

Insights into the Molecular Organization of Lipids in the Skin Barrier from Infrared Spectroscopy Studies of Stratum Corneum Lipid Models

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In order to gain some insight into the molecular organization of lipids in the skin barrier we used Fourier transform infrared (FTIR) spectroscopy to investigate models of the stratum corneum (SC) containing deuterated hexadecanoic acid, cholesterol, and ceramide 2 or ceramide 5. In both models there is clear evidence of separate conformationally ordered domains of ceramide and fatty acids. In addition, these chains are packed in orthorhombic subcells at physiological temperatures. The ceramide headgroup behavior indicates distinct hydrogen bonding patterns between the ceramide 2 and ceramide 5 models. In the ceramide 2 model the amide I mode is split into two components suggesting strong transverse intermolecular hydrogen bonding between headgroups. In contrast, no amide splitting is observed for ceramide 5 although the amide frequencies are indicative of strong hydrogen bonding. These observations on the molecular organization of SC lipids are discussed in terms of skin barrier function. Key words: ceramides; fatty acids; stratum corneum; lipids; domains; FTIR (Accepted July 1, 1999.)

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INTRODUCTION

The skin barrier plays an essential role in human physiology by preventing water loss from the body and protecting the body from external physical, chemical, and biological insults. Barrier function resides in the outer layer of the epidermis, the stratum corneum (SC), which consists of two major structural components; corneocytes and lipids (1). A widely employed analogy for describing SC organization is a brick wall; the corneocyte bricks contribute the major volume of the wall (~80%) surrounded by lipid mortar. A significant portion of barrier function resides in the lipids of the SC (2).

To function as a coherent, almost water impermeable barrier, the lipids of the SC must be organized in ordered gel or crystalline phases. This organization is quite unlike the typical liquid crystalline (L_α) phases of most biological membranes. The composition of the SC is unique, consisting of ceramides, free fatty acids, and cholesterol (2–4). Presumably the barrier function of the SC requires this unusual lipid composition. Furthermore, it seems likely, given the unique composition and particular function of the SC lipids, that molecular organization in the SC may be quite distinct from other biological membranes.

To date, a detailed picture of the molecular organization of

lipids in the SC has not been elucidated. However, it is clear from many studies in the last decade, using a diverse range of physical techniques including X-ray, TEM, DSC, AFM, NMR, and FTIR that SC lipids are organized in lamellar bilayer structures in which the lipid chains are highly ordered (5–12). There are also ceramides covalently bonded to the protein envelope of corneocytes in the SC which interact with the free lipid of the SC matrix and influence macroscopic lipid organization (15, 16).

This paper summarizes some recent work in our laboratory using biophysical FTIR spectroscopy techniques to investigate intramolecular and intermolecular lipid organization in models of the skin barrier. This technique permits the investigation of membrane lipids under conditions of controlled pH and hydration without the use of probe molecules. We have utilized biophysical FTIR spectroscopy techniques as part of an ongoing effort to understand stratum corneum lipid dynamics and organization at a molecular level (13, 14). To this end we have studied samples of pure ceramides (synthetic and isolated), binary and ternary lipid samples in a variety of physical states, including anhydrous samples, hydrated bilayers in H_2O and D_2O , and SC lipid model samples as monolayers at the air/water interface.

This research is an ongoing effort but is already providing us with some interesting insight into intermolecular interactions, both in the headgroup region and in the lipid chains, of different SC models. Some of these findings are discussed below.

EXPERIMENTAL METHODS

Materials

Perdeuterated hexadecanoic acid was purchased from CND Isotopes, Quebec, Canada. Cholesterol, bovine non-hydroxy fatty acid (NFA) ceramide, and bovine hydroxy fatty acid (HFA) ceramide were from Sigma Chemical Co. (St. Louis, MO.) Bovine NFA and HFA ceramides have the same chemical backbone structure as human ceramides 2 and 5, respectively, also known as ceramides [NS] and [AS]. This nomenclature follows the work of Downing and colleagues (17). These two ceramides are among the most abundant SC ceramides and together constitute approximately 45% of total ceramide. For simplicity the nomenclature ceramide 2 and ceramide 5 is used throughout this paper (see Figure 1). The fatty acid chain length distribution is primarily C18:0 and C24:1 for the bovine NFA ceramide and C18:0, C22:0, C24:0, and C24:1 for the bovine HFA ceramide (14).

FTIR spectroscopy: sample preparation and data collection

Ternary lipid samples were prepared by co-dissolving equal molar quantities of ceramide, cholesterol, and fatty acid in $CHCl_3/CH_3OH$. After removing solvent the samples were hydrated in pH 5.5 buffer by repeated vortexing and heating. In general samples are prepared in

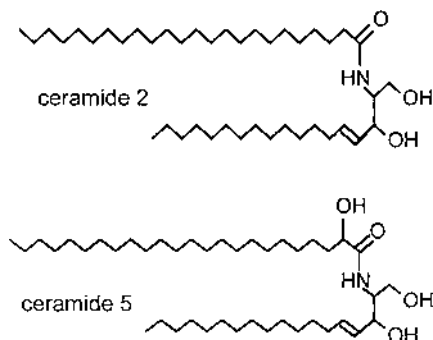


Fig. 1. The chemical structures of ceramides 2 and 5.

IR Spectrum of Lipid SC Model

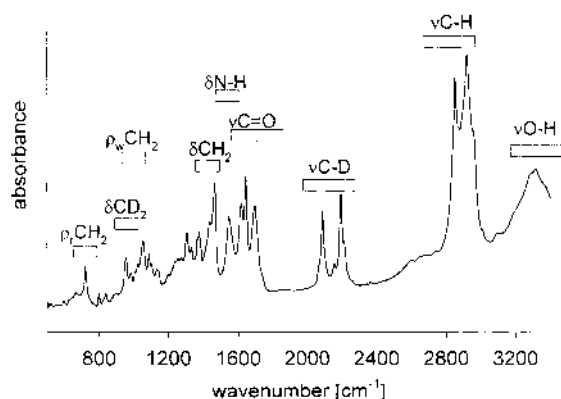


Fig. 2. A representative IR spectrum of SC lipid model. The major modes of interest to the current work are indicated.

the presence of excess buffer to allow the lipids to maximally hydrate. In the case of ceramide systems only a small amount of water is taken up by the sample (especially in comparison to other lipid systems such as phospholipids). Samples were then placed between infrared windows and held in a temperature controlled sample holder. After

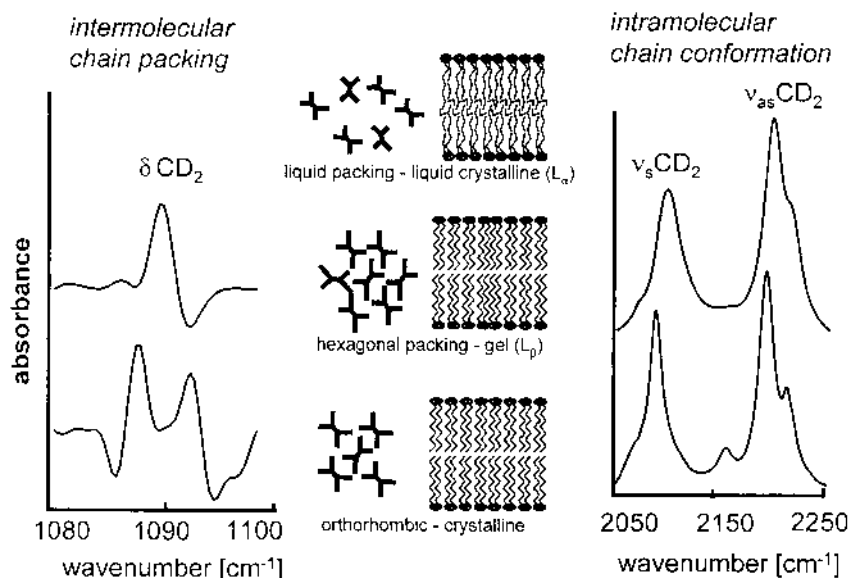
slow heating to 80°C the samples were cooled to 20°C and held for 48 hours before data collection was started.

Fourier transform infrared spectra were collected at 2 cm⁻¹ resolution on a Mattson Infinity spectrometer equipped with a broad band MCT detector and a sample shuttle and kept under dry air purge. Data were analyzed with software written at the National Research Council of Canada and plotted with Sigma Plot (Jandel Scientific). A representative IR spectrum of a model SC containing ceramide, deuterated fatty acid, and cholesterol is displayed in Figure 2. The major bands of interest in these studies are indicated.

IR spectra: chain and headgroup biophysics

In bilayer phases of long chain biological lipids, including phospholipids, fatty acids, and ceramides, the chain methylenes can pack in orthorhombic subcells forming highly ordered phases. In these orthorhombic phases two bands are observed in the IR spectrum for both the methylene rocking and bending vibrational modes. This results from coupling between adjacent methylene groups (of the same isotope) in the orthorhombic subcell. The observation of this factor group splitting permits discrimination of orthorhombic lipid packing from hexagonal packing (where only a single peak is observed). This is illustrated in Figure 3 where the spectra-structure relationships are shown for a deuterated fatty acid as used in our SC lipid models. In the experiments described in this paper the CD₂ bending (scissoring) modes of hexadecanoic acid and the CH₂ rocking modes of ceramide have been monitored in the IR spectra of the SC lipid models. Monitoring these modes provides direct and simultaneous information on fatty acid and ceramide chain packing in the skin lipid models. In addition, the IR spectrum also permits monitoring of intramolecular chain conformational order. The transition from fully extended all-*trans* hydrocarbon chains to disordered chains results in a frequency increase of both the symmetric and asymmetric methylene stretching modes. This is illustrated pictorially in Figure 3. In proteated chains, such as the ceramides used in the SC models, these modes appear at ~2850 and 2920 cm⁻¹, respectively. In deuterated chains, such as the fatty acids used in our SC models, these modes appear at 2090 and 2190 cm⁻¹, respectively. It is therefore possible to monitor separately and simultaneously the conformational order of the ceramide and deuterated fatty acid constituents in these SC models. Differences in ceramide and fatty acid chain length are likely very important to the structure and

Fig. 3. Representative spectra showing the methylene bending mode region of a deuterated lipid bilayer with orthorhombic chain packing and with hexagonal chain packing. Note in the orthorhombic phase the mode splits into two bands (~1085 and 1092 cm⁻¹) whereas only one band is observed in the spectra of the hexagonal or liquid packed chains (~1088 cm⁻¹). The spectra on the right show the methylene stretching mode region of deuterated lipids in an ordered, all-*trans*, bilayer and in a disordered liquid crystalline phase bilayer. Note the frequencies of both the symmetric and asymmetric CD₂ stretching modes (~2090 and ~2190 cm⁻¹, respectively) are higher in the conformationally disordered phase. The inter- and intramolecular arrangements of lipid bilayers are illustrated in the middle of the figure.



organization of lipids in the stratum corneum. This is, in part, the very issue we hope to address by using biophysical FTIR spectroscopy to investigate lipid models of the stratum corneum. In the experiments discussed in this paper there is a distribution of chain lengths in the bovine ceramides while the free fatty acid is always hexadecanoic acid. Ongoing experiments (not yet completed) using synthetic ceramides are systematically varying these parameters. While every model has its limitations we hope that by studying many different models systems we can gain some general insight into stratum corneum lipid organization.

A complete description of the molecular organization of the SC lipid barrier requires an understanding of the molecular interactions in the headgroup regions of ceramides and fatty acids. Indeed, our experiments lead us to believe that intermolecular hydrogen bonding between lipid headgroups is very important to the cohesiveness and integrity of the SC lipid barrier. An additional advantage of using FTIR spectroscopy for lipid biophysical studies is the ability to monitor headgroup interactions in the same spectra as chain interactions, thereby providing a comprehensive picture of all inter- and intramolecular interactions.

The frequencies of the fatty acid carbonyl stretching mode ($\sim 1700\text{ cm}^{-1}$), the ceramide amide I ($\sim 1650\text{ cm}^{-1}$) and the ceramide amide II (1550 cm^{-1}) modes are sensitive to hydrogen bonding and provide information on headgroup interactions in the SC lipid models. In our experience with hydrated model SC lipid samples the hydrogen bonding structures that are formed are very stable, and do not change in the presence of additional water as long as the lipid chains are highly ordered. Figure 4 displays the $1500\text{--}1750\text{ cm}^{-1}$ spectral region of a model SC sample showing the amide I and II headgroup modes of ceramide and the C=O stretching mode of the fatty acid. The figure shows how these bands change as a function of temperature indicating changes in the hydrogen bonding of the polar regions of the lipid bilayers.

The behavior of the headgroup modes can be correlated with inter- and intramolecular chain behavior, and resolved in terms of the ceramide and fatty acid components. This provides a highly detailed picture of lipid organization and dynamics in these models of the skin barrier.

RESULTS AND DISCUSSION

This paper reviews some of our previous FTIR work and describes some recent studies examining, in detail, both the

hydrophobic and hydrophilic regions of lipids in model of the epidermal skin barrier. The experiments described in this paper utilize samples containing deuterated hexadecanoic acid, cholesterol, and either ceramide 2 or ceramide 5 (see Figure 1). As discussed above, in biophysical FTIR spectroscopy studies of such models we can directly and simultaneously monitor chain and headgroup inter- and intramolecular interactions. Thus by studying these skin barrier models we hope to develop a detailed molecular level picture of the complex lipid organization that is critical to a well functioning epidermal barrier.

Ceramide 5/hexadecanoic acid- d_{31} /cholesterol model SC lipid barrier

Hexadecanoic acid behavior

The thermotropic response of the CD_2 bending mode frequencies and the CD_2 asymmetric stretching mode frequency of hexadecanoic acid- d_{31} are plotted in Figure 5A and 5B, respectively. The CD_2 scissoring (Figure 5A) mode is split into two bands indicating orthorhombic domains of hexadecanoic acid. These domains collapse at 50°C . The initial frequency of the CD_2 stretching mode is low (2193 cm^{-1}) indicating the chains are tightly packed with little rotational freedom. The onset of hexadecanoic acid conformational disordering occurs at $\sim 50^\circ\text{C}$ as manifest by the immediate jump in frequency to 2196 cm^{-1} . This is followed by a 1 cm^{-1} increase in frequency between 60 and 70°C . The temperature dependence of the hexadecanoic acid carbonyl frequency is plotted in Figure 5C. The data show a strongly hydrogen bonded carbonyl at temperatures below 50°C , indicated by the frequency of $\sim 1698\text{ cm}^{-1}$, followed by a sharp jump at 50°C to a less hydrogen bonded frequency of 1712 cm^{-1} . The change in acid headgroup bonding occurs with the collapse of the crystalline chain packing and occurs while the chains remain in a highly ordered gel state i.e., before chain disordering.

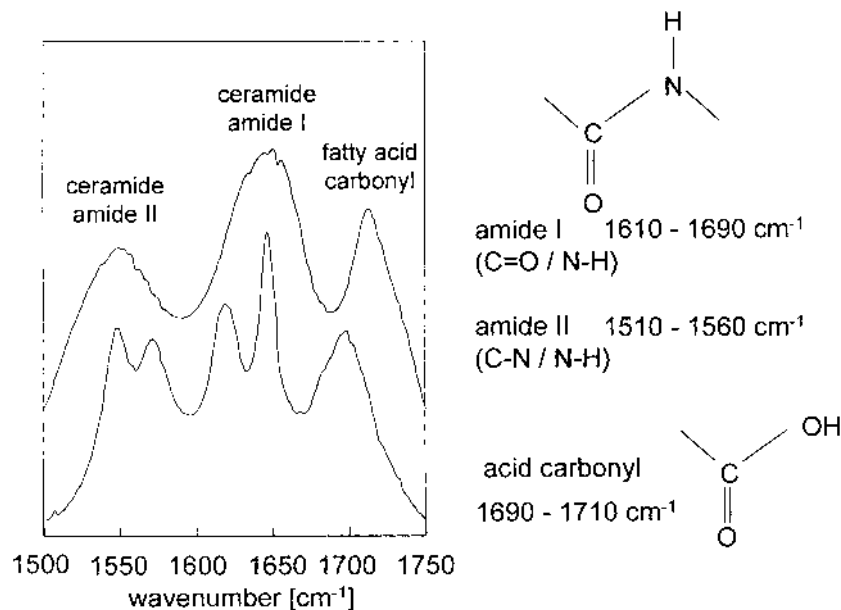


Fig. 4. IR spectra of the $1500\text{--}1800\text{ cm}^{-1}$ spectral region of model SC at low and high temperature. As indicated on the figure these spectra show the amide I and II modes of ceramide and the carbonyl mode of hexadecanoic acid. These modes are illustrated on the right of the figure.

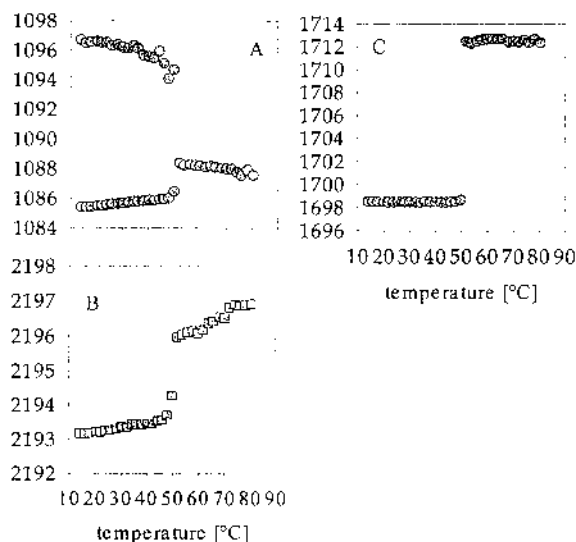


Fig. 5. Hexadecanoic acid-d₃₁ in Ceramide 5 Model SC. (A) The thermotropic response of the CD₂ bending frequencies, (B) the thermotropic response of the CD₂ asymmetric stretching mode, (C) the thermotropic response of the C=O stretching mode.

Ceramide 5 behavior

The thermotropic response of the CH₂ rocking mode frequencies and the CH₂ symmetric stretching mode frequency of ceramide 5 are plotted in Figure 6A and 6B, respectively. The CH₂ rocking mode of ceramide 5 is split into two bands at 722 and 727 cm⁻¹ indicating orthorhombic chain packing. The unusually high frequency of the lower frequency component (722 cm⁻¹) suggests a distorted orthorhombic structure. We have also observed this behavior in pure ceramide 5 samples (14). The initial CH₂ stretching frequency of ceramide 5 is low (2847 cm⁻¹), indicating tight packing in the ceramide 5 chains. Ceramide 5 undergoes a single transition from 2847 to 2853 cm⁻¹ that begins at 50°C and ends at 70°C. The behavior of the ceramide 5 headgroup modes is plotted in Figure 6C and 6D. These modes show very little change in frequency as a function of temperature. The frequencies of the amide I (1632–1634 cm⁻¹) mode and amide II (1538 cm⁻¹) mode are indicative of strong hydrogen bonding.

Discussion of ceramide 5 model studies

The data from the ceramide 5 model reveal that hexadecanoic acid and ceramide 5 pack in separate highly ordered orthorhombic domains. The fatty acid domains are stable to 50°C indicating these domains are probably quite large. Interestingly, in the ceramide 5 model the crystalline domains of hexadecanoic acid and ceramide both collapse at the same temperature (50°C). It may be that although the ceramide 5 chains are conformationally ordered, distorted chain packing increases their susceptibility to disruption by the melting of the hexadecanoic acid chains. In the ceramide 5 model the methylene stretching frequencies provide direct evidence that hexadecanoic acid and ceramide 5 are in fully extended all-*trans* conformations at physiological temperature. The very low values for both the CD₂ and CH₂ stretching mode

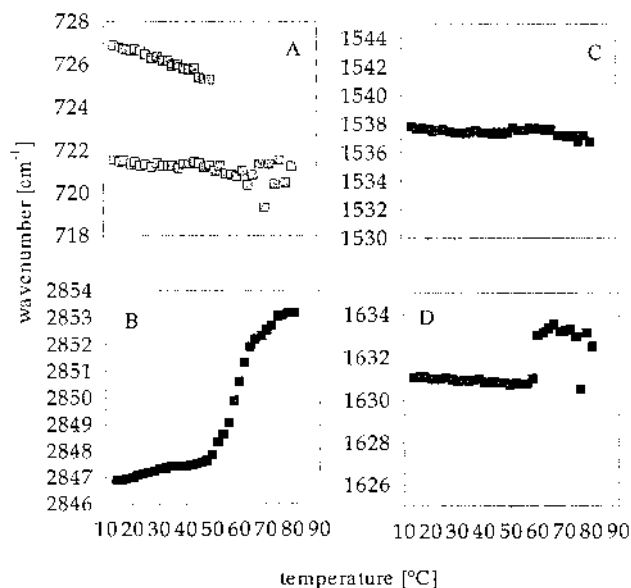


Fig. 6. Ceramide 5 in Model SC. (A) The thermotropic response of the CH₂ rocking frequencies, (B) the thermotropic response of the CH₂ symmetric stretching mode, (C) the thermotropic response of the headgroup amide II mode, (D) the thermotropic response of the amide I mode.

frequencies indicates no rotational freedom and extremely tight packing for both species. The onset of chain disordering ~50°C for both hexadecanoic acid and ceramide 5 may indicate the onset of mixing of these species. However, it could also be coincidental or be due to the melting of domains of one species disrupting the domains of the other species. The sharp phase transitions of hexadecanoic acid and ceramide 5 in this model suggest little mixing with cholesterol.

The headgroup interactions of the ceramide 5 model appear quite straightforward. The carbonyl mode of ceramide 5 (the amide I mode) is not split and maintains a constant frequency of ~1631 cm⁻¹ until 60°C where it increases to ~1634 cm⁻¹ indicating a weakening of hydrogen bonding. The frequency over the entire temperature range, including physiological, is indicative of a strongly hydrogen bonded amide I mode. The amide II mode is also strongly hydrogen bonded but does not show a significant dependence on temperature. Thus the hydrogen bonding of the ceramide 5 headgroup is not effected by changes in the inter- and intramolecular organization of the lipid chains in the model SC. The one clearly significant change in headgroup bonding is a sharp decrease in the acid carbonyl hydrogen bonding that occurs when the crystalline orthorhombic domains of hexadecanoic acid collapse at 50°C. This change occurs immediately before the onset of conformational disorder in the fatty acid chains.

Ceramide 2/hexadecanoic acid-d₃₁/cholesterol model SC lipid barrier

Hexadecanoic acid behavior

The CD₂ scissoring component frequencies are plotted in Figure 7A. The observation of splitting in this mode indicates that the hexadecanoic acid is packed in a perpendicular all-*trans* orthorhombic subcell structure. These modes collapse to

a single peak at 42°C. The thermotropic response of the CD₂ asymmetric stretching mode frequency of hexadecanoic acid-d₃₁ is plotted in Figure 7B. Hexadecanoic acid exhibits a broad melting transition with onset, midpoint, and completion temperatures of 42, 50, and 60°C, respectively. Furthermore, as is clearly shown in Figure 7C, the carbonyl stretching mode of hexadecanoic acid jumps from a strongly hydrogen bonded frequency of 1698 cm⁻¹ to a significantly less strongly bonded frequency of 1712 cm⁻¹ at ~45°C.

Ceramide 2 behavior

The ceramide CH₂ rocking mode is also split into two bands the frequencies of which are plotted as a function of temperature in Figure 8A. This splitting does not collapse until 55°C. The CH₂ symmetric stretching mode frequency of ceramide 2 is plotted in Figure 8B. Ceramide 2 begins its transition at 52°C and ends at 73°C with a T_m of ~65°C. Between 52 and 60°C the ceramide 2 CH₂ frequency increases from 2849 to 2850 cm⁻¹, a range which indicates a loosening of chain packing. The onset of ceramide 2 chain conformational disorder begins at ~60°C. The thermotropic behavior of the ceramide amide II and amide I modes are plotted in Figure 8C and 8D, respectively. The initial frequency of 1548 cm⁻¹ increases to 1553 cm⁻¹ at 45°C (indicating stronger hydrogen bonding) then decreases to 1540 cm⁻¹ at 80°C. It is interesting to note that the increase in amide II frequency occurs while the ceramide chains are highly ordered, but coincides with the collapse of the hexadecanoic acid domains. As shown in Figure 8C, the amide I mode of ceramide 2 splits into two components (~1620 and 1645 cm⁻¹) until 60°C, then collapses to a single peak. We have previously described this splitting behavior in both anhydrous and hydrated ceramide 2 samples (14). Splitting of the amide I mode is often observed in the IR spectra of proteins with extended β-sheet structures and has been known for several decades (18, 19). The splitting indicates coupling between ceramide 2 headgroups.

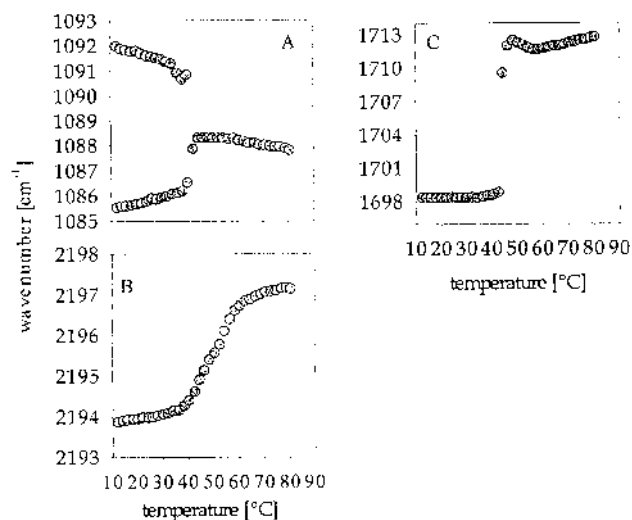


Fig. 7. Hexadecanoic acid-d₃₁ in Ceramide 2 Model SC. (A) The thermotropic response of the CD₂ bending frequencies, (B) the thermotropic response of the CD₂ asymmetric stretching mode, (C) the thermotropic response of the C=O stretching mode.

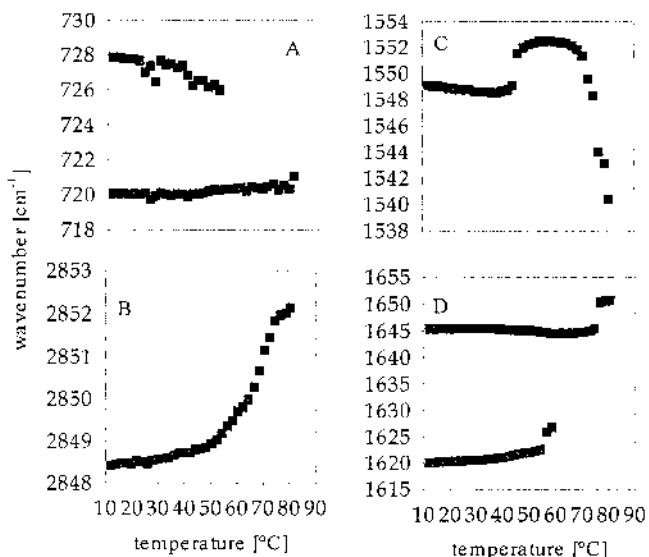


Fig. 8. Ceramide 2 in Model SC. (A) The thermotropic response of the CH₂ rocking frequencies, (B) the thermotropic response of the CH₂ symmetric stretching mode, (C) the thermotropic response of the headgroup amide II mode, (D) the thermotropic response of the amide I mode.

Discussion of ceramide 2 model studies

In the ceramide 2 model the observation of splitting in the CD₂ and CH₂ bending and rocking modes, respectively, provides direct evidence of highly ordered orthorhombic phases. The IR experiment permits direct and simultaneous observation of these coupled modes and thereby allows these structures to be characterized as crystalline orthorhombic phases. Such crystalline phases are consistent with barrier function and provide direct evidence for the presence of lipid domains. The observation of splitting is direct evidence for separate hexadecanoic acid and ceramide 2 domains. Splitting does not occur between molecules of different isotopes and therefore the frequency separation would be reduced in mixed domains of ceramide and hexadecanoic acid. Furthermore, the temperatures at which these crystalline phases collapse are 15°C apart providing further direct evidence for a molecular arrangement in which the fatty acid and ceramide are in separate crystalline domains. The transition temperature from fully ordered to disordered chains is ~50°C for hexadecanoic acid and ~65°C for ceramide 2, therefore both the hexadecanoic acid and ceramide chains are in fully extended all-*trans* conformations at skin physiological temperatures (30–35°C). This is consistent with the water barrier function of lipids in the stratum corneum. The separate melting temperature data provides separate strong evidence that there is some segregation (domains) of these species in the SC model. However, complete immiscibility is not consistent with the data since transition temperatures for both the hexadecanoic acid and ceramide 2 are ~20°C below the pure materials. In addition, the broad nature of the hexadecanoic acid transition suggests that the fatty acid is mixing with another species (the melting of a single species occurs over a range of 2–3°C not 20°C, as in this case). At temperatures below 52°C the splitting of ceramide 2 CH₂ rocking modes indicates that ceramide 2 is still packed in crystalline domains. Since mixing of ceramide 2 with hexadecanoic acid is

probably not responsible for the broad phase transition, it seems likely that cholesterol mixing is producing the broad phase transition.

The data from the Ceramide 2 model provide direct evidence for strong intermolecular headgroup bonding between ceramide 2 headgroups in this model SC sample. We speculate that this intermolecular bonding occurs between opposing headgroups and plays a significant role in providing cohesiveness to the SC barrier. This concept is illustrated in Figure 9. The bonding between ceramide 2 headgroups persists to 60°C, i.e. some 30°C above skin physiological temperatures, and is not disrupted until the onset of intramolecular conformational disorder in the ceramide 2 chains. Both the amide II mode of ceramide 2 and the carbonyl mode of hexadecanoic acid are strongly hydrogen bonded at physiological temperatures. However, when the orthorhombic phases of the hexadecanoic acid chains collapse there is an immediate weakening of hexadecanoic acid headgroup hydrogen bonding as indicated by the increase in frequency of the acid carbonyl. At the same temperature the frequency of the amide II mode also increases indicating stronger hydrogen bonding (bending mode frequencies increase with H-bonding) for the N-H bond of the ceramide 2 headgroup. This stronger interaction persists until the ceramide chains are completely disordered at ~70°C.

CONCLUSION

These experiments provide some insight to the molecular organization of fatty acids and ceramides in two lipid models of the SC. As outlined at the beginning FTIR spectroscopy is an extremely well suited technique for such studies. While the utility of FTIR spectroscopy for investigating lipid chain biophysics is well recognized, the current work illustrates the high level of molecular information concerning headgroup

interactions that is contained within the same IR spectra. It is interesting to consider the structural implications of the headgroup data from these two ceramide models. The molecular structure information provided by the current studies of SC models aids in our attempt at understanding the role of ceramide 2 (ceramide [NS]) and ceramide 5 (ceramide [AS]) in the structural integrity of the SC. While both ceramide species and hexadecanoic acid pack in highly ordered crystalline phases at physiological temperature, the interactions of the headgroups are very different. The coupling and splitting of the amide I mode of ceramide 2 indicates strong bonding between opposing ceramide 2 headgroups which indicates a transverse organization (domain) of ceramide 2 molecules in addition to the lateral orthorhombic chain organization. However, the headgroup interactions collapse with the melting of the ceramide 2 chains. In contrast, the data from the headgroup modes of ceramide 5 indicate that strong headgroup hydrogen bonding is maintained between the lateral assemblies (domains) of ceramide 5 even after conformational disordering of the ceramide 5 chains.

A few years ago Forslind proposed the "domain mosaic model" of the skin barrier (20, 21). In this model the lipids of the skin barrier are organized in domains of ordered lipids bounded by more fluid phase lipids. This hypothesis has been a great influence on our thinking about the lipid biophysics of the SC. Our IR experiments with SC lipid models are consistent in many ways with Forslind's model and clearly show an organization of ordered orthorhombic lipid domains in the physiological temperature range.

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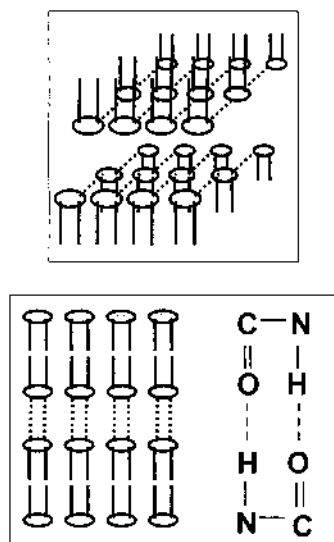


Fig. 9. Possible headgroup hydrogen bonding interactions in the ceramide 5 and 2 SC models. The lateral hydrogen bonding of the ceramide 5 model would add cohesion within the bilayer plane. In contrast, the transverse hydrogen bonding between ceramide 2 molecules could add significant integrity to the barrier structure through the bilayers.

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