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Evidence that Oleic Acid Exists in a Separate Phase Within Stratum Corneum Lipids

Boonsri Ongpipattanakul,[1,2] Ronald R. Burnette,[2] Russell O. Potts,[1] and Michael L. Francoeur[1,3]

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Oleic acid is known to be a penetration enhancer for polar to moderately polar molecules. A mechanism related to lipid phase separation has been previously proposed by this laboratory to explain the increases in skin transport. In the studies presented here, Fourier transform infrared spectroscopy (FT-IR) was utilized to investigate whether or not oleic acid exists in a separate phase within stratum corneum (SC) lipids. Per-deuterated oleic acid was employed allowing the conformational phase behavior of the exogenously added fatty acid and the endogenous SC lipids to be monitored independently of each other. The results indicated that oleic acid exerts a significant effect on the SC lipids, lowering the lipid transition temperature (T_m) in addition to increasing the conformational freedom or flexibility of the endogenous lipid alkyl chains above their T_m. At temperatures lower than T_m, however, oleic acid did not significantly change the chain disorder of the SC lipids. Similar results were obtained with lipids isolated from the SC by chloroform:methanol extraction. Oleic acid, itself, was almost fully disordered at temperatures both above and below the endogenous lipid T_m in the intact SC and extracted lipid samples. This finding suggested that oleic acid does exist as a liquid within the SC lipids. The coexistence of fluid oleic acid and ordered SC lipids, at physiological temperatures, is consistent with the previously proposed phase-separation transport mechanism for enhanced diffusion. In this mechanism, the enhanced transport of polar molecules across the SC can be explained by the formation of permeable interfacial defects within the SC lipid bilayers which effectively decrease either the diffusional path length or the resistance, without necessarily invoking the formation of frank pores.

KEY WORDS: oleic acid; penetration enhancer; Fourier Transform Infrared Spectroscopy (FT-IR); lipid phase-separation transport mechanism.

INTRODUCTION

Previous calorimetric studies with porcine stratum corneum (SC) show that the T_m of the two lipid transitions, which occur between 60 and 70°C, are significantly reduced in the presence of oleic acid (1). This decrease in the lipid-associated T_m is correlated with the amount of oleic acid taken up by the SC, and the *in vitro* flux enhancement of ionically charged molecules, stressing the importance of the lipid pathway in skin transport. Further, the extent of enhancement increases with the concentration of the charged permeant within the applied vehicle, suggesting nonadherence to the classical pH-partition hypothesis. These observations, analyzed in the context of a number of phospholipid references (2–8) describing lateral phase separation, fatty acid phase behavior, and increased ion permeability at the gel–liquid crystal phase transition, suggest that the enhancement of transport across the skin may be related to a microperturbation of the SC lipid bilayer structure. Consequently, a mechanism is proposed in which oleic acid exists in a separate phase contained within the endogenous SC lipids, thereby forming a number of permeable defects at liquid–solid interfaces. This mechanism is also tacitly consistent with reports that increased TEWL occurs in a number of skin disorders characterized by lipid abnormalities, which may involve phase separation (9,10). Grubauer *et al.* (11) in fact, experimentally correlate TEWL to the presence of separate polar and nonpolar lipid phases in mouse skin. In the studies described here, Fourier transform infrared (FT-IR) spectroscopy was employed to investigate specifically the possibility of liquid–solid phase separation within the SC lipid domain in conjunction with oleic acid. A salient aspect of these studies is that the C-D stretching band is located at a lower wavenumber region, allowing the conformational behavior of ^{2}H-oleic acid to be differentiated from that of the endogenous SC lipids (12).

MATERIALS AND METHODS

Preparation of Stratum Corneum (SC) and Extracted Lipids

Full-thickness skin was obtained immediately after sacrifice from pigs weighing 15 to 30 kg. Only the thoracic sections were utilized. The hair was first clipped with standard shears and then dermatomed to a thickness of about 500 μm. As described elsewhere (13), skin sections of about 6 cm^2 were incubated with a 0.5% trypsin buffer (pH 7) to separate the SC from the epidermis. The isolated SC was rinsed briefly with cold hexane, then distilled H_2O, and air-dried before storing in a desiccator.

The lipids were isolated from the SC according to the method described by Wertz *et al.* (14). The SC was extracted successively in three different chloroform–methanol mixtures, each for 2 hr. These mixtures were 2:1, 1:1, and 1:2 chloroform–methanol, respectively. This series was then repeated at 1-hr intervals before finally extracting the SC overnight with methanol. All fractions were combined and evaporated to dryness under N_2. The dried lipid residue was placed under vacuum (~25 in. Hg) until a constant weight was achieved. The lipids were then stored under N_2 at −20°C until needed.

Treatment of the SC and Extracted Lipids with Oleic Acid

For these experiments, an ethanol solution of perdeuterated oleic acid (Cambridge Isotope Laboratories, Woburn, MA) was prepared to a final concentration of 300

[1] Dermal Therapeutics Group, Central Research Division, Pfizer Inc., Groton, Connecticut 06340.
[2] University of Wisconsin, School of Pharmacy, Department of Pharmaceutics, Madison, Wisconsin 53706.
[3] To whom correspondence should be addressed.

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mg/ml. To treat the SC, an aliquot of 30 μl was layered on the apical side of a piece measuring about 4 cm^2. After evaporation of the ethanol, this tissue was carefully placed (same side up) in a 75% RH chamber for at least 24 hr. Subsequently, the SC was rinsed by briefly immersing the sample with forceps into three different solutions of cold ethanol. The excess ethanol was removed by gentle blotting and evaporation before the SC was reequilibrated at 75% RH. Untreated SC samples were also hydrated to 75% RH. In a separate set of experiments, the same treatment process described above was repeated several times using ^{3}H-oleic acid to quantitate the amount taken by the SC. The oleic acid content in SC was then measured by a standard liquid scintillation technique.

The extracted SC lipids were processed by a method similar to that reported by White *et al.* (15). The lipid residue was first dissolved in a small volume of chloroform–methanol (2:1) and dried under N_2 to a thin film. The lipids were dispersed in sufficient quantity of distilled H_2O to give a concentration of 1 mg/ml. This mixture was recycled through several heating/cooling cycles (up to 80°C) to obtain a uniform dispersion. Subsequently, 300 μl of this dispersion was deposited on the FT-IR window and partially dried in a $CaSO_4$ desiccator. The film was rehydrated at 75% RH before use. The treated samples were prepared by dissolving the ^{2}H-oleic acid in the initial chloroform–methanol mixture at a ratio of 1:2 (w/w; oleic acid:SC lipid) to approximate the amount of oleic acid taken up by the intact SC. This approximation assumes that all of the OA was taken up by the SC lipids, which constitute about 15% of the total SC mass (1). The ^{2}H-oleic acid spectrum (Fig. 4) was obtained in a similar manner by placing 20 μl between two FT-IR windows.

FT-IR Spectroscopy

Infrared spectra were recorded with a Nicolet 730 FT-IR spectrophotometer (Nicolet, Madison, WI) equipped with a liquid N_2 cooled MCT detector. To obtain a spectrum at each temperature, three groups of 64 scans were collected at 0.5-cm^{-1} resolution, coadded, and transformed using a Happ-Genzel apodization function. The samples were contained between ZnS (IRTRAN 2, SpectraTech, Stamford, CT) windows mounted in a specially designed heating/cooling cell. The cell was connected to a Lauda low-temperature water circulator (Model RC-6, Brinkman Instruments, Westbury, NY), which was controlled by a separate computer interfaced to the spectrometer workstation. The circulating fluid consisted of a 50% ethylene glycol–H_2O mixture. The ZnS windows were sealed around the edge with electrical tape, and an Alumel–Chromel thermocouple (Omega, Stamford, CT) was inserted through a hole drilled in one sample window to monitor directly the sample temperature to within ±0.2°C. The spectra were collected at 1 to 5°C increments as programmed by the water bath computer. Overall, the rate of temperature increase averaged less than 20°C/hr. To eliminate optical channeling in the frequency domain, the appropriate part of the interferogram was replaced with a straight line before transformation (16). Peak positions were determined using the software supplied by Nicolet, which was based on a polynomial least-squares method (17), or with a center of gravity algorithm (18) allowing frequencies, in all cases, to be determined with an uncertainty of less than 0.1 cm^{-1}.

RESULTS AND DISCUSSION

FT-IR Spectra

An infrared spectrum, spanning from about 3000 to 1900 cm^{-1}, is illustrated in Fig. 1 for a porcine SC sample which had been treated with ^{2}H-oleic acid. Of particular interest for these studies are the C–H and C–D symmetric stretching frequencies found at approximately 2850 and 2090 cm^{-1}, respectively. The significance of these particular bands is that they reflect, on a molecular level, conformational changes of the alkyl lipid chains (19–21). Specifically, as gauche conformers are introduced along the hydrocarbon chain, the stretching frequency shifts to higher values due to steric hindrance of partially eclipsed methylene groups along the lipid molecular axis. The increase in the number of gauche conformers is associated with increased conformational freedom and flexibility of the alkyl chains leading to a reduction in bilayer thickness and segmental order. The inflection midpoint of the temperature-frequency curve, consequently, can be taken as the T_m for the corresponding calorimetric phase transition (22). For these data the inflection point, or T_m, was estimated graphically (12).

Figure 2 illustrates the changes in $\nu_s(CH_2)$ obtained for the oleic acid-treated and the untreated SC as a function of temperature. For these experiments, the average concentration of oleic acid taken up by the SC was determined to be 60 μg/mg SC (SE = 4.0, n = 6). The incorporation of oleic acid did not have a significant effect on the conformational order of the endogenous lipids below the T_m, as there was no statistical difference (α = 0.05) between the $\nu_s(CH_2)$ for the treated and that for the control samples (Table I). Treatment with oleic acid did, however, lower the T_m of the inherent SC lipids by 7.7°C (SE = 1.3, n = 4), in agreement with results obtained by differential scanning calorimetry (1,23). At temperatures above the T_m, higher values ($P < 0.05$) for $\nu_s(CH_2)$ were found in the treated samples, indicating that fluid SC lipids are further disordered in the presence of oleic acid. Similar effects of oleic acid were observed in the extracted SC lipid samples (Fig. 3). There was no difference (α = 0.05) between the conformational order of the treated and that of

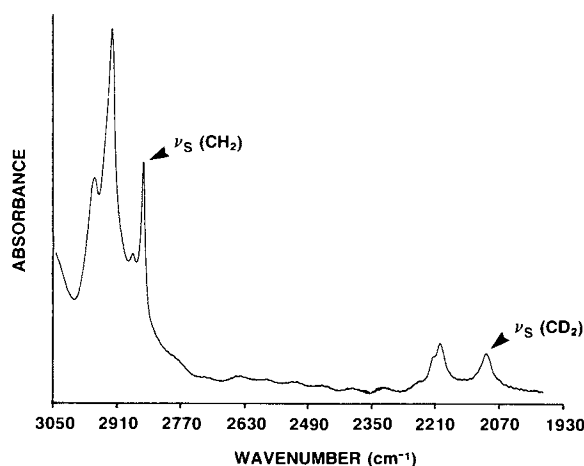

Fig. 1. Partial infrared spectrum of porcine stratum corneum treated with per-deuterated oleic acid. Note the separate C–H and C–D stretching bands at about 2850 and 2096 cm^{-1}.

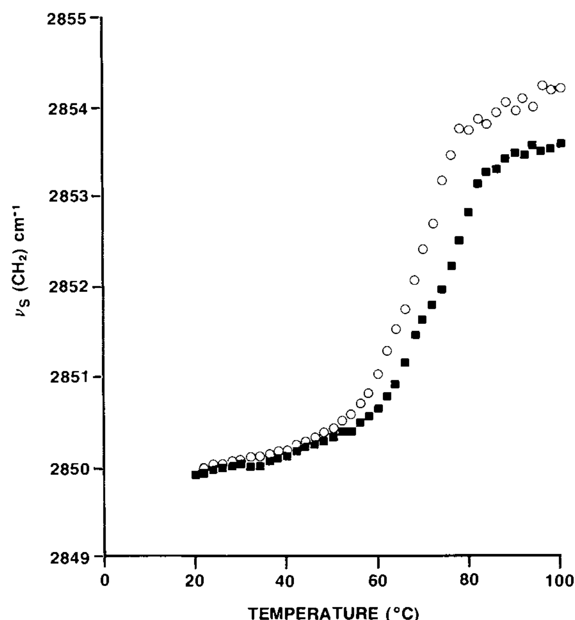

Fig. 2. The change in C–H symmetric stretching frequency, $\nu_s(CH_2)$, for porcine stratum corneum as a function of temperature. Included are the oleic acid-treated and the untreated (control) samples. ■, control; ○, ^{2}H-OA treatment.

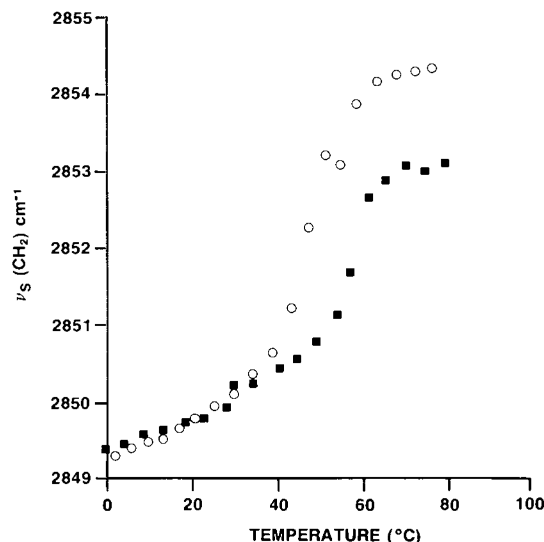

Fig. 3. The change in C–H symmetric stretching frequency, $\nu_s(CH_2)$, for lipids extracted from porcine stratum corneum as a function of temperature. Included are untreated and oleic acid-treated lipid samples. ■, control; ○, ^{2}H-OA treatment.

the control lipids below the T_m, but like the intact SC, the oleic acid-treated extracts showed greater ($P < 0.05$) disorder above the T_m (Table I). The T_m of the extracted lipids was likewise shifted to a lower value ($\Delta T_m = -9.5$°C, SE = 1.25, $n = 3$).

The decrease in T_m for the endogenous SC lipids following treatment with oleic acid can be partially explained on the basis of thermodynamic principles. At the SC lipid phase transition, $\Delta G = 0$ since the respective lipid phases are in equilibrium. Accordingly, T_m will be equal to $\Delta H/\Delta S$, where $\Delta S = S_{\text{final}} - S_{\text{initial}}$. Previous calorimetric experiments have indicated that the $\Delta H_{\text{SC LIPIDS}}$ is only slightly altered by oleic acid (1), suggesting that an increase in ΔS may, in part, account for the reduction in T_m. Indeed, the changes in $\nu_s(CH_2)$ above the T_m suggest that oleic acid increases S_{final} with no effect on S_{initial}. No change in S_{initial} implies that the mechanism by which the transport of polar molecules, at physiological temperatures, is enhanced must be due to subtle microphysical changes within the lipid bilayers, and cannot be ascribed to macroscopic perturbation or fluidization.

Table I. The Symmetric Stretching Frequency, $\nu_s(CH_2)$, for Stratum Corneum and Extracted Lipids Treated with ^{2}H-Oleic Acid: The Temperatures Represent Values Above and Below the Transition (see Figs. 2 and 3)

	Stratum corneum		Extracted lipids	
	30°C	90°C	20°C	75°C
Control	2850.1 (0.1)[a] $n = 8$	2853.4 (0.1)[a] $n = 7$	2849.7 (0.0)[a] $n = 2$	2853.2 (0.2)[a] $n = 2$
Oleic acid treated	2850.1 (0.1) $n = 4$	2854.2 (0.2) $n = 4$	2849.7 (0.1) $n = 2$	2854.2 (0.2) $n = 2$

[a] Numbers in parentheses represent SE.

The increase in conformational disorder or entropy of the endogenous fluid SC lipids may reflect at least two effects. Deuterium NMR and FT-IR studies have shown that the greatest conformational disorder exists toward the middle of the bilayer for fluid phospholipids (24,25). The methylene groups closest to the polar head region remain somewhat ordered, even in the liquid-crystalline state. If this analogy holds here for the SC lipids, it can be speculated that oleic acid exerts its primary effect(s) on the first 6 to 10 alkyl carbons proximal to the polar region of the bilayer. The other possible explanation is that oleic acid may increase the fraction of total lipids which have undergone a fluid phase transition. The latter possibility, though, would suggest that there are normally ordered lipids present in the untreated SC above the observed phase transition. While infrared spectroscopic analysis cannot distinguish between the two effects, X-ray diffraction studies suggest that it is unlikely that these ordered lipids exist at 90°C (15). Thus, these results suggest that oleic acid primarily disorders the alkyl chain near the polar region of the bilayers. No effect by oleic acid on the terminal $-CH_3$ (data not shown) stretching above the T_m support this conclusion.

The net frequency changes for the ^{2}H-oleic acid and the SC lipids are compared in Table II. While the C–H stretching frequency for the SC lipids increases by 4 to 5 cm^{-1}, the corresponding change in the C–D value is less than 1 cm^{-1}. Thus, it is apparent that the oleic acid present in the SC or extracted lipids does not undergo a major phase transition. Furthermore, the actual value of $\nu_s(CD_2)$ at 32°C is approximately 2097 cm^{-1}. As shown by Fig. 4, a frequency of 2097 cm^{-1} corresponds to ^{2}H-oleic acid that is almost fully disordered. Therefore, it can be concluded that the alkyl chains of the ^{2}H-oleic acid within the SC are, on average, in the

Table II. The Symmetric Stretching Frequency, $\nu_s(CD_2)$, for ^2H-Oleic Acid in Treated Stratum Corneum and Extracted Lipid Samples

Temperature (°C)	Stratum corneum	Extracted lipids
20	2096.9 (0.5)[a]	2097.1 (0.0)
30	2097.2 (0.6)	2097.4 (0.2)
40	2097.5 (0.4)	2097.6 (0.1)
50	2097.8 (0.2)	2098.3 (0.4)
60	2098.3 (0.2)	2098.7 (0.0)
70	2098.0 (0.1)	2098.4 (0.0)
80	2098.0 (0.1)	2098.3 (0.1)
90	2097.6 (0.3)	—
100	2097.8 (0.1)	—

[a] Numbers in parentheses represent SE where $n = 2$.

liquid state. It should be noted that this FT-IR analysis cannot measure the long-range order of the liquid oleic acid molecules to establish whether the fatty acid is dispersed in the same plane as the endogenous lipid bilayer or if it induces the formation of a more exotic domain such as a hexagonal II phase. In either case, the ideas of phase-separated defects and enhanced transport would still apply. At this time, the precise composition of the lipid phase containing the liquid oleic acid is not known. It is likely that the oleic acid exists in a heterogeneous phase containing one or more other SC lipid components (e.g., cholesterol) and, as such, would not be expected to manifest a separate cooperative phase transition. Nevertheless, it is unequivocal that the oleic acid molecules in the SC at physiological temperatures are in the liquid state and, therefore, are phase-separated from the endogenous solid lipids.

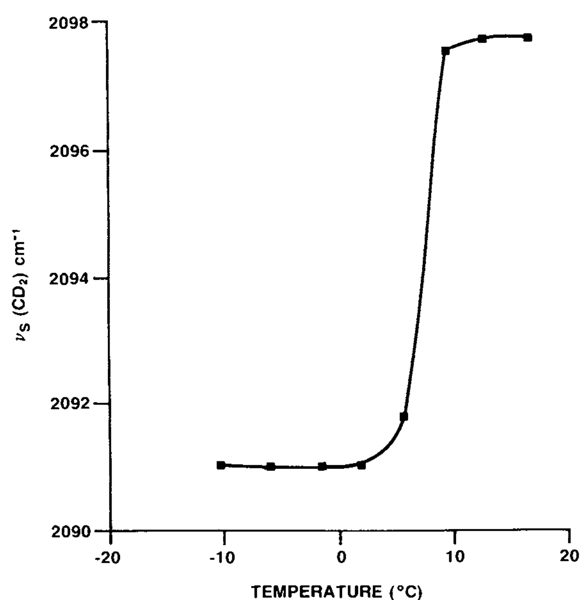

Fig. 4. The change in C–D symmetric stretching frequency, $\nu_s(CD_2)$, for pure ^2H-oleic acid as a function of temperature.

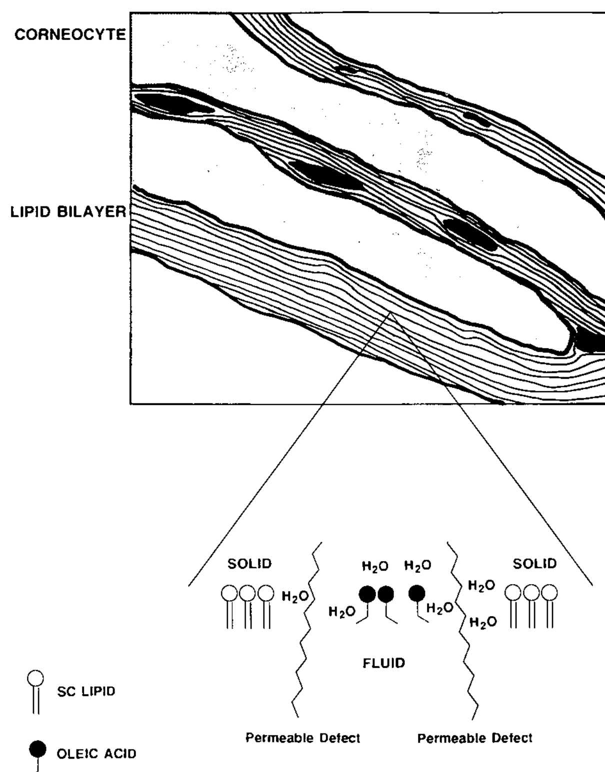

Fig. 5. Simplified schematic representation for one type of permeable defect which could be formed within the lipid bilayers. Stratum corneum illustration is an actual tracing of an electron micrograph (~385,000×), with the dark intercellular patches attributed to desmosomal fragments.

While previous reports have correctly concluded that oleic acid increases the overall alkyl-chain disorder of the SC lipids (23,26), the changes in the $\nu_s(CH_2)$ under those conditions reflect an average contribution from both oleic acid and the endogenous lipids. Hence, the oleic acid must be in the fluid state to account for the overall increase in $\nu_s(CH_2)$ which is observed. The conformational behavior of the SC lipids and the added fatty acid in these FT-IR experiments here was resolved with the use of per-deuterated oleic acid.

Phase Separation Hypothesis for Enhanced Transport

The possible significance of oleic acid, existing as a separate liquid phase in the SC lipids, to increased skin transport can be inferred from the phospholipid literature (2–8). Anomalously high diffusion rates for small ions such as Na^+ and K^+ are reported for various phospholipid systems which are heated to their phase transition temperature (2,5). The enhanced flux of these ions is thought to be related to the formation of permeable defects at the fluid–solid interface of lipids at their T_m. In addition, enhanced ion transport is observed in two component lipid systems which exhibit lateral phase separation (3,4). Given the likely existence of separate liquid and solid phases within oleic acid-treated SC lipids,

and previous results (1) that show a dramatic increase in permeability of charged compounds, it seems reasonable to propose a similar mechanism of enhanced skin transport. For any molecule to diffuse across the SC it must, at some point, encounter the lipid bilayers, as they are the only continuous structure. The formation of some type of permeable interfacial defects (Fig. 5) within the bilayer could explain the enhanced transport by reducing either the diffusional path length or the resistance. Further, the apparent tendency of oleic acid to increase the transport of charged or polar molecules suggests that the defect areas may also be associated with water (1,6). In conclusion, the FT-IR results obtained here with oleic acid are consistent with, but do not prove, this type of mechanism.

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