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Detection of Phospholipid Phase Separation

A MULTIFREQUENCY PHASE FLUORIMETRY STUDY OF 1,6-DIPHENYL-1,3,5-HEXATRIENE FLUORESCENCE*

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Using multifrequency phase and modulation fluorometry and a nonlinear least-squares analysis of lifetime data, we were able to determine the complex decay of 1,6-diphenyl-1,3,5-hexatriene (DPH) in synthetic phospholipid bilayers. Our results showed a monoexponential decay of DPH in the pure isotropic solvents studied, over a wide temperature range, and a double-exponential decay of DPH in phospholipids, both above and below the transition. During the transition, and in mixed-phase phospholipids, a three-component analysis was successfully accomplished, and the pre-exponential factors of the two main components have been shown to be quantitatively representative of the gel and liquid-crystalline phases of the bilayer. The fractional intensity of the shorter lifetime component depends on the modalities of the sample preparation. The factors affecting this component are discussed. From the DPH fluorescence lifetime and from the anisotropy data in *L*- α -dimyristoyl-phosphatidylcholine/*L*- α -dipalmitoyl-phosphatidylcholine mixtures, a phase diagram was independently constructed. Conclusions about the sensitivity and the partition of the probe between gel and the liquid-crystalline phases of the bilayer are derived. Lifetime experiments on DPH in a *L*- α -dilauroyl-phosphatidylcholine/*L*- α -dipalmitoyl-phosphatidylcholine mixture suggested a general method for the determination and quantitation of the two different phases in the bilayer.

vation of the structural and dynamical properties of membranes.

Multiple lifetimes of DPH in mixed phospholipid vesicles have been reported; double-exponential decay of the fluorescence has often been observed from probes inserted in phospholipid bilayers and has been generally attributed to phase domain segregation in membranes (7, 8). In principle, a multiexponential fluorescence decay can be due to heterogeneity in the chemical and structural interactions between solvent and probe or to the intrinsic photophysical properties of the probe molecule. This latter case is not pertinent to DPH which shows a monoexponential decay in most isotropic solvents over a wide temperature range. Thus, a multiexponential decay in pure or mixed phospholipid bilayers may be indicative of different probe environments. Also, DPH shows no preferential partitioning between the gel and the liquid-crystalline phases (9).

The determinations of DPH decay modes in single-phase phospholipids are still controversial. A number of authors using pulse fluorometry have reported a double-exponential decay of DPH in both pure gel and pure liquid-crystalline phase of DMPC and DPPC bilayers (2-4). The reported lifetime values are around 10 and 4.5 ns below the transition temperature and around 8 and 3 ns above the phospholipid transition. On the contrary, by phase fluorimetry, a monoexponential decay has been found for many phospholipid bilayers above the transition temperature (5-7), although a double-exponential decay is still observed below the transition. The different results obtained with the two techniques have been attributed to possible systematic errors inherent to the modulators used in the commercially available phase fluorimeters (10). Also, an unfavorable circumstance is the proximity of the main component lifetime values, below and above the phospholipid transition, corresponding approximately to 10 and 8 ns, respectively. The accurate determination of the decay mode of DPH in pure single-phase phospholipids is a preliminary condition for the use of the DPH decay behavior for the quantitative detection of domain segregation in phospholipid membranes. Furthermore, we need to have the possibility of performing a multicomponent analysis in order to separate the various decay modes in a mixed-phase bilayer.

In this paper, we present a study on DPH emission decay in pure isotropic solvents and in synthetic phospholipid bilayers by means of the powerful new fluorescence methodology of multifrequency cross-correlation phase and modulation fluorimetry (10). Using the instrumentation described by Gratton and Limkeman (11), which allows facile selection of arbitrary light modulation frequencies, we were able to obtain lifetime results with resolution of several picoseconds. This accuracy was necessary to obtain the resolution of close

DPH¹ is currently the most popular hydrophobic probe utilized in studies concerning the structural and dynamical properties of synthetic and natural membrane systems. Information on the physical state of the phospholipid bilayer originates from the observed changes in the fluorescence parameters, *i.e.* polarization and lifetime. The meaning of the derived microviscosity and order-related parameters has been the subject of debate in recent years (1, 2). Furthermore, uncertainties also exist about the exact nature of the basic fluorescence parameters, such as the number and relative weights of the lifetime components (2-6). The characterization of such parameters is of great consequence for the deri-

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¹ The abbreviations used are: DPH, 1,6-diphenyl-1,3,5-hexatriene; DLPC, *L*- α -dilauroylphosphatidylcholine; DMPC, *L*- α -dimyristoyl-phosphatidylcholine; DPPC, *L*- α -dipalmitoylphosphatidylcholine.

lifetime components. Furthermore, this instrument is apparently free of sensible systematic errors. This ideal condition was the basic motivation for the investigation reported in this work. The possibility of resolving the emission into the components corresponding to the probe in the gel and in the liquid-crystalline phase can provide a method to quantitate the concentration of each phase. This determination can be of great relevance in the study of natural membranes. Moreover, the difference in the lifetime value above and below the phase transition can provide information on the change of the physical properties of the membranes.

MATERIALS AND METHODS

DLPC, DMPC, DPPC, and DPH were from Sigma and from Avanti Polar Inc. (Birmingham, AL) and were used without further purification. DPH from Aldrich was also used. In the experimental conditions used in this work, the background phospholipid fluorescence was always below 0.5% of the total fluorescence of DPH-labeled samples. All solvents were of spectroscopic grade and deoxygenated by N₂ bubbling for 30 min prior to each measurement.

Preparation of Labeled Multilamellar Vesicles—Aliquots of DLPC, DMPC, or DPPC solutions in chloroform were evaporated under N₂ with the proper amount of a tetrahydrofuran solution of DPH. The ratio between the probe and the phospholipids was 0.1 μM, 0.1 mM (final concentrations). The dry samples were then resuspended in Dulbecco's phosphate-buffered saline, previously deoxygenated by vigorous N₂ bubbling for at least 45 min, and then were capped under N₂ atmosphere.

Fluorescence Measurements—Steady-state polarization measurements were performed with an SLM 4800 spectrofluorometer using the L format. Excitation wavelength was 360 nm with 1-nm bandwidth. The emission was observed after a Schott cutoff filter, type KV ($\lambda_{em} > 418$ nm). Scattering contribution to the polarization values was always negligible. The polarization values were corrected for the instrument-intrinsic polarization. During the measurements, the samples were continuously stirred by a magnetic device under the cell holder, and the temperature was controlled using a thermostated cuvette holder.

Fluorescence lifetime measurements were performed with the multifrequency phase and modulation fluorometer described elsewhere (11). The instrument operates using the cross-correlation principle introduced by Spencer and Weber (14). The light source is an argon ion laser whose intensity is sinusoidally modulated using a Pockels cell. The modulation frequency can be varied continuously from 1 to 160 MHz. Generally, a set of 8–12 modulation frequencies was used in the range most appropriate for the sample under investigation. For the present study, the frequency range used was from 2 to 90 MHz for DPH in pure solvents and from 2 to 70 MHz for DPH in phospholipids. For each frequency, the phase and the modulation of the fluorescence were measured with respect to a scatter solution (glycogen). The use of a reference fluorophore of known lifetime gave identical results which indicate the lack of geometric or "color" effects in the system (10). Phase and modulation values were collected by an

Apple II computer using an ISS01 interface (ISS Inc., Champaign, IL). The set of phase and modulation data was analyzed by a nonlinear least-squares routine, using a software for the Apple II from ISS Inc. The method has been described by Jameson and Gratton (15) and by Lakowicz *et al.* (16). Data were fitted to a sum of exponential terms, each characterized by a lifetime τ and a fractional intensity f . Pre-exponential factors for the i component were derived from the fractional intensities using the relation $\alpha_i = (f_i/\tau_i)/(\sum_j f_j/\tau_j)$, where the sum is over all the components. The reduced χ^2 value was used to judge the goodness of the fit. Lakowicz *et al.* (16) have discussed the criterion for accepting a decay scheme based on the value of the χ^2 for the instrument used in this work. Generally a value less than 3 is considered acceptable while a value of the order of 10 indicates large deviations between experimental and calculated values. The errors in the parameters can be calculated using the covariance matrix of errors as discussed by Lakowicz *et al.* (16). In this work, excitation from the argon-ion laser was at 351 nm. Emission was observed through a Corning sharp-cutoff filter 3-74. Scattered light from the glycogen solution was observed using an interference filter (PTR Optics) at 351 nm. For the experiments in ethanol at low temperature, a cryostat (Beckman, model 196778), modified for fluorescence, was used. In this case, the internal scattering of the sample viewed using the interference filter at 351 nm was used as reference. Irradiation experiments on DPH-labeled phospholipids and on DPH in pure solvents were performed using a 150-watt Xenon arc lamp (LX150UV, ILC Technology Inc., Sunnyvale, CA) at 25 cm of distance and after a neutral density filter of 0.3 absorbance.

RESULTS

Pure Solvents—The DPH fluorescence lifetime in dodecane was measured at nine different modulation frequencies in the range of 2 to 90 MHz and at three different temperatures. The least-squares analysis of the lifetime values gave a monoexponential decay with a value of approximately 12.2 ns at all the temperatures investigated (Table I). For DPH in cyclohexanol, a monoexponential decay was also obtained, with a lifetime value of 5.6 ns. The DPH decay behavior in ethanol was monoexponential at room temperature but double exponential at -70°C , in accordance with previously reported results (12). Also reported in Table I are the dielectric constant and the viscosity values of the solvents. A correlation between the DPH lifetime values and the dielectric constant of the solvent is evident, as well as the absence of correlation with viscosity, in accordance with previously reported data (13). The double-exponential decay of DPH in ethanol at low temperature has been attributed to photochemical properties of the probe in polar environments (12). This interpretation can also be related to the temperature dependence of the DPH lifetime value in polar hydrocarbon solvents (13). In apolar isotropic solvents, however, the DPH lifetime value is inde-

TABLE I
DPH emission decay in isotropic solvents

Lifetime, τ ; fractional intensity, f ; pre-exponential factor, α ; and solvent dielectric constant, ϵ . Temperature of reported determinations were as follows: for footnote a, 10°C ; for footnote b, 20°C ; for footnote c, 25°C ; for footnote d, -60°C ; and for footnote e, -70°C .

Temperature °C	τ_1 ns	f_1	α_1	τ_2 ns	f_2	α_2	χ^2	ϵ	Viscosity
DPH in dodecane									
-6.0	12.18	1	1				2.59	2.047 ^a	
0.0	12.16	1	1				0.73		
6.5	12.19	1	1				0.70	2.014 ^b	1.35 ^c
DPH in cyclohexanol									
25.0	5.60	1	1				1.57	15.0 ^c	68.0 ^b
DPH in ethanol									
20.0	5.30	1	1				1.89	24.3 ^c	1.2 ^b
-70.0	11.17	0.901	0.361	2.17	0.099	0.639	3.70	41.0 ^d	13.2 ^e

TABLE II
Multiexponential decay of DPH in DPPC vesicles
Comparison between one-, two-, and three-component analysis at selected temperature values below, above, and during the transition. Lifetime, τ ; fractional intensity, f_i ; and pre-exponential factor, α_i .

Temperature °C	τ_1 ns	f_1	α_1	τ_2 ns	f_2	α_2	τ_3 ns	f_3	α_3	χ^2
21.6	8.55	1	1							21.90
	9.35	0.941	0.839	3.03	0.059	0.161				0.11
54.0	6.84	1	1							9.79
	7.31	0.948	0.870	2.67	0.052	0.130				0.22
40.5	7.89	1	1							7.13
	8.30	0.962	0.901	3.00	0.038	0.099				1.13
	9.50	0.392	0.322	7.50	0.578	0.600	3.00	0.030	0.078	0.80

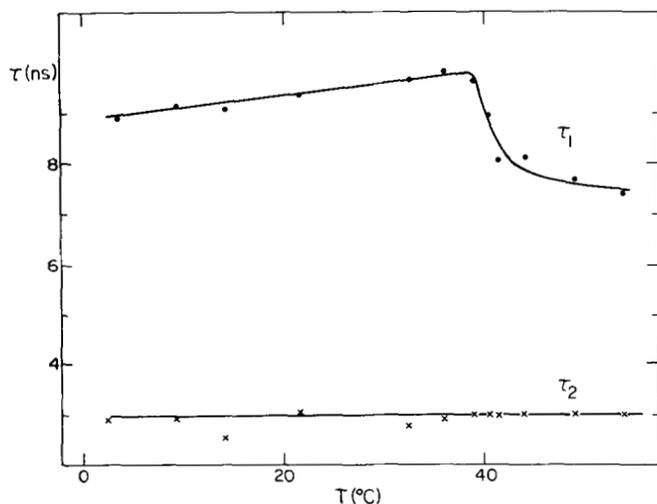


FIG. 1. DPH lifetime values in DPPC vesicles versus temperature. Lifetime data were analyzed for a two-component system.

pendent of temperature, as can be seen also from the dodecane results reported in Table I.

Pure Phospholipids Vesicles—DPH fluorescence lifetimes in DPPC were determined in the range from 2 to 70 MHz and at several temperatures. The lifetime values obtained at the different modulation frequencies in gel phase DPPC membranes were not consistent with a single component decay, as judged by the large value of the χ^2 (Table II). Improved fits, with a significantly lower χ^2 value, were obtained by a two-component analysis. The resulting main component (pre-exponential factor of approximately 0.85) had the value of about 9.5 ns near the transition temperature and was decreased as the temperature decreased. The short lifetime component had the value of 3 ns (Table II). This short lifetime component had constant value with similar pre-exponential factor throughout the temperature range investigated that included measurements below, above, and at the DPPC transition. Below the phospholipid transition temperature for all the samples studied, a small decrease of the 9.5-ns lifetime value with decreasing temperature was observed, in agreement with the increase of the dielectric constant of the environment that is known to affect the DPH lifetime value (Fig. 1). Above the transition temperature, the longer lifetime component was lowered to about 7.5 ns. In the temperature range close to the transition, the main lifetime component was split into two components. A three-component analysis was performed by fixing the lifetime values to 9.5, 7.5, and 3 ns, determined by the measurements above and below the DPPC transition, and solving for the associated pre-exponential factors (Table

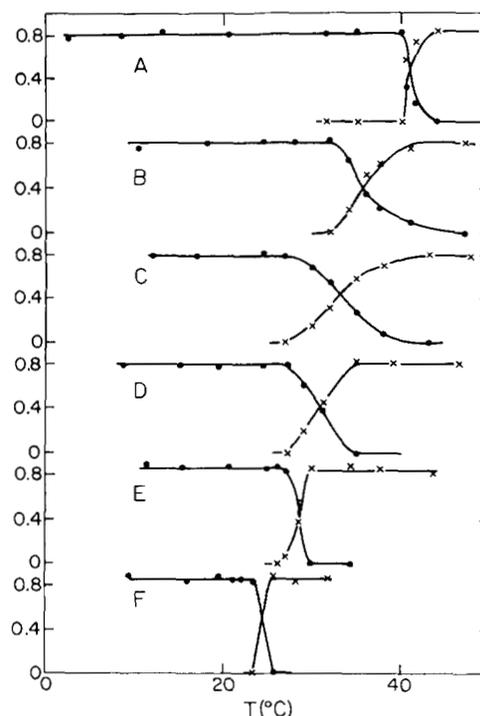


FIG. 2. Temperature variation of the pre-exponential factors of the 9.5- and 7.5-ns lifetime components, α_1 (●) and α_2 (×), respectively, obtained from a three-component analysis of DPH in DMPC/DPPC multilamellar vesicles. The pre-exponential factor of the shorter lifetime component of 3 ns is not shown. DMPC molar fractions from A to F are 0, 0.2, 0.4, 0.6, 0.8, 1.

II). The value of 9.5 ns obtained with the two-component analysis below the transition was assumed to be constant during the transition. The value of 7.5 ns was observed for the lifetime value of a two-component analysis above the transition. Furthermore, the three-component analysis performed fixing the 7.5- and 3-ns values gave the value of 9.5 ns for the third component. Instead, fixing the 9.5- and 3-ns values, the analysis gave the value of 7.5 for the third component. At all the temperatures investigated, the 3-ns component had a pre-exponential factor of approximately 0.15. Increasing the temperature, the 9.5-ns component was progressively substituted by the 7.5-ns component, by the decrease of the former pre-exponential factor and the contemporaneous increase of the 7.5-ns pre-exponential factor from 0 to 0.85 (see Fig. 2A). A complete analysis in terms of three-exponential components is outside the possibilities of the present instrumentation due to the proximity of the 9.5- and 7.5-ns components. However, we succeeded in performing a

partial three-component analysis and in decreasing the χ^2 value with respect to a two-component analysis by simply fixing the values of the lifetimes on the basis of the results obtained at temperatures well above and below the transition range. The transition midpoint obtained with the variation of the pre-exponential factors associated with the 9.5- and 7.5-ns lifetimes was coincident with the known DPPC transition temperature.

Phospholipid Mixtures—Similar results were obtained with DMPC and DMPC/DPPC mixed membranes, with molar ratios of 0.2, 0.4, 0.6, and 0.8, respectively, as can be seen from Fig. 2. For all the mixtures investigated, in pure gel or pure liquid-crystalline phase, the best fits were obtained using the procedure employed for pure DPPC. In the mixtures, the short lifetime component always had the value of 3 ns and a pre-exponential factor of approximately 0.15. The long lifetime component had the value of approximately 9.5 ns below the phospholipid phase transition and of approximately 7.5 ns above that temperature. In the transition range, the three-component analysis was repeated, fixing the lifetime value at 9.5, 7.5, and 3 ns and solving for the pre-exponential factors. The pre-exponential factors decreased to zero for the 9.5-ns component and increased from 0 to 0.85 for the 7.5-ns component as the temperature increased. The transition midpoint obtained by plotting the pre-exponential factors was shifted to higher temperatures as the concentration of DPPC in the samples increased (Fig. 2).

The DPH fluorescence anisotropy plots of DMPC/DPPC mixtures as a function of temperature are reported in Fig. 3. The anisotropy values followed the phospholipid phase transition. The transition was shifted to higher temperatures with the increase in the DPPC concentration in the membrane. In the DMPC/DPPC mixed samples, the transition curve was broadened, but only one transition was ever observed, indicating the miscibility of the two phospholipids (17). In the phase diagram obtained from the anisotropy data reported in Fig. 4, the gel phase line was obtained from the temperatures below which all the lipids are in the gel phase (Fig. 3), and the liquid-crystalline phase line was obtained from the temperatures above which all the lipids are in the liquid-crystalline phase.

A phase diagram is also obtained using the DPH lifetime pre-exponential factors associated with the 9.5- and 7.5-ns

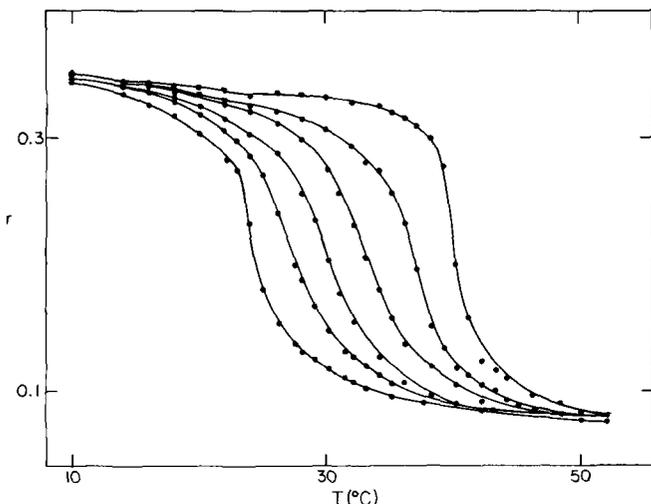


FIG. 3. Steady-state DPH fluorescence anisotropy of DMPC/DPPC pure and mixed multilamellar vesicles as a function of temperature. DPPC molar fractions from the left to the right plot are 0, 0.2, 0.4, 0.6, 0.8, 1.

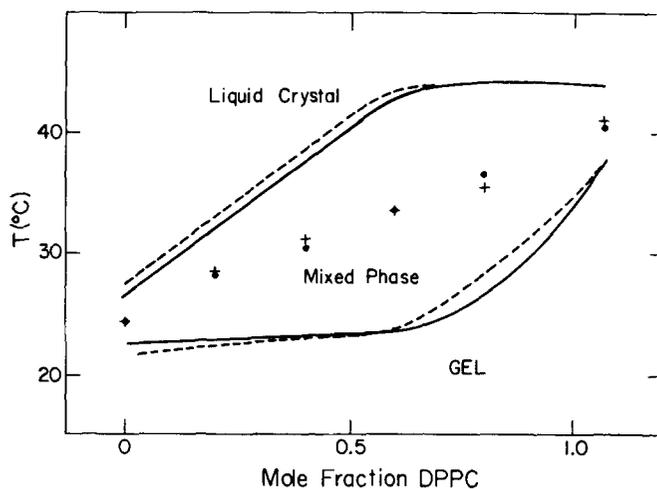


FIG. 4. Phase diagram of DMPC/DPPC mixed bilayers derived from DPH fluorescence anisotropy data (● and dashed line) and from the pre-exponential factors associated to the 9.5- and 7.5-ns components of the DPH decay (+ and solid line).

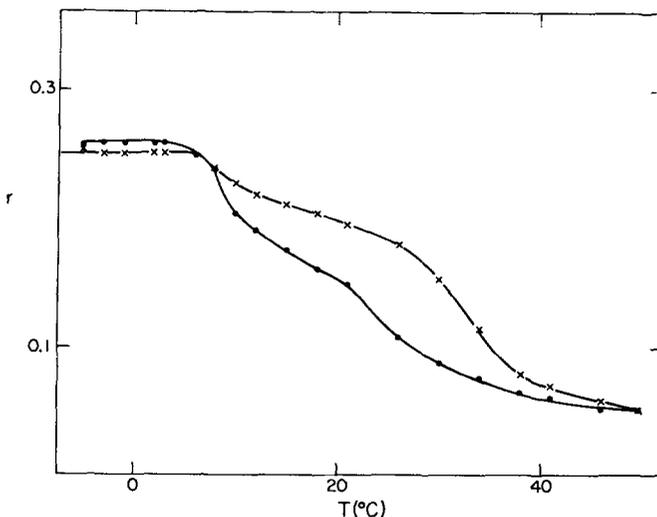


FIG. 5. Steady-state DPH fluorescence anisotropy in DLPC/DPPC mixtures versus temperature. DPPC molar fractions are 0.4 (●) and 0.6 (×).

components, as plotted in Fig. 2. For this phase diagram, the gel phase line was obtained from the first temperature at which a pre-exponential factor for the 7.5-ns component was detected, and the liquid-crystalline phase line was obtained from the last temperature at which the presence of the 9.5-ns component was detected. The two phase diagrams, as determined using the fluorescence anisotropy or the lifetime pre-exponential factors, almost overlap, indicating the absence of preferential partitioning of DPH between the gel and liquid-crystalline phases, in agreement with Ref. 9.

The DPH decay was measured also in a 1:1 molar ratio mixture of DLPC and DPPC. In this mixture, two separate phase domains have been reported, with transitions occurring at two widely separated temperatures (5, 7). In fact, our plots of DPH anisotropy versus temperature also strongly suggested such a phase separation (Fig. 5). Two main transitions were detected, the first involving a DLPC-enriched domain at approximately 8 °C and the second involving a DPPC-enriched domain at approximately 30 °C. The two transitions were present in all DLPC/DPPC mixtures at various concentration ratios (not shown), with a shift of both transition

TABLE III
Least-squares analysis of the decay of DPH in DLPC/DPPC equimolar mixture
Comparison between one-, two-, and three-component analysis at selected temperature values. Lifetime, τ ;
fractional intensity, f ; and pre-exponential factor, α .

Temperature °C	τ_1 ns	f_1	α_1	τ_2 ns	f_2	α_2	τ_3 ns	f_3	α_3	χ^2
-1	9.26	1	1	ns			ns			8.67
	9.66	0.972	0.916	3.00	0.028	0.084				2.80
	10.50	0.704	0.613	7.50	0.281	0.342	3.00	0.015	0.045	1.74
18	8.73	1	1	ns			ns			11.11
	9.24	0.961	0.890	3.00	0.039	0.110				1.82
	10.50	0.566	0.463	7.50	0.411	0.471	3.00	0.023	0.066	0.88
47	7.24	1	1	ns			ns			19.35
	7.97	0.921	0.815	3.00	0.079	0.185				2.32
	10.50	0.142	0.096	7.50	0.790	0.745	3.00	0.068	0.160	2.09

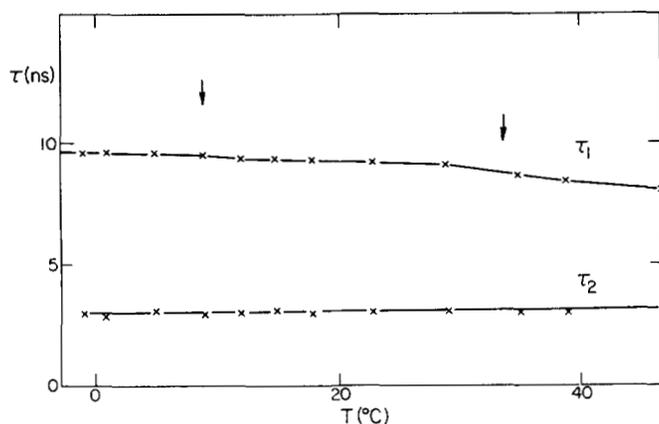


FIG. 6. Results of a two-component analysis of DPH decay in an equimolar DLPC/DPPC mixture versus temperature. The associated pre-exponential factors are approximately 0.9 and 0.1 for τ_1 and τ_2 , respectively.

temperatures following the variation of the two phospholipids' concentration ratio in the mixture. Lifetime data, obtained using the previously employed modulation frequencies set, were analyzed for a two-component system, and the lifetime values obtained were 3 ns, associated to a pre-exponential factor of approximately 0.1, and a longer value around 8 ns, slowly decreasing with increasing temperatures (Fig. 6). The two phospholipid transitions were poorly detectable when the data were fit to only a two-exponential decaying system. The χ^2 value was significantly decreased, using a three-component analysis with the previously employed procedure of fixing the lifetime values to 10.5, 7.5, and 3 ns (Table III). The pre-exponential factors obtained by this analysis impressively followed the two transitions (Fig. 7). With the DLPC/DPPC mixture, the fit was improved, fixing the longest lifetime value to 10.5 ns instead of 9.5 ns, as was the case of DMPC/DPPC samples. The value of 10.5 ns was the average value obtained by the least-squares analysis when the values of the 7.5 and 3 ns were fixed. The pre-exponential factor associated to the 7.5-ns component progressively increased when the temperature was raised, substituting for the 10.5-ns component. In the range between the two main transition temperatures, a plateau was observed, with almost equal pre-exponential factor for the 10.5- and 7.5-ns components. Increasing the temperature, a small increase of the pre-exponential factor associated to the 3-ns component was also observed.

The Origin of the 3-ns Component—The constant value of the short lifetime component, the constancy of its associated

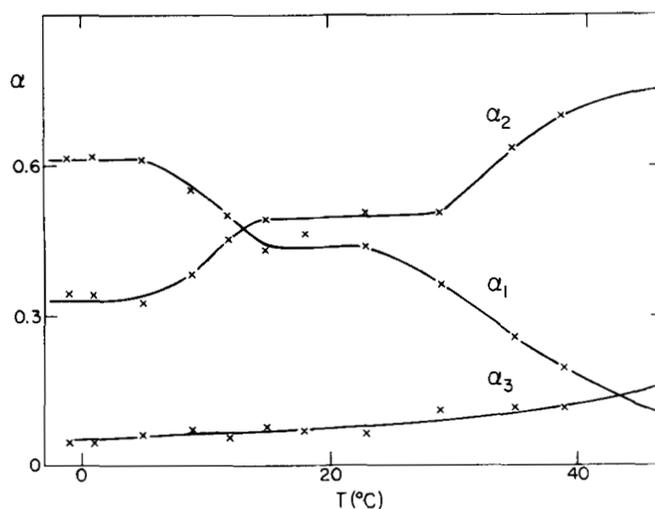


FIG. 7. Pre-exponential factors obtained from a three-component analysis on the DPH decay in DLPC/DPPC equimolar mixture versus temperature. The associated lifetime values are 10.5, 7.5, and 3 ns, for α_1 , α_2 , and α_3 , respectively.

pre-exponential factor, and its insensitivity to temperature variations strongly suggest that the origin of this component is independent from the physical state of the vesicle. The relatively large intensity of this component also shows that the background fluorescence cannot be responsible for its presence. Instead, we observed that the relative amount of the 3-ns component tended to increase as the sample aged. Furthermore, the fractional contribution of this component was smaller when the DPPC from Sigma (reported purity 98%) was substituted by DPPC from Avanti Polar Inc. (reported purity 99.9%). However, we observed that in a sample with an initial very low contribution of the short component fraction, the intensity of the 3-ns component dramatically increased upon exposure of the sample to light at room temperature. In Fig. 8, we report an irradiation experiment performed on a sample of DPPC from Avanti Polar Inc. with an initial negligible amount of the 3-ns component. This experiment shows that upon increasing the irradiation time, the fluorescence intensity decreased (Fig. 8A) and the fractional contribution of the 3-ns component increased (Fig. 8B). However, the decrease of the fluorescence intensity was larger than the increase of the 3-ns fractional intensity. We also observed a fluorescence intensity decrease (Fig. 8A) and the appearance of a second lifetime component with a value around 2 ns irradiating a solution of DPH in cyclohexane

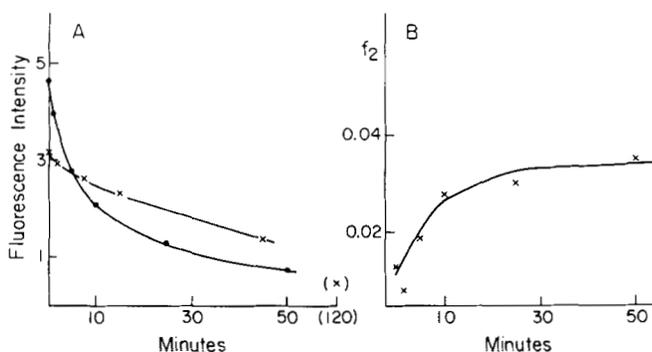


FIG. 8. Irradiation experiment of DPH in DPPC and in cyclohexane. A, DPH in DPPC vesicles (●) and DPH in cyclohexane (×). B, fractional intensity of the 3-ns component versus irradiation time at room temperature in deoxygenated samples. The irradiation procedure is described under "Materials and Methods."

(not shown). Moreover, irradiating DPPC vesicles in the absence of DPH, followed by incubation of the samples with the probe just prior to the measurements, we found that the short component was present in a relevant amount. In a series of tests to investigate the parameters affecting the relative amount of the 3-ns component in phospholipid vesicles, we found that the presence of unsaturated lipids affected the total amount and the rate of increase of the 3-ns component. We believe that the photochemical reaction responsible for the appearance of the 3-ns component is different in a pure solvent than in the phospholipid vesicle. In pure solvents, the reaction involves the DPH molecule directly. Although the rate of the bleaching process is slow, the total amount of bleaching can be considerable; the 3-ns component in the residual fluorescence remains small. Instead, for irradiated vesicles, the photochemical reaction probably involves a non-fluorescence species in the phospholipid moiety which then, in turn, reacts with the DPH. Finally, we noticed that exposing the phospholipid suspension to room light for a few hours is sufficient to produce a fraction of the 3-ns component of about 0.03–0.05.

DISCUSSION

DPH emission in isotropic solvents is known to be monoexponential (13), with a temperature-dependent lifetime in polar hydrocarbon solvents. Moreover, the lifetime value is sensitive to the dielectric constant of the medium but not to the viscosity. A double-exponential decay of DPH has been observed only in ethanol at very low temperature (12). These results were confirmed in the present multifrequency phase and modulation fluorimetry study, as can be seen in Table I. A monoexponential decay was found for DPH in dodecane with a lifetime value which is temperature-independent, in cyclohexanol, and in ethanol at room temperature. The appearance of a double-exponential decay in ethanol at -70°C , consistent with previously reported observations (12), does not pose supplementary problems to the interpretation of the DPH decay behavior in phospholipids, since the temperature range used in membranes studies cannot be lowered below the freezing point of the aqueous suspension. The multiplexponential decay of DPH in membranes can be properly attributed to different probe environments, probably due to microheterogeneity of the dielectric constant.

DPH fluorescence in single phase phospholipids has been reported to be described either by a monoexponential (5–6) or by a double-exponential decay (2–4), using phase or pulsed fluorimetry, respectively. The systematic errors inherent to the use of the Debye-Sears modulator tank (10) together with

the restricted number of modulation frequencies available in the commercial phase fluorometer rendered these results questionable. Our phase fluorimetry study on DPH lifetime in phospholipids showed a double-exponential decay with a short-lived component of 3 ns, associated with a pre-exponential factor around 0.15, and a long-lived component of approximately 10 ns below the phospholipid transition and of 7.5 ns above it. The results did not vary, substituting the glycogen-scattering reference with a fluorescent reference of known lifetime. Our results are in good agreement with determinations by the pulsed technique (2–4). In the DMPC/DPPC samples, the longest lifetime had a value of 9.5 ns below transition of both lipids. In the DLPC/DPPC mixture, a good fit was obtained, fixing the longest lifetime value to 10.5 ns. We attributed this variation to the decrease of the average acyl chain length in the DLPC/DPPC mixture. The dielectric constant decreases as the length of the chain increases in saturated hydrocarbons. The origin of the 3-ns component has been discussed under "Results" and depended on the sample source and irradiation. Since the 3-ns component is easily detectable and quantitated, in principle it can be subtracted from the data. We attributed the 10-ns component to DPH molecules in a gel phase environment and the 7.5-ns component to probe molecules in a liquid-crystalline phase. The decrease of the lifetime value passing through the phospholipid transition cannot be attributed to "microviscosity" changes of the hydrophobic matrix, since DPH lifetime value is not sensitive to the viscosity of the medium (Table I and Ref. 13). We attributed the decrease of the lifetime value from about 10 to 7.5 ns, below and above the transition, to DPH molecules residing in an environment with different dielectric constant.

During the phospholipid transition, a three-component analysis was successfully performed, with a decrease of the χ^2 value, as compared to the two-component analysis. The procedure used for fixing the lifetime values and then searching for the associated pre-exponential factors allowed us to bypass the difficulty inherent to the resolution of a three-component system, in which two of the lifetime values are separated only by a factor of 1.5, as are the lifetime values of the main component of the DPH decay in pure gel and pure liquid-crystal membranes. The pre-exponential factors obtained for the two longer components followed the phospholipid transition and were used to construct a phase diagram of a DMPC/DPPC mixture. This phase diagram was coincident with that obtained by DPH anisotropy values and by other techniques (17), demonstrating the equal partitioning of the probe between the phospholipid phases. In a mixture of DMPC/DPDC, the DPH lifetime variations had been successfully used to distinguish and quantitate the different coexisting phases.

Another phospholipid mixture of DLPC/DPPC at an equal molar ratio that is known to originate separate domains in a wide temperature range (7, 17) was investigated. In this case also, it was possible to detect and to quantitate the two phospholipid phases present using the DPH emission decay. To our knowledge, this is the first study in which different phases have been directly quantitated in a phospholipid mixture showing two distinct transitions.

In conclusion, using the very sensitive technique of multifrequency phase fluorimetry and a powerful method of the nonlinear least-squares for the analysis of the lifetime data, we were able to investigate the multiexponential decay of DPH in phospholipid vesicles. We detected a short lifetime component of about 3 ns, whose existence has been debated in the literature. We traced the physical origin of this com-

ponent to a photolytic process involving the DPH and the phospholipids. Once the origin and the lifetime value of this component was determined, we accounted for its contribution from the samples investigated. We have not concerned ourselves at this time with elucidating the exact chemical species produced in the photolytic reaction. We found that the lifetime of DPH in the gel phase is about 10 ns, whereas in the liquid-crystalline phase it is about 7.5 ns. In a mixed phase, two components were resolved, and their pre-exponential factors were used to quantitate the relative amount of each phase.

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