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# Transbilayer Distribution in Small Unilamellar Phosphatidylglycerol–Phosphatidylcholine Vesicles†

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ABSTRACT: The transbilayer distribution of the phospholipids in small unilamellar vesicles comprised of egg phosphatidylglycerol (PG) and egg phosphatidylcholine (PC) was ascertained by $^{31}P$ NMR. These vesicles, containing 10–75 mol % PG, were formed by sonication (pH 7.6) and fractionated by centrifugation. Data from spectra accumulated in the presence and absence of a paramagnetic shift reagent, $Mn^{2+}$, indicated that the phospholipids are randomly arranged across the bilayer. The absence of compositional asymmetry, which contradicts earlier reports, is also exhibited by small unilamellar vesicles (50 mol % PG) prepared by the rapid ethanol injection method. Control experiments showed that $Mn^{2+}$ did not induce fusion, permeate the vesicles, or cause the phospholipids to migrate across the bilayer. It has been proposed that the transbilayer distribution of charged phospholipids in membranes is a consequence of the different surface charge densities on the opposing sides of the membrane. Our results suggest that it is the difference in the effective polar headgroup volumes of the components rather than the net charge of one component that determines the packing constraints for mixtures of phospholipids with the same acyl chains, at least in highly curved bilayers.

It seems reasonable to expect membrane constituents to be asymmetrically distributed in biological membranes since the different functions performed on opposing sides of the membrane probably require different structural components. In fact, there is much evidence to support the notion that constituents of biological membranes are vectorially arranged within the bilayer [see review by Op den Kamp (1979)]. Generally, the proteins are distributed with an absolute asymmetry while the various classes of phospholipids are present in both monolayers, albeit in unequal amounts.

Several investigators have attempted to understand the intermolecular interactions that might induce the spontaneous formation of asymmetric phospholipid bilayers by studying binary mixtures of phospholipids in small unilamellar vesicles. Elegant studies by Litman (1973, 1974) indicate that egg phosphatidylethanolamine in small unilamellar vesicles (SUV)[1] comprised of this lipid and egg phosphatidylcholine (PC)[1] is preferentially localized in the outer monolayer at low phosphatidylethanolamine concentrations (<10 mol %) and in the inner monolayer at higher phosphatidylethanolamine concentrations. Phosphatidylserine in small vesicles containing phosphatidylcholine also prefers the inner monolayer (Berden et al., 1975), at least at low phosphatidylserine concentrations (Barsukov et al., 1980). For other mixtures, there has not always been complete agreement. Phosphatidic acid in sonicated phosphatidic acid–phosphatidylcholine vesicles was reported by Berden et al. (1975) to be preferentially localized in the inner monolayer while more recent work (Koter et al.,

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[1] Abbreviations used: PC, phosphatidylcholine; PG, phosphatidylglycerol; SUV, small unilamellar vesicles; EDTA, ethylenediaminetetraacetic acid.

0006-2960/81/0420-6415$01.25/0 

1978) demonstrated that over a wide composition range the phospholipids were symmetrically distributed. Published reports concerning the distribution of phosphatidylglycerol (PG)[1] in cosonicated mixtures of this lipid and phosphatidylcholine are even more contradictory. Michaelson et al. (1973) reported that phosphatidylglycerol, in an equimolar mixture, preferred the outer monolayer. When a range of compositions were studied, one group (Massari et al., 1978) claimed that PG was preferentially localized in the outer monolayer only at mole fractions below about 0.4, whereas Lentz et al. (1980) found that it always preferred the outer monolayer. More recently Barsukov et al. (1980) presented data suggesting that phosphatidylglycerol resides preferentially in the inner monolayer at low PG concentrations, with the phospholipid distribution becoming less asymmetric as the PG concentration increases.

In order to gain a better understanding of the factors which affect the transbilayer arrangement of phospholipids, we have reexamined the transbilayer phospholipid distribution in cosonicated mixtures of phosphatidylglycerol and phosphatidylcholine. The vesicles were fractionated by centrifugation before $^{31}P$ NMR and the paramagnetic ion, $Mn^{2+}$, were employed to study the organization of the phospholipids. The experiments were carefully controlled to assure that $Mn^{2+}$ did not induce fusion, permeate the vesicles, or cause the phospholipids to migrate across the bilayer.

## Materials and Methods

*Phospholipid Preparation.* Egg phosphatidylcholine was purchased from Avanti Biochemicals (Birmingham, AL) and used without further purification. Phosphatidylglycerol was synthesized from PC by the transferase activity of phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4). The enzyme, which was isolated from Savoy cabbage and purified through the acetone precipitation step (Yang et al., 1967), was stable for several months when stored as a lyophilized powder at −80 °C. The method used to synthesize PG was essentially that of Comfurius & Zwaal (1977). Upon termination of the reaction, the ether was evaporated at room temperature under a stream of nitrogen, and the remaining aqueous layer was added to 3 volumes of methanol and refluxed for 1.5 h. The reaction mixture was filtered while hot, and the filter was washed with hot methanol. This step was required to separate PG from the enzyme. The lipid was extracted according to Folch et al. (1957) and purified by chromatography on carboxymethylcellulose using chloroform–methanol mixtures for elution. The chromatographically pure PG fractions were combined, washed with acid to remove the residual calcium, and converted into the sodium salt (Castle & Hubbell, 1976). No detectable impurities were observed when PC or PG (0.5 μmol) were loaded on thin-layer silicic acid chromatography plates and developed in $CHCl_3$–$CH_3OH$–$H_2O$ (65:25:4). The phospholipids were dissolved in $CHCl_3$ and stored under argon at −20 °C. The purity of the phospholipids remained unchanged throughout the course of these experiments under these storage conditions.

*Preparation of Small Unilamellar Vesicles.* Small unilamellar vesicles were prepared by probe sonication (Huang, 1969) and rapid ethanol injection (Batzri & Korn, 1973; Nordlund et al., 1981). Mixtures of egg PG and egg PC were evaporated to dryness under argon and lyophilized for 3–4 h in the dark. Phospholipids used to form sonicated SUV were suspended in 50 mM borate buffer (pH 7.6) containing 150 mM NaCl and 1 mM EDTA. For some experiments, the buffer also contained 1 M $K_3Fe(CN)_6$. The phospholipid dispersion (2–3 mL), under a nitrogen atmosphere, was sonicated intermittently at 0 °C for 2–3 min by using a Heat System W-350 sonifier. The maximum sonication time was 30 min. SUV were also prepared by a rapid ethanol injection procedure. Lyophilized phospholipids were dissolved in absolute ethanol (35 μmol of phospholipid/mL) and injected rapidly through a 100-mL Hamilton syringe into rapidly stirring buffer. The resulting vesicle dispersion contained less than 5% ethanol by volume. The dispersion was concentrated to 1–2 mL in an Amicon ultrafiltration device (Lexington, MA) by using a XM-100 membrane. The rapidly stirring dispersion was maintained under nitrogen gas whose pressure did not exceed 25 psi.

A homogeneous population of SUV was prepared from the crude vesicle dispersions by centrifugation for 60 min at 96500*g* (Ti-50 fixed-angle rotor). Only the vesicles found in region III of the supernatant, as described by Barenholz et al. (1977), were used.

*Determination of Vesicle Size Distribution.* The homogeneity of the SUV population was determined by column chromatography on Sepharose CL-4B at 4 °C. A downward flowing column (1.6 × 45 cm) was equilibrated at 4 °C with buffer. The vesicle dispersions (1–2 mL) were loaded onto the column and eluted at 10 mL/h.

*Nuclear Magnetic Resonance (NMR).* The NMR measurements were performed at 59.75 MHz for $^1H$ and 24.15 MHz for $^{31}P$ by using a Jeol FX60Q Fourier transform spectrometer. The spectra contained 4K data points after Fourier transformation. The $^{31}P$ spectra were obtained at 24 °C by using continuous $^1H$ noise decoupling, a 600-Hz sweep width, 1.7-s acquisition time, 2.7-s delay between acquisitions, and a 90° pulse of 16 μs. Longer delay times gave identical results. Between 1000 and 2000 scans were accumulated, so the time per spectrum was 1–2 h. The $^1H$ NMR spectra were obtained at 24 °C by using a 600-Hz sweep width and a 3.4-s acquisition time. A 180°–$\tau$–90° pulse sequence was used to minimize the residual HOD signal (Patt & Sykes, 1972). A total of 64 scans was accumulated for 10 mM samples.

*Preparation of Small Vesicles Containing Trapped Ferricyanide.* Small unilamellar vesicles containing trapped ferricyanide were prepared by probe sonication as described above. A homogeneous vesicle population, freed from external ferricyanide, was obtained by gel filtration on Sepharose CL-4B. The fractionated vesicles were concentrated to 1.5 mL in an Amicon ultrafiltration device with a XM-100 membrane. The rapidly stirring dispersion was maintained under nitrogen gas whose pressure did not exceed 25 psi. The vesicles were then dialyzed against borate buffer (pH 7.6) in $D_2O$ containing 150 mM NaCl and 1 mM EDTA and stored under argon until spectra were taken.

## Results

Binary mixtures of egg PG and egg PC were sonicated in buffer (pH 7.6) and fractionated by centrifugation as described under Materials and Methods. At this pH, the PG head group is negatively charged (p$K$ = 3–5.5, depending on the ionic strength; Sacre & Tocanne, 1977; Watts et al., 1978; Tocanne et al., 1974).

The transbilayer distribution of phospholipids in these vesicles was determined by $^{31}P$ NMR with $Mn^{2+}$ as a broadening reagent (Bergelson, 1978). Two well-resolved resonances are evident in $^{31}P$ NMR spectra of mixed egg PG–egg PC vesicles (Figure 1A). The phosphatidylglycerol resonance, which is downfield from the PC resonance, and the phosphatidylcholine resonance are both composed of two partially resolved peaks arising from packing differences between the two monolayers in the head-group region. When $Mn^{2+}$ is added, there is extensive line broadening of the resonances from

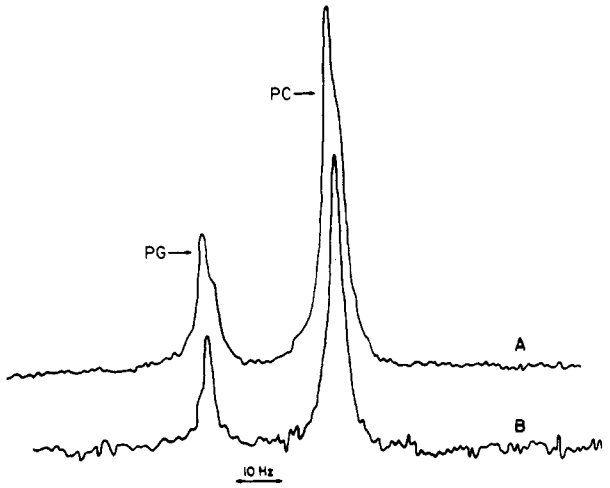


FIGURE 1: 24.15-MHz $^{31}P$ NMR spectra of small unilamellar vesicles comprised of egg phosphatidylcholine and phosphatidylglycerol (29:71) in the absence (A) and the presence (B) of $MnCl_2$ ($Mn^{2+}$/phospholipid = 0.01). The total phospholipid concentration is approximately 30 mM.

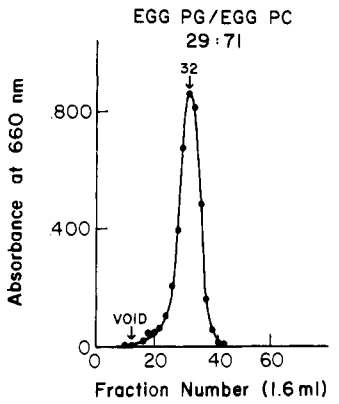


FIGURE 2: Elution profile of sonicated egg PC–egg PG vesicles. Subsequent to the NMR experiments, the vesicles were eluted from a Sepharose CL-4B column, and the phosphorus content of the fractions was determined by a modified Bartlett procedure [see Litman (1973)]. Pure egg PC SUV produce a similar elution profile. The position of the void volume was determined by Blue Dextran 2000.

Table I: Distribution of Egg PG and Egg PC in Small Unilamellar Vesicles

| mol % PG[a] | mol % PG (outside)[b] | mol % PC (outside)[b] | mol % PG (inside)[b] | mol % PC (inside)[b] | $I_{outside}$[c]/$I_{inside}$ |
|---|---|---|---|---|---|
| | | Sonicated SUV | | | |
| 0 | | 69 | | 31 | 2.2 |
| 12 | 12 | 88 | 12 | 88 | 2.2 |
| 29 | 30 | 70 | 27 | 73 | 2.2 |
| 49 | 50 | 50 | 48 | 52 | 2.1 |
| 59 | 60 | 40 | 57 | 43 | 2.6 |
| 74 | 75 | 25 | 71 | 29 | 2.6 |
| | | Ethanol Injection SUV | | | |
| 55 | 56 | 44 | 53 | 47 | 2.0 |

[a] The phospholipid ratios were determined after inorganic phosphate analysis of the iodine-sensitive spots separated by thin-layer chromatography (Litman, 1973) and from the $^{31}P$ NMR integral values. [b] The estimated error in the mole percents, which were determined directly from the spectral integrals, is ±3. [c] The estimated error is ±0.1.

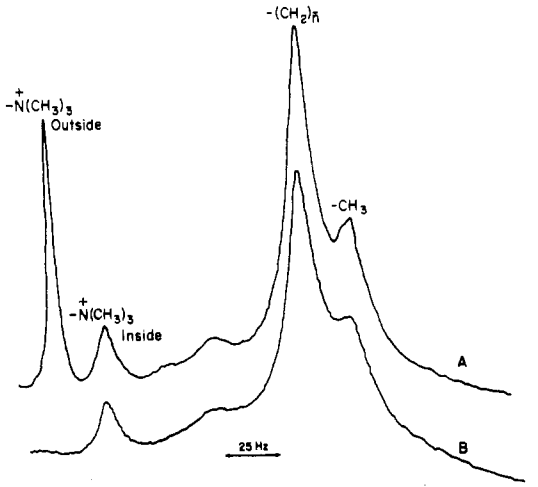


FIGURE 3: 59.75-MHz $^1H$ NMR spectra of egg PC–PG vesicles containing 1 M potassium ferricyanide in the absence (A) and in the presence (B) of $MnCl_2$ ($Mn^{2+}$/phospholipid = 0.01).

the exposed molecules. This is shown in Figure 1B for egg PG–egg PC vesicles (29:71) in the presence of externally added $Mn^{2+}$ at a $Mn^{2+}$/phospholipid ratio of 0.01. Further addition of $Mn^{2+}$ did not decrease the integral value. In every experiment, the unbroadened signal did not decrease over a 24-h period, and the original spectral intensity was restored upon addition of sufficient amounts of EDTA. There was no noticeable increase in turbidity upon addition of $Mn^{2+}$, nor did the elution profile from a Sepharose 4B column, run after the NMR experiment, indicate the presence of larger vesicles (Figure 2). Thus, $Mn^{2+}$ does not penetrate the vesicles or induce fusion.

Table I presents the transbilayer distribution of PG and PC in small vesicles as a function of the phosphatidylglycerol content. For every composition examined, the composition of the inner monolayer was nearly identical with the total phospholipid composition, demonstrating that PG and PC are equally distributed between the two monolayers over a wide composition range. For sonicated SUV, the ratio of the number of molecules exposed to the external medium to the number that is not ($I_{outside}/I_{inside}$) remains constant up to approximately 60 mol % PG. Above this concentration, the ratio increases, indicating that the average vesicle is somewhat smaller. The elution profiles from Sepharose CL-4B confirmed this observation (data not shown). It is interesting to note that phospholipids in small vesicles containing equimolar amounts of PG and PC made by the ethanol injection procedure (see Materials and Methods) are symmetrically distributed. The average vesicle size was slightly larger (Table I) and the preparation was less homogeneous (data not shown).

Transbilayer exchange (flip-flop) in model bilayer systems has been shown by several studies to be a very slow process (Op den Kamp, 1979). However, since rapid exchange can occur under some conditions [reviewed by van Deenen (1981)], we have investigated the possibility that the addition of $Mn^{2+}$ induces changes in the transbilayer distribution on the time scale of the $^{31}P$ NMR experiments. This was done by using $^1H$ NMR and the anionic shift reagent, ferricyanide, which was trapped inside the vesicles during sonication. The vesicles were size fractionated, and the external ferricyanide was removed by molecular sieve chromatography as described under Materials and Methods. A typical $^1H$ NMR spectrum for these small egg PG–egg PC vesicles (10:90) containing 1 M potassium ferricyanide is shown in Figure 3A. The resonance due to the choline *N*-methyl protons of the phosphatidylcholine molecules in the inner monolayer is shifted upfield from the resonance for the same protons in molecules located in the outer monolayer. When a sufficient quantity of $Mn^{2+}$ is added externally, the signal for the exposed molecules is completely broadened, leaving only those resonances representing the

molecules in the inner monolayer (Figure 3B). When the integral of the signal from the *N*-methyl protons in the PC molecules in the inner monolayer in the presence and absence of $Mn^{2+}$ is compared, the extent of phospholipid transbilayer migration can be assessed. In sonicated vesicles containing 10 or 25 mol % PG, the integral value for these protons in the presence of $Mn^{2+}$ was 97–98% of the original integral value. Sonicated egg PC vesicles, used as a control, gave identical results. Thus, $Mn^{2+}$ does not induce detectable amounts of exchange of PG for PC on the time scale of the $^{31}P$ NMR experiment. Larger mole percentages of PG reduced the magnitude of the ferricyanide-induced choline methyl $^1H$ shifts, so that the inner monolayer resonance could not be satisfactorily resolved from that of the outer monolayer. This is presumably due to decreased binding of the ferricyanide to the more negatively charged bilayers. $Mn^{2+}$-induced transbilayer exchange cannot therefore be definitely excluded at the higher PG mole fractions.

Control experiments were done to confirm that preparing the vesicles in the presence of ferricyanide did not affect either the transbilayer distribution of PG and PC or the size, stability, and homogeneity of the vesicles. $^{31}P$ spectra were acquired and analyzed as before. They were also rerun to verify that the permeability barrier of the vesicles remained intact. Futhermore, the molecular sieve chromatography elution profiles of these vesicles were identical with those of vesicles without ferricyanide.

## Discussion

The transbilayer distribution of phospholipids in membranes can be assessed with confidence only when a well-characterized system is studied with a reliable technique. Thus, a homogeneous population of unilamellar vesicles should be examined by nonperturbing probes that penetrate the bilayer in a controlled fashion, if at all. We present the results of experiments designed to ascertain the topological asymmetry of phospholipids in vesicles comprised of egg PG and egg PC. These vesicles were formed by sonication or rapid ethanol injection and were small, unilamellar (Table I), and homogeneous (Figure 2). The transbilayer phospholipid distribution was determined by $^{31}P$ NMR with $Mn^{2+}$ as a broadening regent (Bergelson, 1978). The probe, $Mn^{2+}$, did not penetrate the bilayer (see Results), induce fusion of the vesicles (Figure 2), or facilitate the transbilayer exchange of PG for PC (Figure 3). In this system, using this method, we found no evidence for transbilayer asymmetry. Phospholipids in sonicated vesicles containing 10–75 mol % PG were randomly distributed across the bilayer, as were the phospholipids in small vesicles (50 mol % PG) made by ethanol injection. Since the vesicles were formed by different mechanisms (one dispersive and the other aggregative), the symmetrical phospholipid distribution is probably not a consequence of the mechanism. Similarly, when SUV exhibit transbilayer asymmetry, the asymmetry is present in vesicles made by either method. Nordlund et al. (1981) showed that the transbilayer distribution of egg phosphatidylethanolamine in binary mixtures of that lipid and egg PC was the same in small vesicles made by sonication or rapid ethanol injection.

Our results are not in complete agreement with earlier reports. Michaelson et al. (1973) reported that phosphatidylglycerol was preferentially localized in the outer monolayer at equimolar concentrations of PG and PC. Although they used $^{31}P$ NMR, their spectra indicate a poorly defined vesicle preparation. Since they were not able to resolve adequately the phospholipid resonances, the phospholipid distribution had to be calculated from the peak amplitudes rather than from the more reliable integral values. Massari et al. (1978), using a technique based on the interaction of negative phospholipids with positive metachromatic dyes, concluded that phosphatidylglycerol prefers the outer monolayer at low PG concentrations, with the distribution becoming less asymmetric as the phosphatidylglycerol concentration increased. However, their protocol for vesicle formation and storage and uncertainties in the binding technique make interpretation of their data difficult. Lentz et al. (1980) developed a chemical assay to quantitate the amount of exposed PG. Using fractionated populations of sonicated vesicles, they carefully assessed the transbilayer distribution of PG. Regardless of the PG to PC ratio, phosphatidylglycerol was always found at higher relative concentrations in the outer monolayer. The reason for this discrepancy is difficult to identify. Perhaps the structural integrity of the vesicles was disrupted by the assay. This possibility could not be investigated because the permeability barrier was destroyed within 22 min, presumably a consequence of periodate oxidation. Barsukov et al. (1980) presented data suggesting that phosphatidylglycerol resides preferentially in the inner monolayer at low PG concentrations. As the PG concentration increased, the asymmetry was diminished. Two paramagnetic ions were used to separate the resonances for the *N*-methyl protons in the inner and outer monolayers. One ion, $Pr^{3+}$, was trapped within the vesicles during sonication while the other, $Eu^{3+}$, was added externally before $^1H$ NMR spectra were recorded. Since the effect of the interaction between the positively charged ion and the negatively charged phospholipid on the phospholipid distribution during vesicle formation is not known, the authors' conclusions are probably not applicable to our system. It should be noted that in some systems the transbilayer distribution of the phospholipids is also a function of the fatty acid composition. Lentz & Litman (1978) reported that dimyristoylphosphatidylethanolamine in mixtures with dimyristoyl-PC always preferred the outer monolayer of sonicated vesicles. In our study, the fatty acid composition of the PG and the PC were identical. In two of the cases mentioned above (Massari et al., 1978; Lentz et al., 1980), they were different. Since the effect of fatty acid composition on the PG distribution is not known, this factor represents a possible cause of discrepancy among the various results.

The absence of transbilayer asymmetry in small vesicles containing negatively charged phospholipids is not surprising in light of the recent investigations by Koter et al. (1978) on binary mixtures of egg PC and phosphatidic acid prepared from egg PC. Using $^{31}P$ NMR and the broadening reagent $Co^{2+}$, they showed that phospholipids in fractionated preparations of sonicated vesicles (pH 7.4) were randomly distributed across the bilayer over a wide composition range. The low concentrations of $Co^{2+}$ required did not cause the vesicles to aggregate or fuse.

There is evidence suggesting that some negatively charged phospholipids (PG and phosphatidic acid) are randomly distributed in SUV while others (phosphatidylserine and perhaps phosphatidylinositol) are not. Israelachvili and co-workers (Israelachvili et al., 1977, 1980; Carnie et al., 1979) have attempted to explain this behavior by a theory formulated primarily in terms of the effect of molecular geometry on the free energy of association. Neglecting specific lipid interactions, they hypothesized that the transbilayer distribution of the phospholipids could be predicted by knowing the optimal hydrocarbon–water interfacial areas, the hydrocarbon chain volumes and critical lengths, and the effective head-group length parameters, *D*, of the components. The magnitude of

$D$, which is difficult to estimate, depends on the head-group charge, conformation, and hydration properties. Their approach adequately predicts the asymmetry found in mixtures of cholesterol or lyso-PC with PC since $D$ can be assumed to be near zero for cholesterol or the same as PC for lyso-PC (Carnie et al., 1979). The lack of knowledge about $D$ has prevented its useful application to mixtures of double-chain phospholipids. Moreover, the fact that the direction of the asymmetry in SUV may vary with composition (i.e., egg phosphatidylethanolamine–egg phosphatidylcholine) indicates that $D$ may be a function of the composition or that intermolecular forces such as hydrogen bonding are important.

In general, it seems reasonable to say that the location of the molecules within the bilayers is determined in part by the dynamic shapes of the components, with these shapes being described by superimposing the motional properties of the molecules onto their molecular geometries. Thus one would expect that highly curved bilayers composed of molecules with different time-averaged space requirements will exhibit transbilayer asymmetry, as has been observed. In pure phospholipid bilayers, transbilayer asymmetry should be absent if the molecular packing densities in the inner and outer monolayers are similar or if the components possess equivalent dynamic shapes that are independent of the bilayer composition. The former possibility has been demonstrated in egg PE–egg PC vesicles where the phospholipid asymmetry observed in small unilamellar vesicles was absent in large unilamellar vesicles having the same phospholipid composition (Nordlund et al., 1981). Our results support the second possibility for PG. The absence of asymmetry in SUV comprised of a negatively charged phospholipid and a zwitterionic phospholipid may seem unlikely until the physical properties of the molecules are compared. Spectroscopic studies indicate that the head-group motions are similar (Wohlgemuth et al., 1980), and differential scanning calorimetry provides strong evidence for a high degree of miscibility of the head groups (Findlay & Barton, 1978; Watts et al., 1978). While these studies were peformed in planar bilayers and our results are for highly curved bilayers, the similarities in the motional and structural properties of these molecules suggest that they possess an equivalent dynamic shape. In contrast, phospholipids that form asymmetric bilayers when mixed with PC have different motional properties, e.g., phosphatidylserine (Browning & Seelig, 1980; Wohlgemuth et al., 1980), or structural properties, e.g., phosphatidylserine and phosphatidylethanolamine (see Table I, Israelachvili et al., 1980), than phosphatidylcholine.

In general, the influence of the electrostatic interactions between charged species on their distribution within the bilayer is difficult to assess. However, the dissimilarity in the head-group parameters of phosphatidylserine and PG and the similarity in the same parameters for PG and PC suggest that the negative charge in phosphatidylglycerol is less important than previously appreciated. More attention should probably be given to hydrogen bonding and head-group hydration forces. Recent, Cevc et al. (1980) found a rather large nonelectrostatic contribution to the phase transition temperature shift on titration of phosphatidylglycerol bilayers. They suggested that this temperature shift, which was operationally defined by its independence of the bulk ionic strength at high 1:1 electrolyte concentrations, may be due to head-group–water interactions or the strength of the hydrogen bonding. Further evidence for the importance of hydration forces has been demonstrated by measuring the repulsive force between bilayers in water. Pure egg PC bilayers produced a force that was fit by a exponential function that decreased rapidly with increasing distance. In contrast, PC bilayers containing 10 mol % PG generated a biphasic repulsive force that was dominated by electrostatic interactions at interbilayer distances larger than 25 Å (Cowley et al., 1978; Parsegian et al., 1979). At smaller separations, the stronger repulsive force was independent of surface charge density. Such short-range repulsive forces are thought to arise from the increased order of the water molecules near the bilayer surface. Further investigations are needed to determine how these forces may influence intrabilayer interactions and the resultant transbilayer distribution of phospholipids. Electrostatic interactions will always be important in biological membranes that possess a significant intramembrane potential. The distribution of charged lipids in such membranes can be calculated by combining the Boltzmann relation and the Gouy equation (McLaughlin & Harary, 1974).

Acknowledgments

We thank Drs. B. J. Litman and B. R. Lentz for many stimulating discussions.

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# Interaction of Rhodopsin with Two Unsaturated Phosphatidylcholines: A Deuterium Nuclear Magnetic Resonance Study†

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ABSTRACT: Rhodopsin, prepared free of native lipid, was reconstituted with two unsaturated and specifically deuterated phosphatidylcholines: the minimally unsaturated 1-(16,16,16-trideuteriopalmitoyl)-2-palmitoleoyl-*sn*-glycero-3-phosphocholine [($CD_3$-16:0)(16:1)PC] and a highly unsaturated phosphatidylcholine typical of that found in native rod outer segment (ROS) membranes, 1-(16,16,16-trideuteriopalmitoyl)-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine [($CD_3$-16:0)(22:6)PC]. Deuterium magnetic resonance ($^2H$ NMR) spectra of these membranes and dispersions of the lipids alone were obtained at 23.0 MHz by using the quadrupolar echo technique. The apparent quadrupolar splittings are slightly less and the spectral features are somewhat broadened in the presence of the protein. Moment analyses of these spectra show that in the fluid phase the presence of rhodopsin at near physiological concentrations (1:1 w/w) does not change the average orientational order of either lipid. Rhodopsin does affect the structure of the bilayer, however, by causing an increase in the spread of the distribution of orientational order parameters about the average. Several interesting differences are observed in the phase behavior of the two lipids in the absence of rhodopsin. The orientational order of ($CD_3$-16:0)(16:1)PC and ($CD_3$-16:0)(22:6)PC is markedly different in the phase transition region, even though these two lipids have surprisingly similar phase transition temperatures. The 22:6-containing lipid exhibits a relatively large hysteresis (8–9 °C) in its phase transition, while no hysteresis is observed for ($CD_3$-16:0)(16:1)PC. Further, the phase transition for ($CD_3$-16:0)(22:6)PC occurs over a much smaller temperature range than that for ($CD_3$-16:0)(16:1)PC. Both the hysteresis and sharpness of the phase transition suggest a higher degree of cooperativity in the 22:6 lipid than in the 16:1 lipid. The presence of rhodopsin eliminates this hysteresis and sharp phase transition of the ($CD_3$-16:0)(22:6)PC and produces values of the average orientational order similar to those observed for the ($CD_3$-16:0)(16:1)PC/rhodopsin reconstituted membranes, which are unchanged from the 16:1 lipid alone. Spin–lattice ($T_1$) and quadrupolar echo decay ($T_{2e}$) relaxation times were measured as a function of temperature for the lipids with and without rhodopsin. For all of the samples, $T_{2e}$ is very sensitive to the gel to liquid crystal phase transition, while $T_1$ is relatively insensitive. In the fluid phase, rhodopsin decreases $T_1$ by approximately the same amount (about 2-fold) for both lipids while producing a 2-fold greater decrease in $T_{2e}$ in the membranes reconstituted with the 22:6 lipid as opposed to the membranes prepared with the 16:1 lipid. This implies that rhodopsin differentially alters low-frequency reorientational motions of the two lipids. Overall, these results are consistent with our previous $^1H$, $^{13}C$, and $^{31}P$ NMR studies of native ROS membranes and ROS lipids: rhodopsin does not produce a long-lived, highly ordered population of lipids.

The vertebrate retinal rod outer segment (ROS)[1] disk membrane is the site of visual excitation. In these membranes, the chromophoric protein rhodopsin comprises at least 95% of the integral membrane protein (Krebs & Kuhn, 1977). Rhodopsin is deeply embedded in the (ROS) disk membrane bilayer (Dratz et al., 1979), and the polypeptide chain spans the membrane (Fung & Hubbell, 1978; Nemes et al., 1980). The fatty acids in the ROS membrane phospholipids are strikingly polyunsaturated; in cattle ROS, about 50% of the fatty acids are docosahexaenoic acid (22:6) (Miljanich et al., 1979; Stone et al., 1979). Most of the other fatty acids are the saturated palmitic (16:0) and stearic (18:0) acids. Although there is considerable heterogeneity in the head group and fatty acid composition of the phospholipids in the ROS membrane (Miljanich et al., 1979), it is reasonable to describe a typical phospholipid as having a saturated fatty acid at the *sn*-1 position of the glycerol backbone and a 22:6 fatty acid at the *sn*-2 position.

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[1] Abbreviations used: NMR, nuclear magnetic resonance; ROS, retinal rod outer segment; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; ($CD_3$-16:0)(16:1)PC, 1-(16,16,16-trideuteriopalmitoyl)-2-palmitoleoyl-*sn*-glycero-3-phosphocholine; ($CD_3$-16:0)(22:6)PC, 1-(16,16,16-trideuteriopalmitoyl)-2-docosahexaenoyl-*sn*-glycero-3-phosphocholine; $T_1$, spin–lattice relaxation time; $T_{2e}$, time constant for the decay of the quadrupolar echo.

0006-2960/81/0420-6420$01.25/0 