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INTERACTIONS OF DIVALENT CATIONS OR BASIC PROTEINS WITH PHOSPHATIDYLETHANOLAMINE VESICLES

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Summary

Small unilamellar vesicles have been prepared from phosphatidylethanolamine by sonication of the lipid in aqueous buffers of low ionic strength and high pH. These vesicles and their interactions with various di- and trivalent cations have been characterized using freeze-fracture electron microscopy. Phosphatidylethanolamine from 4 sources was examined: Hens' yolk phosphatidylethanolamine, human grey matter phosphatidylethanolamine, *Escherichia coli* phosphatidylethanolamine and dimyristoyl phosphatidylethanolamine. The phosphatidylethanolamine from natural sources formed spherical, uniform 20—40 nm vesicles while dimyristoyl phosphatidylethanolamine formed larger, 70 × 25 nm, disc-shaped vesicles when sonicated above the phase transition temperature. Fusion of the unilamellar egg phosphatidylethanolamine, *E. coli* phosphatidylethanolamine and human grey matter phosphatidylethanolamine vesicles was induced by dialysis against buffers containing 2.0 mM Ca^{2+} or 3.0 mM Mg^{2+}. The fusion of the vesicles resulted in the precipitation of the lipid and the formation of multilamellar and, in some cases, hexagonal II structures. Dimyristoyl phosphatidylethanolamine vesicles were precipitated at 55°C by 1.0 mM Ca^{2+} or 2.0 mM Mg^{2+}. Treatment of the calcium- and magnesium-precipitated vesicles of hen's egg yolk phosphatidylethanolamine, *E. coli* phosphatidylethanolamine, human grey matter phosphatidylethanolamine and dimyristoyl phosphatidylethanolamine with EDTA resulted in resuspension of the lipid. The specific size and shape of the vesicles formed in this manner depends on the type of phosphatidylethanolamine and ion involved. Dialysis of the Ca^{2+}- and Mg^{2+}-precipitated egg phosphatidylethanolamine vesicles against buffer containing no Ca^{2+}, Mg^{2+} or EDTA also resulted in dissociation of the precipitate and formation again of a new vesicle population. This evidence indicates that the Ca^{2+} and Mg^{2+} are not strongly bound to the phosphatidylethanolamine.

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Egg phosphatidylethanolamine vesicles would fuse in the presence of many di- and trivalent ions. Egg phosphatidylethanolamine vesicles were precipitated by beryllium, aluminum, chromium, manganese, cobalt, nickel, copper, zinc, strontium, cadmium, barium, lanthanium, mercury and lead. The amount of ion required to precipitate the vesicles and the type of structure resulting from the fusion of the vesicles was found to be unique for each ion.

Small unilamellar vesicles prepared from egg phosphatidylethanolamine were reacted with several basic proteins (cytochrome *c*, basic protein from human myelin, protamine, poly-L-lysine and cationically-modified ferritin). The basic proteins also initiated the fusion of egg phosphatidylethanolamine vesicles but these proteins did not fuse egg phosphatidylcholine vesicles nor did normal ferritin initiate fusion. Human myelin basic protein initiated the fusion of dimyristoyl phosphatidylethanolamine vesicles above and below the phase transition of this lipid.

Introduction

Phospholipid vesicles have proven to be valuable in the study of membrane structure and function. They are commonly employed to study phospholipid-ion, -sterol, -protein and -cellular interactions [1—6]. They are becoming important in the study of model membranes. Unfortunately, little is known about one of the most commonly occurring phospholipids, phosphatidylethanolamine. It is found in many mammalian tissues including nervous tissue, mitochondria and red blood cells [7]. It is also found to be the predominant phospholipid in many bacterial plasma membranes; the most notable is *Escherichia coli* [8]. Our knowledge of the role of this phospholipid in membrane biology has been hindered because of unseccessful attempts to produce phospholipid vesicles. Several authors have reported that the soniciation of phosphatidylethanolamine in buffer resulted in the formation of suspensions that tend to spontaneously precipitate from solution [9—11]. In this paper we describe a new method by which stable, small, unilamellar vesicles can be produced from phosphatidylethanolamine. Freeze-fracture electron microscopy has been employed to characterize these vesicles and to describe the results of fusion of phosphatidylethanolamine vesicles produced by the addition of di- and trivalent cations. Not only the physiologically important ions, Ca^{2+} and Mg^{2+}, but all divalent ions as well as basic proteins tested were found to be capable of producing vesicle fusion.

Materials and Methods

Materials

Phosphatidylethanolamine was isolated from 12 1-day-old hens' egg yolks by the method of Bangham, Hill and Miller [12] and purified using silicic acid chromotography by the method of Hanahan, Dittmer and Warrashina [13]. *E. coli* phosphatidylethanolamine was isolated from 30 gm of wet cell paste by the method of Folch, Lees and Stanley [14] and purified on silicic acid as above. Phosphatidylethanolamine was isolated from 300 g of normal human

grey matter as described by Folch [15] and purified using DEAE-chromatography according to Rouser et al. [16]. Dimyristoyl phosphatidylethanolamine was purchased from Fluka A.G. All lipids gave single spots when tested by thin layer chromatography and were considered chromatographically pure. Lipids were dissolved in chloroform at 10 μmol of lipid phosphorus [17] per ml, and 1.0 ml was placed in ampoules, layered over with argon, sealed, and stored at −70°C. Fresh ampoules were opened for each experiment. Protamine, poly-L-lysine and cytochrome *c* were purchased from Sigma chemical Co. Cationically-modified ferritin and 6× recrystallized ferritin were purchased from Miles Laboratories.

Phospholipid vesicles preparation

Small unilamellar vesicles were prepared from 10 μmol of lipid P_i by the following method. Lipid was dried in a test tube under a gentle stream of dry nitrogen gas and the tube evacuated at 0.01 Torr for 30 min to remove all traces of chloroform. 2 ml of a buffer containing 10 mM sodium borate/1.0 mM EDTA at pH 9.2 was added, and the suspension was sonicated in a 100 W bath-type sonicator at 22°C for 1 h. The resulting optically clear suspension was placed in dialysis tubing pretreated by boiling with 20 mM EDTA, and dialyzed against 1 l of a buffer containing 10 mM Tris · HCl (pH 7.2) for 3 h. The vesicle preparation was then centrifuged at 78000 × *g* for 1 h to sediment any large multilamellar vesicles which comprised about 10% of the total lipid phosphorus.

Thin layer chromatography of vesicle preparations

Phosphatidylethanolamine vesicles were sonicated at 22°C and 45°C in Borate/EDTA buffer at pH 9.2, as described earlier. The preparations were extracted by the method of Folch, Lees, and Stanley [14]. The 2 ml vesicle preparation was extracted with 40 ml of chloroform/methanol (1 : 1), placed in a separatory funnel, made to 20% (v/v) with water, shaken well, and the phases allowed to completely separate. The lower chloroform layer was collected and dried in a rotary evaporator and redisolved in 2 ml of chloroform. A phosphorous analysis was determined on this preparation and 20 μl applied to heat-activated thin layer chromatography plates (Absorosil 5, Applied Science Laboratories). The plates were chromatographed in chloroform/methanol/water (65 : 25 : 4). The dried plates were developed with iodine vapor and photographed.

Controls were run with unsonicated phosphatidylethanolamine, as well as egg phosphatidylcholine sonicated in a similar manner as egg phosphatidylethanolamine vesicles.

Electron microscopy

The vesicle suspension was then concentrated to approx. 0.1 ml in Sartorius colloidin membrane, and an equal volume of a solution containing 60% (v/v) glycerol, 10 mM Tris · HCl (pH 7.2) was added. 10-μl aliquots were mounted in gold cups, frozen in Freon 22, and stored in liquid nitrogen. Samples were fractured in Balzer BA 360 M Freeze-Etch Apparatus at −115°C at 10^{-6} Torr, and replicated with carbon-Pt and then with carbon. Replicas were washed with

50% (v/v) sulphuric acid, twice with water, then with Javex (Commercial sodium hypochlorite) and again twice with water, and mounted on bare 75 × 300 mesh copper grids. All replicas were cleaned of any residual lipid by placing them over boiling chloroform and allowing the vapours to condense on the grids, dried and then examined in a Philips EM 300 electron microscope.

Interactions with ions

The minimum concentrations of various di- and trivalent ions required to initiate the fusion of phosphatidylethanolamine vesicles was determined by the following method. A phosphatidylethanolamine vesicle suspension was prepared as described and was dialyzed against 1 l of a buffer containing 10 mM Tris · HCl/1.0 mM cation (pH 7.2) for 3 h. If no changes were observed, buffer was discarded and replaced by 1 l of a solution containing 10 mM Tris · HCl/2.0 mM cation (pH 7.2), and the vesicles allowed to dialyze for another 3 h. In the case of egg phosphatidylethanolamine vesicles, 2.0 mM Ca^{2+} resulted in fusion of the vesicles and precipitation of the lipid. The precipitated lipid was then cooled to 4°C, to facilitate easier manipulation, and pelleted by centrifugation in a clinical centrifuge at 4°C. The supernatant fluid was discarded, and an equal volume of a solution containing 60% (v/v) glycerol/10 mM Tris · HCl (pH 7.2) at 4°C was added to the pellet. The sample was then frozen and fractured as described above. Using this technique the minimum concentrations of Ca^{2+} and Mg^{2+} required to precipitate egg phosphatidylethanolamine, *E. coli* phosphatidylethanolamine, and human grey matter phosphatidylethanolamine vesicles. Dimyristoyl phosphatidylethanolamine was also tested but at 55°C above the phase transition.

Egg phosphatidylethanolamine vesicles were also tested with a variety of di- and trivalent cations. These included the chloride salts of manganese, cobalt, nickel, zinc, strontium, cadmium, barium, lanthanum (La^{3+}) and mercury, which were all tested at pH 7.2. Chromium (Cr^{3+}), copper and lead chlorides were tested at pH 5.5 in buffers containing 10 mM sodium acetate and the appropriate ion. As a comparison, calcium and magnesium were also tested at this pH. Beryllium and aluminum (Al^{2+}) chlorides were tested at pH 5.0 in buffers containing 10 mM sodium acetate.

Effects of EDTA

The calcium- and magnesium-precipated vesicle preparations of egg phosphatidylethanolamine, *E. coli* phosphatidylethanolamine, human grey matter phosphatidylethanolamine, and dimyristoyl phosphatidylethanolamine (55°C) were treated with EDTA in the following manner. The respective calcium- or magnesium-precipitated vesicle preparations were dialyzed against a buffer containing 10 mM Tris · HCl and an EDTA concentration 1.5-fold that of the ion used to precipitate the vesicles at pH 7.2. In all cases treatment with EDTA resulted in dissociation of the precipitate and resuspension of the lipid. The suspensions were then concentrated to approx. 0.1 ml in Sartorius colloidin membrane and prepared for freeze fracture as described above. As a comparison, Ca^{2+}- and Mg^{2+}-precipitated egg phosphatidylethanolamine vesicle preparations were dialyzed for 12 h against 3, 1-l changes of a buffer containing only 10 mM Tris · HCl (pH 7.2). This treatment also resulted in the dissociation of the lipid. The suspensions were concentrated and fractured as described.

Reaction of proteins with phosphatidylethanolamine vesicles

Proteins were dissolved at 5.0 mg/ml in a buffer containing 10 mM Tris · HCl/1.0 mM EDTA (pH 7.2) and 0.1 ml aliquots were added to the vesicle suspension with stirring until flocculation occurred. The suspensions were incubated at 37°C for 15 min and then concentrated to approx. 0.1 ml in Sartorius colloidin membranes and an equal volume of a solution containing 60% (v/v) glycerol/10 mM Tris · HCl/1.0 mM EDTA (pH 7.2) was added and prepared for freeze fracture electron microscopy as previously described.

Results and Discussion

Several authors have reported difficulty in producing stable vesicle suspensions from phosphatidylethanolamine [9–11]. Usually the suspensions spontaneously came out of solution after sonication of the lipid in isotonic buffers at physiological pH. Papahadjopoulos and Miller [10] suggested that presence of intermolecular hydrogen bonds between amine groups and adjacent phosphate residues. These authors suggest that raising the pH of the solution would eliminate hydrogen bonding by removing the charge from the amine group. Philips, Finer and Hauser [18] examined phosphatidylethanolamine head-group conformation uxing X-ray diffraction and found that the head-group was oriented in the plane of the membrane. They stated that this configuration would promote intermolecular hydrogen bonding. This work was verified by Hitchcock et al. [19]. Jendrasiak and Hasty [20] supported the work of Philips, Finer and Hauser in a study of hydration properties of phosphatidylethanolamine. They found that phosphatidylethanolamine hydrated very poorly which suggested the possibility of intermolecular hydrogen bonding. With these studies in mind, we found it possible to prepare stable vesicle suspensions from phosphatidylethanolamine by lowering the ionic strength and raising the pH prior to sonication. Lowering of the pH back to physiological levels using dialysis as described does not seem to alter vesicle morphology. Fig. 1A is a freeze-fracture electron micrograph of an egg phosphatidylethanolamine vesicle suspension at pH 7.2 in 10 mM Tris · HCl. Vesicles prepared from *E. coli* phosphatidylethanolamine, and human grey matter phosphatidylethanolamine were of similar size and shape to those seen in Fig. 1A. Dimyristoyl phosphatidylethanolamine vesicles, on the other hand, were flattened discs as can be seen in Fig. 1B.

Phospholipid purity and effects of sonication

The purity of all phospholipids used in this study was determined by thin layer chromatography. Fig. 2A shows a typical chromatogram of chloroform solutions, egg phosphatidylethanolamine, *E. coli* phosphatidylethanolamine, and human grey matter phosphatidylethanolamine with R_F values of 0.80, 0.79 and 0.83 respectively.

Fig. 2B illustrates the effect of sonication in aqueous buffers in borate/EDTA buffers, at pH 9.2. A small, barely visible spot is present at the origin after the lipids were sonicated at pH 9.2. These spots might indicate free fatty acids due to a slight amount of hydrolysis of the free fatty acids and

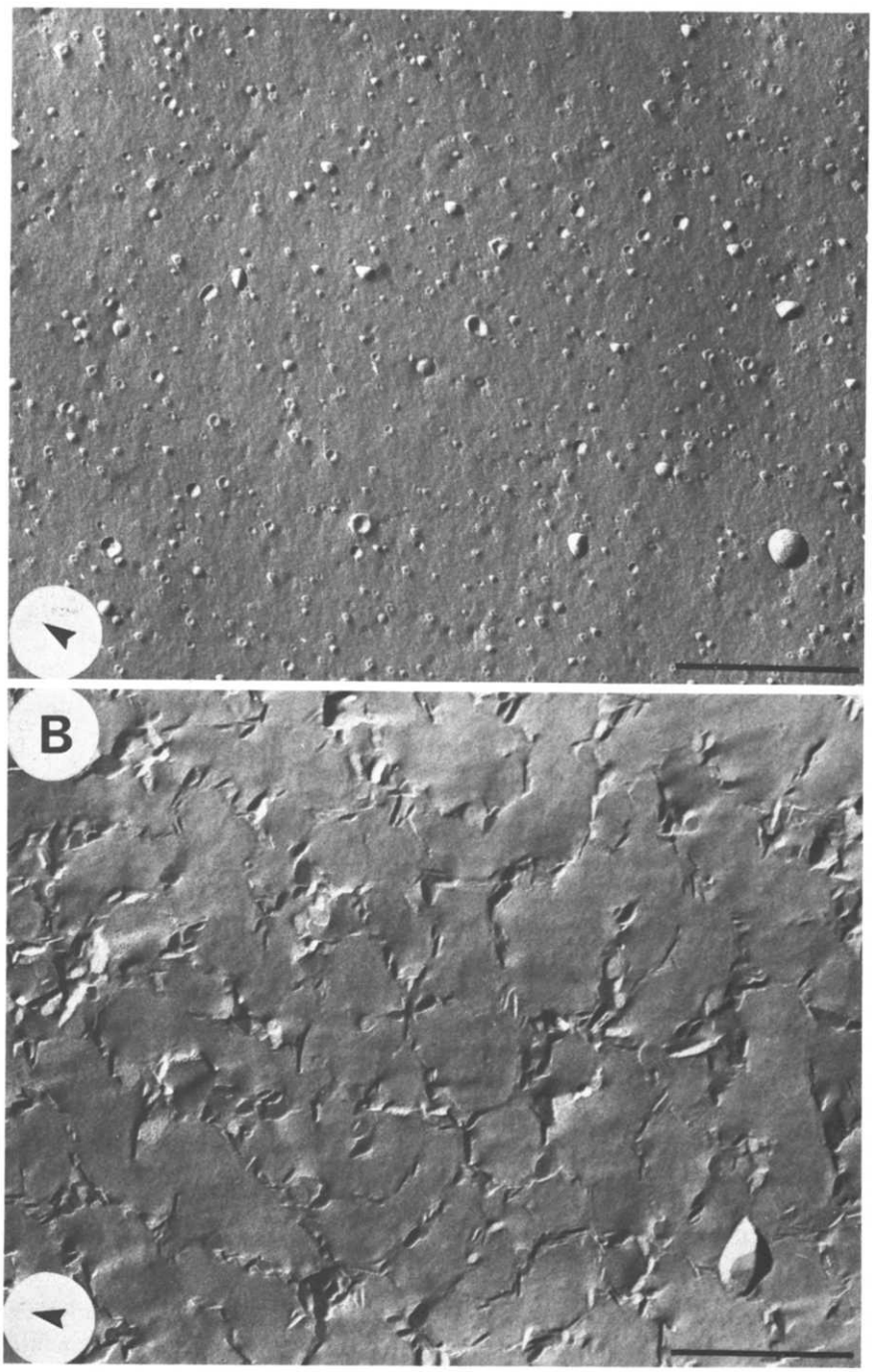

Fig. 1. Small unilamellar vesicles formed by the sonication of phosphatidylethanolamine in aqueous buffer. A. Freeze-fracture of egg phosphatidylethanolamine vesicles in 10 mM Tris · HCl (pH 7.2). The vesicles range in diameter from 20 to 50 nm and are spherical in shape. Magnification 49800 X, and bar represents 500 nm. In all cases the arrow represents the direction of the shadow. B. Freeze-fracture electron microscopy of dimyristoyl phosphatidylethanolamine vesicles. The vesicles are flattened discs approximately 70 X 25 nm. Magnification 49800 X and bar represents 500 nm.

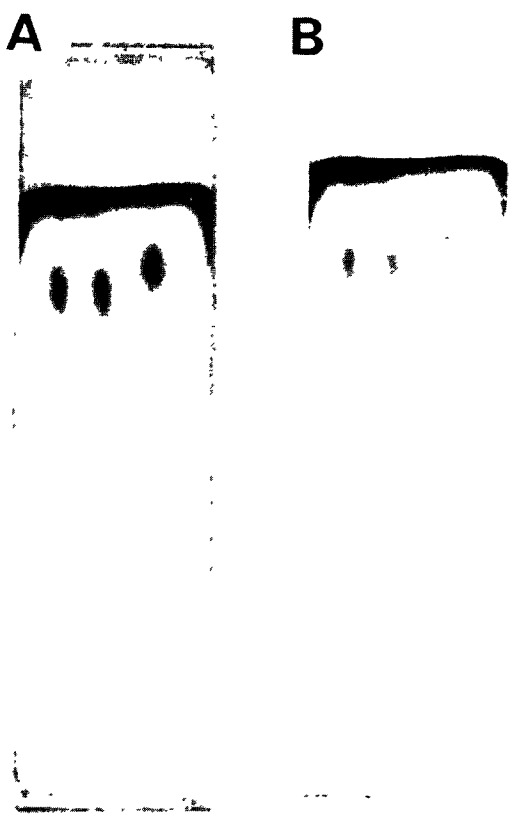

Fig. 2. A. Thin layer chromatography of phosphatidylethanolamines from various sources. From left to right: Egg phosphatidylethanolamine, *E coli* phosphatidylethanolamine, human grey matter phosphatidylethanolamine. B. As above but sonicated at pH 9.2 at 22°C, then dialyzed to pH 7.2.

increased the amount with temperature. However, the corresponding lysocompounds were not observed. We estimated from the density of the spots at the origin and, if lysocompounds were present, they represented less than 10% of the total lipid present. However, it would not be prudent to ignore this observation since it might indicate slight lipid hydrolysis.

Interactions with ions

The interactions of phosphatidylethanolamine with ions have not been characterized. Papahadjopoulos [21] demonstrated the presence of an ionizable group, at pH below 3.5, which was reported as the pKa_1 of the primary phosphate group. A second ionizable group was titratable at a pH greater than 6.5, and was determined to be the amine of the ethanolamine residue. In the same paper, microelectrophoresis indicated a slight negative charge at pH 4.0. Joos and Carr [22] found that phosphatidylethanolamine dispersions did not bind calcium below pH 8.0. Blaustein [23] however, demonstrated the binding of

calcium to phosphatidylethanolamine et pH 6.5. Rojas and Tobias [24], in a monlayer study, demonstrated the binding of calcium to phosphatidylethanolamine at pH 4.1, which roughly corresponds to the ionization of the primary phosphate group. Philips, Finer and Hauser [18] suggested that because the head-group of phosphatidylethanolamine lies in the plane of the membrane, the phosphate group would be available for interaction with calcium. With these studies in mind we examined the interaction of divalent cations with small, unilamellar, sonicated vesicles prepared from phosphatidylethanolamine. Table I lists the minimum concentrations of Ca^{2+} and Mg^{2+} required to precipitate vesicles prepared from egg phosphatidylethanolamine, *E. coli* phosphatidylethanolamine, human grey matter phosphatidylethanolamine and dimyristoyl phosphatidylethanolamine. At room temperature (22°C) the phosphatidylethanolamine vesicles from natural sources were precipitated by dialysis against 2.0 mM Ca^{2+} or 3.0 mM Mg^{2+}. Dimyristoyl phosphatidylethanolamine was precipitated at 55°C by dialysis against 1.0 mM Ca^{2+} or 2.0 mM Mg^{2+}. Egg phosphatidylethanolamine vesicles, when incubated at 55°C, were also precipitated by 1.0 mM Ca^{2+} or 2.0 mM Mg^{2+}. Thus, the lower levels of Ca^{2+} and Mg^{2+} required to precipitate the dimyristoyl phosphatidylethanolamine vesicles seem to be related to the elevated temperature.

The addition of 2.0 mM Ca^{2+} to egg phosphatidylethanolamine vesicles, as described, resulted in fusion of the small unilamellar vesicles and formation of large multilamellar vesicle aggregates. Fig. 3A is a freeze-fracture electron micrography of such a vesicle aggregation. In all samples examined, the lamellar phase was found to predominate after calcium induced vesicle fusion.

Several reports have appeared in the literature describing the fusion of acidic phospholipid vesicles by calcium and magnesium [1,2,25–27]. It has been suggested that this reaction may provide an important in vitro system for the study of membranes. Phosphatidylethanolamine is a weakly acidic phospho-

TABLE I

MINIMUM CONCENTRATIONS OF CALCIUM AND MAGNESIUM REQUIRED TO PRECIPITATE VESICLES PREPARED FROM EGG PHOSPHATIDYLETHANOLAMINE, *E. COLI* PHOSPHATIDYLETHANOLAMINE, HUMAN GREY MATTER PHOSPHATIDYLETHANOLAMINE AND DIMYRISTOYL PHOSPHATIDYLETHANOLAMINE VESICLES

Ion	Vesicle type	Minimum ion concentration required to precipitate (mM)
Calcium	Egg phosphatidylethanolamine, pH 7.2	2.0
	Egg phosphatidylethanolamine, pH 5.5	4.0
	Egg phosphatidylethanolamine, pH 7.2, 55°C	1.0
	E coli phosphatidylethanolamine, pH 7.2	2.0
	Human grey matter phosphatidylethanolamine, pH 7.2	2.0
	Dimyristoyl phosphatidylethanolamine, pH 7.2, 55°C	1.0
Magnesium	Egg phosphatidylethanolamine, pH 7 2	3 0
	Egg phosphatidylethanolamine, pH 5.5	10.0
	Egg phosphatidylethanolamine, pH 7.2, 55°C	2.0
	E coli phosphatidylethanolamine, pH 7.2	3.0
	Human grey matter phosphatidylethanolamine, pH 7.2, 55°C	2.0

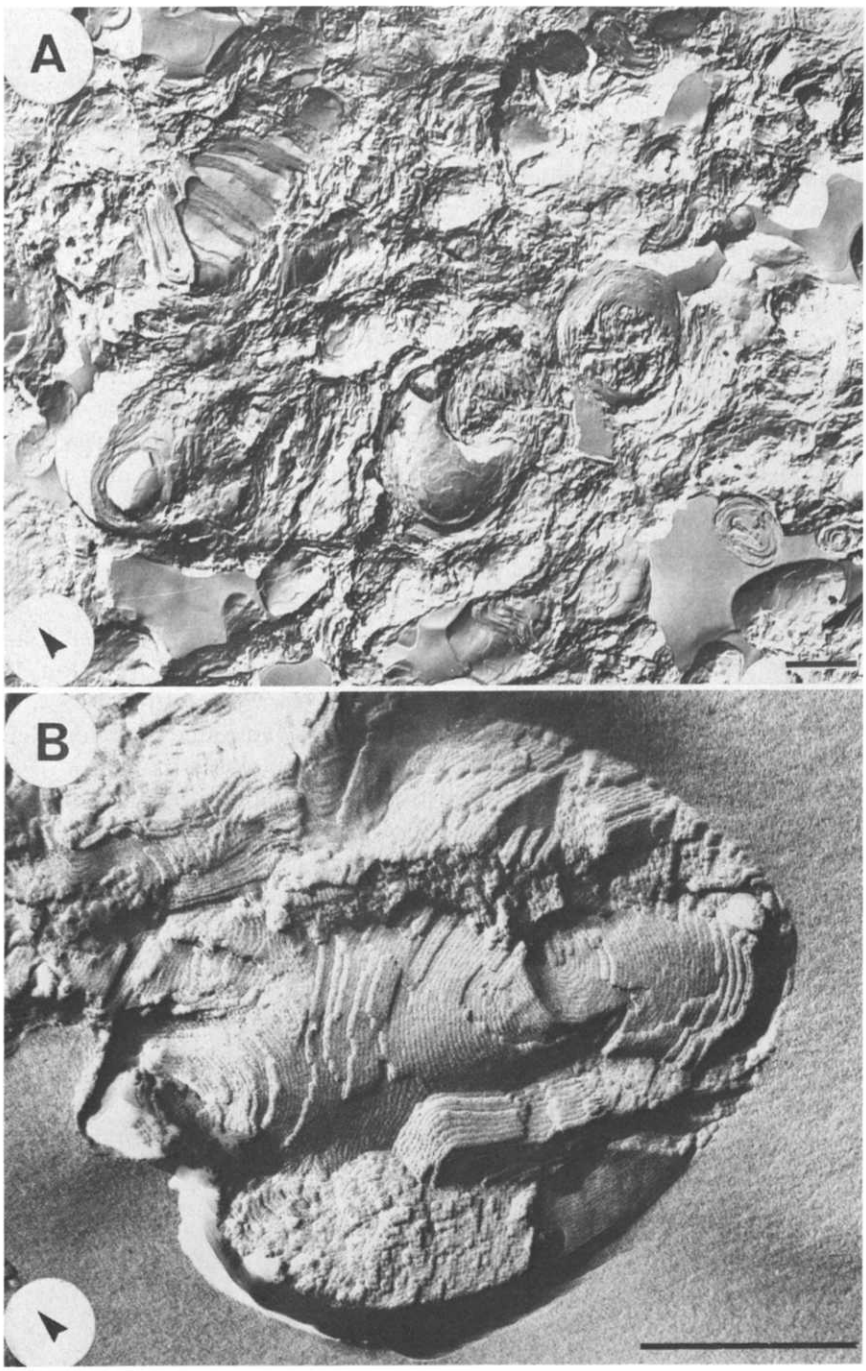

Fig. 3. The addition of calcium (2.0 mM) to small unilamellar egg phosphatidylethanolamine vesicles (Fig. 1) results in fusion and precipitation of the lipid. A. Freeze-fracture of the precipitate reveals the presence of large multilamellar vesicle aggregates. Also note the presence of amorphus areas. Magnification 18900 × and bar represents 500 nm. B. A hexagonal structure also formed from the addition of calcium to egg phosphatidylethanolamine vesicles. Magnification 58500 × and bar represents 500 nm.

lipid when compared to phosphatidic acid or phosphatidylserine [21] which reacts strongly with calcium. It was found that at pH 5.5 egg phosphatidylethanolamine vesicles were precipitated by 4.0 mM Ca^{2+}, although more ion is required at pH 5.5 then at 7.2. It appears that once the primary phosphate group of the phosphatidylethanolamine is ionized, the vesicles will interact with calcium. In some cases hexagonal structures were observed as the result of the fusion of egg phosphatidylethanolamine vesicles by Ca^{2+} or Mg^{2+}. Fig. 3B is a freeze-fracture electron micrograph of such a hexagonal array.

Hexagonal II structures have been reported in phosphatidylethanolamine-water systems by several authors [28—30]. Reiss-Husson [30] demonstrated using X-ray diffraction that egg yolk phosphatidylethanolamine-water systems form hexagonal II structures. At concentrations greater than 79% lipid the hexagonal phase predominated, but at lower lipid concentrations and higher temperatures (25—35°C), the lamellar phase co-existed with the lamellar phase. Rand and Sengupta [31] reported the appearance of hexagonal II structures arising from the addition of Ca^{2+} to a fully hydrated cardiolipin vesicle suspension. Similarly, Papahadjopoulos et al. [27], reported the appearance of hexagonal structures resulting from the precipitation of phosphatidic acid vesicles by magnesium. Our experimental results suggest that, in the case of egg phosphatidylethanolamine vesicles, calcium and magnesium can initiate the formation of hexagonal structures. However, in all cases, the lamellar phase predominated, and the appearance of hexagonal structures were not consisitent from preparation to preparation.

Magnesium also stimulated the fusion of egg phosphatidylethanolamine resulting in the formation of large multilamellar vesicle aggregates similar in morphology to those formed by calcium. Magnesium also caused the formation of hexagonal II structures. However, as in the case of calcium, the appearance of the hexagonal structure was not predictable.

Effects of EDTA

It has been reported [25] that the treatment of calcium-precipitated phosphatidylserine vesicles when treated with EDTA undergo a phase transition resulting in dissociation of the precipitate and the formation of a new vesicle population termed "large unilamellar vesicles". This reaction is considered important because it allows the entrapment of large or sonication labile molecules in liposomes. The treatment of the calcium and magnesium egg phosphatidylethanolamine-precipitated vesicles resulted in dissociation of the precipitate and resuspension of the lipid. Fig. 4A is a freeze-fracture electron micrograph of a calcium-precipitated egg-phosphatidylethanolamine vesicle preparation treated with EDTA. The chelation of the ions formed many larger vesicles, some of which appear to be multilamellar. Similar results were obtained with magnesium-precipitated vesicles. As described, the dialysis of the Ca^{2+}- and Mg^{2+}-precipitated egg phosphatidylethanolamine vesicles against buffer containing no added Ca^{2+} or Mg^{2+} resulted in resuspension of the lipid. Fig. 4B is a freeze-fracture electron micrograph of a typical field observed after this treatment. This evidence indicates that the ions are not strongly bound to the phosphatidylethanolamine membranes.

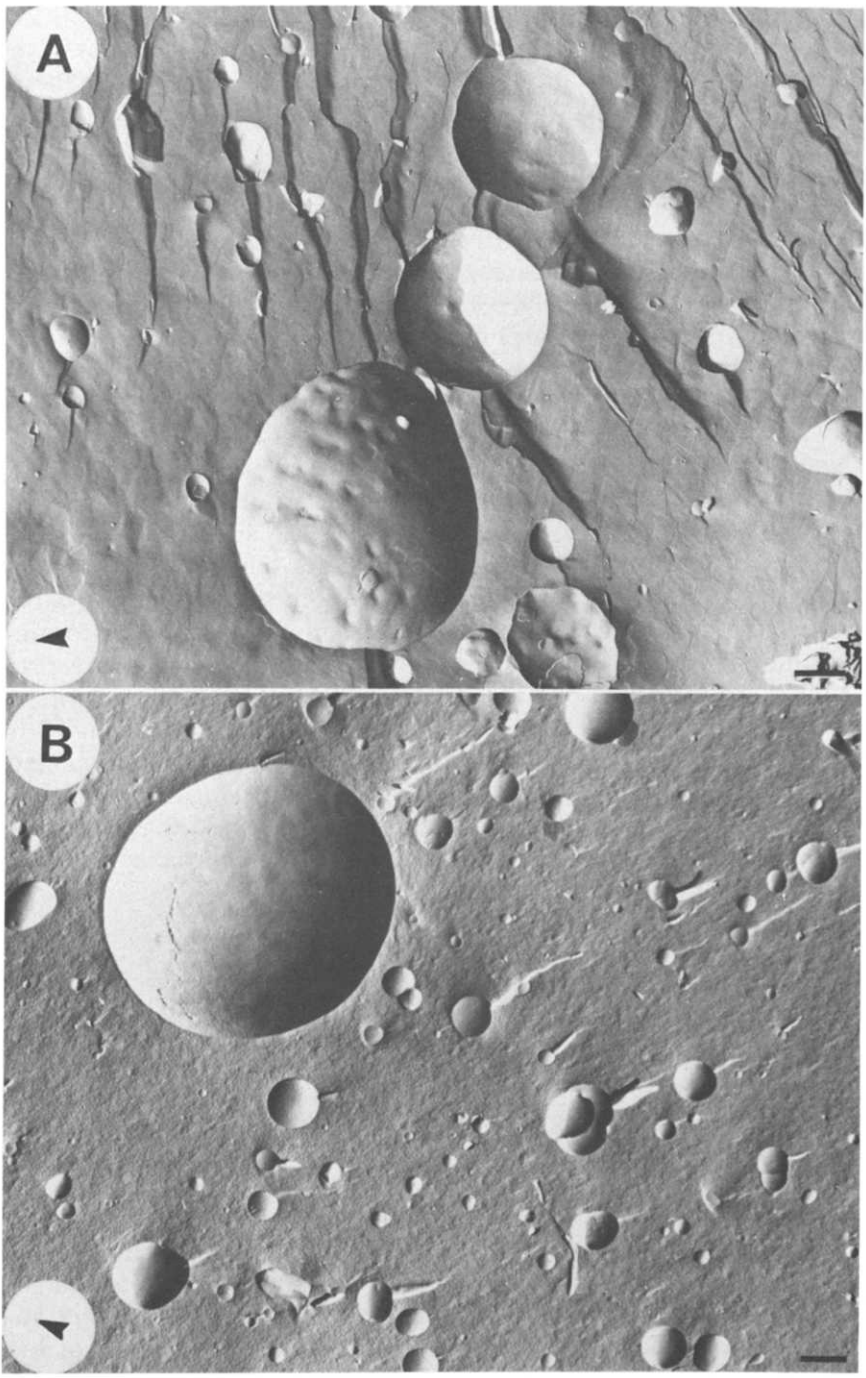

Fig. 4. Removal of the precipitating ion from fused egg phosphatidylethanolamine vesicles results in a structural change. A. The addition of EDTA to a calcium-precipitated egg phosphatidylethanolamine vesicle preparation (Fig. 2). A new vesicle population, heterogenous in size (0.1—2.5 μm) and shape is formed. Magnification 13500 $^{\times}$ and bar represents 500 nm. B Dialysis of calcium-precipitated egg phosphatidylethanolamine vesicles against buffer containing no calcium also results in a structural change indicating that the calcium is only weakly bound to phosphatidylethanolamine membranes

E. coli phosphatidylethanolamine

Calcium (2.0 mM) and magnesium (3.0 mM) were also able to initiate the fusion of *E. coli* phosphatidylethanolamine vesicles. As can be seen with the calcium treatment (Fig. 5A), the precipitate was vesicular and more "sheet-like" in nature than the calcium-precipitated egg phosphatidylethanolamine vesicles. This difference may have been due to differences between the fatty acid composition of egg phospahtidylethanolamine and *E coli* phosphatidylethanolamine. Egg phosphatidylethanolamine has a high content of long polyunsaturated fatty acids [10], while *E. coli* phosphatidylethanolamine possesses shorter chain monounsaturated fatty acids as well as cyclopropane residues [8].

Treatment of the calcium- and magnesium-precipitated *E. coli* phosphatidylethanolamine vesicles with EDTA resulted in dissociation of the precipitate and the formation of a new vesicle suspension as in the case of egg phosphatidylethanolamine. Fig. 5B is a freeze fracture electron micrograph of such a vesicle preparation.

Human gray matter phosphatidylethanolamine

Although the addition of 2.0 mM Ca^{2+} or 3.0 mM Mg^{3+} to human grey matter phosphatidylethanolamine vesicles resulted in fusion of the vesicles, the morphology of the precipitate is difficult to interpret. Although the lamellar phase can be identified, the precipitated preparation lacks an ordered structure. As in the case of egg phosphatidylethanolamine and *E. coli* phosphatidylethanolamine, human grey matter phosphatidylethanolamine has a distinctive fatty acid make-up. Human grey matter phosphatidylethanolamine has been reported to possess a high proportion (47% molar) of fatty aldehyde residues (plasmologen) [7]. In this case the vesicles were much smaller than in the comparable egg phosphaitdylethanolamine and *E. coli* phosphatidylethanolamine treatments.

Dimyristoyl phosphatidylethanolamine

Dimyristoyl phosphatidylethanolamine vesicles were prepared and treated at 55°C to insure that the vesicles were fluid [32]. Dimyristoyl phosphatidylethanolamine vesicles were precipitated at 55°C by 1.0 mM Ca^{2+} or 2.0 mM Mg^{2+}. In each case fusion of the vesicles resulted in the formation of large multilamellar vesicles. Treatment with EDTA resulted in dissociation of the precipitate and formation of the large vesicles.

No hexagonal structures were observed in any experiments with *E. coli* phosphatidylethanolamine, human grey matter phosphatidylethanolamine or dimyristoyl phosphatidylethanolamine vesicles tested with Ca^{2+} or Mg^{2+}.

Other ions

The interaction of egg phosphatidylethanolamine with a variety of divalent and trivalent ions was also examined. Table II lists the ion tested, the experimental pH, and the structural phase observed (lamellar and/or hexagonal). All divalent and trivalent cations tested were able to initiate fusion of the egg phosphatidylethanolamine vesicles. Several ions were tested at pH 5.5 or 5.0 to solubilize salts. Several of the ions stimulated the formation of hexagonal struc-

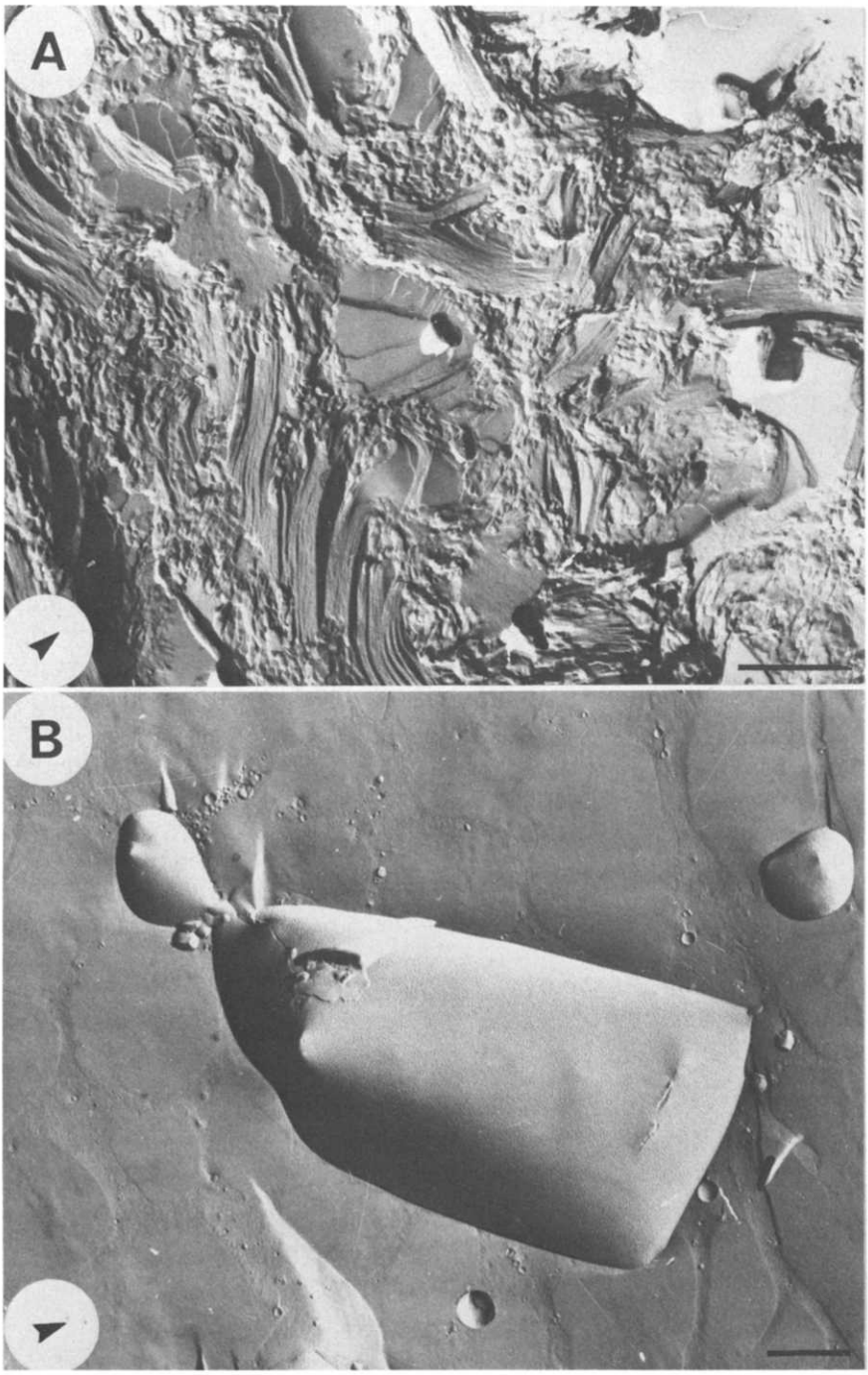

Fig. 5. The interaction of *E coli* phosphatidylethanolamine vesicles with calcium. A The addition of 2.0 mM calcium to *E coli* phosphatidylethanolamine vesicles results in fusion of the vesicles. In this case the multilamellar aggregates are less vesicular in nature then the corresponding egg phosphatidylethanolamine preparation. Amorphus areas can also be seen No hexagonal structures were observed. Magnification 30800 × and bar represents 500 nm B. The addition of EDTA to a calcium-precipitated *E coli* phosphatidylethanolamine vesicle preparation results in the formation of a new vesicle population. Again the vesicles are heterogenous in size and shape. Magnification 22100 × and bar represents 500 nm

TABLE II

MINIMUM ION CONCENTRATION NECESSARY TO CAUSE PRECIPITATION OF EGG PHOSPHATIDYLETHANOLAMINE VESICLE PRECIPITATION

Ion (Chloride salt)	pH	Minimum ion concentration needed to precipitate (mM)	Observed structural phase
Calcium	7.2	2.0	L and H [a]
Calcium	5.5	4.0	L
Magnesium	7.2	3 0	L and H
Magnesium	5.5	10.0	L
Beryllium	5 0	30 0	H
Aluminum (Al^{+3})	5 0	4 0	L
Chromium (Cr^{+3})	5.5	1.0	L and H
Manganese	7.2	2 0	L
Cobalt	7.2	3 0	L
Nickel	7 2	3.0	L
Copper	5.5	3 0	L
Zinc	7.2	2.0	L and H
Strontium	7 2	3.0	L
Cadmium	7 2	2.0	L
Barium	7 2	3.0	L
Lanthanum (La^{+3})	7 2	1.0	L
Mercury	7 2	4.0	L
Lead	5.5	1.0	L

[a] H, hexagonal, L, lamellar

tures. In the case of beryllium, for example, only the hexagonal phase was observed. However, with chromium (Cr^{3+}) and zinc, both hexagonal and lamellar phases were observed. However, in both cases the lamellar phase predominated in all samples tested.

It was not possible to correlate the amount of ion required to precipitate the vesicles and appearance of lamellar or hexagonal structures with the atomic number, radius or hydration enthalpy. In most cases the fusion of the vesicles of which were characteristic of the ion involved, for example, the hexagonal structures obtained from the precipitation of the vesicles by beryllium.

Effect of basic proteins on phosphatidylethanolamine vesicles

Fig. 6A shows such a vesicle population before the addition of a protein solution. Upon the addition of 1.0 mg basic protein extracted from human myelin, there was an immediate flocculation of the mixture. This mixture was then concentrated and examined (Fig. 6B). Large amplitude fusion occurred in the vesicle population showing numerous multilamellar vesicles. No fusion nor aggregation of vesicles prepared from egg phosphatidylcholine occurred with the myelin basic protein.

We decided to examine the behaviour of dimyristoyl phosphatidylethanolamine in the presence of basic proteins above and below the phase transition of the lipid. For vesicle fusion to occur, the lipid must be above its phase transition. Small unilamellar vesicles were prepared from dimyristoyl phosphatidylethanolamine as described previously, except at 55°C which is 7°C above the phase transition of the lipid. It can be seen from Figs. 7A and 7B that vesicles prepared from dimyristoyl phosphatidylethanolamine will fuse either above or

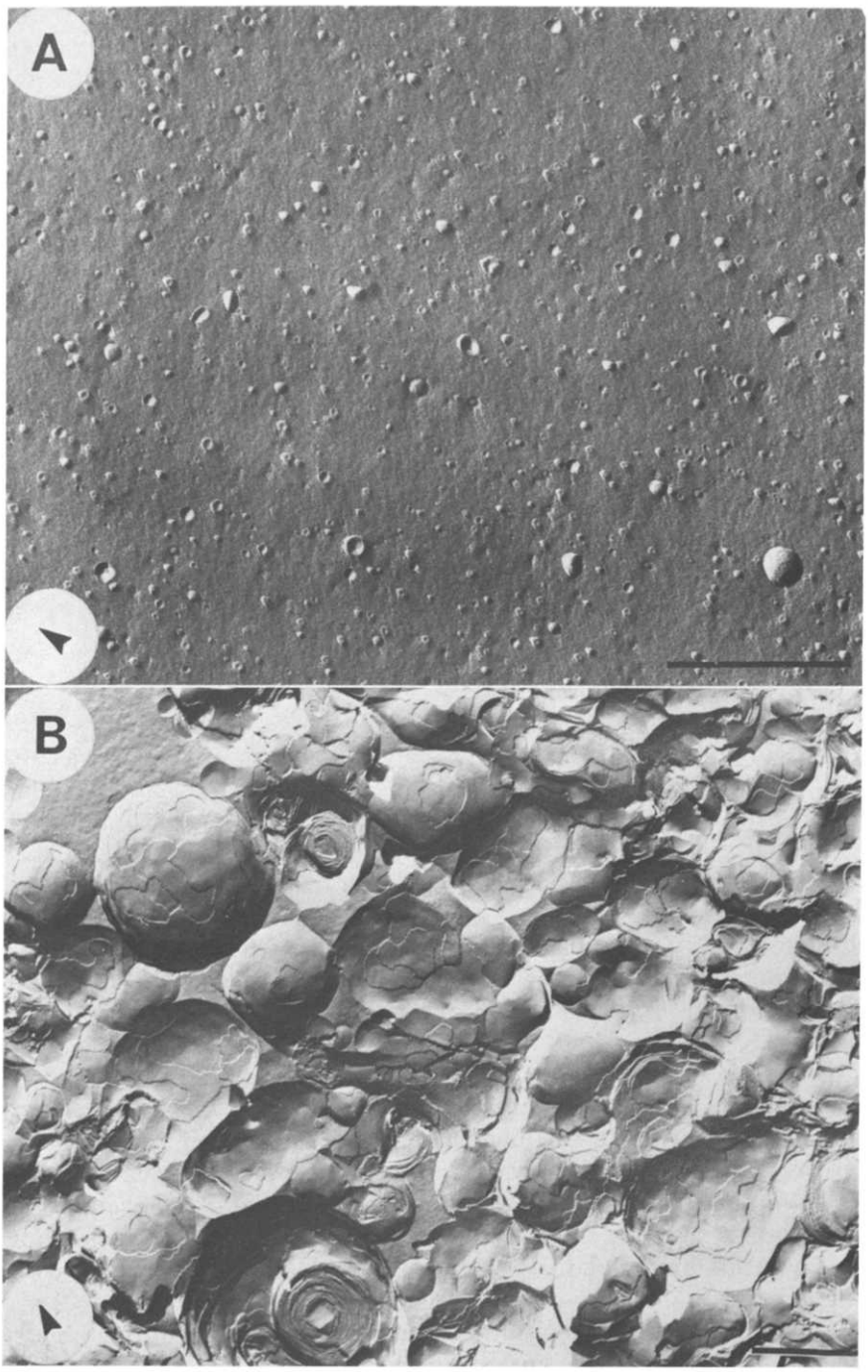

Fig. 6. Freeze-fracture electron micrographs of hens' egg phosphatidylethanolamine. A. A population of small unilamellar vesicles incubated at 37°C for 30 min. Total magnification 49800 × and bar represents 500 nm. B. Multilamellar vesicles after the addition of 1 mg myelin basic protein and incubated at 37°C for 30 min. Total magnification 24900 × and bar represents 500 nm.

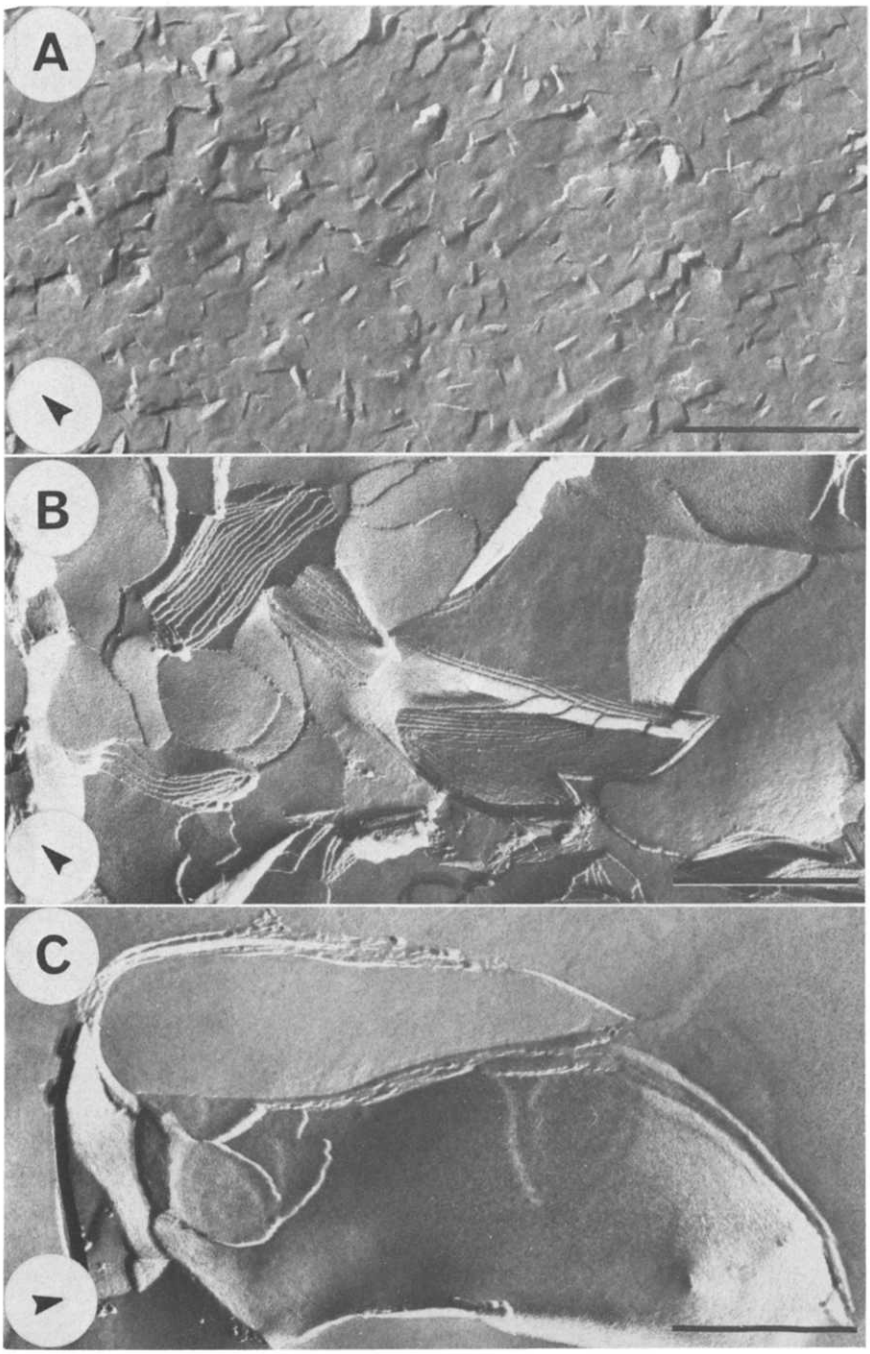

Fig. 7. Freeze-fracture electron micrographs of dimyristoyl phosphatidylethanolamine. A. Small disc-shaped unilamellar vesicles prepared by sonication at 55°C. Total magnification 49800 × and bar represents 500 nm. B. Dimyristoyl phosphatidylethanolamine vesicles treated with 1.0 mg of basic protein and incubated at 55°C for 30 min. Fusion of the vesicles results in the formation of multilamellar "sheet-like" structures. Total magnification 49800 × and bar represents 500 nm. C. Dimyristoyl phosphatidylethanolamine vesicles treated with 1.0 mg of basic protein and incubated at 37°C for 30 min. The addition of the myelin basic protein at this temperature also results in fusion of the small vesicles. Total magnification 49800 × and bar represents 500 nm.

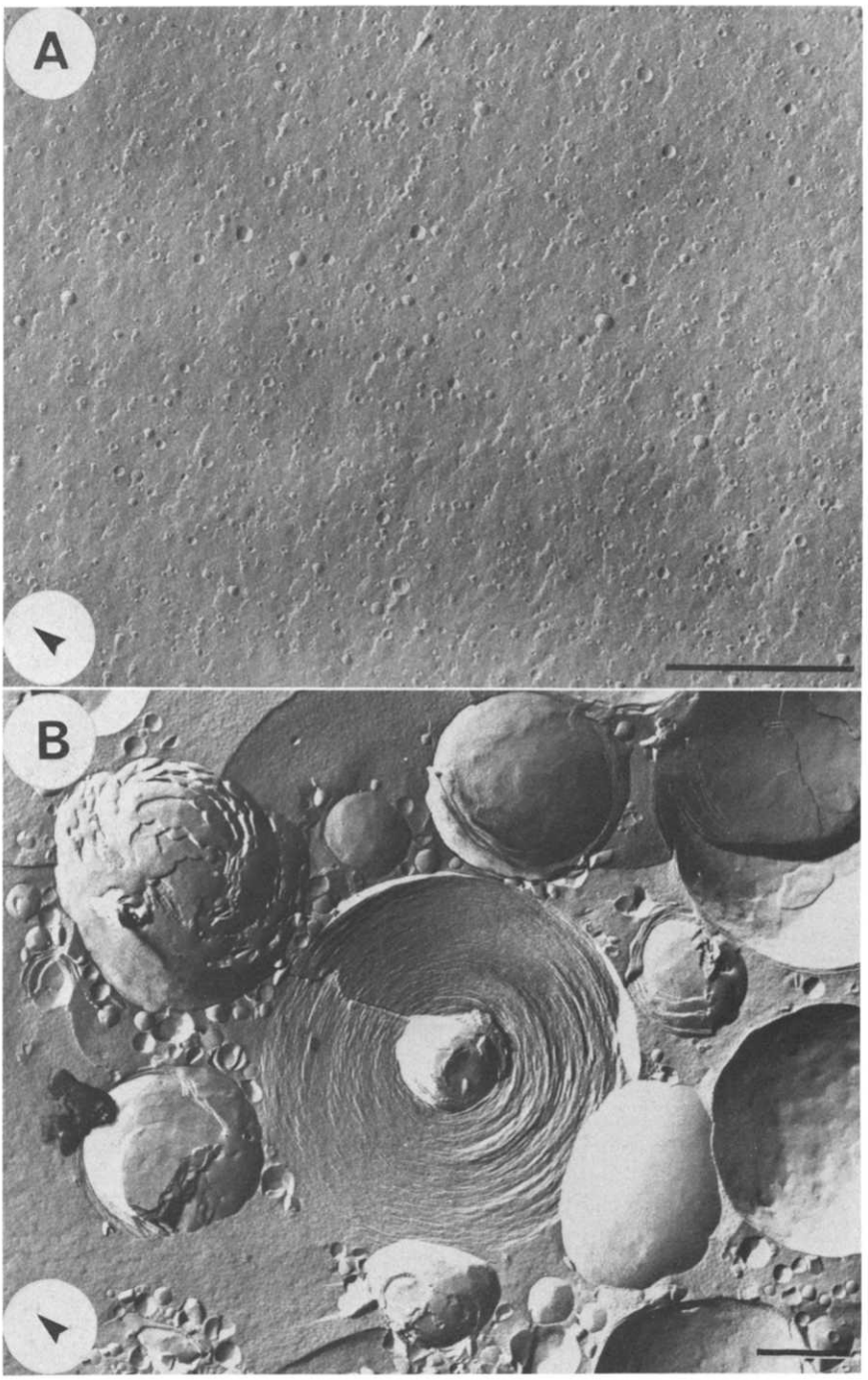

Fig. 8. Freeze-fracture electron micrographs of phospholipid vesicles with 2.0 mg of cytochrome *c* incubated at 37°C for 30 min. A Phosphatidylcholine vesicles. Note the small unilamellar vesicles have not fused. Total magnification 49800 × and bar represents 500 nm. B. Phosphatidylethanolamine vesicles treated as above. Note many multilamellar fused vesicles. Total magnification 24900 × and bar represents 500 nm.

below the reported phase transition with the basic protein from human myelin. Sonicated, small unilamellar vesicles might have a lower phase transition than multilamellar vesicles used to determine the phase transition of dimyristoyl phosphatidylethanolamine. Papahadjopoulos et al. [33] has shown that both the basic protein of myelin and cytochrome *c* decreases the phase transition of the negatively charged dipalmitoyl phosphatidylglycerol but found that poly-lysine increased the phase transition of this lipid.

Since the basic extrinsic protein of myelin mediates vesicle fusion, we thought perhaps another basic extrinsic membrane protein, cytochrome *c*, may also cause phosphatidylethanolamine vesicles to fuse. Fig. 8A shows the results of egg phosphatidylcholine with cytochrome *c*. No fusion occurs. Cytochrome *c* reacted with phosphatidylethanolamine causes the vesicles to fuse, producing large, multilamellar aggregates (Fig. 8B).

We also tried several other non-membrane basic proteins, namely: Protamine (1.0 mg), poly-L-lysine (0.5 mg) [16] and cationically-modified ferritin (1.0 mg) [17]. All these proteins tested cause the fusion of phosphatidylethanolamine vesicles at 37°C. No fusion occurred with phosphatidylcholine vesicles. Unmodified ferritin [17] was also tested with egg phosphatidylethanolamine, and no fusion occurred.

Thus, the fusion of phosphatidylethanolamine vesicles can be mediated by basic proteins which vary widely in size and shape. The basic protein from myelin has been reported to be a fiberous protein 15 × 135 Å [34]. Protamine (molecular weight, 5000), poly-L-lysine (molecular weight, 41000) and cationically-modified ferritin (molecular weight, >450000) can also mediate fusion.

Unlike phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, cardiolipin and phosphatidic acid, which are strongly negatively charged at physiological pH, phosphatidylethanolamine exhibits a weak negative charge. It has been speculated that the orientation of the polar head group lies flat against the bilayer [18,19], and thus causes a partial charge neutralization of the protonated amino group.

It is interesting to speculate on the function of this lipid in biological membranes since it is found in numerous membranes such as the plasmic membrane, myelin and the mitochondrial inner membrane. From our studies with model membrane systems, it might suggest that this lipid may be important in biological membrane fusion such as fertilization, virus-cell membrane interactions and endocytosis in association with either positively charged proteins and/or divalent cations.

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