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Thermotropic Behavior of Monoglucocerebroside–Dipalmitoylphosphatidylcholine Multilamellar Liposomes†

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ABSTRACT: The thermotropic behavior of multilamellar liposomes prepared from mixtures of glucocerebroside and dipalmitoylphosphatidylcholine has been studied by high-sensitivity scanning calorimetry. It is shown that glucocerebroside has a marked effect on the gel–liquid crystalline transition of dipalmitoylphosphatidylcholine. The pretransition seen in pure samples of dipalmitoylphosphatidylcholine is undetectable at small mole fractions of glucocerebrosides (<10%). The main transition is shifted to higher temperatures and becomes broader and less cooperative in the presence of glucocerebroside. The enthalpy change of the main transition decreases with increasing the glucocerebroside content. However, this decrease is not linear with the glucocerebroside/phospholipid mole ratio. Glucocerebroside itself does not show a separate transition in the temperature range of these studies (10–75 °C). The origin of these effects and their dependence on the glucocerebroside content suggest that the in-plane distribution of glucocerebroside molecules is affected by the physical state of the lipid bilayer and by the glucocerebroside/phospholipid mole ratio.

Glycosphingolipids are complex lipids found mostly on the outer surface of plasma membranes. These sugar-containing lipids have been shown to play a major role as surface receptors (Rothman & Lenard, 1977; Karlsson, 1977; Fishman & Brady, 1976). Physicochemical studies also indicate that these lipids increase the stability of cell surface membranes and decrease their permeability (Pascher & Sundell, 1976; Abrahamsson et al., 1977). However, little is known about the molecular organization and the interactions of these lipids with other membrane components. It is our intention to further our knowledge of the interactions of glycosphingolipids, namely, glucocerebrosides, and phospholipids as a first step in understanding the role these lipids play in biological membranes. This paper reports a calorimetric study of the gel–liquid crystalline phase transition of multilamellar liposomes prepared from various compositions of dipalmitoylphosphatidylcholine and glucocerebrosides.

Experimental Procedures

Materials. 1,2-Dipalmitoyl-3-*sn*-phosphatidylcholine (DPPC) was synthesized by the method of Cubero Robles & Van den Berg (1969) as described by Suurkuusk et al. (1976). DPPC was dissolved in spectral grade chloroform (Fisher Scientific Co.) and stored at −20 °C until needed. Glucocerebroside (Glc-Cer), extracted from the spleen of a patient with Gaucher disease, was a gift from the Hadassah Medical School of the Hebrew University, Jerusalem. Glc-Cer was purified by silic acid chromatography and dissolved in a 2:1 ratio of spectral chloroform to methanol and kept at −20 °C until ready for use. The fatty acid composition of the glucocerebroside stock was analyzed by gas–liquid chromatographic procedures. The Glc-Cer sample was first subjected to acid hydrolysis, followed by methylation, as described by Kates (1964). These results are summarized in Table I. The sample consisted mostly of a mixture of saturated, long-chain fatty acids, principally C_{22} and C_{24} which together comprised 65% of the total.

Table I: Fatty Acid Composition of Glucocerebroside

fatty acid[a]	% of total	fatty acid[a]	% of total
$C_{16:0}$	3.90	$C_{23:0}$	13.82
$C_{18:0}$	3.22	$C_{24:0}$	31.07
$C_{20:0}$	5.25	$C_{24:1}$	6.98
$C_{22:0}$	34.62	others	1.14

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

Preparation of Liposomes. Approximately 10 mg of the desired phospholipid–glucocerebroside mixture was placed in a round bottom flask and the solvent was evaporated on a rotary evaporator at 40 °C. The sample was then frozen in an ethanol bath and the mixture lyophilized for at least 4 h. The dry lipid was then heated to 55 °C and suspended in 50

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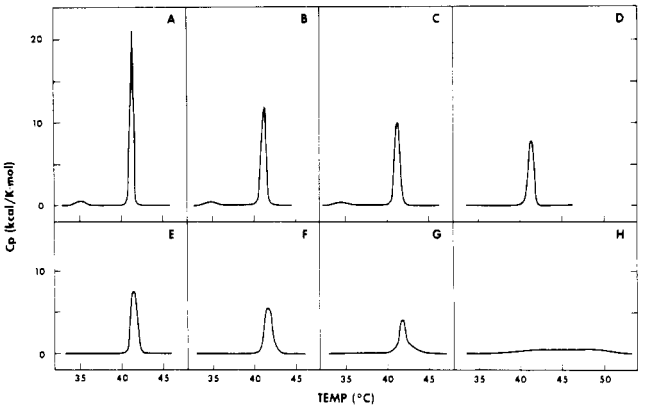

FIGURE 1: Representative calorimetric scans of multilamellar liposomes prepared from different mixtures of dipalmitoylphosphatidylcholine and glucocerebroside. Heat capacity values were calculated per mole of phospholipid. Samples contained (A) 0, (B) 2, (C) 5, (D) 11, (E) 15, (F) 21, (G) 23, and (H) 27 mol % glucocerebroside.

mM KCl at the same temperature. Multilamellar liposomes were prepared by vortexing for 1 min at 55 °C, followed by agitation in a rotating shaker bath for 1 h at 60 °C. The DPPC concentration of each dispersion was measured as inorganic phosphate as described by Bartlett (1959). The Glc-Cer concentration of each dispersion was determined by a modified procedure of the anthrone method for determining glucose concentrations, as described by Dittmer & Wells (1969). The sample was first treated with 1 mL of concentrated phosphoric acid and heated in a boiling water bath for 15 min. After cooling, the anthrone reagent was added and the color was developed by reheating the samples in boiling water for 6 min. The usual total lipid concentration in the calorimetric cell ranged between 7 and 10 mM.

Scanning Calorimetry. A highly accurate scanning calorimeter of the heat conduction type was used for these studies (Suurkuusk et al., 1976). The calorimeter was designed for measuring heat capacities and heat effects accompanying thermally induced transitions of solute molecules in dilute solution. The temperature range for the instrument is 0–75 °C. The total volume of the sample compartment is approximately 0.7 mL. The precision in terms of base line noise is better than ± 25 μcal/K. Absolute temperature determination is better than ± 0.05 °C. All the experiments in this study were made at a scanning rate of 15 °C/h. Digitized C_p data were recorded and stored at constant temperature intervals of 0.1 °C. All calculations were performed in a CDC Cyber 172 computer. A brief description of the calorimeter and calculation of the apparent molar heat capacity of the sample are given in Suurkuusk et al. (1976) and Mountcastle et al. (manuscript in preparation), respectively.

Results

The heat capacity functions of multilamellar liposomes prepared from dipalmitoylphosphatidylcholine (DPPC) and glucocerebroside (Glc-Cer) are shown in Figure 1 for some representative Glc-Cer/DPPC mole ratios. Identical calorimetric traces were obtained after repeated scans of the samples, indicating that the structures formed by glucocerebroside and dipalmitoylphosphatidylcholine do not vary with time and represent an equilibrium distribution of the two components.

Only the characteristic heat capacity maxima associated with the pre and main transition of DPPC were detected by the calorimeter, suggesting that glucocerebroside does not undergo a distinct transition in the temperature range of these studies (10–75 °C). We have been unable to obtain any

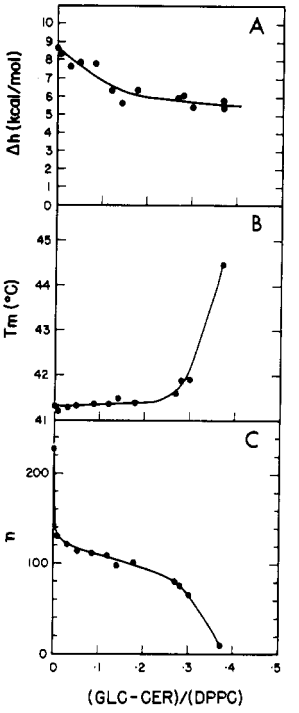

FIGURE 2: (A) The effect of glucocerebroside on the enthalpy change per mole of phospholipid, ΔH, of the gel-to-liquid crystalline transition of dipalmitoylphosphatidylcholine. The ΔH values were calculated by numerical integration of the excess heat capacity functions. (B) Dependence of the main transition temperature, T_m, of the gel-to-liquid crystalline transition of dipalmitoylphosphatidylcholine on the glucocerebroside/phospholipid mole ratio. (C) The effect of glucocerebroside on the apparent cooperative unit size, n, associated with the main gel-to-liquid crystalline transition of dipalmitoylphosphatidylcholine. n was calculated as described in the text.

evidence of a separate glucocerebroside transition either by scanning calorimetry or fluorescence probe techniques. Previously, Clowes et al. (1971) have reported that ox brain galactocerebroside incorporated into egg lecithin liposomes undergoes a separate transition centered around 55 °C. The reasons for this apparent discrepancy with the results of Clowes et al. (1971) are still not clear.

The effect of glucocerebroside on the DPPC gel-to-liquid transition is rather complex and affects all the transition parameters. First, the pretransition is suppressed with relatively small amounts of glucocerebroside (<10 mol %). A similar effect has been observed for the interaction of cholesterol (Estep et al., 1978) and anesthetics (Mountcastle et al., 1978) with DPPC. The origin of this effect is still unclear since it is not possible to distinguish between thermodynamic and kinetic effects due to the fact that the pretransition is a very slow process when compared with the scanning rate of these experiments (Lentz et al., 1978).

Second, the enthalpy change associated with the main DPPC transition decreases in a monotonic fashion with increasing mole fractions of glucocerebroside. This effect is illustrated in Figure 2A, where ΔH per mole of DPPC has been plotted as a function of the glucocerebroside/phospholipid mole ratio. This decrease in ΔH indicates that glucocerebroside interacts with DPPC and that only a decreasing fraction of the total number of DPPC molecules participates in the gel-to-liquid crystalline transition as the mole ratio of glucocerebroside is increased. Qualitatively similar results have been obtained with other molecules such as cholesterol (Estep et al., 1978) and integral membrane proteins (Curatolo et al., 1977). In the present case, however, the observed decrease in ΔH is not linear on the glucocerebroside/phospholipid mole

ratio, as is apparent from Figure 2A.

In the presence of glucocerebroside, the enthalpy change per mole of DPPC associated with the gel-to-liquid transition can be written in the form

$$\Delta H = \Delta H_0 \left(1 - \langle \eta_i \rangle \frac{[\text{Glc-Cer}]}{[\text{DPPC}]} \right) \quad (1)$$

where ΔH_0 is the enthalpy change per mole of DPPC in the pure system and $\langle \eta_i \rangle$ is the mean number of DPPC molecules subtracted from the transition per glucocerebroside molecule (E. Freire and R. L. Biltonen, manuscript in preparation).

Analysis of the data in Figure 2A in terms of eq 1 indicates that, up to 15 mol % of glucocerebroside, approximately two DPPC molecules are subtracted from the transition per glucocerebroside molecule. At higher glucocerebroside concentrations $\langle \eta_i \rangle$ decreases continuously, suggesting that a significant number of glucocerebroside molecules do not interact directly with DPPC. A possible explanation for this phenomenon is that at higher glucocerebroside concentrations relatively large domains of glucocerebroside are formed within the bilayer, thus reducing the average number of glucocerebroside–phospholipid contacts.

The dependence of the main transition temperature on the glucocerebroside/phospholipid mole ratio is shown in Figure 2B. As illustrated in the figure, the transition temperature increases very little with the cerebroside content up to about 20 mol %. This effect should be contrasted with the small decrease in the main transition temperature of DPPC, observed when cholesterol is added to the system (Estep et al., 1978). At higher concentrations of glucocerebroside the transition temperature appears to increase at a very rapid rate. It should be noted that this abrupt increase in T_m occurs in the same Glc-Cer concentration region in which ΔH decreases with a slower rate.

Finally, the cooperativity of the main transition decreases with increasing the glucocerebroside content. This reduced cooperativity is reflected in the broadening of the main DPPC transition and also in the size of the cooperative unit for this transition. The half-height width of the transition increases from 0.4 °C in the pure DPPC to about 10 °C at 27 mol % glucocerebroside. At higher glucocerebroside mole fractions the transition becomes so broad that it cannot be detected calorimetrically. The cooperative unit size, n, may be estimated from the amplitude of the heat capacity maximum ($\Delta C_{p,\max}$) (Mabrey & Sturtevant, 1976; Mountcastle et al., 1978) as follows

$$n = \frac{4RT_m^2(\Delta C_{p,\max})}{\Delta H^2} \quad (2)$$

where R is the universal gas constant. A plot of the calorimetrically calculated cooperative unit vs. the glucocerebroside/phospholipid ratio is shown in Figure 2C. For these calculations the heat capacity curves were normalized to a total area of 8.6 kcal/mol (the enthalpy change of pure DPPC; Suurkuusk et al., 1976) in order to obtain the effective cooperativity associated with those phospholipids undergoing the transition. This calculation assumes that glucocerebroside reduces the effective number of DPPC molecules involved in the transition and that those DPPC molecules participating in the transition contribute 8.6 kcal/mol to the overall enthalpy change. Three regions can be distinguished in this graph. At very low cerebroside concentrations the cooperative unit size decreases rather dramatically from about 250 to 150 DPPC molecules; then, from about 5 to 15 mol % cerebroside the cooperativity of the transition remains practically constant (approximately 100 molecules); and, finally, above 20 mol % glucocerebroside the apparent cooperative unit size decreases continuously.

Discussion

The calorimetric results presented in the preceding section strongly suggest the following mechanism for the interaction of glucocerebroside and dipalmitoylphosphatidylcholine. At low glucocerebroside/phospholipid ratios, each glucocerebroside molecule incorporated into the membrane interacts with two phospholipid molecules. As a result of this interaction these lipid molecules exist in the same configuration at all temperatures and therefore do not participate in the gel–liquid transition. This interpretation is consistent with the observed linear decrease in ΔH at glucocerebroside mole percents smaller than 15. The slight increase in the transition temperature further suggests that the phospholipid molecules interacting with glucocerebroside are in a gellike configuration. This interpretation is also supported by microviscosity measurements using 1,6-diphenyl-1,2,5-hexatriene (DPH) as a fluorescent probe (M. C. Correa-Freire, unpublished results), which indicate that the addition of glucocerebroside increases the anisotropy of the fluid phase by a larger amount than that of the gel phase. As the glucocerebroside/phospholipid mole ratio increases, the mean number of DPPC molecules interacting with each glucocerebroside molecule decreases, suggesting that relatively pure glucocerebroside domains are formed within the bilayer. This process appears to occur at approximately 15–20 mol % of glucocerebroside, as deduced from the dependence of ΔH on the glucocerebroside/phospholipid mole ratio.

The cooperativity of the main DPPC transition decreases very dramatically with as little glucocerebroside as 1 mol %. This decrease is presumably due to a contaminant effect of glucocerebroside on the phospholipid phase. Above 5 mol % and up to 15 mol % glucocerebroside, the cooperative unit size for the main DPPC transition remains essentially constant and finally above 15 mol % the cooperative unit size decreases continuously. This effect appears to be correlated with the observed changes in ΔH and T_m. Presumably, up to 15 mol % glucocerebroside, these molecules are randomly distributed within the bilayer without disrupting the DPPC phase. In this glucocerebroside concentration range the main transition temperature of DPPC increases very slightly from 41.3 to 41.45 °C. Above 20 mol % glucocerebroside, the main DPPC transition broadens considerably and the heat capacity function becomes progressively asymmetric toward the high-temperature side of the transition. This process is accompanied by a rather abrupt shift of the heat capacity maximum toward higher temperatures and a continuous decrease in its amplitude, until it is no longer discernible at approximately 25 mol % glucocerebroside. In this glucocerebroside concentration range the DPPC phase appears to be disrupted and no longer exhibits cooperative melting behavior. This process is presumably due to the formation of large glucocerebroside domains and appears to occur in a rather short concentration interval (20–25 mol % cerebroside).

The above calorimetric results substantiate previous results, suggesting that the addition of glycolipids to model membrane systems markedly increases the rigidity of the phospholipid bilayer (Sharom et al., 1976). Apparently, glycolipid molecules do so by preventing the hydrocarbon chain melting transition of other lipid molecules, as appears to be the case in the glucocerebroside–DPPC system.

Another important aspect of these results is the apparent higher affinity of glucocerebroside for those phospholipid

molecules existing in the rigid, gel configuration. The observed increase in the main transition temperature of DPPC could reflect a better solubility of glucocerebroside in the gel phase than in the liquid crystalline phase of DPPC. If this is so, the formation of pure glucocerebroside domains is more likely to occur in the liquid crystalline phase. Thus, the gel-to-liquid transition may be accompanied by an increase in the average size of glucocerebroside domains. At the present time it is not clear whether the molecular origin of the above effects is due to head-group interactions or fatty acyl chain interactions or a combination of both.

From the point of view of membrane function, the possibility that the in-plane distribution of glucocerebroside molecules might be modulated by the physical state of the lipid bilayer is very interesting and is currently under study in this laboratory. Techniques such as freeze-fracture electron microscopy are being utilized for obtaining independent quantitative assessments of this distribution.

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Gross Structural Changes in Isolated Liver Cell Plasma Membranes upon Binding of Insulin†

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ABSTRACT: The addition of 10^{-9} M insulin to a suspension of rat liver plasma membranes increases the overall lipid microviscosity, $\bar{\eta}$, by about 10–20%. The effect is confined to physiological concentrations of the hormone and is highly specific. The specificity was demonstrated in experiments where insulin analogues were added to liver plasma membranes and where insulin was added to human erythrocyte membranes. In both of these experiments practically no change in $\bar{\eta}$ was detected. Upon in vitro enrichment of the membrane cholesterol, $\bar{\eta}$ exceeded the level mediated by insulin binding, and the addition of 10^{-9} M insulin to the cholesterol-enriched membranes did not further increase $\bar{\eta}$. Concomitant to the increase in $\bar{\eta}$ upon insulin binding, the overall degree of exposure of the membrane protein, presumably to both sides of the membrane, is substantially increased. This effect is in line with the notion of vertical displacement of membrane proteins induced by changes in $\bar{\eta}$. The observed structural modulation can account for the effect of insulin on unrelated membrane responses, as well as for the negative cooperativity of insulin binding.

The concept that the mechanism of action of insulin is mediated through an interaction with the plasma membrane of the target cell has played a pivotal role in the studies on the physiological effects of the hormone (Stadie, 1954; Levine & Goldstein, 1955; Pilkis & Park, 1974; Steiner, 1977). The relevance of the hormone–membrane interaction has received further support in recent years from the extensive studies carried out on the membrane-bound receptor for insulin (Cuatrecasas, 1973; Kahn, 1976; Ginsberg, 1977). Attempts to relate the manifold action of the hormone to the general hypothesis of cAMP serving as the second messenger for the information carried by the hormone molecule (Robison et al., 1971) did not yield unequivocal results (Goldfine, 1977). In addition, evidence has been reported in recent years as to the presence of intracellular binding sites for insulin which would explain its intracellular effects which follow internalization (Goldfine, 1977; Steiner, 1977). In such a complex framework

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