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Swainsonine-induced oligosaccharide excretion in sheep

Time-dependent changes in the oligosaccharide profile

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Urinary oligosaccharides isolated from locoweed-intoxicated sheep were separated and quantified by reversed-phase high pressure liquid chromatography of the perbenzoylated alditols. Mannose-containing oligosaccharides were elevated as early as day 3 of feeding, but maximum levels (approx. 1 μmol/ml) were not attained until after 6 weeks of feeding. The relative abundance of individual oligosaccharides changed over the course of the feeding period. $Man_3GlcNAc_2$ reached a peak on day 3 and then rapidly declined. Two isomers were shown to be present in this fraction and the relative proportions altered with the duration of locoweed treatment. The major isomer present at early time points (< 8 days) co-eluted with synthetic Man(α1–3)[Man(α1–6)]Man(β1–4)GlcNAc(β1–4)GlcNAc, was digested by endo-β-*N*-acetylglucosaminidase D, and is probably derived from the trimannosyl core of complex glycoproteins synthesized prior to locoweed treatment. $Man_3GlcNAc_2$ isolated from day 53 urine was resistant to endo-β-*N*-acetylglucosaminidase D digestion but was cleaved by endo-β-*N*-acetylglucosaminidase H. This isomer has the probable structure Man(α1–3)Man(α1–6)Man(β1–4)GlcNAc(β1–4)GlcNAc, indicative of its origin from hybrid or high-mannose glycoproteins. $Man_5GlcNAc_2$ reached a peak on day 13 and then slowly declined, whereas $Man_4GlcNAc_2$ increased concomitantly. The rapid increase in $Man_5GlcNAc_2$ can probably be attributed to the breakdown of hybrid glycans produced as a result of swainsonine inhibition of Golgi α-D-mannosidase II. The onset of observable clinical signs on day 38 closely correlated with the time point at which the level of $Man_4GlcNAc_2$ exceeded $Man_5GlcNAc_2$. After locoweed feeding was discontinued, the amount of urinary oligosaccharides declined rapidly and reached baseline levels within 12 days.

Ingestion by grazing livestock of certain leguminous plants of the genera *Astragalus* and *Oxytropis* (locoweeds) as well as the Australian genus *Swainsona* induces a chronic neurological disease which resembles mannosidosis (James *et al.*, 1981; Huxtable & Dorling, 1982). Extracts of tissues from affected animals contain high amounts of mannose-rich oligosaccharides (Dorling *et al.*, 1978). The toxic principle, swainsonine and its *N*-oxide (Molyneux & James, 1982), is a potent, reversible inhibitor of lysosomal α-D-mannosidase (EC 3.2.1.24) (Dorling *et al.*, 1980). More recently, swainsonine has also been shown to inhibit strongly Golgi α-D-mannosidase II (Tulsiani *et al.*, 1982), leading to the formation of hybrid glycoproteins in cultured cells (Tulsiani & Touster, 1983*a*; Gross *et al.*, 1983; Kang & Elbein, 1983).

Livestock must consume locoweed for an extended period before signs of poisoning become evident. If locoweed grazing is discontinued before

Abbreviation used: h.p.l.c., high pressure liquid chromatography.

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an animal becomes too emaciated, the animal will recover, but will continue to exhibit typical neurological signs when stressed (James *et al.*, 1981). However, if the intoxication could be detected at an early stage, there is a strong possibility that recovery would be complete (Dorling *et al.*, 1978). Swainsonine is secreted in the milk of affected animals and can cause abortion and birth defects if ingested (James & Hartley, 1977). Hence, early detection of the disease is very important.

We recently reported that a typical pattern of urine oligosaccharides accompanies locoweed intoxication (Warren *et al.*, 1983*a*). In the present work, we have performed a quantitative study of oligosaccharide excretion, and shown that specific oligosaccharides in urine from a 'loco' sheep were significantly elevated by day 3 of feeding, 5 weeks before clinical symptoms became apparent. We also report that characteristic changes in the pattern of excreted oligosaccharides accompany the development and remission of intoxication.

Experimental

Materials

Bio-Gel P-2, AG 50W-X8 and AG 1-X8 were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Benzoic anhydride and 4-dimethylaminopyridine were obtained from Sigma (St. Louis, MO, U.S.A.). Bond Elut disposable extraction columns were purchased from Analytichem International (Harbor City, CA, U.S.A.). H.p.l.c. grade solvents (pyridine, water and acetonitrile) were supplied by Burdick and Jackson (Muskegon, MI, U.S.A.). Raffinose was purchased from Pfanstiehl (Waukegan, IL, U.S.A.). Endo-β-*N*-acetylglucosaminidases D and H were purchased from Miles (Elkhart, IN, U.S.A.). Bovine mannosidosis urine was supplied by Dr. R. D. Jolly, Massey University, Palmerston North, New Zealand. Synthetic Man(α1–6)[Man(α1–3)]Man(β1–4)GlcNAc(β1–4)GlcNAc was supplied by Prof. H. Paulsen, University of Hamburg, Germany.

Feeding experiment

This was performed as described by Warren *et al.* (1983*a*). Briefly, a 1-year-old female sheep was fed locoweed (*Astragalus lentiginosus*), 260g/day, and a control sheep was fed alfalfa. Both sheep were kept under observation and their urine was collected daily. Up to week 5, the behaviour of the two animals was similar but then the locoweed-fed sheep began to develop typical symptoms of locoism (James *et al.*, 1981). On day 51 the locoweed was withdrawn and replaced by alfalfa, after which the animal recovered in about 10 days.

Isolation of oligosaccharides and derivatization

Oligosaccharides were isolated from 1ml of urine as described by Warren *et al.* (1983*a*) and reduced with aqueous $NaBH_4$ (0.5ml, 10mg/ml) overnight. Excess reagent was destroyed by addition of a drop of glacial acetic acid and Na^+ was removed by passage through a column (0.5cm × 2.5cm) of AG 50W-X8 (H^+ form). The eluant was evaporated to dryness and boric acid was removed by repeated addition and evaporation of methanol (0.5ml, three times). Reduced oligosaccharides were benzoylated overnight at 37°C in pyridine (0.5ml) containing 10% (w/v) benzoic anhydride plus 5% (w/v) 4-dimethylaminopyridine as acylation catalyst, as described by Daniel *et al.* (1981). Following benzoylation, each sample was diluted with water (4.5ml) and applied to a C18 Bond Elut extraction column. The column was then washed with 10% (v/v) aqueous pyridine, followed by water (2ml each). Benzoylated oligosaccharides were eluted with acetonitrile (2ml), dried under N_2 and dissolved in acetonitrile for analysis by h.p.l.c.

H.p.l.c. analysis

H.p.l.c. was performed with a Varian Instrument model 5020 (Varian Associates, Palo Alto, CA, U.S.A.) equipped with a 3μm octylsilica column (4.6mm × 100mm) (C8 Microsorb short-one; Rainin Laboratories, Woburn, MA, U.S.A.). The output from a model SF770 variable wavelength detector (Kratos/Schoeffel, Ramsey, NJ, U.S.A.) was connected in series to an Autolabs System 1 computing integrator (Spectra-Physics, Piscataway, NJ, U.S.A.) and a strip-chart recorder. A 15min linear gradient from acetonitrile/water (4:1, v/v) to pure acetonitrile at a flow rate of 1ml/min was employed to elute benzoylated oligosaccharides, which were detected by their absorbance at 230nm. Raffinose was used as an external standard for quantification as described (Daniel *et al.*, 1981).

Treatment with glycosidases

Digestion of samples with endo-β-*N*-acetylglucosaminidases D and H was performed as described previously (Sadeh *et al.*, 1983).

Results

Urinary oligosaccharides from a control sheep and a sheep fed locoweed were monitored by h.p.l.c. during 51 days of treatment and for a further 12 days after locoweed feeding was discontinued. The rapid onset of oligosaccharidiuria after initiation of locoweed feeding is illustrated in Fig. 1. Peaks were identified by comparison of their

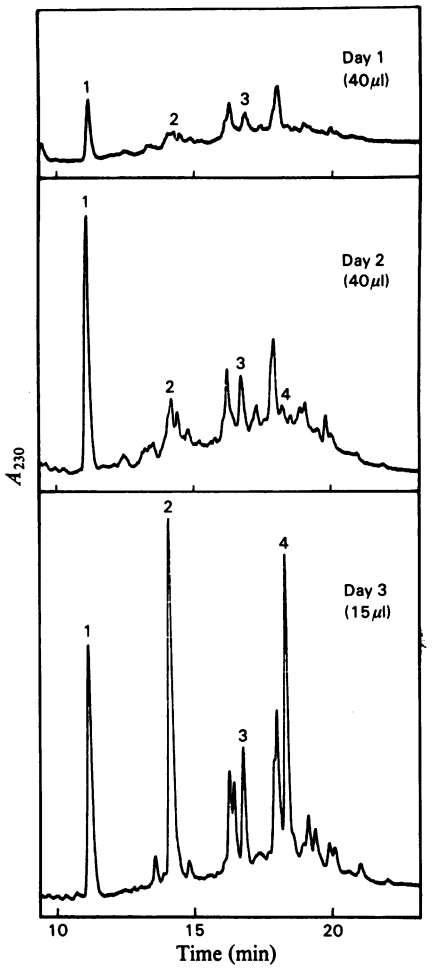


Fig. 1. *H.p.l.c. elution profiles of urinary oligosaccharides after 1, 2 and 3 days of locoweed feeding*
Oligosaccharides were isolated from 1 ml of urine, reduced with $NaBH_4$ and benzoylated as outlined in the Experimental section. Derivatized samples were dissolved in acetonitrile, injected onto a Microsorb C8 column (4.6mm × 100mm) and eluted with a linear gradient of acetonitrile/water (4:1, v/v) changing to pure acetonitrile over 15 min. The volume equivalent of urine injected is indicated on each chromatogram. Peak identification: 1, $Man_2GlcNAc_2$; 2, $Man_3GlcNAc_2$; 3, $Man_4GlcNAc_2$; 4, $Man_5GlcNAc_2$.

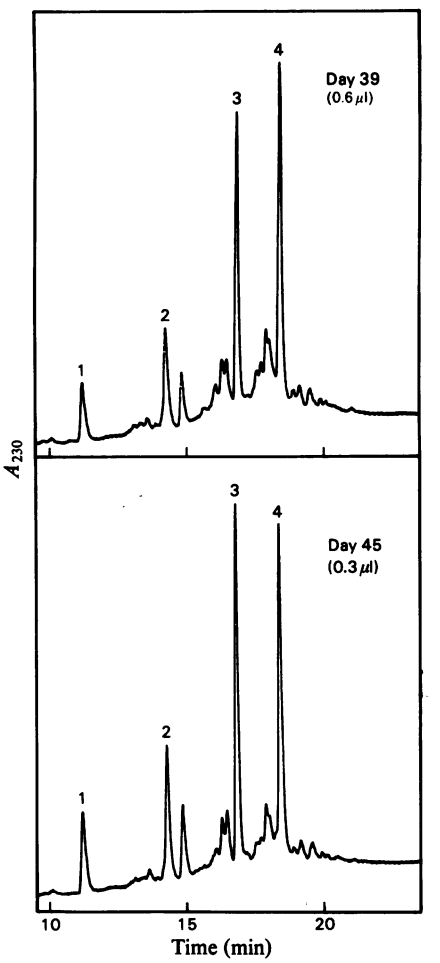


Fig. 2. *H.p.l.c. elution profiles of urinary oligosaccharides after 39 and 44 days of locoweed feeding*
For experimental details and peak identification, see the legend to Fig. 1.

retention times with those of known oligosaccharides from bovine mannosidosis urine (Warren *et al.*, 1983*b*). The concentration of the four major oligosaccharides as a function of treatment is given in Table 1. A low level of each oligosaccharide was present in urine from the control sheep at the three time points tested (Table 1). The most abundant species were $Man_2GlcNAc_2$ (55%) and $Man_3GlcNAc_2$ (26–44%). It is apparent from Fig. 1 that by day 3 of locoweed treatment there has been a substantial increase of each numbered component, the increases in $Man_3GlcNAc_2$ and $Man_5GlcNAc_2$ being most striking. The total urinary oligosaccharide level on day 3 was elevated 5-fold over that observed on day 2 and continued to increase until a maximum of approx. 1 μmol/ml was attained after 6 weeks of treatment.

The locoweed-fed sheep began to develop typical symptoms of locoism around day 38, intoxication becoming severe from day 44. $Man_5GlcNAc_2$ was the most abundant oligosaccharide in urine from the experimental animal from day 5 through to day 39 (Table 1), but $Man_4GlcNAc_2$ was the most abundant by day 45. This change-over is illustrated in Fig. 2. It is note-

Table 1. *Excretion of urinary oligosaccharides as a function of the duration of locoweed treatment*
Oligosaccharides were isolated from 1 ml of urine, reduced with $NaBH_4$ and benzoylated. Derivatized samples were dissolved in acetonitrile and analysed by h.p.l.c. with detection at 230nm. Perbenzoylated raffinose was used as an external standard for quantification as described (Daniel *et al.*, 1981). Abbreviation used: tr, trace (<0.1 nmol/ml).

	Oligosaccharides (nmol/ml)				
Days of treatment*	$Man_2GlcNAc_2$	$Man_3GlcNAc_2$	$Man_4GlcNAc_2$	$Man_5GlcNAc_2$	Total
Locoweed-fed sheep					
1	0.6	tr	0.2	tr	0.8
2	2.8	0.7	0.6	0.2	4.3
3	7.0	8.8	2.3	5.1	23.2
5	11.4	20.2	8.1	24.7	64.4
13	15.0	28.4	42.0	88.1	173.5
31	53.8	74.3	163.8	212.9	504.8
39	42.0	51.7	116.2	125.2	335.1
45	116.3	128.1	292.2	263.0	799.6
(2)	95.6	99.3	145.6	84.7	425.2
(4)	143.4	107.5	104.0	34.1	389.0
(8)	4.4	4.1	2.8	1.2	12.5
(12)	1.2	0.9	0.6	0.3	2.9
Alfalfa-fed control					
3	5.7	2.9	1.3	0.7	10.6
39	2.6	1.2	0.6	0.2	4.7
(12)	1.0	0.8	tr	tr	1.8

* Numbers in parentheses show days after discontinuation of locoweed feeding (day 51).

worthy that the alteration in relative abundance of $Man_5GlcNAc_2$ and $Man_4GlcNAc_2$ approximates to the onset of clinical symptoms.

The early rise in $Man_3GlcNAc_2$ was unexpected. In order to investigate this further, we collected the $Man_3GlcNAc_2$ peaks from urine samples obtained on day 5 and on day 53 and compared their elution in an isocratic solvent system. As shown in Fig. 3(*a*), the two peaks were not identical and presumably represent different isomers, since the peak isolated from day 5 was digested to $Man_3GlcNAc$ by endo-β-*N*-acetylglucosaminidase D but not by endo-β-*N*-acetylglucosaminidase H, whereas the converse was true for the peak isolated from day 53.

After locoweed feeding was discontinued on day 51, the 'loco' sheep recovered rapidly and was apparently normal again in about 12 days. During this period the urinary oligosaccharides declined from 425 to 3nmol/ml, which is within the control range of 5.7 ± 4.5 nmol/ml. This dramatic decline is illustrated in Fig. 4. The major drop occurred between day 4 and day 8 of recovery (from 389 to 12.5nmol/ml).

Discussion

In previous work (Warren *et al.*, 1983*a*), 'loco' sheep urinary oligosaccharides were identified by h.p.l.c. on aminopropyl columns with detection at 190nm. The advantages of the procedure described herein are (*a*) much greater sensitivity (ng versus μg), (*b*) increased resolution, (*c*) reproducibility of retention times, and (*d*) ease of quantification. These advantages have allowed us to do an extended time course of the effects of locoweed on oligosaccharide excretion. We have shown that mannose-containing oligosaccharides were elevated in the urine as early as day 3 of treatment, 5 weeks before symptoms of locoism became apparent on day 38. Thus monitoring urinary oligosaccharides by this procedure could provide a sensitive method for early detection of the disease when locoweed consumption is suspected. This would be of benefit to farmers because affected animals become addicted to locoweed and actively seek out the plant (James *et al.*, 1981).

The h.p.l.c. elution profile of 'loco' sheep urine after sustained ingestion is similar to that obtained from bovine mannosidosis urine, but the levels of $Man_4GlcNAc_2$ and $Man_5GlcNAc_2$ are greatly enhanced. The major oligosaccharide in the bovine urine is $Man_2GlcNAc_2$ (48%); $Man_4GlcNAc_2$ and $Man_5GlcNAc_2$ comprise only 9% and 5%, respectively (P. F. Daniel, C. D. Warren & R. D. Jolly, unpublished work).

The relative abundance of individual oligosaccharides changed over the course of the feeding period, as illustrated in Fig. 5. There was a dramatic decline in $Man_2GlcNAc_2$ and a transient increase in $Man_3GlcNAc_2$, which reached a peak on day 3 and then declined to a steady state level by

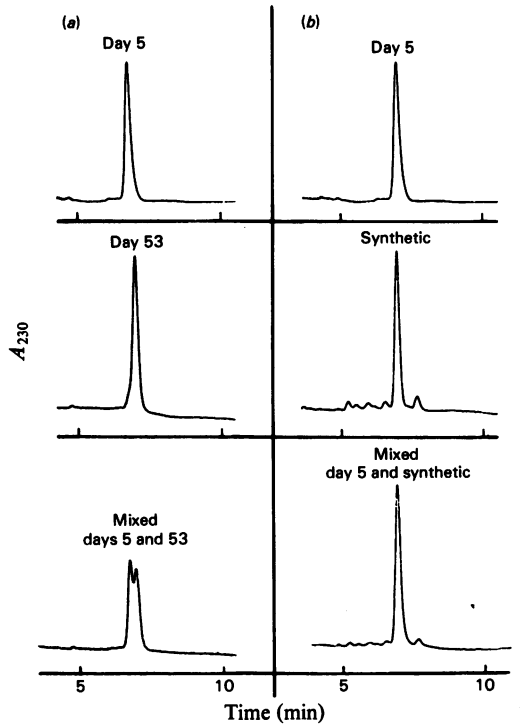


Fig. 3. *Co-chromatography of $Man_3GlcNAc_2$ peaks isolated from 'loco' sheep urine and comparison with synthetic branched $Man_3GlcNAc_2$*

$Man_3GlcNAc_2$ peaks were isolated by preparative h.p.l.c. employing gradient elution as described in the legend to Fig. 1. Aliquots (approx. 100 pmol) were re-analysed isocratically in acetonitrile/water (9:1, v/v). (*a*) Comparison of $Man_3GlcNAc_2$ from days 5 and 53; (*b*) comparison of $Man_3GlcNAc_2$ from day 5 with synthetic Man(α1–3)[Man(α1–6)]-Man(β1–4)GlcNAc(β1–4)GlcNAc.

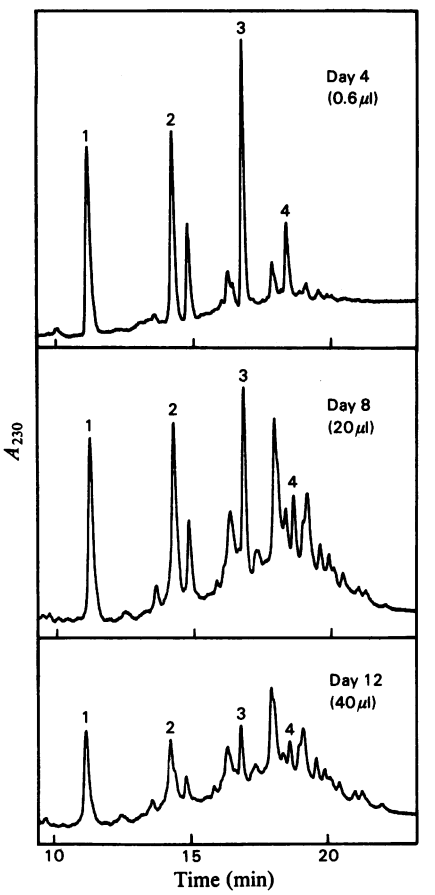


Fig. 4. *H.p.l.c. elution profiles of urinary oligosaccharides after 4, 8, and 12 days of recovery from locoweed poisoning*

Locoweed feeding was discontinued on day 51. For experimental details and peak identification, see the legend to Fig. 1.

day 13. Two isomers were shown to be present in the $Man_3GlcNAc_2$ fraction and the relative proportions altered with the duration of locoweed treatment (Fig. 3*a*). $Man_3GlcNAc_2$ isolated from day 6 urine and earlier is probably derived from the trimannosyl core of glycoproteins synthesized prior to locoweed feeding (see Scheme 1). It is predominantly a single isomer ($Man_3GlcNAc_2$-I) that is endo-β-*N*-acetylglucosaminidase H resistant but is digested by endo-β-*N*-acetylglucosaminidase D and co-elutes with chemically synthesized Man(α1–6)[Man(α1–3)]Man(β1–4)GlcNAc(β1–4)GlcNAc (Fig. 3*b*). A different isomer, $Man_3GlcNAc_2$-II, presumably derived from the breakdown of hybrid and high-mannose glycoproteins via $Man_5GlcNAc_2$, predominates from day 13 on. This isomer has a slightly longer (approx. 6 s) retention time on h.p.l.c., is digested by endo-β-*N*-acetylglucosaminidase H but not by endo-β-*N*-acetylglucosaminidase D and has the probable structure Man(α1–3)Man(α1–6)Man(β1–4)GlcNAc(β1–4)GlcNAc, based on the known specificity of endo-β-*N*-acetylglucosaminidase H (Tai *et al.*, 1977; Trimble *et al.*, 1978).

The rapid rise in $Man_5GlcNAc_2$ excretion (a 25-fold increase between days 2 and 3) is probably due to inhibition of Golgi α-D-mannosidase II activity. In support of this, Tulsiani & Touster (1983*b*) have recently reported a very rapid decrease in rat liver mannosidase II activity to 32% of normal after only 24 h of swainsonine feeding. Inhibition of mannosidase II by swainsonine prevents the formation of complex glycoproteins, by blocking the processing of $GlcNAcMan_5GlcNAc_2$ to $GlcNAcMan_3GlcNAc_2$ (Tabas & Kornfeld, 1978; Harpaz & Schachter, 1980) (see Scheme 1), and has been shown to lead to the production of hybrid glycopro-

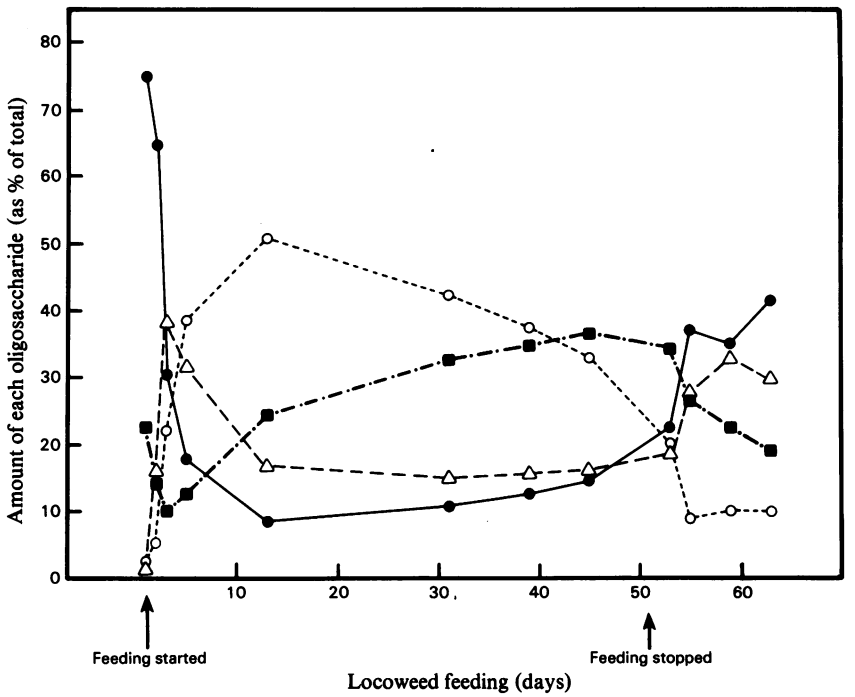
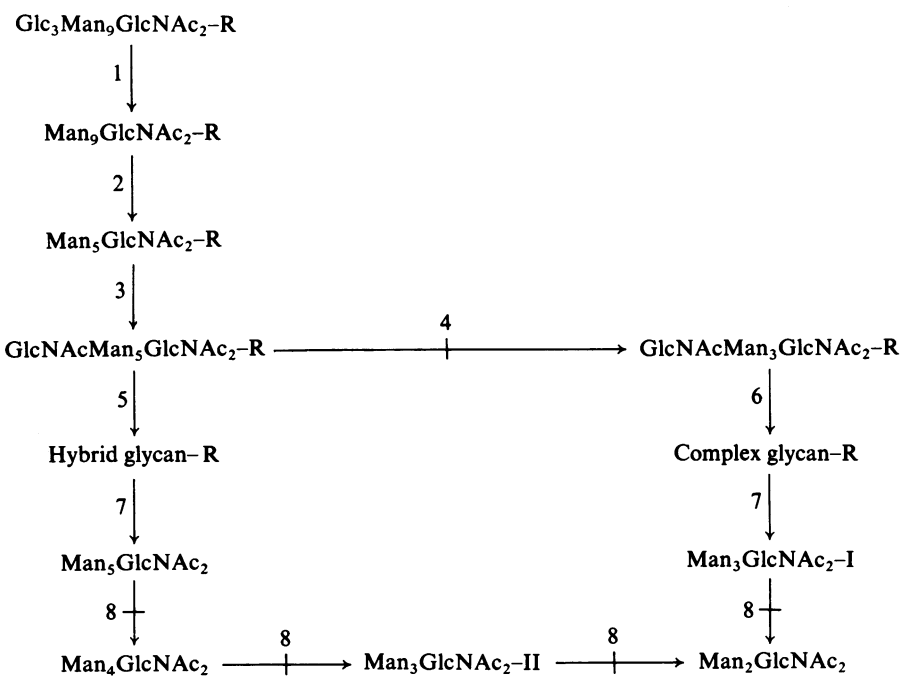


Fig. 5. *Relative oligosaccharide abundance as a function of the duration of locoweed feeding*
The amount of each oligosaccharide from Table 1 is expressed as a percentage of the sum of all four major oligosaccharides present in each urine sample. ●, $Man_2GlcNAc_2$; △, $Man_3GlcNAc_2$; ■, $Man_4GlcNAc_2$; ○, $Man_5GlcNAc_2$.



Scheme 1. *Processing, elongation and degradation of oligosaccharides*
Key: –R, Asn-peptide; 1–4, processing; 5–6, elongation; 7–8, lysosomal degradation; ⊣⟶, steps inhibited by swainsonine *in vitro*.

teins by an alternative pathway (Tulsiani & Touster, 1983*a*; Gross *et al.*, 1983; Kang & Elbein, 1983). In view of this block in the processing pathway, the excretion of large amounts of $Man_4GlcNAc_2$ was surprising. In fact $Man_4GlcNAc_2$ was the most abundant oligosaccharide by day 45, as shown in Fig. 5, and there appeared to be a precursor–product relationship between $Man_5GlcNAc_2$ and $Man_4GlcNAc_2$. The structures of these two oligosaccharides have been determined recently (Sadeh *et al.*, 1983). Presumably hybrid glycoproteins are catabolized normally in the lysosomes to $Man_5GlcNAc_2$. This is then further degraded to $Man_4GlcNAc_2$, but which α-D-mannosidase is responsible is uncertain at present. Tulsiani *et al.* (1982) showed that lysosomal α-D-mannosidase is strongly inhibited by swainsonine *in vitro*, but it is unclear whether the enzyme is completely inhibited *in vivo* since Tulsiani & Touster (1983*b*) subsequently reported an unexpected increase in the level of lysosomal α-D-mannosidase in liver and brain of a swainsonine-treated rat.

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