Divalent Cation-Mediated Interaction Between Cerebroside Sulfate and Cerebrosides: An Investigation of the Effect of Structural Variations of Lipids by Electrospray Ionization Mass Spectrometry

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ABSTRACT Divalent cations mediate a carbohydrate-carbohydrate association between the two major glycolipids, galactosylceramide (GalCer) and its sulfated form, cerebroside sulfate (CBS), of the myelin sheath. We have suggested that interaction between these glycolipids on apposed extracellular surfaces of myelin may be involved in the stability or function of this multilayered structure. A mutant mouse lacking galactolipids because of a disruption in the gene that encodes a galactosyltransferase forms myelin that initially appears relatively normal but is unstable. This myelin contains glucosylceramide (GlcCer) instead of GalCer. To better understand the role of GlcCer in myelin in this mutant, we have compared the ability of divalent cations to complex CBS (galactosyl form) with GlcCer or GalCer in methanol solution by using positive ion electrospray ionization mass spectrometry. Because both the α-hydroxylated fatty acid species (HFA) and the nonhydroxylated fatty acid species (NFA) of these lipids occur in myelin, we have also compared the HFA and NFA species. In addition to monomeric Ca^{2+} complexes of all three lipids and oligomeric Ca^{2+} complexes of both GalCer and GlcCer, Ca^{2+} also caused heterotypic complexation of CBS to both GalCer and GlcCer. The heterotypic complexes had the greatest stability of all oligomers formed and survived better at high declustering potentials. Complexes of CBS with GlcCer were less stable than those with GalCer. This was confirmed by using the free sugars and glycosides making up the carbohydrate headgroups of these lipids. HFA species of CBS and GalCer formed more stable complexes than NFA species, but hydroxylation of the fatty acid of GlcCer had no effect. The ability of GlcCer to also complex with CBS, albeit with lower stability, may allow GlcCer to partially compensate for the absence of GalCer in the mouse mutant.

INTRODUCTION

The role of interactions between carbohydrates, often mediated by divalent cations such as calcium, in cell recognition and adhesion has been the subject of many recent investigations (Eggens et al., 1989; Kojima and Hakomori, 1989, 1991; Misevic and Burger, 1993; Siuzdak et al., 1993; Hakomori, 1991; Kojima et al., 1992; Huang, 1978; Spillmann et al., 1993, 1995; Misevic et al., 1987; Stewart and Boggs, 1993a; Koshy and Boggs, 1996; Zimmerman et al., 1998). Although individually these interactions are relatively weak, they are strengthened by multivalent presentation of carbohydrate on the surface of a membrane or polymer (Yu et al., 1998; Dammer et al., 1995; Spillman, 1994; Bovin, 1996). Carbohydrate-carbohydrate interactions have been suggested to play an accessory role in initiating cell adhesion before the involvement of adhesive proteins (Kojima et al., 1992).

A calcium-mediated association between the carbohydrate headgroups of the two major myelin glycolipids, cerebroside sulfate (galactosylceramide-I^3-sulfate) (CBS) and galactosylceramide (GalCer), has been demonstrated by liposome binding to supported lipid films, vesicle aggregation, and electrospray ionization mass spectrometry (ESI-MS) (Hakomori, 1991; Stewart and Boggs, 1993a; Koshy and Boggs, 1996). These two lipids make up 4 and 23 wt %, respectively, of central nervous system (CNS) myelin and 2.5 and 14 wt % of peripheral nervous system (PNS) myelin (Norton, 1977).

Monohexosyl ceramides in normal vertebrate myelin almost exclusively have galactose as the headgroup. However, a recently created mutant mouse lacking galactolipids because of a disruption in the gene that encodes UDP-galactose:ceramide galactosyltransferase (CGT) produced PNS and CNS myelin containing glucosylceramide (GlcCer) instead of GalCer, at 40% of normal levels of GalCer (Bosio et al., 1996; Coetzee et al., 1996). The myelin also contained the sulfated form of GlcCer in the PNS (Bosio et al., 1998) but not in the CNS (Dupree et al., 1998). These substitutions permitted formation of compact myelin, which initially appeared relatively normal (Bosio et al., 1996, 1998; Coetzee et al., 1996), except for abnormal paranodal loops and interactions with the axolemma in the CNS (Dupree et al., 1998). However, in the CNS there were conduction deficits, and the myelin rapidly degenerated after 6 weeks of age (Bosio et al., 1996, 1998; Coetzee et al., 1996). PNS myelin was more stable (Dupree et al., 1998). These studies indicate that either glycolipids are not necessary for myelin formation or that glucolipids can partially

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compensate for the functions of galactolipids. They indicate further that once myelin is formed, the galactosyl form of these lipids and/or a sulfated form of a glycolipid, either GalCer or GlcCer, are necessary for normal myelin function and stability. Possibly the Ca^{2+}-mediated interaction between GalCer and CBS is involved in adhesion between the extracellular surfaces in compact myelin and the paranodal loops (Hakomori, 1991; Stewart and Boggs, 1993a). These and other possible roles of these lipids in myelin have been reviewed recently (Coetzee et al., 1998; Stoffel and Bosio, 1997).

The fatty acids in the ceramide portion of sphingolipids can be nonhydroxylated or α-hydroxylated. The hydroxy fatty acid (HFA) species of GalCer and CBS are present to the extent of 40–60% in vertebrate myelin (Svennerholm and Stallberg-Stenhagen, 1968). Interestingly, the GlcCer and sulfated GlcCer formed in the CGT mutant mouse were exclusively the HFA species. Lipid vesicle aggregation studies have shown that α-hydroxylation of the fatty acid has a positive effect on the ability of CBS and GalCer to interact with each other in the presence of divalent cations (Stewart and Boggs, 1993a). Although interactions of GlcCer in lipid vesicles with CBS have not been measured as extensively as those of GalCer, they appeared to be much weaker (Hakomori, 1991; Stewart and Boggs, 1993a). Vesicle aggregation studies also showed that the ability of divalent cations to mediate this interaction decreased with increase in ionic radius, with Zn^{2+} having a greater effect than Ca^{2+}.

To better understand the role of GlcCer in myelin formation and stability in the CGT mutant and the role of hydroxylation of the fatty acid of glycolipids in normal myelin, we have examined the intermolecular complexes of HFA and nonhydroxy fatty acid (NFA) species of CBS with HFA and NFA species of GalCer and GlcCer in the presence of Ca^{2+} and Zn^{2+} in methanol solution, making use of the capability of ESI-MS to detect such complexes. Only the galactosyl form of CBS was used, because of the difficulty of obtaining sufficient quantitites of the glucosyl form. Interactions between the free sugars and methyl glycosides in acetronitrile/water were also studied.

The relatively mild ionization conditions employed in ESI-MS allow the complexes existing in solution to be carried to the vapor phase, and their m/z values to be determined. Although complex ions detected under ESI-MS conditions do not always accurately reflect complex formation in solution (Robinson et al., 1996), there are many examples in which they do accurately reflect solution behavior (Smith and Light-Wahl, 1993; Langley et al., 1997; Jorgensen et al., 1998). ESI-MS has recently been used to detect complexes of cations with free sugars (Siuzdak et al., 1994; Fura and Leary, 1993) and divalent cation-mediated glycolipid oligomers (Siuzdak et al., 1993). Our previous work has shown that ESI-MS can be successfully used to investigate the calcium-mediated association between NFA species of CBS and GalCer (Koshy and Boggs, 1996). Use of synthetic lipids with uniform molecular mass permits the unambiguous assignment of the relevant peaks.

MATERIALS AND METHODS

Materials

All reagents and solvents used were either analytical grade or high-performance liquid chromatography grade. Calcium chloride ($CaCl_2.2H_2O$) was purchased from Fisher (Fairlawn, NJ); zinc chloride ($ZnCl_2$) from Aldrich (Milwaukee, WI); methanol from Mallinckrodt (Paris, KY); glucose from Caledon (Georgetown, ON, Canada); galactose from BDH (Poole, England); 1-*O*-methyl-β-D-galactopyranoside, 1-*O*-methyl-β-D-glucopyranoside, and 1-β-D-galactosylsphingosine from Sigma (St. Louis, MO); 1-β-D-glucosylsphingosine from Mattreya (Pleasant Gap, PA); stearic acid from Fluka (Switzerland); stearic acid-d_{35} from CDN isotopes (Point-Claire, QC, Canada); and methanol-d_4 from Cambridge Isotope Laboratories (Andover, MA).

Synthesis of lipids and other compounds

Galactosylceramide (*N*-stearoyl form, NFA-GalCer) and cerebroside sulfate (*N*-D-2-hydroxystearoyl form, HFA-CBS; and *N*-stearoyl form, NFA-CBS) were synthesized as described previously (Koshy and Boggs, 1983). For the synthesis of NFA-d_{35}-galactosylceramide, the procedure followed was identical to that for NFA-galactosylceramide, substituting perdeuterated stearic acid (stearic acid-d_{35}; 99.1 atom % of *D*) for stearic acid. A similar procedure was used for the synthesis of glucosylceramide (*N*-stearoyl form; NFA-GlcCer) from glucosylsphingosine. The *N*-D-2-hydroxystearoyl forms of galactosylceramide (HFA-GalCer) and glucosylceramide (HFA-GlcCer) were synthesized from galactosyl- and glucosylsphingosine, respectively, and D-2-hydroxystearic acid, obtained synthetically as described (Koshy and Boggs, 1983). Briefly, the hydroxyl group of D-2-hydroxystearic acid was protected by acetylation, and the acid was converted to the *N*-hydroxy-succinimide ester in the presence of 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide. The succinimide ester was then reacted with galactosyl- and glucosylsphingosine under the same conditions used for the acid chlorides (Kopaczyk and Radin, 1965). After removal of the protecting group by hydrolysis with methanolic KOH, the products were then purified on a silica gel column, using chloroform/methanol (80:20) as the eluting solvent. Acetylation of lysosulfatide was carried out as follows. Lysosulfatide (2 mg) prepared from bovine brain CBS (sulfatide) (Koshy and Boggs, 1982) was dispersed in 250 ml of 5% sodium bicarbonate solution by vortexing. The mixture was stirred at room temperature, and 3 ml of acetic anhydride was added in 1-ml portions at 5-min intervals, and the pH of the solution was then adjusted to 7 by the careful addition of hydrochloric acid. To this was added 1.5 ml of chloroform/methanol (2:1). The sample was thoroughly vortexed and allowed to stand until phase separation occurred. After the upper phase was discarded, the lower chloroform phase was washed twice with the theoretical Folch upper phase (chloroform/methanol/H_2O 3:48:47) and evaporated to dryness. The residue was further dried under vacuum. Electrospray ionization mass spectra confirmed the identity of the product, and it was used without further purification.

Galactose-3-sulfate was synthesized as the potassium salt by published methods (Archibald et al., 1981). To avoid interference by potassium ions in the mass spectra, the potassium ion was replaced with calcium by repeatedly passing through a column of Biorad AG50W-X8 cation exchange resin (calcium form). 1-*O*-Methyl-d_3-β-D-galactopyranoside was synthesized by an adaptation of the method of Cadotte et al. (1952). A mixture of 100 mg of Bio-Rad AG50W-X8 cation exchange resin (hydrogen form), 70 mg of D-galactose, and 0.7 ml of methanol-d_4 were refluxed in a tightly capped test tube for a period of 18 h. The methanolic solution was then decanted off and evaporated to dryness. 1-*O*-Methyl-d_3-β-D-galactopyranoside was then purified from the mixture of isomers by column chromatography on silica gel, using chloroform/methanol/ammo-

nium hydroxide (65:35:4) as the eluting solvent. The product was finally recrystallized from ethyl acetate.

Mass spectral analysis

The limited solubility of HFA-GalCer and HFA-GlcCer in pure methanol required the preparation of stock solutions of these and the corresponding NFA species at concentrations in the range of 80–120 μM, and the solutions were always warmed to room temperature or slightly higher before use, if stored in the refrigerator. The HFA-CBS and NFA-CBS stock solutions were prepared at concentrations in the range of 350–400 μM, $CaCl_2.2H_2O$ at 6.5 mM, and $ZnCl_2$ at 9.9 mM in methanol. Aliquots of these solutions were appropriately diluted with methanol to give the concentrations required for mass spectral analysis, which were normally 20 μM for the lipids and 200 μM for the salts, to give a mole ratio of neutral glycolipid (glycosylceramide, GCer) to CBS and cation of 1:1:10.

For experiments with the free sugars, methanol was found to give poor spectral quality (perhaps because of strong solvation forces). Therefore, acetonitrile/water (1:1) was used as the solvent in these experiments. All of the sugars were appreciably soluble in this solvent. Stock solutions of methyl-glycosides and acetylated lysosulfatide were similarly made in 50% acetonitrile. Although detectable signals corresponding to the heterodimeric ion $[AcLysCBS.\beta\text{-MeG}.Ca^{2+}\text{-H}]^+$ were observed with equimolar amounts of the acetylated lysosulfatide and β-methyl-glycosides (β-MeG) with an excess of Ca^{2+}, a mole ratio of AcLysCBS/β-MeG/Ca^{2+} of 1:10:10 was used because it resulted in greater signal intensity of the complex ion.

ESI mass spectra were acquired on two different Perkin-Elmer Sciex API triple quadrapole mass spectrometers (one API III and the other API 300) in the positive ion mode. They were calibrated with polypropylene glycol, and one unit resolution was achieved through the mass range 30–2400 in the case of the API III model and 30–3000 in the case of the API 300 model. The only ion detected in a negative ion scan in our previous study was $[NFA\text{-}CBS\text{-}H]^-$ (Koshy and Boggs, 1996). Therefore, negative ion scans were not performed in the present study. Sample solutions were introduced at the rate of 5 μl/min. The declustering potential was varied from 50 V to 250 V. Peak assignments in ambiguous cases were confirmed by collision-induced dissociation (CID) experiments. Unassigned peaks in the spectra shown in the figures are probably due to degradation products or association of solvent with the ions, but have not been identified.

RESULTS

Ca^{2+}-mediated association of HFA-CBS with NFA and HFA species of GalCer and GlcCer

In our previous ESI-MS study of the Ca^{2+}-mediated complexation of CBS and GalCer, we used the NFA species of both lipids. In the present study we also used HFA-CBS and varied the cerebroside component, to either HFA or NFA-GalCer or HFA or NFA-GlcCer. Because the glycolipids were insoluble in water or methanol/water, they were studied in 100% methanol. In general, the ESI mass spectra of mixtures of HFA-CBS with any of the glycosyl ceramides (GCer), especially at low declustering potentials (80–150 V), showed ions corresponding to a number of Ca^{2+} adducts of the cerebrosides (Table 1, lines 1–3), along with the Ca^{2+} complex of HFA-CBS (line 5), and varying proportions of ternary complexes of HFA-CBS with the corresponding cerebrosides and Ca^{2+} (Table 1, lines 6 and 7). In the case of HFA-CBS, the only significant Ca^{2+}-associated peak, other than its ternary complexes with cerebroside and Ca^{2+}, is that of the monomer. A peak due to the Na^+ complex of GCer is also observed, because of the ubiquitous presence of Na^+. These results agree with our previous findings for NFA-CBS and NFA-GalCer (Koshy and Boggs, 1996).

TABLE 1 Complex ions typically observed in the ESI mass spectra of combinations of HFA-CBS with NFA and HFA species of GalCer and GlcCer in the presence of excess Ca^{2+}

		m/z[§]	
Line	Ion composition*[#]	NFA	HFA
1	$[GCer.Ca^{2+}]^{2+}$	383.8	391.8
2	$[2GCer.Ca^{2+}]^{2+}$	747.6	763.8
3	$[3GCer.Ca^{2+}]^{2+}$	1111.6	1136.0[¶]
4	$[GCer.Na^+]^+$	751.0	766.8
5	$[HFA\text{-}CBS.Ca^{2+}\text{-H}]^+$	—	862.8
6	$[2HFA\text{-}CBS.GCer.2Ca^{2+}\text{-2H}]^{2+}$	1226.8	1234.6
7	$[HFA\text{-}CBS.GCer.Ca^{2+}\text{-H}]^+$	1590.0	1606.4

*GCer = GalCer or GlcCer.

[#]Although the dimeric species $[2HFA\text{-}CBS.Ca^{2+}\text{-2H}]$ may exist, being uncharged, it will not be detected in the ESI mass spectrum.

[§]Monoisotopic masses are 727.6 for NFA-GCer, 743.6 for HFA-GCer, and 823.6 for HFA-CBS.

[¶]Very little observed in the case of HFA-GlcCer.

Fig. 1 shows a typical ESI mass spectrum of a mixture of HFA-CBS with HFA-GalCer and Ca^{2+} at a declustering potential of 120 V. As a general rule, under ESI conditions, when the declustering potential is raised, the increased collisional energy brings about increased dissociation of ionic complexes and more fragmentation of susceptible ions in the interface (Fura and Leary, 1993; Baca and Kent, 1992). However, the relative intensities of some stable complexes formed between two lipid molecules have been observed by Siuzdak et al. (1993) and by us (Koshy and Boggs, 1996) to increase in response to increasing declustering potential. Changes in relative peak intensities as a function of declustering potential can thus provide useful information for qualitative comparison of the relative stabilities of various ionic complexes. Because the monomer $[CBS+Ca^{2+}\text{-H}]^+$ ion persists in the spectra with appreciable intensity up to a declustering potential of +250 V, we compare the intensity of the other complex ions to the intensity of this ion.

Table 2 summarizes the changes in peak height ratios with declustering potential of the various complex ions for the four species of cerebroside investigated. In all cases the heterodimeric species $[HFA\text{-}CBS.GCer.Ca^{2+}\text{-H}]^+$ shows a progressive increase in intensity relative to the monomer $[HFA\text{-}CBS.Ca^{2+}\text{-H}]^+$ ion as the potential is raised. Although this increase in the ratio may be due to a decrease in the intensity of the monomer rather than an actual increase in intensity of the heterodimer, all other complex peaks due to homo-oligomers show a progressive decrease in relative intensity when compared to the same CBS monomer ion, at least up to 180 V. The continuous increase in the relative intensity of the heterodimeric ions in the spectrum with an increase in the declustering potential is an indication of their greater stability compared to the other molecular complexes of the cerebrosides with Ca^{2+}. Table 2 indicates some

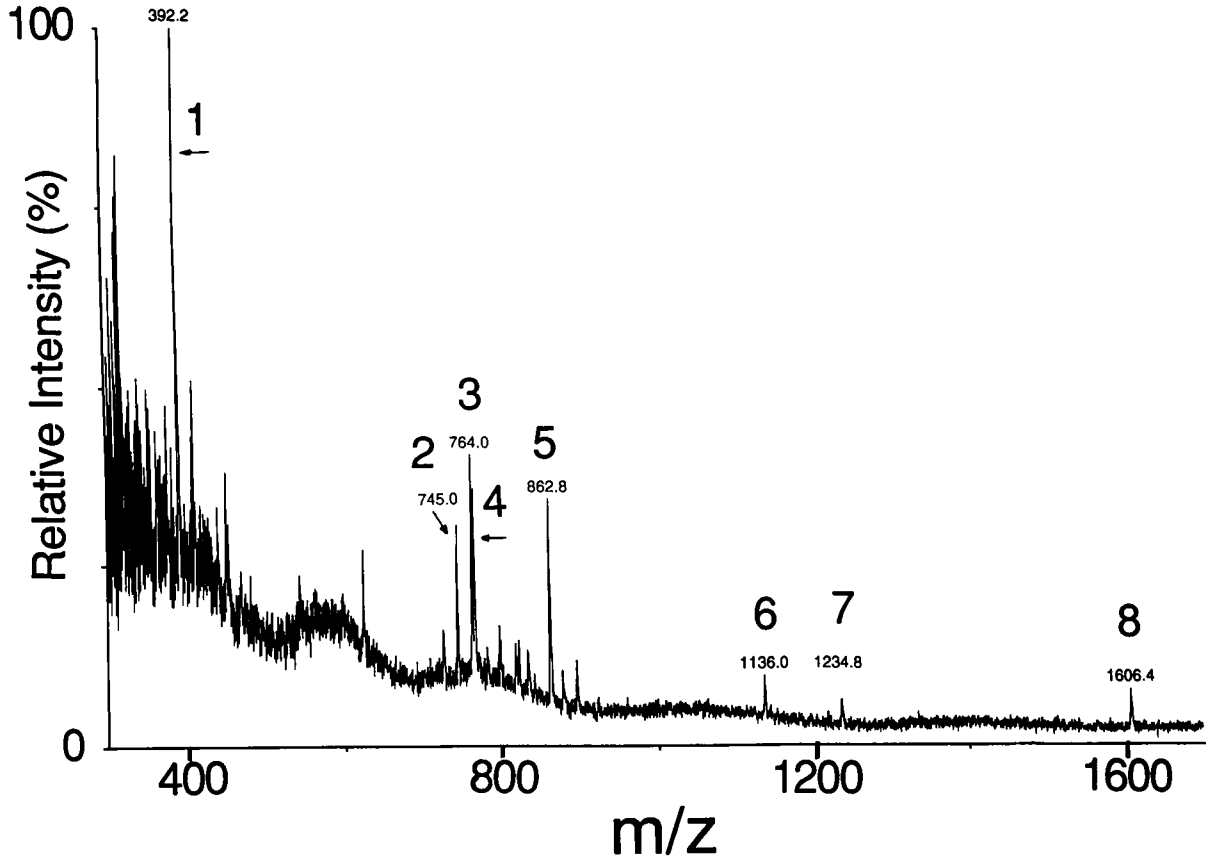

FIGURE 1 ESI mass spectrum of a mixture of HFA-CBS, HFA-GalCer, and calcium chloride in methanol solution in the molar ratio 1:1:10 at a declustering potential of 120 V, showing the various ions normally observable under ESI conditions. The compositions of the ions are as follows: 1. $[HFA\text{-}GalCer.Ca^{2+}]^{2+}$ (392.2), 2. $[HFA\text{-}GalCer.H^{+}]^{+}$ (745), 3. $[2HFA\text{-}GalCer.Ca^{2+}]^{2+}$ (764), 4. $[HFA\text{-}GalCer.Na^{+}]^{+}$ (767), 5. $[HFA\text{-}CBS.Ca^{2+}\text{-}H]^{+}$ (862.8), 6. $[3HFA\text{-}GalCer.Ca^{2+}]^{2+}$ (1136), 7. $[2HFA\text{-}CBS.HFA\text{-}GalCer.2Ca^{2+}\text{-}2H]^{2+}$ (1234.8), and 8. $[HFA\text{-}CBS.HFA\text{-}GalCer.Ca^{2+}\text{-}H]^{+}$ (1606.4). The numbers in parentheses are the observed *m*/*z* values.

differences in the relative abundance in the ESI mass spectrum of the heterodimeric complex of CBS and Ca^{2+} with GalCer and GlcCer. However, these differences may partly depend on uncontrollable variations in the spectrometer interface conditions for different spectra.

To determine more accurately the effect of structural changes in the lipids on the stability of the Ca^{2+}-mediated association between CBS and GCer, competition experiments were performed. Two species of one class of the lipids were combined in equimolar amounts and allowed to compete for an equivalent amount of its complementary lipid class in the presence of excess Ca^{2+} (Table 3). Because GalCer and GlcCer have the same molecular mass, isotopically substituted GalCer containing perdeuterated stearic acid was synthesized. This gave a displacement of 35 mass units for NFA-GalCer from NFA-GlcCer, which permitted the unencumbered observation of their respective complexes with CBS and Ca^{2+} (Fig. 2). In addition to Ca^{2+}-mediated heterodimeric complexes, mixed dimers of GalCer and GlcCer and heterotrimeric complexes of CBS containing both GalCer and GlcCer were also observed at low declustering potentials (Fig. 2).

The amount of one CBS-GCer heterodimer relative to the other in the mixture was compared from the intensity ratios of the two heterodimers observed in each case. Differences in the amount of an ion observed may be due to differences in the amount of the complex formed in solution or to differences in its stability under the ESI conditions. These intensity ratios are shown in Table 3 and were derived from spectra run at high declustering potentials. This minimizes any contribution from weak or nonspecific association occurring at low potentials.

When HFA- and NFA-CBS were present together with NFA-GalCer and Ca^{2+}, the relative intensity of the complex of NFA-GalCer and HFA-CBS was 1.22 times that with NFA-CBS, indicating that the complex with HFA-CBS was formed in greater amounts and/or had greater stability than that with NFA-CBS (Table 3, line 1). This suggested that the fatty acid hydroxyl group of CBS directly or indirectly stabilizes the complex. For the NFA/HFA-GalCer pair, the complex of HFA-CBS with the HFA-GalCer species had greater stability than that with the NFA-GalCer species (Table 3, line 2). In the case of the NFA/HFA-GlcCer pair, however, there was no difference in the relative intensities

TABLE 2 Changes in the relative peak height of various complex ions (compared to [HFA-CBS.Ca^{++}-H]$^+$) with declustering potential of mixtures of HFA-CBS with different species of cerebroside in the presence of excess calcium

Cerebroside species	Declustering potential (V)	Cerebroside monomer with calcium	Cerebroside homodimer with calcium	Cerebroside homotrimer with calcium	CBS/ cerebroside heterotrimer* with calcium	CBS/ cerebroside heterodimer# with calcium
NFA-GalCer	80	12.6	2.8	0.37	0.13	0.08
	100	6.2	1.7	0.33	0.13	0.14
	120	2.8	1.5	0.26	0.14	0.18
	150	0.6	1.4	0.31	0.11	0.29
	180	0.0	1.3	0.32	0.06	0.50
HFA-GalCer	80	7.4	1.3	0.18	0.14	0.11
	100	5.0	1.3	0.23	0.13	0.15
	120	2.6	1.1	0.21	0.13	0.21
	150	0.6	0.9	0.19	0.10	0.24
	180	0.0	0.9	0.23	0.05	0.47
NFA-GlcCer	80	9.4	1.2	0.24	0.11	0.08
	100	3.2	1.1	0.31	0.15	0.10
	120	1.2	0.8	0.27	0.10	0.12
	150	0.3	0.7	0.22	0.07	0.18
	180	0.0	0.6	0.20	0.0	0.27
HFA-GlcCer	80	3.7	0.46	0.0	0.08	0.05
	100	2.6	0.35	0.0	0.10	0.05
	120	1.0	0.33	0.05	0.10	0.07
	150	0.3	0.25	0.0	0.08	0.12
	180	0.0	0.24	0.0	0.0	0.15

*Ion structure given in Table 1, line 6.
#Ion structure given in Table 1, line 7.

of the complex ion peaks of HFA-CBS with NFA-GlcCer and HFA-GlcCer (Table 3, line 3).

The relative stabilities of complexes of HFA versus NFA species with a third lipid (lines 1–3) were not dependent on declustering potential. However, the intensity of the peaks due to the complexes of GalCer-d_{35} with CBS (both NFA and HFA) relative to those due to GlcCer with CBS (lines 4 and 5) increased with an increase in the declustering potential, suggesting that the stabilities of the complexes with NFA-GalCer were greater than those with NFA-GlcCer. This difference in stability of the complex of CBS with NFA-GalCer-d_{35} compared to NFA-GlcCer was maintained even when the ratio of CBS to GCer was doubled (Fig. 2). From these results it is apparent that NFA-GalCer forms more stable complexes with both NFA- and HFA-CBS and Ca^{2+} than NFA-GlcCer does. Although hydroxylation of the fatty acid of GlcCer has little effect, hydroxylation of both CBS and GalCer increases the stability of their Ca^{2+}-mediated complexes with each other.

With a view to further understanding the difference in stability of Ca^{2+}-mediated complexes of GalCer and GlcCer with HFA-CBS, CID analysis was carried out on the

TABLE 3 Comparison of the relative intensities of the heterodimeric ions produced by different pairs of GCer and CBS in competition experiments*

Lipid species compared			Third lipid#	Intensity ratio of the heterodimeric ion peak
Line	A	B	C	AC/BC§
1¶	HFA-CBS	NFA-CBS	NFA-GalCer	1.22 ± 0.08‖ (n = 5)
2	HFA-GalCer	NFA-GalCer	HFA-CBS	1.22 ± 0.06‖ (n = 4)
3	HFA-GlcCer	NFA-GlcCer	HFA-CBS	0.99 ± 0.07** (n = 4)
4	NFA-GalCer-d_{35}	NFA-GlcCer	NFA-CBS	1.56 ± 0.15‖ (n = 5)
5	NFA-GalCer-d_{35}	NFA-GlcCer	HFA-CBS	1.56 ± 0.21‖ (n = 5)

*Four to five different experiments were carried out using two different mass spectrometers.
#C is the third component which forms a heterodimeric complex with Ca^{+2} and either A or B. The mole ratio between A, B, and C was 1:1:1.
§Values were derived from spectra run at the highest orifice potential used (200–250 V).
¶After normalization to the respective [CBS.Ca^{2+}-H]$^+$ ion peak.
‖Values are significantly greater than 1 at $p < 0.01$ by a one-tailed Student's t-test.
**Value is not significantly different from 1 at $p < 0.01$ by a one-tailed Student's t-test.

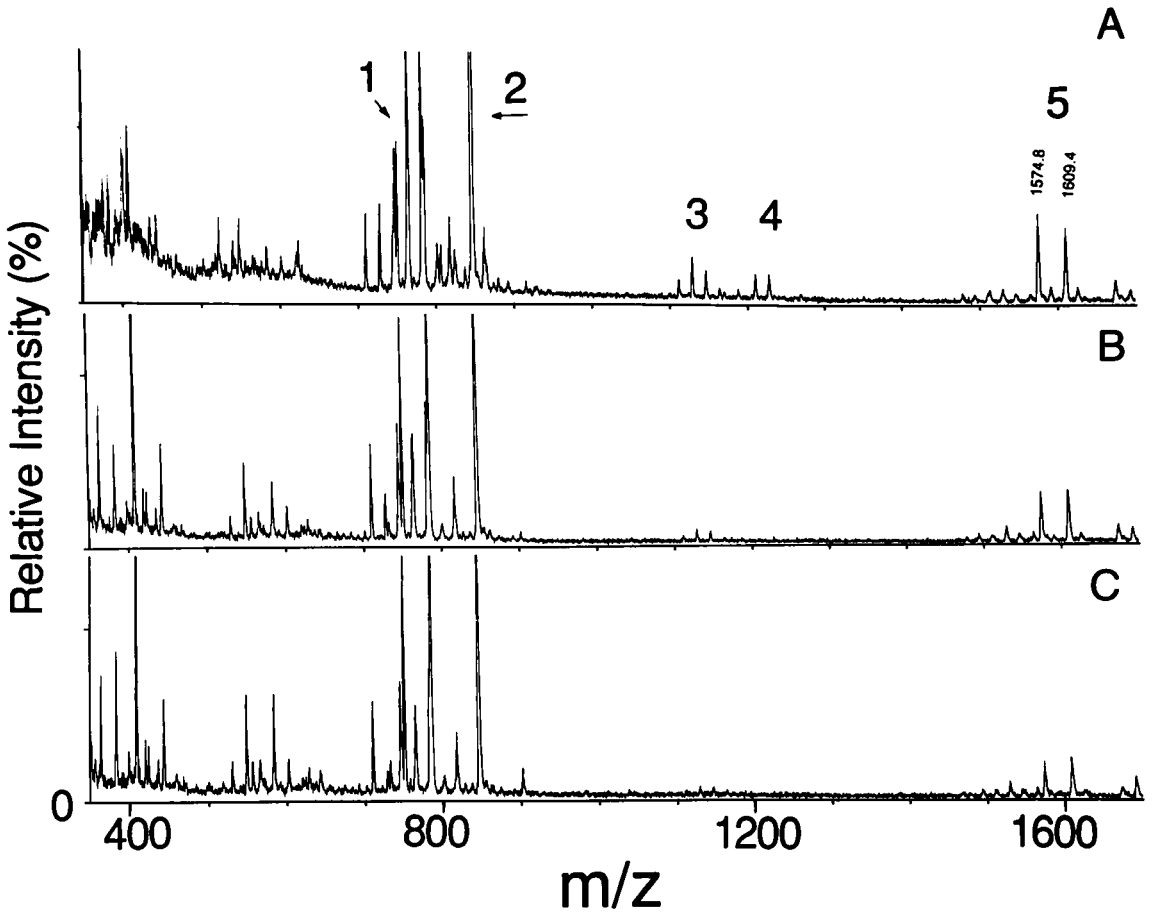

FIGURE 2 ESI mass spectra of a mixture of NFA-CBS, NFA-GalCer-d_{35}, NFA-GlcCer, and calcium chloride in the ratio 2:1:1:10 at declustering potentials of 100 V (*A*), 150 V (*B*), and 180 V (*C*). The peaks of interest are the following: 1. A set of three peaks corresponding to the compositions $[2\text{NFA-GlcCer.Ca}^{2+}]^{2+}$ (747.6), $[\text{NFA-GlcCer.NFA-GalCer-}d_{35}\text{.Ca}^{2+}]^{2+}$ (765), and $[2\text{NFA-GalCer-}d_{35}\text{.Ca}^{2+}]^{2+}$ (783) (peaks at 751 and 786 are the sodium adducts of NFA-GlcCer and NFA-GalCer-d_{35}, respectively); 2. $[\text{NFA-CBS.Ca}^{2+}\text{-H}]^{+}$ (847); 3. a set of three peaks corresponding to the compositions $[3\text{NFA-GlcCer.Ca}^{2+}]^{2+}$ (1111.4), $[2\text{NFA-GlcCer.NFA-GalCer-}d_{35}\text{.Ca}^{2+}]^{2+}$ (1129.4), and $[\text{NFA-GlcCer.2NFA-GalCer-}d_{35}\text{.Ca}^{2+}]^{2+}$ (1146.4); 4. a set of two peaks corresponding to the compositions $[2\text{NFA-CBS.GlcCer.2Ca}^{2+}\text{-2H}]^{2+}$ (1211.0) and $[2\text{NFA-CBS.GalCer-}d_{35}\text{.2Ca}^{2+}\text{-2H}]^{2+}$ (1229.0); 5. a set of two peaks corresponding to the compositions $[\text{NFA-CBS.NFA-GlcCer.Ca}^{2+}\text{-H}]^{+}$ (1574.8) and $[\text{NFA-CBS.NFA-GalCer-}d_{35}\text{.Ca}^{2+}\text{-H}]^{+}$ (1609.4).

heterodimeric complex ions containing Ca^{2+}, CBS, and NFA-GalCer or NFA-GlcCer. Both of them gave on dissociation $[\text{HFA-CBS.Ca}^{2+}\text{-H}]^{+}$ (m/z = 863.2) as the base peak with fragment ions at m/z = 364.2 and at 424.4. While confirming the composition of the parent ions, this also indicates that the fragments most likely arise from the covalent decomposition of the cerebrosides. There were minor differences only in the relative intensities of the fragment ions in the two cases.

Association of HFA-CBS with GalCer and GlcCer in the presence of Zn^{2+}

A similar series of ESI-MS experiments was performed, substituting Zn^{2+} for Ca^{2+}. Because of differences in the polarizing ability and electronegativity of Zn^{2+} as compared to Ca^{2+} (Douglas et al., 1994), more fragmentation of the lipids was observed, even at low declustering potentials. Furthermore, less oligomerization of GCer was observed with Zn^{2+} than with Ca^{2+}, even at a low declustering potential. Table 4 summarizes the major ions of interest identifiable in the spectra. Two novel features of the Zn^{2+} spectra worth mentioning are 1) the appreciable desulfation

TABLE 4 Various complex ions observed for combinations of HFA-CBS with NFA and HFA species of GalCer and GlcCer with Zn^{2+}*

	m/z#	
Ion composition	NFA-GCer	HFA-GCer
$[2\text{GCer.Zn}^{2+}]^{2+}$	759.4	776.0
$[\text{GCer.Zn}^{2+}\text{-H}]^{+}$	790.8	806.8
$[\text{HFA-CBS.Zn}^{2+}\text{-H}]^{+}$	—	886.8
$[\text{HFA-CBS.GCer.Zn}^{2+}\text{-H}]^{+}$	1614.4	1631.0

*Low-intensity peaks corresponding to the heterotrimer $[2\text{HFA-CBS.HFA-GCer.2Zn}^{2+}\text{-2H}]^{2+}$ and the GlcCer trimer $[3\text{NFA-GlcCer.Zn}^{2+}]^{2+}$ were also observed at certain declustering potentials.

#Peak broadening due to the natural isotope distribution in zinc caused slight variations in the observed m/z.

of HFA-CBS to HFA-GalCer by neutral loss of SO_3, and 2) the formation of the Zn^{2+} complexes of the deprotonated cerebrosides $[GCer.Zn^{2+}\text{-}H]^+$. In the case of mixtures of HFA-GCer with HFA-CBS, the latter process is indistinguishable from desulfation because the *m/z* values are identical for the product ions. However, because deprotonation occurred in the case of NFA-cerebrosides, it most likely occurred in the case of HFA-cerebrosides as well.

Changes in the ratio of the intensities of the heterodimeric complex of HFA-CBS with the different cerebrosides and Zn^{2+} to the intensity of the monomer $[HFA\text{-}CBS.Zn^{2+}\text{-}H]^+$ ion with declustering potential are shown in Fig. 3 for the four cerebrosides. In all cases the ratios are significantly higher than those found for calcium (Table 2), but as found for Ca^{2+}, they show a further increase with the declustering potential. At the highest declustering potential employed, 180 V, the only major peaks remaining in the spectra between 700 and 2000 mass units are those due to the heterodimer $[HFA\text{-}CBS.GCer.Zn^{2+}\text{-}H]^+$ and the monomers $[HFA\text{-}CBS.Zn^{2+}\text{-}H]^+$ and $[GCer.Zn^{2+}\text{-}H]^+$ (e.g., Fig. 4). Thus the heterodimeric complexes of HFA-CBS with all four cerebrosides and Zn^{2+} possess considerable stability compared to oligomeric complexes of GCer with Zn^{2+}.

In the presence of Zn^{2+}, the NFA species of both GalCer and GlcCer appeared to form the most stable complexes with HFA-CBS (Fig. 3). HFA-GlcCer and HFA-GalCer appeared to form significantly less stable complexes than the NFA species, in contrast to Ca^{2+}. However, competition experiments between these lipid species in the presence of Zn^{2+} were not performed. These differences could be a result of the increased fragmentation, desulfation, and deprotonation of the lipids that occurred in the presence of Zn^{2+}.

When Zn^{2+} and Ca^{2+} are present in equimolar amounts in the same solution with HFA-CBS and HFA-GalCer, the spectrum is dominated by the calcium complexes of the lipids, perhaps because Ca^{2+} complexes may enter the vapor phase more readily. However, comparison of peak intensity ratios of the heterodimeric complex ion $[CBS.GCer.M^{2+}\text{-}H]^+$ relative to the monomer $[CBS.M^{2+}\text{-}H]^+$ for the respective cations, M^{2+}, will compensate for this difference between the two cations. The relative intensities of the heterodimeric complex ion peaks at $m/z = 1631$ (Zn^{2+}) and 1606.6 (Ca^{2+}), expressed as ratios (Zn^{2+}/Ca^{2+}), at three different declustering potentials, are given in Table 5. This ratio is greater than 1 and increases with declustering potential, indicating greater stability of the Zn^{2+} heterodimeric complex compared to that with Ca^{2+}. Because this pair of lipids, HFA-CBS and HFA-GalCer, may form the least abundant or least stable complex in the case of Zn^{2+} (Fig. 3) and one of the most stable complexes in the case of Ca^{2+} (Table 3, line 2), the relative ability of Zn^{2+} to stabilize complexes of other pairs of the glycolipids should be even greater.

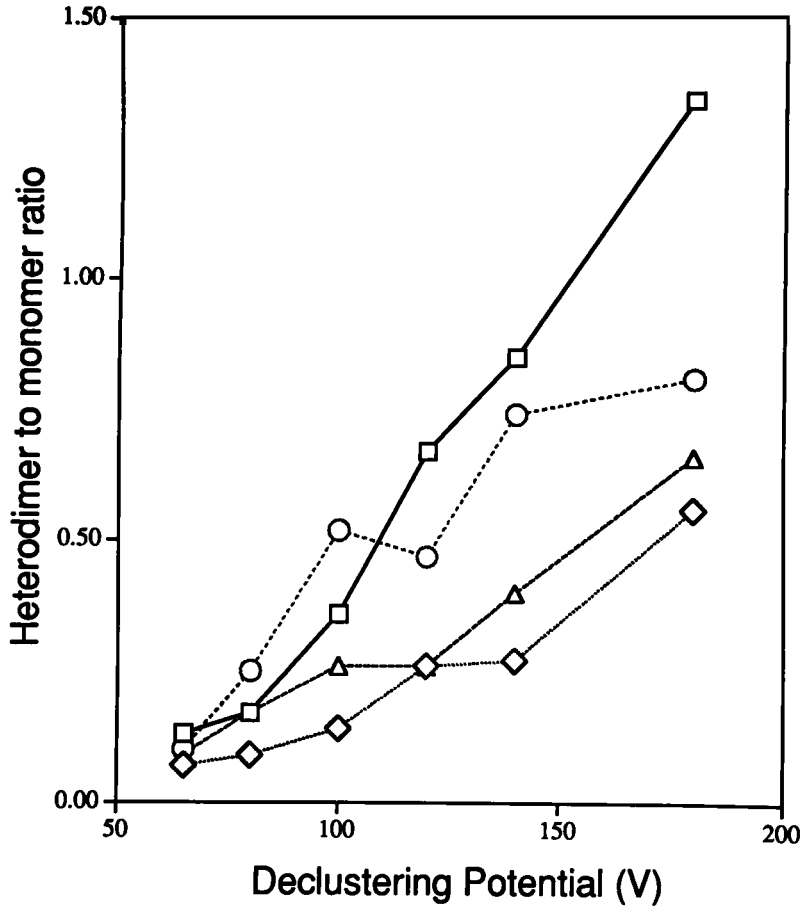

FIGURE 3 The variation of the relative intensity of the peak due to the heterodimeric ion $[HFA\text{-}CBS.GCer.Zn^{2+}\text{-}H]^+$ with declustering potential. □, NFA-GalCer; ◇, HFA-GalCer; ○, NFA-GlcCer; △, HFA-GlcCer.

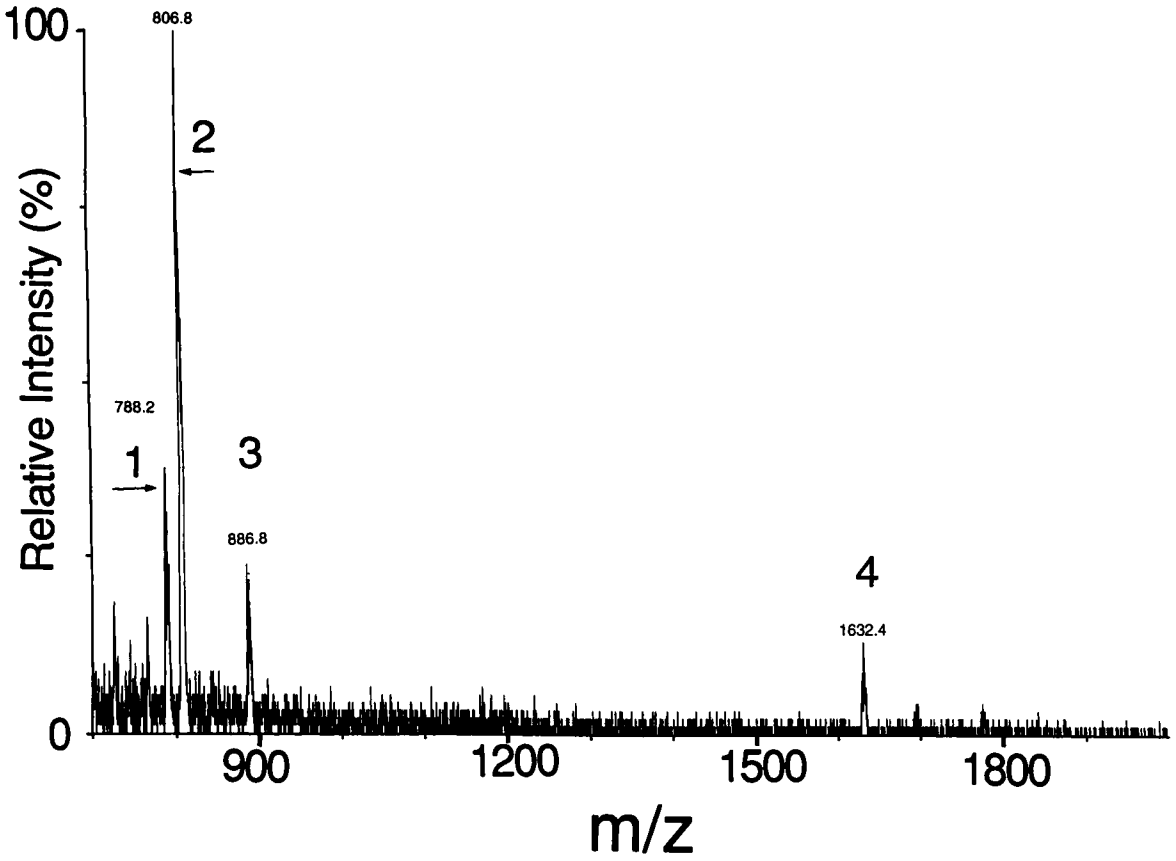

FIGURE 4 ESI mass spectrum of a mixture of HFA-CBS, HFA-GalCer, and zinc chloride in methanol in the molar ratio 1:1:10 at a declustering potential of 180 V. The peaks indicated are: 1. [HFA-GalCer.Zn^{2+}-H-H_2O]$^+$ (788.2), 2. [HFA-GalCer.Zn^{2+}-H]$^+$ (806.8), 3. [HFA-CBS.Zn^{2+}-H]$^+$ (886.8), and 4. [HFA-CBS.HFA-GalCer.Zn^{2+}-H]$^+$ (1632.4). See footnote to Table 4. The numbers in parentheses are the *m*/*z* values.

Association between free sugars or glycosides in the presence of calcium

Although the carbohydrate headgroups in CBS and GCer, through their ability to coordinate with divalent cations, probably provide the major cohesive force in the formation and maintenance of intermolecular complexes between the two lipids, the hydrophobic part of the lipid molecules could also play a role. Therefore, complex formation between Ca^{2+} and the free sugars, galactose-3-sulfate (GalS) and glucose or galactose in acetonitrile/water (1:1), was examined. In the ESI mass spectra of GalS with galactose and glucose, respectively, and $CaCl_2$ at a declustering potential of 100 V (e.g., Fig. 5), the ion [GalS.Ca^{2+}-H]$^+$ and those due to the heterodimeric complexes of GalS and Ca^{2+} with each of galactose and glucose were readily identifiable, along with other Ca^{2+} complexes of the sugars. The peak height ratio for the heterodimeric ion relative to the monomer [GalS.Ca^{2+}-H]$^+$ ion was greater for galactose than for glucose (0.29 for galactose compared to 0.19 for glucose).

TABLE 5 Comparison of the complex-forming ability of Zn^{2+} relative to Ca^{2+}*

Declustering potential (V)#	Ratio of the relative intensity of the heterodimeric complex of HFA-CBS and HFA-GalCer with Zn^{2+} relative to that with Ca^{2+}
100	1.8
120	2.5
150	2.4

*The molar ratio of HFA-CBS/HFA-GalCer/Ca^{2+}/Zn^{2+} was 1:1:10:10.
#At 180 V, spectral quality was poor for Zn^{2+} spectra.

In the case of the free sugars, the hydroxyl group at position 1 of the pyranose ring (anomeric hydroxyl) is also available for coordination. This hydroxyl has two orientations, i.e., α and β, which may provide more favorable geometries for coordination with Ca^{2+}. The use of β-methyl-glycosides (β-MeG) should better mimic the behavior of the lipids. Because there is no easy synthetic access to β-methyl-galactose-3-sulfate, we used lysosulfatide acetylated at the amino group (AcLysCBS) (monoisotopic mass = 582.6) with the natural β-glycosidic structure. This reduces the involvement of van der Waals interactions between the lipid hydrocarbon chains in complex formation and increases the water solubility of the derivatized sugar relative to CBS, allowing an acetonitrile/water mixture to be used rather than methanol. Interaction of the β-galactose-3-sulfate moiety of AcLysCBS with β-methyl-galactoside (β-MeGal) and β-methyl-glucoside (β-MeGlc), respec-

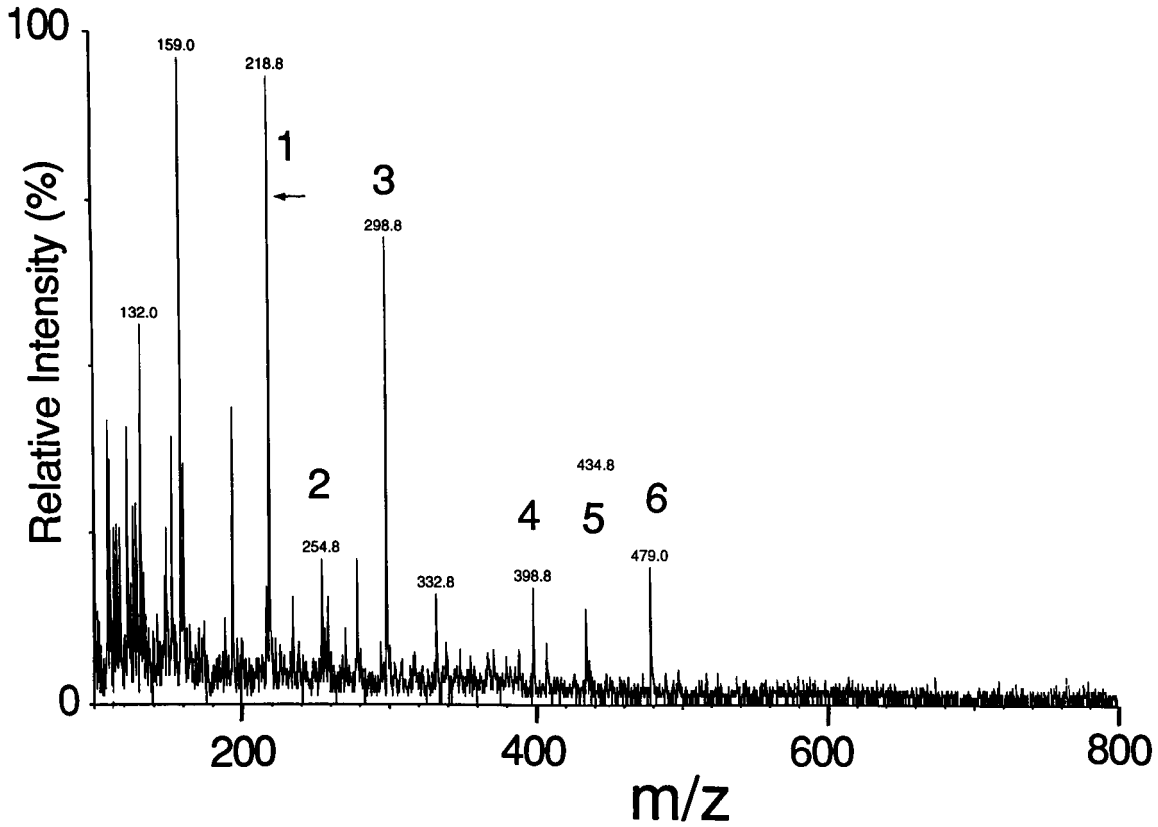

FIGURE 5 ESI mass spectrum of a mixture of galactose 3-sulfate, galactose, and calcium chloride in the molar ratio of 1:1:10, in acetonitrile/water mixture (50:50) at a declustering potential of 100 V. Peaks indicated are 1. $[Gal.Ca^{2+}\text{-}H]^+$ (218.8), 2. $[Gal.Ca^{2+}.Cl^-]^+$ (254.8), 3. $[GalS.Ca^{2+}\text{-}H]^+$ (298.8), 4. $[2Gal.Ca^{2+}\text{-}H]^+$ (398.8), 5. $[2Gal.Ca^{2+}.Cl^-]^+$ (434.8), and 6. $[GalS.Gal.Ca^{2+}\text{-}H]^+$ (479.0). The numbers in parentheses are the *m/z* values.

tively, was examined in a 1:1 acetonitrile/water mixture, as in the case of the free sugars (eg., Fig. 6). The relative intensity of the peak due to the heterodimeric complex $[AcLysCBS.\beta\text{-}MeG.Ca^{2+}\text{-}H]^+$ compared to that of the monomer $[AcLysCBS.Ca^{2+}\text{-}H]^+$ was 0.41 for β-MeGal and 0.29 for β-MeGlc, showing again greater stability of the complex of compounds containing galactose-3-sulfate with galactose compared to glucose.

Deuterium substitution of β-MeGal permitted examination of competition between the two glycosides together in the same solution. The heterodimeric complex ions of AcLysCBS and Ca^{2+} with each of β-d_3-MeGal and β-MeGlc were observed at $m/z = 819$ and 816, respectively, in the ratio 2.8:1.0. This result confirms that between the two neutral sugars, the complex of the 3-sulfated galactose moiety of acetylated lysosulfatide with the galactoside is more stable than that with the glucoside.

Association of glycolipids or glycosides in the presence of monovalent cations

To assess the role of Na^+ in the association of CBS with the cerebrosides, NaCl was substituted for $CaCl_2$. In the case of NFA-GalCer at declustering potentials below 120 V, the intensity of the heterodimeric ion corresponding to the composition $[HFA\text{-}CBS.GalCer.2Na^+\text{-}H]^+$ was barely within the limits of detection. At higher potentials, no peak corresponding to this ion was observed. In the case of HFA-GlcCer, there was no Na^+-mediated association of GlcCer with HFA-CBS at any potential.

However, weak association between AcLysCBS and β-MeGal occurred in the presence of Na^+, K^+, and Li^+, resulting in complexes of the general formula $[AcLysCBS.\beta\text{-}MeGal.2M^+\text{-}H]^+$. Interestingly, in these complexes, two univalent cations take the place of one divalent cation. Judging from relative intensities and the response to increasing declustering potential, the stability of these univalent cation complexes decreased in the order $Li^+ > Na^+ > K^+$ (Table 6). Ca^{2+} was not compared with the univalent cations in the same experiment, but the heterodimeric complex of AcLysCBS with β-MeGal and Ca^{2+} survived at higher declustering potentials than the univalent cation complexes. Furthermore, Na^+ is always present in ESI-MS samples, yet in the samples containing Ca^{2+}, CBS, and GCer, only Ca^{2+} mediates a heterodimeric complex, although Na^+-monomers of GCer are abundant.

DISCUSSION

The results of this study show that both GalCer and GlcCer can form Ca^{2+}-mediated homo-oligomers and that CBS can form Ca^{2+}-mediated heterodimers and trimers with both

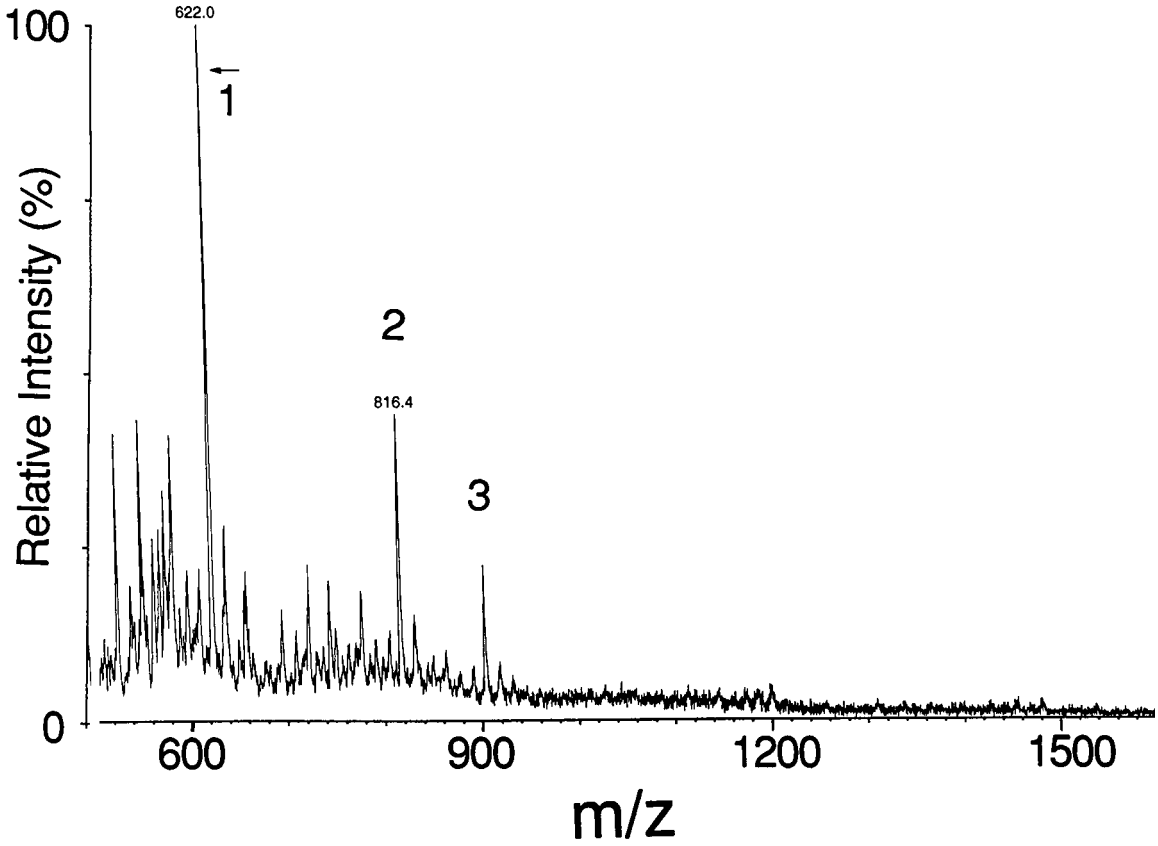

FIGURE 6 ESI mass spectrum of a mixture of acetylated lysosulfatide, β-methylgalactoside, and calcium chloride in the mole ratio 1:10:10 in acetonitrile/water mixture (50:50) at a declustering potential of 60 V, showing the [AcLysCBS.Ca^{2+}-H]$^+$ ion, peak 1 (622) and [AcLysCBS.MeGal.Ca^{2+}-H]$^+$, peak 2 (816.4). The origin of peak 3 (904) is obscure. Although this peak appeared in the spectrum of both β-methyl galactoside and β-methyl glucoside individually, in the competition experiment using β-methyl galactoside-d_3 and β-methyl glucoside, this peak was not observed. CID experiments showed that [AcLysCBS.Ca^{2+}-H]$^+$ is a component of this ion.

GalCer and GlcCer in methanol. Assuming that complex ions observed by ESI-MS reflect complexes that occur in solution, the results show that free sugars and glycosides can also form these complexes in solvent-containing water. Thus water probably does not prevent coordination of the sugar OH groups and sulfate to Ca^{2+}. Complex formation between Ca^{2+} and carbohydrates is well documented and occurs when one or more carbohydrate moieties can provide the requisite number and geometrical arrangement of hydroxyl groups and other oxygens favorable for coordination with the cation (Cook and Bugg, 1977; Whitfield et al., 1993; Angyal, 1989; Rendleman, 1966; Symons et al., 1984; Bugg, 1973).

TABLE 6 Intensities of the [AcLysCBS.β-MeGal.2M$^+$-H]$^+$ ion relative to [AcLysCBS.2M$^+$-H]$^+$ for monovalent cations

	Relative intensity at	
Cation	80 V	100 V
Li^+	0.82	0.26
Na^+	0.26	0.08
K^+	0.12	0

The relative intensity of peaks due to the heterodimeric complex ions varied with structural changes in the glycolipid headgroup and fatty acid. The relative abundance of the complex ions under ESI-MS conditions can be affected by factors other than the relative affinity of the two lipid molecules for Ca^{2+}. Differences in the abundance of different ions may be due not only to differences in the concentration of the complexes in solution, but also to differences in their ability to form ions and/or their stability under ESI-MS conditions. Another factor to be taken into account is the relative propensities of the glycolipids to form larger clusters. A lower intensity of a complex ion peak at low declustering potentials may result from a greater tendency of one of the components to form multimeric clusters. At higher declustering potentials, more of these can dissociate, causing an increase in the relative intensity of smaller oligomers. Thus comparisons of peak intensities must be made with caution, especially in light of the fact that the complex peaks in all cases are generally weaker than the reference peak.

However, the relative intensity of the peaks due to the heterodimeric complex ions increased with an increase in

declustering potential, whereas that of the peaks due to the homo-oligomeric complexes generally decreased. Thus the heterodimeric complexes containing both CBS and GCer are more stable than the homo-oligomers containing GCer only. Electrostatic interactions of Ca^{2+} with the anionic sulfate group, together with hydroxyl groups possessing geometry favorable for coordinating with the calcium, contributed from both CBS and GCer, may stabilize the binding (Cook and Bugg, 1977) and be responsible for the relatively higher stability of the heterodimers containing CBS and GCer compared to homo-oligomers of GCer. CID analysis confirmed the appreciable stability of the heterodimers of CBS with both GalCer and GlcCer, because the collisional energy caused covalent decomposition of the complex in addition to or in preference to noncovalent decomposition (Siuzdak et al., 1993; 1994). The complete lack of formation of $[GCer.Ca^{2+}]^{2+}$ monomers after CID indicates that Ca^{2+} interacts more strongly with the CBS in the complex than with the GCer.

The results show further that although both GalCer and GlcCer are capable of forming divalent cation-mediated complexes with CBS, the stability of the complexes containing GlcCer is less than those containing GalCer, at least with Ca^{2+}. The relatively lower abundance in the ESI mass spectrum of the Ca^{2+}-mediated heterodimeric complex ions containing glucose as compared to galactose, both when the carbohydrates form part of lipid molecules and when they are in the free state, support this conclusion. This is also consistent with the lower degree of Ca^{2+}-mediated interaction of vesicles containing GlcCer with vesicles or lipid films containing CBS, compared to vesicles containing GalCer found earlier (Hakomori, 1991; Stewart and Boggs, 1993a). Greater fragmentation of the lipids with Zn^{2+} made comparisons of its ability to complex different species of the glycolipids less reliable.

β-Glucose and β-methyl-glucoside by themselves are considered to be very weak in their ability to bind divalent cations, because all of their hydroxyl groups are equatorially oriented (Angyal, 1989; Symons et al., 1984). β-Galactose and β-methyl-galactoside, with their axially disposed 4-OH and equatorial 3-OH groups, should have a greater ability to coordinate with Ca^{2+} (Angyal, 1989). However, the evidence presented here from ESI mass spectrometry shows that both GalCer and GlcCer monomers associate with Ca^{2+} in methanol. Furthermore, as part of more complex structures, glucose and galactose can participate in forming a coordination shell around cations (Bugg, 1973) and may form more stable heterotypic complexes with Ca^{2+} and another sugar than they do by themselves. Further work is needed to fully elucidate the structure of the heterodimeric complexes of CBS with the cerebrosides.

Whereas hydrophobic interactions between the glycolipid acyl chains may possibly contribute to complex formation between the glycolipids in methanol, only weak association of the glycolipids occurred in the presence of univalent cations, indicating that such a contribution alone does not cause complex formation. The fact that divalent cation-mediated complex formation occurs between unattached carbohydrate molecules also indicates that interactions between the acyl chains are not essential for complex formation between the glycolipids.

A role of the fatty acid hydroxyl group in stabilizing the divalent cation-mediated complex between CBS and GalCer has been demonstrated by vesicle aggregation studies (Stewart and Boggs, 1993a) and is confirmed here by the ESI-MS results. Hydroxylation of the fatty acid of GlcCer, however, had no effect on the stability of the complex of CBS with GlcCer under ESI-MS conditions. The increased aggregation of lipid vesicles containing the HFA species of CBS and GalCer in our earlier study was difficult to understand because the carbohydrate of the HFA species of glycosphingolipids in phosphatidylcholine/cholesterol lipid bilayers was found to be less accessible to antibody (Crook et al., 1986) and to galactose oxidase (Stewart and Boggs, 1993b) than that of the NFA species. These latter studies suggested that hydroxylation of the acyl chain decreases exposure of the carbohydrate groups on the bilayer surface. This might be due to altered interactions of the glycolipids with the surrounding lipids. However, the present ESI-MS results on the glycolipids in solution show that the fatty acid hydroxyl group must also directly or indirectly stabilize the complex between Ca^{2+}, GalCer, and CBS. This stabilization may counteract the decreased exposure of the HFA species of the glycolipid in a lipid bilayer, permitting greater interaction with the complementary glycolipid in an apposed bilayer. The fatty acid hydroxyl group could be directly involved in stabilization of the CBS-Ca^{2+}-GalCer complex by coordinating to the cation or by forming an intermolecular hydrogen bond with the other lipid. Alternatively, the fatty acid hydroxyl group could indirectly increase the stability of the complex by favorably altering the geometry of the groups involved in the coordination to the cation by steric effects or by affecting intramolecular hydrogen bonding interactions, as shown by FT-IR spectroscopy of NFA and HFA species of CBS and GalCer (Menikh et al., 1997; Lee et al., 1986).

The complex formation detected here by ESI-MS between divalent cations and GalCer and CBS in solution could be due to a head-on *trans* interaction, as might occur between two glycolipids in apposed bilayers, or to a lateral *cis* interaction, which could occur between two glycolipids in the same bilayer. Either *cis* or *trans* or both types of interactions might occur between these two glycolipids in myelin. However, the correlation between the stability of the complexes in solution and aggregation of lipid vesicles for HFA versus NFA species, at least of GalCer, suggests that they reflect head-on *trans* interactions.

Trans interactions could be involved in some way in the adhesion of the extracellular surfaces of myelin. Myelin separates readily at the intraperiod line in hypotonic buffer or at low pH. Divalent cations inhibit this separation better than monovalent cations (Padron et al., 1979; Inouye and Kirschner, 1984), suggesting that they participate in the interactions between the extracellular surfaces of myelin.

Assuming that complex formation observed for GlcCer also reflects a *trans* interaction, the ESI-MS results indicate that GlcCer may be able to at least partially compensate for this putative function of GalCer in myelin. The CBS used in this study was the galactosyl form present in normal myelin. If the glucosyl form of CBS present in PNS myelin of the CGT mutant behaves relatively similarly to the galactosyl form, the ability of GlcCer to complex with Ca^{2+} and CBS may account for the relative normalcy of PNS myelin and peripheral nerve conduction in this mutant compared to its CNS myelin, which lacks a sulfated glycolipid. This is not to deny an important role for adhesive proteins in myelin compaction, such as P0 in PNS myelin (D'Urso et al., 1990) and possibly proteolipid protein (PLP) in CNS myelin (Boison and Stoffel, 1994). Indeed, protein-protein and glycolipid-glycolipid interactions may be redundant or additive mechanisms for adhesion of the extracellular surfaces of myelin, particularly in the CNS. Their combined effects may stabilize myelin.

Between the two divalent cations examined here by ESI-MS, viz. Ca^{2+} and Zn^{2+}, the latter appears to be more effective in promoting association and/or stabilizing the complex between CBS and GalCer, in agreement with our earlier vesicle aggregation studies (Stewart and Boggs, 1993a). Zn^{2+} has also been found to inhibit swelling of myelin at low pH better than Ca^{2+} (Inouye and Kirschner, 1984). It is possible that its interaction with these glycolipids at the extracellular surfaces of myelin plays a role in this inhibition. Although Ca^{2+} is present in higher concentrations in myelin than Zn^{2+}, Zn^{2+} occurs in CNS myelin at a higher concentration than other trace elements (Bourre et al., 1987). Zn^{2+} has a smaller atomic radius than Ca^{2+}, which causes Zn^{2+} to have a higher charge density. It is also more polarizable than Ca^{2+} (Douglas et al., 1994). Thus it could form stronger complexes and fit into smaller cavities.

The weak association between AcLysCBS and β-MeGal in the presence of monovalent cations is in agreement with the known ability of alkali metal cations to form complexes with and bridge carbohydrate moieties (Fura and Leary, 1993; Whitfield et al., 1993; Angyal, 1989; Rendleman, 1966; Polvorinos et al., 1994; Jackobs et al., 1968; Webb et al., 1988). The abundance or stability of the heterodimeric complexes of AcLysCBS and β-MeGal increased in the order $K^+ < Na^+ < Li^+$. The ionic radius decreases and the charge density increases in the same order (Angyal, 1989). Thus, as for divalent cations, a high charge density and a small ionic size promote complex formation.

In conclusion, this study contributes to our understanding of the role of glycolipids in myelin. It shows a correlation between the relative stabilities of the complexes of CBS with GalCer and GlcCer, the lipid composition of CNS and PNS myelin in the CGT mutant mouse, and the relative stabilities of CNS and PNS myelin in this mutant. Although myelin also contains adhesive proteins, this correlation suggests that the divalent cation-mediated carbohydrate-carbohydrate interaction between these lipids may also play a role in myelin stability and function. The ability of GlcCer to also complex with CBS, albeit with lower stability than the complex of GalCer with CBS, may allow GlcCer to partially compensate for the absence of GalCer in the mutant mouse, particularly in the PNS, where sulfated GlcCer is present. The absence of a sulfated glycolipid in the CNS to complex with GlcCer may be involved in the low stability of CNS myelin in this mutant.

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