CEREBROSIDE SYNTHESIS IN GAUCHER'S DISEASE *

By EBERHARD G. TRAMS and ROSCOE O. BRADY

(From the National Institute of Neurological Diseases and Blindness, Bethesda, Md.)

(Submitted for publication April 12, 1960; accepted May 6, 1960)

Familial lipodystrophic conditions, such as Gaucher's, Niemann-Pick, and Tay-Sachs disease, are characterized by the intracellular accumulation of abnormally large quantities of sphingolipids. In Gaucher's disease, the offending lipids are cerebrosides, while Niemann-Pick and Tay-Sachs diseases are characterized by the accumulation of sphingomyelin and gangliosides, respectively. In Gaucher's and Niemann-Pick disease, involvement of the reticuloendothelial system is extensive, and splenomegaly and hepatomegaly are frequently observed. Lieb in 1924 (1) identified the lipid stored in reticuloendothelial cells in a case of Gaucher's disease as a cerebroside. His findings were supported by other investigators (2, 3), and eventually Halliday, Deuel, Tragerman and Ward (4) and Rosenberg and Chargaff (5) presented evidence that the accumulated cerebrosides in Gaucher's disease contained predominantly glucose instead of the high content of galactose usually present in cerebrosides.

It was felt that techniques developed in this laboratory for studies of the biosynthesis of sphingosine and cerebrosides might be useful for investigations of cerebroside formation in tissue samples obtained from patients with Gaucher's disease. The data obtained by Brady and Koval (6), Burton, Sodd and Brady (7), and Cleland and Kennedy (8) indicate that cerebroside synthesis probably occurs in the following fashion: palmitic aldehyde + serine → sphingosine + CO_2; sphingosine + uridinediphosphate galactose → psychosine + uridinediphosphate; psychosine + fatty acid → cerebroside. Since purified enzyme systems which catalyze the biosynthesis of cerebrosides were not available, tissue slices were employed in the present investigations to study the formation of these sphingolipids. Tissue was occasionally available from patients who were splenectomized to arrest the pancytopenia of hypersplenism. It was not possible to obtain control specimens of splenic tissue suitable for metabolic studies from normal children of the same age as the patients with Gaucher's disease. Therefore, the results obtained in spleens from Gaucher's patients were compared with those obtained in two children with Niemann-Pick disease and in one adult case of idiopathic thrombocytopenic purpura.

Experiments were designed to explore several factors that might be thought to contribute to the pathogenesis of Gaucher's disease. Enzymic disturbances conceivably could lead to an alteration of normal processes, causing a replacement of a large proportion of cerebroside galactose by glucose. The metabolism of such an "abnormal" cerebroside might be imagined to proceed at a slower rate than the characteristic galacto-cerebroside. Also, attention was directed to the rate of formation of the total cerebroside molecule compared with the rate observed in tissue samples obtained from patients who did not show signs of cerebroside accumulation.

EXPERIMENTAL METHODS

The spleens used in this study were refrigerated following splenectomy and processed within 15 to 30 minutes. Slices measuring $10 \times 10 \times 0.5$ mm were prepared in the cold and incubated aerobically in Krebs-Ringer bicarbonate solution (9), pH 7.5, for 3 hours at 37° C. The following substrates were added to the incubation mixtures in amounts ranging from 0.5 to 5.0 μmoles per flask: uniformly-labeled glucose-C^{14} (0.89 μc per μmole); galactose-1-C^{14} (0.44 μc per μmole); and sodium acetate-1-C^{14} (5.8 μc per μmole). Each substrate was incubated with the tissue slices in triplicate. Non-incubated samples also in triplicate were used as controls for determining the reliability of the isolation and recovery techniques.

After incubation, the cerebrosides were isolated essentially according to the method described by Radin, Brown and Lavin (10). The contents of the incubation flasks were homogenized with an equal volume of 10 per cent ice-cold trichloroacetic acid and centrifuged. The precipitated material was washed 3 times in the

* The authors express their gratitude to Dr. Allen C. Crocker of the Children's Hospital Medical Center, Boston, Mass., for his interest and help in providing much of the clinical material used in this study.

cold with 5 per cent trichloroacetic acid and once with acetone at −10° C. The residue was then extracted 3 times with chloroform: methanol (2:1) at 45° to 50° C. The chloroform-methanol extract was washed 5 times with 2 vol of water, and sufficient methanol was added to the residual chloroform phase to obtain a clear solution. This solution was decolorized with activated charcoal and chromatographed on Florisil and on a mixed-bed ion exchange column as described by Radin and coworkers (10).

The cerebrosides obtained in this manner were recrystallized 3 times from methanol. Radioactivity was measured in a proportional gas-flow counter. The concentration of cerebrosides was determined by the anthrone reaction for hexoses after hydrolyzing the samples for 3 hours (10). The relative amounts of glucose and galactose in the hexose fractions were determined by incubating the cerebroside hexoses with glucose oxidase. After chromatography on an MB-3 (Rohm and Haas Co.) ion exchange column the galactose was determined and the amount of glucose was then calculated by the difference. Known amounts of glucose and galactose were carried as internal standards throughout the whole isolation procedure and the subsequent determination of galactose to ascertain the accuracy of these methods.

The total cerebroside content was estimated by the isotope dilution technique. A known amount of highly purified radioactive cerebroside obtained by tritiating, according to the method described by Wilzbach (11), was added to tissue homogenates, and the cerebrosides were isolated by the procedures outlined above. Comparison of the specific activity of the recovered cerebroside with that of the added cerebroside, as well as per cent recovery of the added isotope, permitted an accurate measurement of tissue cerebroside levels. The amounts of free glucose and galactose in tissue samples were estimated in trichloroacetic acid extracts of tissues by similar procedures with the use of labeled hexoses.

RESULTS

Accurate measurement of the specific activity of the cerebrosides obtained from the incubation mixtures required that the cerebrosides be obtained in a reasonably pure state. This restriction entailed considerable losses in the purification procedures which had to be determined. For this reason the isotope dilution technique was used to estimate total cerebroside content of samples of splenic tissue. Comparison of the data obtained by the isotope dilution method and colorimetric methods indicated that the latter measured from 31 to 58 per cent of the actual cerebroside content. Data of the levels obtained with the isotope dilution technique and the amount of glucose in the cerebroside hexoses are shown in Table I. The results indicate that considerable amounts of galactose were present in the cerebrosides of Gaucher's spleens as well as in the control spleens. Because the entire procedure was controlled with added standard amounts of glucose and galactose, these data are thought to be relatively reliable.

When the data of the incorporation of the isotopically-labeled precursors were compared, it became apparent that the uptake of each isotope by the tissue samples was essentially the same for all of the cases investigated in this study. The net incorporation of isotopes from the different precursors is shown in Table II. The data presented in the last column are expressed as specific activities of the recovered cerebrosides. These figures do not indicate that splenic tissue from patients with Gaucher's disease synthesized cerebrosides at a slower rate than did the control spleens.

TABLE I

Cerebroside contents of pathological states

Disease	Patient*	Age		Sex	Spleen weight	Total lipids†	Cerebroside content of spleen	Glucose in cerebroside hexoses
		yrs	*mos*		*g*	*% wet wt.*	*% wet wt.*	%
Gaucher's	K.S.[1]	6	2	♂	720	6.05	2.45	53
Gaucher's	S.R.[1]	4	1	♀	304	4.8	1.20	41
Gaucher's	C.R.[1]	5	8	♀	302	4.8	2.40	44
Gaucher's	J.A.[1]	8	10	♂	1,300	6.40	3.54†	32
Niemann-Pick	J.K.[1]	3	4	♂	258	12.9	1.05†	39
Niemann-Pick	C.C.[2]	5	6	♀	235	7.3	0.41	50
Idiopathic thrombocytopenic purpura	M.R.[3]	69		♀	73	‡	0.18	‡

* Superscripts indicate source of patient: [1] Children's Hospital Medical Center, Boston, Mass.; [2] Walter Reed Army Hospital, Washington, D. C.; [3] The George Washington University Hospital, Washington, D. C.

† These data courtesy Dr. Allen C. Crocker, Children's Hospital Medical Center, Boston, Mass.

‡ No analysis performed.

TABLE II

Incorporation of isotopic precursors into cerebrosides

Disease	Patient	Precursor	Incorporation into cerebrosides	
			cpm/g dry wt.	*mμmoles/ μmole*
Gaucher's	K.S.	Glucose-C^{14}	19,840	0.134
		Galactose-C^{14}	17,720	0.680
Gaucher's	S.R.	Glucose-C^{14}	4,210	0.051
		Galactose-C^{14}	6.960	0.163
Gaucher's	C.R.	Glucose-C^{14}	4,890	0.045
		Galactose-C^{14}	7,180	0.167
Gaucher's	J.A.	Galactose-C^{14}	19,800	0.054
		Acetate-C^{14}	21,660	0.120
Niemann-Pick	C.C.	Glucose-C^{14}	12,870	0.640
		Galactose-C^{14}	12,420	1.25
Niemann-Pick	J.K.	Galactose-C^{14}	12,700	0.95
		Acetate-C^{14}	15,500	3.05
Thrombocyt. purp.	M.R.	Glucose-C^{14}	1,850	0.326
		Galactose-C^{14}	4,960	1.087

Marked dilution of recovered isotope had taken place in the Gaucher's spleen by virtue of the larger amount of endogenous cerebrosides (cf. Table I).

Labeled galactose appeared on a molar basis to be a better precursor for cerebroside synthesis than is glucose. It was of considerable interest, therefore, to learn that appreciable portions of the galactose were subsequently isolated as glucose from the cerebroside hexoses (Table III). The amount of isotope derived from labeled hexoses in the ceramide (N-acyl sphingosine) portion of the cerebroside molecule did not exceed 13 per cent in the Gaucher's spleens. Such data might suggest that transglycosylation (12) had occurred during the incubation in contrast to *de novo* synthesis of the entire cerebroside molecule. Accordingly, the data obtained with labeled hexoses as precursors were compared with those found with labeled acetate. If the ceramide portion of the cerebroside molecule were synthesized during the incubation, rather than merely participating in an exchange of labeled hexose, one would expect a significant incorporation of labeled acetate into the sphingosine and fatty acid moieties. This indeed was found to be the case as was indicated by the significant incorporation of labeled acetate which was largely confined to the ceramide portion of the cerebroside molecule. The radioactivity in the ceramide was divided fairly evenly between the fatty acid moiety and sphingosine.

Because of some preference for galactose as a precursor for cerebroside hexoses, an investigation of the relative pool sizes of both free glucose and free galactose was undertaken in some of the tissues studied. The data shown in Table IV indicate that the apparent preferential incorporation of radioactivity from galactose into cerebrosides was not significantly influenced by the dilution of labeled precursor with endogenous glucose.

DISCUSSION

It is apparent that cerebroside synthesis in the spleens of patients with Gaucher's disease proceeds at a fairly active rate. The rate of cerebroside formation was essentially the same as that observed in a control spleen obtained from a patient with thrombocytopenic purpura and was almost identical with the values obtained in the spleens from two patients with Niemann-Pick disease.

TABLE III

Partition of isotopic label in cerebroside constituents

Disease	Patient	Substrate	Percent of label in:			
			Ceramides	Glucose	Hexoses	Galactose
Gaucher's	K.S.	Glucose-C^{14}	1.3	73		25
		Galactose-C^{14}	9	52		39
Gaucher's	C.R.	Glucose-C^{14}	13	75		12
		Galactose-C^{14}	13	59		28
Gaucher's	S.R.	Glucose-C^{14}	12		88	
		Galactose-C^{14}	13		87	
Gaucher's	J.A.	Galactose-C^{14}	6	60		34
		Acetate-C^{14}	59		41	
Niemann-Pick	C.C.	Glucose-C^{14}	10	22		68
		Galactose-C^{14}	13			
Niemann-Pick	J.K.	Galactose-C^{14}	49	35		16
		Acetate-C^{14}	95		5	

TABLE IV

Free hexose pools of pathological spleens

Disease	Patient	Total free hexose/g wet spleen	Glucose
		μmoles	%
Gaucher's	K.S.	2.31	81
Gaucher's	S.R.	3.27	68
Gaucher's	C.R.	4.05	61
Niemann-Pick	C.C.	3.90	36

Even though the possibility exists that some transglycosylation with radioactive hexose may have occurred during the incubation, the finding that radioactive acetate was well utilized as a precursor indicates that there was predominantly *de novo* synthesis of the entire cerebroside molecule.

Two findings were not anticipated at the outset of these investigations. First, the ratio of galactose to glucose in the cerebrosides of Gaucher's spleens was higher than expected from some of the values in the literature. Other investigators have reported that the cerebrosides from Gaucher's tissue contained mostly (4) or exclusively (5) glucose. Our observations do not agree with these findings, but seem to support the data obtained by Montreuil, Boulanger and Houcke (13), who reported a galactose: glucose ratio in cerebrosides of a case of Gaucher's disease as 0.80 in contrast to the ratio of 3.16 obtained in a normal spleen. Similarly, it is apparent that Lieb and Mladenović in an earlier study (14) had isolated a certain amount of galactose-cerebroside from a case of Gaucher's disease.

Second, the finding that galactose appears to be incorporated into cerebrosides at a higher rate than is glucose probably should not have been entirely unexpected, since normal tissue seems to synthesize predominantly the galactoside. Perhaps it is reasonable to assume that the enzymic mechanisms available for the utilization of galactose are preferentially engaged in this process in contrast with the multiplicity of pathways available for the metabolism of glucose. The relatively large amount of incorporated radioactivity from labeled galactose which had been converted to glucose, prior to or during the synthesis of the cerebroside molecule, is indicative of little, if any, change in the enzymes which catalyze the epimerization of uridinediphosphate galactose to uridinediphosphate glucose (15). Consistent with these observations is the fact that galactose tolerance is not impaired in Gaucher's disease (16).

From the data obtained in this study, it is concluded that the biochemical lesion leading to the lipodystrophic syndrome of Gaucher's disease probably does not lie in pathways concerned with the utilization of hexoses. Furthermore, it was shown that *de novo* synthesis of cerebrosides occurs at similar rates in Gaucher's cells and in the control tissues studied, although this does not necessarily imply that cerebosides in the spleen were solely derived by *de novo* synthesis. These observations suggest, however, that the nature of the biochemical defect may be catabolic rather than anabolic, and further investigations should be directed along lines of utilization and destruction of sphingolipids in lipodystrophic states.

SUMMARY

Cerebroside formation in slices of splenic tissue obtained from four patients with Gaucher's disease was compared with the rate observed in two cases of Niemann-Pick disease and one case of thrombocytopenic purpura. The biosynthesis of cerebrosides was examined with the use of labeled glucose, galactose, and acetate as substrates. Galactose was preferentially incorporated into the hexose moiety of the cerebrosides, although 40 to 60 per cent of the incorporated galactose was present as glucose-cerebroside. Significant quantities of galactose-cerebrosides were found in the Gaucher's spleens as well as glucose-cerebrosides. Cerebroside synthesis in Gaucher's disease does not appear to be sufficiently elevated to warrant the conclusion that this lipodystrophic condition is due to an increased rate of sphingolipid formation *in situ*.

REFERENCES

1. Lieb, H. Cerebrosidspeicherung bei Splenomegalie, Typus Gaucher. Hoppe-Seylers Z. physiol. Chem. 1924, **140**, 305.
2. McConnell, J. S., Forbes, J. C., and Apperly, F. L. Notes on chemical studies of a Gaucher spleen. Amer. J. med. Sci. 1939, **197**, 90.
3. Capper, A., Epstein, H., and Schless, R. A. Gaucher's disease. Report of a case with presentation of a table differentiating the lipoid disturbances. Amer. J. med. Sci. 1934,**188**, 84.

4. Halliday, N., Deuel, H. J., Jr., Tragerman, L. J., and Ward, W. E. On the isolation of a glucose-containing cerebroside from spleen in a case of Gaucher's disease. J. biol. Chem. 1940, **132**, 171.
5. Rosenberg, A., and Chargaff, E. A reinvestigation of the cerebroside deposited in Gaucher's disease. J. biol. Chem. 1958, **233**, 1323.
6. Brady, R. O., and Koval, G. J. The enzymatic synthesis of sphingosine. J. biol. Chem. 1958, **233**, 26.
7. Burton, R. M., Sodd, M. A., and Brady, R. O. The incorporation of galactose into galactolipides. J. biol. Chem. 1958, **233**, 1053.
8. Cleland, W. W., and Kennedy, E. P. The enzymatic synthesis of psychosine. J. biol. Chem. 1960, **235**, 45.
9. Elliott, K. A. C. Tissue slice technique *in* Methods in Enzymology, S. P. Colowick and N. O. Kaplan, Eds. New York Academic Press, 1955, vol. 1, pp. 3–9.
10. Radin, N. S., Brown, J. R., and Lavin, F. B. The preparative isolation of cerebrosides. J. biol. Chem. 1956, **219**, 977.
11. Wilzbach, K. E. Tritium-labeling by exposure of organic compounds to tritium gas. J. Amer. chem. Soc. 1957, **79**, 1013.
12. Stetten, M. R. Transglucosylation by a mammalian liver enzyme. J. Amer. chem. Soc. 1959, **81**, 1437.
13. Montreuil, J., Boulanger, P., and Houcke, E. Chromatographie sur papier des constituants glucidiques des cérébrosides d'une rate de Gaucher. Bull. Soc. Chim. biol. (Paris) 1953, **35**, 1125.
14. Lieb, H., and Mladenović, M. Cerebrosidspeicherung bei Morbus Gaucher. Hoppe-Seylers Z. physiol. Chem. 1929, **181**, 208.
15. Maxwell, E. S. The enzymic interconversion of uridine diphosphogalactose and uridine diphosphoglucose. J. biol. Chem. 1957, **229**, 139.
16. Thannhauser, S. J. Lipidoses: Diseases of the Cellular Lipid Metabolism. New York, Oxford Univ. Press, 1950.