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^{2}H NMR Evidence for dynamic disorder in human skin induced by the penetration enhancer Azone

F.R. Bezema[a], E. Marttin[b], P.E.H. Roemelé[b], J. Brussee[a], H.E. Boddé[b,*], H.J.M. de Groot[a]

[a]*Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, P.O. Box 9502, 2300 RA Leiden, Netherlands*
[b]*Center for Bio-Pharmaceutical Sciences, Gorlaeus Laboratories, Leiden University, P.O. Box 9502, 2300 RA Leiden, Netherlands*

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Abstract

Penetration enhancers mediate trans-dermal drug delivery. The penetration enhancer dodecyl-azacycloheptanone (Azone®) was characterized in situ with ^{2}H NMR on human stratum corneum treated with either uniformly deuterated Azone® or penetration enhancer with only the entire chain deuterated, in both cases in propylene glycol. The ^{2}H NMR spectrum at ambient temperature constitutes a single narrow line only 180 Hz wide. This contrasts with the complex lineshapes 1–25 kHz wide commonly observed for methylene deuterons of lipids in a bilayer gel phase. Hence there is no evidence for differences in orientational behaviour between different deuterons at ambient temperature. The narrow line translates into a low overall order parameter $S_{CD} < 0.0008$, for all deuterons, revealing rapid motion of the entire enhancer with correlation times $\tau_c \ll 6 \times 10^{-6}$ s, in all possible directions. At temperatures of about 150 K a ^{2}H NMR pattern characteristic for a random isotropic powder was observed on samples of stacked stratum corneum sheets at different orientations with respect to the magnetic field. This provides strong evidence for random isotropic freezing of the Azone® molecules upon cooling. The ^{2}H NMR data suggest than incubation with Azone® in propylene glycol provokes dynamic structural disorder of the intercellular lamellar lipid structure throughout the stratum corneum and possibly even the creation of fluid domains involving the intercellular lipids, which may be essential for the penetration enhancing effect.

Keywords: Azone®; Gelphase; Isotope labeling; Lipid bilayer; Order parameter; Stratum corneum

1. Introduction

The stratum corneum (SC), the outer layer of the human skin, serves as a barrier against the penetration of water and various solutes. This is attributed to the presence of an extra-cellular lamellar lipid structure surrounding the corneocytes and forming extended sheets in the SC [1]. Transdermal drug delivery used skin penetration enhancers such as Azone® (Scheme 1) in propylene glycol (PG) to increase the permeation across SC for both hydrophilic and hydrophobic drugs and other agents [2–6].

* Corresponding author.

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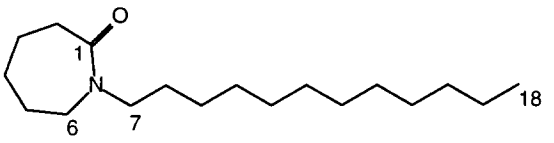

Scheme 1. Dodecyl-azacycloheptanone (Azone®). Uniformly deuterated, deuterons at C_2–C_{18}, chain deuterated, deuterons at C_7–C_{18}.

Different models for the molecular mechanism of penetration enhancement by Azone® exist. The skin penetration characteristics of azacycloheptanones vary with alkyl chain length and are optimal for lengths C_{10}–C_{12}, which match the average length of the hydrophobic tails of skin lipids [2]. Molecular modelling studies have suggested that the polar head group of the Azone® can be positioned in the polar region at a conformational energy cost of only 1 kcal mol^{-1} with respect to the stretched form [2,6,7]. This suggests that the Azone® can be incorporated in the lamellar skin lipid structure with the alkyl chain penetrating into the plane of the lamellar lipid sheets, allowing both rotational and translational diffusion of the enhancer in the lamellar lipid structure. However, the apparent gel–liquid phase transition, which can be monitored with differential scanning calorimetry (DSC), is largely suppressed after treatment with Azone® [8], while freeze fracture electron microscopy (FFEM) studies have provided evidence for pronounced structural disorder of the lipids in the presence of the enhancer/PG mixture.

^{2}H NMR is an established versatile microscopic technique for the study of mobility. The method has been applied extensively to lipid model systems such as bilayers, micelles, hexagonal phases, etc. In a pioneering effort, ceramide skin lipids were assembled into a model system to study skin lipid bilayer formation using deuterated lipids [9]. In this work we address for the first time the dynamics of a penetration enhancer in situ with ^{2}H NMR of deuterated Azone® in genuine, freshly prepared human SC, in order to gain additional insight into the mechanism of medicinal penetration enhancement at the molecular level.

2. Materials and methods

2.1. Synthesis of deuterated Azone®

For the ^{2}H NMR measurements, both uniformly deuterated and alkylchain-deuterated ([7,8,9,10,11,12,13,14,15,16,17CD_2, 18CD_3], Scheme 1) Azone® were prepared. The synthesis of the chain-deuterated Azone® is a subset of the uniform deuteration scheme, and only the latter will be given.

First a solution of 5.15 g cyclohexanol-d_{12} (MDS isotopes, Montreal, Canada) in 25 ml of acetone was treated with 15 ml Jones reagent [10]. After 5 min the mixture was poured into 25 ml of water, extracted with ether, washed with water and dried with $MgSO_4$. Evaporation of the solvent yielded 4.1 g of cyclohexanone-d_{10} as a colourless oil. To a stirred solution of the 4.1 g cyclohexanone-d_{10} in 113 g of polyphosphoric acid, 2.8 g of NaN_3 was added. The reaction mixture was stirred for 16 h at 65°C, cooled on an ice bath, and treated with a 9 M KOH solution (pH 10–12). This mixture was extracted with dichloromethane, washed with water and dried with Na_2SO_4. Evaporation of the solvent yielded a crude product. After purification by flash column chromatography (eluent, EtOAc/MeOH 95:5) 3.32 g caprolactam-d_{10} was obtained, with a m.p. of 58–60°C. Subsequently a solution of 2.2 g dodecanoic acid-d_{23} in 10 ml of THF was slowly added to a soluion of 0.53 g $LiAlD_4$ (MDS isotopes, Montreal, Canada) in 10 ml of dry tetrahydrofuran (THF). After refluxing for 2.5 h the mixture was cooled to room temperature and 0.6 ml H_2O in 10 ml THF, 0.6 ml 15% NaOH in water and 1.8 ml water were sequentially added. The precipitate was filtered off and washed with THF. The filtrate was dried with $MgSO_4$, filtered and evaporated. Dodecanol-d_{25} was obtained (1.65 g) as a colourless oil which solidified upon standing (m.p. $\approx$ 25°C). The dodecanol-d_{25} (1.65 g) was mixed with 0.71 g PBr_3 and two drops of pyridine. The reaction mixture was stirred for 3 h at 150–160°C. After cooling to room temperature 50 ml of H_2O was added and the mixture extracted with ether (3 × 40 ml). The combined organic layers were dried with $MgSO_4$, filtered,

evaporated, and the compound was obtained in 85% yield. Finally dodecyl-azacycloheptanone-d_{35} was prepared by adding a solution which contained 0.82 g ε-caprolactam-d_{10} in 3 ml of dry toluene to a suspension of 0.35 g NaH in 10 ml of dry toluene. The mixture was refluxed for 1 h in a nitrogen atmosphere. Then a solution of 1.81 g dodecylbromide in 2 ml toluene was added. The obtained mixture was stirred for 20 h at room temperature and subsequently refluxed for 32 h. The precipitated NaBr was filtered off and the crude product was obtained by evaporation of the solvent from the filtrate. After flash column chromatography (eluent, EtOAc/MeOH 39:1) pure uniformly deuterated Azone®-d_{35} was obtained in 74% yield.

2.2. Preparation of stratum corneum samples for NMR

For every sample three 6 cm^2 pieces of human SC, obtained from abdominal surgery were prepared on the day of the operation following the methods described by Boddé et al. [2]. The SC was exposed to moist air above a 27% NaBr solution in ^2H depleted water (MSD Isotopes, Montreal, Canada) for 48 h. Different SC samples (18 cm^2 each) were used for the NMR measurements: (a) untreated (basic control for natural abundance; (b) incubated for 24 h at 32°C upon application of 200 $\mu l\ cm^{-2}$ propylene glycol (PG, J.T. Baker Chemicals B.V., Deventer, The Netherlands) on the anatomical SC surface (vehicle control); (c) incubated for about 1 min at 32°C upon application of 0.15 M uniformly deuterated Azone® in PG (200 $\mu l\ cm^{-2}$) on the anatomical SC surface, followed by an immediate triple wash with pure PG to remove superficial Azone® (validation of the washing procedure); (d) incubated for 24 h at 32°C upon application of 0.15 molar deuterated Azone® in PG (200 $\mu l\ cm^{-2}$) on the anatomical SC surface, whereupon both sides of the SC were washed three times with pure PG to remove any Azone® that might still reside on top of the SC. The washing procedure was identical to the one used in (c). To fit in a 5 mm NMR tube while retaining the orientation of the sheets, the SC samples were cut into 3×10 mm^2 pieces, which were stacked and oriented with respect to the magnetic field.

2.3. NMR spectroscopy

61.4 MHz ^2H NMR data were collected with a single pulse or with the wide line solid echo sequence using a Bruker (Karlsruhe, Germany) MSL400 spectrometer equipped with a standard wideline probe where the sample was surrounded by a glass vacuum dewar flask. The temperature was measured with an accuracy of about 3°C using a thermocouple in the dewar flask near the sample.

For recording the solid echo spectra a $\pi/2_{\phi}-\tau-\pi/2_{\phi+2\pi}-\tau$ pulse sequence with quadrature detection and phase cycling was used with a $\pi/2$ pulse length of 2.7 μs and $\tau = 20$ μs. Recycle delays were 0.5 and 2 s for measurements at room temperature and at low temperatures, respectively. The spectra were recorded with a sweep width (SW) of 166 kHz to obtain good resolution of the echo in the time domain prior to Fourier transformation. The probe and sample were allowed 15 min to equilibrate to a newly set temperature before data acquisition was started. The T_2 relaxation time was measured with the Carr–Purcell/Gill–Meibohm pulse sequence. Deuterium lineshapes were simulated using Matlab (The MathWorks, Inc., Massachusetts).

3. Results

The ^2H spectrum collected with a single pulse from a human SC sample incubated with chain-deuterated Azone®, at room temperature and in a magnetic field oriented parallel to the SC sheets, is shown in Fig. 1, curve a. A liquid-type Azone® resonance is observed with a linewidth of 180 Hz. For comparison, the overall linewidth of the resonance of an 0.15 M solution of chain-deuterated Azone® in PG is 40 Hz (Fig. 1, curve b).

In order to check for the possibility of partial orientation of the chain deuterated Azone® molecules, the sample was cryofixed by lowering the temperature to about 150 K, and solid echo specta were taken at angles of $\beta = 90°$ (Fig. 2,

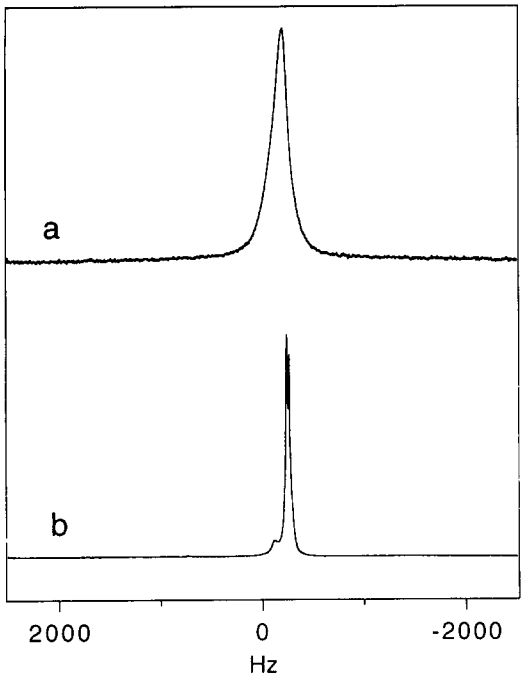

Fig. 1. ^{2}H NMR spectra at room temperature: curve a, human SC pre-treated for 24 h with a 0.15 M solution of chain-deuterated Azone® in PG; curve b, solution of 0.15 M chain-deuterated Azone® in PG.

curve a), $\beta = 54°44'$ (Fig. 2, curve b) and $\beta = 0°$ (Fig. 2, curve c) by rotating the sample tube in the coil. Here β is the angle between the normal of the plane of the SC layers and the magnetic field. The signal is essentially the same at these different angles. The separation $\Delta\nu_{q1}$ of the two outer peaks is 126 ± 1 kHz, while for the inner two peaks $\Delta\nu_{q2} = 36 \pm 2$ kHz.

A freezing transition is observed at $T \approx 228$ K. This is illustrated in Fig. 3, curve b, which shows a solid echo spectrum of uniformly deuterated Azone® in SC at 228 K. At this temperature a narrow resonance is observed at the centre of the spectrum, but in addition two broad peaks with a separation of about 126 kHz are present, indicating that at this temperature at least two phases coexist. For comparison, Fig. 3, curve a shows a solid echo spectrum collected at ambient temperature. The narrow feature in Fig. 3, curve b resembles the response from the sample at room temperature in Fig. 3, curve a, while the broad component in Fig. 3, curve b, resembles the response from the sample at 150 K (cf. Fig. 2, curves a–c).

In order to verify that the observed ^{2}H NMR response was related to the labelled Azone® and not to the natural abundance ^{2}H background of 1.56×10^{-2}%, a sample of SC treated with pure PG was also measured (Fig. 3, curve d). Its spectrum contains essentially only noise. Fig. 3, curve c shows data obtained from a sample that was treated with a solution of uniformly deuterated Azone® in PG which was then immediately washed away with pure PG, to ensure that the Azone® did not have the time to penetrate into the SC. Here also, essentially no signal was measured, so there was no Azone® left on the surface

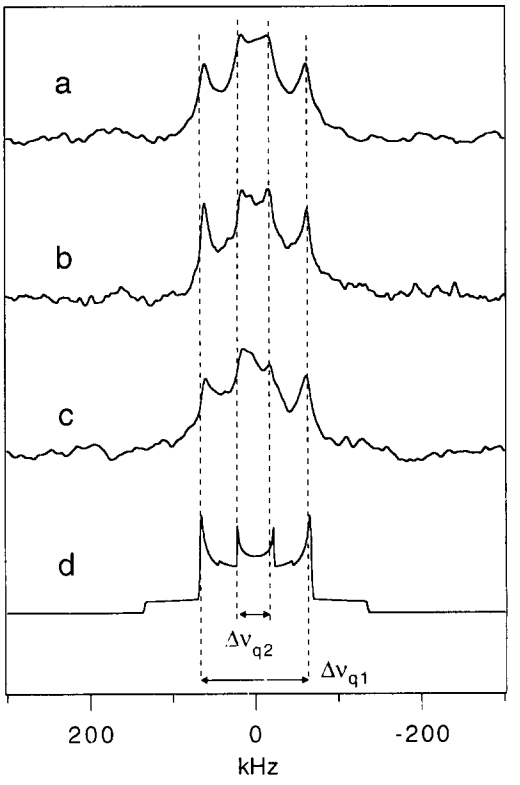

Fig. 2. ^{2}H NMR solid echo spectra of chain-deuterated Azone® pre-treated SC at 150 K at different angles between the normal of the plane of the SC layers and the magnetic field. curve a, $\beta = 90°$; curve b, $\beta = 54°44'$; curve c, $\beta = 0°$; curve d, Plot of the theoretical ^{2}H NMR spectrum $S(\nu)$ using Eq. (1) or 1 methyl- and 11 methylene groups per molecule of Azone®, yielding a superposition of two pake patterns with $\Delta\nu_q = \Delta\nu_{q1} = 126$ kHz and $\Delta\nu_q = \Delta\nu_{q2} = 36$ kHz. Some line broadening was applied to mimic experimental conditions.

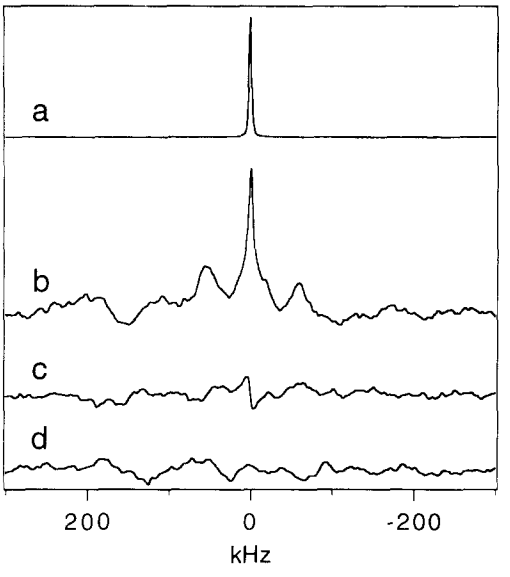

Fig. 3. ^{2}H NMR echo spectra. Curve a, 24 hour incubation of an 0.15 M solution of uniformly deuterated Azone® in PG on SC, room temperature; curve b, 24 h incubation of an 0.15 M solution of uniformly deuterated Azone® in PG on SC at $T = 228$ K; curve c, very short incubation of a 0.15 M solution of uniformly deuterated Azone® in PG on SC, immediately followed by washing; curve d, SC pre-treated for 24 h with PG.

after washing, confirming the efficacy of the washing procedure.

4. Discussion

The ^{2}H NMR can be used to investigate the dynamics of labeled Azone® molecules in SC and the order parameters at the molecular level. The width and shape of the NMR spectrum is mainly determined by the quadrupole interaction of the $I = 1$ ^{2}H nucleus with the microscopic electric field gradient tensor associated with the chemical environment (see, e.g. Ref. [11]). For most aliphatic deuterons the electric field gradient tensor is axially symmetric along the C–D bond vector. An important parameter in ^{2}H NMR is the quadrupole splitting $\Delta\nu_q$ which measures the separation between the two singularities of the familiar ^{2}H lineshape $S(\nu) = S^+(\nu) + S^-(\nu)$ with

$$S\pm(\nu) = (\pm 2\nu/\Delta\nu_q + 1)^{-(1/2)} \quad (1)$$

and ν the observed frequency. Here $S^+(\nu)$ is calculated over the interval $1/2\Delta\nu_q < \nu < \Delta\nu_q$, while $S^-(\nu)$ is calculated for $\Delta\nu_q < \nu < 1/2\Delta\nu_q$. In the case of partially oriented layers

$$\Delta\nu_q = \frac{3}{4}\left(\frac{e^2qQ}{h}\right)S_{CD}\left(\frac{3\cos^2\beta - 1}{2}\right) \quad (2)$$

in which e^2qQ/h is the quadrupole coupling constant in frequency units (169 kHz for aliphatic deuterons) with e the elementary charge, eq the largest principal component of the electric field gradient tensor V_{zz}, Q the quadrupole moment of the nucleus (2.875×10^{-31} m^2 for deuterium) and h is Planck's constant [11]. The scalar effective order parameter

$$S_{CD} = \frac{3\cos^2\theta - 1}{2}. \quad (3)$$

measures the extent of rapid motional time averaging of θ, the angle between the C–D bond vector and the magnetic field. When the bilayer is not oriented or spatial orientation is lost for other reasons, Eq. (2) reduces to

$$\Delta\nu_q = \frac{3}{4}\left(\frac{e^2qQ}{h}\right)S_{CD} \quad (4)$$

In SC treated with deuterated Azone® at room temperature, a single narrow line was observed at different orientations of the sample with respect to the magnetic field (Fig. 1, curve a). The NMR line is 180 Hz wide and is inhomogeneously broadened. The T_2 relaxation time is 9 ms at room temperature which translates via $\Gamma = 1/(\pi T_2)$ into a homogeneous linewidth $\Gamma = 35$ Hz. The upper limit of the order parameter is now given by the ratio of the inhomogeneous linewidth and the linewidth in the case that there is no motion. This means $\Delta\nu_q$ is at most 100 Hz and from Eq. (4) it follows that $S_{CD} = 7.9 \times 10^{-6}$ $\Delta\nu_q < 0.0008$. This reflects rotation of the deuterated probe at room temperature on a timescale which is fast compared with the ^{2}H linewidth [12], in this case with a correlation time $\tau_c \ll 1/(e^2qQ/h) \approx 6 \times 10^{-6}$ s.

Normally, unperturbed SC lipids at room temperature are in a gel state [8,13]. Lipid bilayer gel states have been extensively investigated with ^{2}H NMR. For lipid bilay systems, order parameters

in the range $0.4 > S_{CD} > 0.01$ for methylene deuterons in the hydrophobic tail were reported [14]. Generally, the order parameter decreases going along the hydrophobic chain, yielding a complex lineshape constituting a superposition of pake patterns in multiply labelled systems [15]. Deuterons in the more restricted polar head groups give rise to the larger order parameters up to $S_{CD} = 0.4$ [14]. For instance, SC lipid preparations containing α-CD_2 palmitic acid yield $\Delta\nu_q =$ 35 kHz and $S_{CD} = 0.3$ [9]. If upon entering SC, the deuterated Azone® had inserted in the lipid bilayers without disrupting the gel state, a complex set of scaled powder patterns would have been expected for the ^2H NMR response. In this case the quadrupolar interaction for the methylene deuterons would be only partially averaged, due to anisotropic rotation of the molecules constituting a gel along the aliphatic chain axis. In contrast, the observation of one single narrow line for both the uniformly deuterated Azone® and the penetration enhancer with the entire aliphatic chain deuterated, shows that the deuterated probe molecules approach a liquid-type rather than a gel-type state. This suggests that lipid molecules in the direct vicinity may be in a similar fluid-type state.

To study the possibility of spatial anisotropy with respect to the SC lipid sheets in more detail, the Azone® molecules were cryofixed at about 150 K, and samples were measured at three different orientations with respect to the magnetic field at this temperature. For these experiments chain deuterated Azone® was used to avoid interference from the intrinsically differently orientated C–D bonds in the azacycloheptanone head group of the molecule, as would be the case with uniformly deuterated Azone®. At $T = 150$ K a ^2H NMR static methylene powder-type lineshape with $\Delta\nu_q = \Delta\nu_q^{max} = 126$ kHz is observed (Fig. 2). There is no indication for spatial anisotropy of the Azone® molecules at low temperature, as the separation of the two outer peaks in the spectrum does not vary with the rotation of the sample in the magnetic field.

The observation that the separation of the peaks is independent of the orientation of the SC stack indicates that the Azone® molecules are randomly oriented under the conditions of the 150 K experiment. The NMR signal obtained at either orientation at 150 K strongly resembles a superposition of two random powder patterns with quadrupole splittings $\Delta\nu_q$ of about 126 kHz and 36 kHz respectively. The separation of 126 kHz corresponds to what is expected for static randomly oriented ($S_{CD} = 1$) methylene groups. For a methyl group at the end of an alkyl chain, the quadrupole splitting is scaled with $S_{CD} = 0.36$ to about 36 kHz due to threefold "hopping" at this temperature [16–18]. Because methyl groups tend to freeze only at very low temperatures ($T < 150$ K), the $\Delta\nu_{q2} = 36$ kHz component in the spectra could very well be associated with the end methyl of the Azone®. Comparison with the theoretical lineshape assuming axial symmetry and superposition of two pake patterns (Fig. 2, curve d) showed that the ratio of the integral of the two superpositioned pake patterns is larger than the expected ratio of 3:22 calculated for the methyl and methylene deuterons in the chain-deuterated Azone®. Partial saturation of the methylene NMR signal due to a longer T_1 relaxation time compared to the methyl deuterons at this low temperature can in principle account for this difference.

The results of two important control experiments depicted in Fig. 3, curves c and d also deserve some attention. Fig. 3, curve d shows a spectrum obtained after treating the SC with pure PG. From this data it is obvious that the natural abundance deuterium background signal is not resolved from the noise. The experiment which led to the spectrum shown in Fig. 3, curve c (procedure c) was to validate the washing procedure. The same amount of deuterated PG/Azone® as in procedure (d) was applied, but only for a very short time followed by exactly the same washing procedure as used in procedure (d). The ^2H NMR data collected from this control sample also contain only noise (Fig. 3, curve c). This argues that any deuterium signal observed after the PG/Azone® treatment according to procedure (d) indeed relates only to the Azone® incorporated inside the SC and not to enhancer residing on the SC surface, where it would expectedly be in a liquid state at room temperature.

5. Conclusions

At room temperature the deuterated Azone® molecules exhibit rapid motion inside the SC, isotropic to such an extent that it yields almost a solution-type ^2H NMR spectrum. After cryofixation a random static ^2H NMR powder pattern is obtained, confirming predominantly random orientation of deuterated Azone® in SC.

Provided there is some degree of interaction between the enhancer and the SC lipids, a fluid state may be induced by the Azone®, at least in the direct vicinity of the enhancer molecules. This suggests pronounced molecular disorder at ambient temperatures upon addition of the penetration enhancer, which could very well explain the remarkable reduction of the barrier function of the skin by Azone®. The ^2H NMR observations compare well with FFEM results and small angle X-ray diffraction data that indicated loss of anisotropy in the ultrastructure of the SC lipids, and disorder, in the sense of loss of lamellarity [13]. Whether the rapid, isotropic motion of Azone® in SC only occurs in Azone®-rich droplets in the intercellular domain, or indeed reflects the overall physical state of an intimately mixed Azone®–lipid phase, cannot be ascertained on the basis of the NMR data alone; the FFEM micrographs and the DSC results tend to support the latter explanation [8,13].

The data presented here do not provide additional support for a penetration enhancement mechanism involving individual conformationally perturbed Azone® molecules incorporated into an otherwise intact bilayer, as inferred from molecular modelling studies [2]. In order to further investigate the role of Azone® in the lipid structure perturbation, quanitative structure–activity relationship studies using selectively labeled Azone® isotopomers are currently being carried out.

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References

[1] M.L. Williams and P.M. Elias, The extracellular matrix of stratum corneum: role of lipids in normal and pathological function. CRC Crit. Rev. Therap. Drug Carrier Systems, 3(2) (1987) 95.
[2] H.E. Boddé, M. Ponec, A.P. Ijzerman, A.J. Hoogstraate, M.A. Salomons and J.A. Bouwstra, in K. Walters and J. Hadgraft (Eds.), Skin Penetration Enhancement, M. Dekker, New York, 1993, p. 199.
[3] N.V. Sheth, D.J. Freeman, W.I. Higuchi and S.L. Spruance, Int. J. Pharm., 28 (1986) 201.
[4] H.E. Boddé, M.A.M. Kruithof, J. Brussee and H.K. Koerten, Int. J. Pharm., 53 (1989) 13.
[5] H.E. Boddé, H.L.G.M. Tiemessen, H. Molee and F.H.N. de Haan, in R.C. Scott, R.H. Guy, J. Hadgraft (Eds.), Prediction of Percutaneous Penetration, IBC Technical Services, London, 1990, p. 93.
[6] A.J. Hoogstraate, J. Verhoef, J. Brussee, A.P. Ijzerman, F. Spies and H.E. Boddé. Int. J. Pharm., 76 (1991) 37.
[7] D. Lewis, J. Hadgraft and H.E. Boddé, in R.C. Scott, R.H. Guy, J. Hadgraft and H.E. Boddé (Eds.), Prediction of Percutaneous Penetration/Methods, Measurements, Modelling, ICB Technical Services, London, 1992, p. 355.
[8] J.A. Bouwstra, L.J.C. Peschier, J. Brussee and H.E. Boddé, Int. J. Pharm., 52 (1989) 47.
[9] W. Abraham and D.T. Downing, Biochim. Biophys. Acta, 1068 (1991) 189.
[10] N.J. Doorenbos and M.T. Wu, J. Org. Chem., 26 (1961) 2548.
[11] J. Seelig, Q. Rev. Biophys., 10 (1977) 353.
[12] H.W. Spiess, in Advances in Polymer Science 66, Characterization of polymers in the solid state I, Springer-Verlag, Berlin, 1985, p. 24.
[13] J.A. Bouwstra, G.S. Gooris, J. Brussee, M.A. Salomons-de Vries and W. Bras, Int. J. Pharm., 79 (1992) 141.
[14] U.A. van der Heide, H. Eviatar and Y.K. Levine, in Baranholtz and Lasic (Eds.), Nonmedical Use of Liposomes, CRC Press, Boca Raton, in press.
[15] R.L. Thurmond, G. Lindblom and M.F. Brown, Biochemistry, 32 (1993) 5394.
[16] L.S. Batchelder, C.H. Niu and D.A. Torchia, J. Am. Chem. Soc., 105 (1983) 2228.
[17] K. Beshah, E.T. Olejniczak and R.G. Griffin, J. Chem. Phys., 86(9) (1987) 4730.
[18] A.S. Ulrich, M.P. Heyn and A. Watts, Biochemistry, 31 (1992) 10390.