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POLYMORPHIC PHASE BEHAVIOUR OF DILINOLEOYLPHOSPHATIDYLETHANOLAMINE AND PALMITOYLOLEOYLPHOSPHATIDYLCHOLINE MIXTURES

STRUCTURAL CHANGES BETWEEN HEXAGONAL, CUBIC AND BILAYER PHASES

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The polymorphic phase behaviour of dilinoleoylphosphatidyethanolamine (DLPE) and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) is investigated by freeze-fracture electron microscopy, X-ray diffraction and ^{31}P-NMR. The structures at 5% or less POPC are predominantly inverted hexagonal (H_{II}), whereas at 15% or more POPC, the structure is mostly bilayer (L), interrupted by defects (lipidic particles). A cubic phase structure is observed in the transition range between H and L phases; the cubic arrangement deteriorates at higher temperatures into an amorphous aggregate of spherical units. Both cubic and amorphous structures contribute to the isotropic ^{31}P resonance, with no preference for PC or PE partitioning in the isotropic motion as observed by high resolution NMR. The existence of the cubic phase seems to depend critially on the homogeneity and the degree unsaturation of the phospholipids.

Introduction

Many studies of phosphatidylethanolamine (PE) have shown that, under certain conditions, unsaturated PE prefers nonbilayer structures, such as the inverted hexagonal phase (H_{II}), the lipidic particles and the cubic phase [1–6]. Some of these nonbilayer structures have been implied to be an intermediate state of membrane fusion [2,4,7]. The bilayer-to-nonbilayer transition possibly modulates membrane functions, protein incorporation and activities [3]. This property of PE leads to the proposition that PE may play an important role in controlling fusion and other functions of biological membranes through the destabilization of the bilayer.

When unsaturated PE are mixed with other phospholipids or cholesterol, their phase states are modified by the other components in the mixture. For instance, when phosphatidylcholine (PC) is mixed with PE, the bilayer-forming PC tends to stabilize the bilayer, thereby expanding the bilayer region in the phase diagram of the mixtures [3,5]. In our previous study [3] we found that the bilayer-to-H_{II} transition in soybean PE and egg PC mixtures was a continuous process; the transition region covered a wide area in the phase diagram (from 5 to 25% of egg PC). In this composition and temperature region numerous lipidic particles (LIP) were observed as interbilayer attachment sites [7], together with co-existing bilayers and H_{II} structures. This phase diagram differs from that of

Abbreviations: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DLPE, dilinoleoylphosphatidylethanolamine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine.

a mixture of saturated synthetic PE and PC [8]. Cullis and De Kruijff [5], using ^{31}P-NMR, suggested an isotropic phase as the intermediate phase between the bilayer and the H_{II} structure. In some of their studies, they have observed spectra consisting entirely of isotropic resonances, without signals indicative of either a bilayer or a H_{II} phase. The structure associated with this isotropic signal cannot be defined by ^{31}P-NMR studies alone. On the other hand, an inverted cubic phase has been proposed as a possible intermediate structure between the hexagonal and the lamellar phases in other systems [9,10].

In view of the complications of bilayer to H_{II} phase transition and the interest associated with the instability in the transition range, we undertook a detailed study of the mechanism of bilayer to H_{II} phase transition, using two well defined synthetic lipids, i.e. dilinoleylphosphatidylethanolamine (DLPE) and palmitoyloleoylphosphatidylcholine (POPC). We chose these two lipids because DLPE has sufficiently unsaturated acyl chains to simulate the natural soybean PE, whereas the POPC is very similar to natural egg PC. Both lipids contain known acyl chains and have well defined and reproducible properties.

Methods and Materials

DLPE and POPC were purchased from Avanti Biochemicals (Birmingham, AL). The lipids were desolved in chloroform, mixed in given molar ratios, and stored at −70°C prior to use. The mixed lipids were evaporated under vacuum from nitrogen atmosphere and hydrated in a buffer solution containing 100 mM of NaCl, 2 mM of histidine, 2 mM of Tes, and 2 mM of EDTA at pH 7.4. The lipids were suspended by vortexing. In the mixtures containing high PE molar ratios, it was sometimes necessary to remove the lipids from the glass wall by scraping with a glass rod. The lipids were allowed to remain in suspension for more than an hour to insure uniform hydration. These lipid suspensions were then concentrated by centrifugation and incubated at different temperatures for at least one hour before experiments. No lipid degradation was observed by thin-layer chromatography after experiments.

The samples were divided into three aliquots. One aliquot containing approx. 20 mg was used for ^{31}P-NMR experiments. The remainder was used for X-ray diffraction analysis and for rapid freezing for freeze-fracture study by electron microscopy. ^{31}P-NMR experiments were performed on a Bruker WP-200 Fourier transform spectrometer operating at 81 MHz. Samples in 10 mm NMR tube were allowed to equilibrate at given temperatures for at least 15 min before data collection. All spectra were taken under continuous broadband proton decoupling with a 40° pulse angle. For low-resolution spectra a sweep width of 20 kHz was employed, collecting 2K data points and up to 30000 scans with a 0.15-s delay between pulses. For high resolution spectra of isotropic phases we used a 1000 Hz sweep width, collected 4K data points, and acquired over 1000 scans with a 2-s delay. No appreciable changes in the spectra were noted following up to 6 h of incubation at each temperature. A line broadening of 100 Hz or 1 Hz was employed during signal enhancement for low and high resolution spectra, respectively. The method for X-ray diffraction analysis was given previously [11]. Samples were loaded in X-ray diffraction sample holders with mica windows. Both small- and wide-angle diffractions were recorded using a Frank-type camera, and a line X-ray source was employed. Each exposure required between 10 to 24 h. Procedures for rapid-freezing were also described previously [12]. Briefly small aliquots of the specimen were placed between thin copper plates and rapidly frozen, without cryoprotectant, by dipping the sandwich into liquid propane cooled by liquid nitrogen. The cooling rate exceeded 5000 K/s. This freezing rate might be slightly reduced for samples that were difficult to spread thin. No ice damage was observable, judging from the smooth fractured faces of the aqueous buffer.

Results

Pure DLPE gives a typical H_{II} line shape in ^{31}P-NMR spectra at all experimental temperatures between 10 to 50°C. The spectrum at 20°C is shown in Fig. 1. The X-ray diffraction pattern shows three small-angle lines with a spacing ratio of $1:(1/\sqrt{3}):(1/2)$, typical of hexagonal lattice reflections. Micrographs of DLPE consist of pre-

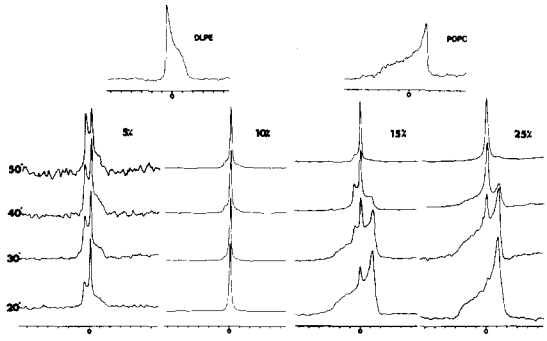


Fig. 1. 81 MHz ^{31}P-NMR spectra of POPC/DLPE mixtures. (Left to right) Mixtures of 5, 10, 15 and 25% of POPC in DLPE taken at the stated temperatures (horizontal). Each mark on the scale represents 10 ppm, 0 being inorganic phosphate. Top inset: Spectra of pure POPC and DLPE at 20°C.

dominantly H_{II} tubes as shown in Fig. 2a. There is little doubt that at temperatures above 10°C, pure DLPE in excess water exists only in the H_{II} form.

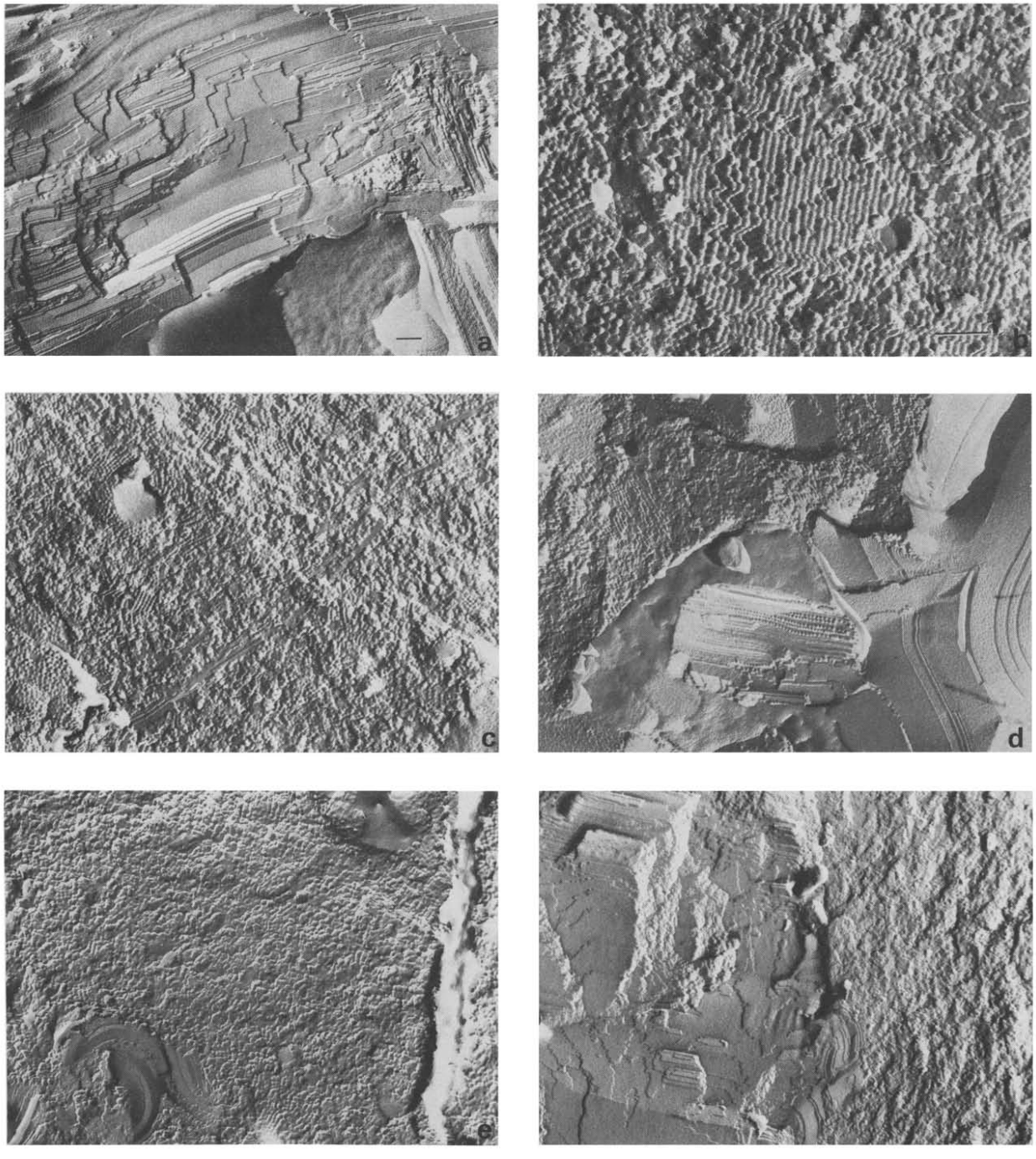
At the composition range between 5% through 15% of POPC in DLPE, the phase state of the mixtures are very complicated. ^{31}P-NMR of 5% of POPC contain a mixture of an isotropic resonance and a component typical of the H_{II} lineshape. The sample is optically translucent. With increasing temperature the H_{II} component seems to grow in relation to the isotropic resonance. At 10 and 20°C the X-ray diffraction pattern consists of five lines. The ratio of spacing of this five lines are $1:(1/\sqrt{2}):(1/\sqrt{4}):(1/\sqrt{6}):(1/\sqrt{8})$. At 40°C, the intensity of two of these lines decreases. The remaining three lines at spacing ratios of $(1/\sqrt{2}):(1/\sqrt{6}):(1/\sqrt{8})$ form a pattern similar to a hexagonal diffraction pattern. An example of the small-angle diffraction pattern is shown in Fig. 3. The five line pattern can be matched with a cubic lattice diffraction index. The possible index numbers are given in Table I. With the limited number of lines we have observed, we could not unambi-

TABLE I

SMALL-ANGLE X-RAY DIFFRACTION SPACINGS OF DLPE/POPC MIXTURES

Brackets indicate diffuse reflections.

POPC/DLPE molar ratio	Spacing (Å)				Assigned indexes		
	10°	20°	40°	50°	Cubic	Hexagonal	Lamellar
0/100	66	60				100	
	37.5	34.5				110	
	33	31				200	
5/95	88				200		
	66	64.5	62	56	220	100	
	48	46	43.5		400		
	38	37	36	33	422	110	
	33	32	30	27.5	440	200	
10/90	(92)	(105)	67			100	
	(53)		39			110	
	(45)		33			200	
15/85			(87)			100	
		55	(51)			110	100
			(43)			200	
		27.5					200
		18.5					300
25/75		59	60	60			100
		29.5	30	30			200
		19.7					300
100/0		66					100
		33					200



giously assign their indices and determine the space group of the crystal lattice. Nevertheless, the additional lines definitely cannot be derived from a hexagonal or a lamellar structure. If the cubic lattice reflection assignment of (200), (220), (400), (422), and (440) is correct, then we may deduce the unit cell dimension from the diffraction pattern. The unit cell dimension varies from 175 Å at 40°C

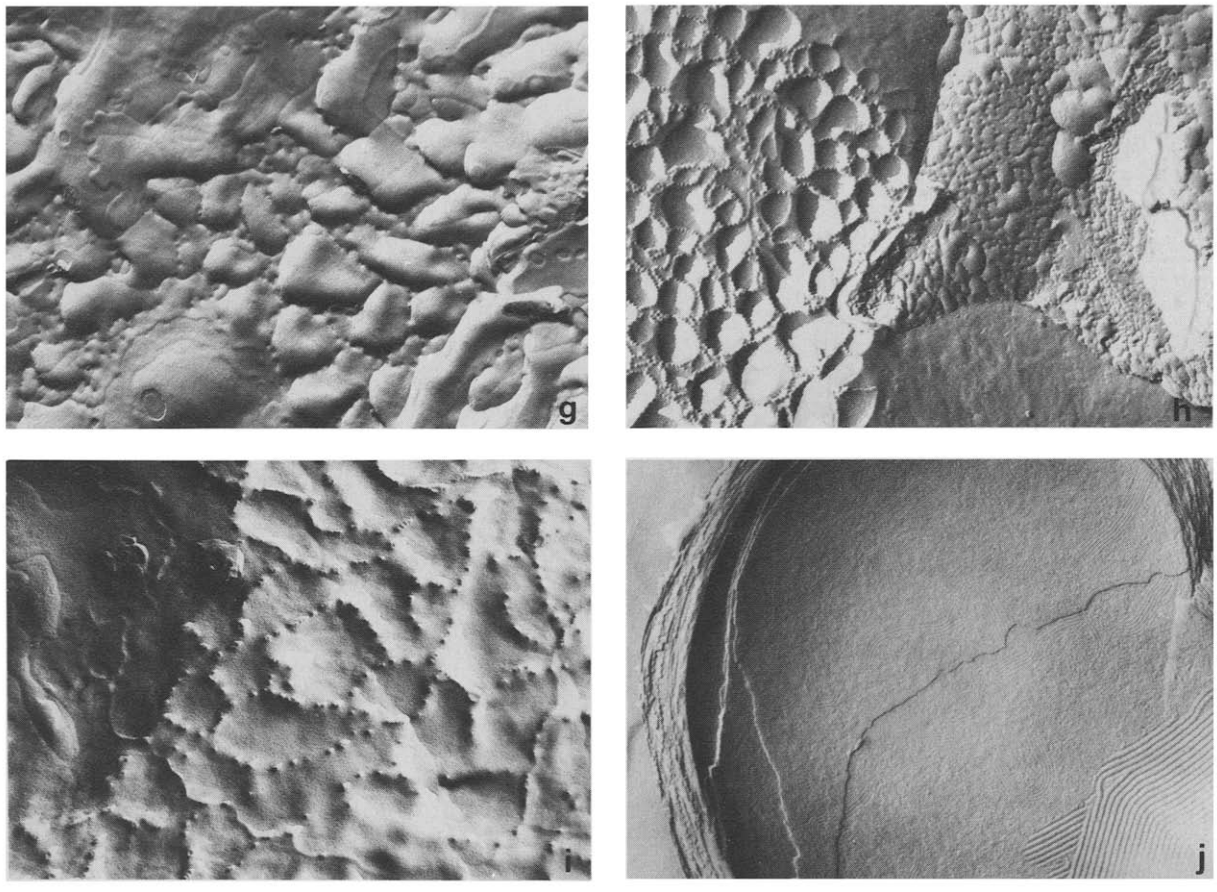


Fig. 2. Freeze-fracture electron micrographs of samples containing different mole percentages of POPC in DLPE. The samples were rapidly frozen at various temperatures as indicated: (a) 0%, 20°C; (b,c) 5%, 20°C; (d) 5%, 40°C; (e) 10%, 20°C; (f) 10%, 40°C; (g) 15%, 20°C; (h) 15%, 40°C; (i) 25%, 40°C; and (j) 100%, 20°C. All micrographs except (b) are at the same magnification. Bars: 100 nm.

to 187 Å at 10°C. At 40°C, the three strongest reflection lines can also be assigned to a hexagonal lattice, with a unit cell dimension of 71 Å. These spacings are summarized in Table I. Freeze-fracture electron microscopy provides the most interesting findings of the molecular organization. At temperatures below 20°C, electron micrographs show predominantly a cubic arrangement of small spherical units. The unit spheres are approx. 180 Å in diameter (Fig. 2b). The cubic structures are not perfectly packaged over the entire fracture face. Between the cubic structures, there are areas where the these spherical units are less organized. We shall refer to this disordered structure as an amorphous phase. In the less ordered areas of the sample, large, bubble-like structures can also be seen, and H_{II} tubes are seen intermixed with cubic structures. A view of this structure is shown in Fig. 2c. As the temperature is raised to 40°C, H_{II} tubes similar to the ones shown in Fig. 2a are commonly seen. In some areas the spherical units are seen to line-up along the ends of H_{II} tubes. In these areas, the H_{II} tubes tend to have a larger diameter than those observed in pure DLPE. An example is given in Fig. 2d. The isotropic resonance observed by ^{31}P-NMR is most likely due to the molecular motion of phospholipids in the cubic structures and/or the less organized, amorphous arrangement of the spherical subunits. X-ray diffraction can detect only the cubic arrangement of these

units. The unit cell dimensions of the cubic phase detected by X-ray diffraction agree with those observed by freeze-fracture electron microscopy. From the above information we may deduce that at 5% of POPC the mixtures consist of both the hexagonal and the cubic structures, depending on the temperature.

At 10% of POPC the structures are similar to those observed at 5% except that the isotropic resonance in ^{31}P-NMR spectra is much more prominent, with a smaller quantity of the hexagonal resonance component seen at higher temperatures. X-ray diffraction patterns range from extremely broad diffraction lines centered around 100 Å at 20°C to a diffuse hexagonal pattern at 40°C. The diffraction lines are much broader than those observed at 5% of POPC. Freeze-fracture electron micrographs support this observation, showing that the predominent features at 10% POPC are disorganized spherical units and amorphous structures intermixed with hexagonal tube structures. The organization of the spherical units are in less ordered cubic arrays as compared to the case of 5% POPC (Fig. 2e). The close proximity and the abundance of these spherical units explain the predominance of the isotropic component in ^{31}P-NMR spectra. The disordered packing could also cause the broad X-ray diffraction bands observed. At higher temperatures (40°C), more H_{II} structure is observed with tubes directly adjoined to the amorphous phase (Fig. 2f). Very few stacks of spheres were seen.

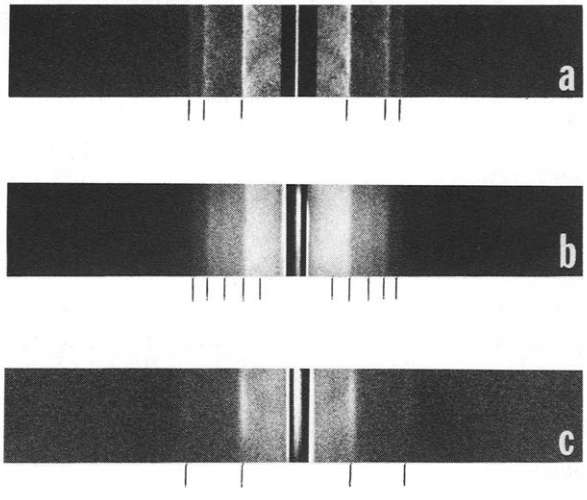


Fig. 3. Small-angle X-ray diffraction of samples containing different mole percentages of POPC in DLPE. (a) 0%, 20°C; (b) 5%, 10°C and (c) 25%, 40°C. Possible indexing of marked lines are given in text. The first line in (b) is visible on the negative.

At 15% POPC the morphology takes a drastic change. At 20°C and below, X-ray diffraction shows only one series of small-angle reflections whose spacing are in a simple ratio of $1:(1/2):(1/3)$ etc., indicating a lamellar repeat structure. ^{31}P-NMR spectra also show a predominantly bilayer type of resonance together with some isotropic component. These observations are again supported by the freeze-fracture electron microscopic evidence. The features observed are predominantly multilamellar structures, but the fracture planes are interrupted by many defects such as those shown in Fig. 2g. These defects in some cases look similar to the lipidic particles reported previously for soybean PE and egg PC [3]. But more often these particles are larger and sometimes show a ring-like structure rather than a cone-like structure. These enlarged particles or plateaus usually line up in rows or in circles. As the temperature is raised to 40°C, the spherical units or particles again appear, although they seldom pack into a cubic structure (Fig. 2h). These spherical units line up also in rows and sometimes they are packed in a random way over a fracture plane. The bilayers are divided into small zones by these rows of spherical particles. On the interface between lipid and aqueous buffer, the lipid dispersion can be seen to bulge in bubbles or small vesicles, but no spherical particles are seen between the buffer and the lipid (Fig. 2h). This seems to indicate that these spherical particles only exist between lipid lamellae and not within a single lipid bilayer. Hexagonal structures are occasionally observed. X-ray diffractions show very diffuse lamellar reflections intermixed with diffuse hexagonal reflections. This observation agrees with NMR results (Fig. 1).

At higher percentages of POPC, the predominant features observed by electron microscopy are multibilayer structures. At 20°C, the extended bilayer is occasionally disturbed by the ring-like or larger structures. The bilayer fracture faces also are marked by ridges. NMR shows only the anisotropic bilayer resonance and X-ray diffraction consists of only lamellar reflections. At higher temperatures, more disruption of the bilayer is

observed (Fig. 2i) and the ^{31}P-NMR spectra shows an increasing isotropic component for 25% POPC at 40°C. This suspension also transforms into two distinct phases, one aqueous and one oil-like. As the concentration of POPC in mixtures increases, disruption of the bilayer becomes less frequent.

At 100% POPC, the lipid supensions consisted of only multilamellar vesicles (Fig. 2h). The fracture faces of pure POPC at 20°C show ripple patterns of two different periodicities, intermixed with jumbled areas. Our POPC sample shows two endothermal peaks as measured by differential scanning calorimetry. The transition spans between $-3°$ and $+30°$, with peaks centering at 6° and 23° (results not shown). The mixed ripple pattern could be a result of P_β and L_α phase separation. The ripple pattern in POPC is similar to those observed by Ververgaert et al. [13].

All observed phase transitions are reproducible. However, whenever transitions between bilayer and isotropic phases are involved, the transition into the isotropic phase is not reversible. As an example, when the 15% and 25% PC samples were cooled from 50 to 20°C, the ^{31}P-NMR spectra were persistently dominated by the isotropic resonance, even after equilibrating overnight at 20°C.

To determine whether PE alone gives rise to the isotropic resonance (attributable to regions of high curvature), a more detailed analysis was undertaken. High resolution spectra were taken in order to resolve the PC and PE resonances. Any phase separation of PE from PC in these structures would give a ratio of intensities unequal to the ratio of the initial PE/PC content. For the 15% and 25% POPC/DLPE mixtures at 40°C, where less than half of the total lipids accounted for the isotropic resonances, we observed that there was a partitioning of PE and PC undergoing isotropic motion equal to the initial PC/PE ratio. This is indicated by the right-hand peak, corresponding to POPC, being 15 and 25% of the total resonance (Figs. 4b and 4d), respectively. Upon heating both samples to 50°and cooling back down to 40°C, where only the isotropic resonance persisted (Figs. 4a and 4c), the ratios of PC/PE in the isotropic phase remained unchanged. It is also interesting to note that the isotropic resonances taken when less than half of the total phospholipids were undergoing isotropic motion had a linewidth 50% broader than the spectra taken when all the phospholipids were undergoing isotropic motion.

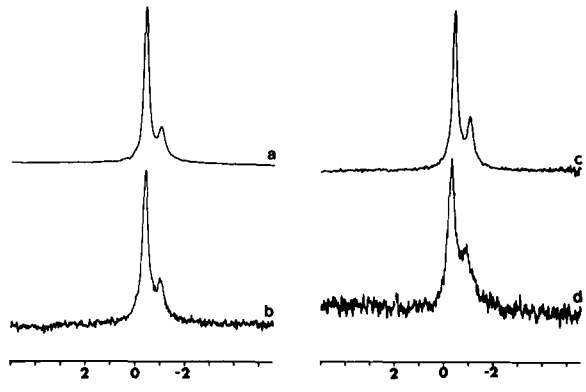


Fig. 4. High-resolution spectra of the isotropic component from POPC/DLPE mixtures containing 15% (b) and 25% (d) of POPE at 40°C. (a) and (c) are corresponding spectra after the sample is cycled back to 40°C from 50°C.

With this knowledge we can roughly divide the phase space of the lipid-water mixture into three zones. In zone I, with the lipid composition consisting of less than 5% of POPC, the structures are primarily hexagonal. In zone II, between 5 and 15% of POPC, lamellar, cubic and hexagonal phases co-exist and intermix. At higher temperatures, the cubic phase becomes less order and gradually turns into a random packing of spherical subunits. It is more appropriate to describe it as amorphous rather than cubic. In zone III, at compositons containing more than 15% of POPC, and below 40 degree, the structure is mainly bilayer, although the bilayers are interupted by various defects in the form of lipidic particles or extended interbilayer contacts. In such a phase diagram, the cubic or the amorphous phase is in the intermediate zone between the pure hexagonal and the pure lamellar phases. The hexagonal phase is very regular and uninterrupted, whereas the lamellar phase is often interrupted by various defects. The proposed phase diagram is shown in Fig. 5. Data from ^{31}P-NMR, X-ray diffraction, and electron microscopy are entered as shown, and hypothetical phase boundaries are drawn in. As can be seen in the phase diagram, results derived from different techniques usually agree.

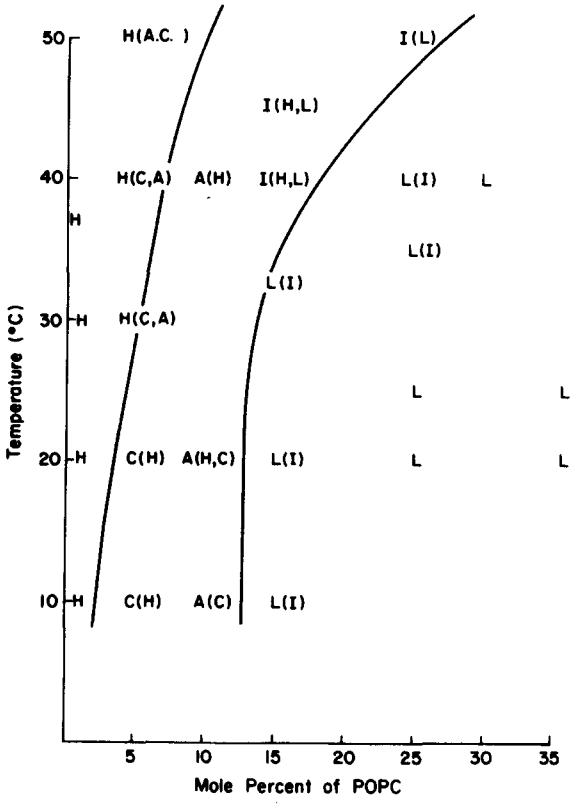


Fig. 5. The partial, empirical phase diagram of POPC/DLPE. Results from ^{31}P-NMR, X-ray diffraction and freeze-fracture electron microscopy are summarized. The predominant morphology at a given temperature and composition are indicated by the following symbols: (H) inverted hexagonal; (C) inverted cubic; (L) lamellar; (A) amorphous and (I) isotropic. The less predominant types are given in parenthesis. Lines indicate approximate divisions between zones of a given predominant structure.

Discussion

Although there have been many studies on the polymorphism of pure PE as well as of phospholipid mixtures containing PE, [3–6,8,14–16], detailed phase diagrams are seldom presented. Many of these studies employed only one or two techniques, and the interpretations could be ambiguous. For instance, ^{31}P-NMR results can not uniquely determine the hexagonal or the bilayer phase, since the molecular motional constraint, the headgroup conformation, contamination by small vesicles, etc., also contribute to the line shape anisotropy [3,11,17]. In some previous studies with ^{31}P-NMR of various PE mixtures, pure isotropic resonances have been obtained [5,15,18]. This pure isotropic phase is sometimes difficult to reconcile with freeze-fracture electron microscopic observations of similar systems reported so far. We decided to apply three complementary techniques to study more closely a mixture, using synthetic lipids that have compositions close to those of the natural phospholipids we used in our last experiment [3]. DLPE constitutes over 35% of the soybean PE, and POPC is the major component of egg PC. This mixture was selected so that we could make comparisons with our previously determined phase diagram.

The phase diagram we obtained with these synthetic phospholipids has some similarity to that which we obtained with soybean PE and egg PC. Both phase diagrams show that high unsaturated PE content and high temperatures favour the formation of an isotropic phase from the lamellar phase, and the H_{II} phase from the isotropic phase. This is expected since the lipid molecular shape becomes more conical than cylindrical with increasing temperature and unsaturation. The main difference is that, in this experiment, we observed for the first time by electron microscopy the predicted cubic phase between the hexagonal and the lamellar zones in the phase diagram. The cubic phase is not always well ordered, especially at high temperatures when it turns into an amorphous structure. It is unlikely that the amorphous structure is a result of ice damage, since the reproducible variation of disorder with temperature and composition was observed both by freeze-fracture and by X-ray diffraction. The appearance of a cubic phase or randomly dispersed lipidic particles as transitional states between the lamellar and the hexagonal phase seems to depend critically on the lipid composition. It seems that the transition from bilayer to hexagonal can take different routes, and the lipidic particle route previously proposed [19] is only one of the possible intermediate structures. In many cases the cubic phase is a stable structure [20]. In fact, from our experience, the transition from the cubic or amorphous phases to the bilayer phase always associates with a large temperature hysteresis. In many cases, incubation at a given temperature for over an hour is required. This was also observed by Cullis and De Kruijff [16] using ^{31}P-NMR.

The cubic phase we reported here seems to consist of spherical subunits that are larger and differ in shape with the conical lipidic particles we and other investigators reported eailer [3,4,7]. In fact we can see in Fig. 2h, that these large spherical subunits co-exist with the conical lipidic particles in the same sample. When both spherical and conical particles are observed at the same area of the replica, the shadowing is invariably of the opposite sense, namely, if one is protruding from the plane, the others are pits (Fig. 2h). We believe that the spherical particles are small spheres containing water as proposed by Larsson et al. [10], which is equivalent to inverted micelles [4,21], whereas conical lipidic particles are the attachement sites of the hydrophobic connections between layers of spheres [7,22]. These hydrophobic connections agree with our previous proposed model for the lipidic particles observed singularly or in rows [3]. The reported pure isotropic resonance [5] can be explained by the cubic or a less orderly amorphous structure containing a high density of spherical subunits. As long as most individual phospholipids can exchange via lateral diffusion with regions of high curvature during the NMR time scale, an entirely isotropic resonance is plausible [5,18]. Both PC and PE contributed to the isotropic resonance as indicated by our high resolution NMR data. This result also agrees with that deduced from thionphospholipid experiments [23]. A detailed molecular organization model of these nonbilayer structures will be given elsewhere. Their relation to the lamellar or hexagonal molecular arrangement can also be explained by our model [24].

The similar and yet different phase diagrams of DLPE/POPC, and of soybean PE/egg PC show that the polymorphic phase change of lipid mixtures is critical not only to the headgroups but also to the homogeneity and the degree of unsaturation of the acyl chains. It is therefore reasonable to believe that subtle changes of local curvature with various phospholipids interplaying in biological membranes could have significant functional implications.

Acknowledgements

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