Characterization of oligosaccharides from the urine of loco-intoxicated sheep

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Two major oligosaccharides were isolated by preparative HPLC from the urine of a locoweed-fed sheep. Analysis by gas-liquid chromatography and mass-spectrometry indicated compositions of $(Man)_4(GlcNAc)_2$ and $(Man)_5(GlcNAc)_2$, respectively. Structures were determined by digestion with α-D-mannosidase and *endo-β-N*-acetylglucosaminidases D and H, and comparison of the products by HPLC with synthetic standards, and oligosaccharides isolated from human mannosidosis urine. Incubation with an *exo-β-N*-acetylglucosaminidase was without effect.

Sheep *Locoweed* *Urine* *Oligosaccharide* *Structure*

1. INTRODUCTION

Studies of glycoprotein biosynthesis [1] require 'high mannose' oligosaccharides, which may be obtained from the urine of patients with mannosidosis [2], but occur therein as complex mixtures. Swainsonine is an inhibitor of lysosomal α-D-mannosidase [3] and of Golgi α-D-mannosidase II [4]. In its presence, cultured hepatocytes [5] or fibroblasts [6] produce glycoproteins having 'hybrid' oligosaccharide chains, but the structure of the oligosaccharides excreted by swainsonine-intoxicated animals has not been reported. The accompanying paper [7] shows that animals fed locoweed, which contains swainsonine and its *N*-oxide [8], excrete oligosaccharides in the urine. The properties and chemical structure of two of the oligosaccharides are described herein.

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Abbreviations: GlcNAc, 2-acetamido-2-deoxy-D-glucose; Man, D-mannose

2. MATERIALS AND METHODS

The source of chemicals and isolation of human mannosidosis oligosaccharides have been described previously [2]. *exo-β-N*-Acetylglucosaminidase and Jack bean α-D-mannosidase were purchased from Sigma (St Louis MO). *endo-β-N*-Acetylglucosaminidases H and D were purchased from Miles (Elkhart IN). Sodium boro[$^3H_4$]hydride (341 mCi/mmol) was obtained from New England Nuclear (Boston MA).

2.1. *Analytical methods*

The analytical methods have been described previously [2]. Reduction of oligosaccharides with sodium borohydride was done as in [9]. Labeled oligosaccharides were prepared by reduction with sodium boro[$^3H_4$]hydride as in [10] and were purified by HPLC. Mass-spectrometry was performed with a Finnigan-MAT 312 double-focusing mass-spectrometer with reverse geometry, fitted with a combined CI/EI ionization source. Samples were converted into pyridinylamine derivatives

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[11] and permethylated prior to desorptive chemical-ionization with ammonia gas as the reagent. GLC was performed with a Perkin-Elmer 900 Gas-chromatograph on a 3-m column, packed with 3% OV-17 on Gas-Chrom Q, 80–100 mesh. Column temperature: 120°C with a gradient of 8°C/min. Samples (300 μg) were hydrolyzed with HCl (1 M, 200 μl) at 100°C for 4 h. The acid was evaporated by passage of nitrogen, followed by addition and evaporation of water (3 × 100 μl). Each sample was dried overnight in vacuo over KOH, subjected to methanolysis, and converted into trimethylsilyl ethers as in [12] prior to the analysis. Preparative HPLC was performed on Micropak NH_2-10 column (8 × 300 mm, Varian Associates, Palo Alto CA) with detection at 190 mm as in [7], or by refractive-index detection with a Differential Refractive Index Detector, Model 79877A (Hewlett-Packard Co., Palo Alto CA). Labeled sugars were detected by collection of fractions (0.5 ml) of the eluent from the HPLC column and monitoring the radioactivity as described before [7]. After enzymic reactions, the samples were filtered by Millipore filter (0.2 μ) and 10–20 μl injected directly onto the HPLC column.

In order to confirm an oligosaccharide structure by chromatographic comparison with a known compound, the two compounds were co-chromatographed under at least three different elution conditions, or by use of two different NH_2-5 columns, which are known to be capable of separating oligosaccharide isomers [9,13].

2.2. *Feeding experiment and collection of the urine*

A 1-year-old female sheep was fed locoweed (*Astragalus lentiginosus*), and the urine collected daily and monitored for oligosaccharide content as described in the accompanying paper [7]. When this showed that loco intoxication was fully established, urine was pooled over a 7-day period, treated with preservative (Noxythiolin), and refrigerated.

2.3. *Isolation and separation of oligosaccharides from loco-sheep urine*

Oligosaccharides were isolated from the loco-sheep urine as described previously [7]. After determination of the sugar content, the mixture was subjected to preparative HPLC with acetonitrile/water (70:30, by vol.; 2 ml/min). Fractions (2 ml) were collected and combined according to the chromatographic profile, which was obtained from the UV and refractive index detectors. The combined fractions were evaporated in vacuo, redissolved in acetonitrile/water (1:1 by vol.), filtered through a Millipore filter (0.2 μ), partially evaporated with nitrogen, and lyophilized. The incompletely separated fractions were subjected to rechromatography.

2.4. *Treatment with glycosidases*

The oligosaccharide or its alditol (1–5 μg) was incubated for 20 h at 37°C in a total volume of 100 μl containing 0.05 M buffer and 5 μl toluene. For *exo*-β-*N*-acetylglucosaminidase (10 milliunits), the buffer was sodium citrate, pH 5. For α-D-mannosidase (0.2 units), the buffer was sodium acetate containing 0.1 M sodium chloride and 0.1 mM zinc sulfate, pH 5. For *endo*-β-*N*-acetylglucosaminidase D (0.5 milliunit), the buffer was citrate–phosphate containing 0.5 M sodium chloride, pH 6. For *endo*-β-*N*-acetylglucosaminidase H (0.1 milliunit), the buffer was citrate–phosphate, pH 6. At the end of the incubation, the mixture was boiled for 3 min and subjected to HPLC analysis. A control was performed under the same conditions with boiled enzyme.

3. RESULTS AND DISCUSSION

Oligosaccharides C and D, the major components of the mixture (fig.1), were analyzed by GLC, MS, and enzymic degradation (table 1). GLC of the per(trimethylsilyl) ethers after acid hydrolysis showed the presence of only D-mannose and 2-acetamido-2-deoxy-D-glucose, and indicated compositions $[Man_4(GlcNAc)_2]_n$ and $[Man_5(GlcNAc)_2]_n$, for C and D, respectively. These compositions were confirmed, with $n = 1$, by the mass spectra of the permethylated pyridinylamine derivatives [11].

Treatment with an *exo*-β-*N*-acetylhexosaminidase, of C and D and the derived alditols, was without effect, whereas the enzyme hydrolyzed *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside, or di-*N*-acetylchitobiose, in the presence of C or D. These results demonstrate the

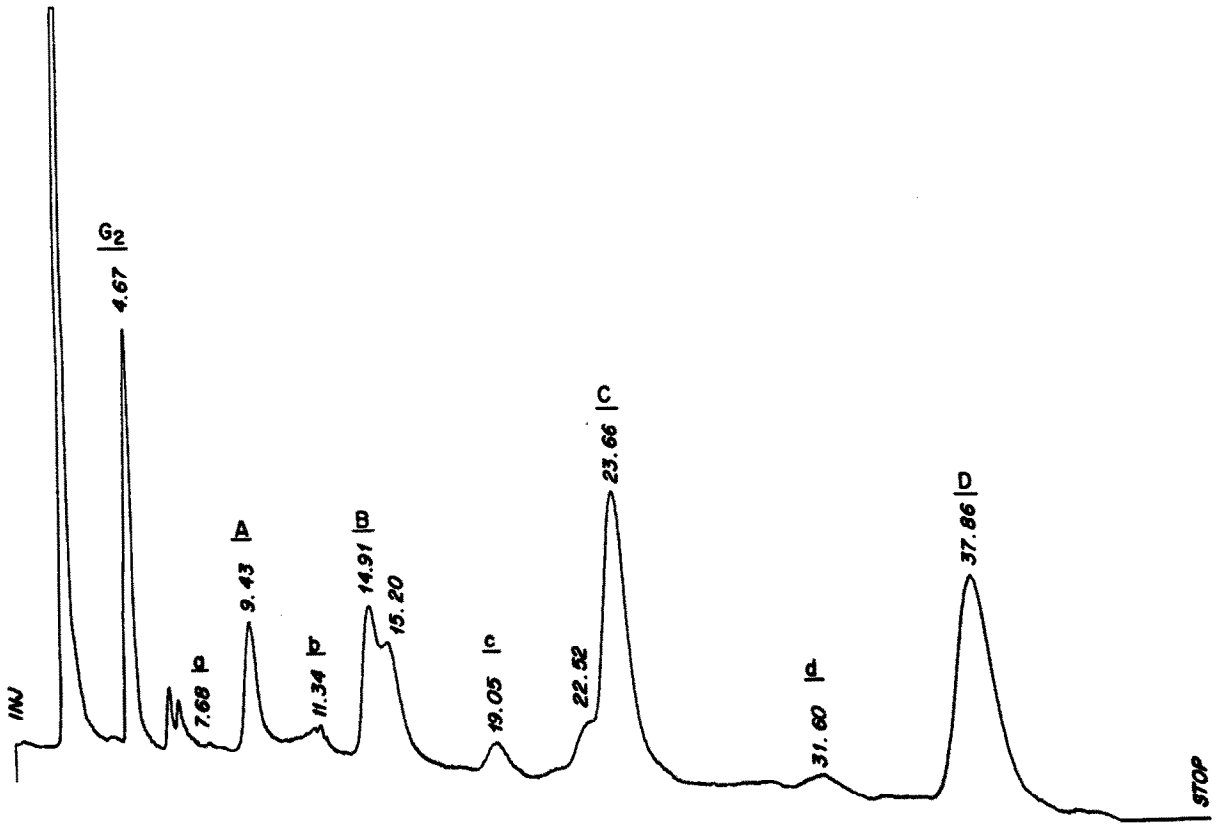

Fig.1. HPLC elution pattern of oligosaccharide mixture from urine of locoweed-fed sheep. Elution was performed with acetonitrile/water (65:35 by vol.), 2 ml/min; di-*N*-acetylchitobiose (G_2) was used as internal standard. The numbers above the peaks are retention times.

absence of a nonreducing, terminal β-D-GlcNAc residue.

C and D and the derived alditols were degraded by α-D-mannosidase. The labeled alditols released a labeled product that cochromatographed with synthetic β-D-Man*p*-(1⟶4)-β-D-Glc*p*NAc-(1⟶4)-D-GlcNAcol [14] (fig.2).

Treatment of oligosaccharides C and D with *endo*-β-*N*-acetylglucosaminidase D or H gave GlcNAc and two new compounds, indicating that C and D contain a di-*N*-acetylchitobiose residue at the reducing end. *endo*-β-*N*-Acetylglucosaminidase D has a specific requirement for a terminal, α-(1⟶3)-linked D-mannopyranosyl group attached to the β-D-mannopyranosyl residue [15], and *endo*-β-*N*-acetylglucosaminidase H requires the presence of the structure α-D-Man*p*-(1⟶3)-α-D-Man*p*-(1⟶6)-β-D-Man*p*-(1⟶4)-β-D-Glc*p*-NAc-(1⟶4)-D-GlcNAc [16]. These requirements and the results of *exo*-*N*-acetylhexosaminidase and α-D-mannosidase treatment support the structures illustrated in scheme 1. To confirm these suggestions, the products of *endo*-β-*N*-acetylglucosaminidase H treatment of C and D were compared by HPLC with $(Man)_4GlcNAc$ (M_4G) and $(Man)_5$-GlcNAc (M_5G) from human mannosidosis urine [2], as described in section 2. The product from C had a T_R similar to that of M_4G, but did not co-chromatograph (fig.3), whereas the product from D co-chromatographed with M_5G (fig.4) under a variety of conditions (see section 2.1). It has been shown that the main component of M_4G from human mannosidosis urine is the linear pentasaccharide [α-D-Man*p*-(1⟶2)]$_2$-α-D-Man*p*-(1⟶3)-β-D-Man*p*-(1⟶4)-D-GlcNAc [17], whereas the major component of M_5G from the same source is the branched hexasaccharide α-D-Man*p*-(1⟶3)-[α-D-Man*p*-(1⟶6)]-α-D-Man*p*-(1⟶6)-[α-D-

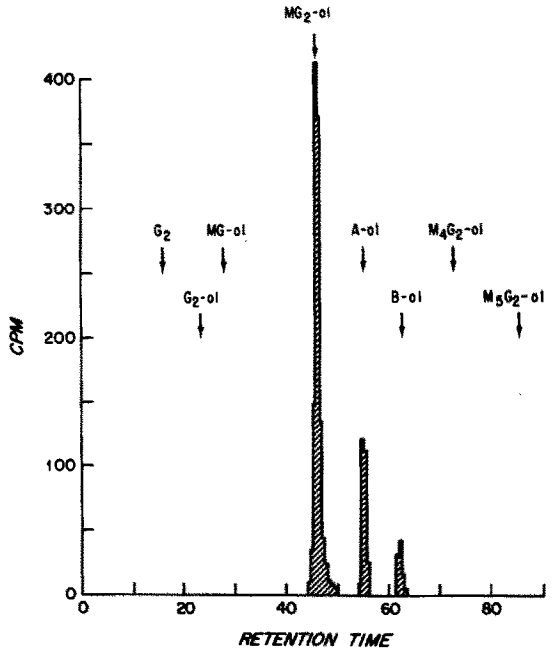

Fig.2. HPLC elution pattern showing the effect of α-D-mannosidase on labeled sugar (M_4G_2ol) derived from C by boro[$^3H_4$]hydride reduction. The products from the enzymic treatment were mixed with unlabeled, synthetic alditols and also with a sodium borohydride-reduced mixture of oligosaccharides from the urine of locoweed-fed sheep, and subjected to HPLC analysis, in acetonitrile/water (2 ml/min), 82:18 (by vol.) for 25 min, and gradient from 82:18 to 65:35 (by vol.) during 30 min. The unlabeled sugars were detected by UV absorbance at 190 mm, while the eluent was collected in fractions (1 ml) and tested for radioactivity. Similar results were obtained from the treatment of the alditol from D with α-D-mannosidase. G_2, di-*N*-acetylchitobiose; G_2-ol, di-*N*-acetylchitobiitol; MG-ol, β-D-Man*p*-(1⟶4)-D-GlcNAc-ol; Mg_2-ol, β-D-Man*p*-(1⟶4)-β-D-Glc*p*NAc-(1⟶4)-β-D-GlcNAc-ol; A-ol and B-ol, derived from oligosaccharides A and B, respectively (fig.1) by sodium borohydride reduction; and M_4G_2-ol and M_5G_2-ol, derived from C and D, respectively, by sodium borohydride reduction.

Table 1

Structure analysis of oligosaccharides C and D from urine of locoweed-fed sheep

Analysis	Oligosaccharide	
	C	D
GLC (Ratio Man to GlcNAc)		
Found	2.2:1	2.7:1
Calc.: $Man_4(GlcNAc)_2$	2.0:1	2.5:1
$Man_5(GlcNAc)_2$		
Mass spectrometry [M^+ (*m/z*)]		
Found	1444	1650
Calc.: $Man_4(GlcNAc)_2$ + H	1444	1650
$Man_5(GlcNAc)_2$ + H		
Enzymic degradation		
α-D-Mannosidase	+	+
endo-β-*N*-Acetylglucosaminidase H	+	+
endo-β-*N*-Acetylglucosaminidase D	+	+
exo-β-*N*-Acetylglucosaminidase	–	–

α-D-Man*p*-(1⟶3)-α-D-Man*p*-(1⟶6)-β-D-Man*p*-(1⟶4)-β-D-Glc*p*NAc-(1⟶4)-D-GlcNAc

3
↑
1

α-D-Man*p*

Compound C

α-D-Man*p*-(1⟶3)-α-D-Man*p*-(1⟶6)-β-D-Man*p*-(1⟶4)-β-D-Glc*p*NAc-(1⟶4)-D-GlcNAc

6 3
↑ ↑
1 1

α-D-Man*p* α-D-Man*p*

Compound D

Scheme 1. Proposed structures of hexa- and heptasaccharides obtained from urine of locoweed-fed sheep

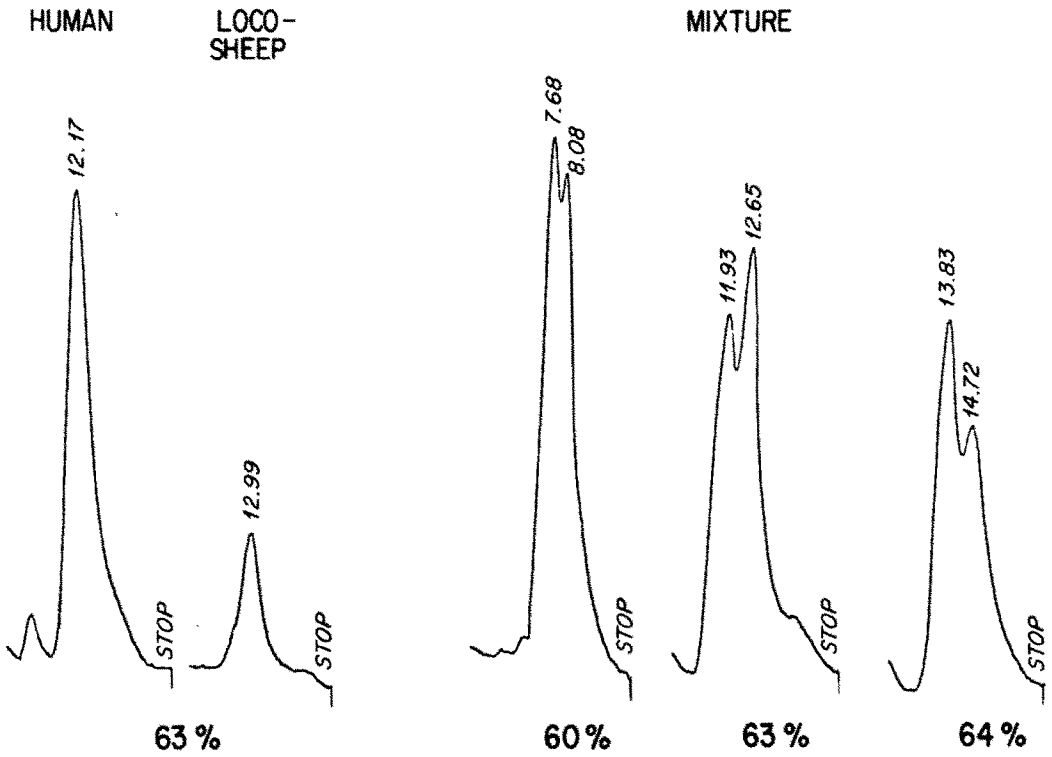

Fig.3. HPLC elution patterns of M_4G from human mannosidosis urine (Human), M_4G derived from C (M_4G_2) by *endo-β-N*-acetylglucosaminidase H treatment (loco-sheep), and mixtures of both compounds. Elution with acetonitrile/water (2 ml/min). The percentage refers to the acetonitrile content.

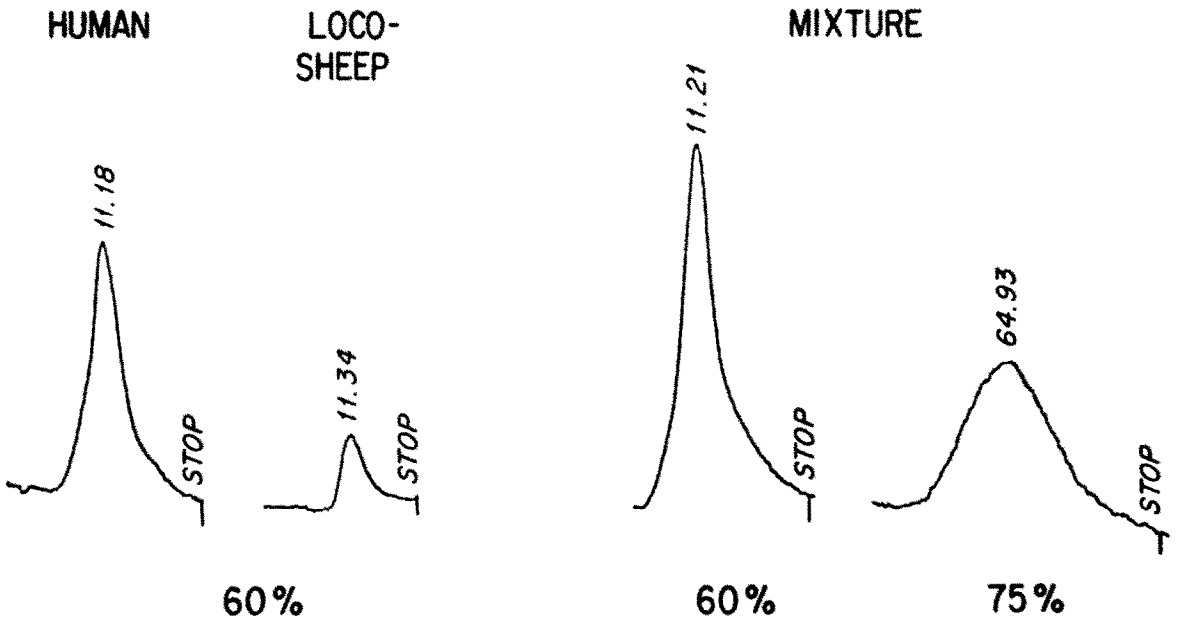

Fig.4. HPLC elution patterns of M_5G from human mannosidosis urine (Human), M_5G derived from D (M_5G_2) by *endo-β-N*-acetylglucosaminidase H treatment (loco-sheep), and mixtures of both compounds. Elution with acetonitrile/water (2 ml/min). The percentage refers to the acetonitrile content.

Man*p*-(1⟶3)]-*β*-D-Man*p*-(1⟶4)-D-GlcNAc [17]. These results corroborate the proposed structures for C and D (scheme 1).

Thus, loco-intoxicated sheep excrete oligosaccharides having structures similar to those of hybrid chains [5,6,18], except that the sialic acid⟶D-galactose⟶GlcNAc chain is absent. If hybrid chains were the precursors of loco-sheep oligosaccharides, *exo*-glycosidases must have cleaved off this external chain during the excretory process. The pattern of oligosaccharides [19] in loco-sheep urine bears distinct resemblance to that of bovine mannosidosis [20], but the levels of the Man_4 and Man_5 compounds are strongly enhanced. This would be consistent with the superimposition of a 'processing' block on a mannosidosis condition. The occurrence of a di-*N*-acetylchitobiose residue in the urine oligosaccharides of swainsonine-induced mannosidosis, has also been observed for goat *β*-mannosidosis [21], and GM_1 gangliosidosis in dogs [22], and contrasts with the presence of a single terminal GlcNAc residue in the oligosaccharides from human storage diseases. The presence of a di-*N*-acetylchitobiose residue makes the compounds from nonhuman storage diseases particularly useful for studies of *N*-glycoprotein biosynthesis.

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