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STUDIES ON LECITHIN–CHOLESTEROL–WATER INTERACTIONS BY DIFFERENTIAL SCANNING CALORIMETRY AND X-RAY DIFFRACTION

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SUMMARY

Addition of cholesterol to dipalmitoyl-L-lecithin in water lowers the transition temperature between the gel and liquid crystalline phase, and decreases the heat absorbed at the transition. No transition is observed with an equimolar ratio of the lecithin with cholesterol. This ratio corresponds to the maximum amount of cholesterol which can be introduced before cholesterol precipitation occurs. Unsaturated lecithins and the lipid extract of human erythrocyte ghosts exhibit similar behaviour. The cholesterol controls the fluidity of the hydrocarbon chains of the phospholipid by disruption of the crystalline chain lattice of the gel phase, and by inhibiting the flexing of chains in the dispersed liquid crystalline phase. The possible implications of this behaviour to the role of cholesterol in biological membranes are discussed.

INTRODUCTION

Recent studies of pure phospholipids using a variety of physical techniques[1,2] have shown that each phospholipid, on heating, exhibits a transition from a crystalline to a smectic phase. The transition temperature depends upon the type of fatty acid chains in the lipid. The addition of water to a given phospholipid lowers this transition temperature until it reaches a characteristic limiting value at a concentration corresponding to the maximum uptake of bound water by the phospholipid gel. The ability to form myelin forms and to disperse lipids occurs above this characteristic temperature (T_c). In order to shed light on the possible role of cholesterol in biological membranes, we use calorimetric and X-ray techniques to investigate the effect of cholesterol on some lecithin–water systems.

EXPERIMENTAL

Materials

Pure synthetic 1,2-dipalmitoyl- and 1,2-dioleoyl-L-lecithin were prepared in this laboratory by the method described elsewhere[2]. Egg yolk lecithin was obtained from fresh hens' eggs by silicic acid chromatography. Total lipid and phospholipid extracts from human erythrocyte membrane were the same as those used for nuclear magnetic resonance studies[3]. The cholesterol used was recrystallised several times from absolute alcohol and the water was twice distilled in glass from alkaline potassium permanganate.

Preparation of mixtures

The two lipid components were first dissolved together in chloroform and the solvent then removed in a stream of dry nitrogen. Last traces of solvent were removed *in vacuo*. Homogeneous mixtures of lecithin, cholesterol and water were prepared by sealing weighed amounts of the components into glass ampoules and centrifuging the material backwards and forwards through a narrow constriction in the centre of the ampoule at a temperature above that required for liquid crystal formation.

Unless otherwise indicated, all studies were made with systems containing 50 wt. % water. Previous studies[2,4] and the results presented show that 50 wt. % water always provides an excess of water present in the system. In this case the variation in the lecithin/cholesterol ratios is the only contributory factor affecting the results.

Differential scanning calorimetry

Samples were sealed into Perkin–Elmer volatile sample capsules and examined on the differential scanning calorimeter DSC-1. A scan speed of 8°/min was used for heating and cooling runs over the temperature range −100° to +100°. Peak areas were measured with a planimeter. Pure water was used as a primary standard for temperature and power calibration. After the runs the sample pans were punctured and the exact water content determined by drying to constant weight at 90° *in vacuo*. Weighings were made on a Cahn electrobalance, Model G.

X-ray diffraction

A Rigaku-Denki low-angle X-ray goniometer, operated *in vacuo* to eliminate air scattering, was used to record diffraction patterns, within the range $0.03 > \sin\theta/\lambda > 0.002$ Å^{-1}, on a photographic film. The incident X-ray beam was collimated by a slit system, and the sample to film distance used was of the order of 25 cm. The X-rays diffracted in the high-angle region were recorded using a Unicam (US26) camera. CuK_α ($\lambda = 1.5418$ Å) radiation was used.

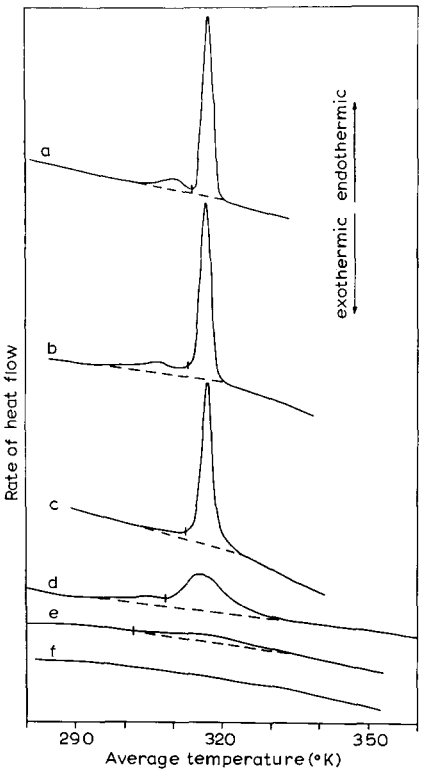
RESULTS

Differential scanning calorimetry (d.s.c.)

The d.s.c. curves between 280 and 360 °K for a series of 1,2-dipalmitoyl-L-lecithin-cholesterol mixtures each containing 50 % by weight of water and varying ratios of lecithin to cholesterol are shown in Fig. 1. As the concentration of cholesterol increases the main endothermic transition remains sharp while a small peak at 35° disappears. This is followed by a profound change in which the main transition becomes broad and decreases in area. When the concentration reaches 50 mole % of cholesterol, no endothermic peak can be observed. The variations in the transition temperature and the heat associated with the transition are shown in Figs. 2a and 2b, respectively.

The variation in the heat absorbed in the ice melting peak for the same series of mixtures is shown in Fig. 2c. The heat absorbed is expressed as cal/g of total material. The concentrations of lecithin and cholesterol are given as mole fraction of the total lipid present. The value of the heat change, in the absence of cholesterol, corresponds to that previously obtained for 1,2-dipalmitoyl-L-phosphatidylcholine at

50 % water[2]. The dashed line corresponds to the expected heat change if cholesterol acted solely as an inert diluent. A significant deviation from this line occurs only after 7.5 mole % cholesterol are added. The heat change decreases to a minimum value at 50 mole % cholesterol.



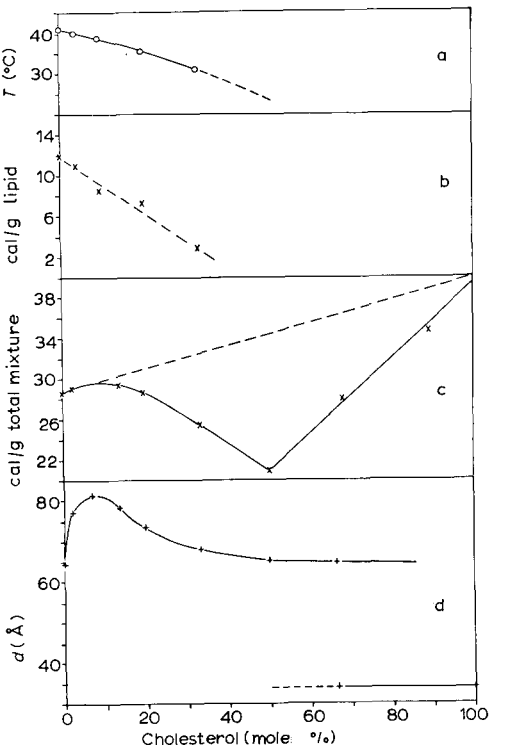


Fig. 1. D.s.c. curves of 50 wt. % dispersions in water of 1,2-dipalmitoyl-L-lecithin–cholesterol mixtures containing (a) 0.0 mole %, (b) 5.0 mole %, (c) 12.5 mole %, (d) 20.0 mole %, (e) 32.0 mole %, and (f) 50.0 mole % cholesterol.

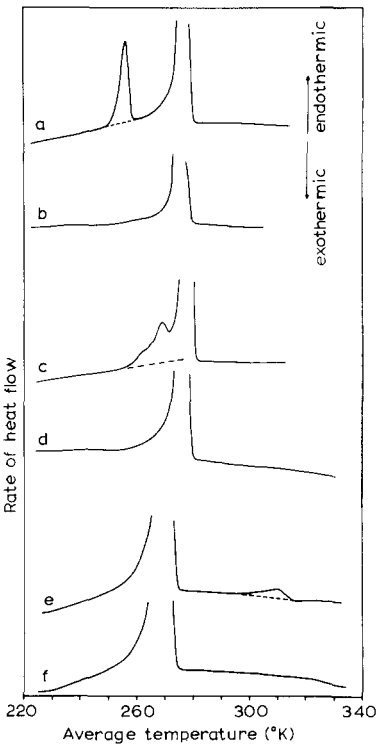
Fig. 2. The variation with 1,2-dipalmitoyl-L-lecithin–cholesterol ratio in 50 wt. % aqueous dispersions of (a) lecithin transition temperature, (b) heat absorbed in the lecithin transition, (c) heat absorbed in the ice transition at 0°, (d) X-ray long spacing (*d*) at 25°.

The limiting transition temperature for dioleoyl lecithin in water is —22°. With a 50 % dispersion in water of an equimolar mixture of dioleoyl lecithin and cholesterol, no endothermic transition associated with the lecithin is observed. The ice melting peak is decreased in size corresponding to an increase in the amount of bound water. Egg yolk lecithin behaves in the same way. The total lipid extract from human erythrocyte ghost and the corresponding phospholipid fraction in 50 % aqueous dispersions show similar results. The heating curve for the total lipid extract shows only an ice melting peak. An additional small endothermic peak is, however, obtained near 25° with the phospholipid fraction. These results are illustrated in Fig. 3.

X-ray diffraction

The 1,2-dipalmitoyl-L-lecithin–cholesterol–water systems show only integral orders of a principal long spacing in the low-angle region. The variation of long spacing

with cholesterol content (constant lipid/water ratio) at 25° is shown in Fig. 2d. Initially there is a large increase in spacing which reaches a maximum at 7.5 mole % cholesterol. There is no observable change in the high-angle Bragg spacing of 4.2 Å. On addition of further cholesterol the long spacing gradually decreases to a constant



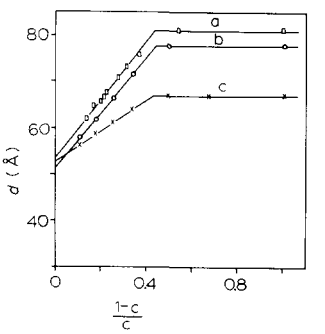


Fig. 3. D.s.c. curves of 50 wt. % dispersions in water of (a) 1,2-dioleoyl-L-lecithin, (b) 1:1 molar ratio of 1,2-dioleoyl-L-lecithin–cholesterol, (c) egg yolk lecithin, (d) 1:1 molar ratio of egg yolk lecithin–cholesterol, (e) phospholipid extract of human erythrocyte ghosts, (f) total lipid extract of human erythrocyte ghosts.

Fig. 4. Variation with water content of the X-ray long spacing (d) at 25° for the 1,2-dipalmitoyl-L-lecithin–cholesterol–water system (c = g lipid per g lipid–water mixture). Curve a, 7.5 mole % cholesterol; Curve b, 12.5 mole % cholesterol; Curve c, 42 mole % cholesterol.

value of 64 Å at 50 mole %. The sharp high-angle spacing increases to 4.45 Å and becomes diffuse. For concentrations greater than 50 mole %, an additional pattern appears with a long spacing of 34 Å attributable to the presence of crystalline cholesterol.

The variation in long spacing with water content is shown in Fig. 4. The system containing 7.5 mole % cholesterol exhibits a linear increase in long spacing with increase in $(1-c)/c$ up to 30 wt. % water ($(1-c)/c = 0.43$), after which no further increase is observed. Examination of the slope of the line and its intercept at $(1-c)/c = 0$ yields directly[5] the density of the lipid* (1.15 g·cm^{-3}) and the thickness of the

* This density is calculated on the basis that the density of the water between the bilayers is 1.00 g·cm^{-3}. Most, if not all, of the water between the bilayers is bound and therefore the density of this water is possibly less than 1.00 g·cm^{-3}. If, for example, a density for bound water of 0.95 g·cm^{-3} is used, the density of the lipid layer is calculated to be 1.09 g·cm^{-3}.

lipid layer (53.5 Å). The system containing 12.5 mole % cholesterol shows similar water uptake and a lipid layer thickness of 51 Å. The behaviour of the system containing 42 mole % cholesterol is somewhat similar in that an increase in long spacing is observed up to 30 wt. % water. The thickness of the lipid layer cannot be directly calculated from Curve c of Fig. 4 because, when the water content is reduced below 30 wt. %, the diffuse high-angle line of 4.45 Å sharpens and approaches 4.2 Å. A structural transition thus occurs on reducing the water content below 30 wt. % for the systems with high cholesterol content. The agreement of the experimental points with a straight line in the region $0 < (1-c)/c < 0.43$ in Fig. 4c is thus merely accidental.

X-ray diffraction from the 50 % dispersion in water of the total lipid extract of human erythrocyte ghosts gives integral orders of a principal long spacing in the low angle region.

DISCUSSION

The observation of only integral orders of a principal long spacing for all systems examined shows that only lamellar structures are formed at 25°. In the absence of cholesterol at 25°, 1,2-dipalmitoyl-L-lecithin in water forms a gel[2] in which the hydrocarbon chains are packed in an hexagonal sub-cell with the chain axes inclined at 58° to the lipid–water interface, as illustrated in Fig. 5a. The lipid and water layers are respectively 46 Å and 18.5 Å thick.

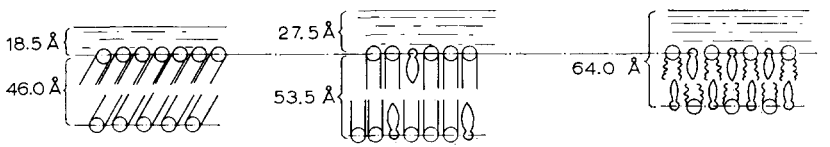


Fig. 5. Schematic representation of 1,2-dipalmitoyl-L-lecithin–cholesterol lamellae in water. (a) 0 mole % cholesterol, (b) 7.5 mole % cholesterol, (c) 50 mole % cholesterol.

The lecithin lamellae can probably accommodate cholesterol molecules more readily if the hydrocarbon chains are perpendicular to the lipid–water interface, but this change in configuration alone could only increase the long spacing by 7 Å. It cannot account for the large increase, 16.5 Å, in spacing when small quantities of cholesterol are added, and there must therefore be an increase in the water layer thickness. The results shown in Fig. 4 (Curve a) confirm this by showing an increase of approx. 9 Å in the water layer thickness to 27.5 A. The sharp line in the high-angle region at 4.2 Å, indicates that, at 7.5 mole % cholesterol, the hexagonal packing of the hydrocarbon chains is not seriously disturbed. The structure proposed for the system at this cholesterol content is shown in Fig. 5b. This concentration probably represents the maximum amount of cholesterol which can be readily accommodated into the hexagonal lattice. (The change of tilt of the lipid to a vertical configuration probably changes the distribution of charges in the polar head group layer, causing the increase in water layer thickness.)

Addition of further cholesterol up to a maximum of 50 mole % causes (see Fig. 2) (a) a reduction in the lipid transition temperature, (b) a reduction of the heat of transition, (c) an increase in bound water, and (d) the X-ray long spacing to

decrease from 81 to 64 Å. These results are consistent with the cholesterol causing a reduction in the cohesive forces between the adjacent hydrocarbon chains of the lecithin. This leads to some fluidisation of these chains as indicated by the diffuse spacing at 4.45 Å. The reduction of long spacing can be accounted for entirely by a reduction in the length of the hydrocarbon chains caused by this fluidisation.

We can infer that the thickness of the water layers is similar for systems with low (7.5 mole %) and high (42 mole %) cholesterol content, because 30 wt. % water is taken up by both systems. The structure of the system containing equimolar amounts of lecithin and cholesterol in water is shown in Fig. 5c. However, as we have mentioned in the X-ray results section, we cannot calculate that dimensions of the lipid and water layers from Curve c, Fig. 4, because the cholesterol, when present in high concentrations, does not completely fluidise the lipid in the presence of less than 30 % water. The important effect of water on this system is shown by the fact that an equimolar mixture of 1,2-dipalmitoyl-L-lecithin and cholesterol in the dry state shows a transition at 56°, and the thermal behaviour of anhydrous mixtures is rather more complex than when water is present. This is not surprising in view of the known polymorphism exhibited by both lecithin[2] and cholesterol[6].

The presence of X-ray spacings attributable to crystalline cholesterol when present in more than 50 mole % with lipid, suggests that this is the maximum amount of cholesterol which can be accommodated in the lecithin lamellae.

The effect of cholesterol is to disrupt the ordered array of hydrocarbon chains in the gel and, when cholesterol and lecithin molecules are present in equimolar proportions, all the chains are in a fluid condition. Our results in the bulk bilayer system are in agreement with the findings of SHAH AND SCHULMAN[7] that cholesterol liquefies solid monolayers of dipalmitoyl lecithin at high surface pressures. However, recent NMR spectroscopic studies on egg yolk lecithin at 33.5° (*i.e.*, well above the lipid transition (T_c) temperature) show a selective reduction in the signal due to the polymethylene chains when cholesterol is added[8]. One explanation given to explain this was that the presence of cholesterol caused some inhibition of the lipid chain motion. Thus, at a particular temperature (T), the presence of cholesterol keeps the hydrocarbon chains of differing phospholipid molecules in an "intermediate fluid" condition. Some lipids which would normally be above their limiting transition temperature ($T > T_c$) may have a certain amount of inhibition of chain motion. (This inhibition may depend upon how unsaturated are the lipid chains[3].) Those lipids which would normally be in a gel condition ($T_c > T$) are, however, given much greater fluidity. We have earlier noted[9] that phospholipids can only be dispersed in water when heated above their transition (T_c) temperature. The presence of equimolar amounts of cholesterol to lipid causes the phospholipid–cholesterol mixtures to be dispersible in water over a much wider temperature range than occurs with the individual phospholipid.

When we consider the relevance of these results to biological membranes, we know that correlations between bulk phase phenomena and discrete bilayers of lipid in any membrane structure must be made with caution[10]. We must not ignore the presence and effects due to the protein of the membrane. However, our results are pertinent to lipid bilayer organisation and how this is affected by the presence of cholesterol and to this extent are relevant to membrane organisation. Our results with the lipid extracts of human erythrocyte membrane encourage us to the view

that a possible role of cholesterol may be to control the fluidity of the hydrocarbon chains of the phospholipids giving a coherent structure stable over a wide temperature range and permitting some latitude in the fatty acid content of the lipids.

The susceptibility of the composition of the fatty acids of some membranes to vary with the environmental temperature[11] and the presence and influence of cholesterol will be examined elsewhere.

APPENDIX

The temperature composition diagram of the lecithin–cholesterol–water system

The temperature–composition diagrams for the dipalmitoyl lecithin–water and egg-yolk lecithin–water[12] systems are essentially equivalent and disposed along the temperature axis according to the melting point of the hydrocarbon moiety. BOURGÈS, SMALL AND DERVICHIAN[4] have obtained the phase diagram for the egg-yolk lecithin–cholesterol–water system at 25°, *i.e.*, somewhat above the transition temperature for this lecithin. This diagram should represent the behaviour of the dipalmitoyl lecithin–cholesterol–water system at an equivalent corresponding temperature above the T_c line. Combining the available data produces the three-dimensional phase diagram illustrated in Fig. 6.

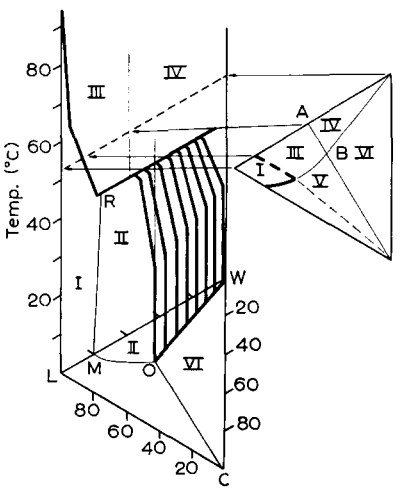


Fig. 6. Temperature–composition diagram for the ternary system 1,2-dipalmitoyl-L-lecithin–cholesterol–water. L, lecithin; C, cholesterol; W, water. Heavy lines are used to indicate the T_c surface. Concentrations expressed as percentage by weight are given along the side C–L for lecithin, along the side W–C for cholesterol, and along the side L–W for water. I, single phase gel region; II, two phase region, gel + water; III, single phase mesomorphic lamellar region; IV, two phase region, mesomorphic lamellar + water; V, two phase region, mesomorphic lamellar + cholesterol; VI, three phase region, mesomorphic lamellar + cholesterol + water.

The percentage of bound water in the series of lecithin–cholesterol mixtures has been calculated from the reduction in the size of the ice melting peaks and is plotted in the form of a ternary composition diagram at 0°. In the concentration range 0 to 7.5 mole % cholesterol there is no change in bound water but an increase in the thickness of the water layer, whereas between 7.5 and 50 mole % cholesterol the bound water increases while the thickness of the water layer remains constant.

Only at 50 mole % cholesterol is the amount of bound water equal to the total amount of water between the lipid bilayers.

A significant feature is the correspondence in the composition of the triple point in the liquid crystalline region (B) and in the gel region (O). The amount of water involved in the triple point composition is clearly related to the maximum amount of water taken up by the lecithin in its liquid crystalline state (A). In the gel state the amount of water taken up by the lecithin alone is considerably less than this and is given by point M at 0° and point R just below the transition temperature.

The point at which the transition temperature reaches a minimum corresponds to the point at which the maximum uptake of bound water occurs.

REFERENCES

1 D. CHAPMAN, P. BYRNE AND G. G. SHIPLEY, *Proc. Roy. Soc. London, Ser. A*, 290 (1966) 115.
2 D. CHAPMAN, R. M. WILLIAMS AND B. D. LADBROOKE, *Chem. Phys. Lipids*, 1 (1967) 445.
3 D. CHAPMAN, J. DE GIER, V. B. KAMAT AND S. A. PENKETT, *J. Mol. Biol.*, 31 (1968) 101.
4 M. C. BOURGÈS, D. M. SMALL AND D. G. DERVICHIAN, *Biochim. Biophys. Acta*, 137 (1967) 157.
5 J. M. VINCENT AND A. SKOULIOS, *Acta Cryst.*, 20 (1965) 432.
6 H. L. SPIER AND K. G. VAN SENDEN, *Steroids*, 6 (1965) 871.
7 D. O. SHAH AND J. SCHULMAN, *J. Lipid Res.*, 8 (1967) 215.
8 D. CHAPMAN AND S. A. PENKETT, *Nature*, 211 (1966) 1304.
9 D. CHAPMAN AND D. J. FLUCK, *J. Cell Biol.*, 30 (1966) 1.
10 E. D. KORN, *Science*, 153 (1966) 1491.
11 D. CHAPMAN, in A. H. ROSE, *Thermobiology*, Academic Press, New York, 1967.
12 F. REISS-HUSSON, *J. Mol. Biol.*, 25 (1967) 363.