ions and the N7 site of the purines.

In vivo, the DNA concentration in nuclei can be favorably compared to the concentration at which the Z form of poly[d(A-C)]·poly[d(G-T)] has been obtained with Ni^{2+} ions. The existence of left-handed DNA sequences that possibly play a role in gene expression (Rich, 1982) may thus be promoted by small amounts of divalent cations such as Ni^{2+}.

Registry No. Poly[d(A-C)]·poly[d(G-T)], 55684-99-6; poly[d(G-C)], 36786-90-0; Ni, 7440-02-0.

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Fast Atom Bombardment Mass Spectrometry of Glycosphingolipids. Glycosphingolipids Containing Neutral Sugars†

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ABSTRACT: Natural and synthetic glycosphingolipids containing neutral sugars have been analyzed by positive and negative ion fast atom bombardment mass spectrometry. Basic structural characterization including saccharide size and sequence and ceramide composition is possible on the basis of the fragment ions observed. The degree of fragmentation could be increased by using higher sample concentrations and lower fast atom beam energies. Commercially available synthetic compounds that had been presumed to be pure were shown to contain homologous fatty acids. Mixtures of glycosphingolipids such as those obtained from Gaucher's spleen and from human erythrocytes can be characterized and quantitated.

The growing interest in the diverse biological functions of cell membrane glycosphingolipids (GSL's)[1] as cell surface antigens, cell–cell recognition sites, and receptors for a variety of signal molecules (Fishman & Brady, 1976; Yamakawa & Nagai, 1976; Hakamori, 1981; Ledeen & Yu, 1982) has prompted the constant search for new procedures for their structural analysis. Mass spectrometry (MS) because of its high sensitivity and speed, has always figured prominently in this regard. First applied to GSL's in 1969 by Sweeley & Dawson (1969) and by Samuelsson & Samuelsson (1969), it has gained rapid recognition as a viable tool in furnishing detailed structural information regarding the carbohydrate composition and sequence and, in some instances, the lipophilic constituents. Previous investigations have relied upon either electron ionization (EI) or chemical ionization (CI) for the

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[1] Abbreviations: GSL, glycosphingolipid; MS, mass spectrometry; EI, electron ionization; CI, chemical ionization; DI, desorption ionization; FAB, fast atom bombardment; DCI, desorption chemical ionization; FD, field desorption; CTH, ceramide trihexoside; TETA, triethylenetetramine; GC, gas chromatography; TFA, trifluoroacetic acid.

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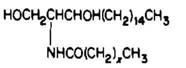
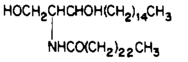
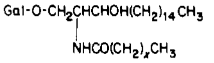
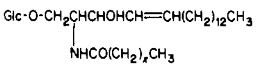
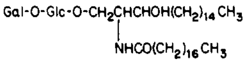
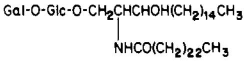
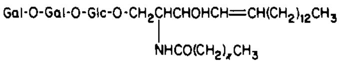
analysis of GSL's as their trimethylsilylated, peracetylated, or permethylated derivatives [Tanaka et al., 1984; Ariga et al. (1982) and references cited therein]. Conversion of the nonvolatile GSL's to their volatile derivatives prior to mass spectrometric analysis requires elaborate sample preparation and purification steps that are time consuming and frequently difficult to achieve in a quantitative manner. Furthermore, derivatization of the parent GSL's invariably increases the molecular weights, resulting in generally less intense spectra in the higher mass ranges required. With the advent of new ionization, particularly desorption ionization (DI), techniques [Busch & Cooks (1982) and references cited therein], it is possible to obtain mass spectra directly from underivatized, large, and involatile biomolecules of low thermal stability. Among these techniques, the recently developed fast atom bombardment (FAB) has been shown to be particularly effective (Barber et al., 1981; Williams et al., 1981). Its usefulness in providing structural information on several naturally occurring compounds, including GSL's, has also been clearly demonstrated (Rinehart, 1982). Mass spectra of polysaccharides with molecular weights to ca. 3000 have been obtained (Forsberg et al., 1982; Dell & Ballou, 1983), and the utility of negative ion spectra for saccharides has been demonstrated (Barber et al., 1982a), as has the efficacy of alternative matrices for oligosaccharides and GSL's (Harada et al., 1982a,b; Arita et al., 1983a,b). FAB has been compared to desorption chemical ionization (DCI) for the analysis of GSL's (Carr & Reinhold, 1982) and has also been compared to field desorption (FD) (Higuchi et al., 1983). In this paper we provide a detailed systematic analysis of several neutral GSL's of increasing complexity and their ceramides, using FAB mass spectrometry. Matrix requirements and homologous mixture analysis are also discussed.

Experimental Procedures

Materials. Synthetic *N*-stearyldihydrosphingosine (ceramide **1**), *N*-lignoceryldihydrosphingosine (ceramide **2**), *N*-stearyldihydrogalactocerebroside (galactosylceramide **3**), *N*-stearyldihydrolactocerebroside (lactosylceramide **5**), and *N*-lignoceryldihydrolactocerebroside (lactosylceramide **6**) were obtained from Miles Laboratory, Elkhart, IN. Glucocerebroside (**4**), isolated from Gaucher's spleen, was obtained from Sigma Chemical Co., St. Louis, MO. Ceramide trihexoside (CTH; **7**) was isolated from human erythrocytes and was kindly provided by Dr. S. Ando, Tokyo Metropolitan Institute of Gerontology, Tokyo, Japan. Structures of these compounds are given in Table I. Glycerol (99.5%) was obtained from Fisher Scientific, Pittsburgh, PA. Tetraethyleneglycol (tetragol), 3-mercapto-1,2-propanediol (thioglycerol, 95%), and triethylenetetramine (TETA, technical) were obtained from Aldrich Chemical Co., Milwaukee, WI.

Mass Spectrometry. FAB spectra of **1**–**6** were run on a VG ZAB-1F spectrometer equipped with a high-field magnet and a VG 11/250 data system. An oscillographic spectrum of **7** was obtained on a Finnigan MAT 731 spectrometer and was manually counted and measured and plotted on a DEC VAX-11 computer. Both spectrometers were equipped with Ion Tech saddle field fast-atom sources. The samples were dissolved in methanol/chloroform (2:1) and, unless otherwise noted, 1 μL of a 4 μg/μL solution was added to ca. 2 μL of the appropriate dispersing solvent on a stainless steel FAB target ribbon. The samples were introduced into the ZAB-1F FAB source and the modified 731 FD/EI source through the FAB/FD probe inlet, in line with the flight axis, and were bombarded with a 9-keV xenon atom beam at 1 mA. Spectra were acquired at a 5 s/decade scan rate with a 3-s interscan delay. From 10 to 20 scans were generally acquired before spectral intensity decreased significantly due to evaporation of the more volatile solvents. The source pressure prior to sample introduction was 2.3 × 10^{-6} Torr. The data system was calibrated with glycerol or neat sodium iodide. Mass defects of greater than 0.5 amu resulted in computer-assigned masses that were 1 amu greater than the expected nominal masses. The spectra have been relabeled with corrected nominal masses for ease of interpretation.

Table I: Compounds Studied and Their Masses

compd	structure[a]	calcd M_r	x
1	 $\mathrm{HOCH_2\underset{\substack{\vert\\ NHCO(CH_2)_xCH_3}}{CH}CHOH(CH_2)_{14}CH_3}$	567	16:0
		539	14:0
2	 $\mathrm{HOCH_2\underset{\substack{\vert\\ NHCO(CH_2)_{22}CH_3}}{CH}CHOH(CH_2)_{14}CH_3}$	651	
3	 $\mathrm{Gal\text{-}O\text{-}CH_2\underset{\substack{\vert\\ NHCO(CH_2)_xCH_3}}{CH}CHOH(CH_2)_{14}CH_3}$	729	16:0
		701	14:0
4	 $\mathrm{Glc\text{-}O\text{-}CH_2\underset{\substack{\vert\\ NHCO(CH_2)_xCH_3}}{CH}CHOHCH{=}CH(CH_2)_{12}CH_3}$	699	14:0
		727	16:0
		755	18:0
		781	20:1
		783	20:0
		797	21:0
		809	22:1
		811	22:0
5	 $\mathrm{Gal\text{-}O\text{-}Glc\text{-}O\text{-}CH_2\underset{\substack{\vert\\ NHCO(CH_2)_{16}CH_3}}{CH}CHOH(CH_2)_{14}CH_3}$	891	
6	 $\mathrm{Gal\text{-}O\text{-}Glc\text{-}O\text{-}CH_2\underset{\substack{\vert\\ NHCO(CH_2)_{22}CH_3}}{CH}CHOH(CH_2)_{14}CH_3}$	975	
7	 $\mathrm{Gal\text{-}O\text{-}Gal\text{-}O\text{-}Glc\text{-}O\text{-}CH_2\underset{\substack{\vert\\ NHCO(CH_2)_xCH_3}}{CH}CHOHCH{=}CH(CH_2)_{12}CH_3}$	1133	22:1
		1135	22:0

[a] Gal = galactose; Glc = glucose.

Quantitation of the FAB data was accomplished by averaging entire spectra from the first 9 to 13 scans, excluding the first scan. The background signal was subtracted before relative compositions were calculated. Averaging of 10 scans resulted in composition values good to ±2%. Averaging additional scans (up to 50 in less volatile matrices) resulted in no S/N improvement, indicating that the noise is chemical rather than statistical. Determination of quantitation accuracy was accomplished by combining mixture **1** and compound **2** in known ratios. These mixtures were analyzed by FABMS, and the *m/z* 568/652 intensity ratios were calculated.

Gas Chromatography. GC was performed on a Varian 3700 gas chromatograph with a 10-m SP-2100 capillary column. Fatty acid methyl esters were prepared by heating 100–200 μg of each mixture for 3 h at 100 °C in 400 μL of 1 N methanolic hydrogen chloride. GC peak areas were obtained with a Spectra Physics Minigrator integrator and were corrected for relative molar sensitivity for comparison with the FAB data.

Liquid Chromatography. A mixture of compounds **1** ($C_{16:0}$ plus $C_{18:0}$) and **2** was separated by reversed-phase high-performance liquid chromatography (HPLC) to yield **1** ($C_{18:0}$ only) and **2** contaminated with column packing material. The HPLC was performed with a Perkin-Elmer Series 1 pump and an LC-75 variable-wavelength detector operated at 205 nm. The mixture (0.5–2 mg), dissolved in chloroform/methanol (2:1), was injected onto a 250 × 4.6 mm column packed with 5-μm Econosphere C18 (Alltech, Deerfield, IL). The solvent was 98:2 methanol/buffer (5 mM formic acid/triethylamine, pH 7.4), and the flow rate was 2 mL/min (Jungalwala et al., 1979).

Table II: Positive and Negative Ions of Glycosphingolipids 1–7

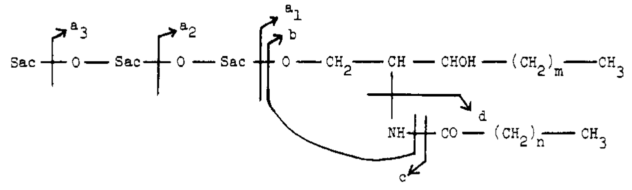
ion[a]	1	2	3	4	4A	4B	4C	4D	4E	4F	4G	4H	5	6	7
Positive Ions															
M + H	568, 540	652 (674)[b,c]	730, 702		700			782	784		810	812	892	976	1134, 1136
M + H − H_2O	550, 522	634			682	710	738	764	766		792	794			1116, 1118
a_1			568, 540										568	652	
a_1 − H_2O			550 (522)		520			602	604		630	632	550	634	(630, 632)
a_2 − H_2O															(792, 794)
a_3 − H_2O															954, 956
b	302	302	(302)												
b − H_2O	284	284	284	282											282
b − $2H_2O$				264											264
c	302	302	464										626	626	(786)
d	284	368	284											368	
Negative Ions															
r			119	119									119	119	119
															221
															263
															383
															425
v			179	179									179	179	179
w					254	282	310	336	338	352	364	366	282	366	
x					280	308	336	362	364	378	390	392	308	392	
y					296	324	352	378	380	394	406	408	324	408	
z_3															970, 972
z_2													728	812	808, 810
z_1			(566)		536	564	592	618	620	638	646	648	566	650	
M − H	566, 538	650, 758[d]	728, 700		698	726	754	780	782	796	808	810	890	974	1132, 1134

[a] See Schemes I and II. [b] Parentheses indicate low intensity. [c] Na adduct. [d] Thioglycerol adduct.

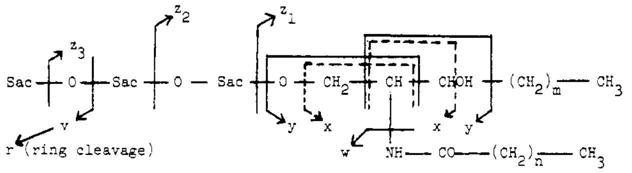
Results

Single Compounds. The unsubstituted ceramide **2** (Table I) was one of the simplest compounds investigated, and its FAB spectra varied with the "matrix" solvent (dispersant) used. The simplest spectra were obtained with tetragol as the matrix. Despite the homology of ceramides **1** (Table I and see Mixtures) and **2**, spectra for the somewhat larger **2** were much harder to obtain and required a different matrix. A loading of 5 μg of **1** in tetragol yielded spectra quite readily, while spectra of **2** could not be obtained until the amount was decreased to 0.5 μg. With 5 μg, **2** did not remain completely dissolved in the tetragol and gave only very transient ions. Moreover, only relatively weak molecular ions could be obtained in glycerol, even at 0.5 μg, and addition of acid (HCl, TFA) did not increase sensitivity or fragmentation. The positive ion spectrum for **2** in tetragol contained an $(M + H)^+$ ion at *m/z* 652, with a peak at *m/z* 634 corresponding to the loss of one molecule of water. The negative ion spectrum in tetragol was simpler still, with only a pseudomolecular ion $(M - H)^-$ at *m/z* 650. A suitable matrix for fragmentation study of positive ions was found in thioglycerol, which, with 1.2 μg of **2**, gave a very strong $(M + H)^+$ peak and intense fragment peaks. In addition to $(M + H)^+$, a weak $(M + Na)^+$ ion was observed at *m/z* 674 as well as loss of water at *m/z* 634. Peaks at *m/z* 302 and 284 are due to the protonated sphingosine ion b (Table II and Scheme I) and its loss of water, respectively. An ion at *m/z* 368 is clearly due to the C_{24} fatty acid amide ion d. The negative ion spectrum of **2** in thioglycerol showed only a relatively weak $(M - H)^-$ peak and an $(M - H + \text{thioglycerol})^-$ peak at *m/z* 758.

Scheme I: Positive Ions



Scheme II: Negative Ions



Cerebroside **5** (Table I) is a dihexoside sphingolipid containing lactose. A distinct $(M + H)^+$ ion is present at *m/z* 892 in the positive ion FAB spectrum (glycerol) with no clear peak due to loss of water. Loss of the galactose unit is not observed under these conditions, but nominal loss of the entire lactosyl group produces the acylsphingosine peak a_1 at *m/z* 568 (ion a_1) and its dehydration product at *m/z* 550. Cerebroside **5** shows loss of the fatty acyl group with hydrogen transfer at *m/z* 626 (ion c). No ions indicative of the sphingosine alone (ion b, *m/z* 302 and 284) were observed under these conditions.

Cerebroside **5** shows a distinct $(M - H)^-$ ion in the negative ion spectrum (TETA) at *m/z* 890. The peak at *m/z* 728 is formed nominally by the loss of a galactosyl unit from M (ion z_2, Scheme II) and that at *m/z* 566 by the further loss of the glucosyl unit with hydrogen transfer or, alternatively, the loss of lactosyl as a unit from M. The monosaccharide is evident at *m/z* 179 (ion v), while the ion r at *m/z* 119 presumably arises from loss of $C_2H_4O_2$ (hydroxyacetaldehyde) from v, a loss noted as typical of sugars (Barber et al., 1982). No corresponding peaks are present for the disaccharide as a unit. The peak at *m/z* 282 can be attributed to the fatty acid amide (ion w) and the ions at *m/z* 308 and 324 to species x and y (Table III and Scheme II):

$$CH_3(CH_2)_{16}CO{-}NH{-}CH{=}CH^-$$
m/z 308 (x)

$$CH_3(CH_2)_{16}CO{-}NH{-}CH{=}COH^-$$
m/z 324 (y)

Table III: Fatty Acid Related Fragments from **4**

fatty acid	fragments (relative intensity)[a] w	x	y
16:0	254 (40)	280 (85)	296 (60)
18:0	282 (27)	308 (47)	324 (26)
20:0	310 (22)	336 (36)[b]	352 (30)[c]
22:1	336 (36)[b]	362 (62)	378 (46)[e]
22:0	338 (51)	364 (99)[d]	380 (75)
23:0	352 (30)[c]	378 (46)[e]	394 (32)
24:1	364 (99)[d]	390 (60)	406 (66)
24:0	366 (31)	392 (93)	408 (60)

[a] w, $CH_3(CH_2)_n(CH{=}CH)_mCONH^-$; x, w—$CH{=}CH^-$; y, w—$CH{=}COH^-$. [b–e] Redundancies.

Cerebroside **6**, a synthetic dihexoside glycosphingolipid like **5**, contains lignoceryldihydrosphingosine as the ceramide. An $(M + H)^+$ ion is observed at m/z 976 in the positive ion spectrum (glycerol). No peak is discernible for the loss of water, nor is any peak observed for the loss of the galactosyl unit. However, loss of lactosyl with hydrogen transfer gives the ceramide peak a_1 at m/z 652 and its dehydration product at m/z 634. A peak at m/z 626 can be attributed to loss of the acyl group with hydrogen transfer from the molecular ion to give the same peak (c) as seen for **5**. The sphingosine ion and its dehydration product (b, m/z 302 and 284) are not observed under these conditions (vide infra). A small peak at m/z 368, which does not fit into the m/z 369 glycerol peak pattern, is probably d, due to the fatty acid amide, as was also observed for **2** where there is no interference from the thioglycerol.

The negative ion spectrum for **6** (TETA) is considerably weaker than the positive ion spectrum. An $(M - H)^-$ ion is observed at m/z 974. Loss of galactosyl, with hydrogen transfer to give m/z 812 (ion z_2), and lactosyl, to give m/z 650 (z_1), is evident. The usual monosaccharide peaks appear at m/z 119 (r) and 179 (v), and the fatty acid amide is present as fragments w, x, and y at m/z 366, 392, and 408. The peak at m/z 367 may be due to a cleavage complementary to the w, x, y series, yielding

$$\text{Gal—O—Glc—O—CH}{=}\text{CH}^-$$

In general then, the sugars can be observed in the negative ion FAB spectra at m/z 179 and 119 (v and r) and at peaks due to successive losses of sugars as a_1, a_2, a_3, etc., the fatty acid amide in the positive (or negative) ion spectra as d (or w, x, y), and the sphingosine in the positive ion spectra as b and $b - H_2O$. The acylsphingosine is found in the positive ion spectrum in ion a_1 and $a_1 - H_2O$, and the entire glycosphingolipid is found as M + H in the positive ion spectrum and as M − H in the negative. Thus, the complete glycosphingolipid is reasonably well characterized by the FAB spectrum (see Table II).

Mixtures. Compound **1** was presumed to be a single compound but was found to contain a mixture of fatty acids by FABMS and GC. The simplest spectra (not shown) were obtained by using tetragol and consisted of $(M + H)^+$ and $(M + H - H_2O)^+$ ions at m/z 568 and 540 and m/z 550 and 522 (weak) in the positive ion spectrum and of $(M - H)^-$ ions at m/z 566 and 538 in the negative ion spectrum. The spectrum of **1** with glycerol as the matrix provided a few identifiable fragments as well as the peaks in the molecular ion region. The pseudomolecular ion of ceramide **1** cleaves, with hydrogen transfer, at the amide linkage to give b, the protonated dihydrosphingosine $[CH_3(CH_2)_{14}CHOHCHNH_2CH_2OH + H]^+$ at m/z 302. The peak at m/z 284 may arise simply by loss of water from b, but it may also result from a contribution by d, $[CH_3(CH_2)_{16}CONH_2 + H]^+$. Since $b - H_2O$ and d have the same elemental composition, a metastable experiment would be needed to make a definite assignment. However, analogous peaks for **2** (cf. above) indicate the m/z 284 peak of **1** probably arises from both routes although the necessary confirmatory homologue peak at m/z 256 is too weak to assign. The negative ion spectrum of **1** (glycerol) gives only transient $(M - H)^-$ peaks at m/z 566 and 538.

Quantitation of the components of **1** by FAB indicated a fatty acid composition of 92:8 (C_{16} to C_{18}) from an average of nine scans of the positive ion spectrum. An average of 10 scans of the negative ion spectrum yielded a composition of 78:22, but the averaged positive ion spectrum was considerably less noisy than the negative and corresponded more closely to the fatty acid methyl ester GC data, which indicated a 93:7 composition.

Cerebroside **3**, like ceramide **1**, was initially presumed to be pure but contained a mixture of fatty acids. It exhibited more extensive fragmentation in the positive ion spectrum when run in glycerol than in tetragol, and the fragmentation was similar to that of **1** in glycerol. The molecular ion region showed $(M + H)^+$ ions at m/z 730 and 702 (glycerol). The glycosphingosine portion of the molecule was indicated by a rather strong peak at m/z 464 (ion c), corresponding to loss of the C_{18} acyl group with hydrogen transfer. Since the m/z 464 peak did not have an associated peak 28 amu lower, the homology must be in the fatty acid portion of the molecule. A major fragment from **3** arises from loss of the galactosyl unit, with hydrogen transfer to the ceramide, presumably to give the $(M + H)^+$ ion for the ceramide (a_1). This now familiar fragmentation, which gives the dehydration ions as well, is observed at m/z 568 and 540 and m/z 550 and 522 (weak) for **3** and its homologue. Ion a_1 would be expected to produce the peaks at m/z 302 (weak) and 284, due, as in **1**, to the sphingosine (ion b) and sphingosine − H_2O. The fatty acid amide should also presumably contribute to the m/z 284 ion, but the low intensity prevented observation of the homologue.

The negative ion spectrum (tetragol) showed mainly $(M - H)^-$ ions for both **3** and its homologue, but a weak peak at m/z 566 was observed for loss of galactose (z_1). The galactosyl anion is observed at m/z 179 (v) and the ring cleavage product, r, at m/z 119.

Quantitation of the components of **3** by FAB indicated an 80:20 (C_{16} to C_{18}) composition based on an average of 20 positive ion scans. An average of 10 negative ion scans indicated a composition of 76:24. The GC data for the fatty acid methyl esters again agreed more closely with the positive ion data, yielding an 85:15 composition. The averaged data also showed a less noisy positive ion spectrum.

Cerebroside **4**, a more complex mixture of glucocerebrosides that are heterogeneous in the fatty acid portion of the molecule, showed exactly the same fragmentation pattern as **3**. The molecular ion region contained peaks for **4A**, **4D**, **4E**, **4G**, and **4H** as well as the corresponding dehydration peaks in the positive ion spectrum (Figure 1a, tetragol). However, **4B** and **4C** were represented only by small dehydration peaks at m/z 710 and 738, and **4F** was not detectable due to background. These compounds were all expected on the basis of evidence provided by GC and CIMS of the trimethylsilyl derivatives (Murata et al., 1978). Loss of glucose with accompanying hydrogen transfer from the dehydrated molecular ions ($a_1 - H_2O$) was observed for **4A**, **4D**, **4E**, **4G**, and **4H** at m/z 520, 602, 604, 630, and 632. The corresponding peaks expected at m/z 548, 576, and 618 for **4B**, **4C**, and **4F** were not large

Table IV: Composition Consistency of FAB Ions from **4**

	GC[a]	negative ion			positive ion				av of positive and negative ions
		M − H	M − H − Glc	av	M + H	M + H − H_2O	M + H − Glc	av	
$C_{16:0}$	17	22	20	21	23	19	20	21	21
$C_{18:0}$	4	4	9	6	2	5	11	6	6
$C_{20:0}$	4	6	4	5	3	6	4	5	5
$C_{22:1}$	3	7	6	6	14	12	11	12	10
$C_{22:0}$	30	25	20	23	20	19	11	19	21
$C_{23:0}$	5	5	4	4	6	8	6	7	6
$C_{24:1}$	17	14	21	18	16	15	14	15	16
$C_{24:0}$	22	18	16	17	15	16	15	15	16
av Δ[b]		2.6	4.2	2.9	4.8	4.5	6.0	4.6	3.8

[a] Totals may be greater than 100% due to rounding of percentages. [b] Determined by averaging the differences between the GC and FABMS data.

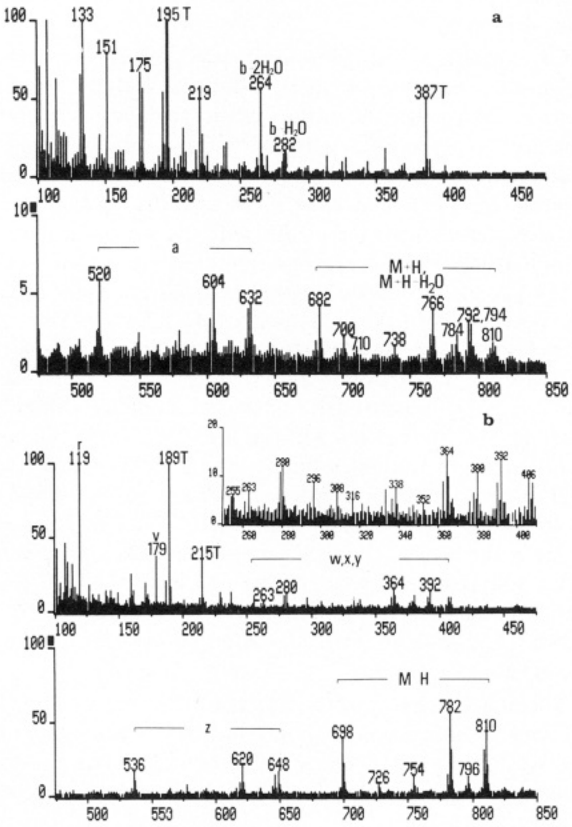


FIGURE 1: (a) Positive ion FAB spectrum of **4** in tetragol (T = tetragol peak); (b) negative ion FAB spectrum of **4** in TETA (T = TETA peak), with fatty acid related fragments expanded in inset.

enough to be distinguished from the background. An intense peak at *m/z* 264 is clearly due to the loss of water from the sphingosine peak b at *m/z* 282, formed by elimination of glucose and loss of the acyl group with hydrogen transfer. No peaks were observed for the fatty acid amides (ion d), but the heterogeneity would split that signal among several peaks, which are presumably unobservable since this fragment ion is usually weak.

The negative ion spectrum of cerebroside **4** was difficult to obtain. In addition to tetragol, diethanolamine and TETA were tried as matrices, chosen primarily by trial and error. The strongest spectrum was obtained in TETA (Figure 1b). In this matrix, $(M - H)^-$ peaks were observed for **4A**, **4D**, **4E**, **4G**, and **4H**, as expected from the positive ion spectrum, and weaker peaks at *m/z* 726, 754, and 796 for **4B**, **4C**, and **4F**. These results generally confirmed Murata's GC and CIMS evidence for the qualitative composition of the mixture. Peaks at *m/z* 536, 564, 592, 618, 620, 638, 646, and 648 are indicative of the loss of the glucosyl unit with hydrogen transfer to give the ceramide ions z_1 from **4A** − **4H**, respectively. The saccharide is also confirmed by peaks at *m/z* 179 and 119 (v and r). Ions related to the fatty acid were not immediately apparent, but by analogy to the spectra of **5** and **6**, a series of peaks (w, x, y) for each of the homologous fatty acids could be discerned. Ions for each series are listed in Table III, and the expanded portion of the spectrum is in Figure 1b. The multiplicity and overlap of these ions prevent them from providing much insight into the nature of the fatty acids or composition of the mixture.

Quantitation of this mixture by FAB was obtained by averaging 13 scans of the negative ion spectrum. This provided a reasonable estimate of the composition (Table IV), which can be compared with GC results on the mixture obtained in this laboratory. Deviations between our FABMS and GC data are similar to the deviations between Murata's CIMS and GC data (Murata et al., 1978), where the greatest difference was 4.5%. Internal consistency was tested by comparing corresponding peaks from an average of 10 and 13 scans from the positive and negative ion spectra, respectively. The data, presented in Table IV, show that any of the sets of peaks can be used to give a rough estimate of composition except that a consistently high value for the $C_{22:1}$ component is noted in the positive ion spectra. The best estimate of composition is obtained, however, from the $(M - H)^-$ ions, which result in the lowest total difference between FAB and GC data and also have the smallest spread of differences. The generally poorer estimates of composition from the positive ion data are at least in part due to the lower signal to background ratio, which in some cases is not much better than 2:1.

Cerebroside **7** is the human erythrocyte CTH, which contains a neutral linear trihexoside. The ceramide is composed of two components, one with a $C_{24:0}$ and the other with a $C_{24:1}$ fatty acid. Both the $(M + H)^+$ ions and their dehydration products are apparent at *m/z* 1134 and 1136 and *m/z* 1116 and 1118 (glycerol), but other peaks above *m/z* 700 are weak. Peaks at *m/z* 954 and 956 correspond to the loss of the first galactose (ion a_3). Peaks at *m/z* 792 and 794 are too weak to be diagnostic for loss of the second galactosyl unit (a_2). The ceramide formed by the loss of the entire trisaccharide is indicated by peaks at *m/z* 630 and 632 (a_1). The sphingosine is indicated by strong peaks at *m/z* 282 (b) and 264. No clear evidence exists to place the variation in the fatty acid portion of the molecule since the ions at *m/z* 786 are too weak to be diagnostic. Analogy to the other compounds studied would place the unsaturation in the fatty acid unit.

The negative ion spectrum (glycerol) provides confirmatory $(M - H)^-$ ions at *m/z* 1132 and 1134 as well as some weaker fragment ions. Loss of one galactosyl with hydrogen transfer is confirmed by peaks at *m/z* 970 and 972 (z_3), loss of a second galactosyl by ions at *m/z* 808 and 810 (z_2), and loss of glucosyl

Table V: Composition of **7** by FABMS

positive ions			negative ions		
	intensity			intensity	
ion	22:1	22:0	ion	22:1	22:0
M + H	57	48	M − H	62	68
M + H − H_2O	48	48	z_1	21	25
a_1	25	25	z_2	25	19
a_2	27	19	z_3	38	48
a_3	328	356			
	48%[a]	52%[a]		48%[a]	52%[a]

[a] Average composition, based on averaging above ions' relative intensities.

at m/z 646 and 648 (z_1). The saccharide units are again evident at m/z 179 and 119 (v and r). The fact that only extremely weak peaks are observed at m/z 341 and m/z 503 suggests that the oligosaccharides' glycosyloxy anions are relatively unstable, preferring to lose glycosyl units down to monosaccharides (m/z 179 and 119). Two ions, at m/z 221 and 263, are probably from the series:

$$\underset{m/z\ 179}{\text{Hex–O}^-} \leftarrow \underset{m/z\ 221}{\text{Hex–O–CH=CHO}^-} \leftarrow \underset{m/z\ 263}{\text{Hex–O–CH=COH–CH=CHO}^-}$$

A similar series is also observed containing two hexoside units:

$$\underset{m/z\ 341}{\text{Hex–O–Hex–O}^-} \leftarrow \underset{m/z\ 383}{\text{Hex–O–Hex–O–CH=CHO}^-} \leftarrow$$

$$\underset{m/z\ 425}{\text{Hex-O–Hex–O–CH=COH–CH=CHO}^-}$$

The relative intensities of the pairs of peaks in the positive and negative ion spectra (Table V) indicate that the mixture of glycosphingolipids has an approximately 1:1 composition, but insufficient material was available to obtain GC data on the fatty acid methyl esters.

Discussion

The positive ion spectra of the glycosphingolipids with neutral sugars were generally more intense than the negative ion spectra, but the negative ion spectra were often easier to interpret. Lower background signals and fewer solvent-related peaks often caused the signal to background ratio to be higher for the negative ion spectra, which facilitated interpretation. Clearly, FAB ionization provides enough fragmentations to determine the basic structure of glycosphingolipids. Successive losses of saccharide units, which were often weak in the positive ion spectra but relatively strong in the negative, yield the sequence of the oligosaccharide portion but do not give any information about the configuration at the anomeric (or other) carbons nor about the location of the saccharide linkages. The isomeric hexoses thus cannot be distinguished. The neutral GSL's that we have studied so far have not allowed us to ascertain what can be determined about branching in the carbohydrate chain. The ceramide peak (due to loss of the oligosaccharide) is generally strong in the positive ion spectrum, and sphingosine peaks in the positive ion spectra and fatty acid amide and related peaks in the negative ion spectra define the nature of those units, allowing unsaturations and homologies to be readily assigned. Information about the position of unsaturations in the lipid chains cannot be extracted from the FAB spectra, however.

FABMS is a good method for obtaining a quick estimate of the relative composition of GSL mixtures since no derivatives need to be made prior to analysis. Although background peaks inherent in the FAB spectrum limit the ability to determine accurate compositions, such information complements that obtained by derivatization and GC and is probably of comparable accuracy.

Table VI: Relative Ion Intensities for Mixtures of **1** and **2**

	ion ratios	
wt ratio (**1**:**2**)[a]	(+) m/z 568:652	(−) m/z 566:650
1:5	0.40	0.41
1:4	0.47	0.28
1:3	0.50	0.57
1:2	0.85	0.48
1:1	1.90	1.60
2:1	3.16	2.24
3:1	3.97	3.16
5.1	7.32	4.96
	1.41 (0.996)[b]	0.97 (0.993)[b]

[a] Compounds were impure (see Experimental Procedures). [b] Slope and coefficient.

The accuracy of FABMS for relative composition determination was tested by analyzing a series of synthetic mixtures of *N*-stearyldihydrosphingosine (**1**) and *N*-lignoceryldihydrosphingosine (**2**). Since both were impure, as described under Experimental Procedures, the actual composition of each mixture is unknown, but the relative ratios of the series is accurate to ca. 0.1%. The results, presented in Table VI, demonstrate a striking linearity with sample size. The negative ion results, however, with a slope about 1, are clearly better than the positive ion results (slope ca. 1.4). The explanation for the positive ion slope is unclear at this juncture, but it is clear (a) that for accurate estimates of composition a working curve of the present sort must be prepared for each mixture and (b) that with such a working curve in hand good estimates of composition can be obtained.

The choice of matrix played an important part in the analysis of the glycosphingolipids. Solubility of the compound in the matrix is a key consideration (Barber et al., 1982b; Meili & Seibl, 1983). Tetraethyleneglycol was fairly useful for ceramides and monoglycosylated sphingolipids. As the saccharide chain increased in length, the compounds became more readily soluble in a more polar solvent like glycerol, due to increased opportunity for hydrogen bonding. The nature of the solvent also affected the intensity of the spectra. While protic solvents (glycerol, thioglycerol, tetragol) generally worked well for positive ion spectra, a basic solvent (triethylenetetramine) produced better results in the negative ion mode. Arita (1983a,b) also noted the utility of a basic matrix (triethanolamine plus tetramethylurea) for negative ion analysis of gangliosides.

Finally, we note that it is possible to alter the relative intensities of fragment and molecular ions in two ways. Other workers (Rudat, 1982) have observed that higher atom beam energies tend to skew the spectrum so that higher masses are favored; we have observed similar beam energy effects with regard to the pseudomolecular ion intensity vs. the intensity of the nearest solvent cluster ion (Figure 2). Moreover, when we reduced the beam energy from 9 to 4 keV, while determining the spectrum of **6**, fragments that were weak or missing at 9 keV (m/z 266, 284, 302, and 368) increased in intensity (Figure 3). The increase in fragmentation is not due to the effects of increased pressure (CAD) because fragmentation does not increase when a 9-keV beam is used and the ZAB source diffusion pump isolation valve is partially closed in order to achieve higher pressures. This apparent relationship between beam energy and fragmentation is the reverse of what might be expected since the input of more energy into the sample and substrate would presumably result in ions with

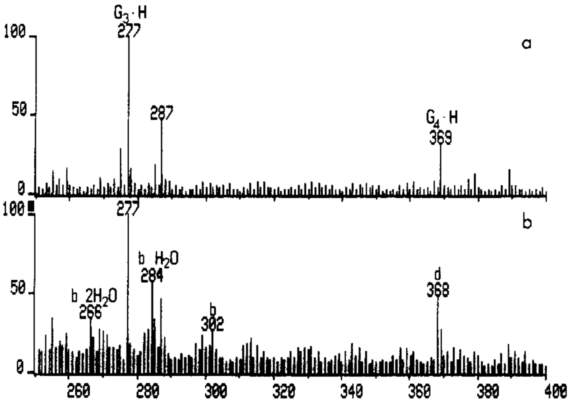


FIGURE 2: Fragmentation of **6** vs. beam energy and pressure: (a) 9-keV Xe at 1.0-mA gun current and 2.3 × 10^{-6} Torr source pressure on ZAB-1F; (b) 4-keV Xe at 1.0-mA gun current and 1.5 × 10^{-5} Torr source pressure on ZAB-1F.

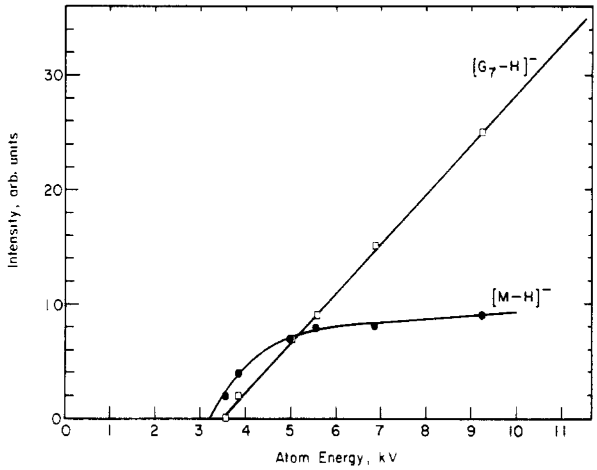


FIGURE 3: Effect of beam energy on solvent cluster vs. molecular ion intensity in negative ion spectrum of **2**. Similar results are obtained in the positive ion mode.

greater or equal, but certainly not less, internal energy. A number of researchers (Macfarlane, 1982; Busch et al., 1983; Stillwell et al., 1983) have discussed the possible role that the matrix plays in making FAB a "soft" ionization technique. A plausible explanation is found in the ability of a solvated gas-phase molecule (or molecular ion) to dissipate excess internal energy through the process of desolvation. Since the weakest bonds that a solvated molecule experiences are the solvation bonds, the excess internal energy is used to desolvate the desorbed cluster, resulting in an effectively less energetic molecular ion. On the basis of Rudat's observations, it may be supposed that a more energetic atom beam will be able to desorb larger molecules and clusters and these larger clusters will contain more solvent molecules to aid in the dissipation of excess internal energy. The M − H curve in Figure 2 will begin to level off when the cluster size is such that desolvation reduces the excess energy below that of the weakest bond in the sample molecule. Further increases in beam energy and, hence, cluster size will have little effect, whereas the G_7 − H peak can be shown by B/E linked scans to contain contributions from desolvation of larger clusters (G_8, G_9, G_{10}, etc.), resulting in the continued linear dependence with primary beam energy.

A related parameter is the concentration of the sample in the solvent. At dilute concentrations, the sample may be surrounded by many solvent molecules as it is desorbed and consequently the molecular ion would have little excess internal energy to promote fragmentation. A more concentrated solution would produce molecular ions that have fewer solvent molecules to dissipate energy or the clusters may contain several sample molecules, in which case the excess internal energy would somehow be partitioned among them. In fact, we have observed just this kind of concentration/fragmentation relationship in obtaining some of the GSL spectra, so that fragment ion intensity (relative to molecular ions) does increase with concentration.

Registry No. **2**, 92545-07-8; **5**, 92621-20-0; **6**, 92621-21-1.

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Involvement of Prolines-114 and -117 in the Slow Refolding Phase of Ribonuclease A As Determined by Isomer-Specific Proteolysis[†]

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ABSTRACT: Using the method of isomer-specific proteolysis (ISP), the cis–trans nature of the peptide bonds involving prolines-114 and -117 in ribonuclease (RNase) has been investigated. These studies involve the pretreatment of RNase first with either a short pepsin pulse or a short mercaptoethanol pulse to irreversibly unfold the protein and then with a short chymotrypsin pulse to quickly cleave the Tyr_{115}–Val_{116} bond so that the chain is suitably trimmed for the subsequent stereospecific cleavage either by aminopeptidase P, to investigate proline-117, or by a proline-specific endopeptidase, to investigate proline-114. The most reasonable interpretation of our results suggests that proline-117 is essentially 100% trans in both the native and unfolded states, so it apparently makes no direct contribution to the slow refolding kinetics of RNase. It is also determined that proline-114 is 100% cis in native RNase and ca. 95% cis in reversibly unfolded RNase so only 5% of the unfolded RNase can be rate limited by trans to cis isomerization of proline-114 during refolding. Careful spectroscopic studies of refolding show that the smallest and slowest of the refolding phases, the ct phase, has the proper amplitude (5%), relaxation time (400 s at 10 °C), and activation energy (17 kcal) for a phase that is rate limited by the trans to cis isomerization of proline-114. Measurements of the kinetics of binding of cytidine 2′-monophosphate during refolding further show that RNase does not become active until proline-114 has isomerized to the native cis configuration. It is concluded that none of the three prolines thus far examined (i.e., prolines-93, -114, and -117) by the ISP method is involved in the formation of a fully active, nativelike intermediate which has "incorrect" proline isomers. The specific structural process which is responsible for the largest of the three slow refolding phases, the XY phase, is still undetermined. Although ISP results on proline-42 are not yet available, it seems possible that this slow phase may be rate limited by a process other than proline isomerization. In unrelated studies, results from chymotrypsin hydrolyses of several short peptides containing the sequence -X-Y-Pro- show that cleavage of an active X–Y bond is very slow when it is immediately adjacent on the amino side of a proline peptide bond. Thus, chymotrypsin cleavage may not be generally useful as the analytical step in isomer-specific proteolysis.

The refolding of ribonuclease (RNase) occurs in both a fast (ca. 20% of the spectroscopic amplitude) and a slow (ca. 80%) kinetic phase, with the end product of each phase being a fully active form of RNase. It is now generally accepted, as proposed some time ago (Brandts et al., 1975), that the fast phase results from refolding of denatured molecules which have all proline residues in the correct native configuration while the slow phase involves refolding of molecules which have one or more incorrect proline isomers. Nevertheless, there are still many unanswered questions concerning the molecular details of the slow phase, since RNase has four proline residues and since certain features of the slow refolding kinetics may not be fully explained by proline isomerization alone. It has often been suggested (Schmid & Baldwin, 1978; Schmid, 1981; Schmid & Blaschek, 1981), for example, that unfolded RNase can be slowly refolded to a nativelike intermediate which has one or more prolines in an incorrect nonnative configuration.

The slow phase of refolding itself is known to be complex. When refolding into solutions containing a low concentration of denaturants, it was shown (Lin & Brandts, 1983c) to consist mainly of a faster phase (i.e., the XY phase) of ca. 50% of the spectroscopic amplitude and a slower phase (i.e., the CT phase) of ca. 25–30% of the amplitude. In addition, careful studies revealed (Lin & Brandts, 1983c) a third very slow phase of ca. 5% amplitude, which will be characterized in more detail in this paper and which will be referred to as the spectroscopic ct phase.

The best approach toward understanding the role of proline isomerization in each of the component processes of the complex slow phase of refolding is to directly measure the isomerization of each of the four prolines by using isomer-specific proteolysis (ISP). Two of these, prolines-93 and -114, are cis in the native protein while the other two, prolines-42 and -117, are trans. In previous work (Lin & Brandts, 1983b), which utilized trypsin as the stereospecific protease, we were able to show that the trans to cis isomerization of proline-93 is the rate-limiting step in the spectroscopic CT refolding phase and that RNase is unable to refold to an active form while proline-93 is in the incorrect trans state.

In a continuing effort to utilize the ISP method for studying the other prolines of RNase, particularly prolines-114 and -117 in the sequence -Gly_{112}-Asn_{113}-Pro_{114}-Tyr_{115}-Val_{116}-Pro_{117}-, proteases such as papain and chymotrypsin have been examined. We found that papain, which has low solubility in aqueous solution, hydrolyzes the Gly_{112}-Asn_{113} bond too slowly

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