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Review

Phospholipid/cholesterol model membranes: formation of cholesterol crystallites

Diana Bach[a,*], Ellen Wachtel[b,1]

[a] Department of Biological Chemistry, Weizmann Institute of Science, Rehovot, 76100 Israel
[b] Chemical Services Unit, Weizmann Institute of Science, Rehovot, 76100 Israel

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Abstract

Experimental data that define conditions under which cholesterol crystallites form in cholesterol/phospholipid model membranes are reviewed. Structural features of the phospholipids that determine cholesterol crystallization include the length and degree of unsaturation of the acyl chains, the presence of charge on the headgroups and interheadgroup hydrogen bonds.

Keywords: Differential scanning calorimetry; X-ray diffraction; Phospholipid; Cholesterol; NMR

1. Introduction

Biological membranes are complex structures containing a wide variety of phospholipid molecules that differ with respect to the structure of the polar headgroup, hydrocarbon chain length and degree of unsaturation (Fig. 1A). In mammalian cell membranes, cholesterol (Fig. 1B) is also found. It is known that cholesterol plays a structural as well as a regulatory role in biomembranes. Cholesterol is non-homogeneously distributed in cell membranes, with high concentration in the plasma membrane and low concentration in the membranes of intracellular organelles. It is also believed that cholesterol asymmetry exists between the two leaflets of the bilayer.

Because of the complexity of biomembranes, studies of the nature and consequences of the interaction of cholesterol with phospholipids have generally been carried out on model membranes composed of well-defined phospholipids to which cholesterol is added. Such studies were first begun almost four decades ago and the phosphatidylcholines (PC) with saturated acyl chains were primarily used. Investigation of mixtures of cholesterol with negatively charged phospholipids was initiated in our laboratory. Phosphatidylserine, which is negatively charged, is abundant in nervous tissue.

A question which was asked in this research pertains to the aggregational state of cholesterol molecules in the membrane. Is cholesterol dissolved among the phospholipid molecules or clustered in small domains; or does cholesterol in certain situations form crystallites?

The following is a review of experimental results relating to cholesterol crystallite formation in cholesterol–phospholipid mixtures. Particular emphasis will be placed on the differences between the solubility of cholesterol in zwitterionic phospholipids and that in negatively charged phospholipids. The biological significance of cholesterol crystallite formation will be addressed elsewhere in this issue (see Jacob and Mason).

2. Detection of cholesterol crystallites

Cholesterol and phospholipids are insoluble in water. In order to prepare mixtures of phospholipids and cholesterol, the two lipids are dissolved in organic solvents. The conventional method uses a chloroform/methanol mixture (2:1,v/v). Ethanol and chloroform only have also been used. The solvents are evaporated under a stream of nitrogen. Remaining solvent is then removed by keeping the sample under high vacuum for at least 3 h. The lipid film is hydrated with buffered salt solution by incubating with frequent vortexing

* Corresponding author. Tel.: +972-8934-4059; fax: +972-8934-4112.
E-mail addresses: bmbach@wicc.weizmann.ac.il (D. Bach), ellen.wachtel@weizmann.ac.il (E. Wachtel).
[1] Fax: +972-8934-4137.

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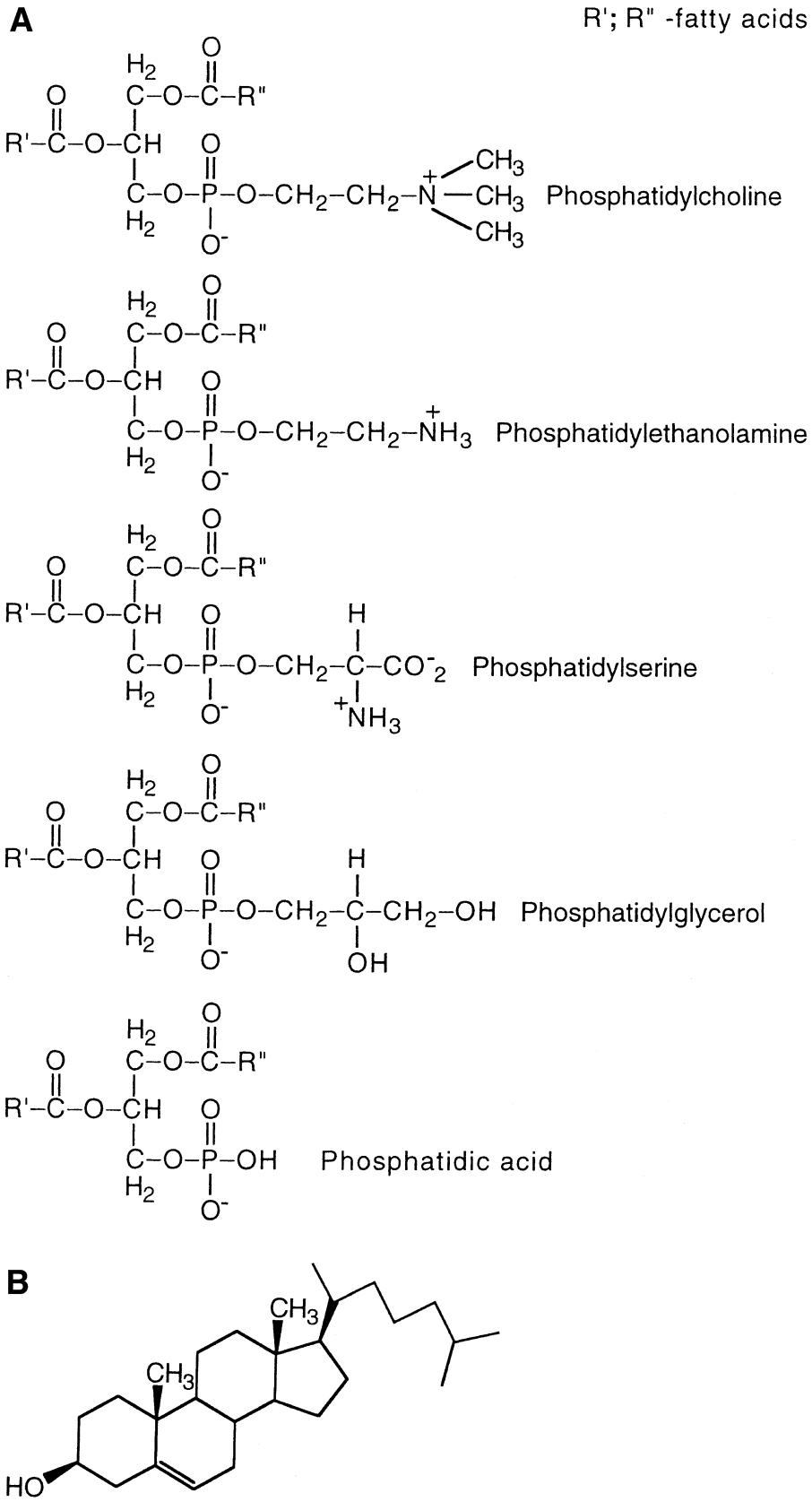

Fig. 1. (A) Chemical structure of phospholipid molecules. (B) Chemical structure of the cholesterol molecule.

for at least 1 h at a temperature approximately 10 °C above the gel–liquid crystalline transition temperature of the phospholipid. This produces multilamellar vesicles. It has been claimed that during solvent evaporation, there may be artifactual separation of the two lipid components, and a new technique has been developed whereby the lipid mixture is sprayed into water, resulting in rapid exchange of the organic solvent by water [1].

For detection of cholesterol crystallites in pure cholesterol and in mixtures with phospholipids, three biophysical techniques have been primarily used. These are differential scanning calorimetry (DSC), X-ray diffraction (XRD) and nuclear magnetic resonance (NMR).

2.1. DSC

At room temperature, anhydrous cholesterol crystals are triclinic (space group $P1$, unit cell dimensions: $a=14.172$ Å, $b=34.209$ Å, $c=10.46$ Å, $\alpha=94.64°$, $\beta=90.67°$, $\gamma=96.32°$ [2]). Upon heating to approximately 37 °C (Fig. 2), the triclinic crystals undergo a polymorphic phase transition to a second triclinic form, in which the length of the a-axis is doubled. The contents of the unit cell increase from 8 to 16 molecules [3,4]. The enthalpy associated with this transition is 0.81 ± 0.21 kcal/mol [4]. In the cooling mode a large hysteresis is detected; the exothermic transition appears at around 20–25 °C [4,5] as seen in Fig. 2. Since the transition is reversible, both the endotherm and exotherm can be used to detect the presence of cholesterol crystallites in mixtures with phospholipids. Additional endothermic transitions of crystalline cholesterol detected in DSC are dehydration of cholesterol monohydrate ($P1$, $a=12.39$ Å, $b=12.41$ Å, $c=34.36$ Å, $\alpha=91.9°$, $\beta=98.1°$, $\gamma=100.8°$ [6]) at about 86 °C and melting of cholesterol crystals at 150–157 °C [4]. When using DSC to quantitate the amount of cholesterol crystals present, the sample should be cycled at least twice to above 86 °C in order to convert all cholesterol crystals to the anhydrous form.

After extended incubation of mixtures of phosphatidylserines (PS) with cholesterol at molar fraction (X(chol)) above 0.3, a new high temperature transition of crystalline cholesterol monohydrate has been identified at 95 °C [7]. It is partially reversible and has been associated with dehydration.

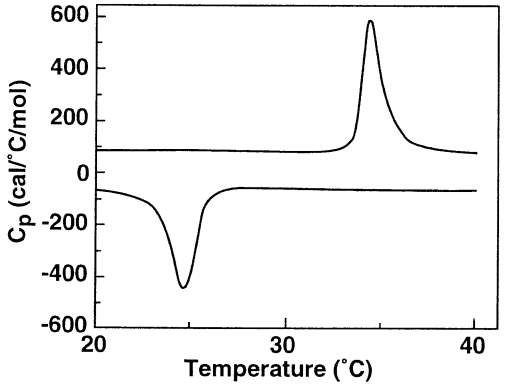

Fig. 2. DSC thermograms of cholesterol dispersed in 0.5 M NaCl, 10 mM Tris, buffer pH 7.4. Cholesterol concentration, 26 mg/ml; scan rate, 0.05 °C/min. The top curve is a heating scan and the bottom curve is a cooling scan. (Reproduced from Ref. [5] by permission of the Biophysical Journal).

2.2. XRD

Both the anhydrous and monohydrate forms of crystalline cholesterol contain a pseudo bilayer structure with repeating distance approximately 34 Å [2,6]. This feature gives rise to a quite strong diffraction peak in small angle XRD at $Q=(2\pi/34)$ Å^{-1} as well as a much weaker second order peak at $2Q$ [6]. The diffraction at 34 Å is useful for detecting the presence of cholesterol crystallites in phospholipid/cholesterol mixtures. However, as the position of the diffraction peak is the same for both anhydrous and monohydrate crystals (010 reflection for anhydrous crystals and 001 for the monohydrate) [8] its appearance alone cannot identify the crystallite form.

In the presence of phospholipids and depending on the type of phospholipid and the ratio of cholesterol to phospholipid, the cholesterol crystallites may either be associated with the phospholipid bilayer or free in solution (see Discussion). In either case, a necessary prerequisite for detection of cholesterol crystallites by the appearance of the 34 Å reflection is a sufficiently large number of pseudo bilayers in the crystal. There is evidence that crystalline cholesterol associated with single lamellae or single shell vesicles does not satisfy this criterion [5]. On the other hand, crystals associated with multilamellar vesicles are detectable. Both anhydrous and monohydrate forms of crystalline cholesterol have specific diffraction peaks in the wide angle region. The dominant peaks are found at 5.75, 5.02 and 4.91 Å for anhydrous cholesterol and at 5.8, 4.7 and 3.8 Å for cholesterol monohydrate. However, these peaks may be weak or difficult to resolve in the presence of phospholipids, so their usefulness in detecting the onset of cholesterol crystallization is somewhat limited.

2.3. NMR

NMR is a nondestructive spectroscopic technique that can differentiate among the magnetic resonances of nuclei with different chemical environments. Spinning the solid sample (MAS) during measurement (e.g. 5 kHz) improves resolution. Guo and Hamilton [9,10] have shown that cross-polarization MAS NMR is an excellent method for detecting cholesterol crystallites in mixtures with lipids. This is so because the efficiency of cross polarization increases as the molecules become more solid-like. When cholesterol is dissolved in the membrane, the ^{13}C resonances of C5 and C18 appear as single peaks, whereas when crystallization occurs, these resonances are shifted and split into doublet

form. Cholesterol crystallites can be detected by MAS NMR even in cases where they are not yet detectable by DSC.

Solid state ^{2}H NMR of selectively deuterated cholesterol/phospholipid mixtures can distinguish between membrane-dissolved cholesterol and phase-separated solid sterol [11].

3. Cholesterol crystallization in zwitterionic phospholipid/cholesterol mixtures

3.1. Phosphatidylcholines (PC)

PC (Fig. 1) are the most common phospholipid in biological membranes. The headgroup is zwitterionic, and due to the absence of hydrogen bond donors, they do not form interheadgroup hydrogen bonds. Studies of cholesterol in model systems initially concentrated on the disaturated chain species. Mixtures of cholesterol with dipalmitoyl phosphatidylcholine (DPPC) were first investigated by Ladbrooke et al. [12] using XRD almost 35 years ago. The mixtures were prepared by codissolving the two components in chloroform, fully drying and then rehydrating with 50% (w/w) water. The homogeneous mixtures were centrifuged through a narrow glass constriction at a temperature above that of the lipid melting transition. It was shown that, under these conditions, cholesterol is soluble in the phospholipid up to a molar ratio cholesterol/DPPC about 1:1. Above this ratio, there is phase separation of crystalline cholesterol (Fig. 3). The experiments presented in Fig. 3 were performed in the lamellar gel phase of the phospholipid; in the lamellar liquid crystalline state the second order of the phospholipid diffraction covers the 34-Å diffraction peak from crystalline cholesterol. Metastable dispersions of DPPC/cholesterol with molar ratio up to 1:4 were studied by Collins and Phillips [13] using DSC. With time (140 days) these mixtures reached an equilibrium state where the molar ratio of the components was 1:1. Excess cholesterol appeared as crystals. This was determined by noting the appearance of the peak at 157 °C and calculation of the associated enthalpy. By employing MAS NMR, Guo and

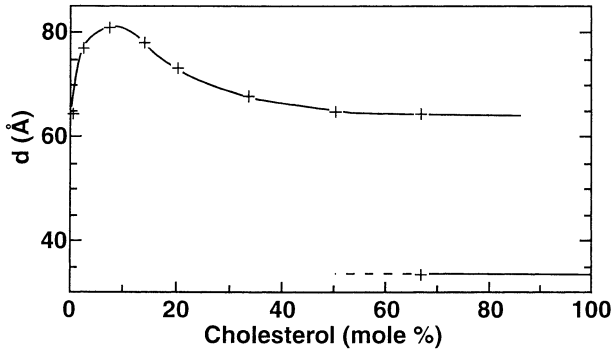

Fig. 3. The variation of the interlamellar spacing (d) of the phospholipid/cholesterol membranes and the appearance of the 34-Å diffraction peak of cholesterol crystallites for DPPC/cholesterol mixtures in 50 wt.% aqueous dispersions at 25 °C. (Taken from Ref. [12]).

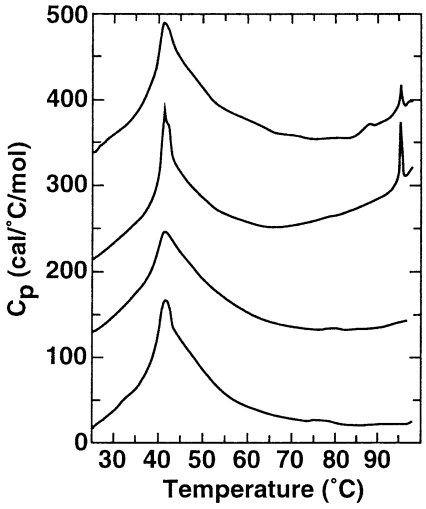

Fig. 4. Heating scans of DPPC with 0.4 mole fraction cholesterol at a scan rate of 0.25 °C/min showing a cholesterol crystallite transition at 95 °C. Top two curves are in the presence of NAP-22 at a protein to lipid ratio of 1:500 with the next to the top curve being the first scan and the top curve being the second heating scan. The bottom two curves are with the same lipid composition but without protein, with the first scan being the one on the bottom. (Reproduced from Ref. [16] by permission of Biochemistry).

Hamilton [9] detected crystalline cholesterol in mixtures with DPPC at ratios of cholesterol to phospholipid above 50 mol%. In mixtures of dimyristoyl phosphatidylcholine (DMPC) with cholesterol, the existence of crystallites was inferred at X(chol) ~ 0.45 using small angle neutron scattering [14] and was detected by XRD at X(chol) 0.5 [15]. In distearoyl phosphatidylcholine (DSPC) cholesterol mixtures no phase separation was observed up to X(chol)-0.51 [15]. Recently Epand et al. [16] presented evidence that protein of neuronal rafts –NAP-22 added to mixtures of cholesterol with DPPC or 1-stearoyl-2-oleoyl phosphatidylcholine (SOPC) induces formation of cholesterol rich domains containing cholesterol crystallites. In Fig. 4 are presented thermograms of DPPC with X(chol)-0.4. The two lower traces are for samples lacking protein while for the two upper ones protein has been added. The appearance of a peak at around 95 °C has been attributed to the high temperature dehydration of crystalline cholesterol monohydrate [16]. The protein itself is very stable and does not undergo denaturation within the temperature range investigated. The peak detected at 95 °C indicates that phase separation of cholesterol has occurred.

Natural PC are heterogeneous with respect to hydrocarbon chain length and degree of unsaturation. Egg PC contains a mixture of acyl chain lengths from 16 to 22 carbon atoms with varying amounts of double bonds. Mixtures of cholesterol with egg PC were prepared by dissolving the components either in chloroform or in ethanol [17]. In samples dissolved in ethanol, cholesterol crystals were detected in freshly prepared samples already at 1:1 molar ratio. In freshly prepared samples in chloroform, cholesterol diffraction was seen only at molar ratio 2:1 cholesterol/PC. However, these

samples were metastable and after 14 days, cholesterol diffraction was seen at 1:1 molar ratio, indicating that phase separation of crystalline cholesterol had taken place. Recently Brzustowicz et al. [11,18] used ^{2}H-NMR and XRD to determine the solubility limit of cholesterol in polyunsaturated PC. In didocosahexaenoyl phosphatidylcholine (DDPC) they found the solubility limit to be 10 mol%; in diarachidonyl phosphatidylcholine (DAPC) cholesterol monohydrate crystallites are detected above 15 mol% cholesterol. In 1-stearoyl-2-arachidonyl phosphatidylcholine (SAPC) the solubility of cholesterol is higher, crystallites are detected above 50 mol%.

Huang et al. [1] have contended that the conventional method of preparing phospholipid/cholesterol mixtures may lead to artifactual demixing of the components. They developed a solvent exchange procedure for preparing these mixtures, producing large unilamellar vesicles. Using this new method together with XRD (observing the 17-Å diffraction peak of cholesterol) they obtained the solubility limit of X(chol)-0.66 for DPPC, 1-palmitoyl-2-oleoyl PC (POPC), dierucoyl PC (DEPC) and dilauroyl PC (DLPC). A possible explanation for this apparent uniformly high solubility is that, as mentioned above, the cholesterol crystals associated with unilamellar vesicles may have a very small number of pseudo bilayer repeats when X(chol) is less than 0.66. Consequently, the authors did not detect diffraction at 17 Å unless cholesterol crystals appeared free in solution.

In a bovine cardiac PC sample prepared by stacking multilamellar vesicles with strong centrifugation and measuring at 37 °C, 93% relative humidity, XRD of cholesterol was observed at a molar ratio of cholesterol to phospholipid 1:1 but not at a molar ratio of 0.5:1 [19]. In a similarly prepared sample of POPC/cholesterol at 20 °C, 74% relative humidity, crystalline cholesterol was observed at a molar ratio of POPC/cholesterol 1:2 [20].

3.2. Phosphatidylethanolamines (PE)

The headgroups of PE (Fig. 1) are zwitterionic; in addition they form intermolecular hydrogen bonds. PE molecules can assemble into three forms: lamellar gel, lamellar liquid crystal and hexagonally packed cylinders, which form at higher temperatures. Mixtures of cholesterol with egg phosphatidylethanolamine (egg PE) or with dielaidoyl phosphatidylethanolamine (DEPE) have been studied. The latter is a synthetic lipid with two C18 chains, each with a trans double bond at position 9,10 [21]. Crystalline cholesterol domains were detected by XRD in the liquid crystalline phase of both phospholipids beginning at X(chol) 0.35–0.4. In Fig. 5 are presented one-dimensional small angle XRD profiles for a mixture of egg PE with X(chol)-0.45 as well as the temperature dependence of the spacings of the diffraction peaks. The interbilayer ($l=1$) spacing equals the thickness of a bilayer plus the interbilayer water. The diffraction from crystalline cholesterol (C) is readily apparent in the lamellar phase but in the hexagonal

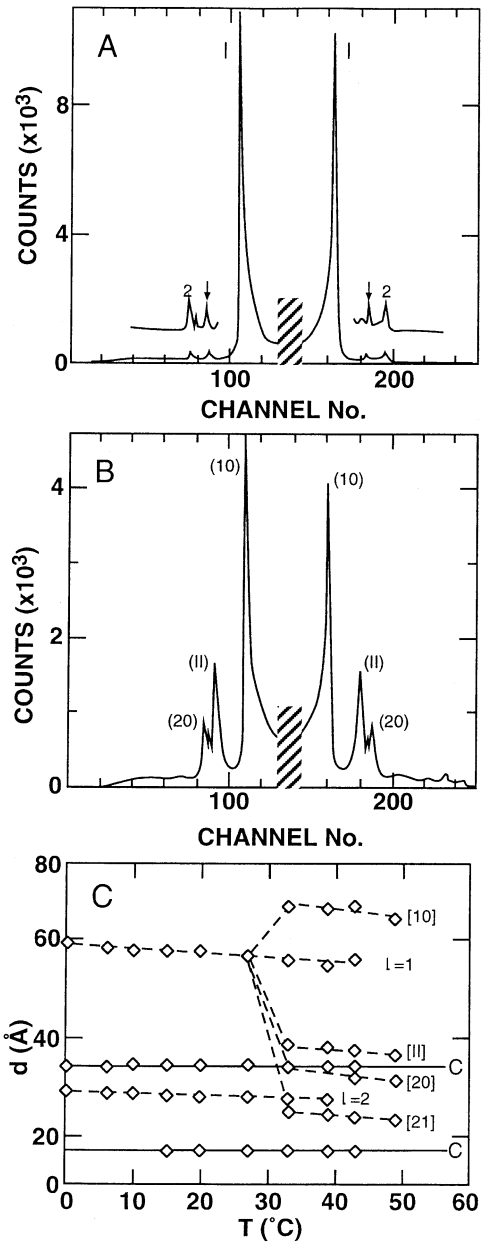

Fig. 5. Characteristic small angle XRD patterns from the egg PE/cholesterol mixture in 0.5 M NaCl, 10 mM Tris, buffer pH 7.4. X(chol)-0.45 at 22 °C (A) and 41 °C (B). Depending on the phase, the numbers indicate either the order of the bilayer spacing or the Miller indices of the two dimensional hexagonal lattice. The arrows indicate the reflections due to the presence of a crystalline cholesterol phase. The hatched region at the center of the pattern denotes the position of the beam stop. (C) The temperature dependence of the positions of diffraction peaks of egg PE/cholesterol mixture X(chol)-0.44. The letter C marks the cholesterol reflection. (Reproduced from Ref. [21] by permission of Biochemistry).

phase it can be distinguished only at high sterol concentrations, due to reflection overlap. Chen and Rand [22] detected the diffraction from cholesterol crystallites at molar ratio cholesterol/dioleoyl phosphatidylethanolamine (DOPE) 3:7 when the phospholipid is in the hexagonal phase. Our work (unpublished) on mixtures of cholesterol with dimyristoyl phosphatidylethanolamine (DMPE) shows that the

onset of cholesterol phase separation as detected by XRD is at X(chol)-0.43 in both the gel and liquid crystalline phases of the phospholipid. Huang et al. [1] found that maximal solubility of cholesterol in 1-palmitoyl-2-oleoyl phosphatidylethanolamine (POPE) bilayers prepared by their solvent exchange technique and detected by monitoring the 17-Å XRD peak of cholesterol crystals is X(chol)-0.51. For reasons outlined in Section 2, this may be an overestimate of the solubility.

4. Cholesterol crystals in mixtures of cholesterol with negatively charged phospholipids

The first research report on the phase separation of cholesterol from membranes of negatively charged phospholipids appeared in 1984 [23]. Negatively charged phospholipids (phosphatidylserines, PS) are abundant in nervous tissue, and it was important to determine how the structure and/or charge of the phospholipid headgroup might influence the interaction with cholesterol. Since that time a number of studies have appeared concerning mixtures of cholesterol with a variety of PS, phosphatidic acid (PA) and phosphatidylglycerols (PG). In the following, we review the data relevant to cholesterol crystallite formation in these model membranes and compare the results with those found for zwitterionic phospholipids.

4.1. Phosphatidylserines (PS)

PS (Fig. 1) are found in majority of mammalian membranes and are the most abundant negatively charged phospholipid. At neutral pH, PS has net charge of −1: one negative charge on the carboxyl group, a second on the phosphate and a single positive charge on the amino group. The headgroups have both hydrogen bond donors and acceptors, and as judged by their relatively high melting temperature, interheadgroup hydrogen bonds are formed. This PS, as with all natural phospholipids, displays hydrocarbon chain heterogeneity both with respect to length and degree of unsaturation. The first mixture in which cholesterol crystallite formation was observed was bovine spinal cord PS/cholesterol [23]. In Fig. 6 are presented thermograms of these mixtures as a function of X(chol). At low cholesterol content, only one peak is seen. This peak is due to the gel–liquid crystalline transition of the phospholipid, either alone or in the presence of solubilized cholesterol. The melting temperature (T_m) of the phospholipid in the mixture decreases about 4 °C upon addition of cholesterol, leveling off at about X(chol)-0.2. Above X(chol)-0.3, a second peak is seen in the thermograms. This peak is ascribed to the polymorphic transition of anhydrous crystalline cholesterol, as the temperature and breath of the transition are similar to those of pure cholesterol (Fig. 6, X(chol)-1; see also Fig. 2 and Refs. [4,5]). The appearance of the cholesterol transition indicates that phase separation into a mixed phosphatidylserine/cholesterol phase and a crystalline cholesterol phase occurs. The phase separation of cholesterol takes place from the liquid crystalline state of the phospholipid, as the transition temperature is above the melting temperature of the phospholipid. According to the Gibbs phase rule, the leveling off of T_m also implies the appearance of an additional phase. As may be seen in Fig. 6, the enthalpy of melting of the phospholipid persists even at X(chol)-0.7, indicating that the interaction of the phosphatidylserine with cholesterol is weaker than in the case of PC [12].

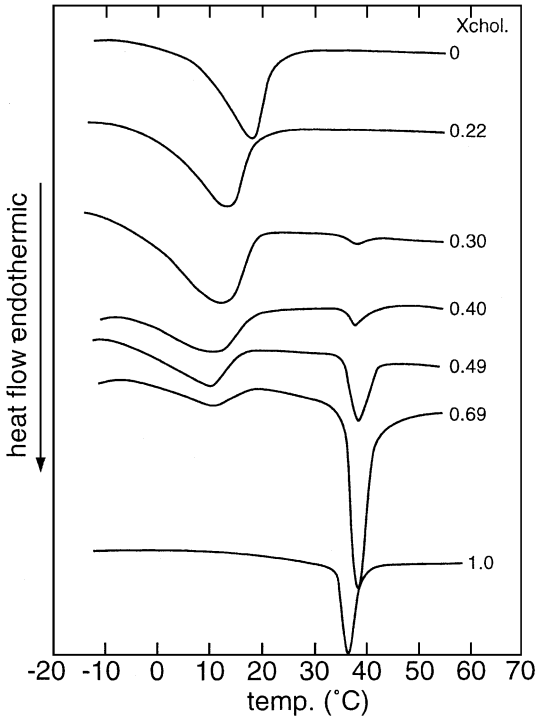

Fig. 6. Thermograms of PS/cholesterol mixtures dispersed in 0.15 M NaCl, 10 mM Tris, pH 7.4 as a function of X(chol). (Taken from Ref. [23]).

The effect of cholesterol on the interbilayer (*d*) spacing and the onset of cholesterol phase separation were determined by XRD [8,24,25]. In Fig. 7a are presented *d* spacings for PS as a function of cholesterol content. At neutral pH, PS forms a lamellar phase with *d* spacing of 71 Å in the gel state and of 60 Å in the liquid crystalline phase. With 0.5 M NaCl, the thickness of the interlamellar water layer is approximately constant between 7 and 30 °C, so changes of *d* spacing with temperature can be used to construct the melting curve of the phospholipid [8].As seen from the figure, the appearance of the specific diffraction from cholesterol crystallites is initially observed at X(chol)-0.31. Cholesterol crystallites can be observed only at temperatures where PS is in the liquid crystalline state; as in the gel state the second order of the phospholipid diffraction covers the 34-Å diffraction peak from cholesterol crystallites.

Additives including calcium ions, lithium ions, and ethanol, as well as protonation, effect the phase separation of cholesterol from PS/cholesterol mixtures [24–26]. In Fig.

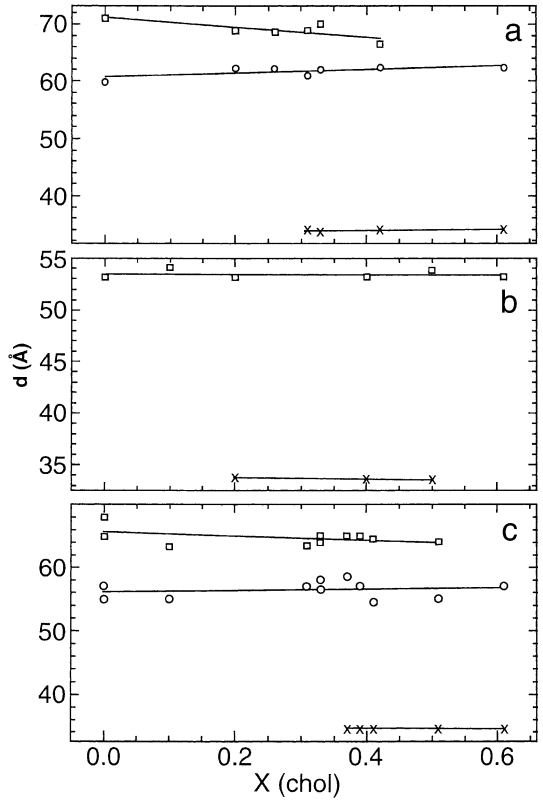

Fig. 7. Comparison of interlamellar spacing d for gel (□) and l.c. (○) phases and appearance of crystalline cholesterol diffraction (×) at 34 Å for (a) spinal cord PS in 0.5 M NaCl, 10 mM Tris, pH 7.4; (b) PS/Ca, molar ratio 1:1.3; (c) protonated PS. (Taken from Ref. [24]).

7b and c are presented *d* spacings for PS with different additives; appearance of the specific diffraction of cholesterol is also noted. It is known that calcium binds to the phosphate group, thereby dehydrating it and inducing isothermal chain crystallization [27]. Interaction of Ca^{++} with PS at a molar ratio 1.3:1 shifts the melting temperature of the phospholipid to above 80 °C and decreases the interbilayer spacing. In Fig. 7b are presented *d* spacings for PS–Ca as a function of cholesterol content; *d* is 53 Å, invariant both with respect to cholesterol content and temperature. Cholesterol crystallites are detected already at X(chol)-0.2. It has been suggested that the decreased miscibility of cholesterol in PS–Ca as compared to PS in NaCl is caused by the marked rigidifying effect of Ca^{++} on both the headgroup region and on the acyl chains of PS [25]. To investigate how the charge may influence cholesterol solubility, the carboxylate group of PS was protonated by lowering the pH to 2.6, thereby producing a zwitterionic phospholipid PS–H. As shown in Fig. 7c the solubility of cholesterol in PS–H is higher than in PS at pH 7.4, becoming more similar to that observed for PE, i.e. appearance of crystallites at X(chol)-0.37.

Lithium ions, a therapeutic agent in mental disorders, affect phase separation of cholesterol from its mixtures with PS [24]. Cholesterol crystallites were detected by XRD in the presence of 1 M LiCl at X(chol)-0.34. Ethanol at 10% (v/v) causes additional coiling of the acyl chains in the liquid crystalline state of PS and increases the interheadgroup spacing [28]. These structural changes apparently permit better accommodation of cholesterol in PS/cholesterol mixtures, resulting in slightly increased solubility of cholesterol in these mixtures. The specific diffraction of cholesterol is observable at X(chol)-0.34 [26].

To evaluate the influence of hydrocarbon chain length on the miscibility of cholesterol, mixtures of cholesterol with dimyristoyl phosphatidylserine (DMPS) or dipalmitoyl phosphatidylserine (DPPS) were studied [24,29]. The onset of appearance of cholesterol crystallites in DMPS/cholesterol mixtures as determined by XRD occurs at molar ratios 2:1 and 1.7:1 in the gel and liquid crystalline states of the phospholipid, respectively [29]. Thermograms of mixtures of DPPS with cholesterol are presented in Fig. 8. As seen in the figure, the presence of cholesterol induces a small downward shift of the melting temperature of the phospholipid up to X(chol)-0.2. At around X(chol)-0.3 a second peak in the thermograms is seen at approximately 37 °C. As noted above, this is the peak of the polymorphic transition

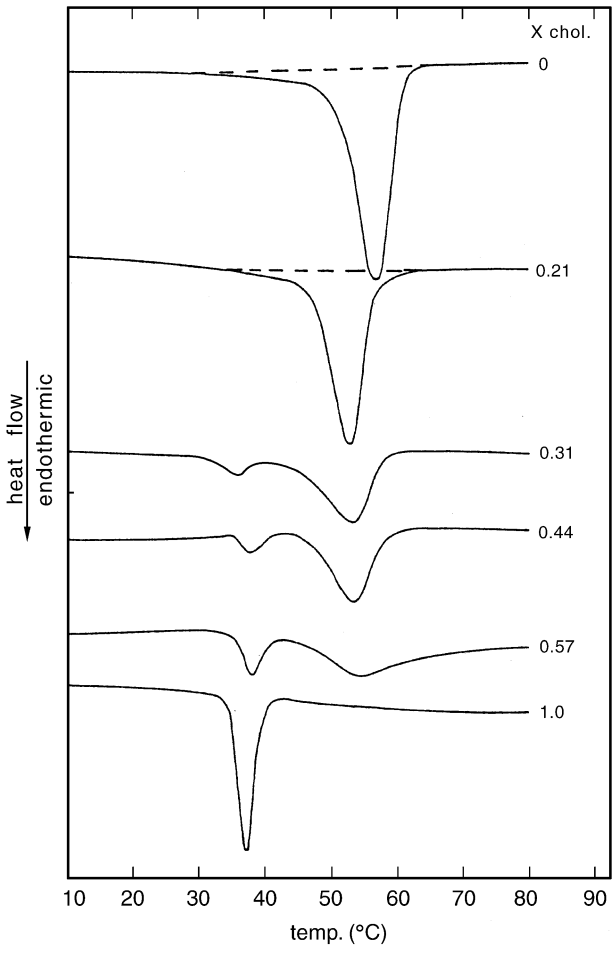

Fig. 8. Thermograms of DPPS/cholesterol mixtures dispersed in 0.15 M NaCl, 10 mM Tris, pH 7.4 as a function of X(chol). Dashed line indicates the base line. (Taken from Ref. [24]).

of anhydrous crystalline cholesterol. At X(chol)-0.57 the melting peak of the phospholipid may still be discerned. These results are similar to those described for mixtures of cholesterol with natural PS except that here the phase separation of cholesterol is from the gel phase of the phospholipid as the melting temperature of the phospholipid is above that of the polymorphic transition of cholesterol. No useful X-ray data were obtained on this system as the second order of the phospholipid bilayer diffraction covers the 34-Å diffraction peak of cholesterol.

The effect of an oleoyl acyl chain (single double bond) in position 2 of the glycerol on the onset of phase separation of cholesterol could be seen in a comparative study of mixtures of cholesterol with 1-palmitoyl-2-oleoyl phosphatidylserine (POPS) [30]. In Fig. 9 are presented the thermograms for POPS/cholesterol mixtures. The interaction with cholesterol is weak. For mixtures of POPS with cholesterol onset of phase separation of cholesterol crystallites is seen at X(chol)-0.36 as determined by XRD (data not shown) and some residual enthalpy of melting is still detected at 1:1 molar ratio. This indicates that the introduction of one unsaturated chain increases cholesterol solubility. However,

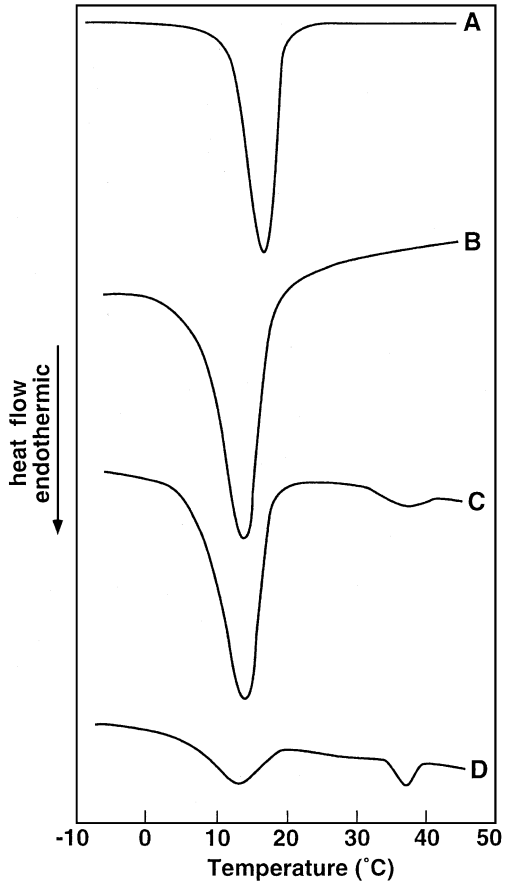

Fig. 9. Thermograms of POPS/cholesterol in 0.15 M NaCl, 10 mM Tris, pH 7.4, plus ethylene glycol 2:1 obtained at a heating rate of 5 °C/min. (A) POPS only; (B) POPS with X(chol)-0.33; (C) POPS with X(chol)-0.36; (D) POPS with X(chol)-0.5. (Taken from Ref. [30]).

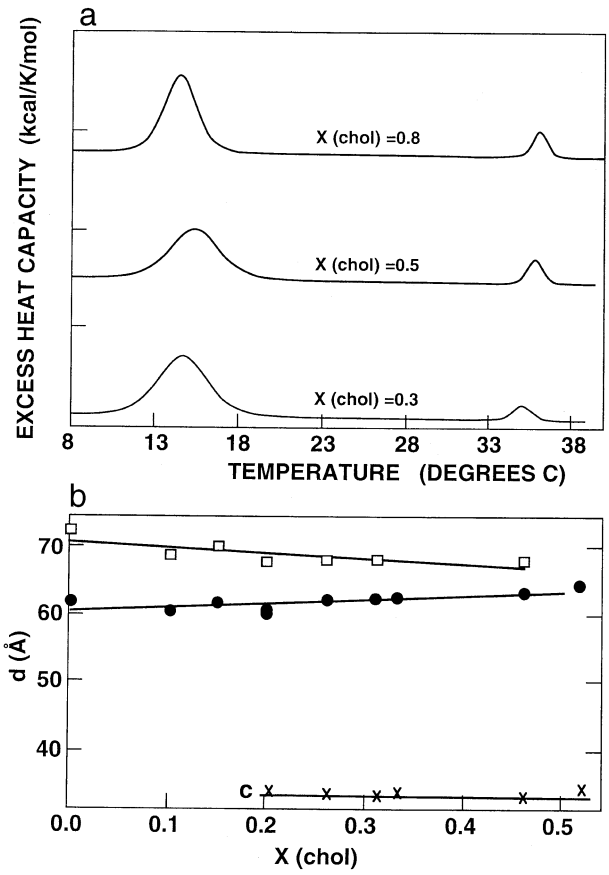

Fig. 10. (a) Thermogram of SOPS/cholesterol mixtures dispersed in 20 mM Pipes buffer, pH 7.4, 1 mM EDTA, 0.15 M NaCl and 0.02 mg/ml NaN_3. Each tick mark on the ordinate represents 1 kcal/mol. (b) Interbilayer spacing (d) of SOPS/cholesterol mixtures dispersed in 0.5 M NaCl 10 mM Tris, pH 7.4, and the appearance of diffraction from crystalline cholesterol as a function of cholesterol content. (□) Below T_m; (●) above T_m; C designates cholesterol diffraction (×). (Taken from Ref. [30]).

retaining the oleoyl acyl chain and increasing the length of the saturated chain at position 1 by 2 methylene groups cause a marked decrease in cholesterol solubility [30]. In mixtures of 1-stearoyl-2-oleoyl phosphatidylserine (SOPS) with cholesterol, the enthalpy of melting of the phospholipid is still seen at X(chol) 0.8 (Fig. 10a) and cholesterol crystallites are already detected at X(chol)-0.2 (Fig. 10b). Mismatch in length between the cholesterol molecule and the phospholipid molecule is believed to decrease cholesterol solubility. Since the length of a cholesterol molecule is approximately 17 Å, the mismatch is larger in the case of SOPS than in the case of POPS.

Recently, McMullen et al. [31] reported the results of DSC studies on mixtures of saturated PS (DMPS, DPPS, distearoyl phosphatidylserine (DSPS)) with cholesterol. McMullen et al. [31] claim that when the solvent evaporation stage of the preparation protocol (see Section 2) was performed at 40–50 °C, cholesterol crystallites were not detected by DSC in these mixtures up to X(chol)-0.5. They attributed the appearance of cholesterol crystals in mixtures prepared with the conventional method to artifactual demix-

ing of the components. However, XRD measurements on POPS/cholesterol mixtures X(chol)-0.45, prepared with or without heating the sample during solvent evaporation, consistently showed the 34-Å peak of cholesterol [7]. Similar results were obtained for mixtures of DMPS, SOPS and natural PS with cholesterol (unpublished results). Furthermore, it has been shown that mixtures of SOPS with cholesterol dissolved in benzene, quickly frozen and lyophilized for several days, show similar properties with respect to phase separation of cholesterol as mixtures dried from chloroform–methanol according to the standard protocol (see Section 2). As benzene is removed from the solid state, the probability that the lipid components will separate during lyophilization is very low.

4.2. Phosphatidic acid (PA)

As seen in Section 4.1, the negative charge of the headgroup reduces the solubility of cholesterol in mixtures with PS as compared to those with PC. The generality of this result was investigated in mixtures of cholesterol with other negatively charged phospholipids, such as PA (see Fig. 1) and PG. For PA, the charge is −1 at neutral pH. As in the case of PS, PA are stabilized by intermolecular hydrogen bonds as judged by their high melting temperature when compared to the melting temperature of the corresponding PC. Mixtures of egg PA with cholesterol in the presence of approximately 10:1 Ca^{++} to PA and also in the absence of calcium ions were investigated by DSC [24]. In both cases the peak of the anhydrous cholesterol polymorphic transition was detected at about X(chol)-0.33. This is then the upper bound for onset of phase separation for these mixtures. An approximately 10 °C decrease in the melting temperature was observed for PA in the presence of X(chol)-0.3—significantly larger than in the case of PS.

4.3. Phosphatidylglycerols (PG)

PG (Fig. 1) also carry charge −1 at neutral pH due to ionization of the phosphate group. However, PG are not stabilized by hydrogen bonds: their temperature of melting is similar to that of the corresponding PC. Studying the miscibility of cholesterol in membranes of saturated PG of increasing acyl chain length can highlight the effect of absence of interhydrogen bonding as well as the systematic increase of acyl chain length on the onset of phase separation of cholesterol crystallites. Mixtures of cholesterol with dimyristoyl phosphatidylglycerol (DMPG), dipalmitoyl phosphatidylglycerol (DPPG), distearoyl phosphatidylglycerol (DSPG) and 1-palmitoyl-2-oleoyl phosphatidylglycerol (POPG) were studied using DSC and XRD [15].

In the DSC experiments it was seen that the melting temperature of PG almost does not change with addition of cholesterol. The enthalpy of melting decreases linearly with increase of concentration of the sterol and vanishes at PG/cholesterol molar ratios which depend on the acyl chain length. In Fig. 11 is presented the onset of appearance of cholesterol crystallites as a function of acyl chain length. Miscibility of cholesterol in PG bilayers is lower than in the corresponding PC bilayers. As seen from the figure, cholesterol phase separation occurs earlier as the length of the acyl chains increases. In mixtures of cholesterol with POPG in the liquid crystalline phase, cholesterol crystallites are detected at X(chol) 0.45. This should be compared to X(chol) 0.36 in the case of POPS which has interheadgroup hydrogen bonds.

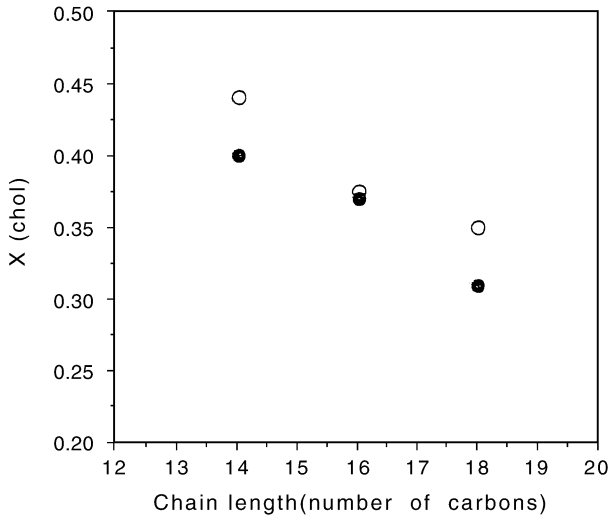

Fig. 11. Onset of cholesterol phase separation as a function of the acyl chain length of fully saturated PG molecules: (●) below T_m; (○) above T_m. (Taken from Ref. [15]).

5. Discussion

We have reviewed experimental data which define conditions under which cholesterol crystallites form in cholesterol/phospholipid model membranes. These data are summarized in Table 1 with respect to the onset of crystalline cholesterol phase separation. For saturated di-C14 hydrocarbon chains, miscibility of cholesterol in phospholipid/cholesterol mixtures decreases in the order: PC>PG ~ PE>PS. It appears that the presence of either a net charge on the headgroup or interheadgroup hydrogen bonding acts to reduce cholesterol solubility. The presence of both factors has a reinforcing effect. For fully saturated PG which have been studied, C14 chains allow maximum solubility. When the acyl chains of PC are polyunsaturated, cholesterol crystallization is clearly promoted.

The physical location of cholesterol crystallites formed in mixtures with phospholipids is not certain. They may be attached to or embedded within the bilayer, and/or may be free in solution. None of the experimental techniques presented above can resolve this ambiguity. We believe that the distribution of crystallites depends on the molar ratio of phospholipid to cholesterol and on the host phospholipid.

Table 1
Onset of phase separation of cholesterol from phospholipid/cholesterol mixtures

Lipid	Charge	Presence of hydrogen bonds	Hydrocarbon chain length	Number of double bonds/hydro-carbon chain	Onset of cholesterol phase separation X(chol)[a]		Reference
					Gel phase	l.c. phase	
DMPC	(±)	no	14	0		0.45	[14]
						0.5	[15]
DPPC	(±)	no	16	0	0.5		[9,12]
DSPC	(±)	no	18	0	>0.5	>0.5	[15]
SAPC	(±)	no	18,20	0,4		0.5	[18]
DAPC	(±)	no	20	4		0.17	[18]
Egg PC[b]	(±)	no	variable	variable		0.45–0.55	[17]
DDPC	(±)	no	22	6		0.10	[11]
DMPE	(±)	yes	14	0	0.43	0.43	(unpublished)
DEPE	(±)	yes	18	1[c]		0.35–0.4	[21]
DOPE	(±)	yes	18	1		0.33[d]	[22]
Egg PE[b]	(±)	yes	variable	variable		0.35–0.4	[21]
DMPG	(−)	no	14	0	0.4	0.44	[15]
DPPG	(−)	no	16	0	0.38	0.37	[15]
DSPG	(−)	no	18	0	0.32	0.35	[15]
POPG	(−)	no	16,18	0,1		0.45	[15]
DMPS	(−)	yes	14	0	0.33	0.37	[29]
DPPS	(−)	yes	16	0	0.3		[24]
POPS	(−)	yes	16,18	0,1		0.36	[30]
SOPS	(−)	yes	16,18	0,1		0.2	[30]
PS[b,e]	(−)	yes	variable	variable		0.3	[23,25]

[a] Molar fraction of cholesterol.
[b] Natural product with variable degree of unsaturation.
[c] Trans double bond.
[d] Phospholipid in hexagonal phase.
[e] PS from spinal cord.

There are few reports which relate either directly or indirectly to this question.

Cholesterol crystallites formed in single shell vesicles of DMPS/cholesterol, X(chol)-0.4, are not detectable by XRD. On the other hand, DSC detects the presence of these crystallites. If the same mixture is not sonicated, thereby retaining multilamellar vesicles, the strong 34-Å reflection from cholesterol crystallites can be detected. When X(chol) is increased to 0.5 in the lipid dispersion, both DSC and XRD detect cholesterol crystallites [5]. One interpretation of these results is that the curvature and/or thickness of the vesicle wall are incompatible with many repeats of the cholesterol pseudo bilayer structure; the crystals are primarily two-dimensional. Therefore, at X(chol)-0.4, when the crystals are associated with the bilayer, no 34-Å diffraction peak is observed. At X(chol)-0.5, three-dimensional crystals begin to appear free in solution (as observed by polarized light microscopy) and then the diffraction is also observed.

Other indirect evidence with bearing on the question of location is that in DMPS/cholesterol mixtures, water binding to DMPS molecules, as determined in DSC, first increases when phase separation of cholesterol starts and then levels off at high cholesterol content when cholesterol crystals appear in solution [32]. The increase in water binding has been attributed to the increased formation of crystalline cholesterol/phospholipid boundaries.

Cholesterol monohydrate, when heated, undergoes dehydration at about 80 °C as detected in DSC profiles. When mixtures of POPS/cholesterol X(chol)-0.3 to 0.9 or SOPS/cholesterol 0.4 to 0.7 are incubated for some weeks at 37 °C, this transition is shifted to about 95 °C and becomes slightly reversible [7]. As this shift in the temperature of dehydration is not detected in pure cholesterol monohydrate, incubated under similar conditions but only in the presence of the phospholipid, we conclude that some cholesterol crystallites must be in contact with phospholipid. Nevertheless, polarized light microscopy of POPS/cholesterol samples X(chol)-0.5 shows that some fractions of the crystallites are free, i.e. visible, in solution, independent of time of incubation.

Two-dimensional XRD patterns from oriented multilamellar samples of POPC/cholesterol, 1:2 molar ratio, show that the lattice vector associated with the 34-Å diffraction peak of cholesterol is parallel to the normal to the plane of the phospholipid bilayers [20]. This finding could indicate that at least some of the cholesterol crystals are embedded in the plane of the bilayer, as the authors have suggested. However, one must also consider the possibility that the plate-like morphology of free cholesterol monohydrate

crystals results in their being oriented between the bilayer surfaces during centrifugation.

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