A method for the quantitative determination of neutral glycosphingolipids in urine sediment

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ABSTRACT A method is described for the isolation and quantitation of six neutral glycosyl ceramides from human urinary sediment. Total lipids were extracted from sediments of 24-hr urine collections, and the glycosyl ceramides were isolated by silicic acid column chromatography followed by thin-layer chromatography. Methanolysis of the individual glycosyl ceramides yielded methyl glycosides which were quantitated as the trimethylsilyl ethers by gas–liquid chromatography.

By this technique, the submicromolar concentrations of six glycosyl ceramides in normal subjects and in individuals with Fabry's disease, an hereditary glycosphingolipid storage disease, were determined. Trihexosyl ceramide (galactosyl-galactosylglucosyl ceramide) and a digalactosyl ceramide accumulated in the urinary sediment of patients with Fabry's disease.

SUPPLEMENTARY KEY WORDS: neutral glycosyl ceramides · thin-layer chromatography · gas–liquid chromatography · trimethylsilyl methyl glycosides · Fabry's disease · trihexosyl ceramide · digalactosyl ceramide

SIBLEY (1) in 1918 first described vacuolated cells in the urine sediment of a patient with Fabry's disease. Morphological and histochemical changes have been observed in the urinary sediment of individuals with other metabolic lipidoses (2–5), degenerative neurological diseases (5), as well as disorders affecting the kidney (6–8).

The cellular elements of the urinary sediment contain the desquamated glomerular and tubular epithelial cells and casts. These cellular elements in the sediment are an indication of kidney pathophysiology and represent an easily obtainable source of abnormally deposited lipids for repeated determinations without trauma to the subject.

Since the measurement of lipids in urinary sediment might be of diagnostic value in the detection of individuals with Fabry's disease and in the identification of variants and heterozygotes with defects in lipid metabolism, an investigation was undertaken to devise a scheme for the identification and quantitation of glycosyl ceramides in urinary sediment. The present paper describes a procedure for the isolation and quantitation of the neutral glycosyl ceramides of urinary sediment of normal subjects and individuals with Fabry's disease. The data presented in a preliminary report (9) are similar to the qualitative findings on urinary sediment glycolipids reported by Kremer and Denk (10) and Philippart, Sarlieve, and Manacorda (11).

MATERIALS AND METHODS

Sediment Collection

Uncatheterized 24-hr urine samples were collected in iced, preservative-free containers and adjusted to pH 6.5–7.0. Urines were tested for the presence of protein, glucose, ketones, and blood using Ames Labstix (Ames Co., Inc., Elkhart, Ind.), and aliquots were taken for creatinine determinations (12). Urinary sediment was collected by centrifugation at 15,000 *g* for 30 min at 4°C in the GSA head of a Sorvall RC2-B centrifuge.

This paper is Michigan Agricultural Experiment Station Journal No. 4755.

Abbreviations: C–M, chloroform–methanol; TLC, thin-layer chromatography; GLC, gas–liquid chromatography; TMSi, trimethylsilyl; gal, galactose; glu, glucose.

The pellets were resuspended in 0.9% NaCl and recentrifuged. The washed pellets were suspended in a total volume of 100 ml of methanol, and enzymes were inactivated by addition of 10 ml of chloroform. The suspensions were stored at −20°C in a 250 ml Erlenmeyer flask under nitrogen until extraction. The supernatant urine was placed in cellulose dialysis tubing (Union Carbide Co., Chicago, Ill.) which had been prewashed in chloroform–methanol (C–M) 2:1 and was dialyzed against polyethylene glycol ("Carbowax," Union Carbide Corporation, New York) to dryness.

Extraction of Sediment Lipids

Chloroform was added to the suspended sediment material to give a final mixture of 200 ml of C–M 1:1. The mixture was stirred with a magnetic bar for 15 min at room temperature and filtered by suction through a medium-frit glass sintered filter into a round-bottomed flask. The residue was suspended and extracted twice again, first with 200 ml of C–M 2:1 and then, after filtration as above, with 100 ml of C–M 7:1 saturated with ammonium hydroxide. The combined extracts were evaporated to near dryness, and the damp residue was extracted from the flask successively with three aliquots of chloroform (total volume 100 ml), two aliquots of methanol (total volume 50 ml), and two aliquots of C–M 2:1 (total volume 50 ml). The combined fractions were filtered through sintered glass into a separatory funnel, and 40 ml of 0.1 M KCl were added. The mixture was shaken and left to stand in the cold (4°C) overnight. The lower phase was removed and evaporated to dryness; the residue of crude lipids was dissolved in 10 ml of C–M 2:1.

The method described was found to be optimal for the extraction of urinary sediment lipids, and further extractions of the residue failed to yield detectable lipid.

Isolation and GLC Quantitation of the Neutral Glycolipids

Chromatography on silicic acid columns separated the total crude lipids into glycolipid, phospholipid, and neutral lipid classes. The six neutral glycosyl ceramides were contained in the glycolipid fraction, which was eluted with acetone–methanol 9:1 (13). No glycosyl ceramides were eluted with the phospholipid or neutral lipid fractions as determined by TLC.

Mild alkali-catalyzed methanolysis was done according to the methods of Vance and Sweeley (13). One-dimensional preparative TLC (14) was carried out using Silica Gel H (Brinkmann Instruments Inc., Westbury, N.Y.). After visualization with iodine vapor, the individual components were eluted as previously described (13). Glycosyl ceramides prepared from red blood cells (13) were cochromatographed as comparison standards. Qualitative TLC analysis, when carried out, was accomplished by streaking 10–25% of the glycolipid fraction from the silicic acid column on a 0.25 mm TLC plate. Glycolipid components were detected by using a 0.2% orcinol spray (15).

Methanolysis of the individual glycolipids and subsequent GLC of the TMSi methyl glycosides were carried out as previously described (13) with the following modifications of Clamp, Dawson, and Hough (16). After the fatty acid methyl esters were extracted with hexane, the HCl was neutralized by the addition of Ag_2CO_3 (ca. 0.5 g) (Mallinckrodt Chemical Co., St. Louis, Mo.). The suspension was shaken at intervals during a period of at least 30 min and then centrifuged at 2000 rpm for 10 min. The supernatant solution containing the methyl glycosides and sphingosine bases was transferred to a Teflon-capped vial (4 ml) and evaporated to dryness under nitrogen. After trimethylsilation of the dry residue with 100 μl of silylating reagent (pyridine – hexamethyldisilazane – trimethylchlorosilane 5:2:1), the TMSi derivatives were injected onto a glass U-shaped column (1.83 m × 0.6 cm) containing 3% SE-30 on 100–120 mesh Supelcoport (Supelco, Inc., Bellefonte, Pa.) maintained isothermally at 160°C. A Barber Colman Model 5000 gas chromatograph (Barber Colman Company, Rockford, Ill.) equipped with a flame ionization detector was used for the analyses. The concentrations of the six neutral glycosyl ceramides were calculated from the observed molar yield of glucose (relative to the internal standard D-mannitol) without correction for losses in isolation. A factor of 1.25 was necessary to correct for the molar difference between total TMSi methyl glucoside and TMSi mannitol as previously reported (13).

RESULTS

Separation of Urinary Sediment Lipids and Isolation of Glycosyl Ceramides

Table 1 shows the concentration of total lipid and the relative proportions of glycolipid, phospholipid, and neutral lipid in urinary sediments from normal subjects and patients with Fabry's disease. These results indicate significant increases in both total fractionated lipids ($P < 0.005$) and glycolipids ($P < 0.01$) in sediment from patients with Fabry's disease.

The individual glycosyl ceramides separated well on TLC, as shown in Fig. 1. Mild alkali-catalyzed methanolysis was effective in removing contaminants prior to this step. No sulfatides were detected by TLC with comparison standards.

To detect the presence of any glycolipid in the urinary supernatant, a supernatant from a patient with Fabry's

TABLE 1 Analysis of Urinary Sediment Lipids from Normal Individuals and From Patients with Fabry's Disease

Donor	Sex	Age	Total Fractionated Lipid		Neutral Glycolipid	Phospholipid	Neutral Lipid
			mg/24 hr sample	*mg/g creatinine*	%	%	%
Normal							
MN	M	35	1.19	0.89	36.1	33.9	29.9
RD	M	25	0.82	0.47	58.6	20.5	20.9
RE	M	40	2.38	1.40	37.6	35.3	26.9
WK	M	40	1.41	0.76	24.4	37.0	38.6
Mean ± MEM			1.39 ± 0.44	0.88 ± 0.26	39.2 ± 9.7	31.7 ± 5.3	29.1 ± 5.2
Fabry's disease							
CL	M	12	3.18	4.32	30.3	22.6	47.1
DL	M	15	6.19	3.35	49.1	17.1	33.9
AG	M	28	3.93	4.42	62.6	18.0	19.4
CB	M	54	11.35	6.47	44.3	15.5	40.3
Mean ± MEM			6.16 ± 2.61	4.64 ± 0.92	46.6 ± 9.3	18.3 ± 2.1	35.2 ± 8.6

TABLE 2 Precision in Glycolipid Analyses of Human Urinary Sediments*

Sample	Aliquot	Galactosyl Ceramide	Glucosyl Ceramide	Digalactosyl Ceramide	Lactosyl Ceramide	Trihexosyl Ceramide	Tetrahexosyl Ceramide
				μmoles/aliquot			
A	1	0.009	0.020	0.138	0.055	1.04	0.056
	2	0.014	0.013	0.149	0.052	1.17	0.116
Mean		0.012	0.016	0.144	0.054	1.10	0.086
B	1	0.015	0.196	0.159	0.133	1.89	0.091
	2	0.048	0.198	0.165	0.112	2.03	0.116
Mean		0.032	0.197	0.163	0.122	1.96	0.104

* Duplicate analyses of equal aliquots from two 24-hr urine samples from one patient with Fabry's disease.

disease was dialyzed and the residue was extracted as described above. The absence of orcinol positive components (TLC) in aliquots of the silicic acid column fractions indicated that the urine supernatant did not contain detectable glycolipids.

Precision and Recovery of the Urinary Sediment Glycolipid Analyses

Two 24-hr urine samples from a patient with Fabry's disease were each divided into equal aliquots before sediment collection, and the concentrations of the glycosyl ceramides from each fraction were determined. These data (Table 2) show the quantitative reliability of the method.

To evaluate the recovery of the glycosyl ceramides from urinary sediment, known amounts of digalactosyl ceramide, trihexosyl ceramide, and a tetrahexosyl ceramide were added to two equal aliquots of a washed sediment (Fabry's disease) that had been previously analyzed for endogenous glycolipids. The results (Table 3) indicate that over 90% of the dihexosyl, trihexosyl, and tetrahexosyl ceramides were recovered.

TABLE 3 Recovery of Added Glycolipids from Human Urinary Sediment

	Digalactosyl Ceramide	Trihexosyl Ceramide	Tetrahexosyl Ceramide
		μmoles	
Observed total glycolipid	0.162	1.890	0.116
Endogenous glycolipid	0.102	1.646	0.095
Added glycolipid recovered	0.060	0.254	0.021
Amount of added glycolipid	0.065	0.272	0.023
% Recovery	92.3	93.4	91.1

Known quantities of digalactosyl ceramide, trihexosyl ceramide (gal–glu 2:1), and urinary sediment tetrahexoside were added to two equal aliquots of urine from a patient with Fabry's disease. Endogenous glycolipids were determined on another aliquot of the same urine.

Analyses of Normal Human and Fabry Urinary Sediment Glycolipids

The concentrations of neutral glycosyl ceramides from normal urinary sediment are given in Table 4. Although the concentrations of neutral glycosyl ceramides in normal urinary sediment were extremely low, the sensitivity of the GLC technique permitted accurate analyses

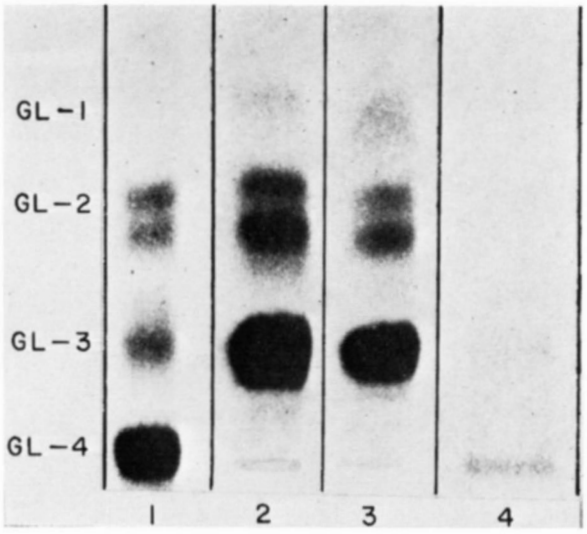

FIG. 1. TLC of urinary sediment glycolipids and red blood cell glycolipid standard. *1*, Red blood cell glycolipids; *2*, sediment glycolipids of M. B., Fabry hemizygote, 47 yr; *3*, sediment glycolipids of C. L., Fabry hemizygote, 12 yr; *4*, sediment glycolipids from a normal male, 40 yr. 25% of each glycolipid fraction from a a 24 hr urine was plated. Developing solvents: C–M–H_2O 100 : 42:6; spray, 0.2% orcinol in H_2SO_4, charred at 100°C. GL-1, cerebrosides; GL-2, dihexosyl ceramides; GL-3, trihexosyl ceramide; GL-4, tetrahexosyl ceramides.

of sediments from 24-hr urine samples from individual donors.

Two dihexosides, digalactosyl ceramide and lactosyl ceramide, were detected. The latter dihexoside was predominant, especially in the urinary sediment of females. Small amounts of glucosyl ceramide, galactosyl ceramide, trihexosyl ceramide, and a tetrahexosyl ceramide (assumed but not proven to be globoside) were also detected in normal urinary sediment.

The concentrations of these glycolipids in subjects with Fabry's disease were considerably different from those of normal individuals (Table 5). Trihexosyl ceramide, which accumulates in body tissues (17, 18) and plasma (19) of patients with Fabry's disease, was the predominant glycolipid. Two dihexosyl ceramides, digalactosyl and lactosyl ceramides, were identified, but, in contrast to normal sediment, the digalactosyl ceramide predominated. The concentrations of tetrahexosyl ceramide and two cerebrosides, glucosyl and galactosyl ceramides, were also elevated. Although galactosyl ceramide was a very minor component in normal sediment, in some of the Fabry sediments the concentrations of galactosyl ceramide and glucosyl ceramide were nearly equal.

TABLE 4 CONCENTRATIONS OF GLYCOSYL CERAMIDES IN NORMAL HUMAN URINARY SEDIMENT

Donor	Sex	Age	Galactosyl Ceramide	Glucosyl Ceramide	Digalactosyl Ceramide	Lactosyl Ceramide	Trihexosyl Ceramide	Tetrahexosyl Ceramide
				μmoles/24 hr urine				
S.T.	M	2	tr	0.022	—	0.018	0.017	0.018
R. D.	M	25	0.022	0.026	—	0.031	0.027	0.016
L. K.	F	19	0.020	0.087	—	0.058	0.028	0.024
K. W.	F	23	tr	0.010	—	0.068	0.026	0.028
E. R.	M	40	0.014	0.054	tr	0.039	0.028	0.015
Mean				0.040		0.044	0.025	0.020

TABLE 5 CONCENTRATIONS OF GLYCOSYL CERAMIDES IN URINARY SEDIMENT FROM PATIENTS WITH FABRY'S DISEASE

Donor	Sex	Age	Galactosyl Ceramide	Glucosyl Ceramide	Digalactosyl Ceramide	Lactosyl Ceramide	Trihexosyl Ceramide	Tetrahexosyl Ceramide
				μmoles/24 hr urine				
R. L.*	M	7	0.035	0.029	0.067	0.019	0.34	0.039
Ro. L.*	M	9	0.019	0.018	0.089	0.050	0.37	0.031
C. L.*	M	12	0.098	0.16	0.86	0.24	2.58	0.64
D. L.*	M	15	0.019	0.072	0.20	0.031	1.35	0.039
R. S.	M	18	0.033	0.062	0.39	0.12	3.76	0.38
A. G.	M	29	0.017	0.039	0.080	0.038	0.87	0.096
M. B.†	M	45	tr	0.043	0.038	0.031	0.24	0.080
R. B.†	M	55	0.067	0.096	0.27	0.085	1.16	0.059
C. B.	M	56	0.071	0.074	0.47	0.40	4.91	0.35
W. R.‡	M		0.014	0.048	0.007	0.031	0.12	0.048
Normal male mean (3)			tr	0.034	tr	0.029	0.024	0.016
Normal mean (5)			tr	0.040	tr	0.058	0.025	0.020

*, † Siblings of respective families.
‡ Fabry variant having no skin manifestations.

DISCUSSION

Glycosyl ceramides have been isolated previously from normal human kidney (20, 21), spleen (22–24), liver (22), brain (25), placenta (26), eye lens (27), cultured skin fibroblasts (28), aorta (29), bone marrow (30), serum (22), leukocytes (31), erythrocytes (13), and plasma (13). In 1960, Hagberg and Svennerholm (32) first reported the presence of glycolipid material in urinary sediment, and in three cases of metachromatic leukodystrophy the level of sulfatide excretion was much greater than normal. Subsequently, Wherrett (33) utilized column chromatography to separate the polar lipid classes of the urinary sediment and found that the molar ratios of total neutral glycolipid to total phospholipid, as estimated by spectrophotometric techniques, were increased 1.5–9-fold in three cases of gargoylism (mucopolysaccharidosis).

In 1969, Philippart et al. (11) identified small amounts of trihexosyl ceramide and a dihexosyl ceramide in normal urinary sediment and showed that their concentrations were greatly increased in urinary sediment from patients with Fabry's disease. An aliquot of the sediment lipid extract was directly used for TLC, and the relative amounts of the two glycolipids were visually estimated by the intensity of the spot after treatment with anthrone. In both of the above studies (11, 33), the individual neutral glycolipids were identified by their migration on TLC, but they were not quantitated, nor were the constituent monosaccharides identified.

The techniques described in this paper, essentially modifications of those of Vance and Sweeley (13), allowed the characterization and quantitation of all the individual neutral glycosyl ceramides from the urinary sediment. The quantitative isolation of the sediment glycolipids presented no major difficulties, and loss of glycolipid by lysis of the cellular sediment elements into the urine supernatant was excluded since no glycolipids could be detected in a urine supernatant from a patient with Fabry's disease by the usual TLC–orcinol technique. Because Svennerholm and Svennerholm (34) suggested that Silica Gel G gave low recoveries of glycolipid with increasing length of the oligosaccharide moiety, Silica Gel H, which has no $CaSO_4$ binder, was utilized for TLC. Excellent separation and recovery of over 90% of the individual glycosyl ceramides were achieved using this latter method.

The methanolysis and GLC techniques gave optimal results as evaluated by both the glycolipid gal:glu ratios and the reproducibility, precision, and recovery experiments using glycolipid standards. The relative proportions of digalactosyl and lactosyl ceramides were calculated from the gal:glu ratios after GLC of the TMSi derivatives of the dihexoside component.

Normal sediment contained relatively small amounts of lipid. Although only trace amounts of the neutral glycosyl ceramides were excreted daily, a single 24 hr urine sample was sufficient for a reliable quantitative analysis. Of the six neutral glycosyl ceramides determined, glucosyl ceramide and one of the two dihexosides (lactosyl ceramide) were the predominant glycolipids in normal sediment, thus confirming the qualitative findings of Wherrett (33).

In Fabry's disease, concentrations of the glycolipid, phospholipid, and neutral lipid fractions were all significantly elevated, a finding that is consistent with the observed increase of cellular material present in the Fabry urinary sediment. Fabry's disease is characterized by the absence of trihexosyl ceramide galactosyl hydrolase (35) and, as expected from previous work (17, 36), trihexosyl ceramide was found to be the predominant glycolipid in the urinary sediment. Digalactosyl was the major type of dihexoside in Fabry urinary sediment, whereas in normal individuals lactosyl predominated and only trace amounts of digalactosyl ceramide could be detected. This agrees with previous work (17, 36) showing an accumulation of digalactosyl ceramide in Fabry kidney.

Since both of the glycolipids which accumulate have the same terminal monosaccharide sequence, namely Gal-(1 → 4)-Gal, a single galactosyl hydrolase might be responsible for their catabolism. Studies on purified trihexosyl ceramide galactosyl hydrolase (37) showed that the enzyme would not cleave the terminal galactosyl residue from lactosyl ceramide, but digalactosyl ceramide was not tested as a substrate. G. Dawson[1] was unable to show activity of the rat trihexosyl ceramide galactosyl hydrolase with digalactosyl ceramide. Therefore, it may be likely that there is a specific digalactosyl ceramide galactosyl hydrolase[2]. This new enzyme might share a common structural or functional subunit (i.e. a polypeptide chain) with the trihexosyl ceramide galactosyl hydrolase. A common defective subunit could then affect the activities of both enzymes, resulting in the accumulation of both substrates. Either explanation is consistent with the single gene mutation postulated for Fabry's disease.

A significant variation in glycolipid concentration was found in both normal controls and patients with Fabry's disease. This presumably reflects the influence of factors such as renal function, rate of renal desquamation, urine volume, and diet. Infection can increase the number of erythrocytes and leukocytes in the sediment, but

[1] Unpublished work.

[2] In a personal communication, R. O. Brady indicated that he has evidence for the occurrence of this enzyme in mammalian tissues.

the amount of glycolipid contributed (13, 31) would be insignificant even in markedly abnormal urines.

Thus, the urinary sediment provides an "indirect biopsy" of the kidney cellular elements. This is supported by the isolation of digalactosyl ceramide from urinary sediment, especially from patients with Fabry's disease. This glycolipid has not been found in other nonneural tissues (19), and is therefore diagnostic for the presence of kidney cells in urinary sediment. In addition, galactosyl ceramide, a major kidney cerebroside (20) which is almost completely absent from erythrocytes and plasma (13), was also found in normal and Fabry sediment. Furthermore, Makita and Yamakawa (38) have shown that nonneural sulfatide occurs mainly in kidney, and Hagberg and Svennerholm (32) have isolated sulfatide from the urinary sediment of normals and patients with metachromatic leukodystrophy. An important future application of this method for kidney indirect biopsy is the biochemical investigation of other diseases which show histochemical lipid accumulation in the kidney (39–45).

The ability to determine abnormal amounts of neutral glycosphingolipid deposition in the sediment provides a useful tool for analyses in metabolic studies (46, 47) since the sediment may be examined repeatedly without trauma to the subject. Chemical evaluation of urinary sediment lipids, as well as plasma lipids (10, 19), can be of diagnostic value for early detection of Fabry's disease and identification of genetic variants and heterozygotes (11, 48).

Further applications of this method include the identification and quantitation of abnormal lipid metabolites in other lipid disorders, especially those in which glycosphingolipids are disturbed, and the continuous monitoring of urinary glycosphingolipids during therapy.

We would like to thank Dr. A. S. Anderson (Minneapolis, Minn.), Dr. Kenneth Caldwell (Montezuma, Iowa), Dr. Robert Collison (Oscaloosa, Iowa), Dr. John Ferguson (Newton, Iowa) and Dr. Rufino Pabico (Buffalo, N. Y.) for samples of Fabry urine, and Dr. John Opitz (Madison, Wisc.) for his warm and generous assistance in locating the Fabry families. The excellent technical assistance of Miss Linda Kern is gratefully acknowledged.

This investigation was supported in part by Research Grants CA 08101, CA 08832, GM 01156, and AM 12434 from the National Institutes of Health, U.S. Public Health Service, a University of Minnesota Medical School General Research Support Grant, and the Graduate School Grant from the University of Minnesota.

Manuscript received 16 June 1969; accepted 11 September 1969.

References

1. Sibley, W. K. 1918. *Proc. Roy. Soc. Med.* **11:** 70.
2. Richterich, R., P. Van Mechelen, and E. Rossi. 1965. *Amer. J. Med.* **39:** 230.
3. Lake, B. D. 1965. *Arch. Dis. Childhood.* **40:** 284.
4. Siegmund, H. 1921. *Verh. Deut. Pathol. Ges.* **18:** 59.
5. Austin, J. H. 1961. *Clin. Res.* **9:** 106.
6. Klahr, S., K. Tripathy, and O. Bolanos. 1967. *J. Clin. Invest.* **46:** 1475.
7. Knepshield, J. H., P. L. Roberts, C. J. Davis, and R. H. Moser. 1968. *Arch. Intern. Med.* **122:** 156.
8. Relman, A. S., and N. G. Levinsky. 1963. *In* Diseases of the Kidney. M. B. Strauss and L. G. Welt, editors. Little, Brown and Company, Boston. 80.
9. Krivit, W., and R. J. Desnick. 1968. *J. Pediat.* **72:** 571. (Abstr.)
10. Kremer, G. J., and R. Denk. 1968. *Klin. Wochenschr.* **46:** 24.
11. Philippart, M., L. Sarlieve, and A. Manacorda. 1969. *Pediatrics.* **43:** 201.
12. Chasson, A. L., H. J. Grady, and M. A. Stanley. 1961. *Amer. J. Clin. Pathol.* **35:** 83.
13. Vance, D. E., and C. C. Sweeley. 1967. *J. Lipid Res.* **8:** 621.
14. Svennerholm, E., and L. Svennerholm. 1963. *Biochim. Biophys. Acta.* **70:** 432.
15. Svennerholm, L. 1956. *J. Neurochem.* **1:** 42.
16. Clamp, J. R., G. Dawson, and L. Hough. 1967. *Biochim. Biophys. Acta.* **148:** 342.
17. Christensen Lou, H. O. 1966. *Acta Pathol. Microbiol. Scand.* **68:** 332.
18. Sweeley, C. C., and B. Klionsky. 1966. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, and D. S. Fredrickson, editors. McGraw-Hill Book Company, New York. 618–632.
19. Vance, D. E., W. Krivit, and C. C. Sweeley. 1969. *J. Lipid Res.* **10:** 188.
20. Makita, A. 1964. *J. Biochem.* (*Tokyo*). **55:** 269.
21. Mårtensson, E. 1966. *Biochem. Biophys. Acta.* **116:** 296.
22. Svennerholm, E., and L. Svennerholm. 1963. *Nature.* **198:** 688.
23. Makita, A., and T. Yamakawa. 1962. *J. Biochem.* (*Tokyo*). **51:** 124.
24. Suomi, W. D., and B. W. Agranoff. 1965. *J. Lipid Res.* **6:** 211.
25. Eeg-Olofsson, O., K. Kristensson, P. Sourander, and L. Svennerholm. 1966. *Acta Paediat. Scand.* **55:** 546.
26. Svennerholm, L. 1965. *Acta Chem. Scand.* **19:** 1506.
27. Feldman, G. L., L. S. Feldman, and G. Rouser. 1966. *Lipids.* **1:** 21.
28. Dawson, G., C. C. Sweeley, R. Matalon, and A. Dorfman. 1969. *Fed. Proc.* **28:** 540.
29. Michalec, C. 1966. *G. Arterioscler.* **4:** 41.
30. Wajda, M. 1965. *Biochem. J.* **95:** 252.
31. Miras, C. J., J. D. Mantzos, and G. M. Levis. 1966. *Biochem. J.* **98:** 782.
32. Hagberg, B., and L. Svennerholm. 1960. *Acta Paediat. Scand.* **49:** 690.
33. Wherrett, J. R. 1967. *Clin. Chim. Acta.* **16:** 135.
34. Svennerholm, E., and L. Svennerholm. 1963. *Biochim. Biophys. Acta.* **70:** 432.
35. Brady, R. O., A. E. Gal, R. M. Bradley, E. Mårtensson, A. L. Warshaw, and L. Laster. 1967. *N. Engl. J. Med.* **276:** 1163.
36. Sweeley, C. C., and B. Klionsky. 1963. *J. Biol. Chem.* **238:** PC3148.
37. Brady, R. O., A. E. Gal, R. M. Bradley, and E. Mårtensson. 1967. *J. Biol. Chem.* **242:** 1021.
38. Makita, A., and T. Yamakawa. 1964. *J. Biochem.* (*Tokyo*). **55:** 365.

39. Horsley, J. S., J. P. Baker, and F. L. Apperly. 1935. *Amer. J. Med. Sci.* **190:** 511.
40. Landing, B. H., F. N. Silverman, J. M. Craig, M. D. Jacoby, M. E. Lahey, and D. L. Chadwick. 1964. *Amer. J. Dis. Child.* **108:** 503.
41. Pick, L. 1929. *Verh. Deut. Ges. Verdauungs-Stoffwechselkrankh.* **8:** 134.
42. Cammermeyer, J., W. Haymaker, and S. Refsum. 1954. *Amer. J. Pathol.* **30:** 643.
43. Perkoff, G. T. 1967. *N. Engl. J. Med.* **277:** 79.
44. Krickstein, H. I., F. J. Gloor, and K. Balogh, Jr. 1966. *Arch. Pathol.* **82:** 506.
45. Schrade, W., E. Böhle, and G. Becker. 1955. *Deut. Arch. Klin. Med.* **202:** 415.
46. Moser, H. W., G. M. McKhann, and A. E. Moser. 1964. *Trans. Amer. Neurol. Ass.* **89:** 229.
47. Lloyd, A. G., P. J. Large, A. M. James, and K. S. Dodgson. 1964. *J. Biochem.* (*Tokyo*). **55:** 669.
48. Desnick, R. J., and W. Krivit. 1968. *Amer. J. Human Genet. Proc.* 18. (Abstr.)