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Research Article

Terpenes and the Lipid–Protein–Partitioning Theory of Skin Penetration Enhancement[1]

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A series of terpenes has been assessed as skin penetration enhancers towards the model polar penetrant 5-fluorouracil (5-FU). Cyclic terpenes were selected from the chemical classes of hydrocarbons (e.g., α-pinene), alcohols (e.g., α-terpineol), ketones (e.g., carvone), and oxides (e.g., 1,8-cineole, ascaridole). Permeation experiments were performed on excised human epidermal membranes and the terpenes varied in their activities; α-pinene only doubled the permeability coefficient of aqueous 5-FU, whereas 1,8-cineole caused a near 95-fold increase. Essential oils, e.g., chenopodium (70% ascaridole), were less effective than the corresponding isolated terpenes. 5-FU is less soluble in the terpenes than in water, and the terpenes did not exert their action by increasing partitioning of the drug into the membranes as illustrated by stratum corneum:water partitioning studies. The penetration enhancers increased drug diffusivity through the membranes, an effect which correlated empirically with the enhancer activities. The principal mode of action of these accelerants may be described by the lipid–protein–partitioning theory; the terpenes interacted with intercellular stratum corneum lipids to increase diffusivity, and the accelerant effects were not due to partitioning phenomena. Keratin interaction was assumed negligible.

KEY WORDS: percutaneous absorption; skin penetration enhancers; terpenes; lipid–protein–partitioning theory.

INTRODUCTION

Transdermal administration of many drugs is often precluded because of the stratum corneum barrier. In attempts to reduce this diffusional barrier, researchers have employed penetration enhancers (or accelerants) which usually disrupt the highly ordered membrane structure (1–6). An established potent enhancer such as dimethyl sulfoxide (DMSO) has limited use; side effects such as sensitization or allergic reactions, an odorous metabolite, and the high concentration required clinically, have prevented widespread commercial use of DMSO (7). An ideal penetration enhancer is pharmacologically inert and cosmetically acceptable and has a specific, immediate yet reversible action (7–9). This study investigates naturally occurring terpenes as enhancers for the cytotoxic agent 5-fluorouracil (5-FU), a model polar penetrant. By employing natural products, some of the side effects associated with synthetic agents may be avoided.

Found in essential oils such as orange, terpenoid compounds have been used as flavorings, perfumes, and medicines, and as such, their toxicities are well documented (10). For example, menthone is approved by the FDA for food use and has an acute dermal LD_{50} in rabbits in excess of 5 g/kg. Oil of ylang ylang (containing geraniol and linalool esters of acetic and benzoic acids) has been used since the 1880s and has recently been employed in aromatherapy. It is extensively formulated into perfumes and is also approved for food use by the FDA. In this study, simple cyclic terpenes were chosen from the broad chemical classes of hydrocarbons, alcohols, ketones, and oxides; see Fig. 1.

MATERIALS AND METHODS

Materials

The terpenes used as received were *d*-limonene and 1,8-cineole supplied by Sigma Chemical Company, α-pinene, 3-carene, terpinen-4-ol, carveol, carvone, pulegone, menthone, α-pinene oxide, limonene oxide, cyclohexene oxide, cyclopentene oxide, and 7-oxabicyclo[2.2.1]heptane provided by Aldrich Chemical Company, α-terpineol and oil of eucalyptus (containing approximately 75% 1,8-cineole) supplied by BDH Chemicals Ltd., piperitone and oil of chenopodium (containing approximately 70% ascaridole) from Field & Co., anise oil (containing approximately 80% anethole) obtained from Thornton and Ross Ltd., and oil of ylang ylang supplied by Schimmel and Co. Ascaridole, the main constituent in oil of chenopodium, was isolated from the oil by fractional distillation *in vacuo* (11). As a first assessment of enhancer activity, all terpenes were used as neat liquids.

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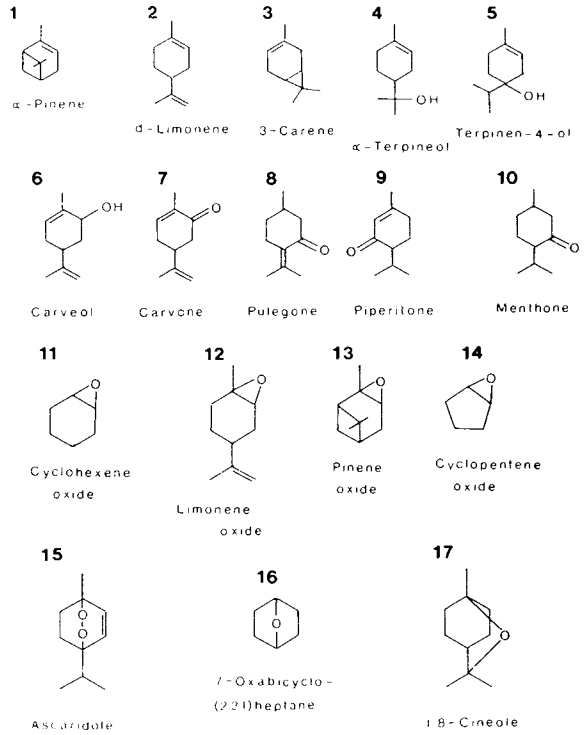

Fig. 1. The structural formulae of terpenes used in this study.

The model polar permeant was 5-[6-^{3}H]fluorouracil (NEN Research Products), radiochemical purity 98%. Unlabeled 5-fluorouracil (Sigma Chemical Company) was used to prepare a saturated aqueous drug solution [10.2 mg/ml at 32 ± 1°C (12)].

Preparation of Human Epidermal Membranes

Caucasian abdominal skin (male and female, 38–88 years) was obtained postmortem. Excess fatty and connective tissues were removed, and the samples stored at −20°C (13). Epidermal membranes were prepared by a heat-separation technique (14); the skin was immersed in water at 60°C for 45 sec, after which the epidermal membrane was teased off the underlying dermis. The tissue was floated on an aqueous solution of 0.002% sodium azide for 36 hr to ensure full hydration of the stratum corneum.

Assessment of Terpene Purities

The terpene purities were assessed by gas chromatography (Perkin Elmer 8320B capillary gas chromatograph) using a flame ionization detector. Aliquots (0.02 μl) of terpenes were injected onto a 50-m BP1 column, using helium as a carrier gas, and the oven was heated from 70 to 300°C at 2°C/min. The purity of the terpenes ranged from 90% for pulegone to 99% for carvone (Table I). No single impurity was present in any of the terpenes at greater than 2%, and such impurities were considered to be at such low thermodynamic activities that their effects on human skin permeability would be insignificant in comparison to the main terpene.

Table I. The Mean Permeability Coefficients (K_p), with Standard Error of the Mean, of 5-FU in Human Epidermal Membranes at 32 ± 1°C Before and After Treatment with a Terpene

Terpene	Purity (%)	K_p (cm/hr × 10^5) Initial (control)	Treated
Hydrocarbons			
d-Limonene	94.1	1.22 ± 0.50	2.57 ± 0.72
α-Pinene	98.1	4.69 ± 1.96	4.74 ± 1.59
3-Carene	95.9	0.82 ± 0.12	3.13 ± 0.65
Alcohols			
α-Terpineol	91.4	5.08 ± 1.55	46.8 ± 7.58
Terpinen-4-ol	95.7	2.46 ± 0.30	25.3 ± 6.31
Carveol	97.6	1.42 ± 0.40	29.0 ± 5.59
Ketones			
Carvone	99.7	1.29 ± 0.42	15.9 ± 4.44
Pulegone	90.7	0.74 ± 0.06	15.5 ± 4.23
Piperitone	97.6	1.62 ± 0.90	47.0 ± 9.82
Menthone	96.1	3.38 ± 1.59	128 ± 46.3
Oxides			
Cyclohexene oxide	99.2	2.86 ± 0.74	6.86 ± 1.99
Limonene oxide	98.4	3.11 ± 0.65	37.3 ± 13.9
α-Pinene oxide	92.7	0.42 ± 0.07	5.84 ± 2.16
Cyclopentene oxide	99.4	1.11 ± 0.22	34.4 ± 12.6
Ascaridole	96.3	2.50 ± 0.37	214 ± 39.1
7-oxabicyclo[2.2.1]-heptane	99.3	4.18 ± 1.14	380 ± 117
1,8-cineole	99.1	2.15 ± 0.30	204 ± 66.0
Oils			
Ylang ylang	—	3.79 ± 1.25	29.6 ± 9.90
Anise	—	2.30 ± 0.34	6.33 ± 0.50
Chenopodium	—	4.33 ± 1.32	93.5 ± 29.1
Eucalyptus	—	2.09 ± 0.42	69.3 ± 13.4

Permeation Experiments

Experiments at 32 ± 1°C used an automated diffusion apparatus with 0.002% aqueous sodium azide as flow-through receptor solution (15).

Fully hydrated epidermal membrane samples, mounted in the cells, were treated with 150-μl aliquots of saturated radiolabeled 5-FU solution placed in the donor compartments, which were covered. Receptor samples (4 ml) were collected every 2 hr for 36 hr, to which 10 ml Scintran cocktail T scintillation fluid was added, and the radiolabeled drug was determined by liquid scintillation counting (Packard Tri-Carb 460 scintillation counter). The permeant solution was then washed from the membrane with 0.002% aqueous sodium azide for 6 hr, which removes over 95% of the drug from the tissue. Aliquots (150 μl) of a terpene were then placed in the donor compartments for a 12-hr treatment, the donor compartment again being covered. After 12 hr the test agent was removed from the membrane and the permeation of radiolabeled 5-FU again monitored to 36 hr to reevaluate the drug permeation. Linear regression analysis of the steady-state diffusion results after the lag time allows evaluation of the permeability coefficient (K_p) of the drug in the membrane before and after terpene treatment. The enhancement ratio (ER) was calculated as (16)

$$ER = \frac{K_p \text{ after application of penetration enhancer}}{K_p \text{ before application of penetration enhancer}}$$

Values reported are mean ratios from a minimum of five replicates.

Partitioning Experiments

The effects of terpenes on 5-FU partitioning into stratum corneum membranes, at 20 ± 1°C, were investigated. Isolated sheets of human stratum corneum were prepared by the method of Kligman and Christophers (14); epidermal membranes were floated overnight on a solution of trypsin (0.0001%, w/v) and sodium hydrogen carbonate (0.5%, w/v) at 37°C. The enzyme digests the viable tissue allowing the remnants to be removed by swabbing. The stratum corneum sheets were floated briefly on water before drying in a desiccator. The membranes were rinsed in ice-cold hexane for 10 sec to remove surface contamination. Samples of dry stratum corneum, approximately 1 cm^2, were weighed and hydrated by floating on aqueous sodium azide for 3 days. The samples were reweighed, equilibrated in a terpene for 12 hr, blotted dry, and placed in a saturated radiolabeled aqueous solution of 5-FU for 4 hr. The membranes were blotted dry and solubilized in 1 ml Soluene-350 tissue solubilizer (Packard). Ten milliliters of Opti Phase HiSafe II (Pharmacia) scintillation fluid and 0.1 ml of glacial acetic acid were added, and the samples stored overnight to allow chemiluminescence to subside. The concentration of 5-FU in the membranes was evaluated by liquid scintillation counting. The concentration of drug remaining in the donor solution after partitioning was determined in duplicate by liquid scintillation counting.

Partitioning of the drug between stratum corneum and water was determined in triplicate, using three tissue samples from different sources for each determination. A control partition coefficient was evaluated by treating the stratum corneum with distilled water for 12 hr instead of a terpene.

This determination of partition coefficients is based on fully hydrated stratum corneum, which provides a more appropriate value than a figure based on the dry tissue weight; in permeation studies the membrane is fully hydrated.

Solubility Studies

Excess crystals of radiolabeled drug were added to 2 ml of the terpenes, heated to 70°C (at which the terpenes are chemically stable) to ensure saturation. The samples were cooled to 20 ± 1°C and equilibrated for 24 hr. The saturated drug concentration was determined in triplicate by liquid scintillation counting of 0.2-ml aliquots in 5 ml Scintran Cocktail T.

Reversibility of Terpene Activity

The reversibility of terpene action was investigated under diffusional conditions. Samples of human epidermal membranes were treated with carveol, menthone, or 1,8-cineole for 12 hr, while control samples were treated with 0.002% aqueous sodium azide solution. The terpenes were then washed from the donor compartments of the diffusion cells and replaced with 0.002% aqueous sodium azide for 24 hr. The sodium azide solution was removed from the treated and control membrane samples and the permeation of radiolabeled 5-FU monitored for 36 hr as before and permeability coefficients determined. The values reported are from six replicates.

RESULTS

Table I shows the mean permeability coefficients of 5-FU before and after treatment of the membrane with a terpene. The mean control value for K_p of 5-FU in the untreated membranes at 32°C is $2.46 \pm 0.29 \times 10^{-5}$ cm/hr (n = 112), agreeing with published data (16,17). The penetration enhancing activity of the terpenes is more clearly demonstrated in terms of the enhancement ratios (Fig. 2).

The unsaturated hydrocarbons show minimal activity compared to the oxygen-containing terpenes, the greatest hydrocarbon ER being only 3.2 for 3-carene. Of the alcohols, carveol has the most activity (ER 20), whereas menthone is the most effective ketone (ER near 40). The oxides range widely, from an ER of 2.4 for cyclohexene oxide to 94.5 for 1,8-cineole. Oil of eucalyptus (containing approximately 75% 1,8-cineole), ER 34.2, is the most effective essential oil but is less potent than the corresponding isolated terpene (1,8-cineole).

Partitioning results are in Table II. The terpenes have no positive effect on drug tissue partitioning, as illustrated by the partition ratio, P_R, where

$$P_R = \frac{\text{partition coefficient after terpene treatment}}{\text{partition coefficient with untreated membrane}}$$

Using the experimentally determined partition coefficients, a diffusion coefficient (D) of 5-FU in the membranes may be calculated by

$$D = \frac{K_p h}{P}$$

where K_p is taken from Table I and h is the membrane thickness (taken as 3×10^{-3} cm). The experimentally determined untreated (control) diffusion coefficient of 5-FU through the membranes is then 0.26×10^{-7} cm^2/hr.

Following membrane treatment with a terpene, the lag time (L) for 5-FU permeation falls (Fig. 3). From L obtained during permeation experiments before and after terpene treatment, an alternative estimate of the diffusion coefficients (D^*) may be deduced from

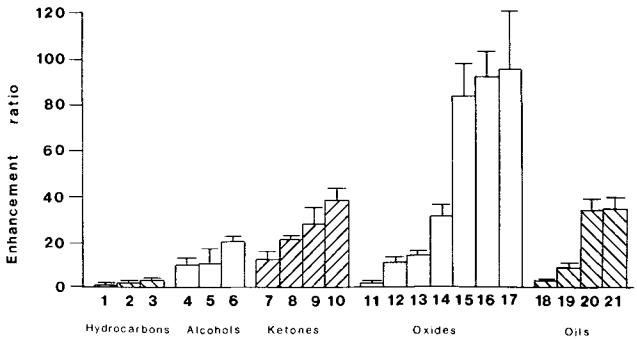

Fig. 2. The penetration enhancing activities of the terpenes expressed as enhancement ratios (with SE). Numbers correlate with those in Fig. 1 and, additionally: 18, anise oil; 19, oil of ylang ylang; 20, oil of chenopodium; and 21, oil of eucalyptus.

Table II. The Effects of Terpenes on the Partitioning of 5-Fluorouracil into Fully Hydrated Human Stratum Corneum Membranes at 20 ± 1°C

Terpene	$P \pm SE^a$	$P_R + SE^b$	D (cm^2/hr, $\times 10^7$)^c
Control	2.88 ± 0.29	1.00	0.26
d-Limonene	2.29 ± 0.17	0.80 ± 0.04	0.34
α-Pinene	2.22 ± 0.58	0.74 ± 0.14	0.64
3-Carene	1.57 ± 0.29	0.59 ± 0.15	0.60
α-Terpineol	1.81 ± 0.25	0.64 ± 0.10	7.75
Terpinen-4-ol	2.57 ± 0.47	0.94 ± 0.23	2.95
Carveol	2.90 ± 0.42	1.04 ± 0.20	3.00
Carvone	1.92 ± 0.53	0.64 ± 0.11	2.48
Pulegone	2.09 ± 0.40	0.73 ± 0.12	2.22
Piperitone	1.45 ± 0.02	0.52 ± 0.05	9.72
Menthone	1.99 ± 0.09	0.71 ± 0.04	19.30
Cyclohexene oxide	1.32 ± 0.40	0.49 ± 0.19	1.56
Limonene oxide	0.88 ± 0.10	0.28 ± 0.05	12.72
α-Pinene oxide	1.09 ± 0.25	0.41 ± 0.12	1.61
Cyclopentene oxide	2.03 ± 0.28	0.73 ± 0.14	5.08
Ascaridole	2.17 ± 0.59	0.73 ± 0.16	29.59
7-oxabicyclo-[2.2.1]heptane	0.88 ± 0.15	0.33 ± 0.08	129.55
1,8-Cineole	0.82 ± 0.02	0.29 ± 0.03	74.63

[a] P = partition coefficient, stratum corneum/water, mean of three replicates, with SE.
[b] P_R = partition ratio = (P/control) = (P/2.88) with SE.
[c] D = mean diffusion coefficient, calculated from K_p in Table I and mean P.

$$D^* = \frac{h^2}{6L}$$

and the relevant partition coefficient may also be calculated

$$P^* = \frac{K_p h}{D^*}$$

These results are in Table III.

The mean untreated (control) diffusion coefficient of 5-FU is 1.54×10^{-7} cm^2/hr (n = 17, SE = 0.06×10^{-7} cm^2/hr), showing good agreement with values calculated from Goodman (18) of 1.50×10^{-7} cm^2/hr. The mean calculated untreated (control) log partition coefficient (stratum corneum/water) is −0.33. The experimentally determined log P (stratum corneum/water), from Table II is 0.46. These values compare to log P octanol/water (often used as an approximation to partitioning into the stratum corneum) of −0.89 (18), confirming that the stratum corneum is a more polar environment than octanol.

The increase in diffusivity of 5-FU in the membranes, calculated from lag-time changes following treatment with the terpenes, may be expressed as a diffusivity ratio, D_R^*, where

$$D_R^* = \frac{\text{diffusivity of 5-FU in membrane after terpene treatment}}{\text{diffusivity of 5-FU in membrane before terpene treatment}}$$

This ratio may be correlated with the terpene penetration

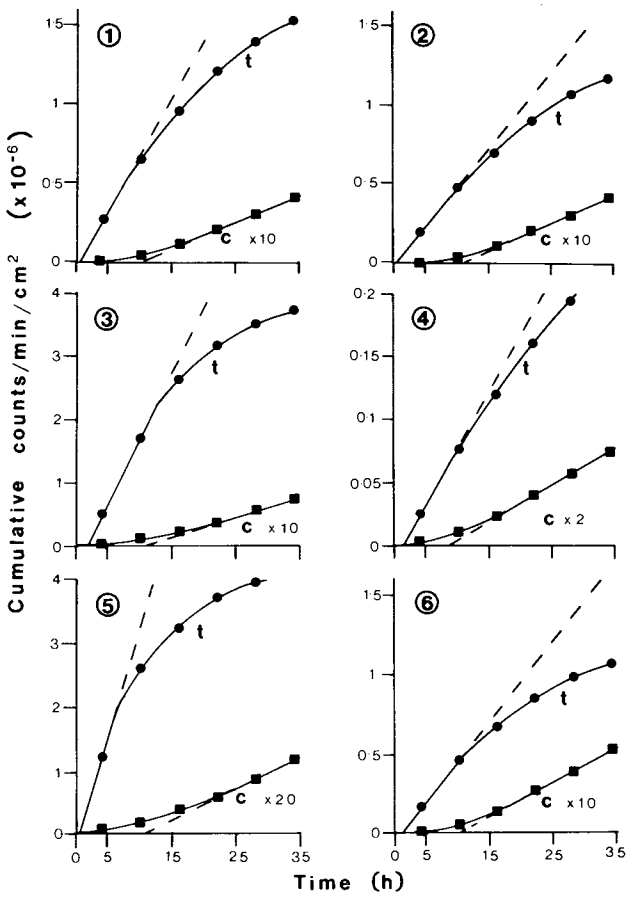

Fig. 3. Typical permeation profiles at 32 ± 1°C of 5-fluorouracil through human epidermal membranes. Membranes treated with (1) oil of chenopodium; (2) carveol; (3) pulegone; (4) α-pinene; (5) 1,8-cineole; and (6) cyclopentene oxide. C, untreated (control) permeation profile, counts multiplied by the factor given; t, permeation profile after terpene treatment. Dotted line is the pseudo steady-state flux; intercepts give the lag times. Every third experimental value plotted.

enhancing activities as illustrated in Fig. 4. The equation of the line, by linear regression, is

$$ER = 5.83\, D_R^* - 7.24, \quad \text{correlation coefficient} = 0.985.$$

The solubility results are also given in Table III. No correlation exists between the partitioning of drug into stratum corneum membranes following terpene treatment and the solubility of 5-FU in the terpenes. 5-FU was much less soluble in all the terpenes than in the donor phase, water.

The log partition coefficients in octanol/water systems give a rank-order approximation to partitioning into the stratum corneum (8). The accelerant activity of the terpenes may be correlated with their log P octanol/water values calculated by the fragment method of Hansch and Leo (19). Examples of this correlation are illustrated in Fig. 5 for the ketones,

$$ER = 23.3 \log P - 45.9, \quad \text{correlation coefficient} = 0.969$$

and epoxides,

$$ER = 4.29 \log P - 4.67, \quad \text{correlation coefficient} = 0.998.$$

Table III. The Calculated Effects of Terpenes on Lag Times, Diffusion Coefficients, and Partition Coefficients of 5-FU Permeating Through Human Epidermal Membranes, with Experimentally Determined Mean Enhancement Ratios and 5-FU Solubilities in the Terpenes[a]

Terpene	L_0 (hr)[b]	D_0^* (cm²/hr, × 10^7)[b]	P_0^{*b}	L (hr)[c]	D^* (cm²/hr, × 10^7)[c]	P^{*c}	D_R^{*d}	P_R^{*d}	ER[e]	Solubility (mg/ml)
d-Limonene	11.6	1.3	0.28	7.8	1.9	0.40	1.5	1.4	2.1	<0.01
α-Pinene	10.5	1.4	0.98	5.5	2.7	0.52	1.9	0.5	1.2	<0.01
3-Carene	8.3	1.8	0.14	2.7	5.6	0.16	3.1	1.1	3.2	<0.01
α-Terpineol	10.8	1.4	1.10	5.8	2.6	5.42	1.9	4.9	9.4	0.56
Terpinen-4-ol	8.6	1.7	0.42	2.0	7.5	1.01	4.3	2.4	10.4	1.88
Carveol	10.8	1.8	0.24	2.6	5.8	1.51	3.2	6.3	20.0	0.23
Carvone	12.3	1.2	0.32	2.9	5.2	0.92	4.2	2.9	12.2	0.10
Pulegone	8.7	1.7	0.13	2.0	7.5	0.62	4.4	4.8	21.2	0.11
Piperitone	12.0	1.2	0.39	1.8	8.3	1.69	6.7	4.3	27.7	0.13
Menthone	10.9	1.4	0.73	1.4	10.7	3.59	7.8	4.9	37.9	0.12
Cyclohexene oxide	7.5	2.0	0.41	3.1	4.8	0.43	2.4	1.0	2.4	0.75
Limonene oxide	9.3	1.6	0.58	3.7	4.1	2.76	2.5	4.8	11.0	0.21
α-Pinene oxide	7.5	2.0	0.06	4.1	3.7	0.48	1.8	8.0	13.7	0.10
Cyclopentene oxide	10.3	1.5	0.23	1.4	10.7	0.96	7.3	4.2	30.9	1.79
Ascaridole	9.5	1.6	0.47	0.6	25.0	2.57	15.8	5.5	82.5	0.24
7-Oxabicyclo-[2.2.1]heptane	12.9	1.2	1.08	0.8	18.8	6.06	16.2	5.6	91.7	3.15
1,8-Cineole	11.9	1.3	0.51	0.7	21.4	2.86	17.0	5.6	94.5	0.07

[a] SE not included for clarity, but similar order to Table II.
[b] L_0 = control (untreated) lag time; D_0^* = control (untreated) diffusion coefficient; P_0^* = control (untreated) partition coefficient.
[c] L = lag time after terpene treatment; D^* diffusion coefficient after terpene treatment; P^* = partition coefficient after terpene treatment.
[d] D_R^* = diffusivity ratio = (D^*/D_0^*); P_R^* = partition ratio (P^*/P_0^*).
[e] Enhancement ratio.

The reversibilities of action of an oxide (1,8-cineole), ketone (menthone) and an alcohol (carveol) were investigated (Table IV). No significant difference exists in the permeability coefficients of the drug in membranes treated with a terpene followed by extensive washing and the pretreated control value (P = 0.10).

DISCUSSION

The experimental design of the permeation studies allows each piece of skin to act as its own control, with the permeability coefficient being monitored before and after treatment with a terpene. This reduces experimental errors arising from biological variability. Human skin remains intact for at least 8 days using this protocol (20), hence significant changes in permeability coefficients are unlikely to result from tissue deterioration. The experimental conditions for drug delivery were maximized as the donor compartments were occluded and the permeant solution was saturated and thus at maximum thermodynamic activity. Fully hydrated epidermal membranes were used, a condition which enhances the permeation of most penetrants including 5-FU (5,21) and, thus, usually provides a stringent test of penetration enhancing activity.

The oxide terpenes provide an interesting series of compounds. The enhancement ratios of the 1,2-oxygen-bridged terpenes (epoxides) are markedly lower than the longer oxygen-bridged terpenes (cyclic ethers) such as cineole, which has a 1,8-linkage. The ER differences may possibly be due, in part, to conformational differences. The six-membered epoxide ring is essentially flat, being in the chair conforma-

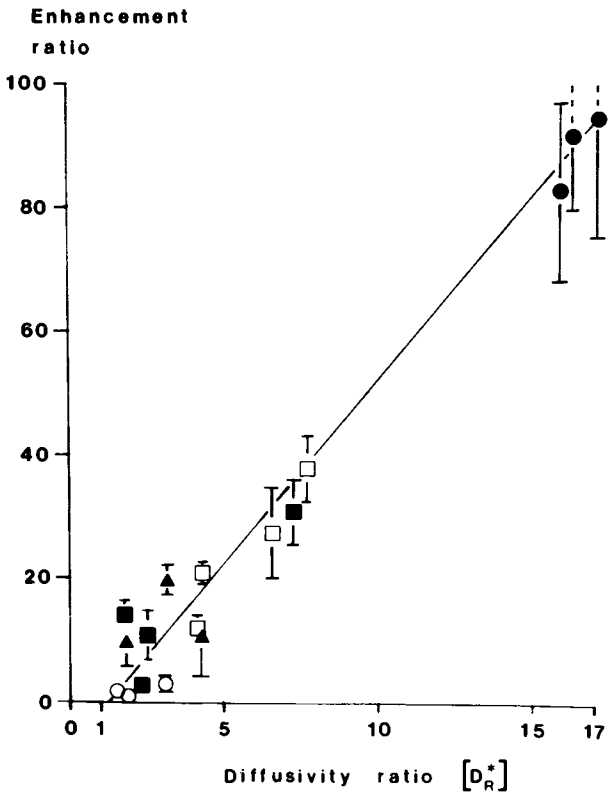

Fig. 4. The relationship between the increased diffusivity of 5-fluorouracil permeating through human epidermal membranes following terpene treatment and enhancement ratios. (●) Cyclic ethers; (□) ketones; (■) epoxides; (▲) alcohols; (○) hydrocarbons.

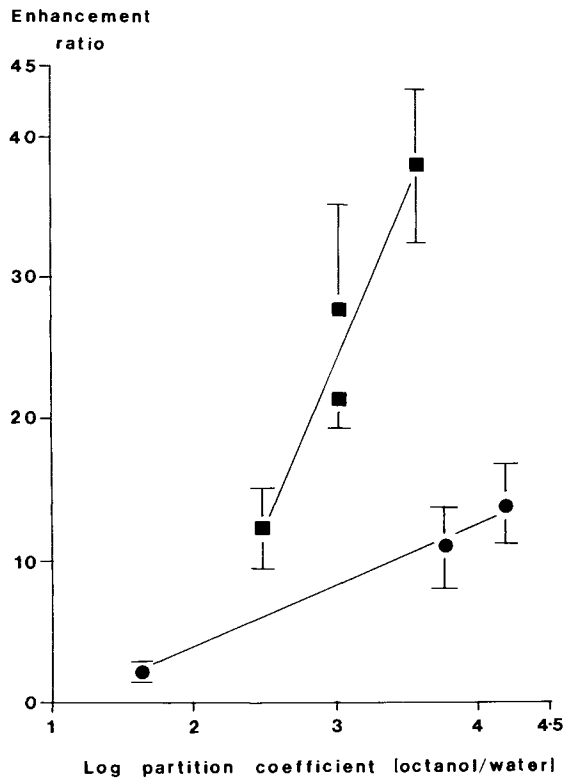

Fig. 5. The relationship between log partition coefficient octanol/water and enhancement ratio for ketone and epoxide terpenes (with SE). (■) Ketones; (●) epoxides.

tion where the ring is relatively free of angular and torsional strain. The hydrocarbon side chains increase the steric bulk of limonene oxide and α-pinene oxide, both of which are significantly better accelerants for 5-FU than the unsubstituted cyclohexene oxide ($P = 0.05$). The cyclic ethers (ascaridole, 1,8-cineole and 7-oxabicyclo[2.2.1]heptane) show significantly greater enhancement ratios than the epoxides ($P = 0.05$) and exist in the less thermodynamically stable boat conformation. As such, the molecules are under considerable torsional and angular strain. No significant difference was found among the activities of the three cyclic ethers ($P = 0.05$), despite ascaridole containing two oxygen atoms and an unsaturated bond, 7-oxabicyclo[2.2.1]heptane containing no hydrocarbon side chains, and 1,8-cineole having a 1,8-oxygen bridge instead of the 1,4-linkage of the other two cyclic ethers. The significantly different ($P = 0.05$) enhancement ratios observed between cyclohexene oxide (ER 2.4) and cyclopentene oxide (ER 30.9) may also possibly be explained in terms of molecular conformations; cyclopentene oxide is under considerable torsional strain when planar, and to relieve this strain somewhat, the molecule takes on a slightly puckered conformation. However, whether or not conformational differences are important in explaining differences in the enhancement abilities of the terpenes remains to be proved.

Table IV. The Mean Permeability Coefficient (K_p), with Standard Error of the Mean, of 5-Fluorouracil Through Human Epidermal Membranes Following Terpene Treatment and Extensive Washing

Treatment	K_p (cm/hr, × 10^5)	Enhancement ratio
Control	3.42 ± 0.34	1.00
Menthone	4.49 ± 0.61	1.31
1,8-Cineole	5.79 ± 1.01	1.70
Carveol	4.93 ± 0.65	1.44

Of the essential oils studied, ylang ylang and anise show mild accelerant activities. Eucalyptus (approx 75% 1,8-cineole) and chenopodium (approx 70% ascaridole) are less effective than the corresponding isolated terpenes, probably because the active constituents are not at maximum thermodynamic activities in the oils.

The activity of 1,8-cineole (eucalyptol) toward several drugs, including procaine, indomethacin, bupranolol, dibucaine, and benzocaine, has been reported (22), although these studies were performed using excised hairless mouse skin, a suspect model for human *in vitro* tissue (12,20,23–25). Eucalyptus oil and camphor increased the total flux of nicotine permeating excised hairless mouse skin (26), and a variety of cyclohexanone derivatives (which show some structural similarities to the ketone terpenes) increased the percutaneous absorption of ketoprofen and indomethacin from gel ointments applied *in vivo* to shaven rats (27). Terpineol and acetyl terpineol are penetration enhancers for the diffusion of prednisolone through hairless mouse skin *in vitro* (28), and the effect of limonene and related compounds on the percutaneous absorption of indomethacin from gel ointments has been investigated in rats (29,30).

A general theory for the modes of action of penetration enhancers, based on molecular and solvent changes in the stratum corneum, has been proposed (for relevant references, see Refs. 5 and 21). According to this theory, accelerants may act by one or more of three main mechanisms: disruption of the highly ordered lipid structure between the corneocytes, so increasing intercellular diffusivity, interaction with intracellular protein to promote permeation through the corneocyte, and increased partitioning of the drug or a coenhancer into the tissue. This concept is formalized as the lipid–protein–partitioning (or LPP) theory (31) and the present results may be rationalized according to this scheme.

The diffusivity ratio calculated from permeation experiment lag times (D_R^*) apparently relates linearly to the enhancement ratio (ER) (Fig. 4), suggesting that the terpenes act, in part, by modifying intercellular lipids, disrupting their highly ordered structure to increase diffusivity. The high log P values (octanol/water) of the terpenes imply that there would be little terpene–keratin interaction within the corneocyte, so that possible protein interaction suggested under the LPP theory should be negligible. The product of the diffusivity and partition ratios calculated from permeation lag times equates to the experimentally determined enhancement ratios after terpene treatment (Table III). However, for the enhancement ratio to be linearly related to the diffusivity ratio, the partition ratio should be constant with a value, given by the slope of the line, of approximately 5. It is clear from Table III that this situation does not exist and we need to consider this contradiction further. Figure 4 may in fact

consist of two point clouds. Examination of the 11 points clustered around the origin shows considerable scatter and a low correlation coefficient with an equation of the best-fit line by linear regression for these points of

$$ER = 3.58\,D_R^* - 0.46, \qquad \text{correlation coefficient} = 0.551$$

Furthermore, in performing the calculations to evaluate D_R^* and P_R^* from experimental lag times, several assumptions were made. It is generally accepted that most molecules permeate through the stratum corneum mainly by a tortuous intercellular route, and it has recently been suggested that the diffusional pathlength for a molecule is approximately 350 μm (32), i.e., some 10-fold greater than the stratum corneum thickness. Thus, the value of h used to calculate partition and diffusion coefficients may be underestimated. However, by taking the ratios of diffusivities and partition coefficients before and after terpene treatment, it is unnecessary to know the exact value for diffusional pathlength, provided we can assume that the pathlength remains constant. This invariance may not in fact be the true situation, as the terpenes may affect the membrane thickness, thus altering the diffusional pathlength. Additionally, the design of the permeation experiments dictates that a concentration gradient of terpene will exist through the tissue (in partitioning experiments, conditions are different) and that the terpenes will clear somewhat from the stratum corneum as the drug permeation is redetermined. Further, human skin is not a simple homogeneous barrier as required by Fick's laws, and the calculations use K_p's which vary 10-fold between skin samples (Table I), a typical phenomenon with human skin. However, by using each piece of tissue as its own control, and expressing results as ratios, the errors due to biological variability of human skin may be minimized. Despite the deviations from Fickian diffusion laws and unavoidable experimental errors, it is clear that the permeation lag time decreases and the diffusivity of 5-FU through the membrane increases following terpene treatment of human epidermal membranes.

The role of partitioning phenomena in the increased drug permeation is less clear. The partition ratios calculated from the lag times suggest that the terpenes increase partitioning of the drug. However, these values may be misleading as previously described. The experimentally determined distribution coefficients using human stratum corneum membranes show that the terpenes exert no positive effect to increase 5-FU partitioning into the tissue (Table II); indeed the reverse may be true. The drug is less soluble in all the terpenes than in water, hence a decrease in drug–tissue partitioning after terpene treatment may be expected. However, 5-FU is, for example, 100 times less soluble in carvone than in water, yet drug partitioning into the terpene-treated stratum corneum is only half that for the aqueous-treated control. With no clear partitioning effects, the terpenes appear to exert their action primarily by increasing the diffusion coefficient of the drug in the membrane. This contrasts with results of Chow *et al.* (33), who found that long-chain fatty acids had no effect on the diffusivity of hydrocortisone through hairless mouse skin and that enhancement entirely arose from partitioning phenomena. However, 5-FU is polar; with lipophilic terpenes, partitioning phenomena may be more important in the acceleration of more lipophilic drugs. A general feature of our investigations is the somewhat poor correlation between diffusion coefficients and partition coefficients calculated from permeation experiments or partitioning determinations (Tables II and III). Such mismatches are common in skin work.

Clearly, the passage of terpene accelerants into the lipid domain of the stratum corneum is essential for activity. This feature is well demonstrated in Fig. 5, where the ERs of the ketone and epoxide terpenes correlate with the calculated log partition coefficient (octanol/water) values, albeit that only three and four points are involved. Such log partition coefficients usually relate linearly to stratum corneum/water values (8). As the partition coefficients of the accelerants increase, so the enhancement ratios increase; the concentration of test agent in the membrane will determine, in part, the scale of lipid disruption and hence the increase in diffusivity of 5-FU.

The reversibility of action of the terpenes was verified. Under diffusional conditions, terpene-treated membranes, after prolonged washing, showed no significant increase in the K_p of 5-FU compared to untreated epidermis (Table IV). This invariance suggests that the terpenes, which are also solvents, do not act by extracting significant amounts of barrier lipid from the tissues. This reversibility is also suggested in Fig. 3, where the fluxes of 5-FU in terpene-treated membranes fall with time as the accelerant washes from the skin, producing the tailing-off effect in the permeation profiles. The donor (drug) solution shows no major depletion during the permeation experiments; less than 3% donor depletion is seen 36 hr after cineole treatment.

In conclusion, our data illustrate the use of naturally occurring terpenes as penetration enhancers in human skin. Of the terpenes evaluated, hydrocarbons were poor accelerants and alcohols and ketones were more effective. The oxides may be subdivided into two chemical classes with the epoxides demonstrating mild accelerant activity, whereas the cyclic ethers are very effective; ascaridole, 7-oxabicyclo[2.2.1]heptane, and 1,8-cineole all induce a near-90-fold increase in the permeability coefficient of 5-FU. The five-membered cyclopentene oxide shows greater penetration enhancing activity than the six-membered cyclohexene oxide. As expected, the essential oils show less accelerant activity than their corresponding isolated chief constituents. The terpenes act by disrupting the lipid structure of the stratum corneum, thereby increasing the diffusion coefficient of the polar drug in the membrane, illustrated by the reduced lag time observed and the increased diffusivity calculated from permeation studies. A linear relationship may exist between the enhancement ratios and the diffusivity increase caused by the accelerants. The terpenes do not increase the partitioning of drug into human stratum corneum, a predictable effect as 5-FU is less soluble in all the terpenes than in water. The high log P values (octanol/water) of the terpenes imply that the penetration enhancers will not significantly modify corneocyte proteins. The terpene action is reversible. The results of this study show that terpene penetration enhancers act by increasing diffusivity via stratum corneum *lipid* disruption, no significant *protein* interaction, and no major *partitioning* alterations, the three major features of the LPP theory of penetration enhancer activity.

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NOMENCLATURE

D	Diffusion coefficient calculated from the experimentally determined partition coefficient
D^*	Diffusion coefficient calculated from permeation experiment lag times
D_R	Diffusivity ratio
ER	Enhancement ratio
h	Membrane thickness
K_p	Permeability coefficient
L	Lag time
LPP	Lipid–Protein–Partitioning Theory
P	Partition coefficient, experimentally determined
P^*	Partition coefficient calculated from permeation experiment lag times
P_R	Partition ratio

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