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ANALYSIS OF THE HEXAGONAL II PHASE AND ITS RELATIONS TO LIPIDIC PARTICLES AND THE LAMELLAR PHASE

A FREEZE-FRACTURE STUDY

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Model systems of phosphatidylethanolamine (PE) and cardiolipin (DPG), as pure components and in binary mixtures with phosphatidylcholine (PC) have been morphologically analysed. The relation between the hexagonal$_{II}$ (H_{II}) phase and lipidic particles as well as between the H_{II} phase and the lamellar phase has been studied. Moreover, the periodicity of the various H_{II} tubes was determined. (1) The periodicity of the H_{II} phase of cardiolipin is dependent on the cation involved. DPG-Ca exhibits the smallest tube to tube distance when compared to Mg^{2+} and Mn^{2+}. Moreover, the DPG-Ca tubes are quite straight, in contrast to the Mg^{2+} and Mn^{2+} tubes, which appear to be frequently curved. (2) H_{II} tubes with two distinct diameters have been observed in H_{II} phase containing lipid mixtures. The thickness of the H_{II} tube is related to the composition of the tube. In the cardiolipin-lecithin system, structural separation of the pure cardiolipin H_{II} phase has been suggested with Mg^{2+} and Mn^{2+}, but not with Ca^{2+}. (3) Models for the H_{II} to lamellar phase transition and for the H_{II} phase to the lipidic particles are presented. (4) Lipidic particles are exclusively found in lipid model systems, which contain H_{II} phase favouring lipids. Morphological evidence is presented which suggests these lipidic particles represent inverted micelles. These observations include: (*i*) there is a strong topological and quantitative relation between H_{II} tubes and lipidic particles, (*ii*) lipidic particles occur densely packed in conglomerates without the presence of a smooth layer.

Introduction

Individual lipid species isolated from biological membranes commonly assume bilayer or hexagonal arrangements on dispersal in excess water [1–3]. In recent years interest in non-bilayer forming lipids has been renewed by the observation that in mixtures of bilayer-favouring lipids and hexagonal$_{II}$ (H_{II}) lipids a new molecular organization, the 'lipidic particle' has been observed [4,5]. Both morphological and physico-chemical evidence strongly suggest that these particles reflect either an inverted micellar structure within one bilayer [4,5] or at the fusion site of two bilayers [6,7]. However, an alternative molecular explanation for the lipidic particle has also been proposed [8] and discussed [9]. On morphological basis it is suggested to be an attachment site between two closely apposed bilayers.

Lipidic particles are involved in fusion of lipidic vesicles [6,7] and may be of relevance for facilitated flip-flop and transmembrane ion-transport [10,11]. The existence of such inverted micelles is strongly connected to the occurrence of phospholipids that can assume the H_{II} phase. Such a relation is based upon the fact that H_{II} phase forming lipids are cone-shaped

Abbreviations: DPG, cardiolipin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DOPC, 1,2-dioleoyl-*sn*-glucero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine.

molecules [13]. This relationship between H_{II} cilinders and lipidic particles is evident from recent freeze-fracture micrographs [7]. It is valuable, therefore, to study the structural aspects of the H_{II} phase both in pure and mixed phospholipid systems and their relation with the lipidic particles.

In the current study, we present a qualitative description of lipid systems containing the H_{II} phase and transitions from this phase to lipidic particles and the lamellar phase, respectively. Secondly, periodicity measurements have been carried out upon the H_{II} phase tubes present in the different systems. This quantitative analysis has been performed on pure cardiolipin samples containing excess divalent cations, e.g. Ca^{2+}, Mg^{2+}, Mn^{2+} or Ba^{2+}. In addition, systems comprised of equimolar mixtures of phosphatidylcholine and cardiolipin complexed with Ca^{2+}, Mg^{2+}, or Mn^{2+}, respectively, pure DOPE and DOPE/DOPC/cholesterol were also studied.

Materials and Methods

Egg phosphatidylcholine was isolated from hen eggs and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (18 : 1_c/18 : 1_c-phosphatidylcholine) and 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (18 : 1_c/18 : 1_c-phosphatidylethanolamine) were synthesized as described previously [12,13]. Cardiolipin (diphosphatidylglycerol (DPG)) was purchased from Sigma (St. Louis, MO, U.S.A.) and cholesterol from Fluka (Buchs, Switzerland). All lipids were chromatographically pure.

Phospholipid samples were prepared as follows. Cardiolipin (2.5 μM) (DPG) or 5.0 μM cardiolipin/DOPC (1/1; molar ratio) was dispersed in 10 mM Tris, 150 mM NaCl, pH 7.4 (HAc) buffer. Liposomes were pelleted and the supernatant discarded. Buffer (0.1 ml) containing 100 mM divalent cations was added to the pure DPG system, whereas buffer with 250 mM cations was added to the mixed PC/DPG systems. The DOPE (10 μM) and DOPE/DOPC/cholesterol (3 : 1 : 2, molar ratio) systems were dispersed in buffer and vigorously shaken at 4°C for 1 h. The milky dispersion was subsequently heated to 30°C (DOPE) or 60°C (DOPE/DOPC/cholesterol). The resulting flocculant suspension contained lipid in the H_{II} phase.

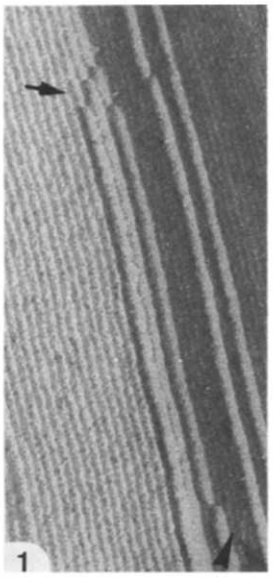
Freeze-fracture electron microscopy was performed according to established procedures [14]. Samples were quenched with the conventional freezing method (in a slush of liquid and solid nitrogen) or with the jet-freezing technique [15], which features an ultra-rapid cooling rate. In the conventional freezing experiments glycerol was added to prevent damage due to freezing. All cardiolipin containing samples were frozen conventionally whereas the DOPE containing samples were frozen by means of the jet-freezer. A thermally controlled specimen holder was developed for quenching of the DOPE samples from temperatures above room temperature [16].

Tube to tube distances were measured at places where parallel lines unambiguously represent H_{II} phase oriented lipid. Care was taken that measurements were performed only on fracture faces oriented in a plane approximately perpendicular to the axis of observation (approx. 50% shadow). Moreover, only the largest values were selected. In this way, an optimal approach of the real H_{II} tube diameter is achieved.

Fluctuations in the magnification of the microscope were kept to a minimum as previously described [17]. Final magnification of the micrographs was checked by photographing a calibration replica (Polaron) after each photographic session.

Results

Both upon addition of divalent cations to pure cardiolipin or PC/DPG and upon increasing the temperature of DOPE or a mixture of DOPE/DOPC/cholesterol the lipid samples flocculate. These precipitates all contain H_{II} phase oriented lipids as visible by freeze-fracture electron microscopy. However, in all these samples a variety of other morphological features are also present. The most conspicious are: (*i*) H_{II} phase cylinders, exhibiting distinct diameters (see Table I). This phenomenon is found in the PC/DPG mixtures containing Mg^{2+} (Fig. 1), Ca^{2+} (Fig. 2), or Mn^{2+} (Fig. 3), as well as in the DOPE sample. (*ii*) The presence of lipidic particles and pits. They may be arranged in a conglomerate or more dispersed but predominantly lined up, frequently going over into H_{II} phase tubes. Examples of lipidic particles in conglomerates are found in the PC/DPG/Ca sample (Fig. 4). Adjacent particles and tubes, arranged in a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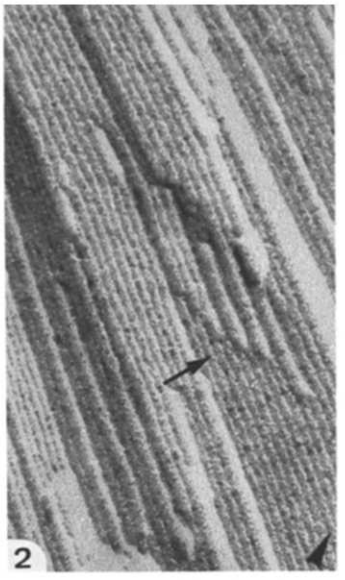
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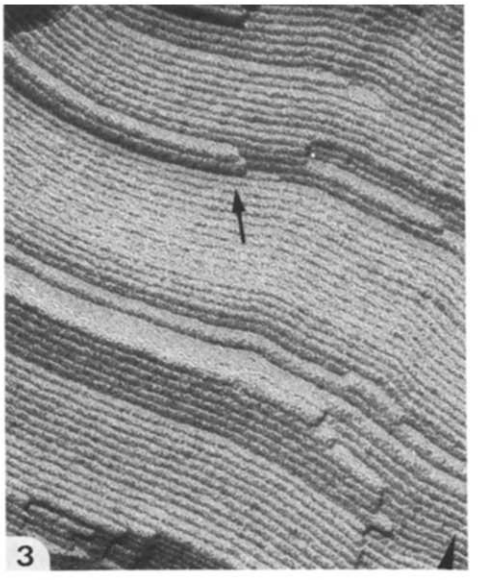
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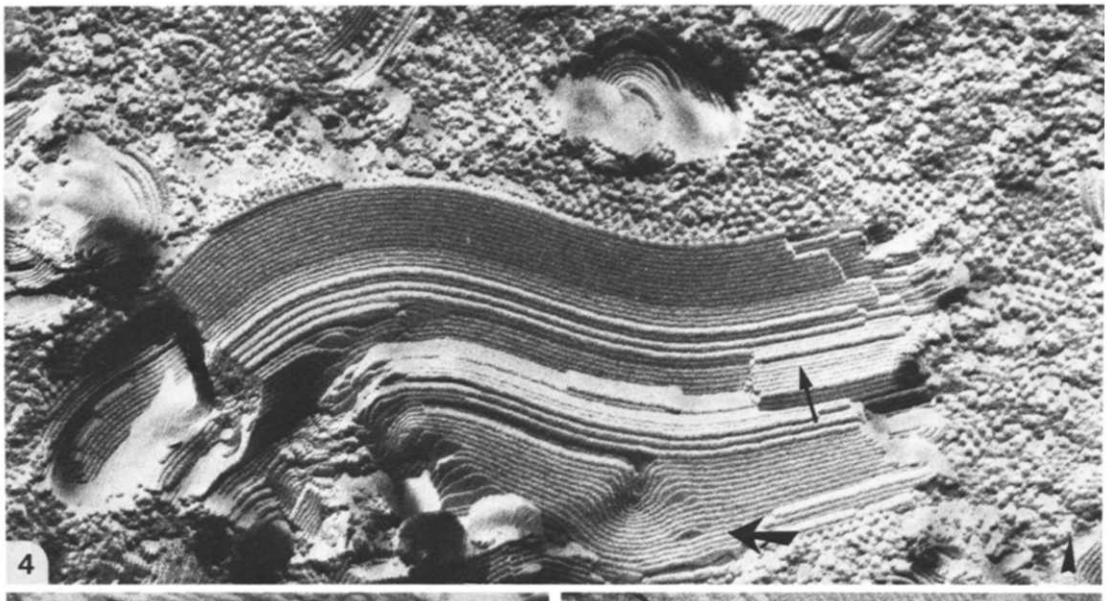
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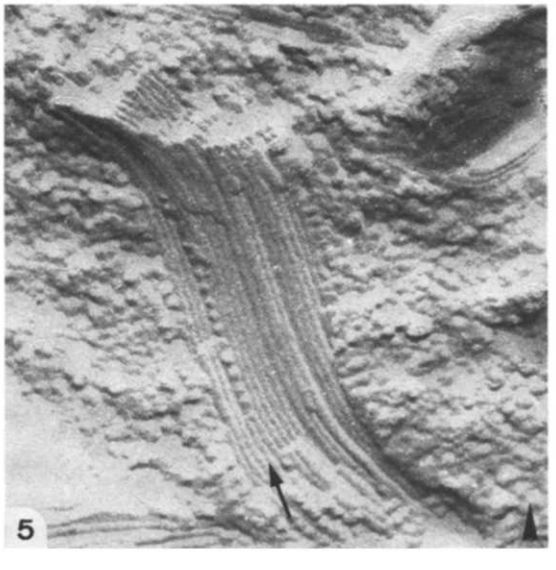
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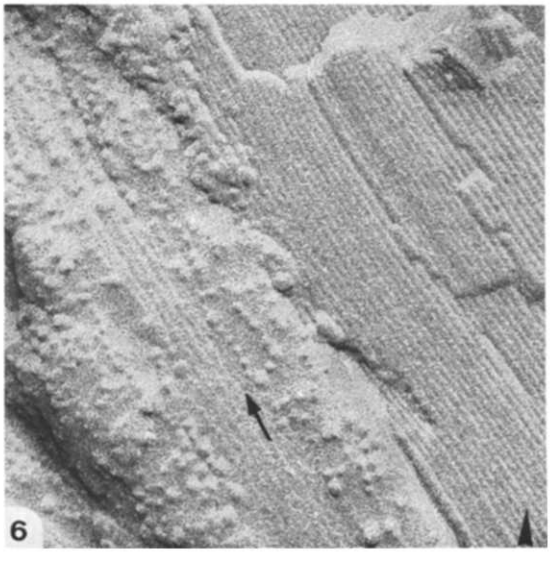
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TABLE I

PERIODICITY MEASUREMENTS OF THE H_{II} PHASE TUBES

H_{II}* indicates the thick type tubes. H_{II}** represents the extra thick tube type, as found in the Ca/DPG/PC system only. The diameter value of the lipidic particles is represented as the mean of the particle and pit diameter.

	Diameter (nm)			
	H_{II}	H_{II}*	H_{II}**	Lipidic particle
DOPE	7.4			
DOPE/DOPC/cholesterol	8.6	–	–	9.5
Ca/DPG	5.2			
Ca/DPG/PC	–	7.3	14.0	8.5
Mg/DPC	6.5			
Mg/DPC/PC	6.4	11.5	–	13.0
Mn/DPG	7.5			
Mn/DPG/PC	7.3	9.7	–	13.0

linear fashion, are found in the PC/DPG systems complexed to Ca^{2+} (Fig. 6) or Mn^{2+}, as well as in DOPE (Fig. 5) and DOPE/DOPC/cholesterol. (*iii*) Smooth fracture faces being continuous with respect to their fracture plane with the H_{II} phase tubes, i.e. the tubes divergate into smooth fracture faces. Examples of this morphology can be found in DPG/Mn, DPG/Mg, DPG/PC with Ca^{2+} or Mn^{2+}, DOPE (Fig. 7) and DOPE/DOPC/cholesterol (Fig. 8).

It is of interest to note that the precipitates of H_{II} phase oriented lipids containing DPG are enclosed from the aqueous medium by a smooth layer, covered with particles or pits (in the case of the PC/DPG/Ca sample, Fig. 9) or with ridges or fissures (seen in PC/DPG/Mg sample, Fig. 10). Ice is found immediately adjacent to this layer.

In Table I we have summarized the periodicity measurements performed on the H_{II} phase tubes. We also estimated the size of the particles and pits. The particle/pit mean values are presented. In the pure cardiolipin samples containing the various divalent cations it is evident that the most condensed tubes are found in the calcium salt sample. Upon studying the morphology, it was observed that in the DPG/Ca sample (Fig. 11) the straightest tubes are found, whereas the tubes in the magnesium and manganese salt samples are frequently curved (Figs. 12 and 13). Moreover, in these latter systems the phenomenon of the H_{II} phase tubes, dilating into smooth fracture faces can be observed. The DPG/Ba salt does not display H_{II} phase tubes when quenched from 20°C. Only densely stacked layers can be seen (Fig. 14). This is in agreement with X-ray diffraction experiments [3]. These observations suggest that Ca^{2+} has the best capacity to induce and maintain H_{II} phase, whereas Ba^{2+} has the lowest capability.

In the PC/DPG mixtures containing Mg^{2+} or Mn^{2+}, two types of H_{II} phase tubes are found, one type having the same diameter as the corresponding tubes in the pure DPG sample, and a larger type one (Figs. 1 and 3). Moreover, areas with lamellar structures are observed. In the PC/DPG/Ca sample the two types of cylinders both have a diameter value larger than that found in the pure DPG sample (Fig. 2), and lamellar structures are only rarely seen.

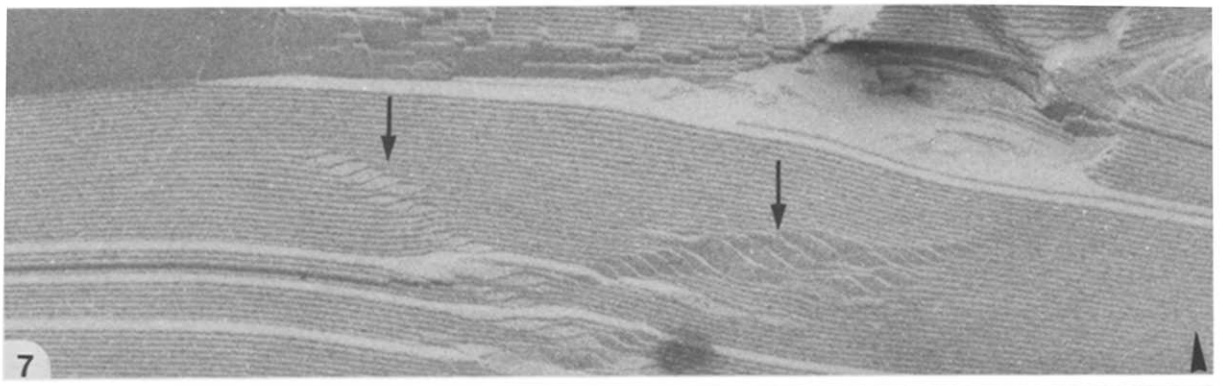
Discussion

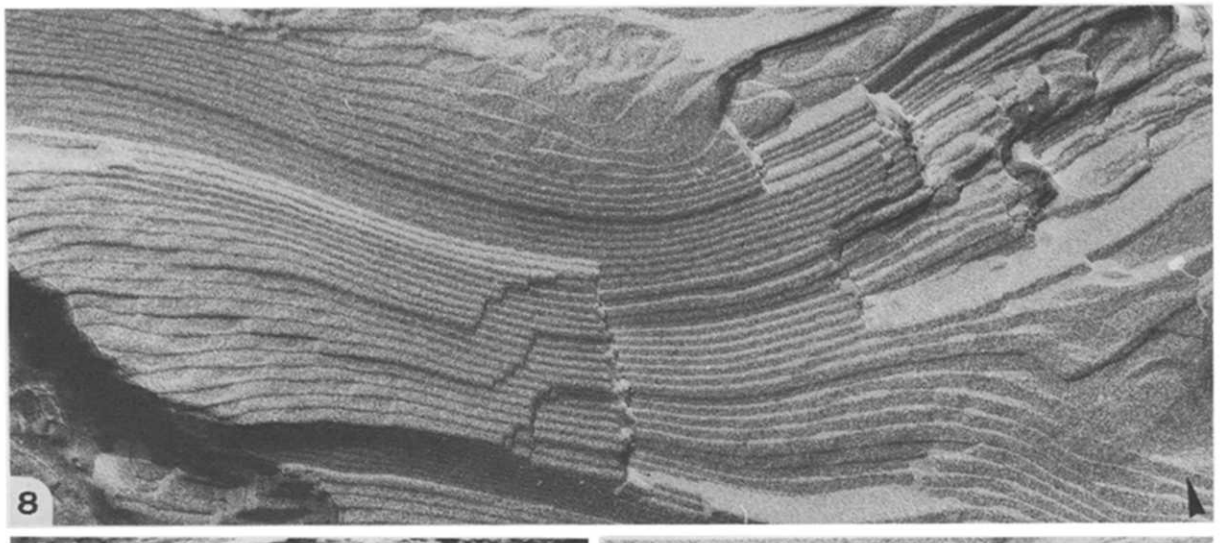
In this paper we have presented a qualitative and morphometric study of the various lipidic phases found in lipid model systems containing H_{II} phase forming lipids. We have been able to extend our studies to the synthetic dioleoylphosphatidylethanolamine containing systems by applying the jet-freezing

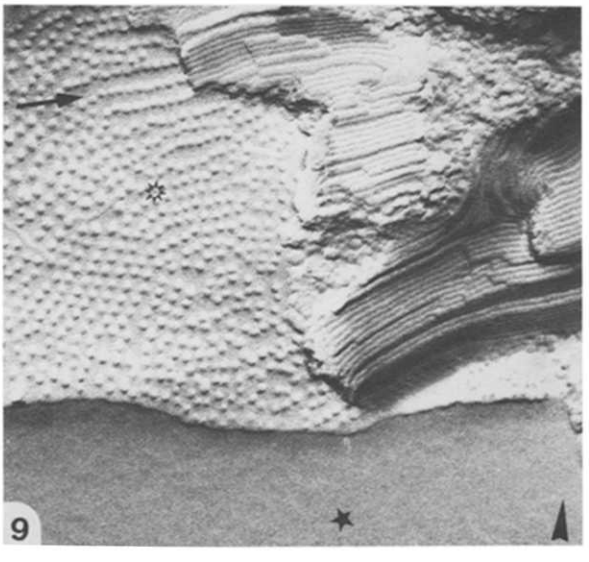
Figs. 1–3. Two types of H_{II} tubes with distinct diameters exist in PC/DPG mixtures. The arrow indicates thick type tubes. Fig. 1: PC/DPG/Mg. Fig. 2: PC/DPG/Ca. Fig. 3: PC/DPG/Mn. Magnification: 200 000×.

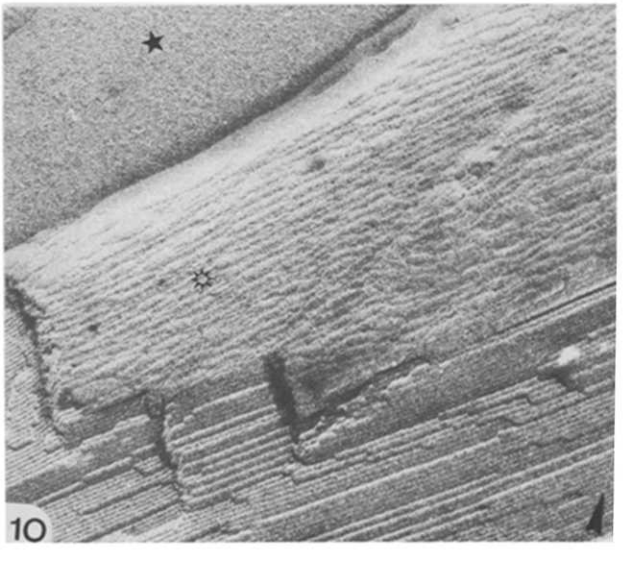
Fig. 4. Representative PC/DPG/Ca sample. H_{II} tubes with two distinct diameters are seen in the middle (small arrow). H_{II} tubes gradually going over in stacked lamellae are present (thick arrow). In the upper right corner there are conglomerates of lipidic particles. A row of particles is oriented parallel to H_{II} tubes at the upper rim of the tubes. Magnification: 100 000×.

Figs. 5 and 6. Adjacent particles and H_{II} tubes arranged in a linear fashion in DOPE (Fig. 5) and in PC/DPG/Ca (Fig. 6). The arrows indicate the particle rows. Magnification: 200 000×.
In all micrographs the arrow in the lower right corner indicates the shadowing direction.

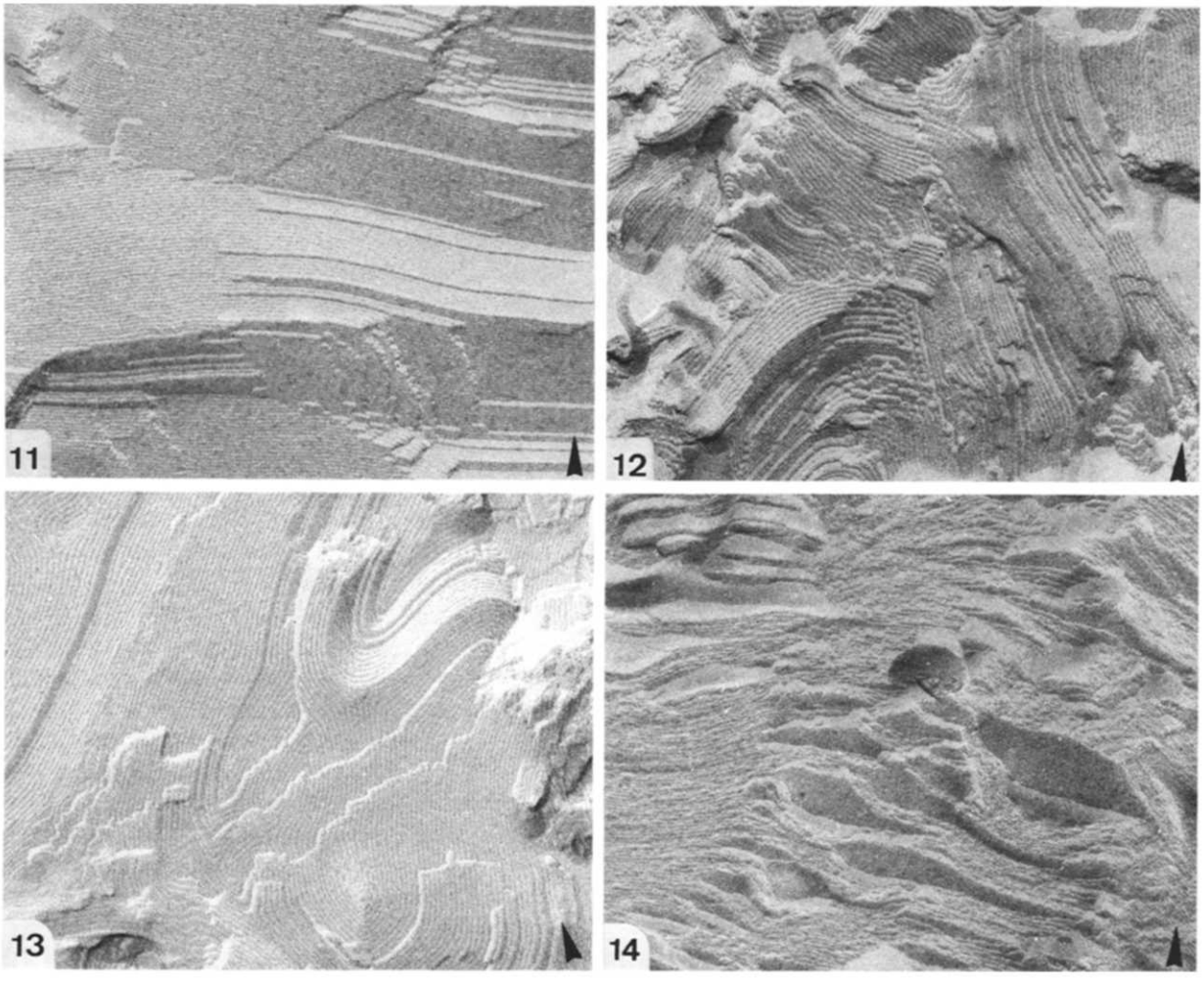








Figs. 7 and 8. Hexagonal II phase tubes, gradually divergating into stacked layers in a DOPE system (Fig. 7) and in DOPE/DOPC/cholesterol (Fig. 8). Arrows indicate the stacked layers. Magnification: 100 000×.



Figs. 11–14. Pure cardiolipin, complexed to Ca^{2+} (Fig. 11), Mg^{2+} (Fig. 12), Mn^{2+} (Fig. 13) or Ba^{2+} (Fig. 14). Ca^{2+}, Mg^{2+} and Mn^{2+} induce H_{II} phase whereas Ba^{2+} shows stacked layers. Note the difference in organisation of the H_{II} tubes in the different salts. Magnification: 100 000×. The arrow in the lower right corner indicates the shadowing direction.

technique. This quenching technique is characterized by its ultra-rapid cooling rates when compared to conventional freezing methods used in freeze-fracture electron microscopy [16]. Under the conditions of the jet-freezing method, DOPE molecules are prevented from undergoing the H_{II} to lamellar phase transition during quenching.

The data obtained from the periodicity measurements (see Table I) are in good agreement with previous studies, e.g. X-ray diffraction [2,3] and freeze-fracture electron microscopy [18]. In the pure cardiolipin system, the tube to tube periodicity is dependent on the divalent cation involved. DPG-Ca tubes have the smallest periodicity and thus the smallest hy-

Figs. 9 and 10. A sheet (open asterisk), enclosing the H_{II} tubes from the aqueous medium (solid asterisk). Fig. 9: In the PC/DPG/Ca system the smooth fractured sheet is covered with particles, sometimes lying in line with H_{II} tubes (arrow). Fig. 10: In the PC/DPG/Mg system, ridges are observed on the sheet. Magnification: 100 000×.
The arrow in the lower right corner indicates the shadowing direction.

drophilic pore. In the cardiolipin/phosphatidylcholine systems, tubes are found with a larger tube to tube spacing than in the corresponding pure cardiolipin system. This result is in agreement with X-ray data obtained from DPG-Ca and PC/DPG/Ca systems [3]. This strongly indicates that in these tubes both cardiolipin and phosphatidylcholine are present, resulting in a larger hydrophilic pore in the tube. This phenomenon is not confined to PC/DPG mixtures, since also larger H_{II} tubes are found in the DOPE/DOPC/cholesterol mixture, when compared to the tubes in the pure DOPE system. In contrast, two of the investigated PC/DPG mixtures (Mg^{2+} and Mn^{2+}) contained a majority of tubes having the same periodicity values as those in the corresponding pure DPG system. This may be explained in that a phase segregation between DPG and PC has occurred, the former adapting the small type H_{II} phase and the latter a lamellar structure. In the cardiolipin-DOPC Ca system such a segregation of the pure DPG-Ca cylinders is not found. In addition, conglomerates of closely packed lipidic particles are exclusively seen in this system.

The relation between the H_{II} phase tubes and the lipidic particles is quite conspicious in many of our systems. The diameter of the lipidic particles, an average of the particle and pit diameters, shows direct correlation with the periodicity of the H_{II} phase tubes. This correlation suggests a structural relationship which can also be found in the micrographs. Rows of particles can be found lying in line with, or parallel to the H_{II} phase tubes. In Fig. 15 we present a model for this structural relationship. Both the H_{II} phase tube and the inverted micelle perform an inverted molecular organisation. In other words, the H_{II} phase cylinder can be considered as a one-dimensional extension of the spherical inverted micelle structure. In this respect it may be noted that in the PC/DPG/Ca system it is strongly suggested from ^{31}P-NMR data that both phospholipid species are present in the inverted micelles [5].

The transition of H_{II} phase tubes, gradually divergating into smooth fracture faces, may be interpreted in the following manner (see Fig. 16).

H_{II} phase lipids are cone shaped. When these lipids undergo the hexagonal to lamellar phase transition, the molecules assume an overall cylindrical shape. Consequently, these lipids prefer a flat layered configuration. Migration from the H_{II} phase tubes occurs, resulting in extended, flattened cylinders. In the DOPE systems this phenomenon may be induced by the decreasing temperature during the quenching procedure. In the case of the cardiolipin containing

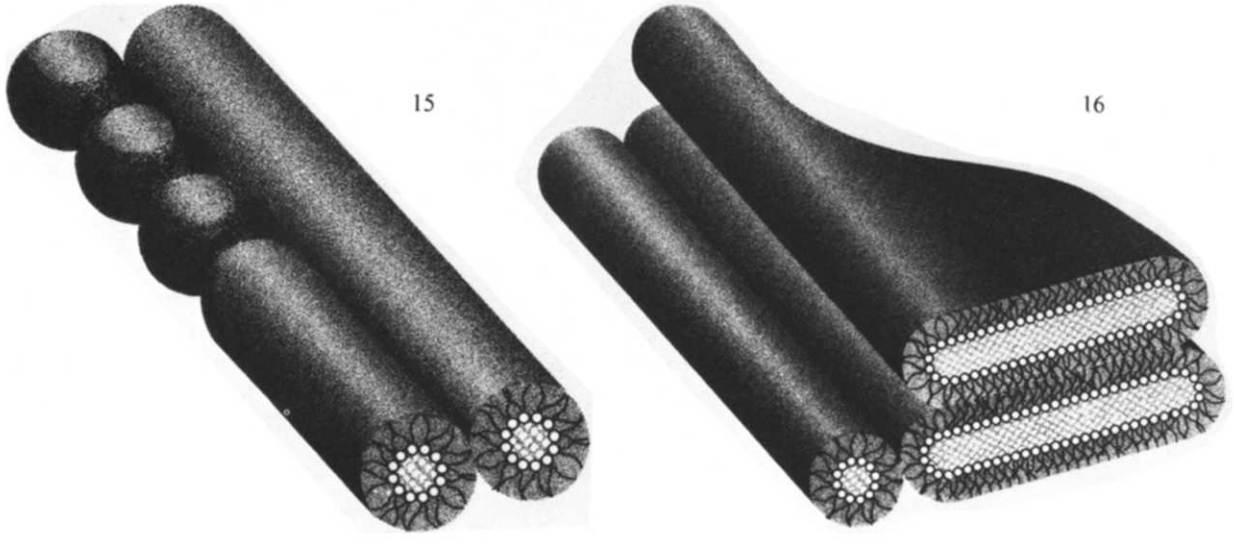


Fig. 15. Model of the relation between H_{II} tubes and the lipidic particles. Both show an inverted molecular organisation.
Fig. 16. Model of the H_{II} to lamellar phase transition. See text for further explanation.

systems a divalent cation gradient rather than a temperature gradient may be the driving force. Alternatively in the PC/DPG mixtures, phase segregation of PC into the smooth layers and DPG in the H_{II} tubes may result.

It has been observed that many of the studied systems containing H_{II} phase tubes are enclosed from the aqueous environment by characteristic morphologies, either by a particle and pit covered smooth fracture face or by a ridge or furrow covered fracture face. The smooth fractured layer may be the reflection of a lipidic monolayer with the acyl chains being oriented towards the hexagonal II phase tubes. The difference in morphology may be explained by assuming that in the case of the particles and pits, inverted micelles are present between the monolayer and the H_{II} phase tubes, whereas in the case of the ridges and furrows the inverted micelles are lacking. It has to be noted that the particles and the ridges are exclusively found on the monolayer facing the aqueous environment whereas the pits and fissures are only seen on the fracture face facing the lipid conglomerate.

Finally, the finding of the morphological relation between H_{II} cylinders and lipidic particles in pure as well as in mixed lipid systems provides additional evidence for the hypothesis that lipidic particles reflect inverted micellar structures.

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