Models of Stratum Corneum Intercellular Membranes: ^2H NMR of Macroscopically Oriented Multilayers

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ABSTRACT Deuterium NMR was used to characterize model membrane systems approximating the composition of the intercellular lipid lamellae of mammalian stratum corneum (SC). The SC models, equimolar mixtures of ceramide:cholesterol: palmitic acid (CER:CHOL:PA) at pH 5.2, were contrasted with the sphingomyelin:CHOL:PA (SPM:CHOL:PA) system, where the SPM differs from the CER only in the presence of a phosphocholine headgroup. The lipids were prepared both as oriented samples and as multilamellar dispersions, and contained either perdeuterated palmitic acid (PA-d_{31}) or [2,2,3,4,6-$^2H_5$]CHOL (CHOL-d_5). SPM:CHOL:PA-d_{31} formed liquid-ordered membranes over a wide range of temperatures, with a maximum order parameter of approximately 0.4 at 50°C for positions C3–C10 (the plateau region). The quadrupolar splitting at C2 was significantly smaller, suggesting an orientational change at this position, possibly because of hydrogen bonding with water and/or other surface components. A comparison of the longitudinal relaxation times obtained at $\theta = 0°$ and 90° (where θ is the angle between the normal to the glass plates and the magnetic field) revealed a significant T_{1Z} anisotropy for all positions. In contrast to the behavior observed with the SPM system, lipid mixtures containing CER exhibited a complex polymorphism. Between 20 and 50°C, a significant portion of the entire membrane (as monitored by both PA-d_{31} and CHOL-d_5) was found to exist as a solid phase, with the remainder either a gel or liquid-ordered phase. The proportion of solid decreased as the temperature was increased and disappeared entirely above 50°C. Between 50 and 70°C, the membrane underwent a liquid-ordered to isotropic phase transition. These transitions were reversible but displayed considerable hysteresis, especially the conversion from a fluid phase to solid. The order profiles, relaxation behavior, and angular dependence of these parameters suggest strongly that both the liquid-ordered CER- and SPM-membranes are bilayers. The unusual phase behavior observed for the CER-system, particularly the observation of solid-phase lipid at physiological temperatures, may provide insight into the functioning of the permeability barrier of stratum corneum.

INTRODUCTION

The permeability barrier of mammalian skin is located within the stratum corneum, the uppermost layer of the epidermis. The stratum corneum is a layered arrangement of terminally differentiated keratinocytes surrounded by intercellular sheets of lipid lamellae (Elias et al., 1983; White et al., 1988). The passage of water and other solutes is mediated by the lipid lamellae. Although the composition of the lamellae is complex and varies between species, the predominant lipids are ceramide, cholesterol, and free fatty acid, which are present in roughly equimolar proportions (Yardley and Summerly, 1981; Yardley, 1983; Elias et al., 1985; Wertz et al., 1987). Triglycerides, cholesteryl esters, and cholesterol sulfate are present at low levels, but the former two lipids have low membrane solubilities (<5 mol%) and are likely located in extramembrane inclusions. There is good evidence that these unusual intercellular membranes are formed by exocytosis of stacked membranes contained within lamellar bodies, intracellular organelles formed by differentiating keratinocytes, and that during this process considerable enzymatic modification of membrane lipid occurs (Grayson et al., 1985; Freinkel and Traczyk, 1985). Of particular interest are the conversions of sphingomyelin and monoglucosylceramides to ceramide by sphingomyelinase and β-glycosidases, respectively (Freinkel and Traczyk, 1985; Chang et al., 1991), and the conversion of phosphoglycerolipid to free fatty acid by phospholipases (Bowser and Gray, 1978).

The lipid modifications that occur during the formation of mature stratum corneum membranes may have relevance to the functioning and maintenance of the permeability barrier. Investigation of the physical properties of stratum corneum intercellular membranes is relatively recent, and stimulated by the evidence that these membranes act as the skin's primary permeability barrier to water (Golden et al., 1987; Potts and Francoeur, 1991). Because most biological membranes are quite permeable to water (Deamer and Bramhall, 1986; Fettiplace and Haydon, 1980), the question arises as to how such barrier requirements are fulfilled. In this context, it is of interest that thermally reversible lipid phase transitions

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Abbreviations used: CS, cholesterol sulfate; CER, bovine brain ceramide (Type III); CHOL, cholesterol; CHOL-d_5, [2,2,3,4,6-$^2H_5$]cholesterol; DSC, differential scanning calorimetry; ESR, electron spin resonance spectroscopy; H_{II}, hexagonal H_{II} phase; IR, infrared spectroscopy; MLV, multilamellar vesicle; OPP, order parameter profile; PA-d_{31}, [$^2H_{31}$]palmitic acid; PA-d_2, [2,2-$^2H_2$]palmitic acid; PEG, polyethylene glycol M_r 8000; SC, stratum corneum; SPM, bovine brain sphingomyelin; T_{1Z}, spin-lattice relaxation time.

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have been observed in the vicinity of 25–75°C in several species of mammalian skin by various methods such as DSC (Rehfeld and Elias, 1982; Golden et al., 1986; Rehfeld et al., 1988), IR (Knutson et al., 1985), ESR (Rehfeld et al., 1988), and x-ray diffraction (White et al., 1988; Bouwstra et al., 1991, 1992). The implication must be that stratum corneum lipids do not exist in a single phase. Furthermore, the x-ray diffraction results reveal a lamellar arrangement for the lipids, and the presence of both crystalline and liquid lipid at physiological temperatures (White et al., 1988; Garson et al., 1991). Many questions remain regarding the types of phases formed by the lipid and whether the presence of proteins and cellular material affects these preferences. To understand better the behavior of the stratum corneum intercellular membranes, we have utilized magnetic resonance techniques, particularly ^{2}H NMR, to study model systems that approximate the in vivo lipid compositions. ^{2}H NMR is a powerful technique for the study of membrane structure and dynamics (Seelig, 1977; Seelig and Seelig, 1980; Bloom et al., 1991), and is particularly suited to the study of complex lipid mixtures, because different components of the system can be studied separately. To model the stratum corneum membranes, we have chosen an equimolar mixture of bovine brain ceramide, cholesterol, and palmitic acid, with deuterium labels present on the latter two components. The ceramide samples were compared with analogous models in which bovine brain sphingomyelin was substituted for ceramide. Because the ceramide is prepared from the same source as the sphingomyelin, the major difference in our models is the absence of the phosphocholine headgroup in the CER-containing samples. Using multilamellar dispersions, we have previously shown that SPM:CHOL:PA-d_{31} forms liquid-ordered membranes over a wide range of temperature and pH, whereas CER:CHOL:PA-d_{31} displays a complex polymorphism, with both solid palmitic acid and liquid-ordered membranes coexisting in the region of physiological temperatures and pH, and an isotropic phase appearing at higher temperatures (Thewalt et al., 1992; Kitson et al., 1994). The results from ^{1}H NMR suggest that the ceramide also participates in the polymorphism. In the present study, we demonstrate that both the SPM- and CER-containing systems can be studied as oriented samples and that important additional information can be obtained in this manner. Furthermore, we observe that CHOL displays the same polymorphic behavior as the PA, forming solid, liquid-ordered, and isotropic phases over the same temperature range. The complex behavior exhibited by CER:CHOL:PA membranes may provide insight into the functioning of the permeability barrier.

MATERIALS AND METHODS

Bovine brain ceramide (Type III) and sphingomyelin were obtained from Sigma Chemical Co. (St. Louis, MO). The acyl chain populations are roughly similar and contain substantial quantities of stearic (18:0), nervonic (24:1), and lignoceric (24:0) amide-linked fatty acids. [2,2-^{2}H$_2$]palmitic acid was obtained from MSD Isotopes (Montreal, Canada). Perdeuterated palmitic acid (PA-d_{31}) was prepared by the method of Hsiao et al. (1974). CHOL-d_5 was a generous gift from Dr. Harold C. Jarrell, Institute of Biological Sciences, National Research Council of Canada. Deuterium-depleted water was obtained from Sigma Chemical Co. (St. Louis, MO).

For the preparation of multilamellar dispersions (MLVs), solutions of lipids were first freeze-dried from benzene:methanol 7:3 (v/v) and then dispersed at 70°C in 100 mM citrate, 150 mM NaCl, 4 mM EDTA, pH 5.2. Oriented samples were prepared essentially as described by Jarrell et al. (1987) using method B. Approximately 40 glass slides, with dimensions varying from 3 × 15 mm to 8 × 15 mm, were cut from microscope cover slides. The plates were stacked in a 10 mm (o.d.) open-ended NMR tube and rinsed several times with methanol, which was removed under high vacuum. The plates were then removed from the NMR tube. Between 40 and 90 mg of a given lipid mixture, dissolved in $CHCl_3$, was evenly applied to the plates, allowing the solvent to dry between applications. The plates were restacked in the NMR tube, and traces of $CHCl_3$ were removed under high vacuum overnight. Hydration of the lipid was accomplished by placing the tube in a sealed 25 ml liquid scintillation vial containing 2 ml of 100 mM HEPES, pH 5.2, prepared in deuterium-depleted water, at 50–65°C for 3–4 days. For CER-containing samples, it was found that better orientation was achieved at 65°C. For some SPM-containing samples, 1–3 tiny drops of buffer were gently placed on the edges of the glass plates before sealing the NMR tube. For some SPM- and all CER-containing samples, it was desirable to control the pH and maintain full hydration on the glass plates. This was accomplished as described by Morrison (1993). After hydration of the lipid, an aliquot (0.3–0.5 ml) of preheated 100 mM citrate, 150 mM NaCl, 4 mM EDTA, pH 5.2, containing 5 wt% PEG (M_r 8000) was carefully pipetted into the NMR tube, directly onto the glass plates, and the sample was incubated at 50°C for at least 2 h before acquiring any spectra. This procedure did not affect the quality of lipid orientation on the plates.

^{2}H NMR spectra were acquired at 46 MHz on a home-built spectrometer using the quadrupolar echo pulse sequence with quadrature detection and phase cycling of all pulses (Sternin, 1985). The ^{2}H NMR $\pi/2$ pulse length was 4.0 μs (10 mm solenoid coil), the interpulse spacing τ was 40–50 μs, and the recycle time varied between 100 ms and 40 s. Spectra were acquired with a dwell-time of 2 μs, and between 1,000 and 400,000 transients were collected for signal averaging. Partially recovered (T_{1Z}) ^{2}H NMR spectra were acquired using the inversion recovery sequence 180_x-τ-90_x-t-90_y-t-ACQ. A modified acquisition sequence was used in which the partially recovered spectra were subtracted from the completely recovered spectrum, so that the spectral intensity decays from a maximum value for short τ to zero intensity for $\tau \gg T_1$. Rotation of the sample in the magnetic field was accomplished using a home-built goniometer with an accuracy of ±1°.

The T_{1Z} values of solid anhydrous PA-d_{31} and CHOL-d_5 are on the order of 12 and 4 s, respectively (J. Thewalt, unpublished observations; Monck et al., 1992; Kitson et al., 1994). The same lipids in gel, liquid-ordered, or liquid-crystalline membranes have T_{1Z} values ranging from 10 to 60 ms. Thus, in a system containing solid lipid, the echo height will vary as a function of the recycle delay (for the same number of acquisitions). This provides a method for quantifying the proportion of solid lipid at any given temperature. A spectrum is first acquired with a recycle delay sufficiently long (100–300 ms) to allow complete relaxation of lipid in the gel or fluid phases, but too short to allow detection of any solid phase lipid. The ratio of echo height/number of scans (EH/ns) for this spectrum is EH_{short} or EH_s. A spectrum is then acquired with a recycle delay sufficiently long (20–50 s) to observe all of the lipid, both that which is fast-relaxing (gel and fluid phases) and that present in the solid phase. EH/ns for this spectrum is EH_{long} or EH_l. Because the long T_{1Z} is on the order of 12 s for PA-d_{31}, spectra acquired with recycle delays of 40 and 50 s have essentially the same value of EH_l, but with recycle delays of 20 s, the observed ratio needs to be multiplied by 1.1 to give the correct EH_l. The fraction of solid phase lipid is then given by the formula % solid = $100C[(EH_l - EH_s)/EH_l]$, where C, which is 31/28 for PA-d_{31} and unity for CHOL-d_5, is a factor to correct for the intensity of solid PA-d_{31} methyl groups that are observed in the 300-ms spectra (because of the rapid rotation of the methyl rotors, they have short relaxation times even in the solid phase).

The quantity of PA-d_{31} or CHOL-d_5 present in the isotropic phase at temperatures of 40°C and above was determined from the FID. In these spectra, the fast decaying broad-line echo rides on top of a slow decaying

component originating from the isotropic phase lipid. A linear extrapolation of this slow-decaying line (in the region near the echo), back to the time corresponding to the peak of the echo (25 μs), is performed. The intensity of the extrapolated line, at the echo peak, is proportional to the quantity of labeled lipid in the isotropic phase, whereas the total echo height is proportional to the quantity of total labeled lipid. For some PA-d_{31} spectra, complete decay of the isotropic phase was not obtained in the FID and, therefore, the echo heights were obtained without baseline-correcting the FID.

RESULTS

Sphingomyelin:cholesterol:palmitic acid

Oriented samples of SPM:CHOL:PA-d_{31} were prepared by hydration of the lipid mixture (on glass plates) in a humid atmosphere at 50–55°C for 3 days, as described in Materials and Methods. ^{2}H NMR spectra acquired at 40°C for three values of the rotation angle θ (0°, 55°, and 90°) are shown in Fig. 1. Essentially complete orientation was achieved, because the presence of unoriented lipid would result in a powder pattern lineshape, which would be easily observed at $\theta = 0$ and 55°. The quadrupolar splittings scale with a $(3\cos^2\theta - 1)/2$ dependence, with maximum values for $\theta = 0°$ (*A*), a collapse of all splittings near the magic angle (*B*), and half-maximal values for $\theta = 90°$ (*C*). This is further illustrated in Fig. 2, which shows values of $\Delta\nu_Q$ obtained from rotation of an oriented sample of SPM:CHOL:[2,2-$^2H_2$]PA between $\theta = 0°$ and 90°. An excellent fit to the data points is given by the function $(3\cos^2\theta - 1)/2$ (*solid line*). This demonstrates that the axis of motional averaging is the normal to the plane of the membranes, which are aligned with the glass plates. The observed angular dependence of the quadrupolar splitting has been previously observed for phospholipids (Mayer et al., 1990) and for glycolipids deuterium-labeled in the carbohydrate headgroups (Jarrell et al., 1987), all of which adopt a bilayer morphology. The spectra are indicative of highly ordered membranes, with maximum values of the order parameter $S_{CD} = 0.4$ in the plateau region (splittings corresponding to positions 3–10 (Fig. 1 *A*). For reasons discussed in detail elsewhere (Ipsen et al., 1987; Bloom et al., 1991), we will refer to the liquid-crystalline state of the membrane having large order parameters as liquid-ordered. Briefly, the liquid-ordered phase is a phase characterized by relatively fast molecular reorientational rates, as in normal fluids, but having relatively large values of orientational order parameters for the acyl chains. Thus, the oriented SPM:CHOL:PA-d_{31} appears to adopt a liquid-ordered phase at this temperature, as is observed with multilamellar dispersions (Thewalt et al., 1992; Kitson et al., 1994).

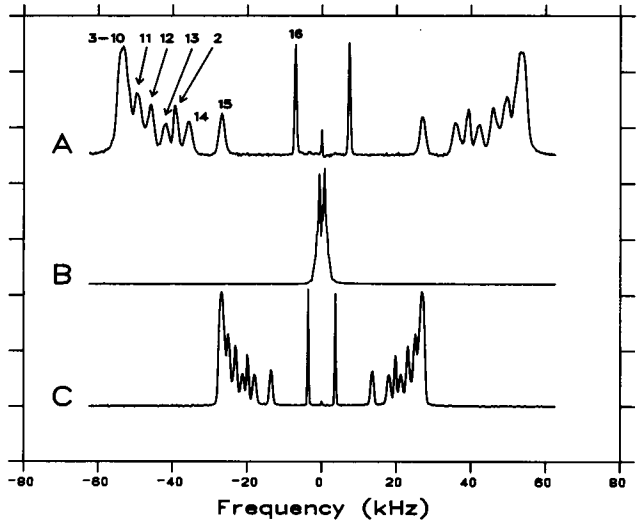

FIGURE 1 ^{2}H NMR spectra of oriented multilayers of SPM:CHOL:PA-d_{31} at 40°C as a function of the angle θ between the normal to the glass plates and the external magnetic field. $\theta = 0°$ (*A*), 55° (*B*), and 90° (*C*). The assignments given in *A* for the labeled positions of PA-d_{31} corresponding to each quadrupolar splitting were obtained by assuming a monotonic decrease in order from C3 to C16; the assignment of C2 was obtained by comparison with the $\Delta\nu_Q$ and T_{1Z} values obtained from a sample of SPM:CHOL:[2,2-$^2H_2$]PA (see Fig. 3). Number of acquisitions = 5000 (*A*), 1000 (*B*), 2000 (*C*).

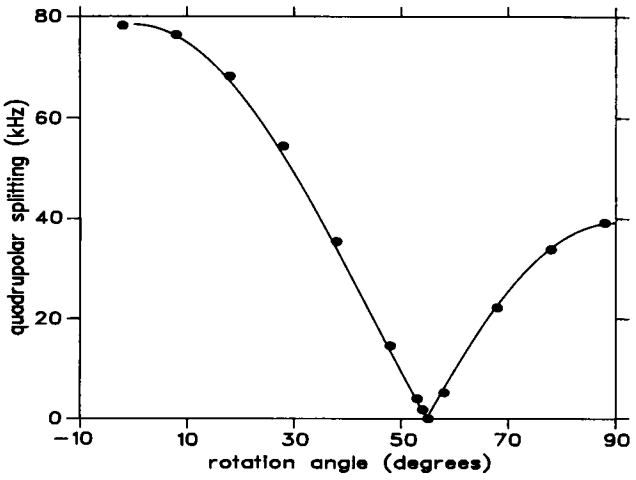

FIGURE 2 Dependence of the quadrupolar splitting $\Delta\nu_Q$ on the angle θ between the normal to the glass plates and the external magnetic field for oriented multilayers of SPM:CHOL:[2,2-$^2H_2$]PA at 40°C. The solid line is a plot of $(3\cos^2\theta - 1)/2$ normalized to $\Delta\nu_Q = 78.4$ kHz for $\theta = 0°$.

The assignments in Fig. 1 were made assuming a monotonic decrease in order from positions 3–16, as observed for POPC-d_{31} (Lafleur et al., 1989). The assignment of position 2 was made by comparison with the $\Delta\nu_Q$ and T_{1Z} values obtained from an oriented sample of SPM:CHOL:PA-d_2 (see Fig. 3 and Table 1). The greatly reduced $\Delta\nu_Q$ value observed for C2 indicates an orientational change at C2, possibly caused by ionic and/or hydrogen bonding interactions with other surface groups or water (Abdolall et al., 1977).

All positions of SPM:CHOL:PA-d_{31} display a T_{1Z} anisotropy, as shown in the partially recovered spectra acquired for $\theta = 0°$ and 90° (Fig. 3). The spectra were acquired for τ values of 1, 50, and 100 ms, where τ is the relaxation delay in the modified inversion recovery sequence (see legend to Fig. 3). The T_{1Z} values of positions 3–10 are significantly shorter for $\theta = 0°$ than for $\theta = 90°$ (Table 1). Similar results have been obtained for the fatty acyl chains of DMPC:CHOL membranes (Mayer et al., 1990).

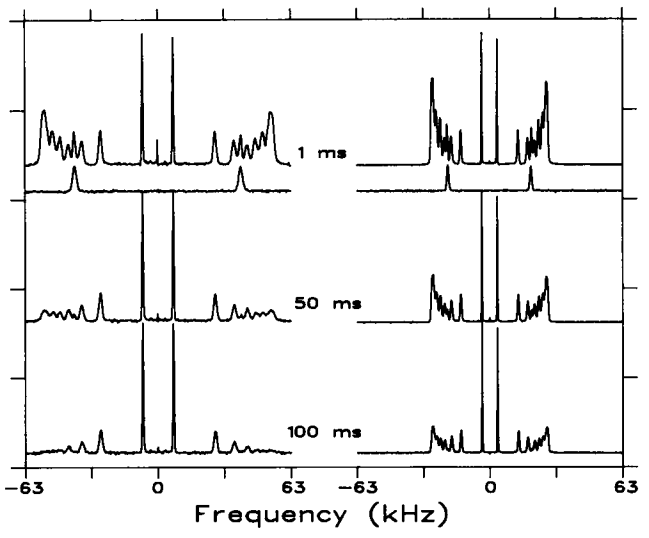

FIGURE 3 Partially recovered (T_{1Z}) ^{2}H NMR spectra for oriented multilayers of SPM:CHOL:PA-d_{31} at 40°C for rotation angles $\theta = 0°$ (*left*) and $\theta = 90°$ (*right*). The spectra were acquired for τ values of 1, 50, and 100 ms, respectively, where τ is the delay in the inversion recovery sequence: 180_x-τ-90_x-t-90_y-t-ACQ. A modified acquisition sequence was used in which the partially recovered spectra were subtracted from the completely recovered spectrum, so that the spectral intensity decays from a maximum value for short τ to zero intensity for $\tau \gg T_1$. The second spectrum from the top in each column was obtained from an oriented sample of SPM:CHOL:[2,2-$^2H_2$]PA at 40°C.

TABLE 1 Quadrupolar splittings $\Delta\nu_Q$ (kHz) and spin-lattice relaxation times T_{1z} (ms) of the methylene groups of SPM:CHOL:PA-d_{31} oriented multilayers at 40°C as a function of the rotation angle θ

	$\theta = 0°$		$\theta = 90°$	
Carbon number	$\Delta\nu_Q$	T_{1z}	$\Delta\nu_Q$	T_{1z}
2	78.8	25.0*	40.0	36.2
3–10	106.6	27.2	53.6	62.6
11	99.0	33.9	50.4	63.1
12	92.0	39.8	46.4	65.0
13	84.2	66.7	42.6	87.2
14	71.4	86.9	36.0	105.1
15	53.9	144.3	27.6	128.0

* The T_{1z} values of SPM:CHOL:[2,2-$^2H_2$]PA (40°C) and CER:CHOL:[2,2-$^2H_2$]PA (50°C), obtained for $\theta = 0°$, were 26.1 and 22.9 ms, respectively.

The oriented samples from which Figs. 1–3 were acquired were formed by hydration of the lipid, on the glass plates, in a humid atmosphere of pH 5.2 (see Materials and Methods). Whether this results in a similar pH on the plates is not known but seems unlikely. This is not crucial for the SPM-containing samples, because multilamellar dispersions form liquid-ordered phases over a wide range of temperature and pH (Thewalt et al., 1992; Kitson et al., 1994). However, the CER-containing samples (discussed below) are extremely pH-sensitive and, therefore, the pH on the oriented sample needs to be known and controlled if meaningful results are to be obtained. Recently, a technique for obtaining fully hydrated oriented samples using polymer solutions has been developed (Morrison, 1993). Essentially, 5 wt% of PEG (M_r 8000) is dissolved in the buffer of choice, and an aliquot is applied to the oriented sample after hydration and orientation of the lipids. The $\Delta\nu_Q$ and T_{1Z} values of oriented SPM:CHOL:PA-d_2, with and without 100 mM HEPES, 150 mM NaCl, 4 mM EDTA pH 5.2 containing 5 wt% PEG, were found to be essentially identical. Thus, the SPM:CHOL:PA-d_{31} system is not sensitive to the solvent pH or to the hydration levels studied.

The temperature dependence of oriented SPM:CHOL:PA-d_{31} was also investigated (data not shown). There is a slight increase in the width of the individual resonances as well as the quadrupolar splittings as the temperature is decreased from 50 to 30°C. Nevertheless, the membranes remain liquid-ordered over this temperature range. Identical behavior is observed with multilamellar dispersions (Thewalt et al., 1992; Kitson et al., 1994).

Ceramide:cholesterol:palmitic acid

In contrast to the SPM-containing systems, multilamellar dispersions composed of equimolar ratios of ceramide, cholesterol, and palmitic acid display a complex polymorphism (Thewalt et al., 1992; Kitson et al., 1994). This is summarized in Fig. 4 for multilamellar dispersions containing PA-d_{31} (Fig. 4 *A–C*) and CHOL-d_5 (Fig. 4 *D–F*). For the present, we will only discuss the PA-d_{31} samples. At 20°C, the majority of the palmitic acid (approximately 80%) is present in a "solid" phase, which gives rise to a very broad ^{2}H NMR spectrum with edges at ±63 kHz. The shoulders at ±126 kHz are difficult to observe (Fig. 4 *C*). This phase has a very long value of T_{1Z} (approximately 12 s) and, therefore, a long recycle delay (in the range of 10–50 s) is required to observe it. As the temperature is increased, the palmitic acid is incorporated into a liquid-ordered lamellar phase, a process

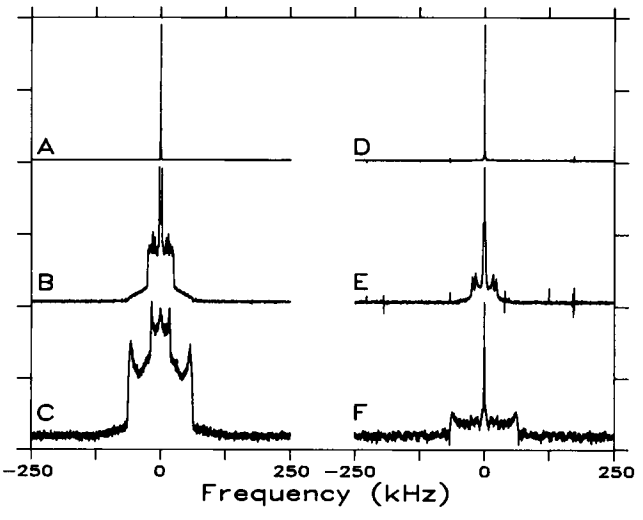

FIGURE 4 ^{2}H NMR spectra for multilamellar dispersions of CER:CHOL:PA-d_{31} (pH 5.2) at 75°C (*A*), 50°C (*B*), and 20°C (*C*), and CER:CHOL-d_5:PA (pH 5.2) at 75°C (*D*), 50°C (*E*), and 25°C (*F*). The spectra were acquired with recycle delays of 50 s (*B*, *C*, *F*), 1 s (*D*, *E*), and 300 ms (*A*).

that is essentially complete by 50°C (Fig. 4 *B*). An isotropic resonance appears at temperatures above 50°C, and by 75°C essentially all of the palmitic acid (94%, see below) has been incorporated into an isotropic (possibly cubic) phase (Fig. 4 *A*) (Lindblom and Rilfors, 1989). These transformations are completely reversible, but display considerable hysteresis, particularly in the reestablishment of the solid phase, which requires time periods on the order of a week or more.

Oriented samples of CER:CHOL:PA-d_{31} were prepared in the same manner as the SPM:CHOL:PA-d_{31}, but in all cases aliquots of PEG-containing buffer (pH 5.2) were applied to the plates to ensure full hydration and controlled pH. The first sample to be investigated was hydrated at 55°C. After addition of the PEG buffer, initial spectra were acquired at 50 and 40°C (not shown), revealing a mixture of isotropic and liquid-ordered phase lipid at both temperatures. A significant fraction of the liquid-ordered lipid was unoriented, especially at 40°C, where the proportion of unoriented to oriented membrane increased over a period of 24 h and then stabilized. The sample was maintained in the temperature range of 40–50°C for a period of 3 days, after which it was cooled to 30°C. At this temperature, over a period of several days, some of the PA-d_{31} remained in the liquid-ordered phase. Fig. 5 shows ^{2}H NMR spectra of oriented CER:CHOL:PA-d_{31}, at 30°C and pH 5.2, as a function of the rotation angle θ (compare with Fig. 1). Essentially the same number of peaks are resolved as with the SPM:CHOL:PA-d_{31} (except for an additional, unassigned large splitting observed in Fig. 5 *A*, which may have been solid phase PA-d_{31} that disappeared as the temperature was increased and was not observed again for spectra acquired with a 300 ms recycle delay). The assignment for C2 was obtained from an oriented sample of CER:CHOL:PA-d_2 (not shown) and has the same relative position as in SPM:CHOL:PA-d_{31}. The presence of a significant proportion of oriented bilayer is apparent in Fig. 5, *A* and *C*. The unoriented material is clearly seen near the magic angle (Fig. 5 *B*), where the oriented splittings have collapsed, leaving a remnant powder lineshape.

Having established that at least partial orientation of the CER:CHOL:PA-d_{31} system could be achieved, it was of interest to investigate whether the oriented samples also displayed the polymorphism observed in multilamellar dispersions. Further studies on the sample shown in Fig. 3 revealed transitions from liquid-ordered to isotropic phase and from liquid-ordered to solid phase, the latter detected by acquisition of spectra at 30°C with recycle delays of 300 ms, 10 s, and 50 s (Table 2). However, because of its variable thermal history, a second oriented sample of CER:CHOL:PA-d_{31} was prepared in which the sample was first hydrated at 65°C (Fig. 6). An initial spectrum, acquired at $\theta = 0°$ and a temperature of 50°C, indicates a well oriented system, although some unoriented material is present (Fig. 6 *A*). Upon cooling to 20°C, the proportion of oriented liquid-ordered lipid decreases (Fig. 6 *B*). Between the acquisition of the spectra in Fig. 6, *B* and *C*, some 66 h elapsed, during which time the oriented peaks broadened considerably, suggesting a transition to a gel-like state (spectra not shown). In addition, the proportion of oriented lipid decreased and a significant quantity of the PA-d_{31} appeared as a solid phase (Table 2). Rotation of the sample to 55° (Fig. 6 *C*) reveals a powder line-

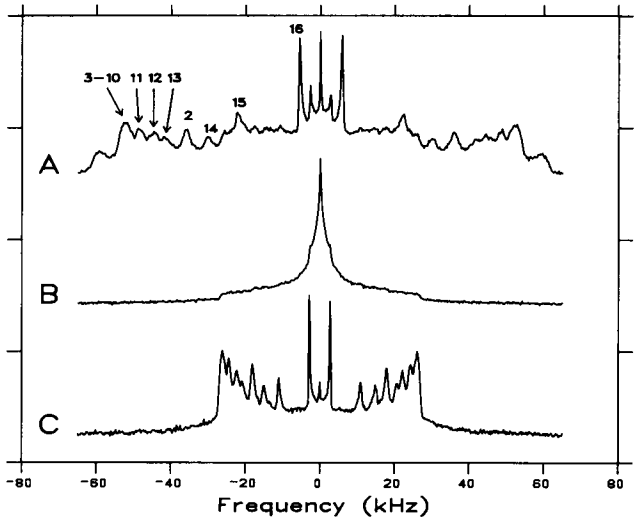

FIGURE 5 ^{2}H NMR spectra of oriented multilayers of CER:CHOL:PA-d_{31} at 30°C (pH 5.2) as a function of the angle θ between the normal to the glass plates and the external magnetic field. $\theta = 0°$ (*A*), 55° (*B*), and 90° (*C*). The pH was maintained by the addition of approximately 0.3 ml of 100 mM HEPES, 150 mM NaCl, 4 mM EDTA pH 5.2 containing 5 wt% PEG (M_r 8000) to the glass plates before data acquisition. The assignments given in *A* for the labeled positions of PA-d_{31} corresponding to each quadrupolar splitting were obtained by assuming a monotonic decrease in order from C3 to C16; the assignment of C2 was obtained by comparison with the $\Delta\nu_Q$ values obtained from a sample of CER:CHOL:[2,2-$^2H_2$]PA (not shown). Number of acquisitions = 10000 (*A*), 2000 (*B*, *C*).

TABLE 2 Quantity of solid and isotropic PA-d_{31} and CHOL-d_5 in oriented samples of CER:CHOL:PA pH 5.2 as a function of temperature

	PA-d_{31}		CHOL-d_5	
T (°C)	% Solid*	Time (h)‡	% Solid*	Time (h)‡
20	7	31	0	31
	46	62	33	63
	54	87	57	88
30	53	17	50	21
	46§	79§		
40	36	26	28	21
50	10	18	11	21
	% Solid¶	% Isotropic¶	% Solid¶	% Isotropic¶
40	36	1.5	28	1.4
50	10	27	11	4.9
60	0	49	0	19
70	0	72	0	41

* The quantity of solid was determined as described in Materials and Methods. The remainder of the lipid is in either a gel or fluid phase (i.e., liquid-ordered or isotropic). For the PA-d_{31}, the short and long recycle delays used were 300 ms and 40 s, respectively. For the CHOL-d_5, the short delay was 100 ms, and the long delay either 20 or 40 s. For the 20-s delay, the ratio of echo height/number of scans was multiplied by 1.1.

‡ Determined from the point at which the temperature was changed and the end of data acquisition.

§ Data from a different sample, with short and long recycle delays of 300 ms and 50 s, respectively.

¶ The remainder of the sample is in the liquid-ordered phase.

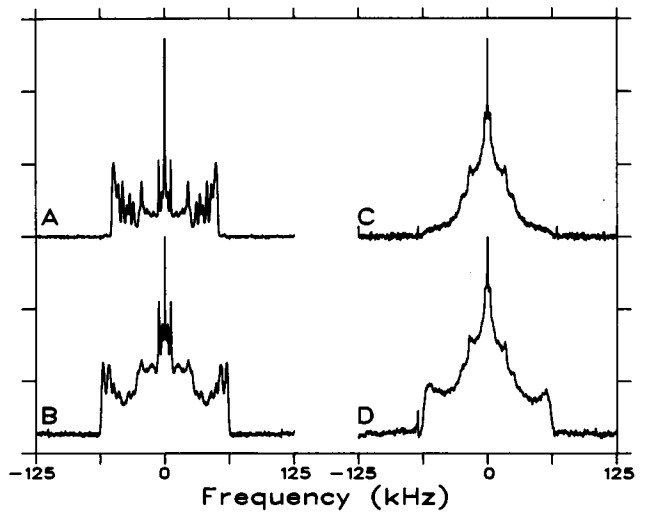

FIGURE 6 ^{2}H NMR spectra for oriented multilayers of CER:CHOL:PA-d_{31} at 50°C for $\theta = 0°$ (*A*), and at 20°C for $\theta = 0°$ (*B*) and 55° (*C*, *D*) with recycle delays of 300 ms (*A–C*) and 40 s (*D*). Number of acquisitions = 10000 (*A–C*) and 1712 (*D*).

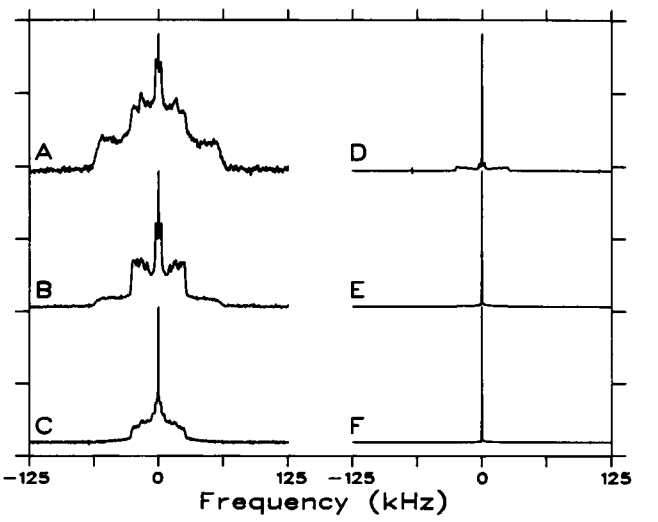

FIGURE 7 ^{2}H NMR spectra for oriented multilayers of CER:CHOL:PA-d_{31} for $\theta = 90°$ (*A*, *B*, *D–F*) and $\theta = 55°$ (*C*) at 30°C (*A*), 40°C (*B*, *C*), 50°C (*D*), 60°C (*E*), and 70°C (*F*). The recycle delays were 40 s (*A*, *B*, *D*) and 300 ms (*C*, *E*, *F*). Number of acquisitions = 1352 (*A*), 2160 (*B*), 10000 (*C*, *E*, *F*), and 1536 (*D*).

shape that resembles an unoriented gel state spectrum (the peaks at ±19 kHz originate from the solid PA-d_{31} methyl groups). The presence of solid PA-d_{31} is shown by comparison of Fig. 6, *C* and *D*, where the latter spectrum was acquired at the magic angle with a recycle delay of 40 s. The solid phase developed slowly over a period of 88 h. Several pairs of spectra with recycle delays of 300 ms and 40 s were acquired in this period, allowing calculation of the percentage of solid phase present as a function of time (see Materials and Methods). This data is listed in Table 2. About 57% of the PA-d_{31} is in the solid phase in Fig. 6 *D*.

The temperature dependence of the CER:CHOL:PA-d_{31} is shown in Fig. 7. After acquisition of the spectra in Fig. 6, the sample was rotated to $\theta = 90°$. The spectrum acquired at 20°C (not shown) is essentially identical to that acquired at 30°C (Fig. 7 *A*). Comparison with spectra acquired at $\theta = 0°$ and 90° revealed the presence of oriented lipid. At 30°C, both solid and gel-like phases are seen to coexist (Fig. 7 *A*), and the proportion of solid is unchanged from 20°C (Table 2). As the temperature is raised to 40°C, the proportion of solid decreases to 36% and the oriented lipid undergoes a transition from gel-like to liquid-ordered (Fig. 7 *B*). That the liquid-ordered phase is a mixture of both oriented and powder lipid is shown by Fig. 7 *C*, which was acquired at $\theta = 55°$ with a recycle delay of 300 ms. As the temperature is further increased to 50°C (Fig. 7 *D*), the solid phase decreases to 12% of the PA-d_{31} and an isotropic phase begins to grow, which accounts for some 27% of the PA-d_{31}. At 60 and 70°C, only isotropic and liquid-ordered phases are observed (Fig. 7, *E* and *F*), with the latter phase accounting for some 28% of the PA-d_{31} at the higher temperature (Table 2). The temperatures at which these transitions occur are in good agreement with those observed in the dispersions (Fig. 4).

A further comparison of the multilamellar dispersions and oriented samples is shown in Fig. 8. The spectra of

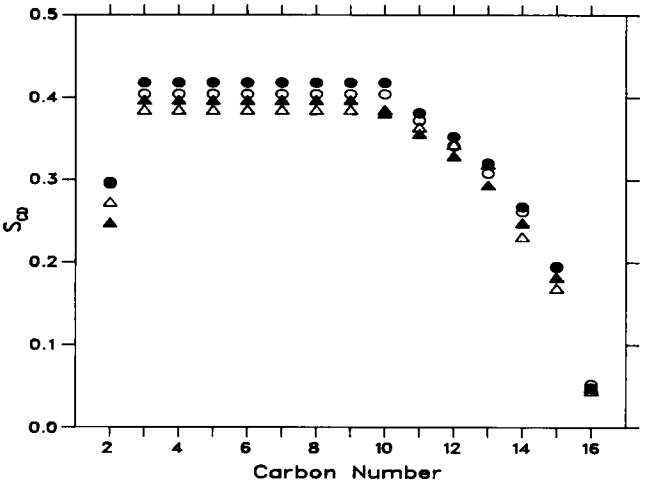

FIGURE 8 Comparison of order parameter profiles obtained at 50°C of SPM:CHOL:PA-d_{31} (○, ●) and CER:CHOL:PA-d_{31} (△, ▲) for multilamellar dispersions (*closed symbols*) and oriented multilayers (*open symbols*).

SPM:CHOL:PA-d_{31} and CER:CHOL:PA-d_{31} multilamellar dispersions, acquired at 50°C, were dePaked according to Sternin et al. (1988), from which the order parameter profiles (OPPs) were obtained. These are compared with the OPPs derived from the oriented samples. For both sample types, the CER-containing bilayers were slightly less ordered than the SPM-containing bilayers. For both the SPM- and CER-containing bilayers, the OPPs obtained from the powder and oriented samples were very similar. All of the OPPs fall within a fairly narrow range of order. Both oriented and powder samples are seen, therefore, to give similar information regarding lipid polymorphism and membrane order.

Ceramide:[2,2,3,4,6-$^2H_5$]cholesterol:palmitic acid

The above data, dealing only with the behavior of the palmitic acid probe in the two lipid systems, reveals nothing about the possible participation of the other components in the observed polymorphism. To address this question, multilamellar dispersions and oriented samples of CER:CHOL-d_5:PA (pH 5.2) were prepared. Temperature-dependent spectra of the former are shown in Fig. 4, and spectra of the latter, obtained at 50°C as a function of rotation angle, are shown in Fig. 9. The latter sample, prepared from approximately 45 mg of lipid, displayed essentially complete orientation. The 2-, 2-, 3-, 4-, and 6-positions were assigned to the observed quadrupolar splittings by comparison with data obtained from PC/CHOL mixtures (Taylor et al., 1981; Dufourc et al., 1984). The outermost resonance originates from positions 3 ($\Delta\nu_Q$ = 49 kHz) and [2,4]axial ($\Delta\nu_Q$ = 47 kHz). The [2,4]-equatorial deuterons have $\Delta\nu_Q$ values of 35 and 30 kHz (minor component), respectively. The innermost splitting (4 kHz) originates from the 6-position. These values are similar to values obtained in egg PC/CHOL (Taylor et al., 1981), DMPC/CHOL (Dufourc et al., 1984), human erythrocyte membranes (Kelusky et al., 1983), and *Acholeplasma laidlawii* strain B membranes (Monck et al., 1993), indicating a similar orientation of cholesterol in the CER:CHOL-d_5:PA membranes. The quadrupolar splittings scale with the same angular dependence as the PA-d_{31} samples (Figs. 1 and 5). When this sample was cooled to 30°C and spectra were acquired for recycle delays of 100 ms and 10 s, a solid phase was not observed over a period of 20 h (spectra not shown). The 100 ms recycle delay is adequate to observe all of the fluid phase cholesterol, because the T_{1Z} values for all positions are less than 14 ms (Dufourc and Smith, 1986). This result is consistent with the hysteresis effect observed for the CER:CHOL:PA-d_{31} (Table 2). That some CHOL was beginning to partition into a solid phase comes from comparison of the ratio of echo height with number of scans obtained for two spectra at 20°C with recycle delays of 100 ms. Over a period of some 20 h, this ratio decreased by 20%, demonstrating that a significant quantity of the liquid-ordered lipid was being removed to the solid state.

To quantify the polymorphism of CHOL-d_5, a sample of CER:CHOL-d_5:PA was prepared under identical conditions to the sample of CER:CHOL:PA-d_{31} listed in Table 2 (the quantity of lipid on the plates was 40 mg, and the hydration temperature was 65°C). Analogous spectra were acquired, as a function of temperature, with long and short recycle delays. All three phases were observed over the same temperature ranges as with the CER:CHOL:PA-d_{31}. The results, listed in Table 2, reveal good agreement between the quantity of solid PA-d_{31} and CHOL-d_5 present at any temperature. However, significant differences are observed in the quantity of isotropic phase lipid present between 50 and 70°C, with a greater proportion of PA-d_{31} partitioning into this phase (Table 2). This is also observed with multilamellar dispersions (Fig. 4 and Table 3).

It should be noted that, at 20°C, the sharp resonances originating from oriented liquid-ordered cholesterol broadened significantly over a period of 24 h, retaining their orientation, suggesting a transformation into a gel-like state. Similar behavior was observed with the CER:CHOL:PA-d_{31}.

Because this sample of cholesterol is only 60% deuterated (H. C. Jarrell, personal communication), a sample containing a larger quantity of lipid (approximately 90 mg) was prepared to obtain better S/N in the solid state. Spectra acquired at 50°C at an angle of 0° gave a powder lineshape with θ = 90° splittings, indicating that the bilayers were not aligned with the glass plates (see Fig. 10 *C* for a representative spectrum). The sample was cooled to 20°C, where solid cholesterol was observed from spectra acquired with a recycle delay of 40 s

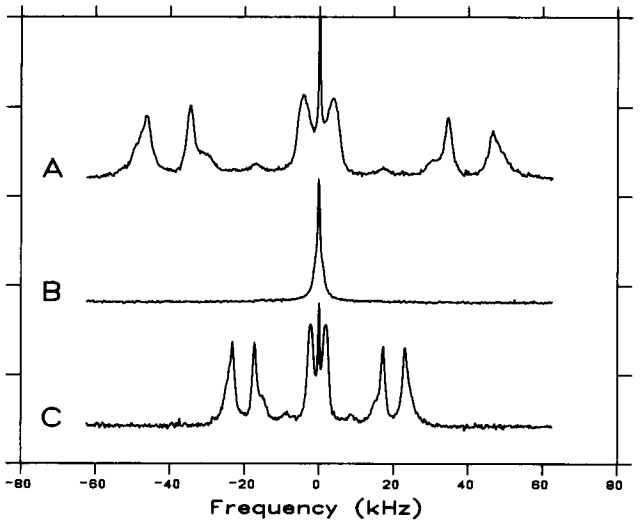

FIGURE 9 ^2H NMR spectra of oriented multilayers of CER:[2,2,3,4,6-$^2H_5$]CHOL:PA at 50°C (pH 5.2) as a function of the angle θ between the normal to the glass plates and the external magnetic field. θ = 0° (*A*), 55° (*B*), and 90° (*C*). The pH was maintained by the addition of approximately 0.3 ml of 100 mM citrate, 150 mM NaCl, 4 mM EDTA, pH 5.2 containing 5 wt% PEG (M_r 8000) to the glass plates before data acquisition. The assignment of positions 2-, 2-, 3-, 4-, and 6- to the observed resonances is given in the text. Number of acquisitions = 350,000 (*A*), 10,000 (*B*), and 60,000 (*C*). The total quantity of lipid applied to the glass plates was approximately 45 mg.

TABLE 3 Quantity of isotropic PA-d_{31} and CHOL-d_5 (percent of total lipid) in multilamellar dispersions of CER:CHOL:PA pH 5.2 as a function of temperature and hydration buffer

	PA-d_{31}		CHOL-d_5
T (°C)	Citrate	HEPES	Citrate
60	54	51 ± 2*	10
65	67	71	28
70	72	85 ± 1*‡	46
75	78	92 ± 2*	62

* The results at 60, 70, and 75°C are the averages of three, two, and two separate experiments, respectively.

‡ At pH 6.2, 57% of the lipid is in the isotropic phase at this temperature (Thewalt et al., 1992).

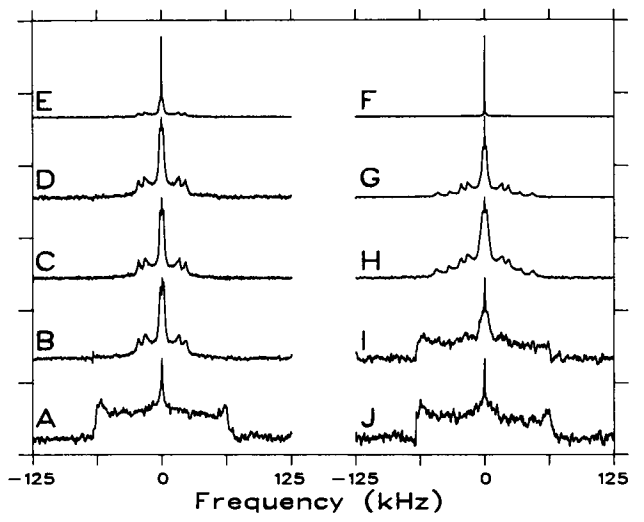

FIGURE 10 ^{2}H NMR spectra of CER:[2,2,3,4,6-$^2H_5$]CH:PA as a function of temperature (pH 5.2). The sample was prepared as an oriented sample, and the spectra were acquired at an angle $\theta = 0°$ between the normal to the glass plates and the external magnetic field. For each spectrum, the temperature and recycle delays were as follows: (*A*) 20°C, 40 s; (*B*) 40°C, 100 ms; (*C*) 50°C, 100 ms; (*D*) 50°C, 20 s; (*E*) 60°C, 100 ms; (*F*) 70°C, 100 ms; (*G*) 50°C, 100 ms; (*H*) 20°C, 100 ms; (*I*) 20°C, 20 s; (*J*) 20°C, 40 s. The spectra were acquired in the above order. Number of acquisitions: (*A*) 5,800; (*B*) 20,000; (*C*) 40,000; (*D*) 2,880; (*E*) 20,000; (*F*) 20,000; (*G*) 400,000; (*H*) 40,000; (*I*) 2,880; (*J*) 1,800.

(Fig. 10 *A*). Fig. 10 *A* is the sum of two spectra acquired over a period of 64 h, but significant solid was present after 32 h. There is no evidence of liquid-ordered lipid at this temperature, although significant quantities are observed at 40°C (Fig. 10 *B*). By 50°C, the sample is completely liquid-ordered, as seen from a comparison of spectra acquired with a recycle delay of 100 ms (Fig. 10 *C*) and 20 s (Fig. 10 *D*). As the temperature is raised to 60°C (Fig. 10 *E*) and 70°C (Fig. 10 *F*), the cholesterol undergoes a liquid-ordered to isotropic phase transition. Of interest is the behavior of the lipids as the same sample was cooled after acquisition of the 70°C spectrum. At 50°C, a pair of quadrupolar splittings with twice the width of the powder splittings is observed, indicating the presence of some oriented bilayers (Fig. 10 *G*). Liquid-ordered-oriented and powder bilayers are observed when the temperature is reduced to 20°C, as shown by the spectrum collected with a recycle delay of 100 ms (Fig. 10 *H*). The solid phase is reestablished rapidly, over a period of 17 h (Fig. 10, *I* and *J*; recycle delay = 20 and 40 s, respectively), over which time the liquid ordered phase seems to disappear (the central resonance observed in Fig. 10 *I* is not present in Fig. 10 *J*). The ratio of echo height to number of scans is greater for the 40-s delay (by a factor of 1.1), indicating that more of the solid phase is observed for the longer delay. These results are in good agreement with multilamellar dispersions (Fig. 4) and with the samples containing less lipid, except that the rate of formation of the solid phase seems to be much faster. This suggests that factors such as the quantity of lipid, or the quality of orientation, affect the kinetics of formation of the solid phase.

DISCUSSION

The stratum corneum, the uppermost layer of mammalian epidermis, is a complex matrix of extracellular lipid and corneocyte cells. The permeability barrier is thought to be mediated mainly by the lipid, which is arranged in lamellae between the cells, in a fashion that has been referred to as a "brick and mortar" model (Elias et al., 1983). The present study is part of an attempt to understand the behavior of the stratum corneum by modeling the lipid component of the permeability barrier. The model we have chosen is an equimolar mixture of ceramide, cholesterol, and palmitic acid and is based on best estimates of lipid composition in the stratum corneum (see Introduction). Because continuous lipid modification is a feature of this complex differentiating epithelium, uncertainty still exists as to the precise lipid composition in the stratum corneum of any given species, anatomical area, or level within the tissue, and detailed analysis is correspondingly difficult. However, the presence of ceramides, cholesterol, and fatty acids as major fractions of stratum corneum lipids is general among mammals and extends to other (e.g., avian) species (Menon et al., 1989). Because the systematic construction of a "complete" phase diagram for a mixture of this complexity is a formidable task, we have chosen a simple model as a starting point. For comparison, we have used an equimolar mixture of sphingomyelin, cholesterol, and palmitic acid. Although the composition of the "precursor" membranes formed in the lamellar bodies is more complex than this (containing such lipids as phospholipids, glucosylceramides and cholesterol sulfate), the choice of this system allows us to examine the effects of a single change in membrane structure, i.e., the removal of the phosphocholine headgroup as sphingomyelin is converted to ceramide. Previous studies from our laboratory (Thewalt et al., 1992; Kitson et al., 1994) in which these mixtures were examined as multilamellar dispersions have established that the SPM-containing membranes form liquid-ordered bilayers over a wide range of temperatures and pH, whereas the CER-containing membranes display a complex polymorphism that is pH-dependent. This study extends our previous work by examining both systems as oriented multilayers. There are several reasons why such an approach would be desirable. First, the study of lipid dynamics is greatly enhanced by studying the orientational dependence of the longitudinal (T_{1Z} and T_{1Q}) and transverse (T_2) relaxation rates (Jarrell et al., 1988; Mayer et al., 1990; Auger et al., 1990; Winsborrow et al., 1991; Monck, 1993). We show that the SPM:CHOL:PA-d_{31} system displays a large T_{1Z} anisotropy. Second, the membranes of the stratum corneum are themselves macroscopically "oriented" by the cellular matrix and, therefore, the oriented samples may be better models in some respects than multilamellar dispersions. Third, the hydration levels of the oriented sample can be varied by increasing the percentage of PEG in the hydrating buffer (Morrison, 1993). This could allow for model conditions that better approximate biological conditions, because it is known

that stratum corneum membranes are fully hydrated at low water contents (approximately 20 wt%) (Golden et al., 1987). Finally, the observation of a $(3\cos^2\theta - 1)/2$ dependence of the quadrupolar splittings with rotation angle, combined with the large order parameters, would demonstrate that the model membranes are lamellar and aligned with the glass plates and, therefore, quite possibly bilayers.

Several lines of evidence suggest that the liquid-ordered lamellae observed in both the SPM- and CER-containing systems exist as bilayers. First, for all oriented samples, the quadrupolar splittings obey a $(3\cos^2\theta - 1)/2$ dependence, indicating that the axis of motional averaging is the normal to the plane of the membrane (Figs. 1, 2, 5, and 9). This has been previously observed in oriented bilayers of phospholipids (Mayer et al., 1990) and glycolipids (Jarrell et al., 1987). Second, the order parameter profiles (OPP) obtained from the resolved quadrupolar splittings of both SPM:CHOL:PA-d_{31} and CER:CHOL:PA-d_{31} at 50°C are similar and characteristic of liquid-ordered bilayers, with a high degree of order ($S_{CD} = 0.4$) in the plateau region (Fig. 8). These results are in good agreement with those obtained from PC:CHOL dispersions. POPC-d_{31} containing 45 mol% CHOL has a maximum order parameter of 0.38 at 30°C (Lafleur et al., 1990). The quadrupolar splitting of 2[6,6-d_2]DMPC containing 40 mol% cholesterol, oriented at $\theta = 0°$ at 35°C (Mayer et al., 1990), is essentially the same as that of the plateau region of SPM:CHOL:PA-d_{31} at 40°C. This correspondence with other model membranes is also observed with the CER:CHOL-d_5:PA. The quadrupolar splittings of CHOL-d_5 in the CER-containing bilayers are similar to those in PC:CHOL mixtures (Taylor et al., 1981; Dufourc et al., 1984) and in biological membranes (Kelusky et al., 1983; Monck et al., 1993). Third, there is good agreement between relaxation data obtained from SPM:CHOL:PA-d_{31} and PC:CHOL mixtures. Both the absolute values of the relaxation times and the magnitude of the T_{1Z} anisotropy are comparable with results obtained with DMPC:CHOL mixtures (Mayer et al., 1990). For the plateau deuterons of SPM:CHOL:PA-d_{31} at 40°C, the T_{1Z} values increase from 27 to 63 ms as the sample is rotated from $\theta = 0$ to 90°, respectively, giving $T_{1Z}(90°)/T_{1Z}(0°) = 2.3$ (Table 1). This result can be understood in terms of a recently published theory for the orientation dependence of T_{1Z} (Morrison and Bloom, 1993, 1994). For 2[6,6-d2]DMPC containing 40 mol% CHOL, the T_{1Z} values range from about 24 to 52 ms for the same rotation, and $T_{1Z}(90°)/T_{1Z}(0°) = 2.2$. This suggests similar rates and types of molecular motion in the SPM:CHOL:PA-d_{31} samples and in liquid-ordered PC:CHOL bilayers.

An interesting feature is seen in Fig. 3 for the spectrum obtained for palmitic acid selectively labeled at the 2,2-position. The 2,2-position has a shorter T_{1Z} than any other position, particularly at $\theta = 90°$. It also has a significantly smaller quadrupolar splitting than those of the plateau deuterons. This almost certainly indicates an orientational change of this position because of interactions with surface components (Abdolall et al., 1977). This is an important point, because other workers have used [2,2-$^2H_2$]palmitic acid to probe lipid mixtures containing ceramides obtained from stratum corneum samples (Abraham and Downing, 1991). For a mixture of CER:CHOL:PA-d_2:CS (38:38:19:5) at 45°C, a quadrupolar splitting of 34 kHz was obtained (Abraham and Downing, 1991). This is identical to our value for CER:CHOL:PA-d_2 at 50°C (data not shown), and somewhat smaller than the value of 40 kHz obtained for SPM:CHOL:PA-d_2 at 40°C (Table 1). This agreement is of particular interest because Abraham and Downing's samples were made from ceramides isolated from stratum corneum.

In the multilamellar dispersions of both CER:CHOL:PA-d_{31} and CER:CHOL-d_5:PA, a complex polymorphism is observed, with solid (crystalline) lipid present at low temperatures (<50°C) and liquid-ordered and isotropic phase lipid coexisting at temperatures above 50°C. This behavior is summarized in Fig. 4. In addition, a significant hysteresis is observed in the formation of the solid phase after the liquid-ordered to isotropic transition, and 3-phase coexistence (solid, gel, and liquid-ordered) is observed at 20°C (Kitson et al., 1994). A key observation of the present study is that the oriented samples display the same polymorphism. The presence of liquid-ordered membranes in the oriented samples in the temperature range of 20–50°C (Figs. 5, 6, and 10), after incubation at 50°C or higher, is clearly a hysteresis effect. The solid phase appears slowly, over a period of days, during which time the liquid-ordered lipid appears to undergo a transition to a gel-like state. The broad solid-phase spectrum is invariant to rotation (Figs. 6 *D* and 7 *A*) and, therefore, is not a highly ordered gel phase. This is also demonstrated by its very long T_{1Z}, which ranges from 4 to 12 s for the solid CHOL-d_5 and PA-d_{31}, respectively (Monck et al., 1993; J. Thewalt, unpublished observations). The T_{1Z} values of gel state lipids are on the same order as the liquid crystalline values (Auger et al., 1990). In all respects, the results obtained with identical oriented samples of CER:CHOL:PA-d_{31} and CER:CHOL-d_5:PA are in good agreement (Table 2). For both PA-d_{31} and CHOL-d_5, the solid, liquid-ordered, and isotropic phases are observed at the same temperatures, and the rate of formation of the solids occurs on the same timescale. The analogous behavior of the labeled cholesterol and palmitic acid demonstrates that the complex polymorphism observed in the CER-samples involves the membrane as a whole, which is consistent with previous ^1H NMR results that suggested that at least the fatty acid and ceramide were involved in the transitions (Thewalt et al., 1992). Unfortunately, our data do not allow us to address the question of whether the CER-membranes are homogeneous or heterogeneous mixtures, either in the liquid-ordered or solid phases, or whether any lateral phase separation occurs during this transition. However, although the PA-d_{31} and CHOL-d_5 are present in similar proportions in the solid phase, it would appear that the isotropic phase is enriched in the PA-d_{31} relative to the CHOL-d_5. At 70°C, some 72% of the PA-d_{31} is found in the isotropic phase, compared with only 41% of the CHOL-d_5 (Table 2). The same trends are observed with multilamellar dispersions, where the observed quantities of isotropic lipid are in good

agreement with the oriented samples (Table 3). Sample-to-sample variation in the proportion of isotropic phase lipid appears to be quite small, on the order of ±2% (Table 3). Slightly greater quantities of PA-d_{31} were found at higher temperatures in the isotropic phase in samples prepared in HEPES buffer (Table 3).

An interesting observation was made with an "oriented" sample of CER:CHOL-d_5:PA containing approximately 90 mg of lipid, in which almost no orientation of lipid was observed (Fig. 10). The appearance of the solid phase was much more rapid (17 h) than in the oriented samples with 40–45 mg of lipid (80 h). This suggests that the rate of appearance of solid membrane, and possibly the final equilibrium levels, may be influenced by such factors as the flatness of the membrane or how well it is oriented on the glass plates. It is tempting to speculate that the lamellar arrangement of stratum corneum lipid is one control mechanism in determining the quantity of solid lipid in vivo. Wertz et al. (1989) have recently demonstrated that covalently bound ceramides are located on the external surface of keratinocytes where, conceivably, they could function as a template for the establishment of "oriented" intercellular lamellar lipid.

In the present study, we have limited our investigation of polymorphism in the CER:CHOL:PA system to an equimolar lipid mixture at a pH of 5.2, which approximates in vivo SC compositions and pH. However, it is worth noting that the observed polymorphism is sensitive to pH and possibly to other parameters such as lipid composition. In a recent detailed study on CER:CHOL:PA-d_{31} MLVs (Kitson et al., 1994), we show that increasing the pH to 7.4 (where the palmitic acid is negatively charged) results in a transition to H_{II} phase at elevated temperatures. This suggests that the polymorphic preference of the membrane is regulated by the charge on the palmitic acid (i.e., size of the headgroup). This may partially explain some differences between our results and other recent studies on SC model systems. Abraham and Downing (1991) used ^2H NMR to characterize lipid polymorphism in SC models prepared from SC ceramides and observed a lamellar to H_{II} transition at temperatures >60°C. However, their system was composed of CER:CHOL:PA:CS in the molar ration 38:38:19:5, and their samples were prepared in water; hence, the pH was not controlled. More recently, Lieckfeldt et al. (1993) used x-ray diffraction to examine the polymorphic behavior of the system CER:CHOL:(fatty acids), where the molar ratio was 27.4:26.2:46.6 and the fatty acids were a mixture of six different species. Although the pH is not recorded, sufficient NaOH was added to neutralize 41% of the fatty acids. This system exhibited a lamellar gel to liquid-crystalline transition at approximately 57°C, followed by a transition to H_{II} phase at 68°C. The CER was required to prevent the crystallization of cholesterol, which formed from CHOL:fatty acid mixtures. The results of the present study demonstrate that solid CHOL can also form in the presence of CER; however, we cannot say that the solid we observe is the same as the crystalline cholesterol observed with x-ray diffraction, because the latter is presumably pure cholesterol, whereas the former may be embedded in a heterogeneous solid. Overall, our results with CER:CHOL:PA appear to be consistent with other SC models when differences in lipid composition and solvent pH are taken into account.

Our results on a simple model system demonstrate that in the range of body temperature, equimolar mixtures of CER:CHOL:PA can exhibit coexistence of solid-, gel-, and liquid-ordered membrane phases. Thus, a lipid mixture approximating that found in stratum corneum is capable of very complex phase behavior. This leads to the question of whether our model bears any resemblance to the lipid lamellae of intact SC. Support for the approach described here comes from DSC, ESR, IR, and x-ray diffraction studies that reveal thermal lipid phase transitions in the range of 25–75°C in a wide variety of stratum corneum preparations (Rehfeld and Elias, 1982; Knutson et al., 1985; Golden et al., 1986; Golden et al., 1987; Rehfeld et al., 1988; Bouwstra et al., 1991). Although some of these transitions are close to the temperatures at which we observe the solid-to-liquid-ordered and liquid-ordered-to-isotropic transitions, we cannot at present suggest a direct correspondence between our data and those of intact stratum corneum. The precise nature of the in vivo transitions has not yet been elucidated, and it is possible that the transition temperatures may be influenced by differences in lipid composition and by the presence of proteins and other lipids (Golden et al., 1986). Further support comes from x-ray diffraction results that reveal a lamellar arrangement for the intercellular lipid domain and the presence of crystalline and liquid lipid coexisting near physiological temperatures (White et al., 1988; Garson et al., 1991). In some studies, at least one unit cell is observed with a repeat distance on the order of 13 nm, suggesting a double bilayer arrangement (White et al., 1988; Bouwstra et al., 1991). This is not necessarily inconsistent with our results, which support a bilayer arrangement for CER:CHOL:PA, because the exact form of the "double bilayer" is not yet known.

The ability of the lipids in our stratum corneum model to partition into a solid phase is highly unusual and perhaps unique behavior, leading to the question of whether this might contribute to the functioning of the permeability barrier in vivo. The permeability of water vapor through stratum corneum is about 1000 times less than through fluid lipid bilayers (Potts and Francoeur, 1991). This has been attributed to the highly tortuous path that water must traverse through the extracellular lipid. The measured path length is some 50-fold greater than the thickness of the stratum corneum (Potts and Francoeur, 1991). Furthermore, both the diffusion coefficients and activation energies for water flux through the stratum corneum are similar to values obtained in fluid phospholipid bilayers (Potts and Francoeur, 1991; Golden et al., 1987), arguing against reduced permeability resulting from lipid composition. In fact, it would appear that variation in lipid content, from that of epidermal stratum corneum to a composition that contains 12% phospholipid, only affects the permeability constants by an order of magnitude (Wertz et al., 1992). The effect of crystalline lipid, then, would be to increase the diffusion pathlength, if solid lipid were

present in appreciable quantities and located in the extracellular matrix. Using x-ray data, Garson et al. (1991) have estimated the size of the solid lipid crystals to be comparable with that of a corneocyte.

It is encouraging that a simple model system provides general agreement with many features of intact SC. Clearly, further work will be required to improve the agreement and to ascertain the effect of proteins and cellular material on the polymorphism observed herein.

CONCLUSIONS

A simple model of mammalian stratum corneum, consisting of CER:CHOL:PA in equimolar proportions, displays complex polymorphism, forming membranes composed of solid and gel/liquid-ordered lipid in the range of physiological temperatures, with an unknown isotropic phase forming at higher temperatures. Our results suggest that the liquid-ordered phase has a bilayer arrangement. At least two components of the membrane partition approximately equally into the solid phase, although we cannot say whether they form a homogeneous or heterogeneous solid mixture. The unusual physical properties of this SC model may provide insight into the functioning of the permeability barrier.

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