Kidney Sulfatides in Mouse Models of Inherited Glycosphingolipid Disorders

Determination by Nano-electrospray Ionization Tandem Mass Spectrometry

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Sulfatides show structural, and possibly physiological similarities to gangliosides. Kidney dysfunction might be correlated with changes in sulfatides, the major acidic glycosphingolipids in this organ. To elucidate their in vivo metabolic pathway these compounds were analyzed in mice afflicted with inherited glycosphingolipid disorders. The mice under study lacked the genes encoding either β-hexosaminidase α-subunit (Hexα−/−), the β-hexosaminidase β-subunit (Hexβ−/−), both β-hexosaminidase α and β-subunits (Hexα−/− and Hexβ−/−), GD3 synthase (GD3S−/−), GD3 synthase and GalNAc transferase (GD3S−/− and GalNAc−/−), GM2 activator protein (GM2a−/−), or arylsulfatase A (ASA−/−). Quantification of the sulfatides, I3SO3−GalCer (SM4s), II3SO3−GalCer (SM3), II3SO3−GalNAc-GalCer (SM2a), and IV3III3(Iv3SO3−)2−GalCer (SB1a), was performed by nano-electrospray tandem mass spectrometry. We conclude for the first time situation in mouse kidneys that: 1) a single enzyme (GalNAc transferase) is responsible for the synthesis of SM2a and GM2 from SM3 and GM3, respectively. 2) In analogy to GD1a, SB1a is degraded via SM2a. 3) SM2a is hydrolyzed to SM3 by β-hexosaminidase S (Hex S) and Hex A, but not Hex B. Both enzymes are supported by GM2-activator protein. 4) Arylsulfatase A is required to degrade SB1a. It is probably the sole sphingolipid-sulfatase cleaving the galactosyl-3-sulfate bond. In addition, a human Tay-Sachs patient’s liver was investigated, which showed accumulation of SM2a along with GM2 storage. The different ceramide compositions of both compounds indicated they were probably derived from different cell types. These data demonstrate that in vivo the sulfatides of the ganglio-series follow the same metabolic pathways with the sulfatides with the replacement of sulfotransferases and sulfatases by sialyltransferases and sialidases. Furthermore, a novel neutral GSL, IV°GalNAcβ−GbCer, was found to accumulate only in Hexα−/− and Hexβ−/− mouse kidneys. From this we conclude that Hex S also efficiently cleaves terminal β1–6-linked HexNAc residues from neutral GSLs in vivo.

The abbreviations used are: SM2a, gangliotriaosylceramide II3-sulfate; ASA, arylsulfatase A (EC 3.1.6.8); C/A, chloroform/acetone; C/M/W, chloroform/methanol/water; GalNAcT, GalNAc-transferase (β1,4-N-acetylgalactosaminyltransferase, UDP-N-acetyl-D-galactosamine:GM3/GD3 β1,4-N-acetyl-D-galactosaminyltransferase (EC 2.4.1.92)); GM2, II3-N-acetyl(or N-glycolyl)-neuraminyl gangliotetraosylceramide; GM1a, II3-N-acetyl(or N-glycolyl)-neuraminyl gangliotetraosylceramide; GD1a, II3,IV3-bis-N-acetyl(or N-glycolyl)-neuraminyl gangliotetraosylceramide; GD1a, II3,IV3-bis-N-acetyl(or N-glycolyl)-neuraminyl gangliotetraosylceramide; GD3 synthase, CMP-sialic acid:GM3/GD3 1,4-sialyltransferase (EC 2.4.99.8); GM1β-galactosidase (EC 3.2.1.23); GM2AF, GM2-activator protein; GSLs, glycosphingolipid(s); Hexα, Hexβ, or S, β-hexosaminidase (A, B, or S) (EC 3.2.1.52); MS, mass spectrometry; nano-ESI-MS/MS, nano-electrospray ionization tandem mass spectrometry; SAP B, sialoprotein activator protein B (saposin B); sialidase, neuraminidase (EC 3.2.1.18); SM, sphingomyelin; sulfatides were abbreviated according to Ishizuka (1), i.e. SM4s, galactosylerceride sulfates, GalCer I3−sulfate; SM4g, seminolipid, galactosyl-1-alkyl-2-acetyl-sn-glycerol I3−sulfate; SM5, lactosylceramide sulfates, LacCer I3−sulfate; SM1a, gangliotettraosylceramide I2III3-sulfate; SB1a, gangliotettraosylceramide II3IV3α-sulfate-bis-sulfate.

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All chemicals and solvents were of p.A. grade. Gold-sputtered boro-silicate glass capillaries, type D, were purchased from Tee Coatings Ltd. (Worcestershire, UK). Lysophosphatidyl (lys-SMs4s), sulfatide (SM4s), GlicCer, GalCer, LacCer, GM3, Gg3Cer, and bovine brain gangliosides were obtained from Matreya Inc. (Chalfont, PA), sphingosylphosphorylcholine, Forsman glycolipid, triethylamine, myristic acid, nonadecanoic acid, heptacosanolic acid, N-hydroxysuccinimide, N,N'-dicyclohexylcarbodiimide, antracene, taurosedosycholate, orcinol, absolute tetrahydrofuran, as well as, absolute N,N-dimethylformamide, both from Merck (Darmstadt, Germany). SCDase (sphingolipid ceramide N-deacylase) from Takara Shuso (Otsu, Shiga, Japan), DEAE-Sephadex A-25 from Amersham Biosciences (Uppsala, Sweden), ammonium solution 25% p.A., LiChrorep RP-18 and LiChrorep Si 60 Silica Gel from Merck (Darmstadt, Germany), and dialysis tubes, Visking type 27/32 from Roth (Karlsruhe, Germany). GM2 was isolated from human GM2 gangliosidosis brain.

Methods

**Purification of Sulfated GSL from Kidney Tissue**—SM3 was extracted from human kidney. SM2a and SB1a were isolated from rat kidney. In general, 100 g of tissue was homogenized on ice in 100 ml of distilled water with an Ultra Turrax T25 basic from IKA Labortechnik (Staufen, Germany) (6 × 2 min of homogenizing at 24,000 rpm with pauses of 2 min in between). The homogenate was centrifuged and subsequently extracted with acetone. The freeze-dried tissue then was dissolved in C/M/W as running solvent systems.

**Quantification of Purified GSL TLC Standards by Anthrone Reaction** (25)—Commercially available, or from tissue isolated and purified, GSLs were dissolved in C/M/W (10/10/1) and once with C/M/W (30/60/8). The combined C/M/W extracts were concentrated and dialyzed against 5 × 5 liters of distilled water. The dialyzed extract was lyophilized, dissolved in C/M/W (30/60/8) and loaded on DEAE A-25 column to separate neutral and acidic lipids. Elution was with a stepwise gradient of 20, 80, 200, 500, and 1000 mM methanolic potassium acetate (KAc). SM3 was eluted with the 200 mM, SM2a with the 80 mM, and SB1a in the 500 mM KAc fraction. The fractions were desalted by dialysis with 5 × 5 liters of distilled water and lyophilized. From the corresponding fractions, the sulfated GSLs were further purified by repeated silica gel flash column chromatography with the appropriate mixtures of n-hexane/isopropyl alcohol/water or C/M/W as running solvent systems.

**Experimental Procedures**

Mutant mice used: Hexa−/− (13), Hexb−/− (15), Gna2a−/− (17), ASA−/− (14) and GD3S−/− as well as GD3S−/− and GalNAC-T−/− ("GM3-only") mice (23). Hexa−/− and Hexb−/− were produced by interbreeding Hexa−/− and Hexb−/− mice as described (16). PCR was employed for genotyping (24).

The human Tay-Sachs liver material was from a girl that died at the age of 2 years and 10 months. The girl was affected with the classical form of Tay-Sachs and symptoms were apparent from 8 months of age including hyperacusis. The control liver material was from a healthy 42-year-old male donor who died in an accident.
transient 96-well microtiter plate. Absorption was measured at 620 nm.

**Synthesis of GSL Standards for Nano-ESI-MS/MS**—Lyso-SM3, lyso-SM2a, and lyso-SB1a as well as lyso-GM3 and lyso-GM2 were obtained by treatment of the purified compounds with SCDase according to Ref. 26. The crude products were purified by silica gel column flash chromatography using an appropriate mixture of C/M/W as running solvent system. Prior to purification 2-hydroxy fatty acids were esterified with acetic anhydride as follows: 2-hydroxy-myristinic acid was dissolved in 200 µl of anhydrous and alcohol-free chloroform and 50 µl of acetic anhydride of 500 mM methanolic potassium acetate. Solvent was evaporated and neutral GSL. Acidic GSL, collected as fraction 2, were eluted with 4 ml of 0.1% N,N-dimethylaminopyridine in chloroform. The reaction mixture was incubated for 60 min at 37 °C. Then 500 µl of toluol were added and the sample dried under a gentle stream of nitrogen. An aliquot of the aqueous fraction 1 was dissolved in 100 µl of anhydrous and alcohol-free chloroform and 4 µl of triethylamine. Then 2 ml of the activated fatty acid were added. The reaction mixture was incubated at room temperature for 2–5 days and monitored by TLC. Upon the long incubation some GSL were also acylated at hydroxyl groups. The reaction was completed in 60 min at 37 °C. When 500 µl of toluol were added and the sample dried under a gentle stream of nitrogen at 37 °C. The sample was dissolved again in 200 µl of toluol and dried again as before.

Fatty acids (65 µmol) were dissolved in 4 ml of dry tetrahydrofuran under nitrogen gas and activated with 0.82 equivalents of dicyclohexylcarbodiimide and 0.93 equivalents of N-hydroxysuccinimide. Reaction took place overnight at room temperature.

For condensation, about 100 nmol of lyso-GSL was dissolved in 2 ml of dry N,N-dimethylformamide and 4 µl of triethylamine. Then 2 ml of the activated fatty acid were added. The reaction mixture was incubated at room temperature for 2–5 days and monitored by TLC. Upon the long incubation some GSL were also acylated at hydroxyl groups. The reaction was completed in 60 min at 37 °C. When 500 µl of toluol were added and the sample dried under a gentle stream of nitrogen at 37 °C. The sample was dissolved again in 200 µl of toluol and dried again as before.

Evaluation of the Nano-ESI-MS/MS Data and Quantification of Lipids—Quantitative spectra were measured with an average mass resolution of 1200 (ion mass/full width half-maximum). Peak height values were calculated. The first mono-isotopic peak of each compound was taken for evaluation. From the peak intensities of the corresponding internal standard lipids a linear trend was calculated. The obtained calibration curve represented the intensity of the internal standard molar amount at a given m/z value. In addition, a linear trend for n + 2 molecular isotopic signal intensities (molecules containing either two 13C-atoms or one 15N-atom, and, thereby, shifted by m/z 2 upwards) was calculated from the measured standard. If necessary, signal intensities were corrected first from influence of n + 2 signal overlap. This overlap appears if lipids, that contain one additional double bond, are present. Then their n + 2 signal overlaps with the main signal of the lipid without this double bond. From the corrected intensity ratio (sample lipid/internal standard trend) and the amount of internal standard added the quantity of the individual molecular species (e.g., SM4s (18:1, 16:0) or SM4s (18:1, 24:1) etc.) was calculated. From the sum of individual molecular species then the amount of a lipid (SM4s) resulted. Endogenous SB1a, GM3, and GM2 were correlated to the sole corresponding standard.

**Extraction of GSLs from Human Liver for Mass Spectrometric Analysis**—For TLC analysis, 50 mg of kidney wet weight were extracted as above using the appropriate volumes. The neutral and acidic GSL fractions were each taken up in 100 µl of C/M/W (10/10/1). Aliquots according to the Figure legends were spotted on TLC plates with a Linomat IV from CAMAG (Muttenz, Switzerland) equipped with a nano-electrospray source operating at an estimated flow rate of 20–50 nL/min. Usually, 10 µl of a samples, dissolved in methanol or methanolic ammonium acetate (5 mM), was filled into a gold-sputtered capillary. The capillary was positioned at a distance of 1–3 mm in front of the cone. The source temperature was set to 30 °C and the spray was started by applying 800–1200 V to the capillary. For each spectrum 20–50 scans of 15–30 s duration were averaged. All tandem MS experiments were performed with argon as collision gas at a nominal pressure of 2 × 10−5 mbar. The parameters for the cone voltage and the collision energy of the different scan-modes are listed in Table I.

### Table I

| Polarity | scan mode | Charge of detected ions | Measured substances | Cone | Collision energy |
|----------|-----------|-------------------------|---------------------|------|------------------|
| [-]      | Precursor ion m/z 97 | (−) | Sulfatides by [HSO4]−/fragment | 70–105 | 60–115 |
| [-]      | Precursor ion m/z 220 | (−) | Sphingolipids containing HexNAC | 125 | 58 |
| [-]      | Precursor ion m/z 405 | (−) | Sphingolipids containing (HexNAC)2 | 125 | 50 |
| [-]      | Precursor ion m/z 322 | (+) | IV–GlcNacβ-Gb-Cer compared to Forssman glycolipid | 125 | 58 |
| Product ion | (−) | Fragments of sulfatides | 70–105 | 40–60 |
| (+)      | Precursor ion m/z 264 | (+) | Neutral sphingolipids containing C18-sph or C18-phyo by fragment from sphingoid base | 35–70 | 44–90 |
| (+)      | Precursor ion m/z 204 | (+) | Sphingolipids containing HexNAC | 55–90 | 65–75 |
| (+)      | Precursor ion m/z 204 | (+) | Sphingolipids containing HexNAC | 75 | 40 |
| (+)      | Product ion | (−) | Fragments of Forssman glycolipid or IV–GlcNacβ-Gb-Cer | 70 | 40 |

**Extraction of GSLs from Human Liver for Mass Spectrometric Analysis**—Human liver GSLs were extracted in analogy to the mouse kidney protocol. Since the Tay-Sachs liver was stored frozen for more than 25 years it lost barely any weight by freeze drying. Therefore GSL concentrations were calculated per mg dry weight. Extraction without MS standards was carried out with 150 mg dry weight, introducing MS standards with 20 mg.

**Determination and Characterization of Sulfatides and GSLs by Nano-ESI-MS/MS**—All analyses were performed with a triple quadrupole instrument (VG micromass (Cheshire, UK) model Quattro II) equipped with a nano-electrospray source operating at an estimated flow rate of 20–50 nL/min. Usually, 10 µl of a samples, dissolved in methanol or methanolic ammonium acetate (5 mM), was filled into a gold-sputtered capillary. The capillary was positioned at a distance of 1–3 mm in front of the cone. The source temperature was set to 30 °C and the spray was started by applying 800–1200 V to the capillary. For each spectrum 20–50 scans of 15–30 s duration were averaged. All tandem MS experiments were performed with argon as collision gas at a nominal pressure of 2 × 10−5 mbar. The parameters for the cone voltage and the collision energy of the different scan-modes are listed in Table I.
obtained after sodium borohydride reduction and peracetylation were analyzed by GC/MS using the instrumentation and microtechniques described previously (29, 30).

RESULTS

Synthesis of Internal Standards for Nano-ESI-MS/MS—For quantification of GSLs by nano-ESI-MS/MS an appropriate internal standard must be applied (24). Therefore, sulfatides and gangliosides with unusual fatty acid composition (myristic acid (14:0), 2-hydroxymyristic acid (h14:0), nonadecanoic acid (19:0), and heptacosanoic acid (27:0)) were synthesized from the corresponding lyso compounds. The latter were prepared enzymatically from the corresponding sulfatides, SM4s, SM3, SM2a, and SB1a, and gangliosides, GM3 and GM2 using SCDAse (for SM2a, see Fig. 2A). Coupling the lyso-GSL to a fatty acid as described under “Experimental Procedures,” we produced SM4s (18:1,14:0), SM4s (18:1,19:0), SM4s (18:1,27:0), SM3 (18:1,14:0), SM3 (18:1,19:0), SM3 (18:1,27:0), SM2a (h18:0,14:0), SM2a (h18:0,19:0), SM2a (h18:0,27:0), SB1a (h18:0,19:0), and recently GM3 (18:1,19:0), GM2 (18:1,14:0), and GM4s (18:1,h14:0). The saposioglycogen of human kidney SM3 consisted solely of C18-sphingosine (data not shown), whereas rat kidney SM2a and SB1a contained a mixture of C18-sphingosine and C18-phosphatidylglycerose as verified by nano-ESI-MS/MS (Fig. 2B).

Densitometric quantification of the product bands of lyso-SM2a from the orcinol/sulfuric acid-stained TLC (Fig. 2A) revealed a ratio of 57:100 for sphingosyl-lyso-SM2a to sphingosyl-lyso-SM2a (h18:0) (7). This was in good agreement with the ratio of the corresponding peaks in the nano-ESI-MS/MS spectrum (Fig. 2B) showing a sphingoid ratio of 59 (18:1):100 (h18:0). Each standard solution was quantified by densitometric scanning of the orcinol/sulfuric acid- or CuSO4/phosphoric acid-stained TLC band. On TLC, standards with C14, C19, or C27 fatty acid migrated sequentially faster as compared with one another (data not shown). For mass spectrometric quantification of each sulfatide, the respective fatty acid derivative standards were mixed in an equimolar ratio. Since the concentration of the different sulfated GSLs, i.e., SM4s, SM3, SM2a, and SB1a, was not identical in murine kidney (Fig. 3, top, lane 3), different amounts of corresponding MS standards were added to the kidney samples. For most samples, 432 pmol of SM4s, 156.6 pmol of SM3, 102.6 pmol of SM2a, and 152.2 pmol of SB1a-MS standards were added (Fig. 4). Correlating the endogenous sulfatide signals to those of the corresponding standards levels of kidney sulfatides were quantified as described under “Experimental Procedures.”

At Higher Collision Energies 2-Hydroxy Fatty Acid-containing Sulfatides Are Measured in the Precursor Ion Mode (m/z – 97) with the Same Abundance as Sulfatides Containing Non-hydroxy Fatty Acids—Since sulfatides SM4s with a 2-hydroxy fatty acid give rise to additional product ions (due to a break between the carboxyl-carbon- and the α-carbon-atom of the fatty acid), this might affect the relative abundances of the common fragments (e.g., [HSO4]− used for quantification) (31, 32). These additional fragments could be detected for SM4s (2hEA) at collision energies of 50–60 eV with not more than 7% of the intensity of fragment m/z – 97 ([HSO4]−). At collision energies of 90–115 eV that were used to quantify SM4s in the precursor ion mode, these fragments were not detectable or had an abundance smaller than 0.2%. For SM3 and SM2a additional fragments due to a 2-hydroxy fatty acid could also be detected at collision energies of 65–70 eV with up to 7% abundance relative to m/z – 97. But none of these fragments appeared at collision energies of 90–115 eV, which were relevant for quantification.

For SM4s a 2-hydroxy fatty acid containing standard SM4s (18:1,h14:0) was synthesized and mixed in an equimolar ratio with SM4s (18:1,14:0), SM4s (18:1,19:0), and SM4s (18:1,27:0). From the peak intensities of the three non-hydroxy fatty acid containing SM4s a linear trend was calculated. At low collision energy (60 eV) the measured intensity of the 2-hydroxy standard SM4s (18:1,h14:0) reached 99% of the linear trend whereas at 90 eV it differed no more than 2% from the linear trend.

Linearity of the Mass Spectrometric Method in Comparison to TLC Densitometry—To test the linearity of the mass spectrometric method, a constant amount of SM4s standard (272 pmol) was mixed in several samples with different amounts of bovine brain sulfatide (8.5 to 17.7 nmol). The values obtained by mass spectrometry as plotted against the amounts used showed that linearity was achieved from 35 to 8830 pmol (Fig. 5). The average concentration evaluated from the 9 data points in this range differed by 1.7% from the theoretical value with a standard deviation of 8%. Since bovine brain sulfatide is a mixture of sulfatides with different ceramide compositions, values obtained for some representative individual sulfatides.
with regard to mouse kidney sulfatide ceramide composition, C18-sphingosine was the most prominent sphingoid with less than 6% of additional C18-phytosphingosine and 60–70% fatty acids of C22- and C24-aliphatic chain length. In addition, fatty acids of C-16, C-18, C-20, C-21, C-23, C-26, and C-28 chain length were also detected. More than 75% of the fatty acids were saturated and the amount of 2-hydroxylated fatty acids, ~60% of the total, was twice as high for SM4s than for SM3 and SB1a with ~30%. 2-Hydroxylation was identified by both, molecular mass in mass spectrometry, as well as, the additional fragments m/z 522, 540, and 568, that appeared in the corresponding product ion spectra of SM4s (data not shown). These fragments have been reported to be characteristic for sulfatide with 2-hydroxy fatty acids (31, 32).

Gg₄Cer, Gb₂Cer, and IV⁴GlcNACβ-Gb₄Cer Accumulate in Hexa−/− and Hexb−/− Kidney—Neutral and acidic GSLs of double mutant Hexa−/− and Hexb−/− mice were isolated. As compared with the wild type mouse, TLC of the neutral GSLs revealed two double and one single bands that stained intensely with orcinol/sulfuric acid indicating the accumulation of three glycolipid components (Fig. 7, lane 1). The upper double band had a TLC migration rate corresponding to Gg₄Cer, and the lower with Gb₂Cer. Both GSLs are known to accumulate in these mice. The lower single TLC band, designated compound X, showed a migration between Forssman glycolipid and Gg₄Cer.

Investigating the neutral GSL fraction in nano-ESI-MS/MS with a precursor ion scan of m/z 264 significantly increased signals for neutral GSL with the sequence Cer-Hex-Hex-NAc (as in Gg₄Cer) and Cer-Hex-Hex-HexNAc (as for Gb₂Cer) were detected, as compared with wild type kidney. m/z +264 represents the protonated and dehydrated C18-sphingosine base, which is obtained as a characteristic fragment of neutral GSLs under these conditions. The ascribed sequence was confirmed from the collision induced fragments obtained from these molecules (data not shown). Scanning for higher neutral GSLs in nano-ESI-MS/MS, we also used a precursor ion scan of m/z +204. m/z +204 represents a protonated and dehydrated HexNAc residue which should be present at the terminus in all storage compounds of this mutant mouse. By both of these scans, signals for a GSL containing 3 Hex and 2 HexNAc residues could be identified that were not present in wild type kidney. Thus, the third accumulating GSL, compound X, contained five sugar residues.

Comparing the collision induced fragments of the protonated storage compound X with that of protonated Forssman glycolipid by nano-ESI-MS/MS indicated that the characteristic fragments were identical (Fig. 8A). From these data the structure for compound X could be assigned as HexNAc-HexNAc-Hex-Hex-Cer.

Since in Forssman glycolipid the terminal HexNAc residue is α-glycosidically linked and not a substrate for β-hexosaminidase, it is assumed not to accumulate in the GM2 gangliosidosis mice. In addition, the TLC band of compound X did not co-migrate with the Forssman lipid standard. Compound X was further analyzed by nano-ESI-MS/MS. Comparing the collision induced fragments of the deprotonated compound X and Forssman glycolipid in the negative product ion mode of nano-ESI-MS/MS, distinct differences were observed. First, the storage compound did not yield a fragment of m/z 154 that appeared in Forssman lipid standard from sheep erythrocytes (Fig. 8B, ii) or from chicken heart (data not shown). Second, a fragment with m/z 322, not present in Forssman lipid, appeared with compound X (Fig. 8B, i). This is a terminal fragment produced by ring cleavage between C2-C3 and C5-oxygen ring of the subterminal HexNAc residue. To ensure that this
fragment was not due to impurities, the neutral GSL fraction was scanned for compound X using this fragment in a nano-ESI-MS/MS precursor ion mode. The storage compound with the same ceramide pattern (C18-sphingosine combined with 16:0, 22:0, 24:1, and 24:0 fatty acids) as described before with a precursor ion scan of \( m/z 220 \), representing the deprotonated terminal HexNAc-residue was again detected (Fig. 8A, iii and iv). Since Forssman glycolipid from chicken heart had a distinctly different ceramide composition, including ceramide (C18-sph,18:0) and (C18-sph,20:0) (Fig. 9A, ii), it was admixed with the GSL fraction containing compound X. Both compounds could be detected when scanning the mixed sample either in nano-ESI-MS/MS total negative ion mode (Fig. 9B, i), or with collision induced fragments (\( m/z 405 \), Fig. 9B, ii, or \( m/z 220 \), Fig. 9B, iii) that appear in the product ion scans of both compounds. In contrast, by scanning with the compound X collision-induced fragment the \( m/z 322 \) only compound X could be detected; no signals for Forssman glycolipid appeared (Fig. 6).
Neutral GSL storage compounds in kidney of Hexa−/−, Hexa+/− and Hexb−/−, and Hexa−/− and Hexb−/− mice. Neutral GSL corresponding to 5 mg of kidney wet weight were separated on TLC with the running solvent chloroform, methanol, 0.2% CaCl₂ (60/35/8) and stained with orcinol/sulfuric acid as described.

![TLC separation of GSLs](Image)

| Mouse kidney | Age (weeks) | SM4s (pmol lipid/mg wet weight) | SM3 | SM2a (pmol lipid/mg wet weight) | SB1a | SM |
|--------------|-------------|-------------------------------|-----|---------------------------------|------|-----|
| Wild type    | 15          | 390                           | 49  | <1.4a                           | 32   | 3000|
| Wild type    | 52          | 380                           | 47  | 1.4a                            | 31   | 4200|
| Gm2α−/−      | 23          | 320                           | 25  | 6.4                             | 22   | 3300|
| Gm2α−/−      | 23          | 370                           | 25  | 7.9                             | 21   | 9200|
| Hexα−/−      | 19          | 340                           | 33  | 230                             | 38   | 3900|
| Hexα−/−      | 20          | 280                           | 41  | 270                             | 33   | 4500|
| Hexb−/−      | 13          | 240                           | 38  | 25                              | 23   | 3500|
| Hexb−/−      | 18          | 310                           | 52  | 25                              | 28   | 3600|
| Hexa−/− and Hexb−/− | 9   | 500                           | 24  | 240                             | 36   | 2000|
| Hexa+/− and Hexb−/− | 15  | 360                           | 41  | 130                             | 39   | 4000|

*No SM2a could be detected and background noise was calculated to 1.39 pmol/mg wet weight.

Fig. 7. Neutral GSL storage compounds in kidney of Hexa−/−, Hexa+/− and Hexb−/−, and Hexa−/− and Hexb−/− mice. Neutral GSL corresponding to 5 mg of kidney wet weight were separated on TLC with the running solvent chloroform, methanol, 0.2% CaCl₂ (60/35/8) and stained with orcinol/sulfuric acid as described. Lanes 1, Hexa−/− and Hexb−/−; 2 and 8, neutral GSL standard, from top to bottom: GalCer (double band), LacCer (double band), Gb3Cer (double band), Gb4Cer (strong band), Lc4Cer, nLc4Cer and Gg4Cer (strong band).

It is known that in mouse kidney a characteristic globo-series glycolipid occurs, Galβ1–4(Fucα1–3)GlcNAcβ1–6(Galβ1–3)Gb4Cer, which could be detected by nano-ESI-MS/MS in wild type and in mutant kidney samples (data not shown). Therefore, it appears highly likely that compound X is an accumulated degradation product of this glycolipid with its remnant N-acetylgalactosamine-terminal core structure IVe-GlcNAcβ1–6-Gb4Cer.

Besides C18-sphingosine and non-hydroxylated fatty acids of C16 up to C24 aliphatic chain length, hydroxy fatty acids, as well as, phytosphingosine were determined in Gb4Cer and Gg4Cer. This explains the appearance of TLC double bands for both of these glycolipids (data not shown).

**SM2A Accumulates in Hexa−/− and Hexb−/− Mouse Kidney**—Separation of the acid GSL fraction on TLC and staining with orcinol/sulfuric acid revealed a new prominent band running at the level of SM2a that does not appear in wild type kidney. No significant increase of a band at the level of GM2 was observed.

Whereas quantification of SM4s, SM3, or SB1a by nano-ESI-MS/MS showed no significant changes in concentrations, a large amount of SM2a (239 pmol/mg wet weight) was identified in kidney from a 9-week-old mutant mouse. This corresponds to an SM2a increase of at least 172-fold as compared with kidney from a wild type mouse. No significant changes in the ceramide compositions of SM4s, SM3, and SB1a, or accumulated SM2a compared with wild type were detected.

**SM2A but Not Neutral GSLs Accumulate in Hexa−/− Mouse Kidney**—TLC analysis of the neutral GSLs of Hexa−/− mice kidney showed no significant differences as compared with wild type (data not shown). In contrast, the acidic GSL component profile was characterized by a prominent band running at the level of SM2a that was not present in lipids of wild type kidney (Fig. 3, top, lane 5). However, no significant increase of a TLC band at the level of GM2 could be observed. Similar to TLC analysis, quantification of the sulfated GSL by nano-ESI-MS/MS revealed no significant changes in SM4s, SM3, or SB1a concentrations (Fig. 6B). However, in the case of kidney from 19- and 20-week-old Hexa−/− mice, large amounts (248 ± 18...
pmol/mg wet weight) of SM2a were detected that corresponded to an average increase of at least 180-fold as compared with the wild type (Table II and Fig. 10). No significant changes in the ceramide compositions of sulfated GSL compared with wild type were detected. The SM2a pattern was similar to that of Hexa/H11002/H11002 and Hexb/H11002/H11002 mice.

Accumulation of Gg3Cer and Gb4Cer but Not IV6GlcNAc1-6Gb4Cer in Hexb/H11002/H11002 Kidney—TLC analysis of the neutral GSLs of kidney from Hexb/H11002/H11002 mice showed storage of Gg3Cer, Gb4Cer, but no accumulation of GlcNAcβ1-6Gb4Cer. There was no significant difference in Gg3Cer and Gb4Cer TLC-band intensities between Hexb/−/− and Hexa/−/− and Hexb/−/− double mutant kidney (Fig. 7).

SM2A Accumulates in Hexb/−/− Kidney—In the case of the Hexb/−/− mouse mutant, TLC of the kidney acidic GSLs showed the appearance of a faint band migrating identically to the SM2a standard (Fig. 3, top lane 6). By nano-ESI-MS/MS, 24.7 ± 0.05 pmol of SM2a per mg wet weight was quantified in a 13- and 18-week-old mutant kidney corresponding to an average increase of at least 18-fold as compared with the wild

![Fig. 8: Fragmentation patterns of compound X from Hexa/−/− and Hexb/−/− and of Forssman glycolipid from sheep erythrocytes by nano-ESI-MS/MS-product ion mode. A, fragments in positive mode: (i) compound X (18:1,24:0) with m/z 1542.9 and (ii) Forssman glycolipid (GalNAc-α1,3-Gb4Cer (18:1,24:0)) with m/z 1542.9. Comparing the fragments of both compounds demonstrates, that the sugar increments are ordered in the same sequence: Cer-Hex-Hex-HexNAc-HexNAc. B, fragments in negative mode (i) compound X (18:1,24:0) with m/z 1540.9 and (ii) Forssman glycolipid (18:1,24:0) with m/z 1540.9. Fragment m/z 322 is generated only by compound X (i) and represents the terminal HexNAc with a fragment of the sub-terminal HexNAc generated by a ring cleavage between C2-C3 and ring O-C1. On the other hand, fragment m/z 154 can only be generated by Forssman glycolipid (ii), indicating that both compounds are not identical. C, structures and fragmentation schemes of compound X and Forssman glycolipid.](https://example.com/fig8.png)
type (Table II, Fig. 10). No significant changes in SM4s, SM3, or SB1a-concentrations were found. No significant differences in the ceramide compositions of sulfated GSL compared with wild type were detected. The SM2a pattern was similar to that of double mutant Hexa−/− and Hexb−/− mice. SM2a pattern was similar to that of double mutant Hexa−/− and Hexb−/− mice.

All Sulfatides Accumulate in Arylsulfatase A-deficient Kidney—The neutral GSLs of ASA−/− mouse kidney were not different from the wild type (data not shown). In contrast, TLC of the mutant mouse kidney acidic GSL fraction showed strong increases in bands co-migrating with SM4s, SM3, and SB1a. All were stained by orcinol/sulfuric acid spray reagent (Fig. 3, bottom, lane 3) and with azur A (data not shown).

In a 11-week-old ASA−/− kidney, 11-, 4.4-, and 15-fold accumulation of SM4s, SM3, and SB1a, respectively, was quantified by nano-ESI-MS/MS. Analysis of a kidney from a 1-year-old ASA−/− mouse demonstrated a further increase in the accumulation of SM4s, SM3, and SB1a to about 80-, 40-, and 60-fold, respectively. However, no further increase in the accumulation of these GSLs was seen in a 2-year-old ASA−/−
Kidney Sulfatides of Mutant Mice

Assay of sphingolipid composition in mutant mouse kidney

Kidney sulfatide concentrations were very similar to those of the 1-year-old ASA/− kidney (Table III and Fig. 11). No significant changes in the ceramide constituent compositions of SM4s, SM3, and SB1a were detected in comparison with wild type.

Mice Deficient in β-GalNAc Transferase Lack SB1A in the Kidney—No significant differences, as compared with wild type, were observed by TLC for the neutral kidney GSLs of GD3S/− and GalNAcT+/+ and GalNAcT−/−, and GD3S/− and GalNAcT−/− mutants (data not shown).

With regard to the acidic GSLs, the TLC profile of the GD3S− and GalNAcT−/− kidney was characterized by the disappearance of a band co-migrating with the SB1a standard (Fig. 3, bottom, lane 7). Quantification of the sulfated GSL in these mutants revealed a 20% decrease of SB1a with a corresponding increase in SM3 in GD3S−/− and GalNAcT−/−/− kidney as compared with GD3S−/− and GalNAcT+/-/+. Kidneys from GD3S−/− and GalNAcT−/− mutants showed an increase in SM3 with SB1a being undetectable (Table IV).

SM2A Accumulates in Addition to GM2 in a Tay-Sachs Patient’s Liver—Acidic GSLs were extracted with and without internal MS standards from a Tay-Sachs patient’s and a control human liver. In both livers, comparable amounts (~10%) of GM3 were detected in agreement with the data published by Nilsson and Svennerholm (33). In addition, significant amounts of SM2a and GM2 could only be detected in the Tay-Sachs liver (Table V). The ceramide composition of all three GSLs, GM3, GM2, and SM2a, was different from each other, whereby that of GM3 and GM2 were comparable to the values reported earlier (33) (Figs. 12 and 13). With 57% GM2 containing stearic acid (GM2(18:1,18:0)) was the main GM2 species. In contrast to this, with 31% SM2a containing a C24:1-fatty acid (SM2a(18:1,24:1)) was the main SM2a species.

DISCUSSION

The acidic glycolipids, because of their negative charge and often complex and seemingly systematic structures appear as particularly enigmatic with regard to their physiological significance. Naturally occurring genetic mutants that affect their metabolism have so far been observed only for deficiencies in catabolizing enzymes causing glycolipid accumulations. For the study of such inherited sphingolipid storage diseases, and more recently, with a more basic view on the possible elucidation of study of such inherited sphingolipid storage diseases, and more significance. Naturally occurring genetic mutants that affect their physiological sig-

Kidney, particularly in the mouse, is the organ in which sulfatides are highly concentrated and have been associated with renal transport and metabolism (1, 34). Therefore, an investigation was initiated to characterize alterations of sulfatides in mutant mouse models. The sulfatide components were characterized and quantified by nano-electrospray tandem mass spectrometry. This technique has already been successfully applied to characterize and quantify different sphingolipids (31, 32, 35–44). Internal standards were synthesized from lyso-sulfatides containing fatty acids of unusual chain length. Lyso-sulfatides were obtained by enzymatic cleavage of fatty acids from the parent GSL compounds using sphingolipid ceramide N-deacylase (SCDase) from Pseudomonas sp. (26). In the case of somewhat alkali-sensitive GSLs such as SM2a and SB1a, the enzymatic method of ceramide fatty acid cleavage offers advantage over the chemical cleavage.

Quantification of sulfatide by mass spectrometry was linear over more than 2 orders of magnitude with R² greater than 0.995 from 18 to 8830 pmol. In contrast, the conventional quantification of GSLs by densitometric scanning on TLC plates was only linear in the range of 100 to 700 pmol (with R² of 0.995). Accordingly, the linear range of quantification by nano-ESI-MS/MS was ~70 times greater than that of densitometric scanning. Furthermore, nano-ESI-MS/MS proved to be more sensitive than chemical staining of GSLs on TLC. In addition, mass spectrometric quantification gives more detailed information about the quantitative ceramide compositions of each individual GSL species, which could vary between different samples. No differences beyond the accuracy of the method could be detected when comparing the mass spectrometric sensitivity of 2-hydroxy fatty acid-containing sulfatide SM4s (18:1,14:0) with sulfatides containing non-hydroxylated fatty acids (collision energy: 90–115 eV). Nevertheless, at lower collision energies (50–60 eV) detection of sulfatides SM4s with a 2-hydroxy fatty acid was about 10% less sensitive than for sulfatides without 2-hydroxy fatty acid. At these energies, additional fragments due to a break between the carboxyl-carbon- and the a-carbon-atom of the fatty acid are produced (31, 32), which seem to influence the abundance of the sulfate fragment. At higher energies (90 to 115 eV) these fragments disappear for all complex sulfatides. Therefore extrapolation of the SM4s data onto SM3, SM2a, and SB1a is justified and all sulfatides can be correlated to standards with non-hydroxy fatty acids.

SB1a was absent in β-GalNAc transferase-deficient mice as expected from an in vitro study suggesting that a single enzyme synthesizes both GM2 and SM2a (45). In the kidneys of these knockout mice, SM3 accumulated to a level comparable with the combined levels of SM3 and SB1a of wild type mouse kidney. The total concentration of these two sulfatides taken together, was also retained at the same level in GalNAcT−/−/− kidneys. Therefore, it appears plausible that SM3 is the precursor for SB1a biosynthesis. First SM3 is converted to SM2a by the action of GalNAcT and, possibly, via formation of SM1a to SB1a. This would be in analogy to the synthesis of GD1a in brain (9). The absence of SB1a in GalNAcT−/− mice is consistent with its structural ganglio-series derivation.

Sulfatides like all other GSL are degraded in the cellular

| Mouse kidney | Age | SM4a | SM3 | SM2a | SB1a | SM |
|--------------|-----|------|-----|------|------|-----|
| ASA/−/−      | 10  | 230  | 48  | ND   | 17   | 3700|
| ASA−/−      | 11  | 2600 | 210 | ND   | 260  | 3400|
| ASA+/-      | 47  | 210  | 87  | ND   | 19   | 3900|
| ASA−/−      | 53  | 18,000 | 1900| ND   | 970  | 4600|
| ASA+/-      | 98  | 310  | 37  | ND   | 30   | 4100|
| ASA−/−      | 95  | 19,000 | 1700| ND   | 890  | 2600|

* ND, not detectable.
lysosomes. For SM4s, the initial step in degradation is the removal of sulfate by the combined action of arylsulfatase A and the activator protein SAP B (10). Thus far this is the only sulfatase known to act on sulfatides (1). Since SM4s, SM3, and SB1a all carry a sulfate group linked to a terminal sugar residue, the cleavage of the sulfate group is expected to be the first step of their biodegradation. It was shown here that in ASA−/− mice that SB1a together with SM4s and SM3 accumulates in the kidney. This finding demonstrates the ability of both, human Hex S and Hex A, to degrade SM2a in the presence of human GM2 activator protein. For these two mutant mice, SM2a accumulation was comparable indicating that Hex B, the only relevant active enzyme isomer expressed in Hexa−/− mice, did not act on SM2a. This is in good agreement with previous in vitro studies (46, 47).

Hexa−/− mice, expressing only an intact Hex S isomer, accumulated 10 times less SM2a than Hexb−/− or Hexa−/− and Hexb−/− mutants. It is, therefore, concluded that Hex S plays an important and necessary role in the in vivo degradation of SM2a. In contrast, Hex A is the pivotal enzyme for the degradation of GM2 (47). Nevertheless, Hex A contributes to SM2a degradation in vivo: Hexb−/− mice left with only Hex S, but no Hex A and Hex B activity, accumulate SM2a to a lesser degree. These findings are in agreement with in vitro studies demonstrating the ability of both, human Hex S and Hex A, to degrade SM2a in the presence of human GM2 activator protein (47).

For the degradation of GM2 in mice two metabolic pathways have been described. One catabolic sequence, in humans the only significant pathway, is the degradation of GM2 to GM3 by Hex A in presence of GM2AP, and further hydrolysis of GM3 to LacCer. Another mode of degradation is the formation of GalCer from GM3, and the activator protein SAP B (10). Thus far this is the only sulfatase known to act on sulfatides (1). Since, in contrast to GM2, Hexa−/− and Hexb−/− mice accumulated SM2a to an equal extent, it is sug-
gested that there is no significant degradative pathway from SM2a to Gg3Cer in mice.

GM2 activator protein stimulates in vivo not only the enzymatic ganglioside hydrolysis but also the degradation of SM2a. This is concluded from the significant accumulation of SM2a in Gm2a/H11002/H11002 mice, which again is in agreement with in vitro results (46, 47). However, as compared with hexosaminide-deficient mutant mice, the accumulation of SM2a was small (roughly 3.5 times lower than in Hexb/H11002/H11002, and 35 times lower than in Hexa/H11002/H11002 and Hexb/H11002/H11002 mice). The degradation of SM2a to SM3 must still be operative in the absence of GM2 activator protein in the Gm2a/H11002/H11002 mice. In conclusion, SM2a is degraded by Hex S and Hex A with both enzymatic hydrolases increased in effectiveness by GM2AP.

SM2a accumulation was also observed in a human Tay-Sachs liver. SB1a has only been described in a human hepatic carcinoma cell line (48). We conclude that this lipid is the degradative precursor of the accumulating SM2a in the human Tay-Sachs liver. Thus, it appears that the human pathway for complex sulfatides is very similar to that of mice. Interestingly, the ceramide patterns of the SM2a and GM2 of the human Tay-Sachs liver are very different. Therefore we conclude that SM2a and GM2 are metabolized in different pools, e.g. different cell types.

With regard to neutral GSLs as storage compounds in gangliosidoses, Gg3Cer and Gb4Cer are known to accumulate in Sandhoff disease and Hexa/H11002/H11002 mice. Only in the Hexa/H11002/H11002 and Hexb/H11002/H11002 double mutant mice was a third neutral glycolipid found to accumulate. Its structure, IV6GlcNAc/H9252-Gb4Cer, was identified by nano-ESI-MS/MS, GC/MS, and permethylation analysis. It can be distinguished from Forssman glycolipid in the negative ESI-MS/MS product ion scan by an additional ring-cleavage fragment (m/z 322). It is assumed that the likely precursor of IV6GlcNAc/Gb4Cer is the typical mouse kidney octaosylceramide Galβ1–4(Fucα1–

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**Table VI**

| Mouse model                                      | Knocked out genes | Remaining β-hexosaminidase (subunit combination) | Accumulation of SM2a in mouse kidney |
|--------------------------------------------------|-------------------|--------------------------------------------------|-------------------------------------|
| Tay-Sachs disease (TSD) (15)                      | Hexa–/–           | Hex B (β/β)                                      | +++                                 |
| Sandhoff disease (SD) (15)                        | Hexb–/–           | Hex S (α/α)                                      | +                                   |
| Combination of TSD and SD (16, 55)                | Hexa–/– and Hexb–/– | None                                             | +++                                 |
| GM2 activator deficiency (AB variant) (17)        | Gm2a–/–           | Hex A (α/β) and Hex B (β/β)                      | +                                   |

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**FIG. 12.** Composition of accumulated SM2a in a human Tay-Sachs patient’s liver as determined by ESI-MS/MS-precursor ion m/z 97 mode.

**FIG. 13.** Composition of GM3 and accumulated GM2 in a human Tay-Sachs patient’s liver as determined by ESI-MS/MS-precursor ion m/z 87 mode. Peaks are labeled according to masses derived by gangliosides containing C18-sphingosine, since this is the main sphingoid in these gangliosides according to Nilsson and Svennerholm (33). Nevertheless, gangliosides with C20-sphingosine (4% in GM3 and 21% in GM1 (33)) and a corresponding smaller fatty acid (shorter by a C2H4-unit) give rise to the identical signals.
3′GlcNacβ1–6Galβ1–3GbCer (49–52). The fact that IVGlcNacβ-GbCer does not accumulate in Hexβ−/− mice, demonstrates that Hex S is able to degrade neutral GSLs with a terminal β1,6-linkage in vivo. In addition, one remaining wild type allele for the hexosaminidase α-subunit in Hexα−/− and Hexβ−/− mice is sufficient to maintain a level of Hex S activity that suffices for the complete degradation of IVGlcNacβ-GbCer (Fig. 11, lane 7). This extends the spectrum of glyco-compound substrates on which Hex S can act to include neutral GSLs with a terminal β1,6-linked N-acetylhexosamine.

The knowledge of all storage compounds and the resulting cellular changes due to GSL accumulation are a prerequisite for a more complete understanding of the pathology of the respective human diseases. And, by analogy, knowledge of all GSL that are missing in mutant mice may be important for a correct interpretation of the respective phenotypes. Furthermore, the accumulation of GSL in pathologic tissues, such as SM2a in human Tay-Sachs liver, demonstrates the existence of certain GSL components in particular organs where they may play special functional roles.

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