Chromatographic Separation from Food Materials of Galactocerebroside and Sulfatide According to their Degree of Hydroxylation and Characterization by ^{1}H and ^{13}C NMR and by IR Spectroscopy

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ADRIAN IGA[a], NICOLETA FLORENTINA PREDESCU[b], SILVIA IGA[a], ALINA NICOLESCU[c], DUMITRU PETRU IGA[a*]

[a] *Faculty of Biology, Splaiul Independentei 95, Bucuresti-5, Romania, R-76201, e-mail pdiga49@yahoo.com*

[b] *LCCF-Bucharest, Roumania*

[c] *"Petru Poni" Institute of Macromolecular Chemistry, Group of Biospectroscopy, Iasi, Roumania*

[*] *corresponding author*

Abstract

The homogenized tissue was extracted with chloroform-methanol and chloroform-methanol-water mixtures and the total lipids were partitioned according to Folch. Lipids of lower phase were chromatographed on Florisil and the mixture of galactocerebrosides and sulfatides was subsequently separated by ion-exchange chromatography on DEAE-Sephadex A-25. Galactocerebrosides and sulfatides were then separated according to their degree of hydroxylation by chromatography on silica gel in gradients of methanol in chloroform. Fractions containing homogenous compounds were mixed, the solvent removed and the respective glycolipids peracetylated by stirring overnight with a pyridine-acetic anhydride mixture. Alternatively, they were perbenzoylated by heating with a mixture of benzoyl chloride-pyridine. ^{1}H and ^{13}C NMR spectra were registered on peracylated compounds by using $CDCl_3$ as solvent. IR spectra were registered on native compounds. The native compounds could be reconstituted from the acylated ones by Zemplen saponification.

Keywords: galactocerebroside, sulfatide, NMR spectroscopy, IR spectroscopy, acetylation, benzoylation, hydroxylation degree

Introduction

Galactocerebroside (ß-D-galactopyranosyl-1'ceramide, GalCer) and sulfatides (ß-D-(sulfo-3)galactopyranosyl-1'ceramide, sulfo-3GalCer, cerebroside 3-sulfate) were discovered by Thudichum in 1874 as important constituents of the nervous tissue [1]. Structure and configuration of sphingosine and ceramide, as constituents of galactocerebroside and sulfatide were elucidated by working on sphingomyelin (ceramide-phosphoryl-choline) and galactocerebroside [2] [3].

The highest concentration and amount of galactocerebrosides and sulfatides is mainly found in especially in the brain. In case of a defective lysosomal enzyme, galactocerebrosidase, galactocerebroside accumulates in the white matter and the result is Krabbe's disease [4]. This disease manifests itself as a neurological disorder characterized by severe myelin loss and mental and motor deterioration in infants [5] [6]. Ester sulfate of cerebroside 3-sulfate is cleaved, in a reaction catalyzed by arylsulfatase A in the presence of a proteic activator, to galactocerebroside and inorganic sulfate; a defect in the enzyme or/and in the activator protein produces the disease called metachromatic leukodistrophy. There are three forms of metachromatic leukodistrophy: late infantile, juvenile, and adult. In advanced stages of the late infantile metachromatic leukodystrophy, the myelin in the nervous system becomes devastated: abnormal metachromatic granules accumulate in the central and peripheral nervous systems and in the kidneys, and are excreted in the urine [7]. Excessive levels of cerebroside sulfates (sulfatides) have been found in the metachromatic tissues [8] [9]. In the case of adult metachromatic leukodystrophy, it was found a relative increase in short chain fatty acids mainly affecting the cerebrosides and also a decrease of unsaturated fatty acids affecting mainly the sulfatides. The whole composition of the sulfatides was altered to a larger extent than that of the cerebrosides and this result was in contrast to results obtained in infantile metachromatic leukodystrophy [10]. The cerebroside sulfate activator (also called SAP-1 or Saposin B) is a small protein involved in the catabolism of cerebroside sulfates and a number of other glycosphingolipids. Cerebroside sulfate activator is believed to function by binding target lipids, extracting them from membranes or

micelles and making them available to water soluble enzymes. The cerebroside sulfate activator is defective in a rare form of metachromatic leukodystrophy and the responsible mutations have been defined [11]. In a mouse model of the human disease metachromatic leukodystrophy, ie arylsulfatase A deficient mice, sulfatide accumulates intracellularly in neurons and astrocytes [12]. A galactocerebroside was isolated and characterized from human aorta [13] while sulfatide was found in porcine plasma [14] and in rooster testis [15].

In spite of numerous papers dealing with the separation of galactocerebroside and sulfatide, they still have a relatively high cost. In this paper, we present a new procedure for their isolation and chemical and spectral characterization.

Materials and Methods

Materials. Reference samples of galactocerebroside and sulfatide, Florisil and DEAE-Sephadex A-25 were from Sigma. Silica gel for column chromatography and thin layer plates of silica gel were from Merck.

Methods. 1.**Activation of chromatographic materials**. Silica gel for column chromatography as well as the plates for thin layer chromatography were activated by heating for 45 min at 120 ^{0}C. Florisil was activated for heating at 140 ^{0}C for four hrs. DEAE-Sephadex A-25 was activated by stirring repeatedly in a mixture of chloroform-methanol-1M sodium acetate 30/60/8, (v/v) and then four or five times in a mixture of chloroform-methanol-water, 30/60/8, (v/v). Then it was suspended in a column containing the latter solvent.

2.**Thin layer chromatography**. Plates were alternatively migrated in the following solvent systems: I, chloroform-methanol-water, 65/25/4 (v/v); II, chloroform-methanol-water, 50/10/1 (v/v); III, chloroform-methanol-acetone-acetic acid-water, 10/2/4/2/1 (v/v); IV, chloroform-methanol-water-concentrated ammonia, 12/8/1/1 (v/v); V, n-propanol-water, 8/2 (v/v).

3.**Homogenization and extraction of total lipids**. Tissue was homogenized in seven volumes of methanol with a Waring Blendor or by grinding with quartz sand in a mortar. 14 volumes of chloroform were added to the suspension and the whole mixture was stirred overnight at room temperature. After filtration, the material on filter was resuspended again in 10 volumes of chloroform-mixture, 1/1 (v/v), stirred overnight and filtered. The procedure was repeated once more on the extracted material by using five vols of a mixture consisting of chloroform-methanol-water, 10/5/1 (v/v). Finally, all filtrates were unified and the chloroform-methanol mixture were adjusted to 2/1, by adding enough chloroform.

4.**Folch partition**. Two solutions were prepared just before Folch partition: one containing chloroform-methanol-0.11 M aqueous potassium chloride, 86/14/1, (v/v) (Folch lower phase, FLP), and the other one containing the same solvents in the ratio 3/48/47 (v/v) (Folch upper phase, FUP). The solution of total lipids was mixed and energetically stirred in a separation funnel with a fifth volume of 0.11 M solution of potassium chloride. The two layers formed were separated and the lower layer was washed two times in a separation funnel with an equal volume of FUP while the upper layer washed successively in the same way with two volumes of FLP. Finally, phases containing solvents in the same ratio were mixed. The solution with higher density contains galactocerebroside, sulfatide, phospholipids, free cholesterol, cholesterol esters, etc. The lighter solution is reach especially in gangliosides.

5.**Florisil chromatography**. Solution of total lower layers of lipids was evaporated to dryness, the residue resumed in the smallest volume of a mixture of chloroform-methanol-water, 10/5/1, (v/v) and applied on a Florisil column in the ratio 2.5-3 g of total lipids per 100 g of Florisil. Elution was made with three vols of chloroform and then with a gradient of methanol in chloroform. Fractions were analyzed by thin layer chromatography (TLC) in solvent I in comparison with reference samples of galactocerebroside and sulfatide.

6.**DEAE-Sephadex A-25 chromatography**. Fractions proved to contain galactocerebrosides and sulfatides were mixed, concentrated to dryness, resumed in a small volume of chloroform-methanol, 1/1 (v/v) and applied on the column. The column was washed with 4-5 volumes of methanol and then with a gradient chloroform-methanol-water, 30/60/8 - chloroform-methanol-M sodium acetate 30/60/8, (v/v). Fractions were analyzed by TLC in solvent I in comparison with reference samples of galactocerebroside and sulfatide.

7.**Silica gel column chromatography**. Fractions containing galactocerebroside from the preceding column were mixed, concentrated to dryness and the residue resumed in a small volume of chloroform-methanol, 2/1 (v/v). Silicagel column chromatography was made by elution with chloroform-methanol, 70/30 containing a small amount of water. Fractions containing sulfatides were mixed, evaporated to dryness and

dialyzed against distilled water or submitted to Folch dialysis against the same solvent. Then sulfatides were submitted to silica gel column chromatography in a similar manner with galactocerebrosides.

Determination of chemical constituents. A small sample containing 0.1-1.0 mg of glycolipid, either galactocerebroside or sulfatide, was heated for 3 hrs in a mixture consisting of ethanol-chloroform-concentrated hydrochloric acid, 7.4/6/3 (v/v) [15]. Lipidic solution was then concentrated to dryness and the residue partitioned between 7 mL water and 0.6 mL chloroform. Aqueous phase served for sugar (antrone) and sulfate (rhodizonate) determination, while lipidic constituents were determined in chloroformic phase [15].

IR Spectra of native compounds were registered in KBr pellets or in an adequate solvent.

NMR Spectra. Fractions containing homogenous compounds were mixed, the solvent removed and the respective glycolipids peracetylated by stirring overnight with an excess of pyridine-acetic anhydride mixture. Alternatively, they were perbenzoylated by heating with a mixture of benzoyl chloride-pyridine. ^{1}H and ^{13}C NMR spectra were registered on peracylated compounds by using $CDCl_3$ as solvent.

The native compounds could be reconstituted from the acylated ones by Zemplen saponification.

Results

Folch partition.In vertebrates brain, cerebroside and sulfatide are abundant enough that they can be distinguished by thin layer chromatography in solvent systems I, III or IV. However, by Folch partition, numerous constituents, that are relatively abundant in this tissue, especially gangliosides, are removed by Folch partition.

Adsorption chromatography on Florisil. As expected, due to its content in magnesium oxide, Florisil retains a great amount and a remarkable diversity of phospholipids. Florisil chromatography can be driven in two ways. (A) By a sharp increase in the polarity of eluents, a mixture of galactocerebroside and sulfatide is obtained (Figure 1). (B) A careful control of solvent polarity during elution would provide fractions of galactocerebroside that are free of sulfatide and fractions containing a mixture of galactocerebroside and sulfatide.

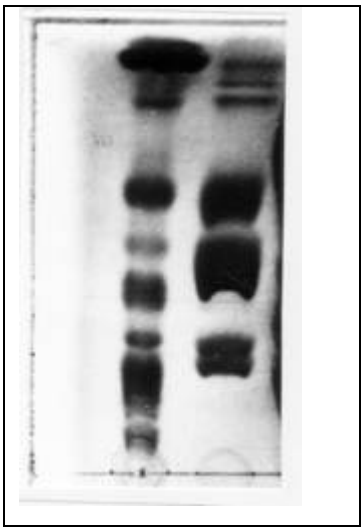


Figure 1. Separation of galactocerebroside and sulfatide by Florisil column chromatography evidenced by TLC. Start 1, lipids of lower layer after Folch Partition; start 2, a mixture of galactocerebrosides and sulfatides separated by Florisil (from top to bottom: traces of cholesterol and triglycerides, galactocerebroside containing normal fatty acids, galactocerebroside containing hydroxy fatty acids, sulfatides containing normal fatty acids, sulfatides containing hydroxy fatty acids). Solvent III: chloroform-methanol-acetone, acetic acid, water, 10/2/4/2/1 (v/v). Visualysation with mostain.

Ion-exchange chromatography. Separation of galactocerebrosides from sulfatides as well as removing of the last traces of ionic impurities from galactocerebrosides fractions are comfortably accomplished by ion-exchange chromatography on DEAE-Sephadex A-25. By this chromatography, galactocerebroside and sulfatide are completely separated (Figure 2).

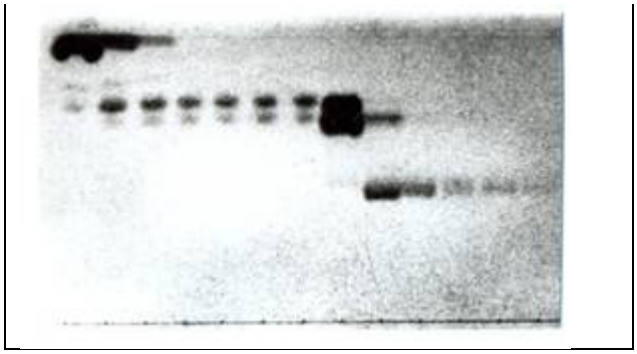


Figure 2. TLC of fractions indicating separation of galactocerebrosides and sulfatides by DEAE-Sephadex A-25. Solvent I, chloroform-methanol-water, 60/25/4, (v/v). Visualysation with mostain.

Chromatography on silica gel. The last traces of impurities were removed from galactocerebroside and sulfatide by silica gel column chromatography (Figure 3). The prerequisite condition for this type of chromatography is that lipid mixture be free of ions (sodium acetate) that were used in the preceding step for desorption from DEAE-Sephadex A-25.

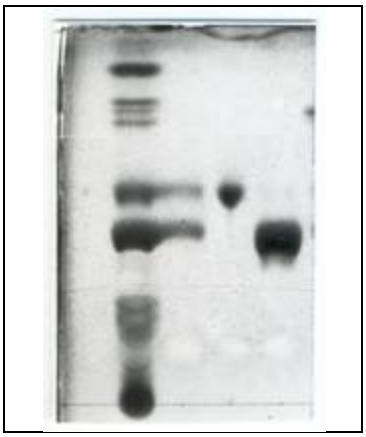
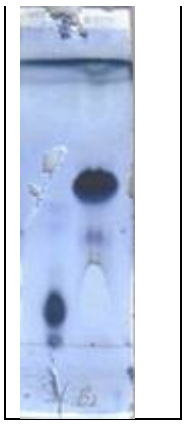


Figure 3. TLC illustrating separation of galactocerebrosides by silica gel chromatography according to hydroxylation degree. Start 1, lipids of lower layer after Folch partition; start 2, galactocerebrosides after Florisil and DEAE Sephadex A-25 chromatography; start 3, galactocerebrosides containing normal fatty acids; start 4, galactocerebrosides containing hydroxy fatty acids. Solvent I, chloroform-methanol-water, 60/25/4, (v/v). Visualysation with mostain.

Consequently, sodium acetate was removed either by dialysis or by gel-filtration chromatography on Sephadex G-25. In this stage, galactocerebroside and sulfatide were relatively pure, so a lower ratio (5-10 mg lipids/g silica gel) could be applied. In fact, it was in this step that galactocerebroside and sulfatide were separated according to their degree of hydroxylation (Figure 3). In this stage, pure chemical species of galactocerebrosides and sulfatides were obtained. They presented a unique spot in solvent systems I-V, by TLC. Galactocerebroside contained D-galactose, sphingosin and fatty acid in the ratio 1:1.05:0.94, and sulfatide contained sulfate, D-galactose, sphingosin and fatty acid in the ratio 1.1:1:1.05:0.94. The four glycosphingolipids isolated were either peracetylated or perbenzoylated (Figure 4).

Figure 4. TLC for benzoylated sulfatide; start 1, sulfatide; start 2, benzoylated sulfatide; solvent, chloroform-methanol-water, 50/10/1, (v/v); visualysation with mostain.

IR Spectroscopy. IR Spectra disclosed the characteristic groups of the two glycosphingolipids. Galactocerebroside contained the following absorption bands: 3500 cm^{-1}, OH groups; 2940 and 2860 cm^{-1}, CH_2; 1650 cm^{-1}, amide linkage. Sulfatides contained very similar absorption bands; moreover it presented absorption at 1240 cm^{-1}, sulfate ester.



NMR Spectra. ^{1}H NMR Spectra of peracetylated galactocerebroside containing hydroxy fatty acids displayed all the signals (in ppm) of constitutive fragments: 0.863 (t, methyl terminal groups of sphingosine and fatty acid), 1.256 (m, methylene groups of sphingosine and fatty acid), 1.979-2.049 (s, methyl group of acetate); 6.343 (d, NH) the signals of the sugar were also present but relatively small, and of this reason they were presented as a detail (Figure 5).

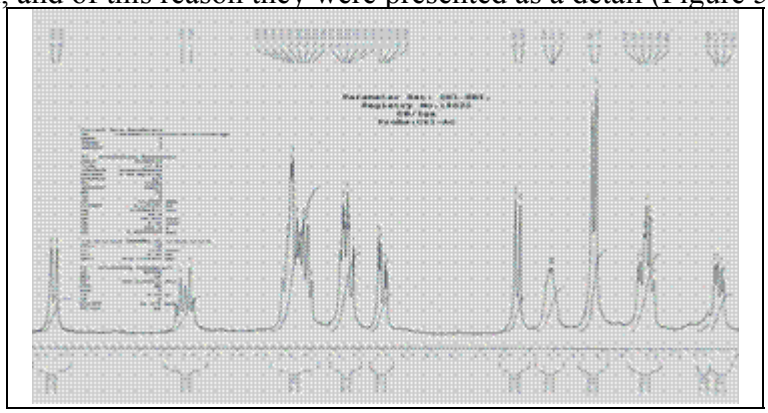


Figure 5. Detail of ^{1}H NMR spectra of peracetylated galactocerebroside demonstrating the sugar moiety. Solvent $CDCl_3$, containing TMS as reference; 400 MHz.

ß-D-Glucopyranoside moiety has been evidenced by the following signals: 4.442 (d, J=7.6 Hz, H-1, ß configuration of D-galactopiranosic ring); 5.296 (H-2); 5.144 (H-3); 5.384 (H-4); 3.921 (H-5); 4.128 (H-6a); 4.307 (H-6b). ^{13}C NMR Spectra (Figure 6) confirmed the above mentioned significances. Ceramide moiety was revealed by the following signals: 14.123 (terminal methyls of sphingosine and fatty acid); 22.702 (last methylene group of sphingosine and fatty acid); 29.722-29.377 (intermediary methylene groups of sphingosine and fatty acid); 73.973 (C-3 of sphingosine); 129.918 (C-5 of sphingosine); 137.350 (C-4 of sphingosine). Sugar moiety (Figure 6) was disclosed by the following signals: 100.902 (C-1); 68.682 (C-2); 73.193 (C-3); 66.968 (C-4); 70.793 (C-5); 61.191 (C-6).

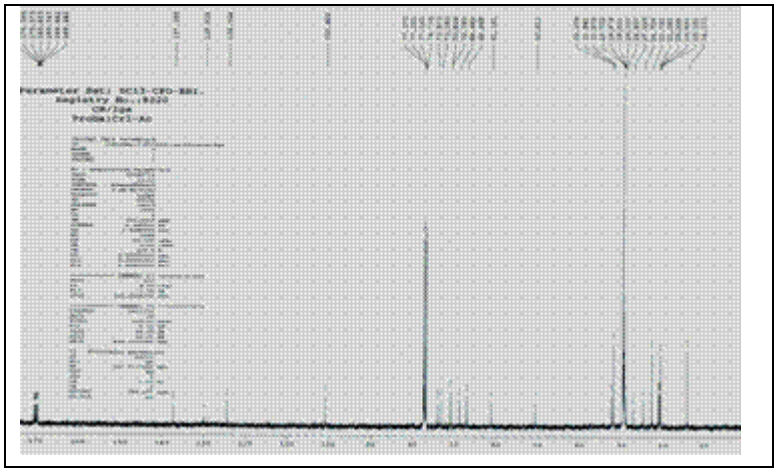


Figure 6. ^{13}C NMR Spectra of peracetylated galactocerebroside demonstrating the presence of ceramide and ß-D-galactopyranoside. Solvent $CDCl_3$, containing TMS as reference; 400 MHz.

IR Spectra and ^{1}H and ^{13}C NMR spectra of sulfatide presented similar signals as galactocerebroside. Sulfate ester could be detected due to its absorption at 1240 cm^{-1} and a diminution of acetate or benzoate signals caused by the substitution of an acetate or benzoate group for a sulfate ester; in case of benzoate ester, phenyl groups could be observed at 7.5-8.5 ppm.

Discussion

In biochemical terms, galactocerebroside and sulfatide play important and essential roles (Figure 7). They are interconvertible by two enzymes that belong to different classes: an arylsulfatase cleaves the sulfatide to galactocerebroside [12] while a sulphotransferase makes the reverse [16]. Sialosylation of galactosylceramide in the presence of a sialosyl transferase produces ganglioside GM4 [17] and sialydase of microbial origin hydrolyses GM4 back to galactocerebroside [18]. Two novel ganglioside lactones were isolated from the brain of

Bryde's whale (*Balaenoptera edeni*) and characterized by secondary ion mass spectrometry and infrared spectroscopy. Two dimensional J-correlated proton NMR spectra disclosed that an inner ester linkage was formed in one lactone between the sialic acid carboxyl group and the C-4 hydroxyl group of the galactopyranosyl residue and another inner ester linkage was formed in the other lactone between the sialic acid carboxyl group and the C-2 hydroxyl group of galactose. The two lactones contained *N*-acetylneuraminic acid and galactose in the molar ratio 1:1 and were converted to GM4 by mild alkali treatment [19].

Enzymes of variable origin, endoglycoceramidases, cleave the linkage between ceramides and oligosaccharides; galactocerebroside and sulfatide are also substrates for these enzymes [20], [21].

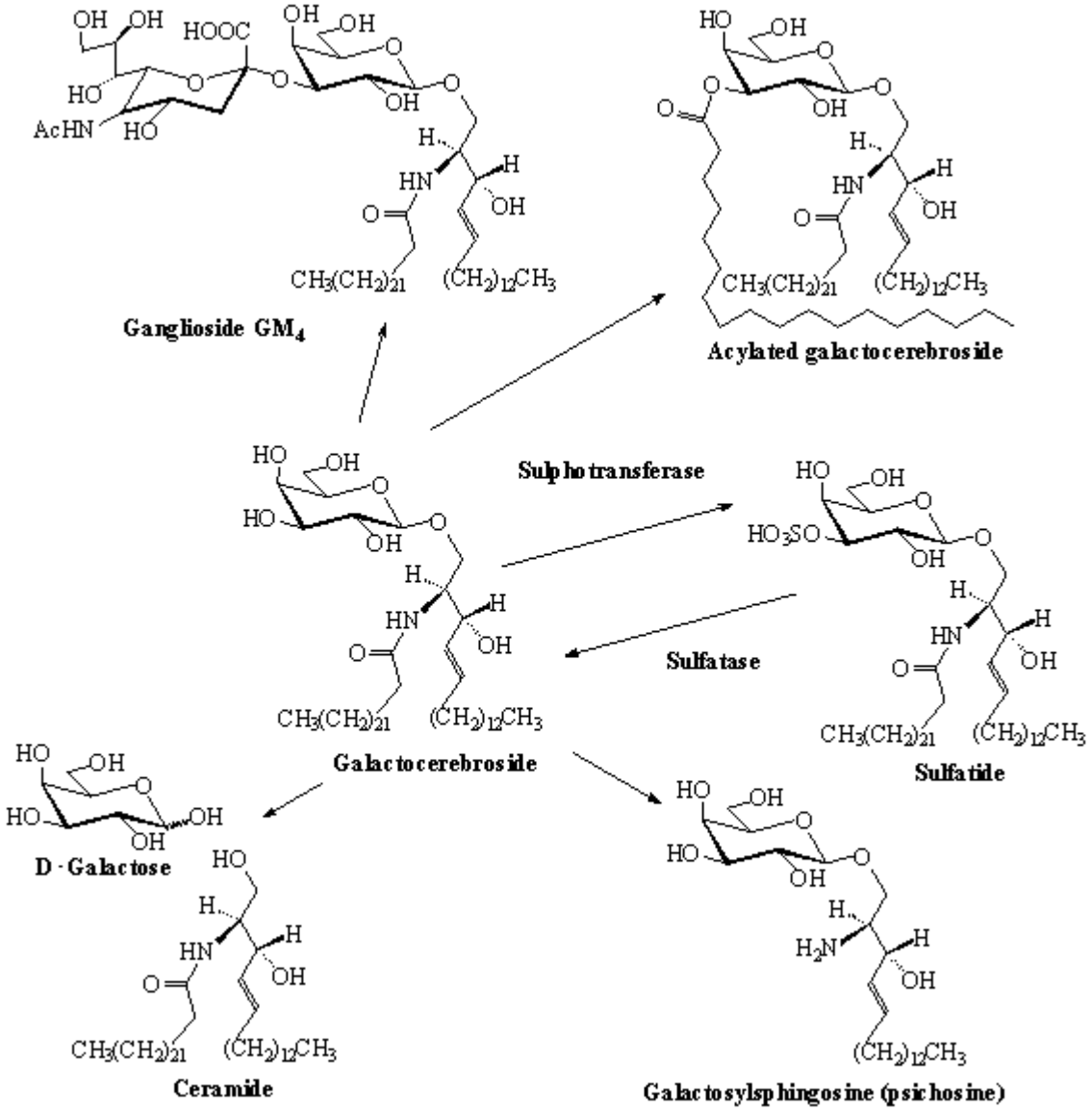


Figure 7. Biochemical transformations suffered by galactocerebroside and sulfatide.

Another enzyme, N-deacylase, accomplishes an interesting reaction: fatty acid is removed and lysogalactocerebroside (psichosine) or lysosulfatide are obtained; the enzyme was isolated and characterized from a marine bacterium, *Shewanella alga* G8 [22].

An increasing array of biochemically modified galactocerebroside and sulfatide were isolated from living tissues. The first report about the existence of fatty acylated galactocerebrosides in brain was presented in 1963 and was referred to cerebron [23]. A few years later, kerasin became involved in this phenomenon [24]. A fatty acylated sulfatide, sulfo-3 (6-fatty-O-acyl)ß-D-galactopyranosyl-1'ceramide, was isolated from equine brain and the chemical structure was characterized by proton NMR and mass spectrometry. The O-acylation site of the acylated sulfatide was determined by NMR, by the down-field shift of protons attached to a carbon having an O-acyl group in comparison with a non-acylated compound. This result was confirmed by analysis of a partially methylated derivative before and after acetalization of the intact sulfatide using gas chromatography-mass

spectrometry. The O-acyl chain length was determined by GLC, disclosing it to be palmitoyl and stearoyl residues as the major fatty acids [25].

Conclusions

1. Column chromatography on Florisil of a mixture of lipids containing galactocerebroside and sulfatide produces an enrichment in the latter compounds due to removing of free cholesterol, cholesterol esters, triglicerides and phospholipids.
2. Ion exchange chromatography on DEAE Sephadex A-25 constitutes an excellent method for the separation of galactocerebroside from sulfatide.
3. Traces of water improve the resolution of column chromatography on silicagel: galactocerebroside and sulfatide are separated according to their degree of hydroxylation.
4. Structure of neutral or acidic glycosphingolipids can be assigned by ^{1}H and ^{13}C NMR; moreover, the degree of hydroxylation can be ascertained by this type of spectroscopy.

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