Characterization of stratum corneum structure in reconstructed epidermis by X-ray diffraction

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Abstract The intercellular lipid regions in the stratum corneum (SC), the outermost layer of the skin, form the major barrier for diffusion of substances through the skin. The barrier function of in vitro reconstructed epidermis is still impaired. With respect to further optimization of the model, it is necessary to characterize its stratum corneum lipid structure. In this study, small and wide angle X-ray diffraction were used to characterize the lipid organization in stratum corneum isolated from 14-day-old reconstructed epidermis. The measurements were carried out at room temperature, and subsequently as a function of temperature between 25°C and 109°C, followed by measurements after cooling to room temperature. The results of the X-ray diffraction measurements revealed the following in reconstructed epidermis. *1)* The lamellar ordering of stratum corneum lipids was much lower than that observed in native stratum corneum. *2)* Crystalline anhydrous cholesterol was present. *3)* Orthorhombic packing was present, but the corresponding reflections were very weak. The orthorhombic packing disappeared between 30°C and 45°C. *4)* A hexagonal packing was present and disappeared between 60°C and 75°C. *5)* Soft keratin is present. *6)* A higher extent of lamellar ordering could be achieved by heating to 109°C and cooling down to room temperature. Analysis of SC lipids revealed the presence of high amounts of triglycerides, the level of which could be decreased by lowering the glucose content. However, modulation of culture medium composition did not significantly affect lipid lamellae structures or hydrocarbon chain packing.—**Bouwstra, J. A., G. S. Gooris, A. Weerheim, J. Kempenaar, and M. Ponec.** Characterization of stratum corneum structure in reconstructed epidermis by X-ray diffraction. *J. Lipid Res.* 1995. **36:** 496–504.

Supplementary key words lipids • human epidermis • cultured keratinocytes • lipid composition

Recent progress in the field of epithelial culture techniques has led to the development of culture systems in which the reconstructed epidermis that is formed exhibits morphological and biochemical differentiation features close to those seen in vivo. In these 'organotypic' culture systems, human keratinocytes are attached to a biological matrix, (e.g., de-epidermized dermis (DED) (1), or collagen matrices populated with fibroblasts (2)), which is lifted to the air–liquid interface. In spite of the fact that under these conditions a stratified epidermis is formed that shares many similarities with native epidermis, its barrier function is deficient (3–6). The finding that the human skin recombinants do not synthesize epidermal barrier lipids in the same proportions (7) as native skin does may account for their disturbed barrier function (reviewed in ref. 8). Recently it has been suggested that the epidermal barrier function not only depends on the composition of stratum corneum (SC) lipids but also on their structural organization (9–11). Therefore studies on characterization of SC lipid structures in human skin recombinants are necessary. Recent studies have revealed anomalies in lamellar structures in the cornified layers, as observed by freeze-fracture electron microscopy (12) and transmission electron microscopy using ruthenium oxide post-fixation technique (13).

The purpose of this study is to elucidate the structure of SC isolated from reconstructed human epidermis by X-ray diffraction. The epidermis was reconstructed on a de-epidermized dermis (RE-DED) in culture media of varying composition. The lipid and protein structures were compared to those found in Living Skin Equivalent (LSE™). Furthermore, stratum corneum lipid composition was determined by high performance thin-layer chromatography (HPTLC).

EXPERIMENTAL

Cell culture

Air-exposed keratinocyte culture grown on DED-RE-DED. Secondary cultures of adult human keratinocytes (ob-

Abbreviations: DED, de-epidermized dermis; RE-DED, epidermis reconstructed on DED; SC, stratum corneum; LSE, Living Skin Equivalent; PBS, phosphate-buffered saline; GM, Genesis medium; SAXD, small-angle X-ray diffraction; WAXD, wide-angle X-ray diffraction; Q, scattering factor; λ, wave length; θ, scattering angle; HPTLC, high performance thin-layer chromatography.

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tained from healthy patients undergoing surgical corrections) cultured using the Rheinwald-Green method (14) were seeded on de-epidermized dermis (DED) and cultured under air-exposed conditions (15) in either of two media. Medium I was a 3:1 mixture of DMEM (4.5 g glucose/l) and Ham's F12 media (final glucose concentration 3.825 g/l), and the stratum corneum (SC) is denoted by SC I RE-DED. Medium II was a 3:1 mixture of DMEM (1.0 g glucose/l) and Ham's F12 media (final glucose concentration 1.20 g/l) and the SC cultured in this medium is referred to as SC II RE-DED. All media were supplemented with 5% HyClone calf serum (Greiner), 0.4 μg hydrocortisone/ml, 5 μg insulin/ml, 1 μM isoproterenol, and 10 ng/ml EGF. In some experiments the Genesis™ medium was used for the reconstruction of the epidermis on DED; this SC is denoted by SC GM RE-DED. The effects of different media composition were examined in three independent experiments with keratinocytes derived from two donors.

Living Skin Equivalent (LSE™) (Organogenesis, Cambridge, MA) was kept overnight in Genesis™ Maintenance Medium after its arrival. The SC of this reconstructed epidermis is denoted by SC LSE.

Isolation of stratum corneum

To separate epidermis from the underlying tissue, RE-DED or LSE were first incubated for 2 h in 0.1% trypsin in PBS. Thereafter the tissue was washed with PBS and the trypsinization was stopped by short incubation of tissue in PBS containing soybean trypsin inhibitor (10 mg/ml). Subsequently, the epidermis was separated from underlying dermal substrate, washed several times with PBS, and reincubated for 2 h in a 0.01% proteinase K in PBS (16). After extensive washing with PBS and with distilled water, stratum corneum was dried under vacuum and stored under nitrogen in the dark until use. Before use, the stratum corneum was hydrated for 24 h over 27% NaBr solution which results in an approximate hydration level of 20% [(weight of hydrated SC − weight of dry SC)/weight of hydrated SC].

Small angle X-ray scattering

All measurements were carried out at the Synchrotron Radiation Source at the Daresbury Laboratory using station 8.2. This station was built as part of an NWO/SERC agreement. The small angle camera was connected with a position-sensitive multiwire quadrant detector. The sample to detector distance was set to 1.80 m. A more detailed description of the experimental set up has been given elsewhere (17).

The SC, approximately 3 mg in weight, was put randomly in a specially designed sample cell with two mica windows. The temperature of this sample cell could be adjusted between 25° and 120°C. All samples were measured for a period of 15 min. Checks for the appearance of radiation damage were performed but proved to be negative (17).

The scattering intensities were plotted as a function of the scattering vector Q defined as $Q = (4 \pi \sin \theta)/\lambda$, in which λ and θ are the wavelength and scattering angle, respectively. The positions of the diffraction peaks are directly related to the repeat distance of the molecular structure, as described by Braggs law $2 d \sin \theta = n \lambda$, in which n is the order of the diffraction peak. In case of a lamellar structure, the various peaks are located at equal interpeak distances, $Q_n = 2 n \pi/d$, Q_n being the position of the nth order peak.

Wide angle X-ray scattering

The diffraction patterns were obtained with the fiber diffraction camera at station 7.2 of the Synchrotron Radiation Source in Daresbury. A more detailed description of the camera was given recently (18). Spacings between 0.3 and 3.5 nm could be detected. In this paper the positions of the reflections will be denoted by their spacings. The primary beam path length through the sample was 1 mm. The temperature of the sample cell could be adjusted between 25°C and 120°C.

Lipid extraction and separation

Human skin and reconstructed epidermis specimens were heated for 1 min at 60°C to separate the epidermis from the dermis. Epidermal lipids were extracted using the method of Bligh and Dyer (19), dissolved in chloroform–methanol 2:1 (v/v) and stored at −20°C under nitrogen until use. The extracted lipids were separated by one-dimensional high performance thin-layer chromatography, as described earlier (7). For quantification, authentic standards (Sigma) were run in parallel. The quantification was performed after charring using a photodensitometer with automatic peak integration (Desaga, Germany).

RESULTS

Wide and small angle diffraction patterns

The following measurements were carried out. *1)* The SAXD and WAXD diffraction profiles of SC I RE-DED, SC II RE-DED, SC GM RE-DED, and SC LSE were measured at room temperature. *2)* The SAXD and WAXD diffraction profiles of SC I RE-DED were measured at various temperatures varying between 25°C and 109°C. *3)* The SAXD and WAXD diffraction profiles of SC I RE-DED and SC II RE-DED heated to 109°C and subsequently cooled to ambient temperature were measured.

Diffraction patterns at room temperature. The SAXD and WAXD diffraction profiles of stratum corneum isolated from RE-DED obtained under identical culture conditions, but by using keratinocytes originating from two different donors, were similar. Therefore, the results of

representative experiments will be presented below. The diffraction curves obtained with SC I RE-DED are depicted in **Fig. 1A**. The curve of SC I RE-DED exhibits a small but clearly detectable peak at Q = 1.1 nm^{-1}, corresponding to a spacing of 6 nm. No higher order reflections of the lipid phase were detected, which made it impossible to attribute this peak to a particular lipid phase. A second sharp peak located at Q = 1.87 nm^{-1} (3.35 nm spacing) was attributed to polycrystalline cholesterol. The presence of anhydrous cholesterol was confirmed by the results obtained by WAXD (see below).

The diffraction curve obtained with SC II RE-DED revealed a very broad shoulder on the scattering curve from which it was not possible to calculate a corresponding spacing. Again a strong crystalline cholesterol peak at 3.35 nm spacing was observed (data not shown).

No peaks were observed on the diffraction curves of SC GM RE-DED and of SC LSE (not shown). It seems that no detectable long range ordering and no detectable crystalline cholesterol was present in SC cultured in GM and in SC isolated from LSE.

The WAXD pattern obtained from SC I RE-DED, depicted in **Fig. 2A,** revealed two diffuse diffraction rings at 0.96 and 0.46 nm, both assigned to soft amorphous keratin. The 0.46 nm diffuse diffraction ring can also be attributed to a lipid liquid phase. The sharp 3.38, 1.70, 1.38, 0.622, 0.575, 0.519, 0.505, and 0.483 nm reflections were assigned to anhydrous crystalline cholesterol (20). The strong diffraction ring at 0.59 nm, characteristic for cholesterol monohydrate (21), was not detected. One additional ring was observed at 0.45 nm. This reflection was occasionally also observed in native human SC and was attributed to triglyceride rich lipid regions (J. A. Bouwstra, G. S. Gooris, A. Weerheim, and M. Ponec, unpublished results). Two diffraction rings were found at approximately 0.415 and 0.377 nm, respectively. The diffraction ring at 0.377 nm was very weak, but detectable. In previous studies (18, 22) these diffraction rings were assigned to a hexagonal (0.412 nm) and an orthorhombic lipid chain packing (0.377 and 0.417 nm). The detection of both rings in SC I RE-DED led us conclude that small amounts of lipids were organized in an orthorhombic lattice, but due to the low intensity of the 0.377 nm ring the major part of the lipids was probably present in a hexagonal or a liquid phase.

In the wide angle diffraction pattern of SC II RE-DED (not shown) 0.96 and 0.46 nm diffuse rings based on liquid-like chain packing and soft keratin were also present. Very weak cholesterol reflections at 3.35, 0.575, and 0.483 nm were found. A weak 0.417 nm reflection attributed to the hexagonal packing of lipids was present. No evidence was found for the presence of crystalline lipids (orthorhombic lattice).

The WAXD pattern of SC GM RE-DED (not shown) revealed two diffuse rings at 0.96 and 0.46 nm based on amorphous keratin and possibly a lipid liquid phase. No

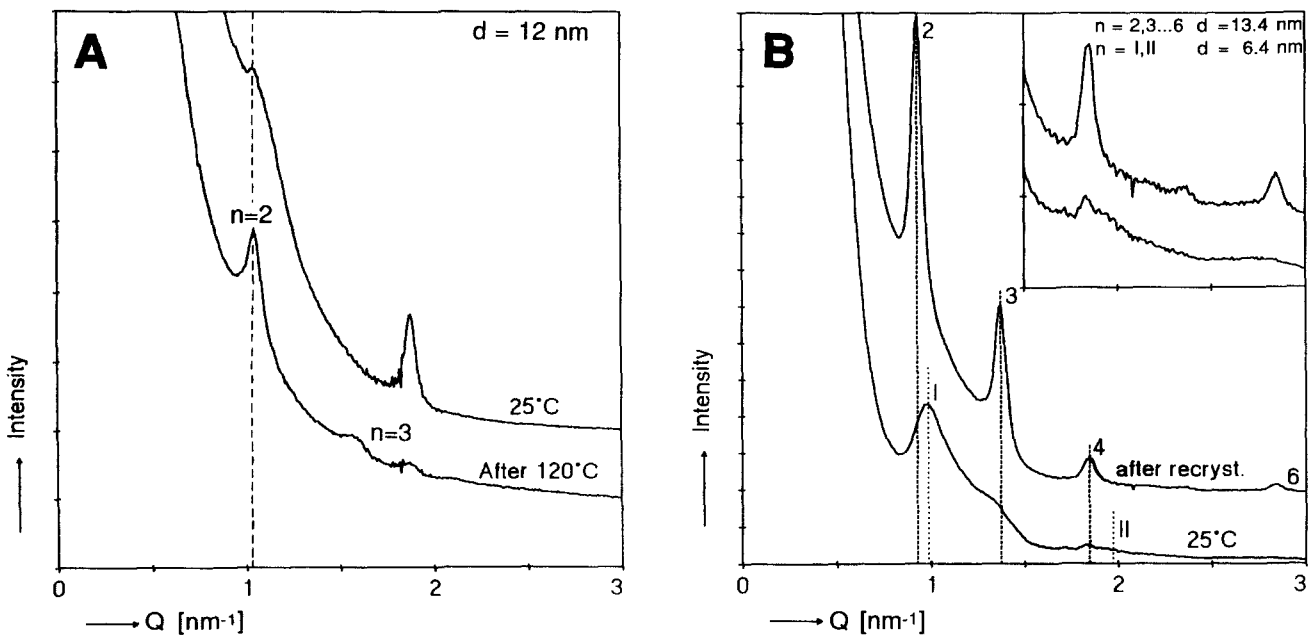

Fig. 1. The SAXD profile of SC measured at room temperature and after recrystallization of lipids. A: SC I RE-DED measured at room temperature and after recrystallization from 109°C; B: native human SC measured at room temperature and after recrystallization from 120°C.

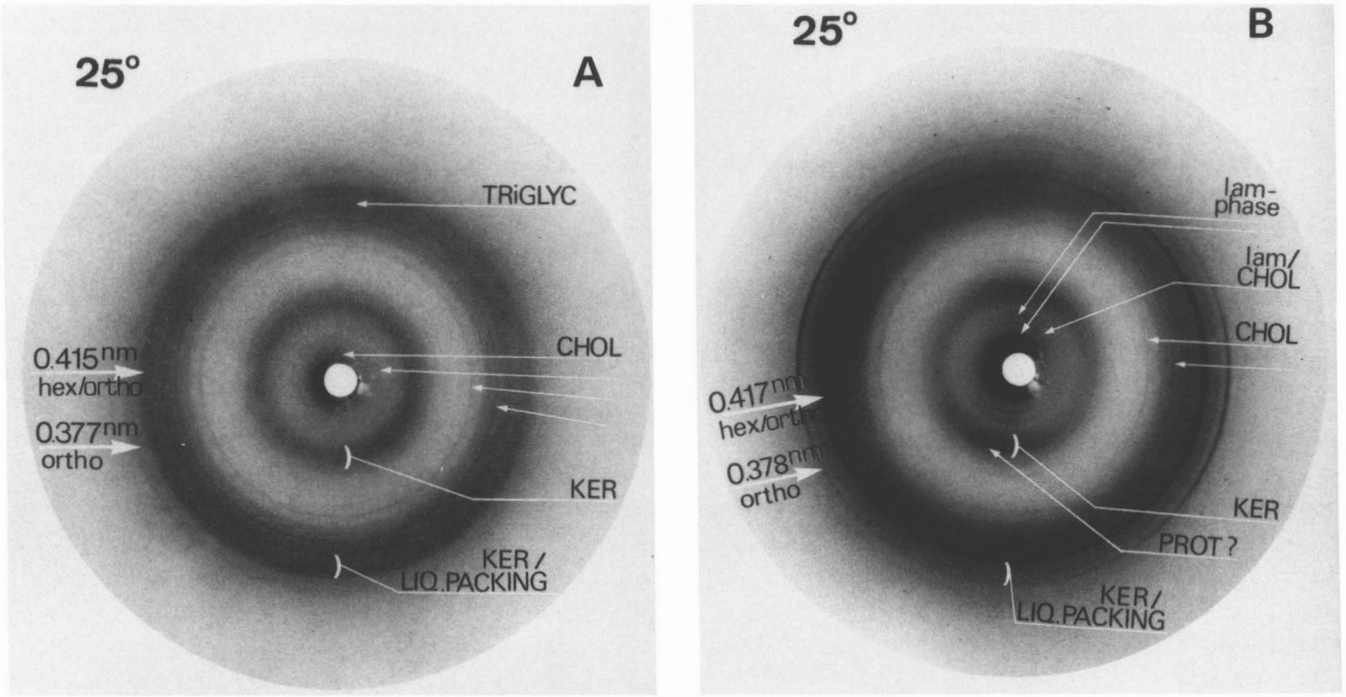

Fig. 2. The WAXD pattern of SC measured at 25°C. A: SC I RE-DED; B: native human SC.

reflections based on crystalline triglycerides, crystalline cholesterol, an hexagonal or orthorhombic chain packing were depicted.

In the WAXD pattern of SC LSE (not shown) diffuse rings were present at 0.96 and 0.46 nm. Furthermore, weak 3.35 nm and 0.575 nm cholesterol reflections were detected. No reflections based on an orthorhombic phase, a hexagonal lipid phase, or crystalline triglycerides could be detected.

Diffraction patterns as a function of temperature. The SAXD curves of SC I RE-DED measured at elevated temperatures are presented in **Fig. 3A.** An increase in tempera-

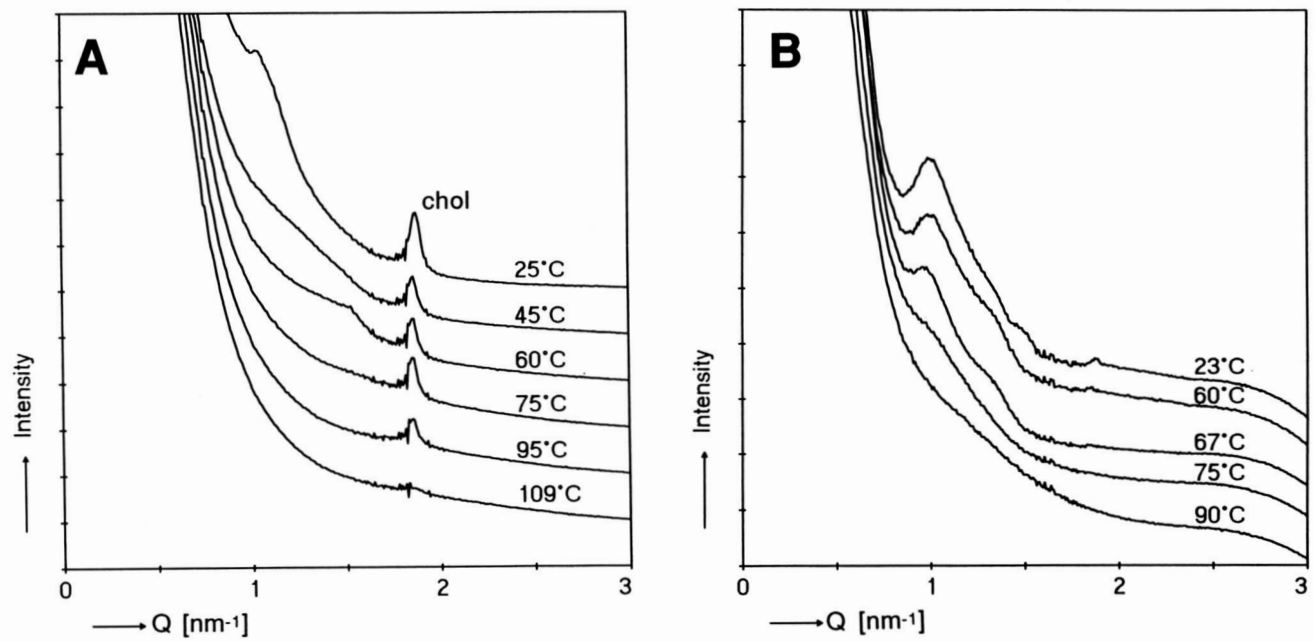

Fig. 3. The SAXD patterns of SC measured as a function of the temperature. A: SC I RE-DED; B: native human SC.

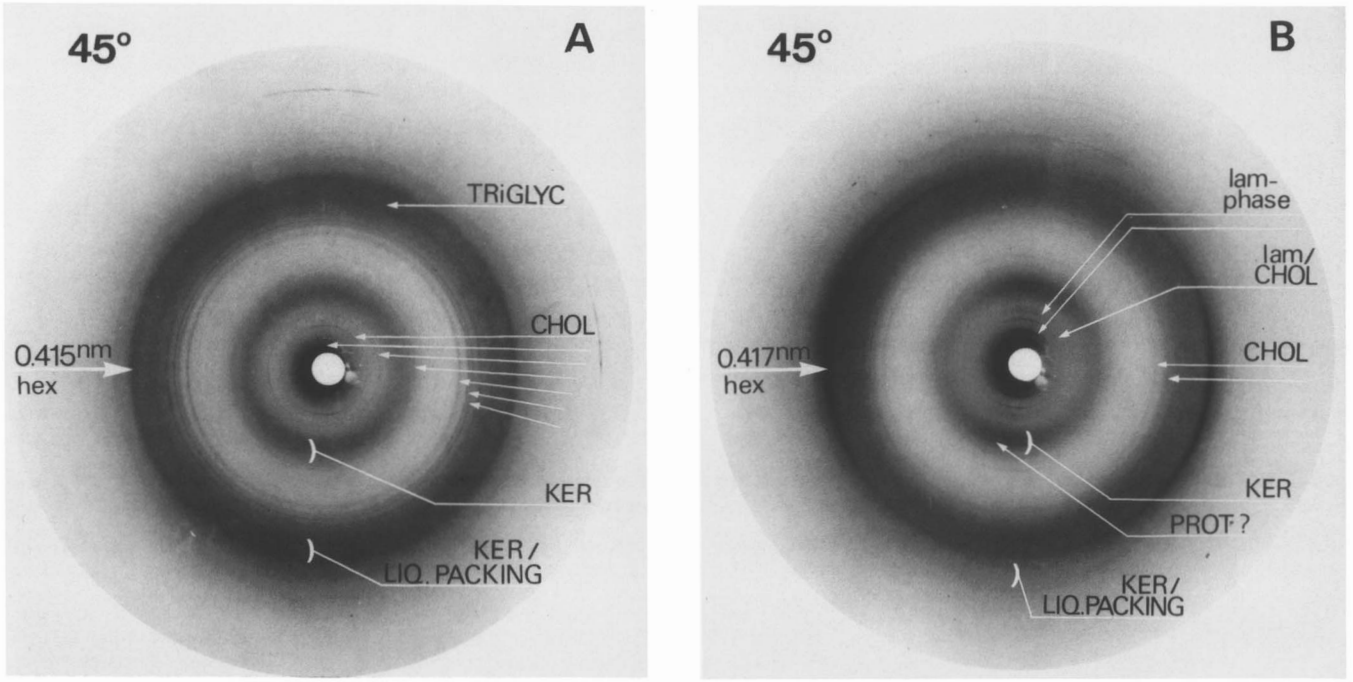

Fig. 4. The WAXD pattern of measured at 45°C. A: SC I RE-DED; B: native human SC.

ture from 25°C to 45°C turned the 1.1 nm^{-1} diffraction peak at the diffraction curve of SC I RE-DED into a shoulder. Further increase in temperature shifted the shoulder to higher Q-values. The shoulder disappeared between 60° and 75°C. Increase in temperature also decreased the intensity of the cholesterol peak. At 109°C only a very weak cholesterol peak was present on the diffraction curve. The changes in the SAXD curves of SC II RE-DED as a function of temperature (not shown) were similar to those found in the SAXD curves of SC I RE-DED. In the temperature range between 25° and 60°C, a shift in the position of the shoulder on the descending scattering curve to higher Q-values and a decrease in intensity of the cholesterol peak were observed. At 75°C only the cholesterol peak was present on the curve.

The WAXD pattern of SC I RE-DED was also measured as a function of temperature (**Fig. 4A**). At 30°C the reflections due to orthorhombic and hexagonal packing were still present together with the reflections based on anhydrous crystalline cholesterol and triglyceride-rich lipid regions; no differences were found between the profiles measured at 25°C and 30°C. The diffraction profiles are therefore not shown. However, between 30°

TABLE 1. Wide angle and small angle X-ray diffraction spacings found at 25°C and 45°C of SC I RE-DED

	Reflections Ascribed to Spacings in nm	
	25°C	45°C
Soft keratin	0.96(s)[a]	0.96(s)[a]
Soft keratin/liquid	0.46(s)[a]	0.46(s)[a]
Orthorhombic/hexagonal	0.415(m)	0.415(m)
Orthorhombic	0.377(vw)	absent
Crystalline anhydrous cholesterol	3.35(s), 1.70(m), 1.38(m) 0.666(vw), 0.622(w), 0.575(s), 0.519(s), 0.483(s), 0.464(vw)	3.43(w), 1.68(m), 1.35(w), 0.83(w), 0.679(w), 0.652(w), 0.607(w), 0.567(s), 0.535(s), 0.518(s), 0.495(s)
Triglycerides	0.45(w)	0.45(w)
Lipid phase	6.0 nm[b]	absent

Abbreviations: s, strong; m, medium; w, weak; vw, very weak.
[a] Diffuse ring.
[b] A specific lipid phase could not be determined from the diffraction curve, but a 12-nm lamellar phase seems to be likely.

and 45°C phase transitions occurred. The reflections found at 45°C are given in **Table 1.** The 0.377 nm orthorhombic reflection disappeared indicative of an orthorhombic-hexagonal transition, which is in the same temperature range as found in native human SC (18). The intensity of the 0.415 nm reflection increased between 30° and 45°C. The anhydrous cholesterol reflections observed at 45°C are given in Table 1. Compared to those found at 25°C, several reflections were slightly shifted and two new reflections appeared at 0.828 and 0.535 nm, respectively. This change in diffraction pattern was based on a polymorphic transition of anhydrous crystalline cholesterol that was also found in the pure substance (20). Between 60° and 75°C, the 0.415 nm reflection disappeared, which is indicative of a hexagonal to liquid phase transition. The 0.45 nm triglyceride reflection was weakened. Further increase in temperature to 90°C decreased the intensity of the cholesterol reflections and the 0.45 nm reflection disappeared. Only the diffuse 0.46 and 0.96 nm rings based on the lipid liquid phase and amorphous keratin were still present on the diffraction pattern. The diffraction pattern at 105°C was not significantly different from that measured at 90°C.

Diffraction patterns after recrystallization of the lipids. The SAXD curve of SC I RE-DED is depicted in Fig. 1A. Diffraction peaks with spacings of 6 nm, 3.9 nm, and 3.35 nm were observed. The peak intensities observed at the SAXD curve of SC I RE-DED were stronger than those observed in the SAXD curve of SC II RE-DED (not shown), and revealed only very weak reflections at 6.0 and 3.35 nm spacings. The peak present at 3.35 nm is probably due to anhydrous crystalline cholesterol, while the 6.0 nm (2nd order) and 3.9 nm (3rd order) spacings indicate a 12 nm lamellar phase. In the WAXD pattern of SC I RE-DED no higher order reflections of the lamellar phase were observed. After crystallization from 120°C only a strong 0.417 nm reflection and the diffuse rings at 0.46 and 0.96 nm were present (data not shown). The 0.377 nm reflection and the cholesterol reflections did not reappear on the diffraction pattern suggesting that the lipids crystallized in either a hexagonal or a liquid-like lipid chain packing.

Stratum corneum lipid composition

Lipids extracted from SC isolated from all different reconstructed epidermis contained all lipid classes present in native skin. As expected, the relative amounts of phospholipids were low and varied between 1 and 4%. In contrast to this, the relative triglyceride content was rather high and varied between various samples tested: SC I RE-DED containing about 34% triglycerides, SC II RE-DED 27% triglycerides, and SC LSE and SC GM RE-DED about 21% triglycerides. The relative amounts of the lanosterol/diglyceride fraction accounted for about 9% in all samples. As recent findings revealed that tri- and diglycerides do not substantially contribute to the regular organization of SC lipids (J. A. Bouwstra, G. S. Gooris, A. Weerheim, and M. Ponec, unpublished observations), the composition of other lipid fractions in the various SC samples were compared (**Table 2**).

DISCUSSION

While our previous studies (12, 13) revealed that reconstructed epidermis mimics its native counterpart to a high extent, the results of the present investigation clearly indicate that the organization and composition of lipids in cultured SC differ to a high extent from the native tissue. Substantial improvement of lipid organization could not be achieved by modulation of culture medium composition or by using fibroblast-populated collagen as a dermal substrate. Evaluation of the SAXD patterns revealed that

TABLE 2. Comparison of stratum corneum lipid composition in various reconstructed epidermis

Lipid Fraction	SC I RE-DED	SC II RE-DED	SC LSE	SC GM RE-DED
			%	
Phospholipids	2.2 ± 1.0	1.9 ± 0.4	1.4 ± 1.0	2.4 ± 0.3
Triglycerides	34.2 ± 7.4	27.5 ± 5.0	21.6 ± 2.4	21.2 ± 3.0
Diglycerides	5.6 ± 2.0	3.9 ± 2.2	4.2 ± 1.8	3.5 ± 2.6
Other lipids	58.0 ± 4.3	66.7 ± 3.8	72.8 ± 4.1	72.9 ± 4.0
Total other lipids	100	100	100	100
Cholesterol sulfate	2.6 ± 1.9	4.8 ± 0.8	6.5 ± 1.9	5.8 ± 1.6
Glycerosphingolipids	0.9 ± 0.5	3.2 ± 0.8	2.3 ± 0.6	1.8 ± 0.7
Ceramides	17.6 ± 4.0	24.1 ± 4.8	23.8 ± 4.2	24.1 ± 3.3
Free fatty acids	5.0 ± 3.9	6.7 ± 3.5	5.9 ± 2.6	6.3 ± 1.6
Cholesterol	72.0 ± 7.5	58.1 ± 4.3	58.9 ± 6.5	58.8 ± 7.6
Cholesteryl esters	1.9 ± 0.9	3.1 ± 0.3	2.6 ± 1.1	3.1 ± 1.6

Lipid composition of stratum corneum (SC) isolated from epidermis reconstructed on DED (RE-DED) in medium containing high glucose concentration (3.8 g/l; medium; SC I RE-DED), low glucose concentration (1.2 g/l; SC II RE-DED), or Genesis™ medium (SC GM RE-DED) or from Living Skin Equivalent™ (SC LSE) were determined after lipid extraction, separation, and photodensitometry (7). The results are given as percentage of total lipids ± SEM (n = 3).

only in SC I RE-DED was a weak 6 nm peak present (Fig. 1A) suggesting long range ordering of SC lipids. However, the position of this peak significantly differed from that found in native human SC (Fig. 1B), where two lamellar phases with periodicities of 6.4 and 13.4 nm were found (17). Whether a lamellar or another lipid phase is present in SC I RE-DED cannot be deduced from the diffraction curve. However, inspection of electron micrographs obtained with RuO_4-fixed SC (13) and electron micrographs obtained by freeze-fracture electron microscopy (12) revealed the presence of lamellae, although their presence was less frequent than observed in native human SC. More information about the lipid organization was obtained after heating SC I RE-DED to 109°C and subsequent cooling to ambient temperature. Under these conditions the recrystallized lipids formed a lamellar phase with a repeat distance of 12.0 nm (Fig. 1A). Similarly, as seen with native human SC, no first order diffraction peak was observed, but in native human SC the recrystallized lipids were organized in a 13.4 nm lamellar phase. As stated above, only a 6 nm peak was observed in the SAXD curve of SC I RE-DED measured at room temperature. This peak was at the same position as the strongest diffraction peak of the 12 nm lamellar phase observed in recrystallized lipids (Fig. 1A). The 12 nm lamellar phase is probably also present in untreated cultured SC, but due to its infrequent occurrence its detection is impossible. In fact, the presence of a 12 nm lamellar phase in cultured SC was confirmed by electron microscopic studies by using RuO_4 post-fixation (13). These studies revealed that lipids in cultured SC were locally organized in multilamellar structures with a periodicity of 12 nm, each unit consisting of alternating electron lucent and electron dense bands (see Fig. 6a, ref. 13). This long spacing lamellar phase was also found in the native human SC (17), the repeat distance being 13.4 nm. However, in native stratum corneum an additional 6.4 nm phase was observed. In recent studies carried out in our laboratory (J. A. Bouwstra, G. S. Gooris, A. Weerheim, and M. Ponec, unpublished results), the phase behavior of mixtures of extracted ceramides and cholesterol prepared at varying molar ratios has been investigated. It appeared that the 13 nm phase was present only at high cholesterol/ceramide ratios, while at lower cholesterol/ceramide ratios (comparable with that found in native SC) two lamellar phases with repeat distances of approximately 13 and 6 nm were found. One can speculate that the presence of only a 12 nm lamellar phase in cultured SC is due to a higher cholesterol/ceramide ratio as compared to native epidermis (see Table 2). Furthermore, the slightly smaller repeat distance (12 nm) found in cultured SC compared to the 13 nm found in native human SC might be due to the presence of relatively higher amounts of shorter fatty acids in cultured SC compared to native SC (7).

Also, lipid chain packing of cultured SC I RE-DED most closely resembles the native human SC. In cultured SC the lipids are partly arranged in an orthorhombic packing, a hexagonal packing, or in a liquid-like packing. Such structures were also found in native human SC, but the intensities of the 0.378 and 0.415 nm diffraction reflections of cultured SC (Fig. 2A) were much weaker than the reflections seen in the native human SC (Fig. 2B). High content of fatty acids with shorter alkyl chains found in cultured epidermis (7) may facilitate liquid packing. However, the corresponding diffuse 0.46 nm ring was obscured by the 0.46 nm diffuse ring based on amorphous keratin. Furthermore, the diffraction pattern of cultured SC depicts many reflections based on anhydrous crystalline cholesterol. The strongest cholesterol reflections were also frequently observed in human SC, but appeared to be much weaker (J. A. Bouwstra, G. S. Gooris, A. Weerheim, and M. Ponec, unpublished results). The presence of numerous cholesterol reflections together with observed high cholesterol content tempt us to speculate that the abnormal lipid structures observed in freeze-fracture electron micrographs of cultured SC (12) may be assigned to cholesterol.

The results of the present study show that all groups of lipids that have been found in native human SC were also present in cultured SC, but their relative lipid content is different. The main differences are the relatively low amount of ceramides and relatively high amounts of triglycerides and cholesterol. The effect of high cholesterol/ceramide ratios on the lipid phase behavior has already been discussed above. Inspection of electron micrographs revealed that a part of triglycerides in cultured SC is stored as intracellular lipid droplets (13). High triglyceride content may also account for the presence of diffraction reflection observed in WAXD patterns. This reflection is occasionally also observed in native human SC (J. A. Bouwstra, G. S. Gooris, A. Weerheim, and M. Ponec, unpublished results) and can be attributed to triglyceride-rich lipid regions for two reasons: *a)* the reflection was only observed in human SC with high triglyceride content, and *b)* the reflections were not present in hexane-extracted SC. Hexane extraction mainly removes di- and triglycerides from native human SC. Based on these observations, we speculated that triglycerides do not significantly contribute to the formation of lipid lamellae. Therefore, when relating SC lipid composition to SC lipid structures, the triglyceride content was not taken into account. As shown in Table 2, the cholesterol content in all cultured SC was high; its relative content being highest (about 72%) in SC I RE-DED. This may also explain why the cholesterol reflections in the WAXD pattern were strongest in SC I RE-DED. Cholesterol reflections were, however, absent in SC GM RE-DED in which cholesterol content was about 58%. Of

all cultured SC, the lipid organization in SC GM REDED was also the best when evaluated by means of electron microscopy (13). The absence of the cholesterol reflections in this SC might be due to *a)* an amorphous state of a part of the cholesterol, or *b)* a higher solubility of cholesterol in the lipid lattice compared to that in native SC, in which even at lower cholesterol contents reflections characteristic for cholesterol are found (J. A. Bouwstra, G. S. Gooris, A. Weerheim, and M. Ponec, unpublished results). One can speculate that differences in fatty acid alkyl chain length might be responsible for the increased solubility of cholesterol, as cultured SC contains fatty acids with shorter alkyl chains (7). The presence of shorter fatty acids might lead to an increased cholesterol solubility.

Important information about the SC lipid organization can be obtained in experiments in which the changes in X-ray diffraction pattern are followed as a function of temperature. Such experiments revealed that upon increasing temperature, lamellar disordering in cultured SC already occurs between 25° and 45°C in contrast to native SC where such changes are observed between 60° and 75°C (Fig. 3B). With respect to the packing of the lipids in cultured SC, the transition from an orthorhombic to a hexagonal packing occurs between 30° and 45°C, which is in the same temperature range as observed for native SC (Fig. 4). The transformation of the lipid packing in cultured SC from hexagonal to a liquid phase occurs at lower temperatures (between 60° and 75°C) than observed in native SC (between 65° and 90°C).

These observations clearly show that the changes in the lipid organization in cultured SC occur at significantly lower temperatures than in native SC. This might have consequences also for drug transport through SC, which is probably strongly affected by the lamellar ordering of the lipids. The effect of orthorhombic to hexagonal transitions on transport rate of ^3H_2O across native human SC appeared to be not so vigorous, as the flux of ^3H_2O (23) across SC did not increase upon passing the orthorhombic-hexagonal phase transition temperature, while between 60° and 75°C (the temperature range at which disordering of the lamellae occur and the hexagonal lateral packing starts to transform to a liquid one) the ^3H_2O flux strongly increased. As diffusion experiments are usually performed at 32°C, one can assume that at this temperature the lamellar phase in cultured SC might already be partly disordered. This may lead to an increased permeability of cultured SC as compared to that measured with native SC. Furthermore, the intensity of hexagonal and orthorhombic lateral packing reflections is less strong as compared with that seen in native human SC. This might be a result of reduced lateral packing ordering and an increased liquid lateral packing leading to an increased transport rate of penetrants as well.

Although a number of similarities are observed between native human and cultured human SC, such as the presence of soft keratin, the presence of a lamellar phase, and the presence of a crystalline phase, some important differences still exist in the SC lipid organization. It is tempting to speculate that the low degree of lipid ordering in reconstructed epidermis can be attributed to deviations observed in lipid composition and to observed perturbance in the extrusion of lamellar bodies at the stratum granulosum–SC interface (13), the process that is the key one regulating the formation and organization of the intercellular lamellae. The high rate of maturation under the in vitro conditions together with suboptimal nutrient supplementation and microenvironmental conditions may account for the low degree of lipid ordering observed in SC of reconstructed epidermis. Further modulations of cultured conditions are therefore necessary to improve the quality of the reconstructed epidermis.

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