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Modes of action of terpene penetration enhancers in human skin; differential scanning calorimetry, small-angle X-ray diffraction and enhancer uptake studies

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Abstract

The mechanisms through which the terpenes, *d*-limonene, 1-8-cineole and nerolidol, increase the permeability of human stratum corneum (s.c.) and the mechanisms underlying propylene glycol (PG)/terpene synergy were investigated using differential scanning calorimetry (DSC), small-angle X-ray diffraction (SAXD) and enhancer uptake studies. DSC experiments identified two major lipid transitions at 72° and 83°C. *d*-Limonene reduced the temperatures of both transitions by approx. 20°C without affecting their enthalpies (ΔH). 1-8-Cineole also reduced the temperatures of both transitions by approx. 20°C but also reduced ΔH for the first major lipid transition; ΔH for the second was unaffected. *d*-Limonene increased the combined entropy change (ΔS) associated with both lipid transitions by 11% whereas 1-8-cineole decreased ΔS by 32%. The decrease in ΔS produced by 1-8-cineole provides evidence that this enhancer is lipid disruptive at normal skin temperature. Nerolidol reduced the transition temperatures of both major lipid transitions by approx. 4°C and also decreased their cooperativity. Reduced bilayer cooperativity indicates that this enhancer also disrupts the intercellular lipids. Lack of a clear baseline prevented accurate measurement of ΔH and ΔS values following nerolidol treatment. SAXD experiments showed that *d*-limonene and 1-8-cineole act to reduce the intensity of lipid based reflections. Decreases in reflection intensities may be linked to a disruption of lipid packing within the bilayers and/or to a disturbance in the stacking of the bilayers. Treatment with nerolidol did not markedly reduce the intensities of the bilayer based reflections. Uptake studies revealed that large quantities of terpenes can be accommodated by the s.c. (mean uptake of *d*-limonene, 1-8-cineole and nerolidol was 8.90%, 26.2% and 39.6% w/w dry s.c.). The possibility that terpene enhancers pool in the s.c. is discussed. DSC and SAXD investigations provided fragmented evidence that PG/terpene synergy may produce enhanced lipid bilayer disruption. Enhancer uptake studies showed that PG does not significantly increase terpene delivery to the s.c. above that provided by application of neat terpenes.

Keywords: Human skin; Penetration enhancers; Terpenes; Differential scanning calorimetry; Small-angle X-ray diffraction; Stratum corneum lipid

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1. Introduction

The outermost layer of the skin, the stratum corneum, is an important barrier, preventing the loss of body fluids and protecting against the absorption of chemical and biological toxins. Clinically, the stratum corneum is of great interest since it is usually the main rate-limiting barrier to drug absorption across the skin (Scheuplein, 1965, 1967) The tissue is composed of highly flattened, keratin-filled cells embedded in a lipid/water matrix. Intercellular lipids are arranged as stacks of bilayers (Madison et al., 1987) which run mainly parallel to the surface of the skin (Garson et al., 1991). Many studies have shown that the intercellular lipid bilayers are vital for the barrier function of the stratum corneum (e.g. Scheuplein and Blank, 1971; Elias et al., 1981; Golden et al., 1987a). It is also argued that they constitute one of the major routes of penetration for topically applied compounds (Albery and Hadgraft, 1979; Boddé et al., 1991).

The excellent barrier properties of this horny layer often preclude the skin as a route for systemic drug delivery (Flynn and Stewart, 1988). One way to extend the range of drugs which may be administered transdermally is to incorporate skin penetration enhancers into formulations. Penetration enhancers are compounds which can increase the permeability of the stratum corneum by either increasing drug diffusivity within the membrane and/or by increasing drug partitioning from the applied formulation into the skin (Barry, 1991). A class of penetration enhancers currently receiving much attention is the terpenes (Williams and Barry, 1991a; Hori et al., 1991; Cornwell and Barry, 1994). Terpene compounds are derived from plant essential oils and combine good penetration enhancing abilities with low skin irritancy and low systemic toxicity. The effects of terpene enhancer treatment on drug diffusivity in the stratum corneum and drug partitioning into the horny layer can be resolved through combining in vitro permeation data with stratum corneum/water partitioning study results (Williams and Barry, 1991a; Cornwell and Barry, 1994). It appears that for hydrophilic drugs, such as 5-fluorouracil, the primary effect of terpene enhancer treatment is to increase drug diffusivity in the horny layer, i.e. to reduce the barrier properties of the skin (Williams and Barry, 1991a; Cornwell and Barry, 1994). For more lipophilic drugs, such as oestradiol, terpenes, in most instances, increase drug diffusivity but also raise drug partitioning into the stratum corneum (Williams and Barry, 1991b). Increases in partitioning are likely to be due to bulk solvent effects since oestradiol is moderately soluble in many of the terpenes (Williams and Barry, 1991b). The mechanisms by which terpene enhancers increase drug diffusivity in the stratum corneum are not fully understood. Differential scanning calorimetry (DSC) measurements have shown that terpene enhancers reduce lipid phase transition temperatures (Williams and Barry, 1989; Cornwell and Barry, 1991). This effect has been taken to imply that they may increase stratum corneum permeability by disrupting the intercellular lipid bilayers . In the present study DSC and small-angle X-ray diffraction (SAXD) measurements investigate the actions of three terpene penetration enhancers, *d*-limonene, nerolidol and 1-8-cineole. These terpenes increase in vitro human skin permeability towards the hydrophilic permeant, 5-fluorouracil, 2-, 23- and 95-fold respectively (Williams and Barry, 1991a; Cornwell and Barry, 1994).

The extent of enhancer uptake into the stratum corneum during enhancer treatment is not often measured. Those uptake studies which have been performed on other lipophilic enhancers often reveal surprisingly high enhancer loadings in the stratum corneum. Francoeur et al., 1990 report oleic acid uptake into porcine stratum corneum from an ethanol/water (2:3) vehicle to be 5–7% w/w of dry tissue weight. Such uptake, the authors note, would, if the enhancer is located predominantly in the lipid domains, involve significant expansion of the intercellular lipids. Schuckler and Lee, 1992 report azone loadings in human stratum corneum of up to 30% w/w. Again, if azone distributed mainly into the lipid domains, this amount would cause considerable lipid expansion. In the present study terpene enhancer uptake is determined after 12 h treatment, to correlate uptake measurements and DSC and SAXD results.

Propylene glycol is frequently used in dermatological formulations as a vehicle or co-solvent. When applied to fully hydrated human skin propylene glycol has only a mild effect on skin permeability (Goodman and Barry, 1988). However, propylene glycol has a marked synergistic effect on the activities of lipophilic skin penetration enhancers (Goodman and Barry, 1988). Recently, propylene glycol has been shown to synergistically improve the in vitro activities of cyclic monoterpene enhancers (Barry and Williams, 1989) and sesquiterpene enhancers (Cornwell, 1993), increasing their penetration enhancing abilities, towards 5-fluorouracil, in most cases approximately 4-fold. In the present study DSC and SAXD experiments compare terpene effects on the structure of the stratum corneum both with and without propylene glycol. Furthermore, enhancer uptake studies measure the effects of propylene glycol on terpene delivery to the stratum corneum.

2. Materials and methods

2.1. Materials

d-Limonene and 1-8-cineole were obtained from Sigma Chemical Co. (St. Louis, MO), and nerolidol and propylene glycol (99%) from Aldrich (Gillingham, UK). Each terpene was tested, in our laboratories, by capillary gas chromatography to be >99% pure.

2.2. Preparation of stratum corneum

Full thickness human abdominal skin samples were obtained post-mortem and stored, on receipt, at −20°C in double-sealed evacuated polyethylene bags (Harrison et al., 1984). Epidermal membranes were prepared by immersing full thickness skin samples, trimmed of subcutaneous fat, in water at 60°C for 45 s and gently peeling off the epidermis (Kligman and Christophers, 1963). Stratum corneum sheets were obtained by floating freshly prepared epidermal membranes on an aqueous solution of 0.0001% trypsin (type III, bovine; Sigma Chemical Co., St. Louis, MO) and 0.5% sodium bicarbonate, for 12 h. Digested material was removed from the underside of the stratum corneum with tissue paper and the isolated sheets were rinsed in an aqueous solution of 0.002% sodium azide. Cleaned sheets were dried on PTFE-coated wire meshes under ambient conditions. Each sheet was rinsed in acetone for 20 s, to remove any sebaceous or subcutaneous fat contamination (Goodman and Barry, 1989), and stored for up to 2 weeks over silica gel, under vacuum. For some DSC measurements lipid extracted stratum corneum was employed. Lipid extraction was performed by immersion of isolated stratum corneum in chloroform:methanol 2:1 for a minimum of 48 h.

2.3. Enhancer treatment

Terpene enhancers were applied as undiluted oils and as solutions in propylene glycol. In order to ensure that the thermodynamic activity of the enhancers in both the neat oils and the propylene glycol solutions was the same the enhancers were applied, whenever possible as saturated solutions in propylene glycol. Nerolidol was fully miscible with propylene glycol, making it impossible to prepare a saturated solution; it was thus applied as a 90% w/w solution in propylene glycol. The treatment procedure was the same for DSC, SAXD and uptake studies. Samples of dry stratum corneum (approximately 10 mg for DSC and 10–15 mg for SAXD and uptake studies) were hydrated for 2–3 days to 20–40% hydration over a saturated solution of potassium sulphate, r.h. 97%. Percentage hydration was defined as ; (wet weight − dry weight)/dry weight. The chosen hydration range (20–40%) is representative of the normal in vivo level of stratum corneum hydration throughout much of the tissue (Potts, 1986). No major changes in the properties of the stratum corneum are expected between 20 and 40% hydration (Goodman and Barry, 1989; Bouwstra et al., 1991).

Hydrated samples were immersed in 1–2 ml of enhancer for 12 h, at 20°C. This treatment time mimicked that used in in vitro diffusion studies (Williams and Barry, 1991a, b; Cornwell and Barry, 1994). For DSC and SAXD experiments,

treated stratum corneum samples were blotted dry with tissue paper prior to immediate analysis. In uptake studies each sample was blotted dry and any enhancer adhering to the surface removed with tissue paper soaked in acetone. Enhancer treatment is commonly performed at room temperature in mechanistic studies such as this (e.g. Golden et al., 1987b; Goodman and Barry, 1989; Bouwstra et al., 1989, 1992a). The behaviour of the intercellular bilayers at 20°C is widely believed to be representative of that at normal surface skin temperature (32°C) since both temperatures are below the major lipid phase transitions at 72°C and 83°C, and the minor lipid transition at 36°C.

2.4. Differential scanning calorimetry

DSC experiments characterising the thermal transitions in untreated stratum corneum used skin from 11 different donors. Donors included 7 females and had a mean age of 75 ± S.D. 6.7 years. Most of these skin samples were also used in terpene enhancer studies. Enhancer studies used skin obtained from 6 donors. Donors included 4 females and had a mean age of 78 ± S.D. 3.8 years. Thermal analysis employed a DSC7 Differential Scanning Calorimeter (Perkin-Elmer, USA) fitted with an Intracooler (Model FC-60-PED, Perkin-Elmer, USA). Stratum corneum samples were hermetically sealed into 75 μl large volume stainless-steel capsules for analysis. The hermetic seal prevented water vaporisation from the tissue at 100°C. Samples were heated from −10 to 140°C. A heating rate of 10°C/min was selected, following detailed studies (Goodman, 1986), to obtain satisfactory endotherm peak resolution with the minimum of temperature-lag effects. In reheat experiments samples were cooled, following the first run, in their hermetically sealed DSC pans, to −10°C and then immediately reheated to 140°C at 10°C/min as before. Since in human stratum corneum one deals with a mixture of lipids or proteins it was most appropriate to measure transition midpoint temperatures (T_ms). T_m values were determined manually and approximate transition enthalpies (ΔH) measured using 'partial areas'.

Changes in transition temperatures and enthalpies upon enhancer treatment were calculated as peak shifts and enthalpy ratios, respectively. Peak shifts were derived by subtracting control T_m values from post-treatment T_m values, i.e. decreases in T_m produced negative peak shifts. Enthalpy ratios were calculated by dividing post-treatment ΔH values by control ΔH values, i.e. decreases in ΔH produced enthalpy ratios of less than one. In both situations, values recorded after enhancer treatment were compared with the mean values obtained from two control samples from the same skin donor, thus reducing the influence of inter-donor variability. A minimum of three post-treatment values were obtained for each enhancer or enhancer formulation, using skin from at least two different donors.

2.5. Small-angle X-ray diffraction

Abdominal skin from two donors was used in SAXD experiments (donor A was unidentified, B, male, aged 24). A control diffraction pattern was obtained from each donor. Each enhancer experiment was performed in duplicate using one stratum corneum sample from each donor.

SAXD experiments were performed in Station 8.2 at the Daresbury Laboratories (Cheshire, UK) using the Synchrotron Radiation Source (SRS). These studies used a beam with a wavelength (λ) of 0.15 nm, and an intensity of 4×10^{11} photons/s when the SRS was operating at 200 mA and 2 GeV. The combination of high beam intensity and camera collimation produced a highly collimated, slightly converging beam, which at the focal spot had a cross-sectional area of 0.4×4 mm^2. The diffraction pattern was recorded on a multi-wire quadrant detector (512 channels, 20 cm radius; built at Daresbury) set approximately 1.8 m from the sample. The repeat distance (d) resolution obtained by the detector was related to 2θ. For example when $\theta = 2.0°$ the resolution was 0.015 nm and when $\theta = 0.7$ the resolution decreased to 0.13 nm. Wet rat tail collagen ($d = 67$ nm) and anhydrous cholesterol ($d = 3.35$ nm) were used as calibration standards. Stratum corneum samples were mounted, randomly orientated, into specially designed sample holders with air-tight seals and

thin mica windows (Bouwstra et al., 1991). The beam pathlength through the sample was 1 mm. Samples were exposed to the beam for 15 min at 21°C. Following collection of the diffraction patterns, the results were corrected for detector channel response and the channel positions calibrated to a 2θ scale. The scatter from the empty sample holder was also subtracted from each of the patterns. The scattering intensities were plotted as a function of the scattering vector $\boldsymbol{Q}$, defined as; $\boldsymbol{Q} = (4\Pi \sin\theta)/\lambda$. Lattice spacings were calculated from $\boldsymbol{Q}$ values using the relationship; $d = (2\Pi n)/\boldsymbol{Q}$, where n represents the order of reflection.

2.6. Enhancer uptake studies

Uptake studies used skin samples from 8 donors. Donors included 7 females and had a mean age of 75.0 ± S.D. 6.0 years. Enhancer uptake was measured by extraction of terpenes from stratum corneum samples following treatment. Extracts were analysed by capillary gas chromatography (GC).

For extraction, cleaned stratum corneum samples were cut into 0.5 cm^2 sections. The sections were mixed in 2-ml glass vials with 1.5 ml of chloroform/methanol (2:1) (Bligh and Dyer, 1959) containing 5 mg/ml N-nonane GC internal standard. The vials were crimp sealed with PTFE coated rubber septa and shaken vigorously for 48 h at 20°C. Enhancer/lipid extracts were filtered through glass micro-fibre filters (Whatman GF/F 0.7 μm pore size, Whatman, UK) to remove any cell debris prior to analysis.

It was anticipated that some enhancer would be lost during the extraction and filtration procedures (i.e. some may have remained adhered to stratum corneum fragments, glass-ware, filter, etc.). To determine the amount of unrecoverable enhancer, untreated samples were spiked with a known weight (5–10 mg) of terpene and extracted using the method described above. The difference between the known spiked amount and the amount determined following GC analysis was taken to be representative of the amount remaining unrecovered. By measuring (in triplicate) the amount unrecovered for each enhancer a small correction was made to uptake values.

Samples were analysed using a 8320B Capillary Gas Chromatograph (Perkin-Elmer, USA) installed with an AS-8300 autosampler (Perkin-Elmer, USA). They were separated on a 25 m BP-5 fused silica capillary column (SGE Inc., Australia) using helium (8 $lb/inch^2$) as the carrier gas. Eluate was analysed by a hydrogen flame-ionisation detector. The oven temperature was programmed to rise from 50°C, at injection, to 275°C at 2°C/min. Chromatograms were analysed using the 8320B chromatograph's own software, enabling measurement of integrated peak areas. A minimum of three replicate experiments were performed for each enhancer using samples from a minimum of two different donors.

2.7. Differential scanning calorimetry

2.7.1. Control traces

DSC analysis of 20–40% hydrated stratum corneum samples revealed four major endothermic transitions; $T1$ (at 36°C), $T2$ (at 72°C), $T3$ (at 83°C) and $T4$ (at 100°C) (Fig. 1, Table 1). The first transition, $T1$, had the smallest ΔH and was only observed in approximately 75% of all samples analysed. $T2$, $T3$ and $T4$ were observed in all samples. All four transitions have been reported by numerous other workers (e.g. Van Duzee, 1975; Golden et al., 1987a; Goodman and Barry, 1989; Bouwstra et al., 1989). In order to characterise these transitions further, DSC traces were obtained for reheated and lipid extracted samples. Heating samples for the second time revealed a broad new transition at approximately 70°C (Fig. 1). This new transition is believed to be a combination of $T2$ and $T3$ (Bouwstra et al., 1989). Contrary to previous studies, $T1$ was removed on reheating. Examination of the DSC traces in the literature, however, reveals that $T1$ is often reduced in size on reheating (Van Duzee, 1975; Goodman and Barry, 1989). Such a reduction in endotherm size may explain the apparent absence of $T1$ in reheated samples in the present study. $T4$ did not appear in reheated samples and thus appeared to be thermally irreversible. DSC traces obtained from hydrated, lipid extracted samples, displayed only the last transition, $T4$; all three other transitions were absent (Fig. 1). This pro-

vides good agreement with other studies which have also shown that $T1$, $T2$ and $T3$ are removed by lipid extraction (Van Duzee, 1975; Goodman and Barry, 1989). The origins of the major lipid transition will be discussed later. Briefly, however, $T1$, $T2$ and $T3$ are associated with phase changes in the intercellular lipid bilayers and $T4$ with further denaturation of intracellular keratin.

During this study it was noted that approximately 50% of 20–40% hydrated control stratum corneum samples exhibited a weak thermal transition at 51°C, T_x (Fig. 1). Since T_x was positioned between $T1$ and $T2$ it was often poorly resolved, making measurement of T_m and ΔH difficult. In some cases its presence was only noted because sample heat flows did not return to baseline values between $T1$ and $T2$. T_x increased in intensity as sample hydration reduced (Fig. 2). It was not observed in hydrated, lipid extracted stratum corneum (Fig. 1) but was present in dry lipid extracted stratum corneum (Fig. 2).

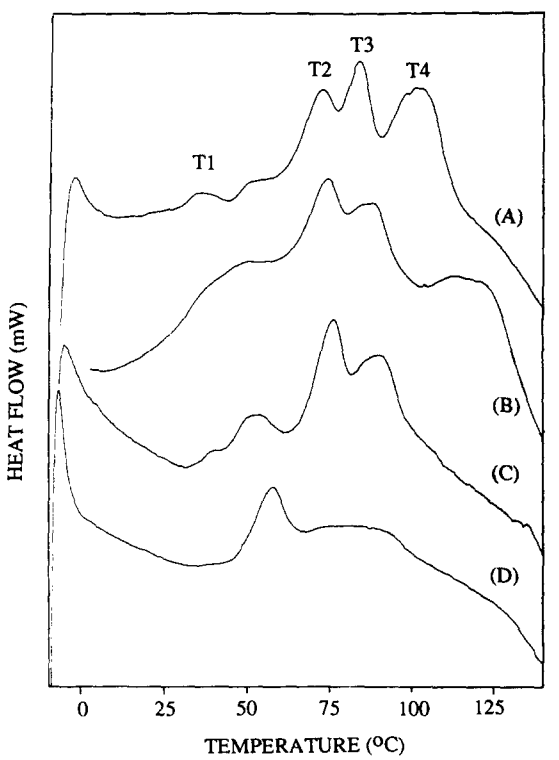

Fig. 1. DSC traces of (A) 20–40% hydrated stratum corneum, (B) reheated sample and (C) delipidised stratum corneum (20–40% hydrated). Endothermic transitions appear as peaks.

Table 1
The transition midpoint temperatures, T_m, and enthalpies, ΔH, of the thermal transitions observed in 20–40% hydrated human stratum corneum (means ± S.D.)

Thermal transition	T_m (°C)	n[a]	ΔH (J/g)	n
$T1$	36 (3.0)	17/23	0.8 (0.4)	22/23
T_x	51 (1.4)	9/23	1.2 (1.0)	12/23
$T2$	72 (1.2)	23/23	5.3 (1.7)	23/23
$T3$	83 (2.1)	23/23	4.1 (0.9)	23/23
$T4$	100 (2.2)	23/23	5.1 (0.9)	23/23

[a]First figure represents the number of samples in which the thermal transition was sufficiently defined to measure T_m or ΔH and the second figure represents the total number of samples analysed. The same 23 samples, obtained from 11 skin donors (see Methods section for details of donors) were used for all the measurements.

2.7.2. *Effects of terpene enhancer treatment*

Following treatment with either *d*-limonene or 1-8-cineole, two major thermal transitions were observed at approx. 50°C and 67°C in DSC traces (Fig. 3 and Table 2 and Table 3). It is likely that treatment with these enhancers acted to reduce the temperatures of the two major lipid transitions, $T2$ and $T3$, by approximately 20°C. Closer examination of lipid transitions revealed that *d*-limonene treatment shifted $T2$ and $T3$ without markedly affecting their enthalpies. The change in ΔH for $T2$ was of low statistical significance (two-sided, paired Student's t-test, $P < 0.20$) and the change in ΔH for $T3$ of no significance ($P > 0.2$). 1-8-Cineole treatment also shifted $T2$ and $T3$ but, in contrast to *d*-limonene, reduced the enthalpy of $T2$ by 50%; the enthalpy of $T3$ was unaffected. The effect of 1-8-cineole treatment on ΔH for $T2$ was statistically significant ($P < 0.01$) whereas the effect on ΔH for $T3$ was not significant ($P > 0.2$). Nerolidol, had different effects on the lipid transitions to the *d*-limonene and 1-8-cineole. Following treatment with this enhancer three thermal transitions were observed at approx. 50, 68 and 80°C (Fig. 3 and Tables 2 and 3). The last two transitions are likely to be $T2$ and $T3$ which are shifted by approx. 4°C, whilst the first transition is likely to be T_x. In addition to shifting

Table 2
Effects of neat terpenes on the transition midpoint temperatures (T_m) of the major thermal transitions in human stratum corneum; $n = 3$ for treated data and n = 6 for control data

Treatment	Control T_m2 (°C)	Treated T_m2 (°C)	Mean T_m2 shift (°C)	Control T_m3 (°C)	Treated T_m3 (°C)	Mean T_m3 shift (°C)	Control T_m4 (°C)	Treated T_m4 (°C)	Mean T_m4 shift (°C)
d-Limonene	73 (0.8)	51 (2.3)	−22 (2.0)****	84 (1.1)	66 (0.7)	−18(1.3)****	98(3.2)	106 (2.9)	+7.9 (2.5)***
Nerolidol	73 (1.0)	68 (1.1)	−4.2 (1.1)****	84 (1.1)	80 (0.4)	−3.6(0.8)****	98 (3.2)	109[a]	+8.2[a]
1-8-Cineole	73 (1.0)	48 (10)	−25 (9.5)***	84 (1.1)	68 (0.5)	−16 (1.4)****	98(3.2)	107[b]	+7.1[b]

[a]n = 1; in two samples *T*4 was not observed following nerolidol treatment.[b]n = 2.Data are given as means ± S.D. The statistical significance of shifts in T_m were tested using a two-sided, paired Student's *t*-test.****$P < 0.01$; ***$P < 0.05$.

Table 3
Effects of neat terpenes on the transition enthalpies (ΔH) of the major thermal transitions in human stratum corneum; $n = 3$ for treated data and n = 6 for control data

Treatment	Control *T*2 ΔH (J/g)	Treated *T*2 ΔH (J/g)	Mean *T*2 enthalpy ratio	Control *T*3 ΔH (J/g)	Treated *T*3 ΔH (J/g)	Mean *T*3 enthalpy ratio	Control *T*4 ΔH (J/g)	Treated *T*4 ΔH (J/g)	Mean *T*4 enthalpy ratio
d-Limone	5.5 (1.6)	6.0 (1.2)	1.1 (0.1)*	4.0 (0.6)	3.9 (0.3)	1.0 (0.2)	4.7 (0.7)	3.1 (1.3)	0.7 (0.4)
Nerolidol	5.7 (1.5)	9.0 (0.3)	1.7 (0.4)**	4.0 (0.6)	9.7 (1.6)	2.4 (0.5)***	4.8 (0.9)	–[a]	–[a]
1–8-Cineole	5.7 (1.5)	2.6 (0.5)	0.5 (0.04)****	4.0 (0.6)	3.7 (0.5)	0.9 (0.1)	4.8 (0.9)	1.6[b]	0.3[b]

[a]Not determined.[b]$n = 2$.Data are given as means ± S.D. The statistical significance of enthalpy changes were tested using a two-sided, paired Student's *t*-test.****$P < 0.01$; ***$P < 0.05$; **$P < 0.10$, *$P < 0.20$; no symbol indicates $P > 0.2$, i.e. no significant difference.

$T2$ and $T3$ to lower temperatures nerolidol also clearly decreased their cooperativity (i.e. it broadened both transitions). Unfortunately, peak enthalpy measurements post-treatment with nerolidol were hampered by the lack of a clearly defined baseline. These measurements indicated that nerolidol may actually increase lipid transition ΔH values. However, in view of the aforementioned baseline problems, these data should be viewed with caution.

Both *d*-limonene and 1-8-cineole treatment shifted the protein-associated endotherm, $T4$, to a higher temperature and broadened the endotherm peak. These effects may be due to tissue dehydration, which characteristically shifts $T4$ to higher temperatures and broadens the endotherm (Fig. 2), and/or to terpene/protein interactions. Nerolidol treatment removed $T4$ in most instances. It is not clear whether this is because of

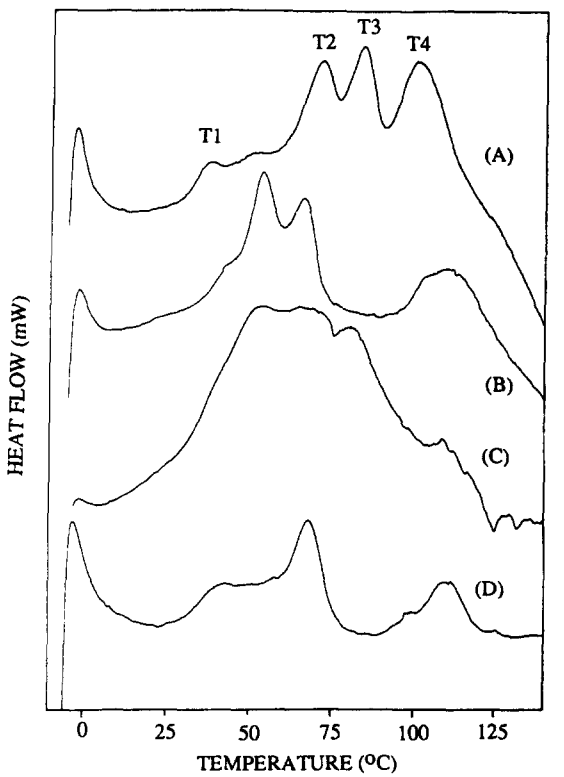

Fig. 2. DSC traces of (A) 20–40% hydrated stratum corneum, (B) 10% hydrated stratum corneum, (C) dry stratum corneum and (D) dry stratum corneum following lipid extraction. Endothermic transitions appear as peaks.

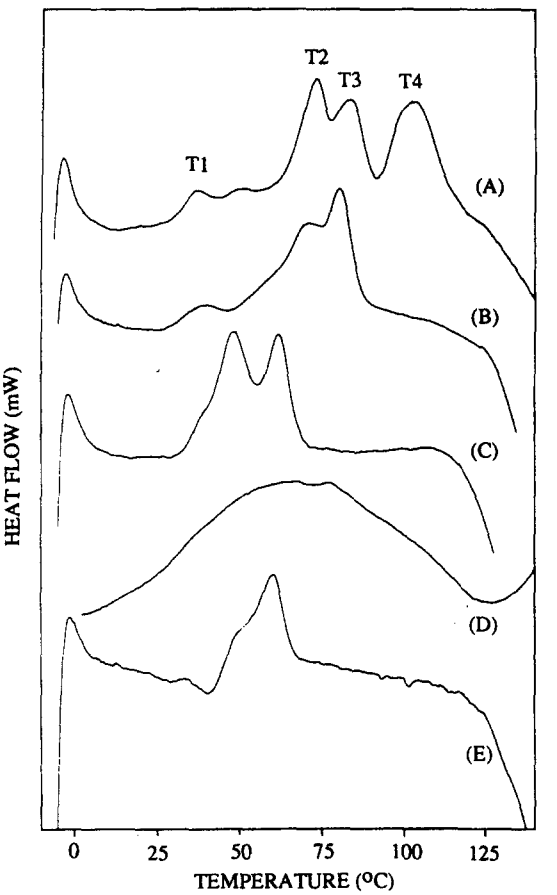

Fig. 3. DSC traces of (A) untreated 20–40% hydrated stratum corneum and stratum corneum treated with (B) *d*-limonene, (C) nerolidol and (D) 1-8-cineole. Endothermic transitions appear as peaks.

(a) tissue dehydration or (b) nerolidol/protein interactions or (c) because $T4$ is obscured by the broadened $T3$.

2.7.3. *Effect of co-application with propylene glycol*

In control experiments propylene glycol treatment reduced the transition temperatures of $T2$ and $T3$ by 2 and 6°C, respectively (Fig. 4 and Table 4); and removed $T4$. Propylene glycol had no significant effect on the enthalpies of $T2$ and $T3$ (two-sided, paired Student's t-test; $P > 0.20$, Table 5). When terpenes were applied in combination with propylene glycol the effects of the enhancers and the vehicle on lipid endotherm shifts were usually approximately additive.

Treatment with *d*-limonene saturated in propylene glycol did not significantly change the enthalpies of *T*2 or *T*3 (two-sided, paired Student's *t*-test, $P > 0.20$). Treatment with 1-8-cineole saturated in propylene glycol, however, did significantly decrease the enthalpies of both *T*2 and *T*3 ($P < 0.01$ in each case). The enthalpy of *T*2 was reduced by over 80% and the enthalpy of *T*3 by approximately 30%. Enthalpy changes following treatment with nerolidol in propylene glycol were impossible to measure since, as in earlier experiments with neat nerolidol, no clear baseline was evident. It was noted, however, that the cooperativities of both *T*2 and *T*3 were further reduced following this treatment.

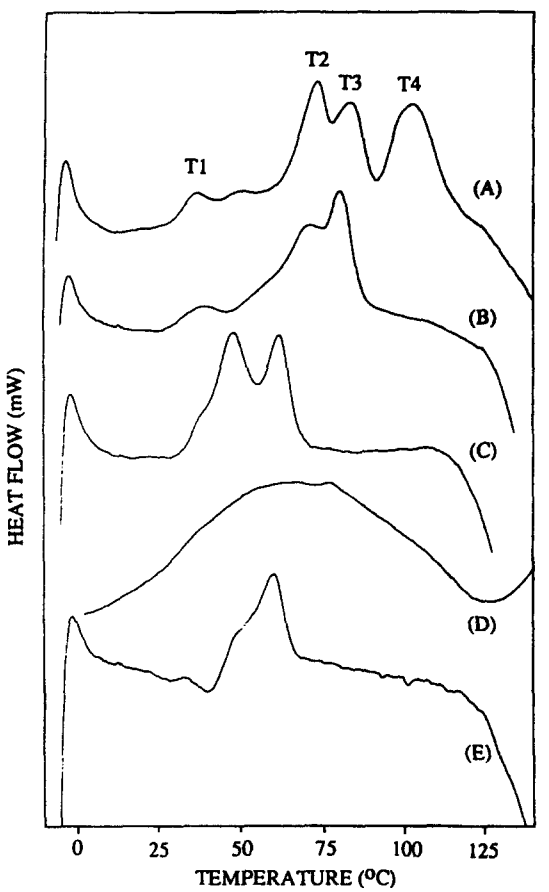

Fig. 4. DSC traces of (A) untreated 20–40% hydrated stratum corneum and stratum corneum treated with (B) propylene glycol, (C) *d*-limonene saturated in propylene glycol, (D) nerolidol 90% w/w in propylene glycol and (E) 1-8-cineole saturated in propylene glycol. Endothermic transitions appear as peaks.

2.8. Small-angle X-ray diffraction

2.8.1. Control measurements

The control scattering profile obtained from donor A, illustrated in Fig. 5, shows diffraction peaks at $Q = 1.00\ nm^{-1}$ and $Q = 1.85\ nm^{-1}$. A shoulder is also evident on the main peak at $Q = 1.35\ nm^{-1}$. This shoulder was not seen in the sample from donor B. The intense peak at $Q = 1.00\ nm^{-1}$ corresponds to a first order reflection from a 6.3 nm spacing. The shoulder at $Q = 1.35\ nm^{-1}$ and the peak at $Q = 1.85\ nm^{-1}$ may correspond to third and fourth order reflections from a mean spacing of 13.8 nm (Bouwstra et al., 1991). The peak at $Q = 1.85\ nm^{-1}$ may also arise from crystalline cholesterol which is known to produce a reflection at $Q = 1.88\ nm^{-1}$ ($d = 3.35$ nm). Reflections attributable to crystalline cholesterol have previously been observed in wide-angle X-ray diffraction (WAXD) studies on human stratum corneum (Garson et al., 1991; Bouwstra et al., 1992b; Cornwell et al., 1994). In all scattering curves, an intense, featureless scatter was noted at $Q < 0.5\ nm^{-1}$. This probably is associated with intracellular keratin since it remains in lipid extracted stratum corneum (Bouwstra et al., 1991).

2.8.2. Effects of terpene enhancer treatment

Following treatment with *d*-limonene and 1-8-cineole, the intensities of the lipid bilayer based reflections were markedly reduced (Fig. 6). Only a weak shoulder at approx. $Q = 1.00\ nm^{-1}$ remained. Nerolidol treatment did not markedly reduce the intensities of lipid based reflections (Fig. 6).

2.8.3. Effect of co-application in propylene glycol

Propylene glycol treatment did not markedly affect small-angle reflections (Fig. 7). This is in good agreement with previous SAXD studies which have also shown no change in lipid bilayer periodicity in human stratum corneum following 24 h treatment with propylene glycol (Bouwstra et al., 1992a). Co-application of the monoterpene enhancers with propylene glycol intensified their

Table 4
Effects of terpenes applied in propylene glycol (PG) on the transition midpoint temperatures (T_m) of the major lipid transitions in human stratum corneum

Treatment	Control T_m2 (°C)	Treated T_m2 (°C)	Mean T_m2 shift (°C)	Control T_m3 (°C)	Treated T_m3 (°C)	Mean T_m3 shift (°C)
PG	71(0.7)	70(1.5)	−2.0(1.4)**	85(0.7)	79(1.1)	−6.1(1.1)****
d-Limonene/PG	73(0.8)	47(1.0)	−26(1.2)****	84(1.1)	62(1.1)	−22(0.7)****
Nerolidol/PG	72(0.9)	65(2.0)	−7.3(1.3)****	84(0.9)	77(0.8)	−7.5(1.4)****
1-8-Cineole/PG	72(0.9)	48[a]	−24[a]	84(1.0)	59(1.4)	−25(1.1)****

[a]n = 2; *T*2 was not observed in one sample following 1-8-cineole treatment.n = 3 for treated data and $n \geq 4$ for control data. Data are given as means ± S.D. The statistical significance of shifts in T_m were tested using a two-sided, paired Student's *t*-test; ****$P < 0.01$; **$P < 0.10$.

effects lipid reflections. The shoulder remaining at $Q = 1.00\ nm^{-1}$ following treatment with either neat *d*-limonene or 1-8-cineole was not present in samples treated with propylene glycol/enhancer mixtures. In fact, no coherent scatter was observed from these samples. Scattering curves from samples treated with nerolidol in propylene glycol showed varied responses to enhancer treatment. Stratum corneum from donor A produced a scattering profile very similar to that observed following neat nerolidol treatment. A sample from donor B, in comparison, showed a reduction in lipid reflection intensities.

2.9. Enhancer uptake studies

2.9.1. Uptake of neat terpenes

Uptake studies revealed that large quantities of terpene are incorporated into the stratum corneum following 12 h treatment in neat enhancers. Uptake of *d*-limonene, 1-8-cineole and nerolidol was 8.90, 26.2 and 39.6% w/w dry tissue weight, respectively (Table 6).

2.9.2. Terpene uptake from enhancer/propylene glycol mixtures

Measurement of uptake from propylene glycol/terpene mixtures showed that propylene glycol does not significantly change the uptake of *d*-limonene ($P > 0.20$; Student's two-sided *t*-test) and significantly reduces that of 1-8-cineole ($P < 0.01$) and nerolidol ($P < 0.10$) into the stratum corneum. This was unexpected since it has long been argued that propylene glycol may exert part of its synergistic effects through increasing enhancer uptake into the skin.

3. Results and discussion

3.1. Thermal transitions in human stratum corneum

In this study DSC measurements have probed the thermal transitions which occur in human stratum corneum. From these data, and information already in the literature, we can build a picture of the likely origins of these transitions.

DSC studies revealed a minor thermal transition at 36°C, termed *T*1 (Fig. 1). This transition was absent following extraction with lipid solvents and is thus lipid based (Fig. 2). *T*1 has, in the past, sometimes been attributed to the melting of surface, sebaceous lipids (Golden et al., 1986). This is no longer thought to be the case, however, since *T*1 remains in the hexane-rinsed samples which are largely devoid of sebaceous lipids (Gay et al., 1994). WAXD measurements on hairless mouse and human stratum corneum indicate that *T*1 represents a transition, within the intercellular bilayers, from an orthorhombic perpendicular to a hexagonal lipid subcell arrangement (i.e. from a 'crystalline' phase to a gel, or L_β, phase) (White et al., 1988; Bouwstra et al., 1992b). In such a transition, tight, crystalline chain packing is lost and chain motion increases slightly, allowing the molecules to rotate a few degrees within the lattice. Recent FTIR measurements, however, detect

Table 5
Effects of terpenes in propylene glycol (PG) on the transition enthalpies (ΔH) of the major lipid transitions in human stratum corneum

Treatment	Control $T2$ ΔH (J/g)	Treated $T2$ ΔH (J/g)	Mean $T2$ enthalpy ratio	Control $T3$ ΔH (J/g)	Treated $T3$ ΔH (J/g)	Mean $T3$ enthalpy ratio
PG	4.6 (0.5)	4.8 (1.2)	1.1 (0.3)	3.9 (0.4)	4.7 (0.9)	1.2 (0.2)
d-Limonene/PG	5.5 (1.6)	5.2 (0.9)	1.0 (0.4)	4.0 (0.6)	3.5 (0.3)	0.9 (0.2)
Nerolidol/PG	6.1 (1.7)	18 (3.0)	3.3 (0.6)****	4.2 (0.7)	10 (0.9)	2.5 (0.4)****
1-8-Cineole/PG	6.4 (1.4)	1.3 (1.1)[a]	0.2 (0.2)[b]****	4.8 (1.2)	3.4 (0.5)	0.7 (0.1)****

[a]Individual data were; 1.8, 2.0 and 0.0 J/g; i.e. $T2$ was not present in one sample following treatment.[b]Individual data were 0.3, 0.4 and 0.0.n = 3 for treated data and $n \geq 4$ for control data. Data are given as means ± S.D. The statistical significance of enthalpy changes were tested using a two-sided, paired Student's *t*-test.****$P < 0.01$; no symbol indicates $P > 0.2$, i.e. no significant difference.

a 'solid-to-fluid' phase transition at $T1$, not an orthorhombic perpendicular to hexagonal subcell rearrangement (Gay et al., 1994). This discrepancy, it has been suggested, may be because the WAXD and FTIR studies detect different subpopulations of lipids within the stratum corneum. $T1$, therefore, may be the sum of two phase changes undergone by two lipid subgroups.

In the present study a thermal transition, T_x, was observed at 51°C in 20–40% hydrated samples. T_x increased in magnitude, relative to the other lipid transitions, as tissue hydration was reduced. It was not present in 20–40% hydrated, delipidised samples, but was clearly visible in dry, delipidised samples. A similar transition was recorded by Van Duzee, 1975 in desiccated human stratum corneum but was not commented on. Recent DSC and FTIR investigations on human stratum corneum have also identified a thermal transition at approximately 55°C (Gay et al.,

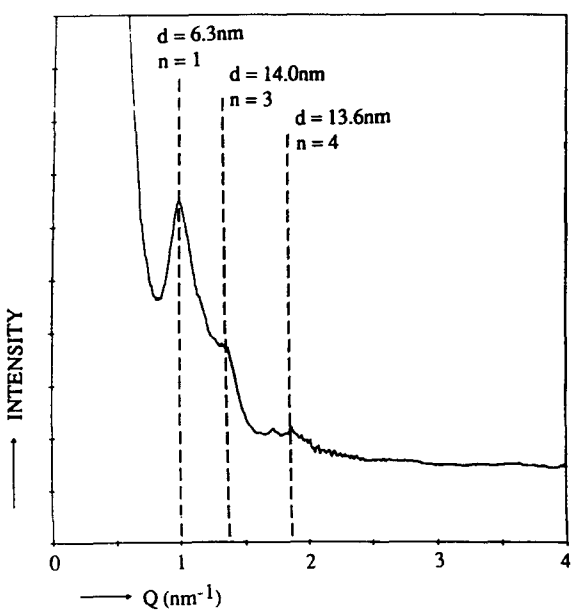

Fig. 5. Small-angle X-ray scattergram obtained from 20–40% hydrated stratum corneum. *d*, unit cell repeat distance; *n*, order of reflection.

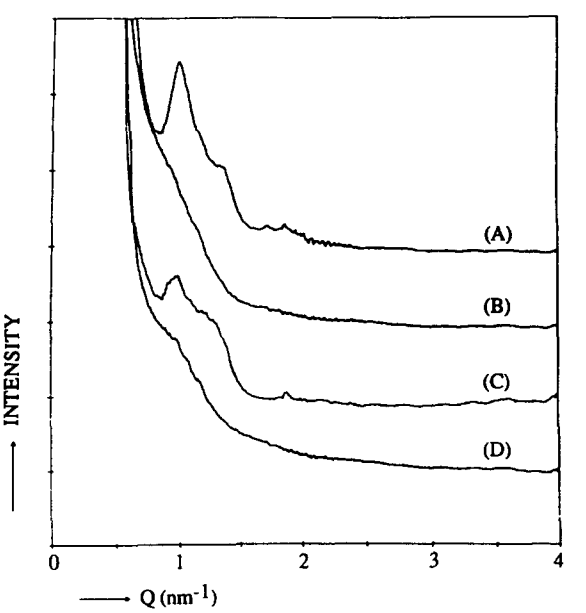

Fig. 6. Small-angle X-ray scattergrams obtained from (A) 20–40% hydrated stratum corneum and stratum corneum treated with (B) *d*-limonene, (C) nerolidol and (D) 1-8-cineole.

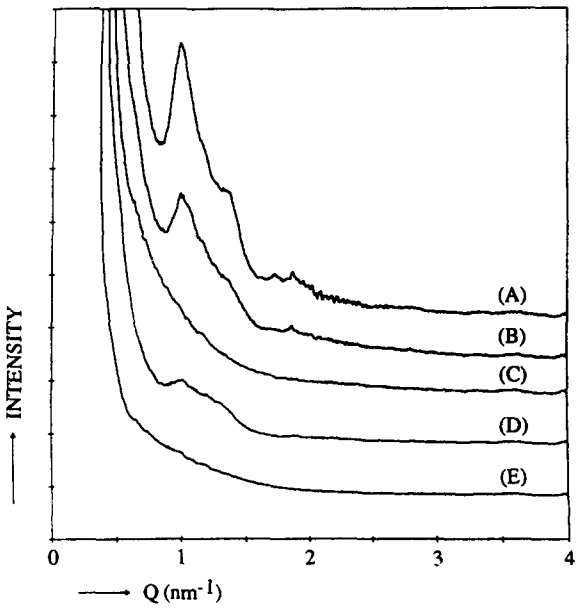

Fig. 7. Small-angle X-ray scattergrams obtained from (A) untreated 20–40% hydrated stratum corneum and stratum corneum treated with (B) propylene glycol, (C) *d*-limonene saturated in propylene glycol, (D) nerolidol 90% w/w in propylene glycol and (E) 1-8-cineole saturated in propylene glycol.

1994). FTIR measurements suggest that his transition is related to a loss of crystalline orthorhombic perpendicular lattice structure. The authors suggest that the 55°C transition may arise from the covalently bound lipids present on the outside of the corneocyte envelope. This would be in agreement with the present study since T_x remained following lipid extraction, indicating that it arises from a protein or protein bound component of the stratum corneum. Further work is required to understand fully the nature of this new transition. It would be useful, for example, to remove covalently bound lipids through hydrolysis of their ester linkages and to repeat DSC scans.

Table 6
Uptake of terpene enhancers into human stratum corneum from neat oils or saturated solutions in propylene glycol (PG)

Enhancer	Mean quantity not extracted (mg)	Mean uptake[a] (% w/w)	Mean corrected uptake[a] (% w/w)
d-Limonene	0.02 (0.02)	8.79 (2.64)	8.90 (2.63)
Nerolidol	0.32 (0.07)	38.0 (5.20)	39.6 (5.16)
1-8-Cineole	0.13 (0.04)	25.6 (3.82)	26.2 (3.81)
d-Limonene/PG	As above	8.96 (3.09)	9.06 (3.09)
Nerolidol/PG	As above	27.9 (5.45)	29.7 (3.16)
1-8-Cineole/PG	As above	18.5 (3.56)	19.1 (3.57)

[a]% w/w dry stratum corneum. $n \geq 3$. Data are given as means ± S.D.

The second major thermal transition, $T2$, observed in DSC traces at approx. 72°C, is removed by lipid extraction and is associated with free-lipids. SAXD measurements show that $T2$ is associated with a disordering of a lamellar lipid phase (Bouwstra et al., 1991). FTIR (Knutson et al., 1987), WAXD (Bouwstra et al., 1992b) and electron-spin resonance studies (Rehfeld et al., 1990) support the view that $T2$ is likely to be associated with a gel (L_β) to liquid crystalline phase (L_α) transition.

The third thermal transition, $T3$, at approx. 83°C, is also abolished by lipid extraction and is also associated with a lipid phase change. FTIR (Knutson et al., 1987) and WAXD (Bouwstra et al., 1992b) studies show that fluidisation of the lipid chains is not complete following $T2$, suggesting that $T3$ is also a gel to liquid crystalline phase transition. Why stratum corneum lipids should melt in two stages is not fully understood. It is possible that phase separation in the lipids of the stratum corneum may give rise to this phenomenon. WAXD experiments on human stratum corneum have shown that tightly packed lipids (orthorhombic perpendicular packing), gel phase lipids, crystalline cholesterol and possibly also liquid crystalline lipids co-exist at normal skin temperatures (Bouwstra et al., 1992b), indicating that lipids are heterogeneously arranged. Furthermore, FTIR studies on porcine stratum corneum have also shown that tightly packed lipids (orthorhombic perpendicular packing) and gel phase lipids co-exist below the first main lipid phase transition (Ongpipattankul et al., 1993), also indicating the presence of different lipid regions. It can be argued that this observed phase-separation in the intercellular lipids causes some areas to 'melt' at a lower temperature than others. Of course the question remains as to which lipid components or lipid microdomains give rise to $T2$ and $T3$. Using lipids extracted from porcine stra-

tum corneum Ongpipattankul et al., 1993 have shown that polar lipids and cholesterol undergo a phase transition at a similar temperature to $T3$ and also that polar lipids alone 'melt' at a temperature similar to that of $T2$.

The fourth thermal transition, $T4$, which remained after lipid extraction and which was observed to be thermally irreversible is usually attributed to further intracellular keratin denaturation (Goodman and Barry, 1989). $T4$ is absent in membrane couplet preparations confirming that it is of intracellular origin (Golden et al., 1986).

3.2. Mechanisms of action of neat terpenes

This study has used DSC measurements to investigate the lipid disruptive effects of terpene enhancers. Phase transitions in single component lipid bilayer systems have been shown to be essentially first-order (see review by Lee, 1987). At the transition temperature, T_m, $\Delta G = 0$. Therefore:

$$\Delta G = 0 = \Delta H - T_m \Delta S \quad (1)$$

thus:

$$\Delta S = \Delta H / T_m \quad (2)$$

Although skin lipids comprise of a complex mixture of components, Eq. (2) has usefully been used to interpret DSC results obtained with stratum corneum (Bouwstra et al., 1989; Ongpipattanakul et al., 1991). In the present study *d*-limonene was observed to reduce lipid transition temperatures without changing ΔH. From the data presented in Table 2, and Eq. (2) we calculate that *d*-limonene treatment increased the combined ΔS associated with the major lipid transitions, $T2$ and $T3$, from approx. 0.027 J/K per g to 0.030 J/K per g (i.e.by approx. 11%). The increase in ΔS produced by *d*-limonene treatment is associated with the decrease in transition temperatures since, according to Eq. (2), ΔS is inversely proportional to T_m. The increase in ΔS after *d*-limonene treatment is probably associated with an increase in entropy of the lipids in the liquid crystalline phase and is characteristic of a freezing-point depression effect. Evidence for freezing-point depression occurring in skin lipids has recently been published by Ongpipattanakul et al., 1991 using the penetration enhancer oleic acid. It is reported, from DSC measurements, that treatment of porcine stratum corneum with oleic acid shifts $T2$ and $T3$ to lower temperatures with no attendant decrease in ΔH. Most importantly, however, parallel FTIR studies showed no change in lipid chain mobility below $T2$ upon oleic acid treatment but, instead, an increase in mobility above $T3$, when the lipids are completely in the liquid crystalline state. Since freezing-point depression usually involves an increase in lipid entropy in the liquid crystalline state, DSC data, with respect to *d*-limonene, provide no direct evidence of a lipid disruptive effect in the gel state at normal skin temperatures.

1-8-Cineole was observed to reduce $T2$ and $T3$ and to reduce ΔH for $T2$. Using the data in Tables 2 and 3 and Eq. (2) we calculate that 1-8-cineole treatment *reduced* the combined ΔS associated with the major phase transitions, $T2$ and $T3$, from 0.028 J/K per g to 0.019 J/K per g (i.e. a decrease of 32%). The reduction in ΔS produced by 1-8-cineole treatment was linked to the reduction in ΔH since, according to Eq. (2), ΔS and ΔH are directly proportional. The calculated reduction in ΔS is most likely to be associated with an increase in the entropy of the lipids in the gel state following 1-8-cineole treatment. A reduction in ΔH for $T2$ is therefore indicative that this enhancer is lipid disruptive at normal skin temperatures. Such an interpretation of the data would be in agreement with DSC studies employing a series of *N*-alkyl azone enhancers (Bouwstra et al., 1989) where decreases in the transition enthalpies of $T2$ and $T3$ were able to be directly correlated to improved skin permeability.

DSC studies thus provide no clear proof that *d*-limonene disrupts the intercellular bilayers, whereas they provide evidence that 1-8-cineole is lipid disruptive at physiological temperatures. This is in good agreement with previous in vitro permeation studies, employing the hydrophilic permeant 5-fluorouracil, which show that 1-8-cineole is 50-fold more potent as an enhancer than *d*-limonene (Williams and Barry, 1991a).

Following nerolidol treatment the cooperativity of the major lipid transitions, $T2$ and $T3$, de-

creased, as seen by the transition peaks coalescing. This decrease in transition cooperativity suggests that lipid chain interactions within the intercellular bilayers have been disrupted by the enhancer, and that the cooperative units within the bilayers have reduced in size. It indicates, therefore, a disordering of the intercellular lipid bilayers by nerolidol at normal skin temperatures. Treatment of porcine stratum corneum with fatty acid penetration enhancers, such as oleic acid, also reduces the cooperativity of $T2$ and $T3$ (Golden et al., 1987b). These changes were similarly interpreted as a lipid disruptive effect of the enhancers at normal skin temperatures. Indeed, the greatest percutaneous flux enhancement for the hydrophilic permeant, salicylic acid, was produced by the enhancers which most reduced lipid transition cooperativity. Unfortunately, the lack of a clear baseline prevented accurate measurement of ΔH and ΔS values following nerolidol treatment. As a result calculation of ΔS was not possible following treatment with this enhancer.

SAXD measurements showed that both *d*-limonene and 1-8-cineole acted to reduce the intensities of lipid bilayer based reflections (Figs. 5 and 6). The reduction in lipid reflection intensities may arise from one, or a combination of two effects. Firstly, the enhancers may increase the random motion of the lipids within the bilayers (i.e. disordering of the first kind (Blaurock, 1982)). Alternatively, the enhancers may disrupt the stacking arrangement of the lipid bilayers (i.e. disordering of the second kind (Blaurock, 1982)). Unfortunately, the weakness of the scattering pattern obtained from stratum corneum samples both before and after monoterpene treatment makes it impossible to distinguish which type of disordering occurs.

The similar reductions in bilayer periodicity produced by *d*-limonene and 1-8-cineole appear to contradict DSC results which show 1-8-cineole to be lipid disruptive and which provide no evidence that *d*-limonene has a lipid disordering effect. SAXD results are also in disagreement with in vitro permeation study data, obtained using the model hydrophilic drug 5-fluorouracil, which show 1-8-cineole to be much more active as an enhancer than *d*-limonene (Williams and Barry, 1991a). The reasons for this are not clear and require further investigation.

In contrast to *d*-limonene and 1-8-cineole, nerolidol had no marked effect on lipid bilayer periodicity. It is possible that this may be because nerolidol phase separates from the majority of the intercellular lipids thus enabling lipid bilayer structures to remain relatively unaffected. Clearly more work is required to investigate this theory.

3.2.1. The possibility of enhancer pooling

Enhancer uptake studies have shown that terpenes are incorporated into the stratum corneum in large quantities when applied as undiluted oils. From the enhancer uptake values obtained it was possible to calculate the ratios of terpene to lipid molecules within treated stratum corneum. It was assumed that lipids make up 10% w/w of the dry weight of the stratum corneum, that the lipid composition was as described by Elias, 1990 and that the ceramide composition was as described by Wertz et al., 1985). The mean molecular weight of stratum corneum lipids was determined to be 487. The molecular weights of *d*-limonene, nerolidol and 1-8-cineole are 136.2, 222.4 and 154.3, respectively. The ratio of *d*-limonene to lipid molecules was calculated to be approximately 3.5:1, that for nerolidol to lipid molecules approximately 12:1, and for 1-8-cineole it was approximately 10:1.

Molar ratios of 3.5:1, 12:1 and 10:1 for *d*-limonene, nerolidol and 1-8-cineole, respectively, suggest that, if the enhancers mixed only with the intercellular bilayers, there would be much more enhancer than lipid material present. Since DSC traces obtained following terpene treatment (Fig. 3) continue to display lipid bilayer transitions, it can be concluded that at least some bilayers remain intact following enhancer treatment. We propose that high enhancer uptake may occur alongside the maintenance of bilayer structures if enhancers pool together somewhere outside the lipid bilayers. This would be in agreement with recent WAXD studies which investigated the effects of the same terpene enhancers on human stratum corneum in vitro (Cornwell et al., 1994). These studies showed that, following enhancer treatment, liquid terpenes phase separated within

the stratum corneum from undisrupted lipid bilayers (i.e. bilayers with orthorhombic perpendicular lipid chain packing and/or hexagonal packing).

The question arises as to where the enhancers might pool. It is possible that a proportion may form microdroplets in the intercellular lipid domains. In agreement with this theory, it has been noted that when a molten mixture of octadecanol (selected as a simple model lipid) and 1-8-cineole is cooled to room-temperature, the mixture solidifies and most of the terpene separates as droplets. Alternatively, or in addition to this mechanism, significant amounts of terpene may distribute into the corneocytes. The log P values for *d*-limonene, nerolidol and 1-8-cineole are 4.54, 3.53 and 3.36, respectively (calculated using the Fragmants method of Hansch and Leo, 1979). If the terpenes behave in a similar manner to the hydrocortisone 21-esters investigated by Raykar et al., 1988, 1-8-cineole and nerolidol would be expected to partition 3- to 10-fold more into the lipid domains relative to the intracellular protein domains, and *d*-limonene 10- to 30-fold more. However, if one considers that the protein domains make up 70–95% of the volume of stratum corneum (Elias and Leventhal, 1979) it becomes apparent that terpene uptake into the protein domains may, in fact, be very significant. As an example, if we assume 1-8-cineole partitions 10-fold less into the protein domains relative to the lipids but the volume of the corneocytes is 10-fold that of the intercellular regions, then total uptake into the corneocytes may be as high as 50%.

An additional point needs to be made regarding these estimates of terpene uptake by stratum corneum. The results in this paper refer to typical partitioning experiments in which the membrane is soaked in the medium. However, in permeation experiments at steady state, either in vitro or in vivo, under ideal conditions (including perfect clearance below the stratum corneum) a linear concentration gradient would develop across the stratum corneum. The terpene would be at the maximum concentration at the input face and zero concentration at the downstream face. Thus the uptake concentrations and molar ratios quoted above would represent those applying in the first layer of the stratum corneum, with zero values for the deepest layer. The average values for whole stratum corneum operating during a steady state diffusional process would be half the above amounts.

3.3. Mechanisms underlying propylene glycol synergy

Control DSC and SAXD measurements investigated the actions of neat propylene glycol on the stratum corneum. DSC experiments showed that propylene glycol treatment reduces somewhat the temperatures of $T2$ and $T3$ without significantly affecting their enthalpies. Using the data in Tables 4 and 5 and Eq. (2) we calculate that propylene glycol increases the combined ΔS for both major lipid transitions from approx. 0.024 J/K per g to 0.027 J/K per g (i.e. by approx. 13%). As discussed above, this is characteristic of a freezing-point depression effect and thus provides no direct evidence that propylene glycol is lipid disruptive at 32°C. In agreement with DSC studies, SAXD experiments showed that propylene glycol had no effect on intercellular lipid bilayer periodicity (Fig. 7). DSC measurements also showed that propylene glycol treatment removes the protein based transition, $T4$. It has been postulated by other workers that this effect may be related to the dehydration of intracellular keratin as propylene glycol replaces bound water (Bouwstra et al., 1989).

One mechanism proposed for terpene/propylene glycol synergy is that of synergistically enhanced lipid disruption. DSC and SAXD data from the present study provide only fragmented evidence in support of such a mechanism. DSC experiments have shown that when terpene enhancers are applied in propylene glycol, the effects of propylene glycol and each of the study terpenes on lipid transition temperature shifts are approximately additive, providing no evidence of synergy. However, propylene glycol was noted to enhance the lipid transition enthalpy reductions produced by 1-8-cineole. We can calculate, using data from Tables 4 and 5, and Eq. (2), that treatment with 1-8-cineole in propylene glycol reduces the combined ΔS associated with the two major lipid

transitions from approx. 0.032 J/K per g to 0.014 J/K per g (i.e. by approx. 55%). This compares with a reduction in the combined ΔS produced by neat 1-8-cineole treatment of approx. 32%. Since propylene glycol alone had no effect on lipid transition enthalpies, and, in fact, acts to increase the combined ΔS by approx. 13%, these results suggest that propylene glycol synergy with 1-8-cineole may occur through enhanced lipid disruption at normal skin temperatures. SAXD experiments showed that propylene glycol enhances the reductions in lipid bilayer periodicity produced by the cyclic monoterpenes, *d*-limonene and 1-8-cineole. Since propylene glycol alone had no marked effect on lipid bilayer periodicity, SAXD data suggest that propylene glycol synergy with *d*-limonene and 1-8-cineole occurs via increased lipid disruption.

Enhancer uptake studies have shown that propylene glycol does not improve enhancer delivery into the stratum corneum. It appears that applying neat terpenes for 12 h saturates the stratum corneum so that further material cannot be absorbed when applied in propylene glycol. Indeed, the delivery of nerolidol and 1-8-cineole was significantly reduced possibly because the propylene glycol itself filled some of the available free volume. Increased uptake of enhancer into the stratum corneum thus cannot account for the synergy observed between the terpenes and propylene glycol.

The mechanisms underlying propylene glycol synergy with terpene enhancers therefore remain unclear. It is possible that the synergy may not arise for some drug molecules from the combined effects of propylene glycol and the terpenes on skin structure. For other molecules, part of the effect may be an increased partitioning of the drug into the stratum corneum. Alternatively, propylene glycol synergy would also arise in part from the extraction of lipids out of the stratum corneum into enhancer formulations over the treatment period. Evidence against such a mechanism, however, comes from a recent DSC study performed with neat terpene enhancers (Yamane et al., 1995) and 1-8-cineole in propylene glycol. These experiments showed that evaporation of volatile terpenes, such as 1-8-cineole and *d*-limonene, from stratum corneum samples following treatment, enables the transition temperatures and enthalpies for the major lipid transitions to return to their control values. If lipid extraction had occurred one would have expected a permanent alteration to the lipid phase transitions.

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