Thermotropic Behavior of Glycosphingolipids in Aqueous Dispersions†

Bruno Maggio,‡ Toshio Ariga,‡ Julian M. Sturtevant,§ and Robert K. Yu*,‡

Department of Neurology, Yale University School of Medicine, and Department of Chemistry, Yale University, New Haven, Connecticut 06510

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ABSTRACT: The thermotropic behavior of 20 chemically related glycosphingolipids (GSLs) of high purity, containing neutral and anionic carbohydrate residues in their oligosaccharide chains, was studied by high-sensitivity differential scanning calorimetry. In general, the polar head group of GSLs appears to be one of the major determinants of their phase behavior. Compared to phospholipids, the presence of the carbohydrate rather than the phosphorylcholine moiety in the polar head group and a sphingosine base in the hydrocarbon portion of GSLs reduces the effect on the transition temperature (T_m) brought about by increasing the number of methylene groups in the amide-linked fatty acyl chains. For simple neutral GSLs, the T_m's were 20–40 °C higher than those of phospholipids with comparable hydrocarbon chains. As the oligosaccharide chain of GSLs becomes more complex, the excess heat capacity, T_m, enthalpy (ΔH_{cal}), and entropy of the transition decrease proportionally to the number of carbohydrate residues present in the polar head group. The T_m and ΔH_{cal} for anionic GSLs were 16–25 °C and 1–3 kcal mol^{-1} lower than those of neutral GSLs with comparable oligosaccharide chains. A linear dependence of ΔH_{cal} with T_m was found. However, the slopes of these plots were different for neutral and for anionic GSLs, suggesting different types of intermolecular organizations for the two. The T_m and ΔH_{cal} were linearly dependent on the molecular area of both neutral and anionic GSLs; this indicated that the influence of the complexity of the polar head group in GSLs for establishing the thermodynamic behavior may be mediated by the intermolecular spacings.

Glycosphingolipids (GSLs)[1] are important constituents of plasma membranes and are particularly abundant in the nervous system (Fishman & Brady, 1976; Hakomori, 1981; Ledeen & Yu, 1982; Ledeen, 1983; Ando, 1983). In myelin, glycolipids such as galactocerebrosides and sulfatides account for 30% of total lipids (Norton, 1977). The nervous system membranes are also unique in having an abundance of gangliosides which are sialic acid containing GSLs (Hakomori, 1981; Ledeen & Yu, 1982; Ledeen, 1983; Ando, 1983). In certain inborn errors of metabolism, the amount of GSLs is abnormally increased (Brady, 1982) whereas in some neurological disorders, such as human and experimental demyelinating diseases, the GSLs can be selectively altered in quality or quantity (Maggio et al., 1972, 1983; Yu et al., 1974, 1982). These alterations in the composition of GSLs invariably lead to an unstable membrane structure. In addition, GSLs have been postulated to play an important role in membrane function, cell–cell interaction, and cell transformation (Cumar et al., 1970; Fishman & Brady, 1976; Hakomori, 1981). They have also been postulated to serve as receptors for toxins, drugs, and natural agonists (Fishman & Brady, 1976; Hakomori, 1981; Ledeen, 1983; Ando, 1983). However, any understanding in molecular terms of the effects of these lipids on the structure and function of cell membranes remains obscure. This is even more so if they are considered as part of multimolecular dynamic ensembles in which the individual molecular properties are no longer exhibited as these properties become modified by intermolecular interactions with other lipids and proteins (Maggio et al., 1981).

Systematic studies of the individual interfacial properties and interactions that several chemically related GSLs can establish with natural and synthetic phospholipids and with proteins at the air–water interface have been previously published (Maggio et al., 1978a,b; Fidelio et al., 1982). These studies have provided a better understanding in molecular terms of the ability of some GSLs to induce changes in membrane permeability, stability, and fusion (Maggio et al., 1977, 1978c, 1983; Monferran et al., 1979) and in neurotransmitter movements in nerve endings (Cumar et al., 1980), as well as information on their possible intermolecular organization in complex lipid–protein interfaces (Maggio et al., 1978b, 1980; Fidelio et al., 1982). These findings have clearly indicated that an important element of the surface behavior of GSLs rests on the type of oligosaccharide chain present in their polar head group (Maggio et al., 1980, 1981; Fidelio et al., 1982).

Reports on the thermal behavior of a limited number of GSLs have been published during the last few years (Sillerud et al., 1979; Bunow, 1979; Freire et al., 1980; Ruocco et al., 1981; Bach et al., 1982a,b; Curatolo, 1982). Most of the studies in this field have dealt with a single or a few isolated compounds from different sources, including commercial samples and mixtures of variable purity, and were performed in different laboratories with various equipment, lipid concentrations, degrees of hydration, and scan rates. All these variations make comparisons of the behavior of different types of GSLs very difficult, and an understanding of the general factors influencing their thermotropic behavior is not possible.

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‡ Department of Neurology.

§ Department of Chemistry.

[1] Abbreviations: GSLs, glycosphingolipids; 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_3Cer, GalNAc(β1–4)Gal(β1–4′)Glc(β1–1)Cer; Gg_4Cer, Gal(β1–3)GalNAc(β1–4)Gal(β1–4)Glc(β1–1′)Cer; Gb_4Cer, GalNAc(β1–3)Gal(α1–4)Gal(β1–4)Glc(β1–1′)Cer; $NeuGcG_{M3}$, NeuGc(α2–3)Gal(β1–4)Glc(β1–1′)Cer; $NeuAcG_{M3}$, NeuAc(α2–3)Gal(β1–4)Glc(β1–1′)Cer; G_{M2}, GalNAc(β1–4)Gal[3–2αNeuAc](α1–4)Glc(β1–1′)Cer; G_{M1}, Gal(β1–3)GalNAc(β1–4)Gal[3–2αNeuAc](α1–4)Glc(β1–1′)Cer; G_{D1a}, NeuAc(α2–3)Gal(β1–3)GalNAc(β1–4)Gal[3–2αNeuAc](α1–4)Glc(β1–1′)Cer; G_{T1b}, NeuAc(α2–3)Gal(β1–3)GalNAc(β1–4)Gal[3–2αNeuAc8–2αNeuAc](β1–4)Glc(β1–1′)Cer; DSC, differential scanning calorimetry.

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In the present work, we have studied systematically and comparatively, by high-sensitivity differential scanning calorimetry, the thermotropic behavior of *dilute* aqueous dispersions of 20 chemically related GSLs of high purity. Several of the compounds differed by only a single type of carbohydrate residue in the oligosaccharide chain of their polar head groups. This study thus provides information on possible influences of the complexity of the polar head group in these lipids on the various thermodynamic parameters involved in their phase transition. Several factors such as differences in the fatty acyl residues and sphingosine base composition, especially in natural samples, as well as the possibilities for adoption of different states of organization for the different GSLs are undoubtedly important to establishing the phase behavior apart from the type of polar head group. Nevertheless, a comparative study of this type provides the general trend regarding the influence of the oligosaccharide chain. In addition, the thermodynamic parameters associated with the phase transition process of several GSLs are reported for the first time. An understanding of these parameters is a prerequisite to the eventual appreciation of the overall organization of GSLs in biological membranes.

Materials and Methods

Materials. Synthetic glycosphingolipids, including *N*-stearoyldihydrogalactosylsphingosine, *N*-stearoyldihydroglucosylsphingosine, *N*-lignoceroyldihydrogalactosylsphingosine, *N*-lignoceroyldihydrolactosylsphingosine, and *N*-stearoyldihydrolactosylsphingosine, were purchased from Calbiochem. Bovine brain GalCer, bovine brain sulfatide, and Gaucher spleen GlcCer were purchased from Analab, North Haven, CT. Gangliosides G_{M2}, G_{M1}, G_{D1a}, and G_{T1b} were isolated and purified from bovine brain by DEAE-Sephadex and Iatrobeads column chromatography as described previously (Ando & Yu, 1977; Ledeen & Yu, 1982). *N*-Acetyl- and *N*-glycolylneuraminic acid containing G_{M3} gangliosides were isolated and purified from adrenal medulla by using a similar procedure (Ariga et al., 1982). Globoside (Gb_4Cer) was isolated from pig erythrocytes (Miyatake et al., 1968). Gangliotetraosylceramide (Gg_4Cer or asialo-G_{M1}) was prepared from a bovine brain ganglioside mixture by mild formic acid desialylation followed by purification on an Iatrobeads column (Kasai et al., 1982). Gangliotriaosylceramide (Gg_3Cer or Asialo-G_{M2}) was obtained from human brain G_{M2} ganglioside by a similar desialylation and purification procedure (Kasai et al., 1982). LacCer was isolated from bovine adrenal medulla as described previously (Ariga et al., 1980). Phrenosin and kerasin were prepared from a human brain GalCer fraction. The separation was achieved by Iatrobeads column (58 × 1 cm) with continuous gradient elution using chloroform–methanol (97:3 and 70:30).

The purity of all the above glycolipids was at least 95% as judged by high-performance thin-layer chromatography and densitometry (Ando et al., 1978; Macala et al., 1983). All organic solvents were freshly distilled before use. All chemicals were of analytical reagent grade.

Preparation of Lipid Dispersions and Scanning Calorimetry. The proper amount of lipid was weighed or pipetted from chloroform–methanol (2:1) solutions into empty glass vials. 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 these lipids were prepared in 0.05 M phosphate buffer, pH 7.0. This buffer was chosen because of its very low enthalpy of ionization (Watt & Sturtevant, 1969). The amount of buffer necessary to give lipid concentrations of 1–2 mg/mL for neutral GSLs (except globoside) and sulfatide and 4–7 mg/mL for globoside and gangliosides was pipetted into vials containing weighed lipids. The samples were subsequently hydrated by heating to 95 °C in a water bath for 5 min and vortexed twice for 1 min each with an interval of about 2 min during which the sample was returned to 95 °C. The lipid dispersions were cooled to room temperature, degassed under vacuum, and loaded into one cell of a Privalov type (DASM-1M) differential scanning calorimeter. The reference cell was filled with degassed buffer. Visually, the dispersions of GSLs with less than three carbohydrate residues appeared with a degree of turbidity similar to that usually obtained with phospholipids but with a tendency to aggregate and settle with time. As the oligosaccharide chain of the polar head group became more complex or if negatively charged residues were present, the turbidity decreased sharply.

Calorimetric scans were usually performed at a nominal rate of 0.5 °C/min; some of the samples were also scanned at 1 °C/min with similar results. The actual scan rate, power calibration, and heater voltages were monitored for each sample. The ΔH_{cal} for each transition was calculated from the area under the excess heat capacity–temperature curve. Repetitive runs were made after the sample was cooled to a particular temperature in the calorimeter. When the availability of the purified GSLs permitted, replicate runs were also made by preparing new samples. A few samples were rescanned after being kept at 4 °C for 2–7 days. After the calorimetric runs, samples of representative GSLs were analyzed by the recently developed high-sensitivity thin-layer chromatographic procedure (Ando et al., 1978; Macala et al., 1983). No evidence of degradation or contamination was found.

Results and Discussion

In our initial experiments, we found that some of the lipids, particularly the natural or synthetic GalCer and LacCer, exhibited a complex behavior if the lipids were dispersed in buffer at room temperature or by heating only to 60–70 °C. These experiments revealed exotherms before or after the main transition and alterations of ΔH_{cal} (the transition enthalpy), C_p(max) (the maximal excess apparent heat capacity), and the values of T_m (the temperature of maximal excess heat capacity) after cooling and rescanning. Also, apparently random variations of the excess heat capacity after the transition were present which led to uncertain base lines. Under these conditions, the reproducibility of the calorimetric scans was usually poor. These effects are probably due to the complex polymorphic behavior of this type of lipid which can undergo interconversions between stable and metastable forms (Freire et al., 1980; Ruocco et al., 1981; Curatolo, 1982), particularly at partial or intermediate degrees of hydration. Hence, in subsequent experiments, the samples were dispersed at high dilution (1–7 mg/mL; see Materials and Methods) in buffer at 95 °C for 5 min, cooled to room temperature, and loaded into the calorimeter. This procedure led to a more stable and reproducible thermotropic behavior for the GSLs tested, with straight base lines (cf. Figure 1) and without the presence of exotherms. These results probably reflect the attainment of more stable or, at least, long-lived structural organization after the above treatment. Under these conditions, 3–13 different calorimetric scans for at least two samples of each individual lipid gave maximum dispersion values corresponding to SEM's of less than ±10% for ΔH_{cal} and C_p(max) and ±0.3 °C for T_m (see Table I). A characteristic of the calorimetric scans, particularly of neutral glycosphingolipids, was that periodic oscillations in the excess heat capacity of about ±0.05 cal K^{-1} g^{-1} were frequently observed

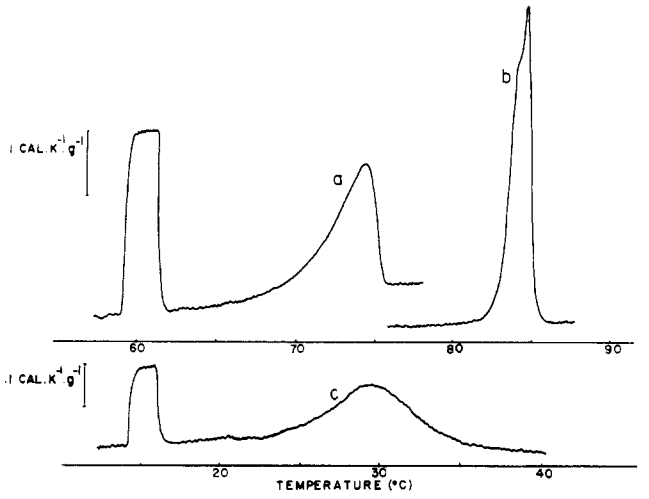

FIGURE 1: Tracings of actual DSC output curves. The samples, scanned at a nominal rate of 0.5 K min^{-1}, were lipid dispersions in 0.05 M phosphate buffer, pH 7.0, of (a) LacCer (2 mg mL^{-1}), (b) *N*-stearoyldihydroglucosylsphingosine (1.8 mg mL^{-1}), and (c) G_{M2} (7 mg mL^{-1}). The rectangular calibration signals were produced by supplying 14.2 mcal of excess energy to the sample cell over a period of 250 s (curve a) or 2.26 mcal over a period of 200 s (curve b).

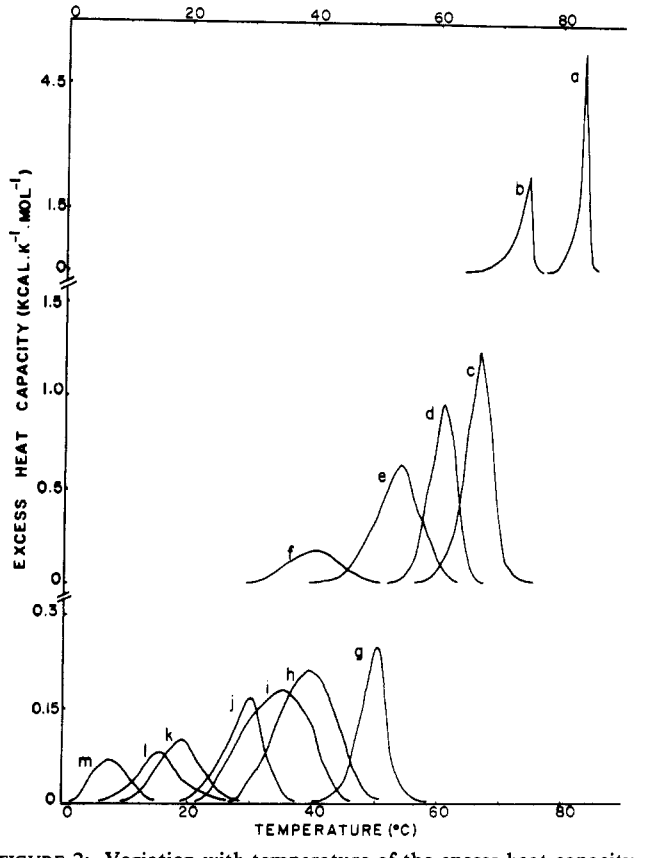

FIGURE 2: Variation with temperature of the excess heat capacity for different glycosphingolipids. (a) GlcCer, 1.87 mg mL^{-1}, scanned from 40 °C; (b) LacCer, 2 mg mL^{-1}, scanned from 30 °C; (c) GalCer, 1.81 mg mL^{-1}, scanned from 30 °C; (d) Gg_3Cer, 0.78 mg mL^{-1}, scanned from 30 °C; (e) Gg_4Cer, 1.7 mg mL^{-1}, scanned from 20 °C; (f) Gb_4Cer, 3.1 mg mL^{-1}, scanned from 10 °C; (g) Sulf, 1.87 mg mL^{-1}, scanned from 20 °C; (h) $NeuGcG_{M3}$, 4.56 mg mL^{-1}, scanned from 10 °C; (i) $NeuAcG_{M3}$, 4.56 mg mL^{-1}, scanned from 10 °C; (j) G_{M2}, 7 mg mL^{-1}, scanned from 10 °C; (k) G_{M1}, 7 mg mL^{-1}, scanned from 1 °C; (l) G_{D1a}, 6 mg mL^{-1}, scanned from 1 °C; (m) G_{T1b}, 6.3 mg mL^{-1}, scanned from 1 °C. The nominal scan rate was 0.5 K min^{-1}.

during the first run but were usually not present on rescanning. This behavior may be due to the tendency of these dispersions to aggregate and settle within the calorimetric cell.

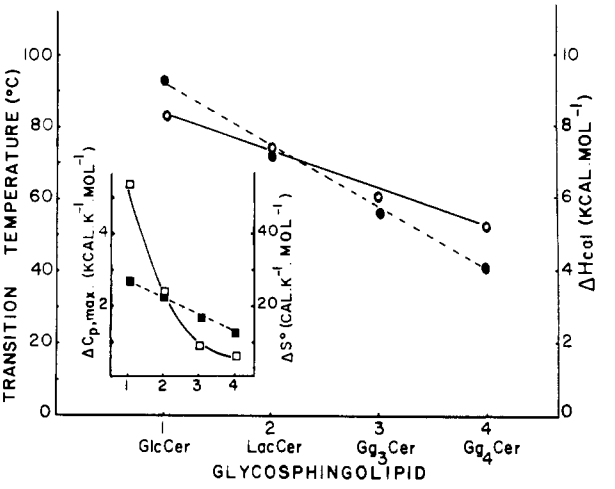

FIGURE 3: Variation of thermodynamic parameters with the complexity of the oligosaccharide chain of neutral glycosphingolipids. The variation of T_m (○), ΔH_{cal} (●), C_p(max) (□, insert), and $\Delta S°$ (■, insert) is shown for neutral GSLs with the number of carbohydrate units in the polar head group indicated on the abscissa. The change of the thermodynamic parameters brought about by an increase of one carbohydrate unit in the polar head group as derived by the slopes of the curves calculated by linear regression analysis of the data is the following: $\Delta T_m = -10.3$ °C; $\Delta\Delta H_{cal} = -1.8$ kcal mol^{-1}; $\Delta\Delta S° = -4.8$ cal K^{-1} mol^{-1}.

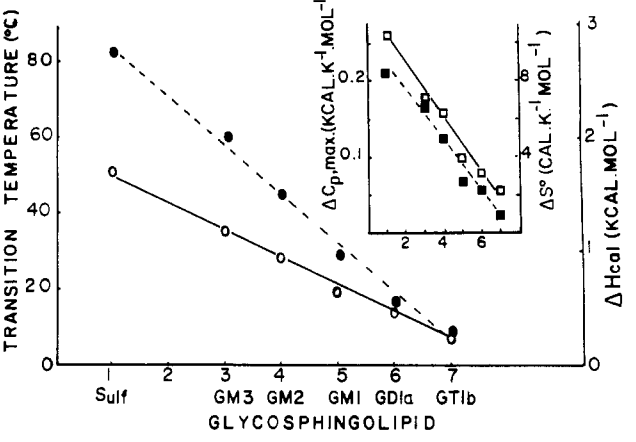

FIGURE 4: Variation of thermodynamic parameters with the complexity of the oligosaccharide chain of anionic glycosphingolipids. The variation of T_m (○), ΔH_{cal} (●), C_p(max) (□, insert), and $\Delta S°$ (■, insert) is shown for anionic GSLs with the number of carbohydrate units in the polar head group indicated on the abscissa. The change of the thermodynamic parameters brought about by an increase of one carbohydrate unit in the polar head group, calculated as in Figure 3, is the following: $\Delta T_m = -7.2$ °C; $\Delta\Delta H_{cal} = -0.5$ kcal mol^{-1}; $\Delta\Delta S° = -1.3$ cal K^{-1} mol^{-1}.

Actual DSC scans for *N*-stearoyldihydroglucosylsphingosine, natural LacCer, and ganglioside G_{M2} are shown in Figure 1. The excess heat capacity as a function of temperature for the different natural and anionic naturally occurring GSLs studied is shown in Figure 2. Table I reports the values for T_m, ΔH_{cal}, C_p(max), and $\Delta T_{1/2}$, the temperature width at half C_p(max), for the phase transitions. Values for $\Delta S°$, the apparent entropy changes of the transitions, calculated as $\Delta H_{cal}/T_m$, are also given for comparative purposes. It is recognized that these values have little or no thermodynamic significance in the cases of the very broad and asymmetric transitions. Since for neutral and anionic glycosphingolipids the state of aggregation is different and gangliosides are not present in lamellar form but in micellar form (Hill & Lester, 1972; Yohe et al., 1976; Sillerud et al., 1979), any comparison regarding the effect of the complexity of the polar head group should therefore be

Table I: Thermodynamic Parameters of the Phase Transitions of Glycosphingolipids

glycosphingolipid	T_m (°C)	ΔH_{cal} (kcal mol^{-1})	ΔC_p(max) (kcal K^{-1} mol^{-1})	$\Delta T_{1/2}$ (°C)	$\Delta S°$ (cal K^{-1} mol^{-1})
		Neutral			
GalCer	66.7 ± 0.2	6.5 ± 0.3	1.30 ± 0.10	4.6 ± 0.1	19.1 ± 1.0
kerasin	67.6 ± 0.1	6.2 ± 0.2	1.10 ± 0.04	3.6 ± 0.1	18.1 ± 0.7
phrenosin	64.9 ± 0.1	5.0 ± 0.4	1.00 ± 0.04	4.2 ± 0.1	14.9 ± 1.0
GlcCer	83.7 ± 0.1	9.5 ± 0.8	5.30 ± 0.50	1.3 ± 0.1	26.6 ± 3.0
LacCer	74.4 ± 0.1	7.2 ± 0.2	2.40 ± 0.03	2.2 ± 0.1	22.8 ± 0.7
Gg_3Cer	60.8 ± 0.2	5.6 ± 0.3	0.97 ± 0.04	5.3 ± 0.2	16.9 ± 0.9
Gg_4Cer	54.0 ± 0.1	4.2 ± 0.2	0.65 ± 0.02	8.5 ± 0.1	12.8 ± 0.8
Gb_4Cer	40.5 ± 0.1	2.0 ± 0.1	0.18 ± 0.01	10.3 ± 0.1	6.4 ± 0.4
N-stearoyldihydrogalactosylsphingosine	83.7 ± 0.1	4.7 ± 0.2	1.50 ± 0.10	2.6 ± 0.1	13.2 ± 0.8
N-stearoyldihydrolactosylsphingosine	74.2 ± 0.1	5.3 ± 0.7	2.80 ± 0.40	1.4 ± 0.1	15.4 ± 2.4
N-lignoceroyldihydrogalactosylsphingosine	84.5 ± 0.1	7.3 ± 0.5	2.90 ± 0.10	2.6 ± 0.1	20.5 ± 1.7
N-lignoceroyldihydrolactosylsphingosine	76.6 ± 0.1	8.3 ± 0.1	5.70 ± 0.01	1.2 ± 0.1	23.9 ± 0.1
N-stearoyldihydroglucosylsphingosine	84.9 ± 0.1	6.9 ± 0.1	4.10 ± 0.10	1.3 ± 0.1	19.2 ± 0.2
		Anionic			
Sulf	50.2 ± 0.1	2.8 ± 0.2	0.30 ± 0.04	4.8 ± 0.1	8.5 ± 0.9
$NeuGcG_{M3}$	39.4 ± 0.1	2.4 ± 0.2	0.21 ± 0.02	11.5 ± 0.1	7.8 ± 0.1
$NeuAcG_{M3}$	35.3 ± 0.3	2.5 ± 0.2	0.18 ± 0.01	13.5 ± 0.2	8.2 ± 0.6
G_{M2}	29.3 ± 0.1	1.5 ± 0.1	0.20 ± 0.01	6.5 ± 0.1	5.0 ± 0.1
G_{M1}	19.3 ± 0.1	0.8 ± 0.1	0.10 ± 0.01	8.0 ± 0.1	2.9 ± 0.2
G_{D1a}	15.2 ± 0.3	0.6 ± 0.1	0.08 ± 0.01	8.0 ± 0.2	2.2 ± 0.2
G_{T1b}	7.3 ± 0.1	0.3 ± 0.1	0.06 ± 0.01	8.0 ± 0.2	1.1 ± 0.2

Table II: Relevant Previously Published Values for Thermodynamic Parameters of the Phase Transitions of Some Glycosphingolipids and Phospholipids

lipid	T_m (°C)	ΔH_{cal} (kcal mol^{-1})	$\Delta S°$ (cal K^{-1} mol^{-1})	ref
dipalmitoylphosphatidylcholine	41.4	8.7	27.8	*b*
distearoylphosphatidylcholine	54.9	10.6	32.4	*b*
dibehenoylphosphatidylcholine	75.0	14.9	42.8	*c*
N-palmitoyldihydrosphingosinylphosphatidylcholine	47.8	9.4	29.3[a]	*d*
N-palmitoylsphingosinylphosphatidylcholine	52.8	17.9	54.9[a]	*d*
N-lignoceroylsphingosinylphosphatidylcholine	48.6	15.3	47.6[a]	*d*
N-palmitoylglucosylerythrosphingosine	87.5	17.1	47.4[a]	*e*
N-palmitoylgalactosylsphingosine	82.0	17.5	48.5[a]	*f*
GlcCer	83.0	13.6	38.2[a]	*e*
GalCer	66.1	6.7	19.8[a]	*g*
	67	6.9	20.9[a]	*h*
kerasin	71.8	15.8	45.8[a]	*g*
	72	16.2	47.0[a]	*h*
phrenosin	66.5	7.7	22.7[a]	*g*
	68	7.3	22.1[a]	*h*
G_{M1}	26–43			*i*
G_{D1a}	12–40			*i*

[a] Values calculated from the data for ΔH_{cal} and T_m, not provided by the original paper. [b] Mabrey & Sturtevant (1976). [c] Phillips et al. (1969). [d] Barenholz et al. (1976). [e] Freire et al. (1980). [f] Ruocco et al. (1981). [g] Bunow (1979). [h] Curatolo (1982). [i] Bach et al. (1982a,b).

made as presented in Figures 3 and 4 for the series of neutral or anionic glycosphingolipids. Even with this consideration, the specific type of structural organization may be different for each glycosphingolipid in these two groups, and very little information exists in this regard. The simpler monohexosylceramides form bilayers while sulfatide can form bilayers and cubic and micellar phases (Abrahamsson et al., 1972; Ruocco et al., 1981). On the other hand, gangliosides form only micellar structures, but even these do not correspond to an identical organization; the shape and eccentricities of ganglioside micelles depend on the particular constitution of the polar head group and hydrophobic portion in each type of ganglioside (Yohe et al., 1976). Therefore, the variation of the type of oligosaccharide chain and any modification of the hydrophobic portion occurring concomitantly to this must be considered as a multiple influence that will undoubtedly affect the state of organization, the hydration, and the thermodynamic parameters for the phase transition process. The lack of information defining in a precise manner the structural organization of the different GSLs below and above their respective transition is an important limitation to interpreting their thermotropic behavior. Obviously, a complete description of the influence of the carbohydrate and hydrocarbon portions of GSLs on their phase behavior must wait till synthetic compounds become available for a chemically related series of GSLs of different and defined complexities in their hydrocarbon and oligosaccharide moieties.

Published values for a few GSLs and some phospholipids are given in Table II for comparison. Our data regarding the T_m's agree well with those reported for some galactosyl- and glucosylsphingosine derivatives containing comparable hydrocarbon chains. Very high values for ΔH_{cal} were, however, reported previously for *N*-palmitoylglucosylsphingosine, *N*-palmitoylgalactosylsphingosine, and kerasin. It was reported that some of these lipids exhibited a metastable behavior (Freire et al., 1980; Ruocco et al., 1981; Curatolo, 1982). The studies were performed on rather concentrated aqueous dispersions in which the amount of lipid was 14% or higher. Under these conditions, it was reported that some glycosphingolipids such as *N*-palmitoylglucosylerythrosphingosine and glycosylceramide from Gaucher spleen (Freire et al., 1980), *N*-palmitoylgalactosylsphingosine (Ruocco et al., 1981), and kerasin and phrenosin (Curatolo, 1982) exhibited complex interconversions between metastable phases. The presence of

various metastable phases and their interconversion depend upon the manner the sample is analyzed, e.g., the rate of heating and cooling. Also, the rate of transition between metastable phases is variable so that the presence or absence of a particular phase depends on the period of time during which the sample is kept at a particular temperature. For these reasons, when metastable behavior is present, it is difficult to compare the thermotropic behavior of different glycosphingolipids. In our experiments, the concentration of lipid never exceeded 1%, and we found that all lipids except the synthetic *N*-lignoceroyl derivatives (see below) were in a stable state at least for a prolonged duration. No exotherms or metastable transitions were found under successive rescanning after cooling the sample at a controlled rate (1 °C/min) in the calorimeter or after taking the sample out of the calorimetric cell and cooling at room temperature before reloading the sample into the cell. In addition, several representative glycospingolipids (GalCer, kerasin, Gg_4Cer, G_{M3}, and G_{Dla}) were scanned after being kept at 4 °C for varying periods between 2 and 7 days, and a reproducible thermotropic behavior was always found. The more stable behavior might conceivably be due to the high dilution of the sample and to the heating step at 95 °C before scanning that may provide better conditions for hydration and/or dispersion of the GSLs (see Materials and Methods).

The T_m's for kerasin and phrenosin were about 4 and 2 °C lower, respectively, than those reported in the literature (Bunow, 1979; Curatolo, 1982), and the values of ΔH_{cal} for kerasin, phrenosin, and GlcCer were also lower in our samples compared to the data reported by the above authors for more concentrated samples. We have no explanation for these discrepancies.

However, it was observed that when the heating step at 95 °C was omitted and a metastable behavior was present, the values of ΔH_{cal} and T_m were higher. One possibility is that differences in the initial phase state or hydration of these lipids may account for the differences in behavior; the degree of dilution of the sample, on the other hand, can certainly affect the possibility for the existence of interconversions between different phase states. For phospholipids, it is well-known that an increase in the proportion of water in the system causes a decrease in the T_m and ΔH_{cal} (Phillips, 1971). The thermodynamic parameters for glycosphingolipids appear to be linearly dependent on and extremely sensitive to small variations of the intermediate spacings (see below). Therefore, differences in the concentration, hydration, or prior treatment of the samples, or the presence of small amounts of impurities may easily lead to apparent discrepancies of the absolute values reported in different laboratories.

CPK space-filling molecular models and limiting molecular area determinations in lipid monolayers (Maggio et al., 1978, 1980) indicate that glucosyl- and galactosylceramides have molecular areas and polar head group sizes similar to those of phosphatidylcholines and sphingomyelins. However, the values of T_m for these GSLs are much higher while the values for ΔH_{cal} are, in general, lower than those of phospholipids with comparable hydrocarbon chains. Apart from the influence of the carbohydrate itself, an increase in the number of methylene groups in the fatty acid will lead to enhanced differences in the length of the two hydrocarbon chains of these molecules. For sphingomyelins, it has been shown that while increasing the *N*-acyl chain from 16 to 18 methylene groups increases the T_m by about 11 °C, the *N*-lignoceroyl derivative, with 24 carbons, exhibits a T_m value about 4 °C lower than that of *N*-stearoylsphingosine (Barenholz et al., 1976). This behavior was ascribed to a probable manifestation of the differences in chain length between the *N*-acyl and sphingosine chains. Natural GSLs contain different fatty acyl residues, and this may affect their phase behavior apart from the type of oligosaccharide chain. However, the fatty acid and long-chain base heterogeneity is relatively small compared to the differences in the polar head group (Yohe et al., 1976; Murata et al., 1978; Ando & Yu, 1984; see also Materials and Methods). Moreover, the composition of the hydrophobic portion is the same in the case of the synthetic GSLs or for some of the neutral GSLs, such as Gg_3Cer and Gg_4Cer, that are obtained by desialylation of a parent ganglioside (see Materials and Methods). The presence of hydroxy fatty acids can influence the T_m and enthalpy of the transition as shown by comparing phrenosin to kerasin [see Table I and Bunow (1979) and Curatolo (1982)]. However, hydroxy fatty acyl residues are not present in GlcCer or in the gangliosides studied here and, as a consequence of their being derived from parent gangliosides, also not in Gg_3Cer or Gg_4Cer. In addition, the data in Table I for synthetic *N*-stearoyl- and *N*-lignoceroylsphingosines linked to the same carbohydrate moiety in the polar head group show that an increase of six methylene groups in the amide-linked fatty acyl chain produces an increase in T_m of only 0.8 and 2.4 °C for the galactosyl and lactosyl derivatives, respectively. By contrast, a decrease of 9.5 and 7.9 °C occurs when the polar head group is a lactosyl residue compared to a galactosyl or glucosyl group with the same hydrocarbon portion. On the other hand, the values of ΔH_{cal} and $\Delta S°$ for *N*-stearoyl and *N*-lignoceroyl derivatives display increases of between 0.44 and 0.50 kcal mol^{-1} and 1.2 and 1.4 cal K^{-1} mol^{-1}, respectively, per methylene group of the fatty acyl chain. These values are very similar to those reported for phosphatidylcholines (Phillips et al., 1969). It seems from all these considerations that the influence of a different chain length of the fatty acyl residue is relatively small compared to that of a different complexity of the polar head group. Comparison of differences in the values of the thermodynamic parameters as a function of the type of oligosaccharide chain can therefore be made. It should, however, be recalled that the different complexity of the oligosaccharide chain also introduces marked differences in the state of aggregation and the thermodynamic parameters will reflect the combination of several influences.

Figures 3 and 4 show that the values of the different thermodynamic parameters decrease linearly as the complexity of the oligosaccharide chain increases or if negatively charged residues are present in the polar head group of the GSL. Ceramide did not form a dispersion in the buffer solution even when exposed to temperatures between 98 and 100 °C for 20 min, and it was not possible to obtain useful calorimetric scans. A continuous increase in the heat capacity was observed in this sample to take place from about 30 to 98 °C, reaching 1 cal K^{-1} g^{-1} at 98 °C where the DSC run was discontinued. Extrapolation of the values in Figure 3 to zero carbohydrate unit (i.e., ceramide) suggests that, if it were possible to hydrate and disperse ceramide in aqueous medium, the transition temperature should be above 94 °C, with a ΔH_{cal} above 11 kcal mol^{-1}.

Both *N*-lignoceroyldihydrogalactosylsphingosine and lactosylsphingosine showed a reproducible transition centered at 70.5 °C that was only present when the samples were cooled to room temperature before rescanning. The values of C_p-(max) and ΔH_{cal} for this transition were, respectively, 3.18 kcal K^{-1} mol^{-1} and 9.24 kcal mol^{-1} for *N*-lignoceroyldihydrogalactosylsphingosine and 5.70 kcal K^{-1} mol^{-1} and 8.34

kcal mol^{-1} for *N*-lignoceroyldihydrolactosylsphingosine. This transition was not present if the samples were cooled only to 55–60 °C before being rescanned. It probably represents a temperature-dependent metastable phase similar to that described earlier for this type of lipid (Freire et al., 1980; Ruocco et al., 1981). This behavior was not further investigated in this work.

The higher T_m of GSLs compared to phosphatidylcholines may be ascribed to some role played by increased possibilities for intermolecular hydrogen bonding interactions. However, the situation is likely to be more complex (see Table I). The presence of an additional OH group in $NeuGcG_{M3}$ compared to $NeuAcG_{M3}$ induces an increase in T_m. On the other hand, the presence of a α-OH group in the amide-linked fatty acid of phrenosin compared to kerasin leads to a decrease of T_m and ΔH_{cal} instead of an increase. The T_m and ΔH_{cal} of *N*-stearoyldihydrogalactosylsphingosine are 1.2 °C and 2.2 kcal mol^{-1} lower, respectively, than the corresponding values for *N*-stearoyldihydroglucosylsphingosine. This difference between galactosyl and glucosyl derivatives with the same hydrocarbon moiety may be related to the different spatial orientation of hydroxyl groups at carbon 4 of the pyranose ring, which may have an influence on the hydrogen bonding capacity as pointed out by Abrahamson et al. (1977). In any case, the contribution of hydrogen bonds to the thermodynamic parameters does not appear to be a simple one, necessarily leading to strengthened intermolecular cohesion. An increase in the number of carbohydrates in the polar head group (which, presumably, ought to increase the possibilities for intermolecular hydrogen bonding) leads to a decrease of the transition temperature and ΔH_{cal} (Table I and Figures 3 and 4). This observation indicates that the intermolecular interactions have actually diminished. In order that an increase in the possibilities for intermolecular hydrogen bonding capacity could lead to an increased intermolecular cohesion, the position and specific orientation of donor and acceptor groups as well as the polar head group size with respect to the hydrocarbon portion appear to be critical. It seems necessary that the hydrogen bonding groups be located very near the hydrocarbon–water interface (Lunden et al., 1977), presumably protected from competition from bulk water since extensive hydrogen bonding to or through the latter is likely to loosen intermolecular cohesion. Also, an optimal stability for an oriented array of GSLs is obtained when the oligosaccharide chain and the hydrophobic portion are of comparable size (Maggio et al., 1978a).

Lower T_m and ΔH_{cal} values were also found for the naturally occurring GalCer compared to the naturally occurring GlcCer (Table I). In this case, the difference is probably caused by the different fatty acid composition (Murata et al., 1978) and because GalCer is actually a mixture of two types of cerebrosides containing a predominance of hydroxy fatty acyl (phrenosin, 67%) and non-hydroxy fatty acyl residues (kerasin, 33%). This heterogeneity leads to a broader and less defined transition than in the case of the more homogeneous GlcCer. It was previously reported (Curatolo, 1982) that kerasin, studied in more concentrated solutions than in our samples, exhibited a metastable behavior depending on the rate of cooling. On the other hand, phrenosin, which contains OH fatty acyl residues, did not exhibit metastability. The lack of metastability of GalCer was tentatively ascribed to the effect of phrenosin preventing kerasin from undergoing metstable behavior. The small peak at 72 °C found for kerasin (Figure 5) might suggest that no more than 15% of this lipid could be in the state of high enthalpy and T_m described by Bunow (1979) and Curatolo (1982) (see Table II). However, we

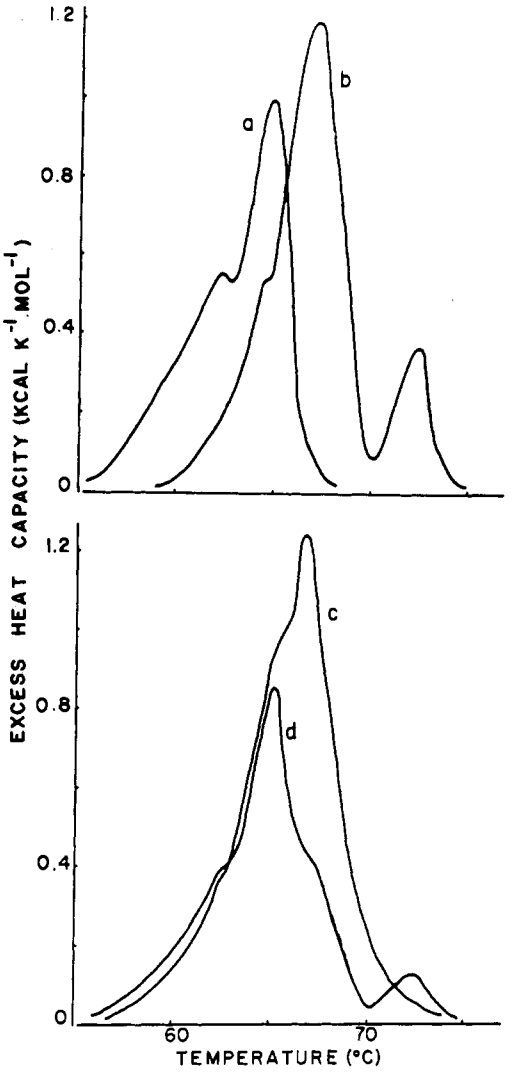

FIGURE 5: Variation with temperature of the excess heat capacity for naturally occurring GalCer. (Upper panel) (a) Phrenosin, 1.55 mg mL^{-1}; (b) kerasin, 1.68 mg mL^{-1}. (Lower panel) (c) GalCer (naturally occurring mixture of 67% phrenosin and 33% kerasin), 1.81 mg mL^{-1}; (d) theoretical DSC curve calculated for an ideal mixture of phrenosin and kerasin in the proportions of the natural sample, on the basis of the individual DSC curves shown in the upper panel. The samples were scanned from 30 °C at a nominal scan rate of 0.5 °C min^{-1}.

should stress that under our conditions of sample pretreatment, lipid concentration, and scan rate (see Materials and Methods), the calorimetric scan was reproducible for several days, with no evidence of interconversions among different states, so that our samples correspond to a stable or, at least, long-lived state as pointed out before. The calorimetric scans for phrenosin and kerasin and the theoretical curve to be expected for an ideal mixture of these in the proportions corresponding to the natural mixture are shown in Figure 5. It can be seen that the experimental curve actually obtained for GalCer is not accounted for by assuming an ideal noninteracting mixture of phrenosin and kerasin. The displacement of C_p(max) toward the kerasin side suggests that an enriched kerasin phase may progressively separate out of the mixture as the sample is heated above about 65 °C.

The polar head group of these lipids appears to have an important influence on both the type of thermodynamically stable aggregate formed and the concomitant phase behavior. The configurational entropy of the polar head group in the absence of intra- or intermolecular constraints should be higher for GSLs containing a greater number of carbohydrate resi-

dues. However, the thermodynamic behavior indicates that the entropy of the transition actually decreases as the complexity of the polar head group increases. This effect is opposite to that induced by an increase of the length of the hydrocarbon portion in phospholipids and *n*-alkanes where the entropy of the transition increases due to the greater configurational disorder possible in longer chains (Phillips et al., 1969; Phillips, 1972). Apart from unfavorable interactions between an increased number of similarly oriented dipole moment vectors in the oligosaccharide chain (Maggio et al., 1978a, 1981), it has been shown that the degree of hydration increases and greater amounts of water become ordered around the polar head group as the oligosaccharide chain of GSLs becomes more complex (Bach et al., 1982a,b). All these factors may induce a decrease of intermolecular interactions by increasing intermolecular spacings due to electrostatic, steric, and hydration repulsive forces.

Comparison of van't Hoff enthalpies, calculated by assuming a two-state process, with the calorimetric enthalpies provides an indication of the cooperativity of the phase transition process (Mabrey & Sturtevant, 1976). The absolute value for the size of the cooperative unit is particularly affected by the presence of small amounts of impurities and other undetermined influences. For this reason, it probably is of little value in the case of natural preparations. However, on a comparative basis, we have observed that there is a definite trend in the variation of the size of the cooperative unit for the phase transition process of neutral and anionic GSLs. Synthetic neutral GSLs showed cooperative units in the range of 55–95 molecules. For the natural neutral GSLs, the cooperative unit decreases as the oligosaccharide chain in the polar head group becomes more complex (the number of molecules participating in a cooperative manner in the phase transition is 59 for GlcCer, 44 for LacCer, 31 for Gg_3Cer, and 27 for Gg_4Cer). Conversely, the cooperative unit for gangliosides increases as the negatively charged oligosaccharide chain contains more carbohydrate residues (the number of molecules cooperatively involved in the ganglioside phase transition is 22 for $NeuAcG_{M3}$, 55 for G_{M2}, 98 for G_{M1}, 137 for G_{D1a}, and 479 for G_{T1b}).

Chemical or conformational differences in the polar head group appear to affect considerably the thermodynamic parameters. In globoside Gb_4Cer, the presence of a different carbohydrate sequence and an α linkage in the oligosaccharide chain confers on this molecule a relatively rigid L-shaped conformation (Yu et al., 1984). On the other hand, the all-β-linked Gg_4Cer assumes a linear conformation (Maggio et al., 1978a). Apart from these differences, the lipophilic portion of Gb_4Cer contains a higher proportion of long-chain fatty acyl residues (22:0 and 24:0) and hydroxylated fatty acids than Gg_4Cer which contains predominantly 18:0 fatty acyl residues and no hydroxylated fatty acids (Taketomi & Kawamura, 1972). On the basis of the results for the synthetic *N*-stearoyl and *N*-lignoceroyl derivatives, an increase in the number of methylene groups of the fatty acyl chain should lead to a slight increase in the T_m and a more noticeable increase in the ΔH_{cal}. On the other hand, a comparison of the data for kerasin and phrenosin suggests that the presence of hydroxylated fatty acyl residues should bring about a small decrease of T_m and ΔH_{cal} (see Table I). The unique conformation in Gb_4Cer is likely responsible, to a large extent, for the lowering of 14 °C in the T_m and 2.2 kcal mol^{-1} in ΔH_{cal} compared to Gg_4Cer (Table I). The substitution of the *N*-acetyl group linked to the sialosyl moiety in G_{M3} for an *N*-glycolyl residue induces an increase of about 4 °C in T_m and 0.3 kcal mol^{-1} in ΔH_{cal}. Similar to

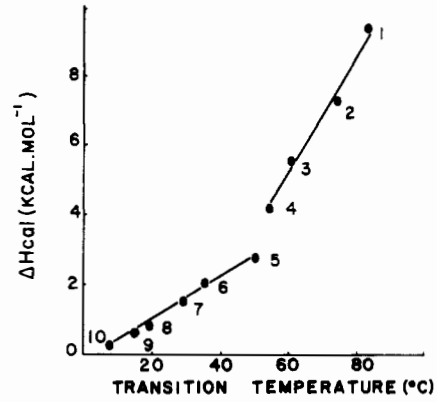

FIGURE 6: Variation of ΔH_{cal} with T_m for different glycosphingolipids. The numbers of the plot represent (1) GlcCer; (2) LacCer, (3) Gg_3Cer, (4) Gg_4Cer, (5) Sulf, (6) $NeuAcG_{M3}$, (7) G_{M2}, (8) G_{M1} (9) G_{D1a}, and (10) G_{T1b}.

anionic phospholipids (McElhaney, 1982), the T_m's and ΔH_{cal}'s for negatively charged GSLs are 16–25 °C and 1–3 kcal mol^{-1} lower, respectively, than those of their neutral analogues.

The dependence of ΔH_{cal} on T_m is linear for both the neutral and anionic GSLs (Figure 6). However, the slopes of the ΔH_{cal} vs. T_m plots are clearly different for the two groups of lipids (0.17 kcal K^{-1} mol^{-1} for neutral GSLs and 0.06 kcal K^{-1} mol^{-1} for anionic GSLs). This and the broader transition occurring with lower T_m and ΔH_{cal} for the negatively charged GSLs probably reflect lower intermolecular forces acting in the different three-dimensional structures adopted. Again, it should be pointed out that the state of aggregation for a particular lipid is also contributing to the variation of the thermodynamic parameters. For the more complex gangliosides, the preferred state of aggregation in dilute aqueous dispersions is the micellar (Hill & Lester, 1972; Yohe et al., 1976; Sillerud et al., 1979) rather than the bilayer type preferred by the simpler GSLs (Abrahamsson et al., 1972; Ruocco et al., 1981). This difference undoubtedly plays an important role in influencing the thermotropic behavior of the two groups of GSLs. In any case, it is interesting that the T_m and ΔH_{cal} for the two groups of GSLs (neutral and anionic) each show a different linear proportionality with the type of oligosaccharide chain in their polar head groups (Figures 3, 4, and 6). As the neutral oligosaccharide chain becomes longer, the thermodynamic parameters approximate the values of the simpler anionic GSLs. Conversely, as the polar head group of the latter becomes simpler, it converges with a different slope to the values of the longer neutral GSLs. For Gg_4Cer, Gb_4Cer, and sulfatide, the values tend to overlap. As far as we know, the state of aggregation of Gg_3Cer and Gg_4Cer has not been studied. However, sulfatide can organize into cubic and micellar phases in addition to bilayers in contrast to the neutral monohexosylceramides which only form bilayers (Abrahamsson et al., 1972), while Gb_4Cer can form tubular structures which probably represent long cylindrical micelles (Pinteric et al., 1973). Information on the structural organization of all GSLs below and above their respective transitions would be desirable but is not available at present. Extrapolation of the plots in Figure 3 to $\Delta H_{cal} = 0$ suggests that stable intermolecular associations are probably not possible for neutral GSLs with oligosaccharide chains containing more than seven neutral carbohydrate units. Similarly, anionic GSLs with six neutral carbohydrate units and one negative charge or anionic GSLs with more than three negative charges in their polar head group would not form stable multimolecular structures (Figure 4).

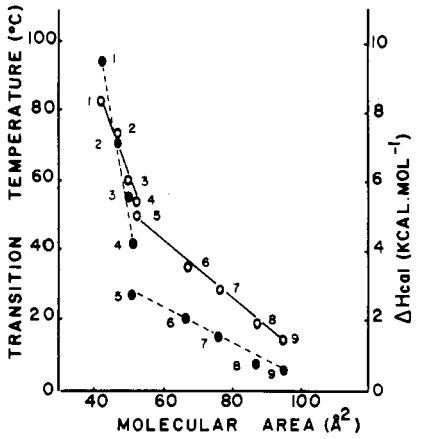

FIGURE 7: Variation of T_m and ΔH_{cal} with the molecular area of glycosphingolipids. The values for T_m (○) and ΔH_{cal} (●) are shown as a function of the molecular area occupied by the GSLs at the air–145 mM NaCl interface, at a lateral surface pressure of 30 mN m^{-1}. The dependence of the thermodynamic parameters on the molecular area was similar in the range of surface pressures from 5 mN m^{-1} up to the collapse pressure of the corresponding lipid. The numbers on the plot correspond to the GSLs indicated in the legend to Figure 6.

On the basis of surface pressure–molecular area isotherms obtained with monolayers at the air–water interface (Maggio et al., 1978a), the approximate range of transition temperatures to be expected for some GSLs was very closely anticipated (Maggio et al., 1981). This may possibly be explained now by the finding that the thermodynamic parameters are linearly proportional to the molecular area of the GSLs (Figure 7). This linear dependence occurs at lateral pressures lower than 10 mN m^{-1} (data not shown), between 10 and 30 mN m^{-1} (Figure 7), and even at the collapse pressure point (i.e., at the limiting cross-sectional molecular area which occurs above 37 mN m^{-1} for any of the GSLs studied). The dependence of the thermodynamic parameters on small variations of the intermolecular packing is striking. According to the slopes of the plot in Figure 7, at a lateral pressure of 30 mN m^{-1} a change of as little as 1 $Å^2$ in the molecular area may bring about a variation in T_m and ΔH_{cal} of 3.0 °C and 0.5 kcal mol^{-1}, respectively, for neutral GSLs and 0.8 °C and 0.1 kcal mol^{-1}, respectively, for anionic GSLs. This suggests that the influence of the complexity of the polar head group in establishing a particular thermotropic behavior may be mediated by the intermolecular spacings, with a corresponding effect on intermolecular interaction energies (Maggio et al., 1981), and concomitantly, the state of aggregation. Extrapolation to $\Delta H_{cal} = 0$ in Figure 7 indicates molecular areas of between 60 and 70 $Å^2$ and between 105 and 110 $Å^2$ for neutral and anionic GSLs, respectively, as limiting values above which no stable bilayer or micellar aggregate would be formed if the lateral surface pressure is maintained at about 30 mN m^{-1} or above. The limiting molecular areas for forming stable bilayers or micelles for neutral or anionic GSLs, respectively, are increased to about 95 and 170 $Å^2$ if the lateral surface pressure is lowered to 5 mN m^{-1} (data not shown).

The phase transition properties of phosphatidylcholines have been described in terms of increased possibilities for rotational isomers in the hydrocarbon chains (Phillips et al., 1969; Phillips, 1977). However, this interpretation does not apply equally well to sphingomyelins (Barenholz et al., 1976) and, according to the data discussed herein, to GSLs. In the present study, the thermodynamic data indicate that the number, type, particular conformation, and hydration properties of the carbohydrate residues in the oligosaccharide chain of GSLs are probably the major factors influencing the thermotropic behavior of this group of lipids. These results agree well with the molecular behavior of GSLs found in monolayers at the air–water interface (Maggio et al., 1978a, 1981). All these features may be of biological significance since the effects of these lipids on the membrane stability (Maggio et al., 1978b,c, 1983; Monferran et al., 1979) and their function as putative receptors of bioactive agents (Fishman & Brady, 1976; Cumar et al., 1982) are all mediated, in molecular terms, through particular residues or conformations of their polar head groups (Maggio et al., 1980, 1981; Fidelio et al., 1982). If these molecular properties can be dynamically modified in certain conditions, leading to changes of the mean molecular area occupied at a biological interface (Maggio et al., 1978b; Monferran et al., 1979; Cumar et al., 1980), the lateral topography and phase state of the membrane are certainly bound to feel the consequences and to influence the cellular function as previously suggested (Cumar et al., 1980; Maggio et al., 1981, 1983).

Registry No. LacCer, 4682-48-8; Gg_3Cer, 35960-33-9; Gg_4Cer, 71012-19-6; Gb_4Cer, 11034-93-8; $NeuGcG_{M3}$, 69345-49-9; $NeuAcG_{M3}$, 54827-14-4; G_{M2}, 19600-01-2; G_{M1}, 37758-47-7; G_{D1A}, 12707-58-3; G_{T1B}, 59247-13-1; kerasin, 536-13-0; phrenosin, 37211-11-3.

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Sendai Virus Induced Leakage of Liposomes Containing Gangliosides†

Yung-shyeng Tsao and Leaf Huang*

Department of Biochemistry, University of Tennessee, Knoxville, Tennessee 37996-0840
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ABSTRACT: Sendai virus induced liposome leakage has been studied by using liposomes containing a self-quenching fluorescent dye, calcein. The liposomes used in this study were prepared by a freeze and thaw method and were composed of phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine (1:2.60:1.48 molar ratio) as well as various amounts of gangliosides and cholesterol. The leakage rate was calculated from the fluorescence increment as the entrapped calcein leaked out of the liposomal compartment and was diluted into the media. It was shown that the target liposome leakage was virus dose dependent. Trypsin-treated Sendai virus in which the F protein had been quantitatively removed did not induce liposome leakage, indicating that the leakage was a direct result of F-protein interaction with the target bilayer membrane. The activation energy of this process was approximately 12 kcal/mol below 17 °C and approximately 25 kcal/mol above 17 °C. Gangliosides GM_1, GD_{1a}, and GT_{1b} could serve as viral receptor under appropriate conditions. Liposome leakage showed a bell-shaped curve dependence on the concentration of ganglioside in the liposomes. No leakage was observed if the ganglioside content was too low or too high. Inclusion of cholesterol in the liposome bilayer suppressed the leakage rate of liposomes containing GD_{1a}. It is speculated that the liposome leakage is a consequence of fusion between Sendai virus and liposomes.

Sendai virus and other paramyxoviruses can interact with a wide spectrum of cells resulting in the following consequences: (a) virus–cell fusion; (b) cell membrane changes which lead to leakage of small intracellular constituents such as ions; (c) swelling of cells; (d) lysis of cells, e.g., hemolysis; (e) cell–cell fusion and/or polykaryon formation. The interrelationships among these processes are of great interest. It has been suggested that cellular permeability change is the

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