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BARRIER CHARACTERISTICS OF MEMBRANE MODEL SYSTEMS CONTAINING UNSATURATED PHOSPHATIDYLETHANOLAMINES

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The barrier characteristics of membrane model systems containing unsaturated phosphatidylethanolamines have been investigated. (1) At increasing temperatures $18{:}1_c/18{:}1_c$-phosphatidylethanolamine liposomes lose their permeability barrier for K^+ as a consequence of the transition from a lamellar to a hexagonal orientation as detected by ^{31}P-NMR and freeze-fracturing electron microscopy. (2) Introduction of $18{:}1_c/18{:}1_c$-phosphatidylcholine in the $18{:}1_c/18{:}1_c$-phosphatidylethanolamine lipid system stabilizes the bilayer structure and the permeability barrier for K^+ and glucose while cholesterol destabilizes. (3) Upon heating of the investigated $18{:}1_c/18{:}1_c$-phosphatidylcholine-$18{:}1_c/18{:}1_c$-phosphatidylethanolamine-(cholesterol) mixtures, structures are formed which give rise to isotropic ^{31}P-NMR signals and which on the basis of freeze-fracture pictures are interpreted as sponge-like structures. Lowering the temperature results in restoration of the barrier function of the lipid structures.

Introduction

Early X-ray [1,2] and recent ^{31}P-NMR [3,4] studies have shown that unsaturated synthetic and naturally occurring phosphatidylethanolamines undergo bilayer to hexagonal (H_{II}) phase transitions at temperatures which are sensitive to the fatty acid composition. In mixtures of unsaturated phosphatidylethanolamines and phosphatidylcholines (with or without cholesterol), depending on the composition and temperature [5–7] large lipid association structures are formed in which part of the phospholipids gives rise to a narrow symmetrical ^{31}P-NMR resonance indicating fast three-dimensional (isotropic) movement [5] of these phospholipid molecules. Freeze-fracturing microscopic studies on the cholesterol-containing mixtures visualized small particles and pits on the fracture faces. Similar ^{31}P-NMR spectra and freeze-fracture morphology were observed in mixtures of phosphatidylcholine with other hexagonal (H_{II}) forming lipids like monoglucosyldiglyceride and cardiolipin in the presence of Ca^{2+} [7,8]. To explain these experimental results a model has been proposed in which part of the lipid is present in inverted micelles located in the hydrophobic interior of lipid bilayer structures. The occurrence of this new type of lipid organization may have relevance to biomem-

branes as comparable ^{31}P-NMR spectra and freeze-fracture morphology were observed in the total lipid extracts of rod outer segment [9] and *Escherichia coli* membranes [10]. Furthermore ^{31}P-NMR spectra of microsomes at 37°C indicate isotropic motion of part of the membrane phospholipids [11,19].

It is generally accepted that the phospholipid organization in biomembranes determines the main barrier function of the interface. In current view these lipids are thought to be orientated in bilayers, but in view of the fact that unsaturated phosphatidylethanolamines are main constituents of many membranes, possibilities for transitions to other orientations as discussed above must be considered. If such transitions do occur they may have consequences for the barrier function of the membrane. Therefore we though it of interest to study the permeability properties of liposomal model systems containing unsaturated phosphatidylethanolamines in relation to temperature-induced polymorphic phase transitions.

Materials and Methods

Cholesterol was purchased from Merck. Egg yolk phosphatidylethanolamine was isolated from the total lipids of egg yolk employing silicic acid column chromatography followed by carboxymethyl-cellulose column chromatography [12]. Phosphatidic acid was prepared from egg lecitin by degradation with phospholipase D [13]. 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 [3,14]. Hexokinase (EC 2.7.1.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) and $NADP^+$ were from Boehringer, Mannheim (F.R.G.); ATP was from Sigma Chemical Co., St. Louis (U.S.A.).

Multilayered liposomes for K^+ leak experiments, containing 4 mol% of egg phosphatidic acid to ensure sufficient trap [15], were prepared by dispersing at 4°C a dry film of 5–30 μmol lipid in 1–2 ml 150 mM KCl, 25 mM Tris–HCl, pH 7.5 . Outside K^+ was removed by dialysis at 4°C against isotonic cholinechloride buffer, 25 mM Tris–HCl (pH 7.5). Small samples (0.5–1.0 μmol lipid) of the dialyzed liposome suspension (kept on ice) were transferred into 10 ml cholinechloride buffer of the desired temperature. Changes in K^+ activities of the outside medium were monitored by a Schott and Gen K^+-glass electrode (Jena Glaswerk, Mainz, F.R.G.) connected to a pH meter (Radiometer, type PHM 26) and a recorder. After measuring the K^+ leak during 20 min Triton X-100 was added to liberate the residual trapped K^+.

Multilayered liposomes for experiments in which we compared K^+ and glucose leak were prepared by dispersing at 4°C a dry film of 5–30 μmol lipid in 1–2 ml 150 mM KCl, 300 mM glucose, 25 mM Tris–HCl (pH 7.5). After dialysis at 4°C against isotonic cholinechloride buffer to remove outside K^+ and glucose, samples of the liposomes suspension were dialyzed at the desired temperature against cholinechloride buffer, to study the effect of heating on K^+ and glucose content.

The dialyzing solution (100 ml) was refreshed after 0, 5, 10, 20, 30, 40, 55, 65, 75 and 85 min. The K^+ and glucose trap inside the liposomes were measured after 50, 60, 70, 80 and 90 min. Glucose trap was measured spectrofotometrically according to the procedure developed by Kinsky et al. [16].

Phospholipid dispersions for ^{31}P-NMR measurements were made by dispersing at 4°C a dry film of 100 μmol lipid in 0.8 ml ^2H_2O containing 100 mM NaCl, 0.2 mM EDTA, 10 mM Tris–HCl (pH 7.5) buffer. ^{31}P-NMR spectra were recorded at 36.4 MHz under conditions of proton decoupling as described before [5], generally a sweep width of 12 kHz and a pulse rate of 0.17 sec using 45° rf pulses was used.

Freeze-fracture electron microscopy was performed as outlined previously [17].

Results

Barrier properties of pure 18:1$_c$/18:1$_c$-phosphatidylcholine- and 18:1$_c$/18:1$_c$-phosphatidylethanolamine systems

At 4°C multilayered liposomes can be prepared when 18:1$_c$/18:1$_c$-phosphatidylcholine or 18:1$_c$/18:1$_c$-phosphatidylethanolamine containing 4% phosphatidic acid are dispersed in 150 mM KCNS or KCl. The phospholipids organize themselves in multilamellar structures as indicated by the typical 'bilayer-shaped' [18] ^{31}P-NMR spectra (Fig. 2A,B). That the structures are multilayered liposomal is demonstrated by typical freeze-fracture faces which is in agreement with the considerable amount of K^+ enclosed in the structures (Table I). The slightly lower trap of 18:1$_c$/18:1$_c$-phosphatidylethanolamine might be caused by the presence of some smaller vesicles which is also indicated by a small isotropic signal which is observed superimposed on the bilayer spectrum (Fig. 2B).

When a small sample of the 18:1$_c$/18:1$_c$-phosphatidylethanolamine liposomes, made and dialyzed at 4°C, is injected in the K^+ leak bath at 40°C nearly all enclosed K^+ is lost within 5 min (Fig. 3). At 40°C the ^{31}P-NMR spectrum of the 18:1$_c$/18:1$_c$-phosphatidylethanolamine system (Fig. 2D) has obtained an opposite asymmetry and a reduced width which is typical for a hexagonal phase [5]. Freeze-fracture pictures as shown in Fig. 1B confirm the formation of such a hexagonal organization. Incubation of the 18:1$_c$/18:1$_c$-phosphatidylcholine liposomes at 40°C on the contrary has no effect on the K^+ trap (Table I, Fig. 3). The ^{31}P-NMR spectrum at 40°C is a typical bilayer spectrum (Fig. 2C). The small isotropic signal which disappeared again upon cooling indicates the presence of some smaller structures. Therefore we conclude that the temperature induced K^+ release from the phosphatidylethanolamine liposomes is a consequence of the transition from a lamellar to a hexagonal orientation.

Barrier properties of 18:1$_c$/18:1$_c$-phosphatidylcholine-18:1$_c$/18:1$_c$-phosphatidylethanolamine mixtures.

Replacement of 18:1$_c$/18:1$_c$-phosphatidylethanolamine molecules by 18:1$_c$/

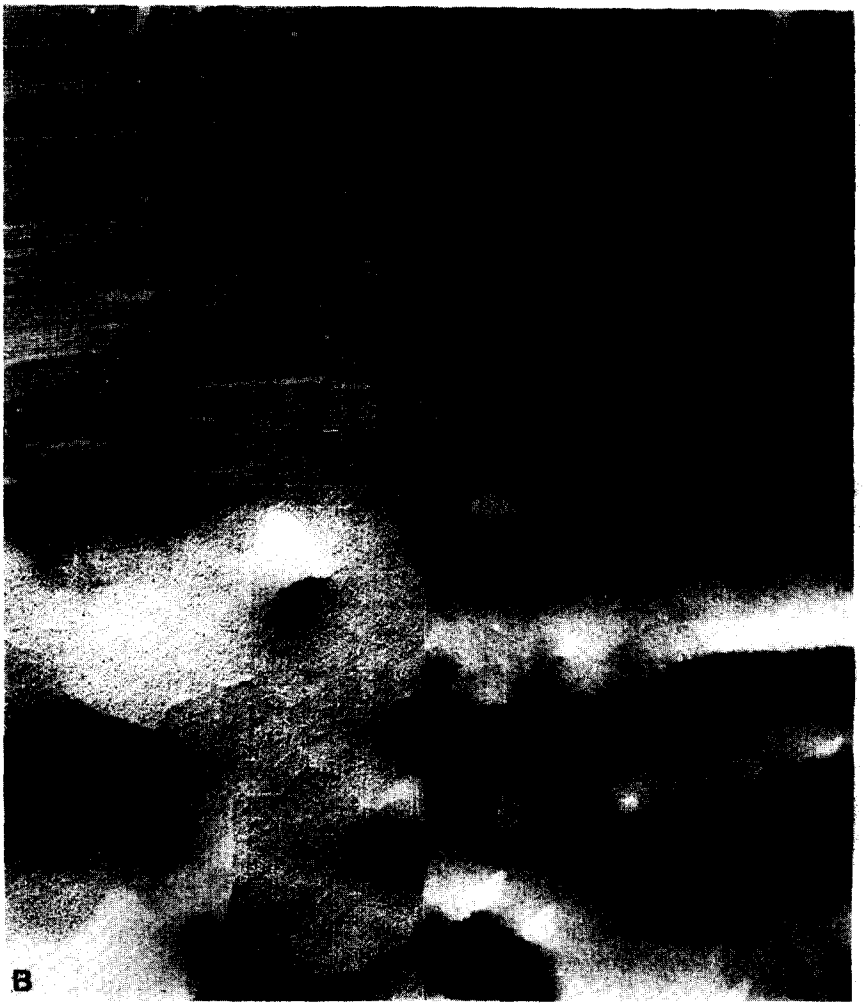

Fig. 1. Freeze-fracture electron micrographs of aqueous dispersions of $18{:}1_c/18{:}1_c$-phosphatidylethanolamine-containing lipid mixtures. A: freeze-fracturing of an aqueous dispersion of $18{:}1_c/18{:}1_c$-phosphatidylethanolamine at 40°C. B: freeze-fracturing of an aqueous dispersion of $18{:}1_c/18{:}1_c$-phosphatidylethanolamine-$18{:}1_c/18{:}1_c$-phosphatidylcholine-egg phosphatidic acid (72 : 24 : 4) at 20°C after being heated till 60°C. C: freeze-fracturing of an aqueous dispersion of $18{:}1_c/18{:}1_c$-phosphatidylethanolamine-$18{:}1_c/18{:}1_c$-phosphatidylcholine-cholesterol-egg phosphatidic acid (51 : 17 : 28 : 4) at 4°C after being heated till 60°C.

$18{:}1_c$-phosphatidylcholine molecules in the $18{:}1_c/18{:}1_c$-phosphatidylethanolamine liposomes stabilizes the bilayer structure [5]. This is apparent from Fig. 4A which shows that replacement of the $18{:}1_c/18{:}1_c$-phosphatidylethanolamine by $18{:}1_c/18{:}1_c$-phosphatidylcholine results in lipid structures which hold full trap of K^+ ions up to 25°C. Above this temperature release of the enclosed K^+ can be noticed;

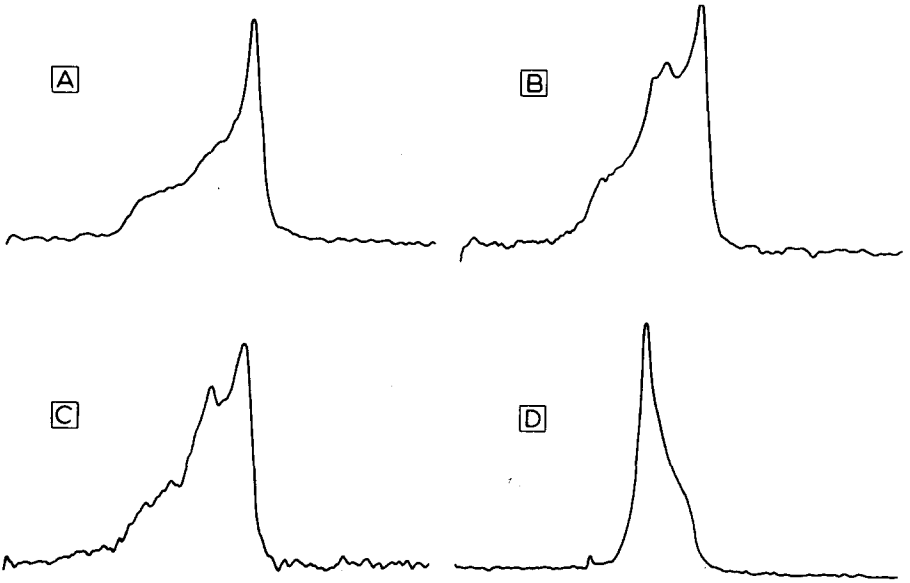

Fig. 2. 36.4 MHz ^{31}P-NMR spectra of aqueous dispersions of 18:1_c/18:1_c-phosphatidylcholine-egg phosphatidic acid (96 : 4) at 4°C (A) and at 40°C (C) and of 18:1_c/18:1_c-phosphatidylethanolamine-egg phosphatidic acid (96 : 4) at 4°C (B) and at 40°C (D).

^{31}P-NMR spectra of this mixture (18:1_c/18:1_c-phosphatidylethanolamine 72%, 18:1_c/18:1_c-phosphatidylcholine 24%, egg phosphatidic acid 4%) are shown in Fig. 5A. At increasing temperatures the spectrum changes gradually from the bilayer phase to a phase which is characterized by the possibility of isotropic motion of the phospholipid molecules. It is unlikely that the narrow line originates from the formation of small vesicles which can undergo rapid tumbling. A first indication in this respect is the fact that the phospholipid dispersion also at elevated temperature is highly turbid. As noted before [6], the

Table I

K^+ trap (nmol K^+/μmol lipid) of aqueous dispersions of lipid mixtures 20 min after injection at the indicated temperatures

	4°C	40°C
18:1_c/18:1_c-Phosphatidylcholine-egg phosphatidic acid (96 : 4)	614	602
18:1_c/18:1_c-Phosphatidylethanolamine-egg phosphatidic acid (96 : 4)	401	54

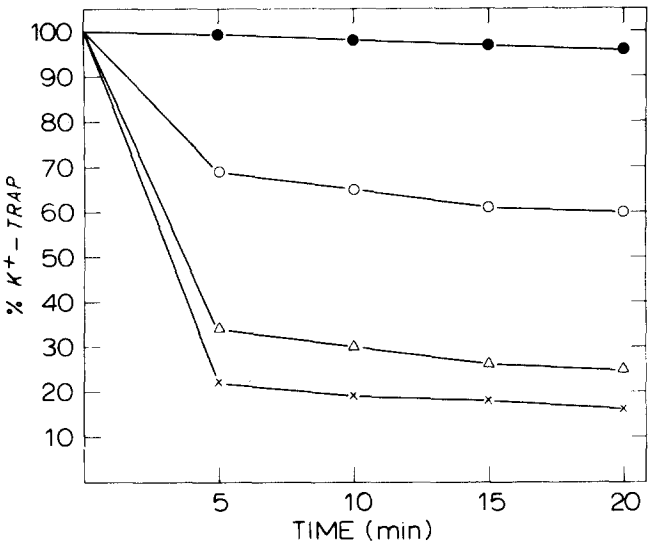

Fig. 3. Time-dependence of the K^+ leak from aqueous dispersions of different lipid mixtures at 40°C. •——•, 18:1_c/18:1_c-phosphatidylcholine-egg phosphatidic acid (96 : 4); ×——×, 18:1_c/18:1_c-phosphatidylethanolamine-egg phosphatidic acid (96 : 4); ○——○, 18:1_c/18:1_c-phosphatidylethanolamine-18:1_c/18:1_c-phosphatidylcholine-egg phosphatidic acid (72 : 24 : 4); △——△, 18:1_c/18:1_c-phosphatidylethanolamine-18:1_c/18:1_c-phosphatidylcholine-cholesterol-egg phosphatidic acid (51 : 17 : 28 : 4).

lipid mixture exhibits a pronounced hysteresis upon cooling: the ^{31}P-NMR spectrum does not change back to the shape characteristic for extended bilayer structure but remains isotropic although at 4°C the line width is two times larger than the line width at 60°C (Fig. 5A). Freeze-fracture pictures of the structures did not show a significant amount of small vesicles but mainly large structures of which a typical example is given in Fig. 1B. On the irregular bilayer face ridges and fissures can be noticed. At lower temperatures the trapping ability of the structure is restored (cf. Fig. 6A) although the amount of trapped K^+ is about half the amount before the heating process. Reheating of the structures shows reproducable effects, at higher temperature release of enclosed K^+ can be noticed again. Identical results (same trapped volume, same dependence of the leak on temperature and time) were obtained when glucose permeability was studied.

Barrier properties of 18:1_c/18:1_c-phosphatidylcholine-phosphatidylethanolamine-cholesterol mixtures

In contrast to phosphatidylcholine cholesterol destabilizes the bilayer structure when introduced to unsaturated lipid systems containing phosphatidylethanolamines [5]. This is understandable from the molecular shape of the cholesterol molecule having a small polar head and a bulky hydrophobic body.

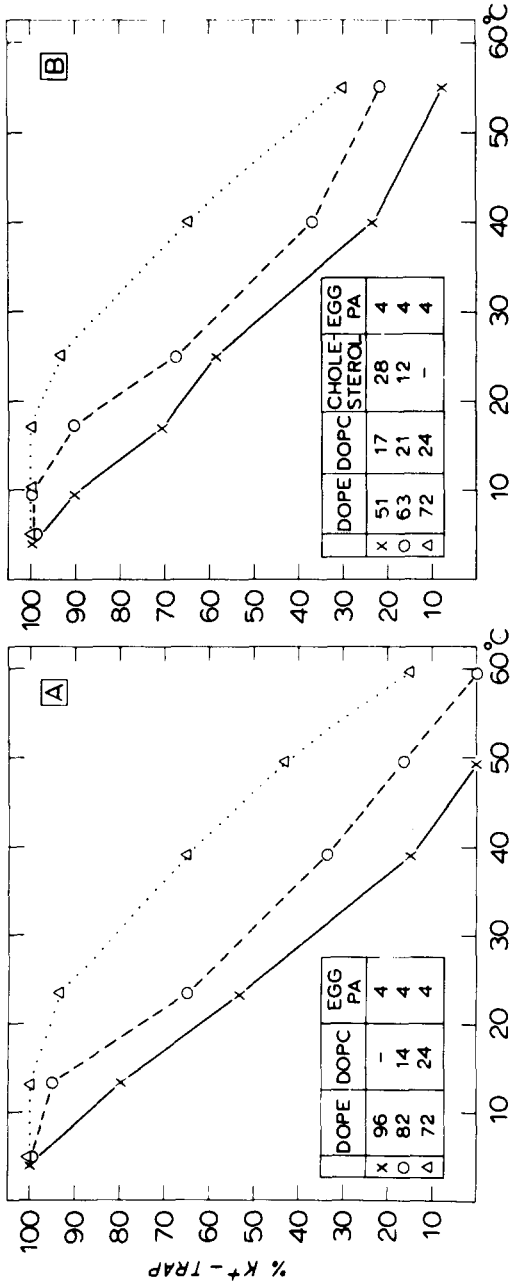

Fig. 4. K^+ trap of aqueous dispersions of different 18:1_c/18:1_c-phosphatidylethanolamine-(DOPE) containing lipid mixtures 20 min after injection at the temperature indicated. A: barrier stabilization by lecithin. B: barrier destabilization by cholesterol.

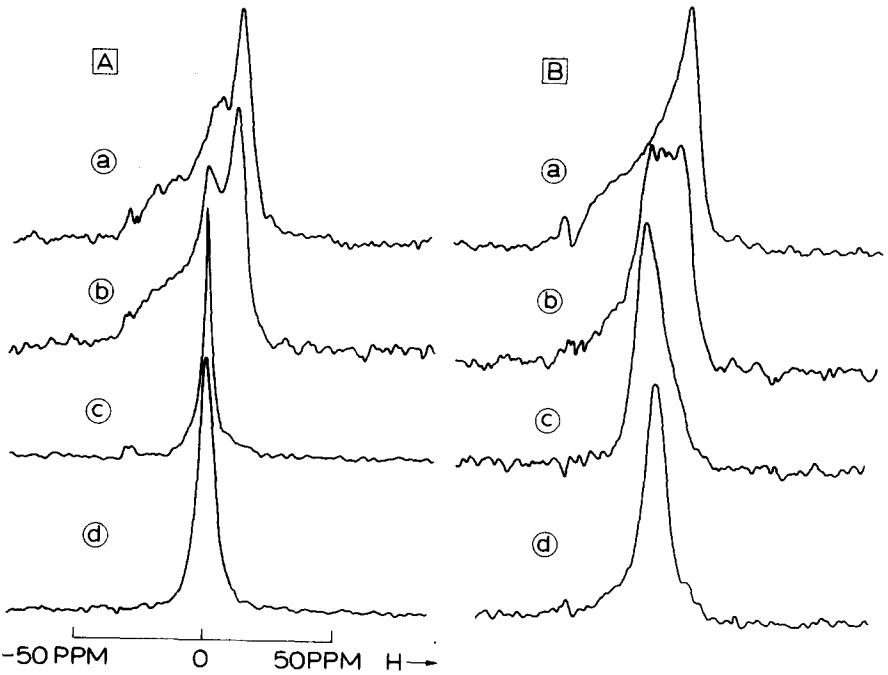

Fig. 5. 36.4 MHz ^{31}P-NMR spectra of (A) 18:1$_c$/18:1$_c$-phosphatidylethanolamine 18:1$_c$/18:1$_c$-phosphatidylcholine-egg phosphatidic acid (72 : 24 : 4) at (a) 20°C, (b) 40°C, (c) 60°C and (d) 20°C after being heated till 60°C; (B) 18:1$_c$/18:1$_c$-phosphatidylethanolamine-18:1$_c$/18:1$_c$-phosphatidylcholine-cholesterol-egg phosphatidic acid (51 : 17 : 28 : 4) at (a) 4°C, (b) 24°C, (c) 40°C and (d) 4°C after being heated till 40°C.

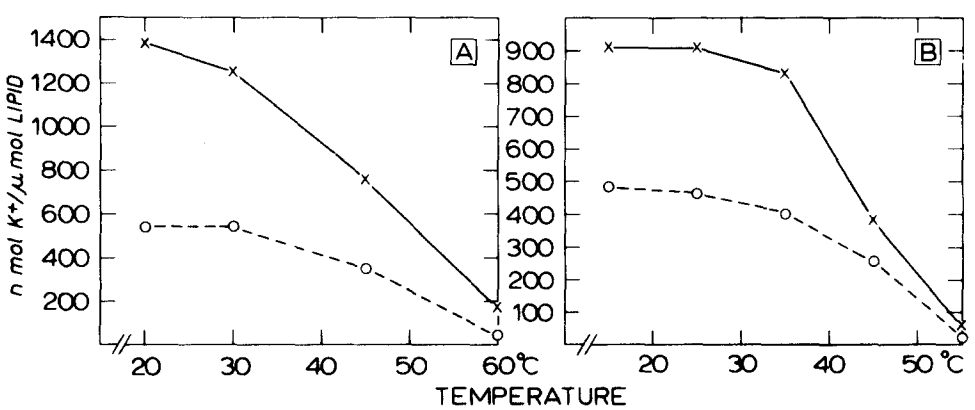

Fig. 6. K^+ trap of aqueous dispersions of (A) 18:1$_c$/18:1$_c$-phosphatidylethanolamine-18:1$_c$/18:1$_c$-phosphatidylcholine-egg phosphatidic acid (72 : 24 : 4) and (B) egg phosphatidylethanolamine 18:1$_c$/18:1$_c$-phosphatidylcholine-cholesterol-egg phosphatidic acid (51 : 17 : 28 : 4) after 50 min dialysis at the temperature indicated. x——x, bilayer condition; o——o, isotropic condition.

Figure 4B shows that with increasing amounts of cholesterol the temperature induced leak shifts to lower temperatures. Figure 5B shows the ^{31}P-NMR spectra of such a cholesterol containing phosphatidylethanolamine-phosphatidylcholine-phosphatidic acid mixture. When the lipids are dispersed at 4°C a spectrum typical for extended bilayers is obtained. When the temperature is increased from 4 till 40°C, the spectrum changes gradually from the bilayer to the hexagonal H_{11} shape. Upon cooling the spectrum turns into one which indicates isotropic motion of the phospholipid molecules. Figure 1C shows freeze-fracture face of this 'isotropic' phase. On the bilayers bulges are visible on which particles are present as was observed previously [7]. Comparable ^{31}P-NMR, freeze-fracturing and leak results were obtained when 18:1_c/18:1_c-phosphatidylethanolamine was substituted by egg phosphatidylethanolamine. Figure 6B shows that heating at 4°C dispersed lipid mixture egg phosphatidylethanolamine-18:1_c/18:1_c-phosphatidylcholine-cholesterol-egg phosphatidic acid 51 : 17 : 28 . 4 causes loss of K^+ as a result of the bilayer-hexagonal H_{11} transition. Compared to the corresponding 18:1_c/18:1_c-phosphatidylethanolamine containing lipid mixture (Fig. 4B) the barrier is maintained till higher temperatures as a consequence of the 15°C higher bilayer-hexagonal H_{11} transition of egg phosphatidylethanolamine [4]. Lowering of the temperature results in restoration of the barrier function although as in the 18:1_c/18:1_c-phosphatidylethanolamine-18:1_c/18:1_c-phosphatidylcholineegg phosphatidic acid mixture the trap is about half the amount before heating. In studies on glucose entrapment in the structures of these lipid

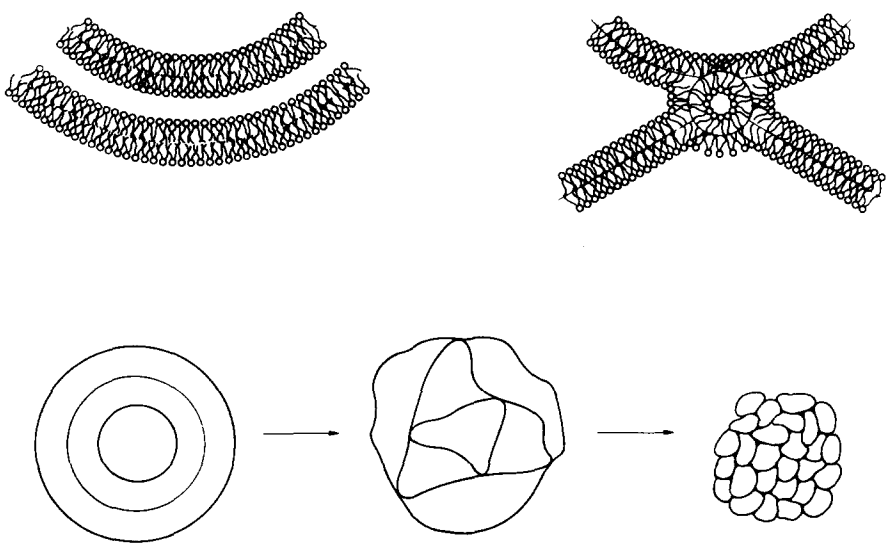

Fig. 7. Spongelike model to explain isotropic movement. The lines between the monolayers in the fusion-model indicate the fracture possibilities.

mixtures identical results were obtained. Both the K^+ and glucose leak were measured as a function of time. After the first checkpoint at 50 min no further release could be measured.

Discussion

The present study clearly demonstrates large differences in membrane properties of $18{:}1_c/18{:}1_c$-phosphatidylcholine and $18{:}1_c/18{:}1_c$-phosphatidylcholamine. Both phospholipids are able to form bilayers which are highly impermeable to K^+ and glucose, but with increasing temperature only those of the latter phospholipid lose their barrier function, as a consequence of a transition from a lamellar to a hexagonal orientation. The transition in our liposomal system of $18{:}1_c/18{:}1_c$-phosphatidylethanolamine occurs over a rather broad temperature range which is in contrast to the transition of pure phosphatidylethanolamine [4] and which might be explained by the presence of 4 mol% phosphatidic acid as an impurity in this system. The bilayer-hexagonal H_{11} transition of $18{:}1_c/18{:}1_c$-phosphatidylethanolamine-4% egg phosphatidic acid is fully reversible; cooling down of the system results in the reformation of liposomal structures. The ability to entrap K^+ and glucose is restored and the ^{31}P-NMR spectrum changes back to a typical bilayer type of spectrum.

When using mixtures of the phosphatidylethanolamine and the phosphatidylcholine different and nonreversible NMR effects are observed. Upon dispersion at 4°C of a mixture of $18{:}1_c/18{:}1_c$-phosphatidylethanolamine, $18{:}1_c/18{:}1_c$-phosphatidylcholine and phosphatidic acid in a ratio 72 : 24 : 4 large liposomal structures are formed which are stable up to 20°C. An increasing leak of K^+ and glucose in the range 20–60°C indicates a destabilization of the original bilayer barrier. In this temperature range the ^{31}P-NMR spectrum of the system changes from the bilayer type to the isotropic type of spectrum. Cooling down again restores the ability to entrap glucose and K^+ but the ^{31}P-NMR spectrum is persistent in indicating isotropic motion of the P. nuclei now also at low temperatures [5]. A possible explanation for the observed phenomena could be that at higher temperatures the bilayers of the system became unstable, forming a intermediary state, which causes K^+ and glucose release and extensive fusion between the concentric bilayers and results in the formation of a sponge like structure as schematically indicated in Fig. 7. The isotropic ^{31}P-NMR signal could be explained by fast lateral diffusion of the lipid molecules in the strongly curved bilayers. The hysteresis upon cooling shows the stability with slower diffusion at lower temperature. The idea of spongelike structure is supported by the freeze-fracture pictures of the isotropic phase which show irregular bulged bilayer surfaces with ridges and fissures. The restored but largely reduced K^+ and glucose trap at lower temperature when compared with original liposome dispersion could be explained by the more condensed lipid bilayer association structure after the fusion process (cf. Fig. 7).

Introduction of cholesterol in the $18{:}1_c/18{:}1_c$-phosphatidylcholine-$18{:}1_c$/$18{:}1_c$-phosphatidylethanolamine-egg phosphatidic acid system destabilizes the bilayer structure in agreement with earlier observations [5]. The bilayer-hexagonal H_{II} transition which is observed upon heating of the cholesterol-containing mixture contrasts with the temperature dependent behaviour of the lipid system without cholesterol. Upon cooling the ^{31}P-NMR spectrum becomes isotropic which again at least in part may be explained by a spongelike network. The electron microscopic pictures show also in this case bulged fracture faces. Furthermore on these fracture faces particles and pits can be noticed, which, as has been pointed out in the introduction, are thought to be formed by inverted micelles in the hydrophobic interior of the bilayer structures. It is obvious that an organization in micelles will contribute to an isotropic type of ^{31}P-NMR signal.

It can be noticed that the particles are organized in strings on top of the bulges, which suggests that the freeze-fracture procedure catches the particles at the contact regions of the bilayers as indicated in Fig. 7. Therefore it is likely that the formation of inverted micelles is important for the fusion process. In this respect it is of importance that the addition of Ca^{2+} to small unilamellar vesicles of an equimolar mixture of egg phosphatidylcholine and cardiolipin induces fusion of these vesicles also in association with the appearance of lipidic particles on the fusion sites [20]. Moreover, it was observed that Ca^{2+} induced formation of particles in multilayered liposomes of the lecithin/cardiolipin is correlated with a rapid transbilayer movement of the lipid molecules and a largely increased ionic permeability of the barrier [21].

The present experiments, however, show that the existence of lipidic particles in the investigated phosphatidylethanolamine-phosphatidylcholine-cholesterol-phosphatidic acid system does not lead to facilitated iontranslocation.

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