Physical Properties and Surface Interactions of Bilayer Membranes Containing N-Methylated Phosphatidylethanolamines†

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ABSTRACT: The structure and physical properties of aqueous dispersions of 1,2-diacyl-*sn*-glycero-3-phosphoethanolamines (PE's) and their N-methylated analogues have been studied by scanning calorimetry, ^{31}P nuclear magnetic resonance, and freeze–fracture electron microscopy. While successive N-methylations of a diacylphosphatidylethanolamine cause only modest decreases in its gel to liquid-crystalline phase transition temperature, the introduction of even a single *N*-methyl group sharply increases the temperature at which the lipid forms a hexagonal II phase. However, ^{31}P nuclear magnetic resonance and electron microscopy show that unlike pure PE species, N-methylated PE's can form a variety of irregular nonlamellar structures at temperatures well below that at which a well-defined hexagonal II phase is formed. The rate of calcium-induced leakage of encapsulated carboxyfluorescein from large unilamellar vesicles composed of dioleoyl- or dielaidoylphosphatidylserine and the corresponding PE is strongly reduced when PE is replaced by N-methylated derivatives. The rate of calcium-induced intermixing of lipids of PE/phosphatidylserine (PS) vesicles steadily decreases as the PE component is successively replaced by its mono-, di-, and tri-N-methylated (phosphatidylcholine) derivatives. By correlating calorimetrically obtained phase diagrams with measurements of vesicle lipid intermixing, we conclude that dielaidoyl-*N*-methylphosphatidylethanolamine, like PE, can support direct interactions between the surfaces of PS/*N*-methyl-PE vesicles without lateral separation of a PS(Ca^{2+})-rich phase, while dielaidoyl-*N*,*N*-dimethyl-PE (and phosphatidylcholine) cannot. When dioleoyl lipids are examined, however, both the monomethyl- and dimethyl-PE species can support lipid intermixing between the membranes of vesicles containing even low amounts of PS. The ability of PE to support PS/Ca^{2+}-initiated interactions between vesicle surfaces is invariably superior to that of its N-methylated derivatives, a fact that appears to reflect the higher hydrogen bond donating ability of unsubstituted PE more than the relatively small size of its head group.

1,2-Diacyl-*sn*-glycero-3-phosphocholine (PC)[1] and 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (PE), the two major zwitterionic glycerolipids of mammalian cellular membranes, differ markedly in their physical properties and in their contributions to membrane function in vivo (Crocken & Nyc, 1964; Hale et al., 1977; Engelhard et al., 1978; Hauser et al., 1981; Schroeder, 1981). These differences reflect substantial differences in two basic properties of PE and PC in aqueous dispersions. First, while the polar head groups of PE and PC adopt similar conformations in lipid bilayers (Seelig et al., 1977; Yeagle, 1978), PE can hydrogen bond to adjacent lipids through its amino and phosphoryl groups, while the PC headgroup, lacking groups that can serve as hydrogen bond donors, is expected to interact more strongly with water than does PE. Accordingly, bilayers that are rich in PE can dehydrate and aggregate much more readily than can bilayers rich in PC (Miller & Racker, 1976; Kolber & Haynes, 1979; Lis et al., 1981; Mantsch et al., 1983; Seddon et al., 1983; Chang & Epand, 1983). Second, PE and PC are usually considered to have different shapes. The effective cross-sectional area of the PE head group is smaller (in relation to the cross-sectional area of the hydrocarbon chains) than that of the PC head group. The smaller effective size of the PE head group has been suggested to be the basis of the greater ability of this lipid to form nonlamellar structures such as the hexagonal II phase (Cullis & de Kruijff, 1979) and to reconstitute proper activity of certain membrane enzymes and transport proteins (Navarro et al., 1984; Jensen & Schutzbach, 1984).

One of the clearest functional differences between PE and PC is seen when the abilities of the two lipids to support membrane fusion are compared. Vesicles containing anionic lipids in combination with PE fuse readily upon addition of divalent cations, even when the anionic species is the minority component, while vesicles comparably enriched in PC fuse poorly if at all (Düzgünes et al., 1981; Sundler et al., 1981). Recent findings suggest that the divalent cation induced fusion

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[1] Abbreviations: CF, 5(6)-carboxyfluorescein; DE, dielaidoyl; DM, dimyristoyl; DO, dioleoyl; EDTA, ethylenediaminetetraacetic acid trisodium salt; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; NMR, nuclear magnetic resonance; PA, 1,2-diacyl-*sn*-glycerol 3-phosphate; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PE-ME, 1,2-diacyl-*sn*-glycero-3-phospho-*N*-methylethanolamine; PE-Me_2, 1,2-diacyl-*sn*-glycero-3-phospho-*N*,*N*-dimethylethanolamine; PE-Me_x, mono-, di-, or trimethylated PE; PS, 1,2-diacyl-*sn*-glycero-3-phosphoserine; Rho-DOPE, *N*-(lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine; T_c, gel to liquid-crystalline transition temperature; T_H, lamellar to hexagonal II phase transition temperature; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TLC, thin-layer chromatography; LUV, large unilamellar vesicle(s).

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of vesicles enriched in PC requires lateral segregation of domains highly enriched in anionic lipids, while the fusion of vesicles rich in PE can occur without such phase separation (Düzgünes et al., 1981; Sundler et al., 1981; Silvius & Gagné, 1984a,b). In order to study in more detail the structural basis for these dramatic differences in the fusion-supporting behavior of PE and PC, we have synthesized saturated and unsaturated PE derivatives of intermediate degrees of N-methylation (PE-Me and PE-Me_2). We have correlated calorimetric, NMR spectroscopic, and freeze–fracture electron microscopic examinations of the phases formed by the methylated PE's, both by themselves and in mixtures with PS, with measurements of the calcium-induced intermixing of the membranes of vesicles formed from these lipids in combination with PS. Our results indicate that successive *N*-methylations of PE change its behavior from a "PE-like" to a "PC-like" pattern in a gradual manner rather than by an abrupt change in lipid properties with one particular methylation step.

Materials and Methods

Materials

Synthetic phosphatidylcholines, phosphatidylethanolamines, and phosphatidylserines were prepared and purified as described previously (Silvius & Gagné, 1984a). Phospholipase D was extracted from savoy cabbage by the procedure of Davidson & Long (1958) as outlined elsewhere (Silvius & Gagné, 1984a). Diethyl ether used for vesicle preparations was redistilled from P_2O_5, stabilized with 0.5% water, and stored in the cold for periods not in excess of 2–4 weeks. All other solvents were redistilled before use. 5(6)-Carboxyfluorescein (Eastman) was purified by charcoal treatment followed by chromatography on Sephadex LH-20 as described by Ralston et al. (1981). All organic reagents and inorganic chemicals used were of at least reagent grade.

Methods

Preparation of N-Methyl- and N,N-Dimethyl-PE's. *N*-Methyl- and *N,N*-dimethylethanolamine (Aldrich) were fractionally redistilled at reduced pressure, converted to the hydrochlorides by bubbling with HCl in dry methylene chloride, and recrystallized from ethanol at −20 °C or methanol at −40 °C, respectively. The hydrochlorides were thoroughly freed from alcohol by prolonged incubation under high vacuum in the presence of P_2O_5.

N-Methylated PE's were prepared from the corresponding PC's by phospholipase D mediated transphosphatidylation essentially as described by Comfurius & Zwaal (1977), using 35% (w/v) methyl(hydroxyethyl)ammonium chloride or 40% (w/v) dimethyl(hydroxyethyl)ammonium chloride in the aqueous phase. The crude isolated lipid products were freed of PA by selective precipitation of this species with barium acetate (~4 μmol/μmol of lipid) from a 2% solution in 1:4 chloroform/methanol. Pure methylated PE's were then isolated by column chromatography as described previously for PE (Silvius & Gagné, 1984a). The purity of the final products was evaluated by TLC in 65:35:2.5:2.5 $CHCl_3$/methanol/H_2O/concentrated NH_4OH [which resolves PE-Me_2 from PE plus PE-Me (Eibl & Nicksch, 1978)] as well as in 50:20:10:10:5 $CHCl_3$/acetone/methanol/acetic acid/H_2O. *N*-Methyl-PE's were also chromatographed and the plates sprayed with ninhydrin reagent; no staining characteristic of a primary amine was seen under conditions where much smaller samples of PE gave a strong positive reaction. Phospholipid was assayed by the procedure of Lowry & Tinsley (1974), but the sample digestion time was prolonged to 2 h to ensure complete lipid digestion.

Calorimetry. Samples of 3–7 μmol of lipid, containing ionophore A23187 at 1 mol/250 mol of lipid if the samples were to be treated with calcium, were lyophilized from benzene. Calcium-free samples were dispersed under nitrogen in 0.85 mL of 200 mM NaCl, 5 mM histidine, 5 mM Tes, and 1 mM EDTA, pH 7.4 (calcium-free buffer), and incubated according to the following schedules: for dioleoyl lipids, 24 h at 0–2 °C; for dielaidoyl lipids, 30 min at 37 °C or 15 min at 40 °C and then 4 h at 20 °C and 16–24 h at 4 °C; for dimyristoyl lipids, 10 min at 45 or 50 °C, 4 h at 20 °C, and 16–24 h at 4 °C. DEPE and DMPE samples were prepared as described previously (Silvius & Gagné, 1984a). Samples containing calcium were prepared by dispersal under nitrogen in 100 mM NaCl, 5 mM histidine, 5 mM Tes, and 1 mM EDTA, pH 7.4 at 37 or 40 °C, followed by addition of $CaCl_2$ (as a 1 M stock) to 30 mM. The samples were then incubated according to the following schedules: for DEPE-Me, 15 min at 40 °C, 12–16 h at 15 °C, ~5 min at 40 °C, and finally 16–24 h at 4 °C; for DEPE-Me_2, 1 h at 37 °C, 12–16 h at 15 °C, ~20 min at 37 °C, and finally 16–24 h at 4 °C. All heating and cooling steps in the preparation of samples with or without calcium were carried out at slow rates (≤0.3 °C/min). The thermograms of samples thus equilibrated were essentially unchanged by further heating/cooling cycles or longer equilibrations, although these were avoided to ensure against sample degradation (monitored by TLC).

Scanning calorimetry and the determination of enthalpies of transition were carried out as described previously (Silvius & Gagné, 1984a) using a Microcal MC-1 high-sensitivity differential scanning calorimeter at a scan rate of 24 °C/h except where otherwise noted.

^{31}P Nuclear Magnetic Resonance. ^{31}P NMR spectra were obtained on a JEOL FX 270 Fourier-transform NMR spectrometer at 109 MHz. Samples were measured in 10-mm tubes on a broad-band probe. Spectra were obtained with a fully phase-cycled Hahn echo, as described previously (Rance & Byrd, 1983), to prevent spectral distortions that are inherent in a normal single-pulse experiment. Fifty-kilohertz spectra were obtained, and no first-order phase corrections were used to assure a flat, undistorted base line for the spectra. A total of 2048 data points were accumulated in the time domain, and 1000 scans were obtained. Temperatures were maintained to ±1 °C. Samples were prepared and incubated as described for calorimetry samples above.

Freeze–Fracture Electron Microscopy. Thoroughly dried films of DOPE and its N-methylated derivatives were suspended by vortexing at 4 °C and under N_2 in 200 mM NaCl, 5 mM histidine, 5 mM Tes, and 0.1 mM EDTA, pH 7.4. Samples were incubated at various temperatures, normally for 1 h and always for at least 30 min. A ~0.1-μL aliquot of sample, sandwiched between thin copper plates, was then rapidly frozen from the incubation temperature by plunging into liquid propane. Details of freeze–fracturing procedures are given in Hui et al. (1983).

Fusion and Leakage Assays. Large unilamellar vesicles (LUV) were prepared as described by Wilschut et al. (1980) by reverse-phase evaporation and pressure filtration through 0.1-μm pore-size Nucleopore membranes. Vesicle lipid intermixing was assayed by the resonance energy transfer based procedure of Struck et al. (1981) or by the modification of this procedure described by Hoekstra (1982). In the former assay, vesicles labeled with 1 mol % each of Rho-DOPE and NBD-PE were incubated with an equal amount of unlabeled vesicles, and in the latter, vesicles labeled with 2 mol % NBD-PE were incubated with an equal amount of vesicles

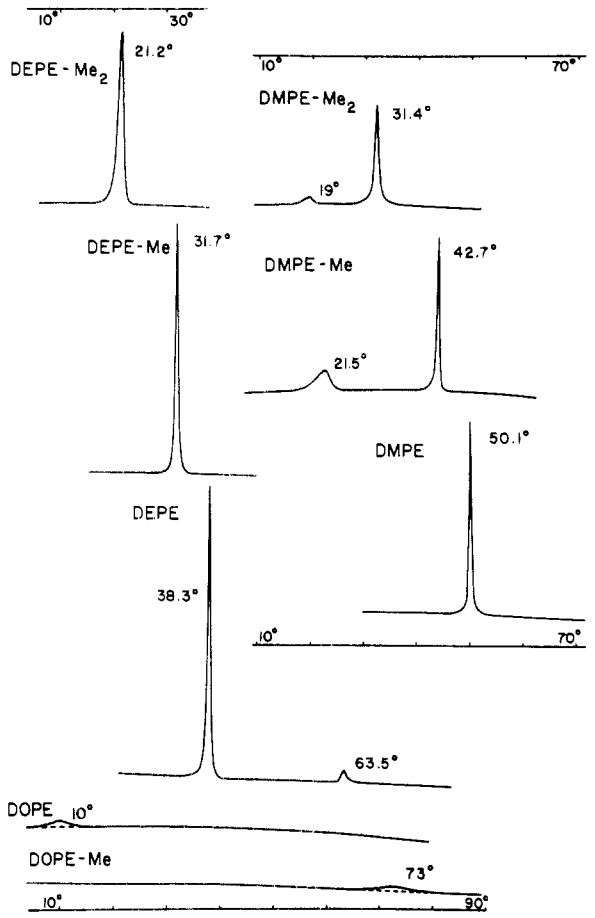

FIGURE 1: Scanning calorimetric thermograms of pure PE's and methylated PE's suspended at 5 mM in calcium-free buffer. All samples were prepared and equilibrated as described under Materials and Methods, with the exception of the DMPE-Me and DMPE-Me_2 samples, which were equilibrated at 2 °C for 40 and 42 days, respectively, prior to calorimetry. The temperatures indicated beside transition endotherms indicate their measured peak temperatures. Dioleoyl and dielaidoyl lipid scans shown were recorded at a scan rate of 25 °C/h, and the dimyristoyl lipid scans were recorded at a rate of 12 °C/h. The enthalpies of the main transitions were determined to be 8.4 ± 0.2, 11.2 ± 0.6, 12.0 ± 0.1, and 7.9 ± 0.5 kcal mol^{-1} for DEPE, DEPE-Me, DEPE-Me_2, and DEPC, respectively, and 5.9 ± 0.6, 8.0 ± 0.4, 7.2 ± 0.3, and 5.7 ± 0.4 kcal mol^{-1} for DMPE, DMPE-Me, DMPE-Me_2, and DMPC, respectively.

labeled with 2 mol % Rho-DOPE. In both procedures, the efficiency of resonance energy transfer corresponding to complete mixing of vesicle lipids was estimated by using vesicles prepared with 0.5 mol % of each label (for the Struck et al. procedure) or 1% of each label (for the procedure of Hoekstra). All assays were performed by using a total phospholipid concentration of 40 μM in 200 mM NaCl, 2 mM histidine, and 2 mM Tes, pH 7.4. Vesicle leakage was measured by the carboxyfluorescein release assay as described by Wilschut et al. (1980).

RESULTS

Thermotropic Properties of Pure Methylated PE's. Heating thermograms are shown in Figure 1 for the N-methylation series DEPE to DEPE-Me_2 and DMPE to DMPE-ME_2, and for DOPE and DOPE-Me. Under our normal conditions of sample incubation, single major endotherms identifiable with gel to liquid-crystalline transitions are seen for most of the dielaidoyl and dimyristoyl species. The temperatures (T_c) and enthalpies (ΔH_m) of these transitions are summarized in the legend to Figure 1. DMPE dispersions incubated at 2 °C for prolonged periods (4–14 days) showed a highly endothermic

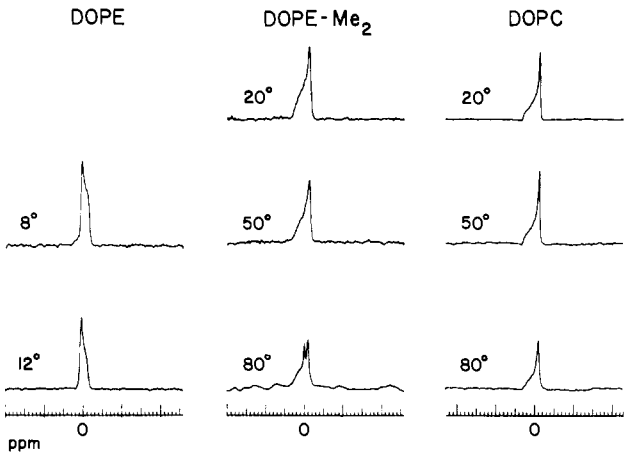

FIGURE 2: ^{31}P NMR spectra recorded for dispersions of DOPE, DOPE-Me_2, and DOPC at various temperatures as indicated. Samples were prepared in calcium-free buffer and were scanned from lower to higher temperatures. Details of spectral collection are given under Materials and Methods. Major divisions of the indicated scale of chemical shifts represent 100 ppm.

transition at 57.3 °C which Mantsch et al. (1983) and Wilkinson & Nagle (1984) have ascribed to a subgel to liquid-crystalline phase transition. By contrast, DMPE-Me and DMPE-Me_2 samples showed no changes in the main transition after incubation at 2 °C for 14–42 days. However, after such prolonged incubations, DMPE-Me and DMPE-Me_2 samples showed additional, broader transitions at ~21.5 °C and at ~19 °C, respectively, as shown in Figure 1.

Minor endotherms are seen above the temperatures of the major transitions and below 95 °C for DEPE, DOPE, and DOPE-Me (Figure 1). These upper temperature transitions were much broader when samples were vortexed above T_c than when the samples were vortexed below T_c and then warmed above this temperature without agitation (compare, for example, the width of the DEPE high-temperature endotherm to those of the DOPE and DOPE-Me endotherms in Figure 1). No analogous high-temperature transitions were seen up to 95 °C for the other lipids whose thermograms are shown in Figure 1, or for DOPE-Me_2 or DOPC.

The thermotropic phase changes of the dioleoyl and dielaidoyl lipids studied were further examined by ^{31}P NMR. Spectral line shapes for the dioleoyl species at various temperatures are shown in Figures 2 and 3. DOPE gives a mixed liquid-crystalline lamellar and hexagonal phase line shape at 8 °C and a pure hexagonal phase line shape at 12 °C (Figure 2), corresponding to the calorimetric observation of an endotherm centered at 10 °C. The spectra of DOPE-Me_2 and DOPC, for which no calorimetric endotherms are seen from 5 to 95 °C, show line shapes indicative of a primarily lamellar organization of the lipids up to at least 80 °C. However, a small isotropic component is also seen in the 80 °C spectrum of DOPE-Me_2. This feature appears much more dramatically in the spectra of DOPE-Me (Figure 3). Above 30 °C, an isotropic signal progressively increases at the expense of the lamellar component in the spectrum of this lipid until at 60 °C an essentially pure isotropic line shape is observed. At and above 70 °C, a component characteristic of a hexagonal phase is seen underlying the isotropic resonance. The appearance of the hexagonal component around 70 °C correlates with the small calorimetric endotherm observed at ~73 °C for DOPE-Me.

The ^{31}P NMR spectra of the dielaidoyl lipids at a given temperature generally resemble those of the corresponding dioleoyl species at temperatures some 50 °C lower. DEPE

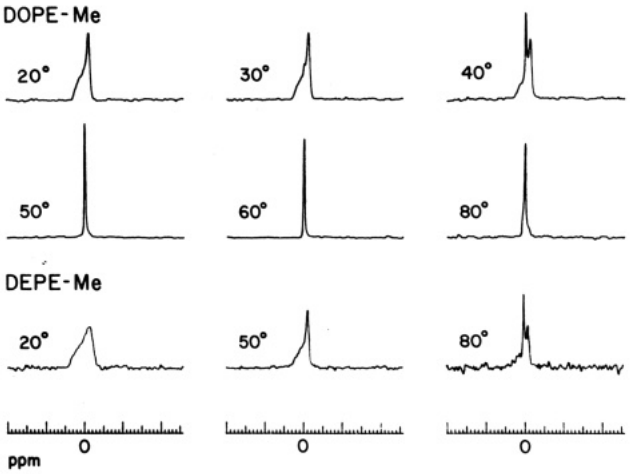

FIGURE 3: ^{31}P NMR spectra recorded for dispersions of DOPE-Me (upper two rows) and DEPE-Me (bottom row) in calcium-free buffer at the indicated temperatures. The spectra shown were obtained by scanning from lower to higher temperatures except in the case of the 20 °C spectrum shown for DEPE-Me, which was recorded ~3 h after recooling from 80 °C. Other details are as in Figure 2.

shows a transition from a lamellar liquid-crystalline to a hexagonal line shape at ~60 °C with no intermediate isotropic line shape (not shown). For DEPE-Me, an isotropic component begins to emerge from the lamellar-type spectrum at ~80 °C as shown in Figure 3. When the sample is recooled to 20 °C, the isotropic component slowly vanishes over a period of a few hours, giving a spectrum indistinguishable from that observed before cycling to 80 °C. The 20 °C spectrum of this lipid is characteristic of a lamellar gel phase, which is consistent with the calorimetric observation of the main phase transition of this lipid at 31.7 °C. DEPE-Me_2 and DEPC give purely lamellar line shapes, similar to those shown for DOPC in Figure 2, at all temperatures from 20 to 80 °C.

To investigate the origin of the isotropic components seen in the ^{31}P NMR spectra of some of the methylated PE's, lipid samples were rapidly freeze–quenched from various temperatures and examined by freeze–fracture electron microscopy. The micrographs shown in Figure 4 illustrate the morphologies of DOPE-Me dispersions at 4, 25, and 50 °C. At 4 °C, DOPE-Me forms bilayer vesicles whose surfaces reveal occasional ridges and furrows (Figure 4A). At 25 °C, the surface deformations of these vesicles have become more extensive, to the point that the bilayer fracture planes are disrupted (Figure 4B), but the lipid remains predominantly in the form of bilayer vesicles. Surface deformations such as those seen in Figure 4A,B have been observed previously in mixed PE/PC systems (Hui et al., 1983) and are usually the precursors of interlamellar connections that lead to the formation of hexagonal II cylinders (Hui et al., 1981). Several large, flat tubelike structures, apparently representing these interlamellar connections, were in fact seen in DOPE-Me dispersions at 25 °C. By contrast, DOPC vesicles showed perfectly smooth surfaces at 25 °C (not shown).

At 50 °C, a temperature at which DOPE-Me dispersions give a largely isotropic ^{31}P NMR spectrum, DOPE-Me vesicles were observed by phase-contrast microscopy to aggregate and ultimately to form very large clumps. These clumps were found by freeze–fracture electron microscopy to contain a variety of lipid structures, as shown in Figure 4C,D. In Figure 4C, lipidic particles can be seen to arise from the surrounding bilayer. The lipidic particles are more variable in size than those previously observed in certain PE/PC mixtures (Hui et al., 1981; Boni & Hui, 1983). The presence of large numbers of these particles is likely the source of the isotropic ^{31}P NMR

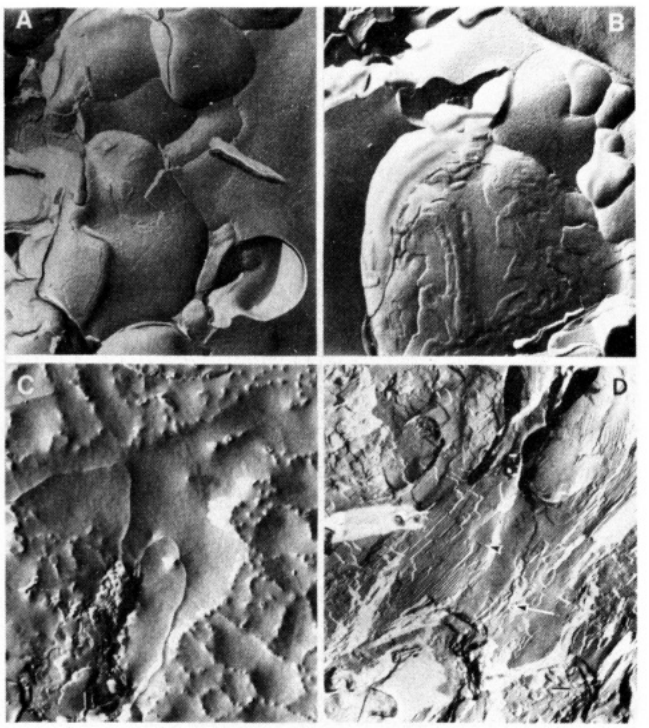

FIGURE 4: Freeze–fracture electron micrographs of DOPE-Me dispersions freeze–quenched from (A) 4, (B) 25, and (C and D) 50 °C. The arrowhead and the arrow in (D) indicate hexagonal II tubes and their precursors forming from lamellar bilayers. Scale bar in (D) = 100 nm. Details of sample and replica preparation are given under Materials and Methods.

resonance observed for DOPE-Me samples at higher temperatures. In Figure 4D, small clusters of hexagonal II cylinders (arrow) appear to develop from the surrounding bilayers through the intermediate formation of larger, flattened tubelike structures (arrowhead) without the involvement of lipidic particles. Nonlamellar structures were retained temporarily after DOPE-Me dispersions were cooled from 50 °C to room temperature, but the dispersions gradually reverted to purely lamellar vesicles after storage at 4 °C for several hours. No small vesicles were observed in DOPE-Me dispersions incubated at any of the temperatures examined. DOPE dispersions incubated at room temperature were found to consist of hexagonal II structures, while DOPE-Me_2 and DOPC formed purely bilayer structures at this temperature (not shown), in agreement with the ^{31}P NMR spectral results.

Mixing of Methylated PE's with PS. Thermograms obtained for representative mixtures of DEPS with DEPE-Me in the absence of calcium are shown in Figure 5. The phase diagram derived from the calorimetric results is shown in Figure 6A, where the procedure described by Mabrey & Sturtevant (1976) has been used to correct the liquidus and solidus lines for the finite transition widths of the pure lipid components. The pronounced low-temperature shoulder seen in the transition endotherms for DEPS/DEPE-Me mixtures is also observed for pure DEPE-Me in the presence of calcium. We believe that the main transition of DEPE-Me may have multiple components, as Chowdhry et al. (1984) have recently reported for DMPE, and that these components may be partly resolved under certain conditions (e.g., in the presence of calcium). Mixtures of DEPS with DEPE-Me_2 give thermograms with single, symmetrical main transition peaks (not shown), from which the phase diagram shown in Figure 6B has been determined.

The simplest picture of the mixing of two lipids predicts that the molar enthalpy of a phase transition in a binary mixture

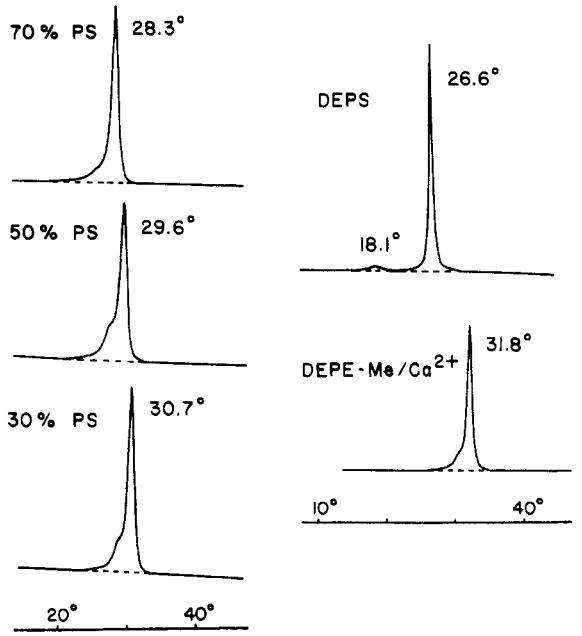

FIGURE 5: Heating thermograms recorded for mixtures of DEPE-Me and DEPS prepared in the absence of calcium and for DEPE-Me in buffer containing 30 mM $CaCl_2$. Details of sample preparation and calorimetric analysis are given under Materials and Methods.

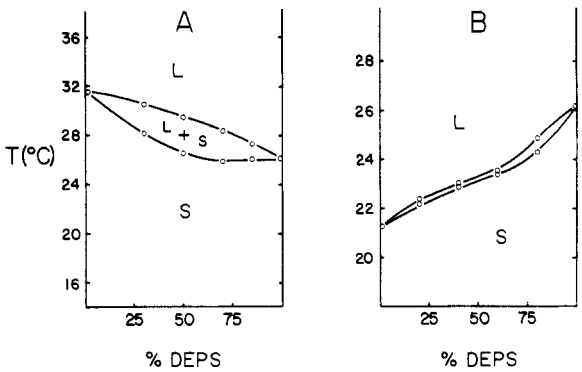

FIGURE 6: Phase diagrams derived from calorimetric thermograms for mixtures of DEPS with (A) DEPE-Me and (B) DEPE-Me_2 in calcium-free buffer. Lipid dispersions were prepared and their thermograms analyzed as described in the text.

will be simply the sum of the molar enthalpies of transition for the pure components multiplied by their mole fractions. This simple relationship was not observed for mixtures of DEPS with DEPE-Me or DEPE-Me_2. Instead, the molar enthalpies of transition for the mixtures fell off rapidly as the mole fraction of DEPS rose from 0 to ~40 mol % and then leveled off at a value comparable to that of pure DEPS (8.3 kcal mol^{-1}) at higher PS contents (data not shown). Therefore, while DEPS does not phase separate from DEPE-Me or DEPE-Me_2 in the absence of calcium, these lipids do not form mixtures that behave as ideal solutions.

The addition of calcium to mixtures of the methylated PE's with DEPS drastically perturbs their thermotropic behavior, as the thermograms shown in Figure 7 illustrate. Many qualitative features of these thermograms, and their variation with PS content, are similar to those previously observed for the DEPE/DEPS (Ca^{2+}) and DEPC/DEPS (Ca^{2+}) systems (Silvius & Gagné, 1984a,b). The interpretation of the calorimetric results for the DEPE-Me/DEPS (Ca^{2+}) system to construct the phase diagram shown in Figure 8A is illustrative and is outlined below.

The results presented above indicate that pure DEPE-Me exists in two states: a lamellar gel phase below 32.2 °C and

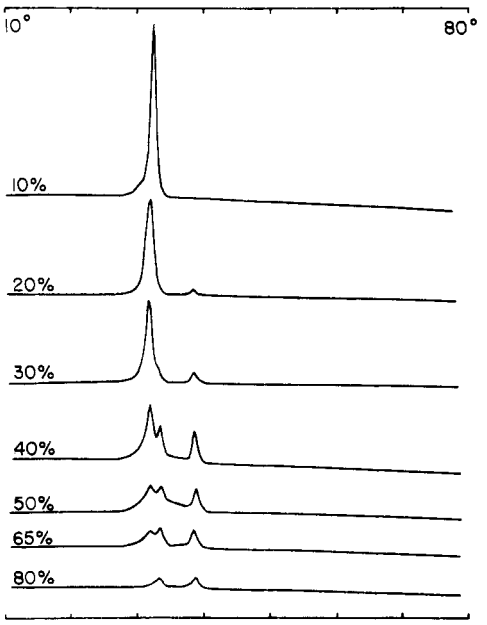

FIGURE 7: Heating thermograms recorded for DEPS/DEPE-Me mixtures, containing the indicated mole percent of DEPS, in the presence of 30 mM $CaCl_2$. Samples were prepared and equilibrated as described under Materials and Methods. The heating thermogram for pure DEPE-Me in calcium-containing buffer is shown in Figure 5 and closely resembles that shown in this figure for the sample containing 10% DEPS in DEPE-Me.

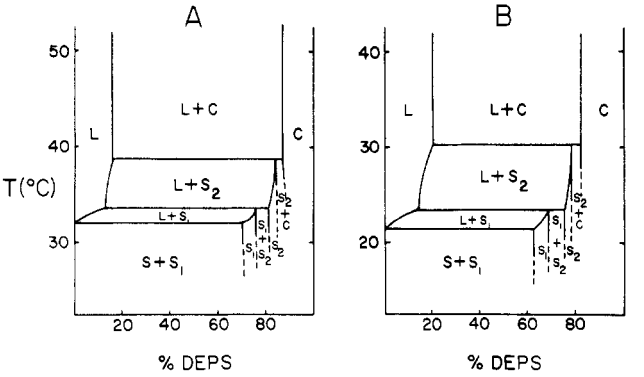

FIGURE 8: Calorimetrically derived phase diagrams for (A) the DEPS/DEPE-Me system and (B) the DEPS/DEPE-Me_2 system in the presence of calcium. The construction of these phase diagrams from the calorimetric results is described in the text.

a liquid-crystalline lamellar phase above this temperature, up to at least 80 °C. The endotherm representing the transition between these states is preserved, essentially unshifted in temperature but with a gradually diminished amplitude, as DEPS is introduced into the lipid mixture at increasing levels (up to ~65 mol %). This calorimetric feature is mapped on the phase diagram as a horizontal line at 32.0 °C, extending from nearly 0 to ~65 mol % DEPS and representing the melting of a gel phase of nearly pure DEPE-Me in equilibrium with a phase richer in DEPS. Two other sharp endotherms occurring at constant temperatures (33.6 and 38.7 °C) in samples of widely varying PS content (~20–85 mol % PS) are also mapped as horizontal lines on the phase diagram. To explain the existence of these additional endotherms over the indicated ranges of PS contents, we must postulate the existence of at least three distinct PS-enriched phases. The most PS rich and temperature stable of these phases is the "cochleate" phase familiar from studies of pure PS/Ca^{2+} systems (Papahadjopoulos et al., 1977; Hauser et al., 1977; Portis et al., 1979; Hauser & Shipley, 1984). The other two postulated PS-rich phases are stable only at lower tempera-

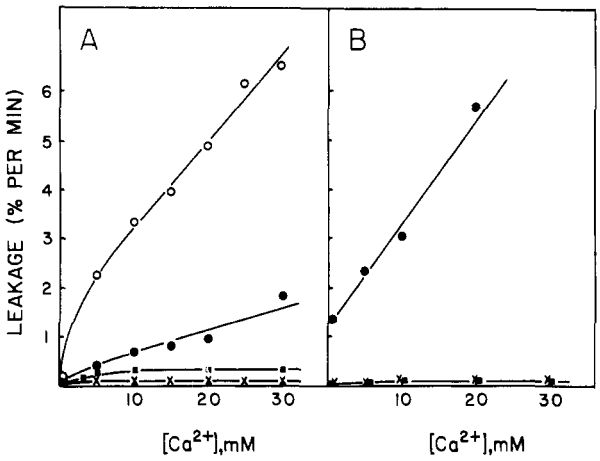

FIGURE 9: (A) Effects of calcium concentration on the leakage of carboxyfluorescein from large unilamellar vesicles containing 15 mol % DEPS in combination with (○) DEPE, (●) DEPE-Me, (■) DEPE-Me_2, or (×) DEPC. (B) Effects of calcium concentration on the leakage of carboxyfluorescein from vesicles containing 15 mol % DOPS together with (●) DOPE-Me, (■) DOPE-Me_2, or (×) DOPC. Details of vesicle preparation and fluorometric assay of carboxyfluorescein leakage are given under Materials and Methods.

tures, decomposing at 33.6 and 38.7 °C to give a mixture of liquid-crystalline lipid (which accounts for the appreciable heat contents of these conversions) and another phase more enriched in PS. The latter two PS-rich phases thus have the character of solid solutions and are designated as S_1 and S_2 on the phase diagram. The final construction of the phase diagram, incorporating the features discussed above, is straightforward and basically as described previously for the DEPE/DEPS (Ca^{2+}) and DEPC/DEPS (Ca^{2+}) systems (Silvius & Gagné, 1984a,b).

The phase diagram for the DEPE-Me_2/DEPS (Ca^{2+}) system (Figure 8B) was constructed from calorimetric results (not shown) essentially in the same manner as that for the DEPE-Me/DEPS (Ca^{2+}) system. Samples containing low mole fractions of DEPS (~10%) in DEPE-Me_2 showed a marked tendency to form metastable solid solutions under conditions where, at true equilibrium, a gel phase rich in DEPE-Me_2 should coexist with one rich in DEPS. This anomaly was never observed in samples containing sufficient DEPS to exhibit phase separation at temperatures where the DEPE-Me_2 component was in a liquid-crystalline state.

Vesicle Leakage Assays. To evaluate the stability of bilayers containing DOPE, DEPE, and their N-methylated derivatives (PE-Me_x), we encapsulated 5(6)-carboxyfluorescein (CF) in ~1000-Å diameter unilamellar vesicles composed of 85 mol % each of PE or methylated PE and 15 mol % of PS with the same acyl chains. Preparations of vesicles containing this level of PS could be ultrafiltered in reasonable yields regardless of which PE or PE-Me_x species they contained. The leakage of carboxyfluorescein from the vesicles, which leads to dequenching of the dye fluorescence, was monitored in the presence of varying levels of calcium ion. Results obtained with DEPS/DEPE-Me_x vesicles at 40 °C, and with DOPS/DOPE-Me_x vesicles at 25 °C, are summarized in Figure 9. The vesicles containing dielaidoyl lipids are relative nonleaky in the absence of calcium. In the presence of calcium, the rate of CF leakage from DEPS/DEPE vesicles is strongly increased, but the leakage rate at any given calcium concentration falls off sharply as DEPE is replaced successively by DEPE-Me, DEPE-Me_2, and DEPC. DOPE/DOPS vesicles are very leaky at 25 °C even in the absence of calcium. No trace of CF could be detected in association with these vesicles after gel filtration to remove extravesicular dye, a process requiring approximately 15 min. DOPE-Me/DOPS vesicles can retain some encapsulated CF during gel filtration but are also markedly leaky, losing ~1% of their contents per minute at 25 °C in the absence of calcium and considerably more when calcium is present (Figure 9B). By contrast, vesicles formed with DOPE-Me_2 or DOPC are relatively nonleaky, losing ≤5% of their contents per hour even in the presence of calcium.

Calcium-Induced Intermixing of Vesicle Membrane Components. The data presented above indicate that vesicles combining PS with various methylated PE species vary widely in their abilities to retain trapped solutes. Accordingly, it was not experimentally feasible to compare quantitatively the rates of fusion of all of these different types of vesicles by using assays of the mixing of vesicles' aqueous contents. As an alternative means to compare the abilities of various (methylated) PE species to promote interactions between the surfaces of different vesicles, we examined the calcium-promoted intermixing of lipids between vesicles containing PS and various methylated PE's. Previous studies of vesicle lipid mixing (Struck et al., 1981; Hoekstra, 1982) have demonstrated that vesicle aggregation alone does not promote rapid intermixing of lipids. Therefore, measurements of lipid intermixing can provide information about the extent to which vesicles form direct surface contacts through which rapid exchange of lipids can occur.

Calcium-induced lipid mixing between unilamellar vesicles of average diameter ~1000 Å was examined by using the assay of Hoekstra (1982), which monitors the development of resonance energy transfer between two membrane-bound fluorophores, NBD-PE and Rho-DOPE, that are initially incorporated into separate vesicle populations. While this procedure proved very useful technically for monitoring accurately the rate of lipid intermixing, it was necessary to confirm initially that the experimentally observed energy transfer from NBD-PE to Rho-DOPE was in all cases a result of a true intermixing of vesicle membrane components and not of simple close apposition of the surfaces of different vesicles containing the two probes. This point was established in three ways. First, in cases where calcium addition to vesicles induced significant energy transfer between the two fluorophores, the extent of energy transfer attained with time was consistently found to be comparable to that expected for complete mixing of the vesicle lipids (estimated as described under Materials and Methods) and was considerably greater than the value expected if only fluorophores at the outer surfaces at the vesicles were interacting. Second, addition of a 2-fold excess of EDTA to vesicles at different times after calcium addition invariably failed to reverse even partially the energy transfer developed at that point, even after prolonged incubation with the chelator. These two tests were applied as routine controls in all experiments. Finally, calcium-induced lipid mixing between vesicle membranes rich in DEPE-Me or DEPE-Me_2 was also assayed by the procedure of Struck et al. (1981), which measures the decrease in energy transfer that results when the lipids of membranes containing both NBD-PE and Rho-DOPE intermix with those of unlabeled membranes. The results obtained by this alternate assay agreed well with those obtained by the procedure of Hoekstra.

As DEPS is combined with increasing amounts of DEPE-Me_2 in LUV, the rate of intermixing of vesicle lipids at any given calcium concentration decreases, as shown in Figure 10A. This inhibitory effect of DEPE-Me_2 on lipid mixing has two components. First, as shown in Figure 11A, increasing the

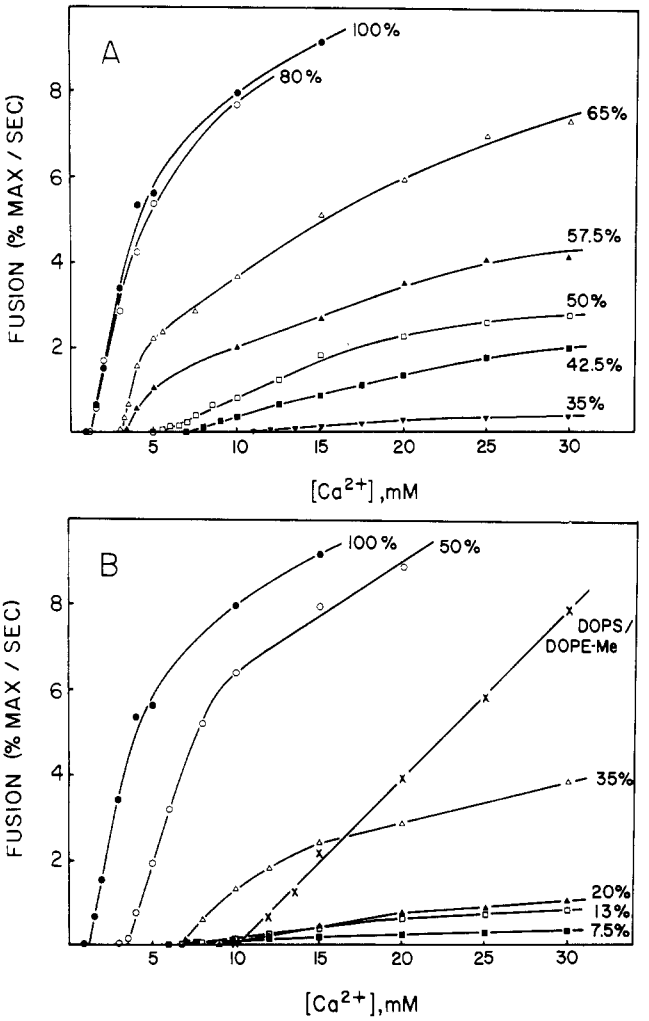

FIGURE 10: (A) Effects of calcium concentration on the rate of lipid intermixing, monitored by the lipid mixing assay of Hoekstra, between vesicles containing the indicated molar percentages of DEPS in DEPE-Me_2. (B) Calcium concentration dependence of lipid mixing for vesicles containing the indicated molar percentages of DEPS in DEPE-Me or 15 mol % DOPS in DOPE-Me (×). Assays were carried out at 40 °C for dielaidoyl lipid vesicles and at 25 °C for dioleoyl lipid vesicles. Details of vesicle preparation and the assay of lipid mixing are given in the text.

proportion of DEPE-Me_2 in the vesicles steadily increases the "threshold" concentration of calcium, the minimum calcium level required to induce significant lipid intermixing. Second, DEPE-Me_2 decreases the steepness of the dependence of the rate of intermixing on the calcium concentration above the threshold. This latter effect is quantified for purposes of discussion as the "efficiency" of intermixing of vesicle lipids, which we define as the initial rate of intermixing at a calcium concentration equal to twice the threshold. In Figure 11B, the efficiency of calcium-induced intermixing of DEPS/DEPE-Me_2 vesicle lipids is plotted as a function of the vesicle composition. The efficiency falls off strongly with increasing proportions of DEPE-Me_2, particularly above ~20 mol %, and is essentially zero at ≥75 mol % DEPE-Me_2.

The effects of DEPE-Me on the PS/calcium-initiated intermixing of lipids between vesicles are considerably different from the effects of DEPE-Me_2. Lipid mixing between vesicles containing equimolar proportions of DEPE-Me and DEPS is nearly as rapid at high calcium levels as is the intermixing of lipids between vesicles composed purely of DEPS (Figure 10B). As shown in Figure 11B, the efficiency of lipid intermixing is considerably greater for 50:50 DEPE-Me/DEPS vesicles

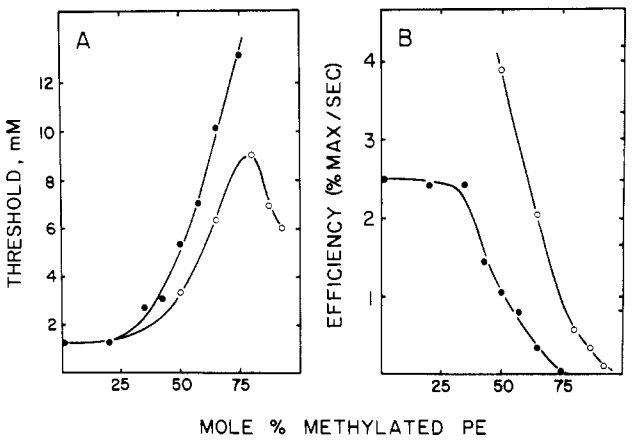

FIGURE 11: (A) Threshold calcium concentrations required to induce intermixing of the lipids of vesicles containing the indicated molar percentages of DEPS in combination with (●) DEPE-Me_2 or (○) $DEPE_t$-Me. (B) Efficiency of calcium-induced membrane intermixing (i.e., the initial rate of lipid mixing at a calcium concentration equal to twice the threshold level) for vesicles containing the indicated proportions of DEPS together with (●) DEPE-Me_2 or (○) DEPE-Me.

than for vesicles containing only DEPS. As the proportion of DEPE-Me in the vesicles increases beyond ~50 mol %, the efficiency falls off steadily. However, significant rates of calcium-induced lipid intermixing are still observed for vesicles containing as much as 92.5 mol % DEPE-Me (the highest proportion tested). The threshold calcium concentration required to induce lipid intermixing between DEPE-Me/DEPS vesicles gradually increases as the proportion of DEPE-Me in the vesicles rises from 0 to 80 mol % (Figure 11A). As the proportion of DEPE-Me increases still further, however, the calcium threshold begins to decrease significantly.

The intermixing of lipids between vesicles containing low molar proportions of DOPS in DOPE-Me or DOPE-Me_2 was also examined as a function of the calcium concentration. Results obtained with vesicles containing 15 mol % DOPS in DOPE-Me at 25 °C are shown in Figure 10B. The threshold calcium concentration for lipid intermixing between such vesicles is comparable to that observed with DEPS/DEPE-Me vesicles of comparable PS content. However, the efficiency of the calcium-induced lipid mixing is much greater for the vesicles containing the dioleoyl lipids. Vesicles containing 15 mol % DOPS in DOPE-Me_2 also showed significant rates of calcium-induced lipid intermixing at 25 °C, with a calcium threshold of 16 mM and an efficiency of 0.7% maximal fluorescence quenching per second (not shown). This efficiency of lipid intermixing is far greater than that measured at 40 °C for vesicles containing 15 mol % DEPS in DEPE-Me_2 (for which no significant intermixing was observed even at 30 mM $CaCl_2$). Interestingly, however, the calcium threshold measured for the dioleoyl lipid vesicles (16 mM) agrees well with that predicted by extrapolating the curve of threshold vs. mole percent PE-Me_2 obtained with DEPS/DEPE-Me_2 vesicles (Figure 11A) to 85 mol % PE-Me_2. Vesicles containing 7.5 mol % DOPS in DOPE-Me_2 showed a very slow and limited intermixing of membrane lipids at 25 °C when 20–30 mM $CaCl_2$ was added (not shown).

Discussion

Previous physical studies of mono- and dimethylated PE's have focused mainly on the lamellar phases of these compounds (Vaughan & Keough, 1974; Casal & Mantsch, 1983; Mulukutla et al., 1984). Our calorimetric results with methylated DMPE's agree with those recently reported by Mulukutla & Shipley (1984), although the kinetics of formation of the subgel

phase in our relatively dilute samples at 2 °C may be somewhat slower than those observed by these authors for samples containing 25–50% (w/v) lipid at −4 °C. The main transition temperatures (T_c) for DEPE and DMPE and their methylated derivatives decrease almost linearly with increasing extent of methylation, with an average decrease in T_c of ~7 °C per added methyl group.

N-Methylation of PE affects the lamellar to hexagonal II phase transition temperature (T_H) much more dramatically than it does T_c. The T_H values for DOPE and DEPE are ~10 and ~63 °C, respectively, while DOPE-Me forms a hexagonal II phase only above ~73 °C and DEPE-Me gives no indication of hexagonal phase formation up to at least 95 °C. However, N-methylated PE's can form structures giving an isotropic ^{31}P NMR resonance at temperatures well below that at which a hexagonal phase is first observed. Similar ^{31}P NMR results have been reported previously for mixtures of unsaturated PE's and PC's (Cullis & de Kruijff, 1978; Hui et al., 1981; Tilcock et al., 1982; Verkleij, 1984). For these systems, X-ray and electron microscopic evidence indicates that the isotropic ^{31}P NMR spectral component results from local deviations of the lipid organization from a planar arrangement at points where adjacent bilayers come into contact (Hui et al., 1981). The freeze–fracture results obtained here with DOPE-Me suggest that the isotropic spectral component observed for the methylated PE's has a similar structural basis.

Our freeze–fracture results suggest that hexagonal II structures evolve from bilayers in DOPE-Me dispersions by two distinct pathways as the temperature is increased. In the first pathway, lipidic particles form at points of interbilayer contact and gradually coalesce to form hexagonal II cylinders (Boni & Hui, 1983; Hui et al., 1983). In the second pathway, irregular surface ripples of the lipid bilayers develop into linear rather than punctate sites of contact between bilayers, forming initially large and irregular tubes which gradually evolve into parallel arrays of hexagonal II cylinders (Hui et al., 1983). While both pathways for a net conversion of bilayer to hexagonal II structures have been observed previously for various PE/PC mixtures (Hui et al., 1981, 1983; Boni & Hui, 1983; Verkleij, 1984), seldom have both pathways been observed for the same system. The ^{31}P NMR spectra recorded for dispersions of DOPE-Me_2 and DEPE-Me at high temperatures resemble those observed for DOPE-Me dispersions at temperatures some 50 °C lower and differ from those recorded for DOPE and DEPE at temperatures around T_H. It is thus likely that the temperature-dependent rearrangements of DOPE-Me_2 and DEPE-Me that occur above ~80 °C are similar in character to those observed for DOPE-Me above ~30 °C. Taken together, our ^{31}P NMR and freeze–fracture results indicate that N-monomethylation of PE allows it to form an extended lamellar phase up to temperatures at least ~20 °C above the T_H of the parent PE species, while N,N-dimethylation stabilizes this phase up to temperatures at least ~70 °C above the T_H of the unmethylated PE.

The calorimetric results obtained in this study, and those reported previously for the DEPE/DEPS and DEPC/DEPS systems (Silvius & Gagné, 1984a,b), indicate that DEPS is miscible with DEPE and all of its N-methylation products in the absence of divalent cations but can phase separate from these lipids in the presence of calcium. At the temperatures at which calcium-induced vesicle lipid intermixing was examined in our studies (37–45 °C), the calorimetrically derived phase diagrams indicate that lateral separation of a PS-rich phase can occur when calcium is added to mixtures containing ≥15% DEPS in DEPE or DEPE-Me, ≥20% DEPS in DEPE-Me_2, or ≥40% DEPS in DEPC. At the same time, significant lipid intermixing can only be induced by calcium addition to vesicles containing ≥25% DEPS in DEPE-Me_2 or ≥40% DEPS in DEPC, while the membranes of vesicles containing ≤15% DEPS in DEPE or DEPE-Me still show appreciable rates of lipid mixing upon addition of calcium (this paper; Silvius & Gagné, 1984a,b). The intermixing of vesicle membranes containing DEPS plus either DEPE-Me_2 or DEPC thus can occur only when the membranes contain sufficient PS to form a laterally segregated PS(Ca^{2+})-rich phase in the presence of calcium. By contrast, vesicles containing low molar proportions of DEPS (≤15 mol %) in DEPE or DEPE-Me show calcium-induced lipid intermixing even though no lateral separation of a PS-rich phase is predicted from the phase diagrams for these lipid mixtures. This result might suggest that PE-Me is qualitatively "PE-like" in its ability to promote close associations between vesicle surfaces, while PE-Me_2 is "PC-like" in that it does not support such associations. However, vesicles containing either DOPE-Me *or* DOPE-Me_2 in combination with low mole percentages of DOPS exhibit significant rates of lipid intermixing in the presence of calcium. The observed differences in the abilities of PE-Me and PE-Me_2 to promote intermixing of vesicle membrane lipids can thus be modified by changing the lipid acyl chains and may be more quantitative than qualitative.

An examination of the effects of various PE-Me_x species on the calcium threshold for lipid mixing between PS-containing vesicles also reveals a gradual progression rather than an abrupt change from PE-like to PC-like behavior with successive methylations of the PE head group. When vesicles are prepared containing a fixed proportion of PS in combination with PE or one of its N-methylation products, the calcium threshold for vesicle lipid intermixing is found to rise steadily as PE is replaced successively by PE-Me, PE-Me_2, and PC (this work; Silvius & Gagné, 1984a,b). The effects of different methylated PE's on the calcium threshold are almost unchanged when dielaidoyl lipids are replaced by dioleoyl species. It thus appears that the calcium threshold for intermixing of vesicle membrane lipids is determined mainly by the types and proportions of lipid head groups in the membrane, while the rate of vesicle membrane intermixing at suprathreshold levels of calcium can be influenced by both the head-group and the acyl chain composition of the vesicle lipids.

N-Methylation of the head group of PE will both increase its steric bulk and decrease its hydrogen-bonding capacity. In principle, either or both of these factors could influence the ability of N-methylated PE's to promote direct interactions between two membrane lipid surfaces. However, we have recently observed that dielaidoylphosphatidyl-3-amino-1-propanol, with a head group of comparable size to that of DEPE-Me but with an unsubstituted amino group, can support calcium/PS-initiated intermixing of vesicle lipids essentially as well as DEPE (J. Steers and J. R. Silvius, unpublished observation). The loss of hydrogen-bonding ability of PE that accompanies methylation of the amino group thus appears to be the major cause of the diminished ability of N-methylated PE's to promote intermixing of membrane components.

The ability of phospholipids to participate in the formation of nonlamellar phases is frequently cited as a factor of importance in determining their ability to support bilayer–bilayer interactions and membrane fusion [see, for example, Verkleij (1984)]. The results of our ^{31}P NMR and vesicle lipid intermixing studies are broadly consistent with this suggestion inasmuch as those PE derivatives that form nonlamellar

structures at relatively low temperatures can support calcium/PS-initiated intermixing of vesicle membranes better than can species that form purely lamellar phases up to high temperatures. However, lipids such as DEPE, DEPE-Me, and DOPE-Me_2 can support calcium/PS-initiated mixing of vesicle membrane components at temperatures far below those at which they form nonlamellar phases as pure compounds. A complete model for the fusion of such vesicles will clearly require as much knowledge of transient fluctuations as of the equilibrium configurations of their constituent lipids (Siegel, 1984).

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Registry No. DEPE, 19805-18-6; DEPE-Me, 96647-98-2; DEPE-Me_2, 96647-99-3; DEPC, 56782-46-8; DMPE, 998-07-2; DMPE-Me, 68755-13-5; DMPE-Me_2, 68755-14-6; DMPC, 18194-24-6; DOPE, 4004-05-1; DOPE-Me_2, 96687-22-8; DOPC, 4235-95-4; DOPE-Me, 96687-23-9; DEPS, 63976-14-7; Ca, 7440-70-2.

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