From: NEW VISTAS IN GLYCOLIPID RESEARCH
Edited by Makita, Handa, Taketomi, and Nagai
(Plenum Publishing Corporation, 1982

# THE SULFOGLYCOLIPID, HIGHLY ACIDIC AMPHIPHILES OF MAMMALIAN RENAL TUBULES

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Recent studies on the structure, distribution and metabolism of sulfoglycolipids of animal tissues showed that they constitute a distinct group of amphiphiles with diverse lipophilic and hydrophilic moieties (1-5). Here will be reported some of our studies on the sulfo-amphiphiles of (A) mammalian renal tissue and (B) cell lines derived from renal tubular epithelia (6). *In vivo* labeling with $^{35}$S-sulfate of animals or culture cells can be performed by intraperitoneal injection and by addition to the medium of the isotope. Many sulfono- and sulfolipids, from 6-sulfoquinovosyldiacylglycerol of chlorella to sulfoglyco- or sulfo-phospholipids of *Halobacterium cutirubrum* and SM2 sulfatide of rat kidney (3, 4) or renal tubular cells (6-9), were discovered in such a manner.

## A. Sulfoglycolipids in mammalian kidneys

### a) Incorporation of $^{35}$S into mouse organs

Intraperitoneal administration of carrier-free $^{35}$S-sulfuric acid to mouse at 5 μCi/g of body weight, labeled SM4g of testis (10) maximally within 24 h. SM4s was not detectable by autoradiography.

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In this article the term 'acidic amphiphiles' is used to designate glycolipids containing a long chain lipophilic moiety, and acidic groups. The symbols for gangliosides are those of Svennerholm. The symbols for sulfoglycolipids are a modification of the above nomenclature system: SM4g, Galβ1-3-*sn*-Gro-1-alkyl-2-acyl-I$^3$-sulfate (seminolipid); SM4s, GalCer-I$^3$-sulfate (galactosyl sulfatide); SM3, LacCer-II$^3$-sulfate (lactosyl sulfatide); SM2, GgOse$_3$Cer-II$^3$-sulfate (gangliotriaosyl sulfatide); SB2, GgOse$_3$Cer-II$^3$, III$^3$-sulfate (bissulfogangliotriaosyl sulfatide).

The age for the most active incorporation into testis, on a gram tissue basis, was 11 to 20 days of age ($7.0 \times 10^5$ cpm/g of testis) (10). The amount of SM4g in rat testis also increased dramatically between 15 and 22 days of age, reflecting the onset of spermatocyte maturation (11). On an individual mouse basis, the most active synthesis of SM4g occurred between 35 ($4.5 \times 10^4$ cpm/mouse) and 42 days ($5.5 \times 10^4$ cpm/mouse) (10), representing the peak of reproductive activities in mouse.

The labeling pattern for sulfolipids in mammalian brain has been well documented (12). The age for the most active incorporation of $^{35}S$ into mouse brain lipids was from 10 to 20 days, 1.2 to $1.4 \times 10^5$ cpm/g of brain, reflecting the onset of myelination. A chase study of $^{35}S$-radioactivities indicated that SM4s was the fairly stable component of myelin with a half life of more than 100 days.

In contrast, the incorporation into kidney of mouse reached the peak at 40 days of age ($4.3 \times 10^5$ cpm/g of kidney), indicating that the highest turnover of kidney sulfolipids in mouse occurred between 35 to 42 days (Ishizuka, I., Handa, S., Suzuki, A., Yamato, K., and Yamakawa, T., unpublished results). However, the weight of kidneys and the total lipid at 42 days, 280 mg and 12 mg/mouse, respectively, continued to increase in parallel with each other until 84 days (470 mg and 22 mg/mouse, respectively). The label decreased to only 16% of the maximum incorporation after 7 days indicating that the half life of kidney sulfolipids was considerably shorter than that of testis and myelin.

b) Incorporation into rat organs

Labeling of sulfolipids in various tissues of rat by intraperitoneally injected $^{35}S$-sulfuric acid has been performed repeatedly. Green and Robinson (13) showed that the sulfolipids of rat (150 g body weight) kidney were labeled with 1 mCi of $H_2{}^{35}SO_4$ maximally at 24 h ($5.4 \times 10^4$ cpm/g of kidney). The radioactivity had decreased to $1.2 \times 10^4$ cpm/g on the 8th day. The incorporations into the lipid fractions of brain (14, 15), kidney (6) and testis (16) have been compared. Finally, Kornblatt (16) showed by an elegant study that SM4g was the major $^{35}S$-containing compound of rat testis and once synthesized practically showed no turnover (16). The presence of SM4s in rat kidney was demonstrated by Kawanami (17). SM4s is distributed in all mammalian kidneys studied. In contrast, SM3 was identified only in humans (18). Recently, rat kidney (4) and cultured renal epithelial cells derived from monkey (JTC-12), dog (MDCK), hog (LLC-$PK_1$) and beef (MDBK) were shown to contain SM3. A third sulfoglycolipid (SM2), which migrated slower than SM3, was identified in both rat kidney (7) and in JTC-12 cells (6) by labeling with radioactive sulfuric acid.

c) Purification of sulfo-amphiphiles from rat kidney

$^{35}$S-labeled sulfolipids (170,000 dpm) were obtained from the kidneys of a rat (300 g) 16 h after intraperitoneal injection of $H_2{}^{35}SO_4$. Elution of radioactive lipids from a column of Iatrobeads was monitored by measurement of the radioactivity (Fig. 1). Peak I and Peak II (total 150,000 dpm) corresponded to cholesterol sulfate and SM4s, respectively. The third peak (11,000 dpm) was found to contain SM2. Among rat organs, the kidney is reported to contain the most cholesterol sulfate, 250-300 μg/g of dry tissue (19), although the sulfated form corresponds to only 1.2% of the total cholesterol.

The elution profile of acidic lipids of unlabeled kidney from a column of DEAE-Sephacel is shown in Fig. 2 (20). The column was eluted with a linear gradient of chloroform/methanol/ammonium acetate (5:10:1, v/v), 0.05 to 0.7 M, 2 liters, followed by 0.7 to 3.5 M, 1.5 liters (not shown in the Fig.). GM4, GM3, SM2 (0.25 M ammonium acetate), SM4s (0.3 M), SM3 (0.35 M), GD3, GT3 and SB2

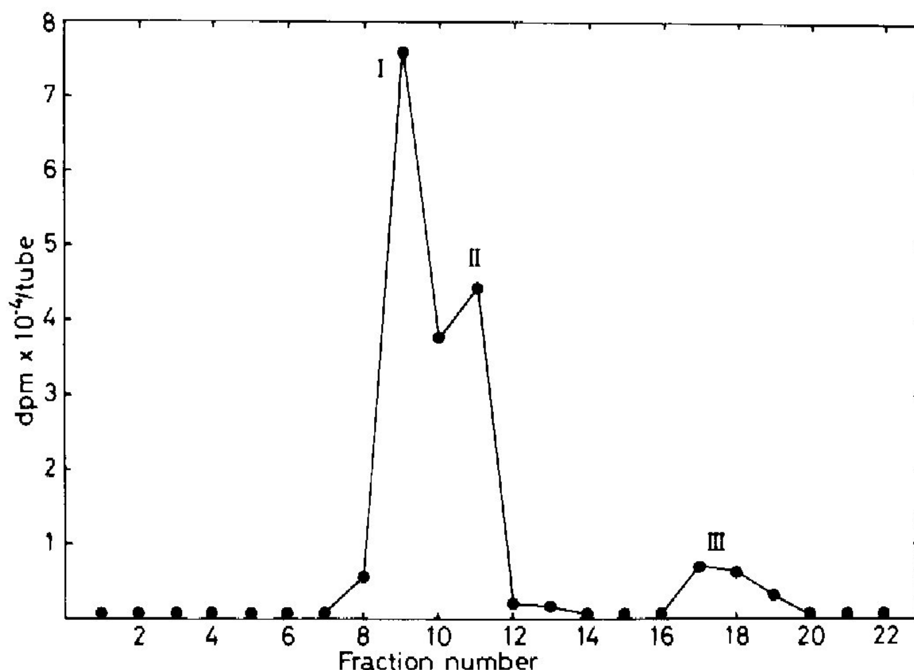



Fig. 1: Elution profile of $^{35}$S-labeled rat kidney sulfolipids from a silica beads column.

$^{35}$S-labeled amphiphiles were obtained from kidneys (1.8 g) of a rat, 16 h after intraperitoneal injection of $H_2{}^{35}SO_4$ (1 mCi). After mild alkaline methanolysis, the total extract was eluted from a column (0.6 × 40 cm) of Iatrobeads using a linear gradient of a total of 350 ml of chloroform/methanol/water, 91:10:1 to 30:70:4 (v/v). Each fraction, 5 ml each, was monitored by radioactivity measurement of $^{35}$S.

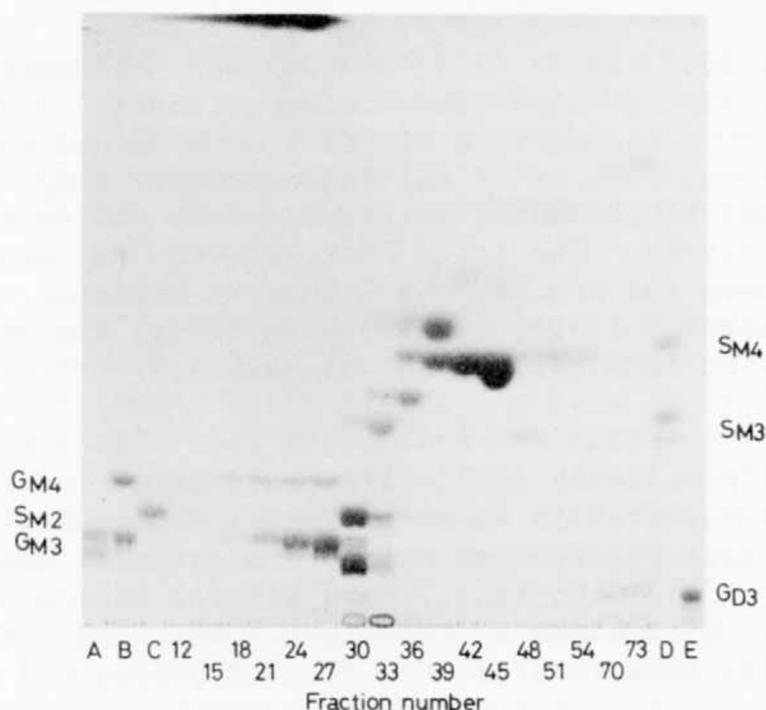



Fig. 2: DEAE-Sephacel column chromatography of alkali-resistant acidic amphiphiles of rat kidney.

Alkali-resistant and acidic lipids were eluted from a column (2 × 30 cm) of DEAE-Sephacel, acetate form, using a linear gradient of 2.0 liters of chloroform/methanol/ ammonium acetate in water (5:10:1, v/v, 0.05 to 0.7 M). Every 3 tubes, 20 ml each, were monitored by TLC with development with chloroform/methanol/acetone/acetic acid/ water (8:2:4:2:1, v/v) and staining with orcinol reagent. Lane A, GM3 from human kidney; lane B, GM4 (upper band) and GM3 (lower band) from rat kidney; lane C, SM2 from rat kidney; lane D, SM4s (upper band) and SM3 (lower two bands) from human kidney; lane E, GD3 from hog kidney cortex.

(2.0 M) were eluted in that order. Apparently the binding of the negative charge of the C-3 sulfate group with DEAE (diethylaminoethyl group) is modified by the presence of the amide group at C-2, which donates hydrogen to the neighboring sulfate group. As a result, the ionic binding force at neutral pH in ammonium acetate (3) or ammonium bicarbonate (7) was reduced so that SM2 appeared earlier than SM4s and SM3 from the column (Fig. 2). This situation is similar to the relation of the elution velocity of GM2 vs. GM3, GD2 vs. GD3 (21) and also GT2 vs. GT3 (22), when these amphiphiles are eluted from a DEAE-Sephadex column with methanolic ammonium acetate.

When the total lipid mixture of boar testis (1) was separated on a DEAE-Sephadex column, diphosphatidylglycerol (cardiolipin) was eluted earlier than SM4g, together with monosialosyl amphiphiles, indicating that the sum of negative charges contributed by two phosphodiester groups of cardiolipin is weaker than a sulfate group in SM4g. Similarly, SB2 was eluted later than GT3 as described above, indicating that the negative charge of the trisialosyl group is weaker than two sulfate groups.

The fractions containing SM2 (Fig. 2, Fractions 29 to 35), SM4s (Fractions 36 to 54), or SB2, respectively, were pooled and the final purification of each sulfoglycolipid was achieved by Iatrobeads column chromatography using a linear gradient of chloroform/methanol/water (3, 4). In a separate experiment, the combined total monosialosyl amphiphile fraction (Fig. 2, Fractions 15 to 35) was chromatographed on a column of Iatrobeads. SM2 was eluted earlier than GM4 and GM3, with some fractions overlapping those of GM4. Therefore it was necessary to degrade GM4 by neuraminidase treatment before the final purification of SM2 (7).

Several sulfolipids were subjected to desulfation by solvolysis in anhydrous dioxane. SM4g (14) and SM4s were rapidly converted to the respective desulfated compounds within 30 min at 100°C. SM2 at the concentration of 0.2 mM, however, was desulfated to about 50% after 4 h at 100°C. At the concentration of 2 mM, SM2 was completely desulfated in 2 h at 100°C (4). Similar results were obtained using a dimethylsulfoxide (DMSO) system. In 10% methanolic DMSO with 4.5 mM $H_2SO_4$ at 80°C, the sulfate esters of SM4g, SM4s, and SM3 as well as the terminal sulfate ester (at C-3 of GalNAc) of SB2 could be cleaved within 30 min, whereas the internal sulfate ester at C-3 of Gal in SM2 and SB2 could only be very slowly cleaved (3). After 3 h, SM2 was converted to a major and a minor product which migrated similarly to AM2 and LacCer, respectively. After 18 h, the proportion of the compound which corresponded to LacCer was found to be larger. When solvolysis of SB2 was performed with 8 mM $H_2SO_4$ in DMSO/methanol (9:1, v/v) at 80°C, more than 50% of SB2 was converted into a compound which migrated similarly to SM2 in 10 min. After 3 h, SB2 was completely converted into compounds which comigrated with SM2, AM2 and LacCer (3). These data suggested that the terminal sulfate ester could be cleaved more rapidly than the internal sulfate ester. The juxtapositioning of the sulfate ester to the proton-donating amide at C-2 of GalNAc may be responsible for the resistance to solvolysis. During solvolysis of SM2 and SB2, partial loss of GalNAc also occurred before the cleavage of the internal sulfate ester. A longer time of reaction with SM2 or SB2 produced a fluorescamine-positive compound, which might be the de-*N*-acetylation product. The fact that a de-*N*-acetylation product was not formed from AM2 ($GgOse_3Cer$) (Ref. 4., Fig. 6) under similar conditions strongly supported the hypothesis that the sulfate ester at C-3 of Gal has a close relationship with the amide group of GalNAc.

B. Sulfo-amphiphiles in cultured renal cell lines

a) Incorporation of $^{35}$S-sulfuric acid

The cell lines derived from monkey (JTC-12) and dog (MDCK) kidneys reacted to parathyroid hormone and vasopressin, respectively (6). Their origins were assigned to renal tubules of cortex and medulla, respectively. These epithelial cell lines not only synthesize SM4s and SM3 *in vivo* but also contain enzymes that catalyze the transfer of sulfate to GalCer and LacCer *in vitro* (8, 23). When JTC-12 cell was incubated with $H_2{}^{35}SO_4$ (5 μCi/ml) for 24 h (6, 23), four sulfate-containing compounds were detected by thin-layer autoradiography of the total lipid extract. These sulfolipids migrated similarly to cholesterol sulfate, SM4s, SM3 and SM2 in neutral, acidic (Fig. 3) and basic solvent systems. These sulfoglycolipids were isolated by column chromatographies on DEAE-Sephadex and silica beads (9). Gas chromatographic analyses of methyl glycosides and partially methylated alditol acetates obtained from these glycolipids suggested that the structures were SM4s, SM3 and SM2 (9). More detailed studies, such as infrared spectroscopy, periodate oxidation and $CrO_3$ oxidation, supported that the structure of SM2 in JTC-12 cells is similar to that from rat kidney (9). MDCK was found to contain cholesterol sulfate, SM4s and SM3, but SM2 was not detected by $^{35}$S-labeling *in vivo* (6, 8).

b) Effect of butyrate

On the addition of butyrate to the culture medium, the radioactivity incorporation into cholesterol sulfate increased in both cell lines (8) (Fig. 3). In JTC-12 cells, the incorporation into SM2 was also stimulated strongly (9). The degree of stimulation was dependent on the dose of butyrate (Table I). In contrast, the incorporation was not affected by the addition of $Bt_2$cAMP or $Bt_2$cGMP, and was significantly decreased by the addition of phosphodiesterase inhibitors, isobutylmethylxanthine (IBMX) or Ro20-1724 in the culture of JTC-12. IBMX and Ro20 elevated the level of cAMP in JTC-12 cells (base value, about 10 pmol/mg protein) to about 30 and 140 pmol/mg, respectively. The effect of butyrate on the ganglioside composition of JTC-12 cells was also examined. In control cells, GM3, GM2 and GM1 were found to be the major gangliosides, and among them, GM2 increased specifically with 2.5 mM butyrate (9). These data suggested that the enzyme which catalyzes the transfer of GalNAc to both SM3 and GM3 was induced by butyrate (9).

In the MDBK (bovine kidney) cell line, a trace amount of radioactivity was incorporated into SM3 by $^{35}$S-labeling *in vivo*. On the addition of butyrate (2.5 mM), the incorporation into SM3 increased about 20 times, suggesting that a LacCer-sulfotransferase was

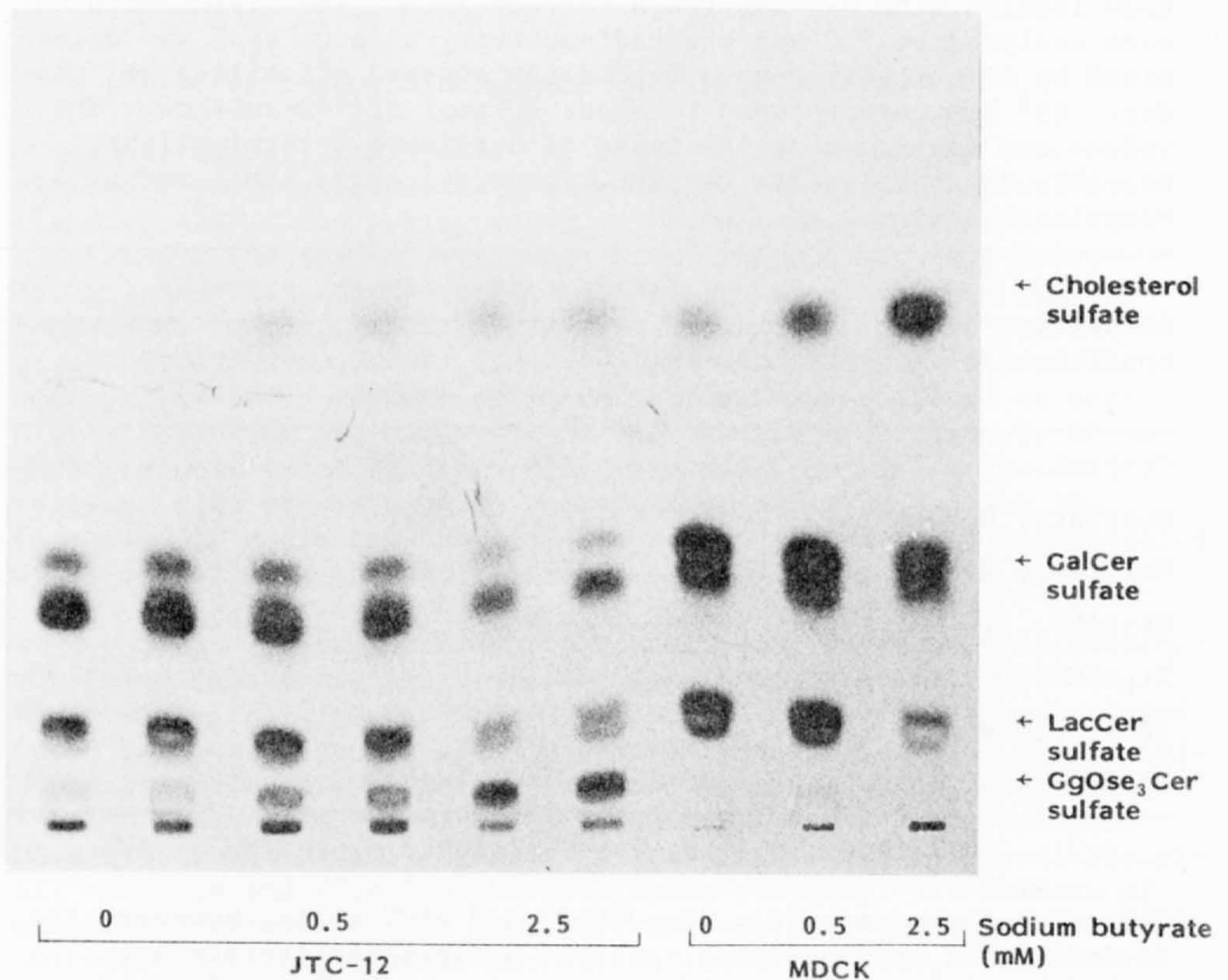



Fig. 3: Thin-layer autoradiogram of lipid extracts from JTC-12 and MDCK cells incubated with or without sodium butyrate.

The cells (nearly at the confluent stage) were preincubated with or without sodium butyrate for 24 h, and $H_2{}^{35}SO_4$ was added and the incubation was continued for a total of 48 h. The lipids were extracted from the cells and analyzed by TLC, developed with chloroform/methanol/acetone/acetic acid/water (10:2:4:2:1, v/v).

Table I. Incorporation of $^{35}$S-sulfate into individual sulfolipids of JTC-12 cells.

JCT-12 cells were incubated with different drugs for 24 h, and then labeled with $H_2{}^{35}SO_4$ for a further 24 h. The lipid extracts were analyzed by TLC and the radioactivity of each spot was determined by scintillation counting of the scraped off silica gel powder. $10^3$ dpm corresponded to about 80 pmol of $^{35}$S-sulfate. The values are expressed as the means of duplicate determinations. $Bt_2$cAMP, dibutyryl cAMP; $Bt_2$cGMP, dibutyryl cGMP; IBMX, isobutylmethylxanthine.

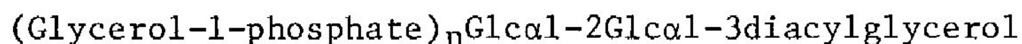
| Incubation conditions | Incorporation, dpm/mg protein | | | | |
|---|---|---|---|---|---|
| | Cholesterol sulfate | GalCer sulfate | LacCer sulfate | $GgOse_3Cer$ sulfate | Total |
| Control | 53 | 3164 | 1048 | 145 | 4949 |
| Butyrate, 0.5 mM | 267 | 2968 | 1479 | 501 | 5801 |
| Butyrate, 2.5 mM | 537 | 2665 | 1023 | 1247 | 5846 |
| $Bt_2$cAMP, 1.0 mM | 73 | 2434 | 1194 | 172 | 4175 |
| $Bt_2$cGMP, 0.1 mM | 49 | 2712 | 1147 | 129 | 4158 |
| IBMX, 0.5 mM | 26 | 1408 | 824 | 57 | 2475 |
| Ro20-1724, 0.5 mM | 46 | 1011 | 538 | 31 | 1919 |

induced by butyrate (24). In JTC-12 and MDCK cells, however, the activities of arylsulfatase, assayed using paranitrocatechol sulfate, were 126.3 ± 9.6 nmol/h/mg protein and 20.1 ± 3.5 nmol/mg, respectively, suggesting that the catabolism of sulfate esters in SM3 and SM4 was not affected. Butyrate has been reported to stimulate LacCer-sialyltransferase activity in tumor cells such as HeLa or KB (see Ref. 9). Also in a human kidney tumor cell line, it was recently shown that the synthesis of cell-associated sulfated glycoprotein was specifically elevated (25).

## C. Conclusion

Acidic amphiphiles are ubiquitous in the plasma membrane of cells in biospheres (5, 26). They contain phosphate (monoester and diester), sulfate, uronic acid and sialic acids. Since the end of the 1960s, a new type of phosphoglycolipid, *sn*-glycerol-1-phosphate linked to C-6 of the external Glc of diglucosyldiacylglycerol, has

been isolated (27) and characterized (28) from streptococci. This compound was later shown to be the precursor of a high molecular weight membrane polymer, lipoteichoic acid, which contains both hydrophilic poly(glycerol-1-phosphate) and anchor glycolipid in the molecule as shown below (20):


(Glycerol-1-phosphate)$_n$Glcα1-2Glcα1-3diacylglycerol

Polysialosyl amphiphiles of brain of poikilotherms have been extensively investigated by the groups of Tettamanti, Avrova and Wiegandt (29). Cod brain was shown to contain gangliosides with trisialosyl (NeuAcα2-8)$_3$-linkages such as GTlc, GQlc and GPlc. The brain of ray (an *Elasmobranch*) and embryonal chicken brain contained, in addition to a high amount of conventional polysialogangliosides, appreciable amounts of hexa- and heptasialosyl amphiphiles (30). To our surprise, hog kidney contained an appreciable amount of GT3-NeuAc (31). This is the first example of an extraneuronal tissue containing glycoconjugates with a (NeuAc)$_3$ residue. The presence of an enzymatic activity catalyzing the formation of a NeuAcα2-8NeuAcα2-8NeuAc linkage was also demonstrated using the crude membrane fraction from hog kidney cortex (31).

Only two sulfate-containing glycolipids, SM4s and 6-sulfoquinovosyldiacylglycerol, had been described before 1964. In 1966, Mårtensson (18) discovered SM3 from human kidney. This sulfoglycolipid has been detected also in human liver (12) and rat kidney (4). Since the discovery of SM4g as the major sulfate-containing glycolipid of mammalian spermatozoa (1), several new sulfoglycolipids have been described. They are: (Glcα1-6)$_2$Glcα1-3(1)-Gro-alkylacyl-III$^6$-sulfate and (Glcα1-6)$_3$Glcα1-3(1)Gro-alkylacyl-IV$^4$-sulfate of mammalian secretions (2); LnOse$_3$Cer-III$^6$-sulfate and LnOse$_4$Cer-III$^6$-sulfate (2) of hog gastric mucosa; sulfated GD3 ($HSO_3$-8Siaα2-8Siaα2-3Galβ1-4GlcCer) and GM2 (GalNAcβ1-4-($HSO_3$-8Siaα2-3)Galβ1-4Glc1-1Cer) from bovine gastric mucosa (32); and SM2 and SB2 from rat kidney (3, 4). The groups of Kochetkov (33) and Bergelson (34) described sulfosialosylceramides containing 8-*O*-sulfated NeuAc in sea urchin.

Now polysulfated, polysialylated and polyphosphated glycolipids constitute a diverse entity of highly acidic amphiphiles. The conventional terms, 'ganglioside,' 'sulfatide' and 'phosphatide' are now ambiguious, when one thinks how 'sulfated GD3,' 'polysialosyl phosphatidic acid' (35) or sulfated phosphatidylglycerol can be designated. Actually, Laine referred to 'phytoglycolipids' which contain uronic acid and phosphate as the 'ganglioside' of plant. Also because SM4g contains no ceramide, and because SB2 contains two sulfate groups, should they not belong to the sulfatide? We propose that the term 'acidic amphiphile' should be used to include all of these categories.

$Ca^{2+}$ and $Mg^{2+}$ have high affinity to acidic lipids (4) and bind to lipids with one negative charge in a 1:2 cation/lipid ratio (phosphatidylglycerol, SM4s and GM1) and to lipids with two net negative charges in a 1:1 cation/lipid ratio (*e.g.*, phosphatidic acid and possibly SB2). Divalent ions bridging between adjacent binding sites have been reported for $Ca^{2+}$ binding to phospholipid and sulfolipid monolayers (36).

Cultured epithelial cell lines of renal tubular origin were characterized by hormone-dependent cellular cAMP formation as follows: JTC-12, monkey cortical collecting tubule; MDCK, canine medullary collecting tubule; LLC-$PK_1$, porcine thick ascending limb of the loop of Henle; and MDBK, bovine distal tubule, definite origin not clear but probably the thick ascending limb (6). Recently, Kurokawa (37) reported that the non-cAMP-dependent stimulation of $Ca^{2+}$ ion reabsorption of rat is localized on the thick ascending limb of the loop of Henle. Nagai (38) demonstrated that the specific activity of $^{35}S$-SM4s in $^{35}S$-labeled mouse kidney was specifically elevated 7 days after hemilateral nephrectomy. Also Zwingelstein (39) found that eel gill contained an appreciable amoung of SM4s. In seawater-adapted eels, the incorporation of $^{35}S$ into gill lipids increased progressively to a high level until 24 h. The specific radioactivities of SM4s of seawater-adapted eels were about five times higher than that of sweetwater eel. Kurachi (40) found that the activation of Factor XI (thromboplastin antecedent) by Factor XII (Hageman factor) to produce a serine protease, Factor XIa, required the presence of SM4s or dextran sulfate instead of kaolin + HMW kininogen. These phenomena involving sulfo-amphiphiles should be resolved on a molecular level.

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