ELSEVIER

Biochimica et Biophysica Acta 1511 (2001) 244–254

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

The influence of two azones and sebaceous lipids on the lateral organization of lipids isolated from human stratum corneum

G.S.K. Pilgram [a,b,*], J. van der Meulen [a], G.S. Gooris [b], H.K. Koerten [a], J.A. Bouwstra [b]

[a] *Center for Electron Microscopy, Leiden University Medical Center, Leiden, The Netherlands*
[b] *Department of Pharmaceutical Technology, Leiden/Amsterdam Center for Drug Research, Leiden University, P.O. Box 9502, 2300 RA Leiden, The Netherlands*

Received 5 July 2000; received in revised form 4 January 2001; accepted 11 January 2001

Abstract

The main problem with topical application of compounds to administer drugs to and regulate drug levels in a human body, is the barrier formed by the intercellular lipid matrix of the stratum corneum (SC). In a search for possibilities to overcome this barrier function, a good understanding of the organization and phase behavior of these lipids is required. SC lipid model studies especially provide a wealth of information with respect to the lipid organization and the importance of certain subclasses of lipids for the structure. Previously, we have shown that electron diffraction (ED) provides detailed information on the lateral lipid packing in both intact SC (G.S.K. Pilgram et al., J. Invest. Dermatol. 113 (1999) 403) and SC lipid models (G.S.K. Pilgram et al., J. Lipid Res. 39 (1998) 1669). In the present study, we used ED to examine the influence of two azones and sebaceous lipids on the lateral phase behavior of lipids isolated from human SC. We established that human SC lipids are arranged in an orthorhombic packing pattern. Upon mixing with the two enhancers the orthorhombic packing pattern was still observed; however, an additional fluid phase became more apparent. In mixtures with sebaceous lipids, the presence of the hexagonal lattice increased. These findings provide a basis for the mechanism by which these enhancers and sebaceous lipids interact with human SC lipids.

Keywords: Electron diffraction; Penetration enhancer; Phase behavior; Sebum; (Skin)

1. Introduction

Since the 1970s the advantage has been recognized to administer drugs to the body by topical application to the skin [3–5]. A problem that has been faced since then, is the low penetration of most compounds through the outermost layer of the skin, the stratum corneum (SC). Therefore, a lot of effort has been put in the development of (trans)dermal drug delivery systems to overcome the SC barrier locally and temporary [6–8]. The SC consists of terminally differentiated keratinocytes, referred to as corneocytes, embedded in a lipid-rich intercellular matrix. The main constituents of this matrix are ceramides (CER), cholesterol (CHOL) and long-chain free fatty acids

Abbreviations: CER, ceramides; CHOL, cholesterol; DAz, dodecyl azone; ED, electron diffraction; FFA, free fatty acids; FTIR, Fourier-transformed infrared spectroscopy; OAz, oleyl azone; SAXD, small-angle X-ray diffraction; SC, stratum corneum; TEM, transmission electron microscope; WAXD, wide-angle X-ray diffraction

* Corresponding author. Fax: +31-71-527-4565;
E-mail: g.s.k.pilgram@bio.uu.nl

0005-2736/01/$ – see front matter
PII: S0005-2736(01)00271-1

(FFA) [9–11]. These lipids, which are organized in crystalline lamellae at room temperature, play a key role in the barrier function of human skin. As the intercellular matrix forms a continuous pathway by which most compounds penetrate the SC, the organization of the lipids in this matrix has drawn a lot of attention. One of the possibilities to increase the permeability of the SC to certain compounds is the (co)application of penetration enhancers. Such studies on drug delivery in the SC mainly focus on two topics: (i) the selection of possible candidates that may act as enhancers and examination of their enhancing activity by flux studies [12,13], and (ii) elucidation of the mechanism of action of penetration enhancers (e.g., interaction with SC lipids) [14–16]. The present study is focused on this second topic.

It has been observed that the permeability of phospholipid membranes and SC lipid lamellae increases considerably during phase transitions induced by raising the temperature [17–20]. Based on these studies, several mechanisms have been postulated that explain the changes in permeability of such lipid layers. One mechanism is the transformation of lipid phases, e.g., crystalline to gel or gel to fluid phases, as a result of which lattice density is reduced and alkyl chain mobility increases. However, this fluidization does not explain the sudden increase in permeability at the phase transition temperature. As in lipid mixtures not all lipids undergo a phase transition simultaneously, different phases may coexist. Therefore, a second mechanism postulated is phase separation, by which the coexistence of phases leads to the formation of grain boundaries. At these boundaries additional penetration pathways are created, which increase the permeability even more than the fluidization of lipids during the phase transition [21–23]. Finally, a third mechanism, occurring in the intercellular matrix of SC, includes disordering of the stacking of lipid lamellae [24,25].

Penetration enhancers are developed to induce similar changes in the intercellular lipids in order to reduce the SC barrier temporary and improve drug penetration. As penetration of drugs also depends on the characteristics of the compound itself, like charge, size, shape and lipophilicity [26,27], the optimal enhancer should be selected in combination with a certain drug. Therefore, it is important to understand the mechanism by which enhancers affect SC permeability.

Wide angle X-ray diffraction (WAXD), Fourier transform infrared spectroscopy (FTIR) and electron diffraction (ED) studies have shown that SC lipids organize in hexagonal (gel phase) and orthorhombic (crystalline phase) lattices [1,28–30]. In intact murine skin a fluid phase has been detected as well, using WAXD [31]. The presence of this phase in human SC has not yet been confirmed, because the reflection of the fluid phase may be obscured by reflections of α-keratin in the diffraction patterns [14,28]. However, FTIR studies revealed a subpopulation of lipids in a liquid phase [30,32].

It is known that azones, like dodecyl azone (DAz) and oleyl azone (OAz) increase SC permeability and affect the lamellar organization of intercellular SC lipids [24,33]. Recently, we studied the influence of these Azones on the lateral packing of intact human SC using ED [34]. Due to the presence of keratin it was difficult to clearly establish the presence of a fluid phase. However, our studies revealed an increase in the number of grain boundaries in the lipid domains, while the orthorhombic packing remained dominantly present. In earlier ED studies, we already showed that the lipid packing of untreated human SC is predominantly orthorhombic; however, in the outer part of the SC the hexagonal lattice is present as well [1]. There are indications that sebum may be responsible for the alterations in the lipid organization in superficial SC layers [29,35,36]. Sebum is excreted on the surface of human skin and consists of triglycerides, (mainly short-chain, unsaturated) FFA, squalene, wax monoesters, CHOL and cholesteryl esters [37]. Some of these components, like short-chain FFA, function as penetration enhancers [15,38–40].

Based on the results from the studies discussed above, we chose to examine the influence of DAz, OAz and sebaceous lipids on the lateral organization of SC lipid models. The use of ED may elucidate the mechanisms of action, including phase transition and the formation of grain boundaries, but not the possible disordering of the lamellar organization. An advantage of using SC lipid models is the absence of intracellular keratin and the possibility to study the lipid organization as a function of enhancer content. The SC models were prepared from lipids

extracted from human SC that were devoid of triglycerides after purification. We could demonstrate that DAz and OAz alter the lipid packing in the SC lipid model in a different way compared to sebaceous lipids.

2. Material and methods

2.1. Extraction and purification of lipids from human SC

Human skin was obtained from eight different female donors, who underwent cosmetic surgery (mamma reduction). The SC was separated from the epidermis by a trypsin treatment [41] and subsequently the SC sheets from the eight donors were pooled for lipid extraction. The SC lipids were extracted using the method of Bligh and Dyer [42] and stored in chloroform/methanol (C/M 2:1, v/v) at −20°C under gaseous nitrogen until use. For purification, the extracted lipids were applied on a silica gel 60 (Merck) column with a diameter of 4 cm and a length of 13.5 cm. By sequential elution, the different lipid fractions were collected in separate glass tubes [41]. One-dimensional high-performance thin-layer chromatography (HPTLC) was used to establish the lipid composition in the tubes [43]. Those containing the CER, FFA and CHOL lipids were mixed, while those containing triglycerides were discarded. The composition of the final mixture was determined again by HPTLC. For quantification, authentic standards were run in parallel consisting of 0.1 μg/μl for each of the components CHOL, bovine Cer III and FFA (Sigma). After charring, the intensities of the bands were determined using a photodensitometer (BioRad GS 710) with automatic peak integration [43].

2.2. Preparation of SC lipid models

Samples were prepared from either purified SC lipids alone, or from SC lipids mixed with DAz, OAz or sebaceous lipids (concentration of 10 μg/μl in C/M 2:1) in the weight ratios 3:1 and 1:1. The azones were synthesized as previously described and have a purity better than 97% as determined by nuclear magnetic resonance [16]. The sebaceous lipids are composed of 29.5% palmitoleic acid, 28% triolein, 26% stearic acid oleyl ester, 12% squalene, 3% cholesteryl palmitate, and 1.5% cholesterol (Sigma). The preparation method of the samples was similar to that for SC lipid models in X-ray diffraction studies [44]. The mixtures were nebulized onto 400-Mesh copper grids as described previously [2]. These grids were covered with a thin carbon film to increase stability of the lipid film. Then the samples were equilibrated to 60°C during 10 min, hydrated in a 50 mM acetate buffer (pH 5) at 60°C, rapidly frozen in liquid nitrogen and subsequently placed in the same buffer at room temperature. To increase the homogeneity of the lipid mixtures, the samples underwent 10 freeze–thawing cycles by alternately freezing at −20°C and equilibrating to room temperature for 1 h. Finally, the grids were plunge frozen in liquid nitrogen cooled ethane and stored in liquid nitrogen until use. The procedure of sample preparation was repeated four times for each lipid mixture.

2.3. Electron diffraction

The examination of grids using ED has been described previously [1,2]. Briefly, grids were mounted into a pre-cooled cryo holder (Gatan, Model 626, Pleasanton, CA) and examined in a Philips EM420 transmission electron microscope (TEM). The TEM operated at 100 kV, with a condenser lens-two aperture of 30 μm, a spotsize of 40 nm, and a beam diameter of approximately 1 or 20 μm^2 from which ED patterns were obtained. These microscope settings allow a low dose necessary to reduce radiation damage of the sample. Good recordings on Kodak electron image films (SO-163, Rochester, NY) could be obtained with exposure times varying between 5 and 20 s.

For each lipid mixture, four samples were examined and the collected ED patterns were interpreted on basis of the pattern (the hexagonal lattice is characterized by 0.41 nm spacings, the orthorhombic lattice by 0.37 and 0.41 nm spacings, the fluid phase by a broad reflection at 0.46 nm) and the appearance of the pattern (spots, arcs or rings). These data were analyzed semi-quantitatively using the chi-square test in the statistical analysis program SPSS 7.5.

3. Results

3.1. Purified human SC lipids

It was shown using HPTLC that the isolation of the extracted human SC lipids has been successful (Table 1). The exogenous (tri)glycerides were removed, while endogenous components, including CHOL, FFA, different classes of CER, and cholesterol sulfate were still present. ED patterns of samples prepared from SC lipids before purification consisted predominantly of a hexagonal lipid organization (data not shown). ED patterns of the samples prepared from the purified SC lipids were collected before and after the freeze-thawing cycles (Fig. 1). It was observed that without homogenizing the sample, the ED patterns mainly consisted of reflections at 0.41 nm, indicative of the hexagonal lattice (Fig. 1A). After the freeze–thawing procedure the ED patterns consisted predominantly (see also Fig. 4) of reflections at 0.41 and 0.37 nm, indicative of the orthorhombic lattice (Fig. 1B), although the presence of the hexagonal lattice cannot be excluded from the ring ED patterns. The presence of a small amount of lipids in the hexagonal phase was confirmed by the recording of ED patterns with reflections located at 0.41 nm only. Furthermore, a broad band centered at 0.46 nm was sometimes present in the ED pattern, which is characteristic of a fluid phase. Finally, reflections appeared occasionally as 3 pairs of double arcs at 0.37 and 0.41 nm (also in SC models mixed with enhancers) (Fig. 1C).

Table 1
Composition of the lipids extracted from eight donors before and after purification

Composition	Before purification	After purification
Triglycerides	76.3	6.9
Cholesterol	5.8	26.3
FFA	6.8	17.4
Ceramides	11.1	49.4
Cer 1	6.5	6.8
Cer 2	18.8	25.5
Cer 3+4	36.5	29.5
Cer 5	18.0	19.5
Cer 6	7.9	6.2
Cer 7	12.3	12.5

The amounts of the different lipid classes are given in percentages. Furthermore, the relative distribution of the ceramide subclasses are shown. The presence of the triglycerides is clearly reduced. The CHOL/CER ratio and the composition of the ceramide subclasses did not change significantly. However, the FFA/CER ratio is reduced after purification. This ratio was restored by adding a mixture of FFA according to the in vivo FFA composition to the purified SC lipid mixture.

3.2. Human SC lipids mixed with DAz and OAz

The purified human SC lipids were mixed with either DAz or OAz in two different weight ratios, viz. SC lipid/enhancer = 3:1 and 1:1. Fig. 2 shows a number of characteristic ED patterns that were re-

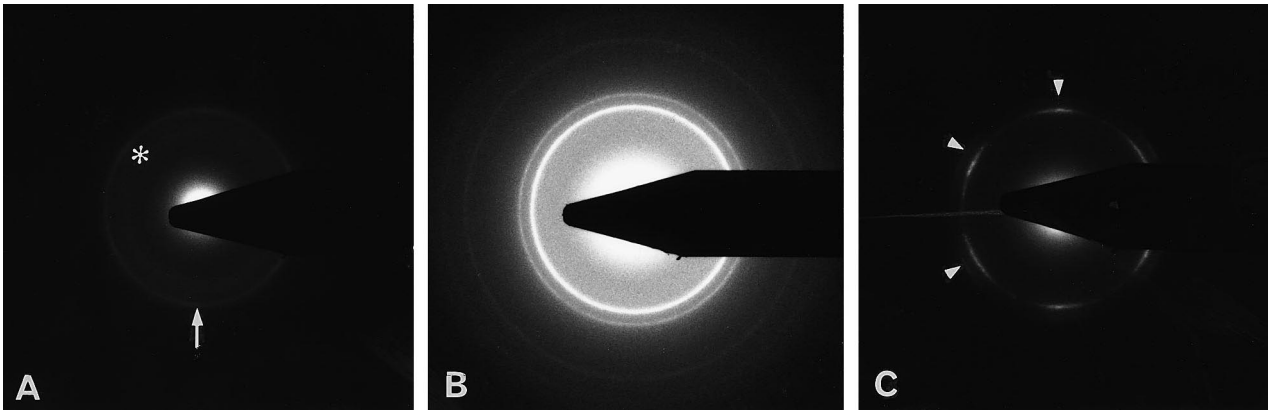

Fig. 1. Characteristic ED patterns from purified human SC lipids. (A) Without treatment, the lipids arrange mainly in a hexagonal lattice, while a fluid phase can also be observed. (B) After homogenizing by freeze–thawing cycles, the lipids form an orthorhombic lattice. Higher order reflections at 0.22 and 0.25 nm are also present. (C) Typical ED pattern that can be explained by three differently oriented orthorhombic lattices rotated over an angle of 60° relative to each other. This pattern present in the SC lipid models is frequently observed in intact human SC (arrow marks the 0.41 nm reflection, arrowhead indicates a reflection at 0.37 nm, and asterisk is located in the broad ring at 0.46 nm indicative of a fluid phase).

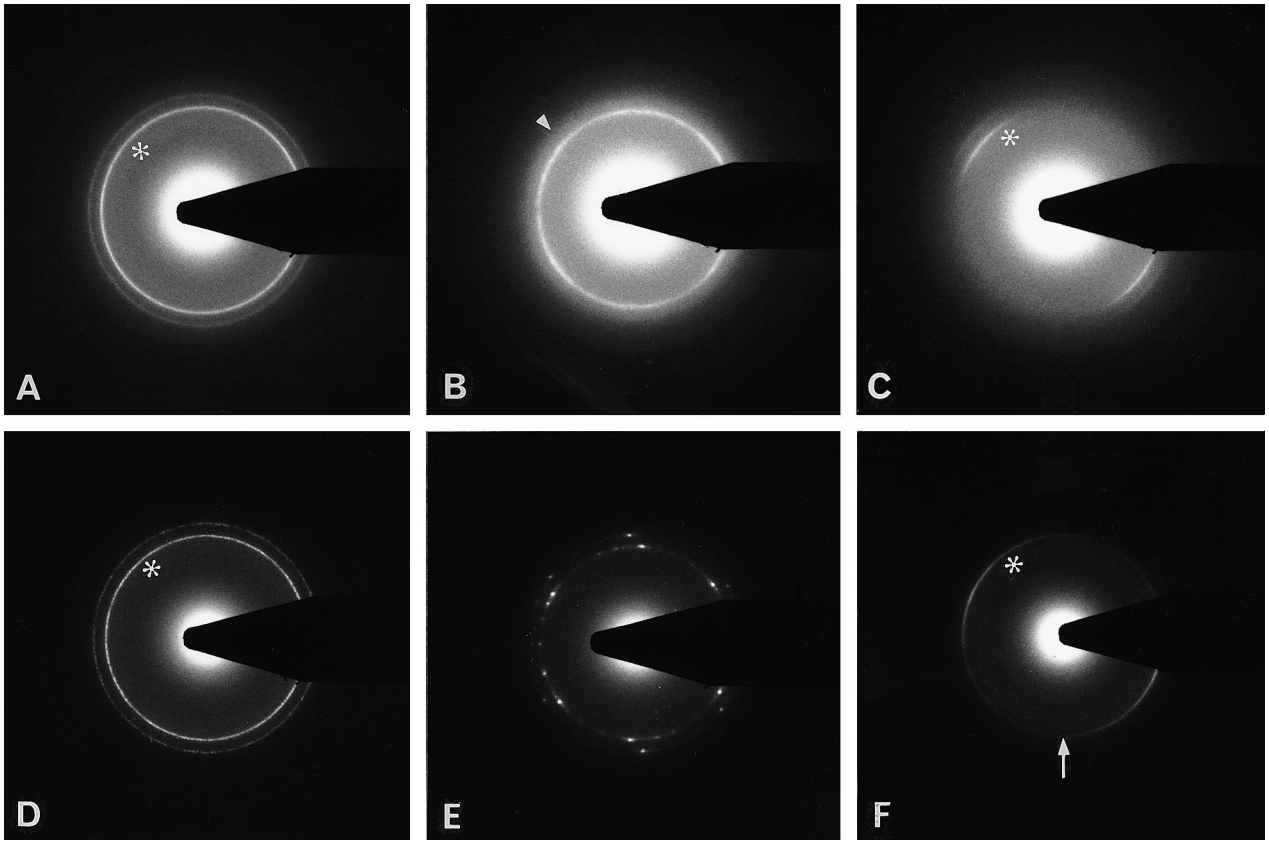

Fig. 2. Characteristic ED patterns from purified human SC lipids mixed with DAz (A–C) and OAz (D–F). (A) Orthorhombic packing and fluid phase. (B) The 0.37 nm reflection is weak. (C) Unidirectionally scattered ED pattern as result of tilting the sample. The fluid phase is not influenced by tilting. (D) Orthorhombic packing and fluid phase; note the spotted appearance of the rings as in powder diffraction patterns. (E) Orthorhombic crystals present in mainly three different orientations. The reflections appear as spots indicating that the crystals are probably small and well defined. (F) Hexagonal packing and fluid phase recorded at 40° (arrow marks the 0.41 nm reflection, arrowhead indicates a reflection at 0.37 nm, and asterisk is located in the broad ring at 0.46 nm indicative of a fluid phase).

corded in these samples. These ED patterns were interpreted on basis of the type of crystal lattice and the appearance of the reflections. Fig. 3 shows the intensity curves of some of these ED patterns in which the shoulder on the right side of the 0.41 nm peak originates from the fluid phase. Fig. 4 shows the relative distribution profile of the ED patterns that were classified into five different categories: ort, orthorhombic; ort*, orthorhombic – however, the hexagonal lattice cannot be excluded; hex, hexagonal; hex*, probably hexagonal – however, the presence of 0.37 nm reflections cannot be excluded; ort+hex, both orthorhombic and hexagonal lattices are present. No significant differences were observed in the ort/hex distribution profile between the mixtures of different weight ratios. However, the category of ort+hex lattices was only recorded in the mixtures with a ratio of SC lipid/enhancer = 1:1 (w/w). With both enhancers, the ED patterns of the mixtures showed predominantly an orthorhombic packing (Fig. 2A,D,E), although regularly the 0.37 nm reflection appeared less prominent (Fig. 2B). Furthermore, a broad band around 0.46 nm indicative of a fluid phase was present more clearly in the mixtures with enhancers (Fig. 2A,D) and the frequency, by which the fluid phase was observed, was significantly increased with approximately 10% compared to control samples (from 8% to 19% and 16% for DAz and OAz, respectively). In mixtures with OAz, the occurrence of reflections as small spots increased from 4% to 20% (Fig. 2D,E), while this feature remained unchanged in mixtures with DAz. Upon tilting the sam-

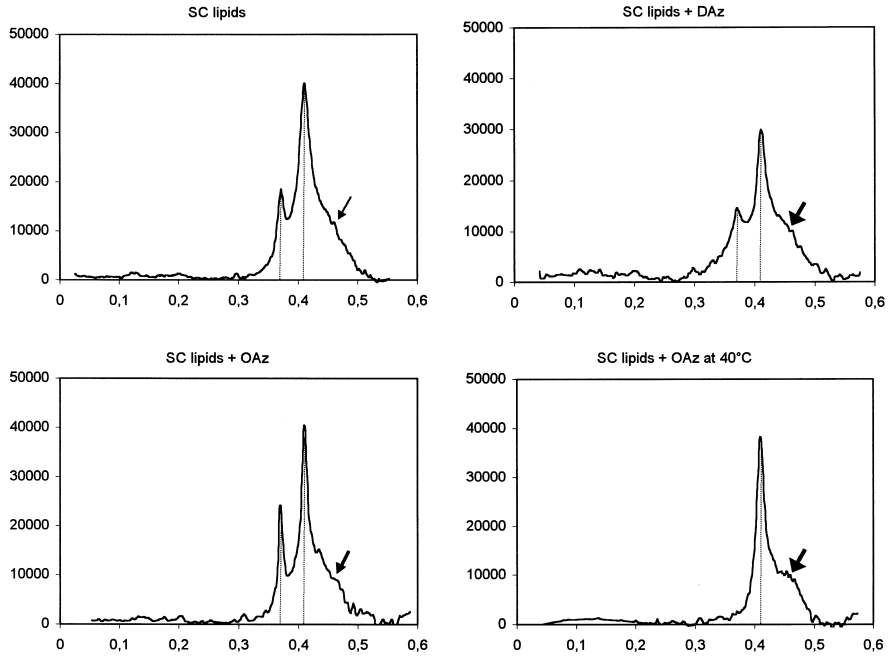

Fig. 3. Intensity curves of the ED patterns from, respectively, Fig. 1B and Fig. 2A,D,F. (A) Purified SC lipids, in which the shoulder from the fluid phase is relatively weak compared to the 0.41 nm peak. (B) SC lipids mixed with DAz, in which the shoulder from the fluid phase is clearly present. (C) SC lipids mixed with OAz, in which the fluid phase can be observed. (D) The fluid phase becomes more apparent in SC lipids mixed with OAz recorded at 40°C. On the *x*-axis the lattice spacing is indicated.

ple, the ED pattern showed unidirectionally scattered reflections at 0.41 and 0.37 nm, while the fluid phase was observed in all directions (Fig. 2C). When examining the samples at elevated temperatures the fluid phase is clearly present from 40°C (Fig. 2F).

3.3. Human SC lipids mixed with sebum lipids

The purified human SC lipids were mixed with sebum lipids in the ratios SC lipid/sebum = 3:1 and 1:1 (w/w). In Fig. 5, some characteristic ED patterns

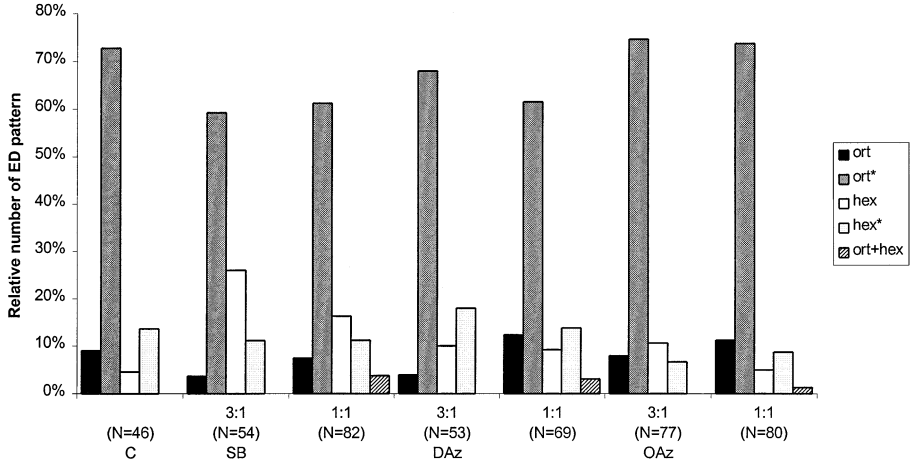

Fig. 4. Distribution profile of the ED patterns recorded in purified human SC lipid models of control samples (C), and mixtures with sebum (SB), DAz and OAz in different weight ratios (SC lipid/enhancer = 3:1 or 1:1). All samples were homogenized by freeze–thawing cycles. The *y*-axis shows the relative number of ED patterns recorded in a certain category. ort, orthorhombic; ort*, orthorhombic – however, the hexagonal lattice cannot be excluded; hex, hexagonal; hex*, probably hexagonal; ort+hex, both lattices are present in the same area selected for diffraction.

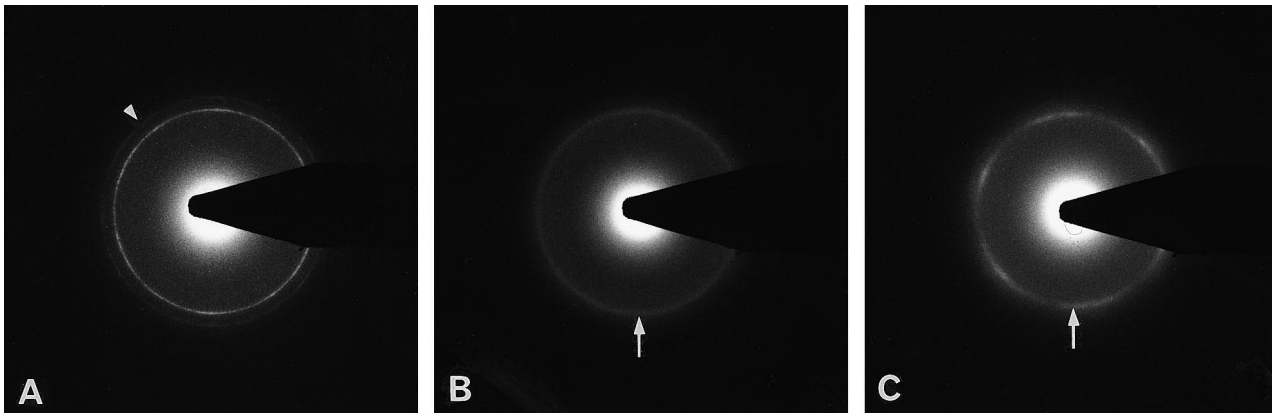

Fig. 5. Characteristic ED patterns from purified human SC lipids mixed with sebaceous lipids in different weight ratios. (A) Orthorhombic packing; however, the hexagonal lattice cannot be excluded. (B) Hexagonal packing, ring pattern. (C) Hexagonal ED pattern in which reflections are separated in arcs.

of these samples are shown. The relative distribution profile of the ED patterns changed upon adding sebaceous lipids to the isolated human SC lipids in that the frequency of the hex-category increased from 5% to 20% (Fig. 4). Thus besides ED patterns that are characteristic of an orthorhombic packing (Fig. 5A), hexagonal ED patterns in which the reflections appeared as rings or arcs (Fig. 5B,C) were regularly observed as well. The occurrence of a broad reflection indicative of the fluid phase and the appearance of reflections as small spots did not change significantly.

4. Discussion

An advantage of using SC lipid models in diffraction studies is the absence of intracellular keratin, which makes it possible to establish the presence of a fluid phase [45]. In intact human SC this reflection is obscured by a reflection of keratin in the diffraction pattern. In a previous ED study of SC lipid models, we used lipids isolated from porcine skin and showed that ED can be applied to obtain detailed information about the lateral packing [2]. The lipid mixtures in that study were not hydrated in order to avoid the formation of ice crystals when examining the samples at −170°C. In this study, however, we mimicked the preparation method of lipid mixtures used in previous WAXD and SAXD studies as closely as possible [44]. We were able by plunge freezing to preserve the lipid organization in SC lipid models that were hydrated using a buffer (pH 5), while keeping the formation of ice crystals limited. The ED patterns revealed that the lipids isolated from human SC were also arranged in an orthorhombic packing in vitro just as in intact human SC. However, the ED patterns often appeared as rings in the SC lipid models. This suggests that rotational misalignment occurs more frequently in comparison with intact SC in which the lipid organization is often maintained over large areas. As a consequence, it is more difficult to establish whether the 0.41 nm reflection in patterns, containing also 0.37 nm reflections, is derived from the orthorhombic lattice only or from the hexagonal packing, as well [2,41]. On the other hand, ED patterns were observed in control samples and mixtures with enhancers that consisted of three pairs of double arcs at 0.41 and 0.37 nm (Figs. 2C and 3E). Similar patterns have more frequently been observed in intact human SC. These patterns were explained by the presence of three orientations of the orthorhombic lattice rotated over an angle of 60° relative to each other [1]. It has been hypothesized that these three orientations are present on top of each other and constitute the lamellar phase with a periodicity of approximately 13 nm [46,47]. Such an organization is in good agreement with molecular models for the SC lipids proposed by Swartzendruber et al. [47] and Bouwstra et al. [46,48]. However, it cannot be excluded that these three orientations are located next to each other within the same lipid layer. These characteristic ED patterns have not been observed in our

previous study on SC lipid models prepared from porcine derived CER [2]. This is most likely related to the hydration state of the lipids in the present study, although differences in the lipid composition between the mixtures prepared from human and porcine SC lipids cannot be excluded.

Upon mixing the isolated human SC lipids with the enhancers DAz and OAz, the lateral lipid packing remained predominantly orthorhombic. Apparently, these enhancers do not provoke a transition from orthorhombic to hexagonal. A similar conclusion was drawn in an earlier study in which these enhancers were applied to intact ex vivo skin [34,49]. However, in samples mixed with DAz and OAz, the fluid phase became more pronounced and could be observed more frequently. Upon tilting, the appearance of the broad reflection at 0.46 nm did not change, which suggests that the fluid phase does not have a lamellar arrangement. These findings suggest that the two azones may form separate domains between the SC lipids, while the orthorhombic lipid organization remains locally present. This is in agreement with another study using SC models composed of CER 3/CHOL/FFA (C16–18), in which it was concluded that the crystalline phase was not affected by incorporation of Azone® [50]. Yet, it cannot be excluded that SC lipids are dissolved in the enhancer-rich phase as was suggested by Engblom et al. [51]. Otherwise, azones may be inserted into the endogenous SC lipids. Such assumptions may be confirmed by the observation that the 0.37 nm reflection of the orthorhombic lattice appeared less apparent several times in mixtures containing the enhancers, suggesting that changes occurred in the organization of the SC lipids. Furthermore, especially in the SC lipid models mixed with OAz, many randomly oriented well-defined crystals were observed, which was indicated by the appearance of spots in the ED patterns. Between these separate crystal domains new penetration pathways may arise, which results in increased permeability. Also in intact SC treated with OAz, the reflections appeared more frequently as spots compared to the reflections in ED patterns of control SC [34].

The effect of the sebaceous lipids on the SC lipid organization differs from that of the two enhancers. These lipids did not promote the presence of a fluid phase. Instead, a hexagonal packing was observed more frequently. It has been suggested by Bonte et al. [35] that sebaceous lipids are highly miscible with lipids extracted from human SC, as was indicated by SAXD studies. In our samples, the CER/CHOL/long-chain FFA ratio, as present in intact SC, may be changed to a mixture in which the relative amount of long-chain FFA has decreased. Since the presence of long-chain FFA is required for the formation of the orthorhombic lattice [41,45], inhomogeneous distribution of long-chain FFA will be in favor of the formation of the hexagonal lattice. Whether only the presence of short-chain (unsaturated) FFA in sebum promotes the hexagonal packing or other (non-polar) components as well, remains a topic for further investigations.

The observation that the appearance of the hexagonal packing is increased in mixtures with sebaceous lipids is consistent with a recent study on intact human SC, in which we demonstrated that in the superficial SC layers the hexagonal packing was more frequently observed, while it was absent in the deeper layers of the SC [1]. We suggested that this observation may be related to the presence of sebaceous lipids. It has been demonstrated that sebum components penetrate into the outer part of the SC [36] and Bonté et al. [35] already suggested that this may alter the endogenous lipid organization. Furthermore, other authors already indicated that some compounds present in sebum act as penetration enhancers [38–40,52,53]. The present study indicates that the enhancing activity is most likely achieved by inducing a transition from the orthorhombic to the hexagonal packing of SC lipids. Such a change in alkyl chain packing and mobility may affect transport of small molecules through lipid bilayers [54]

From the results of the present study, conclusions can be drawn with respect to the mechanisms by which penetration enhancement can be achieved. Golden et al. [55] already stated that penetration enhancers should be sufficiently similar to the endogenous SC lipids to intercallate, but different enough to alter the structure in order to increase SC permeability. The molecular architecture of the Azones, however, is quite different from SC lipids [56]. It has already been observed that these enhancers do not intercallate in the lamellae but disrupt the lamellar organization [24,51]. Therefore, these enhancers are most probably present in a separate fluid phase

and it is unlikely that this phase has a lamellar arrangement. Such a fluid domain may function as a penetration pathway and consequently increase SC permeability. Alternatively, disruptions of the lamellar organization may lead to the presence of separate crystals as seems to be the case in mixtures with OAz. This may result in an increase of grain boundaries, which are also sites where permeability is enhanced.

For the sebaceous lipids an alternative mechanism of changing the SC lipid arrangement may result in an increased permeability. Possibly, sebum components may intercalate between the SC lipids and induce a transition from an orthorhombic to a hexagonal lateral packing. As a result of this phase transition, the mobility of the alkyl chains of the endogenous lipids increases. Whether this indeed leads to an increased permeability remains a subject for further investigation.

Acknowledgements

The authors wish to thank F.E.R. Dubbelaar for technical assistance with the lipid extraction and A.M. Engelsma-van Pelt for assistance with sample preparation. Furthermore, we thank A. Weerheim for providing the curves shown in Fig. 3 and L.D.C. Verschragen for printing the micrographs. This research was supported by a grant (STW, LGN44.3541) of the Dutch Society for Scientific Research (NWO).

References

[1] G.S.K. Pilgram, A.M. Engelsma-van Pelt, J.A. Bouwstra, H.K. Koerten, Electron diffraction provides new information on human stratum corneum lipid organization studied in relation to depth and temperature, J. Invest. Dermatol. 113 (1999) 403–409.

[2] G.S.K. Pilgram, A.M. Engelsma-van Pelt, G.T. Oostergetel, H.K. Koerten, J.A. Bouwstra, Study on the lipid organization of stratum corneum lipid models by (cryo-) electron diffraction, J. Lipid Res. 39 (1998) 1669–1676.

[3] S. Chandrasekaran, J. Shaw, Factors influencing the percutaneous absorption of drugs, Curr. Probl. Dermatol. 7 (1978) 142–155.

[4] T. Higuchi, Design of chemical structure for optimal dermal delivery, Curr. Probl. Dermatol. 7 (1978) 121–131.

[5] J. Shaw, S. Chandrasekaran, Controlled topical delivery of drugs for systemic action, Drug Metab. Rev. 8 (1978) 223–233.

[6] R.H. Guy, Current status and future prospects of transdermal drug delivery, Pharm. Res. 13 (1996) 1765–1769.

[7] J. Hadgraft, Passive enhancement strategies in topical and transdermal drug delivery, Int. J. Pharm. 184 (1999) 1–6.

[8] B.A.I. van den Bergh, I. Salomons-de Vries, J.A. Bouwstra, Interactions between liposomes and human stratum corneum studied by freeze-substitution electron microscopy, Int. J. Pharm. 167 (1998) 57–67.

[9] P.M. Elias, Epidermal lipids, barrier function, and desquamation, J. Invest. Dermatol. 80 (1983) 44–49.

[10] P.W. Wertz, M.C. Miethke, S.A. Long, J.S. Strauss, D.R. Downing, The composition of the ceramides from human stratum corneum and from comedowns, J. Invest. Dermatol. 84 (1985) 410–412.

[11] L. Norlen, I. Nicander, A. Lundsjo, T. Cronholm, B. Forslind, A new HPLC-based method for the quantitative analysis of inner stratum corneum lipids with special reference to the free fatty acid fraction, Arch. Dermatol. Res. 290 (1998) 508–516.

[12] B.B. Michniak, M.R. Player, J.M. Chapman, J.W. Sowell, In vitro evaluation of a series of Azone analogs as dermal penetration enhancers. I, Int. J. Pharm. 91 (1993) 85–93.

[13] R.M. Hanif, Q.E. Ping, F.Z. Muo, Synthesis and effect of two new penetration enhancers on the transdermal delivery of 5-fluorouracil through excised rat skin, Chem. Pharm. Bull. 46 (1998) 1428–1431.

[14] P.A. Cornwell, B.W. Barry, C.P. Stoddart, J.A. Bouwstra, Wide-angle X-ray diffraction of human stratum corneum: Effects of hydration and terpene enhancer treatment, J. Pharm. Pharmacol. 46 (1994) 938–950.

[15] H. Tanojo, E. Boelsma, H.E. Junginger, M. Ponec, H.E. Bodde, In vivo human skin barrier modulation by topical application of fatty acids, Skin Pharmacol. Appl. Skin Physiol. 11 (1998) 87–97.

[16] J.A. Bouwstra, L.J.C. Peschier, J. Brussee, H.E. Bodde, Effect of N-alkyl-azocycloheptan-2-ones including azone on the thermal behaviour of human stratum corneum, Int. J. Pharm. 52 (1989) 47–54.

[17] E.J. van Zoelen, C. Henriques de Jesus, E. de Jonge, M. Mulder, M.C. Blok, J. de Gier, Non-electrolyte permeability as a tool for studying membrane fluidity, Biochim. Biophys. Acta 511 (1978) 335–347.

[18] J. Risbo, K. Jorgensen, M.M. Sperotto, O.G. Mouritsen, Phase behavior and permeability properties of phospholipid bilayers containing a short-chain phospholipid permeability enhancer, Biochim. Biophys. Acta 1329 (1997) 85–96.

[19] T. Ogiso, T. Hirota, M. Iwaki, T. Hino, T. Tanino, Effect of temperature on percutaneous absorption of terodiline, and relationship between penetration and fluidity of the stratum corneum lipids, Int. J. Pharm. 176 (1998) 63–72.

[20] A. Emilson, M. Lindberg, B. Forslind, The temperature effect on in vitro penetration of sodium lauryl sulfate and

nickel chloride through human skin, Acta Derm.-Venereol. 73 (1993) 203–207.
[21] M. Langner, S.W. Hui, Dithionite penetration through phospholipid bilayers as a measure of defects in lipid molecular packing, Chem. Phys. Lipids 65 (1993) 23–30.
[22] T.X. Xiang, B.D. Anderson, Phase structures of binary lipid bilayers as revealed by permeability of small molecules, Biochim. Biophys. Acta 1370 (1998) 64–76.
[23] S.G. Clerc, T.E. Thompson, Permeability of dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine bilayer membranes with coexisting gel and liquid-crystalline phases, Biophys. J. 68 (1995) 2333–2341.
[24] J.A. Bouwstra, G.S. Gooris, J. Brussee, M.A. Salomons-de Vries, W. Bras, The influence of alkyl-azones on the ordering of the lamellae in human stratum corneum, Int. J. Pharm. 79 (1992) 141–148.
[25] H. Tanojo, A. Bos-van Geest, J.A. Bouwstra, H.E. Junginger, H.E. Boddé, In vitro human skin barrier perturbation by oleic acid: thermal analysis and freeze fracture electron microscopy studies, Thermochim. Acta 293 (1997) 77–85.
[26] T.X. Xiang, B.D. Anderson, Influence of chain ordering on the selectivity of dipalmitoylphosphatidylcholine bilayer membranes for permeant size and shape, Biophys. J. 75 (1998) 2658–2671.
[27] R.H. Guy, R.O. Potts, Structure-permeability relationships in percutaneous absorption, J. Pharm. Sci. 81 (1992) 603–604.
[28] J.A. Bouwstra, G.S. Gooris, M.A. Salomons-de Vries, J.A. van der Spek, W. Bras, Structure of human stratum-corneum as a function of temperature and hydration – a wide-angle X-ray diffraction study, Int. J. Pharm. 84 (1992) 205–216.
[29] D. Bommannan, R.O. Potts, R.H. Guy, Examination of stratum corneum barrier function in vivo by infrared spectroscopy, J. Invest. Dermatol. 95 (1990) 403–408.
[30] B. Ongpipattanakul, M.L. Francoeur, R.O. Potts, Polymorphism in stratum corneum lipids, Biochim. Biophys. Acta 1190 (1994) 115–122.
[31] S.H. White, D. Mirejovsky, G.I. King, Structure of lamellar lipid domains and corneocyte envelopes of murine stratum corneum. An X-ray diffraction study, Biochemistry 27 (1988) 3725–3732.
[32] C.L. Gay, R.H. Guy, G.M. Golden, V.H.W. Mak, M.L. Francoeur, Characterization of low-temperature (i.e. $< 65°C$) lipid transitions in human stratum corneum, J. Invest. Dermatol. 103 (1994) 233–239.
[33] A.J. Hoogstraate, J. Verhoef, J. Brussee, A.P. IJzerman, F. Spies, H.E. Bodde, Kinetics, ultrastructural aspects and molecular modelling of transdermal peptide flux enhancement by N-alkylazacycloheptanones, Int. J. Pharm. 76 (1991) 37–47.
[34] G.S.K. Pilgram, A.M. Engelsma-van Pelt, H.K. Koerten, J.A. Bouwstra, The effect of two azones on the lateral lipid organization of human stratum corneum and its permeability, Pharm. Res. 17 (2000) 796–802.
[35] F. Bonte, P. Pinguet, A. Saunois, A. Meybeck, S. Beugin, M. Ollivon, S. Lesieur, Thermotropic phase behavior of in vivo extracted human stratum corneum lipids, Lipids 32 (1997) 653–660.
[36] H.M. Sheu, S.C. Chao, T.W. Wong, J.Y.Y. Lee, J.C. Tsai, Human skin surface lipid film: An ultrastructural study and interaction with corneocytes and intercellular lipid lamellae of the stratum corneum, Br. J. Dermatol. 140 (1999) 385–391.
[37] D.T. Downing, J.S. Strauss, Synthesis and composition of surface lipids of human skin, J. Invest. Dermatol. 62 (1974) 228–244.
[38] R.O. Potts, V.H. Mak, R.H. Guy, M.L. Francoeur, Strategies to enhance permeability via stratum corneum lipid pathways, Adv. Lipid Res. 24 (1991) 173–210.
[39] M.L. Francoeur, G.M. Golden, R.O. Potts, Oleic acid: Its effects on stratum corneum in relation to (trans)dermal drug delivery, Pharm. Res. 7 (1990) 621–627.
[40] R.R. Burnette, B. Ongpipattanakul, Characterization of the pore transport properties and tissue alteration of excised human skin during iontophoresis, J. Pharm. Sci. 77 (1988) 132–137.
[41] J.A. Bouwstra, G.S. Gooris, K. Cheng, A. Weerheim, W. Bras, M. Ponec, Phase behavior of isolated skin lipids, J. Lipid Res. 37 (1996) 999–1011.
[42] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.
[43] M. Ponec, A. Weerheim, J. Kempenaar, A.M. Mommaas, D.H. Nugteren, Lipid composition of cultured human keratinocytes in relation to their differentiation, J. Lipid Res. 29 (1988) 949–961.
[44] J.A. Bouwstra, K. Cheng, G.S. Gooris, A. Weerheim, M. Ponec, The role of ceramides 1 and 2 in the stratum corneum lipid organisation, Biochim. Biophys. Acta 1300 (1996) 177–186.
[45] J.A. Bouwstra, G.S. Gooris, F.E.R. Dubbelaar, A.M. Weerheim, M. Ponec, pH, cholesterol sulfate and fatty acids affect the stratum corneum lipid organization, J. Invest. Dermatol. Symp. Proc. 3 (1998) 69–74.
[46] J.A. Bouwstra, G.S. Gooris, F.E.R. Dubbelaar, A.M. Weerheim, A.P. IJzerman, M. Ponec, Role of ceramide 1 in the molecular organization of the stratum corneum lipids, J. Lipid Res. 39 (1998) 186–196.
[47] D.C. Swartzendruber, P.W. Wertz, D.J. Kitko, K.C. Madison, D.T. Downing, Molecular models of the intercellular lipid lamellae in mammalian stratum corneum, J. Invest. Dermatol. 92 (1989) 251–258.
[48] J.A. Bouwstra, F.E.R. Dubbelaar, G.S. Gooris, M. Ponec, The lipid organisation in the skin barrier, Acta Derm.-Venereol. Suppl. 208 (2000) 23–30.
[49] J.A. Bouwstra, M.A. Salomons de Vries, B.A.I. Van den Bergh, G.S. Gooris, Changes in lipid organisation of the skin barrier by N-alkyl-azocycloheptanones: A visualisation and X-ray diffraction study, Int. J. Pharm. 144 (1996) 81–89.
[50] L. Norlen, J. Engblom, Structure-related aspects on water diffusivity in fatty acid-soap and skin lipid model systems, J. Controlled Release 63 (2000) 213–226.

[51] J. Engblom, S. Engstrom, B. Jonsson, Phase coexistence in cholesterol fatty acid mixtures and the effect of the penetration enhancer Azone, J. Controlled Release 52 (1998) 271–280.
[52] H. Tanojo, J.A. Bouwstra, H.E. Junginger, H.E. Bodde, In vitro human skin barrier modulation by fatty acids: Skin permeation and thermal analysis studies, Pharm. Res. 14 (1997) 42–49.
[53] C.A. Squier, P.W. Wertz, D.M. Williams, A.T. Cruchley, Permeability of oral mucosa and skin with age, in: C.A. Squier, M.W. Hill (Eds.), The Effect of Aging in Oral Mucosa and Skin, CRC Press, Boca Raton, FL, 1994, pp. 91–98.
[54] L.L.M. van Deenen, Chemistry of phospholipids in relation to biological membranes, Pure Appl. Chem. 25 (1971) 25–56.
[55] G.M. Golden, J.E. McKie, R.O. Potts, Role of stratum corneum lipid fluidity in transdermal drug flux, J. Pharm. Sci. 76 (1987) 25–28.
[56] J. Hadgraft, J. Peck, D.G. Williams, W.J. Pugh, G. Allan, Mechanisms of action of skin penetration enhancers/retarders: Azone and analogues, Int. J. Pharm. 141 (1996) 17–25.