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Glycoprotein metabolism in normal and β-mannosidase-deficient cultured goat skin fibroblasts

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Cultured skin fibroblasts established from goats affected with β-mannosidosis, an inherited neurovisceral storage disorder, showed an absence of lysosomal β-mannosidase activity and the corresponding accumulation of a trisaccharide (TS) with the structure Manβ(1→4)GlcNAcβ(1→4)GlcNAc (0.4 μmol/g) and lesser amounts (0.15 μmol/g) of a Manβ(1→4)GlcNAc disaccharide (DS). By using purified storage TS isolated from fibroblasts metabolically labelled with [^{3}H]GlcN, no conversion of TS into DS could be demonstrated in homogenates of affected cells at either lysosomal pH (4.4) or cytosolic pH (6.1), or in the culture medium (pH 7.0) of affected cells. Both TS and DS were secreted into the culture medium by affected fibroblasts. When affected fibroblasts were treated with tunicamycin before labelling with [^{3}H]GlcN, the accumulation of both labelled TS and DS was completely inhibited. Treatment of both affected and normal goat fibroblasts with swainsonine resulted in the inhibition of lysosomal α-mannosidase activity and in the accumulation of the same labelled oligosaccharides in both. The major storage pentasaccharide from both normal and affected swainsonine-treated fibroblasts was sensitive to digestion with α-mannosidase and endo-β-*N*-acetylhexosaminidase D, suggesting a branched mannose structure and a chitobiose core. In the absence of evidence for the existence of unusual *N*-linked glycoprotein-associated chitotriose oligosaccharide structures in affected goat fibroblasts, it must be concluded that degradative pathways for *N*-linked oligosaccharides are similar in both normal and affected goat fibroblasts, and that these pathways differ from catabolic pathways in human fibroblasts.

INTRODUCTION

Goat β-mannosidosis, a recently described autosomal recessive neurovisceral storage disorder, was originally shown to occur in a colony of inbred Nubian goats (Jones & Dawson, 1981; Jones & Laine, 1981). Biochemically, the disease is marked by the absence of lysosomal β-mannosidase activity (Jones & Dawson, 1981), and the concomitant accumulation of Manβ(1→4)GlcNAcβ(1→4)GlcNAc trisaccharide, together with smaller amounts of Manβ(1→4)GlcNAc disaccharide in brain (Jones & Laine, 1981) and kidney (Matsuura *et al.*, 1981) and their excretion in urine of affected animals (Matsuura *et al.*, 1983).

On the basis of the structural characterization of the storage oligosaccharides in goat mannosidosis, it has been presumed that they arise as a result of impaired degradation of *N*-linked glycoprotein-associated oligosaccharides, since the β-mannose linkage is ubiquitous in the core structure of these oligosaccharides (Dawson, 1978). Moreover, the storage oligosaccharides in human sialidosis (Michalski *et al.*, 1977), fucosidosis (Tsay *et al.* 1976), G_{M1}-gangliosidosis (Wolfe *et al.*, 1974), Sandhoff's disease (Ng Ying Kin *et al.*, 1974) and α-mannosidosis (Norden *et al.*, 1973) all terminate in the Manβ(1→4)GlcNAc sequence, whereas analogous enzyme defects in dogs (Warner & O'Brien, 1982), cats (Burditt *et al.*, 1980), cows (Abraham *et al.*, 1983) and goats (Jones & Laine, 1981; Matsuura *et al.*, 1981, 1983) result in major storage oligosaccharides that terminate in the Manβ(1→4)GlcNAcβ(1→4)GlcNAc sequence. It has thus far been unclear as to whether the two storage products in goat β-mannosidosis arise as a result of metabolic interconversion, independently as a result of different endoglycosidase activities, or from partial degradation of heterogeneous core oligosacharide structures. The present work provides definitive evidence for the origin of the storage oligosacharides in impaired *N*-linked oligosaccharide degradation, and suggests the existence of similar pathways of glycoprotein-associated oligosaccharide catabolism in both normal and affected cells, which primarily involves the action of endo-aspartylglucosaminidase. Portions of this work have been presented in abstract form (Hancock *et al.*, 1982).

MATERIAL AND METHODS

Materials

Powdered culture medium, foetal-calf serum, 0.25% trypsin solution, and penicillin/streptomycin were purchased from Grand Island Biological Co. (Grand Island, NY, U.S.A.). Garamycin (gentamycin) was from Schering Corporation (Kenilworth, NJ, U.S.A.). Authentic *N*-acetyl-lactosamine, di-*N*-acetylchitobiose, and tri-*N*-acetylchitotriose were from Sigma Chemical Co. (St.

Abbreviations used: TS, storage trisaccharide Manβ(1→4)GlcNAcβ(1→4)GlcNAc; DS, storage disaccharide Manβ(1→4)GlcNAc.

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Louis, MO, U.S.A.). 4-Methylumbelliferyl glycosides were from Koch–Light (Colnbrook, Slough, Berks., U.K.). Derivatizing reagents and anhydrous hydrazine were from Pierce Chemical Co. (Rockford, IL, U.S.A.), and 3% OV-1 on 100/120 Gas Chrom Q was from Applied Science Laboratories (State College, PA, U.S.A.). Bio-Gel P-2, P-4 and P-6 (−400 mesh) were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Sepharose 6B and concanavalin A–Sepharose 4B were from Pharmacia Fine Chemicals (Piscataway, NJ, U.S.A.). LHP-K t.l.c. plates and 3MM chromatography paper were from Whatman, Incorporated (Clifton, NJ, U.S.A.). Endo-β-*N*-acetylglucosaminidase D (*Diplococcus pneumoniae*) was purchased from Seikagaku Fine Biochemicals (Tokyo, Japan). Exoglycosidases [β-mannosidase from the snail *Helix pomatia*, α-mannosidase and β-*N*-acetylhexosaminidase from jack bean (*Canavalia ensiformis*)] were generously given by Dr. Y.-T. Li, Tulane University, endo-β-*N*-acetylglucosaminidase L was generously given by Dr. Robert Trimble and Dr. Frank Maley, New York Department of Health, and swainsonine was generously given by Dr. Peter Dorling, Murdoch University, Murdoch, Western Australia, Australia 6150. D-[6-^{3}H]Glucosamine hydrochloride (20.2 Ci/mmol) was from New England Nuclear (Boston, MA, U.S.A.). Tunicamycin was from Calbiochem (La Jolla, CA, U.S.A.). All other chemicals and reagents were of reagent grade or better.

Establishment of skin fibroblast cultures and culture conditions

Punch biopsies were collected aseptically from normal and affected goats and transferred in culture medium to the tissue-culture facilities. The tissue was minced with razor blades, treated with 0.25% trypsin solution for 15 min at 37 °C, and then cultured in Matalon's Modified Dulbecco's Medium (Matalon & Dorfman, 1966) supplemented with penicillin/streptomycin, gentamycin, and 10% (v/v) foetal-calf serum. After outgrowth of fibroblasts was observed (usually within 2 weeks), larger pieces of tissue were aspirated and cultures were maintained until near confluency. Subsequently, cells were routinely passaged 1:4 by trypsin treatment at 2–3-week intervals, and maintained in the same medium under CO_2/air (1:9) and at 37 °C. Fibroblasts at fewer than 20 cell-population doublings were maintained for experimental purposes. All experiments were carried out on fibroblast cultures from at least two separate control and affected animals.

Metabolic labelling

At 1–2 weeks after subculturing, unlabelled medium was replaced with medium containing [^{3}H]GlcN at 4–10 μCi/ml. Labelling was continued for 48 h, after which cells were harvested as described below. In experiments involving the characterization of medium-associated glycoconjugates, cells were rinsed three times with unlabelled medium after the 48 h labelling, and the cells were cultured in unlabelled medium for an additional 48 h. In experiments involving tunicamycin treatment, cells were pre-treated with tunicamycin (0.5 μg/ml) for 6 h before labelling, and labelling was continued for only 24 h in both control and tunicamycin-treated cultures. For swainsonine studies, cells were pre-treated with 100 μM-swainsonine for 24 h before addition of [^{3}H]GlcN for a further 48 h.

Harvesting of cells and collection of medium

Before harvesting either labelled or unlabelled cells, the culture medium was aspirated and the monolayer was rinsed three times with phosphate-buffered saline (50 mM-KH_2PO_4/NaOH/0.15 M-NaCl), pH 7.2. Additional buffer was added to the culture dish and the cells were harvested by scraping with a rubber policeman. The cell pellet was obtained after centrifugation of the suspension at 600 *g* for 10 min. Medium-associated glycoconjugates were characterized after centrifugation of culture medium at 600 *g* for 10 min to remove whole cells and debris.

Preparation of the soluble and glycoprotein-associated oligosaccharide cell fractions

To obtain the soluble cell fraction, the cell pellet was suspended in distilled water or 10% (v/v) acetic acid and the cells were disrupted by brief sonication (two 10 s bursts) at power setting 4 with a Model W185 sonifier–cell disruptor (Heat Systems–Ultrasonics, Plainview, NY, U.S.A.). After centrifugation at 100000 *g* for 75 min, the supernatant fraction was used for further characterization of the soluble glycoconjugates. Gel filtration of this fraction was carried out on a 1.5 cm × 100 cm column of Bio-Gel P-2 (−400 mesh), with 10% acetic acid as the eluant. The 100000 *g* pellet was resuspended in 1.2 ml of distilled water and 6.0 ml of chloroform/methanol (2:1, v/v) was added. The phases were mixed thoroughly, and extraction was continued for 30 min at room temperature with frequent mixing (Dawson *et al.*, 1979). The interphase from this extraction was dried, and glycoprotein-associated oligosaccharides were prepared by treatment with anhydrous hydrazine as described by Rush *et al.* (1981). Concanavalin A–Sepharose 4B affinity chromatography of the oligosaccharide fraction was carried out on a 5 ml column, with sequential elution with buffer, 20 mM-α-methyl D-glucoside and 200 mM-α-methyl D-glucoside (Krusius & Finne, 1978). Gel filtration of glycoprotein-associated oligosaccharides was carried out on 1.5 cm × 100 cm columns of Bio-Gel P-4 or P-6 (−400 mesh) (Takasaki *et al.*, 1980), with 10% acetic acid as the eluant.

Characterization of storage oligosaccharides

Carbohydrate composition and quantification of storage oligosaccharides was obtained by g.l.c. of the trimethylsilyl derivatives of the methyl glycosides (Dawson, 1976). T.l.c. of the intact oligosaccharides was carried out on Whatman LHP-K plates, with acetonitrile/water (4:1, v/v) as solvent (Jones & Laine, 1981). Digestions with α-mannosidase, β-mannosidase, β-*N*-acetylhexosaminidase, and endo-β-*N*-acetylglucosaminidases D and L were carried out in 0.1 M-citrate/phosphate, pH 4.4, 0.1 M-sodium acetate, pH 4.3 (endo-β-*N*-acetylglucosaminidase L), or 0.15 M-sodium phosphate, pH 6.5 (endo-β-*N*-acetylglucosaminidase D) for 16 h at 37 °C under toluene. Descending paper chromatography of intact and enzyme-digested oligosaccharides was carried out on Whatman 3 MM paper, using the solvent system butan-1-ol/pyridine/water (6:4:3, by vol.) (Turco *et al.*, 1980). Methylation analysis was carried out exactly as described by Kaizu *et al.* (1982), and partially methylated GlcN derivatives were analyzed by t.l.c., with di-*N*-acetylchitobiose and tri-*N*-acetylchitotriose as standards for terminal and 4-linked *N*-acetylglucosamine residues. Radioactivity was determined on aqueous samples, or on

non-aqueous samples, after suspension in water, by liquid-scintillation spectrometry after the addition of ACS liquid-scintillation-counting cocktail (Amersham Corp., Arlington Heights, IL, U.S.A.).

Hydrolase assays

Lysosomal hydrolase activities were determined by using the 4-methylumbelliferyl derivatives as described by Dawson & Tsay (1977).

RESULTS

Lysosomal hydrolase activities

No detectable β-mannosidase activity was present in affected fibroblast cultures, whereas heterozygotes showed intermediate levels of the activity found in normals (Jones *et al.*, 1984). In contrast, other lysosomal hydrolases were generally elevated in affected as compared with the unaffected fibroblasts (Table 1).

Accumulation of labelled oligosaccharides

After labelling for 48 h with [^{3}H]glucosamine (Fig. 1), a major peak of radioactivity (TS), eluted just ahead of the tetrasaccharide stachyose, was isolated from affected fibroblast samples (Fig. 1*b*); this peak represented approx. 30% of the radioactivity in the 100000 *g* supernatant fraction compared with less than 2% of the radioactivity in normal or heterozygote supernatant fractions (Fig. 1*a*). An additional peak of radioactivity (designated 'DS'), representing approx. 8% of the total (and 25% of storage oligosaccharide) radioactivity, was observed in affected supernatant (less than 2% in the normal) at an elution volume between that of the tetrasaccharide stachyose (a) and the Gal-Glc-Fru trisaccharide raffinose (b). Both TS and DS storage oligosaccharides, which were eluted somewhat anomalously because of the presence of *N*-acetylglucosamine residues, were pooled and subjected to further characterization.

Characterization of the minor (DS) storage oligosaccharide

The carbohydrate composition of DS from affected fibroblasts, as determined by g.l.c. of the trimethylsilyl derivatives of the methyl glycosides, was $Man_1GlcNAc_{1.4}$, at levels of 0.15 ± 0.07 μmol/g fresh wt. (four independent determinations). The mobility on t.l.c. of DS was between that of the standards lactose and glucose near that of *N*-acetyl-lactosamine (result not shown). The disaccharide was not susceptible to digestion with endo-β-*N*-acetylglucosamindase L (data not shown), but was converted into a radioactive product that is co-eluted with GlcNAc (fractions 88–92) (mannose residue not labelled) on Bio-Gel P-2 after digestion with β-mannosidase (Fig. 2*c*); the identity of this digestion product as *N*-acetylglucosamine was further confirmed by descending paper chromatography (results not shown). After methylation and hydrolysis, a major radioactive component corresponding in t.l.c. mobility to 2-*N*-methyl-3,6-di-*O*-methylglucosamine was observed (Fig. 3), whereas no product corresponding in mobility to 2-*N*-methyl-3,4,6-tri-*O*-methyl glucosamine (a non-reducing terminal *N*-acetylglucosamine residue) was observed. Material migrating 1.5 cm from the origin represents partially methylated material (Kaizu *et al.*, 1982). This characterization is consistent with the structure Manβ(1→4)GlcNAc for DS.

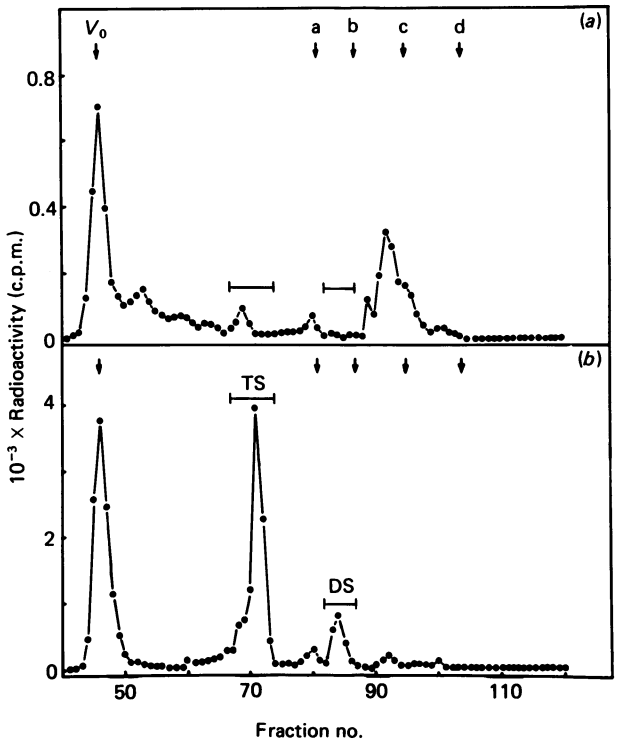

Fig. 1. Bio-Gel P-2 gel filtration of 100000 *g* supernatant fractions

Supernatant fractions (100000 *g*) of [^{3}H]glucosamine labelled normal (*a*) and affected (*b*) goat fibroblasts, in a volume of 1.0 ml, were chromatographed on a column (1.5 cm × 100 cm) of Bio-Gel P-2 (−400 mesh), using 10% acetic acid as the eluant. Fractions (1.2 ml) were collected, and aliquots (0.4 ml) were assayed for radioactivity. Fractions labelled TS and DS and indicated by horizontal bars from affected fibroblasts (*b*) were pooled and further characterized. V_0, albumin; a, stachyose; b, raffinose; c, lactose; d, glucose.

Table 1. Lysosomal hydrolase activities in cultured goat fibroblasts

Hydrolase assays were carried out as described in the text.

Hydrolase	Activity (nmol of 4-methylumbelliferyl glycoside substrate hydrolysed/h per mg of protein)	
	Normal	Affected
α-L-Fucosidase*	330	446
β-D-*N*-Acetyl-hexosaminidase*	761	1717
α-D-Mannosidase*	27	41
β-D-Mannosidase†	6	< 0.1
β-D-Galactosidase*	148	139

* 1 h incubation.
† 24 h incubation.

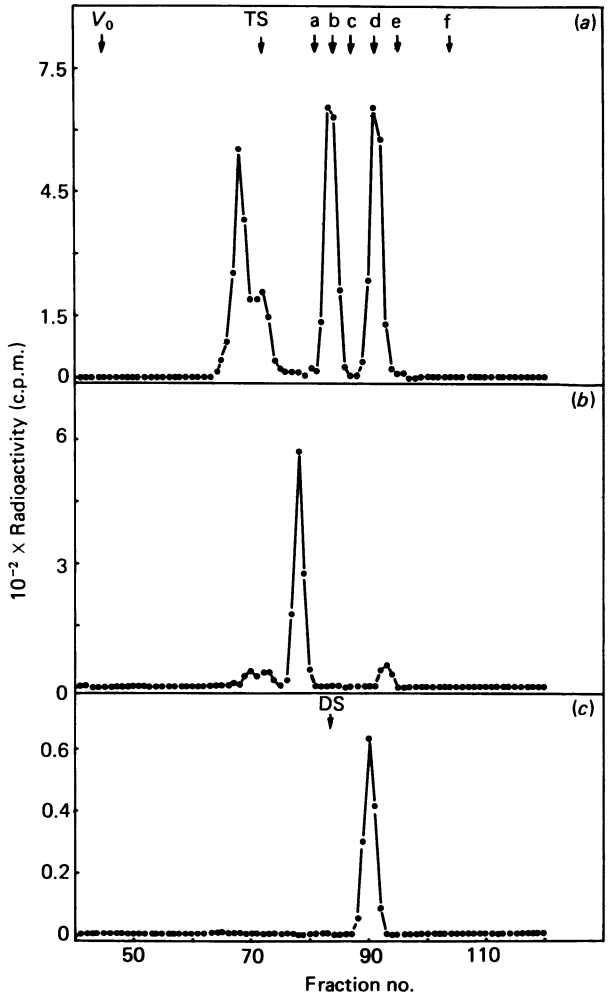

Fig. 2. Bio-Gel P-2 gel filtration of enzyme-digestion products of [^{3}H]glucosamine labelled storage oligosaccharides

Enzyme-digestion mixtures were inactivated by boiling for 2 min, then adjusted to a volume of 1 ml with distilled water and applied to a column (1.5 cm × 100 cm) of Bio-Gel P-2 (−400 mesh), with 10% (v/v) acetic acid as the eluant. Fractions (1.2 ml) were collected and 0.4 ml aliquots were assayed for radioactivity. (*a*) Endo-β-*N*-acetylglucosaminidase L digestion of TS; (*b*) β-mannosidase digestion of TS; (*c*) β-mannosidase digestion of DS. V_0, albumin a, stachyose; b, N-acetyl-lactosamine; c, raffinose; d, GlcNAc; e, lactose; f, glucose.

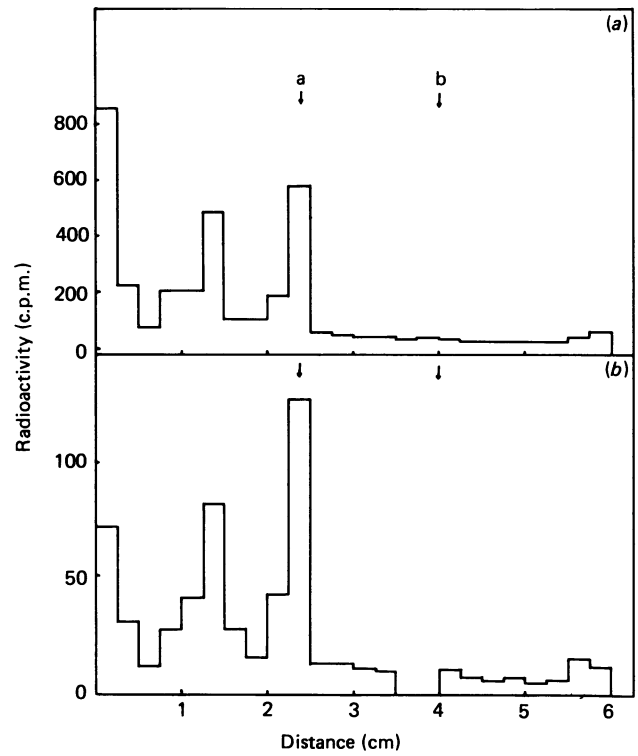

Fig. 3. T.l.c. of partially methylated [^{3}H]glucosamine-labelled storage oligosaccharide hydrolysates

Labelled oligosaccharides and standard di-*N*-acetylchitobiose, tri-*N*-acetylchitotriose, and glucosamine were methylated, hydrolysed, and chromatographed on LHP-K (10 cm × 10 cm) plates in the solvent system acetone/4.5 M-NH_3 (500:9, v/v) as described by Kaizu *et al.* (1982), who cited Hakomori (1964) and Stoffyn *et al.* (1973). Standards were detected with an orcinol/H_2SO_4 spray followed by heating at 120 °C, and radioactivity was determined on 0.25 cm scrapings. (*a*) TS; (*b*) DS. a, 2-*N*-methyl-3,6-di-*O*-methylglucosamine; b, 2-*N*-methyl-3,4,6-tri-*O*-methylglucosamine.

Characterization of the major (TS) storage oligosaccharide

The carbohydrate composition of TS from affected fibroblasts, as determined by g.l.c., was $Man_1GlcNAc_{2.2}$, at levels of 0.4 ± 0.1 μmol/g fresh wt. (four independent determinations). The intact oligosaccharide had a mobility on t.l.c. between that of the standard oligosaccharides raffinose and lactose (result not shown). TS was susceptible to digestion with endo-β-*N*-acetylglucosaminidase L, yielding two major radioactive products which co-chromatographed on Bio-Gel P-2 with DS (or *N*-acetyl-lactosamine) and *N*-acetylglucosamine (Fig. 2). Equal amounts of radioactivity were observed in the peaks co-chromatographing with DS and *N*-acetylglucosamine, and the identity of these products was further confirmed by t.l.c., descending paper chromatography and β-mannosidase digestion (results not shown). The material eluted in fractions 65–70 (Fig. 2*a*) is an unresolved component that also appears in normal supernatants (Fig. 1*a*). The TS storage oligosaccharide was also susceptible to digestion by β-mannosidase, yielding a single major radioactive product (mannose is not labelled) which co-chromatographed with di-*N*-acetylchitobiose (ahead of stachyose) on Bio-Gel P-2 (Fig. 2*b*) and descending paper chromatography (results not shown).

After methylation and hydrolysis of TS, a major radioactive component coresponding in t.l.c. mobility to 2-*N*-methyl-3,6-di-methylglucosamine was observed (Fig. 3*a*), and no product corresponding in mobility to 2-*N*-methyl-3,4,6-tri-*O*-methylglucosamine was observed. This characterization, particularly the susceptibility of TS to endo-β-*N*-acetylglucosaminidase L (Trimble *et al.*, 1979), is consistent with the structure Manβ(1→4)GlcNAcβ(1→4)GlcNAc for TS.

On the basis of the preceding observations, cultured fibroblasts derived from affected animals accumulated storage oligosaccharides having the same structure as

those found in brain, kidney and urine (Jones & Laine, 1981; Matsuura *et al.*, 1981).

Metabolism of TS and DS storage oligosaccharides

No conversion of TS into DS was observed after incubation *in vitro* with affected fibroblast homogenates at either pH 6.1 or 4.4, suggesting that no metabolic interconversion takes place in affected cells in either the cytosolic or lysosomal compartments. This observation was confirmed by analysis of the TS/DS ratio in 100000 *g* supernatant fractions of fibroblasts labelled for 48 h with [^{3}H]glucosamine (the ratio of 3.9:1), compared with cells labelled for 48 h and cultured for an additional 48 h in unlabelled medium, where the ratio was 3.2±0.5.

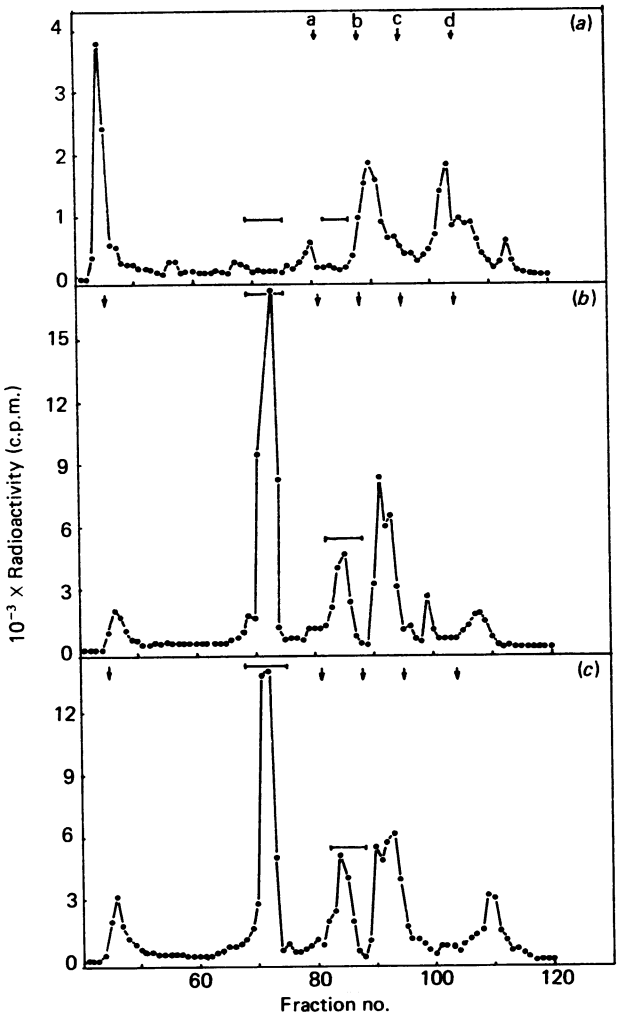

Fig. 4. Bio-Gel P-2 gel filtration of low-M_r metabolites from the culture medium of [^{3}H]GlcN-labelled fibroblasts

Pooled low-M_r material from Sepharose 6B columns, in a volume of 1.0 ml, was chromatographed on a column (1.5 cm × 100 cm) of Bio-Gel P-2 (−400 mesh), 10% acetic acid being used as the eluant. Fractions of 1.2 ml were collected, and 0.4 ml aliquots were assayed for radioactivity. (*a*) Medium from normal fibroblasts; (*b*) medium from affected fibroblasts; (*c*) medium from affected fibroblasts incubated for an additional 48 h with unlabelled affected fibroblasts. V_0, albumin; a, stachyose; b, raffinose; c, lactose; d, glucose. Horizontal bars indicate the expected elution positions of TS and DS.

To complete the metabolic study, conditioned medium from normal and affected cells was fractionated on Sepharose 6B and the peak of radioactivity corresponding to low-M_r metabolites (which were eluted just before Phenol Red) was pooled and re-chromatographed on Bio-Gel P-2, as shown in Fig. 4. No radioactive components corresponding to the elution position of either TS or DS were observed in the medium of normal or heterozygote fibroblasts (Fig. 4). In contrast, both TS and DS were present in the culture medium of affected fibroblasts (Fig. 4*b*), the TS/DS ratio being 4:1. The lack of metabolic conversion of TS into DS was confirmed by incubating medium containing labelled secreted metabolites for an additional 48 h with unlabelled fibroblasts. After gel filtration on Sepharose 6B (where no alterations in the proportion of low-M_r metabolites were apparent), the low-M_r metabolites were pooled and re-analysed by gel filtration on Bio-Gel P-2. As shown in Fig. 4*c*, the TS/DS ratio remained similar to that observed after a single 48 h incubation. The material eluted in fractions 88–100 is a mixture of phosphorylated *N*-acetylglucosamine-containing oligosaccharides previously reported by one of us (Hancock, 1979), whereas the most included material is probably [^{3}H]glucosamine and monosaccharide metabolites. These results suggest that both TS and DS are secreted into the culture medium by affected fibroblasts, and that no extracellular interconversion of storage oligosaccharides or catabolism of glycoproteins takes place under these conditions.

Tunicamycin effects on storage oligosaccharide accumulation in fibroblasts

Tunicamycin inhibited [^{14}C]leucine incorporation into trichloroacetic acid-precipitable material by less than 40%, whereas [^{3}H]glucosamine incorporation into precipitable material was inhibited more than 80%. More detailed studies showed inhibition of *N*-linked oligosaccharide synthesis to be complete, since the residual radioactivity from [^{3}H]glucosamine labelling was incorporated into *O*-linked oligosaccharides, as judged by the absence of any binding to concanavalin A–Sepharose (Table 2) and gel filtration on Bio-Gel P-6 (results not shown).

Table 2. Concanavalin A fractionation of [^{3}H]glucosamine-labelled oligosaccharides

Cells were treated with tunicamycin and swainsonine and oligosaccharides prepared as described in the text, weakly bound (biantennary) material being eluted with 20 mM-α-methyl D-glucoside and strongly bound (mannose-rich) material being eluted with 200 mM-α-methyl D-glucoside.

	Radioactivity (% of total)		
	Unbound	Weakly bound	Strongly bound
Normal	74	20	6
Affected	75	18	7
Normal after tunicamycin	100	0	0
Affected after tunicamycin	100	0	0
Normal after swainsonine	43	47	10
Affected after swainsonine	39	46	15

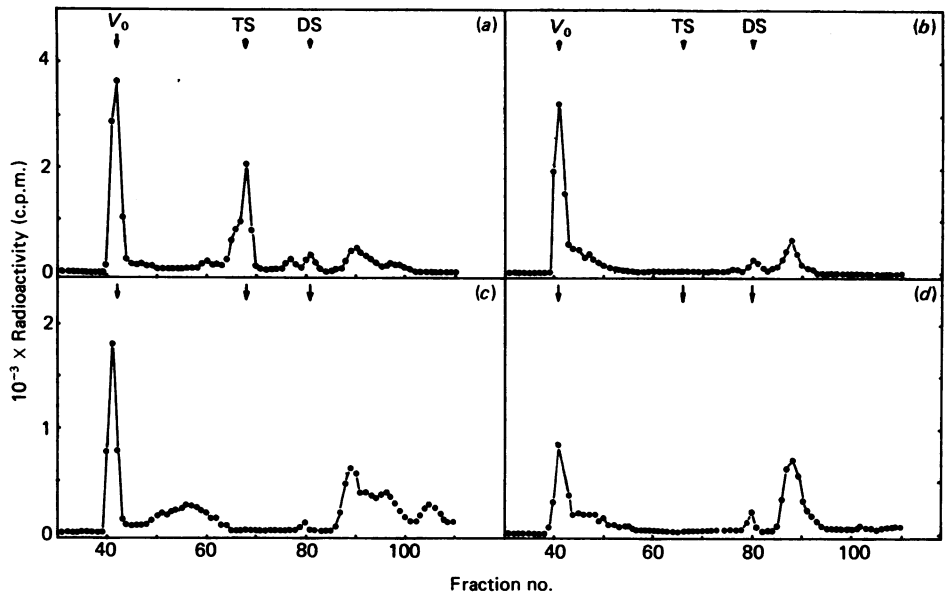

Fig. 5. Bio-Gel P-2 gel filtration of 100000 *g* supernatant fractions from untreated and tunicamycin-treated fibroblasts

100000 *g* supernatant fractions from fibroblasts labelled in the presence and absence of tunicamycin with [^{3}H]glucosamine were chromatographed on a column (1.5 cm × 100 cm) of Bio-Gel P-2 (−400 mesh), with 10% acetic acid as the eluant. Fractions (1.2 ml) were collected, and 0.4 ml aliquots were assayed for radioactivity. (*a*) Untreated affected fibroblasts; (*b*) untreated normal fibroblasts; (*c*) tunicamycin-treated affected fibroblasts; (*d*) tunicamycin-treated normal fibroblasts. V_0 shows the elution position of albumin.

As shown in Fig. 5, the addition of tunicamycin before labelling resulted in the complete inhibition of labelled TS and DS accumulation, confirming their origin in *N*-linked oligosaccharides. Conversely, the addition of tunicamycin to cultures prelabelled with [^{3}H]glucosamine had no appreciable effect on the accumulation of TS and DS. The apparent higher ratio of TS to DS in the 24 h 100000 *g* supernatant fraction of affected cells may have resulted from incomplete equilibration of the label in the relatively short labelling period.

Glycoprotein-associated oligosaccharides of normal and affected fibroblasts

Total glycoprotein-associated oligosaccharides were prepared by hydrazine treatment of chloroform- and methanol-extracted cell pellets. As shown in Table 2, concanavalin A–Sepharose 4B affinity chromatography of oligosaccharides from normal and affected fibroblast glycoconjugates revealed little difference in the proportion of [^{3}H]glucosamine-labelled material unbound (mainly hyaluronic acid, polysialo-oligosaccharides and *O*-linked oligosaccharides), weakly bound (biantennary *N*-linked structures), and strongly bound (high-mannose structures) to the lectin. Moreover, gel filtration of the individual fractions on Bio-Gel P-4 or P-6 columns failed to show any size differences. Although not conclusive, the results of these experiments suggest no major structural differences in the *N*-linked oligosaccharides of normal and affected goat fibroblasts.

Swainsonine effects on storage-oligosaccharide accumulation

Treatment of normal and affected fibroblasts with 100 μM-swainsonine for 24 h resulted in 90–95% inhibition of lysosomal α-mannosidase activity (measured using the 4-methylumbelliferyl substrate), but no measurable inhibition of any other lysosomal glycosidases. Metabolic labelling of fibroblasts (pretreated for 24 h with swainsonine) with [^{3}H]glucosamine for 48 h in the continued presence of swainsonine, followed by fractionation of the 100000 *g* supernatant fractions on Bio-Gel P-4 and P-2, indicated the accumulation of identical storage oligosaccharides in both normal and affected fibroblasts (Fig. 6). On the basis of the elution profile, the major storage oligosaccharide appeared to be a pentasaccharide. This major storage oligosaccharide was susceptible to α-mannosidase digestion (Fig. 7*b*), giving a major product that was co-eluted with TS on Bio-Gel P-2. The storage oligosaccharide was also susceptible to digestion with endo-β-*N*-acetylglucosaminidase H (not shown) and endo-β-*N*-acetylglucosaminidase D (Fig. 7*a*), giving in both cases products that co-eluted with the major swainsonine-induced storage oligosaccharide from human fibroblasts and *N*-acetylglucosamine in equal proportions; the virtual co-chromatography of the larger digestion product with TS strongly suggests a probable $Man_3GlcNAc_2$ composition for the major swainsonine-induced oligosaccharide in goat fibroblasts. Treatment of unfractionated swainsonine-induced labelled oligosaccharides from normal goat fibroblasts with α-mannosidase, followed by gel filtration on Bio-Gel P-2 (Fig. 8), gave a profile of core TS and DS with a similar ratio (3.7:1) observed for similarly treated swainsonine-induced oligosaccharides from affected goat fibroblasts. These results confirm the heterogeneous nature of the swainsonine-induced oligosaccharides, and further support the chitobiose nature of the core structure of the major swainsonine-induced labelled oligosaccharide. Moreover, the TS/DS ratio in untreated metabolically labelled affected fibroblasts (Fig. 1) is similar to the ratio observed for the core structures of swainsonine-induced oligosaccharides (Fig. 8) from either normal or affected

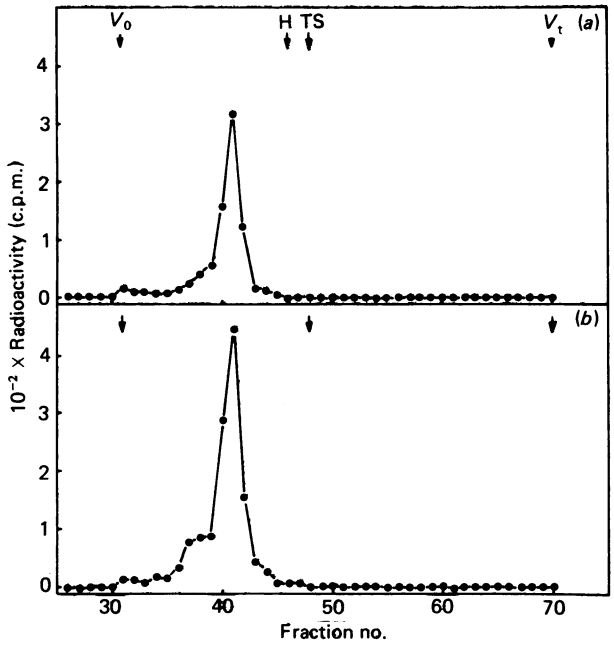

Fig. 6. Bio-Gel P-2 fractionation of 100000 *g* supernatant fractions from swainsonine-treated fibroblasts

Supernatant fractions (100000 *g*) from [^{3}H]glucosamine-labelled fibroblasts pretreated with swainsonine were fractionated by gel filtration on Bio-Gel P-4, and labelled material that was eluted between the V_0 and DS were pooled. The pooled fraction, in a volume of 1.0 ml, was chromatographed on a column (1.5 cm × 100 cm) of Bio-Gel P-2 (−400 mesh), with 10% acetic acid as the eluant. Fractions (1.8 ml) were collected and 0.2 ml aliquots were assayed for radioactivity. (*a*) Affected fibroblasts; (*b*) normal fibroblasts. Further abbreviation: H, tetrasaccharide storage material from swainsonine-treated human fibroblasts.

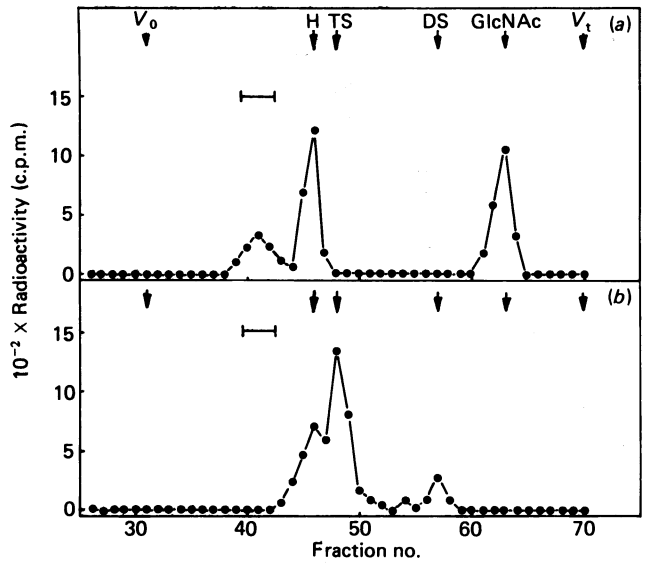

Fig. 7. Bio-Gel P-2 gel filtration of enzyme-digestion products of [^{3}H]GlcN-labelled swainsonine-induced storage pentasaccharide

Enzyme-digestion mixtures were inactivated by boiling for 2 min, adjusted to a volume of 1.0 ml with distilled water, and chromatographed on a column (1.5 cm × 100 cm) of Bio-Gel P-2 (−400 mesh), with 10% acetic acid as the eluant. Fractions (1.8 ml) were collected and 0.4 ml aliquots were assayed for radioactivity. (*a*) Endo-β-*N*-acetylglucosaminidase D digestion of storage pentasaccharide from normal fibroblasts. (*b*) α-Mannosidase digestion of storage pentasaccharide from normal fibroblasts. Abbreviation used: H, tetrasaccharide storage material from swainsonine-treated human fibroblasts. Horizontal bars indicate initial elution volume of storage pentasaccharide (Fig. 6).

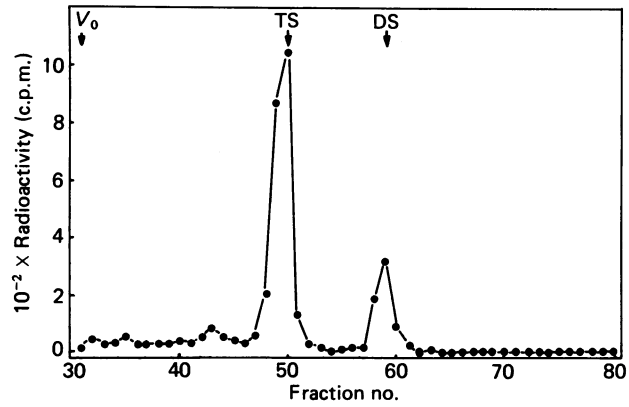

Fig. 8. Bio-Gel P-2 gel filtration of α-mannosidase-digestion products of [^{3}H]glucosamine-labelled swainsonine-induced storage oligosaccharides from normal goat fibroblasts

Enzyme digestion, elution and collection were carried out as described in Fig. 7, with unfractionated [^{3}H]glucosamine-labelled oligosaccharides (fractions 31–50, Fig. 6).

fibroblasts, suggesting identical catabolic endoglycosidase activities in normal and affected fibroblasts.

Fractionation of glycoprotein-associated oligosaccharides derived from swainsonine-treated fibroblasts revealed the expected increase in concanavalin A-binding oligosaccharides (Table 2), and there was no evidence of storage TS and DS in swainsonine-treated affected fibroblasts; these results suggest that both lysosomal and 'processing' α-mannosidases were inhibited by swainsonine under these experimental conditions.

DISCUSSION

We have documented the absence of lysosomal β-mannosidase activity and the accumulation of storage oligosaccharides having the structures Manβ(1→4)GlcNAc and Manβ(1→4)GlcNAcβ(→4)GlcNAc in goat β-mannosidosis fibroblasts, identical with those found in brain and kidney and excreted in urine of clinically affected animals (Jones & Dawson, 1981; Jones & Laine, 1981; Matsuura *et al.*, 1981, 1983). In all the previous studies on heterogeneous whole tissue, the trisaccharide has been observed as the major storage product, although the proportion of disaccharide was somewhat increased in urine. In light of other studies in which the major storage products in canine G_{M1}-gangliosidosis (Warner & O'Brien, 1982), bovine and feline α-mannosidosis (Abraham *et al.*, 1983), and ovine locoweed poisoning (Warren *et al.*, 1983) were characterized as oligosaccharides having two GlcNAc residues in β(1→4) linkage at the reducing terminus, it has been

postulated that the storage trisaccharide in goat β-mannosidosis could arise as a result of primary degradation of *N*-linked oligosaccharides by an endo-β-aspartylglucosaminidase activity. This is in contrast with the primary endo-β-*N*-acetylglucosaminidase activity observed in human tissues (Dawson, 1979), resulting in storage oligosaccharides with only a single GlcNAc residue at their reducing terminus.

In spite of this imposing body of inferential evidence, however, there has been no definitive evidence for the metabolic origin of the disaccharide and trisaccharide storage products in goat β-mannosidosis, or indeed in any of the oligosaccharide storage diseases, and no information as to their possible metabolic interconversion, either intracellularly or extracellularly. In the present paper we have clearly shown that both the TS and DS storage oligosaccharides arise as a result of impaired *N*-linked oligosaccharide degradation, since the accumulation of labelled storage products was completely blocked by the addition of tunicamycin before labelling. In addition, the storage oligosaccharides induced by swainsonine treatment also contained the predominant di-*N*-acetylchitobiose structure. On the basis of the previous work of others (Tulsiani & Touster, 1983; Cenci di Bello *et al.*, 1983), who used chemical quantities of oligosaccharides or [^{3}H]mannose as the isotopic precursor rather than [^{3}H]glucosamine, we would have expected the major storage material to be $Man_5GlcNAc_2$ rather than the observed $Man_3GlcNAc_2$. However, it is possible that we are observing yet another glycosidase difference between human and goat tissue, or that the *N*-linked oligosaccharides labelled with [^{3}H]glucosamine are significantly different from those labelled with [^{3}H]mannose. It seems probable that the inhibition of extralysosomal α-mannosidase activity is incomplete in the cultured goat fibroblasts, giving rise to the $Man_3GlcNAc_2$ storage oligosaccharide; this hypothesis is supported by recent experiments in which the $Man_3GlcNAc_2$ oligosaccharide has been observed as the major storage product in goat fibroblasts treated with swainsonine for 5 days before labelling (L. Hancock, unpublished work). Since no TS or DS accumulation is observed in swainsonine-treated affected goat fibroblasts, however, it is clear that lysosomal α-mannosidase is completely inhibited.

No conversion of TS to DS was observed in cell homogenates at either pH 4.4 or pH 6.1, or in the culture medium (pH 6.8–7.4) of affected fibroblasts, suggesting that the TS and DS storage oligosaccharides arise independently of one another. Although metabolic interconversion might be observed under conditions not used in these experiments, pulse–chase experiments with [^{3}H]glucosamine in which the trisaccharide/disaccharide ratio remained relatively constant would argue against interconversion.

Although the results presented here allow one to focus on the mechanisms of *N*-linked oligosaccharide degradation as the basis for trisaccharide and disaccharide accumulation in goat β-mannosidosis, several questions yet remain to be answered as to the pathways of degradation that could lead to their generation. There has been a report suggesting that a major storage oligosaccharide in the urine of animals suffering from bovine α-mannosidosis has a tri-*N*-acetylchitotriose structure at the reducing terminus (Lundblad *et al.*, 1975), although this is contradicted in an earlier report (Norden *et al.*, 1973). The existence of such *N*-linked oligosaccharides in goat glycoproteins cannot be completely ruled out, and if this were the case, the action of an endo-β-*N*-acetylglucosaminidase on oligosaccharides having chitobiose and chitotriose core structures could be responsible for the generation of the storage oligosaccharides. The accumulation of similar storage produces in swainsonine-treated normal and affected fibroblasts would, however, suggest no induction of endo-aspartylglucosaminidase activity in affected cells. As yet, mammalian endoglycosidases have only been described in a very preliminary manner (Nishigaki *et al.*, 1974; Overdijk *et al.*, 1981), so it is not possible at present to test this directly by an assay *in vitro*. However, recent studies in which we have observed that the catabolism of [^{3}H]glucosamine-labelled human glycoproteins in affected goat fibroblasts yields storage material identical with endogenously derived goat storage TS and DS (Hancock & Dawson, 1985) lends support to the hypothesis that distinct endo-β-*N*-acetylglucosaminidase and endo-aspartylglucosaminidase activities, acting on oligosaccharides having chitobiose core structures, are responsible for the generation of the storage oligosaccharides in cultured goat fibroblasts.

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