Biochimica et Biophysica Acta, 318 (1973) 299–305

BBA 76432

A STRUCTURAL TRANSITION IN EGG LECITHIN–CHOLESTEROL BILAYERS AT 12 °C

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(Received March 1st, 1973)

SUMMARY

The thermal coefficient of expansion of egg lecithin bilayer thickness, αd_l, was measured as a function of its cholesterol content, up to mole ratio lecithin/cholesterol of 1:1, and over the temperature range 0–40 °C. At all cholesterol contents αd_l changes abruptly at approximately 12 °C indicating a structural transition at this temperature. Above 12 °C, αd_l decreases monotonically from $-2 \cdot 10^{-3}$ for pure egg lecithin to $-1 \cdot 10^{-3}$ at mole ratio 1:1. Below 12 °C αd_l is always higher than above 12 °C and shows a sharp, anomalously high value of $-6 \cdot 10^{-3}$ at the mole ratio 2:1. The results have been interpreted as the movement of cholesterol into the bilayer or the formation of lecithin–cholesterol "complexes" at temperatures below 12 °C. Similar studies with phosphatidylinositol containing cholesterol showed no structural transition and lysolecithin containing cholesterol behaved differently giving two lamellar phases in equilibrium.

INTRODUCTION

In previous X-ray diffraction studies on the structure of the lamellar phase of membrane phospholipids and cholesterol[1] we established that cholesterol's position in the bimolecular layer is with the long axis of the steroid nucleus parallel to the hydrocarbon chains and with its hydroxy group oriented toward and close to the polar-group layer. In this and in studies with egg lecithin[2] it was shown that one effect of cholesterol is to increase the thickness of the phospholipid bilayer resulting in a decrease in the area available to the polar group of the phospholipid molecules on the surface of the bilayer from 60 to 50 Å^2, similar to the condensing effect of cholesterol on phospholipid monolayers. The effect was attributed to a decrease in the thermal motion or mobility of the disordered hydrocarbon chains of these phospholipids by the presence of the rigid steroid nucleus. An enormous number of studies on many similar systems using many different techniques, including rather direct measures of "disorder" and "mobility", have confirmed and refined this interpretation (for a recent review see ref. 3). A thickening of the phospholipid bilayer results as well from decreasing the thermal motion of the chains by lowering their temperature, *i.e.* there is a negative thermal coefficient of bilayer thickness of approx. $2 \cdot 10^{-3}$ (ref. 4). The relative magnitude of these two effects is illustrated by the fact that an addition of cholesterol to approx. 33 mole % with egg lecithin, a

mole ratio where its effect is maximal[2], produces a thickening of the bilayer equivalent to lowering the temperature of the pure phospholipid by 30 °C.

Temperature dependent changes in the thickness of lipid bilayers are important to recognize in any temperature studies of their properties. For example, Papahadjopoulos *et al.*[5] in a study of the effect of cholesterol and temperature on the permeability of phospholipid membranes obtained some anomalously high activation energies for some solutes. In an analysis of Arrhenius plots, where activation energy is usually assumed to be independent of temperature, these authors have shown that even a very small temperature dependence of the activation energy can lead to large errors in its value as determined from linearization of the Arrhenius plots. They have therefore attributed some large observed activation energies to the temperature dependent structural changes, *i.e.* thinning of the bilayer, rather than to true changes in activation energy. It was in measuring the temperature dependence of the thickness of egg lecithin bilayers, with and without cholesterol, that we discovered, and report here, a structural transition that occurs in the bilayer at 12 °C and which depends strongly on the lecithin/cholesterol ratio.

MATERIALS AND METHODS

Egg lecithin, prepared according to the method of Singleton *et al.*[6] and cholesterol (Sigma) in chloroform solutions were mixed to give the desired mole ratio, dried under vacuum and finally mixed gravimetrically with 0.1 M NaCl to the desired concentration. The repeat distance d of the resulting lamellar phase was determined by X-ray techniques described previously[1]. For each X-ray sample d was measured as a function of temperature, which was measured at the sample with a thermocouple and controlled with thermoelectric elements to ± 0.2 °C. Similar preliminary experiments were performed with chromatographically pure, Baker's yeast phosphatidylinositol and egg lysolecithin kindly supplied by D. O. Tinker, University of Toronto.

The thermal coefficient of expansion of the repeat distance d of the lamellar phase is given by

$$\alpha_d = \frac{1}{d}\frac{\delta}{\delta T}(d) \tag{1}$$

and was experimentally determined from the slope of the line

$$\ln d = \alpha_d T + \text{constant}$$

The lamellar phase is known to be made up of alternating layers of lipid, of thickness d_l and water, of thickness d_w.
The thickness of the lipid bilayer, d_l is given by
$d_l = \varphi_l d$ where φ_l is the volume fraction of lipid in the sample and

$$\varphi_l = \frac{Cv_l}{Cv_l + (1-C)v_\omega}$$

where C is the weight fraction of lipid in the sample and v_w and v_l are the partial specific volumes of water and lipid respectively.

Using these relationships and attributing the changes in d in any particular sample to changes in the average length of the lipid molecules and to differences in the bulk thermal coefficient of expansion of the lipid and water then

$$\alpha_d = \alpha_{d_l} + K(\alpha_{v_\omega} - \alpha_{v_l}) \tag{2}$$

where the α's are the thermal coefficients of the variables of the subscript, and are similar to Eqn 1, and

$$K = \frac{(1-C)v_\omega}{Cv_l + (1-C)v_\omega} \approx (1-C) \text{ since } v_l \approx v_\omega$$

The first term of the right-hand side of Eqn 2 is the contribution to the thermal coefficient of the repeat distance d resulting from changes in the length of the lipid molecules. The second term is the contribution to α_d which would result from any differences in the cubical or bulk coefficient of thermal expansion of lipid and water. In the absence of a measure of α_{v_l} for the phospholipid–cholesterol mixture, an estimate of the maximum value of this second term is $0.34 \cdot 10^{-3}$, taking $\alpha_{v_\omega} = 0.13 \cdot 10^{-3}$ and $\alpha_{\text{pentane}} = 1.5 \cdot 10^{-3}$, one of the highest thermal coefficients listed[7] and $K = 0.25$. (For paraffin this second term would be $0.14 \cdot 10^{-3}$.) Since the values of α_d measured in this study are from $2 \cdot 10^3$ to $6 \cdot 10^{-3}$, they result primarily from changes in the length of the lipid molecules in the lipid layer, as has been assumed in the past and described in the introduction. For the purposes of further discussion we will assume that

$$\alpha_d \approx \alpha_{dl}$$

an assumption that will not affect the conclusions reached in this study.

RESULTS

All X-ray samples were single lamellar phases with $\varphi_l \approx 0.75$ unless otherwise noted. At this lipid concentration all the water is in the lamellar structure and the lipid layers are separated by approx. 15 Å of water, close to their maximum separation. No changes in the diffuse high-angle line at 4.5 Å were ever observed indicating that there were no detectable changes in the disorder of the hydrocarbon chains of the lecithin.

Fig. 1 shows a plot of ln d *vs* T for egg lecithin and for lecithin/cholesterol mole ratio of 2:1; for both samples $\varphi_l = 0.75$.

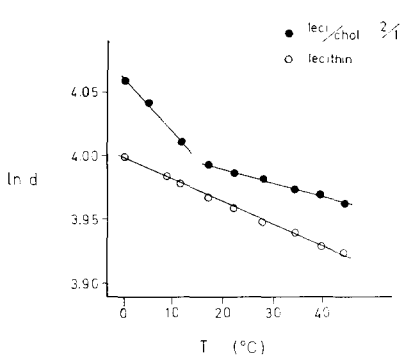
For pure lecithin there is a monotonic increase in thickness of the lipid leaflet as the temperature decreases, the temperature coefficient $\alpha d_l = -2 \cdot 10^{-3}$ is agreement with previously measured values for other similar lipids[4].

In comparing the sample of lecithin/cholesterol (2:1) with pure lecithin in Fig. 1 note (i) at all temperatures the thickness of the lipid leaflet increases when cholesterol is added to egg lecithin, the familiar thickening effect; (ii) above a critical temperature, approx. 12 °C, the slope α_d decreases when cholesterol is added to the bilayer, *i.e.* the effect of increasing T on thinning of the lipid layer is less with cholesterol than without. (iii) below the critical temperature the slope α_d increases

above that of pure egg lecithin, *i.e.* the effect of increasing temperature on thinning is greater with cholesterol than without.

The presence of the break in the curve for lecithin containing cholesterol existed for all concentrations of lipid up to 90%. Inasmuch as the critical temperature is given by the intersection of the two lines and therefore is accurate only to approx. ± 2 °C, no relation was found between it and the concentration of lipid.

Fig. 2 shows the dependence of α_d on the cholesterol content of the lipid. Above the critical temperature, as the amount of cholesterol increases to the limiting amount that lecithin can take the temperature coefficient decreases to a value approximately one-half that for pure lecithin. Below the critical temperature, and as the cholesterol content increases, the absolute value of α_d first increases showing a sharp maximum equal to 3 times the value for pure lecithin at lecithin/cholesterol ratio of 2:1, and then decreases to a value equal to that above the critical temperature at the lecithin/cholesterol ratio of 1:1.



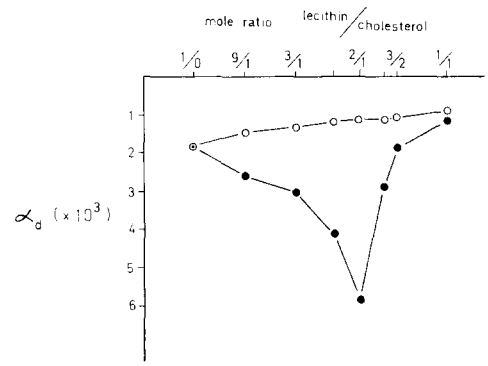


Fig. 1. Plot of logarithm of the repeat distance d of the lamellar phase as a function of temperature. Whenever the egg lecithin contains cholesterol there is a change in the slope of the line, α_d, at approx. 12 °C. α_d is the thermal coefficient of the thickness of the lipid bilayer. leci, lecithin; chol, cholesterol.

Fig. 2. Thermal coefficient α_d as a function of cholesterol content. Above 12 °C, ○——○, α_d decrease to half its value at lecithin/cholesterol, 1:1. Below 12 °C, ●—●, a sharp maximum in the absolute value of α_d is observed at the lecithin/cholesterol ratio of 2:1.

In preliminary attempts to determine the dependence of these phenomena on the nature of the phospholipid the following results were obtained.

(i) The replacement of 10 mole % of the egg lecithin with dipalmitoyl lecithin (Serdary) in a lecithin/cholesterol ratio of 2:1, produced no change either in the values for α_d or the value of the critical temperature.

(ii) Phosphatidylinositol/cholesterol, 2:1 mole ratio, showed no break in the temperature curve over the range 0–40 °C.

(iii) Egg lysolecithin/cholesterol behaved very differently forming two lamellar structures in equilibrium and therefore could not be compared.

DISCUSSION

Changes in α_d above the critical temperature

Since cholesterol inhibits the mobility and disorder of the hydrocarbon chains of egg lecithin and since it is the changes in the disorder with temperature that endows the bilayer thickness with a negative temperature coefficient, it is to be expected that replacing hydrocarbon chains with the rigid steroid nucleus would reduce the value of this coefficient. Indeed above the critical temperature this is the case; α_d being reduced by one-half when one-half of the chains are replaced. Nevertheless the chains are still disordered and the value of the negative coefficient indicates that their "order" and therefore length can be increased with a decrease in temperature. In the context of the thermal studies of Papahadjopoulos *et al.*[5] such changes in bilayer thickness would result in thermally dependent activation energies for solutes permeating such bilayers. Therefore, as suggested by these authors, such activation energies determined from Arrhenius plots would be higher than their true value although closer to it than those determined similarly with pure egg lecithin.

Changes in α_d below the critical temperature

The structural transition observed at 12 °C and its sharp dependence on the cholesterol content were unexpected new results. The increased slopes make the comments about Arrhenius plots even more relevant. Their investigation may lead to new insights into the molecular interactions of these two different membrane lipids. The following comments, based on these new observations, are made in order to develop a working hypothesis about these interactions.

(1) The increase in α_d. Below 12 °C, α_d increases in magnitude, *i.e.* the change in disorder of the hydrocarbon chains with temperature is greater whenever cholesterol is present, in whatever amount, than without it. This change is in the opposite direction than expected as explained above in accounting for the decrease in α_d above the critical temperature. Furthermore, such high absolute values of linear thermal coefficients, up to $6 \cdot 10^{-3}$, are high for any material including rubber and paraffin[7] and higher than the theoretical value of, $-1/T$, for a disordered linear polymer[8]. Therefore we are forced to conclude that α_d, below the critical temperature, does not represent a true linear coefficient as it applies to a single material. Rather it must reflect a conversion from one structure to another, the degree of conversion depending on the temperature.

As a hypothesis to explain such a structural conversion, we suggest that cholesterol could, for example move further into the bilayer as a result of changes in the energy of interaction of the hydroxy group at the polar interface and/or of the cholesterol with the hydrocarbon chains. The steroid nucleus in moving in could effectively inhibit the motion of a longer length of the hydrocarbon chains, generally thought to extend only to about the eighth or tenth CH_2 group when its hydroxy group is at the polar interface[9,10], and in so doing would cause an increase in the length of the hydrocarbon chain and therefore d_l. Some such effect of cholesterol must cause the enhanced increase in d_l, or equivalently the "supercondensing" effect below 12 °C, and a further elaboration of it is given in the following.

(2) The optimum lecithin/cholesterol ratio of 2:1. The lecithin/cholesterol ratio of 2:1, shown to be sharply optimal for α_d below 12 °C, has also been shown to be

optimal for a number of phenomena that include the absolute increase in d_1 (ref. 2) and these studies confirm this at all temperatures 0–40 °C. At this ratio the simple geometrical planar arrangement is that each cholesterol, if uniformly distributed in the plane of the bilayer, is surrounded by six hydrocarbon chains of the lecithins and might be expected to have its optimal effect on them. At lower cholesterol contents, areas containing only hydrocarbon chains exist and would be expected to behave like the hydrocarbon layers of pure lecithin. At higher cholesterol contents cholesterol–cholesterol contacts are made. The optimum ratio observed in the present studies may result from these simple considerations of the planar organization of the constituents, and have recently[14] been invoked to account for the complete elimination of the crystallization of the hydrocarbon chains of dipalmitoyllecithin by cholesterol at this mole ratio. However, it should be noted that, for chain lengths equivalent to those of egg lecithin, unsaturated hydrocarbon chains are required for the condensing effect of cholesterol on monolayers[11], and that egg lecithin contains on the average one unsaturated chain per molecule.

Therefore it is reasonable to add to our hypothesis that the optimal ratio observed for α_d, and which results in a "supercondensation" of the lipid, results from the formation of a "complex" of one cholesterol and two lecithin molecules. The interaction in the complex would be enhanced by the packing of one cholesterol between two bent unsaturated fatty-acid chains. Such a packing was originally suggested by Shah and Schulman[12] as mainly responsible for the condensing effect of cholesterol on monolayers but it has been shown not to be necessary[13]. However, the "cavities" so formed by double bonds may well be required at the lower temperatures where the number of gauche rotations is reduced and the saturated parts of the chains are more extended. At the higher temperatures cholesterol can be accommodated as easily between the disordered saturated chains as well, *i.e.* the cholesterol–chain interaction is higher and no optimum ratio is observed for α_d.

If such complexes are required for the enhanced lateral packing of the molecules observed at the lower temperatures, and which may be related to the movement of cholesterol into the bilayer as suggested above, then the optimum lecithin/cholesterol ratio should change with the ratio of unsaturated/saturated hydrocarbon chains. Preliminary experiments with lecithin, containing 10 mole % dipalmitoyllecithin, and with phosphatidylinositol are inconclusive in the respect. In the former the shift in optimum expected, from 2:1 to 2.2:1, was difficult to detect and higher dipalmitoyllecithin contents resulted in segregation of the lecithins at the lower temperatures. In the latter the change in the phospholipid, both in headgroup and in degree of chain unsaturation (unsaturated/saturated = 2:1), somehow resulted in the elimination of the structural transition at 12 °C.

Darke *et al.*[10] have recently interpreted NMR data of non-equimolar dispersions of egg lecithin and cholesterol on the basis of there being free lecithin and lecithin/cholesterol, 1:1, complexes that have a long life-time (>30 ms). Our results cannot be interpreted on the basis that these complexes become sufficiently long-lived when the temperature is lowered below 12 °C to be observed by X-ray diffraction, because the ratio is wrong. In our experiments the addition of cholesterol above 2:1 disrupts the formation of any complexes and α_d is quickly reduced. Complexes formed below 12 °C appear to be made up of 2 lecithin and 1 cholesterol molecule.

Since the measured values of d and d_1 represent time and space averages over

the area of the lamellar phase, the anomalously high values of α_d can be accounted for by noting that they would be a measure of the degree of movement of cholesterol into the bilayer or of the number of complexes that are formed as the temperature is lowered.

The above hypotheses are being tested and the phenomena observed in a number of phospholipid–sterol systems with a view to understanding these molecular interactions and their relevance to natural membranes.

ACKNOWLEDGEMENTS

We gratefully acknowledge the skilled technical help of Mrs Olga Tkaczyk and the financial support of the National Research Council of Canada through grant No. A4920.

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