Plasmalpsychosine, a Novel Plasmal (Fatty Aldehyde) Conjugate of Psychosine with Cyclic Acetal Linkage

ISOLATION AND CHARACTERIZATION FROM HUMAN BRAIN WHITE MATTER*

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Through a systematic examination of basic (cationic) lipids separated on Folch's lower phase from extracts of human brain by cation exchange chromatography on carboxymethyl Sephadex in a chloroform/methanol mixture, followed by successive chromatographies on Florisil and Iatrobeads columns, five compounds of basic lipids were separated. Two major unknown compounds A and B and a minor unknown compound C were separated, in addition to minor compounds sphingosine and N,N-dimethylsphingosine. This paper describes the isolation and chemical characterization of major unknown compounds A and B, which were found only in the white matter but not in the gray matter of the human brain. Unmodified psychosine (galactosylsphingosine) was essentially undetectable under the experimental conditions. Unknown compounds A and B were identified as novel plasmal (fatty aldehyde) conjugates of psychosine with cyclic acetal linkage at the galactosyl residue of psychosine. Fatty aldehydes were identified as mainly palmital (16:0) and stearal (18:0). Sphingosine was identified as d18:1 sphingosine. Faster migrating compound A had 3,4-cyclic acetal linkage, and slower migrating compound B had 4,6-cyclic acetal linkage (where m is 14 or 16 and n is 12) as shown below.

A

B

Preliminary studies showed that compounds A, B, and C had a weak inhibitory effect on protein kinase C (PKC) and had no cytotoxic effect. In contrast, psychosine displayed a strong cytotoxicity and inhibitory effect on PKC. Therefore, the process controlling the addition or deletion of plasmal cyclic linkage to psychosine could be a crucial step in regulation of PKC, src, or other kinases susceptible to psychosine.

In view of the possible involvement of sphingosine, DMS, and lysoglycosphingolipids in modulating transmembrane signaling (1–5), chemical identification of these compounds occurring naturally in neural tissue is of great interest. Sphingosine and DMS inhibit PKC (1,6) but stimulate epidermal growth factor receptor kinase (7) and src kinase (6). Lyso-GM₃ (II NeuAcLac-sphingosine) strongly inhibits PKC (8) and is detectable as a physiological cell component (9). On the other hand, psychosine inhibits PKC (2,8) and enhances src and ras kinases acting on substrate² but is virtually absent under normal physiological conditions. Based on the fact that all of these lipid modulators have common cationic properties, a procedure for systematic isolation and characterization of cationic lipid through cation exchange chromatography in chloroform/methanol followed by a series of chromatographies on Florisil and Iatrobeads columns has been developed. The major cationic lipids (compounds A and B; Summary), present in the lower phase of Folch's partition and found exclusively in extract of white (but not gray) matter, have been identified as cyclic plasmalogens, aliphatic aldehyde acetas linked at different hydroxyl groups of the galactosyl residue of psychosine (β-D-galactopyranosyl-1-sphingosine). Isolation, chemical characterization, and biological properties of these novel compounds are hereby reported.

MATERIALS AND METHODS

Preparation of Cationic Lipids and Cationic Glycosphingolipids

Extraction and Preparation of Lower Layer—Adult human brain (cerebrum) was dissected and separated into gray and white matter with a razor blade. With careful practice, using a razor blade to scrape the outer layer of cortex, it was possible to obtain a nearly pure gray matter fraction. White matter was considerably easier to prepare, by cutting the brain into vertical sections and separating large areas of white matter. An entire small brain (cerebellum) was also used as a source of extraction. In all cases, the tissue was homogenized in five volumes (i.e., five times volume/weight of wet tissue) of isopropyl alcohol/hexane/water (55:25:20 v/v/v, upper phase removed), filtered.

The abbreviations used are: DMS, N,N-dimethylsphingosine; HPLC, high performance liquid chromatography; EME, enol methyl ether; FAB-MS, fast atom bombardment mass spectrometry; GC-MS, gas chromatography-mass spectrometry; HPTLC, high performance thin layer chromatography; NBA, 3-nitrobenzyl alcohol; PKC, protein kinase C; PMAA, partially methylatedalditol acetate; EGF, [ethylenesbis(oxyethylenenitrilo)tetraacetic acid].

1. M. Abdel-Ghany, M. Ousosky, D. Shalloway, Y. Igarashi, S. Hakomori, and E. Racker, unpublished data.
over a Büchner funnel, and the residue rehomogenized in the same solvent. After filtration, the residue was rehomogenized twice in chloroform/methanol/water (2:1:0.1). All filtrates were pooled, evaporated to dryness, and brought up in chloroform/methanol (2:1) to a suitable volume (50 μl) for Flock's partition (10). For Flock's partition, one-sixth volume of deionized water was added to a chloroform/methanol (2:1) extract solution in a screw-cap container and the contents inverted 20 times. After two phases had resolved (usually 2-3 h), the upper phase was drawn off and replaced with an equal volume of "theoretical upper phase" (chloroform/methanol/water 1:1:60). This was followed by stepwise evaporation to dryness by rotary evaporator.

Separation of Cationic Lipid Fraction by CM-Sephadex Chromatography—The cationic lipid fraction was prepared from the total lower layer lipid by CM-Sephadex chromatography. CM-Sephadex (Sigma, C-25) was carefully washed and equilibrated with the following protocol: It is crucial that the Sephadex be equilibrated properly to achieve effective binding of cationic lipids. The dry resin was washed extensively over a Büchner's funnel in 0.2 N HCl and allowed to soak for several hours in the acid. The resin was then washed extensively with deionized water with intermittent shaking, followed by stepwise washing of methanol/water 20:80, 50:50, 70:30, and 90:10. Subsequently, the Sephadex column was soaked in a solution of 2.0 M aqueous triethylamine/methanol/water (1:1:1) and allowed to sit at room temperature overnight. Triethylamine was purchased from Mallinkrodt Specialty Chemical Co. (St. Louis, MO). Excess triethylamine was removed from the Sephadex by extensive washing in methanol/water 1:1. The equilibrated CM-Sephadex was then washed with 100% methanol followed by chloroform/methanol/water 40:60:5 (solvent A).

To the dried lower phase of brain extract, solvent A was added until the solution became clearly soluble. For the residue derived from 500 g of tissue, it was necessary to add ≥1 liter of solvent. This was passed over a bed volume of 50 ml of equilibrated CM-Sephadex (≈100 ml/kg wet tissue) and allowed to elute by gravity filtration. An additional 2 liters of solvent A was washed through the column, and the total pass-through fraction was collected and saved. The column was then washed with methanol/water 90:10 until the bed volume was equilibrated (Sephadex will shrink slightly). Cationic lipids were eluted using a solution of 0.5 M triethylamine in methanol/water 90:10 (titrated to a pH of 9.25 by gently bubbling CO₂ gas through the solvent). For 500 g of starting tissue, 500 ml of 0.5 M triethylamine was sufficient to quantitatively elute three unknown compounds designated A, B, and C (see Fig. 1A, lanes 2-4), as well as sphingosine separated on TLC (see Fig. 1A, lanes 5) as described in the Fig. 1 legend. In separate tests, this concentration of triethylamine also quantitatively eluted standard psychosine, although psychosine was absent in brain extract. Increasing triethylamine concentration up to 2.0 M did not result in elution of any other detectable species.

Further Purification of Unknown Compounds A, B, and C Using HPLC Iatrohead Chromatography

The 0.5 M triethylamine eluted fraction from CM-Sephadex was evaporated to dryness several times using absolute ethanol to eliminate triethylamine. The fraction was then transferred to screw-cap tube and brought up to a final volume of 2-10 ml of chloroform/methanol 2:1, and 10 μl was chromatographed on HPTLC (Merck) plates in chloroform/methanol/conc. NH₄OH 80:30:2. Fluorescent bands were detectable under UV light after spraying with 0.5% Primulin (Sigma) in 80% acetonitrile or 0.03% fluorescamine (Sigma). With fluorescamine, detectability was enhanced by spraying in advance with triethylamine 1:2-dichloroethane (9:1). It was also possible to detect unknown compounds A, B, C with 0.5% orcinol (Sigma) in 10% sulfuric acid followed by heating in an oven at 130 °C.

To separate unknown compounds A, B, C from the more polar sphingosines and contaminating neutral glycolipids, it was necessary to perform several HPLC gradient runs. This was accomplished using a very nonpolar isopropl alcohol/hexane/water gradient. The long column (0.4x60 cm) packed with Iatroheads (10 μm) was equilibrated as follows. Elution began with a solvent mixture of isopropl alcohol/hexane/water 55:45:5 at 2.0 ml/min; the gradient was increased to isopropl alcohol/hexane/water 55:25:20 over the next 30 min, followed by 80% isopropl alcohol/hexane/water 55:45:5 for 30 min, then 60:40 for 30 min, and hexane 100% for 30 min. The 0.5 M triethylamine fraction for injection into HPLC column was prepared by evaporating to dryness and brought up in 100% hexane in the following manner. For a 2-ml injection, 100 μl of chloroform/methanol 2:1 was added, the cap screwed on tightly, and the sample warmed and sonicated. In most cases, this almost completely solubilized the lipid. To this thick oily solution, 2 ml of 100% hexane was added during sonication. In some cases, a very fine, opalescent precipitate formed, but this never interfered with the injection.

The sample was loaded onto the column and eluted isocratically for 25 min at 0.5 ml/min. Gradient elution was started from this solvent to isopropl alcohol/hexane/water 10:50:81 from 25 to 150 min and continued from this solvent to isopropl alcohol/hexane/water 24:4:7:2 (150-400 min), to 55:45:5 (400-500 min), and to 55:25:20 (500-600 min). Effluent (3 ml/tube) was collected over a fraction collector in 100 tubes, and the tubes were assayed for HPTLC analysis (chloroform/methanol/NH₄OH, 80:20:2). Fractions were pooled based on separation of three detectable bands for unknown compounds A, B, and C, and the fractions containing sphingosine overlap made several HPTLC runs necessary to purify unknown compounds A, B, and C to homogeneity. In this manner sphingosine was also conveniently purified, as well as a slower migrating sphingosine analog (see Fig. 1).

Chemical Degradation of Unknown Compounds A, B, and C

Weak acid treatment as catalyzed by mercuric chloride (0.1% HgCl₂, 1 in 1 N HCl) was performed according to the original method of Feulgen et al. (11); nitriles and formates were detected with 0.5 N HCl in MeOH at 80 °C for 30 min. Weak base hydrolysis was carried out in 0.3 N NaOH in MeOH at 80 °C for 40 min.

Preparation of Long Chain EME Standards

Long chain alcohols (n-hexadecanol and n-octadecanol), purchased from Aldrich, were oxidized to aldehydes using pyridinium dichromate in CH₃CO₂H, according to the method of Corey and Schmidt (12). Identity and purity of products were verified by GC-MS. Aldehydes were converted to EMEs by treatment with 0.5 N HCl, 5 M H₂O 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 N₂ stream at 37 °C to 10 μl, 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 dimethylacetals.

Long Chain Aldehyde Analysis

Lipid samples (400-500 μg) were methanolysed in 2.0 ml of 0.5 N HCl, 5 M H₂O in MeOH for 5.5 h at 80 °C. The methanolysate was cooled and extracted three times with hexane. The combined hexane extracts were evaporated under N₂ stream at 37 °C to 10 μl and then diluted with hexane in a volume of 10-50 μl to provide a dilution for analysis by GC-MS. GC-MS of aliquots of the hexane extractable material were performed using a Hewlett-Packard 5890A gas chromatograph interfaced to an Extrel ELQ 400 quadrupole mass spectrometer. Gas chromatography was performed using a 30-m DB-5 (J & W Scientific, Rancho Cordova, CA) bonded-phase fused silica capillary column (0.25-mm outer diameter, 0.25-μm film thickness; splitless injection; temperature program, 140-250 °C at 4 °C/min). The mass spectrometer was operated in either CI (isobutane, mass range m/z 150-500, scanned once/s) or EI (mass range m/z 50-500, scanned once/s) mode. EME derivatives were identified by characteristic ions and retention times, compared with those of synthetic standards (see previous section), and verified by co-injection when necessary.

Monosaccharide and Fatty Acid Analysis

Lipid samples (50-100 μg) were methanolysed in 1.0 ml of 0.5 N HCl in anhydrous MeOH for 24 h at 80 °C. The methanolysate was cooled and extracted three times with hexane. The combined hexane extracts were evaporated under N₂ stream at 37 °C to 10 μl and then diluted with hexane for analysis by GC-MS in the Conditions described in the previous section. The acidic MeOH lower layer was neutralized by addition of Ag₂CO₃ (≈10 mg) and treated with acetic anhydride (100 μl) for 6 h at room temperature. After centrifugation and removal of the MeOH, the precipitate was washed twice with 1-ml portions of MeOH. The combined MeOH extracts were dried under an N₂ stream. The resulting monosaccharide methyl glycosides were analyzed as their per-O-trimethylsilyl ethers (13, 14) by GC-MS using the Extrel ELQ 400 system described above (DB-5).
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column; splitless injection; temperature program 140-270 °C at 4 °C/min; CI-MS (isobutane mode).

Chemical Derivatizations of Intact Lipids

Lipid samples (≈50 μg) were permethylated by the method of Ciukanu and Kerek (15) as modified by Larson et al. (16), except that equal volumes of MeI and dimethyl sulfoxide were used (100 μl each). The reaction time was 30 min, and MeI was removed by flushing with N₂ for 25 min at 37 °C before partitioning between CHCl₃ and H₂O. After washing three times with H₂O, the CHCl₃ was evaporated to dryness under N₂.

Lipid samples were per-N,O-acetylated with 2:1 pyridine/acetic anhydride (0.5 ml, 20 h, room temperature). The reagents were subsequently de-0-acetylated by the Zemplén procedure (brief treatment with NaOMe in anhydrous MeOH).

Methylation/Linkage Analysis

Linkage positions of substituents on glycosyl residues were determined by permethylation of ≈50 μg of each sample (see previous section). Following hydrolysis, reduction, peracetylation and FAB-MS as described in detail elsewhere (18), except that the analysis was performed on the Extrel EQL 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 partially methylated alditol acetate (PMAA) derivatives made by retention time and characteristic electron-impact mass spectra (19, 20). 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, operated in the accumulation mode at full accumulation voltage (10 kv); xenon beam 6 kV; resolution 3,000. Alligots of sample (≈20 μg) were transferred to a FAB target and suspended in an appropriate matrix. For native lipid samples analyzed by "FAB-MS the matrix was triethanolamine/15-crown-5 (21, 22); the mass range was m/z 100-2,000. Three scans were accumulated for each spectrum. Sodium iodide in glycerol was used as the calibration standard.

Quantitative Analysis of Plasmalopsychosine in Human Brain

The 0.5 M triethylamine fraction from CM-Sephadex columns of six different normal adult human brain preparations were quantitatively analyzed for compounds A, B, and C using LKB densitometric analysis with a laser beam scanner. To obtain an accurate standard curve for densitometry, it was necessary first to isolate weighable amounts of plasmalopsychosine in highly purified form. This was accomplished by preparative TLC followed by HPLC, yielding 1.2 mg of pure plasmalopsychosine (compound B; see Fig. 1A, lane 3) by weight. This sample was used to obtain a standard curve of dilutions which gave absorbance values between 0.10 and 2.5 and which was linear over this range (0.5-5.0 μg).

To assay for absolute amounts of compounds A, B, and C in the 0.5 M triethylamine fractions, a known amount was applied on HPTLC and developed in chloroform/methanol/0.5 N HCl (80:20:2), and the separate bands were subjected to densitometric analysis. Values were converted to μg amounts using the standard curve.

Effects of Unknown Compounds A, B, and C on In Vitro PKC Activity

PKC from mouse brain was prepared by the method of Kikkawa et al. (23). Inhibitory effects of compounds A, B, and C were determined as described previously (6). Briefly, in conical tubes (1.5 ml; Sarstedt) phosphatidylerine (5 μg/tube) and 1,2-diolein (0.05 μg/tube), with or without appropriate quantities of compound A, B, or C, were dissolved in chloroform/methanol, and the lipids were evaporated under an N₂ stream. The lipid mixture was sonicated in 20 mM Tris-HCl buffer (pH 7.5) for 30 min. Liposomes in the tube were supplemented with the reaction mixture, consisting of 25 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 400 μM EDTA, 50 μM EGTA, 500 μM CaCl₂, 200 μM γ-[32P]ATP (2 x 10⁶ cpd); final volume 90 μl. The reaction was initiated by the addition of 10 μl of PKC fraction (1-2 μg of protein), and the reaction mixture was incubated for 10 min at 30 °C. The reaction was terminated by the addition of 1 ml of 25% trichloroacetic acid with 200 μl of 1% bovine serum albumin in 1 mM ATP solution (pH 7.5). The precipitate was centrifuged, washed twice with 1 ml of 25% trichloroacetic acid, dissolved in 1 ml of 1 M NaOH containing 0.1% deoxycholate with slight heating (80 °C for 10 min), and counted in a scintillation counter using ScintiVerse BD solution as a scintillator. The value without phosphatidylerine, 1,2-diolein, or Ca²⁺ was used as a reference blank.

Results

Isolation of Cationic Lipids and Glycolipids from Human Brain and Their Compositional Patterns

The cationic lipid fraction prepared from Folch's lower partition by retention on CM-Sephadex column and elution with 0.5 M triethylamine in chloroform/methanol 2:1 is shown in Fig. 1. Panel A shows the pattern with orcinol-sulfuric acid, with unknown compounds A, B, and C stained purple (lanes 2, 3, and 4, respectively), indicating the presence of carbohydrate. Other bands (including all slow-migrating bands in lanes 1 and 5 for sphingosine) were different in coloration with orcinol-sulfuric acid reaction. All bands were negative with resorcinol-HCl reagent specific for gangliosides. Panel B shows the results of UV fluorescent detection after plates were sprayed with fluorescamine (0.1%). The bands corresponding to unknown compounds A, B, and C were purified further by HPLC and separated by preparative HPTLC.

Fig. 1. HPTLC pattern of cationic lipids adsorbed on CM-Sephadex and eluted with triethylamine in chloroform/methanol mixture. Panel A, TLC was developed in chloroform/methanol/28% NH₄OH (80:20:2). Bands were detected by orcinol-sulfuric acid. Lane 1, total eluate from CM-Sephadex column with 0.5 triethylamine; lane 2, purified unknown compound A; lane 3, purified unknown compound B; lane 4, purified unknown compound C; lane 5, sphingosine. Panel B, the same chromatogram as in panel A. Bands were detected by spraying with triethylamine-dichloroethane (1:9) followed by 0.1% fluorescamine in acetone and were viewed under UV light.
compound B before extensive purification on HPTLC. Characterization and quantitative determination of sphingosine and DMS will be described elsewhere. For either lower or upper phase prepared from gray and DMS, the characterization and quantitative determination of sphingosine (galactosyl-sphingosine) was essentially undetectable.

**Comparison of Gray Versus White Matter and Cerebellum Versus Brainstem**

Equal weights (100 g) of white and gray matter from cerebrum were collected from the same human brain and processed side by side to obtain lower phases. Equal weights of cerebellum and brainstem were also obtained. These samples were extracted, partitioned, and lower phases were passed over CM-Sephadex as described above and eluted with 0.5 M triethylamine. Fig. 3 shows the orcinol staining of various fractions from gray and white matter, cerebellum, and brainstem. Lane 6 clearly shows that the major source of unknown compounds A and B is the white matter of the cerebrum. Unknown compounds A, B, and C were present in human brain white matter but undetectable in gray matter (Fig. 3, lane 9). The composition of unknown compounds A, B, and C in six different adult brains was measured quantitatively as described under “Materials and Methods.” The quantity of each component and total quantity/g, total wet weight, of tissue are summarized in Table I. Unknown compounds A, B, and C were absent in cerebral gray matter, present in trace amounts in the cerebellum, and 10–15% (relative to cerebral white matter) in the brainstem.

**Structural Characterization of Unknown Compounds A and B as Plasmalopsychosine**

**Methanolsysis: Monosaccharide Analysis**—Because the unknown lipids could be stained purple with orcinol-sulfuric acid, indicating the presence of hexose, pentose, or methylpentose, they were subjected to monosaccharide analysis by GC-MS of trimethylsilyl methyl glycosides produced after acidic methanolsysis. In each case, peaks were clearly observed for the usual trimethylsilyl derivatives of galactose (data not shown). No other saccharide peaks were observed, except for a trace (<1%) of glucose detected in the methanolsylate of the uppermost band.

**Methanolsysis: Analysis of Long Chain Aliphatic Aldehydes**—Fatty acid methyl esters were not detected in the hexane wash (upper phase) of the methanolsylates. A number of unknown peaks were observed, whose significance was not at first appreciated. After evaluation of the results of FAB-MS analysis of the intact lipids (see next section), the identity of these peaks was carefully determined and several major components found to correspond to long chain EMEs. Two components were found to be identical in retention times and mass spectra to EMEs prepared by acidic methanolsylation of authentic 16:0 and 18:0 long chain aldehydes. Two other components, having molecular masses 2 atomic mass units less than those synthesized from the 18:0 aldehydes, and having slightly faster retention times, were assumed to correspond to isomeric unsaturated 18:1 species. These four components are identified in the GC-MS reproduced in Fig. 4.

**FAB-MS Analysis of Native Lipids**—FAB mass spectra of the unknown native lipids were obtained in both positive and negative ion modes. The positive ion spectra of A and B are reproduced in Fig. 5, A and B. Observed in both spectra were prominent ions at m/z 684, 710, and 712 (nominal, monoisotopic masses). That these corresponded to pseudomolecular ions [MH]+ was confirmed by obtaining spectra after the addition of sodium acetate to the matrix. Sodiated molecular ions were then observed at m/z 706, 732, and 734 (see Fig. 5C). Further confirmation was provided by negative ion spectra, in which mode pseudomolecular ions [M-H]- could be observed at m/z 682, 708, and 710 (see Fig. 5D). Because these ions correspond to the odd molecular masses 683, 709, and 711 Da, it could be concluded that each species contains an odd number of nitrogen atoms. Interestingly, the negative ion spectra were characterized by the presence of extra peaks apparently associated with the pseudomolecular ions. Each pseudomolecular ion is accompanied by an ion at m/z [M+H]+, along with a less abundant one at m/z [M-H+26]. Such adduct ions were previously observed in the negative ion spectra of semisynthetic lyso- and de-N-acetyl gangliosides, only when triethanolamine was used as the matrix (24, 25). They have been observed only with compounds containing a free amino group and are believed to result from an addition reaction with some component in the matrix, either present as an impurity or formed by decomposition of triethanolamine under the conditions of FAB (25). In this case, the conclusion that the lipids bear a primary amino function is consistent with their detection by fluorescamine on HPTLC plates.

Of some further interest was the observation, in the positive ion spectra, of peaks consistent with dimeric ions. These were found between m/z 1,300 and 1,500 at masses corresponding to EMEs prepared by acidic methanolsylation of authentic 16:0 and 18:0 long chain aldehydes. Two other components, having molecular masses 2 atomic mass units less than those synthesized from the 18:0 aldehydes, and having slightly faster retention times, were assumed to correspond to isomeric unsaturated 18:1 species. These four components are identified in the GC-MS reproduced in Fig. 4.

**Table I**

| Brain no., donor | Wet weight | Compound | Ratio | Total quantity | Plasmalopsychosine/total weight |
|------------------|------------|----------|-------|----------------|-------------------------------|
| 1. 57 yo d      | 550        | A 0.60   | B 1.00 | C 0.10         | 33/60/7                      | 2.1                          | 3.82                          |
| 2. 72 yo d      | 900        | A 0.95   | B 4.00 | C 0.40         | 18/75/7                      | 3.75                         | 4.17                          |
| 3. 66 yo g      | 750        | A 0.78   | B 1.25 | C 0.25         | 34/56/10                     | 2.3                          | 3.07                          |
| 4. 78 yo g      | 400        | A 0.72   | B 1.20 | C 0.25         | 32/53/11                     | 1.72                         | 4.30                          |
| 5. 52 yo g      | 900        | A 0.72   | B 2.20 | C 0.25         | 23/68/8                      | 3.15                         | 3.50                          |
| 6. 76 yo d      | 1,147      | A 0.70   | B 3.10 | C 0.23         | 17/77/6                      | 4.03                         | 3.51                          |
to the possible combinations of the monomeric species, i.e. at m/z [M1+M2+H]+ and [M1+M2+H+2Na]+ (see Fig. 5, A-C). In the negative ion spectra, they were accompanied, again, by adduct ions 26 and 42 units to higher mass (Fig. 5D). To our knowledge, such noncovalent self-associations of glycolipids in FAB spectra have not been noted previously, although Ballou and Dell (26) studied the interaction between long chain alkyl trimethylammonium ions and a natural 3-methylmannosene polymer from Mycobacterium smegmatitis by +FAB-MS. In the positive ion spectra, a second set of ions, observed at mlz 210, 238, and 264, associated with less abundant ions at mlz 282, 300, and 328. The ions at mlz 300, 282, and 264 were observed previously by Hara and Taketomi (27) to be characteristic fragments of unsaturated d18:1 sphingosine in positive mode FAB spectra of psychosines (representing, for galactospsychosine, for example, [M+H-Gal]+, [M+H-Gal-H2O]+, and [M+H-Gal-2H2O]+, respectively). Confirmation of the unknown lipids as derivatives of psychosine and of the possible isomeric relationship between them was provided by degradation experiments monitored by FAB-MS.

**FAB-MS of the Products of Mild Acid Hydrolysis**—Brief treatment of compound A with 0.1 N HCl/HgCl2 yielded a product whose Rf was identical to that of compound B on HPTLC. The *FAB mass spectrum of this product (Fig. 6A) was virtually identical to those of the native untreated compounds A and B, demonstrating an acid-catalyzed transformation of compound A to compound B. On more extended treatment of compound A, or treatment of compound B, a new product was observed, having an Rf identical to that of authentic galactospsychosine. The *FAB mass spectra of these products were virtually identical to those obtained for galac-

topspsychosine (Fig. 6, B-D).

If one accepts the hypothesis that the lipids are novel covalent modifications of psychosine, the following further conclusions can be reached. Given the great relative abundance of the ion at m/z 282 (in Fig. 6, A and B) compared with that at m/z 310 (which may represent a homolog containing d20:1 sphingosine), it is apparent that the differences in mass of the pseudomolecular ions must be largely a result of differences in the mass of the modifying group(s) rather than the occurrence of different sphingosine chain lengths. The modifying groups would have to be such as to add incremental masses of 222, 248, and 250 units to that of the psychosine. Identical differences in mass were also observed in a series of low abundance fragments (m/z 444, 470, 472) found in the spectra of the native lipids (Fig. 5, A and B). These could be analogs of the fragment found at m/z 222 in the FAB mass spectrum of psychosines (27) (see Fig. 6D), which does not appear in the spectra of the native modified lipids. Interestingly, a pair of fragments found at m/z 250 and 252 in the spectrum of psychosines (27) (see Fig. 6D) was also found in the spectra of the native modified lipids (Fig. 5, A and B), whereas there was no set of ions observed with masses incrementally increased as found for the m/z 222 fragment. Although not discussed by Hara and Taketomi (27), the m/z 222 fragment probably originates from the nonreducing end of psychosine and contains the galactose moiety along with a portion of the sphingosine chain including C-1, C-2, and the amino group. This suggested that, in the native lipids A and B, the modifying groups are attached to galactose. On the other hand, the fragment at m/z 250 most likely originates from the sphingosine moiety after elimination of galactose.

Coincidentally, the differences in mass of the pseudomolecular ions correspond to the differences in chain length of the EMEs found by GC-MS of the hexane-soluble methanolysis products, suggesting that these might be chemical transform-

ants of the modifying groups in question. Previously, the structure of a plasmalogen-like form of glycosphingolipid was proposed by Kochetkov et al. (28), in which the 3-OH group of psychosine was modified by attachment of a long chain enol ether. However, in the total absence of any fragments corresponding to loss of the hexose moiety alone, as commonly observed in FAB mass spectra of glycosphingolipids and lysoglycosphingolipids (such as psychosine), it seemed more likely that the modifying group(s) must be attached to the galactose residue rather than to the sphingosine moiety. The idea that these modifications might take the form of enol ethers was also shown to be erroneous by further derivatization experiments followed by FAB-MS.

**FAB-MS of Sequentially Per-N,O-acetylated and De-O-acetylated Lipids**—Peracetylation of the native lipids with acetic anhydride/pyridine resulted in incorporation of four

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**Fig. 3.** HPTLC pattern of cationic lipid from various regions of human brain. Cationic lipids were isolated by chromatography on CM-Sephadex and eluated with 0.5 M triethylamine; TLC was developed in chloroform/methanol/NH4OH (80:20:2). Lanes 1 and 10, standard ceramide monohexosides; lanes 2-5, lower phase without concentration by CM-Sephadex column; lane 2, white matter; lane 3, cerebellum; lane 4, brainstem; lane 5, gray matter; lanes 6-9, 0.5 triethylamine eluate from CM-Sephadex columns of various brain components; lane 6, white matter; lane 7, cerebellum; lane 8, brainstem; lane 9, gray matter. Note that no bands for compounds A or B can be clearly seen without concentration through CM-Sephadex column chromatography.

**Fig. 4.** GC-Cl/MS of long chain methyl enol ethers. A, from methanolysis of compound B; B, from methanolysis of standard n-16:0 and 18:0 aldehydes. Peaks were identified as 1, 16:0; 2a and 2b, isomeric 18:1; and 3, 18:0 methyl enol ethers, having pseudomolecular ion masses of 255, 281, and 283 units, respectively. Peaks marked by an asterisk are impurities common to both samples, probably arising from the derivatization reagents.
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Fig. 5. FAB-MS of native lipids. A, "FAB mass spectrum of compound A in NBA matrix; B, "FAB mass spectrum of compound B in NBA matrix; C, "FAB mass spectrum of compound B in NBA/sodium acetate matrix; D, "FAB mass spectrum of compound B in triethanolamine/15-crown-5 matrix.

acetate groups, as illustrated in Fig. 7, A–C. For compound A (Fig. 7A), pseudomolecular ions [M+Na]+ at m/z 874, 900, and 902 corresponded to the addition of 4 x 42 units to each of the native species. In addition, ions at m/z 792, 818, and 820 were observed, representing [MH-60]+, a facile neutral loss of HOAc. Confirmation of the higher mass group as being the true pseudomolecular ions was confirmed by the addition of sodium acetate to the matrix, as illustrated for the peracetylated compound B (Fig. 7C). A concomitant suppression of the [MH-60]+ ions was observed under this condition. At the lower end of the spectra, the triply unsaturated ion m/z 264 was now the predominant sphingosine fragment. Also observed was an ion at m/z 366, probably representing a single dehydration of the N-Ac, O-Ac sphingosine fragment (m/z 384). This sphingosine fragment can eliminate one and two molecules of HOAc, to yield the ions at m/z 324 and 264, respectively. Elimination of HOAc from the fragment at m/z 366 yields the ion at m/z 306. The origin of the group of odd-mass ions, m/z 469, 495, and 497, is unclear at this time.

As illustrated for compound B (Fig. 7D), de-O-acetylation with MeONa/MeOH resulted in the loss of three O-Ac groups and retention of one N-Ac, confirming again the presence of a reactive amine in the native lipid. Sodiated molecular ions were now observed at m/z 748, 774, and 776. Sphingosine ions were again observed at m/z 324, 306, and 264, representing the singly dehydrated, mono-N-acetylated fragment, the doubly dehydrated, mono-N-acetylated fragment, and the elimination of HOAc from the singly dehydrated fragment, respectively. The dehydrated N-Ac, O-Ac ion at m/z 366 was no longer observed. Similar results were obtained for compound A (not shown).

These results clearly indicated that (a) the 3-OH group of sphingosine is free in the native lipids and (b) the modifying group(s) occupy two hydroxyl positions on the galactose moiety. This could not be accommodated by the attachment of two enol ethers in tandem because the mass increases relative to free psychosine 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. Acetylation of d18:1 sphingosine with 16:0, 18:1, and 18:0 fatty aldehydes would yield the observed molecular weights for the new lipids. This conclusion was confirmed by methylation/linkage analysis, as described below.
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Methylation Analysis by GC-MS—After permethylation, acid hydrolysis, reduction, and acetylation of the native lipids, the resulting partially methylated hexitol acetates were analyzed by GC-MS (Fig. 8, A and B). From compound A, 2,6-di-O-Me-Gal was obtained, while 2,3-di-O-Me-Gal was obtained from compound B. These represent 3,4- and 4,6-linked galactose moieties, respectively, and clearly show that the lipids must be isomeric cyclic acetals derived from psychosine, that in compound A forming a five-membered ring, and that in compound B forming a six-membered ring. The structures are illustrated in the Summary. The product from limited acid treatment of compound A also yielded 2,3-di-O-Me-Gal (Fig. 8C), demonstrating the facile isomerization of the five-membered ring into the more stable six-membered ring. The only uncertainties remaining in the primary structures are the chiralities at the acetal C-1 positions. Until these can be determined unambiguously, we assume an equatorial orientation for the long chain in the six-membered acetal ring, and a pseudo-equatorial orientation for this group in the five-membered ring.

Comparative Effect of Plasmalopsychosine and Psychosine on PKC Activity

Psychosine has been reported previously to inhibit PKC (2, 8). We therefore compared the inhibitory effects of plasmalopsychosine versus psychosine on PKC activity as described.
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FIG. 8. GC-MS analysis of PMAAs from permethylation, hydrolysis, reduction, and acetylation of lipids. A, PMAA from compound A; B, PMAA from compound B; C, PMAA from compound A after brief acid treatment; D, standard galactose PMAAs. Peaks are identified as PMAAs of 1, 2,3,6-tri-O-; 2, 3,4,6-di-2,4,6-tri-O-; 3, 2,3,4-tri-O-; 4, 2,6-di-O-; 5, 4,6-di-O-; 6, 3,6-di-O-; 7, 2,3-di-O-; 8, 6-mono-O-; 9, 3,4-di-O-; 10, 2-mono-O-; and 11, 3 (or 4)-mono-O-Me-Gal.

FIG. 9. Effects of cerebroside, psychosine, and plasmalopsychosine on in vitro brain PKC activity. PKC activity was measured as described under “Materials and Methods” using PKC samples prepared from mouse brain and histone III-S as substrates. Values represent mean ± S.D. from three independent experiments. Abscissa, concentration in µM. •, cerebroside (galactosylceramide). ○, psychosine. ■, plasmalopsychosine (compound B). ▲, plasmalopsychosine (compound A).

Lipid components of cells can be classified as acidic, neutral, or zwitterionic. Acidic lipids include gangliosides, sulfatide, phosphoinositide, and phosphatidic acid. Neutral lipids include neutral glycolipids and neutral glycerides. The majority of phospholipids are zwitterionic. Cationic (basic) lipids such as sphingosine, DMS, and lysoglycosphingolipids are assumed to be present as minor components modulating cellular functions, as described in the Introduction. In this study, white and gray matter of human brain extracts were separated by Folch’s partition and the lower phase subjected to system-
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...adic chemical analysis. The following results were of particular interest. (a) The presence of sphingosine and DMS in both gray and white matter was confirmed. These compounds may not be recovered quantitatively; their chemical characterization and quantitative determination will be described elsewhere. (b) Although galactosylceramide and sulfatide were the major lower phase glycolipids separated according to Folch's partition, psychosine (galactosylsphingosine) and lyso-sulfatide were undetectable. (c) Two major cationic glycolipids detected, initially termed unknown compounds A and B, were identified as plasmal (fatty aldehyde) conjugated with psychosine through 3,4-cyclic acetal and 4,6-cyclic acetal linkage, respectively, at the galactosyl residue of psychosine (see Summary for structures). A relatively minor compound C has been tentatively identified as a 4,6-cyclic plasmal conjugate of psychosine but with t18:0 phytosphingosine in place of d18:1 sphingosine. These three components are hereby collectively termed “plasmalopsynsine.” (d) Plasmalopsynsine, regardless of position of acetal linkage, showed no cytotoxic or cyotyotic effect and weak inhibitory effect on PKC. In contrast, psychosine showed strong cytotoxicity and strong inhibition of PKC.

Two views of a molecular model of 4,6-0-linked plasmalopsynsine are shown in Fig. 10. The model was constructed for illustrative purposes only and is not intended to imply that a single minimum-energy three-dimensional structure can be calculated which would reflect the actual conformation of the molecule in a membrane. On the contrary, preliminary calculations suggest enough linkage flexibility to accommodate a variety of spatial arrangements. The illustration shows a unique structure having two long aliphatic chains oriented in nearly opposite directions. In striking contrast, essentially all known sphingolipids and glycosphingolipids have two long aliphatic chains constrained in parallel; this structure is suited for formation of a lipid bilayer in cell membranes. The unique structure of plasmalopsynsine may allow one aliphatic chain to insert into one lipid bilayer and the second chain to insert into a different lipid bilayer. Alternatively, it may help anchor proteins through hydrophobic interaction.

Kochetkov et al. (28) described siphingolipidogens as a minor component of chromatographically fast migrating cerebroside in brain. The compound was assumed to have a structure with a fatty aldehyde linked via an unsaturated ether bond to the sphingosine C3 hydroxyl group of galactosylceramide, based on infrared spectroscopy (absence of absorption at 1750 cm⁻¹ for ester linkage); fatty aldehydes were identified as p-nitrophenylhydrazide using Wittenberg's method (29). However, extensive studies of multiple fast migrating cerebrosides by Xlenk and Lohr (30), Tanai and co-workers (31, 32), and Kishimoto et al. (33) concluded that all of these fast migrating glycosphingolipids are cerebrosides esterified at various positions of hydroxyl groups with fatty acid. The existence of sphingolipidogens was disproved by a series of subsequent studies (33-35). These previously reported compounds, whether siphingolipidogen or fast migrating ester cerebrosides, showed very different TLC mobility compared with the plasmalopsynsine described in the present study; that is, plasmalopsynsine has much slower migration mobility and has only two aliphatic chains (one sphingosome, one plasmal); also, the orientation of the aliphatic chains linked to galactopyranosyl moiety may be in an entirely opposite direction.

The fatty aldehyde (or long chain aliphatic aldehyde), termed plasmal, was originally discovered by Feulgen and Voit in 1924 (36) and was recognized as a component of a glycerophospholipid termed plasmalogen in 1929 (11). However, the structure of plasmalogen, originally claimed to be a 1,2-cyclic acetal of glycerol, was eventually identified as 1-alkenyl-2-acetyl-3-phosphorylcholine (37-38).

The structure of plasmalopsynsine is now established unambiguously as being a 4,6- or 3,4-cyclic long chain acetal linked to the galactopyranoside moiety of psychosine, by FAB-MS of native, acetylated and deacylated compounds, GC-MS of long chain aldehyde derivatives released by methanolation, and GC-MS of partially methylated galactitol acetates derived from these lipids. It is interesting to note that the cyclic acetal linkage of plasmal, as originally imagined by Feulgen to be present in his plasmalogen, was not found in subsequent studies, but it does indeed exist in the form of these plasmalopsynsines.

In the present study, psychosine was not detectable in normal human brain extract, whereas plasmalopsynsine (compounds A and B) constituted the major cationic glycosphingolipid in these extracts. Interestingly, psychosine is detectable in brains of patients with sphingolipidoses (e.g., Krabbe's disease), as initially reported by Miyatake and Suzuki (40) and confirmed much later by Neuenhofer et al. (41). Because psychosine has a striking cytolytic effect (detergent effect) and inhibits brain PKC activity (2, 8), it is assumed that accumulation of psychosine causes the pathological syndrome associated with sphingolipidoses (2). The presence of plasmalopsynsine in place of psychosine in normal brain, and its weak but clear inhibitory effect on brain PKC, suggests that the cyclic plasmal linkage may serve as the precursor of PKC inhibitor in normal brain. It is possible that plasmalopsynsine is uniquely incorporated into cells and is converted to psychosine and thereby regulates activity of PKC and other protein kinases essential for cellular function. Therefore, enzymatic processes for synthesis or degradation of cyclic plasmal to psychosine could be crucial in regulation or neuronal functions.

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