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The influence of cholesterol 3-sulphate on phase behaviour and hydrocarbon order in model membrane systems

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Cholesterol 3-sulphate (CS) is a component of the intercellular lipid found in the uppermost layer of human epidermis (the 'stratum corneum') and is thought to play an important role in tissue cohesion. In this investigation we have compared the influence of cholesterol (CH) and CS on the gel-to-liquid crystalline phase behaviour, the polymorphic phase behaviour, and the hydrocarbon order profile in selected model membranes. It is shown that in sphingomyelin (SPM) systems, the presence of equimolar amounts of either CH or CS eliminates the gel-to-liquid crystalline transition as detected by calorimetry. Similarly, in 1-palmitoyl,2-oleoyl-phosphatidylethanolamine (POPE) dispersions containing a perdeuterated palmitoyl chain (POPE-d_{31}), it is shown that both CH and CS exert an ordering effect as determined by ^{2}H-NMR techniques, however, CS is less potent at temperatures both above and below that of the main transition for the native phospholipid. Alternatively, in mixed systems containing dioleoylphosphatidylethanolamine (DOPE) and SPM (DOPE/SPM, 6:1 mol/mol) CH promotes thermotropic $L_\alpha \rightarrow H_{II}$ phase transitions, whereas CS stabilizes the bilayer organization. These bilayer stabilization effects can be diminished by addition of Ca^{2+}. These effects are consistent with a larger area per molecule of CS as compared to CH, presumably related to the presence of the negatively charged sulphate moiety of CS.

Introduction

Sulphated sterols are found in many mammalian tissues, but their physiological significance is not always clear [1]. A function suggested for one of these lipid species, cholesterol 3-sulphate (CS), is to 'stabilize' the physical properties of biological membranes. Roberts and his colleagues demonstrated that addition of CS to erythrocyte ghost membranes increased protection from osmotic lysis [2,3], and speculated that such protection was due to increased 'membrane stability' conferred by CS. The same group proposed [4–6] that the hydrolysis of CS within sperm membranes may permit membrane destabilizing events, and so constitute the first essential step in 'capacitation' (the acquisition by sperm through residence in the female genital tract of the capacity to fertilize an ovum). Subsequently, Epand and his colleagues demonstrated that CS stabilized model membranes [7,8] as determined by an increase in the temperature of the $L_\alpha \rightarrow H_{II}$ phase transition, and found also that addition of CS to erythrocyte membranes inhibited haemolysis induced by Sendai virus [9]. Although there is no single definition of 'membrane stability', the term is used broadly to mean resistance of a lipid bilayer structure to disruption. Such disruption may lead variously to lipid phase transitions [10], membrane fusion [11], membrane rupture [12], and increased membrane permeability [13], events that are not necessarily mutually exclusive [14]. From this perspective it may be seen that CS has been shown to increase some measures of model membrane stability, and may conceivably play an analogous role in biological membranes.

This hypothesis has been extended explicitly to epidermis, the epithelial covering of mammalian skin. In human epidermis CS is synthesized and then hydrolyzed during the terminal differentiation of epidermal epithelial cells ('keratinocytes'), and this so-called

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Abbreviations: DOPE, dioleoylphosphatidylethanolamine; EDTA, disodium ethylenediaminetetraacetate; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulphonic acid); H_{II}, inverted hexagonal phase; L_α, liquid crystalline phase bilayer; NaCl, sodium chloride; NMR, nuclear magnetic resonance; POPE, 1-palmitoyl,2-oleoyl-phosphatidylethanolamine; XLRI, X-linked recessive ichthyosis.

'cholesterol sulphate cycle' [15] is associated with the formation of unusual intercellular domains in the outermost layer of human epidermis, the 'stratum corneum' (see Refs. 16 and 17 for reviews). The large lipid component of these domains seems clearly to assume a lamellar organization, but the details of such structures have yet to be determined [18]. Loss of intercellular cohesion ('desquamation') is an essential physiological function in this continuously proliferating tissue and has been observed to be associated with the loss and presumed hydrolysis of CS [19]. Furthermore, the absence of cholesterol sulphatase (arylsulphatase C, EC 3.1.6.2) in the genetic disorder X-linked recessive ichthyosis (XLRI) leads to accumulation of CS within the stratum corneum [20], and is associated with a particular clinical pattern of 'scaling' and thickening of the stratum corneum [21] that represents a disorder of normal desquamation. Taken together, this evidence has led logically to the hypothesis that CS may promote cohesion in the stratum corneum by increasing the stability of intercellular membranes [16], although CS may play other biological roles as well [15].

An obvious test of this hypothesis would be to contrast the effects of cholesterol and cholesterol sulphate on lipid polymorphism in a model system simulating the stratum corneum intercellular membranes. However, the structure and phase behaviour of the relevant lipids (e.g., ceramides) are not well understood, and we have therefore used standard phospholipid model membranes to investigate, in a comparative manner, the influence of CH and CS on gel-to-liquid crystalline behaviour, polymorphic phase behaviour, and hydrocarbon ordering. We sought to extend existing observations in several ways. First, we used relatively high membrane concentrations of CS (12.5–50% mol/mol lipid), since sterols and sterol derivatives may account for up to a third of stratum corneum intercellular lipid [22,23]. Second, we used a mixed system for polymorphism studies, since cholesterol has been demonstrated to have a profound destabilizing effect in mixed systems [24], and since stratum corneum lipid is itself a mixed system. Finally, we have contrasted the effects of CH and CS on hydrocarbon order in the fluid bilayer phase, an area not explored to date.

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Materials and Methods

Egg sphingomyelin (SPM), dioleoylphosphatidylethanolamine (DOPE), and 1-palmitoyl-d_{31}-2-oleoylphosphatidylethanolamine (POPE-d_{31}) were obtained from Avanti Polar Lipids (Alabaster, AL, USA 35007), and cholesterol and cholesterol 3-sulphate from Sigma (St. Louis, MO, USA 63178–9916). Samples were prepared by mixing appropriate volumes from stock solutions in benzene/methanol (7:3, v/v), and then freeze-drying. The resulting white powders were hydrated at room temperature by gentle agitation. Unless otherwise noted the buffer was 150 mM NaCl, 100 mM Hepes, 4 mM EDTA (pH 7.0). For experiments in which the pH was varied, separate solutions were made for each pH. Citrate (100 mM) was employed for pH 4.0–6.0, whereas Hepes (100 mM) was used for pH 7.0 and 8.0. For calcium experiments aliquots of a stock solution of $CaCl_2$ were added to stock buffer solutions of 150 mM NaCl, 100 mM Hepes (pH 7.0) prior to lipid hydration to give the desired molar ratios of Ca^{2+}/CS.

^{31}P-NMR spectra were recorded with broad band proton decoupling at 81 MHz using a Bruker WP 200 NMR spectrometer equipped with temperature control. Before each new run all samples were frozen at −20°C, and then warmed to room temperature. ^{2}H-NMR measurements were made at 46.2 MHz on a home-built spectrometer described elsewhere [25]. The hydrating buffer for these experiments was 150 mM NaCl, 100 mM Hepes, 4 mM EDTA (pH 7.4) prepared in deuterium-depleted water. Spectra were obtained using a quadrupolar echo pulse sequence [26] having times of 300 ms, 50 μs, and 30.5 μs for the recycle delay, interpulse delay, and ring-down delay, respectively. Dwell time was 2 μs. An eight-step phase cycling sequence was used for data acquisition. The probe was tuned for each sample at each temperature. Spectra were 'dePaked' as previously described [27], and a 'smoothed' order profile derived [28].

Differential scanning calorimetry (DSC) was performed on a MicroCal 2 instrument equipped with a MicroCal 1 control unit (MicroCal, Northampton, MA, USA 01060), employing a scan rate of 10 C°/h. All DSC samples had similar thermal histories in that they were frozen after hydration, warmed to room temperature, and after loading into the calorimeter cells were equilibrated overnight at 4°C before heating runs were begun. Lipid concentrations were approx. 20 mg/ml for each sample.

Results

Initial experiments were aimed at comparing the effects of CH and CS on the gel-to-liquid crystalline phase behaviour of lipid bilayer systems, employing SPM. As shown in Fig. 1, increasing concentration of CS within SPM bilayers resulted in progressive inhibition of the co-operative endothermic gel-to-liquid crystalline transition of SPM,and at 50 mol% additive no transition could be observed. Similarly addition of 50 mol% of either CH or CS to SPM bilayers resulted in ^{31}P-NMR spectra characteristic of liquid crystalline bilayer systems over the temperature range 20–60°C (results not shown). Thus CS exerted an effect on SPM

dispersions similar to that previously observed for CH [10,29].

The next set of experiments concerned the influence of CH and CS on the polymorphic phase preferences of mixed lipid dispersions employing DOPE/SPM (6 : 1, mol/mol) as a model system. In previous work [10], it has been shown that SPM can effectively stabilize unsaturated PE, which would otherwise adopt the H_{II} configuration, in a bilayer organization. The addition of CH to such stabilized systems, however, results in reversion to H_{II} structure. These effects are illustrated in Fig. 2. In the absence of SPM, DOPE exhibits a bilayer-to-H_{II} transition as the temperature is increased through the range 5–10°C [30] but for the DOPE/SPM (6 : 1) system this transition is observed at much higher temperatures in the range of 40°C (Fig. 2a). However, the presence of CH in DOPE/SPM/CH (6 : 1 : 1, mol/mol) destabilizes the bilayer organization (Fig. 2b) and appreciable H_{II} phase structure is evident at 21°C. This contrasts strongly with the behaviour of the DOPE/SPM/CS (6 : 1 : 1, mol/mol) dispersion where complete bilayer structure is maintained to temperatures in excess of 40°C, and considerable bilayer structure is still evident at 56°C (Fig. 2c). An H_{II} component and an isotropic component are also evident at 56°C. Such 'isotropic' components are commonly observed for mixed lipid systems containing bilayer and non-bilayer lipids (see, for example, Ref. 31) and can arise from small vesicular structures or other phases such as the cubic organization [32]. The results of Fig. 2 clearly demonstrate the bilayer stabilizing

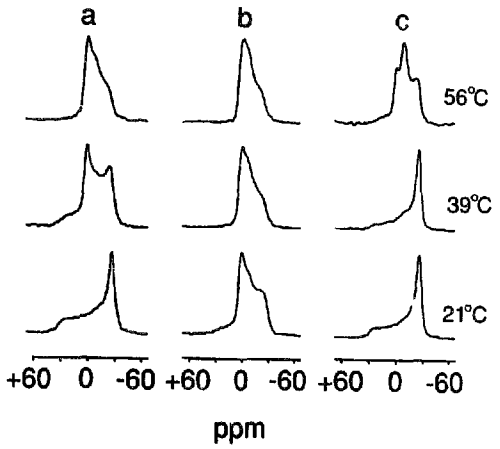

Fig. 2. Temperature dependent phase behaviour of lipid mixtures hydrated in excess buffer (150 mM NaCl, 100 mM Hepes, 4 mM EDTA, pH 7.0). ^{31}P-NMR spectra were derived from (a) DOPE/SPM (6 : 1, mol/mol), (b) DOPE/SPM/CH (6 : 1 : 1, mol/mol), (c) DOPE/SPM/CS (6 : 1 : 1, mol/mol). Addition of cholesterol in (b) lowered the range of the $L_\alpha \rightarrow H_{II}$ transition observed in (a), whereas addition of cholesterol sulphate (c) resulted in a bilayer signal being observed at higher temperatures than for (a) or (b). An isotropic signal was observed at higher temperatures (e.g., 56°C) in dispersions containing CS.

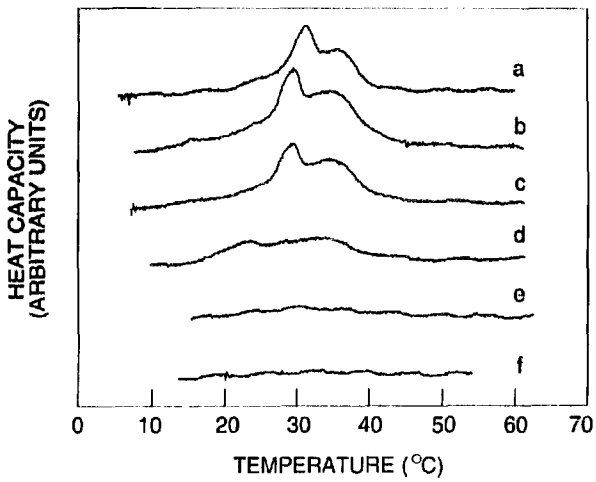

Fig. 1. Differential scanning calorimetry of hydrated egg sphingomyelin (SPM) containing the following concentrations (mol%) of cholesterol sulphate (CS): (a) 0, (b) 5, (c) 10, (d) 20, (e) 40, and (f) 50. Lipids were hydrated in excess buffer (150 mM NaCl, 100 mM Hepes, 4 mM EDTA, pH 7.0) to a concentration of about 20 mg/ml. A broad multi-component endothermic transition is observed for SPM alone between 20 and 40°C. This peak becomes progressively broader with increasing concentration of CS, and is no longer apparent at 50 mol% additive.

capacity of CS which contrasts with the strong destabilizing influence of CH in these mixed systems.

As has been extensively discussed elsewhere [32] the ability of certain lipids to stabilize bilayer organization as compared to H_{II} organization in mixed systems can be attributed to factors leading to an increase in the membrane area at the lipid/water interface as compared to the hydrophobic centre of the bilayer. Thus larger headgroup molecular volumes, increased headgroup charge and increased polar headgroup hydration, are all factors that tend to stabilize the bilayer organization. In the case of CS, the increased size and net negative charge of the sulphate as compared to the hydroxyl group of cholesterol would be expected to serve these functions. Conversely, factors which serve to neutralize the sulphate charge, such as high ionic strength, binding of divalent cations, and protonation of the sulphate would be expected to reduce the bilayer stabilizing capacity of CS. This effect is illustrated by the influence of Ca^{2+} (Fig. 3) where an excess of the divalent cation (Ca^{2+}/CS, 2 : 1, mol/mol) results in demonstrably reduced bilayer stabilizing capacity of CS in the DOPE/SPM (6 : 1) model system. Reducing the pH to 4.0 had no effect on the phase behaviour in this system (not shown), a result consistent with other studies that have found the effective pK_a of CS to be very low, certainly less than 4 [3,33].

Given the probable importance of CS in normal and pathological stratum corneum, it is of interest to extend these studies to compare the influence of CH and CS on the order profile in the hydrocarbon region of bilayer systems. Briefly, it is well-established that

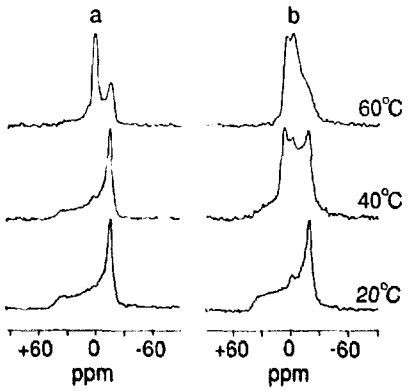

Fig. 3. The effect of calcium ions on thermotropic phase behaviour of DOPE/SPM/CS (6:1:1, mol/mol) as determined by ^{31}P-NMR. The molar ratio of calcium ions present in the hydrating buffer (150 mM NaCl, 100 mM Hepes, pH 7.0) to CS present in each lipid sample was (a) 0, (b) 2. The addition of divalent cations resulted in the emergence of an H_{II} phase as determined by the development of a lineshape having 'reversed asymmetry' [54]. A small isotropic peak is also present at 60°C.

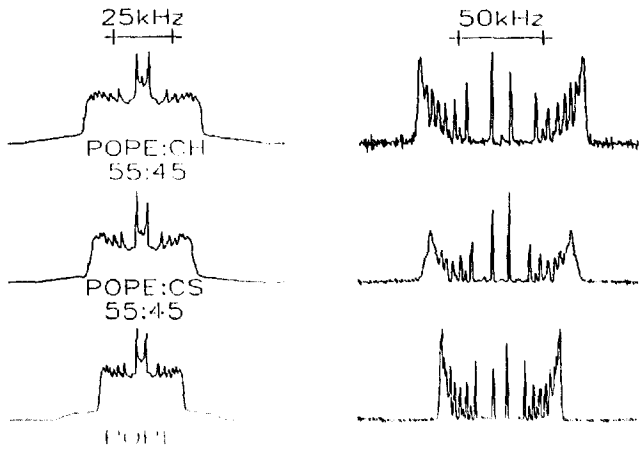

Fig. 4. ^{2}H-NMR powder patterns (left column) and corresponding deconvoluted ('dePaked') spectra (right column) derived at 27°C from dispersions of POPE-d_{31} alone, POPE-d_{31}/CS (55:45, mol/mol), and POPE-d_{31}/CH (55:45, mol/mol). The addition of either CS or CH to POPE-d_{31} results in an increase in spectral width, but the effect of CH is greater.

cholesterol can markedly increase the order in the hydrocarbon region of liquid-crystalline bilayers [34], effects which are related to increases in bilayer thickness [35], decreased membrane permeability [13] and condensing effects on the area per phospholipid molecule [35]. In this regard, phospholipids which prefer H_{II} phase structures in isolation also increase hydrocarbon order in bilayer systems [32]. It is thus difficult to predict the influence on membrane order of a cholesterol derivative which stabilizes the bilayer. In order to perform these experiments, we used a PE which had a perdeuterated palmitoyl chain (POPE-d_{31}).

The addition of 20 mol% (not shown) and 45 mol% (Fig. 4) of either CH or CS to POPE-d_{31} resulted in an increase in the width of the ^{2}H-NMR powder patterns. In order to analyze these effects more convincingly the 'dePakeing' procedure was used to obtain the 'smoothed' orientational order profiles for these dispersions [28]. As shown in Fig. 5 the presence of CS increases the hydrocarbon order appreciably, however, CH is clearly more effective in this regard. ^{2}H-NMR studies were performed on the POPE-d_{31} systems over the temperature range 4–75°C, and a plot of M_1 (the first moment of the spectrum) vs. temperature is shown

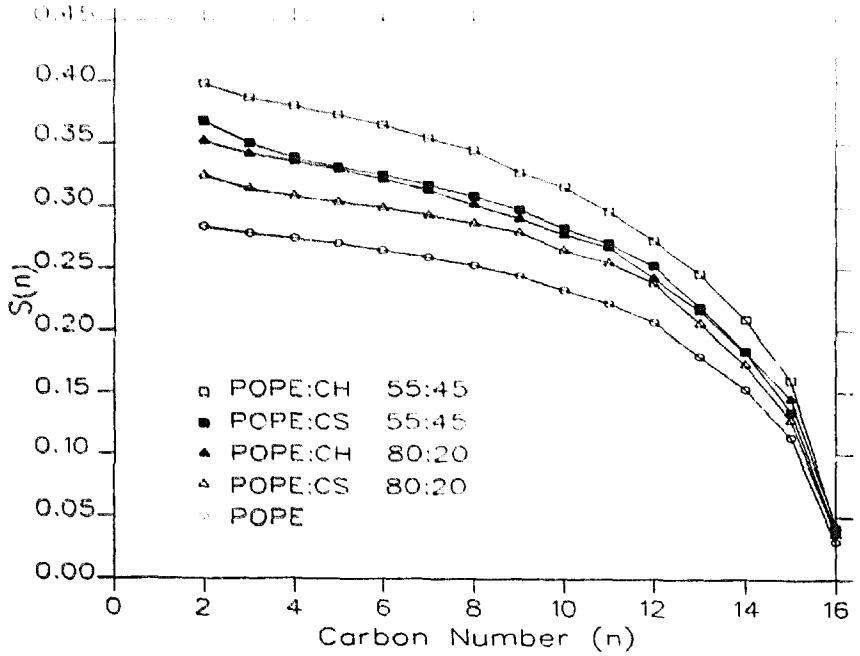

Fig. 5. 'Smoothed' order profiles derived from the dePaked spectra [28] obtained at 27°C. The carbon-deuterium bond order parameter S(n) is plotted against the carbon position on the perdeuterated palmitoyl chain of POPE-d_{31}. At concentrations of both 20 and 45 mol%, the ordering effect of CS is less than that of CH.

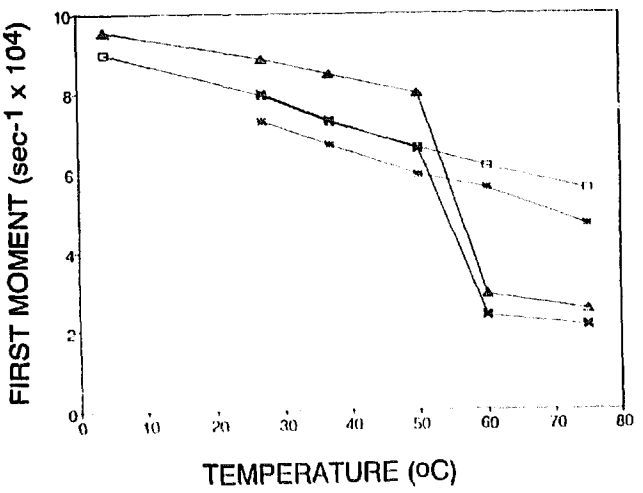

Fig. 6. First moments of ^{2}H-NMR spectra (M_1) are plotted as a function of temperature for the following hydrated mixtures (expressed as mole ratios): POPE-d_{31}/CH 55:45 (triangles), POPE-d_{31}/CS 55:45 (squares), POPE-d_{31}/CH 80:20 (×), and POPE-d_{31}/CS 80:20 (asterisks). The 'step' observed between 50 and 60°C in the plots for the dispersions containing cholesterol corresponds to the spectral narrowing associated with the $L_\alpha \rightarrow H_{II}$ transition.

in Fig. 6. M_1 has been shown to give an accurate representation of the average order for such lipid dispersions [26]. Three points are clear. First, over the interval 4–50°C, the presence of CS results in values of M_1 that are reduced (to a maximum of 17% at 50°C), in comparison with equivalent samples containing cholesterol (consistent with the results of Fig. 5). Second, at higher temperatures (> 50°C) M_1 decreases abruptly between 55 and 60°C for the CH-containing samples, whereas for the CS-containing systems, M_1 decreases only gradually with temperature. The abrupt decrease in M_1 for the CH-containing systems can be attributed to the H_{II} phase formation at temperatures above 60°C; in the absence of CH such a decrease is observed in POPE bilayers between 65 and 72°C [36]. The lack of any such abrupt decrease in M_1 for the CS containing systems is consistent with maintenance of bilayer structure at temperatures above 50°C, again indicating the bilayer stabilizing capacity of CS. Finally, both CH and CS have the ability to maintain a POPE-d_{31} bilayer in a fluid phase, most likely 'liquid ordered' [37], at temperatures below the gel-to-liquid crystalline transition temperature for the native phospholipid (22°C [21]). However, the bilayers containing CS were less ordered than those containing CH, a difference maintained throughout the experimental ranges of temperature and composition.

Discussion

The results of this investigation show interesting similarities and differences between the influence of cholesterol and cholesterol sulphate on the physical properties of lipid systems. Here we discuss the physical basis of these effects and subsequently possible biological interpretations.

The major similarities observed concern the ability of both cholesterol and cholesterol sulphate to inhibit gel phase structures in mixtures with sphingomyelin and POPE, and to increase the order in the hydrocarbon region of fluid bilayer systems as evidenced by the increased order in mixtures with POPE. The ability to inhibit gel phase formation corresponds to inhibition of cooperative crystallization interactions between phospholipids, and is consistent with an ability of both cholesterol and cholesterol sulphate to act as 'spacer' molecules to inhibit such molecular interactions. The ability of cholesterol sulphate to increase hydrocarbon order in fluid bilayers (albeit less effectively than cholesterol), further indicates that the interaction has a similar molecular basis for both sterols. While the details of cholesterol–phospholipid interactions are not fully understood [34,38], the increased order in the liquid-crystalline phase is generally believed to arise from increased straightening of the hydrocarbon arising from interactions of the acyl chains with the rigid sterol ring structure [21]. The so-called 'condensing effect' of cholesterol, in which the average membrane area per polar headgroup is reduced following addition of this sterol to a fluid phospholipid bilayer, is associated with this increase in orientational order [39], and is widely believed to be dependent also on the presence of a β-hydroxyl group at the 3-position of the sterol [21,34,40]. The fact that cholesterol sulphate is somewhat less effective in this capacity can be rationalized as due to a larger effective area of the putative sterol-phospholipid complex in the case of the sulphated species. Elias and his colleagues have suggested that CS may not fulfill a membrane 'fluidizing' role as does CH [16,41], but our evidence and that of Epand and his colleagues [8] is to the contrary. However, the effects of CH and CS on model membranes resembling those of the stratum corneum intercellular spaces remain to be determined.

The major difference between cholesterol and cholesterol sulphate concerns their respective influences on the polymorphic phase preferences of lipids. As illustrated here and elsewhere [24] cholesterol promotes formation of H_{II} phase structures both in this novel DOPE/SPM (6:1) system as well as the POPE dispersions. This contrasts strongly with the ability of cholesterol sulphate to stabilize bilayer organization in both model membrane systems. Epand and his colleagues have found that CS and other similar sterol esters can inhibit H_{II} phase formation in model systems [7–9] regardless of charge. This behaviour can be rationalized according to molecular shape [42,43] or intrinisic curvature [44], where the H_{II} phase formation is inhibited due to an increased membrane area at

the lipid/water interface as compared to the acyl chain region. This can be attributed to intermolecular electrostatic repulsion effects and increased hydration in the case of cholesterol sulphate, and is consistent with the ability of Ca^{2+}, which would reduce the surface charge density in the systems containing cholesterol sulphate, to induce H_{II} organization.

The relation between the physical properties of CS and biological function is unknown, although some detailed models have been presented in the context of sperm capacitation [45] and viral fusion [9]. In mammalian stratum corneum, biological function is significantly determined by two physical characteristics, tissue permeability and tissue cohesion. There is both evidence and speculation that the physical properties of the intercellular lipid lamellae are in some measure directly responsible for both tissue permeability and cohesion, and that the lipid composition of the lamellae in turn determines their physical properties (for an alternative view, see Ref. 46). Williams and Elias [16] have specifically suggested that CS accumulation within intercellular lipid may stabilize the putative bilayer structures and so alter tissue cohesion, and there is other evidence consistent with this hypothesis [19]. Although alteration of intercellular lipid phase behaviour must be considered as a possible mechanism for the orderly loss of tissue cohesion at the skin surface, other possibilities exist [47–50], and there is to our knowledge no convincing evidence that non-bilayer phases occur in this or any other tissue. On the other hand, there is strong circumstantial evidence for the involvement of non-bilayer inverted lipid structures in membrane fusion (and the reverse 'fission') events [14]. In this context, the ability of CS to inhibit fission processes leading to normal orderly loss of the outer layers of the stratum corneum (possibly the last controlled event in epidermal differentiation), could play a role in the pathology of X-linked recessive ichthyosis, and the association in this disorder between altered tissue cohesion and an increase in CS tissue concentration is most obviously rationalized as an effect on lipid phase behaviour. It is also conceivable that modification of membrane lipid is the first step in a more complex process (involving proteins for example) that leads ultimately to loss of intercellular cohesion. In this context we note that Epstein et al. [51] suggested that calcium ions might serve as interbilayer 'bridges' and so stabilize stratum corneum intercellular membranes. The evidence presented here and elsewhere [8,14] shows that the presence of Ca^{2+} will destabilize model membranes containing CS and other negatively charged lipids, as determined by $L_\alpha \rightarrow H_{II}$ transition temperatures and fusion assays. We therefore suggest that the presence of calcium ions in the stratum corneum intercellular spaces [52] will tend to destabilize rather than stabilize membranes containing CS.

Hydrocarbon order in liquid-crystalline bilayers can be directly related to membrane thickness [53] but not as yet to biological function. Differences in order between comparable systems containing CH or CS cannot therefore be related directly to functional characteristics of stratum corneum containing normal or excess mole fractions of CS. However, our observations do establish that a difference in the extent of hydrocarbon motion exists between such liquid-crystalline or liquid-ordered phases, and that those containing CS are less ordered under all conditions so far examined. Of perhaps more importance is the fact that both CS and CH can 'fluidize' a sphingolipid bilayer (in this case SPM) that otherwise would exist in a gel state at body temperature. Since a major component of stratum corneum intercellular lipid consists of various species of sphingolipid, CH and CS may have similar 'fluidizing' effects in vivo. The effects of CH and CS on dispersions of skin ceramides remains to be determined.

In summary, we have found that CS (like CH) has the ability to induce hydrocarbon ordering in lipid bilayers, but that in mixed systems containing sphingolipid and in pure glycerolipid dispersions CS stabilizes while CH destabilizes the bilayer. We propose that in the stratum corneum intercellular lipid, CS may play a role very similar to that of CH in establishing fluid bilayer phases, but at the same time CS may act to preserve membrane integrity by eliminating the destabilizing influence of cholesterol

Acknowledgements

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