USE OF FLUORESCENT PROBES THAT FORM INTRAMOLECULAR EXCIMERS TO MONITOR STRUCTURAL CHANGES IN MODEL AND BIOLOGICAL MEMBRANES

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ABSTRACT 1,3-dipyrenylpropane (PC_3P) and *bis*(4-biphenylmethyl)ether, two molecules that form intramolecular excimers, were embedded in phospholipid vesicles and biological membranes to monitor dynamic properties of membrane lipids. Excimer formation was evaluated from determinations of excimer to monomer emission intensity ratios (I_D/I_M). I_D/I_M values of PC_3P and bis(4-biphenylmethyl)ether were reduced when cholesterol was added to egg lecithin vesicles. PC_3P was sensitive to the temperature-induced crystalline to liquid-crystalline phase transition in dimyristoyl phosphatidylcholine vesicles. For studies of cellular membranes, PC_3P was used exclusively, because the fluorescence of tryptophan residues of membrane proteins interferes with the responses of *bis*(4-biphenylmethyl)ether. Microviscosities of membrane interiors were calculated from standard curves of I_M/I_D plotted against solvent viscosity. Microviscosity values of egg lecithin vesicles and biological membranes, especially those obtained with PC_3P, were more than an order of magnitude lower than values obtained by other techniques. We concluded that the intramolecular process leading to the formation of the excimer is influenced differently in isotropic solvents than in anisotropic environments, such as lipid bilayers. Although distinguishable I_D/I_M ratios can be obtained for different biological membranes (mitochondrial, microsomal, and plasma membranes were studied), this parameter may be phenomenological and not simply related to membrane microviscosity. As such, fluorescent probes that form intramolecular excimers are of value in making qualitative comparisons of different membranes and in studying the relative effects of physical changes and chemical agents on membrane structure. These probes may also be valuable for studying structural anisotropy of biological membranes.

INTRODUCTION

Fluorescent probes have been widely employed for studying the physical properties of lipid bilayer membranes. Much information has been obtained by sensitive fluorescent techniques,

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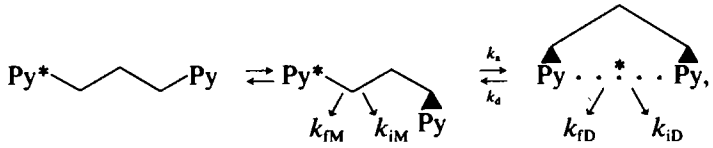
but the limitations of molecular probes embedded in membranes must be realized. Fluorescence polarization, a technique sensitive to the composition of the bilayer (1–3), has revealed phase transitions in phospholipid vesicles (4–7) and microviscosities of complex biological membranes (8, 9). Quantitative estimates of membrane microviscosities by steady-state fluorescence depolarization have, however, been criticized (10–14), because rotational motion of fluorophores, such as 1,6-diphenyl-1,3,5-hexatriene (DPH),[1] is not equivalent in isotropic solvents and in anisotropic lipid bilayers.

Another fluorescent probe approach involves the formation of intermolecular excimers by the association of an aromatic molecule in the first excited singlet state with an unexcited molecule of the same species (15). Birks et al. (16) have shown that the formation of intermolecular excimers is a diffusion-controlled process in a variety of solvents. It is, therefore, viscosity and concentration dependent. The rate of excimer formation competes with fluorescence from excited monomer molecules. A broad structureless emission, red-shifted from the monomer emission, is attributed to the dimer association complex. Pyrene excimer fluorescence has been used to evaluate the microviscosities of the hydrocarbon interiors of lipid micelles (17), phospholipid vesicles (18, 19), and biological membranes (19, 20). However, because of possible heterogeneous distribution of chromophore molecules in the membranes, the technique is of limited value for calculating and providing effective concentrations of the probe. These limitations can be minimized with fluorescent probes that form intramolecular excimers.

In 1965, Hirayama (21) reported that 1,3-diphenylpropane exhibits both monomer and excimer emissions and that the ratio of excimer emission to monomer emission is independent of dilution. This was the first reported observation of intramolecular excimer fluorescence. As in the intermolecular process, the rate of formation of intramolecular excimers competes with monomer fluorescence. The formation of intramolecular excimers, which is thermally activated, involves a conformational transition leading to the stable "sandwich" conformation between excited and unexcited aromatic moieties (22, 23). The conformational transition leading to the formation of an intramolecular excimer of 1,3-dipyrenylpropane (PC_3P) can be schematically depicted as



where Py represents pyrene in the ground state, Py* is pyrene in the excited state, and k_{fM}, k_{iM}, k_{fD}, k_{iD}, k_a, and k_d are rate constants for monomer fluorescence, monomer internal quenching, excimer fluorescence, excimer internal quenching, excimer formation, and excimer dissociation, respectively. The ratio of quantum yields of excimer fluorescence, ϕ_D, to monomer fluorescence, ϕ_M, is related to these rate constants (24) by

[1] *Abbreviations used in this paper:* PC_3P, 1,3-dipyrenylpropane; DPH, 1,6-diphenyl-1,3,5-hexatriene; BSA, bovine serum albumin; THF, tetrahydrofuran; DMPC, 1,2-dimyristoyl phosphatidylcholine; DPDL, 1,2-*bis*[ω-(1-pyreno) decanoyl]-*sn*-glycero-3-phosphorylcholine; I_M, corrected intensity of monomer emission; I_D, corrected intensity of excimer emission.

$$\frac{\phi_D}{\phi_M} = \frac{I_D}{I_M} \cdot x = \alpha \left(\frac{k_a}{k_{fM}}\right)\left(1 + \frac{k_{iD} + k_d}{k_{fD}}\right)^{-1}, \quad (1)$$

where I_D and I_M are intensities of excimer and monomer emission (peak heights), respectively, x is a proportionality constant, and α is the fraction of PC_3P that exists in the conformation from which the excimer can be formed by a single hindered rotation. Because k_{fM} and k_{fD} have generally been found to be independent of temperature (16, 25, 26), the change in log (I_D/I_M) with temperature can be used to determine the activation energy of the conformational transition leading to the formation of the excimer (k_a), providing $(k_{iD} + k_d)/k_{fD} \ll 1$ (27). Cundall and Robinson (28), however, have provided data for benzene in cyclohexane that suggests that, whereas k_{fM} is independent of temperature, k_{fD} increases with increasing temperature. This means that apparent activation energies of k_a determined from plots of log (I_D/I_M) against $1/T$ are slightly overestimated. At low temperatures, where log (I_D/I_M) changes linearly as a function of $1/T$, k_d is negligible (16, 26). According to Birks (25), at low temperatures (below T_{max}), k_{iD} is independent of temperature. The existence of an isoemissive point when temperature is varied indicates that k_{iD} is independent of temperature (22, 24, 26). Isoemissive points have been observed for a number of fluorescent compounds (22, 24) and polymer films and solutions (26) that form intramolecular excimers at low temperatures ($\leq -50°C$). Aqueous dispersions of lipid vesicles, however, cannot be cooled to such temperatures to allow the observation of an isoemissive point. Plots of log (I_D/I_M) against $1/T$ for molecules forming intramolecular excimers have been observed (24, 26) to be linear at temperatures in the range of the isoemissive point and to continue linearly well above the range of the isoemissive point. This suggests that the temperature dependence of I_D/I_M in these ranges are mainly due to a temperature effect on k_a and that the contribution of the temperature dependence of k_{iD} on excimer emission is small in comparison (24). Thus, apparent activation energies for k_a have been calculated from the linear slopes of plots of log (I_D/I_M) against $1/T$ (24, 26, 27, 29).

Goldenberg et al. (24) have demonstrated that the conformational transition necessary for excimer formation depends on the viscous resistance of the solvent. When viscosities are >10 cp, the activation energy for formation of intramolecular excimers approaches the activation energy for viscous flow. Recently, fluorescent probes that form intramolecular excimers have been used to evaluate the microviscosities of aqueous micelles (29–31).

It was our objective to determine whether fluorescent probes that form intramolecular excimers could be used to evaluate the fluidity of lipid bilayer interiors. The major advantage of intramolecular excimer formation compared with intermolecular excimer formation in evaluating physical properties of lipid bilayers is that the former technique is concentration independent. Consequently, lower concentrations of probe are necessary for the detection of excimer emission. This minimizes probe-induced membrane perturbations. For these studies we used the excimer-forming molecules PC_3P and *bis*(4-biphenylmethyl)ether, and we report the usefulness of these probes for studying structural properties of bilayers of phospholipid vesicles and biological membranes. A pyrene derivative was chosen because it fluoresces at wavelengths well above those of the tryptophan residues of membrane proteins. It also has a high quantum yield of excimer fluorescence ($\phi = 0.61$ in methylcyclohexane at room temperature [30]), thereby permitting the use of very small quantities. Also, pyrene is essentially insoluble in water, which reduces interference from unbound probe.

METHODS

Synthesis of PC_3P

Acetyl pyrene was synthesized by a Friedel-Crafts acylation of pyrene (32). Pyrene chalcone (1-pyrenoyl, 2-pyrenylethylene) was synthesized by a cross-carbanion condensation of pyrene carboxaldehyde with acetyl pyrene in ethanol in the presence of catalytic amoungs of NaOH. This step was similar to the synthesis of 1-naphthoyl-2-naphthylethylene as described by Chandross and Dempster (22). Our product, the chalcone precipitate, was filtered and recrystallized from benzene-chloroform (1/1, vol/vol) (melting point [mp]: 225°C [IR]: 1,660, 1,590 cm^{-1}). The chalcone was reduced to pyrenyl-2-pyrenylethyl ketone over Pd-charcoal in tetrahydrofuran (THF). The product of this hydrogenation showed one spot on a thin-layer chromatograph (mp: 174–177°C, IR: 1,600 cm^{-1}). The saturated ketone was reduced to PC_3P by the Huang-Minlon modification of the Wolff-Kishner reduction (33). Recrystallization of the product in hot benzene-hexane (1/1, vol/vol) yielded pale yellow crystals and showed one spot on a thin-layer chromatograph (IR: no carbonyl, mp: 166°C [lit. mp: 163.5°C (34)]).

Synthesis of Bis(4-biphenylmethyl)ether

This compound was formed by the dimethyl sulfoxide–mediated dehydration of *p*-phenylbenzyl alcohol as described by Goldenberg et al. (24).

Membrane Preparations

Phospholipid vesicles were prepared and separated by a procedure similar to that of Huang and Thompson (35). Lipid samples were homogenized in 0.1 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris)-base, pH 8.5, and then sonicated at full power for 2 h in a water-jacketed vessel under a nitrogen atmosphere with a 20-kc Branson Sonic Power Co. (Danbury, Conn.) sonifier. The water jacket was maintained at temperatures above the phase transition temperature of the lipid vesicles being formed. After centrifugation (105,000 *g* for 60 min) to remove titanium fragments and undispersed phospholipids, the sample was concentrated to ~6 ml by ultrafiltration with a PM-10 membrane (Amicon Corp., Lexington, Mass.). Descending gel filtration on Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, N.J.) of the concentrated vesicle dispersion yielded a homogeneous population of single bilayer vesicles. Negatively stained samples (36) of the vesicle fraction were examined in a Hitachi Ltd. (Tokyo) XI electron microscope. Phospholipid concentrations were calculated from inorganic phosphate contents (37). Cholesterol contents were determined with the ferric sulfuric reagent (38).

Mitochondria were isolated from the livers of 200-g inbred male albino rats of the CDF strain (Charles River Breeding Laboratories, Wilmington, Mass.) by differential centrifugation (39) in a medium of 300 mM sucrose, 1 mM ethylenediaminetetraacetate, 1 mM Tris-HCl, pH 7.6. Submitochondrial particles were prepared from freshly isolated mitochondria as previously described (40). Microsomal membranes were sedimented by centrifuging the postmitochondrial supernatant fraction at 20,000 *g* for 15 min and then centrifuging the resulting supernatant at 100,000 *g* for 60 min. Plasma membranes were isolated by the Ray modification of the Neville procedure (41). Membrane protein concentrations were determined by the biuret method (42), using bovine serum albumin (BSA) as a standard. Purity of mitochondrial, microsomal, and plasma membrane fractions were evaluated from measurements of the specific activities of succinate cytochrome *c* reductase (43), nicotinamide adenine dinucleotide phosphate cytochrome *c* reductase (43), and alkaline *p*-nitrophenyl phosphatase (41), respectively.

Labeling of Membranes

Bis(4-biphenylmethyl)ether was added from a stock solution of 2×10^{-2} M in ethanol to a cuvette containing phospholipid vesicles (1.23 mg phospholipid/ml) so that the final concentration of probe was 1×10^{-4} M. At these concentrations of probe and vesicle, the probability of double occupied vesicles is <3% (36, 44), thus contributions from intermolecular excimer emisssion are virtually eliminated. The probe was added to the phospholipid vesicle dispersions and heated at 65°C with stirring for 5 min.

PC_3P was added from a stock solution of 1×10^{-2} M in THF to phospholipid vesicle dispersions, so that the final concentration of probe was 1×10^{-5} M. In this case, the probability of doubly occupied vesicles is <0.3% (36, 44). The mixture was stirred overnight at ~10°C above the phase transition temperature of the phospholipid vesicles. Before measurement of emission spectra, the solution was filtered through a 0.22-μm Millipore filter. When PC_3P was incorporated into phospholipid vesicles, excimer was formed by an intramolecular process, since the ratio of excimer to monomer fluorescence intensity (I_D/I_M) was independent of the concentration of phospholipid vesicles.

Mitochondrial, microsomal, and plasma membrane fractions (1 mg protein/ml) were incubated individually with PC_3P (1×10^{-5} M final concentration) overnight at 4°C. Treated membranes were separated from unincorporated probe molecules by centrifugation at 100,000 *g* for 60 min on a discontinuous sucrose gradient (sample in appropriate buffer, 15% [wt/wt] sucrose, 45% [wt/wt] sucrose). Membrane fractions were collected from the interface of the sucrose solutions.

Fluorescence Measurements

All fluorescence spectra were taken on a Perkin-Elmer Corp. (Norwalk, Conn.) MPF-2A fluorescence spectrometer equipped with a thermostated sample compartment. The temperature was regulated to ±0.3°C with an FTS Systems Inc. (Stone Ridge, N.Y.) Flexicool system, and dry nitrogen was passed over the cell to prevent condensation. The temperature was monitored with a YSI thermistor (tele-thermometer system, Yellow Springs Instrument Co., Yellow Springs, Ohio.) immersed in a separate cuvette. Samples were allowed to equilibrate for at least 15 min at each temperature before fluorescence spectra were recorded. All samples were degassed by bubbling prepurified nitrogen (Airco Industrial Gasses, Airco Inc., Murray Hill, N.J., 99.995%) through the solution for at least 30 min. PC_3P was excited at 333 nm, and excimer/monomer intensity ratios (I_D/I_M) were calculated by comparing the fluorescence intensity at 480 nm to that at 395 nm. *bis* (4-Biphenylmethyl)ether was excited at 270 nm, and I_D/I_M values were calculated by comparing the fluorescence intensity at 380 nm to that at 320 nm. Excitation and emission slit widths were always set at 4 nm. For all calculations of I_D and I_M, corrections were made for contribution of monomer emission at the excimer λ_{max}, and for contribtuion of excimer emission at the monomer λ_{max}. The following equations were used to calculate the corrected ratios of excimer to monomer emissions of PC_3P and of *bis*(4-biphenylmethyl)ether, respectively:

$$\left(\frac{I_D}{I_M}\right)'_{\text{corrected}} = \frac{I_D - (0.0166\, I_M)}{I_M - (0.0156\, I_D)} \tag{2}$$

$$\left(\frac{I_D}{I_M}\right)'_{\text{corrected}} = \frac{I_D - (0.0174\, I_M)}{I_M - (0.0534\, I_D)}. \tag{3}$$

Viscosity Determinations

Viscosities of reference solvents were determined with a series of calibrated Ubbelhode capillary viscometers immersed in a thermostated water bath at 20°C.

Materials

Pyrene (99+% purity), 1-pyrene carboxaldehyde (98+% purity), spectrograde butanol, spectrograde cyclohexane, and THF (gold label, distilled over $LiAlH_4$) were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis. Hexane (high pressure liquid chromatography grade, distilled over CaH_2) was obtained from Fisher Scientific Co., Pittsburgh, Pa. Spectrograde methylcyclohexane and *p*-pentyl *p*-propylbenzoate were obtained from Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y. L-α Egg lecithin, L-α DMPC, BSA, and cholesterol were purchased from Sigma Chemical Co., St. Louis, Mo. Absolute ethanol and *n*-propanol were fractionally distilled over Mg/I_2 to yield fluorescent-free solvent. Fluorescent-free ethylene glycol and glycerol were obtained by treatment with activated carbon (24). All other reagents were of the highest purity commercially available.

RESULTS

A plot of log (I_D/I_M) against $1/T$ of PC_3P in ethanol is shown in Fig. 1. As the temperature is increased, log (I_D/I_M) tends to a maximum (T_{max} = 60°C). Below 0°C, log (I_D/I_M) changes linearly as a function of $1/T$ and depends on the viscosity of ethanol.

I_D/I_M is independent of concentration for molecules that form intramolecular excimers. This characteristic of PC_3P is illustrated in Fig. 2, where emission spectra of pyrene are compared with emission spectra of PC_3P. In Fig. 2 *A*, the spectra have been normalized to the band at 380 nm. As the concentration of pyrene is decreased from 10^{-3} to 10^{-5} M, the broad excimer band at 470 nm nearly totally disappears. However, when PC_3P is diluted from 10^{-5} to 10^{-6} M, the ratio of I_D/I_M remains constant (Fig. 2 *B*), indicating that excimer is formed by an intramolecular process.

A plot of I_M/I_D vs. viscosity for PC_3P dissolved in various solvents and solvent mixtures at 20°C is shown in Fig. 3. At viscosities >10 cp, I_M/I_D increases linearly with solvent viscosity. At viscosities <10 cp, the rate of conformational transition necessary for excimer formation is

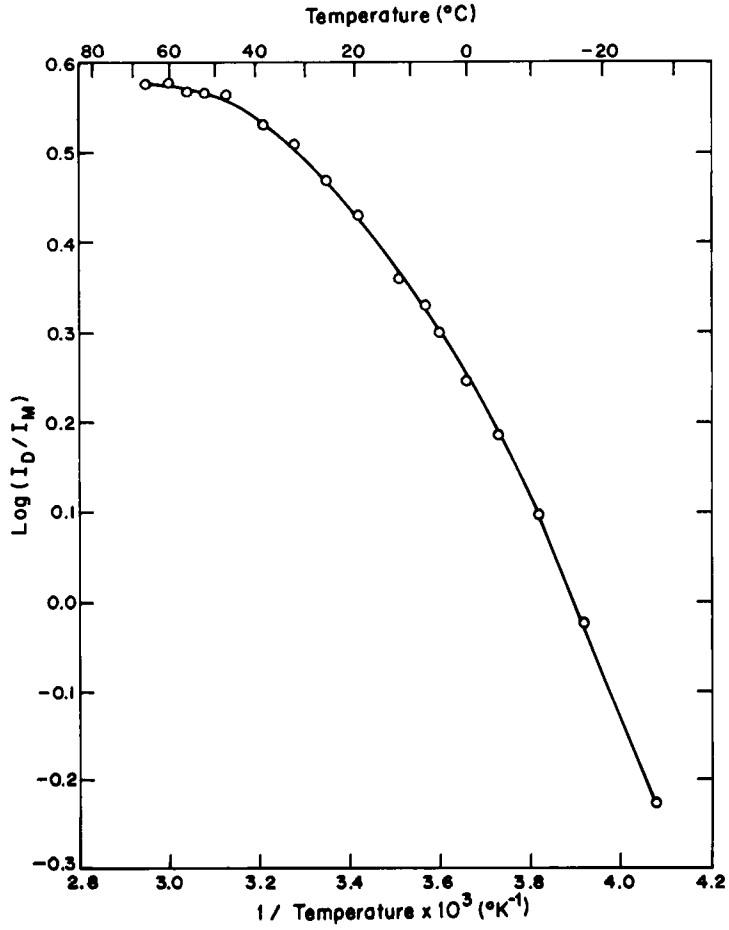


FIGURE 1 Log (I_D/I_M) against $1/T$ for PC_3P (10^{-5} M) in ethanol.

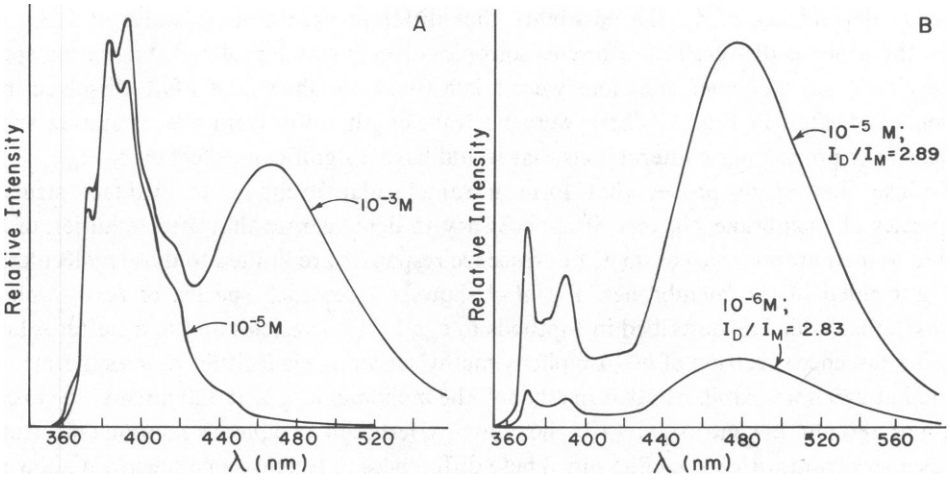


FIGURE 2 Fluorescence spectra of (*A*) pyrene and (*B*) PC_3P in methylcyclohexane at 20°C. Spectra of pyrene were normalized to the band at 380 nm. Concentrations of probes are indicated above each spectrum.

not proportional to solvent viscosity. According to Birks (25), the rate parameters of molecular fluorescence (k_{fM} and k_{fD}) are independent of the solvent except for the influence of the refractive index. Differences in refractive index, however, do not account for the nonlinearity of Fig. 3, since a correlation between I_M/I_D and the refractive indices of those solvents is lacking. Avouris et al. (45) also observed a similar curvature at low viscosities for 1, 3-dinaphthylpropane in ethanol-glycerol mixtures. Although Fig. 3 largely reflects the

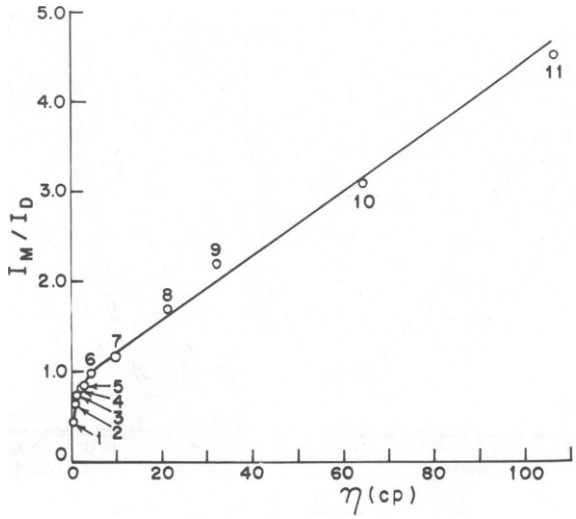


FIGURE 3 I_M/I_D vs. viscosity for PC_3P (10^{-5} M) at 20°C in (*1*) hexane, (*2*) cyclohexane, (*3*) ethanol, (*4*) *n*-propanol, (*5*) *n*-butanol, (*6*) 23/6.2 (vol/vol) ethanol/glycerol, (*7*) 19/10 (vol/vol) ethanol/glycerol, (*8*) 16/13 (vol/vol) ethanol/glycerol, (*9*) 14/15 (vol/vol) ethanol/glycerol, (*10*) 11/20 (vol/vol) ethanol/glycerol, (*11*) 9/20 (vol/vol) ethanol/glycerol.

viscosity dependence of k_a, the possibility that different preexcimeric states of PC_3P exist when the probe is dissolved in different isotropic solvents was considered. Excitation spectra for excimer and monomer emissions were taken (data not shown) of PC_3P dissolved in the solvents indicated in Fig. 3. There were no wavelength shifts from the excitation spectra indicative of preexcimeric interactions that would have a significant effect on I_M/I_D.

To use fluorescent probes that form intramolecular excimers to evaluate structural properties of membrane bilayers, it is necessary to demonstrate that after addition of such probes to membranous suspensions, fluorescence responses are limited to those molecules that are embedded in the membranes. Fig. 4 *A* shows fluorescence spectra of *bis*(4-biphenylmethyl)ether added as described in Methods to egg lecithin vesicles and to a buffer solution. The fluorescence spectrum of *bis*(4-biphenylmethyl)ether in egg lecithin vesicles (spectrum 2) excited at 270 nm is similar to that in ethanol; the monomer λ_{max} is at 320 nm and the excimer λ_{max} is at 380 nm. In contrast, *bis*(4-biphenylmethyl)ether in an aqueous solution (spectrum 1) yields a spectrum with λ_{max} at 335 nm. These differences in fluorescence spectrum allow us to specify the location of the probe. The lack of a band or shoulder at 335 nm in the spectrum of *bis*(4-biphenylmethyl)ether in egg lecithin vesicles indicates that in our preparations there is no fluorescence contribution of the probe from the aqueous phase, i.e., *bis*(4-biphenylmethyl)ether partitions almost exclusively into the bilayer hydrocarbon phase. The peak at 335 nm was not characterized, but we attribute it to a complex aggregate of the probe formed in an aqueous medium.

The addition of PC_3P to an aqueous solution yields a large excimer emission at 480 nm (Fig. 4 *B*, spectrum 1). The probe can be removed from this solution by Millipore filtration (0.22 μm), as shown by the complete absence of fluorescence in the filtrate (Fig. 4 *B*, spectrum 4). The addition of PC_3P to egg lecithin vesicles, as described in Methods, yields a

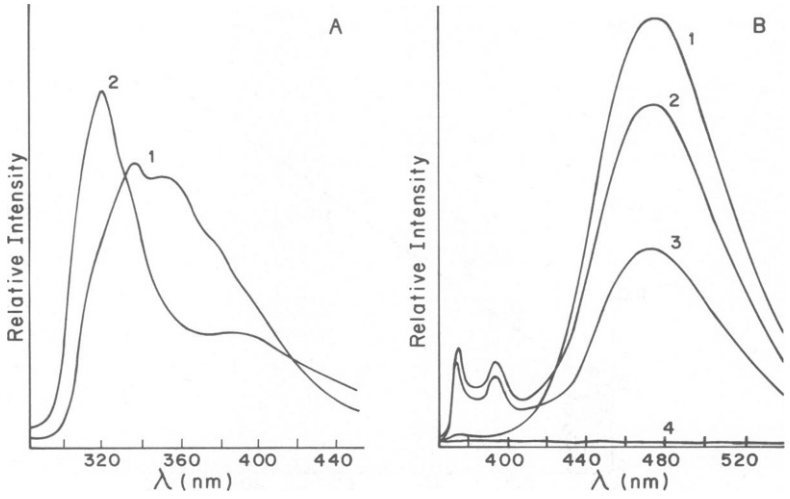


FIGURE 4 Fluorescence spectra of (*A*) bis(4-biphenylmethyl)ether and (*B*) PC_3P in egg lecithin vesicles and in 0.1 M NaCl, 0.01 M Tris-base, pH 8.5, buffer. In *A* and *B*, spectrum 1 is of the probe in buffer and spectrum 2 is of the probe added to the dispersion of egg lecithin vesicles. In *B*, spectrum 3 is of the egg lecithin vesicle dispersion plus PC_3P after Millipore filtration and spectrum 4 is of PC_3P added to buffer and Millipore filtered. For details see Methods.

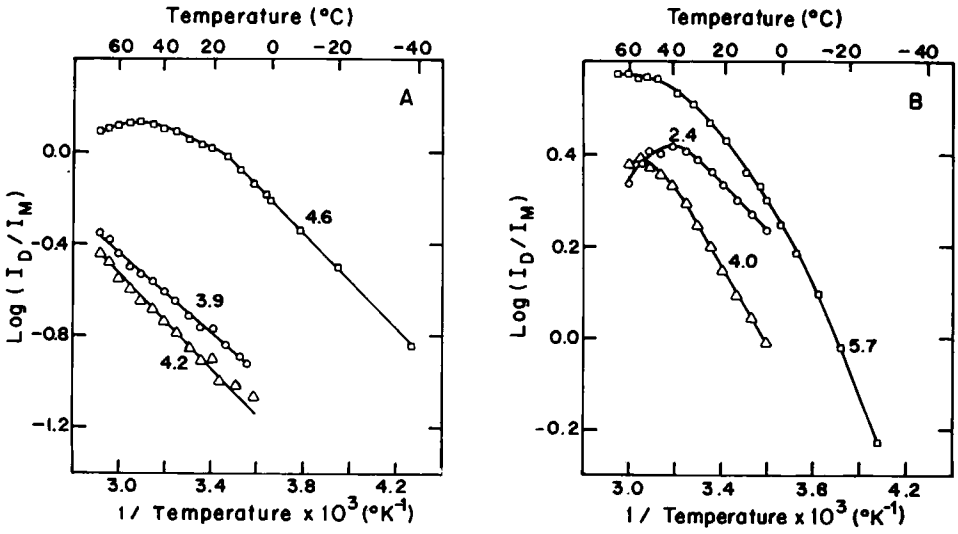


FIGURE 5 Log (I_D/I_M) against $1/T$ for (*A*) *bis*(4-biphenylmethyl)ether and (*B*) PC_3P in (○) egg lecithin vesicles and in (△) egg lecithin-cholesterol vesicles with a lecithin to cholesterol molar ratio of 2. Plots of both probes in (□) ethanol are provided for comparison. The numbers by the plots are the calculated apparent activation energy values in kcal/mol for the intramolecular process leading to the formation of excimers. Apparent activation energy values were calculated from the region of the plots where log (I_D/I_M) changes linearly as a function of $1/T$. Straight lines of best fit were drawn after a least-squares linear regression of the data was performed. For labeling procedures, see Methods.

spectrum (spectrum 2) similar to that of the probe in ethanol. A single filtration of this mixture is sufficient to achieve an emission spectrum (spectrum 3) of PC_3P embedded in the phospholipid bilayer. Subsequent filtrations, even 7 h after the first filtration, altered neither the fluorescence spectrum nor the I_D/I_M ratios. This finding demonstrates that PC_3P is well retained in the membrane.

Plots of log (I_D/I_M) against $1/T$ of *bis*(4-biphenylmethyl)ether and PC_3P embedded in egg lecithin vesicles with and without cholesterol are presented in Fig. 5. Plots of both probes in ethanol are provided for comparison. Log (I_D/I_M) changes linearly as a function of $1/T$ to ~35°C for PC_3P and to >60°C for *bis*(4-biphenylmethyl)ether embedded in these vesicle dispersions. Thus, the temperature range in which k_a governs the excimer yield (and k_d is negligible) is substantially extended for these probes in lipid bilayers, compared with that in ethanol. Goldenberg et al. (24) observed that T_{max} values for *bis*(4-biphenylmethyl)ether and dibenzyl ether are higher when solvent viscosity is increased. Apparent activation energy values were greater for both probes in vesicles enriched with cholesterol. These studies were performed at temperatures above the phase transition temperature of the egg lecithin vesicles, and therefore, the lower I_D/I_M values obtained for the cholesterol enriched vesicles may be explained by the probes' sensitivity to restricted motion of fatty acyl chains caused by the presence of cholesterol (46). Microviscosity values at 20°C for these vesicles were estimated from standard curves [for *bis*(4-biphenylmethyl)ether, reference 27; for PC_3P, Fig. 3] of I_M/I_D plotted against solvent viscosity. The estimated microviscosities of egg lecithin vesicles at 20°C were 15 cp without cholesterol and 24 cp with cholesterol, using *bis*(4-biphenylmethyl)ether, and only ~2 cp and 1 cp for the egg lecithin vesicles with and without

cholesterol, using PC_3P. The low estimated microviscosities, especially those obtained with the pyrenyl probe, reflect high excimer emission intensities, compared with monomer emission intensities. It is possible that I_D/I_M values are not equivalent in solvents and in anisotropic environments such as that of lipid bilayers (see Discussion).

A plot of log (I_D/I_M) against $1/T$ of PC_3P embedded in dimyristoyl phosphatidylcholine (DMPC) vesicles is presented in Fig. 6. A discontinuity similar to that reported by other techniques that monitor this crystalline to liquid-crystalline transition (7, 18, 47, 48) is observed at ~24°C. Thus, intramolecular excimer fluorescence affords a technique that also can monitor such phase transitions.

Fig. 7 presents plots of log (I_D/I_M) against $1/T$ of PC_3P embedded in isolated rat liver mitochondrial, microsomal, and plasma membranes. *bis*(4-Biphenylmethyl)ether could not be used in these studies because of interference in the fluorescence by tryptophan residues of membrane proteins. The important features of this figure are (*a*) PC_3P can become incorporated into biological membranes and yield excimer fluorescence, (*b*) membranes of different physical and chemical properties can be distinguished by their excimer to monomer emission intensity ratios, and (*c*) phase transitions in these membranes are not observed by this technique. The estimated microviscosities of these membrane fractions at 20°C are 1 cp for microsomal membranes, 4 cp for mitochondrial membranes, and 26 cp for plasma membranes. These low values are due to the rather high (compared with solvent) I_D/I_M ratios (see Discussion). The differences in I_D/I_M ratio of these membranes may be related to their different lipid compositions or different phospholipid to protein ratios. To examine this possibility, we added PC_3P to a BSA solution (0.33 mg/ml). This mixture was incubated overnight at 4°C and then filtered through a 0.22-μm Millipore filter in a manner similar to that described in Methods for the preparation of probe-embedded phospholipid vesicles.

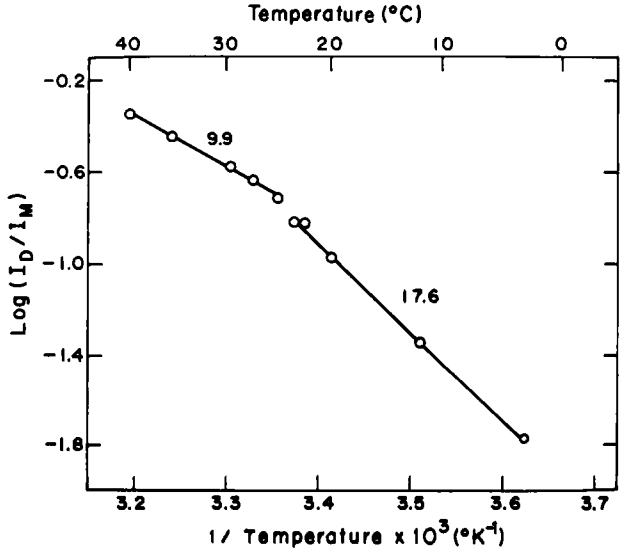


FIGURE 6 Log (I_D/I_M) against $1/T$ for PC_3P in DMPC vesicles. The numbers above the plots are apparent activation energy values in kcal/mol, as described in Fig. 5. For labeling procedures, see Methods.

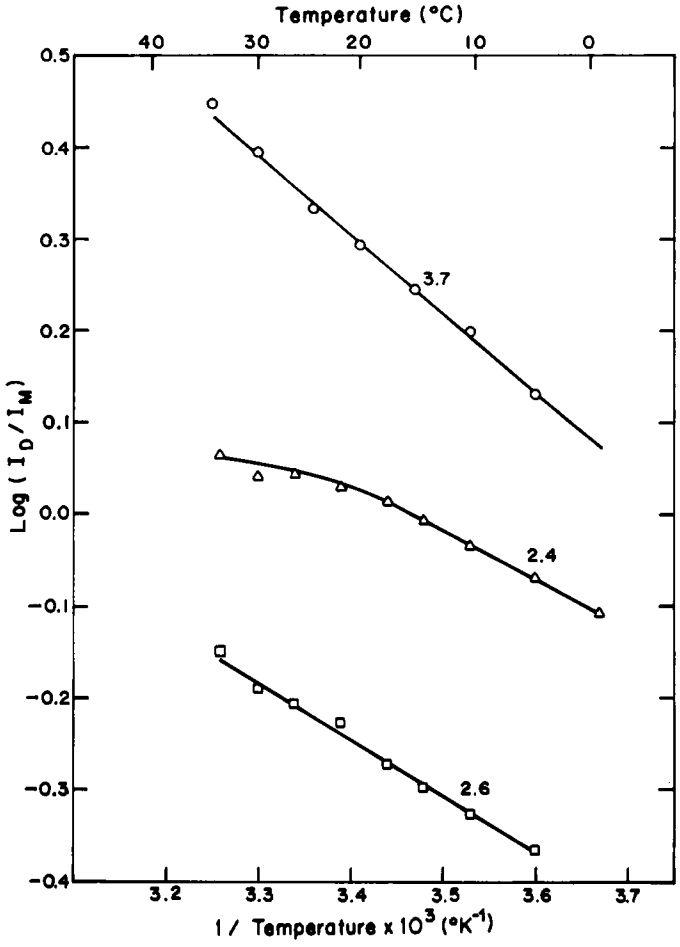


FIGURE 7 Log (I_D/I_M) against $1/T$ for PC_3P in (○) microsomal, (△) mitochondrial, and (□) plasma membranes. The numbers above the plots are apparent activation energy values in kcal/mol, as described in Fig. 5.

Monomer and excimer emissions of PC_3P were observed in this filtrate (results not shown), indicating that PC_3P is capable of interacting with BSA. Thus, some of the fluorescence response obtained with PC_3P embedded in biological membranes may arise from the probe associated with hydrophobic portions of membrane proteins.

Soutar et al. (18) observed that the I_D/I_M ratio of pyrene dissolved in the liquid crystal, *N*-(*p*-methoxybenzylidene)-*p*-butylanaline, decreased by a factor of 10 when the temperature was raised above 43°C, the temperature of the liquid-crystalline to isotropic phase transition. We similarly observed (Fig. 8) a decrease in the I_D/I_M ratio of PC_3P dissolved in *p*-pentylphenyl *p*-propylbenzoate as the temperature was raised to produce the isotropic phase. A further increase in temperature (above 13°C) led to an increase in I_D/I_M, as would be expected for the thermally activated formation of intramolecular excimers of PC_3P in an isotropic solvent. Soutar et al. (18) suggested that the liquid crystalline phase has an orienting effect on pyrene, producing an arrangement favorable to excimer formation. It is our

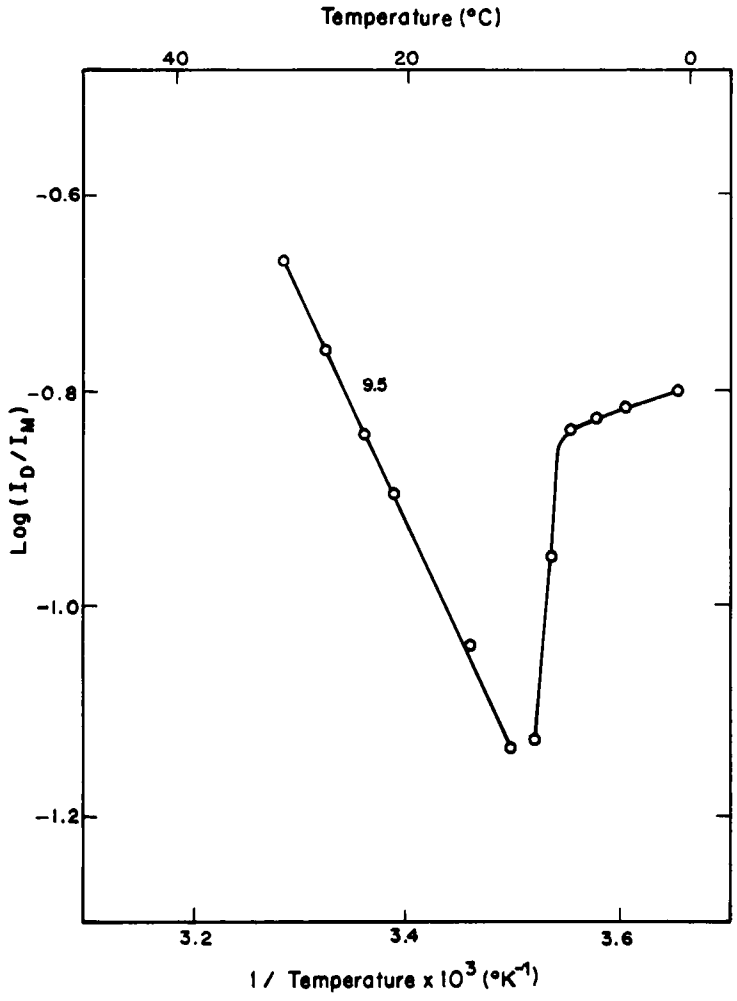


FIGURE 8 Log (I_D/I_M) against $1/T$ for PC_3P in *p*-pentylphenyl *p*-propyl benzoate.

interpretation that the changes in I_D/I_M that we observed are due to the influence of an anisotropic environment on the conformational equilibrium of PC_3P.

DISCUSSION

These studies demonstrate that lipophilic molecules that form intramolecular excimers become embedded in phospholipid vesicles and biological membranes and can be used as probes to monitor dynamic properties of membranes. Such probes are useful for studying membrane structural changes induced by physical or chemical means. PC_3P and *bis*(4-biphenylmethyl)ether, at low concentrations, can be used to monitor changes in phospholipid to cholesterol molar ratios, and PC_3P is sensitive to the temperature-induced crystalline to liquid-crystalline phase transition. PC_3P, unlike *bis*(4-biphenylmethyl)ether, was suitable for

studies of biological membranes. Sunamoto et al. (49) recently synthesized 1,2-*bis*[ω-(1-pyreno)decanoyl]-*sn*-glycero-3-phosphorylcholine (DPDL) and examined its emission from dipalmitoyl phosphatidylcholine bilayers. Although this probe forms intramolecular excimers, its response in lipid bilayers seems to be different from that of PC_3P. Enrichment of lipid bilayers with cholesterol resulted in higher I_D/I_M ratios with DPDL, whereas this ratio was reduced for PC_3P (Fig. 5) in cholesterol-enriched vesicles. Sunamoto et al. (49) suggest that I_D/I_M ratios of DPDL depend more on the probability of formation of "ground-state dimers" and on solvent polarity than on k_a.

The comparison of I_M/I_D values of PC_3P and *bis*(4-biphenylmethyl)ether embedded in egg lecithin vesicles (and of PC_3P in biological membranes) to I_M/I_D values of these probes in solvents of various macroscopic viscosities reveals unusually low "microviscosities" of membrane interiors. Such low "microviscosities" reflect high excimer emission intensities, compared with monomer emission intensities. One explanation for this is that the intramolecular process leading to the formation of the excimer is influenced differently by isotropic solvents and anisotropic environments such as that of lipid bilayers. Consequently, the conformational equilibrium of molecules that form intramolecular excimers, especially PC_3P, may be changed when they are solubilized by anisotropic lipid bilayers (i.e., conformational constraints may be imposed on these probes as a result of their incorporation into the bilayer), changing the value of α (see Eq. 1). α could increase if the conformations from which excimers cannot be formed by a single hindered rotation (i.e., the *trans-trans* form of PC_3P) are of low frequency when PC_3P is embedded in lipid bilayers. Such a change would increase the probability of excimer formation during the lifetime of the excited state. Another possible explanation is that when such probes are embedded in lipid bilayers, the structural anisotropy of this environment inhibits their achieving a random orientation. Consequently, they may be responding to a single membrane viscosity component, possibly one parallel to the orientation of the fatty acyl chains.

Apparent activation energies for the formation of intramolecular excimers were found to be lower in lipid bilayers than in ethanol, which indicates that energy barriers separating the ground state and excimer conformations are smaller in lipid bilayers. Possibly, the solubilization of a probe like PC_3P in a lipid bilayer induces a change in the ground state conformation, producing a transitional state in which the pyrenyl moieties are present in a nearly eclipsed orientation. Thus, formation of an excimer from this state could easily arise via a partially hindered rotation that is less than the rotation necessary for the *trans* to *gauche* isomerization of the uncombined probe.

The observation that the I_D/I_M ratio of PC_3P in the liquid crystal, *p*-pentylphenyl *p*-propylbenzoate, decreases as the temperature is raised to affect the transition from a liquid-crystalline to an isotropic phase supports the contention that structural properties, as well as solvent viscosity, can influence the relative intensities of excimer and monomer emission. In addition, I_D/I_M ratios were much lower for PC_3P in the liquid crystal than in egg lecithin vesicles (Fig. 5) or in biological membranes (Fig. 7). In the liquid crystal, a continuous environment exists in which PC_3P is solubilized. However, in lipid bilayers, incorporation of PC_3P is driven largely by its insolubility in the aqueous environment. To avoid exposure at the polar domains of the bilayer, PC_3P may assume a condensed conformation; one that has an increased probability of forming pyrenyl dimers. Thus, the

thickness of the hydrophobic domain of the membrane may also influence the I_D/I_M ratios of PC_3P.

If the ground-state conformational equilibrium of PC_3P changes in lipid bilayers, then I_D/I_M values are only phenomenological parameters of membranes. Thus, the technique involving fluorescent probes that form intramolecular excimers is inadequate for determining the real "microviscosities" of membrane interiors. The technique may, however, be suitable for monitoring structural changes in membranes. It is not too surprising that the anisotropic environment of the lipid bilayer would influence the conformational equilibrium or orientation of probes that form intramolecular excimers, inasmuch as it has been demonstrated that rotational motions of DPH and spin labels are restricted in lipid bilayers (10–12, 50). Structural anisotropy may influence the formation of intramolecular excimers less in micellar interiors than in phospholipid vesicles, inasmuch as the microviscosities of sodium dodecyl sulfate and cetyltrimethylammonium bromide micelles are similar when determined by fluorescence depolarization (51, 52) and intramolecular excimer fluorescence (29–31).

It is not appropriate to calculate a "microviscosity" value for DMPC vesicles with the standard curve of Fig. 3, since the latter data were obtained at a temperature at which the lipids of these vesicles are in the crystalline phase. However, above the phase transition temperature of these vesicles, I_D/I_M values are much lower than I_D/I_M values of egg lecithin vesicles. The fatty acyl chains of egg lecithin are longer than those of DMPC, and they are nearly 50% unsaturated (53). These compositional differences may influence conformational states of PC_3P embedded in the bilayer. We are examining the effects of chain length and unsaturation on monomer and excimer emissions of PC_3P.

Excimer to monomer emission intensity ratios of PC_3P were different for this probe embedded in isolated plasma, mitochondrial, and microsomal membranes. I_D/I_M ratios were readily distinguishable for the various membrane fractions studied and followed the order microsomal > mitochondrial > plasma membrane. Although the exact nature of these differences is not known, they are probably related to membrane compositional differences, including fatty acid composition, polar head group composition, sterol content, and protein to lipid ratios. These characteristics have been shown to influence properties of membrane lipids (2, 3, 54). Binding of PC_3P to hydrophobic portions of membrane proteins may also account for some of the differences in the I_D/I_M ratio of these membranes.

Fluorescence polarization measurement of DPH embedded in biological membranes has been a popular approach to the study of the fluid properties of membrane interiors and the comparison of membranes in different physiological states (8). Measurement of intramolecular excimer fluorescence of PC_3P may have similar applications, and, therefore, it seems appropriate to evaluate the limitations and advantages of these two fluorescent probe techniques. The primary objective of the use of these techniques has been to quantitatively monitor the lipid fluidity of membranes. However, orientational constraints within the lipid bilayer appear to hinder rotational motion of DPH (10–14), resulting in overestimation of membrane "microviscosities," and solubilization of PC_3P in the membrane seems to influence its conformational equilibrium, resulting in underestimation of membrane "microviscosities." Thus, the usefulness of these probe techniques may be limited to the relative comparison of membranes and to the study of the effects of physical changes and chemical agents on the phenomenological parameters determined by the fluorimetric measurements.

Lentz et al. (55) have shown that the fluorescence anisotropy of DPH embedded in membranes is influenced by scattering depolarization caused by sample turbidity. Although individual corrections for scattering depolarization for each specific membrane can be made, such additional manipulations complicate the fluorescence polarization technique when applied to studies of biological membranes. Corrections for solutions that scatter and depolarize excitation and emitted light are not necessary with PC_3P embedded in membranes, since this technique is independent of the polarization phenomenon. It seems that, although both probe techniques are sensitive to phase transitions of lipid bilayers and changes in cholesterol to phospholipid ratios, for making relative comparisons of different membranes, PC_3P has the advantage of making data analysis simpler. In addition, although both of these probes are highly lipophilic, and, therefore, localize in the hydrophobic interiors of membranes, different relative responses may arise due to differences in the domains in which they become situated.

In comparison to the intermolecular excimer formation process for analyzing structural properties of different membrane systems, PC_3P has the advantage of concentration independence (Fig. 2). Because of this, excimer emission can be detected at much lower concentrations, and the relative excimer emissions from different types of membranes is independent of the level of probe uptake.

In conclusion, we should consider why calculated "microviscosities" of egg lecithin vesicles obtained with PC_3P were an order of magnitude lower than those obtained with bis(4-biphenylmethyl)ether. The rate of conformational transition from the *trans-gauche* state to the excimer state governs the excimer yield and is the viscosity-dependent rate constant (k_a) for the formation of intramolecular excimers (23, 24). The pyrene chromophore of PC_3P is planar and a good intercalating agent. Biphenyl, however, is nonplanar in its ground state. Therefore, when *bis*(4-biphenylmethyl)ether is solubilized in lipid bilayers, the conformations of the biphenyl moieties may impede the conformational transition of this probe to the excimer state. Therefore, we suggest that "microviscosity" values are also related to the degree of intercalation of chromophores in lipid bilayers. Because the lipid bilayer affects the conformational transitions of these probes differently, we expect that fluorescent probes that form intramolecular excimers will provide a new and powerful technique for studying structural anisotropy of biological membranes.

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