A Nuclear Magnetic Resonance Study of Sphingomyelin in Bilayer Systems†

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ABSTRACT: The physical properties of small single-walled vesicles composed of the zwitterionic phospholipid sphingomyelin have been studied using ^1H and ^{31}P nuclear magnetic resonance spectroscopy. The temperature variation of proton line widths and spin-lattice relaxation times and the chemical shift behavior for sphingomyelin vesicles are compared with results previously determined for phosphatidylcholine vesicles. Differences between the two systems are interpreted as indications of the presence of both inter- and intramolecular hydrogen bonding in sphingomyelin bilayers.

There is much evidence to show that the structure and properties of biological membranes are strongly dependent on lipid composition of the component bilayer (for a comprehensive review, see Silbert, 1975). In mammalian membranes, the principal lipids are phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin, and cholesterol (White, 1973). Although the structure and properties of bilayers formed from glycerophosphatides and the effects of acyl chain length, degree of unsaturation, and cholesterol content on membrane organization have become the focus of much research activity (for a recent review, see Lee, 1975), very little work has been carried out on sphingomyelin systems.

The limited information available indicates that the physicochemical properties of glycerophosphatides and sphingomyelin in bilayer arrays may differ in several biologically relevant respects. Perhaps most relevant is the fact that the sphingomyelins of mammalian membranes have their gel to liquid-crystalline phase transition temperatures close to 37 °C, whereas the phospholipids of these membranes are far above their phase transitions at this temperature (Shinitzky and Barenholz, 1974; Shipley et al., 1974; Barenholz et al., 1976).

In this communication, we report the results of ^1H and ^{31}P NMR studies of small single-walled vesicles comprised of a highly purified sphingomyelin prepared from beef brain and mixtures of this material and phosphatidylcholine. The NMR work is supplemented by fluorescence depolarization measurements in these systems of the probe 1,6-diphenyl-1,3,5-hexatriene (DPH).[1]

Materials and Methods

Materials. Beef brain sphingomyelin was prepared, purified, and analyzed as described by Shinitzky and Barenholz (1974). The fatty acid composition is shown in Table I. It is apparent that there are two dominant fatty acids, stearic (C18:0) and nervonic (C24:1).

Phosphatidylcholine was prepared from hen eggs by procedures previously described (Litman, 1973). Egg [*N-methyl*-^2H]phosphatidylcholine ($N^+(CD_3)_3PC$) was prepared from egg phosphatidylcholine as follows. [*N-methyl*-^2H]-Choline iodide was prepared by methylation of ethanolamine with [^2H]methyl iodide. The iodide was replaced by acetate and the choline acetate was purified by chromatography on IR-45 anion-exchange resin (Sears et al., 1976). Phosphatidic acid was prepared from purified egg lecithin using the enzyme phospholipase D from cabbage (Sigma Type I, 19 units/mg) (Dawson, 1967). Special precautions were taken during purification of this material to remove traces of choline and unreacted phosphatidylcholine. Direct esterification of the phosphatic acid with the [^2H]choline acetate to form phosphatidylcholine was carried out according to Aneja and Chadha (1971). The ^1H NMR spectra of lipid vesicles made of the $N^+(CD_3)_3PC$ showed less than 1% of the *N*-methyl proton signal when compared with the spectrum of the original egg lecithin.

Preparation of Small, Single-Lamellar Vesicles. Small, single-lamellar vesicles, homogeneous in size, were prepared following a modification of Huang's procedure (Huang, 1969; Huang and Thompson, 1974). Dispersions of these vesicles were prepared by sonication of the lipid in 50 mM KCl using a Heat Systems W-350 Sonifier. Homogeneous dispersions were then prepared by high-speed centrifugation (Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., Thompson, T. E., and Carlson, E. D., submitted for publication). Sonication of sphingomyelin was carried out at 45 °C, mixtures of sphingomyelin and lecithin were carried out at 10–20 °C, and egg lecithin was carried out at 4 °C. In all cases, the sonication was done under nitrogen.

Replacement of solvent H_2O by D_2O for ^1H NMR studies was achieved by dialysis against 10 volumes of 50 mM KCl in D_2O (Bio-Rad 99.89%). Four changes of the dialysis medium were required for complete exchange.

For fluorescence measurements, 1 μL of 2 mM DPH in tetrahydrofuran was added to one portion of the vesicle dispersion with rapid stirring; the remaining portion was used as a light-scattering blank.

Determination of Phospholipid Concentration. The lipid concentration of each dispersion was measured as inorganic phosphate by the Bartlett (1959) method. At least ten phosphate determinations were made on each sample with a resulting standard deviation of ±1% for small vesicle dispersions.

Fluorescence Depolarization Measurements. Fluorescence depolarization measurements were carried out as previously

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[1] Abbreviations used are: DPH, 1,6-diphenyl-1,3,5-hexatriene; $N^+(CD_3)_3PC$, egg [*N-methyl*-^2H]phosphatidylcholine; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine.

TABLE I: Fatty Acid Composition of Beef Brain Sphingomyelin.

Fatty acid[a]	Wt (% of total)
C14:0	<0.1
C16:0	2.8
C18:0	35.9
C19:0	<0.1
C20:0	0.9
C22:0	4.4
C23:0	3.7
C24:0	9.7
C25:0	4.7
C26:0	1.2
C18:1	<0.1
C24:1	30.8
C25:1	3.9

[a] Given as C*m*:*n* where *m* is the number of carbon atoms and *n* is the number of double bonds.

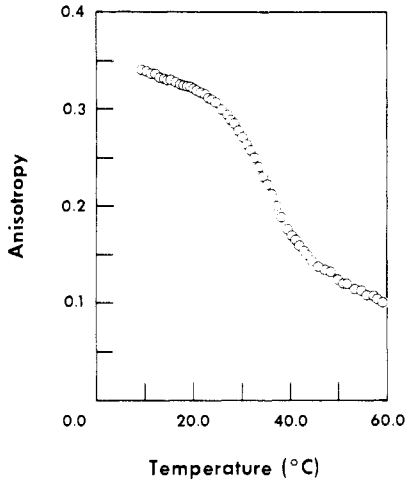

FIGURE 1: The effect of temperature on the fluorescence anisotropy of DPH in beef brain sphingomyelin vesicles. The ratio of DPH to sphingomyelin is 1 to 1000. Sphingomyelin concentration is 1 mM.

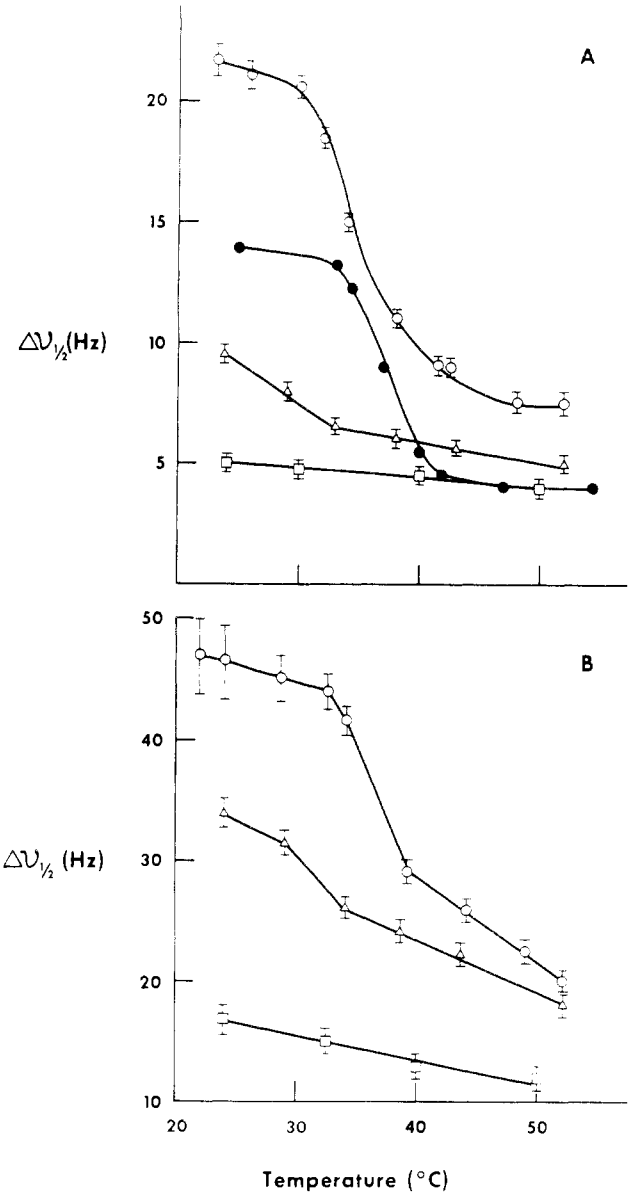

FIGURE 2: (A) The effect of temperature on the line width ($\Delta\nu_{1/2}$) of the choline methyl proton resonance of vesicles composed of: sphingomyelin (○); egg phosphatidylcholine (□); 1:1 mole ratio sphingomyelin/$N^+(CD_3)_3PC^1$ (△); and $DPPC^1$ (●) (data for DPPC taken from Lee et al., 1972). (B) The effect of temperature on the line widths ($\Delta\nu_{1/2}$) of the acyl-chain methylene proton resonances of vesicles composed of sphingomyelin (○); 1:1 mole ratio sphingomyelin/$N^+(CD_3)_3PC$ (△); and egg phosphatidylcholine (□).

described (Shinitzky and Barenholz, 1974; Suurkuusk et al., 1976; Lentz et al., 1976).

NMR Spectroscopy. All measurements were carried out on a JEOL PS-1000 P/EC-100 Fourier transform spectrometer. The desired temperature for each experiment was obtained using JEOL VT/3B temperature control. The temperature (±0.5 °C) was measured using a Yellow Spring Instrument thermistor No. 44016 set in an NMR tube under conditions identical to experimental conditions.

For proton experiments, the instrument was locked on the D_2O resonance. Typical conditions were a spectral width of 1 kHz using 4K data points in the frequency domain. Excellent signal to noise ratios were obtained after averaging 100–1000 transients, depending upon the lipid concentration. A recycle time at least five times the longest T_1 in the sample was used. The 90° pulse width was calibrated at 22.5 μs. No exponential filters were used in transforming the spectra. The relative number of protons was determined from the area of the separated resonances by the integral height and by planimetry. Agreement between the two methods was always better than 97%. The width in Hz of the resonances at half-height was determined assuming a Lorentzian line shape.

Spin-lattice relaxation times (T_1) were determined using 180°-τ-90° pulse sequence (Vold et al., 1968; Levine et al., 1973) with the usual precautions (Farrar and Becker, 1971). The data were collected using the JEOL automatic T_1 software. One hundred to one thousand scans were used to obtain each data point. From 12 to 16 τ values were collected and used in a least-squares analysis of the data (τ is the waiting period between 180° and 90° pulses). In all cases, the linear correlation coefficient for $\ln(A_0 - A_\tau/2A_0)$ vs. τ was greater than 0.996.

^{31}P NMR spectra were determined using the JEOL Fourier transform spectrometer at 40.48 MHz. Experimental conditions are specified under Results. Four thousand data points in the frequency domain were used with pulse repetition rate of about five times T_1. Measurements of the ratio of the number of molecules on the outside of small vesicles to the

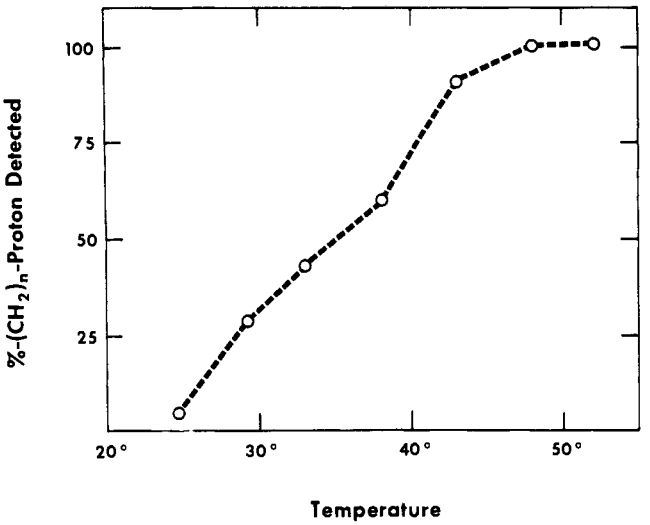

FIGURE 3: The percentage of acyl-chain methylene protons contributing to the observed resonance in sphingomyelin vesicles as a function of temperature.

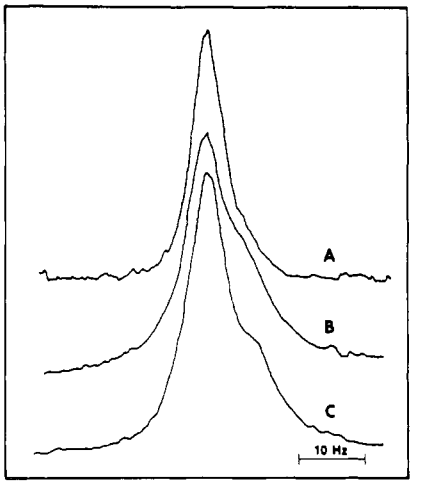

FIGURE 4: Detailed shape of choline methyl proton resonances of vesicles composed of: (A) egg phosphatidylcholine; (B) 1:1 mole ratio sphingomyelin/$N^+(CD_3)_3PC$; (C) sphingomyelin. Temperatures are: (A) 21 °C; (B) 29 °C; (C) 52 °C.

number on the inside were made using both a lanthanide shift reagent (Pr^{3+}) and a broadening reagent (Mn^{2+}). When Pr^{3+} was employed, gated proton decoupling was used to eliminate the differential nuclear Overhauser effect caused by the Pr^{3+} ions (Yeagle et al., 1975).

Results

Fluorescence Polarization and ^1H NMR Line Widths. The anisotropy of the probe DPH in the bilayer of sphingomyelin vesicles is given as a function of temperature in Figure 1. The large change in anisotropy in the range 24–48 °C coincides with the gel to liquid-crystalline phase transition observed calorimetrically for this lipid (Shipley et al., 1974; Barenholz et al., 1976).

The temperature variation of the line widths (width at half-height) of the sphingomyelin choline methyl, acyl-chain methylene, and acyl-chain terminal methyl proton resonances is given in Figure 2A,B. Figure 2A shows the thermotropic behavior of the choline methyl proton line widths in vesicles composed of sphingomyelin, egg phosphatidylcholine, and sphingomyelin and $N^+(CD_3)_3PC$ in a 1:1 molar ratio. Also shown for comparison are data for DPPC taken from Lee et al. (1972). Under some conditions, a second, smaller peak, due to choline methyl protons of molecules on the inside surface of the vesicles, could be partially resolved. For these cases, the line widths given are for the major downfield resonance only. It can be seen from Figure 2A that the temperature dependence of the sphingomyelin choline methyl proton line width is very similar to that shown by the fluorescence anisotropy of DPH in Figure 1. As expected, no transition is observed for egg phosphatidylcholine in this region. In the sphingomyelin–$N^+(CD_3)_3PC$ vesicles, the decrease in the sphingomyelin choline methyl proton line width with increasing temperature is still observed, but its magnitude is considerably smaller. At 50 °C, where the line widths for DPPC, egg phosphatidylcholine, and the equimolar sphingomyelin/$N^+(CD_3)_3PC$ vesicles become nearly equal, pure sphingomyelin still shows a small, but we believe significant, difference. These latter two results also agree with those obtained by the fluorescence depolarization of DPH. Sphingomyelin liposomes have higher microviscosities than egg phosphatidylcholine at all temperatures in the range 0–60 °C (Shinitzky and Barenholz, 1974) and also show a more diffuse phase transition when mixed with egg phosphatidylcholine (Barenholz and Shinitzky, unpublished results).

Similar observations can be made for the methylene and terminal methyl acyl-chain proton line widths, shown in Figure 2B. The sphingomyelin line widths are broader than those of egg phosphatidylcholine at all temperatures in the range studied. For sphingomyelin, the main difference between the thermotropic behavior of the choline methyl and the acyl-chain proton resonances is the "loss" of signal intensity for the latter as the temperature decreases through the onset of the thermal transition, as shown in Figure 3, while the choline methyl proton intensity remains constant. This behavior is also observed for DPPC vesicles (Sheetz and Chan, 1972) and is due to the experimental conditions used in Fourier transform NMR, i.e., a relatively small sweep width and the delay between the end of the pulse and the start of data acquisition (Seiter et al., 1972). This apparent signal loss accounts for the fact that the choline methyl proton resonances appear to be more sensitive to the thermal transition than those of the acyl-chain methylenes. When wide sweep widths and continuous-wave conditions are used for DPPC vesicles below their thermal transition, a methylene line width of 500–1000 Hz is observed (Lichtenberg et al., 1975).

^1H NMR Chemical Shifts and Influence of Shift Reagents. As previously mentioned, the choline methyl proton resonance of sphingomyelin vesicles is composed of two overlapping peaks due to molecules on the outside and inside surfaces of the vesicles. This is illustrated in Figure 4 for sphingomyelin vesicles at 52 °C, as well as for those of egg phosphatidylcholine at 21 °C, and the 1:1 sphingomyelin/$N^+(CD_3)_3PC$ mixed vesicles at 29 °C. These temperatures were chosen on the basis of the fluorescence depolarization measurements, in order to compare bilayers of equal fluidity. An inside–outside splitting of 0.02 ppm can be resolved for egg phosphatidylcholine at 250 MHz and 30 °C (Kostelnik and Castellano, 1973). At 100 MHz, this represents a splitting of 2 Hz, which cannot be resolved. For sphingomyelin, the splitting is 3.5 Hz at 52 °C, and it increases with decreasing temperature. Below the onset of the thermal transition, however, only a small asymmetry can be seen, due to the broadness of the peaks. For the sphingomyelin/$N^+(CD_3)_3PC$ 1:1 mixed vesicles, it is interesting to note that, while the average fluidity of the mixture at 29 °C,

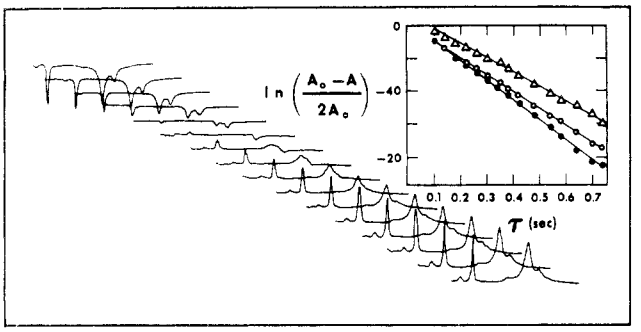

FIGURE 5: The set of partially relaxed spectra used for the determination of the T_1 values for the choline methyl and acyl-chain methylene and terminal methyl protons of 14.8 mM sphingomyelin vesicles at 58 °C. Other conditions are as described under Methods.

as monitored by the DPH microviscosity, is the same as that of pure sphingomyelin vesicles at 52 °C, the broader sphingomyelin resonances observed for the mixture indicate that the sphingomyelin head groups are in a more restricted motional state than the $N^+(CD_3)_3PC$ molecules. The inside–outside splitting for the mixture remains roughly equal to that observed for pure sphingomyelin. Sphingomyelin/phosphatidylcholine interactions will be the subject of a forthcoming publication (C. F. Schmidt, Y. Barenholz, and T. E. Thompson, in preparation).

Additional differences in the choline methyl proton chemical shift behavior of sphingomyelin and phosphatidylcholine can be seen after the addition of a lanthanide shift reagent, Pr^{3+}, to vesicle preparations. For sphingomyelin vesicles, a Pr^{3+}/sphingomyelin ratio of 0.07 was sufficient to shift the resonance due to molecules on the outside of the vesicles 0.6-ppm downfield. In order to achieve the same shift for egg phosphatidylcholine vesicles, it was necessary to bring the Pr^{3+}/egg phosphatidylcholine ratio to 0.2.

The Pr^{3+} shifted spectra can also be used to measure the ratio of the number of molecules on the outside of the vesicles to that of the molecules on the inside. However, it is found that the outside/inside ratios calculated using ^1H NMR are less reproducible and reliable than those determined using ^{31}P NMR. The reasons for this are the presence of other proton resonances in the vicinity of the choline methyls, and the difficulties in defining a unique baseline for the proton spectra.

Phosphorus NMR. The major difference between sphingomyelin and egg phosphatidylcholine in vesicles, as detected by ^{31}P NMR, is the fact that the sphingomyelin ^{31}P resonance is 0.6-ppm downfield from that of egg phosphatidylcholine, measured relative to an external reference. This shift difference increases to 0.7 ppm in methanol and to 1.4 ppm in chloroform. The ^{31}P shifts for lysophosphatidylcholine, glycerylphosphorylcholine, and sphingosine phosphorylcholine were also measured in aqueous solution and found to agree with previous results (Assman et al., 1974). The ^{31}P resonances of the two species are also affected differently by Pr^{3+}. At a Pr^{3+} to phospholipid ratio of 0.01, the ^{31}P downfield shift produced for sphingomyelin is 1.2 times that observed for egg phosphatidylcholine. This parallels the results obtained for the choline methyl proton resonances of these two phospholipids. These shifted resonances can also be used to measure the outside–inside intensity ratio, which is found to be 2.2 ± 0.1 for both sphingomyelin and egg phosphatidylcholine vesicles. The use of Mn^{2+}, a broadening reagent, gives the same result. Two other ^{31}P NMR parameters, the line width and the chemical shift difference between molecules on the outside and

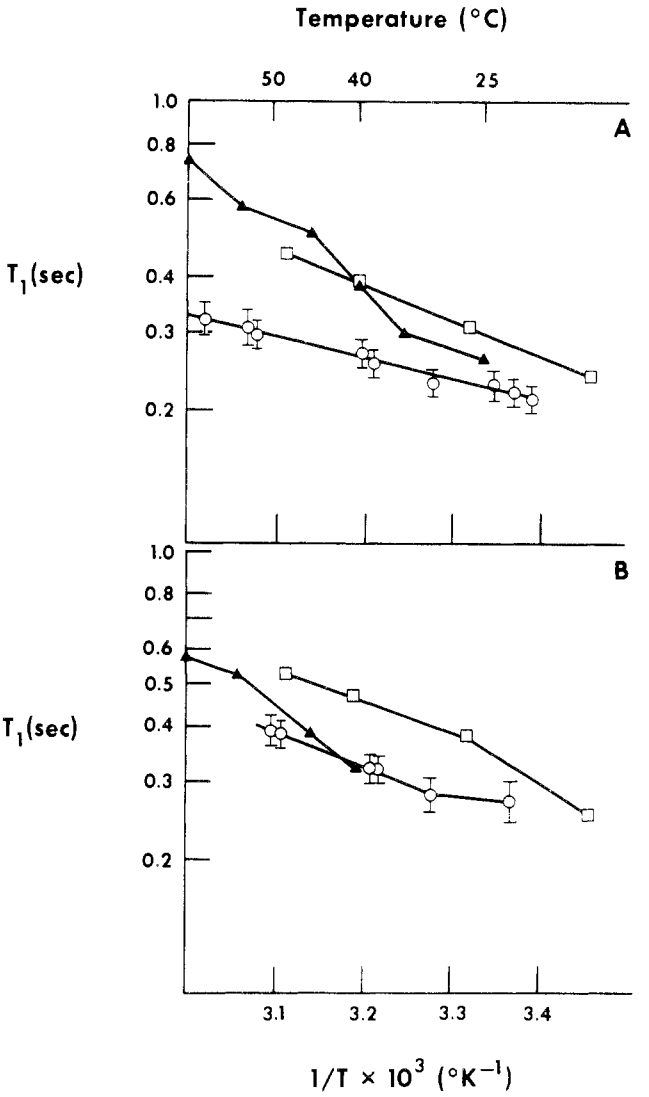

FIGURE 6: Semilog plots of T_1 vs. the reciprocal of the absolute temperature for the (A) choline methyl, and (B) acyl-chain methylene protons of vesicles composed of: (○) sphingomyelin; (□) egg phosphatidylcholine; and (▲) DPPC. The data for egg phosphatidylcholine and DPPC protons were obtained by Lee et al. (1972).

inside vesicles, can be compared at temperatures for which the DPH bilayer fluidities are the same. For sphingomyelin at 55 °C, the line width is 3.7 Hz, and the outside–inside splitting is 4.3 Hz. For egg phosphatidylcholine at 24 °C, the line width is 5.7 Hz and the outside–inside splitting is 4.9 Hz. However, when the differences between these values are viewed in the light of the results (line width, 4 Hz; outside–inside splitting, 5 Hz) obtained for DPPC at 55 °C (McLaughlin et al., 1975), they do not appear to be significant.

As mentioned in the previous section, sphingomyelin/phosphatidylcholine mixtures will be considered in a separate study (C. F. Schmidt et al., in preparation). However, one result is relevant to this study of the separate species, namely, that, for these mixtures, the sphingomyelin appears preferentially on the outside of the vesicles, although the magnitude of this asymmetry is not large.

T_1 Measurements. The spin-lattice relaxation times (T_1) for the choline methyl and the acyl-chain methylene and terminal-methyl protons of sphingomyelin in vesicles were measured over the temperature range 22–58 °C. Figure 5 shows the set of partially relaxed spectra used in the calculation of

TABLE II: T_1 Values for Sphingomyelin–Phosphatidylcholine Vesicles.

Vesicle system	Temp (°C)	$N^+(CH_3)_3$ T_1 (s)	$N^+(CH_3)_3$ E_a (kcal/mol)	$(CH_2)_n$ T_1 (s)	$(CH_2)_n$ E_a (kcal/mol)
Beef brain sphingomyelin	50	0.29 ± 0.02	2.1	0.39 ± 0.02	3.3
Beef brain sphingomyelin/$N^+(CD_3)_3$ egg PC 1:2	50	0.32 ± 0.02		0.41 ± 0.02	
Egg phosphatidylcholine[a]	48	0.45 ± 0.01	3.4	0.53 ± 0.02	3.3
DPPC[a]	54	0.57 ± 0.05	3.9	0.45 ± 0.05	
Beef brain sphingomyelin/cholesterol 3:2	52	0.32 ± 0.02		0.31 ± 0.03	
Egg phosphatidylcholine/cholesterol 2:1[a]	48	0.41 ± 0.02		0.18 ± 0.01	

[a] Data from Lee et al. (1972).

the sphingomyelin T_1 values at 58 °C. Measurements and calculations were made as described under Methods. The T_1 values calculated from Figure 5 are 0.33 ± 0.03 s for the choline methyl protons and 0.40 ± 0.04 s for the methylene protons. The measurements were reproducible to ±10% or better at all temperatures. In all cases, the ln $(A_0 - A)/(2A_0)$ vs. τ plot for the methylene protons, as well as the choline methyl protons, gave a single straight line. Proton T_1 values for egg phosphatidylcholine vesicles were also obtained at 24 °C. The results were found to be in close agreement with those of Lee et al. (1972).

The Arrhenius plot of the choline methyl proton T_1 values is shown in Figure 6A. We have also plotted the data of Lee et al. (1972) for the choline methyl protons of egg phosphatidylcholine and DPPC for comparison. The values obtained for the slopes of these plots are given in Table II.

Arrhenius plots of the T_1 values for the acyl-chain methylene and terminal methyl protons, as well as the corresponding data for egg phosphatidylcholine and DPPC (Lee et al., 1972) are shown in Figure 6B. It should be noted that the sphingomyelin T_1 values are determined from only that part of the resonance observed by Fourier transform NMR.

Also included in Table II are T_1 values at 50 °C for vesicles composed of a 1:2 mole ratio mixture of sphingomyelin and $N^+(CD_3)_3$PC. It can be seen that the sphingomyelin choline methyl proton T_1 is not changed significantly by the addition of 67% $N^+(CD_3)_3$PC. Surprisingly, this is also true for the methylene proton T_1 values, which will include contributions from both components. For this mixture, the T_1 plot can still be fit using a single straight line.

We have also investigated the effect of cholesterol on the sphingomyelin proton T_1 values (Table II), and have found results comparable to those obtained for egg phosphatidylcholine/cholesterol mixtures (Lee et al., 1972); i.e., addition of cholesterol changes the choline methyl proton T_1 slightly, but produces a large decrease in the acyl-chain proton T_1 values. We have also found similar effects on the proton line widths of sphingomyelin (Y. Barenholz, unpublished data).

Discussion

The observed differences in the physical properties of sphingomyelin and phosphatidylcholine can be ascribed, on the molecular level, to one or more of three differences between the two substances: (1) The degree of unsaturation in the fatty acid chains is less for natural sphingomyelins than for natural phosphatidylcholines. If the trans-4 double bond of the sphingosine is taken as part of the interface region, rather than part of the acyl-chain region, then the beef brain sphingomyelin used in this study has an average of 0.35 cis double bond/molecule. Egg phosphatidylcholine, the natural lecithin on which most NMR studies have been done, has 1.3–1.4 double bonds/molecule, depending on the preparation. It is therefore more relevant to compare sphingomyelin to synthetic lecithins such as DPPC, rather than egg phosphatidylcholine. (2). In the region of the molecule which can be considered as an interface between the hydrophobic acyl chains and the zwitterionic phosphorylcholine, the glycerol diester moiety of phosphatidylcholine molecules is replaced by a 2-amido-3-hydroxy-*trans*-4-ene moiety in sphingomyelin. It has previously been suggested that the presence of the NH and OH groups in this moiety can lead to the formation of hydrogen bonds between sphingomyelin molecules which are not possible for phosphatidylcholine molecules (Sundaralingam, 1972; Shinitzky and Barenholz, 1974). An additional possibility is the formation of intramolecular hydrogen bonds between these groups and the phosphate group. (3) The fatty amide chain of sphingomyelin will extend further into the bilayer than the hydrocarbon chain of the sphingosine. Using the average fatty acid chain length of 21 carbons, this difference will be either 7 or 5 carbons, depending on whether or not the *trans*-4-ene carbons are taken as parallel to or perpendicular to the bilayer surface. This asymmetry in the chain lengths is in contrast to the symmetry observed for egg and rat liver phosphatidylcholines; i.e., the longer saturated fatty acids are found to be, on the average, attached to the same molecules as the longer unsaturated chains (Kuksis and Marai, 1967; Trewella and Collins, 1973).

Structural studies (Reiss-Husson, 1967; Small, 1967; Shipley et al., 1974), using x-ray diffraction, emphasize the similarities between sphingomyelin and phosphatidylcholine, particularly if the two are compared in their most relevant physical state, i.e., fully hydrated above the gel to liquid-crystalline phase transition. The surface area per molecule for sphingomyelin is found to be the same as that for DPPC, and the lipid core thickness is about 25% greater, due to the presence of a higher percentage of longer chain fatty acids in sphingomyelin.

Qualitatively, the thermotropic behavior of beef brain sphingomyelin, monitored either by proton line widths or fluorescence anisotropy, can be explained by the lower degree of unsaturation found in beef brain sphingomyelin. The effect of the addition of 15 mol % dioleylphosphatidylcholine to DPPC vesicles (making the "average" number of double bonds/molecule equal to 0.3) is to broaden the DPPC transition such that it closely resembles the sphingomyelin transition (Lentz et al., 1976). However, when the sphingomyelin thermal transition is monitored using differential scanning calorimetry, using two natural and several mixtures of synthetic sphingomyelins, the broad transition can be seen to be composed of two or more partially resolved transitions (Barenholz et al.,

1976). This complex phase behavior suggests that there are specific interactions between sphingomyelin molecules beyond those present between phosphatidylcholine molecules. The most probable source of these additional interactions is hydrogen bonding between molecules. These additional interactions could obviously have a marked effect on the physical properties of sphingomyelin aggregates. Ultimately, one would hope to attribute differences in the physical and biological properties between lecithins and sphingomyelins to one, or to a combination of these molecular differences, thus clarifying the structure-function relationship for the individual species, as well. But, while NMR is particularly well suited to this task, in this initial sphingomyelin study it is not generally possible to prove unequivocally that an observed difference is due to a specific molecular cause(s). Further, more detailed studies, using specifically labeled phospholipids, as well as other physical methods, will be necessary. The results of other physical studies support the idea of strong sphingomyelin-sphingomyelin interactions. For example, sphingomyelin liposomes are found to be less permeable to glucose and water molecules than those of egg phosphatidylcholine (Hertz and Barenholz, 1975).

Line Widths. We believe that the fact that the sphingomyelin line widths are greater than those of egg phosphatidylcholine or DPPC also indicates that there are interactions between sphingomyelin molecules that are not present between phosphatidylcholine molecules. Proton line widths for the acyl-chain resonances have been shown to be primarily due to off axis motions of the chains (Seiter and Chan, 1973; Gent and Prestegard, 1977). However, the conclusion that the broader sphingomyelin lines are due to greater intermolecular interactions must be viewed with some caution, and further justification provided. First, the increased sphingomyelin line widths could be due to intramolecular effects; i.e., the 2-amido-3-hydroxy-*trans*-4-ene moiety, which contains both a double bond and an amide linkage would be expected to be intrinsically more rigid than the glycerol diester moiety, and this rigidity could affect the methylene line widths. However, sphingomyelin methylene proton line widths in methanol solution, where the molecules presumably exist as monomers, are not measurably broader than those of phosphatidylcholines, so we conclude that the broader sphingomyelin lines in vesicles are the result of the aggregation of the molecules. ^{13}C spectra, taken in the same solvents, show the same result more clearly (C. F. Schmidt, unpublished data), because of the greater resolution, intrinsically narrower line widths, and absence of proton couplings obtained in decoupled ^{13}C NMR. Second, the size of the vesicles may influence the observed line widths. The sphingomyelin vesicles used in this study have been shown by correlation light scattering to have an average radius of 118 Å (Goll, J., Barenholz, Y., Litman, B. J., and Thompson, T. E., manuscript in preparation), which can be compared to the egg phosphatidylcholine radius of about 105 Å (Huang and Thompson, 1974). But, while it has been shown that vesicles of an average radius of 500 Å have much broader proton resonances (Sheetz and Chan, 1972), it has not been conclusively shown whether small changes (<100 Å) in the radius measurably affect the proton line widths, if other interactions remain the same.[2] However, we would argue that radius effects are not of major importance here, since sphingomyelin/egg phosphatidylcholine mixed vesicles, which have larger radii than those of pure sphingomyelin, have narrower resonances. We believe that the line widths measured for these mixtures reflect the sphingomyelin line widths and possibly strong sphingomyelin-phosphatidylcholine interactions, even though the phosphatidylcholine methylene protons also contribute. Third, the fact that the acyl methylene resonance is composed of overlapping peaks from the methylenes at different positions along the chain (Feigenson and Chan, 1974), and the possible filtering out of broad contributions to the resonances, due to the delay between the end of the pulse and the start of data acquisition necessary in Fourier transform NMR spectroscopy, makes direct comparison of acyl methylene line widths strictly valid only for continuous-wave spectra extrapolated to zero field strength.

The latter two problems, overlapping resonances and contributions from phosphatidylcholine in sphingomyelin-egg phosphatidylcholine mixtures, are overcome by comparing choline methyl proton resonances and using $N^+(CD_3)_3PC$. However, the greater line widths observed for sphingomyelin may also be due to increased intramolecular interactions, as discussed in the next section.

Chemical Shifts. The most clear-cut differences between sphingomyelin and phosphatidylcholine, as detected by NMR, occur in the results obtained for the head-group region of the molecules, i.e., in the choline methyl and the phosphorus resonances. We believe that these differences can be explained by the presence of an intramolecular hydrogen bond between the phosphate and either the amide or the hydroxyl group. Intramolecular hydrogen bonding in sphingomyelin was first suggested by Shah and Schulman (1967). However, the results on which they based this conclusion were later shown to be due to impurities (Colacicco, 1973). More recently, Henderson et al. (1974) have suggested that, in chloroform-methanol solution, the ^{31}P chemical shift differences between phosphatidylcholine and various other phospholipids, among them sphingomyelin, indicate intramolecular hydrogen bonding. The solvent dependence of this shift difference adds further evidence in support of this idea. As the hydrogen bonding ability of the solvent increases, the shift difference between sphingomyelin and egg phosphatidylcholine decreases. The fact that this shift difference persists in aqueous dispersions indicates that the phosphate water of hydration cannot successfully compete with the intramolecular hydrogen bond when the molecules are in a bilayer, otherwise the sphingomyelin and egg phosphatidylcholine shifts would be equal. As further proof, we note that the ^{31}P chemical shift of dihexanoylphosphatidylcholine, which exists as monomers in aqueous solution at concentrations below 10 mM (Tausk et al., 1974), is 0.1-ppm downfield of that of sphingomyelin in bilayers. The upfield shift of the ^{31}P resonance for phosphatidylcholine in bilayers can then be interpreted as being the result of the packing of the molecules. The mechanism of this shift is most likely due to two factors: the partial exclusion of water from the phosphate region, and/or the effect of bringing the negatively charged phosphates into close proximity. Furthermore, the fact that an intramolecular hydrogen bond makes these mechanisms

[2] Gent and Prestegard (1974) approached this problem using soybean phosphatidylcholine-phosphatidylethanolamine mixtures, for which both size and methylene line width increase with increasing phosphatidylethanolamine content. They argue that the radius increases are the result of head-group interactions, and that the increased line widths observed for the methylene protons are the result of an increase in order caused solely by the increased radii, not directly by the changing head-group interaction. The broadening measured is 0.25 Hz/Å. But, while we accept the general validity of their conclusion that line widths are affected directly by size changes, we feel that unequivocal proof of it could only be obtained using systems where head-group interactions are constant, i.e., phosphatidylcholine mixtures with different radii. Further, fatty acid compositions for phosphatidylcholines and phosphatidylethanolamine are normally found to be different.

inoperative (i.e., the sphingomyelin ^{31}P chemical shift is nearly the same as those of lysophosphatidylcholine and sphingosine-phosphorylcholine, which exist as micelles in aqueous solution (Robinson and Saunders, 1958; Hamori and Michaels, 1971), or dihexanoylphosphatidylcholine and glycerylphosphorylcholine, which exist as monomers) suggests that the water exclusion mechanism is the dominant one, since these various species will obviously have varying degrees of charge repulsion, but all are either fully hydrated or intramolecularly hydrogen bonded. Further experiments are under way to test these hypotheses.

The presence of an intramolecular hydrogen bond for sphingomyelin can be used to interpret the other sphingomyelin-phosphatidylcholine chemical shift differences, if it is further assumed that this bond will change the average confirmation of the choline moiety of sphingomyelin with respect to the bilayer surface. The conformation of the phosphatidylcholine head groups has been a matter of controversy (see Lee (1975) for a summary). However, a consensus seems to be emerging in favor of an extended or nearly extended conformation (Hauser et al., 1976; Jendrasiak and Mendible, 1976) for which the ordering axis is perpendicular to the bilayer surface (Stockton et al., 1974; Gally et al., 1975). The presence of an intramolecular hydrogen bond might be expected to change the orientation of the motional averaging axis away from the perpendicular. If so, one consequence would be to increase steric/electrostatic interactions between the cholines of neighboring molecules, which would in turn cause small chemical shifts and, more importantly, make the observed shifts more sensitive to differences in the packing density of the molecules, e.g., those between molecules on the outside and inside of small vesicles. This line of reasoning will explain why the choline methyl proton outside-inside splitting is greater for sphingomyelin than for phosphatidylcholine, despite the fact that the larger sphingomyelin vesicles will have reduced packing differences.

The increased sensitivity of the sphingomyelin ^{31}P and choline methyl proton resonances to lanthanide shift reagents can also be explained using the idea of a different average head-group conformation. Previous work has shown that the binding site for the lanthanide ions is the phosphate group, and that the effect of the shift reagent on proton resonances decreases with increasing distance of the protons from this binding site (Hauser et al., 1975). Using the relative magnitude of these shifts, along with the effect of lanthanide ions on T_1 values and line widths, Hauser and co-workers have shown that, in the presence of shift reagents, the head group is in an extended conformation (Hauser, 1976; Hauser et al., 1976). They have also suggested that this extended conformation would have a tendency to block the access of the hydrated lanthanide ion to the phosphate. Given these results, we can explain the increased effects of shift reagents on sphingomyelin in terms of a different average head-group conformation, since such a conformation would (1) partially remove the blocking effect of the choline group, and (2) bring the $N^+(CH_3)_3$ group closer, on the average, to the binding site.

T_1 Values. Before discussing the T_1 results for sphingomyelin protons, it is useful to summarize the current picture of head-group motions for phosphatidylcholines. The motion of the choline methyl proton-proton vector has been described by Feigenson and Chan (1974). The fast motions about the averaging axis which determine most of T_1 are most likely due to rotations about the $N-CH_3$ and $N-CH_2$ bonds, while the slower, off-axis motions which determine T_2 and the line width will be the result of rotations about the rest of the head-group bonds. The off-axis motions of a given molecule will be coupled to those of neighboring molecules due to steric interactions, so that the line widths reflect the thermal transition. The frequency dependence of the choline methyl proton T_1 for DPPC above its thermal transition (McLaughlin et al., 1973) can be used to estimate that the slow motion contributes about 10% to the total relaxation rate at 100 MHz. This produces a small inflection in the log T_1 vs. $1/T$ plot for DPPC (Figure 6A). However, there is no detectable inflection in the Arrhenius plot for the sphingomyelin-choline methyl proton T_1 values, which probably reflects the fact that the fast T_1 motion is slower for sphingomyelin than for DPPC, and is thus more effective in producing relaxation, so the slow motion contributes relatively less. It also illustrates that the fast, about-axis motion is affected very little by the packing changes which occur at the thermal transition. More importantly, in order to explain why the sphingomyelin-choline methyl proton T_1 values are at all temperatures less than those of egg phosphatidylcholine or DPPC, we are left with the idea of increased steric hindrances of the fast motion. These hindrances can presumably come only from interactions with neighboring molecules, which again supports the idea of a different average conformation for the choline moiety. Furthermore, the fact that the choline methyl proton T_1 values for sphingomyelin in 67% $N(CD_3)_3PC$ vesicles differ very little from those of pure sphingomyelin vesicles provides further support for the presence of an intramolecular hydrogen bond which is nearly unaffected by changing the intermolecular "environment".

At first glance, the T_1 results for the sphingomyelin acyl methylene protons (Figure 6B) might appear to indicate conclusions somewhat similar to those above. However, we note that (1) the values of Arrhenius plot slopes are close to those of egg phosphatidylcholine, (2) T_1 values for DPPC methylenes are close to those of sphingomyelin, and (3) the frequency dependence of the DPPC methylene T_1 values is greater than that of the choline methyl protons, which shows that the slow, off-axis motions contribute relatively more to the methylene relaxation. Results 1 and 2 indicate that the motional processes which are responsible for methylene proton relaxation are the same for both sphingomyelin and phosphatidylcholines. The larger relative contribution of the slow motion to the methylene T_1 values probably indicates that the small differences between DPPC and sphingomyelin reflect either the effect of increased interface interactions on motions of the sphingomyelin fatty acid chains, as seen in the line-width results, or on the translational motion of the molecules, since intermolecular dipolar relaxation has been shown to contribute about 20% of the total acyl methylene proton relaxation rate for DPPC at 50 °C and 100 MHz (Kroon et al., 1976).

In summary, we have implicated three molecular properties of sphingomyelin vesicles which account for the differences in the NMR results for sphingomyelin and phosphatidylcholine. These are the longer and more saturated acyl chains and, more importantly, inter- and intramolecular hydrogen bonds. We note that all of these effects tend to make sphingomyelin-containing bilayers more rigid than those without, and that they may possibly be responsible for the formation of microdomains in biological membranes.

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