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X-ray Diffraction Analysis of Isolated Skin Lipids: Reconstitution of Intercellular Lipid Domains†

T. J. McIntosh,*,‡ M. E. Stewart,§ and D. T. Downing§

Department of Cell Biology, Duke University Medical Center, Durham, North Carolina 27710, and Marshall Dermatology Research Laboratories, University of Iowa, Iowa City, Iowa 52242

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ABSTRACT: Low- and wide-angle X-ray diffraction were used to determine the structural organization of lipids isolated from the stratum corneum extracellular matrix that forms the major water permeability barrier in mammalian epidermis. Hydrated pig skin ceramides gave a single low-angle reflection of about 62 Å and a wide-angle reflection at 4.15 Å. The addition of either cholesterol or fatty acid, the other major lipid components of the skin stratum corneum extracellular matrix, modified this diffraction pattern, depending on the lipid mole ratios. In the absence of water, lipid mixtures exhibited lipid phase separation, as shown by low- and wide-angle reflections typical of a separate cholesterol phase. However, a hydrated 2:1:1 mole ratio of ceramide:cholesterol:palmitic acid (similar to that found in stratum corneum) produced a diffraction pattern with a single sharp wide-angle reflection at 4.10 Å and low-angle reflections which indexed as the first eight orders of a single repeat period of 130 Å. The repeat period and intensity distribution of the low-angle data were similar to those found in intact stratum corneum [White et al. (1988) *Biochemistry 27*, 3725−3732; Bouwstra et al. (1994) *Biochim. Biophys. Acta 1212*, 183−192]. Higher concentrations of cholesterol or palmitic acid resulted in lipid phase separations. The 130 Å repeat period decreased only about 3 Å as water was removed by incubation in low-relative humidity atmospheres. The 130 Å repeat period depended on the presence of a particular ceramide, *N*-(*ω*-acyloxy)-acylsphingosine, which is found only in the epidermis. In contrast, 2:1:1 mixtures of brain ceramide: cholesterol:palmitic acid gave reflections of 56 and 34 Å. These results indicate that a structure with dimensions similar to those of the lamellar repeating unit found in skin stratum corneum does not depend on the presence of protein but does depend on the presence of specific skin ceramides and appropriate concentrations of cholesterol and fatty acid.

The major water permeability barrier of the skin is provided by its outer layer, called the stratum corneum (SC). The stratum corneum consists of dead cells surrounded by an extracellular matrix containing lipid lamellae. Ceramides are the major lipid constituent of this extracellular matrix, which also contains appreciable amounts of cholesterol and free fatty acids (Downing, 1992). Unlike biological membranes, there are no phospholipids in these lipid lamellae.

In recent years, a number of studies have provided information on the composition and structure of the lipid lamellae in the stratum corneum. Downing and colleagues have isolated seven groups of ceramides from pig epidermis (Wertz & Downing, 1983) and shown by electron micros-

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‡ Duke University Medical Center.
§ University of Iowa.
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copy (Swartzendruber et al., 1989) and NMR (Abraham & Downing, 1991, 1992) that lamellar structures can be formed *in vitro* from mixtures of these ceramides with cholesterol and fatty acids. The structure of the stratum corneum lipid lamellae has also been investigated by both electron microscopy and X-ray diffraction. Electron microscopy (Elias & Friend, 1975; Swartzendruber et al., 1989) showed that extracellular domains contain lamellar structures. White et al. (1988) found that intact stratum corneum from the hairless mouse gave sharp wide-angle X-ray reflections at 3.75 and 4.6 Å and a lamellar X-ray spacing of 130 Å. However, isolated mouse stratum corneum lipids did not produce a single lamellar pattern. They (White et al., 1988) proposed that another component of the stratum corneum, perhaps protein, is necessary for stabilization of the 130 Å lamellar structure. Recently, Bouwstra and colleagues (Bouwstra et al., 1991a, 1992, 1994, 1995a,b; Schuckler et al., 1993) have performed extensive X-ray diffraction studies of the stratum corneum. At physiological temperatures, they have found two lamellar repeating units in stratum corneum, the larger one with a repeat period of 132 Å in pig SC (Bouwstra et al., 1995a,b) and 134 Å in both mouse SC (Bouwstra et al., 1994) and human SC (Bouwstra et al., 1991a) and the smaller one with a repeat period of 60 Å in pig SC (Bouwstra et al., 1995a,b), 61 Å in mouse SC (Bouwstra et al., 1994), and 64 Å in human SC (Bouwstra et al., 1991a). A change in the water content of the samples from 6 to 60% water did not appreciably swell these repeat periods (Bouwstra et al., 1991a). In agreement with the observations of White et al. (1988), Bouwstra et al. (1992, 1994, 1995b) and Schuckler et al. (1993) observed sharp wide-angle reflections in pig, mouse, and human SC near physiological temperatures. Both the sharp lamellar low-angle pattern and the sharp wide-angle reflections disappeared as the temperature was raised above 65−75 °C (Bouwstra et al., 1991a, 1992, 1995a,b). Upon the SC being cooled to room temperature, a single sharp lamellar repeating unit was observed, with a repeat-period of 132−134 Å, depending on the source of the SC (Bouwstra et al., 1991a, 1992, 1995a,b).

In this paper, we analyze the structure of ceramides isolated from the stratum corneum. One objective is to provide additional information on the source of the approximately 130 Å lamellar structure observed from the stratum corneum of several animals. A second objective is to determine the structure of lipid preparations used as models of the intercellular lamellae in the stratum corneum. In particular, we determine (1) whether the 130 Å repeat period can be obtained with purified skin lipids in the absence of protein, (2) whether specific ceramides found in SC lipids are essential for this repeating unit, and (3) the structural role of the other major lipids found in the stratum corneum, cholesterol and free fatty acid. Experiments are performed with lipids isolated from the stratum corneum of pig, since the pig produces little or no sebum which can contaminate lipids isolated from the epidermis (Downing, 1992), and mixtures of these lipids have been developed as models of the intercellular domains in the epidermis (Abraham & Downing, 1992). We also perform diffraction experiments with mixtures of cholesterol, fatty acids, and ceramides isolated from bovine brain membranes, since these lipids have recently been studied by NMR as models of stratum corneum intercellular membranes (Fenske et al., 1994).

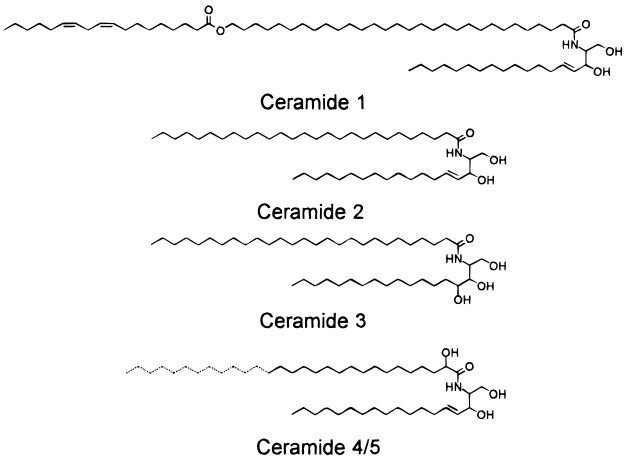

FIGURE 1: Molecular structures of the skin ceramides 1−5.

MATERIALS AND METHODS

Skin ceramides (Figure 1) were isolated from pig epidermis that had been heat-separated as described by Hedberg et al. (1988). Lipid was extracted from the epidermal sheets with 2:1 chloroform:methanol (v:v). After the solvent was evaporated under reduced pressure, the lipid was weighed and redissolved in 2:1 chloroform:methanol at a concentration of 50 mg/mL. Aliquots containing about 35 mg of lipid were applied as streaks to 20 × 20 cm thin-layer chromatography (TLC) plates coated with a 25 mm-thick layer of silica gel (soft layer Adsorbosil plates, Alltech Associates, Inc., Deerfield, IL). The chromatograms were developed to the top first in 190:9:1 chloroform:methanol:acetic acid (v:v:v) and then in 99:1 ether:acetic acid (v:v). Under these conditions, proteins do not move from the origin of the TLC plate. Bands were located by spraying the chromatograms with a 0.2% solution of dichlorofluorescein in ethanol, allowing the plate to dry, and then viewing it under UV light. The silica gel from the areas of the chromatograms containing ceramide 1 (Figure 1) and ceramides 2−5 was scraped off the plates onto weighing paper and transferred to small glass columns. The lipid was then eluted with 2:1 chloroform:methanol. To remove the dichlorofluorescein, the lipids were applied in a reduced volume of solvent to small columns of magnesium hydroxide in Pasteur pipettes and the lipid was eluted with 2:1 chloroform:methanol.

Cholesterol (Chol), palmitic acid (PA), and ceramides isolated from bovine brain (type III) were obtained from Sigma Chemical Co. (St. Louis, MO).

Lipid specimens for X-ray diffraction analysis were prepared by the following procedures. The appropriate mixture of ceramides, cholesterol, and palmitic acid was first codissolved in chloroform:methanol (2:1 v:v). For the preparation of unoriented specimens, the solvent was removed by rotary evaporation, the lipid mixture was hydrated in excess water by heating the lipid:water mixture three times to 80 °C for 30 min, and the hydrated lipid was sealed in an X-ray capillary tube. To obtain oriented specimens, the lipid in chloroform:methanol was placed on a curved glass substrate and the solvent was removed with a gentle stream of nitrogen. This oriented lipid sample was hydrated by covering the lipid film with a drop of water and incubating in an 80 °C oven three times for 30 min each. The oriented specimen on the curved glass substrate was then placed in a controlled relative humidity chamber, as described previously

Table 1: X-ray Diffraction Data from Hydrated Skin Lipids

lipid	low-angle reflections (Å)	wide-angle reflections (Å)
ceramides 1−5 (Cer 1−5)	62	4.15
ceramide 1 (Cer 1)	47	4.18
ceramides 2−5 (Cer 2−5)	58	4.15
cholesterol (Chol)	34, 17 ($d = 34$; $h = 1, 2$)	5.97, 5.38
palmitic acid (PA)	36, 18, 12 ($d = 36$; $h = 1-3$)	4.33, 4.12, 3.75
2:1:1 Cer 1−5:Chol:PA	128, 64, 43, 32, 26, 16 ($d = 128$; $h = 1-5, 8$)	4.10
2:2:1 Cer 1−5:Chol:PA	129, 64, 43 ($d = 129$; $h = 1-3$), 34, 17 ($d = 34$; $h = 1, 2$)	5.97, 4.10
1:1 Cer 1−5:Chol	129, 64, 43 ($d = 129$; $h = 1-3$), 34, 17 ($d = 34$; $h = 1, 2$)	5.97, 4.13
1:1 Cer 1−5:PA	80, 40 ($d = 80$; $h = 1, 2$)	4.12
2:1:1 Cer 2−5:Chol:PA	129, 64, 43 ($d = 129$; $h = 1-3$), 54, 36	–
2:1:1 Cer 1:Chol:PA	95, 47 ($d = 95$; $h = 1, 2$)	–

[a] d is the lamellar repeat period, and h corresponds to the order number of that repeat period.

(McIntosh et al., 1987, 1989). The relative humidity in the chamber was controlled with a saturated salt solution in the sample chamber (McIntosh et al., 1987, 1989).

X-ray diffraction patterns from the lipids were recorded using three different X-ray camera systems. Wide-angle patterns (spacings from 70 to 3 Å, including the reflections near 4 Å arising from the lipid hydrocarbon chains) were recorded from unoriented specimens with a point collimation camera. Low-angle patterns (spacings from 200 to 15 Å) from unoriented specimens were recorded using a mirror−mirror camera, and low-angle patterns from oriented specimens were recorded using a single-mirror, line focus camera (McIntosh et al., 1987, 1989).

RESULTS

Total skin ceramide in excess water gave a single sharp low-angle reflection at 62 Å, plus a strong sharp wide-angle reflections at 4.15 Å, and a very weak reflection at 3.85 Å. Hydrated skin ceramide 1 gave sharp reflections at 47 and 4.15 Å, and the hydrated mixture of skin ceramides 2−5 gave sharp reflections at 58 and 4.15 Å (Table 1).

The major reflections in X-ray patterns from various mixtures of ceramides, cholesterol, and palmitic acid are given in Table 1. In general, the low-angle pattern depended on the type of ceramide in the mixture, as well as on the molar ratio of ceramide:cholesterol:fatty acid. The most interesting result was that obtained with a molar mixture of 2:1:1 total skin ceramides:cholesterol:palmitic acid, similar to the lipid composition of skin (Downing, 1992). This sample gave a diffraction pattern consisting of eight orders of a lamellar repeat period of 128 ± 2 Å (mean ± standard deviation, $N = 3$ experiments) and a single sharp wide-angle reflection at 4.10 Å. Note that the major low-angle and wide-angle spacings in this pattern were different from the corresponding spacings in patterns from any of the component lipids—skin ceramides, cholesterol, or palmitic acid (Table 1). (For this lipid mixture, some patterns also contained a very weak reflection at 54 Å.) A typical low-angle pattern from this mixture is shown in Figure 2. The intensity distribution in this X-ray pattern is similar to that previously observed in patterns from mouse stratum corneum (Bouwstra et al., 1994), in that in both cases the relative intensities of order [$I(h)$] followed the distribution $I(1) > I(2) \approx I(3) \gg I(4) \approx I(5)$.

A mixture containing a higher concentration of cholesterol, namely 2:2:1 skin ceramide:cholesterol:palmitic acid, produced a diffraction pattern with a similar large repeat period

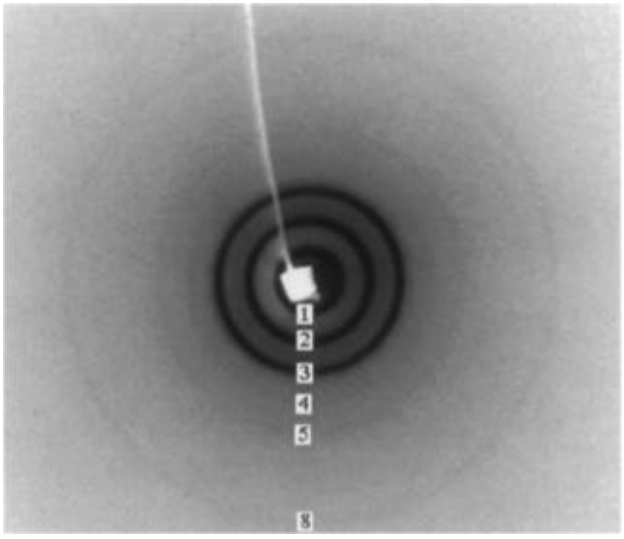

FIGURE 2: X-ray diffraction pattern of a hydrated mixture of total skin ceramides:cholesterol:palmitic acid (2:1:1 molar ratio). Orders 1−5 and 8 of a lamellar repeat period of 128 Å are indicated. A very faint reflection at 54 Å is also observed.

(130 Å) but also contained low-angle (34 Å) and wide-angle reflections (5.97 Å) characteristic of a pure cholesterol phase (Table 1). An equimolar mixture of skin ceramides and cholesterol with no free fatty acid also gave a pattern containing a mixture of the large repeat period (129 Å) and cholesterol reflections. However, an equimolar mixture of skin ceramides and palmitic acid gave a single lamellar repeat period of 80 Å and did not contain reflections near 130 Å.

Experiments were also performed with mixtures containing various components of the skin lipids (Figure 1), including ceramide 1 and a mixture of ceramides 2−5 in the ratio found in the stratum corneum. A 2:1:1 molar mixture of skin ceramides 2−5:cholesterol:palmitic acid produced a diffraction pattern containing several reflections (Figure 3). Three very weak reflections indexed as the first three lamellar orders of a 130 Å repeat period. However, by far the strongest reflection had a spacing of 54 Å, similar to the repeat period obtained from the hydrated skin ceramides 2−5 (Table 1). A 2:1:1 molar ratio of skin ceramide 1:cholesterol:palmiticacid produced a weak reflection at 95 Å, a very strong reflection at 47 Å, and a weak reflection at 34 Å (Table 1). The 95 and 47 Å reflections probably correspond to the first two orders of a 95 Å unit cell, and the 34 Å reflection is undoubtedly from cholesterol. The strong reflection at 47 Å is similar to the reflection observed for hydrated skin ceramide 1. In the case of skin ceramide 1, it

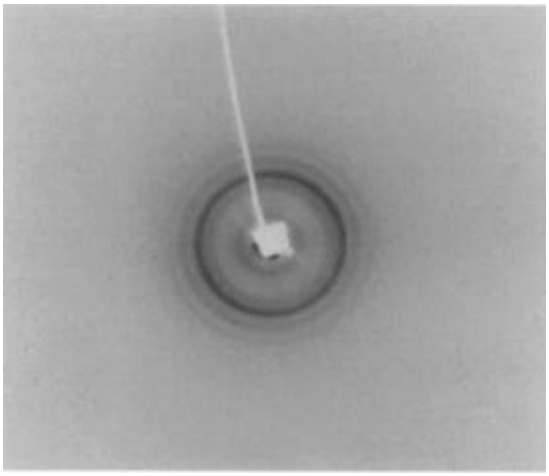

FIGURE 3: X-ray diffraction pattern of a hydrated mixture of skin ceramides (2−5):cholesterol:palmitic acid (2:1:1 molar ratio). The most intense reflection has a spacing of 54 Å. Faint reflections corresponding to the first three orders of a 129 Å repeat and a single faint reflection at 36 Å are also present.

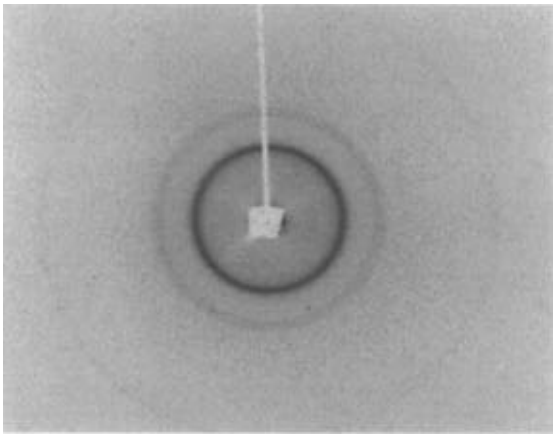

FIGURE 4: X-ray diffraction pattern of a hydrated mixture bovine brain ceramides:cholesterol:palmitic acid (2:1:1 molar ratio). The inner, most intense reflection corresponds to a spacing of 56 Å, and the outer, lighter reflections correspond to spacings of 34 and 17 Å.

is not clear whether this single reflection corresponds to the second order of a 95 Å repeating unit or to the first order of a 47 Å repeating unit.

To determine if the 130 Å periodicity could be obtained with ceramides other than skin ceramides, we performed experiments with ceramides isolated from bovine brain membranes. A hydrated mixture of a 2:1:1 mixture of bovine brain ceramide:cholesterol:palmitic acid gave reflections at 56, 34, and 17 Å (Figure 4), but did not produce a 130 Å reflection.

Additional experiments were performed to determine the hydration properties of the 2:1:1 total skin ceramide: cholesterol:palmitic acid specimen. Within experimental uncertainty, the same repeat period (128 ± 2 Å) was observed when the lipid was hydrated in water or a buffer containing 100 mM NaCl and 20 mM HEPES (pH 7). This repeat period decreased slightly when the sample was partially dehydrated by incubation in relative humidity atmospheres; the repeat period decreased from 128 Å at 98% relative humidity to 125 Å at 32% relative humidity. However, a 2:1:1 skin ceramide:cholesterol:palmitic acid specimen prepared in the absence of water showed cholesterol phase separation, as indicated by the presence of a strong reflection at 34 Å.

DISCUSSION

The X-ray diffraction results (Figure 2 and Table 1) show that the lamellar structure previously observed in skin stratum corneum (White et al., 1988; Bouwstra et al., 1991ab, 1992, 1995a,b) can be obtained with a mixture of lipids similar to that found in the stratum corneum, namely a 2:1:1 molar mixture of skin ceramides, cholesterol, and saturated fatty acid. That is, the X-ray diffraction patterns recorded from this mixture have repeat periods and lamellar intensity distributions similar to patterns recorded from stratum corneum. Moreover, this structure is nearly invariant with water content, as previously observed with skin stratum corneum (Bouwstra et al., 1992). These results imply that the lamellar structure observed in intact stratum corneum is provided by these skin lipids and does not depend on the presence of protein.

The X-ray data given in Table 1 show that the appropriate molar ratio of these lipids is necessary for this 130 Å lamellar repeating unit. In particular, ceramides by themselves or mixtures of ceramides and fatty acids do not produce the 130 Å repeat period, and the presence of the appropriate amount of cholesterol is necessary. However, there appears to be a saturation limit for cholesterol in the 130 Å structure, as 2:2:1 or 1:1:0 mixtures of ceramides:cholesterol:palmitic acid produce X-ray reflections indicative of a separate cholesterol phase (Table 1). White et al. (1988) observed a 131 Å periodicity from intact stratum corneum but obtained complicated patterns with many reflections from lipids isolated from murine stratum corneum. We raise the possibility that at least some of these extra reflections could arise from contamination from sebum lipids. Pig produces little or no sebum (Downing, 1992), and 2:1:1 mixtures of pig stratum corneum ceramides, cholesterol, and fatty acid produce only a single lamellar repeat period (Table 1).

Our experiments also show that the molecular composition of the ceramide mixture is critical to the formation of the 130 Å lamellar phase. Mixtures containing bovine brain ceramide:cholesterol:palmitic acid do not yield this 130 Å repeat period (Figure 4). Previous NMR experiments have indicated that such mixtures form a bilayer structure (Fenske et al., 1994). Our results (Figure 4) are consistent with that interpretation but show that the organization of this bilayer structure is quite different from the lamellar structure found in intact stratum corneum. That is, the reflection at 56 Å is consistent with the first order of a bilayer structure, and the reflections at 34 and 17 Å correspond to the first two orders of a cholesterol phase (Table 1). Moreover, mixtures of skin ceramides:cholesterol:fatty acid lacking the skin ceramide 1 show only weak reflections corresponding to the large repeat 130 Å repeat period (Figure 3 and Table 1). This implies that ceramide 1, with its long ω-hydroxyacid chain (Figure 1), is important to the formation of the 130 Å structural unit.

Our experiments with the 2:1:1 mixture of skin ceramides: cholesterol:palmitic acid provide information on the organization of the lipids in the stratum corneum. The sharp wide-angle spacing at 4.1 Å indicates that the lipid hydrocarbon chains are tightly packed in a gel phase, despite the presence of 25 mol % cholesterol. The experiments from dry and hydrated lipid mixtures indicate that the presence

of at least some water is essential to the stability of the 130 Å lamellar phase. However, in agreement with previous studies that show that the dimensions of lamellar structure of the stratum corneum do not vary significantly with water content (Bouwstra et al., 1991a, 1992), our experiments show that the lamellar repeat period decreases only a few angstroms when the fully hydrated sample is subjected to low relative humidities. Previous experiments have shown that, for bilayers composed of membrane lipids, 32% relative humidity removes most of the water from between adjacent bilayers (McIntosh et al., 1987, 1989). Therefore, our result indicates that only a very small fraction of the 130 Å lamellar repeat period corresponds to an interlamellar fluid space. Since the repeat period of skin ceramide is about 62 Å (Table 1), undoubtedly corresponding to a single bilayer, the 130 Å repeat probably contains two bilayers, as postulated by White et al. (1988). On the basis of model electron density calculations, Bouwstra et al. (1994) consider the possibilities that the 130 Å repeat contains either two or three bilayers. In either case, to produce a 130 Å repeat period (rather than a 62 Å repeat period), the bilayer must be asymmetric. As noted above, White et al. (1988) proposed that the asymmetry might be due to the presence of protein. Our experiments with lipid systems, where proteins have been removed by thin-layer chromatography, indicate that protein cannot be the source of the asymmetry and also show that cholesterol and ceramide 1 must play key roles in the production of the asymmetry in the lamellar structure (Table 1).

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