Calorimetric Properties of Mixtures of Ganglioside G$_{M1}$ and Dipalmitoylphosphatidylcholine*

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The calorimetric properties of the numerically predominant membrane lipid species, the phospholipids, both singly (3) and in binary mixtures (4), are now reasonably well understood. Other lipid components, such as glycolipids, have so far received less attention.

The rich variety of carbohydrate arrangements among the glycolipids equips them well for possible roles in the recognition of molecules by cell membranes. The sialic acid-containing gangliosides (5, 6) are of particular interest, as putative receptors for bacterial toxins (7–10), peptide hormones (11–15), viruses (16–18), and neurotransmitters (19). Variations in the oligosaccharide portions of these glycosphingolipids, coupled with variations in their pattern of occurrence in various tissues, may confer upon those tissues specificity to differing classes of biological stimuli.

At present, we know little about how gangliosides or other receptors may communicate stimulus effects on the cell. In particular, we do not know very much about the effects of the gangliosides themselves on local membrane structure. It is of interest, then, to examine the influence of gangliosides on the physical properties of model membranes. Study of the properties of ganglioside/phospholipid mixtures may also enhance our understanding of the effects of ganglioside accumulation in several gangliosidoses, such as Tay-Sachs disease and G$_{M1}$ gangliosidosis, in which the lack of specific catabolic enzymes causes gangliosides to accumulate in neural tissues (20, 21).

In the present investigation, therefore, we have examined the effects of ganglioside G$_{M1}$, the cholera toxin receptor, isolated in high purity from bovine brain, on the thermotropic behavior of a well studied synthetic phospholipid system, multilamellar dispersions of dipalmitoyllecithin (1,2-dipalmitoyl-sn-glycero-3-phosphocholine, DPPC) in aqueous buffer.

MATERIALS AND METHODS

Ganglioside G$_{M1}$ was purified from bovine brain, starting with neuraminidase treatment of a mixture of gangliosides, according to the method described previously (22). This procedure gave G$_{M1}$ with a fatty acid composition heavily dominated (more than 85%) by octadecanoic acid, C$_{18}$O, the rest being a mixture of C$_{20}$O$_2$, C$_{22}$O, and C$_{24}$O acids (6). The average sphingosine chain length of the G$_{M1}$ was 19 ± 1 carbon atoms; three of these carbon atoms participate in the junction of the oligosaccharide head with the hydrocarbon tails (in analogy with the glycerol in acyl glycerolipids). Thus the two side chains had effective lengths of 18 (octadecanolate) and 16 (sphingosine) carbon atoms. The final purity of the G$_{M1}$ was greater than 99%, as judged by high resolution thin layer chromatography; the only contaminant was less than 1% G$_{M2}$. Synthetic DPPC was obtained from Calbiochem (Lot 501926) and recrystallized from ethanol.

Observations were made on pure DPPC, pure G$_{M1}$, and the following mole fractions of G$_{M1}$ in DPPC: 0.04, 0.08, 0.11, 0.24, 0.30, 0.40, 0.50, 0.54, 0.63, and 0.68. The pure lipids were stored as chloroform: methanol (1:1) solutions at 10 mg/ml at −20°C. The appropriate

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volumes of stock solutions corresponding to a fixed amount, 1 mg, of DPPC and the desired mole fraction of GMI were added to 5 ml pear-shaped flasks, the solvents were removed with a stream of dry nitrogen, and the samples were stored in vacuo overnight at 4°C. The mixed lipids were resuspended by adding 1.5 ml of phosphate buffer (NaCl, 137 mM; KCl, 2.7 mM; Na2HPO4, 6.9 mM; KH2PO4, 1.5 mM; pH 7.34) prewarming to above 50°C, and blending on a Vortex mixer at the same temperature for 1 min. The turbidity of all samples was judged visually.

The samples were loaded into one cell of a Privalov differential scanning calorimeter, and buffer was loaded into the other cell. Runs were initiated at nominal heating rates of 0.5°C min⁻¹ on samples degassed in vacuo. Scans were made over the temperature range from 12-83°C. The scan rate, power calibration, and heater voltages of the calorimeter were monitored for each run.

For each transition the temperature of the transition maximum, Tₘ, and the width of the transition at half-maximum, W⁻¹, were measured. The enthalpy, AH, for each transition was also determined by integrating the scans with a planimeter; for this purpose the base-line was estimated as indicated in Fig. 1. The apparent number of molecules in the cooperative unit, n, was calculated as 29.7/(TₘΔH).

RESULTS

Turbidity of Suspensions—Suspensions of pure DPPC and of all GMI/DPPC mixtures up to a GMI mole fraction (xG) of 0.24 were markedly turbid, by visual inspection. When xG was increased to 0.30, the turbidity of the suspensions abruptly decreased. At xG = 0.40 and above, including xG = 1.00, the suspensions were visually transparent.

Thermotropic Behavior of Pure GMI and Pure DPPC—When differential scanning calorimetry was performed with pure GMI alone in excess water, no thermal transitions could be detected, even at concentrations of 4 mg/ml, over the entire temperature range of these studies (12-83°C).

Calorimetric scans obtained for pure DPPC and a variety of GMI/DPPC mixtures are shown in Fig. 1. The uppermost scan, for pure DPPC, is characteristic of multilamellar dispersions of this phospholipid (3), with a pretransition at 32.7°C and a main transition (Fig. 2) at 41.4°C. The enthalpy of the main transition (Fig. 3) was 36.8 ± 0.9 kJ mol⁻¹, in good agreement with values previously obtained: 36.6 kJ mol⁻¹ by Mabrey and Sturtevant (4) and 36.4 kJ mol⁻¹ by Chapman (1). The width of the main transition at half-maximum was 0.6°C, and the calculated size of the cooperative unit for this transition was 102 ± 9 molecules (Fig. 4). For the pretransition, the width was 3.3°C, and the size of the cooperative unit was about 190 molecules (not shown).

Thermotropic Behavior of GMI/DPPC Mixtures—As GMI was added in increasing amounts to pure DPPC, the excess heat capacity curve remained qualitatively similar up to xG = 0.24, but the temperature of the pretransition gradually increased from 32.7-37°C and that of the main transition (Fig. 2) increased from 41.4-44.3°C. When xG was increased from 0.24 to 0.30, several changes simultaneously occurred in the excess heat capacity curve. The pretransition disappeared, and did not reappear at higher values of xG. Also, the height of the main transition began to decrease sharply, and a new transition appeared at a higher temperature, 48°C. Above xG = 0.30, the temperature of the original main transition remained constant or perhaps decreased slightly (Fig. 2).

Up to xG = 0.24, the calculated enthalpy of the pretransition remained constant, within the error of the measurement, at 3.01 ± 0.38 kJ mol⁻¹. The enthalpy of the main transition also remained constant over this range of xG values (Fig. 3). At values of xG between 0.24 and 0.63, the enthalpy of the original main transition decreased linearly to zero, and the enthalpy of the new transition increased linearly at the same rate, the sum of the enthalpies remaining constant. The standard error of the calculated enthalpies was ±7%.

No transitions were observed below 30°C or above 50°C. All the observed thermotropic transitions were experimentally reversible for at least 2 weeks. In several instances where

Fig. 1. Representative calorimetric heating scans of mixtures of DPPC with ganglioside GMI. Multilamellar dispersions of 1.0 mg of DPPC were prepared containing the indicated mole fractions (xG) of bovine brain GMI, at pH 7.34 in phosphate buffer. Estimated base-lines and resolution of the scans into two curves for xG > 0.30 have been indicated. In the curve for xG = 0.24, representative of mole fractions greater than 0.30, what appears to be a very broad and asymmetric peak at 37-47°C is also shown; at present this peak is unexplained.
Absence of Transitions for Pure GM₁—We have been unable to detect any transitions in calorimetric scans of pure GM₁ over the temperature range from 12–83°C, at concentrations as high as 4 mg/ml. In an earlier calorimetric study of commercial ganglioside mixtures, broad thermotropic transitions at 15 and 39°C were observed (23). If transitions such as these had occurred in our system, they would have been readily detectable. We have not attempted to establish the cause of this apparent discrepancy, but the effects seen in the earlier study might have been due to 1) low water content (less than 85%); 2) possible effects of ganglioside heterogeneity; and, most likely, 3) contamination with other lipid molecules (24).

Interestingly, it has recently been reported that pure monoglycosylcerebroside likewise does not display thermotropic transitions over the range from 10–75°C (25).

Structures of Phospholipid/Ganglioside Mixtures—In order to interpret the calorimetric data obtained with DPPC/GM₁ mixtures it is useful to consider the microscopic structures of the pure components and their mixtures. Whereas phospholipids in aqueous media predominantly form extended bilayers of the lamellar type (1), numerous studies (26–28) have established that in aqueous media, at water contents above 50% by weight, gangliosides form spheroidal micelles. Since phospholipids and gangliosides form sharply contrasting structures, the structures of their mixtures are particularly interesting. Hill and Lester (27) prepared phospholipid/ganglioside mixtures by two different methods and compared the resulting structures by centrifugation and electron microscopy. Using the method of preparation employed in our study, they found that only multilamellar structures were formed at low ganglioside concentrations (up to about 30 mole%); between 65 and 80 mole% only cylindrical micelles were found; and above 80 mole% the micelles became spheroidal. Subsequent investigations have supported the view that at low concentrations, gangliosides (29) and other glycolipids (25,30) are incorporated into the phospholipid structure. Ganglioside incorporation occurs up to a ganglioside mole fraction of about 0.25 to 0.30, at which point a marked physical change occurs (27, 29).

The structure of phospholipid/glycolipid mixtures is determined by the balance between the interaction of head groups (normally repulsive) and the hydrophobic interaction of tail groups (31,32), which in turn depends upon the mean surface/volume ratio of the molecules. The available structures include bilayers extended in two dimensions as lamellae (L), in one dimension as cylindrical micelles (C), or in zero dimensions as spherical micelles (S). For a hydrocarbon region of constant thickness, a convenient first order approximation, the surface/volume ratios of L, C, and S structures are in the proportions L:C:S = 1:2:3. If the mean head group area at the site of maximum repulsive interaction is low, an L structure is formed; for increasing head-group area, C and then S structures are required. Mixtures of pure phospholipids and pure glycolipids may form one, two, or all three structures, depending on their composition and the geometry of the individual molecular species.

Our turbidity observations and calorimetric data indicate that mixtures of pure DPPC and pure GM₁ behave qualitatively like the lecithin/ganglioside mixtures studied by Hill and Lester (27), and as expected from the preceding considerations. At low GM₁ mole fractions (up to 0.24), GM₁ continuously perturbs the DPPC transitions characteristic of the lamellar structure, but does not introduce any new transitions. The abrupt drop in turbidity at xG between 0.30 and 0.40 reveals a sharp decrease in particle size over this range. The
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The appearance of a higher temperature transition at \( x_0 = 0.30 \), and its growth with increasing GM1 mole fraction, at the expense of the original transition, points to the progressive replacement of the lamellar phase by a different structure, presumably the cylindrical micellar phase. At \( x_0 = 0.63 \) and 0.68, the persistence of the higher temperature transition in the absence of the one at lower temperature suggests that only micelles are present.

The addition of GM1 to DPPC increases \( T_c \) for the main transition up to \( x_0 = 0.24 \), and \( T_c \) for the micellar transition, where \( x_0 \) is presumed to be around 0.63, is even higher, implying that GM1 stabilizes the DPPC structure. Stabilization of phospholipids by myristic acid (33) and sphingomyelin (34), and by gangliosides (29) and other glycolipids (25, 30, 35, 36) has been observed by both calorimetric (25, 30, 33, 34) and spin-labeling (29, 34–36) techniques. Such stabilization appears to be due partly to shielding of phospholipid head groups from each other, reducing their repulsive interaction (37). Stabilizing interactions are also thought to take place directly between the ganglioside head groups (29). For mixtures of egg lecithin and sphingomyelin (34) and of DPPC and monoglycerolcereside (25), formation of a 2:1 phospholipid:glycolipid complex at low sphingolipid concentrations has been postulated.

The cooperativity of the main DPPC transition, as reflected in the apparent size of the cooperative unit, \( n \), decreases sharply with the addition of small amounts of GM1, then plateaus (Fig. 4). This behavior closely parallels the effect of glucocerebroside on the cooperativity of DPPC transitions (25). On the other hand, addition of GM1 to the micellar phase at \( x_0 \) values above 0.54 causes an abrupt and unexplained increase in the cooperativity of the high temperature transition.

We have not explored the region of GM1 mole fractions above 0.68; to do this would require using either very large amounts of GM1 or amounts of DPPC so small as to yield unacceptable signal/noise ratios. Thus our data do not bear directly on the existence of two micellar structures.

**Interpretation in Terms of Heterogeneous Equilibrium**

The approach of DPPC/GM1/water systems to thermodynamic equilibrium is dependent on the method of mixing. The penetration of gangliosides into multilamellar structures (27) and the dissociation of ganglioside micelles into monomers (38) are extremely slow processes. Equilibrium DPPC/GM1 mixtures do not readily result from mixing aqueous suspensions of the pure components; but resuspending dried mixtures, as we have done, results in rapid homogeneous dispersion of the ganglioside in the bilayers (27) and produces uniform structures whose physical properties are stable over long periods of time. Our calorimetric scans were completely reproducible after an interval of 2 weeks. Similar stability was noted in a recent calorimetric study of monoglycerolcereside/DPPC mixtures (25). Also, the limiting concentrations of the GM1-rich and DPPC-rich phases should not be strongly temperature-dependent if they are determined mainly by molecular structure. Consequently, the compositions of the two phases should not vary greatly during a calorimetric run at a given mole fraction of ganglioside. The fact that the curves obtained were independent of scan rate confirms this view.

We conclude that the calorimetric behavior of our system closely approximates equilibrium behavior.

A binary system at equilibrium at constant pressure is invariant with respect to the temperature and mole fractions when three phases coexist (39). Addition of either component to such a system alters the relative amounts of the three phases, but does not affect either their compositions or the equilibrium temperature. In our experiments, the DPPC/GM1 system behaved as one would expect for an equilibrium system of two partially miscible components whose conjugate solid phases (DPPC-rich and GM1-rich, respectively) melt at slightly different temperatures. Specifically, the curves in Fig. 2 between \( x_0 \) values of 0.30 and 0.54 may readily be interpreted in terms of two invariant systems of three phases: 1) one at a temperature of 44°C, in which the three phases at equilibrium are ordered bilayers, disordered bilayers, and ordered micelles; and 2) one at a temperature of 48°C, in which the three phases are disordered bilayers, ordered micelles, and disordered micelles.

In summary, we have studied the thermotropic behavior, over the temperature range from 12–83°C, of pure ganglioside GM1, pure synthetic dipalmitoylphosphatidylcholine (DPPC), and 10 mixtures of GM1 and DPPC. At GM1 mole fractions from 0.04 to 0.68. Our principal observations are that 1) pure GM1 shows no thermal transitions; 2) at low mole fractions (up to 0.24), GM1 quantitatively perturbs the excess heat capacity curve for DPPC multilamellar bilayers, which shows a major transition in the temperature range 41–44°C; 3) at moderate mole fractions of GM1 (between 0.30 and 0.54), the transition at 41–44°C gradually diminishes in height and area as the proportion of GM1 increases, being replaced by a new transition at 48°C, whose height and area increase correspondingly; and 4) at still higher GM1 mole fractions (0.63 and 0.68), only the transition at 48°C is observed.

The present data support the interpretation that ganglioside GM1 can be incorporated into bilayers of DPPC only up to mole fractions near 0.25. It appears that at low concentrations GM1 can substitute for phospholipid molecules in the bilayer lattice. At \( x_0 \) values above 0.25 GM1 disrupts the bilayer, with the formation of mixed micelles of phospholipid and ganglioside. Phase transitions are detected over the whole range of ganglioside mole fractions reported here \((0 \leq x_0 \leq 0.68)\), indicating that GM1 can pack well enough into DPPC structures to exhibit cooperative melting. It further appears that GM1 stabilizes the DPPC lattice, possibly by the formation of a specific complex.

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