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Kinetics, ultrastructural aspects and molecular modelling of transdermal peptide flux enhancement by *N*-alkylazacycloheptanones

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Summary

Transdermal delivery is an attractive route to administer peptide drugs. The stratum corneum, however, is a barrier for peptides; hence there is a need for agents to enhance peptide transport. The aim of this study was to investigate the enhancement properties of azacycloheptan-2-ones (Azones) as a function of hydrocarbon chain length. Their ability to enhance the percutaneous penetration of desglycinamide arginine vasopressin (DGAVP) was taken as a criterion. The flux of DGAVP through human stratum corneum was measured. For control experiments the stratum corneum was either untreated or immersed in propylene glycol (PG). The non-enhanced peptide flux through human stratum corneum was 1.6 ± 0.1 nmol/cm^2 per h (peptide concentration in the donor 6.0 mM). Pretreatment with PG or hexyl- or octyl-Azone did not change the flux significantly. However, the permeability increased 1.9-fold after pretreatment with decyl-Azone, 3.5-fold with dodecyl-Azone, and 2.5-fold with tetradecyl-Azone. Electron micrographs taken from freeze-fracture replicas of skin samples treated with either PG or dodecyl-Azone suggest that these treatments do not drastically change the lamellar appearance of the intercellular lipids. Taken together, the morphological and kinetical data suggest that the enhancing effect of Azone is caused by interference with the packing arrangement of the intercellular lamellar lipids in the stratum corneum, most likely by insertion of Azones into the lipid bilayers. In order to assess the capability of the Azone molecule to perturb the lipid arrangement within the bilayers, the degree of conformational freedom in Azone's polar head group was investigated using computer-aided molecular modelling. These calculations indicated that, upon turning the carbonyl moiety towards the interlamellar hydrophilic domain, a 'soup-spoon' conformation can be obtained, which is only 1 kcal/mol away from the minimum energy conformation. This 'soup-spoon' conformation would be highly favourable in order to accomodate the Azone molecule at the interface between the hydrocarbon and polar head group regions, respectively. This conformation might strongly perturb the lipid structure, making it more permeable to the penetrant. Such an effect apparently optimizes around a chain length of about 12 C-atoms.

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Introduction

Peptides are of great therapeutic interest because of their high potency and generally low

toxicity. Peptide drugs, however, cannot be simply administered orally, due to enzymatic degradation in the gastrointestinal tract and hepatic first-pass metabolism (Banga and Chien, 1988; Verhoef et al., 1990). Delivery of these drugs by injection can overcome such problems but requires trained personnel and is often painful. Transdermal delivery of peptides may offer an attractive alternative. This route avoids the aforementioned problems and, furthermore, the transdermal route is favourable because the administration of the drug can be interrupted at will by removal of the topical device. Since the transdermal drug input can be continuous, it is also possible to deliver drugs with short half-lives (e.g., peptides). The application of a transdermal device is easy and painless, thus aiding patient compliance (Barry, 1986; Boddé et al., 1989a).

Recent studies reveal the capability of some peptide drugs to penetrate across the stratum corneum, e.g., thyrotropin-releasing hormone (Burnette and Marrero, 1986), leuprolide (Meyer et al., 1988), des-enkephalin-γ-endorphin (Boddé et al., 1989b), vasopressin (Bannerjee and Ritschel, 1989; Lelawongs et al., 1989) and insulin (Chien et al., 1989; Srinivasan et al., 1989). Because the stratum corneum presents an efficient barrier to transdermal administration of drugs, it seems highly unlikely that application per se to the skin of hydrophilic and high molecular weight compounds like peptides will result in any significant percutaneous transport. Therefore, it will be necessary to improve transdermal transport of peptides by the use of penetration enhancers. *N*-Dodecylazacycloheptan-2-one (Azone) has been shown to be a potent enhancer in transdermal administration of antibiotics, glucocorticosteroids and 5-fluorouracil (Stoughton, 1982). Recently, Azone has also been found to be successful in the enhancement of transdermal transport of peptides (Bannerjee and Ritschel, 1989; Boddé et al., 1989b). The application of undiluted Azone to the human skin surface did not result in any irritation. Moreover, Azone causes only minimal irritation to mucous membranes (Stoughton, 1982).

The mechanism of enhancement of percutaneous drug transport by Azones is still unknown. However, it seems likely that Azone molecules interact with the lipid bilayers in the stratum corneum, and by fluidizing the lipid barrier they may facilitate drug permeation through the intercellular lipid space (Barry, 1987; Boddé et al., 1989c).

The present paper focuses on the transport of the antiamnesic peptide 9-desglycinamide, 8-arginine vasopressin (DGAVP; De Wied et al., 1987) through human stratum corneum in vitro. In addition, the effect of a series of Azones, with aliphatic chain lengths ranging from C6 to C14 was investigated. On the basis of peptide flux studies, freeze-fracture electron microscopy and computer-aided molecular modelling, a mechanism of action is proposed to explain the observed enhancing effects.

Materials and Methods

Chemicals

9-Desglycinamide, 8-arginine vasopressin (DGAVP) citrate was a generous gift from Dr J.W. van Nispen (Organon international, Oss, The Netherlands). The amino acid structure of this neuropeptide (MW = 1412) is:

H-Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-OH

A number of *N*-alkylazacycloheptanones (Azones) were synthesized by the Department of Organic Chemistry (Leiden University, Leiden, The Netherlands), including hexyl-, octyl-, decyl-, dodecyl- and tetradecyl-Azone. The chemical structure of dodecyl-Azone is shown in Fig. 1. Propylene glycol (PG) was obtained from J.T. Baker Chemicals B.V. (Deventer, The Netherlands). All chemicals used were of analytical grade and dissolved in freshly prepared bidistilled water.

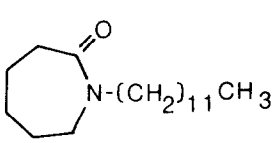

Fig. 1. Chemical structure of *N*-alkylazacycloheptan-2-one (*N*-dodecyl-Azone).

Preparation and analysis of Azones

The *N*-alkylazacycloheptan-2-one derivatives were prepared as follows. 11 g 55–60% NaH dispersion in mineral oil (0.25 mol) was washed twice with 75 ml of petroleum ether 40–60. The petroleum ether was decanted and the NaH was suspended in 250 ml of dry toluene and placed in a three-necked, round-bottomed flask that had a mechanic stirrer and an N_2 gas inlet. 20 g of azacycloheptan-2-one (0.177 mol) dissolved in 200 ml of dry toluene was added in a dropwise manner under stirring in a dry N_2 atmosphere. Subsequently, the reaction mixture was refluxed for 1 h and then cooled to room temperature. Alkyl bromide (0.200 mol) mixed with 200 ml of dry toluene was added in a dropwise manner. The reaction mixture was stirred under nitrogen for 24 h at room temperature, followed by heating to reflux for various periods of time, depending on which alkyl bromide was used. After cooling, formed NaBr was filtered off and the filtrate was concentrated by distillation under reduced pressure. The remaining oil was purified by fraction distillation under reduced pressure. The purity of the compounds was verified by NMR spectroscopy. The yield of product was 50–80%.

Preparation of the skin

Human inguinal skin was obtained from the Dutch Burn Society (Beverwijk, The Netherlands), from a 22 year old male donor. After removal of the subcutaneous fat, the skin was dermatomed at a thickness of about 200 μm and incubated on a filter paper soaked in a 0.2% trypsin (type III; from bovine pancreas) solution in isotonic phosphate buffered saline (PBS, pH 7.4) for 24 h at 37°C. By this splitting technique the stratum corneum can be separated from the underlying epidermis. Because the remaining trypsin might affect the integrity of the stratum corneum and also interfere with peptide stability, the stratum corneum was subsequently treated with a 0.2% solution of trypsin inhibitor (type II-S from soybean; Sigma Chemicals, The Netherlands) in PBS. Then the stratum corneum was dried and stored in a desiccator over sillica gel.

Before use the stratum corneum was punched into circular samples of 14 mm diameter and hydrated to a relative humidity of 50% at room temperature over a PBS solution of pH 7.4 (Tiemessen et al., 1989). To this buffer penicillin and streptomycin were added to prevent bacterial growth, and amphotericin B to prevent fungal growth. Dialysis membrane disks (cut-off value: 5000 Da, Diachema, Switzerland) with a diameter of 18 mm were placed on each side of the stratum corneum to avoid tissue damage. The dialysis membranes did not influence peptide transport through the stratum corneum. When a penetration enhancer was used, the prehydration was followed by pretreatment with the enhancer. For this purpose the stratum corneum samples were submerged in the enhancer solution (PG, as a cosolvent, or a 0.15 M solution of the enhancer in PG) for 24 h at room temperature. Thereafter the pretreated stratum corneum was sandwiched between two dialysis membrane disks.

In vitro penetration equipment

A sandwich of human stratum corneum and dialysis membranes was clamped horizontally in a diffusion cell made of polytetrafluoro ethylene (Tiemessen et al., 1988), with a stirred donor compartment, initially containing 1 ml of a 5 mM solution of the peptide in PBS. The area of diffusion was 0.79 cm^2. The isotonic PBS solution (pH 7.4) was pumped through the acceptor compartment at a rate of 1 ml per h and samples were collected at hourly intervals for 18 or 19 h with a fraction collector. Experiments were performed at 33 °C. Collected fractions were stored at −20 °C until analysis.

Permeation experiments

Permeation experiments were performed using human stratum corneum, either untreated or pretreated with PG or a 0.15 M solution of Azone in PG. The Azones studied were tetradecyl- (C14), dodecyl- (C12), decyl- (C10), octyl- (C8) and hexyl- (C6) Azone, respectively.

DGAVP analysis

Concentrations of DGAVP in the collected acceptor fractions were determined using a sensitive and specific radioimmunoassay (Van Bree et al., 1988). The limit of detection was approx. 200

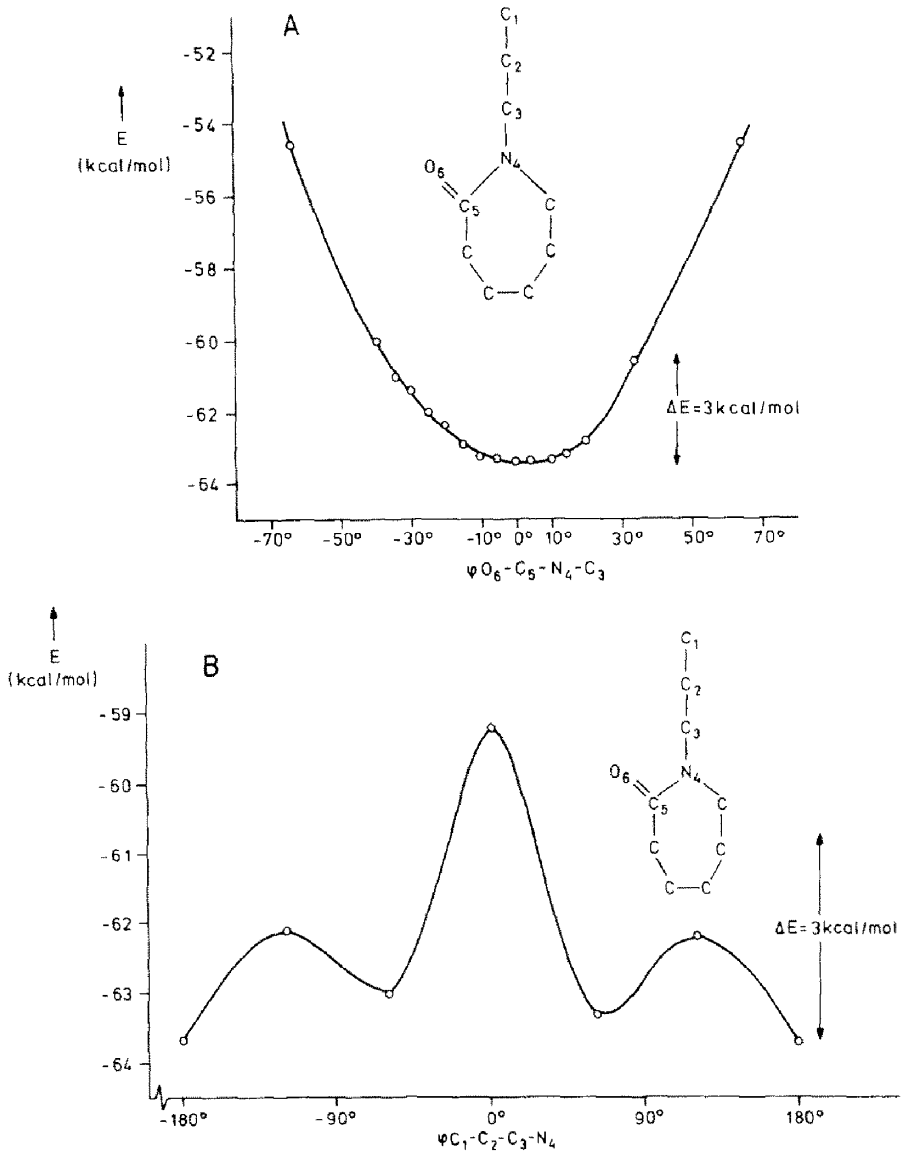

Fig. 2. Conformational energy of *N*-propylazacycloheptan-2-one as a function of the torsion angle of (a) its peptide bond and (b) the C1-C2-C3-N4 moiety.

pg/ml. Inter-assay variation of the procedure was found to be less than 15%.

Because Azones hinder the formation of the DGAVP-antibody complex, the samples were subjected to an extraction with ethyl acetate, in order to remove the enhancer prior to the radioimmunoassay. The recovery of DGAVP from the aqueous phases obtained after extraction was about 90%.

Data analysis

Data were plotted as the cumulative amount of DGAVP collected at the receiver end of the

diffusion cell vs time. The permeability coefficient, P, was calculated from the formula:

$$P = [1/(CA)][dQ/dt] \quad (1)$$

in which C is the concentration difference across the stratum corneum, A (0.79 cm^2) is the effective cross-sectional area available for diffusion and dQ/dt is the steady-state slope of the cumulative penetration curve (Crank, 1975). By dividing the permeability coefficient determined in the presence of an enhancer by that obtained from the untreated control, the enhancement factor (E) was calculated. A one-way analysis of variance was used to test statistical significance of differences.

Electron microscopy

To obtain perpendicular cross fractures, sample holders with a cylindrical shape made of 83.5% silver and 16.5% copper were used, in which the sample can be manipulated very easily. These cylinders form the upper parts of specimen holders normally used for the examination of cell suspensions; they are open at both ends. Enhancer-treated and untreated skin samples were cut into small pieces with a razor blade. The obtained samples were folded in a tunnel-shape and placed in a cylinder with both ends inside the samples; subsequently, they were rapidly frozen in liquid propane (Cryo-jet QFD 020 Balzers, Liechtenstein). Under liquid nitrogen, the cylinders were placed on an adapted specimen stage and placed in the recipient of a freeze-fracture device (BAF 400D, Balzers, Liechtenstein) on a precooled table (−150°C). After evacuation ($p < 10^{-6}$ Torr) the table temperature was increased to −115°C and the skin fractured by the following procedure: the knife edge was adjusted to the same level as the top ends of the cylinders. Fracturing occurred by a single firm forward movement of the knife. Replicas were made by evaporating platinum from a platinum/carbon source (45°, 2.5 nm) and by subsequently depositing a carbon backing layer (90°, 35 nm) on the surfaces in a unilateral direction. Finally, the specimens were removed from the vacuum chamber and cleaned (Holman et al. 1990). The obtained replicas were mounted on copper grids (400 mesh) and examined in a Philips EM 300 electron microscope operated at 80 kV.

Computations

Visualisation and manipulation of Azone structures were performed with the molecular modelling program Chem-X (April 1989 update) running on a VAX 11/785 computer and employing a Pericom MX 7200 color display (Chem-X: Molecular Modelling System. Chemical Design Ltd., Oxford, U.K.). In order to save CPU time the hydrocarbon chain of the Azone molecule was shortened to yield *N*-propylazacycloheptan-2-one. The minimum energy conformation of this Azone analogue and subsequent conformational searches were computed on a Convex C-120 mini-super computer with the semi-empirical molecular orbital MOPAC program (Stewart, 1990). The AM1 Hamiltonian and Pulay's method of convergence were used.

The rotational flexibility between the ring system and the aliphatic side chain was studied as follows. The minimum energy conformation of 1-propylazacycloheptan-2-one calculated in MOPAC was used as a starting point for separate conformational searches also performed in MOPAC over two torsion angles (φ) with steps varying from 5° ($\varphi_{O6\text{-}C5\text{-}N4\text{-}C3}$) to 60° ($\varphi_{C1\text{-}C2\text{-}C3\text{-}N4}$) (for atom numbering see Fig. 2a). The calculation of each step was started from the conformation found in the previous step. All other torsion angles in the molecule were allowed to relax fully.

Results

Penetration experiments

Fig. 3 shows the cumulative permeation-time profile of DGAVP through non-pretreated human stratum corneum. The peptide flux through this tissue is of the order of 1 pmol/h per cm^2 (donor concentration of DGAVP: 5 mM). The corresponding permeability coefficient, calculated from Eqn 1, is $5.3 \pm 0.6 \times 10^{-11}$ cm/s (Table 1).

Pretreatment of the skin tissue with PG, hexyl-Azone and octyl-Azone, respectively, did not significantly affect the transport of DGAVP

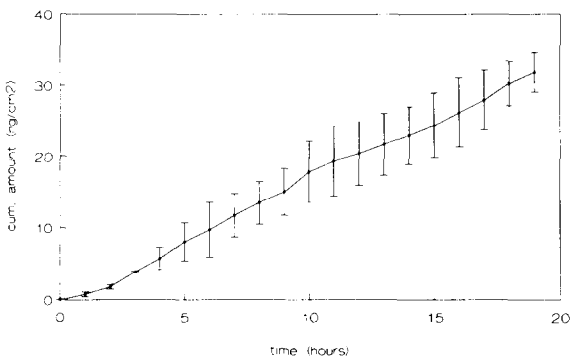

Fig. 3. Cumulative penetration of DGAVP through human stratum corneum as a function of time. Data are the means ± S.D. of 3 experiments.

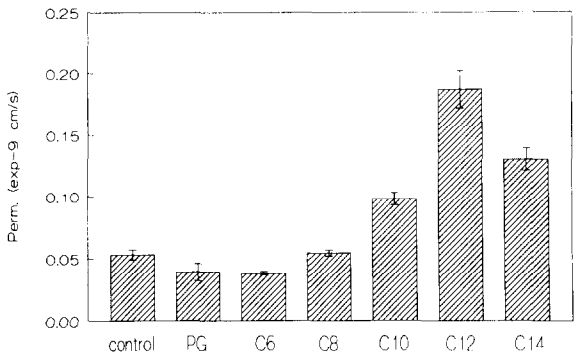

Fig. 4. Bar diagram of the permeability coefficients corresponding to the penetration of DGAVP through human stratum corneum, either untreated (control) or pretreated with various enhancer solutions.

across the stratum corneum in vitro (Table 1 and Fig. 2). However, pretreatment with decyl-, dodecyl- or tetradecyl (myristyl)-Azone increased DGAVP transport through human stratum corneum significantly as compared to the control (Fig. 4). From Table 1, it is evident that dodecyl-Azone yielded the highest enhancement factor, being 3.5, for percutaneous DGAVP transport, whereas those for decyl- and tetradecyl-Azone were lower (1.9 and 2.5, respectively).

Fig. 5 presents transmission electron micrographs taken of freeze-fracture replicas of skin samples treated with PG and *N*-dodecylazacycloheptan-2-one in PG, respectively. The skin samples were cross-fractured such as to reveal the ultrastructure of stratum corneum in a plane roughly perpendicular to the anatomical surface. Fig. 5a shows a corneocyte with intact lipid multilamellar stacks still attached; the cell is surrounded by PG (typical isotropic, granular fracturing pattern), which seems to have intruded into intercellular space in the outermost squamous layer. Typically, neither PG has interfered with the lamellar lipid structure, nor does the corneocyte seem to have taken up PG. Fig. 5b indicates the persistence of lamellar lipid aggregates in the intercellular space, despite the Azone treatment.

TABLE 1

Permeabilities and enhancement factors of the penetration DGAVP through human stratum corneum, untreated or pretreated with enhancers

	Permeability ± S.D. (cm/s) ($\times 10^{-11}$)	Enhancement factor ± S.D.
Control	5.3 ± 2.1	1.00
PG	4.0 ± 0.8	0.75 ± 0.15
C14-Azone	13.0 ± 0.9	2.45 ± 0.18
C12-Azone	18.7 ± 1.9	3.53 ± 0.35
C10-Azone	9.9 ± 0.6	1.86 ± 0.12
C8-Azone	5.4 ± 0.6	1.03 ± 0.11
C6-Azone	3.8 ± 0.2	0.71 ± 0.03

Data represent the mean ± S.D. of 3 experiments.

Computations

The heat of formation of 1-propylazacycloheptan-2-one as computed by MOPAC was −63.92 kcal/mol with $\varphi_{C1\text{-}C2\text{-}C3\text{-}N4}$ being 180° and $\varphi_{O6\text{-}C5\text{-}N4\text{-}C3}$ around 0°. Within an energy window of 3 kcal/mol above the minimum energy conformation these torsion angles could adopt values of $-40° < \varphi_{O6\text{-}C5\text{-}N4\text{-}C3} < +35°$ and $\varphi_{C1\text{-}C2\text{-}C3\text{-}N4} < -30°$ or $> +30°$, respectively. The energy involved in torsion around $\varphi_{O6\text{-}C5\text{-}N4\text{-}C3}$ and $\varphi_{C1\text{-}C2\text{-}C3\text{-}N4}$ is plotted in Fig. 2a and b, respectively, as a function of the torsion angle. The Azone conformation corresponding to the carbonyl group being tilted away from the minimum energy over only 10° is illustrated in Fig. 6. This tilting procedure yielded a 'soup-spoon'-like structure which fitted well within these ranges with $\varphi_{O6\text{-}C5\text{-}N4\text{-}C3} = 10°$ and $\varphi_{C1\text{-}C2\text{-}C3\text{-}N4} = 62°$.

The heat of formation of this particular conformation, as calculated for *N*-propylazacycloheptan-2-one, was −63.08 kcal/mol, less than 1 kcal/mol above the minimum energy level.

Discussion

Barry (1987) has pointed out that propylene glycol might be able to reduce the hydration of

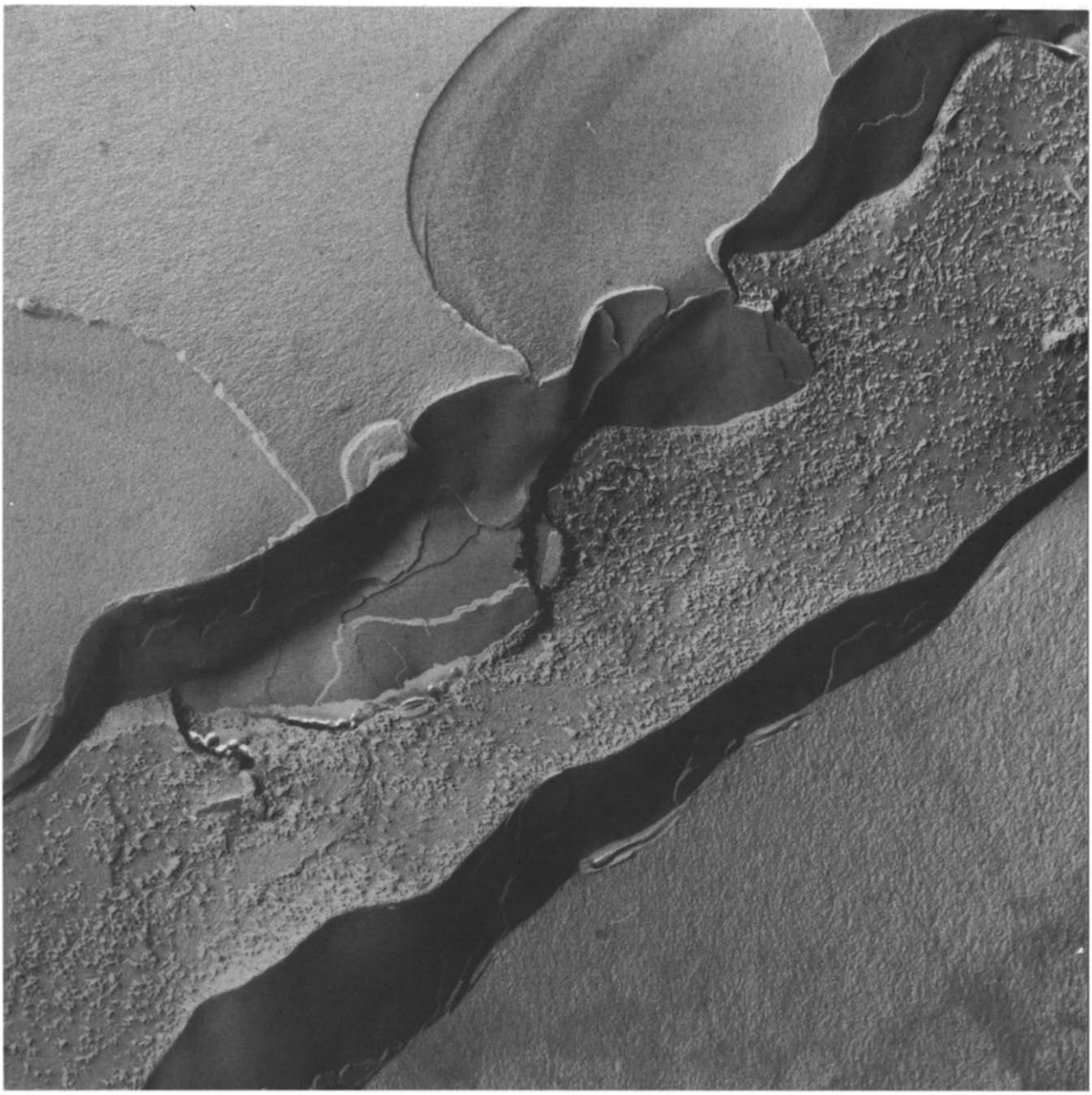

Fig. 5. Freeze-fracture electron micrographs of human stratum corneum treated with: (a) propylene glycol; (b) 0.15 M *N*-dodecylazacycloheptan-2-one in propylene glycol.

human stratum corneum. Since most of the water is presumably accumulated inside the corneocytes (Middleton, 1969; Van der Merwe and Ackerman, 1987), and since propylene glycol apparently does not penetrate into the corneocytes, it may withdraw water from the corneocytes by virtue of its hygroscopic properties. This concept would also agree with the results obtained from thermo-

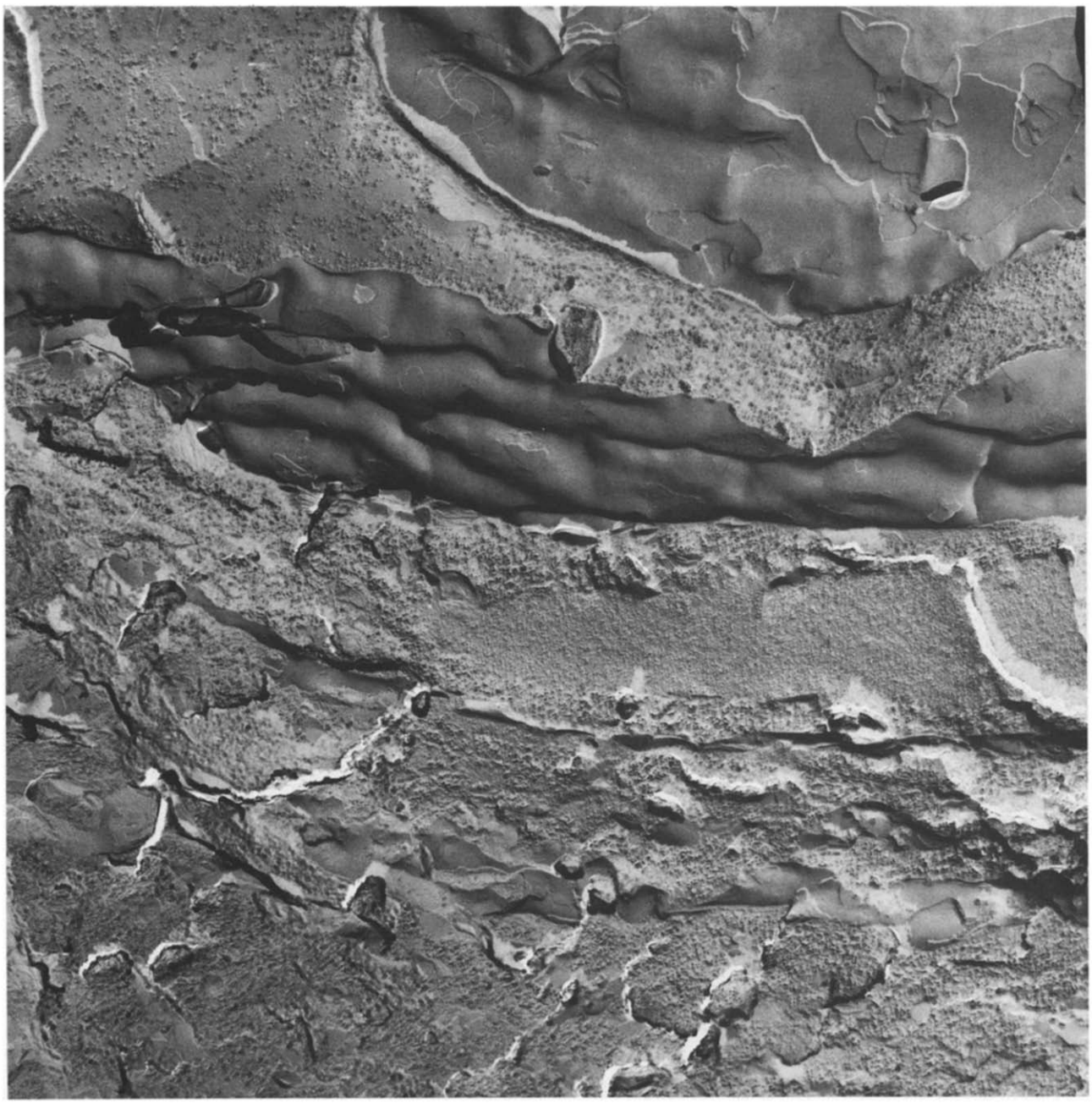

Fig. 5 (continued).

Fig. 6. 'Soup-spoon' conformation with Van der Waals contours of *N*-dodecylazacycloheptan-2-one, obtained through torsion of the peptide bond over 10°, with $\varphi_{C1\text{-}C2\text{-}C3\text{-}N4} = 62°$ (see text).

analytical studies by Bouwstra et al. (1989), which indicated that a major effect of propylene glycol is to make the thermal denaturation peak of the protein in stratum corneum vanish, possibly due to protein dehydration. The mere fact that an intense, 24 h propylene glycol treatment does not significantly change the rate of transport of DGAVP across human stratum corneum makes it very unlikely that the peptide would penetrate across corneocytes, and suggests that DGAVP diffuses primarily along intercellular pathways. The existence of intercellular pathways for hydrophilic compounds through stratum corneum has been unequivocally demonstrated in electron microscopical studies by Boddé et al. (1989c, 1990) and by Potts and Francoeur (1991) in carefully analyzed kinetic studies.

Decyl-, dodecyl- and tetradecyl-Azone appear to be effective enhancers for the percutaneous transport of DGAVP. Previous studies regarding the effect of dodecyl-Azone on in vitro percutaneous transport of DE E, a neuropeptide with a molecular weight comparable to that of DGAVP, resulted in an almost equally high enhancement factor (Boddé et al., 1989b). In a recent review, Wiechers and De Zeeuw (1990) have given an overview of percutaneous flux enhancement by dodecyl-Azone; their compilation of data also included enhancement factors obtained in vivo in man with a variety of drugs and model compounds. Interestingly, the enhancement factors obtained on human volunteers were scattered within a rather narrow range (between 1.2 and 9); the values we obtained with DGAVP and with DE E (Boddé et al., 1989b) lie well within this interval, suggesting our in vitro model to be quite realistic. However, with animal skin pretreated with dodecyl-Azone, much higher permeabilities have been obtained. Using vasopressin as a permeant and dodecyl-Azone as an enhancer, Bannerjee and Ritschell (1989) reported enhancement factors of 70 and 15 for mouse and rat skin, respectively. Within the range of *N*-alkyl-Azones studied, the enhancement effect as a function of the alkyl chain length presents a bell-shaped dependence, the optimum corresponding to *N*-dodecyl-Azone.

Differential thermal analysis has shown a higher mobility of the stratum corneum lipids when *N*-alkyl-Azones with alkyl chains of 8 C-atoms or more were added (Bouwstra et al., 1989). Recent small-angle X-ray studies on the effects of *N*-alkyl-Azones on stratum corneum lipids (Bouwstra et al., 1990) have shown the longer chain Azones (longer than C10) to decrease significantly the degree of order in the lipid lamellar structure, without the lamellar structure disappearing. Altogether, these data indicate that Azones can, at least to some extent, insert into the intercellular lipid bilayers and, depending on the length of their alkyl moiety, distort the lipid structure or packing order to a certain degree. It is most likely this property that causes some of the Azones to enhance effectively the percutaneous peptide flux. The fact that Azones which interact strongly with the intercellular lipids are effective as peptide transport promotors suggests the lipid lamellae to present the principle barriers for peptide diffusion across stratum corneum.

Some investigators have sought an explanation for the mode of action of Azone(s) as penetration enhancers. As a first approximation to the prob-

lem, Barry (1987) proposed dodecyl-Azone to insert into the lipid bilayers and somehow distort either the hydrophobic alkyl chain regions, or the head-group regions, or both. Later on, a spoon-shaped model of Azone was proposed to account for its lipid disordering capability (Boddé, 1989). This model was based on the crucial assumption that the carbonyl moiety belonging to the amide group, being the strongest hydrogen bond former in the Azone molecule, would 'try' to turn itself away from the lipid bilayer and into the polar region as much as possible. This would require a 'twist' in the ring, almost inevitably pushing it upwards so as to create a 'soup-spoon' conformation. This conformation can be obtained by only imposing a torsion around the bond between the carbonyl-carbon and the adjacent nitrogen atom. We have included the corresponding energy calculations and the results shown in Figs 2 and 6 stem directly from the aforementioned assumption. The spoon conformation presented here should be easily attainable, since it is only 1 kcal/mol away from the minimum energy conformation; it would even become energetically favourable, if the carbonyl group were able to form hydrogen bonds with polar groups facing it from an opposite head-group region, or with interstitial water. The soup-spoon conformation not only could provide a rationale for the flux enhancement by Azones, it might also explain why a minimal chain length would be needed: namely, to ensure the required affinity of the Azone for the bilayer to make sure that it will insert. On the basis of compressibility studies on 'Azone-spiked' phospholipid monolayers, Lewis and Hadgraft (1990) recently came up with a spoon-shaped conformation for Azone, but they based their calculations on torsions around different bonds (situated inside the hydrocarbon chain), without making clear why such torsions would be energetically favourable within the anisotropic surroundings of a bilayer.

In conclusion, this study firstly provides evidence that, even in the absence of enhancers other than water, a peptide can cross the human stratum corneum in vitro at a measurable rate. The results furthermore suggest that the peptide (DGAVP) traverses the stratum corneum along intercellular pathways, the intercellular lipid lamellae presenting a strong barrier. Pretreatment of the stratum corneum with enhancers of the Azone type results in a significantly increased peptide flux, the degree of enhancement depending on the Azone hydrocarbon chain length and the effect most likely being due to a distortion of the lipid packing order, possibly triggered by an energetically favoured 'twist' in the Azone molecule causing it to adopt a typical 'soup-spoon' conformation.

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