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Thermotropic behavior of binary mixtures of diplamitoylphosphatidylcholine and glycosphingolipids in aqueous dispersions

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The thermotropic behavior of mixtures of dipalmitoylphosphatidylcholine (DPPC) with natural glycosphingolipids (galactosylceramide, phrenosine, kerasine, glucosylceramide, lactosylceramide, asialo-G_{M1}, sulfatide, G_{M3}, G_{M1}, G_{D1a}, G_{T1b}) in dilute aqueous dispersions were studied by high sensitivity differential scanning calorimetry over the entire composition range. The pretransition of DPPC is abolished and the cooperativity of the main transition decreases sharply at mole fractions of glycosphingolipids below 0.2. All systems exhibit non-ideal temperature-composition phase diagrams. The mono- and di-hexosylceramides are easily miscible with DPPC when the proportion of glycosphingolipids in the system is high. A limited quantity (1–6 molecules of DPPC per molecule of glycosphingolipid (GSL) can be incorporated into a homogeneously mixed lipid phase. Domains of DPPC, immiscible with the rest of a mixed GSL-DPPC phase that shows no cooperative phase transition, are established as DPPC exceeds a certain proportion in the system. One negative charge (sulfatide) or four neutral carbohydrate residues (asialo-G_{M1}) in the oligosaccharide chain of the glycosphingolipids results in phase diagrams exhibiting coexistence of gel and liquid phases over a broad temperature-composition range. Systems containing gangliosides show complex phase diagrams, with more than one phase transition. However, no evidence for phase-separated domains of pure ganglioside species is found. The thermotropic behavior of systems containing DPPC and glycosphingolipids correlates well with their interactions in mixed monolayers at the air/water interface.

Introduction

The polar head group of glycosphingolipids has a major influence on their molecular behavior in biological interfaces [1–3]. Various glycosphingolipids are able to increase the permeability of liposomes in the presence of proteins or neurotransmitters [4], to modify neurotransmitter release and uptake in nerve endings [5] and to induce cell fusion [2]. Several of these effects appear to be dependent on the type of oligosaccharide chain

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Abbreviations: Cer, ceramide (*N*-acylsphingosine); GlcCer, Glc(β1–1′)-Cer; GalCer, Gal(β1–1′)Cer; Sulf, sulfate-I[3]-GalCer; LacCer, Gal(β1–4)Glc(β1–1′)Cer; Gg_4Cer, Gal(β1–3)GalNAc(β1–4)Gal(β1–4)Glc(β1–1′)Cer; G_{M3}, NeuAc(α2–3)Gal(β1–4)Glc(β1–1′)Cer; G_{M1}, Gal(β1–3)GalNAc(β1–4)Gal[3–2αNeuAc](β1–4)Glc(β1–1′)Cer; G_{D1a}, NeuAc(α2–3)Gal(β1–3)GalNAc(β1–4)Gal[3–2αNeuAc](β1–4)Glc(β1–1′)-Cer; G_{T1b}, NeuAc(α2–3)Gal(β1–3)GalNAc(β1–4)Gal[3–2α-NeuAc8–2αNeuAc](β1–4)Glc(β1–1′)Cer; DPPC, dihexadecanoylphosphatidylcholine; DMPC, ditetradecanoylphosphatidylcholine.

present in gangliosides and other glycosphingolipids. This, in turn, influences their individual molecular properties [1,2], interactions with various proteins [7,8], phospholipids and Ca^{2+} [6,9,10] and toxins [11–13]. It has also been reported that the rate of hydrolysis of gangliosides can be affected by the physical state of a mixed ganglioside-DPPC phase [14].

Recently, a systematic analysis of the thermotropic behavior of a series of chemically related glycosphingolipids has been published [3]. These studies have provided strong evidence that the type of polar head group present in a particular glycosphingolipid plays a major role in determining its phase behavior. Very few studies on the thermotropic behavior of mixtures of glycosphingolipids with phospholipids have been made [15–19] and, with few exceptions [15,19], most of the published data only extend over a limited composition range and do not allow the construction of a complete phase diagram. However, a thorough description of the phase properties of a mixed lipid system is only possible in terms of temperature-composition phase diagrams. In this work we have studied by high sensitivity differential scanning calorimetry the thermotropic behavior of mixtures of DPPC with glycosphingolipids containing oligosaccharide chains of various complexities. Mixtures of DPPC with GalCer, phrenosine, kerasine, sulfatide, GlcCer, LacCer, Gg_4Cer, G_{M3}, G_{M1}, G_{D1a} and G_{T1b} in dilute aqueous dispersions were studied over the entire temperature-composition range. Complete phase diagrams and variations with composition of the thermodynamic parameters for the phase transitions are described for all of these systems.

Materials and Methods

The source and purity of the glycosphingolipids used were described previously [3]. DPPC was purchased from Avanti Inc. (Birmingham, AL). Calorimetric studies were performed with a DASM-1M Privalov calorimeter at a nominal scan rate of 0.5 K/min; the actual scan rate was monitored for each run. Some of the samples were also scanned at 1 K/min with similar results. Samples of lipids were premixed in the desired proportions from chloroform/methanol (2 : 1, v/v) or chloroform/methanol/water (2 : 1 : 0.15, v/v) solutions. The solvent was evaporated under N_2 and the dried lipid was heated to 55°C for 1 h and submitted to high vacuum for at least 4 h. Aqueous dispersions of the mixtures were prepared in 0.05 M phosphate buffer (pH 7.0). This buffer was chosen because of its low enthalpy of ionization [20]. The concentration of the lipid dispersions ranged between 1 and 7 mg/ml; for each particular mixture the concentration was chosen taking into account the magnitude of the heat changes involved in relation to the sensitivity of the instrument so as to ensure detection of small transitions in the sample. The range of temperature extended well over the regions where each pure component exhibits its own phase transition. A reproducible, reversible and stable thermotropic behavior was achieved by preparing the lipid dispersions as described elsewhere [3]; no metastable behavior was observed under successive cooling and reheating cycles or after representative samples of each type of glycosphingolipid were kept at 4°C for several days. The noise level and base line stability of the actual calorimetric scans were as described before (Fig. 1 in Ref. 3)

The construction of the phase diagrams was made taking the onset and completion temperatures for the transition. These were obtained as the intersections of the straight lines resulting from extrapolation of the rising and falling parts of the curves of excess heat capacity vs. temperature with the extrapolated base line. Most of the curves were steep enough to allow for an acceptable precision (better than ± 0.3 deg. C) in the estimation of the points of intersection. In the case of the broadest and more oddly-shaped curves there were considerable uncertainties that can amount to as much as 3–8 deg. C for some of the values obtained. The onset and completion temperatures were not corrected for the contributions arising from the finite widths of the transition of the pure lipids to the total transitions (cf. Ref. 21). This type of correction has no meaning in the case of the broadest curves since the phase transition is obviously taking place over a considerable temperature range, especially for the more complex glycosphingolipids [3]. The cooperative units and the ideal phase diagrams were computed from the known transition temperatures and enthalpies of the individual

lipids [3] as described by Mabrey and Sturtevant [21,22].

The composition of the system was expressed in mole fraction units. However, the statement of the actual compositions of the phases present in a binary lipid mixture is not a simple matter. When an appreciable part of either component may show a tendency to remain in the water phase (either as soluble monomers or as part of a different structure in heterogeneous equilibrium), the actual lipid composition in a particular phase depends on the relative volumes of the different lipid and water phases. In all such cases, lipid composition would only be known if the partition coefficients of the components among the various phases were known. These problems limit the validity of expressing the compositions as mole fractions. In this work, and considering the above limitations, the mole fractions should be taken only as representing the relative proportions of the lipids in the whole system.

Results

Neutral glycosphingolipids

The temperature of the pretransition shifts from 35.7°C for pure DPPC to 31°C as the proportion of GlcCer is increased up to a mole fraction of 0.235 (Fig. 1a, inset). The transition enthalpy (ΔH_{cal}) for this process decreases and, at values above a mole fraction of 0.235 for GlcCer, the curve of excess heat capacity vs. temperature for the pretransition becomes broadened beyond detection. The temperature of the main transition (T_m) exhibits only slight increases in this composition range (from 41.4°C for pure DPPC to 41.7°C) and ΔH_{cal} decreases (from 8.6 kcal · mol^{-1} for pure DPPC to 6.2 kcal · mol^{-1}) (Fig. 1a, Fig. 3b). Obviously, the entropy of the main transition (ΔS°) shows a variation similar to that of ΔH_{cal}; at the same time, the cooperative unit (CU) shows a sharp decrease from 280 molecules for pure DPPC to 44 molecules (Fig. 3b).

The T_m increases rather rapidly as the proportion of GlcCer in the system is increased above 0.235 (Figs. 1a and 3a). At mole fractions of GlcCer between 0.350 and 0.683 the excess heat capacity vs. temperature curve becomes very broad and with a correspondingly low maximal excess

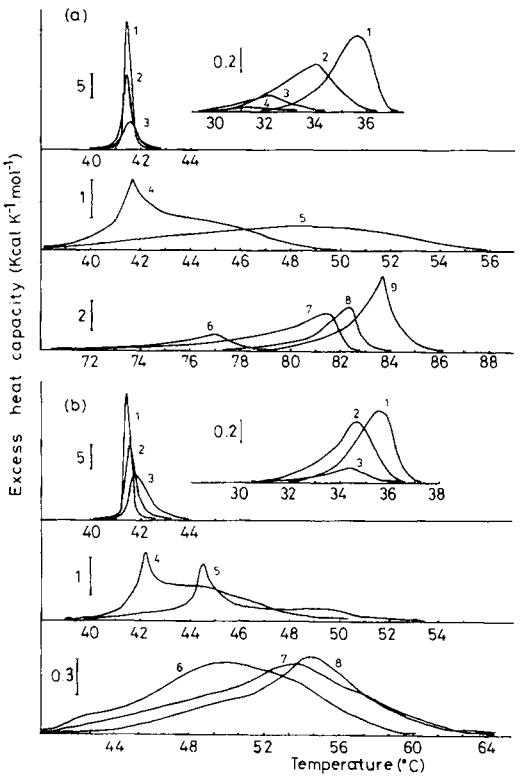

Fig. 1. Variation with temperature of the excess heat capacity of binary mixtures of DPPC with neutral glycosphingolipids. For the system DPPC-GlcCer (a) the concentration of total lipid was 1 to 3 mg · ml^{-1} and the range of temperature scanned was from 25°C to 90°C for samples with the following mole fractions of GlcCer: (1) 0.0; (2) 0.023; (3) 0.093; (4) 0.235; (5) 0.480; (6) 0.680; (7) 0.890; (8) 0.97; (9) 1.0. The inset shows the corresponding pretransition changes. For the system DPPC-Gg_4Cer (b) the concentration of total lipid was 1 to 3 mg · ml^{-1} and the range of temperature scanned was from 25°C to 70°C for samples with the following mole fractions of Gg_4Cer: (1) 0.0; (2) 0.030; (3) 0.060; (4) 0.197; (5) 0.364; (6) 0.697; (7) 0.916; (8) 1.0. The inset shows the corresponding pretransition changes. The vertical bars represent units of excess heat capacity in kcal · K^{-1} · mol^{-1}. The transitions shown are the only ones observed over the range of temperature indicated above. For simplicity, the curves are not extended beyond the transition region.

heat capacity (Fig. 1a); ΔH_{cal} and CU change little in this range (Fig. 3b). Above a mole fraction of GlcCer of 0.88, ΔH_{cal} and ΔS° increase sharply towards the values of pure GlcCer.

Fig. 3a shows that the phase diagram for this system is definitely non-ideal below a mole fraction of GlcCer of 0.48 while approaching a more

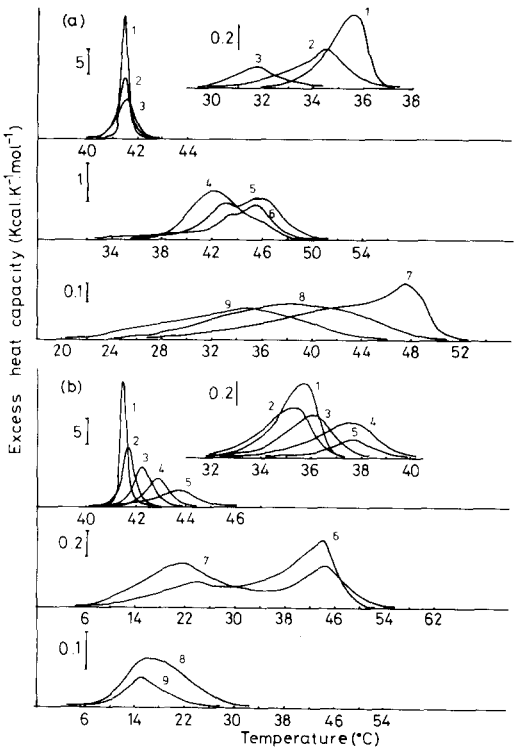

Fig. 2. Variation with temperature of the excess heat capacity of binary mixtures of DPPC and gangliosides. For the system G_{M3}-DPPC (a) the concentration of total lipid was 1 to 4 mg·ml^{-1} and the range of temperature scanned was from 15°C to 60°C for samples with the following mole fractions of G_{M3}: (1) 0.0; (2) 0.006; (3) 0.063; (4) 0.289; (5) 0.477; (6) 0.587; (7) 0.846; (8) 0.960; (9) 1.0. The inset shows the corresponding pretransition changes. For the system G_{D1a}-DPPC (b) the concentration of total lipid was 1 to 6 mg·ml^{-1} and the range of temperature scanned was from 5°C to 60°C for samples with the following mole fractions of G_{D1a}: (1) 0.0; (2) 0.010; (3) 0.042; (4) 0.089; (5) 0.208; (6) 0.350; (7) 0.479; (8) 0.780; (9) 1.0. The inset shows the corresponding pretransition changes. The vertical bars represent units of excess heat capacity in kcal·K^{-1}·mol^{-1}. The transition shown are the only ones observed over the range of temperature indicated above. For simplicity, the curves are not extended beyond the transition region.

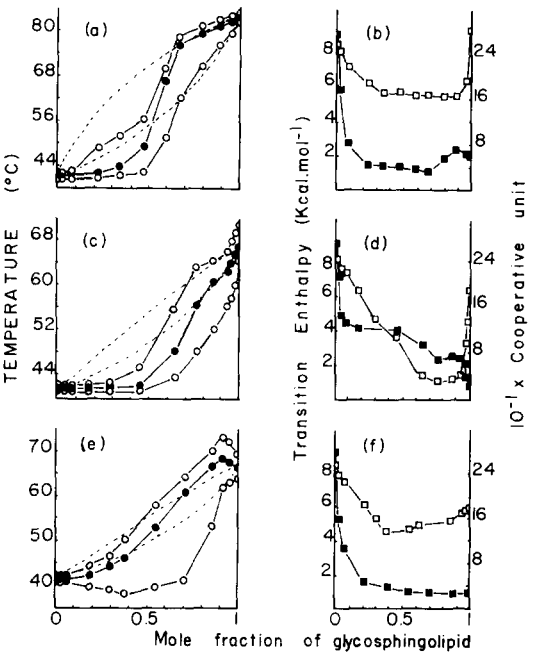

Fig. 3. Phase diagrams and variation of thermodynamic parameters for binary mixtures of DPPC with neutral glycosphingolipids. The variation of the onset and completion temperatures (○), T_m (●), ΔH_{cal} (□) and cooperative unit (CU) (■) with the composition is given for binary mixtures of DPPC with: GlcCer (a, b); GalCer (c,d); kerasine (e,f). The dashed lines show the phase diagrams calculated on the assumption of ideal behavior.

ideal behavior at higher mole fractions. Peritectic behavior, with DPPC immiscible with GlcCer in the gel phase, occurs below a mole fraction of GlcCer of 0.235. Also, liquid-phase immiscibility is apparently present below a mole fraction of GlcCer of 0.1.

The phase diagram for mixtures of GalCer-DPPC and the dependence of ΔH_{cal} and CU on composition is shown in Figs. 3c and 3d, respectively. The CU shows a decrease to about 80–120 molecules between mole fractions of GalCer of 0.1 to 0.95. The values of ΔH_{cal} show a greater decrease than for the system GlcCer-DPPC, reaching much lower values at mole fractions of GalCer between 0.65 and 0.95; in this composition range the excess heat capacity function becomes extremely broadened (not shown). The phase diagram for this system reveals that gel-phase immiscibility may already be present at mole fractions of GalCer below 0.46, with the formation of pure DPPC domains (Fig. 3c). Mixtures of phrenosine with DPPC (not shown) revealed behavior similar to that of GalCer-DPPC. Kerasine appears to be more miscible with DPPC; gel-phase immiscibility for kerasine-DPPC occurs only below a mole fraction of kerasine of 0.15 (Fig. 3e).

The system consisting of LacCer-DPPC shows changes of the pretransition similar to those found

for GlcCer-DPPC. The variation with composition of ΔH_{cal} and CU for the main transition is also similar while the shape of the phase diagram follows more closely that expected for an ideal system. If there is gel-phase immiscibility for this system it can occur only below a mole fraction of LacCer of 0.1 (Figs. 4a and 4b).

A greater miscibility with DPPC was also found for the system containing Gg_4Cer. Liquid-phase miscibility is observed at all proportions and gel-phase immiscibility can only be present below a mole fraction of Gg_4Cer of 0.10. The phase diagram is very broad on both sides of the solidus and liquidus curves calculated for ideal behavior (Fig. 3e). Because of these broad limits, it is difficult to exclude the possibility of formation of rather pure Gg_4Cer domains above 45°C although the lack of a horizontal portion in the solidus or liquidus curves seems to rule out this possibility at mole fractions of this lipid above 0.2. The temperature of the pretransition shows a small shift from 35.7°C to 34.5°C for mole fractilons of Gg_4Cer up to 0.1 and above this proportion it becomes undetectable (Fig. 1b, inset). The changes of ΔH_{cal} and CU for the main transition show the same general behavior as found for the other neutral glycosphingolipids (Fig. 4d).

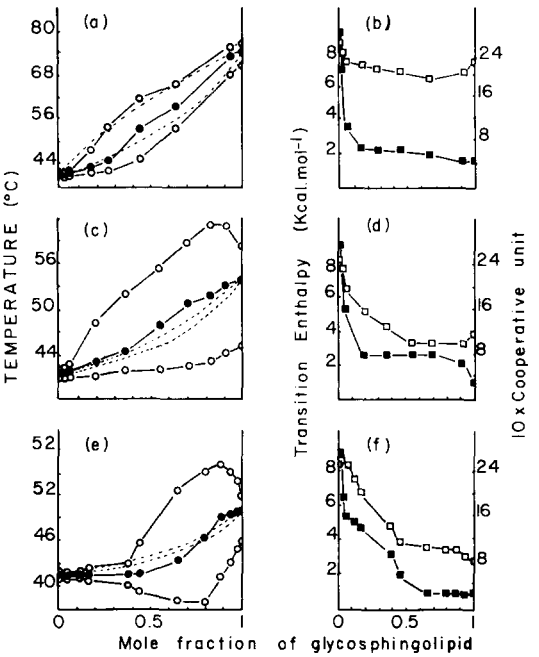

Fig. 4. Phase diagrams and variation of thermodynamic parameters for binary mixtures of DPPC with neutral glycosphingolipids and sulfatide. The variation of the onset and completion temperatures (○), T_m (●), ΔH_{cal} (□) and cooperative unit (CU) (■) with the composition is given for binary mixtures of DPPC with: LacCer (a, b); Gg_4Cer (c, d); sulfatide (e, f). The dashed lines show the phase diagrams calculated on the assumption of ideal behavior.

Anionic glycophingolipids

The T_m of the pretransition for the system Sulf-DPPC decreases to 33°C at a mole fraction of Sulf of 0.123 and it becomes undetectable thereafter. The shape of the phase diagram for the main transition shows that DPPC is rather miscible with Sulf (Fig. 4e). Gel-phase immiscibility may be present only below a mole fraction of Sulf of 0.123; the phase diagram becomes very broad compared to the ideal behavior above a mole fraction of Sulf of 0.4. Because of this, it is not possible to exclude the possibility that pure Sulf domains may exist above a mole fraction of 0.5 and above 46°C. However, these are unlikely since no region of isothermal melting is observed. The variation of ΔH_{cal} and CU with composition shows a behavior similar to that of the neutral glycosphingolipids (Fig. 4f).

The system G_{M3}-DPPC reveals a decrease of the T_m of the pretransition to 31.5°C at a mole fraction of 0.063. The pretransition becomes undetectable above a mole fraction of 0.1 (Fig. 2a, inset). At mole fractions of G_{M3} up to 0.4 the presence of the ganglioside perturbs the main transition of DPPC and the T_m increases slightly and continuously, from 41.4°C to 42.0°C; the ΔH_{cal} shows very small decreases, if any, in this range of composition while the CU decreases abruptly (Fig. 2a, Fig. 5a, b). The lipid dispersions remain with a milky appearance below a mole fraction of G_{M3} of 0.4, but the turbidity decreases progressively above this proportion and the dispersions become visually transparent above a mole fraction of 0.6. Above a mole fraction of 0.4, G_{M3} induces several complex changes in the curve of excess heat capacity vs. temperature. The apparent maximal excess heat capacity shifts to a higher temperature while the peak of 42°C is still discernible (Fig. 2a). As the proportion of G_{M3} increases from a mole fraction of 0.4 to 0.846, the peak between 41°C and 44°C gradually decreases in relation to the

maximal excess heat capacity change that takes place at the higher temperature (about 46°C). Above a mole fraction of 0.7 the curve of excess heat capacity vs. temperature shifts definitely to lower temperatures and towards the values of pure G_{M3} (Fig. 2a). Fig. 5a shows the complete phase diagram. In contrast to what occurs for the systems containing neutral glycosphingolipids or Sulf, the solidus and liquidus curves of the real system are displaced above those calculated for the ideal system at almost all compositions. At proportions of G_{M3} below a mole fraction of 0.06 some gel-phase immiscibility might be present. Also, in contrast to the systems containing neutral glycosphingolipids or Sulf the variation of ΔH_{cal} follows an almost linear variation with composition (Fig. 5b). ΔH_{cal} for the lower temperature transition decreases to zero above a mole fraction of G_{M3} of 0.6 while ΔH_{cal} of the transition process at higher temperature becomes measurable at mole fraction 0.3 and increases at mole fractions of G_{M3} up to 0.7. Above this proportion, a single transition remains and its T_m progressively decreases toward the value of pure G_{M3} as the mole fraction is increased (Fig. 2a, Fig. 5b).

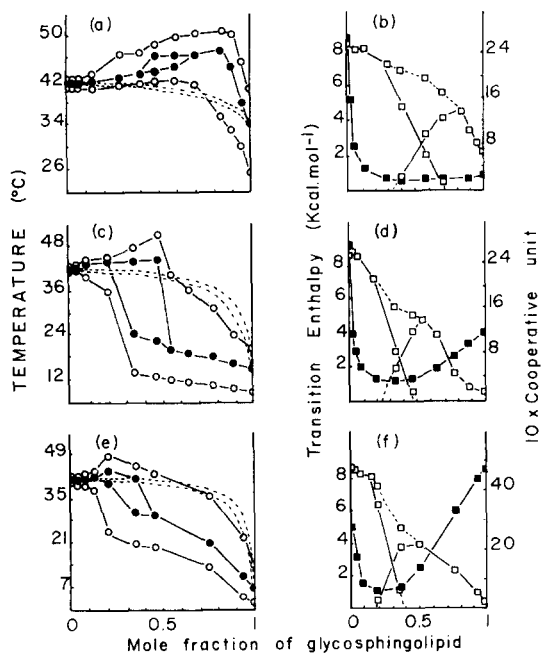

Fig. 5. Phase diagrams and variation of thermodynamic parameters for binary mixtures of DPPC with gangliosides. The variation of the onset and completion temperatures (○), T_m (●), ΔH_{cal} (□) and cooperative unit (CU) (■) with the composition is given for binary mixtures of DPPC with: G_{M3} (a, b); G_{D1a} (c, d) and G_{T1b} (e, f). The dashed lines show the phase diagrams calculated on the assumption of ideal behavior.

A qualitatively similar behavior to that described for G_{M3}-DPPC was found for the mixture G_{M1}-DPPC (not shown). Several of the findings for this system confirm and extend the observations previously reported by Sillerud et al. (1979) [17]. These authors could not detect a transition for pure G_{M1} over the temperature range 12–83°C with a sample containing 4 mg/ml. This was probably due to the low temperature, low enthalpy and low cooperativity of the phase transition of G_{M1}. Employing 7 mg/ml of G_{M1} and a higher sensitivity in the instrument, a broad transition extending from about 12°C to 28°C and centered at 19°C is detected [3].

The curves of excess heat capacity vs. temperature for the system G_{D1a}-DPPC are shown in Fig. 2b. Several differences compared to the mixtures of DPPC and monosialogangliosides are apparent. The T_m of the pretransition first decreases slightly at mole fraction of G_{D1a} of 0.01 and then increases from 35.7°C to 37.5°C as the mole fraction of G_{D1a} increases to 0.208 (Fig. 2b, inset). The T_m of the main transition increases from 41.4°C to 43.7°C in this range. The turbidity of the samples decreases sharply above a mole fraction of ganglioside of 0.2 and the samples become visually transparent above 0.5. Above a mole fraction of 0.25 for G_{D1a} the transition with T_m at approximately 44°C is maintained but a new peak appears at lower temperatures (about 24°C, Fig. 2b). The peak of the lower temperature transition becomes predominant while the peak at 44°C is diminished as the proportion of G_{D1a} increases. The peak with lower T_m is shifted further down towards the T_m of pure G_{D1a} (from 22°C to 15.2°C) as the mole fraction of G_{D1a} increases from 0.350 to 1.00; the peak with higher T_m becomes undetectable at a mole fraction of ganglioside of 0.55. Fig. 5c shows the phase diagram for this system. The variation of ΔH_{cal} is almost linear with composition and the CU decreases to minimal values at mole fractions of ganglioside between 0.2 and 0.5. ΔH_{cal} of the transition occurring at higher temperatures decreases to zero above a mole fraction of

G_{D1a} of 0.5 while the transition occurring at lower temperatures becomes detectable and its enthalpy increases above a mole fraction of G_{D1a} of 0.25 (Fig. 5d).

The behavior of the system consisting of G_{T1b} and DPPC (Fig. 5e, f) is similar to that of G_{D1a}-DPPC. The T_m of the pretransition increases to 37.3°C at a mole fraction of G_{T1b} of 0.128. The dispersions show a marked decrease in turbidity and become visually transparent in the range of mole fractions of G_{T1b} of 0.128–0.254. Above a mole fraction of 0.128, the complex changes occurring in the curve of excess heat capacity vs. temperature are similar to those of the system containing G_{D1a}. The T_m of the main transition increases to 44.5°C at a mole fraction of G_{T1b} of 0.170 while a new transition takes place at a lower temperature. The transition with high T_m remains between 43.5 and 44.5°C but becomes progressively smaller while the transition with low T_m becomes predominant as the proportion of G_{T1b} increases. The low T_m transition shifts toward the values of pure G_{T1b} (from 42.2°C at mole fraction of G_{T1b} of 0.170 to 11°C at a ganglioside mole fraction of 0.93).

Discussion

The occurrence of immiscibility and phase separation of domains of a pure component of a binary mixture can only be ascertained by analysing the variation of the onset and completion temperatures of a phase transition as a function of the composition of the system. The presence of more than one peak in the calorimetric scans without the occurrence of isothermal melting may, however, indicate the formation of immiscible mixtures of components. On the other hand, even if more than one peak is present a system can nevertheless remain homogeneous [21]. According to the phase rule, a horizontal portion in either the solidus or liquidus curves of the phase diagram of a binary mixture which indicates isothermal melting over a certain range of composition is required as evidence for immiscibility and phase separation of a pure component [21,22].

Systems containing neutral glycosphingolipids and sulfatide

The phase diagrams of mixtures of DPPC with the neutral glycosphingolipids GalCer, phrenosine, kerasine, GlcCer and LacCer indicate the presence of peritectic behavior and immiscibility in the gel-phase and, eventually, even in the liquid-phase for some systems at low contents of glycosphingolipid (GSL) (see Fig. 3). The data reveal the formation of domains of pure DPPC immiscible with the rest of a GSL-DPPC phase that becomes enriched in glycosphingolipid as its mole fraction increases (see also Fig. 1). The monohexosylceramides are more miscible with DPPC in the liquid-phase but a certain degree of liquid-liquid immiscibility also becomes apparent at higher proportions of DPPC. Table 1 shows the amount of essentially unmodified DPPC that phase separates as its proportion in the system increases. The number of molecules of DPPC per molecule of glycosphingolipid that are sequestered into a mixed GSL-DPPC phase and do not participate in the DPPC transition is also given for the region of gel-phase immiscibility.

Even at mole fractions of glycosphingolipid where phase separated DPPC domains are present, very small amounts of glycosphingolipid in the system induce noticeable changes. The behavior of the phospholipid becomes modified as revealed by the changes of its pretransition properties. This process is shifted to lower temperatures at very low proportions of glycosphingolipid and the size of the CU of the main transition decreases dramatically. Thus, even the DPPC molecules that phase separate no longer exhibit their usually high cooperativity during the phase change. In the region of gel-phase immiscibility at high content of DPPC, the decrease of ΔH_{cal} suggests that between 1 and 6 molecules of DPPC (depending on the glycosphingolipid, see Table I) are being sequestered into a mixed phase with the monohexosylceramide while the amount of DPPC exceeding this proportion forms separated domains. Correa-Freire et al. [16] found that 2 molecules of DPPC were sequestered by GlcCer over a similar composition range. Our results regarding the general thermotropic changes for mixtures of GlcCer-DPPC agree well with the observation of these authors who carried out a similar study but over a reduced temperature-composition range.

On the other hand, on the glycosphingolipid-rich side of the phase diagrams no immiscibility can be

TABLE I

AMOUNT OF PHASE SEPARATED DPPC IN MIXTURES WITH GLYCOSPHINGOLIPIDS (GSL)

Lipid	Mole fraction of		ΔH_{cal} (kcal·mol^{-1})	Fraction of DPPC phase-separated [a]	Moles DPPC sequestered per mole GSL
	GSL	DPPC			
DPPC	0.000	1.000	8.60	1.000	0.0
GalCer	0.022	0.978	8.15	0.948	2.3
	0.087	0.913	7.50	0.872	1.3
	0.300	0.700	4.70	0.547	1.1
Phrenosine	0.009	0.991	8.40	0.977	2.5
	0.043	0.957	7.70	0.895	2.3
	0.268	0.732	3.90	0.453	1.5
Kerasine	0.016	0.984	8.00	0.930	4.3
	0.064	0.936	7.70	0.895	1.5
	0.209	0.791	6.50	0.756	0.9
GlcCer	0.023	0.977	8.20	0.953	2.0
	0.093	0.907	7.30	0.849	1.5
	0.235	0.765	6.30	0.733	0.9
LacCer	0.008	0.992	8.20	0.953	5.8
	0.040	0.960	7.70	0.895	2.5
Gg_4Cer	0.006	0.994	8.28	0.963	6.2
	0.030	0.970	7.05	0.819	5.9
	0.060	0.940	6.60	0.767	3.7
Sulf	0.020	0.980	8.40	0.977	1.1
	0.123	0.877	7.50	0.872	0.9
G_{M3}	0.006	0.994	8.55	0.994	1.0
	0.063	0.937	8.10	0.932	1.0
G_{M1}	0.006	0.994	8.55	0.994	1.0
	0.049	0.951	8.40	0.944	1.1
G_{D1a}	0.006	0.994	8.55	0.994	1.0
G_{T1b}	0.006	0.994	8.55	0.994	1.0

[a] Corresponds to the region of gel-phase immiscibility.

detected for the systems containing CalCer, phrenosine, kerasine, GlcCer, LacCer, Gg_4Cer and Sulf. This indicates that the phospholipid is readily incorporated into the neutral glycosphingolipid or Sulf phase. As the content of DPPC increases, it perturbs in a continuous manner the thermodynamic properties of the glycosphingolipid but the system remains homogeneous up to a point where more phospholipid molecules are no longer compatible with the restrictions of the mixed GSL-DPPC lattice and pure DPPC domains separate out. The rest of the mixed GSL-DPPC phase at this point shows no cooperative phase transition and the curve of excess heat capacity is broadened beyond detection. This effect is qualitatively similar to that found for mixtures of DPPC with cholesterol. In this case, very little miscibility, if any, exists between pure DPPC and the rest of the DPPC-cholesterol mixture in the gel-phase below a mole fraction of cholesterol of 0.2 [23,24]; the molecules of the mixed DPPC-cholesterol phase exhibit a low cooperativity for the phase transition and the changes of state occur very gradually [24].

A detailed calorimetric and X-ray diffraction

study of mixtures of DPPC with synthetic *N*-palmitoylgalactosylphingosine in samples containing 70% water by weight was recently reported [19]. Two phase transitions were described in this system for mixtures containing more than 23 mol% of glycosphingolipid and this was interpreted as an indication of phase separation of immiscible domains of the synthetic cerebroside above this proportion. For mixtures of natural cerebrosides (GalCer, phrenosine, kerasine, GlcCer) with DPPC, in systems containing above 99% water, the behavior is different and no indication of phase separated pure cerebroside domains can be found. The greater heterogeneity of the hydrocarbon portion in the natural cerebrosides might be responsible for a greater miscibility at high proportions of glycosphingolipid.

For the systems GalCer-DPPC, phrenosine-DPPC, kerasine-DPPC and GlcCer-DPPC the shapes of the phase diagrams are not far from the ideal shapes at mole fractions of glycosphingolipid above about 0.5. This indicates a fairly good intermolecular compatibility in the mixed lattice at these proportions; the compatibility is greater for the system LacCer-DPPC whose behavior is almost ideal at all proportions except below a mole fraction of LacCer of 0.1. An increase of the size of the oligosaccharide chain of the glycosphingolipid to two (LacCer) or four (Gg_4Cer) neutral carbohydrates or the presence of a negative charge (Sulf) appears to have the effect of facilitating the miscibility of the GSL with DPPC (see Fig. 4). However, for all these systems, liquid and gel phases tend to coexist over a broader range of temperature and composition than in the ideal case. GlcCer appears to partition preferentially into the liquid-phase of DPPC while DPPC seems to be distributed preferably into the gel-phase of the glycosphingolipid. This can be inferred from the asymmetry of the curves of excess heat capacity vs. temperature (Fig. 1a) and from the displacement of the curve representing the variation of T_m with the composition toward the solidus or liquidus curves (Fig. 3). For the system GalCer-DPPC (Fig. 3) and phrenosine-DPPC (not shown), LacCer-DPPC, Gg_4Cer-DPPC and Sulf-DPPC (Fig. 4) the molecules of phospholipid and glycosphingolipid appear to be equally partitioned between the liquid and gel-phases; the system kerasine-DPPC (Fig. 3) deviates toward a preferential partition of each of the two lipids into the gel-phase of the other.

ΔH_{cal} values of GlcCer-DPPC and GalCer-DPPC decrease sharply with very small increases of the mole fraction of DPPC; the decrease caused by similar small increases in the proportion of monohexosylceramide is more gradual. This suggests that the long range intermolecular interactions between glycosphingolipid molecules are perturbed considerably by the presence of small amounts of phospholipid into the mixed lattice but the system can remain monophasic. The more gradual changes of the thermodynamic parameters on the DPPC-rich side of the phase diagram with the establishment of phase separation reflects the exclusion of DPPC molecules occurring progressively as its proportion exceeds a certain limit in the mixed lattice. Conformational studies of mixed cerebroside-DMPC systems [25] indicated a greater formation of *gauche* conformers of kerasin when mixed with DMPC together with an expansion of the area occupied by the molecule resulting in a decreased lateral packing density. The latter result is in agreement with studies in lipid monolayers that show an expansion in the area per molecule of a mixed cerebroside-DPPC system [6].

Systems containing gangliosides

The behavior of the systems containing gangliosides is rather different from that observed for those containing neutral glycosphingolipid or Sulf. At very low proportions of ganglioside, (i.e., below a mole fraction of 0.06) it is difficult to eliminate the possibility of some gel-phase immiscibility. Above this proportion the different gangliosides appear to be miscible with DPPC. The main feature in these mixtures is the establishment of a new phase transition. As the proportion of ganglioside increases in the mixture the curves of excess heat capacity vs. temperature become broad and the corresponding transition occurs with very low cooperativity. This causes uncertainties in the estimation of the onset and completion temperatures for the transition and makes it difficult to draw conclusions about the occurrence of isothermal melting and immiscibility. As an example of this problem, it is possible that some gel-phase and liquid-phase immiscibility between mixed ganglio-

side-DPPC domains can exist for the system G_{M3}-DPPC at about 42°C and near 50°C, respectively, for mole fractions of G_{M3} between about 0.4 and 0.7 (Fig. 5a). For the other ganglioside-DPPC systems a similar uncertainty may apply to some of the regions of their corresponding phase diagrams. In any case, however, no isothermal region corresponding to the melting of a pure component is present and no evidence for the existence of phase-separated pure ganglioside domains can be found. Contrary to the results obtained with G_{M1}-DPPC, Bunow and Bunow [15] reported that a region of isothermal gel-phase melting is present in the phase diagram for the system G_{M1}/1-stearoyl-2-oleoylphosphatidylcholine at mole fractions of ganglioside above 0.32. They suggested that this phase separation represents a demixing process with the formation of a micellar aggregate of pure G_{M1} molecules. A scanning calorimetric study of dilute aqueous dispersions of unilamellar DPPC vesicles containing G_{D1a} or G_{T1b} up to 10 mol% incorporated only in the outer membrane surface was recently reported by Myers et al. [14]. These authors found that the presence of G_{D1a} induced a small increase in the T_m of the main transition of DPPC with a broadening of the excess heat capacity curve, a decrease of the CU and practically no change in ΔH_{cal} up to a mole fraction of ganglioside of 0.1. For G_{T1b}, a shoulder at a temperature of about 42.2°C was found for mole fractions of G_{T1b} above 0.06 and up to 0.1. However, this study was not carried out on mole fractions of ganglioside above 0.1. Our findings at low proportions of ganglioside (up to 20 mol% of G_{D1a} and 17 mol% of G_{T1b}) are in agreement with the above data but show that the presence of these gangliosides in nonsonicated multilamellar lipid dispersions induces more marked modifications of the thermodynamic parameters.

It is possible that the two phase transition processes established for the systems containing gangliosides correspond to a phase separation of DPPC-rich and ganglioside-rich mixed domains. However, other profound changes are taking place. The turbidity and the state of aggregation of these systems undergo marked changes at defined proportions for each particular ganglioside. The composition range over which mixed ganglioside-DPPC micellar structures are formed [17,26] coincides with the region in which the complex changes of the phase behavior take place. As previously proposed by Sillerud et al. [17], this type of behavior may be interpreted in terms of the existence of an heterogeneous equilibrium between different types of self-aggregated structures. These may correspond to ordered and disordered bilayer vesicles and micelles whose relative amount in the system varies depending on the temperature and the proportion and type of ganglioside in the mixture. Different types of structures such as vesicles and micelles may coexist in the range of mole fractions of ganglioside of 0.4–0.75 for the system G_{M3}-DPPC (Figs. 5, a, b), of 0.3–0.62 for G_{M1}-DPPC (not shown, see Sillerud et al. [17]), of 0.2–0.55 for G_{D1a}-DPPC (Figs. 5, c, d) and of 0.13–0.35 for G_{T1b}-DPPC (Figs. 5, e, f).

Correlations with studies in lipid monolayers and natural membranes

The results described can be related to previous experiments performed with lipid monolayers and to studies employing natural membrane preparations (for reviews, see Refs. 2, 27, 28). Previous monolayer studies indicated that phase-separated domains of pure ganglioside species in oriented lipid interfaces are unlikely [2,6]. This is probably because of steric hindrances, hydration and electrostatic repulsion forces present in the polar head group of the more complex glycosphingolipids and gangliosides that interfere with the thermodynamic and packing requirements for attaining stable interactions [2,6,9]. A recent systematic study of the thermotropic behavior of a series of glycosphingolipids in dilute aqueous dispersions also indicates that the interactions in the aggregate are weakened as the oligosaccharide chain present in the polar head group becomes more complex [3].

The interaction of different glycosphingolipids with DPPC in lipid monolayers and the intermolecular organization of these mixed interfaces depends on the type of oligosaccharide chain of the glycosphingolipid but is fairly independent of its hydrocarbon portion [6,9]. Our previous [3] and present calorimetric studies also indicate that the thermotropic behavior of the different mixtures is primarily influenced by the type of polar head group of the glycosphingolipids. Mixtures of DPPC

with glycosphingolipids containing a similar hydrocarbon portion [29,30] but with different components in their polar head groups (such as GalCer and Sulf; kerasine and GlcCer; LacCer, Gg_4Cer and G_{M1}) lead to differences in the phase behavior. Conversely, the presence of hydroxylated fatty acyl residues or hydrocarbon residues of different chain length in GalCer and GlcCer (or phrenosine compared to kerasine) [29] does not introduce gross qualitative differences in the phase diagrams of their mixtures with DPPC.

The mixtures of DPPC with G_{D1a} or G_{T1b} are the only ones that exhibit an increase of the T_m of the pretransition of DPPC. The structural changes associated with this process are still not completely understood. However, these are probably related to modifications of the polar head group arrangement, rotations along the molecular axis, and must derive from molecular changes other than a mere change in tilt angle [31]. The increase of the T_m of the pretransition induced by polysialogangliosides, in contrast to the decrease induced by monosialogangliosides and neutral glycosphingolipids, may be related to an increased stabilization provided by more favorable dipole-dipole interactions among fundamental components of their polar head groups [2,6,9]. In this regard, monolayer studies indicated that an optimal matching and complementarity exists between the apparent fundamental dipole moment vectors of the polar head group of polysialogangliosides and DPPC that leads to a thermodynamically more favorable interaction [6,9]. One the other hand, the orientation of the fundamental dipoles in neutral glycosphingolipids and monosialogangliosides are not favorable for interacting with DPPC [2,6]. This correlation with the thermotropic behavior can only apply to the region of composition corresponding to low proportions of gangliosides. At higher proportions the possible establishment of heterogeneous equilibria with the formation of structures with a different organization such as micelles is obviously complicating the phase behavior and this effect probably overcomes any influence that the more subtle changes of intermolecular interactions may induce.

Some of the molecular properties and interactions of these glycosphingolipids are probably related to changes of permeability and stability of natural membrane preparations that occur during membrane fusion [2,28,32,33] and during the release and uptake of neurotransmitters in nerve endings [5,28] whose membrane is particularly enriched in some of the complex gangliosides [34,35]. Important alterations of the content of glycosphingolipids have been reported during demyelinating diseases [27,36,37] and various sphingolipidoses [38]. Changes of the relative proportions of different glycosphingolipids and DPPC induce non-ideal changes of the interfacial stability [6–8] and the permeability of reconstituted model membranes containing phosphatidylcholine, cholesterol and myelin basic protein depends on the relative proportion of anionic glycosphingolipid in the system [27,41]. A variation of these proportions in the membrane could lead to altered membrane structures such as the disruption of neural membranes and the subsequent formation of abnormal 'membraneous cytoplasmic bodies' found in gangliosidoses [39]. An increased permeability, altered response to pharmacological agents, variations of lipid-protein interactions, decreased stability and, in some cases, membrane fusion events may also be expected [2,6–8,27,28,33]. The thermotropic behavior of the different GSL-DPPC systems reported in this work reveals that, in certain regions of the phase diagram, relatively small changes of the lipid composition can modify appreciably the proportion of coexisting gel and liquid phases. The coexistence of these phases extends over a broader range than that expected for an ideal system; this is more apparent for mixtures containing the more complex glycosphingolipids than for those with the more simple monohexosylceramides and it is the former lipids that have a greater effect on the membrane stability [5,28,33]. In addition, the formation of immiscible DPPC domains may be expected if the relative proportions of glycosphingolipids and phospholipid are varied. Due to the presence of lateral interfaces, packing defects and surface density fluctuations [40,41], the coexistence of different domains in different phase states and intermolecular organization, depending on the lipid composition, can be of extreme importance for the structural stability, permeability and for the activity of membrane-bound enzymes in glycosphingolipid-containing membranes [2,6,9,14,42,43].

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References

1 Maggio, B., Cumar, F.A. and Caputto, R. (1978) Biochem. J. 171, 559–565
2 Maggio, B., Cumar, F.A. and Caputto, R. (1981) Biochim. Biophys. Acta 650, 69–87
3 Maggio, B., Ariga, T., Sturtevant, J.M. and Yu, R.K. (1985) Biochemistry 24, 1084–1092
4 Maggio, B., Mestrallet, M.G., Cumar, F.A. and Caputto, R. (1977) Biochem. Biophys. Res. Commun. 77, 1265–1272
5 Cumar, F.A., Maggio, B. and Caputto, R. (1980) Biochim. Biophys. Acta 597, 174–182
6 Maggio, B., Cumar, F.A. and Caputto, R. (1978) Biochem. J. 175, 1113–1118
7 Fidelio, G.D., Maggio, B., Cumar, F.A. and Caputto, R. (1982) Biochem. J. 203, 717–725
8 Fidelio, G.D., Maggio, B. and Cumar, F.A. (1984) Chem. Phys. Lipids 35, 231–245
9 Maggio, B., Cumar, F.A. and Caputto, R. (1980) Biochem. J. 189, 435–440
10 Probst, W., Mobius, D. and Rahmann, H. (1984) Cell. Mol. Neurobiol. 4, 157–176
11 Fishman, P.H. and Brady, R.O. (1976) Science 906–915
12 Cumar, F.A., Maggio, B. and Caputto, R. (1982) Mol. Cell. Biochem. 46, 155–159
13 Mestrallet, M.G., Bennun, F., Maggio, B. and Cumar, F.A. (1984) J. Neurosci. Res. 12, 335–341
14 Myers, M., Wortman, C. and Freire, E. (1984) Biochemistry 23, 1442–1448
15 Bunow, M.R. and Bunow, B. (1979) Biophys. J. 27, 325–337
16 Correa-Freire, M.C., Freire, E., Barenholz, Y., Biltonen, R.L. and Thompson, T.E. (1979) Biochemistry 18, 442–445
17 Sillerud, L.O., Schafer, D.E., Yu, R.K. and Konigsberg, W. (1979) J. Biol. Chem. 254, 10876–10880
18 Bach, D., Miller, I.R. and Sela, B. (1982) Biochim. Biophys. Acta 686, 233–239
19 Ruocco, M.J., Shipley, G.G. and Oldfield, E. (1983) Biophys. J. 43, 91–101
20 Watt, G.D. and Sturtevant, J.M. (1969) Biochemistry 8, 4567–4571
21 Mabrey, S. and Sturtevant, J.M. (1976) Proc. Natl. Acad. Sci. USA 73. 3862–3866
22 Mabrey, S. and Sturtevant, J.M. (1978) Methods Membrane Biol. 9, 237–274
23 Lee, A.G. (1976) FEBS Lett. 359–363
24 Mabrey, S., Mateo, P.L. and Sturtevant, J.M. (1978) Biochemistry 17, 2464–2468
25 Bunow, M.R. and Levin, I.W. (1980) Biophys. J. 32, 1007–1022
26 Bertoli, E., Masserino, M., Sonnino, S., Ghidoni, R., Cestaro, B. and Tettamanti, G. (1981) Biochim. Biophys. Acta 467, 196–202
27 Maggio, B., Cumar, F.A., Roth, G.A., Monferran, C.G. and Fidelio, G.D. (1983) Acta Neuropathiol. Suppl. 9, 71–85
28 Cumar, F.A., Maggio, B. and Caputto, R. (1983) in Neural Transmission, Learning and Memory (Caputto, R. and Marsan, C.A., eds.) IBRO Monograph Series, Vol. 10, pp. 35–47, Raven Press, New York
29 Murata, T., Ariga, T., Oshima, M. and Miyatake, T. (1978) J. Lipid Res. 19, 370–374
30 Ando, S. and Yu, R.K. (1984) J. Neurosci. Res. 12, 205–212
31 Chowdhry, B.Z., Lipka, G., Dalziel, A.W. and Sturtevant, J.M. (1984) Biophys. J. 45, 901–904
32 Maggio, B., Cumar, F.A. and Caputto, R. (1978) FEBS Lett. 90, 149–152
33 Monferran, C.G., Maggio, B., Roth, G.A., Cumar, F.A. and Caputto, R. (1979) Biochim. Biophys. Acta 553, 417–423
34 Ledeen, R.W. (1983) in Handbook of Neurochemistry (Lajtha, A., ed.), Vol. 4, 2nd Edn., pp. 41–90, Plenum Press, New York
35 Ledeen, R.W. and Yu, R.K. (1982) Methods Enzymol. 83, 139–191
36 Cumar, F.A., Maggio, B. and Roth, G.A. (1977) Adv. Exptl. Med. Biol. 83, 505–511
37 Yu, R.K., Ueno, K., Glaser, G.H. and Tourtellotte, W.W. (1982) J. Neurochem. 39, 454–477
38 Brady, R.O. (1982) Annu. Rev. Neurosci. 5, 33–56
39 Terry, R.D. and Weiss, M. (1963) J. Neuropath. Exp. Neurol. 22, 18–55
40 Lee, A.G. (1977) Biochim. Biophys. Acta 472, 285–344
41 Phillips, M.C., Graham, D.E. and Hauser, H. (1975) Nature 254, 154–155
42 Sandermann, H., Jr. (1978) Biochim. Biophys. Acta 515, 209–237
43 Van Deenen, L.L.M., Demel, R.A., Geurts van Kessel, W.S.M., Kamp, H.H., Roelofsen, B., Verkleij, A.J., Wirtz, K.W.A. and Zwaal, R.F.A. (1976) in The Structural Basis of Membrane Function (Hatefi, Y. and Djavadi-Ohmaniance, L., eds.), pp. 21–38, Academic Press, New York