A novel plasmal conjugate of glycosphingolipid having cationic lipid properties was isolated from the white matter of bovine brain. Linkage analysis of galactosyl residue by methylation, liquid secondary ion, and electrospray ionization mass spectrometry of intact and methyalted derivatives, and by $^1$H- and $^{13}$C-NMR spectroscopy, identified the structure unambiguously as an O-acetal conjugate of plasmal to the primary hydroxyl group of glycerol and to the 6-hydroxyl group of galactosyl residue of β-galactosyl 1→1 sphingosine (psychosine). This novel compound is hereby termed “glyceroplasmalopsychosine”; its structure is shown below.

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Long chain aldehydes, hexadecanal and octadecanal, with or without an olefinic double bond, are collectively called plasmal, and are known as a common component of two classes of lipid conjugates, described as follows: (i) the classically well-established phospholipid plasmalogen (1, 2), in which plasmal is conjugated as O-alk-1-enyl (vinyl ether) group to one of the hydroxyl groups of glycerol in phosphatidylethanolamine or phosphatidylcholine1; (ii) the recently found novel type of glycolipid, containing unknown compound; Sph, sphingosine; DQF-COSY, double-quantum-filtered correlated spectroscopy; 2D-HOHAHA, two-dimensional homonuclear Hartmann-Hahn spectroscopy; ROESY, rotating overhauser effect spectroscopy; GM3, NeuAcα2→3Galβ1→4Glcβ1→1Cer (other gangliosides are abbreviated according to Svennerholm’s list (44)).
Gap, PA). Gb4Cer was prepared from human erythrocytes. Other reagents were from Sigma Chemical Co. (St. Louis, MO). Iatrobeads were from Iatron Laboratories Inc. (Kanda, Tokyo, Japan).

Separation of Cationic Lipid Fraction on CM-Sephadex, and Purification of Px by Silica Gel Chromatography—Four steps of chromatography were performed: (i) adsorption and stepwise elution from CM-Sephadex; (ii) gradient elution from CM-Sephadex; (iii) chromatography on Iatrobeads 6RS-8060; (iv) purification on Iatrobeads 6RS-8010. For step (i), Folch’s lower phase of bovine brain white matter extract was applied on a CM-Sephadex column as described previously (3). Cationic lipids were eluted with methanol/water (91:1, v/v) containing 0.5 M triethylamine (pH adjusted to 9.25 by gently bubbling CO₂ gas). The eluted fractions were rotary-evaporated to dryness, and the residual triethylamine was co-evaporated several times with absolute ethanol. The composition of this crude fraction was checked by TLC, and by TLC with ion-blotting with LSIMS (see below). Cationic lipids were found to include unknown component PX in addition to previously known PLPS A, PLPS B, and psychosine. Further purification by step (ii) was performed using a second CM-Sephadex column with a linear gradient in methanol/water (1:1) from 0-1 M triethylamine. The eluate (6 ml per tube) was collected by fraction collector over 80 tubes. The elution was monitored by TLC with the solvent system C/M/acetone/acetic acid/water (8:2:4:2:1), and fractions containing PX were combined. Step (iii): The combined fraction containing PX was further purified by liquid chromatography on a silica bead (Iatrobeads 6RS-8010 column (4.6 × 300 mm) with a linear gradient of C/M/2.5 M NH₄OH (90:10:5 to 75:25:2.5:5). Methods for preliminary characterization of cationic lipids by TLC combined with functional analysis, and final identification of PX by various modes of mass spectrometry, and by ¹H and ¹³C NMR, are described below.

Preliminary Identification of Lipids by TLC with Iron Blotting with LSIMS—This was performed by negative-ion LSIMS on a Concept III mass spectrometer (Shimadzu/Kratos, Kyoto, Japan) (8). Cationic lipid extract developed by one- or two-dimensional TLC was transferred to a polyvinylidenefluoride membrane (Clear Blot Membrane-P, ATTO Co., Tokyo, Japan) by “iron-blotting” (9), and the band on the membrane was excised and placed on a mass spectrometer probe tip with triethanolamine (positive-ion mode) or triethylamine (negative-ion mode) as the matrix, as described previously (8).

TLC of GSLs and Detection of Functional Groups—GSLs in crude extract or in eluate fraction from CM-Sephadex or Iatrobeads chromatography were separated on Silica Gel 60 high-performance TLC plates (Merck, Darmstadt, Germany) in C/M/15 M NH₄OH (80:20:2) (solvent A), C/M/3.5 M NH₄OH (60:40:9) (solvent B), C/M/0.2% CaCl₂ (60:40:9) (solvent C), or C/M/acetone/acetic acid/water (8:2:4:2:1) (solvent D), and visualized by orcinol-sulfuric acid method for hexoses, or fluorescamine reaction for amino group (10).

Determination of Lipids Containing Aldehyde Using Schiff’s Reagent—For detection of plasmal, GSLs separated on TLC in solvent D were sprayed with 2.5 M HCl in 50% methanol to release the aldehyde group and heated at 80 °C for 10 min. After cooling, the plate was momentarily soaked in Schiff’s reagent (reduced colorless Fuchsin in sodium bisulfide) (Sigma) then dried to stain the reducing aldehydes with red. The condition of acid pretreatment required for release of plasmal varied with type of linkage of plasmal conjugate. 2.5 M HCl in 50% methanol and 80 °C for 10 min was sufficient for PLPS. Much weaker acid condition (0.5 M HCl, room temperature, 10 min) was required for PX reactivity, and plasmalogen did not require preliminary acid treatment to get positive reaction, acidity of Schiff’s reagent itself appeared to be sufficient.

Acid Hydrolysis of Acetal Linkage in Cationic GSLs—For acid hydrolysis, 0.5–5 nmol of PX or PLPS was dissolved in 200 µl of 2.5 M HCl in 50% methanol and heated at 80 °C for 30 min. A 200-µl aliquot of acid hydrolysate was neutralized by 100 µl of 5 M NaOH in methanol/water (1:1) and vigorously mixed with 450 µl of C/M (8:1) (11). After centrifugation at 3000 rpm for 5 min, GSLs were recovered from the lower phase, which was analyzed by TLC using the solvent system C/M/10% (CH₃O)₃B (60:35:8) (12). The bands were visualized by permethylation of cosyl residues were determined by permethylation of 50-µg portions of glycolipids followed by hydrolysis, reduction, peracetylation, and GC-MS as described in detail elsewhere (13), except that the analysis was performed on the Profile (Kratos) GC-MS system described above (DB-5 column, J&W Scientific, Rancho Cordova, CA); splitless injection; temperature program 140–250 °C at 4 °C/min, EI/MS mode), with identification of partially methylated alditol acetate made by retention time and characteristic electron impact mass spectra (14, 15).

Positive- and Negative-ion LSIMS of Native and Permethylated PX—LSIMS was performed on a Concept III mass spectrometer fitted with a cesium ion gun. About 0.5 nmol of GSL in 1 µl of C/M (1:2) was mixed with 1 µl of glycerol (positive-ion mode) or triethanolamine (negative-ion mode) as the matrix. Spectra were recorded at an accelerating voltage of 8 kV and at a resolution of 1000–2000 (8, 16). 20-nmol portions of cationic GSLs were derivatized by permethylation, and an aliquot (0.5 nmol) was examined by positive-ion LSIMS. Linked scan spectra were obtained at constant B/E ratio after collision-induced dissociation (CID) with helium introduced into a collision cell (8). The scan speed for both normal and linked scan was 5 s/decade.

Positive- and Negative-ion ESIMS of PX—Measurements were performed using an LCQ DECA ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) equipped with an ESI probe. Sample solution (~5 nmol/ml in methanol) was directly infused into the ion source at a flow rate of 3 µl/min. The source parameters were set to the following values: spray voltage, 5 kV; sheath gas flow rate, 50 in arbitrary units; capillary temperature, 220 °C; capillary voltage, 5 kV. Low energy CID was carried out on the molecule-related ions (MS²) and their product ions sequentially (MS³) using helium gas present in the ion trap. The relative collision energies used ranged from 35 to 45%.

The ESIMS spectra were also run on a TSQ triple-stage quadrupole mass spectrometer (ThermoFinnigan) equipped with an API2 ESI source. The samples were introduced by direct infusion as described above at a flow rate of 5 µl/min. The heated capillary was set at 300 °C, and the spray voltage set at 4.5 kV. Low energy CID MS/MS studies were performed with 25-eV collision energy and 0.3 pascal of argon as the collision gas (8).

NMR Spectroscopy—¹H and ¹³C NMR spectra were obtained at 400 and 100 MHz, respectively, using a GX-400 spectrometer (JEOL, Tokyo) with a probe temperature of 60 °C. The purified GSL was deuterium-exchanged with D₂O, dried over P₂O₅ under vacuum, and dissolved in (CD₃)₂SO/D₂O (98:2). The final concentration was ~4 mm. Chemical shifts were referenced to tetramethylsilane. In DQF-COSY, data matrices of 4096 (2) × 512 (1) points, acquired with a spectral width of 2000 Hz, were zero-filled to 4096 × 1048 points (17, 18). ¹H-detected heteronuclear multiple bond connectivity (HMBC) spectrum (19, 20) was recorded with 256 increments in t₁ and 800 scans were collected for each t₁ experiment. The spectral widths were 2.5 kHz in t₂ (for ¹H) and 5.4 kHz in t₁ (for ¹³C). After zero filling, the time-domain spectrum was transformed to give 2048 × 512 data point matrices with a resolution of 2.4 and 11 Hz/point, respectively.

Conformational Models—The three-dimensional model of PX was built using standard parameters in the CS Chem 3D Pro program (CambridgeSoft). Some ¹H–¹H distances and dihedral angles, which were obtained from nuclear Overhauser effects and coupling constants,
TABLE I
Quantities of PLPS A, PLPS B, Px, and psychosine, in micrograms per gram of wet weight, from bovine and human brain white matter

|       | A       | B       | Total   | Px | Psychosine |
|-------|---------|---------|---------|----|------------|
|       | µg/g wet weight |         |         |    |            |
| Bovine| 0.085   | 0.135   | 0.220   | 0.489 | 0.355      |
| Human | 1.00    | 2.41    | 3.41    | NM   | ND         |

\(^\text{a}\) NM, not measured.  
\(^\text{b}\) ND, not detectable.

RESULTS

Pattern of Cationic GSLs in White Matter of Bovine Brain—
The cationic GSLs adsorbed on CM-Sephadex and eluted with triethylamine were examined on TLC with four different solvents as described under “Experimental Procedures.” Four cationic GSLs and one very minor component were found in bovine brain white matter. Two fast-migrating components showed TLC mobility similar to that of PLPS A and B, and one component had the same TLC mobility as psychosine. Major unknown component Px and a trace quantity of another unknown component (Ux: indicated by the arrow in Fig. 1) were detected. TLC mobility and separation patterns of the above five cationic GSL components, in comparison with separation patterns of reference GSLs (GlcCer, LacCer, Gb4Cer) in four solvent systems are shown in Fig. 1, A–D. Even at the first step of purification, the spots corresponding to PLPS A, PLPS B, and psychosine were identified by TLC with iron-blotting with LSIMS, and Px showed different deprotonated molecules compared with known cationic GSLs (see below).

The quantity of Px in bovine brain white matter is much higher than that of PLPS A or PLPS B. Although the possible presence of Px in human brain was not examined, there are clear differences in cationic GSL composition between human and bovine brain white matter: (i) human brain contains much higher levels of PLPS A and B than bovine brain; (ii) human brain does not contain psychosine (3), whereas bovine brain contains a significant quantity (Table I).

Isolation and Preliminary Characterization of Px—

**Preliminary analysis of the first CM-Sephadex eluate by iron-blotting, followed by negative-ion LSIMS (see “Experimental Procedures”), produced intense deprotonated molecules [M–H]– at m/z 774 and 800 for Px, and at m/z 682, 708, and 710 for PLPS A and PLPS B. The difference of [M–H]– ions between Px and PLPS B (PLPS B) was molecular mass 92 Da, i.e., Px has an additional residue with this mass. Further characterization by functional group analysis, linkage analysis by methylation, various modes of mass spectrometry, and 1H and 13C NMR, was performed for extensively purified Px, as described below.

Repeated cation-exchange chromatography on CM-Sephadex (see “Experimental Procedures”) allowed separation of cationic GSLs in the following order (triethylamine concentration given in parentheses): (i) PLPS A, PLPS B, and Px (0.20–0.23 M); (ii) psychosine (0.23–0.28 M); (iii) unknown, very minor band Ux (0.22–0.25 M). Fractions containing the above components were combined, and further purification was performed using Ionrobes 6RS-8060 and 6RS-8010 with linear gradients of C/M/water and C/M/NH4OH as described under “Experimental Procedures.” Thus, each component was purified to give a single band on TLC in four solvent systems.

Hydrolysis of Px and PLPS, and Methylation Analysis by

**GC-MS—** Px and PLPS B were subjected to acid hydrolysis in 2.5 M HCl in 50% methanol at 80 °C for 30 min. Psychosine was found as a common component, whereas glucosyl-Sph (lyso-GlcCer) was not found, in hydrolysates of Px and PLPS B. Under this condition, Px and PLPS B released an aldehyde group that was clearly detectable with Schiff’s reagent. Alternatively, Px or PLPS band separated on TLC gave red color in situ with Fuchsin reagent, after treatment with HCl.

After permethylation, acid hydrolysis, reduction, and acetylation of the native lipids, the resulting partially methylated hexitol acetates were analyzed by GC-MS. From compound Px, only 2,3,4-tri-O-Me-Gal, but no peak corresponding to 2,3-di-O-Me-Gal or 2,6-di-O-Me-Gal, was detected. These results indicate that Px is substituted only at the 6-hydroxyl position of galactose moiety, in striking contrast to PLPS where two hydroxyl groups are substituted.

Positive- and Negative-ion LSIMS—

By positive-ion LSIMS of Px, an intense protonated molecular [M+H]+ was detected at m/z 802 (Fig. 2A). This is consistent with a structure derived from GSL containing a glycerol, an aldehyde (18:1), a hexose (Hex), and a Sph (d18:1). Less-abundant [M+Na]+ ions, corresponding to other molecular species, were also detected at m/z 776 (16:0/d18:1) and 804 (18:0/d18:1). When NaCl was added to the matrix (0.1% NaCl in glycerol), the [M+Na]+ ions dominated the spectrum, and the [M+H]+ ions were observed at very low intensities. The less abundant fragments (Z anguish at m/z 684, 710, and 712 resulted from elimination of glycerol. Loss of the glycerol and aldehyde unit yields the fragment observed at m/z 462 (Y anguish), which is identical to the [M+H]+ ion obtained from psychosine with the same Sph composition. The most abundant fragment was observed at m/z 282 (Z anguish), which arises from elimination of glycerol, aldehyde, and hexose. By high energy CID on the [M+H]+ ion at m/z 802 (Fig. 2A, lower spectrum), elimination of glycerol was predominant, as demonstrated by the abundance of Z anguish product ion at m/z 710. The peaks at m/z 462 (Y anguish) and 282 (Z anguish) corresponded to psychosine and Sph, respectively. The CID spectra obtained from other molecular species also contained signals at m/z 462 (Y anguish) and 282 (Z anguish). In contrast, the Z anguish ion varied depending on each molecular species, confirming that this fragment contains an aldehyde residue.

The negative-ion LSIMS spectrum of Px (Fig. 2B, upper spectrum) exhibited intense deprotonated molecules [M–H]– at m/z 774 (16:0/d18:1), 800 (18:1/d18:1), and 802 (18:0/d18:1) as well as the Y anguish and Z anguish ions, whereas the Z anguish ion species were absent. By high energy CID of [M–H]– (Fig. 2B, lower spectrum), the Y anguish ion was abundantly produced, whereas the Z anguish ions were absent. The presence of d18:1 (m/z 399 for 18:1/d18:1), which arises from cleavage of the Gal ring with charge retention at the nonreducing terminus, confirmed that Px has a substituent at C-6 of Gal.

To further confirm the number of non-substituent hydroxyls and the presence of O-linked glycerol and single O-linked galactosyl residue (in contrast to the double O-linked galactosyl residue of PLPS), permethylated Px and PLPS B were analyzed by positive-ion LSIMS and ESI (Fig. 2D; see below). Permethylated PLPS B (18:0/d18:1) gave an intense [M]– ion at m/z 794, which corresponds to a structure containing 1 mol each of aldehyde (18:1), di-O-Me-hexose, and mono-O, tri-N-Me-Sph (d18:1). By comparison, [M]– at m/z 928 for permethylated Px was consistent with a structure containing a di-O-Me-glycerol, aldehyde (18:1), tri-O-Me-hexose, and mono-O, tri-N-Me-Sph (d18:1). The difference of [M]– and [M+H]– between native and permethylated Px (928–802 = 126) corresponded to nine methyl groups, confirming the presence of six non-substituent hydroxyls and one free amino group in Px. Furthermore, the

\[^3\] N. Iida-Tanaka, T. Hikita, S. Hakomori, and I. Ishizuka, manuscript in preparation.
fragment ion at $m/z$ 103 supported the presence of di-O-Me-glycerol.

Positive- and Negative-ion ESIMS—Essentially, little difference was observed among positive-ion ESIMS with ion trap (Fig. 2C) and quadrupole (data not shown) analyzers and LSIMS (Fig. 2A) of native Px, except that [M + H]$^+$ ions were always accompanied by their sodium adducts in the ESIMS spectra (Fig. 2C). The negative-ion ESIMS produced only
[M—H]⁻ ions without any major fragment ions (data not shown). The [M⁺] ions for permethylated Px were also clearly detected at m/z 902 (16:0), 928 (18:1), and 930 (18:0) by positive-ion ESIMS (Fig. 2).

The ESIMS/MS (MS²) spectra produced by low energy collision of [M⁺]⁻ and [M⁺]²⁺ of Px (data not shown) were essentially similar to the LSIMS-linked scan spectra with high energy CID, supporting the conclusion that Px has a glycerol-O-aldehyde-O-Sph with major molecular species (aldehyde/Sph) of 16:0/d18:1, 18:1/d18:1, and 18:0/d18:1.

By sequential product ion fragmentation experiment (MS³) with the Z⁻ ion as precursor, an intense product ion corresponding to [Z⁻²Hex] was produced. This unexpected fragmentation by MS³ may be attributed to a rearrangement of the Z⁻ ion and will be described elsewhere.

A minor unidentified component, having a slightly slower migration rate than Px on TLC, was purified and analyzed by positive-ion ESIMS. Although the Y₁ and Z₀ product ions were identical to those obtained from Px, the molecule related ions and Z₀ ions were 16 Da higher as compared with those from Px, suggesting the presence of hydroxylated aldehyde (16OH:0 and 18OH:1)-containing Px as minor molecular species (~5% of total Px).

1H and 13C NMR Spectroscopy—Fig. 3A shows a one-dimensional 1H NMR spectrum of Px. Signals were assigned based on the DQF-COSY and 2D-HOHAHA spectra. A doublet at 4.07 ppm, identified as an anomeric signal of β-Hex (Gal-H1, 3J₁,₂ 5H z), served to identify the hexose as β-galactose a priori, judging from the chemical shift of H₄ (Gal-H₄, 3.59 ppm) and the coupling constant 3J₃,₄ (4 Hz). The characteristic signals at 5.45 (L-H₄) and 5.58 ppm (L-H₅) showed that Px has 4-sphingenine in the lipid moiety. The H₂ multiplet of the long-chain base (L-H₂), however, resonated in a much higher field (~1 ppm higher) than that in GalCer (21), indicating that an amino group at the C2 position is not acylated (22, 23). A triplet at 4.48 ppm (P-H₁), coupled with a methylene signal at 1.49 ppm (P-H₂), was assigned to an acetal proton (-O-CH(-O)-CH₂-) (24). A triplet at 5.32 ppm, attributed to allylic protons, shows the presence of a substantial amount of cis-double bond in the fatty aldehyde. In addition to the signals of Gal, sphingenine, and aldehyde residues, signals ascribed to glycerol were observed in the spectra. All ¹H signals were identified by DQF-COSY, 2D-HOHAHA, and ROESY experiments (25, 26).

4 K. Tadano-Aritomi, T. Hikita, M. Kubota, S. Hakomori, and I. Ishiruka, manuscript in preparation.
In the DQF-COSY spectrum, we found two sets of the H6a and H6b signals of Gal and H1a and H1b of Gro (Fig. 3B), which were significantly distinct from each other. Furthermore, the P-H1 signal was slightly split (Fig. 3A). The fact that one of the H6b quartets at 3.67 ppm has approximately half the peak area of Gal-H1, P-H1, and the principal signals of sphingosine (L-H3, L-H4, and L-H5) (Fig. 3A) ruled out the possibility that Px has two galactosyl residues in a single molecule. It is most probable that Px is a mixture of two plasmal conjugates consisting of a galactosylpsycosine, a fatty aldehyde, and a glycerol residue. Careful study of relations of the residues by ROESY and HMBC experiments (Fig. 3C) indicated that Px consists of two stereoisomers within the structure Gro(1,3)-O-CH(2)CH(2)CH(2)O-6Galβ1-1Sph, which were tentatively named A and B. Fig. 3C shows the two sets of connectivities of the P-H1 protons to Gal-C6 and Gro-C1 carbons. The 1H-chemical shifts of Px at 60 °C are 0.08 ppm, respectively, in terms of B minus A), suggesting that quantities of A and B isomers are in an approximate ratio of 1:1, as indicated by NMR analysis.

**DISCUSSION**

The present study indicates the presence of a significant quantity of a cationic GSL with plasmal conjugate, Px, having highly novel structure, in white matter of bovine brain. Px is characterized by properties similar to those of PLPS A and B, i.e. (i) Px and PLPS A and B are co-eluted with the same solvent (ionic strength 0.20–0.23 m triethylamine in methanol) and separated from psychosine; (ii) TLC behavior similar to that of PLPS in basic, neutral, and acidic solvents; (iii) both Px and PLPS yield psychosine and plasmal on weak acid hydrolysis. On the other hand, Px has certain properties distinct from those of PLPS: (i) Px has an additional polar group showing much slower TLC mobility than PLPS in all solvents tested. (ii) Preliminary negative-ion LSIMS of Px in comparison to PLPS A and B clearly indicated that Px has the higher molecular mass. Px had a molecular mass 92 Da higher in deprotonated ions [M–H] than that of PLPS; this difference in molecular mass corresponds to glycerol. (iii) Px has only one 6-O-substitution, whereas PLPS A has 3,4-di-O-substitution, and PLPS B has 4,6-di-O-substitution on methylation analysis. (iv) The sequence of the components and molecular species of aldehyde are characterized as a glycero-ol-1-aldehyde (16:0, 18:1, and 18:0)-O-Hex-Sph, by LSIMS and ESI-MS of native and methylated compounds. (v) The structure was established unambiguously as a glycerol derivative of PLPS by 1H and 13C NMR.

The novelty of the Px structure lies in the mode of plasmal conjugation, i.e. the way that O-plasmal conjugate is linked at C1 at two primary hydroxyl groups at glycerol and galactose. The glycerol residue may interact with galactosyl residue to achieve steric stability, such as axes of two aliphatic chains, sphingosine and plasmal, are oriented in parallel regardless of the C1 stereoisomer of plasmal. A tentative minimum energy conformational model is shown in Fig. 4. No similar structure has been observed previously in any plasmal conjugated compound. The most common plasmal conjugates are based on O-vinyl ether, i.e. O-alk-1- enyl group linked to one of the hydroxyls of glycerolphosphoethanolamine or glycerophosphocholine, as observed typically in plasmalogen (for review see Refs. 27, 28). Another group of plasmal conjugates is based on cyclic acetal linked to a vicinal dihydroxyl group of galactosyl residue linked to Sph, ceramide, or diglyceride (3–6). We term this compound with novel structure “glyceroplasmalopysychosine.”

The biosynthetic mechanism of either O-cyclic acetal or novel acetal, as now found in Px, is totally unknown at this time. In contrast, biosynthesis of O-alk-1-enyl structure as found in plasmalogen was well established through the 1-alkyl desaturation system, a microsomal mixed-function oxidase (29). Release of free fatty aldehyde (plasmal) appears to be a prerequisite for synthesis of O-acetal conjugates; however, the mechanism for this is still ambiguous. Three biochemical routes have been proposed: (i) action of acyl-CoA reductases in the presence of NADPH; (ii) oxidative cleavage of alkyl glyceride by alkyl mono-oxygenase (tetrahydropterine; Pte-H4-dependent); (iii) specific hydrolyase for plasmalogen (“plasmalogenase”) capable of hydrolyzing O-alk-1-enyl group (30). However, released fatty aldehyde is transient and immediately converted to fatty alcohol or fatty acid, and it is perhaps utilized for O-acetal conjuga-
gation. The entire process may take place in the microsomal membrane.

Although the mechanism of O-plasmal conjugate formation is unknown, it may be an enzymatic reaction coupled with either plasmal-forming reaction i, ii, or iii as above. This concept is supported by the following observations: (i) Specificity of plasmal conjugate always occurs at β-galactosyl but not other sugar residues (3–6). Such specificity cannot be associated with non-enzymatic reaction. (ii) Plasmal conjugate as plasmalocerebroside was detectable by MALDI-TOF of sphingolipid extract from as little as 200 mg of wet weight of Eker rat brain white matter but not from gray matter extract of the same brain. The level of plasmalocerebroside was many times higher in brain from Eker rats with hereditary renal carcinoma (31). This finding indicates that biosynthesis of acetal conjugates is enhanced in pathological processes and rules out the possibility that they are formed during the isolation procedure. (iii) The yield of Px from bovine brain white matter is consistent regardless of amount of material used or whether old (long-term frozen) versus fresh samples are used, i.e. 0.55–0.60 μmol per kg. (iv) The yield of PLPS from human brain is ~10× higher than that from bovine brain, as found in both our previous and present studies. This indicates that a consistent level of plasmal conjugate is synthesized in brain and that the level varies depending on species. (v) No Px or PLPS was yielded when a mixture of plasmal and psychosine was incubated with or without glycerol for various durations, and the yield of PLPS was the same as that directly from the homogenate when human brain white matter homogenate was incubated with plasmal and psychosine or with phosphatidyethanolamine containing plasmalogen.

NMR data indicate that two stereoisomers with regard to the asymmetric C1 carbon of plasmal in Px are detected in a ratio of ~1:1, whereas those of PLPS are found exclusively in one form (“endo type”). This may reflect a difference in stability of plasmal linkage in these two compounds, i.e., the linkage in Px is much more unstable than that in PLPS (see under “Experimental Procedures”: “Determination of Lipids Containing Aldehyde Using Schiff’s Reagent”). Anomeric conversion of sugars as detected by mutarotation depends highly on stability of glycosidic linkage; e.g. N-glycosides are in general much more unstable than O-glycosides, if the same sugar is linked to a similar aglycon. Susceptibility of mutarotation is higher in unstable N-linked structures than in O-linked structures. It is possible that the ratio of Px stereoisomers A and B is different in nascent product compared with those after isolation, although this is difficult to demonstrate at this time. In stereoisomers A and B, general conformational structure is very similar in terms of axes of two aliphatic chains and location of sugars.

In general, lipids can be classified as acidic, neutral, zwitterionic, or catonic based on their ionic properties. Positive ionic properties of cationic lipids (which include psychosine and other lyso-GSLs, Sph, and dimethyl-Sph) are ascribable to the amino group of Sph. Cationic lipids having free Sph amino group, though a minor component, modulate activity of growth factor receptor kinase, protein kinase C, or other membrane-bound signal transducer molecules located at upstream regions of signal transduction pathways. This event leads to up- or down-regulation of key molecules, located downstream, which control transcription. For example, Sph and dimethyl-Sph inhibit PKC (32, 33), activate epidermal growth factor receptor kinase (34) and many other protein kinases that modulate chaperone effect, e.g. Sph-dependent kinase 1 for 14-3-3 protein (35, 36). Another example is activation by PLPS of Trk A tyrosine kinase in PC12 cells, leading to enhancement of mito-

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