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Fluorescence Quenching in Model Membranes. 1. Characterization of Quenching Caused by a Spin-Labeled Phospholipid†

Erwin London‡ and Gerald W. Feigenson*

ABSTRACT: A new method is described to evaluate contact between fluorophors and lipids in model membranes. This method utilizes a nitroxide spin-labeled phosphatidylcholine to quench the fluorescence from a variety of membrane-bound molecules by a static process. The distance dependence of the fluorescence quenching is analyzed in terms of simple models. The analysis shows that quenching of diphenylhexatriene, *p*-terphenyl, and molecules containing tryptophan arises only from spin-labeled phospholipid that is in contact with the fluorophor.

Understanding of molecular interactions in membranes is still at a primitive level. Part of the problem has been that the classically useful procedure of systematically measuring binding constants heretofore has not been feasible in membranes. In this series of three papers, we report a new method, based upon the contact quenching of a membrane-bound fluorophor by a nitroxide spin-labeled lipid, which yields relative binding constants. After the method is characterized in this paper, the second and third papers relate results for the important problem of measuring phospholipid binding to an integral membrane protein.

Our method is based upon literature reports which suggest that the nitroxide free radical quenches fluorophors upon "contact". It is necessary to establish certain details of such quenching in membranes to enable a quantitative binding analysis. In solution, where a collisional process is dominant (Green et al., 1973), the rate of fluorescence quenching of a variety of organic fluorophors by a nitroxide is rapid at fluorophor–quencher separation of $\leq$5–7 Å (Atik & Singer, 1978). Nitroxide fluorescence quenching within micelles is primarily static rather than collisional (Atik et al., 1979), with an indetermined distance dependence. It is expected that quenching within membranes also would be static, but only descriptive information is available, for example, for spin-labeled fatty acid quenching of erythrocyte protein tryptophanyl fluorescence (Bieri & Wallach, 1975) and spin-labeled

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phospholipid quenching of Ca^{2+}ATPase tryptophanyl fluorescence (London & Feigenson, 1978a).

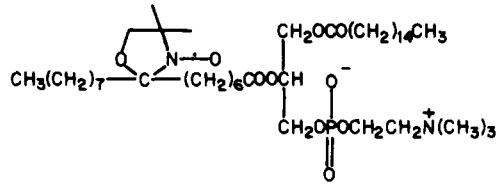
In this communication, we describe the static nature and characterize the distance dependence of quenching of fluorophors in membranes by a spin-labeled phospholipid. In addition, since some of the measurements involve bilayers composed entirely of spin-labeled phospholipid, some relevant physical characteristics of these bilayer vesicles are described.

Materials and Methods

DMPC[1] (1,2-dimyristoyl-*sn*-glycero-3-phosphocholine), cholic acid, Tris, gramicidin A′, DL-tryptophan octyl ester, Sephadex G-50, *Crotalus adamanteus* venom, GPC, oleic acid, and Sepharose 4B-CL were obtained from Sigma. Diphenylhexatriene (DPH), *p*-terphenyl, and tempol (4-hydroxy-2,2,6,6-tetramethylpiperidinyl-1-oxy) were obtained from Aldrich. DLPC and DPPC were obtained from Calbiochem. Pyrene was from Eastman. [^{14}C]Sucrose (20 μCi/μmol) was obtained from ICN. Analytical thin-layer chromatography (TLC) was performed on Adsorbosil-5-P plates (Applied Science) and preparative TLC on silica gel HF-254 (Merck). All other commercially obtained chemicals were reagent grade.

The Ca^{2+}ATPase was purified according to MacLennan (MacLennan, 1970). DOPC was synthesized by acylation of GPC with oleic anhydride (Robles & Van den Berg, 1969) and purified by TLC in chloroform/methanol/water (65:25:4 v/v). Egg PC was isolated from fresh egg yolks (Singleton et al., 1965).

Spin-labeled phospholipid was prepared with a nitroxide-containing moiety on the eighth carbon of the 2-position fatty acyl group. The spin-labeled fatty acid referred to as I-(7,6)-palmitic acid was synthesized by the method of Hubbell & McConnell (1971). Lyso-PC was prepared from egg PC (Chakrabarti & Khorana, 1975). Egg lyso-PC and I(7,6)-palmitic acid were condensed with CDI to form (7,6)PC (Boss



(7,6)PC

et al., 1975). The (7,6)PC was purified by TLC in chloroform/methanol/7.7 N ammonium hydroxide (90:104:11 v/v). (7,6)PC was stored in ethanol at −20 °C.

The PC's used were of ≥98% purity as estimated by TLC in chloroform/methanol/water (65:25:4 v/v). A phosphorus-sensitive spray (Dittmer & Lester, 1964) and subsequent charring were used to detect lipid on thin-layer chromatograms. The purity of cholic acid, subsequent to decolorization with charcoal, was tested by TLC in chloroform/acetone/acetic acid (70:10:10 v/v) and in ethyl acetate/acetic acid/water (85:10:5 v/v). In each solvent system, a single impurity with the mobility of deoxycholic acid was detected by charring and estimated to be 2–5% of the cholic acid. A stock stolution of 10% (w/v) cholic acid was adjusted to pH 8.1 with KOH. The purities of DPH, pyrene, *p*-terphenyl, gramicidin A′, and tryptophan octyl ester hydrochloride were checked with ^{1}H NMR. The chemical shift and spin–spin splitting patterns matched those of standard spectra (Sadtler Standard Spectra, 1969), and no impurity resonances could be detected.

With the exception of the Ca^{2+}ATPase, all fluorophors were incorporated in multilamellar phospholipid vesicles as follows. Stock solutions of tryptophan octyl ester hydrochloride, gramicidin A′, and pyrene in ethanol, DPH in chloroform, and *p*-terphenyl in acetone were prepared to 0.1 mM. Between 0.2 and 1.0 nmol of fluorophor was mixed with 125 nmol of phospholipid dissolved in chloroform or ethanol. A few drops of chloroform were added, and each sample was mixed vigorously. The samples were then dried under a flow of N_2 gas with gentle warming (35–45 °C). Samples were further dried under vacuum for 10 min. Either 2.5 mL of water or 2.5 mL of 20 mM Tris-HCl, pH 7.5 (for tryptophan octyl ester hydrochloride), was added to each sample. The samples were then vigorously mixed until a constant absorbance of 0.05–0.1 was reached at 360 nm (1-cm light path).

The Ca^{2+}ATPase was reconstituted by a cholate dilution method described in detail in the following paper (London & Feigenson, 1981). Unilamellar vesicles were prepared by sonication of aqueous phospholipid suspensions. Tubes containing phospholipid samples were flushed with N_2, sealed, and sonicated to clarity for 15 min to 1 h at 30–45 °C in a bath sonicator.

Fluorescence measurements were obtained by using a Perkin-Elmer MPF-3 spectrofluorimeter. The excitation and emission wavelengths used were 290/340 nm for gramicidin A′, tryptophan octyl ester, and *p*-terphenyl; 290/330 for Ca^{2+}ATPase; 358/430 for DPH; and 335/386 for pyrene (monomer). Nominal excitation and emission bandwidths of 10 nm were used. All fluorescence measurements were made while the sample was maintained at 23–24 °C, unless otherwise noted. Optical rotation measurements were obtained with a Perkin-Elmer 141 polarimeter. ^{1}H NMR spectra were obtained at 79.54 MHz on a Varian CFT-20 NMR spectrometer equipped for variable-temperature operation. Osmotic swelling studies were performed by using a Cary 14 spectrophotometer. Details of osmotic swelling, gel filtration, and phospholipase A_2 studies are given under Results and Discussion.

Results and Discussion

Chemical and Physical Properties of (7,6)PC. (7,6)PC exhibits about the same R_f as natural PC's when chromatographed by TLC. The molecular rotation, M_D, of (7,6)PC in ethanol is 58°, closely matching the value M_D = 59.5° measured for DMPC in ethanol, indicating that (7,6)PC is in the natural L-α form. The ESR spectrum of (7,6)PC exhibits the familiar three-line pattern at about the same magnetic field and with the same hyperfine splitting as other nitroxide-containing molecules such as tempol (Griffith & Waggoner, 1969). The absorbance spectrum of (7,6)PC in ethanol shows two peaks, one in the low ultraviolet [λ_{max} 235 nm (ϵ_{max} 2300)] and another in the visible [λ_{max} ~450 nm (ϵ_{max} ~5–10)]. These values are very close to those observed for other molecules containing nitroxide groups (Green et al., 1973) and indicate that there is an average of one free-radical function for each molecule.

The position of esterification of the spin-labeled fatty acid on the (7,6)PC was determined by phospholipase A_2 digestion. Under relatively mild conditions in which egg PC was digested completely into lyso-PC and fatty acid (Haverkate & Van

[1] Abbreviations used: DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; DLPC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; egg PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine derived from egg; GPC, *sn*-glycero-3-phosphocholine; CDI, *N′,N*-carbonyldiimidazole; P_i, inorganic orthophosphate; I(7,6)-palmitic acid, 2-(6-carboxyhexyl)-2-octyl-4,4-dimethyloxazolidinyl-3-oxy; (7,6)PC, 1-acyl-2-[I(7,6)-palmitoyl]-*sn*-glycero-3-phosphocholine.

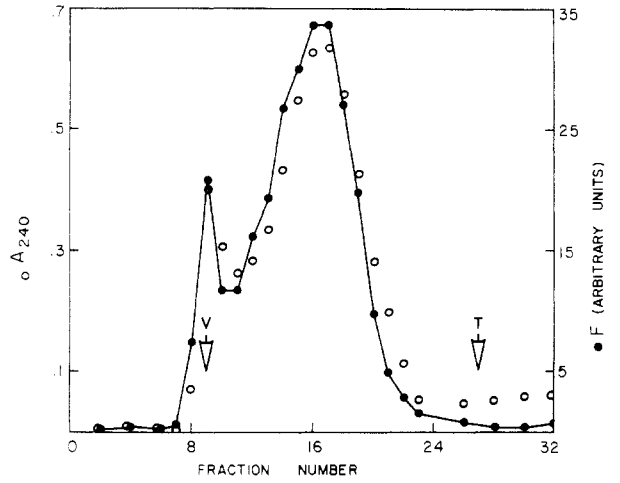


FIGURE 1: Sepharose 4B-CL fractionation of sonicated phospholipids. A 25 × 0.9 cm column bed was employed in a buffer containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 0.02% NaN_3. Fractions of 0.75 mL were collected. Arrow at V mark, void volume; arrow at T, total included volume. (○) Fractionation of 2 μmol of (7,6)PC. Nitroxide absorbance at 240 nm was measured after 75 μL of 10% cholate was added to each fraction to eliminate light scattering. (●) Fractionation of 2.5 μmol of DOPC. Phospholipid was assayed by DPH fluorescence enhancement (London & Feigenson, 1978b). The small shift in base-line optical density of the (7,6)PC vesicles profile was not reproducible.

Deenen, 1965), only a fraction of the (7,6)PC was hydrolyzed. (7,6)PC was hydrolyzed to >95% by modifying these mild conditions by using 50 × the standard concentration of *C. adamanteus* venom and digesting overnight at 37 °C in a tightly capped tube. Lyso-PC and fatty acid were separated by TLC in chloroform/methanol/water (65:25:4 v/v). Lyso-PC was extracted with chloroform/methanol/water (10:10:1 v/v) and then with ethanol/water (4:1 v/v), and fatty acid was extracted with chloroform/methanol/water (10:10:1 v/v). The intensity of the ESR signal, which is approximately proportional to the peak height times the square of the line width, was measured, and the percentage of the total spins in each fraction was calculated. The percentage of spin-label in each position was also measured by the ratio of the intensity of the ESR signal to chemically detected phosphorus (Bartlett 1959; Chen et al., 1956) in lyso-PC, and this ratio was compared to that for intact (7,6)PC. The results from both analyses showed that about 25% of the spin-labeled fatty acid is esterified to the 1 position rather than the 2 position. A similar result was observed after partial digestion using the milder conditions.

The ESR signal of an aqueous dispersion of (7,6)PC produced by hydration of dried (7,6)PC exhibits a single, broad resonance (linewidth ~32 G) in place of the three-line spectrum observed in organic solvent. This broad signal is characteristic of rapid spin exchange and dipole–dipole line broadening, which are expected for a dense local concentration of spins (Träuble & Sackmann, 1972). Sonication of this dispersion has virtually no effect on the intensity of the ESR signal, indicating that sonication does not destroy any of the nitroxide groups. Figure 1 illustrates the Sepharose 4B-CL gel filtration profile of sonicated DOPC and sonicated (7,6)PC. The profiles reflect very similar size distributions. Sonicated egg PC also exhibits a similar profile. The main peak is typical of sonicated unilamellar vesicles, preceded by a few percent of much larger liposomes or aggregated vesicles (Huang, 1969).

Trapping of ^{14}C-labeled sucrose in sonicated (7,6)PC was measured to determine the leakiness of these vesicles. Sonicated (7,6)PC vesicles (10 mM) were prepared in 18 mM [^{14}C]sucrose. After 10-fold dilution into the column buffer, the vesicles were chromatographed on a 15 × 2.5 cm Sephadex G-50 column. There was a peak of trapped sucrose in the void volume containing about 0.5% of total radioactivity. An aliquot of the void volume sample was rechromatographed on Sepharose 4B-CL as described above. Trapped sucrose was detected in both the void volume and the fractions containing small unilamellar vesicles. This confirms that sucrose is trapped in the small vesicles. The half-time of sucrose leakage was estimated to be 6–12 h.

Differential scanning calorimetry did not detect a transition in the range −50 to +50 °C for hydrated (7,6)PC. Thus, this lipid either has an undetectably broad transition or else remains in one phase over this temperature range. ^1H NMR and osmotic shrinking studies were performed to determine whether (7,6)PC bilayer vesicles are in the liquid-crystal or gel phase in the experimental range of temperatures.

In the gel state, due to ineffective averaging of magnetic dipole–dipole interactions, ^1H NMR resonances are extremely broad, as reflected in an apparent loss in signal intensity relative to the liquid-crystal state in the narrow frequency range detected in a high-resolution spectrometer (Sheetz & Chan, 1972). Figure 2B illustrates the temperature dependence of the intensity (measured as peak area) of the ^1H NMR spectra of sonicated DPPC, (7,6)PC/DPPC (1:10 w/w), and (7,6)PC. The phase transition detected for DPPC is 38.5 °C, which is in reasonable agreement with previous studies (Sheetz & Chan, 1972; Lentz et al., 1976). The inclusion of 9 wt % (7,6)PC shifted the apparent phase transition of DPPC no more than 2 °C toward a lower temperature. Pure (7,6)PC is seen to be in the liquid-crystal state in the range 18–70 °C. Partial loss of intensity in (7,6)PC at lower temperatures is an artifact of extreme paramagnetic line broadening by the nitroxide.

Osmotic shrinking studies were undertaken to confirm the ^1H NMR results. When multilamellar vesicles consisting of PC with 4% (w/w) PA are subjected to a hypertonic shock, osmotic shrinking can be measured as the rise in turbidity at 360 nm only if the PC is in the liquid-crystal state (Blok et al., 1976). In the gel state, there is no response to a hypertonic shock. The temperature dependence of the osmotic shrinking response is illustrated for several PC species in Figure 2A. The behavior of (7,6)PC is clearly the same as for DLPC and egg PC, and therefore this lipid also must be in the liquid-crystal state in this temperature range.

Fluorescence Quenching in Model Membranes Containing (7,6)PC. Figure 3A,B illustrates the fluorescence quenching of DPH, *p*-terphenyl, pyrene, tryptophan octyl ester, and the Ca^{2+}ATPase in model membranes containing both egg PC and (7,6)PC. In this figure, fractional fluorescence [F/F_0, i.e., the ratio of fluorescence in a sample with a given fraction (7,6)PC to that in a sample without (7,6)PC] is plotted against the mole fraction of (7,6)PC. Pyrene can form excimers (Birks, 1970), but, at the concentrations of pyrene in the vesicles used in these studies, no excimers were detected, as indicated by a lack of emission at 480 nm. Gramicidin A′ also exhibits a monomer–dimer equilibrium, and calculation of the monomer–dimer ratio from the association constant (Veatch et al., 1975) indicates that under the conditions of these experiments, gramicidin A′ will be almost exclusively in the dimeric form.

For all of the fluorophors tested, fluorescence quenching increases monotonically with increasing mole fraction of (7,6)PC. The curves drop most steeply at low mole fractions of (7,6)PC. The shape and wavelength maxima of the exci-

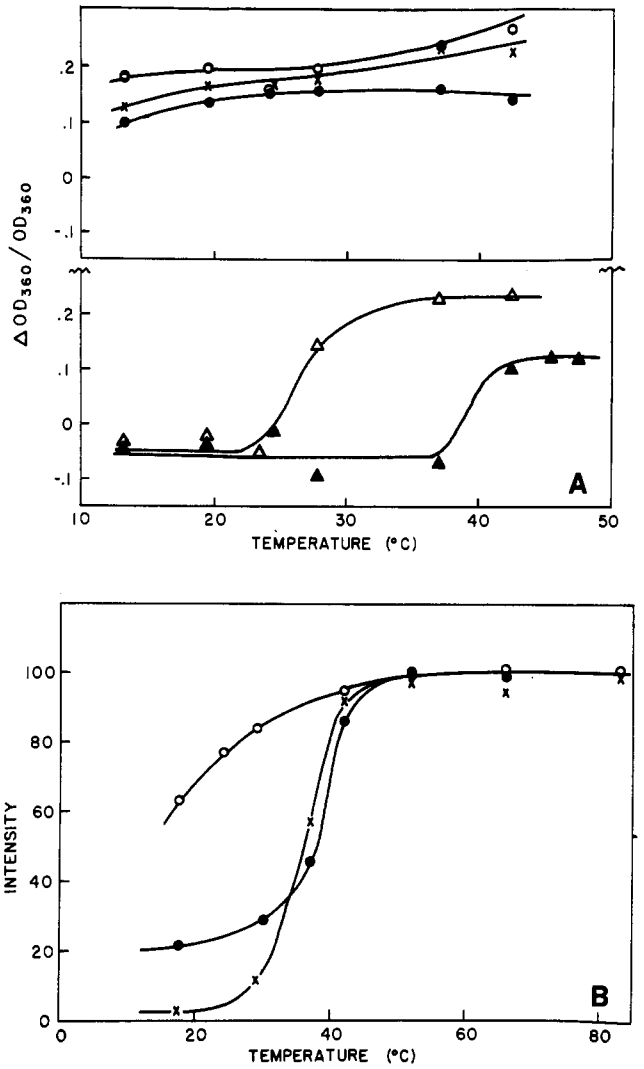


FIGURE 2: Determination of physical state of (7,6)PC. (A) Osmotic shrinking studies. At each temperature, 50 μL of 1 M glucose/20 mM Tris-HCl, pH 7.5, was mixed into 1 mL of a 0.15 mg/mL multilamellar dispersion of PC with 4% DPPA (w/w)/20 mM Tris-HCl, pH 7.5. The fractional rise in optical density, corrected for dilution, was measured after 20 s. (×) DLPC; (●) egg PC; (○) (7,6)PC; (△) DMPC; (▲) DPPC. (B) Dependence of ^1H NMR intensity of sonicated vesicles upon temperature. (●) DPPC; (×) 1:10 (7,6)PC/DPPC (w/w); (○) (7,6)PC.

tation and emission spectra of these fluorophors did not show appreciable shifts in membranes containing a high mole fraction of (7,6)PC relative to spectra in membranes of pure egg PC, indicating that the loss in fluorescence intensity at fixed wavelengths reflects a drop in quantum yield rather than a spectral shift. Previous workers also have shown that in solution the loss of fluorescence intensity observed upon introduction of a nitroxide-containing molecule is due to a drop in quantum yield of the fluorophor (Green et al., 1973; Weiss, 1976).

The fluorescence quenching curves shown in Figure 3A,B for different fluorophors vary both in shape and in maximum quenching. The curves for tryptophan octyl ester, gramicidin A′, and DPH are nearly superimposable. In solution, nitroxide fluorescence quenching is relatively independent of the emission wavelength at ≤500 nm (Green et al., 1973), so that differences in emission spectra are not the cause of the differences in quenching observed in Figure 3A,B. One variable which appears to be correlated with the shape of the fluorescence quenching curves is the fluorescence lifetime: pyrene $\tau_0 \sim 200$ ns (Fischkoff & Vanderkooi, 1975); DPH, $\tau_0 = 8$–10 ns (Lentz et al., 1976; Lakowicz et al., 1979); *p*-terphenyl, $\tau_0 \sim 1$ ns (Berlman, 1971). Figure 3A shows that at a given mole fraction of (7,6)PC fluorescence quenching increases with longer lifetime in this series of fluorophors. Tryptophanyl fluorescence lifetimes commonly fall in the narrow range 3–5 ns (Eftink & Ghiron, 1976a,b), and the fluorescence quenching curves of gramicidin A′ and tryptophan octyl ester are almost identical, yet the Ca^{2+}ATPase exhibits a very different fluorescence quenching curve. This is most readily explained as an effect of lower exposure of tryptophan in the Ca^{2+}ATPase to (7,6)PC than that in small molecules (see details below).

At 100% (7,6)PC fluorescence quenching is nearly complete except for pyrene and Ca^{2+}ATPase. Pyrene fluorescence is not quenched completely by (7,6)PC because of a small amount of pyrene in the water (Galla et al., 1979). The incomplete fluorescence quenching of Ca^{2+}ATPase probably reflects a pool of inaccessible tryptophanyl groups [see London & Feigenson (1981)].

An example of the response of fluorescence quenching to a change in lipid environment is illustrated by the effect of detergent on fluorescence quenching. In Figure 4, the effect of the addition of cholate on the fluorescence of DPH, gramicidin A′, and pyrene is shown. At cholate concentrations below the critical micelle concentration (cmc) some cholate will enter the phospholipid vesicles. The small extent of dilution of (7,6)PC which results would have little effect on the fluorescence quenching because in vesicles of ~50% (7,6)PC Figure 3A,B shows that quenching is only weakly dependent on (7,6)PC concentration. In the mixed lipid/cholate micelles which occur above the cmc, the ratio of (7,6)PC to cholate plus egg PC is less than 3/1000 (mol/mol). The amount of (7,6)PC in contact with fluorophor is then so low that virtually no quenching is observed. Similar observations have been reported for the reversal of quenching of the Ca^{2+}ATPase by detergent (Hardwicke, 1976; London & Feigenson, 1978a).

Dependence of Fluorescence Quenching on Fluorophor–Quencher Separation. The dependence of fluorescence quenching upon the separation of fluorophor and quencher is a critical factor in determining the use of a quenching molecule. In order to probe the local environment of a fluorophor, it is important that quenching molecules directly in contact with fluorophor efficiently be able to quench fluorescence and that those quenching molecules not in contact with a fluorophor be unable to quench its fluorescence.

The lateral diffusion coefficient of lipid in membranes has been found to be 10^{-7}–10^{-8} cm^2 s^{-1} (Devaux & McConnell, 1972; Schlessinger et al., 1976; Sheats & McConnell, 1978). For a fluorescence lifetime shorter than 50 ns, there will be less than one collision between molecules not already in contact. Thus, the fluorescence quenching of tryptophanyls and DPH observed in these experiments must be essentially a static process. The fluorescence quenching results support this conclusion since quenching is temperature independent in the absence of phase separations (see following paper in this issue). Collisional processes in model membranes have been treated in terms of two-dimensional phenomena (Träuble & Sackmann, 1972; Galla & Sackmann, 1974). Below, we treat fluorescence quenching in model membranes as primarily a static process, and quenching is analyzed by both two- and three-dimensional models.

The distance dependence of fluorescence quenching by (7,6)PC is depicted in Figure 5. Here, static quenching is analyzed following Perrin (1924) with

$$F_0/F = e^{V[Q]} \quad (1)$$

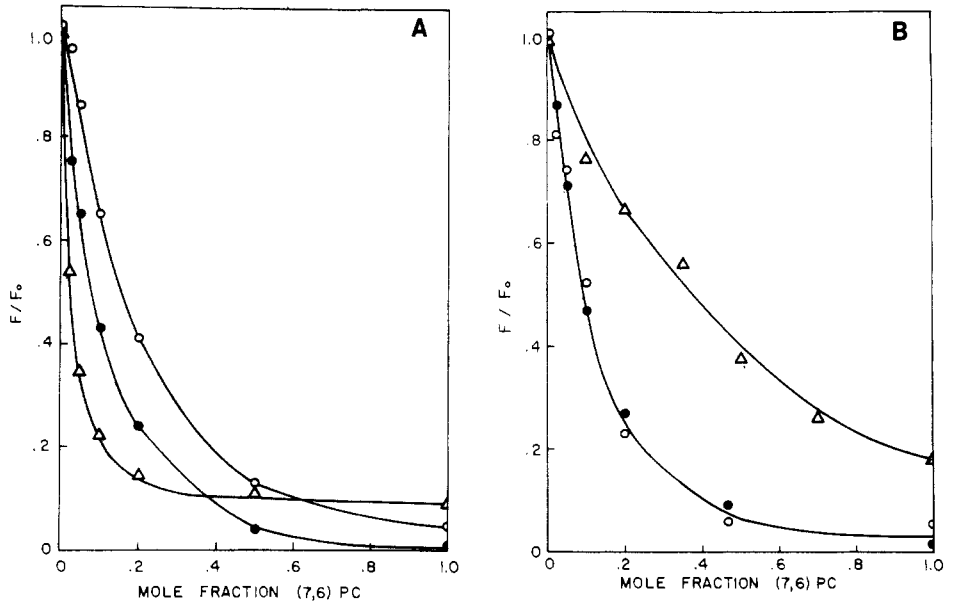


FIGURE 3: Fluorescence quenching of various fluorophors in model membranes composed of (7,6)PC/egg PC. The abscissa is the mole fraction of (7,6)PC in the membrane, and the ordinate is the ratio of fluorescence in the presence of (7,6)PC to that in the absence of (7,6)PC. For details of sample preparation see Materials and Methods. (A) (△) Pyrene; (●) DPH; (○) *p*-terphenyl. Samples containing pyrene were deoxygenated with N_2. (B) (○) Tryptophan octyl ester; (●) gramicidin A′; (△) Ca^{2+}ATPase. Molar ratio of fluorescent molecule to total lipid in samples was 1:156 except for samples containing Ca^{2+}ATPase which was 1:1000.

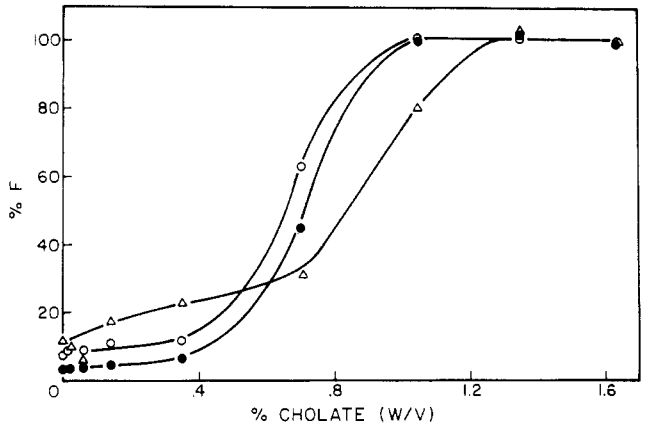


FIGURE 4: Effect of the addition of detergent upon fluorescence quenching of fluorophors in 50 mol % (7,6)PC/egg PC. (△) Gramicidin A′; (○) pyrene; (●) DPH. Molar ratio of fluorescent molecule to total lipid was 1:625 in these samples.

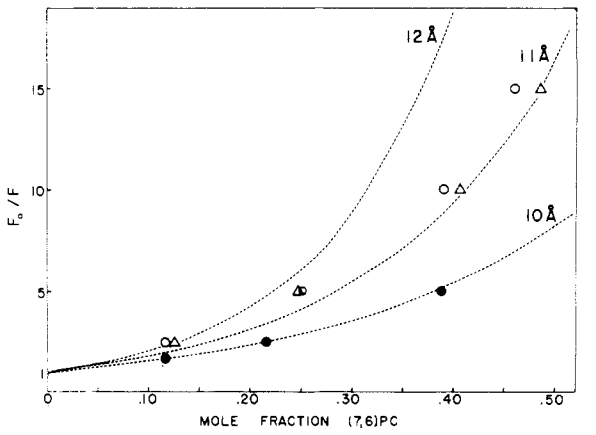


FIGURE 5: Dashed curves are theoretical profiles of static quenching by (7,6)PC for the different values of r_c shown. Experimental points in (7,6)PC/egg PC are for (○) DPH, (△) tryptophan octyl ester and gramicidin A′, and (●) *p*-terphenyl. Molar ratio of fluorophors to total lipid was 1:150.

A hard sphere approximation is used for the parameter V (Birks, 1970):

$$V = (4/3)\pi r_c^3 N' \qquad (2)$$

where r_c is a critical separation between fluorophor and quencher such that the quenching rate changes from zero to infinity at r_c, and N' is Avogadro's number per millimole. An approximate effective molarity in a membrane is calculated corresponding to the mole fraction of (7,6)PC. When the average molecular weight of phospholipid is 800 and when a phospholipid density of 1 g/mL is used for an aqueous dispersion of phospholipid (Huang, 1969; Watts et al., 1978), the number of moles of pure phospholipid per unit volume of membrane is

$$[\text{phospholipid}]_{\text{effective}} = (1/800 \text{ mol/g})(1 \text{ g/mL}) = 1.25 \text{ M}$$

With the assumption that the nitroxide and fluorophor are both trapped in the hydrophobic region of the membrane and that 75% of the total membrane is hydrophobic, for pure (7,6)PC the effective nitroxide concentration is 1.67 M. For a membrane of a given mole fraction (7,6)PC, the molarity of nitroxide is the product of 1.67 M and the mole fraction of (7,6)PC. Small corrections in this simple model should be made for the exact molecular weight and density of lipid in different mixtures, and a different estimate of the hydrophobic portion of a membrane could be made. Larger errors can arise from the implicit assumption of isotropic fluorophor and nitroxide distribution within the hydrophobic region and by neglecting wall effects. Nevertheless, this calculated effective concentration is useful for comparison of solution and membrane properties.

Fluorescence quenching curves shown in Figure 5 were calculated from eq 1 and 2 for different values of r_c, using the corresponding effective molarity of nitroxide discussed above. The value of r_c = 11 Å in Figure 5 corresponds most closely to the experimental data for DPH, tryptophan octyl ester, and gramicidin. For *p*-terphenyl r_c = 10 Å. The surface area occupied by an average phospholipid in the liquid-crystal state is ~65 Å^2 (Small, 1967). This corresponds to an average lateral separation of ~9 Å between the centers of neighboring phospholipids. If, in addition, we take into consideration the possible vertical separation in the bilayer as well as the lateral

separation between nitroxide and fluorophor, it appears as if fluorescence quenching will not occur if a (7,6)PC molecule is separated from these fluorophors by one or more unlabeled phospholipids. On the other hand, a (7,6)PC molecule in contact with a fluorophor should be able to quench fluorescence even if there is some vertical separation of the nitroxide and fluorescent moieties. For (7,6)PC, which has the nitroxide group halfway along the fatty acyl chain, quenching will occur between (7,6)PC molecules and fluorophors located in the same monolayer of the model membranes with only a small region at the center of the bilayer where fluorophor can be quenched by (7,6)PC in either monolayer.

A previous study of fluorescence quenching by nitroxides found a smaller critical interaction distance in solution (5–7 Å; Atik & Singer, 1978) compared to that measured here for quenching by nitroxide in membranes. Several factors that account for this difference must be considered aside from the approximations already discussed. Lateral diffusion during a fluorescence lifetime of 1–10 ns would result in a root-mean-square translation of about 1–4 Å, thereby increasing the effective r_c calculated from eq 1 and 2 for static quenching. In addition, as pointed out by Osborn & Porter (1965), at high viscosity, the longer encounter times result in quenching contributions at longer distances compared to low viscosity.

An alternate model can be developed to describe fluorescence quenching in model membranes. In a hexagonal lattice, there are six immediately neighboring phospholipids around a phospholipid-sized fluorophor located in one monolayer of the model membrane. There are 12 phospholipids in the second layer, separated from fluorophor by the phospholipids immediately around the fluorophor. In addition, there can be about six lipids in the monolayer not containing the fluorophor, at nearly the same distance to fluorophor as the second layer. Next closest in distance to the fluorophor are 18 third layer lipids plus 12 in the opposite monolayer, etc. If we consider hard-sphere or step-function fluorescence quenching, the amount of quenching is related to the probability that a spin-labeled lipid occupies any site in a layer close enough to fluorophor to cause quenching. The number of lattice points which can be occupied by a single (7,6)PC and result in fluorescence quenching will reflect the range of the fluorescence quenching interaction. For a random distribution of phospholipids, the probability that any lattice site is not occupied by (7,6)PC is 1 − [(7,6)PC] where [(7,6)PC] is the mole fraction of (7,6)PC. The probability of fluorescence arising from any particular fluorophor molecule is proportional to the probability that none of the n lattice sites close enough to the fluorophor is occupied by (7,6)PC. Quenching is then given by

$$F/F_0 = (1 - [(7,6)PC])^n \quad (3)$$

We note that this model does not depend on calculation of an effective nitroxide concentration to describe the effect of fluorophor–(7,6)PC separation upon fluorescence quenching.

The experimental data for DPH, tryptophan octyl ester, and gramicidin A′ are compared to theoretical curves calculated for various values of n in Figure 6. The closest fit for the experimental data is $n \approx 6$. This fits quenching arising only from the (7,6)PC molecules in the layer of lipid immediately around the fluorophor, in agreement with the analysis of Figure 5 discussed above. There is some difference in the shape of the theoretical and experimental curves, and this might reflect the effects of several complicating factors described for the previous model. Nonetheless, the fluorophor–nitroxide interaction is short range. This implies that the fluorescence quenching of membrane protein fluorophors will arise only

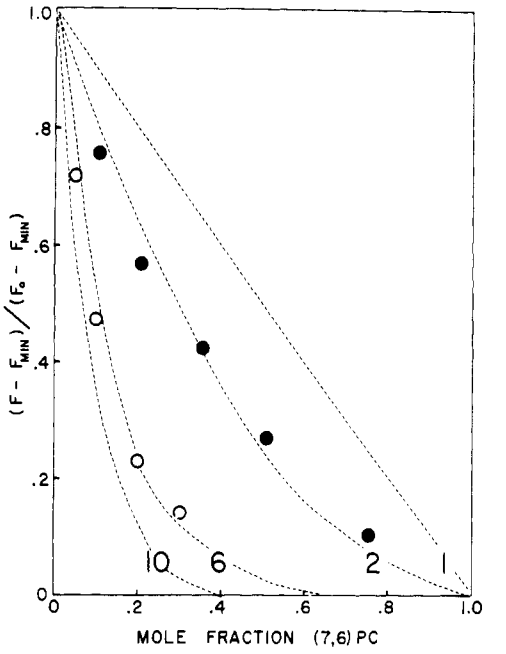


FIGURE 6: Determination of the number of lipid sites around a fluorophor group at which (7,6)PC can quench fluorescence. (○) Fluorescence quenching of DPH, tryptophan octyl ester, and gramicidin in (7,6)PC/egg PC. (●) Fluorescence quenching of Ca^{2+}-ATPase. Dashed lines are theoretical curves for different values of n. See text for details. Molar ratio of organic fluorophors to total lipid was 1:150, of Ca^{2+}ATPase to lipid 1:1000.

from those lipids "bound" to the protein and not from lipids in the remainder of the bilayer. Therefore, fluorescence quenching by spin-labeled lipids can be used to measure binding of lipids to membrane proteins.

The fluorescence quenching of the Ca^{2+}ATPase is also compared to hypothetical curves in Figure 6. The pool of fluorophors which are unquenched in pure (7,6)PC has been taken into account by assuming that they represent a pool of completely inaccessible fluorophors. Fluorescence quenching would then correspond to

$$(F - F_{min})/(F_0 - F_{min}) = (1 - [(7,6)PC]^n) \quad (4)$$

where F_{min} is the residual fluorescence in (7,6)PC. Note that if $F_{min} = 0$, then the left-hand side of eq 4 is just F/F_0. Substituting the values $F_{min} = 0.2$ from Figure 3B and using $F_0 \equiv 1$, one obtains from eq 4 the alternate form (eq 5).

$$F/F_0 = 0.8(1 - [(7,6)PC])^n + 0.2 \quad (5)$$

A good fit to the data for the Ca^{2+}ATPase in Figure 6 is found for $n = 2$. The value of n can be thought of as the number of lipids close enough to an average fluorophor in the protein to result in quenching, as described above for small organic fluorophors, or else a measure of the overlap in the fluorescent domains (i.e., regions) of the protein quenched by two neighboring, bound (7,6)PC molecules. A small value of n indicates a small amount of overlap in domains quenched by neighboring (7,6)PC molecules. The value $n = 2$ is reasonable, considering that a protein fluorophor is not likely to be surrounded by lipids on most sides, unlike a small fluorescent molecule. Similar observations have been made for fluorophors buried in water-soluble proteins (Lakowicz & Weber, 1973; Eftink & Ghiron, 1976a,b).

A value of $n = 4$ was determined for p-terphenyl and $n = 30$–35 for pyrene. The large n found for pyrene probably reflects a collisional component of quenching, and the small n found for p-terphenyl probably results from inefficient quenching by neighboring phospholipids due to the short τ_0 of this molecule.

Conclusions

This study demonstrates that (7,6)PC forms aqueous bilayer membrane vesicles which are similar in physical properties to bilayer membrane vesicles of other phospholipids in the liquid-crystalline state. Fluorescence quenching by the nitroxide moiety of (7,6)PC can be measured for many different fluorophors, including a membrane protein. In model membranes, fluorescence quenching by (7,6)PC of fluorophors with fluorescence lifetimes of ≤ 10 ns is primarily static in nature. The dependence of the fluorescence quenching rate upon fluorophor–quencher separation is such that the fluorescence quenching primarily, and probably exclusively, arises from the nearest spin-labeled lipid neighbors to the fluorophor. Application of fluorescence quenching to problems of model membrane structure and lipid–protein interaction are described in the following papers of this issue.

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