A Carbohydrate–Carbohydrate Interaction between Galactosylceramide-Containing Liposomes and Cerebroside Sulfate-Containing Liposomes: Dependence on the Glycolipid Ceramide Composition†

Robert J. Stewart‡ and Joan M. Boggs*

Department of Clinical Biochemistry, University of Toronto, and Department of Biochemistry, Research Institute, Hospital for Sick Children, Toronto, Ontario, M5G 1X8, Canada

Received March 12, 1993; Revised Manuscript Received July 21, 1993®

ABSTRACT: Galactosylceramide (GalCer) and cerebroside sulfate (CBS) are the major glycolipids found in myelin. They occur in greater concentrations in this membrane than any other. Recently, it was reported that these two glycolipids can participate in a heterotypic carbohydrate–carbohydrate interaction [Hakomori *et al.* (1991) *Glycoconjugate J. 8,* 178]. In the present study, the effect of changes in the ceramide composition of both GalCer and CBS on this interaction has been examined. The interaction was monitored by measuring the aggregation of small unilamellar phosphatidylcholine/cholesterol liposomes containing GalCer with similar liposomes containing CBS, through the increase in optical density at 450 nm. Aggregation depends on the addition of a divalent cation and varies inversely with the ionic radius of the cation. Aggregation occurred at millimolar concentrations of divalent cation and was inhibited and reversed by the addition of EDTA. A lesser degree of homotypic self-aggregation of GalCer and of CBS liposomes also occurred in the presence of divalent cations, but the sum of this self-aggregation was significantly less than the heterotypic interaction between the two types of liposomes. Changes in the ceramide composition of GalCer and CBS significantly affected the extent of their interaction with each other. Increasing the fatty acid chain length of either GalCer or CBS resulted in increased aggregation. Hydroxylation of the fatty acid also increased the degree of aggregation of GalCer and CBS liposomes. These findings indicate that a divalent cation-mediated GalCer–CBS interaction could play a role in cell recognition and membrane adhesion phenomena such as the formation of compact multilamellar myelin. They indicate further that the ceramide composition can influence the receptor activity of the carbohydrate head groups of glycolipids, suggesting that variations in the ceramide composition which occur in myelin in different species, during development, and in some diseases could modulate the formation and maintenance of compact myelin.

Glycolipids are important cell surface molecules found in virtually all cells. The carbohydrate head groups of glycolipids have been shown to be involved in a wide variety of cell surface recognition phenomena including cell adhesion, interactions with microorganisms, and interactions with biological ligands (Hakomori, 1984; Karlsson, 1989). Changes in cell surface glycolipid composition occur during cell growth, development, and differentiation, and during oncogenesis (Hakomori, 1984). Although the membrane concentration and carbohydrate composition of glycolipids can influence cell surface recognition phenomena, changes in the membrane environment of glycolipids and in the glycolipid ceramide composition may also alter their cell surface expression, as detectable by antibody. For example, during oncogenesis, the expression of glycolipids can be altered without any significant change in the glycolipid chemical quantity (Young *et al.*, 1991; Kannagi *et al.*, 1982; Nudelman *et al.*, 1982), and these changes have been associated, at least in part, with changes in glycolipid ceramide composition. Antibody binding to glycolipids in a membrane environment has been shown to increase with increases in the fatty acid chain length of the ceramide group (Alving & Richards, 1977; Yoshino *et al.*, 1982; Crook *et al.*, 1986; Stewart & Boggs, 1990). Hydroxylation of the fatty acid of the glycolipid has been shown to reduce its recognition by antibody (Crook *et al.*, 1986; Stewart & Boggs, 1990). The oxidation of GalCer[1] in a membrane environment by galactose oxidase has also been shown to be similarly dependent upon the fatty acid composition of the glycolipid (Stewart & Boggs, 1991, 1993). In addition, developmental changes (Svennerholm & Stallberg-Stenhagen, 1968; Shimomura & Kishimoto, 1983; Palestini *et al.*, 1990) and tissue specific differences in glycolipid ceramide composition (Karlsson, 1982; Ogawa-Goto *et al.*, 1990) together suggest a functional role for the ceramide structure of glycolipids. These findings imply further that changes in the ceramide composition could modulate cell contact, cell growth and regulation, susceptibility to infection, and immune response as a result of changes in the expression of the glycolipid carbohydrate group.

† This work was supported by grants from the Multiple Sclerosis Society of Canada and the Medical Research Council of Canada. R.J.S. gratefully acknowledges a studentship from the Multiple Sclerosis Society.

* To whom correspondence should be addressed at the Biochemistry Department, Hospital for Sick Children, 555 University Ave., Toronto, Ontario, Canada M5G 1X8. Telephone: (416) 813-5919; FAX: (416) 813-5022.

‡ Present address: Brewing Research Department, Labatt Breweries, 150 Simcoe St., London, Ontario, Canada N6A 4M3.

® Abstract published in *Advance ACS Abstracts,* September 15, 1993.

[1] Abbreviations: GalCer, galactosylceramide; CBS, galactosylceramide I^3-sulfate; nGalCer, bovine brain GalCer; nCBS, bovine brain CBS; HFA-nGalCer, fraction of bovine brain GalCer with hydroxy fatty acids; NFA-nGalCer, fraction of bovine brain GalCer with non-hydroxy fatty acids; HFA-nCBS, fraction of bovine brain CBS with hydroxy fatty acids; NFA-nCBS, fraction of bovine brain CBS with non-hydroxy fatty acids; GluCer, glucosylceramide, prepared from Gaucher's spleen; [^{3}H]DPPC, [*2-palmitoyl*-9,10-^{3}H(N)]-L-α-1,2-dipalmitoylphosphatidylcholine; PC, phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; CHOL, cholesterol; TLC, thin-layer chromatography.

0006-2960/93/0432-10666$04.00/0

Although much attention has been focused on the interactions of glycolipids with protein ligands, it has recently been shown that the carbohydrate moieties of certain glycolipid and glycoprotein molecules can specifically interact with each other (Webb *et al.*, 1988; Eggens *et al.*, 1989; Kojima & Hakomori, 1989, 1991; Hakomori *et al.*, 1991). The list of glycolipids which can participate in carbohydrate–carbohydrate interactions includes the two major myelin glycolipids galactosylceramide (GalCer) and cerebroside sulfate (CBS) (Hakomori *et al.*, 1991). In central nervous system myelin, these two glycolipids comprise 27% (by weight) of the total membrane lipid (Norton, 1977). The interaction between these two glycolipids, therefore, might play a role in the formation of the compacted myelin membrane. Changes in the fatty acid composition of both GalCer and CBS in myelin occur during development (Svennerholm & Stallberg-Stenhagen, 1968; Shimomura & Kishimoto, 1983) and in the demyelinating disease adrenoleukodystrophy (Kishimoto *et al.*, 1985). Variations are also found in different species (Ki *et al.*, 1985; Tamai *et al.*, 1992). Therefore, it is important to determine if changes in the ceramide group of these two glycolipids can influence this specific interaction.

Thus, in the current study we have examined the GalCer–CBS carbohydrate–carbohydrate interaction and its dependence on the ceramide composition of both glycolipids. The interaction has been examined by use of an aggregation assay, where phosphatidylcholine/cholesterol liposomes containing GalCer are added to similar liposomes containing CBS, and the resultant aggregation of the liposomes is monitored by an increase in the absorbance at 450 nm. The effect of changes in the ceramide fatty acid chain length and hydroxylation on the interaction of GalCer and CBS has been studied by using liposomes which contain different molecular species of GalCer and CBS, each with a defined fatty acid chain.

MATERIALS AND METHODS

Lipids. Bovine brain galactosylceramide I^3-sulfate (nCBS) was purified from bovine brain white matter as described (Koshy & Boggs, 1983; Boggs *et al.*, 1988). Glucosylceramide (GluCer) prepared from Gaucher's spleen was obtained from Sulpelco. Bovine brain galactosylceramide (nGalCer), dimyristoylphosphatidylcholine (DMPC), and cholesterol (CHOL) were obtained from Avanti Polar Lipids. Semi-synthetic molecular species of GalCer which contained C16:0, C18:0, and C24:1 fatty acids were purchased from Sigma. Semi-synthetic molecular species of GalCer containing C24:0 or C26:0 and of CBS containing C16:0, C24:1, C24:0, C26:0, or α-hydroxylated fatty acids C16:0h or C24:0h were synthesized and purified as described (Koshy & Boggs, 1983; Boggs *et al.*, 1988; Stewart & Boggs, 1993). Hydroxy fatty acid (HFA) and non-hydroxy fatty acid (NFA) fractions of nGalCer and nCBS were purified by preparative TLC with chloroform/methanol/aqueous ammonium hydroxide(65/25/4 v/v), as the developing solvent (Koshy & Boggs, 1983). The hydroxy and non-hydroxy fatty acid fractions, identified by their comigration with standards and light iodine staining, were eluted from the silica after scraping the band from the plate with chloroform/methanol/water (7/7/1 v/v) as described (Radin, 1972). [*2-palmitoyl*-9,10-^{3}H(N)]-L-α-1,2-Dipalmitoyl phosphatidylcholine ([^{3}H]DPPC), specific activity 58.0 Ci/mmol, was obtained from New England Nuclear.

Reagents and Chemicals. All chemicals used were of reagent grade. Divalent cations were obtained from Sigma ($MnCl_2 \cdot 4H_2O$), Fisher ($CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, $BaCl_2 \cdot 2H_2O$), or Aldrich ($(CH_3COO)_2Zn \cdot 2H_2O$, $(CH_3COO)_2Pb \cdot 3H_2O$). Galactose, *N*-acetylgalactosamine, and glucose were obtained from Sigma. Dextran sulfate was from Pharmacia, and heparin was from Organon, Canada, Ltd., and was lyophilized before use. Galactose 3-sulfate was synthesized by the procedure of Archibald et al. (1981).

Table I: Effect of Variations in Ceramide Composition of GalCer and CBS on Mole Ratio of Glycolipid to PC in Sonicated Liposomes

molecular species of glycolipid	glycolipid/PC (mol/mol)[a]	molecular species of glycolipid	glycolipid/PC (mol/mol)[a]
C16:0-CBS	0.14 ± 0.02	NFA-nCBS	0.10 ± 0.01
C26:0-CBS	0.12 ± 0.01	HFA-nCBS	0.12 ± 0.03
C16:0-GalCer	0.13 ± 0.05	NFA-nGalCer	0.14 ± 0.01
C26:0-GalCer	0.10 ± 0.03	HFA-nGalCer	0.15 ± 0.03

[a] Mean of 3 experiments ± standard deviation. Mole ratio of lipids in sonicated liposomes which remain suspended after centrifugation. The initial mole ratio was 0.2 in all cases.

Preparation of Small Unilamellar Liposomes. Small unilamellar liposomes were prepared for aggregation studies of GalCer-, GluCer-, and CBS-containing liposomes. The liposomes were prepared by mixing chloroform/methanol (2/1 v/v) solutions of individual lipids in a small screw-capped tube to a final mole ratio of DMPC/CHOL/glycolipid of 1.0/0.75/0.2, respectively. Vesicles without glycolipid were also made and contained DMPC/CHOL (1.0/0.75 mol/mol). In order to monitor the liposome concentration, 10^5 cpm of [^{3}H]DPPC were added to each lipid mixture. The chloroform/methanol was evaporated under a nitrogen stream, and traces of solvent were removed by evacuation under vacuum for 2 h. The lipid mixture was then redissolved in benzene and colyophilized (Lin & Huang, 1988). A few glass beads were added to the dried lipids and then hydrated in 100 mM NaCl and 50 mM HEPES, pH 7.4, at a final concentration of 10 mM DMPC, and the tube was tightly capped. The lipid dispersion was incubated in a 90–95 °C water bath for 10 min with intermittent vigorous vortexing. The lipid suspension was then transferred to a large (30 × 300 mm) glass tube, diluted to 1 mM DMPC with 100 mM NaCl and 50 mM HEPES buffer, pH 7.4, and sonicated under nitrogen in 15-s bursts with a Branson cell sonifier disruptor probe sonicator at medium setting for 5 min. The liposome suspensions were transferred to an Eppendorf centrifuge tube, and any larger multilamellar vesicles were removed by centrifugation at 4 °C in an Eppendorf centrifuge for 30 min. The supernatants were transferred to glass test tubes, and the phospholipid concentration of each sample was determined by taking aliquots for liquid scintillation counting. The phospholipid/glycolipid ratio of representative samples was determined by also taking aliquots for sphingosine assay. Sphingosine content was assessed by the method of Naoi *et al.* (1974). The liposomes were then diluted with the 100 mM NaCl and 50 mM HEPES, pH 7.4, to a final PC concentration of 0.1 mM. Initial studies showed that during the preparation of the GalCer and CBS liposomes there was a selective loss of glycolipid resulting in up to 50% reduction of the glycolipid/PC mole ratio, and thus the starting ratio of glycolipid/DMPC of 0.2/1.0 was used in order to obtain a final ratio of at least 0.1/1.0. This loss was variable but did not depend on acyl chain length or hydroxylation of the glycolipid (Table I; also see footnote to Table III and caption to Figure 8). Aggregation was independent of mole ratio over the range 0.10–0.2 although it dropped significantly at a ratio of 0.05 (data not shown).

Liposomes were also prepared by the ethanol injection method, as described (Batzri & Korn, 1973; Eggens *et al.*, 1989). The lipid mixtures were dissolved in 100 μL of hot ethanol, taken up into a Hamilton syringe, and were rapidly injected into 4.0 mL of 100 mM NaCl and 50 mM HEPES,

pH 7.4, which had been preheated to 90 °C. The liposomes were diluted with buffer to reduce the ethanol concentration to 0.75% (v/v). If the liposomes were somewhat opalescent, they were centrifuged in an Eppendorf centrifuge to remove any oligo- or multilamellar vesicles. All samples were adjusted to the same final PC concentration of 0.1 mM by use of the radioactive tracer, [^{3}H]DPPC. However, this method resulted in much greater loss of glycolipid than the sonication method, and aggregation was low.

Aggregation of GalCer and CBS Liposomes. The specific interaction of GalCer (or GluCer) with CBS was examined by determining the increase in the absorption of the suspension due to the aggregation of the liposomes as described by Eggens *et al.* (1989). Three hundred microliters of GalCer or GluCer liposomes was mixed with an equal volume of CBS liposomes, and 35 μL of 100 mM divalent cation ($CaCl_2$ or $MnCl_2$ for most experiments) in 50 mM HEPES, pH 7.4, was added. The mixture was then gently vortexed and incubated at room temperature for 20 min, and the absorbance of the mixture was then measured using a Hitachi U-2000 spectrophotometer at 450 nm, against a buffer blank. Preliminary studies showed that the aggregation of the liposomes was rapid and that maximal aggregation occurred within 20 min. Control samples contained the same mixture of liposomes, but 35 μL of 50 mM EDTA in 50 mM HEPES, pH 7.4, was added instead of $CaCl_2$. Self-aggregation of each type of liposomes was determined by adding 300 μL of 100 mM NaCl and 50 mM HEPES, pH 7.4, to 300 μL of the liposomes, followed by the addition of 35 μL 100 mM Ca^{2+} or Mn^{2+}. Net absorbance due to the specific interaction of GalCer and CBS liposomes was determined by subtraction of the sum of the absorbance due to the self-aggregation of GalCer liposomes plus the absorbance due to the self-aggregation of CBS liposomes from the absorbance obtained when the two liposome types were mixed together in the same tube. In experiments where the effects of different divalent cations on induction of the aggregation of GalCer and CBS were examined, 100 mM solutions of each salt were prepared in 50 mM HEPES, pH 7.4. In the case of lead and zinc salts, acetates were used in place of the chloride salts because of the relative insolubility of the latter. The effect of ionic strength on the relative amount of interaction between GalCer and CBS liposomes was determined by preparation of both types of liposomes in pH 7.4 buffer containing varying concentrations of NaCl (10 mM NaCl, 5 mM HEPES; 100 mM NaCl, 50 mM HEPES; 450 mM NaCl, 50 mM HEPES). The solutions of divalent cations (and EDTA) were prepared in the corresponding NaCl solutions.

The reversibility of the aggregation of GalCer and CBS liposomes was determined by first inducing aggregation by the addition of $MnCl_2$ to the mixture of liposomes to a concentration of 5.5 mM and incubation at room temperature for 20 min. To 600-μL aliquots of aggregated liposomes was added 85 μL of 100 mM EDTA (final EDTA concentration was 12.5 mM) or 85 μL of HEPES buffer, and the absorbance of each was determined at 450 nm. The ability of different carbohydrates (galactose, galactose 3-sulfate, glucose, *N*-acetylgalactosamine, heparin, dextran sulfate, and lysosulfatide) and anions (sodium salts of sulfate, nitrate, and acetate) to inhibit aggregation was determined by addition of 85 μl of the inhibitor solution to achieve a final concentration of 0–50 mM to the mixture of GalCer liposomes and CBS liposomes followed by the addition of the divalent cation solution. The liposomes were then incubated for 20 min at room temperature, and the absorbance at 450 nm was determined.

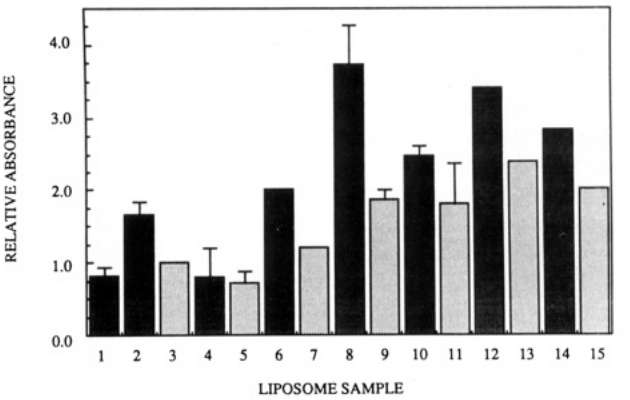

FIGURE 1: Characterization of GalCer–CBS interaction. The absorbance at 450 nm of liposomes containing nGalCer, nCBS, or GluCer, either by themselves or after addition to another set of liposomes, upon the addition of Ca^{2+} or EDTA (both at a final concentration of 5.5 mM) was determined as described in Materials and Methods. The absorbance of each sample was normalized to the absorbance of GalCer liposome plus EDTA which was given a value of 1.0 (column 3). Values are expressed as the mean of 5 experiments ± standard deviation except for PC/CHOL liposomes, which is the mean of 3 experiments. The values for the aggregation of GluCer liposomes from a separate experiment are also shown. Dark bars give values in the presence of Ca^{2+}, light bars in the presence of EDTA. Column 1, DMPC/CHOL liposomes plus Ca^{2+}; column 2, GalCer liposomes plus Ca^{2+}; column 3, GalCer liposomes plus EDTA; column 4, CBS liposomes plus Ca^{2+}; column 5, CBS liposomes plus EDTA; column 6, GluCer liposomes plus Ca^{2+}; column 7, GluCer liposomes plus EDTA; column 8, GalCer liposomes mixed with CBS liposomes plus Ca^{2+}; column 9, GalCer liposomes mixed with CBS liposomes plus EDTA; column 10, sum of GalCer liposomes plus Ca^{2+} and CBS liposomes plus Ca^{2+}; column 11, sum of GalCer liposomes plus EDTA and CBS liposomes plus EDTA; column 12, GluCer liposomes mixed with CBS liposomes plus Ca^{2+}; column 13, GluCer liposomes mixed with CBS liposomes plus EDTA; column 14, sum of GluCer liposomes plus Ca^{2+} and CBS liposomes plus Ca^{2+}; column 15, sum of GluCer liposomes plus EDTA and CBS liposomes plus EDTA.

RESULTS

Characterization of the GalCer–CBS Interaction. If liposomes containing nGalCer were added to liposomes containing nCBS in the presence of a divalent cation, such as calcium, a significant increase in absorbance was observed relative to that in the presence of EDTA (Figure 1). The absorbance of vesicles containing DMPC/CHOL without glycolipid in the presence of Ca^{2+} was much less. The addition of calcium to both nGalCer liposomes and nCBS liposomes individually also increased the absorbance, indicating that it caused some self-aggregation especially of GalCer liposomes. However, when the absorbance due to the homotypic self-aggregation of nGalCer liposomes and nCBS liposomes was summed (column 10), it was significantly less than that which was observed when nGalCer liposomes and nCBS liposomes were mixed together in the presence of calcium (column 8), indicating that a divalent cation-mediated heterotypic interaction between nGalCer and nCBS occurred. The results of eight different experiments are summarized in Table II and indicate a statistically significant difference ($p < 0.005$) between the absorbance due to the aggregation of nGalCer liposomes with nCBS liposomes in the presence of Ca^{2+} and the sum due to self-aggregation of each set of liposomes. A much smaller difference occurred in the presence of EDTA. Liposomes containing GluCer also self-aggregate in the presence of divalent cation. The aggregation between GluCer and CBS liposomes was only a little greater than the sum due to the self-aggregation and may not have been divalent cation mediated since it occurred to a similar extent in the presence of EDTA (Figure 1).

Table II: Characterization of GalCer and CBS Interaction As Measured by the Aggregation of GalCer-Containing Liposomes with CBS-Containing Liposomes[a]

liposome sample[b]	relative absorbance[c]	liposome sample[b]	relative absorbance[c]
nGalCer + Ca^{2+}	1.63 ± 0.18*	nGalCer + nCBS + Ca^{2+}	3.81 ± 0.42***
nGalCer + EDTA	1.0	nGalCer + nCBS + EDTA	1.94 ± 0.17
nCBS + Ca^{2+}	0.80 ± 0.21**	nGalCer + nCBS + Ca^{2+} (SUM)	2.43 ± 0.27
nCBS + EDTA	0.66 ± 0.16	nGalCer + nCBS + EDTA (SUM)	1.66 ± 0.16

[a] Liposomes were prepared containing DMPC/CHOL/nGalCer or nCBS (1.0/0.75/0.2 mol/mol) as described in the Materials and Methods section. [b] The aggregation of different liposome mixtures was monitored by absorbance at 450 nm. To either nGalCer or nCBS liposomes, or to nGalCer and nCBS liposomes mixed together in the same tube, was added 5.5 mM Ca^{2+} or 3 mM EDTA, and the absorbance at 450 nm was determined after 20 min. The samples nGalCer + nCBS + Ca^{2+} (SUM) and nGalCer + nCBS + EDTA (SUM) were determined by summing the values of the absorbance due to GalCer + Ca^{2+} (or EDTA) and CBS + Ca^{2+} (or EDTA) in each experiment. [c] The relative absorbance was determined by normalizing the absorbance of each sample to that of GalCer + EDTA, for each experiment. Values are expressed as the mean ± standard deviation of 8 experiments. * The relative absorbance of GalCer liposomes + Ca^{2+} is significantly higher than the absorbance of GalCer liposomes + EDTA, $p < 0.005$. ** The absorbance of CBS liposomes + Ca^{2+} is significantly higher than that of CBS liposomes + EDTA, $p < 0.025$. *** The absorbance of GalCer liposomes mixed with CBS liposomes in the presence of Ca^{2+} is significantly higher than both the absorbance due to GalCer liposomes mixed with CBS liposomes in the presence of EDTA and the sum of the absorbances due to the self-aggregation of GalCer liposomes and CBS liposomes in the presence of Ca^{2+}, $p < 0.005$.

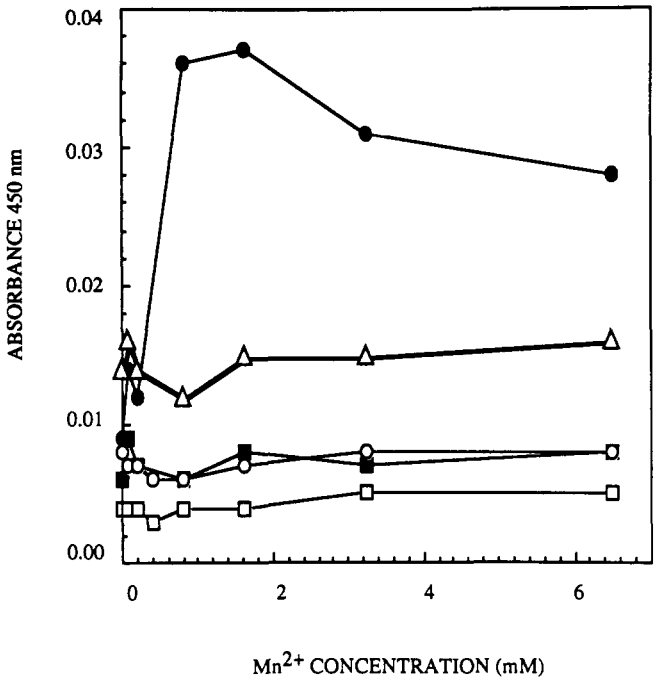

FIGURE 2: Dependence of aggregation on divalent cation concentration. Increasing concentrations of $MnCl_2$ were added to GalCer and CBS liposomes, and the absorbance at 450 nm was determined after 20-min incubation. Values are from a representative experiment. (●) GalCer liposomes plus CBS liposomes; (○) GalCer liposomes; (■) CBS liposomes; (△) sum of absorbance of GalCer liposomes and CBS liposomes; (□) DMPC/CHOL liposomes.

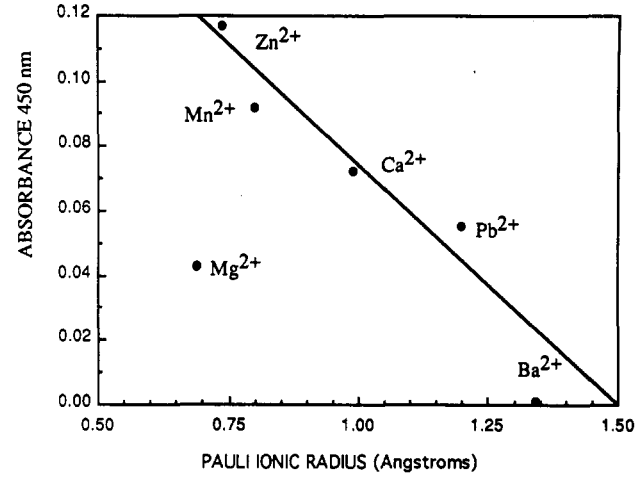

FIGURE 3: Dependence of aggregation on ionic radius of the divalent cation. Various divalent cations were added to GalCer and CBS liposomes at a final concentration of 5.5 mM. For each cation, the net absorbance was determined by subtracting the absorbance due to the self-aggregation of both GalCer and CBS liposomes from the absorbance when the two sets of liposomes were mixed in the same tube. The net absorbance was plotted against the Pauli ionic crystal radius of each divalent cation (Weast & Astle, 1980).

Dependence of GalCer–CBS Interaction on Divalent Cations. The interaction between nGalCer and nCBS depended on the concentration of divalent cation (Figure 2). Significant aggregation was observed only when the cation concentration reached values of 1–2 mM and did not increase with further increases in concentration, as shown for manganese in Figure 2. The sum of the absorbance due to the self-aggregation of nGalCer and nCBS liposomes was less than that due to the heterotypic aggregation between nGalCer and nCBS at all Mn^{2+} concentrations examined (Figure 2).

The dependence of the interaction of nGalCer and nCBS on the nature of the divalent cation was also examined using each divalent cation at a concentration of 5.5 mM (Figure 3). An inverse relation between the ionic crystal radius of the divalent cation and the degree of aggregation was observed, with the exception of Mg^{2+}, which resulted in less aggregation of the vesicles than with Ca^{2+}, despite the smaller radius of Mg^{2+}. In each experiment the total glycolipid concentration was 10–20 μM; thus at a concentration of divalent cation of 5.5 mM there was several hundredfold excess of cation present, indicating that the binding of the cation to the glycolipids was of low affinity. Since Mn^{2+} caused more aggregation than Ca^{2+}, it was used for all subsequent experiments.

The liposomes containing GalCer or CBS were prepared in buffer containing 10, 100, or 450 mM NaCl, and the relative amount of aggregation induced by the addition of Mn^{2+} was compared (Figure 4). The total amount of aggregation increased with increasing ionic strength, but the net GalCer–CBS aggregation, obtained by subtraction of the absorbance due to the self-aggregation of GalCer and CBS liposomes from the absorbance when the two liposomes were mixed, increased significantly only when the NaCl concentration was 450 mM. This increase at high ionic strength indicated that there was some electrostatic repulsion between the liposomes.

Reversibility of Aggregation. The amount of aggregation was not affected by preincubation of GalCer and CBS liposomes mixed together with either galactose or galactose 3-sulfate at concentrations up to 50 mM or with *N*-acetylgalactosamine, glucose, sodium sulfate, sodium nitrate, or sodium acetate, all at concentrations up to 12.5 mM (data not shown). Heparin did not inhibit up to 12.5 mg/mL. However, aggregation was inhibited by dextran sulfate at a concentration of 12.5 mg/mL, indicating that a multivalent sugar is required. This suggests that the galactose–cation-sulfated galactose

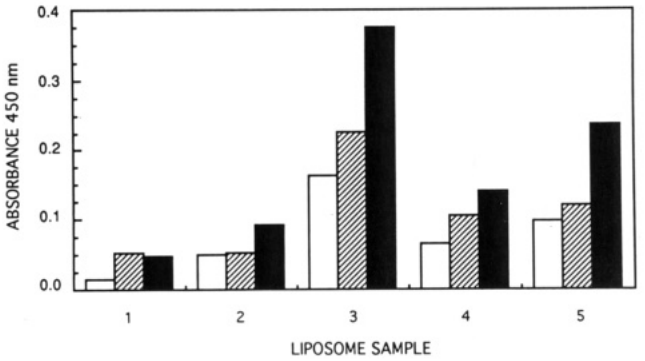

FIGURE 4: Effect of ionic strength. GalCer liposomes and CBS liposomes were prepared in 10 mM NaCl and 1 mM HEPES, pH 7.0 (white bars); 100 mM NaCl and 50 mM HEPES, pH 7.4 (hatched bars); and 450 mM NaCl and 50 mM HEPES, pH 7.4 (solid bars). $MnCl_2$ solutions were also prepared in the corresponding NaCl solutions and were added to a final concentration of 5.5 mM $MnCl_2$. (1) GalCer liposomes; (2) CBS liposomes; (3) GalCer and CBS liposomes mixed together in the same tube; (4) sum of the absorbance due to the self-aggregation of GalCer and CBS liposomes; (5) net absorbance due to the aggregation of GalCer and CBS liposomes obtained by subtracting the values for the sum of the absorbance due to the self-aggregation from the actual absorbance due to GalCer and CBS liposomes mixed together.

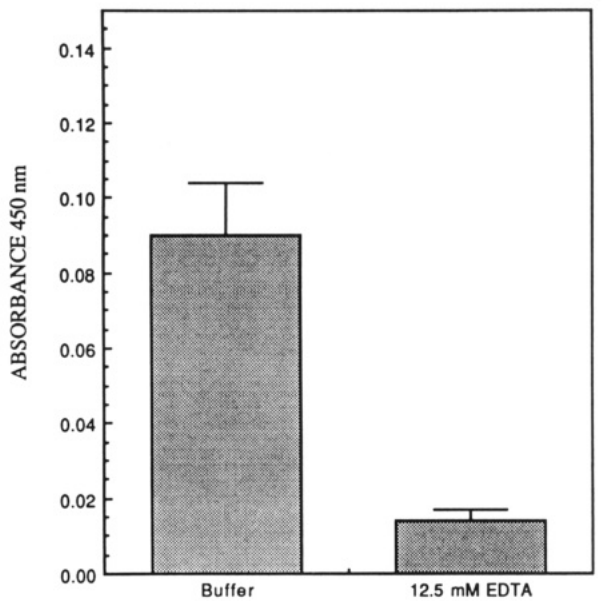

FIGURE 5: Reversibility of the aggregation of GalCer and CBS liposomes. GalCer liposomes were incubated with CBS liposomes in the presence of 5.5 mM Mn^{2+} for 20 min, and aliquots were diluted with either 100 mM NaCl and 50 mM HEPES buffer, pH 7.4, or 100 mM EDTA (to a final concentration of 12.5 mM EDTA), and the absorbance at 450 nm was then determined. Values are expressed as the mean ± standard deviation of 3 different experiments.

interaction is of low affinity. Similar results have been obtained for the interaction of other ligands with carbohydrate (Lindberg *et al.*, 1987). The absorbance was reduced by lysosulfatide at concentrations of 45 μM, but this may have been due to a detergent effect on the liposomes rather than inhibition of aggregation. The aggregation of GalCer and CBS liposomes could be reversed by the addition of EDTA. Figure 5 shows that the absorbance due to aggregated GalCer and CBS liposomes in the presence of 5.5 mM Mn^{2+} is completely reversed by the addition of EDTA to a final concentration of 12.5 mM. The absorbance observed after the addition of EDTA is similar to that observed when the liposomes are mixed together in the presence of EDTA only, indicating that fusion did not occur. Lower concentrations

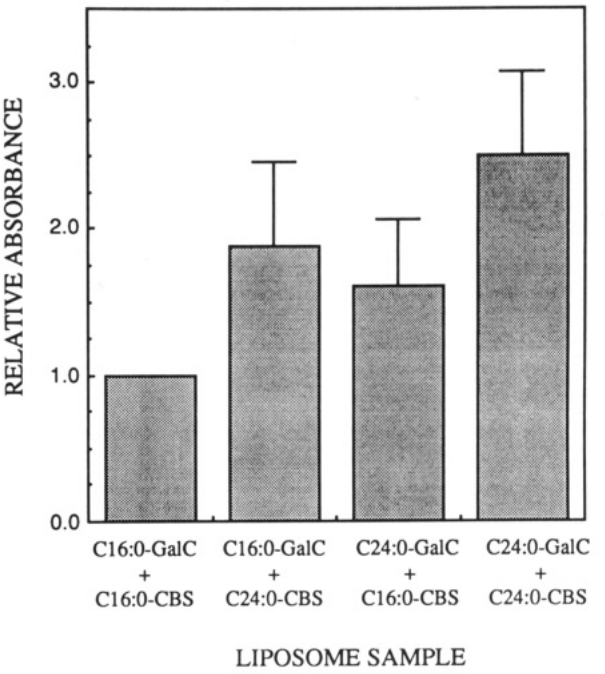

FIGURE 6: Effect of GalCer and CBS fatty acid chain length on the Mn^{2+}-dependent aggregation of GalCer and CBS liposomes. Liposomes were prepared containing molecular species of GalCer and CBS with C16:0 or C24:0 fatty acids. Values are expressed as the mean of 6 experiments ± standard deviation. The aggregation of C16:0-GalCer liposomes with C16:0-CBS liposomes was significantly less than the aggregation of C24:0-GalCer with C24:0-CBS liposomes, $p < 0.005$. The absorbance due to the aggregation of C24:0-GalCer with C24:0-CBS liposomes was also significantly higher than the absorbance due to either C16:0-GalCer + C24:0-CBS or C24:0-GalCer + C16:0-CBS, $p < 0.005$.

of EDTA resulted in a partial reversal of the aggregation of GalCer and CBS liposomes (not shown).

Effect of Fatty Acid Chain Length on GalCer–CBS Interaction. The effect of the fatty acid chain length of either GalCer or CBS on the amount of aggregation was examined by incorporating semisynthetic molecular species of both GalCer and CBS, each with a defined fatty acid chain length, into the liposomes. Self-aggregation increased only a little with acyl chain length (not shown). However, an increase in the fatty acid chain length of GalCer and/or CBS from C16:0 to C24:0 significantly increased the amount of net aggregation of GalCer and CBS liposomes. The interaction was increased if the chain length of just one of the glycolipids was increased, but the interaction was greatest if both glycolipids had long fatty acid chains (Figure 6). Interestingly, a further increase in the chain length of both glycolipids by only 2 carbons from C24:0 to C26:0 increased the aggregation almost 2-fold, while introduction of a *cis* double bond into the C24 chain significantly decreased it (Table III). As summarized in Table III, where data from a number of different experiments is averaged, the relative aggregation of GalCer and CBS liposomes significantly increased with fatty acid chain length and decreased with unsaturation. However, the aggregation of liposomes containing C24:1 molecular species was greater than when the liposomes contained C16:0 molecular species. This increase in aggregation with increase in chain length and saturation was not due to greater incorporation of the longer chain or saturated chain species in the liposomes (Table I, footnote to Table III).

Effect of Fatty Acid Hydroxylation on GalCer–CBS Interaction. The effect of hydroxylation on the interaction of nGalCer and nCBS was examined by using TLC purified HFA and NFA fractions of nGalCer and nCBS (Figure 7).

Table III: Effect of Changes in the Ceramide Composition of GalCer and CBS on the Relative Amount of Liposome Aggregation

fatty acid molecular species of GalCer and CBS compared[a]	relative absorbance at 450 nm[b]
Relative to C24:0-GalCer	
C16:0-GalCer + C16:0-CBS	0.38 ± 0.07* (7)
C24:1-GalCer + C24:1-CBS	0.65 ± 0.12** (3)
C24:0-GalCer + C24:0-CBS	1.0 (7)
C26:0-GalCer + C26:0-CBS	1.86 ± 0.28*** (4)
Relative to NFA-nGalCer	
HFA-nGalCer + HFA-nCBS	1.97 ± 0.36† (6)

[a] The dependence of the Mn^{2+}-induced interaction of GalCer and CBS liposomes on the acyl chain length of the glycolipids when both types of liposomes had the same fatty acid. Absorbance values were normalized to that of C24:0-GalCer plus C24:0-CBS liposomes. The mole ratio of glycolipid to PC in the liposomes was determined for one set of samples to be 0.16 for C24:1-CBS, 0.09 for C24:0-CBS, 0.10 for C26:0-CBS, 0.22 for C24:1-GalCer, 0.18 for C24:0-GalCer, and 0.13 for C26:0-GalCer. In the case of the HFA lipids, the relative absorbance due to the Mn^{2+}-induced aggregation of HFA-nGalCer liposomes with HFA-nCBS liposomes was normalized to that due to the Mn^{2+}-induced aggregation of NFA-nGalCer and NFA-nCBS liposomes. [b] Values are expressed as the mean of n experiment ± standard deviation, relative to the absorbance due to the aggregation of C24:0-GalCer liposomes with C24:0-CBS liposomes, with n shown in parentheses. * Value is significantly lower than the relative absorbance due to the aggregation of C24:0-GalCer and C24:0-CBS liposomes, $p < 0.005$. ** Value is significantly lower than the relative absorbance due to the aggregation of C24:0-GalCer and C24:0-CBS liposomes, $p < 0.0025$. Value is also significantly greater than the relative absorbance due to the aggregation of C16:0-GalCer and C16:0-CBS liposomes, $p < 0.005$. *** Value is significantly higher than the relative absorbance due to the aggregation of C24:0-GalCer and C24:0-CBS liposomes, $p < 0.0025$. † Value is significantly greater than the relative absorbance due to the aggregation of NFA-nGalCer liposomes with NFA-nCBS liposomes, $p < 0.005$.

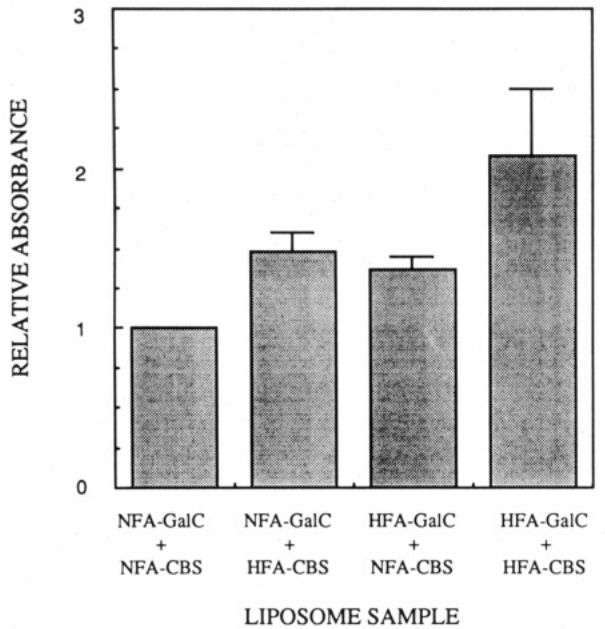

FIGURE 7: Effect of fatty acid hydroxylation of GalCer and CBS. Liposomes were prepared with TLC-purified HFA and NFA fractions of nGalCer and nCBS. Values are normalized to the absorbance of NFA-nGalCer mixed with NFA-nCBS liposomes in 5.5 mM $MnCl_2$ and expressed as the mean of 3 experiments ± the standard deviation. The absorbance of HFA-nGalCer liposomes plus HFA-nCBS liposomes was significantly higher than the absorbance due to the aggregation of NFA-nGalCer with NFA-nCBS liposomes, $p < 0.025$.

Aggregation significantly increased when just one of the glycolipids was hydroxylated, but the interaction was greatest when both were hydroxylated. Overall, the relative absorbance due to the aggregation of HFA-nGalCer and HFA-nCBS was nearly double that due to the aggregation of GalCer and

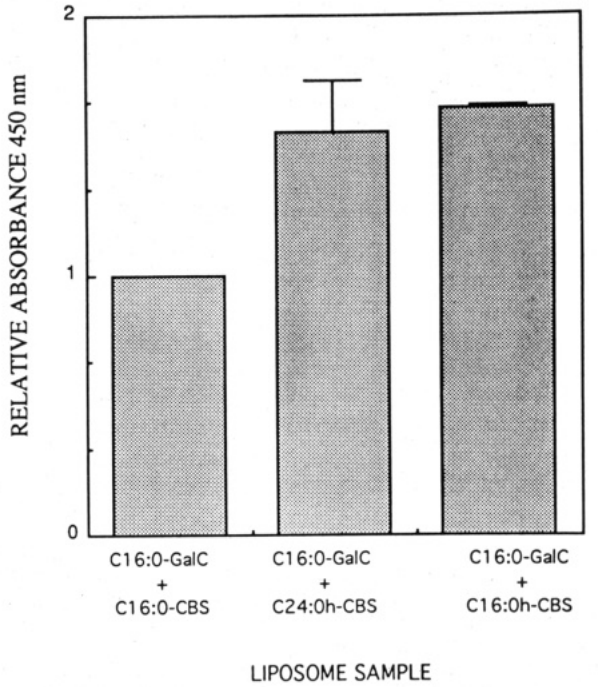

FIGURE 8: Effect of fatty acid hydroxylation and chain length of GalCer and CBS. Liposomes were prepared with C16:0-GalCer and C16:0-CBS, C16:0h-CBS, or C24:0h-CBS. Values are normalized to the absorbance of C16:0-GalCer liposomes mixed with C16:0-CBS liposomes in 5.5 mM $MnCl_2$ and expressed as the mean of two experiments ± the range. The glycolipid to PC ratio in the liposomes was measured for 1 experiment and was 0.16 for C16:0-CBS, 0.9 for C24:0h-CBS, 0.11 for C16:0h-CBS, and 0.14 for C16:0-GalCer.

CBS liposomes containing non-hydroxy fatty acids (Table III). The amount of HFA and NFA species incorporated into the liposomes was not significantly different and could not account for the increased aggregation caused by the HFA species (Table I). Using semisynthetic species of GalCer and CBS, C16:0-GalCer interacted with C16:0h-CBS significantly more than with C16:0-CBS (Figure 8). However, increasing the chain length of the hydroxy fatty acid of CBS from C16:0h to C24:0h did not significantly affect the extent of this interaction. Thus, either an increase in the fatty acid chain length or hydroxylation of the fatty acid increased the amount of aggregation, but the effects of chain length and hydroxylation were not notably additive.

DISCUSSION

The reported carbohydrate–carbohydrate interaction between GalCer and CBS (Hakomori *et al.*, 1991) has been examined further in the present study. The dependence of the divalent cation-induced GalCer–CBS interaction on the ceramide structure of both glycolipids has been examined by measuring the aggregation of liposomes containing GalCer with liposomes containing CBS. The aggregation was due to a heterotypic carbohydrate–carbohydrate interaction, since the amount of aggregation between liposomes containing GalCer and CBS was greater than that due to the self-aggregation of both GalCer and CBS, and no aggregation of vesicles with liposomes lacking glycolipid was found. In addition, CBS was also able to interact with GluCer.

The adhesion between two membranes depends on the balance of the attractive and repulsive forces between the two bilayers (or liposomes). The carbohydrate–carbohydrate interaction of GalCer and CBS provides an attractive force, whereas repulsive forces arise from physical forces between the bilayers such as electrostatic and hydration forces (Helm *et al.*, 1992). The aggregation of GalCer and CBS liposomes

requires addition of a divalent cation, which must increase the attraction between the two sets of liposomes and/or reduce the magnitude of the repulsive forces. Only the CBS vesicles are charged, but the specific interaction between GalCer and CBS was increased in 450 mM NaCl, indicating that there was at least a moderate amount of electrostatic repulsion between the two types of liposomes, perhaps due to the zwitterionic PC molecules.

Many studies have shown that divalent cations are able to interact directly with neutral and anionic carbohydrates, albeit with a relatively low affinity constant on the order of 0.5–5 M^{-1} [*cf.* Angyal (1989) for review]. In some cases conformational changes in the carbohydrate have been noted upon binding with the cation. Both monovalent and divalent cations promote self-aggregation of liposomes of digalactosyldiacylglycerol (Webb *et al.*, 1988). In order to induce interaction of GalCer and CBS, it was necessary to use the divalent cation in great excess, and thus it was present in sufficient amounts to allow low-affinity interactions to occur. At higher concentrations (0.1 M), calcium has been shown to cause self-aggregation of pure CBS liposomes, as well as CBS–PC liposomes (Abramson *et al.*, 1967). It influences the thermotropic behavior of both GalCer and CBS, either in pure form or incorporated into PC bilayers (Boggs *et al.*, 1984; Maggio *et al.*, 1987), and has been shown to bind to the sulfate of CBS (Tupper *et al.*, 1992). However, unlike its effect on acidic phospholipids, it lowers the phase transition temperature of CBS relative to Na^+ (Boggs *et al.*, 1984) and it does not cause phase separation of CBS from PC (Maggio *et al.*, 1987). GalCer has been reported to have weak ionophoretic properties for calcium when added to red cell membranes (Shibuya *et al.*, 1990).

The effect of the divalent cation may be to alter the conformation of the carbohydrate of either or both glycolipids such that they are able to interact directly, or to participate in the formation of a hydrated bridge between the two carbohydrate moieties, as has been shown for the interaction of Ca^{2+} with fucose (Cook & Bugg, 1976). Neutral sugars can chelate divalent cations through sets of hydroxyl groups or other oxygens which substitute for some of the water molecules in the cation hydration shell. Anionic groups of charged sugars may participate in the coordination complex. The inverse relationship between the ionic crystal radius of the divalent cations and their ability to induce the specific aggregation of GalCer liposomes with CBS liposomes suggests that the more hydrated cations interact better with these lipids. They may be better able to form coordination complexes with oxygen atoms of the glycolipids. The poor ability of Mg^{2+} to cause aggregation despite its small ionic radius may be due to the fact that it binds water more tightly than Ca^{2+} (Samoilov, 1957) and thus may not readily exchange it for the sugar oxygens. However, the physical basis for the carbohydrate–carbohydrate interaction of GalCer and CBS is at present unknown, and further experiments are required to answer this question.

An important result from this study is that the interaction between GalCer and CBS was increased when the fatty acid chain length of one or both of the glycolipids was increased. Even an increase in the acyl chain length of both GalCer and CBS by only two carbons, from C24:0 to C26:0, had a large effect. The presence of a double bond in the C24 chain decreased the interaction. Previous studies from our (Crook *et al.*, 1986; Stewart & Boggs, 1990, 1991, 1993) and other laboratories (Alving & Richards, 1977; Kannagi *et al.*, 1982; Nudelman *et al.*, 1982; Yoshino *et al.*, 1982) have shown that an increase in the fatty acid chain length of glycolipids, including both GalCer and CBS, up to 24 carbons increases the accessibility or exposure of their carbohydrate head groups at the bilayer surface to protein ligands, while a double bond in the C24 chain decreases exposure. This may indicate that the saturated acyl chain of the glycolipid, rather than interdigitating across the bilayer, terminates at the bilayer center, thus forcing the carbohydrate head group to protrude above the plane of the membrane surface at least transiently. This increased exposure may account for the increased interaction between the galactosyl and galactosyl 3-sulfate groups when the fatty acid chain length of either GalCer and/or CBS is increased. It may also result in a partial exposure of the acyl chain, which may promote hydrophobic interactions between the two liposome bilayers or reduce the hydration of the bilayer surface, and thereby increase the extent of aggregation. Indeed, Helm *et al.* (1992) have shown that, when the exposure of hydrophobic groups at the bilayer surface is increased, adhesion between bilayers is significantly improved. The reduced exposure of the C24:1 molecular species, compared to C24:0 molecular species, may be due to the former's greater ability to bend and kink in the membrane and, thus, reduce its effective fatty acid chain length.

Although increasing the fatty acid chain length of glycolipids from C24:0 to C26:0 increased the GalCer–CBS interaction even more than the increase from 16 to 24 carbons, our previous study showed that an increase from 24 to 26 carbons caused a decrease in the exposure of GalCer, suggesting that the longer acyl chain of C26:0-GalCer may partially interdigitate across the bilayer center (Stewart & Boggs, 1991, 1993). However, the effect of the C26 chain on the exposure of CBS has not been measured. Furthermore, the effect on the exposure of GalCer was measured using multilamellar liposomes (MLV), whereas the present study used small unilamellar vesicles (SUV), which may not allow the C26:0 molecular species to interdigitate due to the higher packing density of the lipid molecules of the inner monolayer of these vesicles. Thus in SUVs a further increase in the exposure of the carbohydrate head group may occur when the acyl chain length is increased from C24:0 to C26:0.

The effect of hydroxylation of the fatty acid chain in promoting the aggregation of GalCer and CBS liposomes is also in apparent contrast to its previously reported effect in reducing the accessibility of both glycolipids to external ligands (Crook *et al.*, 1986; Stewart & Boggs, 1990, 1991, 1993). The current study has shown that even when a short chain fatty acid of CBS is hydroxylated, the extent of aggregation is increased. This also may be due to differences in the structure of the liposomes used in the earlier study. Alternatively, the hydroxyl group may promote adhesion by its participation in hydrogen bonding even though it decreases exposure. This is supported by the lack of an effect of an increase in chain length of the hydroxylated species on the liposome aggregation. Hydroxylation of the fatty acid of CBS and GalCer promotes lateral intermolecular hydrogen bonding of other hydrogen bonding groups of ceramide, i.e., the sphingosine hydroxyl and the amide moiety (Boggs, 1987; Boggs *et al.*, 1984; Tupper *et al.*, 1992), possibly allowing them to hydrogen bond with the carbonyl or phosphate moieties in phosphatidylcholine. This, in turn, may cause the glycolipid to remain embedded within the bilayer and thereby reduce exposure of the carbohydrate at the membrane surface. However, the sugar of GalCer is oriented parallel to the bilayer, which could expose the fatty acid hydroxyl group on the bilayer surface (Nyholm *et al.*, 1990). This might allow it to hydrogen bond to the carbohydrate of CBS in an apposing bilayer or to participate in chelation with the cation. The fatty acid hydroxyl group

may also improve the affinity of the interaction between the carbohydrate head groups of GalCer and CBS by affecting the conformation of the carbohydrate and/or the binding of the divalent cation. Although only a small change in the conformation of the galactosyl head group has been noted in GalCer molecular species with hydroxy fatty acids by ^{2}H-NMR (Jarrell *et al.*, 1992), the presence of the cation may cause a greater effect.

The influence of glycolipid ceramide composition on the carbohydrate–carbohydrate interactions between different bilayer surfaces demonstrated here suggests that changes in the ceramide composition of glycolipids could play a role in membrane surface recognition events, such as cell adhesion. This interaction between GalCer and CBS may play a role in the formation or maintenance of the myelin membrane. Myelin is a specialized membrane which ensheathes axons of the central and peripheral nervous systems and promotes saltatory conduction along the axon. In vertebrates it has a highly compact structure consisting of concentric layers of membrane wrapping the axon. CBS and GalCer are the major glycolipids of myelin, accounting, respectively, for 3.8 and 22.7 wt % of the total myelin lipid (Norton, 1977). Considering that most if not all of the glycolipid is probably situated on the extracellular face of the myelin membrane (the intraperiod line) and that the extracellular concentration of calcium is in the millimolar range, these two glycolipids may well be involved in the close adhesion of the extracellular surfaces of the myelin membrane.

Indeed, Padrón *et al.*, (1979) have shown that the interaction of divalent cations with some receptor on the extracellular surface plays an important role in the compaction of myelin. When intact peripheral nerve segments were incubated in water or hypotonic buffer, the extracellular surfaces of the myelin membrane were resistant to swelling, separating only very slowly. This swelling could be rapidly reversed by the introduction of isotonic media. If the isotonic media lacked divalent cations, the myelin rapidly reswelled upon readdition of hypotonic media. However, when the isotonic media also contained divalent cations, the resultant recompacted myelin was as resistant to subsequent hypotonic shock as native myelin. This suggests that divalent cations may participate in the interactions between the extracellular surfaces of the myelin membrane. Our work shows that the receptors with which the divalent cation interacts may well be the major glycolipids, GalCer and CBS. Myelin also swells at low pH. Interestingly, Inouye and Kirschner (1984) found that divalent cations inhibited this swelling relative to Na^+ in a similar order, $Zn^{2+} > Ca^{2+} > Ba^{2+}$, as found for divalent cation-induced aggregation of the GalCer and CBS liposomes. Antibody (decomplemented) to GalCer can also induce swelling of myelin, supporting the notion that GalCer has an important role in the maintenance of the compact myelin membrane (Raine *et al.*, 1981).

The extracellular spacing of central nervous system myelin, as measured from the surface of the phospholipid head groups, is 24 Å (Blaurock, 1981). It may be possible for a galactose–Ca^{2+}-sulfated galactose complex to span this extracellular space provided that the sugars are extended into the aqueous phase and are located on a plane above the phospholipid polar head groups. That the latter may occur, at least for the long fatty acid chain species, is suggested by their increased exposure to antibody and galactose oxidase. The length of each sugar is about 6 Å from the O-glycosidic bond and about 9.5 Å from C-3 of sphingosine (Nyholm et al, 1990). The length of a Ca^{2+} bridge between two sugars is about 5 Å (Cook & Bugg, 1975), giving a total distance of 17–24 Å, depending on the vertical location of the glycolipid with respect to the phospholipids. Although the extracellular spacing is greater in peripheral nervous system myelin, making it less likely that a glycolipid carbohydrate–carbohydrate bridge maintains this structure, such an interaction might be important in the initial stages of myelin formation before insertion of proteins.

Even though the interaction of GalCer and CBS may be relatively weak (as compared to a specific protein–protein interaction), the extensive number of interactions theoretically possible due to the high membrane concentration of these lipids could result in an overall strong multivalent interaction. There are approximately 5 nmol of CBS and 30 nmol of GalCer per 100 μg of dry myelin, whereas in the case of the most abundant myelin proteins, proteolipid protein and myelin basic protein, there are only 0.7 and 0.5 nmol per 100 μg of myelin, respectively. Furthermore, myelin basic protein is thought to be located on the intracellular surfaces. Other, myelin proteins are much less abundant. Thus, GalCer and CBS are very likely adhesion molecules at least partly responsible for the compaction of central nervous system myelin membranes at the extracellular face of the membrane. A low-affinity interaction which does not result in fusion would allow the extracellular surfaces to separate and readhere as the myelin sheath grows.

The importance of glycolipids in formation of compact myelin has been noted before on the basis of phylogenetic studies. Earthworm myelin, which contains only traces of glycolipids, has a loosely wrapped, noncompacted form of myelin (Okamura *et al.*, 1985). Shrimp myelin, which has an abundance of GluCer, but lacks GalCer or any sulfated glycolipids, also is loosely compacted (Kishimoto, 1986). It is noteworthy that the space at the extracellular apposition of myelin is wider for teleost than higher vertebrates (Inouye & Kirschner, 1990), and the former have lower proportions of galactolipids than the higher vertebrates (Selivonchick & Roots, 1976; Burgisser *et al.*, 1986). In addition, a phylogenetically lower order of deep sea fish, the Gadiformes, whose myelin has an unusual glycolipid composition, has thin loosely compacted myelin compared to more advanced species. The lipid composition of the Gadiforme species is characterized by a high proportion of GluCer, the fatty acid ester of GluCer, and galactoglycerolipids rather than galactosphingolipids (Tamai *et al.*, 1992), in contrast to higher vertebrates whose myelin contains only trace amounts of these lipids (Selivonchick & Roots, 1976; Burgisser *et al.*, 1986). These studies also suggest that a combination of GalCer and CBS are necessary for compact myelin. Although the present study has shown that there is a limited amount of self-aggregation of GalCer, CBS, and GluCer liposomes, as well as a GluCer–CBS interaction, the greatest interaction occurred between GalCer and CBS liposomes.

Our results not only demonstrate a mechanism by which these two glycolipids might mediate myelin adhesion, but they also suggest that the ceramide moiety of these glycolipids may have a role in its formation. The fatty acid chain length of these two glycolipids and the proportion of hydroxy fatty acids in white matter of higher vertebrates increase with development (Svennerholm & Stallberg-Stenhagen, 1968; Shimomura & Kishimoto, 1983). The proportion of hydroxy fatty acids also tends to increase with the complexity of the nervous system in different species, and species which lack hydroxylated fatty acids have significantly reduced nerve conduction velocities (Ki *et al.*, 1985). The Gadiformes, discussed above, with loosely compacted myelin and unusual glycolipid composition, also have an unusual fatty acid composition. The glycosphingolipids contain mainly C24:1

with a complete absence of hydroxy fatty acids, in contrast to the high proportion of saturated long-chain fatty acids and hydroxy fatty acids in the myelin of higher vertebrates. Thus, the increased GalCer–CBS interaction with increasing fatty acid chain length and hydroxylation reported here suggests that changes in the fatty acid composition of the myelin glycolipids which occur in different species and in higher vertebrates during development may play a role in the maintenance and/or formation of compact myelin. These results may also be related to the pathogenic effect of the very long chain fatty acids which accumulate in myelin lipids in adrenoleukodystrophy.

ACKNOWLEDGMENT

We thank Mrs. G. Rangaraj for technical assistance and Dr. K. M. Koshy for synthesis of molecular species of CBS and galactose 3-sulfate.

REFERENCES

Abramson, M. B., Katzman, R., Curci, R., & Wilson, C. E. (1967) *Biochemistry 6*, 295–304.
Alving, C. R., and Richards, R. L. (1977) *Immunochemistry 14*, 373–383.
Angyal, S. J. (1989). *Adv. Carbohydr. Chem. Biochem. 47*, 1–43.
Archibald, P. J., Fenn, M. D., & Roy, A. B. (1981) *Carbohydr. Res. 93*, 177–190.
Batzri, S., & Korn, E. S. (1973) *Biochim. Biophys. Acta 298*, 1015–1019.
Blaurock, A. E. (1981) *Brain Res.210*, 383–387.
Boggs, J. M. (1987) *Biochim. Biophys. Acta 906*, 353–404.
Boggs, J. M.,Koshy, K. M., & Rangaraj, G. (1984) *Chem. Phys. Lipids 36*, 65–89.
Boggs, J. M., Koshy, K. M., & Rangaraj, G. (1988) *Biochim. Biophys. Acta 938*, 373–385.
Burgisser, P., Matthieu, J.-M., & Waehneldt, T. V. (1986) *Neurochem. Res. 11*, 1261–1272.
Cook, W. J., & Bugg, C. E. (1975) *Biochim. Biophys. Acta 389*, 428–435.
Crook, S. J., Boggs, J. M., Vistnes, A. I., & Koshy, K. M. (1986) *Biochemistry 25*, 7488–7494.
Eggens, I., Fenderson, B., Toyokuni, T., Dean, B., Stroud, M., & Hakomori, S. (1989) *J. Biol. Chem. 264*, 9467–9484.
Hakomori, S. (1984) *Annu. Rev. Immunol. 2*, 103–126.
Hakomori, S., Igarashi, Y., Kojima, N., Okoshi, K., Handa, B., & Fenderson, B. (1991). *Glycoconjugate J. 8*, 178.
Helm, C. A., Israelachvili, J. N., & McGuiggan, P. M. (1992) *Biochemistry 31*, 1794–1805.
Inouye, H. & Kirschner, D. A. (1984) *Biochim. Biophys. Acta 776*, 197–208.
Inouye, H., & Kirschner, D. A. (1990) Phylogenetic aspects of myelin structure. In *Cellular and Molecular Biology of Myelination* (Jeserich, G., Althaus, H. H., & Waehneldt, T. V., Eds.) pp 376–387, Springer-Verlag, Berlin.
Jarrell, H., Singh, D., & Grant, C. W. M. (1992) *Biochim. Biophys. Acta 1109*, 331–334.
Kannagi, R., Nudelman, E. & Hakomori, S. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3470–3474.
Karlsson, K.-A. (1982) in *Biological Membranes* (Chapman, D., Ed.), Vol. 4, pp 1–74, Academic Press, New York.
Karlsson, K.-A. (1989) *Annu. Rev. Biochem. 58*, 309–350.
Ki, P. F., Kishimoto, Y. Y., Lattman, E. E., Stanley, E. F., & Griffin, J. W. (1985) *Brain Res. 345*, 19–24.
Kishimoto, Y. (1986) *Chem. Phys. Lipids 42*, 117–128.
Kishimoto, Y., Moser, H. W., & Suzuki, K. (1985) in *Handbook of Neurochemistry* (Lajtha, A., Ed.) Vol. 10, pp 125–151, Plenum Press, New York.
Koiv, A., & Kinnunen, P. K. J. (1992) *Chem. Phys. Lipids 62*, 253–261.
Kojima, N., & Hakomori, S. (1989) *J. Biol. Chem. 264*, 20159–20162.
Kojima, N., & Hakomori, S. (1991) *J. Biol. Chem. 266*, 17552–17558.
Koshy, K. M., & Boggs, J. M. (1983) *Chem. Phys. Lipids 34*, 41–53.
Lin, H.-N., & Huang, C.-H. (1988) *Biochim. Biophys. Acta 946*, 178–184.
Lindberg, A. A., Brown, J. E., Stromberg, N., Westling-Ryd, M., Schultz, J. E., & Karlsson, K. A. (1987) *J. Biol. Chem. 262*, 1779–1785.
Maggio, B., Sturtevant, J. M., & Yu, R. K. (1987) *Biochim. Biophys. Acta 901*, 173–182.
Naoi, M., Lee, Y. C., & Roseman, S. (1974) *Anal. Biochem. 58*, 571–577.
Norton, W. T. (1977) in *Myelin* (Morell, P., Ed.) pp 161–199, Plenum Press, New York.
Nudelman, E., Hakomori, S.-I., Kannagi, R., Levery, S., Yeh, M.-Y., Hellstrom, K. E., & Hellstrom, I. (1982) *J. Biol. Chem. 257*, 12752–12756.
Nyholm, P-G., Pascher, I., & Sundell, S. (1990) *Chem. Phys. Lipids 52*, 1–10.
Ogawa-Goto, K., Funamoto, N., Abe, T., & Nagashima, K. (1990) *J. Neurochem. 54*, 1486–1493.
Okamura, N., Stoskopf, M., Yamaguchi, H., & Kishimoto, Y. (1985) *J. Neurochem. 45*, 1875–1879.
Padrón, R., Mateu, L., & Kirschner, D. A. (1979) *Biophys. J. 28*, 231–240.
Palestini, P., Masserini, M., Sonnino, S., Giuliani, A., & Tettamanti, G. (1990) *J. Neurochem. 54*, 230–235.
Radin, N. S. (1972) *Methods Enzymol. 28*, 300–306.
Raine, C. S., Johnson, A. B., Marcus, D. M., Suzuki, A., & Bornstein, M. B. (1981) *J. Neurol. Sci. 52*, 117–131.
Samoilov, O. Ya. (1957) *Disc. Faraday Soc. 24*, 141–146.
Selivonchick, D. P., & Roots, B. I. (1976) *J. Thermal Biol. 1*, 131–135.
Shibuya, H., Kawashima, K., Sakagami, M., Kawanishi, H., Shimomura, M., Ohashi, K., & Kitagawa, I. (1990) *Chem. Pharm. Bull. 38*, 2933–2938.
Shimomura, R., & Kishimoto, Y. (1983) *Biochim. Biophys. Acta 754*, 93–100.
Stewart, R. J., & Boggs, J. M. (1990) *Biochemistry 29*, 3644–3653.
Stewart, R. J., & Boggs, J. M. (1991) *Glycoconjugate. J. 8*, 163.
Stewart, R. J., & Boggs, J. M. (1993) *Biochemistry 32*, 5605–5614.
Svennerholm, L., & Stallberg-Stenhagen, S. (1968) *J. Lipid Res. 9*, 215–225.
Tamai, Y., Kojima, H., Saito, S., Takayama-Abe, K., & Horichi, H. (1992) *J. Lipid Res.33*, 1351–1359.
Tupper, S., Wong, P. T. T., & Tanphaichitr, N. (1992) *Biochemistry 31*, 11902–11907.
Yoshino, T., Watanabe, K., & Hakomori, S. I. (1982) *Biochemistry 21*, 928–934.
Young, W. W., Jr., Durdik, J. M., Urdal, D., Hakomori, S. I., & Henney, C. S. (1981) *J. Immunol. 126*, 1–6.
Weast, R. C., & Astle, M. J., Eds. (1980) *CRC Handbook of Chemistry and Physics*, 60th Ed., F-214–215, CRC Press, Inc., Boca Raton, FL.
Webb, M. S., Tilcock, C. P. S., & Green, B. R. (1988) *Biochim. Biophys. Acta 938*, 323–333.