Fluorescence Anisotropy Measurements under Oxygen Quenching Conditions as a Method to Quantify the Depolarizing Rotations of Fluorophores. Application to Diphenylhexatriene in Isotropic Solvents and in Lipid Bilayers†

J. R. Lakowicz,* F. G. Prendergast, and D. Hogen

ABSTRACT: We have measured the fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) as its fluorescence lifetime is decreased by oxygen quenching. Such studies were done on DPH dissolved in the isotropic solvent mineral oil and for DPH embedded in phospholipid vesicles of either dimyristoyl-L-α-phosphatidylcholine (DMPC) or dioleoyl-L-α-phosphatidylcholine (DOPC), each at several temperatures. In order to obtain adequate quenching increased pressures of oxygen had to be used. Oxygen quenching resulted in significant changes in intensity and anisotropy, and these effects were reversible. To control for possible effects of pressure on the systems under study equivalent experiments were performed with nitrogen, argon, or helium forming the gas phase. Under these last-mentioned conditions, changes in intensity and anisotropy were insignificant when compared with those observed with oxygen quenching. The depolarizing rotations of the fluorophore are described by its rotation rate (R) in radians/seconds and its limiting anisotropy at times which are long compared with the fluorescence lifetime, r_∞. This latter parameter provides a measure of the degree to which the fluorophore's environment hinders its rotational diffusion. Oxygen quenching of fluorescence provides a means to vary the fluorescence lifetime simultaneous observation of the steady-state fluorescence anisotropy allows quantitation of both R and r_∞. For DPH in mineral oil at two different temperatures we found that the values of R obtained from this quenching–anisotropy measurement agreed precisely with those obtained from steady-state anisotropy measurements and with the values obtained from differential polarized phase fluorometry (Lakowicz, J. R., et al. (1979) *Biochemistry 18* (preceding paper in this issue)). Additionally, r_∞ was found to be zero. These results indicate that in mineral oil DPH behaves as an ideal unhindered isotropic rotator. In contrast, DPH embedded in lipid bilayer vesicles of DMPC behaves as an isotropic but highly hindered rotator below the phase transition temperature, as is indicated by $r_\infty \simeq 0.33$. Above the phase transition temperature the depolarizing rotations become significantly less hindered, $r_\infty \simeq 0.03$. In DOPC vesicles the depolarizing rotations are unhindered at all temperatures. The temperature profiles of R and r_∞ obtained for DPH in lipid bilayers were in agreement with those observed using differential polarized phase fluorometry. Quenching–anisotropy measurements of the type we have described provide a powerful method for investigation of time-resolved decays of fluorescence anisotropy without the direct use of time-resolved methods. The estimation of membrane microviscosity from steady-state anisotropy measurements assumes that the nature of the depolarizing rotations of the fluorophore in the membrane are identical with those in an isotropic reference solvent. Our results indicate that this assumption is invalid. We estimated the apparent membrane viscosity by three methods: (1) from steady-state anisotropy measurements; (2) from the rotational rate of DPH within its hindered environment; and (3) from the diffusivity of molecular oxygen. Each method yielded a different value with steady-state polarization giving the highest and oxygen diffusivity the lowest. These results show that any quantitative estimate of microviscosity depends critically upon the molecular process used for its estimation.

Fluorescence anisotropy measurements have been useful in the investigation of protein–ligand interactions (Anderson & Weber, 1965; Weber & Daniel, 1966), protein–protein interactions (Levison et al., 1970; Dandliker & de Saussure, 1970), and the phase transitions of lipid bilayers (Cogan et al., 1973; Shinitzky et al., 1971). This usefulness derives primarily from the similarity of the rates of fluorescence emission and the rates of rotational diffusion of proteins or the rates of fluorophore rotation in lipid bilayers.

Diffusional rates are generally sensitive to temperature and solvent viscosity, whereas the rate of fluorescence emission can be insensitive to these factors; consequently one must frequently rely upon variation of temperature and/or viscosity to investigate the process of interest. In the study of proteins with covalently attached or strongly bound fluorophores, it has been often observed that the apparent rotational rate of the protein varies depending upon whether temperature or viscosity is varied (Rawitch et al., 1969; Wahl & Weber, 1967; Bridge & Johnson, 1973). These differences may result from the effects of solutes on the protein structure, or the effects of solvent and temperature on the absorption bands of the fluorophore (Valeur & Weber, 1977); but these difficulties could be avoided if a means were available to vary the fluorescence lifetime without significant perturbation of the processes of interest. Diffusional processes could then be studied without variation of temperature or solvent.

Oxygen quenching of fluorescence provides precisely this opportunity. Quenching by molecular oxygen is known to be a diffusion-controlled collisional process; hence the fluorescence lifetime is decreased in proportion to the decrease in the fluorescence yield. The noncollisional portion of this quenching is generally small, but easily quantified (Lakowicz & Weber, 1973a; Weber & Lakowicz, 1973). As a result the fluorescence lifetime of a fluorophore can be decreased to accurately

† From the Freshwater Biological Institute and Department of Biochemistry, University of Minnesota, Navarre, Minnesota 55392 (J.R.L. and D.H.), and the Department of Pharmacology, Mayo Medical School, Rochester, Minnesota 55901 (F.G.P.). *Received July 5, 1978; revised manuscript received October 30, 1978.* This work was supported by National Institutes of Health Grants ES 01283 (to J.R.L.) and CA 15083 (to F.G.P.), American Heart Association Grant 76-706 (to J.R.L.), a grant from the Muscular Dystrophy Association (MDA-24) (to F.G.P.), and a grant from the Minnesota Lung Association (to J.R.L.). This work was done during the tenure of an Established Investigatorship (to J.R.L.) of the American Heart Association.

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measured values. Relaxation processes as short as 13 ps have been observed using this methodology (Weber & Lakowicz, 1973).

One disadvantage of using oxygen quenching to vary fluorescence lifetimes is the need for a specialized high pressure sample holder. Oxygen pressures of up to 1500 psi or 100 atmospheres are required to dissolve enough oxygen in the aqueous phase to obtain an adequate range of fluorescence lifetimes. Fortunately, these pressures do not result in significant photodecomposition, nor do they perturb the structure or activity of enzymes (Lakowicz & Weber, 1973b). Additionally, our control studies with 100 atm of nonquenching gases indicated that the effects of these pressures on the model membranes were minimal compared with the changes resulting from quenching. Oxygen is transparent in the ultraviolet, allowing ultraviolet excitation of fluorophores. Finally, the maximum concentration of oxygen in water, 0.13 M, does not appreciably alter the solvent composition. We conclude that oxygen quenching provides an ideal method to quantitatively vary the lifetime of fluorophores.

As an example of the usefulness of this quenching method we studied the depolarizing rotations of 1,6-diphenyl-1,3,5-hexatriene (DPH)[1] in an isotropic solvent and in lipid bilayers. Steady-state anisotropy measurements of DPH have been widely used to estimate membrane microviscosity (Lentz et al., 1976). In this estimation one assumes that the depolarizing rotations of the probe in the lipid bilayer are identical with those in a suitable reference solvent (Shinitzky et al., 1971). The choice of the reference solvent can alter the derived microviscosities (Hare & Lussan, 1977). Time-resolved measurements of fluorescence anisotropy do not require this assumption, but it is more difficult to make these measurements and in any event the technique is limited at short times by the finite width of the lamp pulse. To circumvent the difficulties inherent in both steady-state polarization and pulse fluorometric measurements, we propose the use of fluorescence anisotropy measurements under conditions of oxygen quenching. We note that the central difficulty remains, which is the relationship of the observed anisotropy data to membrane microviscosity.

Recent work in this laboratory (Lakowicz & Prendergast, 1978a,b; Lakowicz et al., 1979) and others (Chen et al., 1977; Kawato et al., 1977; Dale et al., 1977; Kinosita et al., 1977) have shown that DPH behaves as a hindered isotropic rotator in lipid bilayers and therefore provides a good test of our methodology. In this paper we discuss the theoretical aspects of the quenching–anisotropy technique and the results obtained for DPH dissolved in an isotropic solvent (mineral oil) or embedded in lipid bilayers prepared from dimyristoyl-L-α-phosphatidylcholine (DMPC) or dioleoyl-L-α-phosphatidylcholine (DOPC) and, finally, illustrate how problematic the assessment of membrane viscosity may be.

Theory

Fluorescence anisotropy measurements yield the rotational rates of fluorophores. For an ideal spherical molecule the steady-state fluorescence anisotropy (r) is described by the Perrin–Weber equation (Perrin, 1929; Weber, 1966)

$$\frac{r_0}{r} = 1 + \frac{R_g T}{\eta V}\tau = 1 + 6R\tau \qquad (1)$$

where R_g is the gas constant, T is the temperature, η is the solvent viscosity, V is the fluorophore's hydrodynamic volume, τ is the fluorescence lifetime, R is the fluorophore's rotational rate in radians/seconds, and r_0 is the steady-state fluorescence anisotropy observed in the absence of depolarizing rotations. Factors which affect R, η, V, or τ will result in changes in the observed anisotropy. Herein lies the value of anisotropy–quenching studies. Examination of eq 1 will show that variation of τ is just as useful as variation of T, η, or V. Dramatic changes of τ can be achieved with virtually no change in solvent composition, save for the presence of a low concentration of oxygen (0.13 M at 100 atm). The measurements so obtained are for times shorter than the fluorescence lifetime and are thus complementary to those obtained by time-resolved decays of fluorescence anisotropy. (The latter method provides information primarily at times longer than the fluorescence lifetime.) Equation 1 may be modified as follows

$$r = (r_0 - r)/(6R)\tau \qquad (2)$$

A plot of $(r_0 - r)/\tau$ vs. r for a free isotropic rotator should be linear with a slope equal to $1/6R$ and an intercept of zero on the r axis. It should be noted that a linear plot is not expected for an anisotropic rotator (Weber, 1973; Teale & Badley, 1970) and such nonlinearities have been observed for tryptophan and perylene in propylene glycol (Lakowicz & Weber, unpublished observations).

The Perrin–Weber equation (eq 1) can be modified to allow for the possibility that the depolarizing rotations are isotropic, but hindered, so that at times long compared with the fluorescence lifetime, the average angular (θ) distribution of the fluorophore is described by a limiting anisotropy, r_∞, such that (Weber, 1978)

$$r_\infty/r_0 = (3\cos^2\theta - 1)/2 \qquad (3)$$

Under these conditions (Lakowicz et al., 1979)

$$r = r_\infty + (r_0 - r)/6R\tau \qquad (4)$$

A plot of r vs. $(r_0 - r)/\tau$ therefore permits r_∞ and $1/6R$ to be obtained from the intercept and slope, respectively.

As is apparent from the above discussion, we require the fluorescence lifetime τ at each measured oxygen pressure. We have not performed these measurements directly. Rather, τ was calculated from the decrease in fluorescence intensity consequent upon oxygen quenching and corrected for the static quenching component.

When the fluorescence is highly quenched ($F_0/F > 5$), the variation of fluorescence intensity resulting from oxygen quenching can be described by a modified version of the Stern–Volmer equation (Vaughn & Weber, 1970)

$$F_0/F = (1 + K_D[Q])(1 + K_S[Q]) \qquad (5)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher (of concentration [Q]), K_D is the dynamic or collisional quenching constant, and K_S is the static quenching constant. Such static components have been observed by others (Rollefson & Boaz, 1948; Boaz & Rollefson, 1950) and appear to be the result of the increased probability at high quencher concentration that a fluorophore is adjacent to the quenching molecule at the moment of excitation (Frank & Vavilov, 1931). This results in instantaneous quenching. The fluorescence lifetime is, however, decreased only by the dynamic component. In this situation τ is obtained from

$$\tau = \tau_0/(1 + K_D[Q]) \qquad (6)$$

where τ_0 is the fluorescence lifetime in the absence of quencher.

[1] Abbreviations used: DPH, 1,6-diphenyl-1,3,5-hexatriene; DMPC, dimyristoyl-L-α-phosphatidylcholine; DOPC, dioleoyl-L-α-phosphatidylcholine.

This was shown to be valid experimentally by Lakowicz & Weber (1973a).

Separate determination of K_S and K_D is achieved by defining

$$K_{app} = \frac{(F_0/F) - 1}{[Q]} = (K_S + K_D) + K_S K_D[Q] \quad (7)$$

where K_{app} is the apparent quenching constant irrespective of mechanism. A plot of K_{app} vs. [Q] yields an intercept of $K_S + K_D$ and a slope of $K_S K_D$. Solution of the quadratic equation yields K_S and K_D.

The observation of a linear plot according to eq 2 and 4 in the lifetime domain is equivalent to an exponential decay of anisotropy in the time domain. In particular, the anisotropy observed at a specific lifetime $r(\tau)$ is given by the Laplace transform of the time-resolved decay of fluorescence anisotropy $r(t)$ according to

$$r(\tau) = \frac{1}{\tau}\int_0^\infty r(t)e^{-t/\tau}dt \quad (8)$$

For an isotropic rotator $r(t) = r_0 e^{-6Rt}$ and application of eq 8 yields the Perrin–Weber equation.

Oxygen quenching anisotropy measurements may provide a means to directly determine time-resolved decays of anisotropy without the use of time-resolved methods. This possibility lies in the use of $r(\tau)$ to recover $r(t)$ by application of an inverse Laplace transform. (If the functional form of $r(t)$ is unknown, or if it is not desirable to assume a functional form, one may use numerical methods to recover $r(t)$.) From oxygen-quenching measurements we may obtain precise $r(\tau)$ values for times shorter than the fluorescence lifetime, precisely the region in which time-resolved methods are weakest due to overlap of the observed fluorescence with the excitation pulse. We have not yet utilized the available methods for numerical inversion of the Laplace transform, and hence we cannot comment on the major sources of error which will be encountered. This inversion was unnecessary in light of the ideal fit obtained when a model for a hindered isotropic rotator was used for analysis of our quenching–anisotropy data. The numerical inversion method should be most valuable for multiexponential decays of anisotropy which result from hindered or unhindered anisotropic rotations.

Materials and Methods

Except where indicated, all experimental conditions and sample preparations were identical with those described in the previous paper (Lakowicz et al., 1979). The concentration of DPH in the mineral oil was 1.5 μM, yielding an optical density at 360 nm of 0.09. The background fluorescence from this solvent was less than 1% of the total DPH fluorescence.

Fluorescence emission spectra and anisotropy measurements were made using an SLM Instruments, Inc., photon counting spectrofluorometer, equipped with a double grating excitation monochromator and Glan-Thompson polarizers. The following conditions were used for all anisotropy and intensity measurements: excitation wavelength 360 nm and a Corning 7-54 filter; emission wavelength 450 nm; excitation and emission band-passes, 8 and 2 nm, respectively. Emission spectra were recorded with both the excitation and emission polarizers in the vertical positions.

Fluorescence anisotropies were obtained from intensity measurements using

$$r = (I_{\parallel}G - I_{\perp})/(I_{\parallel}G + 2I_{\perp}) \quad (9)$$

where G is a correction factor for the monochromator's transmission efficiency for vertically and horizontally polarized light. This value is given by the ratio of the fluorescence intensities of the horizontal to vertical components when the exciting light is polarized in the horizontal direction (Dale et al., 1977). This condition yields an equivalent fluorescence intensity (I) entering the monochromator irrespective of the orientation of the observation polarizer. The total fluorescence intensity, which is required for the calculation of the quenching constants, is given by

$$F = I_{\parallel}G + 2I_{\perp} \quad (10)$$

Fluorescence lifetime measurements were obtained using the phase shift method (Spencer & Weber, 1969) with a modulation frequency of 30 MHz. Polarizers were used to eliminate the effect of Brownian rotations upon the measured fluorescence lifetime (Spencer & Weber, 1970) as described previously (Lakowicz et al., 1979).

Observation of samples subjected to increased oxygen pressures was carried out in a cell similar to that described by Lakowicz & Weber (1973a), except that the temperature is controlled by an external jacket, and the sample is contained in the pressure cell within a 2 × 2 cm (by 4 cm high) cuvette. A magnetic stirring bar rested inside this cuvette, below the light path. All samples were purged with argon and kept under a nitrogen atmosphere until initial fluorescence intensities and anisotropies had been obtained. Data were collected only with increasing pressure since pressure release results in bubbles which interfere with data acquisition. Rapid stirring for 30 min was required to reequilibrate the sample with oxygen at each pressure. When data were to be acquired, the stirrer was operated at slow speed to minimize artifacts due to vortexing and also to decrease any errors due to bleaching of DPH on exposure to light. At the end of each quenching run, the pressure was slowly released and the sample completely removed from the cell and purged with argon. The stirring bar used in the experiment was then removed because we found that the Teflon stirring bars absorb a good deal of oxygen and must be allowed to degas for 24 h after each use. At this point, spectra, intensity, and anisotropy were again measured. In general, we found (even for DPH in DOPC vesicles which was most susceptible to photobleaching) that argon purging restored all spectral properties to those existing prior to initiation of oxygen quenching, except for about 5% of the original fluorescence yield.

Fluorescence spectra collected on highly quenched samples showed Raman scatter centered at 408 nm which was significant relative to the DPH fluorescence. Intensity and polarization artifacts resulting from Raman scatter were avoided by measuring the fluorescence intensities and anisotropies at 450 nm. Scattered light at the excitation wavelength and at 408 nm did not interfere with any of our observations.

With the instrumental arrangement used for these experiments the r_0 value for DPH was found to be 0.362 in propylene glycol at −60 °C; this value was used for all our calculations. It is to be noted that this r_0 value for DPH differs from that reported by us previously (Lakowicz & Prendergast, 1978a,b; Lakowicz et al., 1979). This difference is a result of instrumental optical effects, indicating the need to measure r_0 under the particular experimental conditions being used.

At present we do not know the precise concentration of O_2 in the hydrophobic region of the vesicles. The most unambiguous quantity is the oxygen pressure. Hence we report all quenching constants in psi^{-1}.

Results

Figure 1 shows the fluorescence emission spectra of DPH in DMPC vesicles under an argon atmosphere and when

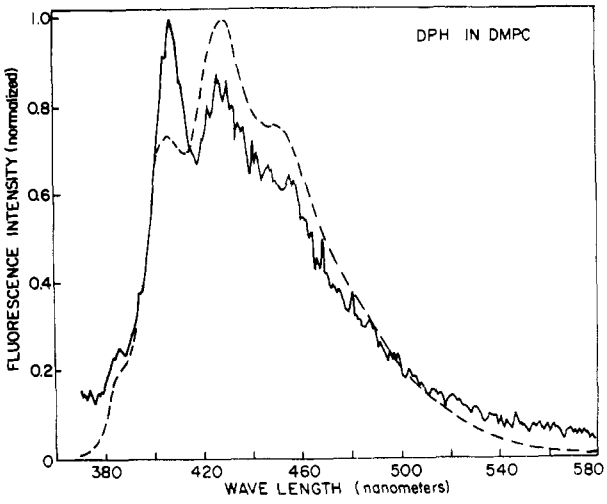

FIGURE 1: Fluorescence emission spectra of DPH in DMPC vesicles. Spectra are shown for DPH-labeled DMPC vesicles for the same sample under an argon atmosphere (---) and after equilibration with 1550 psi of oxygen pressure (—), both normalized to the same maximum intensity. At 1550 psi of oxygen F_0/F is 44.4 at the experimental temperature of 29.6 °C.

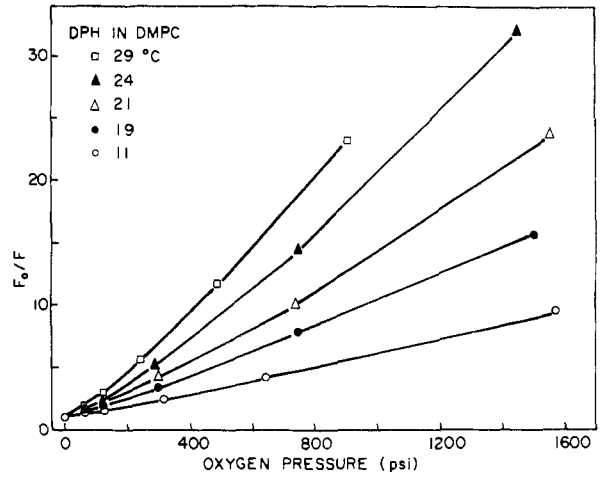

FIGURE 2: Stern–Volmer plots of the oxygen quenching of DPH in DMPC vesicles.

equilibrated with 1550 psi O_2. The Raman peak is apparent at 408 nm, but it is equally clear that this peak does not interfere with our anisotropy measurements at 450 nm. Although not shown, we have observed for DPH in propylene glycol at −60 °C that the fluorescence anisotropy is constant across the emission band, and therefore the anisotropy measurements will not be sensitive to the chosen emission wavelength.

The Stern–Volmer plots for the oxygen quenching of DPH in DMPC vesicles (Figure 2) show a clear upward curvature, especially when F_0/F is greater than 10. We attribute this nonlinearity to a static component defined by K_S (see section on Theory). A plot of K_{app} vs. psi O_2 allowed K_S and K_D to be determined (Figure 3).

Table I summarizes the dynamic and static quenching constants for DPH in the isotropic solvent mineral oil and in DMPC and DOPC vesicles. From these data it is clear that the static component is small, maximally 1–9% of K_D. However, it should also be noted that this small component can result in a sizable difference in the calculated value of τ at high quencher concentrations. For example, for DPH-labeled DMPC vesicles equilibrated with 1550 psi O_2 at 45 °C, the calculated value for τ is 0.60 ns, whereas the τ value

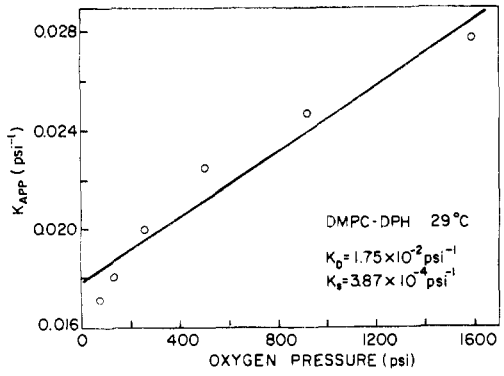

FIGURE 3: Separation of the static and dynamic oxygen quenching constants.

Table I: Quenching Constants for DPH in Mineral Oil and in Phospholipid Vesicles

sample	temp (°C)	τ (ns)[a]	$K_D \times 10^2$ (psi^{-1})	$K_S \times 10^4$ (psi^{-1})
mineral oil	22.3	9.9	2.79	16.70
	44.9	9.9	4.94	14.75
DMPC	3.5	10.1	0.30	1.45
	11.0	9.8	0.41	1.93
	14.3	9.5	0.51	2.79
	19.0	9.3	0.81	1.10
	21.0	9.2	1.01	2.87
	24.0	9.1	1.22	6.08
	24.8	8.9	1.59	5.40
	29.3	8.7	1.75	3.87
	35.0	8.4	1.76	6.56
	45.2	7.8	1.62	14.50
DOPC	5.0	7.0	0.82	1.70
	11.4	6.8	1.04	1.85
	22.3	6.5	1.20	2.70
	31.6	6.1	1.68	0.65
	42.2	5.8	1.89	1.10
	53.0	5.4	1.84	0.70

[a] All samples were purged with argon to remove dissolved oxygen. See Materials and Methods for additional details.

obtained from simply the proportional decrease in fluorescence intensity is 0.32 ns. These differences result in substantial alteration of the appearance of the plots of r vs. $(r_0 - r)/\tau$. In fact, without this static quenching correction, one could easily reach erroneous conclusions concerning the isotropy or freedom of the fluorophore's rotations. We note that it has been previously demonstrated (Lakowicz & Weber, 1973a) that the decrease in fluorescence lifetime due to oxygen quenching, when measured directly under oxygen quenching conditions, is in fact described by the dynamic quenching constants obtained from the procedure described in the Theory section, and not by the proportional decrease in fluorescence intensity.

A plot of r vs. $(r_0 - r)/\tau$ allows computation of R and r_∞. For DPH in mineral oil at two temperatures (Figure 4) it is clear that $r_\infty = 0$ in agreement with the r_∞ values obtained by differential polarized phase fluorometry (Lakowicz et al., 1979). This result indicates that the depolarizing rotations are unhindered. The isotropic nature of the rotations is demonstrated by the linearity of these plots. (For an anisotropic rotator such as perylene, such linearity is not observed because of the differences in the rotational rates for in-plane and out-of-plane rotations (Lakowicz & Weber, unpublished observations).) We note that the rotational rates for DPH obtained from the slopes of this plot precisely agree with those obtained for the same sample by differential polarized fluorometry (Table II) and that the results obtained by the latter methodology also indicate that the depolarizing rotations

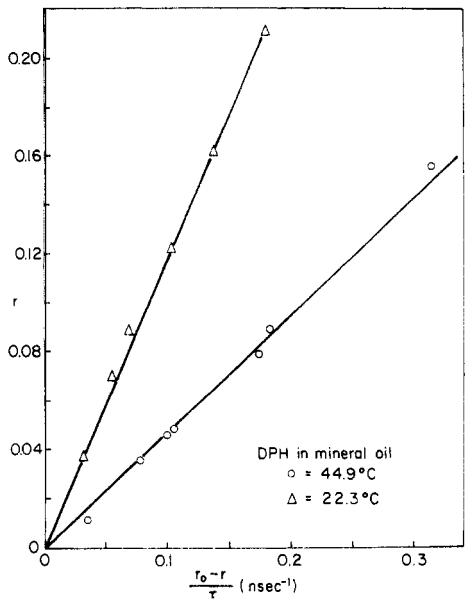

FIGURE 4: Oxygen quenching–anisotropy plot of DPH in mineral oil.

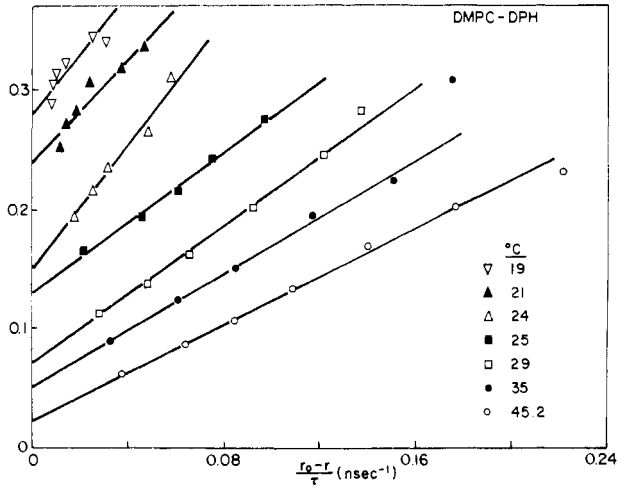

FIGURE 5: Oxygen quenching–anisotropy plots of DPH-labeled DMPC vesicles. Data for 3, 11, and 14 °C are omitted from this plot since r is relatively invariant with quenching, these values being 0.35, 0.35, and 0.33, respectively. Unfortunately, as a result of the small changes in r with quenching, the rotational rates could not be determined at these temperatures.

Table II: Comparison of the Rotational Rates and Limiting Anisotropies for DPH in Mineral Oil as Observed by Oxygen Quenching-Anisotropy Measurements and Differential Polarized Phase Fluorometry

temp (°C)		O_2-anisotropy	differential polarized phase fluorometry
22.3	log R	8.15	8.16
44.9	log R	8.56	8.58
22.3	r_∞	0.00	0.005
44.9	r_∞	0.00	0.005

of DPH in mineral oil are unhindered and isotropic. Such agreement in data from independent methods provides strong evidence for our conclusions that the depolarizing rotations of DPH in mineral oil are isotropic and unhindered.

DPH in DMPC Vesicles. Oxygen quenching–anisotropy studies of DPH-labeled DMPC vesicles (Figure 5) clearly indicate nonzero values for r_∞ as can be seen from the intercepts on the r axis. Thus, in DMPC vesicles, DPH is a hindered rotator. Because of geometric considerations it

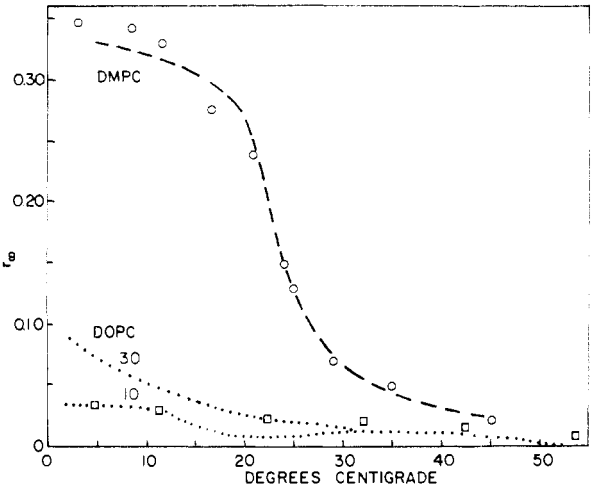

FIGURE 6: Limiting anisotropies (r_∞) as observed by oxygen quenching–anisotropy measurements and differential polarized phase fluorometry. Data are shown for DMPC (○) and DOPC (□) labeled with DPH as observed by the quenching method. For comparison, data are shown which were obtained by differential phase measurements, DMPC (- - -), DOPC (···), the latter at both 10 (lower) and 30 MHz (upper).

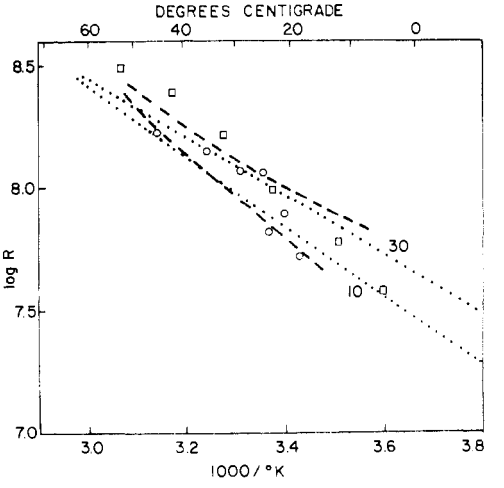

FIGURE 7: Comparison of the rotational rates of DPH as observed from oxygen quenching-anisotropy studies and differential phase measurements. Rotational rates for DPH, as observed by the oxygen quenching–anisotropy method, are shown for DMPC (○) and DOPC (□). For comparison we show these same rates for DPH in DMPC (- - -) and DOPC (···), as observed by differential polarized phase fluorometry. The numbers indicate 10 and 30 MHz data.

appears likely that these hindered depolarizing rotations should still be isotropic (Shinitzky & Barenholz, 1974), and the linearity of the plots lends further support to this view. The data presented in Figure 5 are adequate for construction of temperature profiles for r_∞ (Figure 6) and R (Figure 7) for DPH in DMPC vesicles. DPH is seen to be a highly hindered rotator below the transition temperature, $r_\infty \simeq 0.33$, but the rotations become unhindered above T_c, $r_\infty \simeq 0.03$. Also shown on Figure 6 are the limiting anisotropies obtained by combined use of steady-state anisotropies and differential polarized phase fluorometry (Lakowicz et al., 1979). The data given in Figures 6 and 7 show the excellent agreement in the r_∞ values and the rotational rates (R) determined by the two independent methods.

DPH in DOPC. Oxygen quenching–anisotropy measurements were also carried out for DPH embedded in vesicles composed of the unsaturated phosphatidylcholine-DOPC. The differential polarized phase fluorometric investigations had

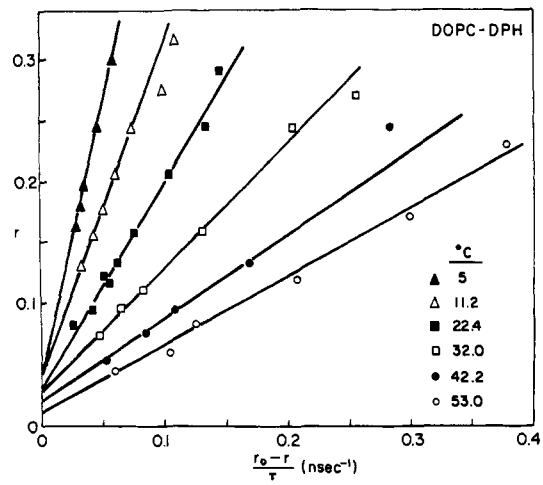

FIGURE 8: Oxygen quenching–anisotropy plot of DPH-labeled DOPC vesicles.

already indicated that the rotations were unhindered, $r_\infty = 0.06$ at 5 °C to $r_\infty = 0.01$ at 45 °C, with no break in the Arrhenius plots for the rotational rate of DPH (Lakowicz et al., 1979). The oxygen quenching–anisotropy data (Figure 8) also indicate unhindered isotropic rotations for DPH in DOPC vesicles. As for DPH in DMPC vesicles good agreement is seen between these r_∞ and R values and those determined by differential polarized phase fluorometry (Figures 6 and 7). We were, of course, concerned about the possible oxidation of DOPC under increased oxygen pressure. However, upon removal of oxygen, the anisotropy values returned to the original values; thus any oxidation which might have occurred was not significant for the anisotropy determination and, therefore, presumably did not significantly perturb the bilayer. In all cases at least 90%, but typically more than 95%, of the original intensity was recovered upon removal of oxygen, and the small loss of fluorescence intensity may be attributed to the photobleaching of DPH which has been noted by other workers (Shinitzky & Barenholz, 1974). The observed bleaching was most significant at higher temperatures and was not increased by the presence of oxygen; it now appears that the problem is greatest when the probe is in an environment of low viscosity. In our experimental arrangement bleaching problems were minimized by use of a sample volume of about 12 mL and continual stirring of the solution during excitation; with smaller sample volumes, and the resulting increased percentage of the sample which is subject to illumination at any one time, it may be necessary to correct the observed intensities for the effects of photobleaching.

Discussion

The data clearly indicate that the measurement of fluorescence anisotropy under oxygen quenching conditions allows the quantitation of the depolarizing rotations of fluorophores in isotropic solvents and lipid bilayers.

In accord with the data obtained by differential polarized phase fluorometry, the results from oxygen quenching–anisotropy experiments indicate a fundamental difference between the rotations of DPH in the solvents and lipid bilayers. The rotations of DPH in the solvents are unhindered and isotropic. In lipid bilayers prepared from DMPC the rotations appear to be isotropic, but are highly hindered below the phase transition temperature of the lipid. The data obtained for DPH in DMPC bilayers were further used to calculate rotational rates of the probe as a function of temperature. An Arrhenius plot constructed from those data did not show an inflexion at the transition temperature of the lipid, meaning that within the average angles through which the DPH is free to rotate, its rotational rate change with temperature shows a single activation energy of 7–9 kcal/mol from 17 to 46 °C.

These data taken with those of the preceding paper (Lakowicz et al., 1979) and the results of Kawato et al. (1977), Chen et al. (1977), Dale et al. (1977), and Kinosita et al. (1977) should remove any doubt regarding the pattern of rotational motion of DPH in lipid bilayers. We should now like to address in considerably greater detail the issue of membrane microviscosity.

The central assumption in the use of steady-state anisotropy measurements to estimate membrane microviscosities is that the depolarizing rotations of the fluorophore in the bilayer are of similar nature to those in an isotropic reference solvent. It is now apparent that this assumption can no longer be accepted as valid and consequently we must reexamine this whole question of "microviscosity". What precisely does the term mean as applied to artificial lipid bilayers or biomembranes?

It is apparent that the problem derives from the restricted nature of DPH rotations in the bilayer. In situations where the motions are less hindered, the steady-state polarization yields apparent microviscosities which are similar to those obtained by differential polarized phase fluorometry and pulse fluorometry. Such a situation is encountered for DPH in DOPC vesicles. Where the lipid system is heterogeneous, however, steady-state polarization alone is unlikely to provide accurate estimates of "microviscosity". For example, in cholesterol-containing bilayers we found that the DPH rotations are hindered at all accessible temperatures (Lakowicz et al., 1979).

To illustrate the difficulties inherent in the qualitative and quantitative assessments of membrane microviscosity, we have used our data to calculate the apparent microviscosity by three independent methods. These are (1) by use of steady-state anisotropy, (2) by use of rotational rates determined from these oxygen quenching–anisotropy measurements, and (3) by relating the oxygen diffusivity to viscosity parameters.

Method I. We may assume that the steady-state anisotropy values reflect the rotational rate of an unhindered fluorophore. This rotational rate is proportional to the absolute temperature T and inversely proportional to the viscosity η. These parameters may be incorporated into a general form of the Perrin–Weber equation

$$r_0/r = 1 + C(r)T\tau/\eta \qquad (11)$$

where $C(r)$ is a parameter related to the effective molar volume of the fluorophore at a given anisotropy value r. For DPH $C(r)$ is relatively invariant with r (Shinitzky & Inbar, 1974). We used the value reported by these workers, 8.6×10^5 P deg^{-1} s^{-1}. (We have not made a correction for the variation of $C(r)$ as r changes, although recently Shinitzky & Barenholz (1978) have reported that $C(r)$ decreases by as much as 20% over the temperature range 273–313 K.)

Method II. The oxygen quenching–anisotropy measurements yield a value for the probe's rotational rate R within its hindered environment. From eq 1 and 11 one may obtain

$$\eta = C(r)T/6R \qquad (12)$$

Hence the probe's rotational rate within the cone may be used to estimate the microviscosity. Equation 12 is equivalent to the classical relationship between rotational rate and viscosity (Weber, 1966) where $C(r) = R_g/V$.

Method III. A parameter implicitly included in the data is the diffusivity of oxygen in the lipid bilayers. Since diffusional processes are inversely related to viscosity, these data provide a third means for calculating the membrane's mi-

croviscosity. The requisite information is contained in the oxygen bimolecular quenching constant, k_D (Lakowicz & Weber, 1973a,b). This constant is related to the dynamic quenching constant K_D by

$$K_D = k_D\tau_0 \tag{13}$$

The bimolecular quenching constant is related to the diffusion constant of oxygen by the Smoluchowski equation (Smoluchowski, 1917)

$$k_D = \gamma 4\pi\sigma_{pq}N(D_p + D_q)/1000 \tag{14}$$

where σ_{pq} is the sum of the molecular radii of the probe (p) and quencher (q), N is Avogadro's number, D is the respective diffusion coefficient, and γ is the quenching efficiency, equal to unity for oxygen. Oxygen is nonpolar and therefore is not expected to localize at the lipid–water interface. In addition, oxygen is much smaller than the lipid molecules which form the environment through which it is diffusing. As a result we feel the Smoluchowski equation, which applies to three-dimensional diffusion, is appropriate for describing oxygen diffusion in a lipid bilayer. Because of the smaller size of oxygen compared with DPH, its diffusion coefficient is dominant in eq 14 and thus the bimolecular quenching constant is directly proportional to the oxygen diffusivity. Calculation of k_D requires knowledge of the concentration of oxygen in the bilayer. We assumed that the oxygen concentration in the bilayer was 6.48-fold higher than in the water phase, and we further assumed that this lipid–water partition coefficient for oxygen is invariant with temperature. (The value of 6.48 is the ratio of the solubilities of oxygen in dodecane and water at 25 °C (Landolt-Bornstein, 1962; Richardson, 1928; Winkler, 1891). Oxygen solubilities in water at various temperatures were obtained from Winkler (1891).) We further must assume that the quenching encounters in the membrane arise from oxygen dissolved in the bilayer at the moment of fluorophore excitation. If this last-mentioned assumption is incorrect or if the partition coefficient is <6.48, the values of k_D we calculate are underestimated, while those of the membrane's microviscosity are overestimated.

With all the above assumptions in mind, the quenching constant K_D in psi^{-1} may be converted to the bimolecular quenching constant k_D in M^{-1} s^{-1} using

$$k_D = \frac{14.7K_D}{6.48[O_2]\tau_0} \tag{15}$$

where $[O_2]$ is the oxygen water solubility at the appropriate temperature in M/atm, and τ_0 is the unquenched fluorescence lifetime in seconds. The membrane's viscosity is then obtained from

$$\eta = \eta_w k_w/k_D \tag{16}$$

where η_w and k_w are the viscosity and bimolecular quenching constant for a probe in a solution of known viscosity. We use water at 25 °C, where $\eta_w = 8.937 \times 10^{-3}$ P and $k_w = 1.2 \times 10^{10}$ M^{-1} s^{-1}, the latter value having been observed for water soluble fluorophores (Lakowicz & Weber, 1973b). This use of η_w and k_w seems appropriate since the efficiency of quenching by oxygen is near unity and does not appear to be solvent dependent.

The data on apparent microviscosity obtained by these three methods are given in Figure 9. The temperature profiles are clearly different with the highest apparent microviscosities being obtained from steady-state polarization and the lowest from oxygen diffusivity. For example, for DMPC at 5 °C these values are 50 and 0.3 P, respectively.

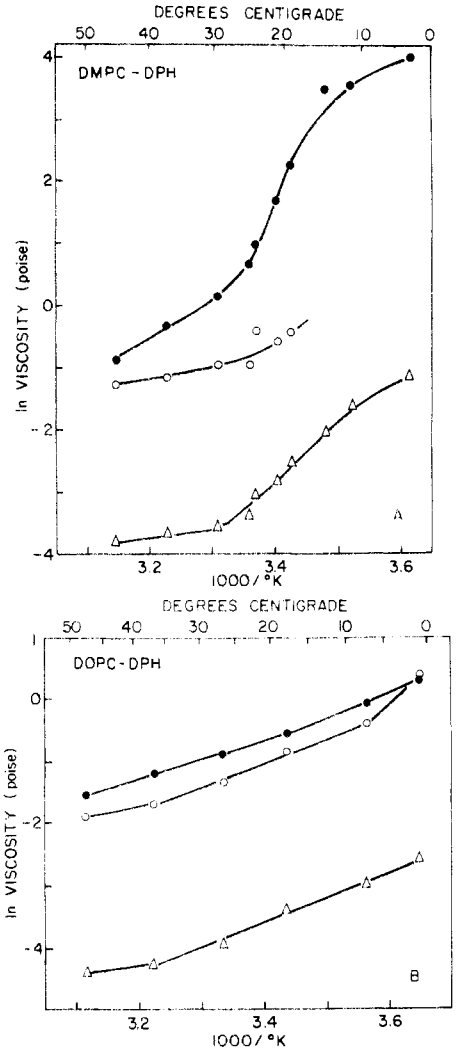

FIGURE 9: Comparison of membrane microviscosities as calculated by three different methods. Data are shown for DMPC (A) and DOPC (B) vesicles labeled with DPH. Microviscosities were calculated from the steady-state anisotropy measurements in the absence of oxygen quenching (●), from the rotational rate of the fluorophore as observed by oxygen quenching-anisotropy measurements (○), and from the diffusivity of oxygen (△). The procedures used are described in the Discussion.

These data indicate that measurements of different physical processes can provide very different estimates of the microviscosity of lipid bilayers. This ought not to be surprising since the methods are all dependent in some manner on a definition of the rotational or translational diffusion of some "probe" molecule. In the previous paper we discussed the fundamental differences between the apparent rotational behavior of DPH as described by steady-state anisotropy or differential polarized phase fluorometry which derive from the geometric restrictions imposed on the probe by the environment. Herein lies the fundamental reason for the differences in microviscosity determined by these two methods, though it is not immediately apparent why the differences should be as large as they are nor why the pattern of change in (apparent) η as given by the curves in Figure 9 should be so different. The fact that the results obtained from the third method are yet more disparate further indicates how dependent apparent η is on the methodology employed. The reasons for the disparity are not completely obvious but may lie in the fact that the diffusional behavior of molecules in solutions become more rapid than predicted by Stokes' law as the size of the diffusing species becomes smaller than the solvent molecules, the effect being

most readily observed for diffusing gases such as O_2 (Osborne & Porter, 1964). There is every reason to believe that we are faced with a similar problem in a bilayer with the additional difficulty that the bilayer is inherently asymmetric.

For these reasons it seems unlikely that a single "microviscosity" parameter can describe the behavior of all diffusing species in a lipid bilayer. It should, however, be possible to describe the size and shape dependence for the rotational and translational motion of foreign molecules in lipid bilayers. One approach could be that adopted by Lakowicz et al. (1977), which employs fluorescence quenching techniques for the study of diffusion in membranes. Carbazole-labeled phospholipids are quenched by a wide variety of chlorinated hydrocarbons, and measurement of these quenching rates for a well-defined series of chlorinated hydrocarbon may provide size and shape profile for diffusional transport in bilayers; such work is currently underway.

The oxygen quenching–anisotropy methodology presented in this paper provides a second means to investigate the rotational diffusion of fluorophores especially at times equal to or shorter than the fluorescence lifetime. It appears likely that these anisotropy data in the frequency domain can be numerically transformed to the time domain and, thus, provide information complementary to the time-resolved measurements of anisotropy. We hope that this approach will be useful in resolving complex decays of anisotropy in which the rotations are neither unhindered or isotropic.

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