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PHASE CHANGES OF CARDIOLIPIN VESICLES MEDIATED BY DIVALENT CATIONS

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Summary

Small unilamellar vesicles were prepared from cardiolipin and produced the hexagonal II phase when dialyzed against $CaCl_2$ or $MgCl_2$. Upon removal of the cation by dialysis against EDTA large unilamellar vesicles were formed. The events of the transition from the lamellar to hexagonal phase and back to the lamellar phase are described.

Introduction

Cardiolipin has been reported to be a major lipid of the mitochondrial inner membrane [1]. The work by Deamer et al. [2] has clearly shown that the calcium salt of cardiolipin is hexagonal as visualized by freeze fracture electron microscopy. Their preparation was filtered and pressed dried so as there was little water present in the sample.

Rand and Sengupta [3] has shown that cardiolipin precipitated by either Ca^{2+} or Mg^{2+} will form a hexagonal structure monitered by X-ray diffraction. These workers suspended the lipid in water and precipitated the lipid with the cation and concentrated the precipitate by centrifugation. Apparently multilamellar vesicles were precipitated by the cation. They concluded that the hexagonal II phase was present with water cylinders packed hexagonally in a hydrocarbon matrix.

We wish to show in this report that small unilamellar vesicles can be prepared from cardiolipin by sonication, and these vesicles will fuse in the presence of either Ca^{2+} or Mg^{2+} to form the hexagonal phase. When the cation is removed by chelation with EDTA, the hexagonal phase reverts to large unilamellar vesicles.

In addition, we wish to show the transition between lamellar and hexagonal

* Reprints of this paper will not be available due to budgetary limitation.

phases by dialysis aginst low calcium concentration, as well as the transition of the hexagonal phase to the lamellar phase by dialysis against low amounts of EDTA.

Materials and Methods

Cardiolipin was purchased from the Sigma Chemical Co. in an ethanol solution. Thin-layer chromatography showed a single spot, and the lipid was considered chromatographically pure. Phosphorus analysis was by the method of Fiske and Subbarow [4].

The lipid solution was evaporated to dryness by a gentle steam of dry nitrogen gas in a test tube and evacuated to 10^{-2} Torr for 30 min to ensure the complete removal of the solvent. A buffer containing 100 mM NaCl, 2 mM histidine, 2 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), and 0.1 mM EDTA, pH 7.2, was added, and the mixture was vortexed for 5 min under dry argon to produce a cloudy suspension. This suspension was sonicated in a bath type sonicator (100 W) for 60 min under dry argon. The resulting suspension was optically clear and is termed small unilamellar vesicles [5]. A 10 μmol aliquot of lipid phosphorous (1 ml) was reduced approx. 0.1 ml in Satorius colloidin bags and glycerol was added to one third of its volume for freeze fracture.

1 ml each (10 μmol lipid phosphorus) of this small unilamellar vesicle preparation was placed in dialysis tubing pretreated by boiling against 20 mM EDTA. These preparations were dialyzed against 1 l of the following buffer containing 100 mM NaCl, 2 mM histidine, 2 mM TES, pH 7.2.

Aliquots of either 1 mM $MgCl_2$ or $CaCl_2$ were added and allowed to equilibrate for 3 h at 22°C and the preparations in dialysis tubing were observed for changes in turbidity. Increased turbidity occurred at 3 mM Ca^{2+} or 4 mM Mg^{2+}, respectively, with complete precipitation of the lipid. The precipitate was collected by centrifugation in a clinical centrifuge for 2 min and 60% (v/v) glycerol in the above buffer was added to approx. 0.2 ml for freeze fracture.

Further experiments were prepared to determine the morphology of the vesicles after removal of the cation by EDTA. Small unilamellar vesicles were precipitated with 3 or 4 mM cation by dialysis, and the dialysis tubing transferred to 1 l of a solution containing 100 mM NaCl, 2 mM histidine, 2 mM TES and 4 mM EDTA, pH 7.2, and dialyzed for an additional 3 h at 22°C. The precipitated lipid went back into solution to produce a cloudy suspension. This suspension was collected by centrifugation for 30 min at 105 000 $\times g$ in a Spinco L-2 centrifuge at 22°C. The clear supernatant was discarded and the pellet resuspended in 0.2 ml 30% (v/v) glycerol buffer for freeze fracture.

To show the transition between the lamellar phase and hexagonal phase, three 1-ml aliquots containing 10 μmol each of lipid phosphorus, of small unilamellar sonicated vesicles were dialyzed against 1 l of the saline, histidine, TES buffer containing 1.0 mM $CaCl_2$. Vesicles were removed after 45, 90 and 135 min and concentrated to approx. 0.1 ml in colloidin bags and prepared for freeze fracture.

To show the transition from hexagonal to lamellar phase, three 1-ml aliquots,

containing 10 μmol each of lipid phosphorus, of small unilamellar sonicated vesicles were dialyzed at 22° C against 1 l of the saline, histidine, TES buffer containing 3 mM $CaCl_2$ for 3 h. The tubing containing the hexagonal precipitate was washed in distilled water and transferred to 1 l of the saline, histidine, TES buffer containing 1.0 mM EDTA and dialyzed at 22° C for 45, 90 and 135 min and concentrated as previously described.

For freeze fractures, aliquots of lipid were placed in gold cups and quickly frozen in Freon 22 and stored in liquid nitrogen. Fractures were preformed in a Balzer BA 360 M apparatus at 10^{-6} Torr or better vacuum at −115° C. Replicas were floated on water, washed for 60 min in Javex, twice with water and mounted on bare 75 × 300 mesh copper grids. Specimens were examined in a Philips EM 300 operated at 60 kV.

Arrows in the lower left-hand corner of the micrograph indicate the direction of the platinum deposit and concave (∩) and convex (∪) surfaces are shown.

Results and Discussion

Cardiolipin can be used to prepare small unilamellar vesicles by sonication of the lipid in a buffer of high ionic strength. The population of these small unilamellar vesicles are rather uniform (Fig. 1). The vesicles measure 20—50 nm. However, the smaller vesicles could result from random fractures through the tops or bottoms of larger vesicles. Thus, the largest measured

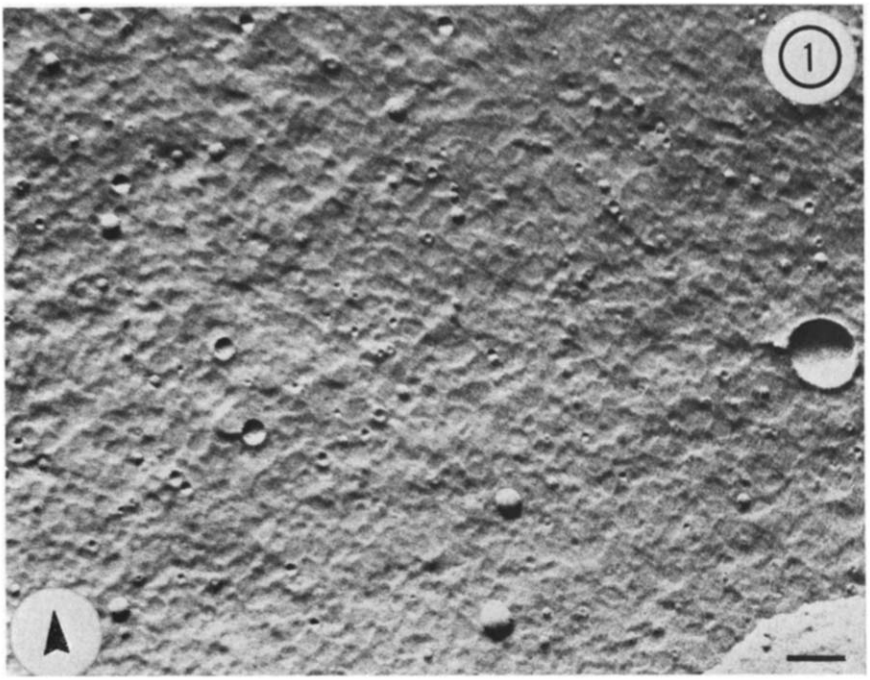


Fig. 1. Small unilamellar vesicles prepared from cardiolipin by sonication. Total magnification 80 000×. Bare represents 100 nm.

vesicles might be a median fracture and may be a more accurate size of the population using the freeze fracture technique.

We have used these small unilamellar vesicles in this series of experiments for several reasons; namely, the vesicles are fully hydrated, have a uniform morphology, and are unilamellar.

If the small unilamellar vesicles are dialyzed against a buffer containing 3 or 4 mM of the chloride salts of either Ca^{2+} or Mg^{2+}, turbidity begins to appear and a white granular precipitate soon settles at the bottom of the dialysis tube. The precipitate can be collected by gentle centrifugation in a clinical centrifuge and examined. Fig. 2 shows the presence of the hexagonal phase. Rods of lipids are present with center to center distances of 8—9 nm while the ends of the rods likewise measure 8—9 nm. These measurements indicate the hexagonal II phase as was previously observed by Rand and Sengupta [3] using the X-ray diffraction technique. These workers used presumably multilamellar vesicles precipitated by the divalent cation. However, we have used small unilamellar vesicles and obtained the same results. These rods are composed of a water center surrounded by the polar lipid head with the cation and the fatty acid chains radiating outward. The length of the rods are quite variable. This observation could result from random fractures between and across the lipid rods. Similar structures were observed by Papahadjopoulos et al. [5] with phosphatidic acid precipitated with Mg^{2+} and by Stollery and Vail [6] with phosphatidylethanolamine precipitated with Ca^{2+}, Mg^{2+}, or Be^{2+}. Our observations verify the observation of Rand and Sengupta [3] using entirely different physical tech-

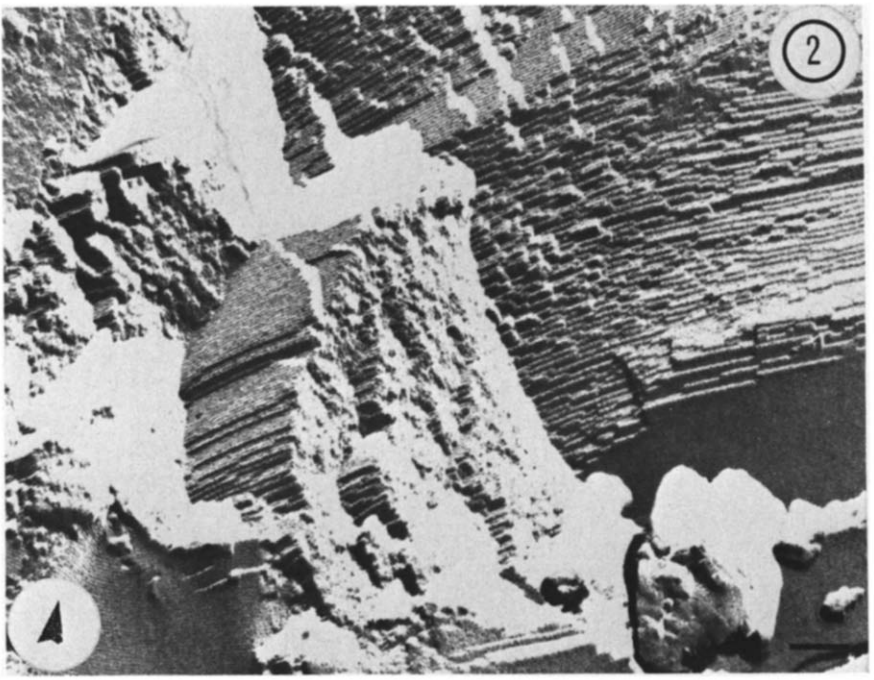


Fig. 2. Hexagonal array produced by dialysis of small unilamellar vesicles against 3 mM $CaCl_2$. Total magnification 80 000X.

niques and similar structures occur whether Ca^{2+} or Mg^{2+} was added to the small unilamellar vesicles.

However, we thought it might be prudent to determine what type of structure would occur if the cation is removed from the hexagonal structure by dialysis aginst EDTA. Papahadjopoulos et al. [7] found that if small unilamellar vesicles prepared from phosphatidylserine were dialyzed against Ca^{2+}, cochleate cylinders resulted and when the cation was removed a population of large unilamellar vesicles resulted. However, if Mg^{2+} was added to these small unilamellar vesicles, no cochleate cylinders were produced and an aggregation of the small vesicles resulted with very little fusion of vesicles.

If cardiolipin was precipitated with 3 or 4 mM of either $CaCl_2$ or $MgCl_2$ and dialyzed against EDTA (4 mM) the precipitate gradually produced a cloudy suspension and could be collected by centrifugation at 105 000 $\times g$ for 30 min. When this latter precipitate was examined using the freeze fracture technique, we observed a population of large unilamellar vesicles (Fig. 3). The vesicle morphology was similar whether Ca^{2+} or Mg^{2+} was used to produce the precipitate. Thus, the removal of the cation from the hexagonal precipitate produced a uniform population of large unilamellar vesicles which measured 150—250 nm. The lamellar phase changed to hexagonal by the addition of the cation. The removal of the cation produced again the lamellar phase.

For us it was difficult to imagine how these transitions could occur; the transition of a 'sheet-like' lamellar phase to a 'rod-like' hexagonal phase and back again to the 'sheet-like' lamellar phase. Could we observe these transi-

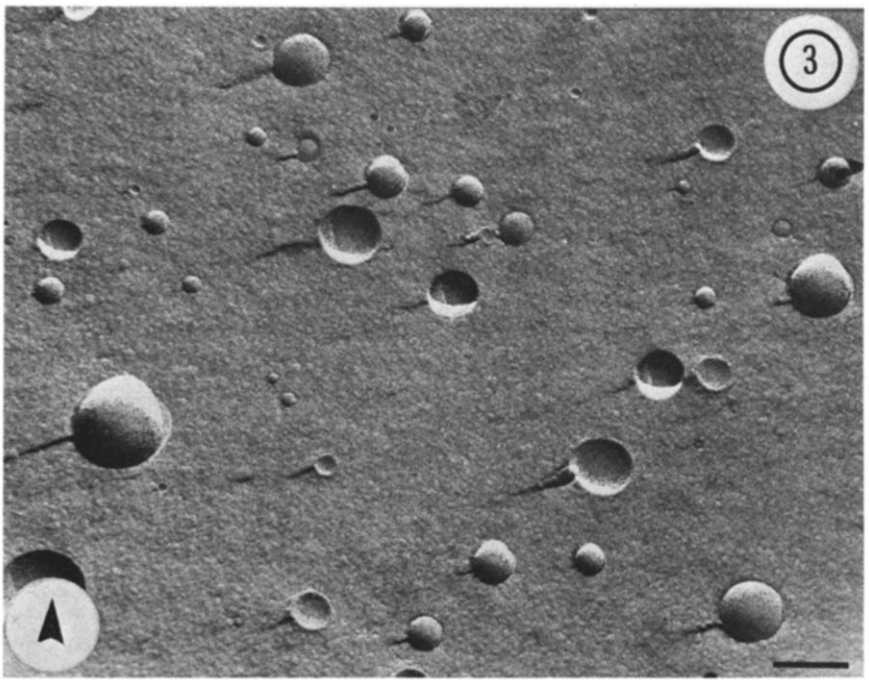


Fig. 3. Large unilamellar vesicles produced by dialysis of the hexagonal phase against 4 mM EDTA. Total magnification 50 000X. Bar represents 200 nm.

tional events using the freeze fracture technique? Perhaps we might dialyze the small unilamellar vesicles against lower amounts of Ca^{2+} and withdraw samples during the change, from lamellar to hexagonal phase. After preliminary experiments, we determined that dialysis against 1 mM Ca^{2+}, removal of the samples after 45, 90 and 135 min incubation at 22°C, removing 1 ml of lipid (approx. 10 mol of lipid phosphorus), concentrate the sample and adding the cryto-protectant, and immediately freeze the samples for freeze fracture would show these events.

Figs. 4A and 4B shows the results of a 45 min incubation against a buffer containing 1 mM $CaCl_2$. The first events appear to be the fusion of the small vesicles (at arrow in Fig. 4A). The larger vesicles from depressions on the convex surfaces while on concave surfaces they appear as invaginations. These structures are presumably sites of the attachment of Ca^{2+}, and these depressions or invaginations appear to be the beginning of lipid tubes.

After 90 min incubation further changes occur (Fig. 5). It appears that more vesicles fuse to form still larger vesicles and tubes begin to form and have a curious linear arrangement on this concave surface. The initiation of one tube, follows closely with the initiation of adjacent tubes to form a linear array. The lamellar phase thus coexists with the hexagonal phase.

After 135 min (Fig. 6) structures appear with centers of hexagonal phase with an intermediate phase towards the outside, as seen as a granular area. This structure results as more and more invaginations occur producing the hexagonal phase and would occur until there is an equilibrium between all the acidic sites of the lipid with the divalent cation and thus form a completely hexagonal phase.

Next, we then wished to determine what events occurred during the transition from the hexagonal phase to the lamellar phase with the removal of the cation. The hexagonal phase was produced by dialysis of the small unilamellar vesicles against a buffer containing 3 mM $CaCl_2$ for 3 h at 22°C. The dialysis tubing was then removed from the dialysate and placed in a buffer containing 1 mM EDTA and samples removed at 45, 90, and 135 min incubation, concentrated as previously described, and immediately frozen for freeze fracture.

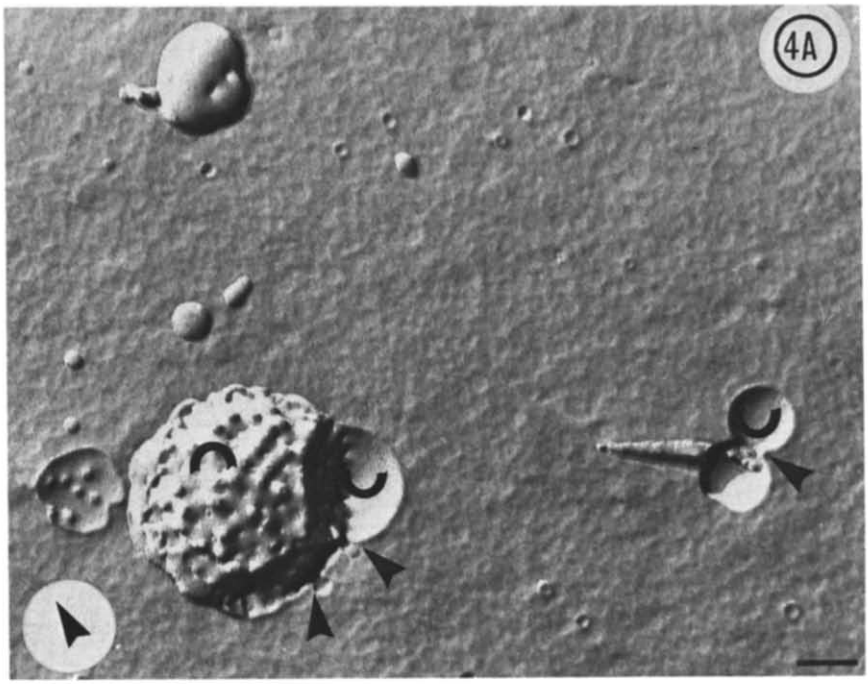
After 45 min incubation (Fig. 7) vesicles in the lamellar phase are present along the edge of an aggregate. The center of the aggregate shows a hexagonal phase surrounded by a heterogeneous granular or mixed phase. This sample is quite similar to Fig. 6.

After a 90 min incubation (Fig. 8) in the buffer containing EDTA, numerous vesicles can be seen at the edge of the aggregate (at arrows) and a large single lamellar vesicle has been released (at double arrows).

After a 135 min incubation (Fig. 9) many vesicles can be seen being formed on the surface of the vesicle. The surfaces of these vesicles are smooth and the surface of the large vesicles are covered with small invaginations.

Thus, it appears from our study that the formation of large unilamellar vesicles is similar to the events of the formation of the hexagonal phase. It appears also that once a critical size of these large unilamellar vesicles is attained they are released. This size appears to be about 200 nm.

The events which lead to the formation of the hexagonal phase from the lamellar phase appears to follow the following sequence: (1) Fusion or the



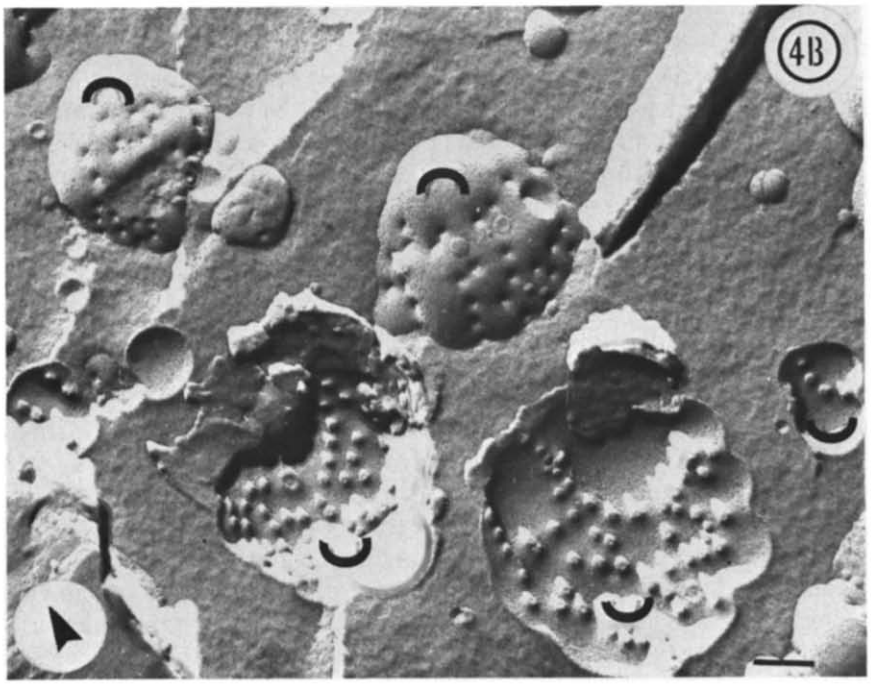


Fig. 4. (A and B) Initiation of the change from the lamellar phase to the hexagonal phase by incubation for 45 min of the small unilamellar vesicles against 1 mM $CaCl_2$. Fusion of the vesicles can be seen at arrows to form larger vesicles. Total magnification 80 000X. Bar represents 100 nm.

Fig. 5. A concave surface of a lipid vesicle showing the linear array of invaginations of the membrane after incubation for 90 min in the presence of a buffer containing 1 mM $CaCl_2$. Total magnification 50 000X. Bar represents 200 nm.

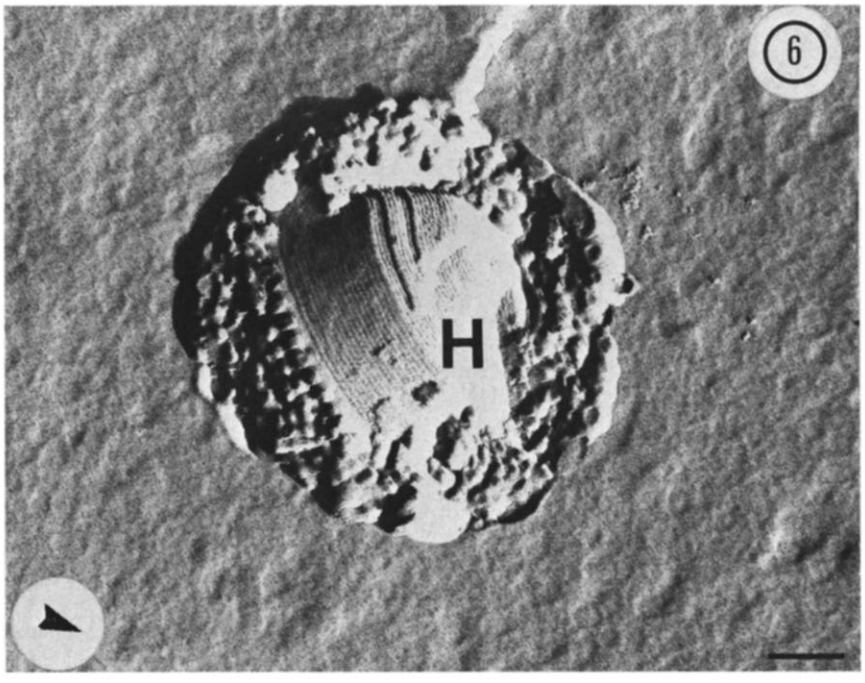


Fig. 6. A vesicle containing a hexagonal center (H) with a mixed hexagonal-lamellar area surrounding the hexagonal area after an incubation for 135 min in a buffer containing 1 mM $CaCl_2$. Total magnification 100 000X. Bar represents 100 nm.

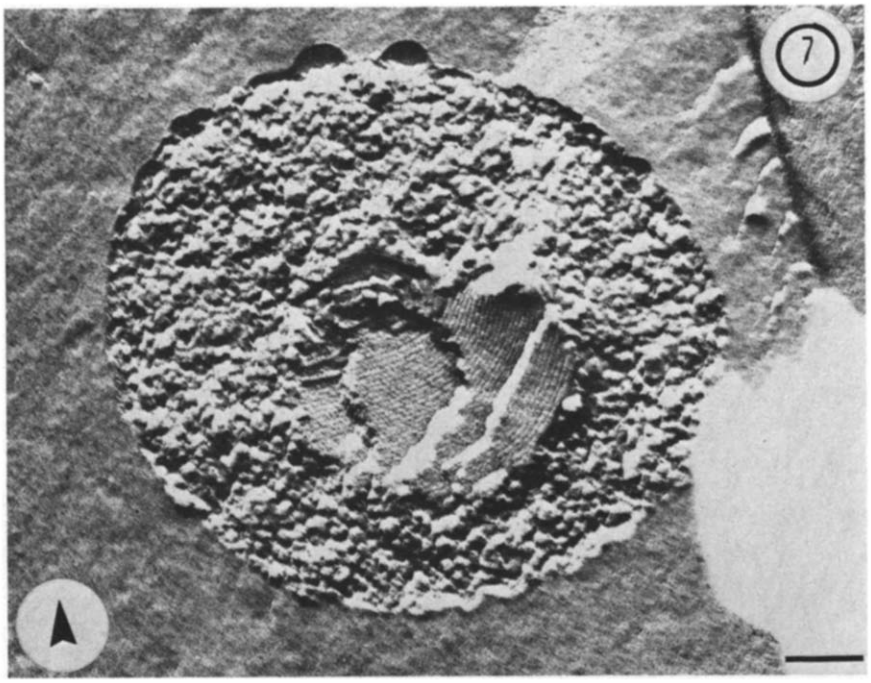


Fig. 7. After the formation of the hexagonal phase with $CaCl_2$, a 45 min incubation without $CaCl_2$ but in the presence of 1 mM EDTA. Total magnification 100 000X. Bar represents 100 nm.

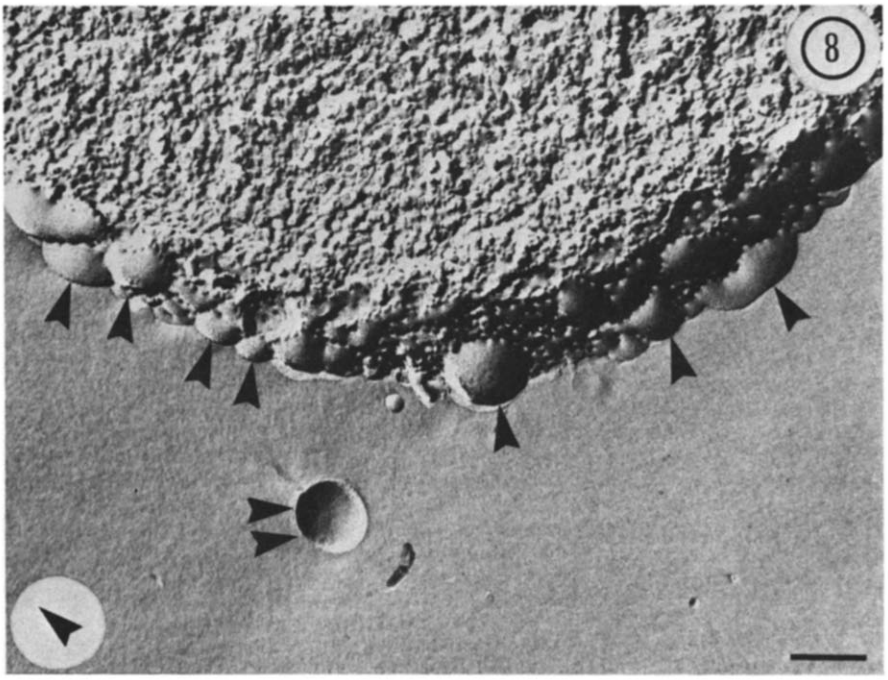


Fig. 8. Conditions as in Fig. 7 except incubation with EDTA for 90 min. Large unilamellar vesicles can be seen (at single arrows) forming at the surface. A vesicle has been released (at double arrows). Total magnification 50 000X. Bar represents 200 nm.

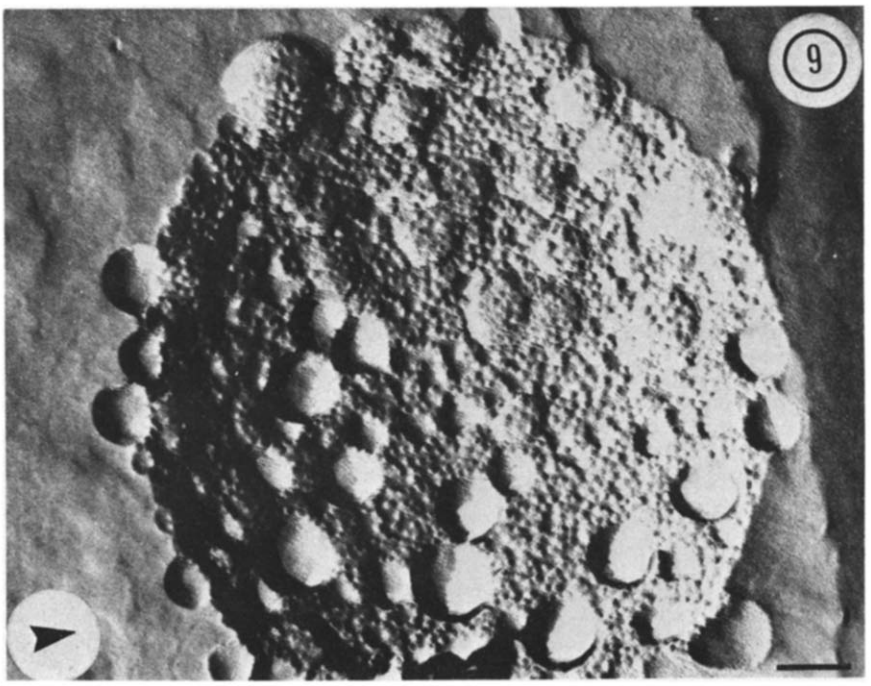


Fig. 9. Conditions as in Fig. 7 except incubation with EDTA for 135 min. Numerous large unilamellar vesicles can be seen in all stages of formation at the surface. Total magnification 50 000X. Bar represents 200 nm.

small unilamellar vesicles mediated by divalent cations. (2) Binding of the cations to the surface of the vesicles to produce invaginations. As more cations bind, tubes are produced from the invaginations which increase in length, as well as more invaginations and tubes are produced. (3) The tubes have water and cations in their centers which decreases the fluidity of the monolayer surface and has a condensing effect on this monolayer. The monolayer of the lipid on the outer side of the bilayer of the tube has low cation concentration since it was originally on the inner surface of the bilayer of the vesicle. As more cation binds to the surface adjacent to the tubes, a new tube would be formed which would be produced from the inner monolayer of the existing adjacent tube and thus form the hexagonal phase.

If the cation is removed from the center of adjacent tubes of the hexagonal structure by EDTA, vesicles are produced on the surface and released, and thus the hexagonal phase then reverts to the lamellar phase.

It is interesting to speculate to what effect cardiolipin might have as a major component of a membrane. If lateral phase separation occurs, within a biological membrane, then areas rich in cardiolipin might be produced. If in these areas hexagonal structures occur in the presence of divalent cations, namely Ca^{2+} or Mg^{2+}, membrane pores might result from the formation of the hexagonal II phase. According to Rand and Sengupta [3] the hexagonal pores for Ca^{2+} would result in 15 Å pores while Mg^{2+} would form pores of 24 Å in diameter. These pores would indeed modify the permeability of a membrane such as the inner mitochondrial membrane.

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