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LIPIDIC INTRAMEMBRANOUS PARTICLES

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I. Introduction

In 1979 we described for the first time the phenomenon of 'lipidic intramembranous particles' or 'lipidic particles' in model membranes [1]. These particles have been called 'lipidic particles' since they were found in pure lipid systems, and thus must originate from a specific lipid organization. Such lipidic particles have been found in a variety of lipid mixtures and in total lipid extracts under physiological conditions in excess water [2–8]. A common feature of these lipid systems is that at least one of the lipids prefers to adopt the hexagonal II (H_{II}) phase when dispersed in pure form.

Abbreviations: PC, Phosphatidylcholine; DOPC, dioleoylphosphatidylcholine; PE, phosphatidylethanolamine; DOPE, dioleoylphosphatidylethanolamine; PS, phosphatidylserine; MGlucDG, monoglucosyldiacylglycerol; DGlucDG, diglucosyldiacylglycerol; MGalDG, monogalactosyldiacylglycerol; DGalDG, digalactosyldiacylglycerol.

In this review I will try to evaluate all the data concerning the phenomenon of 'lipidic particles'. Since it is clear that these particles are closely related to the H_{II} phase and are intermediary structures in the transition from lamellar to H_{II} phase, I will first summarize these aspects. I will then analyze the lipidic particles in more detail, including their occurrence, their morphological appearance, and their distribution on the complementary fracture faces as well as their lateral distribution. Furthermore, I will evaluate the various interpretations with respect to the molecular organization of the lipids in the lipidic particles and their possible involvement in membrane fusion and other processes. Finally, I will discuss the possible relevance of the lipidic particles and related structures for membrane biology.

II. The hexagonal II phase

There is quite a variety of mixtures of both synthetic and naturally occurring lipids in which 'lipidic particles' have been found [1–8]. In principle, the 'lipidic particles' and related structures have to be envisaged as intermediary structures between the lamellar and H_{II} phase [9]. The H_{II} phase was first recognized by X-ray diffraction [10–12] but can also be detected by ^{31}P-NMR [9] and visualized by freeze-fracture electron microscopy [13]. The H_{II} phase, consisting of lipid cylinders with a hydrophilic core, gives rise to distinct fracture planes (Fig. 1) which follows the apolar interfaces and results in a fracture face with a ribbed appearance. Three fracture planes at angles of 60° to each other can be found. If the H_{II} cylinders are cross-fractured, the fracture faces have a particulate appearance. The bilayer phase, on the other hand, exhibits smooth fractured faces except the $p\beta'$-phase found for example in synthetic phosphatidylcholine [14–16]. Liposomes consisting of concentric bilayers show alternating smooth faces as the fracture plane jumps from one bilayer to another (Fig. 1).

IIA. Hexagonal II phase lipids

There are many lipids which adopt the H_{II} phase at physiological temperatures, but prefer the bilayer phase at lower temperatures. For example, unsaturated phosphatidylethanolamine (PE) from eukaryotic cells [17,18] and *E. coli* [19], monogalactosyldiacylglycerol (MGalDG) from chloroplasts [20] and monoglucosyldiacylglycerol (MGlucDG) from *Acholeplasma laidlawii* [3,21] adopt the H_{II} phase at physiological temperatures. The tendency of other naturally occurring lipids, like cardiolipin [13,22,23] and phosphatidic acid [8,24], to form the H_{II} phase is dependent on pH and the presence of divalent cations. These negatively charged phospholipids can also adopt the H_{II} phase in the presence of the local anaesthetics dibucaine and chlorpromazine [8,23]. Cardiolipin adopts the H_{II} phase in the presence of cytochrome *c* [25,26]. Moreover, the H_{II} phase can be induced by gramicidin [27,28], can be modulated in the presence of cholesterol [29], appears in complex lipid mixtures of synthetic lipids [9] and lipid extracts [19,30,31] from biological membranes.

The diameters of the H_{II} cylinders, or tubes, can be calculated from freeze-fracture replicas by measuring the repeat distances of the parallel lines in places where the fracture plane runs perpendicular to the electron beam in the electron microscope [33]. These diameters agree well with those obtained by X-ray diffraction measurements [13]. Table I shows the diameters of the H_{II} phases of the different lipid systems as measured by freeze fracturing and X-ray diffraction. It is of interest to note that the diameters of the cylinders formed by cardiolipin and phosphatidic acid are dependent on the divalent cation involved. It is also noteworthy that the diameters of the H_{II} cylinders in 22 : 6c/22 : 6c-PE and 20 : 4c/20 : 4c-PE are considerably smaller than those of 18 : 3c/18 : 3c-PE (Table I and Fig. 1). These differences in tube diameter are most likely due to the overall dynamic volume (shape concept; see Ref. 9). In this simplistic concept bilayer lipids are considered to exhibit a cylindrical shape whereas lipids preferring the H_{II} phase are considered to exhibit a 'cone' shape compatible with that organization.

In mixed lipid systems one can distinguish cylinders which are identical in diameter to that of the pure H_{II} lipid present in the mixture (Table I). Such a behaviour has been encountered in cardiolipin/egg PC (1 : 1) in the presence of Mg^{2+} or Mn^{2+}. The majority of the H_{II} tubes have the

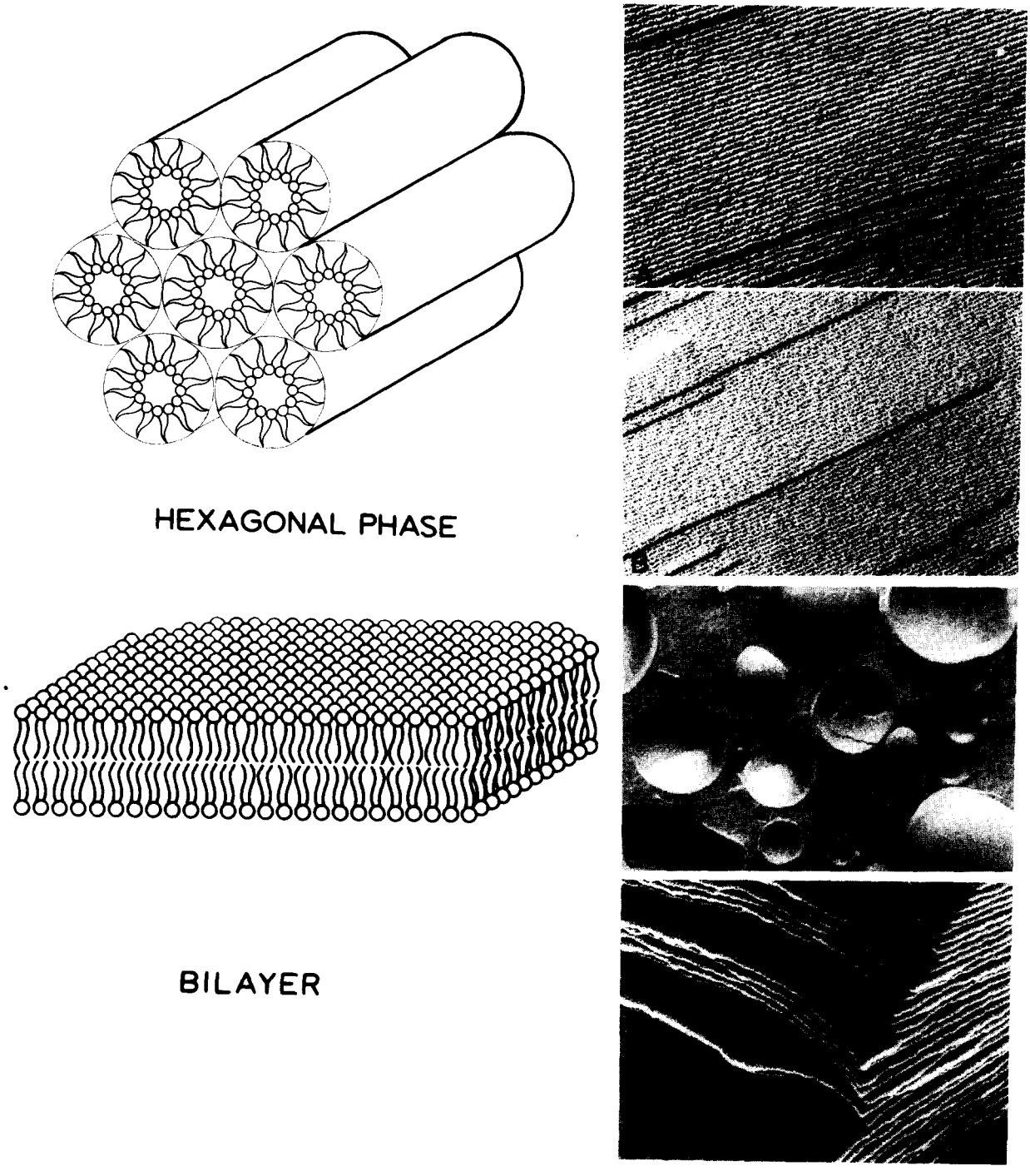


Fig. 1. Schematic drawing and freeze-fracture electron micrographs of the hexagonal II phase (H_{II}) of 18:3c/18:3c PE (A) and 20:4c/20:4c PE (B) and of bilayer phases (C and D) (×100000).

TABLE I

THE DIAMETER OF THE LIPIDIC PARTICLES IS REPRESENTED AS THE MEAN OF THE PARTICLE AND PIT DIAMETER

	Repeat distance (nm) H_{II} phase		Diameter of lipidic particles (nm) (Freeze-fracture)
	Freeze fracture	X-ray	
$18:1_c/18:1_c$-PE (20°C)	7.4[33]	–	–
$18:1_t/18:1_t$-PE (60°C)	–	7.0[a]	–
$18:3_c/18:3_c$-PE (20°C)	7.7[32]	–	–
20:4/20:4-PE (20°C)	4.4[32]	–	–
22:6/22:6-PE (20°C)	4.3[32]	–	–
$18:1_c/18:1_c$-PA, Ca^{2+}-salt (20°C)	5.2[8]	–	–
$18:1_c/18:1_c$-PA, Mg^{2+}-salt (20°C)	5.7[8]	–	–
$18:1_c/18:1_c$-PA, Mn^{2+}-salt (20°C)	7.4[8]	–	–
CL, Ca^{2+}-salt (20°C)	5.2[33], 5.3[34]	5.4[22]	6–8[37]
CL, Mg^{2+}-salt (20°C)	6.5[33]	6.4[22]	–
CL, Mn^{2+}-salt (20°C)	7.5[33]	–	–
CL/egg-PC, molar ratio 1:1, $+Ca^{2+}$ (20°C)	7.3[33], 14.0[33]	7.2[22]	8.5[33]
CL/egg-PC, molar ratio 1:1, $+Mg^{2+}$ (20°C)	6.4[33], 11.5[33]	–	13.0[33]
CL/egg-PC, molar ratio 1:1, $+Mn^{2+}$ (20°C)	7.3[33], 9.7[33]	–	13.0[33]
$18:1_c/18:1_c$-PE, $18:1_c/18:1_c$-PC-Chol, molar ratio 3:1:2 (20°C)	8.6[33]		9.5[33]

[a] Unpublished data (B. de Kruijff).

same diameter as those of pure cardiolipin in the presence of Mg^{2+} or Mn^{2+}. This may be explained by postulating that a phase segregation of cardiolipin and PC has occurred, the former adopting a H_{II} phase orientation and the latter a bilayer structure [33]. This is supported by the fact that lamellar structures, probably enriched in PC molecules, are also encountered in these mixtures.

In most of the systems one finds that the diameter of the H_{II} cylinders is larger than that of the pure H_{II} component. In an equimolar mixture of cardiolipin/egg PC in the presence of Ca^{2+} the diameter of the cylinders is about 7.3 nm, whereas the pure cardiolipin/Ca^{2+} tube is 5.3 nm in diameter. This strongly suggests that in cardiolipin/egg PC systems both lipids are present in the H_{II} tubes [33]. A similar conclusion has been obtained from ^{31}P-NMR using thion-phospholipids [78]. The presence of PC in the cylinders could explain the increase in tube diameter, since this molecule is probably more hydrated and therefore has an overall cylindrical shape. In the cardiolipin/egg PC/Ca^{2+} system one can even find tubes with a diameter of 14.0 nm. From freeze-fracturing one could interpret them as larger H_{II} type cylinders. Other authors have interpreted these 14.0 nm tubes as the complex H_{II} phase, in which the cylinders are in fact bilayers [34]. This interpretation is based on their appearance in thin-section micrographs. However, since lipids cannot be fixed chemically and fixation, dehydration and staining can induce artifacts this preparation method does not justify such a definite conclusion. Larger tubes have also been observed in DOPE/DOPC/cholesterol mixtures. [33].

IIB. H_{II} phase-lamellar transitions in single lipid systems

^{31}P-NMR and differential scanning calorimetric (DSC) studies show that the lamellar to H_{II} transition of pure unsaturated PE is remarkably abrupt,

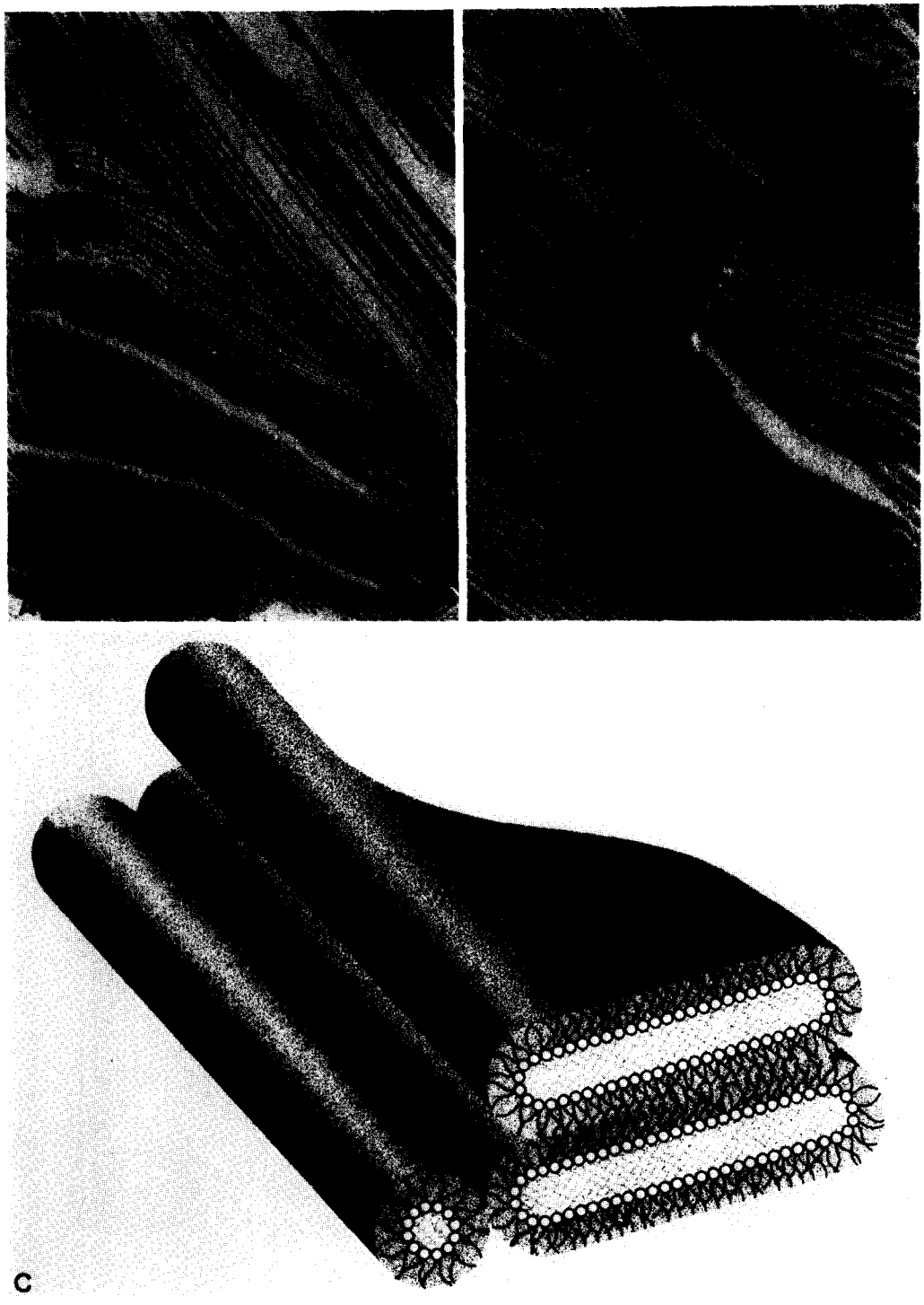


Fig. 2. Transition from bilayer to H_{II} phase and vice versa. Hexagonal II phase tubes diverge gradually into stacked bilayers. (A) DOPE jet-frozen from 20°C [35]. (B) DOPE/DOPC/cholesterol jet-frozen from 30°C [35]. (C) Schematic drawing of the transition from lamellar to H_{II} phase ($\times 100000$).

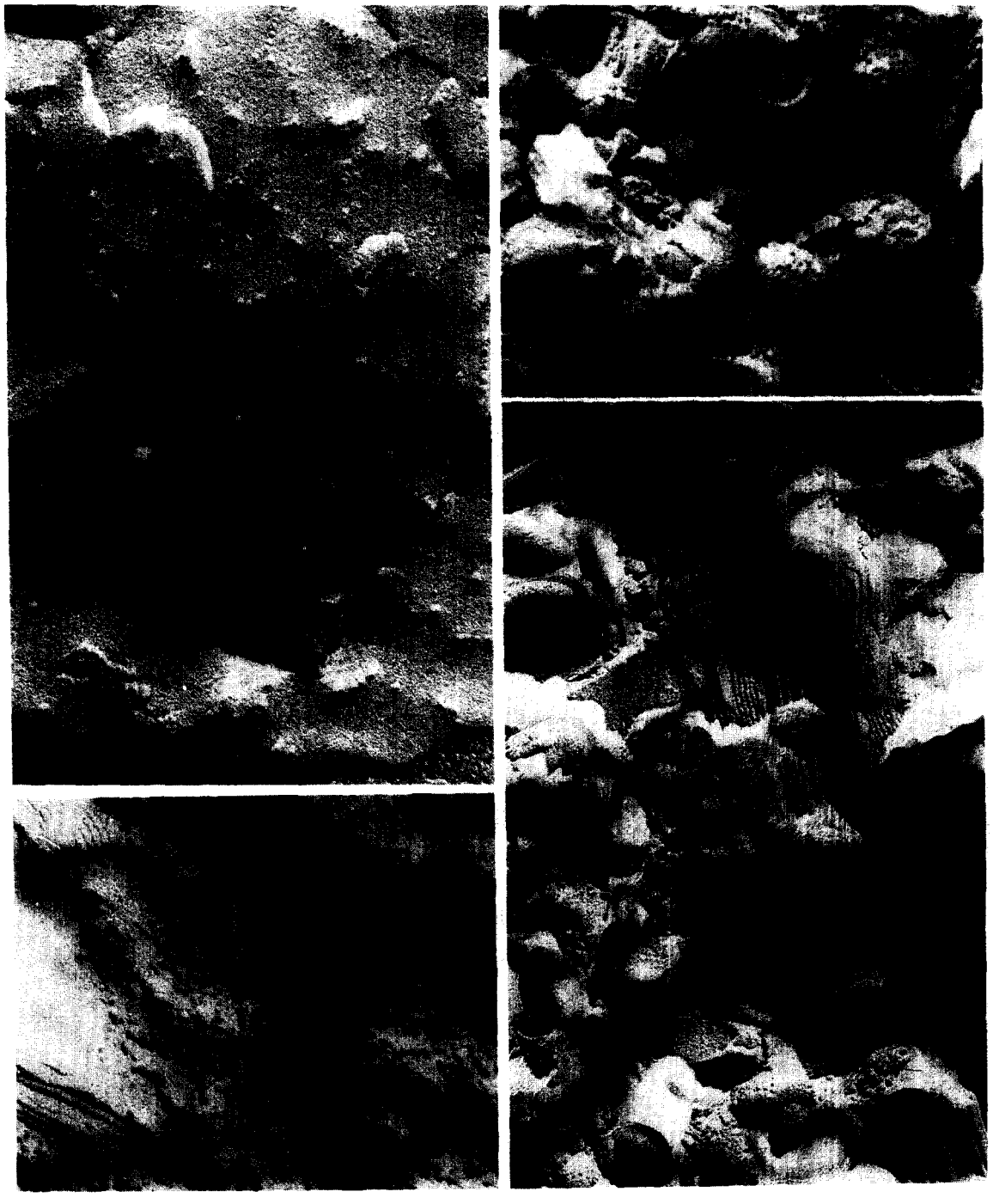


Fig. 3. Lipidic particles in single lipid systems. (A) Egg PE frozen from 40°C (×100000). (B) DOPE jet-frozen from 20°C [35] (×200000). (C, D) Cardiolipin upon addition of Ca^{2+}, jet-frozen from 20°C [37] (×100000).

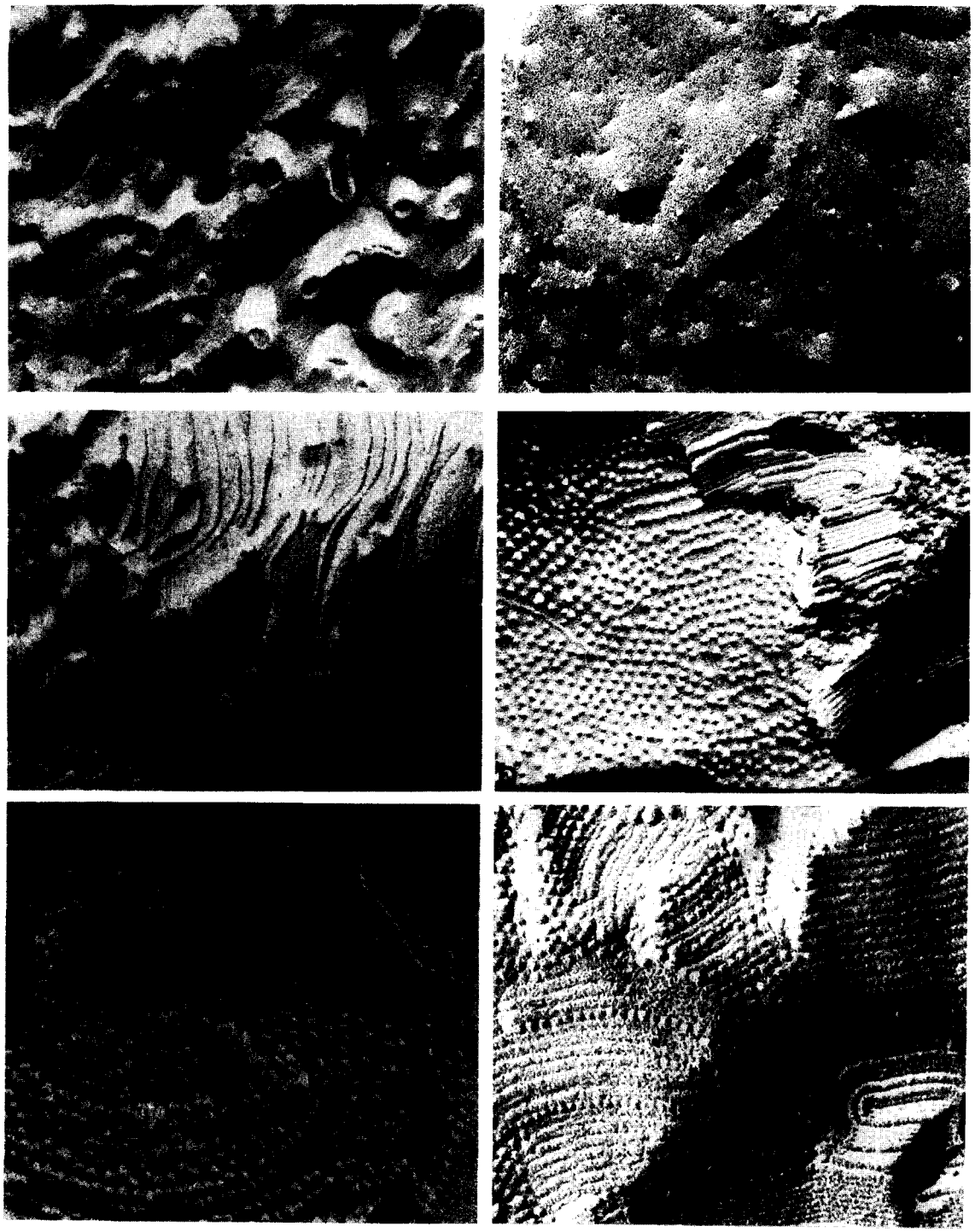


Fig. 4. Lipidic particles in mixed lipid systems: (A) DOPE/DOPC/cholesterol (molar ratio 3:1:2) jet-frozen from 20°C. (B) Cardiolipin/egg PC (molar ratio 1:1) with Ca^{2+} frozen from 20°C [1]. (C) DOPE/DOPC/cholesterol (molar ratio 3:1:2) frozen from 10°C [3]. (D) Cardiolipin/egg PC (molar ratio 1:1) with excess Ca^{2+} frozen from 20°C [35]. (D) Cardiolipin/egg PC (molar ratio 1:1) with Mn^{2+} frozen from 20°C [47]. (F) Cardiolipin/chlorpromazine at pH 6 frozen from 20°C [8] (×100000).

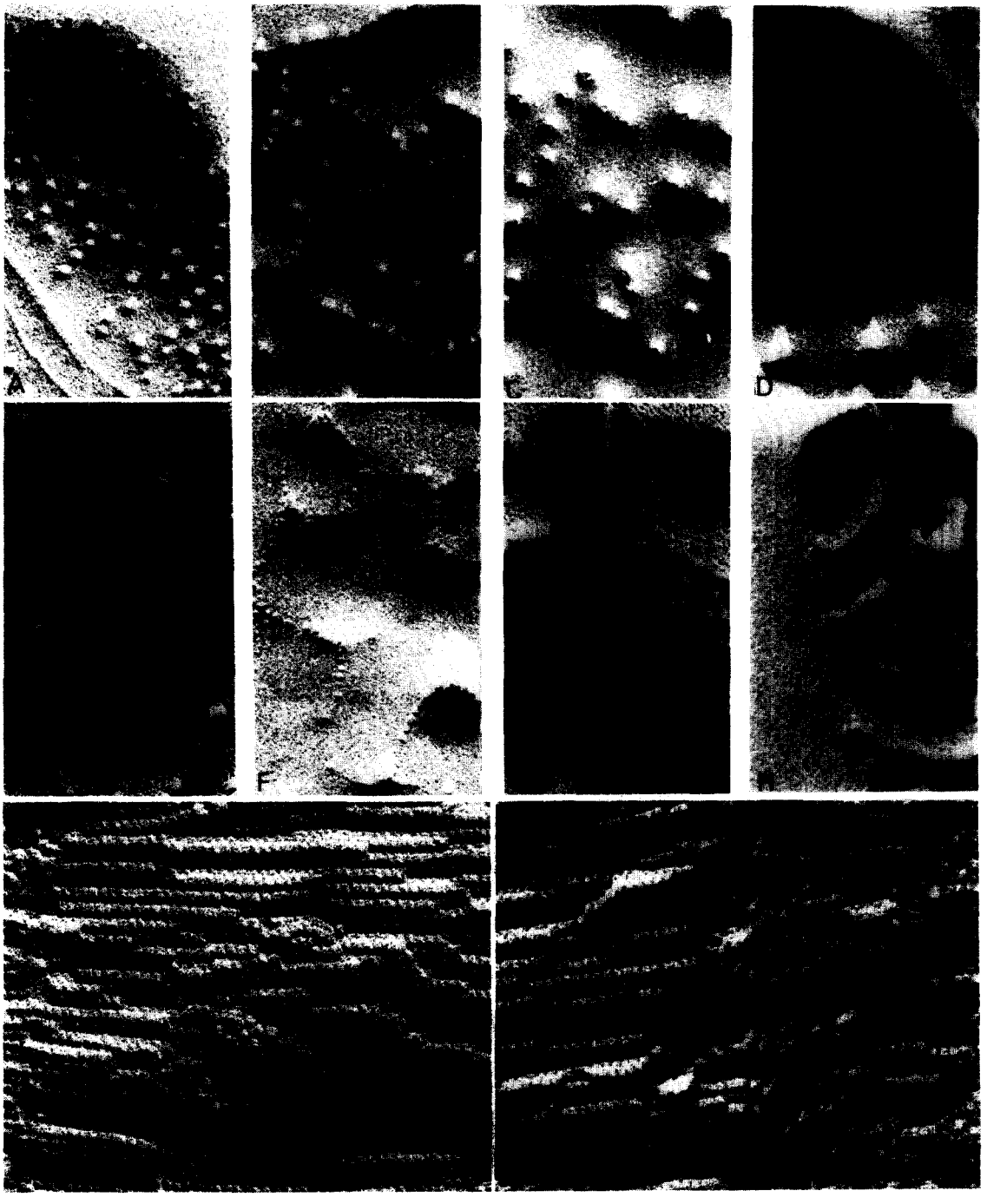


Fig. 5. Types and organization of lipidic particles. (A) DOPE/DOPC/cholesterol [3]. (B) Cardiolipin/egg PC/Ca^{2+} [3]. (C) DOPE/DOPC/cholesterol (molar ratio 3:1:2) frozen from 10°C. (D) DOPE/DOPC/cholesterol (molar ratio 3:1:2) heated up to 60°C for 10 min, cooled down to 4°C and subsequently frozen at 4°C. (E) Cardiolipin/egg PC/Ca^{2+} [3]. (F) MGlucDG/egg PC [3]. (G, H) MGalDG/DGalDG [44]. (J, K) MGlucDG/DGlucDG [61]. (×100000 except G and H which are ×212500). G and H are reproduced with permission from the authors [44].

occurring within a temperature range of only a few degrees [9]. Moreover, DSC also shows that the enthalpy change involved in this structural rearrangement is very small compared to the amount of heat taken up in going from the solid to fluid bilayer configuration [29]. Experiments with ^{31}P-NMR on oriented bilayers of PE suggest that such a transition from bilayer to H_{II} phase proceeds via the formation of long cylinders of the H_{II} phase from the monolayers of two adjacent bilayers. The H_{II} cylinders then lie parallel to the plane of the bilayer [35].

Freeze-fracture experiments on pure PE systems frozen from a temperature just above the H_{II} bilayer transition with fast-freezing devices in the absence and presence of cryoprotectants, show transitions from the H_{II} phase into the bilayer configuration, indicated by the conversion of the H_{II} tubes into smooth fracture faces (Fig. 2A). Lipidic particles, visible at smooth fracture faces of bilayers (Fig. 3A) and within the H_{II} tubes (Fig. 3B) also occur under these conditions.

The fact that bilayer structures and lipidic particles have been found in PE samples frozen above the H_{II}-bilayer transition temperature indicates that the freezing rate was insufficient to prevent the transition from H_{II} to bilayer phase. These intermediary structures can be envisaged as being trapped during the freezing (fixation) process. If the transition from H_{II} to lamellar phase occurs at temperatures higher than about 30°C, even fast-freezing is unable to prevent such phase changes [36].

The presence of lipidic particles between H_{II} phase and bilayer phase may seem to be at variance with ^{31}P-NMR data [35], since the presence of lipidic particles has not been indicated by this method in the pure PE system. Most likely this apparent discrepancy is due to the kinetics of the process.

The intermediary structures encountered in the pure single PE system are probably real structural intermediates in the transitions between the H_{II} and bilayer phases. This has been confirmed with the cardiolipin system. Similar structural transitions from bilayer to H_{II} phase have been found in pure cardiolipin upon addition and removal of Ca^{2+}, respectively (Fig. 3C, D) [2,23,37]. It is of importance to note that ^{31}P-NMR measurements in this case detect an isotropic signal during the transition, which may reflect the presence of lipidic particles [23,37].

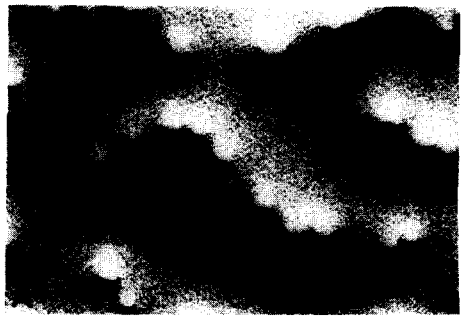
IIC. H_{II} phase-lamellar transition in mixed lipid systems

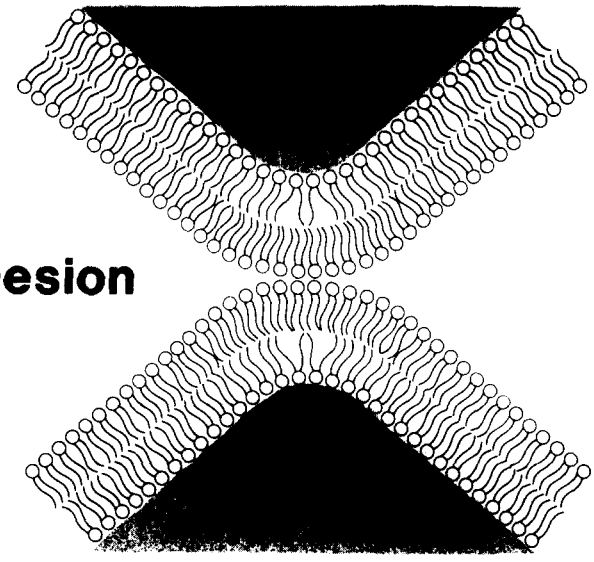
In mixed lipid systems in which one of the lipids prefers the H_{II} phase the situation seems to be more complicated. In most of these systems an isotropic ^{31}P-NMR signal has been detected next to line shapes typical of lipids organized in the lamellar and H_{II} phases. Such mixtures are PE/egg PC [5], DOPE/DOPC/cholesterol [3], cardiolipin/egg PC in the presence of Ca^{2+}, MGlucDG/egg PC [3], total lipid extracts of *E. coli* [19], rod outer segments [30] and inner mitochondrial membranes [31]. Such an isotropic signal found next to bilayer and H_{II} phase spectra indicates isotropic motion of lipids in large structures. This means that a substantial amount of the lipids has an increased motional freedom.

Structural transitions similar to those encountered in the single systems of PE and cardiolipin can be detected in these mixed lipid systems with freeze-fracturing. First, one can detect a transition from H_{II} tubes directly into the lamellar phase (Fig. 2B). These H_{II} tubes flatten out which give rise to smooth fracture faces, which can be explained as a phase transition of lipids that prefer the H_{II} phase and lipids that prefer the bilayer phase (see for a model Fig. 3C and Ref. 33). Second, the transition from bilayer to H_{II} and vice versa can occur with the appearance of lipidic particles and their complementary pits (Fig. 4). The lipidic particles have frequently been found to lie in line with, and also within, H_{II} cylinders (Fig. 4D, E and F). Ridges and their complementary fissures are often encountered (Fig. 4A, B; Fig. 5A, C, D) [3,33,38], which probably reflect small H_{II} tubes or tubular micelles (Fig. 4C). A three-dimensional quasi-crystalline arrangement of particles lying next to the H_{II} and lamellar phase has also been seen. This structure will be discussed in detail in subsection IIIC.

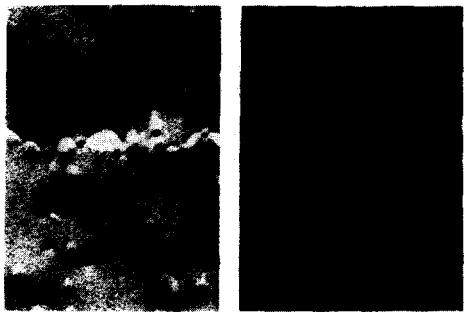
III. Lipidic particles

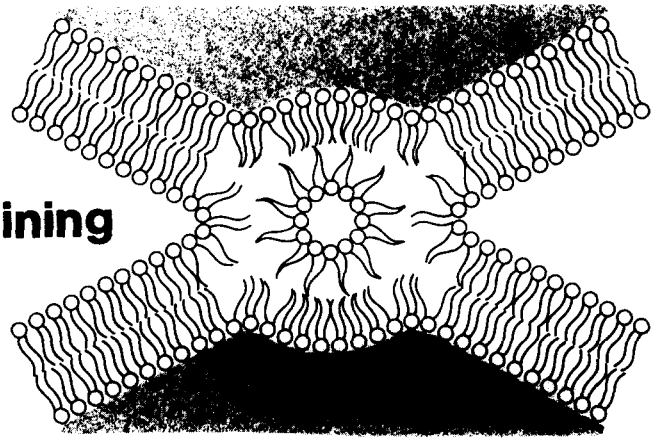
Lipidic particles have only been found in lipid systems in which one or more of the lipids prefer(s)



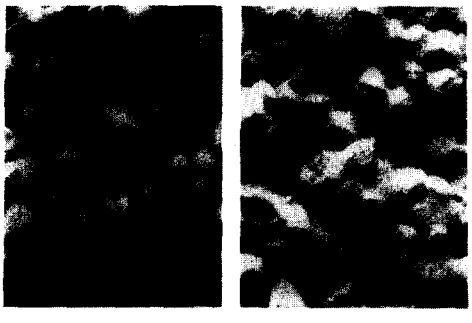


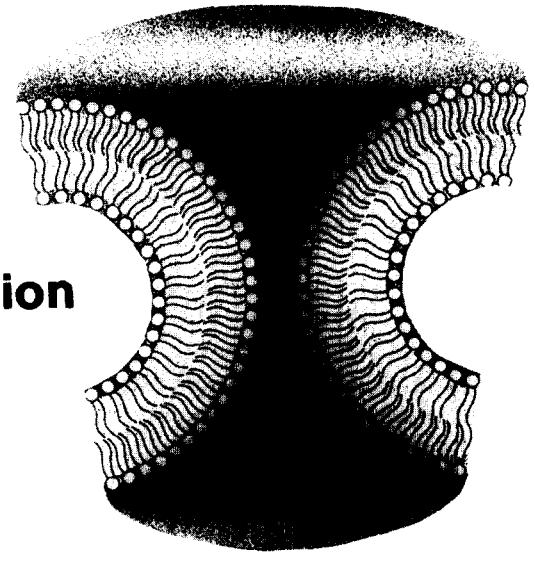
Adhesion





Joining





Fission

to adopt the H_{II} phase. Such lipidic particles can be seen as intermediary structures between bilayer and H_{II} phase and vice versa.

The appearance of lipidic particles is also influenced by the presence of cryoprotectants [38,39] and time [34,40], as well as environmental factors such as temperature [3,9], divalent cations [3,9], pH [8] and ionic strength [56]. The presence of cryoprotectants can decrease the number of particles [34,38–40] and has been shown to change the diameter of the particles in a certain lipid mixture [38]. The reason for this effect is likely a change in the balance of forces existing between the lipid molecules, in particular, hydrogen bonding between lipid and water, and water-water interactions.

IIIA. Type and organization of lipidic particles

Lipidic particles show a large variation in shape, size and organization. One can find lipidic particles as semi-spherical protrusions, either well-defined with respect to the smooth, rather flat, underlying layer (Fig. 5A) or lying on top of volcano-like protrusions of the smooth fracture face (Fig. 5B). Relatively large particles (above 200 Å in diameter) which have small depressions in the center have been encountered (Fig. 5C). One can also find volcano-like protrusions without any distinct structure at the top (Fig. 5D; [5,38,44]). The size of these protrusions is hard to define and they have been described as 'cusps' rather than lipidic particles [5]. The size of the well-defined particles found so far varies from 6 to 14 nm (Table I) and is in general rather homogeneous for one specific lipid mixture: 6–7 nm in MGlucDG/egg PC [3], 10–11 nm in DOPE/DOPC/cholesterol [3] and MGalDG/DGalDG [7] and 10–14 nm in cardiolipin/egg PC systems in the presence of divalent cations [1,33]. In all these lipid systems pits have been found which are the complementary impressions of the particles [1,3].

The transverse distribution of the lipidic particles is remarkable in that the particles are almost exclusively, although not always, located on one fracture face and the pits on the complementary fracture faces (the concave and convex fracture faces, respectively). The reason for this asymmetric distribution of particles and pits is not clear, but it could be related to the orientation of the fracture plane with respect to the curved bilayer.

The lateral distribution of the particles is highly variable. First of all, one observes some single particles, randomly arranged over the underlying face (Fig. 5A). Secondly, rows of particles or pits are encountered (Fig. 5B, E). These rows of particles sometimes form circles (Fig. 5F). Alternatively, a particle row may end up in a tube with a similar diameter (Fig. 5G), suggesting a structural relationship between the particles and these H_{II} tubes. Thirdly, sheets of particles and pits can be observed. The degree of packing of particles can be either loose (Fig. 5G) or closely packed into even ordered arrays (Fig. 5H). Finally, one can find three-dimensional arrays of particles (Fig. 5J, K) in a variety of systems either in the presence or the absence of cryoprotectants [34,38]. The repeat distance can vary from 8–9 nm to 13–16 nm and is dependent upon the type of lipid, water content, temperature and presence of cryoprotectants (see subsection IIIC.).

IIIB. Molecular interpretation of lipidic particles

At present there is still much confusion regarding the molecular organization of lipids in the lipidic particles. This is partly due to the variety in their size, shape (well-defined or 'cusp'-like), organization (two-dimensional and three-dimensional), the asymmetric distribution on concave and convex fracture faces and type of systems studied. Moreover, there are at present no unambiguous electron microscopic or spectroscopic techniques which can give a definite answer.

In all the models presented up to now there is a general consensus that the lipidic particles, cusps, or protrusions are strongly associated with membrane adhesion and membrane fusion or, to be

Fig. 6. Tentative drawings of membrane fusion intermediates and their possible corresponding features as visualized by freeze fracturing. A, B, C, D and E are micrographs taken from the sample DOPE/DOPC/cholesterol (molar ratio 3:1:2) heated up to 60°C for 10 min, cooled down to 4°C and subsequently frozen at 4°C. (×100000).

more precise, with certain stages of membrane fusion. (This does not exclude the possibility that lipidic particles can appear in a single bilayer (see below)). Some investigators [1,3,7] favour the hypothesis that the lipidic particles are inverted micelles, intermediates in the fusion process [45–49] whereas the alternate view does not recognize specific non-bilayer configurations [5,6], but sees the lipidic particles as pre- [4,50] or post- [6] fusion structures. Although these interpretations may appear to be in competition, it is the author's view that they are not mutually exclusive, and may represent different stages (and hence different structures) of membrane fusion.

Figure 6 describes all the sequential steps of membrane fusion. In principle, one can distinguish three different stages: the 'adhesion', the 'joining' and the 'fission' of fusing membranes.

In the 'adhesion' stage, two neighbouring bilayers form polar contact points requiring local dehydration, and charge neutralization, which is characteristic for H_{II} preferring lipids. The bilayers are still intact but the contact points may give rise to deflections in the freeze-fractured membranes because of the local high curvature. Such deflections are most likely not homogeneous in size (diameter) and are not well-defined. Cusp-like particles may reflect this stage and indeed represent intermembrane attachment sites (IMAS model and cusp model) [4,50].

In the second stage, the bilayers 'join', which enables intermixing of their lipids to take place. This may either proceed with an inverted micelle or extended inverted micelle (H_{II} type rod) [45–49] as an intermediate, or without such inverted structures [5]. Fracturing of a 'joining' stage containing an inverted micelle or extended inverted micelle should be consistent with the appearance of well-defined lipidic particles, or ridges, and their complementary pits, or fissures (Fig. 6). For purely morphological reasons, such a lipidic particle is more compatible with the inverted micelle model than is an intermediate 'joining' stage without an inverted micelle, since the lipidic particles encountered in the model systems are rather homogeneous in size and clearly defined in each lipid system. This would not be expected in a model not requiring such inverted structures. The difference in size of the lipidic particles, as encountered in the different lipid systems (Table I), may be due to the different chemical compositions of the inverted micelles.

Many aspects of the lipidic particle phenomenon support the interpretation that such well-defined lipidic particles are inverted micelles. First of all, it accounts for the requirement of at least one H_{II} phase-preferring lipid in particle-containing lipid systems. The cone-shape dynamic volume [9] of these lipids allows packing in a curved structure with the polar headgroups pointing inwards.Secondly, this interpretation is supported by the morphological relation between lipidic particles and H_{II} tubes. Both structures have a similar molecular organization if the particles are considered as a reflection of an inverted micelle. An analogous example of a structural relationship between micellar and hexagonal phase can be found in another area of lipid polymorphism, in that lipids can adopt both micellar and H_I phase in water as does lysolecithin for example [51]. Thirdly, inverted micelles can provide an explanation for the small size and the well-defined hemisphere-like structure of several types of lipidic particles. The alternative interpretation for those well-defined lipidic particles, i.e. the IMAS model [4,6], is not compatible with such a small radius of curvature, since curved bilayers are highly unstable when their diameter is smaller than about 250 Å. This latter radius is found to be the minimal value for stable vesicles. Finally, lipid systems containing inverted micelles can show profound hysteresis of the isotropic ^{31}P-NMR spectrum. This can be explained by considering these semi-long living structures to occur at the point of connection of two 'joining' bilayers [35].

In the last step there will be a 'fission', which can give rise either to the original two vesicles or to real fusion by intermixing of the two aqueous compartments of both vesicles. In the former case the bilayers will temporarily pass a stage similar to that of the intermembrane attachment site, whereas in the latter case, the fracture plane passing through the newly formed aqueous channel will show a marked change from cusp shapes to volcanoes with flat tops. The flat tops arise as a result of fracturing through ice in aqueous pores. These pores will grow as can be observed in certain micrographs; Fig. 5C, Fig. 6D, E and Fig. 7C, D.

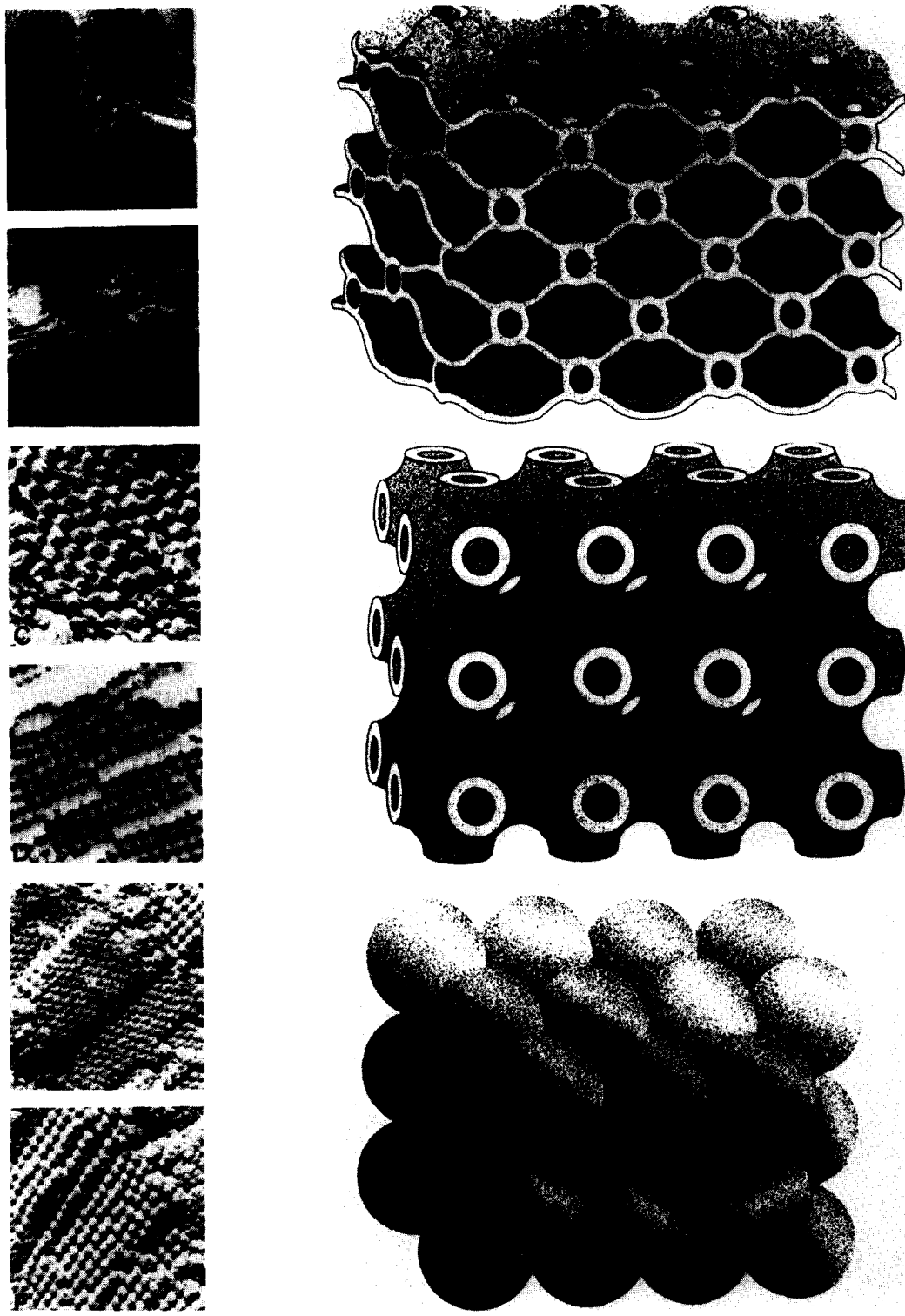


Fig. 7. Tentative drawings of possible intermediate stages from lamellar to quasi-crystalline phase, with their corresponding freeze-fracture features. (A, B and D) DOPE/ DOPC/cholesterol (molar ratio 3 : 1 : 2) heated up to 60°C for 10 min, cooled down to 4°C and subsequently frozen at 4°C. (D, E and F) cardiolipin-chlorpromazine (molar ratio 1 : 1) at pH 6.0 frozen from 20°C [8] (×100000).

It has to be mentioned that particles with small pits may also reflect the 'joining' stage if one assumes that the fracture plane goes under, instead of over, the inverted micelle.

In conclusion, the cusp-like particles may reflect the prefusion stage of adhesion, the more well-defined lipidic particles, the inverted micelles, at the 'joining' stage, and the vulcano-like protrusions a stage just after 'fission' which allows intermixing of the separated aqueous compartments.

IIIC. Lipidic particles and 'cubic' phases

It has been pointed out that one can find lipidic particles organized in a three-dimensional quasi-crystalline arrangement (Fig. 5J, K). This has been found in cardiolipin/egg PC in the presence of Ca^{2+} [46], MGalDG/DGalDG [7,38], cardiolipin/ chlorpromazine [8], dioleolyl-PA/ chlorpromazine [8], dilinoleoyl-PE/PC [42], and in MGlucDG/DGlucDG mixtures [58]. In most of these systems three-dimensional organized particles are found next to regions of H_{II} phase [7,8,38,42,46], however, in a mixture of MGlucDG/DGlucDG (molar ratio 2 : 1) this organization is found exclusively (Fig. 5J, K). It is of great importance to note that this lipid system is optically isotropic. The dimensions of the unit structure (repeat distances) of the particles may depend on the presence or absence of cryoprotectants [38].

This structure has been suggested to be a reflection of spheres, probably inverted micelles, in a closely packed organization [8], or to correspond to lipid micelles sandwiched within a membrane bilayer [7]. The latter interpretation was deduced from optical diffractograms of negatively stained pictures. Recently, both interpretations have been the subject of debate [42,43]. Based on X-ray diffraction data, it has been suggested that these quasi-crystalline structures in fact correspond with an isotropic cubic phase of a type reported in X-ray diffraction studies on soaps and detergents [52] and simple monoglycerides [53].

At present it is very difficult to decide between these interpretations. It is possible that the structural organization of these quasi-crystalline structures may reflect different lipid organizations. As stated before [43], more evidence using other techniques is required before the detailed structure of the aggregates of particles is known with certainty. A more refined analysis of the freeze-fracture pictures to determine the angles of the different fracture planes and to define the unit repeat distance of the particles in the different fracture planes may help to unravel the underlying molecular organization.

To develop a model for the formation of those structures it is informative to discuss morphological features frequently encountered in these systems and their possible significance in the formation of the stacked quasi-crystalline structures. Figure 7 shows a drawing of the possible intermediate stages going from lamellar to quasi-crystalline phase, with their corresponding freeze-fracture morphologies. It has to be noted that this correlation is rather speculative since it is impossible to correlate directly these morphological features with the proposed structures.

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 between the different bilayers. One may then expect to find lipidic particles (inverted micelles) during the 'joining', followed by 'fission' (opening up) as described in Fig. 6. These multiple 'fission' points will result in a structure in which direct contact of the aqueous compartments occurs (these were initially separated) and which is compatible with the cubic structure suggested for monoglycerides [52,53] for MGlucDG/DGlucDG [41] and the etioplast [54]. Further dehydration and fusion of this strongly curved bilayer system may lead to the formation of stacked aggregates of inverted micelles. The morphological difference between this last system and the proposed 'cubic phase' may be indicated by the different angles of the fracture planes, made in the freeze-fracture micrographs. Finally, the lipidic particles may fuse into H_{II} tubes forming a stable H_{II} phase.

IV. Postulated functions for lipidic particles

In nearly every biological membrane large fractions of lipid species are present which, when purified and dispersed in excess water at physiological temperature, adopt the H_{II} phase. This, and the fact that total lipid extracts of several biologi-

cal membranes show H_{II} phase and lipidic particles, has led to the notion that non-bilayer structures are involved in membrane functioning.

IVA. Lipidic particles and membrane fusion

Immediately after the discovery of lipidic particles it was postulated that these structures are directly involved in membrane fusion. Unilamellar vesicles made of a mixture of bilayer and H_{II} phase-preferring lipids will fuse when they are subjected to conditions in which the tendency to form the H_{II} phase is increased [46–49]. In the fusing vesicles, lipidic particles often appear at the site of fusion. Recently, kinetic experiments [57], using an assay in which mixing of aqueous compartments was measured, have demonstrated that the fusion of such unilamellar vesicles is extremely fast (within 1 s). Freeze-fracture experiments using fast-freezing devices reveal that at the earliest moments of vesicle fusion lipidic particles cannot be observed [40,58], though they do appear after several rounds of fusion. This suggests that the lipidic particles are stable structures which for as yet unknown reasons cannot be arrested by freezing at the initial stage of vesicle fusion. It is likely that transiently-formed inverted micelles between unilamellar vesicles might escape detection by freeze-fracture microscopy, since the life-time of such 'joining' structures is very short (likely ms). In multilamellar systems, where one has multiple-point fusion, the life-time of the lipidic particles may be increased. It has also been found that the appearance of lipidic particles is increased by the presence of cryoprotectants which stabilize these intermediate structures [34,38–40].

IVB. Lipidic particles and trans-bilayer transport

Dynamic formation of inverted structures in bilayers can provide a possible mechanism for the transport of lipids and polar solutes [9]. Local compositional fluctuations or agents which trigger H_{II} structure could cause a bilayer invagination, possibly allowed by inverted micelle formation. When the inverted micelle subsequently 'dissolves' in the opposite monolayer, trans-bilayer transport of lipids and polar molecules via the aqueous compartment occurs. Model studies have shown that such events as trans-bilayer movement of lipids (59,60) and transport of divalent cations [3,59], the latter process occurring with cardiolipin and possible phosphatidic acid [8] as ionophores, are associated with the appearance of lipidic particles. It is however difficult to discriminate between lipidic particles occurring within one bilayer and those occurring between two adjacent bilayers. Morphologically, the former should appear as hemi-spheres on a flat smooth bilayer. Model studies on unilamellar vesicles may eventually show whether lipidic particles exist in one bilayer.

V. Possible non-bilayer structures in biology

The fact that almost every biological membrane contains a substantial amount of lipids that can adopt the H_{II} phase upon isolation and the fact that total lipid extracts of several biomembranes do form non-bilayer structures under physiological conditions, strongly suggests a role for non-bilayer structures in biomembranes [19,30,31].

With respect to the occurrence of non-bilayer structures, such as the H_{II} phase and transitional structures from bilayer to H_{II} phase, in particular the lipidic particles, there are at present only fragmentary data and a few indications of their existence in biological membranes in vivo.

VA. H_{II} phase

Lipid inclusions of H_{II} phase have been observed within the lamellar array of rod outer segment membranes [61]. Such structures have also been found in the adrenal cells and the retinal ganglia cells in which liposidosis is chemically induced [62]. They can be induced in vitro in the matrix of isolated mitochondria upon addition of Mn^{2+} [63] and can be found between thylakoid membranes upon heating above 45°C [64]. Although the biological relevance of these structures is as yet unknown, it indicates that the bilayer organization of lipids in biomembranes is not a stable one.

VB. Lipidic particles and related structures

The question is then, 'do lipidic particles or elongated lipidic particles (small H_{II} tubes) have

any functional significance in the native membrane'? From the results obtained from model membrane components such non-bilayer lipidic structures are expected at the point of connection of two membranes either as rather stable 'joining' points or as intermediates in membrane fusion.

The most prominent example of an arrested 'fusion' or 'joining' between membranes in cell biology is the 'tight junction' (see Fig. 8). This structure serves as a barrier for intercellular diffusion [65]. Furthermore, it has been thought to be involved in a diffusion barrier for membrane components, separating the two domains in the plasma membrane i.e. the apical and the basolateral membranes. Indeed it has been demonstrated that these domains appear to have different protein [66] and lipid [67] compositions. From the similar morphological appearance [68,69] and the complementarity aspect [70] it has been proposed that the 'tight junctions' elements or strands reflect inter-bilayer tubes with the lipids in the H_{II} orientation. Since peptides [27,28] and proteins [25,26] can also induce H_{II} phase it is possible that such isolated H_{II} tubes between two membranes are induced and stabilized by specific 'tight junction' proteins.

A similar reasoning could be valid for lines of particles encountered in tubular myelin of developing rat lung [71]. These particles and complementary pits correspond to the intersections of sheets of bilayer membrane i.e. at the contact sites of interwoven bilayer networks.

Another structure which might be of interest in this context is the prolamellar body in the etioblast [72], which transforms into a chloroplast after exposure to light. This structure greatly resembles a cubic phase that has been proposed for monoglycerides in water [53,55] and which is possibly intermediary between the lamellar and H_{II} phase (see subsection IIIC.). Of the various factors which might be responsible for the unique structure of the prolamellar body, the H_{II} phase-preferring MGalDG [20,73] is the most likely. Freeze-fractur-

Fig. 8. Tight junction in epithelium of rat ileum. Fracture planes, jumping from one bilayer to another at joining sites. (×120000).

ing [72] reveals fracture faces with a regular pattern of projections and depressions. Large particles (protuberances) have also been seen. Despite the complexity the freeze-fracture morphology is compatible with that proposed from thin section studies [72].

Contact sites likely occur between the outer and inner membrane of mitochondria. The existence of such contact sites was first suggested on the basis of thin section electron micrographs [74]. Biochemical evidence supporting this proposal comes from studies on the ATP-ADP translocation [75] and the transport of lipid molecules from outer to inner mitochondrial membrane in the presence of Ca^{2+} [76]. Freeze-fracture studies [63] have shown that conditions which favour H_{II} phase formation of lipids (including the addition of Ca^{2+}) induce a patchwork-like structure, as a result of a frequent jumping of the fracture plane between the outer and inner mitochondrial membranes (Fig. 9). Recently, the frequency of jumping has been shown to be correlated to the different functional states of the inner mitochondria. The number of jumps is higher in phosphorylating mitochondria when compared to freshly isolated or energized mitochondria [100]. It has been proposed [63] that these jumps are promoted by a 'joining' of the inner and outer membrane, possibly by an extended inverted micelle. Furthermore, it is of interest to note that apocytochrome *c*, a precursor of cytochrome *c*, also induces such a patchwork fracture behaviour [63]. This may indicate the involvement of contact sites in the import of mitochondrial proteins, as has been suggested from in vitro studies of protein translocation [77].

VC. Membrane fusion

As has been hypothesized [46,78] and discussed above (subsection IVB.), inverted micelles may be envisaged as intermediates during membrane fusion. However, it is clear that lipidic particles—the reflection of such intermediates—have to be considered as rather stable structures or, in other

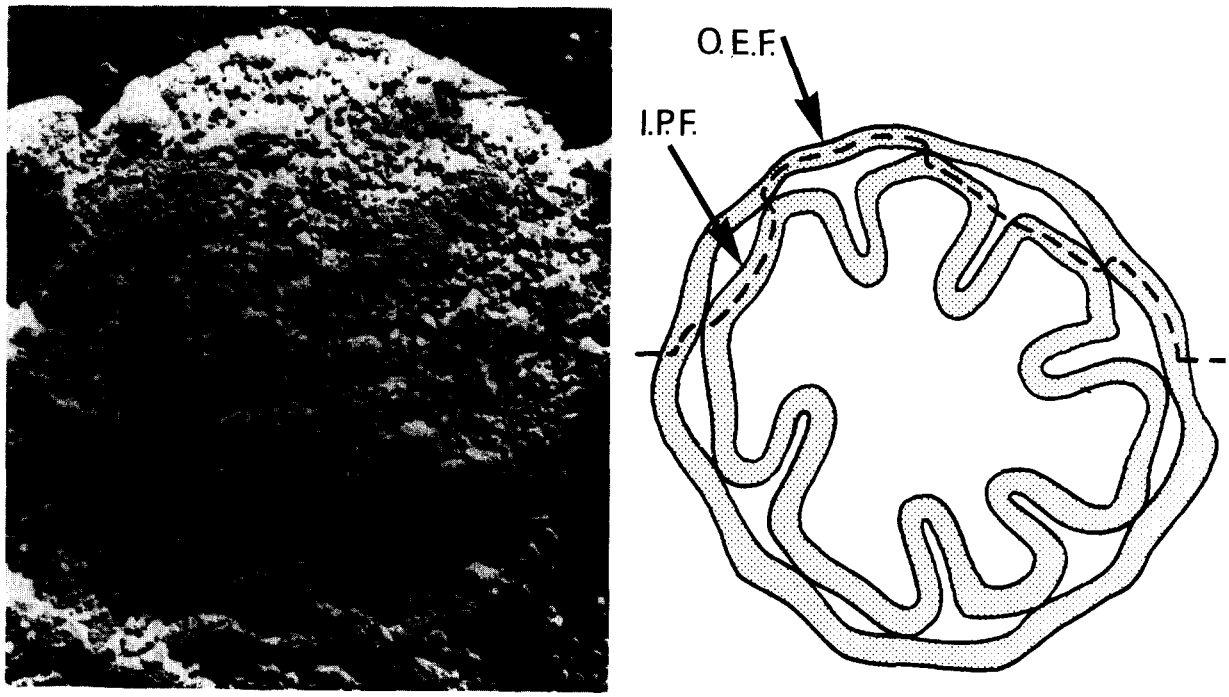


Fig. 9. Patchwork-like structure of mitochondrial membranes upon incubation with Ca^{2+}, Mg^{2+} at 37°C or apocytochrome *c* [63] (×150000) and a schematic drawing of the fracture plane jumping from outer to inner membrane and vice versa. O.E.F. is the exoplasmic fracture face of the outer membrane and I.P.F. is the protoplasmic fracture face of the inner membrane.

words, as arrested 'joining' points between two membranes. This interpretation is supported by the fact that lipidic particles have been visualized subsequent to fusion [40,61] suggesting that such intermediate structures are difficult to trap kinetically during fusion even using fast-freezing rates.

Despite the absence of direct proof for the involvement of inverted micelles or small H_{II} tubes in membrane fusion there are, however, theoretical considerations which strongly support the hypothesis that such H_{II} phase-derived intermediates play a pivotal role in fusion.

First, all membranes investigated so far appear to contain a substantial amount of lipids which prefer the H_{II} phase and if the non-bilayer structure-forming ability is expressed membrane fusion can occur. On the one hand, there may be factors in biological membranes which inhibit the local expression of non-bilayer structures and thus form a blockade for fusion. If this is the case, fusion can only proceed by taking away these blockade factors. Such a blockade might be steric hindrance (by the interaction with the proteins and lipids of the membrane) of the membrane skeleton proteins such as clathrin as has been shown recently [80] or a spectrin-actin-ankyrin-like network on the cytoplasmic site and extracellular matrices on the outside. Intrinsic membrane proteins may also work as blockades by interacting with the lipids in such a way as to suppress the formation of non-bilayer phases. In that respect, it has been recently shown that the intrinsic receptor protein, glycophorin, can stabilize PE [81] and cardiolipin [82] in the bilayer phase. Addition of wheat germ agglutinin induces fusion and the formation of the H_{II} phase, probably by aggregation of the receptor protein [83], a phenomenon considered to be a prerequisite essential for receptor-mediated endocytosis (see for review Ref. 84). Furthermore, changes in lipid-lipid interactions by environmental parameters can induce the non-bilayer lipids to display the capacity to fuse. Among the parameters involved, Ca^{2+} (which has been shown to be essential for exocytosis [85] and the fusion of coated vesicles with the endosome [80]) may be of particular relevance. It is conceivable that Ca^{2+} can induce the non-bilayer phase in the lipids present in the cytoplasmic monolayer of plasma membranes [86–89] and coated vesicles [85]. This is in light of the lipid asymmetry concept [86–88] which predicts that PS and PE are predominantly in the cytoplasmic monolayer, and because Ca^{2+} can trigger lamellar to H_{II} phase transitions in model membranes that mimic the cytoplasmic monolayer of plasma membranes and coated vesicles [27,88].

Since it has been shown that proteins [25,26] and peptides [27,28] can trigger lamellar to H_{II} phase transitions, it is alternatively possible that these substances actively induce non-bilayer structures, and thereby, fusion. In this respect, the fusion of membrane viruses with the lysosomal membrane may be relevant [89]. In that fusion process a viral membrane protein which undergoes a conformational change because of the low pH in the lysosome, might have the capacity to trigger the formation of non-bilayer configurations.

In other fusion mechanisms it has been postulated that special fusogenic lipids such as lysophosphatidylcholine [90], monoglycerides [91] or phosphotidylserine (PS) [92] play a pivotal role. In fact if there is a universal mechanism of the involvement of lipids in membrane fusion it is highly unlikely that one particular type of lipid is required in this process, both in light of high variability of lipid composition in membranes and lipid asymmetry. For instance the role of PS postulated in membrane fusion can only be valid for exocytosis or other fusion events from the cytoplasmic site (Table II), since this phospholipid appears to be almost exclusively located in the

TABLE II

MEMBRANE ASYMMETRY AND MEMBRANE FUSION

2–1, two compartments form one compartment; 1–2, one compartment forms two compartments.

Outside-outside (exoplasmic surface)	Inside-inside (protoplasmic surface)
Cell fusion 2 – 1	*Cell division 1 – 2*
Endocytosis 1 – 2	*Exocytosis 2 – 1*
phagocytose	secretion
pinocytose	
receptor-mediated endocytosis	*Endocytosis 2 – 1*
	coated vesicles - lysosome
	phagosome - lysosome
Exocytosis 1 – 2	
granule formation in Golgi	*Virus budding 1 – 2*
	Pinching off 1 – 2

cytoplasmic monolayer of the plasma membranes [86,87] and coated vesicles [80]. The role of PS during exocytosis is in our concept an indirect one. Upon binding of Ca^{2+} to PS on the cytoplasmic site of the membrane the bilayer stabilization which this negatively charged phospholipid has on PE is lost [93,94]. Thus Ca^{2+} indirectly promotes the non-bilayer behaviour of PE and triggers fusion.

Another point of interest is the amount of lipid (or in other words the bilayer surface area) necessary for membrane fusion. Initial freeze-fracture experiments [95–97] suggested that fusion could proceed only after clearance of the protein particles. It was assumed that regions free of intramembrane particles or at least relatively large exposed bilayer regions were necessary for actual fusion to occur. However, using fast-freezing methods [98,99], no particle clearance could be detected indicating that only a very small area of the lipid bilayer of both fusing membranes is necessary for fusion.

All these notions, (i) that biological membranes contain lipids which can adopt H_{II} phase, (ii) that both fusion and bilayer H_{II} phase transitions can be modulated by similar factors including Ca^{2+}, pH and also peptides etc., (iii) that bilayer H_{II} phase transitions are at the time scale comparable to that of membrane fusion (ms), and (iv) that fusion is probably a local point fusion at which site the lipids have to leave temporarily the bilayer configuration, corroborate the universal hypothesis that H_{II} phase preferring lipids, by virtue of their ability to adopt non-bilayer structures (inverted micelles) are pivotal for fusion of biological membranes.

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