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Interactions of oleic acid and model stratum corneum membranes as seen by ^{2}H NMR

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Abstract

We have investigated the mechanism through which the penetration enhancer oleic acid acts on stratum corneum (SC) model membranes (bovine brain ceramide:cholesterol:palmitic acid, 1:1:1 molar ratio). We used solid state deuterium nuclear magnetic resonance to monitor such multilamellar SC dispersions containing either cholesterol-d_6, palmitic acid-d_{31}, or oleic acid-d_2 as a function of both fatty acid concentration (2:2:1:1 and 1:1:1:1 bovine brain ceramide:cholesterol:palmitic acid:oleic acid) and temperature (18–75 °C). Our results show that below 40 °C, oleic acid (OA) is in an 'isotropic' phase, indicating that it has not incorporated into the lamellar membrane phase. At and above the SC model membrane's crystalline to liquid crystalline melting temperature, T_m = 40–42 °C, OA interacts with lamellar SC membranes with a slight dependence on OA concentration. T_m does not change upon the exposure of the SC model membrane to OA, nor do we see any significant change in membrane chain disorder as monitored by the labelled PA. However, the spectra of both the palmitic acid (PA) and cholesterol SC model membrane components contain an isotropic peak that grows with increasing temperature. Our results thus indicate that oleic acid extracts a fraction of the endogenous SC membrane components, promoting phase separation in the SC membrane system. Reducing the proportion of crystalline lipids and creating more permeable OA-rich domains is a plausible mechanism that explains how OA enhances transdermal penetration.

Keywords: Penetration enhancer; Transdermal drug delivery; Deuterium NMR; Fatty acids

1. Introduction

Forming the outermost layer of the epidermis, the stratum corneum (SC) consists of protein-rich corneocytes embedded in a matrix of lipids. While both components contribute to the remarkable impenetrability of the SC, it is notable that the intercellular lipid membranes are orders of magnitude less permeable than ordinary mammalian membranes (Wester and Maibach, 1995). These barrier properties are essential to the SC's protective role but hinder transdermal drug delivery. In altering the structural organization of lipids, penetration enhancers can increase the SC's permeability and aid the transdermal delivery of drugs.

Oleic acid (OA) is one such penetration enhancer (Barry, 1987; Green et al., 1988; reviewed in Williams and Barry, 2004) that has been demonstrated to increase the flux of exogeneous additives (drugs) through the SC in vitro (Jain and Panchagnula, 2003; Yener et al., 2003; Yamane et al., 1995; Takeuchi et al., 1992; Mak et al., 1990). How OA acts to increase SC permeability, however, is not clear. Some have speculated that *cis*-unsaturated fatty acids such as OA 'fluidize' SC lipids. Differences in lipid packing caused by OA's 'bent' 9,10-*cis*-double bond configuration could perturb the crystalline SC lipids (Takeuchi et al., 1998; Barry, 1987; Golden et al., 1987) and thereby enhance membrane permeability. Indeed, model membranes of extracted SC lipids exhibit a reduction in T_m in the presence of OA as seen by DSC (Walker and Hadgraft, 1991; Yamane et al., 1995) and Fourier transform infrared (FTIR) spectroscopy (Golden et al., 1987), indicating that OA stabilizes the fluid phase.

A growing body of evidence points to phase separation as the mechanism behind OA's action. With simpler saturated lipid systems, differential scanning calorimetry (DSC), and fluorescence experiments have elucidated that *cis*-unsaturated fatty

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acids lower the main phase transition temperature (T_m) as they preferentially partition into the fluid phase (Ortiz and Gomez-Fernandez, 1987; Klausner et al., 1980). Investigations of OA-ceramide mixtures with Fourier transform Raman spectroscopy and DSC showed that OA also decreases T_m of ceramides and promotes phase separation (Wartewig et al., 1998). Using deuterated OA, IR studies reveal the presence of a fluid phase induced by OA that is distinct from the endogenous SC phase both in SC lipid mixtures (Ongpipattanakul et al., 1991) and in human SC in vivo (Naik et al., 1995). Furthermore, visual evidence of OA promoting phase coexistence in SC membrane systems has been obtained by thermal optical microscopy (Walker and Hadgraft, 1991) as well as freeze-fracture electron microscopy (FFEM) (Tanojo et al., 1997). Based on these observations, OA is thought to increase transdermal penetrability by creating a more permeable phase that coexists with the endogeneous SC lipids. The nature of these coexisting phases and the underlying mechanism through which OA enhances percutaneous penetration still remains unclear. How does OA influence the structure and phase behaviour of crystalline SC lipids? Does OA affect the configurational entropy of the SC lipid chains and induce membrane disorder? What is the structural organization of OA–SC mixtures?

To answer these questions, we have investigated the interactions of OA with a model SC membrane consisting of an equimolar mixture of bovine brain ceramide (BBCer), cholesterol (Chol), and palmitic acid (PA) (Kitson et al., 1994) using solid state deuterium nuclear magnetic resonance (^{2}H NMR). This technique allows for the study of local molecular motions in complex membrane systems that are characterized by more than one phase. ^{2}H NMR thus provides information about the phase behaviour and conformational order of the model SC constituents. By incorporating labelled OA-d_2, PA-d_{31}, or cholesterol-d_6 into the SC model membrane and OA mixture, we can monitor the deuterated component independently of the other membrane constituents. We have investigated how the behaviour of cholesterol, palmitic acid, and oleic acid components of the SC model systems, 2:2:1:1 and 1:1:1:1 BBCer:Chol:PA:OA, depend on temperature. Investigating the phase behaviour of lipid mixtures as a function of temperature helps to define emerging trends in physical properties that determine lipid interactions near physiological temperature as well as the dynamics of the relationships between components of a complex membrane (Velkova and Lafleur, 2002; Mendelsohn and Moore, 2000; Kitson et al., 1994). The rationale for choosing these particular compositions was to treat OA as either a replacement for, or an addition to, the SC lipids. In the former case, OA replaces half the PA. In this way, we were able to determine the effect of introducing an unsaturated fatty acid into the SC model membrane without changing the overall ratio of BBCer:Chol:fatty acid. The latter case, where OA is an additive to the well-studied BBCer:Chol:PA SC model membrane, mimics in a controlled way the topical application of OA. Active ingredients in topical application are assumed to be present "in excess" relative to the lipid content of the SC. For example, Francoeur et al. (1990) observed that the maximum effect of OA on the diffusion of piroxicam occurred at an uptake of about 6% (w/w OA/SC). Since the intercellular lipids represent a small fraction of the total weight of the SC, the molar ratio of OA taken up by the SC to the native SC lipids must be quite large. Note that when using topical application the equilibrium concentration of an active ingredient in the SC barrier membranes can be difficult to determine, but in the model membrane this quantity is known. Our results show that for both cases, OA induces phase separation in OA–SC membranes where crystalline SC components coexist with an OA-rich phase whose constituents are rapidly reorienting in all directions.

2. Materials and methods

2.1. Model stratum corneum (SC) membrane

Bovine brain ceramide (Type III, 99% purity) was a product of Sigma Chemical Co. (St. Louis, MO, USA). Cholesterol (≥99% purity, Sigma Grade) and deuterium-depleted water were obtained from Sigma–Aldrich Canada Ltd. (Oakville, Ont., Canada) and salts from BDH Chemical Company (Toronto, Ont., Canada). Cholesterol-2,2,3,4,4,6-d_6 (97–98% purity), palmitic acid-d_{31} (98% purity), and oleic acid-9,10-d_2 (97% purity) are products of Cambridge Isotope Laboratories (Andover, MA, USA). Spectrograde solvents were used (Caledon Laboratories, Georgetown, Ont., Canada). Approximately 40 mg of deuterated compound was used in each sample. Solutions of lipids in benzene:methanol (7:3) were freeze-dried and hydrated at 90–95 °C in a 150 mM NaCl, 4 mM EDTA, and 100 mM citrate buffer (pH 5.2) prepared with deuterium depleted water and thereafter frozen in liquid nitrogen. This freeze-thawing procedure was repeated five times. Samples were equilibrated at the desired temperature before experiments.

2.2. ^{2}H NMR

NMR spectra were obtained on a locally built spectrometer operating at 46.2 MHz using the quadrupolar echo technique (Davis et al., 1976). Data was collected in quadrature with 8-cyclops phase cycling. The 90° pulse length was 4 μs, inter-pulse spacing 40 μs, post time 20 μs, and dwell time 2 μs. The repetition time between acquisitions varied amongst 300 ms, 1 s, and 50 s depending upon the percentage of solid in the sample. The characteristic spectral shape observed for deuterium-labelled lipids is called a Pake doublet (Hsueh et al., 2003). Pake doublets are deconvoluted to produce 'dePaked' spectra (Bloom et al., 1981; Sternin et al., 1983) from which the frequency separation (quadrupolar splitting, $\Delta\nu$) of the doublet corresponding to each deuteron is easily measured. A reflection of the amount of trans-gauche isomerisation along the acyl chains, the magnitude of the quadrupolar splitting is directly related to the CD bond order parameters, S_{CD}, according to $\Delta\nu = (3/2)(e^2qQ/h)S_{CD}$, where $(e^2qQ/h) = 167$ kHz is the static quadrupolar coupling constant (Davis and Jeffrey, 1977). Particular doublets were assigned to deuterons along the carbon chain as described previously (Fenske et al., 1994). In addition to Pake doublets, spectra of SC model membranes often feature a narrow central peak corresponding to deuterium-labelled lipid that reorients

rapidly and isotropically. The fraction of labelled lipid undergoing isotropic motion is equal to the area of this narrow peak divided by the total area. In practice this ratio is obtained from the spectrum prior to Fourier transformation by extrapolating the decaying exponential wave (which corresponds to the isotropic peak) back to echo time $t = 0$ s and dividing this height by the magnitude of the echo peak (which corresponds to the total spectral area).

3. Results and discussion

3.1. Interpreting the ^2H NMR spectra

An introduction to ^{2}H NMR studies of SC lipids was previously presented (Hsueh et al., 2003). In brief, this technique can be used to examine the motion of individual species of labelled molecules, and is particularly suited to studying the phase behaviour of lipids in complex mixtures. The obtained ^{2}H NMR spectra exhibit distinct characteristics depending on the structure and dynamics of the deuterated membrane component. As was previously reported, the SC model membrane undergoes a melting transition at a temperature, $T_m = 40$–$42\,^\circ$C, from a more ordered crystalline state to a liquid crystalline phase where lipids exhibit axially symmetric motion and conformational chain disorder (Kitson et al., 1994; Thewalt et al., 1992). Below T_m, spectra are dominated by two pairs of Pake doublets, one with a quadrupolar splitting of 126 kHz reflecting motionless chains on the NMR timescale, and the other with a splitting of 64 kHz due to the terminal methyl groups. At T_m, the spectrum transforms into a superposition of Pake doublets reflecting the gradient in conformational freedom along the acyl chain. Also, the spectral width decreases by at least a factor of two demonstrating the presence of axially symmetric motion. At higher temperatures ($\sim$52 °C), a second transition occurs where the liquid crystalline phase converts to a phase where membrane components exhibit a high degree of isotropic motion.

3.2. Effects of OA below T_m

In Fig. 1, we illustrate the effect of OA on the SC model membrane at 25 °C. Fig. 1a displays the spectrum of the control SC model membrane, 1:1:1 BBCer:Chol:PA-d_{31}. At this temperature, the spectral peaks at $\pm$63 kHz are characteristic of the crystalline phase and dominate the spectral lineshape. Note that the small peak at 0 kHz is a signal from residual deuterated water and is not due to reorienting PA. Replacing half of the PA with OA, we incorporated this unsaturated fatty acid into the SC model membrane to create a 2:2:1:1 BBCer:Chol:PA-d_{31}:OA mixture. At 25 °C, we observe the emergence of an isotropic component in the spectrum of the PA-d_{31} (Fig. 1b). A more intense isotropic component is seen in the spectrum of 1:1:1:1 BBCer:Chol:PA-d_{31}:OA where OA has been added to the control SC mixture (Fig. 1c). These results reveal how OA affects the SC lipid phase behaviour both when OA is replacing the PA and when added as an extra component to SC model membranes.

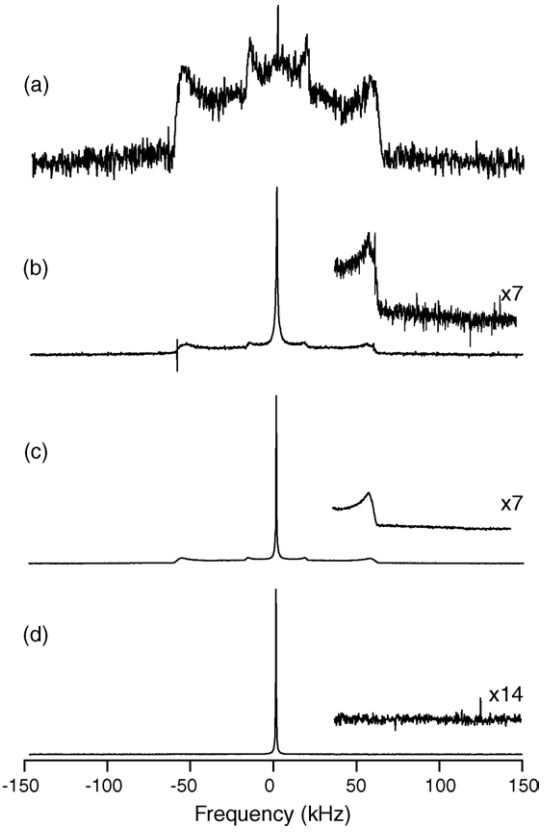

Fig. 1. ^{2}H NMR spectra demonstrating the effects of oleic acid on the SC model membrane at $T = 25\,^\circ$C, pH 5.2. (a) 1:1:1 BBCer:Chol:PA-d_{31} (control); (b) 2:2:1:1 BBCer:Chol:PA-d_{31}:OA; (c) 1:1:1:1 BBCer:Chol:PA-d_{31}:OA; (d) 2:2:1:1 BBCer:Chol:PA:OA-d_2. Expanded regions of the spectra are shown in the insets.

The spectrum in Fig. 1d illustrates the behaviour of the OA in 2:2:1:1 BBCer:Chol:PA:OA-d_2 mixture. This spectrum consists entirely of an isotropic component indicating that OA experiences rapid isotropic motion within a distinct OA-rich phase and thus is not interacting with the crystalline SC membrane. In light of the isotropic component of the PA-d_{31} spectra displayed in Fig. 1b and c, it is likely that this phase also contains some PA. While we see the emergence of an isotropic phase representing $\sim$21% of the PA at 25 °C, PA-d_{31} spectra also retain characteristics of the lamellar phase at this temperature. These observations are consistent with previous FTIR evidence that OA is phase separated from endogenous SC lipids below T_m (Ongpipattanakul et al., 1991). It is clear that in this temperature range, OA remains in a highly isotropic phase and extracts some PA; whether this interaction involves the incorporation of OA into the membrane and formation of an inverted micellar phase between leaflets and/or dissolution of crystalline SC components out of the lamellar membrane remains a question.

3.3. Action at and above T_m

For the 1:1:1 BBCer:Chol:PA-d_{31} control SC model membrane, the onset of the melting transition occurs at 42 °C (Kitson et al., 1994; Thewalt et al., 1992). In the presence of OA, the isotropic component of 2:2:1:1 BBCer:Chol:PA-d_{31}:OA

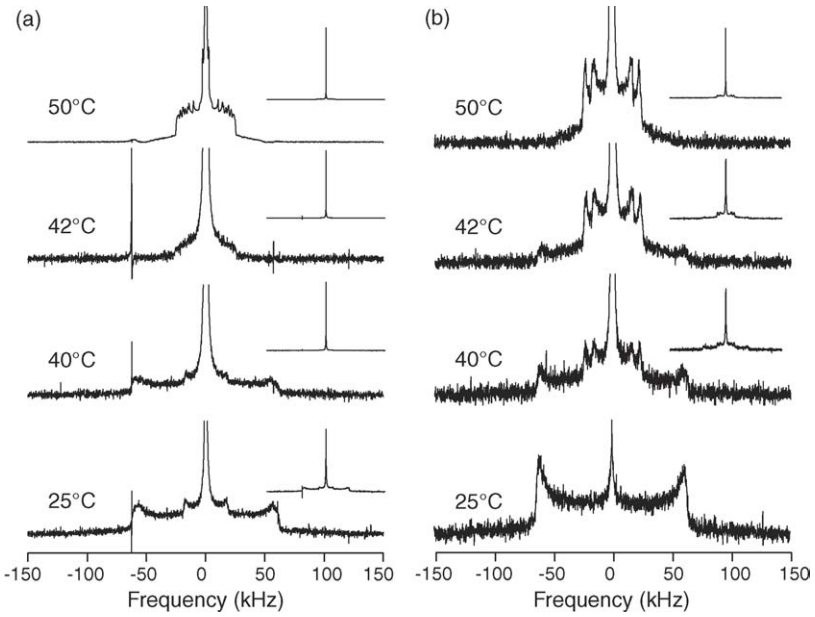

Fig. 2. ^{2}H NMR spectra as a function of temperature, pH 5.2. (a) 2:2:1:1 BBCer: Chol:PA-d_{31}:OA; (b) 2:2:1:1 BBCer:Chol-d_6:PA:OA. The isotropic peak has been truncated to highlight the temperature-dependent spectral changes. Non-truncated spectra are shown in the insets.

spectra that is already visible at 25 °C grows with temperature while the underlying spectrum retains characteristics of the crystalline phase up to 40 °C (Fig. 2a). By 42 °C this crystalline component has melted and spectra exhibit fluid characteristics. At 50 °C, the spectrum is a superposition of an isotropic line and a clearly resolved liquid crystalline component.

Fig. 2b displays spectra obtained using labelled cholesterol. This reveals the presence of a small isotropic component at 25 °C but the spectrum consists almost entirely of a Pake doublet with 126 kHz splitting reflecting crystalline packing. This signal from the crystalline phase is still visible at 42 °C but has disappeared by 50 °C. At 40 °C, there are two Pake doublets with splittings 33.2 ± 0.8 kHz and 46.5 ± 0.4 kHz that signify axially symmetric rotation. As the model membrane system is heated to 50 °C, these Pake doublets become more prominent. Meanwhile the intensity of the isotropic peak grows steadily as shown in the temperature sequence in Fig. 2b.

From the perspective of OA, spectra of 2:2:1:1 BBCer:Chol:PA:OA-d_2 (Fig. 3) illustrate that between 40 and 42 °C, the magnitude of the isotropic component markedly decreases (not shown). At 42 °C, two Pake doublets corresponding to the deuterons at the 9- and 10-C positions emerge in addition to the isotropic line. These Pake doublets indicate that the OA is experiencing axially symmetric motion such as would be expected if it were intercalated in a liquid crystalline membrane. Fig. 3 shows the spectrum of 2:2:1:1 BBCer:Chol:PA:OA-d_2 at 50 °C where the doublets, with splittings 24.7 ± 0.1 and 9.7 ± 0.1 kHz, are better defined. Note that these splittings are much larger than those for the corresponding deuterons of 1-palmitoyl-2-oleoyl-(*sn*)-glycerophosphocholine even at 0 °C (Seelig and Waespe-Šarčević, 1978). An earlier FTIR study of deuterated OA revealed that OA is disordered both below and above T_m of SC lipids, and thus phase separated from the endogenous SC lipids (Ongpipattanakul et al., 1991). In accordance with this study, we observe that most of the OA is in an isotropic phase. In addition, ^{2}H NMR facilitates observations of the small proportion of OA's non-isotropic behaviour.

3.4. Percentage isotropy

Quantifying the percentage isotropy provides further evidence that the isotropic component of the OA-d_2 spectrum decreases dramatically at the transition. Fig. 4 illustrates how OA's percentage isotropy attains a *minimum* of 60% near T_m, demonstrating that its configurational freedom is maximally constrained about this temperature. Plotting the percentage isotropy of PA reveals that this SC component gains

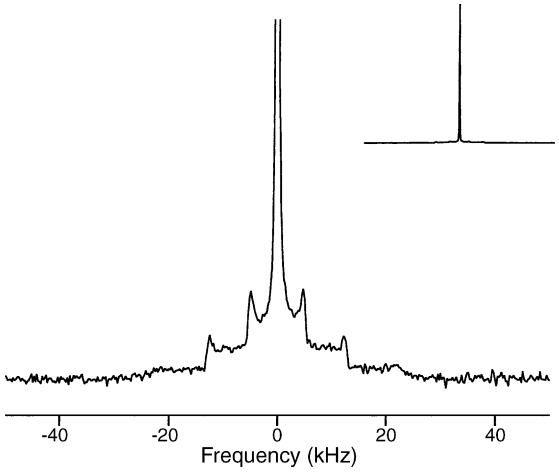

Fig. 3. Spectra of 2:2:1:1 BBCer:Chol:PA:OA-d_2 at $T = 50$ °C, pH 5.2. The isotropic peak has been truncated. The non-truncated spectrum is shown in the inset.

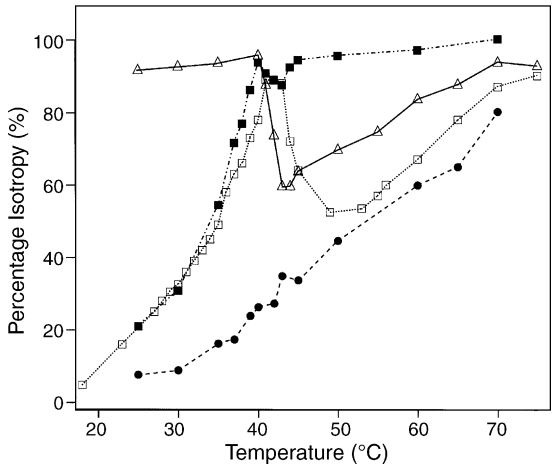

Fig. 4. Temperature dependence of the percentage isotropy. 2:2:1:1 BBCer:Chol:PA-d_{31}:OA (hollow squares); 1:1:1:1 BBCer:Chol:PA-d_{31}:OA (solid squares); 2:2:1:1 BBCer:Chol:PA:OA-d_2 (hollow triangles); 2:2:1:1 BBCer:Chol-d_6:PA:OA (solid circles). Error is estimated to be within ±1% of the value of the percentage isotropy for model membranes containing PA-d_{31} and OA-d_2 and ±3% for those containing Chol-d_6. Lines connecting the data points are for visual aid.

conformational freedom as a function of temperature and displays a *maximum* between 40 and 42 °C. The concomitant *increase* in PA and *decrease* in OA percentage isotropy is a strong indication that OA is participating in the liquid crystalline phase and that PA is simultaneously extracted out of the crystalline phase. Fig. 4 also shows that the percentage isotropy of cholesterol steadily increases with temperature. This is in contrast to the behaviour of PA which displays a marked decrease in the percentage isotropy immediately above T_m and reaches a minimum of 52% at 49 °C before increasing again. Interestingly, the slopes of the percentage isotropy of both cholesterol and PA above 50 °C are similar, suggesting that these two SC components interact. Together with Fig. 2a that shows the absence of any crystalline component of PA at and above 42 °C, we can conclude that the minimum in percentage isotropy is due to the conversion of PA to a liquid crystalline phase. Note that the liquid crystalline fraction of cholesterol is also increasing dramatically around T_m (Fig. 2).

The intensity of the isotropic component in the 1:1:1:1 BBCer:Chol:PA-d_{31}:OA mixture also markedly increases below T_m and attains a maximum at 40 °C. The decrease in the percentage isotropy at temperatures immediately above this maximum is lesser than the reduction observed for 2:2:1:1 BBCer:Chol:PA-d_{31}:OA and extends over a much smaller temperature range (40–45 °C). Above this temperature, PA exhibits highly isotropic behaviour.

Note that the presence of OA does not appreciably decrease T_m of the SC model membrane as monitored by PA-d_{31}. This is contrary to previous evidence that OA reduces the T_m of SC mixtures (Yamane et al., 1995; Ongpipattanakul et al., 1991; Walker and Hadgraft, 1991; Golden et al., 1987). Another study also found that OA/ethanol reduced T_m of porcine SC lipids, but this reduction cannot be isolated from the effects of ethanol (Francoeur et al., 1990).

3.5. The question of SC membrane disorder

Determining the order parameters, S_{CD}, along PA's hydrocarbon chain yields a measure of acyl chain order at 50 °C. For a membrane in the fluid phase, S_{CD} values provide a measure of lipid chain conformational order at sites along the acyl chain. Plotting S_{CD} as a function of carbon position along the acyl chain yields an order parameter profile that typically exhibits a plateau in the upper acyl chain region, where the chains are more ordered. S_{CD} values decrease towards the end of the chain reflecting increased conformational freedom. Fig. 5 illustrates the order parameter profiles of SC membranes in the presence (2:2:1:1 BBCer:Chol:PA-d_{31}:OA, 1:1:1:1 BBCer:Chol:PA-d_{31}:OA) and absence (1:1:1 BBCer:Chol:PA-d_{31}) of OA. In the plateau region, S_{CD} = 0.4 is typical for liquid crystalline membranes containing high concentrations of cholesterol. Little change in S_{CD} due to the presence of OA is observed: the greatest increase is for carbons 12 to 15 where the order parameters differ by no more than 8%. Thus, the PA-d_{31} order parameters for the non-isotropic liquid crystalline phase of BBCer:Chol:PA-d_{31}:OA are nearly insensitive to OA concentration. This observation indicates that PA exists in either a lamellar or isotropic phase and not in an intermediate state. The unchanged PA-d_{31} order parameters also implies that the concentration of OA in the liquid crystalline regions of the membrane is low. This is consistent with other reports of OA/propylene glycol induced lipid extraction of rat abdominal SC by FT-IR/AR (Takeuchi et al., 1993) and phase coexistence induced by OA in SC membranes (Walker and Hadgraft, 1991; Ongpipattanakul et al., 1991; Tanojo et al., 1997). These results appear to differ from previous reports of OA's sterically disordering effects in the superficial SC layers of human forearm in vivo using attenuated total reflectance infrared spectroscopy (Naik et al., 1995). Other studies of OA–SC mixtures have also reported 'disorder' induced by OA (Takeuchi et al., 1998, 1992) but in such IR measurements, the obtained data is an average of the behaviour of OA and SC lipid chains and also an average over any phase coexistence within the membrane. The presence of OA-rich domains that form a phase distinct from the lamellar SC components provides an explanation for OA's

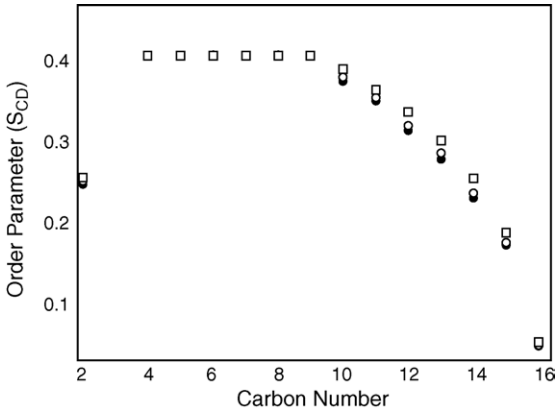

Fig. 5. Order parameter profile at 50 °C, pH 5.2. (a) 1:1:1 BBCer:Chol:PA-d_{31} (squares); (b) 2:2:1:1 BBCer:Chol:PA-d_{31}:OA (hollow circles); and (b) 1:1:1:1 BBCer:Chol:PA-d_{31}:OA (solid circles). Error is estimated to be within ±1%.

penetration enhancing activity and does not require disordering of the SC lipids.

In summary, we show here that OA exhibits highly isotropic behaviour in SC mixtures and promotes the isotropic motion of a portion of the endogenous SC components. At and above T_m, some OA penetrates into the liquid crystalline SC phase while the remaining OA coexists with other SC components in a phase characterized by isotropic behaviour. The increased interaction between OA and SC membranes at this temperature can be understood in considering the major changes in membrane properties that occur at and around the main transition temperature. At and about T_m, lipid bilayers are known to exhibit increased binding to solutes (Trandum et al., 2000) and increased permeability of small ions (Papahadjopoulos et al., 1973; Cruziero-Hansson and Mouritsen, 1988). These observations can be explained by a coexistence of gel and fluid domains near the main phase transition and an accompanying increase in interfacial regions and area per molecule (Cruziero-Hansson and Mouritsen, 1988; Jørgensen et al., 1993; reviewed in Mouritsen et al., 1995). Such alterations in bilayer structure may ultimately influence OA–SC membrane interactions in this temperature region about the main phase transition.

The structural organization of the isotropic, OA-rich phase is still unclear. Isotropic motion could occur in vesicles distinct from the lamellar phase or oily droplets embedded in the lamellae. Rapidly reorienting molecules in oily droplets could serve as a mechanism to transport exogenous lipophilic substances through the SC membrane barrier. Promoting phase separation in this way would also result in an increase in the interfacial area (leaky grain boundaries) between phases (Wu and McConnell, 1973; Cruziero-Hansson and Mouritsen, 1988) and thereby further enhance percutaneous permeability. Future microscopy studies with lipophilic fluorescent probes will provide complementary visual insights into the structure of the non-lamellar phase. Finally, our results show that the complex but mainly crystalline lipid bilayers characteristic of the SC's intracellular membranes can exist in dynamic equilibrium with different structural organizations of lipids. This indicates that the structure, and therefore function, of the SC permeability barrier can be modified by the application of appropriately chosen exogeneous lipids.

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