

Investigation of the Calcium-mediated Association between the Carbohydrate Head Groups of Galactosylceramide and Galactosylceramide I³ Sulfate by Electrospray Ionization Mass Spectrometry*

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Calcium has been shown previously to cause aggregation of phosphatidylcholine/cholesterol liposomes containing galactosylceramide (GalCer) with similar liposomes containing cerebroside sulfate (galactosylceramide I³ sulfate) (CBS), suggesting that it mediates a carbohydrate-carbohydrate association between these two glycolipids. In order to determine if such an association occurs, the noncovalent complexes formed on addition of calcium chloride to GalCer and CBS in methanol were examined by positive and negative ion spray mass spectrometry. Monomeric Ca²⁺ complexes of both lipids were observed. In addition, Ca²⁺ also caused oligomerization of GalCer. Oligomerization of CBS anion was not seen, but dimers would not have been observed, as they would be neutral. However, Ca²⁺ caused heterotypic complexation of GalCer and CBS. Although these heterotypic complexes were of low abundance in methanol compared with the other monomeric and homotypic oligomeric positive ions formed at low declustering potentials, the heterotypic dimer [GalCer-CBS-Ca²⁺-H]⁺ had the greatest stability of all oligomers formed and was the only one to survive at high declustering potentials. Na⁺ did not cause oligomerization of GalCer in methanol indicating that the complexes of GalCer with Ca²⁺ are not caused by van der Waals interactions between the lipid moieties. GalCer and CBS are present in high concentrations in myelin. This Ca²⁺-mediated carbohydrate-carbohydrate interaction, which can bridge apposing bilayers, may be involved in adhesion of the extracellular surfaces of the myelin sheath.

Calcium-mediated interactions between cell surface carbohydrates have recently been implicated as a basis of cell recognition and adhesion and have therefore been the subject of increasing interest (1–8). Carbohydrate-carbohydrate interactions between free sugars and polysaccharides have been known for some time (9–14) and have more recently been investigated among glycolipids in lipid bilayers (1–3, 6, 15–19). The list of glycolipids that have been shown to participate in divalent cation-mediated carbohydrate-carbohydrate interactions includes the two major myelin glycolipids galactosylceramide and cerebroside sulfate (galactosylceramide I³ sulfate) (6,

18). In central nervous system myelin these two glycolipids comprise 27% (by weight) of the total myelin lipid (20). Thus an interaction between these two glycolipids across apposing membrane surfaces might play a role in the formation of the compacted myelin membrane.

x-ray crystallography of a number of divalent cation complexes of simple carbohydrates has provided detailed information concerning the structure of these complexes (cf. Refs. 9 and 10 for reviews). NMR and infrared spectroscopy have complemented this information (11, 12). However, the complex-forming properties of calcium with carbohydrates attached to lipid moieties remain largely unexplored. Most of the evidence for complex formation so far comes from liposome aggregation or lipid binding studies using solid phase presentation of the lipids, either bound to a solid support or in liposomes. In addition, Fourier transform-infrared spectroscopy was used to provide information about the structure of the complex of Ca²⁺ with digalactosyldiacylglycerol and the groups on the carbohydrate that chelate with the divalent cation in a membranous environment in the presence of water (19). The polyvalent nature of presentation in lipid bilayers or on a solid support increases the affinity of the interaction. The difficulty of observing the divalent cation-carbohydrate interactions in solution (especially in aqueous solution) given their weak nature and the solubility characteristics of lipid-bound carbohydrates makes the study of such interactions rather challenging.

Electrospray ionization mass spectrometry (ESI-MS)¹ is a relatively new technique, which can detect the presence of a complex in a solvent in which it is soluble (21–25). The soft ionization conditions employed allow the transfer of complexes present in solution to the gas phase with minimal decomposition (26). ESI-MS has recently been used to detect the divalent cation-mediated complexation of the carbohydrate of some glycolipids, resulting in the homotypic and in some cases heterotypic oligomerization of these lipids in methanol (21). This technique was successful at detecting Ca²⁺ oligomerization of the Le^x oligosaccharide (21), while ¹H-NMR failed to detect metal binding or evidence of oligomerization of the free sugar in water (27).

In this paper we present evidence for the noncovalent association among galactosylceramide, Ca²⁺, and the anion of cerebroside sulfate in methanol solution using electrospray ionization mass spectrometry. Positive ion scans showed that Ca²⁺ caused homotypic oligomerization of GalCer and that it bound to monomers of CBS anion to form a singly charged positive

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¹ The abbreviations used are: ESI-MS, electrospray ionization mass spectrometry; GalCer, galactosylceramide; CBS, acid form of cerebroside sulfate (galactosylceramide I³ sulfate); CID, collision-induced decomposition; Le^x, Lewis X determinant (Galβ1→4-(Fucα1→3)-GlcNAc).

ion. Dimers of CBS anions with Ca²⁺ form a neutral species and are not detected. However, Ca²⁺ caused heterodimerization of GalCer and CBS anion in methanol. Although the heterotypic complexes were of low abundance compared with the others at low declustering potentials, the heterotypic dimer had greater stability than any other complex.

EXPERIMENTAL PROCEDURES

All reagents and solvents used were either analytical grade or high pressure liquid chromatography grade. Calcium chloride (CaCl₂·2H₂O) was purchased from Fisher (Fairlawn, NJ). Methanol was from Caledon (Georgetown, Ontario, Canada), stearic acid from Fluka (Switzerland), 1-β-D-galactosyl sphingosine (psychosine) from Sigma, and oxalyl chloride from Aldrich.

Synthesis of Lipids—Galactosylceramide was synthesized from psychosine by reaction with stearoyl chloride (prepared from stearic acid and oxalyl chloride) in tetrahydrofuran in the presence of aqueous sodium acetate (28). The product was purified by column chromatography on silica gel and finally precipitated from a small volume of chloroform/methanol (90/10) by the addition of excess acetone. The identity of the final product was established as 1-O-galactosyl-2-N-stearoyl sphingosine by thin layer chromatography, differential scanning calorimetry, ¹H NMR spectroscopy, and mass spectrometry.

Galactosylceramide I³ sulfate was synthesized from lysosulfatide (psychosine sulfate) prepared from bovine brain sulfatide and stearoyl chloride as described previously (29). The structure of the product was verified as above. ¹H NMR showed that it was in the NH₄⁺ salt form (data not shown). However, the NH₄⁺ adduct was not detected in the presence of excess Ca²⁺, indicating that the Ca²⁺ completely displaced it from the lipid. When it was converted to the Ca²⁺ salt form and examined by ESI-MS in the absence of excess Ca²⁺, it formed the Na⁺ salt form due to exogenous Na⁺ present in the system. Therefore, it was necessary to study it in the presence of excess Ca²⁺.

Mass Spectral Analysis—Stock solutions of the two lipids were prepared at concentrations of 550 nmol/ml (GalCer) and 385 nmol/ml (CBS) in methanol. A stock solution of CaCl₂·2H₂O in methanol was prepared at a concentration of 6.8 μmol/ml. Aliquots of these solutions were appropriately mixed and made up to the requisite volume to give the final concentrations required for mass spectral analysis. Insolubility of the lipids in water or methanol/water solutions precluded their study in these solvents.

Electrospray mass spectra were acquired on a Perkin-Elmer Sciex API III triple quadrupole mass spectrometer. Both positive and negative ion scans were performed. Concentrations of the lipids were usually kept at 20–50 nmol/ml and the Ca²⁺ concentration at 200 nmol/ml for optimum spectral quality. For CID experiments, higher concentrations of the lipids were used for greater signal strength. The solutions were introduced at the flow rate of 5 μl/min.

RESULTS AND DISCUSSION

Binding of Galactosylceramide to Ca²⁺—The complex-forming property of galactose with Ca²⁺ in crystalline form has been well documented (30). In the crystal structure, Ca²⁺ is coordinated to five hydroxyl groups, contributed by three α-D-galactose molecules, and to three water molecules. Since each galactose can provide only one or two hydroxyl groups for Ca²⁺ binding, single isolated galactose moieties have a low affinity for Ca²⁺ in aqueous solution. However, the Ca²⁺-galactose complex may be more stable in methanol due to the lower tendency of Ca²⁺ to associate with methanol than water (31). In GalCer, the β-conformation of the sugar and the presence of the ceramide aglycone may also modify the binding to Ca²⁺. The positive ion ESI mass spectrum of GalCer (monoisotopic mass 727.6) in the presence of excess Ca²⁺ (Fig. 1) indicates that in addition to the monomeric complex ion of GalCer with Ca²⁺ ([GalCer·Ca²⁺]²⁺), which is found in high abundance, the lipid forms several oligomers as well, of the general formula [nGalCer·Ca²⁺]²⁺. This behavior is similar to that of Le^x-lactosylceramide (21). A small amount of the Na⁺ complex is also present at *m/z* = 750.6.

It is reasonable to assume that the oligomers of GalCer are formed by interaction of the carbohydrate head groups through coordination with Ca²⁺. As will be discussed later, the rela-

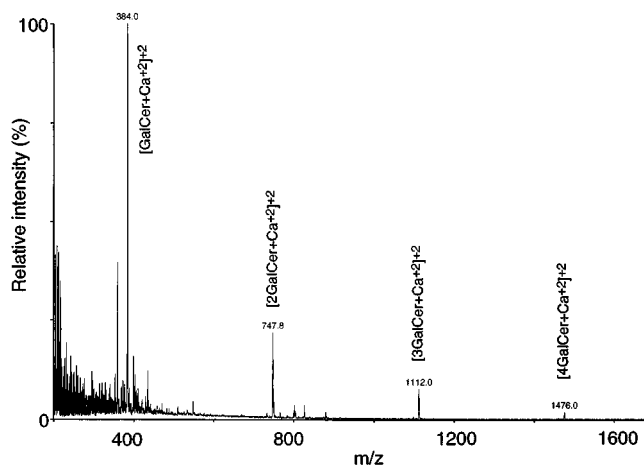


FIG. 1. Positive ion spray mass spectrum of GalCer (50 nmol/ml) with Ca²⁺ (500 nmol/ml) in methanol. The declustering potential was 80 V.

tively higher stabilities of the oligomers, especially the dimer compared with the monomer, lend some support to this suggestion, since the coordination of hydroxyl groups from more than one carbohydrate moiety with the cation has been shown to be the preferred arrangement in the crystal structures of the Ca²⁺ complexes of many carbohydrates including galactose. It should also be noted that in the presence of Na⁺, the monomeric ion [GalCer·Na⁺]⁺ was the primary ion present in the spectrum with only trace amounts of a dimeric species, [2GalCer·Na⁺]⁺ (not shown). There was no evidence of any higher oligomers containing Na⁺. As will be seen later, Na⁺ appears to have a high affinity for GalCer to form the monomeric ion, but even in the absence of Ca²⁺, its ability to promote oligomerization is negligible. This supports the conclusion that the oligomers of GalCer formed in the presence of Ca²⁺ for the most part are not due to Van der Waals interaction between the ceramide moieties. The negative ion spectrum of GalCer in the presence of excess calcium chloride on the other hand showed predominantly a peak due to a monomeric ion at *m/z* = 762.4 (not shown), which was identified as [GalCer·Cl]⁻. It persisted at declustering potentials of -80 to -180 V.

Binding of Galactosylceramide I³ Sulfate to Ca²⁺—In contrast to GalCer, CBS (monoisotopic mass 807.6) in the presence of excess Ca²⁺, under the same conditions as above, gives a single adduct with Ca²⁺ corresponding to the formula [CBS·Ca²⁺-H]⁺ (*m/z* = 846.6) as evident from the positive ion spectrum shown in Fig. 2. It is of lower intensity than that of the complex ions of GalCer. Neutral dimeric species of CBS anion of the type [2CBS·Ca²⁺-2H], although possible, would not be detected. The only ion detected in a negative ion scan was [CBS-H]⁻ in high abundance at *m/z* 806.4 (not shown). Negatively charged trimers of the type [3CBS·Ca²⁺-3H]⁻ have too high an *m/z* ratio to be detected by the mass spectrometer used.

Complex Formation between GalCer and CBS in the Presence of Ca²⁺—In order to detect Ca²⁺-mediated complex formation between GalCer and CBS, solutions containing the two lipids at a concentration of 20 nmol/ml each and Ca²⁺ at a concentration of 200 nmol/ml in methanol were used. The positive ion ESI mass spectrum of the mixture was acquired under similar conditions as for the individual lipids. Fig. 3 shows a representative spectrum at a declustering potential of 80 V. In addition to the Ca²⁺ adducts of the individual lipids as described above, there are hetero-oligomers of the two lipids detectable in the spectrum at *m/z* = 1170.6 ([2GalCer·CBS·2Ca²⁺-2H]²⁺), 1211.0 ([GalCer·2CBS·2Ca²⁺-2H]²⁺), and 1574.2

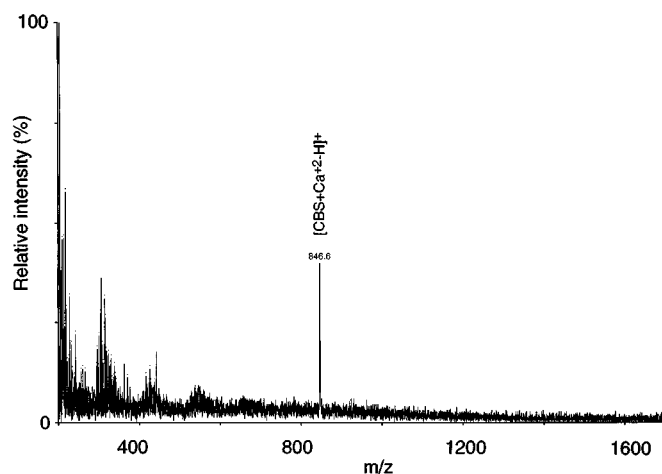


FIG. 2. Positive ion spray mass spectrum of CBS (46 nmol/ml) with Ca²⁺ (500 nmol/ml) in methanol. The declustering potential was 80 V.

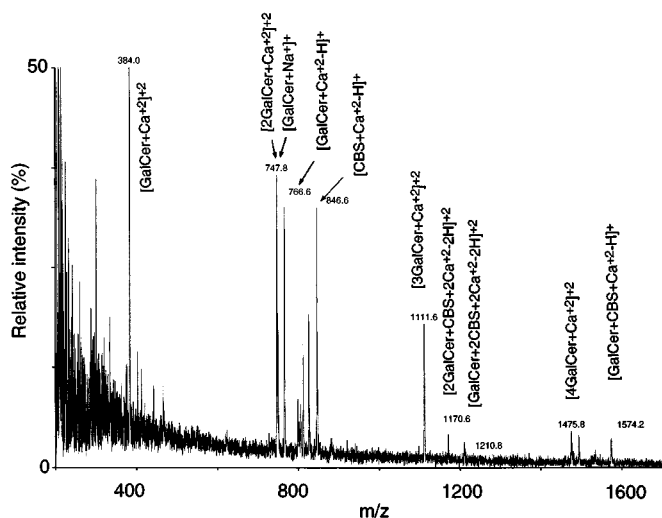


FIG. 3. Positive ion spray mass spectrum of GalCer (20 nmol/ml) plus CBS (20 nmol/ml) plus Ca²⁺ (200 nmol/ml). The declustering potential was 80 V.

([GalCer·CBS·Ca²⁺–H]⁺). The combined intensities of the three peaks containing CBS·GalCer·Ca²⁺ positive ions were approximately 30% of the intensity of the [CBS·Ca²⁺–H]⁺ peak. The relative intensities of the peaks due to the hetero-oligomers were constant at concentrations of each lipid of 5–50 nmol/ml. A hetero-oligomer containing GalCer plus two CBS anions and only one Ca²⁺ would not be detectable since it would be neutral. The negative ion scan of the mixture of the two lipids in the presence of calcium chloride contained only two peaks due to [GalCer·Cl][–] and [CBS–H][–] (not shown). It did not show any peaks resulting from the association of the two lipids. This result is not surprising in view of the fact that the negative ion spectra of the individual lipids showed no Ca²⁺-bound ions.

The Effect of Declustering Potential on the Stability of the Complexes—The declustering potential was varied from +50 to +180 V in order to alter the collisional energy and thus the amount of complexes and oligomers. For these experiments the concentration of GalCer was kept at 20 nmol/ml, while that of CBS was increased to 50 nmol/ml. This gave a relatively more intense peak at $m/z = 846.6$, corresponding to [CBS·Ca²⁺–H]⁺, but had little effect on the intensity of any of the other peaks. With an increase in declustering potential, the various Ca²⁺ adducts of the two lipids disappear. Surprisingly, at 180 V (Fig.

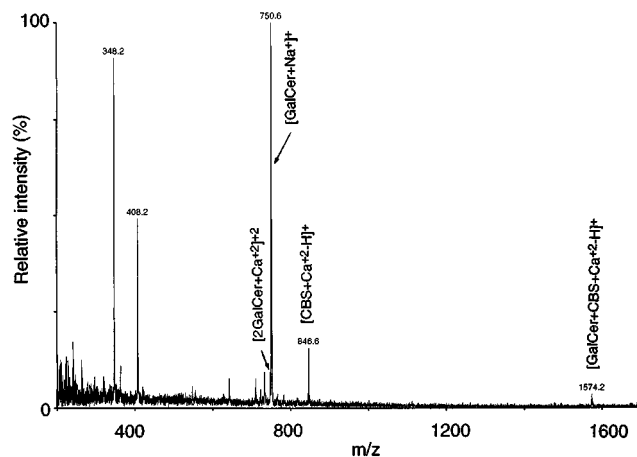


FIG. 4. Positive ion spray mass spectrum of GalCer (20 nmol/ml) plus CBS (50 nmol/ml) plus Ca²⁺ (200 nmol/ml) at a declustering potential of 180 V.

4) the only Ca²⁺ adducts still remaining are [CBS·Ca²⁺–H]⁺ ($m/z = 846.6$), the homodimer of GalCer ($m/z = 747.8$), and the heterodimer [GalCer·CBS·Ca²⁺–H]⁺ ($m/z = 1574.6$). The former two ions are considerably reduced in intensity. As the declustering potential is increased from 50 to 180 V, the first Ca²⁺ complex of GalCer to disappear from the spectrum is the monomer [GalCer·Ca²⁺]²⁺, and the last to survive in vestigial amounts is the dimer [2GalCer·Ca²⁺]²⁺. This may allow a qualitative comparison of their stabilities to be made, the monomer being the least stable, the dimer the most stable, and other oligomers somewhere in between.

Although almost all of the Ca²⁺ adducts of GalCer dissociated at a declustering potential of 180 V (Fig. 4), there is a high intensity peak at $m/z = 750.6$ due to the retention of the Na⁺ adduct. The new peaks at 408.2 and 348.2 are fragment ions, as confirmed by parent ion scans, indicating that under the conditions of the experiment significant fragmentation of the lipids occurs.

The ratio of intensity of the heterodimer to that of the CBS monomer increases with increase in declustering potential as shown in Table I. While it is not clear whether there is actually an increase in the abundance of the heterodimer ion with increase in declustering potential, since the intensity of the monomer shows a gradual decrease, the conclusion that the heterodimer [GalCer·CBS·Ca²⁺–H]⁺ is the most stable of all the Ca²⁺ adducts of either lipid, despite its low abundance, seems inescapable.

The low abundance of this complex in methanol contrasts with liposome aggregation studies, which indicated that the Ca²⁺-mediated heterotypic aggregation of GalCer with CBS anion was greater than the Ca²⁺-mediated homotypic interaction of either. The polyvalent nature of liposomes compared with the monovalent lipids in solution may contribute to the greater heterotypic interaction of liposomes. This difference in behavior may also be due to the relative stability of the Ca²⁺ adducts of GalCer in methanol solution compared with water due to the lower ability of Ca²⁺ to chelate methanol compared with water (31). The greater solubility of galactose and other sugars in methanol containing dissolved calcium chloride than in pure methanol, in which they are virtually insoluble, is well documented (32) and also indicates the formation of Ca²⁺-galactose complexes in agreement with the ESI-MS results.

CID Analysis of the [GalCer·CBS·Ca²⁺–H]⁺ ion—Collision-induced decomposition of the heterodimer of GalCer and CBS was accomplished by selecting the ion at $m/z = 1574.2$ with the mass analyzer and allowing it to collide with argon. The resulting fragments were analyzed in the positive ion mode. Fig. 5

TABLE I
Effect of declustering potential on the ratio of the intensity of the peak due to the heterodimer [GalCer · CBS · Ca²⁺-H]⁺ to that due to the monomer [CBS · Ca²⁺-H]⁺

Declustering potential	Peak height ratio ^a
V	
80	0.04
100	0.06
120	0.08
150	0.16
180	0.20

^a Height of [GalCer · CBS · Ca²⁺-H]⁺
Height of [CBS · Ca²⁺-H]⁺

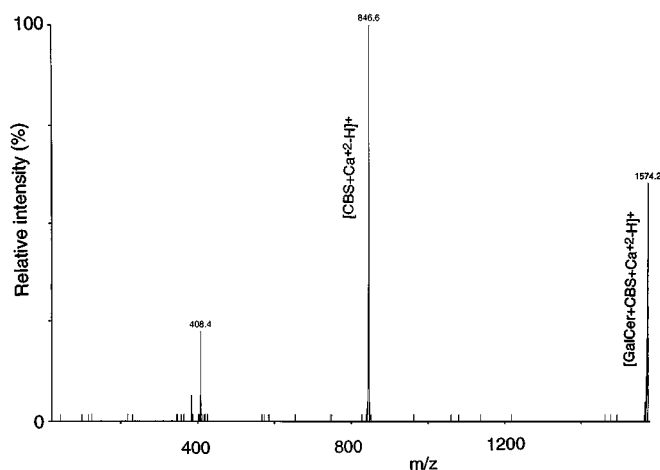


FIG. 5. CID mass spectrum of the heterodimeric complex [GalCer·CBS·Ca²⁺-H]⁺ ($m/z = 1574.2$).

shows the spectrum obtained. The noncovalent dissociation of the complex should result in the formation of both components of the complex, *viz.* the cationized species of GalCer and CBS. The peak at $m/z = 846.6$ corresponding to the ion [CBS·Ca²⁺-H]⁺ is formed by the loss of GalCer (mass = 727.6) from the parent ion. No intact GalCer ions are found in the spectrum, but the ion at $m/z 408.4$ is its decomposition product. The collisional energy thus appears to be entirely utilized for covalent decomposition in preference to noncovalent decomposition of the complex. The occurrence of predominantly covalent dissociation of complexes under CID conditions has been interpreted as the result of the tightness of the binding of the components (21). Thus in addition to confirming that the complex with $m/z = 1574.2$ is a heterodimer of GalCer and CBS with Ca²⁺, the CID experiment also corroborates the finding that this complex is relatively stable, since it undergoes mainly covalent decomposition. However, it is interesting to note that it is the GalCer component of the complex that appears to undergo decomposition while the CBS is unaffected. Thus based on the results of both the CID experiment and the relative tendency of different complexes to dissociate in response to increasing declustering potential, it can be concluded that the heterodimeric complex [GalCer·CBS·H-Ca²⁺]⁺ is more stable than the calcium complex of either lipid alone.

Conclusions—In this study we have demonstrated binding of

Ca²⁺ to each of GalCer and the anion of CBS individually. We have also demonstrated the existence of a specific interaction between GalCer and CBS anion mediated by Ca²⁺ as suggested by the divalent cation-mediated aggregation of phosphatidylcholine/cholesterol liposomes containing GalCer with similar liposomes containing CBS (6, 18). Oligomerization of GalCer, but not of CBS anion, by Ca²⁺ also occurred in methanol. However, the low degree of aggregation of GalCer-containing liposomes by Ca²⁺ compared with the greater degree of aggregation of CBS liposomes with GalCer liposomes suggests that in water this homotypic interaction must be much weaker than the heterotypic interaction and/or that the heterotypic interaction is stronger in water than in methanol. The observation of the heterotypic complex ion [GalCer·CBS·Ca²⁺-H]⁺ in the ESI mass spectrum even under conditions harsh enough to dissociate all other intermolecular clusters points to the specificity of this interaction and stability of the complex.

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