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The structure of cerebrosides in Gaucher's disease*

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SUMMARY

Crystalline cerebrosides were isolated from the spleen of a woman with Gaucher's disease. The cerebrosides accounted for 38 per cent of the total spleen lipids and 0.89 per cent of the fresh spleen. The sugar moiety of the cerebrosides was found to be glucose and its position of attachment was established to be on the primary hydroxyl group of sphingosine. The double bond in the sphingosine moiety had the *trans* configuration. The fatty acids of the cerebroside were determined by gas chromatography and paper chromatography and found to be mainly lignoceric, behenic, and palmitic acid. Small amounts of arachidic and stearic acids were also present.

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Cerebrosides isolated from persons with Gaucher's disease contain predominantly glucose (1 to 5) in place of galactose. Carter and Greenwood (6) have shown that in the case of phrenosin the galactose is attached to the primary hydroxyl group of sphingosine. However, the position of attachment of glucose in the abnormal Gaucher's cerebroside from humans has not been investigated. In addition, the nature of the fatty acids of the Gaucher's cerebrosides has not been sufficiently studied. This paper will elucidate both of these points.

METHODS

A portion of human spleen (310 g. wet weight of a 780 g. spleen) was obtained from a woman (age 50) with Gaucher's disease immediately after surgery. The spleen was minced, washed with isotonic saline, and then extracted with methanol-ether 1:1 (v/v) in the usual way. The yield of total lipid was 7.26 g.

Column chromatography of the spleen lipids was carried out on Florisil[1] (7) and on silicic acid (8). The cerebrosides isolated by chromatography on Florisil were recrystallized several times from acetic acid. Hydrolysis, acetylation, reductive hydrogenolysis, and isolation of diacetylsphingine were carried out by procedures similar to those described by Carter and Greenwood (6). Nitrogen and phosphorus analyses were carried out as described previously (8). Sugar was analyzed by the method of Radin *et al.* (9). Carbon, hydrogen, and acetyl group analyses were done by the Schwarzkopf Microanalytical Laboratory, Woodside, N. Y.

The cerebrosides were hydrolyzed with aqueous HCl by the procedure of Rosenberg and Chargaff (5) and the osazone derivative prepared in the usual manner (10). Paper chromatography of the sugars was carried out by the ascending technique in three solvent systems: (*a*) *n*-butanol-pyridine-water 3:1:1.5 (v/v/v) (upper phase of this mixture was mixed with 1 volume of pyridine); (*b*) ethyl acetate-pyridine-water 30:21:9 (v/v/v); and (*c*) isopropyl ether-formic acid 3:2 (v/v).

The fatty acid methyl esters were analyzed by gas chromatography. The analysis was kindly performed by Dr. M. Kates, of the National Research Council, Ottawa, Canada. The free fatty acids were analyzed by paper chromatography on mineral oil-impregnated paper (11). The solvent system consisted of acetic acid-water 195:5 (v/v) (saturated with mineral oil). Whatman No. 1 filter paper was impregnated by dipping into a 10 per cent (v/v) solution of mineral oil in benzene.

Paper chromatography of diacetylsphingine was carried out on silicic acid-impregnated paper, employing a solvent of *n*-hexane-diisobutylketone 70:60 (v/v) (12).

Infrared spectral analyses were kindly run by Dr. W. B. Mason and Mr. A. Behringer, of the Atomic Energy Project of the University of Rochester, and were made possible by funds from the Atomic Energy Commission. A Perkin-Elmer Model 21 instrument

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[1] Floridin Co., Tallahassee, Fla.

with NaCl prism was used. The spectra were run on pressed KBr disks at a concentration of 5 μg. of compound per 1 mg. of KBr. The compounds were incorporated into the KBr by the lyophilization technique (13).

RESULTS

The lipid content of the spleen on a wet weight basis was 2.35 per cent. The phosphorus content of the lipids was 1.86 per cent. Thirty-eight per cent of the total spleen lipids (or 0.89 per cent of the wet weight spleen) was cerebroside. The nonphosphatides (triglycerides plus total cholesterol) as determined by column fractionation on silicic acid constituted 17 per cent of the total lipids.

The cerebrosides (2.74 g.) obtained by column chromatography on Florisil were found to contain 0.13 g. of phosphatide (determined by P analysis). Three recrystallizations from acetic acid yielded 1.7 g. of crystalline cerebrosides (I) (m.p. 169°-171°C begins to sinter at 80°C). The analysis of cerebroside (I) was as follows: 69.35 per cent C; 11.55 per cent H; 1.76 per cent N; 23.7 per cent glucose. (Calculated values for $C_{46}H_{89}O_8N$ [784.2]: 70.50 per cent C; 11.42 per cent H; 1.78 per cent N; and 23.0 per cent glucose.) Cerebrosides (I) were free from P.

TABLE 1. Gas Chromatographic Analysis of the Fatty Acids of the Spleen Cerebrosides (I)

	moles per cent
C_{24} (lignoceric acid)	46
C_{22} (behenic acid)	27
C_{20} (arachidic acid)	3
C_{18} (stearic acid)	3
C_{16} (palmitic acid)	20

The acetic acid supernatant fluids from the recrystallized (I) were found to contain cerebrosides and phosphatides. Hydrolysis of this fraction showed glucose to be the only sugar present. The fatty acids on these cerebrosides were not analyzed because of the contaminating phosphatide.

Attempts to isolate and purify the cerebrosides by chromatography on silicic acid were unsuccessful. The cerebrosides were eluted with 20 per cent methanol in chloroform along with phosphatidylethanolamine and phosphatidylserine.

The fatty acid methyl esters obtained by hydrolysis of the cerebrosides with methanolic-sulfuric acid were analyzed by gas chromatography. The results are given in Table 1. It is evident that the major components are lignoceric, behenic, and palmitic acid. The fatty acids and sphingosine were isolated in nearly theoretical yields.

The fatty acid methyl esters were converted to the free fatty acids which were then analyzed by paper chromatography (11). The major acids which were observed were lignoceric, behenic, and palmitic. Stearic and arachidic acids were barely detectable on the chromatograms. Hence the paper chromatographic findings are in agreement with the gas chromatographic analyses. The acids were saturated since they gave an essentially negative test with permanganate. Since the cerebroside (I) yielded a pentacetyl derivative rather than a hexacetyl derivative, the presence of an appreciable amount of hydroxy acids is ruled out.

Analytical data on the acetylated (II) and the re-

TABLE 2. Analytical Data on the Acetylated Cerebrosides

	N	Glucose	Acetyl	Glucose/N	Acetyl/N
	per cent	*per cent*	*per cent*		
Calculated for pentacetyl compound $C_{56}H_{99}O_{13}N$ (994.4)	1.41	18.2	21.6	1.0	5.0
Found: Compound (II) m.p. 84°-87°C	1.41	18.2	21.2	1.0	4.93
Calculated for reduced tetracetyl compound $C_{54}H_{99}O_{11}N$ (938.4)	1.49	19.2	18.3	1.0	4.0
Found: Compound (III) m.p. 94°-96°C	1.46	18.5	19.3	0.99	4.3

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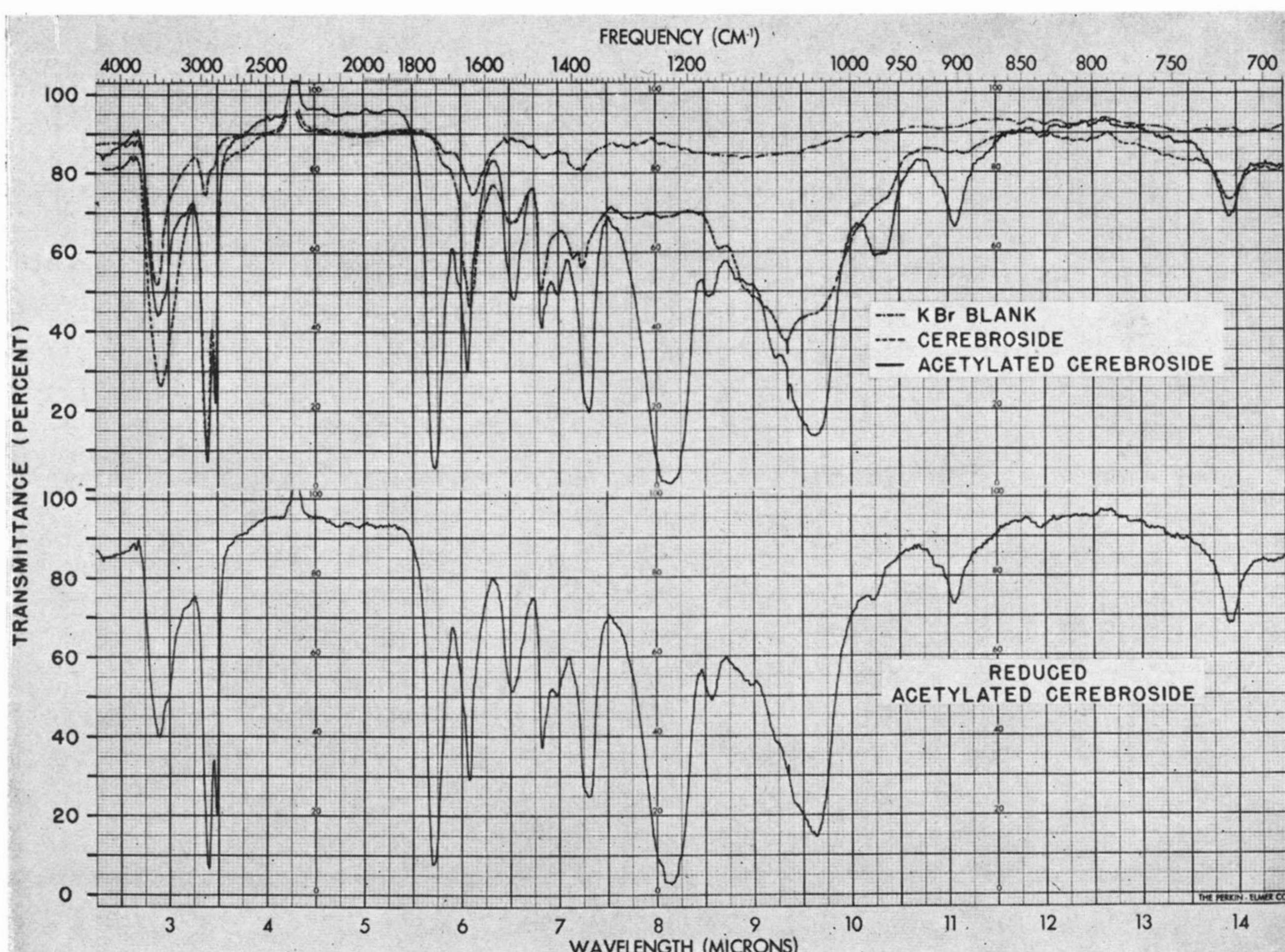

FIG. 1. Infrared spectra of cerebrosides (I), acetylated cerebrosides (II), and reduced acetylated cerebrosides (III). The KBr blank is also shown.

duced acetylated cerebrosides (III) are given in Table 2. In our hands the extent of hydrogenolysis was 84 per cent. (Carter and Greenwood [6] found 40 per cent with phrenosin.) No sugar was liberated during the reduction with hydrogen. The data in Table 2 demonstrate that the acetylated cerebroside (II) was a pentacetyl derivative, but that the reduced derivative (III) was predominantly a tetracetyl derivative but did contain some pentacetyl compound. Hence it is clear that approximately one acetyl group was lost during hydrogenolysis. The reduced compound (III) was a mixture of tetracetyl and pentacetyl derivatives because the extent of hydrogenolysis was 84 per cent.

Hydrolysis of the reduced acetylated cerebrosides (III) (150 mg.) yielded sphingine and some dihydrosphingosine. These were converted to the corresponding diacetylsphingine (IV) and triacetyldihydrosphingosine (V). Crystallization from ethanol yielded 30 mg. of crystalline diacetylsphingine in about 95 per cent purity. (The theoretical yield based on 84 per cent hydrogenolysis would be 39 mg.) Analytical data and properties of the diacetylsphingine (IV) are given in Table 3. The infrared spectra of compounds I to IV and of the authentic sample of diacetylsphingine (kindly donated by Dr. H. E. Carter, of the University of Illinois) are shown in Figures 1 and 2. The

TABLE 3. PROPERTIES OF DIACETYLSPHINGINE

	N	Melting Point	R_f Value
	per cent		
Calculated: $C_{22}H_{43}O_3N$ (369.6)	3.80	105°–106°C*	0.45*
Found: Compound (IV)	3.89	100°–104°C	0.45

* Melting point and R_f value of the authentic compound.

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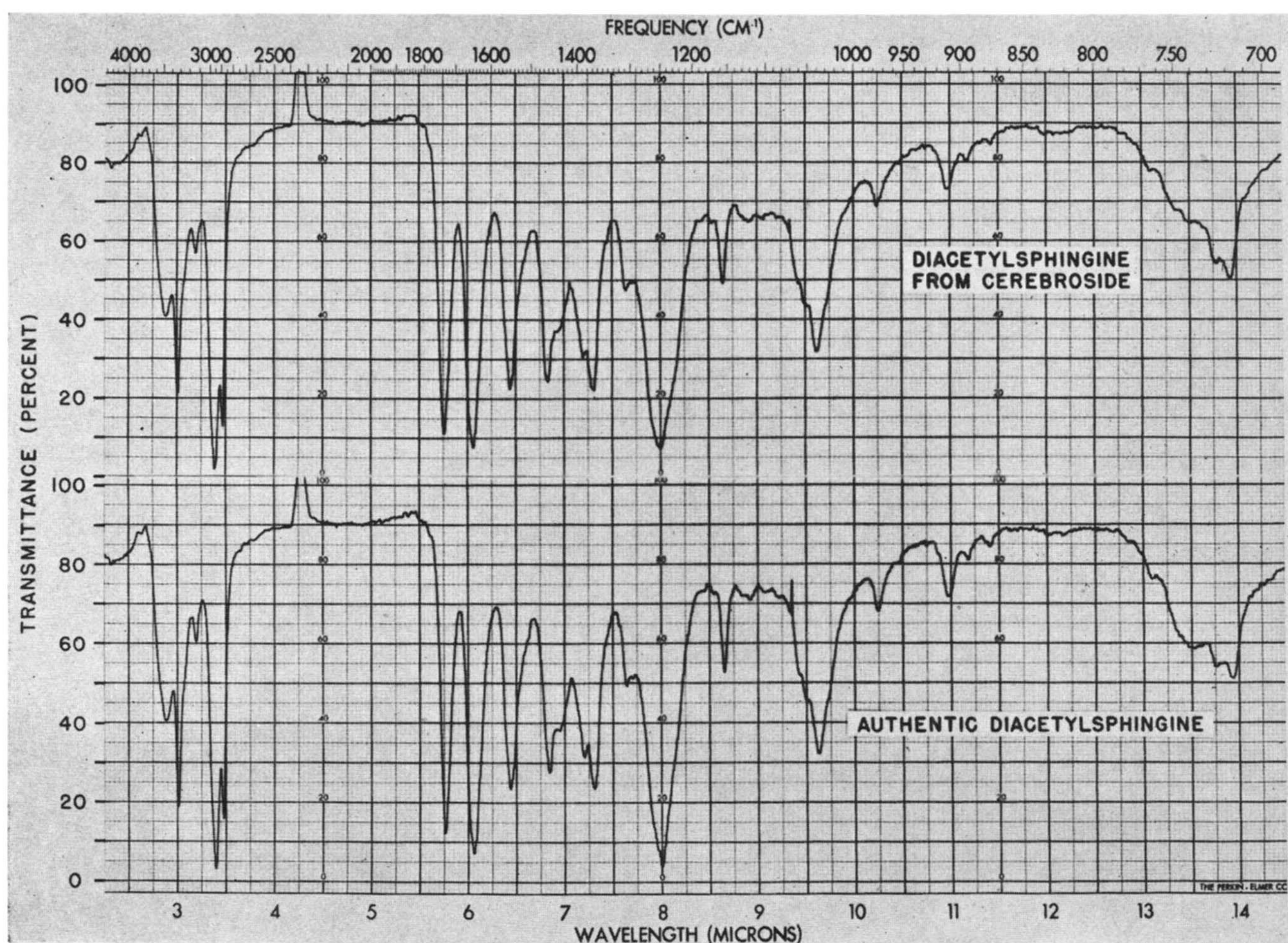

Fig. 2. Infrared spectra of diacetylsphingine (IV), which was isolated from the Gaucher's cerebroside, and of the authentic diacetylsphingine.

spectra of the two compounds are essentially identical. The slightly lower melting point of compound (IV) as compared to the authentic diacetylsphingine was due to the presence of a very small amount of the triacetyldihydrosphingosine (V) (m.p. 100°-102°C) (14).

The diacetylsphingine from the Gaucher's cerebroside had the same mobility as the authentic diacetylsphingine when chromatographed on silicic acid-impregnated paper. The small amount of triacetyldihydrosphingosine in compound (IV) was visible on the chromatograms. These compounds were detected by staining with Rhodamine 6G.

The cerebroside (I) (100 mg.) was hydrolyzed for 90 minutes in 3N aqueous HCl. After extraction of the fatty acids with ether, the aqueous phase was analyzed for sugar by paper chromatography and the osazone derivative was then prepared. The sugar in the spleen cerebrosides migrated exactly as glucose in three different solvents and yielded an osazone which had the same crystal structure as glucosazone.

Galactose was not detected on the chromatograms. However, if 1 to 5 per cent galactose were present, it is doubtful if this small amount could be detected. It is clear then that the presence of small amounts of galactocerebrosides cannot be ruled out. The enzymatic assay for glucose as performed by Rosenberg and Chargaff (5) was not carried out. This enzymatic assay is quite specific for glucose but again it is doubtful whether it could rule out the presence of very small amounts of galactose.

DISCUSSION

Normal cerebrosides contain galactose as the major sugar, whereas cerebrosides in Gaucher's disease contain predominantly glucose (1 to 5). Although Carter and Greenwood (6) have shown that in the case of phrenosin the galactose is linked to the primary hydroxyl group of sphingosine, there are no experimental data to demonstrate that the glucose in the abnormal

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cerebroside is attached to the same position. In view of our observation that galactocerebrosides are hydrolyzed more rapidly with acid than the glucocerebrosides, it seemed important to examine the structure of the Gaucher's cerebroside. Such a study would more fully elucidate the nature of the molecular defect in Gaucher's disease. In addition to the sugar component, a study of the nature of the fatty acids and the configuration of the double bond in sphingosine of the Gaucher's cerebroside was carried out.

The result of these studies conclusively shows that the major sugar in this particular Gaucher's cerebroside is glucose and that it is attached to the primary hydroxyl group of sphingosine. Furthermore, the double bond in the sphingosine moiety has the *trans* configuration, since on reduction the band at 10.3 μ. is markedly diminished (Fig. 1). These structural features are identical to those of the galactocerebroside phrenosin (6, 15) and sphingosine (16, 17).

With regard to the fatty acids on the cerebrosides, this study for the first time gives a detailed analysis of these constituents in Gaucher's disease. It is clear that the cerebrosides are heterogeneous with respect to the fatty acids and are to be considered as a family of compounds. The major cerebrosides in this case are lignoceryl, behenyl, and palmityl derivatives. The stearyl and arachidyl derivatives are minor components. How these acids compare to the acids in normal human spleen cerebrosides cannot be ascertained at the present time since similar detailed studies have not been carried out on normal persons. In our hands attempts to isolate a sufficient amount of pure cerebrosides from the spleens of five normal humans have been unsuccessful because of the small amount of cerebroside which is present.

Recently Rosenberg and Chargaff (5) have reported that a cerebroside isolated from a person with Gaucher's disease might be a behenyl derivative. However, their assumption was based on elementary analysis and on the yield of mixed fatty acid esters. These data cannot distinguish the type of fatty acids present but only give the average chain length of the mixture. In our case, and very likely in theirs, the average fatty acid chain length was 21 to 22 carbon atoms.

The elucidation of molecular diseases must in the final analysis depend on precise chemical data. The work reported here is aimed toward this end.

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