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STUDIES ON MEMBRANE FUSION

II. INDUCTION OF FUSION IN PURE PHOSPHOLIPID MEMBRANES BY CALCIUM IONS AND OTHER DIVALENT METALS

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SUMMARY

The effect of divalent metals on the interaction and mixing of membrane components in vesicles prepared from acidic phospholipids has been examined using freeze-fracture electron microscopy and differential scanning calorimetry. Ca^{2+}, and to a certain extent Mg^{2+}, induce extensive mixing of vesicle membrane components and drastic structural rearrangements to form new membranous structures. In contrast to the mixing of vesicle membrane components in the absence of Ca^{2+} described in the accompanying paper which occurs via diffusion of lipid molecules between vesicles, mixing of membrane components induced by Ca^{2+} or Mg^{2+} results from true fusion of entire vesicles. There appears to be a "threshold" concentration at which Ca^{2+} and Mg^{2+} become effective in inducing vesicle fusion and the threshold concentration varies for different acidic phospholipid species. Different phospholipids also vary markedly in their relative responsiveness to Ca^{2+} and Mg^{2+}, with certain phospholipids being much more susceptible to fusion by Ca^{2+} than Mg^{2+}. Vesicle fusion induced by divalent cations also requires that the lipids of the interacting membranes be in a "fluid" state ($T > T_c$). Fusion of vesicle membranes by Ca^{2+} and Mg^{2+} does not appear to be due to simple electrostatic charge neutralization. Rather the action of these cations in inducing fusion is related to their ability to induce isothermal phase transitions and phase separations in phospholipid membranes. It is suggested that under these conditions membranes become transiently susceptible to fusion as a result of changes in molecular packing and creation of new phase boundaries induced by Ca^{2+} (or Mg^{2+}).

INTRODUCTION

In the preceding paper [1] we reported data on the interaction and molecular mixing of components from pure phospholipid membranes suspended in NaCl solutions and the effect of long chain fatty acids, lysolecithin, a hydrophobic protein and dimethylsulfoxide on the mixing process. We concluded that in many cases where

molecular mixing can be demonstrated the experimental evidence indicates that diffusion of lipid molecules between vesicles is involved rather than fusion of intact vesicles. This is an important consideration since several recent studies have claimed to demonstrate fusion between vesicles based on data that does not exclude molecular diffusion [2–5].

In this paper we present evidence indicating that Ca^{2+} and in some but not all cases Mg^{2+} can induce rapid and extensive fusion between several types of phospholipid vesicles. The original observations on the effect of Ca^{2+} in inducing fusion of mixed phosphatidylserine/phosphatidylcholine vesicles were reported earlier from this laboratory [6]. Evidence of vesicle fusion was based on data obtained by differential scanning calorimetry and by electron microscopy. Subsequently, evidence for Ca^{2+}-induced fusion of lipid vesicles has been obtained by electron spin resonance [4] and by a system involving protein-protein interactions in separate vesicles [7]. Freeze-fracture electron microscopy has also shown that Ca^{2+}-induced fusion of phosphatidylserine vesicles results in the formation of large, spirally folded cochleate lipid cylinders [8].

In this study we report the use of differential scanning calorimetry and freeze-fracture electron microscopy to monitor fusion between vesicles induced by Ca^{2+} and Mg^{2+}. Fusion was observed with both sonicated (unilamellar) and non-sonicated (multilamellar) vesicles but only at a temperature above the solid-to-liquid crystalline phase transition.

MATERIALS AND METHODS

Lipids and preparation of lipid vesicles. All the phospholipids used in this study were synthesized in this laboratory as described in detail elsewhere [9–11] and contained no detectable impurities by thin-layer chromatography on silica gel H and a solvent of chloroform/methanol/7 M ammonia (230 : 90 : 15, v/v). Poly-L-lysine (mol. wt. 17 000) was from Mann Research, Orangeburg, N.Y. Other chemicals used are as before (see ref. 1).

Preparation of multilamellar lipid vesicles (liposomes) was as described in detail in the preceding paper [1] by mechanical shaking in a buffer containing 100 mM NaCl, 2 mM L-histidine, 2 mM *N*-tris (hydroxymethyl) methyl-2-aminoethanesulfonic acid (TES) and 0.1 mM EDTA at pH 7.4. The lipid concentrations used in different experiments are stated in the figure legends. $CaCl_2$ and $MgCl_2$ were added at the concentration stated after the formation of the vesicles. Sonication was used only for the phosphatidic acid dispersions which were examined by freeze-fracture and X-ray diffraction after addition of Ca^{2+} and Mg^{2+}.

The detailed methodology for differential scanning calorimetry was as described before [1, 11] with a Perkin-Elmer DSC-2, at a sensitivity range of 1 mcal/s and heating rate of 5 °C per min. Freeze-fracture electron microscopy was as before [8].

X-ray diffraction. X-ray diffraction analysis was performed by a method described in detail previously [12]. In brief, phospholipid samples were collected by brief centrifugation after precipitation of the original dispersion in buffer by addition of metal ion. They were then transferred to sample holders, in which they were sealed between mica windows, 1 mm apart. The X-ray camera was of the Guinier type, operating in vacuum, and equipped with a bent quartz crystal monochromator which

isolated the CuKα_1 line ($\lambda = 1.54$ Å). The X-ray diagrams were recorded photographically at room temperature. The X-ray diagrams give a number of sharp reflections. The ratio of these spacings indicates the symmetry of the phases present. A lamellar phase is evidenced by spacings in the ratio 1, 2, 3, 4, . . . , while a hexagonal phase is evidenced by spacings in the ratio 1, $\sqrt{3}$, 2, $\sqrt{7}$ . . . . . The magnitude of the observed spacing gives the dimension d, of the repeating unit.

RESULTS AND DISCUSSION

Fusion of phosphatidylglycerol vesicles. Calorimetric studies.

It has been reported that Ca^{2+} and Mg^{2+} at low concentrations produce an

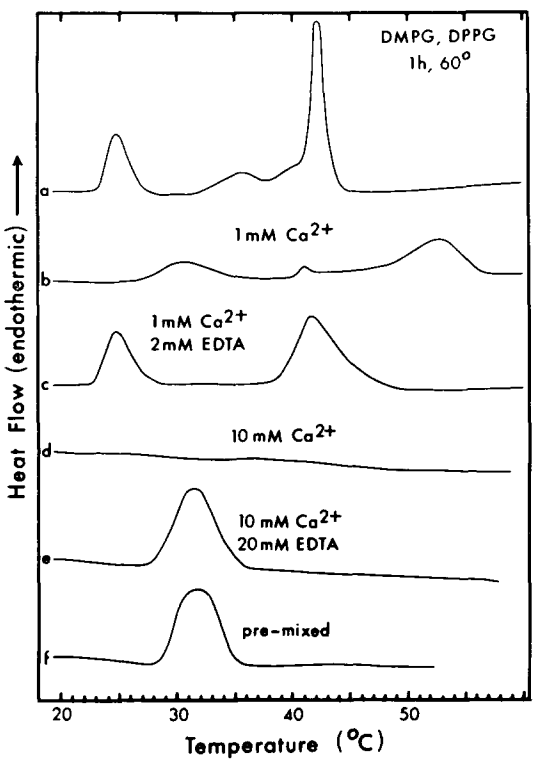

Fig. 1. Effects of Ca^{2+} on the thermotropic phase transitions and mixing of pre-formed vesicles of dimyristoyl phosphatidylglycerol (DMPG) and dipalmitoyl phosphatidylglycerol (DPPG). In this and the following Figs. 2–6 the phospholipids were suspended separately by shaking in 100 mM NaCl buffer, pH 7.4 at 45 °C at a concentration of 5 μmol/ml, and then equilibrated for 30 min at 45 °C. Aliquots from each suspension containing approximately 2 μmol of each lipid were mixed, diluted to a total concentration of 1 μmol/ml with 100 mM NaCl buffer pH 7.4 and incubated (for this experiment) at 60 °C for 1 h under the following conditions: (a) no other additions; (b) 1 mM $CaCl_2$; (c) 1 mM $CaCl_2$ for 1 h, then 2 mM EDTA and additional incubation for 0.5 h at 60 °C; (d) 10 mM $CaCl_2$; (e) 10 mM $CaCl_2$ for 1 h, followed by 20 mM EDTA for 0.5 h; (f) equimolar quantities of dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol, mixed in chloroform and suspended in 100 mM NaCl buffer at 45 °C as above. After the end of each of the above incubations the suspensions were centrifuged at 100 000 $\times g \times$ 15 min at 20 °C. The wet pellets were then transferred to the sample pans of the differential scanning calorimeter and analysed within 1–2 hours.

increase in the phase transition temperature (T_c) of acidic phospholipid membranes [11, 13–16]. It has also been observed that above a threshold concentration of Ca^{2+} (which is different for each phospholipid) the phase transition disappears completely [11], although in some cases a new phase transition at much higher temperatures can be obtained [16]. We have utilized these observations to study the effect of Ca^{2+} on the interaction and fusion of vesicles prepared from dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol.

A thermogram of separate vesicles composed of these two lipids after incubation together for 1 h at 60 °C is shown in Fig. 1 (curve a). The two main endothermic transitions of the two lipids can be clearly distinguished at 24.5 and 42.0 °C (midpoints). As shown in Fig. 3 (curve b) of the preceding paper, the minor transition at 35 °C is the pre-melt of dimyristoyl phosphatidylglycerol. The results indicate that very little, if any, mixing between the two populations of phosphatidylglycerol vesicles incubated in 100 mM NaCl aqueous solutions under these conditions. Mixed vesicles

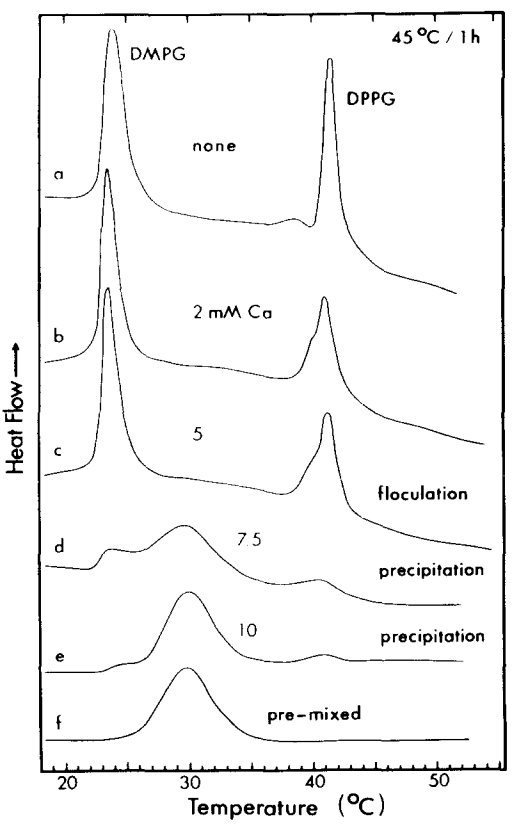

Fig. 2. Effects of different Ca^{2+} concentrations on the mixing of pre-formed vesicles of dimyristoyl phosphatidylglycerol (DMPG) and dipalmitoyl phosphatidylglycerol (DPPG). Conditions as described in legend to Fig. 1 except that the samples were incubated at 45 °C for 1 h under the following conditions: (a) no additions; (b) 2 mM $CaCl_2$, 1 h, then 3 mM EDTA, 0.5 h at 45 °C; (c) 5 mM $CaCl_2$, 1 h, then 6 mM EDTA, 0.5 h at 45 °C; (d) 7.5 mM $CaCl_2$, 1 h, then 8.5 mM EDTA, 0.5 h at 45 °C; (e) 10 mM $CaCl_2$, 1 h, then 12 mM EDTA, 0.5 h, 45 °C; (f) equimolar mixture of the two lipids suspended in 100 mM NaCl buffer.

formed by complete mixing of equimolar proportions of the two components in chloroform have a phase transition at 31.5 °C (Fig. 1, curve f).

When similar populations of phosphatidylglycerol vesicles were incubated in the presence of Ca^{2+} (1 mM), the two main endothermic transitions were still observed, but were shifted to the higher temperatures of 30.5 and 52.5 °C (Fig. 1, curve b). Addition of 2 mM EDTA and further incubation of the mixture at 60 °C for 10 min resulted in the reappearance of the two original transitions at 24.5 and 42.0 °C (Fig. 1, curve c), indicating that the individual phosphatidylglycerol vesicles were still in fairly pure form and had not mixed. When the same type of experiment was performed in the presence of 10 mM Ca^{2+} (1 h incubation at 60 °C) followed by incubation in the presence of 20 mM EDTA (10 min at 60 °C), entirely different results were obtained. Exposure of the vesicles to this higher Ca^{2+} concentration resulted in a complete disappearance of the two peaks (Fig. 1, curve d). This effect of Ca^{2+} in completely eliminating the phase transition appears to involve the establishment of a new crystalline phase with a transition temperature that is much higher [11, 16]. When these vesicles were incubated with EDTA the two original peaks did not reappear and only a single peak at 31.5 °C was found. This indicates that complete mixing of the vesicles had occurred (Fig. 1, curve e).

Freeze-fracture electron-microscopic data on the appearance of these membranes before and after Ca^{2+}-induced mixing will be presented below.

Further experiments on Ca^{2+}-induced mixing of phosphatidylglycerol vesicle membrane components were done in which the vesicles were incubated at 45 °C for 1 h at different Ca^{2+} concentrations before being incubated with EDTA in slight molar excess to the Ca^{2+} present. The results, shown in Fig. 2 (curves a, b and c) indicate that mixing does not occur in the absence of Ca^{2+} or at Ca^{2+} concentrations of 2 and 5 mM, though visible flocculation of the vesicles occurs at the latter concentration. Complete mixing was obtained only at 10 mM Ca^{2+} (Fig. 2, curve e) as indicated by the formation of a single transition with an endothermic peak at a temperature similar to that of the pre-mixed membranes (Fig 2, curve f). The kinetics of the mixing reaction were studied by incubating the two phosphatidylglycerol vesicle populations in the presence of 10 mM Ca^{2+}, at 45 °C for different periods of time. As shown in Fig. 3, addition of EDTA before Ca^{2+} and subsequent incubation for 35 min prevented mixing (curve a). In the presence of Ca^{2+} mixing was not observed at 2.5 min (curve b) or 7 min (curve c) even though extensive aggregation of vesicles occurred as indicated by progressive development of cloudiness and flocculation during this time. However, samples incubated for 20 min exhibited three distinct peaks (curve d). Of these, two were at temperatures identical to those obtained with the individual components, while the third peak occurred at 31 °C, which is the temperature for the transition of the equimolar mixture (curve f). Incubation for 60 min gave only one peak at 31 °C (curve e) indicating that complete mixing of the membrane components had taken place. We conclude from this data that Ca^{2+}-induced molecular mixing involves fusion between phosphatidylglycerol vesicles rather than the exchange of lipid molecules between the vesicles by diffusion.

For technical reasons in all the above experiments excess EDTA was added after the initial incubation with Ca^{2+} because Ca^{2+} abolishes any phase transition with these lipids at these concentrations (Fig. 1) and its subsequent chelation by EDTA is required for the calorimetric analysis of the mixing process. It is of interest,

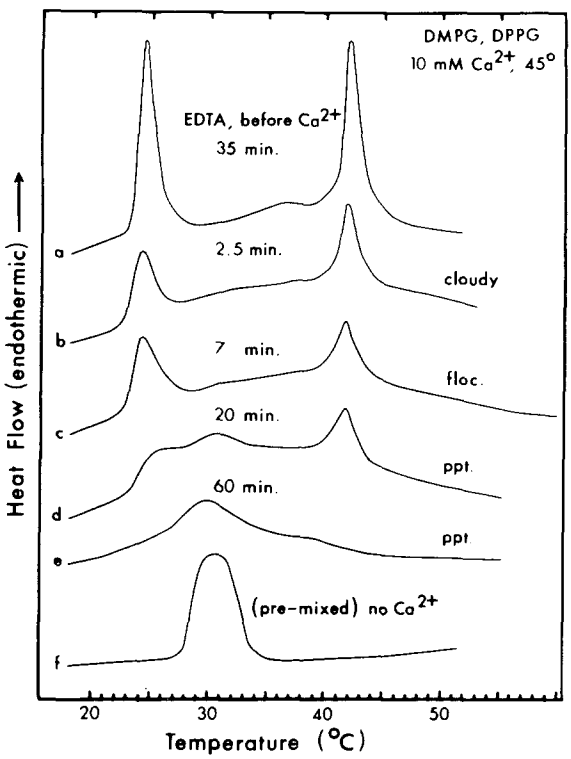

Fig. 3. Effects of Ca^{2+} at different incubation times on the mixing of dimyristoyl phosphatidylglycerol (DMPG) and dipalmitoyl phosphatidylglycerol (DPPG) conditions as described in the legend to Fig. 1 except that incubation was at 45 °C in the presence of 10 mM $CaCl_2$ at the following times, followed by 12 mM EDTA and further incubation at 45 °C for 15 min. (a) EDTA added just before Ca^{2+}, incubated for 35 min; (b) 2.5 min Ca^{2+}; (c) 7 min Ca^{2+}; (d) 20 min Ca^{2+}; (e) 60 min Ca^{2+}, (f) equimolar mixture of the lipids suspended in NaCl buffer. floc, flocculation; ppt. precipitation.

however, that the phosphatidylglycerol membranes are induced to undergo a phase transition both during the initial addition of Ca^{2+} and also during the subsequent chelation of Ca^{2+} by EDTA. It is thus possible that observed mixing of membrane components could be occurring during either of these two steps. The following experiment was designed in order to clarify this point.

Two populations of phosphatidylglycerol membranes were incubated together in the presence of Ca^{2+} (10 mM; 1 h) and then in the presence of EDTA (20 mM; 0.5 h) at different temperatures above and below the T_c of each membrane component. The results are shown in Fig. 4. Curves a, b and c were obtained when both incubations were performed at 60, 45 and 36 °C, respectively. No appreciable mixing was obtained at 36 °C which is below the T_c of dimyristoyl phosphatidylglycerol, but complete mixing occurred at 45 and 60 °C which are both above the T_c of dimyristoyl phosphatidylglycerol. It thus appears that both membrane components must be fluid before molecular mixing can occur. Curves d–g in Fig. 4 were obtained when samples were incubated at 60 °C with Ca^{2+} (10 mM) and the temperature reduced to 36, 26, 20 or 0 °C, respectively, during the subsequent incubation with EDTA. The results

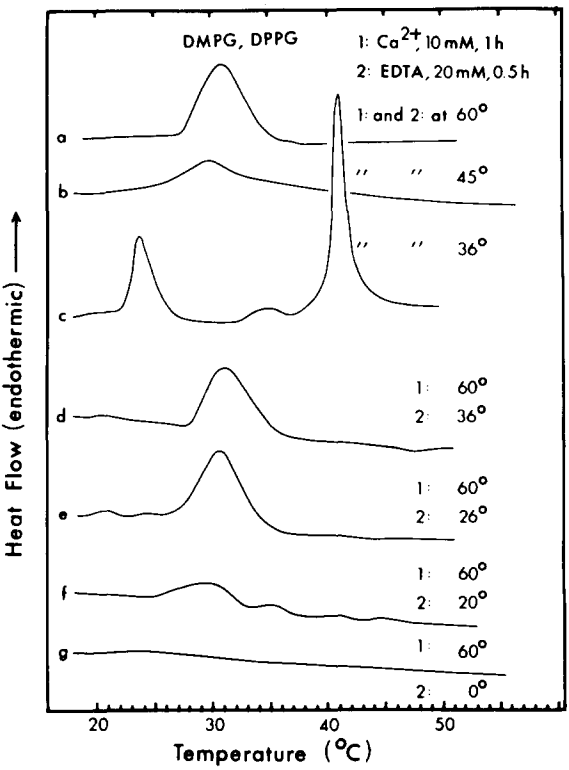

Fig. 4. Effect of temperature on the mixing of pre-formed vesicles of dimyristoyl phosphatidylglycerol (DMPG) and dipalmitoyl phosphatidylglycerol (DPPG) following the addition of Ca^{2+} or EDTA. Preparation of samples and additions as in legend to Fig. 1. All samples were first incubated in the presence of Ca^{2+} (10 mM, 1 h) and then EDTA (20 mM, 0.5 h) at the following temperatures: (a) both Ca^{2+} and EDTA at 60 °C; (b) both Ca^{2+} and EDTA at 45 °C; (c) both Ca^{2+} and EDTA at 36 °C; (d) Ca^{2+} at 60 °C then EDTA at 36 °C; (e) Ca^{2+} at 60 °C then EDTA at 26 °C; (f) Ca^{2+} at 60 °C then EDTA at 20 °C; (g) Ca^{2+} at 60 °C then EDTA at 0 °C.

indicate that complete mixing (single endothermic peak present, melting at approximately 31 °C) had occurred even in the samples incubated with EDTA at 36 and 26 °C. At these temperatures dipalmitoyl phosphatidylglycerol or both the dipalmitoyl phosphatidylglycerol and the mixture of dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol were below their T_c and thus solid. Considerable mixing also occurred in samples incubated with EDTA at 20 °C (curve f) at which temperature all components would be solid. These results indicate that mixing occurs during the initial addition and incubation with Ca^{2+} rather than during the EDTA step. The absence of any transitions in the sample shown in curve g (incubation at 0 °C with EDTA) indicates that the EDTA is not capable of removing the Ca^{2+} from the phosphatidylglycerol membranes, perhaps due to restricted transport through the frozen membranes of the multilamellar system.

As mentioned earlier, gross aggregation and flocculation is readily apparent in the system but is not necessarily related to the occurrence of molecular mixing and fusion. The effect of two other cations which induce aggregation of phosphatidyl-

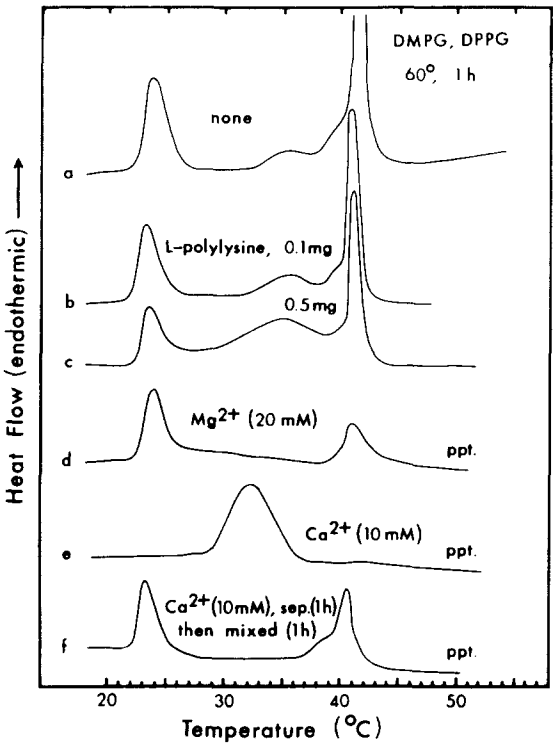

Fig. 5. Effect of polylysine, Mg^{2+} and Ca^{2+} on the mixing of pre-formed vesicles of dimyristoyl phosphatidylglycerol (DMPG) and dipalmitoyl phosphatidylglycerol (DPPG). Conditions as described in legend to Fig. 1. All samples were incubated for 1 h at 60 °C. (a) no other additions; (b) 0.1 mg of poly-L-lysine was added to the incubation mixture containing 2 μmol of each lipid in 2 ml total volume; (c) as in (b) except 0.5 mg poly-L-lysine added; (d) 20 mM $MgCl_2$ during incubation, 1 h, followed by 30 mM EDTA and additional incubation for 0.5 h; (e) same as in (d) except 10 mM $CaCl_2$ followed by 20 mM EDTA; (f) the two vesicle populations were incubated for 1 h, 60 °C, 10 mM $CaCl_2$ separately, then they were mixed, incubated for an additional 1 h at 60 °C, followed by EDTA, 20 mM, 0.5 h. ppt., precipitation.

glycerol vesicles on mixing is shown in Fig. 5. The control shown in curve a was incubated at 60 °C for 1 h and shows negligible mixing of the two components. Curves b and c were obtained by incubating the vesicle populations in the presence of two concentrations of poly-L-lysine which were both effective in producing immediate flocculation and subsequent precipitation of the phosphatidylglycerol vesicles. No appreciable mixing was observed at low concentrations of poly-L-lysine (curve b), but at higher concentrations considerable material melting at intermediate temperatures was found, indicating mixing (curve c). The temperature of the intermediate peak in curve c was higher (35 °C) than that of the equimolar mixture (32 °C), and it is thus difficult to ascertain whether fusion is occurring in this system.

The effect of Mg^{2+} was more clear-cut. As shown in Fig. 5 (curve d) incubation of phosphatidylglycerol vesicles in the presence of 20 mM Mg^{2+} did not produce appreciable mixing, even though it induced precipitation of the vesicles. However, higher concentrations of Mg^{2+} (100 mM) produced approximately 50 % conversion

of vesicle lipids to a 32 °C peak (results not shown). It appears therefore that while aggregation is associated with the fusion of vesicles, it is not automatically followed by fusion.

Further data of interest to the possible mechanism of Ca^{2+}-induced fusion is described in Fig. 5 (curve f). In this case, the phosphatidylglycerol membranes were incubated separately in Ca^{2+} (1 h; 10 mM), then incubated together for an additional hour, before final incubation with excess EDTA (0.5 h). The presence of two distinct peaks in such samples indicates that no appreciable mixing has occurred and that little or no fusion results under these conditions despite the presence of Ca^{2+}. This result provides further support for the above conclusion that Ca^{2+} induced fusion is related to the molecular changes in the membranes and possible phase transitions occurring during the initial addition of Ca^{2+} above a threshold concentration.

In other experiments it was established that the presence of dibucaine (1.0 mM) in a mixture of dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol vesicles completely inhibited fusion induced by Ca^{2+} (12 mM) (results not shown). Incubation of vesicles for 1 h at 45 min with Ca^{2+} alone followed by EDTA produced a single peak at 32 °C, but addition of dibucaine to the same system produced two peaks at −2 and −21 °C, with no intermediate peak being found. This is the expected effect of dibucaine in lowering the T_c of the individual phospholipid components as shown in a recent study from this laboratory [15]. The results indicate that dibucaine can effectively inhibit Ca^{2+}-induced fusion of phosphatidylglycerol vesicles, a conclusion compatible with its inhibitory effects on cell fusion [17].

It was of interest to us to establish whether the above observations on the fusion of phosphatidylglycerol vesicles could also be shown with sonicated, unilamellar vesicles. Addition of either 5 mM Ca^{2+} or 20 mM Mg^{2+} to sonicated populations of phosphatidylglycerol vesicles induced formation of a single peak at a temperature intermediate of the two initial components (results not shown). This indicates that sonicated phosphatidylglycerol vesicles are even more sensitive to fusion by Ca^{2+} and Mg^{2+} since fusion is induced by these ions at concentrations lower than those needed to induce fusion of multilamellar vesicles. Sonicated vesicles of dimyristoylphosphatidylglycerol and dipalmitoyl phosphatidylglycerol were also used to study the effect of poly-L-lysine, with a system identical to that described in Fig. 5 (curve c). Poly-L-lysine (added separately) produced a shift of the T_c of the individual components to 5 °C higher temperatures (from 24 and 41 to 29 and 46 °C, respectively). This is in accord with recent observations on the effect of several proteins and polypeptides on lipid phase transitions [18]. However, when poly-L-lysine was added to a mixture of the dimyristoyl phosphatidylglycerol and dipalmitoyl phosphatidylglycerol vesicles most of the material melted within a broad peak with a midpoint at 37 °C, indicating extensive molecular mixing. Poly-L-lysine at these concentrations also produced extensive aggregation and precipitation of the vesicles. Although this result suggests that fusion of the vesicles has taken place, definitive proof must await further kinetic studies.

Fusion of phosphatidic acid vesicles. Calorimetric studies

Calorimetric observations on two populations of vesicles composed of egg-yolk phosphatidic acid and dimyristoyl phosphatidic acid, both suspended by shaking in 100 mM NaCl buffer at pH 7.4, are shown in Fig. 6. Curves a and b show that each

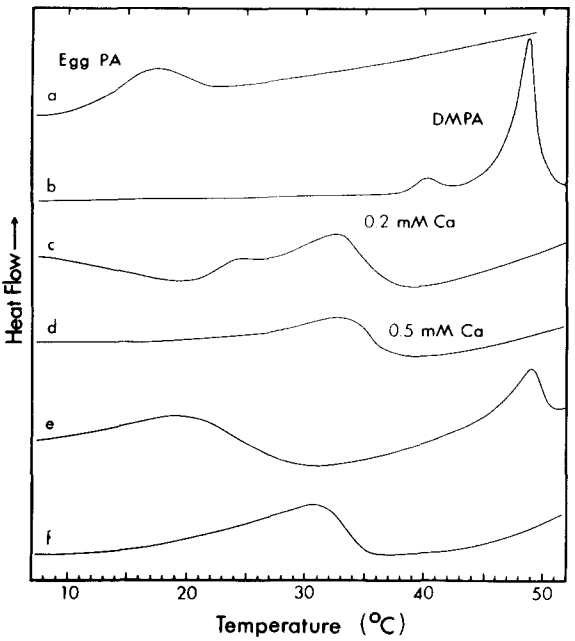

Fig. 6. Effect of Ca^{2+} on the interaction and mixing of pre-formed vesicles of egg yolk phosphatidic acid and dimyristoyl phosphatidic acid. Conditions as in legend to Fig. 1, except that the phospholipids were suspended at 50 °C in 100 mM NaCl buffer, pH 7.4 at a concentration of 2 μmol/ml. These preparations were then incubated further either separately or after mixing equimolar amounts (5–8 μmol each) under the following conditions: (a) egg phosphatidic acid (PA) alone, incubated at 50 °C for 1 h in NaCl buffer at pH 7.4, 0.1 mM EDTA; (b) dimyristoyl phosphatidic acid (DMPA) alone, details as above; (c) A mixture of the two above lipid suspensions dialysed overnight against 500 ml of 100 mM NaCl buffer containing 0.2 mM $CaCl_2$ (no EDTA); (d) same as above in (c), except buffer contained 0.5 mM $CaCl_2$; (e) same as above in (c), except dialysed in same buffer without $CaCl_2$; (f) the two lipids were mixed in chloroform and suspended together in buffer, details as in (a). All samples were centrifuged 100 000 × *g* for 15 min at 20 °C, and the wet pellets transferred within 2 h to the sample pans for scanning calorimetry.

vesicle population has a distinctive T_c, 17.5 °C for egg-yolk phosphatidic acid and 48.8 °C for dimyristoyl phosphatidic acid, while a mixture of the two lipids (1 to 1 molar ratio) has a T_c at 31 ° (curve f). Addition of either Ca^{2+} or Mg^{2+} (5 mM) to a mixed vesicle population and incubation at 50 °C for 1 h produced an intermediate peak at 35 °C indicating that mixing of the vesicle membranes had occurred (results not shown).

In order to study the effect of these cations at low Ca^{2+} bulk concentrations, the mixture was dialysed against 500 ml of 0.2 and 0.5 mM Ca^{2+} at 50 °C overnight. As shown in curves c and d, Ca^{2+} induced complete mixing of the two components at both concentrations, though at 0.2 mM Ca^{2+} a small intermediate peak is also apparent. The control mixture without Ca^{2+} still indicated the presence of the initial components unmixed (curve e). Mg^{2+} gave similar results to Ca^{2+}, producing the intermediate peak at concentrations as low as 0.5 mM (results not shown). In both cases, the vesicles were aggregated (as judged by increased absorbance after dialysis against the appropriate divalent metal (0.2 mM). The data obtained with phosphatidic

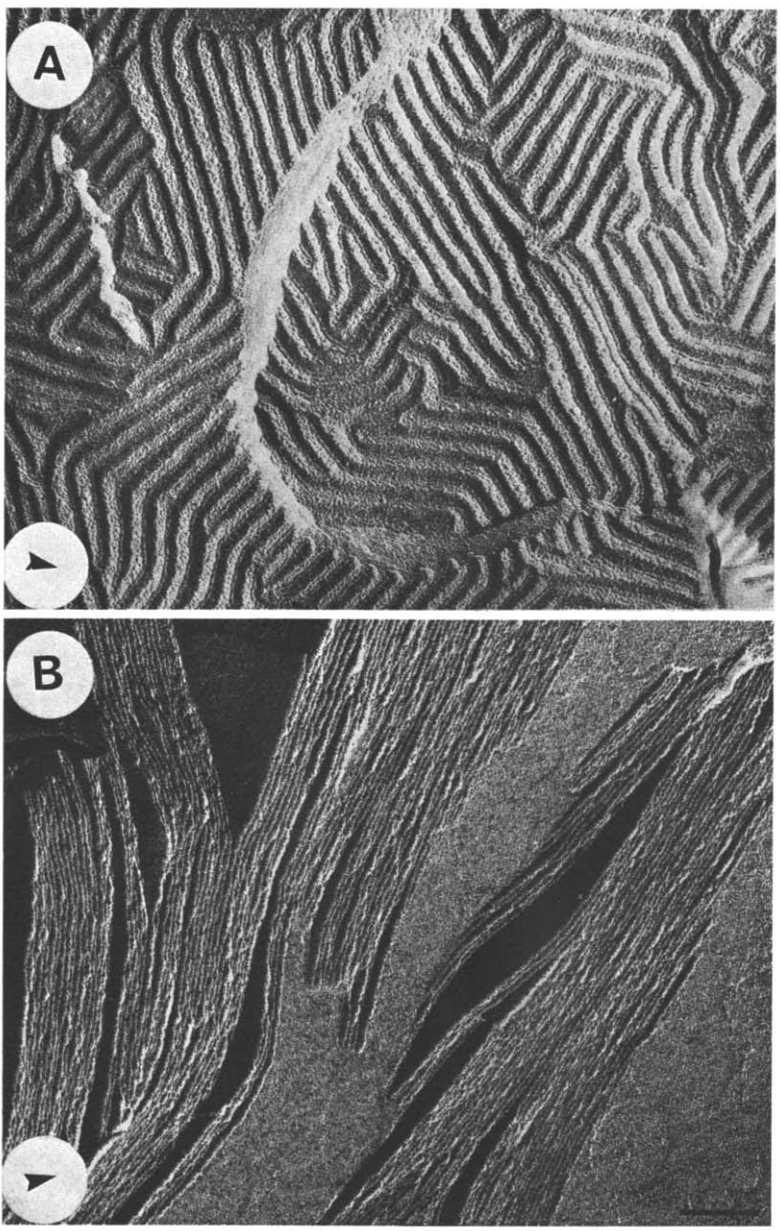

Fig. 7. Freeze-fracture electron microscopy of dimyristoyl phosphatidylglycerol membranes before and after addition of Mg^{2+}. In both cases, the lipid was dispersed (5 μmol/ml) by shaking and equilibrated for 1 h at 37 °C in NaCl buffer pH 7.4. (A) the dispersion was incubated for 1 h at 37 °C, then cooled to 25 °C before quenching; (B) the dispersion was made 20 mM in Mg^{2+}, further incubated for 1 h at 37 °C and then cooled to 25 °C before quenching. In both cases and the samples described in the following figures, the dispersions were centrifuged briefly (Eppendorf microcentrifuge, 5 min). The wet pellets were resuspended in a small volume of 30 % glycerol and then quenched by freezing into freon cooled by liquid nitrogen as before [8]. It was established in control samples that glycerol had no effect on the morphology of the structures obtained. Marker, 0.1 μm. The arrows indicate the direction of the shadowing.

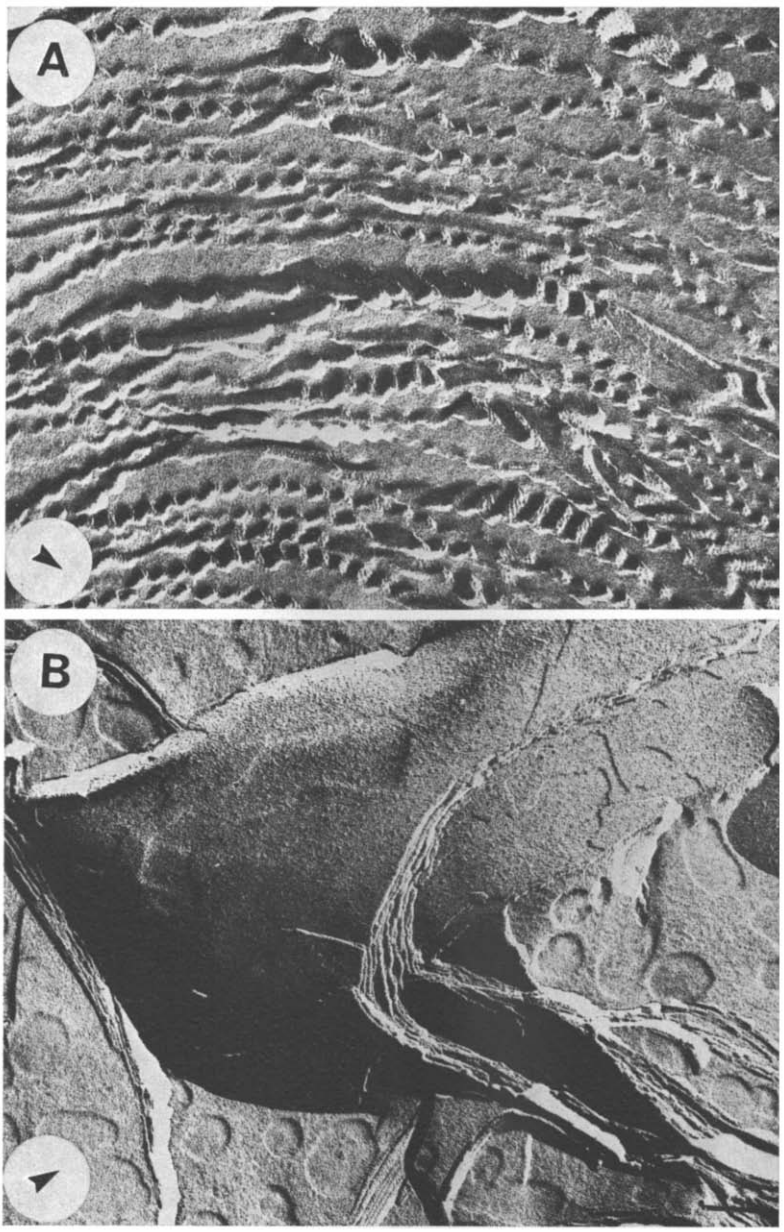

Fig. 8. Freeze-fracture electron microscopy of dimyristoyl phosphatidylglycerol membranes before and after the addition of Ca^{2+}. Details as in legend to Fig. 7. (A) sample same as in Fig. 7A, except the fracture reveals the interior of a multilamellar vesicle instead of the surface. (B) as in Fig. 7B except 10 mM $CaCl_2$ added. Bar, 0.1 μm.

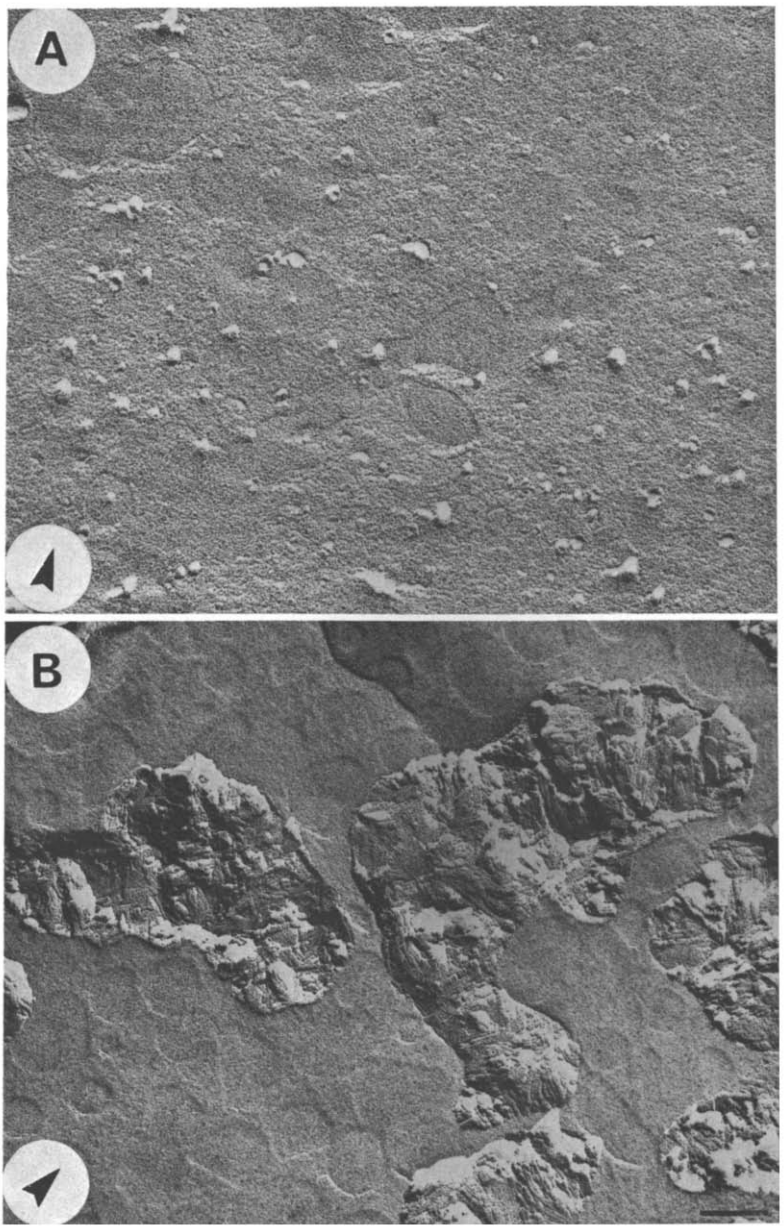

Fig. 9. Freeze-fracture electron microscopy of sonicated vesicles of phosphatidic acid before and after Ca^{2+} at pH 8.0. Egg yolk phosphatidic acid was suspended and sonicated at 37 °C for 1 h in 100 mM NaCl buffer at pH 8.0. The preparation was then treated as follows: (A) concentrated in an Amicon Minicon A-25 concentrator system (Amicon, Lexington, Mass.) at 37 °C for 1–2 h. The suspension was then made 30 % in glycerol and quenched from 25 °C as in Fig. 7; (B) $CaCl_2$ (3 μmol total) was added to the sonicated suspension containing 4 μmol of lipid in 0.6 ml of buffer, and incubated at 37 °C for 1 h. The Ca^{2+} produces immediate flocculation and precipitation. The sample was then treated as in Fig. 7. Bar, 0.1 μm.

acid vesicles, indicate that Ca^{2+} is effective in inducing molecular mixing at concentrations much lower (0.2 mM) than those required to produce the same effect with phosphatidylglycerol vesicles (10 mM). Moreover, Mg^{2+} was equally as effective as Ca^{2+} in the phosphatidic acid system, which again contrasts with the phosphatidylglycerol system in which Mg^{2+} was much less effective. By extrapolation from the rationale established with the phosphatidylglycerol vesicles earlier, we assume that the molecular mixing observed with phosphatidic acid vesicles in the presence of Ca^{2+} and Mg^{2+} is the result of fusion of the vesicles.

Freeze-fracture electron microscopy and X-ray diffraction of vesicle fusion induced by divalent metals

We have recently described the formation of large cochleate cylinders following the fusion of phosphatidylserine vesicles induced by Ca^{2+} [8]. The formation of similar structures have also been reported from dilauryl phosphatidylglycerol in the presence of Ca^{2+} [16, 19] and under certain conditions in the presence of high concentrations of Mg^{2+} [20]. It was considered of interest therefore to examine the morphological changes accompanying Ca^{2+} and Mg^{2+}-induced fusion of the different types of vesicles described in the preceding sections.

Freeze-fracture electron microscopy of non-sonicated dimyristoyl phosphatidylglycerol vesicles revealed large membranous structures with a fracture surface showing a pattern of regularly-spaced striations and corrugations (Fig. 7A and 8A). Incubation of such vesicles in the presence of either 10 mM Ca^{2+} or 10 mM Mg^{2+} resulted in the formation of new membranous structures in which the fracture surfaces are very smooth and closely packed (Fig. 7B and 8B).

Sonicated phosphatidic acid vesicles suspended in 100 mM NaCl buffer at pH 8.0 without Ca^{2+} appear in freeze-fracture micrographs as spherical particles of 200–300 Å diameter (Fig. 9A). Similar structures were seen when phosphatidic acid was sonicated in a similar buffer at pH 6.0. These structures are stable and do not change in appearance upon incubation at 25 °C for several hours. Addition of Ca^{2+} (0.1–0.5 mM) to phosphatidic acid vesicles at pH 8.0 produced immediate flocculation and examination of the flocculated product revealed the presence of large amorphous aggregates (Fig. 9B). Low angle X-ray diffraction data on this material indicates that it is a well ordered lamellar phase with a repeat distance of 54.2 Å. This distance is identical to that obtained by Ca^{2+} addition to bovine brain phosphatidylserine [9]. Chelation of Ca^{2+} by excess EDTA reverses the aggregation but the resulting suspension now contains well defined large multilamellar vesicles with a diameter of approximately 3000 Å, which is at least 10-times larger than the diameter of the original unilamellar vesicles.

The addition of Mg^{2+} to similar sonicated phosphatidic acid vesicles at pH 8.0, produces a precipitate that contains what appear to be large aggregated multilamellar vesicles (Fig. 10A). X-ray diffraction data indicates the presence of two distinct lamellar phases with repeat distances of 54.5 Å and 48.9 Å in this material. Neither the significance nor the morphological correlate of these two phases is apparent to us at present. Addition of excess EDTA to the above preparation reverses the precipitation and the resulting suspension contains what appear in freeze fractures as spherical multilamellar vesicles (Figure 10B) of 1000–3000 Å diameter.

Addition of Ca^{2+} to phosphatidic acid vesicles sonicated in 100 mM NaCl

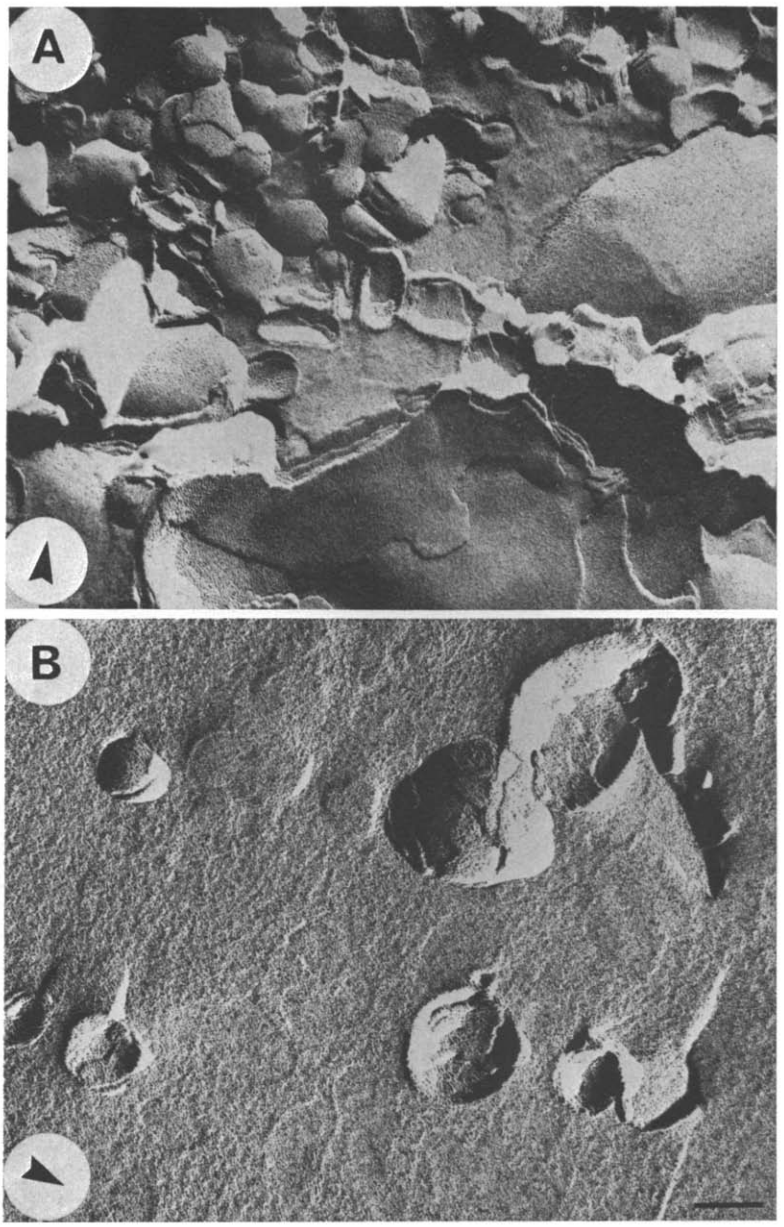

Fig. 10. Freeze-fracture electron microscopy of sonicated vesicles of phosphatidic acid after addition of Mg^{2+} at pH 8.0. Details of preparation as in Fig. 9 except as follows: (A) the sonicated vesicles (4 μmol in 0.6 ml buffer) were incubated after addition of $MgCl_2$ (3 μmol total) at 37 °C (for 1 h, then centrifuged 100 000 $\times g$ for 10 min at 20 °C; (B) as in (A) except the pellet was resuspended in 0.5 ml of buffer containing 4 μmol of EDTA. Bar, 0.1 μm.

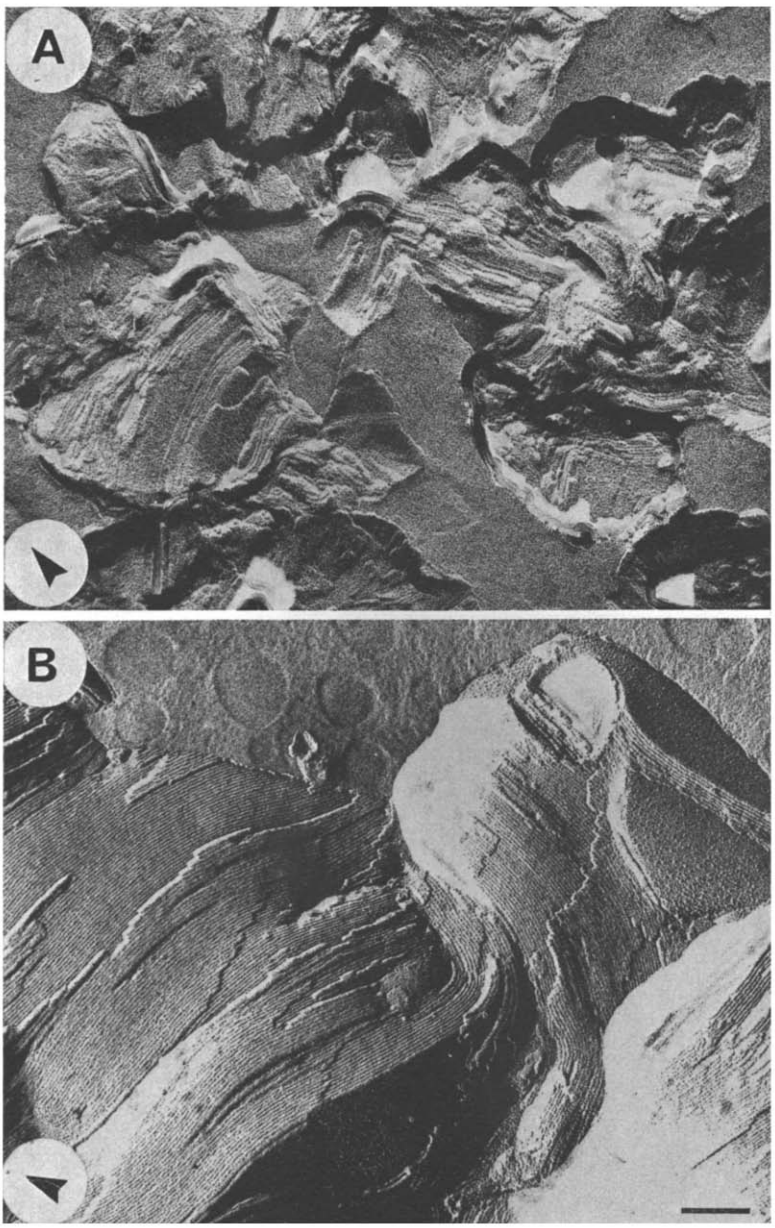

Fig. 11. Freeze-fracture electron microscopy of sonicated vesicles of phosphatidic acid after the addition of Ca^{2+} and Mg^{2+} at pH 6.0. Details of preparation as in Fig. 9, except that the pH of the buffer was adjusted to 6.0. (A) sonicated vesicles (3 μmol lipid in 0.6 ml buffer) were incubated at 37 °C for 1 h after addition of $CaCl_2$ (3 μmol). (B) same as above, except for the addition of $MgCl_2$ (3 μmol). The Mg^{2+} precipitate was centrifuged at 100 000 $\times$ g for 10 min while that of Ca^{2+} was centrifuged in Eppendorf microcentrifuge for 5 min. Samples were then treated as in Figs. 9 and 7. The addition of either Ca^{2+} or Mg^{2+} produces immediate flocculation and precipitation.

buffer at pH 6.0 produces particles (Fig. 11A) that differ markedly to those obtained at pH 8.0. The structures are large and amorphous but with well defined internal layering. X-ray diffraction data indicates the presence of two lamellar phases with repeat distances of 58.3 Å and 49.7 Å. The most striking structures were obtained, however, when Mg^{2+} was added to phosphatidic acid vesicles at pH 6.0 (Fig. 11B). In this case very large structures with highly organized internal layering were found. Although the organization gives the impression of being lamellar, X-ray diffraction data revealed the presence of a single hexagonal phase, with a repeat distance of 58.1 Å. Very few hexagonal phases have been described before for phospholipids in excess water, one of which involved phosphatidylethanolamine at high temperature [21, 22], and the other cardiolipin in the presence of Ca^{2+} [23]. It is of interest to note that earlier freeze-fracture observations of a hexagonal phase preparation [24] show a remarkable similarity to the structures in Fig. 11B.

Despite the fascinating diversity of the structures shown in Figs. 9–11 they share a common origin in arising via the fusion of unilamellar phosphatidic acid vesicles. The formation of these large structures by fusion of small vesicles, and the failure to reverse this structural transformation by EDTA, provide additional evidence to support the conclusions presented earlier on the basis of calorimetric observations.

DISCUSSION

The present results indicate that Ca^{2+} and Mg^{2+} are able to induce extensive molecular mixing of membrane components between lipid vesicles composed of acidic phospholipids. This process is accompanied by drastic structural rearrangement of the vesicles to form new membranous structures. In contrast to the mixing of vesicle membrane components in the absence of Ca^{2+} reported in the preceding paper which in many cases takes place via the diffusion of lipid molecules between the vesicles [1], the mixing of membrane components induced by Ca^{2+} or Mg^{2+} result from the fusion of entire vesicles.

A number of parameters appear to be important in influencing membrane fusion in the present system.

First, the lipids in both of the interacting membranes must be in a "fluid" state at the experimental temperature for fusion to take place. This finding reinforces previous observations from this laboratory in which membrane "fluidity" has been found to influence fusion occurring between vesicles [6] and also the fusion of lipid vesicles with the plasma membrane of living cells cultured in vitro [25].

Second, there appears to be a "threshold" concentration at which Ca^{2+} or Mg^{2+} become effective in inducing membrane fusion. The threshold concentration for each cation varies by several orders of magnitude for different phospholipids ranging from 0.2 mM Ca^{2+} for phosphatidic acid membranes to 10 mM Ca^{2+} for phosphatidylglycerol membranes as reported here, and 1 mM Ca^{2+} for phosphatidylserine membranes as described elsewhere [6]. Different phospholipids also vary markedly in their response to Ca^{2+} and Mg^{2+}. Thus, fusion of phosphatidic acid membranes is induced by Ca^{2+} or Mg^{2+} at similar concentrations but phosphatidylserine and phosphatidylglycerol membranes are both considerably more sensitive to fusion by Ca^{2+}.

Third, the ability of Ca^{2+} and Mg^{2+} to induce vesicle fusion is not due to

simple electrostatic charge neutralization and double-layer effects. Although these effects would be expected to promote vesicle aggregation and to increase the opportunities for contact interactions between apposed membranes, it is clear that aggregation per se is not necessarily followed by fusion. As shown here extensive vesicle aggregation without fusion can be induced by sub-threshold concentrations of both Ca^{2+} and Mg^{2+}.

Fourth, Ca^{2+}-induced fusion is accompanied by a drastic change in the thermotropic properties of the vesicle membranes with resulting crystallization of the lipid acyl chains and the establishment of a phase transition at much higher temperatures. This is equivalent to an isothermal phase transition in single component phospholipid membranes of the type used here but would also correspond to the Ca^{2+}-induced phase separation in a two component membrane similar to that described previously by us in relation to fusion occurring in phosphatidylcholine/phosphatidylserine membranes [6].

It is of interest to note that the threshold concentration of Ca^{2+} required to induce fusion of membranes composed of particular phospholipids corresponds closely to the Ca^{2+} concentration that produces a large increase in the permeability of closed vesicles and planar lipid bilayers of the same composition [26, 27]. It has already been shown that these permeability alterations result from an asymmetric distribution of Ca^{2+} across the membrane, since membranes exposed to Ca^{2+} on both sides are stable and highly impermeable [27]. This therefore raises the obvious possibility that Ca^{2+}-induced membrane fusion might also result from an asymmetric distribution of Ca^{2+} (or Mg^{2+}) across the membrane. The transient membrane instability and structural reorganization accompanying this Ca^{2+} asymmetry may be the important event that renders the membrane susceptible to fusion. Such transient membrane instability may, however, result simply from the phase transitions and separations induced by an increase in the bulk concentration of Ca^{2+} above the threshold, irrespective as to whether Ca^{2+} is added asymmetrically across the membrane or not. In either case, the membranes would become transiently susceptible to fusion as a result of changes in molecular packing and creation of new phase boundaries induced by the interaction with Ca^{2+} [28]. Experimental data on the role of Ca^{2+}-induced phase transitions and separations in triggering membrane fusion in phospholipid vesicles will be presented in the next paper of this series.

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