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Hydration of phospholipid bilayers in the presence and absence of cholesterol

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Abstract

Differential scanning calorimetry (DSC) was used for determining the number of unfreezable water molecules per molecule of phosphatidylserine from bovine spinal cord (PS) or dimyristoyl phosphatidylserine (DMPS) and dimyristoyl phosphatidylcholine (DMPC), alone or in mixtures with cholesterol. It was assumed that the unfreezable water molecules are tightly bound to the phospholipid. It was found that when the phospholipids are in the gel state and in the absence of cholesterol, PS binds 2.5 water molecules, DMPS 3.8 water molecules and DMPC 3.5 water molecules. In the presence of cholesterol the number of water molecules bound increases in the region where phase separation of cholesterol takes place [D. Bach, Chem. Phys. Lipids 35 (1984) 385–392; E.J. Wachtel, N. Borochov, D. Bach, Biochim. Biophys. Acta 1066 (1991) 63–69; D. Bach, N. Borochov, E. Wacktel, Chem. Phys. Lipids, submitted]. © 1998 Elsevier Science B.V.

Keywords: Hydration; Phospholipid bilayer; Cholesterol

1. Introduction

Hydration of phospholipid bilayers was investigated by several biophysical techniques: NMR [1–6], adsorption isotherms [7–9], differential thermal analysis (DTA) [9], IR [10], differential scanning calorimetry (DSC) [11–16]. Most of these studies were performed on zwitterionic phospholipids and the number of unfreezable water molecules which are shown to be tightly bound to the lipid molecule were obtained. However the effect of cholesterol on hydration of zwitterionic phospholipids was not investigated systematically. It was shown that only cholesterol at high concentrations increases the hydration of phosphatidylcholines [3,7,9,12].

Differential scanning calorimetry is especially suitable for determination of the hydration properties of lipid bilayers. From DSC experiments several parameters can be obtained: (1) the number of tightly bound water molecules (unfreezable water) per molecule of lipid in the gel state if the melting temperature of water is below the melting temperature of the lipid investigated or in the liquid crystalline state if it is above the melting temperature of the lipid, (2) the melting temperature of the lipid as a function of hydration, (3) phase separation in some binary lipid mixtures.

Abbreviations: DMPC, dimyristoyl phosphatidylcholine; DMPS, dimyristoyl phosphatidylserine; DPPC, dipalmitoyl phosphatidylcholine; PS, phosphatidylserine natural; DTA, differential thermal analysis; DSC, differential scanning calorimetry; X(chol), molar fraction of cholesterol; ΔH, enthalpy of melting

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We have used DSC for investigation of compositional aspects of lipid hydration [11]. It was shown that the number of unfreezable water molecules generally increases with the size and charge of the polar headgroup and with the degree of unsaturation of the hydrocarbon chains but inter and intramolecular hydrogen bonding may compete with the hydration. For zwitterionic phospholipid dipalmitoyl phosphatidylcholine (DPPC) the number of bound water molecules per lipid molecule is 6, for uncharged and highly hydrogen bonded cerebrosides is 4 (glucocerebroside) and 8 (galactocerebroside) increasing to 20–40 for various gangliosides which possess big charged polar heads.

The objective of the present work was to investigate the hydration properties of phosphatidylserine–cholesterol mixtures (phosphatidylserine natural (PS) and dimyristoyl phosphatidylserine (DMPS)) with an attempt to correlate the number of bound water molecules with the phase separation of cholesterol crystallites.

In a number of publications we have shown that the solubility of cholesterol in negatively charged phospholipids is limited, resulting in phase separation into phospholipid–cholesterol phase and an almost pure cholesterol phase at molar ratio of PS to cholesterol of about 2:1 and lower [17–19]. For comparison we have also investigated mixtures of zwitterionic lipid dimyristoyl phosphatidylcholine (DMPC) with cholesterol. The results presented show that the number of water molecules bound increases in the region of phase separation of cholesterol. Significance of these results will be discussed further.

2. Materials and methods

Dimyristoyl phosphatidylserine sodium salt (DMPS) was purchased from Avanti Polar Lipids (Alabaster, AL). Dimyristoyl phosphatidylcholine (DMPC) was purchased from Sigma (St. Louis MO). Phosphatidylserine (PS) from bovine spinal cord sodium salt-grade 1 was purchased from Lipid Products (South Nutfield, UK). Cholesterol was either from Merck, Darmstadt, Germany (extra pure grade and was recrystallized twice from ethanol) or from Nu-Chek-Prep. (Elysian, MN) and was stored in dark at -18°C.

DMPC, DMPS were dissolved in chloroform: methanol (2:1, v/v), PS was obtained in chloroform: methanol, the solvents were driven off by a stream of nitrogen and the samples were kept under high vacuum for 3 h. The samples were reweighed, dissolved back in chloroform:methanol and for the lipid–cholesterol mixtures appropriate volumes of cholesterol solution in 2:1 chloroform:methanol were added. The solvents were driven off by a stream of nitrogen and the samples were kept under high vacuum for 3 h.

Cholesterol, phospholipids or phospholipid–cholesterol mixtures were weighed on Cahn Model 4100 Electrobalance (Cahn, Ventron Corp. Paramount, CA) directly into aluminum pans of the DSC instrument and kept for 2 h under nitrogen in the compartment of the balance. After drying, the samples were reweighed, appropriate amounts of water added, the pans were sealed and reweighed. The sealed pans were vortexed and incubated for 1/2 h at 50°C. After incubation the pans were reweighed to ensure that no water was lost. For calibration, experiments were performed with various amounts of water only.

For determining the phase separation of cholesterol in PS–cholesterol mixtures various amounts of water were added to lipid mixtures weighing more than 1.6 mg.

The calorimetric measurements were performed on a Du Pont 990 Thermal Analyzer (Du Pont Instruments, Wilmington, DE) equipped with cell base 2. The experiments were performed in a temperature range of -50°C to 50°C at a scan rate of 5°/min, in some cases the scan rate was 2°/min giving similar results. In all the experiments several scans were performed. The measured enthalpy of water melting obtained from the thermograms was calculated per mole phospholipid in the sample and plotted as a function of mole water/mole phospholipid in the sample. The slope of these graphs gives the enthalpy of melting of water and the intercept on *x*-axes gives the number of unfreezable (bound) water molecules per molecule of phospholipid.

3. Results

In the presence of lipids at low hydration the ice melting peak shifts to lower temperatures, becomes

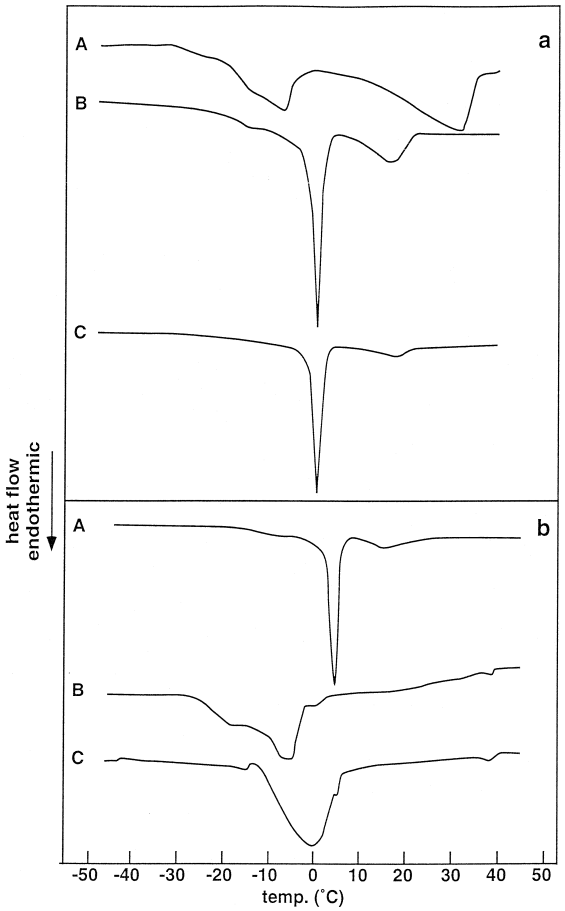

Fig. 1. Thermograms of phosphatidylserine. (a) the phospholipid only at increasing ratio of water to phospholipid: A – H_2O:PS 5:1 (molar ratio), sensitivity 0.4 milical/s/inch; B – H_2O:PS 11:1 (molar ratio), sensitivity 0.1 milical/s/inch; C – H_2O:PS 21:1 (molar ratio), sensitivity 0.2 milical/s/inch. (b) phosphatidylserine–cholesterol mixtures: A – X(chol)-0.21, H_2O:PS 9.1:1 (molar ratio), sensitivity 0.1 milical/s/inch; B –X(chol)-0.37, H_2O:PS 9.4:1 (molar ratio), sensitivity 0.02 milical/s/inch; C – X(chol)-0.50, H_2O:PS 11.5:1 (molar ratio) sensitivity 0.04 milical/s/inch.

broader, and the enthalpy of melting decreases, simultaneously the melting temperature of the lipid (gel–liquid crystalline transition) increases. At very low degrees of hydration the ice melting peak disappears and the melting temperature of the lipid reaches the values of the anhydrous lipid [20,21].

In Fig. 1 are presented thermograms of PS at increasing degree of hydration (1a) or increasing cholesterol content (1b). In trace A (Fig. 1(a)), at low hydration the ice melting peak is shifted to about −7°C. This peak is very broad indicating lowering of water activity with decreasing ratio of hydrating water to lipid as the temperature decreases, but it may also comprise distinct peaks of water hydrating different polar residues. The temperature of the transition of PS (gel–liquid crystalline transition) at this limited hydration is shifted upwards to about 37°C. With increasing water content Fig. 1(a), trace B (H_2O:PS 11:1) the ice melting peak shifts to 0°C and the area of the shoulder corresponding to the melting of the water molecules affected by the lipid is less than a third of the total peak area. This shoulder becomes negligible with respect to the total melting peak in trace C (H_2O:PS 21:1). However the ratio of the area of the shoulder to the area of the lipid peak (gel–liquid crystalline transition) remains the same indicating that the melting temperature of the water molecules affected by the lipid (per lipid molecule) is constant at any water to lipid ratio. At about 10 water molecules per phosphatidylserine molecule the melting temperature of PS shifts down and stays constant as the water content is further increased.

From thermograms of Fig. 1 and additional ones the enthalpy of melting of water per mole lipid was calculated and is presented in Fig. 2 as a function of mole water/mole lipid. Intercept of this graph (the enthalpy of melting = 0) gives the number of tightly bound (unfreezable) water molecules per molecule of

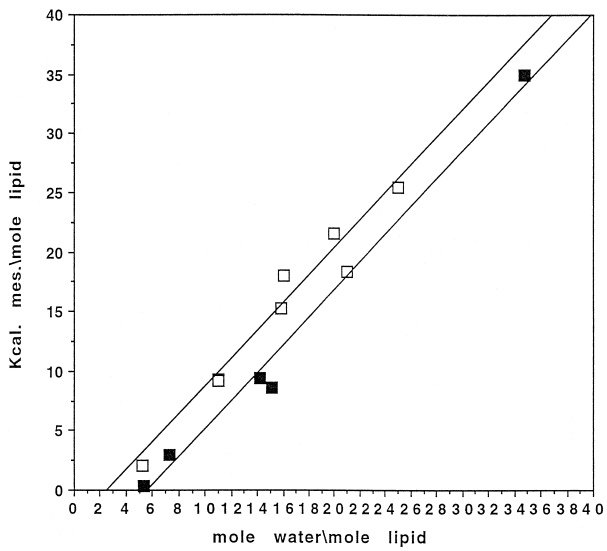

Fig. 2. The measured enthalpy of melting of water (Kcal measured/mole lipid) as a function of the ratio mole water/mole lipid. □ □, PS only; ■ ■, PS–cholesterol mixture; X(chol)-0.6.

phospholipid. For PS, 2.5 water molecules are bound per phospholipid molecule. Slope of the graph of Fig. 2 gives the molar enthalpy of melting of water. The theoretical slope is 1.436 Kcal/mole water. When the experiments were performed with varying amounts of water without the lipid and plotted as on Fig. 2 the slope was 1.51 Kcal/mole, however the slope of Fig. 2 is only 1.186 Kcal/mole indicating that the latent heat of freezing of hydrating water is smaller than that of ordinary water. To determine to what extent the phospholipid influences the enthalpy of melting of ice we have performed these experiments at high ratios of water to phospholipid. Only above 400 water molecules per molecule of PS the enthalpy of melting reached the theoretical value.

In the second step of this study we have investigated the number of unfreezable water molecules in PS–cholesterol mixtures. Pure cholesterol exists in three forms: low temperature anhydrous cholesterol transforming into high temperature anhydrous form at about 38°C and cholesterol monohydrate transforming into the high temperature anhydrous form at about 85°C. Transition from anhydrous cholesterol to cholesterol monohydrate can be achieved by recrystallization from mixture of water ethanol and storing in water or by leaving anhydrous cholesterol crystals in water for 24 h [22].

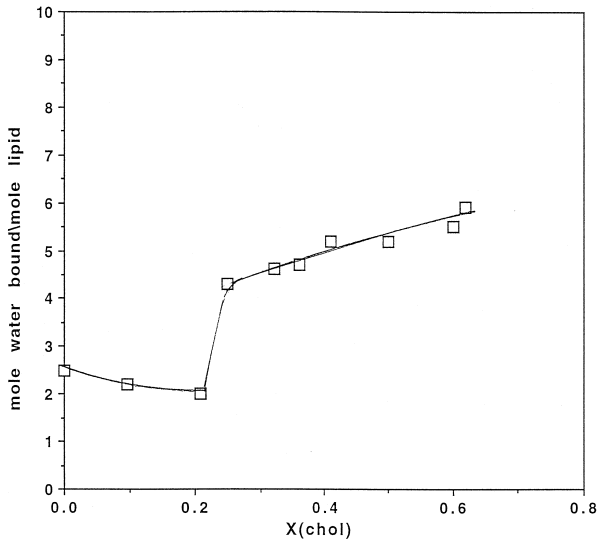

Fig. 3. Hydration of phosphatidylserine-cholesterol mixtures: moles of water bound per mole PS as a function of molar fraction of cholesterol X(chol).

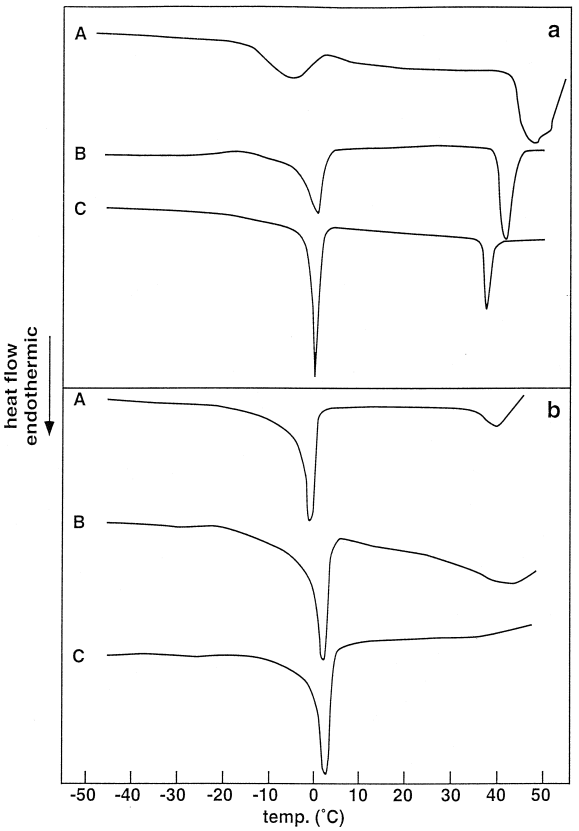

Fig. 4. Thermograms of phosphatidylserine. (a) phospholipid only at increasing ratio of water to phospholipid: A – H_2O:DMPS 6.9:1 (molar ratio), sensitivity 0.04 milical/s/inch; B – H_2O:DMPS 11:1 (molar ratio), sensitivity 0.04 milical/s/inch; C – H_2O:DMPS 20:1 (molar ratio), sensitivity 0.2 milical/s/inch. (b) phosphatidylserine–cholesterol mixtures: A – X(chol)-0.13, H_2O:DMPS 12:1 (molar ratio), sensitivity 0.04 milical/s/inch; B – X(chol)-0.22, H_2O:DMPS 13.2:1 (molar ratio), sensitivity 0.04 milical/s/inch; C – X(chol)-0.35, H_2O:DMPS 14:1 (molar ratio), sensitivity 0.04 milical/s/inch.

We have added varying amounts of water to dry cholesterol and measured the enthalpy of melting of water. No binding of water to cholesterol was detected under the experimental conditions used. In Fig. 1(b) are presented thermograms of PS–cholesterol mixtures at increasing cholesterol content and at almost constant number of water molecules to molecule of PS. The temperature of ice melting is very strongly dependent on the cholesterol content and on the ratio of water to phospholipid as seen by comparing the traces of Fig. 1(b). The shape of these traces is similar to those obtained in the absence of cholesterol. In traces B and C at about 38°C, the peak

corresponding to polymorphic phase transition of cholesterol is also seen, indicating phase separation of cholesterol. We have shown previously that at molar ratio of cholesterol to PS of about 1:2 in the presence of an excess of salt solution phase separation into PS–cholesterol phase and an almost pure cholesterol phase takes place [17,18]. In the present work we have shown that the phase separation takes place also in the presence of limited amount of water. The onset of cholesterol phase separation occurs at about the same ratio.

From thermograms similar to those presented in Fig. 1(b) the ice melting enthalpy (measured per mole of lipid) for each molar fraction of cholesterol was calculated and plotted as a function of mole water/mole lipid. A representative plot is presented in Fig. 2 for X(chol)-0.6. From the intercept of these plots the number of water molecules bound per molecule of PS was obtained and is presented in Fig. 3 as a function of molar fraction of cholesterol. As seen from Fig. 3, the number of water molecules bound increases abruptly at X(chol)-0.25 and then the increase of bound water with X(chol) becomes moderate.

As spinal cord PS contains acyl chains differing in length and degree of unsaturation it was of interest to

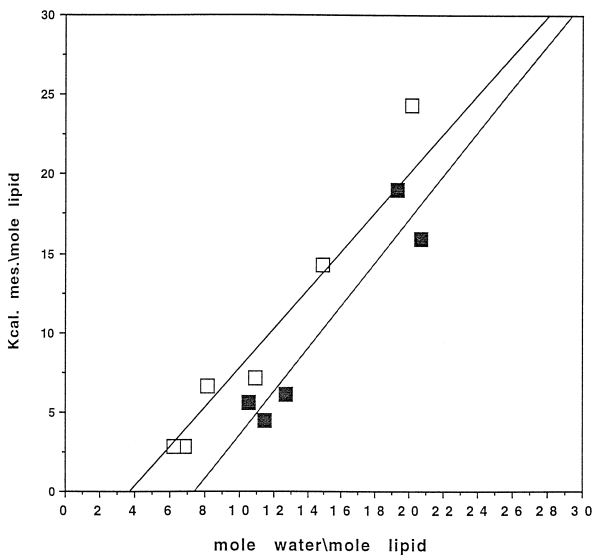

Fig. 5. The measured enthalpy of melting of water (Kcal measured/mole lipid) as a function of the ratio mole water/mole lipid. □ □, DMPS only; ■ ■, DMPS–cholesterol mixture; X(chol)-0.43.

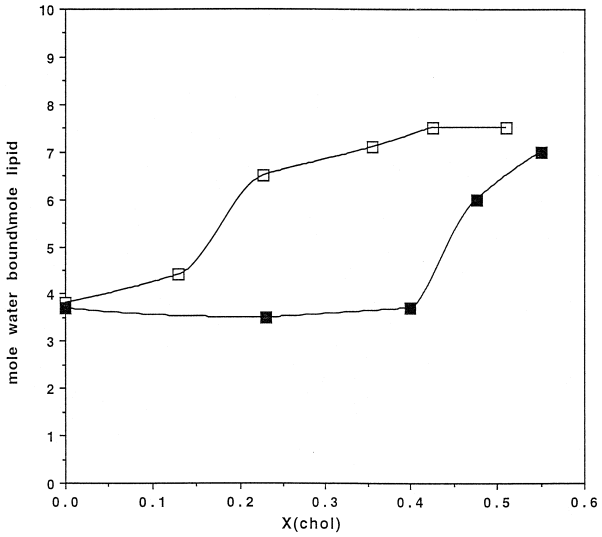

Fig. 6. Hydration of phospholipid-cholesterol mixtures: moles of water bound per mole phospholipid as a function of molar fraction of cholesterol X(chol). □ □, DMPS–cholesterol mixtures; ■ ■, DMPC–cholesterol mixtures.

investigate the water binding to well defined synthetic phosphatidylserine. We have chosen to investigate water binding to dimyristoyl phosphatidylserine (DMPS) alone and to its mixtures with cholesterol.

In Fig. 4 are presented thermograms of water and of dimyristoyl phosphatidylserine (Fig. 4(a) at increasing ratio of water to DMPS and Fig. 4(b) at nearly constant water content for DMPS–cholesterol mixtures). Comparing the thermograms of Fig. 4(a) with those of Fig. 1(a) shows that the effect of DMPS on the ice melting peak is similar to the effect on spinal cord PS-downward shift of the melting temperature and broadening of the peak at low water contents. The melting temperature of DMPS is also affected by the water content.

The enthalpy of melting of water per mole of DMPS was calculated from all thermograms obtained at different water content. Representative thermograms are presented in Fig. 4. In Fig. 5 the measured enthalpy of water melting calculated per mole lipid as a function of mole water/mole lipid is presented. From the intercept of Fig. 5 the number of unfreezable water molecules per DMPS molecule is obtained. This number is 3.8 water molecules and it is assumed that this is the number of water molecules bound to DMPS molecule.

The slope of the graph gives the molar enthalpy of

melting of ice in the presence of DMPS, also here the value obtained (1.23 Kcal/mole) is smaller than the theoretical one. In Fig. 5 is also shown a plot of measured enthalpy of water melting as a function of water per lipid molecule, for a DMPS–cholesterol mixture at X(chol)-0.43.

The plots were drawn for all the DMPS–cholesterol mixtures and the number of water molecules bound/molecule of DMPS were plotted as a function of molar fraction of cholesterol and are presented in Fig. 6.

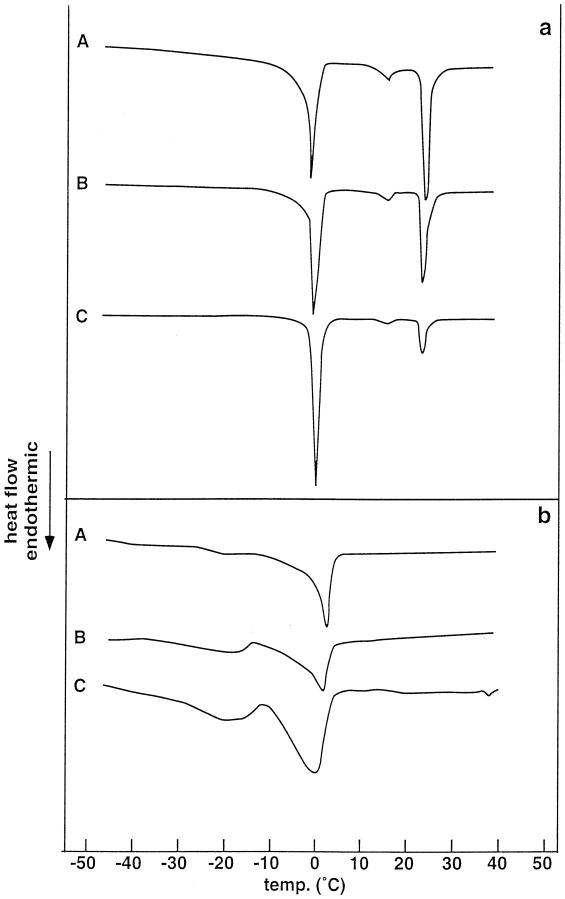

Fig. 7. Thermograms of dimyristoyl phosphatidylcholine. (a) phospholipid only at increasing ratio of water to phospholipid: A – H_2O:DMPC 9.3:1, sensitivity 0.1 milical/s/inch; B – H_2O:DMPC 12.6:1, sensitivity 0.2 milical/s/inch; C– H_2O:DMPC 36:1, sensitivity 0.4 milical/s/inch. (b) dimyristoyl phosphatidylcholine–cholesterol mixtures: (A) – X(chol)-0.4, H_2O:DMPC 14.9:1 (molar ratio), sensitvity 0.1 milical/s/inch; B – X(chol)-0.48, H_2O:DMPC 16.8:1, sensitivity 0.1 milical/s/inch; C – X(chol)-0.55, H_2O:DMPC 14:1, sensitivity 0.04 milical/s/inch.

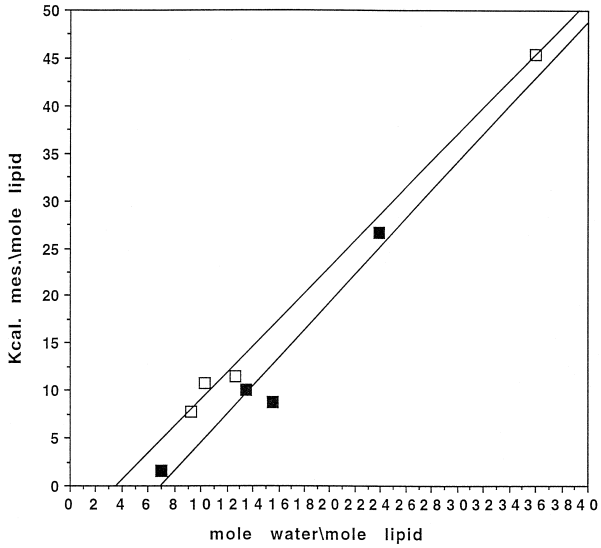

Fig. 8. The measured enthalpy of melting of water (Kcal measured/mole lipid) as a function of the ratio mole water/mole lipid. □ □, DMPC only; ■ ■, DMPC–cholesterol mixture; X(chol)-0.55.

As in the case of natural PS also here around a certain cholesterol content (X(chol)-0.15–0.2) the number of water molecules bound increases abruptly and then levels off.

Phosphatidylserines are negatively charged at neutral pH and the headgroups are connected by intermolecular hydrogen bonding. The phosphatidylserines used in the present study were natural PS from bovine spinal cord and well defined disaturated (di C14)-dimyristoyl phosphatidylserine. For evaluating the effect of the headgroup and hydrogen bonding on hydration of the phospholipid, we have investigated the hydration of zwitterionic phospholipid dimyristoyl phosphatidylcholine and its mixtures with cholesterol.

In Fig. 7 are presented thermograms of DMPC at increasing water content (Fig. 7(a)) and of DMPC–cholesterol mixtures (Fig. 7(b)). Even at the lowest water content (9.3:1 H2O:DMPC) trace A Fig. 7(a) the water melting peak is around 0°C and the main transition of the phospholipid (gel–liquid crystalline transition) is preceded by the pretransition indicating full hydration of the phospholipid and the presence of an excess of free water.

In the phospholipid–cholesterol mixtures (Fig. 7(b)) the melting peak of ice is at around 0°C, but at

lower temperatures another peak or shoulder is seen indicating the melting of at least two types of water.

The graphs of enthalpy of melting of water (Kcal measured/mole lipid) for DMPC alone or for DMPC–cholesterol mixture are presented in Fig. 8. The number of water molecules bound per DMPC molecule given by the intercept of Fig. 8 is 3.5, the slope-enthalpy of melting of ice is near the theoretical value, 1.4 Kcal/mole. Based on this graph and similar ones the number of water molecules bound per molecule of DMPC was calculated and is presented as a function of molar fraction of cholesterol in Fig. 6. The number of water molecules bound stays constant up to X(chol)-0.4 and then increases strongly until it seems to level off at X(chol)-0.5.

4. Discussion

The hydration of phosphatidylcholines was investigated extensively and the number of bound water molecules was determined by different methods. It was reported that egg, dipalmitoyl and dioleoyl phosphatidylcholines bind tightly 9 water molecules per phospholipid molecule [9]. However, different degrees of hydration were also reported. In the gel state dipalmitoyl phosphatidylcholine binds 6–7 molecules of water [11,12]. Dioleoyl phosphatidylcholine binds 9 water molecules per molecule of the phospholipid when in the gel state [16], in agreement with Ref. [9] and 20 molecules in the liquid crystalline state. Recently Faure et al. [5] showed that dimyristoyl phosphatidylcholine binds 4.3 water molecules in the gel state and 9.7 water molecules in the liquid crystalline state. The value of 9.7 is in agreement with 8.6 molecules determined by a different technique and published very recently [23].

In the present study we have also investigated the binding of water to DMPC and found that 3.5 water molecules are bound per phospholipid molecule in the gel state. This result is in agreement with the hydration of DMPC determined by NMR [5].

Finer and Darke [1] investigated by NMR the different hydration shells of egg lecithin, egg phosphatidylethanolamine and spinal cord phosphatidylserine. All the measurements were performed at room temperature when the phospholipids are in the liquid crystalline state. However, they did not determine the number of water molecules bound to PS molecule. In the present work we have investigated systematically by DSC water binding to phosphatidylserines measured as unfreezable water in the absence and in the presence of cholesterol. We have found that PS binds 2.5 water molecules in addition to 1.5 water molecules detected by FTIR in the dry sample before addition of measured quantities of water (unpublished results) and DMPS binds 3.8 water molecules per molecule of phosphatidylserine. The number of water molecules bound per molecule of phosphatidylserines are similar to the number of water molecules bound to DMPC (3.5). This finding is not surprising because inspite that DMPC is zwitterionic and phosphatidylserines are negatively charged the headgroups of the latter are stabilized by interhydrogen bonds, possibly preventing additional hydration due to the presence of negative charge. Phosphatidylethanolamines which are also zwitterionic but with interhydrogen bonds are less hydrated than the corresponding phosphatidylcholines which are also zwitterionic but devoid of hydrogen bonds [7,23].

Another aim of the present study was to determine the hydration of phosphatidylserine–cholesterol mixtures as a function of molar fraction of cholesterol and to try to correlate the hydration with phase separation of cholesterol from these mixtures.

By employing DSC and X-ray diffraction we have shown that solubility of cholesterol in negatively charged phospholipids is limited resulting in phase separation of cholesterol crystallites [17–19,24]. For PS–cholesterol mixtures the onset of phase separation of cholesterol in liquid crystalline state of the lipid as determined by X-ray diffraction takes place at X(chol)-0.3 [18]. Recently we have investigated phase separation in DMPS–cholesterol mixtures and detected the onset of the appearance of cholesterol crystallites at X(chol)-0.32 and 0.35 in the gel and in the liquid crystalline states of the lipid, respectively [19]. In Figs. 3 and 6 is presented the hydration of the phospholipid as a function of molar fraction of cholesterol. In both cases (PS and DMPS) the hydration by tightly bound water molecules determined as the number of unfreezable water molecules per molecule of lipid is measured below the melting temperature of the phospholipid (in the gel state). The abrupt increase of the number of unfreezable water molecules occurs in both cases around X(chol)-0.2 at

cholesterol ratio lower than the one required for cholesterol crystallites to be detected by X-ray diffraction.

The onset of the appearance of cholesterol crystallites as detected by X-ray diffraction is probably preceded by separation of microcrystallites that are undetectable by X-ray diffraction but sufficient for creating interface boundaries. We would like to propose that additional water binding-increase of hydration takes place at the boundary regions of PS–cholesterol and pure cholesterol phases. The abrupt increase in hydration at about X(chol)-0.2 with only a moderate increase with further increase of X(chol) corresponds to the growth of the lipid/cholesterol boundary line. The boundary line is created by the crystallization of cholesterol from the cholesterol solution in the phospholipid. At a critical cholesterol concentration nucleation starts and a large number of microcrystallites with a large specific boundary is formed. Further addition of cholesterol does not increase the interphase boundary proportionally because its increase is compensated by aggregation of the microcrystallites. Thus the interphase boundary increases only moderately and the crystallites become large enough to make the detection by X-ray diffraction possible. To check this point further we have investigated DMPC–cholesterol mixtures.

It is known that phase separation of cholesterol from DMPC–cholesterol mixtures in the liquid crystalline state of the lipid takes place at X(chol)-0.45–0.5 [25,26]. In Fig. 6 the hydration of DMPC–cholesterol mixtures as a function of X(chol) is presented, number of water molecules bound is constant up to X(chol) about 0.45, increasing in the region where phase separation takes place.

At this point it is worth to mention that Ho et al. [27] employing fluorescence techniques detected a continuous increase in hydration for mixtures of cholesterol with monosaturated phosphatidylcholine (palmitoyl-oleoyl phosphatidylcholine) in the range of cholesterol molar fractions of 0–0.25.

We would also like to ask the question why is the tightly bound water unfreezable. The simplest answer is that it never freezes as its vapor pressure at every temperature is lower than that of ice. The other possibility is that the latent heat of transition from bound water to ice approaches zero. This seems to be consistent with the lower than expected latent heat of freezing of the relatively loosely bound subsequent hydration layers. The two possibilities are not mutually exclusive. There is still the possibility that the freezing point decreases with the decreasing ratio of water to lipid with consecutive freezing of water. Thus the transition peak broadens and becomes indiscernible from the background. This is not very likely as each tightly bound water molecule is attached to a specific residue and hence its freezing is completed in a narrow temperature range.

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