

Orientalional Distribution of 1,6-Diphenyl-1,3,5-hexatriene in Phospholipid Vesicles As Determined by Global Analysis of Frequency Domain Fluorimetry Data[†]

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ABSTRACT: Polarized differential phase and modulation ratios were obtained for 1,6-diphenyl-1,3,5-hexatriene (DPH) in 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC), 1,2-dilauroyl-*sn*-glycero-3-phosphocholine (DLPC), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) vesicles by using multifrequency phase fluorimetry. Data were analyzed in terms of both empirical sums-of-exponentials modeling and directly in terms of the orientational distribution functions. The orientational analysis model was used to recover the angular distribution of DPH and the rotational diffusion coefficient in the various membrane model systems throughout the phase transition. A global analysis methodology was utilized to obtain an internally consistent set of parameters that fit all of the data simultaneously. The rank order parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ were extracted from the experimental data, and the angular distribution functions of DPH were calculated. When the time-zero anisotropy (r_0) of several sets of data taken at various temperatures were linked in a single global analysis, better recovery of the fourth rank order parameter $\langle P_4 \rangle$, diffusion constant D , and r_0 was obtained with respect to the unlinked analysis. From these recovered values, a detailed picture concerning the orientational distribution of DPH in membranes as a function of temperature was obtained. The results suggest that a single population of DPH molecules was present in the bilayers with their orientational distributions dependent upon the physical state of the membranes in the pure phases. During the phase transition, a superposition of two populations corresponding to the population of the pure phases was present. As the temperature increased in the transition region, one population was increasing at the expense of the other.

Biological membranes are a complex mixture of different types of lipid and protein molecules. A major goal of membrane research in recent years has been to describe the dynamic and structural properties of membrane components. Since a population of molecules may take up a variety of positions in a membrane, the purpose of this study was to extend methods of analyzing multifrequency phase fluorimetry data to give a complete description of the orientational distribution of a fluorophore in a membrane.

1,6-Diphenyl-1,3,5-hexatriene (DPH)¹ has been used extensively for studying membrane structure and fluidity because of its advantageous structural and spectral properties. It has a rigid rodlike structure and possesses cylindrical symmetry around its long axis. The high extinction coefficient and fluorescence quantum yield in nonaqueous solvents make it possible to use very small amounts in biological studies, thus minimizing perturbations and making DPH particularly attractive for obtaining dynamic and structural information about membrane bilayers. The rotational behavior of DPH as well as its fluorescence lifetime have been used as indicators of the physical state of model and biological membranes. The total intensity decay behavior of DPH in model systems has been described as being predominantly double exponential (Parasassi et al., 1984), although alternative analyses in terms of distributions of lifetimes have been performed (Fiorini et al., 1987; Williams & Stubbs, 1988). Time-resolved

fluorescence anisotropy studies have been used to determine the orientational distribution of DPH in membranes. Several models have been proposed on the basis of consideration of different terms of the angular distribution function and of the dynamics of the DPH molecule. In particular the decay of the emission anisotropy has been analyzed in terms of the parameters r_0 , r_∞ , and ϕ , where r_0 is the anisotropy at time zero, the r_∞ is the anisotropy a long time after excitation or residual anisotropy, and ϕ is the exponential characteristic decay time from r_0 to r_∞ (Lakowicz et al., 1987; Lipari & Szabo, 1980). The term r_∞ is directly related to the coefficient of the second Legendre polynomial and to the order parameter $S^2 = r_\infty/r_0 = \langle P_2 \rangle^2$. In general, an orientational distribution function is fully described by an infinite series. However, due to the dipole transition nature of the fluorescence process, only the $\langle P_0 \rangle$, $\langle P_2 \rangle$, $\langle P_4 \rangle$, and $\langle P_6 \rangle$ terms of the expansion of the orientational distribution function using Legendre polynomials can be obtained. In nonmacroscopically oriented samples, only the $\langle P_0 \rangle$, $\langle P_2 \rangle$, and $\langle P_4 \rangle$ terms can be obtained. The order of the system is described by the $\langle P_2 \rangle$ and $\langle P_4 \rangle$ coefficients, since $\langle P_0 \rangle$ has no angular dependence. The dynamics of the DPH molecule are contained in the diffusional constant D , corresponding to the rotation of the molecule along an axis of the cylinder to which the DPH molecule is modeled. Previous studies have found that all terms $\langle P_0 \rangle$, $\langle P_2 \rangle$, and $\langle P_4 \rangle$ are necessary to describe the orientational distribution

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¹ Abbreviations: DPH, 1,6-diphenyl-1,3,5-hexatriene; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid.

of DPH in bilayers (Van der Meer et al., 1984). The coefficient of the Legendre polynomial terms were found to be temperature dependent. The diffusion constant was also found to be a function of temperature, and it changes abruptly at the phospholipid phase transition temperature (Vogel & Jähnig, 1985). The reconstruction of the orientational distribution function of DPH in bilayers based on the recovered values of $\langle P_2 \rangle$ and $\langle P_4 \rangle$ has shown that there is a substantial contribution to the distribution from molecules parallel to the bilayer surface (Van der Meer et al., 1984). While similar patterns of distributions were found by other independent investigations (Vos et al., 1983; vande Ven et al., 1984; Straume & Litman, 1987a,b), the distribution component parallel to the membrane surface is a quite intriguing observation.

This study focuses on the analysis of differential phase and modulation measurements of DPH obtained throughout the phase transition region. Utilization of a global analysis for the multiple temperature data allows an internally consistent interpretation of the depolarization studies (Beechem & Gratton, 1988). The final results reveal that a continuous distribution of orientations exists in both the gel and fluid phases, with the distribution being dramatically broader in the fluid phase. Although we confirm that it is possible to fit the data with values of $\langle P_2 \rangle$ and $\langle P_4 \rangle$, which apparently indicates that DPH molecules are lying parallel to the membrane surface, we found a mathematically equivalent solution of the orientational distribution that has no contribution in the plane parallel to the membrane surface.

MATERIALS AND METHODS

Materials. DPH was purchased from the Aldrich Chemical Co. (Milwaukee, WI). DPPC, DMPC, and DLPC were from Avanti Polar Lipids, Inc. (Pelham, AL). Glycogen was from ICN Nutritional Biochemicals (Cleveland, OH), and *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) was from the Sigma Chemical Co. (St. Louis, MO). All products were used without further purification.

Preparation of Small Unilamellar Vesicles. Either DPPC, DMPC, or DLPC in chloroform was mixed with DPH in tetrahydrofuran at a 500:1 mole ratio in glass vials. The organic solvents were evaporated under nitrogen and the vials placed under vacuum to remove traces of the organic solvents. The glass vials were filled with 10 mM HEPES, pH 7.0, and 100 mM KCl that had been deoxygenated by bubbling with nitrogen for 30 min. The glass vials were sealed under nitrogen and the final concentration of the phospholipids was 330 mM. The lipids were hydrated for 25 min at 37 °C for DMPC and DLPC and at 45 °C for DPPC. The phospholipid-DPH mixtures were suspended by vigorous vortexing for 30 s followed by sonication for 1.5 h in a sonic cup above the transition temperatures of the phospholipids by using a Heat Systems sonicator (Ultrasonics, Inc., Model W-375) with the output control set at 5. Small unilamellar vesicles were obtained by centrifugation of the suspensions at 50000g for 1 h above the transition temperature of the phospholipids.

Fluorescence Measurements. Fluorescence lifetime and differential polarization measurements were performed with a multifrequency phase fluorometer (Gratton & Limkeman, 1983) equipped with a ISS-ADC interface for data collection. The wavelength of excitation was 325 nm with a helium-cadmium laser as the light source. The modulation frequency set was 2, 4, 6, 10, 20, 30, 50, 70, 85, 100, 125, and 150 MHz at each temperature. The sample temperature was monitored in the sample cell prior to and after each measurement using a digital thermometer. Data were accumulated at each fre-

quency until the standard deviations of the phase and modulation values were below 0.2° and 0.004, respectively. All lifetime measurements were obtained with glycogen in the reference cell and with a polarizer at 35° with respect to the vertical direction in the excitation light beam (i.e., magic angle conditions). For the collection of anisotropy data, differential phase and modulation ratio measurements were collected in the same frequency range used for the lifetime measurements. For each measurement, the polarization correction factors were measured and were close to 1. The emission was measured with a 2-mm thick liquid filter filled with 2 M NaNO₂ and a Corning 3-73 filter. A continuous nitrogen stream flowed through the fluorometer chamber during the measurements.

Data Analysis. The measured differential phases and modulation ratios (which are directly related to the rotational behavior of the probe) are defined as

$$\Delta\phi = \phi_{\perp} - \phi_{\parallel} \quad (1)$$

$$\Lambda = \Lambda_{\parallel} / \Lambda_{\perp} \quad (2)$$

where ϕ and Λ represent the experimentally measured phase lags and AC voltages in the parallel and perpendicular polarization direction, respectively. The experimental measurables are related to the sine and cosine transforms of the impulse response by

$$\Delta\phi = \arctan \frac{D_{\parallel}N_{\perp} - D_{\perp}N_{\parallel}}{N_{\parallel}N_{\perp} + D_{\parallel}D_{\perp}} \quad (3)$$

$$\Lambda = \sqrt{\frac{N_{\parallel}^2 + D_{\parallel}^2}{N_{\perp}^2 + D_{\perp}^2}} \quad (4)$$

$$N = \int_0^{\infty} I_i(t) \sin \omega t \, dt \quad (5)$$

$$D = \int_0^{\infty} I_i(t) \cos \omega t \, dt \quad (6)$$

where N and D are the sine and cosine transforms and the index i refers to the perpendicular and parallel polarized decays. These polarized decays are dependent on both the total intensity decay function $I(t)$ and the anisotropy decay $r(t)$, which describes the rate of rotation of the molecules. The parallel and perpendicular components of the measured response are related to $I(t)$ and $r(t)$ in the following manner:

$$I_{\parallel}(t) = (1/3)I(t)[1 + 2r(t)] \quad (7)$$

$$I_{\perp}(t) = (1/3)I(t)[1 - r(t)] \quad (8)$$

Models Utilized in the Analysis. Four separate analyses of the data have been performed: (1) Empirical nonassociative sums-of-exponential; (2) Empirical associative sums-of-exponential; (3) P_2 - P_4 single experiment target analysis; and (4) P_2 - P_4 global target analysis.

For empirical fitting of single experiment data, one simply utilizes a sums-of-exponentials model for the anisotropy decay part of the intensity decay in the following form:

$$r(t) = r_0 \sum_{i=1}^n \beta_i \exp(-t/\phi_i) \quad (9)$$

Due to the ill-defined nature of fitting sums-of-exponentials models, the number of terms that can be statistically justified by the data is relatively limited. For the multifrequency data collected for this study, single experiment analysis could only support the use of one exponential term plus an r_{∞} constant term.

$$r(t) = (r_0 - r_\infty)e^{-t/\phi} + r_\infty$$

Since the decay of the total-intensity function for magic angle observation was found to be biexponential, two different empirical models can be employed: nonassociative and associative. In the nonassociative model, the lifetime decay components and the rotational correlation times are independent of each other, and all of the cross-terms contained in eqs 7 and 8 are allowed to contribute. In the associative case, the lifetimes and rotational correlation times are not independent, and the cross-terms of eqs 7 and 8 are not utilized.

Analysis of the data in terms of sums-of-exponentials models is rather unsatisfactory for two reasons: numerical instability and lack of a *direct interpretation* of the recovered parameters in terms of molecular properties of the system. For these reasons, the data were further analyzed directly in terms of a *physical model* based on the orientational distribution of DPH molecules within the membrane systems.

Recently, van der Meer et al. (1984) and Ameloot et al. (1984) developed a theoretical framework to determine the orientation of DPH in vesicles. Their procedure allowed the determination of the angular distribution of DPH molecules within the vesicle system. One can describe the equilibrium orientational distribution of DPH in membranes in terms of Legendre polynomials:

$$f(\theta) = (1/2) \sum_n (2n+1) \langle P_n \rangle (\cos \theta) \quad (10)$$

where n is an even number, $\langle P_n \rangle$ is the n th degree Legendre polynomial, and θ is the angle between the normal to the bilayer and the emission moment of DPH. If many $\langle P_n \rangle$'s are known, the distribution is well-defined. Due to the fact that measurements are being performed on macroscopically non-oriented systems only the first one or two order parameters, $\langle P_2 \rangle$ and $\langle P_4 \rangle$, can be extracted from the experimental data. Within this theoretical framework, the analysis of the data is removed from the empirical fitting parameters (elements of the sums-of-exponentials model) to the *physical parameters* that describe the orientational distribution of probes within the bilayer, the diffusion coefficient, and the time-zero anisotropy ($\langle P_2 \rangle$, $\langle P_4 \rangle$, D , and r_0).

These target-fitting parameters are related to the standard sums-of-exponentials models for the anisotropy decay through the following relationships (van der Meer et al., 1984):

$$r(t) = r_0 \sum_{j=1}^3 [g_j \exp(-t/\phi_j) + g_4] \quad (11)$$

where

$$g_1 = 1/5 + 2\langle P_2 \rangle/7 + 18\langle P_4 \rangle/35 - \langle P_2 \rangle^2$$

$$g_2 = 2(1/5 + \langle P_2 \rangle/7 - 12\langle P_4 \rangle/35)$$

$$g_3 = 2(1/5 - 2\langle P_2 \rangle/7 + 3\langle P_4 \rangle/35)$$

$$g_4 = \langle P_2 \rangle^2$$

$$\phi_1 = g_1/[6D(1/5 + \langle P_2 \rangle/7 - 12\langle P_4 \rangle/35)]$$

$$\phi_2 = g_2/[12D(1/5 + \langle P_2 \rangle/7 - 12\langle P_4 \rangle/35)]$$

$$\phi_3 = g_3/[12D(1/5 - 2\langle P_2 \rangle/7 + 3\langle P_4 \rangle/35)]$$

Orientational distribution functions were reconstructed from the resolved $\langle P_2 \rangle$ and $\langle P_4 \rangle$ pairs by using the first three terms of the Legendre polynomial

$$f(\theta) = 0.5[1 + 5\langle P_2 \rangle P_2(\cos \theta) + 9\langle P_4 \rangle P_4(\cos \theta)] \quad (12)$$

where

$$P_2(\cos \theta) = 0.5(3 \cos^2 \theta - 1)$$

$$P_4(\cos \theta) = 0.125(35 \cos^4 \theta - 30 \cos^2 \theta + 1)$$

The use of this equation might result in negative values because it is truncated. Therefore, another form of the equation was used (Berne et al., 1968; Ameloot et al., 1984):

$$f(\theta) = N^{-1} \exp[L_2 P_2(\cos \theta) + L_4 P_4(\cos \theta)] \quad (13)$$

where L_2 and L_4 were calculated according to the $\langle P_2 \rangle$, $\langle P_4 \rangle$ pairs, and N is a normalization constant satisfying the condition

$$N = \int \sin \theta \exp[L_2 P_2(\cos \theta) + L_4 P_4(\cos \theta)] d\theta \quad (14)$$

In anisotropy decay studies, r_0 is a fitting parameter, which when allowed to vary between multiple experiments can result in rather ill-determined recovery of the rotational and orientational dependent parameters ($\langle P_2 \rangle$, $\langle P_4 \rangle$, and the diffusion coefficient D). To alleviate this problem, several sets of data obtained at different temperatures are directly analyzed in terms of an internally consistent r_0 term (i.e., a global analysis). (The only assumption is that r_0 is temperature independent.) Global analysis techniques have been applied to fluorescence decay and anisotropy decay studies (Beechem et al., 1983; Knutson et al., 1983; Arcioni et al., 1987; Lakowicz et al., 1987; Wendler & Holzworth, 1987; Beechem & Gratton, 1988). In these studies, some common parameters (e.g., the fluorescence lifetime or the rotational relaxation time) are linked through multiple experiments. This linking process can result in a more accurate determination of the fitting parameters. As applied to the direct fitting of the orientational distribution, the global analysis greatly improved the recovery of three of the four fitting parameters (D , r_0 , and $\langle P_4 \rangle$), while the sensitivity to the determination of $\langle P_2 \rangle$ remained similar to the single experiment analysis.

The fit for both fluorescence decay and anisotropy decay analyses were judged by the reduced χ^2 value, which is defined by

$$\chi^2 = \sum_{q=1}^{n_{\text{exp}}} \sum_{i=1}^{n(q)} \frac{(\text{data}_{qi} - \text{fit}_{qi})^2}{\sigma_{qi}^2} / (N - m - 1) \quad (15)$$

where N is the number of data points in the entire data surface, σ_{qi} is the standard deviation of the i th data point in the q th experiment, m is the number of total fitting parameters, n_{exp} is the total number of experiments, and $n(q)$ is the total number of data points taken in the q th experiment. Both phase and modulation values were simultaneously included in the fit procedures.

Rigorous error analyses (Beechem et al., 1989) were performed to estimate the uncertainties associated with the values determined for the fitting parameters. In this analysis one systematically fixes a recovered fitting parameter at a series of values and performs an entire nonlinear minimization allowing all of the remaining $m - 1$ parameters to vary while minimizing χ^2 . One can then record the series of minimum chi-square values obtained over a particular range of the i th fitting parameter. This type of error analysis takes into account all of the higher order correlations that may exist between a given set of fitting parameters. The region of the error surface that is explored is determined with an F -statistic criterion at the 67% confidence level. When this type of error analysis was performed, two equivalent global analysis solutions to the orientational distribution of DPH in membrane systems were discovered. This result had been predicted by Ameloot et al. (1984). The physical meaning behind these two solutions will be discussed. All global analyses and error analyses were

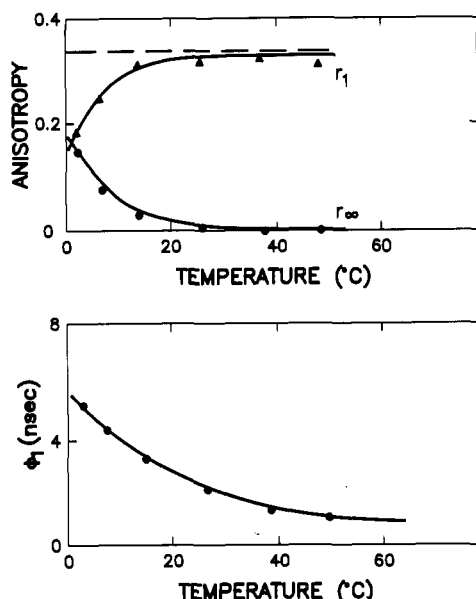


FIGURE 1: Recovered values of r_1 (Δ), r_∞ (\bullet), and ϕ_1 (\bullet) of the DLPC anisotropy decay analyses using the equation $r(t) = r_1 e^{-t/\phi_1} + r_\infty$. The dashed line represents the values of r_0 , which is the sum of r_1 and r_∞ .

performed with Globals Unlimited software package developed at the Laboratory for Fluorescence Dynamics, Department of Physics, University of Illinois.

RESULTS

Fluorescence decay data (at "magic-angle" conditions) were obtained along with the differential phase measurements at the same temperatures on the same sample preparations. The fluorescence lifetime data were resolved in terms of two exponential decays. One of the exponentials had a decay time ranging from approximately 6.5 to 11.0 ns in the temperature range used in the study. This component represented more than 90% of the fluorescence intensity. Another decay component had a short lifetime with a value of approximately 1–2 ns. The longer lifetime component had a value of approximately 10.5 ns below the transition temperature of the phospholipids, and it showed a small increase when the temperature was raised. There was a decrease in the lifetime value just above the transition to a value of approximately 9.0 ns. A further increase of the temperature resulted in a gradual linear decrease of the lifetime value with temperature. These results were similar to what was found with multilamellar vesicles composed of the same phospholipids (Parasassi et al., 1989; Fiorini et al., 1978).

Sum of Exponential Analysis. The empirical analysis of the DPH anisotropy data in terms of the sums-of-exponentials model are shown in Figures 1, 2, and 3. Although this analysis, using only three parameters (r_0 , r_∞ , and ϕ_1), oversimplifies the DPH rotation, the results do demonstrate several important features of the rotational motion of DPH throughout the phase transition. (1) The r_∞ term below the phase transition is large (≈ 0.3) but abruptly decreases to almost zero above the phase transition. (2) The preexponential factor of the faster rotational correlation time behaves in a very complementary fashion; it makes a very small contribution below the phase transition and increases abruptly at and above the phase transition. (3) Examination of the recovered rotational correlation time throughout the phase transition reveals one important point: there are no abrupt changes in the recovered rate, but rather a smooth gradual change throughout the entire phase transition. This type of result is inconsistent with a "two-state" model of the phase transition, where two rotational

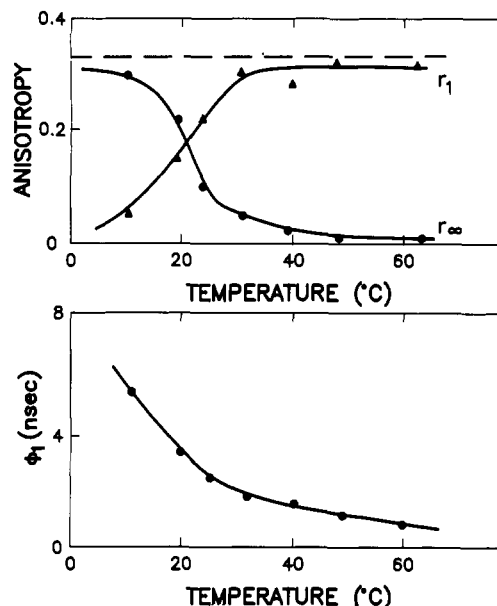


FIGURE 2: Recovered values of r_1 (Δ), r_∞ (\bullet), and ϕ_1 (\bullet) of the DMPC anisotropy decay analyses using the equation $r(t) = r_1 e^{-t/\phi_1} + r_\infty$. The dashed line represents the values of r_0 , which is the sum of r_1 and r_∞ .

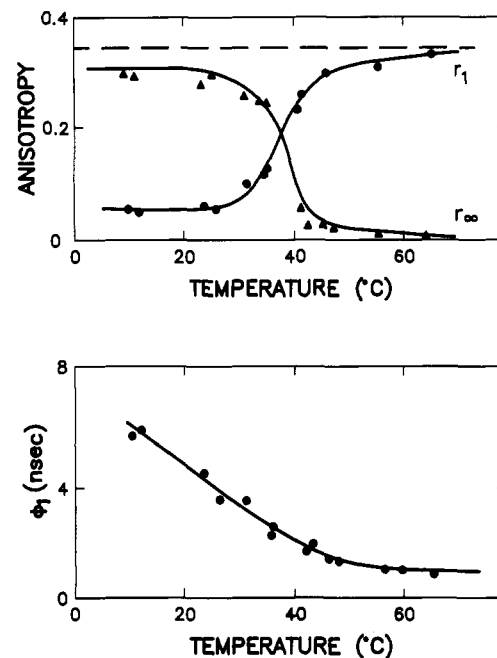


FIGURE 3: Recovered values of r_1 (Δ), r_∞ (\bullet), and ϕ_1 (\bullet) of the DPPC anisotropy decay analyses using the equation $r(t) = r_1 e^{-t/\phi_1} + r_\infty$. The dashed line represents the values of r_0 , which is the sum of r_1 and r_∞ .

environments for DPH exist (one gel and the other fluid) with different "microviscosity" characteristics.

Both associative and nonassociative modeling of the data were performed for these analysis. Each type of analysis resulted in nearly equivalent chi-square values. However, the nonassociative model analysis produced an r_0 term that was not invariant throughout the phase transition region, whereas the associative model resulted in an internally consistent r_0 term. At present, it is unclear if the two lifetime components correspond to two different molecular species with the same r_0 value.

P2/P4 Analysis. The analysis results, in terms of r_0 , r_∞ , ϕ_1 reported above, lead to the question of whether the rotational behavior of DPH throughout the phase transition can be described in terms of change in the orientational distribution

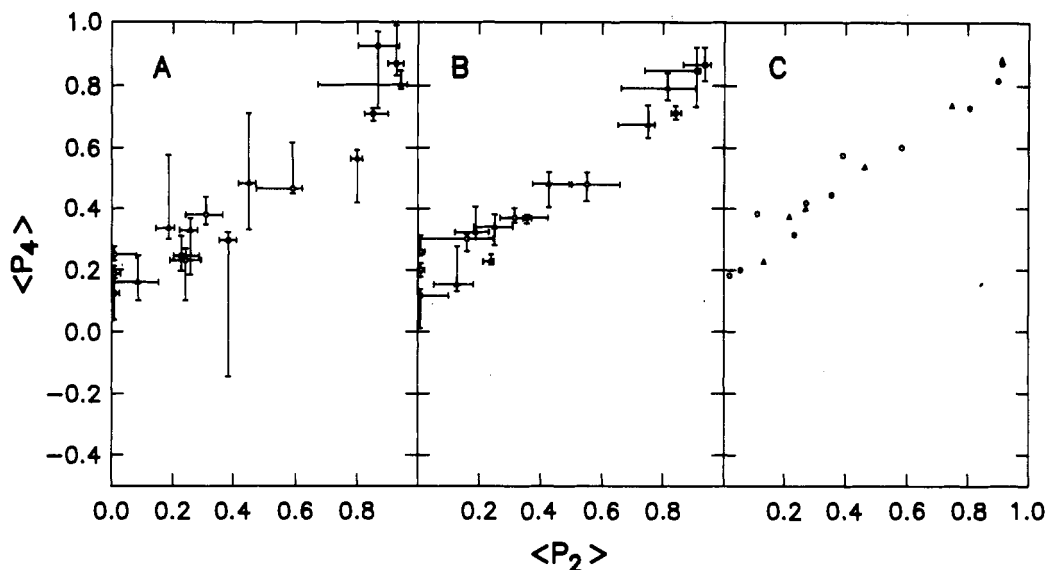


FIGURE 4: $\langle P_2 \rangle$ and $\langle P_4 \rangle$ values for (●) DPPC, (Δ) DMPC, and (○) DLPC. (A) All the fitting parameters, $\langle P_2 \rangle$, $\langle P_4 \rangle$, D , and r_0 , were unlinked in the analyses, i.e., each analysis was performed for the data at one temperature. (B) r_0 was linked in the analyses as one single floating parameter, and the data for one phospholipid at all temperatures were analyzed at one time. (C) r_0 was fixed at a value of 0.380 in the analyses. Because the fits in (C) were much poorer than those in (A) and (B), no error analysis was performed.

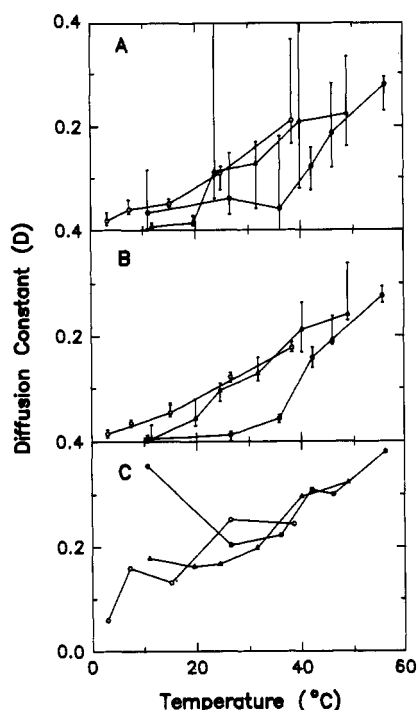


FIGURE 5: Diffusion constant as a function of temperature for (●) DPPC, (Δ) DMPC, and (○) DLPC. (A) All the fitting parameters, $\langle P_2 \rangle$, $\langle P_4 \rangle$, D , and r_0 , were unlinked in the analyses, i.e., each analysis was performed for the data at one temperature. (B) r_0 was linked in the analyses as one single floating parameter, and the data for one phospholipid at all temperatures were analyzed at one time. (C) r_0 was fixed at a value of 0.380 in the analyses. Because the fits in (C) were much poorer than those in (A) and (B), no error analysis was performed.

of DPH within the bilayer upon progressing through the phase transition. To answer this question, the data were reanalyzed by utilizing the theory developed by Van der Meer (1984) and Ameloot et al. (1984). A fit of the data at individual temperatures resulted in rather large uncertainties in these recovered values. However, when a global analysis of the data was performed (by linking the r_0 term over all temperatures), a much more accurate recovery of the orientational distribution parameters as well as the diffusion coefficient resulted (com-

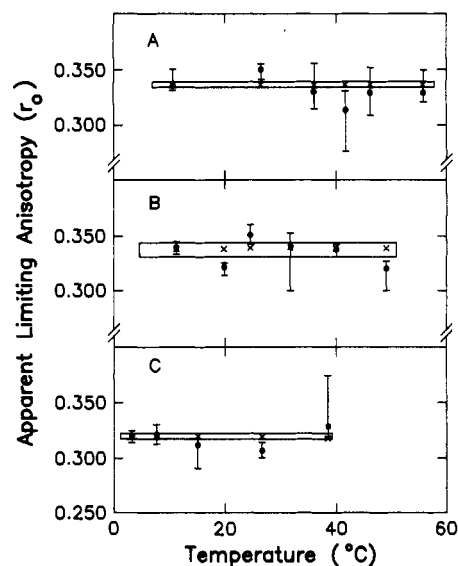


FIGURE 6: Apparent limiting intrinsic anisotropies for (A) DPPC, (B) DMPC, and (C) DLPC obtained by analyzing the data individually and globally. The symbol (●) represents the values determined by analyzing the data at individual temperature. The vertical bars represent the confidence level at the first standard deviation. The symbol (x) represents the values determined by analyzing the data at all the temperatures with r_0 linked as one fitting parameter. The upper and lower boundaries of the hatched areas were the confidence levels at one standard deviation.

pare Figures 4A,B and 5A,B). This analysis is referred to as a global analysis with r_0 linked. Results from the first method of analysis are shown in Figure 4A, 5A, and 6. Results obtained by the second method of analysis are shown in Table I and Figures 4B, 5B, and 6. Since all the parameters were allowed to vary in the analyses, the analyses with r_0 unlinked led to the best optimized values for any single set of data at one temperature. However, setting r_0 variable in an analysis required four fitting parameters for a single fit at each temperature. This resulted in poorer confidence levels on the individual parameters (Figures 4, 5, and 6). Results from global analysis (linking r_0 together for several temperatures) gave very similar values of the fitting parameters; however, much smaller uncertainties were achieved with the values from the global analyses. That is, similar chi-squares were observed

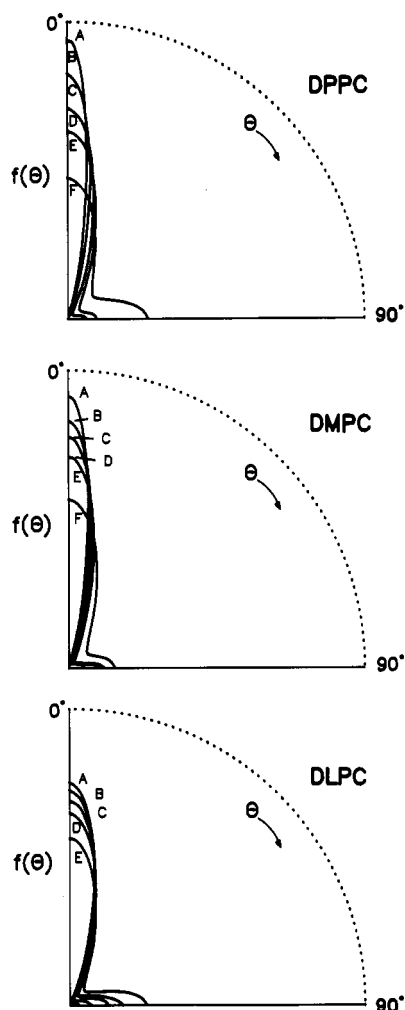


FIGURE 7: Reconstructed angular distributions based on the resolved $\langle P_2 \rangle$, $\langle P_4 \rangle$ pairs. One pair of $\langle P_2 \rangle$, $\langle P_4 \rangle$ values was used to generate a curve according to eq 13. Positive $\langle P_4 \rangle$ values were used for the reconstruction. All curves were normalized with eq 14. The measurements for DPPC vesicles were made at (A) 10.5 °C; (B) 26.5 °C; (C) 36.0 °C; (D) 41.9 °C; (E) 46.0 °C; and (F) 56.3 °C. The measurements for DMPC vesicles were made at (A) 11.0 °C; (B) 19.5 °C; (C) 24.5 °C; (D) 31.4 °C; (E) 39.8 °C; and (F) 49.1 °C. The measurements for DLPC vesicles were made at (A) 3.0 °C; (B) 7.6 °C; (C) 15.2 °C; (D) 26.5 °C; and (E) 38.3 °C.

with the global analyses compared with the unlinked analyses, yet the decreased uncertainties in the recovered values allow a much more rigorous examination of the possible models consistent with the data. The global error surfaces are well-defined but, as will be discussed, reveal the presence of two solutions.

Low values of r_0 (0.30–0.34) for DPH in lipid bilayers have been obtained when the data are analyzed with a free floating r_0 (Dale et al., 1977; Adler & Tritton, 1988). In some other cases, r_0 was assumed to have the same limiting anisotropy as in viscous isotropic solvents and fixed at 0.38–0.40 (Faucon & Lakowicz, 1987; Ameloot et al., 1984; Straume & Litman, 1987a,b). For the phase and modulation data reported here, a free floating r_0 used in the analyses resulted in an r_0 of approximately 0.320–0.340. Analyses with r_0 fixed at 0.380 also were performed. The resulting fits were found to be much poorer, with final global chi-square values of 4.54, 7.32, and 16.93 for DPPC, DMPC, or DLPC vesicles, respectively. Therefore, fixing r_0 at 0.380 in the analyses was not consistent with the data obtained in this study.

The Negative $\langle P_4 \rangle$ Region Solution. When the solution first found by the analysis program below the transition tem-

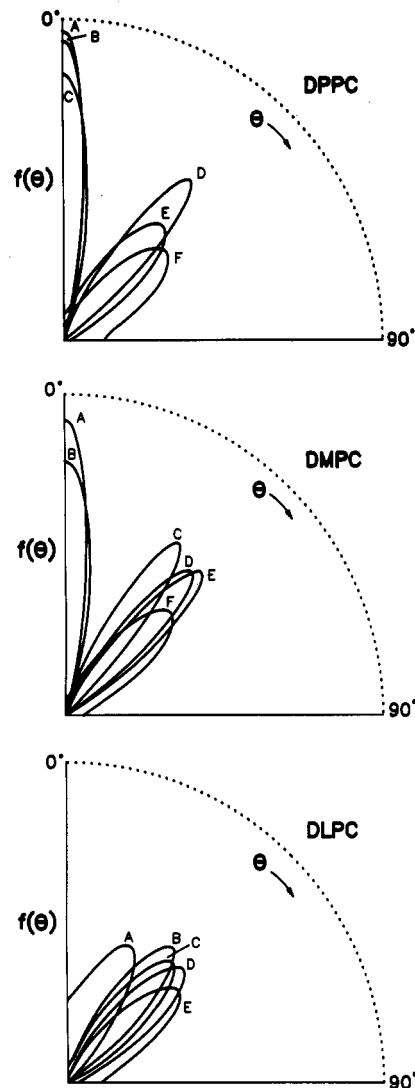


FIGURE 8: Same as for Figure 7, except the negative $\langle P_4 \rangle$ values were used for the reconstruction at and above the transition temperature. Below the transition temperature, there was only one solution for $\langle P_4 \rangle$.

perature is used, the maximum angle of the orientation distributions tend to increase moderately with increasing temperature as shown with DPPC and DMPC vesicles (Figure 7). This also was true for the rotational diffusion constants (Figure 5B). Above the transition temperatures, DPH molecules were oriented at all angles, from just above 0° to 90° (Figure 7).

When the exhaustive error analysis that systematically explores the χ^2 surface was performed, it was found that, for some of the data, the recovered value of $\langle P_4 \rangle$ could take on two distinct values with essentially the same exact chi-square value. This is true for all of the data obtained at and above the phase transition temperatures of the three lipids. There was, however, only one solution for $\langle P_4 \rangle$ below the transition temperatures. These results are consistent with the theoretical study of Ameloot et al. (1984), in which it was determined that a second solution to $\langle P_4 \rangle$ could be obtained at the negative region of the $\langle P_2 \rangle$ – $\langle P_4 \rangle$ plane. The rest of the fitting parameters are not significantly affected by the appearance of the double solutions (i.e., the diffusion coefficient and r_0 are essentially invariant). This second solution reveals that there are actually two equivalent distinct sets of orientational distributions of DPH in membranes at and above the phase transition region that are consistent with the data. These two alternative angular distributions are displayed in Figures 7 and

Table I: Recovered Orientational Distribution for DPH in DPPC, DMPC, and DLPC Vesicles by Global Analysis with r_0 Linked^a

	temp (°C)	$\langle P_2 \rangle$	$\langle P_4 \rangle$	D
DPPC	10.5	0.906 (0.74, 0.91)	0.838 (0.72, 0.92)	0.002 (0.001, 0.008)
	26.5	0.925 (0.905, 0.934)	0.859 (0.81, 0.89)	0.006 (0.004, 0.015)
	36.0	0.844 (0.83, 0.86)	0.709 (0.69, 0.73)	0.040 (0.035, 0.045)
	41.9	0.355 (0.33, 0.375)	0.363 (0.335, 0.369)	0.167 (0.145, 0.165)
	46.0	0.242 (0.21, 0.25)	0.232 (0.217, 0.250)	0.191 (0.185, 0.265)
	56.3	0.003 (0.00, 0.1)	0.123 (0.00, 0.14)	0.284 (0.265, 0.295)
DMPC	11.0	0.815 (0.65, 0.91)	0.784 (0.75, 0.84)	0.003 (0.00, 0.03)
	19.5	0.747 (0.65, 0.77)	0.673 (0.63, 0.74)	0.038 (0.03, 0.08)
	24.5	0.427 (0.37, 0.50)	0.486 (0.40, 0.52)	0.098 (0.07, 0.11)
	31.4	0.249 (0.18, 0.31)	0.339 (0.28, 0.38)	0.132 (0.11, 0.16)
	39.8	0.192 (0.12, 0.23)	0.340 (0.30, 0.41)	0.215 (0.17, 0.27)
	49.1	0.133 (0.05, 0.18)	0.161 (0.13, 0.29)	0.244 (0.23, 0.34)
DLPC	3.0	0.552 (0.53, 0.66)	0.482 (0.415, 0.52)	0.019 (0.018, 0.021)
	7.6	0.320 (0.26, 0.33)	0.373 (0.355, 0.40)	0.037 (0.026, 0.038)
	15.2	0.164 (0.00, 0.25)	0.300 (0.255, 0.32)	0.064 (0.055, 0.072)
	26.5	0.006 (0.00, 0.09)	0.279 (0.27, 0.315)	0.134 (0.11, 0.135)
	38.3	0.003 (0.00, 0.06)	0.186 (0.16, 0.213)	0.185 (0.172, 0.190)

^aThe two values in each set of parentheses indicate the upper and lower boundaries of the confidence level at the first standard deviation. For DPPC, $\chi^2 = 2.32$ and $r_0 = 0.333$ (0.332, 0.340); for DMPC $\chi^2 = 3.28$ and $r_0 = 0.341$ (0.329, 0.346); and for DLPC, $\chi^2 = 2.61$ and $r_0 = 0.318$ (0.315, 0.323).

8. The positive $\langle P_4 \rangle$ solutions represent the orientational distribution of DPH clustered at 0° or 90° from the normal of the membrane, whereas the negative $\langle P_4 \rangle$ solutions predict a unimodal distribution of probes with a maximum between 0° and 90°. It is worth noting that the latter group is located in the area of the $\langle P_2 \rangle, \langle P_4 \rangle$ plane corresponding to the so-called "wobbling-in-a-cone" model.

DISCUSSION

The orientational distribution of DPH in membranes showed distinct differences between the gel phase and fluid phase. A narrow distribution around the membrane normal was found in the gel phase. In the fluid phase, however, there was a continuous distribution over a much wider range (Figures 7 and 8). This was not observed in the study by Ameloot et al. (1984) where two separate populations of molecules were found at low and high angles with no molecules at the intermediate range. Comparison of the fluid phase $\langle P_2 \rangle$ and $\langle P_4 \rangle$ values in the two studies showed that their $\langle P_2 \rangle$ and especially their $\langle P_4 \rangle$ values were higher than those found in this study. Since each distribution is defined by a pair of specific combinations of $\langle P_2 \rangle$ and $\langle P_4 \rangle$, this difference might be responsible for the differences in the distributions. The difference in r_0 values between the two studies was not responsible for the differences in $\langle P_2 \rangle$ and $\langle P_4 \rangle$ values. When r_0 was fixed at 0.380 as in the Ameloot et al. (1984) study, the fits to the data were poor and the $\langle P_2 \rangle$ and $\langle P_4 \rangle$ did not increase significantly (Figure 4C). A lower r_0 value as determined here could be due to another rotational component that was too fast to be detected by either the pulse excitation or the differential phase method. It is also possible that the differences between the two studies were due to differences in sample preparation or differences in the excitation band-pass. In our experiment, we use 325-nm excitation, at which wavelength r_0 is lower with respect to excitation at 350 nm.

Large increases in the values of D were found when r_0 was increased to 0.380 (Figure 5C), which greatly reduced the temperature dependence of D . This would be expected because D reflects the decay rate of $r(t)$ at very early times. Arbitrarily increasing r_0 would result in a higher D (faster decay near $t = 0$) with the intermediate and long time decay patterns remaining largely unchanged (mainly dependent on $\langle P_2 \rangle$ and $\langle P_4 \rangle$).

The confidence levels in the solutions are significantly im-

proved by using the orientational distribution model and by linking all the data together and carrying out a global analysis. The results show that the distribution of DPH in a bilayer is continuous with preferential orientations near the normal in the gel phase and much broader in the fluid phase.

The theory of Van der Meer et al. (1984) is based on the assumption that there is an equilibrium orientational distribution for the DPH molecule in the membrane. The distribution is determined by a potential that tends to preferentially orient the DPH molecules parallel to the membrane normal. The equilibrium is reached by the action of the orienting potential and the Brownian rotational diffusion of the DPH molecules. Fluorescence measurements on nonoriented samples can only determine two terms of the Legendre polynomial expansion due to the symmetry of the dipole transition. Since, in principle, any orientational distribution is represented by an infinite number of terms, the knowledge of only two terms of the expansion will only determine the family of distributions that can be represented by these first two terms. Of course, the recovered distribution must have two orthogonal planes of symmetry. Furthermore, given a pair of values of $\langle P_2 \rangle$ and $\langle P_4 \rangle$, there are an infinite number of possible distributions that have the same values of $\langle P_2 \rangle$ and $\langle P_4 \rangle$. For example, consider a narrow distribution at a given angle with the normal (a delta function) and suppose we describe this distribution using only $\langle P_2 \rangle$ and $\langle P_4 \rangle$. With only this pair of values, a different distribution from the delta function is recovered when the distribution of the form of eq 13 is employed with $\langle P_2 \rangle$ and $\langle P_4 \rangle$ chosen such that this pair of $\langle P_2 \rangle, \langle P_4 \rangle$ values is reproduced. This effect is due to the fact that, in general, a given distribution cannot be described uniquely by using only three terms $\langle P_0 \rangle$, $\langle P_2 \rangle$, and $\langle P_4 \rangle$. The nonuniqueness of the recovered distribution must not be confused with the double solution for the $\langle P_4 \rangle$ coefficient as discussed in the previous sections. The so-called "negative region solution" is a mathematical result that depends on the fact that equal phase and modulation curves can be represented by two different distributions (two different sets of $\langle P_2 \rangle$ and $\langle P_4 \rangle$ values).

In conclusion, the recovery of the parameters $\langle P_2 \rangle$ and $\langle P_4 \rangle$ is greatly improved by using the global analysis method. However, the recovery of the real physical distribution suffers from some fundamental limitations related to the mathematical nonuniqueness of the solution and from the fact that a family of different distributions can be represented by the same values

of $\langle P_2 \rangle$ and $\langle P_4 \rangle$. The negative $\langle P_4 \rangle$ selection provide a unimodal distribution with no concentration of molecules oriented parallel to the membrane surface.

The "negative $\langle P_4 \rangle$ " solution describes an angular distribution that makes it easier to interpret the membrane phase transition than the "positive $\langle P_4 \rangle$ " solution. For the "negative $\langle P_4 \rangle$ " orientational distribution, there is a sudden change in the average orientation of the DPH molecule at the transition temperature. There are two well-defined and different orientational distributions for the gel and liquid-crystalline state. During the transition period, there is an interconversion from one distribution to another which corresponds to a situation in which only two states are present (see Figure 8).

It has been shown by the group of Y. Levine (van Langen et al., 1988) that it is possible to remove the mathematically equivalent double solution by using oriented samples and performing angular resolved anisotropy studies. However, the same group has pointed out that the hydrated samples used for those studies can have a different order and different dynamics compared to a vesicle suspension. Therefore, it is unclear that the ambiguity of the two distributions from suspension studies can be removed by using the results from oriented samples.

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