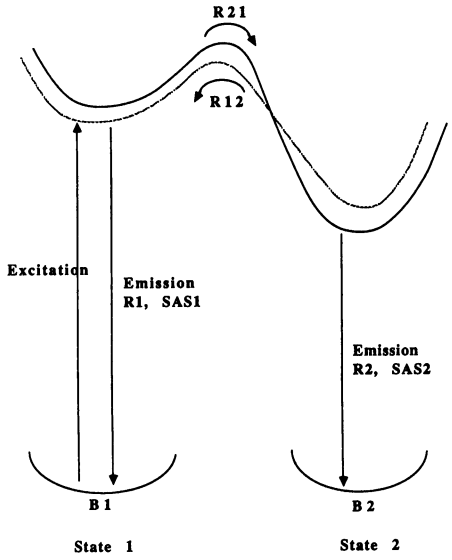

FIGURE 1. Photophysical model for DPH decay. Excitation can only occur from state 1. Both state 1 and state 2 can emit with different rates. $R21$ and $R12$ are the rates of interconversion between state 1 and state 2, respectively. $R1$ and $R2$ are the rates of decay from state 1 and state 2, and $B1$ and $B2$ are the initial population of state 1 and state 2, respectively. The dotted line represents the low temperature modification of the relative position of the two excited states.

respect to the other is contained in a single parameter, $SAS1$ (species associated spectra). If the two states emit with the same spectral contour, $SAS1$ is 0.5, irrespective of the properties of state 1 or state 2. So, $SAS1$ depends only on the spectral overlap and experimental observation conditions.

A similar model for DPHpPC was proposed by Lentz and co-workers to interpret the complex photophysics arising from energy transfer between DPHpPC molecules at high concentrations (Lentz et al., 1988).

The theoretical justification for the two-excited-state model is based on calculation of the excited states of DPH in which it was found that the two excited states S_1 S_2 are very close in energy (see Zannoni et al., 1983, for a review). The relative position of these two states is solvent and conformation dependent. The ground state is totally symmetric ^1A_g while the two excited states are assigned to $^1B_u^*$ and $^1A_g^*$ symmetry. It is likely that the absorption corresponds to the $^1A_g \rightarrow {}^1B_u^*$-allowed transition given the high extinction coefficient ϵ_{340} = 80,000 $M^{-1}cm^{-1}$. This transition, since it is strongly allowed, should also have a relatively short lifetime. The second excited state should have $^1A_g^*$ symmetry and therefore should have a long lifetime due to the $^1A_g^* \rightarrow {}^1A_g$ forbidden transition. The precise mechanisms by which one excited state can interconvert to the other is not clear. The present investigation shows that this process depends on several parameters including the temperature and the nature of the solvent.

MATERIALS AND METHODS

Sample preparation

DPH was purchased from Molecular Probes Inc. (Junction City, OR). Dimyristoyl-, dimyristoleoyl-, and dipalmitoyl-phosphatidycholine (DMPC, DMOPC, DPPC) were from Avanti Polar Lipids, Inc. (Birmingham, AL). Chloroform, cyclohexane, and tetrahydrofuran were high purity from Burdick and Jackson (Baxter Health Care Corp., McGaw Park, IL). Dodecane was from Aldrich Chemical Co. (Milwaukee, WI). Ethanol was from USI Chemical Co. (Cincinnati, OH). All solvents were tested for fluorescence at the wavelengths of interest and used as supplied.

Due to the photosensitivity of DPH, the solvent samples were prepared in darkness by purging with argon for approximately an hour and then sealed in a special grease-free anaerobic cuvette. Samples were always prepared just before use. To check the completeness of purging, the emission intensity with time was measured for each sample before and after lifetime measurements (Fig. 2). Absorption spectra of the samples had an optical density of ≤ 0.1 at 325 nm.

DPH labeled multilamellar phospholipid vesicles were prepared as previously reported (Parasassi et al., 1984). The probe-to-lipid ratio was always in the range of 1/1,000–1/800, and the background fluorescence of the vesicles was $< 0.5\%$ of the total fluorescence. Final phospholipid concentration was 0.1 mM in Dulbecco's PBS.

Fluorescence steady-state measurements

Time-resolved emission was measured using the phase fluorometer described by Gratton and Limkeman (1983). The excitation source was an argon ion laser (λ = 351 nm) or a He-Cd laser (λ = 325 nm). Some of the measurements for DPH in phospholipids were performed on a GREG 200 phase fluorometer (ISS Inc., Champaign, IL) equipped with a xenon-arc lamp, using an excitation wavelength of 360 nm and a 16 nm bandwith. Phase and modulation data were collected using 9–13 modulation frequencies ranging from 2 to 180 MHz.

Lifetime measurements were performed using a reference solution

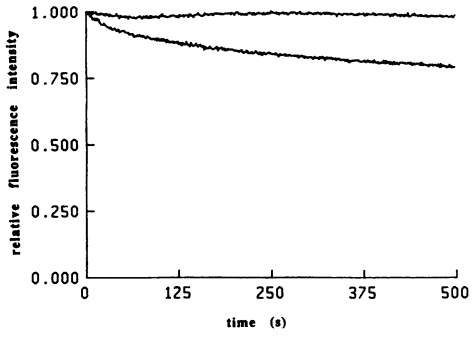

FIGURE 2. Time evolution of the fluorescence intensity of DPH in chloroform. Excitation was at 325 nm, emission at 425 nm, using 8 nm slits. The effect of purging is shown by the difference of the two samples; the upper curve is the deoxygenated sample, and the lower curve is the nondeoxygenated sample. The average power at the cuvette was ~3 mW.

of POPOP in ethanol (τ = 1.35 ns) or a glycogen scattering solution. Emission was observed through different cutoff filters for different excitation wavelengths: KV370 (Schott Glass Technologies Inc., Duryea, PA) for λ_{ex} = 325 nm; 3–74 (Corning Glass Works, Corning, NY) for λ_{ex} = 351 nm; or 420 (Corion Corp., Holliston, MA) for λ_{ex} = 360 nm.

Data were analyzed using the Globals Unlimited software developed at the Laboratory for Fluorescence Dynamics (University of Illinois and Urbana-Champaign) (Beechem and Gratton, 1988). The model contains two decay rates, $R1$ and $R2$ from states 1 and 2; two interconversion rates, $R21$ and $R12$, forward and backward between the first and second state, respectively; one $SAS1$, corresponding to the relative observation of state 1; and two boundary conditions, $B1$ and $B2$, referring to the ground-state population of the two states. For DPH in phospholipid vesicles, the interconversion rate $R12$ was fitted using a lorentzian distribution. For distributed cases, Globals Unlimited software performs a numercial integration using a Romberg algorithm. The minimization program uses the Marquardt-Levenberg algorithm. Derivatives were calculated numerically using the central difference formula and 0.1 relative increments. For the calculation of the reduced χ^2, the standard deviation of phase and modulation was assumed to be 0.2° and 0.004, respectively. The lorentzian model parameters were evaluated using the 67% χ^2 confidence interval routine of the Globals Unlimited software (Beechem and Gratton, 1988).

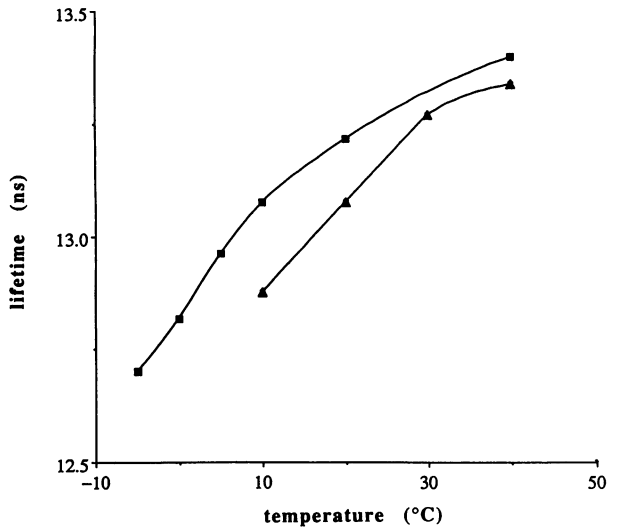

FIGURE 3. DPH lifetime values versus temperature in dodecane (■) and cyclohexane (▲).

RESULTS

DPH decay in isotropic solvents

The decay curves for DPH in different solvents were first analyzed using a single or a double exponential analysis. DPH decay in dodecane and in cyclohexane at temperatures from −5 to 50°C and from 10 to 40°C, respectively, can be described by a single exponential component, with a lifetime value of ~13 ns for both solvents. An unusual increase of the lifetime value was observed with increasing temperature, varying from 12.70 ± 0.02 ns to 13.40 ± 0.07 ns in dodecane and from 12.87 ± 0.05 ns to 13.34 ± 0.04 ns in cyclohexane (Fig. 3). A double exponential model was necessary to describe the DPH decay in ethanol, chloroform, and tetrahydrofuran (Table 1). The shorter component was always associated with a small fractional intensity, varying with temperature and with solvent from ~0.1 to 0.01. High fractional intensities (~8%) for the short-lived component were found in ethanol. The experiment performed in cholorform at low temperature (−28.5°C) showed the appearance of a negative pre-exponential factor, which is considered to be representative of an excited state reaction. Actually, by measuring the DPH decay in chloroform at −28.5°C and selecting the blue and the red edge of the emission with interference filters (λ = 410 and 530 nm, 10-nm bandwith) (Corion Corp.), different fractions were obtained for the two lifetime components (Table 1). In chloroform and in tetrahydrofuran the main component of the DPH decay showed an increase in the lifetime value with increasing temperature.

The two-excited-state photophysical model for DPH fluorescence decay was tested using the Globals Unlimited analysis method (Beechem and Gratton, 1988). The data were fit for all solvents (Table 2) and all temperatures using fixed decay rates of 0.67 and 0.063 ns^{-1} from

TABLE 1 DPH lifetime values vs. temperature in chloroform, ethanol and tetrahydrofuran

	T	τ_1	f_1	τ_2	f_2	χ^2
	°C					
Chloroform						
	−28.5	5.63	1.003	0.02	−0.003	2.37
	−20.0	5.73	1.002	0.10	−0.002	2.25
	−8.0	6.18	0.961	2.44	0.039	0.34
	0.0	6.31	0.968	2.11	0.032	0.88
	20.0	6.47	0.980	1.01	0.020	0.75
	30.3	6.51	0.984	0.67	0.016	0.55
	−28.5					
	(*em* = 410 nm)	5.34	0.962	0.17	0.038	4.30
	(*em* = 530 nm)	5.64	1.012	0.50	−0.012	0.88
Ethanol						
	−40.0	11.91	0.918	5.29	0.082	1.26
	−30.0	11.40	0.965	2.89	0.035	0.90
	−10.0	10.30	0.954	1.85	0.046	1.71
	0.0	9.53	0.948	1.44	0.052	0.82
	10.0	8.41	0.953	0.98	0.047	0.67
	20.0	7.46	0.951	0.81	0.049	4.93
Tetrahydrofuran						
	0.0	9.32	0.993	2.50	0.007	1.03
	10.0	9.74	0.991	1.82	0.009	1.48
	40.0	9.96	0.989	0.87	0.011	5.23

TABLE 2 **Values obtained for DPH in solvents of the forward (*R*21) and backward (*R*12) interconversion rates between state 1 and state 2, of the species associated spectrum of state 1 (*SAS*1) and of the measured average lifetime (τ)**

Solvent	*R*21	*R*12	*SAS*1	$\langle\tau\rangle$	*R*21/*R*12
Dodecane	3.85	0.12–0.09	0.47	13.2	42.79
Cyclohexane	4.45	0.14–0.11	0.47	13.1	44.00
Tetrahydrofuran	4.25	0.40–0.33	0.47	9.5	12.80
Chloroform	3.70	0.97–0.77	0.47	6.2	4.80

	T	*R*21	*R*12	*R*31	*SAS*1	*SAS*2
	°C					
Ethanol						
	−40.0	0.33	0.04	0.27	0.20	0.69
	−30.0	0.31	0.04	0.28	0.20	0.69
	−20.0	0.29	0.03	0.30	0.20	0.69
	−10.0	0.27	0.05	0.31	0.20	0.69
	0.0	0.19	0.05	0.14	0.20	0.69
	10.0	0.26	0.04	0.24	0.20	0.69
	20.0	0.27	0.07	0.33	0.20	0.69

The values of *R*12 are variable with temperature, as reported, and are expressed as nanoseconds^{-1}. The ratio between the two interconversion rates is also reported (*R*21/*R*12). For DPH in ethanol the value of the interconversion rate from state 1 to state 3 (*R*31) and the species associated spectrum of state 2 (*SAS*2) are reported for the different temperatures.

state 1 and from state 2, respectively. The *SAS*1 value was found to be 0.47. In all experiments, in accordance with our model, the initial boundary condition of 1.0 and 0.0 were fixed for the absorption of state 1 and state 2, respectively. The *R*21 values varied with the solvent from 4.45 ns^{-1} in cyclohexane to 3.70 ns^{-1} in chloroform, but were temperature independent. The *R*12 values varied with temperature in the same solvent and showed large variations between different solvents. The ratio between the two rates (*R*21/*R*12) varied from ~40, in dodecane and in cyclohexane, to ~10 in tetrahydrofuran, and to ~5 in chloroform. Higher "apparent" lifetime values correspond to higher *R*21/*R*12 ratios. The χ^2 values obtained using our model were similar to those obtained by performing a two exponential components analysis. The results are summarized in Table 2 and Fig. 4. On the basis of this analysis, the average value and temperature behavior of the lifetime is determined only by the interconversion rates between the two states because they are the only variable parameters in the fitting procedure.

The results in ethanol were different from those obtained in the other solvents. The decay rate from the first state was 0.167 ns^{-1}. To keep the same decay rates obtained for the previous solvents, a third state must be added with the decay rate 0.167 ns^{-1} (Table 2). With the aim of investigating the origin of this third component in ethanol, lifetime measurements were performed by adding 1% (vol/vol) water to the ethanol. The fraction of the third component was observed to increase by a factor of two. For ethanol, the appearance of a third component was then attributed to DPH emission arising from the interaction with a small amount of water, which is present in commercially available absolute ethanol.

DPH decay in phospholipid vesicles

The DPH decay in phospholipids can be described using a sum of discrete exponentials or by a continuous distribution of lifetimes, as previously reported by various authors (Dale et al., 1977; Fiorini et al., 1987, 1988; Parasassi et al., 1984, 1987; Stubbs et al., 1981). Using

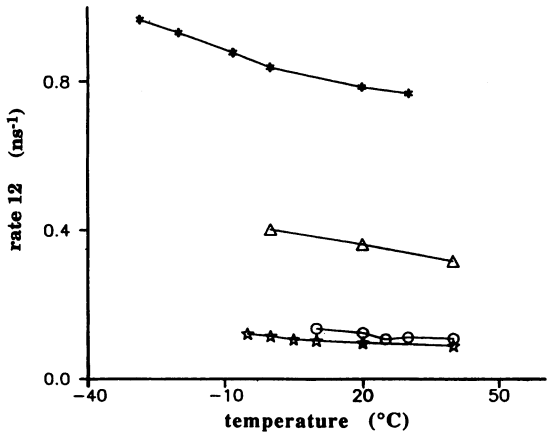

FIGURE 4. Values of the backward interconversion rate, *R*12, from state 2 to state 1 versus temperature for DPH in different solvents. Dodecane (★), cyclohexane (○), chloroform (*), tetrahydrofuran (△).

both analysis models, a short-lived component of relatively small fractional intensity is observed (Fiorini et al., 1987, 1988; Parasassi et al., 1984, 1987). We analyzed the decay data of DPH in various phospholipid multilamellar vesicles after the two-state model described above. The model gave satisfactory fits, resulting in χ^2 values similar to those obtained by the double exponential or the continuous lifetime models. For all phospholipid samples, the decay rates were 0.67 and 0.063 ns^{-1} for state 1 and state 2, respectively, and the $SAS1$ value was 0.47 for all samples. Boundary conditions were $B1 = 1$ and $B2 = 0$. These values were the same as those found in solvents. The $R21$ values were ~ 0.33–1.02 ns^{-1} for all samples. The phospholipid transition could be clearly monitored by the change with temperature of the $R12$ value (Fig. 5)

For the experiments in DMPC and DPPC, better χ^2 values were obtained by distributing the rate $R12$ using a lorentzian distribution (Table 3). The lorentzian distribution provided a better fit to the data than a uniform or a gaussian distribution. On average, the χ^2 decreased by about a factor of two using this distribution analysis. The width of the distribution of $R12$ was a function of temperature and was narrower about the phase transition.

The parameters that describe the DPH fluorescence decay, based on the two-state model illustrated above for the case of DPH in solvents and in phospholipids, can be expressed in terms of apparent fluorescence lifetimes. The results were very similar to those obtained by using two exponential components. In the case of the DPH decay in phospholipids, for a distributed interconversion rate $R12$, an asymmetrical distribution of lifetimes was obtained, with a major component of ~ 10 ns at low temperature and with a second short-lived component of small fractional intensity (Fig. 6).

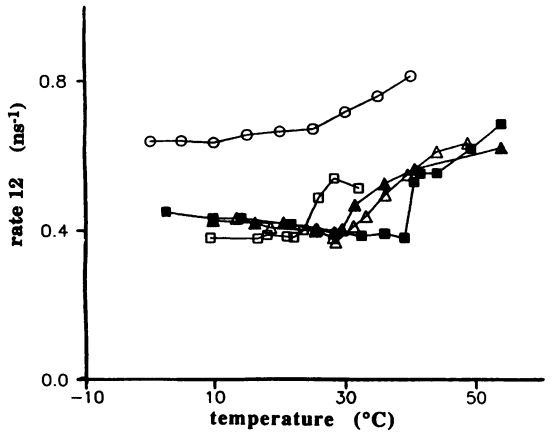

FIGURE 5. Values of the backward interconversion rate, $R12$, from state 2 to state 1 versus temperature for DMPC (□), DPPC (■), DMOPC (○), DMPC:DPPC = 0.6:0.4 (▲), DMPC:DPPC = 0.4:0.6 (△).

DISCUSSION OF THE TWO-EXCITED-STATE MODEL

The fluorescence decay corresponding to the model we propose for DPH is equivalent to a double exponential decay. It is well known that a two-state model always produces two decay rates, which cannot be directly associated with the decay rate of the individual states of Fig. 1. The analytical solution of this model has been reported several times and is given for reader convenience (Alcala et al., 1987)

$$n_1(t) = a_1 e^{-m_1 t} - a_2 e^{-m_2 t} \quad (1)$$

$$n_2(t) = b_1 e^{-m_1 t} - b_2 e^{-m_2 t}, \quad (2)$$

where

$$m_{1,2} = \tfrac{1}{2}\left[\Gamma_1 + \Gamma_2 \pm \sqrt{(\Gamma_1 - \Gamma_2)^2 + 4R_{21}R_{12}}\right] \quad (3)$$

$$\Gamma_1 = R_1 + R_{21}; \quad \Gamma_2 = R_2 + R_{12} \quad (4)$$

$$a_1 = \frac{B_1(\Gamma_1 - m_2) - B_2 R_{12}}{m_1 - m_2} \quad (5)$$

$$a_2 = \frac{B_1(\Gamma_1 - m_1) - B_2 R_{12}}{m_1 - m_2} \quad (6)$$

$$b_1 = \frac{B_2(\Gamma_2 - m_2) - B_1 R_{21}}{m_1 - m_2} \quad (7)$$

$$b_2 = \frac{B_2(\Gamma_2 - m_1) - B_1 R_{21}}{m_1 - m_2}, \quad (8)$$

$n_1(t)$ and $n_2(t)$ represent the excited-state population of state 1 and state 2, respectively. The total fluorescence intensity is given by

$$I(t) = SAS1 n_1(t) + (1 - SAS1) n_2(t), \quad (9)$$

where $SAS1$ is the fractional intensity of the fluorescence due to state 1 in the spectral region used for the fluorescence measurement. For a double exponential decay, a maximum of three parameters can be determined, i.e., two decay rates and one fractional intensity. However, the model of Fig. 1 contains four rates (two decay rates and two interconversion rates), one SAS value, corresponding to the relative observation of one state with respect to the other, and the relative initial population of one of the two states. Then a total of six parameters describe the model. One of the assumptions

TABLE 3 **Values of the forward (*R*21) and the backward (*R*12) interconversion rates of DPH in DMPC and in DPPC vesicles as a function of temperature**

	Discrete				Distributed			
	T	*R*21	*R*12	χ^2	*R*21	*R*12	*W*	χ^2
	°C							
DMPC	15	4.38	0.44	1.37	4.49	0.43	0.21	0.29
	20	4.32	0.43	2.04	4.37	0.43	0.12	1.69
	25	4.44	0.49	1.40	4.55	0.49	0.21	0.51
	30	4.39	0.61	1.18	4.42	0.61	0.08	0.95
	35	4.29	0.64	1.43	4.30	0.64	0.04	1.43
	40	4.44	0.73	1.75	4.60	0.75	0.24	1.06
DPPC	2.5	3.62	0.44	3.03	3.84	0.43	0.31	0.40
	9.8	3.61	0.41	3.54	3.87	0.40	0.30	0.74
	14.0	3.84	0.42	1.80	3.97	0.41	0.23	0.32
	21.6	3.89	0.40	2.13	4.05	0.39	0.25	0.33
	32.5	3.93	0.37	4.05	4.07	0.35	0.23	2.49
	36.0	4.05	0.38	3.05	4.24	0.36	0.27	0.99
	39.0	4.05	0.37	6.49	4.28	0.34	0.33	2.69
	40.5	4.00	0.52	2.27	4.21	0.52	0.30	0.53
	44.0	3.83	0.53	2.77	3.89	0.54	0.12	2.34
	49.3	3.89	0.59	1.15	4.00	0.61	0.17	0.85
	54.0	3.70	0.63	0.74	3.80	0.66	0.17	0.32

Rates are fitted to a discrete two-state model and to a distributed two-state model. Distribution is to a lorentzian function. Rates are expressed in nanoseconds^{-1}, and for the distributed case, the full width at half maximum, *W*, is expressed in nanosecond^{-1}.

of our model is that only state 1 can be excited, i.e., the initial boundary conditions are $B_1 = 1$ and $B_2 = 0$ for state 1 and for state 2, respectively. With respect to the four rates, we can estimate the values of the two decay rates, *R*1 and *R*2. The state which is excited also emits very fast. In the model of two interconverting states, the values of the two decay rates must always be slower than the decay rates found using a double exponential decay analysis. This results from the fact that the interconversion between states can only increase the observed decay rates. We assume that the two decay rates can be identified with the radiative decay rates of the two excited states, which thus can be assumed to be solvent and temperature independent. The value of *R*1 was determined on the basis of the value calculated using the absorption spectrum (Birch and Imhof, 1982; Birks and Birch, 1975; Cundall et al., 1979; Zannoni et al., 1983) and is compatible with our results. The value of *R*2 was chosen as the slower value compatible with all the experiments. It must be emphasized that the values we have used for the decay rates represent only a maximum value. The decay rates can be slower if the interconversion rates are faster. After applying the above assumptions, we have only three fitting parameters, i.e., two interconversion rates, *R*12 and *R*21, and *SAS*1. Inspection of Eq. 5–9 reveals that the values of the *SAS*1 and interconversion rates are strongly correlated.

A fit using the two-state model cannot give better χ^2

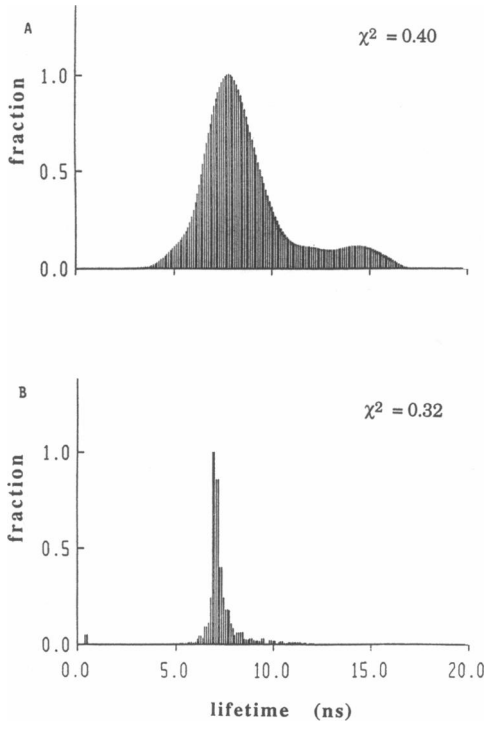

FIGURE 6. Lifetime distributions of DPH in DPPC at 2.5°C (*A*) and at 54°C (*B*) using the two-excited-state model with rate *R*12 distributed with a lorentzian function. The values of the parameters are from Table 3.

values than the double exponential decay fit of the individual curves. The relevance of the model resides in the possibility of specifying that the temperature, the solvent, or the phospholipid physical state should affect only the interconversion between the two states, but not the $SAS1$. With respect to the $SAS1$, its value should depend only on the observational conditions and, hence, is constant for each set of experiments. For different experiments, the $SAS1$ value should also be similar. In this way, all the experimental conditions are contained in a single constant, $SAS1$, whereas the processes influencing the decay rates are reflected by the rate of interconversion between the two states. In summary, although our model is mathematically equivalent to a double exponential decay, we have reduced the total number of fitting parameters when the analysis of the data is performed as a function of temperature, solvent and physical state of the phospholipids. For example, the data for DPH in chloroform at six temperatures will require a total of 18 parameters for a double exponential decay fit. For fitting our model, we used only eight parameters, because the value of the $SAS1$ must be common to all temperatures, and one of the interconversion rates was found to be temperature independent.

Solvents

One of the interesting results from the numerical analysis of our model is that only the rate $R12$ changes with temperature and, consequently, determines the behavior of the system. $R12$ is also more sensitive to different solvents than $R21$. In general, using rate theory, the rate $R12$ (or $R21$) can be expressed in terms of several parameters, including frequency factor, microscopic viscosity, and activation free energy. To explain the observed temperature dependence of $R12$, we can exclude frequency factor and viscosity changes because their behavior is either temperature independent or it goes in the opposite direction. Hence, the free energy barrier must change with temperature. As a consequence, this relative variation of the two-excited state's energy level should also be reflected in a shift of the excitation and emission spectra. In particular, an increase in the rate $R12$ as the temperature is decreased should correspond to a relative increase in the energy of state 2. We can assume that, apart from a common Stoke's shift, which is temperature independent, the excitation spectrum depends on the average energy of state 1. We have measured the excitation and emission spectra of DPH in dodecane at temperatures from −6.8 to 49°C. The emission spectra at the temperature extremes are shown in Fig. 7. We clearly observe a blue shift of the excitation (not shown) and a red shift of the emission as the temperature is increased. In accordance with the predicted behavior based on the rate changes, those spectral shifts should correspond to a relative increase of the rate $R12$ because the barrier is lower at lower temperature. A full quantitative agreement of the spectral shift with the change of the rate $R12$ is more difficult to obtain because the spectral position depends on the transition between the two states, whereas the rate $R12$ depends only on the relative barrier between the two states.

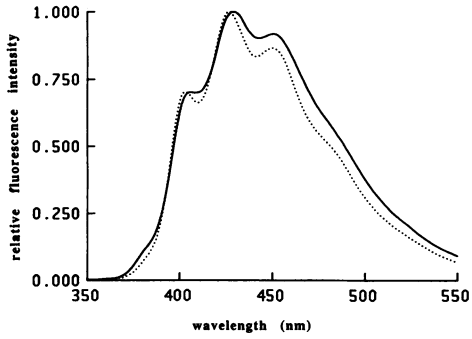

FIGURE 7. Emission spectra of DPH in dodecane at −6.8°C (- - -) and 49°C (——). Excitation was at 325 nm. Excitation and emission bandwidth was 1 nm. Spectra were normalized to the same height.

There are several factors that can affect the average lifetime value of DPH in solvents. The analysis of the decay in several solvents shows that $R21$ is larger than $R12$, which implies that the emission arises mostly from the second excited state. The energy level of state 2, with respect to the ground state, can be evaluated from the center of mass of the emission spectra. However, the average level of the first state is not directly available because we have not identified a specific spectral component of this state. The use of the average excitation energy that can be obtained from the maximum of the excitation spectrum is not appropriate in this case because different solvents can have different Stoke's shifts.

Phospholipids

In the experiments performed in phospholipids, rate $R21$ was much larger than rate $R12$ for all samples and for all temperatures. In general, the rate $R21$ cannot be fixed to a constant value (as was found for solvents) for all temperatures without increasing the χ^2 value by a relatively large amount. However, for vesicles containing a single phospholipid, such as DMPC or DPPC, the rate $R21$ was essentially constant at all temperatures. Instead, $R12$ showed a gradual decrease with increasing temperature in the phospholipid gel phase, and a sudden increase after the phase transition. Above the

phospholipid phase transition temperature, the general trend of the rate $R12$ was to increase with increasing temperature. The change in slope after the transition is not due only to the different influence of the Arrhenius factor. We can estimate that the difference in energy between the two levels corresponding to the second excited state should not exceed 0.41 Kcal/mol, based on the ratio between the two rates. However, an Arrhenius plot of the rate $R12$ of the high temperature data for DMOPC gives an apparent activation energy of the order of 2.6 Kcal/mol. The effect of temperature in the Arrhenius equation is then small, compared to the total activation energy. Therefore, the temperature must affect the overall energy level of the second excited state. We propose that the increase of rate $R12$ is an average increase of the polarity of the membrane as the temperature is increased. In fact, the rate $R12$ is higher in more polar media as determined from the pure solvent studies. One possible source of polarity change is the increase of water penetration above the transition temperature, which has been shown to occur using other techniques (Blechner et al., 1989; Levine et al., 1972; Jendrasiak and Hasty, 1974*a*,*b*; Simon et al., 1982).

Lifetime distributions in phospholipids

We have investigated the effects of the data fits of distributing the rate $R12$. The reason for analyzing the distribution of this rate is because of its sensitivity to different factors such as temperature and polarity. We found no improvement of the fits by distributing rate $R12$ for samples of DPH in solvents. For the phospholipid samples, distribution of the rate $R12$ gave a significant improvement in the quality of the fit. The average χ^2 decreased by about a factor of two. The distribution of lifetime values using symmetric lorentzian distribution has been previously reported (Fiorini et al., 1987, 1988; Parasassi et al., 1987). A common observation in those studies was that the distribution was wider at low temperature below the phase transition and became quite narrow above the transition. The analysis of the distribution of the rate $R12$ shows that the width (0.2/ns) is a substantial part of the center value (0.4/ns). The calculation of the corresponding lifetime distribution gives a width which is temperature dependent, in good agreement with previous results (Fiorini et al., 1987, 1988; Parasassi et al., 1987). Using a lorentzian distribution for the interconverting rate $R12$ results in an asymmetric lifetime distribution (Fig. 6). The distribution is wider at lower temperatures. This effect is due mainly to the relative width of the distribution with respect to its center value. With regard to the physical origin of the distribution width, we observed that above the phase transition the main contribution to the width should arise from microheterogeneity of the local polarity due, perhaps, to different water penetration at different depths of the bilayer. Below the phase transition temperature, the major effect should be related to the density and refractive index microheterogeneity, which seems to be the major affector of the rate $R12$. The distribution of DPH molecules along the membrane normal can be the major source of lifetime heterogeneity in pure phospholipid vesicles. Different DPH molecules will experience a different effective environment, which can be seen as a locally different solvent. Lentz and co-workers (1988) have reported that DPHpPC, which has a unique position with respect to the membrane surface, shows a very narrow distribution of the major lifetime component. As a consequence, in a phospholipid bilayer an effective "solvent properties distribution" can be at the origin of the DPH lifetime heterogeneity.

The two-excited-state model presented here can provide an explanation for some unusual properties of the dependence of the time zero anisotropy (Levine et al., 1972). If we assume that the two-excited-state transition dipoles have different orientations, it is possible to have a fast change of dipole orientation which gives rise to a nonmotional decay of the anisotropy. A similar mechanism has been proposed and experimentally confirmed to explain the value of the time zero anisotropy of tryptophan (Ruggiero et al., 1990).

CONCLUSIONS

Using the two-excited-state model, the spectroscopic properties of DPH in phospholipid vesicles are determined by the interconversion rates $R21$ and $R12$. Because the lifetime of DPH changes with solvent and temperature, and because $R21$ is not very dependent on those variables, then essentially all changes in the observed lifetimes are due to $R12$. To detect phospholipid phase coexistence in membranes, $R12$ and its variation with time must be accurately determined. Changes in $R12$ due to the phospholipid phase transition are very small once the temperature trend has been subtracted (Fig. 5). Such small changes in $R12$ will make it difficult to obtain information about phase coexistence and the dynamics of phase interconversion using DPH as a probe. Furthermore, $R12$ is distributed with a width which is a relevant part of its average value. Another complication is that membrane microheterogeneity also affects the back rate $R12$. But because DPH lifetime is sensitive to local membrane microheterogeneity, DPH can instead be profitably used for the quantitation of this microheterogeneity. The two-excited-state model of DPH fluorescence decay shall require that all spectroscopic

properties of DPH be reconsidered. For example, if the two excited states have different rotational properties, then interpretation of the emission anisotropy decay will be different and its analysis will be more complex than has been previously thought.

We thank Dr. W. W. Mantulin for careful review of this manuscript and useful comments. Data analysis was performed at the Laboratory for Fluorescence Dynamics (LFD) at the University of Illinois at Urbana-Champaign (UIUC). The LFD is supported jointly by the Division of Research Resources of the National Institutes of Health (NIH) and UIUC.

This work was supported by Consiglio Nazionale delle Ricerche (T. Parasassi and G. DeStasio) and by NIH RR03155 (R. M. Rusch and E. Gratton).

Received for publication 7 May 1990 and in final form 4 October 1990.

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