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Deuterium NMR investigation of polymorphism in stratum corneum lipids

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The intercellular lipid lamellae of stratum corneum constitute the major barrier to percutaneous penetration. Deuterium magnetic resonance and freeze-fracture electron microscopic investigation of hydrated lipid mixtures consisting of ceramides, cholesterol, palmitic acid and cholesteryl sulfate and approximating the stratum corneum intercellular lipid composition, revealed thermally induced polymorphism. The transition temperature of bilayer to hexagonal transition decreased as the ratio of cholesterol to ceramides in these mixtures was lowered. Lipid mixtures in which the stratum corneum ceramides were replaced by synthetic dipalmitoylphosphatidylcholine did not show any polymorphism throughout the temperature range used in the present study. The ability of the ceramide-containing samples to form hexagonal structures establishes a plausible mechanism for the assembly of the stratum corneum intercellular lamellae during the final stages of epidermal differentiation. Also, the bilayer to hexagonal phase transition of these nonpolar lipid mixtures could be used to enhance the penetration of drugs through skin.

Introduction

Mammalian stratum corneum (SC) is a highly specialized tissue with the major function of providing a barrier against percutaneous penetration of water and other solutes. SC is made up of fully cornified cells that are embedded in a matrix of extracellular lipid lamellae [1]. These extracellular membranous sheets are believed to constitute the epidermal water barrier [2] and are unique in their lipid composition. The SC lipid lamellae contain no phospholipids, the major bilayer-forming lipids in almost all biomembranes, and are predominantly made up of ceramides, cholesterol, free fatty acids, cholesteryl sulfate and small amounts of some less well characterized nonpolar components [3–5].

The membranous structures of SC originate from the lamellar granules found in the granular layer of the epidermis [6,7]. The lamellar granules discharge their lipid contents in the form of membranous disks, into the extracellular space between the granular and the horny layers. These membranous disks then rearrange to form the extended lamellar sheets of the SC. The membrane fusion processes that lead to this reassembly of the extracellular lipid lamellae of SC are not clearly understood. Formation of intermediary structures during this reassembly process has been proposed [8,9]. This is further complicated by the dramatic biochemical changes that occur in the lipid composition during the final stages of epidermal differentiation [3,4]. Also, the water content, ionic strength and the pH of the tissue change as the granular layer of the epidermis cornifies to form the SC.

As part of our effort to understand the interaction between the component lipids of SC, we have initiated an investigation using solid-state ^{2}H-NMR spectroscopy, which is an ideal technique for investigating the molecular dynamics of lipid aggregates. An understanding of the relationship between the structure and molecular packing of the component lipids would enable us to understand the barrier function on a molecular level. We report here the results from our ^{2}H-NMR

Abbreviations: SC, stratum corneum; DPPC, dipalmitoylphosphatidylcholine; $\Delta\nu_q$, quadrupolar splitting; DSC, differential scanning calorimetry; IR, infrared spectroscopy; FFEM. freeze-fracture electron microscopy.

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investigation of SC lipid mixtures consisting of SC ceramides, cholesterol, cholesteryl sulfate and palmitic acid deuterated at the alpha position. The thermotropic transitions in these lipid mixtures were investigated by following the shape of the ^2H quadrupolar peaks and the magnitude of the quadrupolar splittings as a function of temperature. The presence of significant amounts of saturated free fatty acids in the SC lipid lamellae makes deuterated palmitic acid an appropriate probe molecule, which does not perturb the system. Freeze-fracture electron microscopy was used to examine the morphological changes resulting from the thermotropic transitions.

Materials and Methods

Lipids

Ceramides were isolated by preparative thin-layer chromatography from chloroform:methanol (2:1, v/v) extracts of SC, as described previously [10]. Cholesterol and synthetic L-α-dipalmitoylphosphatidylcholine were purchased from Sigma Chemical (St. Louis, MO). Cholesteryl sulfate was prepared by reaction of cholesterol with excess chlorosulfonic acid in pyridine and purified chromatographically. Palmitic acid deuterated at the alpha position was purchased from MSD Isotopes (Montreal, Canada) and was recrystallized twice from hexane.

Preparation of lipid bilayers

Appropriate volumes of chloroform/methanol (2:1, v/v) solutions of individual lipids were combined to obtain mixtures of the composition shown in Table I. The lipid mixtures were dried under a stream of nitrogen and then under vacuum and were then hydrated in deuterium-depleted water (Aldrich Chemical, Milwaukee) to a final concentration of 50% by weight of lipid. The hydrated mixtures were homogenized by stirring with a glass rod and the homogenous mixtures were then packed in 5 mm NMR sample tubes that were cut to a length of 3 cm. The sample tubes were flushed with dry nitrogen gas and flame-sealed immediately to prevent oxidation of the lipids. The samples contained 70–80 mg of lipid in a sample volume of 100–150 μl. The SC lipid samples were incubated at 80°C and the DPPC-containing samples at 65°C, for 15 min to assure thermal equilibration and homogenization by diffusion and were then cooled to room temperature before spectral acquisition.

TABLE I

Composition of the lipid mixtures used in the present investigation

Mixture	Mol percent of *				
	ceramide	cholesterol	palmitic acid	Chol sulfate	DPPC
1	38	38	19	5	–
2	57	19	19	5	–
3	76	–	19	5	–
4	–	38	19	5	38
5	–	19	19	5	57
6	–	–	19	5	76

* The final mixtures were 50% by weight of lipid in water.

NMR spectroscopy

^2H-NMR spectra were obtained at 46.07 MHz on a Bruker MSL-300 wide bore spectrometer using the quadrupole echo pulse sequence [11]. Complete phase cycling was used to minimize error due to non-orthogonality and gain imbalance of the receiver channels in the quadrature detection mode. The $\pi/2$ pulse length was 2.6 μs for the 5 mm solenoid coil. A pulse interval of 18 μs and a recycle delay of 400 ms were used. Quadrupolar splittings ($\Delta\nu_q$) were measured as the distance between the two horns in the powder pattern spectra. Spectra were acquired at different temperatures, which were controlled by a Bruker variable temperature unit. The system was allowed to equilibrate for 15 min at each temperature before the probe tuning was readjusted. The samples were allowed to equilibrate for 15 min at each temperature before spectral acquisition. Spectra were acquired for the samples both after heating and after cooling to a given temperature.

Freeze-fracture electron microscopy

After NMR spectral acquisition, small amounts of the ceramide-containing samples were incubated at a given temperature for 15 min and then quick-frozen from that temperature in liquid propane at −190°C. The frozen samples were fractured in a Balzars 301 freeze-fracture apparatus and the fractured surface was replicated with platinum-carbon. The Pt-C replicas were cleaned in Clorox bleach for 2–3 h, and rinsed several times in distilled water. The cleaned replicas were examined in a Hitachi H-7000 transmission electron microscope operating at 75 kV.

Results

The composition of different lipid mixtures that were investigated in the present study is shown in Table I. Mixture 1 containing 38 mol percent of ceramides, 38% cholesterol, 19% palmitic acid and 5% cholesteryl sulfate was taken as a close approximation to the in situ lipid composition of SC [3–5]. The mole ratio of cholesterol to ceramides was changed from 1 to 0 in mixtures 1–3. In mixtures 4–6, the SC ceramides were replaced by DPPC, a well characterized, bilayer-forming lipid used as a control in the present investigation.

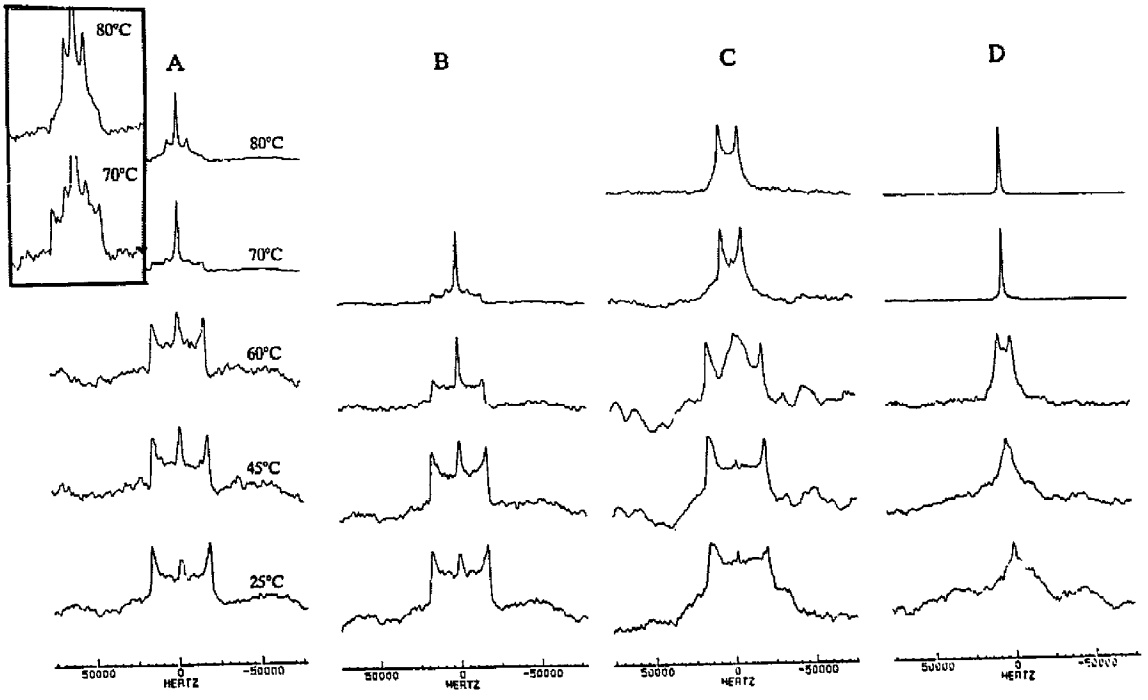

Fig. 1. ^{2}H-NMR spectra of α-CD_2 palmitic acid in the ceramide-containing lipid mixtures shown in Table I, at different temperatures. (A) Mixture 1. The spectra were obtained after heating the sample to the temperature shown. The quadrupolar peaks corresponding to the H_{II} phase are clearly visible in the inset which shows the 70° and 80° spectra at S/N comparable to the lower temperature spectra. (B) Mixture 1. The spectra were obtained after cooling the sample to the given temperature. (C) Mixture 2. (D) Mixture 3. The spectra in C and D were obtained after heating the sample to the given temperature. The quadrupolar peaks with a quadrupolar splitting less than 12–13 kHz correspond to the hexagonal phase.

Fig. 1 shows the ^{2}H-NMR spectra of the α-deuterated palmitic acid in ceramide-containing mixtures from Table I, at different temperatures. Spectra obtained both after heating and cooling the sample to any given temperature as shown in Fig. 1A and 1B, indicate the reversibility of the thermotropic changes observed in this study. For mixture 1 at 25°C, the spectrum shows a powder pattern with a $\Delta\nu_q$ of 34.9 kHz. As the temperature was raised, there was a narrowing of the quadrupolar splitting from 34.9 kHz at 25°C to 31.5 kHz at 60°C. At 70°C, there was an additional pair of quadrupolar peaks with a $\Delta\nu_q$ of 13.4 kHz, while the outer quadrupolar peaks had a $\Delta\nu_q$ of 29.8 kHz. At 80°C, only a remnant of the outer quadrupolar peaks was visible, while the inner pair appeared more intense with a $\Delta\nu_q$ of 12 kHz. The appearance of the inner quadrupolar peaks suggests the formation of a new phase, presumably a hexagonal (H_{II}) phase, with an increased mobility of the acyl chains including the α-methylenes.

^{2}H-NMR spectra obtained at different temperatures from mixture 2 are shown in Fig. 1C. Bilayer to hexag-

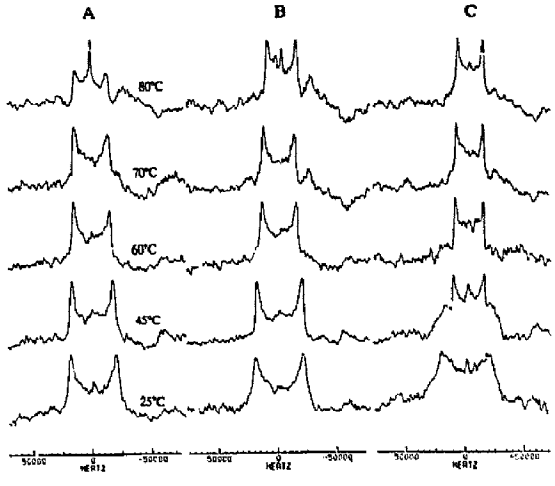

Fig. 2. ^{2}H-NMR spectra of α-CD_2 palmitic acid in the DPPC-containing samples from Table I. (A) Mixture 4. (B) Mixture 5. (C) Mixture 6. There is a monotonic decrease in the quadrupolar splitting values and there is no indication of a hexagonal phase with smaller quadrupolar splitting.

TABLE II

^2H quadrupolar splitting values ($\Delta\nu_q$) as a function of temperature

Temperature (°C)	$\Delta\nu_q$ (in kHz) of mixtures					
	1	2	3	4	5	6
25	34.91	35.40	a	38.33	40.28	41.02
45	33.94	35.16	a	34.67	38.33	26.37
60	31.49	34.42	8.06	30.52	29.05	23.93
70	29.79	12.94	i	28.32	25.88	22.71
	13.43	–	–	–	–	–
80	29.30	11.96	i	26.61	24.17	21.48
	11.96	–	–	–	–	–

[a] anisotropic peak, [i] isotropic peak.

onal phase transition seemed to start around 60°C, and the transition was complete around 70°C. There was a broad central peak at 60°C, which resolved into a pair of quadrupolar peaks corresponding to the hexagonal phase at 70°C. This behavior was seen both during the heating and the cooling cycle and was observed only for samples with composition corresponding to mixture 2 in Table I. The reason for this behavior of mixture 2 is not clear at the present moment. Fig. 1D shows the ^2H-NMR spectra obtained from mixture 3 at different temperatures. A spectrum corresponding to a H_{II} phase was seen at 60°C and it changed to an isotropic peak at 70°C. Fig. 2 shows the ^2H-NMR spectra obtained from DPPC-containing samples from Table I at different temperatures. The DPPC-containing samples did not show the additional pair of quadrupolar peaks that were seen in the ceramide-containing samples. The monotonic decrease in the $\Delta\nu_q$ with the increase in temperature indicates the increase in fluidity of the bilayer. The $\Delta\nu_q$ values from Figs. 1 and 2 are summarized in Table II.

The presence of isotropic peaks in the ceramide-containing samples at higher temperatures, especially in mixtures 1 and 3, could be the result of formation of an isotropic phase which disappeared upon cooling the sample below 60°C. The origin of the central peak in the spectrum from mixture 1 at room temperature is not known. Also, the appearance of the ceramide-containing samples was different from that of the DPPC-containing samples. The former showed signs of bulk melting at temperatures above 70°C, indicated by the oily appearance of the samples, while the latter increased in volume up to about 50% at higher temperatures, with no sign of bulk melting.

Fig. 3 shows freeze-fracture electron micrographs of mixtures 1–3. The lipid mixtures were examined by freeze-fracture electron microscopy by quick-freezing the samples from the high temperatures at which there were indications of formation of a H_{II} phase by these mixtures, as seen by the appearance of the quadrupolar peaks with smaller $\Delta\nu_q$ values in the ^2H-NMR. The closely spaced fracture steps indicate the presence of hexagonal phase [12]. DPPC-containing samples showed smooth fracture faces, corresponding to the lamellar phase, at all temperatures (not shown).

Discussion

The results from the present study indicate that the ceramide-containing mixtures undergo thermotropic transitions that lead to the formation of H_{II} as well as isotropic phases at higher temperatures, while in the

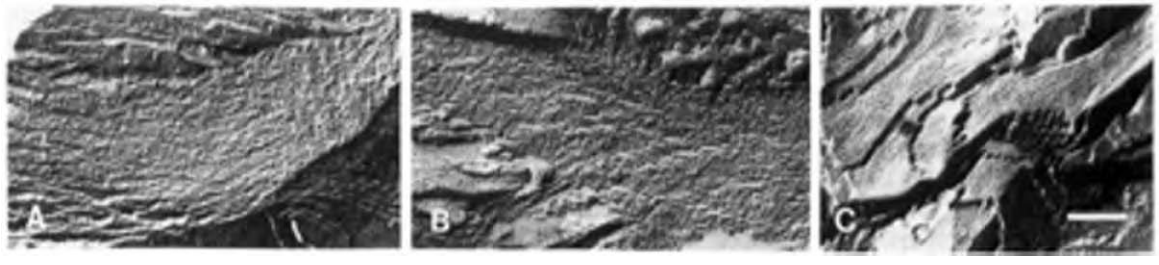

Fig. 3. Freeze-fracture electron micrographs of the ceramide-containing samples from Table I. (A) Mixture 1. The sample was maintained at 80°C, the temperature at which the ^2H-NMR spectrum shows the presence of H_{II} phase in this mixture, for 15 min and then quick-frozen in liquid propane and analyzed by freeze-fracture electron microscopy (FFEM). (B) Mixture 2. The sample was maintained at 70°C for 15 min before analysis by FFEM. (C) Mixture 3. The sample was maintained at 60°C for 15 min before analysis by FFEM. Bar = 100 nm.

DPPC-containing control samples, the constituent lipids remain in the bilayer configuration throughout the temperature range used in the present investigation. Thermotropic transitions of stratum corneum lipids have been investigated both in hydrated tissue as well as in hydrated lipid extracts [13–15]. Several investigators have reported thermal transitions of SC lipids in the temperature range of 60–80°C based on the differential scanning calorimetry (DSC) thermal profiles [13–15]. Also, based on the abrupt increase in the infrared (IR) peak widths and the wavenumber of the C-H absorbance between 60° and 80°C, Golden et al. [13,14] have suggested that the SC lipids undergo thermal transitions in this region. This was attributed to the increase in the gauche conformers due to an increase in the fluidity of the acyl chains undergoing the thermal transition. However, there has been no report of the formation of H_{II} phase in SC lipids as a result of thermotropic transitions. This could be due to the nature of the phase transition in question. While the bilayer to H_{II} transition involves quite dramatic structural rearrangement of monolayers of lipids, it has been shown to be a low enthalpy process [16] and has not been detected by DSC, as in the case of bilayer to H_{II} transitions of some phosphatidylethanolamines [17].

The appearance of a second pair of quadrupolar peaks with smaller $\Delta\nu_q$ for the ceramide-containing mixtures in the present study indicated the presence of H_{II} phase. Theoretically, bilayer to hexagonal transition should result in a decrease in the $\Delta\nu_q$ by a factor of 2 [18]. This is due to the additional averaging that results from the added motion around the axis of the cylinders that are arranged in a hexagonal array in the H_{II} phase. However, the $\Delta\nu_q$ for the inner pair is less than one half of the $\Delta\nu_q$ of the outer pair, as shown in Table II, suggesting some additional mobility of the α-methylenes of the palmitic acid in the H_{II} phase. In the H_{II} phase, the inner aqueous core is surrounded by the lipid head groups with the acyl chains facing outward radially. This would result in a highly disordered hydrocarbon environment and the effect of such disorder apparently is felt even at the α-methylene of the palmitic acid.

Cholesterol has been shown to condense the lipids in the liquid-crystalline phase and fluidize the lipids in the gel phase of lamellar systems [19]. The $\Delta\nu_q$ values shown in Table II for α-CD_2 palmitic acid in the DPPC-containing mixtures is consistent with this dual role of cholesterol. At 25°C, when the lipid mixtures are in the gel phase, mixture 4 containing the highest amount of cholesterol among the three mixtures is also the most fluid, as seen by its smaller $\Delta\nu_q$. At 60°C, when the mixtures are in the liquid-crystalline phase, mixture 4 is the most rigid due to the condensing effect of cholesterol.

Cholesterol is also known to destabilize the bilayer structure and induce the formation of hexagonal phase in unsaturated phosphatidylethanolamine-phosphatidylcholine systems [20]. In the present study, as the ratio of cholesterol to ceramides is changed from 1 to 0 in mixtures 1 to 3, several interesting changes are observed in the ^2H spectra shown in Fig. 1. First, there is a loss of symmetry of the powder pattern at room temperature from mixture 1 to mixture 3. This has been attributed to the increased hydrogen bonding and closer packing of the head groups as the amount of cholesterol is decreased [10]. Second, the temperature at which the quadrupolar peaks corresponding to the lamellar phase are replaced by the quadrupolar peaks corresponding to the hexagonal phase decreases from around 80°C for mixture 1 to around 60°C for mixture 3, suggesting that increasing the ceramide content and decreasing the cholesterol content concommitantly in the SC lipid mixtures, lowers the energy barrier for the bilayer to hexagonal transition. There is a significant increase in disorder in the H_{II} phase in the absence of cholesterol, at least in the region probed by the α-methylenes of the palmitic acid, as reflected by the decrease in the $\Delta\nu_q$ from 12 kHz in mixtures 1 and 2 to 8 kHz in mixture 3.

Recognition of polymorphism and the formation of the H_{II} phase by the ceramide-containing mixtures in the present study is significant to the understanding of the SC membrane function in two important ways. The formation of the SC intercellular membranous structures involves the reassembly of membranous disks that are extruded from the lamellar granules into the extracellular space between the granular and the horny layers of epidermis [6–8]. During this final stage of epidermal differentiation, there is a dramatic change in the composition of the lipids from predominantly phospholipids, glycosylceramides and cholesterol to mainly ceramides, cholesterol, and free fatty acids [3,4]. There is also a reduced water content due to the cornification of the tissue and reduced hydration of the lipids due to the loss of polar and ionic head groups. These physiological factors can regulate the structural preferences of lipids and could cause the bilayer to hexagonal transition isothermally, similar to the thermal transition observed in the present study. The hexagonal phase involves the formation of nonlamellar inverted micelles which are arranged in the form of cylinders in hexagonal arrays. Lipid molecules in a membrane bilayer are believed to undergo momentary departure from bilayer organization at the point of contact and form intermediary structures such as inverted micelles during membrane fusion [21–23]. Thus the ability of SC lipids to form H_{II} structures establishes a plausible pathway for the reassembly of the membranous disks into extended lamellar sheets by membrane fusion processes involving transient nonlamellar intermediates. Freeze-fracture electron microscopic investigation

of the fusion events involving liposomes made from SC lipids has revealed ridges at the fusion sites, which have been suggested to represent intermediary non-lamellar structures [9].

The extraordinarily effective barrier property of the SC intercellular lipid lamellae has been attributed to the highly ordered arrangement of the constituent lipids in the bilayer configuration [24]. Molecules like Azone [25], ethanol [26], or oleic acid [27] are being used to fluidize these bilayers with the aim of enhancing the penetration of drug molecules through SC. The results from the present study indicate that under appropriate conditions, the SC lipid mixtures can be induced to form the H_{II} phase, which has a highly disordered hydrocarbon core and would have very little barrier compared to the highly ordered lamellar phase. Also, this is a reversible transition, which could be induced temporarily by the addition of molecules of suitable geometry, thereby enhance the percutaneous penetration of drugs.

In conclusion, the results presented here indicate that the highly heterogeneous lipid mixture of SC is capable of undergoing a reversible bilayer-to-hexagonal phase transition. A detailed understanding of the lipid polymorphism on the molecular level will lead to a better understanding of the role of the diverse mixture of SC lipids in governing the barrier function of mammalian skin.

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