A Mannose-containing Trisaccharide Isolated from Urines of Three Patients with Mannosidosis*

NILS E. NORDÉN AND ARNE LUNDBLAD

From the Institute of Medical Chemistry, University of Uppsala, Uppsala, Sweden

SIGFRID SVENSSON

From the Department of Organic Chemistry, University of Stockholm, Stockholm, Sweden

PER-ARNE ÖCKERMAN

From the Department of Clinical Chemistry, University Hospital, Lund, Sweden

SEPPO AUTIO

From the Department of Pediatrics, University Central Hospital, Helsinki, Finland

SUMMARY

The urine from three patients with mannosidosis was found to contain abnormally high amounts of mannose-containing oligosaccharides. The highest yield (123 to 430 mg per liter of urine) was obtained of a trisaccharide, which was isolated and characterized. The isolation procedure included ultrafiltration, gel chromatography on Sephadex G-25, purification by preparative zone electrophoresis, and preparative paper chromatography. Sugar analysis showed that the trisaccharide consisted of 2 D-mannose moieties and 1 2-acetamido-2-deoxy-D-glucose residue. The linkages and sequential order of the sugar units were established by methylation analysis of the reduced trisaccharide. The partially methylated sugars obtained on hydrolysis of the methylated trisaccharide alditol were analyzed by gas-liquid chromatography and mass spectrometry. The anomeric configurations were determined by chromium trioxide oxidation followed by methylation analysis. Additional proof for the anomeric natures of the sugar units was obtained by enzymatic hydrolysis studies and by inhibition experiments using a precipitin system with concanavalin A. As a result of these studies, the structure of the trisaccharide could be established as \(\alpha-D\)-mannopyranoside-(1\(\rightarrow\)3)-\(\beta-D\)-mannopyranoside-(1\(\rightarrow\)4)-2-acetamido-2-deoxy-D-glucose. The oligosaccharide is most probably a degradation product derived from the inner core of glycoprotein chains.

In 1967 a new lysosomal storage disease, which has since been called mannosidosis, was discovered (1). The patient presented

* This research was aided by grants from the Swedish Medical Research Council (B72-13X-006B, 19X-2222, B73-15X-2822-06A) and the Medical Faculty, University of Lund, Forstamatbiummans riksföreund and Stiftelsen Solsticken.

† On temporary leave from the Department of Clinical Chemistry, University Hospital, Lund, Sweden.
be detected, and very low activities of other hydrolases were found. Concanavalin A was obtained from Sigma Chemical Company and glycogen from Nutritional Biochemicals Corporation (Cleveland, Ohio). All other chemicals and reagents were reagent grade commercial products.

General Methods—Bacterial growth was prevented by the addition of phenyl mercuric nitrate (30 ml of saturated solution per liter of urine). The urines were filtered and ultrafiltered at 4°C as described by Berggård (10), using Visking 23/32-inch dialysis tubing (Union Carbide Corporation, Chicago, Ill.). This tubing retains protein molecules larger than 10^6 daltons (10). Ultrafiltrates and chromatographic fractions were concentrated by rotary evaporation (water-bath temperature 40°C). Gel chromatography, preparative zone electrophoresis and preparative paper chromatography were carried out as previously described (11, 12), using the following solutions and solvent mixtures: 2 M acetic acid (pH 1.9) (Solvent a), ethyl acetate-acetic acid-water (3:1:1, v/v) (Solvent b), n-propyl alcohol-ethyl acetate-water (6:1:3, v/v) (Solvent c), ethyl acetate-pyridine-water (2:1:2, v/v) (Solvent d), n-butyl alcohol-pyridine-water (6:4:3, v/v) (Solvent e), butyl acetate-acetic acid-water (3:2:1, v/v) (Solvent f), and n-butyl alcohol-acetic acid-water (4:1:5, v/v) (Solvent g).

Analytical Methods—Colorimetric methods for determination of hexose, 6-deoxyhexose, hexosamine, and sialic acid have been described earlier (11, 12). Nitrogen was determined by the ninhydrin procedure (13). The hexosamines were identified by the method of Stoffyn and Jeanloz (14) and N-acetylhexosamines were determined as previously described (12). Monosaccharides were determined as their alditol acetates by gas-liquid chromatography (15) and mass spectrometry (16). For gas-liquid chromatography a Perkin-Elmer 990 instrument equipped with a column packed with 3% ECNSS-M on Chromosorb W (60/100 mesh) or with 3% UC W-98 on Chromosorb Q was used at 190-203°C. For mass spectrometry, a Perkin-Elmer 270 gas-liquid chromatography-mass spectrometry instrument fitted with a column packed with UC W-98 on Chromosorb Q was used at 190°C. Mass spectra were recorded at an ionization potential of 70 e.v., an ionization current of 80 µA, and an ion source temperature of 80°C. The absolute configurations (α or β) of the individual monosaccharides and optical rotations were determined with a Perkin-Elmer 141 polarimeter. Methylation analysis was performed as previously described (17). The presence and position of a β-glycosidic linkage were determined by chromium trioxide oxidation (18) followed by methylation analysis (19). Digestion with α-mannosidase was performed under the conditions described by Li et al. (9). The inhibitory activity of the isolated oligosaccharide in a concanavalin A-glycogen system was measured essentially as described by Goldstein (20). Total nitrogen in the washed precipitates was measured by the ninhydrin method.

Isolation of Oligosaccharides—Concentrated urinary ultrafiltrates were fractionated by gel chromatography on a Sephadex G-25 column as illustrated in Fig. 1. The eluted material was pooled into six fractions (A through F), as indicated. The mannose-rich Fractions C and D were further purified by preparative zone electrophoresis (solvent a, 3.5 volts per cm for 24 hours). The mannose-containing material in both Fractions C and D was stationary. The purified Fractions C and D were studied by paper chromatography (Solvent b) (Fig. 2). At least five components were observed in both Fractions C and D. The main component in Fraction D (R_f values of 0.82) was also present in small amounts in Fraction C, was obtained by preparative paper chromatography in this solvent system. Compound R_f 0.82 was finally passed through a column of Bio-Gel P-2. Hexose assays of eluted fractions revealed a symmetrical peak (R_f 0.32); its corresponding fractions were pooled and the material lyophilized.

RESULTS

Urine samples of the three patients after fasting were fractionated on a Sephadex G-25 column (5 x 130 cm, eluted with distilled water at 4°C). The eluates were analyzed for total hexose, 6-deoxyhexose, and sialic acid. The distribution of

<table>
<thead>
<tr>
<th>Fraction C</th>
<th>Fraction D</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Diagram showing Fraction C and Fraction D with corresponding R_f values for each fraction.]</td>
<td>[Diagram showing Fraction C and Fraction D with corresponding R_f values for each fraction.]</td>
</tr>
</tbody>
</table>

FIG. 1. Gel chromatography on a column (101 X 10 cm, void volume = 2900 ml) of Sephadex G-25, fine, of a urinary ultrafiltrate from a patient with mannosidosis. Fractions (25 ml) were collected and assayed for hexose. The material was pooled and concentrated to give Fractions A through F, as indicated by the vertical lines.

FIG. 2. Fractionation by paper chromatography of gel chromatographic Fraction C and Fraction D. The papers were developed in an ethyl acetate-acetic acid-water system, 3:1:1, and stained with a silver dip reagent.
with no restrictions on food intake. The relative amounts of the different subfractions obtained from healthy persons, described previously (12, 21).

Compound $R_L$ 0.82 was isolated from urines of the three patients are given in Table I. The relative amounts of the different subfractions obtained from nonfasting patients, described in Fig. 1. The 6-deoxyhexose and sialic acid patterns did not differ from those obtained from healthy persons, described previously (12, 21).

For preparative purposes urine could be collected from patients with no restrictions on food intake. The compound was homogeneous in the following paper chromatographic systems: Solvent c (RL 0.99), Solvent d (RL 0.93), Solvent e (RL 0.84), Solvent f (RL 0.78), and Solvent g (RL 0.42). Analytical properties and optical rotations of compound $R_L$ 0.82 varied from 123 to 430 mg per liter of urine (Table I).

The identified monosaccharides, $\alpha$-mannose and 2-acetamido-2-deoxy-$\alpha$-D-mannose, were identical in the three cases. The yields of compound $R_L$ 0.82 were isolated from urines of the three patients by the method outlined under “Experimental Procedure.” The relative molar percentage of the partially methylated hexosaminitols could not be determined, as their response factors are not known.

Analytical properties and optical rotations of isolated oligosaccharides (RL 0.82) are given in Table I.

### Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>Composition</th>
<th>Molar ratios</th>
<th>$[\alpha]^{19}_{D}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-Tetra-O-Me-$\alpha$-Man</td>
<td>30.2</td>
<td>1.10</td>
<td>0.98</td>
</tr>
<tr>
<td>2,3,4,6-Tetra-O-Me-$\beta$-Man</td>
<td>30.2</td>
<td>1.10</td>
<td>0.98</td>
</tr>
<tr>
<td>2,3,5,6-Tetra-O-Me-GlcN(Me)Ac-OL</td>
<td>1.85</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>1,3,5,6-Tetra-O-Me-GlcNAc-OL</td>
<td>2.69</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

### Table II

<table>
<thead>
<tr>
<th>Sugars</th>
<th>$T$ values</th>
<th>Relative molar %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-Tetra-O-Me-$\alpha$-Man</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>2,3,4,6-Tetra-O-Me-$\beta$-Man</td>
<td>1.39</td>
<td></td>
</tr>
<tr>
<td>1,3,5,6-Tetra-O-Me-GlcN(Me)Ac-OL</td>
<td>1.85</td>
<td>+</td>
</tr>
<tr>
<td>1,3,5,6-Tetra-O-Me-GlcNAc-OL</td>
<td>2.69</td>
<td>+</td>
</tr>
</tbody>
</table>

### Table III

<table>
<thead>
<tr>
<th>Sugars</th>
<th>$T$ values</th>
<th>Relative molar %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,3,4,6-Tetra-O-Me-$\alpha$-Man</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>2,3,4,6-Tetra-O-Me-$\beta$-Man</td>
<td>1.39</td>
<td></td>
</tr>
<tr>
<td>1,3,5,6-Tetra-O-Me-GlcN(Me)Ac-OL</td>
<td>1.85</td>
<td>+</td>
</tr>
<tr>
<td>1,3,5,6-Tetra-O-Me-GlcNAc-OL</td>
<td>2.69</td>
<td>+</td>
</tr>
</tbody>
</table>

#### Footnotes

a) Retention times of the corresponding alditol acetates relative to 1,3-di-$O$-acetyl-2,3,4,6-tetra-$O$-methyl-$\beta$-mannitol.

b) Retention times of the corresponding alditol acetates relative to 1,5-di-$O$-acetyl-2,3,4,6-tetra-$O$-methyl-$\beta$-glucitol.

c) The relative molar percentage of the partially methylated hexosaminitols could not be determined, as their response factors are not known.

d) 2,3,4,6-Tetra-$O$-methyl-$\alpha$-mannose.

### Figures

**Fig. 3.** Mass spectrum for the component in the peak at $T_{ECNSS-M} = 1.85$, obtained in methylation analysis of $R_L$ 0.82 after reduction with sodium borohydride.

**Fig. 4.** Mass spectrum for the component in the peak at $T_{ECNSS-M} = 1.85$, obtained in methylation analysis of $R_L$ 0.82 after reduction with sodium borohydride.

**Fig. 5.** Primary fragmentation pattern for a 2-(N-methyl)-acetamido-2-deoxy-$\alpha$-D-glucitol (values for the deuterated analogue are given in parentheses).
The specific optical rotation (+29° to +35°) indicated the presence of one α and one β linkage in the trisaccharide (22). Therefore, the borohydride-reduced oligosaccharide was acetylated and oxidized with chromium trioxide in acetic acid. By this oxidation α-linked sugars are converted into 5-keto-hexulosonates and β-linked sugars are unaffected (18). Sugar analysis after oxidation showed that 45% of the α-D-mannose residues were oxidized. By methylation the oxidized oligosaccharide is cleaved at the oxidized residue and the O-acetyl groups are split off (Fig. 9). Gas-liquid chromatography of the methylated, oxidized oligosaccharide alditol gave only one peak which gave a mass spectrum with ions that are expected for a 2-(N-<br>methylethyl)-acetamido-2-deoxy-1,3,4,5,6-penta-O-methyl hexitol (Fig. 10). The primary fragmentation is depicted in Fig. 11.

Digestion of 50 µg of compound R₈ 0.82 with α-mannosidase (0.1 unit of enzyme per 30 µl of reaction mixture) gave a 50% release of the original mannose content (Fig. 12). Addition of fresh enzyme after 48 hours gave no further release of mannose.

The ability of compound R₈ 0.82 to inhibit the precipitation of concanavalin A by glycogen was compared with that of methyl α-D-mannopyranoside and methyl α-D-glucopyranoside. A 50% inhibition was obtained with 0.25 µmole of compound R₈ 0.82 as compared with 0.20 and 0.70 µmole of the methyl α-D-mannopyranoside and methyl α-D-glucopyranoside, respectively. The only structure possible from the evidence provided...
paper chromatography in five different solvent systems. Sugar methyl-n-mannose and 2,4,6-tri-O-methyl-n-mannose by gas-alditol acetates. The other two components were identified as a 2-(N-inctll-
acetamido-2-deoxy-1, 3,5,6-tetra-O-methyl hexitol.

The isolated oligosaccharide, RL 0.82, was homogeneous by paper chromatography in five different solvent systems. Sugar analysis showed that RL 0.82 consisted of 2 moles of n-mannose and 1 mole of 2-acetamido-2-deoxy-n-glucose. Methylation analysis of the reduced oligosaccharide yielded four components. Two of the compounds were identified as 2,3,4,6-tetra-O-methyl-n-mannose and 2,4,6-tri-O-methyl-n-mannose by gas-liquid chromatography-mass spectrometry of the corresponding alditol acetates. The other two components were identified as a 2-(N-methyl)-acetamido-2-deoxy-1, 3,5,6-tetra-O-methyl hexitol and a 2-acetamido-2-deoxy-1,3,5,6-tetra-O-methyl hexitol by mass spectrometry of their alditol acetate derivatives. The identification of the methoxyl substitution patterns by mass spectrometry, was made using principles previously established for partially methylated alditol acetates (17). Since 2-acetamido-2-deoxy-n-glucose was the only aminosugar present in the oligosaccharide the reduced, methylated aminosugar derivative was oxidized with chromium trioxide in acetic acid and subjected to methylation analysis. This method enabled us to show that the β-linked n-mannose residue was linked to the 2-acetamido-2-deoxy-n-glucose residue. The anomeric nature of the n-mannose residues was also demonstrated by enzymatic hydrolysis and the quantitative precipitin inhibition experiments.

Acknowledgments—The authors are indebted to Mrs. Gunilla Pettersson, Mrs. Elwy Wallin, and Mrs. Birgit Boman for valuable technical assistance.

DISCUSSION

The isolated oligosaccharide, RL 0.82, was homogeneous by paper chromatography in five different solvent systems. Sugar analysis showed that RL 0.82 consisted of 2 moles of n-mannose and 1 mole of 2-acetamido-2-deoxy-n-glucose. Methylation analysis of the reduced oligosaccharide yielded four components. Two of the compounds were identified as 2,3,4,6-tetra-O-methyl-n-mannose and 2,4,6-tri-O-methyl-n-mannose by gas-liquid chromatography-mass spectrometry of the corresponding alditol acetates. The other two components were identified as a 2-(N-methyl)-acetamido-2-deoxy-1, 3,5,6-tetra-O-methyl hexitol and a 2-acetamido-2-deoxy-1,3,5,6-tetra-O-methyl hexitol.
REFERENCES
A Mannose-containing Trisaccharide Isolated from Urines of Three Patients with Mannosidosis

Nils E. Nordén, Arne Lundblad, Sigfrid Svensson, Per-Arne Öckerman and Seppo Autio


Access the most updated version of this article at [http://www.jbc.org/content/248/17/6210](http://www.jbc.org/content/248/17/6210)

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at [http://www.jbc.org/content/248/17/6210.full.html#ref-list-1](http://www.jbc.org/content/248/17/6210.full.html#ref-list-1)