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The role of ceramide composition in the lipid organisation of the skin barrier

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Abstract

The lipid lamellae in the stratum corneum (SC) play a key role in the barrier function of the skin. The major lipids are ceramides (CER), cholesterol (CHOL) and free fatty acids (FFA). In pig SC at least six subclasses of ceramides (referred to as CER 1, 2–6) are present. Recently it was shown that in mixtures of isolated pig SC ceramides (referred to as CER(1–6)) and CHOL two lamellar phases are formed, which mimic SC lipid organisation very closely [J.A. Bouwstra et al., 1996, J. Lipid Res. 37, 999–1011] [1]. Since the CER composition in SC originating from different sources/donors often varies, information on the effect of variations in CER composition on the SC lipid organisation is important. The results of the present study with mixtures of CHOL including two different CER mixtures that lack CER 6 (CER(1–5) mixtures) revealed that at an *equimolar* molar ratio their lipid organisation was similar to that of the equimolar CHOL:CER(1–6) and CHOL:CER(1,2) mixtures, described previously. These observations suggest that at an equimolar CHOL:CER ratio the lipid organisation is remarkably insensitive toward a change in the CER composition. Similar observations have been made with equimolar CHOL:CER:FFA mixtures. The situation is different when the CHOL:CER molar ratio varies. While in the CHOL:CER(1–6) mixture the lamellar organisation hardly changed with varying molar ratio from 0.4 to 2, the lamellar organisation in the CHOL:CER(1–5) mixtures appeared to be more sensitive to a change in the relative CHOL content, especially concerning the changes in the periodicities of the lamellar phases. In summary, these findings clearly indicate that at an equimolar CHOL:CER molar ratio the lamellar organisation is least sensitive to a variation in CER composition, while at a reduced CHOL:CER molar ratio the CER composition plays a more prominent role in the lamellar phases. This observation may have an implication for the in vivo situation when both the CER composition and the CHOL:CER molar ratio change simultaneously.

Keywords: Stratum corneum; Ceramide; Phase behavior; X-ray diffraction

1. Introduction

The mammalian skin is a multilayered tissue that is designed to protect the body against undesired influences from the external environment. The main barrier, which is located in the upper layer of the skin, the stratum corneum (SC) [2], is the major problem for transdermal and dermal delivery of drugs. The SC consists of dead cells enclosed by hydrophobic crystalline lipid lamellar regions. Since the lipids form the only continuous region in the SC

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and consequently the diffusing substances always have to pass these lipid regions, the lipid organisation is considered to be very important for the skin barrier function [3–5].

In previous studies it was found that the lipids are mainly organised in lipid lamellae oriented approximately parallel to the corneocyte surface [6–8]. Furthermore X-ray diffraction and NMR studies revealed that the lateral packing of SC lipids is mainly crystalline or hexagonal [9–14]. Two lamellar phases could be identified with repeat distances of approximately 6 (referred to as the short periodicity phase) and 13 nm (referred to as the long periodicity phase) [10,11,13,14]. Only the latter is present in the skin of all examined species. In contrast to the 6 nm lamellar phase the lipid organisation of the 13 nm lamellar phase is very unusual [15,16]. Both the presence of the 13 nm phase in all species examined and its exceptional lipid organisation are indicative of the importance of this phase for a competent skin barrier function.

The main lipid classes in SC are free fatty acids (FFA), cholesterol (CHOL) and ceramides (CER). SC ceramides consist of at least six ceramides (CER(1–6)) that differ from each other by the head group architecture and by the mean fatty acid chain length. The fatty acid esterified to the amide of the (phyto)sphingosine head group can be either α-hydroxy or non-hydroxy fatty acid [17]. In pig SC the fatty acid chain length varies between 16 (CER 5) and 33 C atoms (CER 1), in which the fatty acids of C24 and C26 chain lengths are most abundantly present.

Information on the relationship between lipid organisation and composition is of great importance to unravel the mechanism controlling the skin barrier function. This is particularly demonstrated in diseased skin, in which an impaired barrier function can often be ascribed to an altered lipid composition and organisation. However, until now only limited information is available about the relation between SC lipid composition and organisation.

The research with native SC on the role of individual lipids on the SC lipid organisation is hampered by the complexity of the native tissue, in which next to lipid lamellae also corneocytes and various protein structures, like desmosomes, are present. In addition, no systematic modulation of SC lipid composition can be achieved with the native tissue. Such studies, however, can be performed with mixtures prepared from isolated SC lipids. Our recent studies with mixtures prepared from CHOL and isolated SC ceramides (CER(1–6)) [1,18] revealed that these lipid mixtures mimic the lipid organisation of intact SC closely. Namely, over a wide CHOL:CER(1–6) molar ratio (ranging from 0.2 to 2) two lamellar phases were formed with periodicities of 12.2 and 5.2 nm, respectively. When long-chain FFA were added (with major fractions being C24 and C26) as present in the SC, the periodicity of the lamellar phases increased slightly to 13.1 and 5.3 nm, respectively, mimicking the SC lipid phase behaviour even more closely. In contrast, when short chain free fatty acids (with major fractions being C16 and C18 that are not present in the SC) were added to ceramides or ceramides: cholesterol mixtures phase separation occurred [19,1,20]. This is most probably due to a mismatch in chain length between free fatty acids and ceramides.

Additional studies revealed that the fraction of lipids that forms the long periodicity phase increases in the presence of CER 1 [16]. Furthermore, the 12 nm lamellar phase was not detected in mixtures of bovine brain ceramides III and IV being similar in molecular structure to CER 2 and CER 3 [21–23].

To examine in more detail the role ceramides play in the SC lipid organisation, in the present study the effect of CER composition on the SC lipid organisation has been further assessed with two different CER mixtures: CER I(1–5) and CER II(1–5). We chose mixtures lacking CER 6, since CER 6 was found to be present in only small amounts in reconstructed human epidermis exerting a deviation in lipid organisation [24]. Compared to CER I(1–5) mixture, the CER II(1–5) mixture contained an increased relative amount of CER 2 and a decreased amounts of CER 3 and CER 4, while the relative amounts of CER 1 and 5 were kept constant. This variation is of interest based on a recently proposed model for the 12.2 nm lamellar phase. In this molecular model it is proposed that the role of CER 2, CER 3 and CER 4 in the 12.2 nm lamellar phase is very similar. In contrast, CER 1 and CER 5 are located at distinct positions in this phase, which makes the role of CER 1 and CER 5 in the organisation of this phase very prominent [16]. The lipid organisation of equimolar

mixtures of CHOL:CER(1–5) and CHOL:CER(1–5):FFA and of CHOL:CER(1–5) mixtures with a reduced amount of CHOL has been studied by X-ray diffraction technique. Furthermore, the results of the present and previous studies have been combined to propose a generalised picture illustrating the effects of variations in CER composition on the SC lipid organisation.

2. Materials and methods

2.1. Isolation of stratum corneum from pig skin

Fresh pig skin was obtained from a slaughterhouse. First, the apical side of the skin was briefly washed with hexane (0.03 ml/cm^2) in order to remove the surface lipids. The SC was separated from the epidermis as described earlier [1].

2.2. Extraction, separation and identification of lipids from stratum corneum

SC lipids extracted using the method of Bligh and Dyer [25], were stored in chloroform:methanol (2:1, v/v) at −20°C under nitrogen until use. Subsequently, the extracted lipids were applied on a silica gel 60 (Merck) column (diameter 2 cm, length 33 cm) and individual lipid classes were isolated by sequential elution [1]. The lipid composition of individual fractions was established by one-dimensional high performance thin layer chromatography, as described before [26].

2.3. Ceramide isolation and preparation of lipid mixtures

Fractions containing individual CER isolated by column chromatography were mixed for preparation of two different ceramide mixtures lacking CER 6: CER I(1–5) and CER II(1–5). The CER I(1–5) and CER II(1–5) fractions originated from SC obtained from the same pig. However, in the CER II(1–5) mixture an additional amount of CER 2 was added, while CER 3 and CER 4 were removed as compared to the CER I(1–5) mixture (Table 1).

The ceramides and cholesterol were mixed in various molar ratios. For calculation of the mean ceramide molecular weight being 700, the data on the ceramide composition and alkyl chain length distributions [17] were used. Approximately 2 mg of lipids were solubilised in 80 μl chloroform/methanol (2:1) at the desired composition and applied on mica. A detailed description is given elsewhere [1]. The applied lipid mixtures were covered with 1–2 ml acetate buffer at pH 5.0 (10 mM) and kept under nitrogen. A pH 5 was chosen, since that is the pH of the skin surface [27]. Mixtures were prepared from CHOL, CER(1–5) and FFA. Long-chain free fatty acids mixed in a molar ratio according to Wertz et al. [28] were used for preparation of the FFA mixture. The following fatty acids were included in the lipid mixtures: C16:0, C18:0, C22:0, C24:0 and C26:0 in a molar ratio of 1:3:42:37:7, respectively.

2.4. X-Ray diffraction measurements

2.4.1. Small and wide angle X-ray diffraction (SAXD)

All measurements were carried out at the Synchrotron Radiation Source at Daresbury Laboratory using station 8.2. This station has been built as a part of a NWO/SERC agreement. The samples were put in a specially designed sample holder with two mica windows [13]. The sample-detector distance was set to 1.7 m. Calibration of the detector was carried out with rat tail and cholesterol. From the scattering angle the scattering vector (Q) was calculated $Q = 4\pi(\sin\theta)/\lambda$, in which λ is the wavelength being 0.154 nm at the sample position. Simultaneously with the small angle X-ray diffraction experiments wide angle X-ray diffraction measurements were carried out using an INEL detector. The distance between the sample and INEL detector was set to approximately 0.25 m. The INEL detector was calibrated with cholesterol and SiO_2. No absolute intensities could be calculated. For this reason the intensity of the tail of the diffraction curves were normalised.

The diffraction curves were plotted as a function of Q. For comparison the curves are staggered and the intensity is given in arbitrary units. From the positions of a series of peaks (Q_n) the periodicity of a lamellar phase was calculated using the equation $Q_n = 2\pi n/d$, in which d is the periodicity and nth order of the diffraction peak.

3. Results

3.1. Long range ordering

3.1.1. CER I(1–5)

The X-ray diffraction curves of mixtures prepared from CER I(1–5) and CHOL at molar ratios of 0.2, 0.4 and 1 are plotted in Fig. 1A,B. The diffraction curve of the 0.2 molar mixture revealed one strong peak at 5.4 ($Q = 1.16$ nm^{-1}) nm and several weaker diffraction peaks located at 12.6 ($Q = 0.5$ nm^{-1}), 6.0 ($Q = 1.05$ nm^{-1}), 4.23 ($Q = 1.49$ nm^{-1}), 3.09 ($Q = 2.03$ nm^{-1}), 2.15 ($Q = 2.92$ nm^{-1}), 1.79 ($Q = 3.51$ nm^{-1}), and 1.55 nm ($Q = 4.05$ nm^{-1}, not shown) (see Table 2). The 12.6 (1st order), 6.0 (2nd order), 4.23 (3rd order), 3.09 (4th order), 2.15 (6th order) and 1.55 (8th order) peaks are based on a lamellar phase with a repeat distance of 12.5 nm. The 5.4 (1st order) nm peak is most probably a first order peak of the 5.4 nm lamellar phase. Recently both lamellar phases have been found in mixtures of CER(1–6) and CHOL [1]. Possibly the 1.79 nm peak is the third order peak of the 5.4 nm phase (see below), although it can also be assigned as the 7th order of the 12.5 nm phase. Increasing the CHOL content to a molar ratio of 0.4 shifted the diffraction peaks based on the 12.5 nm phase to longer spacings and increased the intensities of the peaks based on the long periodicity phase. The diffraction peaks of this phase are now located at 12.8 ($Q = 0.49$ nm^{-1}), 6.1 ($Q = 1.03$ nm^{-1}), 4.24 nm ($Q = 1.48$ nm^{-1}) and 2.14 nm ($Q = 2.94$ nm^{-1}, 6th order). The peaks based on the short periodicity phase were slightly shifted to smaller spacings, being now at 5.25 ($Q = 1.20$ nm^{-1}), 2.65 ($Q = 2.37$ nm^{-1}) and 1.77 ($Q = 3.55$ nm^{-1}) nm. Because an increase in CHOL content shifts the 1.79 nm peak to a smaller spacing being 1.77 nm, this peak is most likely based on the short periodicity phase. Finally two weak peaks at 3.34 nm ($Q = 1.88$ nm^{-1}) and 1.68 nm ($Q = 3.76$nm^{-1}) spacing appeared which could be identified as crystalline CHOL that phase separated. Increasing the CHOL content to a molar ratio of 1 decreased the intensities of the peaks based on the long periodicity lamellar phase slightly and shifted these peaks to a longer spacing being now 13.1 ($Q = 0.48$ nm^{-1}), 6.4 ($Q = 0.98$ nm^{-1}), 4.4 ($Q = 1.42$ nm^{-1}) and 2.23 ($Q = 2.82$ nm^{-1}) nm. The intensities of the peaks based on the short periodicity phase did not change, but were again slightly shifted to smaller spacings being now 5.1 ($Q = 1.23$ nm^{-1}) and 2.63 ($Q = 2.38$ nm^{-1}) nm, respectively. Furthermore the intensities of the peaks assigned to crystalline CHOL increased with increasing CHOL content, indicating that the amount of CHOL present in crystalline form increased.

In the presence of long-chain FFA in an equimolar mixture of CHOL, CER I(1–5) and FFA a slight increase in the repeat distance of the short periodicity phase was observed, while the repeat distance of the long periodicity phase did not change upon addition of FFA (see Table 2). The phase separated CHOL was still present as indicated by the presence of 3.33 and 1.68 nm reflections.

3.1.2. CER II(1–5)

The X-ray diffraction curves of mixtures prepared from CER II(1–5) and CHOL at molar ratios of 0.2, 0.4 and 1 are depicted in Fig. 1C,D. The diffraction curve of the mixture at a 0.2 molar ratio revealed one strong broad diffraction peak at 5.8 nm ($Q = 1.08$

Table 1
Composition of the pig ceramide mixtures used in this study and in previous ones

Ceramides	CER I(1–5)	CER II(1–5)[a]	CER(1–6) [1]	CER(1,2)[b] [31]	CER(2–6)
CER 1	4.7	3.4	7.8	12	–
CER 2	56.7	73.5	55.4	88	43.2
CER 3	18.5	9.0	17.6	–	25.8
CER 4	9.8	5.4	3.6	–	5.5
CER 5	10.3	8.6	9.9	–	19.3
CER 6	–	–	5.6	–	6.3

CER(1–5), CER(1–6) and CER(2–6) are isolated from SC obtained from different pigs. The composition is given in percentage weight.

[a]Weight ratios have been changed by adding CER 2 and removing CER 3 and CER 4.

[b]Ratio chosen such that CER 1:CER 2 weight fraction is similar to that in the CER(1–6) mixture.

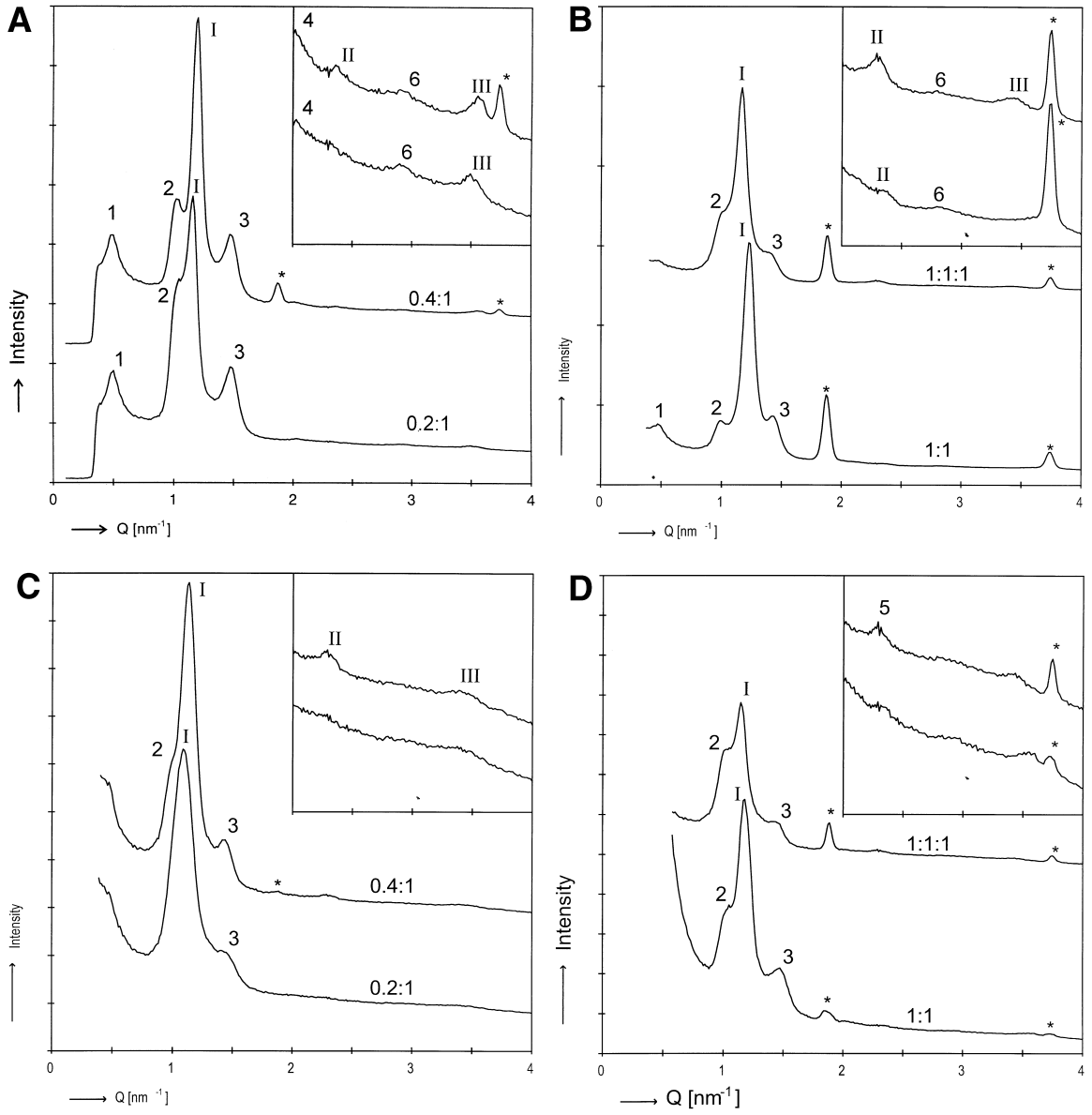

Fig. 1. (A) Small angle X-ray diffraction curves of mixtures of CHOL and CER I(1–5) in a molar ratio of 0.2:1 and 0.4:1. The numbers I, II and III refer to the 1st, 2nd and 3rd order diffraction peaks of a lamellar phase varying in periodicity between 5.3 and 5.4 nm. The numbers 1, 2, 3, 4 and 6 refers to the orders peak of lamellar phase varying in repeat distance between 12.5 and 12.7 nm. * denotes cholesterol. For comparative reasons the curves are staggered and the intensity is plotted in arbitrary units. (B) Small angle X-ray diffraction curves of a CHOL:CER I(1–5) mixture in a molar ratio of 1:1 and a mixture of CHOL, CER I(1–5) and free fatty acids (FFA) in a molar ratio of 1:1:1. The numbers I, II and III refer to the 1st, 2nd and 3rd order of the lamellar phase varying in repeat distance between 5.1 and 5.4 nm, the numbers 1, 2 and 3 refer to the lamellar phase with a repeat distance of approximately 13 nm. * denotes cholesterol. For further explanations see A. (C) Small angle X-ray diffraction curves of mixtures of CHOL and CER II(1-5) in a molar ratio of 0.2:1 and 0.4:1. The numbers I, II and III refer to the 1st, 2nd and 3rd order diffraction peaks of the phase with a repeat distance varying between 5.6 and 5.7 nm. The numbers 2 and 3 refer to the 2nd and 3rd order peaks of the lamellar phase with a repeat distance of approximately 13 nm. CHOL denotes cholesterol. For further explanations see A. (D) Small angle X-ray diffraction curves of a CHOL:CER II(1–5) mixture in a molar ratio of 1:1 and a mixture of CHOL, CER I(1–5) and free fatty acids (FFA) in a molar ratio of 1:1:1. The numbers I, II and III refer to the 1st, 2nd and 3rd order of a lamellar phase varying in repeat distance between 5.3–5.5 nm, the numbers 1, 2 and 3 refer to a lamellar phase with a periodicity of approximately 13 nm. CHOL denotes cholesterol. For further explanations see A.

Table 2
The long range ordering of the CHOL:CER(1–5) and CHOL:CER(1–5):FFA mixtures

Composition	Long periodicity phase	Short periodicity phase	CHOL
CHOL:CER I(1–5)			
0.2:1	**12.5**: *12.6 (1), 6.0 (2), 4.23 (3), 3.09 (4), 2.15 (6), 1.55 (8)*	**5.4**: *5.4 (1), 1.79 (3)*	–
0.4:1	**12.7**: *12.8 (1), 6.1 (2), 4.24 (3), 2.14 (6)*	**5.3**: *5.25 (1), 2.65 (2), 1.77 (3)*	3.34, 1.68
1:1	**13.0**: *13.1 (1), 6.4 (2), 4.4 (3), 2.23 (6)*	**5.1**: *5.1 (1), 2.63 (2)*	3.34, 1.68
CHOL:CER I(1–5):FFA			
1:1:1	**13.1**: *13.1 (1), 6.3 (2), 4.45 (3), 2.25 (6)*	**5.4**: *5.4 (1), 2.72 (2), 1.83 (3)*	3.33, 1.68
CHOL:CER II(1–5)			
0.2:1	**13**[a]: *4.3 (3)*	**5.7**: *5.8 (1), 1.83 (3)*	–
0.4:1	**13**[a]: *6.2 (2), 4.4 (3)*	**5.6**: *5.6 (1), 2.75 (2)*	3.33
1:1	**13**[a]:*6.1 (1), 4.27 (2), 2.13 (6)*	**5.3**: *5.3 (1), 2.65 (2), 1.77 (3)*	3.33, 1.68
CHOL:CER II(1–5):FFA			
1:1:1	**13**[a]: *6.2 (2), 4.4 (3)*	**5.5**: *5.5 (1), 2.73 (2), 1.83 (3)*	3.33, 1.68

The spacings of the diffraction peaks and the periodicity of the lamellar phases are presented in nm. The data are given as follows. The periodicities are given in bold and the spacings are given italics. CHOL is indicated by two reflections of the triclinic lattice.
[a]The periodicity of the 13 nm phase is less accurate due to the broad reflections.

nm^{-1}) and a shoulder at a spacing of 4.3 nm ($Q = 1.45$ nm^{-1}). In addition a very weak peak at a spacing of 1.83 nm ($Q = 3.43$ nm^{-1}) was observed. The 5.8 and 1.83 nm peaks represent the 5.7 nm lamellar phase, while the 4.3 nm phase indicates that a small fraction of lipids formed the long periodicity lamellar phase of approximately 12–13 nm. Furthermore, a strong scattering at low Q value is seen with all CER II(1–5) mixtures. This might be due to a crystallisation of lipid mixtures into small crystals. The small crystal size also limits the number of repeating unit cells (N). Since the width at half maximum of the diffraction peaks is proportional to $1/Nd$ (d is the peak spacing), the broad diffraction peaks in the patterns of the CER II(1–5) mixtures can also be explained by a small crystal size. Increasing the CHOL content to molar ratio of 0.4 shifted the spacing of the strong peak to 5.6 nm ($Q = 1.12$ nm^{-1}). A new peak appeared at 2.75 nm ($Q = 2.28$ nm^{-1}). The 5.6 and 2.75 nm spacings indicate the presence of a lamellar phase with a periodicity of 5.6 nm. In addition two shoulders were observed at 4.4 and approximately 6.2 nm spacing indicating the presence of the approximately 13 nm long periodicity phase. Finally, a very weak diffraction peak was observed at a 3.33 nm spacing indicating that CHOL starts to phase separate at a molar ratio of 0.4. Increasing the CHOL content further to a molar ratio of 1.0 revealed the presence of two lamellar phases with periodicities of 5.3 and approximately 13 nm, respectively. The 5.3 nm phase is based on the diffraction peaks at 5.3 ($Q = 1.18$ nm^{-1}), 2.65 ($Q = 2.39$

Table 3
Variation in CER composition in human SC

	Vicanova (SC) [32]	Vicanova (E) [33]	Ponec (E) [34]	Schreiner (SC) [30]			
CER 1	6.6	9.7	10.2	5.0	9.5	12.3	12.4
CER 2	22.7	20.8	22.1	18.8	18.8	17.7	16.2
CER 3	20.2	20.2	21.9	23.5	15.3	16.4	18.2
CER 4	6.0	7.1	6.9	3.9	7.3	10.2	8.3
CER 5	21.5	19.8	16.7	23.4	20.8	19.7	20.2
CER 6	7.5	6.7	7.5	10.1	8.9	8.0	8.9
CER 7	14.7	15.5	14.7	14.1	19.1	15.8	15.5

All values are given in weight percentages. E indicates CER isolated from epidermis. SC indicates CER isolated from stratum corneum.

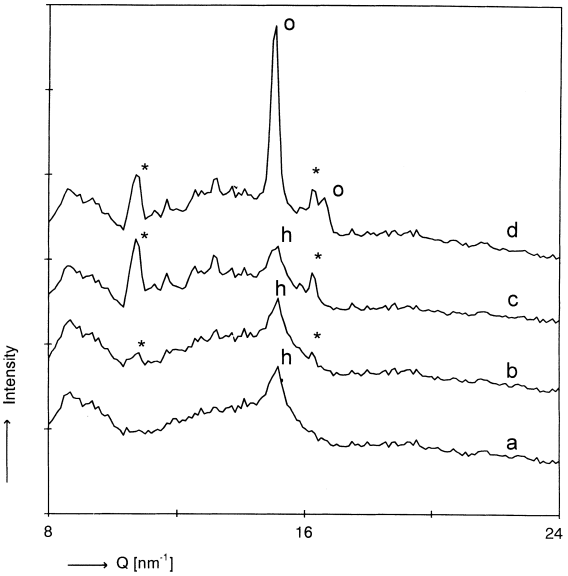

Fig. 2. The wide angle X-ray pattern of the CHOL:CER I(1–5) mixtures in a molar ratio of 0.2:1 (a), 0.4:1 (b) and 1:1 (c) and the wide angle X-ray pattern of the CHOL:CER I(1–5):FFA in a molar ratio of 1:1:1 (d). * denotes reflections based on hydrated crystalline cholesterol, h denotes the reflection characteristic for the hexagonal phase, and o refers to the reflections of the orthorhombic phase. For comparative reasons the curves are staggered and the intensity is given in arbitrary units.

nm^{-1}) and 1.77 ($Q = 3.54$ nm^{-1}) nm, while the 13 nm phase is based on the diffraction peaks at 6.1($Q = 1.03$ nm^{-1}), 4.27 ($Q = 1.59$ nm^{-1}) and 2.13 ($Q = 2.94$ nm^{-1}) nm spacing. The first order diffraction peak of the long periodicity phase was not observed with any mixture prepared from CHOL and CER II(1–5). In addition, two reflection located at 3.33 and 1.68 nm were observed. These reflections can be attributed to the presence of crystalline CHOL.

The addition of FFA resulting in an equimolar mixture of CHOL, CER II(1–5) and FFA revealed a diffraction curve similar to that of the equimolar mixture prepared from CER I(1–5) and CHOL. Only the repeat distance of the short periodicity phase increased slightly to 5.5 nm (see Table 2). The reflections attributed to phase separated CHOL were still present.

3.2. Short range ordering

In this study small sample sizes were used containing mostly typically 1–2 mg of lipids. This resulted in a low signal/noise ratio in the wide angle patterns, which caused the halos at $Q = 8.8$ nm^{-1} and $Q = 14$ nm^{-1} (Fig. 2, only curve a).

3.2.1. CER I(1–5)

The diffraction curve of CHOL:CER I(1–5) mixtures prepared at molar ratios of 0.2, 0.4 and 1.0 revealed one broad peak at 0.416 nm (Fig. 2), which is characteristic for a hexagonal lateral packing. At increased CHOL content no change in spacing was observed. However at molar ratios of 0.4 and 1.0 two additional reflections were present at approximately 0.59 and 0.386 nm indicating that CHOL phase separates at higher CHOL:CER(1–5) molar ratios. The broad reflection at 0.45 nm in Fig. 2 (curves b, c and d) increases in intensity with increasing CHOL content. This is most likely due to the presence of a number of reflections located between 0.45 ($Q = 14$ nm^{-1}) and 0.56 ($Q = 11.2$ nm^{-1}) that can be attributed to crystalline CHOL. Due to the limited resolution of the wide angle wire detector, these CHOL peaks could not be detected separately. The presence of crystalline CHOL is in agreement with observations made with SAXD (Fig. 1A).

The diffraction curve of a mixture prepared from CHOL, CER(1–5) and long-chain FFA at an equimolar ratio is plotted in Fig. 2 (curve d). The diffraction pattern revealed reflections at 0.417 and 0.378 nm indicating an orthorhombic lateral packing. Since the 0.416 nm reflection of the hexagonal lateral sublattice is located at the same position as one of the reflections attributed to the orthorhombic phase, the presence of a hexagonal lateral sublattice cannot be excluded. However, in recent studies using electron diffraction [29] that allows detection of the hexagonal sublattice next to the orthorhombic one due to a very small spot size of the beam, no hexagonal sublattice could be detected in an equimolar CHOL:CER:FFA mixture. The peaks present at approximately 0.586 and 0.385 nm and the broad peak between 11.2 and 14 nm^{-1} indicate phase separation of CHOL in the presence of FFA.

3.2.2. CER II(1–5)

The diffraction pattern of lipid mixtures prepared in the presence of CHOL II(1–5) revealed a similar phase behaviour as observed in the presence of CER I(1–5). A hexagonal lateral packing was observed for

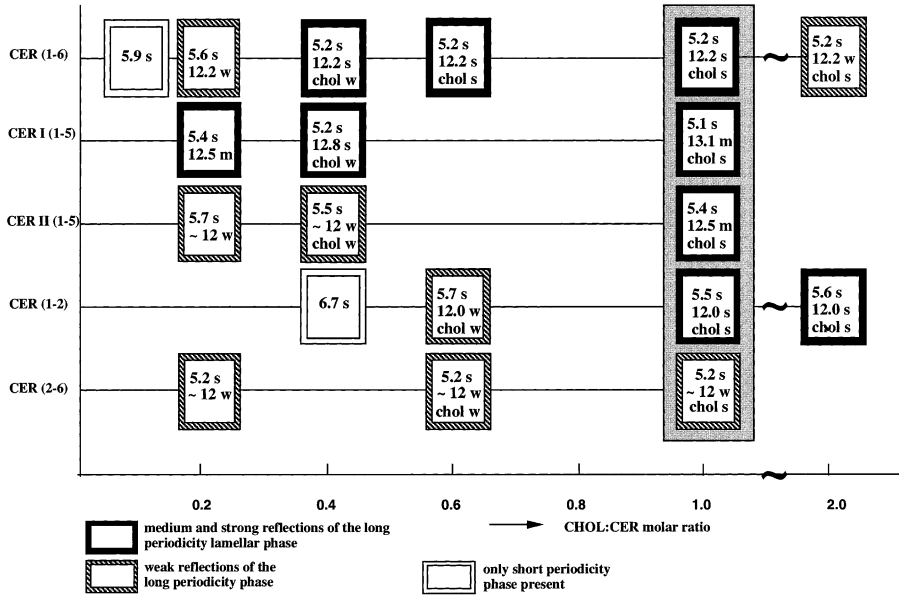

Fig. 3. A schematic overview of the lamellar phases of several CHOL:CER mixtures as function of the molar ratio. * data obtained from [31]. Note the similarity in the lamellar phases of the various CHOL:CER mixtures at an equimolar ratio. The indications weak (w), medium (m) and strong (s) of the long periodicity phase and CHOL denote the intensities of the reflections of these phases compared to the intensities of the reflections attributed to the short periodicity phase.

the mixtures prepared from CHOL and CER II(1–5), while in the presence of FFA an orthorhombic lateral packing was present (not shown).

4. Discussion

Our recent studies in which the lipid organisation of mixtures prepared from CHOL and CER(1–6) has been examined revealed that in CHOL:CER(1–6) mixtures two lamellar phases were present with repeat distances of 5.2 and 12.2 nm, respectively, and that these lipids form a hexagonal lateral packing [1,29]. The formation of these two lamellar phases occurred over a CHOL:CER(1–6) molar ratio ranging from 0.4 to 2, indicating a high insensitivity of these phases toward a change in CHOL content: only the amount of CHOL that phase separated increased with increasing CHOL:CER(1–6) molar ratio. The finding that only three phases coexist at room temperature in this multicomponent system is quite surprising. From our present studies the coexisting of different sublattices cannot be fully excluded. However this seems to be very unlikely, since electron diffraction studies revealed that in equimolar CHOL:CER and CHOL:CER:FFA mixtures only one sublattice is present. The insensitivity towards lipid composition might be of great importance since in native SC, where depending on the skin source, the CHOL:CER molar ratio varies between 0.8 and 1.4 (unpublished results). In intact SC not only the CHOL:CER molar ratio, but also the CER composition may vary (see Table 3) depending on the skin source, such as (i) body site, (ii) donor variation and (iii) skin condition (healthy versus diseased skin). Therefore, the knowledge on the role of individual CERs on the lipid organisation may help us to explain differences observed in the SC barrier function in vivo. In former studies in which we examined the phase behaviour of CHOL:CER(1,2) and CHOL:CER(1–6) mixtures we found that in these equimolar CHOL:CER mixtures two lamellar phases were formed. In contrast, in the equimolar CHOL:CER(2–6) mixture only a very small fraction of lipids formed the 12–13 nm lamellar phase demonstrating the crucial role of CER 1 for the SC lipid organisation. Also results of the present study with equimolar CHOL:CER I(1–5) and CHOL:CER II(1–5) mixtures support the previous findings, since in these mixtures two lamellar phases have been formed with periodicities of approximately 5.5 nm and 12.5 nm, respectively. These findings indicate

that also in the absence of CER 6 two lamellar phases can be formed. The results of our present and previous studies (summarised in Fig. 3), indicate that the lipid organisation of all equimolar CHOL:CER mixtures tested so far (Table 3) is very similar. However, exceptional is the weak 12 nm lamellar phase in the CHOL:CER(2–6) mixture. The results of the previous and present studies suggest that CER 1 plays a distinct role in the organisation of the 12.2 nm phase. In contrast, increasing the CER 2 content, and decreasing the amounts of CER 3 and CER 4 does not drastically affect the lipid organisation. These findings are in agreement with the recently proposed molecular model for the 12 nm phase obtained with crystallographic analysis [16], in which the role of CER 2, CER 3 and CER 4 in the molecular organisation of the 12.2 nm is very similar. The insensitivity toward a change in CER composition can be further extended to equimolar FFA:CHOL:CER mixtures in which also two lamellar phases were formed. This indicates that at an equimolar ratio a remarkable insensitivity exists toward a change in CER content. When we extend these findings to the in vivo situation, in which the CHOL:CER molar ratio is often close to unity we can expect that the lipid organisation in native SC will be insensitive to changes in CER composition with the only exception when the levels of CER 1 are dramatically reduced. However, in the human skin the molecular structure of the various CER slightly differs from that in the pig skin. In human skin both, CER 1 and CER 4 contain a linoleic acid linked to the C31/C33 ω-hydroxy fatty acid. Therefore, in human skin the proportion of lipids forming the 13 nm phase is expected to be reduced when the relative amounts of both CER 1 and CER 4 are low. This is in agreement with findings obtained in a recent study. In this study, in which the relationship between lipid composition and organisation in native human SC has been studied, a decrease in the levels of CER 1 and CER 4 parallels a reduction in the proportion of lipids that forms the 13 nm phase [30].

By summarising all our findings (Fig. 3), we can further conclude that the situation is different when the relative CHOL content is reduced. In both CHOL:CER(1–5) mixtures a gradual change in the repeat distances is observed when the CHOL content is decreasing, indicating that the lipid organisation in the absence of CER 6 is more sensitive toward a change in CHOL content. In addition, in the CHOL:CER(1–6) mixtures the 12–13 nm phase is clearly present already at a molar ratio of 0.2, but for the formation of the 12–13 nm phase in the CHOL:CER II(1–5) and CHOL:CER(1,2) mixtures the CHOL content has to be increased to an 0.4 and 0.6 molar ratio, respectively. Extrapolating these findings to the in vivo situation in which the long periodicity phase is thought to be important for the barrier function. From Fig. 3 we can conclude that the proportion of lipids that form the long periodicity phase may strongly depend on the overall CER composition (Fig. 3) only when both the CHOL:CER molar ratio and the CER composition change simultaneously. We have to keep in mind that the SC lipid behaviour will, next to the relative content of individual SC barrier lipids, also be affected by their molecular structure, such as the hydrocarbon chain lengths of the CER and/or FFA moieties or the presence of non-saturated FFA. Therefore, more detailed studies are needed to unravel the changes in SC lipid composition or structure in diseased skin that lead to barrier abnormalities.

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