Lee, R. H., Brown, B. M., & Lolley, R. N. (1981a) *Biochemistry 20*, 7532–7538.
Lee, R. H., Brown, B. M., Lieberman, B. S., & Lolley, R. N. (1981b) *Abstr. Soc. Neurosci. 7*, 917.
Lee, R. H., Brown, B. M., & Lolley, R. N. (1982) *Biochemistry 21*, 3303–3307.
Lee, R. H., Brown, B. M., & Lolley, R. N. (1984) *Biochemistry 23*, 1972–1977.
Liebman, P. A., & Pugh, E. N. (1979) *Vision Res. 19*, 375–380.
Lolley, R. N., Brown, B. M., & Farber, D. B. (1977) *Biochem. Biophys. Res. Commun. 78*, 572–578.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–270.
Manning, D. R., & Gilman, A. G. (1983) *J. Biol. Chem. 258*, 7059–7063.
Manning, D. R., Fraser, B. A., Kahn, R. A., & Gilman, A. G. (1984) *J. Biol. Chem. 259*, 749–756.
Martin, R. O., & Ames, B. N. (1961) *J. Biol. Chem. 236*, 1372–1379.
Matsumoto, B., & Bok, D. (1984) *Invest. Ophthalmol. Visual Sci. 25*, 1–9.
McGinnis, J. F., & Leveille, P. J. (1985) *Curr. Eye Res. 4*, 1127–1135.
Navon, S., Lee, R. H., Lolley, R. N., & Fung, B. K.-K. (1987) *Exp. Eye Res. 44*, 115–125.
Papermaster, D. S., & Deyer, W. J. (1974) *Biochemistry 13*, 2438–2444.
Papermaster, D. S., Schneider, B. G., & Zorn, M. A. (1978) *J. Cell Biol. 77*, 196–210.
Peterson, G. J. (1983) *Methods Enzymol. 91 (Part I)*, 95–101.
Robb, R. M. (1978) *Invest. Opthalmol. Visual Sci. 17*, 476–480.
Roof, D. J., Applebury, M. L., & Sternweis, P. C. (1985) *J. Biol. Chem. 260*, 16242–16247.
Shuster, T. A., & Farber, D. B. (1984) *Biochemistry 23*, 515–521.
Sitaramayya, A., & Liebman, P. A. (1983) *J. Biol. Chem. 258*, 12106–12109.
Stryer, L. (1985) *Biopolymers 24*, 29–47.
Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.
Wilden, U., Hall, S. W., & Kuhn, H. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1174–1178.
Yau, K.-W., & Nakatani, K. (1985) *Nature (London) 317*, 252–255.
Young, R. W. (1976) *Invest. Ophthalmol. Visual Sci. 15*, 700–725.

Analysis of Long-Chain Bases in Sphingolipids by Positive Ion Fast Atom Bombardment or Matrix-Assisted Secondary Ion Mass Spectrometry†

Yoko Ohashi,‡ Masao Iwamori,‡ Tomoya Ogawa,§ and Yoshitaka Nagai*,‡

Department of Biochemistry, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113, Japan, and RIKEN (Institute of Physical and Chemical Research), Wako-shi, Saitama 351-01, Japan

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ABSTRACT: The structures of long-chain bases are expressed as $[CH_2C(NH_2)=CHR]^+$ (Z^+) in the positive ion mode spectra obtained on fast atom bombardment (FAB) mass spectrometry or liquid-matrix-assisted secondary ion mass spectrometry (SIMS) [Benninghoven, A., Ed. (1983) *Ion Formation from Organic Solids*, Springer, Berlin]. This phenomenon is common to sphingolipids in general: glycosphingolipids [see reviews by Sweeley and Nunez [Sweeley, C. C., & Nunez, H. A. (1985) *Annu. Rev. Biochem. 54*, 765] and Kanfer and Hakomori [Kanfer, J. N., & Hakomori, S. (1983) *Handb. Lipid Res. 3*]] and phosphonosphingolipids [Hayashi, A., & Matsubara, T. (1982) in *New Vistas in Glycolipid Research* (Makita, A., Handa, S., Taketomi, T., & Nagai, Y., Eds.) p 103, Plenum, New York], inclusive. Phytosphingosine compounds show the same type of fragmentation without additional dehydration if a neutral matrix is used. A Z^+ ion is easily detected in the lower mass region (m/z 200–400) as an even mass number fragment ion, and confirmation is made by means of B/E constant and B^2/E constant linked scan techniques [Boyd, R. K., & Beynon, J. H. (1977) *Org. Mass Spectrom. 12*, 163; Boyd, R. K., & Shushan, B. (1981) *Int. J. Mass Spectrom. Ion Phys. 37*, 355; Macdonald, C. G., & Lacey, M. J. (1984) *Org. Mass Spectrom. 19*, 55]. [Principles of linked scannings are explicitly summarized by Jennings and Mason [Jennings, K. R., & Mason, R. S. (1983) in *Tandem Mass Spectrometry* (McLafferty, F. W., Ed.) p 197, Wiley, New York] besides the cited literature.]

Fast atom bombardment (FAB) mass spectrometry and secondary ion mass spectrometry (SIMS), especially in the negative ion mode, have been successfully applied to the identification of the saccharide chain in glycolipids (Arita et al., 1983a,b). However, the ceramide moiety in such glycosphingolipids has been so far described as one unit, the long-chain base and the fatty acid part inclusive, unless chemical cleavage and derivatization for electron ionization (EI) (Sweeley & Nunez, 1985) or desorption chemical ionization (DCI) [in-beam chemical ionization (in-beam CI)] (Markey & Wenger, 1974) mass spectrometry have been previously performed. Recently, Taketomi successfully applied FAB mass spectrometry to determine the long chain base size of chemically deacylated glycosphingolipids (Hara & Taketomi, 1986).

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* Author to whom correspondence should be addressed.
‡ University of Tokyo.
§ RIKEN.

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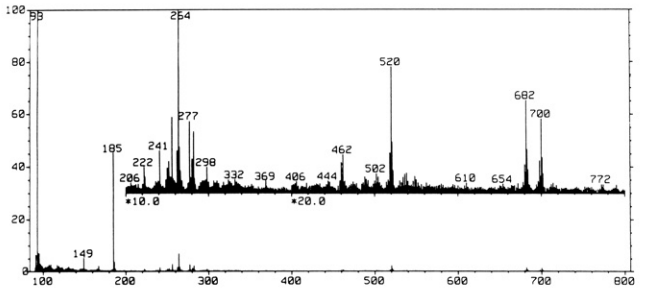


FIGURE 1: (+)FAB spectrum of *N*-palmitoylpsychosine (M_r 699). Matrix: glycerol.

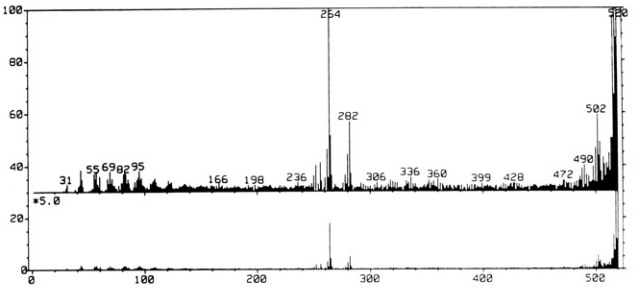


FIGURE 2: (+)FAB B/E constant linked scan spectrum of *N*-palmitoylpsychosine. Precursor ion: m/z 520. Matrix: glycerol.

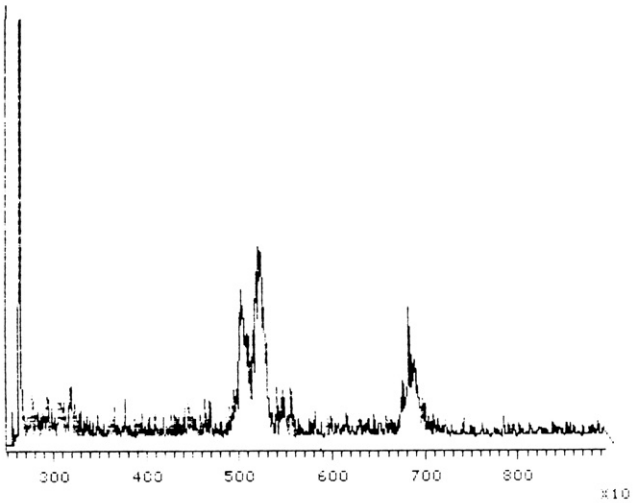


FIGURE 3: (+)SIMS B^2/E constant linked scan spectrum of *N*-palmitoylpsychosine. Daughter ion: m/z 264. Matrix: 3-nitrobenzyl alcohol.

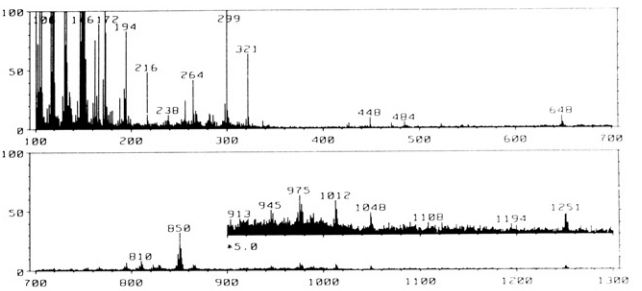


FIGURE 4: (+)FAB spectrum of *N*-cerebronoylpsychosine (M_r 827). Matrix: triethanolamine.

Exceptions are two independent reports describing amide-related ions on underivatized dihydrosphingosine-containing mono- or dihexosyl ceramides, one by Hemling et al. (1984) using FAB and the other by Carr et al. using ammonia DCI (1984).

We noticed, however, that the long-chain base in a sphingolipid is expressed as an even mass number fragment ion, $[CH_2C(NH_2)=CHR]^+$ (Z^+), on positive ion mode secondary ion mass spectrometry (FAB or SIMS) regardless of the saccharide structure or the amide group. Furthermore, structure elucidation by means of the Z^+ ion is applicable not only to glycosphingolipids but also to phosphonosphingolipids.

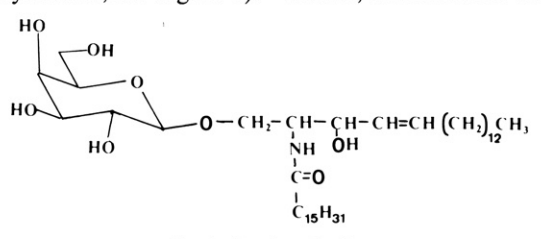
Experimental Procedures

Materials. Brain cerebrosides (ceramide monohexosides, CMH) were prepared from human and bovine brain tissues. *N*-Acylpsychosines were prepared from the bovine brain CMH through subsequent deacylation (Taketomi et al., 1976) and reacylation (Kopaczyk & Radin, 1965) of the material. *N*-Cerebronoylpsychosine (Koike et al., 1985), asialoganglioside G_{M1} (asialo G_{M1} or GA_1),[1] and asialoganglioside G_{M2} (asialo G_{M2} or GA_2) were synthesized as reported previously (Sugimoto et al., 1985). *N*-Lignoceroyldihydrogalactocerebroside (*N*-lignoceroyl-1-*O*-sphinganyl-β-galactoside) was a product of Sigma Chemical Co. and was used without further purification.

Instruments. FAB mass spectra were obtained with a JMS-DX300 or 303 mass spectrometer equipped with a 6-keV Xe^0 FAB ion gun. Most of the linked scan studies were performed by SIMS using a Hitachi M-80B mass spectrometer with an 8-keV Xe^+ primary ion source. No significant difference was noticed between the FAB and SIMS spectra obtained.

Results and Discussion

We noticed previously that all the *N*-acylpsychosines derived from bovine CMH showed an unknown fragment ion at m/z 264 besides the expected MH^+, $(MH - H_2O)^+$, and $(MH -$ hexose$)^+$ of individual molecules in the positive ion mode FAB (Figure 1) or SIMS spectra (Ohashi et al., 1983). Now we have explained this phenomenon by conducting B/E constant linked scan studies on a typical *N*-acylpsychosine (*N*-palmitoylpsychosine; see Figure 2). That is, a cerebroside loses a neutral hexose and then is cleaved at the amide CO–N bond, which leads to the loss of the acyl group, probably in the form of a ketene; meanwhile a molecule of water is also removed at any stage, i.e., before, between, or after the two cleavages. The resultant ion represents the long-chain base as $[CH_2C(NH_2)=CHCH=CH(CH_2)_{12}CH_3]^+$ in the case of 4-sphingenyl compounds, corresponding to m/z 264. The fragmentation mechanism is summarized in Scheme I.



N-palmitoylpsychosine

Confirmation of the fragmentation mechanism was carried out by B^2/E constant linked scanning of the Z^+ ion, which showed precursors at m/z 282, 502, 520, and 682 but not at m/z 700 (Figure 3). This means that the daughter ion does not come directly from MH^+ but through either the dehydration or dehexosylation fragment of MH^+. Reinhold and Carr (1983) and Hemling et al. (1984) reported similar sphingosine-related ions. Both of these papers describe the glycosidic cleavage as occurring on the saccharide side of the glycosidic oxygen. However, the glycosidic cleavage occurs on the ceramide side of the oxygen, forming a precursor ion of the Z^+ ion in our cases (Figures 1, 4, 5, 8, and 9). A line

[1] The ganglioside nomenclature and symbols are based on IUPAC–IUB recommendations (1977) and Svennerholm's system (1963).

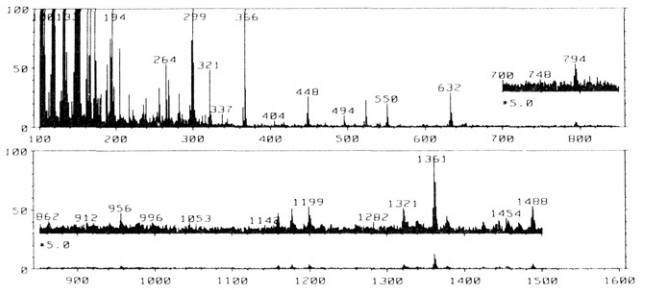
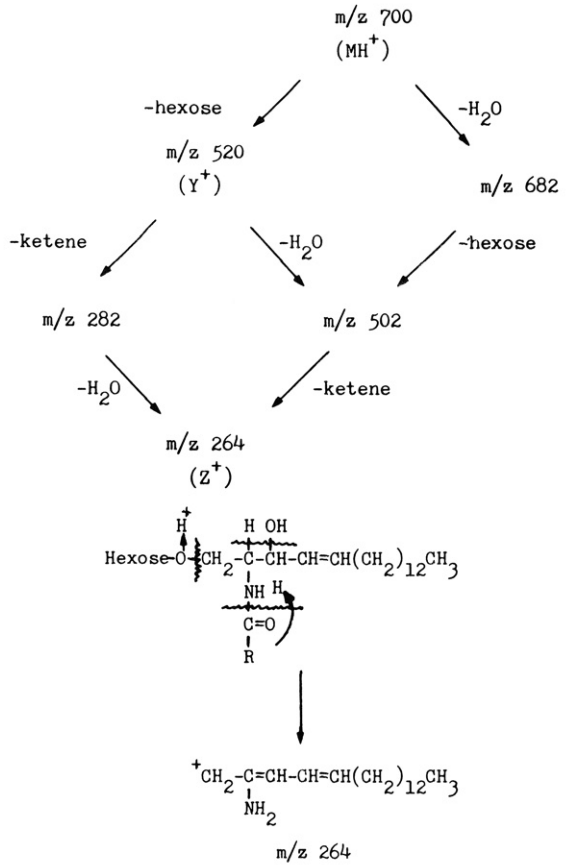


FIGURE 5: (+)FAB spectrum of asialo G_{M1} (M_r 1338). Matrix: triethanolamine.

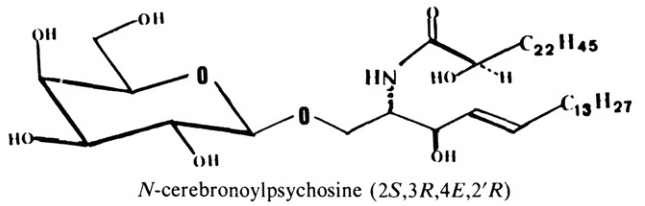
Scheme I: Fragmentation Mechanism for *N*-Palmitoylpsychosine on (+)FAB/SIMS



of evidence is also shown by the linked scannings: The B/E constant linked scan spectrum of *N*-palmitoylpsychosine having MH^+ at m/z 700 as the precursor failed to show a daughter ion at m/z 538 in a meaningful intensity (spectrum not shown), and the B^2/E constant linked scan spectrum, in turn, having the Z^+ ion at m/z 264 as the daughter (Figure 3) showed a precursor at m/z 538 only in insignificant intensity.

Since a Z^+ ion thus expresses the long-chain base structure irrespective of the fatty acid and saccharide moieties, the same m/z value is expected for sphingolipids having the same long-chain base but differing in the other moieties.

Stereoisomers of a cerebroside in which the fatty acid is hydroxylated as 2′*R* and 2′*S* (Koike et al., 1985), respectively,



N-cerebronoylpsychosine (2*S*,3*R*,4*E*,2′*R*)

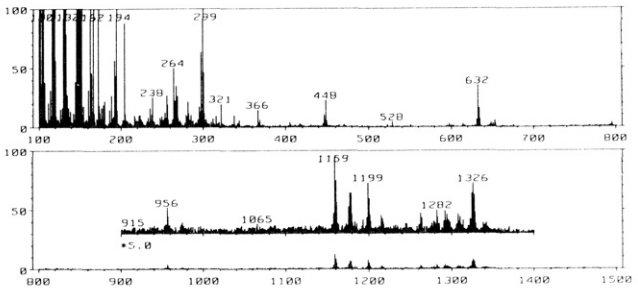
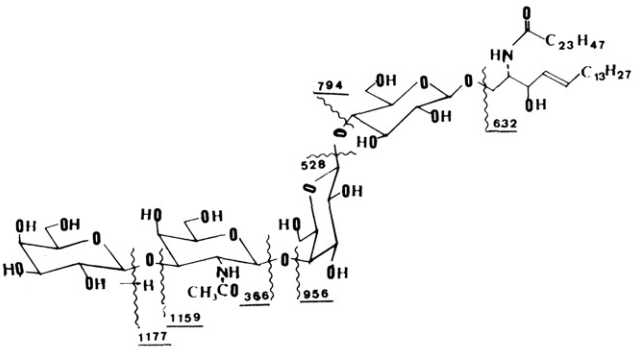


FIGURE 6: (+)FAB spectrum of asialo G_{M2} (M_r 1176). Matrix: triethanolamine.

also showed the same Z^+ ion at m/z 264, because the long-chain base is again d18:1, even though the fatty acid is different from that of *N*-palmitoylpsychosine (Figure 4). The same was true for asialo G_{M1} and asialo G_{M2} (Sugimoto et al.,



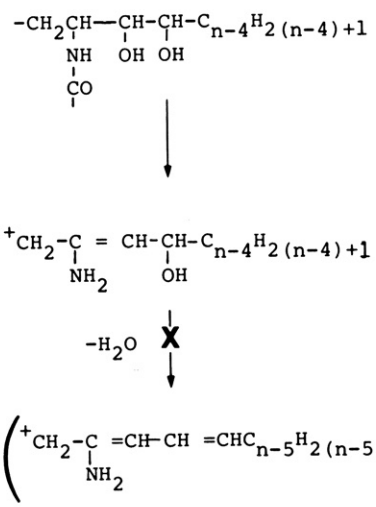
asialo G_{M1} and asialo G_{M2}

1985), both of which have longer saccharide chains extended, and even *N*-acetyl-β-galactosamine, and are also different from the previous examples in the fatty acid moiety (Figures 5 and 6).

Determination of the long chain base length by means of the Z^+ ion is also valid for other types of sphingolipids. For instance, a sphinganine compound, *N*-lignoceroyldihydrogalactocerebroside, in which the long-chain base is d18:0, showed a fragment ion at m/z 266 as the Z^+ ion, signifying $[CH_2C(NH_2)=CH(CH_2)_{14}CH_3]^+$ besides ions at m/z 284, 368, 635, 653, 815 (MH^+ for the most abundant monoisotopes), and 837 (MNa^+). Therefore, an allylic double bond is not essential for the formation of a Z^+ ion. It is noteworthy that the glycosidic cleavage in the sphinganyl compound occurs not only on the expected ceramide side of the glycosidic oxygen (m/z 635) but also on the saccharide side (m/z 653), which does not normally occur in sphingenyl compounds.

The same type of the Z^+ ion formation was shown for phytosphingosine (2-amino-1,3,4-alkanetriol) compounds. The (+)SIMS spectrum of the total ganglioside fraction from *Asterina pectinifera* (Sugita, 1979a,b) showed Z^+ ions in accordance with the natural abundance of the long-chain base, the most abundant Z^+ ion being at m/z 282, $[CH_2C(NH_2)=CHCH(OH)C_{14}H_{29}]^+$, with similar but less intense ions at m/z 254 and 268, when a neutral matrix was used (Figure 7). These major Z^+ ions signify t18:0, t16:0, and t17:0, respectively, and nearly correspond to the long chain base composition determined by means of gas chromatography–mass spectrometry (GC–MS) of the trimethylsilyl (TMS) derivatives of the CMH and ceramide dihexoside (CDH) fractions from the same species and reported by Sugita previously (Sugita, 1977). This means that the fragmentation pattern is the same for these trihydroxylated bases as for dihydroxylated sphingosines in spite of the presence of an additional hydroxyl group, as illustrated at the top of Figure

z^+ ions from Phytosphingosines



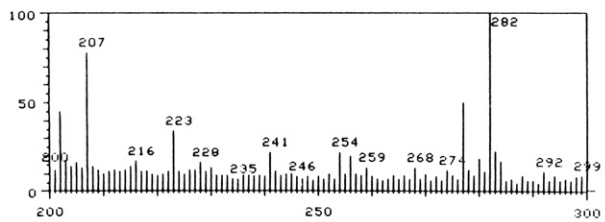


FIGURE 7: (+)SIMS partial spectrum of a total ganglioside fraction extracted from *A. pectinifera*. Matrix: glycerol.

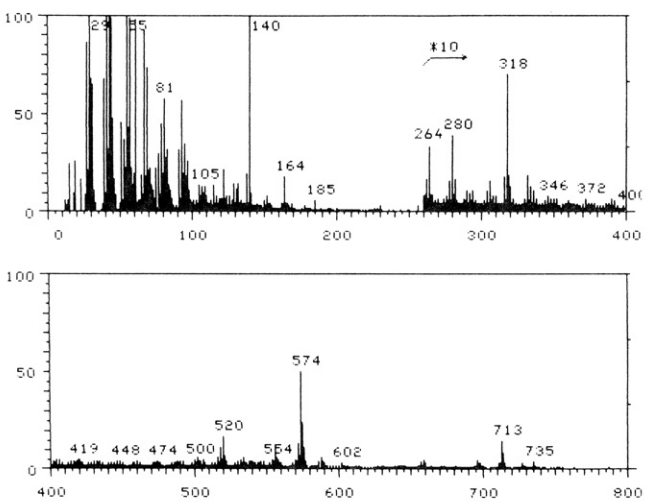
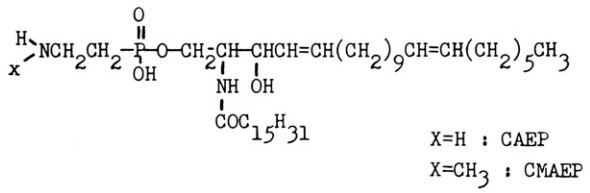


FIGURE 8: (+)SIMS spectrum of ceramide [2-(methylamino)-ethyl]phosphonate (CMAEP) extracted from *Turbo cornutus*. Molecular weights of the two major components: 712 and 658. Matrix: glycerol.

7. The use of a basic matrix such as triethanolamine or diethanolamine, however, led to a different fragmentation pattern, and the Z^+ ions may not be observed at the proper mass numbers.

Further application of this method to determine a long-chain base is exemplified in the cases of unique C–P compounds, phosphonosphingolipids. CMAEP [ceramide [2-(methylamino)ethyl]phosphonate] and CAEP [ceramide (2-aminoethyl)phosphonate] (Matsubara, 1975) behaved similarly to glycosphingolipids on (+)SIMS except that the phosphonolipids showed additional fragment ions expressing the phosphonate groups as $H_3CNH(CH_2)_2P{=}O(OH)_2{\cdot}H^+$ (m/z 140) for CMAEP and $H_2N(CH_2)_2P{=}O(OH)_2{\cdot}H^+$ (m/z 126), together with the sodium adduct $H_2N(CH_2)_2P{=}O(OH)_2{\cdot}Na^+$ (m/z 148), for CAEP. On this particular point, the phosphonolipids show more similarity to phospholipids of which the aminoethyl phosphate moiety is expressed as $[X_3N^+(CH_2)_2OP{=}O(OH)_2]$ (X = H or CH_3) in the (+)SIMS spectrum (Ohashi, 1984). However, another interesting phenomenon is that these phosphonosphingolipids, just like glycosphingolipids, gave Z^+ ions reflecting the natural composition of long-chain bases. As for CMAEP, Matsubara previously reported, on the basis of the results of GC–MS studies, the molecular distribution of the long-chain base, the main components being assigned as d22:2 and d18:1 (Matsubara, 1975). They were both proven in our present study by the appearance of Z^+ ions at m/z 318 and 264, respectively, with several more minor Z^+ ions in the (+)SIMS spectrum. According to Matsubara, the major fatty acid component is 16:0, and the molecular structure given for the most abundant species is (CAEP head group also shown)



The intense ion at m/z 140 in Figure 8 represents the [(methylamino)ethyl]phosphonate moiety previously mentioned. Ions at m/z 713 (MH^+), 574 (MH – phosphonate)$^+$, and 318 (Z^+) form a fragmentation series for a molecule with a d22:2 long-chain base; meanwhile the ions at m/z 659, 520, and 264 form another series for the next abundant molecular species, which has a long-chain base d18:1. Confirmation of the fragmentation pathway was made with the former molecular species by means of linked scan techniques using 3-nitrobenzyl alcohol as the matrix. The B/E constant linked scanning in (+)SIMS specifying the protonated molecule at m/z 713 as the precursor showed daughter ions at m/z 695, 574, and 556. A similar scanning from m/z 574, in turn, gave daughter ions at m/z 556 and 318. Then, B^2/E constant linked scanning specifying the ion at m/z 318 as the daughter ion showed precursor ions roughly at m/z 336, 556, 574, and 695. Therefore, the fragmentation mechanism for the phosphonosphingolipids leading to the Z^+ ion is very similar to that in the case of glycosphingolipids; that is, a Z^+ ion is produced from the protonated molecule through loss of the phosphonate, dehydration, and loss of the ketene. The nature of the ion at m/z 280 has not yet been clarified, but it may be indicative of the presence of another long-chain base of d18:2, whose Z^+ ion, if present, corresponds to m/z 262.

Another example of Z^+ ions expressing the long-chain bases in a phosphonosphingolipid is the case of CAEP (Figure 9). Matsubara (1975) assigned two major long chain base constituents as d18:2 and d16:1 and the fatty acid as 16:0. They are analogues to the structure shown above for the major component of CMAEP, and the molecular weights of the two are 642 and 616, accordingly. The (aminoethyl)phosphonate moiety was assigned by the fragment ion at m/z 126 and the sodium adduct at m/z 148. MNa^+ at m/z 665, (MH – phosphonate)$^+$ at m/z 518, and the Z^+ ion at m/z 262 correspond to a molecular species with a d18:2 long-chain base. The other easily detectable ion series was observed at m/z 639 (MNa^+), 492 (MH – phosphonate)$^+$, and 236 (Z^+), exactly as expected from the assigned structure. Therefore, here again, in the case of phosphonosphingolipids, long-chain bases were expressed as $[CH_2C(NH_2){=}CHR]^+$, analogously to the case of glycosphingolipids.

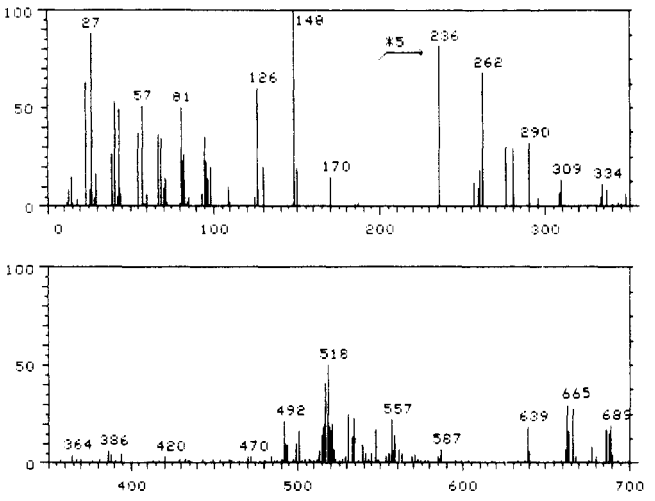


FIGURE 9: (+)SIMS spectrum of ceramide (2-aminoethyl)phosphonate (CAEP) extracted from *Ostrea gigas*. Molecular weights of the two major components: 642 and 616. Matrix: 3-nitrobenzyl alcohol.

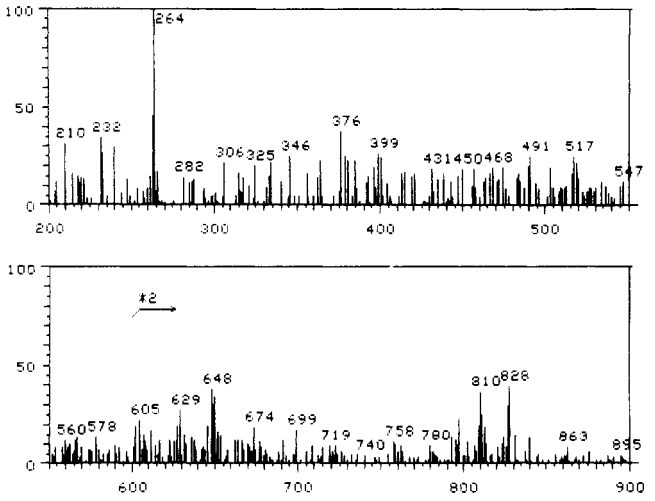


FIGURE 10: (+)SIMS background-subtracted spectrum of a CMH fraction extracted from human brain. Matrix: glycerol.

We tried, next, to determine the long-chain base of an underivatized unknown CMH fraction isolated from human brain by the Z^+ value(s) observed. There was, practically speaking, only one Z^+ ion found at m/z 264 in the (+)SIMS spectrum of the material (Figure 10). This means that the human brain CMH fraction consisted almost solely of one kind of long-chain base of d18:1 above the detection level. This result agrees with the previous report by Karlsson (1970).

Technical Notes for Z^+ Detection. Special care is needed in the selection of the matrix in order to detect the Z^+ ions without ambiguity. A basic matrix may cause misleading fragmentation, and the Z^+ ions may not be observable in the spectrum, especially in the case of a polyhydroxylated long-chain base, even though triethanolamine or diethanolamine is often the matrix of choice for more abundant protonated molecules. A Z^+ ion always has an even mass number; therefore, a non-nitrogenated matrix such as glycerol is generally satisfactory, because the matrix cannot give any even mass numbered ions. Inevitably, however, inadequate mass resolution in linked scannings may pick up a matrix-originated ion in the close neighborhood, and therefore, the matrix may have to be switched to a second choice. We frequently use 3-nitrobenzyl alcohol (Meili & Seibl, 1984) in such a case. This matrix often gives a good protonated molecular intensity of the sample at the early stage of scannings, but it does not have adequate viscosity. It does not generally affect the Z^+ mass region, although it is nitrogen containing. However, special attention is needed for this matrix especially in negative ion mode FAB/SIMS, because it produces a $(M + \text{matrix})^-$ rather than a $(M + \text{matrix} - H)^-$ cluster ion in the spectrum. The presence of nitrogen-containing moieties other than a ceramide, such as sialic acid, *N*-acylhexosamine and (aminoethyl)phosphonate, does not usually disturb the Z^+ manifestation as an even mass numbered ion in a spectrum.

The ion intensity of Z^+ varies markedly, depending not only on the sample concentration but also on the time course of scannings. We noticed that a change in the Z^+ ion intensity is synchronous with those in more direct precursor ions but not necessarily with that in protonated molecule. Therefore, if a spectrum is drawn as the average of several scannings chosen from the standpoint of the protonated molecular intensity, the Z^+ ions may not be sufficiently intense. Careful observation of mass chromatograms for Z^+ (in the case of a known long-chain base) or for a possible precursor ion such as $(MH - \text{hexose})^+$ or $(MH - \text{phosphonate})^+$, if predictable, is essential before specifying the scan-number range for the spectrum.

CONCLUSIONS

The size of a long-chain base of a sphingolipid can be determined by (+)FAB/SIMS with no previous chemical or enzymatic cleavage or derivatization of the sample being required. Indeed, N-acylated natural forms of a long-chain base are preferable to their deacylated derivative in that they show the Z^+ ions more clearly. A ceramide moiety, $-OCH_2CH(NHCOR')CH(OH)R$, is expressed as $[CH_2C(NH_2)=CHR]^+$ regardless of the R composition, and we named the key ion Z^+. It is seen in the spectrum (Kanemitsu & Sweeley, 1986) independently from the polar moiety (glycoside or phosphonate) and the fatty acid amide moiety. Then the fatty acid can be determined by subtracting the contribution of the long-chain base from the Y^+ mass number, where Y^+ represents $[CH_2CH(NHCOR')CH(OH)R]^+$. The relationship is

$$R'CO - H = Y - H_2O - Z$$

or

$$R'CO = Y - Z - 17$$

A few problems still remain for further studies: (1) the distinction of the masses between $-C_nH_{2n-2}(OH)$ and $-C_{n+1}H_{2(n+1)+1}$ in the R component of Z^+ requires an instrument of higher resolution; and (2) the location of unsaturated bonds or detection of a branching in the ceramide still requires GC–MS of the derivatized sample.

Nevertheless, we believe that the determination of the mass number of a long-chain base by means of the Z^+ ions on (+)FAB/SIMS of the underivatized sample has great potential in sphingolipid biochemistry because of its easiness.

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Registry No. Asialo G_{M1}, 71012-19-6; asialo G_{M2}, 35960-33-9; *N*-palmitoylpsychosine, 34324-89-5; *N*-cerebronoylpsychosine, 586-02-7; ceramide [2-(methylamino)ethyl]phosphonate, 91254-90-9; ceramide (2-aminoethyl)phosphonate, 22822-94-2.

References

Arita, M., Iwamori, M, Higuchi, T., & Nagai, Y. (1983a) *J. Biochem. (Tokyo) 93*, 249.

Arita, M., Iwamori, M., Higuchi, T., & Nagai, Y. (1983b) *J. Biochem. (Tokyo) 93*, 319.

Benninghoven, A., Ed. (1983) *Ion Formation from Organic Solids*, Springer, Berlin.

Boyd, R. K., & Beynon, J. H. (1977) *Org. Mass Spectrom. 12*, 163.

Boyd, R. K., & Shushan, B. (1981) *Int. J. Mass Spectrom. Ion Phys. 37*, 355.

Carr, S. A., & Reinhold, V. N. (1984) *Biomed. Mass Spectrom. 11*, 633.

Hara, A., & Taketomi, T. (1986) *J. Biochem. (Tokyo) 100*, 415.

Hayashi, A., & Matsubara, T. (1982) in *New Vistas in Glycolipid Research* (Makita, A., Handa, S., Taketomi, T., & Nagai, Y., Eds.) p 103, Plenum, New York.

Hemling, M. E., Yu, R. K., Sedgwick, R. D., & Rinehart, K. L., Jr. (1984) *Biochemistry 23*, 5706.

IUPAC–IUB Commission on Biochemical Nomenclature (1977) *Eur. J. Biochem. 79*, 11.

Jennings, K. R., & Mason, R. S. (1983) in *Tandem Mass Spectrometry* (McLafferty, F. W., Ed.) p 197, Wiley, New York.

Kanemitsu, K., & Sweeley, C. C. (1986) *Glycoconjugate J. 3*, 143.

Kanfer, J. N., & Hakomori, S. (1983) *Handb. Lipid Res. 3.*

Karlsson, K.-A. (1970) *Lipids 5*, 878.

Koike, K., Sugimoto, M., Nakahara, Y., & Ogawa, T. (1985) *Glycoconjugate J. 2*, 105.

Kopaczyk, K. C., & Radin, N. S. (1965) *J. Lipid Res. 6*, 140.

Macdonald, C. G., & Lacey, M. J. (1984) *Org. Mass Spectrom. 19*, 55.

Markey, S. P., & Wenger, D. A. (1974) *Chem. Phys. Lipids 12*, 182.

Matsubara, T. (1975) *Chem. Phys. Lipids 14*, 247.

Meili, J., & Seibl, J. (1984) *Org. Mass Spectrom. 19*, 581.

Ohashi, Y. (1984) *Biomed. Mass Spectrom. 11*, 383.

Ohashi, Y., Iwamori, M., Ogawa, T., & Nagai, Y. (1983) *Proc. Jpn. Soc. Med. Mass Spectrom. 8*, 213.

Reinhold, V. N., & Carr, S. A. (1983) *Mass Spectrom. Rev. 2*, 153.

Sugimoto, M., Horisaki, T., & Ogawa, T. (1985) *Glycoconjugate J. 2*, C11.

Sugita, M. (1977) *J. Biochem. (Tokyo) 82*, 1307.

Sugita, M. (1979a) *J. Biochem. (Tokyo) 86*, 289.

Sugita, M. (1979b) *J. Biochem. (Tokyo) 86*, 765.

Svennerholm, L. (1963) *J. Neurochem. 10*, 613.

Sweeley, C. C., & Nunez, H. A. (1985) *Annu. Rev. Biochem. 54*, 765.

Taketomi, T., Kawamura, N., Hara, A., & Murakami, S. (1976) *Biochim. Biophys. Acta 424*, 106.

Oxygen Binding Constants for Human Hemoglobin Tetramers†

Stanley J. Gill,* Enrico Di Cera, Michael L. Doyle, Gary A. Bishop, and Charles H. Robert

Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado 80309-0215

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ABSTRACT: High-precision studies of oxygen binding in hemoglobin (HbA_0) solutions at near-physiological concentrations (2–12 mM heme; pHs 7.0–9.1; various buffers) have led to an unanticipated result: an unmeasurably low contribution from the triply ligated species. We have obtained this result from new differential oxygen-binding measurements for human hemoglobin through the use of a thin-layer apparatus, which enables study of solutions at high Hb concentrations. The effect of tetramer dissociation into dimers, which becomes significant at hemoglobin concentrations below 1 mM in heme, is avoided. The analysis of the binding reactions is thus cast in terms of the tetramer-binding polynomial written with overall Adair equilibrium constants which directly reflect the contributions of intermediate ligated species. The unmeasurable contribution of the triply ligated species renders the equilibrium constants of the third and fourth stepwise reactions practically undeterminable.

Since the discovery of hemoglobin's sigmoidal oxygen-binding curve more than 80 years ago (Bohr et al., 1904), efforts to understand the origins of cooperative interaction have resulted in a multitude of experimental findings and theories. A historical review has been given by Edsall (1980). Of particular importance was the establishment of the precise molecular weight of hemoglobin by Adair (1925a) and Svedberg and Fahreus(1926). At that point it became clear that with four oxygen-binding sites there must be four thermodynamic binding reactions, subsequently termed the Adair reactions, describing the stoichiometric oxygenated species (Adair, 1925b). Roughton devoted a significant effort toward the determination of these constants using gasometric procedures (Roughton et al., 1955; Roughton & Lyster, 1965). However, the discovery of the important regulatory properties

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