Calcium-Induced Fusion and Lateral Phase Separations in Phosphatidylcholine–Phosphatidylserine Vesicles. Correlation by Calorimetric and Fusion Measurements†

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ABSTRACT: High-sensitivity differential scanning calorimetry has been used to determine the phase diagrams for mixtures of dielaidoyl- and dimyristoylphosphatidylserine with the corresponding phosphatidylcholines (PC's) in the presence or absence of 30 mM calcium ion. The PC and phosphatidylserine (PS) species of like acyl composition are miscible in the absence of calcium but exhibit a limited lateral phase separation at high calcium levels. At higher temperatures, mixtures containing ≤40% or ≥70% PS in PC are not phase separated at equilibrium in the presence of calcium, while mixtures of intermediate PS content will separate into two phases of these limiting compositions. At lower temperatures, where hydrated solid phases are present, phase separation is more extensive. Parallel studies of the fusion of vesicles containing mixtures of dielaidoyl- or dioleoyl-PS with the corresponding PC species suggest that the calcium-induced fusion of vesicles containing <70% PS in PC is strongly correlated with lateral segregation of PS-enriched domains in the presence of calcium. As such lateral segregation is not thermodynamically favored at vesicle PS contents of ≤40 mol %, vesicles with this range of PS contents (which includes the range of PS levels in most natural fusing membranes) do not fuse. These results, taken together with those reported in the preceding paper [Silvius, J. R., & Gagné, J. (1984) *Biochemistry* (preceding paper in this issue)], raise some question as to whether calcium-induced lipid lateral phase separations play an obligatory role in membrane fusion in vivo.

The interaction between calcium ions and anionic phospholipids has been intensely studied in recent years in view of its possible roles in ion-triggered lateral phase separations in membranes and in membrane fusion (Jacobson & Papahadjopoulos, 1975; Kretsinger & Nelson, 1976; Papahadjopoulos et al., 1979; Liao & Prestegard, 1979, 1981). Phosphatidylserine (PS)[1] in particular has been the object of a variety of studies investigating its physical properties in the presence and absence of divalent metals (MacDonald et al., 1976; Papahadjopoulos et al., 1977; Portis et al., 1979; Browning & Seelig, 1980; Cevc et al., 1981; Hauser & Shipley, 1982) and its role in mediating the Ca^{2+}-triggered fusion of natural membranes and lipid vesicles (Wilschut et al., 1980; Struck et al., 1981; Düzgünes et al., 1981a,b).

In natural membranes, PS invariably coexists with zwitterionic phospholipids, which comprise the major fraction of the total membrane phospholipid. The properties of mixtures of PS with PC, the most thoroughly studied phospholipid head-group species, have been studied by several physical methods, including differential scanning calorimetry (Papahadjopoulos et al., 1974; van Dijck et al., 1978; Hui et al., 1983), electron spin resonance of partition and lipid probes (Ohnishi & Ito, 1974; Luna &McConnell, 1977), freeze–fracture electron microscopy (Luna & McConnell, 1977; van Dijck et al., 1978; Stewart et al., 1979; Hui et al., 1983), and X-ray diffraction, ^{31}P nuclear magnetic resonance, and X-ray microprobe analysis (Hui et al., 1983). To date, however, few studies have examined mixtures of pure synthetic PS and PC species, and only one (van Dijck et al., 1978) has reported results for such a system in the presence of calcium. Studies to date have produced conflicting results regarding the miscibility of PC and PS species in the absence of calcium ion (Luna & McConnell, 1977; van Dijck et al., 1978; Stewart et al., 1979). Further, while there is wide agreement in the literature that PS and PC exhibit phase separation in the presence of calcium ion, in only one study (Hui et al., 1983) have the compositions of the segregated phases at equilibrium been examined in a systematic way.

Studies of the fusion of PC–PS vesicles have shown that PC strongly antagonizes the tendency of PS to mediate fusion in the presence of calcium (Düzgünes et al., 1981a,b; Uster & Deamer, 1981). The mixing of PC and PS, and particularly their tendency to undergo lateral phase separations in the presence of calcium, may therefore be critical determinants of the ability of a lipid membrane of a given PC and PS content to fuse. In this study, we have used high-sensitivity differential scanning calorimetry of mixtures of pure synthetic PS and PC species to determine the miscibilities of these lipids in the presence and absence of calcium. These results, which allow us to determine the ranges of lipid compositions in which lateral phase separations do or do not occur, have been correlated with measurements of the ability of vesicles composed of various mixtures of these lipids to fuse in the presence of calcium. Our results suggest a role for lipid lateral phase separation in the fusion of PS–PC vesicles over a limited range of PS contents (~40–70 mol %), a range that does not include the levels of PS found in most natural membranes. These results, combined with those reported in the preceding paper (Silvius & Gagné, 1984) for PE–PS mixtures, suggest that calcium-induced lipid lateral phase separations may not nec-

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[1] Abbreviations: DE, dielaidoyl; DM, dimyristoyl; DO, dioleoyl; DMPC-d_{54}, 1,2-bis(perdeuteriotetradecanoyl)-*sn*-glycero-3-phosphocholine; DSC, differential scanning calorimetry; EDTA, ethylenediaminetetraacetic acid trisodium salt; NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine; PC, 1,2-diacyl-*sn*-glycero-3-phosphocholine; PE, 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PS, 1,2-diacyl-*sn*-glycero-3-phosphoserine; Tes, *N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; LUV, large unilamellar vesicle(s).

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essarily play an essential role in membrane fusion in vivo.

Materials and Methods

The procedures used for the preparation and purification of pure DMPC, DMPS, DEPC, DEPS, DOPC, and DOPS, for the calorimetric analysis of lipid dispersions, and for the assays of vesicle fusion have been described in the preceding paper (Silvius & Gagné, 1984).

Large unilamellar vesicles (LUV) were prepared for fusion assays by the procedure of Wilschut et al. (1980). Vesicle fusion was assayed by the procedure of Wilschut et al. (1980) or the procedure of Struck et al. (1981) as modified by Hoekstra (1982a), as described in the preceding paper (Silvius & Gagné, 1984). Lipid dispersions for calorimetry were prepared by colyophilization of the lipid components (and, for samples to which calcium was added, the calcium ionophore A23187 at a molar ratio of 1 per 250 lipids) from benzene. The lyophilized mixtures were dispersed by vortexing in 200 mM NaCl, 5 mM Tes, 5 mM histidine, and 1.0 mM EDTA, pH 7.4 ("calcium-free buffer"), or in 100 mM NaCl, 5 mM Tes, and 5 mM histidine, pH 7.4, to which calcium chloride (from a 1 M stock solution) was added to 30 mM after the lipids were first dispersed. The samples were routinely incubated at 45 °C (for DMPS–DMPC mixtures) or 37 °C (for DEPC–DEPS mixtures) for 30 min in calcium-free buffer or for 2 h in calcium-containing medium and then cooled at ≤0.3 °C/min to 20 °C (for dimyristoyl lipids) or 5 °C (for dielaidoyl lipids). Calorimetric scans were then run after 3–6 h for the calcium-free samples, or after overnight incubation at the lower temperature for the calcium-containing samples. Samples were checked by thin-layer chromatography to confirm the absence of lipid degradation products.

Results

Mixing of PC and PS in the Absence of Calcium. Representative heating thermograms for mixtures of DMPC and DMPS, and of DEPC and DEPS, in calcium-free medium are shown in Figure 1. The transition endotherms of the pure lipids [which have been discussed in the preceding paper (Silvius & Gagné, 1984)] are also shown for comparison.

The DMPS–DMPC system has previously been studied by van Dijck et al. (1978) in the presence and absence of calcium, by using DSC at higher scan rates than those employed here (5 °C/min vs. 0.4 °C/min). Our results differ in some significant respects from those reported by these workers. As DMPS is incorporated into DMPC bilayers at progressively higher mole fractions, the main transition peak gradually broadens and shifts upward, and the pretransition shifts upward and decreases in peak amplitude until at 40% PS it overlaps the main transition. As the PS content is increased beyond 40 mol %, the main transition broadens significantly, but in none of the mixtures are two well-resolved transitions observed as reported by van Dijck et al. When the buffer normally used to suspend the lipids was replaced by 100 mM NaCl–25 mM PIPES, pH 6.0, the buffer used by these authors, the transition profiles for PS-rich mixtures were shifted to slightly higher temperatures (~0.5 °C) but were otherwise unchanged in shape (Figure 1A). Therefore, there appears to be no major phase separation in PS-rich mixtures in the absence of calcium when such mixtures are maintained close to equilibrium.

In DEPS–DEPC mixtures, a very similar mixing behavior is seen in the absence of calcium, although there are no observable pretransitions in the mixed-lipid samples. As for the DMPS–DMPC mixtures, a single major transition peak is seen at all mole fractions of PS.

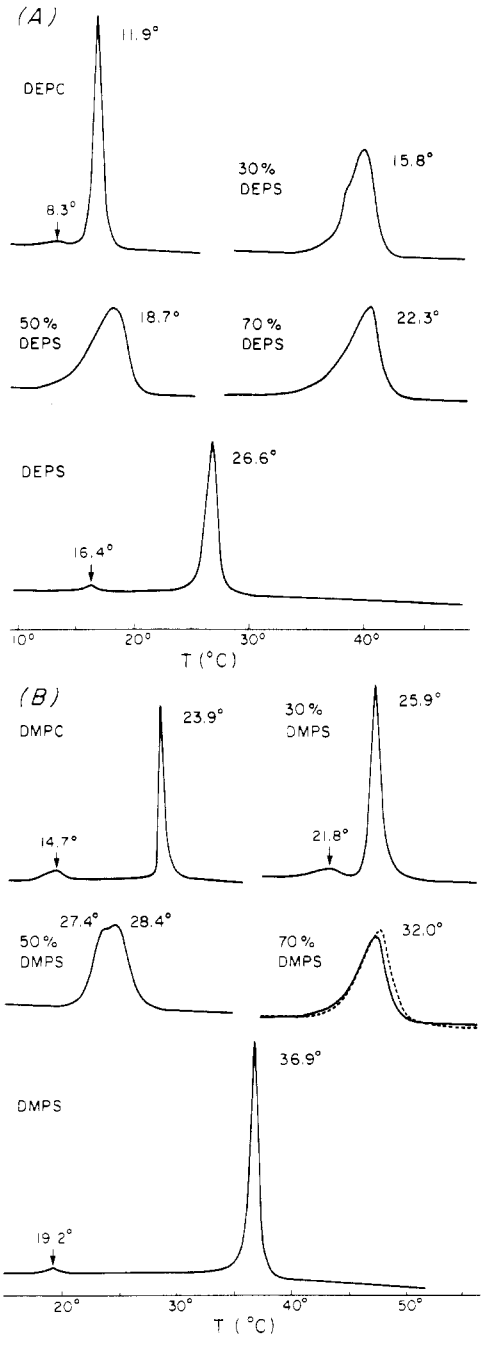

FIGURE 1: Calorimetric thermograms for PC–PS mixtures in calcium-free buffer. Details of sample preparation and conditions for calorimetry are described in the text. Samples were scanned over a temperature range extending from 0–5 °C to 80 °C, of which only the region of the transition is shown. (A) thermograms for DEPC–DEPS mixtures of the indicated PS content. (B) Thermograms of DMPC–DMPS mixtures containing the indicated mole percentages of PS. The dashed curve superimposed on the solid curve for 70% DMPS represents the thermogram obtained for this mixture dispersed in 100 mM NaCl–25 mM PIPES, pH 6.0.

In Figure 2 are shown the phase diagrams derived from the calorimetric results for these two PS–PC systems in the absence of calcium ions. The transition widths have been corrected to account for the finite widths of the transitions of the pure PC and PS species by using the procedure described by Mabrey & Sturtevant (1976). Also shown are the phase diagrams calculated from the measured enthalpies and transition temperatures of the pure compounds with the assumption of ideal mixing. For neither PC–PS system is mixing of the components ideal, but no evidence is seen for gel or liquid-crystalline phase immiscibility.

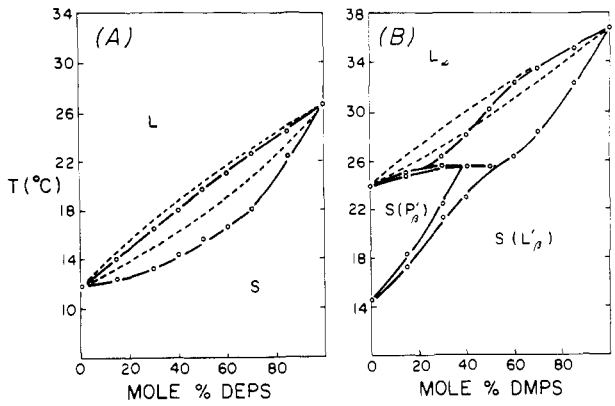

FIGURE 2: Calorimetrically determined phase diagrams for PC–PS mixtures in calcium-free buffer. (A) Phase diagram for the DEPC–DEPS system. One-phase regions are designated by the letters L (liquid crystalline) and S (gel state). (B) Phase diagram for the DMPC–DMPS system. Single-phase regions are identified by the symbols conventionally used for the corresponding phases of pure DMPC, where L_α denotes the liquid-crystalline phase and P_β' and L_β' represent the gel-state phases present above and below the pretransition, respectively.

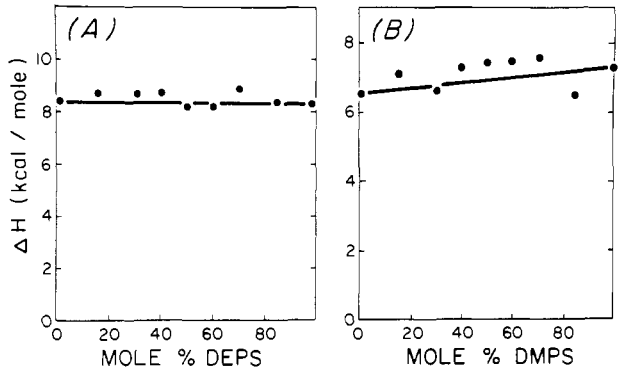

FIGURE 3: Enthalpies of the main (gel to liquid-crystalline) phase transition for PC–PS mixtures in calcium-free buffer. Sample thermograms were integrated, and the transition enthalpies were expressed on a molar basis by assay of the sample phospholipid contents, as described in the text. (A) Transition enthalpies for DEPC–DEPS mixtures. (B) Transition enthalpies for DMPC–DMPS mixtures.

The total enthalpies of the main transitions for the DEPS–DEPC and DMPS–DMPC mixtures are shown as a function of composition in panels A and B, respectively, of Figure 3. In each system, within experimental error, the total enthalpy of the transition varies linearly with increasing mole fraction of PS between the pure PC and the pure PS value. There is no indication of a systematic deviation from this relationship that could be taken as a general characteristic of PC–PS mixtures. As shown below, this behavior, which is consistent with a very simple mixing of the two components in each PS–PC system, is quite different from that exhibited by the same mixtures in the presence of high levels of calcium.

Effects of Calcium on PS–PC Mixing. To allow samples for calorimetric study to equilibrate properly in the presence of high levels of calcium, the calcium ionophore A23187 was added to the lipid phase (at a ratio of 1:250 mol/mol of lipid), and the lipids were dispersed several degrees above the transition temperature of the PS component in buffer containing only monovalent cations before calcium chloride was added to 30 mM. In control experiments, reduction of the calcium level to 20 or 25 mM gave calorimetric results identical with those observed with 30 mM calcium, regardless of sample composition. By varying the conditions of preincubation of the samples before calorimetry, we determined conditions that gave consistent thermotropic behavior from sample to sample

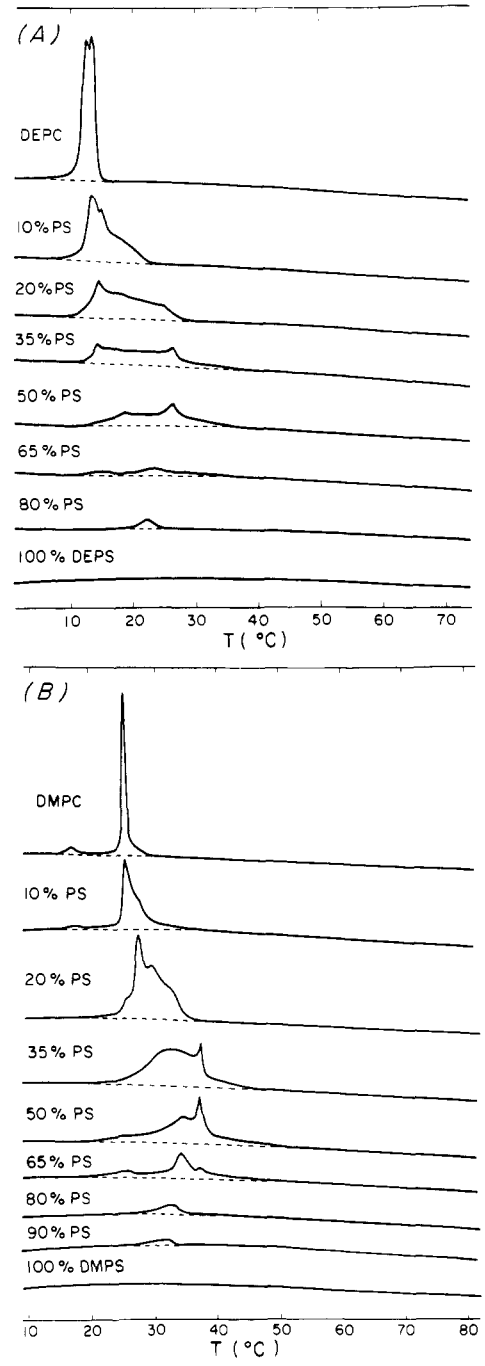

FIGURE 4: Heating thermograms for PC–PS samples in the presence of 30 mM $CaCl_2$, 100 mM NaCl, 5 mM histidine, and 5 mM Tes, pH 7.4. Samples were prepared and preequilibrated prior to calorimetric analysis as described in the text. All samples were scanned up to at least 85 °C; the upper parts of the scan are not shown in (A). (A) DEPC–DEPS mixtures containing the indicated levels of DEPS. (B) DMPC–DMPS mixtures containing the indicated mole percentages of DMPS.

and that appear to allow the system to come to equilibrium (see Materials and Methods).

Representative heating scans for DMPS–DMPC and DEPS–DEPC samples in a high-calcium medium are shown in Figure 4. For both types of mixtures, as the mole fraction of PS in PC is increased, a sharp endothermic component persists near the PC transition temperature up to ~25% PS while a broader component above this peak gradually increases in amplitude and extends to higher temperatures. As the high-temperature edge of this broader component reaches a temperature some 12–13 °C above the PC transition temperature, a small sharp endotherm appears (at ~30% PS in

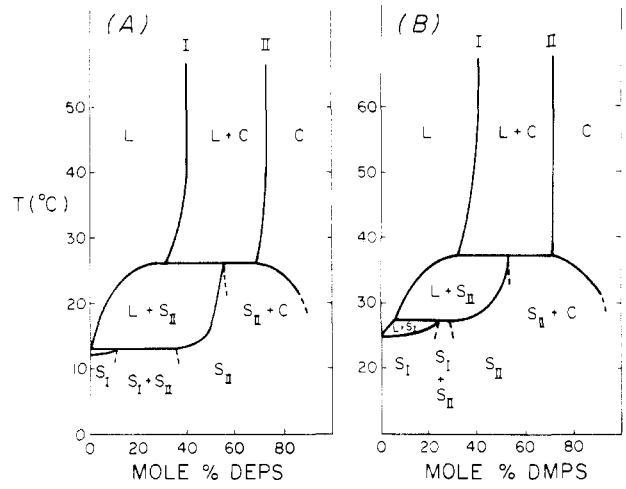

FIGURE 5: Calorimetrically determined phase diagrams for PS–PC systems in the presence of 30 mM calcium ion. The derivation of the phase diagrams from calorimetric thermograms like those shown in Figure 4 is described in the text. (A) The DEPC–DEPS (calcium) system; (B) DMPC–DMPS (calcium) system. Phases are denoted by the following symbols: L, liquid crystalline; C, dehydrated (cochleate) lamellae; S_I, a PC-rich hydrated gel phase; S_{II}, a hydrated solid phase containing higher mole fractions of PS.

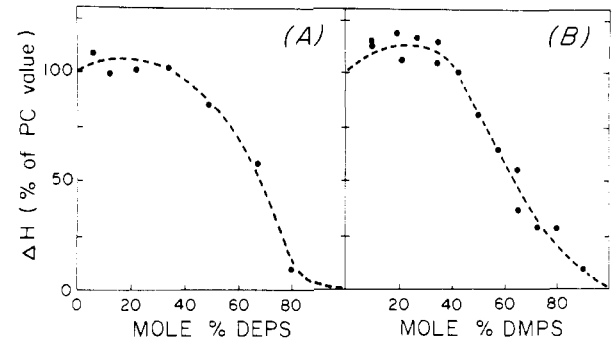

FIGURE 6: Integrated total excess heat absorptions over the temperature range 5–85 °C for PC–PS mixtures in the presence of 30 mM calcium ion. Sample thermograms were integrated, and the sample phospholipid contents were measured to calculate the excess heats on a molar basis, as described in the text. (A) Total molar enthalpies of transition for DEPC–DEPS mixtures; (B) molar enthalpies of transition for DMPC–DMPS mixtures.

both cases). Also, beginning at ~30% PS, we see a still broader endothermic component extending upward from the second sharp endotherm. This complex pattern persists up to ~70% PS, with the upper sharp endotherm rising to a maximum amplitude at ~50% PS and then falling off to disappearance at ~70% PS. At ~70–90% PS, both sharp endotherms and the very broad high-temperature component have vanished, leaving only a small, broad endotherm at lower temperatures.

In Figure 5, these results have been analyzed to construct phase diagrams for the two PC–PS systems. To identify particular structures with various regions of the phase diagrams, we can make use of the freeze–fracture results of van Dijck et al. (1978) for DMPC–DMPS mixtures as well as the known phase behavior of the pure lipid species. Pure DMPC will form hydrated gel-state lamellae at temperatures below 24.9 °C, while pure DMPS will form dehydrated cochleate structures that do not undergo any thermotropic transitions up to at least 90 °C (see above). The conversion of either gel-state lipid or dehydrated cochleate lipid to the liquid-crystalline state is a significantly endothermic process (Rehfeld et al., 1982), and the major endothermic transitions observed by calorimetry will therefore likely be associated primarily with such conversions. To illustrate how the calorimetric results are related to the phase diagram, the case of DMPS–DMPC is described in detail below.

The DMPS–DMPC system in high-calcium medium exhibits two sharp endothermic transitions whose temperature does not vary with composition, one at 27.5 °C from ~10–30% PS and the second at 37.1 °C from ~30–70% PS. These sharp endotherms appear to represent lines of coexistence of three phases: liquid-crystalline lipid cochleates and a solid solution at 37.1 °C; and liquid-crystalline lipid, a solid solution (S_{II}), and nearly pure gel-state PC (S_I) at 27.5 °C. The assumptions that liquid-crystalline and cochleate phases coexist above the 37.1 °C horizontal line and that only lamellar phases are present above and below the 27.5 °C line are based on freeze–fracture electron microscopic and calorimetric results previously reported for DMPC–DMPS and DPPC–bovine brain PS mixtures (van Dijck et al., 1978; Hui et al., 1983). The boundaries of the liquid/S_{II} coexistence region are relatively simple to map from the two three-phase lines, noting the temperature limits of the broad intervening endotherm in samples of various PS contents. The composition at which the right-hand boundary of the liquid/S_{II} coexistence region intersects the 37.1 °C three-phase line can be ascertained by using the prediction of the lever principle that the heat content of the 37.1 °C endotherm will be maximal at this composition. By this criterion, we have mapped the common intersection of the boundaries of the liquid/S_{II} and S_{II}/cochleate regions with the 37.1 °C three-phase line at slightly more than 50% PS. The right-hand boundary of the solid S_{II}/cochleate coexistence region must begin at the right-hand end of the 37.1 °C three-phase line (i.e., at ~70% PS) and can be mapped from this point by observing the small endotherm in the 72.5–90% PS samples. The other boundaries of regions of solid/solid coexistence cannot be reliably determined by calorimetry, and only their origins are indicated in the phase diagram.

To complete the phase diagram, we must assign the boundaries of the liquid-crystal/cochleate phase coexistence region, labeled as lines I and II in Figure 5A,B. I and II must originate at the left- and right-hand limits of the 37.1 °C horizontal line, i.e., at ~30% PS and ~70% PS, respectively. The very broad endothermic component seen by DSC above 37.1 °C in ~35–65% PS mixtures suggests that one or both of the lines I and II must shift significantly to the right (producing an endothermic partial conversion of cochleate to liquid-crystalline lipid) in the temperature range from 37 to ~50 °C. As samples containing ≥72.5% DMPS show no significant excess heat absorption above 37.1 °C, line II appears to be essentially vertical. The extent and amplitude of the upper temperature broad endotherm increase between 35% and 42.5% PS, with little further change at 50% DMPS and a significant and progressive decrease in amplitude at higher PS levels. These results, evaluated with consideration of the predictions of the lever principle, suggest that line I shifts to the right from its origin at ~30% PS and 37.1 °C to 40–45% PS at ~48 °C and ascends nearly vertically from this point. The limit of solubility of DMPS in a liquid-crystalline DMPC bilayer at high calcium concentrations thus appears to be 40–45 mol %, while the solubility of DMPC in the DMPS-rich cochleate phase at higher temperatures is ~30 mol %.

Thermograms of DMPC–DMPS (calcium) and DEPC–DEPS (calcium) mixtures like those illustrated in Figure 4 were integrated over base lines determined by parallel calorimetric scans of buffer without lipid. The total transition enthalpies of various PC–PS mixtures are plotted as functions

of composition in Figure 6A,B. In both cases, the transition enthalpy rises slightly from the value for the pure PC as PS is incorporated in the bilayer up to ~40 mol % and then falls off steadily as the bilayer PS content increases further. However, the transition enthalpy does not simply fall off linearly with increased PS content between 40% and 100% PS. Instead, the decline of the transition enthalpy with increasing PS content appears to change slope at ~80% PS. The data presented in Figure 6 are fully consistent with the phase diagrams shown in Figure 5 if it is assumed that conversions of gel-state and cochleate phases to a liquid-crystalline phase are strongly endothermic, while conversion of a hydrated gel-state bilayer to a cochleate phase is slightly endothermic (Rehfeld et al., 1982). In view of the multiple solid phases and highly nonideal mixing of the PS–PC systems in the presence of calcium, more detailed interpretation of the data of Figure 6 is unwarranted.

The data presented above were obtained with samples incubated with substantially higher calcium concentrations, and for much longer times, than those examined in most of the fusion experiments described below. To determine whether at least qualitatively similar results could be obtained under conditions comparable to those of the fusion assays, we carried out two types of calorimetric experiments. First, equimolar mixtures of DMPC and DMPS were quenched from 45 to 0 °C only 5–20 min after the addition of $CaCl_2$ to 30 mM (i.e., over a time course more closely comparable to that of the fusion assay for these vesicles). Scanning calorimetry of such samples, begun from 5 °C no more than 45 min after sample cooling to 0 °C, showed clear indications of substantial phase separation even in samples preincubated for only 5 min at 45 °C. Preincubation times of <20 min consistently sufficed to produce thermograms with well-developed features indicative of liquid-crystalline/cochleate phase separation, such as the sharp 37.1 °C endotherm representing the upper three-phase line in Figure 5B. In the second type of experiment, a limited number of DMPS–DMPC and DEPS–DEPC samples containing 40–70% PS were equilibrated with lower concentrations of calcium (0.5–15 mM) by dialysis at 45 °C before cooling and calorimetric analysis. These samples invariably exhibited phase separation when the calcium concentration was above the threshold for fusion of vesicles of the particular lipid composition examined, but not when the calcium level was appreciably below the threshold.

Fusion Studies. The fusion of vesicles containing various proportions of DOPS and DOPC at 25 °C, or of DEPS and DEPC at 37 °C, was examined by using the assay of Hoekstra (1982a), which monitors the development of resonance energy transfer between two lipid fluorophores associated with the membranes of initially separate vesicle populations. In Figure 7, the fusion rates for vesicles containing varying proportions of DOPS and DOPC are shown as functions of the calcium concentration. As the mole fraction of PC in the vesicles increases, the rate of vesicle fusion at any fixed concentration of calcium steadily decreases. As the data shown in Figure 8A,B illustrate, this behavior is a consequence of two factors that depend on the PC content of the vesicles. First, as shown in Figure 8A, the threshold calcium concentration for fusion increases with increasing PC content, particularly above ~25% PC. This variation of the calcium threshold for fusion with PC content is similar to but not identical with that reported by Ohki et al. (1982) for aggregation of PC–PS vesicles. Second, the "efficiency" of fusion (defined here as the rate of fusion at a calcium concentration twice the threshold level) decreases dramatically as the PC content is increased, particularly above ~25% PC, and is essentially zero at 60% PC, as shown in Figure 8B. The behavior of DEPC–DEPS vesicles at 37 °C was quantitatively comparable to that of DOPC–DOPS vesicles at 25 °C (except in the absolute rates of fusion) in a more limited number of experiments using the dielaidoyl lipids. For example, the threshold calcium concentration for 50:50 DEPS:DEPC vesicles was 7.5 mM vs. 6.5 mM (interpolated value) for 50:50 DOPS:DOPC vesicles. Likewise, the efficiency of fusion of the 50:50 DEPS:DEPC vesicles was 14% of the value for pure DEPS vesicles, while the interpolated efficiency for 50:50 DOPS:DOPC vesicles was 11% of that for vesicles of pure DOPS.

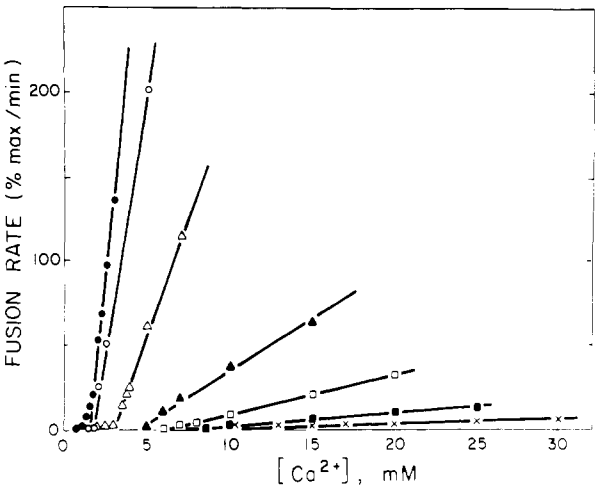

FIGURE 7: Initial rates of fusion of DOPS–DOPC vesicles, determined by the vesicle wall coalescence assay of Hoekstra (1982), at varying calcium concentrations. The percentage of DOPS in the different vesicle preparations was (●) 100%, (○) 77%, (△) 67%, (▲) 57.5%, (□) 52.5%, (■) 45%, and (×) 38%. Initial rates of fusion were measured after injection of calcium into vesicle suspensions (40 μM total lipid concentration) in 200 mM NaCl, 2 mM histidine, and 2 mM Tes, pH 7.4 at 25 °C.

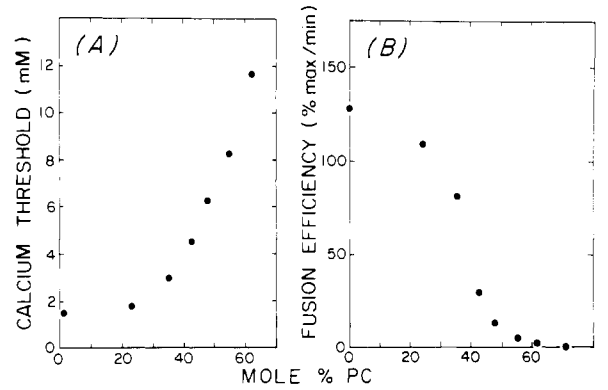

FIGURE 8: (A) Threshold calcium concentrations for fusion of DOPC–DOPS vesicles of the indicated DOPC contents. Fusion was assayed by the procedure of Hoekstra (1982) under the conditions described in the legend to Figure 7. (B) Variation of the efficiency of fusion of DOPC–DOPS vesicles (defined as the rate of fusion at a calcium concentration twice threshold) with DOPC content.

The findings just described are in qualitative agreement with the findings of Düzgünes et al. (1981a,b) for vesicles composed of bovine brain PS and egg PC. These authors, however, reported a complete inhibition of fusion, assayed by the procedure of Wilschut et al. (1980), at 50 mol % PC while we consistently detect a slow but finite rate of fusion at PC contents up to ~60 mol %. To confirm that we were detecting genuine vesicle fusion, two tests were performed. First, we determined that the resonance energy transfer between lipid fluorescent probes that developed when <10 mM calcium was

added to vesicles of high PC content was not reversed by addition of excess EDTA, while the calcium-induced increase in light scattering (an indicator of aggregation as well as fusion) was largely reversed by addition of the chelator. It thus appears that the assay was detecting true fusion and not simply aggregation of the PC-rich vesicles. This conclusion agrees with the report of Hoekstra (1982a) that poly(ethylene glycol)-induced aggregation of two populations of DOPC vesicles, containing the lipid fluorophores in distinct vesicles, does not induce resonance energy transfer. Second, we monitored the fusion of 50:50 DOPS:DOPC vesicles by the contents-mixing assay of Wilschut et al. (1980). By this procedure as well, we measured a slow but finite rate of vesicle fusion at 20 mM and 30 mM calcium (data not shown) although we could not make measurements (including necessary leakage corrections) of sufficient accuracy to permit precise determination of the calcium threshold concentration and efficiency for fusion of these vesicles by this assay. Therefore, it appears that vesicles containing up to 60% of DOPC in DOPS (or DEPC in DEPS) have a genuine (but low) ability to fuse at high levels of calcium.

Discussion

Phase Behavior of PS–PC Mixtures. Certain PC–PS mixtures in which at least the PC component is a single molecular species have been examined previously by calorimetry (Luna & McConnell, 1977; van Dijck et al., 1978; Stewart et al., 1979; Hui et al., 1983), while studies of the fusion of vesicles containing PC and PS have generally utilized lipids of heterogeneous fatty acyl composition. By correlation of the results of these two types of studies, a consistent explanation of the fusion capabilities of PC–PS vesicles of varying composition in terms of the mixing properties of these lipids in the presence and absence of calcium can be developed.

The phase diagrams for DMPS–DMPC and DEPS–DEPC in the presence of only monovalent cations indicate that these lipids are highly miscible in both the gel and liquid-crystalline phases. The phase diagram obtained for the DMPS–DMPC system is very similar in form to that reported for the DPPS–DPPC system by Luna & McConnell (1977) and to the mixing diagram reported by Stewart et al. (1979) for the DPPC–bovine brain PS system. Our phase diagram for DMPS–DMPC in the absence of calcium contradicts the results of van Dijck et al. (1978) in that we find no indication of gel phase immiscibility at high mole fractions of DMPS as did these authors. This discrepancy was not resolved when we used the buffer system employed in the earlier study (which used a sample pH of 6.0 in contrast to the pH of 7.4 used here).

The phase diagrams constructed from the calorimetric data for the DMPS–DMPC and DEPS–DEPC systems in the presence of high levels of calcium (30 mM) are consistent with the results of freeze–fracture electron microscopy of the DMPS–DMPC and bovine brain PS–DPPC systems at high calcium levels (van Dijck et al., 1978; Hui et al., 1983). From our phase diagram for the DMPC–DMPS (calcium) system (Figure 5B), we expect PS–PC samples containing ~40–70% PS to contain both lamellar and cochleate structures at temperatures well above 37 °C. At temperatures well below this point, two solid phases will be present up to ~40% PS, while a solid solution and cochleate structures will coexist from ~60% to 90% PS. The freeze–fracture electron microscopic results of van Dijck et al. (1978) for the DMPS–DMPC system agree with these conclusions. The freeze–fracture results of Hui et al. (1983) for DPPC–bovine brain PS mixtures in the presence of 20 mM calcium show the coexistence of banded vesicular and cochleate structures over the composition range from 30% to over 60% PS. The boundaries of the liquid-crystalline/cochleate coexistence region in this system therefore appear to be similar to those found here for the DEPS–DEPC and DMPS–DMPC systems at high calcium levels. Interestingly, the presence of a sharp endothermic peak at ~42 °C in the thermograms reported by Hui et al. for mixtures containing ~30–70% bovine brain PS in DPPC suggests a possible line of three-phase coexistence in the "phase diagram" for this system at this temperature and range of compositions. This feature of the calorimetric data would suggest the coexistence of liquid-crystalline and cochleate phases from ~30% to 70% PS at 45 °C (i.e., just above the temperature of the three-phase line), as the freeze–fracture results confirm.

The Raman spectroscopic results of Hark & Ho (1980) for the DMPC-d_{54}–bovine brain PS system in the presence of calcium suggest that the phase behavior of this system is also similar to that of the PS–PC systems studied here. These authors reported that a 2:1 DMPC-d_{54}:bovine PS mixture showed a thermotropic solid to liquid-crystalline transition for both components, while a 1:2 mixture of these components remained in a solidlike state throughout the temperature range examined. Such behavior would also be expected for, e.g., the DMPC–DMPS system (see Figure 5B), where a 2:1 PC:PS mixture in excess calcium will change from a mixture of solid phases to a predominantly liquid-crystalline state with increasing temperature, while a 1:2 mixture will consist almost entirely of solid and (presumably solidlike) cochleate phases over a wide range of temperatures. It thus appears that when differences in the transition temperatures of the components of various PS–PC mixtures are allowed for, the phase behavior of the DMPS–DMPC and DEPS–DEPC systems in the presence of calcium is generally representative of that of a variety of other PS–PC systems.

Relevance to Fusion of PC–PS Vesicles. The phase diagrams discussed above indicate that a PS–PC mixture in the absence of calcium and at temperatures well above the transition temperatures of its components will exist as a single fluid phase. Addition of calcium to such a mixture will lead to phase separation into PS-rich and PC-rich domains at equilibrium only if the mixture contains ~40–70% PS. Within this range of compositions, the relative amounts of the PS-rich (cochleate) and PC-rich (liquid-crystalline) phases at equilibrium will vary according to the lever principle, i.e., from 100% cochleate phase at ~70% PS to 0% cochleates at 40% PS. In light of this result, it is striking to note that the efficiency of calcium-triggered fusion of DOPS–DOPC or DEPS–DEPC vesicles (expressed as the initial rate of fusion at a calcium concentration equal to twice the threshold level) declines steeply over the same range of PS contents to a value of essentially zero at $\leq$40% PS. This correlation of the fusion and calorimetric results suggests that the increasing ability of PS–PC vesicles to fuse as their PS content is increased from ~40% to ~70% is a consequence of the increasing thermodynamic potential of such vesicles to phase separate in the presence of calcium, forming PC-rich domains incapable of mediating fusion and PS-rich domains that support efficient vesicle fusion.

The proposed interpretation of our results just presented, in which lateral phase separation plays a necessary role in the calcium-induced fusion of PC–PS vesicles containing <70% PS, would be untenable if it could be demonstrated that fusion could occur in such vesicles without phase separation under certain conditions. Hoekstra (1982b) has in fact reported that the time course of lateral redistribution of NBD-PE in PC–PS

vesicles upon calcium addition is much slower than that of fusion (>6 h vs. a few minutes). However, it is not clear from the results presented how the lateral distribution of NBD-PE, present at low mole fractions (~5%), correlates with the distributions of PS and PC, either at equilibrium or during lateral redistribution of the major lipid species. Our calorimetric observations with PC–PS vesicles incubated for short times with calcium, together with the electron spin resonance studies of Ohnishi & Ito (1974) and the calorimetric and freeze–fracture results of van Dijck et al. (1978), suggest that major lateral phase separations and structural rearrangements can occur within (at most) a few minutes after calcium is added to PC–PS vesicles. This time scale is comparable to that of the fusion assay for vesicles containing <70% PS.

Attempts to separate vesicle fusion from lateral phase separation were no more successful when vesicles containing <70% PS were exposed to calcium concentrations around the fusion threshold prior to calorimetry, as discussed under Results. The available evidence, therefore, is quite consistent with the concept that at least a limited lipid phase separation, forming PS-enriched domains, accompanies the fusion of PC–PS vesicles containing substantially less than 70 mol % PS. Unfortunately, direct and conclusive evidence for a causal link between the two processes is still lacking at present.

Comparison of the phase diagrams and fusion behavior of the PS–PC systems studied here with those for the corresponding PS–PE systems discussed in the preceding paper (Silvius & Gagné, 1984) indicates that PC and PE differ much more in their intrinsic abilities to participate in the formation of "fusing centers" than in their miscibilities with PS. In the absence of calcium, both PC and PE are miscible with PS of similar acyl chain composition in all proportions. The saturating level of PS in liquid-crystalline PE bilayers at high calcium concentrations is 15–20 mol % PS, while in liquid-crystalline PC bilayers, it is ~40 mol % PS. The saturating level of PE in PS cochleate structures appears to be ~20 mol %, and that of PC, ~30 mol %. The differences in PE and PC mixing with PS appear, therefore, to be more quantitative than qualitative. However, a PE bilayer containing even low levels of PS can support very efficient calcium-induced fusion without lateral phase separation, while fusion of PC–PS vesicles will occur only if the level of PS is sufficient to saturate the PC liquid-crystalline phase (at ~40 mol % PS) in the presence of calcium. These considerations raise some question as to whether calcium-induced lateral phase separations play a significant role in the in vivo fusion of membranes of physiological PS content (<30 mol %, even if all of the PS is localized on one side of the membrane). The intrinsic fusion-supporting abilities (and, if multiple species are present, the relative proportions) of the zwitterionic lipid species present may be considerably more important than the exact level of PS in determining whether or not a given membrane is capable of or relatively refractory to fusion.

Registry No. DEPC, 56782-46-8; DEPS, 63976-14-7; DMPC, 18194-24-6; DMPS, 64023-32-1; DOPS, 70614-14-1; DOPC, 4235-95-4; calcium, 7440-70-2.

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