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International Journal of Pharmaceutics 104 (1994) 61–72

international journal of pharmaceutics

An electron spin resonance study: I. Effect of Azone® on 5-doxyl stearic acid-labeled human stratum corneum

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(Received 7 December 1992; Modified version received 10 September 1993; Accepted 13 September 1993)

Abstract

An electron spin resonance study was performed to investigate the effect of Laurocapram (1-dodecylazacycloheptan-2-one, Azone®) on lipid bilayers of human stratum corneum. 5-Doxylstearic acid (5-DSA) was used as a lipid spin label and incorporated with the untreated and treated stratum corneum. Azone® led to a significant change in ESR spectra of human stratum corneum – from strongly immobilized to weakly immobilized spectra. The molecular motion of 5-DSA comprising the Azone®-treated stratum corneum showed an isotropic motion to some extent, which was totally different from the molecular motion of 5-DSA in the untreated stratum corneum. Azone® reduced order parameters (S) by 54–80% at different concentrations. The results suggest that Azone® affected the spin label binding to lipid bilayers and caused an increase in the flexibility and polarity of local bilayers surrounding 5-DSA. The concentration effect of Azone® and influence of ethanol on the stratum corneum were also investigated.

Key words: ESR; 5-Doxylstearic acid; Penetration enhancer; Azone®; Human stratum corneum

1. Introduction

A major barrier for diffusion of chemicals through the skin is its outermost layer, the stratum corneum. The stratum corneum is composed on corneocytes embedded in lipid domains consisting of alternately hydrophilic and lipophilic layers (Barry, 1983; Elias, 1983). In order to extend the variety of drugs that might be administrated via the skin and to increase the percutaneous absorption of drugs, considerable attention has been focused on the mechanism of action of skin penetration enhancers (Goodman and Barry, 1989; Potts et al., 1991).

Many skin penetration enhancers have been proven to interact in some way with stratum corneum lipid structure, generally by increasing the fluidity of the intercellular lipid bilayers (Barry, 1987, 1991). In the lipid regions at least two types of disorder can be distinguished, namely the disorder of the alkyl chains inside one lipid bilayer (a shorter range disorder) and the disorder in the lipid bilayer arrangement (a longer range disorder) (Blaurock, 1982). Over the past

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SSDI 0378-5173(93)E0274-T

few years several physical techniques, such as differential scanning calorimetry (DSC), X-ray diffraction, Fourier transform infrared red (FTIR) spectroscopy, nuclear magnetic resonance (NMR) and electron spin resonance (ESR) spectroscopy have been used to gain more information on the interaction of penetration enhancers with the skin (Goodman and Barry, 1986; Golden et al., 1987; Gay et al., 1989, 1990; Friberg et al., 1990; Mak et al., 1990; Bouwstra, 1991; Ward and Du Reau, 1991; Takeuchi et al., 1992).

ESR spectroscopy of nitroxide spin label has been introduced as a valuable method in the study of the structure of biological membranes, membrane properties and drug-membrane interaction (Sauerheber et al., 1977; Curtain and Gordon, 1984). Spin labels are specifically incorporated with the lipid or lipid part of biological membranes. Thus, each label reflects properties of a different membrane region. ESR spectra of membrane-incorporated spin labels are sensitive to the rotational mobility of the spin labels, the polarity of the environment surrounding the spin labels and the orientation of the spin labels. These effects have been used to advantage in numerous studies of membranes and skins. Some physical conditions (e.g., temperature) and chemicals can perturb biological membranes to yield characteristic changes in their respective ESR spectra (Hubbell and McConnell, 1969; Seelig, 1970; Keith et al., 1973; Cannon et al., 1975).

Although many substances are known as penetration enhancers, a detailed knowledge of the mechanism of action of enhancers is currently developing. Recently, an ESR study of skin penetration enhancer effect on the membrane fluidity in 5-doxylstearic acid-labeled model lipid liposomes (multilayer dipalmitoylphosphatidylcholine) and human skin was conducted by Gay et al. (1989, 1990). The current work investigates the model of action of Laurocapram (1-dodecylazacycloheptan-2-one, Azone®) on the human stratum corneum. 5-Doxylstearic acid (5-DSA) was used as a spin label. In the skin bilayers, this type of spin label interposes itself into lipid bilayers. The unique principal hyperfine axis of the nitroxide is parallel to the long hydrocarbon chains (Hubbell and McConnell, 1969; Curtain and Gordon, 1984). Since the orientation of labels in bilayers must reflect the local molecular structure and should serve as delicate indicators of conformational changes in bilayers, we approached the action of Azone® on the human skin by studying ESR spectra so as to gain more insight into its mechanism of action.

2. Materials and methods

2.1. Materials

5-DSA was purchased from Aldrich. Azone® was generously supplied by Whitby Research Inc. (U.S.A.). All other chemicals and reagents purchased from commercial sources were of analytical grade.

2.2. Preparation of stratum corneum

Human abdominal skin was obtained from fresh cadavers with a dermatome. The dermatomed skin was immersed in a 60°C water bath for 2 min and then the epidermis was separated from the dermis by mechanical removal. The epidermis was placed, stratum corneum side up, on filter paper and floated on a 0.5% trypsin (type II, Sigma), in a Tris-HCl buffer solution (pH 7.4), for 2 h at 37°C. After incubation any softened epidermal parts were removed by mild agitation of the stratum corneum sheets. Samples were dried and stored in a desiccator at −70°C.

2.3. Spin-labeling procedures and pretreatment with the skin penetration enhancer

5-DSA was used as a stearic acid spin labeling agent. The dry stratum corneum samples (a slice of 0.4 cm^2) were incubated in the 5-DSA Tris buffer solution (pH 7.4, 10 $\mu g/ml$) for 2 h at 37°C and then dried under a flow of nitrogen gas for 1 h at 37°C over silica gel (Gay et al., 1990).

For a pretreatment, 5 μl of ethanol containing 25, 100 and 250 μg of Azone®, respectively, was spread on the stratum corneum side of previously prepared skin samples. Similar treatment with

ethanol alone was performed as a control sample. After evaporating the ethanol, all samples were incubated at 37°C for 1 h and then followed by the procedure described above to label with 5-DSA.

2.4. ESR spectral measurement

Three slices of stratum corneum samples previously labeled with 5-DSA were mounted on the flat surface of a modified quartz cell (Wilmad Glass Co., Buena, NJ). The cells are 21 cm in length with an outer diameter of 1 cm. These cells allow thermostatted mineral oil to be passed behind the sample throughout the experiment; the temperature is controlled at 37°C.

A Varian Model E-12 ESR spectrometer (Varian Associates, Palo Alto, CA) and recorder are coupled to a data collection system, Keithley 195A Digital Voltmeter, IEEE interfaced to an IBM-PC. The hyperfine splittings of labeled skin samples were determined with a 100 G scan width, 4×10^2 receiver gain, 16 min scan time and 0.3 s time constant. A microwave power setting of 2 mW was used for all samples and temperatures were controlled to 37 ± 0.5°C. Each sample was scanned three times and the ESR parameters from each spectrum were averaged to give a single estimate for that sample. Under these conditions, the ESR spectrum of 5-DSA spin label in pH 7.4 Tris buffer solution was also measured and is shown in Fig. 1.

The order parameters were calculated according to Griffith and Jost (1976), and Hubbell and McConnell (1971):

$$S = (A_{\parallel} - A_{\perp})/\left[A_{zz} - 1/2(A_{xx} + A_{yy})\right] \times (a'_o/a_o) \quad (1)$$

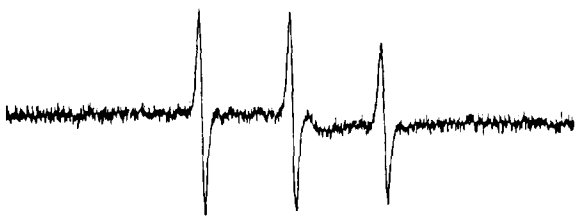

Fig. 1. ESR spectrum of 5-doxylstearic acid in Tris buffer solution (pH 7.4).

where $2A_{\parallel}$ is identified with the outer maximum hyperfine splitting, $2A_{max}$ and $A_{\perp}$ is obtained from the inner minimum hyperfine splitting, $2A_{min}$ (shown in Fig. 2a), using the following corrections:

$$A_{\perp} = A_{min}(G) + 1.4\left[1 - (A_{max} - A_{min})/(A_{zz} - A_{xx})\right]$$

a'_o is the isotropic hyperfine splitting coupling constant for the nitroxide molecule in the crystal state.

$$a'_o = 1/3(A_{xx} + A_{yy} + A_{zz}) \quad (2)$$

The values used to describe the rapid anisotropic motion of membrane-incorporated probes of fatty acid type are:

$$(A_{xx}, A_{yy}, A_{zz}) = (6.1, 6.1, 32.4)\ \text{G}$$

Similarly, the isotropic hyperfine coupling constant for the spin label in the membrane (a_o) is given by:

$$a_o = 1/3(A_{\parallel} + 2A_{\perp}) \quad (3)$$

a_o values are sensitive to the polarity of the environment of the spin label, since increases in the a_o value reflect an increase in the polarity of the medium.

The order parameter (S) provides a measure of the flexibility of the spin label in the membrane. It follows that $S = 1$ for a highly ordered system and $S = 0$ for a completely isotropic motion. Increases in the order parameter reflect decreases in the segmental flexibility of the spin label and conversely decreases in the order parameter reflect increases in the flexibility.

In some circumstances, when the motion of the label appears to be predominantly isotropic, the well-known order parameter formalism no longer applies (Curtain and Gordon, 1984). An empirical parameter, the rotational correlation time (τ), can be used to give a measure of motion in the region probed by the spin label (Keith et al., 1970; Marsh, 1981). The equation is:

$$\tau = 6.5 \times 10^{-10} W_o\left[(h_o/h_{-1})^{1/2} - 1\right] \quad (4)$$

where W_o is the line width in G; h_o and h_{-1} are the heights of the mid- and high-field lines, re-

spectively, on a first-derivative absorption spectrum (see Fig. 2b). The expression based on line broadening and heights assumes isotropic motion. Here, it should be noted that, in some ESR spectra of Azone®-treated stratum corneum, the net motion of 5-DSA is not fully isotropic and may result in inaccuracies in τ calculations. Therefore, both S and τ values are listed in Table 2 for purposes of comparison.

3. Results and discussion

3.1. Concentration effect of 5-DSA on the ESR spectra of human stratum corneum

Human stratum corneum was labeled with two concentrations of 5-DSA in pH 7.4 Tris buffer solution (10 and 20 μg/ml) to investigate the action of label concentration on the ESR spectra of stratum corneum (Fig. 3). The ESR spectra

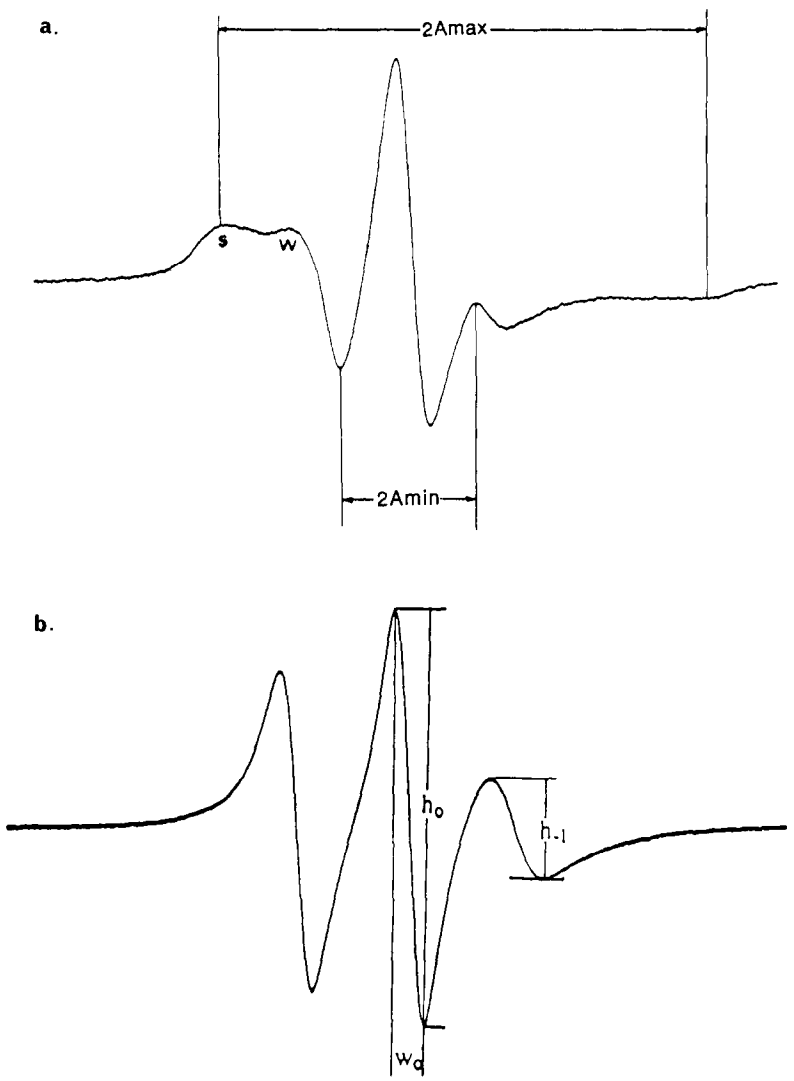

Fig. 2. ESR spectra of 5-DSA labeled human stratum corneum treated with Azone®. Order parameter S (a) and rotational correlation time τ (b) were calculated based on the indicated splittings, line width and line height.

were recorded at 5 and 24 h after labeling for each concentration. The immobilized spectra of spin labeled stratum corneum may be interpreted in terms of a stratum corneum-incorporated 5-DSA molecule undergoing rapid, anisotropic motion about its long molecular axis. A 'fluid' or 'liquid-line spectral component appeared in addition to the immobilized spectra in both concentrations at 5 h after labeling, and more of the fluid components were clearly observed at a higher concentration of 5-DSA. This result indicates that spin label partitioned between a nonpolar membrane lipid (immobilized) environment and an aqueous (fluid) environment. The outer hyperfine splittings ($2A_{max}$) were calculated from the ESR spectra. At a lower concentration of 5-DSA, A_{max} values are 31.6 and 32.7 G at 5 and 24 h, respectively; at a higher concentration of 5-DSA, the corresponding A_{max} values are 30.7 and 32.7 G. The A_{max} values were the same at 24 h for both concentrations of 5-DSA and A_{max} decreased with an increase in the probe concentrations at 5 h. In addition, a higher concentration of 5-DSA caused a broadening of the inner hyperfine splitting (A_{min}) as observed. Because all of the membrane-incorporated spin label molecules participate in nitroxide radical interactions which not only broaden line width and $2A_{min}$ but also increase the flexibility of spin labels when a high concentration of spin label is used, such an 'apparent' increase in fluidity with spin label concentration is not the result of probe-mediated perturbation in the skin structure or bilayers. We can attribute this alteration to extensive probe-probe interactions in the stratum corneum labeled with higher probe concentration

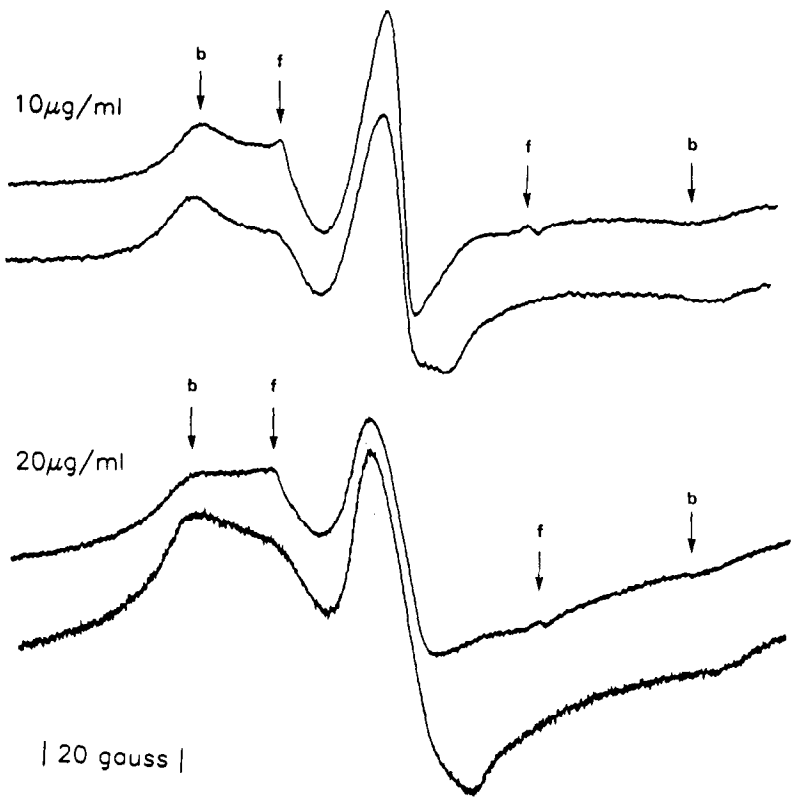

Fig. 3. ESR spectra of human stratum corneum labeled with two concentrations of 5-DSA (10 and 20 μg/ml) at 37°C, recorded at 5 and 24 h after labeling. The binding spectral components are indicated by arrows b and the fluid spectral components are designated by arrows f.

(Sauerheber et al., 1977; Curtain and Gordon, 1984).

Probe-probe interaction may obscure the nature of 'intrinsic' membrane properties. The presence of a fluid component will superpose over the immobilized spectra at higher concentrations of spin label. The ESR parameter obtained from the corresponding ESR spectra affords the most accurate description of the membrane fluidity if lower probe concentrations are used. Therefore, it is desirable to use a spin label concentration at which radical interactions are negligible. In the present study the 5-DSA concentration was fixed at 10 μg/ml.

3.2. Ethanol effect on the ESR spectra of human stratum corneum

An ESR study of human stratum corneum previously treated with ethanol for 1 h was performed to estimate the effect of ethanol vs untreated samples. The ESR spectra of 5-DSA labeled stratum corneum treated without or with ethanol as a function of labeling time are given in Fig. 4 and Fig. 5, respectively. It is evident that two spectral components can be seen in these two figures: the bound components (arrows b) and the fluid components (arrows f). The fluid components are especially clear at 5–8 h after labeling and the line width of the fluid component is narrow (Fig. 4). These spectra indicate that 5-DSA partitioned between a non-polar (immobilized) environment and an aqueous (fluid) environment. With the diffusion of 5-DSA into stratum corneum, the fluid components became smaller while bound components were more prominent. The fact that no fluid components were observed at 24 h indicates that this component was present in a polar environment on the surface of the stratum corneum. In addition, it is clear that

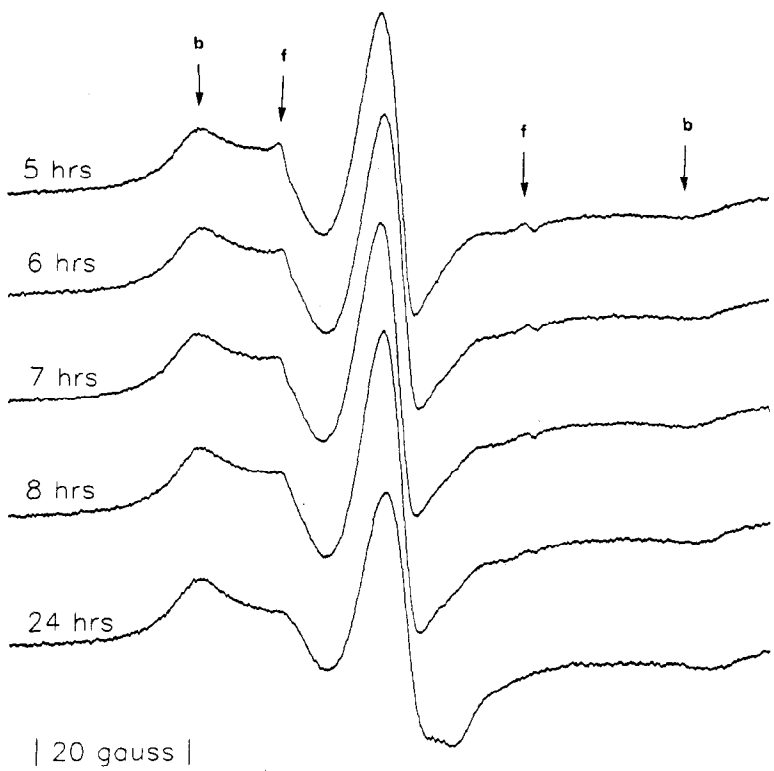

Fig. 4. Time dependence of ESR spectra of 5-DSA labeled human stratum corneum at 37°C, recorded from 5 to 24 h after labeling. The binding spectral components are indicated by arrows b and the fluid spectral components are denoted by arrows f.

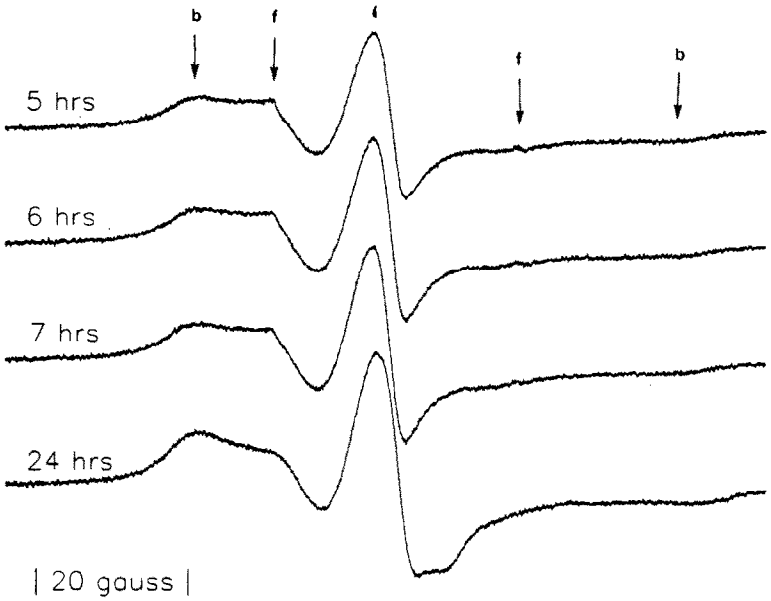

Fig. 5. Time dependence of ESR spectra of 5-DSA labeled human stratum corneum previously treated with ethanol at 37°C, recorded from 5 to 24 h after labeling. The binding spectral components are indicated by arrows b and the fluid spectral components are designated by arrows f.

more bound components were present as observed in ESR spectra of untreated stratum corneum. The difference in peak heights of these bound components between treated and untreated samples is shown in Fig. 6. The binding extents of 5-DSA in these two different skin environments were obviously different. The bound components in untreated stratum corneum slightly increased (increase index: 13.6%) with increased labeling time; while the bound components in ethanol-treated stratum corneum greatly increased from 5 to 24 h (increase index: 80%). Furthermore, the comparison of bound components at 5 and 24 h shows that the bound component in ethanol-treated stratum corneum at 5 h was 2.2-times less than that in the untreated sample and 1.4-times less at 24 h. Ethanol-induced changes in ESR spectra were shown as a weakening of the bound components and a delay of bindings. Table 1 gives the ESR parameters as a function of labeling time. Small increases in order parameters of 5-DSA labeled stratum corneum indicate that 5-DSA took time to enter the stratum corneum. The polarities (a_o) of both untreated and ethanol-treated stratum corneum were almost unchanged. This suggests that 5-DSA did not change the polarity of the environment of lipid bilayers during its partition into the stratum corneum. In fact, our experiments show that all ESR parameters tend to be constant after 24 h of

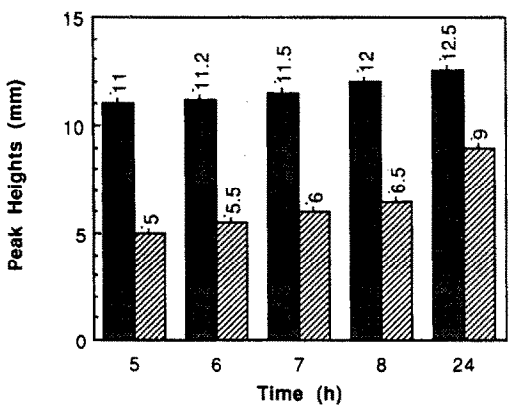

Fig. 6. Plot of peak heights of bound components vs labeling time. Ethanol effect on the 5-DSA binding to lipids was compared with the binding ability of untreated stratum corneum. Mean ± SE ($n = 3$). (■) Untreated stratum corneum; (□) ethanol-treated stratum corneum.

Table 1
ESR parameters of 5-DSA labeled human stratum corneum

Treatment	Time [b] (h)	$2A_{max}$ (G)	$2A_{min}$ (G)	Polarity a_o [c]	Order parameter [d] S
No	5	63.31	15.11	16.16	0.80
	6	64.03	14.75	16.16	0.82
	7	64.39	14.75	16.22	0.82
	8	64.75	14.39	16.16	0.84
	24	65.83	13.67	16.10	0.87
EtOH [a]	5	62.59	15.47	16.16	0.78
	6	63.31	15.11	16.16	0.80
	7	64.03	14.75	16.16	0.82
	8	64.75	14.39	16.16	0.84
	24	65.11	13.87	15.98	0.86

[a] Stratum corneum samples were treated with ethanol.
[b] ESR spectra were recorded at 37°C at the indicated time after labeling with 5-DSA.
[c] Calculated using Eq. 3. Values ±0.05 G.
[d] Calculated using Eq. 1. Estimated uncertainty is values ±0.01.

labeling. $2A_{max}$ values (Table 1) have been shown to be sensitive to the flexibility of the local environment around the spin label (Griffith and Jost, 1976; Birrell et al., 1982). Results show that $2A_{max}$ values of untreated stratum corneum are larger than those of ethanol-treated stratum corneum at any time and a plot of $2A_{max}$ values vs time shows a linear relationship between 5 and 8 h for both samples. The decreased $2A_{max}$ induced by ethanol resulted in smaller order parameters. Ethanol reduced the order parameter by 10 and 1% at 5 and 24 h, respectively.

5-DSA is used as a lipid spin label and known to provide information about the physical state of membrane lipids. Lipids in a fluid bilayer (or monolayer) environment can be distinguished from lipids bound to protein by using 5-DSA spin label (Sinha and Chignell, 1979; Birrell et al., 1982). The ESR spectra of 5-DSA-incorporated human stratum corneum show two spectral components: the bound component (b) is characteristic of lipid associated with the hydrophobic surfaces of proteins and the fluid component (f) is a fluid bilayer-like component (Jost et al., 1977; Jost and Griffith, 1980). Especially noteworthy is that the binding of 5-DSA to stratum corneum and order parameters (S) are a function of labeling time. When the ESR parameters are used to estimate the membrane properties it is important to record the time when the spectra are taken since different values may be obtained at different times. Ethanol had an obvious effect on the behavior of spin label bound to lipid bilayers (less immobilization as shown in Fig. 6). Decreases in $2A_{max}$ values and order parameters (S) could be related to an increase in the freedom of motion of the spin label, greater freedom being associated with the fluidity of local lipid layer environments surrounding the spin label. However, an obvious increase in bound components and order parameters of ethanol-treated stratum corneum at 24 h demonstrates that the influence of ethanol on these properties of stratum corneum decreased with time. Therefore, the ethanol-induced change in lipid bilayers of stratum corneum can be considered to be significant and this effect diminishes with time, which might be responsible for a temporary ethanol-bilayer perturbation in physical effects (hydrogen-bonds, hydrophobic, etc.).

3.3. Azone® effect on the ESR spectra of human stratum corneum

Azone® has been investigated extensively and its promoting effect on the drug permeability through the skin has also been approved. In general, 1–10% of Azone® in topical formulations produces an obvious penetration enhancement for many drugs. However, the effect of Azone® on drug permeability has a limitation when the concentration reaches a certain level (Lambert et al., 1989).

Three Azone® concentrations were used. The ESR spectra of human stratum corneum treated with 25 μg Azone® were measured at 37°C as a function of labeling time (shown in Fig. 7). Clearly, the ESR spectra were totally different from those of 5-DSA labeled stratum corneum and ethanol-treated stratum corneum. No fluid components and only a little bound components could be seen. With the diffusion of 5-DSA into the stratum corneum, the spin label still showed a weak binding to lipid bilayers and the motion of spin label was found a small anisotropy. Fig. 8 and

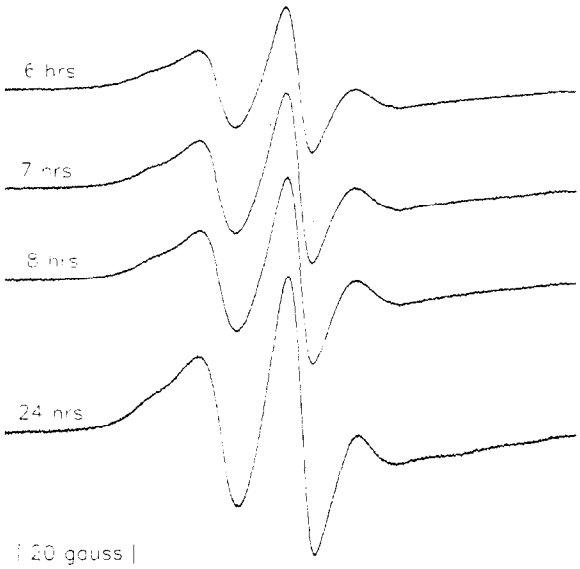

Fig. 7. Time dependence of the ESR spectra of 5-DSA labeled human stratum corneum previously treated with 25 μg Azone® at 37°C, recorded from 6 to 24 h.

Fig. 9 illustrate the ESR spectra of stratum corneum treated with 100 and 250 μg Azone®, respectively. In comparison to 25 μg Azone® treatment, their ESR spectra were similar and displayed a more markedly reduced anisotropy. There were almost no bound components (b) observed.

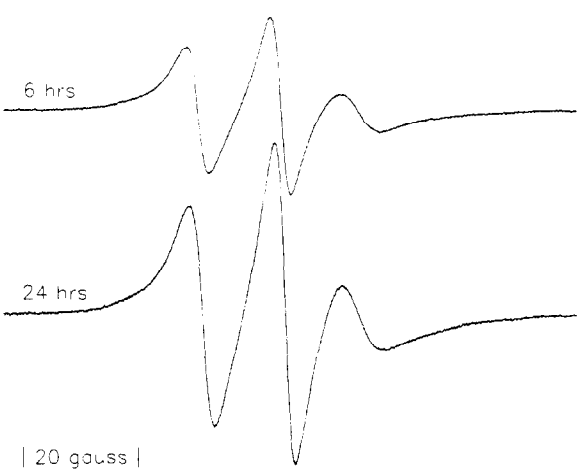

Fig. 8. ESR spectra of 5-DSA labeled human stratum corneum previously treated with 100 μg Azone® at 37°C, recorded at 6 and 24 h.

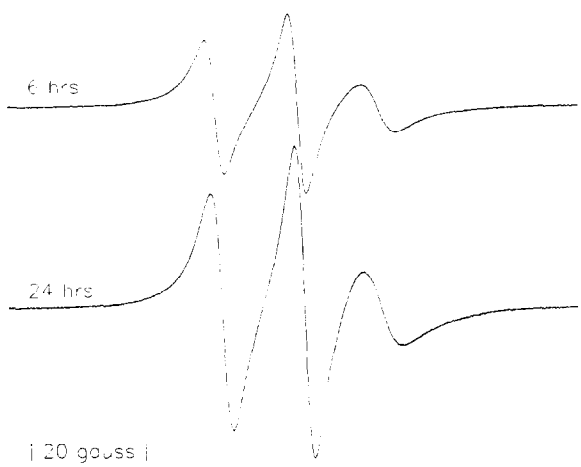

Fig. 9. ESR spectra of 5-DSA labeled human stratum corneum previously treated with 250 μg Azone® at 37°C, recorded at 6 and 24 h.

The corresponding parameters obtained from the ESR spectra at three concentrations of Azone® are summarized in Table 2. The molecular freedom of motion is related to order parameter (S) and rotational correlation time (τ) of nitroxide spin-labeled molecule as defined by Eq. 1 and 4. Because it is difficult to define the

Table 2
ESR parameters of 5-DSA labeled human stratum corneum treated with three concentrations of Azone

Treatment [a]	Time [b] (h)	Rotational correlation time [c] $\tau(\times 10^{-9}$ s)	Polarity a_o [d] (G)	Order parameter [e] S
25 μg	6	4.59	15.17	0.41
	7	5.17	14.92	0.39
	8	5.24	14.80	0.38
	24	6.35	14.68	0.39
100 μg	5	2.83	14.07	0.18
	24	3.68	13.77	0.19
250 μg	5	2.14	14.19	0.15
	24	2.83	13.95	0.17

[a] Stratum corneum samples were treated with the indicated amount of Azone.
[b] ESR spectra were recorded at 37°C at the indicated time after labeling with 5-DSA.
[c] Calculated using Eq. 4. Estimated uncertainty is values ±0.2.
[d] Calculated using Eq. 3. Values ±0.05 G.
[e] Calculated using Eq. 1. Estimated uncertainty is values ±0.01.

molecular motion of 5-DSA in the Azone-treated stratum corneum system, both τ and S values are listed in Table 2 for comparison. The isotropic splitting parameter a_o reflects the polarity of the local surroundings of the spin label in a membrane and increases with increasing polarity. When a lower concentration was used, the rotational correlation time τ increased as a function of time and a_o values showed a slight decrease. In the case of higher concentrations, although the τ values still showed an increasing trend, these values were obviously smaller compared with τ values at a lower concentration. There was a small decrease in a_o values. The corresponding order parameters were in a good agreement with τ values. Azone® reduced the order parameter (S) by as much as 54% at a low concentration (25 μg) applied and by 78 and 80% at higher concentrations (100 and 250 μg, respectively). In addition, note that the general trend is that the τ and S values decreased with an increase in the Azone® concentration. Comparing τ and S values at 6 and 24 h, it was found that both of them at low concentrations were 2-fold greater than those at higher concentrations (100 and 250 μg). These results suggest that Azone® led to a fluid environment in the stratum corneum and this action increased as the concentration of increased.

Previous studies have shown that the structural features of compounds that induce changes in the skin permeability include an alkyl chain of around 8–16 carbon atoms and a polar head group (Quan et al., 1989, 1990). Azone®, which has a polar head group and a long alkyl side chain, is thought to incorporate into structured lipids with the ring structure lying in the plane of polar head groups. The presence of such a seven-membered ring forces apart the alkyl chains of the skin lipids so as to have more free space in which to move, hence inducing the disorder and change in skin permeability (Hadgraft et al., 1992). The present results show that Azone® caused a conversion of the strongly immobilized labels (bound components) to weakly immobilized labels. This suggests that Azone® caused a change in the conformation of 5-DSA that was buried within the lipid bilayer matrix. From 6 to 24 h after labeling, an obvious increase in rotational correlation time τ and a slight decrease in polarity a_o induced by 25 μg Azone® indicate that Azone caused an increase in the flexibility and polarity of the local lipid bilayer of stratum corneum surrounding 5-DSA, but this small concentration effect on lipid bilayers diminished with time. Clearly, at a high concentration (250 μg), decreased τ values indicate that 5-DSA was surrounded by a more flexible lipid environment and Azone led to a major polar environment. Since the orientation of the spin label in the bilayer must reflect the conformational changes of local molecular structure, it is clear that one of its effects was to cause a conformational change in lipid bilayers of stratum corneum. Although it is impossible to directly compare the effect of Azone® on the polarities (a_o), the ESR spectra show that the polarities surrounding 5-DSA in the treated skin system were greatly different from those in the untreated skin system. In addition, concentration effects on the ESR spectra of stratum corneum demonstrate that there was no direct proportionality to the amount applied. In other words, the flexibility of 5-DSA significantly increased from 25 to 100 μg Azone®, and then showed a small change from 100 to 250 μg Azone®. Such a saturation of the enhancement effect has been observed in some permeation experiments in which Azone® was used as an enhancer (Lambert et al., 1989).

4. Conclusions

In conclusion, the following points have been established:

First, when 5-DSA was incorporated into human stratum corneum, it partitioned between a non-polar environment and a polar (aqueous) environment. Two spectral components could be observed from ESR spectra recorded at 37°C. The bound component (immobilized) arises from lipid associated with the hydrophobic surfaces of proteins and the fluid component is a fluid bilayer-like component probably associated with labels that are in a polar environment on the membrane surface. The data also show that the binding of 5-DSA to stratum corneum is a function of

time. The effect of ethanol on the ESR spectra of the stratum corneum is significant but small. Ethanol affects the spin label binding to the lipid bilayer in the stratum corneum and causes a small increase in the flexibility of the local bilayer around the spin label. However, this effect decreases with time.

Second, some ESR parameters (e.g., order parameter S, rotational correlation time τ and polarity a_o) afford the most accurate description about changes in the membrane fluidity, function and property, if a low spin label concentration is used. The use of high concentrations of spin label will lead to radical interactions and may prevent the measurement of intrinsic membrane properties. Therefore, it is important to select an effective concentration of spin label and to minimize probe-probe interactions to ensure that intrinsic properties of the membrane system will be discerned from the ESR spectra obtained.

Third, Azone® produced a significant change in ESR spectra of 5-DSA labeled human stratum corneum – from a strongly immobilized to a weakly immobilized spectrum. This suggests that Azone® induced a change in the conformation of 5-DSA that was buried within the lipid-protein matrix. Furthermore, it can be regarded as a conformational modification of lipid bilayers. Meanwhile, the weakly immobilized label may be associated with the label that is in a more polar and fluid environment. In addition, it was also found that the effect of Azone® on both the flexibility and polarity of the spin label was not in direct proportion to the concentration applied.

Spin labels have proven to be valuable investigative tools for studies of membrane structure and function. As shown in this study the interpretation of ESR spectra of the Azone® effect on 5-DSA labeled human stratum corneum is not straightforward. The detection of the perturbation effects induced by penetration enhancers depends upon the spin labels used. The real actions of the enhancers are difficult to determine completely by using a limited number of spin labels. It should be worthwhile to explore a variety of spin labels and skin systems in order to gain a more complete understanding of the action of penetration enhancers.

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