SHORT COMMUNICATIONS

Preparation of Psychosines (1-O-Hexosyl Sphingosine) from Cerebrosides

ABSTRACT

A convenient method for large or small scale preparation of psychosine from cerebroside has been developed by adaptation of published procedures. Cerebroside is refluxed with butanol and aqueous KOH, then the KOH is removed with perchloric acid. The fatty acids are removed by extraction with hexane and the excess perchloric acid is removed by partitioning between chloroform, ethanol, and water.

INTRODUCTION

The first practical psychosine preparation from cerebroside was described by Klenk in 1926 (1) and modified by Carter and Fujino (2). (Cerebroside is galactosyl ceramide and glucosyl ceramide; psychosine, in analogy with this custom, is used for galactosyl sphingosine and glucosyl sphingosine.) The latter method involves refluxing with dioxan and aqueous barium hydroxide, but, in our hands, this gives rise to obstructive foaming and coalescence of the lipid-base complex into hard lumps. We found that only the cerebroside containing hydroxy acids appears to be hydrolyzed under these conditions. The yield is poor and the final product, psychosine sulfate, does not recrystallize as originally described (2). It is said to be hygroscopic (3).

The method of isolating the psychosine, which involves precipitating the base as the sulfuric acid salt $(Psy)_2H_2SO_4$, has led to an unfortunate terminology which has caused many individuals to assume the salt is actually a sulfate ester. The salt ought to be called psychosinium sulfate.

A marked improvement came from Taketomi and Yamakawa (4), who refluxed the cerebroside for 2 hr in 1 N KOH in butanol-water 90:10. The KOH was removed by washing with water, and the fatty acid was removed with a silica gel column. We found a serious problem with emulsions in the alkaline partition; this was particularly difficult with large samples. Attempts at removing the KOH with other partition systems gave unsatisfactory results, and we finally resorted to dialysis (5). This method is tedious with large samples, and the dialysis bags sometimes break, so we investigated the recent method of Cumar, et al. (6). These workers refluxed cerebroside with 1 N KOH in 2-methoxyethanol-water 70:30 for 6 hr, removed the KOH with perchloric acid, and removed the fatty acid with a Florisil column. This method has the disadvantage of calling for a peroxide-prone solvent. We found a foaming problem when refluxing, but this could be controlled by addition of a little octanol. However, there was an appreciable amount of sphingosine formation, and removal of the relatively nonvolatile solvent necessitated desiccation for some time over sulfuric acid.

It was decided to combine the advantages of these two methods, the use of butanol for hydrolysis and the use of perchlorate for removing the alkali. We found that we could remove the fatty acid by a simple solvent partition, leaving almost pure psychosine. The 2 hr recommended initially for the hydrolysis was found to give incomplete hydrolysis, so the period was extended. A final chromatographic purification step can be avoided for many

purposes.

METHODS AND RESULTS

Galactosyl ceramide was prepared from crude sphingolipids (7) with a Florisil column (8). By using a relatively tall column (4.7 x 210 cm), we were able to isolate rather pure material. The column was packed dry, using 2000 g Florisil that had been dried at 100 C overnight and hydrated with 160 ml water. The packing was wetted with chloroform until most of the air bubbles had been removed, then washed with 500 ml chloroform-methanol 88:12. A 50 g sample of mixed sphingolipids, prepared by solvent extractions (7), was added as a solution in 2500 ml of the same chloroform-methanol mixture and rinsed in with 500 ml more solvent. Elution was carried out with 6000 ml chloroform-methanol 82:18, then with 6000 ml of a 72:28 mixture. Air pressure (ca. 10 psi) was used to drive the solvents at ca. 1000 ml/hr and 800 ml fractions were collected with a modified Technicon fraction collector (9). (A good deal of sulfatide can be obtained by further elution with a 60:40 mixture.) Fractions found to contain cerebroside by thin layer chromatography (TLC) were pooled and lyophilized from benzene.

Glucosyl ceramide was prepared from a Gaucher patient's spleen, following a similar procedure. The sphingolipid concentrate was made from a total lipid extract by alkaline methanolysis (5).

Cerebroside hydrolysis was carried out in a 100 ml round-bottom flask containing 28 g KOH dissolved in 40 ml water. To this was added 10 g cerebroside and 360 ml n-butanol; the flask joint was wiped clean, and refluxing was carried out for 4 hr with the use of an oil bath held at ca. 125 C. A soda lime tube protected the alkali. The level of the oil was kept just below the level of the flask's liquid. One experiment in which the KOH was not first dissolved in the water led to a very dark mixture.

The hydrolysate was diluted with 400 ml methanol, and ca. 120 ml of 5 N $HClO_4$ was added to bring the pH to ca. 7 (overacidification was corrected with KOH). The potassium perchlorate was removed by filtration through a Celite-coated glass funnel (600 ml size) and rinsed with 400 ml methanol. The methanol rinse was used to complete the transfer of the filtrate to a 2 gal glass bottle. Some fatty acid precipitates when the methanol is added.

To remove the fatty acids, we acidified with 5 N perchloric acid (ca. 26 ml, to yield pH 3-4) and added 1350 ml water and 2700 ml hexane. The mixture was mixed well by shaking the bottle in a swirling motion then left until the upper layer cleared. The hexane was sucked off with an aspirator (it can be saved for preparation of hydroxy fatty acids if galactosyl ceramide was used). We removed residual fatty acids with a similar extraction with 1350 ml hexane; 200 ml methanol was added to prevent emulsification.

The perchloric acid was now removed by adding 2 N NaOH (ca. 42 ml to yield pH ca. 10) and 1350 ml chloroform to form the partition system. The mixture was swirled, and, when the lower layer cleared, the upper layer was sucked off and discarded. The lower layer was washed twice more with 1350 ml portions of methanol-water 1:1. The clear lower layer then was evaporated to dryness under vacuum with the aid of benzene. Since small amounts of butanol could be detected by odor, the psychosine was left in a vacuum desiccator over sulfuric acid for a while. The lipid then was transferred to a small flask with benzene and lyophilized again. The yield was ca. 4.3 g with either type of cerebroside.

TLC of the product with chloroform-methanol-water-ammonium hydroxide 70:30:4:1 shows only psychosine, with just a trace of sphingosine, when examined with bromothymol blue, ninhydrin, and a charring spray (10). (A very small spot, presumably dihydropsychosine, can be seen just below the primary spot.) The psychosine gives characteristic colors with the different sprays: blue or blue + white with the pH indicator and pink with ninhydrin. Acylation with fatty acids has yielded cerebrosides, which were identified by TLC on silica gel and silica gel-borate plates, and by their IR spectra. Elemental analysis of a sample by Spang Microanalytical Laboratory, Ann Arbor, Mich., yielded the values, 62.25% C and 10.07% H (theor.: 62.45% C and 10.25% H).

The time required for each partition step varied according to the degree of shaking but was generally 1-5 hr. We usually did not wait for both layers to clear before discarding the unwanted layer. In one trial, we found that several hundred mg of KCl was useful in speeding the clearing of the methanol-water washes. Of course, when one works on a smaller scale, it is possible to speed the washing steps by centrifugation. It is not recommended that any solvent volume ratios given in the above procedure be changed as emulsion or recovery problems might arise. Because the purification steps involve solvent partitioning primarily, the method is particularly suited to small scale preparations as well as to large scale work.

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In Vitro Desaturation of 1-^{14}C Linoleic Acid in Novikoff Hepatoma

ABSTRACT

The lipid fatty acid pattern of normal liver, host liver, and Novikoff hepatoma was determined by gas liquid chromatography, and Δ6-desaturase activity for linoleic acid was measured in the microsomal fractions. The results showed that, in Novikoff hepatoma, there is a correlation between the low content of arachidonic acid and the low activity of Δ6-desaturase, a key enzyme in the biosynthetic pathway of this acid.

INTRODUCTION

Earlier studies carried out in our laboratories (unpublished results) have shown that the lipid fatty acid pattern of normal liver no longer occurs in Novikoff hepatoma. The main features of the fatty acid composition in this tumor are the relatively high oleic acid content, concomitant with a relatively low amount of arachidonic acid. Similar disturbances, especially with respect to the oleic acid content, also were described in other experimental hepatomas (1-3).

The present experiment was performed to find out if the low level of arachidonic acid content in the Novikoff hepatoma is related to changes in the activity of the Δ6-desaturase, a key enzyme in the biosynthetic pathway of this unsaturated fatty acid (4-5).

EXPERIMENTAL PROCEDURES

The Novikoff hepatoma was maintained by intraperitoneal implants into Holtzman rats. Normal liver was obtained from rats of the same breed. Fatty acid composition of total lipids from normal liver, host liver, and Novikoff hepatoma was determined by gas liquid chromatography (GLC) (6) in columns packed with 15% diethylene glycol succinate on Chromosorb WAW (100-120 mesh) at 180 C. The activity of the desaturating enzyme was measured in the microsomal fractions separated by differential centrifugation at 105,000 x g (7). The assay conditions for desaturating activity were as follows: 5 mg microsomal protein were incubated in an open test tube with 100 nmoles diluted labeled fatty acid (1-^{14}C linoleic acid, 57.0 mC/mmole, 99% radiochemically pure, Radiochemical Center, Amersham, England, diluted to a specific activity of ca. 1.7 mC/mmole with the corresponding unlabeled pure fatty acid). The incubation was performed in a Dubnoff Shaker at 37 C for 30 min in a total volume of 1.5 ml 0.15 M KCl, 0.25 M sucrose solution containing, in μmoles: adenosine 5′-triphosphate, 2; coenzyme A, 0.1; nicotinamide adenine dinucleotide, reduced form, 1.2; $MgCl_2$, 7.5; glutathione, 2.2; sodium fluoride (NaF), 62; nicotinamide, 0.5; and phosphate