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Fluorescence Lifetime Distributions of 1,6-Diphenyl-1,3,5-hexatriene in Phospholipid Vesicles†

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ABSTRACT: The fluorescence emission properties of 1,6-diphenyl-1,3,5-hexatriene (DPH) in 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine and 1,2-dimyristoyl-3-*sn*-phosphatidylcholine multilamellar vesicles have been measured by using multifrequency phase fluorometry. The fluorescence decay of DPH in the phospholipid vesicles has been analyzed by assuming either that the decay is made up of a discrete sum of exponential components or that the decay is made up of one or more continuous distributions of lifetime components. The fit of the decay curve using exponentials required at least two terms, and the reduced χ^2 was relatively large. The fit using a continuous distribution of lifetime values used two continuous components. Several symmetric distribution functions were used: uniform, Gaussian, and Lorentzian. The distribution function that best described the decay was the Lorentzian. The full width at half-maximum of the Lorentzian distribution was about 0.6 ns at temperatures below the phase transition temperature. At the phospholipid phase transition and at higher temperatures, the distribution became quite narrow, with a width of about 0.1 ns. It is proposed that the lifetime distribution is generated by a continuum of different environments of the DPH molecule characterized by different dielectric constants. Below the transition temperature in the gel phase, the dielectric constant gradient along the membrane normal determines the distribution of decay rates. Above the transition, in the liquid-crystalline phase, the translational and rotational mobility of the DPH molecule increases, and the DPH experiences an average environment during the excited-state lifetime. Consequently, the distribution becomes narrower. The physical interpretation of the continuous distribution of lifetime values is based on the heterogeneity of the molecular environments of the DPH molecule, and this better describes the observed decay than the use of a discrete number of exponential components.

During the past several years, there has been an increasing interest in the study of the physical properties of lipid bilayers. A variety of spectroscopic techniques have been employed for the investigation of the structure and dynamic properties of synthetic and natural membranes. In particular, fluorescence techniques have been used to investigate lateral and rotational diffusion in lipid bilayers to quantitate the amount of gel and liquid-crystalline phases. Fluorescence studies generally use probes embedded in the membrane. Also, parinaric acid isomers have been used (Sklar et al., 1975, 1977a,b; Wolber & Hudson, 1981; Parasassi et al., 1984). 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] and its derivatives are perhaps the most commonly used probes to investigate the physical-structural properties of membranes at the molecular level (Shinitzky & Barenholtz, 1975; Lentz et al., 1976a,b; Chen et al., 1977; Cranney et al., 1983). DPH is a very sensitive probe, and it has a large partition coefficient for the lipid with respect to the solvent. DPH locates in the membrane interior,

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[1] Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; TMA-DPH, 1-[4-(trimethylammonio)phenyl]-6-phenyl-1,2,5-hexatriene; DPH-PC, 2-[3-(diphenylhexatrienyl)-propanoyl]-3-palmitoyl-L-α-phosphatidylcholine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; POPOP, 1,4-bis(5-phenyl-2-oxazolyl)benzene.

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probably perpendicular to the membrane plane. The precise location of DPH within the membrane is still debated, although it seems to distribute in quite a wide range along the membrane normal (Davemport et al., 1985).

The rotational behavior of DPH as well as the fluorescence intensity and lifetime has been used as indicators of the physical state of the membrane. Recently, several researchers have determined the fluorescence lifetime behavior of DPH using different techniques (Barrow & Lentz, 1985; Chen et al., 1977; Zannoni et al., 1983; Parasassi et al., 1984). In particular, the DPH lifetime has a different value in the gel phase than in the liquid-crystalline phase of DPPC, DMPC, and other phospholipid vesicles. The general picture which is emerging is that at temperatures below the phase transition the lifetime value varies from about 9 to 11 ns depending on the physical composition of the membrane. As the temperature is raised, the average lifetime value increases slightly until the phase transition is reached, where there is an abrupt drop in the lifetime (Barrow & Lentz, 1985; Parasassi et al., 1984). Accurate measurements below the phase transition show that the decay is not a single exponential but that there is a short lifetime component of about 3 ns of variable intensity depending on the preparation and perhaps the technique employed for the lifetime measurement (Parasassi et al., 1984). Above the phospholipid phase transition, the average lifetime value drops to about 8 ns and shows an approximately linear decrease as the temperature is increased. Also, the fluorescence decay is better approximated by a single-exponential component (Barrow & Lentz 1985). The gradual increase in the lifetime value in the gel phase when the temperature increases up to the phase transition has not been explained. Also, the physical origin of the lifetime change at the phase transition is unclear.

Although the origin of the lifetime change is unknown, the sensitivity of DPH lifetime to the physical state of the membrane can be applied in principle for the determination of the relative amount of solid and liquid-crystalline phases in the membranes. In mixed phospholipid systems, in which there are lateral phase separations, lifetime analysis gives a reasonable description of the system in terms of the gel to liquid-crystalline ratio using the assumption that the decay can be represented by the sum of discrete exponential terms (Parasassi et al., 1984). The determination of the relative amount of the gel and liquid-crystalline phases is obtained by assigning the value of the DPH lifetime in the two phases to be 10.5 and 7.5 ns, respectively. The preexponential factor associated with each lifetime gives the amount of each phase. It is assumed that DPH has an equal partition coefficient in the two phases. However, this approach failed when the same analysis was applied to natural membrane systems. For example, for plasma membranes from LM cells, this kind of analysis gave a large amount (>50%) of gel phase at temperatures as high as 50 °C (E. Gratton, S. Wang, and M. Glaser, unpublished observations). Since this result seemed to be paradoxical (Gilmore et al., 1979a,b; Welti et al., 1981), we felt that the origin of the lifetime values in a membrane should be further evaluated to better understand the factors that affect them.

The DPH lifetime is strongly dependent upon the dielectric constant of the medium (Zannoni et al., 1983). On the other hand, the lifetime value is independent of the macroscopic viscosity. In a synthetic membrane system, the dielectric constant is a function of several parameters, such as phospholipid composition, packing, temperature, and water penetration. The latter factor produces a dielectric constant gradient across the membrane. In principle, the average DPH lifetime can be predicted on the basis of the dielectric constant because of the linear relationship between dielectric constant and fluorescence lifetime (Zannoni et al., 1983). Furthermore, since the dielectric constant is a function of the position in the membrane, a wide range of lifetime values of DPH is possible. From these simple considerations, the usual lifetime analysis performed in terms of one or two exponential terms, which correspond to well-defined molecular surroundings of the DPH molecule, may be an oversimplification. In this paper, a different approach was followed, one which intuitively should better describe the real physical situation. The basic assumption was that there was a continuous distribution of decay rates. Since the DPH molecules exist in a variety of different positions along the membrane normal and because they undergo rapid rotational and translational motions, they can experience a range of environments, each of which is characterized by different lifetime value. In the following sections, the mathematical basis for this new "distributional" approach is described and used to analyze the decay of DPH in large, multilamellar vesicles at different temperatures.

During the past several years, we have developed a new frequency domain technique for the measurement of fluorescence decay times. These techniques have the advantage of an extremely high resolving power (Gratton et al., 1984; Jameson et al., 1984; Gratton & Limkeman, 1983) and are based on the use of sinusoidally intensity modulated light for excitation. The measurement of the phase delay and demodulation factor of the emission with respect to the excitation is performed over a wide range of modulation frequencies. The equivalent of the Fourier transform of the time decay is obtained by using this technique. One distinctive advantage of multifrequency phase fluorometry over the correlated single photon counting technique is that there is no deconvolution for the instrumental response since the equivalent of the ideal pulse response is obtained. Furthermore, the measurement of the phase delay and of the modulation ratio is performed with the same absolute error at all frequencies. In this work, many different modulation frequencies were used, and phase and modulation values were collected over a wide frequency range. Also, a quite long integration time was used to increase the accuracy of each measurement. These features have been important factors for the determination of continuous lifetime distributions.

Materials and Methods

DPPC and DMPC were obtained from Avanti Polar Lipids Inc. (Birmingham, AL) and were used without further purification. The background fluorescence was checked prior to each measurement, and it was less than 0.5% of the fluorescence when DPH was added. DPH was obtained from Aldrich Chemical Co. (Milwaukee, WI). Multilamellar liposomes were formed by drying the lipids under N_2 and resuspending them, above the lipid phase transition, in 10 mM HEPES/100 mM KCl, pH 7.4, above the transition temperature of the lipids to a final concentration of 400 μM. The lipid suspension was sonicated with a Branson bath sonicator for 3 min to disperse the lipids. DPH, previously dissolved in tetrahydrofuran, was then added to reach a DPH to phospholipid molar ratio of 1:1000. The suspension was then incubated for 1 h in the dark. Lifetime measurements were performed with the multifrequency phase fluorometer described by Gratton and Limkeman (1983) equipped with an ISS-ADC interface for data collection and analysis. The wavelength of excitation was set at 325 nm (UV line of an HeCd Liconix Model 4240N laser). The modulation frequency set employed was 2, 5, 7,

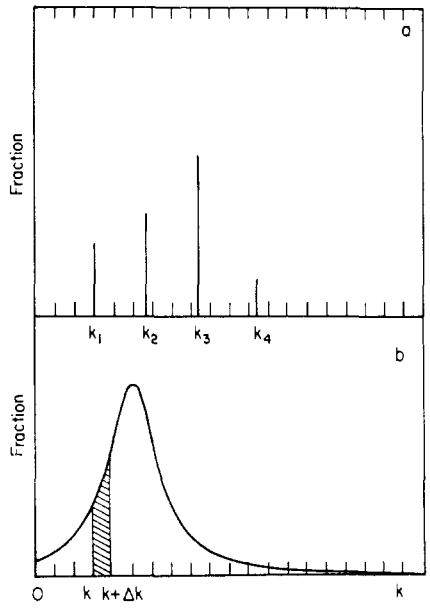

FIGURE 1: Representation of a multiexponential decay (a) and of a continuous lifetime distribution (b).

10, 15, 20, 25, 30, 50, 70, and 100 MHz. Data were accumulated until the standard deviations of phase and modulation values at a given frequency were below 0.1° and 0.002°, respectively. The sample temperature was controlled by using an external bath circulator (Neslab Model Endocal Instruments Inc., Portsmouth, NH). The actual sample temperature was measured at the sample cell prior to and after each measurement using a digital thermometer (Omega Engineering, Inc., Stamford, CT). All lifetime measurements were obtained by using POPOP in ethyl alcohol in the reference cell. The assumed lifetime of the POPOP was 1.35 ns (Lakowicz et al., 1981). The emission was measured by using a high-pass type RG370 filter from Janos Technology (Townshend, VT) which showed negligible fluorescence. The data analysis was performed by using a nonlinear least-squares routine for the multiexponential fit (Lakowicz et al., 1984) and a routine based on the simplex method for the lifetime distribution analysis (Caceri & Cacheris, 1984).

Derivation of Continuous Distribution of Lifetime Values

In the time domain, the fluorescence decay is usually described as a sum of discrete exponentials:

$$I(t) = \sum a_i e^{-k_i t} \tag{1}$$

using the normalization condition $\sum a_i = 1$. The decay rates and preexponential factors can be represented as in Figure 1a where every vertical line at the rate k_i has a length a_i. Similarly, a continuous function could be used to describe the contribution of the decay rate k to the total intensity $I(t)$, where each slice of the graph represented in Figure 1b gives the contribution of the molecules which decay with the rate between k and $k + \delta k$ to the total intensity. In mathematical form, this can be written

$$I(t) = \int_0^\infty a(k) e^{-kt}\, dk \tag{2}$$

where $a(k)$ is an arbitrary function, with the normalization condition

$$\int_0^\infty a(k)\, dk = 1 \tag{3}$$

We note that the set of discrete lifetimes illustrated in Figure 1a might be well represented by the single continuous distribution of lifetimes illustrated in Figure 1b. This need not always be the case.

In frequency domain fluorometry, the phase and modulation (AC/DC ratio) of the emission are measured in relation to the phase and modulation of the excitation. The phase shift (P) and the modulation ratio (M) are defined by the relationships:

$$P = \tan^{-1} [S(\omega)/G(\omega)] \tag{4a}$$

$$M^2 = S^2(\omega) + G^2(\omega) \tag{4b}$$

where

$$S(\omega) = \sum a_i \omega/(\omega^2 + k_i^2) \tag{5a}$$

$$G(\omega) = \sum a_i k_i/(\omega^2 + k_i^2) \tag{5b}$$

where ω is the angular frequency of light modulation. The $S(\omega)$ and $G(\omega)$ functions are usually written in the form

$$S(\omega) = \sum f_i \tau_i \omega/(1 + \tau_i^2 \omega^2) \tag{6a}$$

$$G(\omega) = \sum f_i/(1 + \tau_i^2 \omega^2) \tag{6b}$$

using the relations

$$\tau_i = 1/k_i \tag{7}$$

and

$$f_i = a_i/k_i \qquad \sum f_i = 1 \tag{8}$$

The fractions f_i represent the contribution to the total fluorescence intensity of the molecules which decay with a rate k_i.

With the continuous approach, the functions $S(\omega)$ and $G(\omega)$ can be written as

$$S(\omega) = \int_0^\infty [f(\tau)\omega\tau/(1 + \omega^2\tau^2)]\, d\tau \tag{9a}$$

$$G(\omega) = \int_0^\infty [f(\tau)/(1 + \omega^2\tau^2)]\, d\tau \tag{9b}$$

where $f(\tau)$ is an arbitrary function of τ. The normalization condition is

$$\int_0^\infty f(\tau)\, d\tau = 1 \tag{10}$$

Once $S(\omega)$ and $G(\omega)$ are given by eq 9a,b, the values of phase and modulation can be obtained from eq 4a,b. In frequency domain fluorometry, a measurement provides a set of values of $P(\omega)$ and $M(\omega)$ at different values of the modulation frequency ω.

The purpose of the fitting routine for the case of the distribution analysis is to determine the function $f(\tau)$ which minimizes the reduced χ^2. A program provided by ISS INC, La Spezia, Italy, was used for the distribution fit. For both the exponential and the distributional analyses, the programs minimize the reduced χ^2 defined by

$$\chi^2 = \sum\{[(P_m - P_c)/s^P]^2 + [(M_m - M_c)/s^M]^2\}/(2n - f - 1)$$

where the suffixes c and m indicate the calculated and measured values, respectively, n is the number of modulation frequencies employed, f is the number of free parameters, and s^P and s^M are the standard deviations of each phase and modulation measurement, respectively. In phase fluorometry, the standard deviations of the phase and modulation measurements are independent of the modulation frequency and constant for each measurement, and hence factorize out of the χ^2 expression (Gratton et al., 1984). Since the minimum of

the χ^2 is independent of a common multiplicative factor, it is convenient, vis-à-vis the speed of calculations, to use a common fixed value for the standard deviation. Consequently, the χ^2 value for a given fit is multiplied by an arbitrary factor. The important parameter is thus the change in the χ^2 value upon use of different functions for the fit rather than the absolute value of the χ^2. Calculated values of phase P and modulation M were obtained by using eq 4a,b and 9a,b. In eq 9a,b, the function $f(\tau)$ is replaced by a sum of exponentials or by a distribution function depending on the case investigated. If $f(\tau)$ is an arbitrary function, the task of solving the integral eq 9a,b is quite difficult. Several researchers have shown that the problem can be solved for an arbitrary function $f(\tau)$ provided that a certain degree of "smoothness" of $f(\tau)$ is assumed (Provencher et al., 1978). Instead of numerically solving the integral eq 9a,b, we have used a specific function for $f(\tau)$ which can be described by using a small number of parameters. In this case, the integral problem reduces to the determination of the parameters that describe the function, which can be accomplished by using standard statistical analysis. In particular, some simple functional relationships defined for positive values of τ were used, such as

uniform

$$f(\tau) = A \qquad \text{from } C - W/2 \text{ to } C + W/2$$

$$f(\tau) = 0 \qquad \text{elsewhere}$$

Gaussian

$$f(\tau) = Ae^{2.75(\tau-C)^2/W^2}$$

Lorentzian

$$f(\tau) = A/\{1 + [(\tau - C)/(W/2)]^2\}$$

The essential parameters for the fit are the center position C and the width W of the distribution. The constant A can be obtained from the normalization condition. For some of the above functions, the integral in eq 2 or 9a,b can be analytically evaluated. Notice that the integrals in eq 2, even for the simple uniform distribution, give a nonexponential decay. For example, the uniform distribution gives

$$I(t) = \int_{k_1}^{k_2} e^{-tk}\, dk = (1/t)[(e^{-k_1 t} - e^{-k_2 t})/(k_2 - k_1)]$$

This is a true nonexponential form since it contains a $1/t$ term. If, instead of using a uniform distribution, a triangular distribution is used, the decay contains a term $1/t^2$. In preliminary analyses, the use of a particular distribution function was purely phenomenological. For DPH in multilamellar vesicles, a Lorentzian distribution gave a better fit than other distribution functions.

Data for a uniform, Gaussian, and Lorentzian distribution were simulated by using eq 2, and then a fit was attempted by using a sum of exponentials. The decay rates obtained by the fit reproduced the simulated data well if three to four exponentials were used, although the results depended on the width of the distribution. For example, with a Lorentzian distribution, the pertinent variable was the width:center ratio. A ratio of 0.02 already gave a bad fit using a single exponential (reduced $\chi^2 > 2.5$); for a ratio of 0.05, two exponentials had to be used to obtain a reduced $\chi^2 < 2$. Figure 2 shows simulated data for a Lorentzian distribution with Gaussian noise added to the values of P and M as obtained by eq 9a,b and 4a,b. The fit using a Lorentzian distribution recovered the original distribution. The solid lines in Figure 2 correspond to the fit using two exponentials. The value of χ^2 for the latter case was 1.98. For this simulation, a width:center ratio of 0.05

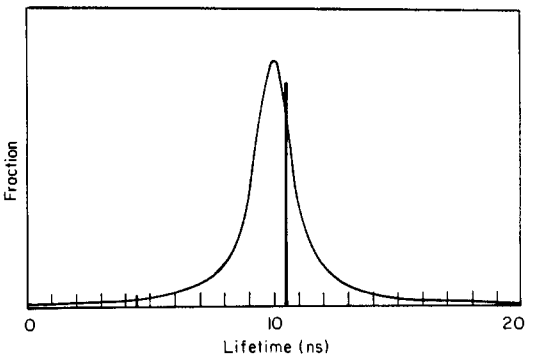

FIGURE 2: Simulated Lorentzian distribution centered at 10 ns and having a width of 1 ns. The vertical bars correspond to the lifetime and fractional intensities recovered by using an analysis with two exponentials.

was used. The values of the major and minor lifetime components depended on the frequency set used for the simulation. In the case of Figure 2, a frequency set was used which gave more weight to the shorter lifetime values.

The simulation described above demonstrates that the result of fitting the data which are derived from a distribution of lifetime values using discrete exponentials is the arbitrary generation of lifetime components. However, when many exponentials are used, the reduced χ^2 can be as good as that obtained by using the true distribution due to the inherent random errors of the experimental data. How can a true distribution be distinguished from a multiexponential decay? Statistical methods can be used to test a given model, and in several cases, this will show, on the basis of χ^2, that one model is better than others. Other information can be used, such as temperature behavior, to determine which model may be more appropriate. Due to the inherent limitation of present day instrumentation, it may be impossible to decide if a decay is multiexponential or distributed, on the basis of the value of χ^2 only.

The difficulty of defining the exact distribution of lifetimes is complicated by the possibility of having multimodal distributions. Several distribution functions can be added, such as uniform, Gaussian, Lorentzian, etc, with a given fractional contribution to the total measured decay. By proper addition of functions of different center and width, an arbitrary shaped function can be described. Clearly, when this method is used, the number of parameters is inflated. The real motivation to use continuous lifetime distributions arises from physical intuition rather than from a statistical–mathematical demonstration. In those cases where a distribution of environments for a fluorophore is expected, it seems preferable to describe a decay using a continuous lifetime distribution which requires only two parameters rather than using the sum of two or more exponentials which requires three or more parameters and which has no direct physical interpretation.

Results

The decay of DPH emission was studied in DPPC and DMPC vesicles. The measurements described in Parasassi et al. (1984) were repeated, but data were collected at many different frequencies, from 2 to 100 MHz, and temperatures from 7 to 70 °C. The experimental data were analyzed by using a sum of exponentials and either a single or a dual continuous lifetime distribution.

Exponential Analysis. Below and above the transition temperature of DPPC multilamellar liposomes, the fluorescence decay was analyzed by using two-exponential components. The analysis obtained by using a sum of exponentials

Table I: Analysis of the Fluorescence Emission Decay of DPH in DPPC Assuming the Decay either Is Made of Discrete Exponential Components or Is Made of a Lorentzian Distribution of Lifetimes[a]

	exponential analysis				Lorentzian distribution					
T (°C)	τ_1	f_1	τ_2	χ^2	C_1	W_1	f_1	C_2	W_2	χ^2
8.9	10.31	0.93	2.00	14.45	10.02	0.66	0.89	2.00	8.67	3.23
17.1	10.48	0.95	1.86	5.19	10.27	0.83	0.96	1.59	0.77	1.13
24.5	10.64	0.95	1.88	4.00	10.44	0.76	0.97	2.00	1.95	1.27
31.5	10.61	0.96	1.79	4.00	10.56	0.36	0.94	2.00	5.04	1.23
36.1	10.86	0.95	2.00	2.63	10.75	0.53	0.96	1.88	0.10	1.41
39.6	10.14	0.93	2.21	2.59	10.23	0.10	0.90	3.20	2.47	1.23
40.4	8.76	0.93	1.98	1.00	8.75	0.10	0.93	2.08	0.34	1.00
41.8	8.41	0.94	1.54	5.50	8.44	0.10	0.91	1.96	2.82	3.77
42.9	8.32	0.93	1.66	1.95	8.36	0.10	0.91	2.13	2.01	0.91
44.8	8.20	0.92	1.76	1.14	8.18	0.10	0.92	1.83	0.35	1.04
50.9	7.46	0.92	1.41	1.41	7.46	0.10	0.92	1.45	0.12	1.54
56.3	6.86	0.92	1.32	1.36	6.80	0.18	0.92	1.30	0.36	0.95
60.5	6.18	0.91	1.23	2.00	6.08	0.26	0.92	1.11	0.10	1.32

[a] τ_1, τ_2, lifetime in nanoseconds; f_1, fractional intensity; C_1, C_2, center of the distribution in nanoseconds; W_1, W_2, full width at half-maximum of the distribution in nanoseconds; χ^2, reduced χ^2.

Table II: Analysis of the Fluorescence Emission Decay of DPH in DMPC Assuming the Decay either Is Made of Discrete Exponential Components or Is Made of a Lorentzian Distribution of Lifetimes[a]

	exponential analysis				Lorentzian distribution					
T (°C)	τ_1	f_1	τ_2	χ^2	C_1	W_1	f_1	C_2	W_2	χ^2
6.7	10.42	0.97	2.95	2.90	10.16	0.86	1.00			2.90
14.4	10.90	0.92	5.00	5.86	10.30	0.83	1.00			3.41
18.1	10.37	0.99	1.78	2.23	10.30	0.34	0.99	1.00	1.14	1.73
20.0	10.60	0.94	4.47	4.77	10.69	0.10	0.87	6.94	1.53	4.04
22.2	10.48	0.94	4.09	1.27	10.40	0.10	0.95	3.98	0.10	1.24
23.4	10.17	0.94	4.92	1.63	10.13	0.10	0.93	5.26	0.54	1.60
25.2	9.39	0.96	2.96	2.18	9.38	0.10	0.95	3.55	0.94	2.45
30.3	8.90	0.96	2.58	1.82	8.90	0.10	0.96	3.01	0.85	1.80
39.7	8.03	0.97	2.22	1.23	8.01	0.10	0.97	2.56	0.96	1.13
50.0	7.04	0.98	0.96	2.36	7.02	0.10	0.98	0.79	1.12	2.33

reproduces the results reported in Parasassi et al. (1984) and also in Barrow and Lentz (1985). The result of the fit gave a short-lifetime component of about 1–2 ns which remained constant in value and fractional intensity at all temperatures and a long-lifetime component (fractional intensity of 0.95) of about 10.5 ns below the transition (Table I). This longer lifetime was slightly affected by temperature, showing a small increase for temperatures below the transition of the phospholipids and a large decrease just above the transition to a value of about 8.5 ns. A gradual decrease of the lifetime value was observed after the temperature was further increased to 60 °C. Following the procedure described in Parasassi et al. (1984), a three-component analysis was used in the temperature region near the DPPC transition by fixing the lifetime values to 2, 10.5, and 7.5 ns, and the preexponential factors were calculated. With the use of this analysis, a progressive decrease of the preexponential factor associated with the 10.5-ns component was obtained and a parallel increase of the 7.5-ns preexponential factor. The preexponential factor of the 2-ns component showed no change as the temperature was varied.

The exponential analysis of the DPH fluorescence lifetime in DMPC multilamellar liposomes showed a similar pattern with a drop of the major component lifetime at the transition temperature using two-component analysis (Table II). In the temperature range near the transition, a three-component analysis was used, and again a progressive decrease of the preexponential factor of the long component was observed while the preexponential factor of the intermediate component increased. The physical model that is assumed in order to perform the above three-component analysis (neglecting the short-lifetime component of obscure origin) is that two phases coexist during the transition and that at the transition midpoint half of the DPH molecules, during the excited-state lifetime, are experiencing an environment which is gellike and the other half have a liquid-crystalline-like surrounding. The model suggested by a two-component analysis is, on the other hand, that a single environment exists for a DPH molecule. During the phase transition, the vesicle undergoes a homogeneous change with no clusterization of gel and liquid-crystalline parts. One problem with the exponential analysis is that the fit shows a slightly larger χ^2 below the phase transition than above the transition, suggesting that the two-component model may not be adequate to describe the DPH decay in the entire temperature range.

Distributional Analysis. The same set of data previously analyzed using two- and three-exponential components was also analyzed by using a sum of two continuous distributions of lifetime values characterized by a Lorentzian shape centered at a decay time C and having a width W. DPH in both DPPC (Table I) and DMPC (Table II) showed a broad lifetime distribution below the transition temperature and a very narrow one above the transition (Figure 3). The reduced χ^2 obtained by using the distributional analysis was relatively constant below and above the phospholipid phase transition as it should be if the analysis accurately describes the experimental data. The mean reduced χ^2 for DPPC was about a factor of 3 better using the dual Lorentzian analysis as compared to the dual exponential analysis for temperatures below the phase transition. The two models gave approximately the same fit for DPPC above the transition (Table I) or for DMPC above the below the transition (Table II). When the Student's F test (Bevington, 1969) was used, the better fit obtained by the Lorentzian analysis below the DPPC phase transition was found to be significant. The lifetime distribution was very narrow above the phase transition (Figure 3B and Tables I and II), and the distribution was almost indistinguishable from an exponential term. A simulated Lorentzian

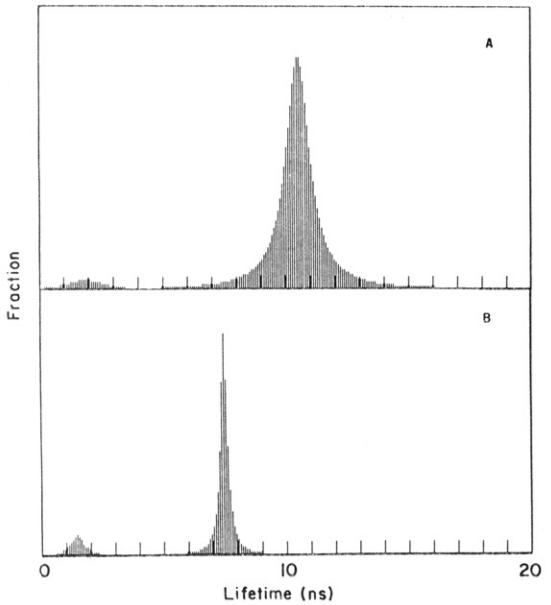

FIGURE 3: Distributional analysis using two Lorentzian functions for DPH in DPPC at 15 °C (A) and 42.8 °C (B).

lifetime distribution characterized by a width of 0.1 ns and centered at 10 ns, given the noise level of the measurements, gave a similar χ^2 values using a single-exponential decay or a Lorentzian distribution. The value of 0.1 ns given in Tables I and II is a lower limit imposed by the program to prevent divergence, and it is equivalent to an exponential component. Figure 3A shows that the major component of the distribution had an average lifetime value of 10.4 ns, which was nearly the same value observed for the long component using a double-exponential analysis.

Discussion

The distributional analysis gives a satisfactory fit to the experimental results and the opportunity of better insight into the detailed environment of the probe. The fits using Lorentzian lifetime distributions are as good or better than those using the sum of several exponentials. One of the limitations of the distributional analysis is the use of symmetric distributions. There is no reason to believe that the "real" lifetime distribution is symmetric. However, the analysis is particularly simple and rapid using this approximation. In most of the cases investigated, it is necessary to include a second distribution centered at shorter lifetime values. At present, it is not clear whether this second distribution component of relatively low amplitude represents a real contribution to the distribution which then should be "bimodal" or if this component is an artifact which resulted from the assumption of a symmetric distribution. One possibility is that there is a long tail in the distribution at shorter lifetimes (see Figure 3A). This problem is being investigated further using nonsymmetrical distributions.

The heterogeneity of vesicle preparations cannot be responsible for the observed lifetime distribution. DPH in vesicles of different sizes can have lifetime values differing by as much as 1.5 ns. However, if in each vesicle size the lifetime is a single exponential, the size heterogeneity will give rise to a lifetime distribution confined in 1.5 ns. The addition of single exponentials over a range of 1.5 ns gives a distribution which is indistinguishable from a single exponential.

If we make the assumption that the decay of DPH fluorescence is realistically described by Lorentzian distributions, this leads to the reasonable explanation for the behavior

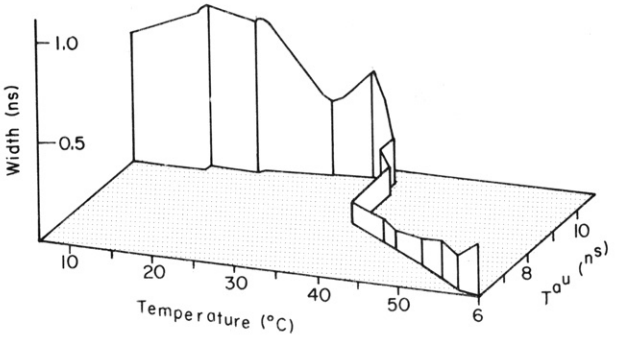

FIGURE 4: Three-dimensional representation of the major component of the distributional analysis of the decay of DPH in DPPC multilamellar vesicles as a function of temperature.

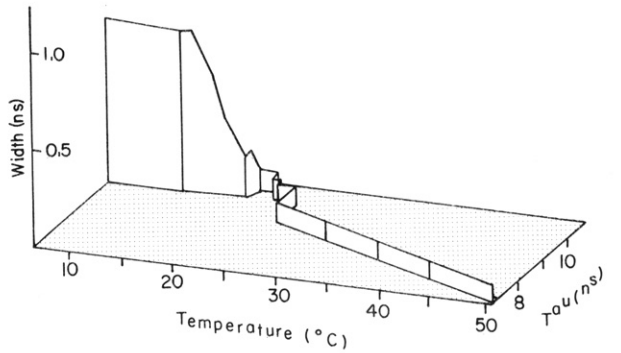

FIGURE 5: Three-dimensional representation of the major component of the distributional analysis of the decay of DPH in DMPC multilamellar vesicles as a function of temperature.

of the lifetime. The present analysis shows that below the phase transition the lifetime distribution is broad (Figures 4 and 5), in accordance with the observation that the decay is nonexponential below the phase transition (Barrow & Lentz, 1985). Also, for temperatures above the phospholipid transition, the distribution is narrow in accordance with the observed quasi-exponentiality at those temperatures. Two different ingredients are important to qualitatively explain the origin of the distribution of lifetime values and its evolution as a function of temperature. First, it is assumed that there is a distribution of environments for the DPH molecules. The DPH lifetime is sensitive to the dielectric constant of the medium where it is embedded (Zannoni et al., 1983). For nonpolar solvents, there is a linear relationship between the lifetime and the dielectric constant. The lifetime ranges from 15.7 ns in *n*-hexane ($\epsilon_r = 1.89$) to 6.1 ns in benzene ($\epsilon_r = 2.28$). In polar solvents, the lifetime is shorter. For example, in ethyl alcohol, the lifetime value is around 6 ns, and in water, the fluorescence is virtually quenched. Since DPH can be located at different positions along the membrane normal, and since the "polarity" of the membrane is also a function of the distance from the membrane surface, a range of lifetime values should exist for DPH in phospholipid vesicles. Second, if the rate of translational diffusion is comparable or faster than the fluorescence lifetime, then the DPH molecule will rapidly average over the different environments. The average lifetime should then be shorter since the DPH molecule will experience an environment with a "faster" decay rate during its fluorescence lifetime. Also, the distribution should be narrower since only the average environment should determine the lifetime value. In support of this interpretation is the observation that a fluorophore such as DPH-PC has a homogeneous lifetime (Barrow & Lentz, 1985; E. Gratton, unpublished results), probably due to a more fixed location along the membrane normal or to a more fluid environment in the middle

of the bilayer. Instead, a molecule such as TMA-DPH has a broad distribution (R. Holmes, unpublished results) since the DPH moiety resides in a region with a large polarity gradient or a more viscous environment near the surface. The second mechanism then assumes that there is an interconversion between different environments. When the rate of interconversion is fast, such as should be the case in the liquid-crystalline phase, the DPH molecule rapidly averages different surroundings during the excited-state lifetime. The lifetime distribution thus narrows as the temperature increases through the phase transition and does so in a manner that parallels its rotational behavior. In conclusion, it is proposed that the observed lifetime distribution depends on the different locations of the DPH molecules and the temperature dependence of the distribution width is related to the mobility of the DPH molecules.

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