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CALCIUM-INDUCED FUSION OF PROTEOLIPOSOMES AND PROTEIN-FREE LIPOSOMES

EFFECT OF THEIR PHOSPHATIDYLETHANOLAMINE CONTENT ON THE STRUCTURE OF FUSED VESICLES

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Summary

The acidic phospholipid cardiolipin was shown to be very efficient in promoting calcium-induced fusion of proteoliposomes. The degree of fusion was dependent on the phosphatidylethanolamine content of the vesicles. Addition of $CaCl_2$ to proteoliposomes containing phosphatidylcholine and cardiolipin but without phosphatidylethanolamine did not induce fusion. Fusion of cytochrome oxidase vesicles, containing less than 50 mol% phosphatidylethanolamine resulted in monolamellar vesicles with a diameter of about 200 nm. The vesicles could be induced to fuse further by establishing an osmotic pressure across their membranes. When proteoliposomes containing more than 50 mol% phosphatidylethanolamine were fused, large vesicles with a diameter exceeding 1 μm were formed. They appeared in the electron microscope as a mixture of multilamellar and monolamellar vesicles. Fusion of corresponding liposomes resulted in formation of even larger structures appearing as dense multilamellar bodies and paracrystalline honeycomb-like lattices.

Introduction

Membrane fusion is a necessary event in many biological processes such as neurotransmission, secretion and phagocytosis [1]. Understanding the mechanism of fusion will no doubt contribute to research on these processes. For this

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

purpose, much research effort has been invested into elucidating the mechanism of fusion of artificial membranes such as liposomes. Recently, this area has received additional interest with the realization that membrane fusion could become a useful tool in research and therapy. Liposome-cell fusion has been explored as a means of introducing various substances trapped in vesicles into cells. A few noted examples are the microinjection of cyclic AMP into mouse cells [2] and hexosaminidase into Tay Sachs leukocytes [3].

Proteoliposomes of lipid composition similar to biological membranes seem preferable as models in research of membrane fusion. Various lipids were shown to induce fusion of artificial lipid membranes. For example, Papahadjopoulos et al. [4] have studied extensively the fusion of liposomes containing high proportions of phosphatidylserine. Ca^{2+} induced these liposomes to grow by fusion into large cochleate cylinders. Martin and MacDonald [5] have demonstrated fusion of basic liposomes containing stearylamine with cells. However, most of the lipid compositions used in these studies are not suitable for functional incorporation of membrane proteins into liposomes. High concentrations of phosphatidylserine and stearylamine [6] cause denaturation of membrane proteins. Recently, Miller and coworkers [7,8] have shown that phosphatidylserine-containing proteoliposomes can be induced to fuse by calcium. The fusion was dependent on the vesicles' curvature. The largest vesicles obtained by fusion were of a diameter of 100—200 nm. These vesicles do not fuse any further, unless an osmotic gradient across the liposome membrane is established with the internal osmotic pressure higher than the external.

In the present study, cytochrome oxidase proteoliposomes containing phosphatidylethanolamine, phosphatidylcholine and cardiolipin were used. This composition is similar to that of the inner mitochondrial membrane. The content of phosphatidylethanolamine determines both the extent of the fusion and the structure of the ensuing vesicles.

Materials and Methods

Materials. Phosphatidylcholine, lysophosphatidylcholine and cardiolipin were purchased from Sigma Chemical Co. Phosphatidylethanolamine was purified from soybean phospholipids [9]. All phospholipids used in this work exhibited single spots upon analysis by thin-layer chromatography. Cytochrome *c* oxidase was prepared from beef heart mitochondria according to a modification of the procedure described [6,10]. The enzyme was further treated to exchange the Tween-80 bound to the enzyme with cholate. For this purpose the enzyme (10 mg/ml) was dissolved in potassium cholate (1%) and precipitated with ammonium sulfate (35% saturation). This procedure was repeated twice. Mitochondrial hydrophobic protein, 'crude F_0', was prepared from beef heart as described [11,12].

Preparation of proteoliposomes and liposomes. Liposomes were prepared by drying the phospholipid solution under a stream of nitrogen gas. The phospholipids were redissolved in ether, dried and resuspended in buffer to a concentration of 25 mM phosphate. The suspension was sonicated in a bath type sonifier (80 W, 50 Hz) until samples were clear. The pH was readjusted to 7.6

whenever necessary. The buffer used throughout the work was 128 mM KCl, 32 mM NaCl, 0.5 mM EDTA in 20 mM Hepes (pH 7.6). Cytochrome *c* oxidase vesicles (1 mg protein and 20 mg lipids/ml) were reconstituted either by direct incorporation [13] or by cholate dialysis [6]. Hydrophobic protein vesicles were prepared by cholate dialysis [12].

Assays. Cytochrome *c* oxidase activity was determined polarographically essentially as described [16]. The assay medium consisted of the buffer described above, cytochrome *c* (1 mg/ml) and 20 mM ascorbic acid. Assays were made in the presence and in the absence of uncoupler (FCCP 2 μg/ml). The degree of activation by uncoupler seemed a convenient parameter of efficient proton pumping by the enzyme. This parameter was termed '% activation' and was defined as $[(O_{(+)} - O_{(-)})/(O_{(+)})\ (\times 100)]$ where $O_{(+)}$ and $O_{(-)}$ are oxygen consumption rates in the presence of uncoupler and in its absence, respectively. This parameter was preferred over the parameter 'control ratio' as it relates more directly to the efficiency of incorporation of the enzyme.

Phospholipids were determined by total ashing of samples, hydrolysis in 0.5 N HCl and phosphate analysis [14]. Vesicles and phospholipid concentrations were expressed as mM phosphate. Turbidity of suspensions was measured as the absorbance at 310 nm in a Zeiss photocolorimeter. For measurements of permeability and inner volume, the proteoliposomes were prepared in the buffer described above containing 0.08 M potassium ferricyanide. After addition of 0.12 M ascorbic acid the absorption of the ferricyanide was measured at 410 nm in a Gilford 2400 spectrophotometer. The permeability and volume of the vesicles were estimated from the initial rate of absorption reduction and the total change, respectively.

Fusion of proteoliposomes was assayed either with the electron microscope or according to the procedure described by Miller and Racker [7], in which proteoliposomes containing either hydrophobic protein (2.5 mg protein/20 mg lipid) or cytochrome oxidase (1 mg protein/20 mg lipid) were prepared by the cholate dialysis procedure. The assay of fusion is based on the fact that the hydrophobic protein, by virtue of its ability to make the liposomes permeable to protons [13] stimulates the rate of oxygen consumption catalyzed by cytochrome oxidase. The rate of oxygen consumption of cytochrome oxidase vesicles (10 μg protein/ml) was assayed with or without hydrophobic protein (25 μg/ml) vesicles. To induce fusion, $CaCl_2$ was added and after 6 min, the rate of oxygen consumption was again determined. Finally, valinomycin (1 μM) and FCCP (1 μg/ml) were added to measure the fully uncoupled rate. The extent of fusion was expressed as 'fusion factor', the fraction of cytochrome oxidase vesicles which have become uncoupled by fusion with hydrophobic protein vesicles.

Electron microscopy. Negative staining with phosphotungstic acid or ammonium molybdate of proteoliposomes containing acidic phospholipids proved unsatisfactory. Therefore, these vesicles were treated essentially as described by Castle and Hubbel [15]. Proteoliposomes were fixed with 0.5% OsO_4 for 30 min at room temperature, applied to carbon-coated collodion grids, and stained with 1% uranyl acetate in 50% ethanol for 5 min.

Samples for thin sectioning were pelleted by centrifugation at 30 000 × *g* for 30 min. The pellets were fixed overnight with OsO_4 (2%) in ice-cold buffer,

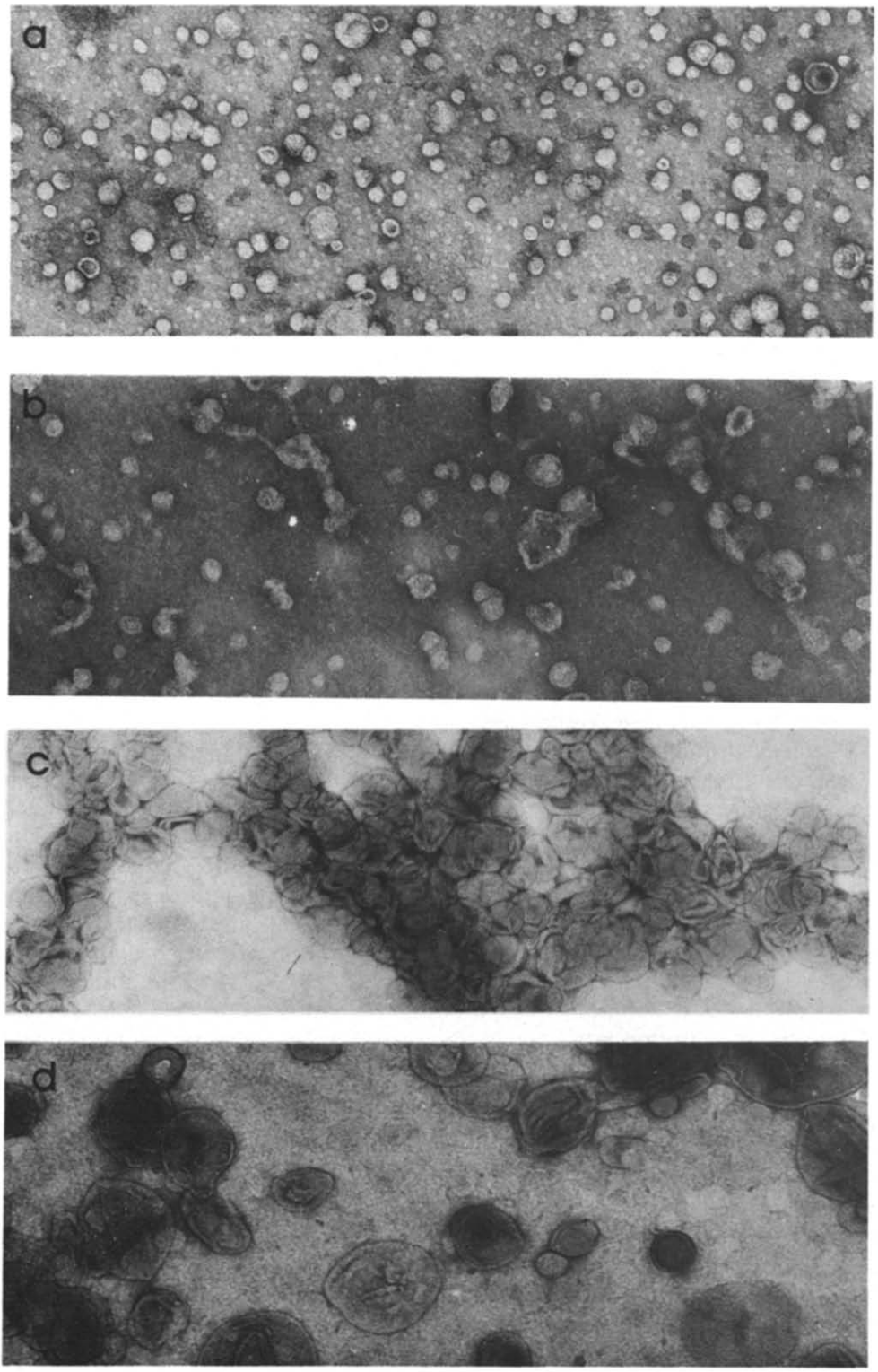
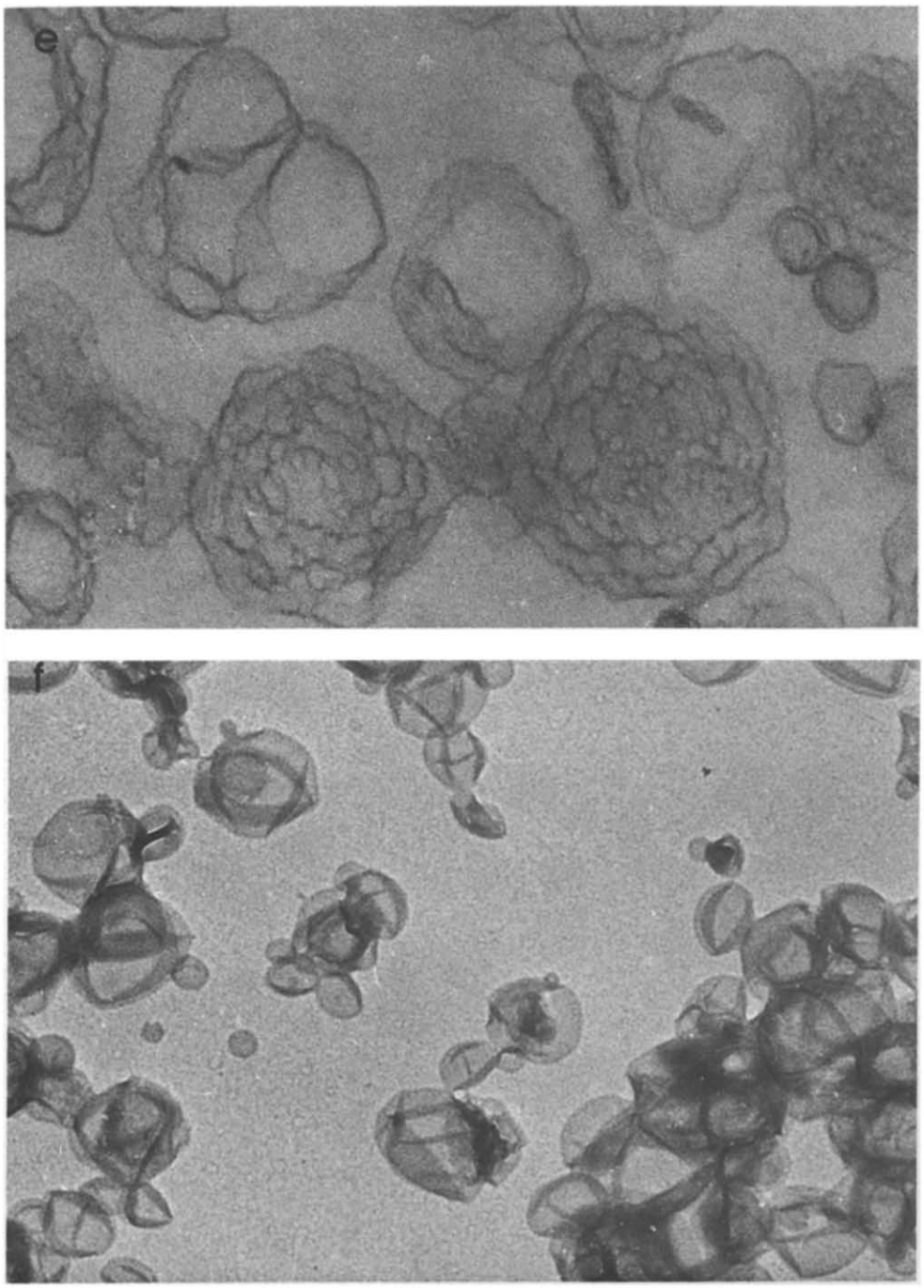
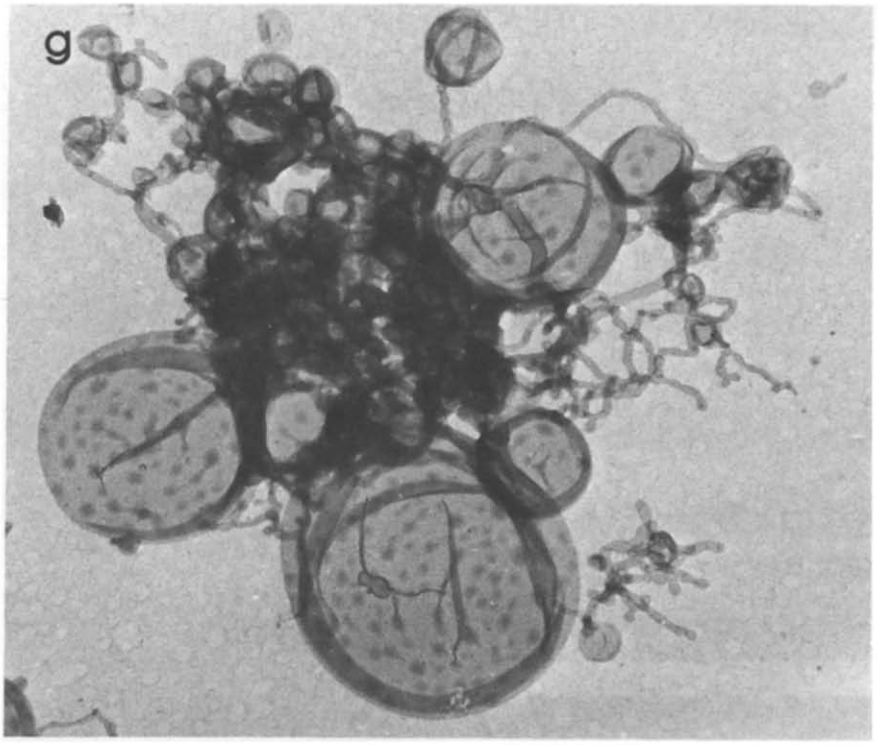


Fig. 1. Effect of phosphatidylethanolamine content on fusion of cytochrome oxidase proteoliposomes; electron microscopy. Liposomes were prepared from constant level of cardiolipin (30 mol%) and varying



ratios of phosphatidylethanolamine and phosphatidylcholine. The lipids (25 μmol P_i) were dried down, resuspended in 1 ml buffer and sonicated to clarity. Cytochrome *c* oxidase (1 mg/ml) was incorporated directly into the liposomes. Samples of the resulting proteoliposomes were diluted six fold into buffer, in the absence of 20 mM $CaCl_2$ (a) or in its presence (b—h). After incubation for 1 h at room temperature, EDTA (40 mM) was added to all suspensions. Samples were withdrawn and processed for either thin sectioning (e) or negative staining. The phosphatidylethanolamine content (in mol%) of the liposomes was: (a) 40; (b) 0; (c) 20; (d) 40; (e and f) 50; (g) 60; (h) 70. The magnification is ×100 000 in a—e and ×50 000 in f—h. For parts g and h, see next page.



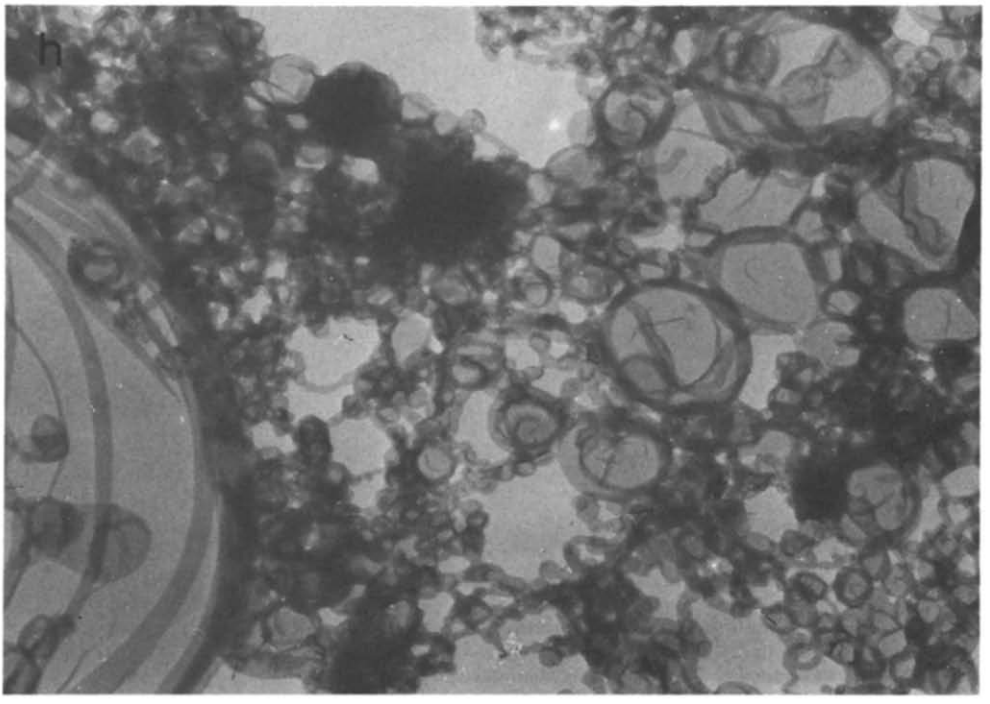


Fig. 1g and Fig. 1h.

dehydrated, embedded in Epon and sliced. The slices were stained with uranyl acetate (1.5% in ethanol) and with lead citrate 0.2% in (0.01 N NaOH).

Results

Fusion of proteoliposomes

We have studied the role of phosphatidylethanolamine in promoting fusion of proteoliposomes. For this purpose, cytochrome *c* oxidase vesicles were prepared with a constant amount of the acidic phospholipid, cardiolipin and varying ratios of phosphatidylethanolamine and phosphatidylcholine. All suspensions prepared were clear. They consisted of a rather uniform population of small unilamellar vesicles with a diameter varying between 20 and 40 nm (Fig. 1a). All the preparations were active in proton pumping as evidenced by enhancement of their oxygen consumption rate in presence of uncouplers (Fig. 2).

The effect of Ca^{2+} on proteoliposomes varied with their phosphatidylethanolamine content. Calcium had no demonstrable effect on vesicles lacking phosphatidylethanolamine. Neither fusion nor aggregation of the vesicles was observed. The size of the vesicles (as observed in the electron microscope) did not increase (Fig. 1b), and the turbidity of the vesicle suspension was only minimally changed upon addition of calcium (Fig. 2). On the other hand, upon addition of calcium to vesicles containing 10—40 mol% phosphatidylethanolamine, the suspension became turbid. The turbidity was partially dispersed by EDTA, probably through disaggregation of clumps. The remaining turbidity was due to the presence of proteoliposomes with a diameter of up to 300 nm (Figs. 1c and 3). This fusion could also be monitored by the more sensitive procedure developed by Miller and Racker [7], according to which cytochrome oxidase proteoliposomes were made to fuse with proteoliposomes containing mitochondrial hydrophobic protein. Upon formation of liposomes containing both proteins, the latter protein, which has been shown to form a proton channel, stimulated the cytochrome oxidase activity (Fig. 2). The fusion, assayed as stimulation of proton pumping activity, could be prevented by first fusing each type of vesicle separately. Prefusion of only one of the proteoliposome types caused only partial inhibition. Thus fusion of vesicles containing low amounts of phosphatidylethanolamine is dependent on their curvature. This is similar to the phenomenon reported by Miller et al. [8] for vesicles containing phosphatidylserine and high amounts of phosphatidylethanolamine.

Both type of vesicles could be induced to undergo further rounds of fusion by establishing an osmotic gradient across the liposome membrane, with the internal osmotic pressure higher than the external (Fig. 4 and Miller et al. [8]). The resulting cardiolipin-containing proteoliposomes appeared multilamellar and had diameters exceeding 1 μm.

Alternatively, such large structures could be produced by fusion of proteoliposomes containing phosphatidylethanolamine in amounts exceeding 50 mol%. Addition of calcium to such proteoliposomes resulted in the formation of a highly turbid suspension, which was only partially cleared by EDTA. Observation by the negative-staining technique revealed a heterogeneous

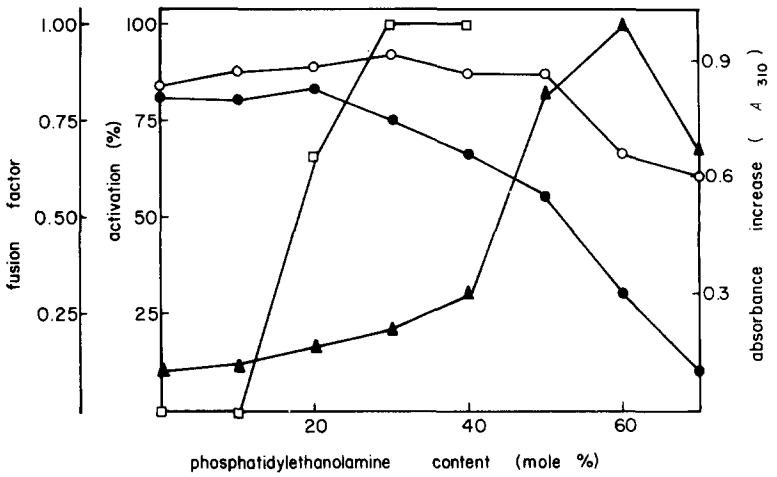


Fig. 2. Effect of phosphatidylethanolamine content on fusion of cytochrome oxidase; biochemical parameters. Cytochrome oxidase proteoliposomes were prepared as described in legend of Fig. 1. In addition, similar proteoliposomes containing mitochondrial hydrophobic protein (2.5 mg/ml) were produced. The effect of $CaCl_2$ was studied as in Fig. 1. The final samples were diluted to phospholipid concentrations of 0.1 mM in buffer and were assayed for percent activation (○, without $CaCl_2$; ●, with $CaCl_2$) and for turbidity of the suspension at 310 nm (▲). The turbidity results are the difference between the measurements with and without $CaCl_2$. Fusion was assayed by the procedure of Miller and Racker [7] as described under Materials and Methods. The fusion factor could not be determined with vesicles containing more than 40 mol% phosphatidylethanolamine, as $CaCl_2$ alone caused severe uncoupling of cytochrome oxidase proteoliposomes.

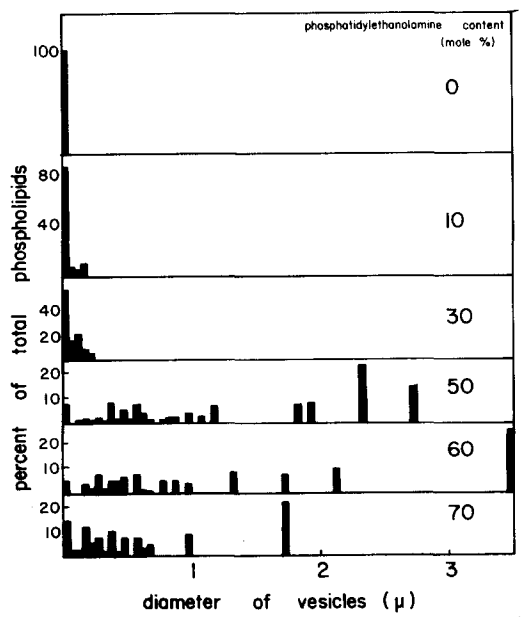


Fig. 3. Size distribution of vesicles formed by Ca^{2+}-induced fusion of proteoliposomes. Experimental conditions as in Fig. 1. The diameters of vesicles were measured in electron micrographs of negatively stained vesicles. The fraction of the total phospholipids present in each size group was calculated as follows: the amount of phospholipids was assumed to be equivalent to the area of the vesicles. The area was calculated with the assumption that they are single walled. As this is not the case, the area of the larger vesicles is underestimated.

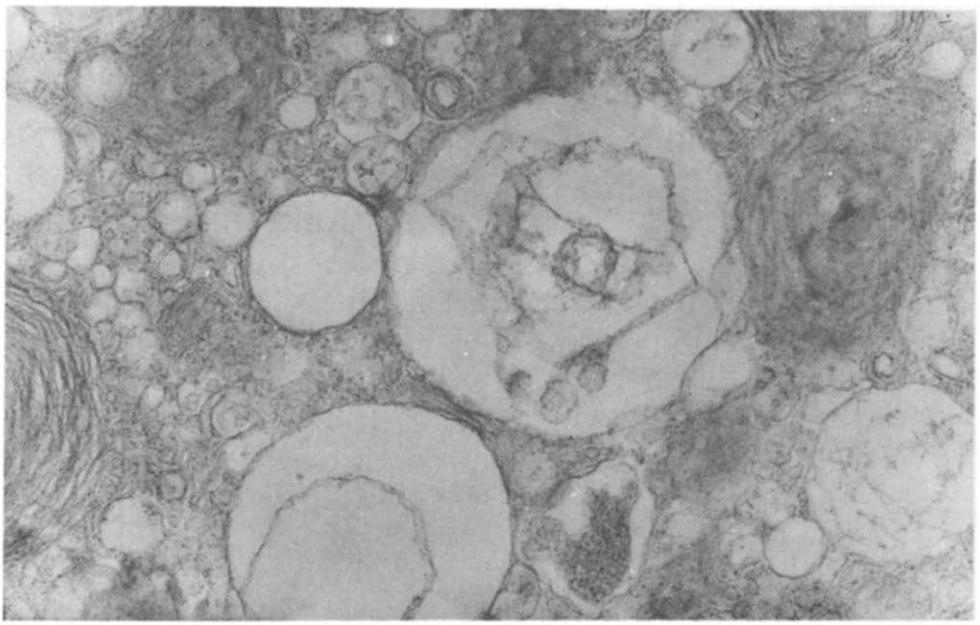


Fig. 4. Osmotically promoted fusion; electron microscopy. Liposomes were prepared from cardiolipin (40 mol%), phosphatidylcholine (45 mol%) and phosphatidylethanolamine (15 mol%). The lipids (25 μmol) were dried down, resuspended in 1 ml buffer containing 0.3 M sucrose and sonicated to clarity. Cytochrome oxidase (1 mg/ml) was incorporated into the liposomes. The vesicles were incubated at a concentration of 4 mM P_i in the same medium in the presence of $CaCl_2$ (20 mM). After 1 h at room temperature an equal amount of buffer was added, thereby diluting the sucrose in the medium. $CaCl_2$ was added to adjust its concentration and samples were processed for thin sectioning after an additional 1 h incubation.

population of vesicles with most of the phospholipid material present in vesicles with diameters exceeding 1 μm. A small fraction of the proteoliposomes remained with diameters of 30—50 nm. Thin sectioning of the same samples (Fig. 1e) revealed the picture observed by negative staining to be misleading. Some of the vesicles appearing after thin sectioning mainly as smooth round collapsed sacs, were actually complex structures of either multilamellar vesicles or aggregates of smaller vesicles trapped in large monolamellar structures. Some of the vesicles appeared as single-walled large proteoliposomes.

Fusion of these cytochrome oxidase vesicles, rich in phosphatidylethanolamine resulted in apparent loss of proton pumping capacity. The activity of these vesicles assayed in the absence of uncouplers increased sharply with fusion and was enhanced only to a small extent by their addition (Fig. 2). However, the maximal activity assayed in the presence of uncouplers was not affected appreciably by the fusion.

The inner volume and permeability of the vesicles were studied before and after fusion by trapping potassium ferricyanide in them and monitoring both the rate and the extent of its reduction by external ascorbic acid. The vesicles' permeability increased sharply upon addition of calcium while the trapped volume increased only 2—10 times. Addition of EDTA did not affect the trapped volume, but decreased the permeability to 2—3 times the permeability of unfused vesicles.

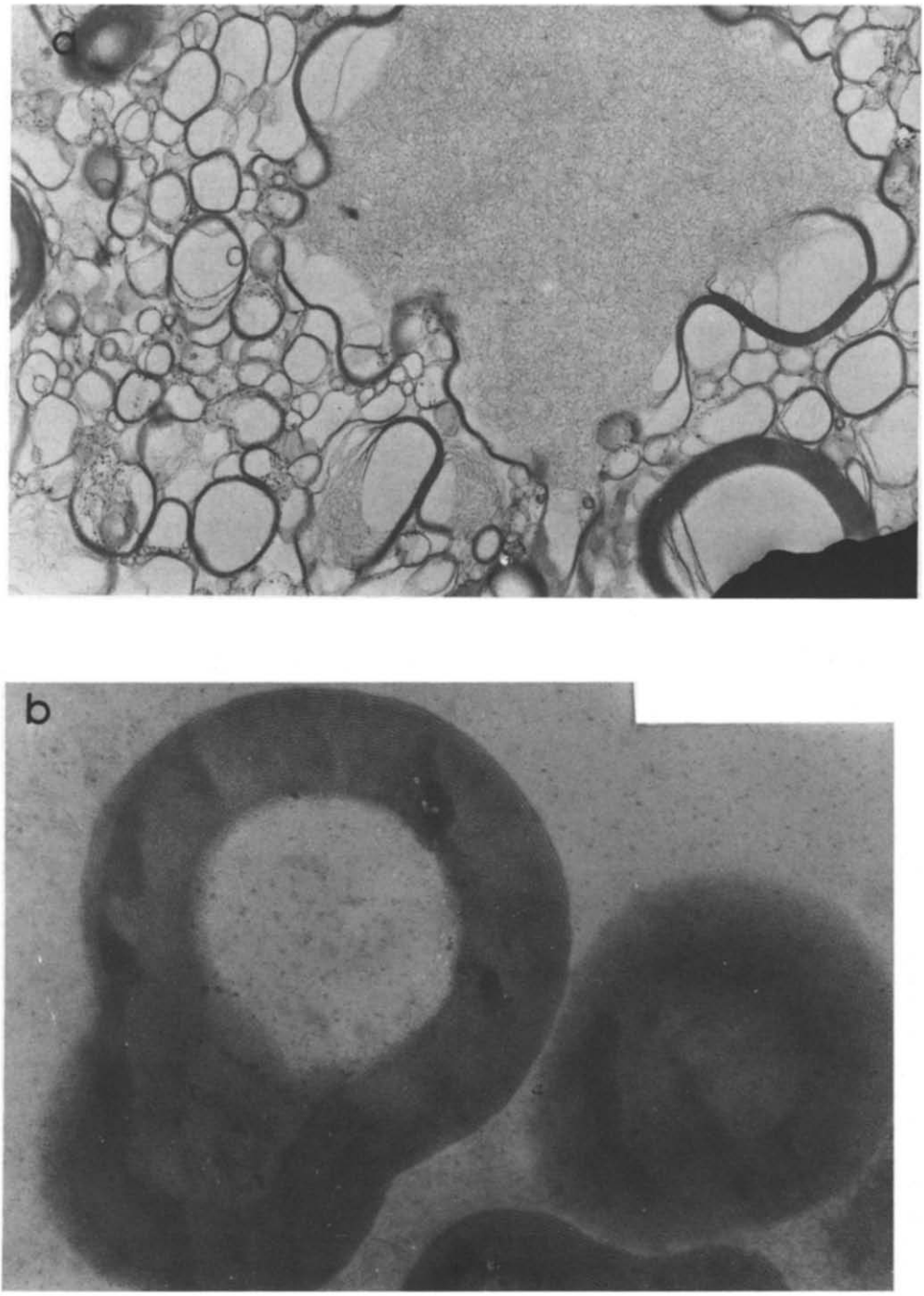
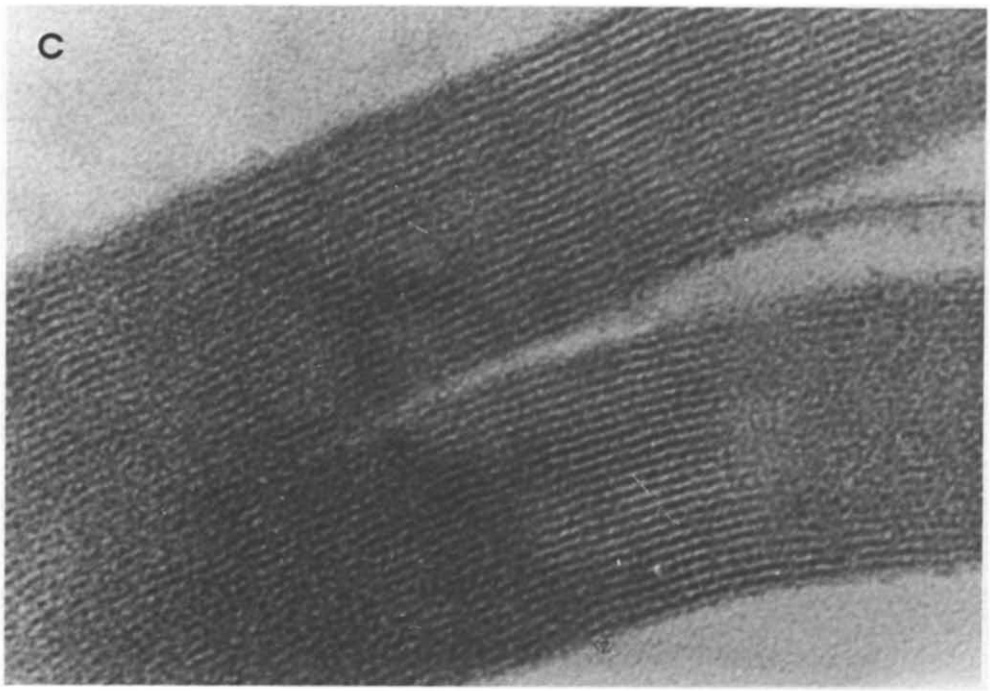


Fig. 5. Fusion of protein-free liposomes. Liposomes containing phosphatidylethanolamine, phosphatidylcholine and cardiolipin (60, 10 and 30 mol%, respectively) were prepared as described under Materials and Methods. The liposomes (4 mM) were incubated for 1 h at room temperature in presence of $CaCl_2$ (20 mM). Samples were processed for thin sectioning either directly (a—c) or after addition of 40 mM EDTA and further incubation for 20 min (d—f). The magnifications in the pictures are: (a) X17 500; (b) X140 000; (c) X440 000; (d) X60 000; (e) X100 000; (f) X300 000. For part f see page 192.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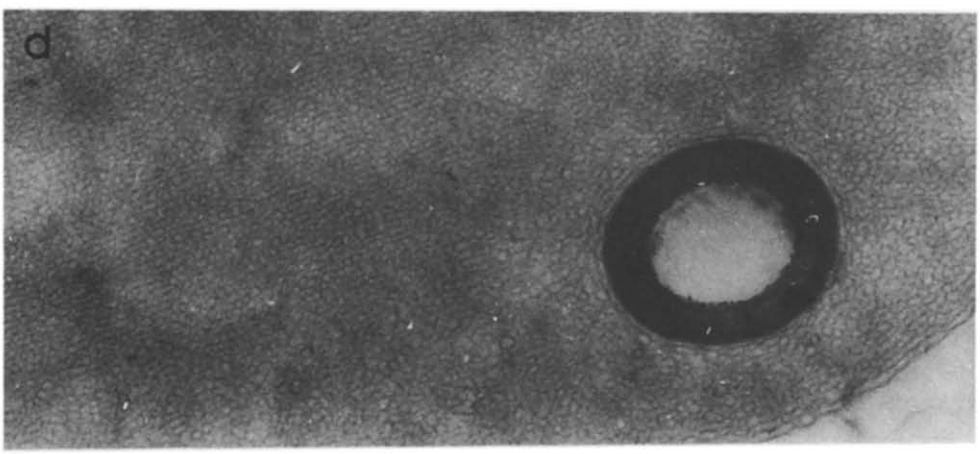
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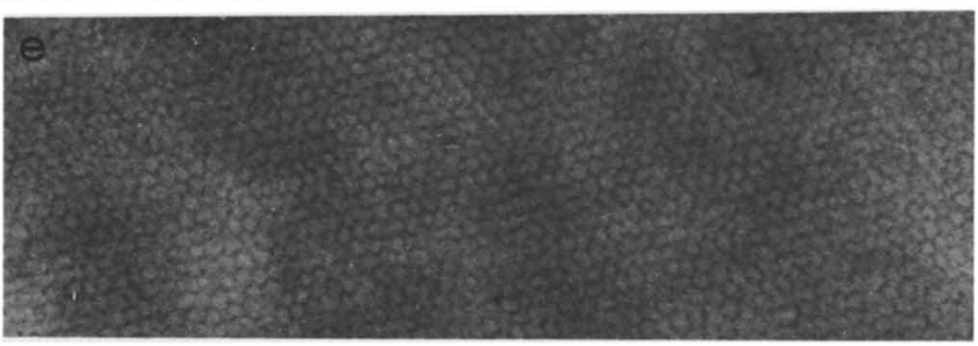
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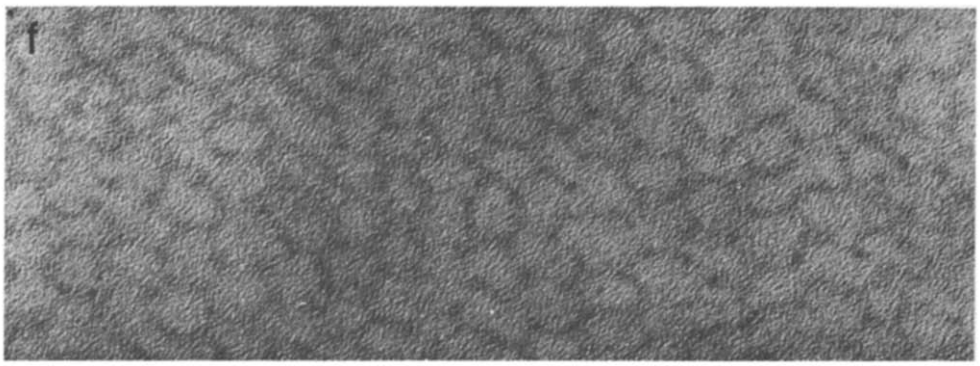

Fig. 5f.

The fusion proceeded rapidly at room temperature and was virtually over within 20 min. There was a positive correlation between the increase in the turbidity of the proteoliposome suspension, their size as measured by the electron micrographs and the loss of activation capacity by uncouplers. The fusion was a temperature-sensitive phenomenon, it proceeded slowly at 10°C and was virtually abolished at 0°C. The growth of the proteoliposomes by fusion was totally dependent on metal ions. In their absence, the proteoliposomes' size and activity were stable for days. The Ca^{2+} concentration affected mainly the final size of the vesicles and not the rate of the fusion process. The calcium concentration required for maximal effect was about 15 mM. Lysophosphatidylcholine, known to promote fusion of cells, was checked for its effect on calcium-induced fusion of proteoliposomes. Instead of promoting fusion, lysophosphatidylcholine actually inhibited it. Its presence in the proteoliposomes inhibited the size increase of the vesicles, the appearance of turbidity, and the loss of activation capacity. Lysophosphatidylcholine improved the incorporation of cytochrome oxidase into the liposomes. The activation by uncouplers of the enzyme incorporated into lysophosphatidylcholine-containing liposomes was more pronounced in both fused and unfused vesicles.

Fusion of protein-free liposomes

In order to assess the effect of the enzyme incorporated into the vesicles on their fusion, we have studied the fusion of protein-free liposomes. The fusion of these liposomes was also dependent on both phosphatidylethanolamine content and Ca^{2+}. Protein-free liposomes containing low phosphatidylethanolamine concentration behaved similarly to proteoliposomes of the same lipid composition upon addition of Ca^{2+}. A distinct effect was noted when liposomes of high phosphatidylethanolamine content were fused. Upon addition of Ca^{2+}, the liposomes aggregated to form clumps, visible to the naked eye, which precipitated within 2 min. The aggregate could be partially dispersed by EDTA. Electron microscopy revealed that these fused vesicles were larger in diameter than the corresponding fused proteoliposomes. Observation of thin sections revealed a variety of forms (Fig. 5). The most outstanding was a multilamellar dense structure with a repeating unit of 4.5 nm consisting of a 2 nm dense band and a 2.5 nm lighter band. These compact structures were only partially disrupted by EDTA. In addition, one could observe large aggregates of small

vesicles and large monolamellar vesicles with diameters of up to 5 μm. When the liposomes were fixed after treatment with EDTA, the prevalent structure was a honeycomb-like lattice (Fig. 5). The size of the repeating hexagonal unit was about 20 nm. A similar structure was observed occasionally also before the addition of EDTA.

Discussion

Cardiolipin, the acidic phospholipid employed throughout the work, seems most suitable for fusion of proteoliposomes. It has been shown to be a most efficient phospholipid both for incorporation of several membrane proteins [6,16], and for promoting fusion of proteoliposomes. Miller and Racker [7] have shown that the phosphatidylserine-promoted fusion of proteoliposomes is dependent on the presence of phosphatidylethanolamine in the vesicles. We have observed a similar effect with cardiolipin-promoted fusion. In the absence of phosphatidylethanolamine, cardiolipin, at concentrations suitable for functional incorporation, does not induce fusion or significant aggregation. Vesicles with phosphatidylethanolamine ratios of up to 40 mol% are induced by Ca^{2+} to form proteoliposomes with a diameter of 100—200 nm. This fusion process is dependent on membrane curvature. Larger vesicles (greater than 1 μm) were formed upon fusion of proteoliposomes containing higher phosphatidylethanolamine ratios. Thus, a combination of cardiolipin with high levels of phosphatidylethanolamine induced the most rapid and extensive fusion of proteoliposomes. Liposomes containing higher concentrations of acidic phospholipids were not suitable for proteoliposome fusion, as their dense negative charge denatured proteins incorporated into them.

Cardiolipin probably promotes fusion by a mechanism similar to that proposed for phosphatidylserine or phosphatidic acid [4,17,18]. However, cardiolipin is more efficient and this might relate to its being the only acidic phospholipid undergoing bilayer-hexagonal shift upon addition of divalent ions [19]. This hexagonal structure may promote fusion of adjacent membranes, by destabilizing the structure of the original membrane.

Phosphatidylethanolamine may play an active role in destabilization of the membrane. It has been reported [20,21] that phosphatidylethanolamine undergoes transition from bilayer to hexagonal arrangement, even in the presence of up to 45 mol% phosphatidylcholine. Since the phosphatidylcholine content of the vesicles employed in this work is low, it is obvious that upon crystallization of the cardiolipin, the phosphatidylethanolamine left in the fluid phase undergoes a shift to hexagonal arrangement. In presence of divalent ions, cardiolipin forms a crystalline domain leaving behind a fluid phase, containing the remainder of the lipids.

The multilamellar structures obtained by fusion of cytochrome oxidase vesicles are probably cochleate in nature rather than consisting of onion-skin structures. One expects that in the onion-skin structures only a minor part of the proteins and lipids would be exposed to the medium. However, the fusion of the proteoliposomes did not cause a reduction in exposed amino groups or loss of cytochrome oxidase activity.

Protein-free liposomes containing cardiolipin and high phosphatidylethanol-

amine concentrations fuse to form various large structures, such as an aggregate of small vesicles, a dense multilamellar large structure and a paracrystalline honeycomb-like body. The dense multilamellar structure has a repeating unit of 4.5 nm and is reminiscent of the phosphatidylcholine pulmonary surface lubricant structures [22] and artificially produced dense structures obtained by hydrating saturated phosphatidylcholine [22,23]. Papahadjopoulos et al. [24] have observed that upon fusion of pure phosphatidylserine liposomes large cochleate cylinders were formed. The latter could be unfolded by chelating reagents to form large flattened sacs. The dense structure observed by us might be also cochleate structures. However, in our hands, the multilamellar structures were only partially disrupted by chelating the Ca^{2+} with EDTA.

The most striking structure obtained by fusion of protein-free liposomes is a honeycomb-like lattice. This structure appears to be a condensation and organization product of the aggregates of small vesicles also observed as fusion products. The honeycomb-like structure could be the result of artefactual vesicularization and organization of multilayer vesicles during the preparation of the samples for observation in the electron microscope. Doggenweiler and Heuser [25] have reported that fixation with osmium tetroxide caused vesicularization of myelin sheath membranes. We do not favor this possibility, as prefixation with glutaraldehyde not only did not abolish the honeycomb-like structures, but actually seemed to preserve them better. Glutaraldehyde cross-links amino groups and thus is expected to prevent reorganization of membranes containing 60 mol% phosphatidylethanolamine. Furthermore, similar honeycomb-reminiscent structures have been observed in hydrated phosphatidylcholine [22] and phosphatidylethanolamine samples [20].

The presence of cytochrome oxidase in liposomes has a dual effect on their fusion, it inhibits to some extent the growth of the vesicles and it interferes with the formation of dense structures such as the multilamellar body or the honeycomb-like lattice.

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