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Influence of structural modifications on the phase behavior of semi-synthetic cerebroside sulfate

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Cerebroside sulfate (galactosylceramide I^3-sulfate) containing α-hydroxy lignoceric acid (C24:0h-CBS), nervonic acid (C24:1-CBS), or hexacosanoic acid (C26:0-CBS) was prepared by a semi-synthetic procedure and studied by differential scanning calorimetry. The phase behavior of these species in 2 M KCl was compared to that of shorter chain length hydroxy and non-hydroxy fatty acid species reported earlier. All three of the new lipids undergo metastable phase behavior similar but not identical to the other species. In addition, the metastable phase behavior of all of the non-hydroxy fatty acid species was found to be more complex than previously thought, with several phases of high transition temperatures and enthalpies possible. Fatty acid hydroxylation inhibits the transition from the metastable to some of the more stable phases. It also significantly increases the phase transition temperatures of both the metastable and stable phases indicating that it contributes to the hydrogen bonding network formed between the lipid molecules and helps overcome the lateral repulsive effect of the negatively charged sulfate. The C-15 *cis* double bond significantly lowers the temperature and enthalpy of the phase transition indicating that it increases the lateral separation of the lipid molecules and decreases the intermolecular hydrogen bonding interactions. However, it does not prevent formation of a more stable phase. By comparing the effect of various structural modifications reported here and earlier it could be concluded that fatty acid chain length has little effect on the phase transition temperature and enthalpy. This suggests that the forces between the lipid molecules may be dominated by head group interactions rather than interactions between the lipid chains. However, fatty acid chain length has a significant effect on the tendency of the hydroxy fatty acid species to form the more stable phase. The ease of formation of the stable phase increases with increase in chain length. Thus an increase in chain length helps overcome the kinetic barrier to stable phase formation presented by hydroxylation of the fatty acid.

Introduction

Cerebroside sulfate (galactosylceramide I^3-sulfate) is present in a number of membranes but occurs in the highest amount in myelin where it is 3.8% of the total lipid [1,2]. In fact it is the major

Abbreviations: DSC, differential scanning calorimetry; CBS, cerebroside sulfate.

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charged glycolipid in myelin. Suggestions have been made that it may be involved in Na^+/K^+-ATPase function [2], opiate binding [3], adhesion of cells to the basement membrane [4], and platelet aggregation [5]. It consists of a number of different species since both the fatty acid chain and the sphingosine base can vary. The fact that such structural modifications to the ceramide portion can occur suggests that these different species have roles to play in the membrane.

In the brain the fatty acid length of cerebroside sulfate (CBS) varies from 14 to 26 carbons in length but is predominantly 24 carbons [6]. The fatty acid can be monounsaturated and/or α-hydroxylated. The major non-hydroxy fatty acid is 24 : 1 while the major hydroxy fatty acid is 24 : 0. The ratio of hydroxy to non-hydroxy fatty acids in adult myelin CBS is 0.25–0.8 : 1 and it increases during development in the human [6,7] and rat [8]. This ratio tends to increase with the complexity of the nervous system in different species [9], but myelin from some species of Urodela completely lacks the hydroxy fatty acid forms of CBS and cerebroside [10]. The velocity of nerve conduction is significantly reduced in this species relative to that in species which possess the hydroxy fatty acid forms, suggesting a relationship between the presence of the hydroxy fatty acid forms and nerve conduction. In the kidney the hydroxy to non-hydroxy fatty acid ratio in CBS is 0.7–2.0 : 1 [8,11].

The sphingosine base of glycosphingosine base of glycosphingolipids can vary from 14 to 22 carbons in length, can be unsaturated, and can have up to three free hydroxyl groups. A relationship between the number of free hydroxyl groups on the fatty acid and sphingosine chains of sphingolipids in membranes and the degree of chemical or physical stress experienced by the membrane has been noted [2]. An increase in the hydroxy to non-hydroxy fatty acid ratio and of the base chain length (from 18 to 20 carbons) of glycosphingolipids of *Neurospora crassa* occurs with increase in growth temperature indicating that the structure of these lipids can be altered in response to changes in the environment [12], and suggesting that the hydroxy fatty acid must contribute greater order and stability to the membrane allowing it to function at a higher temperature.

Deranged metabolism of the very long chain fatty acids of sphingolipids is a primary event in the pathogenesis of the hereditary demyelinating disease adrenoleukodystrophy. The amounts of C25 and C26 fatty acids in sphingolipids of myelin and other cell membranes are increased by 2–5-times [13,14]. This increased content of sphingolipids containing long chain fatty acids may have deleterious effects on the cell membranes.

Investigation of the properties of different molecular species of a lipid can help to understand their roles in the membrane in health and disease and the structure-function relationship. In the present study we have synthesized cerebroside sulfate containing α-hydroxy -lignoceric acid (C24 : 0h-CBS), nervonic acid (C24 : 1-CBS), and hexacosanoic acid (C26 : 0-CBS) and studied their phase behavior by differential scanning calorimetry. They are compared to the hydroxy and non-hydroxy fatty acid species of palmitoyl-CBS and stearoyl-CBS and the non-hydroxy fatty acid species of lignoceroyl-CBS reported earlier [15,16], in order to understand the effect of fatty acid chain length, unsaturation, and hydroxylation on the phase behavior of this lipid. In the accompanying paper we use a fatty acid spin label to investigate the structure of the phases formed by these lipids [25].

Materials and Methods

Bovine brain cerebroside sulfate was prepared from an acetone precipitated, ether insoluble fraction of bovine brain lipids, rich in cerebroside and sulfatide, purchased from Avanti Polar Lipids Inc. (Birmingham, AL), by an adaptation of the method of Svennerholm and Thorin [17]. Cerebroside sulfate containing palmitic acid (C16 : 0-CBS), α-hydroxy palmitic acid (C16 : 0h-CBS), stearic acid (C18 : 0-CBS), α-hydroxy stearic acid (C18 : 0h-CBS), lignoceric acid (C24 : 0-CBS), α-hydroxy lignoceric acid (C24 : 0h-CBS), nervonic acid (C24 : 1-CBS), and hexacosanoic acid (C26 : 0-CBS) were prepared by deacylation of bovine brain CBS and reacylation as described with some modifications [15,18]. In bovine brain CBS the sphingosine base is 94% dihydroxy 18 : 1, 4% dihydroxy 18 : 0, 2% dihydroxy 18 : 2, and 1% dihydroxy 16 : 0 [19]. Palmitic acid was purchased

from Fluka (Switzerland), stearic acid from BDH, lignoceric acid from Aldrich, and nervonic acid and hexacosanoic acid from Sigma Chemical Co. (St. Louis, MO). Nervonyl chloride was prepared by the reaction of oxalyl chloride with nervonic acid in the presence of pyridine [20]. D-2-α-Hydroxy species of palmitic, stearic, and lignoceric acids were prepared as reported previously [15].

After reacylation, most of the excess free fatty acid was removed by extraction of the crude dry lipid with diethyl ether. The product was then applied to a column of DEAE-cellulose and successively eluted with 9 bed volumes chloroform/methanol (9 : 1), 10 bed volumes of chloroform/methanol (9 : 1) containing 0.02% acetic acid and finally with 10 bed volumes of chloroform/methanol (4 : 1) containing 10 mM ammonium acetate and ammonium hydroxide (2 ml concentrated NH_4OH/100 ml) [21]. The last solvent caused quantitative elution of the sulfatide. It was then further purified by chromatography on silica gel as described previously [15]. In the case of C24 : 0h-CBS, a small amount of the free hydroxy fatty acid remained tenaciously bound to the lipid as judged by its calorimetric behavior. This was removed by partitioning the lipid between ethanol/water (3 : 1) and hexane and removing the hexane phase. This procedure was repeated until the upper hexane phase left no detectable residue on evaporation and there was no further change in the calorimetric behavior. The lipids were chromatographically pure and contained the expected amount of galactose and sulfate, determined by the methods of Kushwaha and Kates [22] and Kean [23], respectively. All available evidence indicates that the natural D-*erythro* configuration of the sphingosine base has been conserved in the synthetic species of CBS. Nonaka and co-workers [24], using stronger conditions for alkaline hydrolysis of natural CBS than we used [18], showed by GC-MS studies that there was no change in the stereochemistry of the sphingosine base. The TLC behavior of the lyso-CBS and synthetic species of CBS produced by our procedures corroborates the conclusions of Nonaka et al.

Calorimetric measurements were made on a Perkin-Elmer DSC-2 differential scanning calorimeter (DSC) equipped with a Perkin-Elmer data station. The lipid was dispersed at a concentration of 0.7–1.0 mg lipid/0.05 ml Hepes buffer (10 mM) at pH 7.4, containing 1 mM EDTA and 2 M KCl (unless otherwise noted). The sample was centrifuged in an Eppendorf bench centrifuge and the pellet was loaded into an aluminum DSC pan. The lipid concentration in the pan was less than 1 mg/10 μl. C26 : 0-CBS did not pellet well so it was dispersed in 15 μl and the sample was transferred to the DSC pan without centrifuging. Heating and cooling rates of 1.25–10 C°/min were used. Most samples were scanned from −3°C to 97°C unless otherwise noted. The scans were reproducible after many heating and cooling cycles. The temperature of maximum heat capacity was defined as the phase transition temperature, T_m. The transition temperatures on cooling given in the tables were corrected for instrumental hysteresis by addition of 4.5 C° to the observed values when using a cooling rate of 10 C°/min. The scans in the figures show the transition temperature actually obtained. The peak areas were determined by use of the data station and the amount of lipid in the pan was determined by galactose and sulfate analysis [22,23] as described previously [15]. Some samples were also measured on a high sensitivity calorimeter (Microcal MC-1, Amherst, MA). The lipid was dispersed in the buffer at a concentration of 1 mg/1.2 ml and 0.96 ml of the suspension was loaded into the sample cell. However, all data in the figures and tables were obtained using the Perkin-Elmer DSC.

Results

We reported earlier that other synthetic species of CBS containing C16 : 0, C18 : 0, C24 : 0, C16 : 0h, and C18 : 0h acyl chains undergo metastable phase behavior [15,16]. On cooling from the liquid crystalline phase the sample freezes into a metastable phase A which then goes through another exothermic transition into a more stable phase B. The transition to a more stable phase may occur either while cooling further or during reheating, depending on the lipid species and the type and concentration of cation present. In the present study we show that this behavior is even more complex than reported earlier and compare

it to that which occurs in some additional species. The lipids are compared primarily in the presence of 2 M K^+ because this results in sharper transitions on cooling and increases the rate of formation of the stable phase of the hydroxy fatty acid species.

Metastable phase behavior of C24 : 0h-CBS

The DSC scans of C24 : 0h-CBS shown in Fig. 1 indicate that it also undergoes metastable phase behavior. However, this lipid does not go into its stable phase until it is reheated as is evident from the large exotherm in the heating scan in Fig. 1 A. A sample scanned in a Microcal calorimeter at 0.18 C°/min has a very similar thermogram. On cooling (using a Perkin-Elmer DSC), the lipid goes into the metastable phase A in two transitions of lower temperature and lower enthalpy as shown in Fig. 1 B (See Tables I and II for T_m values corrected for instrumental hysteresis.) The sum of the enthalpies on cooling (7 kcal/mol) and the exothermic transition on heating (10 kcal/mol) are equal to the enthalpy of the transition of the stable phase to the liquid crystalline phase (17 kcal/mol). The two transitions on cooling are reminiscent of the behavior of C24 : 0-CBS on freezing from the liquid crystalline to the metastable phase [16]. Unlike the hydroxy fatty acid species, however, the non-hydroxy fatty acid species go into a more stable phase on cooling in a third exothermic transition. The enthalpy of this third exothermic transition on cooling for C24 : 0-CBS is 7.5 kcal/mol, less than that of the exothermic transition on heating for C24 : 0h-CBS.

The appearance of the cooling scan and ratio of the two transitions shown in Fig. 1B is identical at cooling rates from 10 C°/min down to 1.25 C°/min. If the sample is cooled only to 52°C before reheating, the same two lower temperature, lower enthalpy transitions are observed on heating (Fig. 1C). The ratio of the two peaks observed on heating also does not depend on heating rate. Thus the transition from the liquid crystalline phase to the metastable phase is reversible, providing the sample has not been supercooled. Both the melting and freezing transitions occur in a two stage process which is fast compared to the greatest heating and cooling rates used. C24 : 0-CBS also goes into the metastable phase in a two-stage process but the shorter chain length species of CBS do so in a single step [16].

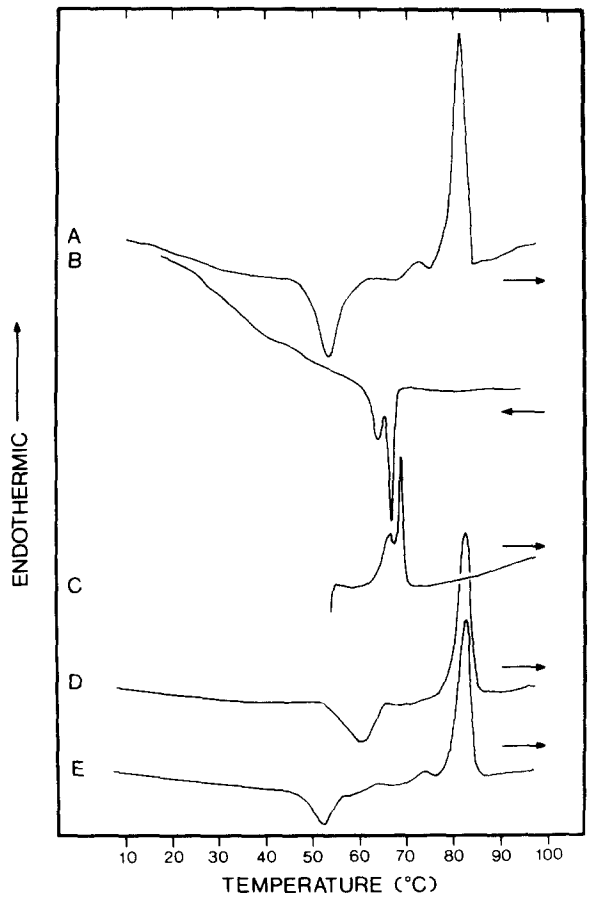

Fig. 1. Thermograms of C24 : 0h-CBS in the presence of 2 M K^+ showing evidence of metastability. (A) Heating scan from −3°C after rapid cooling (40 C°/min); (B) cooling scan; (C) heating scan after rapid cooling to 52°C. (A-C) were scanned at 5 C°/min and are plotted at a sensitivity setting of 0.3 mcal/s. (D) Heating scan at 10 C°/min from −3°C after rapid cooling; (E) heating scan at 10 C°/min from −3°C after cooling at 1.25 C°/min. Sensitivity settings for (E) and (F) are 0.8 mcal/s. Cooling scan at 1.25 C°/min not shown but resembles that in (B). The same sample was used for all scans. All scans are reproducible if the same conditions are used. Cooling scan has not been shifted to correct for instrumental hysteresis but the temperatures given in the tables have.

However, the appearance of the heating scan obtained after supercooling does depend to some extent on heating rate and on the cooling rate of the cooling scan just before it. A small transition observed at 72°C on heating at 5 C°/min (Fig. 1 A) and slower rates is barely detectable on heating

at 10 C°/min (Fig. 1 D, scanned after cooling at 40 C°/min). However, this transition is present in a scan at 10 C°/min obtained after cooling at 1.25 C°/min (Fig. 1 E). Cooling at a slow rate also causes the exothermic transition on reheating to occur at a lower temperature than after fast cooling (compare Fig. 1 D and E). This indicates that some molecular rearrangement of the lipid occurs during the slow cooling scan even though no other transitions are observed by calorimetry. The temperature of the exothermic transition also decreases with decrease in heating rate, irrespective of the cooling rate, indicating that formation of the stable phase can occur at lower temperatures if given sufficient time.

Metastable phase behavior of C26 : 0-CBS

The first heating scan of C26 : 0-CBS at a rate of 10 C°/min generally has one transition at 71.5 °C. Sometimes two smaller transitions at 68.8 °C and 74.8 °C are also observed as shown in Fig. 2 a. On reheating scans only the two lower temperature transitions are observed (labeled 1 and 2 in Fig. 2 b). The sample can be completely converted to the phase giving the third higher temperature transition (peak 3 in Fig. 2 a) by cooling slowly (1.25 C°/min) followed by reheating at the same rate to 70 °C and incubating at that temperature for 30 min. If the sample is then cooled back to a low temperature and rescanned, it undergoes a single transition at 74.8 °C as shown in Fig. 2 d. The enthalpy of this transition is 1.2-times greater than that of the scan shown in Fig. 2 b. Scanning in a Microcal DSC at a rate of 0.042 C°/min shows the two upper temperature transitions.

As with C18 and C24 species of CBS, there is pronounced hysteresis on cooling. Three other transitions are observed on cooling as shown in Fig. 2 c, resembling cooling scans of C24 : 0-CBS [16]. The temperature of the first (peak 1′) is several degrees below the lowest temperature transition (peak 1) observed on heating (See Table I for T_m values on heating and Table II for T_m values on cooling after correction for instrumental hysteresis). The total enthalpy on cooling at 10 C°/min is similar to that on heating in scans such as those shown in Fig. 2 a and b. The first and second higher temperature transitions on cooling

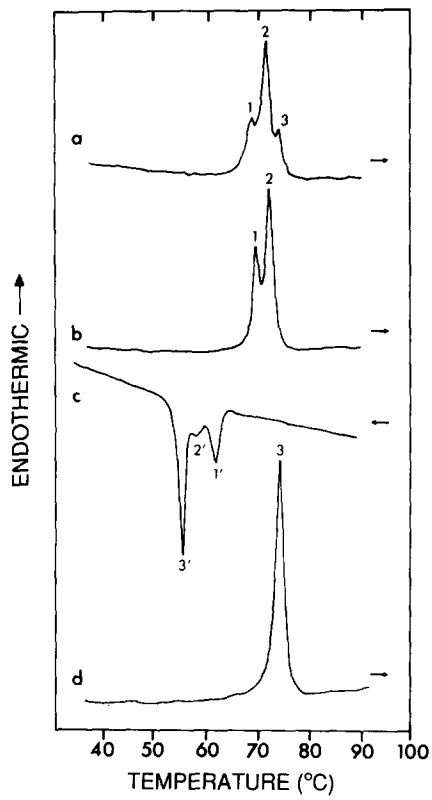

Fig. 2. Thermograms at 10 C°/min of C26 : 0-CBS in 2 M K^+ showing evidence of metastability. (a) First heating scan; (b) repeated heating scan after rapid cooling; (c) cooling scan; (d) heating scan from −3 °C after cooling from the liquid crystalline phase to −3 °C at 1.25 C°/min, heating at 1.25 C°/min to 70 °C and incubating at that temperature for 30 min. Sensitivity setting for all scans was 0.8 mcal/s. All scans are reproducible if the same conditions are used. The same sample was used for all scans. Cooling scan has not been shifted to correct for instrumental hysteresis but the temperatures given in the tables have.

(1′ and 2′) probably represent the two stage transition from the liquid crystalline phase to the metastable gel phase A seen for the hydroxy fatty acid and non-hydroxy fatty acid species of C24 : 0 CBS, while the third, lowest temperature transition (3′) represents the transition from the metastable phase A to a stable phase as for C24 : 0-CBS. The temperature of this transition 3′ increases with decrease in heating rate indicating that the process giving rise to this transition is kinetically limited, as occurs also for C24 : 0-CBS. The temperature difference between the first and third peaks is less than for C24 : 0-CBS at all cooling

rates, resulting in overlap between the second and third transitions even at a fast cooling rate of 10 C°/min.

Thus this lipid has several metastable and stable phases. Although the phases giving the two lower temperature transitions on heating are also metastable with respect to the phase giving transition three, they are all stable with respect to the metastable phase A formed immediately on cooling from the liquid crystalline phase, in peaks 1′ and 2′. Therefore, the three phases giving the three transitions observed on heating (peaks 1, 2, and 3) will all be called stable B phases. The stable phase which is formed in transition 3′ on cooling and presumably gives peak 1 on heating will be called B_1 while the more stable phases which give peaks 2 and 3 will be called B_2 and B_3.

Metastable phase behavior of C24 : 1-CBS

On heating and cooling at 10 C°/min C24 : 1-CBS undergoes a single, reversible transition at 44° C with an enthalpy of 4.2 kcal/mol (Fig. 3 A, B). Cooling at rates down to 1.25 C°/min has no effect on the appearance of the cooling scan or the subsequent heating scan. However, heating at a slower rate, 1.25 C°/min, allows the lipid to go through an exothermic transition at 43° C into another more stable phase. This more stable phase goes into the liquid crystalline phase at 50° C, as shown in Fig. 3C. Incubation at 42° C for 30 min or storage at −20° C for 4 days also allows the lipid to go into this more stable phase as shown from 10 C°/min heating scans in Fig. 3 D and E, respectively. Subsequent heating scans are identical to Fig. 3 A. Since the transition giving peak 1 on heating in Fig. 3 A is identical to that on cooling, it is assumed that this is a transition from a metastable phase A to the liquid crystalline phase. The more stable phase giving peak 2 will be called B_1.

The maximum enthalpy observed for the stable phase of C24 : 1-CBS is 6.9 kcal/mol. However, the conversion to the stable phase is never complete following any of the treatments used, since a small peak at 44° C remains as shown in Fig. 3 C–E. From the heat absorbed by this peak the proportion remaining in the metastable phase and the proportion converted to the stable phase can be calculated. The enthalpy of the stable phase if complete conversion had occurred can thus be estimated at 8.4 kcal/mol.

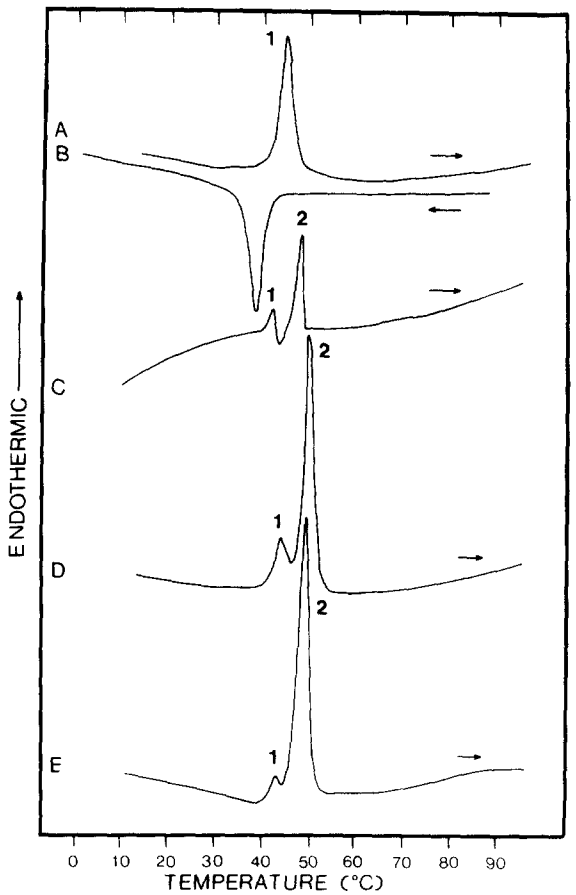

Fig. 3. Thermograms of C24 : 1-CBS in the presence of 2 M K^+ showing evidence of metastability. (A) Heating scan at 10 C°/min; (B) cooling scan at 10 C°/min; (C) heating scan at 1.25 C°/min; (D) heating scan at 10 C°/min after 30 min incubation at 42°; (E) heating scan at 10 C°/min after storage at −20° C for 4 days. All samples heated from −3° C after rapid cool (40 C°/min) except (D) and (E). Sensitivity setting for (A, B, D, E) was 0.5 mcal/s while that for (C) was 0.2 mcal/s. The same sample was used for all scans. Cooling scan at 1.25 C°/min not shown but was similar to (B). All scans are reproducible if the same conditions are used. The same sample was used for all scans. Cooling scan has not been shifted to correct for instrumental hysteresis but the temperatures given in the tables have.

Further studies on metastable phase behavior of other species of CBS

Because of the ability of C26 : 0-CBS to go into several different stable phases with successively

increasing phase transition temperature, we investigated the ability of C16:0, C18:0 and C24:0-CBS to do so. The latter two had been found to undergo this kind of behavior at lower K^+ concentrations [16] but at 2 M K^+ they had given single transitions at a heating rate of 10 C°/min, and it was assumed that this was the highest temperature phase transition possible for these species. However, by appropriate sample treatment, the C16:0, C18:0, and C24:0 species can all be converted to phases with higher transition temperatures and enthalpies than those reported previously.

The scan shown in Fig. 4 a for C18:0-CBS is that obtained on repeated heating after fast cooling. It occurs at a higher temperature and has a greater enthalpy than the transition to the metastable phase observed on cooling [16]. Thus the sample goes into the stable phase B_1 during the broad exothermic transition at about 60°C. If cooled slowly (1.25 C°/min) it goes into this phase in a second exothermic transition (ΔH = 4–5 kcal/mol) on cooling. However, if the sample is heated at a slower rate it gives a transition at a 2 C° higher temperature. In order to obtain a 10 C°/min heating scan of the sample in this more stable phase, B_2, for comparison to that in Fig. 4 a, the sample was cooled at 1.25 C°/min, heated at 1.25 C°/min to 64°C and incubated at that temperature for 30 min. It was then cooled back and reheated at 10 C°/min to give the scan shown in Fig. 4 b. The enthalpy is similar to that in Fig. 4 a but the transition temperature is 2 C° higher. If the sample was treated similarly but incubated at 64°C for 60 min, it also has a shoulder at an even higher temperature as shown in Fig. 4 c, indicating that some of the sample has gone into a third stable phase, B_3. The sample is almost completely converted to phase B_3 by similar treatment followed by incubation at 64°C for 12 h, as shown in Fig. 4 d. The change in enthalpy during this phase transition is 1.2-times greater than that of the lower temperature phase transitions shown in Fig. 4 a and b.

If C24:0-CBS is cooled rapidly from the liquid crystalline phase it is in a stable phase B_1 which gives a single high enthalpy transition at 68.2°C when heated at 10 C°/min. However, if heated at 1.25 C°/min it goes into a second stable phase B_2

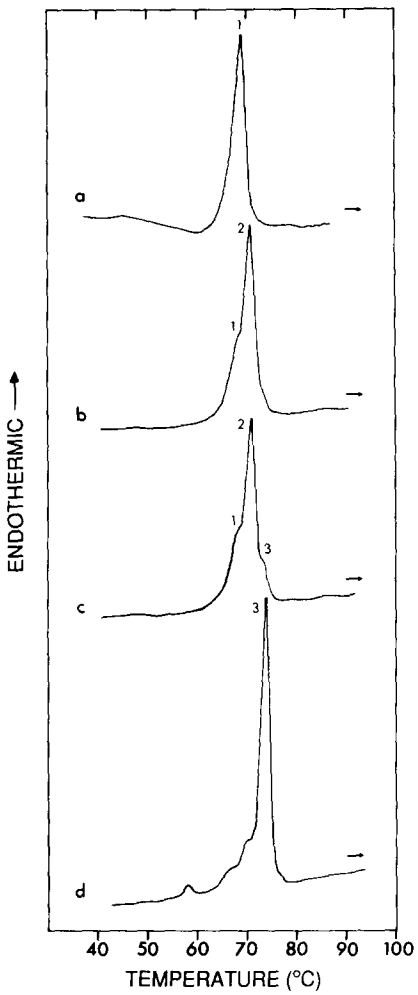

Fig. 4. Thermograms on heating from −3°C at 10 C°/min of C18:0-CBS in 2 M K^+. (a) Repeated heating scan after rapid cooling from the liquid crystalline phase; (b) heating scan after cooling from the liquid crystalline phase to −3°C at 1.25 C°/min, reheating at 1.25 C°/min to 64°C and incubating at that temperature for 30 min; (c) as in (b) except the sample was incubated at 64°C for 60 min; (d) as in (b) and (c) except the sample was incubated at 64°C for 12 h. The same sensitivity setting was used for all scans. All scans are reproducible if the same conditions are used.

while scanning. This occurs in an exothermic transition following a small endothermic transition at 66.9°C (not shown). It can be completely converted to this phase by cooling at 1.25 C°/min, heating at 1.25 C°/min to 66.9°C and incubating at that temperature for 60 min. Cooling back to −3°C and scanning at 10 C°/min gives a single peak at a 3 C° higher temperature and with a

TABLE I

EFFECT OF STRUCTURAL MODIFICATIONS TO CEREBROSIDE SULFATE ON THERMODYNAMIC PARAMETERS OF THE TRANSITIONS OF THE STABLE PHASES

Temperatures from DSC heating scans at 10 C°/min in the presence of 2 M K^+. Enthalpies were similar at slower rates. ΔH_F is the enthalpy of the first lowest temperature transition (of state B_1 for the non-hydroxy fatty acid forms and of the only stable state B for the hydroxy fatty acid forms) and ΔH_h is that of the highest (B_2 or B_3), after complete conversion to the phase undergoing that transition. Both values are the change in enthalpy during the transition from a stable phase to the liquid crystalline phase.

Lipid	T_m (°C)				ΔH_F (kcal/mol)	ΔH_h (kcal/mol)
	B_1	B_2	B_3	B		
C16:0-CBS	64.0				13.6±0.4	
C18:0-CBS	66.4	68.5	71.8		13.8±2.0	16.4
C24:0-CBS	68.2	71.1			15.5±0.2	18.8
C26:0-CBS	68.8	71.5	74.8		16.0±1.0	18.4
C24:1-CBS	50.3				8.4±0.5 [a]	
C16:0h-CBS				75.6	9.9±0.6 [a]	
C18:0h-CBS				83.4	14.3±0.3	
C24:0h-CBS				81.5	17.4±2.0	

[a] Estimated value if complete conversion had occurred as discussed in text.

change in enthalpy 1.2-times greater than that obtained by cooling rapidly and scanning at 10 C°/min (Table I). No evidence of a third transition has been observed for this species of CBS. A sample run on a Microcal DSC at heating rates down to 0.09 C°/min undergoes the two transitions just described.

TABLE II

EFFECT OF STRUCTURAL MODIFICATIONS TO CEREBROSIDE SULFATE ON THERMODYNAMIC PARAMETERS OF THE TRANSITION FROM THE LIQUID CRYSTALLINE PHASE TO THE METASTABLE PHASE

From DSC cooling scans at 10 C°/min in the presence of 2 M K^+. Temperatures have been corrected for instrumental hysteresis, which is 4.5 C° at this cooling rate.

Lipid	T_m (°C)	ΔH (kcal/mol)
C16:0-CBS	63	8.6±0.7
C18:0-CBS	61.9	8.3±1.2
C24:0-CBS	62, 65 [a]	7.6±1.4 [a]
C26:0-CBS	63, 67 [b]	5.1±0.6 [b]
C24:1-CBS	44.2	4.1
C16:0h-CBS	64.5	5.6±0.6
C18:0h-CBS	67.0	7.0±0.3
C24:0h-CBS	67, 69.7 [a]	7.6±1.2 [a]

[a] For two transitions similar to those shown in Fig. 1b. Total ΔH for both is given.

[b] Temperatures are given for the two higher temperature transitions shown in Fig. 2c, but ΔH is given only for the highest temperature transition because the second is not well enough resolved from the lowest temperature metastable to stable state transition. Therefore, it cannot be compared to the enthalpy values for the other lipids.

C16:0-CBS goes through a single phase transition on heating with similar transition temperature and enthalpy as on cooling. Thus it is assumed to be in the metastable A phase. On heating at 1.25 C°/min two transitions are observed with the second 1 C° higher than the first (not shown). Incubation at the temperature of the first transition causes it to be completely converted to a more stable phase with a transition temperature 1 C° higher and a transition enthalpy 1.5-times greater than that formed after cooling from the liquid crystalline phase (Table I). This will be called a B_1 phase. This additional metastable phase behavior is not observed for any of the hydroxy fatty acid species. The phase transition temperatures and enthalpies of the various stable phases of these lipids are given in Table I.

Comparison of thermodynamic parameters and phase behavior of different forms of CBS

The effect of fatty acid chain length, unsaturation and hydroxylation on the phase transition temperatures and enthalpies of the stable B phases and the metastable A phase of CBS is indicated in Tables I and II, respectively. The thermodynamic

parameters for the metastable phase A are obtained from cooling scans in the presence of 2 M K^+. The C16 : 0 species is assumed to also go into the metastable A phase on cooling even though the enthalpy of its transition is a little greater than that found for the liquid crystalline to metastable phase transitions of the other lipids.

The phase transition temperatures and enthalpies of the metastable and stable phases do not depend greatly on chain length. The enthalpy of the stable phase B_1 is similar for the C16 : 0 and C18 : 0 species and a little less than that of the longer chain C24 : 0 and C26 : 0 species. The enthalpy of the most stable phases of the longer chain species is also a little greater than that of the C18 : 0 species. The enthalpies of the metastable and stable B phases of the hydroxy fatty acid species increase with chain length more than those of the non-hydroxy fatty acid species and are generally a little less than those of the non-hydroxy fatty acid species of corresponding chain length. However, hydroxylation significantly increases the phase transition temperature for all fatty acid chain lengths, particularly for the stable phase B. It increases the temperature of the metastable phase A by about 5 C° (less for C16-CBS) and that of the stable phase by 10–11 C°, if similar chain lengths are compared. The *cis* double bond at position C-15 in a chain of 24 carbons, decreases the phase transition temperature of the metastable and stable phases relative to C24 : 0-CBS by 18–21 C°. It also decreases the enthalpy of the metastable and stable phases by 50%.

The fact that the transitions to the stable phases only occur in a certain temperature range, if given sufficient time, indicates that there are kinetic barriers to these transitions. Hydroxylation of the fatty acid appears to increase the kinetic barrier to the transition from the metastable phase A to a stable phase, if the rate of formation of B for the hydroxy fatty acid species is compared to the rate of formation of B_1 for the non-hydroxy fatty acid species. The hydroxy fatty acid species go into their stable B phases only on heating, and only in K^+. The rate of formation of their stable phases increases with increase in K^+ concentration [16]. In Na^+ they remain in the metastable A phases under all conditions while the non-hydroxy species still go into their B_1 phases. However, a comparison of the transition temperatures suggests that the B phase for the hydroxy fatty acid species in K^+ may be more comparable to the most stable B_2 and B_3 phases of the non-hydroxy fatty acid

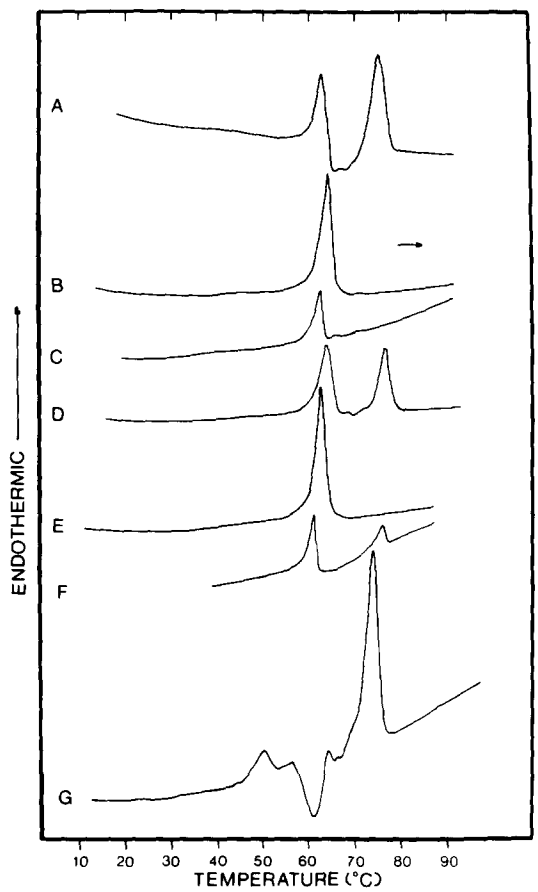

Fig. 5. Thermograms of different chain length species of the hydroxy fatty acid species of CBS showing evidence that rate of transition to stable phase increases with chain length. (A–D) C16 : 0h-CBS in 2 M K^+; (E, F) C18 : 0h-CBS in 0.5 M K^+; (G) C24 : 0h-CBS in 0.5 M K^+. All are heating scans from −3 °C following rapid cool (40 C°/min) except (A) and (D). (A) First scan at 10 C°/min after preparation of sample; (B) subsequent scan at 10 C°/min; (C) subsequent scan at 1.25 C°/min; (D) scan at 10 C°/min after incubation at 62 °C for 6 h, (E) reheating scan at 10 C°/min; (F) reheating scan at 1.25 C°/min; (G) reheating scan at 10 C°/min. Sensitivity settings in mcal/s were (A, B, D) 0.8; (C) 0.3; (E) 0.8; (F) 0.3; (G) 0.3. All scans except (A) are reversible if the same conditions are used. The small peak at 50 °C for C24 : 0h-CBS appeared at K^+ concentrations less than 2 M and in the presence of other cations. It may be related to a similar peak of unknown cause which occurred before the main transition of C24 : 0-CBS at low (0.01 M) but not higher K^+ concentrations (see Fig. 7a in Ref. 16).

species, than B_1. If the rate of formation of these phases is compared, it could be argued that in K^+, C18 : 0h and C24 : 0h go into their most stable phase more readily than the corresponding non-hydroxy fatty acid species.

The rate of formation of the stable B phase by the hydroxy fatty acid species depends on chain length. C16 : 0-CBS goes into the stable B phase only in the presence of high concentrations of K^+ (2 M) and only on heating at very slow rates. In the first heating scan after preparation of the sample in 2 M K^+, two endothermic transitions corresponding to the metastable and stable phase transitions are observed (Fig. 5 A). However, following this first scan the lipid does not go into the stable phase on heating at rates as low as 1.25 C°/min, as shown in Fig. 5 A, B for heating scans at 10 C°/min and 1.25 C°/min, respectively (following 10 C°/min cooling scans). Only after incubation at 62° C, the temperature of the metastable transition, for 6 h, does it go partially into the stable phase as indicated by the two endothermic transitions in Fig. 5 D.

In contrast, in the presence of 2 M K^+ both C18 : 0h-CBS and C24 : 0h-CBS go completely into the stable B phase in an exothermic transition on heating at 10 C°/min. Since the ease of stable phase formation by these two species of CBS appears similar at this cation concentration, they are compared in Fig. 5 at 0.5 M K^+. In the lower concentration of K^+, C18 : 0h-CBS does not go into the stable phase at a heating rate of 10 C°/min (Fig. 5 E). It does so partially only at a slower heating rate of 1.25 C°/min (Fig. 5 F). However, C24 : 0 h-CBS still goes completely into its stable phase in an exothermic transition on heating at 10 C°/min in 0.5 M K^+ (Fig. 5 G). Thus the ease of formation of the stable B phase by the hydroxy fatty acid species increases with increase in fatty acid chain length.

Discussion

The new results reported in this study, the effect of α-hydroxylation of lignoceric acid, the effect of a C-15 *cis* double bond in the C-24 chain, and the effect of an increase in chain length to 26 carbons, on the phase behavior of CBS permit comparison with results on other species of CBS reported earlier. Like the other species, C24 : 0h-CBS, C24 : 1-CBS, and C26 : 0-CBS undergo metastable phase behavior where they all go into a phase with a lower transition temperature and enthalpy on cooling from the liquid crystalline phase. This metastable phase transforms into a more stable phase which has a higher phase transition temperature and enthalpy. For the non-hydroxy fatty acid species with saturated chains of 18 carbons or longer, this so-called stable phase is also metastable. As each phase melts it can transform into another more stable phase with a somewhat higher transition temperature and enthalpy.

The small effect of fatty acid chain length on the phase transition temperature of CBS is in agreement with results on cerebroside [26] and sphingomyelin [27,36]. However, it differs from the behavior of a series of highly asymmetric species of phosphatidylcholine. An increase in the length of the longer acyl chain of these lipids by 2 carbons increases the phase transition temperature by 10–14 C° and the enthalpy by 2 kcal/mol [28]. The similarity in thermodynamic parameters for the longer chain length species of CBS suggests that the forces between the lipid molecules in these phases may be dominated by head group and hydrogen bonding interactions rather than van der Waals interactions between the lipid chains, especially for the more stable phases. The transition temperatures of the various stable phases of the non-hydroxy fatty acid species are spaced at 2–3 C° intervals. Similar results were observed by Cevc et al. [29] for dimyristoylphosphatidylserine at low pH and probably represent different degrees of dehydration. Dehydration, as a result of intermolecular hydrogen bonding, may also be involved in formation of the different stable phases of CBS. It is noteworthy that C18 : 0, C24 : 0, and C26 : 0 all have transitions at 68–69° C and 71–72° C. In addition C18 : 0 CBS has a lower temperature transition while C26 : 0 CBS has a higher one. The tendency to go into the phases with higher transition temperatures increases with increase in chain length. It appears that as the chain length increases, the lower temperature transitions are lost and higher temperature transitions are gained. An increase in chain length and increased van der Waals interactions between the lipid molecules may facilitate the interactions between the head groups.

Hydroxylations of the fatty acid significantly increases the phase transition temperature of both the metastable and stable phases indicating that it contributes to the hydrogen bonding network formed between the galactose hydroxyls and sphingosine amide and hydroxyl group, as occurs in crystals of ceramide and cerebroside [30,31]. Hydroxylation of the fatty acid also causes condensation of ceramide monolayers [32]. Although hydroxylation increases the phase transition temperature of the metastable phase of cerebroside (from cooling scans of stearoyl species in Ref. 20), Curatolo and Jungalwala found that hydroxylation decreases the phase transition temperature of the stable phase of cerebroside [26], in contrast to its effect on CBS. It is interesting that although CBS generally has a lower phase transition temperature than cerebroside, the T_m of the stable phase of the hydroxy fatty acid species of CBS in 2 M K^+ is similar to that of the non-hydroxy fatty acid species of cerebroside and greater than that of the hydroxy fatty acid species of cerebroside [26]. This suggests that hydroxylation of the fatty acid of CBS combined with the charge shielding effects of high K^+ concentrations almost completely counteracts the repulsive effect of the negatively charged sulfate and strengthens the hydrogen bonding interactions to a degree nearly as great as that which occurs for cerebroside, at least in the gel state.

The 19 C° lower temperature and decreased enthalpy of the phase transition of C24 : 1-CBS indicates that the C-15 *cis* double bond has some disordering effect, probably decreasing participation of the lipid in intermolecular hydrogen bonding by causing increased lateral separation. The phase transition temperatures of the nervonic acid containing species of cerebroside and sphingomyelin are 13 C°C and 16 C°, respectively, below those of the lignoceroyl species, although the enthalpy of cerebroside is not affected [26,27], in contrast to the effect on CBS. This *cis* double bond, located in a physiologically natural position along the chain does not prevent transformation of CBS into a more stable phase. Similar results were found for the mixture, C24 : 1/C22 : 0-cerebroside isolated from bovine brain [26]. Lofgren and Pascher [32] found that nervonic acid had little or no perturbing effect on the packing of ceramide in monolayers (relative to stearic acid). They attributed this to axial displacement of the shorter sphingosine chain, giving it an effective bilayer penetrating length of only 13–14 carbons [33]. This would place the C-15 double bond past the end of the sphingosine chain where it might interfere less with molecular packing and thus not prevent increased head group interactions in the stable phase of CBS.

The greater effect which fatty acid hydroxylation and a C-15 *cis* double bond has on the phase transition of CBS compared to cerebroside may be a result of the weaker intermolecular hydrogen bonding interactions in CBS caused by the repulsive effect of the negatively charged sulfate. Intermolecular hydrogen bonding interactions may be so strong in the gel phase of cerebroside that they dominate the effect of other structural modifications. However, hydroxylation of the fatty acid may also increase intermolecular hydrogen bonding in the liquid crystalline phase of these lipids. In the case of cerebroside, this would stabilize the liquid crystalline phase relative to the gel phase and may be the cause of the decrease in transition temperature found for hydroxy fatty acid species of this lipid by Curatolo and Jungalwala [26].

As reported earlier for shorter chain length hydroxy fatty acid species of CBS [16], hydroxylation of C24 : 0-CBS inhibits but does not prevent transformation from the metastable phase to a stable phase. An increase in fatty acid chain length counteracts the inhibitory effect of the hydroxy fatty acid on stable phase formation. Similar results have been found for hydroxy fatty acid species of cerebroside recently [26]. An increase in chain length may decrease the kinetic barrier created by the fatty acid hydroxyl group or it may contribute a stabilizing effect which compensates for the kinetic barrier of the hydroxyl group. The fact that the different species of CBS can form their more stable phases only after melting of the metastable phases indicates that some molecular rearrangement must occur to form the stable phase. This rearrangement may require molecular rotation since it is inhibited by the fatty acid hydroxyl group, which participates in intermolecular hydrogen bonding. These interactions would be weakened at the transition of the metastable phase.

Thus although chain length has little effect on the phase transition temperature, it has a significant effect on the tendency of sphingolipids to form more stable phases. As shown in the following paper [25] it also affects the structure of the stable phase. For this reason alterations in the fatty acid chain length as well as hydroxylation may have significant effects on the behavior and function of sphingolipids in membranes.

The structure of the metastable and stable phases of CBS is investigated in the following paper using fatty acid spin labels. The natural species of bovine brain CBS was recently shown by X-ray diffraction to be lamellar both above and below the transition temperature [34], in contrast to an earlier study in which it was reported to form micelles at high water contents [35]. The calorimetric behavior observed for the synthetic species as well as the fact that they appear as turbid suspensions in water are consistent with a lamellar organization. This has recently been confirmed by X-ray diffraction (Stinson, R. and Boggs, J.M., unpublished data). The similar calorimetric behavior observed at low concentrations (0.1 wt%) by high sensitivity calorimetry is consistent with a lamellar organization at high water contents also.

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