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ISOLATION AND PARTIAL CHARACTERIZATION OF A NOVEL SULFOGLYCOSPHINGOLIPID AND GANGLIOSIDE GM4 FROM RAT KIDNEY

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SUMMARY A novel sulfoglycolipid was isolated from the lipid extract of rat kidney by a procedure involving mild alkaline methanolysis and column chromatographies on DEAE-Sephadex and silicic acid. The component carbohydrates were galactose, glucose and galactosamine in equimolar amounts. Infrared spectroscopy, solvolysis and permethylation studies suggested that the glycolipid was GalNAc(1→4)Gal(1→4)GlcCer sulfated at the C3 hydroxyl of galactose. Ganglioside GM4 was separated from the above sulfoglycolipid on the column of DEAE-Sephadex A-25. The yields of sulfoglycolipid and GM4 were 18.7 and 12.9 nmol/g wet tissue, respectively.

INTRODUCTION The acidic lipid fraction of an extract of rat kidney labeled by intraperitoneal injection of $Na_2{}^{35}SO_4$ (1) was found to contain, in addition to cholesterol sulfate and galactosyl sulfatide (S-GalCer), a sulfolipid that migrated slower than S-GalCer on silica gel TLC (2). Detailed studies of this compound showed that this sulfolipid differed from the sulfate ester of lactosylceramide (S-LacCer). This paper describes the isolations of this novel sulfoglycolipid (Sulfoglycolipid A) and ganglioside GM4, and partial characterization of the former.

MATERIALS AND METHODS The mixture of acidic lipids from monkey brain and GalNAcβ1→4Galβ1→4Glcβ1→1Cer ($GgOse_3Cer$) from guinea pig erythrocytes were supplied by Drs. S. Ando (Tokyo Metropolitan Institute of Gerontology) and Y. Seyama (University of Tokyo), respectively. 4-Hydroxysphinganine and eicosasphingenine were prepared from cerebroside of rice bran (3) and disialogangliosides of human brain, respectively. A mixture of 2-hydroxy fatty acids with carbon numbers of 14, 16 and 18 was purchased from Supelco, Bellefonte. Other glycolipids, including S-GalCer, S-LacCer and ganglioside GM3 with *N*-acetylneuraminic acid were as described previously (5). The acetates of 2,4,6-tri-*O*-methylgalactitol, 2,6-di-*O*-methylgalactitol, 2,3,6-tri-*O*-methylglucitol and 4,6-di-*O*-methyl-2-*N*-methylacetamidogalactitol were prepared in this laboratory from the oligosaccharide of ganglioside GT1b (6). Other acetates of partially methylated alditols were prepared as will be described elsewhere. All other compounds and organic solvents were of analytical grade unless otherwise specified.

Abbreviations and nomenclature: TLC, thin-layer chromatography: GLC, gas-liquid chromatography. Abbreviations for gangliosides follow the nomenclature system of Svennerholm and those of other lipids follow that of the IUPAC-IUB Commission of Biochemical Nomenclature (20).

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Forty-six Wistar rats, weighing about 150 g, were sacrificed by decapitation and their kidneys (54 g) were removed and rapidly frozen at -30°C. ^{35}S-labeled sulfolipids (129000 dpm) were obtained from the kidneys of a rat 16 h after injection of $Na_2{}^{35}SO_4$ (1 mCi), as described previously (1). Labeled and unlabeled kidneys, respectively, were extracted at the same time with chloroform/methanol by the method of Folch et al. (7) as described previously (1). After evaporation of the solvent, the total lipid extract was treated with 0.2 N NaOH in methanol at 37°C for 60 min. The reaction mixture was neutralized with methanolic hydrogen chloride and desalted by passing it through a column of Sephadex G-25 (superfine). The first three column volumes, containing the total lipid, were concentrated to dryness, dissolved in a mixture of chloroform/methanol/water (10:10:1, by vol.), and fractionated on a column of DEAE-Sephadex A-25 (bicarbonate form). The neutral glycolipids were eluted from the column with the same solvent mixture, and the acidic lipids with a linear gradient of 0.05 to 0.5 M ammonium bicarbonate in chloroform/methanol/water (10:10:1, by vol.). After destroying the contaminating gangliosides by incubating the mixture with *Clostridium perfringens* neuraminidase (EC 3.2.1.18) (8), the pooled crude Sulfoglycolipid A was applied to a column of porous silica gel (Iatrobeads, Iatron Co., Tokyo) and material was eluted with a linear gradient of chloroform/methanol/water, 90:10:1 to 40:60:3 (by vol.). The elution was monitored by TLC and determination of hexose by the anthrone-sulfuric acid method (9) and also by assaying radioactivity in a liquid scintillation counter (1).

TLC was performed on HPTLC 60 plates (Merck). The lipids were separated with solvent system I, chloroform/methanol/water (60:35:8, by vol.) and II, chloroform/methanol/conc. ammonia/water (60:35:1:7, by vol.) (5). The hexose-containing lipids were detected by spraying the plate with 0.2% orcinol in 2 M sulfuric acid, and gangliosides by spraying it with resorcinol reagent (10).

Methyl esters of fatty acids and methylglycosides were obtained by methanolysis of glycolipids with 1.2 M anhydrous methanolic hydrogen chloride (1). GLC analyses of trimethylsilyl derivatives of methylglycosides were performed on a column of 3% OV-101 programmed at 2°/min from 150 to 260°C, with mannitol as an internal standard (1). Methyl esters of fatty acids were separated on a column of 10% EGSS-X at 185°C. Sphingoid bases were obtained by hydrolysis in aqueous methanolic hydrogen chloride and analyzed as the *N*-acetyl-*O*-trimethylsilyl derivatives on 3% OV-101 at 240°C.

Permethylation of glycolipid was performed as described by Stellner et al. (12). The acetates of partially methylated alditols were separated on columns of 3% SP-2340 (Supelco), 3% OV-101 and 3% OV-17 on Gas-chrom Q (100-120 mesh) programmed from 160 to 250°C.

Solvolysis of the isolated Sulfoglycolipid A (about 400 nmol) was performed in 0.2 ml of anhydrous dioxane (Merck) at 100°C for 2 h (1). After removal of the solvent, the residue was redissolved in a small volume of chloroform/methanol/water (10:10:1, by vol.) and applied to a small column of DEAE-Sephadex A-25 (acetate form), and desulfated neutral glycolipid was eluted with the above solvent mixture.

Infrared spectra were recorded as described previously (13) in a Type IRA-2 infrared spectrophotometer (Japan Spectroscopic Co., Tokyo).

RESULTS AND DISCUSSION Elution of acidic lipids from the column of DEAE-Sephadex was monitored by measurement of radioactivity. The sulfolipids were separated into three fractions, of which the second (40000 dpm) and the third (78000 dpm) were found to contain S-GalCer and cholesterol sulfate, re-

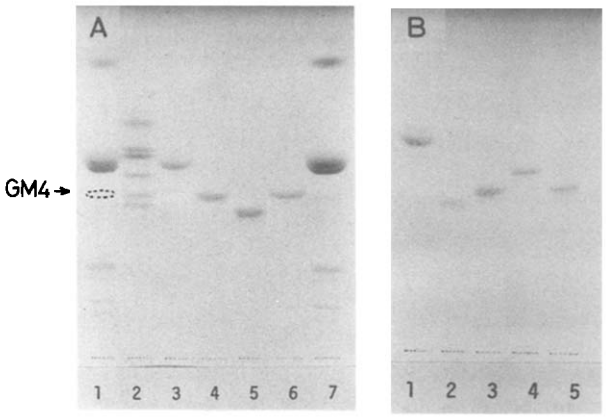

Fig. 1. TLC of Ganglioside X and Sulfoglycolipid A isolated from rat kidney.
Plate A developed with chloroform/methanol/water (60:35:8, by vol.). Lanes 1 and 7, the mixture of acidic lipids from monkey brain; lane 2, mixture of standards: GalCer, LacCer, $GbOse_3Cer$, $GbOse_4Cer$ and $LnOse_4Cer$ from top to bottom; Lanes 3, 4, 5 and 6, S-GalCer, Sulfoglycolipid A, GM3 and Ganglioside X, respectively, from rat kidney.
Plate B developed with chloroform/methanol/conc. ammonia/water (60:35:1:7, by vol.). Lane 1, S-GalCer from rat kidney; lane 2, $GgOse_3Cer$ from guinea pig erythrocytes; lanes 3, 4 and 5, Sulfoglycolipid A, Ganglioside X and GM3, respectively, from rat kidney.
Spray: orcinol reagent

spectively. TLC in solvent system I and staining first with iodine vapor and then with orcinol-sulfuric acid spray, showed that three hexose-containing lipids were present in the first fraction (11000 dpm), which was eluted with about 0.13 M ammonium bicarbonate in chloroform/methanol/water (10:10:1, by vol.). Of these lipids, that with the highest mobility (Ganglioside X) also stained with resorcinol-spray and was indistinguishable from GM4 in solvent system I and II (Fig. 1). The major portion of this ganglioside was eluted earlier than the first peak of radioactivity with 0.08 M ammonium bicarbonate. The second lipid, with a similar Rf value to Ganglioside X in solvent system I, did not stain with resorcinol reagent. In the basic solvent system II, this component migrated slower than Ganglioside X (Fig. 1B) and exactly coincided in position with the first peak of radioactivity. Therefore, it was named Sulfoglycolipid A. The slowest migrating material, which also stained with resorcinol reagent and appeared indistinguishable from ganglioside GM3, was not analyzed further.

Table 1. Carbohydrate compositions of Ganglioside X and Sulfoglycolipid A from rat kidney

Glycolipid	Molar ratio			
	Gal	Glc	GalNAc	NeuAc
Ganglioside X	1.0			1.04
Sulfoglycolipid A	1.0	1.03	1.01	

In order to remove contaminating gangliosides, the crude Sulfoglycolipid A was treated with neuraminidase. On a column of porous silica gel, monohexosylceramide and dihexosylceramide, which were probably derived from gangliosides, were eluted earlier than Sulfoglycolipid A. The peak of Sulfoglycolipid A, monitored by determination of hexose, coincided with the peak of radioactivity. As shown in Fig. 1, purified Ganglioside X and Sulfoglycolipid A were found to be homogeneous by staining with iodine vapor and orcinol-sulfuric acid.

GLC analysis of the methylglycoside showed that the Ganglioside X contained equimolar amounts of galactose and neuraminic acid (Table 1). Permethylation analysis gave 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetylgalactitol as the only partially methylated alditol acetates, confirming that this substance was ganglioside GM4. Octacosanoic acid (24:0) constituted about 75% of the fatty acids, and 4-sphingenine (d18:1) constituted about 85% of the sphingoid bases (Table 2). The yield of GM4 was 12.9 nmol/g wet tissue.

The presence of GM4 in the central nervous system of mammals has been well established (14, 15) and its extraneuronal occurrence (16) has so far been considered as exceptional. However, the results presented here suggest that a small amount of GM4 is also present in rat kidney.

The infrared spectrum of the Sulfoglycolipid A (Fig. 2) was very similar to that of S-GalCer, except for the much larger absorption of OH (3600-3100 cm^{-1}) relative to those of $-CH_2-$ (2920 and 2840 cm^{-1}). The absorptions at

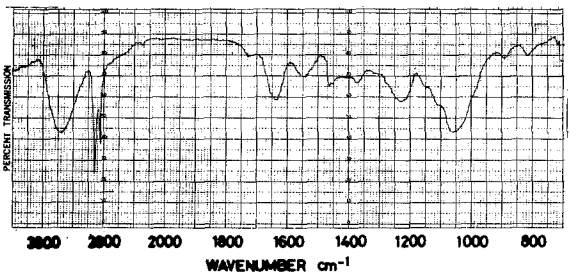

Fig. 2. Infrared spectrum of Sulfoglycolipid A from rat kidney.

1230 and 815 cm^{-1} indicated the presence of an equatorial sulfate ester of hexose (13). The absorption at 890 cm^{-1} indicated the presence of at least one beta-anomeric configuration of hexopyranoside (13).

By GLC, the Sulfoglycolipid A was shown to contain equimolar amounts of galactose, glucose and galactosamine (Table 1). The acetates of partially methylated alditols obtained from Sulfoglycolipid A contained approximately equimolar amounts of the acetates of 2,3,6-tri-*O*-methylglucitol, 2,6-di-*O*-methylgalactitol and 3,4,6-tri-*O*-methyl-2-deoxy-2-*N*-methylacetamidogalactitol.

These results indicate that the sulfate ester is attached to an equatorial hydroxyl of galactose (C3) or glucose (C4) and that *N*-acetylgalactosamine is situated at the nonreducing end. After solvolysis and DEAE-Sephadex column chromatography, the desulfated glycolipid, showed similar mobility to $GgOse_3Cer$ on TLC in solvent system I. The disappearance of 2,6-di-*O*-methyl-1,3,4,5-tetra-*O*-acetylgalactitol and the appearance of 2,3,6-tri-*O*-methyl-1,4,5-tri-*O*-acetylgalactitol in the permethylation-acetolysis product after solvolysis suggested that the sulfate was attached to C3 hydroxyl of galactose. According to the above results, the structure of Sulfoglycolipid A was tentatively assigned as 2-acetamido-2-deoxygalactopyranosyl(1→4)[3→sulfo]-galactopyranosyl(1→4)glucopyranosylceramide.

The major fatty acids present in Sulfoglycolipid A, in order of their abundance, were 24:0, 16:0 and 22:0. 4-Hydroxysphinganine (t18:0) was the pre-

Table 2. Fatty acids and sphingoid bases in Ganglioside X and Sulfoglycolipid A

Short-hand formula	Fatty acid (%)		Sphingoid bases (%)			
	Ganglioside X	Sulfoglycolipid A	Ganglioside X		Sulfoglycolipid A	
			di [1]	tri	di	tri
16:0		13.8			0.9	
16:1		3.2	1.6			
18:0	3.0	5.2				70.7
18:1		5.1	85.3	8.7	18.9	4.2
18:0h [2]		8.3				
20:0		6.9			0.8	
22:0	5.8	12.4				
23:0	1.8	8.1				
24:0	74.4	33.8				
24:1	11.9					
Un-identified	3.1	3.2	4.4		4.5	

1) di, dihydroxy base and tri, trihydroxy base
2) 2-hydroxy octadecanoic acid

dominant sphingoid (Table 2). The concentration of Sulfoglycolipid A (18.7 nmol/g wet tissue) was about 10% of that of S-GalCer (182 nmol/g wet tissue).

Mammalian sulfoglycolipids have been classified into sulfoglycosphingolipids (S-GalCer and S-LacCer) and sulfoglycoglycerolipids such as seminolipid (13, 17). Recently, a lactoneotetraosylceramide sulfated on C6 of *N*-acetylglucosamine and 3-sulfogalactosyl(1→4)galactosyl(1→4)glucosylceramide were isolated and characterized from hog gastric mucosa (18, 19). However, there has been no reports on a sulfolipid containing galactose, glucose and galactosamine such as that detected in this study. A sulfoglycosphingolipid with a similar carbohydrate composition to Sulfoglycolipid A was also discovered in epithelial cell lines (2, 5) originated from cortical tubules of monkey kidney and the thick ascending limb of porcine renal tubules (unpublished observations, cf. Fig. 5 of ref. 5). Thus this sulfoglycolipid may be an essential membrane component of renal tubular cells of mammals.

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