Glycosphingolipid levels in an unusual neurovisceral storage disease characterized by lactosylceramide galactosyl hydrolase deficiency: lactosylceramidosis

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Abstract The glycosphingolipid composition of brain and visceral tissue from a patient with an unusual neurovisceral lipid storage disease, characterized by a lactosylceramide galactosyl hydrolase deficiency, was determined. Analyses of erythrocytes, plasma, bone marrow cells, urine sediment, and liver biopsy from the patient were compared with those of normal infantile controls. Abnormally high levels of lactosylceramide (GL-2a) were found in these samples. Subsequent studies on spleen, liver, kidney, lymph nodes, and adrenal gland confirmed this finding and clearly showed that the metabolism of hematoside (G_{M3}) and glucosylceramide (GL-1a) was also affected. The accumulation of GL-1a and G_{M3} was most pronounced in spleen, but it was not of the order seen in the spleens of patients with Gaucher's disease that were studied for comparison. Since the disease was primarily neurological in nature, fresh-frozen brain was also studied. The level of GL-2a in gray matter was equal to that of galactosylceramide (GL-1b), and elevated amounts of GL-1a, asialo-G_{M2}, G_{M2}, and G_{M3} were also found; the only major abnormality in white matter was the accumulation of GL-2a and lesser amounts of the gangliosides G_{M3} and G_{M2}. Chemical and enzymic evidence suggests the use of the term "lactosylceramidosis" for this disease.

Supplementary key words · lactosylceramide · hematoside · glucosylceramide · neurovisceral glycosphingolipid storage disease

THE GLYCOSPHINGOLIPIDOSES have been biochemically characterized by isolation and identification of the accumulating lipid in tissue obtained postmortem. In this way the specific accumulation of glucosylceramide (GL-1a) was found to be characteristic of Gaucher's disease (1, 2); trihexosylceramide (GL-3) of Fabry's disease (3–5); G_{M2} of Tay-Sachs disease (6); globoside (GL-4) and G_{M2} of the visceral form of Tay-Sachs disease (Type II or O-variant) (7); G_{M1} of generalized gangliosidosis (8, 9); and galactosylsulfatide (GL-1bS) of metachromatic leukodystrophy (10). All these inherited defects have subsequently been attributed to the deficiency of specific glycosidases.

Studies on biopsy material from a patient with a primary neurological disorder, associated with slight hepatosplenomegaly and foam cells in the bone marrow, revealed a marked elevation of lactosylceramide and a deficiency of lactosylceramide galactosyl hydrolase (11) as the apparent primary metabolic defect. This has subsequently been confirmed both chemically and enzymically in autopsy tissue and fibroblasts cultured from skin and bone marrow (12); furthermore, the parents were shown to be heterozygotes for this enzyme deficiency (12). Since improvements in analytical techniques have made it possible to carry out such studies on very small amounts

Abbreviations: gal, galactose; glc, glucose; galNAc, *N*-acetylgalactosamine; NANA, *N*-acetylneuraminic acid; ceramide, 2-*N*-acylsphingosine; GL-1a, glc(1→1)ceramide; GL-1b, gal(1→1)-ceramide; GL-2a, gal(1→4)glc-ceramide; GL-2b, gal(1→4)gal-ceramide; GL-1bS, (SO_3H→3)gal-ceramide; GL-2aS, (SO_3H→3)-gal(1→4)glc-ceramide; GL-3a, gal(1→4)gal(1→4)glc-ceramide; GL-4a, galNAc(1→3)gal(1→4)gal(1→4)glc-ceramide; G_{M1}, asialo-G_{M2}, G_{M2}, G_{M3}, G_{D1a}, G_{D1b}, G_{D2}, G_{D3}, and G_{T1a} are the standard Svennerholm ganglioside nomenclature; OH, α-hydroxy fatty acids; N, other saturated/unsaturated fatty acids; C–M, chloroform–methanol; A–M, acetone–methanol; TLC, thin-layer chromatography on precoated silica gel plates; GLC, gas–liquid chromatography; TMSi, trimethylsilyl.

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of material (2 ml of packed cells, 5 ml of fluid, or 50 mg dry wt of tissue), the initial chemical studies were carried out on erythrocytes, plasma, bone marrow cells, urine sediment, and on brain and liver samples obtained at biopsy. These values were compared with the glycosphingolipid composition of control tissue determined in this laboratory and with values from previous studies on blood (13) and other tissue (1–5, 10, 13–15) from patients with lipid storage in visceral organs. Autopsy material has recently been obtained from this patient, allowing a much more detailed study of the tissue glycosphingolipid concentrations. These have substantiated the initial findings and have also revealed the effect of a catabolic block on the metabolism of both hematoside (G_{M3}) and glucosylceramide (GL-1a); the metabolism of these glycosphingolipids is compared with that in various forms of Gaucher's disease, since the two diseases are closely related metabolically. Substantial differences were found, and it seems reasonable to regard lactosylceramidosis as a separate genetic disease.

MATERIAL

The pathological liver and brain used in the initial study were obtained from S.H. by open biopsy; autopsy samples were obtained approximately 15 hr after death. Control samples of tissues were obtained from young females (2–7 yr) at autopsy; all tissues were kept frozen at −40°C prior to analysis. The diagnosis of the new disease lactosylceramidosis was made on the basis of analyses in these tissues and the reduced level of lactosylceramide galactosyl hydrolase in liver and cultured fibroblasts.

The patient with lactosylceramidosis (S.H.) appeared to develop slowly but normally for the first 25 months of life; after this time, rapidly accelerating neurological deterioration became evident. By the age of 34 months she had lost all her former skills, cerebellar ataxia was pronounced, and dementia progressed rapidly; retinal degeneration was extensive, but there was no cherry-red spot. Examination of the frontal lobe brain biopsy revealed neurons with swollen apical dendrites; other cells appeared normal. Although the disease was primarily neurological, foam cells were found in the bone marrow and this suggested a lipid storage disease. There was slight hepatosplenomegaly, and the visceral storage of lipid material was confirmed at autopsy. The child died at the age of 50 months.

METHODS

Extraction of glycosphingolipids

Blood and marrow aspirate (5–8 ml) were carefully separated into cells and plasma by the method of Vance and Sweeley (16); leukocytes plus immature cell fractions were discarded, and the cells were checked for degree of leukocyte contamination by routine hematological methods. Freshly collected urine from the patient and from normal individuals (24-hr samples) was centrifuged at 10,000 rpm for 30 min; the supernatant was discarded. Urine sediment was pooled, washed with distilled water, and lyophilized so that results could be expressed on either a dry weight or a 24-hr collection basis. The autopsy brain sample was separated into gray and white matter; all other tissue was washed extensively with 0.15 M NaCl to remove as much blood as possible. All tissue was homogenized by hand in 20 vol of 0.15 M NaCl; the length of homogenization varied with the individual tissues, and cooling was often necessary. Digestion of these homogenates with proteolytic enzymes such as trypsin and papain at pH 8 did not increase the yield of glycosphingolipids. Fluid, cells, or homogenates were then extracted with 300 vol of C–M 2:1 and stirred for a further 2 hr at room temperature. After filtration and further extraction of the insoluble residue with 250 ml of C–M 2:1, 0.2 vol of water was added (17). The two phases were separated either by aspiration of the upper phase or by the conventional separatory funnel technique; both phases were concentrated to dryness in 1-liter round-bottomed flasks.

Isolation of gangliosides from the upper (aqueous) phase

Chloroform (2 ml) and 0.6 N NaOH in dry methanol (2 ml) were added to the residue. After 1 hr at room temperature (18) the solution was transferred to a tube, and the flask was washed with 3 × 2 ml of C–M 1:1; these washings were all combined. Concentrated HCl (0.1 ml) and water (1.0 ml) were added to neutralize the mixture, which was then dialyzed against several changes of distilled water for 24 hr. The biphasic dialysate was dried and the residue was taken up in a small volume of C–M (1:1). The lipids were subjected to ascending TLC in C–M–2.5 N NH_4OH 60:40:9; the plates were developed two times in the same direction. Glycosphingolipids were visualized by nonspecific staining with iodine vapor, and a record of all TLC plates was made by exposure to diazo projection paper (B. K. Elliot Co., Pittsburgh, Pa.) and subsequent development with ammonia vapor. The areas of the TLC plate which corresponded to standard glycosphingolipids were scraped with a razor blade and transferred to a sintered funnel; the glycosphingolipids were quantitatively eluted with C–M–water 10:4:0.5 (20 ml) and concentrated to dryness. A known amount of the internal standard D-mannitol was added (0.02–0.2 μmole), and anhydrous methanolysis (1.0 N HCl in 2.0 ml of methanol) was carried out in Teflon-stoppered vials at 80°C for 18 hr (16, 19). After

neutralization with solid silver carbonate and re-*N*-acetylation with acetic anhydride (19), fatty acid methyl esters were removed with hexane (16) and the methanol solution was evaporated to dryness. The methyl glycosides were converted to trimethylsilyl derivatives (16, 19) by addition of pyridine–hexamethyldisilazane–trimethylchlorosilane 10:2:2 (20 μl). This gas–liquid chromatographic procedure enables one to detect and quantitatively estimate galactose, glucose, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid in a single 30-min analysis; fucose, mannose, *N*-acetylglucosamine and other possible components can also be detected in this analysis. The technique is particularly useful when it is necessary to distinguish between glycosphingolipids with very similar R_F values such as GL-3a and asialo-G_{M2}; the latter is readily identified by the presence of galNAc and a gal/glc ratio of 1:1. The Hewlett-Packard 402 gas chromatograph was equipped with a 6 ft × $^1/_8$ inch I.D. U-shaped glass column packed with 3% OV-1 on Supelcoport (100–120 mesh). Temperature programming from 160 to 240°C was carried out at 2°C/min; if no hexosamine or sialic acid was present the analyses were conducted isothermally at 170°C.

Isolation and estimation of individual lipids

Neutral lipids, phospholipids, and glycosphingolipids in the lower phase were initially separated by silicic acid chromatography (16). Neutral lipids, eluted with chloroform (150 ml), were further resolved by TLC on precoated silica gel plates (Quantum Industries, Fairfield, N.J.), using hexane–diethyl ether–acetic acid 80:20:2. Cholesterol was estimated by GLC on 1% OV-1 at 210°C using cholestane as the internal standard. After alkaline methanolysis of the crude glycosphingolipid fraction eluted with A–M 9:1 (200 ml) and dialysis of the entire reaction mixture as described above, the individual neutral glycosphingolipids were separated by ascending TLC in the solvent system C–M–water 11:4:0.6 (Fig. 1). Glycosphingolipids were estimated as described above. Phospholipids, eluted with methanol (150 ml), were separated by two-dimensional TLC (20) and visualized, sequentially, with iodine and ninhydrin.

Identification of sulfatides

Galactosylsulfatide (GL-1bS) was identified by its R_F value on TLC, cochromatography with authentic myelin GL-1bS, the presence of only galactose TMSi methylglycosides upon GLC analysis, and equimolar amounts of sulfate (21), galactose, and fatty acid. Liver GL-1bS contained normal and α-hydroxy fatty acids in the ratio 6:4, and showed the two characteristic bands on TLC; in normal liver the amount of sulfatide is less than 0.01 μmole/g fresh weight, but we have recently found large amounts in a patient with a disease of the "I-cell" type and this has been a useful standard. Lactosylsulfatide (GL-2aS) has an R_F value midway between GL-1bS and GL-3a (Fig. 2) and can be further identified by the equimolar amounts of galactose, glucose, sulfate, and fatty acid. Quantitative fatty acid analyses were carried out by GLC on 15% DEGS using a $C_{19:0}$ fatty acid methyl ester standard; α-hydroxy fatty acids were detected by their increased relative retention times after conversion to the TMSi ether derivative.

Identification of galactosylceramide and digalactosylceramide

Galactosylceramide (GL-1b) isolated from brain or kidney yields two bands in the TLC solvent system described. The upper "cerebroside" band in all human visceral organs contains mainly glucose and normal fatty acids; the lower band, when present, contains mainly galactose. For most tissues the galactosylceramide content was estimated from the amount of galactose in total "cerebroside" region. In humans, digalactosylceramide (GL-2b) appears to be present only in kidney (4, 14, 15) and intestine[1] in significant amounts. Contrary to the report of Miras, Mantzos, and Levis (22), it is not a constituent of human leukocytes. Since GL-2a and GL-2b are separated from GL-1bS in the solvent system described, the amount of GL-2b is based on the galactose:glucose ratio of the dihexosylceramide region. Pure GL-2b isolated from the kidney of a patient with Fabry's disease was used as a standard.

RESULTS

By using GLC it is possible to reliably estimate the level of glycosphingolipids in 1–2 ml of packed erythrocytes, 50 mg dry weight of urine sediment or fibroblasts, and 0.2–0.5 g fresh weight of biopsied tissue. This enables meaningful chemical analyses to be performed on small samples from a patient and is invaluable in the diagnosis of glycosphingolipidoses; in this case we were able to subsequently confirm these analyses on tissue samples obtained at autopsy.

The lactosylceramide isolated from these samples was identified by its R_F value on TLC and the presence of galactose, glucose, sphingosine, and fatty acid in the ratio 1:1:1:1. The presence of a (1 → 4) linkage was confirmed by direct probe mass spectrometry of the fully trimethylsilylated glycosphingolipid (23, 24). Other glycosphingolipids were identified by their R_F values, the

[1]Human small intestine contains digalactosylceramide (GL-2b) and lactosylceramide (GL-2a) in the ratio 3:5, together with GL-1a and GL-1b in the ratio 1:2, GL-1bS, GL-3a, GL-4a, G_{M3}, G_{D3}, G_{M2}, G_{M1}, and fucose-containing glycosphingolipids with structures analogous to blood group antigens. This explains why this tissue is a good source of lysosomal glycosyl hydrolases.

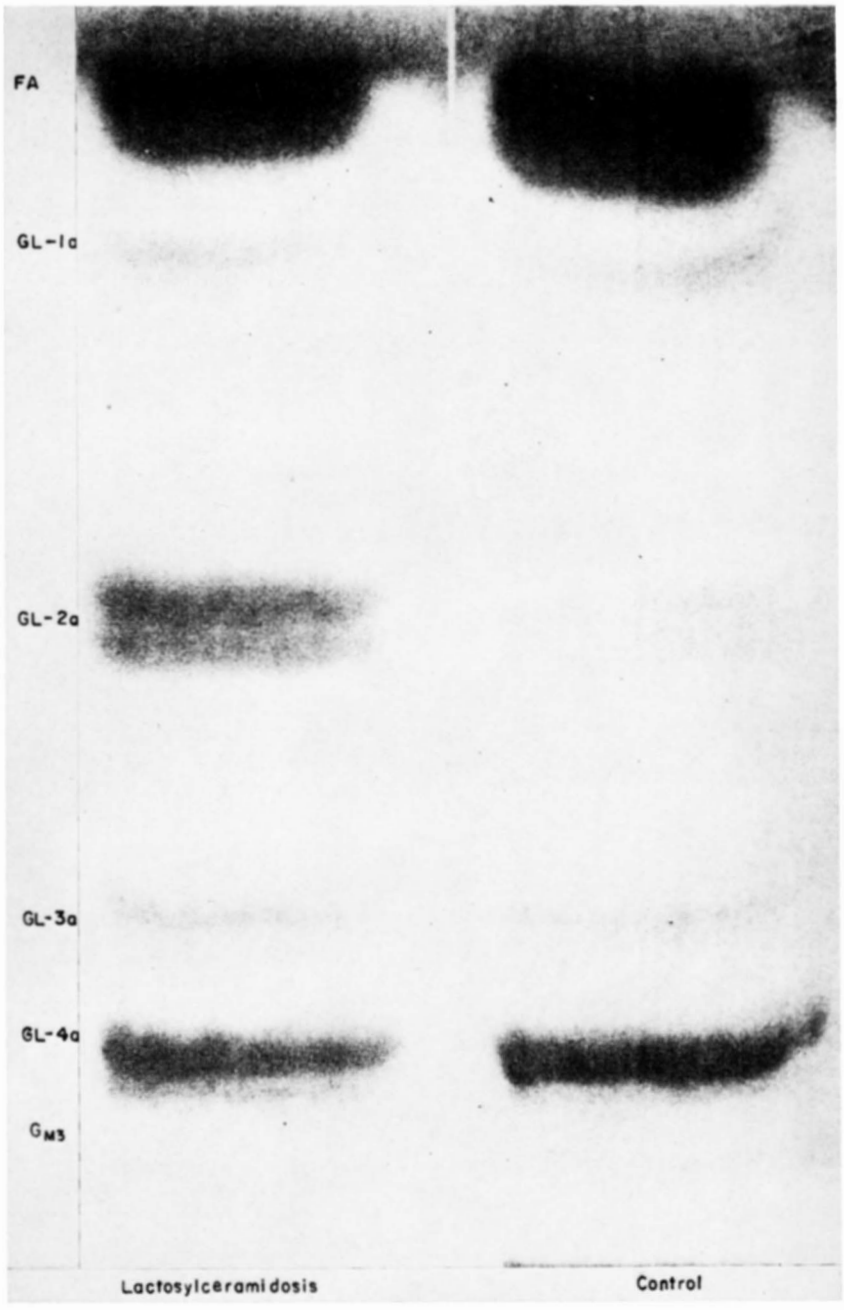

FIG. 1. Thin-layer (silica gel) chromatographic separation of the major glycosphingolipids from normal human and the patient's (S.H.) erythrocytes, using the solvent system C–M–water 110:40:6. Individual glycosphingolipids were visualized with iodine. In all samples studied, the cerebroside region contained glucosylceramide (GL-1a) and small amounts of galactosylceramide (GL-1b). The two bands designated lactosylceramide (GL-2a) differed only in the chain length of their fatty acid moiety. The ganglioside phase contains more G_{M3} and a glycosphingolipid containing gal:glc:glcNAc:NANA (2:1:1:1) whose structure is unknown. The fucoglycosphingolipid blood group substances are undetectable in this amount (3 ml) of erythrocytes.

ratio of galactose to glucose, and the presence or absence of galNAc or NANA.

Glycosphingolipids in blood

Analyses of the glycosphingolipid content of red cells (Table 1) and plasma (Table 2) from young females were consistent and indistinguishable from those of adults. Red cells from S.H. (four samples collected at monthly intervals) were characterized by a threefold elevation of GL-2a (Fig. 2), a slight elevation of glucosylceramide, and normal levels of the other two major glycosphingolipids. Red cells from the hetero-

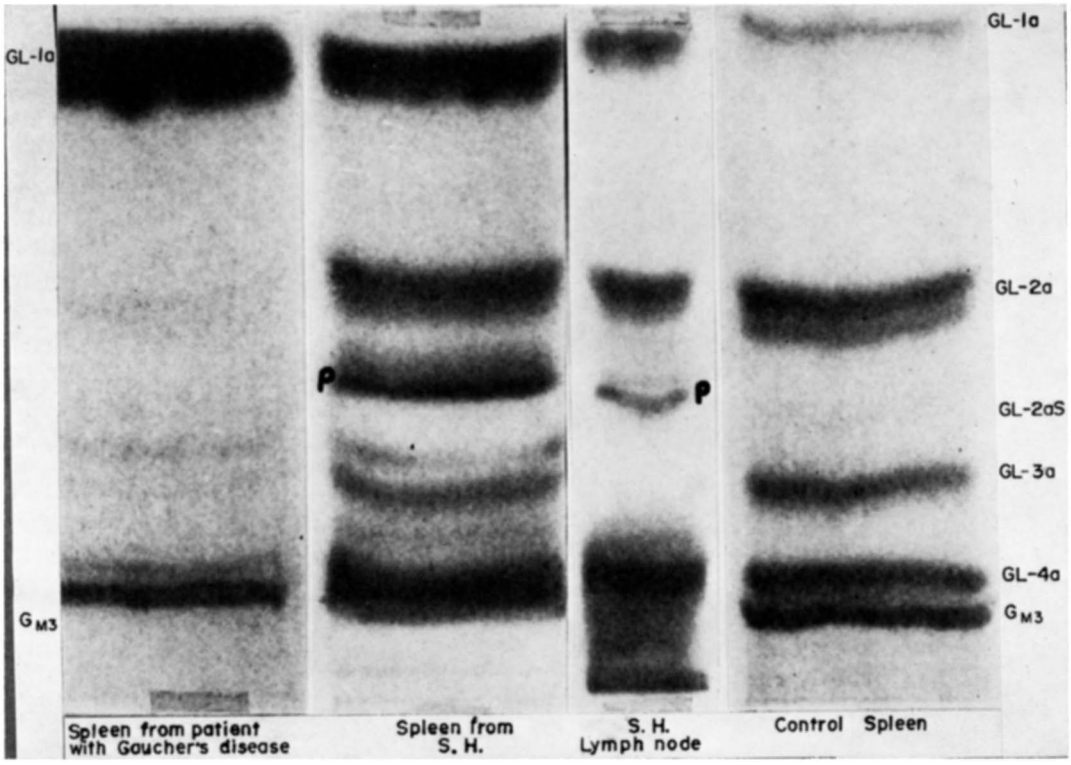

FIG. 2. TLC (precoated silica gel) of the major glycosphingolipids from human spleen and lymph node. The major differences between normal, Gaucher, and S.H. spleen are readily apparent. The solvent system used was C–M–water 110:40:6. The fractions marked *P* (pigment) in lymph node and spleen from S.H. were not glycolipid in nature.

zygous parents were examined (Table 1), but the glycosphingolipid concentrations could not be distinguished from normals, apart from a slight elevation of GL-2a in the mother. Plasma (Table 2) from S.H. contained a threefold elevation of GL-2a; other glycosphingolipids, including G_{M3} (25), were apparently within the normal range. Once again the heterozygotes were normal with respect to the concentrations of plasma glycosphingolipids.

Glycosphingolipids in bone marrow

Bone marrow from the patient (S.H.) was found to contain foam cells, and analysis of the marrow cells revealed an elevated level of GL-2a as the only major lipid abnormality (Table 3); normal marrow cell glycosphingolipid concentrations were identical to mature erythrocytes, apart from an increased level of GL-2a; G_{M3} (26) did not appear to be elevated. Although S.H. marrow cells contained 70% more GL-2a than controls, variations in leukocyte and reticulocyte contamination could invalidate this as a diagnostic test, since GL-2a is the major glycosphingolipid in these cells (27). Bone marrow cells were isolated from patients with leukemias and, as anticipated, high levels of GL-2a were found (Table 3). There was no evidence for GL-2b (22), and one must conclude that GL-2a is the major constituent of peripheral leukocytes. Three individual analyses on cells from a patient with acute myelocytic leukemia showed this to be the only exception, since the level of glycosphingolipids was identical to erythrocytes, apart from a slight reduction in the level of GL-3a.

TABLE 1. Erythrocyte glycosphingolipid composition in lactosylceramidosis, in heterozygotes for this disease, and in normal young females

Glycosphingolipid	Normal Female[a]	Patient (S.H.)	Mother of S.H.	Father of S.H.
		μmoles/100 ml		
GL-1a	1.14 ± 0.5	2.00	1.80	0.98
GL-1b	0.12 ± 0.1	0.10	0.11	0.12
GL-2a	1.40 ± 0.2	5.21	1.98	1.26
GL-3a	1.18 ± 0.2	1.15	1.27	1.55
GL-4a	8.37 ± 1.0	8.24	8.57	9.15
G_{M3}	0.35 ± 0.1	0.39	0.41	0.36

[a] Mean ± SEM, eight subjects.

Glycosphingolipids in kidney and urine sediment

Initial studies were carried out on urine sediment since kidney was not available until autopsy; the presence of GL-2b (28) and GL-1bS in urine sediment suggests that it is at least partly renal in origin. The urine collected from the patient (S.H.) was essentially free from leukocytes and was characterized by a threefold elevation of

TABLE 2. Plasma glycosphingolipid composition in lactosylceramidosis, in heterozygotes for this disease, and in normal young females

Glycosphingolipid	Normal Female[a]	Patient (S.H.)	Mother of S.H.	Father of S.H.
		μmoles/100 ml		
GL-1a	0.99 ± 0.2	1.41	0.86	1.24
GL-1b	0.10 ± 0.1	0.11	0.11	0.12
GL-2a	0.47 ± 0.1	1.83	0.39	0.31
GL-3a	0.25 ± 0.1	0.22	0.21	0.22
GL-4a	0.28 ± 0.1	0.39	0.14	0.17
G_{M3}	0.23 ± 0.1	0.30	0.29	0.21

[a] Mean ± SEM, four subjects.

GL-2a (Table 4). Since normal sediment contains virtually undetectable levels of glycosphingolipids (27), the control normal sediment was obtained from 20 liters of pooled adult male urine (0.6 g dry wt). Although urine sediment contains qualitatively the same glycosphingolipids as kidney, the shorter-chain glycosphingolipids, GL-1a and GL-2a, predominate; this could reflect degradation or the presence of epidermal tissue, which seems to contain predominantly GL-1a.[2]

The glycosphingolipids of S.H. kidney presented a much more complex pattern of elevation, since many of the glycosphingolipids, including GL-2a, were substantially elevated (Table 4); the sulfatide level in kidney was normal.

Glycosphingolipids in liver

Livers from females of different ages contained the same glycosphingolipids in approximately the same concentrations (Table 5); the analytical figures were in agreement with those reported (29) during the course of these studies, with the exception that higher levels of G_{M3} were found. Samples of frozen liver were washed in saline to remove as much blood as possible, but it is still possible that some of the globoside found may have been derived from erythrocytes, where it is present in relatively large (0.1 μmole/ml) concentration. A single analysis on the liver biopsy from the patient with lactosylceramidosis (S.H.) showed an eightfold elevation of lactosylceramide together with a sixfold elevation of glucosylceramide and a twofold elevation of G_{M3}; this was confirmed in studies on autopsy material. For comparison, liver from a patient with the infantile form of Gaucher's disease was studied; there was a more than 200-fold elevation of glucosylceramide and only minor elevations of the other glycosphingolipids.

Glycosphingolipids in other visceral tissue; spleen, lymph nodes, and adrenal gland

In all three tissues, three glycosphingolipids, GL-1a, GL-2a, and G_{M3}, were substantially elevated (Table 6 and Fig. 2). The elevation of GL-1a was most pronounced

TABLE 3. Bone marrow cell glycosphingolipid composition in normal young females, in a patient with lactosylceramidosis, and in leukemic patients

Glycosphingolipid	Normal Female[a]	Patient (S.H.)	AML	AUL[b]	Histiocytosis-X	CML
			μmoles/100 ml			
GL-1a	1.58 ± 0.4	1.24	1.56	1.97	2.47	0.33
GL-1b			0.34	0.65	0.15	0.30
GL-2a[c]	4.15 ± 0.5	6.85	1.38	6.56	11.30	7.91
GL-3a	1.10 ± 0.1	1.38	0.87	0.86	2.72	0.92
GL-4a	8.61 ± 0.9	10.10	10.60	6.65	7.55	5.70
G_{M3}	0.35 ± 0.1	0.39				

Abbreviations: AML, acute myeloblastic leukemia; AUL, acute undifferentiated leukemia; CML, chronic myelocytic leukemia.

[a] Mean ± SEM, four subjects.

[b] Means of seven patients.

[c] There was no evidence for the presence of digalactosylceramide in human leukocytes.

TABLE 4. Kidney and urine sediment glycosphingolipids in the patient (S.H.) and controls

Glycosphingolipid	Normal Kidney	Patient's Kidney	Normal Urine Sediment	Patient's[a] Urine Sediment
	μmoles/g fresh wt		*μmoles/g dry wt*	
GL-1a	0.08 ± 0.03	0.16	0.52	0.43
GL-1b	0.07 ± 0.02	0.18	0.09	0.08
GL-2a	0.05 ± 0.01	0.13	0.18	0.54
GL-2b	0.04 ± 0.01	0.04	0.11	0.06
GL-1bS	0.17 ± 0.03	0.13	0.19	0.20
GL-2aS	0.04 ± 0.01	0.06	0.09	ND[b]
GL-3a	0.10 ± 0.02	0.30	0.17	0.06
GL-4a	0.39 ± 0.08	0.35	0.22	0.25
G_{M3}	0.04 ± 0.02	0.09	0.03	ND
G_{D3}	0.02 ± 0.01	0.02	ND	ND

[a] Values expressed as μmoles of glycosphingolipid/24-hr urine collection have been reported previously (33).

[b] Not determined.

[2] Human skin was removed at operation from the neck of a patient with Hodgkin's disease.

TABLE 5. Liver glycosphingolipids in lactosylceramidosis, Gaucher's disease, and controls

Glycosphingolipid	Normal Female	Patient (S.H.)	Gaucher's Disease (Infantile)
	μmoles/g fresh wt		
GL-1a	0.06 ± 0.02	0.39	11.40
GL-1b	<0.01	0.01	0.01
GL-2a	0.06 ± 0.01	0.45	0.26
GL-1bS	<0.01	0.01	0.01
GL-3a	0.03 ± 0.01	0.08	0.05
GL-4a	0.02 ± 0.01	0.02	0.04
G_{M3}	0.18 ± 0.04	0.39	0.77
G_{D3}	0.03 ± 0.01	0.04	0.04

in the spleen, which is where GL-1a characteristically accumulates in Gaucher's disease. For comparison, we examined the spleen glycosphingolipids from a 4-yr-old patient with this disease; a massive elevation of GL-1a was found together with a smaller elevation of G_{M3}. The accumulation of GL-1a was even more pronounced in an adult with a milder form of this disease. Thus lactosylceramidosis can readily be distinguished from Gaucher's disease by examination of the spleen, and further, the accumulation of GL-2a and G_{M3} was more pronounced in the massively infiltrated lymph nodes and adrenal gland (Table 6).

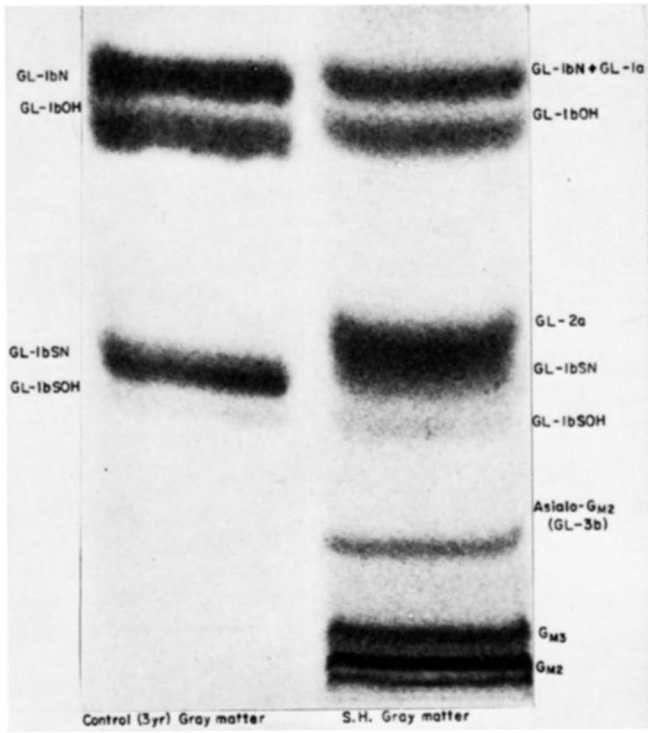

FIG. 3. TLC (precoated silica gel) of the "lower phase" glycosphingolipids from normal and S.H. gray matter. The solvent system used was C–M–water 117:40:6; the increased chloroform content was necessary to adequately resolve GL-2a and GL-1bS.

Glycosphingolipids in brain

Since this disease (lactosylceramidosis) was primarily neurological in nature, it was important to identify the material which was accumulating in the apical dendrites. Initial studies on fragments of frontal lobe obtained at biopsy revealed a significant elevation of GL-2a (11). This was confirmed in gray and white matter (Fig. 3) obtained at autopsy from the patient. When compared with the mean values obtained from three infantile brains (3–6 yr), increased levels of other glycosphingolipids such as GL-1a, G_{M3}, asialo-G_{M2}, and G_{M2} were found in gray matter (Table 7). However, the elevation of GL-2a was the most pronounced and this was also true of white matter, where GL-2a was virtually the only neutral glycosphingolipid present in abnormal amounts (Table 7). Fatty acid analyses of the gray matter neutral glycosphingolipids showed the high level of stearate ($C_{18:0}$) characteristic of gangliosides (Table 8). The fatty acid composition of GL-1a, which cochromatographed with GL-1bN, was different and comparable to the cerebrosides and sulfatides. The GL-2a which accumulated in white matter had a much lower content of stearate than that in gray matter (Table 9); the higher content of longer-chain fatty acids (>$C_{18:0}$) was more like the fatty acid pattern of cerebrosides and the pattern found

TABLE 6. Glycosphingolipids in adrenal gland, lymph node, and spleen of patient S.H. and controls

Glycosphingolipid	Control Adrenal Gland	S.H. Adrenal Gland	Control Lymph Node	S.H. Lymph Node	Control Spleen	S.H. Spleen	Chronic Gaucher Spleen[a]	Chronic Gaucher Spleen[b]
				μmoles/g fresh wt				
GL-1a	0.08	0.09	0.08	0.45	0.09 ± 0.03	1.03	5.30	10.0
GL-2a	0.03	0.22	0.02	0.27	0.13 ± 0.02	0.48	0.23	0.09
GL-2aS	<0.01	0.01				0.04		
GL-3a	0.03	0.08	0.09	0.04	0.04 ± 0.01	0.09	0.03	0.03
GL-4a	0.01	0.05	0.06	0.02	0.04 ± 0.01	0.14	0.03	0.04
G_{M3}	0.21	0.41	0.14	0.49	0.07 ± 0.02	1.13	0.33	0.48
G_{D3}	0.08	0.06	0.05	0.05	0.02 ± 0.01	0.04	0.05	0.06

[a] This spleen was removed from a 4-yr-old Asian male with the noncerebral (chronic) form of Gaucher's disease.
[b] This spleen was removed from a 63-yr-old Ashkenazi Jewish male with chronic Gaucher's disease.

TABLE 7. Glycosphingolipids isolated from gray and white matter

Glycosphingolipid	Gray Matter		White Matter	
	Control	S.H.	Control	S.H.
	μmoles/g fresh wt			
GL-1a	0.04	0.22	0.01	0.03
GL-1bN	0.59	0.13	3.16	4.07
GL-1bOH	0.67	0.27	4.79	3.77
GL-2a	0.02	0.38	<0.01	0.32
GL-1bSN	0.24	0.11	0.89	1.15
GL-1bSOH	0.23	0.09	0.96	1.37
ASG_{M2}		0.06		0.03
G_{M3}	<0.01	0.20	<0.01	0.08
G_{M2}	0.02	0.20	<0.01	0.12
G_{M1}	0.15	0.15	0.04	0.20
G_{D1a}	0.26	0.20	0.06	0.08
G_{D1b}	0.18	0.07	0.03	0.05
G_{D2}	0.02	0.06	0.01	0.04
G_{D3}	0.04	0.02	<0.01	0.02
G_{T1a}	0.18	0.11	0.05	0.09

in the GL-2a stored in the patient's visceral organs (Table 9). The major ganglioside differences were the increased amounts of G_{M3}, G_{M2}, and G_{D2} present (Fig. 4) in both gray and white matter; gas–liquid chromatographic analysis of gangliosides gave values of approximately 530 μg of NANA/g fresh weight of normal gray matter, which is lower than the value of 700–800 μg determined by colorimetric assay (8). This may reflect a low recovery of ganglioside from the TLC plate or an overestimation by the colorimetric assay.

Neutral lipids and phospholipids

Thin-layer chromatographic examination of neutral lipids and phospholipids in tissue from this patient revealed no major variation from control samples. Specific attention was given to sphingomyelin, the lipid which accumulates in Niemann-Pick disease; the concentration in S.H. liver was 1.9 mg/g wet weight compared with a mean of 1.8 mg/g wet weight in three control livers.

Mucopolysaccharides

The individual mucopolysaccharide levels in urine were determined by Dr. R. Matalon, Department of Pediatrics, University of Chicago; the relative amounts and total concentration were within normal limits.

DISCUSSION

The glycosphingolipidoses are characterized by specific catabolic enzyme deficiencies which often result in the accumulation of abnormal amounts of a specific glycosphingolipid in tissues such as brain, kidney, or liver. GLC has been shown (11–13, 16, 19) to give reliable quantitative analyses of carbohydrates in very small amounts of biological material and, in addition, can be used to identify the glycosphingolipid from the ratio of constituent monosaccharides. The precise chemical analysis of glycosphingolipid levels in various tissues can be used not only for diagnosis of the sphingolipidoses, but can provide information about the dynamic relationships between individual glycosphingolipids. Further, detailed chemical analyses are always a prerequisite for metabolic studies designed to determine the origin of the accumulating glycosphingolipid in such diseases (30, 31). The finding of elevated levels of lactosylceramide (GL-2a) in patient S.H. with the present study was the first indication that she had a hitherto undescribed sphingolipidosis.

The significantly elevated level of lactosylceramide (GL-2a) in the patient's erythrocytes is in contrast to the situation in Fabry's disease, in which the erythrocytes contain normal amounts of GL-3a (13). However, the findings are in accord with Gaucher's disease, in which GL-1a and other glycolipids such as GL-4a are significantly elevated (13). The level of GL-2a in both erythrocytes and plasma from the presumed heterozygotes was within the normal range. However, in the patient an elevation of plasma GL-2a, comparable to that of GL-3a

TABLE 8. Fatty acid composition of glycosphingolipids in S.H. gray matter

Fatty Acid	GL-1a/1bN	GL-1bOH	GL-2a	GL-1bSN	GL-1bSOH	Asialo-G_{M2}	G_{M3}	G_{M2}
				% of total fatty acids				
16:0[a]	2.5	15.0	2.2	5.9	14.5	2.2	3.4	3.3
17:0	8.4	0.4		0.5	2.5	0.1	0.5	0.4
18:0	37.4	40.8	86.5	41.7	42.2	85.4	79.5	86.5
18:1	0.9	6.4		0.1	0.7			0.7
20:0	3.5	3.1	5.3	5.1	3.7	3.4	8.1	6.0
21:0		0.2		0.1	0.3	0.1	0.4	
22:0	31.4	18.8	1.0	9.7	18.4	5.0	4.5	2.3
23:0	1.9	1.3	0.5	4.6	2.0	1.0	0.5	0.3
24:0	7.3	8.6	4.5	27.2	13.4	2.8	3.9	0.4
24:1	6.7	2.0		2.0	0.7			0.1
25:0				3.1				

[a] Number of carbon atoms: number of double bonds.

TABLE 9. Major fatty acids of lactosylceramide (GL-2a) stored in white matter, spleen, lymph node, liver, and adrenal gland of patient S.H.

Fatty Acid	White Matter	Spleen	Lymph Node	Liver	Adrenal Gland
			% of total fatty acids		
16:0[a]	4.5	3.0	12.4	4.2	5.2
16:1	0.2		0.8		
17:0	0.5	0.6	1.4	1.2	1.2
17:1		0.7	0.6	2.0	2.1
18:0	24.6	5.7	12.8	4.9	5.4
18:1	2.4	1.5	4.6	1.4	2.2
20:0	3.4	6.2	4.9	5.0	3.4
21:0	0.1	0.1	0.1	0.2	0.1
22:0	11.4	27.1	15.3	29.7	26.6
23:0	6.0	10.0	4.6	11.6	14.0
24:0	27.5	32.3	25.2	31.2	28.9
24:1	17.0	12.8	17.3	8.6	10.9
25:0	2.0				
25:1	0.4				

[a] Number of carbon atoms: number of double bonds.

in Fabry's disease (15) and GL-1a in Gaucher's disease (13), was found. Plasma and erythrocyte analyses are therefore of diagnostic value in the study of glycosphingolipidoses. Since it has been suggested (32) that erythrocyte glycosphingolipids cannot react with antibodies until a trypsin-sensitive coating is removed, erythrocytes and other tissues were digested with proteolytic enzymes prior to extraction. Although there was some preliminary evidence that yields of neutral lipids and phospholipids were significantly increased, there was no increase in the yield of glycosphingolipids. However, the technique did seem to have value in reducing the amount of erythrocyte pigment carried through to the TLC stage and merits further investigation.

The concentration of glycosphingolipids in bone marrow cells has not been previously reported, but the results showed that they were essentially the same as mature erythrocytes (16, 26, Table 1), apart from increased levels of GL-2a. This could be partly attributed to the presence of large numbers of leukocytes and reticulocytes, since leukemic bone marrow cells contained extremely high levels of GL-2a, and this appears to be the major glycosphingolipid constituent of human leukocytes (27). The greater heterogeneity of bone marrow cells suggests that a much more precise fractionation technique may be needed before one can reliably diagnose lactosylceramidosis on the basis of the GL-2a level in these cells. An alternative approach would be to purify the foam cells, which, in the case of this patient

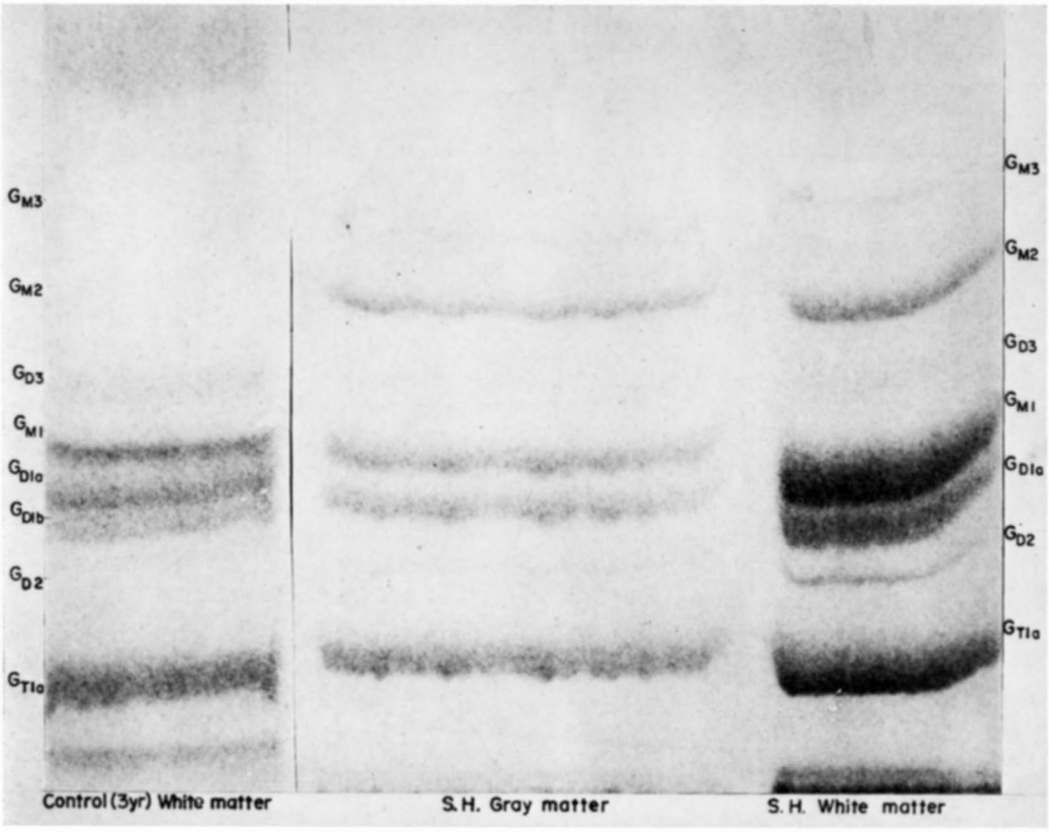

FIG. 4. TLC (precoated silica gel) of the gangliosides derived from the patient's (S.H.) gray and white matter. The solvent system used was C–M–2.5 N NH_4OH 60:40:9, and two sequential developments were necessary to obtain adequate resolution. The identity of each ganglioside was determined from its R_F value compared with ganglioside standards and the ratio of the constituent monosaccharides. The GLC analyses gave absolute identification of all but G_{D1a} and G_{D1b}.

(S.H.), should contain GL-2a plus some GL-1a and G_{M3}.

Urine sediment is the cellular debris from renal tissue, glomerular tubules, and urinary tract and can be considered, on morphological and chemical grounds (the presence of GL-2b, GL-1bS, and GL-2aS), to be equivalent to a kidney biopsy (33, 34). Since lactosylceramide (GL-2a) is a component of normal kidney, a 24-hr urine sediment was analyzed for glycosphingolipids and a 2–3-fold elevation of GL-2a was found; other glycosphingolipid levels were normal. Samples from the patient were collected by catheterization, and routine examination revealed little trace of leukocyte contamination; thus, it was felt that the GL-2a was actually being derived from renal tissue. Examination of fresh kidney material obtained at autopsy confirmed the elevation of GL-2a together with other glycosphingolipids such as GL-1a, GL-1b, GL-3a, and G_{M3}. A chemical diagnosis would not, therefore, be possible if only kidney were available because of the overall disturbance in glycosphingolipid metabolism. However, it is of interest that kidney was the only organ in which an increased amount of GL-3a was found and, indeed, kidney is the major site of GL-3a accumulation in Fabry's disease.

Since there was some suggestion of hepatosplenomegaly prior to autopsy, a liver biopsy was obtained at age 34 months. Once again the marked elevation of GL-2a was accompanied by increased amounts of GL-1a and G_{M3}. A severe GL-2a β-galactosyl hydrolase deficiency was found in the patient's liver (11). Subsequent chemical analyses of liver obtained at autopsy confirmed the high levels of GL-2a and the increased levels of GL-1a and G_{M3}. This may be contrasted to Gaucher's disease, in which the massive elevation of GL-1a in the liver is accompanied by a much smaller increase in GL-2a, GL-3a, GL-4a, and G_{M3}, and to Fabry's disease, in which only GL-3a accumulates (3, 4, 15). Pathological studies on patient S.H. revealed foamy Kupffer cells, and the elevation of these three glycosphingolipids appeared sufficient to account for the clinical findings. Although not as impressive as the accumulation of GL-1a in Gaucher liver, the specific elevation of GL-2a, and to a lesser extent GL-1a and G_{M3}, serve to distinguish lactosylceramidosis from other related disorders such as Hurler's disease, in which a nonspecific elevation of all glycosphingolipids is found.[3]

Increased amounts of G_{M3}, GL-2a, and GL-1a have been reported (35) in gray matter of patients with Niemann-Pick disease, type C. This is probably a heterogeneous group of severe neurovisceral storage diseases characterized by a 2–9-fold elevation of sphingomyelin in liver but no sphingomyelinase deficiency.[4] The disease warrants further investigation with regard to its relationship to the glycosphingolipidoses, but liver glycosphingolipid levels appear normal.[4] The level of sphingomyelin in S.H. liver as measured by the method of Bartlett (36) was within the normal range quoted by Kwiterovich, Sloan, and Fredrickson (29).

Autopsy examination revealed lipid-infiltrated lymph nodes and a moderately enlarged spleen; analyses showed that this could be attributed to elevated levels of GL-1a, GL-2a, and G_{M3}. In Gaucher's disease, there is massive accumulation of GL-1a in the spleen, but only moderate elevations of the two catabolic precursors GL-2a and G_{M3}; this was demonstrated by analyses of spleens from an adult patient with chronic Gaucher's disease and a 4-yr-old child with the nonneurological juvenile form. The rather specific accumulation of GL-1a in spleen (1, 2) is in contrast to the widespread accumulation of GL-3a in Fabry's disease (15), and it is of interest that the greatest accumulation of GL-1a occurred in the spleen of the patient (S.H.). Lymph node and adrenal gland showed the most specific elevation of GL-2a, although the elevation of G_{M3} was still prominent.

We have previously shown (12) that GL-2a is present in threefold excess in skin fibroblasts and, as in the visceral organs, the level of G_{M3} was greater than normal. However, the GL-1a level did not appear to be significantly elevated. Enzymic studies on the fibroblasts showed significant (10–15%) residual activity, which presumably accounts for the relatively late onset of the disease.

Assuming that there is only one mutant enzyme in this disease, a block in the catabolism of GL-2a could result in the ancillary accumulation of immediate metabolic precursors such as G_{M3}; this is seen in Gaucher's disease to a slight extent (Tables 5 and 6). It is interesting that another immediate precursor of GL-2a, namely GL-3a, does not accumulate in this disease (apart from the kidney) but a third possible precursor, asialo-G_{M2}, was significantly elevated in brain (Table 7). The location of GL-2a as the common metabolite of at least three glycosphingolipid catabolic pathways explains the severe neurovisceral nature of this disease.

Since we have no evidence for a significant β-glucosidase deficiency (12), the accumulation of GL-1a is difficult to explain in terms other than of reduced synthesis of GL-2a. There is some precedent for this in the ganglioside storage diseases such as Tay-Sachs disease (37), in which one finds considerable amounts of possible biosynthetic precursors of G_{M2} (GL-1a, GL-2a, asialo-G_{M2}, and G_{M3}) in the gray matter. Since the spleen of a

[3] Liver from patients with Hurler's disease contains elevated levels of GL-2a (0.17–0.28 μmole/g fresh wt), but GL-1a (0.11–0.15), GL-3a (0.10–0.13), GL-4a (0.05–0.09), and G_{M3} (0.30–0.45) are also greatly increased; this is presumably a secondary effect of the massive dermatan and heparan sulfate accumulation.

[4] H. R. Sloan, personal communication. Examination of liver glycosphingolipids from four patients with Niemann-Pick type C disease revealed no abnormalities.

4-yr-old patient with typical Gaucher's disease contained at least five times as much GL-1a as our 4-yr-old patient (S.H.) and minimal elevations of GL-2a and G_{M3} as reported previously (38), the two diseases can be readily distinguished. The relationship between the severely neurovisceral infantile Gaucher's disease and the purely visceral "chronic" type has not yet been completely resolved, and it would be interesting to study GL-2a metabolism in the patients tentatively classed as "juvenile Gaucher with neurological symptoms."

Since lactosylceramidosis is primarily neurological in nature, gray and white matter were analyzed for glycosphingolipids and an accumulation of GL-2a was detected. Fatty acid analyses showed that GL-2a was metabolically related to gangliosides in that $C_{18:0}$ was approximately 90% of the total fatty acid; this was also true of G_{M2}, G_{M3}, and asialo-G_{M2}. The accumulation of GL-2a in white matter was specific, since GL-1a was not significantly increased and asialo-G_{M2} was not detected; G_{M2} and G_{M3} were present but their concentration was comparable to that in other pathological brains where the biochemical defect remains unknown. A 20–30-fold elevation of GL-2a was found in gray matter, and its concentration was equal to "cerebroside" (GL-1b). In addition, increased levels of other related metabolites such as GL-1a, G_{M3}, asialo-G_{M2}, and G_{M2} were also found. This accumulation of glycosphingolipid appears sufficient to account for the unusual storage bodies seen in the neuronal apical dendrites of this patient and could be explained on the basis of a block in GL-2a catabolism.

This is by no means the first time that GL-2a has been found in excessive amounts in gray matter. There are two reports of its accumulation in patients with Krabbe's leukodystrophy (39, 40) (although this was not confirmed by Eto and Suzuki [41]) and in late infantile amaurotic idiocy[5] (42). Further, its presence in Tay-Sachs brain, together with GL-1a, asialo-G_{M2}, and massive amounts of G_{M2}, has been well documented (37). Pilz, Sandhoff, and Jatzkewitz (43) reported some studies on a formalin-fixed brain which showed accumulation of GL-2a, G_{M3}, and G_{M2}, and they considered the possibility that the primary metabolic defect involved either GL-2a or G_{M3} catabolism. Their patient also showed evidence of visceral involvement (44) and was diagnosed as a form of Niemann-Pick disease. Finally, one must consider the case of "atypical" Gaucher's disease reported in 1962 (45, 46); it was stated that there was no glycosphingolipid accumulation but that GL-2a was the major glycosphingolipid in spleen. Subsequent work has shown this to be the case in normal spleen, and because of the absence of further analyses, the biochemistry of this particular case remains enigmatic and should not be considered in terms of a defect in GL-2a catabolism.

A specific reduction in GL-2a galactosyl hydrolase activity has been demonstrated in this patient (S.H.), and by analogy to some other glycosphingolipidoses one would expect a specific accumulation of GL-2a throughout the body. Preliminary studies on samples of blood, marrow, and urine, collected with minimal trauma to the patient, confirmed this. However, subsequent analyses on the major body organs obtained at autopsy revealed a more complicated picture, in that there was substantial accumulation of G_{M3} (the catabolic precursor of GL-2a) and GL-1a (the anabolic precursor of GL-2a). Our knowledge of the in vivo metabolism of glycosphingolipids is somewhat limited, and a detailed study of such diseases, using systems such as cultured skin fibroblasts where conditions can be rigidly controlled, is necessary. GL-2a accumulated in both gray and white matter, and the presence of both asialo-G_{M2} and G_{M3} suggests that there might be two pathways for the metabolism of G_{M2} in brain. Finally, the absence of chemical evidence for an inborn error of mucopolysaccharide, phospholipid, or neutral lipid metabolism supports the concept that this rare disease is a glycosphingolipidosis involving the inability to catabolize GL-2a in the normal manner. Since this glycosphingolipid is an intermediate in both globoside (visceral) and ganglioside (neurological) metabolism, the clinical picture of a severe neurovisceral storage disease is in keeping with the biochemical findings.

I would like to thank Dr. Arthur O. Stein, who was in charge of the patient (S.H.) and arranged for the samples of fluid and biopsies, Dr. Emily Pang for samples of bone marrow aspirate, Dr. K. Suzuki (Philadelphia, Pa.) for the Gaucher liver sample, Drs. P. N. Madarnas and A. N. Kales, University of Chicago, for the Gaucher spleen samples, and Mr. John Oh for excellent technical assistance.

This investigation was supported by U.S. Public Health Service Mental Retardation Research Center grant HD 04583 and grant (RR-305) from the General Clinical Research Centers Program of the Division of Research Resources, National Institutes of Health.

Manuscript received 7 December 1970 and in revised form 9 August 1971; accepted 21 October 1971.

[5]Gray matter from the patient with Bielschowsky's disease contained GL-1a, 0.06; GL-2a, 0.04; GL-3a, 0.01; and GL-4a (or asialo-G_{M1}), 0.01 μmole/g fresh wt, in addition to the normal cerebroside, sulfatide, and ganglioside components.

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