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Changes in lipid organisation of the skin barrier by *N*-alkyl-azocycloheptanones: a visualisation and X-ray diffraction study

J.A. Bouwstra*, M.A. Salomons-de Vries, B.A.I. van den Bergh, G.S. Gooris

Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, Leiden University, P.Box 9502, 2300 RA Leiden, Netherlands

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1. Introduction

The main function of the outermost layer of the skin, the stratum corneum, is to provide a barrier for evaporation of water from the underlying viable cell layers and to protect the organism against undesirable substances from the environment. The stratum corneum consists of dead flattened cells embedded in a lamellar lipid matrix. For most substances the main barrier resides in the intercellular lipid domains. The major lipid classes found in the stratum corneum are long-chain ceramides, long-chain free fatty acids and cholesterol (Schurer and Elias, 1992). Successful percutaneous delivery of drugs relies strongly on an adequate reduction of the barrier properties of the stratum corneum, which in principle can be achieved by penetration enhancers. Among the extensively studied penetration enhancers are oleic acid (Green and Hadgraft, 1987; Ongpipattanakul et al., 1991; Tanojo et al., 1993;), oleyl surfactants, terpenes (Williams and Barry, 1991a,b; Cornwell et al., 1993), alcohols, DMSO, azone (Morimoto et al., 1986; Sheth et al., 1986; Wotton et al., 1985; Wiechers et al., 1990; Ruland and Kreuter, 1992; Schuckler and Lee, 1992) and analogues of azone (Hadgraft, 1993; Michniak et al., 1993a,b).

The structure of stratum corneum has been studied by various methods, such as differential thermal analysis, small and wide angle X-ray diffraction and electron microscopy. One of the methods to visualize the ultrastructure of stratum corneum is electron microscopy in combination with ruthenium tetroxide (RuO_4) postfixation. The use of RuO_4 allows the visualization of the lipid bilayers. Since mainly saturated lipids are present in the stratum corneum this cannot be achieved by using osmium tetroxide (OsO_4) as fixing agent. In the past several papers appeared using this method, especially in studying the mor-

* Corresponding address. Tel.: +31 71 274208; fax: +31 71 274277.

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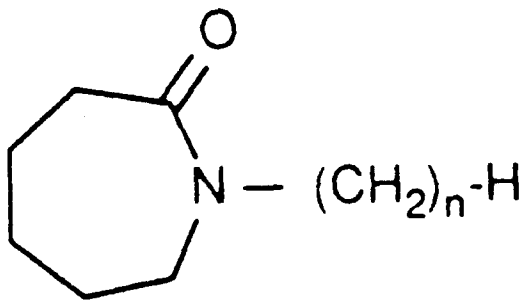

Fig. 1. The molecular structure of the series of *N*-alkyl-azones. The number of C-atoms in the hydrocarbon chain is denoted by *n*.

phology of diseased skin (Hou et al., 1991; Gadially et al., 1992; Fartasch et al., 1992) and in studying the correlation between barrier impairment and changes in lipid organisation (Holleran et al., 1991; Man et al., 1993).

In recent studies on lipid perturbation small-angle X-ray diffraction (Bouwstra et al., 1992a), differential thermal analysis (Bouwstra et al., 1989), freeze fracture electron microscopy and deuterium NMR (Bezema et al., 1993) were used in order to investigate the lipid pertubation induced by a series of *N*-alkyl-azocycloheptan-2-ones (*N*-alkyl-azones) including azone. The structure of the *N*-alkyl-azones is given in Fig. 1. In this series the hydrocarbon chain varied systematically between hexyl and hexadecyl. Furthermore oleyl-azone was included in the studies. This series allowed a systematic investigation of the relationship between the molecular structure of the azone derivatives and their lipid-barrier perturbation properties.

In the study described in this paper wide angle X-ray diffraction and electron microscopy in combination with RuO_4 has been used to further elucidate the mechanisms involved in increased absorption of drugs by this series of *N*-alkyl azones.

2. Material and methods

2.1. Isolation of stratum corneum and treatment with alkyl-azones

Abdomen or mammary skin obtained from the hospital was dermatomed to a thickness of 200 μm. Then the skin was put on a filter paper at 4°C overnight that was soaked in a buffered 0.1% trypsin solution (phosphate buffered saline solution, pH 7.4). Then the stratum corneum was incubated for 1 h at 37°C. The stratum corneum was mechanically separated from the epidermis and treated with an 0.1% trypsin inhibitor in PBS, pH 7.4. Finally the stratum corneum was stored in a desiccator over silica gel. Before use the stratum corneum was hydrated over 27% NaBr salt solution.

Treatment with PG or 0.15 M *N*-alkyl-azones in PG was carried out for a period of 24 h. The *N*-alkyl-azones studied were hexyl-azone, octyl-azone, decyl-azone, dodecyl-azone, hexadecyl-azone and oleyl-azone.

2.2. Wide angle X-ray diffraction

Measurements were carried out at the synchrotron radiation source in Daresbury using station 7.2. A description of the experimental set-up is given elsewhere (Bouwstra et al., 1992a). The diffraction patterns were recorded on a photographic film which allows two dimensional detection. The position of the diffraction ring is denoted by the corresponding spacing (d), which is defined as $d = \lambda/(2d \sin \theta)$. λ is the wavelength of the X-rays and θ is the scattering angle.

2.3. Freeze substitution electron microscopy in combination with RuO_4

The tissue was postfixed with 5% glutaraldehyde and then with 0.2% w/w RuO_4 in a cacodylate buffer (pH 6.8). After postfixation the stratum corneum was quenched using a metal mirror in combination with the plunching method (KF 80, Reichert Jung Austria). Then freeze substitution was carried out in a CS-Auto (Reichert-Jung, Austria) at a temperature of -90°C for 48 h. The substitution medium was pure methanol containing 1% w/w OsO_4, 0.5% uranyl-acetate and 3% glutaraldehyde. After freeze substitution, the temperature was raised to -45°C, and the

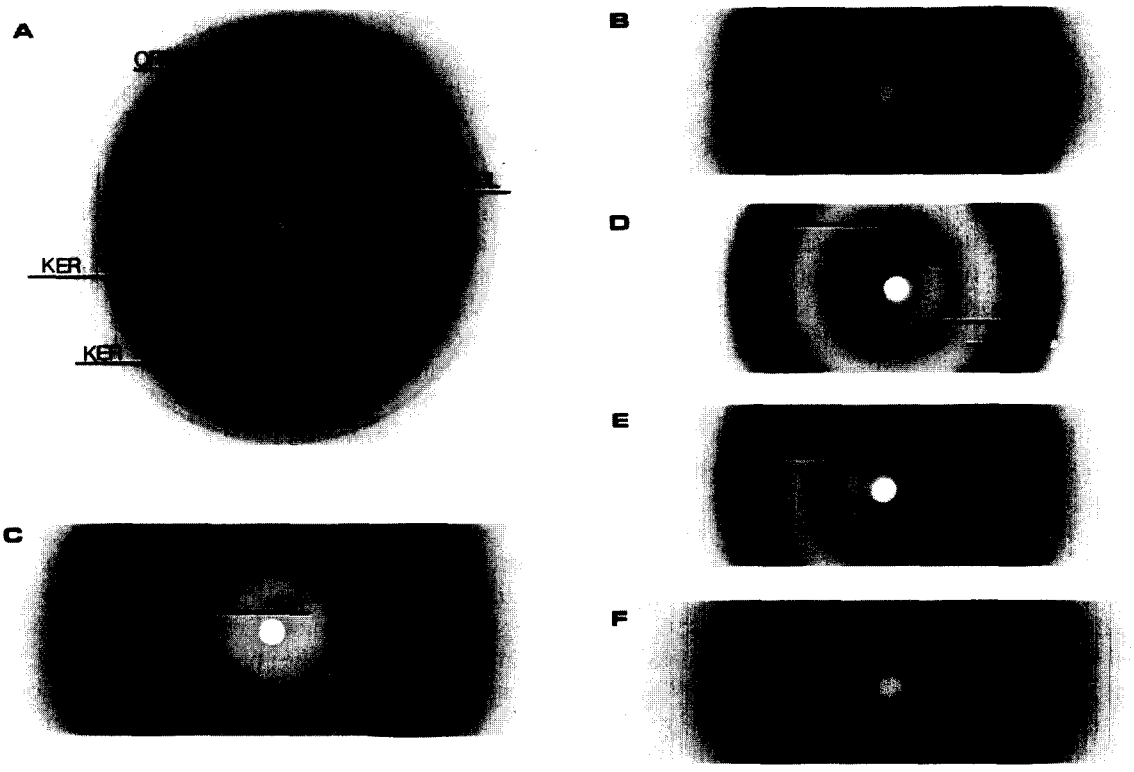

Fig. 2. The WAXD pattern of human stratum corneum, (A) untreated, (B) PG treated stratum corneum, (C) chloroform/methanol-extracted stratum corneum treated by PG, and (D) stratum corneum treated by hexyl-azone, octyl-azone in PG, dodecyl-azone in PG, hexadecyl-azone in PG and oleyl-azone in PG.

methanol was replaced gradually by lowicryl HM20. The polymerization was carried out by exposure of the monomer mixture to UV light. The samples were sectioned and visualized in an 201 analytical electron microscope (AEM) or a 410 AEM (Phillips, The Netherlands).

3. Results

3.1. Wide angle X-ray diffraction

The WAXD patterns are shown in Fig. 2. The pattern of untreated stratum corneum displayed two very strong reflections at 0.415 nm and 0.375 nm, respectively, and a weak reflection at 0.241 nm, see Fig. 2A. The 0.415 and 0.378 nm reflections reveal the presence of an orthorhombic structure (Bouwstra et al., 1992b), while the 0.415 nm and 0.241 nm reflections strongly indicate the presence of an hexagonal lateral packing. Occasionally weak reflections based on crystalline cholesterol were present in the diffraction pattern. Furthermore two broad strong diffraction rings were present at 0.96 and 0.46 nm spacings indicative for soft keratin. The presence of α-keratin cannot be fully excluded, since the diffraction pattern of stratum corneum oriented parallel to the primary beam revealed a slightly non-isotropic 0.96 nm ring (Garcon et al., 1991; Bouwstra et al., 1992b).

After treatment with PG an additional high intensity ring at 0.46 nm spacing was observed in the wide angle X-ray diffraction pattern, the appearance of the ring and its high intensity was different from the 0.46 nm ring in the pattern of untreated stratum corneum, as displayed in Fig. 2B. The 0.46 nm strong diffraction ring was not present in the diffraction pattern of PG alone and could therefore not be ascribed to the solvent

only. It might be that a substantial amount of PG penetrates into the corneocytes after which PG forms a complex with keratin or that PG interacts with the corneocyte envelope. Both events may result in the additional diffuse strong diffraction ring at 0.46 nm, at approximately the same spacing at which one of the amorphous soft keratin reflections is located (see above). To check these hypotheses chloroform/methanol extracted human stratum corneum, which does not contain lipids except for those linked to the corneocyte envelope, was treated with propylene glycol and the wide angle diffraction pattern was measured. This diffraction pattern is shown in Fig. 2C. Again a strong 0.46 nm ring was observed, similar to that present in the diffraction pattern of PG treated human stratum corneum (see Fig. 2B). The similarity in appearance of the 0.46 nm ring between PG treated stratum corneum and PG treated chloroform/methanol-extracted SC resulted in the conclusion that the strong 0.46 nm ring is mainly due to this PG-keratin or a PG-corneocyte envelope interaction and not to interactions between lipids and PG. However it cannot be ruled out that a small part of the lipids might be extracted from the lipid lamellar phases without changing their lamellar periodicity and form a mixture with PG, or that a small amount of PG is intercalated in the hydrocarbon regions of the lipid bilayers in the stratum corneum, leading to a swelling of the lateral packing of a part of the lipids. Both events, however, should not lead to a change in periodicity of the lamellar phases (Bouwstra et al., 1992a) and therefore are expected to have a minor influence on the intensity of the 0.46 nm diffraction ring.

Apart from the additional ring no changes in the diffraction pattern were found after treatment with alkyl-azones and PG, see Fig. 2(D,E) except for the disappearance of the higher order reflections based on the lamellar phases. This is in agreement with the findings obtained by SAXD (Bouwstra et al., 1992a), that revealed that after treatment with *N*-alkyl-azones with a longer hydrocarbon chain than six C atoms, the various diffraction peaks based on the lamellar phases disappeared which is indicative for a disordering of the lamellae. The diffraction rings based on the orthorhombic and hexagonal hydrocarbon packing were still present and the corresponding spacings did not change significantly. No conclusion can be drawn about changes in the relative amount of lipids packed in the orthorhombic and hexagonal sublattices, since PG is an excellent X-ray absorbance, that reduces the intensity of the reflections. In other words a part of the orthorhombic phase might have been disappeared but this can only be detected using quantitative measurements. This was not possible with the diffraction patterns available.

3.2. Electron microscopy in combination with RuO_4 postfixation

Electron micrographs of untreated stratum corneum are shown in Fig. 3. In large areas of the intercellular spaces the lipid lamellae (LL) repeating units were characterized by two broad translucent bands and one narrow translucent band between which electron dense bands were located. The pattern was similar to that found in mouse (Hou et al., 1991) and pig (Swartzendruber, 1992) stratum corneum. The periodicity of the broad-broad-narrow unit was approximately 13 nm,

Fig. 3. Electron micrographs of RuO_4 post-fixed stratum corneum of untreated stratum corneum. The corneocytes (C), lipid bilayer regions (LL) and desmosomes are illustrated. In the inset of the Figure the broad-broad-narrow sequence of the electron trans-lucent bands, which form the structural unit, is displayed.

which corresponded to the periodicity of one of the 2 lamellar phases found by small angle X-ray diffraction (Bouwstra et al., 1991). The broad-narrow-broad structural unit most likely consists of two or three bilayers. From model calculations based on the intensities of various X-ray diffraction peaks the existence of two or three bilayers in one structural unit has also been made very likely (Löfgren and Pascher, 1977; Bouwstra et al., 1994). In some regions the broad-narrow-broad sequence was not present, which might be caused by (i) an artefact due to poor penetration of RuO_4 in the stratum corneum, (ii) a non-perpendicular sectioning direction to the lamellae, or (iii) another lipid lamellar phase that might be present. However, the spacings in these regions did not correspond with the 6.4 nm phase found using small angle X-ray diffraction. Furthermore intercellular grey regions were found. In these regions lipid bilayers could only be visualized along the cell boundaries. In the intercellular spaces also desmosomes that interrupt the lipid bilayers were present. Inside the corneocytes the keratin filaments could be visualized. These filaments were aligned mainly parallel to the stratum corneum surface.

After treatment with PG or hexyl-azone in PG, no significant changes in the lipid lamellar stacking was observed, see Fig. 4(A,B). The broad-narrow-broad lipid bilayer sequence was still present and the periodicity of this structural unit did not change compared to the pattern observed in untreated stratum corneum. This is in agreement with the results obtained with SAXD (Bouwstra et al., 1992a). Treatment with octadecyl-azone and dodecyl-azone in PG resulted in a loss of the lamellar stacking in large regions in the stratum corneum as displayed in Fig. 4(C,D). In these regions the single lamellae (SL) consist of two broad electron lucent bands between which a narrow electron dense band is located. This change in appearance strongly indicates that besides a loss in lamellar stacking also a rearrangement of the lipids inside the broad-narrow-broad sequence of the structure occured. This might include e.g. a change in the interdigitation of the lipids, a change in the lipid headgroup arrangement, or a change in the lateral packing. A change in lipid lateral packing was not observed by WAXD, but in case of WAXD no conclusion could be drawn about changes in relative intensities of the diffraction rings and thus about the relative amounts of lipids forming a hexagonal or a crystalline lateral packing. Furthermore, if a small amount of lipids has been transformed to a liquid lateral packing this change cannot be detected by WAXD, since the liquid lateral packing reflection at 0.46 nm is obscured by one of the keratin reflections or by the broad reflection observed after PG treatment (see above). Close to cell boundaries intercellular lipid bilayers (L) were often still present. One clear difference was observed between treatment with octyl-azone and dodecyl-azone. After treatment with the former frequently stratum corneum regions were observed, in which the intercellular lipid organisation was similar to that of the non-treated control, while after treatment with dodecyl-azone regions with an intact lamellar stacking similar to that in the control were only occasionally present.

Treatment with hexadecyl-azone in PG resulted also in a disordering of the lamellar stacks. However, the appearance of the individual lamellae was different from that observed after treatment with octyl-azone and dodecyl-azone. After treatment with hexadecyl-azone single lamellae consisting of just one electron translucent band were found as displayed in Fig. 4E, indicating a slightly different mechanism of lipid pertubation than using dodecyl-azone.

After treatment with oleyl-azone (Fig. 4F) almost no bilayers were present in the intercellular regions, indicating that most of the lipids were dissolved. Even along the corneocyte envelope no lipid bilayers could hardly be detected, strongly suggesting that oleyl-azone disrupts also the corneocyte lipid envelope. The corneocyte envelope itself also displayed a different appearance. It is not a straight boundary, but in those parts of the intercellular regions at which no desmosomes are present the distance between the cells is enlarged.

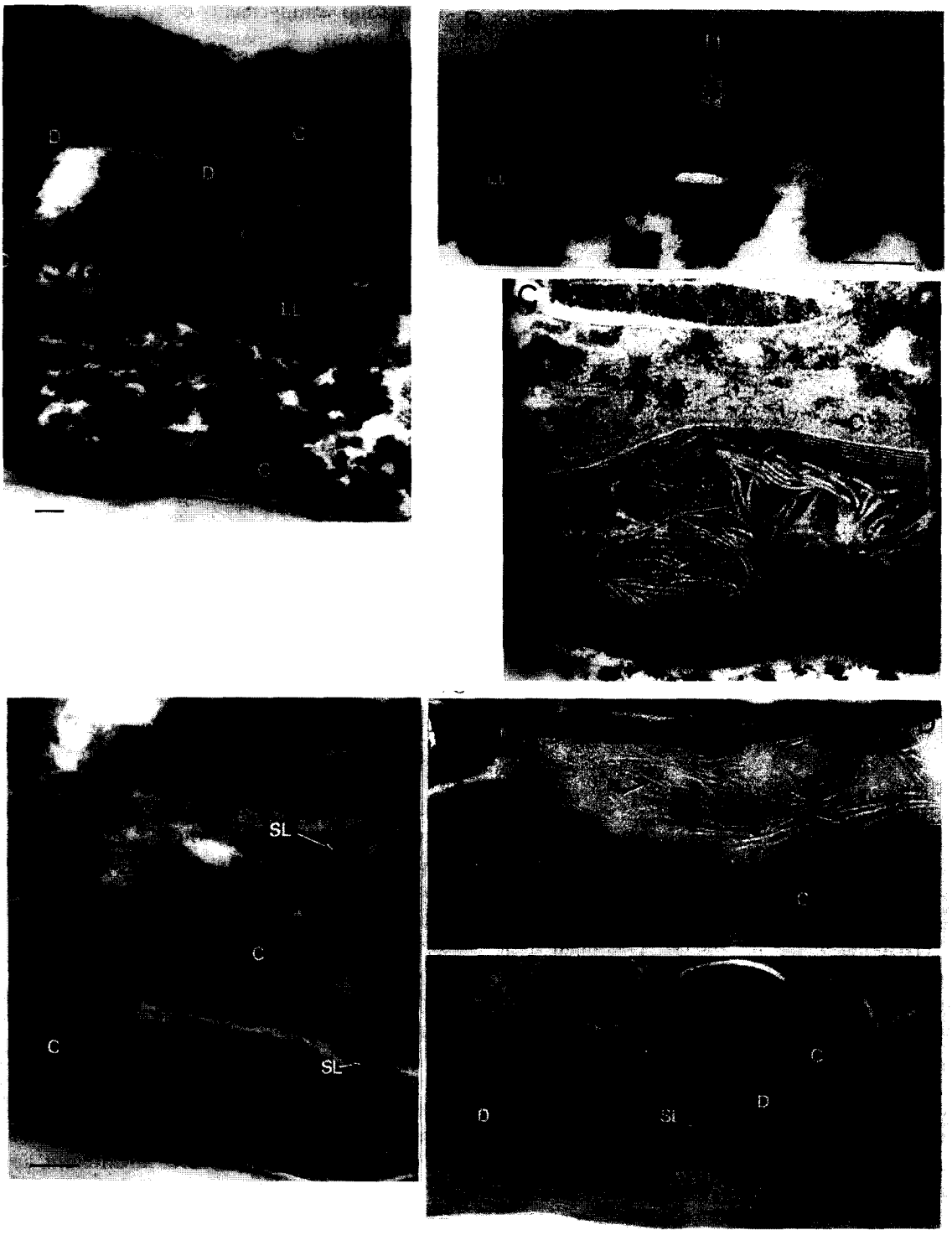

Fig. 4. Electron micrograph of (A) PG-treated stratum corneum, (B) hexyl-azone in PG treated stratum corneum, (C) octyl-azone in PG treated stratum corneum, (D) dodecyl-azone in PG treated stratum corneum, (E) hexadecyl-azone in PG treated stratum corneum and (F) oleyl-azone in PG treated stratum corneum. The corneocytes (C), lipid bilayers regions (LL), desmosomes (D) and single lamellae (SL) are illustrated. Bar = 100 nm.

4. Discussion

The unperturbed lipid organisation of human stratum corneum is characterized by a series of lamellae in the intercellular regions aligned mainly parallel to the stratum corneum surface. These lamellae are most probably interconnected by strong hydrogen bonds, which are most likely present between the ceramide head groups, but free fatty acids and cholesterol might also be involved in this network of hydrogen bondings. These hydrogen bondings and the presence of crystalline and hexagonal lateral packings are most probably very important for the barrier function of the skin.

In this study it was shown that PG does not change the ordering and periodicity of the lamellae in human stratum corneum as determined by electron microscopy. This was also found in previous studies using freeze fracture electron microscopy (Bezema et al., 1993) and small angle X-ray diffraction (Bouwstra et al., 1992a). The presence of a strong reflection with a spacing of 0.46 nm, which was absent in untreated stratum corneum is due to either PG which is absorbed in the corneocytes or to PG that interacts with the cornified envelope, since chloroform/methanol treated stratum corneum revealed a similar strong reflection at 0.46 nm spacing. The results obtained in this study are also confirmed by another study: after treatment with PG as observed by differential scanning calorimetry (Bouwstra et al., 1989) the protein peak at 107°C disappeared, which is also a strong indication that PG interacts with proteins/keratin. That the presence of the 0.46 nm reflection is not based on a phase separation between a mixture of PG and lipids on the one hand and organized lipids on the other hand is confirmed by studies using high performance thin layer chromatography (HPTLC). Using HPTLC we could not find any indications that PG extracts lipids and thus mix with lipids from the stratum corneum. An alternative mechanism to explain the presence of the 0.46 nm reflection is intercalation of PG in the hydrocarbon regions of the lamellae without swelling of the lamellae. A small amount of PG might be present in the lamellar phases, since PG decreased the lipid transition temperatures as observed by differential scanning calorimetry (Bouwstra et al., 1989). However, it is unlikely that large amounts of PG were absorbed in these phases without changing the lamellar periodicity.

The mechanisms by which *N*-alkyl-azones interact with human stratum corneum and change the barrier properties is very complicated and depends on the alkyl chain length. The influence of hexyl-azone in PG on the structure of human stratum corneum was similar to that of PG. Using hexyl-azone no increased transport of drugs was found either (Hoogstraate et al., 1991), which correlates excellent with the data presented in this paper. However, when stratum corneum was pretreated with octyl-azone in PG, the electron microscopic studies revealed disordering of the lamellae, which confirmed results of earlier studies using SAXD (Bouwstra et al., 1992a). The electron microscopic studies clearly displayed that still lamellae are present in the intercellular regions in the stratum corneum and that disordering was more easily induced in the center of the intercellular spaces than close to cell boundaries, where lamellae were still present. Furthermore the micrographs revealed that the appearance of the lamellae after treatment with octyl- and dodecyl-azone in PG differed from that of untreated or PG treated stratum corneum. The structural units consisting of a sequence of broad-narrow-broad-translucent bands were replaced by disordered lamellae that consist of only two broad trans-lucent bands between which one narrow electron dense band was located. Stacking of the latter should lead to a sequence of only broad electron trans-lucent bands between which electron dense bands would be located. Although treatment with hexadecyl-azone also resulted in a disordering of the lamellae as revealed by SAXD (Bouwstra et al., 1992a), the appearance of the single lamellae was different as can be inferred from the results presented in this paper. These small differences in membrane appearance after pretreatment of e.g. dodecyl-azone and hexadecyl-azone is not yet understood. After treatment with oleyl-azone almost no single lamellae were left in the intercellular spaces. It seems that most of the lipid bilayers have been disrupted by this enhancer. Further-

more it was striking that even the corneocyte envelope changed in appearance and that the lipid envelope could not be visualized. It is remarkable that although the stacking, i.e. the cohesion between the various lamellae, completely or partly disappeared, the individual lamellae, although changed in appearance, are still present. It seems that as soon as *N*-alkyl-azones show an affinity towards bilayer regions (octyl-azone and longer *N*-alkyl-azones) these *N*-alkyl-azones disturb the cohesion between the lamellae. An important part of this cohesion may be formed by the inter-lamellar hydrogen bondings, which seems to be disrupted by these enhancers. Furthermore, it is very likely that the enhancer phase separates from the endogeneous lipids present in the bilayers, since the 0.415 and 0.375 nm diffraction rings were still present in the diffraction pattern after enhancer treatment and it is expected that a large amount of enhancer is present in the skin (Schuckler and Lee, 1992).

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