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Dioxygen diffusion in the stratum corneum: an EPR spin label study

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The stratum corneum, the outer 10 μm of the skin, serves as a permeability barrier regulating the transport of molecules between the body and the environment. The purpose of this study is to understand this permeability barrier function as it pertains to the diffusion of molecular oxygen. The stratum corneum was investigated with EPR spectroscopy following inoculation with a stearic acid spin probe. The presence of paramagnetic molecular oxygen results in the broadening of the EPR spectral lines of the spin probe. The rate of oxygen diffusion across the stratum corneum, and then the oxygen diffusion coefficient, $D(O_2)$, was determined by studying this line-broadening as a function of time. $D(O_2)$ in human stratum corneum was found to be $3 \cdot 10^{-7}$ cm^2/s at 37°C with an activation energy of approx. 44 kJ/mol. The application of the permeation-enhancing chemicals, DeMSO and DMSO, to the stratum corneum increased $D(O_2)$ two- to three-fold.

Introduction

The skin is the organ most exposed to the atmosphere, yet its exceptionally low permeability to most molecules enables it to regulate the transport of molecules between the body and the environment. The barrier function of the skin is attributed to the stratum corneum (SC), the outer 10 μm of the epidermis. The SC is composed of a close-packed array of flattened epidermal cells that are partially dehydrated and filled with keratin protein. Thin layers of interstitial lipids are found between the cells. These lipids are the remains of the epidermal cellular membranes after differentiation and keratinization and are then imbedded in the SC [1,2]. These lipids are dispersed in a two-dimensional bilayer consisting primarily of free fatty acids, cholesterol and ceramides [3].

Michaels and his collaborators [4] proposed a 'brick and mortar' model for the SC with the keratinocytes as the bricks and the lipids as the mortar. Their model suggests that a molecule traversing the SC must travel through the lipid matrix via a tortuous pathway around the keratinocyte 'bricks'. They suggest that the order in the lipid region may prevent efficient permeation of even lipophilic molecules [4]. It has been proposed that permeation through the lipid matrix can be increased by the application of chemicals that increase the disorder in the lipid region [5–8].

It is of interest to study the transport of molecular oxygen across the skin. The efficient barrier function of the SC prevents the skin from imbibing oxygen as readily as the lung. It is, therefore, necessary to investigate the transport of oxygen across the SC and to find a method to increase the rate of this transport. Labeling the SC with a paramagnetic nitroxide spin probe enables us to study the SC using electron paramagnetic resonance, EPR spectroscopy. The ability to differentiate between spin probes in different sample environments proves to be especially useful when studying heterogeneous systems like the SC. The EPR linewidth of the probe is sensitive to probe dynamic motions [9] and to the presence of dissolved oxygen. Spin-label oximetry allows one to use magnetic resonance to indirectly detect oxygen in biological systems [10–14]. The time-dependence of the probe linewidth due to spin exchange with oxygen can be observed at a constant magnetic field as a decrease in the lineheight of the second derivative EPR spectrum [15]. In this study we used the time evolution of the second derivative EPR spectrum to determine the molecular oxygen diffusion coefficient in SC lipid model systems, human SC

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and porcine SC. We did this both in the presence and absence of permeation enhancing chemicals.

Most solute diffusion coefficient measurements in the SC membrane use a diffusion cell consisting of two reservoirs separated by the SC membrane. Our ability to make measurements within the SC membrane results in three advantages. First, the experiment is completed in minutes instead of hours or days. Second, the diffusion coefficient we measure is independent of defects or pores that may exist in the sample of SC membrane. Third, the EPR spectrum of the solute within the membrane can provide valuable mechanistic information about the interaction of the solute with this heterogeneous membrane. In this study, for example, we show that the DSA spin probe solute is largely immobilized in the SC while the solute oxygen molecule diffuses freely in the SC lipid.

Materials and Methods

The 17-doxylstearic acid (d_8 ^{15}N 17-DSA) spin probe used in this study was a gift from John Windle (USDA, Albany, CA, USA). This probe was enriched in ^2H and ^{15}N in order to increase EPR spectral resolution and sensitivity [16]. SC sheets were prepared from porcine and human skin by the trypsin treatment as previously described [17]. The SC sheets were then vacuum-dried at room temperature and rinsed twice with cold hexane to remove any sebaceous lipids. The samples were kept under a nitrogen atmosphere and stored at −10°C until used.

Two SC lipid model systems (saturated and unsaturated) were prepared in the mol ratios reflective of the composition of normal human SC [3]. Palmitic acid and tripalmitin were the fatty acid and triglyceride used in the saturated model while 1:1 (w/w) mixtures of palmitic and oleic acids and tripalmitin and triolein were the fatty acids and triglycerides used in the unsaturated model. The cholesterol and ceramides were common to both models. This lipid composition is assumed to be representative of the interstitial lipids in the SC. The fatty acid spin label, d_8 ^{15}N 17-DSA, was added directly to this mixture (0.02% by mass). The lipids were melted and thoroughly mixed. These samples were stored under argon and kept frozen (−70°C) until needed. The unsaturated mixtures were prepared in a dark room and stored in opaque vials. SC lipids were obtained by extracting human SC sheets using the method of Bligh and Dyer [18]. The d_8 ^{15}N 17-DSA spin probe was added to these lipids in a methanol solution and mixed in the liquid phase. The lipid mixtures were vacuum dried at 10^{-2} torr overnight. This low pressure is required to remove water and residual solvents from the samples. The extract was stored under argon and kept frozen (−70°C).

To increase the signal from SC samples, we increased the SC thickness. SC sheets were fused together (top to bottom) by cutting samples of the same dimensions, placing them on top of each other and holding them between two quartz plates overnight. The d_8 ^{15}N 17-DSA spin probe was applied to the SC sheets from a 10 mM solution in ethanol using an aerosol spray gun. All SC samples treated with this label were equilibrated at 37°C for three days to distribute the probe uniformly through the SC sample. The SC samples were mounted on one side of the exterior surface of a quartz flat cell that had been made hydrophobic by siliconization [19]. The cells used were Wilmad aqueous flat cells (#812, Wilmad Glass, Buena, NJ, USA). The cells allow thermostated mineral oil to be passed through the cell to maintain the sample temperature. The entire sample cell was enveloped by a Teflon sleeve which allowed a flow of gas (hydrated O_2 or N_2) to pass over the sample.

The sample cell was placed in the center of the TE-102 cavity of a Varian Model E-12 X-band EPR spectrometer (Varian Associates, Palo Alto, CA, USA). Spectra were recorded at 5 mW incident microwave power with a modulation amplitude of 20% or less of the spectral linewidth to prevent artificial broadening due to over modulation. The thickness of the SC sample was determined gravimetrically from the area and density (assumed to be 1.2 g/cm^3). The EPR spectrometer is coupled to a data collection system, a Keithley 195A Digital Voltmeter (Keithley Instruments, Rochester, NY, USA), IEEE interfaced to an IBM-386 clone. The data were acquired using the Collect data acquisition program by Hart Scientific (Hart Technologies, Pleasant Grove, UT, USA).

The oxygen diffusion was measured via a gas-switch experiment. The sample was initially bathed in a nitrogen atmosphere. The nitrogen gas had been bubbled through saturated sodium chloride to maintain 75% relative humidity in the samples. A valve and valve driver, (General Valve, Fairfield, NJ, USA) was used to rapidly change (0.1 s) the gas to paramagnetic oxygen to start the experiment.

The d_8 ^{15}N 17-DSA probe concentration in the skin lipids is assumed to be constant and uniform across the thickness, l (cm), of the sample. The EPR spectrometer is set to the second derivative mode and is tuned to the peak lineheight, Y'', of the narrowest EPR signal of the probe in the lipid environment. $W(N_2)$ and $W(x,t)$ are the peak-to-peak linewidths (gauss) of the probe in the absence of oxygen and in the presence of oxygen, respectively. $W(x,t)$ is a function of time and of the probe position in the sample:

$$W(x,t) \approx W(N_2) + kD_{O_2}[O_2(x,t)] \quad (1)$$

where x is the axis normal to the sample plane, $0 < x < l$. $D(O_2)$ is the oxygen diffusion coefficient in the SC

(cm^2/s) and $[O_2(x,t)]$ is the oxygen concentration (mol/l) at a depth x and a time t. The constant of proportionality, k, is approx. $2.3 \cdot 10^7$ gauss l s/cm^2 per mol [13]. Using standard solutions to the diffusion equation [20] we average the lineheight over the sample thickness to obtain the time response of the observed lineheight, using $Y'' = k'W^{-3}$ [15]. The resulting integral, evaluated with the Mathematica software package (Wolfram Research, Champaign, IL, USA), gives a multiterm expression that is fit to the data to determine the rate (s^{-1}).

Fig. 1 shows an example of a kinetic curve for a porcine SC sample. The rate obtained from these plots of the average lineheight as a function of time was used to determine the oxygen diffusion coefficient (cm^2/s) according to Ref. 20:

$$D_{O_2} = 4l^2(\text{rate})/\pi^2 \quad (2)$$

Permeation enhancers were applied to the samples (20% by weight) using the same aerosol method used to apply the d_8 ^{15}N 17-DSA spin probe. Gas-switch experiments of samples treated with the relatively volatile enhancer dimethylsulfoxide (DMSO) were performed immediately after application and repeated every hour for 4 h. Samples treated with the large molecular weight enhancers oleic acid, decylmethylsulfoxide (DeMSO) and 1-dodecylazacycloheptan-2-one (Azone) were incubated overnight in a nitrogen atmosphere at 37°C and experiments were done every day for four days.

Results

Initial studies were performed on synthetic lipid model systems as proposed by Lampe et al. [3]. A marked difference was observed between the spectra of the saturated and unsaturated lipid mixtures (Fig. 2). The saturated lipid mixture revealed a much larger linewidth in nitrogen than did the unsaturated mixture

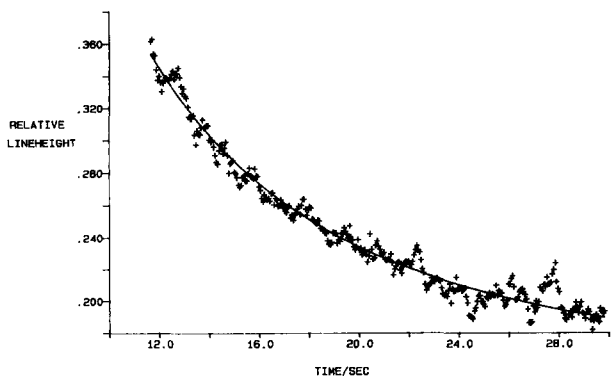

Fig. 1. The kinetic curve of the decrease in the second derivative lineheight as a function of time as oxygen diffuses into porcine SC.

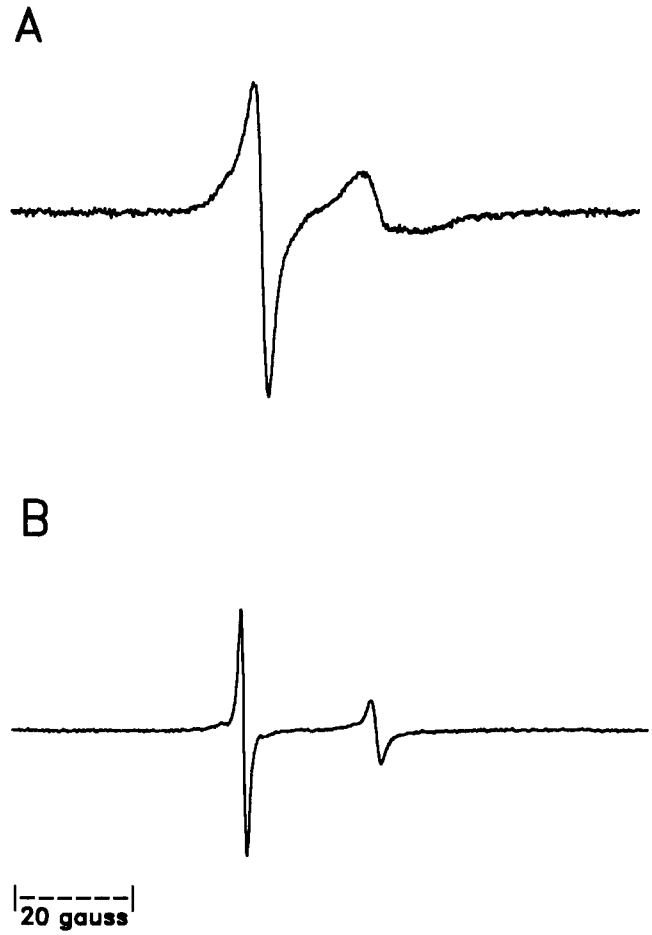

Fig. 2. The EPR spectra of d_8 ^{15}N 17-DSA labeled SC model systems. (A) Saturated lipid model; (B) unsaturated lipid model.

(3.5 vs. 0.8 gauss). This larger linewidth is characteristic of a spin probe in a less fluid environment. The narrow linewidth of the unsaturated spectrum as compared to the saturated spectrum is consistent with previous studies showing that unsaturation results in less efficient packing and thus, increased fluidity (the inverse of the viscosity) [21].

This increased fluidity in the unsaturated mixture resulted in an increased oxygen diffusion coefficient, $D(O_2)$. The $D(O_2)$ for the unsaturated lipid system at 37°C was measured to be seven times larger than that for the saturated lipid system ($D(O_2) = 0.2 \cdot 10^{-5}$ vs. $0.03 \cdot 10^{-5}$ cm^2/s. This is consistent with studies showing increased SC permeability with increased lipid fluidity [5–8,21–25].

Permeation-enhancing chemicals were added to the saturated lipids in an attempt to increase the oxygen diffusion coefficient. Measurements of the linewidths in nitrogen showed no linewidth change with the application of the lipophilic enhancers, oleic acid and azone, and these enhancers produced no change in the $D(O_2)$. A small decrease in the spectral linewidth in N_2 (3.0 vs. 3.5 gauss) was observed in the presence of the sulphoxide based enhancers, DeMSO and DMSO. The pres-

TABLE I

The diffusion coefficients of oxygen in the stratum corneum and SC model systems

All values are determined at 75% RH and 37°C.

Biomedia	$D(O_2)$ (cm^2/s) ($\times 10^5$)	Permeation enhancer	$D(O_2)$ (cm^2/s) ($\times 10^5$)
Saturated lipids	0.03	azone	0.04
		oleic acid	0.03
		DeMSO	0.07
		DMSO	0.05
Unsaturated lipids	0.2	azone	0.2
		DeMSO	0.44
		DMSO	0.46
Human SC	0.03	oleic acid	0.03
		DeMSO	0.06
		DMSO	0.09
Porcine SC	0.007		

ence of these enhancers increased the $D(O_2)$ by a factor of two. This increase of $D(O_2)$, was similar to the increase of $D(O_2)$ in the unsaturated lipids observed in the presence of these enhancers (diffusion coefficients are shown in Table I).

The effect of temperature (20–50°C) on the diffusion coefficient of oxygen was studied using porcine SC. The Arrhenius plot is shown in Fig. 3. The activation energy of $D(O_2)$ was found to be approx. 44 kJ/mol. Values reported for the activation energy of water diffusing through the SC range from 21 kJ/mol [26] to 71 kJ/mol [25].

The EPR spectrum of the d_8 ^{15}N 17-DSA spin probe in the heterogeneous SC possesses two distinct probe sites (Fig. 4). The narrow two line pattern represents spin probe in a fluid environment allowing rapid and approximately isotropic motion ($\tau_c \leqq 1$ ns). The broad underlying spectrum is that of spin probe reporting from a rigid environment and experiencing restricted motion ($\tau_c \geqq 20$ ns).

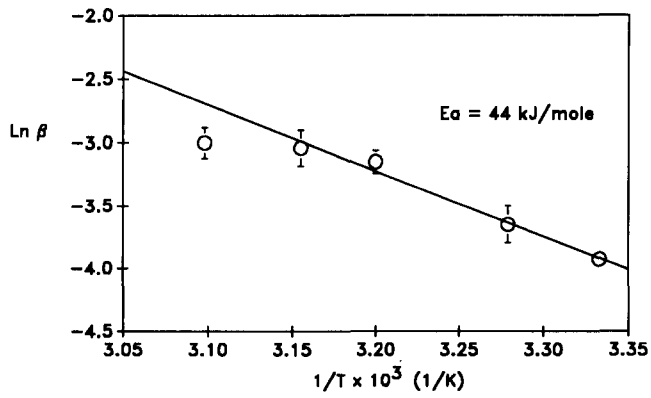

Fig. 3. Arrhenius plot of the oxygen diffusion rate constant for porcine SC, plotted as the natural logarithm of the rate vs. the reciprocal absolute temperature.

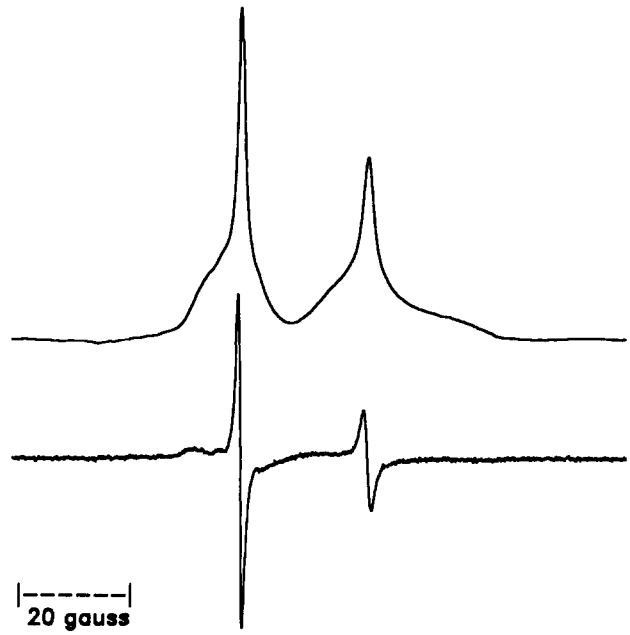

Fig. 4. The first derivative and adsorption spectra of d_8 ^{15}N 17-DSA labeled porcine stratum corneum. Note how the immobilized signal dominates the integrated spectrum.

The hyperfine splitting is a function of the polarity of the environment in which a spin probe resides [27]. Both the isotropic hyperfine splitting and the linewidth for the narrow lines observed in the spectrum of the labelled SC correlate to that of the extracted SC lipids. This infers that the probe molecules responsible for this SC signal are reporting from a lipid environment.

The first derivative EPR spectrum discriminates against broad lines and thus, this spectrum is dominated by the narrow lipid lines (Fig. 4). However, when we integrate this spectrum we find that the spectral intensity of the probe reporting from a region of restricted motion is much larger than that due to probe reporting from the lipid region. This has important implications in the mechanism of the SC barrier function.

The oxygen diffusion coefficient, $D(O_2)$, for human SC was found to be $3 \cdot 10^{-7}$ cm^2/s at 37°C. This value is approximately four times that observed for porcine SC ($7 \cdot 10^{-8}$ cm^2/s. However, the linewidth in nitrogen of the fluid line in porcine SC was the same as that for human SC (0.9 gauss).

The oxygen diffusion coefficient for human SC was increased 100% in the presence of DeMSO and 200% in the presence of DMSO. There was no change in the $D(O_2)$ with the application of oleic acid. The oxygen diffusion coefficients for human SC, saturated lipids and unsaturated lipids, both with and without permeation enhancers, are given in Table I.

Discussion

By studying the dioxygen diffusion across the SC we were able to determine this important diffusion coefficient. We were also able to learn something about the role of the lipid and protein domains in the SC. Small has shown that lipid subphases arise due to variations in the packing of heterogeneous lipid systems [21]. The EPR probe linewidth in nitrogen of the fluid lipid signal in human SC was found to be quite narrow (0.9 gauss). This is very close to the value obtained for the very fluid unsaturated lipid model system (0.8 gauss). This indicates that at least some of the lipids of the SC are not rigid as has been postulated [4]. Our EPR results correlate nicely with X-ray studies showing the existence of liquid alkyl chains [28,29]. This fluidity permits rapid transport of small lipophilic molecules across the SC [30].

This rapid diffusion through the lipids implies that barrier function of the SC cannot be entirely due to rigidity of the lipid region. The keratinocytes of the SC must contribute to this barrier function. We propose that the role of the proteins in the SC is two-fold. In the transport of lipophilic molecules the proteins serve as obstacles to diffusion. Secondly, the keratinocytes appear to bind polar solute molecules (e.g., nitroxides, Fig. 4). These solute molecules may not diffuse while bound, which could explain why long lag times have been observed in classical diffusion cell studies of small polar molecules in the SC [31].

Molecular oxygen is lipophilic [32] and therefore, its diffusion through the SC is well described using the 'brick and mortar' model proposed by Michaels et al. [4]. In this model the proteins serve as barriers forcing oxygen to travel a tortuous diffusion pathway. The effect of this tortuosity is seen in the difference between the oxygen diffusion coefficients in the unsaturated lipid model and the human SC.

The linewidth of the probe in the fluid domain in the SC is comparable to the unsaturated lipid mixture. This makes the unsaturated mixture, rather than the saturated mixture, the obvious choice as the fluidity model for the SC lipids. The oxygen diffusion coefficient for the unsaturated lipids was found to be seven times larger than that for the human SC ($2 \cdot 10^{-7}$ vs. $3 \cdot 10^{-8}$ cm^2/s). Recalling that the diffusion coefficient is dependent upon the square of the mean distance traveled per unit time, the tortuosity produced by the keratin increases the distance traveled by the oxygen by approx. $\sqrt{7}$. The reduced $D(O_2)$ observed for porcine SC suggests increased tortuosity in this species.

We have found that the diffusion of oxygen across the SC is rapid. This is due to the lipophilicity of oxygen [32], the fluidity of the lipids, and because oxygen is not bound by the SC proteins. It is clear that in order to further increase the diffusion of oxygen across the SC the lipids must be further disrupted. We have shown that this disruption can be achieved through the application of dimethyl sulphoxide, DMSO, and decylmethyl sulphoxide, DeMSO. This increase in transcutaneous oxygen diffusion may be useful, for example, to satisfy the oxygen demand during epidermal regeneration.

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