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COCHLEATE LIPID CYLINDERS: FORMATION BY FUSION OF UNILAMELLAR LIPID VESICLES

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Summary

Freeze-fracture electron microscopy was used to study the morphological changes occurring following the addition of Ca^{2+} to sonicated preparations of phosphatidylserine in aqueous NaCl buffer. Before the addition of Ca^{2+}, preparations contained only small (200—500 Å diameter) spheroidal vesicles. After the addition of Ca^{2+} (10 mM) and incubation for 1 h at 37°C preparations contained only large (2000—10 000 Å) apparently multilamellar structures many of which were cylindrical in shape. The lamellae in these cylinders appear to be folded in a spiral configuration. Addition of EDTA to these preparations produced large, closed, spherical, unilamellar vesicles. We suggest the name cochleate lipid cylinders for the spiral structures and propose that they are formed by fusion of unilamellar vesicles into large sheets which fold spirally to form cylinders.

We have recently reported biochemical and ultrastructural observations on the fusion of unilamellar vesicles prepared from various classes of phospholipid [1]. Negative-staining electron-microscopic data reported in this previous paper showed that fusion of unilamellar phosphatidylserine vesicles (diameter 300—500 Å) created large pleomorphic vesicles with diameters ranging from 2000—12 000 Å. In this communication we present freeze-fracture observations on changes in the morphology of phosphatidylserine vesicles incubated under similar conditions to those reported previously as causing vesicle fusion [1]. The results extend our previous electron-microscopic findings and demonstrate that vesicles can undergo substantial structural rearrangements following addition of Ca^{2+}. The new structures created under conditions of vesicle fusion appear to be similar to those observed in previous freeze-fracture studies with dilauryl phosphatidylglycerol in the presence of Ca^{2+} and Mg^{2+} [2, 3,

4]. The conditions for the formation of the structures reported here, however, are quite different than those employed in the earlier studies.

We propose that fusion of unilamellar phosphatidylserine vesicles in the presence of Ca^{2+} creates large planar lamellae which roll up to form cylinders. We suggest the term cochleate cylinders (Greek: κοχλίασ = snail with spiral shell) for these structures, which appear to be formed from spirally folded lipid bilayers. Incubation of cochleate cylinders with Ca^{2+}-chelating agents results in a further structural transformation to create very large closed unilamellar vesicles. These findings therefore suggest a potential new method for creating very large unilamellar vesicles suitable for studies on the properties of lipid bilayers [5] and the interaction of unilamellar vesicles with cultured cells [6, 7, 8]

Phosphatidylserine was chromatographically pure and was isolated from bovine brain as described before [9]. All chemicals were either analytical reagents or of the highert purity available commercially [1].

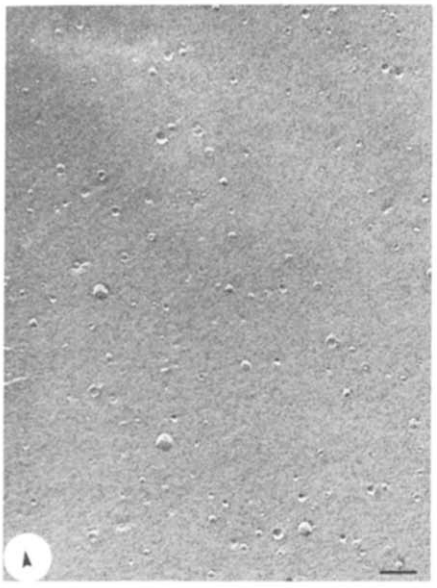
Unilamellar vesicles of phosphatidylserine were prepared as described before with a bath-type sonicator in 100 mM NaCl, 2 mM Histidine, 2 mM *N*-tris-(hydroxymethyl)-methyl-2-aminoethanesulfonic acid, 0.1 mM EDTA, adjusted to pH 7.4.

The sonicated dispersion, initially containing 5 μmol per ml of phosphatidylserine was centrifuged at 100 000 × *g* for 1 h at 20°C to eliminate large particles. The supernatant, containing usually 90—95% of the original phosphate, was used for the addition of Ca^{2+}. For freeze-fracture analysis the supernatant was concentrated in an Amicon Minicon A-75 concentrator system (Amicon, Lexington, Mass.) for 2—3 h at room temperature to one-tenth of the original volume. The concentrated dispersion was then mixed with one half volume of glycerol just before freeze-fracture. The lipid samples after the addition of Ca^{2+} and EDTA were centrifuged (Eppendorf microcentrifuge, 5 min) and the pellets resuspended and re-centrifuged in the same buffer containing 30% glycerol by volume at 22—25°C immediately before freezing.

Freeze-fracture of vesicle populations was performed in a Balzer BA360 apparatus as before [10], after freezing from room temperature into Freon 22 cooled by liquid nitrogen.

A preparation of sonicated vesicles of phosphatidylserine was used to obtain Fig. 1. This is a view typical of all fields examined and contains round (spheroidal) particles in the range of 200—500 Å, with smooth surfaces. This is in accord with images obtained with other lipids in several laboratories as recently reviewed [5].

Addition of Ca^{2+} to sonicated phosphatidylserine preparations at a concentration of 1 mM or higher, produces a white flocculate which slowly settles to the bottom of the test tube. Fig. 2 was obtained from such precipitates after incubation of the phosphatidylserine vesicles (1 μmol/ml) in the presence of added $CaCl_2$ (10 mM for 1 h at 37°C). It shows large apparently multilamellar structures of differing size and shape but also a large number of long tubular structures. Fractures of these tubular structures are shown at different angles in Figs 3 and 4. Examination of the folding of the lamellae in these structures reveals a spiral configuration resulting in "unmatched" layer(s) on one side of the particle (Fig. 3). Negatively stained preparations of the same system with uranyl acetate [1] revealed particles with a "stepped" structure (Fig. 5A), which we



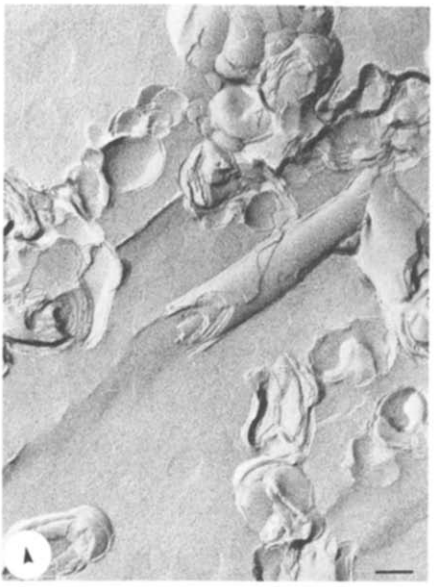


Fig.1. Freeze-fracture electron microscopy of sonicated phosphatidylserine vesicles in 100 mM NaCl buffer, pH 7.4. Magnification X 50 000. Bar: 0.1μm (1000 Å) Direction of shadowing is indicated by arrows in lower left-hand corner.

Fig.2. Freeze-fracture electron microscopy of sonicated phosphatidylserine vesicles in NaCl buffer, after addition of $CaCl_2$ (10 mM). Magnification X 50 000. Bar: 1000 Å.

interpret as corresponding to the "unmatched" lamellae seen on the cochleate cylinders by freeze-fracture.

Addition of EDTA (15 mM) to such preparations following incubation with Ca^{2+} (10 mM) produced immediate clearing of the flocculent. After further incubation in the presence of EDTA for 0.5 h at 37°C, the majority of the lipid was sedimentable by brief centrifugation (Eppendorf, 5 min). Fig. 6 shows typical freeze-fractures obtained from these pellets. The particles have now become very large and rounded with smooth surfaces. Where the fracture plane cuts through such particles, large spaces without structure are revealed, with occasional smaller vesicles completely enclosed inside. Negative staining of identical preparations revealed structures with a "creased" and collapsed bag-like appearance (Fig. 5B).

An interpretation of the sequence of structural transformations seen in Figs 1—6 is given in Fig. 7.

As described previously, addition of Ca^{2+} to sonicated phosphatidylserine vesicles (Fig. 7A) induces a large increase in permeability [11] with loss of both Na^+ and Cl^- [12] indicating rupture of the vesicles with loss of the initially captured ions and water. The resulting membranes (Fig. 7B) contain phosphatidylserine and Ca^{2+} at a 2 to 1 molar ratio [13] and the phospholipid aliphatic acyl chains are in a crystalline state [14]. It has also been shown previously that this rupture of phosphatidylserine membranes is caused by the presence of Ca^{2+} only on one side of the membrane, possibly as a result of asymmetry in the membrane surface charge [15]. The instability caused by "crystalliza-

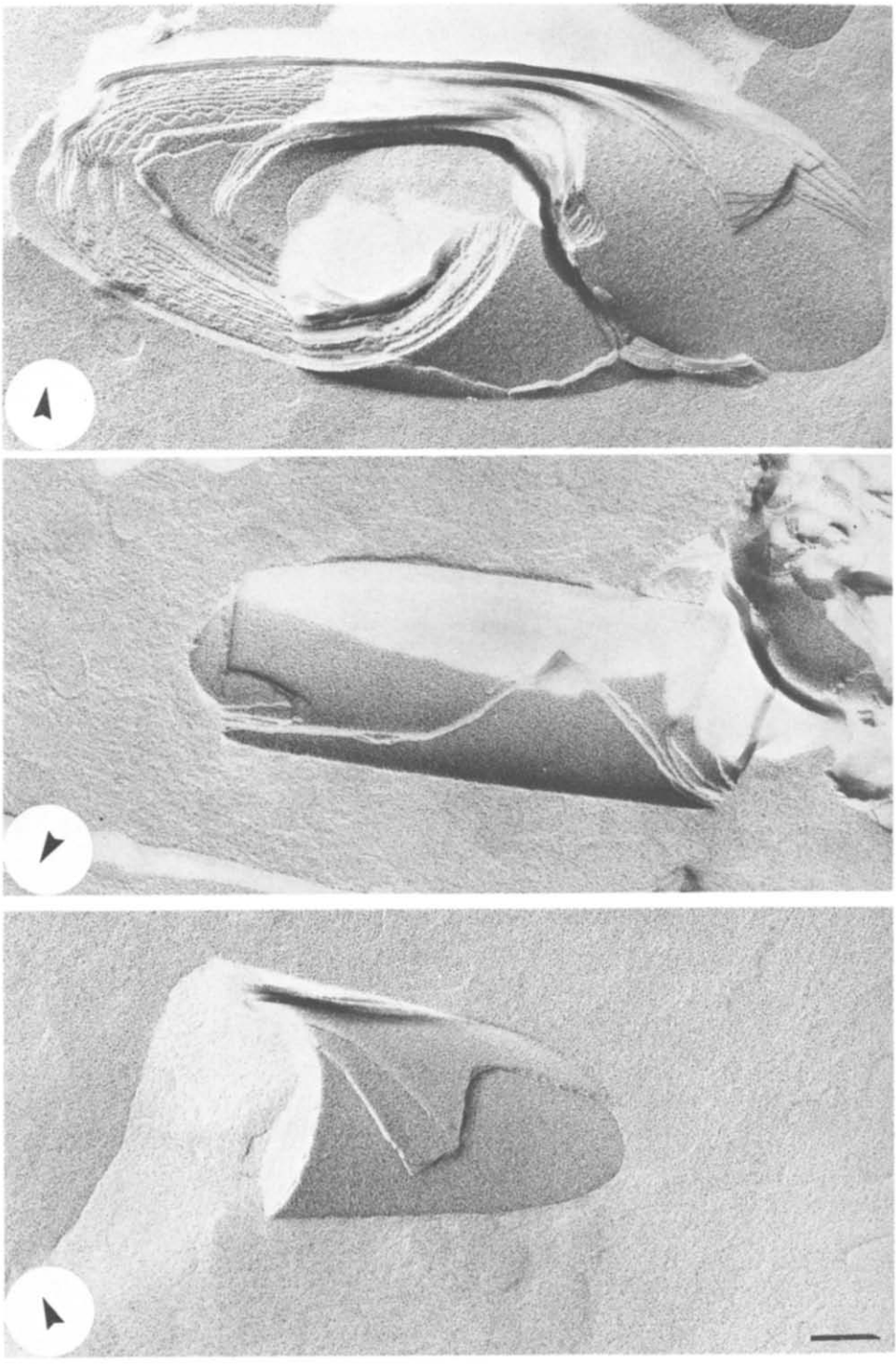


Fig.3. Freeze-fracture electron microscopy of sonicated phosphatidylserine vesicles after the addition of Ca^{2+}, showing side views of individual cochleate cylinders. Details in Figs 1 and 2. Bar: 1000 Å.

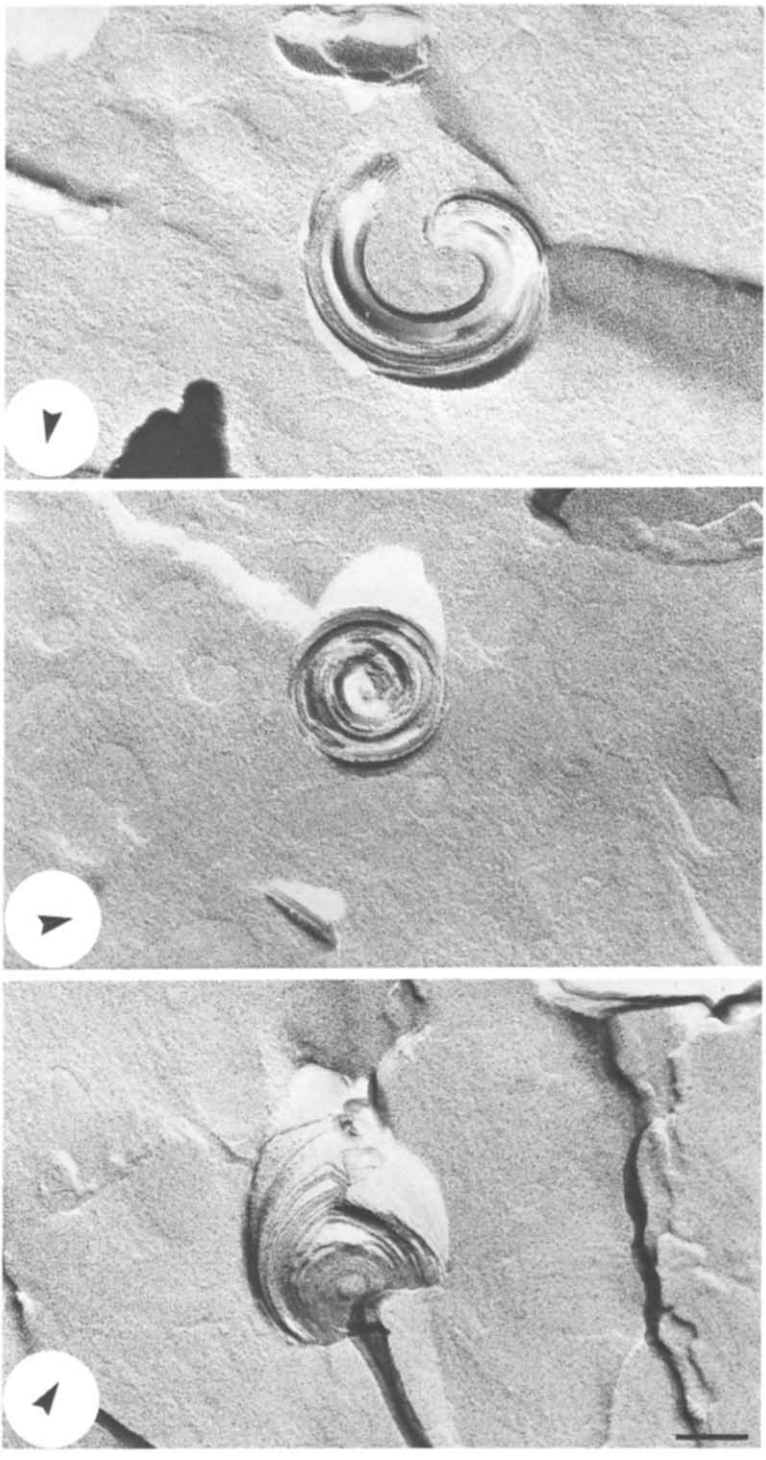


Fig.4. Freeze-fracture electron microscopy of sonicated phosphatidylserine vesicles after addition of Ca^{2+}, showing cross sectional views of individual cylinders. Experimental details as in Figs 1 and 2. Bar: 1000 Å.

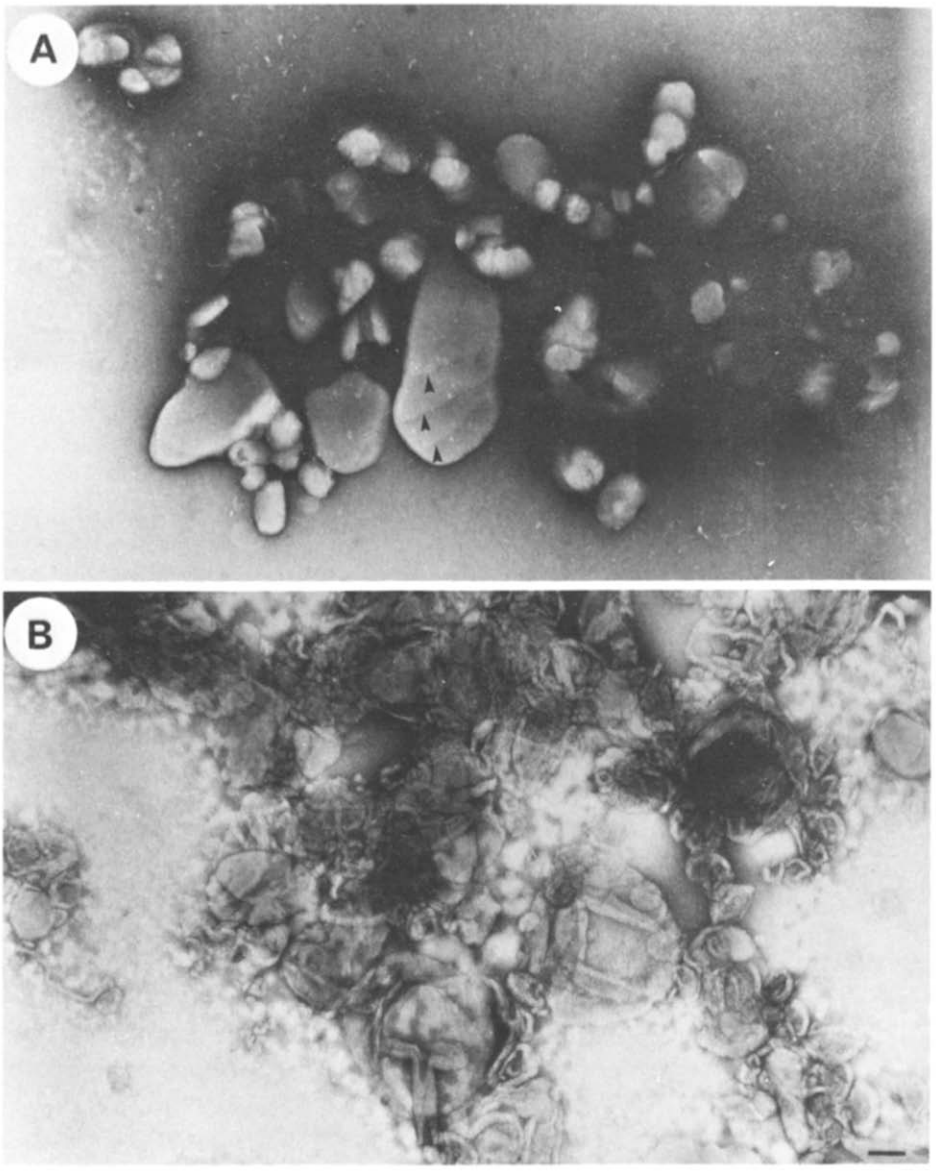


Fig.5. Negative stain electron microscopy of sonicated phosphatidylserine vesicles. A: after addition of Ca^{2+}. B: After the addition of Ca^{2+} as above and then EDTA (15 mM). Samples were stained with uranyl acetate. Arrows indicate the appearance of "stepped" structures. Experimental details as in Figs 1 and 2 and methods. Bar: 1000 Å.

tion" of the outer monolayer might at least be partly responsible for "rupturing" of the vesicles and their subsequent fusion.

We postulate that the collapsed small vesicles (Fig. 7B) fuse into large planar sheets (Fig. 7C) which eventually coil around an initial point of folding (Fig. 7D) to form cochleate cylinders (Fig. 7E). In this case, Ca^{2+} acts both to crystallize the individual lamellae, and to produce a tightly folded apparently multilamellar system with 54 Å repeat distance [9]. This repeat distance is con-

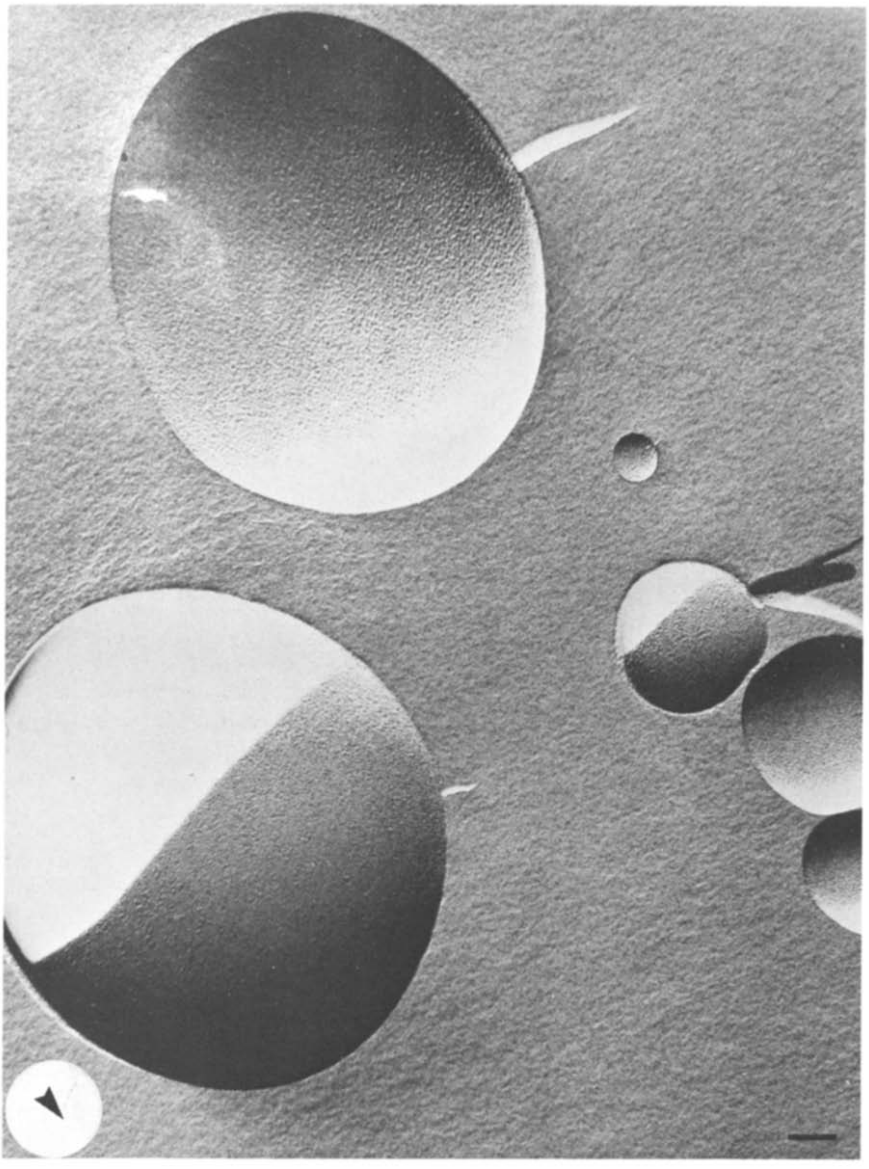


Fig.6. Freeze-fracture electron microscopy of sonicated phosphatidylserine vesicles after addition of Ca^{2+} (10 mM) as in Fig. 2, and then EDTA 15 mM). Total magnification × 65 000. Bar: 1000 Å.

siderably smaller than that observed in other multilamellar lipid systems such as phosphatidylserine and phosphatidylcholine dispersed in 0.1 M aqueous KCl [9, 16, 17] and indicates an interlamellar attraction which may promote the spiral formation. This process, however, might be dependent on lipid concentration. Thus, in more concentrated lipid suspensions, attractions between neighboring lamellae may lead to the formation of planar multi-bilayers in preference to cochleate cylinders or cochleate cylinders formed by the "spiralling" of multiple apposed bilayers. The edges of the cochleate cylinders could also provide

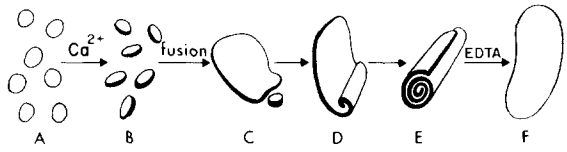


Fig.7. Schematic representation of the effect of Ca^{2+} on sonicated phosphatidylserine vesicles leading to the formation of cochleate cylinders. A: sonicated vesicles in NaCl buffer before addition of Ca^{2+}, shown as spheroidal particles with an aqueous interior and a single lipid bilayer shell. B,C and D are suggested intermediate steps involving the aggregation and fusion of the spheroidal vesicles into cochleate cylinders. B represents the step in which Ca^{2+} ruptures the vesicles forming flat bilayer disks. C indicates fusion of the disks into large sheets in order to minimize hydrocarbon-water contact. D indicates the beginning of the folding of the flat sheet into a cylinder. E: Cochleate cylinders formed by spiral folding of the continuous planar membrane sheets shown in C and D. F: Large unilamellar vesicles created by unfolding of the cochleate spiral membranes following incubation with EDTA. In addition to the sequence outlined in steps B to E several other processes could contribute to the formation of the cochleate cylinders shown in E: (1) Aggregation of unilamellar vesicles could precede the rupture and fusion steps; (2) Cochleate cylinders could be formed by the spiraling of several separate bilayer sheets together as shown in Fig. 4 (top); (3) Although the initial formation of cochleate cylinders might require membrane sheets of a "critical" size, the further growth of these cylinders could proceed by continuous fusion with unilamellar vesicles.

"nucleation" sites for further growth of the cylinders by fusion with small unilamellar vesicles.

The addition of EDTA to cochleate cylinders chelates Ca^{2+}, restoring negative charge and "normal" fluidity to the membranes. These new conditions would affect the balance of attractive and repulsive interactions between bilayers, apparently leading to a new preferred arrangement in which single bilayers form large enclosed spherical vesicles (Fig. 7F). This process could perhaps involve first the unfolding of the bilayers of the cochleate cylinders into planar sheets which seal by fusion at the "edges" to form very large closed unilamellar vesicles.

Our proposal that the elongated phospholipid cylinders observed here, and perhaps those observed in earlier studies [2—4], are spirally folded cochleate structures, applies only to the structures obtained with acidic phospholipids in the presence of divalent metals. These new observations are not contradictory to the well-established view that when phospholipids are suspended in aqueous salt solutions containing monovalent cations at temperatures above their gel-to-liquid-crystalline phase transition they form closed multilamellar structures composed of concentric and separate bilayer lamellae [18]. The proposal that cochleate structures are formed from one continuous membrane that spirals around itself, provides a new interpretation on the mechanism by which Ca^{2+} can produce apparently multilamellar large structures from small unilamellar vesicles. It is an interesting question whether the tendency of acidic phospholipid membranes to form cochleate cylinders in the presence of bivalent metals might contribute to our understanding of the process of myelination, where the membranes of Schwann cells are wrapped spirally a number of times around the nerve axon [19, 20].

The observations reported here also indicate that formation of large unilamellar vesicles can be accomplished by removing Ca^{2+} from cochleate cylinders using EDTA. We anticipate that such large vesicles will be a useful experimental alternative to either the large multilamellar [18] or the small unilamellar vesicles [9], particularly in studies on model membranes where

capture of large macromolecules and large radius of curvature are important requirements.

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