# Sphingomyelin Multiple Phase Behavior as Revealed by Multinuclear Magnetic Resonance Spectroscopy†

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ABSTRACT: $^{31}P$ and $^{13}C$ nuclear magnetic resonance (NMR) spectra are employed to study the phase behavior of bovine brain sphingomyelin as a function of temperature. The $^{31}P$ NMR data suggest that, while at low temperatures sphingomyelin can form a lamellar phase, at physiological temperatures and higher the lamellar phase is unstable, and a new phase, best described as a hexagonal phase, is formed. Egg phosphatidylcholine is suggested to play an important role in stabilizing bilayers in natural membranes. Cholesterol also exhibits a sphingomyelin bilayer-stabilizing ability. The $^{13}C$ NMR spectra suggest a gelling of the hydrocarbon chains of sphingomyelin at low temperature. Thus, bovine brain sphingomyelin undergoes both a gel to liquid-crystalline phase transition and a lamellar to nonlamellar transition.

Phospholipids from many natural and synthetic sources are capable of forming lamellar phases (bilayers) in an aqueous environment. The phosphatidylcholines constitute a class of phospholipids with this ability. X-ray (Levine and Wilkins, 1971) and neutron-diffraction (Büldt et al., 1978) data have provided information about the lamellar structure. The fundamental physical properties of phosphatidylcholine and mixtures of other natural phospholipids have supported the modern concept of the phospholipid bilayer as a basic component of cell membranes.

Another major phospholipid of cell membranes, phosphatidylethanolamine, when unsaturated, forms at physiological temperatures and in an aqueous environment, not a pure lamellar phase but rather a mixture of lamellar and hexagonal phases (Reiss-Husson, 1967). Recently, Cullis and de Kruijff (1978) presented an analysis of $^{31}P$ NMR spectra of phosphatidylcholines and phosphatidylethanolamines in unsonicated aqueous dispersion which suggested a simple method for distinguishing the lamellar from the hexagonal phase without perturbing the system. Because of the additional motional averaging possible in the hexagonal phase, via diffusion around the cylinders, the $^{31}P$ NMR spectra are distinctly different and become diagnostic for the presence or absence of bilayers.

The calorimetric data for sphingomyelin are quite different than for phosphatidylcholine (Barenholz et al., 1976; Untracht and Shipley, 1977), sphingomyelin exhibiting more complex phase transitions. However, the structures of the phases formed by pure bovine brain sphingomyelin over a wide range of temperatures do not appear to have been previously studied in detail. At low temperatures, lamellar phases have been found (Reiss-Husson, 1967; Untracht and Shipley, 1977).

It was, therefore, the purpose of this project to exploit the $^{31}P$ NMR approach of Cullis and de Kruijff (1978) to examine the phase behavior of sphingomyelin. The results indicate that the phase behavior is, indeed, complex; not only can sphingomyelin exist in a gel and a liquid-crystalline phase but also in a lamellar and nonlamellar phase. The presence of a small amount of phosphatidylcholine in the sphingomyelin is shown to stabilize the lamellar phase, which suggests an important bilayer-stabilizing role for phosphatidylcholine in membranes. Cholesterol is, likewise, shown to stabilize the lamellar phase.

## Materials and Methods

Bovine brain sphingomyelin (SPM[1]) was purchased from Avanti Biochemicals (Birmingham, Ala.) or was isolated from bovine brain white matter (Shinitzky and Barenholz, 1974) (the latter was a gift from the laboratory of Dr. T. E. Thompson). Lots I and II correspond to Avanti lots SM8-11 and SM8-15. Egg phosphatidylcholine (PC) was purchased from Avanti and cholesterol from General Biochemical. Thin-layer chromatography of about 0.5 mg of the lipids from Avanti in chloroform/methanol/water (65:25:4) on regular and reversed-phase silica plates revealed only a single spot.

Lipid mixtures were prepared for the following experiments by colyophilization or drying under a stream of nitrogen. The lipids were hydrated by the addition to the dried lipid of an excess of the appropriate aqueous solution, either 0.1 M NaCl, $H_2O$, or deionized, distilled water and no salt. In some cases, spectra were obtained of the lipid at room temperature (25–26 °C) *without* any preheating of the sample. The samples were then heated in the NMR instrument to the desired temperature using a JEOL VT-3B temperature controller. The temperature was measured before and after each measurement with a thermistor (YSI no. 44106) in the probe, and when the temperature varied by more than 2 °C the measurement was discarded. For some experiments, between each measurement the sample was allowed to cool to room temperature while the NMR probe reached equilibrium. For others, the sample was kept at the appropriate temperature in a water bath. The sample was allowed to equilibrate in the probe for 15 min before beginning each measurement.

$^{31}P$ NMR spectra were obtained on a JEOL PS-100/EC-100 Fourier transform spectrometer at 40.48 MHz. Broadband decoupling (20 W) was employed to reduce the dipolar contributions to the $^{31}P$ resonance (Niederberger and Seelig, 1976). Scans (10 000 to 100 000) were collected for each sample with intervals between accumulations of 0.1 s. Fifty to sixty milligrams of material in 10-mm tubes with about 0.8 mL of water or salt solution was used. Windows of 20-kHz were

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[1] Abbreviations used are: SPM, sphingomyelin; PC, phosphatidylcholine; NMR, nuclear magnetic resonance.

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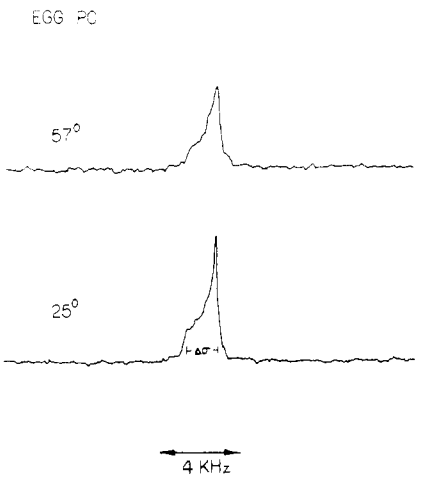


FIGURE 1: 40.48-MHz $^{31}P$ NMR spectra of an unsonicated dispersion of egg phosphatidylcholine in 0.1 M NaCl and $H_2O$ (10% $D_2O$), 60 mg/mL.

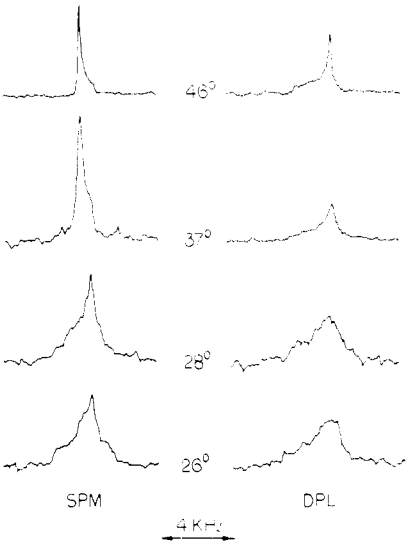


FIGURE 2: Temperature dependence of $^{31}P$ NMR spectra of an unsonicated dispersion of bovine brain sphingomyelin (SPM) and dipalmitoylphosphatidylcholine (DPL). Spectra represent first heating of hydrated sample, as described in text. Both lipids were measured at 50 mg/mL, in 0.1 M NaCl, $H_2O$ (10% $D_2O$).

employed with 1K data points (ADC time = 0.0255 s) and without the RF crystal filter. Pulse widths of 7 $\mu$s were used (20 $\mu$s = 90°) and a total 100-$\mu$s delay before acquisition. Ten percent $D_2O$ in the sample provided an internal lock for the instrument. Exponential filters were used for sensitivity enhancement, which produced 75 Hz of broadening or less. $^{13}C$ NMR spectra were obtained at 25.15 MHz in 10-mm tubes with broad-band proton decoupling. Data points (8K) in the time domain were collected for a display of 5 kHz. A 5-kHz RF crystal filter was employed for sensitivity enhancement. Repetition rates of 1 s and pulse widths of 10 $\mu$s (20 $\mu$s = 90°) were employed. Sample concentrations of 100 mg/mL were used, and data were collected for 2–3 h to obtain a sufficient signal to noise ratio.

## Results

*$^{31}P$ NMR.* The $^{31}P$ NMR spectrum of an unsonicated aqueous dispersion of egg PC is given in Figure 1. At 25 °C the spectrum exhibits residual chemical-shift anisotropy of about 45 ppm. This value represents the difference between the upfield shoulder and the downfield shoulder, $\Delta\sigma$ in Figure 1. This proton-decoupled spectrum contains little dipolar contribution and demonstrates the ability at this field strength of modest decoupling power to remove dipolar contributions from $^{31}P$ resonances of phospholipids above their gel to liquid-crystalline phase transition temperature. This spectrum resembles closely spectra obtained previously and is characteristic of phospholipid in a bilayer phase [see Seelig (1978) for a review]. Also in Figure 1 is the $^{31}P$ NMR spectrum of egg PC at 57 °C. Elevation of the temperature by 22 °C does not alter the spectral shape, and little difference is noted in the residual chemical-shift anisotropy.

Figure 2 presents $^{31}P$ NMR data for a dipalmitoylphosphatidylcholine (DPL) and sphingomyelin at several temperatures. At 26 °C, DPL is below its gel to liquid-crystalline phase transition temperature of about 42 °C. On this instrument the strength of the proton decoupler is not sufficient to completely remove the dipolar contributions, and the residual chemical-shift anisotropy is not distinct. However, generally, the shape of the resonance is similar to that of egg PC and reflects the bilayer nature of this unsonicated aqueous dispersion of DPL. As the temperature is raised, the spectral shape does not change, although the dipolar contributions decrease. An increase in temperature causes an increase in motional rate of the phospholipids, thus reducing the strength of the dipolar contributions to the resonance shape, and the decoupler, at or above the phase-transition temperature, is sufficient to remove the remaining dipolar interaction, as in the egg PC sample of Figure 1. Once again, the residual chemical-shift anisotropy changes only a little, decreasing somewhat as the temperature is increased, as previously noted (Cullis, et al., 1976).

Figure 2 also presents the $^{31}P$ NMR data for an unsonicated aqueous dispersion of bovine brain sphingomyelin. The treatment of the sample is important to a description of the results. Initially, dry sphingomyelin was dispersed in 0.1 M NaCl and $H_2O$ solution (with about 10% $D_2O$ for the internal lock on the spectrometer), and dispersion was accomplished by mechanical agitation at room temperature, without sonication, as for the phosphatidylcholine samples. The initial spectrum at 26 °C, with *no prior heating* of the sample, is shown at the bottom of Figure 2. This spectrum approximates that for dipalmitoylphosphatidylcholine at the same temperature. The shape of the spectrum closely resembles the bilayer spectra of phosphatidylcholine, and the peak maximum is in the same position as that for phosphatidylcholine, about 11- to 12-ppm upfield of the isotropic chemical shift of these phospholipids. As the temperature is raised to 28 °C, the spectrum becomes narrow but retains the same general shape and position of the maximum.

At 37 °C, a dramatic change has taken place. The observed spectrum resembles a composite of the bilayer-type spectrum and a new spectrum. The nature of the new component is clear at 46 °C, where it alone appears. At this temperature, the sign of the chemical-shift anisotropy is reversed and the observed residual anisotropy is approximately half that observed for egg PC.

These spectral changes are not completely reversible. When the sample is returned to 26 °C, the spectrum that results most resembles the original spectrum at 37 °C. The appearance of a spectrum characteristic of bilayer does not dominate even at 5 °C, although general broadening of the resonance does occur, probably due to an increase in strength of dipolar interactions from immobilization. A mixture of phases exists with the high-temperature phase still prominent. This behavior was found to be repeatable with bovine brain sphingomyelin from two independent sources (see Materials and Methods). If the

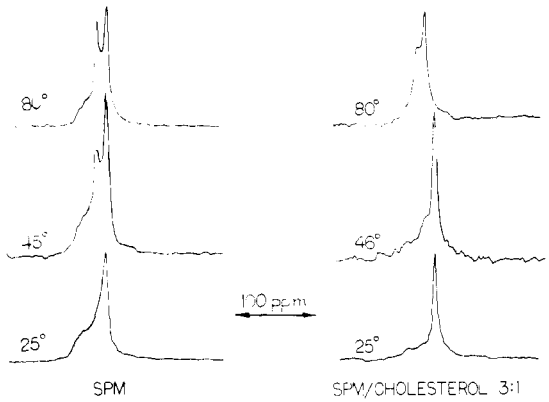


FIGURE 3: Temperature dependence of $^{31}P$ NMR spectra of an unsonicated dispersion of lot II (see text) sphingomyelin with and without 25 mol % cholesterol at 100 mg/mL sphingomyelin in distilled, deionized water.

sample is not preheated, the room-temperature spectra have the appearance of bilayer phase, which is lost upon heating and not regained upon returning to room temperature. Once heated above about 35 °C, all observed changes with temperature are reversible.

Prolonged incubation of a preheated sample at 25 °C had no effect. The $^{31}P$ NMR spectrum appeared the same at 25 min of incubation as at 24 h and at 2 days of incubation.

Since some of the published calorimetry data was obtained in salt-free solutions, a sample of sphingomyelin was dispersed in distilled, deionized water, and the $^{31}P$ NMR spectra were measured as a function of temperature. Exactly the same behavior was observed as for sphingomyelin dispersed in 0.1 M NaCl.

All of the above results were found to be repeatable with sphingomyelin from lot I from Avanti and with sphingomyelin obtained as a gift as described under Materials and Methods, and the two sphingomyelins behaved similarly. However, a second lot from Avanti purchased later exhibited somewhat different behavior. For future discussion, this latter lot will be referred to as lot II. The temperature dependence of the $^{31}P$ NMR spectra of lot II was measured at 25, 35, 45, 55, and 80 °C. Representative spectra appear in Figure 3. As in lot I, a new, nonlamellar phase develops at high temperature, but this time the temperature of the transition is much higher than before and the temperature at which the transition is complete is not readily accessible. This contrasts to the results with lot I, where the upper end of the transition between the lamellar and nonlamellar phase is in the region of 45 to 50 °C, whereas the lower end of the transition is not readily accessible. Thus, while similar, broad phase transitions take place in all bovine brain sphingomyelins measured, the temperature ranges of the transitions vary. Variable behavior of sphingomyelins has been noted before (Barenholz et al., 1976).

Figure 3 also shows the temperature behavior of a sphingomyelin (lot II) dispersion containing 25 mol % cholesterol. In this case, no significant change in the $^{31}P$ NMR spectra is observed from 25 to 60 °C. Only at 80 °C is a change in spectral shape evident. Cholesterol also shows here some ability to stabilize sphingomyelin bilayers, though it does not produce as strong an effect as egg phosphatidylcholine.

Similar experiments to those performed with pure sphingomyelin were performed with a 4:1 (mole ratio) sphingomyelin (lot I)/egg PC mixture. Since 80% of the phosphorus in this sample is in sphingomyelin, the $^{31}P$ resonance still arises predominantly from sphingomyelin. The temperature dependence of the $^{31}P$ spectra of this mixture is shown in Figure 4.

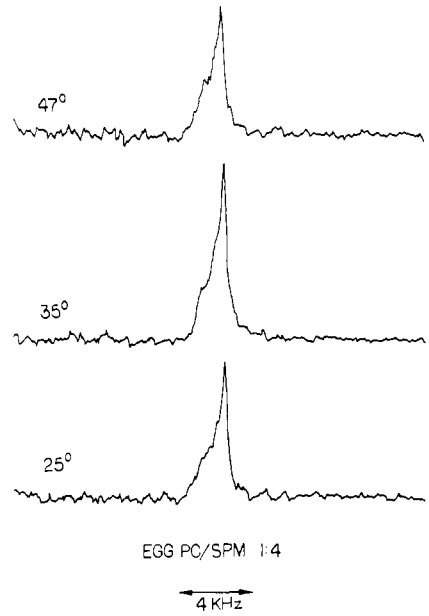


FIGURE 4: Temperature dependence of $^{31}P$ NMR spectra of an unsonicated dispersion of bovine brain sphingomyelin (lot I) containing 20 mol % egg phosphatidylcholine.

The spectra at all temperatures resemble that of PC, with no transitions observed to a nonbilayer phase.

Since the shape of the $^{31}P$ NMR spectrum of pure sphingomyelin at high temperature (see Figure 2) resembles closely the theoretically expected shape for a hexagonal phase, the sphingomyelin (lot I) was subjected to a test designed to distinguish between the hexagonal and bilayer phases. Because of its paramagnetism, $Gd^{3+}$ broadens strongly without shifting the $^{31}P$ resonances of phospholipid head groups to which it has access. In the case of multilayered bilayers, such as obtained with egg phosphatidylcholine, only the outside layer of phospholipid is affected, because the concentric bilayers are impermeable to the cation. Therefore, addition of $Gd^{3+}$ to unsonicated egg phosphatidylethanolamine dispersions should have little effect on the $^{31}P$ spectra. This proved to be the case for the addition of approximately 2 mM $Gd^{3+}$ to a 1-mL dispersion containing about 60 mg of egg PC.

In the hexagonal phase, the phospholipid head groups are all exposed to the addition of cation via the water channels. Therefore, the addition of $Gd^{3+}$ should strongly broaden the resonances and cause a dramatic decrease, if not disappearance, of the $^{31}P$ spectral intensity. The results of such an experiment with sphingomyelin at 41 °C are shown in Figure 5. The addition of about 2 mM $Gd^{3+}$ does broaden a majority of the resonance easily. However, a small part is left unbroadened, which is not eliminated by an increase of the $Gd^{3+}$ concentration to 4 mM . Evidently, a small portion of the phospholipid is protected from interaction with the lanthanide ion. The solution remains milky in appearance at all temperatures so that small structures do not seem to be formed as a predominant species. The chemical shift of the unbroadened resonance at 4 mM $Gd^{3+}$ is slightly upfield of the resonance maximum in the absence of $Gd^{3+}$ and appears to be situated at the isotropic chemical shift of sphingomyelin.

*$^{13}C$ NMR.* Figure 6 shows the $^{13}C$ NMR spectrum of bovine brain sphingomyelin in $CDCl_3$. This spectrum is similar to that observed for egg phosphatidylcholine (Godici and Landsberger, 1975), except in the region of carbon–carbon double bonds. The downfield resonance in that region of the sphingomyelin spectrum arises from the double-bonded carbon at-

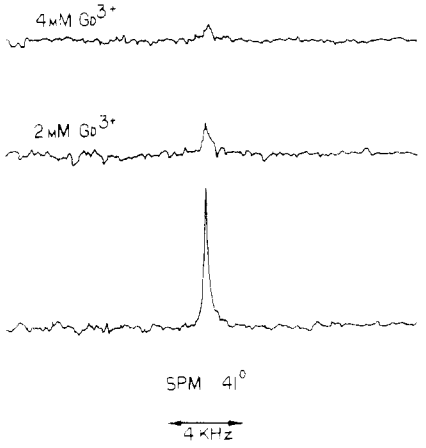


FIGURE 5: $^{31}P$ NMR spectra of the addition of $Gd^{3+}$ (aq) to an unsonicated dispersion of bovine brain sphingomyelin (lot I) at 41 °C. Bottom scan is in absence of $Gd^{3+}$ (aq).

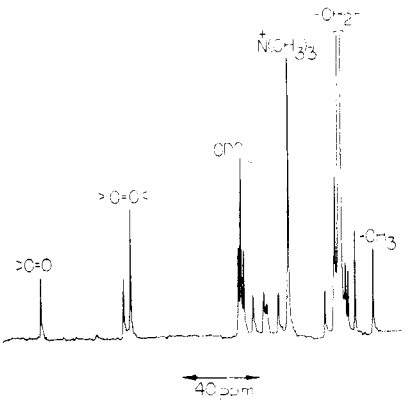


FIGURE 6: $^{13}C$ NMR spectrum of bovine brain sphingomyelin in $CDCl_3$ at 25.15 MHz.

tached to position 1 of glycerol. The upfield resonance in the sphingomyelin corresponds to the low-field resonance in the egg phosphatidylcholine spectrum. Since multiple double bonds do not occur in sphingomyelin, there is no peak which corresponds to the upfield double-bond resonance in egg phosphatidylcholine.

Figure 7 shows the temperature dependence of the $^{13}C$ NMR spectra of unsonicated dispersions of sphingomyelin (lot II). Strong broadening of the resonance is reduced when the temperature is raised to 35 °C and remains unchanged at higher temperatures. The strongest effect appears in the resonances from the hydrocarbon chains, both the methylene and the double bond carbon resonances. Similar spectra were observed for lot I sphingomyelin, except that the spectra were more severely broadened at 24 °C, and at 42 °C and higher, when the $^{31}P$ NMR indicated little lamellar phase remained, the *N*-methyl resonance became dramatically narrower.

## Discussion

Unsonicated aqueous dispersions of phospholipids produce anisotropic $^{31}P$ resonances (Horwitz and Klein, 1972; Sheetz and Chan, 1972). The resonance shape, when the dipolar contributions to the line width are removed, arises from the anisotropy of the $^{31}P$ chemical-shift tensor. A conceptual description of this chemical-shift tensor begins with a crystal of a phosphorus-containing compound. Since there is no fast rotation of the molecule in the crystal, the observed chemical shift is a function of the declination of the crystal with respect to the applied magnetic field (Kohler and Klein, 1976). This

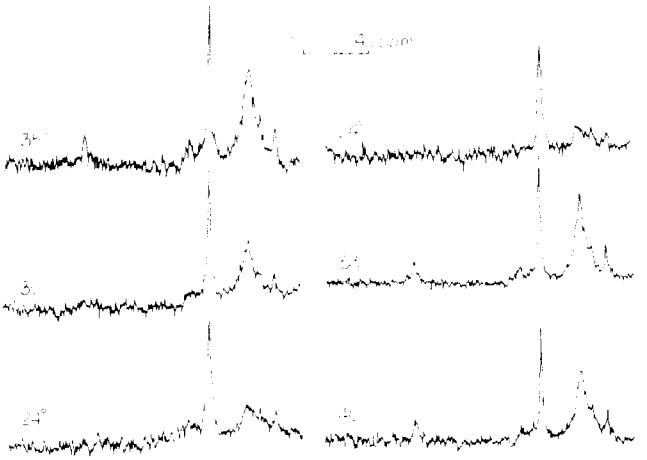

FIGURE 7: Temperature dependence of the $^{13}C$ NMR spectra of an unsonicated dispersion of lot II bovine brain sphingomyelin in distilled, deionized water. Upper right-hand spectrum represents a return to 24 °C after heating to 53 °C. The tall, sharpest peak arises from *N*-methyl carbons, and the next strongest peak is due to methylene carbons.

is because the shielding of the nucleus, which determines the chemical shift, is dependent upon the direction from which the nucleus is approached. Since this is a three-dimensional dependence, the chemical shift is described by a tensor, and it is anisotropic because the shielding around the phosphorus is anisotropic. When a powder is made of the crystal, the chemical-shift tensors are randomly oriented with respect to the applied magnetic field, and the resonance intensity is spread over the full range of the chemical shift possible, around 200-ppm for phospholipids (Seelig, 1978).

The spectrum in Figure 1, however, covers only about 45-ppm. The onset of rapid rotation about an axis (the director) perpendicular to the bilayer surface, as well as some additional motion of the director, occurs upon hydration of the phospholipids (Griffin, 1976). This rapid rotation partially averages the chemical-shift tensor so that the powder spectrum seen (reflecting nonoriented bilayers of phospholipid) is reduced by motion from the powder spectrum of the dry material (Kohler and Klein, 1977).

The above considerations apply to phospholipids in a bilayer configuration. In the hexagonal phase, an additional degree of motional averaging results from lateral diffusion of phospholipids on the surface of the cylinders of that phase. For a phospholipid otherwise experiencing the same degree of motional freedom in the head group as in a bilayer phase, the observed anisotropy will exhibit the opposite sign and one-half the magnitude, as the theoretically calculated spectra of Cullis and de Kruijff (1978) demonstrate. Such a result is obtained with the $^{31}P$ NMR spectra of phosphatidylethanolamine (Cullis and de Kruijff, 1978), where X-ray data have demonstrated the existence of a hexagonal phase (Reiss-Husson, 1967).

The $^{31}P$ NMR spectra of sphingomyelin may be contrasted with those of phosphatidylcholine. The latter exhibit the shape expected for bilayers which phosphatidylcholine in unsonicated aqueous dispersions is known to form. An increase in temperature above any phase transition causes little change in the chemical-shift anisotropy observed, so the motional order of the phosphate is undergoing only small changes (Cullis et al., 1976), although the rate of motion is increasing as reflected in $T_1$ and $^{31}P\{^1H\}$ NOE data (Yeagle et al., 1977). Below the phase transition, dipolar contributions to the line width increase because of the slower rates of motion in the gel state of the

dipalmitoylphosphatidylcholine, but the spectral shape does not change significantly. It remains consistent with the bilayer phase.

In contrast, the sphingomyelin spectra change dramatically with temperature. When first hydrated at room temperature, sphingomyelin exhibits a spectrum characteristic of bilayer. Increasing the temperature of the sample to 40 or 50 °C (or much higher for lot II) causes sphingomyelin to exhibit a spectrum characteristic of a hexagonal phase. A complete return to a bilayer-type spectrum is not accomplished by returning the temperature to 25 °C. (However, temperature changes in these experiments occurred rapidly, and the system may not have been in equilibrium.) Once the sample has been heated, the temperature may be changed and all spectral changes are reversible. At intermediate temperatures, in preheated samples, composite spectra are observed, representing probably a mixture of at least two phases of the phospholipid.

The experiments with $Gd^{3+}$ further characterize the high-temperature phase. Though $^{31}P$ NMR spectra above 37 °C resemble closely that expected from a hexagonal phase, to the best of our knowledge, a hexagonal phase has not been previously reported for sphingomyelin. Addition of $Gd^{3+}$ to sphingomyelin at 41 °C readily broadened most of the $^{31}P$ resonance, indicating most of the lipid was available for interaction with the shift reagent. This broadening is inconsistent with a phase of concentric bilayers, such as is formed in unsonicated dispersions of phosphatidylcholine, unless the bilayers are permeable to cations. Since sonicated vesicles of sphingomyelin are impermeable to cations (Schmidt et al., 1977), this possibility is unlikely. Most, if not all, of the phosphates in a hexagonal phase are accessible to externally added cation, so that these results are consistent with the hexagonal phase.

While both the shape of the $^{31}P$ NMR resonance and the behavior with respect to $Gd^{3+}$ are consistent with the existence of a hexagonal phase for sphingomyelin, these data do not in themselves firmly establish the structure of the high-temperature phase reported here for bovine brain sphingomyelin. Such an unequivocal determination awaits a detailed X-ray study. However, it does appear clear that pure bovine brain sphingomyelin bilayers are not stable at high temperatures or even at physiological temperatures in some sphingomyelins, since the $^{31}P$ NMR spectra observed and the behavior of $Gd^{3+}$ are inconsistent with bilayer structure as exhibited by phosphatidylcholine.

While in a bilayer form as identified by the $^{31}P$ NMR data, sphingomyelin exhibits $^{31}P$ NMR spectra similar to phosphatidylcholine. Since $^{31}P$ NMR of unsonicated systems has been shown to be sensitive to head-group conformation (Seeling, 1978), these results suggest that the polar head group of sphingomyelin is in a conformation similar to that of phosphatidylcholine. The same conclusion was reached studying sonicated vesicles of sphingomyelin (Yeagle et al., 1977; Yeagle, 1978).

More detailed insight into the multiple phase behavior of sphingomyelin is obtained from the $^{13}C$ NMR measurements. The resonances arising from the methylene carbons and the carbons involved in carbon–carbon double bonds provide information on the motional state of the hydrocarbon chains of the phospholipids. In a gel state, these resonances would be expected to be severely broadened, as is observed for phosphatidylcholine below its phase-transition temperature (Levine et al., 1972). Thus, the observation of broad hydrocarbon chain resonances of 24 °C and subsequent narrowing of the resonances with increase in temperatures is suggestive of a gelling of the hydrocarbon chains at low temperature. The degree of broadening suggests the temperature of this transition is lower for lot II sphingomyelin, while the $^{31}P$ data suggest the nonbilayer component attributed to the hexagonal phase is attained at higher temperature for lot II sphingomyelin. Thus, for lot II sphingomyelin a clear distinction can be made between the lower temperature regions where an apparent gel to liquid-crystalline transition takes place and the higher temperature region where an apparent bilayer to hexagonal transition takes place. In the sphingomyelin studied that was not from lot II, these two temperature regions overlap considerably, leading to a complex phase behavior.

The complex phase behavior of sphingomyelin observed here could be expected to manifest itself in calorimetric scans. The differential scanning colorimetry data are more complex than the data for dipalmitoylphosphatidylcholine, and it was concluded that the special rules that are useful in ordering the behavior of various glycerophospholipids are not applicable to sphingomyelin (Barenholz et al., 1976). The results obtained here suggest why. Not only is there a gel to liquid-crystalline phase transition, but also a lamellar to nonlamellar phase transition must be considered. The large temperature range in which these latter two phases coexist is consistent with the broad transitions observed in the differential scanning calorimetry data. Further understanding of the details of this multiphase behavior should come from $^{31}P$ NMR and X-ray studies of pure, synthetic sphingomyelins.

The multiple phase behavior of bovine brain sphingomyelin recalls the behavior of unsaturated phosphatidylethanolamines. Recent $^{31}P$ NMR data have been found consistent with X-ray data, implicating both bilayer and hexagonal phases for phosphatidylethanolamine (Cullis and de Kruijff, 1978). As with sphingomyelin, the bilayer phase appears favored at low temperatures, and at physiological temperatures a mixture of phases occurs.

Egg phosphatidylcholine affects strongly the behavior of bovine brain sphingomyelin. Inclusion of as little as 20 mol % egg phosphatidylcholine eliminates the propensity of the sphingomyelin to form the nonlamellar phase in the physiological temperature range, nor is it evident up to 63 °C. Egg phosphatidylcholine affects phosphatidylethanolamine similarly (Cullis and de Kruijff, 1978). These results suggest a crucial role for phosphatidylcholine in natural membranes. Phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin are three of the most common phospholipids and together constitute a majority of the phospholipid species in many membranes. Natural versions of two of these phospholipids have shown a tendency to form nonbilayer phases when in pure form at physiological temperature. Egg phosphatidylcholine, however, and other phosphatidylcholines studied, form stable bilayers. Phosphatidylcholine-containing natural membranes which have been examined by $^{31}P$ NMR, including erythrocyte ghosts (McLaughlin et al., 1975), vesicular stomatitus viral membranes (Moore et al., 1977), chromaffin granule membranes (McLaughlin et al., 1975), and bovine retinal rod outer segment membranes (Albert, Yeagle, and Litman, manuscript in preparation) exhibit spectra characteristic of bilayers. Therefore, phosphatidylcholine may be a crucial structural phospholipid in creating and maintaining the phospholipid bilayer which has been found in some membranes and has been hypothesized to be an essential component of many membranes.

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# Fatty Acyl Chain Order in Lecithin Model Membranes Determined from Proton Magnetic Resonance†

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ABSTRACT: Proton magnetic resonance ($^1$H NMR) has been used to compare the local orientational order of acyl chains in phospholipid bilayers of multilamellar and small sonicated vesicular membranes of dipalmitoyllecithin (DPL) at 50 °C and egg yolk lecithin (EYL) at 31 °C. The orientational order of the multilamellar systems was characterized using deuterium magnetic resonance order parameters and $^1$H NMR second moments. $^1$H NMR line shapes in the vesicle samples were calculated using vesicle size distributions, determined directly using electron microscopy, and a theory of motional narrowing, which takes into account the symmetry properties of the bilayer systems. The predicted non-Lorentzian line shapes and widths were found to be in good agreement with experimental results, indicating that the local orientational order (called "packing" by many workers) in the bilayers of small vesicles and in multilamellar membranes is substantially the same. This result was found to be true not only for the largest $^1$H NMR line associated with the nonterminal methylene protons but also for the resolved $^1$H NMR lines due to the $\alpha$-$CH_2$ and the terminal $CH_3$ positions on the acyl chain. Analysis of the vesicle $^1$H NMR spectra of EYL taken with different medium viscosities yielded a value of approximately $4 \times 10^{-8}$ cm$^2$ s$^{-1}$ for the lateral diffusion constant of the phospholipid molecules at 31 °C.

Current efforts to understand the details of structure and function of biological membranes have prompted many studies of lipid bilayer model membranes. A variety of different physical studies on both lecithin lamellar dispersions and ultrasonically generated lecithin vesicles have established beyond any reasonable doubt that a crystalline (solid) to liquid-crystalline (fluid) phase transition occurs. Calorimetric measurements (Chapman et al., 1967) show that the principal contribution to the entropy change which occurs with the onset of membrane fluidity is associated with the decrease of orientational order of the hydrocarbon chains in the membrane lipids. Quantitative measurements of "order parameters" associated with different positions along the hydrocarbon chains have been provided by electron-spin resonance (ESR)[1] spin-label experiments (Gaffney and McConnell, 1974; Schreier-Murcillo et al., 1973) and nuclear magnetic resonance (NMR)

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[1] Abbreviations used: NMR, nuclear magnetic resonance; DPL, dipalmitoyllecithin; EYL, egg yolk lecithin; ESR, electron spin resonance; rf, radiofrequency; FT, Fourier transform; FID, free induction decay; POL, 1-palmitoyl-2-oleoyllecithin.

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