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THE OCCURRENCE OF LIPIDIC PARTICLES IN LIPID BILAYERS AS SEEN BY ^{31}P NMR AND FREEZE-FRACTURE ELECTRON-MICROSCOPY

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Summary

A new type of lipid organization is observed in mixtures of phosphatidylcholine with cardiolipin (in the presence of Ca^{2+}), monoglucosyldiglyceride and phosphatidylethanolamine (in the presence of cholesterol). This phase is characterised by an isotropic ^{31}P NMR signal and is visualised by freeze-fracturing as particles and pits on the fracture faces of the lipid bilayer. As the most favourable model for this phase we propose the inverted micelle sandwiched in between the two monolayers of the lipid bilayer.

Introduction

The bilayer concept of biological membranes is generally accepted. However, it is a remarkable fact that each biological membrane contains lipids which in isolated form do not adopt a bilayer phase. Examples include, cardiolipin (in the presence of Ca^{2+}) [1,2], monoglucosyldiglyceride [3] and unsaturated phosphatidylethanolamines [4—10] which in fully hydrated form prefer the hexagonal H_{II} phase. The presence of these lipids in a membrane will tend to destabilize the bilayer structure and possibly allows the occurrence of non-bilayer phases in the membrane. ^{31}P NMR has proven to be a powerful technique to elucidate the polymorphic phase behaviour of phospholipids [2,6—11]. Phospholipids in a bilayer configuration show an asymmetrical spectrum with a high-field peak and a low-field shoulder whereas phospholipids in the H_{II} phase display spectra with a reversed asymmetry and half the spectral width (see Fig. 1, in Ref. 10). In investigations on the phase behaviour of mixtures of unsaturated phosphatidylethanolamines with phosphatidylcholines and cholesterol an 'isotropic' phase was observed intermediate between the bilayer

and the hexagonal H_{II} phase [7,8]. This macroscopically large phase had a narrow symmetrical ^{31}P NMR signal indicating effectively isotropic motion of the phospholipid molecule which for instance is possible by fast lateral diffusion in an inverted micellar structure. Recently, freeze-fracture electron microscopy visualised the presence of small (100 Å diameter) lipidic particles of an inverted micellar nature in Ca^{2+}-containing mixed phosphatidylcholine-cardiolipin bilayers [12]. In this study we describe the ^{31}P NMR characteristics and freeze-fracture morphology of several lipid mixtures in which one component favours the bilayer and the other the hexagonal H_{II} phase in order to understand the 'isotropic phase' in structural terms, i.e. lipidic particles.

Experimental

Cardiolipin was purchased from Sigma (St. Louis, U.S.A.) and cholesterol from Fluka (Buchs, Switzerland). Egg phosphatidylcholine was isolated from hen eggs and 1,2-dioleoyl-*sn*-glycerol-3-phosphorylcholine ($18:1_c/18:1_c$-phosphatidylcholine) and 1,2-dioleoyl-*sn*-glycerol-3-phosphatidylethanolamine ($18:1_c/18:1_c$-phosphatidylethanolamine) were synthesized as described before [6,13]. 3-(*O*-α-D-glucopyranosyl)-1,2-diglyceride (monoglucosyldiglyceride) was isolated from membranes of *Acholeplasma laidlawii* cells grown on a medium supplemented with elaidic acid ($18:1_t$) as described before [14]. The fatty acid composition of this lipid in mol% was 12 : 0 (4.3%), 14 : 0 (7.0%), 16 : 0 (19.0%), 18 : 0 (1.2%), $18:1_t$ (64.3%), 18 : 2 (1.1%), and unknown fatty acids 3.1%. All lipids were chromatographically pure. Phospholipids dispersions were made as described before [7] by dispersing at 30°C a dry film of 50—100 μmol lipid in 1.3 ml 15% ^2H_2O containing 100 mM NaCl, 0.2 mM EDTA, 10 mM Tris-HCl (pH 7.0) buffer. Small vesicles were prepared by ultrasonication [15] and large unilamellar vesicles were made as described elsewhere [12] using the ether evaporation method of Deamer and Bangham [16]. ^{31}P NMR spectra were recorded at 36.4 MHz under conditions of proton decoupling as described before [7], generally a sweep width of 12 kHz and a pulse rate of 0.17 s using 45° radio frequency pulses was used. To enhance the signal to noise ratio, the free induction decay in these experiments was multiplied by an exponential function resulting in an 50 Hz line broadening. To obtain 'high-resolution' spectra of isotropic phases a sweep width of 1200 Hz and a pulse rate of 1.7 s using 90° radio frequency pulses was used. In these experiments the exponential multiplication of the free induction decay resulted in a 2 Hz line broadening. Freeze-fracture electron microscopy was performed as outlined previously [17]. Glycerol was added to the samples to prevent freeze damage.

Results and Discussion

Phosphatidylcholine-cardiolipin (Ca^{2+})

In the absence of Ca^{2+} an aqueous dispersion of an equimolar mixture of egg phosphatidylcholine and cardiolipin consists of bilayers as evidenced by ^{31}P NMR (Fig. 1A) and freeze-fracturing (Fig. 1E). Addition of increasing amounts of Ca^{2+} to the sample results in the appearance of a sharp signal at the reso-

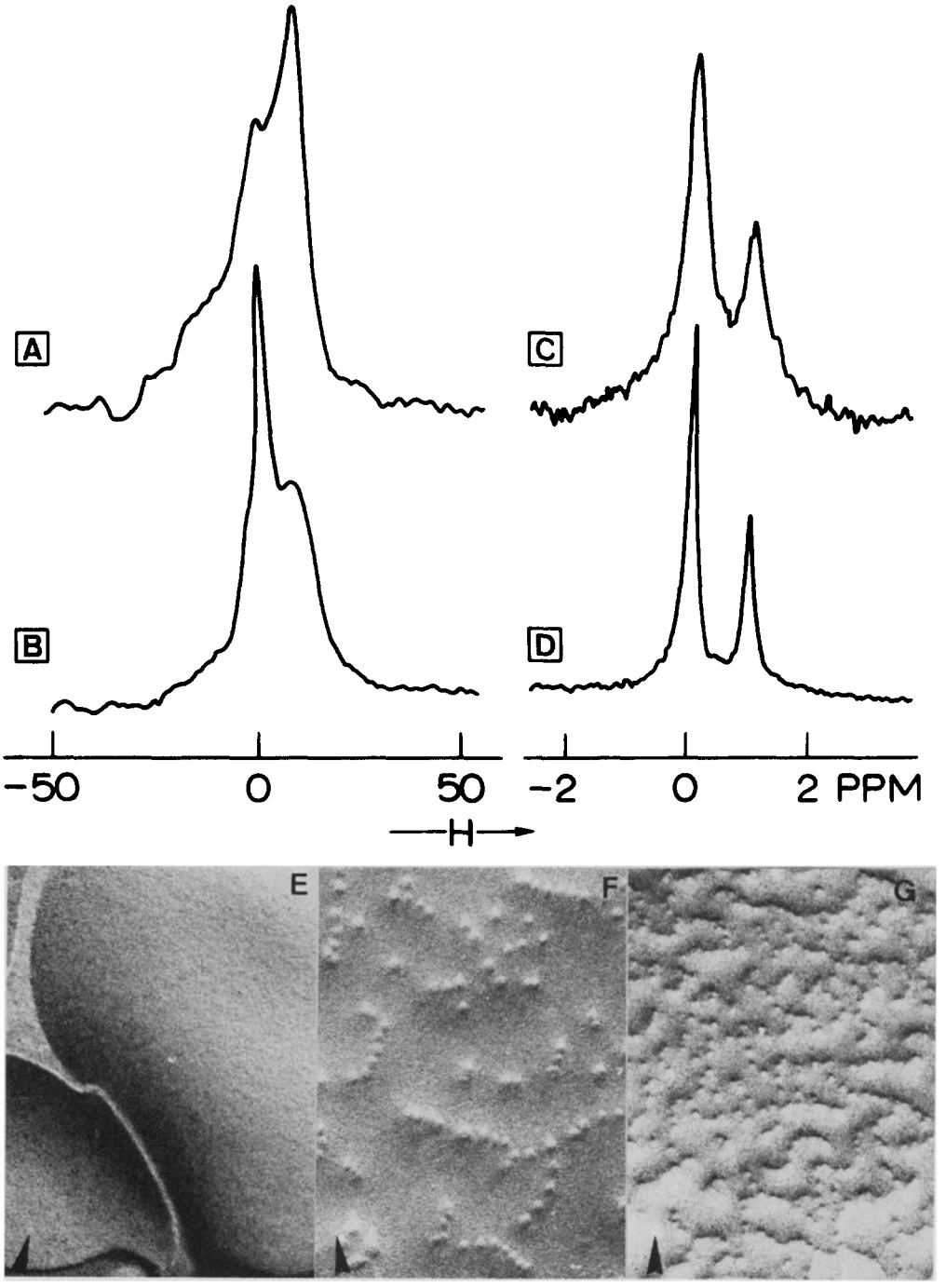

Fig. 1. 36.4 MHz ^{31}P NMR spectra and freeze-fracture morphology at 30°C of egg phosphatidylcholine-cardiolipin mixtures in the absence and presence of Ca^{2+}. (A) ^{31}P NMR spectrum, and (E) freeze-fracturing of an aqueous dispersion of an equimolar mixture of phosphatidylcholine and cardiolipin (100 mM total phospholipid). (B) ^{31}P NMR spectrum, and (F, G) freeze-fracturing of the same sample after the addition of an aliquot of 100 mM $CaCl_2$ solution (final Ca^{2+} concentration in the sample 10 mM). (F) and (G) show, respectively, particles and pits associated with the lipid bilayer. (C) ^{31}P NMR 'high-resolution' spectrum of the isotropic phase observed in large unilamellar phosphatidylcholine-cardiolipin-Ca^{2+} vesicles. (D) ^{31}P NMR spectrum of sonicated mixed phosphatidylcholine-cardiolipin (1 : 1) vesicles (25 mM total phospholipid). Magnification of the micrographs about 100 000×; the arrow in the freeze-fracture pictures indicates the direction of shadowing.

nance position of phospholipids undergoing isotropic motion (Fig. 1B). Freeze-fracturing reveals that the sample mainly consists of bilayers in which numerous small particles and pits of a diameter of 100 Å and 70 Å, respectively, are present (Fig. 1F and G). These particles and pits are predominantly organized in strings. Small vesicles are not observed. A minor fraction of the material was organized in the hexagonal H_{II} phase which also is indicated in the ^{31}P NMR spectrum (Fig. 1B) by the shoulder at 4 ppm (compare refs. 2 and 7) suggesting that by the addition of Ca^{2+} a small fraction of the cardiolipin in the sample is converted to the hexagonal H_{II} phase. The particles and pits observed in Fig. 1F and G are very similar to those observed by freeze-fracturing in large unilamellar equimolar phosphatidylcholine-cardiolipin (Ca^{2+}) vesicles [12]. The ^{31}P NMR spectrum of these vesicles also consists of a broad bilayer component and a narrow isotropic signal (spectrum not shown). These experiments strongly suggest that the narrow ^{31}P NMR signal originates from these particles. To obtain information on the chemical composition of these particles the isotropic component in the ^{31}P NMR spectrum of these large unilamellar vesicles was recorded under 'high-resolution' conditions (Fig. 1C, note the difference in scale with Fig. 1B). The spectrum of the isotropic phase is composed of two signals with an intensity ratio of 2 : 1. These signals are not separated in Fig. 1B because of the large line broadening applied to the free induction decay. The high-resolution spectrum of sonicated phosphatidylcholine-cardiolipin (1 : 1) vesicles also consists of two signals with an intensity ratio of 2 : 1 (Fig. 1D). The low-field peak originates from cardiolipin since this contains two phosphorus atoms/molecule whereas the high-field peak originates from phosphatidylcholine, since its chemical shift is identical to what is observed for pure phosphatidylcholine vesicles. It can be concluded from these experiments that the chemical composition of the isotropic phase is identical to the overall composition of the sample.

Phosphatidylcholine-monoglucosyldiglyceride

X-ray analysis has shown that the glycolipid monoglucosyldiglyceride isolated from *A. laidlawii* cell membranes forms a hexogonal H_{II} phase in excess water [3]. This can also be visualized by freeze-fracturing. Independent on the fracture direction long striations with a 60 Å periodicity are observed (Fig. 2C) which are characteristic for the hexagonal H_{II} phase [2,18–20].

In the presence of an equimolar quantity of egg phosphatidylcholine at 4°C the only structures observed are bilayers as shown by ^{31}P NMR (Fig. 2A) and freeze-fracturing (Fig. 2D), demonstrating that due to the bilayer-stabilizing ability of phosphatidylcholine also the monoglucosyldiglyceride has adopted a bilayer configuration. It is a general observation that membrane lipids prefer at higher temperatures non-bilayers phases [3,5–9,21]. This can be illustrated with the phosphatidylcholine-monoglucosyldiglyceride dispersion. At temperatures up till 50°C the ^{31}P NMR spectrum of the sample remained unchanged, but at 55°C a narrow signal appeared in the spectrum which grew in intensity with increasing temperature such that at 60°C the entire spectrum consists of this narrow line (Fig. 2B), demonstrating that (virtually) all phosphatidylcholine molecules undergo (nearly) isotropic motion. During this transition no visible change occurred in the milky, liposomal like, dispersion. Upon cooling the iso-

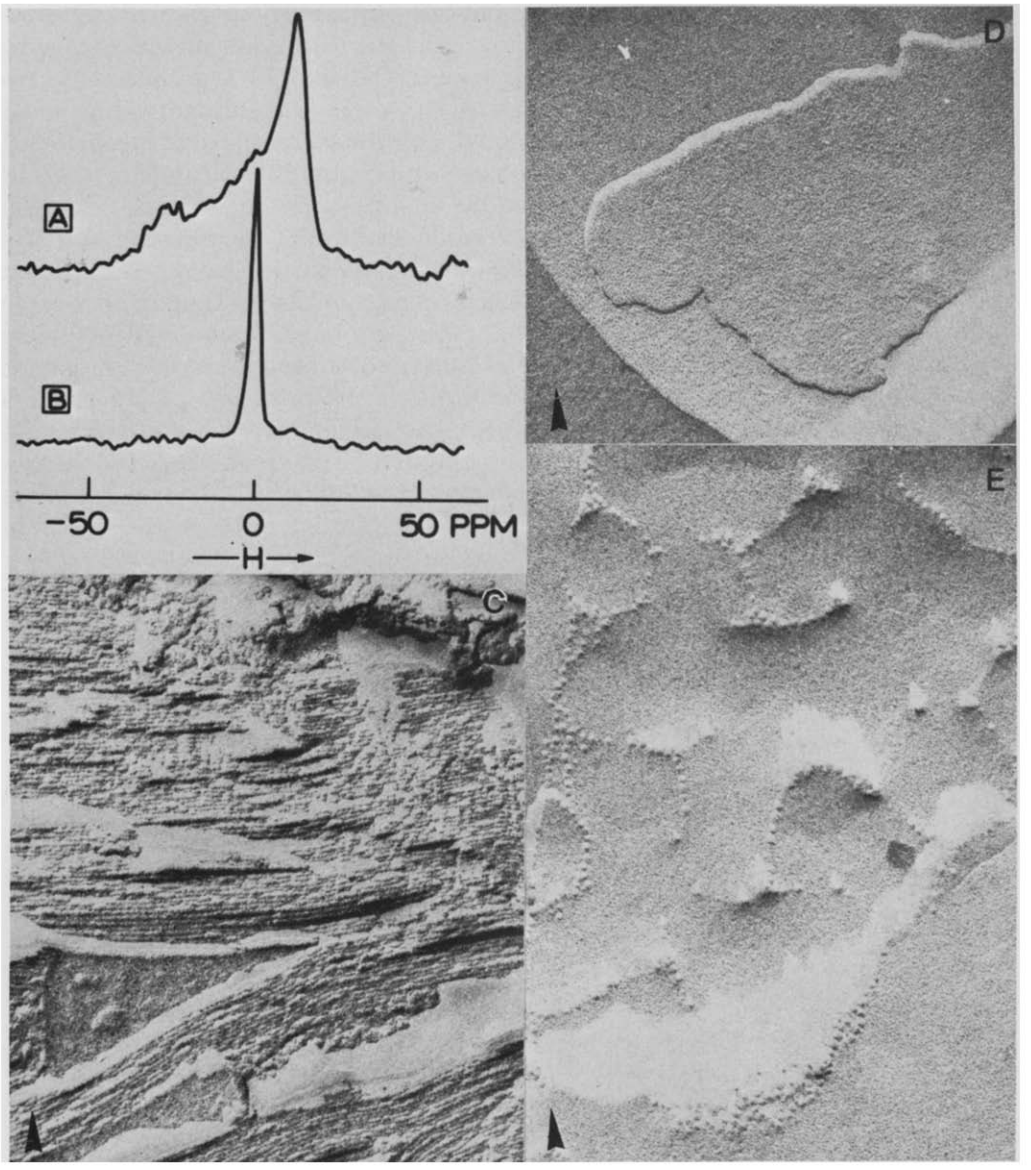

Fig. 2. 36.4 MHz ^{31}P NMR spectrum and freeze-fracture morphology of an equimolar egg phosphatidylcholine-monoglucosyldiglyceride dispersion. (A) ^{31}P NMR, and (D) freeze-fracturing of the sample at 4°C. (B) ^{31}P NMR at 20°C, and (E) freeze-fracturing (at 30°C) of the same sample after being heated till 60°C. (C) Freeze-fracturing of a monoglucosyldiglyceride dispersion at 4°C. In (E) pits are visible at the bottom part of the micrograph. Magnification of the micrographs about 100 000X.

tropic phase displayed strong hysteresis as was noticed previously for phosphatidylethanolamine-containing lipid mixtures [8]. Down to 20°C the narrow line was observed, below that temperature the spectrum changed back to the shape characteristic of bilayer structure. When the sample was quenched from 30°C, after being heated till 60°C the fracture face showed extended bilayers on which, string wise organized, particles and pits of a diameter of about 70 Å

and 40 Å, respectively, were present (Fig. 2E). These results again strongly suggest that a close correlation exists between the occurrence of these particles on the fracture face and the presence of a narrow ^{31}P NMR signal at the chemical shift position of isotropic motion.

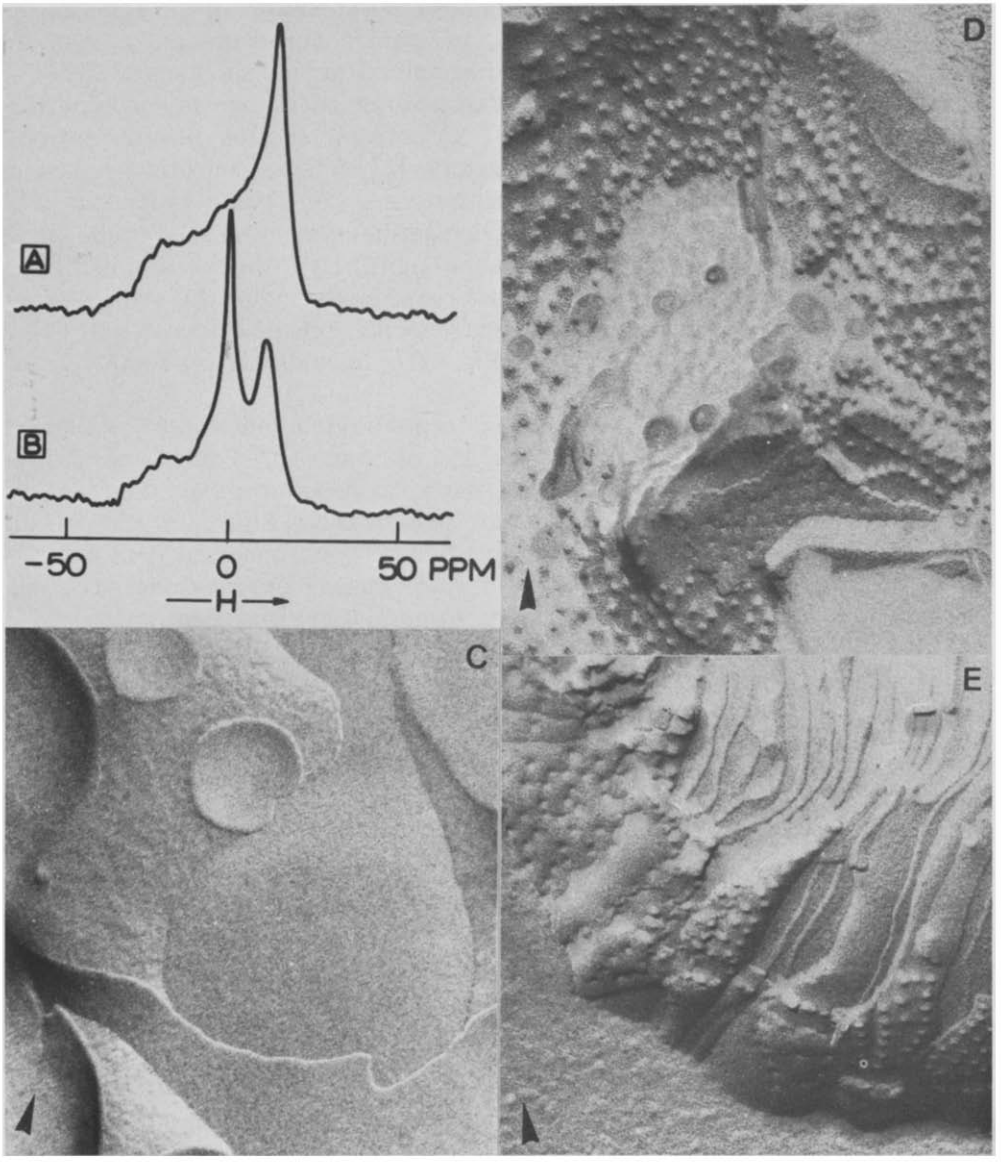

Fig. 3. ^{31}P NMR and freeze-fracturing of an aqueous dispersion of 18 : 1_c/18 : 1_c-phosphatidylethanolamine : 18 : 1_c/18 : 1_c-phosphatidylcholine : cholesterol (3 : 1 : 2). (A) ^{31}P NMR, and (C) freeze-fracturing of the sample at 10°C. (B) ^{31}P NMR and (E) freeze-fracturing of the sample at 10°C after being heated till 60°C. Magnification of the micrographs about 100 000X.

Phosphatidylethanolamine-containing mixtures

Dependent upon temperature, thermal history and composition aqueous dispersions of phosphatidylethanolamine, phosphatidylcholine and cholesterol have ^{31}P NMR spectra characteristic of the bilayer, the hexagonal H_{II} and the 'isotropic' phase [6—9]. Fig. 3 illustrates this for the sample 18 : 1_c/18 : 1_c-phosphatidylethanolamine : 18 : 1_c/18 : 1_c-phosphatidylcholine : cholesterol (3 : 1 : 2). An aqueous dispersion of these lipids at 0°C displays a ^{31}P NMR spectrum (Fig. 3A) and a freeze-etch morphology (Fig. 3C) characteristic of the lamellar phase. At increasing temperatures the following spectral changes were observed: from 15 till 35°C the spectrum changed gradually from the bilayer to the hexagonal H_{II} shape and remained further unchanged till 60°C at which temperature part of the signal was observed in a narrow symmetrical peak at the resonance position typical of isotropic motion of the phospholipid molecule. Upon cooling the hexagonal H_{II}—bilayer transition reversibly occurred but in agreement with previous studies [6—9] and in analogy with the phosphatidylcholine-monoglucosyldiglyceride system this isotropic phase exhibits a pronounced hysteresis. Upon cooling till 10°C the spectrum still contains the isotropic component (Fig. 3B). Freeze-fracturing of this sample shows extended bilayers on which numerous particles and pits of a diameter of 110 Å and 80 Å, respectively, are present (Fig. 3D). In addition on some bilayers ridges are present (Fig. 3E).

Different phosphatidylethanolamine/phosphatidylcholine and phosphatidylethanolamine/phosphatidylserine (with and without Ca^{2+}) mixtures showed complex ^{31}P NMR and freeze-fracture characteristics. Broad symmetrical ^{31}P NMR spectra (similar to the spectrum presented in Fig. 2 of Ref. 8) and irregular bilayer surfaces with ridges and fissures were observed (van Echteld, C.J.A., Gerritsen, W.J. and Verkley, A.J., unpublished observations). The relation of these structures with the particles is under active investigation.

Nature of the particles

The central observation of this study is that for a number of different lipid mixtures next to the classical bilayer a new type of structural organisation of the lipids is observed which is characteristic by fast isotropic motion of the lipid molecules (correlation time approx. 10^{-6} s) and which can be visualized by freeze-fracturing as small particles and corresponding pits with a uniform size which are associated with the lipid bilayer.

The lipid mixtures displaying this behaviour have as a common characteristic in that at least one of the components prefers the hexagonal H_{II} phase, e.g. cardiolipin in the presence of Ca^{2+}, monoglucosyldiglyceride and unsaturated phosphatidylethanolamine. The ability of lipids to adopt the bilayer or hexagonal H_{II} phase depends on the geometry of the molecule. Cylindrical molecules prefer the bilayer and cone-shaped molecules (polar head group being smaller end of the cone) prefer the hexagonal H_{II} phase. The formation of the particles in mixtures of these lipids must be the result of a delicate balance of molecular shapes. This is well illustrated in the strong temperature dependence of the particle formation. In the case of monoglycosyldiglyceride and phosphatidylethanolamines-containing mixtures these particles are formed when the temperature is raised by only a few degrees. Below this transition temperature

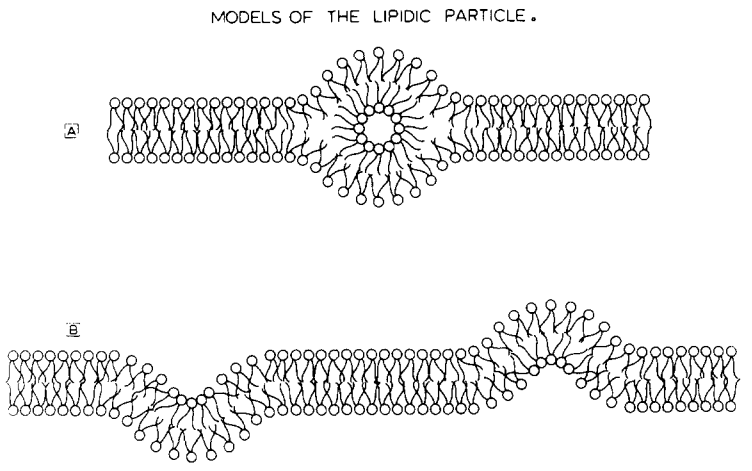

Fig. 4. Models of the lipidic particles; (A) The inverted micelle model. (B) The bulged-bilayer model. The bilayer thickness and the size of the particle is drawn to scale.

all lipids prefer the bilayer phase but because of decreased chain order at higher temperatures the cone character of the lipid molecules increases beyond a critical value which results into a structural reorganisation such that part of the lipids can adopt an inverted structure which is thermodynamically more stable. A striking analogy is found in the bilayer—hexagonal H_{II} transition of natural phosphatidylethanolamines [9]. This transition involving massive reorganization of the lipids occurs over a narrow temperature and requires only a small amount of energy [9]. The two most likely models for the structure of the particles are shown in Fig. 4. In one model part of the lipids is organized in an inverted micelle sandwiched in between two monolayers (Fig. 4A). In the alternative model the lipid bilayer is bulged such that hemi-spheres with a small radius of curvature are present on either side of the bilayer (Fig. 4B). In both models part of the lipids can undergo (nearly) isotropic motion. In model A this can be achieved by tumbling of the inverted micelle in the bilayer and/or by lateral diffusion of the lipids in the inverted micel or alternatively by fast lateral diffusion in the curved monolayers surrounding the inverted micelle. In model B lateral diffusion of the lipids in the hemi-sphere provides the isotropic averaging of the ^{31}P chemical shift anisotropy. Both models account for the observation that the fracture face consists of a bilayer on which particles and pits are present. In view of the geometrical shapes of the lipid molecules it can be speculated that cone-shaped molecules will prefer a location in areas of inward curvature, e.g. in the inverted micelle of model A or in the inner monolayer of the hemi-sphere of model B whereas the cylindrical-shaped molecules will be preferentially located in the curved monolayers around the inverted micelle or alternatively in the outer monolayer of the hemi-sphere. Both lipids can be associated with the particle as is clearly demonstrated in the high-resolution spectrum of the isotropic phase in the phosphatidylcholine-cardiolipin (Ca^{2+}) system (Fig. 1C).

For all three systems tested the amount of phospholipid observed in the isotropic ^{31}P NMR signal is much larger than the fraction of the lipids organized in particles as detected by freeze-fracturing. This strongly suggests that rapid

transitions (exchange frequence 10^{-5}—10^{-6} s) of phospholipids between the bilayer and the particle occur.

Further evidence for a rapid exchange of lipids between the bilayer and the particle can be derived from the ^{31}P NMR line widths. The ^{31}P NMR line width of phospholipids in sonicated vesicles depends to a large extent on vesicle tumbling and lateral diffusion of the lipids [15,22]. An increase in vesicle size, decreases the tumbling rate and broadens the resonance. The chemical shift anisotropy and thus the structure in the phosphate region of the polar head group is similar for lipids organized in bilayers or in the hexagonal H_{II} phase [7,11]. It can therefore be assumed that also for other isotropic phases the line width will primarily depend on vesicle tumbling and lateral diffusion. The line width of the cardiolipin and phosphatidylcholine resonance in the isotropic phase is at least 2—3 times larger than the line width of the corresponding resonances of those lipids in sonicated vesicles (Fig. 1A) which have an average size of approximately 250 Å as detected by freeze-fracturing. Using the theory developed by Saffman and Dellbrück [23] as applied by Cullis [22] on vesicle systems and by taking an inverted micelle size of 60 Å [twice the length of a lipid molecule of 20 Å and an aqueous compartment of 20 Å which can be taken for the size of the aqueous channel in hexagonal H_{II} phase [7]] it can be calculated for model A that even for an infinite large bilayer viscosity (e.g. no tumbling can occur) lateral diffusion in the inverted micelle should be at least two orders of magnitude lower than in the case of the normal bilayer to account for the observed line width. The same applies for model B. Such a very low lateral diffusion rate seems unlikely. Therefore other mechanisms should be responsible for the observed line broadening. Exchange of lipids between the particle and the bilayer is the most attractive mechanism. For the inverted micelles the exchange most likely occurs via the model presented in Fig. 6 of Ref. 7. In model B lateral diffusion of lipids in the bulged bilayer can cause this exchange broadening.

At present no absolute evidence for one of the two models of the particle is available. However, in view of the following three arguments we strongly favor the model of the inverted micelle.

(I) The particles and pits have an uniform size in each system, e.g. for phosphatidylcholine-cardiolipin (Ca^{2+}) about 85 Å, for phosphatidylcholine-glucosyldiglyceride 55 Å and for 18 : 1_c/18 : 1_c-phosphatidylcholine-18 : 1_c/18 : 1_c-phosphatidylethanolamine-cholesterol (3 : 1 : 2) 95 Å which is close to the expected size of the inverted micelle. For the hemi-sphere model the bilayer is very strongly curved which is unlikely because for instance the average size of sonicated mixed phosphatidylcholine-phosphatidylethanolamine vesicles is 250 Å and increases with increasing phosphatidylethanolamine concentration [24].

(II) It is difficult to explain in model B the profound hysteresis observed in the temperature-induced particle formation because the hemi-sphere is part of a continuous bilayer. For the inverted micelle model the strong hysteresis can be more easily understood since the transition involves the conversion from a three to a two dimensional phase. In mixed phosphatidylcholine-lysophosphatidylcholine mixtures structural transitions involving micellar phases also show a strong hysteresis (van Echteld, C., de Kruijff, B. and de Gier, J., unpublished results).

(III) In model B the barrier properties of the bulged bilayer should be similar to particle-free bilayers. In the inverted micelle model due to the dynamical nature of the system the lipids and possible also the aqueous compartment within the inverted micelle will rapidly move across the bilayer (see Fig. 6 of Ref. 7). Therefore bilayer permeability should be greatly increased. Addition of 0.4 mM $MnCl_2$ to large unilamellar equimolar phosphatidylcholine-cardiolipin (Ca^{2+}) vesicles (100 mM phospholipid) broadens both the isotropic and also the bilayer component of the spectrum beyond detection. In contrast, addition of 0.4 mM $MnCl_2$ to sonicated equimolar phosphatidylcholine-cardiolipin vesicles (25 mM total phospholipid) broadens 62% of the total signal beyond detection. The remaining 38% of the intensity originates from the inner monolayer of these vesicles. The phosphatidylcholine-cardiolipin signal intensity ratio of the inner monolayer was identical to the signal intensity ratio of the total vesicle dmonstrating that the vesicle had a symmetrical phospholipid composition. These experiments show that the phosphatidylcholine-cardiolipin bilayer is impermeable to Mn^{2+} but that this cation can move rapidly through the particle-containing bilayer.

Several speculations can be made on the biological implications of the finding that alternative lipid phases can occur in conjugation with the lipid bilayer. At present we only would like to point out that isotropic motion of the phospholipids in a biomembrane, e.g. the metabolically very active microsomal membrane have already been independently reported by two different laboratories [25,26]. Furthermore the observation that lipidic particle also occur in the total lipid extract of inner mitochondrial and *Escherichia coli* membranes (de Kruijff, B. and Verkleij, A.J., unpublished observations) strongly suggests a wide-spread occurrence of this new type of membrane lipid organization.

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