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AN INTERACTING SPIN LABEL STUDY OF LATERAL EXPANSION IN DIPALMITOYLLECITHIN–CHOLESTEROL BILAYERS**

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SUMMARY

The lateral expansion in dipalmitoyllecithin–cholesterol multibilayer films has been investigated using interacting pairs of cholestane spin labels. The abrupt lateral expansion occurring in dipalmitoyllecithin at the phase transition has been directly demonstrated, as also has the extremely high lateral expansivity occurring in the region of lateral phase separation of dipalmitoyllecithin–cholesterol bilayers. A higher lateral expansivity is found above the dipalmitoyllecithin phase transition than below it. The measured increase in lateral molecular separation at the phase transition correlates well with the change in X-ray short spacing, and also with other, indirect, dimensional measurements on the change in area per molecule in the bilayer. Cholesterol is found to have opposite effects on the lateral molecular packing in dipalmitoyllecithin bilayers above and below the phase transition. These opposing effects are also manifest in a reduction, with increasing cholesterol composition, of the total lateral expansion over the transition region. The measured lateral separations are used to calculate the decrease by cholesterol of the intermolecular contribution to the latent heat absorbed over the transition region. The calculations are consistent with the calorimetric measurements of the total latent heat. These results suggest that the observed decrease in the calorimetric onset temperatures for lateral phase separations could be attributed to the decreased lateral expansion in the bilayers containing cholesterol. The broadening of the transition region could also be correlated with a decrease in the cooperativity of intermolecular interactions resulting from the increased intermolecular spacing induced by cholesterol below the transition.

INTRODUCTION

Lateral molecular separation is one of the principal factors governing the strength of intermolecular interactions in phospholipid bilayers [1]. It thus strongly

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influences both the structure and functional characteristics of phospholipid bilayers and membranes, such properties as the liquid crystal phase transition point [2], permeability [3], and rate of phospholipid lateral diffusion [4, 5]. Of almost equal importance is the lateral expansivity of the bilayer which will also affect functional characteristics: ease of insertion of proteins and new membrane, activity of transport systems and binding of ligands and substrates.

In the present work, the method of interacting spin label pairs [6, 7] has been used to measure the lateral expansion, as a function of temperature, in bilayers of both dipalmitoyllecithin and various dipalmitoyllecithin–cholesterol mixtures. The spin label pair method is a sensitive probe of the lateral molecular separation within the bilayer [6], and has been previously used to demonstrate the molecular origin of the fluidizing and condensing effects of cholesterol on lecithin bilayers [7]. Investigations of spin label separation have been made at the phase transition of dipalmitoyllecithin and in the lateral phase separation region of dipalmitoyllecithin–cholesterol mixtures, as well as in the states above and below the transition regions. Comparison with X-ray measurements on dipalmitoyllecithin bilayers [8, 9] enables a quantitative correlation to be established between changes in spin label spacing and changes in lipid lattice constant. This facilitates comparison between the present interacting spin label data on the effects of cholesterol and those obtained by differential scanning calorimetry [10, 11]. Correlations are suggested between the changes in latent heat of transition, transition width and onset temperature for lateral phase separation, and the observed changes in molecular spacing within the bilayer.

EXPERIMENTAL

Oriented multibilayers were prepared in flat, quartz EPR cells (J. F. Scanlon, Co., Whittier, Calif.) as described previously [6, 7, 12] by evaporating a chloroform solution of lipids plus spin label. The spin label concentration was chosen to give best resolution of the interacting spin label spectra. In practice this was found to be in the region of 8 mole % cholestane spin label relative to lipid plus cholesterol. The multibilayer films were hydrated with 0.15 M NaCl for at least 1 h. Immediately prior to the experiments the films were drained and the cells then sealed with a small amount of hydrating solution remaining in the cell. All films measured were thus fully hydrated and contained excess aqueous phase. EPR spectra were recorded on a Varian E-9 spectrometer with the magnetic field perpendicular to the plane of the multibilayer film. A Varian variable temperature controller (V-4540) was adapted to accommodate the larger flat cells by using a quartz sleeve cavity insert. Temperature was measured with a copper–constantan thermocouple placed in the cavity in contact with the sample cell.

Dipalmitoyl L-α-lecithin was obtained from Supelco Inc., Bellefonte, Pa., and egg L-α-lecithin from Lipid Products, S. Nutfield, U.K. Cholesterol from Steraloids, Pawling, N.Y. was recrystallized from methanol. Lipid fraction 1 was extracted from the white matter of bovine brain [13]. Total lipid was extracted according to ref. 14 from erythrocyte ghosts [15] prepared from outdated human blood. The cholestane spin probe, 3-spiro-[2′-(*N*-oxyl-4′,4′-dimethyloxazolidine)]-cholestane was prepared as described by Keana et al. [16].

RESULTS

The temperature variation of cholestane spin label pair spectra has been investigated in oriented multibilayers of dipalmitoyllecithin with varying proportions of cholesterol. Fig. 1 gives typical EPR spectra of dipalmitoyllecithin plus 20 mole% cholesterol multibilayers at various temperatures. The spectra consist of strong central lines arising from single, non-interacting spin labels, and smaller, more widely separated, outer lines which come from the interacting spin label pairs (see refs 6 and 7). The characteristic three line hyperfine splitting of the central lines, although broadened, shows that the multibilayers are well ordered [12, 17, 18]. The spin label pair lines are seen to be well resolved from the central lines and their splitting decreases steadily with increasing temperature, corresponding to a decrease in the lateral separation of the two interacting spin-labelled molecules. The splitting of the pair lines is determined solely by the magnetic dipole–dipole interaction, and thus provides an extremely sensitive and direct measure of the lateral molecular separation. In a previous paper [7] it was shown that the splitting of the lines, ΔH, with the magnetic field oriented perpendicular to the plane of the bilayers is related to the lateral separation, r, of the spin labels in the plane of the bilayers, by the following relation:

$$\Delta H = \frac{3}{2}\frac{g\beta}{r^3} \tag{1}$$

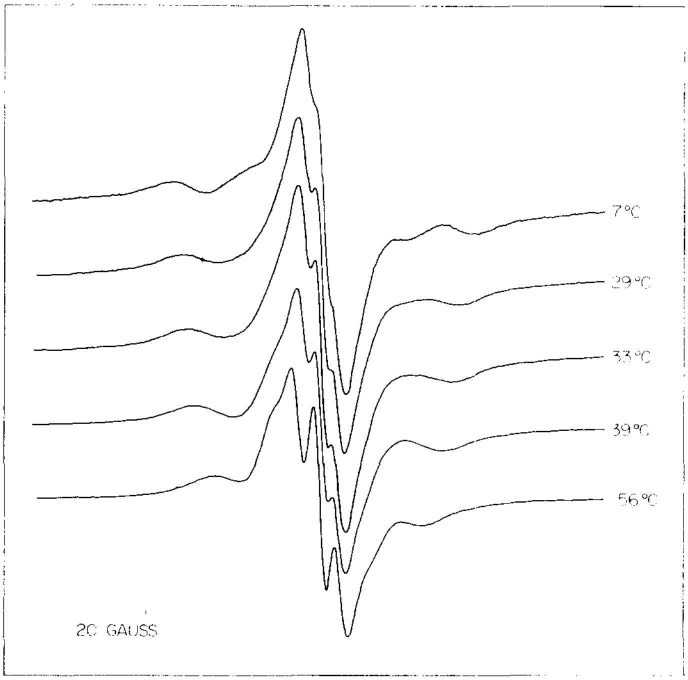
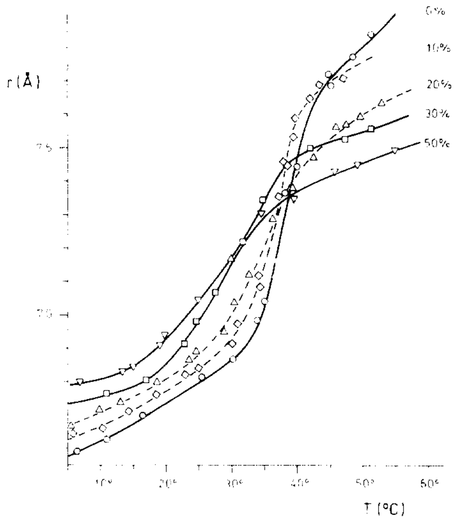


Fig. 1. EPR spectra of interacting cholestane spin label pairs in multibilayers of dipalmitoyllecithin plus 20 mole% cholesterol at various temperatures. The magnetic field is oriented perpendicular to the plane of the multibilayers. The pair lines are the two smaller, outermost lines which are symmetrically split from the intense, central, isolated spin label lines.



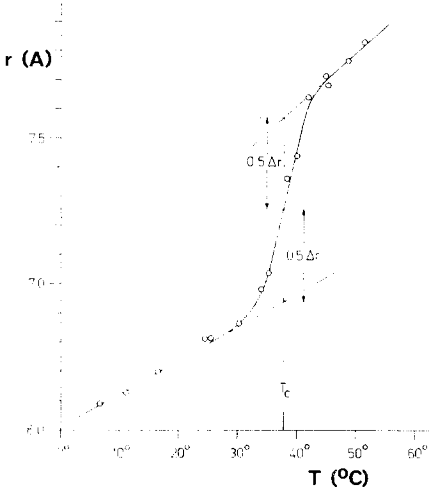


Fig. 2. Variation in spin label separation with temperature in multibilayers composed of dipalmitoyllecithin with varying proportions of cholesterol. (○—○), dipalmitoyllecithin; (◇—◇), dipalmitoyllecithin plus 10 mole % cholesterol; (△—△), dipalmitoyllecithin plus 20 mole % cholesterol; (□—□), dipalmitoyllecithin plus 30 mole percent cholesterol; (▽—▽), dipalmitoyllecithin plus 50 mole % cholesterol.

Fig. 3. Variation in spin label separation with temperature in multibilayers of dipalmitoyllecithin, indicating the method of measurement of the transition temperature, T_c, and the discontinuity in lateral separation, Δr, at the phase transition. The lateral expansivity above and below the transition is also apparent.

The temperature variation of the pair line splitting with the magnetic field perpendicular to the bilayer plane has been investigated in multibilayers of dipalmitoyllecithin with varying proportions of cholesterol from 0 to 50 mole %. The variation of the spin label molecular separation has been calculated using Eqn 1 with a g-value [7] of $g = 2.0058$, corresponding to the perpendicular orientation. The results are given in Fig. 2 for the temperature range 0–60 °C, which includes the gel to liquid crystal phase transition of dipalmitoyllecithin. There is clearly an abrupt change in lateral molecular spacing at the phase transition of dipalmitoyllecithin and a higher rate of lateral expansion of the bilayer above the phase transition than below it. This is seen more clearly in Fig. 3 which is for dipalmitoyllecithin alone, and also indicates the method of measuring the various parameters. Increasing proportions of cholesterol (Fig. 2) progressively diminish both the size and the sharpness of the change in molecular separation at the transition. The apparent transition temperature is also depressed by increasing amounts of cholesterol.

Oriented multibilayers composed of: egg lecithin plus 50 mole % cholesterol; lipids from the white matter of bovine brain; and lipids extracted from the membrane of human erythrocytes, have also been investigated. Fig. 4 gives the temperature variation of the lateral spin label spacing in these systems. All three systems contain similar proportions of cholesterol and are seen to have very similar pair separations, which also resemble quite closely the results for dipalmitoyllecithin plus 50 mole % cholesterol of Fig. 2.

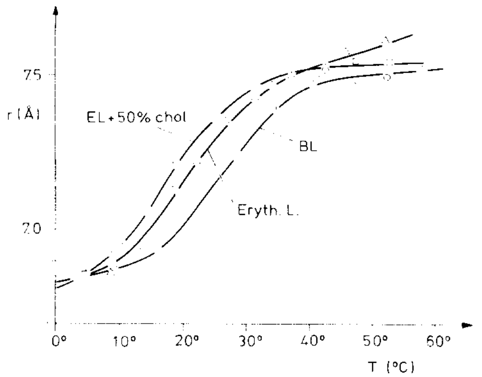


Fig. 4. Variation in spin label separation with temperature in multibilayers composed of: bovine brain white matter lipids (○—○); human erythrocyte lipids (△—△); egg lecithin plus 50 mole % cholesterol (□—□).

DISCUSSION

The results of Figs 2 and 3 clearly demonstrate a discontinuity in the lateral molecular spacing in dipalmitoyllecithin bilayers at the gel to liquid crystal phase transition. This abrupt lateral expansion on transition to the fluid state has been predicted previously [19, 20] by combination of measurements of bilayer volume [19, 21] and X-ray long spacing [8, 20]. The spin label measurement is a direct demonstration of the discontinuity in molecular spacing, and does not rely on the assumptions which must be made about orientation and degree of hydration of the polar headgroups and thickness of the aqueous layer between the bilayers, in interpreting the other dimensional measurements. The origin of the increase in lateral spacing is almost certainly the increased rotational isomerism in the lipid chains [19, 22] which occurs on transition to the fluid state. This conclusion is supported by the known location of the cholestane spin label probe in the bilayer [ref. 17 and Schreier-Muccillo, S., Marsh, D. and Smith, I. C. P., in preparation]. Other spin label measurements [17, 23, 24] have demonstrated that this rotational isomerism is the molecular basis of the fluidity of phospholipid bilayers and membranes.

Fig. 2 demonstrates the various effects which cholesterol has on the lateral molecular spacing in dipalmitoyllecithin bilayers. It is evident that cholesterol has opposite effects on the molecular spacing above and below the phase transition. This supports earlier interacting spin label results [7], obtained with egg lecithin and dipalmitoyllecithin bilayers above and below their phase transitions, respectively, which suggested that the fluidity regulating mechanism of cholesterol lies in the different effects which cholesterol has on bilayer packing in the gel and fluid phases. In the fluid phase, above T_c, cholesterol decreases the lateral molecular separation; the increased close packing decreases the bilayer fluidity since intermolecular steric effects decrease the possible rotational isomerisms in the lipid chains. In the gel phase, below T_c, cholesterol increases the lateral molecular spacing; the relaxation of close packing increases the bilayer fluidity because there is increased possibility of rotational isomerism in the lipid chains. These effects have been attributed to the rigidity and irregular shape of the cholesterol molecule [7].

The effects of cholesterol observed above and below the transition also have their effect in the phase transition region. Increasing proportions of cholesterol decrease the change in lateral spacing over the transition region, broaden the width of the transition region, and depress the temperature about which the transition region is centred. The decrease in lateral expansion at the transition can be directly related to the effects of cholesterol on the states of packing above and below the transition as discussed above. The broadening of the transition may be attributable, at least in part, to the effect of cholesterol below the transition in reducing the close packing of the lipid chains and hence lowering the cooperativity of the transition [11, 25, 26]. The depression in transition temperature by cholesterol can be correlated with the decrease in lateral expansion at the transition which will result in a decreased enthalpy of transition. These points are discussed in more detail below.

The spin label separations found in Fig. 4 for egg lecithin plus 50 mole% cholesterol, bovine brain white matter lipid and erythrocyte lipid, and in Fig. 2 for dipalmitoyllecithin plus 50 mole % cholesterol, are all rather similar. Since the bilayers have considerably different lipid contents but approximately similar cholesterol composition, it would appear that the lateral molecular separations are dominated by the high cholesterol content. Comparison with the results of Fig. 2 suggests that not only is cholesterol capable of regulating the fluidity of phospholipid bilayers and natural membranes, but it also stabilizes the membrane against lateral expansion (see also ref. 17). Regarding the comparison of the forms of the temperature variations in Figs 4 and 2, it is known that dipalmitoyllecithin alone has a phase transition at 41 °C [8]. Broad phase transitions have been detected in the phospholipid extract of human erythrocyte ghosts in the range 25–40 °C [10], and in decholesterolized bovine brain myelin lipids in the temperature range 25–50 °C [27]. Egg lecithin, on the other hand, has a broad calorimetric transition in the region −12–+5 °C [10]. However, a structural transition has been detected in egg lecithin–cholesterol bilayers at +12 °C [28]. This suggests that cholesterol in large amounts may have a considerable effect on the shape of the temperature variation. Any apparent transitions must not necessarily be regarded as an attenuated property of the lipid chains, but rather as a property of the lipid–cholesterol mixture.

Relation between pair spacing and lipid spacing

In order to make any quantitative comparisons it is necessary to know the relationship between the spin label probe separations and the lateral separations of the lipid molecules in the bilayer. It has previously been demonstrated [7] that the observed spin label separations correspond to adjacent probe molecules with no intervening lipid chains. Fig. 5 indicates schematically the way in which an adjacent pair of interacting cholestane spin labels can be incorporated into a triangular lattice of closely packed lipid chains [4]. The measured pair separation, r, can be approximated by the distance between the nitrogen atoms of the spin label nitroxide groups (see ref. 7). In Fig. 5 the lipid chain packing is indicated corresponding to a pair separation of $r = 6.8$ Å, which is that obtained in the range well below the transition region. Clearly some distortion of the lipid lattice must take place, though this will become less on approaching the transition. The main conclusions which can be drawn from Fig. 5 are that the spin labels occupy approximately substitutional positions for phospholipid molecules (as might be expected from their similar molecular areas) and

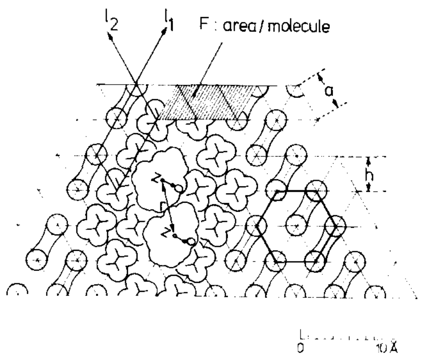


Fig. 5. Pair of cholestane spin labels, with surrounding lecithin chains, incorporated into the triangular lattice formed by closely packed hydrocarbon chains of the lipid molecules. A spin label occupies an approximately substitutional position for a phospholipid molecule. The spin label separation here is $r = 6.8$ Å.

that changes in their separation r, should reflect, in a very direct manner, the changes in the lipid lattice constant, a. Thus, although the absolute values of the spin label separation are a function of the close packing of the spin labels, the changes in r may be quantitatively related to the changes in lipid lattice constant, a. Thus the lateral expansion at the phase transition in dipalmitoyllecithin bilayers, deduced from Fig. 3 is: $\Delta r(T_c) = 0.63 \pm 0.03$ Å. This is to be compared with the change in lattice spacing: $\Delta a(T_c) = 0.5$ Å, deduced from the difference in X-ray short spacings, $h = 4.2$ and 4.6 Å found for phospholipids in their gel and liquid crystalline states, respectively [8]. The agreement is quite good when it is born in mind, that the X-ray short spacing in the fluid phase corresponds to the centre of a rather diffuse diffraction band. It seems that the spin label pair method may be used with some confidence to measure changes in lattice constant which are not readily detectable by X-ray methods.

Molecular areas

The value of $\Delta r(T_c)$ for dipalmitoyllecithin can be used to estimate the area of bilayer surface occupied per lipid molecule in the fluid phase above T_c. The lipid lattice constant below T_c is known from the X-ray short spacing [8] (corresponding to a sharp diffraction) to be $a = 4.84$ Å. Using $\Delta r(T_c) = 0.63$ Å gives a value of $a = 5.47$ Å for the lattice constant immediately above T_c. The area per lipid molecule in the plane of the lattice indicated in Fig. 5 is given by $F_0 = \sqrt{3}\, a^2$, which yields a value of 51.9 Å^2. If it is then assumed that the lipid chains have a net tilt of 30° relative to the bilayer normal, as is the case below T_c, then the projected area in the plane of the bilayer: $F = F_0/\cos 30$, is 60 Å^2.

This value can be compared with two other estimates of the molecular areas calculated from different dimensional data. Using the known composition at limiting hydration and the measured X-ray long spacing, Tardieu [29] has calculated the effective anhydrous thickness of dipalmitoyllecithin bilayers in the fluid (Lα) phase to be $d_L = 41$ Å. This calculation assumes a value for the partial specific volume of dipalmitoyllecithin of $\bar{v}_L = 1.018$ cm^3/g. The area per molecule in the fluid phase can then be calculated using the molecular weight of the lecithin, M_r:

$$F = 2M_r\bar{v}_L/d_L N \qquad (2)$$

where N is the Avogadro number [8, 30]. This yields a value of $F = 60.5$ Å^2. The second calculation utilizes the measured increase in the bilayer volume of 2 % at the phase transition of dipalmitoyllecithin [19, 21], combined with measurement of bilayer thickness. This is essentially the method used by Träuble and Haynes [19]. The anhydrous bilayer thickness in the gel ($L\beta^1$) phase has been determined for dipalmitoyllecithin to be $d_L = 47.4$ Å ($\bar{v}_L = 0.948$ cm^3/g) [9]. Using Eqn 2 this yields a value of 48.6 Å^2 for the molecular area below T_c [9]. If it is assumed that the polar groups contribute 8 Å to the anhydrous bilayer thickness [19], then the molecular volume of the lipid chains is 1,915 Å^3 below T_c, and $33 \times F$ above T_c where F is the molecular area above T_c. Assuming that the 2 % volume increase occurs solely in the hydrocarbon chains one then calculates $F = 59.2$ Å^2. This is in reasonable agreement with the result $F = 58$ Å^2 of Träuble and Haynes [19]*.

The agreement obtained between the spin label measurements and the two other methods is particularly satisfying in view of the extrapolation involved on the one hand, and the difficulties involved in determining the limiting degree of hydration. [9, 29] and the fraction of the bilayer occupied by the polar groups, on the other [19]. It seems clear that the discontinuity in pair separation measured in Fig. 3 corresponds to a lateral expansion of the bilayer in which the surface area increases from 48 Å^2/molecule to 60 Å^2/molecule on transition from the gel to the fluid state. These results also further justify the use of the interacting spin label pair method in measuring changes in lateral lipid separation.

Lateral expansivity

In Fig. 6 the discontinuity in pair spacing at the phase transition, $\Delta r(T_c)$, and the expansivities, dr/dT, in the various temperature domains are plotted as a function of cholesterol composition. The values are deduced from the data of Fig. 2 according

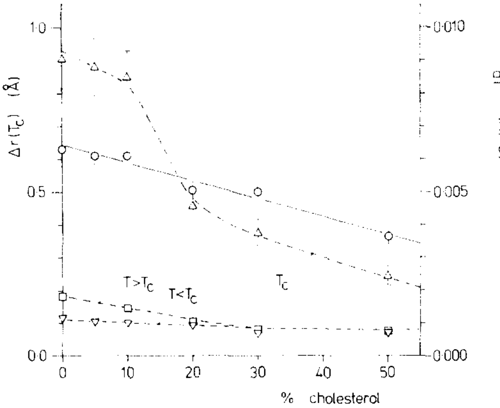


Fig. 6. Variation with cholesterol composition of the parameters governing lateral thermal expansion in dipalmitoyllecithin–cholesterol multibilayers. (○—○): $\Delta r(T_c)$, discontinuity in lateral spin label separation at the phase transition, T_c. (- - -): dr/dT, expansivity of the lateral spin label separation: at T_c (△ - - - △); above T_c (□ - - - □); below T_c (▽ - - - ▽).

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* The method employed here is to be preferred to that of Träuble and Haynes [19] since use of the anhydrous bilayer thickness removes some of the problems regarding the different degrees of hydration in the gel and liquid crystalline states (see refs 8, 9 and 29).

to the method indicated in Fig. 3. The confidence limits in Fig. 6, are an estimate of the accuracy of measurement. Reproducibility was found within these ranges. The values of $\Delta r(T_c)$ decrease with increasing percentage of cholesterol. As mentioned above, this is a result of the different effects which cholesterol has on bilayer packing above and below the transition.

The rate of change of spin label separation with temperature, or expansivity dr/dT, in the temperature region below T_c is relatively little affected by cholesterol composition. Examination of Fig. 5 suggests that this may well mean that the temperature dependence of the pair separation in the region well below T_c is dominated by the misfit of the spin label pair into the lipid lattice. Lateral expansion of the lipid lattice allows a better fit of the interacting spin labels into the lattice which could give rise to a rapid rate of increase in pair separation which is also relatively insensitive to cholesterol composition. If this were the case the spin labels would register a higher rate of lateral expansion below T_c than that of the lattice. The expansivity above T_c is sensitive to cholesterol composition and is considerably greater, for low cholesterol, than that below T_c. The looser packing in the fluid state means that this will be a more reliable measure of the lateral expansion of the lipid lattice than that below T_c. The decrease in expansivity with increasing cholesterol supports this view; the rigid cholesterol molecules inhibit the creation of further rotational isomers in the lipid chains [17]. If the arguments advanced above are correct: that the values of dr/dT are an overestimate for the expansivity of the lipid lattice below T_c, then there is a very large increase in the lateral expansivity on going to the fluid phase, by a factor of at least two, and probably considerably more. This enhanced expansivity corresponds to an increased lateral compressibility which could facilitate the binding of ligands, conformational changes in embedded proteins, etc.

The measured expansivity in the phase transition region, T_c, is seen to be much larger than in the regions either above or below T_c. The extremely high apparent expansivity of dipalmitoyllecithin alone corresponds to the abrupt lateral expansion at the phase transition. Addition of cholesterol, at concentrations greater than 10 mole% leads to a reduction in the expansivity, but the values are still much greater than those found either above or below the transition region. In dipalmitoyllecithin–cholesterol mixtures the transition region is in a state of lateral phase separation [31, 32] in which areas of fluid and gel phase lecithin coexist in equilibrium (the phase transition in dipalmitoyllecithin can be considered as a limiting condition of this state [3]). The high lateral expansivity can be easily understood in terms of lateral phase separation: increasing temperature results in the progressive conversion of gel phase to fluid phase with the attendant increase in molecular area corresponding to the abrupt increase observed at the dipalmitoyllecithin phase transition. Corresponding to the high thermal expansivity in the transition region there must clearly also exist a high isothermal compressibility [33]. As pointed out by Shimshick and Mc Connell [31], such a high lateral compressibility could facilitate the insertion of proteins into the membrane and enhance the activity of transport systems and other membrane bound enzymes, as well as enhancing the binding of ligands and substrates. It seems quite likely that the function of cholesterol in some biological membranes could be to maintain the membrane in a condition of lateral phase separation.

The observed increase in width of the transition region observed both in Fig. 2 and in calorimetry [10, 11] on the addition of cholesterol, can be understood in terms of a reduction in cooperativity of the transition by cholesterol. Phillips and Finer [25] have related the calorimetric transition widths to the size of the effective cooperative unit [26, 34] which undergoes an all-or-none transition. The cholesterol dependence of these results is similar to that found in Fig. 6 for the lateral expansivity in the transition region. (There is some evidence for a similar dependence of r, but unfortunately the data is not sufficiently accurate to resolve the non-linearities.) This suggests some connection between the degree of cooperativity and lateral molecular separation. Phillips and Finer [25] have interpreted the decreasing size of the effective cooperative unit in terms of the removal of lecithin molecules into regions of lecithin–cholesterol "complex" which do not undergo any phase transition. An alternative explanation is that suggested above: that the relaxation of close packing of the lipid molecules by cholesterol below the phase transition (Fig. 2 and ref. 7) reduces the range of the cooperative interaction between chains and hence the size of the effective cooperative unit.

The expansivities of Fig. 6 may be used to calculate the linear coefficients of expansion for the lipid lattice constant, if it is assumed that changes in r correspond to changes in a (see above):

$$\alpha_L = \frac{1}{a}\frac{dr}{dT} \tag{3}$$

This yields a value of $\alpha_L(T>T_c) = 3.4 \cdot 10^{-3}\ ^\circ C^{-1}$ for the lateral expansion of dipalmitoyllecithin bilayers above the phase transition. This can be contrasted with the measured transverse expansion coefficient $\alpha_L(T>T_c) = -3 \cdot 10^{-3}\ ^\circ C^{-1}$ for human brain lipids in the fluid phase, determined by X-ray methods [35]. The effective lateral coefficient below T_c is $\alpha_L(T<T_c) = 2.4 \cdot 10^{-3}\ ^\circ C^{-1}$, but as mentioned above the pair measurement probably overestimates the expansion of the lipid lattice in this temperature range. This value is in fair agreement with one deduced from spin label exchange interaction [4] in a dipalmitoyllecithin–spin labelled androstanol system of $\alpha_L(T<T_c) = -\frac{1}{2}\hat{W}_{ex} \times d\hat{W}_{ex}/dT = 3.6 \cdot 10^{-3}\ ^\circ C^{-1}$. (Above T_c the spin label exchange interaction becomes diffusion controlled [4].) These two values may be compared with the value of $\alpha_L(T<T_c) = 3.7 \cdot 10^{-4}\ ^\circ C^{-1}$ for dipalmitoyllecithin deduced from the X-ray short spacing measurement of Melchior and Morowitz [21], confirming that the interacting spin label measurements are an overestimate for the pure lipid below T_c. Comparison with the spin label results above T_c, shows that there is a very considerable increase in expansivity above T_c.

Transition temperatures and enthalpies

The transition temperatures, deduced from the pair data as indicated in Fig. 3, are plotted as a function of cholesterol composition in Fig. 7. The results are compared with the calorimetric transition temperatures determined by Ladbrooke et al. [10] which correspond to the onset of the lateral phase separation [37]. Similar calorimetric results have been obtained by Hinz and Sturtevant [11] which were interpreted as an increase in transition width. There is a clear correspondence between the calorimetric and spin label pair transition temperatures. The latter are displaced to lower temperatures by the high spin label concentration (approx. 10 mole%); this depression becomes relatively less important at high cholesterol. The correlation between the two sets of transition temperatures implies that the spin label pair separation is sensitive to the point at which lateral phase separation is initiated. This is not surprising, since the misfit of the spin label pair into the lipid lattice (Fig. 5) in the gel phase, favours partitioning of the spin label pair into the fluid phase. A similar effect has been observed for the phase partitioning of cholesterol by de Kruyff et al. [36].

The increase in lateral lipid separation may be used to estimate the decrease in intermolecular interaction energy at the transition. End effects are difficult to calculate, but a calculation of the chain length dependence of the intermolecular interaction can be attempted using the methods with which Salem [1] successfully estimated the heat of sublimation at 0 °K of crystalline hydrocarbons. In this method the interchain dispersion energy is given by:

$$W_{\mathrm{disp}} = -\frac{1.24 \cdot 10^3}{D^5} \ (\mathrm{kcal/mole\ per\ CH_2}) \qquad (4)$$

where D is the separation of the two chains (in Å). The interchain repulsive potential is approximated by the empirical expression based on experimental molecular potentials:

$$W_{\mathrm{H-H}} = +\frac{33.2}{d^{6.18}} \ (\mathrm{kcal/mole\ per\ CH_2}) \qquad (5)$$

where d is the distance (Å) between the centres of the two interacting hydrogen atoms. The net change in intermolecular interaction energy, at the transition, per CH_2 group per chain pair is then given by:

$$\Delta H_{\mathrm{int}} = \Delta W_{\mathrm{disp}} + \Delta W_{\mathrm{H-H}} \qquad (6)$$

where Δ signifies the change in each quantity at T_c.

The calculation of ΔH_{int} for the various cholesterol compositions has been made using the spin label pair data, taking the lipid lattice constant for 0 % cholesterol below T_c as 4.84 Å, and the values of $\Delta r(T_c)$ from Fig. 6, and the differences in r with cholesterol below T_c from Fig. 2 to calculate all other lattice constants. The lipid chains have been assumed to be centred on a triangular lattice as indicated in Fig. 5. The summation of W_{disp} has been taken to the third shell of neighbouring chains and $W_{\mathrm{H-H}}$ calculated for the hydrogen atoms in immediate contact. It has been assumed that $W_{\mathrm{H-H}} = 0$ above T_c, since the repulsive interaction is very short range, and intermolecular separations above T_c are outside the range of applicability of Eqn 5.

The calculated values of ΔH_{int} are given in Fig. 7, and are compared with the calorimetrically determined values for the chain length dependence of the total enthalpy change, ΔH, at the transition. The chain length dependence of the calorimetric enthalpy has been calculated by a combination of the data for dipalmitoyllecithin of Ladbrooke et al. [10] and for dimyristoyllecithin of Hinz and Sturtevant [11]*.

The trends in the calculated intermolecular contribution, ΔH_{int}, correlate reasonably well with the measured total enthalpy change, ΔH. The total enthalpy change is given by:

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* The data of Hinz and Sturtevant [11] for dipalmitoyllecithin–cholesterol differs somewhat from that of Ladbrooke et al. [10]; the latter values have been used here since the former values for dipalmitoyllecithin alone have been subsequently revised [26]. However, it seems from the trends of the Hinz and Sturtevant data, that ΔH may decrease somewhat more rapidly with cholesterol composition than indicated in Fig. 7.

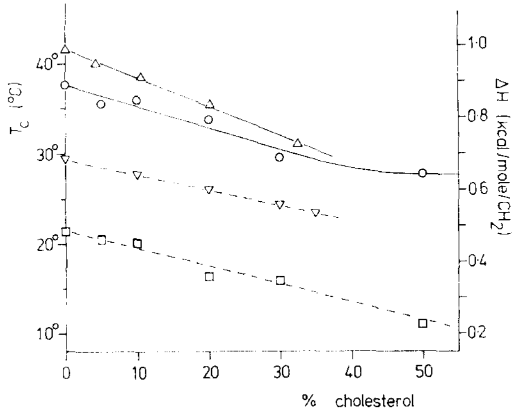

Fig. 7. Variation with cholesterol composition of thermodynamic parameters involved in the phase transition of dipalmitoyllecithin–cholesterol multibilayers. (—): transition temperature, T_c; (△ – △) calorimetric data (from ref. 10); (○ – ○) spin label pair measurement. (- - -): enthalpy change, ΔH, per CH_2 group, per chain, at the transition; (▽ – – ▽) calorimetric measurement, derived from the data for C16 and C14 lecithins (from refs 10 and 11, respectively); (□ – – □) calculated contribution from intermolecular interactions, ΔH_{int} derived from the spin label pair data, $\Delta r(T_c)$, of Fig. 6.

$$\Delta H = \Delta H_{\text{int}} + \Delta U + p \cdot \Delta V \tag{7}$$

where ΔU is the change in internal energy of the lipid chains arising from the increased rotational isomerism in the fluid state. The term $p \cdot \Delta V$ is negligible for $p = 1$ atm and the measured changes in bilayer volume [19, 21] at the transition. In a previous paper a statistical mechanical calculation has been used to estimate ΔU for dipalmitoyllecithin alone [22]; this yields a value of 0.174 kcal/mole per CH_2 per chain which is in reasonable agreement with the value of 0.188 kcal/mole deduced from Fig. 7. The variation of ΔU with increasing cholesterol composition is difficult to estimate, since it requires a specific model of the way in which the rigid cholesterol molecules restrict the lipid chain conformations [17]. It is clear, however, that ΔU will decrease with increasing cholesterol which would be in line with the decrease in ΔH being steeper than indicated in Fig. 7. It thus seems that a consistent explanation of the way in which ΔH decreases with increasing cholesterol can be obtained in terms of the decreased intermolecular contribution arising from inhibited lateral expansion and decreased intramolecular contribution arising from immobilization of the chains. Both effects arise from the influence of cholesterol on the rotational isomerism of the lipid chains.

The decrease in T_c, the temperature for the onset of lateral phase separation, with increasing cholesterol composition can be understood qualitatively in terms of the decrease in ΔH with increasing cholesterol composition. For a first order transition the change in free energy is zero, thus:

$$\Delta H = T_c \cdot \Delta S \tag{8}$$

where ΔS is the change in entropy at the transition. Thus changes in ΔH will be reflected by changes in T_c; a decrease in ΔH will be accompanied by a decrease in T_c if the accompanying changes in ΔS do not fully compensate it. The transitions in dipalmitoyllecithin–cholesterol bilayers are not first order, but it seems that similar features are governing the onset of lateral phase separation. The loss of cohesive energy on transition to the higher entropy state is not so great because of the reduced lateral expansion and thus the lateral phase separation is initiated at a lower temperature. The entropy gain is smaller, because the chain motion in the fluid phase is inhibited by cholesterol, but this effect is presumably dominated by the decreased change in interchain and intrachain energy. Shimshick and McConnell [32] have determined phase diagrams for lecithin–cholesterol mixtures by determining the solubility of the TEMPO spin label. This method does not detect the same onset of lateral phase separation [37] as detected in the calorimetric measurements [10, 11]; the discontinuity in TEMPO solubility apparently occurs at the temperature at which maximum melting rate is observed in calorimetry [11].

Finally, it has been suggested that "complex" formation takes place between lecithin and cholesterol [38]. The present results do not give any direct evidence for complex formation, nor did the phase diagram studies of Shimshick and McConnell [32]. The interacting spin label results would be consistent with complex formation if it is assumed that the complex is at least partially solvated in the remaining lipid and that this gives rise to the observed effects on molecular packing.

CONCLUSIONS

The findings of this paper may be summarised as follows:

(1) A discontinuity in spin label separation of $\Delta r = 0.63$ Å is found at the phase transition in dipalmitoyllecithin bilayers. This corresponds to an abrupt lateral expansion from 48 Å^2 to 60 Å^2 in the bilayer surface area per molecule, in agreement with X-ray and dilatometric measurements.

(2) A very high expansivity, dr/dT, is found in the region of lateral phase separation of dipalmitoyllecithin–cholesterol bilayers, which corresponds to the high lateral compressibility in this state.

(3) The lateral expansion coefficient estimated in the fluid phase ($\alpha_L = 3.4 \cdot 10^{-3}\ {}^\circ C^{-1}$) could be up to an order of magnitude greater than that in the gel phase ($\alpha_L = 3.7 \cdot 10^{-4}\ {}^\circ C^{-1}$, deduced from X-ray measurements), though still much less than that observed in the regions of lateral phase separation.

(4) Cholesterol is found to relax the lipid close-packing in bilayers below the phase transition, and to tighten the packing above the phase transition. Correspondingly the increase in lateral molecular separation over the transition region is reduced by cholesterol. These changes in packing form a molecular basis for the fluidity-regulating mechanism of cholesterol, which is also consistent with the calorimetric data.

(5) Lateral expansion in lipid bilayers with high cholesterol content (e.g. erythrocyte and brain myelin) appears to be dominated by the attenuating effect of cholesterol.

It appears that cholesterol can have either a structural or a functional role in different biological membranes. In membranes with high cholesterol content, the cholesterol will stabilize the membrane against changes in fluidity and the disruptive effect of high lateral expansion. In membranes with lower cholesterol content, the cholesterol could serve to maintain the membrane in the functionally significant state of lateral phase separation.

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