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CHOLESTEROL-PHOSPHOLIPID INTERACTIONS

THE ROLE OF THE 3β-POSITION IN MEMBRANE ORDERING AND INTERMEMBRANE EXCHANGE

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The role of the 3β-hydroxy substituent of cholesterol in sterol-lipid interactions has been examined by incorporating the 3β-thiol analogue, thiocholesterol into egg phosphatidylcholine membranes. Thiocholesterol concentration reaches a maximum at 19% on a molar basis. The degree of phospholipid ordering, as judged by a cholestane spin probe, is significantly weaker than cholesterol but is concentration-dependent up to 20 mol%, a concentration that correlates well with that for the maximum thiocholesterol incorporation into liposomes. The apparent rate constants for exchange between liposomes and erythrocytes of cholesterol and thiocholesterol are indistinguishable. The results suggest a role of hydrogen bonding between the 3β-hydroxy group of cholesterol and phospholipids in determining the concentration and membrane ordering properties of cholesterol.

Introduction

In recent years a considerable effort has been directed toward understanding the functional role of cholesterol in biological membranes. Although a wide variety of processes in a number of different organisms respond to changes in cholesterol level [1–3], detailed analysis of how these cholesterol-dependent activities are regulated is lacking. Based largely upon the condensing and fluidizing effects of cholesterol on phospholipid fatty acyl chains [4–6], one model that has emerged suggests that the physical state of the lipid milieu of membrane proteins is altered in response to changes in cholesterol content. In agreement with this suggestion is the altered organization of erythrocyte proteins [7,8] that accompany microviscosity changes induced by cholesterol depletion and enrichment. However, an alternative to this indirect mechanism of action for cholesterol, namely a direct protein-cholesterol interaction, is suggested by the recent findings of Bloch and coworkers [9,10]. Under conditions where bulk membrane viscosity was unaffected, cholesterol augmented the growth of *Mycoplasma capricolum* in concert with lanosterol; neither steroid supported normal growth when added alone. Thus, to understand more clearly the distinguishing characteristics of direct or indirect cholesterol effects more information is needed regarding cholesterol-

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phospholipid and cholesterol-protein interaction at the molecular level.

Thiocholesterol (cholest-5-ene-3β-thiol) is a cholesterol derivative which may shed light on both interactions. Firstly, as a thiol analogue of cholesterol, the nature of the 3β-position may be examined to characterize phospholipid-cholesterol interactions at that site. Secondly, as a reactive group capable of forming disulphide linkages, thiocholesterol may also be a useful probe for cholesterol-protein interactions. Thus, as a first step in exploring this analogue, we have incorporated thiocholesterol into egg phosphatidylcholine bilayers and compared both its rate of exchange and its effect upon the order parameter of a spin-labelled cholestane to that of equal concentrations of cholesterol. An earlier attempt to measure the ordering effect of thiocholesterol on the steroid probe was obviated by reduction of the probe by the thiol moiety [11]. We have found that whereas the maximum concentration of thiocholesterol obtained in egg phosphatidylcholine bilayers is less than that of cholesterol, and its ordering effect on the cholestance spin probe is significantly weaker than that of cholesterol, the apparent rate constant for intermembrane exchange of cholesterol and thiocholesterol are indistinguishable. The role of hydrogen bonds in cholesterol-phospholipid interactions is discussed.

Materials and Methods

Using methods adapted from Cooper et al. [12] and Huang et al. [13], sterols were incorporated into egg phosphatidylcholine liposomes by sonication. Briefly, chloroform solutions of cholesterol (Sigma) or thiocholesterol (Eastman) and egg phosphatidylcholine (ovolecithin, Sigma) were mixed and the solvent evaporated under a nitrogen stream. The lipids were dried further by storing 6–18 h in vacuo over P_2O_5 and subsequently hydrated with 0.01 M Tris-HCl buffer, pH 8.0, containing 0.1 M NaCl plus, in some preparations, 0.1 M ethylenediaminetetraacetic acid (EDTA) or 0.01 M 2-mercaptoethanol. Sufficient buffer was added to give an egg phosphatidylcholine concentration of 4 mg/ml. Sonication proceeded under nitrogen at 0°C for 60 min using an Artek Sonic 300 Dismembrator, operating at approx. 100 W.

After sonication, 4 ml of a 2% solution of bovine serum albumin was mixed with each 10 ml of sonicate, and the mixture centrifuged at $44000 \times g$ for 30 min to remove undispersed lipid and metal particles released from the probe.

To determine the molar proportions of phospholipid and cholesterol, lipids were extracted from the $44000 \times g$ supernatant fraction by the method of Bligh and Dyer [14]. Samples were taken in duplicate from this extract to assay sterol and phospholipid content by the methods of Zlatkis and Zak [15] and Bartlett [16], respectively.

In sterol-exchange experiments thio[4-^{14}C] cholesterol was synthesized from [4-^{14}C]cholesterol (New England Nuclear) by first preparing the 3β-tosyl derivative, as described by Wallis et al. [17]. Thio[4-^{14}C]cholesterol was then prepared essentially as described by King et al. [18] but scaled down to milligram quantities. The product had characteristics identical to those of an authentic thiocholesterol standard (Eastman) in terms of melting point, optical rotation and mobility in two thin-layer chromatographic systems. Retention of the 3β-configuration of the thiol group was confirmed by comparing NMR spectra of the product with thiocholesterol, cholesterol and epicholesterol.

Erythrocytes were prepared from whole human blood (Type A^+) obtained from the Red Cross by first centrifuging at $1600 \times g$ for 10 min. The 'buffy coat' was removed along with the plasma and the packed cells washed twice in 5 mM Tris-HCl buffer, pH 7.4, containing 0.1 M KCl, 0.06 M NaCl, 10 mM glucose, 100 units penicillin (Gibco) per ml and 100 μg/ml streptomycin (Gibco). Suspensions of erythrocytes (10% v/v) were prepared in this buffer and equilibrated to 37°C and then mixed with an equal volume of liposomes prepared in the same buffer. Duplicate samples were removed at various times and mixed with 40 vol. ice-cold 5 mM phosphate buffer, pH 8.0. The erythrocyte membranes were collected on glass fiber filters (Whatman, GF/F) and separated from liposomes by washing the filter with two successive aliquots of 5 mM phosphate buffer, pH 8.0. Filters were then counted. Contamination by liposomes alone was less than 5% and duplicates averaged $\pm 10\%$.

Oriented multibilayer films were prepared by drying down a solution of egg phosphatidylcholine containing a known mole % of sterol and cholestane spin probes as described in detail elsewhere [19]. Controls containing egg phosphatidylcholine and the spin probe only, were prepared each day.

The films were dried overnight under vacuum and hydrated for 5 min with Ringer's buffer. Electron spin resonance spectra were taken at 23°C, immediately after each film was drained to minimize signal loss due to probe reduction. For each concentration of cholesterol and thiocholesterol, five to ten films were prepared. The data are given as the averages for the replicate samples. Representative spectra are shown in Fig. 1. Order parameters for the spin probe were calculated in the manner described by Smith and Butler [19]; $S = (T'_{\parallel} - T'_{\perp})/(T_{\parallel} - T_{\perp})$ where $T'_{\parallel}$ and $T'_{\perp}$ are indicated on Fig. 1, and $(T_{\parallel} - T_{\perp})$ was taken as 13 G due to rapid axial rotation of the probe. Measurements of the order parameter were reproducible to within 0.02 units for the replicate samples.

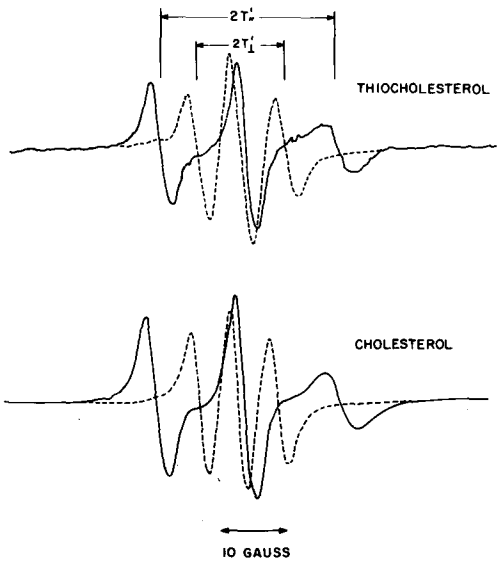

Fig. 1. ESR spectra (9 GHz) of the cholestane spin probe (1% of total lipid) in oriented multibilayer films of egg phosphatidylcholine containing 20 mol% of thiocholesterol or cholesterol, 25°C. The dashed and solid spectra were obtained with the plane of the film perpendicular and parallel, respectively, to the applied magnetic field.

Results

From the analysis of five preparations of liposomes, in which the molar proportions of thiocholesterol to egg phosphatidylcholine were initially one to one, it was found that in the resultant sonicate the corresponding molar ratio was 0.23 (Table I). Since it has been reported that the proportions of cholesterol to egg phosphatidylcholine under similar conditions yielded a molar ratio close to unity [12], and that oxidation of thiocholesterol to the disulphide might occur during sonication, EDTA and/or 2-mercaptoethanol were added to minimize thiol oxidation. However, despite the presence of these antioxidants, either alone or in combination, no enhancement of the thiocholesterol content of the liposomes was apparent. Table I also shows the molar ratio of thiocholesterol to egg phosphatidylcholine resulting from mixing either an excess of thiocholesterol over phospholipid, or a mixture with the reverse proportions. In both these cases no appreciable enhancement of thiocholesterol content was evident. Thus, there is a limiting molar ratio of 0.23:1 (19 mol%) for the thiocholesterol content of egg yolk phosphatidylcholine vesicles, a value considerably less than that reported for cholesterol under similar conditions [12].

The order parameters derived from the ESR spectra are shown in Fig. 2. The data for cholesterol itself are in agreement with those reported earlier [2,20]; a steep linear increase in ordering occurs with increasing cholesterol concentration up to a molar ratio of 1:1. At higher cholesterol concentrations the solubility limit is exceeded, and the ordering effect levels off. With thiocholesterol the ordering effects are much smaller, and reach a maximum at 20 mol% sterol, decreasing slightly with further increase in sterol concentration.

Interactions between the sterols and phospholipid were next examined from the standpoint of their exchange rates between liposomes and erythrocyte membranes. As seen in Fig. 3 the time course of cholesterol and thiocholesterol uptake by erythrocytes are best described by a single curve. The apparent rate constant from this curve is 0.35 h^{-1} and the half time is 1.98 h. Thus, in contrast to the different solubilities of cholesterol and thiocholesterol and their different ordering effects

TABLE I

MOLAR PROPORTIONS OF THIOCHOLESTEROL AND EGG PHOSPHATIDYLCHOLINE IN LIPOSOMES

Additions	Thiocholesterol : phospholipid ratio (mol/mol)	
	Initial	Final
None	1.0 : 1.0	0.23 ± 0.03 [a]
None	1.0 : 5.0	0.17
None	5.0 : 1.0	0.27
0.1 M EDTA	1.0 : 1.0	0.23
0.01 M 2-mercaptoethanol	1.0 : 1.0	0.24
0.1 M EDTA + 0.01M 2-mercaptoethanol	1.0 : 1.0	0.22

[a] Mean ± S.D. of five determinations.

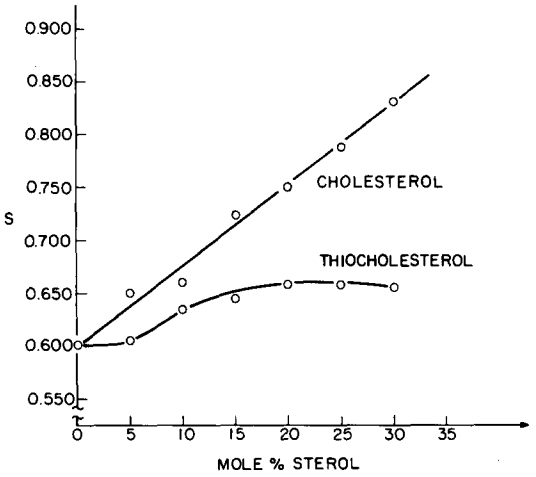

Fig. 2. Dependence of the order parameter of the cholestane spin probe on the content of cholesterol or thiocholesterol in oriented multibilayer films of egg phosphatidylcholine.

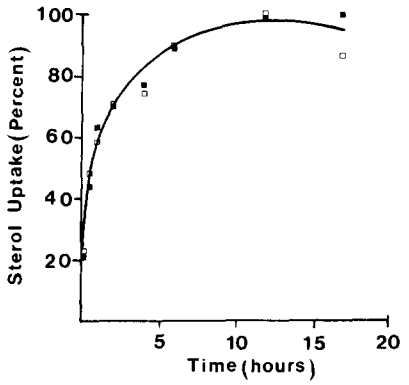

Fig. 3. Sterol uptake by human erythrocytes. The sterol uptakes are normalized as a percentage of the maximum incorporation obtained. Maximal uptakes were 1.83 and 27.3 nmol sterol per 20 μl packed cells for thiocholesterol and cholesterol, respectively. ■, cholesterol; □, thiocholesterol.

on lipid, the rates of exchange of the two sterols are the same.

Discussion

Thiocholesterol and cholesterol differ only in the nature of the group at the 3β-position and therefore any differences in phospholipid interactions are due to either different properties of thiol and hydroxy groups or different membrane locations. The latter possibility appears to be ruled out by the fact that Huang et al. [13] were able to titrate the sulphydryl groups of phosphatidylcholine/thiocholesterol liposomes, suggesting that the 3β moiety of thiocholesterol, like the hydroxy group of cholesterol, lies close to the surface of the bilayer. With respect to their different properties, hydrogen bonds between the 3β-hydroxy groups of cholesterol and phospholipid carbonyl groups have been suggested by Huang [21] based upon steric considerations and such a possibility is supported by ^{13}C and ^{1}H-NMR studies [22–24]. Since sulphur is less electronegative than oxygen, thiocholesterol may be less likely to form hydrogen bonds with membrane phospholipids. In the present work differences in sterol solubility and membrane ordering effects may therefore be the result of differing hydrogen bonding properties. Huang [21] also concluded that the 3α-isomer of cholesterol, epicholesterol, was less likely to form hydrogen bonds. Since hydrogen bonding appears to be lacking for both thiocholesterol and epicholesterol, their interactions with phospholipids should share a number of common properties. Indeed a survey of the

literature indicates that the maximum concentration obtained for epicholesterol in lipid bilayers (25 mol% [25]) is close to that of thiocholesterol (Table I). Epicholesterol also showed virtually identical effects to thiocholesterol upon water permeability of liposomes [26]; the order parameter of 12-doxylstearic acid was very similar for both sterols [11].

The comparison of the properties shared by epicholesterol and thiocholesterol suggests the participation of a 3β-hydroxy group (i.e. hydrogen bonds) in establishing the sterol content, physical state and permeability of the phospholipid bilayer. However, in recent years this concept has been questioned in the light of reports that favour non-polar sterol-fatty acid interactions. Such a notion arises from studies in which the contribution of hydrogen bonds has been precluded by the use of either diether phospholipids or sterols lacking a 3β-hydroxy group. Since these non-hydrogen-bonded analogues displayed properties similar to their hydrogen-bonded counterparts with respect to permeability [27] and condensing effects [28], the suggestion was made that non-polar interactions should be considered as contributory factors in cholesterol-lipid interactions. Whether the parallel effects of thiocholesterol and epicholesterol can be explained by considering non-polar and polar interactions as mutually exclusive contributions remains to be evaluated.

The fact that there is a correlation between the maximum solubility of thiocholesterol and the concentration at which the order parameter attained maximum values (both at 20 mol%) deserves further comment. Recent calorimetry studies [29] reveal that the heat capacity curves for phosphatidylcholine/cholesterol mixtures could be resolved into two components (one narrow and one broad) below 20 mol% but only the broad component is present at high cholesterol contents. Similar observations have also been reported for sphingomyelin/cholesterol mixtures [30]. Conflicting views as to the origin of these two endotherms have emerged. On the one hand it is proposed that sterol rich phases segregate from those enriched in phospholipid; the latter phase gives rise to the narrow endotherm [30]. On the other hand separation of sterol-phospholipid complexes of significantly different composition may occur [29]. Whatever the explanation it is clear that different lipid-sterol interactions occur below 20 mol% than at higher cholesterol concentrations. In keeping with this suggestion the activation energy for cholesterol exchange from liposomes with less than 20 mol% is about twice that for vesicles with higher cholesterol concentration [31].

The significance of the fact that the upper limit to thiocholesterol content and membrane ordering both occur at 20 mol% remains uncertain. However, defining the interactions between thiocholesterol and phospholipid that take place above and below this value must await a greater understanding of the origin of narrow and broad components of cholesterol-phospholipid mixtures. Hydrogen bonding between lipids and the 3β-position may be an important consideration in this analysis.

Intermembrane exchange of cholesterol has been examined in several biological systems [32] but there is as yet no complete agreement upon mechanisms. While one model invokes a collision complex and transient fusion of donor and acceptor membranes [33] another scheme proposes a desorption of sterol into the medium followed by a diffusion-controlled transfer to the acceptor membrane and presumably a resorption of the sterol [34]. The degree of similarity between rates of cholesterol and thiocholesterol exchange are difficult to explain in the absence of a clearly defined exchange mechanism. However the apparent rate constant for exchange is in agreement with published values [34,35]. There have been no previous reports of thiocholesterol exchange between membranes, although recently it was reported that thiocholesterol undergoes rapid transbilayer movement [36]. This latter observation may indicate a greater hydrophobicity of thiocholesterol that is consistent with the reduced hydrogen bonding (i.e. polar) interactions of this sterol. However, with respect to the exchange characteristics of thiocholesterol the role of hydrogen bonding remains to be evaluated.

Differences between thiocholesterol and cholesterol regarding membrane ordering and solubility are contrasted with similar rates of exchange between liposomes and erythrocyte membranes. The possibility that hydrogen bonds may account for these contrasting effects is currently under investigation.

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