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The routes of penetration of ions and 5-fluorouracil across human skin and the mechanisms of action of terpene skin penetration enhancers

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Summary

The activation energies for ion movement and 5-fluorouracil diffusion across human epidermis in vitro have been measured to be 4.08 ± 0.16 and 20.6 ± 1.56 (mean $\pm$ SE) kcal mol^{-1}, respectively. These data imply that ions travel across human epidermis largely through aqueous shunt routes and that 5-fluorouracil, a polar non-electrolyte, travels mainly through the intercellular lipid bilayers in the stratum corneum. Treatment of human epidermis with terpene penetration enhancers has been shown to increase electrical conductivity. The increase in ion transport suggests that terpenes open new polar pathways across the stratum corneum. A correlation between increases in ion transport and previously reported increases in 5-fluorouracil penetration suggests that terpene enhancers may create micro-pores in the intercellular lipids through which both ions and polar drugs may pass.

Terpene compounds, isolated from plant volatile oils, are well established penetration enhancers for human skin. It has been shown that both mono- and sesquiterpene enhancers increase the percutaneous absorption of 5-fluorouracil, a polar non-electrolyte (log $P_{oct/w} = -0.92$), primarily by increasing drug diffusivity within the stratum corneum (Williams and Barry, 1990a; Cornwell and Barry, 1991). Differential scanning calorimetry (DSC) experiments indicate that the increase in drug diffusivity is accompanied by disruption of the intercellular lipid barrier (Williams and Barry, 1990b; Cornwell and Barry, 1992).

Unfortunately, shifts in the lipid phase transition temperatures following terpene enhancer treatment do not correlate with changes in skin permeability towards 5-fluorouracil. The lack of correlation may indicate that terpene enhancers have additional mechanisms of action. It is possible, for example, that terpene enhancers create highly permeable micro-pores in the intercellular lipid bilayers through which 5-fluorouracil is able to pass.

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The present study uses electrical conductivity measurements to investigate the possibility that terpene enhancers open up new polar pathways. The electrical conductivity of human epidermis in vitro is determined before and after treatment with terpene enhancers. Increases in conductivity are related to increases in skin permeability towards 5-fluorouracil to investigate whether the creation of polar pathways can account for terpene activities.

To validate ion transport as a suitable probe for detecting polar channels in the stratum corneum the activation energy, E_{act}, for ion transport across untreated human epidermis is investigated. It is assumed that if ions travel largely via polar pathways through the human epidermis then E_{act} will be similar in magnitude to E_{act} for ion transport through water.

Finally, it is of interest as to whether 5-fluorouracil itself travels through aqueous shunt routes or through the stratum corneum. E_{act} for 5-fluorouracil diffusion across untreated human epidermis is therefore also determined.

For electrical conductivity measurements, human abdominal skin was obtained post-mortem and stored frozen in double-sealed evacuated polythene bags (Harrison et al., 1984). Epidermal membranes were prepared following the heat-separation method of Kligman and Christophers (1963) avoiding the use of hairy samples. Samples of epidermis were mounted into glass side-by-side diffusion cells (diffusional area = 2.27 cm^2). Both donor and receptor compartments were filled with 5 ml of an aqueous solution of 0.9% w/v sodium chloride and the membranes hydrated for a minimum of 3 days. The conductivity of the epidermal membranes with respect to Na^+ and Cl^- was measured with an a.c. half-bridge circuit. The circuit balanced the electrical properties of the skin with series capacitance and resistance decades. An oscillator produced an a.c. voltage with a frequency of 10 Hz and an amplitude of 100 mV. The risk of causing skin damage with a peak voltage of 100 mV was minimal. Kasting and Bowman (1990) demonstrated that d.c. current densities over 15 $\mu A\ cm^{-2}$ cause skin damage. In order to attain a root mean square current density of 15 $\mu A\ cm^{-2}$ in this study sample impedances would need to have fallen to below 2.1 $k\Omega\ cm^{-2}$. Fortunately, this was never the case. Skin conductivities were measured using two stainless-steel electrodes introduced via the donor and receptor sampling ports and set 2 cm from each side of the membrane.

It has been shown that the equivalent circuit for human skin is a parallel resistance and capacitance (Yamamoto and Yamamoto, 1976). When low frequency a.c. current is applied to such a circuit the majority of the current movement occurs across the resistive component. In this study the skin impedance measured at 10 Hz was used an approximation to the true skin resistance. The skin impedance, Zf, was calculated using the capacitance, C, and resistance, R, values obtained from the half-bridge circuit at balance point, and the formula:

$$Zf = \left(R^2 + (1/2\pi fC)^2\right) \quad (1)$$

where f is the driving frequency (10 Hz). The inverse of the calculated impedance, or the skin conductivity, was used in this study as a measure of ion flow across the membranes. The electrical conductivity is analogous to the membrane permeability coefficient for passive diffusion (Dugard and Scheuplein, 1973).

For the measurement of E_{act} for ion transport the control conductivity of each fully hydrated membrane was measured at 20°C. Each diffusion cell was then placed into a stirred water bath which had been cooled to 5°C. Sample membranes were heated to 50°C in ten 5°C steps, pausing to equilibrate for 15 min at each temperature. Following the final temperature step at 50°C the cells were cooled to 20°C. Control conductivity measurements were taken at 20°C to check for irreversible membrane damage.

The membrane conductivity, G ($S\ cm^{-2}$), is related to the absolute temperature, T (K), through the Arrhenius relationship which when logarithmically transformed becomes:

$$\ln G = -E_{act}/RT + \ln G_o \quad (2)$$

where R is the gas constant (8.31451 $J\ K^{-1}\ mol^{-1}$ or 1.98589 $cal\ K^{-1}\ mol^{-1}$) and G_o is the

pre-exponential term. Plotting ln G against $1/T$ produces a linear plot with a gradient of $-E_{act}/R$ from which E_{act} for ion transport across human epidermis can be calculated.

For the penetration enhancer studies the stratum corneum face of each membrane was treated for 12 h at 20°C with 1 ml of test enhancer. The treatment period aimed to mimic the treatment time in previous in vitro permeation studies employing 5-fluorouracil (Williams and Barry, 1990a; Cornwell and Barry, 1991). The monoterpene compounds investigated were: d-limonene, *R*-(−)-carvone, (−)-menthone, *R*-(+)-pulegone, terpineol, ascaridole and 1,8-cineole. A single sesquiterpene, nerolidol, was also used. After enhancer treatment the membrane surfaces were rinsed twice with acetone (Eagle, 1990). The acetone was immediately removed by rinsing the donor compartments with distilled water. The 0.9% v/v sodium chloride solution was then replaced in both sides of each cell and the membranes equilibrated for 1 h before the post-treatment conductivity measurement.

For the determination of the activation energy for 5-fluorouracil diffusion across human epidermis, sample membranes were prepared as described earlier and mounted into side-by-side diffusion cells (diffusional area = 2.27 cm^{-2}). Both donor and receptor compartments were filled with 5 ml of an aqueous solution of 0.002% w/v sodium azide and hydrated for 2 days. Each cell was then placed into a stirred water bath and equilibrated at 25°C. Pseudo-steady-state 5-fluorouracil absorption was achieved by replacing the azide solution in the donor compartment with 5 ml of a 20 mg ml^{-1} aqueous solution of 5-fluorouracil and allowing diffusion to take place for 20 h. The drug solution was radiolabelled with 0.02 mCi ml^{-1} 5-[6-^{3}H]fluorouracil (NEN Dupont). The pseudo-steady-state permeability coefficient for 5-fluorouracil was measured at eight temperature steps between 25.0 and 46.3°C, pausing for a minimum of 5 h at each temperature before beginning sampling. The receptor compartments were sampled every 40 min for 4 h at each temperature step. Since 5-fluorouracil permeated very slowly donor depletion was not significant. Following the final temperature step the membranes were re-equilibrated at 25.0°C for 20 h and the permeability coefficient re-determined to check for irreversible membrane damage.

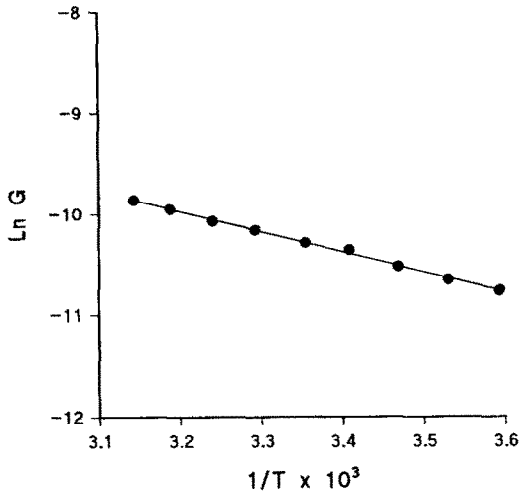

Fig. 1. Typical Arrhenius plot used to determine the activation energy for ion transport through human epidermis in vitro. The membrane conductivity is denoted by G (S cm^{-2}) and the absolute temperature by T (K).

The permeability coefficient, K_p (cm h^{-1}), is related to the absolute temperature, T (K), through the Arrhenius relationship, which when log transformed becomes:

$$\ln K_p = -E_{act}/RT + \ln K_{po} \quad (3)$$

Plotting ln K_p against $1/T$ produces a linear plot with a gradient of $-E_{act}/R$ from which E_{act} for 5-fluorouracil diffusion across human epidermis can be calculated.

Typical Arrhenius plots obtained for ion transport and 5-fluorouracil diffusion are illustrated in Figs 1 and 2, respectively. In both cases the plots were linear over the temperature ranges selected indicating that no significant structural alterations or phase transitions had occurred within the epidermal membranes. Membrane damage was recorded using damage ratios, i.e., the ratio of either the K_p or G after and before the temperature sweep. The damage ratios for the electrical conductivity and 5-fluorouracil diffusion experiments were 0.93 ± 0.03 and 2.10 ± 0.26 (mean ± SE), respectively. In each instance the increases in K_p or G due to irreversible damage

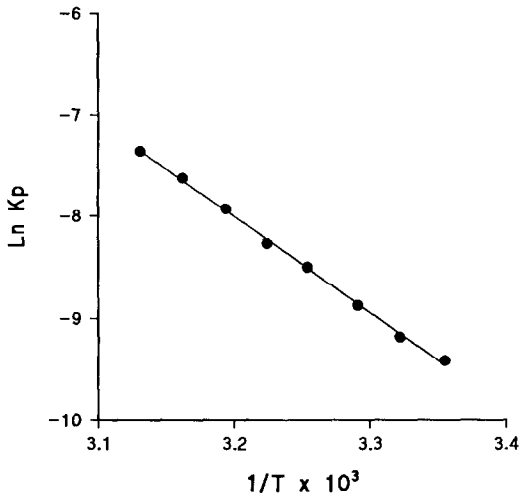

Fig. 2. Typical Arrhenius plot used to determine the activation energy for 5-fluorouracil diffusion across human epidermis in vitro. The membrane permeability coefficient is denoted by K_p (cm h^{-1}) and the absolute temperature by T (K)

were small in relation to the observed increases over the entire temperature sweep.

The E_{act} for ion movement across human epidermis in vitro was determined to be 4.08 ± 0.16 kcal mol^{-1} (mean $\pm$ SE; $n = 4$). This value correlates well with the E_{act} for ion movement through water (3.6 kcal mol^{-1} (Glasstone, 1942)) and suggests that ion movement across human epidermis occurs through aqueous shunt pathways, such as the sweat ducts and hair follicles. The value for E_{act} obtained in this study is lower than the E_{act} for ion transport across phosphatidylcholine bilayers between 10 and 30°C, 10.7 kcal mol^{-1} (Pagano and Thompson, 1968). This suggests further that it is unlikely that ion transport in human skin occurs largely via the intercellular lipid bilayers in the stratum corneum. A previous study has measured the E_{act} for ion transport across human epidermis using electrical conductivity and reports an E_{act} of 14.6 kcal mol^{-1} (Allenby et al., 1969). We feel, however, that these data were possibly miscalculated. Recalculation of E_{act} from the figure illustrating the variation of impedance with temperature produces a value of approx. 3–4 kcal mol^{-1} between 30 and 60°C, a value very similar to that obtained in the present study. The conclusion reached by our study, that ion movement occurs largely through aqueous shunt pathways, is supported by studies from other workers. Using a metal film electrode and also a fine silver wire electrode, Grimnes (1984) has located the major sites for ion transport across human skin in vivo to be at the appendages. Burnette and Ongpipattanakul (1988) have similarly located routes for ion transport to be at the appendages using microelectrodes, as have Cullander and Guy (1991) using a vibrating probe electrode.

The E_{act} for 5-fluorouracil diffusion across human epidermis was determined to be 20.6 ± 1.56 kcal mol^{-1} (mean $\pm$ SE; $n = 5$). This value is 3–4-fold higher than the E_{act} for the diffusion of non-electrolytes through water (Stein, 1967). Unlike ions, therefore, 5-fluorouracil does not appear to travel across human epidermis mainly through aqueous shunt routes. In fact, E_{act} for 5-fluorouracil diffusion is more comparable with E_{act} for the diffusion of non-electrolytes across lipid membranes (Stein, 1967). The results of this study, therefore, further suggest that 5-fluorouracil travels across the intercellular lipid bilayers in the stratum corneum. The high E_{act} values associated with traversing lipid bilayers are believed to arise from, (1) the high energy required to pull molecules free of hydrogen bonds in the aqueous phase and (2) the high energy required to produce sufficient free-volume within the viscous bilayer interiors. Free-volume in lipid bilayers is believed to be produced by the random 'kinking' of hydrocarbon chains (Traübe, 1971). Many other non-electrolytes have similarly high E_{act} values for diffusion across human skin. Polar alcohols (ranging from ethanol to pentanol) have E_{act} values of approx. 16.5 kcal mol^{-1} (Blank et al., 1967). Long chain alcohols (hexanol to octanol) have E_{act} values of approx. 8–10 kcal mol^{-1} (Blank et al., 1967). The E_{act} for glyceryl trinitrate diffusion is 21.8 kcal mol^{-1} (Boddé et al., 1990) and for water diffusion is 17.2 kcal mol^{-1} (Golden et al., 1987). The parity of published data with the results of this study suggests that most non-electrolytes travel across human skin via the intercellular lipids in the stratum corneum.

The effects of terpene treatment on the conductivity of human epidermis are illustrated in

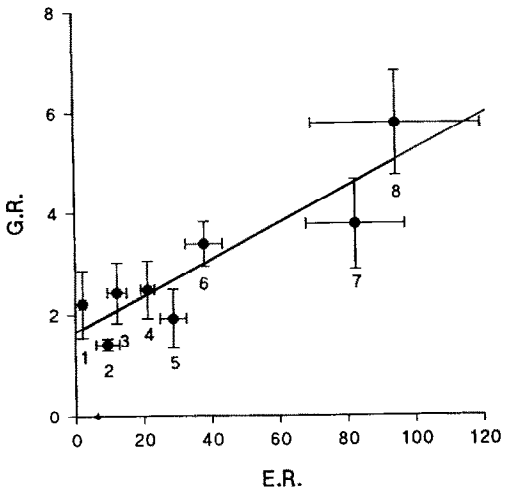

Fig. 3. Relationship between previously reported terpene enhancement ratios, ER (Williams and Barry, 1990a; Cornwell and Barry, 1991), and electrical conductivity ratios, GR. The identities of terpene enhancers are: (1) d-limonene, (2) terpineol, (3) *R*-(−)-carvone, (4) *R*-(+)-pulegone, (5) nerolidol, (6) (−)-menthone, (7) ascaridole, (8) 1,8-cineole. GR values represent means ± standard errors, $n \geq 5$.

Fig. 3. The activity of each enhancer is described by a conductivity ratio. The conductivity ratio is the quotient of the conductivity before and after enhancer treatment. The significant increases in conductivity following treatment with most enhancers suggests that new polar channels are being opened up in the stratum corneum, the major barrier to ion transport in human skin (Yamamoto and Yamamoto, 1976). Fig. 3 further reveals that increases in conductivity are correlated ($r = 0.8957$) to reported increases in membrane permeability towards 5-fluorouracil, although unfortunately there is an uneven spread of the data, with clustering at the low end of the scales. Clustering of the data was unavoidable since terpene enhancers which have enhancement ratios for 5-fluorouracil between 40 and 80 have not been identified. The correlation suggests that terpene enhancers may create new polar pathways in the stratum corneum through which both ions and 5-fluorouracil may pass. Since DSC studies have shown that terpene enhancers disrupt the intercellular lipids in the stratum corneum (Williams and Barry, 1990b; Cornwell and Barry, 1992), the most likely site for polar pathway formation is at the lipid bilayers. It is interesting to note that increases in membrane conductivity following terpene enhancer treatment are smaller than the increases in membrane permeability towards 5-fluorouracil. This may be because ions travel mainly via the aqueous shunt routes and are therefore relatively unaffected by increases in stratum corneum permeability. 5-Fluorouracil penetration, by contrast, has been shown to occur mainly through the stratum corneum and is thus more sensitive to changes in stratum corneum permeability.

The proposal that lipophilic penetration enhancers create polar channels in the intercellular lipids is not new. Polar channels have been suggested to form on oleic acid treatment when, contrary to pH-partitioning theory, piroxicam flux was observed to increase with the proportion of ionised drug (Francoeur et al., 1990). Subsequent Fourier transform infrared studies have suggested that polar channels (or ion permeable defects) may be formed through lateral phase separation between oleic acid and the indigenous lipids (Ongpipattanakul et al., 1991). Similarly, an increase in skin permeability towards the ionised form of salicylic acid following treatment with decylmethyl sulphoxide has been reported by Cooper (1982). Since decylmethyl sulphoxide has been shown in DSC studies to cause lipid disruption (Goodman and Barry, 1989) it is possible that it too forms polar channels in the intercellular lipids.

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