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TEMPERATURE-DEPENDENT MORPHOLOGICAL AND PHASE BEHAVIOR OF SPHINGOMYELIN

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Summary

Aqueous dispersions of bovine brain sphingomyelin were studied as a function of temperature. ^{31}P-NMR, X-ray diffraction, and negative-stain and freeze-fracture electron microscopy were used to determine the morphology and phase structure at several temperatures. ^{31}P-NMR indicated a change in phase structure with an increase in temperature. Evidence was found only for the lamellar phase at all temperatures studied with X-ray diffraction. Electron microscopy unexpectedly revealed the spontaneous development of small unilamellar vesicles at elevated temperatures, consistent with the ^{31}P-NMR data, in the absence of any outside disturbances.

Recently, it has become possible to examine the bilayer structure of model and biological membranes with ^{31}P nuclear magnetic resonance (^{31}P-NMR), providing a new view of membrane structure to complement those already in use. Using this approach, erythrocyte ghosts [1], vesicular stomatitus virus membranes [2], chromaffin granule membranes [1] and bovine rod outer segment membranes (Albert, A.D., Yeagle, P.L. and Litman, B.J., unpublished data) have been examined and the majority of phospholipids shown to be in a bilayer structure. However, while pure phosphatidylcholine exhibits ^{31}P-NMR spectra characteristic of the bilayer, pure phosphatidylethanolamine from some sources [3] and diphosphatidylglycerol with calcium [4] show ^{31}P spectra characteristic of the hexagonal phase. ^{31}P-NMR thus provides important clues to the phase structures of phospholipids.

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The purpose of this study is to characterize the phase behavior of natural sphingomyelin. From the data presented here and earlier [5], it is apparent that natural sphingomyelin undergoes an unusual phase change in a temperature range around physiological temperature, a phase change which is inhibited by phosphatidylcholine. Based on ^{31}P-NMR data, this phase change was first interpreted as indicating the formation of a hexagonal phase. In this study, using a variety of experimental approaches, we demonstrate that this is not the case.

Experimental Procedure

Bovine brain sphingomyelin was purchased from Avanti Biochemicals (lot SM8-16) as was the soybean phosphatidylethanolamine.

It was characterized as follows. Two-dimensional thin-layer chromatography showed a single spot. The first dimension was chloroform/methanol/ammonia (65 : 25 : 5, v/v) and the second dimension chloroform/acetone/methanol/acetic acid/water (6 : 8 : 2 : 2 : 1, v/v). The plate was developed with ninhydrin and sulfuric acid charring. The single spot was only seen by charring. The sphingomyelin was hydrolyzed in acidified methanol at 75° C for 1 h. This material was analyzed in the same two-dimensional solvent system, showing several spots, including one ninhydrin positive spot of relatively slow migration (probably representing the sphingosine base). The sphingomyelin was also base hydrolyzed in KOH/methanol for 45 min at room temperature. This material showed only a single spot on two-dimensional thin-layer chromatography that did not react with ninhydrin. These data are consistent with a pure sphingomyelin preparation.

A sample of egg sphingomyelin (lot ESM12) was also purchased from Avanti and used without further characterization.

Human erythrocyte sphingomyelin was obtained from erythrocyte ghosts prepared according to the method of Dodge et al. [6]. The total lipid extract obtained by Folch extractions of the ghosts was separated into its components on a silicic acid column. The sphingomyelin fraction was identified on thin-layer chromatography (chloroform/methanol/water; 65 : 25 : 4, v/v) using an authentic sphingomyelin sample. While this material appeared to be pure by thin-layer chromatography, fatty acid analysis indicated some small contamination by phosphatidylcholine, since minor amounts of linoleate were observed.

For fatty acid analysis of sphingomyelin, methyl esters of fatty acids were formed by acid hydrolysis in anhydrous methanol at 75° C for 1 h. The methyl esters were extracted with ether and separated on a Varian V 2100 gas chromatograph using 4 f diethyleneglycol succinate column (15% HI-EFF-IBP). Methyl esters of stearic acid, α-hydroxylpalmitic acid, α-hydroxystearic acid and α-hydroxyeicosonioc acid were co-injected as standards. The remaining components were identified according to their relative retention times.

Sphingomyelin samples were prepared for all the measurements by hydrating lyophilized sphingomyelin in 100 mM NaCl or in deionized water. No difference in behavior in these two solvents has been observed.

^{31}P-NMR spectra were obtained on a Varian XL-100 spectrometer, equipped

with a Nicolet FT package. 20 kHz spectra were obtained with 1K data points and continuous broadband proton decoupling, in 10-mm tubes. ^{13}C-NMR spectra were obtained on the same instrument, with 4K data points, 5 kHz spectra width and continuous broadband decoupling.

X-ray diffraction data were recorded in a Frank-type camera using Kodak no screen X-ray film. The X-ray source was nickel-filtered copper Kα, generated in a Jerryl-Ash microfocusing unit. Fully hydrated samples of sphingomyelin suspension were sealed either in glass tubes (Supper) in room temperature experiments, or in an aluminum sample-holder with mica windows for controlled temperature experiments. The temperature of the specimen in the latter case was regulated by a thermoelectric module, controlled by a thermal-sensor power supply. The temperatures of the specimen were maintained to within 0.5° C over a usual exposure time of 12 h. The specimen and the holder were equilibrated in a Blue M oven for 15 min before loading to eliminate differential thermal expansion between the sample and the holder which might lead to the rupture of the window.

For negative-stain electron microscopy, the sample, pipets, carbon-coated grids and staining solution (2% ammonium molybdate) were equilibrated in a Blue M oven for 15 min to ensure that the specimen temperature remained within 2° C of the set value during the entire negative-stain process. The negatively stained specimen was viewed in a Siemens 101 microscope at a magnification of 55 000 ×.

Freeze-fracture specimens were thermally equilibrated as described previously by Stewart et al. [7]. The specimen was quenched from the desired temperature in slush Freon 22. The frozen specimen was fractured and replicated in a Polaron E7500 unit under a vacuum of $5 \cdot 10^{-7}$ Torr. The replicas were cleaned in chloroform vapor and viewed in a Siemens 101 microscope at magnifications up to 55 000 ×.

Results

Previous ^{31}P-NMR data had shown a considerable change in the motional averaging of the phosphate of sphingomyelin in unsonicated dispersions as a function of temperature [5]. The same kind of data were obtained here with a new lot of sphingomyelin. This material behaved very similarly to that labelled lot I previously reported, as is seen in Fig. 1.

At low temperature, the ^{31}P-NMR spectra of bovine brain sphingomyelin (previously referred to as lot I) resemble that of several phosphatidylcholines previously reported. The proton dipolar coupling has been reduced so that the residual chemical shift anisotropy dominates the spectra. This has been shown to be characteristic of bilayer structure [8]. As the temperature is increased, a broad isotropic resonance grows at the expense of the anisotropic bilayer resonance.

In order to gain a better understanding of the origins of the temperature-dependent changes in the ^{31}P-NMR spectra, X-ray diffraction data were obtained on this sphingomyelin at temperatures where the ^{31}P-NMR data indicated bilayer structure. At 25° C, the X-ray diffraction pattern shows a series of small-angle lines at a spacing ratio of 1, 0.5, 0.33, . . . , indicating a

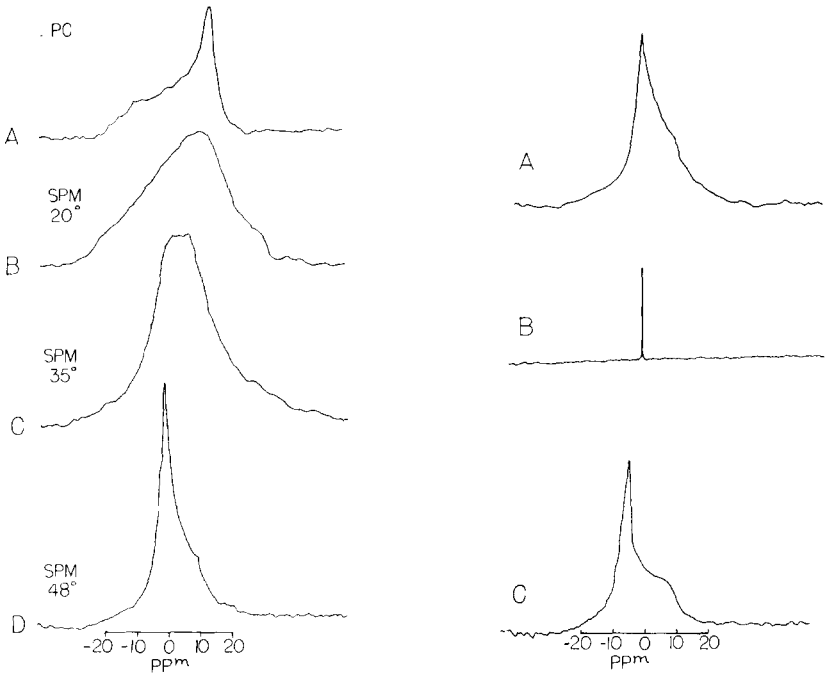


Fig. 1. 40 MHz ^{31}P-NMR spectra of egg phosphatidylcholine and bovine brain sphingomyelin in 0.1 M NaCl. (A) Phosphatidylcholine (PC) at 25°C; (B) sphingomyelin (SPM) at 20°C; (C) sphingomyelin at 35°C; (D) sphingomyelin at 48°C. The sphingomyelin is that referred to in the text as lot I.

Fig. 2. 40 MHz ^{31}P-NMR spectra of bovine brain sphingomyelin and soy phosphatidylethanolamine in 0.1 M NaCl. (A) Unsonicated sphingomyelin at 48°C; (B) Sonicated sphingomyelin (sonicated to clarity in a probe sonicator at 4°C); (C) unsonicated soy phosphatidylethanolamine at 30°C.

one-dimensional crystalline lattice. The repeat distance is 74 Å. Wide-angle diffraction consists of a single line of spacing 4.2 Å. This pattern is characteristic of a lamellar phase with the hydrocarbon chain packing in the gel state (i.e., L_β or L'_β). The diffraction patterns taken with the specimen maintained at 51°C are similar, except that the low-angle repeat spacing changes to 62.5 Å, and the high-angle line is replaced by a 4.6 Å band, indicating hydrocarbon chain packing is now in the liquid-crystalline state. The one-dimensional lattice repeat at the low-angle diffractions rules out the possibility of any significant portion of the sample being in a hexagonal phase (H). The finding is consistent with those reported by Shipley et al. [9] and Khare and Worthington [10] using sphingomyelins of different fatty acid compositions.

Since X-ray diffraction detected only the lamellar phase throughout the temperature range measured, negative-stained electron micrographs were obtained to see if any structural differences were evident between specimens stained and dried at 25 and 55°C. These results are shown in Fig. 3. At 25°C, mostly multilammellar or larger unilamellar vesicles are seen. At 55°C, a very different collection of vesicles is observed. Some multilamellar liposomes can still be seen, but interspersed among them are a large number of small disk-shaped or cup-chaped structures, about 500 Å in diameter.

Freeze-fracture electron micrographs provided similar results. At 25°C,

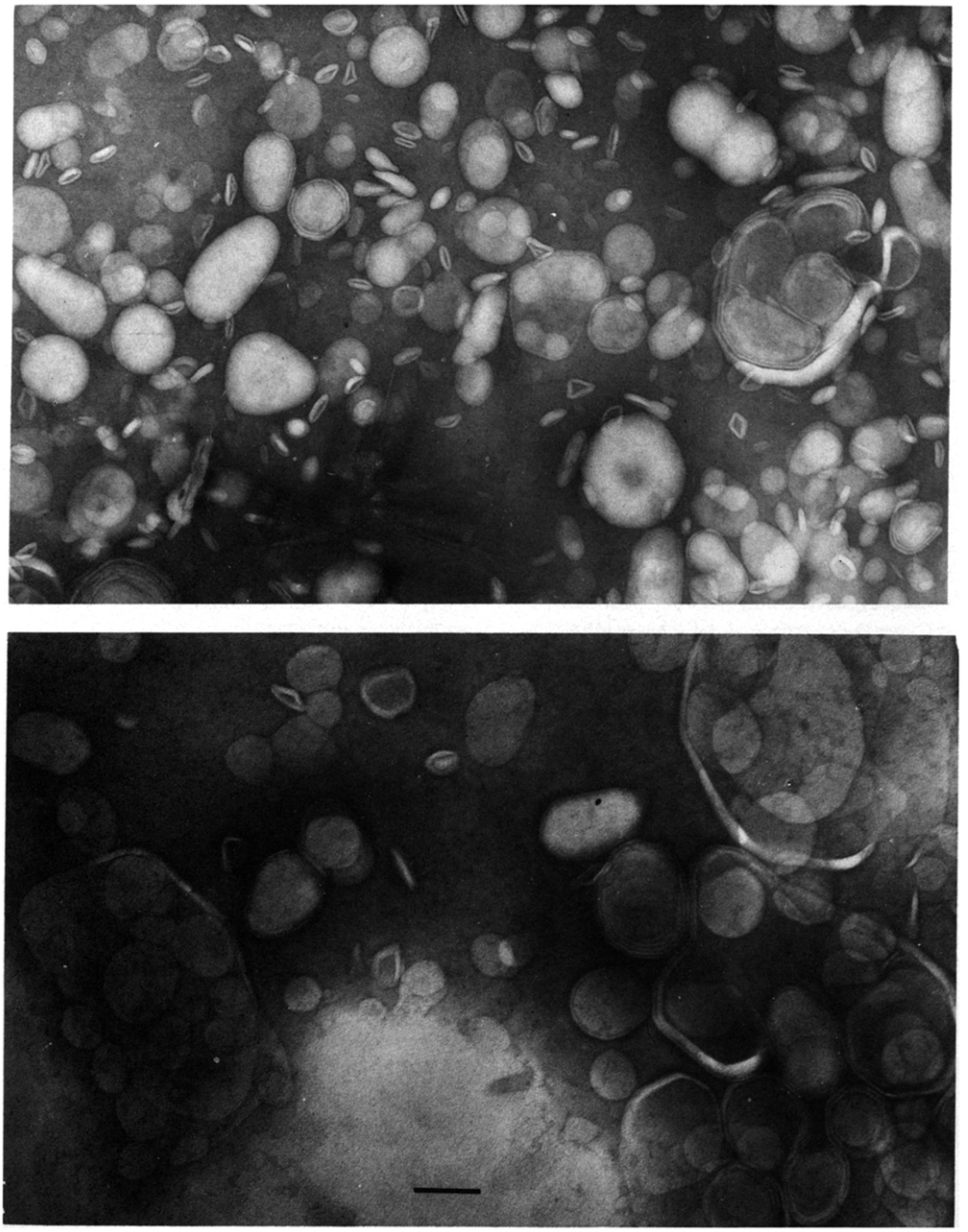
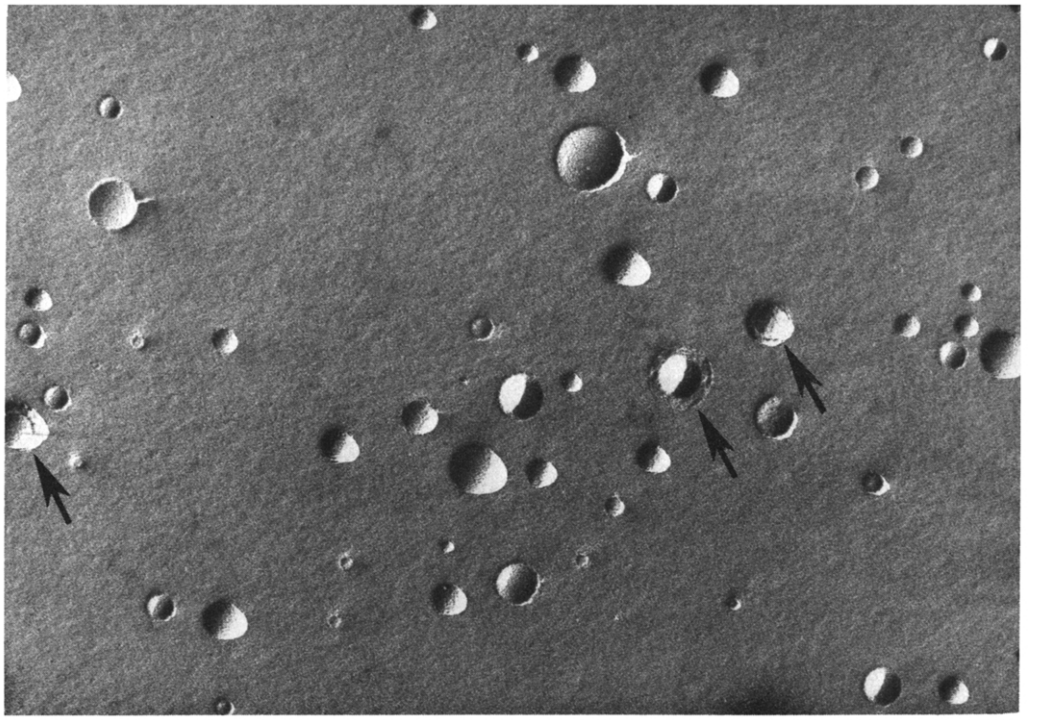


Fig. 3. Negative-stain (with 2% ammonium molybdate) electron micrographs of an aqueous suspension of lot L bovine brain sphingomyelin. (A) Typical view of samples stained and dried at 55°C showing a high percentage of shell-like unilamellar vesicles; (B) typical view of samples stained and dried at 25°C showing a high percentage of large multilamellar vesicles. Bar = 100 nm.

most of the lipids are in the form of multilamellar vesicles the surface of which is covered with banded patterns, a characteristic of the P_{β} phase [7]. At 55°C, however, the majority of the lipid is in the form of small vesicles 500 ± 50 Å (S.D.) in diameter (Fig. 4). The few remaining large vesicles show a jumbled



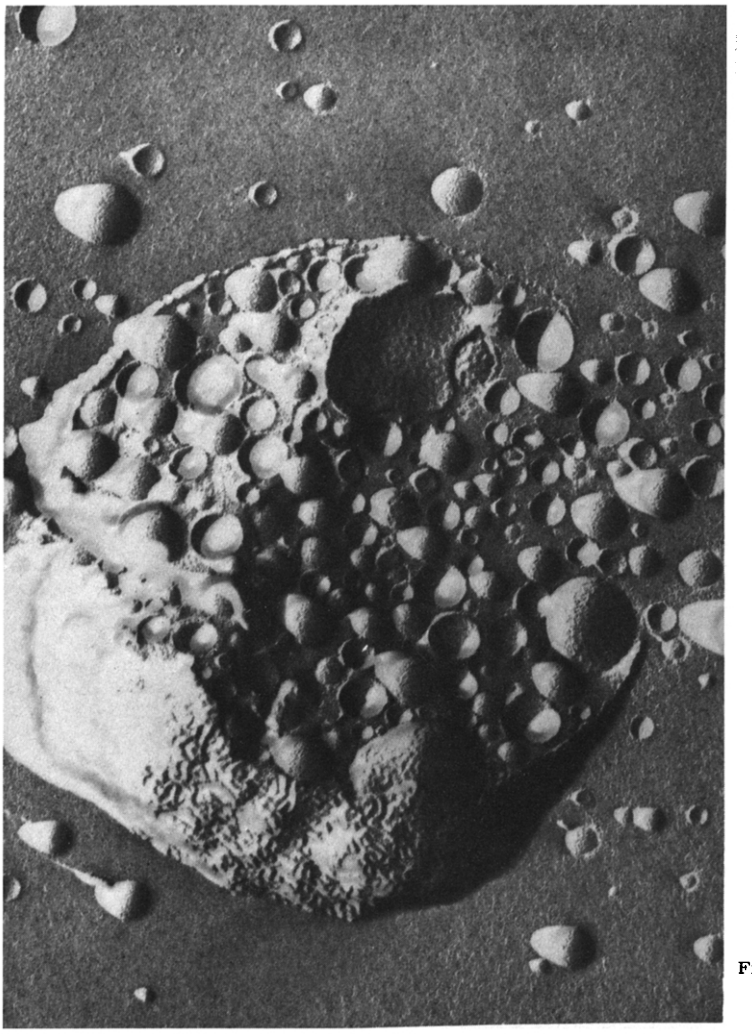


Fig. 4. A, top; B, bottom.

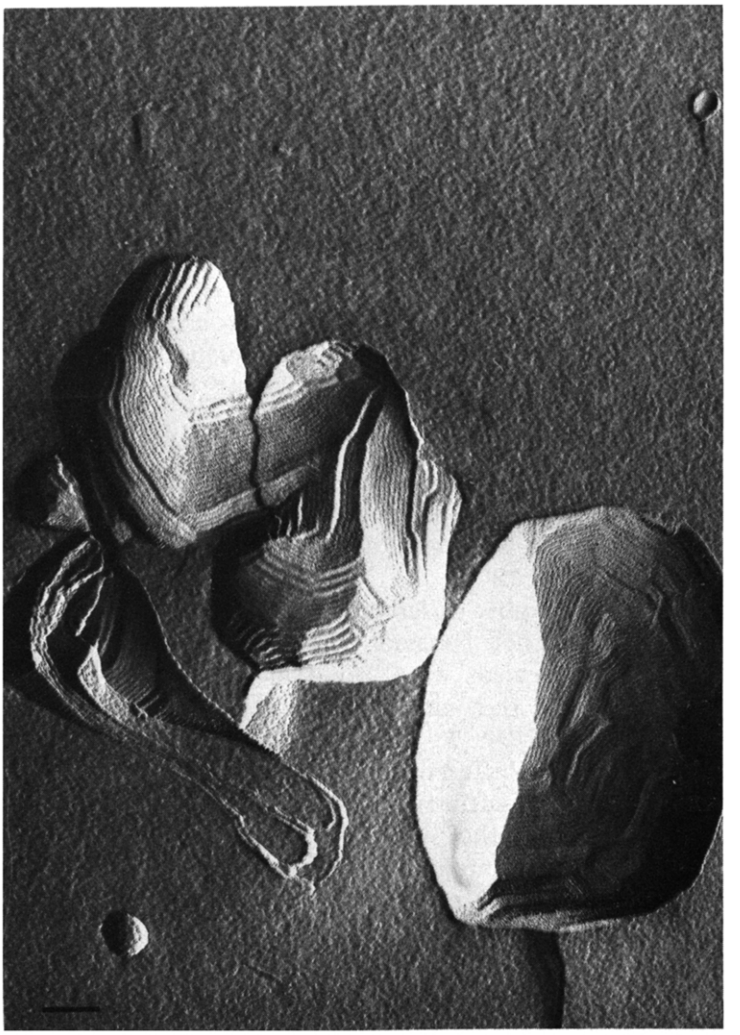


Fig. 4. Freeze-fracture electron micrographs of aqueous suspensions of lot I bovine brain sphingomyelin samples freeze-quenched from 58°C show small unilamellar vesicles either in free suspension (A), or within a large vesicle (B). The larger fracture faces show 'jumbled' patterns typical of a phospholipid in the L_α state. Shell-like vesicles ares indicated by arrows. Samples freeze quenched from 25°C show predominantly large multilamellar vesicles (C). The banded pattern seen on the fracture face is a characteristic of a phospholipid in the P_β phase. Bar = 100 nm.

pattern on the surface, characteristic of lipids in the liquid-crystalline (L_α) phase [7]. Inside some of these large vesicles there are numerous small vesicles of the same diameter as the free ones (Fig. 4C).

It is likely that the shell-like vesicles that predominate in negative-stained micrographs and the small vesicles that predominant in freeze-fracture micrographs of the high-temperature sample are the same. Their diameters are very similar. Some of these vesicles might have collapsed during the negative-staining process. On the other hand, in the freeze-fracture micrographs, some small vesicles have a broad rim which may be interpreted as a rim of a shell. The two

sides of the shell have the same sense of curvature.

As a control, some of the sphingomyelin referred to previously as lot II sphingomyelin [5] was examined. ^{31}P-NMR spectra showed little change as a function of temperature until reaching nearly 80°C, when a second component was introduced into the spectrum [5]. At 25 and 55°C, ^{31}P-NMR was consistent with bilayer structure, in vesicles large enough to tumble sufficiently slowly so as not to average motionally the residual chemical shift anisotropy. X-ray diffraction results with this sphingomyelin gave lamellar repeats of 56 Å at both temperatures. Wide-angle diffraction produced a single line at 4.3 Å at 25°C and no reflection was observed 56°C. Electron micrographs at the two temperatures show multilamellar or large unilamellar liposomes at both temperatures. No appearance of the small disk-like structures described above is apparent.

Two other samples of sphingomyelin were also looked at with ^{31}P-NMR. A sample of egg sphingomyelin at 30 and 50°C did not show any evidence of unusual phase behavior; a normal bilayer spectrum was obtained. A sample of human erythrocyte sphingomyelin, however, did show substantial development of the isotropic phase as low as 32°C.

Since the sphingomyelins behave quite differently, it was expected that their fatty acid content might influence their behavior. The analysis is presented in Table I. Essentially the same fatty acids were found in each sample, but in different relative amounts. The lot II bovine brain sphingomyelin has as its dominant components, 18 : 0 and 24 : 1, which qualitatively agrees with previous analyses [11,12]. Lot I is different in that it contains some α-OH fatty acids (which comigrated with authentic standards), even though thin-layer chromatography demonstrated that it was pure sphingomyelin, and very low content of 24 : 1. The erythrocyte sphingomyelin also has a low content of 24 : 1. Whether this difference can be related to the difference in

TABLE I

SPM, sphingomyelin; RBC, red blood cells.

Fatty acid	% content lot I SPM	% content lot II SPM	% content RBC SPM
14:0	trace	trace	trace
15:0	trace	trace	—
16:0	10	trace	19
16:0 (αOH)	12	trace	trace
17:0	trace	trace	trace
18:0	20	18	7
18:0 (αOH)	17	trace	trace
18:1	—	—	7
18:2	—	—	9
19:0	trace	trace	—
20:0	trace	trace	trace
22:0	17	5	9
22:1	—	5	—
24:0	12	5	18
24:1	6	52	17

behavior cannot be determined until detailed work with synthetic sphingomyelins can be done.

According to the previous interpretation of the ^{13}C-NMR spectra of sphingomyelin in C^2HCl_3, the downfield resonance in the carbon-carbon double bond region arises from the double bond in sphingosine [5]. As such, it is indicative of the dihydrosphingosine/sphingosine ratio. ^{13}C-NMR spectra of the two bovine brain sphingomyelins discussed above indicated that the two sphingomyelins had about the same dihydrosphingosine content; sensitivity limitations prevented a more quantitative determination of the composition. The other resonance in that region reflected the greater unsaturation in the type II sphingomyelin.

The result reported previously with the paramagnetic lanthanide ion [5] is not observed on any of the present lots of sphingomyelin. Since all the reported observations were repeatable, the difference in behavior must have arisen due to differences in the various lots. Unfortunately, the older lots are no longer available for comparison.

Discussion

Previous ^{31}P-NMR results with bovine brain sphingomyelin suggested that an increase in temperature induced changes in the phase structure of some but not all sphingomyelins which were evident in the ^{31}P-NMR spectra at or above 37°C [5].

The structure of the high-temperature phase of bovine brain sphingomyelin could not be identified with certainty from the ^{31}P-NMR spectra. Cullis and de Kruijff [3] recently presented an analysis of ^{31}P-NMR spectra of unsonicated dispersions of phosphatidylcholine and phosphatidylethanolamine, which was consistent with the known structures of the phases these two lipids formed at various temperatures. Many kinds of phosphatidylcholine are known from X-ray diffraction studies to form the lamellar phase. The residual ^{31}P chemical shift anisotropy seen in unsonicated dispersions of phosphatidylcholine is the motionally averaged result when the chemical shift tensor of the phosphate is rotated rapidly about one axis in a lamellar phase.

In the hexagonal phase which some phosphatidylethanolamines form, an additional degree of motional averaging occurs from rapid diffusion about the cylindrical structures of that phase. This produces both theoretically and experimentally a ^{31}P-NMR spectrum with a residual chemical shift anisotropy of half the magnitude and of the opposite sign compared to the bilayer phase, if all other properties of the headgroup are the same [8].

The spectra of the high-temperature 'phase' of sphingomyelin have a lineshape which resembles that of the hexagonal H_{II} phase of unsaturated phosphatidylethanolamines. However, as can be seen from Fig. 2, the chemical shift position of the main peak does not correspond to that of the phosphatidylethanolamine hexagonal H_{II} phase, but is similar to that of phospholipids undergoing isotropic motion. X-ray data show no hexagonal phase present, above or below the temperature range in which the ^{31}P-NMR spectra indicate a structural change is taking place, although a phase transition from the gel to the liquid-crystalline state does occur.

The electron micrographs provide an explanation for these data. In and above the temperature region in which a structural change is evidently taking place, the electron micrographs reveal the development of unusual, small disk-shaped structures or vesicles, about 500 Å in diameter. The structure of these small vesicles is not clear at this time. They appear to be different from the large multilamellar structures normally associated with unsonicated phospholipids. Since they are much smaller than the multilamellar liposomes seen at lower temperatures, the small vesicle must have a significantly shorter rotational correlation time. Calculations indicate that a rotational correlation time in range of 10^{-4} to 10^{-5} s could be expected which is sufficiently rapid to introduce an additional mechanism for averaging the chemical shift tensor of the phosphate. It would be expected in this case to see only a broad isotropic ^{31}P resonance arising from these small vesicles, with little of the residual chemical shift anisotropy remaining. Therefore, if the small vesicles give rise to a broad but isotropic ^{31}P resonance and the large liposomes still show the broader resonance with the chemical shift anisotropy expressed, the summation of these two components would give a resonance with the shape observed in Fig. 1. The continued existence of a fraction of large, multilamellar liposomes would provide a source for the lamellar reflections in the X-ray data.

It is intriguing to find that small unilamellar vesicles are formed spontaneously as the temperature is increased through the transition point. The formation of unilamellar vesicles is seen to start at least in part inside large multilamellar vesicles (Fig. 3) where materials are less susceptible to mechanical disruption and surface tension. The 500-Å vesicles formed, therefore, seem to be a preferred morphology of some sphingomyelins above the transition temperature.

Since all sphingomyelin samples studied here contained a variety of fatty acids, the 'phase transition' is not expected to be a well defined one. Nevertheless, ^{31}P-NMR, X-ray diffraction and electron microscopy results demonstrated a gradual but definite structural change in the lot I bovine sample. The change occurs both in vesicle morphology and in molecular packing, i.e., from large multilamellar vesicles to small unilamellar vesicles, as well as from gel to liquid-crystalline state. In the same temperature range, the transition is less complete with the lot II specimen, as shown by results from all three experimental methods. The results reported here may provide clues to the unusual calorimetric behavior of the natural sphingomyelins [10,11].

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References

1 McLaughlin, A.C., Cullis, P.R., Hemminga, M.A., Hoult, D.I., Radda, G.K., Ritchie, G.A., Seeley, P.J. and Richards, R.E. (1975) FEBS Lett. 57, 213
2 Moore, N.F., Patzer, E.J., Wagner, R.R., Yeagle, P.L., Hutton, W.C. and Martin, R.B. (1977) Biochim. Biophys. Acta 464, 234
3 Cullis, P.R. and de Kruijff, B. (1978) Biochim. Biophys. Acta 507, 207
4 Cullis, P.R., Verkleij, A.J. and Ververgaert, P.H.J.T. (1978) Biochim. Biophys. Acta 513, 11
5 Yeagle, P.L. Hutton, W.C. and Martin, R.B. (1978) Biochemistry 17, 5745
6 Dodge, J.T., Mitchell, C. and Hanahan, D.J. (1962) Arch. Biochem. Biophys. 100, 119
7 Stewart, T.P., Hui, S.W., Portis, A.R. and Papahadjopoulos, D. (1979) Biochim. Biophys. Acta 556, 1
8 Seelig, J. (1978) Biochim. Biophys. Acta 515, 105
9 Shipley, G.G., Avecilla, L.S. and Small, D.M. (1974) J. Lipid Res. 15, 124
10 Khare, R.S. and Worthington, C.R. (1978) Biochim. Biophys. Acta 514, 239
11 Barenholz, Y., Suurkuusk, J., Mountcastle, D., Thompson, T.E. and Biltonen, R.L. (1976) Biochemistry 15, 2441
12 Untracht, S.H. and Shipley, G.G. (1977) J. Biol. Chem. 252, 4449