Mass spectrometry of neutral, mono- and disialoglycosphingolipids

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ABSTRACT Microgram quantities of complex glycosphingolipids were fully trimethylsilylated and analyzed by mass spectrometry. Reproducible ratios of the intensities of certain sugar fragment ions to the total intensity of ions characteristic of the sphingolipid bases were used to determine the number of monosaccharides in the glycosyl moiety and how many of them were unsubstituted at C-3. *N*-Acetylated hexosamine residues were readily detected and further characteristic fragment ions appeared if they were the terminal residues of the oligosaccharide chain. It was also possible to distinguish between the *N*-glycolyl and *N*-acetyl forms of neuraminic acid and to determine the number of sialic acid residues present in the lipid. Considerable information about the fatty acid and long-chain base composition was obtained from the same mass spectral analysis. It has been concluded that reliable structural information can be obtained from small amounts (less than 50 μg) of a purified glycosphingolipid.

SUPPLEMENTARY KEY WORDS fully trimethylsilylated glycosphingolipids · structure determination · comparison of fragment intensities · neutral hexoses · acetamidohexoses · *N*-glycolyl- and *N*-acetylneuraminic acids · fatty acids · sphingolipid bases

GLYCOSPHINGOLIPIDS are of considerable interest because of their implication in neuronal function, their possible role in the antigenicity of cell surfaces, and their accumulation as a result of a number of well-defined inborn errors of metabolism. Although a partial structure for glucosylceramide isolated from the spleen of a patient with Gaucher's disease was proposed 40 years ago, it is only in recent years that the structures of other major glycosphingolipids have been determined. This was largely due to work in the laboratories of Kuhn, Wiegandt, Svennerholm, Klenk, Yamakawa, and others (Table 1). The first step in their structural studies was usually to determine the monosaccharide composition by a combination of acid hydrolysis and paper chromatography. Subsequently, the sequence of monosaccharide residues in either the intact glycosphingolipid or the liberated oligosaccharide unit (1, 2) was determined by partial acid hydrolysis (3, 4) with specific exoglycosidases, or by thin-layer chromatography (5). Additional studies involving periodate oxidation and permethylation-GLC of the residual fragments (6, 7) proved to be of considerable value in locating the position of the glycosidic linkages. Their anomeric configuration was determined either by optical rotation (3), infrared (8) and n.m.r. spectroscopy (9), or the use of glycosidases of known anomeric specificity (3).

Thus, if one has several milligrams of a purified glycosphingolipid there are a number of elegant analytical techniques available to determine the carbohydrate composition, sequence, positions of substitution, and stereochemistry. However, it has become apparent that many biologically active glycosphingolipids cannot be isolated in sufficiently large amounts to be studied in this way, and an alternative approach must be used. The introduction of GLC for the analysis of sugars as their trimethylsilyl (TMSi) (10–12) or acetyl (13) derivatives enables quantitative analyses to be carried out on the microscale. Subsequent studies of the mass spectrometric fragmentation pathways of hexoses (14), sphingolipid bases (15, 16), and ceramides (17, 18) suggested that a combination of the two techniques might be used to determine the structure from a few micrograms of the pure glycosphingolipid. Recent communications from this and other laboratories (19–23) showed that simple

Abbreviations: NANA, *N*-acetylneuraminic acid; NGNA, *N*-glycolylneuraminic acid; galNAc, *N*-acetylgalactosamine; gal, galactose; glc, glucose; TMSi, trimethylsilyl.

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TABLE 1 STRUCTURES OF MAJOR HUMAN GLYCOSPHINGOLIPIDS

Designation	Structure
GL-1a	glc-ceramide
GL-1b	gal-ceramide
GL-2a	gal (1→4) glc-ceramide
GL-2b	gal (1→4) gal-ceramide
GL-3	gal (1→4) gal (1→4) glc-ceramide
GL-4	galNAc (1→3) gal (1→4) gal (1→4) glc-ceramide
G_{M3} (NANA)	NANA (2→3) gal (1→4) glc-ceramide
G_{D3} (NGNA)	NGNA (2→8) NGNA (2→3) gal (1→4) glc-ceramide
G_{M2}	galNAc (1→4) [NANA (2→3)] gal (1→4) glc-ceramide
G_{M1}	gal (1→3) galNAc (1→4) [NANA (2→3)] gal (1→4) glc-ceramide
G_{D1a}	NANA (2→3) gal (1→3) galNAc (1→4) [NANA (2→3)] gal (1→4) glc-ceramide
G_{D1b}	gal (1→3) galNAc (1→4) [NANA (2→8) NANA (2→3)] gal (1→4) glc-ceramide

glycosphingolipids could be made sufficiently volatile for analysis by mass spectrometry and that it was possible to interpret the fragmentation patterns in structural terms. In the present report, we have extended our preliminary studies to include complex glycosphingolipids from a variety of mammalian sources. Some precautions are necessary in these analyses and there are limitations that must be recognized; these problems are discussed. Nevertheless, it is possible that mass spectrometry will be of considerable value in the structural study of glycosphingolipids.

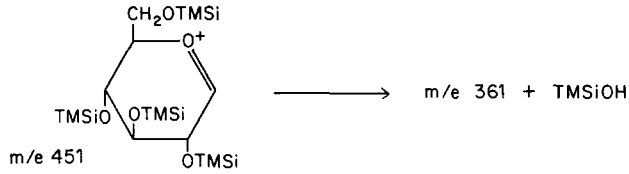
MATERIALS AND METHODS

Glucosylceramide (GL-1a) was isolated from a Gaucher spleen, galactosylceramide (GL-1b) from bovine spinal cord (Supelco, Inc., Bellefonte, Pa.) and human brain, lactosylceramide (GL-2a) and globoside (GL-4) from human and macaque (monkey) erythrocyte stroma, digalactosylceramide (GL-2b) and galactosylgalactosylglucosylceramide (GL-3) from Fabry kidney, and *N*-glycolylhematosides from horse erythrocytes. We are grateful for generous gifts of the following: gangliosides G_{M1} and G_{M2} from Dr. Saul Roseman, Johns Hopkins University, Baltimore, Md.; *N*-acetylhematoside from Dr. R. H. McCluer, Ohio State University, Columbus, Ohio; and the gangliosides G_{D1a} and G_{D1b} from Dr. Liselotte Hof, University of Chicago, Chicago, Ill.

All mass spectrometric analyses were performed in the direct probe inlet of an LKB 9000 single-focusing mass spectrometer with an ionizing voltage of 70 ev and an ion source temperature of 290°C. The trimethylsilylation reaction was carried out as described previously (19) and the TMSi derivatives were volatilized in the ion source at temperatures of 120°C–180°C.

RESULTS

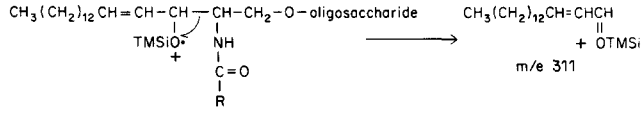
The mass spectra of GL-1a and GL-1b were identical in the mass range studied (m/e 50 to 1000), except for the intensities of ions that consisted partially of the fatty acid moieties. The base peak in these mass spectra occurred at m/e 361, which is an ion commonly found in the mass spectra of TMSi derivatives of carbohydrates (14). It is derived by the initial formation of m/e 451 from molecular ion, followed by the loss of trimethylsilanol (TMSiOH).



A two-carbon fragment, m/e 204, is also characteristic of the TMSi derivatives of carbohydrates and originates from C-2:C-3 and C-3:C-4 (14).

It is absent from the mass spectra of compounds in which the hydroxyl group on C-2 or C-3 is substituted or has been replaced (14).

Cleavage of the long-chain base between the carbons bearing the amide and TMSiO groups, as shown below, is also a typical fragmentation in the mass spectra of *N*-acyl bases (16–18).



Several relatively intense peaks result from this fragmentation, depending on whether the base is sphingosine (m/e 311), sphinganine (m/e 313), or 4-hydroxysphinganine (m/e 299).

The major peaks in the mass spectrum of glucosylceramide are shown in Fig. 1, which is also typical of galactosylceramide. It can be seen that the ratio of m/e 204 to m/e 311 is close to 2.0. This is the ratio of an ion which is typical of the carbohydrate residues (m/e 204)

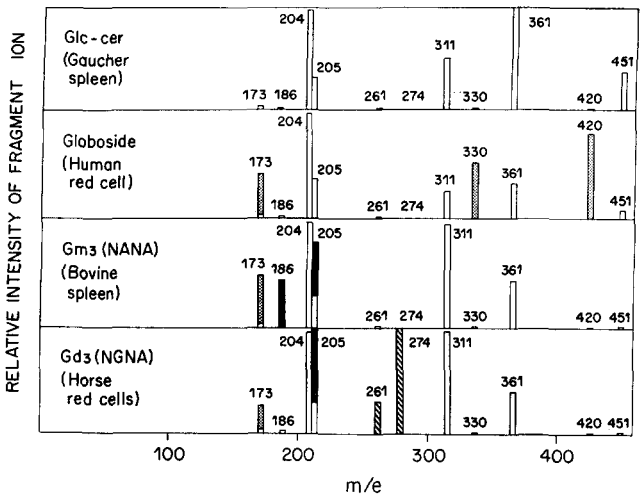


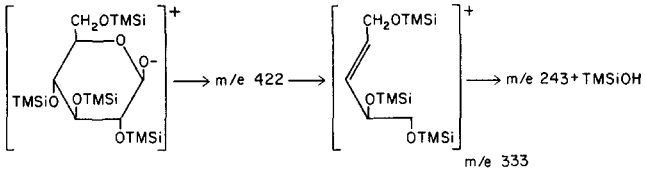
FIG. 1. Bar graph of important fragment ions used in the assignment of structures to glycosphingolipids. Intensities are expressed relative to m/e 204 and may exceed 100% in some cases. Details about the nature of these fragment ions are given in the text.

to one which is typical of the long-chain base. Henceforth the ratio is referred to as m/e 204 *rel*. If there are two or more long-chain bases in the lipid, m/e 204 *rel* should be calculated from the sum of "base" ions such as m/e 311, 313, etc. The observed value of m/e 204 *rel* can be used to count the number of unsubstituted hexose residues, assuming that there are no structural factors influencing the ion yields from these fragmentations. The results (Table 2) obtained with other neutral glycosphingolipids suggest that this is a valid assumption, since m/e 204 *rel* increased with the number of hexose residues in lactosylceramide and digalactosylceramide and was still higher in the trihexosylceramide (GL-3). Its value was lower with human red cell globoside (GL-4), however, which is consistent with the structure. Since the terminal galNAc residue has an OTMSi group on C-2 replaced by an $NHCOCH_3$ group and the glycosidic linkage of the *N*-acetylgalactosamine is to C-3 of the adjacent galactose residue, neither of these monosaccharides is capable of yielding m/e 204, and the value of m/e 204 *rel* is therefore the same as that for a dihexosylceramide.

Similarly, the relative intensities of other ions could also be correlated with the number of monosaccharides present in the lipid, using ratios of their intensities to those derived from the long-chain base. The peak at m/e 103 is derived from C-6 of the sugars and is particularly useful since it is not affected by substitution at C-2, C-3, or C-4.

$$CH_2 = \overset{+}{O}TMSi \qquad m/e\ 103$$

The value of m/e 103 *rel* was therefore directly proportional to the number of monosaccharides even when *N*-acetylhexosamine was part of the chain (Table 2). The fragmentation of a hexose residue that involves the stepwise losses of C-1, a trimethylsilyloxy radical, and TMSiOH gives peaks at m/e 422, 333, and 243 (14).



The value of m/e 243 *rel* was also proportional to the number of hexose and *N*-acetylhexosamine residues; although C-2 and C-3 are involved in this transition, substituents such as CH_3CONH must be preferentially lost since the observed relative value for this ion was not dependent on the nature of the monosaccharides (galNAc vs. hexose) nor on the position of the glycosidic linkages (to C-3 vs. C-4).

A peak formed from C-1 with rearrangement of a TMSiO group from C-3 is found in the mass spectra of TMSi carbohydrates at m/e 191 (14).

$$TMSiO-CH=\overset{+}{O}-TMSi \qquad m/e\ 191$$

Its relative intensity also increased with the number of hexose units in the series from GL-1 to GL-3, but was only marginally greater in the mass spectrum of human red cell GL-4, as compared with that of GL-3. It is

TABLE 2 THE EFFECT OF INCREASING OLIGOSACCHARIDE CHAIN LENGTH, (1→3) GLYCOSIDIC LINKAGES, AND HEXOSAMINES OR SIALIC ACID ON RELATIVE INTENSITIES OF MAJOR FRAGMENT IONS*

m/e†	GL-1a	GL-2a	GL-3	GL-4	G_{M1}	G_{M2}	G_{M3}
103 *rel*	0.7	1.1	1.6	2.7	3.1	1.1	1.2
191 *rel*	0.4	0.6	0.9	1.0	0.8	0.3	0.4
204 *rel*	1.8	4.0	6.2	3.6	4.0	0.9	0.9
243 *rel*	0.3	0.7	1.0	1.2	0.8	0.5	0.4
271 *rel*	0.5	0.7	1.11	1.3	0.9	0.3	0.5
361 *rel*	4.8	3.2	3.7	1.7	1.2	0.2	0.4

* A sufficiently large number of analyses for statistical treatment of data has not been obtained. The variation in ratios for six mass spectra of GL-3 was ±5%.

† The ratio of intensity for a given peak to that of m/e 311 and other ions for long-chain base, if present in the mass spectrum, is referred to as the *rel* value of the peak.

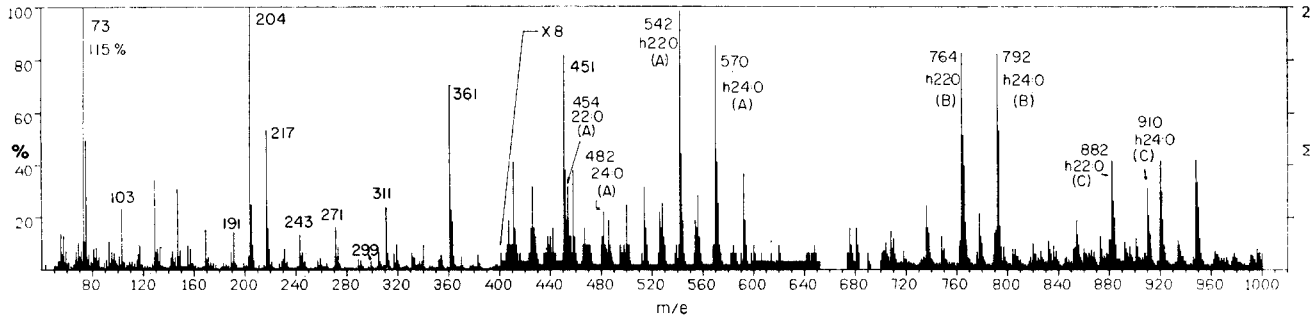
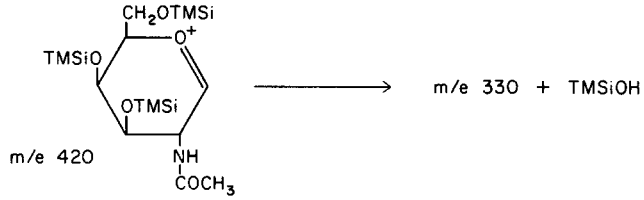


FIG. 2. Partial mass spectrum of the TMSi derivative of GL-2b (digalactosylceramide from Fabry kidney). The molecular weight of the species containing sphingosine and lignoceric acid would be 1549.

proposed that the galNAc (1→3) gal linkage prevents the formation of m/e 191 from the substituted galactose residue since there is no TMSiO group for the rearrangement. The value of m/e 191 *rel* should accordingly be the same for GL-3 and GL-4, both of which have three reactive monosaccharides.

The actual relationships of the ions discussed so far can be compared in the partial mass spectrum of the TMSi derivative of digalactosylceramide, shown in Fig. 2.

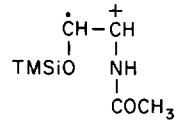
The large peaks located at m/e 451 and 361 in the mass spectra of GL-1, GL-2, and GL-3 were apparently derived predominantly from terminal hexose residues since m/e 361 *rel* was nearly the same for all three lipids (Table 2). The analogous ions observed at m/e 420 and 330 in the mass spectrum of the trimethylsilyl derivative of *N*-acetylgalactosamine (14) were very intense in the mass spectrum of the GL-4, whereas m/e 361 *rel* was decreased as compared with GL-1 (Fig. 1) and the other lipids with terminal hexose residues.



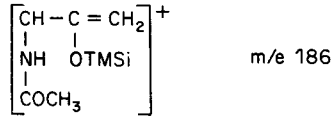
For another glycosphingolipid that contains *N*-acetylgalactosamine in an internal position, asialo G_{M1}, the values of m/e 420 *rel* and 330 *rel* were low, while those for m/e 451 *rel* and 361 *rel* were in the range of those found for GL-1 through GL-3. The results therefore provide a convincing argument for the use of these ions to deduce the nature of the terminal substituent in the oligosaccharide chain.

A comparison of the mass spectra of asialo G_{M1} and the parent ganglioside, G_{M1}, showed that substitution of *N*-acetylneuraminic acid at C-3 of the internal galactose residue in G_{M1} reduced the value of m/e 204 *rel* from about 6 in the asialo G_{M1} (three unsubstituted hexose units) to about 4 for G_{M1}, which contains only two unsubstituted hexose units. The values of m/e 204 *rel* were less than 1 for Tay-Sachs ganglioside (G_{M2}) and hematoside (G_{M3}), and it has been assumed that some unknown structural factor accounts for the fact that m/e 204 *rel* was less than that for glucosylceramide, since they all have the same single unsubstituted glucose residue.

The gangliosides give peaks at m/e 173, 186, and 205 that are related to ions that are characteristic for *N*-acetylneuraminic acid residues (Table 3). The fragment at m/e 173 has been shown to consist of a TMSiO group, an acetamido group, and two carbons of the sugar in studies of the mass spectra of *N*-acetylhexosamine derivatives (14); it is an intense ion with both 2-acetamido-2-deoxygalactose and 3-acetamido-3-deoxyglucose.



The same two-carbon unit occurs in *N*-acetylneuraminic acid and, hence, m/e 173 can arise either from sialic acid residues of the *N*-acetyl type or from the *N*-acetylhexosamine residue in gangliosides. The related ion at m/e 186 can also be formed from either *N*-acetylneuraminic acid or *N*-acetylhexosamine, but m/e 186 *rel* was much higher in G_{M3} (one NANA and no galNAc) than it was in GL-4 (one galNAc and no NANA), as seen in Fig. 1.

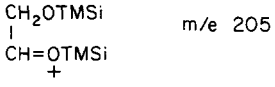


The peak at m/e 205 in the mass spectra of neutral glycosphingolipids can be accounted for entirely by the isotopic forms of the ion at m/e 204. In gangliosides, however, a fragment at m/e 205 can also be formed by the loss of C-8 and C-9 from the sialic acid residues, and m/e 205 *rel* was higher in all of the mass spectra of gangliosides than it was in those of GL-1, GL-2, and GL-3 (Table 3 and Fig. 1).

TABLE 3 RELATIVE INTENSITIES OF MAJOR FRAGMENT IONS IN MASS SPECTRA OF GANGLIOSIDES

m/e	Human G_{M3}	Equine G_{M3}	Equine G_{D3}	Human G_{M1}	Human G_{D1a}	Human G_{D1b}
103	184.0	106.0	228.1	76.9	237.2	117.0
173	29.3	17.1	29.0	10.3	44.1	15.0
186	34.3	—	—	24.1	44.1	27.0
204	100.0	100.0	100.0	100.0	100.0	100.0
205	56.2	67.2	101.7	53.3	88.2	54.0
217	91.0	87.0	100.0	98.0	97.0	98.0
261	0.2	1.5	44.2	0.1	—	—
274	0.9	59.3	200.5	0.8	—	—
311	140.1	88.7	164.2	12.8	88.0	18.0
339	—	—	—	12.8	64.1	29.0
361	51.1	24.1	43.0	30.8	22.1	36.0
363	45.8	20.0	21.1	7.3	—	—
204 *rel**	0.7	1.1	0.6	4.0	0.7	2.2

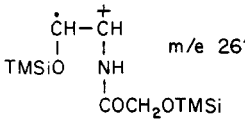
* The ratio of intensity for a given peak to that of m/e 311 and other ions for long-chain base, if present in the mass spectrum, is referred to as the *rel* value of the peak.



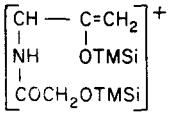
m/e 205

Gangliosides containing two NANA groups on different sugars, such as G_{D1a}, had a higher m/e 205 *rel* than those of monosialogangliosides (Table 3). This peak was equally intense in the mass spectra of gangliosides with the two NANA residues attached to each other (G_{D1b} and G_{D3}), and it is not possible, therefore, to use the value of m/e 205 *rel* to differentiate between the two types of disialogangliosides.

The mass spectra of mono- and disialohematosides isolated from equine erythrocytes were compared with those of *N*-acetyl- and *N*-glycolylhematosides from bovine spleen. As expected, a peak was found at m/e 205 in all of the mass spectra. Fragment ions at m/e 173 and 186 were characteristic of the hematoside with a NANA residue, as described above, while there was a shift in the location of these fragments by 88 mass units to m/e 261 and 274 in the *N*-glycolyl forms (Fig. 1 and Table 3).



m/e 261



m/e 274

It was previously reported (24) that equine hematoside contained the *N*-glycolyl form exclusively, and the mass spectral analysis has confirmed this result.

Fibroblasts cultured from biopsied human skin are capable of biosynthesizing at least five major glycosphingolipids which correspond in chromatographic behavior and chemical composition to GL-1a, GL-2a, GL-3, GL-4, and G_{M3}.[1] Mass spectrometry confirmed the gross features of these structures, the presence of *N*-acetylneuraminic acid rather than *N*-glycolylneuraminic acid in the G_{M3} (normal fibroblasts), and gave the fatty acid composition and sphingolipid base composition. The major ion intensities of GL-4 from the fibroblasts are compared with those of GL-4 from human and monkey erythrocytes in Table 4.

TABLE 4 RELATIVE INTENSITIES OF MAJOR FRAGMENT IONS IN THE MASS SPECTRA OF GLOBOSIDES OF DIFFERENT ORIGIN

m/e*	Human Red Cell Globoside	Porcine Red Cell Globoside	Monkey Red Cell Globoside	Human Fibroblast Globoside
103 *rel*	2.7	2.3	3.5	3.9
173 *rel*	1.3	1.0	1.5	1.1
191 *rel*	1.0	0.7	1.0	1.0
204 *rel*	3.6	2.8	4.2	3.5
205 *rel*	1.1	0.9	1.3	1.1
217 *rel*	3.5	2.3	3.7	3.8
243 *rel*	1.2	0.8	1.1	0.8
271 *rel*	1.3	1.0	1.1	0.9
330 *rel*	1.9	1.4	1.5	0.8
420 *rel*	3.2	1.3	1.9	0.9

* The actual intensities of ions for long-chain base (relative to m/e 204) were 27.9%, 35.3%, 23.6%, and 28.9% for human red cell, porcine red cell, monkey red cell, and human fibroblast, respectively. The ratio of intensity for a given peak to that of m/e 311 and other ions for long-chain base, if present in the mass spectrum, is referred to as the *rel* value of the peak.

In almost all of the glycosphingolipids studied, sphingosine (m/e 311) was the major long-chain base. Sphinganine (m/e 313) was a minor component as a rule, although it accounted for about 9% of the mixture in fibroblast GL-4. Eicosasphing-4-enine (m/e 339) was a significant component of the long-chain base fraction of glycosphingolipids of neural origin (Table 5), where the amount was from 23% in G_{M2} to 61% in G_{D1b}. The extent to which brains of different ages (25)

[1] Dawson, G., C. C. Sweeley, R. Matalon, and A. Dorfman. Unpublished results.

TABLE 5 ESTIMATION OF SPHINGOLIPID BASE COMPOSITION BY MASS SPECTROMETRY

Glycosphingolipid	Sphingosine	Eicosasphing-4-enine
	%	
G_{D1b}*	39	61
G_{D1a}*	43	57
G_{M1}*	50	50
G_{M2}*	77	23
G_{M3}†	>95	<5
GL-1b†	>95	<5
GL-1a†	>95	<5
GL-2a†	>95	<5
GL-3†	>95	<5
GL-4†	>95	<5

* Glycosphingolipids of neural origin.
† Glycosphingolipids of visceral origin.

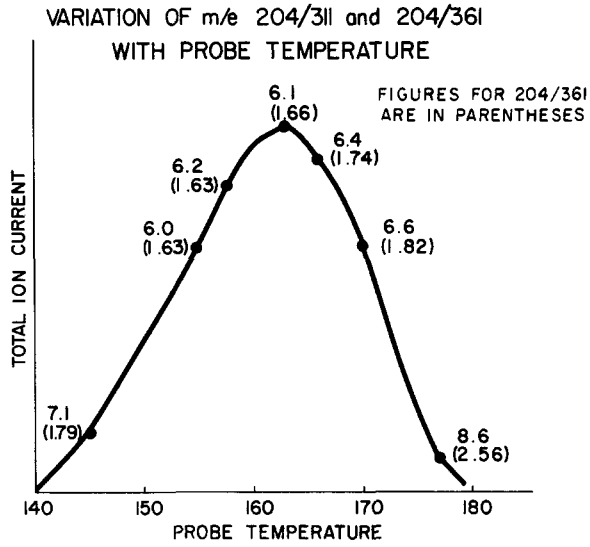


FIG. 3. Mass spectrometric analyses of the TMSi derivative of GL-3 (galactosylgalactosylglucosylceramide), showing the variation of relative intensities of m/e 204/311 (m/e 204 *rel*) and m/e 204/361 with temperature of the direct probe inlet. Conditions are described in the text.

may have caused some of the differences was not examined. The GL-3 isolated from the kidney of a patient with Fabry's disease had a long-chain base composition different from that of GL-2b from the same kidney. The GL-3 contained mainly sphingosine, with small amounts of hexadecasphing-4-enine (m/e 297) and nonadecasphing-4-enine (m/e 325). The mass spectrum of the TMSi derivative of GL-2b showed a peak at m/e 311 and an additional peak at m/e 299 for 4-hydroxysphinganine, which constituted about 15% of the total long-chain base content as previously found (26, 27). The peaks at m/e 299 and 311 are clearly visible in the mass spectrum of this lipid (Fig. 2).

There are four major types of fragmentation involving the fatty acid moiety of the glycosphingolipid, as discussed previously (19) and shown in Table 6. Of these, the Type B ions (m/e 592 to 704) had the highest relative intensities in compounds containing normal fatty acids. By taking mass spectra at the maximum intensity of total ion current it was possible to obtain a reasonable estimate of the fatty acid composition. Furthermore, α-hydroxy acids were easily detected by the homologous series corresponding to Types A, B, and C fragmentation, as shown in Fig. 2. These peaks are 88 mass units higher than the parent peaks for normal fatty acids, and are derived in the same manner but contain the α-OTMSi group on the fatty acid residue. A unique series of peaks was also observed with sphingolipids containing α-hydroxy acids. As previously observed by Hammarström, Samuelsson, and Samuelsson (28) with mass spectra of synthetic ceramides containing α-hydroxy acids, loss of the fatty acyl group and the TMSiO group on C-2 is a prominent fragment. Minor peaks from m/e 327 to 411 in Fig. 2 represent this homologous series.

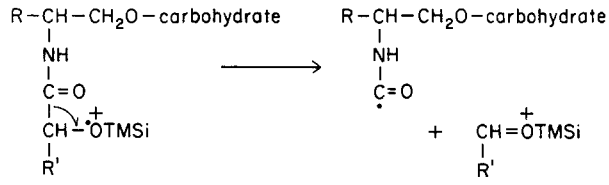


TABLE 6 MASS SPECTRAL FRAGMENT IONS FROM THE FATTY ACIDS OF GLYCOSPHINGOLIPIDS

Homologous Series	m/e*	Structure
Type A		$[RCONH(TMSi)CHCH_2O]^+$
Normal fatty acids	370–482	
α-Hydroxy acids	458–570	
Type B		$[CH_3(CH_2)_{12}CH{=}CHCH(OTMSi)CH(NHCOR)CH_2]^+$
Normal fatty acids	592–704	
α-Hydroxy acids	680–792	
Type C		$[CH_3(CH_2)_{12}CH{=}CHCH(OTMSi)CH(NHCOR)CH_2OCH{=}OTMSi]^+$
Normal fatty acids	710–822	
α-Hydroxy acids	798–910	
Type D		$[R{-}CH{=}OTMSi]^+$
α-Hydroxy acids	299–411	

* Ranges for the homologous series of fatty acids are from 16:0 to 24:0.

Since differences in volatility gave partial resolution of glycosphingolipids according to fatty acid chain length during evaporation in the direct probe inlet of the mass spectrometer (19), an attempt was made to determine whether this was a factor in the reproducibility of relative intensities of ions used to deduce the structure of the oligosaccharide moiety. The intensities of three major fragments (m/e 204, 311, and 361) in the mass spectrum of GL-3 are given in Fig. 3 as a function of the probe temperature. Very good agreement was observed for duplicate mass spectra recorded at any particular time, but the difference in intensities for mass spectra at the extremes of temperature is readily apparent. We have concluded that analyses should be made when total ion current has reached approximately maximum levels.

DISCUSSION

In an analysis of the structure of a glycosphingolipid by mass spectrometry it is assumed that the characteristic ions produced by fragmentation of the long-chain base have about the same probability of formation irrespective of the nature of the oligosaccharide unit. The total intensity of fragment ions for sphingosine, sphinganine, 4-hydroxysphinganine, etc., can be compared, therefore, with the intensities of fragment ions that are derived from the carbohydrate moieties in the molecule. Since the intensities of the latter are affected by the positions of glycosidic linkages, some predictions can be made regarding the length of the carbohydrate chain and the positions of glycosidic bonds. Further, the commonly occurring 2-acetamidohexoses, 6-deoxyhexoses, and sialic acids can be distinguished from neutral hexoses and can be assigned to terminal or internal positions in the oligosaccharide. Although it is not possible to distinguish between individual hexoses such as glucose, galactose, and mannose from mass spectral data, GLC provides this information. It is essential, therefore, that the results of gas–liquid chromatographic analyses of the carbohydrate constituents be used in conjunction with mass spectrometry in the assignment of a structure.

The relationships of intensities of fragment ions that are of greatest use were established by comparisons of the mass spectra of authentic reference compounds. The observed values are likely to be dependent on the conditions used for the analysis. It is necessary, for example, to elute the material from the direct probe tube at a relatively slow rate so that mass spectra can be recorded at the apex of the total ion intensity profile (Fig. 3), thus eliminating anomalies due to changing concentration and possibly those that are drastically affected by temperature. It is possible, however, that even with this precaution the results we have obtained will be considerably different from those in which another mass spectrometer is used, especially if the ratios of intensities are dependent on ion source temperature and probe design. At any rate it will probably be necessary to establish the relative intensities of ions such as m/e 204, 205, 243, 330, and 361 with reference samples of glucosylceramide and globoside as a check before analyzing unknown compounds.

With these possible limitations of the technique in mind, the analysis gives considerable insight into the structure of a glycosphingolipid and requires only a few micrograms of purified material. The total number of monosaccharide residues in simple glycosphingolipids from GL-1 to G_{M1} is directly proportional to the value of m/e 103 *rel*. This ratio would certainly be sensitive to (1→6) glycosidic linkages but, to date, this linkage has never been found in a glycosphingolipid. The values of m/e 243 *rel* and 271 *rel* often parallel those of m/e 103 *rel* but appear to be more susceptible to variations in operating conditions. The value of m/e 204 *rel* is also indicative of the number of monosaccharide units but it was sensitive to (1→3) linkages (Fig. 1). A comparison of the values of m/e 103 *rel*, m/e 204 *rel*, and gas–liquid chromatographic analyses should therefore provide strong evidence for the presence of (1→3) linkages in the oligosaccharide chain. Further confirmation of a (1→3) linkage can then be found in the value of m/e 191 *rel*, although this is not a completely reliable indicator when used alone. At present, it is not possible to distinguish between (1→2) and (1→4) linkages by mass spectrometry.

Substances with terminal hexose residues are characterized by high values for m/e 451 *rel* and 361 *rel*, which are relatively constant for GL-1, GL-2, and GL-3, as well as G_{M1}. On the other hand, substances with a terminal *N*-acetylgalactosamine residue, such as GL-4 and G_{M2}, have high values of m/e 420 *rel* and 330 *rel*. These pairs of ions provide strong evidence for the nature of the terminal monosaccharide residue in glycosphingolipids of the types presently known, and it is probable that their relative intensities would be significantly changed in compounds with branched structures (other than gangliosides).

In the gangliosides, sialic acid is invariably terminal unless two residues are linked together as in the disialogangliosides G_{D1b} and G_{T1a}. All of the gangliosides studied had significant peaks at m/e 186 (NANA) or m/e 274 (NGNA) and less specific fragments at m/e 173 (NANA) or m/e 261 (NGNA), which are derived from sialic acids as well as *N*-acylhexosamines. The relative values of three diagnostic ions, m/e 173 *rel*, m/e 186 *rel*, and m/e 205 *rel*, increased progressively in the series asialo G_{M1}, G_{M1}, and G_{D1a}, which contain one, two, and three acetamido sugars, respectively. When gas–liquid chromatographic analysis of the carbohydrate constituents has shown that a neuraminic acid is present in the gly-

cosphingolipid, the nature of the *N*-acyl group can be deduced reliably from the mass spectrum, and relative intensities of the ions above can be interpreted in terms of the number of sialic acid residues. A parallel analysis of several standards will probably be necessary to confirm the ratios we have observed for the gangliosides.

The fatty acid composition of the glycosphingolipid can also be determined by mass spectrometry of the lipid. The structures of Types A, B, and C fragments which involve the fatty acid moiety were discussed previously (19). It was found that the intensities of peaks in these series were sensitive to the chain length of the fatty acids and the temperature of the direct probe; best results were therefore obtained when the data from several mass spectra, recorded at different temperatures, were averaged.

The most intense series of fragment ions observed with molecular species containing normal fatty acids were those of Type B. When GL-2b was examined, however, the series of peaks at m/e 458 to 570 (Type A plus 88 mass units) was of somewhat greater intensity than those at m/e 680 to 792 (Type B plus 88 mass units), as shown in Fig. 2. These two series and an additional one at m/e 798 to 910 (Type C plus 88 mass units) represent the major ions from molecular species containing α-hydroxy acids. The Type A ions have the same empirical formulas as those proposed for major fragments in the mass spectra of trimethylsilyl derivatives of synthetic ceramides containing normal (17) or α-hydroxy acids (28). The ions were believed to be formed from the ceramides by simple cleavage of the bond between C-2 and C-3 of the long-chain base, with charge retention on the amide nitrogen. In the glycosphingolipids, however, we have proposed that the Type A fragments are formed by the loss of the carbohydrate portion, followed by rearrangement of a TMSi group and cleavage of the long-chain base as described previously (28, 29). Types B and C ions are not formed from simple ceramides but they have been observed in mass spectra of TMSi cerebrosides obtained by combined GLC–mass spectrometry (20), and analogous ions were reported for acyl derivatives of cerebrosides (23).

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References

1. Hakomori, S-I. 1966. Release of carbohydrates from sphingoglycolipids by osmium-catalyzed periodate oxidation followed by treatment with mild alkali. *J. Lipid Res.* **7:** 789–792.
2. Wiegandt, H., and G. Baschang. 1965. Die Gewinnung des Zuckeranteiles der Glykosphingolipide durch Ozonalyse und Fragmentierung. *Z. Naturforsch.* **20b:** 164–166.
3. Kuhn, R., and H. Wiegandt. 1963. Die Konstitution der Ganglio-N-tetraose und des Gangliosids G_I. *Chem. Ber.* **96:** 866–880.
4. Svennerholm, L. 1962. The chemical structure of normal human brain and Tay-Sachs gangliosides. *Biochem. Biophys. Res. Commun.* **9:** 436–441.
5. Wiegandt, H. 1970. Gangliosides of extraneuronal tissue. *In* Chemistry and Metabolism of Sphingolipids. C. C. Sweeley, editor. North-Holland Publishing Co., Amsterdam. 198–204.
6. Kuhn, R., and H. Wiegandt. 1964. Die Konstitution der Ganglioside G_{II}, G_{III} und G_{IV}. *Z. Naturforsch.* **18b:** 541–543.
7. Hakomori, S-I. 1964. A rapid permethylation of glycolipid and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. *J. Biochem. (Tokyo).* **55:** 205–208.
8. Makita, A., and T. Yamakawa. 1962. Biochemistry of organ glycolipid. I. Ceramide oligohexosides of human, equine and bovine spleens. *J. Biochem. (Tokyo).* **51:** 124–133.
9. Sweeley, C. C., P. D. Snyder, Jr., and C. E. Griffin. 1970. Chemistry of glycosphingolipids in Fabry's disease. *Chem. Phys. Lipids.* **4:** 393–408.
10. Sweeley, C. C., R. Bentley, M. Makita, and W. W. Wells. 1963. Gas–liquid chromatography of trimethylsilyl derivatives of sugars and related substances. *J. Amer. Chem. Soc.* **85:** 2497–2507.
11. Vance, D. E., and C. C. Sweeley. 1967. Quantitative determination of the neutral glycosyl ceramides in human blood. *J. Lipid Res.* **8:** 621–630.
12. Clamp, J. R., G. Dawson, and L. Hough. 1967. The simultaneous estimation of 6-deoxy-L-galactose (L-fucose), D-mannose, D-galactose, 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine) and *N*-acetylneuraminic acid (sialic acid) in glycopeptides and glycoproteins. *Biochim. Biophys. Acta.* **148:** 342–349.
13. Kim, J. H., B. Shome, T.-H. Liao, and J. G. Pierce. 1967. Analysis of neutral sugars by gas–liquid chromatography of alditol acetates: application to thyrotropic hormone and other glycoproteins. *Anal. Biochem.* **20:** 258–274.
14. DeJongh, D. C., T. Radford, J. D. Hribar, S. Hanessian, M. Bieber, G. Dawson, and C. C. Sweeley. 1969. Analysis of trimethylsilyl derivatives of carbohydrates by gas chromatography and mass spectrometry. *J. Amer. Chem. Soc.* **91:** 1728–1740.
15. Karlsson, K.-A. 1965. Studies on sphingosines. X. Use of trimethylsilyl ethers for the gas chromatography and mass spectrometry of sphingosines. *Acta Chem. Scand.* **19:** 2425–2427.
16. Polito, A. J., T. Akita, and C. C. Sweeley. 1968. Gas chromatography and mass spectrometry of sphingolipid bases. Characterization of sphinga-4,14-dienine from plasma sphingomyelin. *Biochemistry.* **7:** 2609–2614.
17. Samuelsson, B., and K. Samuelsson. 1969. Gas–liquid chromatography–mass spectrometry of synthetic ceramides. *J. Lipid Res.* **10:** 41–46.
18. Casparrini, G., E. C. Horning, and M. G. Horning. 1969. Gas phase analytical separation and structural study of ceramides. *Chem. Phys. Lipids.* **3:** 1–10.
19. Sweeley, C. C., and G. Dawson. 1969. Determination of

glycosphingolipid structures by mass spectrometry. *Biochem. Biophys. Res. Commun.* **37:** 6–14.
20. Samuelsson, K., and B. Samuelsson. 1969. Gas–liquid chromatography–mass spectrometry of cerebrosides as trimethylsilyl ether derivatives. *Biochem. Biophys. Res. Commun.* **37:** 15–21.
21. Karlsson, K.-A., B. E. Samuelsson, and G. O. Steen. 1969. Mass spectrometry of polar complex lipids; analysis of a sulfatide derivative. *Biochem. Biophys. Res. Commun.* **37:** 22–27.
22. Casparrini, G., M. G. Horning, and E. C. Horning. 1969. The characterization of phospholipids by gas phase analytical methods. *In* Drugs Affecting Lipid Metabolism. W. L. Holmes, L. A. Carlson, and R. Paoletti, editors. Plenum Press, New York. 597–608.
23. Horning, M. G., G, Casparrini, and E. C. Horning. 1969. The use of gas phase analytical methods for the analysis of phospholipids. *J. Chromatogr. Sci.* **7:** 267–275.
24. Klenk, E., and G. Padberg. 1962. Uber die Ganglioside von Pferdeerythrocyten. *Z. Phys. Chem.* **327:** 249–255.
25. Rosenberg, A., and N. Stern. 1966. Changes in sphingosine and fatty acid components of the gangliosides in developing rat and human brain. *J. Lipid Res.* **7:** 122–131.
26. Karlsson, K.-A., and E. Mårtensson. 1968. Studies on sphingosines. XIV. On the phytosphingosine content of the major human kidney glycolipids. *Biochim. Biophys. Acta.* **152:** 230–233.
27. Karlsson, K.-A. 1970. On the chemistry and occurrence of sphingolipid long-chain bases. *In* Chemistry and Metabolism of Sphingolipids. C. C. Sweeley, editor. North-Holland Publishing Co., Amsterdam. 6–43.
28. Hammarström, S., B. Samuelsson, and K. Samuelsson. 1970. Gas–liquid chromatography–mass spectrometry of synthetic ceramides containing 2-hydroxy acids. *J. Lipid Res.* **11:** 150–157.
29. Polito, A. J., J. Naworal, and C. C. Sweeley. 1969. Determination of the structures of sphingolipid bases by combined gas chromatography–mass spectrometry. *Biochemistry.* **8:** 1811–1815.