Calorimetric Investigation of the Complex Phase Behavior of Glucocerebroside Dispersions†

E. Freire,* D. Bach, M. Correa-Freire, I. Miller, and Y. Barenholz

ABSTRACT: The thermotropic behavior of aqueous dispersions of glucocerebroside from Gaucher's spleen has been investigated by differential scanning calorimetry. These results indicate that glucocerebroside undergoes two distinct phase transitions centered at 47 and 83 °C, respectively. The high-temperature transition is associated with the main gel–liquid crystalline transition and has an enthalpy change of 13.6 kcal/mol of lipid; this transition is not rapidly reversible and the liquid crystalline phase supercools to a metastable gel phase. The low-temperature transition is exothermic with an enthalpy change of −6.3 kcal/mol and involves a transformation of the metastable gel phase to a more highly ordered gel conformation, without involving changes in the conformational state of the hydrocarbon chains. The behavior of these transitions as a function of the amount of water suggests that the origin of the metastability is related to a hydration–dehydration process of the cerebroside molecule. Experiments with synthetic D-*erythro*-*N*-palmitoylglucocerebroside revealed the same thermotropic behavior. These glucocerebroside transitions are irreversible and together define a unidirectional cycle in which each state of the molecule can only be reached by completing the entire cycle.

Glucocerebroside (glucosylceramide) accumulates in abnormally high quantities in cells of the reticuloendothelial system of Gaucher's patients. This accumulation is the result of a subnormal activity of the enzyme glucocerebrosidase. In normal human spleen the concentration of glucocerebroside ranges from 0.06 to 0.28 mg/g wet weight, whereas in Gaucher's patients it rises to 3–40 mg/g wet weight. The lipid-storing cells have distinctive morphological and histological features and are called Gaucher's cells. The main source of the accumulating glucocerebroside is the catabolism of gangliosides and other glycosphingolipids that are degraded via the formation of glucosylcerebroside as an intermediate (Brady, 1978). Even though the reason for the accumulation of glucocerebroside is well understood, no satisfactory explanation exists as to why the accumulation of this compound is expressed in such complicated pathology. It is very likely that the physical properties of glucocerebroside and its effects on membrane structure and function are related to the physiological manifestations of the disease.

Glucocerebroside is a nonsoluble swelling amphipate and as such it is related to other membrane phospholipids. Contrary to the case of glycerophospholipids and sphingomyelins (Lee, 1977; Barenholz & Thompson, 1980), only a few physicochemical studies on cerebroside systems exist in the literature. Previously (Correa-Freire et al., 1979) we reported the results of a calorimetric investigation of the effects of glucocerebroside from Gaucher's spleen on the thermotropic behavior of dipalmitoylphosphatidylcholine liposomes. The results indicated that the mixing of these molecules is not ideal and that glucocerebrosides tend to separate into compositional domains above 15 mol %. Recently, Skarjune & Oldfield (1979) have presented nuclear magnetic resonance evidence of a hydrogen bond network in the polar head-group region of aqueous galactocerebroside dispersions. This observation agrees with our previous results and provides a possible molecular mechanism accounting for the tendency of cerebroside to segregate into compositional domains. In this paper we present a detailed calorimetric study of the thermotropic behavior of glucocerebroside from Gaucher's spleen and synthetic D-*erythro*-*N*-palmitoylglucocerebroside.

Experimental Procedures

Materials. Glucocerebroside was extracted from a biopsy of the spleen of a patient with Gaucher's disease and purified by silicic acid chromatography as described previously (Correa-Freire et al., 1979). The fatty acid composition of the glucocerebroside stock was analyzed by gas–liquid chromatographic procedures after acid hydrolysis and methylation of the sample as described by Kates (1964). The sample consisted mostly of a mixture of saturated, long-chain fatty acids, mostly $C_{22:0}$ and $C_{24:0}$, which together comprised 65% of the total. A complete report of the analysis has been published elsewhere (Correa-Freire et al., 1979). *N*-Palmitoylglucocerebroside (D-*erythro*-*N*-palmitoylsphingosine glucoside) was prepared by condensation of palmitic acid with psychosine (D-*erythro*-sphingosine glucoside) using *N*,*N*-dicyclohexylcarbodiimide (A. Dagan, and Y. Barenholz, unpublished results). The purity of the sample was better than 99% based on thin-layer chromatography.

Scanning Calorimetry. The calorimetric measurements were performed in a Du Pont 990 differential scanning calorimeter equipped with a cell base II and a homemade cooling device. Heating rates of 10, 5, 2, 1, and 0.5 °C/min and sensitivities of 0.4 and 0.2 mcal/(s·in.) were used. The dried glucocerebroside samples were placed in aluminum pans and weighed before adding the desired amount of 50 mM KCl solution; usually 2–5 mg of lipid was used per experiment. The calorimetric pans were then sealed as previously described (Bach et al., 1977). In this way calorimetric scans as a function of scanning rate and water content were obtained.

Results

In Figure 1 a typical sequence of glucocerebroside calorimetric scans has been plotted as a function of temperature. The scanning rate in these experiments was 5 °C/min. Curve A shows the characteristic calorimeter trace of a freshly prepared aqueous dispersion of glucocerebroside; this scan is characterized by the presence of a well-defined endothermic

† From the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908 (E.F., M.C.F., and Y.B.), the Department of Membrane Research, The Weizmann Institute of Science, Rehovot, Israel (D.B. and I.M.), and the Department of Biochemistry, The Hebrew University, Jerusalem, Israel (Y.B.). *Received March 6, 1980.* Supported by grants from the National Institutes of Health (GM27244, GM-26894, GM-23573, HL17576, and NS02967) and U.S.–Israel Grant BSF-1688.

0006-2960/80/0419-3662$01.00/0

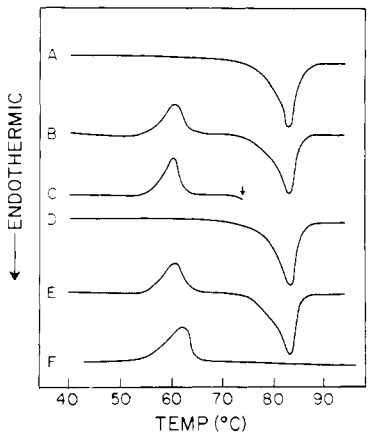


FIGURE 1: Successive calorimetric heating scans (A through E) and cooling scan (F) of an aqueous dispersion of glucocerebroside from Gaucher's spleen. The scanning rate in these experiments was 5 °C/min. The detailed explanation of the labels is in the text.

Table I: Thermodynamic Parameters Associated with the Phase Transitions of Glucocerebroside[a]

system	transition[b]	ΔH (kcal/mol)	T_m (°C)
glucocerebroside from Gaucher's spleen (50 mM KCl)	I	−6.3	61
	II	13.6	83
	III[c]	−7.1	63
glucocerebroside from Gaucher's spleen (dried sample)	II	12.1	105
N-palmitoylglucocerebroside (50 mM KCl)	I	−5.6	51
	II	17.1	87.5
	III[d]	−11.5	

[a] From calorimetric scans at a scanning rate of 5 °C/min. [b] The assignment of the transitions is defined in eq 1. [c] From calorimetric cooling scans at scanning rate of 5 °C/min. [d] Calculated by completing the entire cycle.

peak centered at 83 °C that is typical of the lipid hydrocarbon chains' melting transition. The enthalpy change for this transition is 13.6 kcal/mol. Curve B represents the second scan of the sample; the striking feature in this experiment is the appearance of a clearly distinguishable exothermic transition at 61 °C in addition to the main endothermic peak at 83 °C. This exothermic transition is characterized by an enthalpy change of −6.3 kcal/mol. In curve C the third calorimetric scan is shown; as in the case of curve B the exothermic peak is also present, indicating that this transition can be reproduced after a complete scan of the sample. In this case, however, the calorimeter was stopped before the endothermic transition took place, and the sample was cooled down to room temperature to initiate a new scan. In this new scan (curve D) the exothermic peak was absent and only the main transition endotherm at 83 °C could be detected by the calorimeter. This result indicates that the exothermic transition is irreversible and that its appearance is dictated by the prior occurrence of the main lipid transition. In fast heating scans (10 °C/min) a small endotherm preceded the exothermic peak. Additional experiments (curve E) showed that the entire process is perfectly reproducible after successive scans of the sample. In cooling scans (curve F) only a single exothermic peak centered at 63 °C could be detected by the calorimeter. The enthalpy change for this transition was −7.1 kcal/mol or about equal in magnitude but opposite in sign to the sume of the enthalpy changes in heating scans (see Table I). The location and the shape of this peak were strongly dependent on the scanning rate, indicating that this transition is relatively slow. Incubation of the sample at room temperature (20 °C)

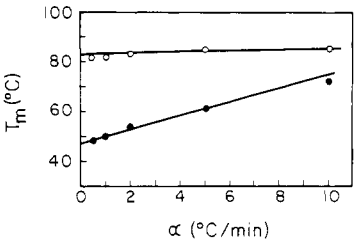


FIGURE 2: Scanning rate dependence of the transition temperatures of the endothermic (○) and exothermic (●) transitions of glucocerebroside from Gaucher's spleen.

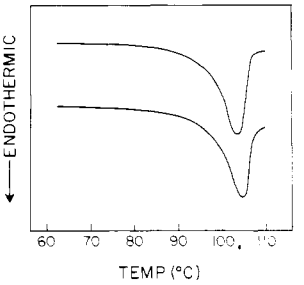


FIGURE 3: Successive calorimetric heating scans of dry glucocerebroside samples from Gaucher's spleen. The scanning rate of these experiments was 5 °C/min.

for a period of ~12 h or more resulted in the disappearance of the exothermic peak. This observation indicates that the liquid-crystalline phase supercools to a metastable phase and that this metastable phase is responsible for the exothermic transition observed in heating scans.

The kinetic behavior of these transitions was examined by performing calorimetric experiments at various scanning rates. In Figure 2 the transition temperatures T_{m_1} and T_{m_2} of the exothermic and endothermic transitions, respectively, have been plotted as a function of the scanning rate. The endothermic peak was, within experimental error, independent of the scanning rate; this was not so for the exothermic transition. T_{m_1} increased with the scanning rate, indicating that the exothermic transition is a slow process. Analysis of the data, as described by Lentz et al. (1978), yielded a characteristic mean relaxation time of 3.5 min and an extrapolated transition temperature at zero scanning rate of 47 °C.

In order to investigate the molecular origin of the exothermic transition, we performed calorimetric scans with dry glucocerebroside samples. These scans are shown in Figure 3. Under these conditions the main transition was shifted to higher temperatures (105 °C) and the enthalpy change was 12.1 kcal/mol, compared to the value of 13.6 kcal/mol obtained for the hydrated samples. In the absence of water, no exothermic peak was ever observed, indicating that this transition is coupled to a hydration–dehydration process. Since addition of water lowers the transition temperature, it can be concluded that the number of water molecules bound to glucocerebroside is larger in the liquid crystalline than in the gel phase.

The thermotropic behavior of glucocerebroside was also investigated as a function of the amount of water present in the sample. In samples containing less than 18% water (w/w), the first scan was not very reproducible and showed a small exotherm at 56 °C followed by the 83 °C endotherm; the enthalpy changes for these peaks were very small (−0.4 and 1.6 kcal/mol) and most probably reflected a nonuniform distribution of water in the sample. Successive scans of the sample corrected this anomalous behavior and produced a pattern of transitions similar to the one observed for the fully

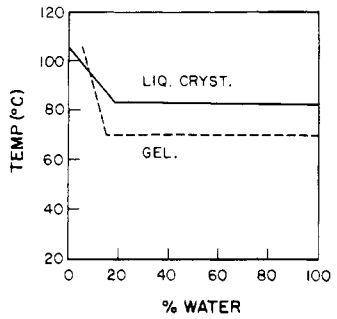


FIGURE 4: Phase diagrams for glucocerebroside from Gaucher's spleen as a function of the amount of water (solid line). The dotted line is the phase diagram for galactocerebroside–water obtained previously by Abrahammson et al. (1972).

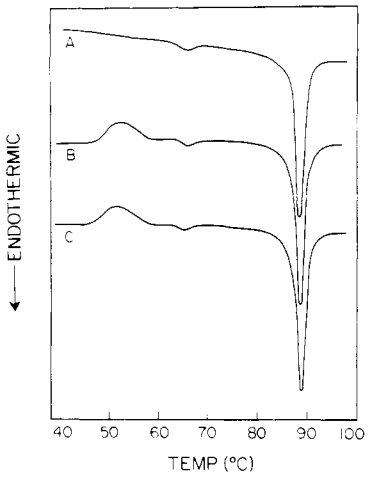


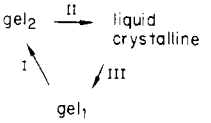
FIGURE 5: Successive calorimetric heating scans of aqueous dispersions of synthetic D-*erythro*-*N*-palmitoylglucocerebroside. The scanning rates of these experiments was 5 °C/min.

hydrated samples. In Figure 4 the transition temperature of the main endothermic peak has been plotted as a function of the amount of water. This figure defines the phase diagram for natural glucocerebroside–water. Also shown in the figure is the corresponding phase diagram for galactocerebroside–water obtained previously by Abrahammson et al. (1972) using X-ray diffraction techniques. As can be seen in the figure, the two phase diagrams are completely similar, differing only in the absolute value of the temperature scale.

The thermotropic behavior of synthetic *N*-palmitoylglucocerebroside was completely analogous to that of glucocerebroside extracted from Gaucher's spleen, despite the fact that the natural sample was a mixture containing fatty acid chains of various lengths (Correa-Freire et al., 1979). As shown in Figure 5, the calorimetric heating scans of *N*-palmitoylglucocerebroside are characterized by the presence of a very sharp endotherm centered at 87.5 °C. The enthalpy change for this transition is 17.1 kcal/mol. This main endotherm was preceded by a small pretransition at 65 °C similar to that observed in calorimetric scans of synthetic phosphatidylcholines. As in the case of natural glucocerebroside, an exothermic peak centered at 51 °C appeared on the second scan of the sample. This exothermic peak was characterized by a ΔH value of −5.6 kcal/mol and had a similar behavior to the one observed for natural glucocerebroside. In Table I we have summarized the thermodynamic parameters associated with this sequence of transitions.

Discussion

In this paper we have investigated the thermotropic behavior of glucocerebroside from Gaucher's spleen and synthetic D-*erythro*-*N*-palmitoylglucocerebroside using differential scanning calorimetry. From a thermodynamic point of view the above experiments suggest the following mechanism for the entire sequence of glucocerebroside transitions:



(1)

In this scheme, transition II represents the main endothermic peak involving the melting of the hydrocarbon chains. After the sample is cooled to room temperature (20 °C), the liquid crystalline state undergoes a transition (III) to a metastable gel state (gel_1). At room temperature this metastable gel state slowly decays to a more ordered gel conformation (gel_2) over a period of ~12 h, thus satisfying the requirement that the appearance of the exothermic peak is due to the existence of a kinetically limited process. This exothermic process can be greatly accelerated by increasing the temperature, as demonstrated by the appearance of the 47 °C exothermic peak in the calorimetric heating scans (transition I). Under our experimental conditions each of the individual transitions in the above scheme is irreversible. The immediate implication of this scheme is that any given state of the molecules can only be reached by completing the entire cycle, i.e., the cycle is unidirectional. Transitions I–III are thermodynamically irreversible as deduced from the sequence of scans in Figure 1 and by the fact that transition III is the only one which can be detected in the cooling mode.

From a molecular point of view this sequence of transitions can be interpreted as follows: the main gel–liquid-crystalline transition of aqueous dispersions of glucocerebroside is a complex process involving both the melting of the hydrocarbon chains and a hydration process associated with the head groups. Below the phase-transition temperature, the cerebroside head groups are presumably immobilized by an internal network of hydrogen bonds; in the liquid-crystalline phase this internal network is at least partially lost and the available hydrogen bond valences are satisfied with water molecules, thus increasing the degree of hydration of glucocerebroside and presumably the average intermolecular distance. This hydration process is probably facilitated by the increased area available to the head groups resulting from the expansion of the lipid molecules induced by the melting of the chains. This interpretation of the main transition is consistent with recent deuterium nuclear magnetic resonance studies of *N*-palmitoylgalactocerebroside (Skarjune & Oldfield, 1979) and is supported by the unusually high value of the enthalpy change observed for this transition and by the fact that addition of water to dried cerebroside samples results in a lowering of the transition temperature.

Rapid cooling of the liquid-crystalline sample to temperatures below the main transition temperature results in the uncoupling of the melting and hydration processes and the formation of a metastable gel phase. The formation of this metastable phase involves a reordering of the hydrocarbon chains and presumably no changes in the degree of hydration. The enthalpy value of −7.1 kcal/mol obtained for this transition is consistent with the above interpretation and in agreement with the results of Bunow (1979) for different fractions of bovine galactocerebroside. The metastable gel phase decays exothermally to a more highly ordered gel conformation by re-forming the internal network of hydrogen bonds between the cerebroside head groups. The similarities of the enthalpy values observed for the synthetic and natural

glucocerebroside samples suggest that this exothermic transformation does not involve a major rearrangement of the hydrocarbon chains.

The existence of metastable phases and irreversible processes in association with the phase behavior of supramolecular lipid structures has received little attention, and their biological implications are still unknown. Recently, however, Estep et al. (1980) have presented evidence of a metastable gel phase in *N*-stearoylsphingomyelin and studied the thermodynamics and structural characteristics of these conformations. These authors concluded that the gel phase of these lipids is more highly ordered than that of other phospholipids and undergoes a gel–liquid-crystalline transition that is not rapidly reversible. The resulting liquid-crystalline phase supercools below the phase-transition temperature, giving rise to a metastable gel phase that slowly converts to a highly ordered gel phase. It must be noted, however, that the pattern of transitions obtained for *N*-stearoylsphingomyelin differs from that of glucocerebroside. In the case of *N*-stearoylsphingomyelin, the metastable phase is also capable of undergoing the gel–liquid-crystalline transition as demonstrated by the appearance of a second endothermic peak associated with the melting of the metastable phase (Estep et al., 1980). In the case of glucocerebroside the metastable phase transforms into the stable gel phase during the exothermic transition. These differences in behavior suggest that the sources of metastability for these two systems are different. In the case of glucocerebroside, the metastable behavior is most likely associated with the head groups, as deduced from the calorimetric data and by the fact that both natural glucocerebroside and synthetic *N*-palmitoylglucocerebroside show this behavior. On the other hand, it is quite conceivable that the origin of the metastable behavior in *N*-stearoylsphingomyelin is associated with the packing of the hydrocarbon chains, since *N*-palmitoylsphingomyelin and *N*-lignocerylsphingomyelin do not show a metastable behavior (Estep et al., 1980).

References

Abrahamsson, S., Pascher, I., Larsson, K., & Karlsson, K. A. (1972) *Chem. Phys. Lipids 8*, 162–179.
Bach, D., Bursuker, I., & Goldman, R. (1977) *Biochim. Biophys. Acta 469*, 171–179.
Barenholz, Y., & Thompson, T. E. (1980) *Biochim. Biophys. Acta* (in press).
Brady, R. O. (1978) in *The Metabolic Basis of Inherited Disease* (Stanbary, J. B., Wyngmarden, J. B., & Fredrickson, D. F., Eds.) pp 731–796, McGraw-Hill, New York.
Bunow, M. R. (1979) *Biochim. Biophys. Acta 574*, 542–546.
Correa-Freire, M. C., Freire, E., Barenholz, Y., Biltonen, R. L., & Thompson, T. E. (1979) *Biochemistry 18*, 442–445.
Estep, T. N., Calhoun, W. I., Barenholz, Y., Biltonen, R. L., Shipley, G. G., & Thompson, T. E. (1980) *Biochemistry 19*, 20–24.
Kates, M. (1964) *J. Lipid Res. 5*, 132.
Lee, A. G. (1977) *Biochim. Biophys. Acta 472*, 285–344.
Lentz, B. R., Freire, E., & Biltonen, R. L. (1978) *Biochemistry 17*, 4475–4480.
Skarjune, R., & Oldfield, E. (1979) *Biochim. Biophys. Acta 556*, 208–218.

Characterization of the Pretransition in 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine by Fourier Transform Infrared Spectroscopy†

David G. Cameron, Hector L. Casal, and Henry H. Mantsch*

ABSTRACT: Fourier transform infrared spectroscopy has been used to study the infrared-active acyl chain vibrational modes of fully hydrated multibilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (L-DPPC) over the temperature range 0–55 °C. Frequencies, bandwidths, and other spectral parameters were measured as a function of temperature for the methylene scissoring, rocking, and wagging modes, as well as for the C–H stretching modes, and they were used to monitor the packing of the acyl chains. Particular emphasis was placed on determining the nature of the pretransition event. It is shown that between 36 and 38 °C the spectral changes are indicative of a phase change in the acyl chain packing from an orthorhombic to a hexagonal subcell. It is also concluded that in the gel phase, at all temperatures below the main transition, the acyl chains are predominantly in all-trans conformations and that the temperature-dependent variations of spectral parameters result from changes in interchain interactions.

The phospholipid 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (L-DPPC) forms bilayer structures when hydrated, and has been used extensively as a model for more complex biomembranes. The bilayer exists in two distinctly different phases. The nature of the liquid-crystalline phase, particularly the state of the acyl chains at temperatures above 42 °C, has been well characterized by vibrational spectroscopy (Wallach et al., 1979, and references therein) and by ^{2}H NMR (Seelig & Seelig, 1974; Davis, 1979). In this phase, the acyl chains contain large numbers of gauche conformers, which vary rapidly in number and location, leading to the description of it as a fluid state.

The detailed organization of the bilayer in the gel phase is less clearly understood. Extensive studies on multibilayers by techniques such as X-ray diffraction (Tardieu et al., 1973;

† From the Division of Chemistry, National Research Council of Canada, Ottawa, Ontario, Canada K1A OR6. *Received January 16, 1980.* Issued as NRCC No. 18178. H.L.C. is supported in part by the University of Ottawa.

0006-2960/80/0419-3665$01.00/0