Lipids of the spleen in Gaucher's disease

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SUMMARY Thin-layer chromatography (TLC) was used to analyze lipids of eight spleens of patients with Gaucher's disease. Four non-Gaucher spleens were also analyzed. Phospholipid concentrations are not increased in Gaucher spleens, while several classes of neutral lipids are moderately increased.

Acid hydrolysis followed by hexose determination was applied to total lipids and to separated fractions. Specific oxidase reactions demonstrated that glucose present is primarily in ceramide glucoside and ceramide lactoside, and galactose is primarily in ceramide lactoside. No ceramide galactoside was detected. The two hexoses found account for the anthrone-positive material in the lipid extract. Two TLC spots seen for Gaucher ceramide glucoside differ only with respect to fatty acid chain length. Long-chain base is all C_{18}-sphingosine. Small amounts of hydroxy acids are present in ceramide lactoside, but not in ceramide glucoside. Ceramide lactoside is present in similar amounts in Gaucher and non-Gaucher spleens.

KEY WORDS spleen · lipids · cerebrosides · fatty acids · man · Gaucher's disease · galactose oxidase · thin-layer chromatography · glycosphingolipids

CERAMIDE GLUCOSIDE, or glucocerebroside, has been well established as the major lipid which accumulates in the spleen in Gaucher's disease (2, 3). However, studies have generally involved reprecipitation of ceramide glucoside or other procedures which could result in the loss of small amounts of other glycolipids. Recent reports (4, 5) have indeed claimed the presence of varying amounts of ceramide galactoside and of ceramide digalactoside, in addition to ceramide glucoside in Gaucher spleens. The significance of a possible accumulation of ceramide galactoside lies partly in the fact that this lipid has thus far been found in large amounts only in the nervous system of higher animals. Also, the infantile form of Gaucher's disease is accompanied by mental deficiency. There is some question concerning changes in cerebral lipids in the infantile form of the disease (6, 7). Ceramide digalactoside has also been tentatively identified as an accumulating lipid in the kidney in cases of Fabry's disease (8) and has been found as a minor constituent in the brain in Tay-Sachs disease (9).

In order to clarify the nature and amounts of the ceramide monosaccharides and ceramide disaccharides in Gaucher spleens, these lipids were isolated chromatographically. Reprecipitation of isolated lipids was avoided. Previous reports (10, 11) from this laboratory described the direct reaction of ceramide monosaccharide fractions from silicic acid columns with the enzyme galactose oxidase in a mixed solvent system. Using spinal cord ceramide galactoside as a standard, Gaucher ceramide monosaccharide was found to contain less than 1% ceramide galactoside.

The present communication reports examination of 12 spleens (8 Gaucher, 4 non-Gaucher) for various lipid components, including ceramide monosaccharides and disaccharides. Glycolipids were determined by the direct galactose oxidase method and, after hydrolysis, by glucose oxidase, galactose oxidase, and anthrone determinations.

We were also interested in establishing the possible presence of hydroxy acids in Gaucher ceramide glucoside, since they have been reported in ceramide glucoside in normal spleen, serum, and liver (12). The fatty acid compositions of the glycolipids and the structure of long-chain base (sphingosine) from Gaucher ceramide glucoside are also reported.

EXPERIMENTAL METHODS

Isolation of Lipids

Portions of spleens removed surgically from patients with Gaucher's disease were kindly supplied by Drs. Allen

A preliminary report on certain phases of this work has appeared previously (1).

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Crocker and Robert Burton. Non-Gaucher spleens obtained at autopsy were made available by Dr. Robert Hendrix. The tissues had not been fixed; they were stored in the frozen state prior to extraction of lipids by the method of Folch et al. (13). Subsequent extraction in a Soxhlet apparatus for 2 hr with chloroform–methanol 2:1 resulted in isolation of less than 0.1% of additional anthrone-positive material.

Quantitative isolation of lipid components from the washed Folch extract was achieved by thin-layer chromatography (TLC) unless otherwise stated. Silica Gel G layers 250 μ thick were prepared according to Stahl (14). The lipid (10–20 mg) was applied as a streak across a 20 × 20 cm TLC plate with a microsyringe. Developing solvents used for TLC are given in Results.

Separated bands were usually located on TLC plates by spraying with 0.04% bromothymol blue in 0.01 N KOH. Ceramide glucoside and ceramide lactoside could be located by spraying with water. The bands were scraped from the plates and packed by suspension in appropriate elution solvents into glass columns (8 mm o.d.) and eluted with 40 ml of solvent per g of Silica Gel G. Effluent solutions were filtered through medium sintered glass filters. Except in the case of the phospholipids, the effluent solutions were washed with 0.2 volume H_2O and then twice with 0.2 volume of equilibrated aqueous phase to remove traces of spray reagent and contaminants present in the silica gel.

Efficiency of elution was checked by application of the lipid from individual eluted bands to separate TLC plates. Chromatography and elution of the latter plates resulted in recovery of 93–102% of the applied lipids as measured by the analtyical procedures described below. Identification of all lipids except "hydrocarbons" and cholesterol ester was checked by co-chromatography with authentic material.

Quantitative Determination of Lipids

Lipids which were determined gravimetrically were first lyophilized from benzene to constant weight. The non-hydrolytic galactose oxidase method for intact ceramide galactoside has been outlined previously (10). Galactose oxidase analyses of free hexose were performed by the same method using aqueous solutions. Galactose oxidase was prepared by the method of Avigad et al. (15). Glucose oxidase determinations of glucose utilized "Glucostat" reagent (prepared by Worthington Biochemical Co., Freehold, N.J.). Each determination with the oxidase systems was corrected by means of a "peroxide blank" containing the complete system minus oxidase. After the hydrolysis described below, anthrone determinations were made by the method of Radin et al. (16).

For hydrolytic glycolipid determinations the sample (containing 0.5–1.2 mg of glycolipid) was heated at 100° in a sealed tube with 3 ml of aqueous 3 N HCl. Hydrolysis time for determination of hexose in spleen glycolipids was 400 min. The aqueous solution was washed three times with 1 ml of diethyl ether. The ether extract was washed with 1 ml of water. The combined aqueous phase was taken to dryness under reduced pressure at 37°, and the residue was treated twice with small amounts of methanol to remove all traces of HCl. The dried material was dissolved in a known volume of water, and aliquots were taken for analysis of free sugars by the above methods. Hydrolysis of purified glycolipids resulted in linear standard curves in the range of glycolipids determined.

Data from the hydrolytic determinations were corrected for less than theoretical yields of hexose on the basis of results of concurrent hydrolyses of purified glycolipids. Purified ceramide glucoside was used to correct glucose oxidase and anthrone data for Folch extracts and ceramide monosaccharide samples. Purified ceramide lactoside was used for ceramide disaccharide fractions and for galactose oxidase analyses of Folch extracts. Representative correction factors obtained from triplicate determinations of purified glycolipid are: glucose in ceramide glucoside, 1.52; glucose in ceramide lactoside, 1.54; galactose in ceramide galactoside, 1.59; galactose in ceramide lactoside, 1.56.

Samples of each of these purified glycolipids were added to samples from the respective lipid fractions from several different spleens. Lipid mixtures prepared in this manner were hydrolyzed, and recoveries of purified glycolipids as hexose were determined. Also checked were recoveries of galactose from purified ceramide galactoside when the latter was added to Folch extracts and to ceramide monosaccharide fractions before hydrolysis.

Weights of neutral esters agreed with ester determinations by the hydroxamate method of Rapport and Alonzo (17). Cholesterol and cholesterol ester weights were checked with Liebermann-Burchard reagent prepared by Hycel Incorporated, Houston, Texas. Total phosphorus was determined by the method of Bartlett (18). Amounts of individual phospholipids were calculated from these values assuming only palmitic acid to be present. Phospholipid in the washed Folch extract was calculated assuming a molecular weight of 725.

Preparation of Purified Glycolipids

Authentic samples of cytolipin H (ceramide lactoside) and of ceramide glucosyl galactosyl galactoside were the generous gifts of Drs. M. M. Rapport and C. C. Sweeley respectively.

For the preparation of purified ceramide glucoside, the washed Folch extract from spleen "J. A., Gaucher"

was fractionated on Florisil. The 4:1 chloroform-methanol (C–M) eluate was dried, then applied as a suspension in chloroform to a silicic acid column (110 mg of lipid, 8 g of "BioRad" silicic acid in a 7 mm i.d. column). The column was eluted with 0–6% methanol in chloroform, employing a gradient. The ceramide glucoside, detected by TLC, was eluted at about 4% methanol. Ceramide glucoside purified in this manner showed only two spots on TLC (see below) and its infrared spectrum was identical with that published for ceramide glucoside by Rosenberg and Chargaff (19). Assuming a fatty acid chain length of 20.86 (see below) and no dihydrosphingosine, the empirical formula is $C_{44.86}H_{86.72}O_8N$ (molecular weight: 768.2). Analysis:

Calculated:	C, 70.14; H, 11.38; N, 1.82
Found:	C, 69.33; H, 11.25; N, 1.78

Isolation of ceramide glucoside for examination of fatty acids and long-chain base was accomplished by direct TLC separation from crude lipid.

Purified ceramide lactoside was also prepared from spleen "J. A., Gaucher." The washed lipid extract was taken to dryness, saponified in 0.5 N KOH in 50% aqueous ethanol (1 mg of lipid per ml) for 16 hr at 37°, acidified to pH 4–5, taken to dryness under reduced pressure, taken up in C–M 2:1, and applied to a TLC plate. The saponification was necessary to remove phosphatidyl serine and phosphatidyl ethanolamine, which overlap ceramide lactoside in the solvent system used. The purified ceramide lactoside fatty acids had an average chain length of 20.1. Assuming dihydrosphingosine to be absent, this corresponds to an average molecular weight of 916.6. After acid hydrolysis, equal amounts of glucose and galactose were found by analysis of the aqueous phase with glucose oxidase and galactose oxidase. Further evidence for the lactose sequence is the observation that the substance co-chromatographed with authentic cytolipin H and that it released ceramide glucoside during hydrolysis (100–200 min) in 3 N HCl but no ceramide galactoside. For hexose determinations, TLC of glycolipids was done without prior saponification since phospholipids did not interfere.

Purified ceramide galactoside was obtained from beef spinal cord using Florisil fractionation (20). This material has a molecular weight of 825, based on the nonhydrolytic galactose oxidase method.

GLC of Fatty Acids and Long Chain Aldehydes

For the characterization of the long-chain base, ceramide glucoside was subjected to methanolysis in 2 N HCl, followed by periodate oxidation of the resulting base, and gas–liquid chromatography (GLC) of the long chain aldehyde thus formed. The method of Sweeley and Moscatelli (21) was followed, except that catalytic hydrogenation of carbon–carbon double bonds was performed directly on the ceramide glucoside since recovery of the aldehyde after hydrogenation was low. The same methanolysis procedure was used to prepare fatty acid methyl esters from ceramide glucoside and ceramide lactoside for GLC. Dimethoxypropane (22) was used to insure complete conversion to methyl esters.

GLC analyses were performed on an F & M Model 609 flame ionization gas chromatograph. The stainless steel column, 36 inch × 1/8 inch o.d., contained 15% diethylene glycol succinate polyester on Gas Chrom Z (Applied Science Labs, Inc., State College, Pa.). Fatty acid methyl esters were identified and determined quantitatively by comparison with known amounts of authentic methyl esters. Long-chain aldehydes were compared with authentic palmitaldehyde and stearaldehyde (K & K Laboratories, Inc., Jamaica, N.Y.).

RESULTS

Lipid Composition of Gaucher and Non-Gaucher Spleens

Table 1 summarizes the amounts of total lipid in each spleen as well as amounts of each of three major classes: neutral lipids, ceramide glycosides, and phospholipids. In addition to the expected large amount of ceramide glucoside responsible for most of the larger content of ceramide glycoside in Gaucher spleens, there was also a significantly greater amount of neutral lipids.

Neutral Lipids

To obtain the data in Table 2, a Florisil column (24) was used to separate neutral lipid classes. It was necessary to follow the course of the elution by means of TLC to insure purity of fractions. In this case, lipids were determined gravimetrically. In later experiments, TLC was used alone to determine the neutral lipids. Values obtained gravimetrically with a microbalance directly from the TLC fractions of one Gaucher and one non-Gaucher spleen agreed closely with values given in Table 2. The TLC developing solvent was 1,2-dichloroethane (25) and chloroform was the eluting solvent. All material migrating faster than cholesterol ester was termed hydrocarbon. The weights of cholesterol ester, triglyceride, and free cholesterol from the TLC plates also agreed with hydroxamate and Liebermann-Burchard values obtained on the weighed materials.

Phospholipids

Since the question had arisen (25) of variation in amounts of individual spleen phospholipids in Gaucher's disease, these compounds were separated and determined. It can be seen in Table 3 that there is little difference from the non-Gaucher values. The sums of the values in Table 3

TABLE 1 TOTAL LIPIDS OF HUMAN SPLEEN

Patient, Diagnosis, Age, Sex, Spleen Weight	Total Lipids	Neutral Lipids	Ceramide Glycosides	Phospholipids	% Lipid Recovered*
			mg/g wet spleen		
A. W., atherosclerosis, 69 yr, female	19.9	4.1	0.9	—	—
F. C., diabetes-hypoglycemia, 64 yr, male	22.3	4.2	1.5	15.1	93
C. M., polycythemia vera, 66 yr, male	22.1	—	1.1	—	—
J. W., normal, traumatic death, 7 yr, female, 57 g	21.0	4.6	1.2	14.3	96
J. A., Gaucher, 9 yr, male, 1300 g	65.9	7.0	30.5	16.8	82
S. S., Gaucher, 3 months, male	46.1	—	17.1	—	—
E. B., Gaucher, 3 yr, female	65.8	—	23.2	—	—
K. S., Gaucher, 6 yr, male, 720 g	64.9	7.3	28.9	15.7	80
D. P., Gaucher, 11 yr, female	42.8	—	13.5	—	—
S. K., Gaucher, 11 yr, male	57.6	—	24.8	—	—
D. S., Gaucher, 7 months, male	50.0	—	15.2	—	—
K. Sa., Gaucher, 4 yr, male	70.1	—	27.5	—	—

Total lipids were determined gravimetrically using the washed Folch extract. Amounts of neutral lipids are sums of separately determined values for individual classes of neutral lipids (see Table 2). Amounts of ceramide glycosides are also sums of separately determined values (see Table 5). Phospholipids are estimated from total phosphorus in the Folch extract.

* Ceramides were determined in only J. W. and J. A. (Table 5). Ceramides may account for 1–2% of lipid not recovered in other spleens.

vary from 91 to 111% of the estimated total phospholipids in the Folch extracts (Table 1) and this is probably within the experimental error. In addition, 1–2% of the lipid phosphorus could be eluted from the origin of the TLC plates after chromatography. Erroneous values, lower than those listed, were obtained for lecithin and ethanolamine phospholipid in portions of spleens which had been frequently frozen and thawed.

Acid Hydrolysis of Purified Glycolipids

As observed by Rosenberg and Chargaff (19), concentrations of HCl below 3 N caused incomplete hydrolysis of ceramide glucoside and higher concentrations caused rapid destruction of glucose. Using the hydrolytic procedure outlined above, we could not recover the expected amount of glucose. Instead, a maximum of 70% of this amount was found by glucose oxidase determinations for hydrolysis times between 200 and 400 min, and even less for shorter or longer periods. Similar results were obtained with glucose and galactose oxidase determinations after hydrolysis of ceramide galactoside and ceramide lactoside. Free galactose or glucose, subjected to the conditions of the hydrolysis (1.3 μmoles of hexose in 3 ml of 3 N HCl), showed recoveries by the oxidase methods which decreased with time to about 70% at 400 min.

When glucose, galactose, or the purified glycolipids were subjected to hydrolysis conditions for 100–400 min, results of anthrone determinations were 3–6% above the values expected on the basis of oxidase determinations.

TABLE 2 NEUTRAL LIPIDS OF HUMAN SPLEEN

Patient	Hydrocarbon	Cholesterol Ester	Triglyceride	Free Cholesterol
		mg/g wet spleen		
Non-Gaucher				
A. W.	0.5	0.3	0.3	3.0
F. C.	0.4	0.3	0.7	2.8
J. W.	0.4	0.3	0.4	3.5
Gaucher				
J. A.	0.6	0.3	0.9	5.2
K. S.	1.3	0.4	0.8	4.8

Lipids from 5 g of spleen were separated by Florisil column chromatography (23) and determined gravimetrically.

Recovery of Glycolipid Hexose from Spleen Lipid Hydrolysates

The 400 minute hydrolysis procedure was applied to samples each containing a mixture of spleen lipid sample and an appropriate purified glycolipid (0.5 mg) as outlined in Methods. Analyses were made with glucose oxidase, galactose oxidase, and anthrone, and data were compared with those for spleen lipid fractions without additions. Values for the samples to which purified glycolipid had been added never differed from expected values by more than 5%.

It was also desirable to determine the *minimum* amount of ceramide galactoside which could be detected by galactose oxidase analysis of ceramide monosaccharide hydrolysates. Ceramide monosaccharide fractions used for this purpose were from 1.20 g of each of two different non-Gaucher spleens and from 0.16 g of each of two different Gaucher spleens. Each of the four fractions was hydrolyzed alone and a duplicate of each was hydrolyzed after addition of 0.01 μmole of purified ceramide galactoside. The addition of ceramide galactoside was noted by an elevation of 0.008–0.013 OD units above the peroxide blank. This difference was not seen in hydrolysates of spleen ceramide monosaccharide fractions to which the galactoside had not been added (Δ_{OD} = 0.000–0.003).

TABLE 3 PHOSPHOLIPIDS OF HUMAN SPLEEN

Patient	"Phosphatidic Acid"	Ethanolamine Phospholipids	Serine Phospholipids	Lecithin	Sphingomyelin
			mg/g wet spleen		
Non-Gaucher					
F. C.	0.62	5.06	0.51	5.85	2.12
J. W.	0.45	5.31	0.46	6.98	2.72
Gaucher					
J. A.	0.71	4.83	0.42	6.17	3.10
K. S.	0.84	4.99	0.48	5.93	2.91

Compounds were separated on neutral TLC plates without binder using C–M–acetic acid–water 50:25:7:3 (26). Eluting solvent was C–M–water 7:7:1. Quantities were calculated from total phosphorus analyses. Phosphatidic acid migrates near the solvent front and may contain some polyglycerol phosphate or cardiolipin.

Since <0.01 μmole of ceramide galactoside is therefore present in these samples, we conclude that the spleens contain <0.008 μmole of ceramide galactoside per g wet non-Gaucher spleen and <0.07 μmole per g wet Gaucher spleen.

Similarly the minimum detectable amounts of ceramide galactoside in the ceramide monosaccharide fractions were determined for the nonhydrolytic galactose oxidase method (10). In fractions from four spleens (two Gaucher) it was possible to detect 0.003 and 0.05 μmole of ceramide galactoside per g wet spleen in non-Gaucher and Gaucher spleens respectively. Thus the high degree of specificity of galactose oxidase permitted the detection of one part of ceramide galactoside in the presence of as much as 500 parts of ceramide glucoside using either the hydrolytic or the nonhydrolytic method.

Hexose in Lipid Fractions

Hexose determinations in lipid fractions are summarized in Table 4. Fractions were separated by TLC using C–M–water 24:7:1 as developing solvent (27) and C–M 2:1 as eluting solvent. Areas eluted corresponded approximately to R_F 0.45–0.75 for ceramide monosaccharide and 0.32–0.40 for ceramide disaccharide. Ceramide glucoside gave two spots with R_F 0.64 and 0.67, while ceramide galactoside gave 3 major spots with R_F 0.52, 0.54, and 0.59. Ceramide lactoside gave two spots with R_F 0.34 and 0.36. After hydrolysis, determinations were made with galactose oxidase and glucose oxidase. As seen in the last two columns on the right, most of the glucose present in the total extract could be accounted for as glucose in the ceramide monosaccharide and ceramide disaccharide fractions of all spleens, while somewhat less of the total galactose in the lipid extract was similarly accounted for. There was insufficient galactose in any ceramide monosaccharide fracton to be detectable. Paper chromatography and TLC on cellulose of sugars obtained from the ceramide monosaccharide fractions revealed no galactose spots.

Glycolipids

In Table 5, the weights of ceramide, ceramide glucoside, and ceramide lactoside present in 1 g of fresh spleen are listed. The level of ceramide lactoside is not very different in Gaucher and non-Gaucher spleens, whereas in the two spleens analyzed, the Gaucher spleen had about twice as much ceramide as the non-Gaucher spleen.

The quantity of ceramide galactoside was also determined in TLC ceramide monosaccharide fractions using

TABLE 4 HEXOSE IN LIPID FRACTIONS

Patient	(*a*) Folch Extract		(*b*) Ceramide Monosaccharide	(*c*) Ceramide Disaccharide		$\frac{b+c}{a} \times 100$	
	Glucose	Galactose	Glucose	Glucose	Galactose	Glucose	Galactose
			μmoles hexose per g wet spleen			%	%
Non-Gaucher							
A. W.	1.35	1.13	0.24	0.84	0.81	80	72
F. C.	1.75	1.72	0.21	1.45	1.49	95	87
C. M.	1.37	1.38	0.13	1.09	1.12	89	81
J. W.	1.06	0.98	0.37	0.50	0.44	83	45
Gaucher							
J. A	39.3	1.7	37.6	0.81	0.87	98	53
S. S.	22.2	1.1	21.4	0.74	0.70	99	64
E. B.	31.6	0.9	29.2	0.86	0.84	95	89
K. S.	37.8	0.7	36.9	0.63	0.60	99	86
D. P.	18.9	0.7	17.1	0.40	0.42	93	71
S. K.	34.5	1.1	31.3	0.92	1.01	93	91
D. S.	20.2	1.4	18.8	0.86	0.89	97	64
K. Sa.	35.6	1.3	34.4	1.22	1.23	100	92

Lipids were separated by TLC. After hydrolysis, determinations were made with galactose oxidase and glucose oxidase.

TABLE 5 CERAMIDE GLYCOSIDES OF HUMAN SPLEEN

Patient	Ceramide	Ceramide Glucoside	Ceramide Lactoside
		mg/g wet spleen	
Non-Gaucher			
A. W.	—	0.18	0.76
F. C.	—	0.16	1.32
C. M.	—	0.10	0.99
J. W.	0.43	0.28	0.45
Gaucher			
J. A.	0.88	28.9	0.74
S. S.	—	16.4	0.68
E. B.	—	22.4	0.79
K. S.	—	28.3	0.58
D. P.	—	13.1	0.37
S. K.	—	24.0	0.84
D. S.	—	14.4	0.79
K. Sa.	—	26.4	1.12

Ceramide was determined gravimetrically after isolation by TLC with C–M 19:1 as developing solvent (24) and as eluting solvent. Ceramide glucoside and ceramide lactoside were calculated from glucose oxidase values after hydrolysis of ceramide monosaccharide and ceramide disaccharide TLC fractions respectively. Small corrections for fatty acid chain lengths were made on the basis of mean chain lengths of J. W. (non-Gaucher) and J. A. (Gaucher) glycolipids.

the nonhydrolytic galactose oxidase method. All spleens contained less than the minimum detectable amount.

Composition of Ceramide Glucoside from Gaucher and Non-Gaucher Spleens

Ceramide glucoside from either Gaucher or non-Gaucher spleens forms two distinct spots on TLC. This separation was at first thought to be due to the different chromatographic effects of unsubstituted and of hydroxy fatty acids in the cerebroside, as has been reported in normal spleens by Svennerholm and Svennerholm (12). An alternative explanation is suggested by the findings of Jatzkewitz and Pilz (28), who reported the double spot observed on TLC of brain sphingomyelin to be due to differences in chain lengths of the fatty acids. Attempts to detect hydroxy acids in either Gaucher or non-Gaucher ceramide glucoside were unsuccessful. Samples of each of the two spots of Gaucher ceramide glucoside and of unseparated non-Gaucher ceramide glucoside were examined by two methods: (*a*) methanolysis and TLC in petroleum ether–diethyl ether 7:3 (24), and (*b*) the cupric chelate method (22). At the concentrations employed, these methods would detect hydroxy fatty acids corresponding to 0.5% and 4% respectively of the fatty acids in the ceramide glucoside sample.

When the materials from the two Gaucher ceramide glucoside spots were subjected to methanolysis and GLC of the methyl esters, a striking difference was seen in the fatty acid compositions. As shown in Table 6, the lower spot, which represents 21.5% of the total ceramide glucoside, contained predominantly palmitic and stearic acids. The upper spot, which represents 78.5% of the ceramide glucoside, contained predominantly fatty acids of longer chain length, particularly 22:0, 23:0, and 24:0. Presumably the two spots observed on TLC of non-Gaucher ceramide glucoside separate for the same reason.

Further studies were directed at establishing whether the long-chain base in the two spots of Gaucher ceramide glucoside was the same for both. Carter et al. (29) had shown the long-chain base from Gaucher ceramide glucoside to be D-*erythro*-sphingosine. To determine the chain length of the base and the percentage of dihydrosphingosine, samples corresponding to each of the two spots for Gaucher ceramide glucoside were examined as outlined under Experimental Methods. Long-chain base was solely C_{18}. C_{20}-Sphingosine, which occurs in brain (30), was absent from Gaucher ceramide glucoside. The base from the upper spot included 4% dihydrosphingosine; from the lower spot, 3% dihydrosphingosine. These spots both contained glucose as the sole sugar and the IR spectra of both were identical with that of the total ceramide glucoside fraction, including a small peak at 11.2 μ for a β-glucosidic linkage (19). Thus, the only difference between these two spots is in the fatty acid chain lengths.

TABLE 6 FATTY ACIDS OF CERAMIDE GLUCOSIDE FROM HUMAN SPLEEN

Patient	Fatty Acid											
	14:0	16:0	17:0	18:0	18:1	19:0	20:0	21:0	22:0	23:0	24:0	24:1
	mole % of total fatty acids recovered											
Non-Gaucher												
A. W.	0.6	26.1	0.4	5.8	5.1	0.5	3.3	0.7	14.3	8.7	19.2	15.2
J. W.	0.8	33.0*	0.9	9.4	7.4	1.5	2.2	1.0	11.0	4.6	10.3	17.9
Gaucher												
6 spleens (pooled)	0.5	23.5	0.7	5.3	0.1	0.2	3.8	0.1	20.7	13.5	21.9	9.7
J. A.	0.4	22.7	0.6	5.3	0.1	0.2	5.3	0.6	27.5	12.0	22.6	2.7
J. A. (upper spot)	0.1	6.5	0.3	4.2	0.0	0.1	5.8	0.9	33.8	14.2	29.9	3.8
J. A. (lower spot)	0.6	79.1	1.8	10.6	0.7	0.3	4.1	0.0	2.6	0.1	0.0	0.0

* Trace of 16:1 detected in addition.

TABLE 7 FATTY ACIDS OF CERAMIDE LACTOSIDE FROM HUMAN SPLEEN

Patient	Fatty Acid											
	14:0	16:0	17:0	18:0	18:1	19:0	20:0	21:0	22:0	23:0	24:0	24:1
	mole % of total fatty acids recovered											
A. W., non-Gaucher	0.3	44.7	0.3	2.2	0.0	0.0	4.7	0.0	13.8	5.1	26.4	2.5
J. W., non-Gaucher	0.6	47.4	0.3	5.5	0.1	0.0	4.1	0.0	12.2	5.0	21.9	3.0
J. A., Gaucher	0.4	38.3	0.2	4.1	0.0	0.0	3.4	0.0	15.5	6.2	30.3	1.5

Fatty Acids of Ceramide Lactoside from Gaucher and Non-Gaucher Spleens

There was no striking difference in the fatty acid distributions in ceramide lactoside of Gaucher and non-Gaucher spleens (Table 7). The results from non-Gaucher spleens are in reasonable agreement with those of Makita and Yamakawa (31), except that the latter did not detect nervonic acid.

A small amount of hydroxy fatty acid in ceramide lactoside was detected by TLC. However, judging by comparison of spot size on TLC with authentic methyl esters of hydroxy fatty acids, the hydroxy fatty acids amount to less than 5% of the total fatty acids of ceramide lactoside. No hydroxy acids were found in neutral lipid or phosphoglyceride fractions. The presence of hydroxy acid in total hydrolysates of rat spleen has been reported (22). It is likely that the two spots of approximately equal intensity (from either Gaucher or non-Gaucher spleens) seen on TLC of ceramide lactoside are also due to difference in populations of fatty acids.

DISCUSSION

The spleen concentrations of total lipid presented are in good agreement with reported values for Gaucher spleens (5, 6) and for normal spleens (6). The ceramide glucoside values correspond reasonably well with reported glycolipid values (25).

It may be seen in Table 2 that the increase of neutral lipid in Gaucher's disease is due to a general increase in several classes of neutral lipids, rather than a large increase in any one class. Values in Table 2 for free cholesterol and cholesterol esters agree with total cholesterol determinations done on the crude lipid extract and generally with reports from other laboratories (6, 32, 33).

In contrast to our findings, Speer et al. (32) report the level of glycerides plus cholesterol esters in normal spleens to be considerably above the level of free cholesterol. However, their values for glycerides plus cholesterol esters were obtained by weighing the entire lipid fraction that was eluted from a silicic acid column before free cholesterol. This early fraction probably contained materials we classified as hydrocarbons (23).

Substantial amounts of triglycerides were obtained from both Gaucher and non-Gaucher spleens. Mono- and diglycerides were also seen on TLC, but the amounts present were insufficient for quantitative determination. The amount of triglyceride detected in the spleens decreased with time if the spleens were not kept frozen or if the lipid extract was stored for long periods in chloroform–methanol solution. This instability may explain the reported absence of mono-, di-, and triglycerides from Gaucher spleens (34). Values for phospholipids confirm data reported previously by other authors (6, 32, 33). It is of interest that a decrease in phosphatidyl ethanolamine and sphingomyelin has been reported in Gaucher erythrocytes (35).

An anthrone determination was performed on each hydrolyzed sample listed in Table 4. Each pair of glucose oxidase–galactose oxidase values was compared with the anthrone value on the same hydrolyzed sample. The amount of glucose plus galactose estimated enzymatically and by the anthrone method agreed within 9%, except in ceramide disaccharide from Gaucher spleens, for which the variation was somewhat larger. This provides a check of the analytical methods as well as an indication that other anthrone-reacting materials are not present in significant amounts. In the ceramide disaccharide fraction, the ratio of glucose to galactose was nearly unity (0.91–1.14) in each of 12 spleens analyzed. The presence of significant amounts of ceramide digalactoside in this fraction is unlikely.

The large amount of ceramide glucoside in the Gaucher spleens was, of course, expected. Similar (5), as well as somewhat lower amounts (33, 36), have been reported.

Previous reports of the amount of cerebrosides in non-Gaucher spleens are those of Ottenstein et al. (36), who reported 1–5 mg/g of dry spleen (0.2–1.0 mg/g wet weight, assuming 80% moisture content), and Svennerholm (33) who reported an average of 1.75 mg/g of dry spleen.

The absence of galactose in the hydrolystates of the ceramide monosaccharide fractions confirms the results of the experiment using intact glycolipid and galactose oxidase. Glucose has previously been reported to be the sole sugar in purified cerebroside from normal spleens (31, 37). Reports of the presence of galactocerebro-

side (ceramide galactoside) in Gaucher spleens (5, 7) may be due to failure to completely separate cerebrosides (ceramide monosaccharides) from cytosides (ceramide disaccharides). Our attempts to separate ceramide monosaccharides and disaccharides using Florisil columns (20) were not successful. The best results were obtained using C–M 4:1 for elution of ceramide monosaccharides, and subsequently C–M 1:1 to elute ceramide disaccharides, but some overlapping was still observed. These difficulties were overcome by the use of TLC instead of Florisil. Since ceramide galactoside and ceramide glucoside are readily separated by TLC, this method can be employed for a simple presumptive identification of ceramide monosaccharide.

The conclusions that ceramide galactoside (4, 5) and ceramide digalactoside (4) are present in Gaucher spleens appear to be the result of analytical errors. The combined use of glucose oxidase, galactose oxidase, and a relatively nonspecific reagent such as anthrone is recommended in glycolipid analyses.

Our use of the HCl hydrolytic method (19) gave low yields of glucose. This seems to be due in part to a higher ratio of HCl to sample than that used by Rosenberg and Chargaff (19). However, even with the recommended times and ratio of HCl to sample, we obtained a low yield with purified ceramide glucoside (79% of theoretical). Since our procedure gave linear relationships between sample weights and optical densities, and since recovery studies were satisfactory, the use of a correction factor is justified.

The Gaucher ceramide glucoside had a higher proportion of saturated longer-chain fatty acids and less of the unsaturated fatty acids than non-Gaucher ceramide glucoside (Table 6). Thus it seems that there is a qualitative, as well as a quantitative difference, between Gaucher ceramide glucoside and non-Gaucher ceramide glucoside. However, it is possible that unsaturated fatty acids have been lost from the Gaucher spleens during a several-year period of storage in the frozen state. The non-Gaucher spleens were stored in a frozen state for one month or less before analysis. Marinetti et al. (2) have reported a partial separation of fatty acids of Gaucher ceramide glucoside, and Radin and Akahori (38) have reported the presence of nervonic acid (24:1).

Our lipid analyses do not account for about 0.3 μmole of galactose (in the washed Folch extract) per g of non-Gaucher spleen, and a similar amount is unaccounted for in the Gaucher spleens. This probably represents small amounts of ceramide glucosyl digalactoside, complex glycolipids, and sulfatides (33). It is not likely that these trace galactolipids are greatly increased in amount in Gaucher spleens.

It has been suggested that the accumulated ceramide glucoside of Gaucher spleen is related to erythrocyte glycolipid (3, 39, 40). The lack of a ceramide glucosidase (cerebrosidase) which is part of a degradative pathway has been proposed (3). The possibility that ceramide glucoside is formed from a neuraminic acid-containing lipid in the erythrocytes (40) is unlikely since neuraminic acid does not occur in human erythrocyte lipid (41). The major erythrocyte glycolipid, ceramide glucosyl galactosyl galactosyl *N*-acetyl galactosamine (globoside) (42) is a more likely precursor. The possibility that a biosynthetic enzyme which converts ceramide glucoside to higher ceramide saccharides is lacking must also be considered.

The following is an alternative possibility: no degradative enzyme is absent from the spleen, but glycolipids accumulate there as a result of an unknown primary cause, involving one or more of the blood elements. If the globoside or other complex lipid is cleaved sequentially, each product is progressively less soluble. As tissue lipid levels increase, deposition in reticulum foam cells occurs at a rate in excess of hydrolytic degradation. This process would cause preferential deposition of the less polar (saturated and long-chain fatty acids) ceramide glucosides in Gaucher's disease, as is indeed observed.

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