Substrate Specificities of Rat Kidney Lysosomal and Cytosolic α-D-Mannosidases and Effects of Swainsonine Suggest a Role of the Cytosolic Enzyme in Glycoprotein Catabolism*

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Swainsonine is a potent inhibitor of lysosomal α-D-mannosidase, causes the production of hybrid glycoproteins, and is reported to produce a phenocopy of hereditary α-mannosidosis. We now report that the effects of swainsonine administration in the rat are different in two respects from those found in other animals thus far studied. (i) Swainsonine caused the accumulation of oligosaccharide in kidney and urine but not in liver or brain. (ii) The accumulated oligosaccharides were mainly Man(α1-3)[Man(α1-6)]Man(β1-4)GlcNAc, Man(α1-3)(6)-[Man(α1-3)]Man(β1-4)GlcNAc, and Man(α1-6)(6)-[Man(α1-3)]Man(β1-4)GlcNAc. Analogous branched Man₄ and Man₅ structures are found in pig and sheep tissues, but they are N, N'-diacetylchitobiose derivatives.

The substrate specificities of rat kidney lysosomal and cytosolic α-D-mannosidases were investigated because in one type of hereditary α-mannosidosis, that occurring in man, the major storage products are linear rather than branched oligosaccharides. The lysosomal enzyme showed much greater activity toward linear oligosaccharides than toward the branched oligosaccharides induced in the kidney by swainsonine. On the other hand, cytosolic α-D-mannosidase preferred the branched oligosaccharides, a result suggesting that this mannosidase might be inhibitable by swainsonine and that the enzyme might play a normal role in glycoprotein catabolism. Swainsonine was indeed found to inhibit this enzyme at relatively high concentrations (100 μM swainsonine), and concentrations of this magnitude were in fact found in the cytosol of kidney swainsonine-fed rats. The kidney cytosolic α-D-mannosidase levels were reduced in these rats and, more important, the accumulated oligosaccharides were present mainly in the cytosol rather than in lysosomes. These results point to possible involvement of cytosolic α-D-mannosidase in glycoprotein degradation in the rat.

Investigation of glycoprotein metabolism and of the functioning of α-D-mannosidases has been facilitated by the availability of the plant toxin, swainsonine (1-9), which is found in plants of the genus Swainsona and in spotted locoweed (Astragalus lentiginosus). It is a potent inhibitor of liver lysosomal α-D-mannosidase (4, 10) and Golgi mannosidase II (4). The inhibition of the latter enzyme explains the ability of the alkaloid to induce the formation of glycoproteins containing N-linked oligosaccharides of the hybrid type in place of those of the complex type (4-8). Moreover, the ingestion of swainsonine-containing plants produces, in grazing animals, a neurological condition that has been characterized as a phenocopy of the hereditary lysosomal storage disease, α-mannosidosis (1, 10, 11). These animals accumulate mannose-rich oligosaccharides in their tissues. In the pig, both swainsonine and locoweed induced the accumulation of branched oligosaccharides in all tissues examined (12).

The structures of the oligosaccharides that accumulate in α-mannosidosis patients and in swainsonine toxicosis are different. In human α-mannosidosis the major storage oligosaccharides contain linearly linked mannose residues (13-15). The origin of the linear compounds is unclear at present, since the specificities of known lysosomal enzymes do not explain their formation. Also unclear is the role of cytosolic α-D-mannosidase. We report herein substrate specificity studies on rat kidney lysosomal and cytosolic α-D-mannosidases and swainsonine-induced changes in rat kidney which strongly suggest an important role of the cytosol in the metabolism of glycoproteins in the rat.

EXPERIMENTAL PROCEDURES

Materials and Methods—Swainsonine, isolated from Rhizoctonia leguminicola as previously described (2), was generously provided by Dr. Harry P. Broquist of Vanderbilt University. Glc(1H)Man₆GlcNAc was a gift from Drs. S. C. Hubbard and P. Robbins of Massachusetts Institute of Technology. Uniformly labeled [3H]mannose-oligosaccharides, namely Man₆GlcNAc, Man₅GlcNAc, Man₄GlcNAc, Man₃GlcNAc, and Man₂GlcNAc, were prepared by our published procedure (8). GlcNAc(1H)Man₄GlcNAc was prepared by incubating (1H)Man₃GlcNAc with UDP-GlcNAc, MgCl₂, swainsonine, and rat liver Golgi membranes as described (8). Branched (1H)Man₅GlcNAc was prepared by treatment of GlcNAc(1H)Man₄GlcNAc with purified Golgi mannosidase II for 24 h at 37 °C (19) followed by digestion with jack bean β-N-acetylglucosaminidase. The resulting oligosaccharide eluted from a Bio-Gel P-4 column at the position of Man₅GlcNAc (4, 8). This oligosaccharide quantitatively bound to a ConA-Sepharose 4B column (12) and was eluted with 0.1 M α-methylmannoside. These results strongly suggest that this oligosaccharide has the predicted branched structure (i.e. Man(α1-3)(6)-[Man(α1-3)]Man(β1-4)GlcNAc).

Linear (1H)mannose-labeled oligosaccharides, namely Man(α1-2)Man(α1-3)Man(α1-4)GlcNAc and Man(α1-3)Man(α1-4)GlcNAc, which are two of the three major oligosaccharides.

1 The abbreviations used are: ConA, concanavalin A; PNP, p-nitrophenyl; (1H)Man-mannosidase, hydrolytic activity with (1H)Man-labeled oligosaccharides as substrates. The subscript OT indicates NaB₃[3H]Juced oligosaccharides.

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when extensively digested with jack bean mannosidase and resolved on a high resolution Bio-Gel P-4 column, yielded one product (Fig. 1B, Peak I), other than free mannose, which eluted at the position of Man\textsubscript{GlcNAc} and presumably has the structure of GlcMan\textsubscript{GlcNAc}.

The oligosaccharides present in Peak I and Peak II did not bind to a ConA-Sepharose 4B column (12). In addition, when the oligosaccharide alcohols were subjected to acetylation and then resolved on a high resolution Bio-Gel P-4 column (4, 8), more than 95% of the radioactivity eluted at the position of free \textsuperscript{[3H]}mannose (data not shown). The manner in which oligosaccharides in Peak I and Peak II are formed (by the trehalosidase of GlcMan\textsubscript{GlcNAc}) and an oligosaccharide with enzymes of known specificity), the failure of the oligosaccharides to bind ConA-Sepharose 4B, and their insensitivity to acetylation all indicate that the structures of the oligosaccharides in Peak I and Peak II (Fig. 1C) are Man(al-2)Man(al-3)Man(j31-4)GlcNAc, and Man(al-2)Man(al-3)Man(31-4)GlcNAc, respectively. The oligosaccharide in Peak I appears to be a single specie. However, minor contamination of Man\textsubscript{GlcNAc} (Peak II) by Man\textsubscript{GlcNAc} cannot be ruled out. The formation of Man\textsubscript{GlcNAc} and perhaps a little Man\textsubscript{GlcNAc} is due to the combined activities on Man\textsubscript{GlcNAc} (Peak I) of endoplasmic reticulum \textalpha-D-mannosidase (21), Golgi mannosidase IA (19, 22) and Golgi mannosidase IB (19) present in the liver microsomes. All of these enzymes are \textalpha,\textalpha-specific and are rather insensitive to swainsonine (4, 21).

Swainsonine Administration—Male Wistar rats (170-200 g body weight) from Harlan Industries Inc. were administered swainsonine in their drinking water as described (23). In all experiments in which the effect of swainsonine on enzyme and oligosaccharide levels was determined, the swainsonine-fed animals were compared to the age-matched control rats given tap water.

Preparation of Kidney Extracts and Organelles—The following preparations were done according to Shibko and Tappel (24) except that the animals were not fasted. Rats were stunned by a blow to the head and killed by decapitation. The kidneys were quickly excised, cut into small pieces, and homogenized in 0.45 M sucrose containing 0.68 mM EDTA, pH 7.0. The mitochondrial-lysosomal fraction obtained as described (25) was suspended in 1 volume of detergent solution (10 mM potassium phosphate buffer, pH 7.0, containing 0.5% Triton X-100 and 0.25 M NaCl) of the initial kidney weight. Cytosolic fraction was prepared by homogenizing the kidney in 1 volume of the above sucrose solution. The homogenate was centrifuged at 105,000 x g for 60 min and the supernatant (cytosolic fractions) was removed by aspiration. Subcellular fractions, namely mitochondrial-lysosomal, microsomal, and cytosolic fractions, from the kidney of controls and rats administered swainsonine were prepared as described (24).

Enzyme Assays—PNP-mannosidase activity was assayed in 100 mM sodium acetate with 4 mM \textalpha-D-mannosidase as substrate at pH 4.4 (lysosomal) or pH 6.0 (cytosolic) as described (23) by incubating 5 \mu l of the kidney fraction (mitochondrial-lysosomal suspension or cytosolic fraction) with the appropriate buffer and substrate in a total volume of 0.5 ml. Following incubation for 30 min at 37 °C, the reaction was stopped by adding 1.0 ml of 3M H\textsubscript{2}SO\textsubscript{4} (25). One unit is the amount of enzyme which catalyzed the release of 1 \mu mol of p-nitropheno1-h at 37 °C.

Oligosaccharide-clearing activity ((\textsuperscript{[3H]}Man)\textsubscript{GlcNAc} activity) was assayed by measuring the hydrolysis of \textsuperscript{[3H]}mannose-labeled oligosaccharide in 100 mM sodium acetate buffer at pH 5.0 (lysosomal) or pH 6.0 (cytosolic). In method A, 5 \mu l of the kidney fraction was incubated with the buffer and 3000 cpm of the labeled oligosaccharide in a total volume of 0.1 ml. Following incubation for 2 h at 37 °C, the reaction was stopped by heating the samples at 105 °C for 5-7 min. In method B, 15 \mu l of the kidney fraction was incubated as above in a total volume of 0.05 ml. The reaction was stopped as above following incubation at 37 °C for 30 min. Free \textsuperscript{[3H]}mannose was separated from oligosaccharide by gel filtration on a column of Bio-Gel P-2 and quantitated as described (19). One unit of \textsuperscript{[3H]}mannose activity is the amount of enzyme which catalyzed the release of 1000 cpm of \textsuperscript{[3H]}mannose/h. Protein was assayed by the fluorometric method (26) using bovine serum albumin as standard.

Swainsonine Assay—Swainsonine in the subcellular fractions obtained from the kidney of a rat administered the alkaloid was quantitated by comparing the inhibition of Golgi mannosidase II (27) prepared by a known volume of heat-inactivated (105 °C) kidney fraction with that of standard swainsonine solutions.

Urine Collection and Preparation of Urinary Oligosaccharide—Twenty-four-hour urine samples were obtained from rats kept in metabolic cages. A few drops of toluene were added in the collecting beakers to prevent growth of microorganisms. The clear supernatant obtained by centrifuging the urine at 1600 x g for 30 min was deproteinized by mixing with 3 volumes of ethanol followed by centrifugation. The crude oligosaccharide from the deproteinized
Lysosomal and Cytosolic α-D-Mannosidases

TABLE I
Effect of swainsonine on oligosaccharide content of rat tissues and urine

| Animal group* | Tissue          | Control | Swainsonine | 1        | 2        | 3        | 4        |
|---------------|----------------|---------|-------------|----------|----------|----------|----------|
|               |                | mg hexose/g tissue | %          | %        | %        | %        | %        |
| Liver         |                | 10,628  | 9,943       | 94       | 94       | 105      | 106      |
| Kidney        |                | 203     | 1,607       | 792      | 788      | 705      | 116      |
| Brain         |                | 205     | 198         | 97       | 101      | 125      | 91       |
| Urine         |                | 11,565  | 15,540      | 134      | 140      | 145      | 102      |

*The animal groups are: 1, swainsonine for 1 week; 2, swainsonine for 2 weeks; 3, swainsonine for 4 weeks; 4, swainsonine for 4 weeks, and then no swainsonine for 1 week. The dose of swainsonine was 5 μg/ml of drinking water.

urine was precipitated with 2 volumes of ether (12).

Preparation and Characterization of Tissue Oligosaccharides—Crude oligosaccharide from whole tissues as well as from kidney subcellular fractions was prepared as described from our laboratory (12). In brief, crude oligosaccharide was precipitated by ethanol/ether, dried, and then suspended in 0.3 ml of 0.1 M acetic acid (12). The suspension was assayed for total hexose by the phenol/sulfuric acid method (28), scaled down 5-fold, with mannose as standard. The crude oligosaccharide was fractioned by gel filtration on Bio-Gel P-4 and purified by ConA-Sepharose 4B chromatography and high resolution Bio-Gel P-4 column chromatography (12). The purified oligosaccharides were analyzed for neutral sugars and hexosamine (29, 30). The oligosaccharides were labeled with NaB[3H]4 (31) and the swainsonine-fed rat was assayed for hexose and then no swainsonine for 1 week. The dose of swainsonine was 5 μg/ml of drinking water.

FIG. 2. Fractionation of oligosaccharide from tissues and urine of control and swainsonine-fed rats. Male Wistar rats (200 g body weight) were administered swainsonine (5 μg/ml drinking water for 4 weeks) as described under "Experimental Procedures." The ethanol/ether-precipitated oligosaccharide was prepared from 1.5 g of tissues (A, liver; B, kidney; and C, brain) and 24 h urine (D) from the control ○ (a) and the swainsonine-fed rat ● (b). The oligosaccharide was suspended in 0.3 ml of 0.1 M acetic acid and applied to a Bio-Gel P-4 column (0.8 × 136 cm, −400 mesh) equilibrated with 0.1 M acetic acid. Fractions (0.5 ml) were collected at a flow rate of 2.5 ml/h. Aliquots from each fraction were assayed for hexose by the phenol/sulfuric acid method (28). The column exclusion volume (Ve) was determined using bovine serum albumin. The standards are: M2N, (Man)2GlcNAc; M2N, (Man)GlcNAc; M2N, (Man)GlcNAc; M2N, (Man)GlcNAc; and M–N, ManGlcNAc.

RESULTS

Effect of Swainsonine Administration on Oligosaccharide Content of Rat Tissues and Urine—Comparison of the total hexose levels in the deproteinized ethanol/ether precipitate showed that only kidney, and perhaps urine, but not liver or brain, from swainsonine-fed rats contained higher levels of total hexose than the age-matched control rats. As shown in Table I, total hexose is essentially unchanged in the swainsonine-fed rat liver and brain, but the kidney of experimental animals contained nearly 8-fold higher oligosaccharide content than the control kidney. The ethanol/ether precipitate from the experimental rat urine showed somewhat higher levels of total hexose (134–146%) than the control rat urine (Table I).

Fractionation of the ethanol/ether-precipitated crude oligosaccharide from the urine and tissues of control and swainsonine-fed rats on a Bio-Gel P-4 column showed that only kidney and urine, but not liver or brain, of experimental animals showed accumulation of oligosaccharides (Fig. 2).

Three oligosaccharides were separated and purified from the experimental rat kidney fractions 90–115 (Fig. 2) by ConA-Sepharose 4B and then high resolution Bio-Gel P-4 column chromatography. When the pooled oligosaccharides were applied to a ConA-Sepharose 4B column (1.5 × 18 cm) as previously described (12), less than 10% of the oligosaccharide passed unadsorbed into the effluent. This unadsorbed oligosaccharide, when applied to the Bio-Gel P-4 column, eluted as a sharp peak at the position of standard Man,GlcNAc (Fig. 3A) and had the composition of M2N,GlcNAc (oligosaccharide B) (Table II). The nearly 90% of the original oligosaccharide that adsorbed to the ConA-Sepharose column, when eluted with 250 mM α-methyl-mannoside and applied to the Bio-Gel P-4 column, separated into two peaks (Fig. 3B). Sugar analyses of Peak I (oligosaccharide C) and Peak II (oligosaccharide A) yielded the composition Man,GlcNAc and Man,GlcNAc, respectively (Table II).

It was observed that the total amount of crude oligosaccharide in kidney is dependent on the amount of swainsonine administered. The rats fed 5 μg of swainsonine/ml of drinking water showed 3- and 8-fold higher oligosaccharides in kidney
after 2 and 5 days, respectively; the rats fed 20 or 50 μg of swainsonine/ml showed 5- and 15-fold increases (Fig. 4). On the other hand, administration of higher concentration of the alkaloid had no effect on the liver or brain oligosaccharide (data not shown). It may be noted here that we have previously shown (9) that the relative amounts of the three oligosaccharides accumulating in the experimental kidney are dependent on the concentration of swainsonine administered.

**Structures of Kidney Oligosaccharides A, B, and C**—The structures of the three oligosaccharides isolated from the kidneys of swainsonine-fed rats were established by first reducing them with NaB[3H]₄ and then subjecting the labeled oligosaccharide alcohols to various treatments which yielded the following results. (i) The three oligosaccharides were sensitive to jack bean α-1,2-mannosidase treatment, resulting in the production of a substance which eluted from a Bio-Gel P-4 column at the position of Man-GlcNAcoT. Together with the compositional data, these results indicate that each oligosaccharide contained only 1 GlcNAc residue (Table II). (iii) Rat liver Golgi mannosidase II had no effect on these oligosaccharides. However, oligosaccharides B and C, but not A, were sensitive to Golgi mannosidase II after they were N-acetylgalactosaminylated (8), a result indicating that B and C are Man₃ or Man₄ derivatives. (iv) Following acetylation, which rather selectively cleaves 1,6 linkages, the labeled products from oligosaccharides A, B, and C eluted from a Bio-Gel P-4 column at the position of Man₃GlcNAcoT. Together with the compositional data, these results indicate that the three oligosaccharides accumulating in the kidney of the swainsonine-fed rat have the structures:

- **Oligosaccharide A**: Man(α1-3)[Man(α1-6)]Man(β1-4)GlcNAc; oligosaccharide B, Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc; and oligosaccharide C, Man(α1-3)[Man(α1-6)]Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc. (Fig. 5). It is noteworthy that oligosaccharides A and C bind to a ConA-Sepharose 4B column, in conformance with their assigned structures, which have 2 interacting mannose residues attached to a single mannose residue (34, 35), whereas oligosaccharide B does not bind to this adsorbent. Oligosaccharides B and C are similar to the oligosaccharides isolated from swainsonine- and locoweed-fed pig brain and kidney (12) and from urine of locoweed-fed sheep (32), except that the rat kidney oligosaccharides do not contain N,N'-diacyctyldihiose at the reducing end.

**Substrate Specificities of Rat Kidney Lysosomal and Cytosolic α-1,2-Mannosidases**—The activities of kidney lysosomal
and cytosolic \( \alpha\)-\(d\)-mannosidases are shown in Table III. Surprisingly, the \( \alpha\)-\(d\)-mannosidase present in the mitochondrial-lysosomal fraction showed low activity toward high mannose oligosaccharides (Man\(_n\)GlcNAc to Man\(_n\)GlcNAc). Even the branched oligosaccharides which accumulate in the kidney of rats administered swainsonine, namely Man\(_n\)GlcNAc, Man\(_n\)GlcNAc and Man\(_n\)GlcNAc, were poor substrates. However, the linear oligosaccharides were good substrates for this enzyme. There was complete hydrolysis of both \( \alpha\)-1-2 and \( \alpha\)-1-3 linkages in linear Man\(_n\)GlcNAc, whereas there was much less cleavage of \( \alpha\)-1-2 linkages in high mannose oligosaccharides and of the \( \alpha\)-1-3 linkage in branched Man\(_n\)GlcNAc. On the other hand, with cytosolic \( \alpha\)-\(d\)-mannosidase the high mannose oligosaccharides (Man\(_n\)GlcNAc to Man\(_n\)GlcNAc) and two of the three oligosaccharides accumulating in swainsonine toxicity were better substrates than the linear oligosaccharides (Table III). The difference in the substrate specificities of the rat kidney lysosomal \( \alpha\)-\(d\)-mannosidase and cytosolic \( \alpha\)-\(d\)-mannosidase is also evident from the time course of the hydrolyses of the smaller oligosaccharides (Fig. 6). Although the limited supply of mannose-labeled substrates available for these specificity studies did not permit variations in substrate concentrations, the results obtained do show substantial differences with the two enzymes, especially in activity toward Man\(_n\)GlcNAc, which was cleaved only by the cytosolic enzyme.

It was of interest to determine the structures of products formed by the action of cytosolic \( \alpha\)-mannosidase on oligosaccharide. Table IV gives the results of an experiment on Man\(_n\)GlcNAc. After extensive action of kidney cytosolic fraction on this substance, the reaction products were isolated by gel filtration on Bio-Gel P-2, and then fractionated on a ConA-Sepharose 4B column. The purified oligosaccharide products were subjected to acetylation, and the degradation products separated on a high resolution Bio-Gel P-4 column. The acetylation experiments indicated that the Man\(_n\)GlcNAc had been hydrolyzed by mannosidase to Man\(_n\)Man\(_\beta\)6,6GlcNAc in 56% yield, Man\(_n\)Man\(_\beta\)1-4GlcNAc in 29% yield, and Man\(_n\)Man\(_\beta\)1-6Man\(_\beta\)4GlcNAc in 15% yield.

**TABLE III**

| Substrate | Structure | Lysosomal \( \alpha\)-\(d\)-mannosidase* | Cytosolic \( \alpha\)-\(d\)-mannosidase* |
|-----------|----------|--------------------------------------|--------------------------------------|
| Man\(_n\)GlcNAc | Several isomers | 11.2 16.8 33.4 57.3 | % [\(\text{H}\)]Man\(_n\)mannose released |
| Man\(_n\)GlcNAc | Several isomers | 6.2 11.1 22.6 53.2 | % [\(\text{H}\)]Man\(_n\)mannose released |
| Man\(_n\)GlcNAc | Several isomers | 4.7 7.8 17.4 51.7 | % [\(\text{H}\)]Man\(_n\)mannose released |
| GlcNAcMan\(_n\)- | M | 2.9 3.8 13.4 44.3 |
| GlcNAc | M | 3.0 6.5 13.2 47.2 |
| Man\(_n\)GlcNAc | M | 5.8 11.4 6.4 31.6 |
| Man\(_n\)GlcNAc | M | 7.2 16.3 6.2 15.7 |
| Man\(_n\)GlcNAc | M | 24.4 73.2 3.6 12.4 |
| Man\(_n\)GlcNAc | M | 29.8 65.3 1.4 6.3 |

* Aliquots from the rat kidney mitochondrial-lysosomal suspension, containing 1.5 units of PNP-mannosidase activity, were incubated at 37 °C with the oligosaccharide at pH 5.0 as described under method A of "Experimental Procedures." Preliminary studies indicated that hydrolysis of the oligosaccharides was twice as high at pH 5.0 than at pH 4.4.

"Incubation time."

"These oligosaccharides accumulate in the kidney of the rat administered swainsonine.

"These oligosaccharides accumulate in the brain and are excreted in the urine of the patients with \( \alpha\)-mannosidosis.

**Effect of Swainsonine on Cytosolic \( \alpha\)-\(d\)-Mannosidase—**We have previously reported that swainsonine is a potent inhibitor of rat liver lysosomal \( \alpha\)-\(d\)-mannosidase and Golgi mannose-d-mannosidase II (4). Although the alkaloid caused 50% inhibition of these two enzymes at a concentration of 0.2 \(\mu\)M, it had little or no effect on liver cytosolic \( \alpha\)-\(d\)-mannosidase even at a concentration of 10 \(\mu\)M (4). We now report that at a much higher concentration the alkaloid inhibits both the PNP-mannosidase and [\(\text{H}\)]Man-mannosidase activities present in the kidney cytosol. As shown in Fig. 7, 50% inhibition of the cytosolic \( \alpha\)-\(d\)-mannosidase activity is caused by 100 \(\mu\)M swainsonine, a concentration that might have biological relevance in spite of its being 500 times higher than required to cause
FIG. 6. The time course of hydrolysis of [3H]Man-oligosaccharides by kidney lysosomal and cytosolic α-D-mannosidases. Approximately 3000 cpm of each of the five oligosaccharides was incubated at 37 °C with aliquots from a rat kidney mitochondrially-lysosomal suspension (pH 5.0) or cytosolic fraction (pH 6.0), each containing 1.5 units of PNP-mannosidase activity, in a total volume of 0.1 ml. The reaction was stopped at the indicated time by heating the samples in boiling water (5–7 min). Free [3H]mannose was quantitated after separation from labeled oligosaccharide on a column of Bio-Gel P-2 (19). M, Man; N, GlcNAc.

Table IV
Products formed from action of rat kidney cytosol on [3H]Man₅GlcNAc

| Product | Acetolysis products | Assigned structure |
|---------|---------------------|-------------------|
| Products not adsorbed on ConA-Sepharose | | |
| Man₅GlcNAc (15%) | 0° | 30 | 54 | 16 | Man(α1-3)Man(α1-6)Man(β1-4)GlcNAc
| Man₅GlcNAc (66%) | 92 | 0° | 0° | 8 | Man(α1-3)Man(β1-4)GlcNAc
| Product adsorbed on ConA-Sepharose and eluted with α-MeMan | | |
| Man₅GlcNAc (29%) | 58 | 0° | 0° | 42 | Man(α1-6)
| | | | | Man(β1-4)GlcNAc
| | | | | Man(α1-3)
| Original substrate, Man₅GlcNAc | 38 | 0° | 36 | 26 | Man(α1-6)
| | | | | Man(α1-3)
| | | | | Man(β1-4)GlcNAc
| | | | | Man(α1-3)

*Less than 5%.

*If acetolysis were completely specific for 1,6 linkages, the amount of labeled cleavage products obtained would allow 15–20% of Man(α1-6)Man(β1-4) to be present in the fraction analyzed. Since acetolysis is not that specific, considerably less is present with the Man(α1-3)Man(α1-6)Man(β1-4)GlcNAc.

Effect of Swainsonine Administration on α-D-Mannosidase Levels in Rat Kidney—Since the swainsonine inhibition of lysosomal α-D-mannosidase (27) and cytosolic α-D-mannosidase is largely reversible, it occurred to us that, if enzyme assays were carried out at different dilutions, the levels of enzymatic activities found might give evidence for the presence of inhibitory amounts of swainsonine in the kidney fractions. Table V shows the results obtained in assaying oligosaccharide cleavage by cytosolic fractions from swainsonine-fed and control animals. Method A, employing the more diluted extracts (20-fold dilution), gave higher enzymatic levels only for the swainsonine-fed animals than did Method B (2-fold dilution). The data strongly suggest that the cytosolic mannosidase is in fact inhibited in the intact kidney and that the inhibition is due to swainsonine.

Subcellular Localization of Oligosaccharides and Swainsonine in the Kidney of the Swainsonine-fed Rat—The kidney of the swainsonine-fed rat shows a rapid and massive accumulation of mannose-rich oligosaccharides (9). Since lysosomal

D. R. P. Tulsiuni and O. Touster, unpublished results.
Fig. 7. Effect of swainsonine on kidney cytosolic α-D-mannosidase. Rat kidney cytosolic fraction was assayed for α-D-mannosidase activity using 4 nM p-nitrophenyl α-D-mannosidase or ~3000 cpm of [3H]Man₆GlcNAc in 100 mM sodium acetate buffer, pH 6.0, the total volumes being 0.5 and 0.1 ml, respectively. The enzyme was preincubated (15 min at 0-4°C) with varying concentrations of swainsonine in the buffer before addition of substrate and incubation at 37°C for 60-120 min. Released p-nitrophenol or [3H]mannose was quantitated as described (19).

### DISCUSSION

It has long been assumed that lysosomal α-D-mannosidase, together with hydrolases in this organelle for other glycosidic linkages, has a central role in the catabolism of glycoproteins containing asparagine-linked oligosaccharides. The assumption is based on the accepted role of lysosomes in catabolism and on the fact that oligosaccharides accumulate in the lysosomes of animals and humans with an inherited deficiency of α-D-mannosidase. At the same time, there has been no clue as to the function of cytosolic α-D-mannosidase. Substrate specificity studies on the two enzymes have been limited (36, 37) and, perhaps in part for this reason, it has been difficult to determine why particular oligosaccharides are found in mannosidosis tissues and urine (13-18).

In the present discussion, the term “cytosol” is used operationally, designating the high speed supernatant solution from a tissue homogenate prepared by a mild homogenization procedure. Nonetheless, the presence of uncharacterized microvesicles is possible. Alternatively, some type of fragile vesicles (other than lysosomes) may have been extensively ruptured during homogenization. Perhaps the best approach to investigating these possibilities would be to carry out...
electronic microscopic immunocytochemical analysis. This point is especially important because current views of glyco-
plasmic reticulum mannosidase raise the possibility that the former is a proteolytic product of the latter. While this possibility cannot be precluded at the present time, it is noteworthy that, whereas the endoplasmic reticulum mannosidase is inactive toward GlcNAcMan,GlcNAc (21), the cytosolic enzyme readily hydrolyzes this substrate (Table III).

Since the present work has relevance to the abnormal oligosaccharide catabolism in hereditary α-mannosidosis, some brief comments on this disease are in order. In the α-
mannosidosis of man, the accumulated tissue oligosaccharides are predominantly linear in structure and contain one GlcNAc (16, 18). Bovine α-mannosidase is accompanied by the excretion of oligosaccharides, half of which appear to be branched and half of which are Man₃GlcNAc₂ (38). Very recently the excreted oligosaccharides in feline α-mannosidosis have been reported to be branched in structure, Man₅GlcNAc₂ being the predominant urinary substance (39). Three points should be made. (i) The species differences make enzymatic interpretations more difficult to reach. (ii) Since urinary oligosaccharides may result from subsequent modification of accumulated tissue oligosaccharides, their structures may not be reliable indicators of the nature of the enzymatic deficiencies in tissues. (iii) The feline storage disease appears to be more complex than the others. In addition to the deficiency of lysosomal α-D-mannosidase, the liver and kidney cytosolic mannosidase were depressed to one-third of normal (40). Tissue analyses in the cat were not reported.

In the present work we show that swainsonine administration causes accumulation of oligosaccharides in rat kidney and urine, but not in liver or brain. The three oligosaccharides isolated from the kidneys of swainsonine-fed animals were shown to be branched-chain structures of the composition Man₃GlcNAc, Man₅GlcNAc, and Man₆GlcNAc. The pentamannosyl compound is probably derived from the hybrid type glycoproteins produced as a result of the inhibition of Golgi mannosidase II by swainsonine, with the other compounds presumably resulting from residual mannosidase action. Man₃GlcNAc and Man₅GlcNAc have the same oligomannose structure as in the two major oligosaccharides found in the brain and kidney of pigs fed swainsonine or locoweed, the oligosaccharides from this species containing a diacetyltobiase residue in place of the single GlcNAc (12). This difference is undoubtedly due to differences in amount, or action, of mammalian endoglycosidase which, until recently, has been found to be localized in the cytosol (41-45), but not the lysosomes. A lysosomal di-N-acetylchitobiose with substrate specificity different from that of the cytosolic enzyme has recently been reported (46). The cytosolic localization of the longer known enzyme obviously raises the question as to whether glycoprotein degradation occurs in the cytosol, which contains all of the required enzymes, namely neuraminidase (47, 48), β-galactosidase (49, 50), N-acetyl-β-hexosaminidase (51), α-D-mannosidase (52, 53), and a β-mannosidase (54), as well as endoglycosidase. Therefore, we examined the substrate specificity of both lysosomal and cytosolic α-D-mannosidase of rat kidney, the only tissue in the rat which showed oligosaccharide accumulation during swainsonine administration.

The comparative study of the two mannosidases disclosed sharp and unexpected differences in substrate specificity. Whereas the lysosomal enzyme readily hydrolyzed linear oligomannose compounds, it had little activity toward high mannose oligosaccharides or toward the three branched oligosaccharides which accumulated during swainsonine administration. While these results are consistent with the accumulation of linear compounds in human mannosidosis, they raise the question as to how the high mannose, branched oligosaccharides are hydrolyzed normally during catabolism. These compounds were found to be better substrates for the cytosolic α-D-mannosidase, which was rather ineffective in cleaving the linear oligosaccharides. These results also suggested a normal role for the cytosol in oligosaccharide degradation and that swainsonine might be inhibiting cytosolic α-
D-mannosidase, thereby causing accumulation of the three storage compounds.

Although we had earlier found that, as compared to the lysosomal enzyme and mannosidase II, cytosolic mannosidase is relatively insensitive to swainsonine (4), a new test with comparatively high alkaloid concentrations showed that it is 50% inhibited by 100 μM swainsonine. Moreover, the level of cytosolic mannosidase activity in the kidney of swainsonine-fed rats was depressed, particularly when concentrated tissue samples were used for assays to counteract the reversibility of inhibition by dilution. This experiment suggested that swainsonine was in fact the cause of the depressed enzyme levels. Furthermore, the concentration of swainsonine required to produce the depressed levels of cytosolic mannosidase in kidney was reasonably similar to that actually found in kidney cytosol, in which the administered swainsonine is highly concentrated. Whether the decreased cytosolic enzymatic activity is sufficient to cause oligosaccharide accumulation cannot be answered at this time, since it would depend in part on the load of oligosaccharide and its structure as well.

Finally, as predicted from the above findings, subcellular localization of the accumulated oligosaccharide in the kidney of the swainsonine-fed rat disclosed that the oligosaccharide was present mainly in the cytosol, rather than in a fraction containing lysosomes. The concentration of both oligosaccharide and swainsonine in the cytosol, the inhibitory effect of swainsonine on cytosolic α-D-mannosidase, the depressed levels of cytosolic mannosidase in the swainsonine-fed rat, and the structures of the accumulated oligosaccharides all suggest that the swainsonine-induced storage in rat kidney is a result of events occurring in the cytosol. The presence of substantial endoglycosidase in the cytosol (41-45) is consistent with this conclusion, and it is relevant that this enzyme is reported to show a marked preference for branched as compared to linear oligosaccharides (45).

What, then, are the different roles of the cytosol and lysosomes in glycoprotein breakdown? One possibility is that each compartment has a different enzymatic system, the cytosolic system involving branched oligosaccharides and the lysosomal one involving the linear oligosaccharides. Among the possible mechanisms for the production of the linear compounds within lysosomes are the following: (i) action of the newly discovered lysosomal endoglycosidase followed by cleavage of high mannose oligosaccharides by unknown exo-
and/or endo-α-D-mannosidase; (The existence of a new α-D-mannosidase in lysosomes has, in fact, been suggested to be present in fibroblasts, this enzyme being inhibited by swain-
sonine but not absent in hereditary mannosidosis (55).) (ii) cleavage by α-D-mannosidase(s) of high mannose oligosaccharides which are temporally blocked, perhaps by Glc, at the 1,3-antenna; and (iii) catabolism of glycoproteins containing linear oligosaccharides (e.g. rat liver cathepsin B (56)).

It is conceivable that oligosaccharide hydrolysis generally begins in the cytosol and that linear products are then trans-

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ferred to lysosomes for further cleavage. In one experiment bearing on this question, we found that cytosolic mannosidases produced from branched Man<sub>3</sub>GlcNAc a mixture containing only 29% branched Man<sub>3</sub>GlcNAc and Man<sub>2</sub>GlcNAc. No transport system is currently known by which the latter two substances might enter lysosomes. However, there is now evidence for transport systems in lysosomal membranes, for example, for cysteine (57) and N-acetylenuraminic acid (58).

It is particularly noteworthy that in human mannosidosis branched Man<sub>α</sub> derivatives are only very minor excretory products and are not found in the tissues of patients (13). If these Man<sub>α</sub> products of the breakdown of complex glycoproteins, the most abundant N-linked glycoproteins of mammalian tissues (59), were normally handled by lysosomal α-D-mannosidase, they should be major storage products. In fact, neither the lysosomal nor cytosolic mannosidases studied in the present work, or even a new brain α-mannosidase recently reported (60), utilize branched Man<sub>3</sub>GlcNAc very well.

It is evident that the major degradative pathways for asparagine-linked oligosaccharides have not as yet been clearly delineated. The results of our study of rat kidney indicate that subsequent investigations should deal with possible involvement of cytosolic as well as lysosomal enzyme systems. In particular, the substrate specificities of the glycosidases in the two cellular compartments are of particular relevance.

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