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EFFECTS OF CHOLESTEROL ON THE PROPERTIES OF EQUIMOLAR MIXTURES OF SYNTHETIC PHOSPHATIDYLETHANOLAMINE AND PHOSPHATIDYLCHOLINE

A ^{31}P NMR AND DIFFERENTIAL SCANNING CALORIMETRY STUDY

P.R. CULLIS [a,*], P.W.M. VAN DIJCK [a], B. DE KRUIJFF [b] and J. DE GIER [a]

[a] *Biochemistry Department and* [b] *Laboratory of Molecular Biology, State University of Utrecht, Transitorium 3, Padualaan 8, Utrecht (The Netherlands)*

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Summary

1. The interaction of cholesterol with equimolar mixtures of 18 : 1_c/18 : 1_c phosphatidylethanolamine and 16 : 0/16 : 0 phosphatidylcholine has been investigated employing differential scanning calorimetry and ^{31}P NMR. At temperatures where lateral phase separation occurs cholesterol interacts preferentially with the (gel state) 16 : 0/16 : 0 phosphatidylcholine component, in agreement with previous studies (van Dijck et al. (1976) Biochim. Biophys. Acta 455, 576—587). Bilayer structure is maintained in such situations, and the effects do not therefore arise due to a preference of cholesterol for phospholipids in bilayer or non-bilayer phases. Further, the ^{31}P NMR results indicate that when both species are in the liquid crystalline state the preferential interaction of cholesterol with the phosphatidylcholine component is maintained.

2. The addition of cholesterol to (liquid crystalline) equimolar mixtures of 18 : 1_c/18 : 1_c phosphatidylethanolamine and 16 : 0/16 : 0 phosphatidylcholine stabilizes the bilayer phase. Alternatively, cholesterol destabilizes the bilayer phase in equimolar mixtures of 18 : 1_c/18 : 1_c phosphatidylethanolamine and 18 : 1_c/18 : 1_c phosphatidylcholine, reducing the temperature at which non-bilayer phases are formed. Intermediate cholesterol concentrations encourage formation of a phase which possibly has cubic structure, whereas higher cholesterol contents (and higher temperatures) encourage formation of the hexagonal (H_{11}) phase.

3. The temperature-dependent polymorphic phase behaviour of equimolar mixtures of 18 : 1_c/18 : 1_c phosphatidylethanolamine and 18 : 1_c/18 : 1_c

* Present address: Biochemistry Department, University of British Columbia, Vancouver V6T 1W5, Canada.

phosphatidylcholine exhibits a very pronounced hysteresis which is progressively reduced by addition of cholesterol.

Introduction

Recent investigations employing differential scanning calorimetry have suggested that cholesterol prefers to be associated with (saturated) phosphatidylcholines in mixed phosphatidylethanolamine-phosphatidylcholine model membrane systems [1]. These and associated observations [2] are of importance with regard to possible heterogeneous distributions of cholesterol in biological membranes induced by preferential interactions with particular phospholipid classes. However, it has also been shown recently [3] that phosphatidylethanolamine/phosphatidylcholine mixtures exhibit rather complex polymorphic phase behaviour, which may depend strongly on such details as fatty acid composition and cholesterol content. It could be suggested that the preference of cholesterol for phosphatidylcholines may arise because of a preference for bilayer or non-bilayer environments, which may co-exist in these mixed systems. In this work we have therefore investigated such possibilities employing ^{31}P NMR techniques, which have been previously shown to be sensitive to the polymorphic phase of aqueous dispersions of phospholipid [3—6].

In addition, in recent work [3] employing a naturally occurring phosphatidylethanolamine it was shown that cholesterol has remarkably different effects on the mesomorphic phase assumed by phosphatidylethanolamine-phosphatidylcholine dispersions, depending on the degree of unsaturation of the phosphatidylcholine component. It is of obvious interest to extend such observations to systems consisting of well defined species of phospholipid, and such studies are also described here.

Materials and Methods

1,2-Dioleoyl-*sn*-glycero-3-phosphorylethanolamine ($18:1_c/18:1_c$ phosphatidylethanolamine) was synthesised as described previously [4]. 1,2-Dipalmitoyl-*sn*-glycero-3-phosphorylcholine ($16:0/16:0$ phosphatidylcholine) and 1,2-dioleoyl-*sn*-glycero-3-phosphorylcholine ($18:1_c/18:1_c$ phosphatidylcholine) were also synthesised according to previously established methods [7]. Cholesterol was obtained from Fluka (Buchs, Switzerland).

Aqueous dispersions of phospholipids were formed from a chloroform solution containing appropriate amounts of phospholipid (total 50—100 mg), from which the chloroform was evaporated under N_2 and subsequent overnight storage under vacuum. The lipids were then hydrated by addition of 0.7 ml ^2H_2O (2 mM EDTA/25 mM Tris-acetic acid, p^{2}H 7.0) and exhaustive vortex mixing. It should perhaps be emphasized that the lipid dispersion thus obtained was fully hydrated, and that a large excess of the aqueous buffer was present.

Differential scanning calorimetry experiments were performed as detailed by van Dijck et al. [1], with the exception that ethyleneglycol was not added to the aqueous phospholipid dispersion.

^{31}P NMR spectra were obtained employing a Bruker WH-90 spectrom-

eter operating at 36.4 MHz in the Fourier transform mode. Temperature control and proton decoupling facilities were available. All spectra were obtained in the presence of maximum power (20 W) broad band proton decoupling. Free induction decays were obtained from ≈10 000 transients employing a 45° radio frequency pulse and 0.17 s interpulse time.

Results

The influence of increasing cholesterol content on the differential scanning calorimetry properties of 18 : 1_c/18 : 1_c phosphatidylethanolamine alone (Figs. 1A and 1C) and equimolar mixtures of 18 : 1_c/18 : 1_c phosphatidylethanolamine and 16 : 0/16 : 0 phosphatidylcholine (Figs. 1B and 1D) is illustrated in Fig. 1. It is to be noted that the temperature(s) and enthalpies of the transitions of pure 18 : 1_c/18 : 1_c phosphatidylethanolamine and the equimolar mixtures of 18 : 1_c/18 : 1_c phosphatidylethanolamine and 16 : 0/16 : 0 phosphatidylcholine are very similar to those observed previously [1], where the lipid dispersions contained 50% volume ethyleneglycol. Thus ethyleneglycol does not perturb the transition behaviour of these lipid dispersions, in contrast to the situation when unsaturated phosphatidylcholines are

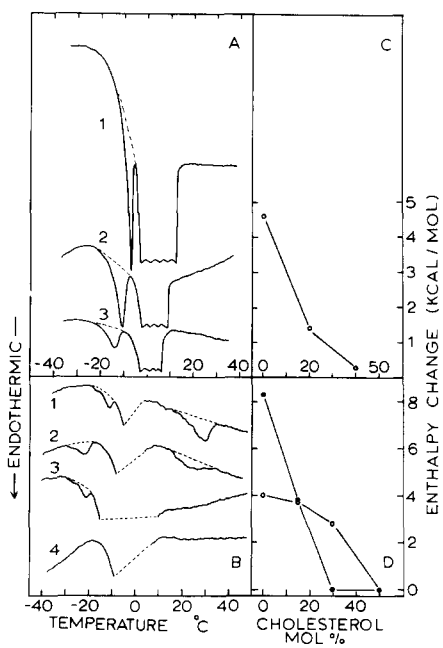


Fig. 1. (A, C) Thermotropic properties of aqueous dispersions of 18 : 1_c/18 : 1_c phosphatidylethanolamine containing the following amounts of cholesterol (mol %): 1, 0%; 2, 20%; 3, 40%. (B, D) Thermotropic properties of equimolar mixtures of 18 : 1_c/18 : 1_c phosphatidylethanolamine and 16 : 0/16 : 0 phosphatidylcholine containing: 1, 0%; 2, 15%; 3, 30% and 4, 50 mol % cholesterol. Heating scans only are shown. The scans in A were obtained by scanning through the ice-water transition, whereas those in B were obtained by scanning up to 2—5°C, at which temperature the ice was allowed to melt isothermally. The latter procedure prevented the end of the ice-water transition from overlapping the transition corresponding to the 16 : 0/16 : 0 phosphatidylcholine peak. In the enthalpy plots the open circles refer to the 18 : 1_c/18 : 1_c phosphatidylethanolamine whereas the closed circles refer to 16 : 0/16 : 0 phosphatidylcholine.

present (van Echteld, C., unpublished). The addition of increasing amounts of cholesterol to 18 : 1_c/18 : 1_c phosphatidylethanolamine is observed to result in an incremental reduction in the enthalpy of the hydrocarbon transition, which is effectively abolished at ≈50 mol % cholesterol, in broad agreement with previous studies of other well-defined phosphatidylcholines [8—10] and phosphatidylethanolamines (see ref. 1 and references therein). Further, the presence of cholesterol is observed to decrease the transition temperature. This effect, which has been observed for other species of phosphatidylethanolamine [1], is not understood. It is intriguing to note that cholesterol also lowers the temperature at which the bilayer to hexagonal (H_{II}) phase transition occurs for 18 : 1_c/18 : 1_c phosphatidylethanolamine [3].

Lateral phase separation may exist in equimolar mixtures of 16 : 0/16 : 0 phosphatidylcholine and 18 : 1_c/18 : 1_c phosphatidylethanolamine, as two distinct hydrocarbon phase transitions are observed by calorimetry (Fig. 1B). Increasing the cholesterol content reduces the enthalpy of the 16 : 0/16 : 0 phosphatidylcholine hydrocarbon transition before that of the 18 : 1_c/18 : 1_c phosphatidylethanolamine component (Figs. 1B and 1D), clearly illustrating the previously noted [1] preferential association of cholesterol with the 16 : 0/16 : 0 phosphatidylcholine, even though this component has the higher transition temperature.

In the light of recent studies [3] which show that phosphatidylcholine-phosphatidylethanolamine dispersions may exhibit complex polymorphic phase behaviour and, in particular, that 18 : 1_c/18 : 1_c phosphatidylethanolamine alone and in the presence of cholesterol may adopt the hexagonal (H_{II}) phase, it is clearly important to establish that bilayer structure is maintained when lateral phase separation occurs. Otherwise the preferential association of cholesterol for phosphatidylcholine may result from a non-specific preference for phospholipids in the bilayer phase. Thus the polymorphic phase behaviour of the equimolar mixtures of 18 : 1_c/18 : 1_c phosphatidylethanolamine and 16 : 0/16 : 0 phosphatidylcholine in the presence of varying amounts of cholesterol was examined employing ^{31}P NMR. As shown in Fig. 2, at 3°C (i.e. where conditions of lateral phase separation prevail) the vast majority of the phospholipids give rise to ^{31}P NMR spectra with a low field shoulder which are characteristic [3,11] of the bilayer phase. (A small spectral component characteristic of the hexagonal (H_{II}) phase [3], visible in the spectrum from the sample containing 15 mol % cholesterol possibly arises from some heterogeneity in the lipid distribution). Further, the broad ^{31}P NMR spectra with distinct 'wings' observed in the absence of cholesterol may be attributed to the presence of gel-state phosphatidylcholine. Gel-state phospholipids exhibit markedly broader ^{31}P NMR spectra [4,11—13], due mainly to increased dipolar interactions, the effects of which are not removed by proton decoupling of the powers available here (Cullis, P.R. and de Kruijff, B., unpublished). The addition of 15 and 30 mol % cholesterol is observed to decrease these effects, until at 30 mol % cholesterol the lineshape observed is characteristic of bilayer liquid crystalline phospholipids. These results indicate that the cholesterol first associates with the higher melting 16 : 0/16 : 0 phosphatidylcholine component, in full agreement with the calorimetric study.

At 40°C (above the transition temperature of the 16 : 0/16 : 0 phosphatidyl-

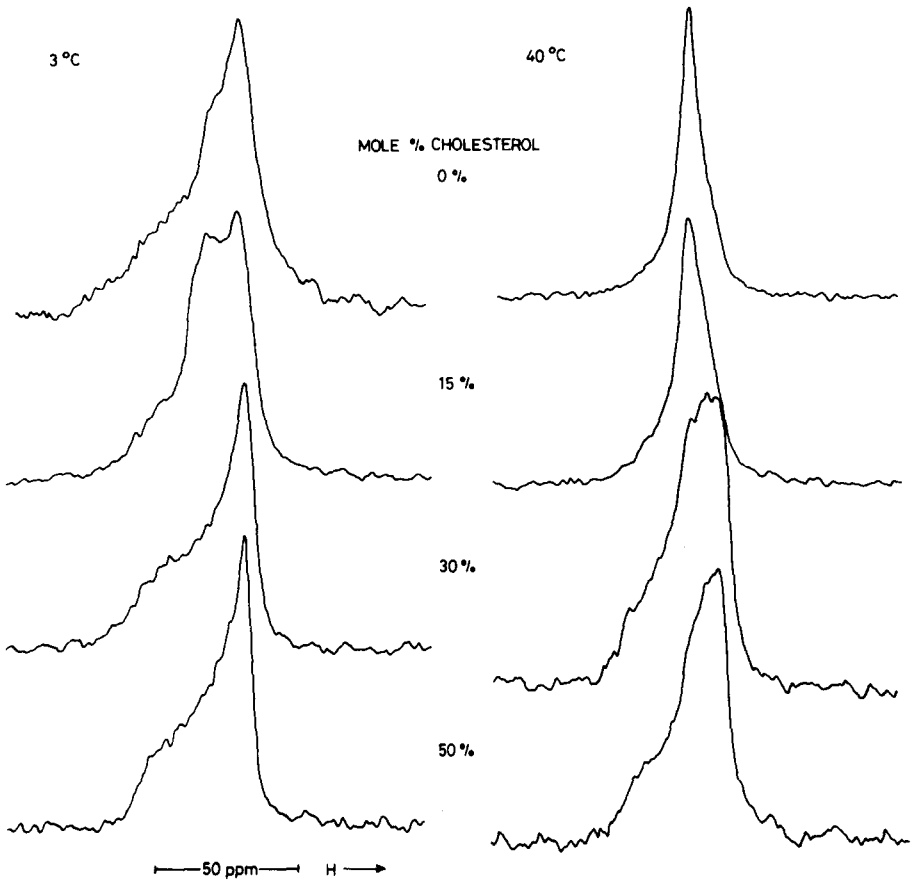


Fig. 2. 36.4 MHz ^{31}P NMR spectra obtained at 3 and 40° C from equimolar mixtures of 18 : 1_c/18 : 1_c phosphatidylethanolamine and 16 : 0/16 : 0 phosphatidylcholine in the presence of various amounts of cholesterol. All dispersions contained 10 mM Tris-acetic acid (p^2H 7.0) and 2 mM EDTA.

choline) the ^{31}P NMR spectra arising from equimolar mixtures of 18 : 1_c/ 18 : 1_c phosphatidylethanolamine and 16 : 0/16 : 0 phosphatidylcholine in the absence and presence of up to 15 mol % cholesterol exhibit a hybrid shape which cannot be clearly identified with bilayer or other phases. Such spectra arise from phospholipids which have available some possibility of (incomplete) isotropic motional averaging, which may be due to lipids primarily in a bilayer configuration experiencing rapid exchange with a small component in a phase such as inverted micellar, rhombic or cubic [14]. Alternatively, the bilayers may have a highly irregular topography, providing local regions with a radius of curvature on the order of 100 Å. The important point is that the addition of 15 mol % cholesterol is not observed to induce a further departure from bilayer structure, which is consistent with cholesterol also being preferentially associated with the (liquid crystalline) 16 : 0/16 : 0 phosphatidylcholine. This is because cholesterol encourages formation of non-bilayer phases for 18 : 1_c/18 : 1_c phosphatidylethanolamine [3], and the presence of cholesterol-phosphatidylethanolamine interactions in these mixed systems would be expected to result in a greater tendency to adopt non-bilayer phases.

The addition of more than 15 mol % cholesterol on the other hand is observed to produce more normal 'bilayer' spectra with a clearly defined low field shoulder, which is consistent with the previously noted [3] ability of cholesterol to stabilize bilayer structure in liquid crystalline systems containing unsaturated phosphatidylethanolamine and saturated phosphatidylcholine.

It is of interest to examine the converse of this effect, concerning the ability of cholesterol to destabilize bilayer structure when unsaturated phosphatidylcholines are present [3], employing well-defined lipid species. The effects of adding increasing amounts of cholesterol to equimolar mixtures of 18 : 1_c/18 : 1_c phosphatidylcholine and 18 : 1_c/18 : 1_c phosphatidylethanolamine at 30°C are illustrated in Fig. 3. The addition of 30 mol % cholesterol is observed to cause a slight reduction in the definition of the low field shoulder, suggesting increased motion available in the phosphate region, whereas the addition of 50 mol % cholesterol (Fig. 3d) causes a complete change to ^{31}P NMR spectra characteristic of the hexagonal (H_{II}) phase, with a small component in a phase (inverted micellar, rhombic or cubic) where effectively isotropic motional averaging occurs.

The temperature dependence of the ^{31}P NMR spectra arising from equimolar mixtures of 18 : 1_c/18 : 1_c phosphatidylethanolamine and 18 : 1_c/18 : 1_c phosphatidylcholine in the presence of 0 and 50 mol % cholesterol is illustrated in Fig. 4. In the absence of cholesterol a change to a phase characterized by the possibility of isotropic motional averaging is observed, which is effectively com-

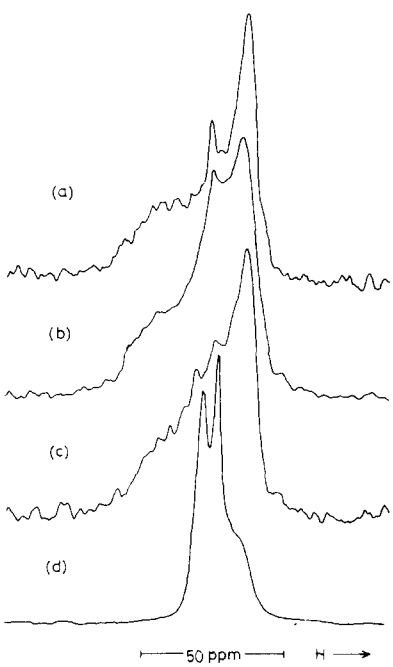


Fig. 3. 36.4 MHz ^{31}P NMR spectra obtained at 30°C from equimolar mixtures of 18 : 1_c/18 : 1_c phosphatidylethanolamine with 18 : 1_c/18 : 1_c phosphatidylcholine in the presence of a 0%, b 15%, c 30% and d 50 mol % cholesterol. All dispersions contained 10 mM Tris-acetic acid (p^{2}H 7.0) and 2 mM EDTA.

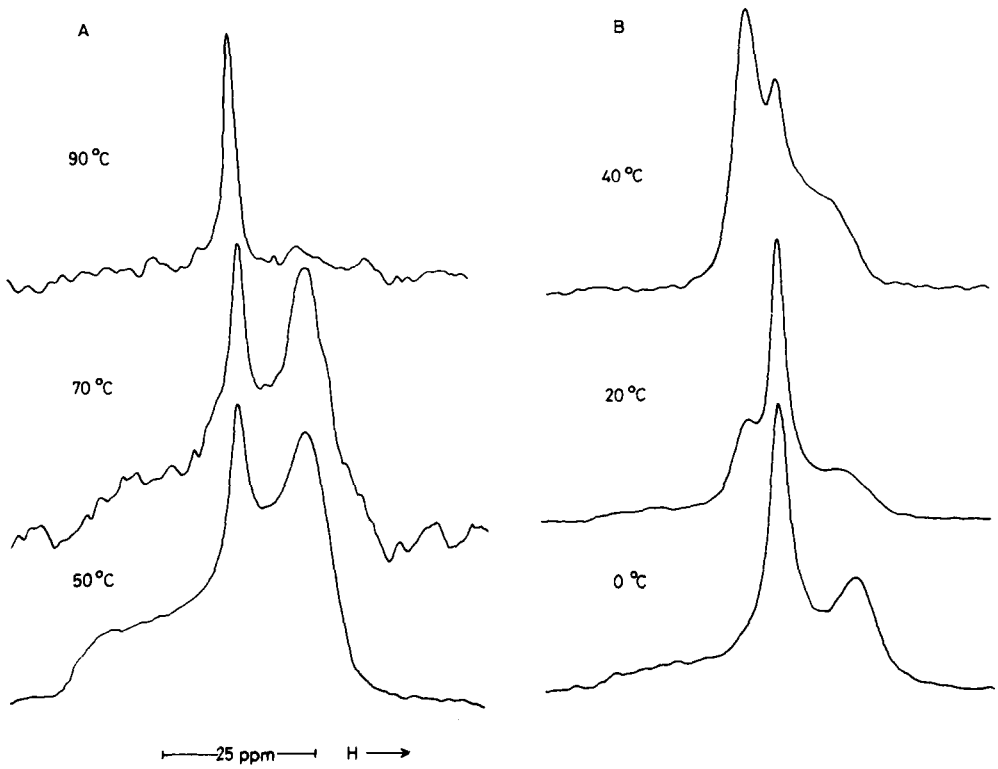
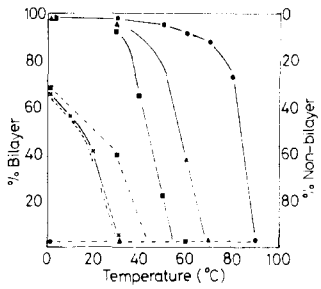


Fig. 4. 36.4 MHz ^{31}P NMR spectra obtained from equimolar mixtures of 18 : 1_c/18 : 1_c phosphatidylethanolamine and 18 : 1_c/18 : 1_c phosphatidylcholine in the presence of A 0 and B 50 mol % cholesterol. All dispersions contained 10 mM Tris-acetic acid (p^2H 7.0) and 2 mM EDTA.

plete at 90°C. At this point the appearance of the sample had changed considerably, becoming considerably less opaque. Alternatively in the presence of 50 mol % cholesterol a considerable portion of the phospholipid (≈40%) is in the 'isotropic averaging' phase even at 0°C (Fig. 4b). As the temperature is increased to 20°C the component in the bilayer phase decreases and a large component in the hexagonal (H_{11}) phase is apparent. Finally, at 40°C the amount of lipid in the phase giving rise to the narrow spectral component is markedly diminished, and over 95% of the lipid has assumed the hexagonal (H_{11}) phase. The bilayer destabilizing capacity of cholesterol in such systems is therefore revealed as a decrease in the temperature at which non-bilayer phases are observed, and a promotion of the hexagonal (H_{11}) phase at high (equimolar) cholesterol. These effects are detailed more precisely in Fig. 5 where it is shown that the addition of 15, 30 and 50 mol % cholesterol progressively decreases the temperature at which the bilayer to non-bilayer transitions occur.

During the course of these experiments a pronounced hysteresis of the temperature-dependent polymorphic phase behaviour of these lipid dispersions was observed. An extreme example of this is shown in Fig. 6 for an equimolar



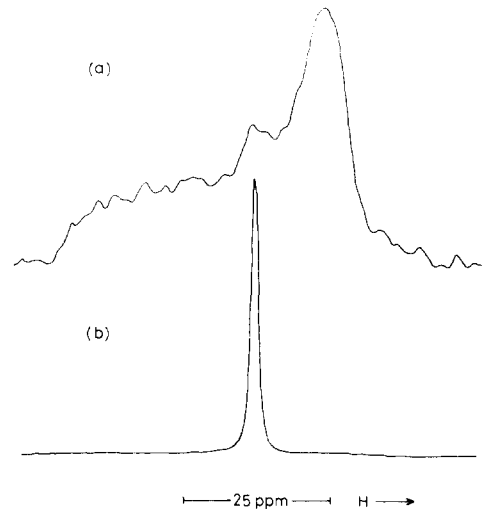


Fig. 5. The amount of phospholipid in bilayer or non-bilayer phases in equimolar mixtures of 18 : 1_c/18 : 1_c phosphatidylethanolamine and 18 : 1_c/18 : 1_c phosphatidylethanolamine as a function of cholesterol content and temperature. •, 0 mol % cholesterol; ▲, 15 mol % cholesterol; ■, 30 mol % cholesterol; X, 50 mol % cholesterol. The solid lines indicate the behaviour on heating the sample from 0°C, whereas the dashed lines indicate the behaviour on cooling from a temperature sufficiently high to induce effectively complete transformation to a non-bilayer phase.

Fig. 6. 36.4 MHz ^{31}P NMR spectra obtained from an equimolar mixture of 18 : 1_c/18 : 1_c phosphatidylethanolamine and 18 : 1_c/18 : 1_c phosphatidylcholine at 30°C (a) before and (b) after heating to 90°C for 30 min. The sample contained 10 mM Tris-acetic acid (p^{2}H 7.0) and 2 mM EDTA.

mixture of 18 : 1_c/18 : 1_c phosphatidylethanolamine and 18 : 1_c/18 : 1_c phosphatidylcholine in the absence of cholesterol at 30°C, before (Fig. 6a) and after (Fig. 6b) heating to 90°C for 30 min. The narrow spectrum obtained after heating corresponds to lipids in the inverted micellar, cubic or rhombic phase. This phase was quite stable, as storage at 4°C for 4 days did not cause reversion to the bilayer phase. The bilayer phase could be restored however by briefly freezing the dispersion at −70°C, which also caused the visual appearance of the sample to revert from semi-translucent to that of normal liposomes. The hysteresis for the samples containing various amounts of cholesterol is indicated in Fig. 5 by the dotted lines, which indicate the amount of non-bilayer phase obtained on cooling the sample from a temperature where all the lipid had assumed a non-bilayer phase. It may be noted that the hysteresis effects were much less pronounced at higher cholesterol contents.

Discussion

The studies presented here confirm previous results [1] indicating a preferential association of cholesterol with phosphatidylcholine in phosphatidylcholine/phosphatidylethanolamine mixtures exhibiting lateral phase separation, and clearly show that this preference does not result from a preference of cholesterol for phospholipids in the bilayer phase. Further, the ^{31}P NMR results are also fully consistent with the suggestion [1] that cholesterol is preferentially associated with the phosphatidylcholine in situations where both phospholipid components are in the liquid crystalline state. These observations are

relevant to the equilibrium distribution in biological membranes such as the erythrocyte membrane where asymmetric transbilayer distributions of various phospholipid classes have been demonstrated [15], and would suggest that cholesterol is preferentially located in the outer monolayer.

Preferential associations of cholesterol with different classes of phospholipid in mixed systems may be suggested to arise in order to obtain optimal packing in the hydrocarbon region. In this context it is useful to think of lipid components as having a characteristic (dynamic) 'shape', which may be cone shaped (where the polar region assumes a smaller cross-sectional area than the hydrocarbon region) of 'inverted cone' shaped, where the opposite situation prevails. Previous work indicates that unsaturated phosphatidylethanolamines [3,16] and cholesterol [3,16,17] are cone shaped (previously referred to as 'wedge' shaped [3]) as indicated by their tendency to adopt or induce non-bilayer phases such as the hexagonal (H_{11}). Alternatively, phosphatidylcholines may be considered to have an inverted cone shape as indicated by their ability to induce bilayer structure in systems containing phosphatidylethanolamine [3]. Thus the preference of cholesterol for phosphatidylcholine in mixed phosphatidylcholine-phosphatidylethanolamine bilayer systems may be suggested to arise because of the optimal packing obtained between the complementary cone and inverted cone shapes of cholesterol and phosphatidylcholine, respectively.

The ability of cholesterol to stabilize the bilayer phase in aqueous dispersions of 18 : 1_c/18 : 1_c phosphatidylethanolamine and 16 : 0/16 : 0 phosphatidylcholine and to destabilize the bilayer phase when 18 : 1_c/18 : 1_c phosphatidylcholine is present, is fully consistent with previous results employing phospholipids from natural sources [3] and again may be rationalized on the basis of different molecular shapes [3]. A major point of interest concerns the structure of the non-bilayer phase observed in the presence of 18 : 1_c/18 : 1_c phosphatidylcholine which gives rise to narrow symmetric ^{31}P NMR spectra. It may be noted in these systems that as the temperature is raised, the sequence of phases observed is bilayer, 'isotropic', and hexagonal (H_{11}) (see Fig. 4). X-ray studies [14] on phosphatidylcholine systems with low water contents show that as the temperature is increased, lamellar, cubic and hexagonal (H_{11}) phases are observed in turn. Further, the observation that the cubic phase is translucent [18] correlates with the appearance of the samples which give the narrow ^{31}P NMR spectra. It is therefore likely that the 'intermediate' phase observed here and elsewhere [3] is of cubic structure. However, it is possible that rhombic or tetragonal [14] phases may be present as they have been observed as intermediates between bilayer and hexagonal (H_{11}) phases as the temperature is increased or the water content decreased. An unambiguous answer therefore awaits detailed X-ray studies. However, the extreme hysteresis exhibited by these systems could be consistent with a high temperature cubic structure, as the three-dimensional nature of this phase would not be expected to allow easy transitions to and from the bilayer phase. This is in contrast to the two-dimensional hexagonal (H_{11}) phase for which transitions to the bilayer phase are observed which exhibit little or no hysteresis [3,4,6]. A related point that should be emphasized is that the phase behaviour of mixed lipid systems may be very strongly dependent on the thermal history of the sample.

Acknowledgements

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