International Journal of Pharmaceutics, 79 (1992) 141–148
Elsevier Science Publishers B.V.

IJP 02620

The influence of alkyl-azones on the ordering of the lamellae in human stratum corneum

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(Received 7 May 1991)
(Modified version received 31 July 1991)
(Accepted 23 August 1991)

Key words: Azone; Lamellar structure; Small angle X-ray scattering; Stratum corneum

Summary

The effect of a series of alkyl-azones (*N*-alkylazocycloheptane-2-one) on the structure of human stratum corneum has been studied by small angle X-ray scattering. Treatment with alkyl-azones having alkyl chains greater than six carbons in length resulted in the disordering of the lamellae in the stratum corneum. The results could be correlated with those obtained by thermal analysis. The remaining thermal transition after treatment with longer alkyl chain azones is probably due to the lipids associated with the proteins.

Introduction

The intercellular regions in the stratum corneum consist of lipids that are arranged in a lamellar phase (Elias and Friend, 1975). The lipids which constitute the lamellar phase are, e.g. ceramides, cholesterol, glycerides and alkyl acids. The intercellular route is supposed to be the main pathway by which drugs and other substances diffuse across the stratum corneum. For this reason it is very important to obtain information on the structure of the intercellular lipids and the changes in this structure induced by penetration enhancers. Using small-angle X-ray scattering (SAXS), one of the first studies on the structure of the lipids in human stratum corneum was carried out by Friberg and Osborne (1985). They observed a single broad diffraction peak. Assuming a lamellar phase, a repeat distance of approx. 6.0 nm was calculated. White et al. (1988) studied the structure of the lipids in murine stratum corneum. They observed a series of sharp diffraction peaks which were based on a lamellar phase with a repeat distance of 13.1 nm. Bouwstra et al. (1991a) found a repeat distance of 6.5 nm for human stratum corneum, but could not exclude a distance of approx. 13 nm. Garson et al. (1991) found two repeat distances of 6.5 and 4.4 nm in human stratum corneum. In more recent studies, it appeared that the scattering curve of human stratum corneum could be explained by

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two unit cells with repeat distances of 6.4 and 13.4 nm, respectively (Bouwstra et al., 1991b).

Several techniques were used to investigate the influence of penetration enhancers on the structure of lipids in the stratum corneum. Thermal analysis was used to study the influence of water (Golden et al., 1987), dimethyl sulfoxide (Goodman and Barry, 1986) and oleic surfactants (Golden et al., 1988) on the phase transitions. Fourier transform infrared spectroscopy (FTIR) was used to study the effect of oleic acid and D_2O on the C-H stretching absorbance (Mak et al., 1991).

Several investigations were carried out on the effect of alkyl-azones on the penetration route (Boddé et al., 1989), thermal transitions (Bouwstra et al., 1989) and penetration enhancement. Dodecyl-azone in combination with propylene glycol (PG) favoured the intercellular transport of $HgCl_2$ (Boddé et al., 1989), while hexyl-azone did not change the distribution of $HgCl_2$ between corneocytes and lipid regions. In another study, an increase in penetration enhancement was observed using longer alkyl-azones (Hoogstraate et al., 1991). The increase in penetration enhancement is accompanied by a decrease in the total enthalpy of two lipid phase transitions. The influence of dodecyl-azone on the bilayers was studied using DPPC liposomes (Beastall et al., 1988), which are supposed to be a model system for the bilayers in the stratum corneum. It was concluded that intercalation of dodecyl-azone resulted in a decrease in the diffusional resistance of the bilayers.

This paper describes the influence of alkyl-azones on the lamellar structure in human stratum corneum measured by small-angle X-ray scattering (SAXS).

Materials and Methods

Preparation of the samples

Human mamma or abdomen skin obtained after surgical operation was dermatomed to a thickness of approx. 200 μm. The stratum corneum was separated from the epidermis by digestion with a 0.1% trypsin solution for 14 h.

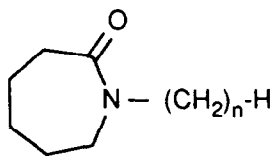

Fig. 1. The structure of alkyl-azones, in which n represents the number of C atoms in the alkyl chain.

The stratum corneum was washed and dried over silica gel. Before use it was equilibrated above 27% w/w NaBr solution to achieve a hydration level of 20% w/w.

Synthesis of alkyl-azones

The synthesis of the alkyl-azones was carried out as described before (Bouwstra et al., 1989). The purity of the azones was checked by NMR and appeared to be better than 98%. The structure of alkyl-azones is shown in Fig. 1.

Pretreatment of human stratum corneum with alkyl-azones

Pretreatment was carried out by immersing the stratum corneum in a solution of propylene glycol (PG) or alkyl-azone in PG (0.15 M solution) for a period of 24 h. The alkyl chain length varied between 6 and 16 atoms. In another series of experiments stratum corneum was heated to 90°C and cooled down in order to recrystallize the lipids, after which the stratum corneum was pretreated with alkyl azones in PG as described above.

Small angle X-ray scattering (SAXS)

All measurements were carried out at the Synchrotron Radiation Source at Daresbury's Laboratories using station 8.2. This station has been built as part of an NWO/SERC agreement. The camera produces a highly collimated beam with a cross-section of 0.4×4 mm^2 at the sample position. With the SRS operating at 200 mA and 2 GeV the X-ray intensity is approx. 4×10^{11} photons/s with $\lambda = 0.15$ nm at the sample position. Smearing of the diffraction pattern due to the finite size of the X-ray beam is negligible. The sample-to-detector distance can be set between 0.2 and 4.5 m enabling studies of systems with

repeat distances $0.4 < d < 100$ nm. For data collection a multiwire, position-sensitive quadrant detector was used. This detector can handle count rates up to 250000 s^{-1}. The detector system spatial resolution is 0.5 mm. This detector definitely improves the signal-to-noise ratio at higher diffraction angles compared with a previously used linear detector (Bouwstra et al., 1991a). For all the experiments the sample-to-detector distance was set to 2.0 m. The diffraction patterns were normalized with respect to synchrotron beam decay and absorption of the sample. Background subtractions and corrections for positional inhomogeneity in the detector sensitivity were performed as well. No smoothing algorithms were applied to the data. Calibrations were performed with the help of a wet rat tail collagen sample with a repeat distance of 67 nm.

The stratum corneum, approx. 5 mg in weight, was put in a specially designed temperature-controlled sample cell. Scattering curves were collected for 15 min. The scattering intensities are plotted as a function of the scattering vector Q defined as $Q = (4\pi \sin \theta)/\lambda$, in which θ is the scattering angle and λ the wavelength.

Results and Discussion

After pretreatment of stratum corneum with alkyl-azones in combination with PG a check on the lipid loss was performed. No lipids could be detected in the alkyl-azone/PG solution using thin-layer chromatography (Ponec et al., 1988).

In Fig. 2 the scattering pattern of untreated human stratum corneum hydrated to 20% w/w is shown. The scattering pattern of 40% w/w hydrated stratum corneum has been added to this plot, since at this hydration level the diffraction peaks are more pronounced. The scattering curve is characterized by a high intensity at $Q < 0.8$ nm^{-1} and a broad diffraction peak at $Q = 0.98$ nm^{-1}. The broad diffraction peak clearly exhibits a shoulder on the right-hand side, indicating that it actually consists of two partially unresolved peaks. At $Q = 1.85$ nm^{-1} a weak diffraction peak could be detected. The weak diffraction peak also exhibits a shoulder on the right-hand side.

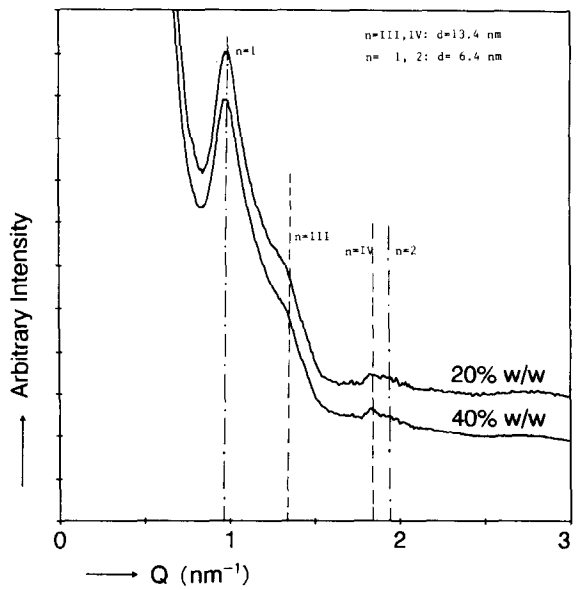

Fig. 2. The scattering curve of untreated stratum corneum hydrated to 20 or 40% w/w.

A lamellar phase results in a scattering curve with Bragg reflections located at equidistant positions in reciprocal space. The position of the n-th order diffraction peak is directly related to the repeat distance d (length of the unit cell) according to the following equation:

$$Q_n = 2n\pi/d$$

In recent studies (Bouwstra et al., 1991b), it has been shown that the presence of the two diffraction peaks can be explained by the existence of two lamellar arrangements with repeat distances of 6.4 and 13.4 nm, respectively. The main peak of the strong diffraction doublet and the shoulder of the weak diffraction peak originate from a unit cell with a repeat distance of 6.4 nm, while the shoulder of the strong diffraction doublet and the main position of the weak diffraction doublet correspond to a unit cell with a repeat distance of 13.4 nm. These conclusions were based on experiments considering recrystallization of the lipids in the stratum corneum which showed a lipid arrangement of only one unit cell with a repeat distance of 13.4 nm.

In Fig. 3a and b, the scattering curves of stratum corneum treated with PG or alkyl-azones in combination with PG are shown. After treatment with PG no significant changes were observed in the scattering curve, even the position of the main diffraction peak showing no shift, which implies that no swelling of the lamellae occurred. A similar behaviour was observed upon hydration (Bouwstra et al., 1991a,b). Changes in hydration level from 6 to 40% w/w did not change the main peak position of the strong diffraction doublet, which is quite remarkable. Treatment with hexyl-azone in combination with PG decreased the intensity of the strong diffraction doublet. The main peak as well as the shoulder decreased in intensity indicating that hexyl-azone interacts with the lipids in both unit cells. Treatment with octyl-azone and longer alkyl chain azones resulted in the almost complete disappearance of the main diffraction doublet. Only a shoulder was observed on the descending scattering curve. Alkyl-azones with 8 or more C atoms in the alkyl chain produced a much stronger disordering of the lamellae than did hexyl-azone. This diference in interaction observed using SAXS can be related with changes observed in the phase transitions after treatment with alkyl-azones (Bouwstra et al., 1991a).

In Fig. 4 the change in the thermal transitions of stratum corneum is shown after treatment with a whole series of alkyl-azones. The thermal behaviour of untreated human stratum corneum exhibits four transitions (Golden et al., 1987, 1988; Bouwstra et al., 1989). The first and second transition appeared at 37 and 70°C, respectively. Both peaks in the DTA curve were ascribed to reversible phase transitions of the lipids in the stratum corneum. The third transition (87°C) is probably due to lipids associated with proteins in the stratum corneum. This transition was only reversible in the cases where heating did not denature the proteins. The fourth irreversible transition located at 120°C is due to denaturation of the proteins in the stratum corneum. Treatment with PG resulted in a shift of the transition temperature of the two lipid transitions originally located at 70 and 87°C to lower temperatures, indicating that PG interacts with the intercellular lipids. No change in the total enthalpy involved in these two transitions was observed. In fact treat-

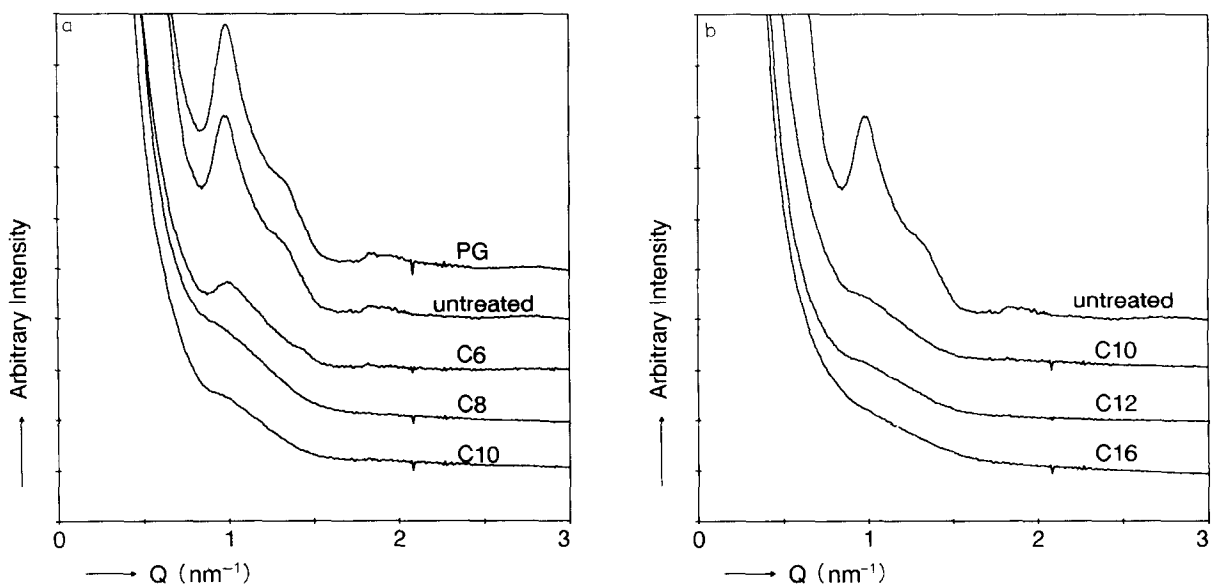

Fig. 3. (a) The scattering curve of human stratum corneum after treatment with hexyl-azone (C_6), oxtyl-azone (C_8) or decyl-azone (C_{10}) in combination with propylene glycol. (b) The scattering curve of human stratum corneum after treatment with decyl-azone (C_{10}), dodecyl-azone (C_{12}) or hexadecyl-azone (C_{16}) in combination with propylene glycol.

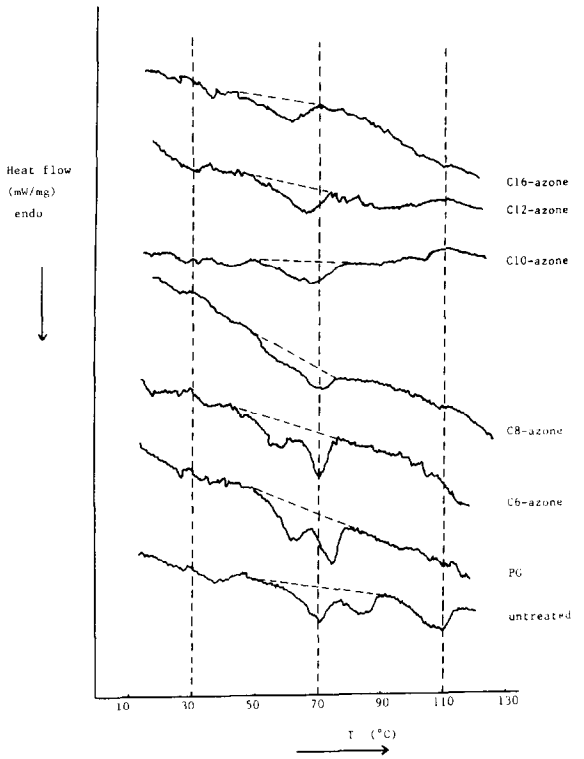

Fig. 4. The thermal behaviour of stratum corneum after treatment with a whole series of alkyl-azones in combination with PG.

ment with PG does not influence the scattering curve, but it does decrease the transition temperature of the lipids. The same behaviour has been found in increasing the hydration level of the stratum corneum. An increase in hydration level to 40% w/w did not result in a swelling of the bilayers, while a decrease in the transition temperatures of the intercellular lipids was observed. Using both techniques a sharpening of the peaks was observed after PG treatment or at increasing hydration level to 40% w/w.

After treatment with octyl-azone in combination with PG the two peaks in the DTA curve were not completely separated. The curve between the two transitions did not return to the baseline. The area under the peak, which is a measure for the enthalpy involved in the transition, decreased significantly (Bouwstra et al., 1989). Treatment with decyl-azone and longer alkyl-azones results in a single peak and in a further shift of the peak to lower temperatures. From the shape of the peak it is not clear whether both transitions still occur and whether the enthalpy involved in these transitions decreased or one of the two transitions disappeared. With respect to the transition originally located at 70°C the temperature of the resulting single peak after decyl-azone treatment was higher than the transition temperature after treatment with octyl-azone. Since an upward jump in temperature is not very likely, it is probable that the lipid transition originally located at 70°C has disappeared, while the lipid transition originally located at 87°C has shifted to lower temperatures. More evidence for the correctness of this hypothesis can be found in the SAXS curves that were obtained at higher temperatures. These scattering curves are shown in Fig. 5. The scattering curve measured at 60°C does not differ significantly from that recorded at 25°C, indicating that no detectable disordering of the lamellae occurred during the lipid transition at 40°C. It appears that this transition is due to a change from crystalline state bilayers to gel state bilayers. At 75°C the main diffraction peak and the weak diffraction peak completely disap-

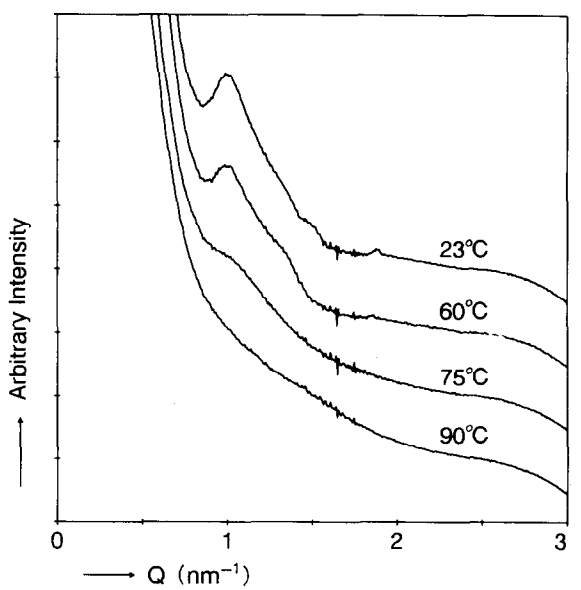

Fig. 5. Scattering curves after heating to various temperatures as indicated. At 75 °C only a shoulder on the descending scattering curve is observed.

peared. Only a shoulder on the descending scattering curve remained at $Q = 1\ nm^{-1}$. It appears that a phase change took place. Since no new diffraction peaks appeared the most likely explanation is a disordering of the lamellar structure. The origin of the shoulder on the descending curve still present at 75°C is not fully understood, but the thermal analysis results indicate that it is caused by lipids associated with proteins in the stratum corneum. This has been confirmed by SAXS, since after recrystallization of the lipids a reheating of the stratum corneum did not exhibit the shoulder on the scattering curve found in the first heating experiment at 75°C. It appears that the shoulder at the scattering curve is influenced by the denaturation of the protein and is therefore indeed due to the lipids associated with the proteins. The presence of only a residual shoulder on the scattering curve implies that the long-range order completely disappeared. In fact, the shoulder in the scattering curve could possibly be caused by the presence of one well-ordered lipid layer, which might be the corneocyte lipid envelope. At 90°C, which is just above the third thermal transition, the shoulder disappeared confirming the hypothesis that the shoulder is due to lipids linked to the proteins in the stratum corneum. If one now returns to Fig. 3a and b, a similar shoulder at the descending scattering curve is observed after treatment with alkyl-azones with more than six C atoms in their alkyl chain. This strongly indicates that the third thermal transition, which can be related to the shoulder in the descending scattering curve, is still present after treatment with longer alkyl chain azones while the second thermal transition disappeared, indicating that the remaining thermal transition is due to the lipids which are assumed to be associated with proteins. This also implies that the alkyl-azones in combination with PG only influence the lipids which are not associated with the proteins. Hoogstraate et al. (1991) showed that after treatment with dodecyl-azone in PG, a lamellar structure is still present in the intercellular spaces. This apparent discrepancy can be explained in two ways. The first explanation is that after treatment with dodecyl-azone, there are still lamellae present but the long-range order has completely disappeared. The second is based on the difference in treatment. For visualization, the stratum corneum was treated on the apical side, while in the experiments described in this paper the treatment was carried out by soaking the stratum corneum in the alkyl-azone/PG solution. Very recent results obtained using the wide-angle X-ray diffraction (WAXD) technique showed that the two reflections corresponding to distances of 0.415 and 0.378 nm are still present after treatment with dodecyl-azone in PG, although the intensity of the reflections might be decreased. The treatment was carried out by soaking stratum corneum in the solution for 12 h. The two reflections are based on an orthorhombic and/or hexagonal lateral packing of the hydrocarbons in the bilayers. These findings confirm the existence of bilayers after treatment with alkyl-azones in PG.

In a series of experiments the stratum corneum was heated to 90°C, and cooled down to room temperature to recrystallize the lipids. After recrystallization the stratum corneum was pretreated with alkyl-azones in PG. The results are shown in Fig. 6. Bouwstra et al. (1991b) showed

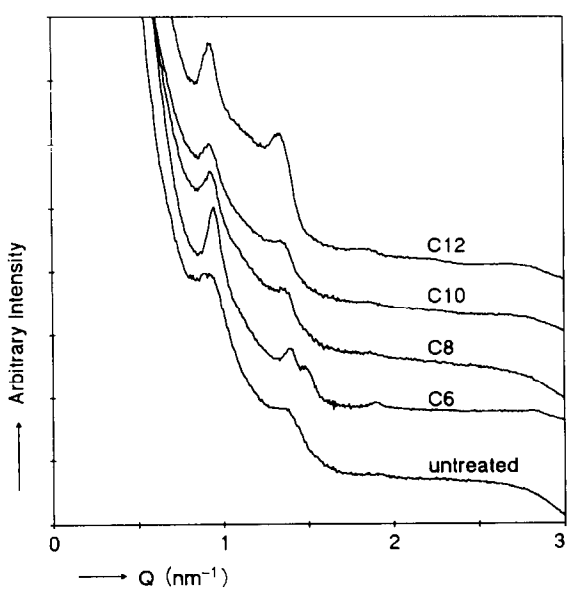

Fig. 6. Scattering curves of human stratum corneum after recrystallization of the lipids and treatment with hexyl-, octyl-, decyl- and dodecyl-azone.

that recrystallization of the lipids revealed one lamellar structure with a repeat distance of 13.4 nm. The diffraction peaks of untreated stratum corneum are broader than found previously (Bouwstra et al., 1991b), indicating that the long-range order of the lipid lamellae is less pronounced. After pretreatment with azones it appeared that no significant disordering occurred, which is quite remarkable. It seems that the interaction between alkyl-azones and the lipid bilayers changed after recrystallization of the lipids, but the origin of the change in interactions is not yet known. A similar change in interaction between pig stratum corneum and detergent after heating was also observed by Wertz et al. (1989). They suggested that it might be caused by a change in the interactions between the protein bound lipid envelopes of different cells. A possibility is the forming of more compact bilayers or larger lipid regions which makes it more difficult for alkyl-azones to intercalate. More experiments must be carried out in order to elucidate this phenomenon.

Conclusions

The influence of alkyl-azones on the long-range ordering of the lamellae in human stratum corneum depends on the length of the alkyl chain. Azones with a hydrocarbon chain length of more than six carbon atoms induce a disordering in the lipid structure of human stratum corneum, whereas after treatment with hexyl-azone no large differences in the structure could be detected. A similar jump in behaviour has also been observed in other studies. From in vitro toxicity studies, it could be concluded that hexyl-azone was less toxic than alkyl-azones (Ponec et al., 1989) with 8 or more C atoms in their hydrocarbon tail. A study in which the the penetration enhancement of alkyl-azones (Hoogstraate et al., 1991) was investigated revealed a significant effect of enhanced penetration of DGAVP after pretreatment of human stratum corneum with decyl-, dodecyl- and tetradecyl-azone, while hexyl- and octyl-azone had only a minor influence on the transport of DGAVP.

The exact mechanism behind the observed difference in behaviour of the alkyl-azones is not yet known, but it appears that it is not caused by a difference in intercalation in the stratum corneum bilayers, since recent experiments using skin lipid liposomes strongly indicate that hexyl-azone (Bouwstra and Salomons-de Vries, unpublished results) is also intercalated in bilayer regions in liposomes. It seems that the way in which the alkyl-azones are intercalated and the resulting distortion also play important roles.

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