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Effects of 1,8-cineole on the dynamics of lipids and proteins of stratum corneum

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Abstract

The interaction of a potent percutaneous penetration enhancer, 1,8-cineole, with the stratum corneum (SC) and DPPC membranes was investigated by electron paramagnetic resonance spectroscopy (EPR) of spin-labeled analogs of stearic acid (5-DSA) and androstanol (ASL). The EPR spectra of lipid derivatives spin probes structured in stratum corneum tissue of neonatal rat containing of 0.1–10% (v/v) 1,8-cineole in the solvent indicate an abrupt increase in membrane fluidity at around 1% 1,8-cineole. These spectra of stratum corneum membranes are characterized by the presence of two spectral components differing in mobility. Component 1 was attributed to the spin labels H-bonded to the headgroups, while component 2 possibly arose from spin labels H-bonded to water molecules or temporally non-hydrogen-bonded. With the addition of 1,8-cineole, the spin probes were transferred from the motionally more restricted component 1 to the more mobile component 2, suggesting that 1,8-cineole causes ruptures in the hydrogen-bonded network of the membrane–water interface, with consequent displacements of spin probes towards the hydrophobic core. 1,8-Cineole increased the rotational diffusion rates of component 2, whereas no significant mobility changes were observed in component 1. The EPR spectra of maleimide derivative spin label (6-MSL) covalently attached to stratum corneum proteins indicate that 1,8-cineole does not alter the dynamics of protein backbones. Instead, this terpene only increases the solvent's ability to 'dissolve' and mobilize the nitroxide side chain, which is in agreement with its low irritation response.

Keywords: Stratum corneum; EPR; Spin label; Lipid dynamics; Terpene; 1,8-Cineole

1. Introduction

The transdermal administration of drugs is a more viable alternative to the oral route due to the low metabolic activity of skin compared to that of the gastrointestinal tract and liver. However, the effectiveness of transdermal drug delivery depends on the drug's ability to penetrate the skin in sufficient amounts to reach therapeutic levels. The primary barrier to exogenous chemical absorption is the stratum corneum (SC), the outermost layer of the skin, which is comprised of corneocytes embedded in a multilamellar lipid domain. Corneocytes are keratin-filled dead cells, entirely surrounded by crystalline lamellar lipid regions (Bouwstra and Honeywell-Nguyen, 2002). These anucleated squamous cells contain an insoluble cell envelope of cross-linked proteins, which reduces absorption of drugs into the cells (Bouwstra and Honeywell-Nguyen, 2002), and a cell lipid envelope composed mainly of ω-hydroxyceramides covalently bound to the periphery of the cell envelope (Wertz and Downing, 1987). The intercellular region contains a complex lipid mixture in continuity with the cell lipid envelope, which self-assembles into an ordered multilayered structure known as lipid lamellae, consisting mainly of ceramides, free fatty acids, cholesterol and cholesteryl sulfate (Gray et al., 1982).

The most widely implemented approach to increase percutaneous absorption is the use of penetration enhancers, which ideally cause a temporary reversible reduction in the barrier function of the SC to facilitate safe and effective drug delivery

Abbreviations: SC, stratum corneum; 5-DSA, 5-doxyl stearic acid; ASL, 17β-hydroxy-4′,4′-dimethylspiro(5α-androstane-3,2′-oxazolidin)-3′-yloxyl; 5-DMS, 5-doxyl methyl stearate; 6-MSL, 4-maleimido-1-oxyl-2,2,6,6-tetramethylpiperidine; DPPC, dipalmitoyl-3-phosphatidylcholine; EPR, electron paramagnetic resonance; NLLS, non-linear least-squares fitting program

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through the skin. It is generally agreed that penetration enhancers may increase the permeability of a drug by affecting the intercellular lipids of the SC via extraction or fluidization (Yamane et al., 1995a,b) and/or by increasing the partitioning of the drug into the SC (Gao and Singh, 1998) and/or changing conformations within the keratinized protein component (Williams and Barry, 2004).

Terpenes are volatile and fragrant substances constituents of essential oils, which contain only carbon, hydrogen and oxygen atoms and are commonly used in flavorings, perfumes, and medicines. The terpenes 1,8-cineole and L-menthol applied at 5% (w/v) in 66.6% ethanol as a vehicle were recently reported to show an enhancing effect on percutaneous Zidovudine (AZT) absorption across rat (Narishetty and Panchagnula, 2004) and human (Narishetty and Panchagnula, 2005) skin, reaching drug permeation levels close to those required for therapeutically effective blood concentrations. Oxygen-containing terpenes were reported to increase the permeation enhancers of several polar and non-polar drugs such as 5-fluorouracil (Cornwell and Barry, 1994), morphine hydrochloride (Morimoto et al., 2002) propranolol hydrochloride (Hori et al., 1991), imipramine hydrochloride (Jain et al., 2002), indomethacin (Okabe et al., 1989), hydrocortisone (El-Kattan et al., 2000), tamoxifen (Gao and Singh, 1998), and haloperidol (Vaddi et al., 2002a). However, there are few studies on the mechanisms of permeation enhancement by terpenes in SC, using mainly Fourier transform infrared spectrophotometry (FTIR) and differential scanning calorimetry (DSC) (Narishetty and Panchagnula, 2004, 2005; Vaddi et al., 2002b).

Electron paramagnetic resonance (EPR) spectroscopy of fatty acid spin labels has been employed as an efficient strategy to provide information about the interactions of drugs with the lipid bilayers (Queirós et al., 2005; Couto et al., 2005; Alonso et al., 1995, 1996, 2000) and SC proteins (Couto et al., 2005). Recently, it has been shown that the permeation enhancer 1-methyl-2-pyrrolidone acts as an extractor rather than a fluidizer of SC lipids and also promotes more mobilized and solvent-exposed protein conformations (Couto et al., 2005). In this work, this methodology was employed to gain further insights into the effect of 1,8-cineole on the biophysical properties of the SC and DPPC membranes.

2. Materials and methods

2.1. Preparation of SC membranes

SC membranes of newborn Wistar rats less than 24 h old were prepared as described previously (Queirós et al., 2005; Couto et al., 2005; Alonso et al., 1995, 1996, 2000). After the animal was killed, its skin was excised and fat removed by rubbing in distilled water. The skin was left for 5 min in a desiccator containing 0.5 l of anhydrous ammonium hydroxide, after which it was floated on distilled water with the epidermal side in contact with the water for 2 h. The epidermal side was placed in contact with a filter paper and SC sheet carefully separated from the remaining epidermis. Subsequently, it was transferred to a Teflon-coated screen, washed with distilled water and allowed to dry at room temperature. The membranes were stored with 1 l of silica gel in a desiccator under a moderate vacuum.

2.2. Preparation and spin labeling of vesicles membranes

Lipids were dissolved in chloroform/methanol (2:1) together with the spin label, in a lipid/spin label ratio of (100:1), and dried under a nitrogen stream. The residual solvent was removed by vacuum drying the tube overnight, after which the lipid film was hydrated with the same buffer used for SC. The DPPC samples were stored at 50 °C and vortexed several times.

2.3. Spin labeling and treatment of intact SC

The three lipid spin labels: 5-DSA, having the nitroxide radical moiety (doxyl) in the fifth carbon atom of the acyl chain, the methylated derivative 5-DMS and the steroid androstane (ASL) (Fig. 1) were purchased from Sigma Chem. Co. (St. Louis, MO). To block the sulfhydryl groups of the SC tissue, thus preventing nitroxide reductions in the presence of ethanol at high concentrations, the SC samples were incubated for about 15 h in a solution of 50 mM *N*-ethyl maleimide (NEM) (Sigma Chem. Co.). A small aliquot (1 μl) of stock solution of spin label in ethanol (5 mg/ml) was put on a glass plate and, after the ethanol evaporated, the SC membrane (3 mg) was suspended with about 50 μl of acetate-buffered saline (10 mM acetate, 150 mM NaCl and 1 mM EDTA, pH 5.1) on the same site where the spin label was placed and gently stirred with a small stick for about 10 min. After spin labeling, the SC membranes were incubated for 90 min in mixtures of buffer–1,8-cineole (0–10%, v/v); the buffer of some of the SC samples contained 50% ethanol. Finally, the intact SC membrane was introduced into a capillary tube for EPR measurements.

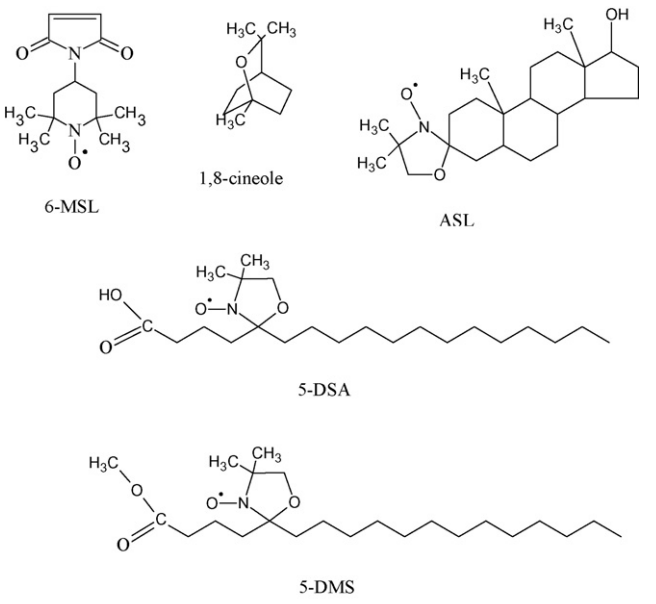

Fig. 1. Chemical structures of the 1,8-cineole and the lipid spin labels used in this work.

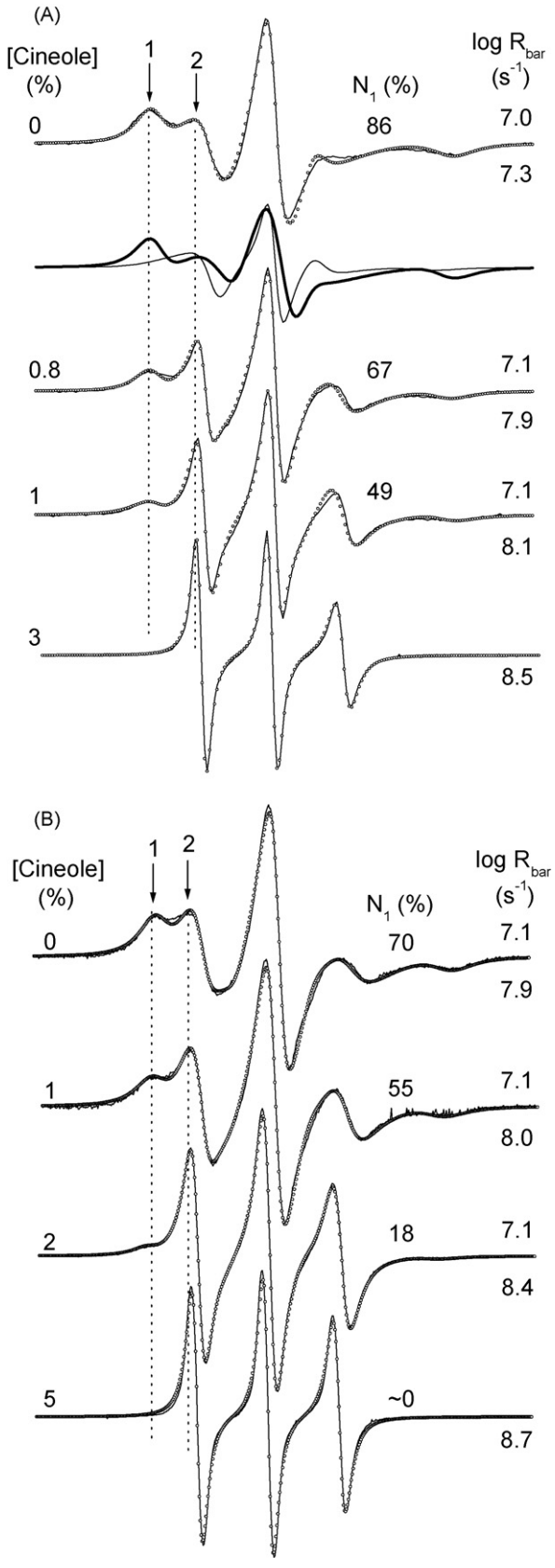

Fig. 2. Experimental (line) and best-fit (empty circles) EPR spectra of spin labels 5-DSA (A) and androstanol (B) in stratum corneum (pH 5.1) at several 1,8-cineole concentrations. The best-fit spectra in this study were obtained by NLLS fitting using a simulation model with one or two spectral components. The arrows and vertical dashed lines indicate the magnetic field positions where the features of these two components are more clearly visible. The two superimposed spectra are components 1 (thick line) and 2 (fine line) simulated for the control spectrum. The figure also indicates the fitting results: N_1 is the percentage of spin labels in component 1; the rotational diffusion coefficients ($\log R_{bar}$) for components 1 and 2 are indicated above and below each spectrum, respectively. The total scan range of the magnetic field was 100 G.

2.4. Spin labeling of SC proteins

The spin label maleimide-nitroxide derivative (6-MSL) was purchased from Sigma Chem. Co. An intact piece of SC (~3 mg) was incubated for 30 min in acetate-buffered saline (10 mM acetate, 150 mM NaCl and 10 mM EDTA), pH 5.1, followed by a second incubation of 15 min in the buffer with 2 mM spin label. The SC membrane was dried on filter paper and incubated in 2 ml of buffer under moderate agitation for 2 min. This process was repeated five times in order to eliminate free unbound labels. After spin labeling, the SC membrane was incubated for 90 min in the buffer containing 5% (v/v) 1,8-cineole and introduced into the capillary tube for EPR measurements.

2.5. EPR spectroscopy

A Bruker ESP 300 spectrometer equipped with an ER 4102 ST resonator and operating in the X-band (9.4 GHz) was utilized in these investigations. The operating conditions of the equipment were microwave power of 20 mW; modulation frequency of 100 KHz; modulation amplitude of 0.6–1.0 G; magnetic field scan of 100 G; sweep time of 168 s; detector time constant of 41 ms. The temperature was controlled within 0.3 °C by a nitrogen stream system (Bruker, Rheinstetten, Germany). EPR spectra simulations were performed using the NLLS program developed by Freed and co-workers (Schneider and Freed, 1989; Budil et al., 1996). This program, which allows a single spectrum to be fitted with two components having different motilities and magnetic tensor parameters, gives the relative populations and the associated rotational diffusion rates. Similarly to previous studies (Queirós et al., 2005; Couto et al., 2005), these parameters were determined based on a general analysis of the overall spectra obtained from this work and, once determined, all the EPR spectra were simulated using the same pre-established values.

3. Results

3.1. EPR spectra of spin-labeled SC samples

Fig. 1 shows the molecular structures of 1,8-cineole and spin labels used in this work. The experimental (line) and best-fit (open circles) EPR spectra of spin label analogs of stearic acid (5-DSA) and androstanol (ASL) structured in the SC membranes are shown in Fig. 2 at the indicated 1,8-cineole concentrations. These EPR spectra clearly reveal the coexistence of two spectral components (except for the higher 1,8-cineole concentration, where only one component is present), each one indicating a distinct motional state of spin probes. Using line-shape simulation,

it was possible to separate these components, determining their respective fractions in the composed spectrum as well their mobility parameters. The origin of these two components has been discussed in previous work (Queirós et al., 2005) but its interpretation is not yet well established. The more restricted motional component 1 was attributed to a spin label population H-bonded to the polar interface of the membrane, while the more mobile component 2 was ascribed to the non-H-bonded spin labels more deeply inserted in the membrane.

As Fig. 2A indicates, the relative population of component 1 decreased gradually with increasing terpene concentration, whereas component 2 showed the opposite effect. Indeed, at a higher terpene concentration, all of the 5-DSA appears to have shifted to the hydrophobic region, since no strongly immobilized signal corresponding to component 1 was observed. A similar behavior is also visible in Fig. 2B, which shows the complete transfer of ASL spin labels from the membrane's polar interface to the hydrophobic region at 5% of 1,8-cineole. Fig. 2A and B also depict the rotational motion parameters $R_{bar}1$ and $R_{bar}2$ obtained from the fitting of EPR spectra, which reflect the mobility states of components 1 and 2, respectively.

3.2. Lipid chain dynamics and partition of spin labels in SC

The fraction of component 1 in the EPR spectra of 5-DSA and the rotational diffusion parameters $R_{bar}1$ and $R_{bar}2$ plotted versus the 1,8-cineole concentration in the SC samples are shown in Fig. 3. Note the $R_{bar}1$ values, which remained essentially constant over the entire terpene concentration range while, at around 1% of 1,8-cineole, the $R_{bar}2$ underwent large increases with the concomitant displacement of spin labels from component 1 to 2. Additionally, Fig. 3 shows that the presence of 50% ethanol in the solvent did not significantly alter the plot behaviors. Similar results were obtained for the spin probes 5-DSA and ASL (data not shown).

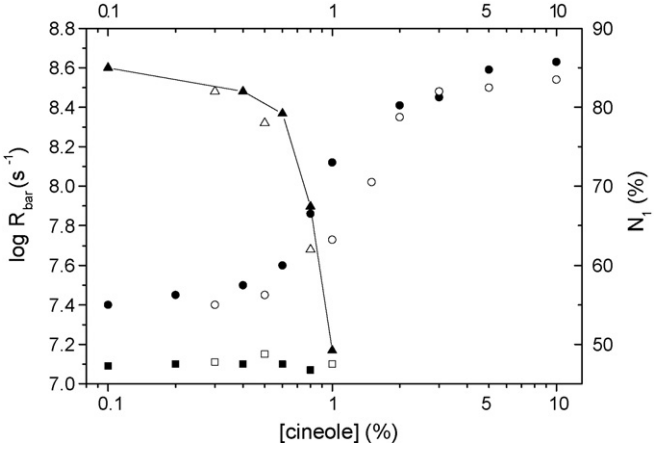

Fig. 3. EPR parameters of spin-labeled lipid 5-DSA in stratum corneum as a function of 1,8-cineole concentration. These parameters were obtained by EPR spectra simulation, considering the resolution of one or two spectral components of spin labels in the membrane. The rotational diffusion coefficient, R_{bar}, is presented for components 1 (squares) and 2 (circles) and the fraction of spin-label population in component 1 (N_1) is given in percentage (triangles). The empty symbols indicate the results for samples containing 50% ethanol (v/v) in the solvent.

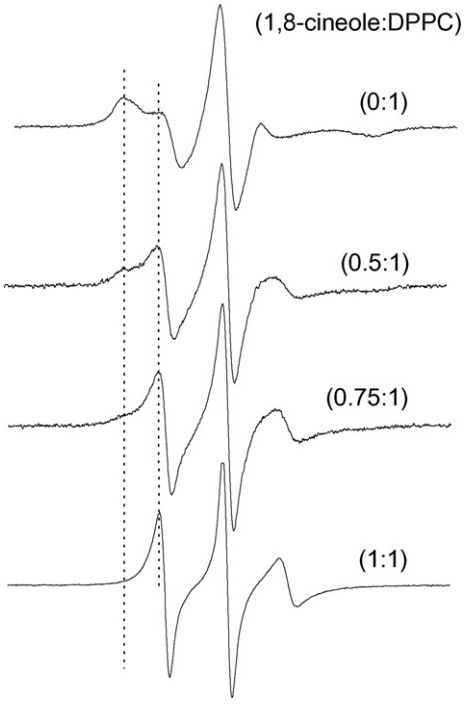

Fig. 4. EPR spectra at 30 °C of spin label 5-DMS in DPPC for several molar ratios of 1,8-cineole:DPPC. The total scan range of the magnetic field was 100 G.

To investigate the molecular relationship of 1,8-cineole:lipids in which the fluidizing effects take place in DPPC membranes, new experiments were conducted using the spin label 5-DMS. Fig. 4 presents the experimental EPR spectra of 5-DMS probe for several molar ratios of terpene:lipids. As can be observed, the line-shape of these spectra are very sensitive to the amount of terpene in the DPPC membranes, which exhibit a significant change in fluidity beginning at a molar ratio of 0.5:1.

3.3. Dynamics of SC proteins

Fig. 5 depicts the EPR spectra of 6-MSL attached to SC proteins in the sulfihydryl groups. These spectra were also simulated allowing for two components to achieve the best least-squares fits. Previous works (Alonso et al., 2001, 2003) showed that these two components result from a thermodynamic equilibrium between two nitroxide populations with very different mobility states. The less mobile component prevails when the nitroxide side chain adopts a "bent" conformation, contacting protein sites through a hydrogen bond involving the oxygen atom of the nitroxide radical, while the mobile component is generated by the spin labels in contact with the solvent, reflecting polarities within the range typical for aqueous systems. Thus, the rotational motion parameter $R_{bar}1$ reflects the segmental motion of the backbone, since the spin labels of the less mobile component are tethered in the protein, whereas $R_{bar}2$ reflects the rotational motion of the nitroxide side chain in the aqueous phase and

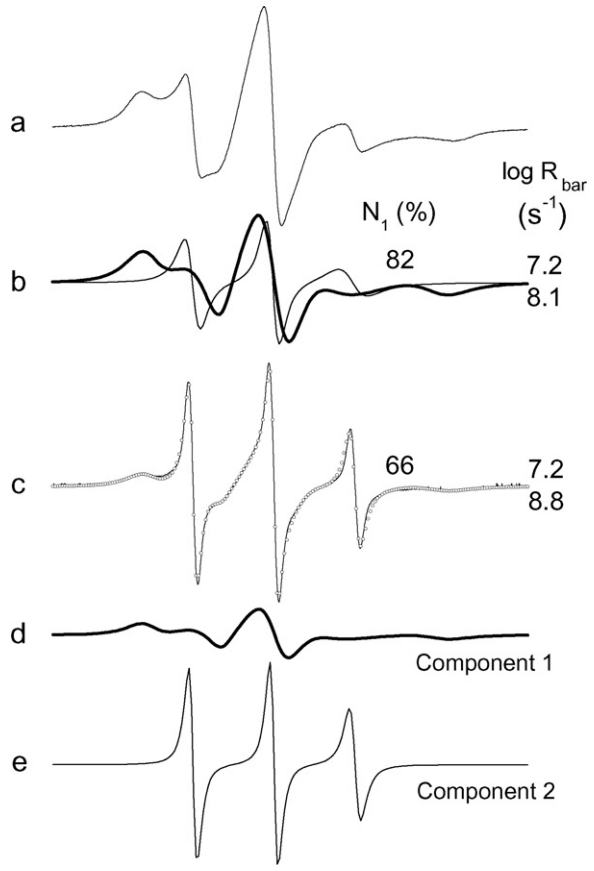

Fig. 5. (a) EPR spectrum of maleimide derivative spin label 6-MSL attached to stratum corneum proteins. (b) Superposition of spectral components 1 (thick line) and 2 (fine line), which are present in spectrum (a). The best-fit spectra of these two components were obtained using the NLLS program. (c) Experimental (line) and best-fit (empty circles) EPR spectra of 6-MSL in stratum corneum after an incubation of 90 min with 5% 1,8-cineole. (d and e) Components 1 and 2, respectively, that describe the spectrum (c).

can therefore be affected by the microviscosity in the vicinities of sulfhydryl groups. The presence of 5% 1,8-cineole in the solvent did not change the protein mobility, as deduced from $R_{bar}1$-parameter values (Fig. 5), but increased both the mobility and fraction of nitroxide side chain free in the solvent (greater $R_{bar}2$ and smaller N_1).

4. Discussion

The amphipathic compound 1,8-cineole is a small molecule with the ability to incorporate into the SC and DPPC membranes, drastically affecting their dynamic organizations. The effect begins at concentrations of around 1% 1,8-cineole in SC and molecular ratios 1,8-cineole:DPPC of (0.5:1). Considering that SC contains approximately 20% of lipids (dry weight), and that the mean molecular weight of SC lipids is about 600 g, one can obtain the molecular ratio 1,8-cineole:SC lipid of (3:1) corresponding to 1% 1,8-cineole in SC (v/v, 50 μl solvent for 3 mg SC). In molecular terms, this finding indicates that the minimum 1,8-cineole concentration necessary to induce fluidity changes is six-fold greater for SC than for DPPC, suggesting that the 1,8-cineole in SC tissue is also partitioned in the hydrophobic sites of proteins.

The irritation response to chemical permeation enhancers has been found to correlate with the denaturation of SC proteins, suggesting the use of protein conformation changes to map permeation-enhancer safety *in vitro* (Karande et al., 2005). Previous works (Alonso et al., 2001, 2003) have demonstrated that the EPR spectra of SC proteins spin-labeled with 6-MSL are highly sensitive to temperature and conformational changes induced by urea (Alonso et al., 2001) or hydration (Alonso et al., 2003). Here, the finding that 1,8-cineole does not change the dynamics of SC-protein backbones and only increases the mobility of the nitroxide side chain is an indication of the inability of 1,8-cineole to induce conformational changes in SC proteins, which is consistent with its low irritation potential. In fact, many terpenes, including 1,8-cineole, menthol, α-terpineol and menthone, are claimed to be GRAS (generally recognized as safe) materials. Cineole, for instance, has been included in the group of terpenes showing no change or very slight irritation through a cytotoxic study of cultured human skin cells (Kitahara et al., 1993).

ATR-FTIR studies using a simple model of SC lipids have indicated that the presence of 1,8-cineole and L-menthol reduces the non-hydrogen-bonded amide I stretching frequency (Narishetty and Panchagnula, 2005), suggesting that terpenes act mainly in polar lipid headgroups and break inter- and intralamellar hydrogen-bonding networks (Narishetty and Panchagnula, 2005). In addition, differential scanning calorimetry has revealed a decrease in the endothermic transition temperature of SC lipids induced by treatment with terpenes (Yamane et al., 1995a,b). This is congruent with our EPR data, which showed gradual increases in the membrane dynamics with increasing 1,8-cineole concentration. In addition, the similar EPR results for SC and DPPC membranes suggest that the 1,8-cineole effect is related to the general properties of the membrane and not to its specific phases.

It is well known that phospholipid bilayers can exhibit dramatic increases in permeability to small water-soluble molecules in the regions of two-phase gel–liquid crystalline coexistence (Bramhall et al., 1987; Clerc and Thompson, 1995; Kraske and Mountcastle, 2001). This phenomenon can be attributed to an increase in permeability at the domain boundaries (Clerc and Thompson, 1995; Sparr and Wennerström, 2001), in which two phases coexist at equilibrium. The suggested mechanisms for this high permeability have focused on density fluctuations in the boundary regions between the coexistent phases (Clerc and Thompson, 1995). These lateral density fluctuations at the phase boundaries increase the probability of defect or transient hole formation that cause maximum permeability of the bilayer at T_m (Clerc and Thompson, 1995). In this context, the suggested mechanism for 1,8-cineole would be as a promoter of fluid phase in the membrane. Increasing the 1,8-cineole concentration in the membrane may increase the fluid-phase domains as well as their mobility states. However, based on previous studies (Queirós et al., 2005) of the formation of two-component EPR spectra, we will now attempt to give a more detailed description of the EPR results and the effects of 1,8-cineole.

A remarkable heterogeneity in the SC membrane dynamics of several spin-labeled lipids was observed by EPR spectroscopy and studied in a previous work (Queirós et al., 2005). The coexistence of two spectral components indicated two classes of probes differing in mobility. An interpretation was given based on the ability of the spin labels to participate in intermolecular hydrogen bonding in the membrane. Components 1 and 2 have been technically called strongly and weakly immobilized components, respectively, in relation to the time scale of the EPR experiments with nitroxides ($\sim 10^{-8}$ s). Component 1 corresponds to a class of motionally restricted spin probes hydrogen-bonded to the polar headgroups (rigid structure), and the more mobilized component 2 is composed of spin labels hydrogen-bonded with water molecules (mobile structure) or temporarily non-hydrogen-bonded and more deeply inserted in the hydrophobic core. Similarly, a bimodal distribution of the fluorescent probe 4′-dimethylamino-3-hydroxyflavone (probe F) in phospholipid bilayer has been previously described, where two probe locations characterized by different polarities and hydrations were identified and attributed to the ability of the probe to participate in intermolecular H-bonding (Klymchenko et al., 2004).

The analysis of spin-labeled lipids by EPR spectroscopy can provide complementary information on the effects of terpenes on SC. By adding 1,8-cineole to the solvent, spin labels 5-DSA and ASL both indicated gradual increases in the fluidity of SC membranes through an interchange of these spin probes from component 1 (less mobile) to component 2 (more mobile), besides a concomitant increase in the mobility of the latter spectral component. The disappearance of the strongly immobilized component (here denoted component 1) of 5-DSA probe in excised guinea pig dorsal skin was reportedly induced by the presence of 1% L-menthol in combination with 15% ethanol (Kitagawa et al., 1998). In this work, the spin probe ASL was selected mainly because its EPR spectra at room temperature for SC present two well-resolved spectral components allowing us to monitor slight variations in the relative fractions of these components. Even for 5-DSA, which has a strong tendency to form component 1, the 1,8-cineole was able to promote the formation of component 2. For DPPC, the methylated derivative 5-DMS was used, taking into account its tendency to form both components at 30 °C. The overall behavior identified by EPR spectroscopy is consistent with the proposition that terpenes enhance transdermal permeations of drugs by transforming SC lipids from highly ordered orthorhombic perpendicular subcellular packing to less ordered hexagonal subcell packing (Narishetty and Panchagnula, 2005). Furthermore, it has also been proposed that L-menthol can disperse throughout the SC, inserted mainly in hexagonal hydrocarbon chain packing, disrupting the regular organization of these structures (Obata et al., 2006). However, the spectral analysis performed in the present work revealed similar effects of 1,8-cineole on SC and DPPC membranes, suggesting that the hypothetical relationship between terpene effects and membrane phases requires a more in-depth investigation.

A recent study (Anjos et al., 2007) showed that ethanol does not significantly alter the fluidity of SC membranes or the relative fractions of components 1 and 2, and that it acts as a lipid extractor at high concentrations (above 30%). The oxygen-containing terpene L-menthol with 20% ethanol in the solvent presented an effect similar to that of 1,8-cineole at temperatures above 24 °C; however, below this temperature, the effect of L-menthol on membrane fluidity was moderated. The present work revealed that the effects of 1,8-cineole on SC membranes may occur without the presence of 50% ethanol in the solvent. Furthermore, DPPC membrane measurements enable us to demonstrate that the effects of 1,8-cineole are similar for both membranes and that a minimum molecular ratio of around 0.5:1 1,8-cineole:DPPC is necessary for significant interactions of spin-labeled lipids to be detected by EPR spectroscopy. It is worth of mentioning that rat skin is more permeable to many drugs than human skin and that the abilities of 1,8-cineole and L-menthol to enhancer the pseudosteady state flux of AZT were less pronounced in human SC (Narishetty and Panchagnula, 2005), suggesting that higher concentrations of these terpenes are necessary to produce the same effects in human SC.

The terpene 1,8-cineole has a functional moiety able to form hydrogen bonds. However, due to its small size and highly hydrophobic character, this molecule is expected to be distributed throughout the interior of the membrane, especially at higher concentrations as those normally used. As a consequence, this molecule may reach the center of the bilayer, where it would increase the local mobility and volume. Thus, the polar groups of 1,8-cineole molecules introduced into the central region of membranes attract more polar lipid headgroups from the membrane–water interface to the interior of the bilayer. This process could cause ruptures in the hydrogen-bonded network of the polar interface, facilitating the partition and, hence, the permeation of small polar molecules through skin.

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