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Thermotropic behavior of phosphatidylcholine–glucosyl ceramide mixtures: Effects of phospholipid acyl chain composition and interaction with water

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Abstract

The thermotropic behavior of multilamellar vesicles composed of mixtures of dimyristoyl phosphatidylcholine–glucosyl ceramide and of egg phosphatidylcholine–glucosyl ceramide was investigated using differential scanning calorimetry. Macroscopic demixing of the lipid components occurred when multilamellar vesicles were prepared from mixtures of glucosyl ceramide and egg phosphatidylcholine by conventional methods. This problem was overcome by a technique based on spray drying of the lipid mixture. The results obtained for the two systems are compared with data available for dipalmitoyl phosphatidylcholine–glucosyl ceramide mixtures (*Biochemistry* 22 (1983) 3497–3501). All three phosphatidylcholines perturb the complex thermotropic behavior of glucosyl ceramide. The data suggest that the interference with intermolecular interactions among glycosyl ceramide molecules by phospholipid molecules is related to the molecular miscibility of the two components. This is strongly dependent on the acyl chain composition of the phosphatidylcholine and the water activity of the ambient aqueous phase.

Keywords: Differential scanning calorimetry; Thermotropic behavior; Glucosyl ceramide–phosphatidylcholine; Liposomes; Phase transition and separation; Gaucher's disease

1. Introduction

The polymorphism of glucosyl and galactosyl ceramides (cerebrosides) in aqueous dispersion is well established [1–9]. It is characterized by the presence of metastable and stable gel allomorphs which differ in their head group conformation [4,5]. The transformation from the metastable to the stable gel allomorph is a kinetic phenomenon. The unique polymorphism results primarily from hydration–dehydration processes. It involves head group–head group interactions which affect hydrocarbon chain packing [6–11]. This polymorphism is not, however, affected by the length of the normal saturated acyl chain of the cerebroside [1]. Introduction of an hydroxyl group at the α-position of the ceramide fatty acid imposes a

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kinetic barrier and perturbs the transformation of the cerebroside to the stable gel allomorph [9].

Previously we have demonstrated that concentrations of dipalmitoyl phosphatidylcholine (DPPC) as low as 5 mol% affect the polymorphism of glucosyl ceramide (GlcCer) [1]. These results suggest miscibility of DPPC and GlcCer at high mole fractions of the glycosphingolipid [1,12]. However immiscible domains exist at intermediate mole fractions of GlcCer [1,12,13] Similar, though quantitatively different observations have been described for galactosyl ceramide–DPPC mixtures [9,12,14].

Published studies have focussed primarily on systems comprised of cerebroside and dipalmitoyl phosphatidylcholine. In this study we examine the behavior of mixtures of glucosyl ceramide and two other phosphatidylcholines of different acyl chain composition, dimyristoyl phosphatidylcholine and egg phosphatidylcholine. We also attempt to assess the importance of water in the interaction between components by examining the thermotropic behavior of the mixed lipid systems dispersed in water and in water–ethylene glycol mixtures.

2. Materials and methods

2.1. Reagents

Grade I egg phosphatidylcholine (PC) was purchased from lipid products, South Nuffield, England: Puriss grade L-α-dimyristoyl phosphatidylcholine (DMPC) was purchased from Fluka-Buchs, Switzerland. D-*Erythro*-glucosyl ceramide (GlcCer) was prepared from a spleen of a Gaucher's disease patient and characterized as described elsewhere [13]. The purity of all lipids was greater than 99% as assessed by thin layer chromatography, loading 0.5–1 mg lipid on a 2 cm strip. All other reagents were of analytical grade or better.

2.2. Preparation of multilamellar liposomes

The conventional method for preparation of multilamellar liposomes requires the complete removal of the organic solvent to form a thin layer of the dry lipid on the wall of the container. The lipid is then dispersed in the desired aqueous solution at a temperature above the range of the gel-to-liquid crystalline phase transition [15–17]. When liposomes are prepared from a mixture of lipids, it is important to prevent macroscopic demixing of the lipid components upon removal of the organic solvent. This has customarily been accomplished by selecting a suitable mixture of organic solvents such as chloroform and methanol [1,17,18]. This approach was used successfully in preparing liposomes from DMPC–GlcCer mixtures. To do this, solutions of DMPC and GlcCer in chloroform–methanol (2:1, v/v) were mixed to give the desired concentration of lipids. The solvent was removed either by a stream of nitrogen or by flash evaporation under reduced pressure. To ensure complete solvent removal, a vacuum (0.1 mmHg) was then applied for 3 hours. The dry mixture was ground and weighed directly into the aluminum pans of a DuPont-990 differential scanning calorimeter. The desired aqueous solution (either 50 m*M* KCl or 25 m*M* KCl in ethylene glycol–water, 1:1, v/v) was then added in excess and the pans were hermetically sealed. Heating scans were repeated on each sample until no further change in the thermogram was obtained. Homogeneity and reproducibility of the dispersion was assured by the extent of identity of differential scanning calorimetric thermograms obtained for multiple preparations of each composition. With DMPC–GlcCer mixtures, reproducibility was satisfactory, as was the case with DPPC–GlcCer mixtures [1]. However, reproducible thermograms could not be obtained for liposomes prepared as described above from egg PC–GlcCer mixtures. Dispersing the dry lipid mixture in bulk and then heating, all prior to addition to the calorimeter pans [12,19], did not improve the reproducibility. To overcome this difficulty a novel dispersal method based on a spray drying step was developed. The egg PC and GlcCer were mixed in chloroform–methanol (2:1, v/v). The lipid solution was then placed in a glass spray gun designed to use a very small volume of solution (shown in Fig. 1). Either low pressure nitrogen or air was applied to spray the solution

onto a glass plate at a slow 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 then scraped off the plate using a microscope slide and collected in glass test tubes for drying in vacuum (0.1 mmHg) for 3 h. The dry lipid mixture was placed in preweighed calorimeter pans, and liposomes were prepared as previously described for the DMPC–GlcCer mixtures. Thompson and coworkers have demonstrated that our spray method, but not the routine bulk solvent evaporation method, gave reproducible mixing of other glycolipids with various phosphatidylcholines [20].

2.3. Calorimetric measurements

Calorimetric measurements were performed on a DuPont-990 differential scanning calorimeter equipped with cell base II and a specially constructed cooling device as described elsewhere [1,6]. The calibrated mode, and a heating rate of 5°C/min were used. Scanning was performed after thermal equilibration at ~5°C and 90°C for heating and cooling modes, respectively for aqueous medium, and ~30°C and 90°C for heating and cooling modes in medium containing ethylene glycol. Only thermograms obtained after the second heating scan were used for data evaluation. This ensures that the lipids went through the complete cycle of phase transitions, as is clear from Figs. 2a (curves F,G) and 2b (curves A,B) which demonstrate the presence of an endotherm and exotherm upon heating [1,6]. The standard deviation of T_m and ΔH was less than $\pm 10\%$ for DMPC–GlcCer mixtures. For egg PC–GlcCer mixtures the standard deviation of the T_m was less than $\pm 10\%$. The standard deviation of ΔH for endotherm 2 was less than $\pm 10\%$ for all mixtures. For endotherm 1 and the exotherm, the standard deviation for ΔH varied between $\pm 5.0\%$ for 80 mol% GlcCer to $\pm 24.0\%$ for 50 mol% GlcCer. The higher standard deviation is related to the low ΔH.

For transformation from weight fraction to mole fraction, see the legend to Fig. 4.

3. Results

3.1. Thermograms in aqueous medium

Heating mode thermograms obtained for various mixtures of DMPC–GlcCer and egg PC–GlcCer dispersions in 50 m*M* KCl are shown in Figs. 2A and 2B. Freeze-fracture electron microscopy performed by M. Allietta and T.W. Tillack, Department of Pathology, University of Virginia School of Medicine, clearly showed all mixtures of lipids examined to be organized in multilamellar structures (data not shown). At a concentration of GlcCer equal to or greater than 90 mol%, the thermograms obtained in the heating mode for both systems are characterized by two distinct endotherms flanking an exotherm, which suggests the coexistence of stable and metastable gel allomorphs [1,6]. The high temperature endotherm (endotherm 2—see Fig. 2A for identification) is reminiscent of the melting of the pure crystalline phase (stable gel phase) of GlcCer to the liquid crystalline phase [1,6].

It should be stressed that the close proximity and a possible overlap between the low temperature endotherm (endotherm 1) and the exotherm (see Figs. 2A, 2B, 3A, 3B) makes it difficult to get "clean" values for ΔH of both processes and accurate T_m values of endotherm 1. The reason is that the exothermic crystallization of GlcCer to

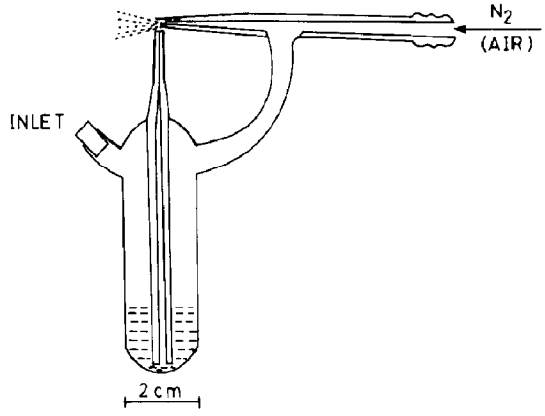

Fig. 1. Schematic drawing of the spraying device used for lipid mixing required for the preparation of egg PC–GlcCer multilamellar liposomes (see Section 2).

form the stable gel allomorph, which is a kinetically controlled process, starts during the low temperature endotherm (endotherm 1). Thus the measured ΔH and T_m values of endotherm 1 are only apparent and are dependent on the existence and extent of the exothermic transition (see Figs. 2A, 2B, 3A, 3B). Comparison of the heating with the cooling thermograms reveals a strong hysteresis, since the T_m of the cooling exotherm is 8–20°C lower than the high temperature heating endotherm (endotherm 2), depending on GlcCer mole fraction (see Fig. 2C). The low temperature end of the cooling exotherm at a scanning rate of 5°C/min coincides with the onset of the low temperature endotherm (endotherm 1) obtained during heating (Fig. 2C). The hysteresis in the heating–cooling loop indicates that the transition from the stable gel state to the liquid crystalline state is irreversible. As shown previously [1,6,11], lowering the scan rate of cooling has almost no effect on the T_m of pure GlcCer dispersed in aqueous KCl. However, in the presence of ethylene glycol, the T_m obtained is reduced with decreasing temperature. From its extrapolation to a zero scanning rate, a T_m of about 7°C below T_m of endotherm 2 was obtained. In the presence of

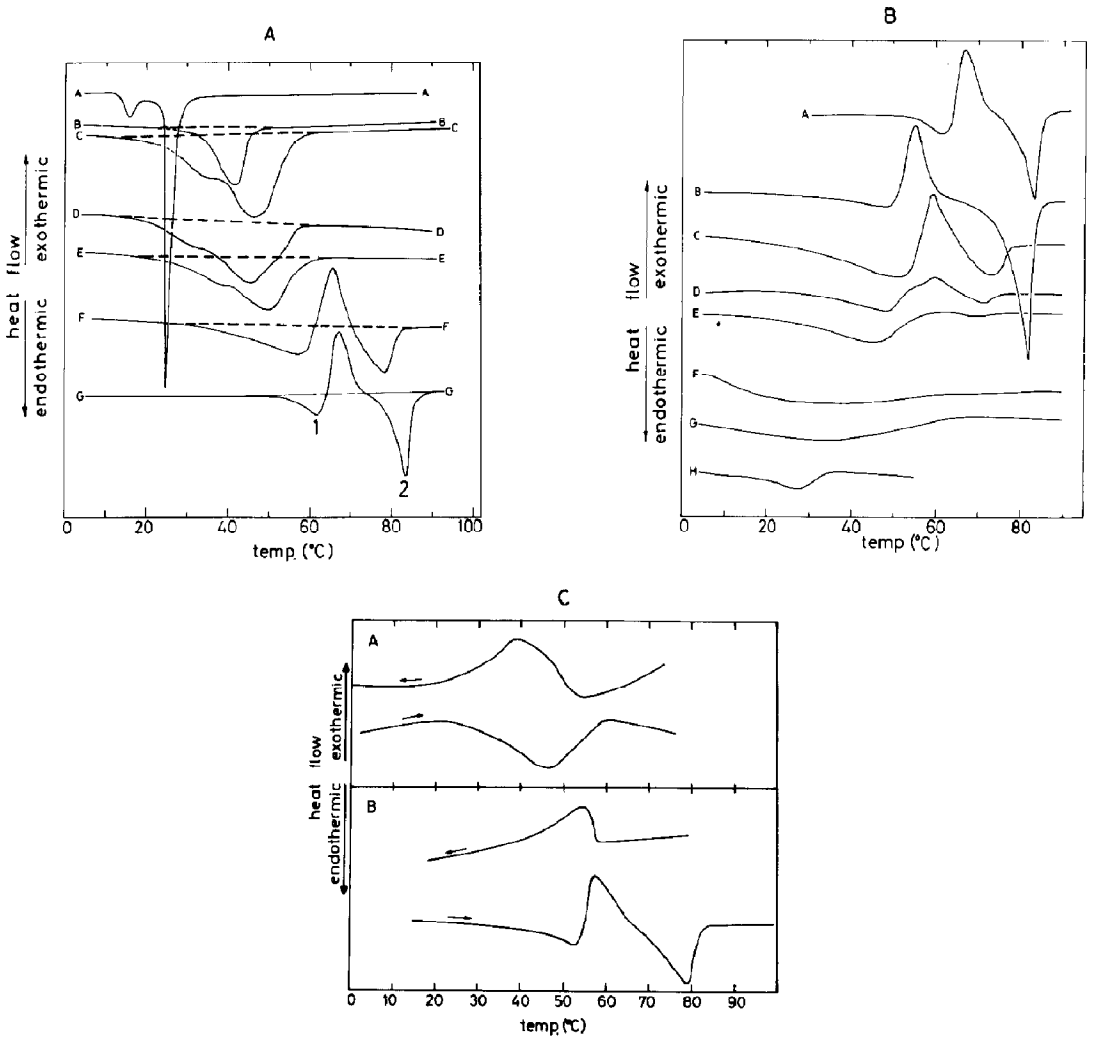

Fig. 2. Thermograms of aqueous dispersions of PC–GlcCer mixtures. All systems were dispersed in 50 mM KCl. For details see Section 2.

Fig. 2 (continued)
(A) DMPC–GlcCer mixtures

Thermogram	Amount total lipid (mg)	X_{GlcCer} (weight fraction)	Sensitivity (mcal s^{-1} $inch^{-1}$)
A	1.8	0.00	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

Base line is indicated for thermograms B–G.
The labels of 1 and 2 refer to endotherm 1 and endotherm 2 (see text).

(B) egg PC–GlcCer mixtures

Thermogram	Amount total lipid (mg)	X_{GlcCer} (weight fraction)	Sensitivity (mcal s^{-1} $inch^{-1}$)
A	1.4	1.00	0.20
B	1.7	0.91	0.10
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

(C) Thermograms of DMPC–GlcCer mixtures in the cooling and heating modes

Thermogram	Amount total lipid (mg)	X_{GlcCer} (weight fraction)	Sensitivity (mcal s^{-1} $inch^{-1}$)
A	1.6	0.50	0.04
B	1.7	0.96	0.10

DMPC, no hysteresis is observed. Presumably the DMPC molecules intercalate between the glucosyl ceramide molecules, abolishing the "stable state" and hysteresis.

A detailed comparison of the thermograms for DMPC–GlcCer (Fig. 2A) with those for egg PC–GlcCer (Fig. 2B), clearly shows interesting differences between the two systems. The most striking difference is the concentration of phosphatidylcholine required to abolish the complex thermotropic behavior characteristic of GlcCer. Thus, whereas this behavior of GlcCer is abolished at about 25 mol% DMPC (Fig. 2A), about 50 mol% egg PC is required to produce a similar result (Fig. 2B). In an earlier paper we showed that 15 mol% DPPC abolished the complex behavior reminiscent of pure GlcCer [1]. In the 46–70 mol% GlcCer range in DMPC-containing systems, 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 (compare Fig. 2A and 2B). In the egg PC–GlcCer systems, broad endotherms of low enthalpic value with temperature maxima between 10–50°C are characteristic of the concentration range 10–84 mol% egg PC (Fig. 2B).

3.2. *Thermograms in ethylene glycol*

Curatolo showed that 50% ethylene glycol abolishes the exothermic peak of *N*-palmitoyl galactosyl ceramide [9]. Indeed, increasing the concentration of ethylene glycol shifts the exothermic peak of galactocerebrosides to high

temperatures until they merge with endotherm 2 [11]. In the case of pure GlcCer, the effect of ethylene glycol is to split the exotherm into two parts, as seen in Figs. 3A and 3B, with the resulting peak at a higher temperature. This splitting, although not fully understood, may be related to a lower level of hydration as well as to two competitive interactions: ethylene glycol–GlcCer and water–GlcCer, producing more than one metastable phase. The splitting is abolished by mixing the GlcCer with a low mole fraction of either DMPC or egg PC (Figs. 3A and 3B). This observation supports our previous assumption that a low mole fraction of PC affects head group–head group interactions between GlcCer molecules, and that these interactions are related to the interaction of the solvent with the head groups [1]. Although ethylene glycol affects the

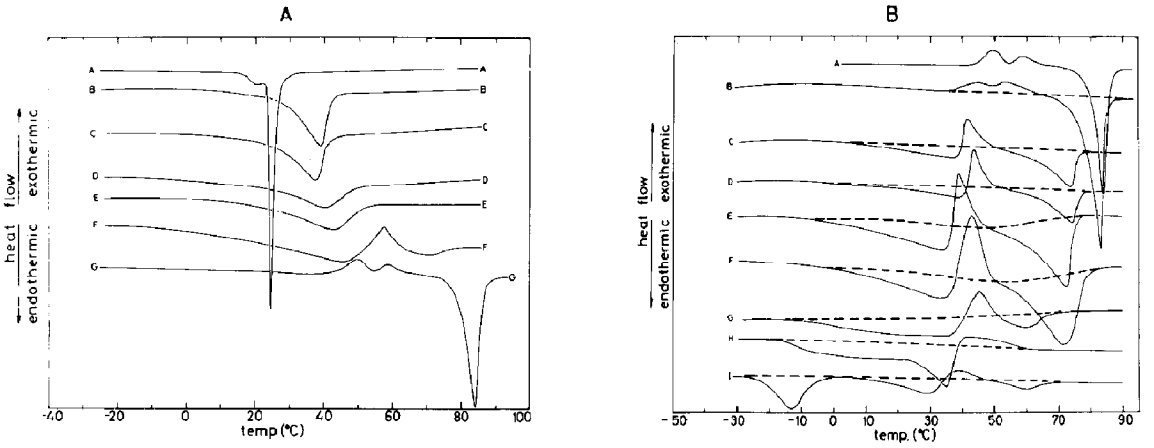

Fig. 3. Effect of ethylene glycol on the thermotropic behavior of PC–GlcCer mixtures. All systems were dispersed in water–ethylene glycol, 1:1 (v/v) containing 25 m*M* KCl. For details see Section 2.

(A) DMPC–GlcCer mixtures

Thermogram	Amount total lipid (mg)	X_{GlcCer} (weight fraction)	Sensitivity (mcal s^{-1} $inch^{-1}$)
A	1.4	0.00	0.1
B	1.0	0.33	0.1
C	1.9	0.44	0.1
D	1.6	0.58	0.1
E	1.4	0.70	0.1
F	1.4	0.90	0.1
G	2.0	1.00	0.2

(B) Egg PC–GlcCer mixtures

Thermogram	Amount total lipid (mg)	X_{GlcCer} (weight fraction)	Sensitivity (mcal s^{-1} $inch^{-1}$)
A	2.0	1.00	0.20
B	1.7	0.91	0.10
C	1.5	0.83	0.10
D	1.6	0.70	0.10
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

interaction of PC with the GlcCer, as monitored by the thermotropic behavior of both DMPC–GlcCer and egg PC–GlcCer systems, this effect is much more pronounced in the case of the egg PC–GlcCer, as can be seen by comparing Figs. 3A and 3B. In the presence of ethylene glycol, the thermotropic features characteristic of pure GlcCer are fully preserved even when egg PC and GlcCer are present in equimolar amounts, and they are still observable at 84 mol% egg PC. In the presence of 50% ethylene glycol, the heating scan can be studied at temperatures below the freezing point of water. In such scans, the gel-to-liquid crystalline transition of egg PC is observed as another endotherm at a temperature lower than that of endotherm 1. This new endotherm is referred to as endotherm 0 (Figs. 2A and 3B). In systems containing 16 and 32 mol% GlcCer, two distinct endotherms exist: endotherm 0 which characterizes the melting of domains enriched in egg PC, and the higher temperature transition reflecting melting of domains composed of a GlcCer and egg PC mixture. No endotherm 0 is observed above 40 mol% GlcCer, suggesting the disappearance of egg PC-enriched domains due to egg PC–GlcCer mixing.

3.3. Effect of DMPC mole fraction

The relationship between lipid composition, solvent composition and the apparent T_m (the temperature of the maximum or minimum for endotherms and exotherms, respectively) is shown in Figs. 4A and 5A. At high molar fractions of GlcCer, two endotherms, one at high temperature referred to as endotherm 2 (□) and the other at lower temperature referred to as endotherm 1 (○), and one exotherm (△) are displayed in the heating mode of DMPC–GlcCer, while only one exotherm is seen in the cooling scans even at high GlcCer concentration (see also Fig. 2C). The latter is identical with the gel to liquid crystalline phase transition of the mixed phase.

Figures 4A and 5A show that endotherm 2 exists in the range 0–22 mol% DMPC for aqueous dispersion and 0–38 mol% in the presence of 50% ethylene glycol. The apparent T_m of this

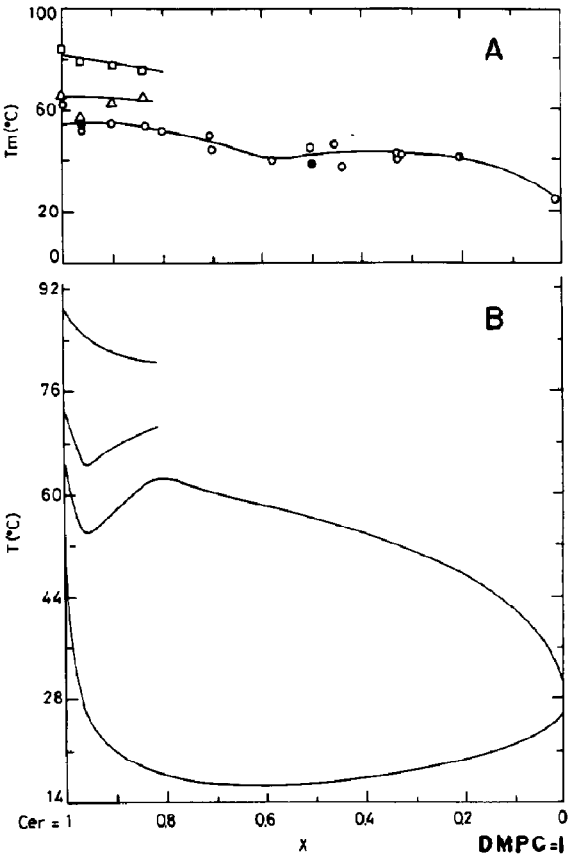

Fig. 4. (A) The effect of the mole fraction of DMPC on the apparent T_m of DMPC–GlcCer mixtures prepared in 50 m*M* KCl. Transformation from weight fraction to mole fraction was done based on DMPC, egg PC and GlcCer molecular weight of 714, 796 and 800, respectively. This was confirmed by phosphorus (PC) and nitrogen (GlcCer) determinations. (○) endotherm 1, heating mode; (□) endotherm 2, heating mode; (△) exotherm, heating mode; (●) exotherm, cooling mode. (B) Phase diagram of DMPC–GlcCer system in 50 m*M* aqueous KCl (based on Fig. 2A).

endotherm is only slightly affected by an increase of DMPC concentration in the presence and absence of ethylene glycol. The apparent T_m of endotherm 1 in aqueous dispersions remains almost constant until the disappearance of endotherm 2, then the apparent T_m values decrease with increasing concentration of DMPC. Similar apparent T_m values were obtained upon heating or cooling (compare empty circles with full circles in Fig. 4A). This suggests a reversible transition of a mixed DMPC–GlcCer phase. Its composition

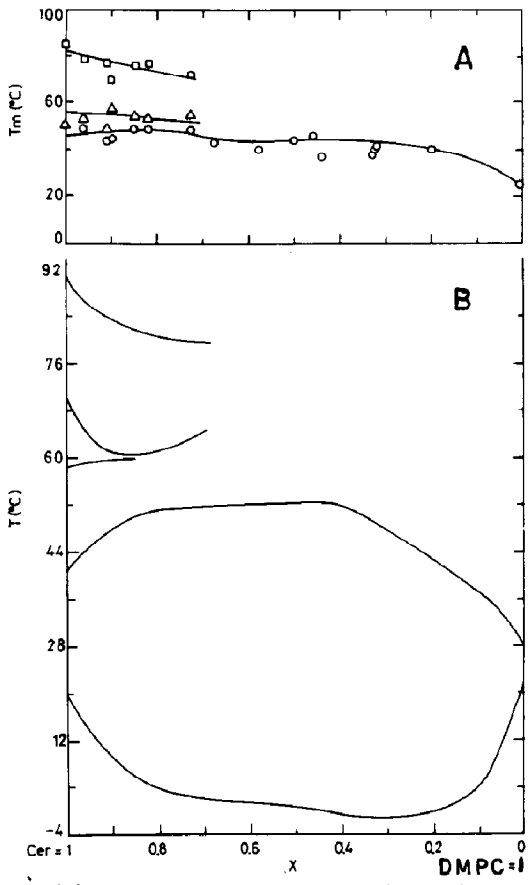

Fig. 5. (A) The effect of DMPC mole fraction on the apparent T_m of DMPC–GlcCer mixtures prepared in 50 mM KCl (aqueous)–ethylene glycol, 1:1 (v/v), based on Fig. 2A. (○) endotherm 1, heating mode; (□) endotherm 2, heating mode; (△) exotherm, heating mode. (B) Phase diagram of DMPC–GlcCer system in 50 mM KCl–ethylene glycol, 1:1 (v/v), based on Fig. 3A.

is changing continuously with the changing ratio between the two components. The shoulder at the lower temperature end of the endotherm may reflect some immiscibility (see also Fig. 2). For systems in 50% ethylene glycol, the apparent T_m values of endotherm 1 are continuously decreasing with increasing concentrations of DMPC.

In the case of the GlcCer–egg PC system, the unique feature is the very broad endotherm 1, with its apparent T_m decreasing drastically with increasing egg PC fraction. This effect is more pronounced in aqueous phase than in the presence of 50% ethylene glycol (Figs. 3A, 3B), again suggesting that hydration is an important factor in the miscibility between the phospholipid and the cerebroside.

4. Discussion

Simple glycosphingolipids in excess water exhibit a complex thermotropic behavior [1,2,6–8,14,21,22]. The thermotropic behavior of pure GlcCer is characterized by two distinct endotherms bracketing an exotherm in the heating mode; a configuration signaling the presence of stable and metastable allomorphs. Similar behavior has also been demonstrated for sulfatides [21]. It should be noted, however, that Maggio et al. [19], using the high-sensitivity Privalov-type DASM-1M differential scanning calorimeter in the heating mode, detected only one endotherm without an exotherm. All other studies on the thermotropic behavior of glycosphingolipids were obtained using low sensitivity differential scanning calorimeters such as the Perkin-Elmer DSC-2 or DuPont 990 [1,6,7,8,9,10]. These require 50 to 100-fold higher lipid concentrations and usually faster scanning rates. This discrepancy reflects differences in experimental conditions and possibly a comparison between equilibrium and non-equilibrium systems [17]. The latter are the subject of this study.

In this study we are dealing with a kinetic phenomenon of the transition from the metastable to the stable state and how it is affected by interaction with water and bilayer composition. This transition proceeds at a very slow rate at room temperature ($t_{1/2}$ of several hours). The half time decreases strongly with increasing temperature to 2 min at 50°C, 30 s at 60°C and 12 s at 70°C [6]. As one decreases the scanning rate, the transition peak is shifted to lower temperature and its height decreases until eventually it merges with the base line. Previous studies [4,5] suggest that the existence of both metastable and stable gel allomorphs reflects differences in the degree of hydration of the hexosyl ceramide polar head group in the bilayer array. These differences in hydration can be attributed to intermolecular interactions between hexosyl ceramide head groups.

The carbohydrate moieties [23,24], and possibly the interface region [25] of the sphingolipid molecules, are involved in this interaction.

The involvement of hydration was demonstrated by altering the solvent structure and lowering water activity through the addition of 50% ethylene glycol. A comparison of the phase diagrams obtained in the presence and absence of 50% ethylene glycol for the DMPC–GlcCer system (Figs. 4B and 5B) makes it clear that in the presence of 50% ethylene glycol, the GlcCer-rich phase is retained at higher DMPC mole fractions than in pure aqueous phase. Ethylene glycol seems to increase the immiscibility of DMPC and GlcCer within the liquid crystalline phase. This effect is even more pronounced for the egg PC–GlcCer system (compare Figs. 2B and 3B), in which the nonideality in the mixing of the two components is larger. Indeed in the presence of ethylene glycol, both behaved like partially hydrated systems and resembled our previous data on DPPC–GlcCer systems below water saturation [1]. It is worth noting that although addition of ethylene glycol affects the hydration it seems also to interact with the gluco- and the galactocerebrosides, affecting them in a different way [11].

It is also possible that interdigitation of the methylene chains of GlcCer molecules in opposing monolayers which form the bilayer may be a contributing interaction [26–28]. Such interdigitation may be expected in bilayers containing GlcCer due to the large disparity in length between the two methylene chains of the ceramide moiety of most GlcCer molecules [2,13]. Such interdigitation was demonstrated even in bilayers containing low mol% of glycosphingolipid [28].

All the interactions are modified by perturbing either the head group or interface regions of the bilayer. This was done by replacement of the normal acyl chain of the sphingolipid with bulkier α-hydroxy acyl chains [8,21], by methylation of its carbohydrate moiety (Frank, Barenholz and Thompson, unpublished results), or by the presence of DPPC molecules in the glycosphingolipid array [1,14,22].

The effect of bilayer composition was studied by comparing the effect of PC of differing acyl chain composition on the intermolecular interactions between GlcCer molecules [6]. DMPC and egg PC (this study) and DPPC [1] were compared. The fact that abolition of the complex themotropic behavior of GlcCer in aqueous phase requires 15 mol% DPPC [1], 25 mol% DMPC and 50 mol% egg PC shows the perturbation to be dependent on PC chain length and degree of unsaturation. This dependence must reflect the degree of nonideality of mixing of each of these three phosphatidylcholines with GlcCer, in agreement with the differences in their T_m. Similar results were described before for mixtures of various PCs [29] or mixtures of PCs with sphingomyelins [25]. Our study stresses again the generality of this phenomenon.

Looking at the three GlcCer–PC systems in detail, one sees that DPPC mixes best with GlcCer and hence the intermolecular interactions between GlcCer molecules are perturbed at low concentrations of DPPC. Egg PC mixes least well with GlcCer and permits the existence of a GlcCer-rich phase up to 50 mol% PC. The nonideality of mixing is correlated with the magnitude of the difference between the gel-to-liquid crystalline phase transition temperatures of GlcCer and the phosphatidylcholines; that is, the smaller the difference, the less nonideal the mixture. The marked nonideality of the egg PC–GlcCer system is also indicated by the difficulty in preventing macroscopic demixing, as discussed in Section 2.

The three systems also differ at the other side of the phase diagram, at higher PC mole percentages. For the DPPC–GlcCer system gel phase, immiscibility leading to phase separation occurs above 60 mol% DPPC [1,12,13]. Such phase separation can be detected between 40% and 80% DMPC for aqueous dispersions of DMPC–GlcCer (Figs. 4A, 4B), but not in the presence of 50% ethylene glycol (Figs. 5A, 5B). The differences between the systems of DMPC–GlcCer and DPPC–GlcGer are summarized in the phase diagrams (compare Fig. 4B of this work with Fig. 7B of Barenholz et al. [1]).

The fact that egg PC appears to mix with GlcCer very nonideally may have clinical implications in Gaucher's disease [30,31]. In this disease, deposits of GlcCer accumulate in certain tissues [31]. It is possible that the reason that deposits

occur reflects the fact that GlcCer at higher concentrations is immiscible in membrane phospholipids. The majority of these phospholipids have an acyl chain composition similar to egg PC [32]. The formation of GlcCer deposits is further abetted by the fact that at physiological temperatures, GlcCer is in the gel phase and the spontaneous transfer rate of this compound between phospholipid bilayers is essentially zero [33].

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Preliminary data of this study are described in the Proceedings of the 9th International Conference of Thermal Analysis (ICTA), Jerusalem, Israel, 21–25 August, 1988; see ref. [2].

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