Lipid mixtures prepared with well-defined synthetic ceramides closely mimic the unique stratum corneum lipid phase behavior

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Abstract Lipid lamellae present in the outermost layer of the skin, the stratum corneum, form the main barrier for the diffusion of molecules through the skin. The presence of a unique 13 nm lamellar phase and its high crystallinity are characteristic for the stratum corneum lipid phase behavior. In the present study, small-angle and wide-angle X-ray diffraction were used to examine the organization in lipid mixtures prepared with a unique set of well-defined synthetic ceramides, varying from each other in head group architecture and acyl chain length. The results show that equimolar mixtures of cholesterol, free fatty acids, and synthetic ceramides (resembling the composition of pig ceramides) closely resemble the lamellar and lateral stratum corneum lipid organization, both at room and higher temperatures. Exclusion of several ceramide classes from the mixture does not affect the lipid organization. However, complete substitution of ceramide 1 (acylceramide with a sphingosine base) with ceramide 9 (acylceramide with a phytosphingosine base) reduces the formation of the long periodicity lamellar phase. This indicates that the head group architecture of acylceramides affects the lipid organization. In conclusion, lipid mixtures prepared with well-defined synthetic ceramides closely mimic the unique stratum corneum lipid phase behavior.

Supplementary key words barrier • X-ray diffraction • lipid organization

The barrier function of the skin is predominantly attributed to its outermost layer, the stratum corneum, which efficiently protects the body from desiccation and percutaneous penetration of compounds. The stratum corneum consists of several layers of overlapping corneocytes embedded in a matrix of lipids. It has generally been accepted that the intercornocyte lipids, with a very specific composition and organization, play an important role in the skin barrier function. Unlike biological membranes, phospholipids are nearly absent in the stratum corneum, and the major constituents are ceramides, cholesterol, and long-chain free fatty acids. Ceramides are structurally heterogeneous, and together with cholesterol and free fatty acids they exhibit quite different phase behavior than phospholipids and other more widely studied biological membrane lipids.

Ceramides are composed of a long-chain sphingosine (S), phytosphingosine (P), or 6-hydroxysphingosine (H) base with variations in the position and number of hydroxyl groups and double bonds (1–4). Through amide bonding, long-chain nonhydroxy (N) or ω-hydroxy fatty acids (A) with varying acyl chain lengths (predominantly 24 and 26 carbon atoms) are linked to the sphingoid bases. In pig stratum corneum, the exceptions are ceramide 1 (CER1) (EOS) and CER5(AS). The former contains an ω-hydroxy fatty acid with a chain length of 30–32 carbon atoms to which linoleic acid is linked (EO), whereas CER5(AS) contains a fatty acid with a chain length of only 16–18 carbon atoms. In human stratum corneum, three ceramides [CER1(EOS), CER4(EOH), and CER9(EOP)] have an exceptionally long molecular structure, whereas CER5(AS) has a fatty acid chain length of ~24 carbon atoms.

Several studies have been performed to provide insight into the complex lipid architecture underlying the skin barrier function. Electron microscopy studies demonstrated that the intercellular lipids in the stratum corneum have a lamellar organization with a repeating pattern of ~13 nm, consisting of a broad-narrow-broad sequence of electron-
lucent bands (5, 6). X-ray diffraction studies demonstrated the presence of two coexisting lamellar phases in human stratum corneum: the short periodicity phase (SPP), with a repeat distance of \( \sim 6 \) nm, and the long periodicity phase (LPP), with a repeat distance of \( \sim 13 \) nm (7–9). The molecular organization of the LPP together with its predominantly orthorhombic lipid packing in the presence of substantial amounts of cholesterol are exceptional and therefore are considered to play an important role in the maintenance of a competent skin barrier.

Mixtures prepared with cholesterol, free fatty acids, and ceramides isolated from human or pig stratum corneum closely mimic the lipid organization found in the stratum corneum (10–12). It has been demonstrated that, in particular, cholesterol and ceramides are important for the formation of the LPP, whereas free fatty acids are required for the crystalline (orthorhombic) character of the lateral lipid packing. As natural ceramides contain various subclasses of ceramides with varying acyl chain lengths, it is practically impossible to selectively remove individual ceramides in quantities. Therefore, detailed studies of the effects of the molecular structures of individual ceramides on stratum corneum lipid organization are only possible with mixtures based on synthetic ceramides, as their composition can be accurately chosen and systematically modified. The prerequisite to replace natural ceramides (natCER) with synthetic ceramides (synthCER) is that their phase behavior reflects that of the stratum corneum. In previous studies, we demonstrated that equimolar mixtures of cholesterol, free fatty acids, and a limited number of synthCER, namely CER1(EOS), CER3(NP), and bovine brain CER type IV (referred to as \( \Sigma \)CERIV), mixed in a 1:7:2 ratio, closely resemble the lamellar and lateral stratum corneum lipid organization, both at room and higher temperatures (13, 14). CER(EOS) and CER(NP) contain fatty acids of a uniform acyl chain length, whereas \( \Sigma \)CERIV contains fatty acids with varying acyl chain lengths, with C18 and C24 as the most abundantly present (14). Mixtures prepared with CER(EOS), CER(NP) and \( \Sigma \)CERIV are denoted as synthCER I mixtures. It has been demonstrated that proper lipid organization in synthCER I mixtures can only be achieved with an optimal CER(NP):\( \Sigma \)CERIV ratio in the presence of CER(EOS) (14, 15).

In spite of many similarities, some differences are also observed between mixtures prepared with natCER and synthCER I. The main difference is the presence of one or two additional phases with repeat distances of 3.7 and 4.3 nm, respectively. These phases have never been observed in mixtures containing natCER and can be ascribed to crystalline CER(NP), present in separate domains. Another difference is the repeat distance of the LPP, which is slightly shorter (\( \sim 12 \) nm) in synthCER mixtures than in natCER mixtures (\( \sim 13 \) nm). In addition, the relative intensities of the reflections attributed to the LPP in mixtures prepared with synthCER differ slightly from those observed in mixtures prepared with natCER (13). This in-

![Fig. 1. The molecular structures of the synthetic ceramides (synthCER) used in this study.](image-url)
dicates that the localization of the lipids within the LPP in mixtures prepared with synthCER might differ slightly.

Currently, we have at our disposal a unique set of synthesized ceramides that closely mimic the ceramide composition of pig stratum corneum (Fig. 1). In addition, we have synthetic CER9 (EOP), which is present in human stratum corneum but not in pig stratum corneum. CER(EOP) is structurally similar to CER(EOS) but contains an additional hydroxyl group at the sphingoid backbone. Each of the synthCER has a well-defined head group architecture and acyl chain length. Mixtures prepared with the well-defined synthCER are referred to as synthCER II. Because the synthetic counterpart of CER(AS) (acyl chain length of 16 carbon atoms) is not available, all synthCER II mixtures were prepared with CER(NP) with an acyl chain of 16 carbon atoms [CER(NP)C16]. The aim of the present study is 3-fold and can be summarized in the following questions: i) Is it possible to mimic the stratum corneum lipid phase behavior with mixtures prepared with well-defined synthCER at room and higher temperatures? We will particularly focus on the role of free fatty acids, the presence of additional phases, and the intensity distribution of the diffraction peaks of the LPP. ii) Is the formation of the LPP also enhanced when CER(EOS) is gradually substituted with CER(EOP)? iii) Is it possible to reduce the number of synthCER classes in the mixture without affecting the formation of the lamellar phases?

To answer these questions, the phase behavior of various equimolar cholesterol (CHOL):synthCER II:FFA mixtures was studied. First, a mixture was studied that closely mimics the composition of the ceramides in pig stratum corneum (10, 16). Subsequently, the synthCER II composition was gradually changed (Table 1) to elucidate the role various synthCER play in the formation of the LPP.

### Materials and Methods

**Materials**

Palmitic acid, stearic acid, arachidic acid, behenic acid, tricosanoic acid, lignocerinic acid, cerotic acid, and cholesterol were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). CER(EOS) C30-linolate, CER(NS)C24, CER(NP)C24, CER3(NP)C16, CER(AS)C24, CER(AP)C24, and CER(EOP) C30-linolate were generously provided by Cosmoferm B.V. (Delft, The Netherlands). All organic solvents used were of analytical grade and manufactured by Labscan Ltd. (Dublin, Ireland).

| Fraction | CER(EOS) | CER(NS) | CER(NP)C24 | CER(AS) | CER(NP)C16 | CER(AP) | CER(EOP) |
|----------|----------|---------|------------|---------|------------|---------|----------|
| synthCER II1  | 0.15 | 0.51 | 0.16 | 0.04 | 0.09 | 0.05 | —         |
| synthCER II2  | 0.10 | 0.51 | 0.16 | 0.04 | 0.09 | 0.05 | 0.05     |
| synthCER III1 | 0.05 | 0.51 | 0.16 | 0.04 | 0.09 | 0.05 | 0.10     |
| synthCER III2 | —     | 0.60 | 0.19 | 0.04 | 0.11 | 0.06 | —         |
| synthCER III3 | 0.15 | 0.53 | 0.17 | —     | 0.10 | 0.05 | —         |
| synthCER III4 | 0.15 | 0.54 | 0.17 | 0.04 | 0.10 | —     | —         |
| synthCER III5 | 0.15 | 0.57 | 0.18 | —     | 0.10 | —     | —         |
| synthCER III6 | 0.15 | 0.25 | 0.29 | 0.06 | 0.16 | 0.09 | —         |

synthCER, synthetic ceramides.

### Preparation of the lipid mixtures

For the preparation of the CHOL:synthCER:FFA mixtures, the following fatty acid mixture was used: C16:0, C18:0, C20:0, C22:0, C23:0, C24:0, and C26:0 at molar ratios of 1.3, 3.3, 6.7, 41.7, 5.4, 36.8, and 4.7, respectively. This is similar to the composition of the free fatty acids in native stratum corneum (17). Appropriate amounts of individual lipids dissolved in chloroform-methanol (2:1) were combined to yield mixtures of ~1.5 mg dry weight at the desired composition at a total lipid concentration of 7 mg/ml. A Camag Linomat IV was used to spray the lipid mixtures onto mica. This was done at a rate of 4.3 μL/min under a continuous nitrogen stream. Subsequently, the samples were equilibrated for 10 min at the optimal temperature (70°C for equimolar CHOL:synthCER:FFA mixtures and 85°C for equimolar CHOL:synthCER mixtures) and hydrated with an acetate buffer at pH 5.0. Finally, the samples were homogenized by 10 successive freeze-thaw cycles between −20°C and room temperature, during which the samples were stored under gaseous argon.

### Small-angle X-ray diffraction

All measurements were performed at the European Synchrotron Radiation Facility (Grenoble, France) using station BM26B (18). The X-ray wavelength and the sample-to-detector distance were 1.24 Å and 1.7 m, respectively. Diffraction data were collected on a two-dimensional multiwire gas-filled area detector. The spatial calibration of this detector was performed using silver behenate. The samples were mounted in a sample holder with mica windows. Static diffraction patterns of the lipid mixtures were obtained at room temperature for a period of 5 min. The temperature-induced phase changes were investigated by collecting diffraction patterns, during which the temperature of the sample was increased from 25°C to 95°C at a rate of 2°C/min. Each successive diffraction curve was collected for a period of 1 min.

Small-angle X-ray diffraction provides information about the larger structural units in the sample, namely, the repeat distance of a lamellar phase. The scattering intensity I (in arbitrary units) was measured as a function of the scattering vector q (in reciprocal nm). The latter is defined as $q = (4\pi \sin \theta)/\lambda$, in which $\theta$ is the scattering angle and $\lambda$ is the wavelength. From the positions of a series of equidistant peaks ($q_n$), the periodicity, or d-spacing, of a lamellar phase was calculated using the equation $q_n = 2\pi/d$, $n$ being the order number of the diffraction peak.

### Wide-angle X-ray diffraction

Wide-angle X-ray diffraction provides information about the lateral packing of the lipids within the lamellae. Wide-angle X-ray diffraction data were collected on a microstrip gas chamber detector with an opening angle of 60° (19). The sample-to-detector distance was 36 cm, and the X-ray wavelength was 1.24 Å. The spatial calibration of the detector was performed with a Si:CHOL mixture.
The small-angle and wide-angle X-ray diffraction data were collected simultaneously.

RESULTS

**Importance of free fatty acids for proper lamellar organization**

The role of free fatty acids for the formation of the lamellar phases was investigated using a synthCER mixture that closely mimics the composition of the ceramides in pig stratum corneum (synthCER II; see Table 1 for detailed composition). The corresponding diffraction patterns are illustrated in Fig. 2. The diffraction pattern of the equimolar CHOL:synthCER II:FFA mixture reveals the presence of five reflections (q = 0.52, 1.03, 1.56, 2.08, and 3.09 nm⁻¹) that can be ascribed to a 12.2 nm phase (LPP). The reflections at q = 1.18 and 2.36 nm⁻¹ correspond to the first- and second-order diffraction peaks of a lamellar phase with a periodicity of 5.3 nm (SPP). The two reflections at 1.87 and 3.74 nm⁻¹ reveal the presence of crystalline cholesterol in separate domains.

In the absence of free fatty acids, three major changes are observed in the diffraction pattern. i) The reflections attributed to the LPP are markedly reduced in intensity. In addition, an increased peak width is observed, indicating a reduced ordering of the lipids within the LPP. ii) Three reflections at 0.89, 1.77, and 3.57 nm⁻¹ reveal the presence of a phase with a repeat distance of 7.1 nm. The exact composition and nature of this phase is not known. iii) The broad reflection at 1.30 nm⁻¹ might be ascribed to the SPP with a reduced periodicity of 4.8 nm. However, no higher order reflections can be detected, which makes the interpretation uncertain.

**Substitution of CER(EOS) with CER(EOP) in equimolar CHOL:synthCER II:FFA mixtures**

To investigate whether an additional hydroxyl group at the sphingoid head group of CER(EOS) influences the formation of the LPP, mixtures were prepared in which CER(EOS) was gradually substituted with CER(EOP) (Fig. 1), thereby maintaining the molar ratio between the remaining synthCER constant. The lipid organization in mixtures prepared with 10% CER(EOS) and 5% CER(EOP) (synthCER II₁) and vice versa (synthCER II₃) closely resembles that obtained with the mixture prepared with 15% CER(EOS) only (synthCER II₄). Five diffraction peaks indicate the presence of the LPP, whereas the SPP is identified by three equidistant peaks. In addition, two reflections indicate the presence of crystalline cholesterol. The diffraction pattern of the mixture prepared with 5% CER(EOS) and 10% CER(EOP) (synthCER II₅) is illustrated in Fig. 3A.

Complete substitution of CER(EOS) with CER(EOP) (synthCER II₄) results in decreased relative intensities of the reflections attributed to the LPP compared with those of the SPP (Fig. 3A). In addition, a slightly increased peak width at half-maximum of the LPP reflections is observed, indicating a reduced ordering of the lipids. No peaks can be detected that might be ascribed to crystalline CER(EOP) in separate domains (15).

As observed in previous studies (15, 20), the characteristic LPP is not present in mixtures prepared in the absence of any acylceramide (synthCER II₃). Instead, three strong reflections indicate the presence of a 5.4 nm lamellar phase (SPP). In addition, crystalline cholesterol is present in the mixture (Fig. 3B).

**Effect of reducing the number of individual synthCER in equimolar CHOL:synthCER II:FFA mixtures**

To elucidate whether the number of synthCER classes can be reduced without affecting the lipid phase behavior, the organization in mixtures prepared in the absence of CER(AS), CER(AP), or both (synthCER II₆₋₈) was studied. The choice to exclude CER(AS) and CER(AP) from the mixture is based on the low relative amounts of these synthCER in the synthCER II mixture. In addition, one mixture was examined in which the amount of CER(NS) in the synthCER fraction was decreased to 50% of its original amount (synthCER II₉).

Exclusion of CER(AS), CER(AP), or both from the synthCER mixtures does not affect the phase behavior of the lipid mixtures (Fig. 4; only the curve of the lipid mixture prepared with synthCER II₆ is presented). Five equidistant peaks suggest the presence of the LPP (12.2 nm), whereas the SPP (5.4 nm) is indicated by three reflections. In addition, phase-separated cholesterol can be detected. The in-
The diffraction pattern of the equimolar CHOL: synthCER II:FFA mixture, in which the relative amount of CER(NS) is decreased to 25%, is also shown in Fig. 4. A decrease in CER(NS) content results in the appearance of a weak reflection at q = 1.45 nm\(^{-1}\). Although no higher order reflections can be detected, this most likely corresponds to a phase with a repeat distance of 4.3 nm, ascribed to crystalline CER(NP) in separate domains (13, 20). The formation of the LPP and SPP is not affected.

**Phase behavior as a function of temperature**

The lipid phase behavior of the equimolar CHOL: synthCER II:FFA mixture has been examined in the temperature range from 25°C to 95°C (Fig. 5A). Each curve represents the mean of the lipid phases present during a temperature shift of 2°C. The diffraction pattern at 25°C reveals a number of reflections that can be ascribed to the presence of a LPP and a SPP, with periodicities of 12.2 and 5.4 nm, respectively, similar to that shown in Fig. 2. Between 25°C and 55°C, no significant changes are observed in positions or intensities of the LPP and SPP reflections. However, a further increase in temperature gradually decreases the peak intensity of the SPP, which disappears at ~61°C. The reflections of the LPP disappear at ~65°C, whereas the two reflections attributed to crystalline cholesterol disappear at ~53°C.

At ~45°C, a new phase is formed, of which only one reflection can be detected that gradually shifts to q = 1.37 nm\(^{-1}\) (repeat distance of 4.6 nm) at 63°C. The reflection drastically increases in intensity up to 71°C, being the most prominent peak in the diffraction pattern at higher temperatures. Between 91°C and 95°C, the reflection disappears.

**Lateral packing of the lipids**

A hexagonal lateral packing is characterized by a strong 0.41 nm reflection in the wide-angle X-ray diffraction pattern.
tern, whereas the diffraction pattern of an orthorhombic packing is characterized by two strong 0.41 and 0.37 nm reflections, located at \( q/H_1 \) 15.40 and 17.12 nm, respectively. The diffraction pattern of the equimolar CHOL:synthCER II 1:FFA mixture monitored as a function of temperature is plotted in Fig. 5B. The 0.408 and 0.367 nm peaks indicate an orthorhombic lateral packing. Between 33°C and 35°C, the 0.367 nm reflection disappears, indicating an orthorhombic-to-hexagonal phase transition. The 0.408 nm reflection first decreases in intensity. However, a further increase in temperature increases the intensity of the 0.408 nm reflection slightly, indicating a metastable-to-stable phase change. Similar results were obtained with isolated stratum corneum (J. A. Bouwstra and G. S. Gooris, unpublished results). A disappearance of this peak is observed at 65°C, which is at the same temperature at which the LPP disappears in the small-angle X-ray diffraction pattern. In addition, two reflections of crystalline cholesterol can be detected (\( q = 10.98 \) and 16.58 nm\(^{-1}\)), both of which disappear at ~53°C.

**Relative intensities of LPP reflections**

The relative intensities of the various reflections of the LPP in six equimolar CHOL:synthCER II 1:FFA mixtures have also been determined to obtain information on the relative electron density distribution in the characteristic 13 nm lamellar phase. The results provided in Table 2 show that the relative intensities of the peaks attributed to the LPP show the same trend in equimolar CHOL:CER:FFA mixtures prepared with either synthCER I, synthCER II 1, or pigCER. However, the values obtained with synthCER II 1 mixtures resemble more closely those of pigCER mixtures than those of the synthCER I mixture.

| Order | CHOL:synthCER II 1:FFA | CHOL:synthCER I:FFA | CHOL:pigCER:FFA |
|-------|-----------------------|---------------------|-----------------|
| 1     | 1.0                    | 1.0                 | 1.0             |
| 2     | 2.55 ± 0.15            | 2.94 ± 0.36         | 2.55 ± 0.28     |
| 3     | 0.50 ± 0.03            | 0.37 ± 0.06         | 0.81 ± 0.10     |
| 4     | 0.13 ± 0.02            | 0.15 ± 0.02         | 0.09 ± 0.01     |
| 5     | 0.02 ± 0.01            | —                   | 0.05 ± 0.01     |
| 6     | —                      | 0.04 ± 0.03         | 0.08 ± 0.03     |

CHOL, cholesterol; LPP, long periodicity phase. For scaling, the intensity of the first-order diffraction peak of all phases was set equal to 1. The values are presented as averages \( \pm SD \) (n = 6). Because of (partial) overlap with more prominent peaks in the diffraction pattern, the sixth and seventh order of the LPP could not all be determined in the synthCER I and synthCER II mixtures, respectively.

**DISCUSSION**

Lipid organization in mixtures prepared with well-defined synthCER

The results of the present study convincingly show that the lipid organization in mixtures prepared with cholesterol, synthCER II, and free fatty acids closely resembles that of natCER mixtures, as both the LPP and SPP are present and a small fraction of cholesterol phase separates into crystalline domains (10–12, 21). Also, at increased temperatures, many similarities can be observed in lipid phase behavior between equimolar CHOL:CER:FFA mixtures containing synthCER II 1 or natCER (10, 11): i) between 33°C and 35°C, a phase transition from an orthorhombic to a hexagonal lateral packing occurs; ii) the LPP and SPP disappear at ~65°C and 61°C, respectively; iii) at higher temperatures, an additional phase is formed with a repeat distance of 4.6 nm (synthCER II 1) or 4.3 nm (natCER); and iv) the hexagonal lateral packing disappears at 65°C, which is the same temperature at which the LPP disappears. This suggests that either an orthorhombic or a hexagonal lateral packing is a prerequisite for LPP forma-
tion, which confirms the findings with natCER: when the fraction of lipids in a liquid phase is too high, the formation of the SPP is increased at the expense of the LPP (21).

Influence of the gradual substitution of CER(EOS) with CER(EOP) on LPP formation

CER(EOS) promotes the formation of the LPP more efficiently than CER(EOP), indicating that the additional hydroxyl group of CER(EOP) and the simultaneous loss of the 4,5-desaturation inhibits the formation of the LPP. When the diffraction patterns of the lipid mixtures containing either synthCER II or synthCER I (15) are compared, two important differences are noted: i) CER(EOP) promotes the formation of the LPP more efficiently in the synthCER II mixtures than in the synthCER I mixtures; and ii) in the synthCER II mixtures, the maximum solubility of CER(EOP) in the lamellar phases is increased compared with that of the synthCER I mixtures, as in the latter mixture CER(EOP) partially phase separates. Both observations illustrate that the lipid organization in synthCER II mixtures, in which the overall head group variability is increased, is less sensitive to variations in the acylceramide head group architecture. Interestingly, in both synthCER mixtures, the partial substitution of CER(EOS) with CER(EOP) does not significantly affect the lipid organization.

Effect of diminishing the number of synthCER on phase behavior

The exclusion of CER(AS) and CER(AP) from the synthCER II mixture (synthCER II0) changes the relative amounts of CER(EOS), CER(NS), CER(NP)C24, and CER(NP)C16 to a 15:57:18:10 molar ratio. The composition of the synthCER I fraction prepared with 15% CER(EOS) is CER(EOS), CER(NP)C24, and ΣCERIV in a 15:66:19 molar ratio. The ratio between the two most abundantly present acyl chain lengths in ΣCERIV is C24:C18 at 2:1. Therefore, the molar ratios of the various synthCER in synthCER II0 and synthCER I are very similar, with CER(NS) or CER(NP) as the most abundantly present. Whereas extensive phase separation of CER(NP) is observed in mixtures prepared with synthCER I, no additional phases attributed to CER(NS) can be detected in mixtures prepared with synthCER II. The difference between CER(NS) and CER(NP) is the presence of an additional hydroxyl group in CER(NP) and the simultaneous loss of the 4,5-desaturation (Fig. 1). The additional hydroxyl group enables strong intermolecular head group hydrogen bonding, which is most likely the driving force for the molecular organization of CER(NP) in separate domains (22). It is striking that in the synthCER II0 mixture, prepared with a relative CER(NS) content of 25%, a small reflection indicates the presence of an additional 4.3 nm phase, which can be ascribed to crystalline CER(NP) in a V-shaped formation (20, 23, 24). This is caused by the increased relative amount of CER(NP)C24 in synthCER II0. In a previous study, in which the optimal CER(NP)-to-ΣCERIV ratio for the formation of the LPP was established, phase separation of CER(NP) was also observed when the relative amount of CER(NP) increased (14).

Comparison of the phase behavior of natCER mixtures versus synthCER II mixtures

In spite of many similarities, two differences in phase behavior are also observed between natCER mixtures and synthCER II mixtures. The first difference is the slightly reduced repeat distance of the LPP in mixtures prepared with synthCER II compared with mixtures prepared with natCER. The periodicity of the LPP in equimolar CHOL:natCER mixtures prepared with isolated human or pig ceramides is very similar, being 12.8 and 12.2 nm, respectively (10, 11). The addition of free fatty acids to those mixtures slightly increases the LPP periodicity from 12.8 and 12.2 nm to 13.0 and 13.3 nm, respectively. However, the repeat distance of the LPP in synthCER II or synthCER I mixtures is always ~12.2 nm, irrespective of the presence of free fatty acids. Although we can speculate that this might be related to differences in the crystallinity or acyl chain length of the ceramides, further studies are required to unravel this phenomenon.

Second, the results of the present study reveal that free fatty acids are required for proper lipid organization in synthCER II mixtures, as in the equimolar CHOL:synthCER II mixture a marked reduction in the formation of the LPP is observed and an additional phase with a repeat distance of 7.1 nm is formed. This strongly suggests that the presence of free fatty acids with varying acyl chain lengths facilitates the incorporation of the synthCER into the lamellar phases in the lipid mixtures. The major difference between natCER and synthCER II is the great variation in acyl chain length in natCER versus the well-defined chain length in synthCER II. The importance of chain length variation for proper LPP formation was already demonstrated in synthCER I mixtures by systematically increasing the relative amount of free fatty acids or by increasing the amount of ΣCERIV (chain length variation) at the expense of CER(NP) (uniform chain length). An increased relative amount of FFA or ΣCERIV initially enhances the formation of the LPP. However, when the amount exceeds a certain optimal amount, the formation of the LPP is reduced and the SPP dominates. This suggests that for proper lipid organization, a certain optimal chain length variation should be present in the mixture, either in the FFA fraction or in the synthCER fraction. However, additional studies should be performed to further elucidate this point.

In conclusion, the results of the present study demonstrate that one can generate lipid mixtures with well-defined synthCER that closely mimic the lamellar and lateral organization in native stratum corneum. As their composition can be accurately chosen and modified, synthCER mixtures provide insight into the importance of the molecular structures of individual CER in relation to lipid organization. Skin disorders are frequently characterized by a defective permeability barrier and an aberrant lipid profile compared with healthy skin. Therefore, synth-
CER mixtures are an attractive tool to study in detail the effects of altered lipid composition on the lamellar and lateral lipid organization.

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