A sulfated neolactotetraosylceramide has been isolated from the lipid extract of hog gastric mucosa. Isolation procedure involved extraction of lipids with 0.4 M sodium acetate in methanol/chloroform/water, alkaline methanolysis, column fractionation on DEAE-Sephadex, separation of the acetylated acidic glycolipids on silicic acid column, and thin layer chromatography. By partial acid hydrolysis, sequential hydrolysis with specific glycosidases, periodate oxidation, and methylation analysis of the native and desulfated compound, the structure of this glycolipid is proposed to be: Galβ1 → 4GlcNAc(6 → SO3)β1 → 3Galβ1 → 4Glc1 → 1 ceramide.

The sulfate ester derivatives of carbohydrates present in glycoconjugates of hog gastric mucosa are represented by sulfatoglycosphingolipids and sulfated glycoproteins (1-5). In sulfated glycoproteins the sulfate ester group is located at 6-hydroxyl group of the N-acetylglucosamine residue proximal to protein core, and the carbohydrate portion of these compounds shows a considerable degree of variance with respect to sugar composition, length of oligosaccharide chains, and degree of branching (4, 5). In sulfatoglycosphingolipids the sulfate ester group is situated at 3-hydroxyl group of the terminal galactosamine residue and the carbohydrate portion of these compounds consist of galactose and glucose (1-3).

Recent improvements in the procedures of extraction and preparation of glycolipids have led to isolation of a complex glycosphingolipid with carbohydrate structures resembling those found in glycoproteins (6-9). By employing a newly developed extraction procedure (6) we found in hog gastric mucosa a new sulfatoglycosphingolipid containing N-acetylgalactosamine. This report describes the isolation and characterization of this glycolipid.

**EXPERIMENTAL PROCEDURES**

Materials—Frozen hog stomachs used for mucosa preparation were purchased from Pel-Freez Biological, Inc. DEAE-Sephadex A-25 was supplied by Pharmacia and silicic acid Bio-Sil A (100 to 200 mesh) by Bio-Rad Laboratories. Silica gel HR plates, 250 nm coating thickness, were from Analtech, Inc. Long chain bases were from Miles Laboratories and standard fatty acid methyl esters from Applied Science Laboratories. Enzymes, β-N-acetylgalactosaminidase, α-galactosidase, and β-galactosidase, were donated by Drs. S. C. Li and T. T. Li, Tulane University. Methyl ethers of erythro-O-methylgalactose and 2,3,6-tri-O-methylglycero was prepared from lactose. The 2,4,6-tri-O-methylgalactose and 3,4,6-tri-O-methyl-N-methylacetamidoglucose were supplied by Dr. K. Moyer, Columbia University. The 3,6-di-O-methyl-N-methylacetamidoglycoside and 3,4,6-tri-O-methyl-N-methylacetamidoglucose were prepared from acid-degraded chitin (5). The 4,6-di-O-methyl-N-methylacetamidoglucose was derived from the methylated hyaluronic acid (5).

In addition to the standards listed above, a set of partially methylated amino sugars was obtained from Dr. P. A. J. Gorin, Prairie Regional Laboratory, Saskatoon, Canada. All other reagents were of commercial origin.

Isolation and Purification of Glycolipid—Hog gastric mucosa scrapings obtained from several fresh stomachs were dried with acetone, homogenized with 10 volumes of chloroform/methanol (2:1), and filtered through a sintered glass funnel. The insoluble residue was then stirred at room temperature with 10 volumes of 0.4 M sodium acetate in methanol/chloroform/water (60:30:8). After 24 h the mixture was filtered and the residue was once again extracted with the above mixture. The combined filtrate was evaporated to dryness and treated for 1 h at 37°C with 0.2 M NaOH in methanol. The sample was evaporated to one-fourth volume, taken up in an excess of water, dialyzed, and lyophilized (6). The crude glycolipid fraction contained in the lyophilisate was dissolved in a small volume of methanol/chloroform/water (60:30:8) and applied to a column (2.5 x 10 cm) packed with DEAE-Sephadex (A-25, acetate form) (10). The neutral glycolipids were eluted from the column with 2100 ml of the above solvent mixture. Sulfated glycolipids and gangliosides were then eluted with 2400 ml of 0.4 M sodium acetate in methanol/chloroform/water (60:30:8). Lipids in this fraction were dialyzed, lyophilized, and acetylated with acetic anhydride/pyridine (2:3) at room temperature for 48 h. Material recovered from the acetylation mixture by evaporation with toluene was dissolved in 1,2-dichloroethane with the aid of sonication and applied to a column (1.2 x 30 cm) equilibrated with the same solvent. The column was developed first with 400 ml of 1,2-dichloroethane/acetone (1:1), 700 ml of acetone, 700 ml of methanol/glycol (9:1), and finally with 800 ml of methanol. An aliquot of each fraction was decanted with sodium methoxide (11). Dialyzed, dried, dissolved in a small volume of methanol/chloroform/water (60:30:8), and monitored for gangliosides and sulfated glycolipids by thin layer chromatography in chloroform/methanol/2.5 M ammonia (60:35:8).

Further purification of acetylated sulfated glycolipid, mainly present in the acetone fraction, was accomplished by preparative thin layer chromatography in chloroform/methanol/chloroform/water (60:10:9:1). The investigated glycolipid in its acetylated form was also examined for purity on thin layer plates developed in chloroform/methanol/water (76:25:2) and 1,2-dichloroethane/methanol/water (80:25:2).

Analytical Methods—The thin layer chromatographic visualization with orcinol and rhodizonate reagents was used for sulfated glycolipids.
glycolipids, resorcinol for gangliosides and ninhydrin for long chain bases. The iodine vapors were employed for preparative purposes. Methyl esters of fatty acids and methyl glycosides were obtained by methanolysis of the glycolipid in 1.2 m methanolic HCl at 80°C for 24 h (12). The alditol acetates and long chain bases were obtained from the glycolipid according to Yang and Hakomori (13). Gas-liquid chromatography analyses were performed with Beckman GC-65 instrument equipped with glass columns (150 x 2 cm) packed with 3% SE-30 on chromosorb W, AW, DMCS (80 to 100 mesh), and packed with 1% ECNSS-M on Gas Chrom-Q. The trimethylsilyl derivatives of methyl glycosides and long chain bases, and fatty acid methyl esters were analysed on SE-30 columns (6). The alditol acetates were analysed on ECNSS-M columns (6). Purified glycolipid was also examined for sulfate ester by infrared spectroscopy (1) and assayed for sialic acid (14) and sulfate (15).

**RESULTS**

**Glycolipid Isolation** - The lipids extracted from 400 g of dry mucosa scrapings with sodium acetate in methanol/chloroform/water, after alkaline methanolysis and DEAE-Sephadex column chromatography, yielded 3.62 g of crude acidic glycolipids. This fraction was acetylated and chromatographed on silica gel column. The 1,2-dichloroethane and 1,2-dichloroethane/acetone eluates from the column contained gangliosides. The latter fraction also contained small quantities of di- and trihexose sulfatides, these were characterized previously (1). The acetone eluate contained the studied sulfatoglycolipid. This component was further purified from the residual ganglioside contaminants by the repeated preparative thin layer chromatography in several different solvent systems. The yield of purified glycolipid (Fig. 1) was 2.6 mg.

**Structure Elucidation** - The infrared spectra of isolated glycolipid resembled that of neutral glycosphingolipids except for the additional presence of strong absorption at 1240 cm⁻¹ and a peak at 820 cm⁻¹. The absorption at 1240 cm⁻¹ indicated the presence of sulfate ester group, whereas peak at 820 cm⁻¹ indicated that primary hydroxyl group of carbohydrate was sulfated (20).

The native glycolipid, but not its desulfation product, reacted as sulfatide in the assay procedure of Kean (14) and contained 0.9 mol of sulfate/1 mol of glucose. Acid solvolysis (desulfation) of the isolated glycolipid resulted in its conversion to a neutral compound which showed enhanced migration on thin layer chromatography (Fig. 1). Gas-liquid chromatography of the alditol acetates and methyl glycosides, formed from the carbohydrate portion of the glycolipid, established the absence of galactose, glucose, and N-acetylgalactosamine.

**Table I**

<table>
<thead>
<tr>
<th>Glycolipid</th>
<th>Molar ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gal</td>
<td>Glc</td>
</tr>
<tr>
<td>Native</td>
<td>1.92</td>
</tr>
<tr>
<td>Native after periodate</td>
<td>1.05</td>
</tr>
<tr>
<td>β-Galactosidase degraded</td>
<td></td>
</tr>
<tr>
<td>Glycolipid</td>
<td></td>
</tr>
<tr>
<td>Glycolipid after periodate</td>
<td></td>
</tr>
</tbody>
</table>

**Fig. 1.** Thin layer chromatogram of the sulfatoglycosphingolipid purified from hog gastric mucosa. 1, acetylated native glycolipid; 2, acetylated glycolipid after β-galactosidase treatment; 3, desulfated native glycolipid; 4, deacylated native glycolipid. Conditions: Silica Gel HR, 250 nm, developed in chloroform/methanol/water (70:25:2). Visualization: orcinol spray.
in the molar ratio of 2:1:1 (Table I). Sialic acid, N-acetylgalactosamine and fucose were not detected. These results clearly indicate that the glycolipid contained a tetrahexosyl chain to which 1 residue of sulfate was attached.

The sequential arrangement of sugar units and anomeric configuration of glycosidic bonds, in the tetrasccharide chain of the studied glycolipid was elucidated with the aid of specific glycosidases. The native and desulfated glycolipid were resistant to the action of α-galactosidase and β-N-acetylgalactosaminidase, but both were hydrolyzed to ceramide trihexoside by β-galactosidase. Neither β-N-acetylgalactosaminidase nor α-galactosidase and β-galactosidase hydrolyzed further the ceramide trihexoside derived from native glycolipid (Fig. 1). The ceramide trihexoside, formed from desulfated glycolipid, was readily converted to ceramide dibeheside (Gal → Glc → ceramide) after incubation with β-N-acetylgalactosaminidase. The ceramide dibeheside was further degraded to glucosylceramide by β-galactosidase. The results of enzymatic degradation presented above indicate that the desulfated glycolipid has the structure: Galβ → GlcNacβ → Galβ → Glc → ceramide. Resistance of the ceramide trihexoside, derived from the native glycolipid, to further degradation by glycosidases suggests that the native glycolipid contained sulfated ester group situated at the subterminal N-acetylglucosamine residue.

The attachment of the sulfate ester group to N-acetylgalactosamine as well as the type of linkages between the sugars in studied glycolipid were established by periodate oxidation and methylation analysis. The molar ratios of the sugar components of the native and β-galactosidase degraded sulfated glycolipid, following the periodate oxidation, are shown in Table I. Oxidation with periodate of native glycolipid resulted only in the loss of 1 galactose residue, whereas in β-galactosidase degraded sulfated glycolipid N-acetylgalactosamine was destroyed. These results suggest that the subterminal N-acetylglucosamine residue in the native glycolipid was substituted at C-4 or C-6 by galactose and at C-6 by sulfate ester group. The permethylated desulfated glycolipid gave rise to partially methylated alditol acetates of 2,3,4,6-tetra-O-methylgalactitol, 2,4,6-tri-O-methylgalactitol, and 3,6-di-O-methyl-N-methylactamidoglucitol. The results clearly indicate that subterminal N-acetylglucosamine unit in the oligosaccharide chain of the intact glycolipid is substituted at C-4 by galactose and at position C-6 by sulfate ester group.

The fatty acid and long chain base composition of the studied glycolipid is given in Table II. Hexadecanoate, octadecanoate, docosenoate, and tetracosanoate were the principal fatty acids. Analyses of long chain bases revealed that C18 sphingosine constitutes over 94% of total bases present in this glycolipid.

### DISCUSSION

The sulfatoglycosphingolipids of hog gastric mucosa extracted from the tissue with chloroform/methanol or present in the organic phase of buffered tetrahydrofurans consist of a mixture of galactosyl sulfatide, lactosyl sulfatide, and triglycosyl sulfatide (1). Recent improvements in the procedures of extraction and preparation of glycolipids have led to isolation of a complex glycosphingolipid which exhibit considerable solubility in the aqueous systems (6-9, 17, 22). The procedure developed in our laboratory for the isolation of such compounds involves extraction of the mucosa with 0.4 M sodium acetate in methanol/chloroform/water (6). Examination of the acidic glycolipids, contained in such an extract, resulted in the isolation of a new sulfated glycosphingolipid carbohydrate portion of which consisted of glucose, galactose, and N-acetylgalactosamine.

On the basis of the experimental results obtained from partial acid hydrolysis, sequential degradation of saccharide chain with specific glycosidases, periodate oxidation, and permethylation study, the structure of this new sulfated glycolipid is proposed to be: Galβ1 → 4GlcNacβ6(SO3)β1 → 3Galβ1 → 4Glc1-1 ceramide.

To our knowledge, sulfatoglycosphingolipids containing sulfated N-acetylgalactosamine have not been previously described in mammalian gastric mucosa. However, N-acetylgalactosamine 6-sulfate was found in blood group (A + H) sulfated glycoproteins of hog gastric mucosa (4, 5). The presence of N-acetylgalactosamine 6-sulfate in glycoproteins and glycosphingolipids of hog gastric mucosa indicates similarities in the biosynthetic pathways of glycoproteins and glycosphingolipids. It also indicates that the same enzyme system may be responsible for the transfer of the sulfate from adenosine 3'-phosphate 5'-phosphosulfate to glycolipid and glycoprotein acceptors. It may also suggest existence of a single sulfatase involved in the degradation of these two types of compounds. The existence of glycolipids with carbohydrate structures (7, 9, 23, 24) identical to those found in glycoproteins (4, 5, 25, 26) further indicates that in glandular epithelial tissue the same oligosaccharides may be linked to a lipid or protein core.

Although the presence of sulfatoglycosphingolipids and sulfated glycoproteins in mucosal tissue is well established, little is known about their function. Sulfated polysaccharides have been shown to inhibit peptic proteolysis in vitro (27). Therefore, sulfated glycolipids in the stomach may protect the gastric mucosa from autodigestion by the acid and pepsin present in gastric juice. The high content of sulfatoglycosphingolipids in the tissues of increased sodium metabolism indicates that these lipids may serve as the cation receptor in sodium ion transport (28). Other functions of sulfatides, although as yet undemonstrated, may include imparting of selective permeability and control of cell-cell contact.
REFERENCES
Isolation and characterization of the sulfated neolactotetraosylceramide from hog gastric mucosa.

B L Slomiany and A Slomiany


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