Novel Modification of Glycosphingolipids by Long-Chain Cyclic Acetals: Isolation and Characterization of Plasmalocerebroside from Human Brain†

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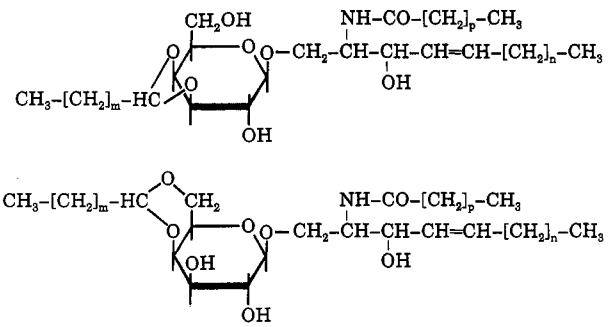
ABSTRACT: A glycosphingolipid component of human brain, having long-chain cyclic acetals, has been isolated and characterized. This compound incorporates a novel type of natural glycan modification, in which a long-chain aliphatic aldehyde is conjugated through a cyclic acetal (plasmal) linkage to the galactosyl moiety of cerebroside. In addition to components normally observed by gas chromatography–mass spectrometry (GC–MS) following methanolysis of cerebroside (fatty acid methyl esters, methyl α- and β-galactosides, sphingosine), this compound produced 16:0, 18:0, and 18:1 fatty aldehydes, unequivocally identified as their enol methyl ether derivatives. Results of positive ion fast atom bombardment mass spectrometry (FAB-MS) of the native compound, and GC–MS of partially methylated hexitol acetates derived from the permethylated derivative, were consistent with structures of galactocerebroside having 3,4- and 4,6-linked cyclic plasmal substituents, as shown.



A class of fatty O-acylated galactocerebrosides has been known since the early 1960s to be present in brain tissue. Collectively termed "ester cerebrosides", these compounds are considerably less polar than unmodified cerebrosides and were initially characterized as having much higher mobility (R_f) than cerebrosides on thin-layer chromatography (TLC)[1] (Kochetkov et al., 1962; Norton & Brotz, 1963). Locations of fatty acids have been identified as 3- or 6-OH of galactose, as well as 3-OH of the SPN moiety (Kishimoto et al., 1968; Klenk & Lohr, 1967; Tamai et al., 1967; Tamai, 1968). Another type of glycosphingolipid, having a fatty aldehyde (plasmal) component linked as an enol ether to the 3-OH of SPN, was reported by Kochetkov et al. (1963) and given the name "sphingoplasmalogen". Subsequent studies in other laboratories failed to confirm the presence of such plasmal conjugates (Klenk & Doss, 1966; Kishimoto et al., 1968; Tamai, 1968). During an investigation of low-polarity glycolipids from human brain, an acid-labile, base-stable minor component was detected and isolated by successive chromatographic separations on columns of Florisil and Iatrobeads (silica gel), followed by preparative TLC. In contrast to the majority of faster-migrating glycolipids, which were identified as fatty acid esters of cerebroside, the new component was found be a novel fatty aldehyde conjugate of cerebroside, characterized by formation of either 3,4 or 4,6 cyclic acetal linkages to the β-galactopyranosyl residue. This structure is analogous to "plasmalopsychosine" as recently characterized in this laboratory (Nudelman et al., 1992). In this paper, we describe the isolation and characterization of the plasmal component, which is structurally distinct from the ester cerebrosides as well as the putative sphingoplasmalogen previously reported.

Materials and Methods

Reagents. Dimethyl sulfoxide was purchased from Pierce (silylation grade; Rockford, IL). All other solvents were HPLC grade, purchased from Burdick & Jackson (Muskegon, MI), J. T. Baker (Phillipsburg, NJ), or Fisher Scientific (Fairlawn, NJ). Methyl iodide (Fisher) was redistilled and stored under N_2 at 4 °C. Galactosyl cerebrosides were purchased from Sigma Chemical Co. All other chemicals were of analytical reagent grade.

Chemical Degradations. Acid lability of glycolipids was assessed by hydrolysis of samples in MeOH–aqueous 0.3 N

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[1] Abbreviations: CMH, ceramide monohexoside; EME, enol methyl ether; +FAB-MS, positive ion fast atom bombardment mass spectrometry; FAME, fatty acid methyl ester; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; PMAA, partially methylated alditol acetate; SPN, sphingosine; TLC, thin-layer chromatography.

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HCl (1:1 v/v; 90 °C, 10 min), followed by Folch's partitioning and examination of the lower phase by HPTLC. Alternatively, the samples were hydrolyzed in MeOH–aqueous 0.1 N HCl containing 1% $HgCl_2$. Long-chain aldehydes released by this method were detected by fuchsin–sulfurous acid reagent, giving the so-called "plasmal reaction" (Feulgen & Voit, 1924; Thierfelder & Klenk, 1930; Fuelgen & Grünberg, 1938). Susceptibility to base was assessed by treatment with 0.3 N NaOH in MeOH (80 °C, 40 min).

Extraction and Fractionation of Low-Polarity Human Brain Glycolipids. Human brain tissue was homogenized with 5 volumes of 2-propanol–hexane–water (55:25:20) and filtered through a Büchner funnel. The residue was subjected to rehomogenization in the same volume of the same solvent. The extracts were pooled, evaporated to dryness in a rotary evaporator, and subjected to Folch partition using 1 L of chloroform–methanol (2:1) and 166 mL of water/100 g original wet weight of tissue. The lower phase was subjected to Folch partitioning (3×) with "theoretical upper phase" [chloroform–methanol–aqueous 0.2% KCl (10:10:1)], and the remaining lower phase was evaporated to complete dryness before subsequent chromatographic steps. Cationic lipids were removed by chromatography on carboxymethyl-Sephadex as described previously (Nudelman et al., 1992). Anion-exchange chromatography on (diethylamino)ethyl-Sephadex was performed according to the procedure of Yu and Ledeen (1972). These ion-exchange steps were followed by chromatography on Florisil (Sigma; 60–100 mesh). A large column (bed volume 1 L/1 kg original tissue) was prepared and equilibrated in pure hexane. The dried lower phase was suspended in hexane (1 L/200 g original tissue), passed over the Florisil column, and exhaustively washed with 4 L of hexane. The Florisil column was then eluted with 2 L of hexane–dichloroethane (2:1), then with 2 L of dichloroethane, and finally with 1 L of dichloroethane–acetone (1:1). The final eluate contained a fast-migrating, orcinol-stainable component found to be acid-labile and base-stable under the conditions described in the previous section. Further purification was carried out by HPLC on Iatrobeads (6RS-8010, Iatron Laboratories, Tokyo, Japan) columns. The crude dichloroethane–acetone lipid fraction was loaded in pure hexane on a preparative column (1.0 × 45 cm) and eluted by gradient to 2-propanol–hexane–water (55:40:5) at 1 mL/min for 3 h, with fractions collected into 200 tubes. The acid-labile glycolipid component was eluted in tubes 130–154. The pooled fraction, considered to contain most of the acid-labile glycolipid, was again purified by Iatrobeads chromatography, loaded on the column in pure hexane, and eluted with a shallow gradient to 2-propanol–hexane (30:70) (1.0 mL/min, 3 h). The fractions containing the acid-labile glycolipid were again pooled and further purified on a long Iatrobeads column (0.5 × 60 cm), loaded in pure hexane, and eluted by gradient to 2-propanol–hexane–water (50:40:5) (0.5 mL/min, 3 h). The final purification was performed by preparative silica gel HPTLC [chloroform–methanol–28% NH_3 (90:10:1 v/v)].

Preparation of Long-Chain EME Standards. Long-chain alcohols (1-hexadecanol and 1-octadecanol), purchased from Aldrich (Milwaukee, WN), were oxidized to aldehydes using pyridinium dichromate in CH_2Cl_2, according to the method of Corey and Schmidt (1979). Identity and purity of products were verified by GC–MS. Aldehydes were converted to EMEs by treatment with 0.5 N HCl–5 M H_2O in methanol at 80 °C for 5.5 h. The methanolysate was cooled and extracted three times with hexane. The combined hexane extracts were evaporated under N_2 stream at 37 °C to ≈10 μL and then diluted with hexane for analysis by GC–MS as described below. Under these conditions, production of EME derivatives was favored over conversion to long-chain dimethyl acetals.

Fatty Acid, Aldehyde, and Monosaccharide Analysis. Fatty acids were estimated as FAMEs liberated by methanolysis (1.0 mL of 0.5 N HCl in anhydrous methanol, 80 °C, 24 h) of about 30–50 μg of lipid. Fatty aldehydes released during the same procedure were converted to long-chain EMEs as described above. Both of these components were extracted from the methanolysate, prior to neutralization, by partitioning three times with approximately equal volumes of hexane. The combined hexane extracts were reduced in volume under N_2 stream at 35–40 °C to ≈1–2 μL and then taken up in a volume of hexane (10–50 μL) providing a suitable dilution for analysis by GC–MS. GC–MS of aliquots of the hexane-extractable material was performed using a Hewlett-Packard (Palo Alto, CA) 5890A gas chromatograph interfaced to an Extrel (Pittsburgh, PA) ELQ 400 quadrupole mass spectrometer. Gas chromatography was performed using a 30-m DB-5 (J & W Scientific, Ranch Cordova, CA) bonded-phase fused silica capillary column (0.25-mm o.d., 0.25-μm film thickness; splitless injection; temperature program, 150–290 °C at 4 °C/min). The mass spectrometer was operated in either CI (isobutane; mass range 150–500 u, scanned once per second) or EI (mass range 50–500 u, scanned once per second) mode. EME and FAME derivatives were identified by characteristic ions and retention times, verified by coinjection with synthetic and commercial standards when necessary.

The remaining acidic MeOH lower layer was neutralized by addition of Ag_2CO_3 (≈10 mg) and treated with acetic anhydride (100 μL) for 6 h at room temperature. Following centrifugation and removal of the MeOH, the precipitate was washed two times with 1-mL portions of MeOH. The combined MeOH supernatants were dried under N_2 stream. The resulting monosaccharide methyl glycosides were analyzed as their per-*O*-(trimethylsilyl) ethers (Sweeley et al., 1963; Laine et al., 1963) by GC–MS using the Extrel ELQ 400 system described above [DB-5 column; splitless injection; temperature program, 140–270 °C at 4 °C/min; CI-MS (isobutane) mode].

Methylation/Linkage Analysis. Lipid samples (≈50 μg) were permethylated by the method of Ciucanu and Kerek (1984), as modified by Larson et al. (1987), except that equal volumes of methyl iodide and DMSO were used (100 μL each). The reaction time was 30 min, and methyl iodide was removed by flushing with N_2 for 25 min at 37 °C prior to partitioning between $CHCl_3$ and H_2O. After washing three times with H_2O, the $CHCl_3$ was evaporated to dryness under N_2.

Linkage positions of substituents on glycosyl residues were determined by permethylation as above, followed by hydrolysis, reduction, peracetylation, and GC–MS as described in detail elsewhere (Levery & Hakomori, 1987), except that the analysis was performed on the Extrel ELQ 400 GC–MS system described above (DB-5 column; splitless injection; temperature program, 140–250 °C at 4 °C/min; EI-MS mode), with identification of PMAA derivatives made by retention time and characteristic electron-impact mass spectra (Bjorndal et al., 1970; Jansson et al., 1976). Identifications were confirmed by comparison with PMAAs in known standard mixtures.

Fast Atom Bombardment Mass Spectrometry. $^+$FAB-MS was performed on a JEOL (Tokyo, Japan) HX-110/DA-5000 mass spectrometer/data system at full acceleration voltage (10 kV); xenon beam, 6 kV; mass range, 3000; resolution, 3000.

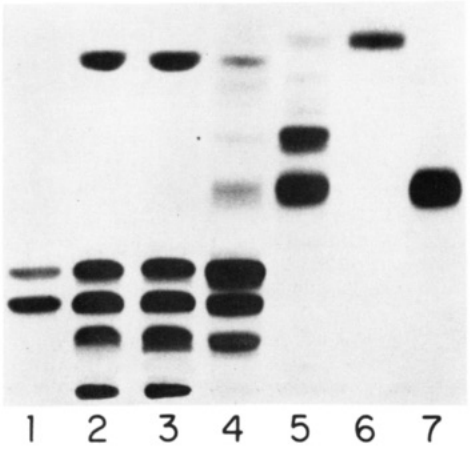

FIGURE 1: HPTLC pattern of various low-polarity glycosphingolipids from Folch's lower phase prepared from human brain. About 5–10 μg of lipid spotted per lane. Silica gel (Merck) plates developed in chloroform–methanol–28% NH_3 (80:20:2 v/v). Bands were visualized with 0.5% orcinol–10% H_2SO_4 reagent. Lane 1: Standard CMH (cerebroside); upper band, CMH with non-hydroxy fatty acids; lower band, CMH with hydroxy fatty acids. Lane 2: Lower phase obtained on Folch's partition. Lane 3: Pass through of total lower phase unabsorbed by carboxymethyl-Sephadex. Lane 4: Partially purified low-polarity fraction obtained following column chromatography on Florisil [eluted by dichloroethane–acetone (1:1 v/v)]. Lane 5: Fraction 47–58 eluate from final Iatrobeads chromatography; darkest bands are ester cerebrosides 1 and 2. Lane 6: HPTLC purified "plasmalocerebroside". Lane 7: Purified ester cerebroside 2.

Aliquots of sample (≈20 μg) in MeOH were transferred to a FAB target and suspended in 3-nitrobenzyl alcohol matrix. Three scans were accumulated and summed for each spectrum. KI/CsI was used as the calibration standard. Nominal, monoisotopic (^{12}C) masses are reported for simplicity.

Results

Isolation and Preliminary Characterization of an Acid-Labile Glycosphingolipid Present in the Fast-Migrating Fraction. The presence of an acid-labile, base-stable, low-polarity glycolipid was noticed as a consistent component of brain extract. This component, found in the lower phase following Folch's partitioning (Figure 1, lane 2), was unabsorbed by either carboxymethyl-Sephadex or (diethylamino)ethyl-Sephadex chromatography (the latter procedure was used only during the initial characterization of the unknown component and was omitted in later preparations). On chromatography through a Florisil column, the unknown component eluted in the dichloroethane–acetone (1:1) fraction (Figure 1, lane 4). The crude low-polarity lipid fraction was further purified by repeated HPLC on Iatrobeads columns. The resulting material was free of cerebroside and all slower-migrating glycosphingolipids (Figure 1, lane 5). A final purification by HPTLC yielded a homogeneous fraction, free of cholesterol and ester cerebrosides, as shown in Figure 1, lane 6. The new compound had a higher R_f than either cholesterol (the band migrating just below it in Figure 1, lanes 2–4) or ester cerebrosides. The yield of the unidentified compound from three preparations (totaling ≈1700 g wet weight of brain tissue) was ≈0.5 mg.

In Figure 2 are illustrated the reactivities of various purified low-polarity glycolipid fractions to acid and base treatments. The fraction having the highest R_f (lane 4), previously uncharacterized, was clearly identified by its extreme lability to acid (lane 5) and stability to base treatment (lane 6). By contrast, purified ester cerebroside fractions were found to be considerably more stable to acid, but labile to base treatment (lanes 7–12).

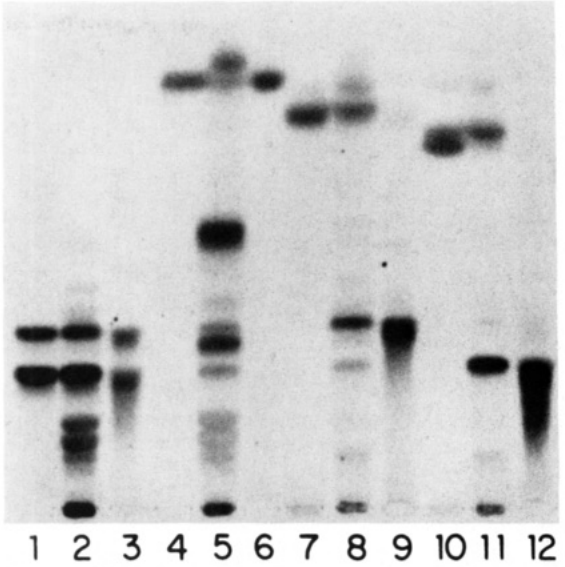

FIGURE 2: HPTLC pattern of cerebroside (CMH), plasmalocerebroside, and ester cerebroside, with degradation by weak acid and weak base. Development and visualization as in Figure 1. Lane 1, CMH; lane 2, CMH degraded by 0.3 N HCl–MeOH; lane 3, CMH–0.3 N NaOH; lane 4, plasmalocerebroside; lane 5, plasmalocerebroside treated with 0.3 N HCl in MeOH; lane 6, plasmalocerebroside treated with 0.3 N NaOH in MeOH; lane 7, ester cerebroside 1; lane 8, ester cerebroside 1 treated with 0.3 N HCl in MeOH; lane 9, ester cerebroside 1 treated with 0.3 N NaOH in MeOH; lane 10, ester cerebroside 2; lane 11, ester cerebroside 2 treated with 0.3 N HCl in MeOH; lane 12, ester cerebroside 2 in 0.3 N NaOH in MeOH.

When acid hydrolysis of the newly identified low-polarity component was carried out in the presence of $HgCl_2$, the presence of aldehyde was detected by the classical "plasmal reaction" (violet color with fuchsin–sulfurous acid reagent). The presence of long-chain aldehydes was confirmed by the results of GC–MS analysis following methanolysis (see below).

Monosaccharide, Fatty Acid, and Fatty Aldehyde Analysis by GC–MS after Methanolysis. Monosaccharide analysis was carried out by GC–MS of trimethylsilyl methyl glycosides produced following acidic methanolysis of the unknown component. Three peaks were clearly observed for the usual trimethylsilyl derivatives of galactose (data not shown). No other monosaccharide peaks were observed.

Fatty acid analysis was performed by GC–MS analysis of the hexane extract of the methanolysate. In addition to peaks corresponding to FAMEs (principally 16:0, 18:1, 18:0, and 24:1), there were observed a number of unknown peaks not detected in the methanolysate of standard cerebroside or ester cerebroside. The identity of a number of these peaks was determined by comparison of retention times, along with EI and CI mass spectra, to authentic long-chain EMEs. They were identified specifically as EMEs derived from 16:0, 18:1, and 18:0 fatty aldehydes. These components are identified, along with the FAMEs, in the GC–MS reproduced in Figure 3.

$^{+}$FAB-MS Analysis of Native Lipid. A FAB mass spectrum of the unknown native lipid was obtained in the positive ion mode. The spectrum (reproduced in Figure 4) was characterized in the lower mass end by the fragments at m/z 282 and 264, which correspond in both mass and relative abundance to the SPN-related ions derived by de-N-acylation and dehydration of ceramide [W′ and W″, respectively, in the nomenclature of Domon and Costello (1988)], as commonly found in $^{+}$FAB and FAB-collision-induced dissociation spectra of cerebrosides having d18:1 SPN (Hemling et al., 1984; Ohashi et al., 1987; Domon & Costello, 1988). Ceramide ions

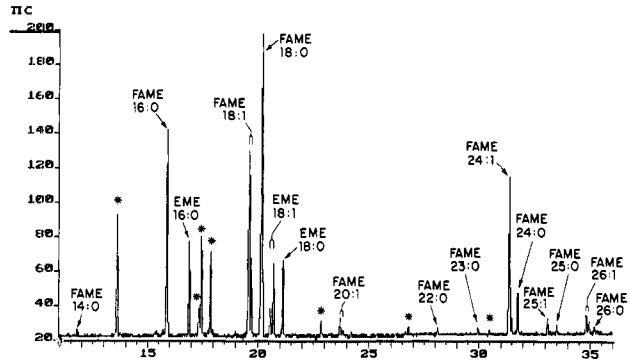

FIGURE 3: GC–EI/MS of long-chain FAMEs and EMEs from methanolysis of unknown lipid component. Ordinate, total ion current; abscissa, time (min). Peaks were identified as marked. Peaks marked by asterisk are unidentified impurities.

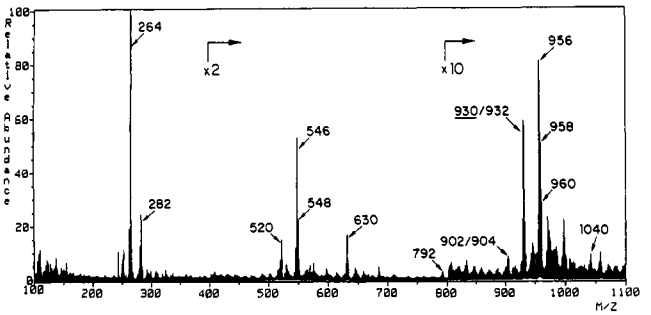

FIGURE 4: $^{+}$FAB mass spectrum of unknown lipid component in 3-nitrobenzyl alcohol matrix. Peaks are labeled with nominal, monoisotopic (^{12}C) masses.

(Y_0) were found most abundantly at m/z 520, 546, 548, and 630, corresponding to compositions having d18:1 SPN N-acylated primarily with 16:0, 18:1, 18:0, and 24:1 fatty acids. These would be expected on the basis of the FAME analysis (previous section; Figure 3). A small peak consistent with a cerebroside $[MH]^+$ was observed at m/z 792 (corresponding to Hex-Cer with d18:1 SPN and 24:1 fatty acid). The primary group of pseudomolecular ions $[MH]^+$ were found at m/z 930, 932, 956, 958, and 960. Note that the even mass numbers observed correspond to odd molecular weights, and therefore to compounds containing an odd number of nitrogen atoms. In analogy to psychosine acetal structures determined previously (Nudelman et al., 1992), these pseudomolecular ion species were hypothesized to correspond to cerebrosides which have been modified by long-chain fatty aldehydes attached in cyclic acetal linkages to vicinal hydroxy groups of the galactose moiety. As determined by analysis of the GC–MS peaks corresponding to long-chain EMEs (previous section; Figure 3), these aldehydes would be primarily 16:0, 18:1, and 18:0 species. The observed pseudomolecular ion abundances therefore reflect a complex distribution according to the proportions of both fatty acid and fatty aldehyde moieties of different lengths found in the lipid. For example, the most abundant pseudomolecular ion at m/z 956 would correspond to a galactocerebroside acetal having d18:1 SPN, 18:1 fatty acid, and 18:1 fatty aldehyde. The ion at m/z 930 could correspond to either d18:1 SPN, 18:1 fatty acid, and 16:0 aldehyde or d18:1 SPN, 16:0 fatty acid, and 18:1 aldehyde. Other ions in the cluster represent other possible combinations of the most abundant fatty acid and aldehyde species (all with d18:1 SPN). No fragments were observed corresponding to loss of hexose alone from the molecular ions, nor were there any indications of attachment of modifying groups to the 3-OH of SPN. The conclusion that the compounds are cerebrosides

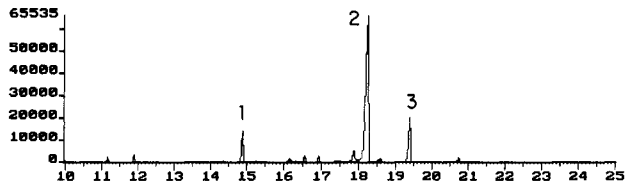

FIGURE 5: GC–MS analysis of PMAAs from permethylation, hydrolysis, reduction, and acetylation of unknown lipid. The GC–MS is plotted as a composite of relevant structural ions. Ordinate, summation of selected ion intensities; abscissa, time (min). Peaks are identified as PMAAs of (1) 2,3,4,6-tetra-*O*-, (2) 2,6-di-*O*-, and (3) 2,3-di-*O*-Me-Gal.

modified by acetal linkage to vicinal hydroxy groups of galactose was confirmed by methylation/linkage analysis, as described below.

Methylation Analysis by GC–MS. Following permethylaiton, acid hydrolysis, reduction, and acetylation of the native lipid, the resulting partially methylated hexitol acetates were analyzed by GC–MS (Figure 5). The primary component detected was 2,6-di-*O*-Me-Gal, along with small peaks corresponding to 2,3-di-*O*-Me-Gal and 2,3,4,6-tetra-*O*-Me-Gal. These results clearly indicated that the modifying group(s) occupies(occupy) two hydroxyl positions on the galactose moiety. This could not be accommodated by attachment of two enol ethers in tandem, since the mass increases relative to free cerebroside would have to be twice those observed. The only modification consistent with the $^{+}$FAB-MS and other data appeared to be attachment of long-chain aldehydes as cyclic acetals. The di-*O*-Me peaks represent 3,4- and 4,6-linked substituents, respectively, on galactose and show that the lipid fraction must be comprised of isomeric cyclic acetals derived from cerebroside, mostly in a five-membered 3,4-linked ring, with some six-membered 4,6-linked ring. The small trace of 2,3,4,6-tetra-*O*-Me-Gal is consistent with the low-abundance pseudomolecular ion detected for unsubstituted cerebroside. These linkages were also found in separate components of the psychosine acetals determined previously (Nudelman et al., 1992). The only uncertainties remaining in the primary structures are the chiralities at the acetal C-1 positions and the location and configuration of double bonds in the fatty aldehyde chains. Until the former can be determined unambiguously, we assume an equatorial orientation for the long chain in the six-membered acetal ring and a pseudoequatorial orientation for this group in the five-membered ring. As for the latter, it may be some time before the double bond positions can be analyzed in detail; however, cis-Δ^9 and cis-Δ^{11} 18:1 aldehydes have been identified in plasmalogens isolated from human brain and placenta (Leupold, 1950; Klenk et al., 1952; Debuch & Winterfeld, 1970).

Discussion

A number of studies have been performed previously to characterize "faster-running" (Tamai, 1968) cerebrosides, i.e., a series of minor glycosphingolipid components showing nonpolar properties, including R_f values greater than those of cerebroside in normal-phase thin-layer chromatographic analysis. The majority of these compounds have been identified as cerebrosides esterified with fatty acids of differing chain lengths and at different positions. Fatty *O*-acyl linkages have been reported at 3- or 6-OH of galactose, or 3-OH of SPN (Kishimoto et al., 1968; Klenk & Lohr, 1967; Tamai et al., 1967; Tamai, 1968). Kochetkov et al. (1963) observed a minor cerebroside component in bovine brain tissue which appeared to be a fatty aldehyde conjugate. Since fatty aldehyde was detectable in the hydrolysate of "sphingolipid 3"

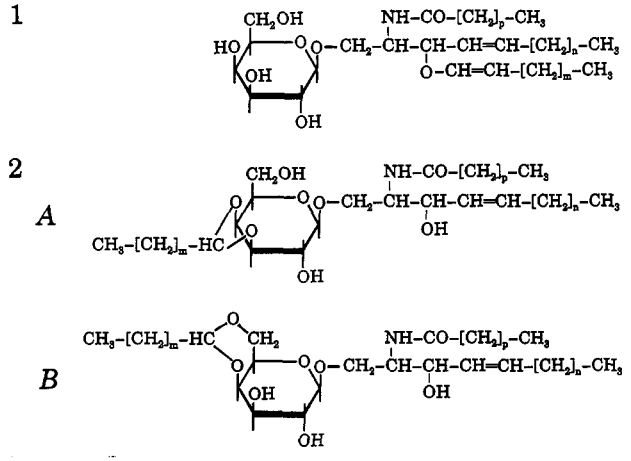

FIGURE 6: Structures of plasmalogen-type structures proposed for low-polarity glycosphingolipids. (1) "Sphingoplasmalogen" structure proposed by Kochetkov et al. (1963) for "sphingolipid 3" of bovine brain; (2) "plasmalocerebroside" structures proposed herein for fast-migrating components of human brain. (A) Galactocerebroside 3,4-*O*-cyclic fatty acetal; (B) galactocerebroside 4,6-*O*-cyclic fatty acetal.

by the method of Wittenberg et al. (1956), the structure was proposed to be as shown in Figure 6-1 and termed "sphingoplasmalogen".

Results of the present study indicate the presence of fatty aldehyde conjugates of cerebroside, but having structures clearly different from that proposed for sphingoplasmalogen by Kochetkov et al. (1963). A combination of $^{+}$FAB-MS and methylation analysis indicated conjugation of the fatty aldehyde to galactose at the 3,4- or 4,6-OH groups through cyclic acetal linkages as shown in Figure 6-2. Heterogeneity in both fatty acid and aldehyde moieties was observed by GC–MS analysis as well as in the $^{+}$FAB mass spectrum. These compounds were isolated as a single band on HPTLC, having a higher TLC mobility than cholesterol; they could not be separated according to differential substitution by any chromatographic system currently available in this laboratory.

Long-chain aliphatic aldehydes (plasmals) are found in animal tissues almost exclusively as a component of plasmalogen, a form of glycerophosphatide in which the fatty aldehyde is linked, in place of the more usual fatty *O*-acyl group, to the 1-hydroxyl group of glycerol through an enol ether linkage. Plasmal linkages, unlike fatty esters, are labile under acidic conditions but stable to alkali. Discovered by Feulgen and Voit (1924), plasmalogens were considered to be a source of the plasmal reaction (violet coloration from oxidation of sulfite-bleached fuchsin) observed in cytoplasm (thus the term plasmal*ogen*). The structure of plasmalogen was originally proposed by Feulgen to be a fatty aldehyde cyclic acetal linked to the 1,2-OH groups of glycerol, linked in turn by the 3-OH group to phosphoryl choline (Feulgen & Bersin, 1939). This cyclic plasmal structure was generally accepted for many years and was presented in many biochemistry textbooks. Later, the structure was carefully reinvestigated and determined to possess an "enol–ether" rather than cyclic acetal linkage, i.e., 1-alkenyl-2-acyl-3-(phosphorylcholinyl)-glycerol (Klenk & Debuch, 1963). Very recently, we reported a novel lipid, termed "plasmalopsychosine", in which fatty aldehyde is linked at the galactopyranosyl residue of psychosine through a cyclic acetal linkage (Nudelman et al., 1992). We now report a similar structural modification present in galactosylceramide. These recent studies indicate the presence of a "classical" cyclic fatty acetal structure, as originally considered by Feulgen for plasmalogen, which nevertheless differs significantly from any class of lipid modification previously proposed by having an aldehyde conjugated to the sugar moiety of a glycosphingolipid. The closest structural analog in carbohydrate chemistry would be the pyruvate ketal modification commonly found attached to residues of bacterial and seaweed polysaccharides (Hirase, 1957; Gorin & Ishikawa, 1967; Bennett & Bishop, 1977; Nimmich, 1979; Sviridov et al., 1980). Clearly, their physicochemical properties and functional roles would have to be completely distinct.

The yield of the novel cerebroside derivatives amounted to ≈0.3 μg/g (wet weight) of brain tissue, which is about 4–5 orders of magnitude lower than the concentration of galactocerebroside (1–2%). By contrast, the yield of plasmalopsychosines was found to total 3–4 μg/g of brain tissue (Nudelman et al., 1992). However, the latter conjugates were more easily purified, since they could be separated from crude Folch's lower phase fractions by ion-exchange chromatography on carboxymethyl-Sephadex. Considerably greater losses of plasmalocerebroside could be expected from the multiple HPLC steps used in its purification. More accurate quantitation of these, and other sphingolipids of extremely low abundance, is currently under investigation.

Taken by itself, the low yield of plasmalocerebroside might prompt the suggestion of artifactual formation during tissue extraction. However, when one also considers the yield of the analogous psychosine derivatives, isolated in 10-fold amounts although native psychosine itself was undetectable, the possibility of artifact seems much less likely. Moreover, the concentration of free long-chain aldehydes in tissues is essentially zero (Klenk & Debuch, 1963). Finally, acidic conditions which would be required to catalyze the acetalation reaction (or transacetalation from conventional plasmalogens) were avoided during the extraction and purification of these lipids. The conditions required for formation of these plasmaloglycosphingolipids in vivo, as well as their functional significance, if any, remain open to speculation.

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References

Bennett, L. G., & Bishop, C. T. (1977) *Can. J. Chem. 55*, 8–16.

Bjorndal, H., Hellerqvist, C. G., Lindberg, B., & Svensson, S. (1970) *Angew. Chem., Intl. Ed. Engl. 9*, 610–619.

Ciucanu, I., & Kerek, K. (1984) *Carbohydr. Res. 131*, 209–217.

Corey, E. J., & Schmidt, G. (1979) *Tetrahedron Lett. 1979*, 399–402.

Debuch, H., & Winterfeld, M. (1970) *Hoppe-Seyler's Z. Physiol. Chem. 351*, 179–183.

Domon, B., & Costello, C. E. (1988) *Biochemistry 27*, 1534–1543.

Feulgen, R., & Voit, K. (1924) *Pfluegers Arch. 206*, 389–410.

Feulgen, R., & Grünberg, H. (1938) *Hoppe-Seyler's Z. Physiol. Chem. 257*, 161–172.

Feulgen, R., & Bersin, T. (1939) *Hoppe-Seyler's Z. Physiol. Chem. 260*, 217–245.

Gorin, P. A. J., & Ishikawa, T. (1967) *Can. J. Chem. 45*, 521–532.

Hemling, M. E., Yu, R. K., Sedgwick, D., & Rhinehart, K. L. (1984) *Biochemistry 23*, 5706–5713.

Hirase, S. (1957) *Bull. Chem. Soc. Jpn. 30*, 68–70.

Jansson, P.-E., Kenne, L., Liedgren, H., Lindberg, B., & Lonngren, J. (1976) *Chem. Commun., Univ. Stockholm 8*, 1–75.
Kishimoto, Y., Wajda, M., & Radin, N. S. (1968) *J. Lipid Res. 9*, 27–33.
Klenk, E., & Debuch, H. (1963) *Prog. Chem. Fats Other Lipids 6*, 1–29.
Klenk, E., & Doss, M. (1966) *Hoppe-Seyler's Z. Physiol. Chem. 346*, 296–298.
Klenk, E., & Lohr, J. P. (1967) *Hoppe-Seyler's Z. Physiol. Chem. 348*, 1712–1714.
Klenk, E., Stoffel, W., & Eggers, H. J. (1952) *Hoppe-Seyler's Z. Physiol. Chem. 290*, 246–251.
Kochetkov, N. K., Zhukova, I. G., & Glukhoded, I. S. (1962) *Biochim. Biophys. Acta 60*, 431–432.
Kochetkov, N. K., Zhukova, I. G., & Glukhoded, I. S. (1963) *Biochim. Biophys. Acta 70*, 716–719.
Laine, R. A., Esselman, W. J., & Sweeley, C. C. (1963) *Methods Enzymol. 28*, 159–167.
Larson, G., Karlsson, H., Hansson, G. C., & Pimlott, W. (1987) *Carbohydr. Res. 161*, 281–290.
Leupold, F. (1950) *Hoppe-Seyler's Z. Physiol. Chem. 285*, 182–200.
Levery, S. B., & Hakomori, S. (1987) *Methods Enzymol. 138*, 13–25.
Nimmich, W. (1979) *Z. Allg. Mikrobiol. 19*, 343–347.
Norton, W. T., & Brotz, M. (1963) *Biochem. Biophys. Res. Commun. 12*, 198–203.
Nudelman, E. D., Levery, S. B., Igarashi, Y., & Hakomori, S. (1992) *J. Biol. Chem.* (in press).
Ohashi, Y., Iwamori, M., Ogawa, T., & Nagai, Y. (1987) *Biochemistry 26*, 3990–3995.
Sviridov, A. F., Arifkhodzhaev, K. A., Chizhov, O. S., & Kochetkov, N. K. (1980) *Bioorg. Khim. 6*, 165–186.
Sweeley, C. C., Bentley, R., Makita, M., & Wells, W. W. (1963) *J. Am. Chem. Soc. 85*, 2497–2507.
Tamai, Y. (1968) *Jpn. J. Exp. Med. 38*, 65–73.
Tamai, Y., Taketomi, T., & Yamakawa, T. (1967) *Jpn. J. Exp. Med. 37*, 79–81.
Thierfelder, H., & Klenk, E. (1930) *Die Chemie der Cerebroside und Phosphatide*, p 76, Springer Verlag, Berlin.
Wittenberg, J. B., Korey, S. R., & Swenson, F. H. (1956) *J. Biol. Chem. 219*, 39–47.
Yu, R. K., & Ledeen, R. W. (1972) *J. Lipid Res. 13*, 680–686.

Gramicidin Channels That Have No Tryptophan Residues†

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ABSTRACT: In order to understand how aromatic residues modulate the function of membrane-spanning proteins, we examined the role of the four tryptophans in gramicidin A (gA) in determining the average duration and permeability characteristics of membrane-spanning gramicidin channels; the tryptophan residues were replaced by tyrosine (gramicidin T, gT), tyrosine *O*-benzyl ether [gramicidin T(Bzl), gT(Bzl)], naphthylalanine (gramicidin N, gN), and phenylalanine (gramicidin M enantiomer, gM⁻). These analogues form channels with durations and conductances that differ some 10- and 16-fold, respectively. The single-channel conductance was invariably decreased by the Trp → Yyy replacement, and the relative conductance alterations were similar in phosphatidylcholine (DPhPC) and monoglyceride (GMO) bilayers. The duration variations exhibited a more complex pattern, which was quite different in the two membrane environments: in DPhPC bilayers, gN channels have an average duration that is ~2-fold longer than that of gA channels; in GMO bilayers, the average duration of gN channels is about one-tenth that of gA channels. The sequence-dependent alterations in channel function do not result from alterations in the channels' peptide backbone structure, because heterodimers can form between the different analogues and gramicidin A, and there is no energetic cost associated with heterodimer formation [cf. Durkin, J. T., Koeppe, R. E., II, & Andersen, O. S. (1990) *J. Mol. Biol. 211*, 221]. The alterations in permeability properties are consistent with the notion that Trp residues alter the energy profile for ion permeation through long-range electrostatic interactions.

Despite advances in molecular biology and electrophysiology, the molecular mechanisms that underlie ion channel function to remain elusive because only limited structural information is available. General questions concerning channel function can therefore with advantage be examined using appropriate prototypes, such as the channel-forming linear gramicidins. In integral membrane proteins, for example, Trp residues seem to be localized at or near the membrane/solution interface or in the transport path (Chattopadhyay & McNamee, 1991; Henderson et al., 1990; Michel & Deisenhofer, 1990). But the structural or functional significance of this arrangement remains obscure.

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