DETECTION OF HINDERED ROTATIONS OF 1,6-DIPHENYL-1,3,5-HEXATRIENE IN LIPID BILAYERS BY DIFFERENTIAL POLARIZED PHASE FLUOROMETRY

J. R. LAKOWICZ, *Freshwater Biological Institute and Department of Biochemistry, University of Minnesota, Navarre, Minnesota 55392, and*
F. G. PRENDERGAST, *Department of Pharmacology, Mayo Medical School, Rochester, Minnesota 55901 U.S.A.*

ABSTRACT Differential polarized phase fluorometry has been used to investigate the depolarizing motions of 1,6-diphenyl-1,3,5-hexatriene (DPH) in the isotropic solvent propylene glycol and in lipid bilayers of dimyristoyl-L-α-phosphatidylcholine (DMPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC), and other phosphatidylcholines. Differential phase fluorometry is the measurement of differences in the phase angles between the parallel and perpendicular components of the fluorescence emission of a sample excited with sinusoidally modulated light. The maximum value of the tangent of the phase angle (tan Δ_{max}) is known to be a function of the isotropy of the depolarizing motions. For DPH in propylene glycol the maximum tangent is observed at 18°C, and this tangent value corresponds precisely with the value expected for an isotropic rotator. Additionally, the rotational rates determined by steady-state polarization measurements are in precise agreement with the differential phase measurements. These results indicate that differential phase fluorometry provides a reliable measure of the probe's rotational rate under conditions where these rotations are isotropic and unhindered.

Rotational rates of DPH obtained from steady-state polarizaton and differential phase measurements do not agree when this probe is placed in lipid bilayers. The temperature profile of the tan Δ measurements of DPH in DMPC and DPPC bilayers is characterized by a rapid increase of tan Δ at the transition temperature (T_c), followed by a gradual decline in tan Δ at temperatures above T_c. The observed $\tan\Delta_{max}$ values are only 62 and 43% of the theoretical maximum. This defect in $\tan\Delta_{max}$ is too large to be explained by any degree of rotational anisotropy. However, these defects are explicable by a new theory that describes the tan Δ values under conditions where the probe's rotational motions are restricted to a limiting anisotropy value, r_∞. Theoretical calculations using this new theory indicate that the temperature dependence of the depolarizing motions of DPH in these saturated bilayers could be explained by a rapid increase in its rotational rate (R) at the transition temperature, coupled with a simultaneous decrease in r_∞ at this same temperature. The sensitivity of the tan Δ values to both R and r_∞ indicates that differential phase fluorometry will provide a method to describe more completely the depolarizing motion of probes in lipid bilayers.

INTRODUCTION

Measurements of the steady-state fluorescence polarization of probes embedded in lipid bilayers have been widely used to estimate the microviscosity of the hydrophobic regions of both natural and model cell membranes (Bashford et al. 1976; Cogan et al. 1973; Jacobson and Wobschall, 1974; Moore et al. 1976; Shinitzky et al. 1971; Shinitzky and Inbar, 1974). All these estimates of microviscosity assume that the depolarizing rotational motion of the fluorophores in lipid bilayers are equivalent to those in isotropic solvents. This assumption is likely to be in error since lipid bilayers are inherently anisotropic and are therefore likely to provide an environment that hinders diffusion motions selectively.

Diphenylhexatriene (1,6-diphenyl-1,3,5-hexatriene, DPH) has become widely used in microviscosity studies as a result of its favorable fluorescence polarization and spectral properties (Shinitzky and Barenholz, 1974), but perhaps more importantly as a result of the dramatic changes in fluorescence polarization that occurs at the solid-to-liquid phase transition of lipid bilayers (Lentz et al. 1976*a*,*b*). These highly temperature-dependent polarization changes appear to be unique to DPH. Other fluorescent probes, such as 1-anilino-8-naphthalene sulfonic acid, perylene, 9-vinylanthracene, 2-methylanthracene, 12-anthroyl stearate, and *N*-phenyl-1-naphthylamine, undergo less dramatic changes in the fluorescence polarization at the transition temperature when embedded in lipid bilayers.

The high fluorescence anisotropy value observed for DPH in the absence of depolarizing rotations indicates that the axes of the absorption and emission transition moments are parallel (Shinitzky and Barenholz, 1974) and probably lie along the long axis of the fluorophore. As a result, only rotations which displace this axis will be depolarizing, and it appears that depolarization is governed by a single rotational rate. For these reasons, steady-state polarization measurements using DPH are considered to reflect accurately the microviscosity of lipid bilayers.

Recently, Chen et al. (1977) have used time-resolved decays of fluorescence anisotropy to demonstrate that DPH undergoes only hindered torsional motions below the phase transition temperature of DMPC vesicles, and a highly nonexponential decay of anisotropy above this temperature. These results indicate an environment in the lipid bilayer which does not permit free probe rotation, even though these limited rotations may be isotropic. Hence, the determinations of membrane microviscosities are likely to be in error since such determinations compare polarization values of a probe undergoing unhindered isotropic rotations with the polarization values for a probe undergoing hindered rotations.

We clearly require a more detailed understanding of the types of probe motion responsible for fluorescence depolarization in order to interpret these data in terms of membrane microviscosity. Additionally, the molecular details of depolarizing rotations in bilayers should reflect to some extent the molecular organization and the segmental motions of the fatty acyl chains.

THEORY

The theory of differential phase fluorometry for isotropic and anisotropic depolarizing rotations has been recently described by Weber (1977). DPH is thought to be an isotropic rotator. Under these conditions the tangent of the differential phase angle (tan Δ) is given by

$$\tan \Delta = \frac{(2R\tau)\omega\tau r_0}{(1/9)(1 + 2r_0)(1 - r_0)(1 + \omega^2\tau^2) + (2R\tau)/3)\ (2 + r_0) + (2R\tau)^2}. \quad (1)$$

The rotational rate of the fluorophore may be obtained from the quadratic form of the equation.

$$(2R\tau)^2 + (2R\tau)[(2 + r_0)/3 - |r_0/\tan \Delta|\ \omega\tau] + m(1 + \omega^2\tau^2) = 0$$

$$m = (1/9)(1 + 2r_0)(1 - r_0). \quad (2)$$

R is the rotational rate of the probe in radians per second, r_0 the anisotropy value in the absence of rotational diffusion, ω the circular modulation frequency, and τ the fluorescence lifetime. For isotropic rotations the maximum value for tan Δ is a function of r_0, ω, and τ only, such that

$$\tan \Delta_{max} = \frac{3\omega\tau r_0}{(2 + r_0) + 2[(1 + 2r_0)(1 - r_0)(1 + \omega^2\tau^2)]^{1/2}}. \quad (3)$$

The above equations apply to spherical molecules, or to molecules whose depolarizing rotations are isotropic. Under these conditions the tan Δ_{max} values will agree with that predicted by Eq. 3. Anisotropic rotations, or a population of isotropic rotators with more than a single rotational rate, results in tan Δ_{max} values less than those predicted by Eq. 3. The studies of Mantulin and Weber (1977) demonstrate the usefulness of differential phase fluorometry in the detection of anisotropic rotations.

Theoretical calculations have shown that one should not expect defects in the differential tangent resulting from anisotropic rotations to exceed 25%. However, our studies of DPH in vesicles of dimyristoyl-L-α-phosphatidylcholine (DMPC) or dipalmitoyl-L-α-phosphatidylcholine (DPPC) showed tangent defects of about 40 and 60%, respectively. We deduced that such defects could result from hindered torsional motions of DPH. Weber (1977)[1] has recently obtained solutions for the tan Δ under such conditions. If the rotations are limited to a nonzero anisotropy value (r_∞) at times long compared to the fluorescence lifetime, then the parallel ($I_\parallel(t)$) and perpendicular ($I_\perp(t)$) components of the fluorescence emission are given by[1]

$$I_\parallel(t) = (1 + 2r_\infty)e^{-t/\tau} + 2(r_0 - r_\infty)e^{-(1/\tau + 6R)t}, \quad (4)$$

$$I_\perp(t) = 1 - 2r_\infty)e^{-t/\tau} - (r_0 - r_\infty)e^{-(1/\tau + 6R)t}. \quad (5)$$

[1] Weber, G. 1977. Personal communication.

From these equations Weber has obtained

$$\tan \Delta = \frac{\omega\tau(r_0 - r_\infty)(2R\tau)}{\left[\begin{array}{c}(1/9)(1 + 2r_0)(1 - r_0)(1 + \omega^2\tau^2) \\ + (2R\tau/3)[2 + r_0 - r_\infty(4r_0 - 1)] + (2R\tau)^2(1 + 2r_\infty)(1 - r_\infty)\end{array}\right]}, \quad (6)$$

and

$$\tan \Delta_{max} = \frac{\omega\tau(r_0 - r_\infty)}{\left[\begin{array}{c}(1/3)[2 + r_0 - r_\infty(4r_0 - 1)] \\ + (2/3)[(1 + 2r_0)(1 - r_0)(1 + 2r_\infty)(1 - r_\infty)(1 + \omega^2\tau^2)]^{1/2}\end{array}\right]}, \quad (7)$$

For $r_\infty = 0$ these equations reduce to those applicable to isotropic rotations.

Rotational rates of probes may also be determined by steady state polarization measurements. In this case the Perrin-Weber equation was utilized

$$r_0/r = 1 + 6R\tau, \quad (8)$$

where r is the fluorescence anisotropy in the presence of rotational diffusion. Fluorescence polarization (P) and anisotropy values are interchangeable with

$$P = 3r/(2 + r). \quad (9)$$

METHODS

Instrumentation

Fluorescence lifetimes and differential tangents were measured by the phase shift method (Spencer and Weber, 1969, 1970), using a light modulation frequency of either 10 or 30 MHz. A schematic drawing of the *T*-format differential phase fluorometer is shown in Fig. 1. Fluorescence lifetimes were determined from the phase angle of the fluorescence emission compared with the phase angle of the exciting light when scattered at right angles by a glycogen suspension. For these lifetime measurements only a single photomultiplier tube was used. The effects of depolarizing Brownian rotations on the observed lifetimes was eliminated by setting the excitation and emission polarizers at 0° and 55° from the vertical, respectively (Spencer and Weber, 1970). All measurements were performed on equipment obtained from SLM Instruments, Inc., Urbana, Ill. The instrumental conditions were: excitation wavelength, 360 nm; excitation filter, Corning 7-54; emission filter, Corning 3–73 (Corning Glass Works, Corning, N.Y.) and 2 mm of 1 M $NaNO_2$. Steady-state anisotropy values were obtained directly in the subnanosecond spectrofluorometer with all the radio frequency electronics and light modulation turned off. At −57°C in propylene glycol we found $r_0 = 0.392$ for DPH. This r_0 value was used in all our calculations.

Differential tangent values were obtained by measuring the phase difference when one emission polarizer was rotated 90° from the parallel to the perpendicular orientation. This phase difference was always measured relative to the second photomultiplier, which constantly observed the phase of the perpendicular emission. These measurements were continued until a satisfactory average was obtained.

In measurements of the differential tangent, systematic errors which could result from the detection system being sensitive to the polarization of the fluorescent signal must be avoided. We

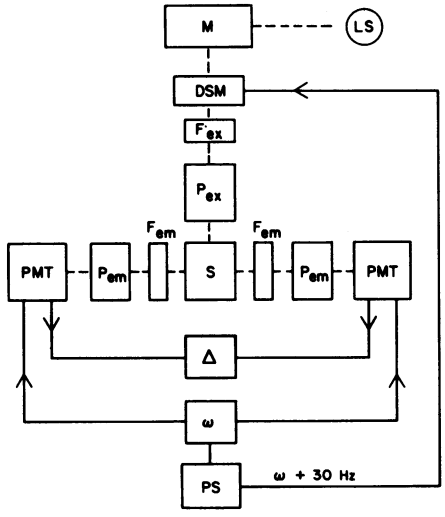

FIGURE 1 Schematic of a differential phase fluorometer. *LS*, light source; *M*, monochromator; *DSM*, Debye-Sears ultrasonic light modulator; F_{ex}, excitation filter; P_{ex}, excitation polarizer; *S*, sample; F_{em}, emission filter; P_{em}, emission polarizer; *PMT*, phototubes; *PS*, phase shifter for adding 10 or 30 Hz to the high-frequency input of the light modulator; Δ, low-frequency electronics to measure the phase of the cross-correlated fluorescence emission.

determined that such systematic errors were not significant in our measurements. Upon excitation of our samples with horizontally polarized light, the phase difference between the vertically and horizontally polarized components of the emission within experimental limits was zero. These control measurements were performed with each sample at a temperature which yielded the maximum differential tangent.

Materials

DMPC, DPPC, and dioleoyl-L-α-phosphatidylcholine (DOPC), synthetic lipids obtained from Sigma Chemical Company (St. Louis, Mo.), were used without further purification. Chromatography of these phospholipids on silica in chloroform/methanol/water (65:25:4) and ethyl ether/benzene/ethanol/acetic acid/H_2O (40:40:20:8:4) showed a single spot by both phosphate and dichromate char staining.

The phospholipid vesicles were prepared by addition of benzene solutions of the probe and lipid to a stainless steel beaker. The probe-to-lipid molar ratio is 1:500. The benzene was evaporated by gently warming the solution while maintaining a constant flow of argon over the materials. Buffer (0.01 M Tris, 0.05 M KCl, pH = 7.5) was added to the dried lipid to establish a concentration of 0.17 mg lipid/ml buffer. Sonication was effected with a Heat Systems model 350 sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.) at 200 W using a 0.5-inch diameter tip. The temperature of the solution was maintained near 40°C during the 15-min sonication period. This preparation was annealed for 1 hr at 40°C and then centrifuged at 48,000 *g* for 90 min. Unsonicated bilayers were prepared in an identical fashion except that the sonication and centrifugation steps were eliminated, and the sample was agitated on a vortex mixer for 10 min after addition of buffer. Phosphate assays (Kates, 1972) performed on these preparations indicate a phospholipid concentration of at least 90% of the expected concentration.

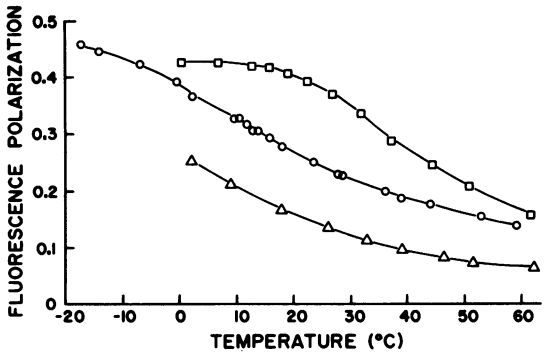

FIGURE 2 Steady-state polarization values of DPH-labeled lipid bilayers and solutions. These measurements were made on the same samples used in the tan Δ measurements, directly in the subnanosecond spectrofluorometer, as is described in the Methods section. Data are shown for DPH in propylene glycol (○), DOPC (Δ), and DMPC/cholesterol, 3/1, (□).

No significant fluorescent impurities were observed in unlabeled vesicles prepared in an identical manner. Additionally, no scattered light at the excitation wavelength, or at the wavelength for Raman scatter, was observed through the filters used for the lifetime and differential phase measurements.

The optical density of DPH in propylene glycol was 0.4 at 360 nm. A blank solution of propylene glycol showed no significant fluorescence at equivalent instrumental conditions.

Figs. 2 and 3 show the temperature profiles of the steady-state polarization values obtained from our vesicle preparations. These results are comparable to those obtained by others.

RESULTS

Fluorescence lifetimes and differential tangent values for DPH in propylene glycol are shown in Fig. 4. The agreement of the maximum observed tan Δ value with that predicted from Eq. 3 (see Table I) indicates that the depolarizing rotations of DPH in propylene glycol are isotropic. Additionally, these rotations must be unhindered since

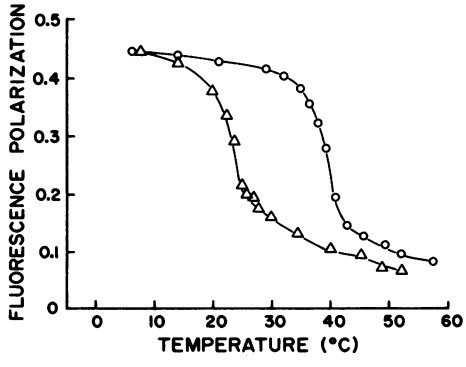

FIGURE 3 Steady-state polarization measurements of DPH-labeled lipid bilayers. Data are shown for DPH in DMPC (△) and DPPC (○) bilayers.

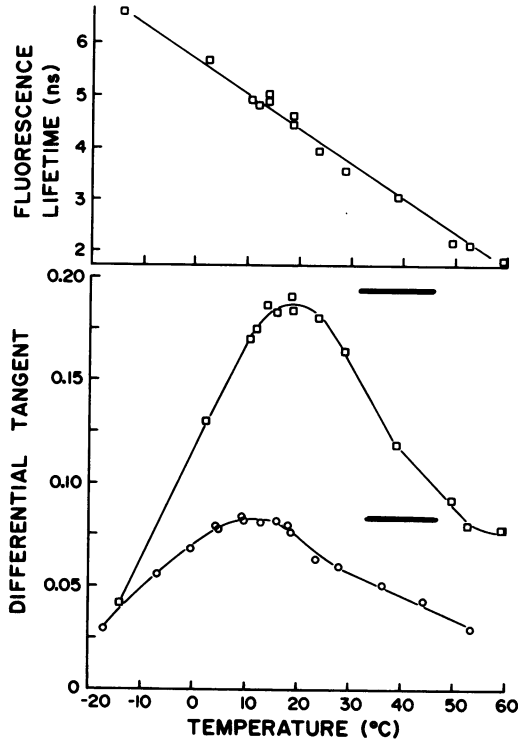

FIGURE 4 Fluorescence lifetimes and differential tangent values of DPH in propylene glycol. Data are shown for 10 (○) and 30 (□) MHz. The optical density of DPH at 360 nm was 0.40. The solid bars indicate tan Δ_{max} for an isotropic rotator with r_0 = 0.392 and a fluorescence lifetime equal to the observed lifetime at the temperature of the maximum observed differential tangent (10 MHz, 5 ns; 30 MHz, 4.3 ns).

a nonzero value of r_∞ also results in a decrease in tan Δ_{max} (Fig. 5). In Fig. 6 we compare the rotation rates obtained from steady-state polarization measurements (Eq. 8) with those obtained from the differential phase measurements (Eq. 2). These data show that there is precise agreement between steady-state polarization and differential phase fluorometric estimates of rotational rates, and validate the assertion that in solvent such as propylene glycol DPH does indeed behave as a free isotropic rotator. Additionally, it is clear that in such situations the extrapolation to a value for the microviscosity of the medium may be quite valid.

The differential phase measurements for DPH in lipid bilayers prepared from saturated phosphatidylcholine bilayers are distinctly different from the results obtained for DPH in isotropic solvents. Figs. 7–9 show the temperature dependence of tan Δ for single-lamellar DMPC vesicles, multilamellar DMPC bilayer, and single-lamellar DPPC vesicles, respectively. The dominant characteristics of all these data are (*a*) a rapid increase in tan Δ at the transition temperature, followed by a decrease in tan Δ above this temperature, and (*b*) failure of tan Δ to reach its theoretical maximum. In agreement with steady-state polarization measurements by ourselves and others (Lentz

TABLE I

DIFFERENTIAL PHASE MEASUREMENTS OF DPH IN PROPYLENE GLYCOL AND LIPID BILAYERS

Sample	Frequency	τ*	$\tan \Delta_{max}$		Percent of theory
			Observed	Calculated‡	
	MHz	*ns*			%
Propylene	10	5.0	0.082	0.081	101
glycol	30	4.3	0.185	0.195	95
DMPC	10	8.8	0.084	0.136	68
	30	8.7	0.188	0.302	62
DMPC§	10	8.5	0.073	0.132	62
	30	8.5	0.170	0.298	52
DPPC	10	8.4	0.057	0.131	43
DOPC	10	7.7	0.105	0.127	83
	30	6.9	0.200	0.274	73
DMPC/cholesterol	10	8.8	0.025	0.135	19
(3:1)	30	7.9	0.068	0.288	24

*Fluorescence lifetime observed at the temperature of maximum differential tangent.

‡For unhindered isotropic rotations (Eq. 3).

§Unsonicated, multilamellar bilayers. All other lipids are sonicated to form single-lamellar vesicles.

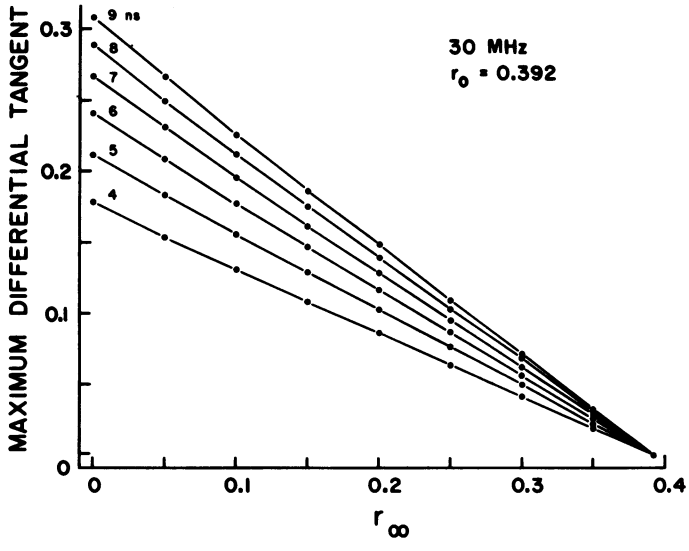

FIGURE 5 Maximum theoretical differential tangent for an isotropic rotator in a hindred environment. The plot is meant to simulate DPH at 30 MHz. Data for the plot were derived from Eq. 7 with r_0 set at 0.392 and the fluorescence lifetimes indicated on the figure.

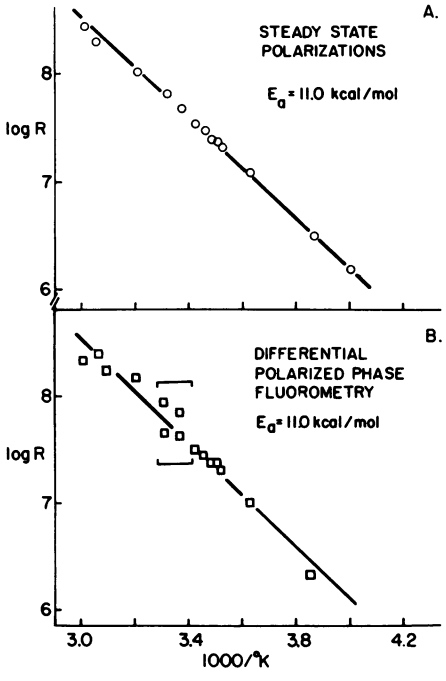

FIGURE 6

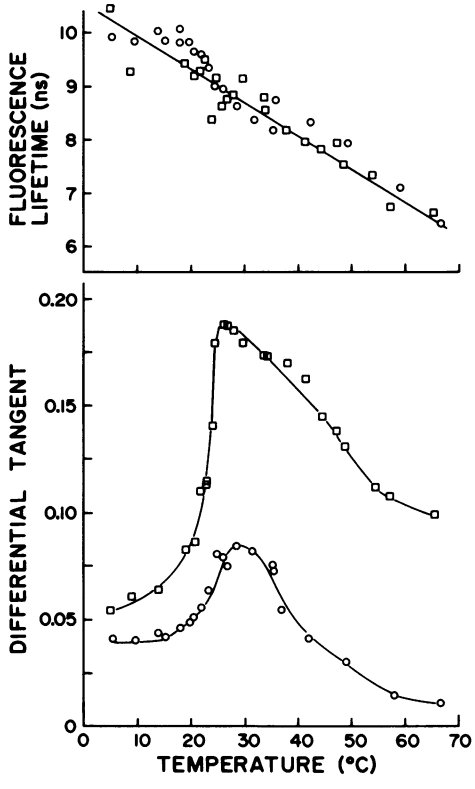

FIGURE 7

FIGURE 6 Rotational rates of DPH in propylene glycol. Rotational rates were calculated by both steady state polarization measurements (A, o) and by differential polarized phase fluorometry (B, □). The duplicate points between the bars on part B are a result of the two possible solutions to Eq. 2. Near tan Δ_{max} the choice of the proper solution is unclear. However, the correct choice is often unimportant since the rotational rates are similar.

FIGURE 7 Fluorescence lifetimes and differential tangents for DPH in small, single-lamellar bilayers of DMPC. 10 (o) and 30 (□) MHz data are shown.

et al., 1976*a*, *b*), the transition, as observed by differential phase measurements, is sharper in the large, multilamellar bilayers than in the small single-lamellar vesicles.

A summary of the observed and theoretical tan Δ_{max} values is provided in Table I. The observed values of tan Δ max in DMPC and DPPC vesicles are only about 62 and 42%, respectively, of that expected for a free isotropic rotator. In contrast to the results obtained for DPH in propylene glycol, we could not obtain agreement between the rotational rates observed by steady-state polarization measurements with those observed by differential phase measurements when we assumed DPH was an unhindered isotropic rotator. Indeed, the observed tangent defects in the lipid bilayers are too large to be explained by any degree of rotational anisotropy (Weber, 1977). However, as can be seen from Fig. 5, the maximum differential tangent decreases to

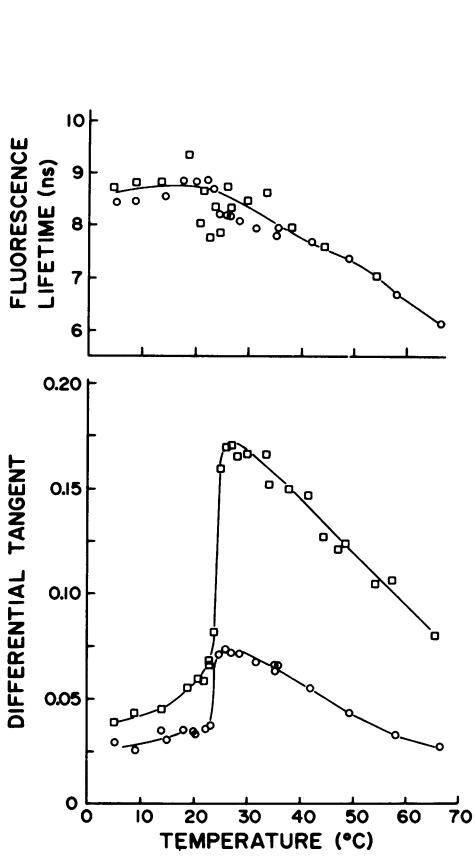

FIGURE 8

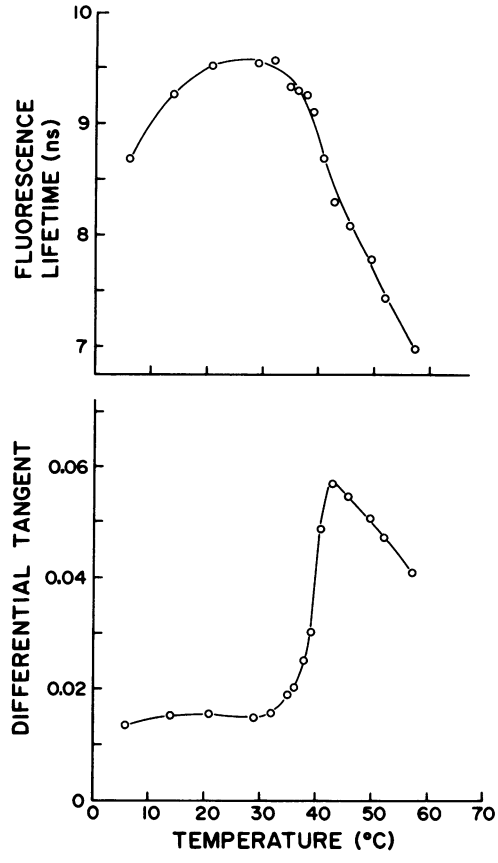

FIGURE 9

FIGURE 8 Fluorescence lifetimes and differential tangets for DPM in large, multilamellar bilayers of DMPC. 10 (○) and 30 (□) MHz data are shown.
FIGURE 9 Fluorescence lifetimes and differential tangets for DPH in small, single-lamellar bilayers of DPPC. The data shown were collected at 10 MHz.

zero in an approximately linear fashion as r_∞ increases from zero to r_0. Hence for $r_\infty - 0.5\ r_0$ one obtains an approximate 50% tangent defect. Thus, an anisotropic environment that limits the probe's rotations to a nonzero anisotropy could easily result in tangent defects of the magnitude we observed in DMPC and DPPC bilayers.

Figs. 10 and 11 show the tan Δ/temperature profiles for DPH in vesicles of DOPC and DMPC/cholesterol (3:1), respectively. The depolarizing rotations of DPH in DOPC vesicles appear less hindered and more rapid than in vesicles of the saturated phosphatidylcholines. These results are inferred from the small tangent defect observed in these bilayers (Table I), and the occurrence of the maximum tan Δ at low temperatures. The rotational motion of DPH in cholesterol-containing bilayers appears to be greatly hindered and less rapid than in bilayers without cholesterol. The

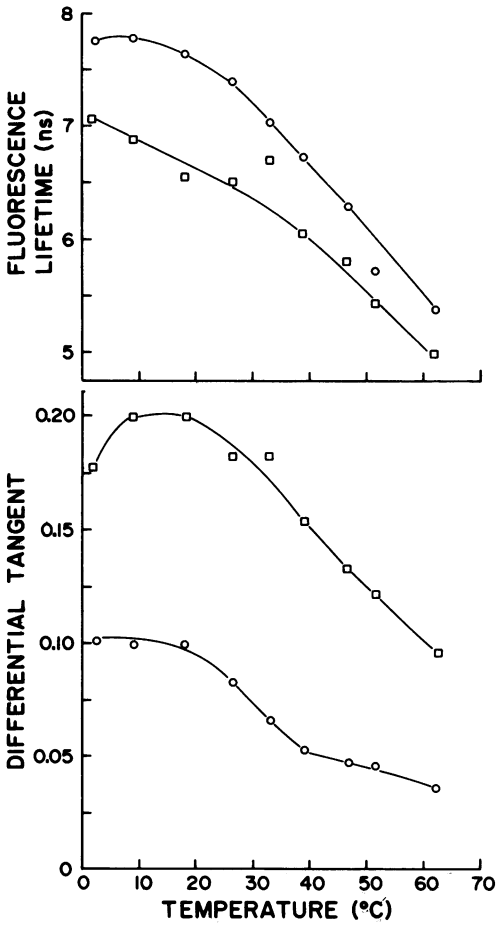

FIGURE 10

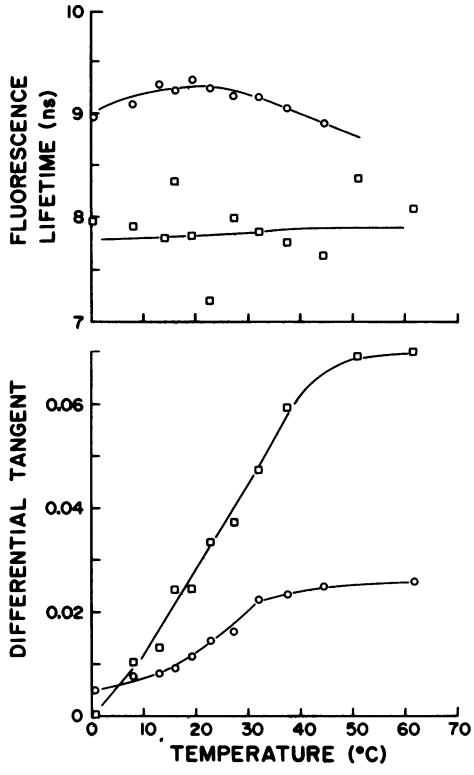

FIGURE 11

FIGURE 10 Fluorescence lifetimes and differential tangents for DPH in DOPC vesicles. 10 (o) and 30 (□) MHz data are shown.

FIGURE 11 Fluorescence lifetimes and differential tangents for DPH in DMPC/cholesterol vesicles. The molar ratio of DMPC to cholesterol is 3/1. 10 (o) and 30 (□) MHz data are shown.

maximum differential tangent is only about 20% of the theoretical maximum, and this value occurs only at high temperatures.

DISCUSSION

What characteristics of the depolarizing motions of DPH could result in the temperature profiles for tan Δ observed in lipid bilayers? The time-resolved polarization studies of Chen et al. (1977) indicate that DPH has a nonzero limiting anisotropy below the transition temperature of DMPC, and that this r_∞ value decreases above the transition temperature. Additionally, steady-state polarization measurements have

indicated that the rotational rate of DPH increases rapidly through the phase transition, and that the temperature dependence of this rotational rate is similar at temperatures far above and below this temperature. However, in light of the data presented in this paper which indicates a hindered environment for DPH in bilayers, this apparent increase in the rotational rate may be primarily a result of a decrease in r_∞ coupled with a smoothly changing rotational rate. The observed temperature profiles could be the result of a simultaneous increase in R and decrease in r_∞.

In an attempt to understand these data more fully, we devised a mathematical model. We assumed a transition temperature of 22°C; we further assumed that this transition was Gaussian with a standard deviation (σ) of 5°C. At a temperature T the fraction of the transition which is complete is given by the area ($A(T)$) under the normal curve from low temperatures to T. Thus,

$$A(T) = (1/\sigma\sqrt{2\pi}) \int_{-\infty}^{T} \exp\{-1/2[(T - Tc)/\sigma]^2\}\, dT. \tag{10}$$

Two further assumptions were made. First, r_∞ was assumed to decrease from 0.3 for temperatures far below T_c, to 0.1 for temperatures far above T_c, according to

$$r_\infty = 0.3 - 0.2\,A(T). \tag{11}$$

The activation energy for DPH rotations was assumed to be 10 kcal/mol at temperatures far from the transition temperature, and R was assumed to increase by one additional order of magnitude as a result of the phase transition. Hence,

$$\log R = -Ea/2.303\,R_g T + C + A(T). \tag{12}$$

The pre-exponential factor C (equal to 14.407) was chosen to obtain $\log R = 7.5$ at $T = T_c$. R_g is the gas constant and T the absolute temperature.

The temperature profile of the tan Δ values expected under these conditions was calculated with Eq. 6, and is shown in Fig. 12. No attempt was made to adjust the functional forms of R and r_∞ so as to minimize deviations from our experimental results. We feel the most important point of this calculation is that our simple model for the behavior of DPH in a restricted environment reproduces the general characteristics of the observed temperature profile of DPH in lipid bilayers prepared from saturated phosphatidylcholines. These characteristics are a rapid increase in tan Δ at $T = T_c$, followed by a decrease in tan Δ at higher temperatures, and maximum differential tangents of approximately 50% of the value expected for $r_\infty = 0$.

The shapes of these tan Δ temperature profiles are sensitive to the temperature dependence of both R and r_∞. Suppose, for example, the phase transition results in a rapid increase in R (Eq. 12) but no change in r_∞. Under these conditions the result of a constant nonzero r_∞ value is a decrease in tan Δ_{max} and a rather symmetrical temperature profile of decreased half-width (Fig. 13). (These profiles are symmetrical when tan Δ is plotted versus $1/T$.) It should be noted that an assumed constant r_∞ value results in much poorer simulations of the experimental data. Because tan Δ is sensitive

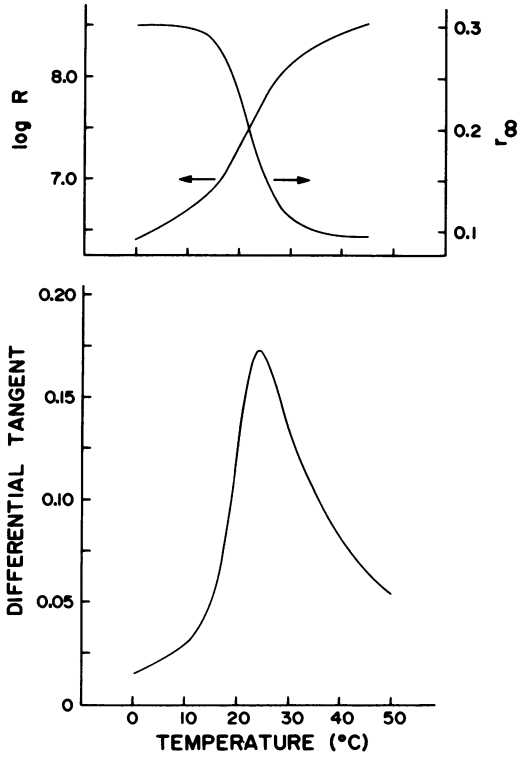

FIGURE 12

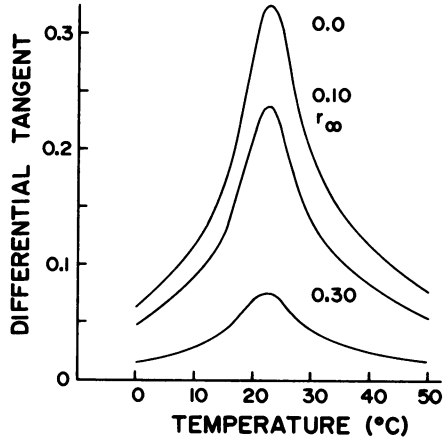

FIGURE 13

FIGURE 12 Model calculations of the differential tangent of a fluorophore in a restricted environment. The top panel shows the values of log R and r_∞ obtained under the assumptions described in Eq. 9–12. The bottom panel shows the expected differential tangent values (Eq. 6) under these conditions, and the following representative values $\omega = 2\pi \times 30$ MHz, $\tau = 10$ ns, and $r_0 = 0.392$.

FIGURE 13 Model calculations of the differential tangent of a fluorophore in a restricted environment. For these calculations we assumed that the temperature dependence of log R was identical to that given for Fig. 12. r_∞ was assumed to be independent of temperature. The r_∞ values used are shown on the figure.

to both R and r_∞ it appears likely that further experimentation and data analysis will allow us to determine more precisely the nature of the rotational motions responsible for fluorescence depolarization of DPH in lipid bilayers. Combined analysis of both steady-state and differential phase data promises to provide even greater insight.

The data we have provided illustrate the difficulties inherent in the extrapolation of DPH fluorescence polarization data to estimation of membrane microviscosity. These difficulties are determined by geometric constraints imposed on the molecule in the lipid bilayer and are unavoidable. To some considerable extent the further development and refining of a theory of hindered and/or anistropic rotations may allow us to describe better the molecular motions of this probe in membranes. However, experi-

ments such as we have described should also be valuable in helping us to select better fluorescence probes whose depolarizing rotations in lipid bilayers are truly similar to those in homogenous solvents.

Our special thanks are due to Professor G. Weber for having supplied us with the theory of hindered rotations (as yet unpublished), and for his continuous generous support of this work.

We thank the Freshwater Biological Research Foundation, and especially its founder Mr. Richard Gray, Sr., without whose assistance this work would not have been possible. Additionally, we acknowledge the generous support of the American Heart Association, Mayo Foundation, and the National Institutes of Health grant ES GM 01238-01A1 to J.R.L. and CA 150 83-00 to F.G.P. J.R.L. is an Established Investigator of the American Heart Association.

Received for publication 8 December 1977.

REFERENCES

BASHFORD, C. L., C. G. MORGAN, and G. K. RADDA. 1976. Measurement and interpretation of fluorescence polarizations in phospholipid dispersions. *Biochim. Biophys. Acta.* **426**:157–172.

CHEN, L. A., R. E. DALE, S. ROTH, and L. BRAND. 1977. Nanosecond time-dependent fluorescence depolarization of diphenyl hexatriene in dimyristoyllecithin vesicles and the determination of "microviscosity." *J. Biol. Chem.* **252**:2163–2169.

COGAN, U., M. SHINITZKY, G. WEBER, and T. NISHIDA. 1973. Microviscosity and order in the hydrocarbon region of phospholipid and phospholipid-cholesterol dispersions determined with fluorescent probes. *Biochemistry.* **12**:521–527.

JACOBSON, K., and D. WOBSCHALL. 1974. Rotation of fluorescent probes localized within lipid bilayer membranes. *Chem. Phys. Lipids.* **12**:117–131.

KATES, M. 1972. Techniques in Lipidology. Elsevier North-Holland, Inc., New York. 355–356.

LENTZ, B. R., Y. BARENHOLZ, and T. E. THOMPSON. 1976*a*. Fluorescence depolarization studies of phase transitions and fluidity in phospholipid bilayers. 1. Single component phosphatidylcholine liposomes. *Biochemistry.* **15**:4521–4528.

LENTZ, B. R., Y. BARENHOLZ, and T. E. THOMPSON. 1976*b*. Fluorescence depolarization studies of phase transitions and fluidity in phospholipid bilayers. 2. Two component phosphatidylcholine liposomes. *Biochemistry.* **15**:4529–4537.

MANTULIN, W. W., and G. WEBER. 1977. Rotational anisotropy and solvent-fluorophore bonds: an investigation by differential polarized phase fluorometry. J. Chem. Phys. **66**:4091–4099.

MOORE, N. F., Y. BARENHOLZ, and R. R. WAGNER. 1976. Microviscosity of togavirus membranes studied by fluorescence depolarization: influence of envelope proteins and the host cell. *J. Virol.* **19**:126–135.

SHINITZKY, M., A. C. DIANOUX, C. GITLER, and G. WEBER. 1971. Microviscosity and order in the hydrocarbon region of micelles and membranes determined with fluorescent probes. *Biochemistry.* **10**:2106–2113.

SHINITZKY, M., and Y. BARENHOLZ. 1974. Dynamics of the hydrocarbon layer in liposomes of lecithin and sphingomyelin containing dicetylphosphate. *J. Biol. Chem.* **249**:2652–2657.

SHINITZKY, M., and M. INBAR. 1974. Difference in microviscosity induced by different cholesterol levels in the surface membrane lipid layer of normal lymphocytes and malignant lymphoma cells. *J. Mol. Biol.* **85**: 603–615.

SPENCER, R. D., and G. WEBER. 1969. Measurements of subnanosecond fluorescence lifetimes with a cross-correlation phase fluorometer. *Ann. N.Y. Acad. Sci.* **158**:361–376.

SPENCER, R. D., and G. WEBER. 1970. Influence of Brownian rotations and energy transfer upon the measurements of fluorescence lifetime. *J. Chem. Phys.* **52**:1654–1663.

WEBER, G. 1977. Theory of differential phase fluorometry: detection of anisotropic molecular rotations. *J. Chem. Phys.* **66**:4081–4091.

NOTE ADDED IN PROOF

We have recently determined that one can combine steady-state anisotropy measurements with the differential phase measurements to obtain unique solutions for both R and r_∞. (Lakowicz and Prendergast, *Science*, in press). These calculations indicate that DPH behaves as a highly hindered rotator below the lipid's transition temperature ($r_\infty \simeq 0.33$), and that these rotations become less hindered above this temperature ($r_\infty \simeq 0.05$). The rotational rate R does not change dramatically at the transition temperature. However, because of the high value of r_∞ below the transition temperature, the errors in the calculated values of R are large below T_c. Overall these observations indicate that the observed steady-state anisotropy values are determined primarily by the degree to which the rotations are hindered, and not by the fluorophore's rotational rate. We conclude that the assumption that the depolarizing rotations of DPH in isotropic solvents are the same as in lipid bilayers is not correct, and hence advise caution in the extrapolation of anisotropy measurements to the membrane's microviscosity.

DISCUSSION

GEACINTOV: The theory of differential phase fluorometry used in your paper applies to the case of a single exponential decay. We have seen in the previous paper, however, the decay of fluorescent molecules in liposomes described by several exponentials (Badea et al., page 197). How does your analysis apply to such cases of nonexponential decay?

LAKOWICZ: We have addressed ourselves to that question and have several answers. Firstly, our decays are not as nonexponential as those in the previous paper. The decays of DPH and lipid bilayers are generally quite a bit closer to a single exponential. Secondly, we observe data at two different frequencies, both 10 and 30 MHz, and we have found precise agreement in the calculated values of the limiting anisotropies (r_∞) at these two frequencies. We feel, but are not certain at this time, that this agreement indicates the appropriateness of a harmonic method in this case. Thirdly, only small differences are observed in the lifetimes measured at these two frequencies by both the phase shift and the demodulation method. The maximum difference we have observed is 1.7 ns between two different frequencies. The differences do not, when propagated into the final results, significantly affect the results compared to the errors in the differential tangent measurements.

GEACINTOV: I am satisfied that in your case the analysis is probably right, but what about other situations in which there is nonexponential decay? Would it be possible to modify the theory to take such cases into account?

LAKOWICZ: Yes. We are presently working on a method that gives an independent determination of the time-resolved decays of anisotropy without actually time-resolving the decays. I think this method will be very powerful for complex decays. We measure steady-state anisotropies in the lifetime domain, rather than in the time domain. We vary the lifetime by oxygen quenching. This allows us to investigate primarily the short side of the decay, and to collect very precise data. The data we collect are the Laplace transforms of the time-resolved decay. This independent method yields results consistent with our harmonic method. Additionally, data collected at both 10 and 30 MHz agree. This result indicates our harmonic method is appropriate.