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Effect of *N*-alkyl-azocycloheptan-2-ones including azone on the thermal behaviour of human stratum corneum

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Summary

In this study the thermal behaviour of human stratum corneum is investigated using *N*-alkyl-azacycloheptan-2-one with a varying number of carbon atoms in the alkyl chain. Throughout this article these substances will be referred to as C_n azones, in which n stands for the number of C atoms in the alkyl chain. The experiments have been carried out using differential thermal analysis (DTA). Untreated stratum corneum shows 4 transitions of which 3 are reversible and one is irreversible. The latter is due to protein denaturation, the former are due to gel–liquid state transitions of the lipids. Treatment with C_n azones in combination with propylene glycol shifts the lipid transition peaks, normally found at 345K and 360K, to lower temperatures; treatment with C_{12} azone decreases the enthalpy involved in the transitions, whereas treatment with C_6 azone affects only the temperature of two transitions and not the enthalpy. A decrease in enthalpy is related to an increase in fluidity of the lipid bilayers; there seems to be a close parallel between the change in enthalpy and the change in permeability of stratum corneum.

Introduction

For most substances, the rate-determining step for penetration through the skin is situated in the outermost layer of the skin, the stratum corneum (Scheuplein, 1965). The stratum corneum consists of a regular array of protein-rich cells, the corneocytes, which are embedded in lipids arranged in a lamellar structure consisting of hydrophobic and hydrophilic layers. The diffusion of drugs occurs either through the intercellular space or partly through the cells and partly through the intercellular space. The diffusion can be increased by penetration enhancers such as DMSO, urea, and C_n azones. The latter are more effective in combination with polyalcohols (Hadgraft, 1984). Although penetration enhancement is described extensively in literature (Hadgraft, 1984; Woodford and Barry, 1986; Boddé et al., 1988), the mechanism of action of enhancers is still unclear. To obtain more insight into these mechanisms it is recommended that a variety of techniques should be used in order to build up a complete picture of penetration enhancement. In this study differential thermal analysis (DTA) is used. A comparison of the results presented here with electron microscopy and kinetic data will be given in a forthcoming paper.

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From the literature (Wilkes et al., 1973; van Duzee, 1975; Golden et al., 1986) it is known that stratum corneum shows two types of thermal transitions: the denaturation of proteins and the gel–liquid transitions of the alkyl chains of the lipid bilayers. The latter are reversible transitions, the former is an irreversible one. In a temperature range of 280K to 400K at least 4 thermal transitions can be distinguished. The peaks at 315K and 345K originate from gel–liquid transitions of the lipid bilayers. This may be concluded from X-ray diffraction data (Wilkes et al., 1973) and the reversible character (van Duzee, 1975) of the transitions. The transition at 385K is an irreversible one and is therefore ascribed to the denaturation of proteins. The origin of the transition at 360K is still a point of discussion: although the transition is a reversible one, thermal pretreatment does clearly affect the position of the peak (van Duzee, 1975).

An interesting feature is the effect of penetration enhancers on the thermal behaviour of stratum corneum; that is, on the transition temperature and on the enthalpy of transition of the lipids in the 340–360K range. Golden et al. (1987) studied the effect of *cis*- and *trans*-octadecanoic acid on these transitions and observed a correlation between a decrease in transition temperature and an increase in motional freedom of the hydrocarbon chains. The increased motional freedom corresponded with an increased permeability of stratum corneum at physiological temperatures.

In this study we have focused our attention on C_n azones in combination with propylene glycol. To investigate the effect of C_n azones on the stratum corneum the thermal behaviour of stratum corneum treated with a number of azones with variable chain length (C_6 to C_{16}) in propylene glycol was measured. Fresh human skin was used throughout the study.

Materials and Methods

Synthesis of C_n azones

The azones were prepared by a modified synthesis described in the literature (Nelson Research, 1976).

11 g of 55–60% NaOH dispersed in mineral oil (0.25 mol) was washed twice with 75 ml petroleum either 40–60. The petroleum ether was decanted and the NaH was suspended in 250 ml dry toluene and placed in a 1 liter three-neck roundbottom flask provided with a mechanical stirrer and a N_2-gas inlet. 20 g azacycloheptan-2-one (0.177 mol) dissolved in 200 ml dry toluene was added dropwise under stirring in a dry N_2-atmosphere. After the addition the reaction mixture was refluxed 1 h and then cooled to room temperature. Alkylbromide (0.200 mol) mixed with 200 ml dry toluene was added dropwise. The reaction mixture was stirred under nitrogen for 24 h at room temperature followed by heating to reflux for a specified period of time (*A* h; see Table 1) depending on the alkylbromide used. After cooling the NaBr formed was filtered off and the filtrate was concentrated by distillation under reduced pressure. The purity of the compounds, being better than 97%, was controlled by NMR spectroscopy. The yield of the product was 50–80%.

Sample preparation

Fresh human skin obtained from breast or abdominal surgery was prepared immediately upon arrival on the day of operation. The stratum corneum was separated from fresh skin in two steps. In the first step the dermis was mechanically removed from the epidermis using an electrodermatome set at 120 μm thickness. In the second step the stratum corneum was removed from the epidermis by digestion in a 0.1% trypsin solution in PBS for one night at 37 °C. The stratum corneum was washed and dried over silica in

TABLE 1

The period (A hours) the mixture of toluene-alkylbromide and azocycloheptan-2-one was stirred and the distillation conditions thereafter (b.p. = boiling point)

Alkyl group	A (hours)	b.p. (K/mm Hg)
Hexyl	8	398–399/18
Dodecyl	12	386–387/0.3
Hexadecyl *	40	–

* Solid product resulted; crystallisation from petroleum ether 40–60 yielded the product, m.p. 35 °C.

a vacuum desiccator and subsequently hydrated to 20% over a saturated solution of NH_4Cl. Treatment with propylene glycol or 10 w/w% C_n azones in propylene glycol was accomplished by soaking the stratum corneum in the solutions for 24 h. The residual amount of propylene glycol solution was removed by blotting the sample. The thermal analysis experiments were performed with approximately 20 mg hydrated stratum corneum. The measurements were carried out in hermetically closed pans at a heating rate of 2K/min in a temperature range from 280K to 400K. The measurements were carried out with a DTA apparatus of Maple Instruments. In the case of differential thermal analysis (DTA), the difference in temperature between sample and reference pan is measured keeping the heat flow to reference and sample pan equal. This method differs from that used in differential scanning calorimetry (DSC), in which the difference in heat flow to the reference and the sample pan is measured keeping the two pans on the same temperature.

The samples were weighed before and after the experiments to check the water loss during the measurements.

Results

Fig. 1 shows the thermal behaviour of abdominal stratum corneum and mamma stratum corneum. A comparison is also made between mamma stratum corneum from two different sources (A and B). Because mixtures are involved in the thermal transitions the position of the top of the peak is taken as the transition temperature rather than the onset of transition as is ordinarily done in case of pure substances. The variability in thermal behaviour between abdomen and mamma stratum corneum is comparable to the variability in thermal behaviour among mamma stratum corneum samples from different sources; the lipid transitions around 345K and 360K have the same intensity and the same position in all four DTA runs, whereas the transitions around 315K and 385K are only produced occasionally. Mamma stratum corneum from one and the same source shows minor differences in thermal behaviour. This can be caused by minor differences in hydration: only the peak around 385K in the two scans indicated by mamma B in Fig. 1 differ in temperature and intensity. This is the transition of which the enthalpy change is most hydration dependent (van Duzee, 1975). Due to the small differences in thermal behaviour of stratum corneum from different origin it is recommended that in comparing the thermal behaviour of treated and untreated stratum corneum always stratum corneum from the same donor should be used.

The origin of the peak at 360K is still under debate. Van Duzee (1975) and Golden et al. (1986) extracted lipids from stratum corneum and measured the thermal behaviour of the lipids and the residual stratum corneum separately. Van Duzee found two transitions in the residual stratum corneum and concluded that the transition at 360K was due to denaturation of protein. He also based his conclusions on the effect of urea on the thermal behaviour of the transition at 360K; urea only influenced the thermal behaviour of that transition if the stratum corneum was pretreated with acetone which destroyed the cell membranes. From this it was concluded that this peak is caused by protein denaturation in intercellular spaces. Golden found one peak in the extracted lipids and concluded that the transition at 360K is a lipid transition but due to the interactions with proteins this transition is shifted to higher temperatures. In this study the origin of the 360K peak was investigated by determining the change in enthalpy and temperature of the lipid transitions at 345K and 360K after thermal pretreatment. Fig. 2 shows the effect of thermal pretreatment on these transitions. In the first scan the stratum corneum was heated from 280K to 360K: only the transitions at 345K and 360K are detected. In the second heating scan the stratum corneum was reheated to 403K, which is above the denaturation temperature of the proteins. The thermal behaviour of the stratum corneum in the second scan resembled that of the first scan. In the third scan the stratum corneum was again heated to 403K. In this heating scan only one peak was observed at a transition temperature of 345K. The enthalpy (7.2 + 0.5 J/g) involved in this transition was, within the experimental accuracy, equal to the sum of the

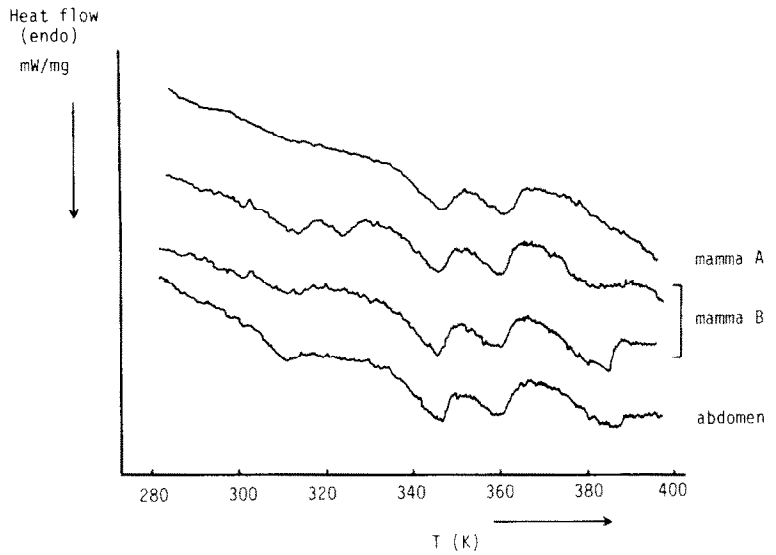

Fig. 1. Thermal analysis of stratum corneum containing 20% water.

enthalpies involved in the two separate transitions of the first and second scan. Therefore the transition at 360K shifted to approximately 345K; due to the denaturation of the proteins, as a result of the second heating scan, no distinction can be made between transitions which originally appear at 345K and 360K. Although these experiments are carried out with stratum corneum pretreated with propylene glycol the same behaviour holds for untreated stratum corneum: the experiments (the second and third scan) have been reproduced with untreated stratum corneum. It could be argued that the observed downward shift of the 360K peak might be due to water loss. However, this is not very likely for at least two reasons. In the first place, water loss during the experiment would result in an enthalpy change involved in both transitions: the enthalpy and temperature of transition both depend on the water content of stratum corneum. Secondly, water loss should result in an increase in both transition temperatures (Golden et al., 1986); only a decrease in transition temperature is observed. Van Duzee (1975) already measured the effect of thermal pretreatment

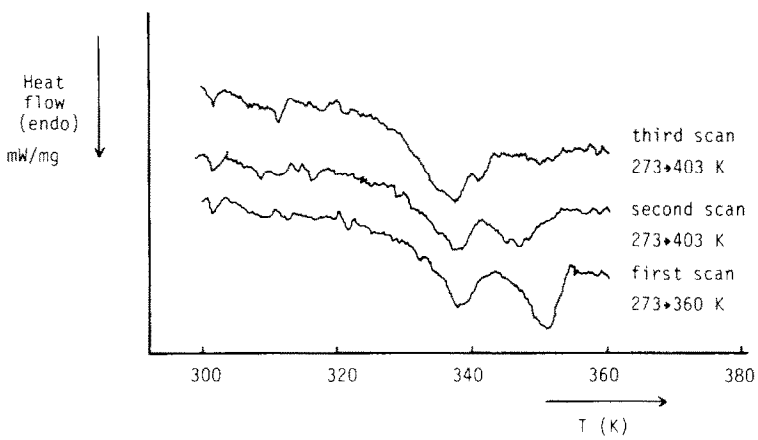

Fig. 2. Gel–liquid state transitions of lipids in stratum corneum pretreated with propylene glycol. Influence of pretreatment.

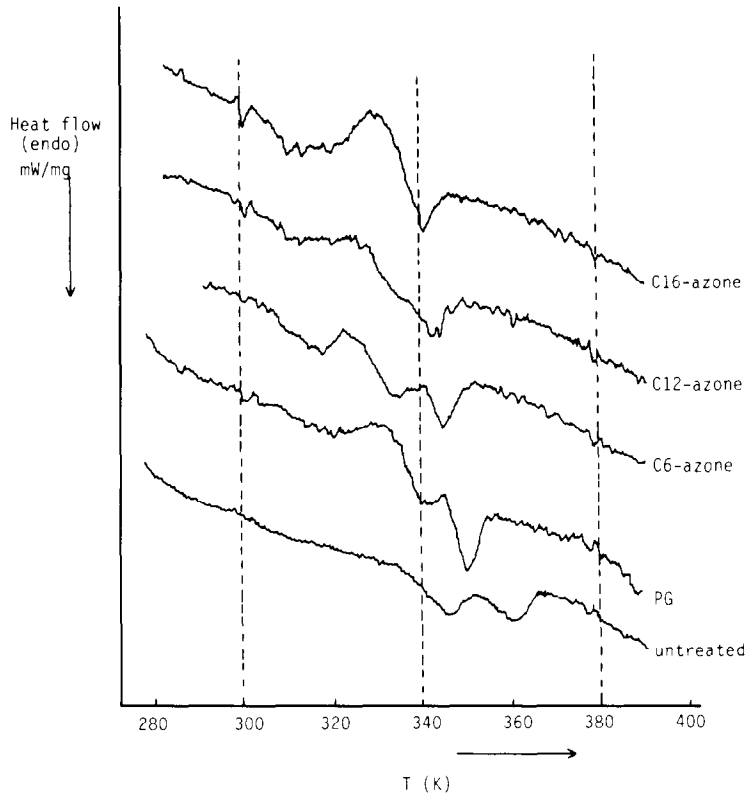

Fig. 3. Influence of azones and propylene glycol on the thermal behaviour of stratum corneum.

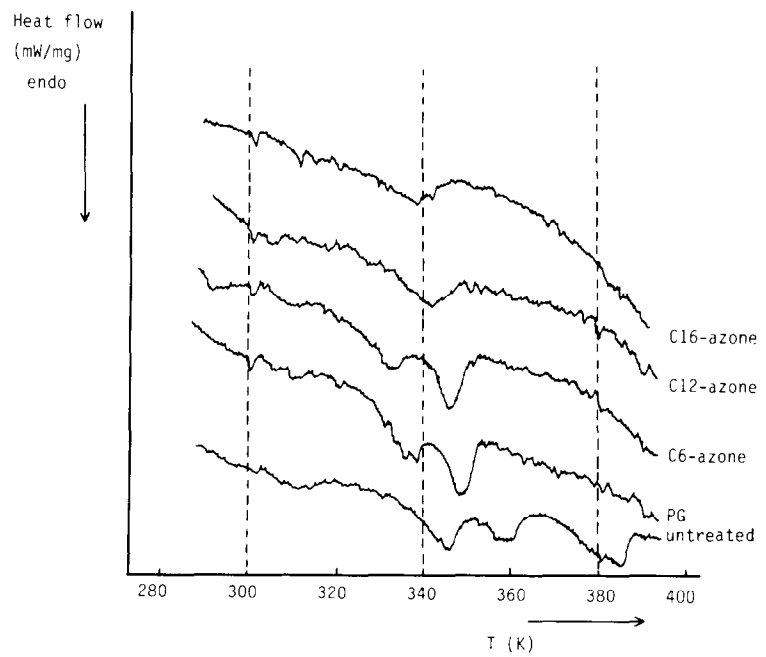

Fig. 4. Influence of azones and propylene glycol on the thermal behaviour of stratum corneum. Equilibration: 10 h at 320K.

on the two lipid transitions. Due to the low sensitivity of his apparatus he could not measure the enthalpy of transition and therefore he concluded that the peak at 360K vanishes after the first heating scan from 280K to 430K. The results of our experiments are in agreement with the hypothesis of Golden at al. (1986) who suggested, as described above, that the transition at 360K is caused by lipids that have an interaction with proteins, but more experiments must be done to prove this hypothesis fully.

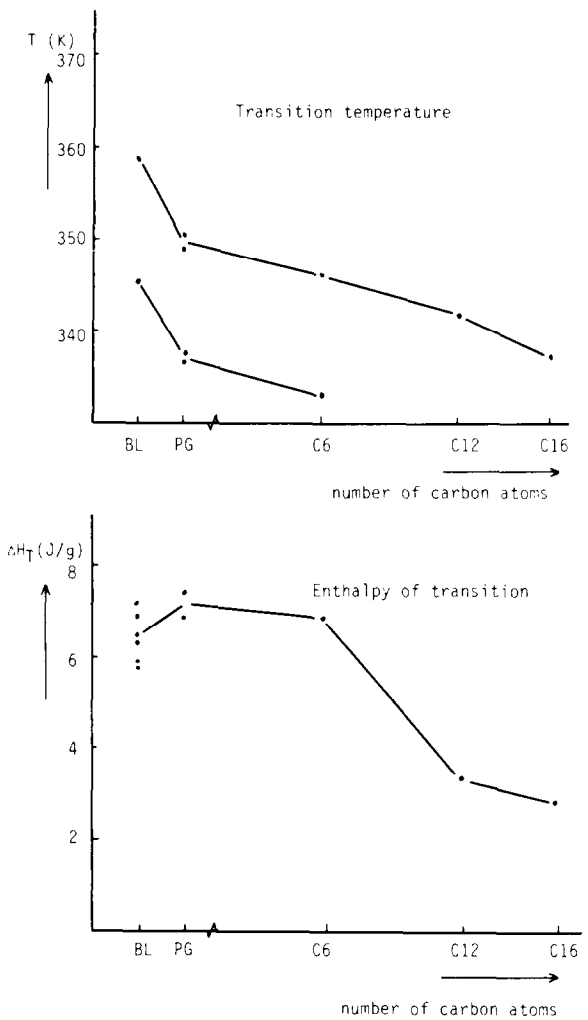

Fig. 5. Influence of azones and propylene glycol on ΔH_T and T_T of the gel–liquid transitions of the lipid bilayers.

The thermal behaviour of treated stratum corneum is shown in Fig. 3. All experiments have been carried out with stratum corneum from the same donor. Untreated stratum corneum shows two peaks at 345K and 360K. Treated stratum corneum shows a downward shift of both transition temperatures. Besides the decrease in transition an exothermal peak is observed as soon as stratum corneum is treated with propylene glycol. Such exothermal peaks, which appear just below the endothermal transition temperature, are a general phenomenon (Billmeyer, 1962), also encountered in other materials such as polymers; they are caused by a reorientation of molecules to achieve a more stable packing (compare e.g. crystallisation of partly amorphous polymers just before the temperature of melting). Because the exothermal transition alters the area under the endothermal peak, it is not possible to calculate the enthalpy involved in the endothermal transition. In order to allow the stratum corneum to reach the most stable state and to avoid an exothermal transition upon heating, the scan is preceded by a stabilisation period just below the temperature region of the exothermal peak. The results of these experiments are shown in Fig. 4, which clearly shows that the exothermal peak has indeed disappeared.

A remarkable feature concerning the thermal behaviour is the absence of the protein-denaturation peak of all treated stratum corneum samples. This peak is only found in untreated stratum corneum. Probably this is due to a decrease in water hydration of the stratum corneum caused by treatment with propylene glycol; the peak is more pronounced as the water content of stratum corneum increases.

From the heating curves, the enthalpy of transition and the transition temperatures were determined. Because the curve between the two observed peaks does not return to the baseline, the total enthalpy involved in the transitions is calculated. In Fig. 5 the values are plotted as a function of the number of C atoms in the alkyl side chains of the azones. The enthalpies of transition are given in Table 2. From Fig. 5 and Table 2 it is clear that the enthalpy of transition decreases abruptly between C_6 and C_{12} azone, whereas the temperature of both transitions decreases continu-

TABLE 2

The total ΔH_T involved in the separated gel–liquid transitions at around 345K and 360K after treatment with azones and propylene glycol. The error introduced by the baseline choice is approximately 0.3 J/g

Pretreatment	H_T(J/g)
–	6.5
propylene glycol (PG)	7.2
C_6 azone/PG	6.9
C_{12} azone/PG	3.3
C_{16} azone/PG	2.9

ously. It is not clear whether the two peaks turn into one broad endothermal peak or that the peak at 345K disappears and the peak at 360K broadens and shifts to lower temperatures. To find out which hypothesis is the right one more experiments must be carried out.

Discussion

Goodman and Barry (1986) did not find any lipid melting peak in the DTA scan of stratum corneum after treatment with C_{12} azone. However as a vehicle for C_{12} azone they used a PBS buffer with 0.1% Tween 80. It could be possible that using this solution most of the lipids are extracted from stratum corneum during the pretreatment and that therefore no lipid transitions have been found.

A question which may arise is: what is the meaning of the results presented here for physiological temperatures? To answer this question, we firstly have to consider the dependence of the enthalpy of transition and the transition temperature on the number of carbons in the alkyl chains of the C_n azones. Due to this dependence it is justified to assume that the azones are incorporated in the lipid bilayers of stratum corneum. A second answer to this question requires further detail. For pure substances the following thermodynamic relation holds:

$$\Delta H_T = T_T \Delta S_T$$

where ΔH_T stands for the enthalpy difference between gel and liquid state, ΔS_T refers to the entropy of transition and T_T is the transition temperature. Although, of course, we do not deal with pure substances but with a mixture of many components it is interesting to use this equation to discuss the effects of azones on T_T and ΔH_T.

In the first place a decrease in the transition temperature should lead to an increase in ΔS_T. This increase would be about 7%, comparing the transition temperatures of stratum corneum treated with C_{16} azone and untreated stratum corneum. Secondly, a decrease in the ΔH_T value leads to a decrease in the ΔS_T. This decrease would be approximately 50%, comparing stratum corneum C_{16} azone treated and untreated stratum corneum. Therefore the total effect of the decrease in enthalpy and temperature leads to a decrease in entropy difference of approximately 46%. We recall, that ΔS_T is the difference in entropy between the liquid and gel state, which means $\Delta S_T = S^l - S^g$. It is reasonable to suppose that C_n azones in gel-state bilayers have more effect on the structure than azones in the liquid-state bilayers. Therefore it is most likely that the major effect of C_n azones on stratum corneum involves an increase in S^g, the entropy of the gel state. An increase in the entropy at the gel state means an increase in the mobility of the alkyl chains in the bilayer and this in turn results in a decrease in the resistance for diffusion of molecules. Therefore we may conclude that in this case a decrease in transition enthalpy reflects an increase in fluidity of the lipid bilayers. These considerations are confirmed by kinetic experiments (Boddé, 1988). The permeation of nitroglycerin through stratum corneum is strongly increased if the latter is pretreated with a C_{12} azone/propylene glycol mixture. Pretreatment with C_6 azone/propylene glycol or propylene glycol does not change the permeability of stratum corneum. Therefore the ΔH_T decrease involved in the lipid transitions of stratum corneum treated with C_{12} azone corresponds to an increase in permeability. From this we may conclude that not the decrease in transition temperature, but the transition enthalpy drop is the key parameter which correlates to the permeability rise.

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