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NEGATIVELY CHARGED PHOSPHOLIPIDS AND THEIR POSITION IN THE CHOLESTEROL AFFINITY SEQUENCE

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Summary

The effects of low concentrations of cholesterol in mixtures of a negatively charged phospholipid (phosphatidylserine or phosphatidylglycerol) and another phospholipid (phosphatidylcholine, sphingomyelin or phosphatidylethanolamine) have been studied by differential scanning calorimetry. Only mixtures which showed a gel phase miscibility gap have been employed. It was demonstrated that in mixtures with phosphatidylethanolamine, cholesterol was preferentially associated with the negatively charged phospholipid, regardless whether this species represented the component with the high or with the low transition temperature in the mixture. In mixtures of a negatively charged phospholipid and phosphatidylcholine, cholesterol associated with the negatively charged phospholipid; when the phosphatidylcholine was the species with the low transition temperature, cholesterol had an affinity for the phosphatidylcholine and for the negatively charged phospholipid as well. Cholesterol, in a mixture of sphingomyelin with a high and phosphatidylserine with a low transition temperature, was preferentially associated with sphingomyelin.

From these experiments it is concluded that phospholipids show a decrease in affinity for cholesterol in the following order: sphingomyelin >> phosphatidylserine, phosphatidylglycerol > phosphatidylcholine >> phosphatidylethanolamine.

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Introduction

Calorimetric studies on model membrane sytems have proven to be very useful in the study of sterol-lipid interactions. It has been shown by differential scanning calorimetry that cholesterol shows a preference for fluid species, when it is present in bilayers of binary phosphatidylcholine mixtures, that undergo lateral phase separation. This was deduced from a selective decrease of the enthalpy change of one of the peaks in the calorimetric scan. In all mixtures studied, due to the lateral separation in fluid and solid domains in the bilayer, cholesterol was found to be associated with those molecular species that undergo a transition at the lower temperature [1,2]. This view was supported by freeze-fracture electron microscopic observations [3].

However, in mixtures of phospholipids with differences in the polar headgroup, fluidity was no longer the predominant factor determining the cholesterol association. In mixtures of phosphatidylcholine and phosphatidylethanolamine it was demonstrated that, regardless of whether the phosphatidylcholine was the higher or the lower melting component in the mixture, the presence of low concentrations of cholesterol caused a preferential reduction in the enthalpy change of the phosphatidylcholine transition [4]. This was taken as evidence that cholesterol is preferentially associated with the phosphatidylcholine molecules, even at temperatures where both the phospholipid species are in the liquid-crystalline state. This hypothesis was strongly supported by ^{31}P nuclear magnetic resonance data on a mixed phosphatidylcholine/phosphatidylethanolamine system [5].

Further studies, employing binary mixtures of phosphatidylcholine, phosphatidylethanolamine and sphingomyelin, yielded results which were interpreted in an affinity sequence of the neutral phospholipids for cholesterol. The sequence was found to be: sphingomyelin > phosphatidylcholine > phosphatidylethanolamine [6]. An identical sequence had been found when phospholipid dispersions were tested in their ability to deplete tissue culture cells of their cholesterol [7].

With respect to negatively charged phospholipids no clear conclusions could be drawn. To date several suitable molecular species of phosphatidylserine and phosphatidylglycerol have become available through the enzymatic conversion procedure described recently [8]. Furthermore some practical aspects in the thermotropic behaviour of synthetic unsaturated negatively charged phospholipids have been solved [10]. Therefore, in this paper the position of negatively charged phospholipids in the cholesterol affinity sequence has been investigated in detail.

Materials and Methods

All synthetic phospholipids used, besides the phosphatidylcholine species, were converted from phosphatidylcholines, which had been synthesized by Ank Lancée, Marion Tieman and Fred Neys according to established procedures [10,11]. 1,2-Dioleoyl-*sn*-glycero-3-phosphorylcholine (18 : 1_c/18 : 1_c-glycerophosphocholine), and 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine (16 : 0/16 : 0-glycerophosphocholine) were used. 1,2-Distearoyl-*sn*-glycero-3-phos-

phorylcholine (18 : 0/18 : 0-glycerophosphocholine) was obtained by hydrogenation of 18 : 1_c/18 : 1_c-glycerophosphocholine.

1,2-Dioleoyl-*sn*-glycero-3-phosphorylethanolamine (18 : 1_c/18 : 1_c-glycerophosphoethanolamine), 1,2-dielaidoyl-*sn*-glycero-3-phosphorylethanolamine (18 : 1_t/18 : 1_t-glycerophosphoethanolamine), 1,2-dielaidoyl-*sn*-glycero-3-phosphorylglycerol (18 : 1_t/18 : 1_t-glycerophosphoglycerol), 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylglycerol (16 : 0/16 : 0-glycerophosphoglyerol), 1,2-dioleoyl-*sn*-glycero-3-phosphorylserine (18 : 1_c/18 : 1_c-glycerophosphoserine) and 1,2-dimyristoyl-*sn*-glycero-3-phosphorylserine (14 : 0/14 : 0-glycerophosphoserine) were obtained by phospholipase D-catalyzed base exchange as described before [8,9,13,14]. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphorylethanolamine (16 : 0/16 : 0-glycerophosphoethanolamine) was obtained from Sigma. Bovine brain sphingomyelin was fractionated by high performance liquid chromatography by Wouter Geurts van Kessel as described before [15]. The long chain fraction which was used in the calorimetric experiments consisted of 37% 18 : 0, 13% 20 : 0, 14% 20 : 1, 14% 24 : 0 and 14% 24 : 1 as fatty acids (average molecular weight of the sphingomyelin was 750).

The negatively charged phospholipids were converted to their (di-) sodium salt as described before [9] or, in case of 18 : 1_c/18 : 1_c-glycerophosphoserine by a treatment as described by Findlay and Barton [10].

Mixtures of phospholipids in equimolar ratios (with or without 20 mol% cholesterol) were mixed in organic solution followed by evaporation of the solvent. Dispersion of the lipids was achieved by agitation for 10 min at 60°C under nitrogen of 5—10 μmol of phospholipid in 1.5 ml of buffer, using a Griffin flask shaker and a thermostated waterbath. The buffers which were used consisted of: 25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 7.0 (A), or 25 mM glycine/NaOH, 150 mM NaCl, 5 mM EDTA, pH 9.5 (B). Buffer B was only used with mixtures containing phosphatidylserine. In all other cases buffer A was used.

The liposomal suspensions were concentrated by centrifugation (10 min, 37 500 × *g*). Specimens of the wet pellets were analyzed on a Perkin-Elmer DSC 2, cooled by an Intracooler I. The apparatus was operated at heating and cooling rates of 5°C/min and was calibrated as described before [2]. The amount of phospholipid present in the sample was determined by phosphorus analysis according to Böttcher et al. [16]. The peak areas were measured by using an electronic planimeter (Hewlett-Packard). The standard deviation in the enthalpy change value was 5 and 10% for pure lipids and lipid mixtures, respectively.

Results

The preferential abolishment by cholesterol of the phase transition of one of the two phospholipid species in a mixed phospholipid bilayer has been interpreted as a preferential association of cholesterol with that given type of phospholipid. However, it cannot be excluded that upon evaporation of the organic solvents in the preparation of a mixed lipid film prior to hydration a non-homogeneous film may be formed as a consequence of differences in solubility of the several lipid species present. This would yield different lipid populations

one of which might contain most of the cholesterol. To test this possibility two types of experiments have been performed.

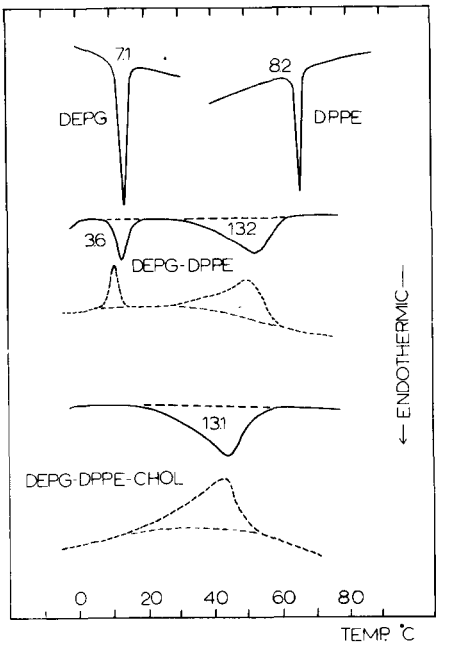
An equimolar mixture of 18 : 1_c/18 : 1_c-glycerophosphoethanolamine and 16 : 0/16 : 0-glycerophosphocholine in the presence of 20 mol% cholesterol was dissolved in benzene and lyophilized prior to hydration. Another sample of the same lipid mixture was prepared according to the normal procedure but the lipids were randomized by sonication followed by freezing and thawing which fused the vesicles to multilayered structures. Both controls showed a thermotropic behaviour which was identical to that reported previously [4,5]. Similar control experiments have been performed for other combinations of phospholipid including those reported in the next sections. All controls yielded results comparable to the results obtained after the normal evaporation and hydration procedure. These experiments confirm that the preferential reduction by cholesterol of one of the two transitions in such mixtures as seen by calorimetry can indeed be interpreted in terms of differences in affinity of certain phospholipids for cholesterol.

Mixtures of negatively charged phospholipids with phosphatidylethanolamine

Phosphatidylethanolamine has a poor affinity for cholesterol compared to the choline-containing phospholipids; therefore firstly mixtures of negatively charged phospholipids and phosphatidylethanolamine have been investigated. As outlined before [4], mixtures displaying a gel phase miscibility gap are most suitable for the study of cholesterol affinity differences.

When 18 : 1_t/18 : 1_t-glycerophosphoglycerol and 16 : 0/16 : 0-glycerophosphoethanolamine (ΔH values of 7.1 and 8.2 kcal/mol for the individual components, respectively) were mixed in an equimolar ratio two fully separated transitions were observed by differential scanning calorimetry (Fig. 1). Values for the enthalpy change of 3.6 and 13.2 kcal/mol were found, indicating that a large fraction of the phosphatidylglycerol molecules (about 50%) was cocrystallizing with the high melting phosphatidylethanolamine molecules. The presence of 20 mol% cholesterol caused a complete disappearance of the lower transition peak whereas the upper one was not affected (13.1 kcal/mol). This experiment (Fig. 1) shows that cholesterol was preferentially associated with the lower melting species which was the phosphatidylglycerol.

As shown in Fig. 2 an equimolar mixture of 18 : 1_c/18 : 1_c-glycerophosphoethanolamine and 16 : 0/16 : 0-glycerophosphoglycerol (ΔH values of 5.0 and 9.1 kcal/mol, respectively, for the individual components), showed gel phase immiscibility albeit incomplete. However, comparing this experiment with the previous one, we have the reverse situation in that the phosphatidylethanolamine is the molecular species with the lowest transition temperature. In the heating curve, due to the incomplete phase separation, the phosphatidylethanolamine-rich part of the transition occurred in the region of the ice-water transition; however, in a cooling curve, in which the ice-water transition displays some super cooling, this part of the curve could be visualized. These incompletely separated transitions were estimated to be 2 and 8 kcal/mol in enthalpy change for the lower and higher transition, respectively. The same mixture in the presence of 20 mol% cholesterol gave two transitions which were fully separated. The enthalpy change of the low-temperature transition,



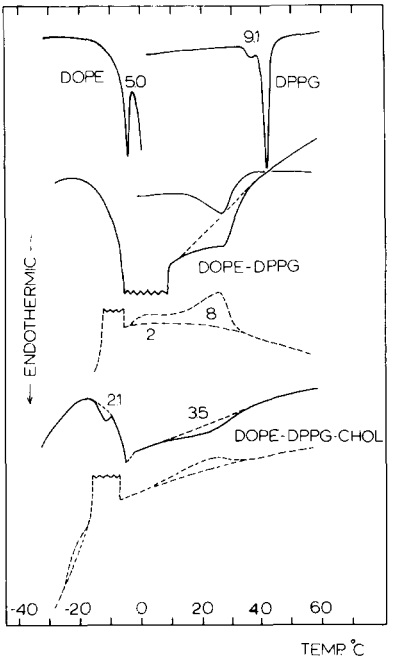
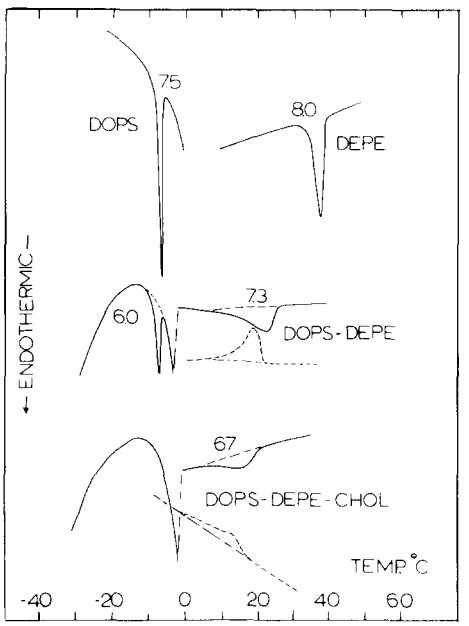


Fig. 1. Influence of 20 mol% cholesterol on the thermotropic behaviour of an equimolar mixture of 18 : 1_t/18 : 1_t-glycerophosphoglycerol (DEPG) and 16 : 0/16 : 0-glycerophosphoethanolamine (DPPE). The lipid samples were prepared in buffer A as described in Materials and Methods. · · · · · ·, cooling scans.

Fig. 2. Influence of 20 mol% cholesterol (CHOL) on the thermotropic properties of an equimolar mixture of 18 : 1_c/18 : 1_c-glycerophosphoethanolamine (DOPE) and 16 : 0/16 : 0-glycerophosphoglycerol (DPPG). The lipid samples were preapred in buffer A as described in Materials and Methods. Of DOPE-DPPG two high-temperature parts of the heating curve are shown. One has been obtained by continuous scanning from —35 to 60°C, the other scan has been interrupted at about 0°C to allow the ice to melt isothermally before the rest of the curve has been recorded. A similar interruption has been used to obtain the heating curve of DOPE-DPPG-CHOL. · · · · · ·, cooling scans.

which occurred at a temperature comparable to that of pure 18 : 1_c/18 : 1_c-glycerophosphoethanolamine, was 2.1 kcal/mol. The high-temperature transition was reduced to 3.5 kcal/mol. From the two phenomena, enlargement of the miscibility gap and a preferential reduction of the enthalpy change of the high-melting component, encountered in the above experiment, it can be concluded that in this situation cholesterol also showed a stronger affinity for phosphatidylglycerol than for phosphatidylethanolamine.

In mixtures of phosphatidylserine and phosphatidylethanolamine similar features were observed. An equimolar mixture of 18 : 1_c/18 : 1_c-glycerophosphoserine (ΔH 7.5 kcal/mol) and 18 : 1_t/18 : 1_t-glycerophosphoethanolamine (ΔH 8.0 kcal/mol) showed complete phase separation with ΔH values of 6.0 and 7.3 kcal/mol for the lower and upper transition, respectively. The rather broad transition of the pure 18 : 1_t/18 : 1_t-glycerophosphatidylethanolamine is caused by the pH of 9.5, which is in the vicinity of the amine group pK. When an identical mixture of phosphatidylserine and phosphatidylethanolamine, but now containing 20 mol% cholesterol was scanned, the low-temperature transition of the phosphatidylserine molecules was completely abolished;



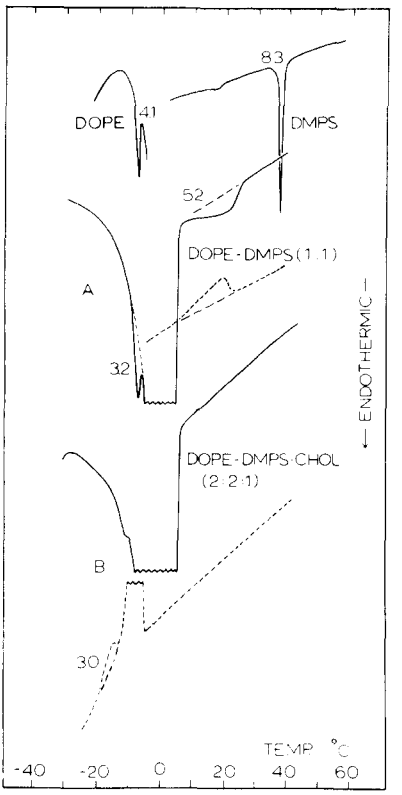


Fig. 3. Influence of 20 mol% cholesterol on the thermotropic properties of an equimolar mixture of 18 : 1_c/18 : 1_c-glycerophosphoserine (DOPS) and 18 : 1_t/18 : 1_t-glycerophosphoethanolamine (DEPE). Samples were prepared in buffer B as described in Materials and Methods. The heating scans have been interrupted at about 0° C to allow isothermal ice melting. · · · · · ·, cooling scans.

Fig. 4. Influence of 20 mol% cholesterol on the thermotropic properties of an equimolar mixture of 18 : 1_c/18 : 1_c-glycerophosphoethanolamine (DOPE) and 14 : 0/14 : 0-glycerophosphoserine (DMPS). Samples were prepared in buffer B as described in Materials and Methods. · · · · · ·, cooling curves.

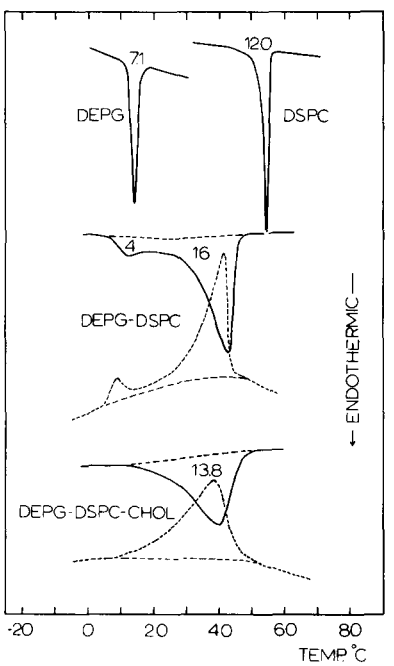
the ΔH value of the high-temperature transition of the phosphatidylethanolamine molecules remained unchanged (6.7 kcal/mol).

From this experiment, shown in Fig. 3, a preferential association of cholesterol with the lower melting species which is the phosphatidylserine is concluded. The reverse experiment is depicted in Fig. 4. 18 : 1_c/18 : 1_c-Glycerophosphoethanolamine (ΔH 4.1 kcal/mol) was mixed in an equimolar ratio with 14 : 0/14 : 0-glycerophosphoserine (ΔH 8.3 kcal/mol). This yielded liposomes showing gel phase immiscibility; two transitions were apparent with ΔH values of 3.2 and 5.3 kcal/mol for the low and high-temperature transition, respectively. It has to be noted that a pretransition does occur in the case of the pure phosphatidylserine. This is also the case in 16 : 0/16 : 0-glycerophosphoglycerol (Fig. 1) and 14 : 0/14 : 0-glycerophosphoglycerol. The absence of a pretransition in similar preparations in previous studies either was caused by the presence of ethyleneglycol [13,17] or was due to hysteresis [9,13]. A mixture of 18 : 1_c/18 : 1_c-glycerophosphoethanolamine, 14 : 0/14 : 0-glycerophospho-

serine and cholesterol in a 2 : 2 : 1 molar ratio displayed a low-temperature transition with no change in ΔH value whereas the high-temperature transition of the phosphatidylserine molecules was completely abolished. This again indicates that cholesterol shows a stronger preference for phosphatidylserine than for phosphatidylethanolamine.

Mixtures of negatively charged phospholipids with phosphatidylcholine

In order to study the effects of cholesterol in a mixture of phosphatidylglycerol and phosphatidylcholine, in which the phosphatidylglycerol species is the lower melting component, 18 : 1_t/18 : 1_t-glycerophosphoglycerol (ΔH 7.1 kcal/mol) and 18 : 0/18 : 0-glycerophosphocholine (ΔH 12.0 kcal/mol) were employed. The equimolar mixture (Fig. 5) showed incomplete phase separation; the two peaks were estimated to be 4 and 16 kcal/mol in enthalpy change. Again, as in the case of an equimolar mixture of 18 : 1_t/18 : 1_t-glycerophosphoglycerol and 16 : 0/16 : 0-glycerophosphoethanolamine (Fig. 1), this indicated that some of the phosphatidylglycerol cocrystallized with the high-melting component. In the calorimetric scan of this phosphatidylglycerol/



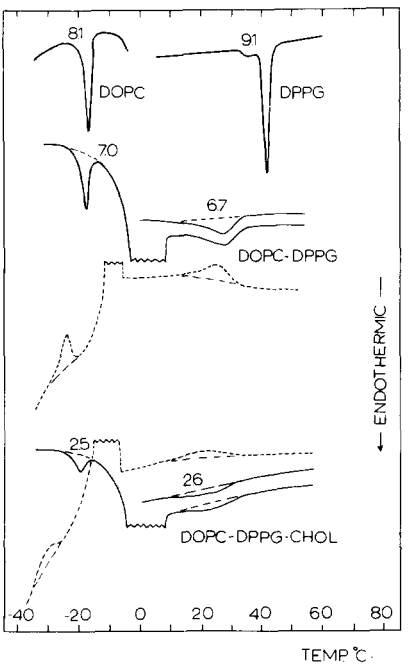


Fig. 5. Influence of 20 mol% cholesterol on the thermotropic properties of an equimolar mixture of 18 : 1_t/18 : 1_t-glycerophosphoglycerol (DEPG) and 18 : 0/18 : 0-glycerophosphocholine (DSCP). The samples have been prepared in buffer A as described in Materials and Methods. · · · · · ·, cooling scans.

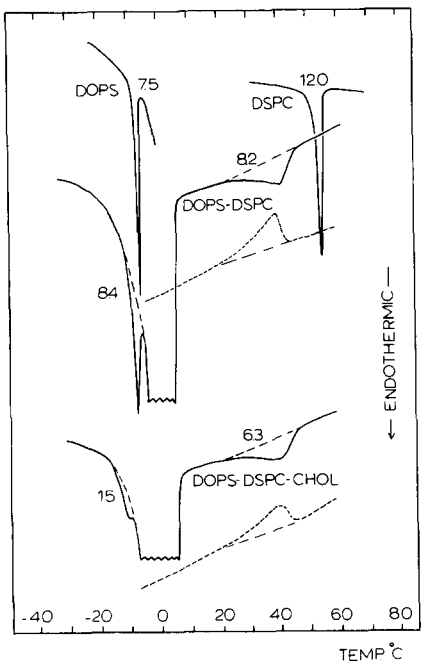
Fig. 6. Influence of 20 mol% cholesterol on the thermotropic properties of an equimolar mixture of 18 : 1_c/18 : 1_c-glycerophosphocholine (DOPC) and 16 : 0/16 : 0-glycerophosphoglycerol (DPPG). The samples have been prepared in buffer A as described in Materials and Methods. · · · · · ·, cooling scans. The heating curves include high-temperature tracings obtained by interrupting the heating scan at about 0°C to allow the ice to melt isothermally.

phosphatidylcholine mixture, in the presence of 20 mol% cholesterol, the lower part of the transition was absent whereas the high-temperature part of the transition was not affected (13.8 kcal/mol). Cholesterol was therefore preferentially associated with the lower melting phosphatidylglycerol molecules in this mixture.

For the experiment, in which phosphatidylglycerol was the higher melting component, 18 : 1_c/18 : 1_c-glycerophosphocholine (ΔH 8.1 kcal/mol) and 16 : 0/16 : 0-glycerophosphoglycerol (ΔH 9.1 kcal/mol) were used. The differences in the transition temperature and enthalpy change of 18 : 1_c/18 : 1_c-glycerophosphocholine compared to a previous study [4] can be attributed to ethyleneglycol (van Echteld, C., de Kruijff, B. and de Gier, J., unpublished results). An equimolar mixture of the molecular species mentioned above displayed complete phase separation (Fig. 6). The enthalpy change values of the low and the high-temperature transitions were 7.0 and 6.7 kcal/mol, respectively. Since there was the possibility that part of the high-temperature transition was obscured by an overlap with the ice-water transition this part of the curve was also recorded starting from a temperature at which the water was supercooled. No differences were detected. Introduction of 20 mol% cholesterol in this mixture caused a reduction in the enthalpy change values of both transitions. ΔH values of 2.5 and 2.6 kcal/mol for the low and high-temperature transition, respectively, were found, indicating an association of cholesterol with the phosphatidylglycerol as well as with the phosphatidylcholine molecules.

Mixtures of phosphatidylserine and phosphatidylcholine were also studied. In the case of phosphatidylserine as the low temperature melting species, 18 : 1_c/18 : 1_c-glycerophosphoserine and 18 : 0/18 : 0-glycerophosphocholine (ΔH values 7.5 and 12.0 kcal/mol, respectively) were used. The equimolar mixture (Fig. 7) showed complete phase separation; 8.4 and 8.2 kcal/mol were found for the ΔH values of the low and high-temperature transition, respectively. Incorporation of 20 mol% cholesterol in this mixture gave rise to a severe reduction in the enthalpy change of the low-temperature transition (ΔH 1.5 kcal/mol); the high-temperature transition was not affected greatly (ΔH 6.3 kcal/mol). From this experiment it can be concluded that, for this combination of lipid species, cholesterol is preferentially associated with the lower melting phosphatidylserine component. Comparable effects were observed when cholesterol was introduced in an equimolar mixture of 18 : 1_c/18 : 1_c-glycerophosphoserine and 16 : 0/16 : 0-glycerophosphocholine; the results were not qualitatively affected when the experiments were carried out at pH 7.0 instead of pH 9.5 (data not shown).

In experiments, in which the phosphatidylserine molecules represented the high-melting component, the results obtained were similar to those where phosphatidylglycerol was used as the higher and phosphatidylcholine as the lower melting component. Mixing 18 : 1_c/18 : 1_c-glycerophosphocholine (ΔH 9.0 kcal/mol) and 14 : 0/14 : 0-glycerophosphoserine (ΔH 8.3 kcal/mol) in equimolar amounts yielded a tracing with two separated lipid transitions of 7.8 and 8.0 kcal/mol for the low and the high temperature respectively (Fig. 8). As above the ice-water transition was shown not to obscure part of the phosphatidylserine transition. Introduction of 20 mol% cholesterol in the mixture



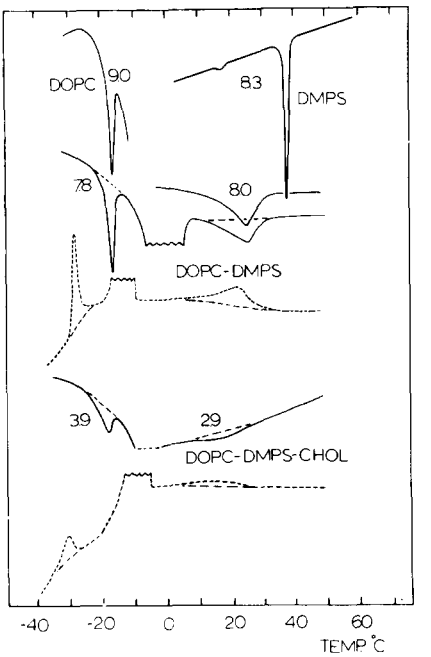


Fig. 7. Influence of 20 mol% cholesterol on the thermotropic properties of an equimolar mixture of 18 : 1_c/18 : 1_c-glycerophosphoserine (DOPS) and 18 : 0/18 : 0-glycerophosphocholine (DSCP). The samples have been prepared in buffer B as described in Materials and Methods. · · · · ·, cooling curves.

Fig. 8. Influence of 20 mol% cholesterol (CHOL) on the thermotropic properties of an equimolar mixture of 18 : 1_c/18 : 1_c-glycerophosphocholine (DOPC) and 14 : 0/14 : 0-glycerophosphoserine (DMPS). The samples have been prepared in buffer B as described in Materials and Methods. The heating curve of DOPC-DMPS-CHOL has been interrupted at about 0° C to allow isothermal melting of the ice. · · · · · ·, cooling curves.

partially eliminated both transition peaks; the enthalpy change values decreased to 3.9 and 2.9 kcal/mol for the low and high-temperature transition, respectively. Here in this experiment cholesterol is associated with both phospholipid species.

Mixtures of phosphatidylserine and sphingomyelin

Fig. 9 shows the thermotropic behaviour of an equimolar mixture of 18 : 1_c/18 : 1_c-glycerophosphoserine and long-chain sphingomyelin (ΔH values for both individual components 7.3 kcal/mol). Two clearly separated transitions were observed with enthalpy change values of 3.6 and 10.0 kcal/mol for the low and high-temperature transitions, respectively. This indicates, as was also shown for other mixtures, that part of the 18 : 1_c/18 : 1_c-glycerophosphoserine molecules cocrystallized with the sphingomyelin molecules. In the presence of 20 mol% cholesterol the enthalpy change value of the low-temperature transition is unaffected (4.0 kcal/mol) but the value for the high-temperature transition had decreased to 4.0 kcal/mol, which indicates that in this mixture cholesterol was preferentially associated with the sphingomyelin molecules.

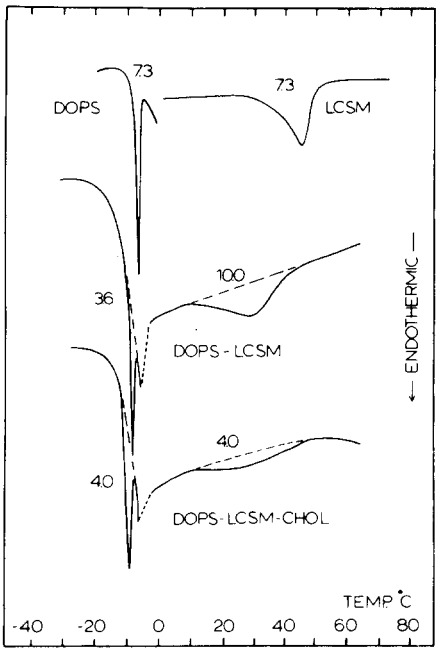


Fig. 9. Influence of 20 mol% cholesterol on the thermotropic properties of an equimolar mixture of 18 : 1_c/18 : 1_c-glycerophosphoserine (DOPS) and a long-chain fraction of sphingomyelin molecules as fractionated by high performance liquid chromatography from bovine brain sphingomyelin (LCSM). The samples were prepared in buffer A as described in Materials and Methods. The curves have been interrupted at about 0° C for isothermal melting of the ice.

Discussion

The results of the present study clearly demonstrate that, in binary mixtures of a negatively charged phospholipid and phosphatidylethanolamine, cholesterol is associated with the negatively charged phospholipid species, regardless of whether or it comprises the low or the high-melting component in the mixture. In the case of mixtures with phosphatidylcholines the situation is more complex. When the negatively charged phospholipid is the low-melting component in the mixture, cholesterol is exclusively associated with this molecule. However, when the phosphatidylserine or phosphatidylglycerol is the high-melting component, cholesterol is associated with the phosphatidylcholine as well. Recently the effects of the erythrocyte membrane-spanning protein glycophorin on the thermotropic properties of synthetic phospholipids have been studied [18]. When glycophorin was introduced into a bilayer composed of an equimolar mixture of 12 : 0/12 : 0-glycerophosphocholine and 14 : 0/14 : 0-glycerophosphoserine a behaviour comparable to cholesterol was found in that both lipid transitions partly were abolished. This behaviour can be explained in the following way. In binary mixtures of phospholipid which have identical polar headgroups and show lateral phase separation, there is a strict preference of cholesterol for fluid lipid over gel phase lipid [1,4]. However, in mixtures of phospholipids with different polar headgroups, cholesterol affinity differences

play the determinant role [4—6]. Apparently both mechanisms play a role in the phosphatidylcholine/phosphatidylserine (or phosphatidylglycerol) mixtures. From these results it can be concluded therefore, that both phosphatidylglycerol as well as phosphatidylserine show a stronger preference for cholesterol than does phosphatidylcholine. However, the differences in affinity are rather small. The result is, that the affinity of cholesterol for a liquid-crystalline phosphatidylcholine is about equal to that for a gel phase negatively charged phospholipid.

It is known from a previous study [6], in which mixtures of sphingomyelin and phosphatidylcholine have been investigated, that cholesterol preferentially associates with sphingomyelin. When the sphingomyelin species represented the high-melting and phosphatidylcholine the low-melting component, no effects on the enthalpy change value of the phosphatidylcholine were found before the sphingomyelin transition had been abolished completely. As the negatively charged phospholipids, phosphatidylserine and phosphatidylglycerol, do not show such an exclusive association with cholesterol, when they are mixed with phosphatidylcholine, this suggests that sphingomyelin shows a stronger preference for cholesterol than do the negatively charged phospholipids. The result of the experiment, in which a high-melting sphingomyelin and a low-melting phosphatidylserine have been mixed, fully supports this statement.

As the miscibility properties of phospholipid mixtures are strongly dependent both on the nature of the acyl chains as well as on the structure of the polar headgroup the amount of one phospholipid being present in the gel phase of the other will vary from one phospholipid mixture to another. This of course will influence the observed affinities for cholesterol. By using a variety of molecular species for a given combination of phospholipid classes the extent of gel phase immiscibility can be varied. It was demonstrated (data not shown) that the presence of a variable amount of one phospholipid in the gel phase of the other one only affects the affinity of that phospholipid in a quantitative way.

In summary the present experiments show a decrease in affinity for cholesterol in the following sequence: sphingomyelin >> phosphatidylserine, phosphatidylglycerol > phosphatidylcholine >> phosphatidylethanolamine *. This sequence is in good agreement with previous data [4,6]. The relative affinities of the negatively charged phospholipids for cholesterol are not known.

It is of interest to note that cholesterol influences the miscibility properties of binary phospholipid mixtures, which show phase separation, in that cholesterol either induces cocrystallization or enlargment of the miscibility gap. This can be appreciated in the following manner. Cholesterol preferentially interacts with one of the molecular species in the binary mixtures. Interaction with cholesterol, as was demonstrated recently by calorimetry, gives rise to a gel phase separation of cholesterol-free domains and lipid domains which are restricted in their cooperativity (perturbed) by the presence of lipid-sterol complexes [19,20]. This is comparable to calorimetric features which have been found for a Folch-Lees protein-containing bilayer [21]. Thus a preferen-

* The sign >> is used for affinity differences without fluid-solid competition problems in the mixtures.

tial interaction of a phospholipid with cholesterol leads to a depletion of that phospholipid in the unperturbed phospholipid pool, which may change the miscibility properties of the phospholipid mixture. This change will be dependent on the phase diagram of the mixture and on the mol fraction of cholesterol in the bilayer. A similar induction of lateral phase separation due to preferential interactions with particular phospholipids has recently been described for proteins [22,23].

The detailed mechanism behind these differences in affinity for cholesterol is still unclear. Since many different molecular species of the several phospholipid classes have been employed, an exclusively hydrophobic interaction cannot explain the observed affinity differences. An alternative may be found in the hypothesis that cholesterol and phospholipid interact through hydrogen bonds between polar groups in the phospholipid and the 3β-OH group in the cholesterol molecule [24,25]. These interactions may be mediated through the bound water region [26]. Differences in the ability of the phospholipids to form hydrogen bridges would explain the observed differences in affinity for cholesterol. However, there are some considerations which do not support this hypothesis. Firstly, phosphatidylserine/phosphatidylethanolamine mixtures have been studied here at both pH 9.5 as at pH 7.0. The amine group of phosphatidylethanolamine is at pH 9.5 in the vicinity of its pK which may affect the bound water system. Since no qualitative differences in affinity for cholesterol were found at pH 9.5 compared to pH 7.0 the importance of the hydrogen belt theory is questionable. Secondly Findlay and Barton [10] have shown that in a mixed phosphatidylcholine/phosphatidylglycerol (Ca^{2+}) bilayer cholesterol is preferentially associated with the phosphatidylglycerol without any evidence of a fluid-solid competition for the cholesterol. This would indicate that a Ca^{2+} salt of phosphatidylglycerol shows a stronger preference for cholesterol than does the Na^{+} salt. As the presence of Ca^{2+} is known to dehydrate the polar region of a negatively charged phospholipid [27,28] the bound water seems to be, at least in these cases, of little importance. A better explanation may be found in the overall geometry of the phospholipid and cholesterol molecules [5,28]. It is energetically favorable for cholesterol, a cone-shaped molecule, to associate with inverted cone-shaped molecules like phosphatidylcholine and negatively charged phospholipids. Phosphatidylethanolamine is also cone shaped and, as expected, it shows the lowest affinity of the phospholipids studied so far. It is not easy to explain the noticable differences in affinity between the choline-containing phospholipids sphingomyelin and phosphatidylcholine. One can emphasize the free OH group of sphingomyelin and the effect it will have on the amount of bound water [6], but there may also be a geometry factor involved. Besides the very long-chain fatty acid, sphingomyelin posesses a rather short hydrocarbon chain originating from the sphingosine backbone. This might make the molecule as a whole a more inverted cone-shaped molecule than a phosphatidylcholine molecule.

In order to obtain more details about the molecular mechanism which lead to these differences in cholesterol affinity and about the biological significance of the cholesterol affinity sequence for the structure and function of biomembranes further studies are needed.

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