Isolation and characterization of glucosylsphingosine from Gaucher's spleen

Srinivasa S. Raghavan, Richard A. Mumford, and Julian N. Kanfer

Eunice Kennedy Shriver Center for Mental Retardation, Walter E. Fernald State School, Waltham, Massachusetts 02154, and Neurology Research, Massachusetts General Hospital, Boston, Massachusetts 02114

Abstract Glucosylsphingosine has been isolated for the first time as a natural constituent from Gaucher's spleen. On thin-layer chromatography, it migrates with authentic glucosylsphingosine, yielding a positive color reaction with ninhydrin for the amino group and with α-naphthol–sulfuric acid for the carbohydrate residue. *N*-Acylation with palmitic acid gave rise to glucosylceramide, which was cleaved by purified glucosylceramide:β-glucosidase to ceramide. Gas–liquid chromatography of the trimethylsilyl derivative showed a retention time similar to authentic glucosylsphingosine. Gas–liquid chromatographic analysis of the trimethylsilyl derivatives after methanolysis revealed the presence of only glucose and C_{18}-sphingosine. Mass spectral data further supported the structural identity with glucosylsphingosine.

Supplementary key words Gaucher's disease · thin-layer chromatography · gas–liquid chromatography · β-glucosidic linkage

Gaucher's disease is a typical sphingolipidosis characterized by the accumulation of glucosylceramide in the reticuloendothelial system (1–7). A decreased activity of the enzyme glucosylceramide:β-glucosidase is well documented (8–10), and this deficiency could conceivably be related to the increased amount of tissue glycosphingolipid. Recently, this laboratory reported a diminution in the ability of tissue samples of patients with Gaucher's disease to hydrolyze glucosylsphingosine (11). Preliminary evidence was obtained for the possible presence of a glucosylsphingosine-like material in Gaucher spleen tissue. The large-scale isolation of this compound and its unequivocal chemical characterization as glucosylsphingosine is the subject of this report. This is the first isolation and identification of glucosylsphingosine from a mammalian tissue.

METHODS

All solvents used were reagent grade (Fisher Scientific Co., Fair Lawn, N.J.), and they were freshly distilled before use. Standard glucosyl- and galactosylsphingosine were obtained from authentic glucosyl- and galactosylceramide (5), respectively, by the alkaline degradation procedure of Taketomi and Yamakawa (12) and were purified as previously described (11).

Two adult Gaucher spleens were used in the present studies. One was obtained at autopsy from Massachusetts General Hospital and was kindly donated by Dr. Hugo Moser. The second spleen was obtained soon after surgical removal by Dr. Joseph M. Ford of Roosevelt Hospital, New York City. The tissues were kept frozen at −40°C in a Revco freezer and were thawed just prior to use.

50 g of tissue was homogenized in 100 ml of distilled water at 4°C in a Lourdes homogenizer (model 700, Volu-Mix, Vernitron Medical Products, Carlstadt, N.J.) and filtered through cheesecloth. The residue was rehomogenized in another 50 ml of water and filtered again to remove the connective tissue. The filtrates were combined and lyophilized.

The dry tissue, approximately 10 g, was mixed with 20 ml of water and uniformly dispersed by sonication in a bath-type sonicator (Heat Systems-Ultrasonics, Inc., Plainview, N.Y.). 80 ml of methanol was added, and the mixture was homogenized. The homogenate was transferred to a flask, and the homogenizer vessel was rinsed with another 60 ml of methanol. This suspension (160 ml) was heated at 60°C for 30 min, and then 280 ml of chloroform was added. The mixture was stirred for 1 hr at room temperature then passed through a sintered glass funnel, and the residue was washed with 50 ml of warm chloroform–methanol 2:1. The chloroform–methanol extract was processed by one of two methods to obtain glucosylsphingosine.

Method 1

The extract was taken to dryness and the total lipids

Correspondence should be addressed to Dr. Julian N. Kanfer, E. K. Shriver Center, 200 Trapelo Road, Waltham, Mass. 02154.

were dissolved in 6 ml of chloroform and 60 ml of 0.1 M sodium methoxide in methanol. The solution was left for 1 hr at room temperature, and 114 ml of chloroform was added. The chloroform–methanol solution was subjected to a modified partitioning (13) procedure in which all solvent mixtures were prepared in 0.05 N NaOH. The final lower phase from this alkaline washing was taken to dryness, and about 2 g of a crude sphingolipid mixture plus fatty acid methyl esters was obtained.

Silicic acid column chromatography. 150 g of Unisil (Clarkson Chemical Co., Williamsport, Pa.) was slurried in chloroform and poured onto a glass column (5 cm ID). The crude sphingolipids dissolved in chloroform were applied to this column. The nonpolar lipids were removed with 4.5 l of chloroform. The column was then eluted with 8 l of acetone–methanol 9:1 to obtain a mixed glycosphingolipid fraction.

Chromatography on Dowex 50 (H^+). Dowex AG 50W-X8 (200–400 mesh), H^+ form (Bio-Rad Laboratories), was washed three times for 15 min by stirring with twice the volume of 0.5 N methanolic HCl, followed by methanol until the washings were neutral to pH paper and also free from chloride ions. Approximately 25 g of washed Dowex was slurried in methanol and poured onto a column (2.5 cm ID). The acetone–methanol 9:1 fraction from the Unisil column, dissolved in 100 ml of methanol–chloroform 9:1, was passed through the Dowex column and washed with 500 ml of the same solvent. This procedure removed all the neutral sphingolipids, and the glucosylsphingosine was then eluted from the column with 500 ml of 0.1 N methanolic HCl.

Second silicic acid column chromatography. The 0.1 N methanolic HCl fraction from the Dowex column along with glucosylsphingosine is contaminated with a small amount of yellowish pigment from the resin. This pigment is effectively removed on a second silicic acid column. Unisil, 10 g, slurried in chloroform was packed into a column of 1.5 cm ID, and the glucosylsphingosine-containing sample was applied in chloroform and washed with 300 ml of the same solvent. This was followed by successive elution with 300 ml each of 5%, 10%, 20%, and 50% methanol in chloroform, and then 100% methanol. The applied glucosylsphingosine was recovered in the 20% methanol fraction and was practically devoid of contamination arising from the Dowex resin.

Method 2

The second method for the isolation of glucosylsphingosine from Gaucher spleen is based on the procedure reported earlier from this laboratory (11). It is a simpler and less time-consuming procedure than method 1. The chloroform–methanol 2:1 extract of lyophilized spleen tissue was subjected to the alkaline washing procedure described earlier. Sufficient methanol was added to the lower chloroform phase to make a 2:1 ratio of chloroform–methanol. This solution was extracted first with 0.2 vol of 0.1 N aqueous HCl followed by 0.4 vol of methanol–0.1 N aqueous HCl 1:1. The two acidic upper phases were combined and made alkaline (pH 9.0) by the addition of 10 N NaOH; this solution was extracted twice with equal volumes of a solvent consisting of chloroform–methanol–water 86:14:1. The lower chloroform phases were combined and evaporated to dryness to obtain crude glucosylsphingosine.

Silicic acid column chromatography. The crude glucosylsphingosine dissolved in chloroform was applied to a 10-g Unisil column (2 cm ID) and eluted successively with 200 ml each of 5%, 10%, 20%, and 50% methanol in chloroform, and 100% methanol. The glucosylsphingosine was obtained in the 20% methanol fraction and was more than 90% pure as judged by thin-layer chromatography. When assayed with the trinitrobenzene sulfonic acid procedure (14), the yield was found to be 5.3 μmoles/100 g of wet spleen tissue.

Silica gel column chromatography. Final purification was accomplished on a column (2 cm ID) containing 16 g of silica gel (0.05–0.2 mm, extra pure, 70–325 mesh, E. Merck, cat. no. 7754) slurried in chloroform–methanol 1:1. The gel was then extensively washed with chloroform, and the column was attached to a fraction collector. The glucosylsphingosine obtained from 100 g of tissue was dissolved in 3 ml of chloroform–methanol–concentrated NH_4OH 90:10:1 and applied to the column. The silica gel was eluted with chloroform–methanol–concentrated NH_4OH 14:5:1, and 2-ml fractions were collected at the rate of 1.26 ml/min. The first fraction was collected as soon as the ammoniacal solvent appeared from the column. The column was conveniently monitored by spotting aliquots of each fraction on a silica gel G thin-layer chromatography plate and charring with a sulfuric acid spray (11). Aliquots of the positive fractions were then subjected to analytical thin-layer chromatography, and the glucosylsphingosine-containing fractions were pooled for further studies.

GLC analysis. An aliquot of the purified material from the silica gel column was methanolyzed in 1 ml of 1 N methanolic HCl in a sealed tube for 18 hr at 70°C (15). The reaction mixture was then neutralized by shaking vigorously with 100 mg of silver acetate powder (16), and the sample was centrifuged. The insoluble residue was washed twice with 1 ml of methanol to ensure complete recovery of methyl glycosides and sphingosine bases. The combined methanolic solution was taken to dryness, and the residue was mixed with 100 μl of pyridine–hexamethyldisilazane–trimethylchlorosilane 10:4:2 (v/v) to obtain the trimethylsilyl (TMS) derivatives, as previously described (17).

Gas–liquid chromatographic analyses were carried out

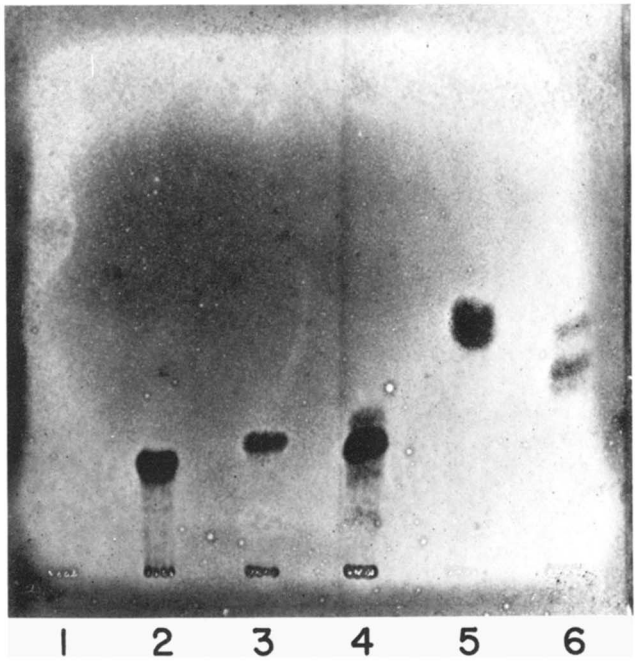

Fig. 1. Thin-layer chromatography on silica gel G (Analtech) plates. The solvent system was chloroform–methanol–concn NH_4OH 14:5:1 (v/v). Carbohydrate-positive spots were identified by α-naphthol–sulfuric acid spray (29). The plate was then heated at 100°C for a few minutes. Lane 1, standard sphingosine; lane 2, standard galactosylsphingosine; lane 3, standard glucosylsphingosine; lane 4, sample from Gaucher's spleen obtained from second silicic acid column; lane 5, standard glucosylceramide; lane 6, standard galactosylceramide.

with a Hewlett-Packard 7620A research chromatograph using a flame ionization detector attached to an electronic integrator capable of recording retention times and relative peak areas. An aliquot of the TMS derivatives of the methanolyzed sample was injected in chloroform into a 3% OV-1 column. The temperature was maintained initially at 160°C for 8 min to complete the separation of sugars present in the sample, after which the temperature was raised to 200°C at the rate of 10°/min and held constant for the separation of sphingosine bases (18).

TMS derivatization was also performed directly upon glucosylsphingosine, and an aliquot of this sample in chloroform was injected into a 3% OV-1 column maintained at 250°C.

Acylation of glucosylsphingosine

Fatty acyl chloride was prepared according to the procedure of Pinter, Hamilton, and Muldrey (19), and the acylation of glucosylsphingosine was performed as described by Shapiro and Flowers (20). 10 μmoles of either nonradioactive or 1-^{14}C-labeled palmitic acid (50 μCi) (New England Nuclear, Boston, Mass.) was dissolved in 0.5 ml of oxalyl chloride and incubated at 37°C in a tightly stoppered flask for 15 min. The sample was then evaporated to dryness gently under suction by warming in a 50°C water bath. This operation was repeated and the sample was dried over P_2O_5 in a vacuum desiccator.

1 μmole of glucosylsphingosine dissolved in 10 μl of 1 N acetic acid and 200 μl of freshly distilled tetrahydrofuran was transferred to the fatty acyl chloride taken in 200 μl of 50% sodium acetate (trihydrate) in a 5-ml round-bottomed flask. The flask was closed and the mixture was stirred with a magnetic flea for 4 hr at room temperature; the sample was then taken to dryness, dissolved in chloroform–methanol 2:1, and subjected to the partitioning procedure of Folch, Lees, and Sloane Stanley (13). The final lower chloroform phase was taken to dryness, dissolved in 2 ml of 0.1 M methanolic sodium methoxide, and incubated for 1 hr at 37°C. Sufficient chloroform was then added so that the solution was chloroform–methanol 2:1, and the solution was subjected to the partitioning procedure of Folch et al. (13). The lower phase was taken to dryness, redissolved in a small amount of chloroform, and applied to 1 g of Unisil packed into a column (0.7 cm ID) in chloroform and eluted with 50 ml of the same solvent. The chloroform fraction was discarded and the column was eluted with 50 ml of acetone to remove the glucosylceramide.

Linkage determination

In order to determine the nature of linkage of the car-

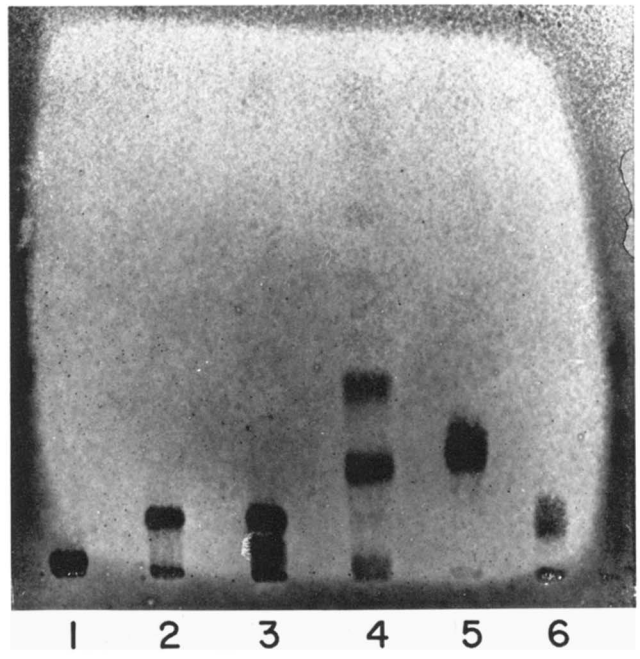

Fig. 2. Thin-layer chromatography on borate-impregnated silica gel G plates. The solvent system was chloroform–methanol–concn NH_4OH 14:5:1 (v/v). The spots were identified as described in Fig. 1. Lane 1, standard galactosylsphingosine; lane 2, standard glucosylsphingosine; lane 3, sample from Gaucher's spleen obtained from second silicic acid column; lane 4, acylation product of the material in lane 3 with palmitic acid; lane 5, standard glucosylceramide; lane 6, standard galactosylceramide.

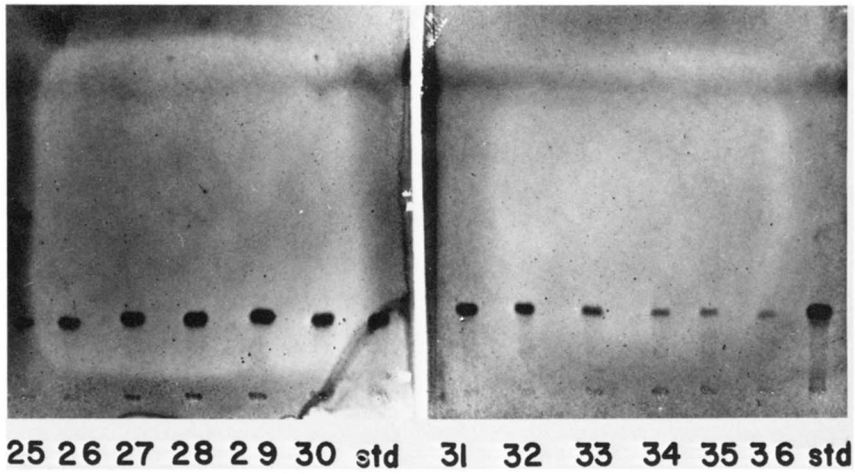

Fig. 3. Thin-layer chromatography on silica gel G plates. The solvent system was chloroform–methanol–concn NH_4OH 14:5:1 (v/v). The spots were identified as described in Fig. 1. Lanes 25–36 represent fractions from silica gel column chromatography; std, standard glucosylsphingosine.

bohydrate residue to the sphingosine base, authentic glucosylsphingosine and the sample isolated from Gaucher spleen were acylated with [1-^{14}C]palmitic acid as described above. The acylated product (cerebroside) was finally purified by preparative thin-layer chromatography on a silica gel G plate, using chloroform–methanol–water 65:25:4 as the solvent system. Glucosylceramide:β-glucosidase purified from calf spleen by affinity chromatography (21) was employed for determining the nature of glycosidic linkage in the sample. The reaction mixture consisted of 0.05 μmole of labeled substrate (sp act, 5 μCi/μmole), 0.75 mg of sodium taurocholate (Pfanstiehl Chemical Corp., Waukegan, Ill.), 0.25 mg of Cutscum (Fisher), and 5 μg of enzyme protein in 0.2 ml of 0.1 M citrate buffer, pH 5.0, containing 1% Triton X-100. The mixture was incubated for 6 hr, and the reaction was stopped by the addition of 5 ml of chloroform–methanol 2:1. The solution was washed (13), the lower phase was taken to dryness, and a sample was applied to a silica gel G plate, which was then developed in chloroform–methanol–acetic acid 90:2:8 (22). The areas corresponding to standard nonhydroxy fatty acid ceramide and glucosylceramide were scraped off and transferred to counting vials to which 0.5 ml of water and 15 ml of Aquasol (New England Nuclear) were added. Radioactivity was determined in a Tri-Carb liquid scintillation counter, model 3380.

RESULTS

A typical thin-layer chromatogram of the material isolated from Gaucher spleen by method 1 is shown in **Fig. 1**. The bulk of this material (lane 4) obtained from the second silicic acid column migrated like glucosyl- and galactosylsphingosine standards (lanes 2 and 3, respectively), which are not well resolved in this system but are well separated from the cerebroside standards (lanes 5 and 6). It was both ninhydrin and carbohydrate positive, suggesting the presence of both an amino group and a carbohydrate residue.

In order to distinguish between the two psychosines, the material was subjected to thin-layer chromatography on a borate-impregnated silica gel G plate (**Fig. 2**). The compound from Gaucher spleen (lane 3) migrated with glucosylsphingosine standard (lane 2) and is well separated from galactosylsphingosine standard (lane 1). This compound, after acylation with palmitoyl chloride and subsequent purification (lane 4), cochromatographed with glucosylceramide standard (lane 5), which is well separated from the galactosylceramide standard (lane 6). The additional spot above glucosylceramide in lane 4 was carbohydrate negative and was probably a contaminant arising from the components of the acylation reaction mixture.

Method 2 produces a purer glucosylsphingosine than method 1 because it avoids the use of the Dowex resin, which introduces contaminants. Final purification by chromatography on the silica gel column gave fractions containing only glucosylsphingosine, as shown in **Fig. 3**. This material appeared at fraction 25 and was completely recovered by fraction 36.

The gas–liquid chromatographic patterns from the methanolyzed samples of authentic glucosylsphingosine (*A*) and the purified sample (Fig. 3) isolated from Gaucher spleen (*B*) are shown in **Fig. 4**. Both appear to be identical and show only α-methyl glucoside (major) and β-methyl glucoside (minor), designated as peak 1, and C_{18}-sphingosine, designated as peak 2. There was a complete absence of C_{18}-*O*-methyl sphingosines, C_{18}-dihy-

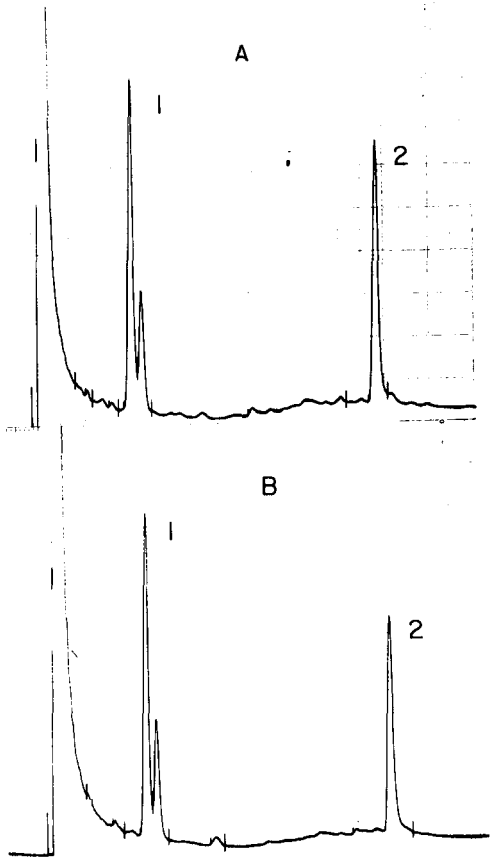

Fig. 4. Gas–liquid chromatography of TMS derivatives of the methanolyzed products of standard glucosylsphingosine (*A*) and purified sample (Fig. 3) from Gaucher spleen (*B*). Peak 1 represents α (major) and β (minor) methyl glucosides. Peak 2 represents C_{18} sphingosine. Conditions of operation are described in the Methods section.

drosphingosine, or other homologs. The absence of *O*-methyl sphingosine in our sample agreed with the findings of Taketomi and Kawamura (23) for the methanolysis of lysosphingolipids. The carbohydrate-to-base ratio was the same as that found in the authentic material employed as standard, showing that the material from Gaucher spleen contains glucose and sphingosine in equimolar amounts.

The gas–liquid chromatographic patterns of the directly silylated authentic glucosylsphingosine (*A*) and the purified sample (Fig. 3) from Gaucher spleen (*B*) are shown in **Fig. 5**. The sample gave a single peak coinciding with the standard material, also suggesting that the material isolated from Gaucher spleen is glucosylsphingosine.

The nature of glycosidic linkage was established by chemically converting the glucosylsphingosine isolated from Gaucher tissue and authentic standard to glucosyl-*N*-[1-^{14}C]palmitoyl ceramide. These were then tested as substrates for enzymatic hydrolysis by a purified glucosylceramide:β-glucosidase obtained from calf spleen. It is seen from **Table 1** that 67–70% of both samples was hydrolyzed by the enzyme to yield labeled ceramide. Because of the specificity of this enzymic preparation, it is concluded that the glucosidic bond has the β configuration. Little, if any, hydrolysis was found to occur in either the boiled enzyme or buffer controls.

DISCUSSION

Previous studies from this laboratory demonstrated a deficiency in the activity of glucosylsphingosine:β-glucosidase in Gaucher spleen tissue and skin fibroblasts (11). These studies provided preliminary evidence for the presence of a glucosylsphingosine-like material in Gaucher's spleen that was undetectable in normal or several pathological spleen tissue samples. The present communication provides evidence for the identification of this compound as β-glucosylsphingosine.

Two different procedures were used for the isolation of this material in an attempt to eliminate the possibility of it being an artifact. The cerebrosides, as well as most sphingolipids, are rather stable to alkali, and extended refluxing under appropriate conditions is required to cleave the amide-linked fatty acid in order to obtain glucosylsphingosine (24, 25). Treatment with mineral acid results

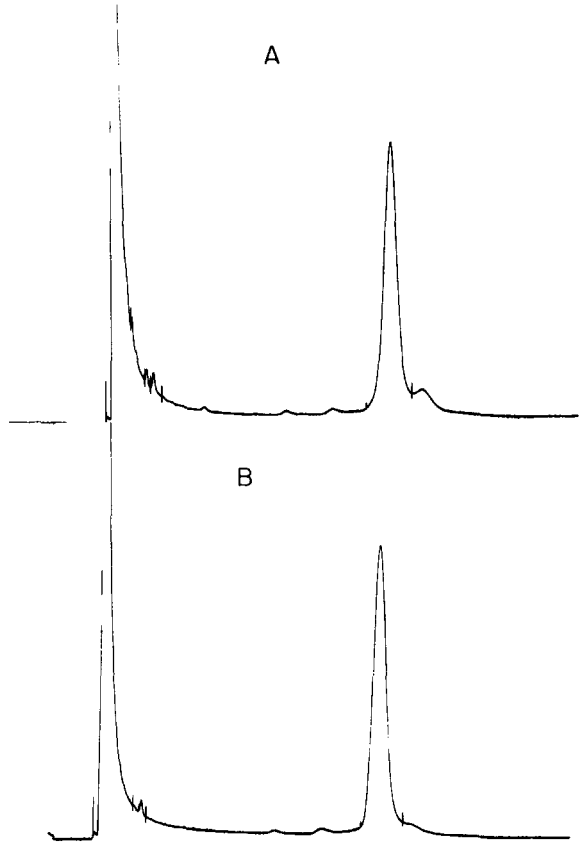

Fig. 5. Gas–liquid chromatography of TMS derivatives of standard glucosylsphingosine (*A*) and purified sample (Fig. 3) from Gaucher spleen (*B*). Conditions of operation are described in the Methods section.

TABLE 1. Hydrolysis of labeled glucosylceramide prepared from Gaucher spleen and from authentic glucosylsphingosine by purified glucosylceramide:β-glucosidase[a]

Substrate	Ceramide Formed[b]
Labeled glucosylceramide from authentic glucosylsphingosine	
Buffer control	0.3
Boiled enzyme	0.25
Experimental	67
Labeled glucosylceramide from Gaucher spleen	
Buffer control	0.09
Boiled enzyme	0.07
Experimental	70

[a] The assay is based upon the appearance of material comigrating with nonhydroxy fatty acid ceramides on thin-layer chromatography.

[b] Values are percentages of total counts originally incubated that chromatographed with ceramide.

in the cleavage of the glycosidic bond yielding ceramide; the amide linkage is then hydrolyzed only after heating for several hours (24, 25). The isolation procedures used in the present study avoid such drastic treatments. Glucocerebroside (500 mg) carried through these protocols does not break down spontaneously to give even a trace of psychosine.

Cleland and Kennedy (26) reported that galactosylsphingosine is soluble in chloroform at an approximate concentration of 50 nmoles/ml, and below this it is not extracted into an aqueous phase if the pH is alkaline. They also showed that due to the free amino group it is adsorbed on a cation exchange resin and is not eluted with aqueous 3 N HCl but is removed with 0.05 N methanolic HCl. These observations provided the basis for the isolation by method 1. The lipids extracted with chloroform–methanol 2:1 were subjected to mild alkaline treatment in order to eliminate lipids containing only ester-linked fatty acids because amide-linked fatty acids would be unaffected under these conditions. The partitioning procedure was modified in order to maintain glucosylsphingosine in the chloroform phase due to the alkaline pH and to remove the other water-soluble materials. The glucosylsphingosine was specifically adsorbed onto Dowex 50 H^+ resin but the neutral sphingolipids were not retained by the column. The psychosine was then recovered from the resin with dilute methanolic HCl.

The mild alkaline treatment was eliminated in method 2 because the chloroform–methanol 2:1 extract was directly subjected to the alkaline partitioning to maintain the psychosine in the lower phase while the water-soluble materials were removed. The glucosylsphingosine was then extracted into an aqueous acidic phase and thus freed from the bulk of the lipid, which remained in the chloroform phase. The psychosine was then simply extracted under alkaline conditions into chloroform.

The identification of the material as glucosylsphingosine, independent of which method of isolation employed, is a result of the following evidence. (*1*) Borate thin-layer chromatography demonstrated comigration with authentic glucosyl- and not galactosylsphingosine (Fig. 2, lane 3); (*2*) *N*-acylation and borate thin-layer chromatography showed comigration with authentic glucosyl- and not galactosylceramide (Fig. 2, lane 4); (*3*) gas–liquid chromatography of the TMS derivative directly showed retention time similar to standard glucosylsphingosine (Fig. 5); (*4*) gas–liquid chromatography of the TMS derivatives from methanolyzed samples gave only glucose and sphingosine in equimolar quantities (Fig. 4); (*5*) *N*-acylation with [^{14}C]palmitic acid yielded a labeled glucosylceramide that was substrate for a highly purified glucosylceramide:β-glucosidase (Table 1); and (*6*) gas–liquid chromatographic–mass spectral data (provided by Dr. Charles C. Sweeley, Michigan State University, East Lansing, Mich.) on authentic glucosylsphingosine and a sample isolated from Gaucher spleen agree with each other, establishing the structure.

The amount of glucosylsphingosine present in Gaucher spleen has been found to be 5.3 μmoles/100 g of wet tissue after isolation according to method 2. This value may not reflect the amount actually present in tissue because the isolation procedures were designed to provide a highly purified material for positive identification and chemical characterization. The quantity is undoubtedly small compared with the amount of glucosylceramide accumulating in Gaucher spleen (4–7). The presence of this material in Gaucher spleen in isolatable amounts while being undetectable in normal tissue clearly suggests that it may be present in the tissue due to the deficiency of glucosylsphingosine:β-glucosidase (11). This is the first time that glucosylsphingosine has been reported to be present in a mammalian tissue.

The origin and function of glucosylsphingosine is yet to be established. There is no evidence for the enzymic de-*N*-acylation of glucocerebroside to yield glucosylsphingosine. The in vitro synthesis of glucosylsphingosine from UDPglucose and sphingosine has been demonstrated to occur in brain tissue (27). The possibility exists that this may be a metabolic precursor for glucosylceramide (28).

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