A Model Membrane Approach to the Epidermal Permeability Barrier†

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ABSTRACT: The permeability barrier of mammalian skin is found in unusual intercellular domains in the upper layers of the epidermis, and is composed mainly of three lipid classes: ceramide, cholesterol, and free fatty acid. These are organized as lamellae, but the details of lipid organization are not precisely known. To examine the relationship between lipid composition and phase behavior, aqueous dispersions of bovine brain ceramide, cholesterol, and perdeuterated palmitic acid were examined by ^{2}H NMR and compared to analogous systems in which sphingomyelin replaced ceramide. The sphingomyelin systems give rise as expected to a stable fluid lamellar signal over the temperature range 20–75 °C and pH 5.2–7.4, whereas the ceramide dispersions show complex polymorphism as a function of both temperature and pH. Prominent features of the ceramide dispersions containing cholesterol are phase coexistence and the presence of a "solid" phase in which molecular motion is more inhibited than in a classical phospholipid gel phase: T_{1z} measurements indicate that lateral diffusion of the palmitic acid probe effectively does not occur. In the absence of cholesterol, a fluid lamellar signal is not observed, but the appearance of a "solid" signal is also influenced by the pH. In the presence of cholesterol, a fluid lamellar signal is present at 50 °C, and the ^{2}H NMR order parameter profile is very similar to that derived from the analogous sphingomyelin dispersions. We interpret these results as evidence that the lipid composition of stratum corneum intercellular membranes will confer physical properties that are considerably different from those of the vast majority of mammalian cell membranes, and speculate that such organization is critical to physiological function.

To achieve control of the internal environment, animals require a permeability barrier (particularly to water) at body surfaces in contact with the external world. Skin makes up most of this barrier, which, in the restricted case of terrestrial mammals (although not necessarily only these), is provided by a lipid domain located between the cells of the outermost layer of the epidermis [the "*stratum corneum*" (SC)] (Williams & Elias, 1987; Wertz *et al.*, 1987). There is convincing evidence that these lipid domains are organized as lamellae, possibly bilayers (White *et al.*, 1988; Bouwstra *et al.*, 1991; Landmann, 1988) having features in common with biological membranes. However, there is also evidence that these structures differ from most biological membranes with respect to lipid composition, physical organization, and function, and the relationships among these three membrane attributes are in our view central to important aspects of *stratum corneum* physiology, pathology, and pharmacology (*e.g.*, transepidermal transport and tissue cohesion). No doubt other factors, particularly proteins, are also critical [see, for example, Lundström and Egelrud (1991)]. While the story is certainly complex, our focus is solely on the membrane lipids, for whose physiological importance there is considerable evidence (Potts & Francoeur, 1990).

In order to compare membranes of the SC to other biological membranes, we emphasize three distinctive features of the former:

(i) The majority of the lipids (at least in human, pig, and neonatal mouse) are of three classes: ceramides, sterols (and sterol derivatives), or free fatty acids (Yardley & Summerly, 1981; Williams & Elias, 1987). Ceramides are sphingolipids similar in structure to sphingomyelin and glycosphingolipids (such as the cerebrosides), but with a hydroxyl function substituting for the larger phosphorylcholine or sugar headgroup. Thus, SC membrane composition is unusual relative to other biological membranes in being devoid of lipids having relatively large headgroups containing either sugars or phosphorus. [This may not be true of some marine mammals in which there are significant amounts of glycosphingolipid within the SC (Elias *et al.*, 1987), but it is not yet clear that the functional properties of this tissue are the same as for terrestrial mammals.]

(ii) There is evidence that SC lipids exhibit complex phase behavior *in vivo* and *in vitro* (White *et al.*, 1988), and X-ray diffraction studies of both mouse (White *et al.*, 1988) and human (Bouwstra *et al.*, 1991) SC suggest that the lamellar repeating structure of at least some of these intercellular regions is not one bilayer, but two.

(iii) Direct measurements of SC water permeability (Potts & Francoeur, 1990) gave values several orders of magnitude smaller than would be expected for conventional biological membranes (Fettiplace & Haydon, 1980).

Taken together, the available data are consistent with direct relationships existing between tissue characteristics (*e.g.*, water permeability) and the unusual lipid composition and phase behavior of SC intercellular membranes, although such correlations are in debate (Potts & Francoeur, 1991). To examine such possible relationships, a direct experimental

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approach is required, and this is difficult to achieve in studies of intact tissue.

The use of membrane models is well established as an approach to understanding physiological membrane events in physical terms (Gruner *et al.*, 1985). A pertinent example is the study of the connection between membrane fusion and the tendencies of certain membrane lipids to adopt nonbilayer phases *in vitro* (Cullis *et al.*, 1986). In such studies, ^{2}H NMR has been found to be a useful probe of membrane organization and behavior (Seelig & Seelig, 1980). Deuterium can be substituted for hydrogen on the molecular species of interest (in this case, a lipid), and information can be derived about different regions within a complex system. Furthermore, the experimental NMR time scales open to investigation (through various relaxation measurements for instance) allow the study of classes of molecular motion, and the method is therefore well suited to studying local molecular motions in complex membrane systems where more than one phase occurs and where long-range order may not exist. Such information does not replace that derived by other methods (such as X-ray diffraction) but is complementary to it (Bloom *et al.*, 1991). Because the lipid composition of the SC intercellular lipid domain is complex, and the phase behavior of such lipid domains is almost certainly also complex, we believe that ^{2}H NMR studies of suitable model membranes will prove useful. Indeed, Abraham and Downing (1991, 1992) have recently published the first such studies. Despite these, and other notable exceptions (Parrott & Turner, 1993; Wiedmann & Salmon, 1991; Lieckfeldt *et al.*, 1993; Löfgren & Pascher, 1977), there is very little published work on the phase behavior of systems containing ceramides (as distinct from, *e.g.*, lipids based on ceramides such as sphingomyelin or monoglucosylceramide), and we believe that this field is still at an early stage of development. As one logical point of departure, we chose to determine the relationship between lipid composition (particularly with respect to lipid classes) and lipid phase behavior (as determined by ^{2}H NMR) in hydrated lipid dispersions having compositions modeled on the SC intercellular membranes. Because there have been so few studies of ceramide, we began by using the commercially available material derived from bovine brain. In addition, we wished to compare the behavior of our ceramide system with one in which sphingomyelin was the relevant sphingolipid: this mimics in an approximate way the changes that occur in lipid classes during epidermal differentiation. A consistent feature of this process is the enzymic alteration in molecular "shape" of various lipid species, such that typical membrane headgroups are much reduced in size. This will be considered more fully below. Thus, our goal at this stage is to examine the effects of gross changes of both lipid class and the aqueous phase on this complex system.

The model membrane composition we have used to mimic that found in SC intercellular membranes is bovine brain ceramide, cholesterol, and palmitic acid in equimolar proportions. This mixture is a simple yet not unrealistic starting point in which the major SC lipid classes are represented. It is important to note that the reported properties of these lipids found *in vivo* vary; for example, a free fatty acid fraction of 33 mol % exceeds that found in some studies [*e.g.*, see Hedberg *et al.* (1988)] but not others (Gray *et al.*, 1982).

Our preliminary results (Thewalt *et al.*, 1992a) showed that the removal of the sphingomyelin headgroup has a dramatic effect on membrane phase behavior. Here we extend this work to examine the effect of pH on the phase behavior of these lipid mixtures. As well, we examine in greater detail the nature of the "solid" phase observed at physiological temperatures in the SC model membrane system. Progression to studies using material derived from skin is an obvious next step.

MATERIALS AND METHODS

Lipids and Sample Preparation. Palmitic acid, cholesterol, sphingomyelin, and ceramides were obtained from Sigma Chemical Co. (St. Louis, MO) and used without further purification. Sphingomyelin and ceramide (type III) were obtained from bovine brain, and the acyl chains were mainly stearic (18:0), nervonic (24:1), and lignoceric (24:0). Perdeuterated palmitic acid (PA-d_{31}) was prepared by the method of Hsiao *et al.* (1974).

Lipids were mixed together by weight, dissolved in benzene/methanol, 7:3 (v/v), and freeze-dried. The resulting white powders were hydrated in excess (about 7:1 w/w) using buffers that varied according to the desired pH. Each solution was prepared in deuterium-depleted water and contained 150 mM NaCl, 4 mM EDTA, and 100 mM HEPES (for pH 7.4), MES (for pH 6.2), or citrate (for pH 5.2). Approximate molecular weights used were 660 for ceramide and 824 for sphingomyelin. Hydration was performed at about 70 °C, and the resulting dispersions were frozen in liquid nitrogen and then heated to at least 75 °C 5 times. Samples were frozen prior to use, and then allowed to equilibrate at room temperature for at least 1 h before beginning the NMR measurements.

NMR Methods. ^{2}H NMR spectra were obtained using the quadrupolar echo technique (Davis *et al.*, 1976) with a home-built spectrometer (Davis, 1979; Sternin, 1985) operating at 46.2 MHz. The typical spectrum resulted from 400–10 000 repetitions of the two-pulse sequence with a 90° pulse length of 4 μs, an interpulse spacing τ = 50 μs, and a dwell time of 2 or 5 μs. The delay between acquisitions was varied in the range 300 ms to 50 s, and data were collected in quadrature with Cyclops 8-cycle phase cycling. First and second moments were calculated from the Fourier-transformed spectra (Davis, 1983). T_{1z} relaxation measurements were performed using the saturation recovery and stimulated echo techniques (Bloom *et al.*, 1992). The saturation recovery method is a quadrupolar echo pulse sequence with varying repetititon times: we used 19 different repetition times in the range 800 ms to 50 s. The stimulated echo pulse sequence used was $90^\circ_x - \tau_1 - 90^\circ_x - \tau_2 - 90^\circ_x - t$ where τ_1 = 20 μs and τ_2 was varied from 1 to 30 s (seven values in all).

^{2}H NMR order parameter profiles (the carbon deuterium orientational order parameter S_{CD} *vs* position along the deuterated palmitoyl chain) were calculated from those spectra which displayed axial symmetry characteristic of a fluid lamellar phase. These spectra, which were superpositions of Pake doublets, were dePaked (Sternin *et al.*, 1983), and smoothed order parameter profiles (Lafleur *et al.*, 1989) were then calculated. (See, however, Results for a discussion of the anomalously small splitting displayed by deuterons attached to C-2 of the palmitic acid in some samples.)

^{2}H NMR spectral subtractions were carried out between the Fourier-transformed spectra of Cer/Ch/PA-d_{31} (1:1:1 mol/mol) or Cer/PA-d_{31} (2:1 mol/mol) and PA-d_{31} alone (all in excess buffer) in order to determine the residual spectral shape of PA's signal in the mixtures once the solid PA signal was removed.

RESULTS

The SPM/Ch/PA-d_{31} sample gave rise to a ^{2}H NMR spectrum showing axially symmetric motion of the fatty acid

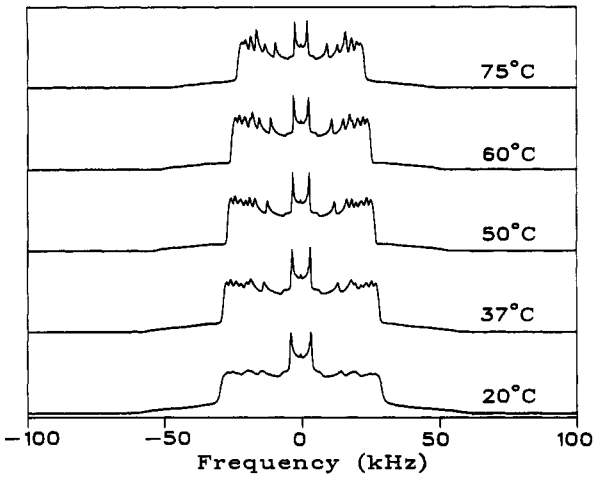

FIGURE 1: Temperature dependence of the ^2H NMR spectrum of an equimolar mixture of SPM, Ch, and PA-d_{31} dispersed at pH 5.2. Repetition time 0.3 s.

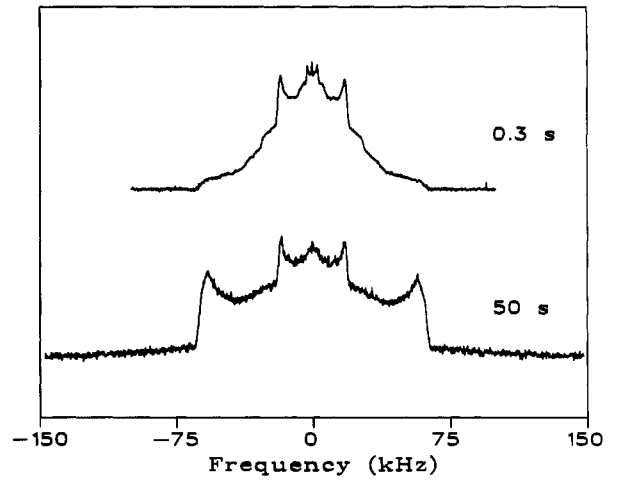

FIGURE 2: Effect of increasing the time between scans (from 0.3 to 50 s) in the ^2H NMR quadrupolar echo experiment to allow a return to equilibrium magnetization for an equimolar mixture of Cer, Ch, and PA-d_{31} dispersed at pH 5.2, T = 20 °C.

(as typically found in lamellar liquid-crystalline phases) between 20 and 75 °C at pH 5.2 (Figure 1). The characteristic feature of the signal derived from a fluid bilayer is that the spectrum consists of a superposition of Pake doublets having quadrupolar splittings that decrease for labeled positions located toward the end of the acyl chain, reflecting their increasing conformational freedom ("disorder"). (Another characteristic of a fluid bilayer, the rapid lateral diffusion of lipids in the bilayer plane, is not immediately inferred from the spectrum.) It is important to note that this line shape, although characteristic of a fluid bilayer phase, is not unique to that phase but is also observed in nematic liquid crystals (Delikatny & Burnell, 1990). Other techniques, especially X-ray diffraction, are necessary to distinguish between bilayer and other lamellar structures. However, sphingomyelin dispersions have been studied by these methods, and the results are indeed consistent with a bilayer organization (McIntosh *et al.*, 1992). The significant point to note is that over the temperature range 20–75 °C and the pH range 5.2–7.4, the spectrum was consistent with a highly ordered fluid bilayer phase. In fact, the degree of chain ordering and the relative temperature-independence of the spectrum lead us to speculate that the bilayer is in the "liquid ordered" (*lo*) phase which is commonly observed in phosphatidylcholine/cholesterol mixtures at high cholesterol concentrations (Vist & Davis, 1990; Thewalt *et al.*, 1992b). The significance of the pH range is that a pH on the order of 5 may be closer to that found in the SC than is the "physiological" value of about 7.4 (Behrendt & Green, 1971). However, for all samples of SPM/Ch/PA-d_{31} (1:1:1 mol/mol), the same phase behavior was observed under all experimental conditions.

In contrast to the sphingomyelin system, comparable experiments with ceramide mixtures gave rise to quite a different range of spectra, and in no case did any of the Cer/Ch/PA-d_{31} (1:1:1 mol/mol) dispersions show *lo* lamellar line shapes over the entire temperature range 20–75 °C. At temperatures below 50 °C, the spectra were clearly complex at pH 5.2, 6.2, and 7.4. Several approaches were used for further analysis. By inspection, we determined that there was within the spectrum (*e.g.*, Figure 2, top) a doublet having a quadrupolar splitting of about 36 kHz, a value known to occur for the freely rotating methyl groups of solid (crystalline) hydrocarbons (Valic *et al.*, 1979). If this were the case, the rest of such a solid signal would be much broader, and the "edges" of the doublet containing the remaining methylene resonances would have a splitting of 126 kHz. However, due to the extremely slow spin–lattice relaxation observed in this phase, the usual repetition times of 0.3 s that are sufficient for fluid phases are much too short to observe this signal. By lengthening the repetition times to 50 s (Figure 2, bottom) to allow complete relaxation of the magnetization to equilibrium between scans, there was indeed an increase in signal intensity, forming a Pake doublet with "edges" at ±63 kHz and weak shoulders extending to ±126 kHz. This broad Pake doublet in fact accounted for much of the spectral intensity under these conditions. The proportion of signal originating from the solid fatty acid can be simply calculated by comparing the quadrupolar echo intensity (which is directly proportional to the number of deuterons in the sample provided the time between scans is sufficiently long to allow a full return to equilibrium) at short and long repetition times. At short times, almost all of the echo comes from the fluid portion of the fatty acid [although the methyl group signal (3/31 of the solid signal) from the solid also contributes]. This signal can be labeled Echo Height (short) or EH_s. At long times, all of the signal (EH_l) is detected, and the proportion of the solid component is thus $EH_{sol} = {}^{31}/_{28}[(EH_l - EH_s)/EH_l]$. For Cer/Ch/PA-$d_{31}$ at pH 5.2, this calculation shows that the proportion of solid fatty acid at 20 °C is 80%. (If anything, this is an underestimate since the solid spectrum is so broad that the wings of the spectrum are inadequately excited by the 4-μs "90°" pulses.) To further characterize the "solid" phase, we performed longitudinal (T_{1z}) relaxation measurements on this signal. We employed two different methods to measure T_{1z}: saturation recovery and stimulated echo, the latter being sensitive to lateral diffusion (Bloom *et al.*, 1992). Using the saturation recovery method, $T_{1z} = 11.7 \pm 0.3$ s, and using the stimulated echo technique, $T_{1z} = 12.5 \pm 0.6$ s. Thus, the ratio (stimulated echo/saturation recovery) of the two T_{1z} measurements is 1.07 ± 0.08, indicating that there was no diffusion (more precisely, no observable contribution due to diffusion of the deuterated PA to a different orientation with respect to the magnetic field) on this time scale.

Figure 3 shows the temperature dependence of ^2H NMR spectra obtained from equimolar dispersions of Cer/Ch/PA-d_{31} at two selected pH values. At pH 7.4 (Figure 3a), line shapes characteristic of a liquid-ordered phase were observed at 37, 50, and 60 °C. At 75 °C, the spectrum is dramatically narrowed, representing an inverted hexagonal (H_{II}) phase. The ^2H NMR powder pattern line shape for the H_{II} organization has been discussed elsewhere (Lafleur *et al.*,

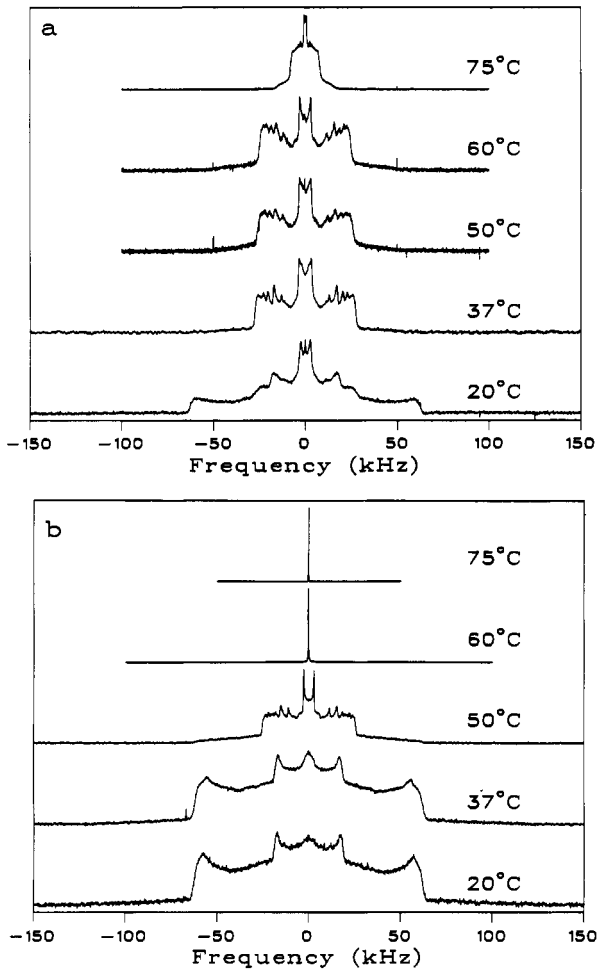

FIGURE 3: Temperature dependencies of ^2H NMR spectra of equimolar dispersions of Cer, Ch, and PA-d_{31} at (a) pH 7.4 and (b) pH 5.2. Repetition time 50 s at temperatures ≤50 °C, 1 s at 60 and 75 °C.

1990), but the essential feature (in comparison to that derived from a fluid lamellar phase) is that the width of the frequency spectrum is reduced by more than a factor of 2, largely due to extra averaging of the quadrupolar interactions by the rapid diffusion of lipid molecules around the long axis of the cylinders. The spectrum at 20 °C was by inspection a superposition of a solid phase and a more fluid phase. The shape of the residual fluid phase spectrum will be discussed later.

At pH 5.2 (Figure 3b), the results again demonstrated thermotropic polymorphism but differed from those obtained at pH 7.4 (although very little from those obtained at pH 6.2), with the exception of the lamellar liquid-ordered line shape observed at 50 °C. At higher temperatures (60 and 75 °C), there was a progression from the liquid-ordered phase to an isotropic phase, with no detectable intervening H_{II} phase. Further identification of this "isotropic" phase was not attempted by NMR, but in other systems such signals are compatible with several lipid organizations in which there is complete averaging of the quadrupolar interaction on the NMR time scale due to rapid molecular motion either having no preferred orientation as in, *e.g.*, micellar structures or having cubic coordinates as in cubic structures. At 20 and 37 °C, at least 80% of the signal available from the PA-d_{31} probe was contained in the solid phase spectrum.

The results so far presented show clearly that the three-component system (Cer/Ch/PA-d_{31}) exhibits complex phase behavior that is not observed when sphingomyelin replaces

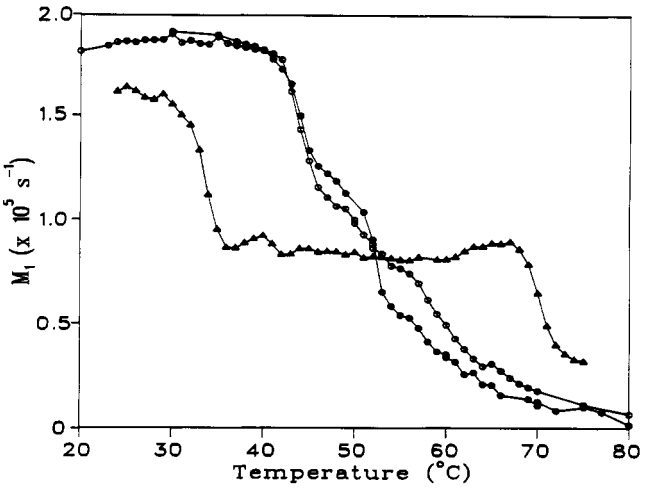

FIGURE 4: Average width of the ^2H NMR spectrum (M_1) as a function of temperature and pH. Closed circles, pH 5.2; open circles, pH 6.2; closed triangles, pH 7.4. Repetition time 50 s at all temperatures where the sample contained some solid component, then 1 s at higher temperatures. (In practice, the quadrupolar echo intensity was checked every few degrees at the 1-s repetition time toward the completion of the solid to fluid transition, and when the echo intensity differed by ≤1% between the short and long repetition times, a 1-s repetition time was used for all higher temperatures.)

ceramide as the sphingolipid. Because our initial observations were at five arbitrarily selected temperatures, we obtained a more detailed perspective on the thermal behavior of the system by measuring the first moment of the distribution (M_1) at many more temperatures. Since the general spectral features were already known, transitions between the various phases could then be observed as a function of temperature. Three such plots representing the three pH values employed are shown in Figure 4. At pH 5.2 and 6.2, the curves are very similar, and show abrupt decreases in M_1 between 42 and 46 °C which correspond to the transition from the complex spectra, showing evidence for several phases (but dominated by a "solid" component), to one represented by the fluid phase line shape. A further abrupt decrease in M_1 was observed for the pH 5.2 sample in the region of 51–54 °C, and this was associated with the appearance of an isotropic signal, which grew as a proportion of the total signal until at 80 °C virtually no fluid lamellar phase remained. A similar phenomenon occurred at pH 6.2, but the decrease in M_1 in the neighborhood of 50 °C is less abrupt. There is an obvious gross difference between these plots and that found for the pH 7.4 sample. Although there was clearly a significant solid component reflected in the large value of M_1 at lower temperatures, this decreased abruptly over the range 33–37 °C, corresponding to the appearance of a characteristically fluid signal. The further decrease in M_1 beginning at about 68 °C reflects the transition to the H_{II} phase already noted. Between these two transition regions, the spectral shape remained nearly constant, except for a small central component with no resolved splitting that appeared at about 45 °C and then disappeared at 63 °C.

For all dispersions containing cholesterol, a characteristic and consistent feature was the presence of a spectrum corresponding to a liquid-ordered phase at 50 °C. To examine this more fully, the powder pattern spectra were "dePaked" (Sternin *et al.*, 1983). Using the well-established (Lafleur *et al.*, 1989) method to obtain the "smoothed" order parameter profiles, it was apparent that the profiles obtained differed from those normally observed for phospholipid membranes. Instead of a profile consistent with monotonically decreasing quadrupolar splittings from C-2 to C-16, the PA-d_{31} profiles had a definite kink in the C-13 to C-15 region. To determine

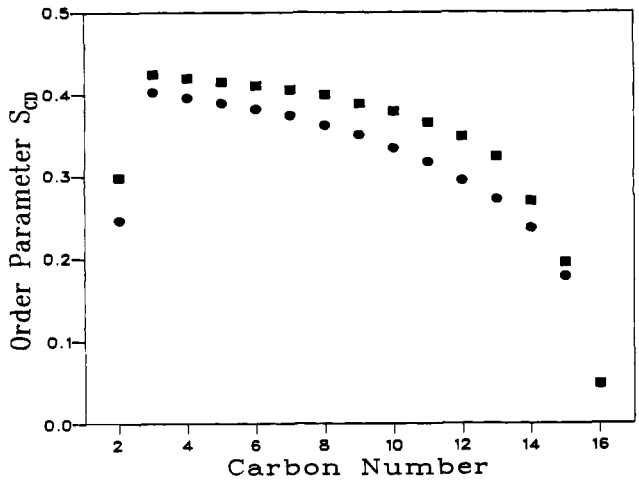

FIGURE 5: ^2H NMR order parameter profiles for equimolar dispersions of SPM/Ch/PA-d_{31} (squares) and Cer/Ch/PA-d_{31} (circles) at pH 5.2 and T = 50 °C. See the text for an explanation of the reduced C-2 splitting.

the source of this kink, we measured the temperature dependence of the quadrupolar splittings for all resolved peaks in the dePaked spectra of SPM/Ch/PA-d_{31}, pH 5.2, dispersions, which are fluid bilayers over the range 20–75 °C (see Figure 1). The separation of one pair of peaks (apart from the narrow methyl splitting) had a very small temperature dependence. This resonance was assigned to the C-2 deuterons using comparisons with published data in other membrane systems. For instance, in *sn*-2 chain deuterated dimyristoylphosphatidylcholine and dimyristoylphosphatidylethanolamine (Oldfield *et al.*, 1978; Marsh *et al.*, 1983), a nearly constant quadrupolar splitting as a function of temperature has been observed for the C-2 methylene due to this molecular segment having a different time-averaged orientation with respect to the bilayer normal than do methylenes more distant from the glycerol backbone. As well, deuterated decanol in dipalmitoylphosphatidylcholine (DPPC) bilayers has a smaller order parameter for C-2 than for the "plateau" (Thewalt *et al.*, 1986). This feature was subsequently confirmed in our sphingolipid mixture using selectively deuterated (at C-2) palmitic acid (D. Fenske, J. Thewalt, M. Bloom, and N. Kitson, unpublished experiments). The small C-2 order parameter was a constant feature of all fluid phase line shapes for PA-d_{31} in sphingolipid dispersions, and this result is being studied in more detail using oriented samples.

Figure 5 compares the order parameter profiles at 50 °C of SPM/Ch/PA-d_{31} and Cer/Ch/PA-d_{31} at pH 5.2, using the smoothed order parameter program to determine the quadrupolar splittings of the doublets and then reassigning the C-2 resonance, assuming that the remaining methylenes have monotonically decreasing order parameters. The two mixtures have remarkably similar order parameter profiles (their average values of S_{CD} differ by only 8%) considering the disparities between them at other temperatures. This implies that the Cer/Ch/PA-d_{31} dispersion forms bilayers at 50 °C of similar thickness to the SPM/Ch/PA-d_{31} bilayers (Seelig & Seelig, 1980).

In all the Cer/Ch/PA-d_{31} 1:1:1 mixtures studied, at room temperature there were clearly spectral components present that could not be attributed to the solid palmitic acid phase. To simplify analysis of these complex spectra, we first made simpler two-component samples consisting of Cer/PA-d_{31} 2:1 (mol/mol) at pH 6.2 and 7.4, in order to eliminate the effect of cholesterol. The results are shown in Figure 6. At pH 7.4 (Figure 6a), the signal progressed from a gel-like spectrum at

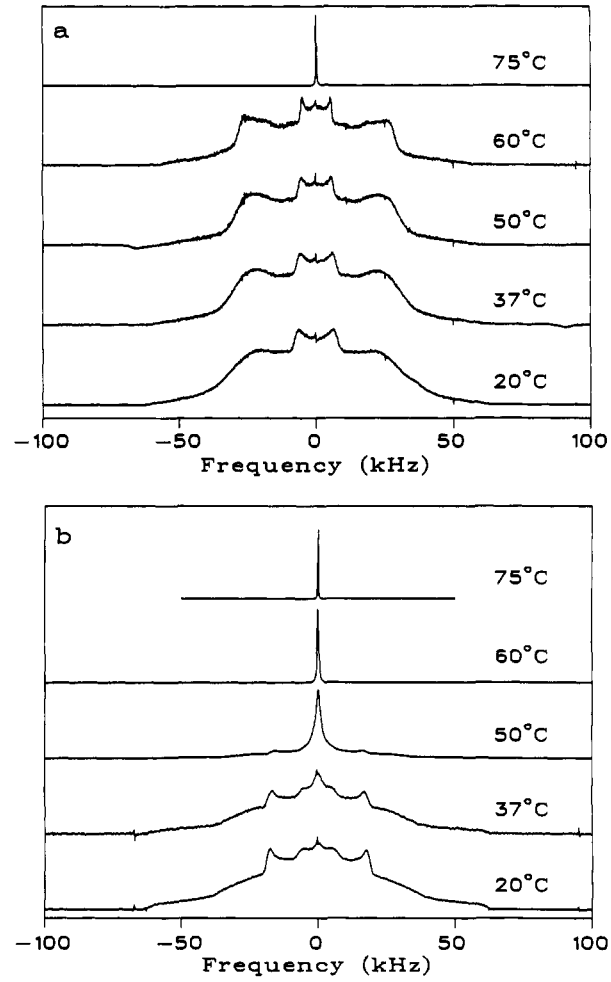

FIGURE 6: Temperature dependences of ^2H NMR spectra of Cer/PA-d_{31} (2:1 mol/mol) dispersions at (a) pH 7.4 and (b) pH 6.2. Repetition time, 0.3 s.

20 °C to an isotropic peak at 75 °C. The 20 °C spectrum is not absolutely typical of gel-phase bilayers in that there are prominent, very broad "edges" in the spectrum at about ±25 kHz—these become sharper as the temperature is increased to 60 °C. The quadrupolar splitting of the central doublet at 20 °C is, however, consistent with that found for the terminal methyl groups of gel phase bilayers (approximately 12 kHz). At 60 °C, this central doublet is too sharp for a gel phase lipid bilayer but has a splitting much too wide (approximately 10 kHz) to be consistent with those derived from even a very ordered fluid phase (compare, for instance, the 60 °C spectrum of SPM/Ch/PA-d_{31} shown in Figure 1, whose central doublet has a splitting of approximately 6 kHz). One possible explanation for this observation is that the hydrocarbon chains may be interdigitated to some extent in the Cer/PA-d_{31} membranes. At no temperature studied were the spectra compatible with a *lo* lamellar phase. This contrasts sharply with the results in Figures 1 and 3 in which a primarily *lo* spectrum is always present at one or more temperatures, and we conclude that cholesterol is essential in forming a fluid phase in these unusual three-component systems containing ceramides. There is no evidence of a solid component in the Cer/PA-d_{31} 2:1 dispersion at pH 7.4, since no Pake doublet in the vicinity of ±18 kHz is visible even at 20 °C. In contrast, the 20 °C spectrum derived from Cer/PA-d_{31} 2:1 prepared at pH 6.2 (Figure 6b) contains clear evidence that more than one phase was experienced by the probe. There is a significant solid component (indicated by the doublet with a frequency separation of approximately 36 kHz) and also a gel-like

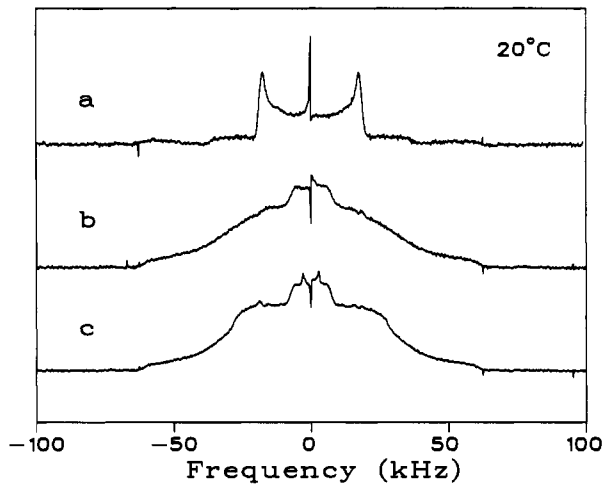

FIGURE 7: (a) PA-d_{31} dispersed in buffer at pH 6.2. (b) The 20 °C spectrum in Figure 6b of Cer/PA-d_{31} (2:1) minus the contributions to the spectrum from solidlike PA-d_{31} shown in spectrum a. (c) The 20 °C spectrum of Cer/Chol/PA-d_{31} (1:1:1) at pH 6.2 (which is very similar to its pH 5.2 spectrum shown in Figure 3b) minus the contributions from solidlike PA-d_{31} shown in spectrum a.

spectrum. Lengthening the repetition time resulted in growth of the solid component, similar to the behavior shown for Cer/Ch/PA-d_{31} (1:1:1) in Figure 2 (results not shown). With increasing temperature, there was progression to an isotropic peak (with no intervening *lo* phase), but the temperature of this transition was reduced in comparison to that at pH 7.4. Thus, the coexistence of different phases at room temperature was dependent on pH, and the most pronounced effect of removing cholesterol from the samples appeared to be the elimination of the *lo* phase.

With these results in hand, we used subtraction procedures to remove identifiable elements of the more complex spectra observed for Cer/Ch/PA-d_{31} (1:1:1 mol/mol) and Cer/PA-d_{31} (2:1 mol/mol) at 20 °C (pH 6.2). The results are shown in Figure 7. For these experiments, we were primarily interested in the spectral shape of the residual fluid phase component, and thus a short repetitition time (300 ms) was used in all cases, resulting in a large increase in the signal-to-noise ratio compared with Figure 3. When PA-d_{31} was dispersed in buffer at pH 6.2, the spectrum obtained (Figure 7a) was typical of a "solid" or crystalline sample, and did not differ from that obtained using the anhydrous powder (not shown). In such a spectrum, the terminal methyl groups are relatively free to rotate and give rise to a Pake doublet having a quadrupolar splitting of about 36 kHz, and the remaining methylene components (in a perdeuterated sample) give rise to a broader doublet that has "edges" at ±63 kHz and "shoulders" at ±126 kHz. After subtraction of this spectrum from that obtained under the same conditions for Cer/PA-d_{31} (2:1 mol/mol), the resulting spectrum (Figure 7b) had features typical of a gel phase as described above, specifically, a central broad doublet having a splitting of about 12 kHz superimposed on a broad featureless mass of methylene resonances that has shoulders at ±63 kHz. Thus, there is evidence that for the two-component mixture of ceramide and palmitic acid, two phases exist at room temperature, one a solid and the other a gel. (The presence of other phases cannot be completely excluded, however, since the probe is attached to only one of the components.) When the crystalline spectrum associated with solid PA-d_{31} was subtracted from the more complex three-component mixture (Cer/Ch/PA-d_{31} at 20 °C), the resulting spectrum had features of more than one phase (Figure 7c). In addition to features of the gel phase as found in Figure 7b, there was an additional Pake doublet at ±3 kHz and "edges" at about ±28 kHz which may be associated with an L_α or *lo* component. A more definitive interpretation is not possible without analogous experiments having the probe on the ceramide or cholesterol components. Although the behavior of the ceramide and cholesterol components has not been observed directly, we conclude that the ^{2}H NMR spectral features that do not represent a solid must be representative of lipid phases in which ceramide and cholesterol participate, since they are not observed for palmitic acid alone. Similarly, the extent to which ceramide and cholesterol may participate in a solid phase cannot yet be determined quantitatively. However, our previous finding (Thewalt *et al.*, 1992a) indicated on the basis of a comparison between ^{1}H NMR and ^{2}H NMR that the palmitic acid probe is a reasonably good reporter of the lipid ensemble.

DISCUSSION

Although ceramides form the basis for common membrane sphingolipids such as sphingomyelin, they have so far been found rarely in nature. The SC intercellular membranes have an unprecedented lipid composition for a mammalian cellular membrane, and there is considerable evidence that the ceramide and free fatty acid component lipids are produced during epidermal differentiation by enzymatic modification of more typical membrane lipids such as sphingomyelin (Freinkel & Traczyk, 1985), β-glucosylceramide (Chang *et al.*, 1991), and phosphoglycerolipid (Bowser & Gray, 1978). This suggests to us that the more differentiated membrane containing ceramide, cholesterol, and free fatty acid has properties more suited to its role as a permeability barrier than are other mammalian membranes, and our working hypothesis is that lipid composition, phase behavior, and biological function are intimately related.

Hydrogen ion concentration is of interest because the pH in stratum corneum is unlikely to be in the usual "physiological" range in the vicinity of 7.4. There are in fact no direct measurements of pH in SC intercellular spaces, but surface measurements of various types have consistently shown a value to be less than 6, and usually in the range 4.5–5.5 (Behrendt & Green, 1971). Hydrogen ion concentration is known to affect the phase behavior of charged lipids, and this has been rationalized as an effect on molecular "shape" (Gruner *et al.*, 1985). In our experiments, variation in pH over the range 5.2–7.4 also affects the phase behavior of dispersions containing ceramide and palmitic acid, and we attribute this to the extent of protonation of the palmitic acid carboxyl group, since this is the only ionizable headgroup present. In contrast, the dispersions of SPM, cholesterol, and palmitic acid were virtually insensitive to these pH changes, and this may in turn be attributed to the dominant effect of the phosphorylcholine headgroup present in sphingomyelin as has been previously observed in the "stabilizing" effect of sphingomyelin on membrane bilayers (Cullis & Hope, 1980).

It is interesting to compare the pH effects in three temperature regions: well below 50 °C, near 50 °C, and above 50 °C. Near room temperature, one major distinction among samples is the extent of "solid" phase reported by the PA-d_{31} probe: this is less for pH 7.4 than for the lower pH values of 6.2 and 5.2 which are for practical purposes indistinguishable. It has previously been observed that the apparent pK_a for free fatty acids in a phospholipid membrane may be above 7 and the ionization state of charge lipids can certainly influence their molecular packing properties, an obvious example being an increase in the gel-to-liquid-crystalline phase transition

temperature with decreasing ionization of charged lipids (Tocanne & Teissié, 1990). Our evidence is consistent with these observations in that at the lower pH values, more of the PA-d_{31} probe is in the "solid" phase, and the transition to the fluid lamellar phase occurs at higher temperatures. The details of this transition remain to be determined, but it is clear that in these systems the temperature range over which a solid lipid phase is found is influenced by the pH of the hydrating medium.

A fluid lamellar signal is found at 50 °C in all dispersions. As shown in Figure 5, the "smoothed" order parameter profiles of SPM/Ch/PA-d_{31} and Cer/Ch/PA-d_{31} are very similar at pH 5.2. However, the pH has an obvious effect on the temperature range over which a fluid lamellar signal is observed, as determined by the temperature of transition to nonbilayer phases. As can be appreciated from Figures 3 and 4, a transition to a nonbilayer phase represented by an isotropic line begins just above 50 °C at pH 5.2, whereas a single fluid lamellar phase is still present at 67 °C at pH 7.4, and the transition beginning at 68 °C is to a spectrum compatible with the H_{II} phase. Thus, the fluid lamellar phase appears to be favored at higher pH values, whereas in more acidic environments this phase is found in a much narrower temperature range, with a solid phase predominating at lower temperatures, and nonbilayer phases at higher temperatures. We conclude that the pH of the hydrating medium is an important consideration in a study of ceramide dispersions containing free fatty acid, and suggest that tissue hydrogen ion concentration may also influence lipid packing of SC intercellular membranes *in vivo*. It follows that other factors known to affect phase behavior such as water and divalent cation concentrations (Gruner *et al.*, 1985) may also influence the organization of these ceramide dispersions. Abraham and Downing (1992) found that the state of hydration did indeed alter ceramide phase behavior in their models, although such differences have been minimal in tissue measurements (Golden *et al.*, 1987). Given this evidence, and the relatively small headgroups associated with Cer/Ch/PA system, we suspect that these lipids are fully hydrated at low water concentrations. Our studies have been conducted in excess water, and therefore our model systems are almost certainly fully hydrated. The influence of water on SC lipid phase behavior *in vivo* is unknown but of considerable interest.

The effect of cholesterol in the ceramide dispersions is unusual in comparison with effects reported with other membrane lipids. First, addition of cholesterol to ceramide dispersions to approximately 33 mol % does not at room temperature result in fluid membranes as is the case for sphingomyelin, and solid signals are observed both by ^{2}H NMR and by ^{1}H NMR (Thewalt *et al.*, 1992a). On the other hand, characteristic fluid lamellar line shapes are not observed unless cholesterol is present. In dispersions of Cer/PA-d_{31} (2:1 mol/mol), a typical fluid lamellar line shape is not found (Figure 6); at lower temperatures, the signals are compatible with gel or coexisting gel and solid phases which with increasing temperature undergo transitions to nonbilayer phases represented by isotropic lines. It is of interest that a pH effect is again evident: a solid phase is obviously present at pH 6.2, and the transition to the isotropic phase occurs at a lower temperature than for the sample at pH 7.4. As with dispersions containing cholesterol, a more acidic hydrating medium appears to promote the development of both a solid phase at lower temperatures and a nonbilayer phase at higher temperatures. Inclusion of cholesterol changes the nature of phase coexistence at the lower temperatures, and as shown in the subtraction experiments as well as in Figure 2, there is evidence for the presence in these systems of a fluid lamellar phase that is absent in samples not containing cholesterol. The behavior of the bulk of cholesterol in ceramide dispersions that are solid (e.g., Figure 3) is not known, but in preliminary experiments using deuterium-labeled cholesterol, we have found a large proportion to be "solid" also under the same conditions (D. Fenske, J. Thewalt, M. Bloom, and N. Kitson, unpublished experiments).

The nature of the solid phase in the Cer/Ch/PA-d_{31} dispersions is of considerable interest since there is evidence for the existence of "crystalline" lipids in SC *in vivo* (White *et al.*, 1988; Bouwstra *et al.*, 1992). Several lines of evidence suggest that the motion in the solid phase we observe is more inhibited than in a conventional phospholipid or sphingolipid gel phase. First, as discussed in detail above, the ^{2}H NMR spectrum is typical of an anhydrous powder, rather than a gel (although a coexisting gel-like spectrum is observed both with and without cholesterol at the lower pH values). Second, comparison of T_{1z} by stimulated echo and saturation recovery methods indicates that there is no measurable lateral diffusion of the probe in this phase, whereas in a gel phase such diffusion is at most 10^{-11} cm^2/s (MacKay, 1981), and in a fluid lamellar phase, 10^{-8} cm^2/s (Houslay & Stanley, 1982). Small (1984) has described three classes of hydrocarbon chain packing: (1) a very tight or "specific" chain–chain interaction which packs in a rectangular lattice and in which motion is very restricted; (2) a "nonspecific" interaction which packs as a hexagonal or "near-hexagonal" lattice and in which motion is partially restricted; and (3) a liquid interaction which packs hexagonally and in which axial rotation and trans–gauche isomerization readily occur. In this scheme, our solid phase would correspond to "specific" chain–chain interactions, and a rectangular packing arrangement would be predicted. X-ray diffraction experiments are in progress to examine chain packing in our ceramide dispersions, but the interesting possibility arises that ceramides can pack tightly with palmitic acid in a nonhexagonal lattice, and that such packing may also be possible with cholesterol. If so, the lipid modifications known to occur in mammalian epidermis might well have the effect of converting fluid precursor membranes to membranes which when established in SC intercellular spaces contain lipids including cholesterol that are packed very tightly and in which lateral diffusion and rotational diffusion are orders of magnitude less than in the vast majority of conventional membranes. Such an arrangement would to our knowledge be unprecedented in mammalian membranes, and would have profound consequences for the physical properties of such structures, permeability being an obvious example.

In the published work most similar to ours, Abraham and Downing (1991) used ^{2}H NMR to examine ceramide phase behavior. Direct comparison of results is difficult due to differences in experimental detail, e.g., biological source of ceramide, lipid composition of samples, nature of the dispersing medium, nature of the deuterated probe, and details of spectrum acquisition. The use in their study of epidermal ceramide (as opposed to bovine brain ceramide) is a clear advantage in creating models of SC lipid organization, but despite this and other differences, we believe that the similarities in results are striking. First, they report a bilayer to H_{II} transition above 60 °C for their dispersion of ceramide/Ch/PA-d_2/cholesterol sulfate (CS) 38:38:19:5 (mol/mol), that we find for our Cer/Ch/PA-d_{31} (1:1:1 mol/mol) dispersion beginning about 68 °C at pH 7.4. Second, a transition to a phase represented by an isotropic peak is observed in their

ceramide sample that did not contain cholesterol, and such a transition is observed also in our samples (Cer/PA-d_{31} 2:1 mol/mol) at pH 7.4 and 6.2. Third, phase coexistence is observed in their ceramide/PA-d_3/CS 76:19:5 (mol/mol), particularly at low water content (Abraham & Downing, 1992). An important difference is the absence in their observations of a "solid" phase such as we observe, but differences in experimental protocol may account for this.

Other studies of ceramide phase behavior are even less directly comparable, but confirm that it is complex. Addition of cholesterol in increasing amounts to bovine brain hydroxyceramide dispersions was shown not to produce the flattening and broadening of the main endothermic transition as is characteristic of other membrane phosphoglycero- and sphingolipids, but results in multiple smaller peaks suggesting phase coexistence (Wiedmann & Salmon, 1991). Similarly, White *et al.* (1988) found that dispersion of extracted SC lipids in water resulted in phase coexistence over a wide temperature range, and between 20 and 45 °C, these included a crystalline phase and a fluid phase. These dispersions could not be indexed as a lamellar phase, however. Finally, Löfgren and Pascher (1977) found, in monolayer studies of semisynthetic ceramides, that both the nature of hydrocarbon chains and the ceramide headgroup hydroxyls influenced the ability of these lipids to pack as crystals. Whether cholesterol can pack in crystals with ceramides remains to be determined.

Comparison of our results to those derived from tissue is of interest. The literature cannot be comprehensively summarized here, but some findings have been consistent and are similar to those reported in our model studies. First, DSC studies of stratum corneum from various mammalian species have shown reversible endothermic transitions in the region of 30–75 °C that can be attributed to lipid in the intercellular domains (Knutson *et al.*, 1985). The existence of such transitions implies that the intercellular lipids are not organized as homogeneous fluid bilayers and that more rigid domains are present. Although such domains may well be gel phase bilayers, X-ray diffraction studies of mammalian SC have provided evidence also for crystalline lipids [see Bouwstra *et al.* (1992) and references cited therein]. These findings correspond to our observation of "solid" lipid signals, and it is of interest that in a study of human SC Bouwstra *et al.* (1991) reported a lipid transition at approximately 40 °C. The authors suggest that this may correspond to an orthorhombic-to-hexagonal phase transition, which would be consistent with the transition between "solid" and fluid lamellar phases that we observe beginning at 42 °C at pH <7. Although such correspondence may be fortuitous, it illustrates that the behavior of a highly simplified system such as ours may not be very different from that observed *in vivo*, and that the major features of lipid organization may be attributed to the classes of lipid present. Thus, the possibility exists that lipid phases observed in SC *in vivo* are determined to a considerable extent by the lipid modifications known to occur during epidermal differentiation.

An issue to be resolved is the ability of ceramide dispersions to form lamellar structures at all. The very small hydrophilic headgroups associated with the Cer/Ch/PA membranes make the prediction of lipid bilayers on the basis of the "shape hypothesis" (Israelachvili *et al.*, 1980) somewhat tenuous. However, Abraham and Downing (1992) found by thin-section electron microscopy that lamellar structures are indeed formed, and our results, particularly at 50 °C, show that the sphingomyelin dispersions, which are undoubtedly bilayers, give rise to spectra and ^{2}H NMR order parameter profiles that are very similar to those found in analogous ceramide dispersions. Further studies on our systems by means of ^{2}H NMR of oriented samples and X-ray diffraction are in progress.

CONCLUSION

Our results demonstrate that ceramide phase behavior differs from that of more conventional membrane sphingolipids and phosphoglycerolipids in several ways. First, in combination with cholesterol and fatty acids, phase coexistence is common over the temperature range 20–75 °C, and complex polymorphism is observed. Second, a "solid" phase is present at $T < 42$ °C in which lipid motion is extremely inhibited, and preliminary evidence suggests that this phase may include cholesterol. Taken together with published studies from SC, these findings support the hypothesis that the SC intercellular membranes contain domains of crystalline lipids, an organization unprecedented in mammalian membranes. Such crystallization may in turn have implications for the organization of an associated aqueous phase, as has been shown to be the case for DPPC (Ruocco & Shipley, 1982). Since the intercellular membranes of SC have been shown to provide most of the barrier to diffusion of various molecular species across the skin, the existence within these membranes of significant crystalline lipid domains would be expected to have a considerable effect on the nature of such diffusion, and therefore on the barrier properties of the SC. A similar proposal has been made recently by Forslind (1994).

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