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Glycerol-, Inositol-, and Reducing End Hexose-containing Oligosaccharides in Human Urine

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Ten previously unreported oligosaccharides have been purified from the urines of human subjects using a combination of gel filtration, ion exchange, and thin-layer chromatographies. Their structures were determined by direct probe mass spectrometry, methylation analysis, and proton NMR spectroscopy of the permethylated oligosaccharide alditols.

On the basis of composition, the oligosaccharides could be divided into three groups. Five oligosaccharides containing glycerol were characterized as glucosylα1-1'glycerol; glucosylβ1-1'glycerol; galactosylβ1-1'glycerol; glucosyl-1-1'(fucosyl-1-2')glycerol and/or fucosyl-1-1'(glucosyl-1-2')glycerol; and glucosyl-1-1'(galactosyl-1-2')glycerol or galactosyl-1-1'(glucosyl-1-2')glycerol. Four inositol-containing oligosaccharides were characterized as galactosylβ1 (fucosylα1)inositol, *N*-acetylgalactosaminylα1 (fucosylα1)inositol, fucosylα1-2galactosylβ1 (*N*-acetylgalactosaminylα1)inositol and fucosylα1-2galactosylβ1-4-*N*-acetylglucosaminylα1(*N*-acetylgalactosaminylα1)inositol. Finally, galactosylα1-3(fucosylα1-2)galactosylβ1-6galactosylα1-4(fucosylα1-3)glucose, an oligosaccharide with glucose at its reducing end, was tentatively identified. The significance and possible origins of the carbohydrate structures are discussed.

Oligosaccharides of human urine are reflective of normal and abnormal biosynthetic and degradation processes of tissue glycoconjugates. Lundblad *et al.* have reported extensively on these human urinary oligosaccharides and have found structures which are related to blood groups A, B, and O oligosaccharides, from both secretor and non-

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secretor individuals [1-5]. They also have analyzed women's urine taken during pregnancy and lactation [6-8], and urine from people maintained on a lactose diet [9]. Thus, many of the urinary oligosaccharides have been well characterized and their structures are detailed in a review [10].

Recently, methodology for the isolation and characterization of oligosaccharides from the urine of patients with abnormal glycoconjugate metabolism, including patients with lysosomal storage diseases, has been greatly improved by employing the techniques of gel filtration chromatography, gas-liquid chromatography-mass spectrometry (GC-MS), high resolution proton and (^{13}C) nuclear magnetic resonance spectroscopy (NMR), exoglycosidase digestion, and high performance liquid chromatography (HPLC) [11-15]. Utilization of these methods has led to the discovery and characterization of urinary oligosaccharides that contain both *myo*-inositol and terminal fucose [16-18]. Interest has been growing in these inositol-containing oligosaccharides because some of them are structurally similar to glucose-containing blood group oligosaccharides, except that inositol is substituted for the reducing glucosyl residue in the oligosaccharide. The biosynthesis, metabolism and biological functions of these substances are, however, as yet unknown.

In the present investigation of human urinary oligosaccharides, five new glycerol-containing oligosaccharides, four inositol-containing oligosaccharides and one reducing end hexose-containing oligosaccharide are described. Separation and complete or partial structural elucidation have been achieved by thin-layer chromatography (TLC), capillary and packed column gas chromatography (GC), gas chromatography-mass spectrometry (GC-MS), proton NMR and methylation analysis.

Materials and Experimental Procedures

Urines

Morning urine was collected from male individuals (unknown secretor status) of ages 23, 29 and 36 with blood types A, B and O, respectively; no attempt was made to control the diets of the donors. A few ml of toluene were added to the urine-containing bottles and the urine samples were stored frozen at -76°C until analysis.

Isomalto-oligosaccharide Standards

The isomalto-oligosaccharide standards, Glcα1-(6Glcα1)$_n$-6Glc, were obtained by partial acid hydrolysis of Dextran T-70, MW = 68 500 (Pharmacia, Sweden) as described by Kobata [12]. Dextran (100 mg) was heated in 1 ml of 0.1 N HCl at 100°C for 4 h. The partially hydrolyzed mixture was then passed through an ion-exchange column (Dowex 1 X4, acetate form, 1 cm × 10 cm), lyophilized and separated on a Bio-Gel P-4 column.

Partially Methylated Alditol Acetate Standards

The partially methylated alditol acetate derivatives were made by permethylation, hydrolysis and acetylation of oligosaccharides or polysaccharides according to the proce-

dures of Hakomori *et al.* [19, 20] and Björndal *et al.* [21]. Fucose, galactose, glucose, lactose, melibiose and stachyose were from Sigma, U.S.A.. Gentiobiose and Yeast galactan were from Koch Light, U.K.. Okra polysaccharide was extracted and purified from vegetable okra as described by Whistler and Conrad [22]. Laminarin was a gift of Dr. Robert Bandurski. The reference amino sugar derivatives were made from glucosamine hydrochloride, galactosamine hydrochloride and chitin (Sigma) [23].

Colorimetric Assays

Total hexose was determined by means of the phenol-sulfuric acid reaction [24]. Creatinine was determined by a picric acid method [25].

Gel Filtration Chromatography

Gel filtration chromatography was carried out on 5 cm × 94 cm Bio-Gel P-2 (100-200 mesh) and 2 cm × 160 cm (minus 400 mesh) Bio-Gel P-4 columns at room temperature. Water containing 0.02% sodium azide was used as the eluting solvent in both cases. Fractions of 16 ml and 1.8 ml were collected from the Bio-Gel P-2 and P-4 columns, respectively.

Thin-Layer Chromatography (TLC)

TLC of the oligosaccharides was performed on silica gel thin-layer plates (0.25 mm) (Uniplate, Analtech Inc., U.S.A.) using acetonitrile-water 4:1 (v/v) as the solvent. The plates were developed twice and the sugars were visualized by orcinol-sulfuric acid spray [26]. TLC of permethylated oligosaccharide alditols was performed on Silica G60 plates (0.25 mm) (Merck, W.Germany), developed twice with benzene-methanol 12:1 (v/v) as the solvent [27]. Preparative and analytical TLC of oligosaccharides or of their permethylated alditols were done under identical conditions. When preparative plates were run, the silica powder scraped from the plate was put into a pasteur pipette, and the oligosaccharides or their permethylated derivatives were eluted from the silica using methanol-water 10:1 (v/v) and chloroform-methanol 2:1 (v/v), respectively.

Gas Chromatography (GC)

GC analyses were performed on a Hewlett-Packard 5840 gas chromatograph equipped with flame ionization detectors. Packed glass columns and fused silica capillary columns were run under the following conditions: (i) 0.25 mm × 12 m fused silica capillary column with chemically bonded 5% phenyl methyl silicone (DB-1), 0.1 μm thickness (J & W Scientific, Inc. U.S.A.), temperature programming from 150°C to 330°C at a rate of 5°C/min for permethylated oligosaccharide alditols; (ii) 2 mm i.d. × 1.5 ft glass column packed with Dexsil-300, temperature programming from 130°C to 330°C at a rate of 5°C/min (this column was also used for permethylated oligosaccharide alditols); (iii) 2 mm i.d. × 6 ft glass column packed with 3% OV-225 at 150°C, 170°C and 190°C oven temperatures (isothermal); (iv) 2 mm i.d. × 6 ft glass column packed with 3% ECNSS-M at 170°C and 190°C oven temperatures (isothermal). Columns (iii) and (iv) were used for the analysis of partially methylated alditol acetates.

Gas Chromatography - Mass Spectrometry (GC-MS)

The GC-MS analyses were performed on a Hewlett-Packard 5985 GC-MS system using Dexsil-300 (column (ii)) for permethylated oligosaccharide alditol analyses, and OV-225 (column (iii)) for partially methylated alditol acetate analyses. The GC conditions were the same as those described above in the GC analysis section. Since both the Dexsil-300 and DB-1 (equivalent to SE-30) are non-polar stationary phases and have similar McReynolds constants [28], they gave similar separations of the sugars in the various samples. This was proven by TLC preparation and subsequent examination of the retention sequence on the two columns, and by direct probe mass spectrometry. Mass spectra were recorded at an ionization potential of 70 eV and with an ion source temperature of 200°C.

Direct probe mass spectrometry of the permethylated oligosaccharide alditols was carried out under the same conditions used in the GC-MS analyses.

Proton NMR

The proton NMR spectra of permethylated oligosaccharide alditols were obtained in a Brucker WM-250 spectrometer using a 5 mm proton probe in $CDCl_3$ solvent at 27°C. The line width was 4 100 Hz and the pulse width was 3 μs. For spectra from only a few μg of sample, solvent peak suppression techniques were used and several thousand scans were accumulated. The chemical shifts are referred to internal trimethylsilane (TMS) or to the chloroform peak (7.24 ppm with respect to internal TMS).

Isolation of Oligosaccharides

Fresh urine equivalent to 1 200 mg creatinine (500-1 000 ml) was concentrated to about 50 ml by rotary evaporation in a 37°C water bath. It was then fractionated on the 5 cm × 94 cm Bio-Gel P-2 column. Protein, and urea plus salts eluted in the exclusion and retention volumes, respectively. The oligosaccharide-containing fractions were pooled, and were passed through a 2 cm × 20 cm cation exchange resin column (Dowex 50 X8, H^+ form) and an anion exchange resin column (Dowex 1 X4, OH^- form) in series, to remove ionic components. The neutral oligosaccharides were further fractionated on the Bio-Gel P-4 column (2 cm × 160 cm, minus 400 mesh) eluting with water containing 0.02% sodium azide, and collecting fractions of 1.8 ml. Aliquots (50 μl) from each fraction were taken for phenol-sulfuric acid assay. In reference to the isomalto-oligosaccharide standards, fractions eluting in the disaccharide to hexasaccharide regions of the column were pooled (Fig. 1), passed over Dowex 50 X8 (H^+ form) and Dowex 1 X4 (OH^- form) columns (1 cm × 10 cm) and lyophilized.

Reduction of Carbohydrate

Samples (0.1-1 mg) were reduced with 500 μl sodium borohydride (10 mg/ml in 0.05 N NaOH) or with 300 μl sodium borodeuteride (10 mg/ml in 0.05 N NaOH) at room temperature overnight. Acetic acid was added to destroy excess reagent. The solution was freed of solvent and boric acid by repeated evaporation with 1 M acetic acid followed

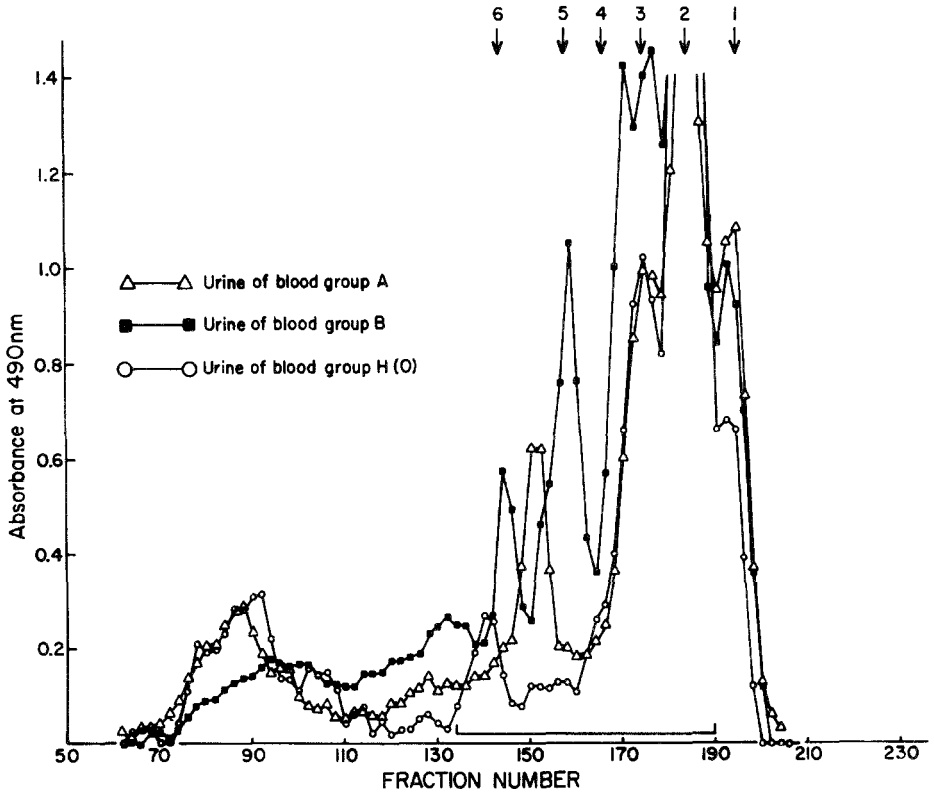

Figure 1. Gel filtration chromatography of neutral oligosaccharides from human urines of individuals with blood groups A, B and O on Bio-Gel P-4, as described in Methods. All urine samples contained 1 200 mg creatinine. The hexose content was determined by phenol-sulfuric acid assay. Numbers above the profile refer to elution positions of linear isomalto-oligosaccharide standards. The bar near the bottom of the graph indicates which fractions were pooled.

by methanol. The reduced carbohydrates were purified by passing them over Bio-Rad AG 50 X4 (H^+ form) column.

Methylation Analysis

The oligosaccharide alditols (100-200 μg) were methylated by the method of Hakomori [19] as modified by Stellner *et al.* [20] and the methylated oligosaccharide alditols were purified by chloroform-water partition. The chloroform phases were carefully dried under a nitrogen stream at room temperature. The dried residues were then redissolved in dry chloroform and were applied to a pasteur pipette column of silica (Iatrobeads, Iatron Laboratories Inc., Japan). After discarding the first five bed volumes of chloroform

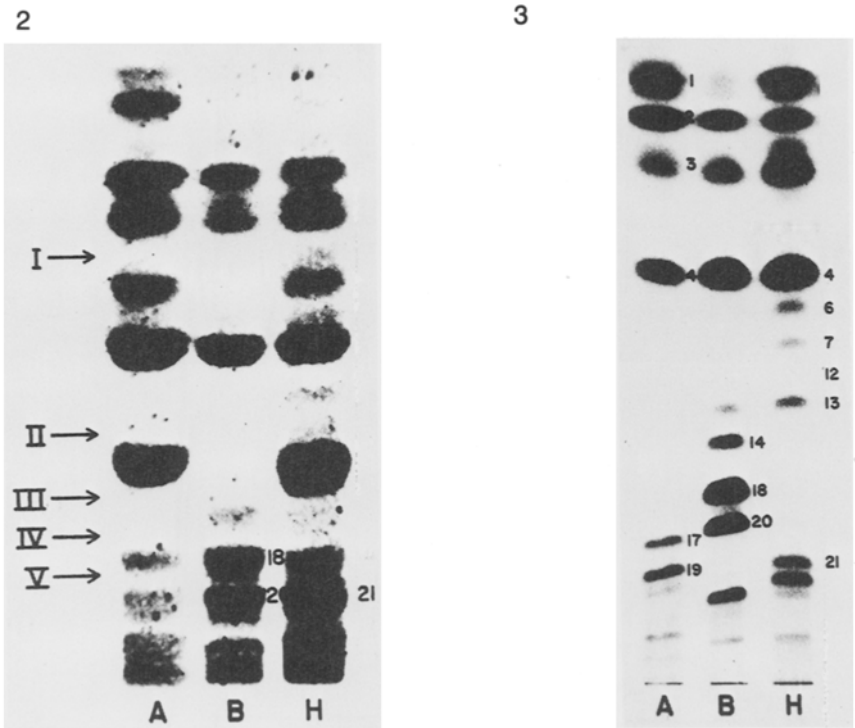

Figure 2. Thin-layer chromatography of neutral oligosaccharides from human urines of individuals with blood groups A, B and O. Chromatograåhy was performed as described in Methods. A, B and H refer to the blood types of the donors. Band numbers denote the same sugar compounds as those shown in Figs. 3 and 4. Roman numerals refer to linear isomalto-oligosaccharide standards.

Figure 3. Thin-layer chromatography of permethylated oligosaccharide alditols from human urines from blood group A, B and O donors. Conditions were as described in Mehtods, using benzene-methanol 12:1 (v/v) as the solvent. The plates were developed twice and sugars were visualized by orcinol-sulfuric acid reagent spray. The A, B, H and numbers are the same as in Fig. 2.

washes, the permethylated sugar alditols were eluted by chloroform-methanol 4:1 (v/v). The eluant was dried, and the permethylated sugar alditols were dissolved in 50-100 μl chloroform.

Hydrolysis and Acetylation

The permethylated oligosaccharide alditols and permethylated oligosaccharides were subjected to hydrolysis and acetylation following the procedure of Stellner [20].

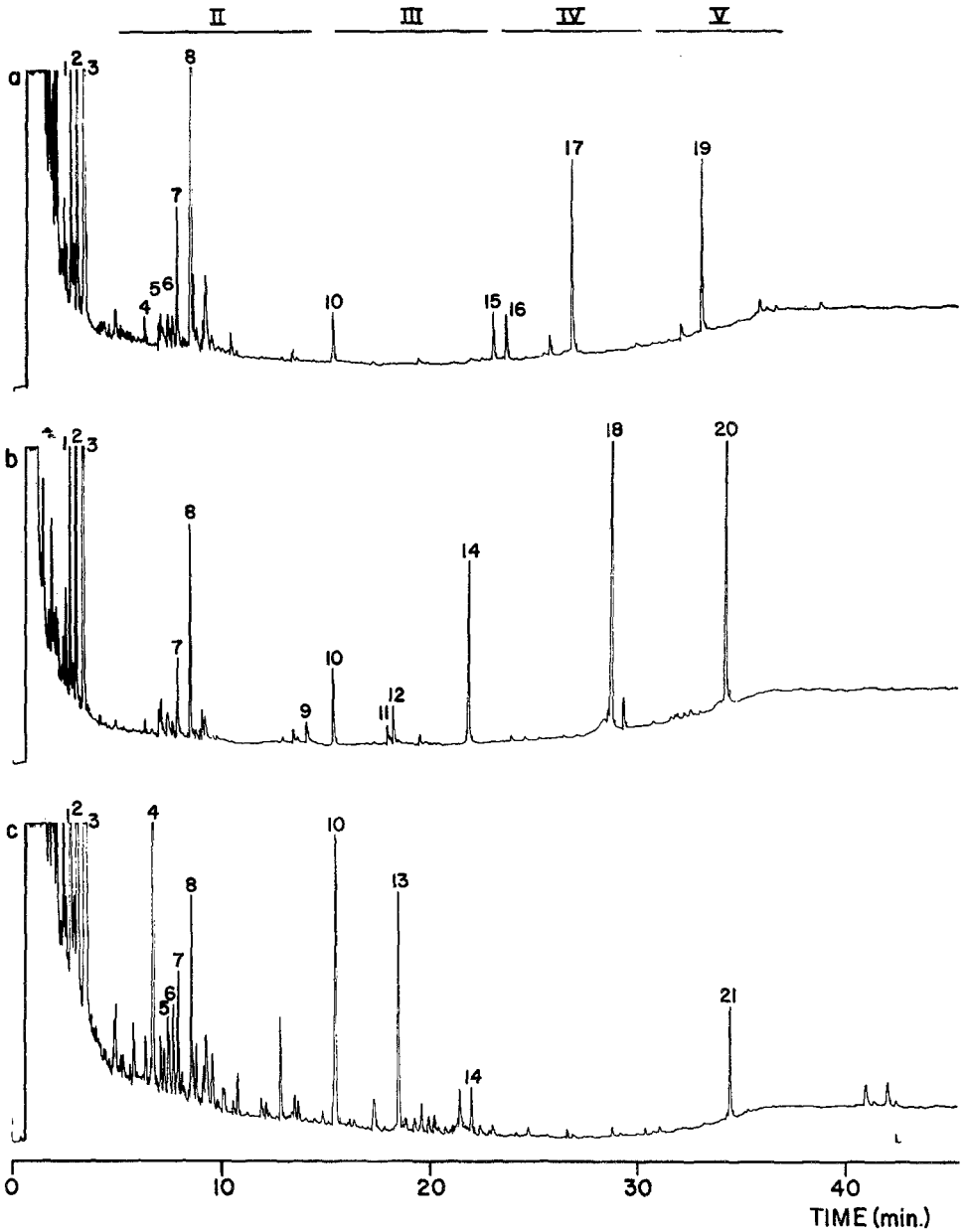

Figure 4. Gas chromatography of permethylated oligosaccharide alditols of the di- to hexasaccharide region of Fig. 1 from human urines from blood group A, B and O donors. A 12 m fused silica capillary column (DB-1) was used. Conditions are described in Methods. Roman numerals above the profile are the regions where di- (II), tri-(III), tetra-(IV) and pentasaccharides (V) eluted. (a) oligosaccharides from urine with blood group A; (b) oligosaccharides from urine with blood group B; (c) oligosaccharides from urine with blood group O. The numbers denoting each peak are the same as those in Table 2 and in all subsequent figures.

Results

Isolation of Neutral Urinary Oligosaccharides

The Bio-Gel P-4 profiles of the neutral urinary oligosaccharides from A, B and O blood group individuals are shown in Fig. 1. The gel filtration profiles from these groups are clearly different, suggesting that distinctly different oligosaccharides are present in the urines. However, the sizes of the carbohydrate moieties could only be estimated because of the different effects of hexosyl, deoxyhexosyl, and *N*-acetylhexosaminyl residues on Bio-Gel P-4 retention behaviour [29]. Fractions containing oligosaccharides with mobilities between those of isomaltose and isomaltohexaose were pooled, as shown in Fig. 1. The oligosaccharides were then analyzed by TLC before (Fig. 2) and after (Fig. 3) sodium borohydride reduction and permethylation. Both TLC systems resolved the oligosaccharides more completely than did Bio-Gel P-4. However, the permethylated oligosaccharide alditols gave sharper bands than their underivatized counterparts; this was especially true for the larger oligosaccharides.

The purity of individual permethylated oligosaccharide alditols separated by TLC was checked by GC or direct probe MS. GC-MS, proton NMR and methylation analyses of the permethylated oligosaccharide alditols gave information about their composition, glycosyl sequences and anomeric linkages. The detailed results of these analyses are given below.

Fig. 4 shows the GC profiles of the permethylated oligosaccharide alditols from the urine of blood types A, B and O subjects. These capillary GC profiles represent oligosaccharides ranging in size from disaccharide to hexasaccharide. To assure their purity, the urinary oligosaccharides were also separated by Silica G60 TLC as their permethylated

Table 1. Structures of previously reported neutral urinary oligosaccharides identified in this study

Peak No. in Fig. 4	Assigned Structure	Reference
4	Fuc 1-2 Glc	10
5	Fuc—Inositol	15
6	Hex—Inositol	18
10	Gal 1-6 Gal 1-4 Glc	31
13	GalNAc 1-3 Gal 2 Fuc 1	32
18	Gal α1-3 Gal β1-4 Glc 2 3 Fuc α1 Fuc α1	4
21	GalNAc α1-3 Gal β1-4(3) Glc 2 3(4) Fuc α1 Fuc α1	5

Table 2. Neutral urinary oligosaccharides separated on a fused silica capillary gas chromatographic column (DB-1) in their permethylated alditol forms.

Peak No. in Fig. 4	RI	Amount in urine with Blood Group A	B	(mg/l) H(O)	Assigned Structure	Comments
1	1826	0.9	2.3	1.6	Glcα1-1'Glycerol	++
2	1865	1.8	3.5	2.6	Glcβ1-1'Glycerol	++
3	1908	6.0	12.7	5.0	Galβ1-1'Glycerol	++
7	2206	0.56	0.31	0.50	(Fuc)Glc-O-CH_2 (Glc)Fuc-O-CH CH_2-OH	+,+++
8	2229	2.8	1.7	0.83	(Glc)Gal-O-CH_2 (Gal)Glc-O-CH CH_2OH	+,+++
14	3375	0.01	0.73	0.19	Galβ1—Inositol | Fucα1	++
16	3499	—	0.02	—	GalNAcα1—Inositol | Fucα1	++
17	3810	0.78	—	0.02	Fucα1-2Galβ1—Inositol | GalNAcα1	++
19	—	0.70	—	0.01	Fucα1-2Galβ1-4GlcNAcα1—Inositol | GalNAcα1	++
20	—	—	2.09	0.02	Galα1-3Galβ1-6Galα1-4Glc 2 3 | | Fucα1 Fucα1	++

* Retention indices were calculated by linear interpolation between the appropriate pairs of flanking hydrocarbons.
\+ Methylation analysis has been done.
\+ + Both methylation and proton NMR analysis were carried out.
\+ + + Exact position of sugar residues is unknown.

alditol derivatives. Summarized in Table 1 are the structures of 7 urinary oligosaccharides previously reported by other investigators. Shown in Table 2 are the approximate yields (assuming the response factors of all oligosaccharides were identical), the assigned structures or partial structures of each new oligosaccharide and their Kovat Retention Indices (RI) on GC. The standard deviations of RI values were 9.4 and 5.7 units for 2 200 and 3 800, respectively.

The 10 fractions listed in Table 2, which correspond to the peaks in the GC profile of Fig. 4, can be divided into three groups on the basis of their compositions:
(1) five glycerol-containing oligosaccharides (peaks 1-3, 7 and 8),
(2) four inositol-containing oligosaccharides (peaks 14, 16, 17 and 19),
(3) one reducing end hexose-containing oligosaccharide (peak 18).
In addition to GC-MS and direct probe MS analysis of the intact permethylated alditols, all 10 fractions (1-3, 7, 8, 14, 16-19) were subjected to methylation analysis and 8 fractions

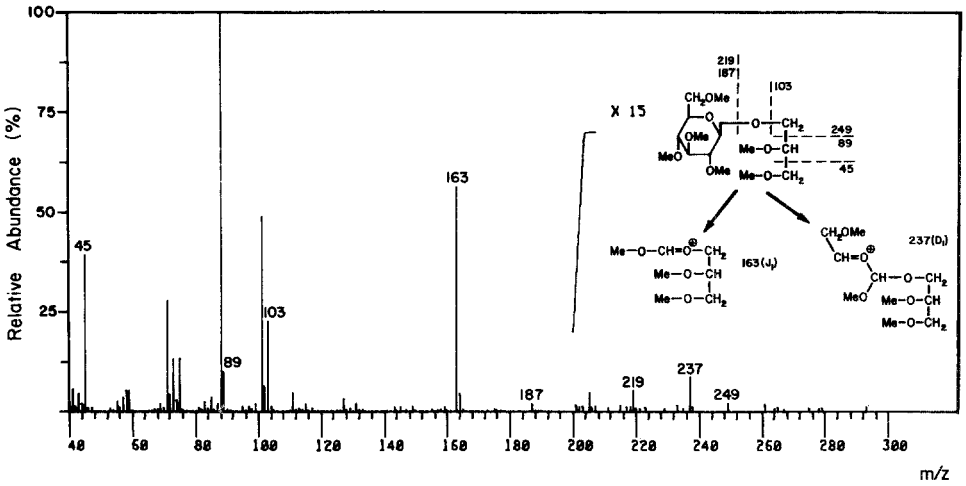

Figure 5. Mass spectrum of intact permethylated glucosyl-1-1'glycerol (peak 2 in Fig. 4).

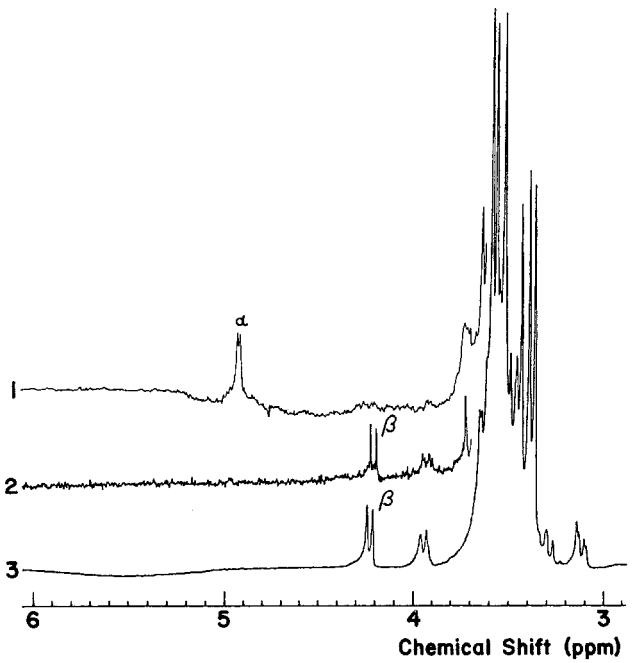

Figure 6. Proton NMR of permethylated glycerol-containing sugars (peak 1, 2 and 3 in Fig. 4).

Table 3. Molecular ratio of alditol acetates obtained from hydrolysates of permethylated oligosaccharide alditols and permethylated oligosaccharides.

Peak No. in Fig. 4	Methyl Ester[a]									
	T-Fuc	T-Glc	T-Gal	T-GalNAc	1,2-Gal	1,6-Gal	1,4-GlcNAc	1,2,3-Gal	1,3,4-Glc	D-Inositol
Glycerol-containing oligosaccharides										
1		1.0								
2		1.0								
3			1.0							
7	1.0	0.95								
8		0.74	1.0							
Inositol-containing oligosaccharides										
14	0.74		0.79							1.0
16	0.8			0.5						1.0
17	0.7			0.3	0.8					1.0
19	0.6			0.8	0.8		0.3			1.0
Reducing end hexose-containing oligosaccharides										
20	1.4		1.0			1.1		0.85	0.8[b]	

[a]Abbreviations. 2,3,4-tri-*O*-Me-1,5-di-*O*-Acetylfucose; T-Fuc: 2,3,4,6-tetra-*O*-Me-1,5-di-*O*-Acetylglucose; T-Glc: 2,3,4,6-tetra-*O*-Me-1,5-di-*O*-Acetylgalactose; T-Gal: 2-Deoxy-2-*N*-Me-3,4,6-tri-*O*-Me-1,5-di-*O*-Acetylgalactosamine; T-GalNAc: 3,4,6-tri-*O*-Me-1,2,5-tri-*O*-Acetylgalactose; 1,2-Gal: 2,3,4-tri-*O*-Me-1,5,6-tri-*O*-Acetylgalactose; 1,6-Gal: 2-Deoxy-2-*N*-Me-3,6-di-*O*-Me-1,4,5-tri-*O*-Acetylglucosamine; 1,4-GlcNAc: 4,6-di-*O*-Me-1,2,3,5-tetra-*O*-Acetylgalactose; 1,2,3-Gal: 2,6-di-*O*-Me-1,3,4,5-tetra-*O*-Acetylglucose; 1,3,4-Glc: tetra-*O*-Me-di-*O*-Acetylinositol; D-Inositol.

[b]By analysis of permethylated oligosaccharides.

(1-3, 14, 16-19) were also analyzed by proton NMR to determine their anomeric configurations. The results of these determinations and the subsequent oligosaccharide structures which were arrived at will be discussed in the following sections.

Structural Analysis of Glycerol-containing Oligosaccharides

The five glycerol-containing oligosaccharides gave GC peaks 1, 2, 3, 7 and 8 (Fig. 4). The mass spectra of Fractions 1, 2 and 3 were identical; the glucosylβ1-1'glycerol mass spectrum is shown in Fig. 5. The ions at m/z 219 and 187 arise from a non-reducing terminal hexose, while m/z 103, 89, 45 and 249 indicate the existence of a glycerol moiety. In addition, the J_1 fragment ion at m/z 163 and D_1 fragment ion at m/z 237 further indicate a linkage between glycerol and a hexose. Only one hexose and one anomeric proton were found in each fraction by methylation analysis and proton NMR (cf. Tables 3 and 4, and Fig. 6). These results allowed the structures of Fractions 1, 2 and 3 to be assigned as glucosylα1-1'glycerol, glucosylβ1-1'glycerol, and galactosylβ1-1'glycerol, respectively.

The mass spectrum of Fraction 7 (Fig. 4) is given in Fig. 7. Fragment ions at m/z 189 and 157 are from a terminal deoxyhexose and fragment ions at m/z 219 and 187 are from a terminal hexose. Cleavages between the carbon atoms in the glycerol backbone give rise to fragment ions at m/z 45, 249, 263, 277, and 307; J_1 ions are at m/z 337 and 367. The ion

Table 4. Proton NMR detection of anomeric protons of permethylated oligosaccharide alditols

Peak No. in Fig. 4	Structure	Chemical Shifts (ppm)	Observed Coupling Constant $J_{1,2}$ (Hz)	Assigned Anomeric Conformation
Glycerol-containing oligosaccharides				
1	Glc α1-1′ Glycerol	4.93	3.35	α
2	Glc β1-1′ Glycerol	4.21	7.91	ß
3	Gal β1-1′ Glycerol	4.22	7.63	ß
Inositol-containing oligosaccharides				
14	Gal β1—Inositol | Fucα1	5.20 4.33	4.16 7.50	α ß
16	GalNac α1—Inositol | Fuc α1	5.18 5.32	4.10 4.6	α α
17	Fuc α1-2 Gal β1—Inositol | GalNAc α1	5.18 5.32 4.32	3.6 4.6 7.9	α α ß
19	Fuc α1-2 Gal β1-4 GlcNAc α1—Inositol | GalNAc α1	5.19 5.18 4.32 5.31	3.6 3.6 7.9 3.6	α α ß α
Reducing end hexose-containing oligosaccharide				
20	Gal α1-3 Gal β1-6 Gal α1-4 Glc 2 3 | | Fuc α1 Fuc α1	5.20 5.19 4.3 5.4 4.9	3.8 3.8 7.9 3.3 4.2	α α ß α α

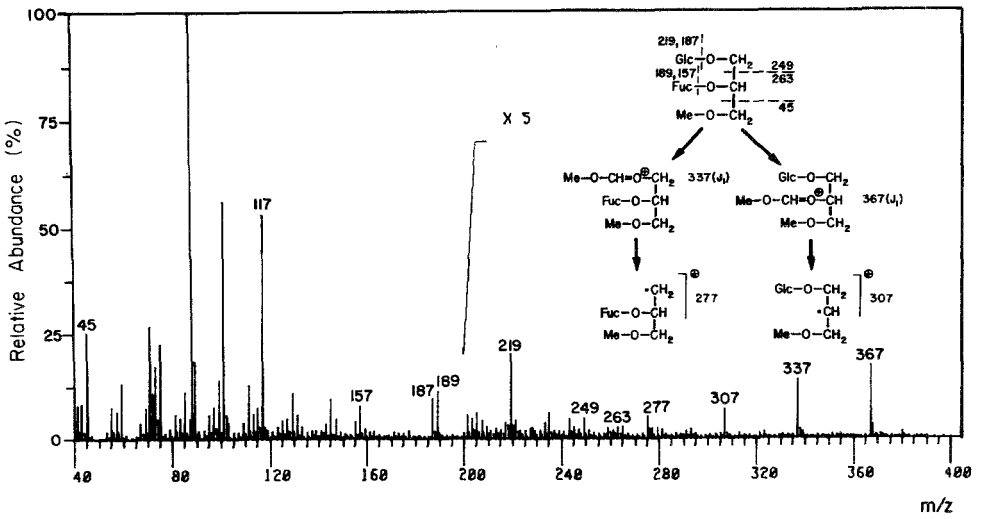

Figure 7. Mass spectrum of intact permethylated glucosyl-1-1′(fucosyl-1-2′) glycerol (peak 7 in Fig. 4).

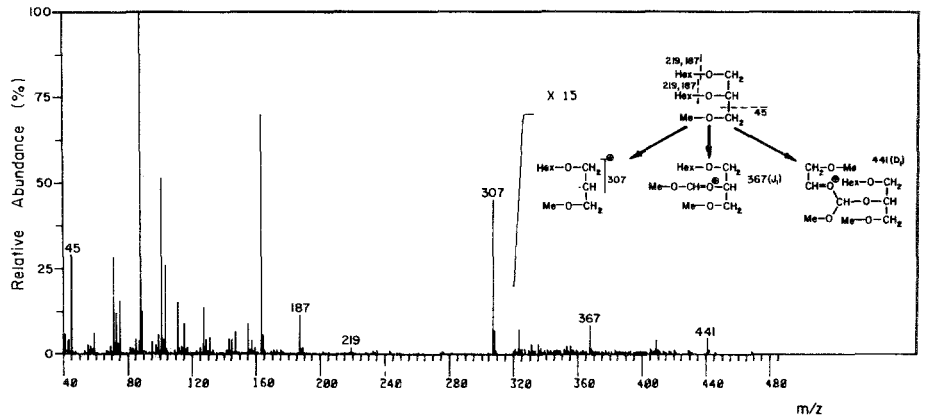

Figure 8. Mass spectrum of permethylated glucosyl(galactosyl)-1-1′[galactosyl(glucosyl)-1-2′]glycerol (peak 8 in Fig. 4).

at m/z 117 probably results from one of the J_1 ions as it has the composition $[CH_3OCH=OCH=CHOCH_3]^+$. Methylation analysis demonstrated that the hexose was glucose, and that the deoxyhexose was fucose. Thus, there are two possible structures of this compound, glucosyl-1-1′(fucosyl-1-2′)glycerol and fucosyl-1-1′(glucosyl-1-2′)glycerol, which cannot be distinguished.

Fig. 8 is the mass spectrum of Fraction 8. As with Fraction 7, cleavages occurred at the glycosyl linkage or between the carbon atoms of glycerol. The J_1 ion at m/z 367 and D_1 ion at m/z 441, and the results of methylation analysis (Table 3), revealed that there are two terminal hexoses (glucose and galactose). Thus, this molecule has one of the alternative structures, glucosyl-1-1′(galactosyl-1-2′)glycerol or galactosyl-1-1′(glucosyl-1-2′)glycerol, as shown in Fig. 8, or is a mixture of the two substances.

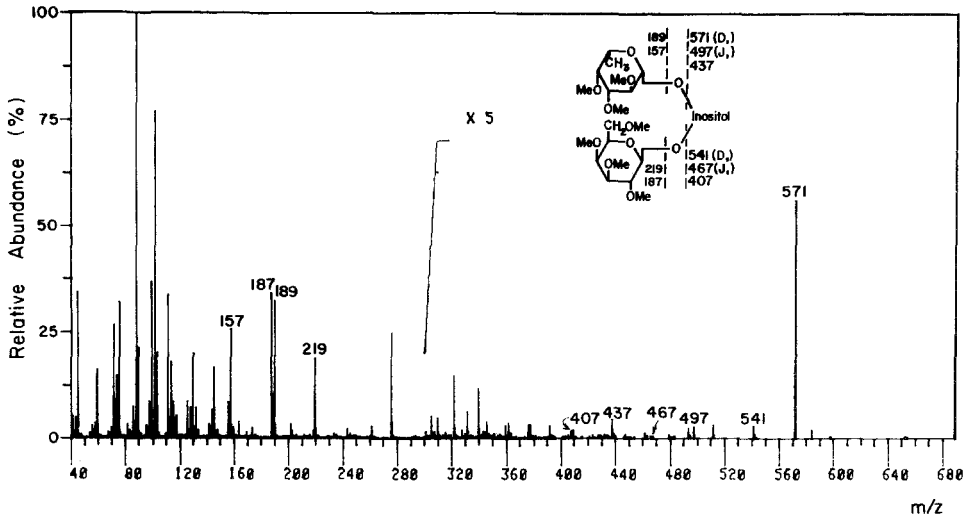

Figure 9. Mass spectrum of fucosyl galactosyl inositol (peak 14 in Fig. 4).

Structure Analysis of Inositol-containing Oligosaccharides

Four new inositol-containing oligosaccharides were found. The mass spectrum of Fraction 14 is shown in Fig. 9. In addition to a terminal deoxyhexose (fragments m/z 189 and m/z 157) and a terminal hexose (fragments m/z 219 and 187), the fragment ions at m/z 407 and 437, and the J_1 and D_1 ions at m/z 467 and 497, and 541 and 571, respectively, indicate that one inositol moiety must be linked to two sugars through glycosyl linkages. Methylation analysis of Fraction 14 indicated the presence of terminal fucose (T-fuc), terminal galactose (T-gal) and di-substituted inositol (D-inositol), in a molecular ratio of 0.74:0.79:1.0 (see Table 3).

Fig. 10 shows the proton NMR spectrum of Fraction 14. Chemical shifts near 5.2 ppm and 4.3 ppm indicate the presence of one α- and one β- anomeric linkage. Since the fucosyl α-proton has a chemical shift near 5.2 ppm (see below), the tentative partial structure should be
Galβ(Fucα)inositol.

The mass spectrum of the permethylated intact alditol of Fraction 16 (Fig. 11) was similar to that of Fraction 14 except that Fraction 16 gave an m/z 478 fragment ion and a J_1 ion at m/z 538 instead of peaks at m/z 437 and 497. These ions, which differ by 41 mass units, and the fragment ions at m/z 260 and 228 indicate that there must be an *N*-acetylhexosamine that is terminally linked to the inositol. Methylation analysis confirmed that the hexosamine was terminal and that it was GalNAc (see Table 3). The presence of two signals at 5.2 - 5.4 ppm and the absence of a signal near 4.3 ppm in the proton NMR spectrum of Fraction 16 (Fig. 10) revealed that the terminal GalNAc had an α- anomeric linkage to the inositol.

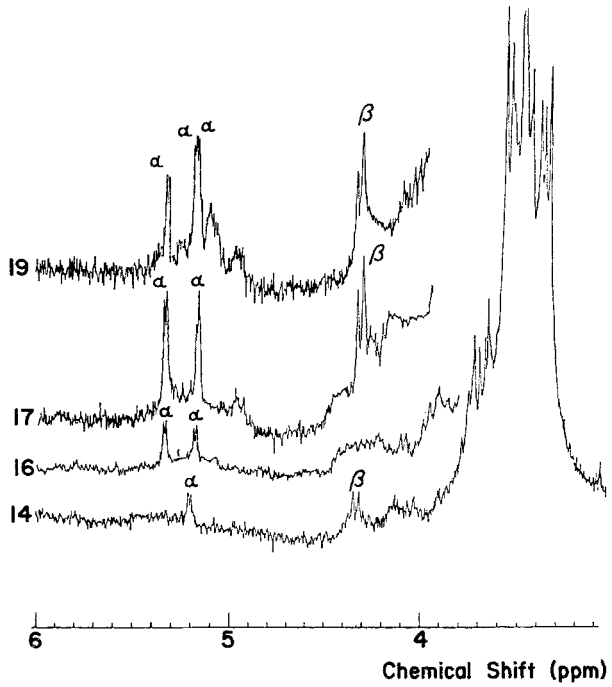

Figure 10. Proton NMR of permethylated inositol-containing oligosaccharides (peak 14, 16, 17 and 19 in Fig. 3).

Figs. 12 and 13 are the mass spectra of the intact permethylated alditols of fractions 17 and 19, respectively. Both have a terminal deoxyhexose (m/z 189 and 157) and a terminal *N*-acetylhexosamine (m/z 260 and 228). The abA_1 fragments in Fig. 12 and Fig. 13 are the same (m/z 393, 361). The ion at m/z 638 is an $abcA_1$ fragment of Fraction 19 (Fig. 13), and m/z 606 results from the loss of methanol. The abA_1 and $abcA_1$ fragments (Fraction 19) suggest a linear array of these sugar residues. Ions resulting from cleavages of the glycosidic bonds between the sugar and inositol moieties were also seen in Fraction 17 (m/z 611) and in fraction 19 (m/z 478 and 446). Results of methylation analysis of these oligosaccharides are given Table 3; the anomeric region of the proton NMR spectrum is shown in Fig. 10 and is summarized in Table 4. Comparison of the chemical shifts and coupling constants for Fractions 16, 17, and 19 indicated a β1-2Gal linkage in fraction 17 and an α1-4GlcNAc linkage in Fraction 19.

Structural Analysis of Oligosaccharides Containing Hexose at the Reducing End

Eight oligosaccharides with reducing-end hexoses were found in this investigation of human urine. Our results (data not shown) indicated that five of the structures were the same as previously reported compounds (cf. Table 1); two oligosaccharides could not be characterized due to the small sample size.

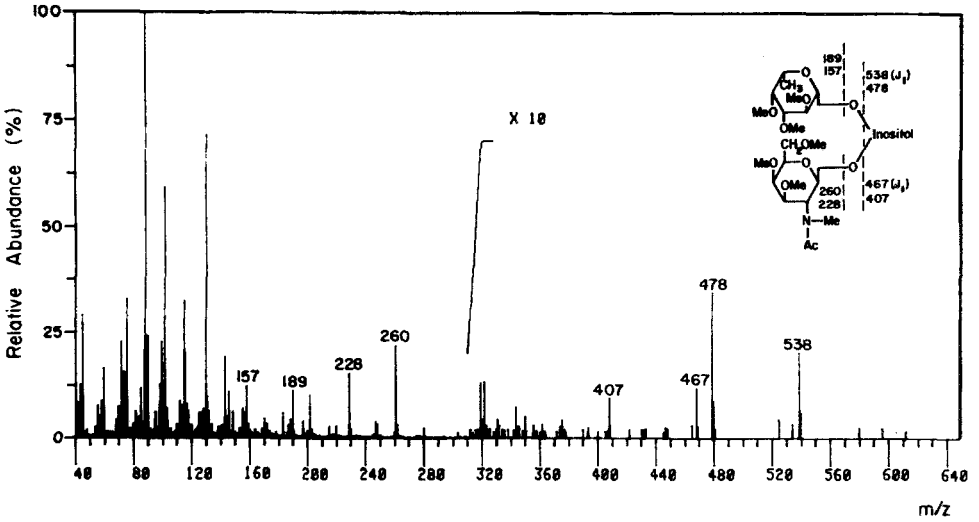

Figure 11. Mass spectrum of permethylated fucosyl *N*-acetylgalactosaminyl inositol (peak 16 in Fig. 4).

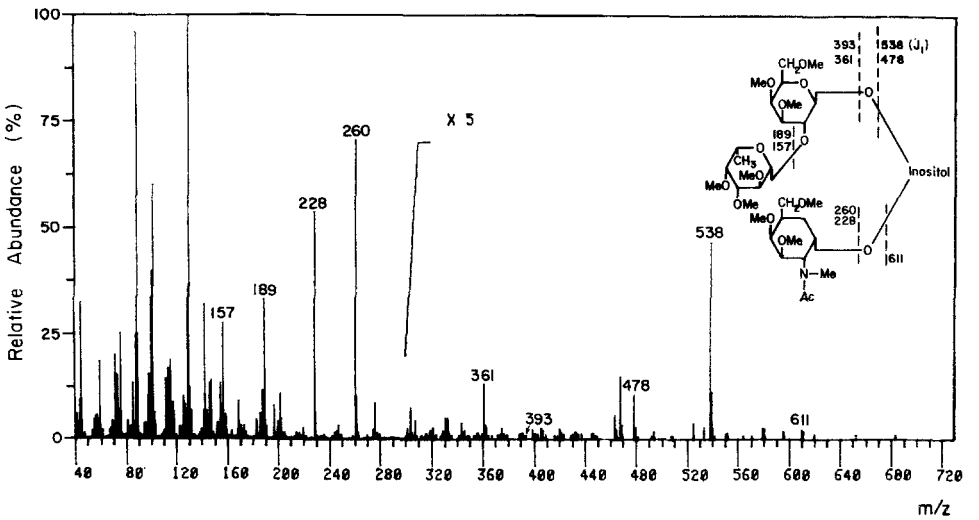

Figure 12. Mass spectrum of permethylated inositol-containing tetrasaccharide (peak 17 in Fig. 4).

Fig. 14 is the mass spectrum of the intact permethylated hexasaccharide alditol (Fraction 20 in Fig. 4). The ions at m/z 189 and 157 indicate the existence of a terminal deoxyhexose. The fragment ion at m/z 409 further demonstrates that the deoxyhexose is linked to a reducing terminal hexose alditol which has a second carbohydrate side-chain. The fragment ion at m/z 919 is formed by cleavage at the backbone of the di-substituted alditol and fragment ions m/z 597 and 565 (elimination of methanol) indicate that this branch contains a three sugar residue. Loss from m/z 597 of a substituent at C-3 gives an ion at m/z 361. By methylation analysis (Table 3), a 4,6-di-*O*-methyl-1,2,3,5-tetra-acetyl-ga-

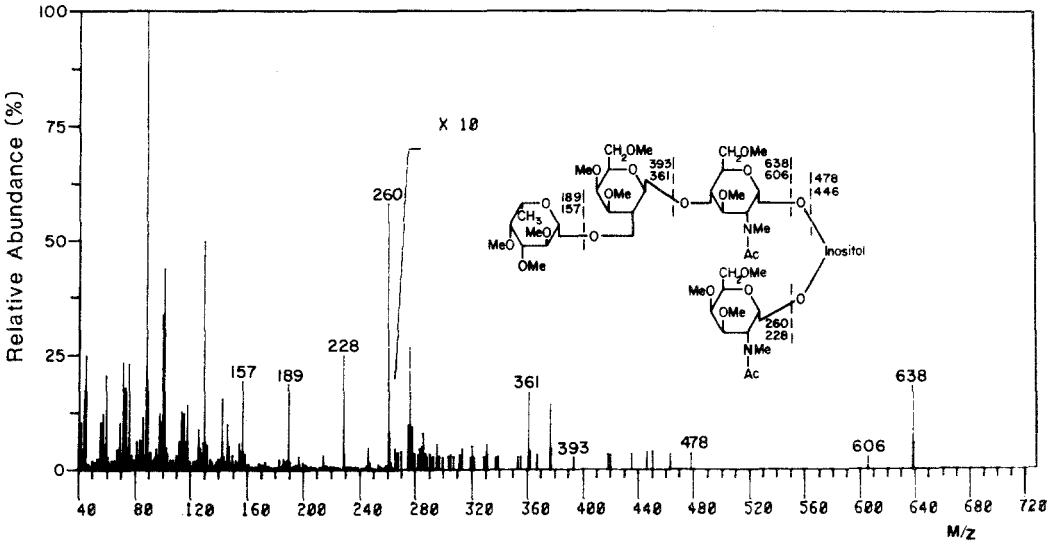

Figure 13. Mass spectrum of permethylated inositol-containing pentasaccharide (peak 19 in Fig. 4).

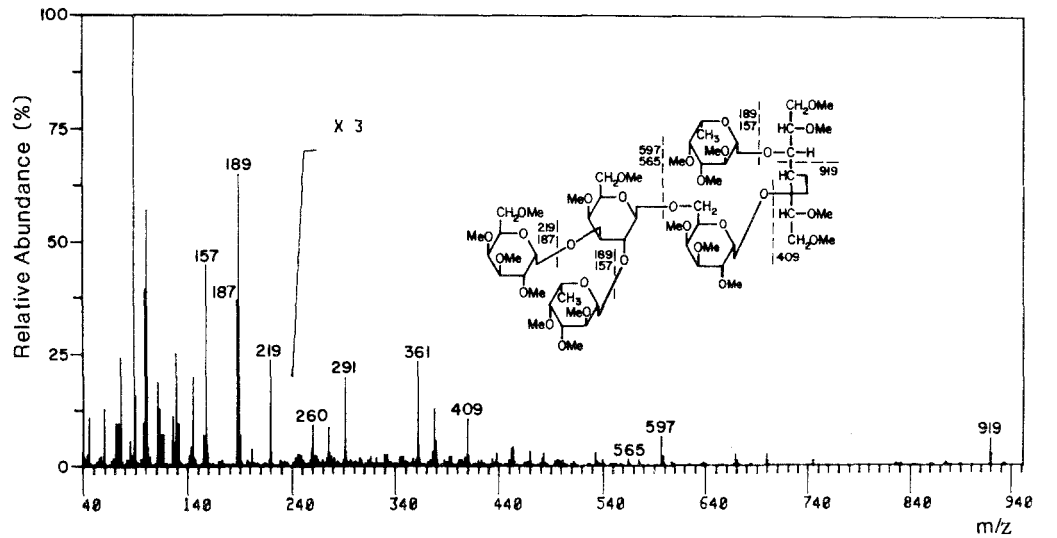

Figure 14. Mass spectrum of permethylated reducing end hexose hexasaccharide (peak 20 in Fig. 4).

lactose was found, confirming the presence of the branched structure. The proton NMR spectrum of Fraction 20 contained signals at about 5.2 ppm for two α-fucosyl linkages. Chemical shifts near 4.9 and 5.4 ppm were also apparent, indicating that the non-reducing terminal galactose is α-linked, as is the internal galactose that is linked to glucose. A signal at 4.3 ppm arose from the β-linked di-substituted galactose. Thus, Fraction 20 is a previously unreported oligosaccharide with the tentative structure shown in Table 2 and Fig. 14.

Discussion

In this investigation, the structures of ten previously unreported urinary oligosaccharides are detailed. Their structures were determined by a combination of GC, GC-MS, proton NMR spectroscopy, and methylation analysis. Three different types of oligosaccharide were found: five oligosaccharides terminated with glycerol, four terminated with inositol, and one had a hexose at its reducing terminus. While inositol-containing oligosaccharides have been previously reported in urine [10], the finding of glycerol-containing oligosaccharides is new.

To our knowledge nothing is known about the biosynthesis, tissue origin, and possible functions of these glycerol- and inositol-containing carbohydrate structures. However, it is well known that glycerol and inositol are constituents of phospholipids found in cell membranes. Perhaps these oligosaccharides are derived from novel "glycolipids" with fatty acid hydrophobic moieties and oligosaccharides such as those recovered from urine. Such "glycolipids" could have functions similar to those of glycosphingolipids or might have completely unrelated functions. The finding of oligosaccharides with inositol substituted for glucose in both type A and B individuals [7, 18] suggests that, similar to glycosphingolipids, these oligosaccharides may play some role in the expression of blood group specificities on the cell surface.

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NMR spectra were obtained at the NMR Facility in the Department of Chemistry at Michigan State University.

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