Characterization of human liver 3-O-β-D-glucopyranuronosyl-cholesterol by mass spectrometry and nuclear magnetic resonance spectroscopy[1]

Isobel A. Muhiudeen,* Theodore A. W. Koerner, Bo Samuelsson,*** Yoshio Hirabayashi,[2,*] Rita DeGasperi,* Su-Chen Li,[3,*] and Yu-Teh Li***

Department of Biochemistry* and Department of Pathology,** Tulane University School of Medicine, New Orleans, LA 70112, and Department of Medical Biochemistry, Faculty of Medicine,*** University of Göteborg, Göteborg, Sweden

Abstract We have isolated an unusual acidic glycolipid which was detected in the lower phase of the Folch partition of the total lipid extract of human liver during a routine isolation of glycosphingolipids. With the solvent systems commonly used for thin-layer chromatography of glycosphingolipids, this glycolipid has a mobility similar to $GbOse_3Cer$, one of the major glycosphingolipids in human liver. Free cholesterol was released from this glycolipid upon treatment with β-glucuronidase. The electron impact mass spectrum of the permethylated derivative of this glycolipid showed an intense peak at m/e 369 which is consistent with the cholesterol part of the molecule. It also showed m/e 233 and 201 which are derived from the permethylated glucopyranuronosyl residue. The final proof of the structure was accomplished by high resolution NMR spectroscopy which revealed the presence of β-linked glucopyranuronosyl residue and cholesterol. Thus, the structure of this acidic glycolipid was conclusively established to be 3-O-β-D-glucopyranuronosyl-cholesterol.—**Muhiudeen, I. A., T. A. W. Koerner, B. Samuelsson, Y. Hirabayashi, R. DeGasperi, S-C. Li, and Y-T. Li.** Characterization of human liver 3-O-β-D-glucopyranuronosyl-cholesterol by mass spectrometry and nuclear magnetic resonance spectroscopy. *J. Lipid Res.* 1984. **25:** 1117–1123.

Supplementary key words cholesterol glucuronide • glycosphingolipids

While studying the glycosphingolipid composition of human liver and small intestine, an unusual acidic glycolipid was encountered (1). By thin-layer chromatography (TLC) this compound was found to have a mobility similar to $GbOse_3Cer$ but did not produce the characteristic color of typical neutral and acidic glycosphingolipids with spray reagents normally used to reveal these compounds. Since this glycolipid was isolated by the same procedure used for the isolation of gangliosides, subsequent analysis was undertaken to distinguish this compound from other "usual" acidic glycosphingolipids such as sulfated glycosphingolipids and gangliosides. Further analysis of this glycolipid showed that it did not contain a ceramide, sialic acid, or sulfate moiety. The structure of this glycolipid was established to be 3-O-β-D-glucopyranuronosyl-cholesterol through high-field proton NMR and mass spectrometry and through the hydrolysis of the glycolipid with β-glucuronidase. While this work was in progress, Hara and Taketomi (2) reported the isolation and partial characterization of a similar glycolipid from human liver.

EXPERIMENTAL PROCEDURES

Materials and methods

Glycolipids were analyzed by thin-layer chromatography using precoated silica gel 60 plates purchased from E. Merck, Darmstadt, Germany. The following solvent systems were used: A) chloroform–methanol–water 60:35:8 (v/v); B) chloroform–methanol–water

Abbreviations: TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; $GbOse_3Cer$, globotriaosylceramide; $GbOse_4Cer$, globotetraosylceramide; FAB, fast atom bombardment; EI, electron impact; Me_2SO-d_6, dimethyl-d_6 sulfoxide; D_2O, deuterium oxide; AHMRV, five-spin system in which all protons are coupled vicinally and linearly; (AB)(HI)M(RV), first-order seven-spin system which contains three geminally-coupled paired protons (AB, HI, and RV), each coupled vicinally to one of the other pairs or to the single proton M in the sequence shown in the symbol; other symbols are used in a similar manner.

[1] Part of this work is taken from the dissertation submitted by I.A.M. to Tulane University in partial fulfillment of the requirements for the Ph.D. in Biochemistry, 1981.

[2] Present address: Department of Biochemistry, Shizuoka College of Medicine. Shizuoka, Japan.

[3] To whom reprint requests should be sent.

65:25:4 (v/v); C) chloroform–methanol–acetic acid–formic acid–water 35:15:6:2:1 (v/v); and D) chloroform–methanol–water–concentrated ammonium hydroxide 60:35:6:2 (v/v). The lipids and glycolipids were visualized using the following spray reagents: resorcinol reagent (3), diphenylamine-aniline reagent (4), anisaldehyde spray (5), orcinol reagent (6), and cupric acetate reagent (7). The molybdenum blue reagent (8) was used to detect phosphate esters. The following glycolipid standards were isolated in this laboratory: galactosylceramide from human brain, lactosylceramide from bovine erythrocytes, $GbOse_3Cer$ and $GbOse_4Cer$ from human erythrocytes. Cholesterol and bovine sulfatide were purchased from Supelco. Iatrobeads were purchased from Iatron Co., Ltd., Tokyo, Japan. Limpet β-glucuronidase (620 units/mg) was the product of Sigma Chemical Company. Human liver was obtained at autopsy from the Charity Hospital morgue, New Orleans, Louisiana. The purified glycolipid was permethylated according to the procedure of Hakomori (9).

Isolation of the unusual acidic glycolipid (*I*)

Human liver (7.3 kg) was homogenized in 30 l of 0.05 M sodium phosphate buffer, pH 7.0, and centrifuged at 6000 *g* for 30 min. The insoluble residue (5.45 kg) was used as a source of glycolipids and was left to stand overnight in 16 l of acetone (4°C). The residue was then homogenized, filtered, and resuspended in 10 l of acetone. The acetone-treated residue was homogenized with 10 volumes each of chloroform–methanol in the ratio as follows: 2:1 (v/v); 1:1 (v/v); and 1:2 (v/v). The combined extracts were evaporated to dryness and dissolved in 4 l of chloroform–methanol 2:1 (v/v). One-liter aliquots of this extract were partitioned with 200 ml of 0.1 M KCl according to the method of Folch, Lees, and Sloane Stanley (10). *I* was found to partition to the lower phase. After washing the lower phase twice with the theoretical upper phase (10), the lower phase was evaporated to dryness and resuspended in 2 l of chloroform–methanol 2:7 (v/v). The lower phase extract was divided into three portions and each was chromatographed on a DEAE cellulose (Whatman DE-52) column (3 × 38 cm) as described by Chien et al. (11). The fractions eluted with the solvent containing 0.01 M sodium acetate from each column were pooled, evaporated to dryness, dialyzed against water, and lyophilized. The lyophilized residues were dissolved in 500 ml of chloroform and divided into two portions; each portion was applied to a column (3 × 38 cm) of Bio-Sil A (Bio-Rad Laboratories) which had been equilibrated with chloroform. After washing each column with 500 ml of chloroform, the elution was carried out by a stepwise increase in the concentration of methanol: 1.5 l of chloroform–methanol 96:4 (v/v), 1.5 l of chloroform–methanol 93:7 (v/v), 2 l of chloroform–methanol 9:1 (v/v), and 750 ml each of chloroform–methanol 2:1 and 1:1 (v/v). The fractions which were eluted with chloroform–methanol 93:7 (v/v) and chloroform–methanol 9:1 (v/v) were found to contain *I*. These fractions were pooled, evaporated to dryness, and dissolved in a small volume of chloroform–methanol–water 83:16:0.5 (v/v). The sample was applied to an Iatrobeads column (1.1 × 90 cm) as described by Momoi, Ando, and Nagai (12). The elution profile is shown in **Fig. 1.** Fractions 90 to 120, which were found to contain *I*, were then pooled, saponified, and partitioned according to the method of Folch et al. (10). The lower phase which contained *I* was evaporated to dryness, dialyzed, lyophilized, and further purified by preparative TLC (13). By this procedure, about 4 mg of pure *I* was obtained from 7.3 kg of fresh human liver.

Treatment with β-glucuronidase

I (10 μg) was incubated at 37°C overnight with 400 units of limpet β-glucuronidase in 200 μl of 10 mM sodium acetate buffer, pH 5.0, containing 200 μg of sodium taurodeoxycholate. The reaction was stopped by adding 1 ml of chloroform–methanol 2:1 (v/v). After the partition, the lower phase was dried and analyzed by thin-layer chromatography.

Mass spectrometry

Direct probe mass spectrometric analysis of *I* was carried out using a ZAB-HF mass spectrometer (VG, Manchester), equipped with a standard FAB source and a conventional EI source. In the FAB mode (positive ions) the sample was loaded in glycerol containing 25% of conc. ammonia–methanol 1:10 (v/v) by volume. Xenon was used as the bombarding gas and the gun was operated at 7kV. The handling of sample in the EI mode has been described by Karlsson (14).

NMR spectroscopy

Chromatographically pure *I* (1.0 mg) was exchanged with D_2O, lyophilized, then dissolved in 0.5 ml of Me_2SO-d_6-D_2O (98:2 v/v), as previously described (15). The Fourier-transformed proton nuclear magnetic resonance spectrum of the resulting sample was then obtained at 500 MHz and 30°C using a Bruker WM-500 spectrometer (NMR Instrument Center, Yale University, New Haven, CT).

RESULTS AND DISCUSSION

General properties of *I*

Although this compound was recovered in the lower phase of a Folch partition along with other neutral

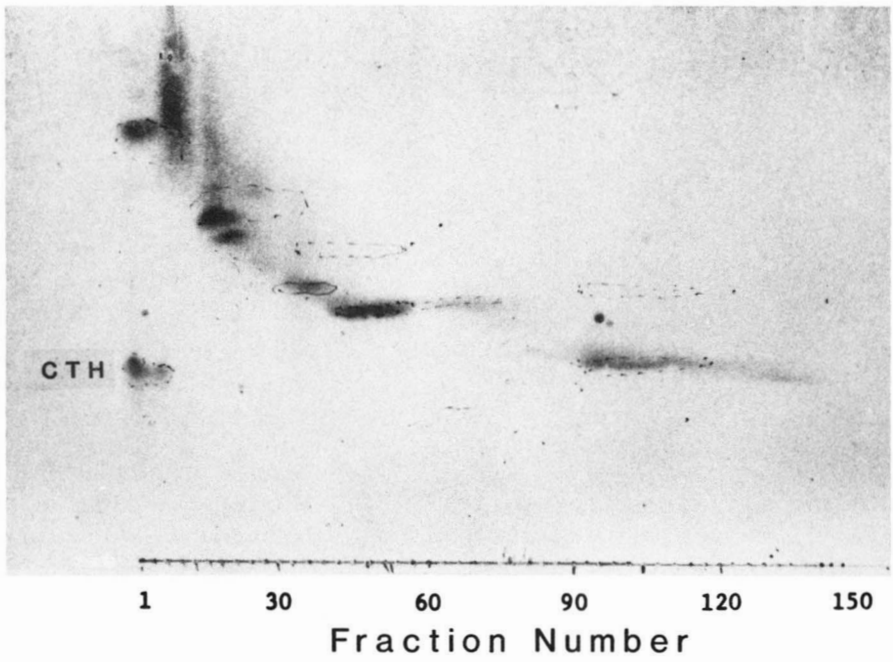

Fig. 1. Elution profile of *I* from Iatrobeads column. The sample was eluted from the column with a gradient of increasing polarity as described in Experimental Procedures. CTH, the standard ceramide trihexoside ($GbOse_3Cer$), externally added to Fraction 1; *I* was eluted in fractions 90 to 120.

glycosphingolipids, it was retained by a DEAE cellulose column together with gangliosides and sulfoglycosphingolipids. Fig. 1 shows the elution profile of the Iatrobeads chromatography; *I* was found to have a TLC mobility close to that of $GbOse_3Cer$. The fractions containing *I* (fractions 90 to 120) were pooled and further purified by preparative TLC. The final preparation was found to move as a single band using solvent systems A, B, C, and D. As shown in **Fig. 2A,** *I* was found to have a TLC mobility close to $GbOse_3Cer$ using solvent system A. It also migrated close to $GbOse_3Cer$ in solvent system D. However, when solvent system C was used, *I* did not behave like a typical glycosphingolipid, and moved much faster than $GbOse_3Cer$ (Fig. 2B). *I* was also found to

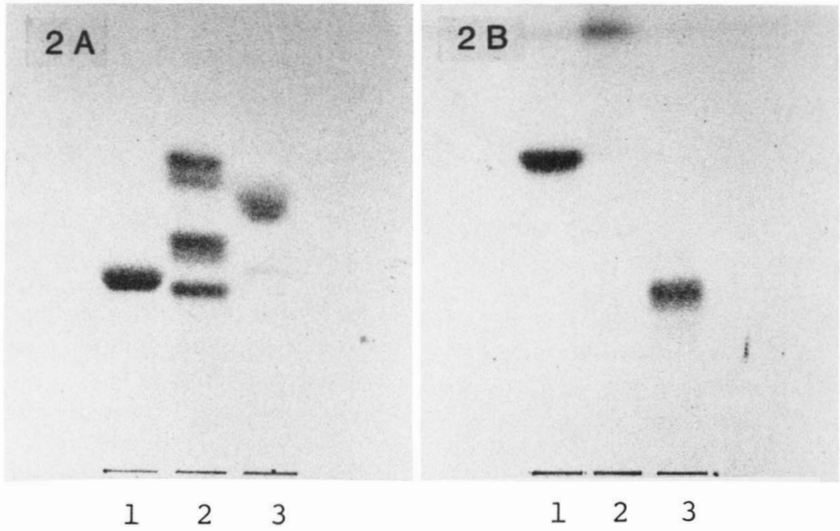

Fig. 2. Thin-layer mobility of *I*: A, lane 1, *I*; lane 2, standards (from the top, galactosylceramide, lactosylceramide, $GbOse_3Cer$; lane 3, galactosylceramide sulfate. The plate was developed using solvent system A and sprayed with the diphenylamine/aniline reagent; B, lane 1, *I*; lane 2, cholesterol; lane 3, $GbOse_3Cer$. The plate was developed using solvent system C and sprayed with the diphenylamine/aniline reagent.

exhibit significantly different staining properties compared to typical glycosphingolipids. For example, with the diphenylamine/aniline reagent, *I* stained with an atypical light blue color compared to other glycosphingolipids which stain grayish blue. Using the anisaldehyde reagent, it stained a yellowish green compared to the typical green obtained with other glycolipids. *I* was also found to react with resorcinol; again the typical purple color was not obtained.

This compound was efficiently hydrolyzed by β-glucuronidase in the presence of sodium taurodeoxycholate and cholesterol was released (**Fig. 3,** lane 2). In the absence of the bile salt, the rate of hydrolysis was found to be extremely slow (Fig. 3, lane 3).

Mass spectrometry of *I*

The mass spectrum, EI mode, of *I* in its permethylated form is shown in **Fig. 4.** The very intense peak at m/e 369 is consistent with a cholesterol part of the molecule and m/e 233 and 201 (233–32) are derived from a permethylated glucuronic acid residue (16) (see formula in Fig. 4). No significant molecular ions of the suggested molecule were found.

The permethylated *I* gave, however, under FAB conditions (positive ions) a mass spectrum (not shown) which, in addition to the above mentioned peaks, contained additional peaks at m/e 619, 636, 641. These could be interpreted as $M + H^+$, $M + NH_4^+$, and $M + Na^+$ for the structure in Fig. 4. Furthermore, the FAB spectrum of the non-derivatized, native molecule gave only one significant peak, at m/e 563, which is $M + H^+$ for the structure in Fig. 4 in non-methylated form.

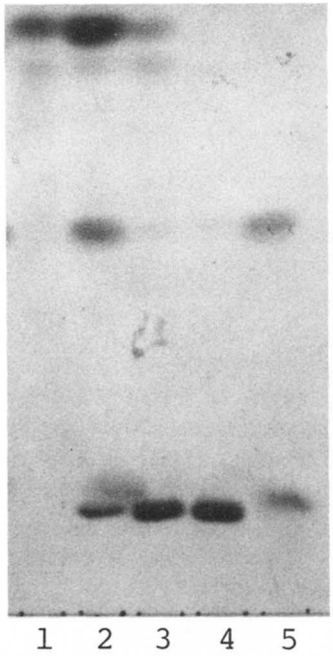

Fig. 3. Treatment of *I* with β-glucuronidase: lane 1, cholesterol standard; lane 2, *I* + β-glucuronidase + sodium taurodeoxycholate; lane 3, *I* + β-glucuronidase; lane 4, *I*; lane 5, sodium taurodeoxycholate. Detailed incubation conditions are described in Experimental Procedures. The plate was developed with solvent system B and dipped in cupric acetate reagent.

To reassure the correct interpretation of m/e 369 in Fig. 4, this peak was subjected to peak matching (permethylated derivative, EI mode) and the mass was determined to be 369.3507 ± 5 ppm at a resolving power of 30,000 (10% valley definition). The theoretical value for the proposed cholesterol fragment, $C_{27}H_{45}$, is 369.3521.

NMR analysis

Examination of the proton NMR spectrum of *I* (**Fig. 5, Table 1**) reveals two distinct subspectra. The first is comprised of a doublet and four doublet-of-doublets at 4.250, 2.910, 3.135, 3.144, and 3.365 ppm, respectively. No resonances are observed between 3.5 and 3.9 ppm, a region in which H-6 protons of glycoside residues resonate. Since no other glycon resonances are observed, the above 5-proton AHMRV subspectrum is constitutionally specific for one pyranuronosyl residue. The sequence of coupling constants for this subspectrum ($J_{1,2}$7.8, $J_{2,3}$8.6, $J_{3,4}$8.9, and $J_{4,5}$9.0) is configurationally specific for β-glucopyranosyl stereochemistry. Since *I* was isolated from human tissue, it can be assumed to belong to the D-monosaccharide family. Thus, the first subspectrum must have the structure of a β-D-glucopyranosyl residue. Further details of the assignment of glycoside residue structure via proton NMR data have been presented by Koerner et al. (15).

The second subspectrum is comprised of the following four parts (see Fig. 5 and Table 1): *1*) a 5-proton series: 1.961 ppm (2H), ca. 3.47 ppm (1H in the glycosidic ether region), 2.368 ppm (1H) and 2.119 ppm (1H) assigned to . . . (HI)M(RV) of an (AB)(HI)M(RV) pattern; *2*) a 4-proton series: 5.324 ppm (1H in olefinic region), 1.833, 1.815, 1.775 ppm (3H) assigned to an A(HI)M pattern; *3*) five methyl resonances, two of which are not coupled (CH_3–CR_3) and three of which are coupled once (CH_3–CHR_2); and, *4*) a 21-proton multiplet between 1.54 and 0.99 ppm. Inspection of the structure in Fig. 5 will reveal that these four subspectral parts are consistent with the following structural fragments of cholesterol: *1*) The *A* ring protons; *2*) H-6, H-7, and H-8 of the *B* ring; *3*) the five methyl groups at Ch-18, Ch-19, Ch-21, Ch-26, and Ch-27; and, *4*) all remaining methylene and methine resonances. Thus, the second subspectrum must be due to cholesterol. This interpretation is consistent with the results obtained by mass spectrometry. Since cholesterol contains only one hydroxyl group at Ch-3 and the fact that the proton spectrum of *I* is attributable completely to a β-D-gluco-

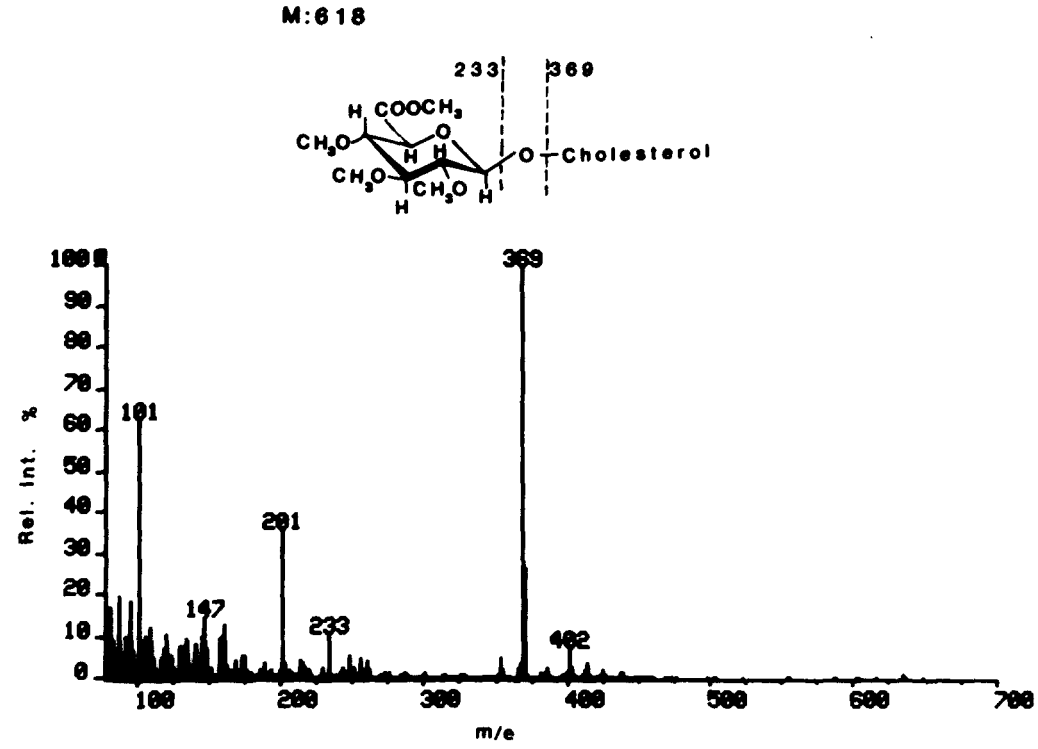

Fig. 4. Mass spectrum of permethylated *I* with a simplified formula for interpretation. The spectrum was recorded in the EI mode using a direct inlet probe under the following conditions: accelerating voltage 8kv; trap current 100 μA; ion source temperature 160°C.

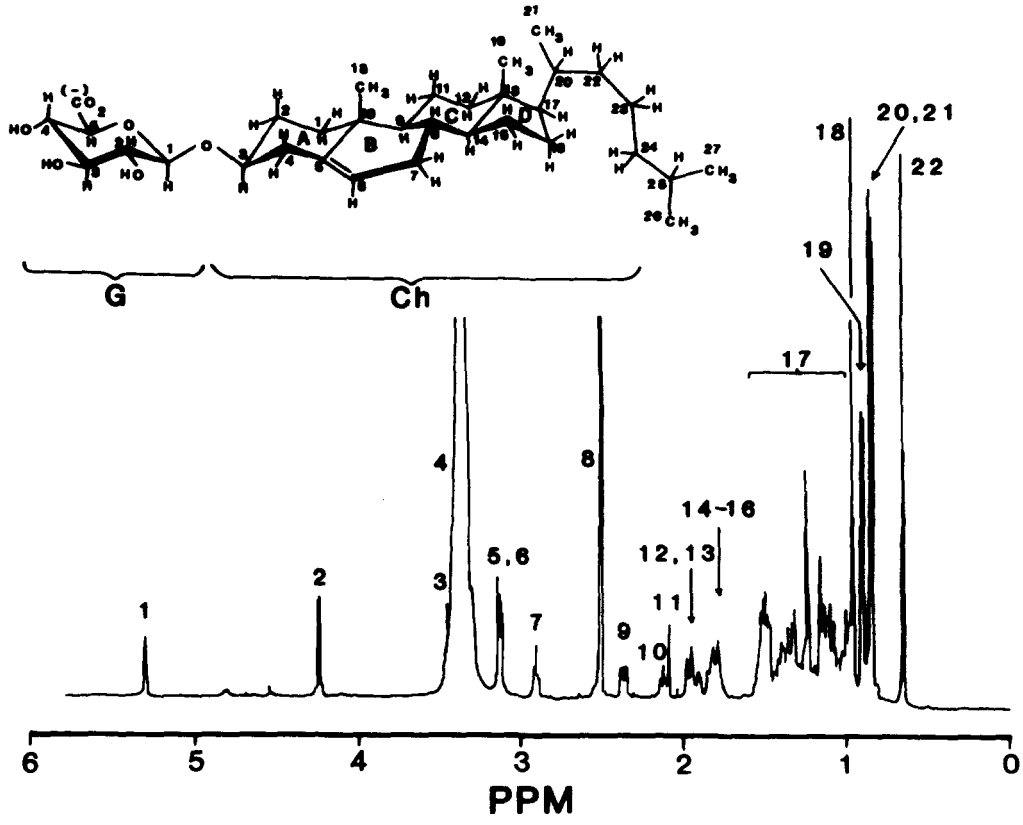

Fig. 5. Structure, symbolism, and numbering of *I* and proton NMR spectrum of *I* obtained at 500 MHz in Me_2-SO-d_6-D_2O. The resonances are numbered from low to high field strength. For the assignment of each resonance, see Table 1.

TABLE 1. Proton NMR data for acidic glycolipid *I* obtained at 500 MHz and 30°C in Me_2SO-d_6-D_2O (98:2, v/v)

Numbered Resonance(s)[a]	Chemical Shift (±0.001 ppm)[b]	Coupling Constant(s) (±0.6 Hz)	Assignment[c]
1	5.324	$J_{6,7a}$5.1, $J_{6,7b}$ 0.6	Ch-6
2	4.250	$J_{1,2}$7.8	G-1
3	ca.3.47	[d]	Ch-3
4	3.365[e]	[d]	G-5, HOD
5	3.144	$J_{2,3}$8.6, $J_{3,4}$8.9	G-3
6	3.135	$J_{3,4}$8.9, $J_{4,5}$9.0	G-4
7	2.910	$J_{1,2}$7.8, $J_{2,3}$8.6	G-2
8	2.508[e]		Me_2SO-d_5
9	2.368	$^2J_{4ax,4eq}$ − 11.8, $J_{3,4eq}$2.5	Ch-4eq
10	2.119	$^2J_{4ax,4eq}$ − 11.8, $J_{3,4ax}$10.6	Ch-4ax
11	2.088	—	Acetone
12	1.961	[d]	Ch-2a,b
13	1.928	[d]	
14	1.833	[d]	
15	1.815	[d]	Ch-7a,b;Ch-8
16	1.775	[d]	
17	1.54–0.99	[d]	21 cholesterol methine and methylene protons[f]
18	0.960	Singlet	Ch-18
19	0.897	$J_{20,21}$6.5	Ch-21
20	0.848	$J_{25,26}$6.6	Ch-26[g]
21	0.843	$J_{25,27}$6.6	Ch-27[g]
22	0.654	singlet	Ch-19

[a] Resonance(s) are numbered from low to high field strength, as shown in Fig. 5.
[b] Chemical shifts are referenced to internal tetramethylsilane.
[c] Ch, cholesterol; G, β-glucopyranuronosyl; ax, axial; eq, equatorial.
[d] Couplings obscured by overlapping with other resonances.
[e] Resonance G-5 assigned by elevating the sample temperature to 80°C.
[f] Integration: 21 ± 2; assigned to this subspectrum; Ch-1a,b; Ch-9; Ch-11a,b; Ch-12a,b; Ch-14; Ch-15a,b; Ch-16a,b; Ch-17; Ch-20; Ch-22a,b; Ch-23a,b; Ch-24a,b; and Ch-25.
[g] Assignments may be interchanged.

pyranuronosyl and cholesterol moieties indicates that the complete structure of *I* must be 3-O-β-D-glucopyranuronosyl-cholesterol.

This report describes the complete structural elucidation of 3-O-β-D-glucopyranuronosyl-cholesterol isolated from human liver. This compound was first detected during a study of the ganglioside composition of human small intestine and liver (1). Although a thorough characterization of glycolipids from human liver has been carried out by Nilsson and Svennerholm (17), the existence of this compound was not revealed. By high resolution proton NMR spectroscopy, mass spectrometry, and enzymatic hydrolysis, the fine structure of this glycolipid has now been unequivocally established. This glycolipid was previously detected in human plasma and urine by using β-glucuronidase (18, 19), however, no attempt was made to establish its complete fine structure. One of the earliest reports on 3-O-β-D-glucopyranuronosyl-cholesterol describes the chemical synthesis and characterization by thin-layer chromatography, infrared spectroscopy, and enzymatic hydrolysis (20). Later, 3-O-β-D-glucopyranuronosyl-cholesterol and derivatives were synthesized by Schneider and Bhacca (21). Recently, Hara and Taketomi (2) described the partial characterization of cholesterol glucuronide from human liver by gas–liquid chromatography, infrared spectroscopy, and enzymatic hydrolysis. The thorough spectral characterization as presented in this report will allow the unambiguous identification of this compound when encountered in future glycolipid analysis.

The significance of the presence of 3-O-β-D-glucopyranuronosyl-cholesterol in human tissues and its physiological function remains unknown. Free cholesterol is mainly found in plasma membranes where it constitutes about 45% of the total lipid. The molecule is inserted deeply into the bilayer, with the β-hydroxyl group orientated toward the polar headgroup region (22). If this glucopyranuronosyl derivative of cholesterol is part of the membrane components, it may have a profound effect on membrane properties.

Supported by Grants PCM 82-19489 from the National Science Foundation, NS 09626 from the National Institutes of Health, and Grants No. 3967 and 6521 from Swedish Medical Research Council. Dr. Y-T. Li is a recipient of the Javits Neuroscience Award.

Manuscript received 9 May 1984.

REFERENCES

1. Muhiudeen, I. A. 1981. Glycolipids of human small intestine and liver. Ph.D. Thesis, Tulane University, Louisiana.

2. Hara, A., and T. Taketomi. 1982. Isolation and determination of cholesterol glucuronide in human liver. *Lipids.* **17:** 515–518.
3. Svennerholm, L. 1957. Quantitative estimation of sialic acids. II. A colorimetric resorcinol-hydrochloric acid method. *Biochim. Biophys. Acta.* **24:** 604–611.
4. Harris, G., and I. C. MacWilliams. 1954. Dipping technique for revealing sugars on paper chromatograms. *Chem. Ind. Lond.* 249.
5. Stahl, E. 1967. Duennschicht-chromatographie. 2nd edition. Springer-Verlag, Berlin, Germany. 817.
6. Svennerholm, L., J. E. Månsson, and Y-T. Li. 1973. Isolation and structural determination of a novel ganglioside, a disialosylpentahexosylceramide from human brain. *J. Biol. Chem.* **248:** 740–742.
7. Fewster, M. E., B. J. Burns, and J. F. Mead. 1969. Quantitative densitometric thin-layer chromatography of lipids using copper acetate reagent. *J. Chromatogr.* **43:** 120–126.
8. Dittmer, J. C., and R. L. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. *J. Lipid Res.* **5:** 126–127.
9. Hakomori, S. 1964. A rapid permethylation of glycolipid, and polysaccharide catalyzed by methylsulfinyl carbanion in dimethyl sulfoxide. *J. Biochem. (Tokyo)* **55:** 205–208.
10. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipides from animal tissues. *J. Biol. Chem.* **226:** 497–509.
11. Chien, J. L., S-C. Li, R. A. Laine, and Y-T. Li. 1978. Characterization of gangliosides from bovine erythrocyte membranes. *J. Biol. Chem.* **253:** 4031–4035.
12. Momoi, T., S. Ando, and Y. Nagai. 1976. High resolution preparative column chromatographic system for gangliosides using DEAE-Sephadex and a new porus silica, Iatrobeads. *Biochim. Biophys. Acta.* **441:** 488–497.
13. Kundu, S. K. 1981. Thin-layer chromatography of neutral glycosphingolipids and gangliosides. *Methods Enzymol.* **72:** 185–204.
14. Karlsson, K-A. 1976. Microscale fingerprinting of blood-group fucolipids by mass spectrometry. *In* Glycolipid Methodology. L. A. Witting, editor. American Oil Chemists' Society, Champaign, IL. 97–122.
15. Koerner, T. A. W., J. H. Prestegard, P. C. Demou, and R. K. Yu. 1983. High resolution proton NMR studies of gangliosides. 2. Use of two-dimensional nuclear Overhauser effect spectroscopy and sialylation shifts for determination of oligosaccharide sequence and linkage sites. *Biochemistry.* **22:** 2687–2690.
16. Thompson, R. M., and N. Gerber. 1976. Separation of permethylated isomeric glucuronides by gas chromatography and analysis of the mass spectra. *J. Chromatogr.* **124:** 321–329.
17. Nilsson, O., and L. Svennerholm. 1982. Characterization and quantitative determination of gangliosides and neutral glycosphingolipids in human liver. *J. Lipid Res.* **23:** 327–334.
18. Wade, A. P. 1970. The estimation of cholesterol sulphate and cholesterol glucuronide in plasma. *Clin. Chim. Acta.* **27:** 109–116.
19. Winter, J. S. D., and A. M. Bongiovanni. 1968. Identification of cholesterol sulfate in urine and plasma of normal and hypercholesterolemic subjects. *J. Clin. Endocrinol. Metab.* **28:** 927–930.
20. Nagayama, F., A. Saito, and D. R. Idler. 1966. The synthesis of cholesterol glucopyranosiduronate. *Can. J. Biochem.* **44:** 1109–1114.
21. Schneider, J. J., and N. S. Bhacca. 1969. Synthesis and characterization of cholesterol-β-D-glucopyranosiduronic acid and derivatives. *J. Org. Chem.* **34:** 1990–1993.
22. Houslay, M. D., and K. K. Stanley. 1982. Dynamics of Biological Membranes. J. Wiley & Sons, New York. 71–72.