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Thermotropic Behavior of Aqueous Dispersions of Glucosylceramide–Dipalmitoylphosphatidylcholine Mixtures†

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ABSTRACT: Differential scanning calorimetry has been carried out on aqueous dispersions of multilamellar liposomes formed from mixtures of glucosylceramide and dipalmitoylphosphatidylcholine. The phase diagram constructed from data obtained from dispersions in excess water in the cooling mode is simpler than the diagram obtained from data obtained in the heating mode. The essentially horizontal portion of the solidus curve up to 60% glucosylceramide indicates lateral phase separation in the gel phase. The existence of a metastable region of glucosylceramide seen in the heating scans at system concentrations greater than 90% of this component is reminiscent of the complex thermal behavior of pure glucosylceramide dispersions in excess water.

Gluco- and galactocerebrosides form lamellar arrays in aqueous solutions and exhibit a complex thermotropic behavior (Bunow, 1979; Bunow & Levin, 1980; Freire et al., 1980; Ruocco et al., 1981; Curatolo, 1982). It is generally believed that this behavior, diagramed in Figure 1 (Freire et al., 1980), is due primarily to head group–head group interactions and a hydration–dehydration process involving the head groups (Freire et al., 1980; Ruocco et al., 1981; Skarjune & Oldfield, 1982). The hydrogen-bond network, demonstrated by NMR[1] studies (Skarjune & Oldfield, 1979, 1982), is altered in the

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[1] Abbreviations: PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; GlcCer, D-*erythro*-glucocerebroside; TLC, thin-layer chromatography; T_m, temperature at which maximum heat flow is observed; NMR, nuclear magnetic resonance.

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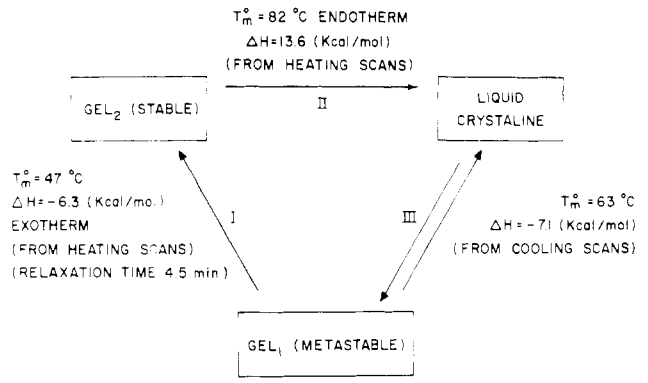

FIGURE 1: Schematic representation of complex thermotropic behavior of pure glucosylceramide dispersions in excess water [based on Freire et al. (1980)].

unidirectional cycle of thermotropic phase transitions, thereby causing further changes in hydrocarbon-chain packing. This results in two polymorphic forms in the gel state, one of which is metastable and the other stable. The stable form has a larger enthalpic change associated with its gel to liquid-crystalline phase transition than does the metastable form (Freire et al., 1980; Ruocco et al., 1981; Curatolo, 1982).

Since pure cerebrosides can exist as both metastable and stable gel-phase polymorphs, can these polymorphs exist in bilayers in which the cerebroside is mixed with other lipids? Although structural and dynamic aspects of the interactions of cerebrosides with synthetic and natural phosphatidylcholines have been investigated by several groups (Clowes et al., 1971; Tkaczuk & Thorton, 1979; Neuringer et al., 1979; Correa-Freire et al., 1979, 1982), the effect of phosphatidylcholine on the polymorphisim of the cerebroside has not been examined. This paper addresses this question.

Materials and Methods

L-β,γ-Dipalmitoylphosphatidylcholine (DPPC), purest grade, was purchased either from Fluka (Buchs, Switzerland) or from Sigma (St. Louis, MO). D-*erythro*-Glucocerebroside (GlcCer) was prepared and characterized as described elsewhere (Correa-Freire et al., 1979). The purity of both lipids was greater than 99% on the basis of TLC (loading 0.5–1 mg of lipid). All other reagents were of analytical grade or better. To prepare aqueous dispersions of DPPC–GlcCer mixtures, both 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 h. 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.

Calorimetric measurements were performed on a Du Pont 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·in.) were used in most measurements (Freire et al., 1980).

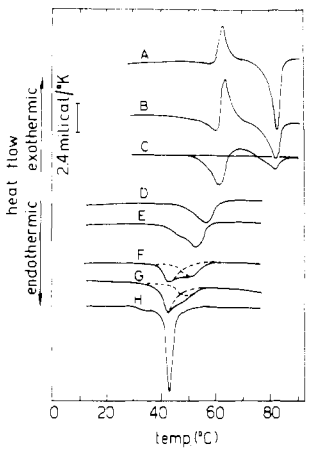

FIGURE 2: Thermograms obtained in heating mode for aqueous dispersions of GlcCer–DPPC mixtures in excess water: (A) pure GlcCer, 1.4 mg; (B) GlcCer–DPPC, 96.7/3.3 (total lipid), 1.5 mg; (C) GlcCer–DPPC, 90/10, 2.2 mg; (D) GlcCer–DPPC, 65/35, 1.6 mg; (E) GlcCer–DPPC, 56/44, 2.1 mg; (F) GlcCer–DPPC, 39/61, 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 in (C) indicates the base line.

Results

Thermotropic Behavior of Aqueous Dispersions of GlcCer–DPPC Mixtures. Recently, we have demonstrated that D-*erythro*-glucocerebroside, 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 (Freire et al., 1980). During the first heating scan of pure glucocerebroside, 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 (Figure 2A). When working in the cooling mode, only one exotherm is observed, which is asymmetrical and is centered at about 62 °C (Figure 3A). Thermograms A of Figures 2 and 3 can be explained by the transitions of glucocerebroside diagramed in Figure 1 and discussed in detail by Freire et al. (1980).

Representative heating scans of various GlcCer–DPPC mixtures in aqueous 50 mM KCl are shown in Figure 2. 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 2b). 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. This lower temperature endotherm is comparable to the 58 °C endotherm for pure GlcCer seen in Figure 2A,B. At 35% DPPC, the 82 °C endotherm, as well as the exotherm, has disappeared. In the range of 35–44% DPPC (Figure 2E), 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. (1979). The ΔH 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.

Figure 3 shows thermograms obtained in the cooling mode for the DPPC–GlcCer mixtures used to obtain the data in Figure 2. In general, these thermograms are simpler than

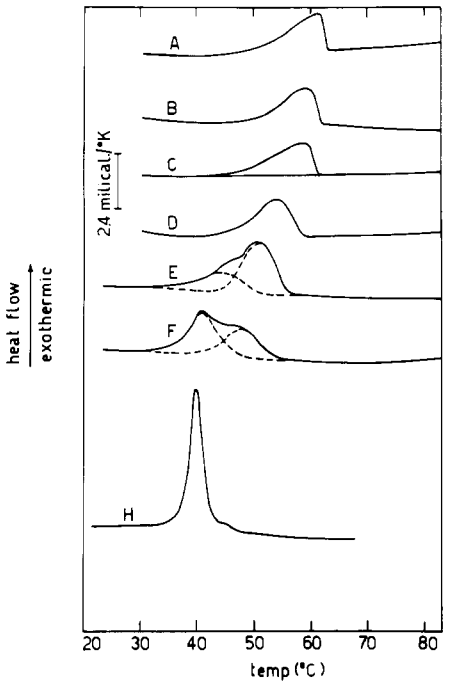

FIGURE 3: Thermograms obtained in cooling mode for aqueous dispersions of GlcCer–DPPC mixtures in excess water. Compositions are the same as for correspondingly lettered curves in Figure 2. Cooling rate was 5 °C/min. The solid line in (C) indicates the base line.

those obtained in the heating mode. For the pure GlcCer dispersion, only a single exotherm centered at 63 °C (ΔH = −7.1 kcal/mol) is observed (Figure 3A; Freire et al., 1980). The ΔH of the exotherm is equal in magnitude but opposite in sign to the sum of enthalpy changes observed in a heating scan. Increasing the DPPC content of the GlcCer–DPPC mixtures does not cause major changes in the shape of the thermograms up to 35% DPPC. Although the ΔH of this exotherm is constant, the temperature of the maximum value of C_p decreases from 59 °C for pure GlcCer to 55 °C for 40% DPPC. At this concentration, a second exotherm appears, which becomes pronounced at 85% DPPC as it did in the heating scan of this mixture.

Figure 4 shows the compositional dependence of the enthalpy changes in the heating and cooling modes. As expected, ΔH in the heating mode is equal in magnitude but opposite in sign to the enthalpy change obtained in the cooling mode:

$$\sum \Delta H^{\text{cool}} = -(\sum \Delta H^{\text{heat}}_{\text{endo}} + \sum \Delta H^{\text{heat}}_{\text{exo}})$$

$$\sum \Delta H^{\text{heat}}_{\text{endo}} = \Delta H_{85} + \Delta H_{59} + \Delta H_{41}$$

Here, the numerical subscript refers to the T_m of the transition. Endo and exo refer to endothermic and exothermic processes, respectively.

The absolute value of the total enthalpy change in both heating and cooling modes is 10 ± 0.8 cal/g up to 40% DPPC. At concentrations of DPPC above this, the total enthalpy change increases with increasing DPPC concentration to reach a value of 12.0 ± 0.5 cal/g for pure DPPC. This value is in good agreement with published data (Estep et al., 1979; Silvius, 1982).

The three endotherms in the heating mode are markedly affected by the relative content of glucocerebroside and DPPC. $\Delta H^{\text{heat}}_{85}$ is linearly reduced in magnitude and extrapolates to ΔH = 0 at 15% DPPC. This is paralleled by a linear reduction in $\Delta H^{\text{heat}}_{\text{exo}}$ to 0 at the same percent DPPC. In addition, this linear change is parallel to the increase in ΔH of the 59 °C endotherm. This effect also reaches a plateau at 15% DPPC.

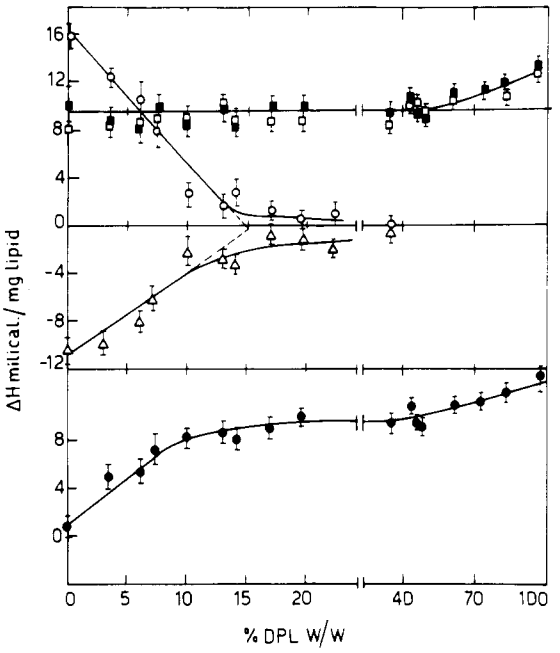

FIGURE 4: Enthalpy of transition as a function of composition of aqueous dispersions of GlcCer–DPPC mixtures: (■) sum of enthalpies in heating mode; (□) transition enthalpy in cooling mode; (○) enthalpy of 85 °C peak; (△) enthalpy of exothermic peak in heating mode; (●) sum of enthalpies of 59 and 41 °C peaks.

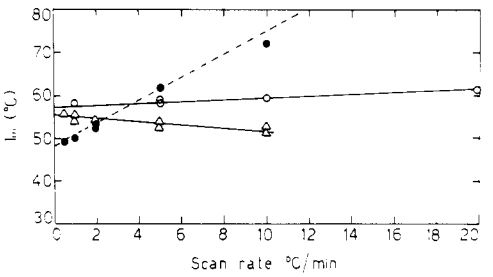

FIGURE 5: T_m as a function of scanning rate for an aqueous dispersion of a mixture of GlcCer–DPPC, 75/25 wt %: (○) heating mode; (△) cooling mode; (●) pure glucosylceramide, heating mode.

Above 40% DPPC, the 59 °C endotherm splits, and the shoulder at lower temperature increases with increasing DPPC content (see Figure 4B). This suggests phase separation of GlcCer-rich domains with T_m = 59 °C from DPPC-rich domains that have their T_m in the temperature range of 41–45 °C.

Effect of Scanning Rate. Figure 5 is a plot of the midpoint temperatures of the endotherms obtained in the heating mode and the single exotherm obtained in the cooling mode as a function of scanning rate for a GlcCer–DPPC mixture, 75/25, in excess water. For comparison, T_m for pure GlcCer obtained in the heating mode is also shown (Freire et al., 1980). It is apparent that, in contrast to the pure GlcCer, T_m values for the GlcCer–DPPC mixture are independent of the scanning rate over the range examined.

Effect of Water Content. The thermotropic behavior of glucocerebroside (Freire et al., 1980) and galactocerebroside (Ruocco et al., 1981) is dependent on the water content of the system. In the stable anhydrous state no exotherm appears upon heating. The phase diagram of pure GlcCer as a function of water content suggests saturation at 18% water (Freire et al., 1980), whereas saturation occurs at 40% water in pure DPPC (Chapman, 1973). Studies of the effect of water content on the thermotropic behavior of GlcCer–DPPC mixtures were performed with a system of GlcCer–DPPC,

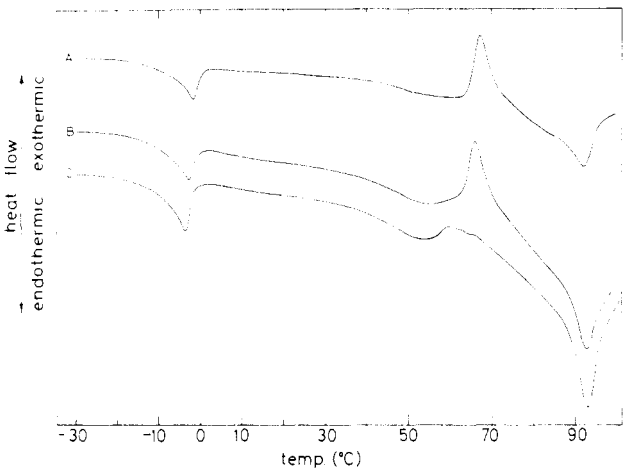

FIGURE 6: Heating thermograms of aqueous dispersions of GlcCer–DPPC mixture (84.6/15.4) at different water contents: (A) 13.8 wt % water; (B) 11.6 wt % water; (C) same system as in (B) after slow cooling.

15.4/84.6 (wt %). Experiments were carried out well below the water saturation level for both pure components at 9, 11.6, 13.8, and 15 wt % water. From the relatively large content of water frozen at 0 °C, it was clear that during the first scan of each system most of the water behaved as free water. Repeated scanning reduced the amount of free water until after five scans reproducible thermograms were obtained. All data were obtained after five or more scans. At all water contents, less than 15% of the water froze at 0 °C as free water.

Thermograms obtained at low water content differ markedly from those obtained for the same GlcCer–DPPC mixture in excess water. Figure 6A shows a thermogram (heating scan) of a GlcCer–DPPC mixture, 84.6/15.4, at 13.8% water. It is characterized by a small endotherm at 0 °C (freezing of free water), a small lipid endotherm at 62 °C, and a major endotherm at 93 °C. There is also an exotherm. This thermogram is very similar to the one obtained for pure GlcCer in 18% water or less. It is apparant that GlcCer–DPPC interactions depend on the water content of the system in the region where water is not in excess.

Comparison of the water endotherms at 0 °C for curves B and C in Figure 6 suggests that the stable low-temperature form of GlcCer–DPPC is less hydrated than the metastable form. However, the difference in hydration is only about 0.3 mol of water/mol of total lipid. Because the difference is small, it can only be observed at low water content due to experimental limitations. Although it is clear that the state of the lipid is related to the hydration, it is not clear whether the degree of hydration or the structure of the hydrated form is the more important factor.

Discussion

One of the principal differences between the thermotropic behavior of pure DPPC and of pure GlcCer in excess water is that DPPC has a reversible gel to liquid-crystalline phase transition with a single stable gel phase whereas GlcCer exhibits a metastable gel-phase polymorph in addition to a stable form. Pure GlcCer displays an irreversible cycle of transitions from a stable gel state to a liquid-crystalline phase to a metastable gel phase as illustrated in Figure 1. This cycle involves changes in the packing and organization of the acyl chains as well as the head groups. In addition, the enthlapy change, equal to 13.6 kcal/mol, is larger than that obtained for the gel to liquid-crystalline phase transition of phosphatidylcholines. The transformation from the liquid-crystalline

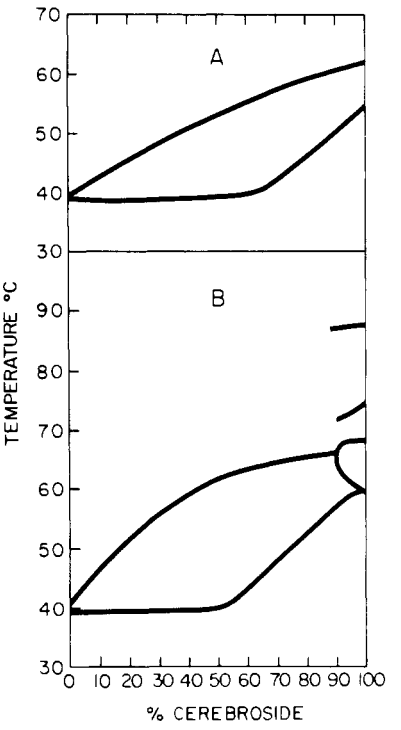

FIGURE 7: Phase diagrams for GlcCer–DPPC mixtures dispersed in excess water: (A) cooling mode; (B) heating mode.

to the metastable gel state is a kinetically limited process having a mean relaxation time of about 3.5 min (Freire et al., 1981). Similar behavior has been reported for galactocerebrosides (Ruocco et al., 1981; Curatolo, 1982). For gluco- and galactocerebrosides, this complex thermotropic behavior is not dependent on the length of the cerebroside acyl chain. However, in galactocerebrosides containing 2-hydroxyacyl chains, the complex cyclic thermotropic behavior disappears and is replaced by a reversible gel to liquid-crystalline phase transition with a value of ΔH similar to that obtained for a comparable phosphatidylcholine. It appears that the liquid-crystalline phase is more hydrated than that of the stable gel phase as suggested by the lowering of the T_m in the presence of water (Ruocco et al., 1981; Freire et al., 1980; Skarjune & Oldfield, 1982). The relative state of hydration of the two gel-phase polymorphs is not yet clear. Ruocco et al. (1981) have suggested that the stable gel polymorph is more hydrated than the metastable gel polymorph; however, this suggestion appears to be at odds with the observation that in the absence of water only the stable phase exists and that the ΔH of the dehydrated sample is similar to that of the stable gel polymorph.

Figure 7 shows the phase diagrams for DPPC–GlcCer obtained from cooling (A) and heating scans (B). The phase diagram obtained from cooling scans is much simpler. The almost horizontal portion in the solidus line up to about 60% cerebroside indicates a significant degree of phase separation, in agreement with the results obtained previously by Correa-Freire et al. (1979). The same feature is observed in the phase diagram obtained from heating scans. The additional feature in this phase diagram is the existence of the metastable region of glucocerebroside at concentrations greater than 90%. Apart from this difference, the phase diagrams obtained from heating or cooling scans are identical within experimental error.

It is clear that DPPC has a marked effect on the complex thermotropic behavior of GlcCer. This effect is more pronounced in the heating scans than in the cooling scans. The phase diagram constructed from cooling scans, which represent

one part of the unidirectional cycle for pure GlcCer, reflects the presence of a broad phase transition from 100% GlcCer to 90% DPPC. The broadness of the transition is at least in part due to the heterogeneous acyl chain composition of the GlcCer (Correa-Freire et al., 1979). On the basis of the cooling scans, the temperature range and T_m of this exotherm are much lower than the values obtained for the GlcCer endotherm obtained upon heating the pure system. This may be a result of the slow rate of relaxation to the stable gel polymorph, which may be further decreased by the presence of DPPC. This situation is similar to that reported for DL-*erythro*-*N*-stearoylsphingomyelin (Estep et al., 1980). This interpretation is supported also by the heating scans. On the basis of the linear reduction in $\Delta H^{heat}_{82,endo}$, ΔH^{heat}_{exo}, and $\Delta H^{heat}_{59,endo}$ (Figure 4), it appears that each DPPC molecule affects 6 molecules of GlcCer. Above 16.5% DPPC, the thermograms obtained for the heating scans resemble those obtained for the cooling scans, and only reversible transitions are seen. It is possible that during cooling GlcCer molecules are trapped in the metastable gel state and cannot reach the stable gel state. That this might be the case is reasonable since energy is required for the transformation from the metastable gel to the stable gel form. During cooling, this energy is not available, and as a result, the transformation to the stable gel phase cannot occur. The fact that the solidus lines are unaffected by the bilayer composition for a broad composition range suggests the presence of more than one gel phase and possibly at least partial phase separation between GlcCer-rich domains and DPPC-rich domains (Figure 7). For the mixtures with a low GlcCer content, there is good agreement between these data and those published previously (Correa-Freire et al., 1979).

The metastable behavior of GlcCer disappears at a DPPC concentration of about 15 mol %. Since the metastable behavior of GlcCer has been attributed to the formation of a hydrogen-bond network between the sugar head groups, this result suggests that DPPC may be acting as a "spacer" between the cerebroside molecules, thus preventing the formation of the hydrogen-bond network. The fact that DPPC abolishes the metastable behavior of GlcCer in an almost linear fashion also suggests that at low DPPC mole fractions, the DPPC molecules are not randomly distributed but intercalated among cerebroside molecules so as to minimize the number of DPPC–DPPC contacts.

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Registry No. DPPC, 63-89-8.

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