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COMPARATIVE STUDY ON THE PROPERTIES OF SATURATED PHOSPHATIDYLETHANOLAMINE AND PHOSPHATIDYLCHOLINE BILAYERS: BARRIER CHARACTERISTICS AND SUSCEPTIBILITY TO PHOSPHOLIPASE A_2 DEGRADATION

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Comparative studies on bilayer systems of saturated phosphatidylcholines and phosphatidylethanolamines revealed a maximum in ionic permeability in phosphatidylcholine bilayers at the temperature of the gel to liquid-crystalline phase transition but such an increase in permeability was not detectable in bilayers of phosphatidylethanolamine. Furthermore, it was found that at the phase transition temperature the phosphatidylcholine bilayers are subject to rapid hydrolysis by pancreatic phospholipase A_2 whereas phosphatidylethanolamine bilayers are not. These differences are discussed in view of detailed information on the molecular organization in the gel and liquid crystalline phases of the two phospholipid classes.

Keywords: phosphatidylcholine; phosphatidylethanolamine; phase transition; bilayer permeability.

Introduction

Gel to liquid-crystalline phase transitions, have a profound effect on the properties of lipid bilayers. For pure lecithin bilayers it has been demonstrated that ionic permeability [1–3], susceptibility for pancreatic phospholipase A_2 degradation [4] and rate of transbilayer movement of lipid molecules [5] are markedly increased in the temperature range at which the hydrocarbon phase transition occurs. Two possible explanations have been given for the enhanced permeability in the vicinity of the gel to liquid-crystalline phase transition temperature. (i) The increase in permeability can be associated with the presence of boundary lipid. The mismatch in packing of the lipid molecules at the ordered-fluid boundaries results in an enhanced diffusion via these domain-boundaries [2, 3]. (ii) The increase in permeability can be correlated with the enhanced lateral compressibility of the lipid molecules at the phase transition temperature resulting from the decrease in area per molecule going from the liquid-crystalline to the gel state. Lateral density fluctuations associated with this high lateral compressibility enhance

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permeability by opening short-lived cavities via which solutes can pass the bilayer [6–8]. The irregularities in lipid packing and the increased lateral compressibility also may enhance the action of phospholipases and facilitate the transbilayer movement of lecithin molecules.

In contrast to unsaturated phosphatidylethanolamines which can undergo a temperature dependent transition from a lamellar to a hexagonal (H_{II}) organization [36], saturated phosphatidylethanolamines form stable bilayer systems [14] in which gel to liquid-crystalline phase transitions can be monitored. It is a matter of interest to know whether these phase transitions are accompanied with similar effects on the barrier properties of the bilayers as noticed in comparable lecithin bilayers.

In the present study we report on K^+-permeability of bilayer systems of dimyristoyl phosphatidylethanolamine and mixtures of this lipid with dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine in the vicinity of the phase transition and on the temperature-dependent susceptibility of these systems to phospholipase A_2 degradation. Structural information on these systems was gained by using freeze-fracture electron microscopy (EM), ^{31}P-NMR, Raman spectroscopy and differential scanning calorimetry (DSC).

Materials and Methods

1,2-Dimyristoyl-*sn*-glycero-3-phosphorylcholine (DMPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC), 1,2-dimyristoyl-*sn*-glycero-3-phosphorylethanolamine (DMPE), 1,2-dielaidoyl-*sn*-glycero-3-phosphorylethanolamine (DEPE) and 1,2-dioleoyl-*sn*-glycero-3-phosphate (18 : 1_C/18 : 1_C-phosphatidic acid) were synthesized as described previously [14–16]. Unilamellar vesicles containing 4 mol% of 18 : 1_C/18 : 1_C-phosphatidic acid to prevent aggregation of the vesicles (etherosomes) were prepared according to the ether-evaporation method described by Deamer and Bangham [17]. The lipids were dissolved in an ether/methanol/water (90 : 10 : 4) mixture to a concentration of 2.5 mM and 3.0 ml of this solution was injected into 4 ml buffer (150 mM KCl, 25 mM Tris–HCl (pH 7.5) for the K^+-leak experiments; 100 mM NaCl, 20 mM Tris–HCl, 0.2 mM EDTA (pH 7.0) for the ^{31}P-NMR experiments) at 58°C. The dispersion was filtrated through a 1.2 μm Millipore filter to remove multilamellar vesicles and subsequently centrifuged at 35 000 × *g* for 45 min. The etherosomes were resuspended and dialyzed overnight at room temperature against the buffer in which they were prepared to remove traces of ether. Multilayered liposomes for K^+-leak experiments, containing 4 mol% of 18 : 1_C/18 : 1_C-phosphatidic acid to ensure sufficient trap [18], were prepared by dispersing at 60°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^+ of liposomes and etherosomes was removed by dialysis

at 4°C against isotonic choline-chloride buffer, 25 mM Tris–HCl (pH 7.5). Small samples (0.5–1 μmol lipid) of the dialyzed suspension (kept on ice) were transferred into 10 ml choline-chloride buffer of the desired temperature. K^+-efflux was measured with a combination of a K^+-selective glass electrode (Philips, No. G 15 K) and a reference electrode (Philips, No. R 44/2 SD 1), connected to a pH 26 Radiometer (Copenhagen, Denmark). The amount of trapped K^+ was determined by lysing the lipid structures with Triton X-100. Differential scanning calorimetry experiments were performed as described by van Dijck et al. [19], with the exception that ethyleneglycol was not added to the lipid suspension. Freeze-fracture electronmicroscopy was performed as outlined previously [24]; glycerol was added to the samples to prevent freeze-damage. ^{31}P-NMR spectra were recorded at 36.4 MHz under conditions of proton decoupling as described before [23]. Signal intensities were determined by integration. Precautions were taken to ensure direct comparability of the spectra.

The apparatus used for Raman spectroscopy consists of a Coherent radiation CR-18 Argon Ion Laser, Iobin Yvon HG-2S double monochromator with Philips 56 TVP photomultiplier detection, photon counting application and stripchart recording. Samples were thermostated in a brass cell and were examined in the transverse mode using 400–500 mW of 5287 Å laser radiation for excitation. The uncertainty of the temperature at the sample was estimated to be 2–3°C. The spectral region of interest was scanned five times and the resulting spectra averaged using a Promeda minicomputer. Spectra resolution was 2–3 cm^{-1}. All data reported are for heating cycles.

Phospholipase experiments were done according to Op den Kamp et al. [4] by dispersing 5 μmol phospholipid in 20 ml 100 mM NaCl, 1 mM $CaCl_2$, 10 mM Tris–HCl (pH 7.2) above the phase transition. Liposome suspension (400 μl) was incubated with 10 μg of highly purified pig pancreatic phospholipase A_2 in a total volume of 0.5 ml at different temperatures for 30 min. The reaction was terminated by the addition of 0.5 ml 0.2 M EDTA and the reaction products were extracted according to Bligh and Dyer [21]. In control experiments the incubation was carried out in the absence of phospholipase A_2 and 10 μg of this enzyme were added after the addition of EDTA. Lipid extracts were chromatographed on silica plates with chloroform/methanol/water/ammonia (90:54:5.5:5.5) as developing system. After being visualized with iodine, the compounds were scraped off the plates and assayed for phospholipid phosphorus [22].

Results

Multilayered liposomes were prepared of DMPC, DPPC and DMPE and also of equimolar mixtures of DMPE with DMPC and DPPC, respectively.

Phosphatidic acid (4 mol%) was added to the lipids and lipid mixtures to ensure sufficient trap in the multilayered structures. Also in the preparation of large unilamellar vesicles by the ether injection method phosphatidic acid was added routinely. It was found that the negative phospholipid prevents aggregation of the vesicles and enhances the solubility of DMPE/DMPC and DMPE/DPPC mixtures in ether. Attempts to prepare large unilamellar vesicles of pure DMPE were unsuccessful. Even in the presence of 4 mol% phosphatidic acid the phospholipid appeared insoluble in the ether solvent. The bilayer systems we used for permeability and phospholipase A_2 degradation studies were characterized by various techniques.

Freeze-fracture EM studies

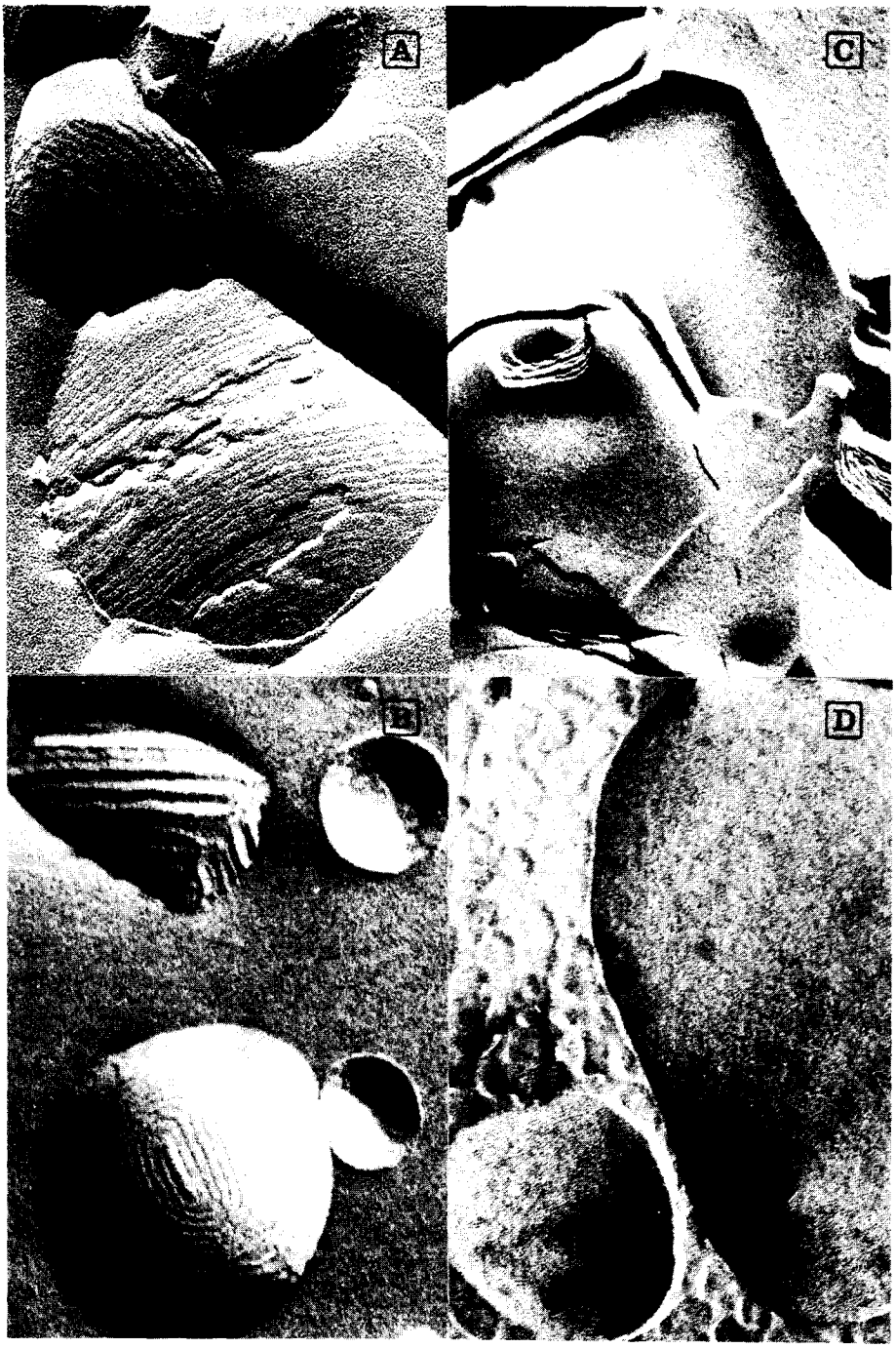
Figure 1 shows electron micrographs of some pure and mixed lipid systems. Freeze-fracturing of liposomal (Fig. 1A) and etherosomal (Fig. 1B) preparations of DMPC (containing 4 mol% phosphatidic acid) quenched from a temperature (20°C) intermediate between the main transition and pretransition temperature of the lipid, reveals band patterns characteristic for the $P_{\beta'}$ phase [24, 25]. As described previously [11], fracture faces of DMPE liposomes are smooth (Fig. 1C). DMPC/DMPE etherosomes (containing 4 mol% phosphatidic acid) also display smooth fracture faces when quenched from room temperature (Fig. 1D). So the introduction of 50% DMPE causes the disappearance of the typical $P_{\beta'}$ ripples in DMPC bilayers [11].

^{31}P-NMR measurements

The bilayer organization of the various model membrane systems was confirmed by ^{31}P-NMR spectra showing a broad asymmetric signal with a high field peak and a low field shoulder characteristic for extended bilayer organizations.

As became apparent from permeability studies (compare e.g. Fig. 4) mixed etherosomes of DMPE/DMPC and DMPE/DPPC exhibit a lower trap when compared with large unilamellar vesicles of the pure lecithin. This could be explained by somewhat smaller diameters of the vesicles or by the existence of multilayered liposomes in the system of mixed phospholipids. In order to discriminate between the two possibilities we studied the effect of addition of Mn^{2+} ions. The validity of the use of paramagnetic ions to

Fig. 1. Freeze-fracture electron micrographs of liposomes and etherosomes quenched from 20°C: (A) DMPC liposomes containing 4 mol% phosphatidic acid; (B) DMPC etherosomes containing 4 mol% phosphatidic acid; (C) DMPE liposomes; (D) DMPC/DMPE (1:1) etherosomes containing 4 mol% phosphatidic acid. Magnification: ×100 000.


A
C
B
D

determine the amount of polar head groups facing the outside medium in saturated phosphatidylethanolamine and phosphatidylcholine bilayer systems has been demonstrated in earlier studies from our laboratory [38–40]. The paramagnetic ion is known to broaden the signals coming from the phospholipid molecules in the outside monolayer and under the conditions used these broadened signals are not measured. It appeared that the signal intensity of both DPPC and DPPC/DMPE vesicles are affected to the same extent. A decrease of the signal intensity to about 55% of its original value indicated that both preparations contained mainly large unilamellar vesicles and only a minor amount of multilayered liposomes.

DSC experiments

In Fig. 2 DSC curves of dispersions of pure lipids and lipid mixtures are presented. All experiments were done at a scan rate of 5.0 K min^{-1}. In heating scans of aqueous dispersions of pure DMPC (Fig. 2A) and DPPC (Fig. 2B) two thermal transitions can be observed: a low enthalpy pretransition, and a high enthalpy main transition. However, in scans of pure DMPE (Fig. 2C), no pretransition was observed, both at scan rates of 5.0 and 1.25 K min^{-1}. All these DSC results agree with previous findings [9, 10].

In the past a third phase transition, centered at about 18°C, was reported from a DSC study on a multilamellar dispersion of DPPC that had been stored at 0°C for 3 days [26]. We could not detect a subtransition in samples of highly (HPLC) purified DPPC that had been stored at 4°C for 5 days. However, heating of an aqueous dispersion of DMPE which was stored for 5 days at 4°C, revealed besides the main transition, two additional endothermic peaks: a small one at 30°C and one at 40°C (Fig. 2D). These additional transitions could only be observed in the first heating scan after the storage at 4°C. In the first cooling scan and in subsequent heating and cooling scans the subtransitions were absent. As yet, the nature of these two additional transitions is unclear. Barrier properties, freeze-fracture morphology and ^{31}P-NMR spectra of aqueous dispersions of DMPE before and after storage at 4°C were shown to be identical.

Incorporation of 50 mol% DMPE into multilayered liposomes of DPPC results in the disappearance of the pretransition of the lecithin whereas the main transition slightly broadens and shifts to a somewhat higher temperature (Fig. 2E). The DMPC-pretransition is also eliminated upon addition of 50 mol% DMPE whereas a considerable broadening of the main transition and a shift to higher temperature occur (Fig. 2F). In the system of the lecithins (multilayered liposomes and etherosomes) containing 4 mol% phosphatidic acid the pretransitions were broadened and could not be observed. The main transitions were only slightly broadened and the maximum of the peak shifted to lower temperature by about one degree.

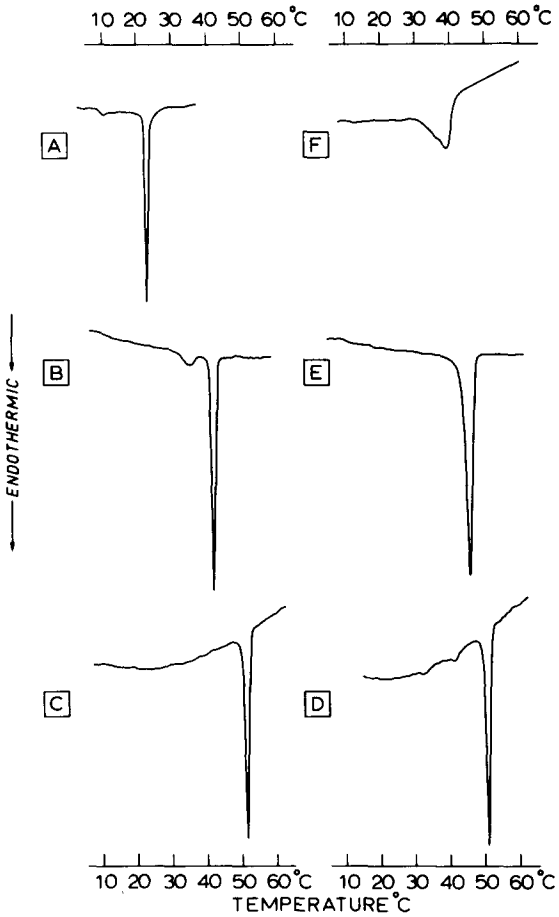


Fig. 2. DSC heating curves of aqueous dispersions of pure lipids and mixtures of phosphatidylcholine and phosphatidylethanolamine: (A) DMPC; (B) DPPC; (C) DMPE; (D) DMPE after being stored at 4°C for 5 days; (E) DPPC/DMPE (1:1); (F) DMPC/DMPE (1:1).

Raman studies

The use of Raman spectroscopy to monitor phospholipid hydrocarbon chain conformation in one and multicomponent phospholipid systems is well documented [27]. The C–C stretching vibrations in the 1050–1150 cm^{-1} range are sensitive to *trans-gauche* isomerization in the hydrocarbon chains. The vibration near 1130 cm^{-1} arises from chain segments in the all-*trans* conformation. Formation of *gauche* rotamers leads to a decrease in intensity of the 1130 cm^{-1} band and a concomitant increase of a band near 1100 cm^{-1}.

The intensity ratio I (1130/1100) is thus a useful measure of phospholipid fluidity. Accordingly, Gaber and Peticolas [27] have developed semiquantitative Raman order parameters which correlate variations in the C–C spectral region with the number of CH_2 groups in the all-*trans* conformation.

The temperature variation in I (1130/1100) for DMPC and DMPE liposomes is shown in Fig. 3. For both lipids a gradual decrease of I (associated with the non-cooperative formation of *gauche* rotamers) is observed prior to T_c (22°C for DMPC, 50°C for DMPE). At T_c both curves show a discontinuity but the drop in intensity ratio (i.e. the formation of *gauche* rotamers) is considerably larger for DMPC than for DMPE. These findings are in good agreement with the results of a similar study done by Mendelsohn and Taraschi [28, 29] on the analogous couple DPPC/DPPE and it can therefore be concluded that the number of *gauche* rotations per hydrocarbon chain formed at the phase transition is larger for phosphatidylcholines than for the corresponding phosphatidylethanolamines.

Permeability studies

Figure 4A shows the release of K^+ from multilayered liposomes of DMPC and DMPE as a function of temperature. As shown before the liposomes of DMPC loose all their enclosed K^+ when incubated at temperatures in the vicinity of the gel to liquid-crystalline phase transition [1]. In contrast, such an increase in permeability could not be noticed in liposomes of DMPE. Also, upon incubation at 49°C where DSC scans showed a sharp transition the phosphatidylethanolamine liposomes were fully capable of retaining

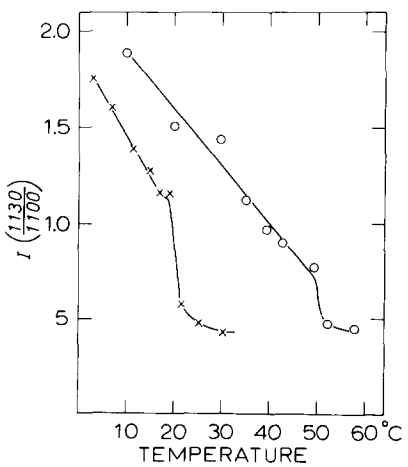


Fig. 3. Temperature-induced variation of the intensity ratio I (1130/1100) for DMPC (×—×) and DMPE (○—○) liposomes.

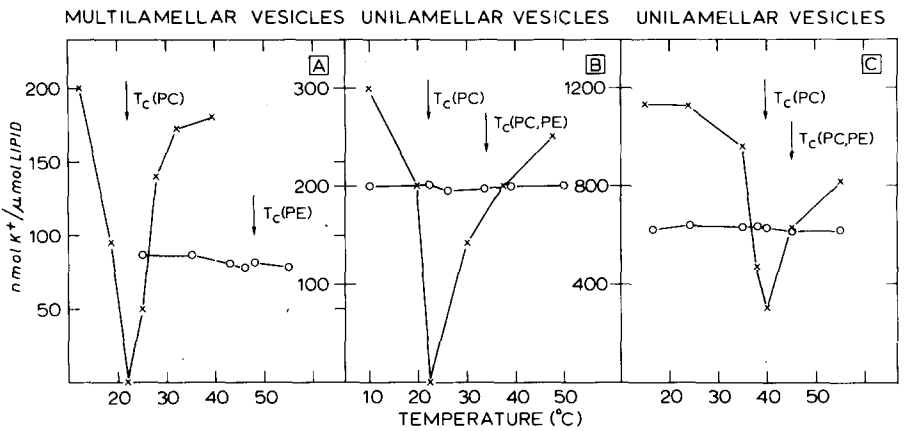


Fig. 4. K^+-trap of different 4 mol% phosphatidic acid containing liposomal and etherosomal preparations 2 min after injection at the temperature indicated: (A) DMPC (×—×) and DMPE (○—○) liposomes; (B) DMPC (×—×) and DMPC/DMPE (1 : 1) (○—○) etherosomes; (C) DPPC (×—×) and DPPC/DMPE (1 : 1) (○—○) etherosomes.

their enclosed K^+. Unilamellar vesicles of DMPC and DPPC showed a rapid release of enclosed K^+ when incubated at the phase transition temperature of the systems. Introduction of increasing concentrations of DMPE into the bilayers of these systems was found to suppress the K^+-leak. Figures 4B and C show that in equimolar mixtures of DMPC/DMPE and of DPPC/DMPE the temperature dependent maxima had disappeared completely although these systems have easily detectable phase transitions at 34°C and 45°C, respectively. The permeability maximum of DPPC etherosomes was also eliminated after incorporation of 25 mol% DEPE. DSC scans of the DPPC/DEPE 3 : 1 mixture revealed only one sharp peak at about 38°C.

Phospholipase experiments

It has been shown that the action of phospholipases on lipid bilayers is strongly enhanced by irregularities in the packing of the substrate molecules [37]. This conclusion has especially been based on the fact that saturated phosphatidycholines can be degraded by pancreatic phospholipase A_2 only in the temperature region of the gel to liquid-crystalline phase transition, so under conditions where liquid and solid domains co-exist in the plane of the membrane [4]. Irregularities in the packing of the lipid molecules at the border of these liquid and solid domains highly favor the insertion of the enzyme into the interface. In view of the remarkable differences in barrier properties of the investigated saturated phosphatidylcholines and DMPE in the vicinity of T_c it seemed to us of interest to examine the action of

pancreatic phospholipase A_2 towards bilayers of DPPC, DMPE and DPPC/DMPE 1:1 as a function of temperature. The results of these experiments are presented in Fig. 5. An aqueous dispersion of DPPC was rapidly degraded by phospholipase A_2 from pig pancreas only near the transition temperature of the lipid (Fig. 5A). In contrast incubations of DMPE liposomes with the phospholipase did not show such a maximum in breakdown at the phase transition (Fig. 5b). Above 40°C the bilayers were only slightly accessible to the enzyme whereas below 40°C the hydrolysis proceeded at a somewhat larger rate. The temperature dependent hydrolysis profile of an equimolar mixture DPPC/DMPE is given in Fig. 5C. Between 43°C and 49°C, the temperature range of the phase transition of this co-crystallizing mixture, both lipids were hardly degraded. Also, in the liquid state the bilayer is resistent to the enzyme attack. Surprisingly, however, it can be noticed that in the gel state a rapid breakdown of the mixed lipid system was observed. Lipid analysis showed that both DPPC and DMPE were almost completely converted to their lyso compounds.

Discussion

The present results show that a gel to liquid-crystalline phase transition has important implications for barrier properties in phosphatidylcholine bilayers but not in phosphatidylethanolamine bilayers. Since, as mentioned in the introduction, the increase in passive permeability at the phase transition of lecithin bilayers has been correlated with the boundaries between regions of different degree of disorder and concomitant lateral compressibility, it is necessary to discuss differences between bilayers of the two phospholipid classes in this respect.

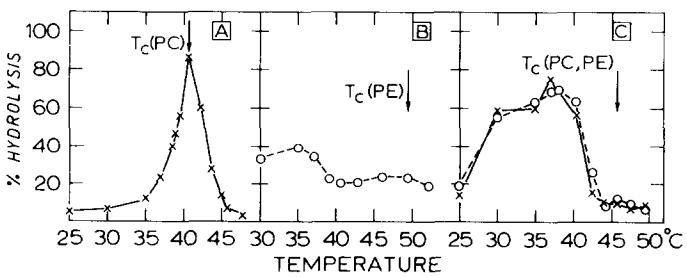


Fig. 5. Temperature-dependent hydrolysis of aqueous dispersions of DPPC (A), DMPE (B) and an equimolar mixture of DPPC and DMPE (C). After 30 min of incubation with phospholipase A_2 phospholipids and lysophospholipids were separated and determined quantitatively. The data are expressed as the percent of the initial amount of lipid which is hydrolyzed in 30 min.

The concept lateral compressibility originates from monolayer studies and is defined as

$$C = \frac{-1}{A}(\delta A/\delta \Pi)_T$$

in which A is the surface area per molecule and Π the surface pressure [20]. In compression isotherms of monolayers of saturated phosphatidylcholines and phosphatidylethanolamines pressure dependent transitions from a liquid expanded to a liquid condensed film can be noticed, which are characterized by a large decrease in area per molecule at slight variation in pressure. Comparison of the Π–A curves of e.g. DPPC and DMPE, films in which the transition occurs at equal pressures, shows that the decrease in area is considerably less for DMPE [30]. However, at equal temperature the transitions of comparable species of the two phospholipid classes occur at quite different pressures. When films of different temperature are compared so that the pressures are identical, the differences are very small, certainly at pressures assumed to exist in bilayers [31]. Therefore, it is not likely that the differences in permeability can be related to data obtainable from monolayer experiments. Furthermore, it can be doubted that the monolayer transitions can be compared with the gel to liquid-crystalline phase transitions in the bilayer organizations; probably they are not first-order transitions.

The lateral phase transitions in bilayers of pure DMPC, DPPC and DMPE are well characterized. X-Ray and freeze-fracture electronmicroscopy have revealed important differences between the gel to liquid-crystalline transitions in bilayers of phosphatidylethanolamines and phosphatidylcholines which are illustrated in Fig. 6. In the gel state of DMPE bilayers the paraffin chains are organized in an array perpendicular to the bilayer surface (L_β phase) [12]. In the gel phase of DPMC and DPPC the chains are tilted [13]. Below the pretransition the lecithin bilayers are smooth ($L_{\beta'}$ phase) but at temperatures between the pretransition and the main transition a rippled [32] structure is apparent ($P_{\beta'}$ phase). The present freeze-fracture and DSC results support the idea that an impurity of 4 mol% phosphatidic acid does not drastically change the different phase organizations. Only hysteresis effects in the $P_{\beta'} \Rightarrow L_{\beta'}$ transitions of the phosphatidylcholine bilayers at the temperature of pretransition seem to be the consequence.

In a recently published excellent review by Hauser et al. [34] it is pointed out that differences in the organization of bilayers of phosphatidylcholine and phosphatidylethanolamine are caused by differences in the packing of the headgroup. In the phosphatidylethanolamine bilayers the polar headgroups form a very compact lattice that is stabilized by strong electrostatic interaction and hydrogen bonding between the ammonium nitrogen

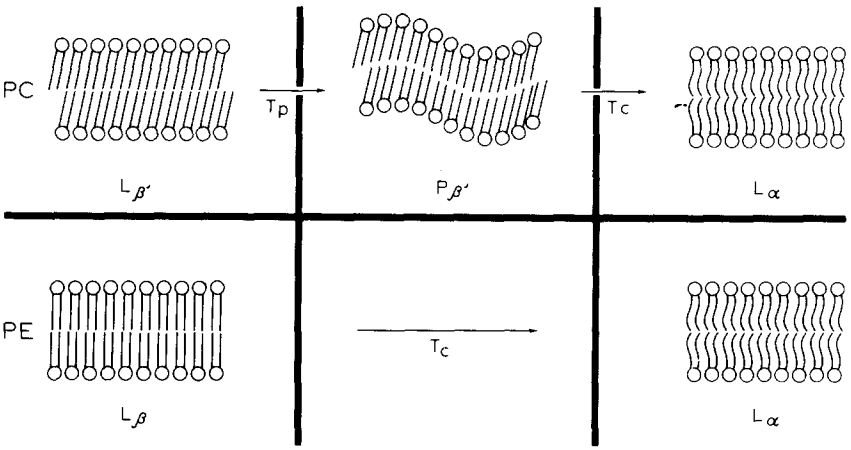


Fig. 6. Molecular organization of phosphatidylcholine- and phosphatidylethanolamine bilayers as a function of temperature.

and the phosphate oxygens. The fully methylated ammonium groups in phosphatidylcholines do not allow direct hydrogen bonding. Instead, the interaction is stabilized by incorporation of water molecules. Despite the larger headgroup spacing an efficient packing is realized by tilting of the chains. At the phase transition temperature an easy expansion of the phosphatidylcholine bilayer occurs, whereas, due to the constraint caused by the hydrogen bonding, such an expansion is inhibited in the phosphatidylethanolamine bilayer. This is supported by dilatometric experiments showing that the change in molecular lipid volume at the main transition is much larger for DMPC than for DMPE [33]. In agreement with this are the results obtained from Raman experiments (compare Fig. 3 and Refs. 28 and 29), which indicate that the increase in *gauche* rotamers at the phase transition is considerably larger for the phosphatidylcholines than for the corresponding phosphatidylethanolamines. The large difference in molecular order of co-existing liquid and solid patches probably explains the maxima in permeability and phospholipase attack which happen in phosphatidylcholine but do not occur in bilayers of phosphatidylethanolamines.

It is a remarkable finding that an equimolar mixture of DPPC and DMPE is subject to rapid hydrolysis by phospholipase A_2 only at temperatures well below the phase transition. In this respect it is of interest that a phase diagram constructed by Blume et al. [35] indicates partial immiscibility in the gel state for DPPC/DMPE mixtures. Boundaries between DMPE rich gel patches with perpendicularly oriented chains and DPPC gel patches with tilted chains could furnish sufficient disorder in packing to enable the phospholipase to attack.

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