Comparison of the Leakage of Carboxyfluorescein from Symmetric- and Asymmetric-Acyl Chain Phosphatidylcholine Vesicles

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INTRODUCTION

A somewhat unusual characteristic of phospholipids is the presence of acyl chains appreciably different in length (1–8). Specifically, if the acyl chains differ in length, then the gel phase bilayer has a structure where the shorter acyl chain meets the longer acyl chain in the opposing monolayer of the bilayer. That is, the sn-1 acyl chain meets the sn-2 acyl chain in the central portion of the bilayer. This has been shown to occur for many phosphatidylcholines. The partial interdigitated structure is perhaps not unexpected, since the sn-1 acyl chain is thought to meet the sn-2 acyl chain in the opposing bilayer even with phospholipids which have acyl chains of equal number of carbon atoms.

However, a unique structure is obtained when the acyl chains differ in effective length by a factor of two (5). Here a mixed-chain, partial interdigitated bilayer is formed which is characterized by the two shorter acyl chains of the phospholipids of the opposing leaflets of the bilayer meeting in the center and the long acyl chains extending across the entire thickness of the bilayer. In addition, this is structurally distinct in that one head group provides the equivalent projected area of three acyl chains instead of two as in the symmetric-acyl chain phospholipids in the membrane. Although the structure has been identified by X-ray diffraction and the gel-to-liquid crystalline phase transition has been characterized by thermal analysis (1–8), no studies have been conducted to investigate the permeability of these bilayers. In the present report, the leakage of the water-soluble dye, carboxyfluorescein, has been determined at several temperatures about the phase transition of the symmetric and asymmetric phospholipids.

MATERIALS AND METHODS

Phospholipid Synthesis. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphoylcholine (16/16 PC) was synthesized by a modified procedure of Mason *et al.* (9) and purified by preparative HPLC on radially pack silica gel columns using a mobile phase of 65:35:5 (v/v) chloroform:methanol:water (10). The asymmetric phosphatidylcholine was synthesized by acylation of the lyso derivative, obtained by reaction of the symmetric lipid with phospholipase A_2 (from *Crotalus adamanteus;* Sigma, St. Louis, MO). The phospholipid was purified by silica column chromatography (Bio-Sil A, 100–200 mesh, Bio-Rad, Richmond, CA) in a graded methanol/chloroform solvent system. To incorporate a negative charge on the bilayer surface with minimally affecting the phase transition, a portion of the phosphatidylcholines was converted to the corresponding phosphatidylglycerol (PG) by exchange of the head groups using phospholipase D as described by Papahadjopoulos *et al.* (11). The resulting phosphatidylglycerol was separated from the phosphatidylcholine by silica column chromatography.

The purified, asymmetric phospholipid moved as a single spot on thin-layer chromatography (TLC) with a mobile phase of 65:35:5 chloroform:methanol:water, indicating the absence of any lyso derivative. The PC was mixed with the PG in chloroform, and ^{31}P nuclear magnetic resonance (NMR) spectroscopy (12) was used to quantify the relative lipid concentration. Additional lyophilized PC was then added to bring the mole ratio of phosphatidylcholine to phosphatidylglycerol to 9:1.

Vesicle Preparation and Characterization. Phospholipid vesicles were prepared as follows (13). A 10-mg sample of lyophilized phospholipid was hydrated with 2 ml of a 50 m*M* carboxyfluorescein [5(6)-carboxyfluorescein, mixed isomers; Sigma] solution which was adjusted to pH 7.5. A uniform size distribution of vesicles was formed by passing the dispersion through filters in a series of decreasing pore sizes (2, 1, 0.6, and 0.2 μm) with an Extruder (Lipex, Biomembranes Inc. Vancouver, BC, Canada) under nitrogen pressure, while the Extruder was placed in a water bath to maintain the temperature above the phase transition of the phospholipids. It was also found to be necessary to anneal vesicles composed of 16/16 PC by incubating in a water bath above the phase transition to obtain the minimum leakage. Incubation of the asymmetric phospholipid vesicles had no effect on the release characteristics. The size distribution of the vesicles was determined with a Nicomp 370 sizer (HIAC/RAYCO Instrument, Menlo Park, CA). The number of lipids exposed to the external solution was quantified for 16/16 PC by ^{31}P NMR as described previously (14). The vesicles composed of 20/12 PC were also analyzed by freeze-fracture electron microscopy without any cryoprotectant (15).

Thermal Analysis. High-sensitivity differential scanning calorimetry (DSC) was performed on both the multilamellar and the extruded vesicles using a MC-2 ultrasensitive scanning calorimeter (MicroCal, Northampton, MA). Samples were prepared with a phospholipid concentration of 2 mg/ml.

Leakage Studies. The extent of leakage of carboxyfluorescein was determined by a modification of the procedure of Ohno *et al.* (16). The external dye solution was removed by passing a 200-μl aliquot of the vesicle dispersion described above through 4 ml of Sephadex G-50 loaded in a

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disposable 5-ml pipette. The mobile phase consisted of 50 m*M* sodium phosphate buffer, pH 7.5, and 2 m*M* EDTA made isotonic with 160 m*M* mannitol. This results in an estimated osmotic pressure difference of 150 mOsm across the lipid bilayer, which should enhance the leakage rate. Of the excluded volume, 2 ml was collected and rapidly dispersed into 18 ml of buffer identical to the mobile phase of the column thermally equilibrated to the desired temperature. This was taken as the start of the release. Thereafter 1-ml aliquots were taken at periodic time intervals, and the fluorescent intensity was determined both before and after the addition of 200 μl of 5% sodium dodecyl sulfate (SDS). The percentage release was taken as the ratio of the fluorescent intensity before and after solubilization of the vesicles along with corrections for the initial intensity and the dilutional effect of the SDS as described (16).

RESULTS

The results of the DSC of the multilamellar dispersions are shown in Fig. 1. Lipid dispersions of 16/16 PC produced a relatively sharp endotherm with a peak at 41.6°C and a broader endotherm with a peak at 35°C, which are in good agreement with accepted values of the gel-to-liquid crystalline and intermediate phase transition temperatures (3). For 20/12 PC, only one endotherm was observed, with an estimated phase transition of 34.1°C. This transition temperature is slightly higher (33.2°C) and more narrow than that reported by Xu and Huang (3) for the gel-to-liquid crystalline phase transition of this asymmetric phospholipid. These results provide strong evidence for the purity and structural integrity of the multilamellar dispersions.

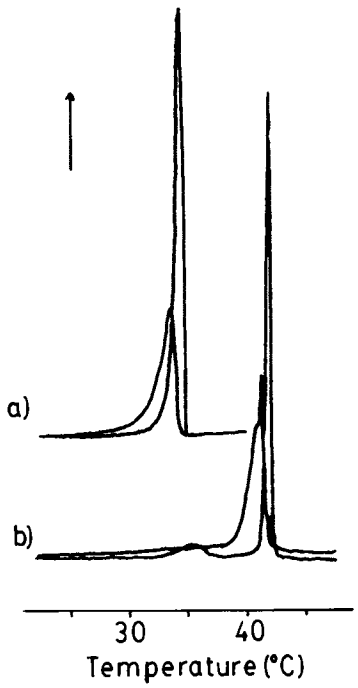

Fig. 1. High-sensitivity differential scanning calorimetric results of multilamellar dispersions (sharper and higher phase transition temperature) and extruded (broader and lower temperature) vesicles of (a) 20/12 PC and (b) 16/16 PC obtained at a scan rate of 20°/hr.

Thermal analysis of extruded vesicles resulted in endotherms which were broadened relative to that obtained with the multilamellar systems as shown in Fig. 1. For 16/16 PC, the endotherm rises from the baseline at about 38°C. In addition, two peaks are evident, with the lower-temperature peak appearing as a shoulder with a peak temperature of 40.7°C. The higher-temperature peak is sharper and has a peak temperature of 41.1°C. For the 20/12 PC extruded vesicles, the peak transition was lowered from 34 to 33°C, but only one peak was observed.

The method of vesicle preparation is believed to have led to a uniform distribution of sizes in the vesicles. The mean diameter for 16/16 PC was 220 ± 90 nm and that for 20/12 PC was 210 ± 85 nm. The concentration of vesicles actually used in the release studies is too low for determining the size, so these results are based on experiments conducted with higher vesicle concentrations. No effect of concentration on the resulting size of the vesicles has been seen (17).

From the ^{31}P NMR, the addition of excess manganese ions to 16/16 PC resulted in a 30% reduction in the intensity of phosphorus moiety of the phospholipid relative to that in the internal standard. This indicates that no more than 60% of the vesicles are unilamellar, since for vesicles with diameters of 220 nm, there is approximately 10% more lipids in the outer bilayer relative to the inner bilayer, in agreement with the results found by Cullis and his co-workers (14). Alternatively, this corresponds to two bilayers for each vesicle assuming a uniform 220-nm diameter and 50-Å water space intervening between the two bilayers.

Finally, freeze fracture was performed with MLVs of 20/12 PC, with a representative result shown in Fig. 2. No continuously smooth planes are observed, which is an indication that the bilayer structure is such that fracture does not readily occur through the center of the bilayer. Similar results are observed with partial interdigitated and mixed-chain partial interdigitated phospholipid systems (3).

The percentage release of carboxyfluorescein was determined for each type of lipid at temperatures about the phase transition. This technique relies on the quenching of the fluorescence of carboxyfluorescein when contained within the vesicle and the subsequent observation of fluorescence when carboxyfluorescein is released and the concentration is diluted by about four orders of magnitude. For both types of vesicles, little release was seen at a temperature of 25°C. However, for 16/16 PC at 42°C, there was relatively rapid release over a 2-hr time period (Fig. 3). This result is in agreement with the recent study by Ohno *et al.* (16) and McKersie *et al.* (18), who monitored the release of carboxyfluorescein from sonicated vesicles composed of 16/16 PC. At longer times, the extent of release observed at 48°C was somewhat reduced relative to that at 42°C. Also in Fig. 3, the percentage release of carboxyfluorescein from 20/12 PC vesicles is given. At 33°C, which corresponds to the phase transition, there was release but at a slower rate. The percentage release at 48°C as a function of time was similar to that obtained at 33°C.

Figure 4 contains a compilation of the percentage release at 1 hr as a function of temperature for the two types of vesicles, with the arrows indicating the phase transition temperature. It is apparent that the vesicles composed of 16/16

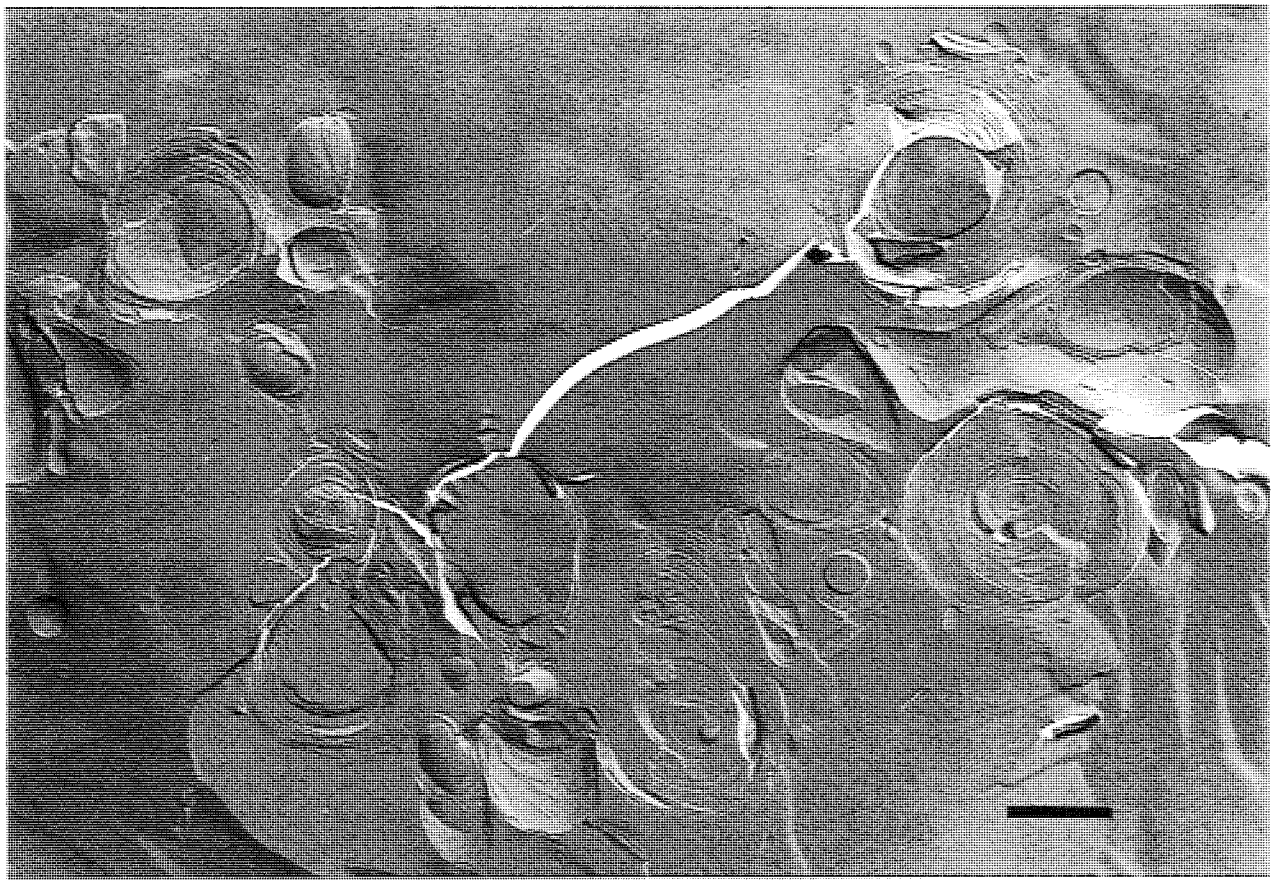

Fig. 2. Freeze-fracture micrograph of 20/12 PC. The sample was frozen in liquid propane from 37°C without cryoprotectant. The bar represents 800 nm. Cross fractures, which were predominant below the T_m as well (data not shown), result from the loss of a bilayer midplane through which fracture faces are revealed.

PC have greater release at temperatures of 37°C and greater. Conversely, 20/12 PC vesicles have lower release at all temperatures relative to the symmetric acyl chain phosphatidylcholine system under study.

DISCUSSION

The leakage of water-soluble compounds about the phase transition of symmetric-acyl chain dipalmitoylphosphatidylcholine has been studied rather extensively (11,19–25). In addition, several investigators have provided theoretical interpretations accounting for this behavior of membranes (26–28). These computational models have generally elaborated upon the original suggestion put forth by Papahadjopolous *et al.* (11) that the boundary domains between the gel and the liquid crystalline lipids are responsible for the leakage. Recent success has been obtained by correlating the observed leakage with the area of the disordered region between the gel and the liquid crystalline lipids (28). The distinct feature of this model was that although this intermediate permeable area is temperature dependent, the magnitudes of the permeabilities were not temperature dependent. It should be clear, however, that the nature of the transbilayer movement of water-soluble compounds may occur by a mechanism unrelated to transport of solutes across lipid bilayers in the gel or liquid crystalline state.

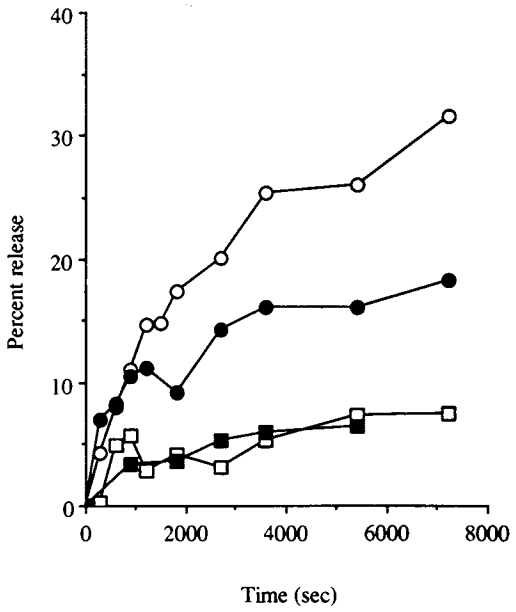

Fig. 3. Percentage release of carboxyfluorescein as a function of time from vesicles composed of 16/16 PC at temperatures of (○) 42°C and (●) 48°C and vesicles of 20/12 PC at temperatures of (□) 34°C and (■) 48°C.

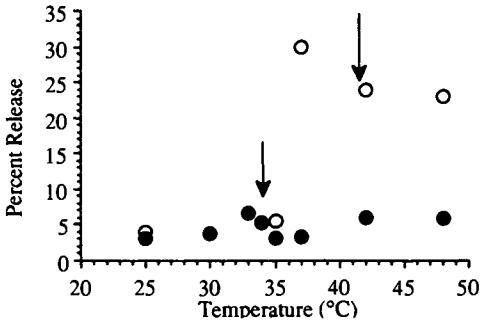

Fig. 4. Percentage release of carboxyfluorescein at 1 hr as a function of temperature for vesicles composed of (○) 16/16 PC and (●) 20/12 PC. The arrows indicate the midpoint of the gel-to-liquid crystalline phase transition.

The leakage results for 16/16 PC in this study are qualitatively consistent with those found with other water-soluble compounds as well as those studies which have specifically determined the release of carboxyfluorescein from 16/16 PC (16,18). Quantitative agreement is not expected, since these studies used sonicated vesicles in the absence of osmotic gradients, whereas in this report extruded vesicles were used along with an estimated osmotic gradient of 150 mOsm. With these experimental differences, it is of interest to note that McKersie *et al.* (18) found the rate of release of carboxyfluorescein was constant with time and that complete release was observed. Kinetic data presented by Ohno *et al.* (16) indicated a more exponential release profile with a variable plateau of percentage released. The latter results were more in line with the type of release patterns seen in this study; see Fig. 3.

Although it is not possible to resolve the differences between the results of McKersie *et al.* (18) and those of Ohno *et al.* (16), the release pattern observed in this study may indicate the polydispersity of the size distribution of the vesicles. The explanation is that small vesicles are inherently more leaky due either to different areas of intermediate lipid domains or to intermediate domains that are more permeable. The latter may be a result of the smaller radius of curvature, which is known to disrupt the packing of the lipid acyl chains (29). This is also reflected in the high-sensitivity DSC as shown in Fig. 1. After extrusion, the phase transition temperature is lowered and broadened. Previous investigators had shown that the phase transition was not appreciably affected unless the vesicle diameter was less than about 70 nm (29). Obviously, with extrusion and the resulting polydispersity, the phase transition is altered even with average diameters of greater than 200 nm.

With vesicles composed of asymmetric lipids, relatively little release is observed at all temperatures experimentally explored. Even at the phase transition, only about 5–10% of the encapsulated dye is released after 2 hr. In contrast to the 16/16 PC system, these lipid vesicles would appear to exhibit intermediate domains between the gel and the liquid crystalline state lipids which are not either sufficiently large or sufficiently extensive to allow readily the passage of carboxyfluorescein. The most reasonable explanation would appear to rest with the packing of the acyl chains and/or head groups in these asymmetric lipid bilayers.

Huang and co-workers (8) have examined an extensive series of phosphatidylcholines and found that those lipids in a class of $0.44 < \Delta CL/L < 0.56$ form mixed-chain partial, interdigitated lipid bilayers. For the 20/12 PC used in this study, the value of $\Delta CL/L$ is $0.56 = [19 - (11–1.5)]/19 = 0.50$, where ΔCL is the difference in the acyl chain length between the short and the long chain after correction for the shorter sn-2 chain (1.5 carbon units) and L is the length of the longer acyl chain less the carbon in the carbonyl moiety (e.g., see Ref. 5). Thus, 20/12 PC would be expected to form a partial mixed-chain interdigitated bilayer. The two interesting features of this type of interdigitation are that it is structurally more ordered than noninterdigitated bilayers and the interdigitation appears to persist even in the liquid crystalline phase (3).

From the perspective of drug delivery, these differences in permeability may be exploited for controlled drug release. Specifically, for 16/16 PC, appreciable leakage occurs at temperatures above the phase transition. However, the 20/12 PC represents a resilient membrane system that appears to be relatively resistant to leakage at all temperatures.

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