Splenic lipids in Gaucher's disease

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ABSTRACT Column chromatography (on cellulose, silicic acid, and Florisil) and thin-layer chromatography were employed for the separation and purification of lipid fractions from normal and Gaucher spleens. A new hydrolysis procedure, followed by paper chromatography, was used for identification of sugar moieties. A nonhydrolytic combined colorimetric procedure, with anthrone and orcinol, was used for the estimation of glucose and galactose separately in glycolipids. The limitations of this method were examined.

Spleens from two control subjects and three patients with Gaucher's disease have been examined in detail. In all Gaucher spleens, the predominant feature was the massive acccumulation of glucocerebroside; neutral ceramide oligohexoside levels were probably within the normal range, as were other neutral lipids and phospholipids. In one case examined for gangliosides, these were increased twentyfold.

One Gaucher spleen, in which others had reported that the stored "cerebroside" contained predominantly lactose as the saccharide moiety, has been examined in detail and it has been established that the stored material was, in fact, glucocerebroside, ceramide lactoside levels not being significantly elevated.

In a further nine cases glucose was the major sugar detected in the splenic lipids.

SUPPLEMENTARY KEY WORDS cerebroside · ceramide dihexoside · ganglioside · glycolipid · lipidosis · sphingolipid

GAUCHER'S DISEASE is a rare, genetically determined disorder characterized by the accumulation of glycolipid in the cells of the reticuloendothelial system. In the so-called "adult" form the patients are usually at least 1 yr old when they first manifest symptoms and, characteristically, only the spleen, bone marrow, lymph nodes, and Kupffer cells of the liver are involved, the patient showing no signs of neurological or pulmonary involvement. The lipid stored in the spleen was long ago identified as a cerebroside but, until relatively recently, much confusion existed as to the nature of the hexose moiety in the cerebroside deposited in Gaucher's disease. During recent years, however, more refined chromatographic and analytical techniques have been developed. In recent analyses of Gaucher spleens, virtually all the hexose in the cerebroside has been found to be glucose (1). The glycolipid profile of normal spleen was elucidated by Svennerholm and Svennerholm (2); the nature of the fatty acid and sphingosine moieties of the splenic glycolipids in Gaucher's disease do not differ significantly from the normal (3, 4).

Parke (5, 6) found lactose in the "cerebrosides" of the spleen removed from a patient with Gaucher's disease; the glucose:galactose ratio in the splenic lipids of about 1:1 suggested that most, if not all, of the carbohydrate moiety initially present was lactose. Rosenberg (7) described an atypical case of Gaucher's disease; of the total glycolipid hexose, including gangliosides, only 10% was glucose, the remainder being galactose. The main neutral glycolipid fraction isolated appeared to be a cytoside, or ceramide dihexoside, containing equal amounts of glucose and galactose. Philippart, Rosenstein, and Menkes (4) reported, in addition to the characteristic elevation of glucocerebroside, a slight increase of ceramide dihexoside and a significant increase in the ganglioside in Gaucher's disease.

We report here examination of the splenic lipids from twelve cases of Gaucher's disease, including that reported by Parke (6) in which storage of ceramide dihexoside instead of glucocerebroside was suggested.

MATERIAL

The material investigated consisted of portions of the surgically removed spleens of eleven patients with Gaucher's disease and of two "normal" controls, and the spleen, removed at autopsy, of a patient with the

Abbreviation: C–M, chloroform–methanol; TLC, thin-layer chromatography.

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TABLE 1 MATERIAL INVESTIGATED

Case	Age at Splenectomy		Spleen Weight	Portion No.	Storage
	yr	*mo*	*g*		
Controls					
A	50	—	110	—	Frozen
B	3	—	—	—	Frozen
Gaucher					
S. M. H.	31	—	2600	1	Preserved 3 mo
				2	Preserved 11 yr
				3*	Preserved 3 mo
				4	Frozen
				5	Frozen
				6–12	Preserved 13 yr
R. I.	15	—	1127	—	Preserved 2 yr
J. L.	6	—	628	—	Frozen
G. B.	1	10	689	1	Preserved 3 mo
				2	Preserved 3 mo
				3	Preserved 3 mo
				4	Preserved 3 mo
S. M.	1	3	—	—	Frozen
R. W.	7	—	732	—	Fresh
I. P.	12	—	—	—	Preserved 4 yr
H. H.	Adult		—	—	Frozen
D. T.	3	3	597	—	Frozen
A. F.	21	—	1350	—	Preserved 20 yr
H. J.	12	—	2350	—	Preserved 37 yr
K. C.†	—	4	50	—	Preserved 22 yr

* Large infarcted region.
† Acute infantile type; spleen obtained at autopsy.

acute infantile variety of Gaucher's disease. Relevant details are given in Table 1. In all twelve patients with Gaucher's disease, histological examination of the spleen confirmed the diagnosis made earlier on clinical grounds. Apart from K. C., none of the patients showed signs of neurological involvement, but G. B. died 1 yr 3 months after splenectomy and, at autopsy, enormous numbers of Gaucher cells were found in the lung parenchyma and alveoli. Clinical accounts have been published of three of these patients: S. M. H. (8), H. J. (9), and K. C. (10). The spleen of S. M. H. was investigated chemically by Parke (5, 6) and a preliminary account of our findings in this case, in D. T., and in K. C. has appeared (11).

Control A was a normal adult apart from carcinoma of the stomach; the spleen was removed in the course of total gastrectomy. Control B was a normal child apart from hiatus hernia; splenectomy was performed to allow free mobilization of the diaphragm for oesophagoplasty.

A portion of spleen R. W. was examined 2.5 hr after surgical removal, without freezing or preservation of any kind. Where indicated in Table 1, portions of the spleens were frozen within 3 hr of removal and stored at −40°C until examined. Preserved tissues were kept either in a neutral solution of 4% formaldehyde in 0.9% sodium chloride, or in Kaiserling solution (8% formaldehyde, 3% potassium acetate, 1.5% potassium nitrate) until examined.

Several of the spleens had infarcted areas. S. M. H., in particular, had many yellowish necrotic areas, 1–2 cm across, and one large infarct, 5 cm in diameter, which was examined chemically.

Glycolipid Standards

Galactocerebroside. Prepared from ox brain white matter (12).

Glucocerebroside. Prepared from Gaucher spleen tissue (S. M. H.) by a similar method. The final material was recrystallized twice from ethanol and dried in vacuo. White needles; mp 169–169.5°C; $[\alpha]_{589}$ at 20°C, −11.2° (c = 9% in pyridine). Analysis (Weiler and Strauss, Oxford, England) gave the following results. Found: C, 70.24%; H, 11.40%; N (Kjeldahl), 1.83%; glucose, (anthrone) 23.0%. Calculated: C, 70.14%; H, 11.38%; N, 1.82%; glucose, 23.4%. The calculated values were obtained by assuming a molecular weight of 768.2 (3).

Hydrolysis and paper chromatography revealed glucose as the only sugar. A thin-layer chromatogram of the cerebroside is shown in Fig. 1; the two bands of glucocerebroside ran to a different position from galactocerebroside.

Ganglioside Fraction from Normal Adult Human Brain. A lipid extract was prepared from cortical gray matter and the gangliosides were extracted by shaking with potassium chloride solution (13). The ganglioside fraction was purified and concentrated tenfold by dialysis against distilled water and ultrafiltration.

Glycolipid Fractions from Human Erythrocyte Stroma. A lipid extract was prepared from the stroma obtained

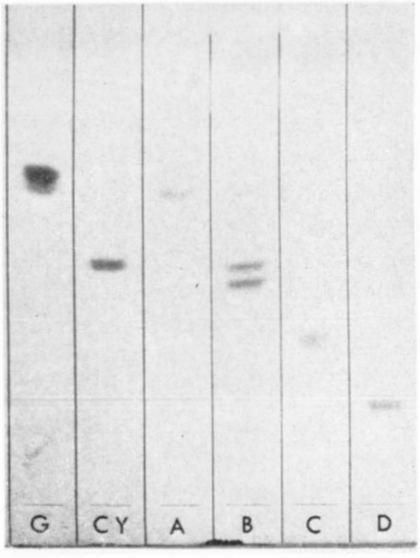

FIG. 1. Thin-layer chromatogram of glycolipids. *G*, glucocerebroside standard; *CY*, *N*-lignoceryl cytolipin H; *A*, *B*, *C*, *D*, glycolipid fractions from human erythrocyte stroma. Developing solvent: C–M–water 65:25:4; spray, 50% sulfuric acid.

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from 8 pints of packed cells, obtained from the Oxford Regional Hospital Board blood bank. After mild alkaline hydrolysis to destroy labile phospholipids (14) and acidification to pH 4–5, the lipids were extracted into C–M 2:1 and the glycolipid fractions separated by silicic acid column chromatography followed by quantitative TLC. Four glycolipid fractions (*A–D*) were obtained (Fig. 1). The major fraction, *D*, was further purified by recrystallization from ethanol–acetone 1:1. Yield of fraction *D:* 66 mg (mp 240°C). Analysis gave the following results. Found: C, 58.14%; H, 9.51%; N (Kjeldahl), 2.0%; glucose, 12.5%; galactose, 26.2%; hexosamine, (15), 11.6%. Calculated (assuming lignoceric acid as the fatty acid): C, 60.98%; H, 9.41%; N, 2.09%; glucose, 13.4%, galactose, 26.9%; hexosamine, 13.4%. The glucose and galactose values were determined by the combined anthrone–orcinol procedure described below. On hydrolysis and paper chromatography, this fraction gave spots corresponding to glucose and galactose in the ratio of approximately 1:2. After partial hydrolysis with 0.2 N HCl for 3 hr at 100°C, a spot corresponding to *N*-acetyl-D-galactosamine was identified. Degradation of the products of complete hydrolysis with ninhydrin and pyridine (16) yielded one spot on the paper chromatogram corresponding to lyxose, which indicated the presence of galactosamine. From these results, fraction *D* has been identified as aminoglycolipid or globoside [*N*-acetyl-galactosaminoyl-(1 → 3)-galactosyl-(1 → 4)-galactosyl-(1 → 4)-glucosyl ceramide] (17).

The other three fractions, *A*, *B*, and *C*, have been tentatively identified as, respectively, ceramide monohexoside (predominantly glucocerebroside), ceramide dihexoside (containing one glucose residue and one galactose), and ceramide trihexoside (containing one glucose residue and two galactose), on the basis of TLC, hexose analyses, and paper chromatography of hydrolysis products.

Ceramide Dihexoside. Synthetic *N*-lignoceryl cytolipin H was obtained from Yeda Research and Development Co., Ltd., Rehovoth, Israel.

METHODS

The sequence of operations is illustrated in Fig. 2.

Extraction of Lipids

Fixed material was first washed in running water for 36 hr. A sample of tissue was dried to constant weight at 70°C to give the water content. The remainder was homogenized with C–M 2:1 and filtered, and the residue was thoroughly washed with C–M 2:1. Proteolipid was split and the lipid freed from protein contamination (18). The extract was dried to constant weight in vacuo at room temperature to give the total lipids. Nonlipid material and gangliosides were then removed by chromatography on cellulose (19).

Silicic Acid Chromatography of Total Lipids

Immediately before use, solvents were degassed under reduced pressure. Silicic acid (Mallinckrodt, 100 mesh) was elutriated to remove fine particles, dried, and activated at 120°C for 24 hr in a stream of dry nitrogen. A column, 27–60 cm high and containing 20–45 g of silicic acid, was washed through with 200–300 ml of C–M 98:2. Lipid (7–8 mg/g of adsorbent) was dissolved in a small volume of the same solvent, 0.5–1 g of silicic acid was added to the solution, and the mixture was applied to the column. Elution with C–M 98:2 (fraction *I*), was followed by C–M 96:4 (fractions *Ia* and *II*), and then by gradient elution with C–M 96:4 changing gradually to C–M 15:85 (fractions *III–VIII*) and finally methanol; flow rate was 25 ml/hr throughout. Each 100 mg of total lipid required 100 to 150 ml of C–M 98:2 before no further solid was eluted in fraction *I*. Other cuts were made on the basis of hexose and phosphorus analyses (25) of the eluate.

Determination of Individual Lipids

Neutral Lipids. Fraction *I* was separated into its components by chromatography on Florisil (20). Hydrocarbon was eluted with hexane; and cholesteryl esters with 5%, triglyceride with 10%, and cholesterol with 25% ether in hexane. A rough estimate of the amount of hydrocarbon and triglyceride was obtained by weighing the appropriate dried fraction. The appropriate fractions were analyzed for cholesterol and cholesteryl esters by the method of Leffler (21).

Cerebroside. 10–20 mg of fraction *II* was purified by passage through a Florisil column 0.56 × 4 cm (22, 23). Cerebroside was eluted with 50 ml of C–M 2:1. In three separate experiments, the recoveries of pure glucocerebroside were 98%, 100%, and 100%, respectively.

Ceramide Dihexoside. 20–25 mg of fraction *III* was purified on a Florisil column 0.56 × 5 cm. Traces of cerebroside were eluted with C–M 9:1 (15 ml). Ceramide dihexoside was then eluted with 150 ml of C–M 2:1 and analyzed for glucose and galactose by the combined anthrone–orcinol procedure described below. In three separate experiments, the recoveries of synthetic *N*-lignoceryl cytolipin H were 95%, 98%, and 100%, respectively.

Lecithin and Sphingomyelin. A combination of fractions *VI*, *VII*, and *VIII*, containing approximately 25–50 μg of lipid phosphorus, was separated into its components by quantitative TLC (24) on Silica Gel G in C–M–water (65:25:4). The bands were made visible with

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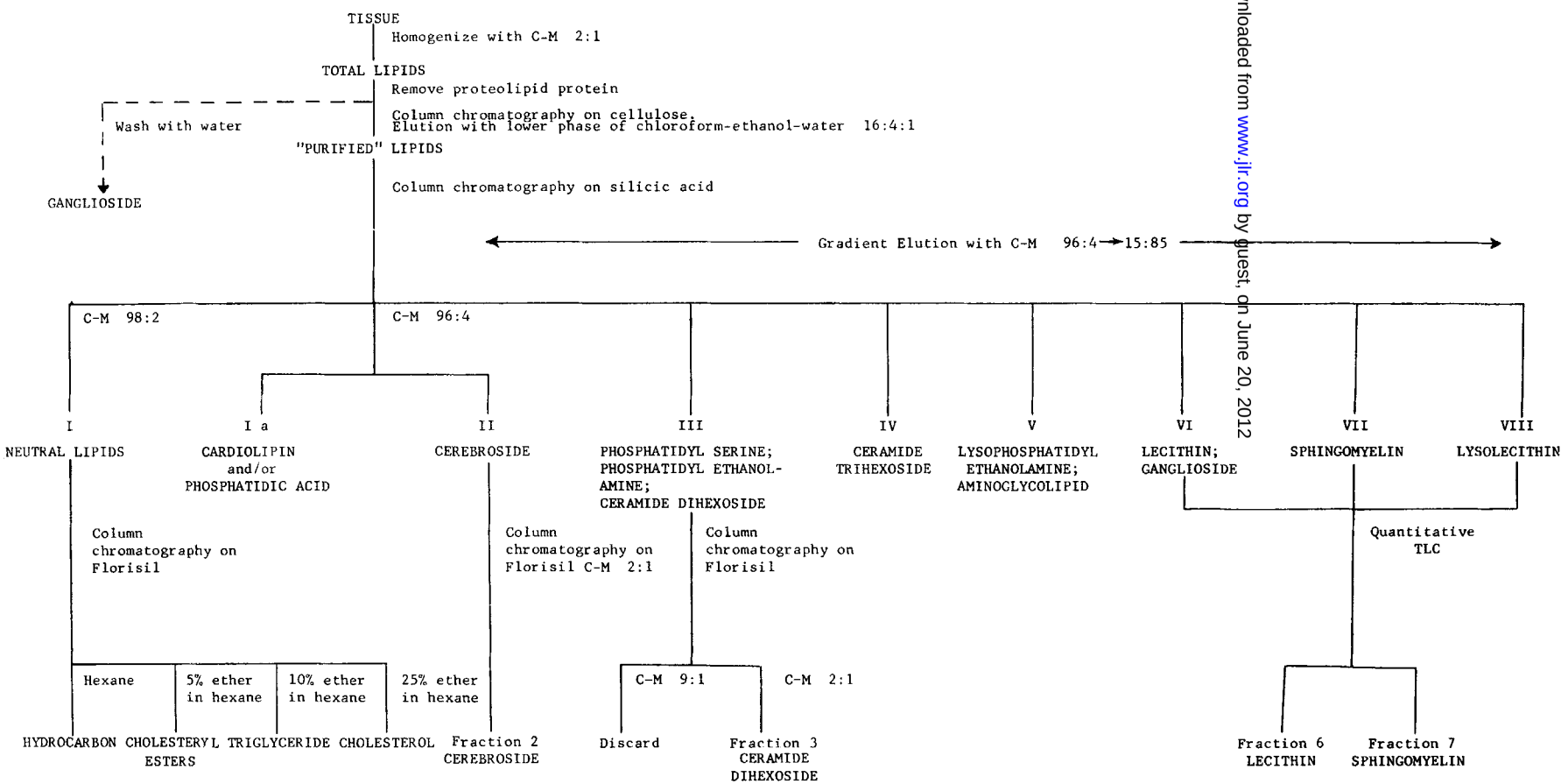

FIG. 2. Scheme for separation and purification of tissue lipids.

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iodine and scraped off into small columns. The lecithin band was eluted with 20 ml of C–M 1:1 (fraction 6) and the sphingomyelin with 15 ml of C–M 2:1 followed by 15 ml of C–M 1:4 (fraction 7).

Glucose and Galactose. These sugar residues in glycolipids were estimated by the nonhydrolytic anthrone procedure (23) and the nonhydrolytic orcinol method (12). For each unknown, several glucose and galactose standards were carried through both procedures in triplicate and standard curves were drawn each time (Fig. 3). From these curves were derived the weight of galactose,

$$\{D_a (x_o - y_o) - D_o (x_a - y_a)\}/(G_oD_a - G_aD_o)$$

and the weight of glucose,

$$\{G_o (x_a - y_a) - G_a (x_o - y_o)\}/(G_oD_a - G_aD_o)$$

where x is the OD of unknown, y the intercept on the OD axis, G the OD of a galactose standard, D the OD of a glucose standard, and subscripts o and a refer to orcinol and anthrone reactions, respectively.

Gangliosides. A portion of the protein-free lipid extract was shaken with $^1/_5$ volume of water and the lower phase washed four times by shaking with ideal upper phase (cf. 13). Use of dilute salt solutions in place of water resulted in the larger part of the ganglioside remaining in the organic phase. The combined aqueous phase was analyzed for sialic acid with Bial's orcinol reagent (26). In this method, interference from other carbohydrates is reduced by a blank determination in which the sialic acid is selectively destroyed.

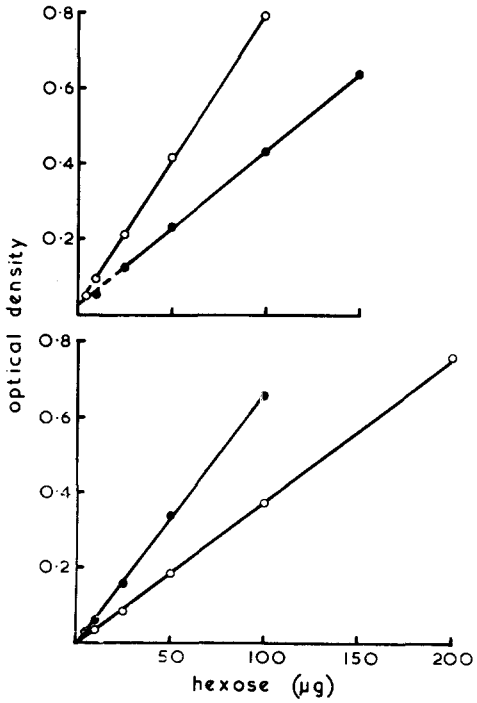

FIG. 3. Calibration curves for glucose (●) and galactose (○) after treatment with anthrone (bottom) and orcinol (top) reagents.

TLC of Lipids

Qualitative TLC was on Silica Gel G (Merck) in C–M–water 65:25:4 (27) or C–M–35% aqueous ammonia 18:6:1 (28). Gangliosides were separated in *n*-propanol–water 7:3 (29).

Spray reagents used were 50% sulfuric acid (charring at about 110°C), ninhydrin, Dragendorff reagent (27), and Bial's orcinol reagent.

Identification of Hexose in Glycolipids

Acid Hydrolysis of Lipids. Method A: Total hydrolysis with sodium dodecyl sulfate was carried out as previously described (11). This method was later abandoned in favor of Method B. Method B: It was found that pure glucocerebroside, galactocerebroside, and cytolipin H were easily soluble in warm, aqueous 3 M trichloroacetic acid. Lipid samples containing about 1 mg of glycolipid were heated with 1 ml portions of 3 M trichloracetic acid, made 2 N in HCl, for 2 hr at 100°C. The solution was cooled, diluted, and extracted twice with an equal volume of ether, and the aqueous phase was passed through a column of strong-base anion-exchange resin in the acetate form (De-Acidite FF, SRA 63, Permutit Co., Ltd., London). The effluent was evaporated to dryness and taken up in 1 ml water and aliquots were used for paper chromatography.

Paper Chromatography. This was descending, one-dimensional chromatography in the upper phase of pyridine–ethyl acetate–water 1:2:2 (30). It proceeded on Whatman No. 52 paper for 24 hr ("over-run" technique). The paper was sprayed with aniline phthalate reagent (31); chromatograms were viewed in daylight and under UV light (3650 A). Quantification (±25%) was by visual comparison with the appropriate standards run alongside.

In some experiments, fermentation with a selected strain of baker's yeast was used prior to paper chromatography to destroy glucose and so reveal small amounts of nonfermentable sugars which were undetectable in the presence of very large amounts of glucose.

RESULTS

Extraction with 20 volumes of cold C–M 2:1 removed virtually all the lipids from these spleens, including the fresh (unfrozen) spleen of case R. W. Further extraction for 2 hr with boiling C–M 2:1, containing 5% water, resulted in the extraction of only traces of additional lipid material (R. W., H. H., and J. L).

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Chromatography on Cellulose

All fractions obtained by cellulose chromatography were examined by TLC and it was found that all glycolipids except ganglioside and all the major phospholipids except phosphatidyl serine were eluted exclusively in the lipid fraction, i.e., with the water-poor phase of chloroform–ethanol–water 16:4:1. A small amount of ganglioside was eluted in the lipid fraction. Considerable amounts of free amino acids were present in all three fractions.

Chromatography on Silicic Acid

The typical elution pattern of lipids from a normal spleen (A) is shown in Fig. 4, and that of a Gaucher spleen (J. L.) in Fig. 5. In each case the neutral lipid fraction had been previously eluted with C–M 98:2.

Four neutral glycolipid peaks were identified: glucocerebroside (peak *II*), ceramide dihexoside (peak *III*), ceramide trihexoside (peak *IV*), and aminoglycolipid (peak *V*). These were identified by hydrolysis and paper chromatography, by anthrone and orcinol analyses, and by TLC alongside standard material. The glycolipid of peak *VI* represented the residual ganglioside that was present in the lipid fraction after cellulose chromatography. TLC showed the complete absence of the "neutral" lipids, cholesterol, cholesteryl esters, triglycerides, and free fatty acids from fraction *II* (cerebroside). Similarly, provided a large enough volume of C–M 96:4 was used and this was followed by a very slow initial gradient, the last portions of fraction *II* were completely free from ceramide dihexoside and the earlier portions of fraction *III* almost completely free from cerebroside, even in the case of Gaucher spleens where the very large quantities of glucocerebroside might have been expected to contaminate more polar fractions. The ceramide

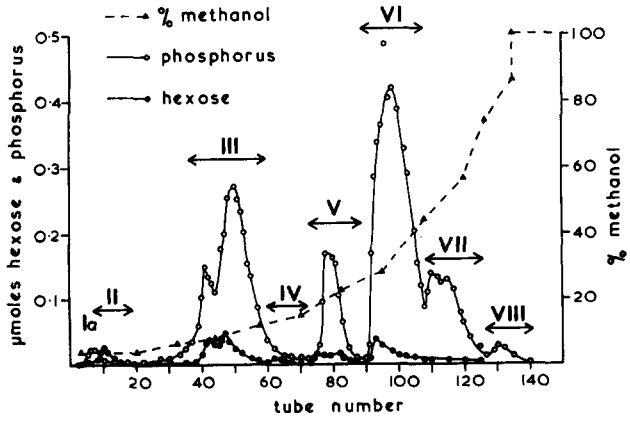

FIG. 4. Silicic acid chromatography of lipids from normal spleen A. Hexose (as glucose) and phosphorus (as phosphate) are expressed as μmoles/g wet weight. Fraction *I*, eluted with C–M 98:2, is not shown.

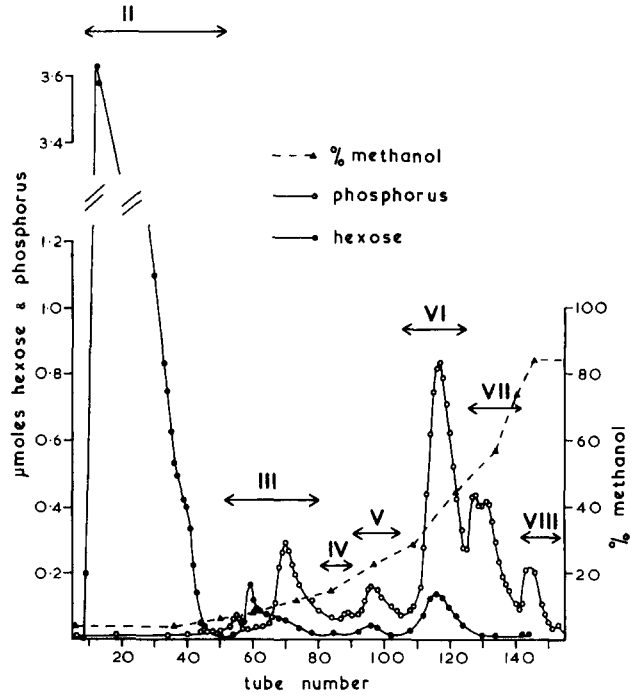

FIG. 5. Silicic acid chromatography of lipids from Gaucher spleen J.L. Hexose (as glucose) and phosphorus (as phosphate) are expressed as μmoles/g wet weight. Fraction *I*, eluted with C–M 98:2, is not shown.

dihexoside, fraction *III*, isolated from both normal and Gaucher spleens contained glucose and galactose in equal amounts. On TLC in three different solvents it ran to the same position as cytolipin H but, unlike the standard, gave a double band—as did the ceramide dihexoside fraction from erythrocyte stroma. The two components were partly separated by silicic acid column chromatography (Fig. 4).

The phospholipid of peak *Ia* has been tentatively identified by TLC as cardiolipin and (or) phosphatidic acid. The remaining phospholipid fractions are phosphatidyl ethanolamine and phosphatidyl serine (peak *III*), lysophosphatidyl ethanolamine (peak *V*), lecithin (peak *VI*), sphingomyelin (peak *VII*), and lysolecithin (peak *VIII*). Identification was by TLC alongside authentic material, reactivity on TLC with ninhydrin (fractions *III* and *V*), and the Dragendorff reagent (fractions *VI*, *VII*, and *VIII*), and paper chromatographic identification of ethanolamine (fractions *III* and *V*) and serine (fraction *III*) in the hydrolysis products.

Determination of Glucose and Galactose

With anthrone, Beer's law was generally obeyed both with galactose and with glucose; with some batches the straight lines did not pass through the origin. With orcinol, OD plotted against amount of galactose or glucose gave a straight line not passing through the origin. The

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intercept on the OD axis (y_o) was the same for both glucose and galactose curves; this was also true for the anthrone reaction when y_a was not zero. Different amounts of galactocerebroside and glucocerebroside gave optical densities identical with those given by equivalent amounts of galactose and glucose, respectively, both for anthrone and orcinol reactions.

The sensitivity and precision of the method are shown in Table 2; the accuracy with which mixtures of known composition could be analyzed is shown in Table 3. The method could be scaled down by a factor of five without difficulty.

The use, and limitations, of the method in establishing the composition of a purified lipid are illustrated in Table 4; the method gives a much more accurate estimate of total hexoses than of the galactose:glucose ratio, particularly when this is far from unity.

Extracts not purified with Florisil turned brown on heating with phosphoric acid; "blank" reactions, with orcinol or anthrone omitted, absorbed strongly at 510 and 625 mμ, respectively. As Radin (23) found, it was not possible to correct for this nonspecific absorption by subtracting a blank value, but Florisil removed virtually all the interfering chromogens.

The sensitivity of paper chromatography for detecting glucose and galactose is illustrated in Table 5. Accuracy of quantitative estimation by this method is ±25%.

Stability of Sugar Moieties to Hydrolytic Procedures

Method A gave a quantitative recovery of glucose from glucocerebroside and galactose from galactocerebroside, as far as could be determined by paper chromatography.

Method B gave 76 ± 5.9% recovery of glucose from glucocerebroside (24 samples) and 90.4 ± 2.6% recovery of galactose from galactocerebroside (8 samples), as determined by the anthrone method before and after hydrolysis. A 2 hr hydrolysis time, a temperature of 100°C, and a solution 2 N in HCl and 3 M trichloracetic acid were found to be optimal in method B.

Lipids of Human Spleen

The lipid content of normal and Gaucher spleens is shown in Table 6. Phosphorus was determined on the lipid fraction after cellulose chromatography and therefore excludes any lipid phosphorus retained in the ganglioside fraction. Since a small amount of ganglioside was eluted from cellulose in the lipid fraction, sialic acid analyses were performed prior to chromatography on cellulose.

In both normal and Gaucher spleens, only one major ganglioside band was detected by TLC. This ran to the same position as the monosialoganglioside G_{M3} (hematoside) from gray matter of normal human brain.

TABLE 2 SENSITIVITY AND PRECISION OF DETERMINATION OF GLUCOSE AND GALACTOSE WITH ANTHRONE AND ORCINOL

Sugar	Wt	Reagent	Final Vol	No. of Replicates	Optical Density
	μg		ml		
Glucose	100	Anthrone	7	12	0.699 ± 0.0054*
Galactose	100	"	7	12	0.388 ± 0.0068
Glucose	100	Orcinol	7	12	0.438 ± 0.0050
Galactose	100	"	7	12	0.792 ± 0.0024

* SD.

TABLE 3 COMPARISON OF ACTUAL GLUCOSE AND GALACTOSE VALUES IN KNOWN MIXTURES WITH VALUES DETERMINED BY THE ANTHRONE AND ORCINOL PROCEDURE

Added*			Found			Recovery of Total Hexose
Galactose	Glucose	Total	Galactose	Glucose	Total	
	μg			μg		%
10	10	20	5.8	13.3	19.1	95
10	20	30	7.1	22.5	29.6	99
10	40	50	7.9	41.7	49.6	99
10	80	90	6.3	79.8	86.1	96
25	10	35	25.1	9.7	34.8	99
25	20	45	25.7	18.6	44.3	98
25	40	65	24.3	40.7	65.0	100
25	80	105	25.2	80.0	105.2	100
50	10	60	48.0	11.8	59.8	100
50	20	70	49.7	19.6	69.3	99
50	40	90	49.5	39.2	88.7	99
50	80	130	49.8	81.3	131.1	101
100	10	110	98.0	12.0	110.0	100
100	20	120	101.0	18.9	119.9	100
Lactose†		25	12.2	12.7	24.9	95
"		50	26	26	52	99
"		75	40	38	78	99
"		100	51	53	104	99

* Final volume 7 ml.
† 100 g of lactose ≡ 105 g of galactose + glucose.

Esterified cholesterol was calculated as the oleate. Results for glucocerebroside were calculated by assuming a molecular weight of 768.2; the figures for ceramide lactoside were calculated from the total hexose (the sum of the glucose and galactose values) by assuming a molecular weight of 916.6 (3). The amounts of glycolipid in peak *IV* and peak *V* were too small for accurate determination, but the quantities in the Gaucher spleens appeared to be no larger than in the normal (cf. Figs. 4 and 5).

The total glycolipid in Gaucher spleen S. M. and four widely separated parts of Gaucher spleen G. B. was determined by the nonhydrolytic anthrone method on the total, washed lipid extract after passage through Florisil (cf. 12). The results, calculated as glucocerebroside, are shown in Table 7; paper chromatography had shown glucose to be virtually the only sugar moiety present.

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TABLE 4 COMPARISON OF THEORETICAL GLUCOSE AND GALACTOSE VALUES IN GLYCOLIPID STANDARDS WITH VALUES DETERMINED BY THE ANTHRONE AND ORCINOL PROCEDURE

	Theoretical			Determined		
	Glucose	Galactose	Total Hexose	Glucose	Galactose	Total Hexose
	%			%		
Synthetic *N*-lignoceryl cytolipin H	18.5	18.5	37.0	17.8	19.7	37.5
Galactocerebroside*	0	22.2	22.2	−1.3	23.4	22.1
Glucocerebroside†	23.4	0	23.4	22.2	1.2	23.4

* Theoretical value calculated by assuming lignoceric acid as fatty acid.
† Theoretical value calculated by assuming mol wt of 768 (3).

TABLE 5 SENSITIVITY OF PAPER CHROMATOGRAPHY FOR DETECTION OF SUGAR MOIETY OF ORGAN GLYCOLIPID

Sugar	Minimum Detectable	Minimum Concentration Detectable	Maximum Permissible Ratio of the Other Hexose
	μg	*μg/g wet spleen*	
Galactose	0.2	0.7	Glu:Gal = ∝*
Glucose	0.4	1.4	Gal:Glu ≃ 80:1†

Glu, glucose; Gal, galactose.
* Glucose destroyed by fermentation with selected strain of yeast or with glucose oxidase.
† For quantification (as opposed to detection) of glucose by this method the maximum ratio of galactose to glucose was rarely higher than 20:1.

Identification of Hexose in Glycolipids. All cerebroside and ceramide dihexoside fractions were subjected to hydrolysis (method B) and paper chromatography. In all cases, the cerebroside fraction showed only glucose, while the ceramide dihexoside fraction showed equal amounts of glucose and galactose.

Complete Hydrolysis of Folch Lipid Extracts (32)

In all cases of Gaucher's disease, glucose was the major sugar detected but several specimens contained appreciable amounts of galactose. Fructose, previously reported (11), may have been an artifact derived from glucose during neutralization of the acid hydrolysis mixture. The relative amounts of glucose and galactose found are shown in Table 8 (the accuracy of paper chromatographic estimation is no better than ±25%). The three portions of spleen S. M. H. recorded in Table 8 came from different parts of the spleen.

A further seven specimens from spleen S. M. H., selected from widely separated, noninfarcted parts of the spleen which had been preserved for 13 yr, were analyzed by hydrolysis method B without purification of the lipid extract. Since glucose was virtually the only sugar present in all cases and only traces of galactose could be detected, it was clear that the major glycolipid must be glucocerebroside and that only small amounts of ceramide lactoside could be present. This was confirmed by TLC of the total lipid extracts.

Partial hydrolysis of the total lipids of some spleens (11) yielded small amounts of a nonfermentable, neutral substance, with the R_f value and color reaction of lactose, accompanying larger amounts of glucose and galactose. Like lactose, this minor component was acid-labile.

DISCUSSION

Phospholipids and gangliosides are destroyed by prolonged preservation in formalin whereas cholesterol and cerebrosides are stable (33–36). The stability of cerebroside and ceramide dihexoside is illustrated by the close agreement of the values obtained on the two S. M. H. extracts (1 and 2) from tissues which had, respectively, been kept at −40°C and stored in formalin for 11 yr. Measurements of individual phospholipids or gangliosides were carried out on fresh frozen tissue only.

Chromatography on cellulose was used instead of extraction with water or salt solutions for the removal of nonlipid components from lipid extracts (cf. 37, 38). Since it was shown by TLC that all the neutral glycolipids and all the major phospholipids with the exception of a little phosphatidyl serine were present exclusively in the lipid fraction, and since the presence of a certain amount of gangliosides, etc. did not interfere with subsequent procedures, the method was considered adequate for our purpose.

The procedure finally adopted for the fractionation of lipids on silicic acid gave complete separation of the "neutral" lipids from glycolipids and phospholipids and of the five classes of glycolipids (cerebrosides, ceramide dihexosides, ceramide trihexosides, aminoglycolipids, and gangliosides) from one another. The most polar phospholipids were less completely separated; although sharp peaks were obtained, there was some overlap and it was not possible to isolate quantitatively sphingomyelin, lecithin, or lysolecithin in a pure state without a further separation by TLC.

Both the hydrolysis procedures A and B led to adequate recovery of monosaccharide units from the spleen glycolipids when the sugars were to be examined by paper chromatography. Hydrolysis procedure B is the

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TABLE 6 LIPID CONTENT OF SPLEEN

	Gaucher's Disease					Normal	
	S.M.H.1 (p 3 mo)	S.M.H.2 (p 11 yr)	S.M.H.3* (p 3 mo)	J. L. (f)	R. I. (p 2 yr)	A (f)	B (f)
	mg/g wet weight						
Water	756	766	837	764	837	792	790
Total lipids†	72	67	55	76	33	33	60
Total phospholipid	17.7	1.0	9.1	17.5	5.5	11.2	16.9
Lecithin	—	—	—	6.4	—	4.3	—
Sphingomyelin	—	—	—	2.2	—	1.3	—
Hydrocarbons	1.2	0.4	0.6	0.5	0.5	0.3	0.4
Triglyceride	<0.1	0.3	0.5	0.8	<0.1	0.5	1.5
Cholesterol (free)	4.4	3.3	11.6	4.1	2.7	2.4	4.3
Cholesteryl ester	0.12	0.15	2.5	0.13	0.24	0.51	0.27
Glucocerebroside	39.8	38.1	19.5	23.8	10.6	0.06	0.20
Ceramide lactoside	0.62	0.55	0.31	0.24	0.28	0.18	0.32
Ganglioside (as *N*-acetylneuraminic acid)	—	—	—	0.299	—	0.013	—

p, preserved in formalin; f, fresh frozen.
* Infarcted region.
† The values for total lipids include proteolipids.

TABLE 7 GLUCOCEREBROSIDE CONTENT OF TWO GAUCHER SPLEENS

	S.M.	G.B.			
	mg/g wet weight				
Portion	—	1	2	3	4
Glucocerebroside*	33.2	30.1	33.7	30.3	26.5

* By nonhydrolytic anthrone determination on a purified extract; glucocerebroside assumed to be the only chromogen present and to have a mol wt of 768.2.

TABLE 8 RELATIVE AMOUNTS OF GLUCOSE AND GALACTOSE RELEASED ON HYDROLYSIS OF TOTAL LIPIDS OF GAUCHER SPLEENS, ESTIMATED BY PAPER CHROMATOGRAPHY

	Portion No.	Glu:Gal Ratio
S. M. H.	3*	8:1
	4	6:1
	5	16:1
D. T.		6:1
H. H.		11:1
I. P.		20:1
J. L.		>20:1
R. W.		20:1
A. F.		>20:1
H. J.		>20:1
S. M.		14:1
G. B.	1	25:1
	2	18:1
	3	10:1
	4	15:1
K. C.		15:1

Glu, glucose; Gal, galactose.
* Large infarcted region.

first homogeneous (single-phase) hydrolysis of a lipid in a predominantly aqueous medium; it may have applications outside the glycolipid field. The nonhydrolytic anthrone and orcinol methods, applied to individual fractions obtained by silicic acid chromatography, enabled the cerebroside and oligosaccharides to be identified and quantitatively determined. When an extract of total spleen lipids was passed through Florisil, nonhydrolytic assay with either anthrone or orcinol gave a reasonably good measure of the total hexose but errors were exaggerated when the two were combined for the calculation of the glucose:galactose ratio and, in contrast to single fractions from silicic acid, the method cannot be used in this context.

In all the cases of Gaucher's disease examined, glucocerebroside was the predominant lipid in the spleen, reaching concentrations up to 200 times those in normal spleens. No galactocerebroside was found, although minute traces accompanying the much larger amounts of glucocerebroside might not have been detected. This is in agreement with the majority of recent work on Gaucher's disease. A marked diminution has been found in the activity of the glucocerebroside-cleaving enzyme in the spleen and white blood cells of patients with Gaucher's disease (39–43). As suggested by Radin (44), the most probable source of the glucocerebroside is globoside from the plasma membranes of erythrocytes undergoing destruction in the spleen. Globoside, the major glycolipid of these membranes, is presumably degraded to ceramide trihexoside, which is further degraded to ceramide lactoside and thence to glucocerebroside (45–47).

The search for ceramide oligohexosides was the main object of this investigation. The concentrations of ceramide dihexoside in the three Gaucher spleens examined were only slightly higher than in the two normal spleens (Table 2). Suomi and Agranoff (3) found equal amounts of ceramide lactoside in normal and Gaucher spleens, while Philippart, Rosenstein, and Menkes (4) considered the amount in Gaucher spleen slightly elevated.

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The other glycolipid fractions (*IV* and *V*) have been identified as ceramide trihexoside and aminoglycolipid, respectively. The ceramide trihexoside and aminoglycolipid were present in only very small amounts in both normal and Gaucher spleens; in no case was there any evidence of a significant increase of these materials in Gaucher's disease (Figs. 4 and 5). In agreement with the report of Svennerholm and Svennerholm (2), the major glycolipid of normal spleen was the ceramide dihexoside whereas, in erythrocyte stroma, the major glycolipid was the aminoglycolipid, globoside.

The ganglioside of both Gaucher spleen and normal spleen was identified as G_{M3} (monosialo ceramide dihexoside, hematoside) in agreement with Svennerholm (48) and Philippart et al. (4). The amount found in the one Gaucher spleen examined by us was elevated more than 20-fold; Philippart et al. (4) also reported an elevation of the amount of G_{M3} in the spleen in Gaucher's disease, although the elevation was less in their cases. This ganglioside, the origin of which is unknown, is presumably a precursor of ceramide lactoside and glucocerebroside and so would contribute to the main lipid stored in Gaucher's disease, but it seems unlikely that G_{M3} is the major source of glucocerebroside in spleen. The accumulation of G_{M3} in Gaucher spleens may perhaps be explained by an inhibiting effect of glucocerebroside on the spleen neuraminidase acting on this ganglioside.

During extraction, purification, and hydrolysis of Gaucher spleen lipids for paper chromatography of the monosaccharide moieties, any procedure which might tend to fractionate the lipids was avoided. Thus differential loss of any glycolipid species was prevented. The quantities of galactose and glucose so found were in agreement with the amounts of glucocerebroside, ceramide oligosaccharides, and ganglioside found by chromatographic analysis. In all these spleens glucocerebroside is obviously by far the major glycolipid stored, other glycolipids contributing minor amounts of both galactose and glucose. The trace of lactose found in partial hydrolysates presumably arose from these other glycolipids.

Case S. M. H. of the present paper is the case reported by Parke (5, 6) to store large amounts of ceramide dihexoside (i.e., lactoside) as the predominant glycolipid in the spleen. Our results on this same spleen are completely at variance with his. Ten widely separated parts of the spleen, some fresh, some preserved, yielded mainly glucose on hydrolysis of the lipids; although the amounts of glucose and galactose varied in different parts of the spleen, glucocerebroside plainly everywhere far outweighed all the other glycolipids combined. The lipids of three different parts of this spleen, including the large infarcted area, examined by chromatography on silicic acid columns, contained only the normal traces of lactoside as well as large amounts of glucocerebroside. It would seem that, if Parke's findings are correct, he must have examined an exceptional part of the spleen. Parke hydrolyzed the isolated glycolipids with 2 N H_2SO_4 at 100°C for 3 hr or with 3 N HCl at 100°C for 1.5 hr; although the evidence he gives would seem to put the identification of lactose beyond doubt, it is difficult to see how any lactose could have survived intact after either of these hydrolytic procedures. We found that when lactose was heated at 100°C in 3 N H_2SO_4 for 10 min it was entirely destroyed, only glucose and galactose being detectable on paper chromatography.

The concentrations of the sugar-free lipids in the three Gaucher spleens are close to those found for the two normal spleens which, in turn, agree well with published results both for the normal range and for Gaucher's disease (3, 4, 49, 50). An exception was the large infarct in the spleen of case S. M. H. which contained large amounts of cholesterol and cholesteryl ester. The moderate increase in cholesterol content of Gaucher spleens reported by Suomi and Agranoff (3) may perhaps be explained by the presence of minor infarcts.

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