Effect of Head Group on Phospholipid Mixing in Small, Unilamellar Vesicles: Mixtures of Dimyristoylphosphatidylcholine and Dimyristoylphosphatidylethanolamine†

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ABSTRACT: The phase behavior of small, single-walled vesicles composed of mixtures of 1,2-dimyristoyl-3-*sn*-phosphatidylcholine (DMPC) and 1,2-dimyristoyl-3-*sn*-phosphatidylethanolamine (DMPE) has been studied using the depolarization of the fluorescence of 1,6-diphenyl-1,3,5-hexatriene to follow phase changes within the phospholipid bilayer. A partial phase diagram for this system has been constructed using previously established techniques. In addition, the distribution of phosphatidylethanolamine between the two monolayers of these small highly curved vesicles was determined by chemical assay with 2,4,6-trinitrobenzenesulfonic acid. These data, along with observed temperature-induced changes in light scattering, have been interpreted in terms of a hypothetical phase diagram. It is concluded from this phase diagram that phosphatidylethanolamine/phosphatidylcholine mixtures show increased immiscibility in the gel phase of small, single-walled vesicles compared to large multilamellar vesicles. This is probably due to the gel phase instability of highly curved structures of high phosphatidylethanolamine concentration. The data also demonstrate that the difference between the phosphatidylcholine and phosphatidylethanolamine head groups is not sufficient to induce lateral phospholipid domain formation in the L_α phase of the small vesicles. Previous studies have shown that small differences in the fatty acid chains of phosphatidylcholines were sufficient to bring about this effect. DMPE is found to distribute asymmetrically in the two monolayers of the DMPE–DMPC vesicles. It is concluded that the nature of the asymmetric distribution of DMPE between the two monolayers of these small, single-walled vesicles is the result of the combined properties of acyl side-chain constituents and head-group packing requirements of the phospholipids involved.

The mixing of different phospholipids has been studied mainly in large, multilamellar vesicles of the type described by Bangham (1968). Such studies have led to general conclusions about the effects of acyl chain length and the phospholipid head group on the lateral mixing and phase behavior of phospholipids in a membrane (Shimshick and McConnell, 1973; Chapman et al., 1974; Lentz et al., 1976b). However, the phase behavior of small, single-lamellar vesicles composed of mixtures of phosphatidylcholines recently has been shown to differ considerably from that of large, multilamellar vesicles of similar composition (Lentz et al., 1976b). In particular, phase diagrams for such small, highly curved vesicles suggest that lateral domain formation may be likely in mixtures of phosphatidylcholines incorporated into these structures. Because regions of comparable curvature are not uncommon in biological membranes (Thompson et al., 1974), an understanding of the peculiar properties of these model membranes may have particular relevance to an understanding of the function of biological membranes. Interest in small, unilamellar vesicles as possible drug-delivery vehicles (Tyrrell et al., 1976) also demands a careful examination of the physical properties of these structures as a function of lipid composition.

It is our intent to extend earlier studies of phospholipid mixing in small, single-lamellar vesicles to obtain an understanding of the effect of phospholipid head-group structure on the phase behavior of highly curved membranes. To this end, we report here the behavior of mixtures of 1,2-dimyristoyl-3-*sn*-phosphatidylcholine (DMPC[1]) and 1,2-dimyristoyl-3-*sn*-phosphatidylethanolamine (DMPE) incorporated into small, single-walled vesicles. Except for the differences in the head group, these two glycerophosphatides have identical molecular structure. In future studies, mixtures of phosphatidylcholines with sphingomyelins, phosphatidylglycerols, and phosphatidylserines will be considered.

A potentially important aspect of phospholipid mixing within the bilayers of small, single-lamellar vesicles is the possibility of transbilayer compositional asymmetry introduced by differences in packing between the two halves of the bilayer. Litman (1973) first reported an asymmetric distribution of egg yolk phosphatidylcholine and egg yolk phosphatidylethanolamine between the two monolayers of small, single-walled vesicles. Although there is no evidence for an asymmetric distribution of saturated, synthetic phosphatidylcholines in such model membrane vesicles (Lentz et al., 1976b), transbilayer asymmetry is sure to play a role in determining the phase behavior of DMPC/DMPE small, single-lamellar vesicles.

Materials and Methods

Synthetic Phospholipids. DMPC was synthesized by the method of Robles and Van den Berg (1969) and purified by silicic acid chromatography, ethylenediaminetetraacetic acid treatment, and recrystallization, as described previously (Lentz et al., 1976a). Purified DMPC was stored under Ar atmo-

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[1] Abbreviations used: DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; DMPE, 1,2-dimyristoyl-3-*sn*-phosphatidylethanolamine; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DPPE, 1,2-dipalmitoyl-3-*sn*-phosphatidylethanolamine; DPH, 1,6-diphenyl-1,3,5-hexatriene; TNBS, 2,4,6-trinitrobenzenesulfonic acid.

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sphere as a solution in spectral or "HPLC" grade chloroform and was found to be indefinitely stable when so stored.

DMPE was purchased from Fluka AG, Switzerland, and found to contain approximately 50% (w/w) of compounds that did not chromatograph as phosphatidylethanolamine. Therefore, the commercial product was purified by chromatography on DEAE-cellulose (Rouser et al., 1965).

The purity of DMPC was established by thin-layer chromatography on Quantum Q5 plates activated for 1 h at 100 °C before use, using the solvent chloroform–methanol–water (65:25:4). As described by Suurkuusk et al. (1976), heavy loading of the chromatograms was employed in order to detect impurities present at the level of only 1% contamination. Iodine staining of the chromatogram showed only one heavy spot at the R_f of standard saturated phosphatidylcholine and a second very faint spot having an R_f slightly larger than phosphatidylcholine. No other spots were observed. The level of this impurity was estimated to be about 1% by scraping the chromatography plate and detecting phosphate by a modification of the method of Chen et al. (1956). The purity of DMPE was determined by thin-layer chromatography on E. Merck F254 precoated plates, using chloroform–methanol–water (65:25:4). Plates were loaded at the 1-μmol level and stained with both ninhydrin and iodine.

Single-Lamellar Vesicles. Mixtures of DMPC and DMPE were prepared in chloroform and dried overnight under vacuum. Phospholipid suspensions were then prepared by adding the aqueous phase and agitating for 1 min at 55–60 °C; this is above the phase transition of both phospholipid components. The aqueous medium contained 50 mM KCl (ultrahigh purity, Heico) dissolved in glass-distilled water (Lentz et al., 1976a). Small, single-lamellar vesicles were prepared in 50 mM KCl by subjecting a phospholipid suspension to ultrasonic irradiation at 55–60 °C, using a Heat Systems W-350 sonifier. Large phospholipid structures were removed from the sonicate by ultracentrifugation (Barenholz et al., 1977). The appropriate centrifugation conditions were determined by centrifuging vesicle preparations at increasingly higher speeds and for increasingly longer times until the resulting small vesicles had a constant ratio of outer monolayer amino groups to total amino groups (see "Determination of Phosphatidylethanolamine Distribution" in this section). The appropriate conditions varied from 168 000*g* for 90 min, determined for pure phosphatidylcholine vesicles by Barenholz et al. (1977) using autocorrelation light scattering, to 168 000*g* for 15 min for 70 mol % DMPE vesicles. Barenholz et al. (1977) demonstrated that phospholipid vesicles prepared in this way were of uniform small size. At all times, except during the centrifugation procedure, the vesicle samples were maintained above their phase-transition temperature. The pH of vesicle suspensions prepared in this way was in the range 6.5–7.0.

Determination of Phosphatidylethanolamine Distribution. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was used to determine the phosphatidylethanolamine in the outer monolayers or in the entire vesicle sample, according to a modification of the originally reported procedure (Litman, 1973). All assays were carried out at 55 °C, so as to be above the phase transitions of both phospholipids. The outer-surface phosphatidylethanolamine content was determined by the following procedure. An aliquot of an aqueous vesicle solution (containing no more than 0.25 μmol of amino groups) was diluted to a final volume of 0.6 mL with the vesicle buffer solution. An addition of 0.2 mL of 0.8 M $NaHCO_3$ (pH 8.5) was made, and the vesicle sample was mixed. A 20-μL aliquot of 1.5% TNBS was added to this solution. The sample was mixed and allowed to incubate in the dark for 30 min at 55 °C. After the incubation period, 0.4 mL of 1.2% Triton X-100 in 1.5 N HCl was added to the sample, followed by mixing and storage in the dark. The absorbance at 410 nm was read within an hour after acidification. The total vesicle phosphatidylethanolamine content was determined by the following procedure. An aliquot of an aqueous vesicle solution (containing no more than 0.25 μmol of amino groups) was diluted to a final volume of 0.6 mL with the vesicle buffer solution. An addition of 0.2 mL of 1.6% Triton X-100 in 0.8 M $NaHCO_3$ (pH 8.5) was made and the sample mixed. A 20-μL aliquot of 1.5% TNBS was then added to this solution. The sample was mixed and allowed to incubate in the dark for 30 min at 55 °C. After the incubation period, 0.4 mL of 0.4% Triton X-100 in 1.5 N HCl was added to the sample, followed by mixing and storage in the dark. The absorbance at 410 nm was read within an hour of acidification. The absorbance at 410 nm was linear with concentration of the amino group to at least 1.5 absorbance units, with a slope of 4.52 absorbance units per micromole of amino groups.

Fluorescence Measurements. Details of the fluorescence measurements are described elsewhere (Lentz et al., 1976a) and we shall limit our description to a simple definition of the fluorescence-derived quantities used in this paper. The anisotropy of DPH fluorescence was taken as a measure of the hydrocarbon chain motion in the hydrophobic region of the bilayer, according to the interpretation of Kawato et al. (1977). The "microviscosity" was calculated from the anisotropy, and the estimated fluorescence lifetime of DPH (Lentz et al., 1976a). As noted previously (Lentz et al., 1978), "microviscosity" need not be related to bulk viscosity but should be understood only to be a convenient parameter describing DPH motion. The derivative of the natural logarithm of "microviscosity" with respect to reciprocal temperature is referred to as the "microviscosity activation energy" (Lentz et al., 1978) and is expected to be constant for a single-component, single-phase system (Lentz et al., 1976a). The depolarization of DPH fluorescence was recorded for each sample in cooling scans at an average scan rate of 25 °C/h. All temperature scans were cooling scans, since storage of vesicles with a high DMPE content below their phase transition resulted in nearly instantaneous vesicle flocculation and concomitant increase in 90° light scattering (see Results). Phase-transition ranges of the vesicle samples were determined from plots of fluorescence anisotropy vs. temperature and from plots of the effective "microviscosity" within the bilayer (Lentz et al., 1976a) against the reciprocal absolute temperature. In addition, plots of the "microviscosity activation energy" vs. temperature were used to better define the transition (Lentz et al., 1978). In general, all three methods gave comparable results. When differences arose, weight was given to the "microviscosity" or the "microviscosity activation energy", since these methods have shown closest correspondence to differential scanning calorimetric results (Suurkuusk et al., 1976; Lentz et al., 1978).

Results

Figure 1 illustrates two of the methods used here to detect the phase transition in DMPE/DMPC small, single-lamellar vesicles. Figure 1A shows the rapid drop in DPH fluorescence anisotropy associated with the phase transition in two DMPE/DMPC vesicle samples. Data points taken as defining the limits of the phase transition are indicated by arrows. In Figure 1B, the "microviscosity activation energy" is plotted vs. temperature, and the delimiting temperatures of the phase change are indicated as the temperatures at which the "activation energy" rises above the noise of the base line. This figure also illustrates the dual peaks observed for many of the small

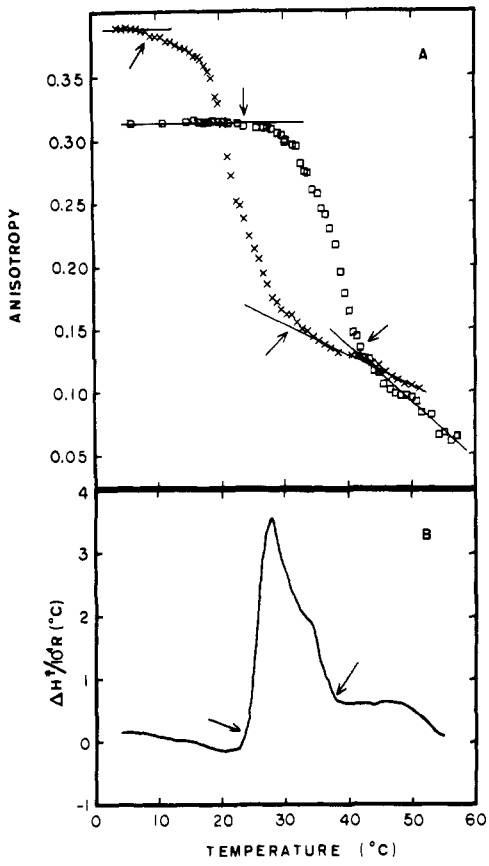

FIGURE 1: Phase changes in small, single-lamellar vesicles composed of DMPE/DMPC mixtures. (A) Detected by changes in DPH fluorescence anisotropy: (X) 12 mol % DMPE; (□) 66 mol % DMPE; arrows indicate the delimiting temperatures of the phase transition (see text). (B) Detected by changes in the "microviscosity activation energy", derived from DPH fluorescence anisotropy. Curve is for 35 mol % DMPE, with arrows indicating the delimiting temperatures of the phase transition (see text). The main peak is at 27–28 °C; the shoulder is at 33–34 °C.

vesicle samples consisting of mixtures of DMPE and DMPC. The results in Figure 1B are for a mixture containing 35 mol % DMPE, which shows a main peak in its phase transition at 27–28 °C and a clear shoulder at 33–34 °C. In Figure 2A, we have plotted the temperatures that correspond to the main peak and the shoulder for each of the mixtures studied. It should be noted that there is a peak or shoulder at 26 to 28 °C in nearly every mixture studied (the 19 and 27 mol % DMPE samples had no definable shoulder). The delimiting temperatures for the phase changes in each phospholipid mixture are plotted in Figure 2B. The nearly horizontal portion of this phase diagram between about 30 and 70 mol % DMPE is particularly noteworthy (see Discussion).

Because of the very broad nature of the phospholipid phase transition, the delimiting-temperature plots in Figure 2B do not have the appearance of classical phase diagrams (Marsh, 1935). This problem has been noted previously by Lee (1975), Lentz et al. (1976b), and Mabrey and Sturtevant (1976). The latter authors suggested that the solidus and liquidus curves be arbitrarily raised and lowered sufficiently to make the phase transitions of the pure phospholipids appear sharp, in order to aid in the interpretation of the phase diagram. This procedure has been applied to the data in Figure 2B to yield the solid curves shown in Figure 2C. This hypothetical, idealized phase diagram shows the phase transitions of mixtures of DMPE and DMPC to be barely broader than that of pure DMPC up to almost 20 mol % DMPE, then to broaden more in the range 20–35 mol % DMPE, and, finally, to broaden appreciably

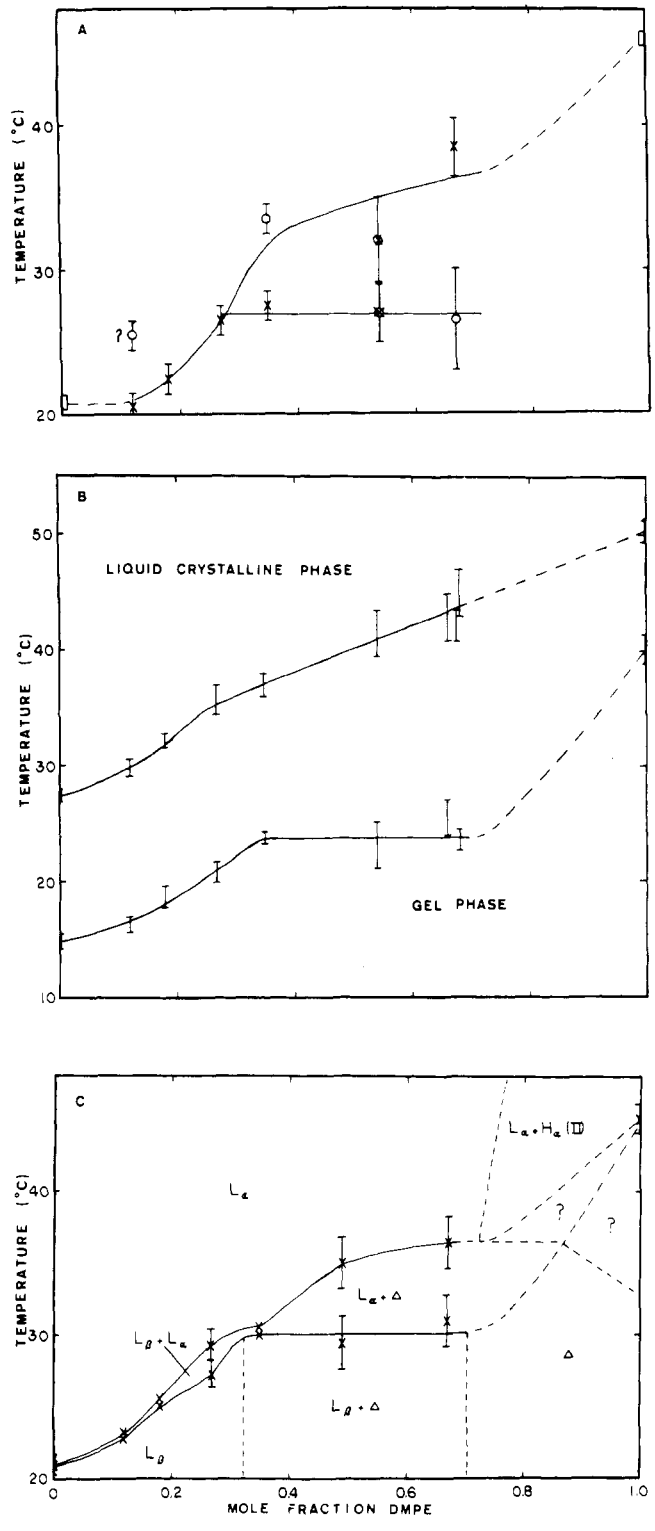

FIGURE 2: Phase behavior of DMPE/DMPC small, single-lamellar vesicles. (A) Positions of main peaks (X) and shoulders (O) in plots of activation energy through phase changes in DMPE/DMPC small, single-lamellar vesicles (see Figure 1). The main peak and shoulder for the 54 mol % sample were ill resolved: (□) the peak position in pure DMPC and the estimated peak position that might be expected to occur in small vesicles composed of DMPE (data from Chapman et al., 1974). (B) Plot of delimiting temperatures of the phase change in small vesicles vs. vesicle composition. (C) Small vesicle hypothetical phase diagram. Portions of the "solidus" and "liquidus" curves (—) were derived from the data in Figure 2B by adding 6.5 °C to the points on the solidus curve and subtracting 6.5 °C from the points on the liquidus curve. This procedure yields the correct peak temperature for the phase transition in pure DMPC small, single-lamellar vesicles (Lentz et al., 1976a). Error estimates (±1 standard deviation) are shown only when they do not obscure the position of the data points. The dashed curves are implied by our and others' data, as described in the text.

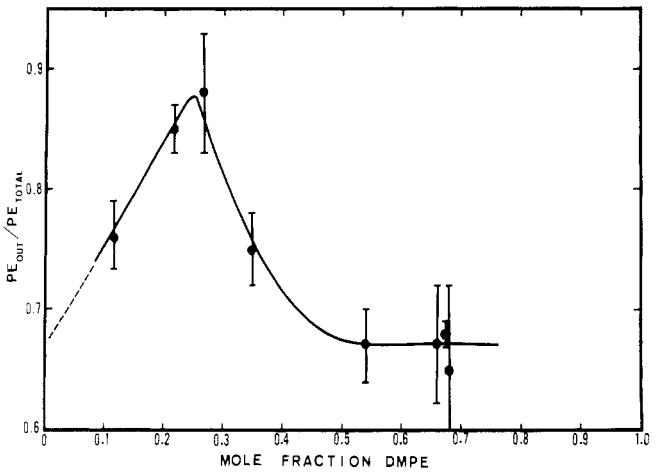

FIGURE 3: Ratio of the outer monolayer phosphatidylethanolamine to the total phosphatidylethanolamine as a function of the small, single-walled vesicle composition. Errors are represented as ±1 standard deviation.

TABLE I: Mole Fractions of DMPE in the Outer and Inner Monolayers of DMPE/DMPC Vesicles.

mol % DMPE	X_{DMPE}^{out}	X_{DMPE}^{in}	$\frac{X_{DMPE}^{out}}{X_{DMPE}^{in}}$
0.12 ± 0.003	0.14 ± 0.004	0.084 ± 0.007	1.7 ± 0.2
0.22 ± 0.002	0.28 ± 0.02	0.107 ± 0.007	2.7 ± 0.4
0.27 ± 0.01	0.36 ± 0.02	0.098 ± 0.03	3.7 ± 1.3
0.35 ± 0.01	0.39 ± 0.01	0.28 ± 0.02	1.4 ± 0.1
0.54 ± 0.02	0.54 ± 0.02	0.56 ± 0.04	1.0 ± 0.1
0.66 ± 0.02	0.66 ± 0.04	0.68 ± 0.06	1.0 ± 0.1
0.68 ± 0.06	0.69 ± 0.04	0.68 ± 0.10	1.0 ± 0.2
0.68 ± 0.02	0.66 ± 0.05	0.74 ± 0.07	0.9 ± 0.2

beyond 35 mol % DMPE, in which region the solidus curve is horizontal. This broadening suggests considerable nonmiscibility of DMPE and DMPC in small, single-lamellar vesicle bilayers at higher mole fractions of DMPE. The dashed curves in Figure 2C have been constructed according to a number of additional observations (see Discussion).

The partitioning of DMPE and DMPC between the two monolayers of small, single-lamellar vesicles is illustrated in Figure 3, where the mole ratio of outer monolayer DMPE to total DMPE is plotted as a function of the composition of the vesicles. This ratio is seen to increase with increasing mole percent DMPE, with the maximum effect being observed at about 25 mol % DMPE. At higher mole percentages of DMPE, the ratio of outer monolayer to total phosphatidylethanolamine decreased and eventually approached 0.67. A similar value was obtained by extrapolating the data at low mole percentages of DMPE to pure DMPC. This value is close both to the value of 0.7 assumed by Litman (1973) to reflect the geometry of egg yolk phosphatidylcholine/phosphatidylethanolamine vesicles and to the range of values later calculated for the egg phosphatide mixture using data obtained from quasielastic light-scattering measurements (Goll et al., 1978). Although we cannot determine precisely the size of our vesicles, if we assume that 0.67 is the ratio of total phospholipids in the outer monolayer to the total phospholipids in the vesicle, it becomes possible to calculate the mole fraction of DMPE and DMPC in each of the two monolayers of the vesicles, using the following relationship:

$$X_{DMPE}^{out} = \frac{RX_{DMPE}}{0.67}$$

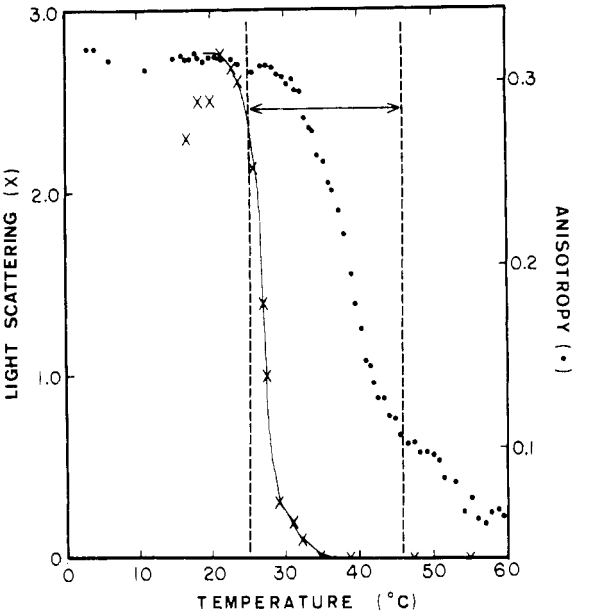

FIGURE 4: Variation of the 90° light scattering (X) and DPH fluorescence anisotropy (●) as a function of temperature through the phase change in DMPE/DMPC (66:34) small, single-walled vesicles. Intensity of scattered light at 430 nm is recorded in arbitrary units. Incident light was centered at 360 nm with a 6-nm width at half-height.

Here, R is the ratio of outer monolayer DMPE to total DMPE (i.e., from Figure 3) and X_{DMPE} is the mole fraction of DMPE for a given small, single-lamellar vesicle preparation. Values for X_{DMPE}^{out} and X_{DMPE}^{in}, calculated in this way, are tabulated in Table I. In a 27 mol % DMPE vesicle, DMPE partitions nearly four times more to the outer than to the inner monolayer.

It is worthy of note that small, single-lamellar vesicles containing large amounts of DMPE appeared to become very unstable within the temperature range in which they underwent phase separation. The evidence for this is the very large, irreversible increase in 90° light scattering observed as the vesicle sample was cooled (see Figure 4). Below 35 mol % DMPE, this increase in scattering was not associated with appreciable depolarization of the incident light. This behavior is not inconsistent with the behavior of pure phosphatidylcholine small vesicles (Lentz et al., 1976a). At and above 35 mol % DMPE, however, the scattered light was appreciably depolarized and the sample became noticeably flocculent below the phase transition. These observations would be consistent with the occurrence of a structural change in DMPE-rich small vesicles that would change the shape of the vesicles, while the low DMPE data could be explained by a simple change in the refractive index of the vesicles induced by the phase change. In addition, the flocculation of DMPE-rich vesicles was very rapid and clearly visible, as opposed to the slow ($t_{1/2} \sim 5.4$ h) and barely perceptible structural changes previously reported for phosphatidylcholine small vesicles held below their phase transition (Suurkuusk et al., 1976). The rapid increase in light scattering seen in Figure 4 did not occur, however, until the phase separation, as detected by the fluorescence depolarization of DPH, was nearly complete (Figure 4). These results argue that the structural changes associated with the increase in 90° light scattering did not affect the onset of phase changes within the vesicles, as reported by fluorescence depolarization spectroscopy. However, it is reasonable to presume that the temperature of completion of the phase separation on cooling should have been influenced by these structural changes.

Discussion

Our results support the picture of incomplete miscibility of DMPE and DMPC within the gel phase of small, single-lamellar vesicles. This picture is based on both the shape of the phase diagram of Figure 2B and the analysis of peak positions in plots of "microviscosity activation energy" vs. temperature (Figure 2A). If the phase rule applies to our small vesicle dispersions, then the invariant, horizontal boundary line in Figure 2B must separate two two-phase regions, one above and one below the line (Yeh, 1970). In large, multilamellar vesicles composed of mixtures of DMPE and DMPC, Chapman et al. (1974) reported a phase diagram having a nearly horizontal solidus line between 0 and about 30 mol % DMPE. In studies of mixtures of 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) with 1,2-dipalmitoyl-3-*sn*-phosphatidylethanolamine (DPPE) incorporated into large, multilamellar vesicles, Shimshick and McConnell (1973) and Lee (1975) reported similar nearly horizontal solidus curves extending from 0 to ~30 and ~20 mol % DPPE, respectively. Although Lee cautioned against interpreting such delimiting-temperature plots as phase diagrams for microscopic systems of limited extent, both he and Shimshick and McConnell ascribed the observed horizontal solidus line to immiscibility of the two lipids in the gel phase. In addition to the implications of the shape of our phase diagram, the existence of two overlapping peaks for some vesicle preparations implies the coexistence of two domains in one of the phases present. In addition, the persistence of the 26–28 °C peak for all lipid mixtures above 27 mol % DMPE indicates that at least some gel-phase lipid domain having this phase transition temperature is formed in each single-lamellar vesicle sample containing more than 27 mol % DMPE. The fact that this peak was the only peak detected at 27 mol % DMPE suggests that the composition of these persistent gel-phase domains is not far from 27 mol %.

These results, as well as our light-scattering results, can be explained by the hypothetical phase diagram given in Figure 2C. At low DMPC content, the "solid" phase is pictured as being of the L_β type, as has been suggested for the "solid" phase of pure DMPC small vesicles (Hui, 1976). Above about 30–35 mol % DMPE, a solid mixture is implied, as discussed above. The DMPE-rich component of this solid mixture (called Δ in Figure 2C) is of unknown structure but is presumed to be the phase responsible for the dramatic increase in depolarizing 90° light scattering in DMPE-rich small vesicles. The "liquid" phase is presumed to be of the L_α type up to about 70 mol % DMPE, beyond which composition we were unable to form stable, small, single-walled vesicles. For pure phosphatidylethanolamines, the "liquid" phase has been reported to be of the L_α or H_{II} types (Junger and Reinauer, 1969; Tinker and Pinteric, 1971). For this reason, we have postulated two "liquid" phases and, of necessity (Yeh, 1970; Marsh, 1935), the existence of a phase boundary separating the L_α region from the "liquid mixture" region. The reader may qualitatively verify this model by performing an imaginary cooling experiment at any composition of vesicles using the hypothetical phase diagram of Figure 2C and the tie-line method (Yeh, 1970; Marsh, 1935) to determine the phase composition of the system at any temperature. Such a "thought-experiment" demonstrates this model to be capable of explaining all of our observations, except the 25 °C shoulder observed in the "microviscosity activation energy" curve for 12 mol % DMPE (see "?" in Figure 2A). We have no satisfactory explanation for this shoulder, except to note that we have observed a similar shoulder in pure DMPC preparations that behaved as a homogeneous, small vesicle population by light-scattering criteria (Lentz et al., 1976a). Since the shoulder is not defined in the 19 mol % DMPE sample, it may be a feature of DMPC-rich small vesicles that fortuitously occurs close to the temperature range in which the 30–35 mol % DMPE species undergoes its phase change.

Although our tentative phase diagram does imply the existence of nonmixing gel-phase domains, the nature of the gel-phase nonmixing and the reasons for it would appear to be very much different in small vesicles than in large, multilamellar vesicles. Previously determined phase diagrams for multilamellar vesicles (Chapman et al., 1974; Shimshick and McConnell, 1973) suggested the existence of complex gel-phase structures below 30 mol % DMPE, likely involving two coexistent gel phases. If more recent conclusions from studies of DMPC/dimyristoylphosphatidylserine mixtures can be applied to DMPE/DMPC mixtures, this complex gel-phase structure likely results from the requirement for a P_β' phase at low DMPC content (Luna and McConnell, 1977). By contrast, the tendency of small vesicles to flocculate (see Figure 4) suggests that the reason for the existence of "solid mixtures" in our system might be due to an instability of highly curved bilayers containing any "solid" domain with more than about 30–35 mol % phosphatidylethanolamine. If this interpretation is correct, then the Δ phase in Figure 2C may not be a small vesicle form but rather a solidlike mesophase resulting from cooling liquid-crystalline small vesicles. This would make it incorrect to interpret Figure 2C as a "small vesicle" phase diagram. Indeed, since Δ may not be reconverted into the small vesicle L_α phase without sonication, the "solidus" line of Figure 2B or 2C may not be a true equilibrium phase line, at least at high DMPE content (greater than 30–35 mol %).

While doubts exist as to the interpretation of the "solidus" line in Figure 2C, no anomalous behavior was noted for this system in the neighborhood of the "liquidus" line. A notable feature of the liquidus line is the lack of any indication of liquid-phase immiscibility within the L_α state, such as was implied by the phase diagrams for mixtures of phosphatidylcholine species of different fatty acid content incorporated into small vesicles (Lentz et al., 1976b). These results imply that liquid-crystalline phase inhomogeneities in highly curved membrane regions would be more likely to be induced by differences in the fatty acid moiety of constituent phospholipids than by differences in the polar head-group regions, at least for two phospholipids as similar as phosphatidylcholine and phosphatidylethanolamine. This possibility is under further experimental investigation.

The asymmetric distribution of DMPE and DMPC between the two monolayers of small, single-lamellar vesicles also speaks to the contribution of acyl chains in determining the spatial distribution of phospholipids within highly curved membrane bilayers. The distribution of phosphatidylethanolamine obtained in the present study is very much different from the distribution that obtains in small vesicles composed of egg yolk phosphatidylethanolamine/phosphatidylcholine mixtures (Litman, 1973, 1974). Litman found a disproportionation of egg phosphatidylethanolamine toward the outer monolayer only at very low mole percentages of phosphatidylethanolamine (<10 mol %), while at higher mole percentages phosphatidylethanolamine he found an appreciable enrichment of phosphatidylethanolamine in the inner monolayer. Our data (Figure 3) show no significant disproportionation of DMPE to the inner monolayer at any mole percent DMPE. An equally dramatic difference is the fact that the maximum disproportionation of DMPE to the outer monolayer occurs in our studies at about 25–30 mol % DMPE, a shift to much higher phosphatidylethanolamine content relative to the egg phospholipid studies. These differences must clearly be due to the

differences in the acyl side-chain compositions of the phospholipids employed in this and the previous study of Litman.

There are at least two possible sources for these differences. First, egg yolk phospholipids contain a large proportion of unsaturated acyl side chains. This should result in the egg phosphatidylethanolamine having a more severe wedge shape (small polar head group and large acyl chain cross sectional areas) than the synthetic DMPE at comparable temperatures. Litman has proposed that the disproportionation of egg phosphatidylethanolamine to the inner monolayer of small vesicles may be due to the wedgelike shape of this molecule (Litman, 1973). If so, it is understandable that DMPE would not be so strongly driven to the inner monolayer as the egg phospholipid. This would allow other factors, such as a slightly negatively charged head group at neutral pH, to force the DMPE to the outer monolayer. It could then be argued that only at high DMPE content would its slight wedge shape provide sufficient driving force to overcome the charge effect and drive the phosphatidylethanolamine to the inner monolayer. This picture also explains the outer-monolayer enrichment of egg phosphatidylethanolamine observed by Litman (1974) at very low phosphatidylethanolamine content. A second possible explanation for the different asymmetry of the egg and synthetic systems is that the more highly unsaturated acyl chains of egg phosphatidylethanolamine may better accommodate this lipid, as opposed to egg phosphatidylcholine, to the packing constraints of a small vesicle inner monolayer. However, Litman (1975) observed that the acyl chain compositions of egg phosphatidylethanolamine were identical for lipid located in either monolayer of the mixed egg phosphatide small vesicles. This observation argues against the second explanation for rationalizing the different asymmetries observed in egg and synthetic phosphatidylcholine/phosphatidylethanolamine small vesicles but does not necessarily preclude it.

The current data do not distinguish between the two possibilities outlined above. It is worth noting, however, that the fatty acid composition of phosphatidylcholine/phosphatidylethanolamine small, single-lamellar vesicles does have an effect on the asymmetric distribution of phospholipids across the bilayer. Thus, it is not possible to measure the transbilayer distribution of phospholipids of one fatty acid composition and extrapolate this distribution to a similar pair of phospholipids with different fatty acid compositions. The extent of this effect is revealed by the fact that the recent theoretical treatment of Israelachvili et al. (1977) predicts, mainly on the basis of phospholipid head-group differences, exactly the opposite asymmetric distribution of phosphatidylethanolamine as observed here.

Although the asymmetry in the distribution of DMPE is substantial, Figure 2C demonstrates that the effect of this asymmetry on the breadth of the phase change is slight compared to the effect of the inhomogeneities in the gel phase. This observation is peculiar to this system and cannot be used as an argument to justify the failure to investigate phospholipid asymmetry when studying the phase behavior of multicomponent, small vesicles.

In summary, we conclude from this study: (1) That the miscibility of DMPC and DMPE in the gel phase of small, single-lamellar vesicles is more severely limited than within the gel phase of large, multilamellar vesicles, probably due to the instability of gel-phase domains having a high DMPE content within highly curved lipid bilayers. (2) That the difference between the phosphatidylethanolamine and phosphatidylcholine head groups is not sufficient to induce lateral phospholipid domain formation in the lamellar liquid-crystalline phase of small, single-lamellar vesicles. Previous studies had shown that small differences in acyl side-chain length were, however, sufficient to bring about this effect in highly curved vesicles composed of phosphatidylcholine mixtures. (3) That the distribution of phosphatidylethanolamine and phosphatidylcholine species between the monolayers of small, single-lamellar vesicles is modulated by both the acyl side-chain composition and packing requirements of the head groups of the mixed phospholipids.

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Lateral Diffusion of a Hydrophobic Peptide, *N*-4-Nitrobenz-2-oxa-1,3-diazole Gramicidin S, in Phospholipid Multibilayers†

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ABSTRACT: Lateral diffusion of *N*-4-nitrobenz-2-oxa-1,3-diazole gramicidin S (NBD-GS) in phospholipid multibilayers has been studied by the method of fluorescence recovery after photobleaching. At 24 °C the diffusion coefficient (D) in egg phosphatidylcholine (EPC) multibilayers is 3.5×10^{-8} cm^2/s. Addition of equimolar cholesterol to EPC bilayers reduced D to 1.4×10^{-8} cm^2/s. In dimyristoylphosphatidylcholine (DMPC) multibilayers, the phase transition was observed as an 100-fold change in D occurring at about 22 °C. Increasing the gramicidin S (GS) membrane concentration lowered the phase transition temperature (T_m) while broadening the transition range. The transition curves constructed with the D's for NBD-GS were correlated with differential scanning calorimetry data for identical GS/DMPC mixtures. Calorimetric data for these mixtures gave multiple melting components at high GS/DMPC but when GS was mixed with dipalmitoylphosphatidylcholine (DPPC), only a single melting component was observed with a slightly decreased T_m at high GS/DPPC. However, at these high ratios, fluorescence images of NBD-GS indicated incomplete incorporation of the peptide into DPPC; this was verified by quantitating the amount of GS associated with multilamellar lipid vesicles following fractionation on an agarose column or on a sucrose gradient. In contrast, no evidence for such incomplete mixing was obtained for GS/DMPC mixtures in either multibilayers or multilamellar vesicles. Addition of cholesterol to DMPC multibilayers reduced D above T_m, raised D below T_m, and also tended to force the NBD-GS out of the bilayers. At 50 mol % cholesterol, fluorescence microscopy showed that the NBD-GS was forced to reside in the domain boundaries of the multibilayer structure. In contrast, 50 mol % cholesterol did not displace NBD-GS in EPC multibilayers as evidenced by uniform fluorescence distribution. The effect of incorporated GS on lipid mobility was studied by measuring the diffusion coefficient of NBD-phosphatidylethanolamine (NBD-PE) in EPC multibilayers at 25 °C. At an apparent concentration of 20 mol % of GS, the D of NBD-PE was reduced by a factor of 1.5 from the value obtained ($\sim 4 \times 10^{-8}$ cm^2/s) in the absence of GS. The relationship of the measured lateral diffusion to the molecular size and lipid "viscosity" is discussed.

Gramicidin S (GS)[1] is a hydrophobic, cyclic decapeptide having a molecular weight of 1141. It has been the subject of numerous structural and synthetic studies, although the mechanism of its antibiotic action is not fully understood (for review, see Kato & Izumiya, 1974). As part of our program to investigate the lateral mobility of various molecules and macromolecules in cell membranes, we have studied the diffusion of this peptide in phospholipid multibilayers by the method of fluorescence recovery after photobleaching (FRAP). This method has been described in detail in the recent literature (Jacobson et al., 1977; Koppel et al., 1976; Edidin et al., 1976). A major purpose of these studies on model membranes is to understand factors governing the lateral diffusion of phospholipids, peptides, and proteins in membranes of defined lipid composition in order to provide a base line with which to compare and interpret the lateral mobility results obtained from measurements on the membranes of single, living cells (see, for example, Edidin et al., 1976; Jacobson et al., 1976, 1977; Schlessinger et al., 1976, 1977; Axelrod et al., 1976b). Studies of lipid diffusion in multibilayers (Wu et al., 1977) and black lipid membranes (Fahey et al., 1977; Wolf et al., 1977) as well as the diffusion of stearoylated dextrans bound to black lipid membranes (Wolf et al., 1977) have already been reported. In this report, the first to our knowledge on peptide lateral mobility in lipid bilayers, we present data on the effect of a bilayer phase transition and the effect of cholesterol on the lateral diffusion of NBD-GS in lipid multibilayers. We also studied the effect of gramicidin S on the lateral diffusion of a fluorescent phospholipid analogue.

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[1] Abbreviations used: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; EPC, egg phosphatidylcholine; GS, gramicidin S; NBD-GS, *N*-4-nitrobenz-2-oxa-1,3-diazole gramicidin S: NBD-PE, *N*-4-nitrobenz-2-oxa-1,3-diazolephosphatidylethanolamine; diI-C_{18}(3), 3,3′-dioctadecylindocarbocyanine iodide; T_m, gel to liquid crystalline phase transition temperature; FRAP, fluorescence recovery after photobleaching; % R, percent recovery; $\tau_{1/2}$, half-time for recovery; w_s, laser spot radius at specimen plane; T_B, time of photobleaching; D, diffusion coefficient; MLV, multilamellar vesicles; DSC, differential scanning calorimetry; Tempo, 2,2,6,6-tetramethylpiperidinyl-1-oxy.

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