Phase behavior of artificial stratum corneum lipids containing a synthetic pseudo-ceramide: a study of the function of cholesterol

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Abstract The phase properties and structural characteristics of stratum corneum (SC) lipid lamellae have been a subject of considerable interest. To clarify the individual role of the stratum corneum constituent lipids, such as ceramides, free fatty acids, and cholesterol, we investigated the thermotropic properties and aggregation structures of a pseudo-ceramide/stearic acid (1/1 mole ratio)–cholesterol system, which is a simplified model for the natural lipids. Differential scanning calorimetry (DSC) detected decreases of melting entropies (ΔS_m) by the incorporation of cholesterol into both anhydrous and hydrated equimolar mixture of pseudo-ceramide (SLE) and stearic acid. Moreover, there was a linear relationship between the cholesterol content and the melting entropies in the region of 0–33 mol% cholesterol for both the anhydrous and hydrate lipids. In addition, as the concentration of cholesterol increased, a liquid lateral packing (4.5 Å) appeared in the wide-angle X-ray diffraction and the intensity of a hexagonal packing (4.15 Å) decreased. The results from the present study strongly follow the idea that cholesterol can regulate the mobility of hydrocarbon chains of the natural stratum corneum lipid bilayer, which is primarily responsible for the barrier properties.–**Mizushima, H., J-I. Fukasawa, and T. Suzuki.** Phase behavior of artificial stratum corneum lipids containing a synthetic pseudo-ceramide: a study of the function of cholesterol. *J. Lipid Res.* 1996. **37:** 361–367.

Supplementary key words stratum corneum lipids • lipid bilayer • pseudo-ceramide • cholesterol • stearic acid

One of the most vital functions of the stratum corneum (SC), the outermost layer of the skin, is known to provide a barrier against water loss through the skin. It was reported that removal of the SC resulted in an approximate 100-fold increase of the epidermal water loss (1, 2). The effective barrier function of SC has been attributed to a highly ordered structure of lipid bilayers observed in the intercellular space of SC (3–5). Therefore, the mechanism for the self-assembly of the lipid bilayer as well as their physicochemical properties has been a subject of considerable interest (6–10). The SC lipid (SCL) lamellae is predominantly made up of ceramide, free fatty acids, cholesterol, and cholesteryl esters, and the presence of ceramides has been suggested to be the basis for the structural organization of SCL in bilayers (3, 11). Although a number of reports have appeared on the phase behavior of ceramides and their function in the SCL membranes (8, 12, 13), there has not been a systematic detailed study of the structure–function relationships of ceramides. Part of the difficulty may be due to the use of mixtures of various sources of natural ceramides.

Recently, Imokawa and co-workers (14–17) synthesized a pseudo-ceramide, named sphingolipid E (SLE), which had a molecular structure analogous to that of the natural non-hydroxy fatty acid ceramide of type 2 (18). They examined the dermatological applications of SLE to the dry and scaly skin, and found that the synthetic ceramide exhibited water-retaining properties similar to those of the natural lipids. The results have prompted us to investigate the physicochemical properties of SLE, which has a well-defined molecular structure. Studying the intermolecular interaction of SLE and other major components of SCL, fatty acids, cholesterol, and cholesteryl esters, we successfully prepared stable bilayers from SLE and long-chain saturated fatty acids (stearic acid and/or palmitic acid) in anhydrous systems (19–21). However, lipid mixtures whose saturated fatty acid had been replaced by oleic acid failed to form bilayers and were in rigid crystalline states (21). These results suggest that fatty acids as well as ceramides are essential to the self-organization of the natural SCL and that stable bilayer formation depends considerably on

Abbreviations: ΔH_m, melting enthalpy; ΔS_m, melting entropy; T_m, melting point; DSC, differential scanning calorimetry; SCL, stratum corneum lipids; SLE, sphingolipid E.

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fatty acid molecular structure. In addition, previous studies (21) have shown that incorporation of cholesterol into an anhydrous equimolar mixture of SLE and stearic acid caused a marked decrease of melting entropy. While the effect of cholesterol on the thermotropic properties of SLE/stearic acid system can be attributed to the disorder of the molecular packing, there is limited information available on a molecular mechanism of this phenomenon. Moreover, effect of hydration on the phase behavior of SLE/stearic acid/cholesterol system has not been studied.

We therefore focused on the role of cholesterol in this simplified model system, and reinvestigated the detailed thermotropic behavior and structural characteristics of SLE/stearic acid(1/1 mole ratio)–cholesterol system with and without water. Thus, the object of the present study is to elucidate the role of cholesterol on the phase properties of SCL as well as the influence of water for bilayer-forming capacity.

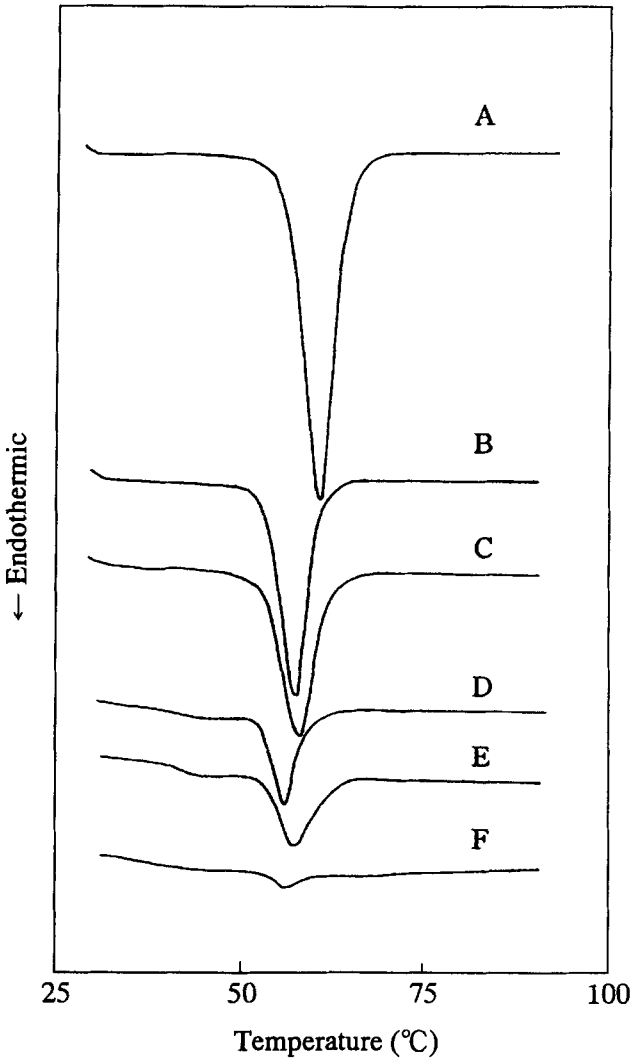
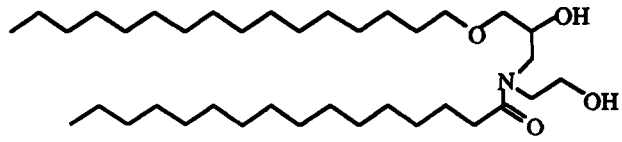


Fig. 1. DSC profiles of anhydrous sphingolipid E (SLE)/stearic acid/cholesterol mixtures (SLE/stearic acid = 1:1 mole) with weight fraction of cholesterol being (A) 0, (B) 0.10, (C) 0.20, (D) 0.30, (E) 0.40, and (F) 0.50.



Scheme 1.

MATERIALS AND METHODS

Materials

The pseudo-ceramide (**Scheme 1**), N-(3-hexadecyloxy-2-hydroxypropyl)-N-2-hydroxyethyl hexadecanamide (sphingolipid E; SLE) was obtained from Kao Co. (Tokyo, Japan) and the purity was greater than 99% as measured by high performance liquid chromatographic analysis. Stearic acid and cholesterol of reagent grade were purchased from Wako Pure Chemical Industry (Osaka, Japan). Chloroform and water were of spectrophotometric grade.

Preparation of samples

Appropriate amounts of lipids containing SLE were dissolved in chloroform at 30–40°C. The choloform solutions were evaporated under a stream of nitrogen by using a rotary evaporator and were dried in vacuo for more than 24 h. The water contents of the anhydrous lipid mixtures were checked by Karl Fischer's (22) method and were proven to be less than 0.1%. Hydration of samples was achieved by heating the lipid mixtures and water (lipid concentration = 50 wt %) at 80°C for 30 min with vigorous agitation. Then the samples were cooled to 25°C and maintained at that temperature for 7 days or more, after which all measurements were started.

Differential scanning calorimetry (DSC)

DSC analyses were carried out on a DSC-100 instrument (Seiko Instrument & Electronics Ltd., Tokyo, Japan). The lipid mixture (10–20 mg) was put into a silver DSC capsule (Seiko). The capsule was sealed and placed in the DSC cell along with a vacant reference. Then the sample was heated usually from 20°C to 100°C at 2°C/min. The melting point (T_m) was determined by extrapolation to the baseline of the most rapid rise in the excess heat capacity curve as a function of temperature. The melting point enthalpy (ΔH_m) was determined by integrating the area under the curve from a plot of the excess heat capacity as a function of temperature. In the aqueous lipid mixtures, the ΔH_m calculation was based on the weight of the added lipids and that of water

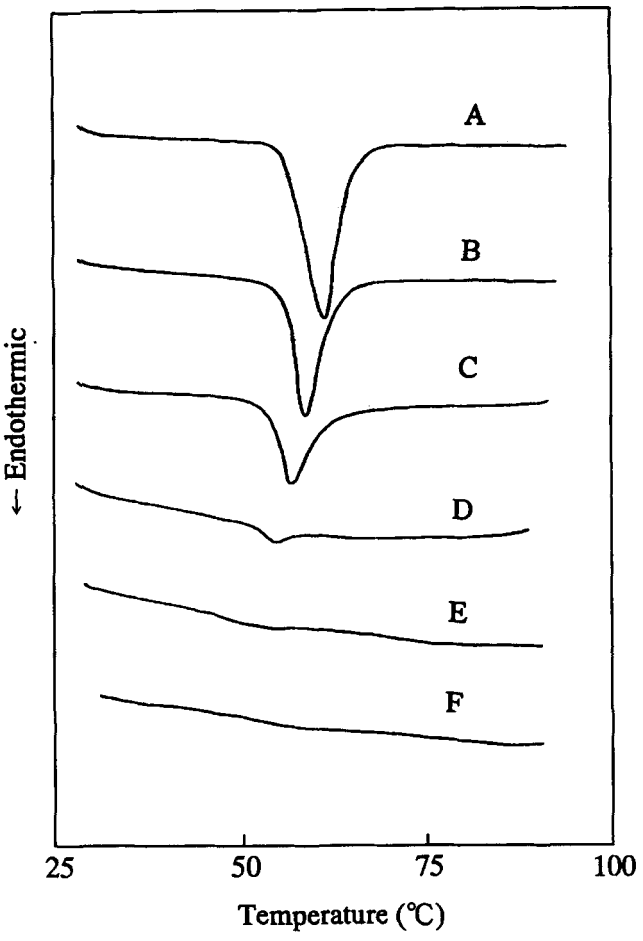


Fig. 2. DSC profiles of hydrated sphingolipid E (SLE)/stearic acid/cholesterol system (SLE/stearic acid = 1:1 mole) with weight fraction of cholesterol being (A) 0, (B) 0.10, (C) 0.20, (D) 0.30, (E) 0.40, and (F) 0.50.

was neglected. The melting entropy (ΔS_m) was calculated from the equation: $\Delta S_m \approx \Delta H_m/T_m$.

X-ray diffraction

The aggregation structure of lipids was identified by powder X-ray diffraction with a wide-angle diffractometer, XD-7A (Shimadzu Co., Kyoto, Japan). Cu-Kα radiation (λ = 1.54 Å) was used in X-ray diffractometries. The lipid mixtures were placed on the X-ray diffraction glass plate in a sample holder and then characterized at 25°C. The d-spacings (Å) were converted from the diffraction angles (degree) by the Bragg equation: $n\lambda = 2d \sin\theta$.

RESULTS

Differential scanning calorimetry

DSC profiles of anhydrous and hydrated mixtures of SLE/stearic acid (1/1 mole ratio)-cholesterol system with weight cholesterol content being 0–50 wt% are presented in **Fig. 1** and **Fig. 2,** respectively. The effect of the heating rate was examined on the thermal profiles for some anhydrous samples. In the range of 0.5 to 2.0°C/min, the heating rate had no effect on the general form of the thermograms. Therefore, DSC measurements were performed usually at 2.0°C/min as described before. All thermograms were analyzed in order to estimate the melting point (T_m) and the melting enthalpy (ΔH_m), and the results are summarized in **Table 1.** Addition of cholesterol into the anhydrous and hydrated equimolar mixtures of SLE and stearic acid produced marked changes in the DSC thermal profiles. In the anhydrous system, as cholesterol content was increased, the endothermic peak at around 60°C, corresponding to the melting of α-crystal (21), shifted to a lower temperature and there was a progressive reduction in the enthalpy change by melting (ΔH_m). At a cholesterol content of 33 mole% (30 wt%), an approximate composition in the natural SCL without cholesteryl esters, cholesteryl sulfate, and other minor constituents (6), the melting temperature (T_m) was 5°C below the value of the cholesterol free lipids and the melting enthalpy (ΔH_m) was reduced by 70% (Fig. 1 and Table 1).

On the other hand, by the incorporation of cholesterol into the equimolar mixture of SLE and stearic acid, there was a change in the hardness of the sample. The equimolar mixture without cholesterol was a hard wax-like solid. However, after increasing the cholesterol concentration, the samples became softer.

In the aqueous system, trends of the thermotropic changes by the incorporation of cholesterol were basically the same as in the anhydrous mixtures, while the value of melting enthalpy (ΔH_m) was smaller than that of the corresponding anhydrous mixture (Fig. 1, Fig. 2, and Table 1). At cholesterol concentrations > 33 mol%, the endothermic peak was extremely decreased and broadened (Fig. 2, curves E and F).

To estimate the degree of order of molecular packing, the entropy changes by melting (ΔS_m) were calculated from the ΔH_ms and the melting points, and plotted as a function of mole fraction of cholesterol (**Fig. 3**). The ΔS_m values of both the anhydrous and hydrate mixtures were decreased markedly by the incorporation of cholesterol. The ΔS_ms of the hydrated lipids were smaller than those of the corresponding anhydrous mixtures over the range of 0–53 mol% cholesterol. Moreover, there were linear relationships between the ΔS_m and mole fraction of cholesterol over the range of 0–33 mol% cholesterol, with a slope of -358.0 (r = 0.99) in the hydrate system (closed circles in Fig. 3) and that of -283.7 (r = 0.97) in the anhydrous mixtures (open circles in Fig. 3). The difference of ΔS_m between the anhydrous mixture (open circles in Fig. 3) and the hydrated lipids (closed circles) was increased progressively from 13 J/K • mol at 0 mol%

TABLE 1. Melting point (T_m) and melting enthalpy (ΔH_m) of SLE/stearic acid/cholesterol system[a]

	Anhydrous		Hydrated[b]	
Cholesterol	T_m	ΔH_m	T_m	ΔH_m
Wt%	°C	KJ/mol	°C	KJ/mol
0	60.4	43.9	62.4	39.9
5	58.7	41.5	61.1	34.8
10	57.2	35.6	60.4	23.9
15	57.9	32.6	59.9	19.5
20	57.2	24.4	59.2	12.8
25	55.0	18.9	58.8	5.5
30	55.4	13.1	58.1	1.9
35	55.2	11.9	57.5	1.4
40	56.6	11.9	56.6	0.2
45	55.5	.3.5	56.6	0.2
50	55.2	2.1	57.2	0.4

[a]SLE/stearic acid = 1/1 mole ratio.
[b]Water content = 50.0 wt %.

cholesterol to 34 J/K • mol at 33 mol% cholesterol. At cholesterol concentrations > 33 mol%, there was a further decrease in the ΔS_m for the anhydrous mixture and another endothermic peak at around 40–45°C was detected (Fig. 1, curves D, E, F). This peak may be related to the polymorphic transition of phase-separated cholesterol (12, 23). On the other hand, for the hydrate lipids, there was no significant change in the range of 33–53 mol% cholesterol.

The entropy decrease by the incorporation of cholesterol into the equimolar mixture of SLE and stearic acid as well as the hydration could be attributed to the disorder of the molecular packing (21).

X-ray diffraction

X-ray diffraction experiments were performed at 25°C to determine the structures below the calorimetric transitions. The X-ray patterns of the anhydrous mixtures of SLE/stearic acid (1/1 mole ratio)–cholesterol (0–50 wt%) are shown in **Fig. 4** and the hydrated system is shown in **Fig. 5.** The X-ray diffraction pattern of the anhydrous equimolar mixture of SLE/stearic acid demonstrated a strong single peak at 4.15 Å in the short spacing region (Fig. 4, A). The strong diffraction peak at 4.15 Å is based on a hexagonal packing of alkyl chains, and indicates that the lipid mixture is in the α-form at 25°C (21, 24). The incorporation of cholesterol into the SLE/stearic acid system caused a marked decrease of the diffraction intensity of the hexagonal packing at 4.15 Å for both the anhydrous and hydrated system. In the range of 11–33 mol% (10–30 wt%) cholesterol, a diffuse reflection centered at 4.5 Å appeared and overlapped the strong peak at 4.15 Å (Fig. 4, B, C, D). As the cholesterol content was increased, the diffuse reflection, indicating a liquid-like packing of the hydrocarbon chains, increased gradually. At cholesterol concentrations > 33 mol%, there were several weak diffraction peaks in the short spacing region (2 θ = 10–20 degree) as well as the diffuse reflection. The diffractions at 5.19 Å and 5.65 Å, based on the crystalline cholesterol (9, 23), were detected (Fig. 4, E and F) together with the hexagonal diffraction peak at 4.15 Å. For all samples, there was a diffraction at around 16 Å (2 θ = 5.5 degree), although the precise assignment of this reflection has not been made.

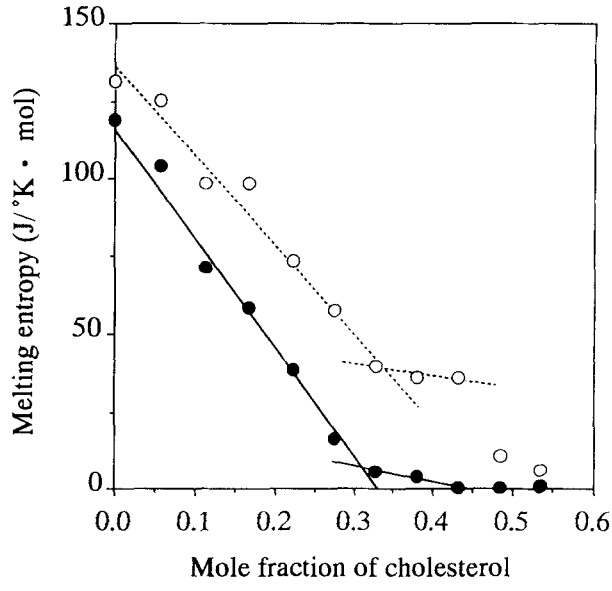


Fig. 3. Melting entropy of SLE/stearic acid/cholesterol system (SLE/fatty acid = 1/1 mole). The closed circles (●) indicate the melting entropy of hydrated lipids and the open circles (◪) the melting entropy of anhydrous mixtures.

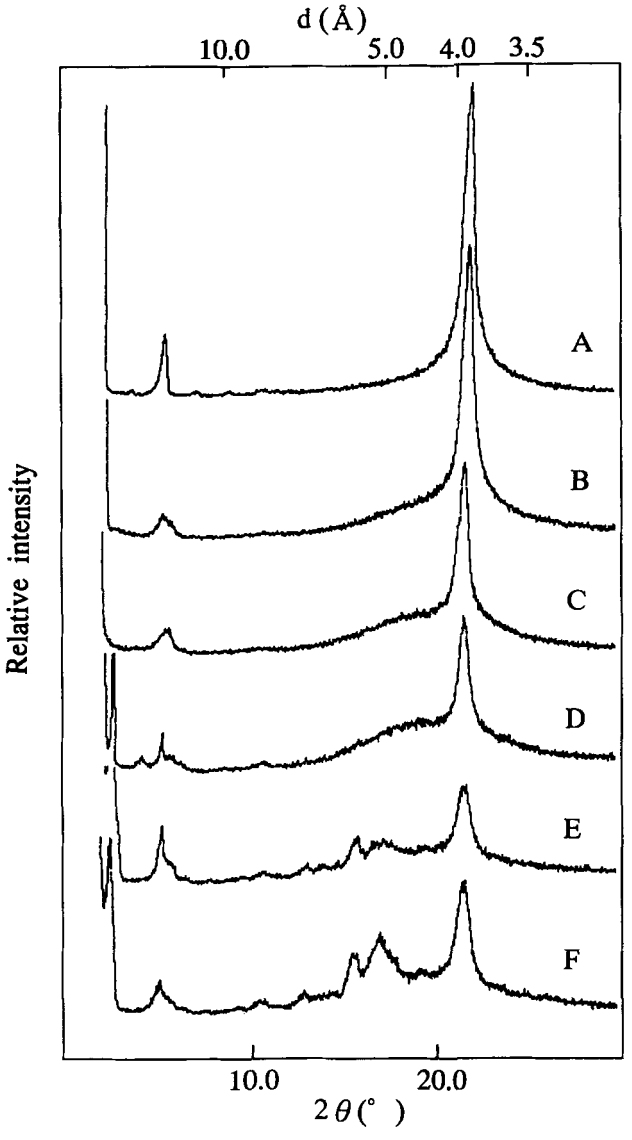


Fig. 4. X-ray diffraction patterns at 25°C of anhydrous SLE/stearic acid/cholesterol mixtures (SLE/stearic acid = 1:1 mole) with weight fraction of cholesterol being (A) 0, (B) 0.10, (C) 0.20, (D) 0.30, (E) 0.40, and (F) 0.50.

The hydrated mixture of SLE/stearic acid (1/1 mol ratio) also demonstrated a single diffraction peak at 4.15 Å (Fig. 5, A), while the relative intensity of the diffraction was smaller than that of the corresponding anhydrous sample. The X-ray pattern of the hydrated mixture containing 0–53 mol% (10–50 wt%) cholesterol was very close to that of the corresponding anhydrous lipids (Fig. 4 and Fig. 5). When the cholesterol concentration was 11–33 mol% (10–30 wt%), a diffuse halo at around 4.5 Å together with a sharp diffraction at 4.15 Å was observed (Fig. 5, B, C, and D). The results showed that hydration did not change the aggregation state of SLE/stearic acid (1/1 mole ratio)–cholesterol system.

DISCUSSION

The incorporation of cholesterol into the equimolar mixture of the pseudo-ceramide and stearic acid resulted in a drastic change of the thermotropic properties and the structural characteristics. In contrast to the natural SC lipids (7, 25, 26) and several model systems containing naturally occurring ceramides (12, 13, 27), the DSC thermal profile of SLE/stearic acid (1/1 mole ratio)-cholesterol system was quite simple. Increasing the cholesterol concentration, the endothermic peak, due to the melting of α-form at around 55–60°C, decreased progressively and there was a linear relationship between the melting entropy and cholesterol concentration in the range of 0–33 mol%. As the magnitude of the ΔS_m associated with the T_m reflects the degree of disorder of the molecular packing below the T_m (21), decrease of the ΔS_m in the SLE/stearic acid/cholesterol system implies disorder in the molecular arrangement. Concurrently, wide-angle X-ray diffraction measurements detected the decrease in intensity of the hexagonal packing reflection (4.15 Å) and the appearance of a liquid-like packing (4.5 Å). Previous investigators have shown the existence of a hexagonal (4.14 Å spacing) and liquid lateral packing (4.6 Å spacing) in pig stratum corneum (10). The aggregation structure of SLE/stearic acid (1/1 mole ratio)–cholesterol system would be very close to that of the natural system. The results have successfully shown that the ΔS_m decrease of the bilayer by the addition of cholesterol could be attributed to the enhancement of molecular motion of the hydrocarbon chains of constituent lipids (7). Cholesterol is an important regulator of the physical properties of biological membranes and membrane function (28, 29). Classically, the role of cholesterol in the phospholipid bilayer (gel to liquid crystal transition) is to diminish the enthalpy change by increasing the gel-state hydrocarbon chain packing disorder while increasing the chain packing order in the liquid crystalline state (28). Therefore, the behavior of cholesterol in this gel phase of the equimolar mixture of SLE and stearic acid seems to be comparable to those of the biological systems mentioned above. Recently, cholesterol is proposed to interact favorably with phospholipid chains in an extended conformation and to disturb the acyl-chain conformational order in the crystalline (gel) state (30). Abraham and Downing (8) investigated the dynamic aspects of hydrated lipid mixtures consisting of natural ceramides, cholesterol, palmitic acid, and cholesteryl sulfate, approximating the SC lipid composition, by using deuterium magnetic resonance and freeze-fracture electron microscopy. They found that there was a loss of symmetry of the powder pattern as the amount of cholesterol was decreased. This has been attributed to the increased hydrogen bonding and closer packing of the head

groups. Thus, the ΔS_m decrease of the SLE/stearic acid bilayer by the addition of cholesterol should be attributed to the enhancement of molecular motion of the hydrocarbon chains of constituent lipids, and to the decreased hydrogen bonding and looser packing of the head groups (7, 8). In addition, the linear relationship between the melting entropy and the cholesterol concentration (0–33 mol%) may be reflected in a constant increase of free volume for molecular motion formed by the incorporated cholesterol in the α-gel phase.

At cholesterol concentrations > 33 mol%, X-ray measurements detected several diffractions in the short spacing region (2 θ = 10–20 degree). Among them, the diffraction peaks of the anhydrous mixtures at 5.19 Å and 5.65 Å, would be assigned to the crystalline cholesterol (9, 24). However, pure SLE crystal having four diffraction peaks at 4.24, 4.13, 3.78, and 3.62 Å (19–21) was not observed. From this observation, it is believed that phase separation of crystalline cholesterol from the gel phase occurred at cholesterol concentrations > 33 mol%. In model systems of pig ceramides and cholesterol in buffer solution, cholesterol monohydrate starts to phase-separate at a ceramide/cholesterol molar ratio of 0.6 (24), but our results suggest that cholesterol is miscible up to the ratio of ceramide/cholesterol being 1.0 in the presence of fatty acid. This is presumably due to the strong interaction, such as hydrogen bonds, between fatty acid and cholesterol (25). On the other hand, another diffraction peak was found at around 16 Å (2 θ = 5.5 degree) for all samples. Assuming this peak is the 3rd order diffraction of lamellar planes, the lamellar periodicity should be 48 Å, which is a reasonable distance of the bilayer. Small-angle diffraction experiments must be done to examine the precise lamellar periodicity and to elucidate other features in the short spacing region (2 θ < 10 degree).

On the other hand, as demonstrated by the entropy decrease by melting and also shown by the decrease in intensity of hexagonal reflection in X-ray diffraction profiles, hydration had little effect on the aggregation structure of SLE/stearic acid-cholesterol system. However, the molecular motion of lipids was enhanced by hydration. These results suggest the existence of strong interaction, such as hydrogen bonds, among SC constituent lipids. The difference of ΔS_m between the anhydrous and hydrated system was progressively increased when the concentration of cholesterol increased. One interpretation of these results would suggest that the incorporation of cholesterol caused a increase of bound water in the bilayer. The water added to the bilayer is primarily confined to the polar region where it inserts among the head groups and loosens the molecular packing (26). This decrease in packing lowers attractive forces among the hydrocarbon chains, and hence, decreases the ΔS_m. Therefore, the increase of the amount of bound water in the bilayer should cause a decrease of the ΔS_m. To clarify the exact role of cholesterol in the natural SC lipid bilayer, we must more fully understand the phase behavior of the natural ceramides/fatty acids/cholesterol system. Through the study of such a model system, we may hope to establish the principles governing the interaction of cholesterol with other SC constituent lipids. Finally, the pH of the hydrated lipids is considered to be an important factor determining their aggregation states. In the range of pH 4.5–5.5 as the normal condition of the skin, fatty acids do not exist as acids but are partially saponified (31). Thus, the effect

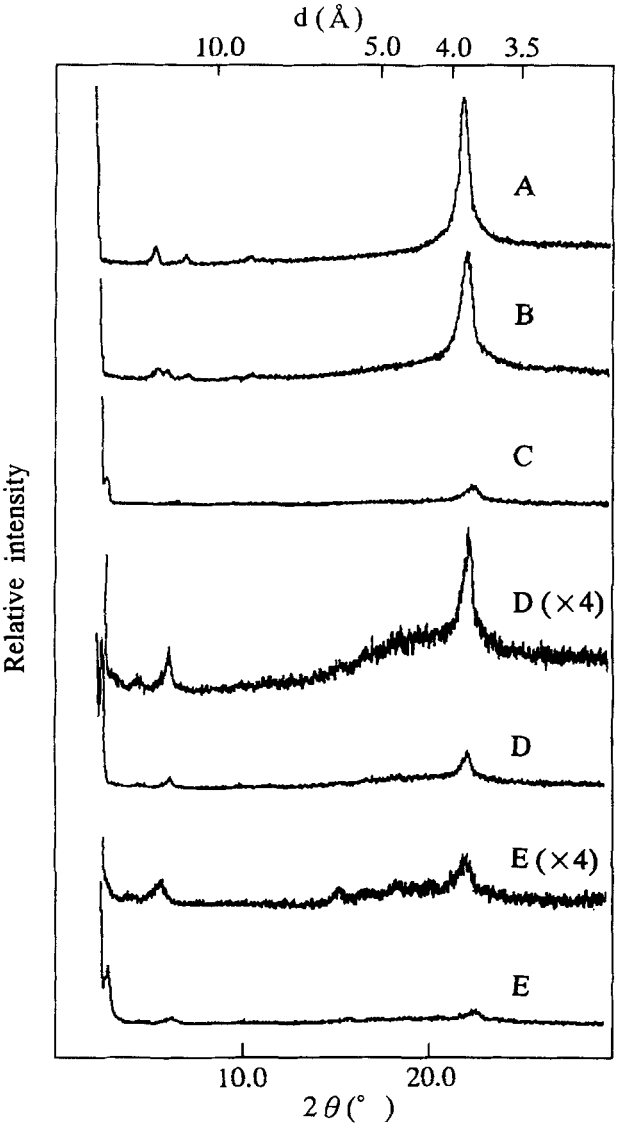


Fig. 5. X-ray diffraction patterns at 25°C of hydrated SLE/stearic acid/cholesterol mixtures (SLE/stearic acid = 1:1 mole) with weight fraction of cholesterol being (A) 0, (B) 0.10, (C) 0.20, (D) 0.30, and (E) 0.40.

of the pH of the hydrated mixtures consisting of SLE, stearic acid, and cholesterol on the thermotropic phase behavior and structural characteristics must be examined.

In conclusion, we found that incorporation of cholesterol into both the anhydrous and the hydrated equimolar mixture of pseudo-ceramide and stearic acid enhanced the mobility of hydrocarbon chains of lipids. Increase in fluidity of the hydrocarbon chains of SC lipid bilayer results in an increased transepidermal water loss (7). The results presented here strongly support the idea that cholesterol can regulate the mobility of hydrocarbon chains of natural SC lipid bilayer, which is primarily responsible for SC barrier properties. Moreover, cholesterol was suggested to be miscible in SC lipid bilayers up to the molar ratio of ceramide/cholesterol being 1.0 in the presence of fatty acid.

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