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Induced mannosidosis-excretion of oligosaccharides by locoweed-intoxicated sheep

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Daily urine samples were collected from a locoweed-fed sheep, and the oligosaccharide content examined by thin-layer and liquid chromatography. An unusual pattern of urine oligosaccharides was observed, which appears to be characteristic of loco intoxication. Changes in the pattern could be correlated with the onset of visible disease, which occurred approximately 5 weeks after the typical urine sugars were first detected. HPLC showed that these sugars consisted of two homologous series of oligosaccharides containing one and two residues of 2-acetamido-2-deoxy-D-glucose, respectively.

Locoweed *Sheep* *Urine* *Oligosaccharide* *HPLC pattern*

1. INTRODUCTION

Locoweed intoxication is a severe problem affecting livestock in the USA [1]. Toxic substances in locoweed (*Astragalus* and *Oxytropis* species) are swainsonine (1,2,8-indolizidinetriol) and its *N*-oxide [2], which are α-D-mannosidase inhibitors. Therefore locoism can be considered an induced mannosidosis. The clinical signs, consisting mainly of depression, emaciation, and neurological disturbances [1], resemble those of the inherited disease [3], but do not appear until animals have ingested the plant for an extended period, and even then may be hard to recognize. Meanwhile, milk from affected animals can cause abortion and birth defects when ingested [4]. Most tissue lesions resulting from locoweed intoxication can be repaired without permanent damage if the animals are removed from loco-infested areas before the onset of clinical signs [5]. Early detection of the condition, apart from its economic and toxicological importance, would therefore also help to make available a reversible, animal model for mannosidosis. We have found that the identification of specific oligosaccharides in urine is an early indicator of locoweed intoxication.

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

2. MATERIALS AND METHODS

The sources of the chemicals have been described before [6]. AG 50 W-X8(H^+) and AG 1-X8(HCO_2^-) resins, and Bio-Gel P-6 and P-2 were obtained from Bio-Rad Laboratories (Richmond CA). Bovine serum albumin was purchased from Miles Laboratories (Elkhart IN). D-[^{14}C]Mannose was obtained from New England Nuclear (Boston MA). 2-Acetamido-2-deoxy-D-glucose and di-*N*-acetylchitobiose were purchased from Sigma (St Louis MO).

2.1. *Analytical methods*

De-ionized water was used in all procedures. Carbohydrate was assayed by the phenol/sulfuric

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acid method [7]. Thin-layer chromatography (TLC) was performed on Merck precoated plates of Silica gel 60 F254, 0.25 mm thick (E. Merck AG, Darmstadt, FRG) in solvent: chloroform/methanol/water (10:10:3 by vol.), two or three elutions, with drying between each elution. Compounds were detected on the TLC plates with the anisaldehyde reagent [8]. Gel filtration was performed on a column (2 × 21.5 cm) of Bio-Gel P-6 (200–400 mesh) in water with bovine serum albumin and D-[^{14}C]mannose as standards. Fractions (1.5 ml) were collected and monitored for sugars on a TLC plate. Ion-exchange filtration was performed on coupled columns (0.5 × 2.5 cm) of (a) AG 50 W-X8(H^+) and (b) AG 1-X8(HCO_2^-). High-pressure liquid chromatography (HPLC) was performed with a Varian instrument, model 5020 (Varian Associates, Palo Alto CA) equipped with a Hibar II column (4.6 × 250 mm), packed with 10 μ Lichrosorb-NH_2 (EM Reagents, Cincinnati OH) or a Spherisorb NH_2-5 μ column (4.6 × 250 mm) (C.S.C. Inc., Ville Mont-Royal, Quebec, Canada). For internal standards, 2-acetamido-2-deoxy-D-glucose and di-*N*-acetylchitobiose were used. Preparation of solvents and samples for HPLC, detection at 190 nm, and recording of chromatograms were performed as in [6]. Radioactivity was determined with a Packard liquid scintillation spectrometer, Model 3255, using Hydrofluor (National Diagnostics, Somerville NJ). Creatinine was assayed, by a picric acid kinetic method, with a Beckman ASTRA analyzer.

2.2. *Feeding experiment*

A 1-year-old, female sheep (White Face X Suffolk) was fed locoweed (*Astragalus lentiginosus*, 260 g/day). A similar sheep was fed alfalfa. The two sheep were kept under observation and their urine was collected daily. Up to the 5th week, the behavior of the two animals was similar, and both appeared to be healthy. Around the 38th day, the locoweed-fed sheep began to develop typical symptoms of locoism [1] and, from the 44th day, intoxication became so severe that the animal gradually stopped eating and drinking, while the control sheep remained normal. On the 51st day of the experiment, the locoweed was withdrawn from the sick sheep and replaced by alfalfa, after which the animal recovered slowly, becoming apparently normal again in about 10 days.

2.3. *Isolation of the oligosaccharides*

For routine analysis, urine (1 ml) was applied to a column (1.2 × 14 cm) of Bio-Gel P-2 (200–400 mesh) and eluted with water. The first 6 ml of eluent were collected and submitted to ion-exchange chromatography. The resins were washed with water (3 × 2 ml) and the combined eluates evaporated to dryness. The residue was dissolved in 1 ml of water, and analyzed by TLC and HPLC. For quantitative analysis, urine (10 ml) was treated with activated charcoal (Darco G-60, 3 g) and stirred at room temperature for 2.5 h. The charcoal was filtered off (Celite), stirred with ethanol/water (1:1, by vol., 20 ml), and filtered off again. The combined filtrates were evaporated to dryness. The residue was dissolved in 1 ml of water, and submitted to gel filtration on Bio-Gel P-6, followed by ion-exchange filtration as described earlier. The sample was dissolved in 1 ml of water and assayed for carbohydrate content. Values were corrected for creatinine content (see below).

2.4. *Routine analysis*

Urine (1 ml), collected daily from loco and control sheep, was monitored for oligosaccharide content (see section 2.3); 25 μl out of 1 ml of the oligosaccharide solution were injected onto the same HPLC column and eluted with acetonitrile/water (3:2) at a flow rate of 2 ml/min. Areas of peaks were integrated by the autointegrator. A further 1 ml from the same day was assessed for creatinine. The areas were corrected per mg of creatinine.

3. RESULTS AND DISCUSSION

Oligosaccharides began to appear in the urine of the locoweed-fed sheep after a few days of feeding. The amount of excreted carbohydrate increased rapidly, reaching a level of 110–130 mg per mg excreted creatinine (about 1.3–1.8 mg/ml urine) by the time the animal was severely intoxicated (6th week) (fig.1,2). After withdrawal of locoweed, urine carbohydrate returned to levels similar to those of the control sheep, and after about 2 weeks the animals were difficult to distinguish on the basis of urine oligosaccharides.

The oligosaccharides from urine of the intoxicated sheep were monitored as described in section 2, and HPLC analysis showed four major peaks

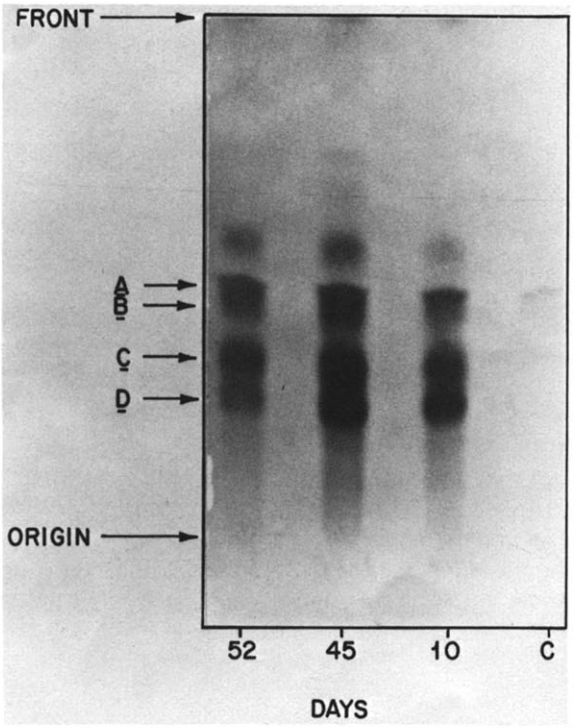

Fig.1. TLC of loco-sheep urine oligosaccharides. Locoweed was withdrawn on the 51st day. (C) Control; 10, 45, 52, days of feeding. A, B, C, D refer to the HPLC peaks (fig.3,4).

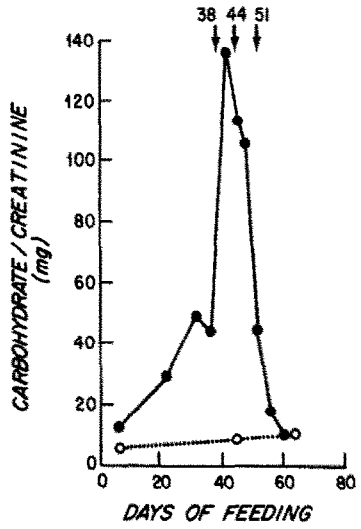

Fig.2. Daily carbohydrate content (mg), in urine, corrected for creatinine (mg). (●---●) Loco-sheep; (○---○) control sheep; 38, 44, 51, critical days of the intoxication.

(fig.3A–D). The peak areas all increased initially, but not to the same extent. Peaks C and D increased most rapidly, and later peak D decreased relative to peak C. The pattern of oligosaccharide HPLC peaks or spots on TLC was apparently related to the extent of intoxication, as judged by the behavior of the animal (figs.1–3). None of the peaks were found in chromatograms from the control urine.

When HPLC was performed with an NH_2-5 μ column, capable of superior resolution, it became obvious that the major oligosaccharides, corresponding to peaks A–D, are accompanied by a minor series (a–d, fig.4). The major compounds (C and D) did not co-chromatograph with $(Man)_4GlcNAc$ and $(Man)_5GlcNAc$, respectively, isolated from human mannosidosis urine [6], but minor compounds (c,d) did have similar elution times (fig.4), under a variety of conditions. In the accompanying paper [10], the oligosaccharides corresponding to peaks C and D have been isolated and shown to be $(Man)_4(GlcNAc)_2$ and $(Man)_5(GlcNAc)_2$, respectively, and a comparison of the HPLC peaks with those from bovine mannosidosis urine [11] suggested that both sets of peaks correspond to oligomers of D-mannose and 2-acetamido-2-deoxy-D-glucose. When values for logarithm of retention times were plotted against the apparent number of D-mannose residues [6], two parallel straight lines resulted, showing that the oligosaccharides corresponding to major peaks A–D, and minor peaks a–d, belong to two different homologous series.

These experiments show that characteristic oligosaccharides are excreted in the urine during locoweed intoxication. The sugars are $(Man)_n(GlcNAc)_2$, with lesser amounts of $(Man)_nGlcNAc$, where n is mainly 2–5. For a rationalization of the excretion of these compounds on the basis of the probable mechanism of locoweed intoxication and inhibitory action of swainsonine [9], see [10].

In preliminary studies, a similar pattern of oligosaccharides has been observed in the urine of two other loco-intoxicated sheep and two pigs. We believe that the detection of the oligosaccharides is a new, general way of identifying locoism in

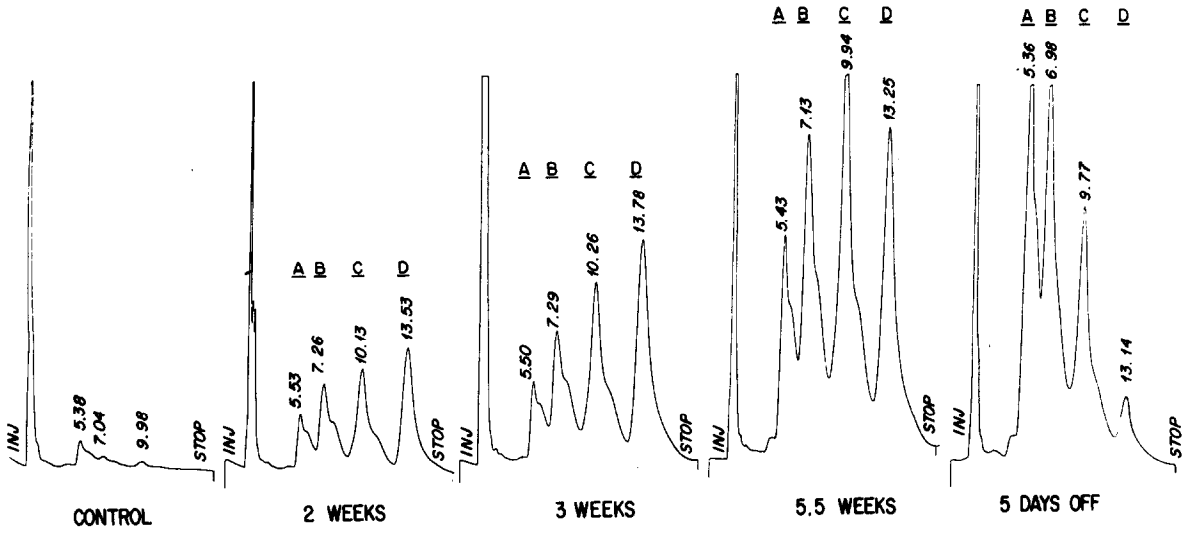

Fig.3. HPLC patterns of oligosaccharides from urine of loco-sheep and control sheep. Control: the profile of oligosaccharides from urine of control sheep was unchanged during the feeding experiment; 2–5 weeks refers to the period from the start of locoweed feeding; 5 days off refers to the 5-day period after withdrawal of locoweed. The numbers above the peaks are the retention times.

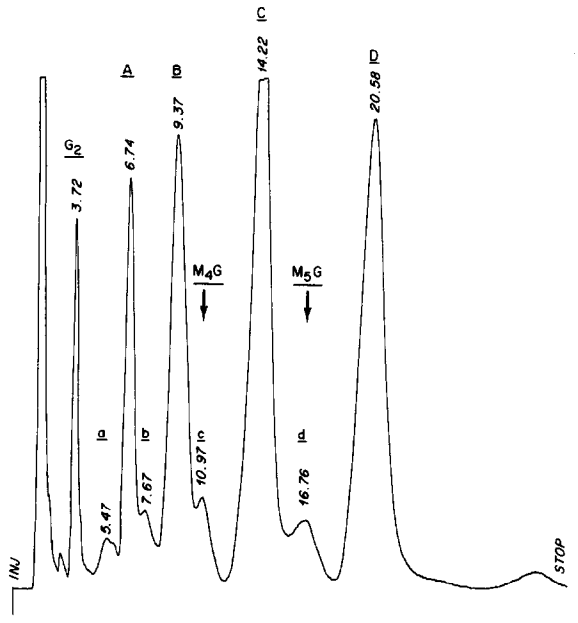

Fig.4. Detailed HPLC pattern of oligosaccharide mixture from urine of locoweed-fed sheep (Sperisorb NH_2-5μ, acetonitrile/water, 65:35, by vol.; 2 ml/min). G_2, di-*N*-acetylchitobiose served as internal standard; M_4G and M_5G refer to elution positions of $(Man)_4GlcNAc$ and $(Man)_5GlcNAc$ isolated from human mannosidosis urine, respectively.

animals, and that inspection of the chromatographic pattern will yield useful data on the extent of the intoxication.

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REFERENCES

[1] James, L.F., Hartley, W.J. and Van Kampen, K.R. (1981) J. Am. Vet. Med. Assoc. 178, 146–150.
[2] Molyneux, R.J. and James, L.F. (1982) Science 216, 190–191.
[3] Jolly, R.D., Slack, P.M., Winter, P.J. and Murphy, C.E. (1980) Aust. J. Exp. Biol. Med. Sci. 58, 421–428.

[4] James, L.F. and Hartley, W.J. (1977) Am. J. Vet. Res. 38, 1263–1265.
[5] Van Kampen, K.R., Rhees, R.W. and James, L.F. (1978) in: Effects of Poisonous Plants on Livestock (Keeler, R.F., Van Kampen, K.R. and James, L.F. eds) pp.465–471, Academic Press, New York.
[6] Warren, C.D., Schmit, A.S. and Jeanloz, R.W. (1983) Carbohydr. Res. 116, 171–182.
[7] Ashwell, G. (1966) Methods Enzymol. 8, 93–95.
[8] Dunphy, P.J., Derr, J.D., Pennock, J.F., Whittle, K.J. and Feeney, J. (1967) Biochim. Biophys. Acta 136, 136–147.
[9] Tulsiani, D.R.P., Harris, T.M. and Touster, O. (1982) J. Biol. Chem. 257, 7936–7939.
[10] Sadeh, S., Warren, C.D., Daniel, P.F., Bugge, B., James, L.F. and Jeanloz, R.W. (1983) FEBS Lett. 163, 104–109.
[11] Warren, C.D., Daniel, P.F., Sadeh, S., James, L.F. and Jolly, R.D. (1983) Abstr. Int. Symp. Glycoconjugates, 7th, Lund-Ronneby, Sweden.