Structure of sulfatides biosynthesized in vitro

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ABSTRACT Starting from galactose-14C-labeled phrenosine and 3′-phosphoadenosine-5′-phosphosulfate, radioactive sulfatides have been obtained in vitro with a biosynthetic system similar to the one described by McKhann and Ho (Ref. 6). It has thus been proved that exogenous cerebrosides can act as acceptors of sulfate. The specific radioactivity of the synthetic phrenosine used as precursor was sufficiently high to permit the proof of the structure of the resulting sulfatides to be done by methylation on an amount estimated at 0.1 μg. The sulfate group was found only at C-3 of galactose, the position at which it is located in sulfatides isolated from tissues. This observation indicates the specificity of the sulfotransferase involved in the in vivo synthesis of sulfatides.

SUPPLEMENTARY KEY WORDS sulfotransferase - micromethylation - phrenosine-14C synthesis

ALTHOUGH direct sulfation of cerebrosides is an easily conceived pathway for the biosynthesis of sulfatides, its unequivocal demonstration has been difficult to obtain.

Radin, Martin, and Brown (1) and Hauser (2) have shown that the in vivo incorporation of galactose-14C (1) and glucose-14C (2) is faster into cerebrosides than into sulfatides of rat brain. Moreover, the specific activity of the cerebrosides was higher, with most of the radioactivity being in the galactose moiety. These experiments are consistent with the idea that cerebrosides are precursors of sulfatides. They do not permit, however, the conclusion that sulfation occurs at the level of the complete cerebroside molecule and not at an earlier stage, for example, through galactose sulfate, UDP-galactose sulfate, or psychosine sulfate.

Goldberg (3) showed that extracts of liver, kidney, and brain catalyze the incorporation of sulfate-35S from 3′-phosphoadenosine-5′-phosphosulfate-35S (PAPS) into lipids which were probably sulfatides. However, under the conditions used by this author sulfation of psychosine or cerebrosides could not be observed.

More recently, Balasubramanian and Bachhawat (4) described the formation of sulfatides when radioactive PAPS was added to brain preparations. However, they emphasized the point that exogenous cerebrosides do not seem to act as sulfate acceptors and proposed that the acceptor is a protein-bound, galactose-containing substance, present in their enzyme preparation. At about the same time, McKhann and coworkers (5, 6) succeeded in showing that, when a microsomal fraction of rat brain or kidney was used as the enzyme source, the addition of exogenous cerebrosides resulted in a threefold increase in incorporation of 35S into sulfatides from PAPS.

Cumar, Barra, Maccioni, and Caputto (7) provided further evidence that exogenous cerebrosides accept sulfate-35S from PAPS. They also showed that other glycolipids and water-soluble, galactose-containing substances were sulfated by brain preparations from young rats. Indirect evidence was obtained consistent with the assumption that the sulfate was attached at C-3 of galactose in sulfatides under the experimental conditions used.

Because of the seemingly contradictory findings of Balasubramanian and McKhann and the absence of definite proof for the structure of the newly formed sulfatides, owing to the very small amounts of material produced, the present work was undertaken. In order...
to demonstrate whether exogenous cerebrosides can act as precursors in the biosynthesis of sulfatides and whether the sulfate becomes attached to the galactose moiety at C-3 as it is in naturally occurring sulfatides, phrenosine, synthesized from \(^\text{14}C\)-labeled galactose, and PAPS, rather than unlabeled cerebroside and PAP\(\text{3S}\), were used in experiments analogous to those described by McKhann and Ho (6) and the structure of the product was established by methylation.

**MATERIALS**

Silica gel for column chromatography, grade 950, 60–200 mesh, was obtained from Davison Chemical, Baltimore, Md. Thin-layer chromatography was done on plates coated with Silica Gel G (Uniplate; Analtech, Inc., Wilmington, Del.). PAP\(\text{3S}\) was purchased from New England Nuclear Corp., Boston, Mass. \(\delta\)-Galactose-1-\(^{14}C\) was obtained from International Chemical and Nuclear Corp., Irvine, Calif. Radioautography was done on Kodak No-Screen Medical X-ray film. Elemental analyses were performed by Midwest Microlab, Inc., Indianapolis, Ind.

**EXPERIMENTAL**

The labeled cerebrosides were synthesized by condensation of 3-O-benzoyl-ceramide with radioactive tetra-O-acetylgalactosyl bromide (8). The 3-O-benzoyl-ceramide was synthesized through the 1-O-trityl derivative starting from ceramide prepared by degradation of bovine brain phrenosine (9). It thus had the natural complement of fatty acids and sphingosine bases characteristic of these cerebrosides.

**Preparation of Phrenosine and Cerasine**

The cerebrosides were obtained from a washed lower phase of a lipid extract of crudely dissected bovine brain white matter (10). The solvents were evaporated under vacuum and the residue (10 g) was treated with alkali and acid according to Schmidt, Benotti, Hershman, and Thannhauser (11), in order to hydrolyze phosphatides and plasmalogens. The insoluble portion was collected on a filter (Whatman No. 54 paper) under a slight vacuum and partially dried by suction overnight. The brown paste was slurried in 10–20 times its weight of \(\pi\)-propanol at room temperature and the suspension was kept for 24 hr. The nearly white insoluble fraction was collected on a filter and washed with \(\pi\)-propanol; the yield was 3.6 g. TLC showed that this material consisted mainly of phrenosine and a smaller amount of cerasine. Separation and purification of the cerebrosides was done by chromatography on a silicic acid–Celite column essentially according to the procedure of Carter, Rothfus, and Gigg (9). The sample was applied to the column in solution in a minimum volume of hot chloroform–methanol 10:1 to which enough Celite analytical filter aid was added to make a soft paste. In this way, phrenosine, which crystallizes on cooling, did not clog the top of the column. Elution was carried out with chloroform–methanol 10:1 and the collected fractions were monitored by TLC. Fractions containing cerasine or phrenosine, respectively, were pooled and evaporated, and the solids were recrystallized from hot methanol. TLC indicated that the resulting cerebrosides were free of contaminants. They were identified by the characteristic GLC pattern of the fatty acids (12), TLC of the sphingosine bases (13), and quantitative estimation of the galactose (14) adapted to a microscale. 1 mole of hexose was found per mole of cerebrosides. Phrenosine gave the infrared spectrum characteristic for these cerebrosides (8).

**Preparation of Ceramide from Phrenosine**

Phrenosine ceramide was obtained from phrenosine by the procedure of Carter et al. (9). This involved periodic acid oxidation, reduction with sodium borohydride, and hydrolysis with weak acid without isolation of intermediate products. The ceramide was purified by column chromatography on 200 times its weight of silica gel. The column was prepared in chloroform. Pure chloroform slowly eluted an unidentified impurity; phrenosine ceramide was then eluted with chloroform–methanol 19:1. Phrenosine ceramide was shown to be homogeneous by TLC. Its fatty acid and sphingosine patterns determined by GLC and TLC were identical with those of the phrenosine used as starting material, and no carbohydrate was detected on TLC by the anthrone–sulfuric acid reagent.

**1-O-Trityl-ceramide**

To a solution of phrenosine ceramide (1 g) in anhydrous pyridine (55 ml), chlorotriphenylmethane (500 mg) was added. The mixture was shaken until it was homogeneous and it was then heated at \(70^\circ\)C for 20 hr. After cooling, a small piece of ice was added to decompose the excess reagent, and after 1 hr the solution was poured onto crushed ice. The mixture was filtered through a layer of Celite; the collected gummy precipitate was washed with water and redissolved on the filter with 250 ml of chloroform–methanol 2:1. The solution was evaporated to dryness under vacuum and the remaining water was codistilled with toluene–ethanol 1:1. The oily residue was chromatographed on a column of 100 g of silica gel prepared in benzene. Pure benzene eluted triphenyl carbinal, and benzene–ether 9:1 eluted the trityl ether as an oil. The yield was 1.210 g (88%). The product appeared homogeneous by TLC in benzene–ether 4:1;
The following procedure was devised in order to prepare

3-O-Benzoyl-N-(2'-O-benzoyl-acyl)-1-O-

trityl-sphingosine

To an ice-cold solution of 1-O-trityl-ceramide (1.07 g) in anhydrous pyridine (9.8 ml), benzoyl chloride (0.59 ml) was added. The mixture was kept for 18 hr at room temperature. A small piece of ice was then added and after 1 hr the mixture was poured onto crushed ice. When the ice had melted, the oily product was extracted with ether and the solution was washed once with cold 2 N hydrochloric acid, twice with cold saturated sodium bicarbonate, and once with water. After drying over anhydrous sodium sulfate, the solution was evaporated, leaving an oil which gave a single spot on TLC in benzene-ether 9:1.

3-O-Benzoyl-N-(2'-O-benzoyl-acyl)-sphingosine

The preceding product (1.0 g), without further purification, was heated for 2 hr on a boiling water bath in 50 ml of 90% acetic acid. The resulting clear solution was evaporated to dryness, and the last traces of acid were eliminated by codistillation with toluene-ethanol 1:1. The semicrystalline product was shown by TLC in benzene-ether 9:1. O.D.

Analysis: C₅₅H₉₁O₆N; calculated: C, 76.93; H, 10.49

(assuming the fatty acid to be hydroxylinoceric acid);

found: C, 76.90; H, 10.54

Preparation of Tetra-O-acetyl-galactopyranosyl-

1-¹⁴C Bromide

The following procedure was devised in order to prepare this labeled compound with high specific activity, starting from submilligram amounts of galactose-¹⁴C without dilution.

Conical tubes, 11 cm long, were made from 12-mm-o.d. borosilicate glass tubing pulled at one end to form a 2.5-cm-long, tapered, thick-walled bottom and constricted at the other end by turning it in the flame until the opening was reduced to 5 mm in diameter. The lip thus formed prevents splashing of the radioactive contents when the tubes are agitated with a Vortex mixer.

In such a tube the solution of galactose-1-¹⁴C (165 µCi in 540 µg) was evaporated to dryness under a stream of nitrogen, and the residue was dried under vacuum. Acetic anhydride (0.16 ml) and, after cooling in an ice bath, 60% perchloric acid (about 3 µl) were added. The tube was stoppered with a rubber stopper, shaken occasionally, and kept at 40°C for 1 hr. The solution was then cooled to 0°C, saturated with anhydrous HBr, and kept at room temperature for 2 hr. The gas was bubbled through the solution by way of a glass capillary which passed through a plug of glass wool.

In order to perform the extraction and washings of the acetyl bromogalactose with minimum decomposition, four conical tubes of the model described above, and containing 2 ml of water each, were precooled in an ice bath. 1 ml of chloroform was added to the reaction mixture cooled to 0°C, followed by 2 ml of ice-cold water. The tube was agitated with a Vortex mixer for 2 to 3 sec and the contents were centrifuged for a few seconds to separate the phases. The lower phase was aspirated as rapidly as possible, using a pipette with capillary tip adapted to a syringe, and transferred into the first tube containing water. These operations were repeated four times. The lower phase was transferred into a tube containing four beads of 8-mesh CaCl₂ for drying. A second extraction was carried out in the same way with 1 ml of fresh chloroform added to the first tube and transferred through the series. The last lower phase was combined with the first chloroform extract. After drying, the chloroform solution was transferred into another tube and evaporated to dryness under a stream of dry nitrogen at room temperature; the residue was kept under vacuum at -10°C in a desiccator containing CaCl₂ and NaOH.

Condensation of 3-O-Benzoyl-N-(2'-O-benzoyl-acyl)-

sphingosine with Tetra-O-acetyl-galactopyranosyl-1-

¹⁴C Bromide

Nitromethane (100 µl) and benzene (100 µl) were combined in a test tube (4 cm long and 0.5 cm i.d.) containing a small magnetic flea. The tube was placed in an aluminum block kept at 100°C on a magnetic stirrer-hot plate. When the volume of solvents was reduced to one-half, the heating block was cooled to 40°C. After addition of mercuric cyanide (2 mg) and ceramide derivative (2.5 mg) the acetyl bromogalactose, redissolved in 50 µl of dry benzene, was added. The tube was stoppered and the mixture was stirred for 24 hr at 40°C. After cooling, the contents were transferred with 1 ml of ether into a conical tube containing 1 ml of cold saturated NaHCO₃. The mixture was briefly agitated and then centrifuged; the lower aqueous phase was discarded. A second washing was done in the same way and a third with pure water. After drying over
anhydrous Na₂SO₄, the ether was evaporated under a stream of nitrogen and the residue was dried under vacuum.

Decaylation
To the dry material, suspended in 1 ml of methanol, 20 μl of 1 n barium methoxide in methanol was added. The mixture was kept at 4°C overnight and then for 8 hr at room temperature. One drop of glacial acetic acid was added, the solvents were evaporated under nitrogen, and the residue was extracted three times with 1 ml of chloroform–methanol 2:1. After evaporation of the solvents, the product was purified by preparative TLC on Silica Gel G in n-propanol–5 N NH₄OH 4:1. The phrenosine band, localized by radioautography, was scraped off and eluted with chloroform–methanol 2:1. The product also cochromatographed with authentic phrenosine on Silica Gel G in chloroform–methanol–concentrated ammonia 4:1:0.02. Its infrared spectrum was identical to that of the natural phrenosine used as starting material.

Preparation of PAPS
The sulfate-activating system was prepared from Anheuser-Busch yeast according to the method described by Robbins (16). However, since in our preparation dialysis of the final enzyme solution resulted in nearly complete loss of activity, this step was omitted. The undialyzed solution could be kept for at least a week at -10°C without loss of activity. With this enzyme system the preparation of PAPS was carried out essentially as described by Robbins (17), except that PAPS was separated from other anions by elution from a column of AG 1-X8, 200-400 mesh, with a concentration gradient of NaCl (18).

Preparation of Sulfotransferase
The enzyme preparation used was the supernatant solution of microsomes, sonicated in sodium deoxycholate and obtained from the kidneys of adult rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) by the method of McKhann and Ho (6).

Biosynthesis of Galactose-1-14C-labeled Sulfatides
McKhann and Ho (6) carried out the biosynthetic reaction in a reaction mixture containing an excess of acceptor cerebrosides and a level of PAPS which, although not explicitly stated, must have been very low. Since for the eventual characterization of the methylated galactose, derived from the biosynthesized sulfatides, it was essential to use phrenosine of very high specific activity as acceptor, preliminary experiments were performed using PAPS to test different concentrations of the two substrates. The incubation conditions selected were: phrenosine, 1–2 × 10⁴ cpm, approximately 20 nmoles, suspended in 50 μl of 1% Brij 96; imidazole–HCl buffer, 0.1 M, pH 7.4; K₂SO₄, 0.8 mM; PAPS, 0.45–1.35 mM; and sulfotransferase preparation, 50 μl. The total volume was 0.5 ml. Incubation was for 2 hr at 37°C and was stopped by the addition of 10 ml of chloroform–methanol 2:1.

After addition of 50 μg of sulfatides, the extract was washed once with 0.88% aqueous KCl and once with theoretical upper phase containing 0.375% KCl (10). The lower phase was dried and chromatographed on Silica Gel G plates in n-propanol–5 N NH₂OH 4:1. After localization by radioautography, the sulfatide area was scraped from the plate and the lipid was eluted from the silica gel with several portions of chloroform–methanol 1:1, with warming at 37°C. The product also cochromatographed with authentic sulfatides in chloroform–methanol–concentrated ammonia 4:1:0.02, a system in which sulfatides travel slower than cerebrosides. In the propanol–ammonia solvent system the opposite is true. Eluted sulfatide samples from several incubations were combined for methylation.

Methylation
The radioactive sulfatides, mixed with an additional 250 μg of unlabeled sulfatides from bovine brain, were methylated with methyl iodide in the presence of dimethylsulfinyl carbaniion (19); the methylated hexose was isolated as previously described (20), except that the whole procedure was carried out on a scale four times smaller. The methylated galactose was identified by comparison on TLC with standards of tetramethylgalactopyranose and the four isomeric trimethyl galactopyranoses. The plates were developed with acetone–water–concd. ammonia 250:3:1.5; after drying they were subjected to radioautography. The spots were visualized with aniline phthalate or anthrone–sulfuric acid spray reagents. The standards were run in separate lanes adjacent to the unknown or, alternatively, 2,3,4,6-tetramethyl galactose and 2,3,4-, 2,3,6-, and 3,4,6-trimethyl galactoses were mixed with the unknown; the nonradioactive 2,4,6-trimethyl galactose originating from the added sulfatides constituted an internal standard (Fig. 1).

RESULTS AND DISCUSSION
Only one radioactive spot was observed on the radioautogram of the unknown. It coincided in position and shape with 2,4,6-trimethyl galactose (Fig. 1). No radioactivity was detected at the level of the other standards. This indicates that C-3 of galactose became blocked as the result of the incubation with PAPS and sulfotransferase, since phrenosine yields only 2,3,4,6-tetramethyl galactose on methylation.
Radioactivity incorporated into the sulfatide fraction starting with synthetic radioactive ceramide are in use as starting material. The fact that sulfate was attached to the unknown was revealed by radioautography and anthrone-H₂SO₄ reagent, giving spots of identical shape and location with both methods of detection.

It is thus established that under the conditions used by McKhann and Ho (6) exogenous phrenosine can accept sulfate from PAPS to form sulfatides. The yield of this reaction is very small, about 0.1%, based on the radioactivity incorporated into the sulfatide fraction (i.e., 2400 cpm out of 1.8 × 10⁶ cpm in the phrenosine used as starting material). The fact that sulfate was attached to C-3 of galactose, the position it occupies in natural sulfatides (21, 22), indicates the specificity of the sulfotransferase involved. Analogous experiments starting with synthetic radioactive ceramide are in progress.

The present demonstration does not exclude the proposition of Balasubramanian and Bachhawat (4) that protein-bound cerebrosides might be the natural precursors of sulfatides.

It is of technical interest that the proof of structure could be performed on an amount of product on the order of 0.1 nmole, corresponding to about 20 ng of galactose. This was made possible by the use of labeled phrenosine of high specific activity, the miniaturization of the chemical manipulations, and the fact that the methylation procedure of Hakomori (19) readily goes to completion. This first example opens the way towards the determination methods for brain cerebrosides, hydroxy fatty acids, and unsaturated and saturated fatty acids. J. Lipid Res. 1: 72–78.

