Thermal Behavior of Fractionated and Unfractionated Bovine Brain Cerebrosides†

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ABSTRACT: Bovine brain cerebrosides have been fractionated into 2-hydroxy fatty acid containing cerebrosides (HFA-CER) and nonhydroxy fatty acid containing cerebrosides (NFA-CER). The thermal behavior of NFA-CER, HFA-CER, and unfractionated cerebroside model membranes has been studied by differential scanning calorimetry. When NFA-CER is cooled at rates ≥2.5 °C/min, subsequent heating runs exhibit metastable behavior: a low enthalpy *exotherm* is observed at ~50 °C (ΔH = −(1–3) cal/g), followed by a high enthalpy *endotherm* at 72 °C (ΔH = 16–17 cal/g). Systematic variation of cooling/heating protocols indicates that NFA-CER possesses two low-temperature states, one metastable and the other stable. Cooling from the liquid-crystalline state results in formation of the metastable low-temperature polymorph I, which must transform into the stable low-temperature polymorph II before the liquid-crystalline state can be reached again. By analogy with recent X-ray studies of synthetic *N*-palmitoylgalactosylsphingosine [Ruocco, M. J., Atkinson, D., Small, D. M., Skarjune, R. P., Oldfield, E., & Shipley, G. G. (1981) *Biochemistry 20*, 5957], it is proposed that metastable polymorph I is dehydrated relative to stable polymorph II. HFA-CER displays no metastability and exhibits a reversible thermal transition at ~68 °C (ΔH = 7.3 cal/g). The thermal behavior of unfractionated cerebrosides is similar to that of HFA-CER, exhibiting a single reversible transition at ~67 °C (ΔH = 6.9 cal/g). These results suggest that a function of hydroxy fatty acids in brain cerebrosides may be to prevent metastable dehydration in the cerebroside-rich myelin membrane.

The integrity of natural membranes relies upon the interaction of a wide variety of polar and nonpolar lipids and proteins. A great deal is known about relatively few of the membrane lipids, with the vast majority of knowledge confined to phosphatidylcholine and cholesterol. The phospholipids, particularly phosphatidylcholine, appear to play a structural role and, via their control over membrane fluidity, may also affect membrane protein function. The glycolipids have been less well studied but may in the long run prove to be of greater functional importance, since the variety of glycosyl polar groups found in this class of compounds suggests a role in extracellular recognition. Furthermore, the simplest glycolipid, galactocerebroside (or its plant and bacterial counterpart galactosyldiglyceride), is found in large quantities only in certain highly specialized membranes, such as those of myelin, the intestinal brush border, chloroplasts, and certain strains of influenza virus (Norton, 1975; Hauser et al., 1980; Benson, 1964; Huang, 1976). This simple glycolipid appears to play some special structural role in these systems. The observation that some glycolipids form nonlamellar phases may also have functional significance (Abrahamsson et al., 1972; Curatolo et al., 1977).

Bovine brain cerebrosides can be divided into two general classes: those which contain amide-linked 2-hydroxy fatty acids (HFA-CER)[1] and those which contain amide-linked nonhydroxylated fatty acids (NFA-CER), with the former predominating. It has been suggested by Pascher and colleagues that the function of 2-hydroxy fatty acids in glycolipids is to increase lateral interactions in the membrane plane via hydrogen bonding, thus imparting greater stability (Pascher, 1976; Lofgren & Pascher, 1977; Pascher & Sundell, 1977). In the present study, the thermal behavior of NFA-CER, HFA-CER, and unfractionated cerebrosides (U-CER) from bovine brain has been determined by using differential scanning calorimetry (DSC). NFA-CER is found to undergo metastable phase behavior, while HFA-CER and U-CER do not.

Materials and Methods

Cerebrosides were extracted from fresh bovine brains by using the procedure of Radin (1976). Alkenyl ether and ester linkages were cleaved by iodinolysis and alkaline methanolysis, respectively (Radin, 1976). Cerebrosides were then isolated by chromatography on diethylaminoethylcellulose according to Rouser et al. (1976) (to remove sulfatides) and subsequently on silicic acid. Cerebrosides were eluted from silicic acid with ~10% methanol in chloroform. NFA-CER and HFA-CER were separated by preparative thin-layer chromatography on silica plates using $CHCl_3/CH_3OH/H_2O$ (65:25:4) as eluent (Kishimoto, 1978).

Cerebroside samples in 2:1 $CHCl_3/CH_3OH$ were dried under N_2 in Perkin-Elmer DSC sample pans (50 μL capacity), desiccated under a vacuum overnight, hydrated with distilled deionized H_2O, and sealed. The cerebroside concentration was ~14 wt %. Scanning calorimetry was carried out by using a Perkin-Elmer DSC-2 scanning calorimeter.

Results

Differential scanning calorimetry traces of multilamellar dispersions of U-CER, HFA-CER, and NFA-CER are presented in Figure 1. For each cerebroside fraction, a cooling run (5 °C/min) is presented, followed by the subsequent heating run (5 °C/min). U-CER exhibits one major endothermic transition on heating and cooling (Figure 1a,b). HFA-CER exhibits similar behavior on heating but displays two overlapping components on cooling (Figure 1c,d). The

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[1] Abbreviations: HFA-CER, 2-hydroxy fatty acid containing cerebrosides; NFA-CER, nonhydroxy (*n*-acyl) fatty acid containing cerebrosides; U-CER, unfractionated cerebrosides; DSC, differential scanning calorimetry.

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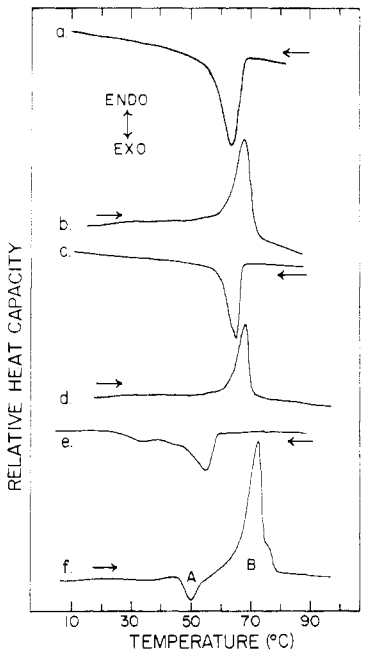


FIGURE 1: Differential scanning calorimetry traces of multilamellar dispersions of unfractionated bovine brain cerebrosides (a, b), HFA-CER (c, d), and NFA-CER (e, f). Runs a, c, and e are cooling runs, while runs b, d, and f are their respective subsequent heating runs. All thermal scans were run at a heating/cooling rate of 5 °C/min.

Table I: Transition Temperatures and Enthalpies[a]

sample	T_i (°C)	T_m (°C)	T_f (°C)	ΔH (cal/g)
U-CER	49	67	72	6.9
HFA-CER	52	68	73	7.3
NFA-CER				
transition A	45	50	58	−2.5
transition B	58	72	79	16.2

[a] For 5 °C/min heating runs, following 5 °C/min cooling runs.

behavior of NFA-CER is quite different, exhibiting two transitions on heating: (A) a small exotherm at ~50 °C and (B) a major endotherm at ~72 °C (Figure 1f). Significant supercooling is observed for NFA-CER, and the cooling exotherm is complex (Figure 1e). The initial (T_i), maximal (T_m), and final (T_f) temperatures for the observed transitions are presented in Table I, in addition to the individual transition enthalpies (ΔH). The T_m's for U-CER (67 °C) and HFA-CER (68 °C) are approximately equal, while the T_m for the major NFA-CER endotherm (72 °C) is ~5 °C higher. The T_f for transition A of NFA-CER and T_i for transition B are approximate, since these transitions probably overlap. A striking difference is observed between the ΔH for the major heating endotherm B of NFA-CER (16.2 cal/g) and that of HFA-CER (7.3 cal/g) or U-CER (6.9 cal/g).

The small exotherm A in the NFA-CER heating trace is indicative of the presence of a metastable low-temperature state which spontaneously undergoes an exothermic transition on heating to a more stable state. This metastable behavior was investigated by heating through the exotherm A, stopping at a temperature (59 °C) just before the major endotherm B, and then cooling (Figure 2a,b). No transitions were observed during the cooling run, and a subsequent heating run (Figure 2c) reveals only the major endotherm B. Thus transition A is irreversible.

The metastable behavior of NFA-CER was further investigated by varying the cooling rate and observing the effect upon subsequent heating runs at 5 °C/min. Heating runs at

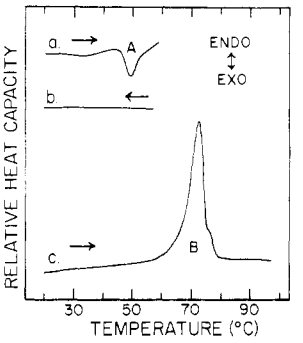


FIGURE 2: Differential scanning calorimetry of NFA-CER, demonstrating the irreversibility of heating exotherm A. The sample was (a) heated from −3 to 59 °C, (b) immediately cooled to −3 °C, and then (c) immediately heated to 97 °C. The heating/cooling rate was 5 °C/min.

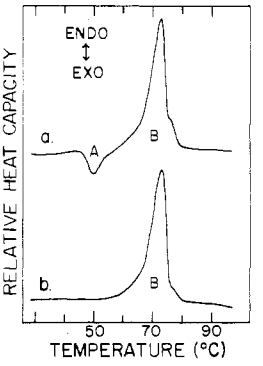


FIGURE 3: Heating scans of NFA-CER at 5 °C/min, which follow either a 5 °C/min cooling scan (a) or a 0.6 °C/min cooling scan (b).

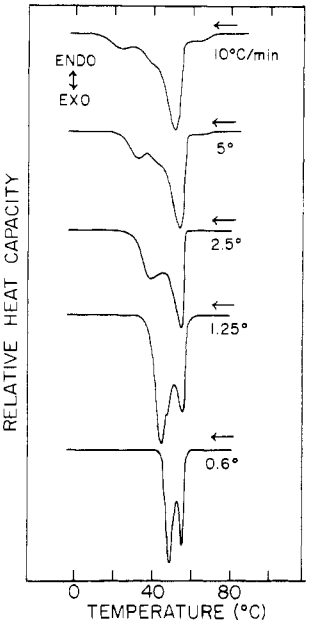


FIGURE 4: Cooling behavior of NFA-CER, at various cooling rates. The relative areas of the thermal profiles as shown do not reflect the relative enthalpies; see Table II for enthalpy as a function of cooling rate.

5 °C/min are presented in Figure 3 which follow cooling runs at either 5 (Figure 3a) or 0.6 °C/min (Figure 3b). The exotherm A is not observed in the heating run which follows a 0.6 °C/min cool. Thus, slow cooling results in formation of the stable low-temperature state exclusively. Examination of cooling runs at various rates is instructive. In Figure 4 are presented cooling traces obtained at rates varying from 10 to 0.6 °C/min. At 10 °C/min the cooling behavior of NFA-CER is complex, exhibiting a very broad (12–72 °C) exotherm

Table II: Enthalpy Data for NFA-CER as the Cooling Rate Is Varied[a]

cooling rate (°C/min)	ΔH (cal/g)		
		subsequent heating run	
	cooling run	transition A	transition B
10	−7.7	−2.8	16.2
5	−8.3	−2.5	16.2
2.5	−9.3	−1.5	16.0
1.25	−11.8	0	17.9
0.6	−13.6	0	17.6

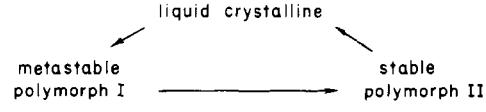
[a] The cooling rate was varied; all heating runs were at 5 °C/min.

composed of at least four components. The major exotherm, at 53 °C, is supercooled ~20 °C from the major heating endotherm B. At 5 °C/min the cooling curve is similar in shape but has decreased in overall breadth (22–72 °C); the major exotherm is at 55 °C. As the cooling rate is further decreased to 0.6 °C/min, the following effects are noted: (i) the exotherm at 53–55 °C remains constant in temperature, but becomes sharper, and (ii) the broad lower temperature components decrease in transition range and finally coalesce into a relatively sharp transition at 50 °C. In Table II are presented enthalpy data for the cooling runs at various rates and for subsequent heating runs at 5 °C/min. As the cooling rate is decreased, the enthalpy of the complex cooling exotherm increases, while the enthalpy of the heating exotherm A in the subsequent heating run decreases. Following cooling runs at rates of 1.25 °C/min or less, the exotherm A is not observed in the subsequent heating runs. The enthalpy of the heating endotherm B appears to be larger when the previous cooling rate was 1.25 °C/min or slower. This effect may not be significant because it may simply reflect base-line changes due to the possible partial overlap of the low enthalpy exotherm A with endotherm B.

Discussion

The observation of metastability in NFA-CER confirms a previous report (Bunow, 1979) and is similar to observations on glucocerebroside from Gaucher's spleen (Freire et al., 1980) and on synthetic gluco- and galactocerebrosides containing an *N*-palmitoyl acyl chain (Freire et al., 1980; Ruocco et al., 1981). Glucocerebroside from Gaucher's spleen contains only nonhydroxylated *n*-acyl groups (Correa-Freire et al., 1979); thus the cerebroside types which exhibit metastability are characterized by the absence of hydroxylated fatty acids. The 2-hydroxy fatty acid containing cerebrosides from bovine brain, on the other hand, have been shown in the present study to exhibit no metastability. Whole brain cerebrosides, which contain predominantly HFA-CER, also exhibit no metastable behavior.

In the present study, the metastable behavior of NFA-CER has been extensively characterized. This behavior can be schematically summarized as



The evidence for this scheme is as follows. As shown in Figures 1 and 2, a heating run which follows a fast cooling run (5 °C/min) exhibits an exotherm at ~50 °C. The evolution of heat by the sample at this transition indicates that a portion of the sample is transformed from a metastable state (polymorph I) into a more stable state (polymorph II). Figure 2 shows that this transition is irreversible; thus after being heated through transition A (but not through B), the entire sample is in the polymorph II form. Subsequent heating through the major endotherm B results in transformation of polymorph II into the liquid-crystalline (fluid acyl chain) state. Figure 3 demonstrates that the heating exotherm A is absent after a slow cool, indicating that a slow cool results in the formation exclusively of polymorph II. This behavior is further clarified by examination of the cooling behavior as the cooling rate is varied. Figure 4 demonstrates that a major transition occurs on cooling at 53–55 °C, whose temperature maximum is essentially independent of cooling rate. When the sample is cooled at 10 °C/min, other broad features are observed at lower temperatures. These features are observed at progressively higher temperatures when the cooling rate is slower and coalesce into one relatively sharp peak at ~50 °C when the cooling rate is 0.6 °C/min. The most reasonable interpretation of these observations is that the invariant cooling exotherm at 53–55 °C reflects the liquid crystalline → metastable polymorph I transition. The cooling transitions at lower temperatures represent the metastable polymorph I → stable polymorph II transition. Since the temperature dependence of the polymorph I → polymorph II transition varies with cooling rate, it is kinetically limited; i.e., it occurs at a rate which is slower than the experimental cooling rate. The rate dependence of the cooling transition enthalpy is also instructive. As shown in Table II, the enthalpy on cooling becomes larger as the cooling rate is decreased. At the slowest cooling rate studied, 0.6 °C/min, the cooling enthalpy (−13.6 cal/g) approaches the enthalpy of the major heating endotherm B (17.6 cal/g) of the subsequent heating run. Furthermore, the enthalpy of the small heating exotherm A decreases with increasing rate of the previous cooling run and disappears when the previous cooling rate is ≤1.25 °C/min. This indicates that after a slow cool the sample is entirely in the polymorph II state. However, for all cooling/heating protocols studied, the total exothermic ΔH (cooling ΔH, or cooling ΔH plus ΔH of subsequent heating exotherm A) is less than the endothermic ΔH for heating transition B. Thus we conclude that a portion of the polymorph I material converts into polymorph II noncooperatively under the conditions studied. In summary, cooling NFA-CER from the liquid-crystalline state results first in formation of metastable polymorph I, which is subsequently transformed into the stable polymorph II, either on slow cooling or on subsequent heating. The major heating endotherm at 72 °C is the stable polymorph II → liquid-crystalline phase transition and is analogous to the gel → liquid-crystalline phase transition of phospholipids. The metastable thermal behavior of NFA-CER is similar to that observed for synthetic palmitoylgalactocerebroside by Ruocco et al. (1981). In an elegant X-ray diffraction and DSC study of the synthetic cerebroside, these workers have demonstrated that the metastable low-temperature form is dehydrated and undergoes a kinetically limited rehydration to the stable low-temperature form.[2]

The enthalpy of the major heating endotherm B of NFA-CER is approximately twice that observed for synthetic phospholipids (Ladbrooke & Chapman, 1969; Hinz & Sturtevant, 1972). Such high enthalpy ordered → liquid-crystalline transitions have been observed for palmitoylgalactocerebroside and also for stearoylsphingomyelin (Ruocco et al., 1981; Estep et al., 1980). It appears that sphingolipids are capable of forming highly ordered gel states which may be stabilized by

[2] Polymorphs I and II in the present study correspond to the metastable A form and the stable E form, respectively, of Ruocco et al. (1981).

intermolecular hydrogen bonds involving the amide group, the sphingosine C-3 hydroxyl group, and, in the case of cerebroside, the glycosyl head group.

HFA-CER from bovine brain exhibits no detectable metastability and exhibits little supercooling (~3 °C). Thus HFA-CER does not undergo any kinetically limited transition behavior which might be associated with hydration/dehydration phenomena. The gel ↔ liquid-crystalline transition is completely reversible and exhibits a ΔH (~7 cal/g) which is similar to that for phosphatidylcholine and less than half of the ΔH observed for NFA-CER (16–17 cal/g). Unfractionated cerebrosides exhibit thermal behavior which is similar to that observed for HFA-CER. HFA-CER is the predominant fraction (~60%) of whole brain cerebroside (O'Brien & Rouser, 1964) and apparently dominates the phase behavior of the natural mixture.

A molecular interpretation of the large difference in ΔH between NFA-CER and HFA-CER must be discussed in terms of initial and final states. The entropy change ΔS ($=\Delta H/T$) for HFA-CER is less than the ΔS for NFA-CER. It is possible that the increased hydrogen-bonding capability of HFA-CER (via the 2-OH acyl group) stabilizes the liquid-crystalline state. Alternatively, the presence of the hydroxyl branch in HFA-CER may cause a disruption of acyl chain packing in the gel state. Neither possibility can be strictly eliminated, but it is unlikely that the addition of one more hydrogen bond in the liquid-crystalline state of HFA-CER could account for the observed large difference in ΔH between HFA- and NFA-CER. We conclude that the presence of the 2-OH group in HFA-CER prevents the formation of the unusually stable gel state observed for *n*-acylcerebrosides and for stearoylsphingomyelin.

The function of amide-linked hydroxy fatty acids in bovine brain cerebroside is unknown. However, the present results suggest that one function of the 2-OH acyl group may be to prevent metastable cerebroside phase behavior. In most membranes, cerebroside content is very low, and thus the presence of metastable domains of cerebroside may not be a significant problem. In myelin, however, cerebrosides make up ~20% of the membrane lipid. Furthermore, studies of viral membranes have indicated that glycolipids are found preferentially in the external monolayer (Stoffel & Sorgo, 1976), and recent studies suggest that cerebroside is similarly situated in myelin (Linington & Rumsby, 1980). Thus the outer monolayer (at the external apposition or intraperiod dense line) of the myelin membrane may contain as much as 40% cerebroside. At this high cerebroside content, it is possible that cerebroside metastability could become problematic, particularly since the maximum solubility of cerebrosides in phosphatidylcholine model membranes is ~30% (W. Curatolo, unpublished results). Since cerebroside metastability has been shown to involve dehydration (Ruocco et al., 1981), its occurrence in mature myelin would most probably be destructive. The possible function of HFA-CER in preventing such metastability is speculative at this time, since myelin contains a variety of other glycolipids and phospholipids and, most importantly, ~30% cholesterol. The interactions of these membrane lipids with cerebrosides have not yet been investigated.

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