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THE METASTABILITY OF GLUCOSYL CERAMIDE IN AQUEOUS PHASE: EFFECT OF HYDRATION AND PHOSPHATIDYLCHOLINES OF VARIOUS CHAIN LENGTH

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ABSTRACT

The thermotropic behavior of aqueous dispersions of glucosylceramide and mixtures of (i) dimyristoylphosphatidylcholine-glucosylceramide; (ii) dipalmitoylphosphatidylcholine-glucosylceramide; and (iii) egg phosphatidylcholine-glucosylceramide were investigated using differential scanning calorimetry. All these systems form a lamellar array in aqueous phase. Aqueous dispersions of glucocosylceramide exhibit complex thermotropic behavior due to the presence of metastable and stable gel allomorphs. This polymorphism results primarily from hydration-dehydration processes which involve head group-head group interactions. All three phosphatidylcholines disturb the complex thermotropic behavior of glucosylceramide. The data suggest that the interference with the intermolecular interactions among the glycosphingolipid molecules by phospholipid molecules is strongly dependent on the acyl chain of the phosphatidylcholine. This effect is directly related to phospholipid-glycosphingolipid molecular miscibility which is determined from the minimal mole % phosphatidylcholine required to abolish the complex thermotropic behavior of the cerebroside. Based on this criteria we found the following order of efficacy, dipalmitoylphosphatidylcholine > dimyristoylphosphatidylcholine >> egg-phosphatidylcholine (16.5 > 22.5 >> 60 mole %, phosphatidylcholine, respectively). Reducing the level of water below its saturation or replacing 50% of the water by ethylene glycol reduces this effect of the phosphatidylcholine. This supports the assumption that hydration and hydrogen bonding are involved. Phase separation occurs throughout all the phase diagrams of the egg phosphatidylcholine-glucosyl ceramide. This was not the case for the systems of the two disaturated phosphatidylcholines. The system of egg phosphatidylcholine-glucosyl ceramide may be of relevance to Gaucher's disease which is characterized by major accumulation of glucosyl ceramide in various organs.

INTRODUCTION

The polymorphism of polar lipids is a well established phenomenon which can be explained by their amphipatic nature and by the fine details of their molecular structure (for review, see Israelachvili, 1, and Rilfors et al., 2). They may undergo various types of phase transformations. Some of these like the micellar to lamellar and lamellar to hexagonal type II involve major alterations in the organization of the assembly and are of low enthalpic changes. Others include the transformation between the various states of the lamellar organization, such as the liquid

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crystalline, gel and crystalline states (for review see 3,4). The transformations between the above states are well characterized and since they involve enthalpic changes, they can be detected calorimetrically and characterized according to various parameters of their thermograms.

Recently, more attention is given to the presence of metastable states which are history dependent and lead to a complex thermotropic behavior. These may be related to interdigitation between the lipid molecules present in the two monolayers forming the lipid bilayer due to chain disparity between the two hydrocarbon chains of these lipids (for review see 4). It may also be a result of changes in head group-head group interaction due to hydration-dehydration processes (5-10).

Cerebrosides (the subject of this paper) are good examples for the latter category. Cerebroside (N-Acyl hexosyl ceramides) when dispersed in aqueous solutions form lamellar assay. These dispersions exhibit a polymorphism leading to a complex thermotropic behavior which results in unidirectional cycle of thermotropic phase transitions (5-10).

Work of ours (5-7) and others (8-10) demonstrate that this complex thermotropic behavior is unaffected by the length of the normal acyl chain of the cerebroside. However, in cerebrosides having acyl chains containing groups which interfere with the tight packing of the molecules, a kinetic barrier is imposed to completion of the cycle (10). In this mini-review, we will describe our work on systems containing D-erythro glucosyl ceramides, a major product of the metabolic degradation of gangliosides which accumulates extensively in the course of Gaucher's disease (11).

Firstly, we will describe the thermotropic behavior of the aqueous dispersions of the pure glucosyl ceramide focussing on the data which led us to propose the unidirectional cycle of thermotropic phase transitions. Then, the effect of the presence of phosphatidylcholines differing in their acyl chains on this complex thermotropic behavior will be demonstrated and analyzed in terms of microscopic miscibility.

MATERIALS AND METHODS

Dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC), purest grade, was purchased from Fluka (Buchs, Switzerland) and from Sigma (St. Louis, MO). Grade I hen egg phosphatidylcholine (egg PC) was purchased from Lipid Products, South Nutfield, England. D-Erythro glucosylceramide (GlcCer) was prepared and characterized as described elsewhere (13). The purity of all lipids was greater than 99% on the basis of thin layer chromatography (loading 0.5-1 mg of lipid per 2 cm strip). All other reagents were of analytical grade or better. To prepare aqueous dispersions of DPPC- GlcCer or DMPC-GlcCer mixtures, the lipids were initially dissolved in a chloroform-methanol solvent (2/1 v/v). The solvent was removed by prolonged exposure to a stream of nitrogen or by reduced-pressure flash evaporation. The complete removal of solvent traces from the samples was accomplished by using high vacuum (0.1 mmHg) for 3 hours. The dry mixtures were pulverized and then weighed into the aluminum pans of the differential scanning calorimeter, and the desired amount of 50 mM KCl solution was

added. The pans were then hermetically sealed. There were indications that during evaporation of the organic solvents, partial demixing of the two components occurred. The remixing during the grinding of the material before dispersion in the aqueous solution was probably not complete at room temperature and was completed only at elevated temperatures. Therefore, heating scans were repeated with each sample until no further change in the thermogram occurred. Macroscopic demixing of the two components occurred when this process was applied to prepare egg PC-GlcCer dispersions (7).

To overcome this difficulty, a novel dispersion method was developed. The egg PC and GlcCer were mixed in chloroform:methanol 2:1 (vol:vol). The lipid solution was then placed in a glass spray gun, designed to use a very small volume of solution. Either low pressure nitrogen or air was applied to spray the solution onto a glass plate at a low rate. The solvents were completely removed from the micro-droplets collected on the glass plate by a stream of nitrogen. The dry lipid mixture was placed in preweighed calorimeter pans and liposomes were prepared as described above for the DPPC-GlcCer and DMPC-GlcCer mixtures.

Calorimetric measurements were performed on a DuPont 990 differential scanning calorimeter, equipped with a cell base II and a specially constructed cooling device. The calibrated mode was employed. A heating rate of 5°C/min and sensitivities of 0.1 and 0.2 mcal S^{-1} $inch^{-1}$ were used in most measurements (5).

RESULTS

Chemical Characterization of the Glucosyl Ceramide

Table I describes the acyl chain composition of D-Erythro glucosyl ceramide purified from a spleen biopsy from a Gaucher's disease patient (for details see 12).

Table I: FATTY ACID COMPOSITION OF GLUCOSYLCERAMIDE

Fatty Acid[a]	% of total	Fatty Acid[a]	% of Total
C16:0	3.90	C23:0	13.82
C18:0	3.22	C24:0	31.07
C20:0	5.25	C24:1	6.98
C22:0	34.62	Others	1.14

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

The data show clearly that most of the molecules have acyl chains much longer than the paraffinic chain contributed by the sphingosine base and therefore will exhibit major chain disparity which may lead to interdigitation between the two monolayers forming the lipid bilayer.

THERMOTROPIC BEHAVIOR OF AQUEOUS DISPERSIONS OF GlcCer.

Glucosylceramide, extracted from a biopsy of the spleen of a patient with Gaucher's disease and dispersed in aqueous solution (excess water), has a complex thermotropic behavior (5). During the first heating scan of pure GlcCer, only one endotherm with a T_m at 82°C and an enthalpy change of 13.6 kcal/mol is observed. During subsequent heating scans an exotherm appears. The T_m of the exotherm, which is dependent on the scanning rate, is between 47 and 70°C in the scanning-rate range of 0-10°C/min. There are also traces of another endotherm centered at temperatures slightly lower than the exotherm (Fig. 1, Curve A; Fig. 4, Curve A; Fig.

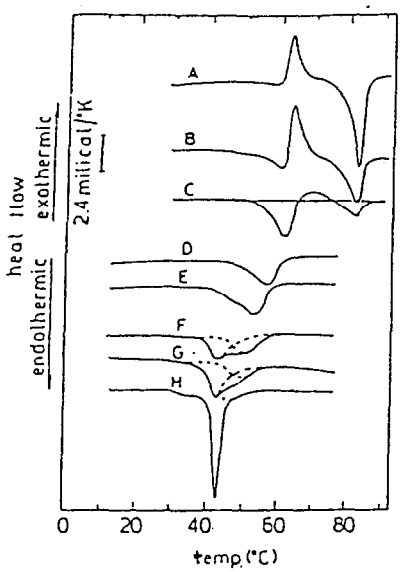

Figure 1: Thermograms obtained in heating mode for aqueous dispersions of GlcCer-DPPC mixtures in excess water:
(A) pure GlcCer, 1.4 mg;
(B) DPPC, 96.7/3.3 (total lipid), 1.5 mg;
(C) GlcCer-DPPC, 90/10,2.2 mg;
(D) GlcCer-DPPC, 65/35, 1.6 mg;
(E) GlcCer-DPPC, 56/44, 2.1 mg;
(F) GlcCer-DPPC, 39/61, 1.8 mg;
(G) GlcCer-DPPC, 26/74, 1.6 mg;
(H) GlcCer-DPPC, 15/85, 1.8 mg.
Compositions are given in weight percent. Heating rate was 5°C/min. The solid line (C) indicates the base line.

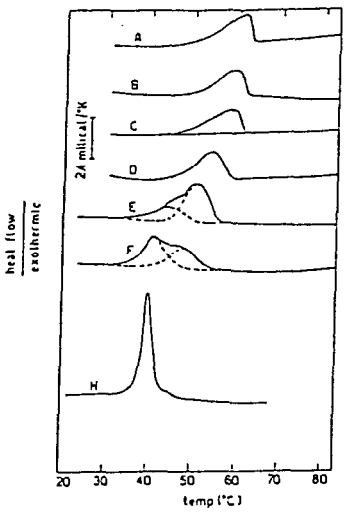

Figure 2: Thermograms obtained in cooling mode for aqueous dispersions of GlcCer-DPPC mixtures in excess water. Compositions are the same as for correspondingly lettered curves in Figure 1. Cooling rate was 5°C/min. The solid line in (C) indicates the base line.

5, Curve G). Comparison between heating and cooling scans show major hysteresis since in the cooling mode, only one exotherm is observed, which is asymmetrical and is centered at about 62°C (Fig. 2, Curve A), namely 20°C below the T_m of the main endotherm present in the heating scans. These thermograms can be explained by the transitions of glucosylceramide diagrammed in Figure 3 and discussed in detail by Freire et al. (5).

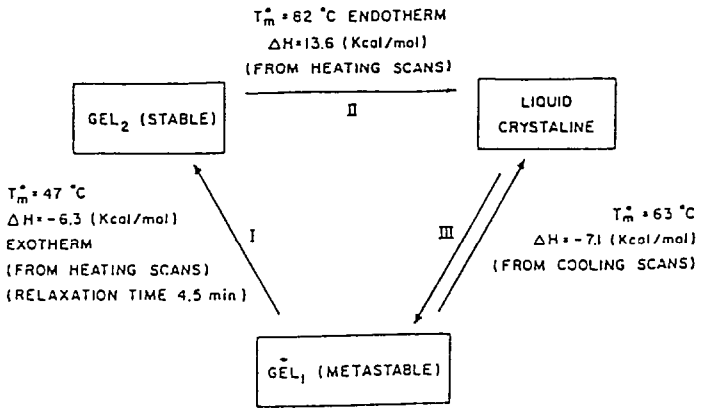

Figure 3: Schematic representation of complex thermotropic behavior of pure glucosylceramide dispersions in excess water [based on 5].

EFFECT OF SCANNING RATE

A relaxation time of 4.5 minutes for the transformation of metastable to stable gel allomorph was determined from the linear curve (6) describing the T_m of the exotherm (heating mode) as a function of scanning rate (in range of 0.5-10°C/min).

EFFECT OF HYDRATION

The complex thermotropic behavior is dependent on water content of the cerebroside. Under anhydrous conditions, no exotherm appears upon heating. The phase diagram of glucosyl ceramide-water shows saturation at 18% water (5). This value is much lower than the saturation level of most phospholipids (for review, see 13), suggesting that GlcCer has a lower degree of hydration than PC. Lowering the activity of water by mixing it 1:1 (vol/vol) with ethylene glycol affects the GlcCer thermotropic behavior. It causes the above exotherm which relate to the transformation between the stable and metastable gel allomorphs to split into two peaks (Fig. 6, Curve A). This again supports the role of hydration/dehydration in the cycle of phase transitions (Fig.3).

INTERACTION OF GLUCOSYLCERAMIDE WITH PHOSPHATIDYLCHOLINES

Representative heating scans of various GlcCer-DPPC, GlcCer-DMPC and GlcCer-egg PC mixtures in aqueous 50 mM KCl are shown in Figures 1,4,5, respectively. On the basis of

calorimetric scans in the heating mode, it is clear that DPPC at concentrations as low as 3.3% has a marked effect on the thermotropic behavior of GlcCer (Figure 1, Curve B). With increasing DPPC concentrations, the heating scans show a decrease in the ΔH of the 82°C endotherm paralleled by an increase in the ΔH of the 59°C endotherm.

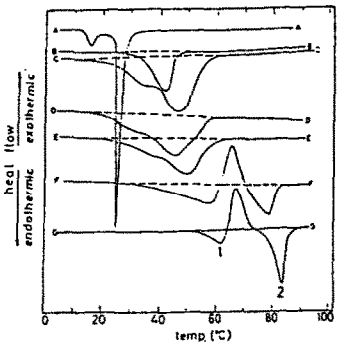

Figure 4: Thermograms of aqueous dispersions of GlcCer-DMPC mixtures. All systems were dispersed in 50 mM KCl. For details, see Methods.

Thermogram	Amount Total Lipid (mg)	X-GlcCer (Weight Fraction)	Sensitivity (mcal sec^{-1} $inch^{-1}$)
A.	1.8	0	0.1
B.	1.5	0.33	0.1
C.	1.7	0.46	0.04
D.	1.6	0.50	0.04
E.	1.3	0.70	0.04
F.	1.6	0.90	0.1
G.	1.4	1.00	0.2

This lower temperature endotherm is comparable to the 58°C endotherm for pure GlcCer seen in Figure 1, Curve A, B. At 16% DPPC, the 82°C endotherm, as well as the exotherm, has disappeared. In the range of 35-44% DPPC (Figure 1, Curve E), the appearance of a shoulder on the endotherm is observed. This composite endotherm can be resolved into two peaks following the procedure of Estep et al., (14). The H of the lower temperature transition increases with increasing DPPC content. At 85% DPPC, the thermogram closely resembles that for pure DPPC; however, the width of the peak at half-height is larger, and ΔH is slightly smaller than the corresponding values obtained for pure DPPC (6,12).

GlcCer DMPC behaves similarly (Figure 4), although slightly higher mole % DMPC than DPPC are required to abolish the complex thermotropic behavior of the cerebroside. The egg PC-GlcCer system behaves differently. Very high mole % (>50%) egg PC are required to abolish

the complex thermotropic behavior of GlcCer compared with 16.5 and 22.5 mole % DPPC and DMPC, respectively. Compare Figures 1, 4 and 5).

In the 46-70 mole % GlcCer range in DMPC systems containing glycosphingolipid, a shoulder is evident on the low temperature side of the single remaining endotherm. No such shoulder is evident in the egg PC-GlcCer systems. This shoulder probably corresponds to the transition of a phase enriched with DMPC. In the egg PC-GlcCer systems broad endotherms of low enthalpic

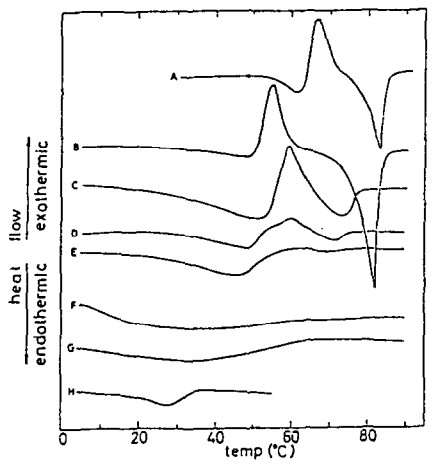

Figure 5: Thermograms of aqueous dispersions of GlcCer-Egg PC mixtures in 50mM KCl.

Thermogram	Amount Total Lipid (mg)	X GlcCer (Weight Fraction)	Sensitivity (mcal sec^{-1} $inch^{-1}$
A.	1.4	1.00	0.2
B.	1.7	0.91	0.1
C.	1.5	0.83	0.04
D.	1.1	0.70	0.04
E.	1.4	0.60	0.04
F.	1.5	0.39	0.02
G.	2.2	0.32	0.04
H.	1.5	0.16	0.02

value with temperature maxima between 10-50°C are characteristic of the concentration range 10-84 mole % egg PC (Figure 5).

These data suggest major quantitative differences in the miscibilities of GlcCer with disaturated PCs and PC having unsaturated acyl chains at position 2. These differences become more pronounced when water activity is lowered by the presence of ethylene glycol (50% by volume). Even at 85 mole % egg PC the thermotropic characteristics of GlcCer are still retained (Fig.6).

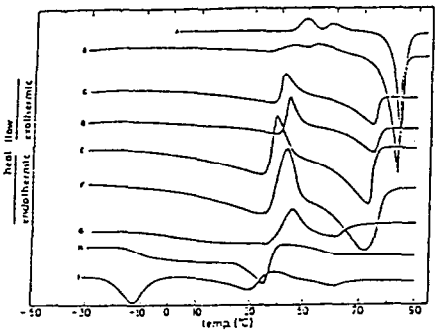

Figure 6: Effect of ethylene glycol on the thermotropic behavior of GlcCer-Egg PC mixtures:

Thermogram	Amount Total Lipid (mg)	X GlcCer (Weight Fraction)	Sensitivity (mcal sec^{-1} $inch^{-1}$)
A.	2.0	1	0.2
B.	1.7	0.91	0.1
C.	1.5	0.83	0.1
D.	1.6	0.70	0.1
E.	1.6	0.60	0.04
F.	2.1	0.50	0.04
G.	1.6	0.39	0.04
H.	1.9	0.32	0.02
I.	1.6	0.16	0.04

This latter suggests that the interaction of the lipid components with water molecules have a major effect on the molecular miscibility of the lipid components. The molecular immiscibility of GlcCer and egg PC in the presence of ethylene glycol is also demonstrated by the presence of low T_m (below 0°C) endotherm (upon heating) at mole fraction GlcCer equal to or lower than 30 mole % (Curve H and I, Fig. 6), reminiscent of the endotherm of egg PC.

DISCUSSION

Recently, it became clear that many polar lipids, when dispersed in aqueous phase in lamellar array, tend to demonstrate a metastable behavior which is characterized by a process of slow relaxation. This group includes phosphatidylcholines, sphingomyelins and cerebrosides (3,4). The latter process is the transformation between metastable and stable (crystalline) phase allomorphs. This transformation explains the large hysteresis which occurs in the heating-cooling cycle of the system. In the case of sphingomyelins and phosphatidylcholines, it seems that the disparity between the two paraffinic chains is the main cause of metastability (4,15). In the case of cerebrosides, head group-head group interactions are also involved. This interaction is disturbed by water molecules. The extent of hydration determines if stable gel allomorph can be formed.

Phosphatidylcholines, when present in the bilayer, together with GlcCer interfere with the head group-head group interaction between GlcCer molecules. This interference is dependent on molecular miscibility between the PC and the GlcCer. The better the miscibility, the larger is the interference. Phase diagrams obtained from the thermograms of mixtures of the above three PCs with GlcCer show that miscibility of GlcCer with the disaturated PCs is much better than with the egg PC (6,7). Therefore, in egg PC, the GlcCer retains its thermotropic characteristics throughout most of the phase diagram. Reducing the activity of water by mixing it with ethylene glycol reduces this miscibility even further. The fact that egg PC appears to mix with GlcCer very non-ideally may have clinical implications in Gaucher's disease (11) since the accumulation of GlcCer deposits in patient tissue may reflect the immiscibility between the GlcCer and the native phospholipids which resemble egg PC in their acyl chain composition.

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