Bis-sulfoglycosphingolipid Containing a Unique 3-O-Sulfated N-Acetylgalactosamine from Rat Kidney*

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A novel sulfoglycosphingolipid containing two sulfo ester groups was isolated from the lipid extract of rat kidney. The isolation procedure involved extraction of lipids with chloroform/methanol, alkaline methanolysis, and column chromatographies with DEAE-Sephadex and silica beads. By infrared spectroscopy, proton magnetic resonance spectroscopy, periodate oxidation, solvolysis of chromium trioxide oxidation, and methylation analysis of the native and partially degraded compounds, the structure of this glycolipid is proposed to be (HSO₃)₃GalNAcβ1-4(HSO₃)₃Galβ1-4Glcβ1-Cer. The presence of a unique 3-O-sulfated N-acetylgalactosaminol monosaccharide is confirmed by gas chromatography-mass spectrometry. The yield of this sulfoglycolipid was 11.2 nmol/g of tissue, which was about half of that of monosulfoglycolphosphatidylcholine from rat kidney.

Next to nervous tissue, the highest concentrations of sulfoglycolipids occur in the kidney. However, sulfoglycosphingolipids with a longer carbohydrate chain than sulfo-LacCer had never been found in kidney tissue. Our recent studies on sulfoglycolipids of rat kidney established that this tissue contained not only sulf-GalCer and sulfo-LacCer, but also more complex sulfoglycolipids composed of galactose, glucose, and N-acetylgalactosamine. One of them was found to be monosulfoglycolipid (3.5). The second compound (sulfoglycolipid P) was found to contain two sulfo ester groups in the molecule (6). This report describes the isolation and complete characterization of sulfoglycolipid P from rat kidney.

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2 The abbreviations and trivial names used are: sulfo-LacCer, sulfo-galactosylceramide (LacCer-1'-sulfate); TLC, thin layer chromatography; PMR, proton magnetic resonance spectroscopy; GC, gas liquid chromatography; GC-MS, gas chromatography-mass spectrometry; sulfo-GalCer, sulfo-galactosylceramide (GalCer-1'-sulfate); monosulfo-GosCer, monosulfoglycolphosphatidylcholine (GalCer-1'-sulfate); bis-sulfo-GosCer, bis-sulfoglycolphosphatidylcholine (GosCer-1',2'-sulfate); Foman glycolipid, globopentanseryceramide (GosCer); GM3, β-N-acetylneuraminyllactosylceramide (II NeuAcGosCer); GM2, β-N-acetylgalactosaminyllactosylceramide (II NeuAcGosCer); GM1, β-N-acetylgalactosaminyllactosylceramide (II NeuAcGosCer); GM0, β-N-acetylgalactosaminyllactosylceramide (II NeuAcGosCer); GM1, β-N-acetylgalactosaminyllactosylceramide (II NeuAcGosCer); GM2, β-N-acetylgalactosaminyllactosylceramide (II NeuAcGosCer). Some abbreviations were used instead of N-acetylneuraminylactosylceramide (II NeuAcGosCer).

MATERIALS AND METHODS
Materials — Water rats were obtained from a commercial source. The mixture of acidic lipids from rat liver, monosulfoglycolipid from rat kidney (13), GlcCer from human kidney (17), and Foman glycolipid, globopentanseryceramide were prepared in this laboratory. Methyl ethers of N-acetylgalactosylpyranosides, 1,4,6-tri-O-methyl-2-N-acetylgalactosyl-pyranosides, and sulfates of galactose, lipid A, and lipid B were prepared from Forssman glycolipid and from the oligosaccharides of ganglitolipids (10). Sulfoglycolipids including GlcCer, Cer, and the standards of fatty acids, sphingolipid and sterol mixtures of partially methylated alditol acetates were prepared by the authors. TLC. Sodium gel 60 HPTLC plates (17), thin-layer chromatography (10), and thin-layer chromatography-mass spectrometry (19) were performed with type Kieselgel 60 F₂₅₄ that is available from Waverly Press. Request Document No. 82M-496, cite the authors, and include a check or money order for $1.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
Isolation of Sulfoglycolipid P—Kidneys (200 g) were removed from Wistar rats (6 to 7 weeks old) of either sex, and extracted in three steps with: 1), 19 volumes of chloroform/methanol (2:1, v/v); 2), 10 volumes of chloroform/methanol/0.88% KCl (60:20:9, v/v); and 3), 10 volumes of 40 mM sodium acetate in chloroform/methanol/water (30:60:8, v/v) (5, 20).

The clear upper phase was concentrated to 1/10 volume, neutralized with methanolic hydrogen chloride, the reaction mixture was partitioned in the Folch system (5, 21, 22). The yield of sulfoglycolipid P on galactose basis was determined to be 11.2 nmol/g of tissue by GLC.

Infrared and Proton Magnetic Resonance Spectroscopy—The IR spectrum of sulfoglycolipid P, recorded in chloroform/methanol (2:1, v/v) at 55 °C, was similar to that of Gg0se:Cer (Fig. 1, A and B). In the basic solvent system (II), sulfoglycolipid P migrated slower than monosulfo-GgOse:Cer, but similarly to GgOse:Cer (Fig. 1). The yield of sulfoglycolipid P on galactose basis was determined to be 11.2 nmol/g of tissue by GLC.

The final purification of sulfoglycolipid P was achieved by column chromatography using latrobeads (0.6 × 70 cm) and a linear gradient with a total of 800 ml of chloroform/methanol/water, 75:25:1.5 to 45:60:3 (v/v). Isolated sulfoglycolipid P was examined for purity by TLC in Solvent Systems I, II, and III, and found to be a homogeneous band which stained with orcinol, but not with acid molybdate or resorcinol, indicating the presence of hexose and the absence of phosphate or sialic acid in the molecule. In the neutral and acidic solvent systems (I and III), sulfoglycolipid P migrated slower than GgOse:Cer and monosulfo-GgOse:Cer (Fig. 1, A and B). In the basic solvent system (II), sulfoglycolipid P migrated slower than monosulfo-GgOse:Cer, but similarly to GgOse:Cer (Fig. 1C). The yield of sulfoglycolipid P on galactose basis was determined to be 11.2 nmol/g of tissue by GLC.

RESULTS

Isolation of Sulfoglycolipid P—Kidneys (200 g) were removed from Wistar rats (6 to 7 weeks old) of either sex, and extracted in three steps with: 1), 19 volumes of chloroform/methanol (2:1, v/v); 2), 10 volumes of chloroform/methanol/0.88% KCl (60:20:9, v/v); and 3), 10 volumes of 40 mM sodium acetate in chloroform/methanol/water (30:60:8, v/v) (5, 20).

The third extract was concentrated, dialyzed, and lyophilized. The lyophilized extract was combined with the first and the second extracts and concentrated to dryness. The residue was treated for 1 h at 37 °C with 0.2 M NaOH in methanol. After neutralization with methanolic hydrogen chloride, the reaction mixture was partitioned in the Folch system (5, 21, 22). The clear upper phase was concentrated to 1/10 volume, dialyzed, and lyophilized. The lyophilized sample was combined with the lower phase, made up to 500 ml of chloroform/methanol/water (5:10:1, v/v) by the addition of solvents, and stained with orcinol reagent.

The plates were developed with: A, chloroform/methanol/0.2% CaCl₂ (60:35:7, v/v/v); B, chloroform/methanol/concentrated ammonia/water (60:35:1:7, v/v/v), and C, chloroform/methanol/0.2% CaCl₂ (60:35:7, v/v/v). The PMR spectrum of sulfoglycolipid P, recorded in chloroform/methanol (2:1, v/v) at 55 °C, was similar to that of monosulfo-GgOse:Cer (Fig. 4 of Ref. 5). The signals at 4.81 ppm (J = 8.0 Hz) and 4.50 ppm (J = 7.4 Hz) were reasonably ascribed to the anomeric protons of the terminal β-N-acetyl-galactosamine and GalNAcβ1-4-linked β-galactose, respectively, since these chemical shift values were close to those of monosulfo-GgOse:Cer (5). The α-proton of the GalNAcβ1 moiety shifted by only 0.10 ppm by attachment of the sulfate ester. By recording the spectra in dimethyl sulfoxide at 25 °C, signals at 4.46 ppm (J = 7.9 Hz), 4.30 ppm (J = 7.4 Hz), and 4.20 ppm (J = 7.7 Hz) were observed. Similar results were

Fig. 1. Thin layer chromatogram of native and partially degraded sulfoglycolipid P. The plates were developed with: A, chloroform/methanol/0.2% CaCl₂ (60:35:7, v/v/v); B, chloroform/methanol/concentrated ammonia/water (60:35:1:7, v/v/v), and C, chloroform/methanol/0.2% CaCl₂ (60:35:7, v/v/v), and stained with orcinol reagent. Lane 1, the mixture of acid lipids from rat brain (A and C) or ganglioside GM₁ (B); Lane 2, monosulfo-GgOse:Cer from rat kidney; Lane 3, sulfoglycolipid P; Lane 4, DSP-I; Lane 5, DSP-II; Lane 6, DSP-III; Lane 7, LacCer (upper bands) and GgOse:Cer (lower band). DSP, desulfated sulfoglycolipid P (see text).
obtained when spectra were recorded at 60 °C. When these values were compared to those obtained in chloroform/methanol, the GalNAc proton shifted to a higher magnetic field by 0.34 to 0.35 ppm and the Gal proton shifted by 0.20 ppm. These values in dimethyl sulfoxide were close to those of β-N-acetylgalactosamine, GalNAcβ1-4-linked β-galactose, and Galβ1-4-linked β-glucose, respectively, in GgOse3Cer recorded in dimethyl sulfoxide at 110 °C (25).

Composition Analysis—GLC of the trimethylsilylated methylglycosides, formed from the carbohydrate portion of sulfoglycolipid P, established the presence of equimolar amounts of galactose, glucose, and N-acetylgalactosamine. The colorimetric analyses showed the presence of 2 mol of sulfate ester groups and 1 mol of sphingoid base (Table 1). The result of the analysis of sulfate was in good agreement with the much larger absorption of the sulfate ester group (1240 and 820 cm⁻¹) in the IR spectrum (Fig. 2 of Ref. 6). The compositions of fatty acids and sphingoid bases in sulfoglycolipid P resembled those in monosulfo-GgOse3Cer from rat kidney (5). The major nonhydroxy fatty acids were 24:0 and 22:0, and the proportion of 2-hydroxy fatty acids was only 3.2% of total fatty acids. The peaks of nonhydroxy and 2-hydroxy fatty acids were identified by comparing their retention times of those with standards, and by mass chromatograms. 4-Hydroxysphinganine accounted for 81.7% of the total sphingoid bases present in Sulfoglycolipid P (Table II). The mass spectrum of the trimethylsilyl derivative of 4-hydroxysphinganine in sulfoglycolipid P was close to that of 4-hydroxysphinganine in ganglioside GM1 from bovine kidney (19). These data, together with those of IR and PMR spectroscopy, were consistent with a triglycosylceramide containing two sulfate ester groups.

Methylation and Periodate Oxidation of Native Sulfoglycolipid P—It has been established that sulfate esters survive the methylation procedure (5). Also with sulfoglycolipid P, it was confirmed that methylation was complete and the desulfation during the process was minimal as judged on TLC compared with peremethylated monosulfo-GgOse3Cer as well as GgOse3Cer. The permethylated native sulfoglycolipid P,

### Table I

**Effect of periodate oxidation and chromium trioxide oxidation on monosaccharide composition of native and modified sulfoglycolipid P**

The methyl glycosides were obtained by the anhydrous methanalysis of the glycolipids and analyzed by GLC, as described in the text. For the conditions of periodate oxidation, solvolysis, and chromium trioxide oxidation, see the text. Colorimetric procedures were used for sulfate (12) and sphingoid base (13) determination. The molar ratio was calculated on glucose as 1.0, except for chromium trioxide oxidation, where the molar ratio was calculated on glucose without oxidation as 1.0 with myo-inositol as the internal standard.

| Sulfoglycolipid P | Galactose | Glucose | N-Acetylgalactosamine | Sulfate | Sphingoid base |
|-------------------|-----------|---------|-----------------------|---------|---------------|
| Peridate oxidation | Native | 0.97 | 1.0 | 1.04 | 1.79 | 0.98 |
| | + | 1.11 | 1.0 | 1.17 | ND | ND |
| | DSP-1 | 0.96 | 1.0 | 0.98 | ND | ND |
| | DSP-II | 1.02 | 1.0 | 0 | ND | ND |
| | DSP-III | 0.98 | 1.0 | 0.92 | ND | ND |
| CrO₃ oxidation | | | | | | |
| | DSP-1 | 0.13 | 0.17 | 0.07 | ND | ND |

* DSP, desulfated sulfoglycolipid P; for details, see text.

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### Table II

**Fatty acid and sphingoid base composition of sulfoglycolipid P**

The values are given as weight per cent. The nonhydroxy fatty acid methyl esters were run on a 10% EGSS-X column. The hydroxy fatty acid and sphingoid base were run as their trimethylsilyl derivatives on a 3% OV-101 column. Peaks were identified by comparing their retention times with those of standards and also by GC-MS.

| Component | Nonhydroxy | 2-Hydroxy | Sphingoid base |
|-----------|------------|-----------|---------------|
| Total | 94.1 | 3.2 | 2.7 |
| 16:0 | 4.5 | Trace* | 4-Hydroxysphinganine |
| 18:0 | 2.8 | 0.6 | 8.4 |
| 20:0 | 8.6 | 1.5 | |
| 22:0 | 20.0 | Trace | |
| 23:0 | 16.2 | 1.1 | |
| 24:0 | 42.0 | Trace | |

* Fatty acid compositions below 0.5% are designated as trace.

### Table III

**Partially methylated alditol acetates derived from the native and modified sulfoglycolipid P**

Peaks were identified by gas chromatography and gas chromatography-mass spectrometry, as described in the text. Gas chromatography was performed on columns of 3% SP-2340, 3% OV-17, and 3% OV-101. The molar ratio was calculated on 2,3,6-tri-O-methylglucitol acetate as 1.0.

| Hexitol and hexosaminol acetates | Approximate molar ratio of the components |
|---------------------------------|-----------------------------------------|
| Native | DSP-I | DSP-II | DSP-III |
| 2,3,6-Tri-O-methylglucitol (1,4,5-tri-O-acetate) | 1.0 | 1.0 | 1.0 |
| 2,6-Di-O-methylgalactitol (1,3,4,5-tetra-O-acetate) | 1.18 | 1.04 | |
| 2,3,6-Tri-O-methylgalactitol (1,4,5-tri-O-acetate) | | | 0.86 |
| 2,3,4,6-Tetra-O-methylgalactitol (1,3,5-di-O-acetate) | | | 0.98 |
| 4,6-Di-O-methyl-2-N-methylacetamidogalactitol (1,3,5-tri-O-acetate) | | | 0.90 |
| 3,4,6-Tri-O-methyl-2-N-methylacetamidogalactitol (1,5-di-O-acetate) | | | 1.08 |

* DSP, desulfated sulfoglycolipid P; for details, see text.

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after acetylation, reduction, and acetylation, gave three major peaks on GLC, which co-chromatographed with the acetates of 2,3,6-tri-O-methylglucitol, 2,6-di-O-methylgalactitol, and 2,4,6-di-O-methyl-2-N-methylacetamidogalactitol, in approximately equimolar proportion (Table III). Retention times of these three peaks were identical to those of standards by GLC using three different columns, SP-2340, OV-101, and OV-17. These peaks were also analyzed by mass spectrometry using a combined gas chromatograph-mass spectrometer. Fig. 2A shows the mass chromatogram of partially methylated alditol acetates obtained from Forssmann glycolipid. Although the acetates of 2,3,6-tri-O-methylgalactitol, 2,3,6-tri-O-methylglucitol, and 2,4,6-tri-O-methylgalactitol could not be separated completely on the column of OV-101, these peaks could be distinguished on the mass chromatogram using m/z 161 and 162 (inset in Fig. 2A). Because NaB₃H₆ was used in the reduction step instead of NaBH₄, the characteristic ion at m/z 161 and 162, which was derived from the structure containing C-1 to C-3 of the 4-substituted hexose, shifted to m/z 162 (18). The ion at m/z 161, which was derived from the structure containing C-4 to C-6 of the 3-substituted hexose, was present in

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*ND, not determined.
Bis-sulfogangliotriaosylceramide of Rat Kidney

FIG. 2. Mass chromatogram of partially methylated alditol acetates derived from sulfoglycolipid P. A, Forssman glycolipid as the standard compound; B, native sulfoglycolipid P; C, desulfated sulfoglycolipid P (DSP-I). Identified peaks are acetates of 1) 2,3,6-tri-O-methylgalactitol, 2) 2,3,6-tri-O-methylglucitol, 3) 2,3,6-tri-O-methylgalactitol, 4) 2,6-di-O-methylgalactitol, 5) 3,4,6-tri-O-methyl-2-N-methylacetamidogalactitol, and 6) 4,6-di-O-methyl-2-N-methylacetamidogalactitol, determined with a Shimadzu model 6020 auto-GC-MS apparatus with SCAP 1123 data system. Gas chromatography was performed on a column (2.6 mm x 1 m) of 3% OV-101 coated on GasChrom Q (100 to 120 mesh) programmed from 150 to 240 °C at the rate of 2 °C/min. Mass chromatograms were obtained at m/z 118, 159, 161, 162, 185, 203, 205, 233, 243, and 275. Ordinate, ion intensity (figures preceded by an asterisk indicate the amplification of the intensity); abscissa, retention time.

Peak 3 of Fig. 2A. By comparing the retention times and mass chromatograms of the two peaks of 4-substituted hexitol acetates (Peaks 1 and 2 in Fig. 2A) from Forssman glycolipid with that of 2,3,6-tri-O-methylglucitol acetate from oligosaccharide of GTlb Peaks 1 and 2 from Forssman glycolipid were identified as 2,3,6-tri-O-methylglucitol acetate and 2,3,6-tri-O-methylgalactitol acetate, respectively. The presence of m/z 162 in Peak 2 of native sulfoglycolipid P (Fig. 2B) and desulfated product (Fig. 2C, see below) indicated the presence of 4-substituted hexose in native sulfoglycolipid P. The mass spectra obtained from Peaks 2 and 4 in native sulfoglycolipid P were also identical to acetates of 2,3,6-O-methylglucitol and 2,6-di-O-methylgalactitol, respectively, prepared from the oligosaccharide of GTlb and Forssman glycolipid.

The fragment ions derived from partially methylated hexosaminitol acetates, m/z 117 and 159 (Peaks 5 and 6 in Fig. 2A), were observed in Peak 6 of native sulfoglycolipid P (Fig. 2B). The specific fragments for the acetate of 4,6-di-O-methyl-2-N-methylacetamidogalactitol, m/z 243 and 275 (Peak 6 in Fig. 2A), were also observed in Peak 6 of native sulfoglycolipid P (Fig. 2B). The mass spectrum of Peak 6 of sulfoglycolipid P (Fig. 3A) was identical to 4,6-di-O-methyl-2-N-methylacetamidogalactitol acetate prepared from Forssman glycolipid and the oligosaccharide of GTlb. The mass spectrum also agreed with that of synthetic 4,6-di-O-methyl-2-N-methylacetamidoglucitol acetate (26) or 4,6-di-O-methyl-2-N-methylacetamidogalactitol acetate prepared from monosialosylopentahexaosylceramide (27), except that these authors used NaBH₄ in the reduction step.

These data confirmed the presence of a 3-substituted N-acetylgalactosamine as well as a 3,4-substituted galactose and a 4-substituted glucose in sulfoglycolipid P. Results of permethylation, together with the IR spectrum, indicated that the sulfate ester groups are attached to two of the three equatorial hydroxyls which are located on C-3 of N-acetylgalactosamine, C-3 of galactose, and C-4 of glucose.

GLC of the methanolized products, after periodate oxida-
tion of native sulfoglycolipid P, showed that all the sugar components were resistant to periodate oxidation (Table I). This was in contrast to the case of monosulfo-GgOse3Cer, where N-acetylgalactosamine was completely destroyed under similar conditions (5). The resistance of 4-substituted glucose suggested that glucose was linked to a sphingoid base (5). Therefore, the possible attachment of the sulfate ester group to C-4 of glucose was ruled out. The survival of galactose and N-acetylgalactosamine indicated that the C-3 hydroxyls of both galactose and N-acetylgalactosamine were occupied. These results of periodate oxidation were in agreement with those of the permethylation study. Since the possible attachment of the sulfate ester at C-4 of galactose was ruled out by the result of IR spectroscopy (the absence of an axial sulfate ester group), it was suggested that the sulfate ester groups were attached to the C-3 hydroxyls of both N-acetylgalactosamine and galactose. Accordingly, it was reasonable to conclude that the C-4 hydroxyl of galactose was substituted by N-acetylgalactosamine and that C-4 of glucose was substituted by galactose.

From these results, the structure of sulfoglycolipid P was tentatively assigned as GalNAc-4Gall-4GlcCer sulfated at C-3 of both N-acetylgalactosamine and galactose.

Analyses of Partially Degraded Sulfoglycolipid P—To confirm the point of attachment of the sulfate ester groups, sulfoglycolipid P was subjected to desulfation by solvolysis in dimethyl sulfoxide/methanol (9:1, v/v) containing 8 mm H2SO4 (5). TLC of the products, after reaction at 80 °C for 10 min, showed that about 50% of sulfoglycolipid P was converted to a compound (desulfated sulfoglycolipid P-I, DSP-I) which migrated similarly to monosulfo-GgOse3Cer. After 30 min, intact sulfoglycolipid P was not detected and only the band which migrated similarly to monosulfo-GgOse3Cer was observed on TLC. In a parallel experiment, monosulfo-GgOse3Cer was not desulfated at all under similar conditions.

After 5 h, sulfoglycolipid P was converted to compounds which co-migrated with monosulfo-GgOse3Cer (DSP-I), GgOse3Cer (DSP-II), and LacCer (DSP-III), respectively. DSP-I, DSP-II, and DSP-III in the reaction mixture were separated by Iatrobeads column chromatography. By TLC in Solvent Systems I, II, and III, purified DSP-I, DSP-II, and DSP-III migrated similarly to monosulfo-GgOse3Cer, GgOse3Cer, and LacCer, respectively (Fig. 1).

GLC of the methylglycosides showed that DSP-I and DSP-II contained equimolar amounts of galactose, glucose, and N-acetylgalactosamine. DSP-III contained only galactose and glucose, indicating that a part of sulfoglycolipid P was converted to a GalNAc-free glycolipid composed of galactose and glucose in the molar ratio of 1:1 (Table I). These results established the presence of N-acetylgalactosamine on the nonreducing end.

The permethylated DSP-I, after acetylation, reduction, and acetylation, gave three peaks corresponding to the acetates of 2,3,6-tri-O-methylglucitol, 2,6-di-O-methylglactitol, and 3,4,6-tri-O-methyl-2-N-methylacetamidogalactitol (Table III). The mass chromatogram (Fig. 2C) showed that the specific fragment ions for 3,4,6-tri-O-methyl-2-N-methylacetamidogalactitol acetate (m/z 203 and 205, Fig. 2A) as well as the fragments for hexosaminitol acetate (m/z 117 and 159) were observed in Peak 5 of DSP-I. The mass spectrum of Peak 5 prepared from DSP-I (Fig. 3B) was identical to that of 3,4,6-tri-O-methyl-2-N-methylacetamidogalactitol acetate from Forsman glycolipid. The disappearance of 4,6-di-O-methyl-2-N-methylacetamidogalactitol acetate (Peak 6 in Fig. 2B) and the appearance of 3,4,6-tri-O-methyl-2-N-methylacetamidogalactitol acetate (Peak 5 in Fig. 2C) in the permethylation acetylation product of DSP-I indicated that one of the sulfate ester groups was attached to C-3 of the terminal N-acetylgalactosamine.

The permethylated DSP-II gave rise to partially methylated aldolite acetates of 2,3,6-tri-O-methylglucitol, 2,3,6-tri-O-methylgalactitol, and 3,4,6-tri-O-methyl-2-N-methylacetamidogalactitol (Table III). The presence of 2,3,6-tri-O-methylgalactitol acetate instead of 2,6-di-O-methylgalactitol acetate in the permethylation acetylation product of DSP-II was indicative of the attachment of the second sulfate ester group at C-3 of the galactose residue. The permethylated DSP-III gave two peaks corresponding to the acetates of 2,3,6-tri-O-methylglucitol and 2,3,4,6-tetra-O-methylgalactitol, indicating that N-acetylgalactosamine was attached to the C-4 hydroxyl of the subterminal galactose (Table III). Oxidation with the periodate of DSP-I resulted in only the loss of N-acetylgalactosamine (Table I), supporting the results of the permethylation study.

From these results, the structures of the desulfated products were proposed to be GalNAc1-4Gal-1-4GlcCer sulfated at C-3 of both N-acetylgalactosamine and galactose. From these  data, the complete structure of sulfoglycolipid P was proposed to be: (HSO3-3)GalNAcβ1-4(HSO3-3)Gal/β1-4Hc81-1Cer (Fig. 4).

**DISCUSSION**

Recent studies on the structure and distribution of sulfoglycolipids indicate that the number of sulfoglycolipids is increasing to form a group of acidic glycolipids comparable to gangliosides (23, 28, 29). The N-acetylgalactosamine-containing sulfoglycolipids were reported in hog gastric mucosa (20), and sialic acid-containing sulfoglycolipids were found in sea urchin (30, 31) and in bovine gastric mucosa (32, 33). We discovered a sulfoglycosphingolipid containing N-acetylgalactosamine from rat kidney (3-5). However, there has been no report so far of a sulfoglycolipid which contains more than one sulfate ester group in the molecule. On the basis of the experimental

![Fig. 4. The structure of sulfoglycolipid P. The ceramide moiety was shown as N-(n-tetracosanoyl)-4-hydroxysphinganine since this structure represents the major component. For variations of the fatty acids and sphingoid bases see Table II.](image-url)
results described in this paper, the structure of the new sulfoglycolipid (sulfoglycolipid P) is a bis-sulfated triglycosylceramide with the following structure: (HSO3-3)GalNAcPl-monosulfo-GgOse3Cer from rat kidney (5).

The chemical shift values of bis-sulfo-GgOSe3Cer recorded in dimethyl sulfoxide at 60 °C were close to those of GgOse3Cer obtained in dimethyl sulfoxide at 110 °C (25). It was suggested that the effect of a 3-O-sulfated N-acetylgalactosamine may be responsible for the resistance of the internal sulfate ester group to solvolysis. The partial cleavage of the terminal N-acetylgalactosamine sulfate to the proton-donating amide at C-2 of N-acetylgalactosamine was much more labile than the sulfate ester group at C-3 of the internal sulfoglycolipid, which was about 6 and 50 mol %, respectively, of sulfogalactosylceramide from rat kidney (5). The desulfation profile of bis-sulfo-GgOSe3Cer in dimethyl sulfoxide showed that the sulfate ester group attached to the C-3 hydroxyl of the terminal N-acetylgalactosamine was much more labile than the sulfate ester group at C-3 of the internal sulfoglycolipid. It was suggested that the effect of the 3-sulfate ester group at the galactose for the resonance of the anomeric proton of the N-acetylglucosaminylsulfate in bis-sulfogalactosylceramide was negligible in comparison with the effect of sulfated galactose in gangliosides.

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