Characterization of Two Mannose-Containing Oligosaccharides Isolated from the Urine of Patients with Mannosidosis†

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ABSTRACT: Patients with mannosidosis were recently found to excrete a number of mannose-containing oligosaccharides. The preponderant one was a trisaccharide, identified as α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-D-GlcNAc (185–430 mg/l. of urine) (N. E. Nordén, Lundblad, A., Svensson, S., Ockerman, P. A., and Autio, S. (1973), *J. Biol. Chem. 248*, 6210). One tetra- and one pentasaccharide have now been isolated from the urine of the same patients (about 60 mg each per liter of urine). Structural studies, including sugar analyses, methylation analyses, and enzymatic degradation, revealed the following structures: α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-D-GlcNAc and α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-D-GlcNAc.

Mannosidosis is a lysosomal storage disease characterized by the tissue accumulation (Öckerman, 1967, 1969) and urinary excretion (Nordén *et al.*, 1973c; Autio *et al.*, 1973) of mannose-containing compounds. The enzymatic defect involves α-mannosidase, an enzyme occurring in at least three different forms called A, B, and C. It was demonstrated that the A and B forms of α-mannosidase are missing in liver tissue from patients with mannosidosis (Carroll *et al.*, 1972). It has recently been shown that at least five different mannose-containing oligosaccharides are excreted in the urine in these patients. The preponderant oligosaccharide (185–430 mg/l. of urine) was a trisaccharide with the following structure, α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-D-GlcNAc[1] (Nordén *et al.*, 1973b). This paper reports the isolation and complete characterization of a tetra- and a pentasaccharide also excreted in large amounts in these urines. The previously isolated trisaccharide was found to be an integral part in the two new oligosaccharides.

Experimental Procedure

Materials. Urine was collected from three patients with mannosidosis. The clinical description of these patients (P. S., A. S., and H. K.) has been reported by Autio *et al.* (1973). The urine specimens, which were collected from each patient with no dietary restrictions, were the same as those used for isolation of the trisaccharide; R_L 0.82 (Figure 4).

Preservation. Bacterial growth was prevented by the addition of phenylmercuric nitrate (30 ml of saturated solution/l.).

Analytical Methods. Ultrafiltration of filtered urine samples was performed at 4° with Visking $^{23}/_{32}$-in. dialysis tubing (Union Carbide Corp., Chicago, Ill.) and a negative pressure of 660 mm (Berggård, 1962). Gel chromatography was carried out using a Sephadex G-25 (fine) column (11.5 × 114 cm; void volume = 4500 ml) and a Bio-Gel P-2 (minus 400 mesh) column (1.5 × 87 cm; void volume = 39 ml).

Preparative zone electrophoresis in 2 M acetic acid was done using the block method described by Kunkel (1954) on Pevikon C 870 (Müller-Eberhard, 1960). Whatman No. 1 papers were used for descending paper chromatography in the following solvent systems: ethyl acetate–acetic acid–water (3:1:1, v/v) (a), *n*-propyl alcohol–ethyl acetate–water (6:1:3, v/v) (b), ethyl acetate–pyridine–water (2:1:2, v/v) (c), *n*-butyl alcohol–pyridine–water (6:4:3, v/v) (d), butyl acetate–acetic acid–water (3:2:1, v/v) (e), *n*-butyl alcohol–acetic acid–water (4:1:5, v/v) (f), ethanol–ethyl acetate–water (6:1:3, v/v) (g), and *n*-propyl alcohol–ethyl acetate–water (42:35:23, v/v) (h). Papers were stained with a silver dip reagent (Smith, 1960).

Colorimetric methods were used for determining total hexose (Scott and Melvin, 1953) and hexosamine (Rondle and Morgan, 1955).

Sugar analysis was performed by glc (Sawardeker *et al.*, 1965) and mass spectrometry (Chizhov *et al.*, 1966). The hexosamine present was also identified in the way described by Stoffyn and Jeanloz (1954) and *N*-acetylhexosamine es previously described by Lundblad (1967). Optical rotation was measured in a Perkin-Elmer 141 polarimeter. Methylation analysis was carried out as described by Björndal *et al.* (1970) and Nordén *et al.* (1973b).

For mass spectrometry a Perkin-Elmer 270 glc-mass spectrometry instrument fitted with a column packed with UC W-98 on Chromosorb Q or OV 225 (S.C.O.T. column) was used at 190°. Mass spectra were recorded at an ionization potential of 70 eV, an ionization current of 80 μA, and an ion source temperature of 80°.

Enzymatic Degradation Experiments. Oligosaccharides were enzymatically degraded with α-mannosidase under the conditions reported by Li (1967). The enzyme was prepared, from Jack bean meal, by the method of Snaith and Levvy (1968). The enzymatic activities were measured and one unit of enzyme activity was defined according to Li and Lee (1972). In this preparation no β-mannosidase activity was detected, using *p*-nitrophenyl β-D-mannopyranoside as the substrate. In a typical experiment the oligosaccharide (20 mg) was digested with α-mannosidase (1–25 units) in 10 ml of 0.1 M acetate buffer (pH 4.6) for 1 hr at 37°. The reaction

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[1] Abbreviations used are: Man*p*, mannopyranoside; GlcNAc, 2-acetamido-2-deoxyglucose; glc, gas–liquid chromatography; R_L, $R_{lactose}$; Me, methyl; Ac, acetyl.

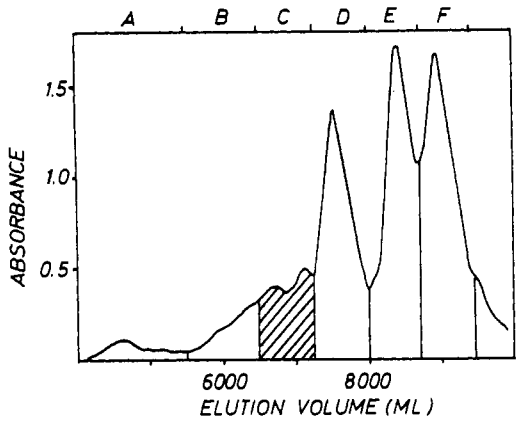

FIGURE 1: Gel chromatographic distribution of hexose-containing material in ultrafiltrates of urine from a patient (H. K.) with mannosidosis; 100 ml of concentrated ultrafiltrate was applied. The fractionation was performed on a Sephadex G-25 (fine) column (11.5 × 114 cm); elution rate, 100 ml/hr; eluent, distilled water with preservative. Trisaccharide R_L 0.82 has previously been isolated from fraction D (Nordén *et al.*, 1973b).

mixture was then heated at 100° for 5 min and desalted by passage through two different ion exchangers (Dowex 50 (H^+) and Dowex 3 (OH^-)). The digests were fractionated by preparative paper chromatography (solvent c).

Results

Isolation of the Oligosaccharides. Urinary ultrafiltrates from the three patients were concentrated ten times and applied to a Sephadex G-25 column. The eluates were analyzed for total hexose. A representative fractionation is shown in Figure 1. The mannose-rich fraction C was pooled and purified further by preparative zone electrophoresis. The mannose-containing material was stationary. Further fractionation of this material from all three patients by preparative paper chromatography with solvent a gave identical results and yielded at least five components (Figure 2A) with the following R_L values: 0.46, 0.21, 0.15, 0.10, and 0.05. Compounds R_L 0.46 and R_L 0.21 were further purified on papers using system g (Figure 2B). A minor component was removed from compound R_L 0.21 and R_L 0.46. These minor components were designated R_L 0.21b and R_L 0.46b. The main components were called R_L 0.21a and R_L 0.46a. All four components were finally passed through a column of Bio-Gel P-2. Hexose analyses of eluted fractions revealed symmetrical peaks. The yields of the four components are given in Table I.

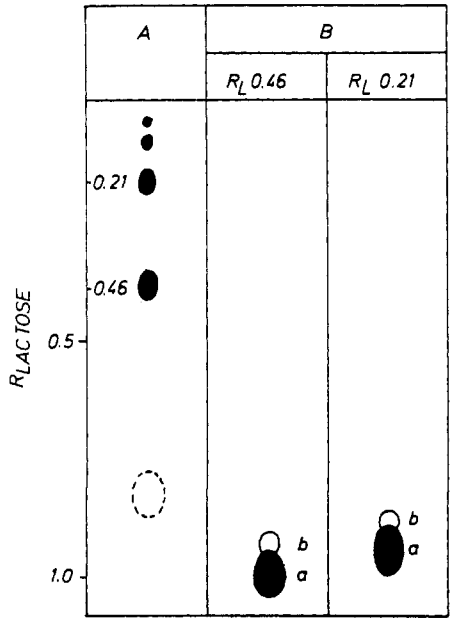

FIGURE 2: (A) Fraction C after electrophoresis in 2 M acetic acid further fractionated by paper chromatography (Whatman 1) solvent a. (B) Further fractionation of compounds R_L 0.21 and R_L 0.46 on papers developed with system g.

TABLE I: Analytical Properties of Isolated Oligosaccharides.

Oligosaccharide	Yield (mg/l.)	Composition D-Man (%)	Composition D-Glc-NAc (%)	Molar Ratios D-Man	Molar Ratios D-Glc-NAc	$[\alpha]_D^{20}$ (deg)
R_L 0.21a	60	76.1	23.2	4.0	1.0	+49
R_L 0.46a	58	68.1	28.0	2.9	1.0	+48
R_L 0.21b	3.7	38.1	20.3	2.3	1.0	N.D.
R_L 0.46b	2.7	63.2	37.2	2.1	1.0	+31

Characterization of the Oligosaccharides. The oligosaccharides R_L 0.21b and R_L 0.46b were obtained in minute amounts only and were therefore characterized only by sugar analyses (Table I). These compounds were not studied further. The main oligosaccharides R_L 0.21a and R_L 0.46a were homogeneous as judged by paper chromatography in five solvent systems (Table II). Yields, analytical data, and optical rotations for R_L 0.21a and R_L 0.46a isolated from one patient (A. S.) are given in Table I. R_L 0.21a and R_L 0.46a yielded on acid hydrolysis D-mannose and 2-amino-2-deoxy-D-glucose in the relative molar proportions 3.9:1.0 and 2.9:1.0, respectively. The absolute configurations of the sugars were not determined, but there is good reason to assume that they both have the D configuration in analogy with the same sugar components isolated from the trisaccharide R_L 0.82 (Nordén *et al.*, 1973b) (Figure 4).

Methylation analysis of the oligosaccharides, after reduction with borohydride, gave the results shown in Table III. In order to determine the sequential order of the D-mannose residues in the oligosaccharides, they were subjected to enzymatic hydrolysis by α-mannosidase. The trisaccharide R_L 0.82 was treated in the same way. The oligosaccharides obtained from each oligosaccharide after digestion with α-mannosidase for 1 hr at 37° were fractionated by paper chromatography (Figure 3). By comparison of chromatographic mobilities it was inferred that all the components in the hydrolysate of R_L 0.82 occurred in the digests of R_L 0.21a and 0.46a. Furthermore, by the same criterion, all the components in the R_L 0.46a digest were in the enzymatic digest of R_L 0.21a. The only monosaccharide found in the enzymatic hydrolysates was D-mannose. The oligosaccharides X_3 (R_L

TABLE II: Paper Chromatographic Properties of Compound R_L 0.46a and R_L 0.21a (R_L Values in Different Solvent Systems).

Oligosaccharide	R_L Value in Solvent b	c	d	g	h
R_L 0.46a	0.82	0.48	0.39	1.05	0.66
R_L 0.21a	0.63	0.20	0.12	0.93	0.48

TABLE III: Methyl Ethers Obtained in the Methylation Analysis of the Reduced Oligosaccharides.

Sugars	*T* Values		Relative Molar Proportion[c]					
	UC W-98[a]	OV 225[b]	R_L 0.21a	R_L 0.46a	X_2 (R_L 0.21a)	X_3 (R_L 0.21a)	X_2 (R_L 0.46a)	X_3 (R_L 0.46a)
2,3,4,6-Tetra-*O*-Me-Man	1.00	1.00	1.0	1.0	+	1.0	+	1.0
3,4,6-Tri-*O*-Me-Man	1.39	1.82	1.9	1.0				
2,4,6-Tri-*O*-Me-Man	1.39	1.90	1.0	1.0		0.9		0.9
1,3,5,6-Tetra-*O*-Me-GlcN(Me)Ac-ol[d]	1.85				+	+	+	+
1,3,5,6-Tetra-*O*-Me-GlcNAc-ol	2.65				+	+	+	+

[a] Retention times of the corresponding alditol acetates relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-mannitol. [b] Retention times of the corresponding alditol acetates relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [c] The relative molar proportion of the partially methylated hexosaminitols could not be determined as their response factors are not known. [d] 1,3,5,6-Tetra-*O*-Me-GlcN(Me)Ac-ol = (*N*-methyl)-2-acetamido-2-deoxy-1,3,5,6-tetra-*O*-methyl-D-glucitol.

0.46a), X_2 (R_L 0.46a), X_3 (R_L 0.21a), and X_2 (R_L 0.21a) (see Figure 3) were isolated by preparative paper chromatography. The yields, optical rotations, and sugar analyses of these oligosaccharides are given in Table IV. After reduction with sodium borohydride the four oligosaccharides were subjected to methylation analyses (Table III).

Discussion

The oligosaccharide R_L 0.46a was homogeneous as judged by paper chromatography in five solvent systems and it appeared as a single peak on Bio-Gel P-2. Sugar analysis demonstrated that it consisted of three D-mannose units and one 2-acetamido-2-deoxy-D-glucose moiety. The sugars were separated and qualitatively analyzed by glc and characterized by mass spectrometry. Methylation analyses of the reduced R_L 0.46a showed one nonreducing terminal D-mannopyranose residue, two chain D-mannopyranose residues linked at the 2 and 3 positions, respectively, and one reducing terminal 4-linked 2-acetamido-2-deoxy-D-glucose residue. The relative order of the two internal D-mannose units and the anomeric configurations of the D-mannose residues were determined by partial enzymatic digestion of R_L 0.46a with α-mannosidase followed by isolation of the oligosaccharides formed. X_2 (R_L 0.46a) consisted of 1 mol of D-mannose as nonreducing terminal and 1 mol of 1→4-linked 2-acetamido-2-deoxy-D-glucose as the reducing end which was demonstrated by sugar and methylation analyses. Judging from the optical rotation, $[\alpha]_D$ −3.3°, the disaccharide contained a β link. The structure of X_2 (R_L 0.46a) is therefore β-D-Man*p*-(1→4)-D-GlcNAc. X_3 (R_L 0.46a) consisted of 2 mol of D-mannose and 1 mol of 2-acetamido-2-deoxy-D-glucose and had the same optical rotation and the same chromatographic mobility in solvent system c as the trisaccharide R_L 0.82 previously isolated. Methylation analyses established the nature of the link between the sugar residues in X_3 (R_L 0.46a). The optical rotation, $[\alpha]_D$ 28°, strongly suggested that one of the D-mannose units was α linked and that the other was β linked. The β-linked D-mannose residue must be linked to the 2-acetamido-2-deoxy-D-glucose residue as in the disaccharide X_2 (R_L 0.46a). According to the above findings the structure of X_3 (R_L 0.46a) is α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-D-GlcNAc.

The results of sugar and methylation analyses in combination with the characterization of the tri- and disaccharides X_3 (R_L 0.46a) and X_2 (R_L 0.46a) show that the structure of R_L 0.46a is α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-D-GlcNAc. The anomeric configuration of the terminal D-mannopyranose residue is evident from the action of α-mannosidase and further supported by the optical rotation of the tetrasaccharide compared with that of the trisaccharide R_L 0.82. The oligosaccharide R_L 0.21a was homogeneous by paper chromatography in five solvent systems, and it appeared as a single peak on Bio-Gel P-2. Sugar and

TABLE IV: Analytical Properties of Oligosaccharides Obtained after Enzymatic Degradation of R_L 0.21a and R_L 0.46a.

Oligosaccharide	Yield (mg) from 20 mg of Oligosaccharide	Composition (mol %)		$[\alpha]_D^{20}$ (deg)
		D-Man	D-GlcNAc	
X_2 (R_L 0.21a)	4.2	0.9	1.0	−6.0
X_3 (R_L 0.21a)	6.1	2.0	1.0	+27
X_2 (R_L 0.46a)	1.7	0.9	1.0	−3.3
X_3 (R_L 0.46a)	9.6	2.1	1.0	+28

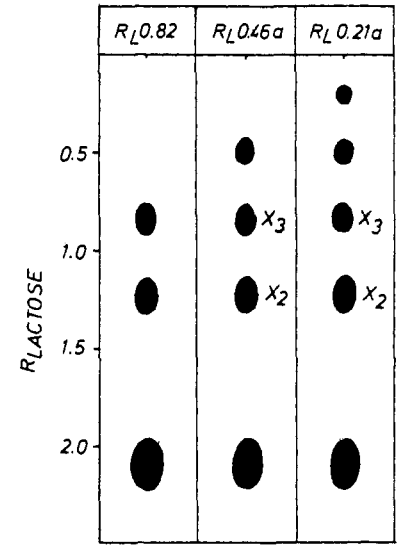

FIGURE 3: Fractionation by paper chromatography in solvent c of desalted digests obtained after treatment of compounds R_L 0.21a, R_L 0.46a, and R_L 0.82 with Jack bean α-mannosidase. For identification of different degradation products, see text.

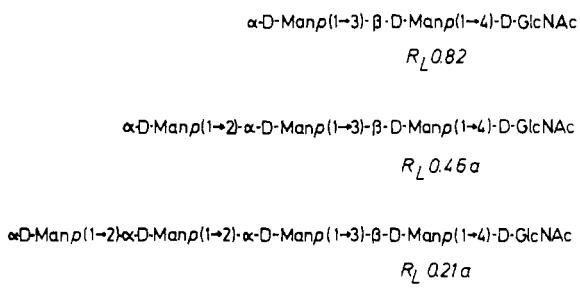

FIGURE 4: Established structures of the oligosaccharides isolated from urine of patients with mannosidosis.

methylation analyses of reduced R_L 0.21a showed that it consisted of one terminal D-mannopyranose residue, two chain D-mannoses linked at the 2 position, one chain D-mannose residue linked at the 3 position, and one reducing terminal of 4-linked 2-acetamido-2-deoxy-D-glucose residue.

Two oligosaccharides X_3 (R_L 0.21a) and X_2 (R_L 0.21a) were isolated from an α-mannosidase digest of R_L 0.21a. The above methods used to characterize the latter oligosaccharides showed the structures of these two oligosaccharides to be the same as those of X_3 (R_L 0.46a) and X_2 (R_L 0.46a). From the results of sugar and methylation analyses together with the identification of the tri- and disaccharide obtained by enzymatic hydrolysis it is concluded that R_L 0.21a has the structure α-D-Man*p*-(1→2)-α-D-Man*p*-(1→2)-α-D-Man*p*-(1→3)-β-D-Man*p*-(1→4)-D-GlcNAc. The anomeric configurations of the two outermost D-mannose residues are based on their susceptibility to hydrolysis by α-mannosidase.

The origin of the isolated oligosaccharides is not known. It is known, however, that the innermost part of several glycoprotein carbohydrate chains is built up of D-mannose and 2-acetamido-2-deoxy-D-glucose residues (Gottschalk, 1972). A variable number of D-mannose moieties are linked in a linear or branched fashion to a 2-acetamido-2-deoxy-D-glucose unit. The latter sugar is then linked, either directly or *via* a second 2-acetamido-2-deoxy-D-glucose residue, to asparagine in the protein chain. Lee and Scocca (1972) recently showed that the innermost mannose unit is linked β-(1→4) to the following 2-acetamido-2-deoxy-D-glucose moiety in several glycoproteins investigated. The urinary mannose containing oligosaccharides probably derive from the internal parts of several glycoprotein carbohydrate groups. When such glycoproteins are catabolized, the peptide portion is apparently hydrolyzed by proteolytic enzymes in the lysosomes. The linkage between the carbohydrate portion and asparagine is cleaved by 2-acetamido-1-β-(L-aspartamido)-1,2-dideoxy-D-glucose amidohydrolase. A deficiency of this enzyme results in an excretion of 2-acetamido-1-*N*-β-L-aspartyl-2-deoxy-β-D-glucopyranosylamine in the urine with the same order of magnitude as that of the trisaccharide R_L 0.82 in mannosidosis (Jenner and Pollitt, 1967). The carbohydrate portion is probably degraded stepwise from the nonreducing end by different *exo*-glycosidases. In patients with mannosidosis the high excretion of oligosaccharides, all of which contain an α-mannosidic linkage at their nonreducing end, is explained by the lack of α-mannosidases A and B. The yields of the different compounds probably reflect the abundance or the metabolic turnover rate of that particular sequence within different tissues and serum glycoproteins. Two mannose-containing oligosaccharides from brains of Angus calves with mannosidosis have previously been isolated and partially characterized (Nordén *et al.*, 1973a). It is remarkable that none of the bovine mannosidosis oligosaccharides was observed in the urines from the human cases and were thus different from the oligosaccharides depicted in Figure 4.

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