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AN INTERACTING SPIN LABEL STUDY OF THE FLUIDIZING AND CONDENSING EFFECTS OF CHOLESTEROL ON LECITHIN BILAYERS*

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SUMMARY

The molecular origin of the fluidizing and condensing effects of cholesterol has been investigated using interacting spin label pairs in multibilayer films of various lecithins. The spin label pair method is a probe of the lateral separation of molecules within the bilayer. The cholestane spin probe separation is found to increase with increasing cholesterol composition in dipalmitoyllecithin bilayers and to decrease with increasing cholesterol composition in both egg and dioleoyllecithin bilayers. These changes in close-packing of the molecules within the lecithin bilayers correspond respectively to the fluidizing and condensing (and rigidifying) effects of cholesterol. The measured decrease in lateral separation corresponding to fluidization of the dipalmitoyllecithin–cholesterol bilayers correlates reasonably well with the latent heat and change in volume at the liquid crystal transition of pure dipalmitoyllecithin. The size of the decrease in lateral separation in egg lecithin bilayers indicates that the condensing effect of cholesterol arises from both molecular interaction with the lecithin chains and the existence of molecular cavities within the lecithin chain region of the bilayer.

INTRODUCTION

Cholesterol is a major component of natural membranes such as erythrocyte membranes and the myelin sheath. The different effects of cholesterol on hydrated phospholipids above and below their liquid crystal transition temperature have led to the suggestion that cholesterol has a structural role in stabilizing the membrane fluidity to changes in temperature and fatty acid composition[1]. On a macroscopic scale, cholesterol is found to have a condensing effect[2,3] on lecithins in their liquid crystalline phase and to remove the sharp gel–liquid crystal transition of lecithins normally in the gel phase[1]. At a molecular level, spin probe studies have shown that cholesterol has an orienting and rigidifying effect[4–8] on lecithins in the liquid

Abbreviation: CSL, cholestane spin label (3-*spiro*-(2′-(*N*-oxyl-4,4′-dimethyloxazolidine))-cholestane).

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crystalline phase and a fluidizing effect[5,6] on lecithins in the gel phase. In the present study, the method of interacting spin label pairs (Marsh, D. and Smith, I. C. P., ref. 26) is used to determine the molecular origin of these condensing, rigidifying and fluidizing effects. The interacting pairs of spin label probes monitor the lateral molecular separation within the bilayer. The difference in origin of the effect of cholesterol on lecithins above and below their liquid crystal transition is found to arise from the different effect of cholesterol on the packing of the bilayers, as reflected by the cholestane spin probe separation.

EXPERIMENTAL

Oriented phospholipid multibilayers were prepared in a flat, quartz EPR cell from a chloroform solution of lipids *plus* spin label. The system has been described in detail elsewhere[9,10]. The multibilayer films were hydrated for at least 30 min (and up to 24 h in the case of dipalmitoyllecithin) with 0.15 M NaCl and drained immediately prior to EPR examination. Preparations were formed with various concentrations of spin label. The concentration which gave an approximately 8 mole percent doping of spin label in the final multibilayers was found to give best resolution of the interacting spin label spectra. The EPR spectra were examined at room temperature on a Varian E-9 spectrometer, with the magnetic field parallel and perpendicular to the plane of the multibilayer film.

Egg lecithin was obtained from Lipid Products, Epsom, U.K. Dipalmitoyl-L-α-lecithin and dioleoyl-L-α-lecithin were obtained from Supelco, Inc., Bellefonte, Pa. Cholesterol from Steraloids, Pawling, N.Y. was recrystallized from methanol. The cholestane spin label (CSL) 3-*spiro*-(2′-(*N*-oxyl-4′4′-dimethyloxazolidine))-cholestane, was prepared according to Keana *et al.*[11].

RESULTS

A typical spectrum of 8 mole percent cholestane spin label in oriented multibilayers of dipalmitoyllecithin+20% cholesterol is given in Fig. 1. The spectra

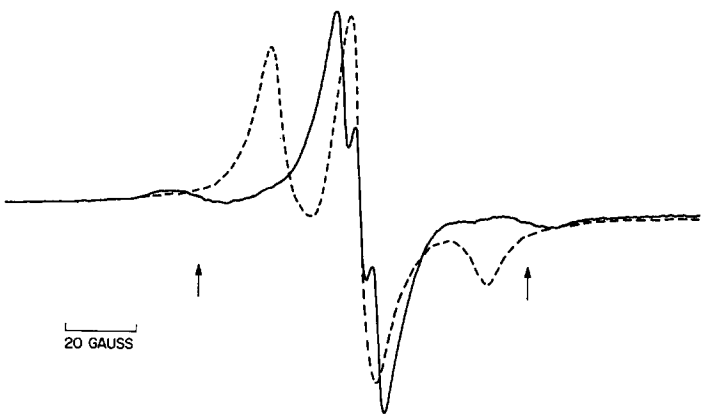

Fig. 1. EPR spectrum of 8 mole percent CSL in hydrated multibilayers of dipalmitoyllecithin +20% cholesterol. Solid lines, magnetic field perpendicular to the bilayer plane; dotted lines, magnetic field parallel to the bilayer plane. The arrows indicate the lines arising from interacting spin label pairs.

consist of strong central lines arising from single, non-interacting spin labels and, in the perpendicular direction, well-resolved lines arising from pairs of closely-spaced interacting spin labels (Marsh, D. and Smith, I. C. P., ref. 26). The configuration of an interacting spin label pair (*P*) as opposed to a single, isolated spin label (*I*) is indicated diagrammatically in Fig. 2. Confirmatory evidence of the identity of the pair lines is afforded by the concentration dependence of their relative intensity. Table I shows that, as the spin label concentration is increased, the intensity of the pair lines increases relative to that of the isolated spin label lines.

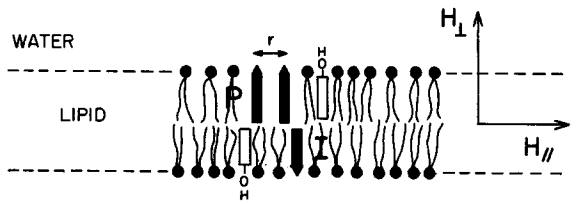

Fig. 2. Schematic illustration of the configuration of an interacting spin label pair, *P*, and isolated pin label, *I*, in a phospholipid bilayer. The two orientations of the applied magnetic field, parallel and perpendicular ($H//$, $H_\perp$) to the plane of the bilayer are indicated.

The hyperfine structure of the isolated spin label reports the orientation or amplitude of anisotropic motion of its molecular environment[10,12], whereas the pair lines are split by their magnetic dipole–dipole interaction which is a sensitive probe of their lateral molecular separation (Marsh, D. and Smith, I. C. P., ref. 26). The anisotropy of the hyperfine structure of the isolated label shows that the multibilayers are well-ordered[4,10]. The magnetic dipole–dipole splitting of the pair lines is inversely proportional to the cube of the lateral separation of the spin labels, *r*, (see Fig. 2). In Appendix I (Eqn 8), it is shown that the pair line splitting in the perpendicular direction is given by the relation:

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

TABLE I

VARIATION IN RELATIVE LINEHEIGHT

Variation in relative lineheight (defined as ratio of height of low field pair line in Fig. 1 to height of central isolated label line) with spin label concentration in preparative solution. Cholestane spin label in dipalmitoyllecithin + 20% cholesterol, hydrated multibilayers.

CSL concn·10³ (*M*)	h_r
3	0.075
1	0.027
0.333	0.003

In principle the pair spectra in the parallel direction should, in addition, give information on the geometry of the lateral arrangement of molecules within the bilayer. In practice, the lines are distributed over too large a region to have observable intensities (see Appendix I and Marsh, D. and Smith, I. C. P., ref. 26).

Multibilayers of dipalmitoyllecithin, egg lecithin and dioleoyllecithin were studied with varying proportions of cholesterol from 0 to 50 mole percent. Typical spectra from hydrated multibilayers of dipalmitoyllecithin, egg lecithin and dioleoyl-

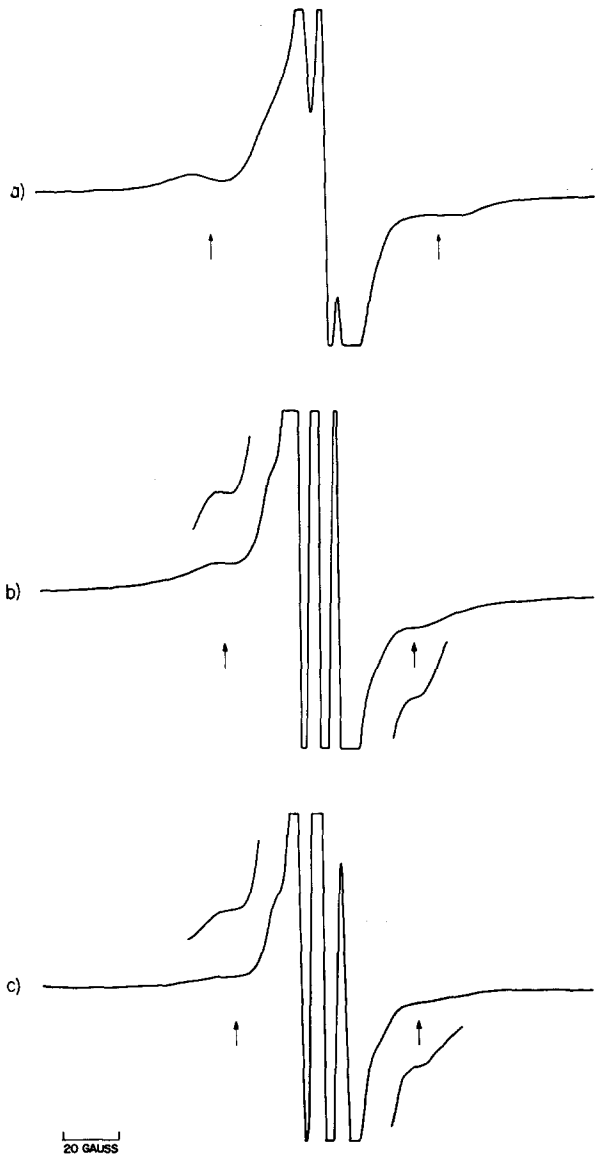

Fig. 3. EPR spectra of interacting spin label pairs in hydrated multibilayers of different lecithins. (a) Dipalmitoyllecithin + 30% cholesterol, (b) egg lecithin + 30% cholesterol, (c) dioleoyllecithin + 30% cholesterol.

lecithin with 30 mole percent cholesterol are given in Fig. 3. The spectra in the parallel direction, although not shown, were routinely examined to ensure that the films were well-ordered. Clearly the pair lines of egg lecithin and dioleoyllecithin are much more closely spaced than in dipalmitoyllecithin, corresponding to more widely spaced spin labels. This also means that the lines are not as well-resolved in egg lecithin and dioleoyllecithin, indeed they are not observed at all for low cholesterol composition because the spin probe separation is then so large that the pair lines lie under the more intense central lines. The resolution improves as cholesterol composition increases because the spin probe separation decreases with increasing cholesterol in egg lecithin and dioleoyllecithin bilayers. The EPR spectra of spin probe pairs in dipalmitoyllecithin bilayers with various other cholesterol concentrations are given in Fig. 4. The pair EPR line separation is seen to decrease with increasing cholesterol composition corresponding to an increase in molecular spacing within the bilayer.

The variation in spin label separation (r) with cholesterol composition is given in Fig. 5. This was calculated using Eqn 1 and a g-value[4] of 2.0058 in the perpendicular direction. The confidence limits are an estimate of the resolution of

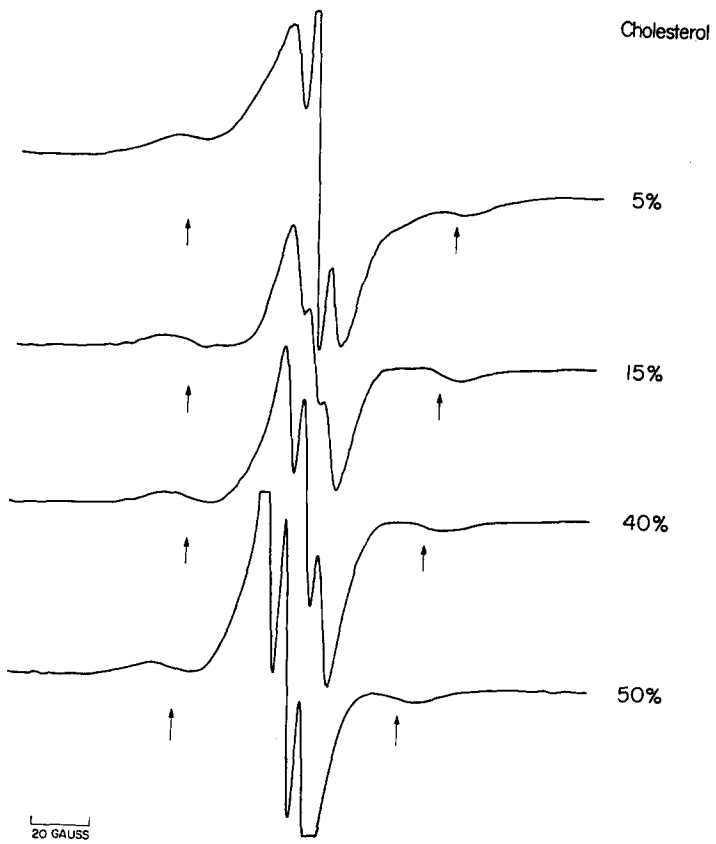

Fig. 4. EPR spectra of interacting spin label pairs in hydrated dipalmitoyllecithin multibilayers of varying cholesterol composition.

the lines. Reproducibility was found within this range. A very clear distinction is apparent between dipalmitoyllecithin which is below the liquid crystalline transition at room temperature and egg lecithin or dioleoyllecithin which are both in the liquid crystalline state. A limited amount of data is included for dry dipalmitoyllecithin which suggests that even in this quasi-solid state cholesterol has a similar effect on molecular separation.

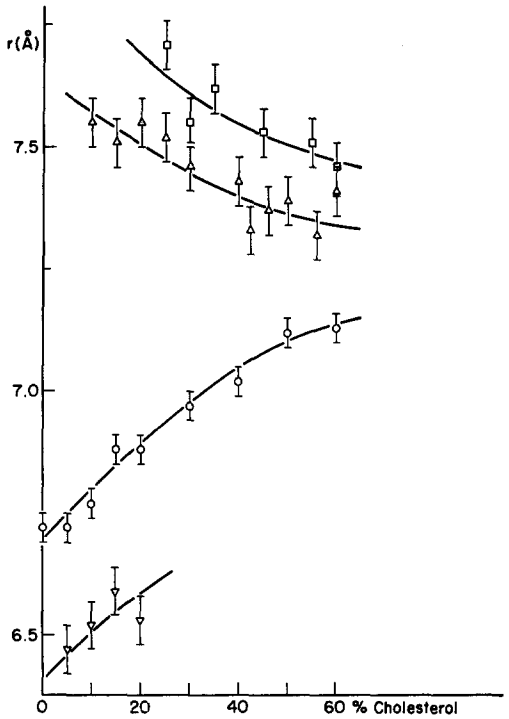

Fig. 5. Variation in spin label separation with the cholesterol composition of multibilayers prepared from different lecithins. ▽, dry dipalmitoyllecithin; ○, hydrated dipalmitoyllecithin; △, hydrated egg lecithin; □, hydrated dioleoyllecithin.

DISCUSSION

The sensitivity of the spin label spacing, *r*, to lateral packing within the bilayer has already been demonstrated for various phospholipid systems (Marsh, D. and Smith, I. C. P., ref. 26). The cholestane spin label spacing reflects packing within the hydrocarbon chain region of the bilayer, because the isotropic hyperfine splitting constant of the non-interacting spin probes indicates that they are anchored close to the polar–hydrocarbon interface with the body of the probe in the hydrocarbon phase. Fig. 5 shows that the difference between dipalmitoyllecithin (the lecithin below its liquid crystal transition point) and egg lecithin or dioleoyllecithin (the lecithins in a liquid crystalline phase) is one of molecular packing in the hydrocarbon region of the bilayer. The origin of the different effects which cholesterol has on lecithins in the two different phases can also be seen to arise from the entirely opposite ways in which cholesterol changes the packing in the hydrocarbon chain region of the hydrated bilayer.

The separations in dipalmitoyllecithin and egg lecithin or dioleoyllecithin approach one another more closely as the bilayer properties are increasingly modified by the cholesterol component. However, the separations are still significantly different at 50% cholesterol composition reflecting the degree of unsaturation of the lecithin chains. Results previously found for other phospholipids (sphingomyelin, phosphatidylethanolamine and beef brain lipid (Marsh, D. and Smith, I. C. P., ref. 26) with 50% cholesterol lie within those for dipalmitoyllecithin and egg lecithin.

The tight packing observed in dipalmitoyllecithin is allowed by the completely saturated hydrocarbon chains and gives rise to large dispersion forces between the chains and correspondingly high liquid crystal transition point. The looser packing found in egg lecithin must arise from the steric effects of the unsaturated fatty acid chains. Dioleoyllecithin which has a double bond in each fatty acid chain gives rise to slightly larger separations than in egg lecithin. The intermolecular steric effect of the double bond reduces hydrocarbon chain contacts and results in a more fluid, liquid crystalline phase.

The addition of cholesterol to dipalmitoyllecithin is seen to relax the close-packing in the bilayer, presumably by forcing apart the lecithin hydrocarbon chains to accommodate the irregularly shaped cholesterol molecules. The looser packing of the lecithin chains reduces the dispersion forces between them, and the irregular packing allows greater steric freedom. This gives rise to the fluidization observed by other spin label probe techniques[5,6].

Cholesterol is seen to cause tighter packing in egg lecithin and dioleoyllecithin bilayers. This corresponds to the cholesterol condensing effect[2,3]: the decrease in area of cholesterol-unsaturated lecithin monolayers below that deduced from additivity of the molecular areas found in monolayers of the pure components. There are two extreme models for the molecular mechanism of the condensation effect[13]: the interaction model and the cavity model[14]. The interaction model states that the effective molecular area of cholesterol is the same as in a monolayer of pure cholesterol and the effective area of the lecithin molecule is reduced by the interaction of cholesterol with its hydrocarbon chains. The cavity model states that the effective area of the lecithin molecule is the same as in a monolayer of pure lecithin and the effective area of the cholesterol molecule is reduced by its incorporation into molecular cavities, which exist within the loosely packed hydrocarbon chain region of the lecithin. In its simplest form the cavity model would imply that molecular separation would remain unchanged with increasing cholesterol composition. This is clearly not the case for egg lecithin and dioleoyllecithin, and shows that some interaction does take place between cholesterol and the lecithin molecules, as indicated by other spin probe measurements[4–8]. However, it should be noted that the experimental scatter does not preclude a constant separation in egg lecithin up to 25% cholesterol indicating some cavity-like condensation. A more detailed quantitative analysis below yields a hybrid model of "cavities with interaction".

In order to make more detailed quantitative use of the results of Fig. 5, it is first necessary to investigate the nature of the interacting spin label complex which gives rise to the observed pair spectra. Fig. 6 gives scale projections of a space-filling model of the cholestane spin label. The Z–X projection, along the long molecular axis, gives the transverse dimensions which are relevant to lateral separations within the bilayer. The unpaired electron which gives rise to the EPR spectrum is

localized in a $2p\pi$ orbital on the nitrogen atom. The closest and most extreme molecular contact radii are seen to be 3.2 Å and 5.0 Å, respectively, from the centre of the nitrogen atom. The extended nature of the $2p\pi$ orbital (Fig. 6) introduces an uncertainty of ≤ 1 Å into the separation, r. However, this is a systematic error and so does not affect the changes in separation. One significant consequence of this elongated orbital is that it effectively extends the radius of closest contact by bringing the unpaired electron closer to certain parts of the molecular surface.

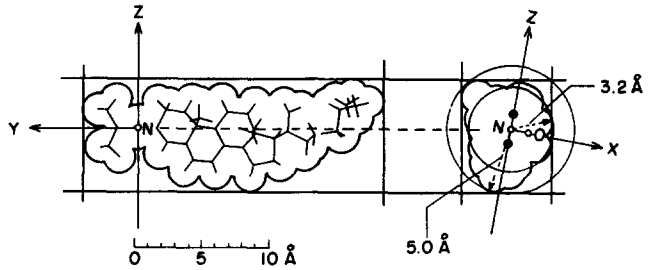

Fig. 6. Projections of space-filling models of the cholestane spin label, showing the nitroxide principal axes and lateral distances of closest approach. The two full dots, ●, indicate the regions of maximum free electron density in the nitrogen $2p$ orbital.

The most important conclusion to be deduced from Fig. 6 is that the observed separations all correspond to spin label pairs situated in adjacent molecular sites. No spectra are seen from interacting spin labels with an intervening phospholipid molecule since the minimum separation in this case would be about 11 Å. Such spin probe pairs undoubtedly do exist but their spectra cannot be resolved by this method since they lie under the strong central lines. The minimum possible spin probe separation is clearly 6.4 Å, corresponding to close contact. This separation agrees with the extrapolated value for dry dipalmitoyllecithin without cholesterol, as might be expected for a quasi-solid structure. The existence of adjacent cholestane spin probe pairs demonstrates that pair-wise clustering of cholesterol should be possible without disrupting bilayer structure. However, the relative intensity of occurrence of spin label pairs versus single spin labels (as indicated by the line heights of Table I) is less than would be expected from a completely statistical distribution, indicating that spin label pairing is somewhat disfavoured energetically. Clustering of cholesterol has been observed in erythrocyte membrane[15] where membrane protein is, of course, also present.

Three possible types of spin label configuration which would give rise to the observed range of separations are given in Fig. 7. These are: face-to-face, side-to-face and side-to-side. A further possibility is that at the larger separations the spin probes are rotating rapidly about their long axes, as is found for the isolated probe[4,5,7–10]. Phospholipid chains are associated with the spin label pairs in Fig. 7, diagrammatically indicating the way in which pair separation reflects the lateral phospholipid organization. The relationship between the changes in spin label separation and the changes in phospholipid separation clearly depends on the geometry of the lateral molecular arrangement within the bilayer. Monolayer experiments[14] indicate that the

molecular areas of dipalmitoyllecithin and cholesterol are approximately equal, suggesting a 1:1 substitution of cholesterol for lecithin in the mixed bilayer. Accordingly, it is assumed in the following discussion that the change in phospholipid separation is that recorded by the cholestane spin probe pair. This assumption will be less valid in the case of egg lecithin or dioleoyllecithin bilayers whose molecules are in a more expanded form than those of dipalmitoyllecithin.

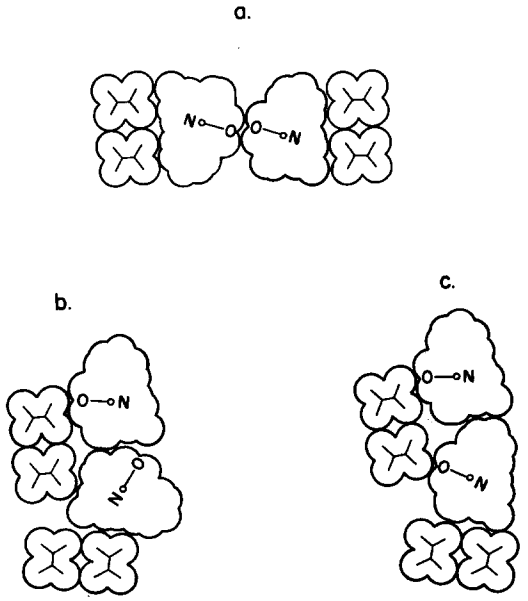

Fig. 7. Possible lateral configurations of the spin label pairs. The associated phospholipid matrix is indicated schematically by the disposition of phospholipid hydrocarbon chains.

One of the clearest effects of the change in molecular separation with increasing cholesterol is the decrease in dispersion energy between the hydrocarbon chains of dipalmitoyllecithin. This results in a transition from the gel to the liquid crystalline state. The variation in dispersion energy with hydrocarbon chain separation has been shown to be[16]:

$$W_{\text{disp}} = \frac{1250}{D^5} \text{ kcal/mole, per } CH_2 \text{ group} \tag{2}$$

The change in spin label separation of hydrated dipalmitoyllecithin is 0.4 Å on going from 0 to 50% cholesterol. Assuming the lipid chain separation to be 4.2 Å at 0% cholesterol[1], then the decrease in dispersion energy on going from 0 to 50% cholesterol, allowing for pair-wise interaction of lipid chains, is 12.4 kcal/mole. In view of the uncertainties in calculating dispersion energies, this agrees reasonably well with the measured latent heat of 8.8 kcal/mole at the liquid crystal transition of pure dipalmitoyllecithin[1]. This comparison ignores the thermal energy required to heat dipalmitoyllecithin to its transition point (41 °C) and also the fact that dipalmitoyllecithin with 50% cholesterol is only in an intermediate state of fluidity.

The calculation also assumes the dispersion interaction between lipid chains to be approximately equal to that between lipid and cholesterol.

The liquid crystal phase transition in pure aqueous dipalmitoyllecithin is accompanied by changes in the bilayer dimensions. The bilayer thickness decreases by 5 Å (ref. 17) and its volume increases by 1.4%[19]. It is thus possible to calculate that the mean area per dipalmitoyllecithin molecule increases from 48 Å^2 (ref. 17) to 58 Å^2 (ref. 18). Assigning a square area to each dipalmitoyllecithin chain (which corresponds to square packing if one assumes a circular cross-section for the chains), the change in area per molecule at the phase transition corresponds to an increase of 0.48 Å in the length of the side of the square. This implies an increase in separation of 0.48 Å between lipid chain centres, correlating quite well with the 0.4 Å increase in spin probe separation on going from 0 to 50% cholesterol composition of mixed dipalmitoyllecithin–cholesterol bilayers.

The mean area per molecule of pure, aqueous egg lecithin bilayers is 72 Å^2 (ref. 19), which corresponds to a monolayer surface pressure of 22 dynes/cm (refs 2, 19). Assuming a square area for each egg lecithin chain, as was done above for dipalmitoyllecithin, this corresponds to a square area per chain of side 6.0 Å. The observed decrease in spin label separation on going from pure egg lecithin to egg lecithin with 50% cholesterol is 0.3 Å. If we assume that this corresponds to a decrease in area per egg lecithin molecule caused by interaction with cholesterol, the resultant decrease in the length of the side of the lipid chain cross-section is 0.3 Å. The square cross-sectional area of the lipid chains thus decreases to 32.5 Å^2 to accommodate the cholesterol molecules, the distance between chain centres remaining unchanged. The predicted area per egg lecithin molecule at 50% cholesterol would then be 65 Å^2. The area per molecule in a condensed, pure cholesterol monolayer is 40 Å^2 (ref. 2). Thus the mean area per molecule for the mixed monolayer predicted on an interaction model would be $1/2\,(65+40)=52.5$ Å^2. This is to be compared with the observed value of 49 Å^2 for an egg lecithin + 50% cholesterol monolayer[14]. The mean area per molecule in the mixed monolayer, assuming additivity would be $1/2\,(72+40)=56$ Å^2. The predicted condensation of 3.5 Å^2 is thus insufficient to explain the whole of the observed condensation of 7 Å^2. These calculations would suggest that there is an approximately 50% contribution to the condensation from a molecular cavity effect. It was mentioned above that the change in spin probe separation might underestimate the effects on the phospholipids in egg lecithin bilayers. A repetition of the above calculations assuming a 50% greater effect on the phospholipids than recorded by the spin probes yields a predicted interaction condensation of 5.2 Å^2 which is still significantly smaller than that measured. Clearly the cavity effect is important.

In conclusion, the interacting spin label method has shown that the molecular origin of the fluidity-regulating mechanism of cholesterol in lecithin bilayers is one of lateral packing. Semi-quantitative agreement with the macroscopic effects of condensation and fluidization is obtained from the interacting spin labels, using simple models. Extrapolation of these results to real biological membranes is possible within the framework of the fluid-mosaic model[20]. In this case the molecular packing may also be modified by interaction with membrane protein which could produce further regulation of the membrane fluidity.

It is interesting to contrast the above results of the spin label pair method with other work recently published on interacting spin labels in which the interacting spin

label pairs were not specifically resolved[21–24]. The latter investigations yielded important information regarding the lateral diffusion[21,24] and clustering[24] of the spin-labelled lipid molecules, whereas the pair method has given precise information on the lateral spatial separation of individual spin-labelled molecules. Clearly the different methods are complementary in their application.

APPENDIX I

The magnetic dipole–dipole interaction is represented by the Hamiltonian:

$$\hat{\mathscr{H}}_D = \frac{g^2\beta^2}{r^3}\left[\underline{\hat{S}}_1 \cdot \underline{\hat{S}}_2 - 3\frac{(\underline{\hat{S}}_1 \cdot \underline{r})\,(\underline{\hat{S}}_2 \cdot \underline{r})}{r^2}\right] \tag{3}$$

where $g\beta\underline{\hat{S}}_1$, $g\beta\underline{\hat{S}}_2$ are the magnetic moments of the two interacting nitroxides, $\underline{\hat{S}}_1$, $\underline{\hat{S}}_2$ being their spins ($S_1 = S_2 = \frac{1}{2}$). $\underline{r}$ is the vector in the plane of the bilayer joining the paramagnetic centres of the two spin labels, as shown in Fig. 2. The two interacting spins couple to form a triplet state of total spin: $S = S_1 + S_2 = 1$. Expressed in terms of the triplet quantization, the spin Hamiltonian of the interacting spin label pair is (see *e.g.* ref. 25):

$$\hat{\mathscr{H}}_s = g\beta\boldsymbol{H}\hat{S}_z + D[\hat{S}_z^{\,2} - \tfrac{1}{3}S(S+1)] \tag{4}$$

where

$$D = \frac{\frac{3}{4}\,g^2\beta^2}{r^3}(1 - 3\cos^2\theta) \tag{5}$$

and θ is the angle between the magnetic field direction (Z-axis) and the pair axis, $\underline{r}$. The off-diagonal (non-secular) terms of Eqn 3 have been omitted in Eqn 4, since they do not contribute to the dipolar energy in first order. The EPR transitions, $\Delta M_s = \pm 1$, within the triplet state of the interacting pair can be derived from the energy levels of Eqn 4 to be:

$$h\nu = g\beta\boldsymbol{H} \pm D \tag{6}$$

where ν is the microwave frequency. Hence the splitting of the pair lines is:

$$\Delta\boldsymbol{H} = 2\frac{D}{g\beta} \tag{7}$$

Thus, for the magnetic field perpendicular to the plane of the phospholipid bilayer ($\theta = 90°$):

$$\Delta\boldsymbol{H} = \frac{3}{2}\frac{g\beta}{r^3} \tag{8}$$

which can be used to calculate the spin label separation from the experimental pair line splitting. With $\theta = 0°$, *i.e.* the magnetic field parallel to the pair axis, the splitting becomes twice that of Eqn 8 and in the opposite sense. For the magnetic field parallel

to the plane of the phospholipid bilayer one can expect spin label pairs to have a range of θ-values from 0 to 90°. The pair spectrum is then spread over a range of approximately 160 G and is too weak to be detected.

In the case of rapid lateral diffusion of molecules within the bilayer, the dipolar splitting with the magnetic field parallel to the bilayer plane would be motionally averaged. The averaged splitting would be half that observed in the perpendicular direction and in the opposite sense. In this case too, the pair lines would be unobservable in the parallel direction since they would lie wholly under the intense central lines from the non-interacting spin labels (see Fig. 1). For rapid lateral averaging, the lateral diffusion rate would have to be greater than the dipolar anisotropy, *i.e.* greater than approximately $3 \cdot 10^8$ s^{-1}. This is an order of magnitude greater than the rates observed so far[21,24].

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REFERENCES

1 Ladbrooke, B. C., Williams, R. M. and Chapman, D. (1968) *Biochim. Biophys. Acta* 150, 333–340
2 de Bernard, L. (1958) *Bull. Soc. Chim. Biol.* 40, 161–170
3 Van Deenen, L. L. M., Houtsmuller, U. M. T., De-Haas, G. H. and Mülder, E. (1962) *J. Pharm. Pharmacol.* 14, 429–444
4 Lapper, R. D., Paterson, S. J. and Smith, I. C. P. (1972) *Can. J. Biochem.* 50, 969–981
5 Schreier-Muccillo, S., Marsh, D., Dugas, H., Schneider, H. and Smith, I. C. P. (1972) *Chem. Phys. Lipids* 9, in the press
6 Oldfield, E. and Chapman, D. (1972) *Biochem. Biophys. Res. Commun.* 43, 610–616
7 Hsia, J. C., Schneider, H. and Smith, I. C. P. (1970) *Biochim. Biophys. Acta* 202, 399–402
8 Butler, K. W., Schneider, H. and Smith, I. C. P. (1971) *Biochim. Biophys. Acta* 219, 514–517
9 Butler, K. W., Dugas, H., Smith, I. C. P. and Schneider, H. (1970) *Biochem. Biophys. Res. Commun.* 40, 770–776
10 Smith, I. C. P. (1971) *Chimia* 25, 349–360
11 Keana, J. F. W., Keana, S. B. and Beatham, D. (1967) *J. Am. Chem. Soc.* 89, 3055–3056
12 Smith, I. C. P., Marsh, D. and Schreier-Muccillo, S. (1973) in *Free Radicals in Molecular Biology and Pathology*, Vol. I (Pryor, W. A., ed.), Academic Press, New York, in preparation
13 Weiner, N. D. and Felmeister, A. (1970) *J. Lipid Res.* 11, 220–222
14 Shah, D. O. and Schulman, J. H. (1967) *J. Lipid Res.* 8, 215–226
15 Murphy, J. R. (1965) *J. Lab. Clin. Med.* 65, 756–774
16 Salem, L. (1962) *Can. J. Biochem. Physiol.* 40, 1287–1298
17 Chapman, D., Williams, R. M. and Ladbrooke, B. D. (1967) *Chem. Phys. Lipids* 1, 445–475
18 Traüble, H. and Haynes, D. H. (1971) *Chem. Phys. Lipids* 7, 324–335
19 Small, D. (1967) *J. Lipid Res.* 81, 551–557
20 Singer, S. J. and Nicholson, G. L. (1972) *Science* 175, 720–731
21 Devaux, P. and McConnell, H. M. (1972) *J. Am. Chem. Soc.* 94, 4475–4481
22 Sackmann, E. and Traüble, H. (1972) *J. Am. Chem. Soc.* 94, 4482–4491
23 Sackmann, E. and Traüble, H. (1972) *J. Am. Chem. Soc.* 94, 4492–4498
24 Sackmann, E. and Traüble, H. (1972) *J. Am. Chem. Soc.* 94, 4499–4510
25 Carrington, A. and McLachlan, A. D. (1967) *Introduction to Magnetic Resonance*, pp. 116–130, Harper and Row, New York, Evanston
26 Marsh, D. and Smith, I. C. P. (1972) *Biochem. Biophys. Res. Commun.* 493, 916–922