Glycopeptide Storage in Skin Fibroblasts Cultured from a Patient with α-Mannosidase Deficiency

GRACE CHEN TSAY, GLYN DAWSON, and REUBEN MATALON

From the Departments of Pediatrics and Biochemistry, Joseph P. Kennedy, Jr., Mental Retardation Research Center, The University of Chicago, Chicago, Illinois 60637

ABSTRACT Patients with mannosidosis, an inherited deficiency of lysosomal α-mannosidase, accumulate large amounts of mannose-rich oligosaccharides (the "core" of the carbohydrate units of many glycoproteins) in brain and liver and excrete these partial degradation products in their urine. A profound α-mannosidase deficiency was demonstrated in fibroblasts cultured from a skin biopsy obtained from a child with mannosidosis. Further, abnormal glycopeptides rich in mannose and similar to oligosaccharides found in the patient's urine were isolated from fibroblast extracts by a variety of chromatographic procedures and by virtue of their binding to a concanavalin A-Sepharose 4B affinity column. This storage material contained mannose, *N*-acetylglucosamine, and asparagine in the ratio 3:1:1 together with a few other amino acids and had a molecular weight of approximately 1,100. There was no evidence for excretion of storage material by mannosidosis fibroblasts or for any abnormality in cell surface glycoprotein composition. The glycopeptide nature of the storage material isolated from cultured skin fibroblasts may be attributed to the low level of *N*-aspartyl-β-glucosamindase (EC 3.5.1.—) activity in these cells.

INTRODUCTION

Three inherited human diseases, G_{M1} [1]-gangliosidosis (β-galactosidase deficiency) (1), G_{M2}-gangliosidosis type II (Sandhoff-Jatzkewitz; β-*N*-acetylhexosaminidase deficiency) (2), and fucosidosis (α-fucosidase deficiency) (3) result in defective glycosphingolipid and glycoprotein catabolism; two others, mannosidosis (α-mannosidase deficiency) (4) and aspartylglucosaminuria (5), involve only glycoprotein catabolism. The accumulating glycosphingolipids in the first three diseases have been characterized (6–8), but the structures of the products of partial glycoprotein degradation have not been completely elucidated. We have isolated a glycopeptide similar in structure to the linkage region of skeletal keratan sulfate and blood group-active glycoproteins (Gal-GlcNAc-Gal-GlcNAc-Gal-GlcNAc-[Gal]-GalNAc-Thr-Ser/Pro) from liver (9) and material with the tentative structure: Gal-GlcNAc-Man-[Gal-GlcNAc]-Man-Man-GlcNAc from brain [2] of two different patients with G_{M1}-gangliosidosis. This oligosaccharide in brain appears similar to that isolated by Wolfe, Senior, and Ng Ying Kin (10) from the liver of three patients with G_{M1}-gangliosidosis type 1, Gal-GlcNAc-Man-[Gal-GlcNAc-Man]-Man-GlcNAc. In addition, we have isolated material with the tentative structure: GlcNAc-Man-[GlcNAc]-Man-Man-GlcNAc from the brain of a patient with G_{M2}-gangliosidosis type II (Sandhoff's disease).[2] An equimolar ratio of mannose and *N*-acetylglucosamine was also found in a mixture of oligosaccharides isolated from the urine of a patient with Sandhoff's disease (11), but no structures were proposed. Further, pentasaccharide with the structure Man-Man-Man-Man-GlcNAc (together with a related tetrasaccharide and trisaccharide) has been purified from the urine of patients with mannosidosis by Norden et al. (12–14) and in this laboratory (15). From our present knowledge, one can envision sialo-

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[1] *The abbreviations used in this paper:* AcNeu, *N*-acetylneuraminic acid; G_{M1}, Gal β(1→3)-GalNAc β(1→4) [AcNeu α(2→3)-]-Gal-β(1→4)-Glc-ceramide; G_{M2}, GalNAc β(1→4) [AcNeu α(2→3)-]-Gal β(1→4)-Glc-ceramide; GLC, gas-liquid chromatography.

[2] Tsay, G. C., and Dawson, G. Unpublished observations.

glycoprotein catabolism occurring by the sequential action of neuraminidase, α-fucosidase, β-galactosidase, *N*-acetyl-β-glucosaminidase, and α-mannosidase (16).

G_{M1} and G_{M2}-gangliosidoses, fucosidosis, and mannosidosis are inherited in an autosomal recessive manner and are clinically similar (ultimately involving severe mental retardation, hepatosplenomegaly, and skeletal abnormalities) to the lipid and mucopolysaccharide storage diseases. Skin fibroblasts cultured from patients with such inherited disorders manifest the diagnostic enzymic defect (17–21), but glycolipid accumulation in fibroblasts has only been observed in G_{M2}-gangliosidosis type II (21). Studies on fibroblasts from patients with fucosidosis (22) using [^{3}H]fucose as a precursor clearly indicated a marked abnormality in glycoprotein catabolism and suggested that [^{14}C]mannose could be used to demonstrate a similar defect in fibroblasts from patients with mannosidosis. This paper describes studies on the metabolism of complex carbohydrates in fibroblasts cultured from both a patient with mannosidosis, a presumed heterozygote, and both normal and pathological controls (α-L-iduronidase deficiency [Hurler's disease]).

METHODS

Materials. α-Methyl-D-mannoside and concanavalin A (jack beans, grade IV) were obtained from Sigma Chemical Co., St. Louis, Mo. Sepharose 4B was obtained from Pharmacia AB, Uppsala, Sweden. D-[1-^{14}C]-Mannose (sp act 50 mCi/mmol) was obtained from New England Nuclear (Boston, Mass.), and 0.25% trypsin was purchased from Grand Island Biological Co. (Grand Island, N. Y.). Glycosphingolipid and mucopolysaccharide standards were prepared in this laboratory. α-Mannosidase from jack bean meal was a generous gift from Dr. Yu-Teh Li, University of Tulane, New Orleans.

Cell culture. All fibroblast cultures were maintained in modified Eagle's medium (23) supplemented with 10% fetal calf serum and 10% calf serum as previously described (23). Cells reached confluency in 3–4 wk and were then harvested mechanically after storage at 4°C for 1 h.

Enzyme assays. Cells were freeze-thawed 3 times and sonicated with a sonifier (model W-185-E [Heat Systems-Ultrasonics, Inc., Plainview, N. Y.] for 4 × 20 s at 100 W) in distilled water. Aliquots (10 μl) of a 600-*g* supernatant solution were assayed with the appropriate 4-methylumbelliferyl glycoside (21) at pH 4.4 (pH 3.8 for α-mannosidase and pH 5.2 for α-fucosidase). 2-Acetoamido-1-(β-L-aspartamido)-1,2-dideoxy-β-D-glucose aspartamido hydrolase (EC 3.5.1.—) assays were carried out according to the method of Mahadevan and Tappel (24).

Isolation of [^{14}C]glycopeptides from cultures of human skin fibroblasts. Cells were grown for 2 wk before labeling for 4 days with [^{14}C]mannose (5 μCi/10 ml medium per Falcon 100-mm tissue culture dish [Falcon Plastics, Division of BioQuest, Oxnard, Calif.]). Sonicated cells were centrifuged at 8,000 *g* for 10 min, and the supernate was applied to a column of concanavalin A coupled to Sepharose 4B (previously equilibrated in 0.02 M sodium phosphate buffer, pH 7.0, containing 1 M NaCl) and packed into small columns (4.6 × 1.5 cm) by the procedure of Lloyd (25). Nonbinding material was eluted at 4°C with 50 ml of the above buffer. Bound material was eluted with 50 ml of 0.1 M α-methyl-D-mannoside in the same buffer. Both fractions were desalted on Sephadex G-10 (135 × 1.0 cm). Their carbohydrate composition was determined by gas-liquid chromatography (GLC) (26), and radioactivity was determined by liquid scintillation counting using a Tri-Carb Spectrophotometer (model 3375, Packard Instrument Co., Inc., Downers Grove, Ill.) and the toluene-based counting mixture as described previously (27).

Isolation and purification of storage material. The harvested fibroblasts (approx. 8×10^7 cells) were disrupted with a sonifier as described above and centrifuged at 8,000 *g* for 10 min. The aqueous extract was applied to a column of Sephadex G-50 (1.0 × 180 cm) equilibrated with water. 5-ml fractions were collected, and a small aliquot (0.1 ml) was assayed for hexose; appropriate fractions were pooled for sugar analysis by GLC (26). The major low molecular weight fraction was further chromatographed on Sephadex G-15 (or Bio-Gel P-2) with some suggestion of partial resolution into two fractions. After final purification by paper chromatography in *n*-butanol-acetic acid-water, 12:3:5 (37), the material was analyzed by GLC (26) and amino acid analyzer. Molecular weight was estimated by the Sephadex G-25 method of Bhatti and Clamp (28) using the ^{14}C-labeled glycopeptide to facilitate its detection in the column eluant.

Treatment of glycopeptides with α-mannosidase. Purified [^{14}C]glycopeptide fractions obtained by affinity chromatography were incubated with a jack bean meal α-mannosidase that hydrolyzes a variety of Man α-Man linkages (1 U of enzymes/200 μl of reaction mixture) according to the method of Li and Li (29). Liberated [^{14}C]mannose was detected by monitoring the effluent for radioactivity after applying samples to Sephadex G-10 (135 × 1.0 cm) previously equilibrated with 0.1 N acetic acid.

Mucopolysaccharide metabolism. Fibroblasts were digested with papain, and individual glycosaminoglycan classes were isolated by a combination of Dowex (Dow Chemical USA, Midland, Mich.) column chromatography and cellulose acetate electrophoresis (30). Monolayer cultures of normal and mannosidosis fibroblasts were stained for metachromasia with toluidine blue O as described previously (30).

Glycosphingolipid metabolism. Extracts of the cultured cells were assayed for lysosomal enzymes associated with glycosphingolipid metabolism as previously described (21). Glycosphingolipids were isolated from cells cultured from skin biopsies obtained from the proband and his parents by previously published procedures and analyzed by GLC (21, 26). Approximately 1% of the ^{14}C-labeled complex carbohydrate material of the cell cochromatographed with the various glycolipid fractions.

RESULTS

The patient with mannosidosis (α-mannosidase deficiency) was first examined at the age of 6 mo by Dr. A. S. Aylsworth (University of North Carolina, Chapel Hill) because of a history of recurrent respiratory infections. At the age of 2 he showed signs of slight psychomotor retardation, some speech and hearing abnormalities, and increasing skeletal abnormalities indicative of a storage disease of the mucopoly-

TABLE I

Lysosomal Hydrolase Levels in Fibroblasts Cultured from the Patient with Mannosidosis and Related Disorders

Enzyme	Mannosidosis	Mannosidosis heterozygote	Normal	Fucosidosis	Hurler's disease
		μmol p-nitrophenol liberated/mg protein per h			
α-Mannosidase	0.04	0.14	0.27	0.31	0.29
β-*N*-Acetylhexosaminidase	6.7	9.1	6.0	7.5	5.3
β-Galactosidase	0.36	0.51	0.21	0.36	0.40
β-Glucuronidase	0.18	0.22	0.16	0.21	0.26
β-Glucosidase	0.21	0.21	0.26	0.13	0.19
α-Galactosidase	0.07	0.08	0.06	0.10	0.05
α-Fucosidase	0.21	0.22	0.19	0.02	0.20
Arylsulfatase A*	2.67	3.68	2.28	3.46	2.06
Arylsulfatase B*	2.55	3.48	2.38	3.20	2.18

All assays were carried out with the appropriate *p*-nitrophenyl substrate in 0.1 M acetate-NaCl or 0.1 M citrate-phosphate buffer at the appropriate pH (3.5–5.2).

* Micromoles nitrocatechol/milligram protein per hour.

saccharidosis type.[8] Urinary mucopolysaccharides were found to be normal, but abnormal mannose-rich oligosaccharides were present (15). Enzymic analysis of extracts of skin fibroblasts cultured from the patient revealed a profound deficiency of α-mannosidase activity as the only abnormality (Table I). α-Mannosidase activity in fibroblasts cultured from patients with mucopolysaccharide storage diseases types I through VI and related lysosomal storage diseases was found to be normal with the exception of "I-cell" disease where previous studies in many laboratories predicted the low (30% of normal) level observed.

Demonstration of abnormal storage by labeling with [^{14}C]mannose. Fibroblasts from the patient with mannosidosis, when labeled with [^{14}C]mannose for 4 days, incorporated 4 to 6 times as much label as did fibroblasts of comparable density and passage number from both controls, the mannosidosis heterozygote, and Hurler's disease. When [^{14}C]mannose-labeled cell extracts from controls and the patient with mannosidosis were applied to a concanavalin A-Sepharose 4B affinity column, approximately one-third of the total label was bound in each case (Fig. 1) although the specific activity was 6 times greater in the case of mannosidosis. The amount and composition of [^{14}C]labeled unbound material (cell glycoprotein and mucopolysaccharide) (fraction I) was the same in both normal and mannosidosis fibroblasts, but the bound material (fraction II) only contained detectable sugar in the case of mannosidosis (Table II). Quantitative analysis of this bound material showed it to be rich in mannose and to constitute 70% of the total carbohydrate (Table II) although it contained only 33% of the radioactive label (Fig. 1). This presumably reflects dilution by preexisting storage material. Its composition and amount are comparable to that of the storage material isolated on a larger scale by conventional chromatographic procedures. Purified α-mannosidase liberated 38% of the [^{14}C]mannose label in 23 h from the bound (storage) material, confirming the presence of at least one terminal α-mannose residue.

Characterization of the storage material in mannosidosis. The supernatant solution obtained from four plates of cells (approximately 8×10^7 cells) was separated into four fractions (with respect to total hexose content) after Sephadex G-50 chromatography (Fig.

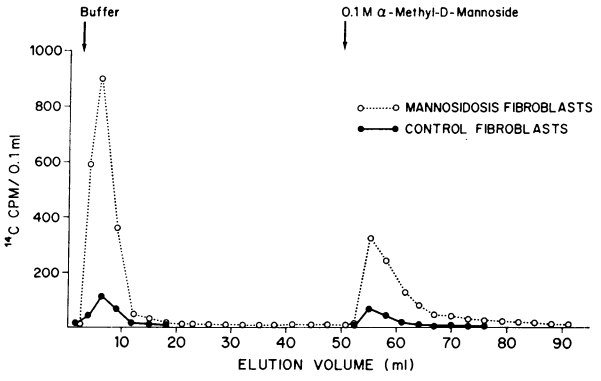

FIGURE 1 ^{14}C-complex carbohydrates isolated from normal and mannosidosis fibroblasts by affinity chromatography on a concanavalin A-Sepharose 4B column (1.5 × 4.6 cm). Fraction I was eluted with 0.02 M sodium phosphate buffer pH 7.0 containing 1 M NaCl and fraction II was eluted with 0.1 M α-methyl-D-mannoside in the same buffer.

[8] A complete clinical summary of this patient will be published elsewhere by A. S. Aylsworth, A. Dorfman, and R. Matalon.

TABLE II

Carbohydrate Composition of ^{14}C-Labeled Material Isolated from Mannosidosis Fibroblasts by Concanavalin A-Sepharose Affinity Chromatography

Sugar	Fraction I*	Fraction II
	mol/mol GlcNAc	
Mannose	1.4	2.7
Galactose	1.7	0.5
N-Acetylglucosamine	1.0	1.0
N-Acetylgalactosamine	0.3	tr
Sialic acid	1.0	ND
Total μmol/3 $\times$ 10^7 cells	0.42	1.26

The ^{14}C-complex carbohydrates‡ isolated from mannosidosis fibroblasts were applied to a concanavalin A-Sepharose 4B column (1.5 $\times$ 4.6 cm). Fraction I was eluted with 0.02 M sodium phosphate buffer, pH 7.0, containing 1 M NaCl (50 ml), and fraction II was eluted with 50 ml of 0.1 M α-methyl-D-mannoside in the same buffer (Fig. 1).

* Fraction I was present in normal cells and had a similar composition, but fraction II was undetectable.

‡ At least 80% of incorporated [^{14}C]mannose was present in the 8,000-*g* supernatant fraction used in these studies.

2). Fraction I probably represents soluble glycoprotein and resealed vesicle glycoproteins and had a similar composition in preparations from both the mannosidosis patient, control fibroblasts, and the mannosidosis heterozygote (Table III). In Hurler fibroblasts the unusual composition (high GalNAc) results from the presence of large amounts of high molecular weight dermatan sulfate. Mannosidosis fibroblasts contained much larger amounts of fractions II through IV (3.37 μmol/9 $\times$ 10^7 cells vs. 0.43 μmol in the heterozygote and 0.35 μmol in the control), and this appeared to be enriched in mannose. Fractions II and III were isolated in highest yield from the mannosidosis fibroblasts and contained other sugars in addition to Man and GlcNAc. Discrepancies between colorimetric and GLC assays were attributable to the presence of variable amounts of glucose. Fraction IV was undetectable in any of the controls and contained only mannose and GlcNAc in the approximate ratio 3:1. This ratio was similar to that previously found (15) for the major component in the urine of this patient. Fraction IV was further purified by Bio-Gel P-2 and paper chromatography and a molecular weight estimation by Sephadex G-25 chromatography (28) (using glucose, raffinose, a G_{M1}-gangliosidosis glycopeptide of molecular weight 1,700, and blue dextran as standards) gave a value of 1,100. A combination of sugar and amino acid analysis indicated that the material was glycopeptide in nature and contained mannose, *N*-acetylglucosamine, and asparagine in the ratio 3:1:1 (Table IV). Incubation of this material with α-mannosidase indicated the presence of α-linked mannose as the nonreducing end-group sugar.

Glucosaminylasparaginase activity in cultured cells. Cell extracts were found to have a low specific activity with respect to this carbohydrate-peptide (GlcNAc-Asn) cleaving enzyme when compared to solid tissues such as kidney or other cultured cell strains such as rat astrocytoma RGC-6 (Table V). Although some activity was detectable in vitro, the low level of activity may be related to the fact that the storage material in the cells is glycopeptide in nature whereas the urinary excretion material is mainly oligosaccharide (12–15).

Mucopolysaccharide (glycosaminoglycan) metabolism. Although the cells appeared metachromatic after staining with toluidine blue O, the total glycosaminoglycan content of 10 confluent plates of cultured fibroblasts (approximately 150 mg dry wt) from the patient was only 0.6 mg, which is within the normal range (0.4–0.6 mg). The distribution of glycosaminoglycan species

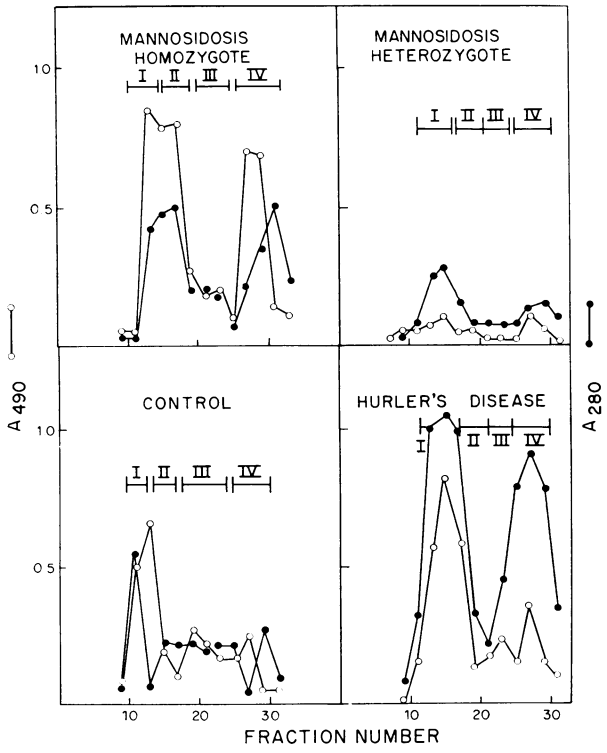

FIGURE 2 Chromatographic profiles of material isolated from a water-soluble extract of normal, mannosidosis heterozygote, Hurler's disease, and mannosidosis homozygote fibroblast cells. Cells (8×10^7) were grown in fresh modified minimal essential medium for 3 wk, harvested, frozen and thawed 3 times, and disrupted by sonication, and the 8,000 *g* supernatant solution was applied to a Sephadex G-50 (1.0 $\times$ 180 cm) column equilibrated with water. Fractions of 5 ml were collected, and aliquots were assayed for protein (1.0 ml) (A_{280} ●——●) and carbohydrate (0.25 ml) (phenyl sulfuric acid method, A_{490} ○——○).

TABLE III

Carbohydrate Composition of Material Isolated from 8 × 10^7 Human Skin Fibroblasts

Patient	Mannose	Galactose	GalNAc	Sialic acid	Total carbohydrate
		mol/mol GlcNAc			*μmol/8 × 10^7 cells*
Fraction I					
Mannosidosis	1.6	1.5	tr	0.3	2.03
Mannosidosis heterozygote	2.0	1.4	0.1	0.6	0.63
Hurler's disease	0.8	0.5	1.4	0.2	3.39
Normal	1.4	1.0	tr	0.7	0.96
Fraction II					
Mannosidosis	2.0	0.5	tr	0.2	1.33
Mannosidosis heterozygote	0.8	0.5	0.7	tr	0.43
Hurler's disease	1.4	0.8	1.1	0.5	1.72
Normal	1.8	1.0	tr	tr	0.18
Fraction III					
Mannosidosis	5.0	1.8	tr	tr	1.54
Mannosidosis heterozygote	—	—	—	—	tr
Hurler's disease	3.0	1.3	1.0	tr	0.21
Normal	1.6	tr	tr	tr	0.09
Fraction IV					
Mannosidosis	3.4	0.2	tr	tr	0.50
Mannosidosis heterozygote	—	—	—	—	tr
Hurler's disease	—	—	—	—	tr
Normal	0.9(?)	tr	tr	tr	0.08(?)

Complex carbohydrates were isolated from sonicated solution of human skin fibroblast cells (8 × 10^7) after growth in modified minimal essential medium for 3 wk. Four fractions were separated on Sephadex G-50 column chromatography (Fig. 2); fraction I represents material with the highest molecular weight.

was normal (hyaluronic acid 68%; chondroitin 4/6-sulfates, 16%; heparan sulfates, 2%; dermatan sulfate, 14%). The essentially normal levels were consistent with the absence of mucopolysaccharidiuria in this patient (15).

Glycosphingolipid metabolism. Analysis of the glycosphingolipids revealed a normal pattern (21). No evidence for mannoglycolipids was obtained, and individual glycolipids were not labeled to any significant

TABLE IV

Composition of Purified Glycopeptide from Fraction IV Mannosidosis Fibroblasts

Sugar	Molar ratio*
Mannose	2.8
Galactose	tr
N-Acetylglucosamine‡	1.0
Asparagine	1.0
Glutamine	1.0
Glycine	1.0
Serine	0.8
Alanine	0.8
Threonine	0.5

* This composition indicates a molecular weight of approximately 1,150 compared to 1,100 as determined by the column chromatography method of Bhatti and Clamp (28).

‡ Total carbohydrate, 0.48 μmol/10^8 cells.

TABLE V

Glucosaminylasparaginase Activity in Mammalian Tissues*

Tissue	Activity
	nmol GlcNAc liberated/mg protein per h
Human skin fibroblasts	5±3
Mannosidosis fibroblasts	3
Mouse RAG fibroblasts	8
RGC-6 rat astrocytoma	270
NB41A mouse neuroblastoma	52
Human kidney	59
Rat kidney	64

* GlcNAc-Asn (2-acetamido-1-*N*-(4′-L-aspartyl)-2-deoxy-β-D-glucopyranosylamine) was used as the substrate.

extent after growth in the presence of [^{14}C]mannose. Less than 10% of the [^{14}C]mannose incorporated into cellular components was found in lipid fractions, 55% in neutral lipid, 30% in phospholipid, and 15% in glycolipid (although the latter was shown by subsequent thin-layer chromatography (26) to be mainly due to glycoprotein contamination). Hydrolysis of the [^{14}C]-mannose-labeled glycopeptide, followed by paper chromatography, revealed at least 90% of the label to be in mannose after a 4-day labeling period.

DISCUSSION

Normal human liver α-mannosidase exists in at least three forms (14, 31, 32) separable by DEAE-cellulose chromatography. In mannosidosis liver, the A and B forms (optimum pH 4.4) were shown to be absent, and the residual activity was due to the presence of the C form (optimum pH 6.0) in normal amounts. In this study, normal human skin fibroblast α-mannosidase showed pH optima at 3.5–4.0 and 6.5. α-Mannosidase activity in fibroblasts from the proband, when assayed with 4-methylumbelliferyl-α-D-mannoside, was less than 1% of normal activity at pH 3.5. The peak of activity at pH 6.0, which may be equivalent to the α-mannosidase C localized to the Golgi in human liver (33), was detectable in the mannosidosis fibroblasts. Thus, the lysosomal α-mannosidase deficiency in fibroblasts appears similar to that reported in liver, and cultured cells can be used for both prenatal and postnatal diagnosis of mannosidosis. The generalized elevation of other related glycosylhydrolases reported in mannosidosis liver (34) did not manifest itself in cultured fibroblasts.

As yet, nothing is known of the substrate specificity of the defective enzyme, although during the course of this work Nordén, Lundblad, Öckerman, and Jolly reported the presence of a trisaccharide (13) with the structure

$$\text{Man } \alpha(1 \rightarrow 3) \text{ Man } \beta(1 \rightarrow 4) \text{ GlcNAc.}$$

Nordén et al. and this laboratory have isolated a pentasaccharide and tetrasaccharide (12–15) with a terminal Man $\alpha(1 \rightarrow 2)$ Man linkage from the urine of four patients with mannosidosis. The trisaccharide may occur as an oligosaccharide unit of renal glomerular basement membrane collagen (35), a fact which could account for the preponderance of this material in urine. Since the deficiency of lysosomal α-mannosidase as assayed with the synthetic substrate appears virtually absolute, the secretion of a variety of oligosaccharides with mannose as the nonreducing end group rather than a single storage material must be explained on the basis of glycoprotein heterogeneity. Of further interest is the existence of a nonhuman model for mannosidosis in Angus cattle (13, 36) in which α-mannosidase activity is greatly diminished, and the storage material (tetrasaccharide) apparently has the structure Man-GlcNAc-Man-GlcNAc (13).

The effect of the lysosomal α-mannosidase deficiency on glycoprotein metabolism was demonstrated by labeling cells with [^{14}C]mannose and comparing the level of incorporation into mannose-rich material. Advantage was taken of the presence of terminal α-mannose residues in the storage material to achieve partial purification by affinity chromatography with concanavalin A. Concanavalin A is specific for terminal (and possibly internal) α-mannose, α-glucose, and α-GlcNAc residues, and of these only α-mannose residues are found in glycoproteins. Although the affinity column method showed a striking difference between normal and mannosidosis cells, for larger scale isolation it was found to be more convenient to isolate the low molecular weight storage material by disrupting the cells and fractionating the extract on a combination of Bio-Gel P-10 and P-2 columns. When this was done, a pure fraction containing approximately three mannose residues and one GlcNAc residue, together with 1.0 mol of asparagine and other amino acids such as glutamate, glycine, serine, and threonine, was obtained. The molecular weight was found to be 1,100 (calculated from composition as 1,150 to 1,200), and our inability to remove the amino acids suggested that it was glycopeptide in nature. The lack of material has prevented a complete characterization of the sugar sequence, and one can only infer that the linkage region involves GlcNAc and Asn linked by an amide bond by virtue of the resistance of the GlcNAc-Asn linkage to complete hydrolysis by 1.0 N HCl in methanol at 80°C for 20 h (26, 37). From these studies and the ability of α-mannosidase to liberate two out of three mannose residues, we can propose the structure

$$\text{Man } \alpha\text{-Man } \alpha\text{-Man } \beta\text{-GlcNAc-Asn-peptide}$$

for the storage material in mannosidosis fibroblasts.

The sequence $(\text{Man})_4$ $(\text{GlcNAc-GlcNAc})_{1\text{-}2}$-Asn has been reported to be the core of the oligosaccharide units of many mammalian glycoproteins (37–40), and one of the endoglycosidases which cleave the sugar-peptide bond (4-L-aspartylglucosylamine amido hydrolase) has been isolated from various tissues (41, 42) including hog kidney (43). The endo-β-*N*-acetylglucosaminidase specific for GlcNAc $\beta(1 \rightarrow 4)$ GlcNAc-Asn has thus far only been isolated from microorganisms (44). Measurement of *N*-aspartyl-β-glucosaminidase (EC 3.5.1.—) in human skin fibroblasts indicated that activity in fibroblasts is less than 10% of that in kidney or other cultured cells such as C-6 rat astrocytoma. However, since previous in vitro studies with

N-aspartyl-β-glucosaminidase from a variety of tissues have indicated that the enzyme will only act on asparaginylglycopeptides (41–43), the observed low level of *N*-aspartyl-β-glucosaminidase activity in vivo may not be the whole explanation as to why the storage material is glycopeptide in nature rather than oligosaccharide as found in other tissues and organs. It is also possible that glycopeptide storage in fibroblasts could be attributed to steric hinderance of proteolytic enzymes by the undegraded carbohydrate moiety (in this case three mannose residues and one GlcNAc residue).

In conclusion, mannosidosis appears to result from lysosomal accumulation of partially degraded cell glycoprotein. Despite the clinical resemblance to patients with mucopolysaccharide and sphingolipid lysosomal storage diseases, there was no evidence for abnormalities. Other lysosomal hydrolases connected with the catabolism of complex carbohydrates had normal or greater than normal activity, indicating that a deficiency of α-mannosidase was the only genetic defect. The most striking chemical abnormality was the storage of a glycopeptide containing 3 mol of mannose and 1 mol of *N*-acetylglucosamine. We have shown that this storage material can arise by *de novo* synthesis although it is possible that in vivo the additional load of exogenous mannose-containing material from the diet may exacerbate the condition. Together with G_{M1}-gangliosidosis, G_{M2}-gangliosidosis type II, fucosidosis, and aspartylglycosaminuria, mannosidosis may be considered to be an inborn error of glycoprotein metabolism.

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