Carbohydrate Composition and Sequence Analysis of a Derivative of Brain Disialoganglioside by Mass Spectrometry, with Molecular Weight Ions at *m/e* 2245. Potential Use in the Specific Microanalysis of Cell Surface Components†

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ABSTRACT: Mass spectrometry has been applied on derivatives of disialoganglioside (hexaglycosylceramide) of brain tissue to test a microscale analysis of carbohydrate structure. Completely methylated derivative (mol wt 2214) gave intense ions for the two methylated sialic acids and also ions corresponding to several or all of the six sugars, in addition to ceramide ions. After reduction of this derivative with $LiAlH_4$ (amide groups of ceramide and amino sugars were reduced to amines, and ester groups of sialic acids to alcohols), ions were recorded corresponding to the complete carbohydrate chain plus the fatty acid, with loss of one or two of the reduced sialic acids. By trimethylsilylation of the two alcohol groups a very informative spectrum was obtained, including molecular weight ions at *m/e* 2245 (M − 1). A particularly intense peak was found at *m/e* 1965, corresponding to the complete carbohydrate chain and the fatty acid. Several series of primary and rearrangement ions allowed a conclusive interpretation concerning the sequence and branching of the carbohydrate chain. This glycolipid is the largest organic molecule so far analyzed in the gas phase with structural information on the complete molecule.

A variety of glycosphingolipids exists in different animal species and organs (Wiegandt, 1971; Hakomori, 1973). They are considered to be cell surface components and many have serologic specificities as represented by the ABH and Lewis blood groups or the Forssman hapten (Hakomori, 1973), and recently the carcinoembryonic antigen (CEA) has been identified among glycosphingolipids (Watanabe and Hakomori, 1973). The possible involvement of these substances in normal and tumor cell dynamics has been strengthened by a number of recent works (see the review by Hakomori, 1973).

Cell surface antigens may be detected and characterized by very sensitive immunological methods. However, due to the lack of chemical methods for a corresponding structural analysis of these minor components, very little is known about the chemical basis of the large number of serologically typed components, like different blood subgroups and transplantation antigens (see, for instance, Aminoff, 1970). Very recently considerable progress has been made in structural and immunological characterization of H and Lewis active glycoproteins isolated in gram quantities from ovarial cysts of individual donors (Rovis *et al.*, 1973a,b). However, a corresponding preparative source of lipid-bound, membrane-associated blood group determinants is not known. We have considered mass spectrometry as a potential method for a structural microanalysis of cell surface antigens of glycolipid nature. The high sensitivity and diversity of fragmentation principles (Waller, 1972) render this method of primary interest. However, substances exceeding molecular weights around 1000 have not been easily analyzed. Theoretically, several times larger molecules might be treated, provided optimal chemical derivatives have been chosen. In several papers (Karlsson, 1973; Karlsson *et al.*, 1974a–d) we have demonstrated the suitability of methylated glycolipids of different kinds for carbohydrate composition and sequence analysis. In the present paper mass spectra of disialoganglioside of brain tissue will be discussed. This molecule was selected for several reasons at this stage of development. Firstly, the structure is known due to the work of Kuhn and Wiegandt (Wiegandt, 1971). Secondly, this molecule is rather large, being outside the range of presently analyzed substances. Thirdly, the presence of both hexoses and amino sugars of two kinds makes the model glycolipid representative for most cell surface antigens of this nature. At present, still larger blood group active glycolipids of the kind discovered recently (Hakomori *et al.*, 1972) are being investigated in this laboratory.

Materials and Methods

Preparation of Ganglioside Derivatives. The glycolipid has been isolated before from bovine brain and completely characterized including stereochemistry (Kuhn and Wiegandt, 1963). The preparation in the present case followed the original procedure in the final steps, and the identity was shown by comparison with the reported chromatographic characteristics. The glycolipid showed one distinct spot after development with several thin-layer chromatographic solvents. The lipophilic part has been shown to contain only stearic acid as fatty acid and about equal amounts of sphingosine and its C_{20} homolog (Wiegandt, 1971). The molecular species reproduced in the chemical formulas is the higher homolog, and the detailed carbohydrate structure given is that of Kuhn and Wiegandt.

The pure glycolipid (93 mg) was methylated in one step according to the procedure of Hakomori (1964), with the following volumes of reagents and solvents: 5 ml of dimethyl sulfoxide, 1.5 ml of a 10% solution of methylsulfinyl carbanion, and 2 ml of methyl iodide. The product was purified from solvent and reagent products on a column of silicic acid (5 g) by elution with increasing proportions of methanol in chloroform. The pure methylated glycolipid was eluted with 2% (by vol-

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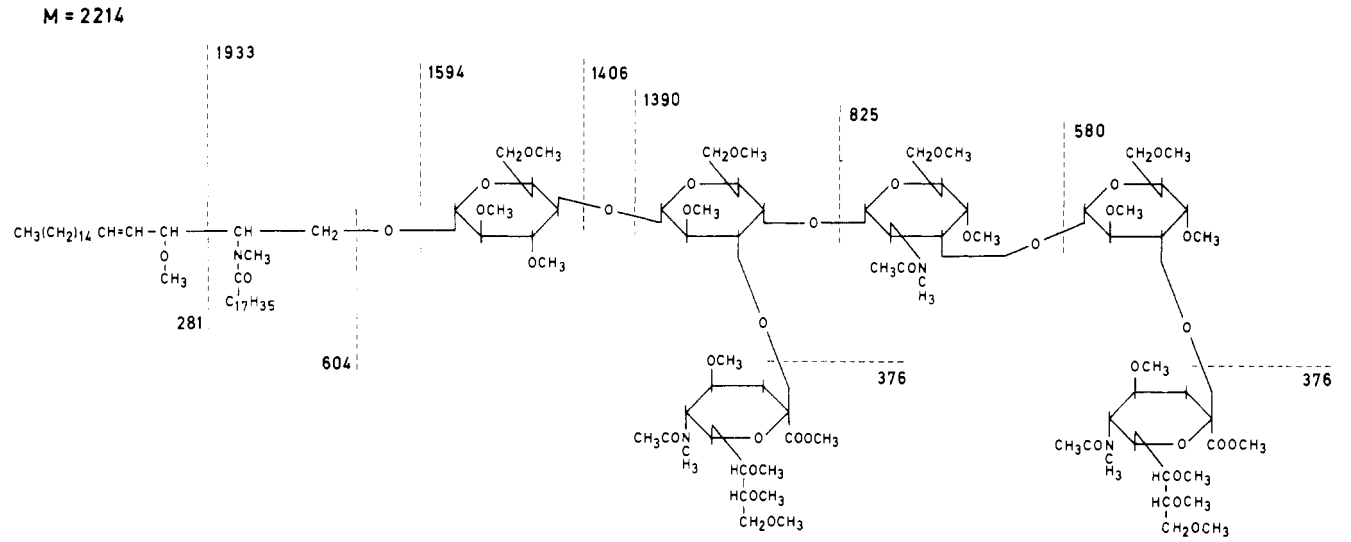

FIGURE 1: Chemical formula of methylated disialoganglioside of brain with indications of fragmentation points. The detailed formula is based on the work by Kuhn and Wiegandt (1963).

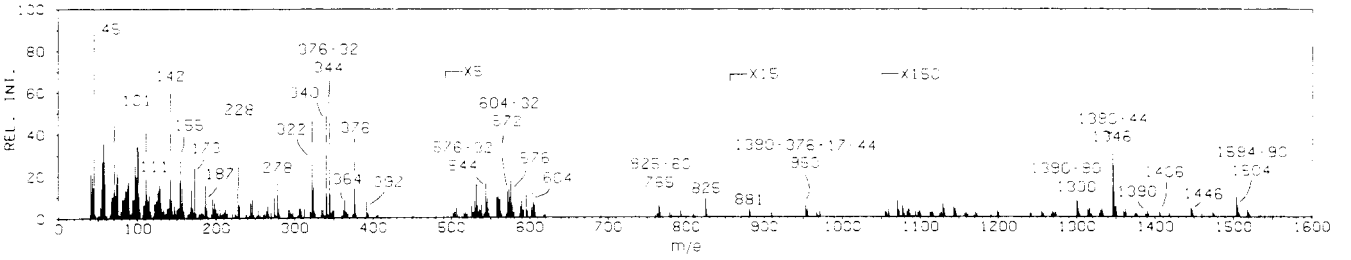

FIGURE 2: Mass spectrum of methylated disialoganglioside of brain. The conditions of analysis were: electron energy, 35 eV; acceleration voltage, 5 kV; trap current, 500 μA; probe temperature, 350°; and ion source temperature, 310°. The formula for the interpretation is shown in Figure 1 with indications of primary fragments. Several secondary fragments have been shown in the spectrum. Those indicated at *m/e* 344, 544, and 572 have probably been produced by a loss of methanol from the corresponding primary ions, but the identity and origin of the peaks at *m/e* 765, 953, 1346, and 1504 are only tentative.

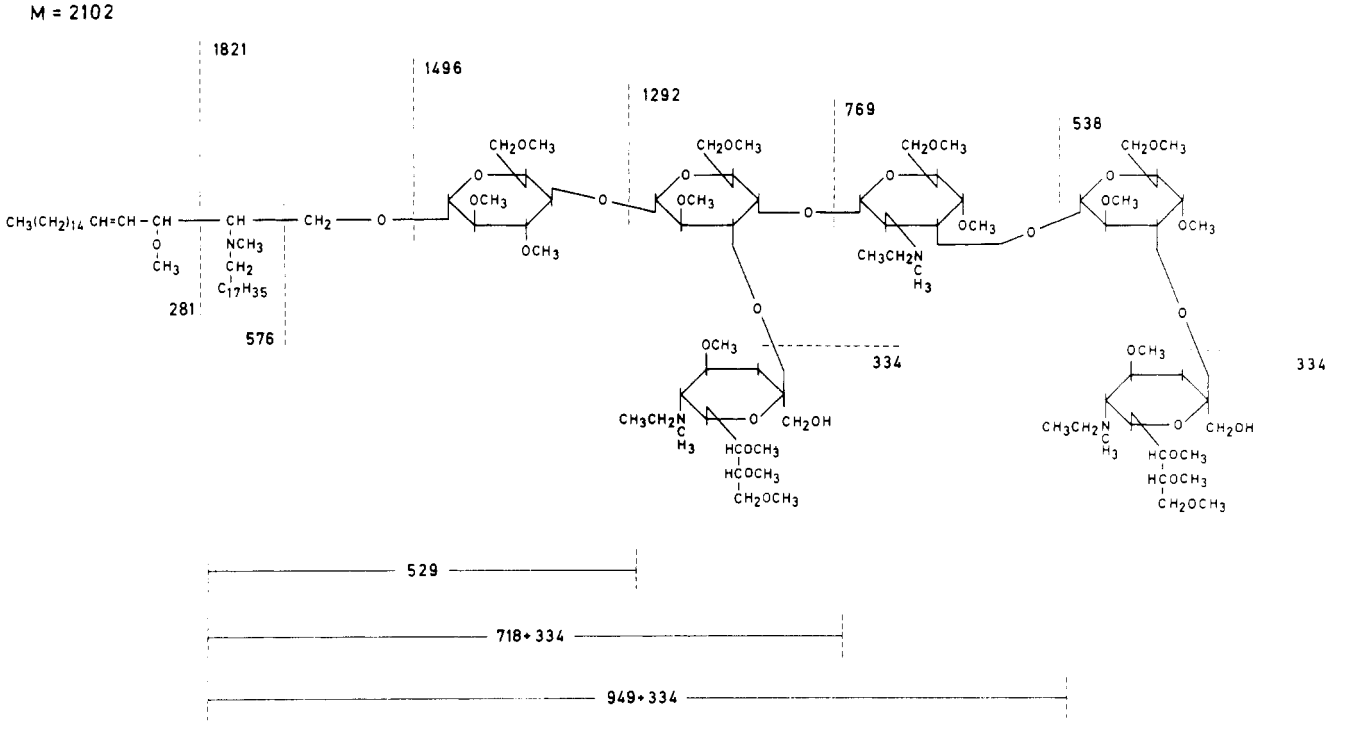

FIGURE 3: Chemical formula of methylated and reduced disialoganglioside of brain. The amide groups of ceramide and the amino sugars have been reduced to amines and the sialic acid esters to the corresponding alcohols (compare Figure 1).

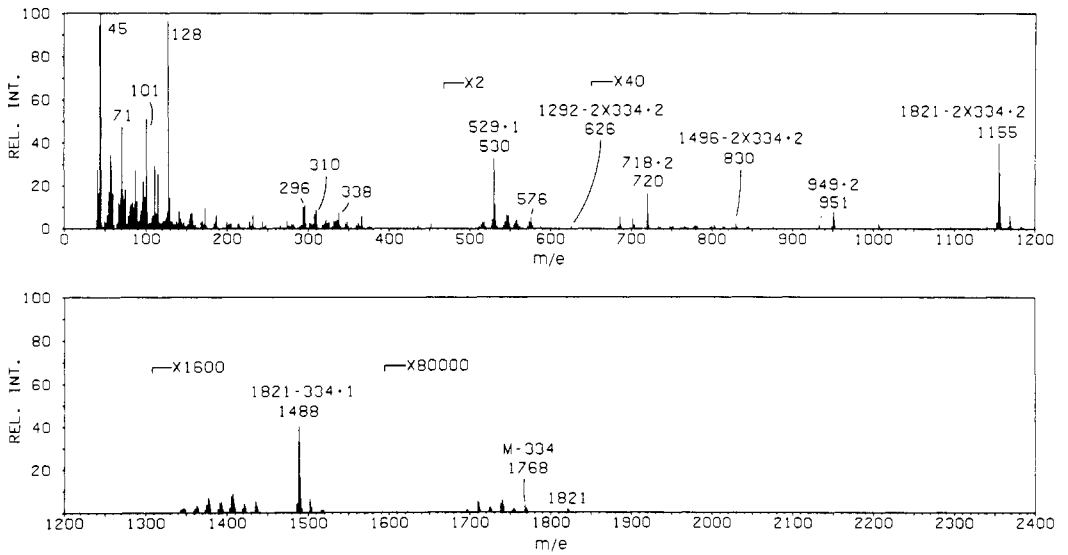

FIGURE 4: Mass spectrum of methylated and reduced disialoganglioside of brain. The conditions of analysis were: electron energy, 30 eV; acceleration voltage, 4.7 kV; trap current, 500 μA; probe temperature, 310°; and ion source temperature, 300 °. The formula for the interpretation is shown in Figure 3 with indications of primary fragments. The abundant fragments at *m/e* 1488 and 1155 most probably arise by a loss of one or two sialic acids and retention of hydrogens at the ketosidic oxygens. A similar explanation may be given for the small peaks at *m/e* 626 and 830. Rearrangement ions containing the fatty acid and part of the carbohydrate chain are found at *m/e* 530, 720, and 951. The origins of these have been suggested below the formula of Figure 3.

ume) methanol in chloroform and showed one distinct band on thin-layer chromatography on silica gel G using 7% (by volume) methanol in chloroform as solvent and a charring procedure for detection (R_F value 0.6).

Part of the glycolipid (15 mg) was reduced for 4 hr at room temperature in 1.5 ml of diethyl ether with 15 mg of $LiAlH_4$ and magnetic stirring. The product was homogeneous on thin-layer chromatography using the same conditions as for the methylated glycolipid (R_F value 0.2).

The methylation and reduction have also been scaled down to micro amounts of material, and chromatographic purification has proved to be unnecessary. For fucolipids we have treated about 100 μg of glycolipid, and the mass spectra obtained have shown complete substitution and reduction. A detailed model study on the important conditions of these derivatizations will be given elsewhere.

Silylation was done in hexamethyldisilazane-trimethylchlorosilane-pyridine 2:1:10 (by volume) for 1 hr at room temperature.

Mass Spectrometry. The apparatus was MS 902 (AEI Ltd., Manchester, England) and the direct inlet system used was equipped with a separate probe heater. The conditions of analysis are given in the legends for the figures. Usually about 10 μg of glycolipid is used for each analysis.

Results

Three different derivatives of the disialoganglioside were analyzed by mass spectrometry, the completely methylated derivative (Figures 1 and 2), the methylated and reduced derivative (Figures 3 and 4), and the trimethylsilyl ether of the methylated and reduced derivative (Figures 5 and 6). The structures of several of the ions in the lower mass region have been suggested in an earlier paper (Karlsson *et al.*, 1974b) and corresponding mass spectra of the major monosialoganglioside of brain have been discussed before (Karlsson, 1973; Karlsson *et al.*, 1974c).

The structure of the methylated ganglioside is shown in Figure 1 and the mass spectrum recorded in Figure 2. The methylation procedure alkylates hydroxyls as well as carboxyl (of the sialic acids) and amide groups. The highest masses recorded are around *m/e* 1500 (mol wt 2214), and these probably correspond to rearrangement ions with all six sugars. Similar ions are found for the five terminal sugars in the interval 1300–1406. However, this information is not diagnostic for the actual structure. The sialic acids give abundant primary ions at *m/e* 376, and a very intense peak at *m/e* 344 due to a loss of methanol. These ions are important for the differentiation of *N*-acetyl- and *N*-glycolylneuraminic acid. The latter should have given peaks at *m/e* 406 and 374 (to be published). Nerve tissue has not yet been shown to contain *N*-glycolylneuraminic acid (Wiegandt, 1971). The terminal trisaccharide is found at *m/e* 825, and the unknown ion at *m/e* 765 (825 − 60) has not been identified but an analogous peak was found in the spectra of the methylated monosialoganglioside (Karlsson *et al.*, 1974c). The intense peak at *m/e* 228 is derived from the hexosamine (Karlsson *et al.*, 1974b), and would probably not have been produced if the amino sugar were substituted with one of the sialic acids.

Several abundant ions are derived from the lipophilic ceramide. The two peaks at *m/e* 364 and 392 contain rearrangement ions of sphingosine and its C_{20} homolog, respectively (Karlsson *et al.*, 1974b). Very intense peaks are found for the fatty acid (C_{18}), at *m/e* 322 and 340, and the combinations of long-chain bases and fatty acid give primary ions at *m/e* 576 and 604, respectively.

The reduction with $LiAlH_4$ of the methylated ganglioside produces alcohols of the sialic acid methyl esters and amines of the amides (of the ceramide and of the amino sugars); see Figure 3. The mass spectrum of this derivative is shown in Figure 4. The intensities of peaks in the higher mass region are very low (see magnification figures in Figure 4), in contrast to the silylated derivative (see below). The heaviest ion at *m/e* 1821 corresponds to the complete carbohydrate chain plus the fatty acid, and *m/e* 1768 is due to a loss of one of the reduced sialic acids from the molecular ion. Two distinct peaks at *m/e* 1488 and 1155 are produced by a loss of one or two sialic acids from *m/e* 1821. However, a specific ion for the sialic acid (*m/e* 334) is not apparent. Some carbohydrate sequence ions are found at

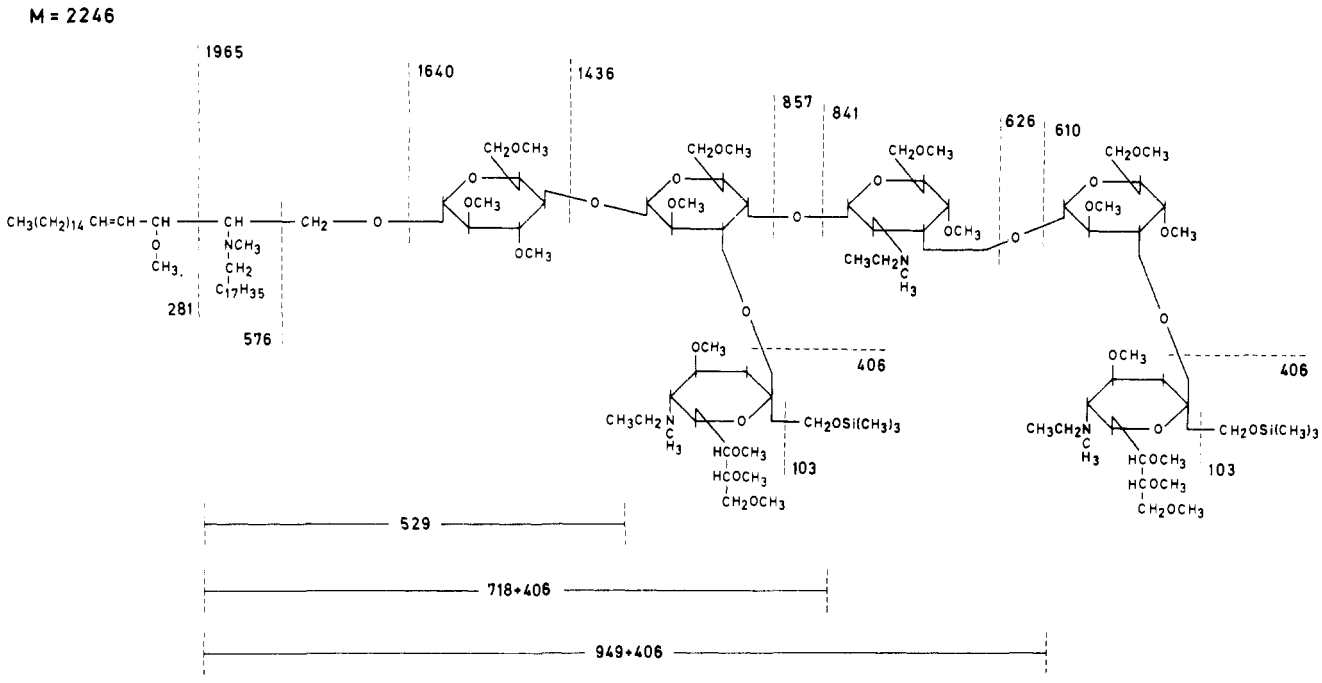

FIGURE 5: Chemical formula of the trimethylsilyl derivative of methylated and reduced disialoganglioside of brain. The alcohol groups of the reduced sialic acids have been silylated (compare Figure 3).

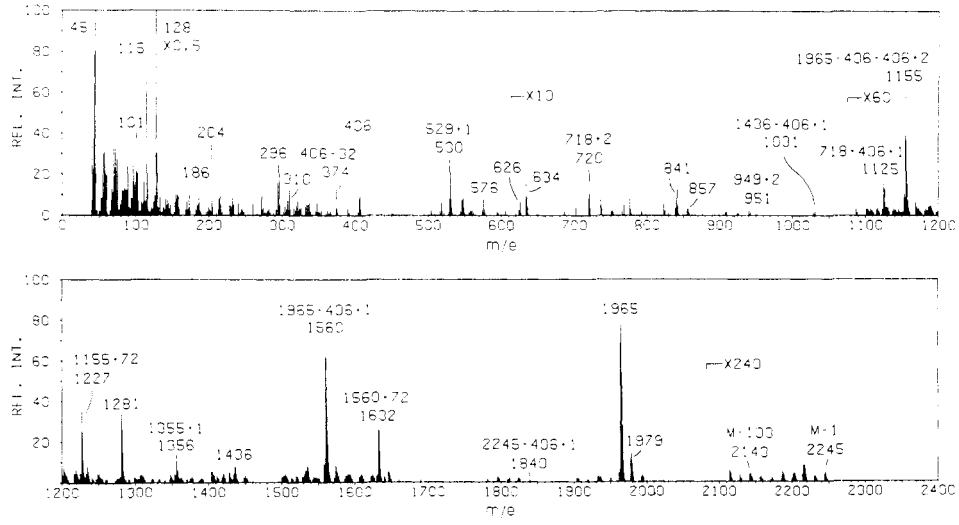

FIGURE 6: Mass spectrum of the trimethylsilyl derivative of methylated and reduced disialoganglioside of brain. The conditions of analysis were: electron energy, 30 eV; acceleration voltage, 3.2 kV; trap current, 500 μA; probe temperature, 320°; and ion source temperature, 290°. The formula for the interpretation is shown in Figure 5 with indications of primary fragments. The abundant fragments at *m/e* 1560 and 1155 are most probably formed by a loss of one or two of the sialic acids and retention of hydrogens at the ketosidic oxygens (compare Figure 4). The two peaks by 72 mass units heavier than these, at *m/e* 1632 and 1227, may be due to a transfer of a silyl group (mass 73) from the leaving sialic acid derivative to the alcohol groups (formerly ketosidic oxygens). The origins of the rearrangement ions at *m/e* 530, 720, 951, 1125, and 1356 (949 + 406 + 1) have been suggested below the formula of Figure 5.

m/e 530, 720, and 951 (compare Figure 3).

The stabilizing effect of trimethylsilyl substituents of the reduced sialic acids was demonstrated before for simpler gangliosides (Karlsson *et al.*, 1974c) and is illustrated in Figures 5 and 6 (compare Figures 4 and 6 for relative intensities of peaks). In this case, the highest mass peak corresponds to the molecular weight (M − 1 at *m/e* 2245 and the lower homolog at *m/e* 2217). As for less complex gangliosides (Karlsson, 1973; Karlsson *et al.*, 1974c) a loss of a trimethylsilyloxymethyl group gives a peak at *m/e* M − 103 (2143). Very abundant are the ions at *m/e* 1965, with the complete carbohydrate chain plus the fatty acid. This is similar to the same derivatives of all earlier analyzed glycolipids (Karlsson, 1973; Karlsson *et al.*, 1974a-d), and this easily defined peak is conclusive for the fatty acid composition and the ratio of carbohydrates (three hexoses, two sialic acids, and one hexosamine). This information from 10–20 μg of material is not possible to obtain with conventional methods, which at present need one separate procedure for each component. In addition, two very intense peaks are due to a successive loss of the two modified sialic acids (at *m/e* 1560 and 1155), confirming the basic tetrasaccharide structure. The two relatively abundant ions by 72 mass units heavier than these two ions (1560 + 73 − 1 at *m/e* 1632 and 1155 + 73 − 1 at *m/e* 1227) are most probably a result of a transfer of the trimethylsilyl groups from the leaving sialic alcohols to the ketosidic oxygens.

Of great interest are the ions with sequence information. A series of rearrangement fragments is indicated below the formula in Figure 5 and has been found for other glycolipids (Karlsson *et al.*, 1974a-d). The intense peak at *m/e* 530 (Figure 6) is evidence for hexose next to ceramide and that this hexose is not substituted with sialic acid. The peak at *m/e* 720 is also strong and gives the second sugar as hexose substituted at two points (718 + 2), indicating a branched carbohydrate chain. The uptake of one hydrogen at each linkage oxygen seems to be a rule for these rearrangement ions. The peaks at *m/e* 1356 (949 + 406 + 1) and 1125 (see fragment below the formula of Figure 5) are evidence for hexosamine and sialic acid bound at these branching points, and also that the sialic acids are not bound to each other as for an isomeric disialoganglioside of brain (Wiegandt, 1971). The terminal trisaccharide sequence is found from the ions at *m/e* 406, 626, 841, and 857.

As for the reduced derivative with free hydroxy groups (Figure 4), fatty acid peaks (*m/e* 296 and 310) and ceramide peaks are found (*m/e* 548 and 576 for the two homologs).

Discussion

There is an increasing need for specific microanalytical tools in the chemical characterization of cell surface antigens of carbohydrate nature. The unique accessibility of gram quantities of secreted soluble glycoproteins with blood group activity (see Rovis *et al.*, 1973a,b) from individual persons has no correspondence in the case of sugars bound to membrane lipid. The fragmentation method illustrated in the present paper is considered to be of potential interest in the primary chemical analysis of surface antigens of glycolipid nature. This has been illustrated earlier for successively more complex glycolipids (Karlsson *et al.*, 1974a-d), including the Forssman glycolipid hapten (Karlsson *et al.*, 1974a). In the present case conclusive information on the type, ratio, and sequence of the components of a branched-chain hexasaccharide was obtained, containing three different types of sugars (hexose, hexosamine, and sialic acid). The methylation and reduction are possible to perform on as little as 100 μg of glycolipid and a mass spectrum is usually obtained from 10 μg of material, although the recording of heavier ions of very low abundance, as in Figure 4, may need 40-80 μg. The composition and sequence information thus obtainable should be of great value for the subsequent obligate degradative studies on positions of binding and stereochemistry, and also for screening purposes when only limited material is available. Concerning the degradation of permethylated sugars containing amine functions there are still important difficulties in obtaining good recoveries (see Stellner *et al.*, 1973). We do not consider six sugars the upper limit for mass spectrometry of glycolipids. The weight of the sialic acid in the most suitable derivatives (Figure 6) is 406, which corresponds to about two methylated fucose residues (2 times 189) or two methylated hexose residues (2 times 219). The disialoganglioside analyzed thus corresponds in total mass to an octaglycosylceramide if the sialic acids were exchanged with fucose or hexose, and we have preliminary evidence that still larger molecules of the type suggested by Hakomori and coworkers (Hakomori *et al.*, 1972) are possible to obtain in the gas phase and resist temperatures above 350°. Mass spectra of a number of ABH and Lewis active glycolipids will be reported elsewhere. We are at present investigating the direct inlet analysis of mixtures of methylated and reduced glycolipids. By a successive increase in probe temperature, components with increasing carbohydrate chains are evaporated with a computer-based recording of spectra. By a programmed retrieval of sequence-specific fragments, it should be possible to obtain chemical information even on mixtures of glycolipids of normal and malignant tissues, at present of great biomedical interest (see Hakomori, 1973).

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