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FLUIDITY AND OXYGEN PENETRATION OF LIPID VESICLES STUDIED BY FLUORESCENCE PROBES

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Abstract—An analysis of the temperature dependence of *trans*-stilbene fluorescence yield in dipalmitoyl lecithin vesicles is used to obtain activation energies. The results are interpreted in terms of bilayer fluidity through and above the phase transition. Oxygen quenching of the fluorescence of pyrenebutyric acid (incorporated in dipalmitoyl lecithin and egg lecithin vesicles) is reported as a function of temperature and bulk oxygen concentration. Above the bilayer phase transition, quenching rates (determined by oxygen quenching) decrease with decreasing temperature. A reduction in oxygen quenching is observed through the dipalmitoyl lecithin phase transition.

INTRODUCTION

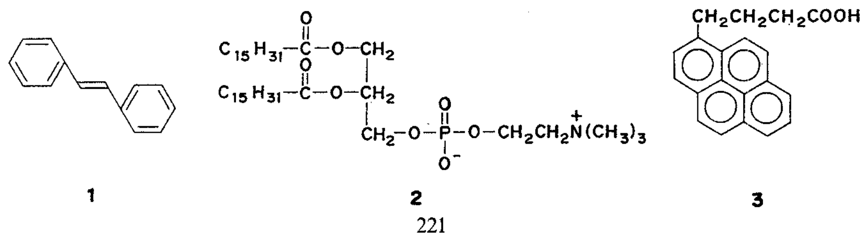
Fluorescence techniques have been used to study membrane and model membrane systems (Radda and Vanderkooi, 1972; Azzi, 1975). The emission properties and quenching behavior of incorporated fluorescence probes or inherent fluorescent chromophores may provide information about membrane structure and permeability. Membrane fluidity has been examined by fluorescence polarization (e.g. Cogan *et al.*, 1973; Jacobson and Papahadjopuolos, 1975; Vanderkooi *et al.*, 1974) and probe diffusion (Soutar *et al.*, 1974; Vanderkooi and Callis, 1974; Galla and Sackman, 1974). We report a new method to study membrane fluidity based on the viscosity dependence of *trans*-stilbene **1** fluorescence yield (Saltiel and D'Agostino, 1972). An analysis of the temperature dependence of *trans*-stilbene fluorescence yield in dipalmitoyl lecithin (DPL) **2** vesicles is used to obtain activation energies. The results are interpreted in terms of bilayer fluidity through and above the phase transition.

This paper also reports the use of oxygen quenching of pyrenebutyric acid (PBA) **3** fluorescence lifetime to study the oxygen penetration of lipid bilayers. Oxygen quenching of fluorescence has been established as a method to determine the oxygen penetration of biological systems (Vaughan and Weber, 1970; Lakowicz and Weber, 1973). This technique can be applied to examine the effects of the temperature and bilayer phase transition on oxygen penetration of lipid bilayers and intact membranes. Thus, we examined the temperature dependence of oxygen quenching of PBA incorporated in DPL and in egg lecithin vesicles. We find that oxygen penetration of the lipid bilayer decreases with decreasing temperature with a substantial reduction through the phase transition of DPL. These results agree with those obtained by Fischkoff and Vanderkooi (1975) who studied the temperature dependence of oxygen quenching of pyrene incorporated in lipid bilayer vesicles and in erythrocyte membranes.

MATERIALS AND METHODS

Dipalmitoyl *d*-1-α phosphatidyl choline (Sigma) and egg lecithin (Sylvana) were used without purification. Both compounds were checked for emitting impurities and none were found. Phosphate buffer solutions were prepared from reagent salts. *trans*-Stilbene (Aldrich, 98%) was recrystallized from ethanol and norite (2×, m.p. 184–185°C).

To prepare lipid vesicles containing a probe, a stock solution of probe in ethanol was prepared and the solvent evaporated in a test tube such that the residual solid would give $A_{334.5} \sim 0.08$ (PBA) and $A_{313} \sim 0.2$ (*trans*-stilbene) upon addition of buffer solution. DPL was weighed out into the test tube containing the probe; egg lecithin was added in ethanol solution and the solvent evaporated. Phosphate buffer (0.1 *M*, pH 7.0, 0.1 *M* NaCl) was added to the test tube containing the probe and lipid. The final concentration of lipid was 20 mg/10 mℓ buffer solution. Solutions were heated to approximately 60°C to disperse the lipids. The lipid dispersions were sonicated for 5 min (Biosonik III, maximum power for regular probe), with cooling (ice bath) for egg lecithin. If the dispersions were cloudy after standing for 5 min, sonication was repeated. When the samples were clear, they were centrifuged (Beckman L3-50 Ultracentrifuge, 60,000 *g*, 4°C) for 30 min. The supernatant solution was stored in the refrigerator and



measurements were made within 24 (egg lecithin) or 48 (DPL) h of preparation.

The fluorescence quantum yield ϕ_f for stilbene in DPL vesicles was determined relative to *trans*-stilbene (3-methyl pentane, $\phi_f = 0.05$; Saltiel and D'Agostine, 1972), naphthalene (cyclohexane, $\phi_f = 0.23$), and triphenylene (cyclohexane, $\phi_f = 0.18$) (Berlman, 1965). The activation energies were derived from least-squares slopes and are averages of three different runs.

The lipid dispersions were saturated with gas by direct bubbling or by passing a stream of gas over the sample. The 40% oxygen/nitrogen was from Matheson (unanalyzed). All gases were used without purification. Fluorescence lifetimes were determined using single photon counting (Ware, 1971). A description of the apparatus has been published (Geiger and Turro, 1975). Fluorescence spectra and intensities were recorded on a Perkin-Elmer MPF-2A or MPF-3L spectrophotometer.

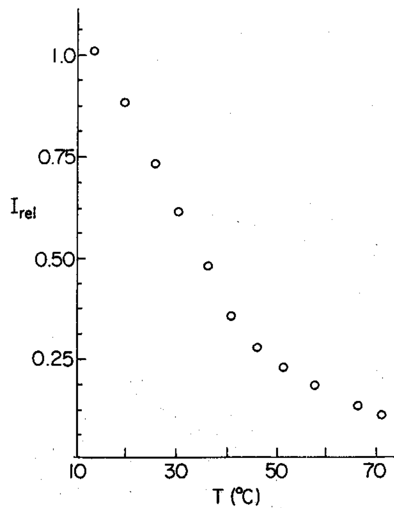


Figure 1. Temperature dependence of *trans*-stilbene fluorescence intensity.

RESULTS AND DISCUSSION

trans-*Stilbene fluorescence in lipid vesicles*

The relative fluorescence intensity (I_{rel}) of *trans*-stilbene in sonicated DPL dispersions was measured as a function of temperature. I_{rel} decreases with increasing temperature (Fig. 1). A change in the temperature dependence of I_{rel} appears to occur above 42°C, around the phase transition of DPL ($T_c = 41°$). The absolute fluorescence quantum yield of *trans*-stilbene was determined to be 0.07 ± 0.02 at 26.5°C. The relative fluorescence intensities reflect relative quantum yields because the spectral shape is constant with temperature. Knowing the absolute quantum yield at one temperature, the absolute quantum yield of *trans*-stilbene fluorescence as a function of temperature was obtained from the fluorescence intensities.

The temperature dependence of *trans*-stilbene fluorescence (1) predicts that plots of ln $(1/\phi_f - 1)$ vs 1/T should be linear for $k_{isc}/k_f \ll 1$, i.e.

$$\left(\frac{1}{\phi_f} - 1\right) = \frac{k_{isc}}{k_f} = \frac{A\,e^{-E/RT}}{k_f} \qquad (1)$$

(Saltiel and D'Agostino, 1972). Our experimental data for the temperature dependence of *trans*-stilbene fluorescence in DPL vesicles can be fit by two straight lines when plotted as $(1/\phi_f - 1)$ vs 1/T (Fig. 2). The activation energy E derived from the data in Fig. 2 above 47°C is 4.2 ± 0.9 kcal/mol; between 25° and 42°, $E = 7.0 \pm 0.3$ kcal/mol. ϕ_f does not depend strongly on viscosity below 0.1 cP. The activation energy in fluid organic solvents (2.7 kcal/mol) can thus be taken as the temperature dependent contribution to ϕ_f (Saltiel and D'Agostino, 1972). Thus, the activation energy below the phase transition ($T_c = 41°$) has a substantial viscosity dependent contribution. The activation energy above the phase transition appears to have a small viscosity contribution, which is consistent with an effective viscosity greater than that of fluid solution. Fluorescence polarization measurements (Cogan *et al.*, 1973; Vanderkooi *et al.*, 1974; Jacobson and Papahadjopoulos, 1975) and probe diffusion studies (Razi Naqvi, *et al.*, 1974; Soutar *et al.*, 1974; Vanderkooi and Callis, 1974; Galla and Sackman, 1974) suggest similar viscosity properties for lipid bilayers.

Oxygen quenching of PBA in lipid vesicles

The fluorescence lifetime of PBA was measured in sonicated lipid dispersions saturated with nitrogen, air, 40% oxygen/nitrogen, and nitrogen as a function of temperature. The measured PBA lifetimes in vesicles are characteristic of a hydrophobic environment; the nitrogen saturated values are much longer in DPL (τ = 190 ns) and in egg lecithin (τ = 150 ns) than in water (τ = 115 ns) at room temperature. The lifetime values in vesicles approach the lifetime in degassed cyclohexane (τ = 250 ns). Also, PBA fluorescence lifetime in vesicles is not reduced upon addition of Br^-,

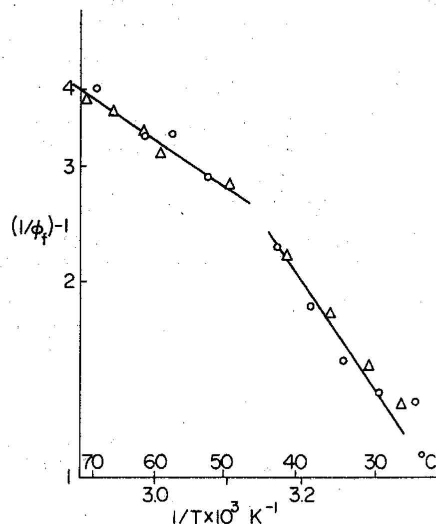
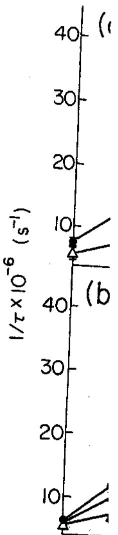


Figure 2. Arrhenius plot for *trans*-stilbene fluorescence in DPL vesicles.



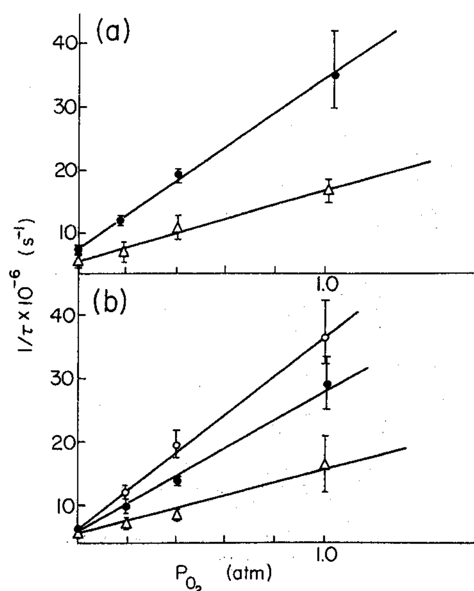


Figure 3. Oxygen quenching of PBA fluorescence lifetime in (a) egg lecithin vesicles: ● 38°C, △ 5°C; (b) DPL vesicles: ● 55°C, ○ 45°C, △ 35°C.

a known quencher of PBA fluorescence. The lipid bilayer, which is impermeable to ions, apparently protects the incorporated PBA from bromide ion quenching.

Our quenching data were treated by plotting the reciprocal lifetime vs the ratio of oxygen to nitrogen according to Eq. 5. Straight lines were obtained in

$$1/\tau = 1/\tau_0 + C P_{O_2} \qquad (5)$$

water, DPL, and egg lecithin (Fig. 3). The slopes, given by C in Eq. 5, reflect both changes in oxygen diffusion and solubility and can be thought of as apparent quenching rate constants. These slopes decrease with temperature in all cases (Table 1). Oxygen solubility in water increases with decreasing temperature (Handbook of Physics and Chemistry, 1972); the decrease in relative quenching rates must be attributed to decreased oxygen diffusion rates. The reduction of relative quenching rates with temperature in vesicles is also most likely related to decreased diffusion rates.

In DPL vesicles, a noticeable reduction in the slopes of the quenching plots is observed around T_c. Oxygen diffusion rates are expected to decrease because of the decreased fluidity associated with the phase transition.

We have assigned the observed changes in oxygen quenching of PBA lifetime at T_c to changes in oxygen diffusion. It is possible that a change in interaction of PBA with the lipid bilayer at T_c could account for the results. Galla and Sackman (1974, 1975) have found evidence for clumping of pyrene derivatives in the gel phase (below T_c) if the probe concentration is greater than 0.1 mol % in the lipid bilayer. The concentration of PBA in the bilayer is less than 0.06 mol % in our work. Additional evidence that PBA does not interact differently with the bilayer below T_c is available. The lifetime of PBA in deaerated DPL dispersions is constant at 180 ns from 55 to 35°C. The emission spectrum shape remains constant through the phase transition. Additionally, the exponential decay of the lifetime is fit well by one exponential both above and below T_c, suggesting a homogeneous distribution of PBA in the bilayer.

The oxygen quenching results suggest the phase transition in DPL occurs between 40 and 45°C. The break in the *trans*-stilbene fluorescence activation energy plot occurs between 42 and 47°C. It is possible that the presence of *trans*-stilbene in the vesicles shifts the phase transition. It is also possible that the phase transition we observe is slightly higher than the literature value ($T_c = 41$°C) due to impurities in the DPL.

CONCLUSIONS

The oxygen quenching results and the *trans*-stilbene fluorescence study are consistent with the model of bilayer behavior through and above the phase transition (Papahadjopoulos and Kimelberg, 1973). Fluidity, as suggested by the *trans*-stilbene fluorescence study, increases above the phase transition. In general, diffusion increases with bilayer fluidity. Therefore, it is reasonable to link the oxygen quenching behavior to oxygen diffusion in the bilayer. Below the phase transition, both oxygen diffusion and fluidity are reduced.

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Table 1. Oxygen quenching of PBA in lipid vesicles and water

DPL T	$C \times 10^{-6}$ s^{-1} atm^{-1}	Egg lecithin T	$C \times 10^{-6}$ s^{-1} atm^{-1}	Water T	$C \times 10^{-6}$ s^{-1} atm^{-1}
55°	32 ± 4	38°	27 ± 4	65°	21 ± 3
50°	29 ± 6	27°	16 ± 4	25°	15 ± 4
45°	20 ± 2	16°	17 ± 4	5°	9 ± 4
40°	11 ± 2	5°	11 ± 2		
35°	9 ± 2				
30°	10 ± 2				

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