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A Calorimetric Study of the Thermotropic Behavior of Aqueous Dispersions of Natural and Synthetic Sphingomyelins†

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ABSTRACT: A recently developed differential scanning calorimeter has been used to characterize the thermotropic behavior of aqueous dispersions of liposomes containing sphingomyelin. Liposomes derived from sheep brain sphingomyelin exhibit a broad gel–liquid crystalline phase transition in the temperature range of 20–45 °C. The transition is characterized by maxima in the heat capacity function at 31.2 and 37.1 °C and a total enthalpy change of 7.2 ± 0.4 kcal/mol. Beef brain sphingomyelin liposomes behave similarly but exhibit heat capacity maxima at 30, 32, and 38 °C and a total enthalpy change of 6.9 kcal/mol. The thermotropic behavior of four pure synthetic sphingomyelins is reminiscent of multilamellar lecithin liposomes in that a single, sharp, main transition is observed. Results obtained for liposomes containing mixtures of different sphingomyelins are complex. A colyophilized mixture of *N*-palmitoylsphingosinephosphorylcholine, *N*-stearoylsphingosinephosphorylcholine, and *N*-lignocerylsphingosinephosphorylcholine in a 1:1:1 mol ratio exhibits a single transition with a T_m below that observed for the individual components. On the other hand a 1:1 mixture of *N*-stearoylsphingosinephosphorylcholine and 1-palmitoyl-2-oleylphosphatidylcholine exhibits three maxima in the heat capacity function. It is clear from these results that the thermotropic behavior of sphingomyelin-containing liposomes is a complex function of the exact composition. Furthermore, it appears that the behavior of the liposomes derived from natural sphingomyelins cannot be explained in terms of phase separation of the individual components.

Considerable information relating to the physical properties and molecular organization of glycerophospholipids in bilayer vesicles (Huang, 1969; Suurkuusk et al., 1976; Lee, 1975) or multilamellar liposomes (Bangham et al., 1967; Hinz and Sturtevant, 1972; Chapman, 1968; Ladbrooke and Chapman, 1969) has been obtained from calorimetric studies of their thermotropic behavior. However, little similar information about sphingomyelin liposomes is available. This latter type of liposome is particularly intriguing in that the sphingomyelins undergo thermotropic phase transitions in the physiological temperature range (Shinitzky and Barenholz, 1974; Shipley et al., 1974).

Shipley et al. (1974) have recently reported that an aqueous dispersion of mixed sphingomyelins derived from bovine brain undergoes a complex series of thermally induced transitions, exhibiting several maxima in the heat capacity function in the temperature range of 30–45 °C. Those workers suggested that the distinguishable transitions may be the result of phase separation of the components, although strong evidence for this proposal was lacking.

In this communication calorimetric results on the thermotropic behavior of aqueous dispersions of sphingomyelins derived from sheep and bovine brain, of four essentially pure synthetic sphingomyelins, of a 1:1:1 colyophilized mixture of three of the synthetic sphingomyelins, and of mixtures of a pure sphingomyelin with 1-palmitoyl-2-oleyl-L-α-phosphatidylcholine are reported. The results obtained with the naturally occurring sphingomyelins are in essential agreement with those of Shipley et al. (1974). However, the results obtained with the pure sphingomyelins demonstrate that the special "rules" governing the thermotropic behavior of glycerophospholipids do not apply to the sphingomyelins; that the phase transition characteristics of sphingomyelin-containing liposomes are a complex function of the exact composition; and that it is unlikely that the thermotropic behavior of natural sphingomyelin liposomes is the result of phase separation of the components.

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TABLE I: Fatty Acid Composition of Sheep Brain and Bovine Brain Sphingomyelin.

Fatty Acid[a]	% of Total	
	Sheep Brain	Bovine Brain
C_{14}:0		<0.1
C_{16}:0		2.8
C_{18}:0	65.5	35.9
C_{19}:0	0.4	<0.1
C_{20}:0	5.5	0.9
C_{22}:0	7.9	4.4
C_{23}:0	3.9	3.7
C_{24}:0	10.9	9.7
C_{25}:0	<0.1	4.7
C_{26}:0	<0.1	1.2
C_{18}:1	<0.1	<0.1
C_{24}:1	5.9	30.8
C_{25}:1	<0.1	3.9

[a] Designated C_m:n where m is the number of carbon atoms and n the number of double bonds in the fatty acid side chain.

TABLE II: Sphingosine Base Composition in Various Sphingomyelin Preparations.

Sphingomyelin	% of Total	
	Sphingosine	Dihydrosphingosine
Sheep brain sphingomyelin	84	16
Bovine brain sphingomyelin	89	11
N-Palmitoylsphingosine-phosphorylcholine	98	2
N-Stearoylsphingosinephosphorylcholine	95	5
N-Lignocerylsphingosine-phosphorylcholine	95	5
N-Palmitoyldihydrosphingosinephosphorylcholine	<0.5	>99.5

Experimental Section

Materials and Preparation of Liposomes. Sheep and beef brain sphingomyelins were prepared, purified, and analyzed as described by Shinitzky and Barenholz (1974). Their fatty acid analysis is summarized in Table I. Analysis of the sphingosine bases showed that more than 96% of the total bases were normal 18 carbon atom chains. The relative amounts of sphingosine and dihydrosphingosine are given in Table II.

N-Palmitoylsphingosinephosphorylcholine, *N*-palmitoyldihydrosphingosinephosphorylcholine, *N*-stearoylsphingosinephosphorylcholine, and *N*-lignocerylsphingosinephosphorylcholine were of the D,L erythro configuration and a gift of Professor D. Shapiro, Weizmann Institute of Science, Rehovot, Israel. All the synthetic sphingomyelins were found to be chromatographically pure. Analysis (Shinitzky and Barenholz, 1974) showed that the amount of the desired fatty acid was greater than 99.8% for all synthetic sphingomyelins. Analysis of the sphingosine bases, summarized in Table II, showed that the various sphingomyelins were composed primarily of sphingosine with small amounts (<5%) of dihydrosphingosine.

1-Palmitoyl-2-oleyl-L-α-phosphatidylcholine was prepared and purified as described by Lentz et al. (1976). Fatty acid analysis demonstrated that the lecithin was composed of greater than 98% palmitic acid and oleic acid in the first and second positions, respectively.

Approximately 10 mg of the desired phospholipid or phospholipid mixture was lyophilized from benzene solution. The dry lipid was then suspended in 1 ml of 50 mM KCl at 70 °C. Multilamellar liposomes were prepared by vigorous stirring of the aqueous KCl solution for 1 min at 70 °C followed by agitation in a rotating shaker for 1 h at 60 °C. The H_2O used to prepare the solutions was deionized, distilled from alkaline $KMnO_4$, and glass-redistilled. Extra-pure KCl (Heico, Inc.) and ultrapure sucrose (Schwarz/Mann) were used for the preparation of the solutions. The lipid concentration of each dispersion was measured as inorganic phosphate as described by Bartlett (1959). At least ten phosphate determinations were made on each sample with a resulting standard deviation of ±1%.

Scanning Calorimetry. A newly developed differential scanning calorimeter of the heat conduction type (Ross and Goldberg, 1974) was used for these studies. The calorimeter was designed for measuring heat capacities and heat effects accompanying thermally induced transitions in dilute solution. The temperature range for the instrument is 0–75 °C, and scanning rates from 3 to 50 °C/h can be selected. The total volume of the sample compartment is approximately 0.7 ml. The precision in terms of baseline "noise" is better than ±25 μcal/°C. Absolute temperature determination is better than ±0.05 °C.

All experiments were begun by placing the sample in the calorimeter and cooling it to about 0 °C. The heat capacity was then measured during heating at a rate of 15 °C/h. Details of the construction and operation of the calorimeter will be described in a future publication. A brief description of the calorimeter and calculation of ΦC_p are given in Suurkuusk et al. (1976).

Drop Heat Capacity Calorimetry. The heat released upon changing the temperature of a sample from some temperature, T, to a reference temperature, T_r, was measured using a drop heat capacity calorimeter constructed by Alvarez (1973) based on the design of Konicek and Wadso (1971). The sample, contained in an 0.8-ml stainless steel ampule, was incubated for approximately 30 min in a furnace maintained at temperature $T \pm 0.0001$ °C. It was then dropped into the calorimeter at temperature T_r which resulted in a temperature rise, ΔT, in the calorimeter cell. This temperature rise produced a time-dependent voltage proportional to the temperature rise. The total heat released is calculated from

$$Q_s = \epsilon \int_0^\infty V \mathrm{d}t$$

where ϵ is the calibration constant of the calorimeter, and t is time. Q_s is equal to the enthalpic difference between the sample at temperature T and T_r. The excess enthalpy is then calculated by subtracting the enthalpy difference of an equivalent amount of buffer:

$$\Delta H_{ex}(T) = [Q_s - Q_{buffer}]/M$$

where M is the number of moles of phospholipid in the ampule.

The above experiment was repeated at approximately 0.5 °C intervals by raising the temperature of the furnace incrementally to provide a set of $\Delta H_{ex}(T)$ data over the temperature range 20–45 °C. ϵ was determined by electrical calibration using a 50-Ω heater located in the calorimeter cell. Details of the construction and calibration of the calorimeter and of the calculation can be found in Alvarez (1973).

Fluorescence Measurements. 1,6-Diphenyl-1,3,5-hexatriene (DPH)[1] was incorporated into bilayers of multilamellar lipo-

[1] Abbreviation used is: DPH, 1,6-diphenyl-1,3,5-hexatriene.

somes at a mole ratio of 1:1000 as described by Shinitzky and Barenholz (1974). Fluorescence measurements were made on a modified Perkin-Elmer MPF 3 spectrofluorometer as described in Suurkuusk et al. (1976). The temperature of the sample chamber was controlled to ±0.10 °C by means of a Lauda MK-2 thermoregulated bath. When fluorescence depolarization was determined as a function of temperature the sample in the cuvette was heated to 55 °C and then cooled at a rate of 20 °C/h. Fluorescence intensity and temperature measurements were made continuously during cooling. DPH was excited at 360 nm and fluorescence recorded at 430 nm using instrument filter 39 as a cutoff for wavelengths below 390 nm.

The general theory of the fluorescence probe depolarization technique as applied to membrane structure has been outlined by Cogan et al. (1973). The use of DPH as a fluorescent probe specific for the hydrophobic region of lipid bilayer has been described by Shinitsky and Barenholz (1974). The microviscosity was calculated as a function of temperature according to the Perrin equation as described by Shinitzky et al. (1971). The lifetime of DPH, estimated from the fluorescence intensity as described by Shinitzky and Barenholz (1974), was measured at 10, 15, 25, and 40 °C using the phase modulation technique (Spencer, 1970). Additional details of this technique, as applied to the study of thermotropic transitions of phospholipid vesicles, are described in Suurkuusk et al. (1976).

Results

Natural Sphingomyelins. A recent calorimetric study of the thermotropic behavior of multibilayered liposomes prepared from bovine brain sphingomyelin has demonstrated that, if the H_2O content is greater than about 50%, such liposomes exhibit complex transition characteristics centered at approximately 37 °C (Shipley et al., 1974). These studies were performed at the extremely high scanning rate of 300 °C/h. Comparison of cooling and heating scans indicated sample hysteresis. Thus, it is possible that these results do not apply to a reversible, equilibrium situation.

Our scanning calorimetric results obtained for a dilute (~2.5%) aqueous suspension of sheep brain sphingomyelin liposomes at a scanning rate of 15 °C/h are shown in Figure 1A. Curve A was obtained with a freshly prepared suspension. Curve B (solid line) was obtained after storing the sample in the cold for approximately 1 week, reloading the calorimeter, and repeating the experiment. The open circles represent a repeat of experiment B after cooling the sample to ~0 °C in the calorimeter. In all three experiments two distinct maxima in the heat capacity function, ΦC_p, are observed at 31.2 ± 0.1 °C and 37.1 ± 0.1 °C.

The results in Figure 1A are values of the absolute apparent heat capacity of the lipid in the aqueous suspension. The difference in ΦC_p at 20 °C calculated from experiment A and B is 50 cal/mol deg. This is equivalent to an absolute error of about 8×10^{-4} cal/deg in these experiments. This error most probably represents the accuracy with which the sample ampule can be loaded. The difference in ΦC_p at 20 °C, calculated from the two repeat experiments in which the sample was maintained in the calorimeter, is 5 cal/mol deg or about 8×10^{-5} cal/deg. This error is a more realistic estimate of the absolute precision of the calorimeter.

The enthalpy change, ΔH_{ex}, for the gel–liquid crystalline transition of sheep brain sphingomyelin was calculated by integration of the ΦC_p curve from 15 °C to temperature T.

$$\Delta H_{ex} = \int_{15\,^\circ C}^{T} (\Phi C_p(T) - \Phi C_p(15\,^\circ C))dT$$

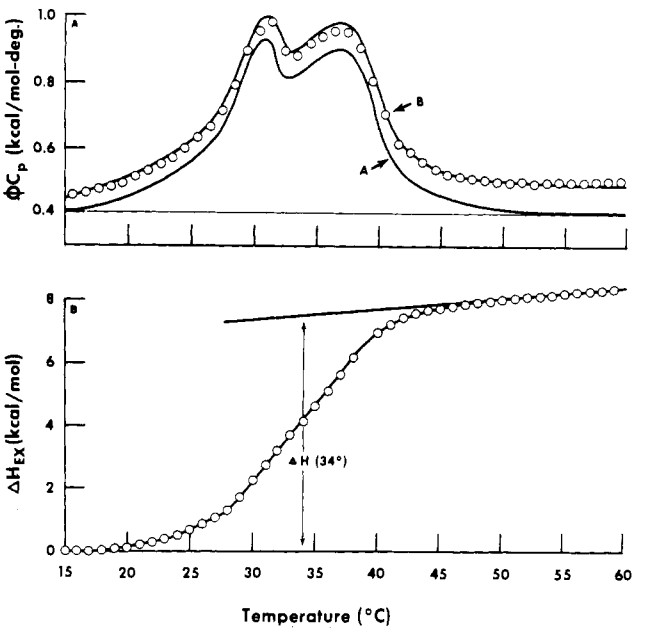

FIGURE 1: (A) ΦC_p vs. temperature for sheep brain sphingomyelin liposomes (concentration = 30 mg/ml). (B) ΔH_{ex} vs. temperature as calculated from the results in Figure 1A and described in the text. The size of the circles correspond to errors in ΦC_p of ±10 cal/mol deg and of 100 cal/mol in ΔH_{ex}. The solid line in A is the heat capacity baseline used to calculate ΔH_{ex}. The solid line in B is the extrapolated representation of ΔH for the transition.

Such a calculation is equivalent to estimation of the area between the ΦC_p curve and the heat capacity baseline. An example is shown for curve A in Figure 1A. The results of such a calculation for the repeat of experiment B (Figure 1A) are shown in Figure 1B where ΔH_{ex} is plotted vs. temperature. In this particular experiment an apparent positive heat capacity change for the transition was observed, i.e., $\Phi C_p(15\,^\circ C) < \Phi C_p(60\,^\circ C)$, such that ΔH for the transition appears to be temperature dependent. The magnitude of ΔC_p was not strictly reproducible and therefore in order to compare ΔH values from different experiments the limiting value of ΔH_{ex} at high temperature was extrapolated back to 34 °C, the mean of the two transition temperatures, as indicated by the solid line shown in Figure 1B. This correction amounted to less than 1 kcal/mol in all experiments.

An index of the sharpness of the transition is the magnitude of the maximum heat capacity change due to the transition, a number that is proportional to the total enthalpy change and the degree of cooperativity. This has been calculated for sheep brain sphingomyelin liposomes at 31 °C by:

$$\Delta C_{p,max} = \Phi C_{p,max} - \tfrac{1}{2}(\Phi C_p(15\,^\circ C) + \Phi C_p(60\,^\circ C))$$

For these three experiments $\Delta C_{p,max} = 0.520 \pm 0.003$ kcal/mol deg.

The thermodynamic quantities associated with the phase transition of sheep brain sphingomyelin are summarized in Table III and demonstrate the reproducibility of the experiment. These results clearly show that the gel–liquid crystalline transition is reversible. In addition, the drop calorimetric and fluorescence results, to be discussed below, support the conclusion that at the scanning rate employed in this study the lipid dispersions are at thermodynamic equilibrium at all temperatures.

Results with 1% (w/w) aqueous suspensions of bovine brain sphingomyelin liposomes are shown in Figure 2A and 2B. The apparent heat capacity, ΦC_p, vs. temperature function (Figure

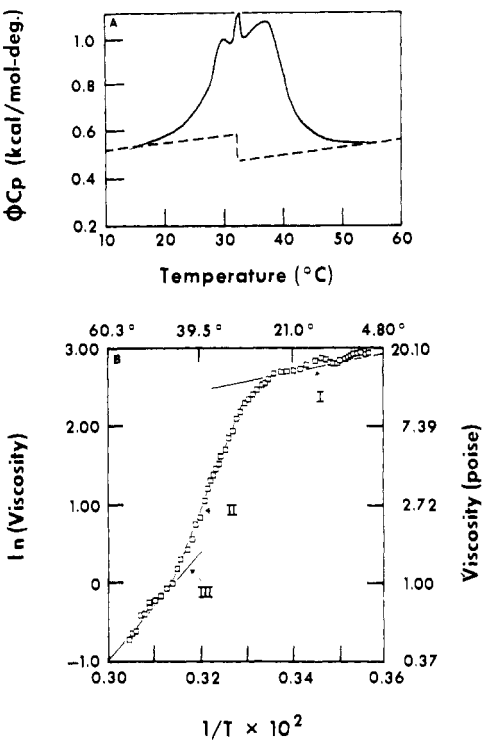

FIGURE 2: (A) ΦC_p vs. temperature for bovine brain sphingomyelin liposomes (concentration = 23 mg/ml). The broken line is the heat capacity baseline used to calculate ΔH. (B) Microviscosity of bovine brain sphingomyelin liposomes as a function of temperature. I, II, III indicate specific regions of curve differing in activation energy as discussed in the text.

2A) exhibits three maxima at approximately 30, 32, and 38 °C. The estimated enthalpy change for the transition is 6.9 kcal/mol. using the heat capacity baseline shown in Figure 2A. Although the basic features of the transition for bovine brain sphingomyelins were generally reproducible, all preparations did not clearly show three heat capacity maxima; in some instances the first transition only appeared as a shoulder. The thermodynamic parameters for two preparations are summarized in Table III. While these quantities show good agreement with one another, indicating the general reproducibility of the thermotropic behavior of sphingomyelin liposomes, the detailed shape of the transition curve appears to depend upon the preparation.

A plot of the microviscosity vs. T^{-1} exhibits three distinct "activation energy" regions as shown in Figure 2B. These results can be correlated with the ΦC_p results in the following manner. Region I, with E_A = 2.4 kcal/mol, corresponds to the temperature dependence of the microviscosity of the liposomes in the low-temperature ($T < 20$ °C) gel state. Region III, with E_A = 12.8 kcal/mol, corresponds to the temperature dependence of the microviscosity in the liquid-crystalline state ($T > 45$ °C). Region II with the largest apparent E_A corresponds to the phase transition region (20 °C $\leq T < 45$ °C) and as such the apparent E_A is primarily a reflection of temperature-dependent structural changes of the liposomes. It is to be noted that the microviscosity function is related to the integral of the ΦC_p function and generally is of insufficient precision to demonstrate the detailed features of the transitions (i.e., the apparent existence of three distinct melting temperatures). It is clear, however, that the temperature-dependent changes of ΦC_p truly reflect temperature-dependent changes in bilayer structure as measured by the fluorescence anisotropy in a manner similar to that observed for dipalmitoylphosphatidylcholine.

TABLE III: Thermodynamic Parameters for the Gel-Liquid Crystalline Transition of Sheep Brain and Bovine Brain Sphingomyelins.

Species		T_{M1} (°C)	T_{M2} (°C)	T_{M3} (°C)	ΔH (kcal/mol)	$\Delta C_{p,max}$ (kcal/mol deg)
Sheep[a]	1	31.0	37.0		7.1	0.523
	2	31.2	37.1		7.6	0.521
	3	31.3	37.1		6.8	0.516
Bovine[b]	1	30.4	32.5	38.4	6.9	0.580
	2	(~30)[c]	32.4	37.0	6.6	0.550

[a] The sets of parameters refer to three calorimetric experiments with the same preparation. 1 was freshly prepared, 2 was a repeat of 1 after a week's storage in the cold, and 3 was a repeat of 2 immediately after cooling (see Figure 1). [b] The two sets of parameters refer to two experiments with different liposome preparations using the same material. [c] Shoulder observed at ~30 °C.

The phase-transition characteristics of both bovine brain and sheep brain sphingomyelins were also studied using a drop heat capacity calorimeter. This calorimeter measures the quantity of heat released from a sample as the temperature is changed from some value T to a reference temperature $T_r < T$. If the thermal behavior of the sample is reversible, this heat quantity is equal to the integral of the ΦC_p function from T_r to T. Results obtained for a bovine brain sphingomyelin dispersion using the drop calorimeter were in essential agreement with the scanning calorimeter results. For example, the existence of three maxima in ΦC_p was observed at the same temperatures and the estimated heat change for the overall transition was in agreement with that calculated from the scanning calorimeter experiment. Because the drop calorimeter experiments were limited to a temperature range of 20–45 °C and because the results were of limited precision, quantitative comparison of the two sets of results cannot be made. Nevertheless, the comparison of the two types of measurements clearly demonstrates that the ΦC_p data truly reflect a reversible equilibrium state of the system. As such, these results have true thermodynamic meaning. Drop calorimeter results with an aqueous dispersion of sheep brain sphingomyelin were also similar to those obtained with the bovine brain preparation.

Synthetic Sphingomyelins. Our results with a heterogeneous preparation of natural occurring sphingomyelins are consistent with the results reported by Shipley et al. (1974). In order to provide a better basis upon which to develop an interpretation of the thermotropic behavior of natural sphingomyelins, calorimetric studies with four preparations of essentially pure synthetic sphingomyelins were undertaken. These studies demonstrate that the thermotropic behavior of a mixture of sphingomyelins is a complex function of the exact composition of the mixture and cannot be easily interpreted in terms of the behavior of the isolated components.

ΦC_p of *N*-palmitoyldihydrosphingomyelin as a function of temperature is shown in Figure 3A. This sphingomyelin exhibits a single, somewhat asymmetric transition at 47.8 °C characterized by ΔH = 9.4 kcal/mol and a transition width at half-height, $\Delta T_{1/2}$, of 1.8 °C. This behavior is reminiscent of multilamellar dipalmitoylphosphatidylcholine except for the absence of an enthalpically smaller transition at lower temperature, 34.6 °C (Suurkuusk et al. 1976; Hinz and Sturtevant, 1972; Ladbrooke and Chapman, 1969; Steim, 1968).

ΦC_p of *N*-palmitoylsphingomyelin, *N*-stearoylsphin-

TABLE IV: Thermodynamic Parameters for the Gel–Liquid Crystalline Transition of Some Sphingomyelin-Containing Liposomes[a]

Preparation	T_M (°C)	ΔH (kcal/mol)	$\Delta C_{p,max}$ (kcal/mol-deg)	$\Delta T_{1/2}$ (°C)
N-Palmitoyldihydrosphingosine-phosphoryl choline	47.8	9.4	4.7	1.8
N-Palmitoylsphingosine-phosphorylcholine	41.3	6.8	8.6	0.8
N-Stearoylsphingosine-phosphorylcholine	52.8	17.9	5.0	2.7
N-Lignocerylsphingosinephos-phorylcholine[b]	48.6	15.3	7.1	1.8
1:1:1 mixture of *N*-palmitoyl-, *N*-stearoyl-, and *N*-lignoceryl-sphingosinephosphorylcholine	39.5	6.8	1.5	2.6
2:1 mixture of *N*-stearoyl-sphingosinephosphoryl-choline and 1-palmitoyl-2-oleylphosphatidylcholine	47.6	9.8	1.3	5.6

[a] Parameters for only the most prominent transition are reported. [b] A small transition (ΔH = 1.9 kcal/mol) is also observed at 42.6 °C.

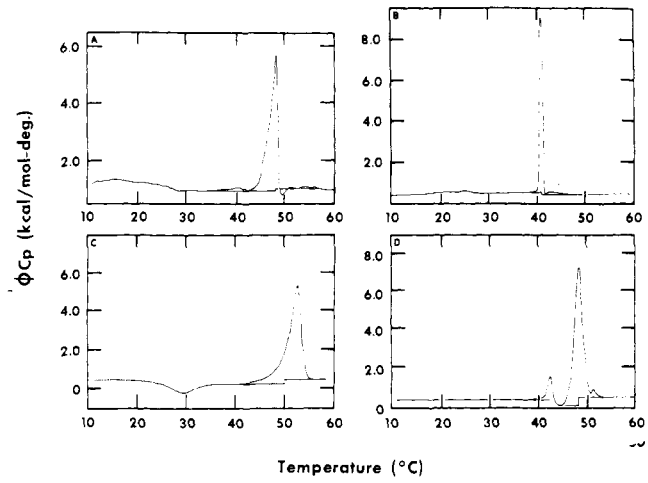

FIGURE 3: ΦC_p vs. temperature for several synthetic sphingomyelin liposome preparations. (A) *N*-Palmitoyldihydrosphingosinephosphorylcholine (concentration = 1.2 mg/ml); (B) *N*-palmitoylsphingosinephosphorylcholine (concentration = 10 mg/ml); (C) *N*-stearoylsphingosinephosphorylcholine (concentration = 10 mg/ml); (D) *N*-lignocerylsphingosinephosphorylcholine (concentration = 10 mg/ml). The straight line segments are the assumed heat capacity baselines used to calculate ΔH.

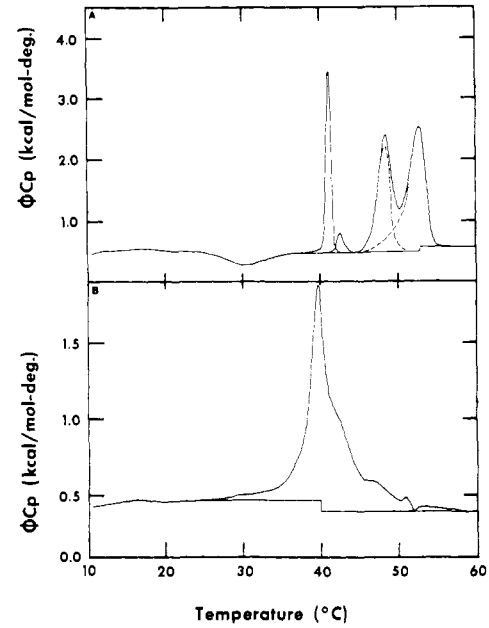

FIGURE 4: ΦC_p vs. temperature for 1:1:1 *N*-palmitoylsphingosinephosphorylcholine and *N*-stearoyl- and *N*-lignocerylsphingosinephosphorylcholine liposomes. (A) mixed; (B) colyophilized. The broken curves in A are the estimated contributions of the pure sphingomyelins to ΦC_p assuming each is behaving independently and were calculated from the results shown in Figure 3B–D and the composition of the mixture. The straight line segments are the assumed heat capacity baselines used to calculate ΔH.

gomyelin, and *N*-lignocerylsphingomyelin as a function of temperature are shown in Figure 3B, 3C, and 3D, respectively. *N*-Palmitoylsphingomyelin exhibits a single maximum in ΦC_p at 41.3 °C, ΔH = 6.8 kcal/mol, and $\Delta T_{1/2}$ = 0.8 °C. *N*-Stearoylsphingomyelin exhibits a single heat capacity maximum at 52.8 °C with a corresponding ΔH = 17.9 kcal/mol. Although no low temperature transition is observed, a reproducible (see Figure 3C) exothermic process is observed at about 30 °C. Since all temperature-induced transformations which occur at thermodynamic equilibrium must be endothermic ($\Delta H > 0$), this must be a process in which the system is maintained by kinetic barriers in a high-energy state at low temperature, but as the temperature is raised the lower energy state becomes accessible within the time-frame of these experiments. This unusual effect is reproducible and is under further investigation.

The *N*-lignocerylsphingomyelin exhibits two maxima in the ΦC_p function at 42.6 and 48.6 °C with associated enthalpy changes of 1.9 and 15.3 kcal/mol, respectively. The $\Delta T_{1/2}$ of the two transitions are 1.4 and 1.8 °C, respectively.

The behavior of the synthetic *N*-palmitoylsphingomyelin and dihydrosphingomyelin liposomes at low temperatures suggests the possibility of a temperature dependent process at these temperatures. Although the magnitudes of these effects are rather small, it is interesting that recent x-ray scattering studies of the sphingomyelin indicate the presence of a broad transition at about 25 °C (G. Shipley, personal communication).

The thermodynamic parameters for the gel–liquid crystalline transition of the synthetic sphingomyelins are summarized in Table IV. While there appears to be a direct correlation between the magnitude of the enthalpy change for the main transition and T_M, no simple correlation between the molecular structure and thermodynamic quantities appears to exist.

Mixtures of Synthetic Lipids. The following experiments were performed to make a preliminary assessment of the transition characteristics of defined mixtures of sphingomyelin-containing liposomes. In Figure 4A is shown the ΦC_p function of a 1:1:1 molar mixture of the *N*-palmitoyl-, *N*-stearoyl-, and *N*-lignocerylsphingomyelin dispersion. The broken line is the transition curve calculated assuming each component behaved independently. The similarity between the experimental curve and calculated curve clearly shows that under these conditions, or even after a week of incubation, these liposomes do not fuse. However, if such a mixture is colyophilized and then suspended in a 50 mM KCl solution the ΦC_p function shown in Figure 4B is obtained; a single maximum at 39.5 °C with a corresponding ΔH = 6.8 kcal/mol and $\Delta T_{1/2}$ = 2.6 °C is observed. This surprising result demonstrates that such a mixture behaves thermotropically as a "homogeneous"

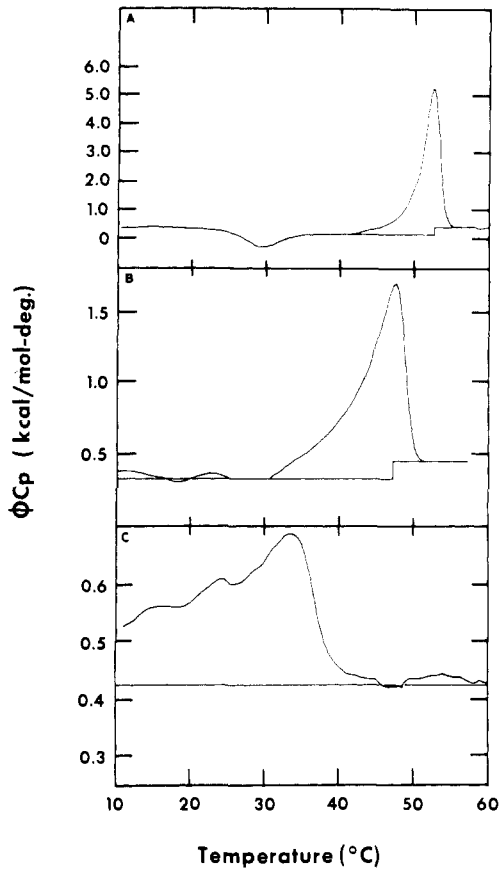

FIGURE 5: ΦC_p vs. temperature for liposomes formed by colyophilization of *N*-stearoylsphingosinephosphorylcholine and 1-palmitoyl-2-oleylphosphatidylcholine. (A) Sphingosine only, same as Figure 3C; (B) 2:1 mole ratio; (C) 1:1 mole ratio. Total concentration $\simeq$10 mg/ml in all cases. The straight line segments are the assumed heat capacity baselines used to calculate ΔH.

phase whose characteristics are *not* a simple function of the properties of the components.

Calorimetric experiments in which *N*-stearoylsphingomyelin and 1-palmitoyl-2-oleylphosphatidylcholine were mixed in varying molar ratios, colyophilized, and dispersed in 50 mM KCl solution showed that the thermotropic behavior of the resulting liposomes was markedly dependent upon the exact composition. The ΦC_p function of a dispersion of pure sphingomyelin is shown in Figure 5A. For a 2:1 sphingomyelin to phosphatidylcholine mixture the ΦC_p function, shown in Figure 5B, exhibited a single, but highly asymmetric, transition curve with a T_m of 47.6 °C, ΔH = 9.8 kcal/mol, and $\Delta T_{1/2}$ = 5.6 °C. These characteristics are similar to, but not identical with, those obtained for the pure sphingomyelin liposome preparation. In fact the magnitude of ΔH, calculated per mole of total lipid, is about that which would be expected if only the sphingomyelin component experienced a gel–liquid crystalline transition. It thus appears that lateral phase separation of the two components has occurred, and the observed transition is that due only to a sphingomyelin phase slightly "contaminated" by the phosphatidylcholine.

The ΦC_p function for a 1:1 sphingomyelin–phosphatidylcholine mixture, shown in Figure 5C, is rather complex. Three maxima in the ΦC_p function are observed in the range of 30–60 °C. The individual transitions are unresolved and the ΦC_p function is qualitatively similar to those observed for liposomes derived from natural sphingomyelins and is distinct from any obtained for liposomes prepared from pure sphingomyelins or the pure phosphatidylcholine. In this regard it is important to note the liposomes prepared from this pure phosphatidylcholine undergo a distinct phase transition at about 4 °C without exhibiting transitions at higher temperatures.

Discussion

The results on the thermotropic behavior of aqueous dispersions of natural mixtures of sphingomyelins confirm the earlier work of Shipley et al. (1974). It is clear that such liposomes containing two major sphingomyelin species undergo a complex series of transitions exhibiting up to three maxima in the ΦC_p function in the physiological temperature range (30–40 °C). It appears, however, that this behavior is a property of the mixture which cannot be simply related to the thermotropic characteristics of the individual components. In particular it seems unlikely that phase separation of the major components has occurred. This conclusion is based upon the fact that liposomes derived from the natural sphingomyelins did not exhibit any transition-like behavior in the temperature range (45–55 °C) where the synthetic *N*-stearoyl- and *N*-lignocerylsphingomyelins undergo a gel–liquid crystalline phase transition.

The thermotropic behavior of the four pure sphingomyelin species is reminiscent of the gel–liquid crystalline transition of homogeneous glycerophospholipid multilamellar liposomes in that they exhibit a single distinct and sharp transition. However, the monotonic relationship between the carbon length of the fatty acid side chain and the melting temperature which holds for the glycerophospholipids does not obtain for the sphingomyelin liposomes. Although increasing the *N*-acyl chain length from 16 to 18 increases the T_m by about 11 °C, the *N*-lignoceryl species exhibits a T_m value between that for the *N*-palmitoyl and *N*-stearoyl derivatives. This is to be contrasted with the relative melting temperatures obtained for the analogous pure glycerol phospholipids: dipalmitoylphosphatidylcholine (T_m = 41.2 °C); dielaidoylphosphatidylcholine (T_m = 5 °C); distearoylphosphatidylcholine (T_m = 58.2 °) (Hinz and Sturtevant, 1972; de Kruijff, et al., 1975). The different behavior for the sphingomyelins is probably a manifestation of the difference in length between the acyl chain and sphingosine chain and related details of specific molecular packing requirements in the bilayer.

The effect of incorporation of a trans double bond at position 4 of the sphingosine moiety appears to have little effect on the transition character. For example, the difference in T_m between *N*-palmitoylsphingomyelin and dihydrosphingosine is only 6.5 °C. This difference in T_m is much smaller than observed for the effect of a cis double bond in position 9–10 of the fatty acid of lecithin when either both chains are unsaturated or only when the acyl chain in position 2 is unsaturated. This apparent anomaly is most likely the result of the intrinsic differences in character between cis and trans double bonds and that the trans bond in sphingomyelin is located at the polar-apolar interface rather than deep in the apolar region. Barton and Gunstone (1975) have described similar effects resulting from variation in the position of a cis double bond in the acyl chains in lecithins.

The total enthalpy changes associated with the gel–liquid crystalline transitions of the synthetic *N*-stearoyl- and *N*-lignocerylsphingomyelins are unusual in their magnitudes. Comparison of their large calorimetric ΔH values (15–18 kcal/mol) with corresponding van't Hoff heats suggests that their cooperative melting unit is rather small (~20–30) compared to similar estimates (70–200) (Hinz and Sturtevant, 1972; Suurkuusk et al., 1976) made for either *N*-palmitoyl-

sphingomyelin or the glycerophospholipids. The validity of this conclusion and the assessment of its significance must await further experimental work.

The observation that multilamellar liposomes made of a colyophilized mixture of the synthetic sphingomyelins in a 1:1:1 mole ratio exhibited a single, sharp transition at a temperature below that of the T_m values for the individual species was surprising. With the glycerophospholipids such mixtures exhibit distinct transitions with a T_m in the range of the T_m values of the individual components (Lentz, et al., 1976; Chapman et al., 1975). Thus, it is clear that the phase transition characteristics of mixed sphingomyelin liposomes are not simple functions of the composition. In this particular case it appears that the mixed liposome forms a homogeneous phase in which all three components participate equally; no phase separation occurs and the thermotropic behavior is a manifestation of the unique character of the mixed, but homogeneous, bilayer phase. In any case it is clear that the special rules which apply to mixtures of the glycerophospholipids do not apply to the sphingomyelins.

The results of our studies on mixtures of *N*-stearoylsphingomyelin and the 1-palmitoyl-2-oleylphosphatidylcholine provide another interesting case. If the sphingomyelin is the major component, the thermotropic behavior of the mixed liposome is similar to that of the pure sphingomyelin except for a pronounced asymmetric broadening of the transition curve and a 5 °C reduction in the T_m (see Figure 5B). It is important to note that the total enthalpy change for the transition is approximately equal to that which would be observed from the melting of the *N*-stearoylsphingomyelin alone when calculated on the basis of the sphingomyelin content. These results are consistent with the conclusion that phase separation of the two components has occurred and that the melting behavior of the mixture is due to the melting of the sphingomyelin phase slightly contaminated by the lecithin.

In the case of an equal mole-ratio mixture of *N*-stearoylsphingomyelin and lecithin, however, the thermotropic behavior is not similar to either of the individual components. It appears that a distinct mixed phase or phases exist in the liposome and the thermotropic behavior of this dispersion is a manifestation of the unique molecular characteristics of these mixed phases.

These results clearly show that the phase characteristics of sphingomyelin-containing liposomes are complex and cannot be understood in terms of the rules which have been applied to other types of liposomes previously studied. In addition it is clear that changes in the exact composition of sphingomyelin-containing liposomes, as might occur in natural membranes during aging or the development of atherosclerosis (Portman, 1969; Eisenberg et al. 1969), can have a pronounced effect on the phase characteristics of the system. Insofar as sphingomyelin-containing membranes may undergo such phase transitions in the physiological temperature range, changes in composition of such membranes will indeed influence the exact position of the phase equilibrium which in turn may have a profound effect on the biological functioning of the system.

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