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THE IMPORTANCE OF THE PHOSPHOLIPID BILAYER AND THE LENGTH OF THE CHOLESTEROL MOLECULE IN MEMBRANE STRUCTURE

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Summary

The properties of mixtures of phosphatidylcholine and analogues of cholesterol bearing side chains of varying lengths were examined by a variety of methods. The incorporation of the analogues into sonicated liposomes and their effect on the rate of osmotic shrinking of multilamellar liposomes were determined. The ordering of a steroid spin label was studied in an oriented multibilayer system and the effect of the analogues on the phase transition of dipalmitoyl phosphatidylcholine monitored using the spin label TEMPO (2,2,6,6-tetramethylpiperidine-*N*-oxyl). Mixtures of analogues and phospholipid were also studied in monolayers.

In all the bilayer systems studied cholesterol caused the greatest 'rigidifying' effect, the analogues with shorter or longer side chains being less effective. However, in the monolayer experiments the length of the sterol molecule was found to be much less critical. It is suggested that cholesterol is anchored in position in a phospholipid bilayer by virtue of the molecule being the precise length required to maximise interactions between neighbouring molecules without disturbing the bilayer structure.

Introduction

The occurrence and function of cholesterol in membranes has attracted much attention in recent years. Cholesterol is widely distributed in animal membranes and is usually found in larger amounts in the plasma membranes of cells rather than in the intracellular membranes. Studies of both natural and model membranes lead to the general conclusion that cholesterol tends to

rigidify membrane phospholipids above their phase transition [1]. Specific interactions of cholesterol with membrane proteins have also been reported [2—4] and cholesterol has also been shown to cause reversible inactivation of a membrane enzyme [5].

The molecular characteristics required for cholesterol to exert most of its effects are a planar ring system with a 3β-hydroxyl group [1]. A side chain is also necessary for maximum effect [1] and we have shown from the ordering of two steroid spin labels in egg phosphatidylcholine liposomes that the length of the side chain is critical for the maximum ordering effect of the sterol to be observed. An increase or decrease of one or more carbons in the side chain length caused a detectable reduction in the ordering of the spin labels when compared with the ordering caused by cholesterol [6,7]. In order to establish the significance of the effect of the sterol side chain more precisely we have examined the properties of mixtures of phospholipids and the cholesterol analogues with modified side chains which we described previously by a variety of methods.

The experiments included determining the ability of sonicated egg phosphatidylcholine liposomes to incorporate the cholesterol analogues and measuring the effect of the cholesterol analogues on the rate of osmotic shrinking of multilamellar egg phosphatidylcholine liposomes by a stopped flow technique. A more physically significant measure of the ordering of the cholestane spin label (3-spiro(2′-(*N*-oxyl-4′,4′-dimethyloxazolidine))cholestane than obtained previously [6,7] was obtained using oriented planar egg phosphatidylcholine multibilayers. The effect of the sterol on the melting of dipalmitoyl phosphatidylcholine was studied by the dependence on temperature of the partitioning of the spin label TEMPO (2,2,6,6-tetramethylpiperidine-*N*-oxyl) into liposomes containing sterol and dipalmitoyl phosphatidylcholine. To determine the importance of a bilayer system a study was also carried out on the surface pressure-area characteristics of sterol-egg phosphatidylcholine monolayers. It was hoped that these experiments would throw further light on the factors which affect the fit of cholesterol into biological membranes.

Materials and Methods

Sterols and egg phosphatidylcholine were obtained as reported previously [6—8]. The cholesterol analogues are referred to by the number of carbon atoms in each analogue (cholesterol = C_{27}) and their structures are given in Fig. 1. Dipalmitoyl phosphatidylcholine was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks., England and cholesterol oxidase from B.D.H. Chemicals Ltd., Poole, Dorset, England. The cholestane spin label (Fig. 1) was obtained from Syva, Palo Alto, CA and TEMPO was synthesised by oxidation of 2,2,6,6-tetramethylpiperidine with *m*-chloroperbenzoic acid (following ref. 9), both obtained from Aldrich Chemical Co. Ltd., Gillingham, Dorset, England. Dicetylphosphate was obtained from Sigma, Kingston-upon-Thames, Surrey, England.

Incorporation of sterols in egg phosphatidylcholine dispersions. These experiments were carried out as described by Stevens and Green [10] using an equimolar mixture of sterol and phospholipid to form the dispersions. Sonica-

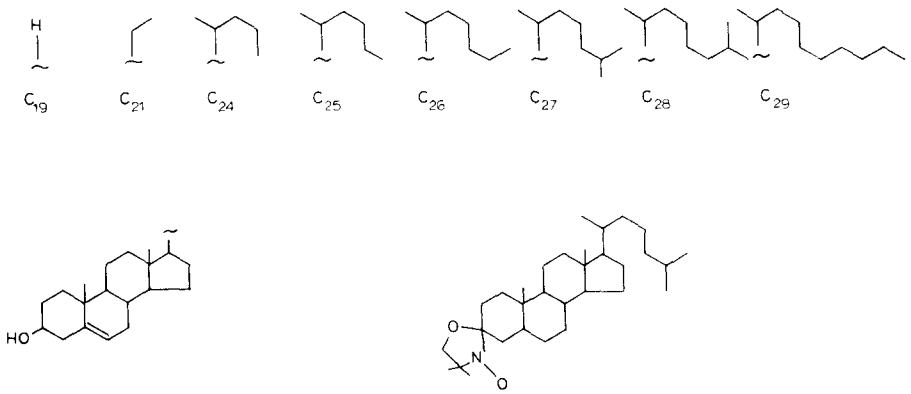


Fig. 1. Structures of steroids used in experiments.

tion was performed as described by Stevens and Green using maximum power of a Rapidis 150 sonifier (Ultrasonics Ltd., Shipley, Yorks., England). The composition of the sonicated liposomes was determined after centrifugation by extraction of the lipids in the supernatant with chloroform/methanol (2 : 1, v/v) and estimation of phosphate by the method of McClare [11]. Sterols were estimated either by GLC or enzymically with cholesterol oxidase.

Osmotic shrinking of egg phosphatidylcholine liposomes. Liposomes were prepared as described previously [6] from a mixture of 72 mol% phospholipid, 24 mol% sterol and 4 mol% dicetylphosphate. The lipids were suspended in 0.05 M KCl adjusted to pH 7.2 and the suspension placed in one reservoir of a stopped flow apparatus (Nortech, Salisbury, England). A solution of 0.18 M KCl, pH 7.2, was added to the other reservoir. Equal volumes of the two solutions were rapidly mixed [12] and the shrinking was observed at 450 nm and recorded on a storage oscilloscope coupled to a transient recorder (Datalab, Mitcham, Surrey, England). The rates of osmotic shrinking are expressed as the initial change (δT) in transmittance in the first second divided by the total change (ΔT), which is complete within 50 s. This allows different preparations of liposomes to be compared since the overall rate of shrinking has been shown to be very sensitive to the amount of dicetylphosphate present in the liposome [13]. Measurements were made for all the sterols over a temperature range of 25—45° C.

Ordering of the cholestane spin label in oriented multibilayers. Oriented multibilayers of sterol and egg phosphatidylcholine (30 : 70 mol%) containing 1 mol% of the cholestane spin label were prepared from hydrated films of the lipids as described by Seelig [14]. Spectra were obtained at room temperature of the films oriented perpendicular and parallel to the magnetic field in a Varian E4 ESR spectrometer. The order parameter S_{mol} was calculated by the method of ref. 14.

Melting transition of dipalmitoyl phosphatidylcholine. Mixtures of sterol and dipalmitoyl phosphatidylcholine were suspended in 0.15 M KCl by vortex mixing above 45° C to give a total lipid concentration of 0.1 M. TEMPO was added to give a concentration of 5 mM. ESR spectra were recorded on an Varian E4 spectrometer fitted with a Varian variable temperature accessory.

The temperature in the cavity was calibrated using a NiCr/NiAl thermocouple and the heating rate was 16°C per h. No hysteresis was observed on cooling, and settling of the liposomes during the experiment was prevented by stirring the mixture within the sample cell in the cavity at intervals with a nylon bristle. The mol fraction of the spin label present in the membrane phase was estimated as the parameter f as described by Shimshick and McConnell [15].

Monolayer experiments. A Langmuir trough 15 cm wide made of polytetrafluoroethylene (PTFE) was used for measurements of the surface pressure-area relations at the air-water interface. Monolayers were spread between PTFE barriers from solutions in chloroform. After an interval of 15 min one barrier was removed and the monolayer compressed against a calibrated flexure balance [16]. Freshly prepared double distilled water was used in the trough and maintained at a temperature of 22.5°C. The compression was continuous (4 cm/min) and the pressure-area curve was recorded automatically in a period of not more than 5 min. Lower rates of compression gave identical results.

Results

Incorporation of sterols in egg phosphatidylcholine dispersions

The ability of sterols to be incorporated into liposomes of egg phosphatidylcholine depends on the structure of the sterol [1]. Using testosterone esters as analogues of cholesterol, it was shown that the incorporation of these analogues into liposomes was affected by the length of the acyl side chain and was maximal when the side chain contained eight carbons, giving a compound approximately the same length as cholesterol [10]. We have performed similar experiments with our cholesterol analogues, which are more closely related to cholesterol than the testosterone esters. The results are given in Table I.

The results show that whilst all the analogues tested with shorter side chains than cholesterol can be accommodated into liposomes to the same extent as cholesterol, the C_{29} sterol is only incorporated up to a molar composition of 30 mol% sterol. The C_{29} analogue is much longer than cholesterol and if fully extended would penetrate into the neighbouring layer of the bilayer. This increase in bulk probably makes it difficult for the C_{29} sterol to pack into a phospholipid bilayer above the concentration found in this experiment. Most of the experiments reported in this paper were carried out using a sterol/phos-

TABLE I

MOLAR COMPOSITIONS OF SONICATED LIPOSOMES FORMED FROM CHOLESTEROL ANALOGUES AND EGG PHOSPHATIDYLCHOLINE

Results are means of 2—5 determinations.

	Sterol					
	19	21	24	26	27	29
Composition (molar ratio sterol/phospholipid)	1.07 ± 0.1	0.94 ± 0.1	0.93 ± 0.1	0.95 ± 0.15	1.0 ± 0.15	0.42 ± 0.1

pholipid molar ratio of about 3 : 7 in order to focus attention on the sterol-phospholipid interaction.

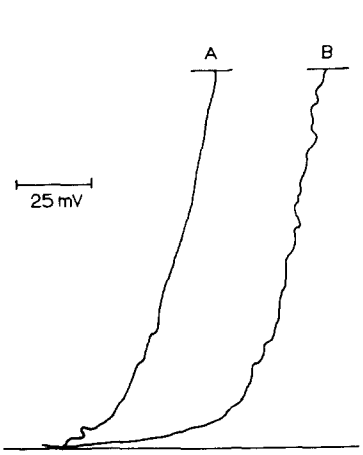
The significance of the maximum incorporation of the C_{29} analogue observed may be that above a molar ratio of sterol/phospholipid of 1 : 2 each sterol molecule is, on average, no longer surrounded by a ring of phospholipid molecules. The shell of nearest neighbour molecules must now include some sterol as well as phospholipid [18] and sterol-sterol interactions become important. Sterol-sterol interactions for the C_{29} analogue in a phospholipid bilayer may be very unfavourable and may place a limit on the amount of this compound that can be incorporated in a sonicated liposome.

Osmotic shrinking of egg phosphatidylcholine liposomes

Jain et al. [13] and Bittmann and Blau [12] have examined the effect of cholesterol on the rate of osmotic shrinking of egg phosphatidylcholine liposomes by a stopped flow technique. Cholesterol was found to substantially reduce the rate of shrinking. We have compared the effects of our analogues with cholesterol in liposomes containing a small amount of dicetylphosphate. This is used to increase the internal volume of the liposomes so that a significant change can be observed on shrinking [13]. The shrinking was followed by the change in transmittance at 450 nm of the liposome suspension since the light scattering of suspension has been shown to be proportional to the size of the liposome [19] (Fig. 2).

Fig. 3 shows the results obtained at 25° C and Table II the rates of shrinking obtained at a range of temperatures expressed as a fraction of the rates obtained for egg phosphatidylcholine alone. This was necessary since the absolute values varied somewhat with each batch of egg phosphatidylcholine used.

It is clear from Fig. 3 that only cholesterol causes a substantial reduction in the rate of shrinking and the other sterols, which are equally rigid, have



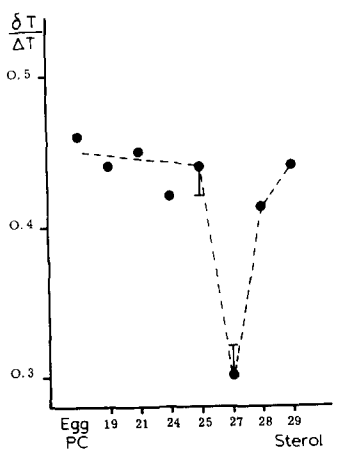


Fig. 2. Osmotic shrinking of egg phosphatidylcholine liposomes containing 4% dicetylphosphate at 22° C. A, shrinking during 1 s; B, shrinking during 50 s.

Fig. 3. Rates of osmotic shrinking ($\delta T/\Delta T$) of liposomes at 25° C. PC, phosphatidylcholine.

TABLE II

RATES OF OSMOTIC SHRINKING OF LIPOSOMES CONTAINING CHOLESTEROL ANALOGUES RELATIVE TO LIPOSOMES OF EGG PHOSPHATIDYLCHOLINE ALONE

Ratios ± 0.05, means of 2—4 determinations.

Temperature (°C)	Sterol						
	19	21	24	25	27	28	29
25	0.96	1.07	0.91	0.98	0.65	0.89	1.07
30	0.96	0.92	0.94	0.94	0.75	1.00	0.98
35	0.91	0.93	0.89	0.89	0.82	1.03	0.91
45	0.93	0.97	0.93	0.99	0.86	1.05	0.90

essentially no effect. Table II shows that the effect of cholesterol occurs over a temperature range of 20° C, although it becomes relatively less as the temperature increases, as one would expect as the membrane becomes more fluid. If the rate of shrinking can be regarded as being dependent on the fluidity of the membrane, or the ability to undergo rotation about C-C single bonds in the fatty acyl chains [20], these experiments show that cholesterol packs into a phospholipid bilayer in such a way as to maximally restrict rotational motion of single bonds in the fatty acyl chains resulting in a more rigid membrane and a liposome which is more resistant to osmotic shock.

Ordering of the cholestane spin label in oriented multibilayers

Our previous experiments [6,7] used steroid spin labels incorporated into liposomes and the ordering of the lipids was characterised by an approximate order parameter using the frequently adopted formalism of Hubbell and McConnell [21]. It has been pointed out recently that this procedure is not always valid and that a more reliable way of estimating the molecular order in a bilayer is to use the oriented bilayer method [22]. We have applied this system to our cholesterol analogues and the results obtained using the cholestane spin label are given in Table III.

The maximal ordering effect of cholesterol is again apparent. All the sterols caused ordering which was significantly less than cholesterol, although the bilayers containing the analogues with longer side chains than cholesterol showed rather greater ordering than those with the shorter side chains. In view of the other experiments presented here where the C_{28} and C_{29} analogues are

TABLE III

ORDER PARAMETER S_{mol} FOR CHOLESTANE SPIN LABEL IN ORIENTED EGG PHOSPHATIDYLCHOLINE MULTIBILAYERS CONTAINING CHOLESTEROL ANALOGUES

Values ± 0.02, means of 2—5 determinations. PC, phosphatidylcholine.

	Sterol							
	Egg PC	19	21	24	25	27	28	29
S_{mol}	0.713	0.765	0.809	0.797	0.773	0.892	0.841	0.847

shown to be less effective than the short chain analogues in reproducing the effect of cholesterol, it may be that this higher degree of ordering reflects the fact that a planar bilayer is more able to accommodate the longer side chains than the curved bilayer of the closed vesicles used in the other experiments.

Melting transition of dipalmitoyl phosphatidylcholine

The partitioning of a small spin label such as TEMPO into membranes has been widely used as a probe for the membrane fluidity and for the detection of possible phase transitions. Mixtures of cholesterol and different phosphatidylcholines have been studied by this method and phase diagrams for the binary systems derived from the results [15]. These experiments showed that cholesterol reduces the fluidity of the membrane and, in agreement with differential scanning calorimetry studies [1], broadens the phase transition of the pure phospholipid. The membrane fluidity is characterized by the parameter f, which is an estimate of the mol fraction of the spin label in the hydrophobic membrane phase. This parameter is plotted as a function of temperature in Fig. 4 for two mixtures of dipalmitoyl phosphatidylcholine and sterol (10 mol% sterol and 30 mol% sterol). The curves show that all the sterols caused a broadening of the phase transition of dipalmitoyl phosphatidylcholine and all, including the C_{19} sterol, removed the initial increase in f observed between 30 and 40° C, but again cholesterol shows the greatest effect. At a molar composition of 10% sterol the effect of the C_{19} analogue is very small whilst all the other sterols with shorter side chains than cholesterol have a similar effect to cholesterol. However the sterols with side chains longer than cholesterol can be seen to cause a relative increase of the partitioning of TEMPO into the membrane consistent with an increase in its fluidity. A similar trend is observed at a molar composition of 30% sterol. Here cholesterol causes the greatest reduction of solubility of TEMPO in the membrane and all the other sterols cause lesser, but significant effects.

Experiments such as these have been interpreted in terms of the reduction in the cooperativity of the melting of the fatty acyl chains of the phospholipid

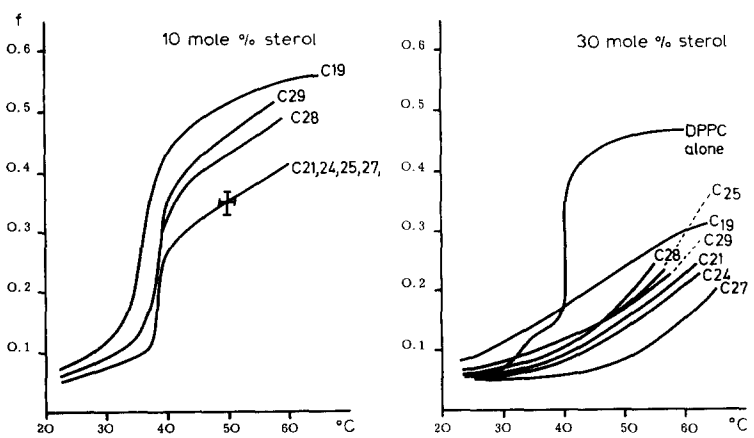


Fig. 4. Partitioning of TEMPO into dipalmitoyl phosphatidylcholine liposomes containing cholesterol and cholesterol analogues.

TABLE IV

PARTITIONING OF TEMPO INTO STEROL-DIPALMITOYL PHOSPHATIDYLCHOLINE LIPOSOMES AT 55°C

Values ± 0.02, means of three determinations.

Composition	Sterol							
	19	21	24	25	26	27	28	29
10 mol% sterol	0.54	0.39	0.37	0.40	0.40	0.39	0.46	0.48
30 mol% sterol	0.27	0.19	0.17	0.22	0.15	0.14	0.23	0.19

due to the sterol. Such effects have been quantified [23], but the model used requires that the melting transition should be symmetrical, which is approximately the case for some mixtures of phospholipids but is clearly not the case here over the temperature range studied. A more empirical approach is to use the value of the TEMPO parameter f at 55°C, a temperature well above the phase transition temperature of dipalmitoyl phosphatidylcholine (41°C), as an index of the fluidity of the membrane and hence the effect of the sterol on the membrane. These values are given in Table IV. The results in Table IV agree well with the oriented multibilayer experiments and the osmotic shrinking experiments with cholesterol again causing the greatest effect. At low sterol concentrations a much shorter side chain is as effective as cholesterol in limiting the fluidity of the membrane. The C_{19} analogue, however, has little effect at 10 mol% composition and the least effect at 30 mol% indicating that reductions in fluidity due to the sterol ring system alone only become significant at higher mol fractions of sterol. These results suggest that at low sterol concentrations the ring system plus a short side chain is sufficient to reduce the cooperative interactions between phospholipid fatty acyl chains to an extent similar to cholesterol and it is only at higher concentrations of sterol that the full side chain is required to restrict the motion of a greater number of phospholipid molecules and a larger part of the fatty acyl chains.

In the oriented multibilayer experiments the C_{28} and C_{29} analogues caused greater ordering of the cholestane spin label than the sterols with side chains shorter than cholesterol. However the partitioning of TEMPO into liposomes containing the C_{28} and C_{29} sterols shows that these liposomes are significantly more fluid than those containing cholesterol and that their fluidity is greater than that due to the inclusion of the C_{25}, C_{24} and C_{21} sterols. It is possible that this greater fluidity in the liposomes is due to the effect of the long side chains of the C_{28} and C_{29} analogues perturbing the packing of the molecules in the curved bilayers and that the planar bilayer used in the ordering experiments is more able to accommodate the sterols with long side chains.

Monolayer experiments

One of the most striking demonstrations of the effect of cholesterol on the molecular packing of lipids can be obtained from studied of monolayers [1,17]. Surface pressure-area measurements of spread films show that cholesterol causes a condensation of a phospholipid monolayer since the mean area per molecule in a mixed cholesterol-phospholipid monolayer is less than

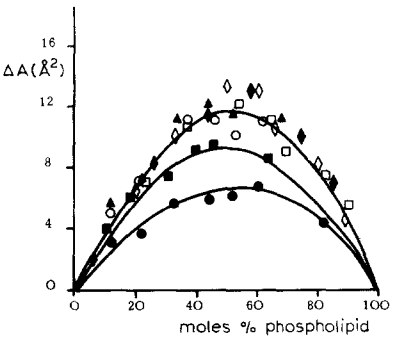


Fig. 5. Deviation from ideal behaviour (ΔA) of mixed sterol-egg phosphatidylcholine monolayers at 22.5° C and 12 dynes/cm surface pressure. ●, C_{19}; ■, C_{21}; ▲, C_{24}; ♦, C_{26}; ○, C_{27}; □, C_{28}; ◇, C_{29}.

would be expected if the molecular packing in the mixed monolayer were the sum of the packing in monolayers of the two lipids separately.

In Fig. 5 we show the deviation of the observed mean molecular area ΔA, for mixed monolayers of cholesterol analogues and egg phosphatidylcholine from that which would be observed if the sterol caused no condensation effect on the monolayer. At a surface pressure of 12 dynes/cm egg phosphatidylcholine had a mean molecular area of 86 Å^2 and cholesterol 41 Å^2. All the sterols above and including the C_{24} sterol caused a substantial condensation of the monolayer which was about the same as that produced by cholesterol. Weaker effects were shown by the C_{21} and the C_{19} analogues, showing that the full condensation requires a side chain of moderate length and that the ring system alone is not sufficient, as had been observed before [1]. The C_{28} and C_{29} sterols here behaved no differently from cholesterol. This is reasonable since in a monolayer the long side chains of these sterols can extend freely in space above the subphase. This observation together with the absence of a weaker effect from the C_{25} and C_{24} analogues, which had been observed in the other experiments, suggests that a bilayer system is required before the full effect of cholesterol can be expressed.

Recently the behaviour of cholesterol-phosphatidylcholine monolayers has been reexamined [28] using a different approach from that reported here and used by other workers [1,17]. It was shown that if monolayers are prepared in such a way that they are at equilibrium with the subphase, the condensing effect described here can be partially attributed to the formation of specific cholesterol-phospholipid complexes of molar composition 1 : 2. The molecular nature of such complexes is unknown but presumably complex formation would cause a reduction in the degrees of freedom of rotation of carbon-carbon bonds in the fatty acyl chains of the bound phospholipid and this would result in the overall changes in 'rigidity' of the membrane which we and others have observed by other methods. Thus according to this interpretation cholesterol would form the most stable complex with phosphatidylcholine of all the sterols tested, leading to the most rigid membrane. There is, however, no evidence to suggest that biological membranes are at equilibrium and the importance of a cholesterol-phospholipid complex in vivo remains to be established.

Discussion

Optimum effect of cholesterol

The experiments reported here show in variety of systems that the maximum effect of a sterol on the physical properties of a phospholipid membrane is only observed with cholesterol. In addition to the structural requirements already established [1] it is clear that the sterol molecule must also have a precise length, that of cholesterol (or of the C_{26} analogue which is equivalent in length).

It has been demonstrated by NMR experiments that the effect of cholesterol on the motion of the methylene groups in the fatty acyl chains of the phospholipids is restricted to the region of the chains that is in contact with the ring system, the region occupied by the side chain being more fluid [24]. Our results show that for this to be the case a complete side chain is required to anchor the sterol in the bilayer. The position occupied by the sterol is thought to be close enough to the ester carbonyl groups of the phospholipid to allow hydrogen bonding between these groups and the 3β-hydroxyl group of cholesterol. The strength of this hydrogen bond may depend upon the sterol having the correct length to fit precisely into the phospholipid bilayer. This fit is close to that revealed by X-ray diffraction and neutron scattering studies of egg phosphatidylcholine-cholesterol mixtures [25,26].

In these experiments no difference was observed between the C_{26} sterol and cholesterol. Both of these compounds have the same length so this would be expected if the effects of cholesterol on phospholipids is due to the overall length of the molecule. This is in contrast to a membrane enzyme system, the rat liver microsomal cholesterol 7α-hydroxylase, which is being studied in our laboratory. This enzyme system has been shown to discriminate between cholesterol and the C_{26} analogue, presumably by virtue of a more specific substrate binding site [2].

The monolayer experiments show that the precise restrictions on the packing of cholesterol and phospholipid are only fully effective in a bilayer system where the juxtaposition of two molecular layers further limits the degrees of motional freedom open to the lipid molecules.

These results apply only to egg phosphatidylcholine or dipalmitoyl phosphatidylcholine bilayers, which have been the most widely studied [1], but may not be relevant to phospholipids with other headgroups.

C_{28} and C_{29} analogues

The long side chains of these analogues give the sterol molecules a length such that when fully extended they would be likely to penetrate into the adjacent half of a phospholipid bilayer. This would be likely to cause an increase in the fluidity of the bilayer due to the distortions that would be caused in the packing of the lipid molecules. Such an effect has already been observed [7] at molar compositions of 40 and 50% sterol and can be also seen to occur at lower sterol concentrations from the experiments reported here. At compositions as low as 10 mol% sterol the C_{28} and C_{29} analogues fluidise dipalmitoyl phosphatidylcholine liposomes more than any of the other sterols except the C_{19} analogue which suggests that this perturbing effect of the long

side chains does not require a highly condensed membrane and is caused by the individual sterol molecules.

C_{19} analogue

The effect of the sterol ring system alone can be judged from the results obtained with the C_{19} analogue, androst-5-en-3β-ol. Most previous studies have been carried out with the saturated sterol androstan-3β-ol and show that the sterol ring system alone cannot mimic the effect of cholesterol [1]. The present results together with those reported previously [6,7] show that our C_{19} analogue can cause a small but detectable effect. This is particularly clear in the experiments with the steroid spin labels and in the TEMPO partitioning experiments. Here the C_{19} analogue at 10 mol% sterol appears to abolish the initial increase in partitioning of TEMPO into the membrane below the phase transition temperature. This pretransition has been attributed to a change in the structure of the headgroup region of the bilayer [27] and it appears that further effects on the melting transition of dipalmitoyl phosphatidylcholine require some side chain structure.

Conclusions

Overall the experiments reported here make it clear that the fit of cholesterol into membranes containing phosphatidylcholine must be very precise indeed. Cholesterol appears to have the exact length required to maximise interactions between neighbouring molecules without disturbing the bilayer structure which is essential for the effects which are characteristic of cholesterol to be observed. The evolutionary significance of this in relation to the role of cholesterol and related plant sterols in cell membranes is a fascinating question.

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