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EFFECT OF CHOLESTEROL AND WATER ON THE RIGIDITY AND ORDER OF PHOSPHATIDYLCHOLINE BILAYERS

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SUMMARY

1. The effect of hydration and cholesterol on the structure of multibilayers of egg lecithin and dipalmitoyllecithin was studied by the use of a rigid spin labelled derivative of cholestane as a molecular probe.

2. The spin label was found to orient nearly perpendicular to the lamellar plane in the dry state for both egg lecithin and dipalmitoyllecithin. Hydration causes a decrease in orientation and an increase in the rotational frequency of the spin label about its long axis Hydrated egg lecithin is in a lamellar liquid crystalline phase with a fluid lattice structure while hydrated dipalmitoyllecithin is in a less fluid, gel-like phase.

3. Cholesterol improves the orientation and decreases the fluidity of the bilayer lattice structure in the hydrated state up to 50 mole% in egg lecithin and up to 25 mole% in dipalmitoyllecithin. However, addition of 33–50 mole% cholesterol to dipalmitoyllecithin increases the fluidity of the bilayer. This is attributed to a gel to liquid crystalline phase transition in dipalmitoyllecithin

4. The effects of water and cholesterol on orientation of the bilayer components are correlated with X-ray diffraction results on the thickness of phospholipid bilayers. The results suggest that cholesterol controls the passive permeability of cells by regulating the membrane thickness and fluidity.

INTRODUCTION

Phospholipids and cholesterol are important components of biological membranes. The proportion of various types of lipids as well as the hydrocarbon fatty acid chains on the phospholipids may determine the permeability properties and other functions of membranes. Model membrane studies can provide valuable information about the interactions between various membrane components and their contribution to membrane structure.

Electron spin resonance (ESR) spectroscopy has been successfully used to study the orientation and motion of the lattice components in phospholipid dispersions and planar multibilayers[1–10]. In this study we used the spin-labelled steroid

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3-doxylcholestane (Fig. 1) to probe changes in the order of planar multibilayers. Since this is a rigid molecule with the plane of the oxazolidine ring perpendicular to the plane of the steroid nucleus, its orientation and motional freedom within the bilayer can be readily derived from its resonance spectra[1–3].

The purpose of this study was to investigate (a) the effect of saturation of the fatty acid chains of phosphatidylcholine on the order and fluidity of the bilayer and (b) the effect of cholesterol. The lipid films were examined in the dry and hydrated state in order to correlate the results with data from X-ray diffraction and other techniques. Dipalmitoyllecithin (with two saturated fatty acids) and egg lecithin (mostly with one saturated and one unsaturated fatty acid) were chosen for this study since results are available from X-ray diffraction[11–16], Raman spectroscopy[17], nuclear magnetic resonance[18], and previous ESR studies[1, 2, 4, 8–10].

MATERIALS AND METHODS

Chemicals

Egg lecithin and dipalmitoyllecithin were purchased from Serdary and Mann Research Laboratories. 3-Spiro-[2′-(*N*-oxyl-4′, 4′-dimethyloxazolidine]-cholestane (to be designated 3-doxylcholestane according to the nomenclature of Jost *et al.*[9] was prepared by the procedure of Keana *et al.*[19]. Solvents and chemicals used were reagent grade.

Preparation of lipid film

Thin lipid films containing a spin label to lecithin mole ratio of 1:150 were prepared by evaporating under reduced pressure a chloroform–methanol solution of the lipids in a flat quartz ESR cell. The dry film was left under vacuum throughout measurement of the spectra. In order to obtain a well-oriented film of egg lecithin at low cholesterol concentrations it was often necessary to redissolve the film in the cell with a small amount of anhydrous ether. The film was then formed by evaporating the ether under reduced pressure. In order to obtain good films of dipalmitoyllecithin it was necessary to heat the prepared film above 80 °C by pouring hot water on the surface of the quartz cell while connected to the vacuum pump and then allowing it to cool back to room temperature. The dry film was hydrated by introducing an aqueous solution of 0.15 M NaCl. Several films were made at each cholesterol concentration until reproducible results were obtained with ESR spectral parameters indicating relatively uniform orientation of the probe in bilayers.

ESR measurement

All spectra were recorded at room temperature on a Varian E-6 ESR spectrometer. The magnetic field was calibrated with Fremy's salt, $a_n = 13.091$ G[20]. The spectra were recorded with the plane of the film parallel and perpendicular to the magnetic field and the spectra were recorded as the first derivative of the absorption peaks

RESULTS

The hyperfine tensor components of 3-doxylcholestane have been determined in a multibilayer film of dipalmitoyllecithin[2]: $T_{zz} \cong 32$ G, $T_{xx} \cong T_{yy} \cong 6$ G. The

orientation of these components with respect to the spin label is shown in Fig. 1. Orientation of the spin label in the lipid film can be detected by measuring the spectrum with the magnetic field perpendicular and parallel to the normal of the bilayer plane. Changes in the fluidity of the bilayer can be estimated from the rotational freedom of the probe about its long axis in the lamellar structure.

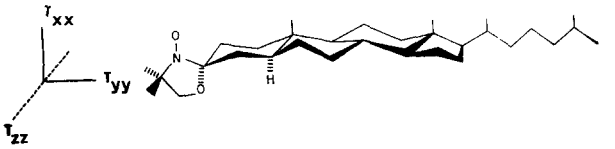


Fig. 1. Cholestane spin label and orientation of hyperfine tensor components T_{zz} is parallel to the $p\pi$ orbital of the N→O bond and is perpendicular to the long axis. T_{xx} and T_{yy} are perpendicular to the $p\pi$ orbital. T_{yy} is parallel to the long axis and T_{xx} is perpendicular to the long axis

If the long axis of the probe is preferentially aligned with the normal of the bilayer and the effective axial rotational frequency is less than 73 MHz (*i.e.* $T_{zz} - T_{xx}$) the maximum splitting of $a_\perp$ should measure approximately 32 G due to the lack of rotational averaging between T_{zz} and T_{xx} tensor components while $a_{||}$ should measure 6 G. The lack of averaging of the tensor components results in asymmetric peak intensities in the parallel orientation even though the splitting constant may be very close to that of the single crystal value $T_{yy} \cong 6$ G. Rapid axial rotational motion causes a decrease in $a_\perp$ from 32 G to approximately 19 G (*i.e.* $\frac{1}{2}(T_{zz} + T_{xx})$, and a more symmetric spectrum in the parallel orientation. Thus a decrease in the ratio of the peak heights in the parallel orientation (M_{+1}/M_0) from a maximum of 1.0 indicates a decrease in fluidity. The peak height ratio also depends on the distribution of orientations and homogeneity of the bilayer. A broad distribution of

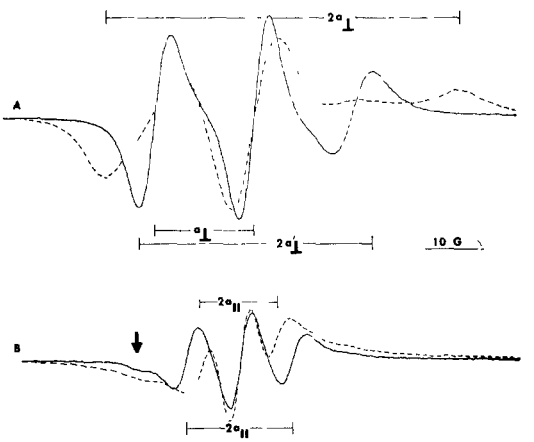


Fig 2 ESR spectra of spin-labelled cholestane in dry and hydrated multibilayers of egg lecithin recorded when the magnetic field is (A) perpendicular to the normal to the bilayer plane (perpendicular orientation) and (B) parallel to the normal to the bilayer plane (parallel orientation) The solid line spectrum is for the hydrated films and the dotted line spectrum is for dry films $2a_\perp$ in the dry film and $2a_\perp'$ in the hydrated film were measured as the maximum separation between the extreme peaks $a_\perp$ in the hydrated film was measured between the midpoints of the low field and centre peaks $2a_{||}$ was measured between the midpoints of the low and high field peaks

orientations in a poorly ordered bilayer causes the peaks to broaden and M_{+1}/M_0 decreases. Changes in rotational frequency of the probe in an intermediate range are qualitatively indicated as $a_\perp'$ values as designated in Fig. 2. The value of $a_\perp'$ increases from 20 to 32 G as the effective rotational frequency decreases to less than 73 MHz

If the long axis of 3-doxylcholestane is not perfectly aligned with the normal of the bilayer, $a_{||}$ will increase from 6 G and $a_\perp$ will decrease. An increase in $a_{||}$ can thus be used as a measure of the disorder in the bilayer[2,3]. Disorder in the bilayer due to tilting and rapid isomerization of the hydrocarbon chains will allow the spin label to undergo complicated anisotropic motion through a range of orientations. The precessing model[9] developed by Seelig[6] can account for the observed hyperfine splitting constants when the disorder is not too great. This model assumes the long axis of the spin label (T_{yy}) precesses rapidly about the normal of the bilayer at an angle β given by

$$\beta = \arccos \frac{(-4S_3 + 1)^{\frac{1}{2}}}{3} \quad (1)$$

where S_3 is the order parameter of the z axis and can be determined experimentally

$$S_3 = (a_{||} - a_\perp)/(T_{zz} - T_{xx}) \quad (2)$$

Thus a change in the order of the lattice structure can be monitored as a change in β provided that the axial motion is rapid enough.

Hydration of egg lecithin

The ESR spectra for pure egg lecithin multibilayers in the dry and hydrated states are shown in Fig. 2. In the dry state $a_{||} = 6.9$ G and $a_\perp = 30.7$ G indicating that the spin label is oriented with its long axis nearly perpendicular to the plane of the bilayers and is relatively immobilized. In a previous study orientation of 3-doxylcholestane in dehydrated egg lecithin films was not obtained[1]. Hydration causes $a_{||}$ to increase to 9.4 G and $a_\perp$ to decrease to 17 5 G indicating a decrease in orientation of the probe and an increase in the rotational frequency about its long axis. This reflects a phase transition from a lamellar crystalline phase in the dry state to a lamellar liquid crystalline phase in the hydrated state in agreement with X-ray diffraction studies (T_c* occurs at –15 °C, ref. 21). Hydration increases β, the amplitude of fluctuation of the probe about the normal of the bilayer, to 30 2°.

Cholesterol effect

The ESR spectra of the spin label in egg lecithin (66 mole%): cholesterol (33 mole%) are shown in Fig. 3. In the dry state $a_\perp = 32$ 3 G and $a_{||} = 6.7$ G and in the presence of excess water $a_\perp = 19.8$ G and $a_{||} = 6$ 5 G. Cholesterol improves the orientation of the lattice components in the hydrated bilayer as can be seen by a decrease in $a_{||}$ (Fig. 4) and increase in M_{+1}/M_0 (Fig. 6) with increase in cholesterol up to 33 mole%. Cholesterol causes an isotropic component in the spectrum (seen in Fig. 2 as a small shoulder indicated by an arrow) to disappear and this contributes to an increase in M_{+1}/M_0.

Greater than 25 mole% cholesterol in the bilayer causes the high field peak to broaden as can be seen in Fig. 3 and increases $a_\perp$ above 19 G indicating a reduction

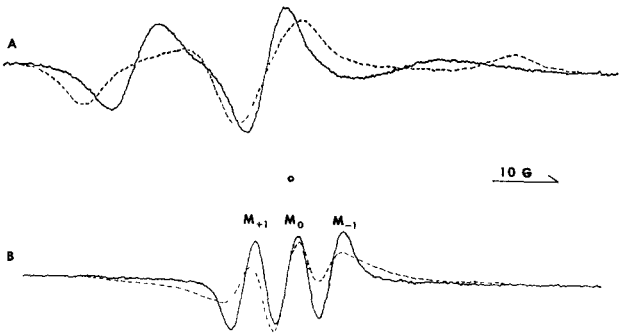


Fig. 3 ESR spectra of spin-labelled cholestane in dry (dotted line spectrum) and hydrated (solid line spectrum) multibilayers of egg lecithin (66 mole)% : cholesterol (33 mole%) in (A) the perpendicular orientation and (B) the parallel orientation The hyperfine splitting constants were measured as for Fig 2 The peaks in the parallel orientation are designated M_{+1}, M_0, M_{-1}

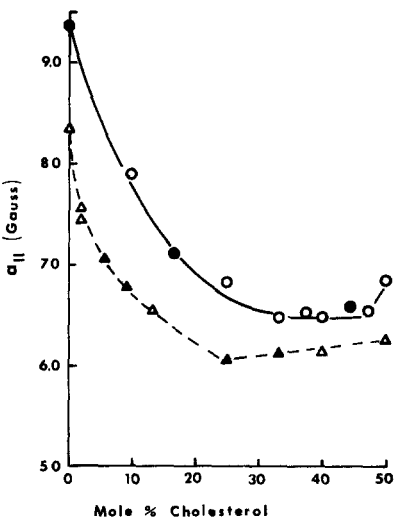


Fig 4 Dependence of a_{II} on the concentration of cholesterol in hydrated films of egg lecithin (○—○), and dipalmitoyllecithin (△---△) Two values at each cholesterol concentration were plotted if similar Otherwise the best film was used. A filled symbol represents two approximately equal or equal values

in the frequency of rotational motion of the probe about its long axis to less than 73 MHz. This increase in hyperfine splitting in the perpendicular orientation is a measure of the decrease in fluidity of the bilayer produced by cholesterol. However, due to the broadening of the high field peak $a_\perp$ can be measured as the average separation of the low and high field peaks only up to 25 mole% (Table II). The separation between the low and center field peaks could be used as a measure of $a_\perp$ (Table I) but does not take into account the broadening of the high field peak and the asymmetry of the spectrum. Thus the separation between the low and high field extrema, $a_\perp'$ (Table I) gives a more realistic measure of the decrease in fluidity with increase in cholesterol (Fig. 5). The decrease in fluidity produced by cholesterol is also indicated by the decrease in M_{+1}/M_0 above 37 mole% cholesterol

TABLE I

ESR PARAMETERS OF HYDRATED EGG LECITHIN–CHOLESTEROL BILAYERS

Mole% cholesterol	$a_{\parallel}$ (G)	$a_{\perp}$ (G)	$a_{\perp}'$ (G)	M_{+1}/M_0
0	9 36	17.5	20 5	0 64
	9.39	17.5	20 6	0 62
9 9	7 89	18.4	21 7	0 76
16 6	7 08	18 9	22 7	0.76
	7 13	18 8	22 9	0 82
25 0	6 82	19 2	23 9	0 84
33 3	6 47	19.8	24 9	1.02
37 5	6 52	20 1	26 1	1.02
40 0	6 47	20 1	26 6	0 95
44 4	6 58	20.0	26 5	0 89
	6 60	19.6	26 2	0 77
47 3	6 55	20 1	26.5	0 90
50 0	6 84	20 1	26.6	0 81

TABLE II

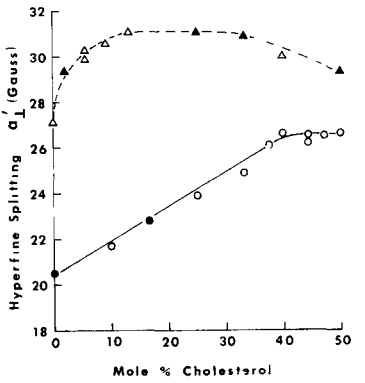
ESR PARAMETERS OF HYDRATED EGG LECITHIN–CHOLESTEROL BILAYERS

Mole% cholesterol	$a_{\parallel}$ (G)	$a_{\perp}$ (*av*) (G)	S_3	β
0	9 36	17 41	−0 310	30 2°
9 9	7 89	18 31	−0 401	21 2°
16 6	7 08	18 77	−0.450	15 0°
25 0	6 82	19 15	−0 474	10 9°

(Fig. 6) while the orientation remains constant as judged by the behavior of $a_{\parallel}$ (Fig. 4).

Eqn 1 can be applied to calculate β for hydrated bilayers only up to 25 mole% cholesterol due to the reduction in frequency of rotational motion below 73 MHz at higher cholesterol concentrations. Cholesterol decreases β continuously from 30 2° in pure egg lecithin to 10.9° at 25 mole%. The results are summarized in Table II.

The mole ratio of egg lecithin to cholesterol = 2:1 (33 mole% cholesterol) for maximum orientation has also been observed in X-ray diffraction work[14] for maximum long spacing. However, other ESR studies[2] and monolayer studies on the condensation effect of cholesterol[22] indicate that the maximum effect occurs



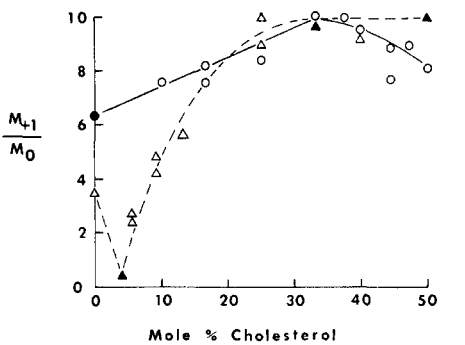


Fig 5 Dependence of the hyperfine splitting in the perpendicular orientation $a_\perp'$ on the concentration of cholesterol in hydrated lipid films of egg lecithin (○—○), and dipalmitoyllecithin (△---△) A filled symbol represents two approximately equal or equal values $2a_\perp'$ was measured as the maximum separation between the extreme peaks

Fig 6 Dependence of the ratio of the peak heights (M_{+1}/M_0) on the concentration of cholesterol in hydrated lipid films of egg lecithin (○—○), and dipalmitoyllecithin (△---△) A filled symbol represents two approximately equal or equal values

at 25 mole% cholesterol. This difference may be due to the types of fatty acids and extent of saturation in the egg lecithin used.

Hydration of dipalmitoyllecithin

The ESR spectra of pure dipalmitoyllecithin in the dry and hydrated states are shown in Fig. 7. The hyperfine separations in the dry state, $a_\perp = 31.4$ G and $a_{||} = 7.0$ G indicate that the long axis of the probe is oriented nearly perpendicular to the bilayer plane and is immobilized. In a previous ESR study of dipalmitoyllecithin, orientation in multibilayers could only be obtained when appreciable amounts of cholesterol were present[2]. The effect of hydration is much smaller than it was for egg lecithin. The value of $a_{||}$ increases to 8.34 G indicating some decrease in orientation. However, $a_\perp' = 27$ G while for hydrated egg lecithin $a_\perp' = 21$ G. Thus the probe is relatively immobilized in hydrated dipalmitoyllecithin indicating a gel-like phase as has been observed from X-ray diffraction studies (T_c* occurs at 41 °C[12, 21]).

Cholesterol effect

The behavior of hydrated dipalmitoyllecithin in the presence of cholesterol is more complex than for egg lecithin since cholesterol causes a broad gel to liquid crystalline phase transition over a wide temperature range[12, 17].

Cholesterol improves the orientation of the probe up to 25 mole% as indicated by a decrease in $a_{||}$ and increase in M_{+1}/M_0 (Figs 4 and 6). A small amount of cholesterol causes the appearance of a peak in the spectrum in the perpendicular orientation due to a contribution from T_{zz}. This indicates disruption of the bilayer lattice in some regions or the presence of another phase and is reflected by the very low values of M_{+1}/M_0 for 2–6 mole% cholesterol. Addition of more cholesterol causes these peaks to disappear and M_{+1}/M_0 increases. The ESR spectra for dry

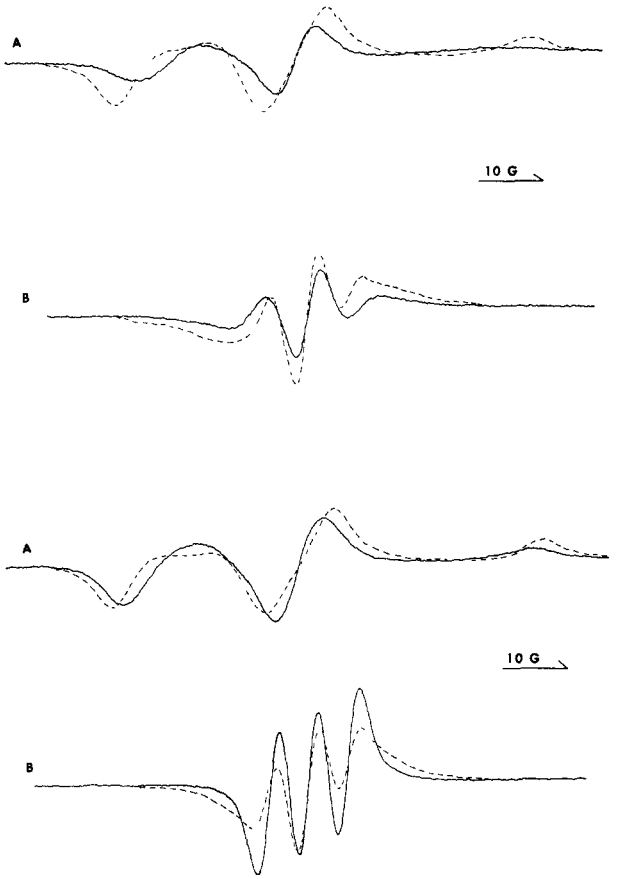


Fig. 7 ESR spectra of spin labelled cholestane in dry (dotted line spectrum) and hydrated (solid line spectrum) multibilayers of dipalmitoyllecithin in (A) the perpendicular orientation and (B) the parallel orientation. The hyperfine splitting constants were measured as for Fig 2

Fig 8. ESR spectra of spin-labelled cholestane in dry (dotted line spectrum) and hydrated (solid line spectrum) multibilayers of dipalmitoyllecithin (75 mole%) cholesterol (25 mole%) in (A) the perpendicular orientation and (B) the parallel orientation The hyperfine splitting constants were measured as for Fig. 2.

and hydrated films of dipalmitoyllecithin (75 mole%):cholesterol (25 mole%) are shown in Fig. 8. In the hydrated state $a_{\perp}' = 31$ G indicating restricted mobility of the probe. Hydration does not increase the rotational motion of the probe significantly until the cholesterol concentration is greater than 33 mole%. Since the rotational frequency of the probe is less than 73 MHz at all cholesterol concentrations β cannot be calculated. However, the minimum value for $a_{\parallel} = 6.04$ G reached at 25 mole% cholesterol indicates nearly perfect orientation of the spin label.

The change in $a_{\perp}'$ with addition of cholesterol can be seen in Fig. 5 and illustrates the large difference in fluidity between hydrated bilayers of egg lecithin–cholesterol and dipalmitoyllecithin–cholesterol (Tables I and III). In egg lecithin $a_{\perp}'$ increases up to 40 mole% cholesterol and levels off due to a decrease in fluidity

TABLE III

ESR PARAMETERS OF HYDRATED DIPALMITOYLLECITHIN–CHOLESTEROL BILAYERS

Mole% *cholesterol*	$a_{\parallel}$ (*G*)	$a_{\perp}$ (*G*)	$a_{\perp}'$ (*G*)	M_{+1}/M_0
0	8 34	18 9	27 1	0 35
2 0	7 41	18 4	29 3	0 03
	7 56	17 8	29 5	0 04
5·7	7 08	21 2	29 9	0 24
	7 04	20 2	30 3	0 27
9.1	6 77	20 5	30 7	0 48
	6 77	21 0	30 5	0 42
13 2	6 54	21 6	31 1	0 56
25 0	6 04	22 0	31 1	1 01
	6 04	21 4	31 2	0 90
33 3	6 12	21 8	31 0	0 97
	6 13	21 7	30 8	1 03
40 0	6 15	21 4	30 0	0 93
50 0	6 26	20 7	29 3	1 01

produced by cholesterol. In dipalmitoyllecithin $a_{\perp}'$ increases initially due to the improvement in orientation produced by cholesterol but above 33 mole% cholesterol $a_{\perp}'$ decreases and approaches the value for egg lecithin–cholesterol mixtures. This is interpreted as an increase in fluidity of the bilayer, reflecting a gel to liquid crystalline phase transition at high cholesterol concentrations. An increase in fluidity is also indicated by the fact that M_{+1}/M_0 remains constant at 1.0 for dipalmitoyllecithin while it decreases for egg lecithin at high cholesterol concentrations. A small increase in $a_{\parallel}$ above 25 mole% cholesterol probably indicates a decrease in orientation of the probe due to the increased fluidity of the membrane lattice.

DISCUSSION

The ESR spectra reflect the state of the membrane bilayers and indicate that hydrated egg lecithin is in a lamellar liquid crystalline phase while dipalmitoyllecithin is in a lamellar gel phase. Hydrated bilayers of dipalmitoyllecithin are better oriented and less fluid at all cholesterol concentrations than films of egg lecithin. This is due to the presence in egg lecithin of an unsaturated fatty acid with an intrinsic bend at the *cis* double bond which decreases the van der Waals interactions between the hydrocarbon chains and results in a lower melting point for egg lecithin.

X-ray diffraction studies on egg lecithin[11,15] indicate that there is a decrease in bilayer thickness and an increase in average surface area per lipid molecule with increasing water content. Cholesterol increases the bilayer thickness and decreases

the average surface area per molecule[11,14]. The increase in fluidity upon hydration of egg lecithin and decrease upon addition of cholesterol can account for these changes Thinning of the bilayer could be caused by tilting of the hydrocarbon chains or by kinks in the chains due to *gauche* rotations about the carbon–carbon bonds. Assuming that the chains are completely extended and tilted at the angle β, the bilayer thickness in the presence and absence of cholesterol can be calculated, giving a difference of 5.3 Å, which is of the same order as that seen by X-ray diffraction[11].

However, the high fluidity of the hydrocarbon interior detected by other spin labels[4,7–9] indicates rapid changes in configuration of the fatty acid chains due to interconverting *gauche–trans* isomeric states. Kinks in the hydrocarbon chains due to *gauche* rotations would decrease their effective length, increase the average surface area per molecule and allow 3-doxylcholestane to move within the cone defined by β. Cholesterol reduces the probability of *gauche* rotations by restricting the freedom of motion of the hydrocarbon chains and thus increases their effective length, increases the packing of the lattice components and improves the orientation of the probe. NMR studies have shown that cholesterol inhibits the motion of the methylene protons of the chains[18].

In dipalmitoyllecithin up to 25 mole% cholesterol improves the orientation in the bilayer while more than 33 mole% cholesterol causes an increase in the fluidity. Raman spectroscopy[17] shows that this increase in fluidity can be interpreted as an increase in *gauche* rotations in the hydrocarbon chains. Large amounts of cholesterol must disrupt the cohesive forces between the extended hydrocarbon chains of the gel phase. The improvement in orientation up to 25 mole% cholesterol followed by the fluidizing effect of cholesterol at concentrations greater than 33 mole% indicates that cholesterol might produce an initial increase in bilayer thickness while large amounts would decrease it. X-ray diffraction studies[12] have shown an increase in long spacing in dipalmitoyllecithin up to 7 5 mole% cholesterol with a decrease at higher concentrations.

The increase in fluidity above 33 mole% cholesterol is interpreted as the beginning of the phase transition. Engelman and Rothman[16] have also detected a phase boundary in dipalmitoyllecithin–cholesterol mixtures near 33 mole% cholesterol by X-ray diffraction. Below 33 mole% both the dipalmitoyllecithin gel phase and the mixed liquid crystalline phase were present. We cannot interpret the spectra in terms of a mixture of phases; however, our results do not rule out this possibility. They propose a model for the mixed phase containing 33 mole% cholesterol in which each cholesterol is surrounded by lipid hydrocarbon chains and the steroid nuclei are randomly oriented about their long axes in order to account for the lack of long range order in the plane of the bilayer. However, the increase in motional freedom of the probe in the presence of greater than 33 mole% cholesterol indicates an increase in fluidity of the hydrocarbon chains even near the polar head group region. This could account for the lack of long range order in the plane of the bilayer of the mixed phase. However, the fact that we also observe the maximum effect of cholesterol on egg lecithin at 33 mole% lends support to this model for the packing of cholesterol in both lipids.

The increased fluidity of egg lecithin bilayers relative to dipalmitoyllecithin and the decrease in fluidity produced by cholesterol correlates well with the effects

of these lipids on the permeability of mycoplasma cell membranes and liposomes[23–27]. The increase in permeability seen with increase in fluidity can be explained by the resultant decrease in thickness of the bilayer as well as by an increase in the solubility of the permeant in the bilayer. Kinks in the fluid hydrocarbon chains create transient pockets in the bilayer which could facilitate the passage of small molecules[28,29].

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