Electron Diffraction Provides New Information on Human Stratum Corneum Lipid Organization Studied in Relation to Depth and Temperature

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The outermost layer of mammalian skin, the stratum corneum, provides the body with a barrier against transepidermal water loss and penetration of agents from outside. The lipid-rich extracellular matrix surrounding the corneocytes in the stratum corneum is mainly responsible for this barrier function. In this study (cryo-) electron diffraction was applied to obtain information about the local lateral lipid organization in the extracellular matrix in relation to depth in human stratum corneum. For this purpose, stratum corneum grid-strips were prepared from native skin *in vivo* and *ex vivo*. It was found that the lipid packing in samples prepared at room temperature is predominantly orthorhombic. In samples prepared at 32°C the presence of a hexagonal packing is more pronounced in the outer layers of the stratum corneum. Gradually increasing the specimen temperature from 30 to 40°C induced a further transition from an orthorhombic to a hexagonal sublattice. At 90°C all lipids were present in a fluid phase. These results are in good agreement with previously reported wide angle X-ray diffraction and Fourier transformed infrared spectroscopy studies. We conclude that the lipids in human stratum corneum are highly ordered throughout the stratum corneum and that electron diffraction allows monitoring of the local lipid organization, which contributes to the understanding of stratum corneum barrier function.
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The epidermis is a multilayered epithelium that provides terrestrial animals with a barrier between the body and the environment. The main function of this barrier is the protection against desiccation and the penetration of microbes and harmful chemicals, which is realized by the stratum corneum (SC). The SC is the outermost nonviable layer of the epidermis and consists of flat, keratinized corneocytes surrounded by lipid lamellae that fill the continuous extracellular matrix. The lamellar organization of the extracellular matrix can be visualized in a transmission electron microscope after staining of transverse SC sections with ruthenium tetroxide (RuO_4) (Swartzendruber *et al*, 1995; Van der Meulen *et al*, 1996) or by preparation of replicas from freeze-fractured SC (Elias and Friend, 1975; Van Hal *et al*, 1996). Small angle X-ray diffraction studies on human and pig SC have shown that these lipid lamellar phases have a periodicity of 13.2–13.4 nm and 6.0–6.4 nm (White *et al*, 1988; Bouwstra *et al*, 1991). By means of wide angle X-ray diffraction (WAXD) lattice spacings of 0.415 nm and 0.375 nm were found. These spacings could be attributed to the orthorhombic (crystalline) packing, which is characterized by both the 0.415 nm and 0.375 nm reflections (Garson *et al*, 1991; Bouwstra *et al*, 1992). From these studies it remained impossible to exclude the presence of the hexagonal (gel) packing, which is characterized by only the 0.415 nm reflection. The aim of this study is to investigate whether next to the orthorhombic sublattice also a hexagonal lateral packing is present and if so whether these sublattices are localized in specific areas of the SC from untreated healthy human skin.

The reason why it has not been possible using WAXD to establish the presence of a hexagonal lipid packing in SC is that bulk quantities of SC are required. For the same reason, it has not been possible to establish whether the lipid organization changes with depth in the SC. Owing to overlap in lattice spacings, the hexagonal reflections in the diffraction patterns are always obscured by the orthorhombic ones (White *et al*, 1988). In contrast to this, electron diffraction (ED) allows the determination of the lateral lipid packing by selecting areas of about 1 μm^2 in much smaller samples. As a consequence, reflections can be recorded from one single or a few differently oriented crystals, in which molecular areas for the alkyl chains are approximately 0.20 nm^2 and 0.18 nm^2 for the hexagonal and orthorhombic unit cells, respectively (Small, 1986). This results in ED patterns containing diffraction spots or arcs that allow to distinguish between the hexagonal and orthorhombic packing arrangements and to obtain information about the orientation of the lattice. This method may thus be suitable to obtain insight in the local lipid packing in relation to depth in the SC.

In a previous study, we have shown that (cryo-) ED can be applied to obtain structural information about the lipid organization of human SC prepared by the so-called grid-stripping method (Pilgram *et al*, 1998a). An advantage of this method is that samples can be obtained from human skin *in vivo* in a noninvasive manner.

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Abbreviations: ED, electron diffraction; SC, stratum corneum; WAXD, wide angle X-ray diffraction.

Furthermore, like the tape-stripping technique, this method offers the possibility to prepare samples in relation to depth. In a study on SC resembling lipid models, we showed that it is possible to exclude the presence of a hexagonal packing by selecting areas as small as 1 μm^2, although both the 0.37 and 0.41 nm reflections were present in the ED patterns (Pilgram *et al*, 1998b).

In this study, we applied ED to both cryo-fixed transverse cryo-sections and grid-strips prepared from human skin *in vivo* (healthy volunteers) and *ex vivo* (excised skin from cosmetic surgery) in order to investigate the lipid packing and the effect of SC orientation. The grid-strips were either cryo-fixed after equilibration at room temperature or at 32°C to approach the *in vivo* skin temperature. This was done, because studies on SC lipid phase behavior have shown that close to the *in vivo* skin temperature there is a lipid phase transition between 35 and 40°C (Rehfeld and Elias, 1982; Gay *et al*, 1994; Bouwstra *et al*, 1997), whereas many studies on the SC lipid packing have been performed at room temperature or at temperatures above 40°C (Bouwstra *et al*, 1992; Chen and Wiedmann, 1996). The transition from an orthorhombic to hexagonal lipid packing and the transition from gel state to a fluid phase could also be studied directly by heating the samples in the specimen holder from room temperature to 90°C while under examination.

MATERIALS AND METHODS

Transverse cryo-sections of SC Human SC was isolated by a trypsin treatment from dermatomed skin, which proceeded as follows. Human skin was obtained from cosmetic surgery and dermatomed to a thickness of 200 μm. The dermatomed skin was soaked overnight in a solution of 0.1% trypsin in phosphate-buffered saline (pH 7.4) at 4°C. After incubation at 37°C during 1 h, the SC could be removed from the epidermis mechanically. The SC was placed in 0.1% trypsin inhibitor in water (purified by Millipore) for ⩾ 30 s and consequently stretched and washed twice in water. The SC was dried to the air on a small-mesh wire netting. Before use, the SC was stored in a desiccator over silica gel.

Small pieces of isolated SC were coiled and mounted on a sectioning holder (Wijdeveld *et al*, 1996). These samples were cryo-fixed in liquid nitrogen cooled propane using a plunge-cooling device. To remove the excess propane, the holders were blotted on cold filter papers. Until use, the samples were stored in liquid nitrogen.

Cryo-sections were cut perpendicular to the SC in a Reichert FCS cryo-microtome (Vienna, Austria). The sections with a thickness of about 60 nm were collected on 400 mesh copper grids (Stork Veco, Eerbeek, the Netherlands) and pressed on the grid with a cooled bar. To avoid undesired background scattering, no supporting film was present on the grids. The grids were stored in liquid nitrogen. Before examination in a Philips EM 400T transmission electron microscope (TEM) (Eindhoven, the Netherlands), the samples were freeze-dried overnight in a freeze-drying device (Polaron) at –80°C.

In order to visualize the extracellular lipid layers, samples were exposed at room temperature to RuO_4 vapour during 3 min by placing them next to a drop of 0.5% RuO_4 in purified water under a Petri dish.

Grid-strips The preparation method of the grid-strips has been described elsewhere (Pilgram *et al*, 1998a). In short, specially prepared grids containing Avery T406 glue (glue-grids) on the gridbars were placed on either *in vivo* skin obtained from the flexor forearm of five healthy volunteers or on freshly obtained *ex vivo* skin of five donors. The *ex vivo* skin samples were derived from cosmetic surgery. The glue-grids were pressed firmly with a spatula and SC was stripped from the skin upon removing the grid (grid-strip). The corneocytes that adhere to the glue on the gridbars partly float over the holes of the grid and are covered with lipid layers. In this way, the lipid organization on the protrusions can be studied in the TEM.

In order to study the lateral lipid packing in relation to depth, cell layers of the SC were removed using the conventional tape-stripping method to reach a certain depth at which three grid-strips were taken. These grid-strips were examined by ED. From *in vivo* skin, grid-strips were collected at strip numbers 3, 6, 10, 15, 20, and 30 and from *ex vivo* skin at depths 3, 6, 10, and 17. These grid-strips were either fixed by rapid freezing in liquid nitrogen cooled propane after equilibration at room temperature, or after equilibration at 32 ± 2°C during 15 min. Using this procedure, the lipid organization present just before cryo-fixation was maintained. All grid-strips were stored in liquid nitrogen until use.

Ultrathin sections from biopsies Biopsies were taken from *ex vivo* skin before and after stripping using a punch with a diameter of 2 mm in order to study the amount of SC removed during stripping. The tissue was fixed in 1.5% glutar aldehyde in 0.1 M cacodylate buffer during 1 h at room temperature. The biopsies were washed twice in phosphate-buffered saline and postfixed in 1% osmium tetroxide in Millonig phosphate buffer during 1 h at 4°C. After rinsing, the biopsies were dehydrated in a graded series of ethanol. The biopsies were embedded in Epon (LX-112, Ladd, Burlington, VT) and polymerization of the Epon occurred overnight at 60°C. Ultrathin sections (60 nm) were cut using a Reichert-Jung Ultracut E ultramicrotome (Vienna, Austria) and collected on 100 mesh copper grids with a Pioloform film coated with a thin carbon layer. The sections were stained with uranyl acetate and lead hydroxide, before examination in the TEM.

Electron diffraction When a sample containing repetitive structures is studied in the TEM, the electron beam will be scattered and interference of diffracted waves occurs at specific angles. As a result, intensity maxima will arise in the detection plane according to Bragg's law. The reflections contribute to a diffraction pattern and provide information about the unit cell dimensions of a crystalline lattice and its orientation (for more information see Misell and Brown, 1987).

Grid-strips were mounted in a precooled cryo-holder (Gatan Cryo Transfer System, Model 626, Pleasanton, CA) and inserted into the TEM. Before examination, the samples were freeze-dried at –80°C in the TEM during 30 min to remove superficial ice that may have formed during cryo-transfer. In this way, the interference of ice-crystal reflections was avoided. During ED, the TEM operated at 100 kV, which corresponds to a wavelength of 0.0037 nm. The samples were studied at –170°C using low beam intensity (dose rate approximately 10 e^- per nm^2 per s) in order to minimize radiation damage. To obtain information about the lateral lipid packing of a sample, several ED patterns were collected from different areas with a diameter of 1 μm (about 1 μm^2) or 5 μm (about 20 μm^2). These patterns were recorded on Kodak Electron Microscope films 4489 at a camera length of 290 mm using exposure times varying from 5 to 20 s. To prevent overexposure of the negatives, the undiffracted electron beam (central spot) was blocked using a beamstop.

From each donor (both *in vivo* and *ex vivo* skin), ED patterns were compared of samples that were obtained at different depths in SC and prepared at room temperature or at 32°C. Information about the influence of temperature on the SC lipid organization was obtained from samples that were exposed to temperature series. For that purpose, the temperature of the specimen-holder was gradually increased from 20 to 90°C in steps of 5°C and ED patterns were recorded at each temperature after equilibration for 10 min. In the region between 30 and 40°C the stepsize was 2°C.

Spacings of the reflections in the ED patterns were calculated using the formula $Rd = \lambda L$ deduced from Bragg's law. From this formula it is clear that there is a reciprocal relationship between the spacing (d) in a lattice and the distance of a recorded reflection to the central beam spot (radius R). The known ED pattern of gold was used to calibrate the constant factor λL, in which λ is the wavelength of electrons and L the camera length. Then the diameter (2R) of reflections was measured on the recordings using a vernier caliper gauge to determine the spacing in the lattice with an accuracy of ± 0.005 nm (Pilgram *et al*, 1998b).

RESULTS

Grid-strips are favorable for ED with respect to the orientation of SC lipids as compared with cryo-sections Corneocytes and extracellular matrix could clearly be distinguished in the unstained transverse cryo-sections (**Fig 1*a***). Structures that might be attributed to remnants of desmosomes (corneodesmosomes) were also observed. The lipid lamellae in the extracellular matrix became visible after staining freeze-dried cryo-sections with RuO_4 (**Fig 1*b***).

Faint ED patterns were recorded of the unstained cryo-sections. These patterns consisted of two opposite arcs at 0.41 nm or had opposite arcs at 0.37 and 0.41 nm (**Fig 1*a***, *inset*). The 0.41 nm reflection can be attributed to both the hexagonal and the orthorhombic lattice, whereas the presence of the 0.37 nm reflection is indicative of an orthorhombic lattice.

In grid-strips, ED patterns could be obtained in which reflections were present in either all directions (ring or spot patterns) symmetrically around the central spot, or in only one direction. Areas in which no clear reflections could be distinguished were also encountered. The unidirectionally scattered ED patterns (data not

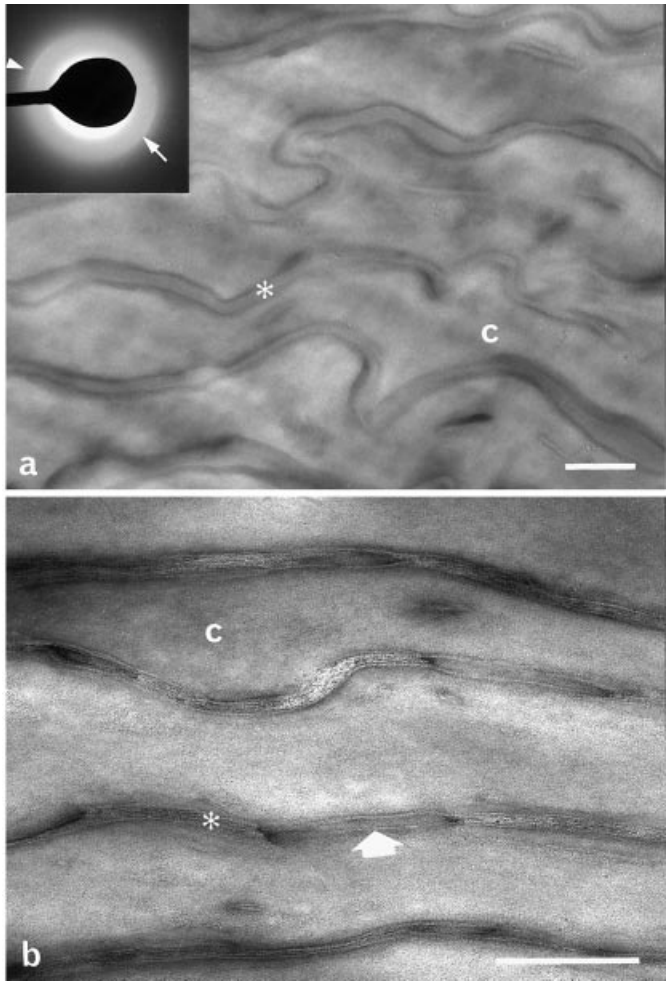

Figure 1. Transverse cryo-sections of isolated human SC in which intercellular domains and corneocytes can clearly be distinguished. (*a*) Electron micrograph of an unstained cryo-section. The inset shows a unidirectionally scattered ED pattern of a cryo-section in which it is uncertain whether only the orthorhombic sublattice is present or the hexagonal as well (the *arrow* indicates a 0.41 nm reflection and the *arrowhead* marks a 0.37 nm reflection). (*b*) The cryo-section has been freeze-dried and exposed to RuO_4 vapor during 3 min, thereby revealing the lipid lamellae (C is corneocyte, *marks the extracellular matrix, and the *big arrow* indicates corneodesmosomes). *Scale bar*: 200 nm.

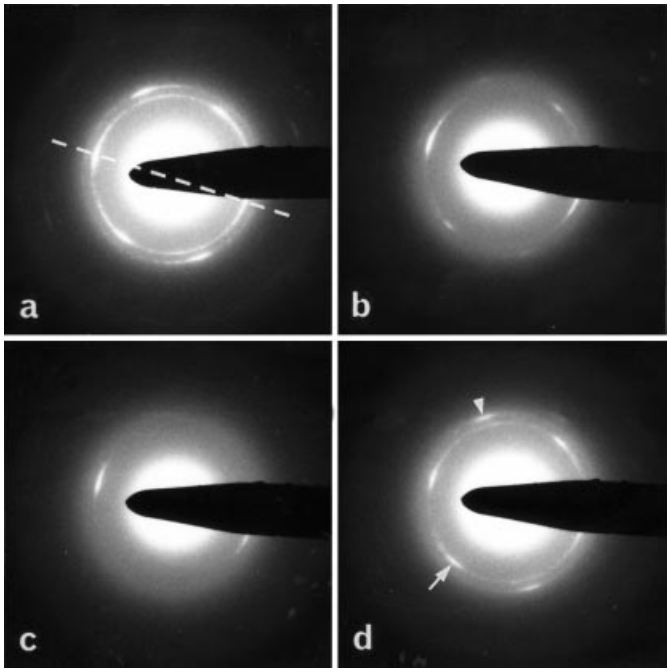

Figure 2. Tilting series obtained at the same area shows that reflections begin to disappear already at small tilting angles. (*a–d*) ED patterns obtained at tilting angles, respectively, 0°, 10°, 30° and 0°. In (*a*) the tilt axis is indicated by a dashed line. In (*d*) the *arrow* indicates a 0.41 nm reflection and the *arrowhead* marks a 0.37 nm reflection, which became restored after returning to the original position.

shown) were similar to the ED patterns of parallel cryo-sections. These patterns could also be obtained upon tilting the specimen-holder in the TEM. To achieve this, an area was selected in which all reflections were present at 0° tilting and which was oriented in such a way that the tilting axis ran perpendicular to a set of lattice planes (**Fig 2*a***). Then the reflections from this set of planes remained, whereas the other reflections vanished gradually resulting in unidirectional scattered patterns (**Fig 2*b***, ***c***). When the specimen holder was returned to its original position, the ED patterns were restored (**Fig 2*d***). In this perpendicular orientation it became possible to exclude the hexagonal lattice in the presence of the orthorhombic packing based on the location of the reflections.

Figure 3(***a***) shows schematically how the orientation of the lipid layers with respect to the incident electron beam influences the ED pattern. The findings that SC cryo-sections give rise to unidirectionally scattered ED patterns, whereas the reflections in ED patterns from grid-strips can be distributed symmetrically around the central spot, are in agreement with results obtained by Garson *et al* (1991) and Bouwstra *et al* (1992). They performed WAXD experiments on sheets of SC oriented perpendicular or parallel with respect to the X-ray beam and found that the diffraction patterns were circular or arc shaped, respectively. In this study, we observed that a tilting angle of 10° already results in the disappearing of reflections in ED patterns from grid-strips. This indicates that the lipid layers must be properly oriented with respect to the incident electron beam in order to obtain ED patterns that provide unambiguous information on the type of sublattice. As corneocytes on (grid-) strips are generally rippled (Pilgram *et al*, 1998a), the orientation of the lipid layers may not always be perpendicular. This explains why in some areas ED patterns could be recorded without clear reflections or with unidirectional scattered reflections only. Nevertheless, areas with a proper lipid orientation were sufficiently present on grid-strips in contrast to the transverse cryo-sections. Therefore, grid-strips were chosen to study the SC lipid organization as a function of depth.

SC lipid packing is preserved during cryo-fixation The grid-strips were studied under cryo-conditions to reduce damage of the lipid packing caused by the electron beam. Therefore, a proper cryo-fixation was required. To study whether such a fixation procedure could affect the lateral lipid packing of human SC, samples were studied at room temperature as well. In order to minimize radiation damage at room temperature, the recording time had to be decreased. Comparing the ED patterns obtained at room temperature with those obtained at –170°C, we concluded that the lipid organization was similar. This finding corresponds well to the results obtained using SC lipid models (Pilgram *et al*, 1998b). In an additional experiment, grid-strips were equilibrated at 50°C just above the orthorhombic–hexagonal transition temperature and subsequently cryo-fixed. It was found that all lipids were packed in a hexagonal lattice (data not shown), confirming that the cooling rate in our cryo-fixation procedure was rapid enough to avoid changes in the lipid organization. The main difference between the frozen and unfrozen samples is the increased stability of the sample at –170°C, due to which the ED patterns fade away much slower than during examination at room temperature.

SC lipid packing is gradually increased upon raising the specimen temperature To study the transition from ortho-rhombic to hexagonal packing, and from hexagonal to liquid

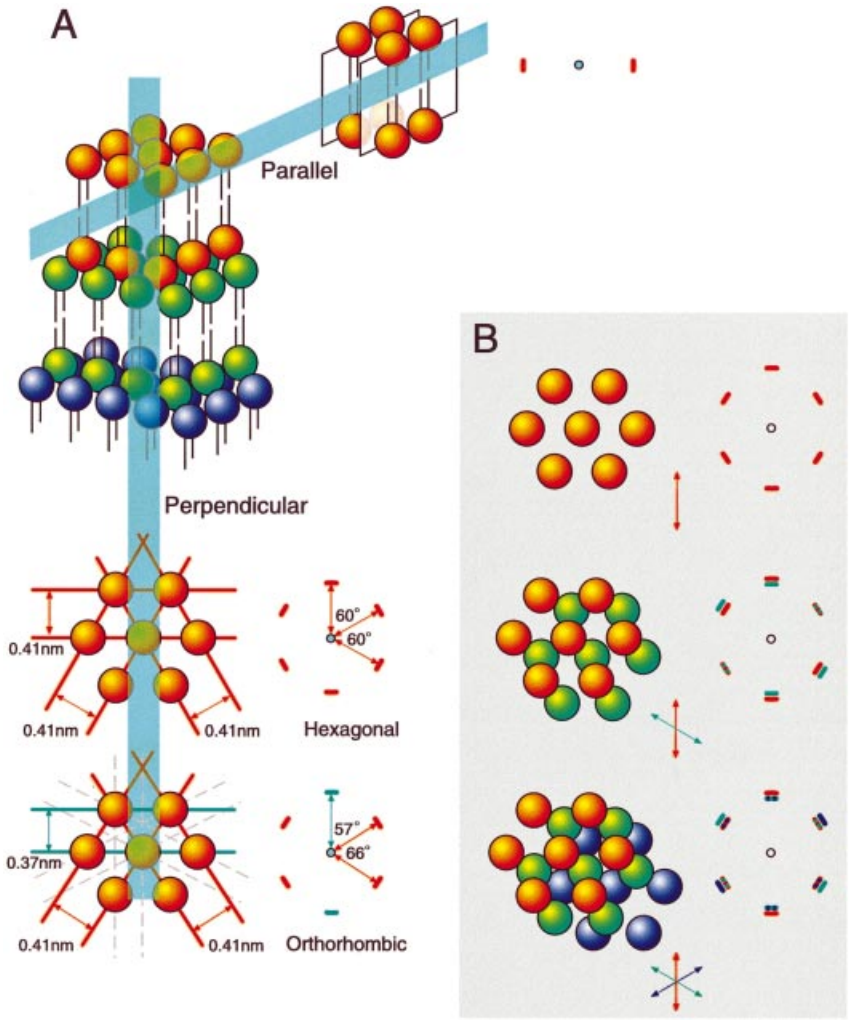

Figure 3. Schematical representation of the possible lipid orientations with respect to the incident electron beam and the effect of 3 superimposed orthorhombic lattices on the ED pattern. (*A*) Incident electron beam (*in blue*) running parallel to the basal plane of lipid lamellae and another running perpendicular with respect to the lipid layers. Next to the lattices are the corresponding ED patterns depicted in which the central green spot is the undiffracted electron beam. In the parallel configuration of the lipids, as in cryo-sections, only one set of repetitive lattice planes (indicated by the vertical rectangles) of a crystal can be oriented properly with respect to the electron beam. These lattice planes give rise to reflections (*in red*) resulting in a unidirectionally scattered ED pattern (*top right*). In this case, only the 0.41 nm reflections are present, which are characteristic to both the orthorhombic and hexagonal lattice. When the 0.37 and 0.41 nm reflections are both present (**Fig 1**, *inset*), these must be derived from at least two crystals. For this reason, it remains unclear whether only the orthorhombic packing is present (in two orientations to reveal both lattice planes with a spacing of 0.37 nm and 0.41 nm) or the hexagonal packing as well. In the perpendicular orientation, as in grid-strips, more sets of lattice planes (indicated by colored lines) from one crystal may give rise to reflections in the detection plane simultaneously. The colored arrows represent the spacing of the sets of repetitive lattice planes and indicate the direction of the corresponding paired reflections in the ED pattern. The reflections at 0.41 nm are colored in red and those at 0.37 nm are shown in green. The hexagonal ED pattern is characterized by three pairs of reflections at 0.41 nm with an interplanar angle of 60°, whereas the orthorhombic ED pattern has two pairs of reflections at 0.41 nm and one pair at 0.37 nm at interplanar angles of 66° and 57°, respectively. This allows distinction between the two lattices. In the orthorhombic lattice, the dashed lines indicate additional sets of lattice planes that give rise to the reflections present in **Figs 2**(*a*) and **4**(*a*). (*B*) Top view on an orthorhombic lattice in which the incident electron beam is perpendicular to the plane of the paper (like in *A bottom left*). The direction of the 0.37 nm reflections is indicated by an *arrow*. Additional orientations give rise to the formation of double arcs in the ED pattern. Note that the striped reflections at 0.41 nm consist of an overlap from two sublattices. The colors of the reflections correspond to the colors of the lattices. The three differently oriented lattices may be present on top of each other (this picture) or next to each other in the same lamellae.

packing, the ED patterns were recorded during increasing the temperature of the sample gradually from room temperature to 90°C. It was found that up to 30°C (**Fig 4***a*) the lipid packing was mainly orthorhombic (see also below). Above 30°C the intensity of the 0.37 nm reflection started to decrease. Between 35°C and 40°C the spacing of this reflection increased (**Fig 4***b*) and finally disappeared at higher temperatures. The observation that the 0.37 nm reflection moved towards the 0.41 nm reflection indicated that the packing density decreased gradually. Above 40°C only the 0.41 nm reflection remained (**Fig 4***c*). At about 80°C the 0.41 nm reflection became less distinct and at 90°C only a broad reflection at 0.46 nm remained, indicating that all lipids were in the fluid phase (**Fig 4***d*). The observed phase transition temperatures were similar to those reported in literature (Bouwstra *et al*, 1992; Gay *et al*, 1994).

SC lipids in gel state were recorded more often at physiologic temperature than at room temperature ED patterns were collected in all grid-strips from *in vivo* and *ex vivo* SC cryo-fixed at room temperature and at 32°C to approach the physiologic skin temperature. Representative ED patterns are shown in **Fig 5**. No differences were observed between ED patterns from *in vivo* and *ex vivo* SC. The diffraction patterns mostly consisted of concentric rings or opposite arcs/spots at both 0.41 and 0.37 nm. A very characteristic pattern that was regularly detected in *in vivo* and *ex vivo* samples at both temperatures consisted of three pairs of double arcs at 0.37 and 0.41 nm (**Fig 4***a*). **Figure 3**(*b*) shows schematically how such an ED pattern can arise. Furthermore, faint reflections at 0.22 and 0.25 nm were recorded occasionally (**Figs 2***a* and **4***a*). These reflections can be attributed to other sets of lattice planes of the same crystal as indicated in **Fig 3**(*a*). The spacings are in agreement with calculated values and with the spacings established by WAXD.

The ED patterns of samples prepared at room temperature could be explained mainly by an orthorhombic lattice on the basis of the location of 0.41 and 0.37 nm reflections (**Fig 5***a*). When the ED patterns consisted of many differently oriented reflections, it became more difficult to exclude the hexagonal lattice (**Fig 5***b*). These patterns were mainly recorded when an area of 20 μm^2 was selected for diffraction instead of an area of 1 μm^2. In some spot patterns, however, it was still possible to relate the observed 0.41 nm reflections to the corresponding 0.37 nm reflections (**Fig 5***c*). Only a few patterns were recorded that could exclusively be attributed to the hexagonal lattice. ED patterns lacking the reflections at 0.37 nm, however, could be recorded more often in samples equilibrated at 32°C. When the 0.41 nm reflections were separated in six arcs with an interplanar angle of 60°, such ED patterns could unambiguously be attributed to the hexagonal lattice (**Fig 5***d*).

All recordings of *in vivo* and *ex vivo* skin were classified according to their ED pattern into four categories: orthorhombic (ort), orthorhombic in which hexagonal cannot be excluded (ort*), hexagonal (hex), and patterns that are probably hexagonal (hex*). The relative distribution of the ED patterns in these categories was similar for *ex vivo* and *in vivo* skin (**Fig 6**). The data in **Fig 6** are

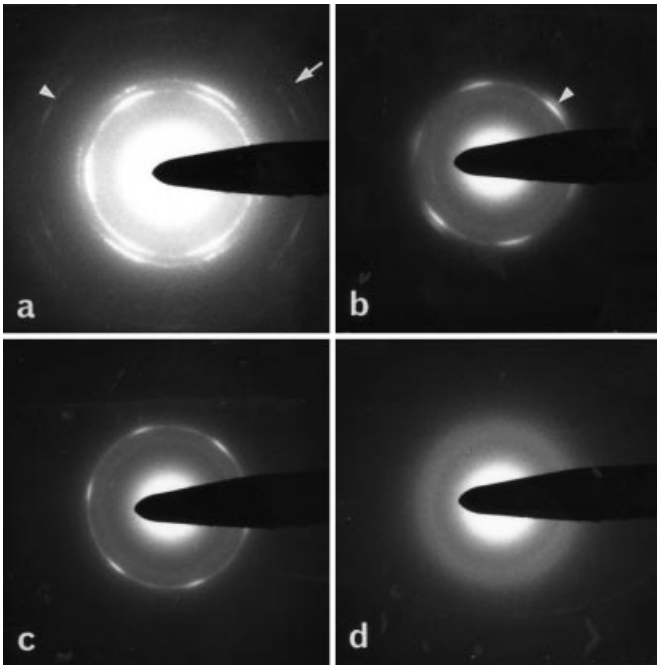

Figure 4. Temperature series showing the lipid phase transitions obtained by increasing the temperature of the sample during examination. (*a–d*) Obtained at, respectively, room temperature, 35°C, 60°C, and 90°C. (*a*) Typical ED pattern that can be explained by three orientations of the orthorhombic lattice. This pattern consisting of three pairs of double arcs has been recorded frequently throughout both *in vivo* and *ex vivo* human SC and is explained schematically in **Fig 3(*b*)**. Furthermore, the *arrowhead* marks a reflection at 0.24 nm at an interplanar angle of 90° with respect to a 0.37 nm reflection, whereas the *arrow* indicates a reflection at 0.22 nm (see **Fig 3*a***). (*b*) Shows that the original 0.37 nm reflection is moving gradually towards the 0.41 nm reflection, indicated by the *arrowhead*. (*c*) Shows a hexagonal lipid packing in which the interplanar angle of the reflections is 60°. (*d*) All lipids are in a fluid phase, characterized by a broad band around 0.46 nm.

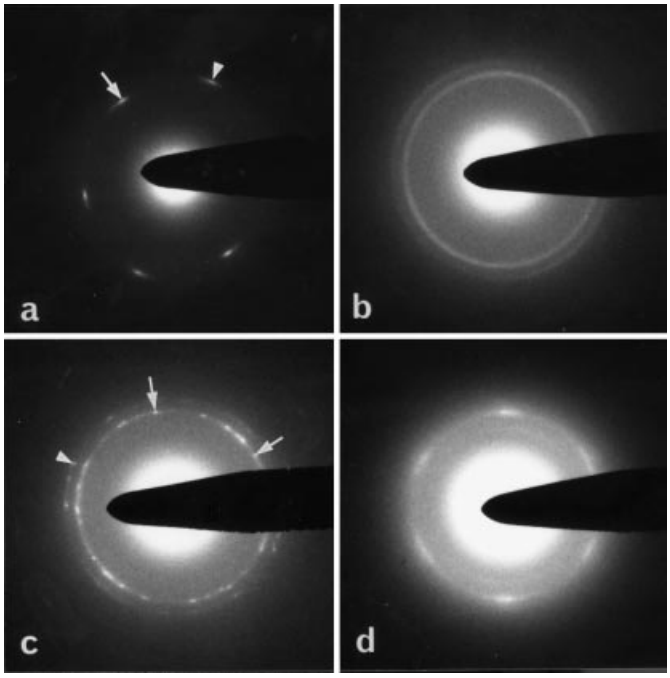

Figure 5. Characteristic ED patterns of both *in vivo* and *ex vivo* human SC. (*a*) Shows an orthorhombic sublattice that can be found throughout the SC. (*b*) shows two rings at 0.37 and 0.41 nm in which the hexagonal lattice cannot be excluded, like in WAXD patterns. (*c*) Shows an ED pattern in which reflections of the hexagonal lattice may be obscured by orthorhombic ones. All reflections in the inner ring at 0.41 nm, however, correspond to 0.37 nm reflections in the outer ring (one orientation is indicated). (*d*) Shows a hexagonal lattice that is mainly found in the outer layers of the SC. (The *arrows* indicate 0.41 nm reflection and the *arrowheads* mark 0.37 nm reflection.)

plotted as a function of temperature and show that the percentage of ED patterns attributed to the hexagonal lattice increased at 32°C compared with room temperature.

The hexagonal lipid packing is mainly present in the outer layers of human SC Whereas several studies have reported that from *in vivo* human skin up to 30 tape-strips can be taken before the deepest layers of the SC have been reached (Pinkus, 1951), most of the SC from *ex vivo* skin was already removed in 17 tape-strips (**Fig 7**). This is in agreement with observations by Pellet *et al* (1997).

The ED patterns were plotted as a function of depth in *ex vivo* SC (number of grid-strip) in order to get an overview of the ED patterns recorded in the grid-strips. It appeared that ED patterns attributed to the hexagonal packing were recorded mainly in the third and sixth grid-strips both at room temperature and at 32°C (**Fig 8**). A similar profile was found for *in vivo* SC. This observation indicates that variations in the lipid ordering exist in relation to depth.

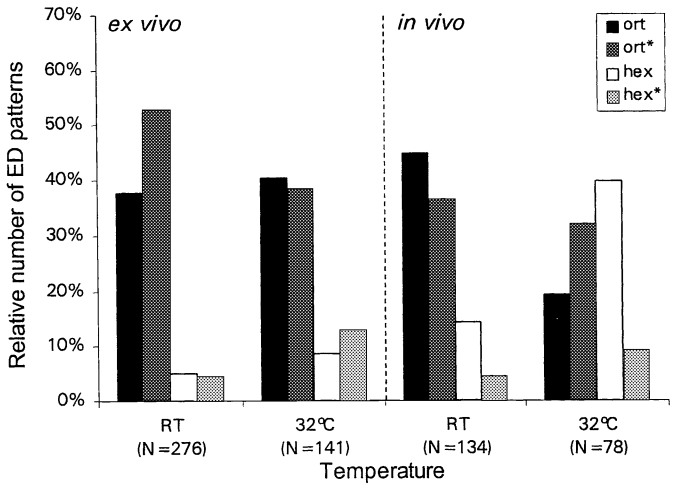

Figure 6. Distribution of the hexagonal and orthorhombic lattices in relation to temperature in human SC *ex vivo* and *in vivo*. The number of recordings (N) shown at the x-axis is set at 100% for each data set. The percentage of ED patterns per category is shown along the y-axis (ort, orthorhombic; ort*, orthorhombic, however, the hexagonal lattice cannot be excluded; hex, hexagonal; hex*, probably hexagonal).

DISCUSSION

As it has been established that the extracellular matrix of the SC plays a key part in the barrier function of mammalian skin, many studies have been carried out to investigate the SC lipid organization. It has been shown that the presence of ceramides, cholesterol, and long-chain free fatty acids is very important for the unique ordering of SC lipids (Bouwstra *et al*, 1996, 1998; McIntosh *et al*, 1996; Pilgram *et al*, 1998b) and that many skin diseases can be imputed to aberrant lipid compositions resulting in a decreased barrier (Holleran *et al*, 1994; Motta *et al*, 1994; Lavrijsen *et al*, 1995). For the development of (trans) dermal drug delivery systems the aim is to disturb the skin barrier locally and temporarily. Therefore, agents are being tested for their penetration enhancing activities without causing irritations or long-lasting damage (Boelsma *et al*, 1996). In addition, human skin equivalents are presently being developed that should provide a good alternative for these tests to replace the use of animal models or *ex vivo* human skin (Ponec *et al*, 1997; Di Nardo *et al*, 1998; Doucet *et al*, 1998). All these studies require a thorough understanding of the lipid organization in the SC. Techniques that provide information on the lipid organization are

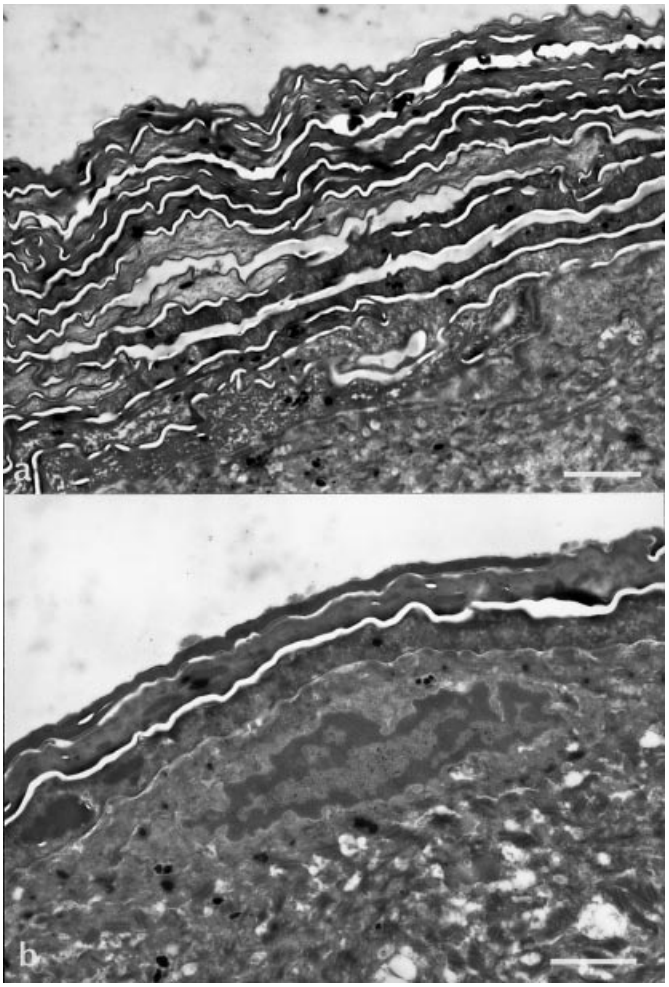

Figure 7. The amount of SC removed after tape-stripping of human epidermis from *ex vivo* skin. (*a*) Shows the epidermis before stripping and (*b*) shows that two or three layers of corneocytes remained after removing 17 tape-strips. *Scale bar.* 2 μm.

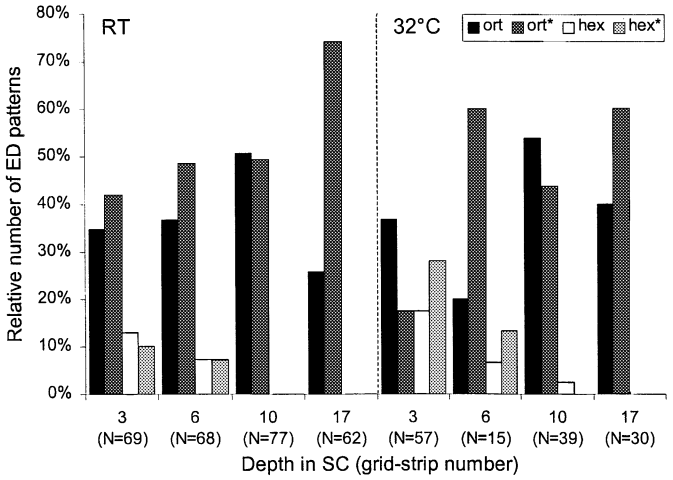

Figure 8. Distribution of the hexagonal and orthorhombic lattices in relation to depth in *ex vivo* human SC equilibrated to room temperature or 32°C. The number of recordings (N) shown at the x-axis is set at 100% for each depth indicated by grid-strip numbers 3, 6, 10, and 17. The percentage of ED patterns per category is shown along the y-axis (ort, orthorhombic; ort*, orthorhombic, however, the hexagonal lattice cannot be excluded; hex, hexagonal; hex*, probably hexagonal).

WAXD, small angle X-ray diffraction, Fourier transformed infrared spectroscopy, differential scanning calorimetry, and nuclear magnetic resonance imaging. With the exception of attenuated total reflection Fourier transformed infrared spectroscopy, these methods cannot provide information about the local lipid organization at a micrometer scale and require relatively large quantities of SC. Furthermore, these techniques are invasive in the *in vivo* situation as skin biopsies are needed. Therefore, it was decided to develop a suitable, noninvasive SC preparation method that enabled us to apply ED as a method to establish the local lipid packing (Pilgram *et al*, 1998a). In this study, ED has been shown to provide detailed information about the lipid organization in *in vivo* and *ex vivo* human skin, which is supplementary to the methods used thus far.

The ED patterns from *in vivo* and *ex vivo* SC grid-strips could predominantly be attributed to the orthorhombic lattice. This finding is supported by findings of Chen and Wiedmann (1996), who used atomic force microscopy to study the surface of tape-strips at ambient temperature; however, they did not report whether a small fraction of lipids is packed in a hexagonal array. In our study, ED patterns consisting of 0.41 nm reflections only were also recorded. Hereby, the presence of a hexagonal lipid organization has been confirmed in human SC by ED. In samples equilibrated at 32°C, this hexagonal packing was even more pronounced. As the transition from orthorhombic to hexagonal occurs approximately between 35°C and 40°C, it might be possible that at 32°C a part of the lipids is already in the gel phase. This polymorphic phase behavior has also been observed by Fourier transformed infrared spectroscopy in SC lipid mixtures (Ongpipattanakul *et al*, 1994) and mouse SC (Krill *et al*, 1992).

A very interesting finding is the presence of the hexagonal packing mainly in the outer part of the SC. Assuming that the sample temperature of 32°C approaches the *in vivo* skin temperature, we conclude that the main lipid organization in human SC *in vivo* is orthorhombic and that in the outer layers of the SC the lipids are partly present in a hexagonal packing. According to Long *et al* (1985) and Lavrijsen *et al* (1994), the relative amount of ceramides in the SC does not change with depth and therefore cannot account for the change in lipid packing. The presence of the hexagonal packing can neither be attributed to changes in temperature or cholesterol sulfate content. Although these factors can increase the fluidity of the lateral lipid packing, their gradient in SC (Long *et al*, 1985; Cox and Squier, 1986) is opposite with respect to the occurrence of the hexagonal packing. A possible explanation may then be the influence of sebum present on the surface of human SC (Downing and Strauss, 1982). Indeed, Bommannan *et al* (1990) showed that the amount of lipids was increased in the outer SC layers due to mixing of intercellular lipids with sebaceous lipids. This may lead to an increase in short-chain free fatty acids (Bonté *et al*, 1997), which are present in sebum. Bouwstra *et al* (1996) showed by WAXD that the addition of short-chain free fatty acids to ceramide–cholesterol lipid mixtures does not induce a change from hexagonal to orthorhombic packing in contrast to long-chain free fatty acids. Whether compounds in sebum indeed account for this change in lipid packing has to be a subject for future research.

In a previous study using lipid model systems, it was found that depending on the size of the area selected for diffraction the ED patterns consisted of rings or arcs (Pilgram *et al*, 1998b). These patterns were explained by the number of orientations of the unit cells of the lipid lattices that were present in the irradiated area. In the SC grid-strips, ED patterns consisting of separate arcs could be frequently recorded in both the 1 and 20 μm^2 areas. This leads to the conclusion that the lipids in the extracellular matrix of human SC maintain a certain orientation over a larger area compared with the lipids in our lipid model system. Possibly, this is related to the presence of the covalently bound lipids in the corneocyte envelope, which may function as a template for the extracellular lipid orientation (Swartzendruber *et al*, 1989).

A peculiar ED pattern that was frequently recorded in SC *in vivo* and *ex vivo*, and at both temperatures (room temperature and 32°C) was hexagonally shaped and consisted of three pairs of double arcs (**Fig 4*a***). Although this seemed unusual at first sight, this pattern

can be explained from the orthorhombic lattice only. We hypothesize that such a pattern is formed by three orientations of the orthorhombic lattice in the area selected for diffraction. These three orientations have rotated over an angle of approximately 60° relative to each other, so that the inner arcs represent an overlap of two orthorhombic crystals, whereas the outer reflections are located just behind the inner reflections. This results in the formation of double arcs shown schematically in **Fig 3**(***b***). The three orientations may be present in one lipid layer and represent lateral misalignment, or can be present in successive lipid layers. Thus far, we were not able using ED to distinguish between these two possibilities. The first explanation, however, seems to be unfavorable for establishing a good barrier function. It is known from studies on phospholipid membranes that penetrants may more easily pass through sites at which phase separation occurs (Langner and Hui, 1993; Xiang and Anderson, 1998). This route of penetration has also been suggested for penetration of compounds through extracellular SC lipids (Ongpipattanakul *et al*, 1991; Bouwstra *et al*, 1994). To explain the second possibility, one lipid layer should form a template for the adjacent layer and somehow cause a change in orientation of approximately 60°, which then occurs in the next layer as well. The three orientations of the orthorhombic lattices may fit in the lamellar ultrastructure often referred to as the broad–narrow–broad sequence (Madison *et al*, 1987; Kuempel *et al*, 1998) and in the molecular model proposed by Bouwstra *et al* (1998) concerning the three successive layers that constitute the 13 nm lamellar phase.

In conclusion, we have shown that the grid-stripping method in combination with ED enables us to study minor changes in the lipid organization of human SC in a noninvasive way. In future, this method can provide important information about the lipid organization in relation to SC barrier function, when applied to studies on transdermal drug delivery systems, human skin equivalents, and skin diseases in which the extracellular lipids are involved.

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