Increased urinary excretion of glycosphingolipids in familial hypercholesterolemia

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Abstract The content of glycosphingolipids (GSL) was studied in the urinary sediments (24-hr specimens) from seven normal subjects, a patient with Fabry's disease, and five homozygotes with familial hypercholesterolemia (FH). Normal urinary sediments contained very small amounts of GalCer, GlcCer, $GaOse_2Cer$, LacCer, $GbOse_3Cer$, and $GbOse_4Cer$. In Fabry urinary sediment, the levels (nmole glucose/24 hr) of $GaOse_2Cer$ and of $GbOse_3Cer$ were 389 and 550, respectively. In urinary sediments from the FH subjects, the mean contents (nmol glucose/mg protein per 24 hr) of GlcCer, GalCer, and LacCer were 2.7, 1.9, and 15.8 times higher, respectively, than in normals. The mean contents (μg/mg protein per 24 hr) of total cholesterol and phospholipid in the urinary sediment of FH (1.1 and 224, respectively) and normals (0.8 and 220) were similar. The mean contents of GlcCer, GalCer, and LacCer, expressed in terms of the cholesterol content of urinary sediment (nmol glucose/μg cholesterol per 24 hr), were increased 3.4-, 1.6-, and 5.4-fold, respectively, in the FH homozygotes. Of the five FH homozygotes, only one, who had undergone a portacaval shunt and was also receiving lipid-lowering therapy, had a normal value of LacCer. The other four FH homozygotes had levels of LacCer that were 3- to 55-fold higher (nmol glucose/mg protein per 24 hr) and 5.5- to 7.3-fold higher (nmol glucose/μg cholesterol per 24 hr) than the mean of the normals. One homozygote underwent plasma exchange therapy that reduced both the baseline urinary (nmol glucose/24 hr) and plasma (nmol/100 ml) LacCer levels from 86 to 7 and from 1491 to 852, respectively. Eleven days after plasma exchange, the urinary LacCer levels approached pre-exchange levels (59 nmol glucose/24 hr). The data indicate that there is an abnormality of GSL metabolism associated with familial hypercholesterolemia and that the urinary excretion of GSL can be modified by plasma exchange therapy.—**Chatterjee, S., C. S. Sekerke, and P. O. Kwiterovich, Jr.** Increased urinary excretion of glycosphingolipids in familial hypercholesterolemia. *J. Lipid Res.* **23:** 513–522.

Supplementary key words hyperlipidemia • hyperlipoproteinemia • plasma exchange • cholesterol • lipid storage disease • Fabry's disease • gas–liquid chromatography • thin-layer chromatography

Familial hypercholesterolemia (FH) is a disorder of low density (beta) lipoprotein (LDL) metabolism that is inherited as an autosomal dominant trait (1). FH heterozygotes have elevated plasma total and LDL cholesterol levels that are expressed early in life and that in adulthood lead to tendon and tuberous xanthomas and premature ischemic heart disease (1–3). FH homozygotes have levels of plasma total and LDL cholesterol that are very high (600–1,000 mg/dl and 500–900 mg/dl, respectively) (1), and they acquire planar and tuberous xanthomas and atherosclerosis of the coronary vessels, aorta, and aortic valve in childhood (1). Cultured fibroblasts from FH homozygotes either lack a functional LDL receptor on the cell surface (receptor-negative), or the receptor has markedly diminished binding activity (receptor-defective), or is incapable of internalizing bound LDL (internalization defect) (4). These defects in LDL receptor activity are associated in vitro with decreased proteolysis of LDL, faulty regulation of hydroxymethylglutaryl CoA reductase, and decreased stimulation of cholesterol esterification (4). In vivo studies of LDL metabolism in FH have shown that the elevated plasma LDL levels are associated with a decreased fractional catabolic rate (5, 6).

Glycosphingolipids (GSL) are complex, sugar-containing lipids that are constituents of cell membranes and are involved in cell-cell interaction and ion transport, and are receptors for cholera toxin, thyrotrophin, and perhaps fibronectin (7–12). Blood group GSL confer antigenic properties (13) to red blood cells. Plasma GSL are primarily carried on LDL with smaller amounts on high density (alpha) lipoproteins (HDL) (14, 15). We had previously found an absolute increase (nmol/mg protein) of GSL on LDL in homozygous FH (14). In

Abbreviations: FH, familial hypercholesterolemia; LDL, low density (beta) lipoproteins; HDL, high density (alpha) lipoproteins; VLDL, very low density (prebeta) lipoproteins; GSL, glycosphingolipids; TLC, thin-layer chromatography; GLC, gas–liquid chromatography. The terminology used for the individual glycosphingolipids was: GlcCer (GL-1a) = Glc-β-(1 → 1′)-Cer; GalCer (GL-1b) = Gal-β-(1 → 1′)-Cer; LacCer (GL-2a) = Gal-β-(1 → 4)-Glc-β-(1 → 1′)-Cer; $GaOse_2Cer$ (GL-2b) = Gal-β-(1 → 4)-Gal-β-(1 → 1′)-Cer; $GbOse_3Cer$ (GL-3a) = Gal-α-(1 → 4)-Gal-β-(1 → 4)-Glc-β-(1 → 1′)-Cer; $GbOse_4Cer$ (GL-4a) = GalNAc-β-(1 → 4)-Gal-β-(1 → 4)-Gal-β-(1 → 4)-Glc-β-(1 → 1′)-Cer; $GbOse_5Cer$ (GL-5a) = GalNAc-α-(1 → 3)-GalNac-β-(1 → 3)-Gal-α-(1 → 4)-Gal-β-(1 → 4)-Glc-β-(1 → 1′)-Cer; II $SO_3LacCer$ = Gal_3SO_3-β-(1 → 4)-Glc-β-(1 → 1′)-Cer; II^3-NeuAc-LacCer (G_{M3}) = NeuAc-α-(2 → 3) Gal-β-(1 → 4) Glc-β-(1 → 1′)-Cer.

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TABLE 1. Plasma lipid and lipoprotein cholesterol levels in five homozygous FH patients at baseline and on the day of their 24-hr urine collection

Homozygous FH Patient		Total Cholesterol	HDL Cholesterol	LDL Cholesterol	VLDL Cholesterol	Total Triglycerides	Lipoprotein Pattern
		mg/100 ml					
D.P.[a]	Baseline 24-hr urine	804	33	735	36	242	IIb
D.D.[a]	Baseline	654	31	609	14	111	IIa
	24-hr urine	287	20	253	14	65	IIa
B.A.[b]	Baseline	903	43	845	15	164	IIb
	24-hr urine	969	25	957	ND	147	IIb
M.C.[c]	Baseline	997	89	801	ND	112	IIa
	24-hr urine	480	ND	ND	ND	68	IIa
T.B.[b]	Baseline	760	39	641	80	233	IIb
	24-hr urine						
	1) 8 days before first exchange	770	42	698	30	152	IIb
	2) 3 days after exchange	335	23	274	38	128	IIb
	3) 11 days after exchange	600	38	513	49	74	IIa

[a] Performed in the laboratory of the Molecular Disease Branch, NIH, Bethesda, MD. (1, 27).
[b] Performed in the Johns Hopkins laboratory (27).
[c] Baseline values are those previously published (21); subsequent values were provided by Dr. David Bilheimer.
ND—not determined.

one family with a homozygous FH child, the GSL in cultured fibroblasts were increased when the cells were grown in serum that contained lipoproteins (16). This does not appear to be true of the fibroblasts from some FH receptor-negative patients (17).

Urinary sediment is a source of exfoliated cellular elements from the kidney and urinary tract and has been used to study the excretion of GSL in a number of lipid storage disorders (18). The GSL content in the urinary sediment was studied to determine if the elevated GSL observed in the plasma lipoproteins and cultured fibroblasts of FH homozygotes might also be found in the urine. The data indicate that the urinary sediment from the majority of FH homozygotes contains increased neutral GSL, particularly LacCer, and that, further, the urinary content of GSL can be modulated with treatment by plasma exchange. A preliminary account of this work has appeared (19).

MATERIALS AND METHODS

Patient population

The five FH homozygotes studied developed planar and tendon xanthomas in the first decade and had markedly elevated plasma total and LDL cholesterol levels before treatment (**Table 1**). B.A. and T.B. were followed at the Johns Hopkins Lipid Clinic, D.D. and D.P. at the National Institutes of Health, and M.C. at the University of Texas Southwestern Medical Center. At the time the urine samples were collected, only one (M.C.) was being treated with lipid-lowering agents (cholestyramine 8 g, p.o. bid and niacin 400 mg, p.o. tid). Each FH homozygote was on a diet low in cholesterol and saturated fat and enriched in polyunsaturated fat (20). M.C. had also undergone a portacaval shunt procedure in 1974 (21). Urine from D.D. was obtained on two occasions, 14 days and 10 days after previous plasma exchange treatments, while that from T.B. was obtained before institution of plasma exchange therapy, and then 3 and 11 days, respectively, after plasma exchange. Seven normal controls of comparable ages and sexes, and one patient (I.O.) with Fabry's disease, were also studied. Each was consuming an ad lib diet. Each FH homozygote was "receptor-negative" (4), as judged by assays of LDL binding, internalization and degradation and cholesterol esterification in cultured fibroblasts.[2]

Urine specimens

Urine specimens were collected over 24 hr. All subjects were studied as outpatients except D.P., B.A., T.B., and I.O., who were studied as inpatients.

Preparation of urinary supernatants, sediments, and homogenates of the sediments

Urine specimens were centrifuged at 16,319 *g* for 30 min in an angle head rotor (GSA 5.75) at 4°C in a

[2] Fibroblasts from T.B., D.D., and B.A. were characterized in the Johns Hopkins laboratory (22, and unpublished data). Cells from D. P. were studied in the Molecular Disease Branch of the National Institutes of Health (E. Schaefer, personal communication). Fibroblasts from M. C. have been previously characterized (21). Cells from D. D. have also been studied by the Dallas group (J. Goldstein, personal communication).

Sorvall RC-2B centrifuge. The supernatants were removed by aspiration, dialyzed against water, neutralized, and freeze-dried. The pellets from the urinary sediments were pooled, washed three times with 0.9% NaCl phosphate buffered saline, pH 7.4 (140 ml), and centrifuged. The pellets were resuspended in glass-distilled water and homogenized. Aliquots of the homogenates were solubilized in 1 N NaOH and the protein concentration was determined (23). The remainder of the homogenates were freeze-dried and stored frozen.

Extraction of lipids from homogenates and supernatants of urinary sediments

Total lipids were extracted from freeze-dried homogenates of urinary sediments by vigorous homogenization and extraction with hot chloroform–methanol 2:1 (v/v), 10 ml/mg protein, in a Potter-Elvehjem homogenizer. The lipid extracts were filtered on a sintered disc funnel, and the non-lipid residue was extracted further with hot chloroform–methanol 1:2 (v/v) containing 5% H_2O (10 ml/mg protein). The lipid extracts were pooled, dried by flash evaporation, and stored in a vacuum desiccator. Water-soluble contaminants were then removed from the lipid extracts using the procedure of Folch, Lees, and Sloane Stanley (24).

Isolation of GSL

GSL were isolated from the other lipids in the lower phase of the Folch partition by silicic acid column chromatography (25). The acetone–methanol fraction containing the GSL and minor phospholipid contaminants was subjected to alkaline methanolysis, neutralized, and taken to dryness. The dried residues were solubilized in chloroform–methanol 2:1(v/v) and subjected to thin-layer chromatography (TLC) on plates of silica gel H developed in chloroform–methanol–water 100:42:6 (v/v) and dried in air. The individual GSL were identified, after TLC, with iodine vapour and the areas corresponding to standard GSL were scraped and eluted with organic solvents (25). The eluates were pooled and dried by flash evaporation.

Assessment of purity of individual GSL

Aliquots of the individual GSL were dissolved in chloroform and subjected to TLC (silica gel H). The TLC plates were developed in chloroform–methanol–water 100:42:6 (v/v), dried, and sprayed with aniline-diphenylamine reagent. The authenticity of the GSL from the urinary sediment from a FH subject (T.B.) (Table 1) was also assessed using the following TLC systems: solvent A, chloroform–methanol–water 100:42:6 (v/v) (silica gel H); solvent B, chloroform–methanol–water–ammonium hydroxide 65:25:3:1 (v/v) (silica gel G); solvent C, chloroform–methanol–water 117:40:6 (silica gel H); solvent D, chloroform–methanol–water 65:25:4 (v/v) (silica gel H); and solvent E, chloroform–methanol–water 65:24:4 (v/v) (silica gel G). The GSL standards that were used for comparison with the GSL in the urinary sediment were GlcCer, GalCer, LacCer, $GaOse_2Cer$, II SO_3 LacCer, $GbOse_3Cer$, $GbOse_4Cer$, $GbOse_5Cer$, II^3 NeuAc-LacCer (G_{M3} ganglioside), ceramides, and digalactosyldiglyceride. The thin-layer plates were dried, stained with aniline-diphenylamine reagent, and the migration of the GSL was measured.

Recovery of GSL from urinary sediments

A known amount of authentic LacCer was added to urinary sediments of known GSL composition. The recovery of LacCer was assessed by GLC (see below) at three separate steps: during extraction of lipids from urine sediments, following silicic acid column chromatography, and following elution from TLC plates. Seventy-five percent of LacCer standard was recovered from the urinary sediment, while the LacCer added to the total lipid extracts followed by silicic acid column chromatography, or after TLC, was completely recovered.

Gas–liquid chromatography (GLC) of GSL

The GSL were quantified by GLC as described previously (25) and expressed in terms of the glucose content except for GalCer. Fatty acid methyl esters and methyl sphingosines were obtained from the GSL by acid-catalyzed methanolysis (26) and were analyzed by GLC (14). Mannitol was used as an internal standard.

Plasma lipids and lipoproteins

The plasma concentrations of total cholesterol and triglycerides and of LDL cholesterol, HDL cholesterol, and very low density (prebeta) lipoprotein (VLDL) cholesterol, and urinary sediment cholesterol were determined using the methods of the Lipid Research Clinics Program (27). HDL cholesterol was determined in the supernate after the precipitation of apoB-containing lipoproteins from plasma with heparin-sulfate and manganese-chloride (27). Plasma was centrifuged without density adjustment at 10°C for 18 hr at 105,000 *g*. The top fraction ($d < 1.006$ g/ml) was removed from the bottom fraction ($d > 1.005$ g/ml) by tube slicing and VLDL cholesterol and LDL cholesterol were determined (27).

Phospholipids in urinary sediments

The total phosphorus content in the lipid extracts of the urinary sediments was determined by the method of Chen, Toribara, and Warner (28). The phospholipid content was estimated by multiplying the phosphorus content by 25.

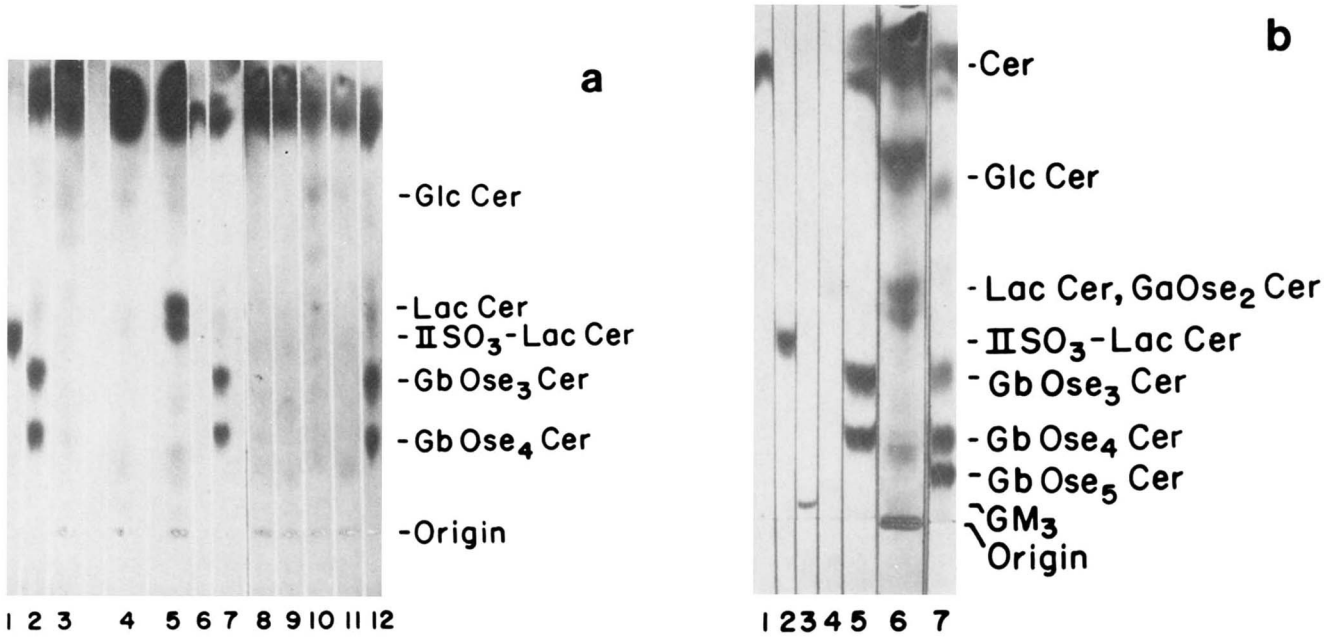

Fig. 1. Thin-layer chromatography (TLC) of glycosphingolipids (GSL) from the urinary sediments of normal and familial hypercholesterolemic (FH) subjects. a.) Twenty-four-hr urinary sediments were collected from normal subjects and a FH subject, T.B., and the GSL fraction was isolated as described in Methods. Individual GSL from urinary sediments (representing equivalent amounts of protein) and GSL standards were separated further by TLC (silica gel H) using chloroform–methanol–water 100:42:6 (v/v). The plate was then dried in air and exposed to iodine vapours. Lane 1, II SO_3-LacCer standard; Lanes 2, 7, 12, a mixture of normal human kidney neutral GSL; Lanes 3, 4, 8, 9, 10, 11, GSL from the urinary sediment of normal subjects; Lane 5, GSL in the urinary sediment of a FH homozygote (T.B.). b.) Twenty-four-hr urinary sediments were collected from a FH homozygous subject, B.A., and processed as described above and in Methods. GSL from urinary sediments and GSL standards were separated by TLC using chloroform–methanol–water 117:40:6 (v/v). In this case, the GSL had not yet been subjected to alkali-catalyzed methanolysis. The TLC plates were dried and exposed to iodine vapours. Lane 1, ceramide standard; Lane 2, II SO_3-LacCer standard; Lane 3, II^3 NeuAc-LacCer standard; Lane 4, $GaOse_2Cer$ standard; Lane 5, human kidney GSL mixture; Lane 6, GSL in urinary sediment from a FH homozygous subject (B.A.); Lane 7, horse kidney neutral GSL mixture.

RESULTS

Characterization of GSL from urinary sediments and supernates

Only trace amounts of neutral GSL were seen by TLC in normal urinary sediments (**Fig. 1a**). In contrast, two intensely stained GSL, one which co-migrated with GlcCer and GalCer and the other with LacCer, were observed in fractions from four of the five FH homozygotes (Fig. 1a, b). Consequently, spots corresponding to these GSL were scraped, eluted, and prepared for further study by TLC and GLC (see Methods). A systematic study of the other neutral GSL, $GbOse_3Cer$ and $GbOse_4Cer$, was not performed. GSL were not detected by TLC in the supernates of the urinary sediments from normal or FH homozygous subjects, indicating that most of the urinary GSL was associated with cellular elements in the sediment.

The major GSL found in the urinary sediment of one FH homozygous subject (T.B., Table 1) was isolated by TLC and found to co-migrate with human kidney LacCer from normal and with $GaOse_2Cer$ from the urinary sediment of a patient with Fabry's disease. The molar ratio of glucose, galactose, and sphingosine of this GSL was 1.00:1.47:0.89, suggesting that it was a mixture composed of LacCer and $GaOse_2Cer$. After plasma exchange therapy, the observed molar ratio (glc:gal:sphingosine) of this GSL was 1.0:1.15:0.90. The molar ratios (glc:gal:sphingosine) for this diglycosylceramide isolated from other FH subjects were, D.P. (1.00:1.35:0.9); D.D. (1.00:1.08:0.92); B.A. (1.00:1.02:0.85); and M.C. (1.00:0.95:0.90), respectively. The other major GSL in the urinary sediment of FH patients was a monoglycosylceramide. We did not separate GlcCer from GalCer; instead, since the GlcCer and GalCer standards both co-migrated with the urinary monoglycosylceramide by TLC, the individual contributions of the sugar moieties, Glc and Gal, were considered as if they were derived from either GlcCer or GalCer, respectively.

Fatty acids of LacCer derived from urinary sediments of a normal and FH homozygous subject

The fatty acid compositions of the major urinary diglycosylceramide in a homozygous FH subject (T.B., Table 1) and in a normal subject were similar (**Table**

TABLE 2. Fatty acid composition of diglycosylceramide in the urinary sediment of a normal human subject and a familial hypercholesterolemic homozygous subject

Fatty Acid	Normal	Homozygote
	% of total	
16:0	5.71	2.6
18:0	12.29	2.4
20:0	7.62	6.7
21:0	0.40	0.3
22:0	30.0	35.8
22:1	2.6	1.8
23:0	2.5	4.7
24:0	29.4	33.5
24:1	9.48	12.2

Fatty acids are presented as chain length:number of double bonds. The relative amount of each fatty acid is given as a percentage of the total area found on gas–liquid chromatograms. Fatty acids of diglycosylceramide were derived from one normal subject (D.O.) and a familial hypercholesterolemic homozygous subject (T.B.). The results are an average of duplicate analyses.

2). The major fatty acids were C22:0 and C24:0 (Table 2).

Characterization of urinary sediments from normal subjects

The total volume (ml), protein (mg), cholesterol (μg), and the content of the neutral GSL, GlcCer, GalCer, and LacCer in the urinary sediments from the 24-hr samples of seven normal subjects are summarized in **Table 3**. The urine volume ranged from 450 to 1,230 ml, the protein content from 6.1 to 31.0 mg and the cholesterol content from 4 to 14 μg. Urinary sediment from normal females contained a higher mean content of protein (28.4 mg) than that from normal males (9.4 mg). The total volume, however, was somewhat higher in the males (827 ml) than in the females (696 ml). This suggested that the urinary sediment of females contained a greater amount of cellular material than that of males. The content of the GSL (nmol/24 hr) ranged from 7.0 to 50.0 for GlcCer, 5.0 to 15.0 for GalCer, and 3.2 to 19.2 for LacCer (Table 3). The mean contents (nmol/24 hr) of GlcCer and LacCer were higher in the females (35.8 and 12.8) than in the males (23.0 and 5.4), while the mean levels of GalCer (9.5 and 10.0, respectively) were similar. The mean cholesterol contents, 10.8 and 10.5 μg, were also similar. The GSL data were therefore expressed as nmol/mg protein and the data for males and females were compared. There were no significant differences in the mean levels (nmol/mg protein) of GlcCer (2.9 for males versus 1.3 for females, $t=1.28$, $P = 0.26$), GalCer (1.3 for males versus 0.4 for females, $t = 1.57$, $P = 0.26$) or LacCer (0.6 for males versus 0.5 for females, $t = 0.73$, $P = 0.50$). Therefore, for the purposes of Tables 5 and 6, the data from the normal males and females were combined.

Characterization of 24-hr urinary sediments from homozygous FH subjects

In the five FH homozygous patients, the volume of the urine varied from 500 to 2,500 ml, the protein content from 3.1 to 32.8 mg, and the cholesterol content from 2.0 to 35.0 μg (**Table 4**). The neutral GSL content (nmol/24 hr) varied from 18.1 to 242.4 for GlcCer, 0.7 to 37.6 for GalCer, and from 8.3 to 247.5 for LacCer. The total content of these three GSL varied from 27.1 to 527.5 (Table 4).

Comparison of content of GSL in the urinary sediments from normal and FH subjects

The content of GSL in the urinary sediments from 24-hr urine collections, expressed as nmol/24 hr, has been used previously to compare normals with subjects with lipid storage diseases (18, 29–33). Using this ap-

TABLE 3. Characterization of 24-hr urine sediments from normal subjects

			24-hr Urine			Neutral Glycosphingolipid Content			
Subject	Sex	Age	Volume	Protein	Cholesterol	GlcCer	GalCer	LacCer	Total
		yr	*ml*	*mg*	*μg*	*nmol/24 hr*			
CO	F	11	755	30.2	4	7.0	5.0	16.0	28.0
DO	F	13	770	23.0	13	50.0	10.0	12.0	72.0
GE	M	11	450	10.1	9	14.0	7.0	7.0	28.0
PK	M	11	800	12.0	N.D.	20.0	8.0	6.0	34.0
TL	M	11	1,230	6.1	12	35.0	15.0	3.2	53.2
CS	F	32	520	29.4	12	44.0	8.0	19.2	71.2
SB	F	32	740	31.0	14	42.0	15.0	4.0	61.0

In this and subsequent tables, 24-hr urinary sediments were collected from normal human subjects and the GSL were isolated and separated by preparative thin-layer chromatography as described in Methods. The individual GSL were next subjected to alkali-catalyzed methanolysis and neutralization, and dried under nitrogen. The dry residues were resuspended in 0.75 N methanolic HCl. Mannitol was added as an internal standard. Following acid-catalyzed methanolysis, the methylglycosides were TMSi derivatized and quantified by gas–liquid chromatographic procedures described in Methods.

TABLE 4. Characterization of 24-hr urine sediments from homozygous familial hypercholesterolemic subjects

Subject	Sex	Age	Twenty-Four Hour Urine			Neutral Glycosphingolipid Content			
			Volume	Protein	Cholesterol	GlcCer	GalCer	LacCer	Total
		yr	*ml*	*mg*	*μg*	*nmol/24 hr*			
DP	M	5	500	12.5	2.0	31.6	2.8	18.9	53.3
DD[a]	F	42	2,500	32.8	35.0	242.4	37.6	247.5	527.5
BA	F	15	800	16.1	5.0	100.8	19.5	42.7	163.0
MC	F	15	800	28.0	27.0	18.1	0.7	8.3	27.1
TB	F	11	800	3.1	9.0	31.5	16.0	85.7	133.2

[a] A repeat sample on this patient gave the following results: volume (1,275 ml); protein (22.0 mg); cholesterol (20.0 μg); GlcCer, GalCer and LacCer (nmol/24 hr) of 150.3, 47.0, and 178.3, respectively.

proach, the mean levels (nmol/24 hr) of GlcCer, GalCer, and LacCer were 2.8-, 1.6-, and 8.4-fold higher in the FH homozygotes than in the normals (Tables 3 and 4). Two of the homozygotes (D.D., B.A) had elevated GlcCer's and three (D.D., B.A. and T.B.) had increases in both GalCer and LacCer. The total amounts of these GSL (nmol/24 hr) in three FH homozygotes (D.D., B.A., T.B.) were considerably above the normal range of 28.0 to 71.2. One homozygote (M.C.) had a total of these three GSL that was below the normal range, while the total GSL in the other, D.P., was within the normal range.

Due to the skewness of the data on GSL in the urinary sediment of the FH homozygotes (Table 4), the contents of the individual and total GSL in the normals (Table 3) were compared with those in FH using a non-parametric test of significance. Using a Mann-Whitney U test (34), the data were ranked and analyzed for each variable in Tables 3 and 4. The differences between normals and FH were statistically significant (P value = 0.015) for LacCer only.

Because of the individual differences in the urine volume and protein in the urinary sediments, GSL content in the 24-hr urinary sediments of the two groups was next compared, using the normal range of nmol of GSL/mg protein as the criterion of abnormality. This approach takes into consideration differences in the amount of cellular material in the 24-hr urinary specimens. The mean content (nmol/mg protein) of GlcCer, GalCer, and LacCer in the FH homozygotes' urinary sediment was 2.7, 1.9, and 15.8 times higher, respectively, than that in the normals (**Table 5**). Three of the five FH homozygotes (D.D., B.A., T.B.) had values of GlcCer outside the normal range, while only one (T.B.) had a value of GalCer above the normal range. Of the five homozygotes, only one (M.C.) had a value of LacCer (0.3 nmol/mg protein) within the normal range (Table 5); the other four FH homozygotes had values of LacCer that were 3- to 55-fold higher than the mean of the normal controls. Thus, the elevation of LacCer in urinary sediment was the most striking and consistent finding in these FH homozygotes. The mean total content of the urinary sediment GSL of the FH homozygotes was 4.8-fold higher than that of the normal controls. The amount of $GaOse_2Cer$ in the urinary sediment of the patient with Fabry's disease was 388.7 nmol glucose/24 hr, a level that was 40-fold higher than the mean content of LacCer in normals (Table 3); the content of $GbOse_3Cer$ in the sediment of this patient was 550 nmol glucose/24 hr.

Comparison of content of cholesterol and GSL in the urinary sediments from normal and FH subjects

The mean and range of the cholesterol content (μg/ml protein) in the urinary sediment of the two groups were similar (**Table 6**). The mean GSL content of GlcCer, GalCer, and LacCer (nmol GSL/μg cholesterol) was increased 3.4-, 1.6-, and 5.4-fold, respectively, in the urinary sediment of the FH homozygotes (Table 6). Only

TABLE 5. Comparison of the glycosphingolipid content in the 24-hr urine sediment of normal and homozygous FH subjects

	GlcCer	GalCer	LacCer	Total
	nmol/mg protein			
Homozygous familial hypercholesterolemic subjects (N = 5)	5.4 (3.9) (0.6 to 10.2)	1.5 (2.1) (0.03 to 5.2)	7.9 (11.3) (0.3 to 27.6)	14.4 (1.0 to 43.0)
Normals (N = 7)	2.0 (1.7) (0.2 to 5.7)	0.8 (0.8) (0.2 to 2.5)	0.5 (0.2) (0.1 to 0.7)	3.2 (0.9 to 8.7)

TABLE 6. Comparison of the cholesterol content and the glycosphingolipid content per μg cholesterol in the 24-hr urine sediment of normal and homozygous FH subjects

	Cholesterol	Glycosphingolipids:Cholesterol			
		GlcCer	GalCer	LacCer	Total
	μg/mg protein	*nmol/μg cholesterol*			
Homozygous familial hypercholesterolemic subjects (N = 5)	1.1 (1.1) (0.2 to 2.9)	9.4 (8.3) (0.7 to 20.2)	1.6 (1.4) (0.03 to 3.9)	7.0 (3.8) (0.3 to 9.5)	18.0 (1.0 to 32.6)
Normals (N = 6)	0.8 (0.7) (0.1 to 2.0)	2.8 (0.9) (1.6 to 3.8)	1.0 (0.3) (0.7 to 1.3)	1.3 (1.4) (0.3 to 4.0)	5.1 (3.12 to 7.00)

Urinary sediments from 24-hr urine were collected from FH homozygous subjects and processed, and the GSL content of GalCer, GlcCer, and LacCer were determined as described in Methods. The total lipid extract was applied to silicic acid columns and the neutral lipid fraction was eluted with chloroform. The neutral lipid fraction was dried by flash evaporation and the total cholesterol content was measured as described in Methods.

one homozygote (M.C.) had a value of LacCer (0.3 nmol/μg cholesterol) within the normal range, and the other four FH homozygotes had values that were 5.5- to 7.3-fold higher than the mean of the normals. The mean total content of these three GSL (nmol/μg cholesterol) was 3.5-fold higher than that of the normal controls (Table 6).

Comparison of content of total phospholipid and GSL in the urinary sediments from normal and FH subjects

The phospholipid contents (μg/24 hr) in the urinary sediment of normal and FH homozygote subjects were also similar (**Table 7**). Among normals there was a 3-fold (0.1–0.34 nmol GSL/μg phospholipid) variation in the amount of GSL excreted, while the FH homozygotes had almost a 10-fold variation (0.19–2.37 nmol GSL/μg phospholipid) in GSL excretion. One FH homozygote, M.C., excreted 0.19 nmol GSL/μg phospholipid, a level within the normal range. The mean GSL content of the urinary cells, expressed per μg phospholipid, was almost 6-fold higher in the FH homozygous group than in normals.

Effect of plasma exchange on urinary LacCer levels in FH

The content of LacCer in the urinary sediments of one patient (T.B.) was measured before and after plasma exchange (Tables 1 and 8). Three days after plasma exchange the content of urinary LacCer fell to within the normal range (**Table 8**). This was accompanied by a significant fall in the plasma total and LDL cholesterol levels (Table 1) and by a 57% fall in the plasma total LacCer level (nmol/100 ml plasma) from 1,491 to 852. By 11 days after plasma exchange, the content of LacCer had increased to well outside the normal range, without any substantial change in the protein or cholesterol content in the sediment (Table 8). The urinary GSL of one other patient (D.D.) were measured 2 weeks and 10 days after her previous plasma exchanges (Table 4), and these values were well outside the normal range at that time.

DISCUSSION

GLC was used to identify and measure the major neutral GSL in the urinary sediments from 24-hr sam-

TABLE 7. Comparison of the total phospholipid content and the glycosphingolipid content per μg phospholipid in the 24-hr urine sediment of normal and homozygous FH subjects.

Subject	Total Phospholipid	nmol GSL/μg phospholipid
	μg/24 hr	
Normal		
CO	280	0.10
DO	300	0.24
GE	180	0.16
PK	200	0.17
TL	158	0.34
Mean	224	0.20
FH Homozygotes		
DD[a]	223	2.37
DD[b]	218	1.73
MC	140	0.19
TB	300	0.44
Mean	200	1.18

[a] First sample of 24-hr urine.
[b] Second sample of 24-hr urine.

Urinary sediments from 24-hr urine were collected and processed as described in Methods. Following extraction of lipids and a partition after Folch et al. (24), the neutral lipid, glycolipid, and phospholipid fractions were isolated from the lower phase by silicic acid column chromatography, as described in Methods. The GSL content of GalCer, GlcCer, and LacCer were then determined as described in Methods. The total phospholipid fraction was dried by flash evaporation and the total phosphorus content was measured (28). The phospholipid content was obtained by multiplying the phosphorus value by 25.

TABLE 8. Effect of plasma exchange on the levels of lactosylceramide, protein, and cholesterol in the 24-hr urine sediments of a familial hypercholesterolemic homozygous subject (T.B.)

	LacCer	Protein	LacCer	Cholesterol	LacCer
	nmol/24 hr	*mg/24 hr*	*nmol/mg protein*	*μg/24 hr*	*nmol/μg cholesterol*
Before plasma exchange	85.7	3.1	27.7	9.0	9.5
Three days after plasma exchange	6.6	10.1	0.7	4.6	1.4
Ten days after plasma exchange	59.3	14.0	4.2	6.0	9.9
Normal range	3.2 to 19.2	6.1 to 31.0	0.1 to 0.7	4 to 14	0.3 to 4.0

Twenty-four hour urine samples were collected from a FH homozygous subject, T.B., before plasma exchange, 3 days after plasma exchange, and 10 days after plasma exchange. The urinary sediments were prepared and quantitative analyses of LacCer, protein, and cholesterol were carried out as described in Methods.

ples of five receptor-negative FH homozygotes and seven controls. In agreement with others (18, 29–33, 35), we found that GlcCer was the major GSL in normal urinary sediment. The levels of GlcCer and GalCer in our normal subjects agreed closely with those of others (18, 29, 33); our normal values of LacCer were similar to one (32), but lower than those of another study (18). In agreement with Desnick, Sweeley, and Krivit (33), we found higher GSL levels in urinary sediments from normal females than males. These differences were presumably due to a greater amount of cellular exfoliation in females than in males; our data on the GSL content in normal urinary sediment extends those of others, since we corrected for differences in the amount of cellular material present in the sediment by determining its content of protein, cholesterol and phospholipid.

The most significant finding of this study was the considerable elevation (expressed as nmol/mg protein, nmol/μg cholesterol, or nmol/μg phospholipid) of GlcCer and LacCer in the urinary sediments from most of the receptor-negative homozygous FH patients studied. The greatest increase occurred in LacCer, a consistent finding with the exception of one FH homozygote (M.C.) (6, 21). This patient differed from the other five in that she was the only one who was receiving lipid-lowering medication and who had undergone a portacaval shunt. Either one, or both, of these therapeutic measures might alter the GSL content of the urinary sediment. Cholesterol-lowering therapy in one of our patients (T.B.) by plasma exchange did transiently lower the LacCer content of the urinary sediment.

Urinary sediment contains cellular elements derived from kidney cells, epithelial cells from urinary tract, and, to a lesser extent, erythrocytes and leukocytes (18, 29–33, 35). The kind and amount of GSL vary in different cells. For example, in renal cells, $GbOse_3Cer$ and $GbOse_4Cer$ are the major GSL while LacCer is the major GSL of leukocytes. The finding that the major GSL in normal urinary sediment was GlcCer, with lesser amounts of LacCer, is not incompatible with a significant proportion of the cells being renal in origin. Apparently, catabolism of the complex GSL occurs and there is a decreasing order of GSL from the more complex to the simpler GSL in normal urine (18). Further, kidney is the only visceral organ that contains appreciable amounts of GalCer (36). In normal urinary sediments, ratios of GlcCer to GalCer of 1.4 to 1.0, 3.4 to 1.0, and 5.0 to 1.0 have been found previously (18, 35, 36); the average ratio in our normals was 3.1 to 1.0. This observation, combined with the fact that the fatty acid pattern of LacCer resembled that of normal kidney (36), indicates that a substantial proportion of the urinary sediment was derived from renal cells. This tenet was confirmed by examining the cellular elements in the urine from three FH homozygotes (T.B., B.A., and D.D.) who had high urinary GSL levels. Proximal tubular cells, transitional kidney epithelial cells, and a few neutrophils and leukocytes were found by light microscopy. GSL from erythrocytes and kidney are enriched in $GbOse_4Cer$. Our failure to find significant amounts of $GbOse_4Cer$ in the urinary GSL indicates that few erythrocytes were present in the urinary sediment and this was confirmed by light microscopy. We have been able to find appreciable levels of $GbOse_3Cer$ (80.5 nmol glucose/24 hr urine) in the urinary cells of one FH homozygous subject, D.D. Our data, however, do not exclude the possibility of some contribution of neutrophils (37–40) (or other white blood cell types) to the urinary sediment GSL. In agreement with Desnick et al. (18, 33), loss of GSL from the sediment into the urinary supernatant by lysis of cellular elements was excluded, since only trace amounts of GSL were found in the supernatants. This latter finding also suggests that the HDL previously found in urine by Segal et al. (41) did not contribute significantly to the urinary GSL.

Other factors may contribute to variations in the levels of GSL in the urinary sediment. These include differences in renal function, site and amount of renal desquamation, urine volume, and diet (18, 33). These factors were controlled, in part, by expressing the data in terms of the protein, cholesterol, or phospholipid content of the urinary sediment. All the FH patients were on similar

diets, low in cholesterol and saturated fats and enriched in polyunsaturated fats. The control subjects were on ad lib diets. We have therefore not excluded the possibility that the therapeutic diet in the homozygotes was associated with increased GSL excretion. We think this possibility unlikely, however, especially in view of the change in urinary LacCer content following plasma exchange therapy in one of the homozygotes (T.B., Table 8). Significant proteinuria (35) and urinary tract infections (31, 35) can produce increases in urinary GSL in both the supernatant and urinary sediment. None of our patients had proteinuria or urinary tract infections. Tests of renal function (plasma creatinine, blood urea nitrogen, and complete urine analysis) were normal in each FH homozygote studied.

Increased GSL in urinary sediments have been found in a number of lipid storage disorders (18) and in G_{M1} gangliosidosis (42). Thus, the increased excretion of GSL in receptor-negative FH homozygotes is not specific for FH. LacCer was the major GSL that was increased, a finding similar to that in G_{M1}, gangliosidosis and "lactosyl ceramidosis," a lipid storage disease variant of Niemann-Pick disease, due to sphingomyelinase deficiency (43, 44).

Plasma exchange therapy of FH subjects decreases plasma total and LDL cholesterol levels (45, 46). We found that a decrease in the plasma levels of LDL cholesterol (2.55-fold) and LacCer (1.75-fold) was accompanied by a substantial decrease (12.98-fold) in the urinary LacCer level into the normal range (Tables 1, 8). The decrement in the urinary LacCer level cannot be solely ascribed to a fall in the LDL cholesterol concentration, since the levels of other lipoproteins, such as HDL, and of the plasma proteins are also lowered during plasma exchange.

The relation between our observations of elevated GSL in cultured fibroblasts (16), plasma LDL (14), and urinary renal cells from FH homozygotes is not known. Further, these elevations in GSL have not been observed in all FH homozygotes (17) nor in each "receptor-negative" patient studied here (M.C., Table 1). A systematic study of FH heterozygotes to determine if they have a less pronounced urinary GSL elevation has not been performed, although both parents of T.B. (Table 1) had urinary GSL values that fell within the normal range (data not shown). While the fundamental genetic defect(s) in FH clearly involve an absent or faulty LDL receptor (4), this abnormality is associated with a number of metabolic derangements that occur as a consequence of the defect. A functional LDL receptor may be required for the normal regulation of GSL metabolism in renal cells, or alternatively, the elevated plasma LDL molecules may enter the cells through a receptor independent pathway (47). Further studies may elucidate the etiology of the storage of LacCer in the kidney cells of these patients and of the possible role of the LDL receptor in GSL metabolism.

We thank Dr. David Bilheimer for the urine sample from M.C., Dr. Richard Gregg and Dr. Ernest Schaefer for the urine samples from patients D.P. and D.D., and Dr. Hugo Moser for the urine specimens from patient I.O. Dr. Prabodh Gupta provided assistance with the cytological examination of the urine. The expert technical assistance of Ms. Hazel Smith and Ms. Carol McGeeney's efforts in typing this manuscript are gratefully acknowledged. Supported in part by a contract, NO1-HV1-2158L with the National Heart, Lung, and Blood Institute and by grant GRC-RR-52 from the General Clinical Research Center, Program of the Division of Research Resources, National Institutes of Health.

Manuscript received 27 May 1981, in revised form 9 November 1981, and in re-revised form 4 January 1982.

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