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'Lipidic particle' systems as visualized by thin-section electron microscopy

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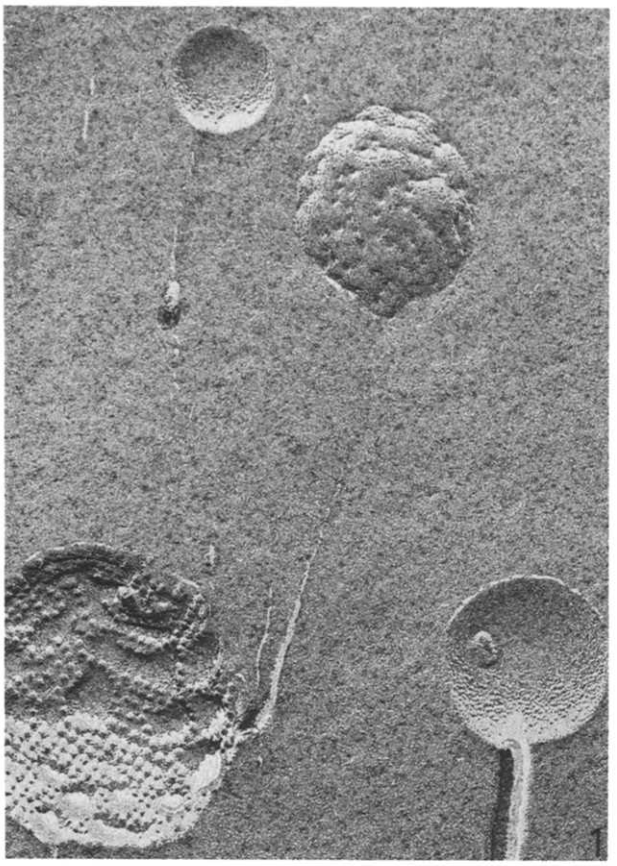
Lipidic phases, containing 'lipidic particles' (cardiolipin/egg lecithin in the presence of Ca^{2+} and egg lecithin/dioleoylphosphatidylcholine/cholesterol) have been investigated with an alternative thin-section method, using rapid cryofixation and freeze substitution according to Müller et al. (Müller, M., Marti, T. and Kriz, S. (1980) in Proc. 7th Eur. Congr. on Electron Microscopy (Brederoo, P. and De Priester, W., eds.), pp. 720–721) in combination with low temperature embedding in Lowicryl HM20 according to Humbel et al. (Humbel, B. and Müller, M. (1984) in Proc. 8th Eur. Congr. on Electron Microscopy (Csanady, P., Röhlich, P. and Szabo, D., eds.), pp. 1789–1798).With this method one can visualize a honeycomb structure with local fusion (joining) points between bilayers which is compatible with the structures hypothesized for lipidic particle-containing systems.

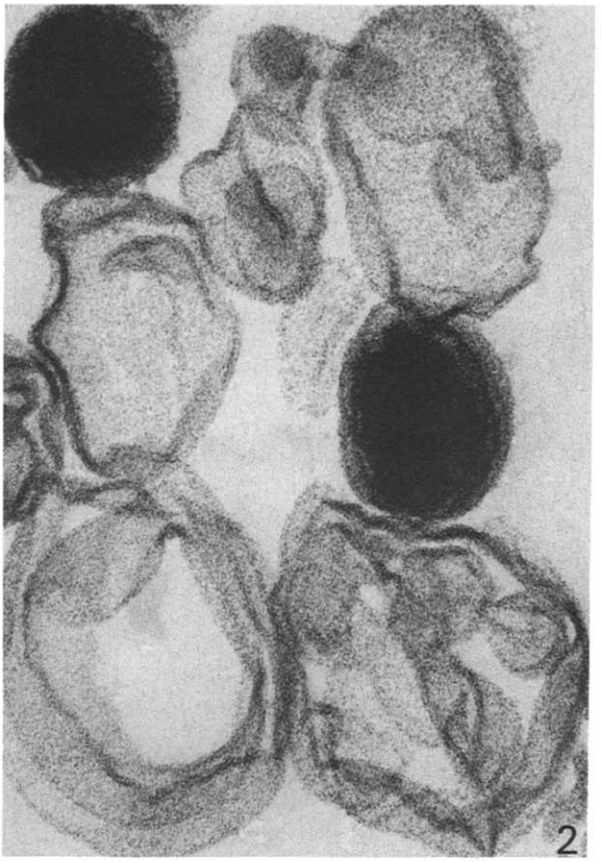
Lipid intramembraneous particles have been found in a variety of lipid mixtures and in total lipid extracts under physiological conditions in excess water (see for review Ref. 3). A common feature of these systems is that at least one of the lipids prefers to adopt the hexagonal H_{II} phase, when dispersed in pure form. It is now the general consensus that these lipidic particles are strongly associated with membrane fusion or, to be more precise, with certain stages of fusion [3]. So it is hypothesized that the well-defined particles reflect inverted micelles at the nexus of two joining membranes. If one starts off with a multilamellar structure, conditions which promote the conversion of lamellar to H_{II} phase will lead to multiple fusion points of lipidic particles between the different bilayers, which leads to a 'honeycomb' structure [4]. Such a 'honeycomb' structure is also compatible with ^{31}P-NMR results which show an isotropic spectral component in these lipid systems [5]. This isotropic signal which is characteristic for phospholipids undergoing rapid motion, contains contributions from lipids organized in the lipid particles but most likely also originates from lipid molecules diffusing laterally along the highly curved bilayers with which these particles are associated [5].

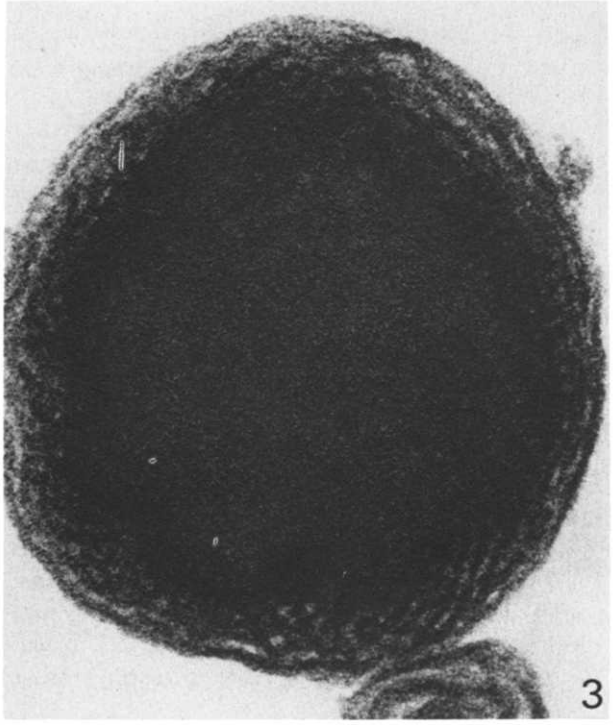
In this report we have investigated some of the systems which exhibit multiple lipidic particles with freeze-fracture electron microscopy, by means of thin-sectioning after cryofixation and freeze substitution according to Müller and co-workers [1,6] in combination with low temperature embedding [2] in Lowicryl HM20 [7].

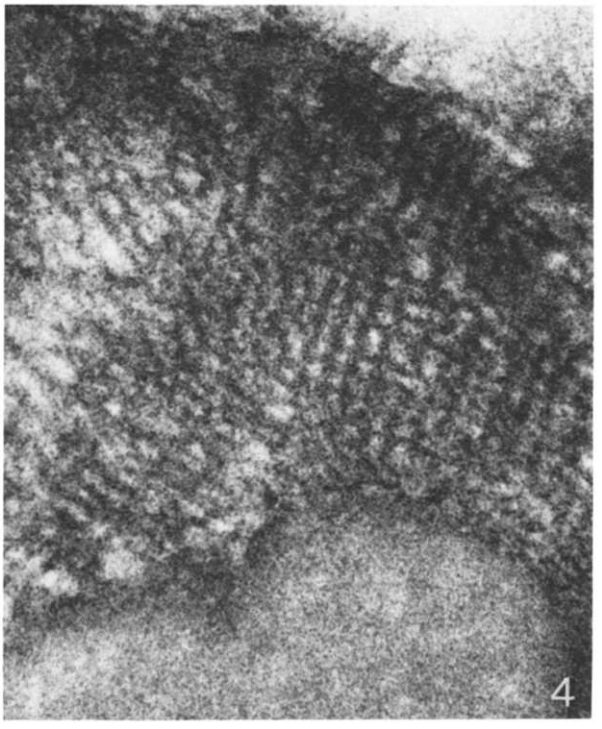
Cardiolipin was purchased from Sigma (St. Louis, U.S.A.) and cholesterol from Fluka (Buchs, Switzerland). Egg phosphatidylcholine (egg PC) was isolated from hen eggs and 1,2-dioleoyl-*sn*-glycerol-3-phosphorylcholine ($18:1_c/18:1_c$-phosphatidylcholine or DOPC) were synthesized as described before [8]. All lipids were chromatographically pure. Lipid dispersions were made by dispersing the lipid mixture at 30°C, in 15 μmol lipid in 1.0 ml 100 mM NaCl/0.2 mM EDTA/10 mM Tris-HCl (pH 7.0) buffer.

The specimen were frozen in a propane jet









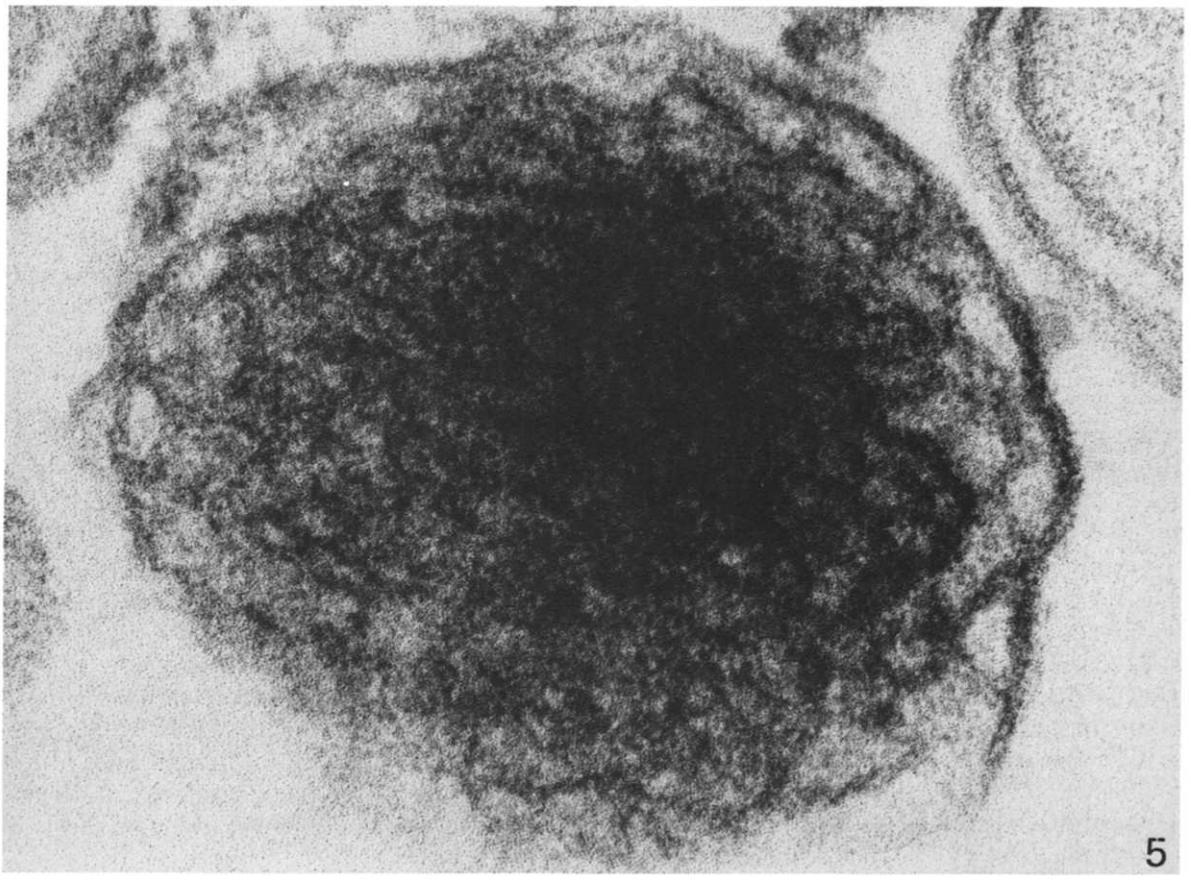


Fig 1–5 Electron micrographs of an equimolar mixture of cardiolipin/egg lecithin after the addition of an aliquot of 100 mM $CaCl_2$ solution (final Ca^{2+} concentration in the sample 10 mM). Freeze-fracturing (Fig 1) and thin sectioning of cryofixed and freeze substitution according to Muller et al [1] and Humbel et al [2] (Figs 2–5) Magnification (1) 100000×, (2, 3 and 4) 170000× and (5) 350000×.

without the addition of cryoprotectants according to Müller et al. [6,9] and freeze-fractured in a Balzers BAF 300 according to standard procedures. For thin-sectioning, identically cryofixed samples were fractured under liquid nitrogen and immersed into the substitution medium consisting of methanol with 1% OsO_4, 0.5% UO_2Ac, 3% glutaraldehyde and 3% water. After the sample was left for 8 h each at −95°C, −60°C and −30°C, the substitution medium was replaced by pure methanol. Low temperature embedding in the hydrophobic HM20 resin [7] and ultraviolet polymerization were performed at −30°C using the equipment outlined in Humbel et al. [2]. Thin sections were stained with uranyl acetate and lead citrate.

In the absence of Ca^{2+} an aqueous dispersion of an equimolar mixture of egg phosphatidylcholine and cardiolipin consists of bilayers as evidenced by freeze-fracture electron microscopy and ^{31}P-NMR. Addition of increasing amounts of Ca^{2+} to the sample leads to the appearance of isotropic signal and hexagonal H_{II} type spectra with ^{31}P-NMR and the appearance of lipidic particles and corresponding pits (see Fig. 1). Also hexagonal II phase and 'cubic phase' of stacked particles arrays were found as described before [10] (not shown).

Thin sectioning of these samples according to the classical procedure (fixation with glutaraldehyde and OsO_4, dehydration, embedding in epon or araldite, staining with uranyl acetate and lead citrate) did not show any interpretable images.

The reason for this is likely due to the fact that most of the lipids are in fact extracted during the dehydration step. Thin sections of rapidly frozen, freeze-substituted and low temperature embedded samples, however, show clearly defined images [11–14]. The reason for this is most probably the fact that during the freeze substitution with methanol, especially in the presence of uranyl acetate, there is hardly any loss of phospholipids as found by thin-layer chromatography and phosphorus analysis (not shown). Figs. 2–5 show an equimolar mixture of cardiolipin and egg PC, upon addition of 10 mM Ca^{2+}. One can observe multilamellar structures with more than one triple-layered structures or bilayers next to the structures where the triple-layer forms a network in which there are many contact, joining or fusion points. In the centre of these network-like structures one frequently encounters homogenously stained material without defined structural details (Figs. 3 and 4). The latter material most probably represents the hexagonal II phase and/or the 'cubic' phase of stacked particles. In the absence of Ca^{2+} only multilamellar structures similar as in Fig. 2 and as shown in Ref. 14 are present.

We have also investigated a mixture of DOPE/egg PC/cholesterol (3 : 1 : 2, molar ratio), which by means of freeze-fracture exhibits lipidic particles, hexagonal II phase and the cubic phase of stacked particles [10,15]. By thin sectioning of freeze-substituted samples as described in the Experimental section, we obtained similar honeycomb structures and homogeneously stained material as found for cardiolipin/egg lecithin in the presence of Ca^{2+} (as shown in Figs. 2, 3, 4 and 5).

The most interesting result of this study is that we are able to visualize in an alternative way the lipid phases which exhibit 'lipidic particles'. The network-like structure is in fact compatible with the honeycomb structure with 'arrested fusion points' as suggested from the freeze-fracture images of this structure [3,4]. One might argue that during the substitution the original structure has been altered and the visualization by positive contrast is a matter of interpretation. However, the fact that phospholipids are not extracted during the freeze substitution and since this structure with local fusion points between the different bilayers is compatible with the freeze-fracture structure is strongly favouring the reality of the structure as visualized by this alternative thin section technique using freeze substitution. In that light this technology is possibly a more reliable method than conventional thin sectioning for the visualization of the lipid organization in biological and model membranes.

At last it has to be noted that the honeycomb structures seen by this method strongly resemble some biological structures, i.e. tubular myelin [16] and the prolamellar body in the etioplast [17] (see for discussion Ref. 3).

References

1 Muller, M., Marti, T. and Kriz, S. (1980) in Proc. 7th Eur. Congr. on Electron Microscopy (Brederoo, P. and De Priester, W., eds.), pp. 720–721, Leiden
2 Humbel, B. and Muller, M. (1984) in Proc. 8th Eur. Congr. on Electron Microscopy (Csanady, P., Rohlich, P. and Szabo, D., eds.), pp. 1789–1798, Budapest
3 Verkleij, A.J. (1984) Biochim. Biophys. Acta 779, 43–63
4 Cullis, P.R., De Kruijff, B., Hope, M., Verkleij, A.J., Nayar, R., Farren, S.B., Tilcock, C., Madden, T.D. and Bally, M.B. (1984) in Membrane Fluidity in Biology (Aloia, V., ed.), Chapter 2, Vol. 1, pp. 39–81, Academic Press, New York
5 Cullis, P.R., De Kruijff, B., Hope, M.J., Nayar, R. and Schmidt, S.L. (1980) Can. J. Biochem. 58, 1091–1100
6 Muller, M. and Moor, H. (1984) Proc. 42nd Ann. Meeting of the Electron Microscopy Society of America, pp. 6–9, San Francisco Press, San Francisco
7 Carlemalm, E., Garavito, R.M. and Villeger, W. (1982) J. Microsc. (Oxford) 126, 123–143
8 Van Deenen, L.L.M. and De Haas, G.H. (1964) Adv. Lipid Res. 2, 168–229
9 Muller, M., Meister, N. and Moor, H. (1980) Mikroskopie 36, 129–140
10 Van Venetie, R. and Verkleij, A.J. (1981) Biochim. Biophys. Acta 645, 262–269
11 Muller, M. (1981) in Membrane Proteins (Azzi, A., Brodbeck, U. and Zahler, P., eds.), pp. 252–256, Springer Verlag, Berlin
12 Zumbuehl, O. and Weder, H.G. (1981) Biochim. Biophys. Acta 640, 252–262
13 Schwendener, R.A., Asanger, M. and Weder, H.G. (1981) Biochem. Biophys. Res. Commun. 100, 1055–1062
14 Hauser, H., Gains, N. and Muller, M. (1983) Biochemistry 22, 4775–4781
15 De Kruijff, B., Verkleij, A.J., Van Echteld, C.J.A., Gerritsen, W.J., Mombers, C., Noordam, P.C. and De Gier, J. (1979) Biochim. Biophys. Acta 555, 200–209
16 Chi, E.Y. and Lagunoff, D. (1979) Proc. Natl. Acad. Sci. USA 75, 6225–6229
17 Simpson, D.J. (1978) Carlsberg Res. Commun. 43, 145–170