The Effect of Two Azones on the Lateral Lipid Organization of Human Stratum Corneum and Its Permeability

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Purpose. Investigation of the relationship between changes in human SC lipid organization induced by N-alkyl-azocycloheptane-2-one and SC permeability to the model compound $HgCl_2$.

Methods. Human dermatomed skin was treated with propylene glycol (PG), oleyl-Azone (OAz) or dodecyl-Azone (DAz) in 0.15 M PG. Untreated skin served as control. The lateral lipid organization was studied by electron diffraction. Hg was measured on tape-strips by X-ray microanalysis and in the acceptor phase by atom absorption spectrometry.

Results. In control and PG treated samples, the lipid packing was mainly orthorhombic, while a small fraction was hexagonal. In OAz and DAz treated samples, the orthorhombic lipid organization remained, however, the hexagonal packing was recorded less frequently. The amount of Hg decreased as a function of depth in all SC samples, however, the penetration profile increased significantly upon OAz treatment. The cumulative amount of Hg in the acceptor phase of OAz treated samples also increased significantly compared to control and PG treated samples.

Conclusions. The increased penetration of Hg into OAz treated skin could not be related to an orthorhombic-hexagonal phase transition. Alternatively, phase separation of OAz and/or formation of grain boundaries might affect SC permeability, hereby increasing Hg penetration. A similar mechanism is proposed for DAz.

KEY WORDS: barrier function; cryo-electron microscopy; electron diffraction; enhancer; penetration; skin.

INTRODUCTION

The main problem that is faced in the field of the development of (trans)dermal drug delivery systems is the relatively low permeability of human stratum corneum (SC) to most chemical substances. The SC forms the outermost layer of the skin and both the unique composition and structure of this layer account for the formation of a protective barrier that is able to maintain homeostasis of the human body. Yet, under certain circumstances interference may be desired to recover this balance by means of drug administration.

Since the '70s, the advantage to deliver drugs topically has been recognized (1) for those drugs that are metabolized in the gastro-intestinal tract or the liver. Furthermore, using transdermal drug delivery, a more continuous plasma level of a drug can be reached.

Several approaches have been examined to overcome the SC barrier function temporarily, like the use of supersaturated solutions (2), co-application or pretreatment with penetration enhancers (3–5), lipid vesicles as drug carriers (6), iontophoresis for charged drugs (7), or a combination of methods (8). Each one of them has been shown to improve to a certain extent the permeation of different classes of drugs or model compounds of various size or lipophilicity.

Several penetration pathways have been postulated for drugs to enter the skin, the trans- and intercellular pathway through the SC and the shunt-route through hairfollicles and sweat glands. As the follicles and glands only comprise 0.1% of the surface area of the skin, this route has generally not been considered to contribute significantly to passive diffusion of most compounds. Therefore, the importance of intercellular lipids for SC permeability with respect to the interfollicular pathway will be considered in this study.

Human SC consists of corneocytes embedded in an intercellular matrix of lipids. This matrix is mainly composed of ceramides, cholesterol and long-chain free fatty acids, which are organized in two lamellar phases with a short periodicity of 6 nm and a long periodicity of 13 nm (broad-narrow-broad sequence) (9). With respect to the interfollicular pathway, in which drugs always have to cross the SC, the compounds will also have to cross these lipid lamellae. Therefore, it is of importance to understand the behavior of SC lipids in detail. Human SC contains both orthorhombic (crystalline state), and hexagonal (gel state) domains (10). Whether the lipids are also present in a fluid phase is still uncertain. Although the orthorhombic lattice predominates (11), phase boundaries must occur at sites where two or more lipid phases are present. It has been hypothesized that penetration enhancers increase SC permeability by affecting SC lipid ordering (12) (i.e., formation of enhancer-rich domains (13) and/or induction of orthorhombic to hexagonal, or orthorhombic to fluid phase transitions (5,14)) and/or by creating phase separation (mismatching) leading to an increased number of grain boundaries.

From phospholipid studies is known that the lipid chain packing properties influence the degree of permeability (15). Furthermore, grain boundaries are shown to be sites where permeability is increased (16). These mechanisms indeed seem to play a role in SC permeability. Ogiso *et al.* (17) showed that raising the temperature led to increased penetration of terodiline, mainly as a result of increased intercellular lipid disorder. It has also been shown that depending on its concentration (18) oleic acid can be present in a separate fluid phase (4,19). The consequent SC lipid disordering (phase separation and SC lipid fluidization) may lead to the increased permeability of human skin.

In previous studies, we showed that using electron diffraction (ED) local information on SC lipid packing can be obtained

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ABBREVIATIONS: AAS, atom absorption spectrometry; DAz, dodecyl-Azone; ED, electron diffraction; OAz, oleyl-Azone; PG, propylene glycol; WAXD, wide-angle X-ray diffraction; SAXD, small-angle X-ray diffraction; SC, stratum corneum; SEM, scanning electron microscope; TEM, transmission electron microscope; XRMA, X-ray microanalysis.

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that is supplementary to information from WAXD studies (11,20). Therefore, in this study we applied ED to investigate human SC lipid organization upon treatment with two well known enhancers, oleyl-Azone (OAz) and dodecyl-Azone (DAz) (Azone®). It was hypothesized that this method may elucidate the effect of these enhancers on SC lateral lipid packing. As a model compound mercurychloride ($HgCl_2$) was applied to the same samples to examine SC permeability. $HgCl_2$ was chosen, as it is known from studies by Bodde *et al.* (21) that it permeates the SC mainly via the intercellular matrix. By means of X-ray microanalysis (XRMA) the presence of Hg in SC was studied, and by means of atom absorption spectrometry (AAS) Hg was also measured in the acceptor phase.

MATERIALS AND METHODS

Preparation of Human Skin and Application of Penetration Enhancers

Human skin was freshly obtained from cosmetic surgery. Subcutaneous fat was removed with a scalpel and the skin was subsequently cleaned with destilled water. Then the skin was dermatomed to a thickness of approximately 250 μm. Circular pieces of skin with a diameter of 22 mm were prepared and each one was placed on a supporting dialysis membrane (5000 MWC, Dianorm, Germany) in a Franz-type diffusion cell with the apical side facing the donor chamber. The acceptor chamber was filled with 3.5 ml PBS and separated from the donor chamber by the skin sample. The temperature of the acceptor chamber was 36°C to reach physiological temperature of 32°C near the skin surface.

A skin sample was either left untreated (control), treated with 250 μl/cm^2 propylene glycol alone (PG), or with one of the two enhancers, DAz or OAz, at a concentration of 0.15 M in PG. These treatments lasted 4.5 hours or 24 hours. After this treatment, grid-strips (22) were collected or the skin samples were used for the permeability studies prior to collection of (tape-and grid-) strips (see below).

Application of $HgCl_2$ to Human Skin in Franz-Type Diffusion Cells

All the Hg permeation experiments were performed with skin from 5 different donors. In each of these experiments, the 3 treatments were carried out in duplo in 6 Franz-type diffusion cells. The skin samples were pretreated with either PG, or OAz in PG during 4.5 hours, or left untreated (control), as described in the previous section. After treatment, the solutions were removed and the surface of the skin was washed with PBS. Then 1.5 ml/cm^2 of 5% $HgCl_2$ solution was applied to the donor chamber of each diffusion cell. The diffusion time lasted 17.5 hours, so the duration of the experiments did not exceed 24 hours. The acceptor phase was stirred continuously to maintain homogeneous distribution of Hg. Then the skin was removed and samples were collected by tape- and grid-stripping, as described below.

Tape- and Grid-Stripping

All samples were stretched on a piece of polystyrene covered with parafilm. By stretching the skin, it was attempted to reduce the problem of furrows during stripping (23). The skin surface was dried with a tissue before stripping. Tape-strips of 1 cm^2 (cellotape, Beiersdorf) were placed on the skin, pressed slightly and carefully removed using tweezers. The 3rd, 6th, 9th, 12th, and 15th tape-strip were fixed on SEM-holders coated with carbon-tape. These tape-strips were sputtercoated with carbon (Balzers) before examination in the scanning electron microscope (SEM).

Grid-strips were collected by alternating tape-strips with grid-strips at depth 3, 6, 10 and 17. The preparation of grid-strips has been described elsewhere (22). The grid-strips were equilibrated to 32°C to approach skin surface temperature and subsequently cryo-fixed by plunging the grid-strips rapidly into liquid nitrogen cooled propane. Using this procedure, the lipid organization present just before cryo-fixation was maintained. The grid-strips were stored in liquid nitrogen until use.

Electron Diffraction

Grid-strips were mounted in a pre-cooled cryo-holder (Gatan Cryo Transfer System, Model 626, Pleasanton, California) and inserted into a Philips EM 420 transmission electron microscope (TEM). 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 $e^-/nm^2 \cdot s$). ED patterns from areas with a diameter of 1 μm (about 1 μm^2) or 5 μm (about 20 μm^2) were recorded on Kodak Electron Microscope films 4489 at a camera length of 350 mm using exposure times varying from 5 to 20 seconds. To prevent overexposure of the negatives, the undiffracted electron beam (central spot) was blocked using a beamstop.

Spacings of the reflections in the ED patterns were calculated using the formula $Rd = \lambda L$ deduced from Bragg's Law. According to this formula 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. The diameter (2R) of reflections was measured on the recordings using a vernier caliper gauge to determine the spacing in the lattice.

Figure 1 shows schematically the alkyl chain arrangement of SC lipids in hexagonal and orthorhombic lattices, including their corresponding ED patterns.

X-Ray Microanalysis

The carbon-coated tape-strips were placed in a Philips 525M SEM equiped with an X-ray detector (Tracor Northern, Z-max 30 series). The SEM operated at 20 kV, spotsize 50 nm and the free working distance was kept at 9.7 mm. An X-ray spectrum was collected from an area of 30 × 50 μm and regions of interest were defined at 2000–2400 keV for the Hg Mα peak and at 9800–12000 keV for the Hg Lα peak. In each sample, 5 areas were measured that contained one layer of corneocytes.

Atom Absorption Spectrometry

The concentration of Hg in the acceptor phase of 3.5 ml was determined using a Flow Injection Atom Absorption Spectrometer (Perkin Elmer 3100 AAS). Before running the

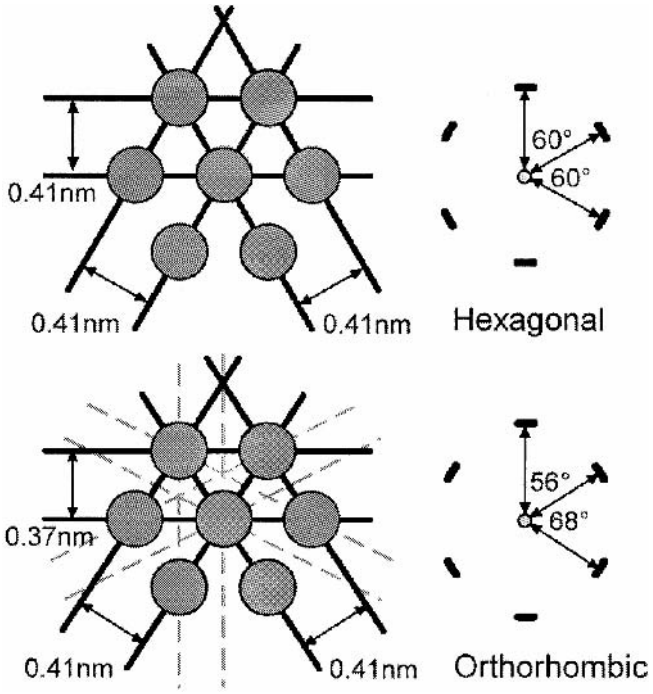

Fig. 1. Schematic drawing of the lateral packing of alkyl chains (black circles) in SC lipid lamellae, and their corresponding ED patterns. In the hexagonal array the spacings between the lattice planes (black lines) that are indicated by the arrows are 0.41 nm. The orthorhombic array is tighter, as a result of which the spacing is smaller in one direction. Note the reciprocal relationship between lattice spacing and distance of a reflection in the ED pattern with respect to the undiffracted central spot.

samples, the AAS was calibrated using Hg standards. The samples were diluted as needed and subsequently 3 ml 2.0 M HCl was added to 2.5 ml of the (diluted) sample. The concentrations (in μg/L) were corrected for the dilution factor. Then the total amount of Hg in the acceptor phase could be calculated.

RESULTS

Human SC Lipid Organization after Treatment with OAz, DAz, and PG

In Fig. 2, ED patterns are shown of control human SC and skin treated with OAz, DAz and PG during 4.5 and 24 hours. The hexagonal packing (gel state) of the lipids is characterized by reflections with a spacing of 0.41 nm. In ED patterns of single crystals, these reflections have an interplanar angle of 60°. A single crystal of the orthorhombic lattice is characterized by 2 pairs of reflections at 0.41 nm and one pair at 0.37 nm (Fig. 2F). All recordings were classified into one of the four categories: orthorhombic (ort), orthorhombic in which the hexagonal packing cannot be excluded (ort*), hexagonal (hex), and patterns that are probably hexagonal (hex*). In this latter group, the presence of 0.37 nm reflections cannot be excluded due to some background scattering. In the class of ort*, many orientations of the orthorhombic lattice may obscure 0.41 nm reflections of a hexagonal lattice, which therefore cannot be excluded (Fig. 2B). Besides these classes of patterns, areas were present that show faint diffraction patterns, consisting of only a broad band centered at 0.46 nm. As it is difficult to decide whether these patterns are only derived from amorphous or α-keratin, or from liquid state lipids as well, these patterns are not included in the graph. The relative distribution of the ED patterns in the four categories are plotted as a function of treatment (Fig. 3). From this graph it is clear that the orthorhombic packing prevailed under all circumstances. However, after 4.5 hours of treatment the hexagonal packing seemed to be decreased in the OAz and DAz treated samples and after 24 hours the hexagonal lattice was no longer observed. The ED patterns from PG treated samples showed a very similar profile compared to the control samples.

Close examination of the types of ED patterns showed that in those derived from OAz and DAz treated samples (Fig. 2C+F) reflections appeared more often as spots (increase of 10–20%) compared to the control and PG treated samples. These spots could be attributed to differently oriented orthorhombic lattices, which indicates that the lipids are present in several orientations as smaller crystallites. Moreover, in ED patterns from samples treated with enhancers the broad band could regularly be observed more clearly in the presence of sharp orthorhombic reflections (Fig. 2E+F).

Penetration of $HgCl_2$ in *Ex Vivo* Human Skin

Figure 4 shows the distribution of Hg in human SC in relation to depth as measured by XRMA on tape-strips. After an initial increase from strip 3 to 6, the amount of Hg decreased significantly with depth. Control and PG treated samples did not differ significantly, while the amount of Hg in OAz treated samples increased significantly ($p < 0.001$). The P/B ratio also showed a decrease with depth, while the background remained relatively constant, which indicates that the penetration profile was related to changes in amount of Hg present on a tape-strip and not to changes in sample thickness.

To study whether the increased amount of Hg in the SC was also related to increased permeation of Hg through the SC, the accumulated amount of Hg in the acceptor phase was measured by AAS. It appeared that the mean amount of Hg in the acceptor phase from control and PG treated samples was 92 μg (0.46 μmol ± sem 0.18) and 152 μg (0.76 μmol ± sem 0.24), respectively, which was not significantly different. Yet, the acceptor phase obtained from the OAz treated skin contained 811 μg (4.04 μmol ± sem 0.96) on average, which was significantly different from the control ($p < 0.001$). This shows that not only the amount of Hg in the SC increases upon treatment with OAz, but the amount that crosses the SC as well (enhancement ratio 8.8).

DISCUSSION

Several studies have reported on the penetration enhancing activity of azones on various compounds applied to mammalian skin. However, their influence on SC lateral lipid organization remains to be elucidated. The mechanisms by which SC barrier function is altered, may vary for each penetration enhancer, although it has often been observed that the intercellular lipid organization of SC is involved.

In this study, we chose OAz and DAz as penetration enhancers, since it is known that these enhancers increase penetration of various compounds (24) and moreover affect the lamellar lipid organization (25). Harrison *et al.* (26) showed by FTIR that Azone® increases fluidity of SC lipids. Engblom

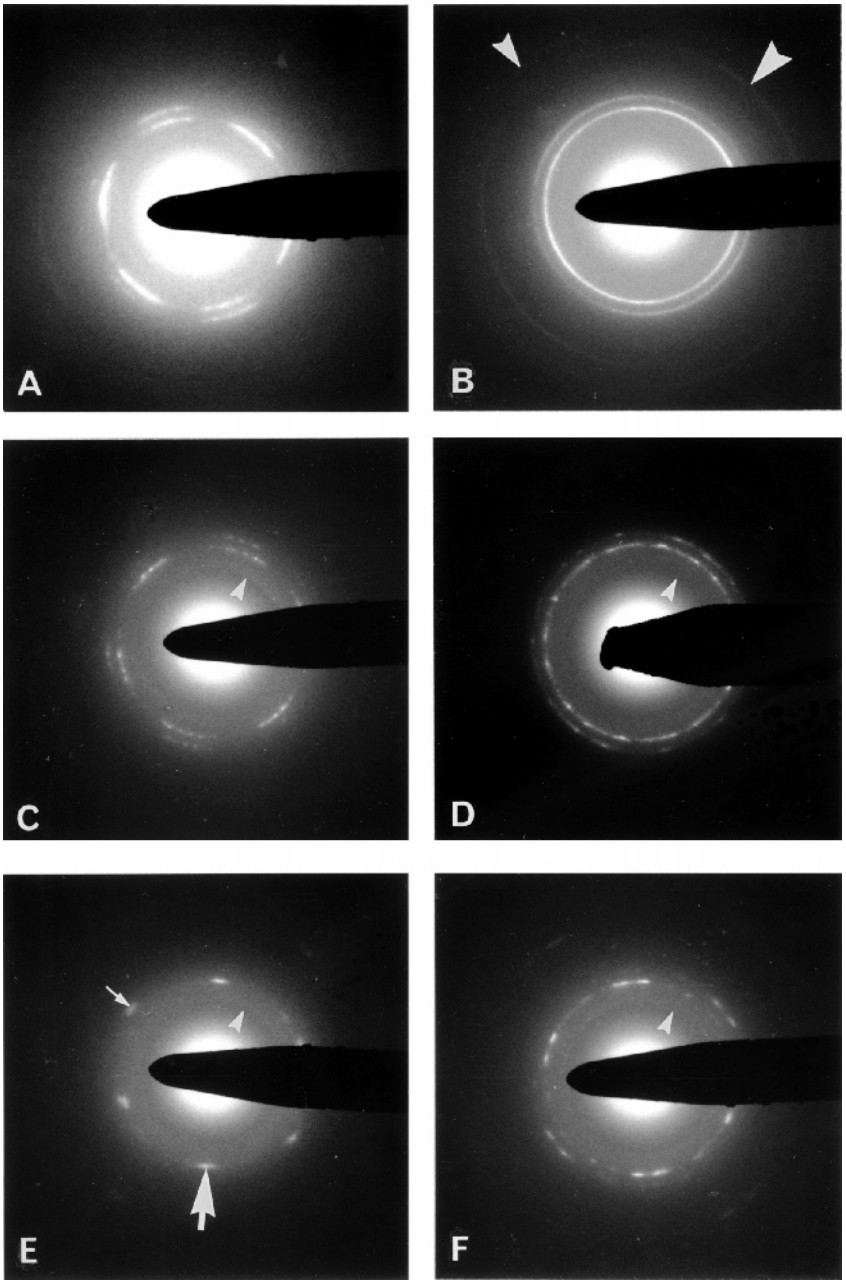

Fig. 2. ED patterns obtained from *ex vivo* human SC. (A) Control sample showing three orientations of the orthorhombic lattice, (B) PG treated sample showing many orientations of the orthorhombic lattice, however, the hexagonal lattice cannot be excluded (the arrowheads indicate the 0.24 nm (big) and 0.22 nm (smaller) reflections), (C) OAz treated sample showing mainly three orientations of the orthorhombic lattice, however, the reflections appear as spots, (D) OAz treated sample showing many random orientations of the orthorhombic lattice in which the hexagonal packing is difficult to exclude, (E) DAz treated sample showing one orientation of the orthorhombic lattice and a faint broad diffraction line (the arrows indicate the 0.40 nm (big) and 0.37 nm (small) reflections), (F) DAz treated sample showing a few orientations of the orthorhombic lattice in slightly different angles. In figures C-F, a faint broad ring with a sharp edge (smallest arrowheads) can be distinguished, which may be attributed to a fluid phase.

et al. (12) showed that Azone® solubilizes cholesterol in cholesterol-fatty acid mixtures and may even deplete the mixture from cholesterol.

Recently, we developed a method to study the local SC lipid organization of both *in vivo* and *ex vivo* (human) SC by ED (22). As in WAXD, information can be obtained about the lateral lipid organization which may be orthorhombic (solid-state) and/or hexagonal (gel-state). An advantage of ED is that overlap of reflections can be avoided when single or a few lipid crystals are selected (20). So far, this has not been possible

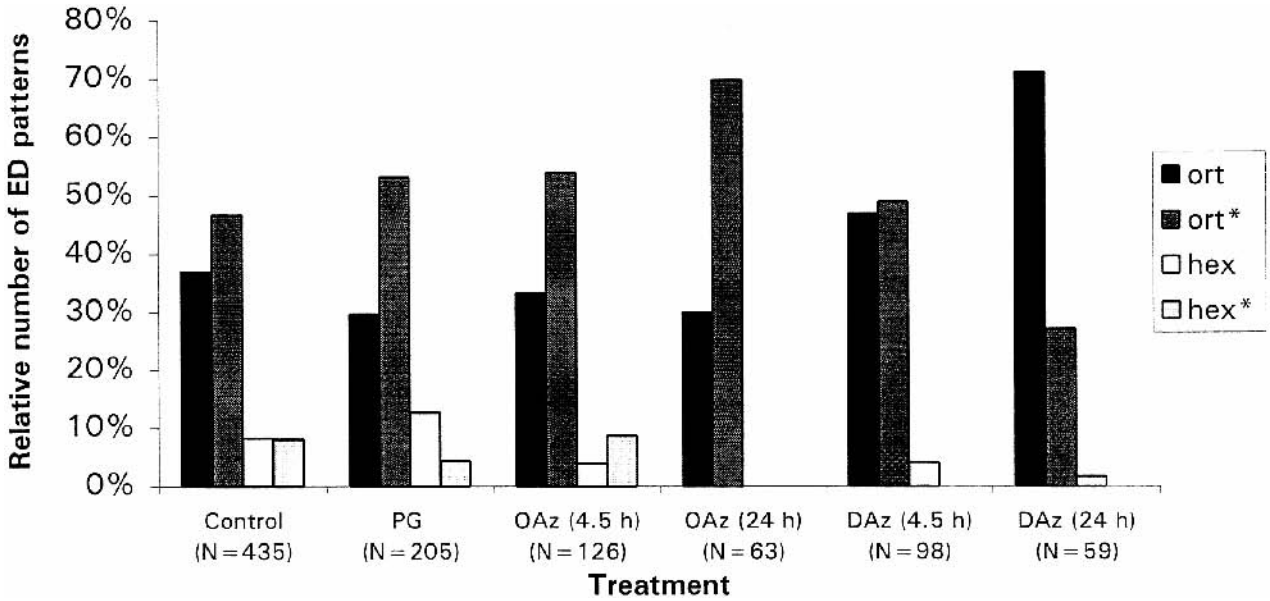

Fig. 3. Distribution of the orthorhombic and hexagonal lattices in *ex vivo* human SC in relation to pretreatment with OAz, DAz, or PG. The percentages of ED patterns recorded per category for a specific treatment are shown along the y-axis (ort, orthorhombic; ort*, orthorhombic, however, the hexagonal lattice cannot be excluded; hex, hexagonal; hex*, probably hexagonal).

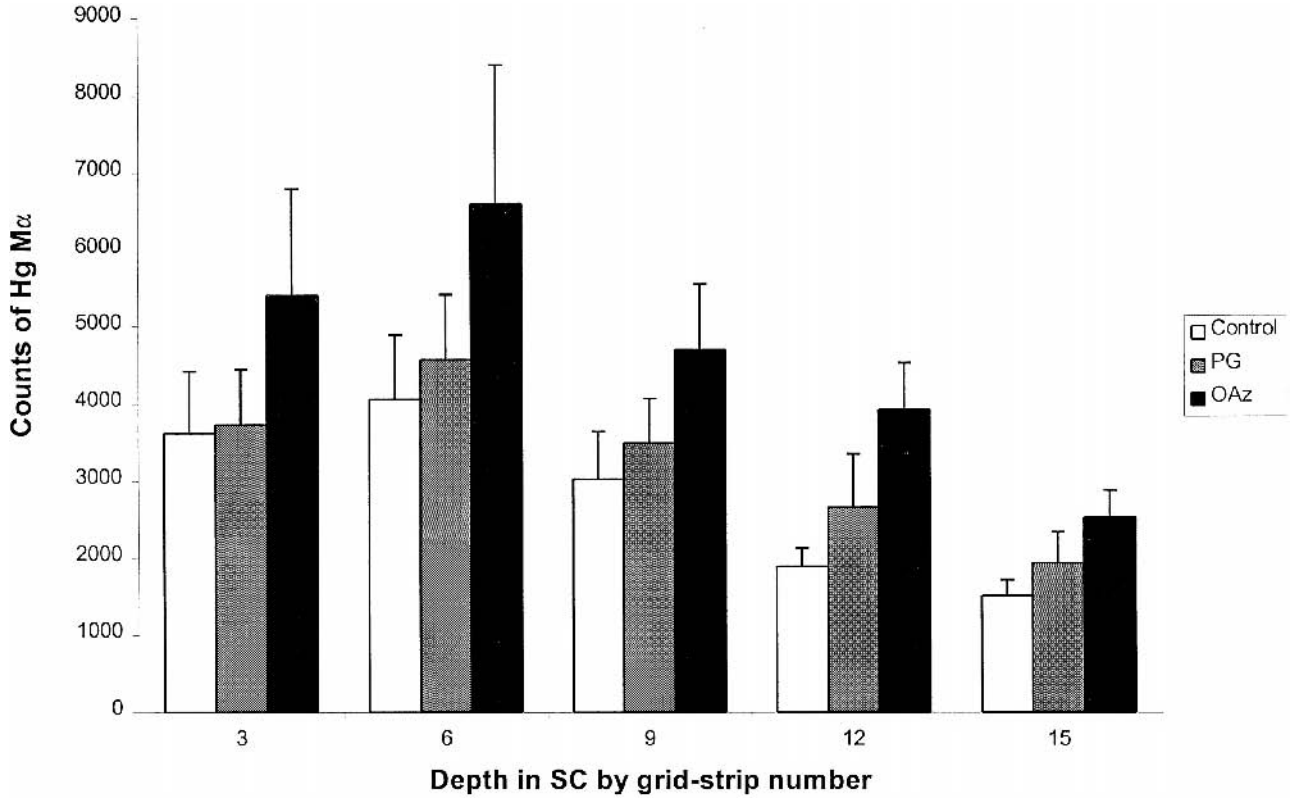

Fig. 4. Penetration profile of Hg across *ex vivo* human SC. The y-axis reflects the X-ray counts produced by electrons that underwent a transition from the N to the M shell of the Hg atom (Mα lines). The bars represent an average of the XRMA measurements in 5 experiments performed in duplo and the error bar indicates the 95% confidence interval (2* s.e.m.). The control and PG treated samples do not differ significantly, whereas the OAz treated samples showed a significant increase in Hg penetration.

by WAXD, because bulk quantities of SC are required. As ED appeared to be able to monitor SC lipid packing accurately, it was hypothesized that supplementary information could be obtained on the mechanism by which penetration enhancers may affect the lateral SC lipid organization.

In this study, it was found using ED that the main lateral lipid organization remained orthorhombic in PG, OAz and DAz treated skin, like in control samples. No transition from orthorhombic to hexagonal packing was observed, in fact, rather the opposite seemed to occur. The hexagonal lattice was detected less frequently in the OAz and DAz treated samples, whereas in the PG treated samples its occurence was comparable to control samples. As the hexagonal lattice was mainly detected by ED in superficial SC layers (11), which might be related to the presence of sebum lipids (27), it can be hypothesized that a possible removal of the sebum by treatment with enhancers might remove the hexagonal lateral packing.

Furthermore, the shape of the ED patterns seemed to be changed; using the same spotsize for diffraction, the reflections appeared more often as spots that could be ascribed to differently oriented crystals. This indicates that the crystal size has been reduced and thus the number of grain boundaries must be increased, which might be caused by a disruption of the endogeneous lipid lamellar stacking by the enhancers, as has been noticed by Bouwstra *et al.* (13). From the present study it is clear that both OAz and DAz did not induce an orthorhombic to hexagonal phase transition.

The detection of a transition from an orthorhombic to a liquid lateral packing, however, is more difficult using ED or WAXD, since the broad reflection from amorphous- or α-keratin obscures the reflection characteristic for lipids in a liquid phase. Furthermore, PG seems to interact with intracellular keratin and/or with the corneocyte envelope by which the reflection at 0.46 nm in a WAXD pattern becomes more pronounced (13). For these reasons, small amounts of lipids in a liquid phase may not be detectable. Moreover, the relation between peak intensity and amount of lipid in a crystalline phase is difficult to determine, since a reduction in peak intensity might be due to a crystalline-liquid transition or a smaller amount of ordered lipid material. Yet, a broad reflection could be observed in ED patterns of enhancer treated samples that may be attributed to liquid state lipids and/or phase separated enhancers. Thus treatment with OAz or DAz might induce the formation of a liquid phase, whereas areas containing unaltered endogeneous lipids remain present to a lesser extent.

From other enhancers, like terpenes (28) and oleic acid (29) it is known that although these enhancers change SC permeability, the endogeneous lipids also maintain their lateral packing at least locally. Cornwell *et al.* (28) suggested that apparently the Van der Waals forces of attraction between the alkyl chains are strong enough to prevent intercalation of significant numbers of the enhancer molecules between the SC lipids within the lamellae. However, this does not mean that the lamellar organization is still intact, as was shown for the azones (9,13). Instead of mixing with endogeneous lipids, the enhancers may be present in a separate phase, meaning that a fluid phase of enhancer-rich domains co-exists in the SC lipid matrix. This is shown to be the case for oleic acid using FTIR (29), at least at certain mole ratios (18). Tanojo *et al.* (4) showed by FFEM that oleic acid affected the lamellar lipid organization as well. Phase separation was also suggested as a mechanism of enhancement for terpenes. Using SAXD, some of the terpenes were shown to affect the lamellar stacking (3), whereas WAXD did not provide direct evidence that terpenes disrupted the lateral packing (28). In a similar way, ED only provided indirect evidence for a change in lipid organization induced by the azones, while changes in the lamellar arrangement have been observed (9).

From the Hg penetration studies, it is clear that OAz influenced SC permeability and XRMA reveals that Hg distribution in SC changes. In all cases there was an initial increase of Hg in the tape-strips from strip 3 to 6. A possible explanation for this finding may be that Hg accumulates in the apical corneocytes that act as a reservoir. This has also been observed in transverse SC sections by Bodde *et al.* (21). From strip 6 to 15 there was a significant decrease in the amount of Hg on tape-strips. It was found that this distribution for PG treated samples was not significantly different from the control, whereas in OAz treated samples the distribution profile of Hg had increased significantly. Moreover, the accumulative amount of Hg in the acceptor phase from OAz treated samples showed a significant increase compared to control and PG treated samples. As Hg has been shown to cross the SC via the intercellular pathway, these findings indicate that changes in lipid organization may be induced by OAz which increased SC permeability. Langner and Hui (16) showed that membrane permeability to ions increases dramatically close to phase transition temperatures, due to the formation of grain boundaries that allow ions to cross the bilayers. Similar routes through hydrophilic microchannels are proposed for ions crossing the SC lipid lamellae (30). Possibly, OAz induces phase separation, either by (partly) disturbing the endogeneous lipid ordering by which mismatches of crystallites occur or by inducing phase separation due to the formation of enhancer rich areas. By this, permeability of the SC to Hg may be increased. The same mechanism may be true for DAz.

In future, we will therefore study isolated human SC lipids by ED in order to investigate whether a fluid phase can be detected in SC lamellae. An advantage of such models may be that if such a liquid phase is present, it will be detectable more clearly in the absence of keratin filaments.

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