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Increased Urinary Excretion of Keratan Sulfate in Fucosidosis

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Summary: In two children exhibiting the clinical symptoms of fucosidosis, the diagnosis was biochemically ascertained by the demonstration of a profound α-*L*-fucosidase deficiency in cultured skin fibroblasts. The non-dialysed urines of these fucosidosis patients were separated into two fractions by chromatography on Biogel P-2. The first fraction containing the glycosaminoglycans was further fractionated on Dowex 1 × 2 by stepwise elution with increasing NaCl concentrations. Keratan sulfate-chondroitin sulfates attached to the same peptide core were assayed and characterised mainly in the fractions eluted with 1.25, 1.5, 2.0 and 3.0 mol/l NaCl. Whereas the excretion of normal children of the same age was found to be 0.77 μmol glucosamine equivalents per day in the 2 mol/l and 3 mol/l NaCl fraction, the two patients excreted 6.7 (M. C.) and 3.5 (M. S.) μmol glucosamine equivalents per day, respectively. Since keratan sulfate contains α-fucose at the non-reducing terminal, this increase in excretion of long chain keratan sulfate in fucosidosis could result from impaired degradation of keratan sulfate, due to the α-fucosidase deficiency.

Erhöhte Ausscheidung von Keratansulfat im Urin bei der Fucosidose

Zusammenfassung: Bei zwei Kindern mit dem klinischen Bild einer Fucosidose wurde die Diagnose durch den Nachweis eines α-*L*-Fucosidasemangels in kultivierten Hautfibroblasten biochemisch bestätigt. Die nicht dialysierten Urine dieser Fucosidose-Patienten wurden durch Chromatographie an Biogel P-2 in zwei Fraktionen getrennt. Die höher molekulare Fraktion, welche die Glykosaminoglykane enthielt, wurde weiterhin an Dowex 1 × 2 durch Elution mit ansteigenden NaCl-Konzentrationen getrennt. Hauptsächlich in den Fraktionen, die mit 1,25 mol/l, 1,5 mol/l, 2,0 mol/l und 3,0 mol/l NaCl eluiert wurden, haben wir Keratansulfat-Chondroitinsulfate bestimmt und charakterisiert, die denselben Peptidcore besitzen. Während die normale Keratansulfatausscheidung gleichaltriger Kinder in der 2 und 3 mol/l NaCl-Fraktion einen Mittelwert von 0,77 μmol Glucosamin-äquivalente pro Tag aufweist, wurden vom ersten Patienten 6,7 und vom zweiten Patienten 3,5 μmol Glucosaminäquivalente pro Tag ausgeschieden. Da Keratansulfat am nicht-reduzierenden Ende α-Fucose enthält, führen wir diese vermehrte Ausscheidung von langkettigen Keratansulfaten beim Enzymdefekt der α-Fucosidase auf den unvollständigen Abbau des Keratansulfats zurück.

Introduction

Fucosidosis is an inborn error of complex carbohydrate metabolism, first described by *Durand* et al. in 1966 (1). By now, more than 20 patients have been observed allowing the definition of a clinical picture exhibiting progressive neurodegeneration, mental retardation, organomegaly, and skeletal abnormalities (1, 2). A severe type of the disease (type I) has been differentiated from a less severe form (type II) based on differences in the time of onset and in phenotype (1, 2). *Van Hoof & Hers* demonstrated a deficiency of the lysosomal enzyme α-*L*-fucosidase (EC 3.2.1.51) in liver and other tissues of fucosidosis patients, whereas other lysosomal hydrolases were normal or even increased (3, 4).

Due to the enzymatic defect, there is an abnormal accumulation of fucose-containing oligosaccharides and glycolipids in the liver of such patients (4, 5). Conclusive evidence for a defect in the catabolism of

glycosaminoglycans, however, has been lacking so far. In keratan sulfate, a substantial part of the polysaccharide chains contain a fucosyl residue at the nonreducing terminus, as found by gas chromatography/mass spectrometry-studies after permethylation of keratan sulfates from bovine cornea and human rib cartilage (6), as well as human knee joint cartilage and bovine tracheal cartilage (7, 8). It was therefore of interest, to investigate the keratan sulfate excretion in the urine of patients with fucosidosis.

Materials and Methods

Determination of lysosomal enzyme activities in cultured skin fibroblasts

Fibroblast cultures from fucosidosis patients M. S. (type I; 8 years of age; patient described by *Voelz* et al. (8a)) and M. C. (type II; 11 years of age) were established and maintained according to published procedures (9). The fibroblasts were homogenized, and the activities of lysosomal enzymes determined, as described previously (10, 11).

Separation, isolation, and determination of urinary glycosaminoglycans

Chromatography on Bio Gel P-2 and Dowex 1 x 2

Twenty-four hour urine samples were collected from fucosidosis patients M. S. and M. C., and from 7 healthy children, and kept at – 20 °C until processed. The specimens were concentrated by rotary evaporation at 40 °C to between 1/4 and 1/10 of the original volume, adjusted to an ethanol concentration of 100 ml/l and subsequently chromatographed on a column of Bio Gel P-2 (4.8 × 80 cm; fig. 1). The column was equilibrated and eluted with ethanol/water (volumes, 100 ml + 900 ml) and 20 ml-fractions were collected. Uronic acid determination (carbazole method) of each fraction showed the presence of two peaks, the first appearing in the excluded volume and representing the glycosaminoglycans, and a second retarded peak consisting mainly of glycosaminoglycan-free uronides. Whereas the fractions of the first peak yielded both glucosamine as well as galactosamine upon hydrolysis, no hexosamines were found in the second peak. The glycosaminoglycan fractions (1. peak) were pooled, concentrated to approximately 100 ml and loaded onto a Dowex 1 × 2 column (2 × 20 cm; Cl^--form), which was then eluted stepwise with 200 ml-portions of 0.15, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00, and 3.00 mol/l NaCl. The eluate was collected in 7.5 ml-fractions, which were analyzed for their uronic acid content (fig. 2). Uronic acid-positive fractions of each elution step (as identified by its conductivity) were combined, concentrated as much as possible, brought to an ethanol concentration of 100 ml/l, and then desalted individually on a Bio Gel P-2 column, which was equilibrated and eluted with ethanol/water (volumes, 100 ml + 900 ml). The desalted glycosaminoglycan fractions (figs. 1 and 2) were then analyzed for their constituents as described in the following paragraph.

Analyses of constituents

Uronic acid was determined using the carbazole reaction of *Dische* (12) as modified by *Bitter & Muir* (13). The galactose content of the glycosaminoglycans was measured both enzymatically with galactose dehydrogenase (14), and by gas chromatography using the alditol acetate method (15), after prior hydrolysis of the samples with 1 mol/l HCl at 105 °C for 3 h. Fucose, mannose, and xylose were also determined by gas chromatography using the alditol acetate method. Sulfate was determined turbidimetrically as $BaSO_4$ (16). Glucosamine and galactosamine, together with amino acids, were determined after hydrolysis for 15 hours in 3 mol/l HCl at 105 °C using an amino acid analyzer (TSM, Technicon). No difference in the yield of amino acids was found on hydrolysis for 20 hours in 6 mol/l HCl at 105 °C. The elution program of the analyzer was modified in such a way as to allow the determination of glucosamine, galactosamine, hydroxyproline, and other amino acids in less than 2½ hours (17). N-sulfate was determined according to *Lagunoff* et al. (18).

Electrophoresis

The glycosaminoglycan fractions obtained by Dowex chromatography were subjected to electrophoresis on cellulose acetate in 0.05 mol/l barium acetate buffer, pH 7.0. The duration of electrophoresis was 40 min at a potential gradient of 20 V/cm.

Results

The results of the determinations of lysosomal hydrolases in the skin fibroblasts of patients M. S. and M. C. are shown in table 1. There was a profound deficiency of α-*L*-fucosidase activity in both patients, whereas the activities of β-*D*-glucuronidase, β-*D*-glucosidase, α-*D*-galactosidase, α-N-acetyl-*D*-glucosaminidase, β-N-acetyl-*D*-glucosaminidase, and α-*D*-mannosidase were within normal limits. The activities of arylsulfatase A and acid phosphatase, and in one case also of β-*D*-

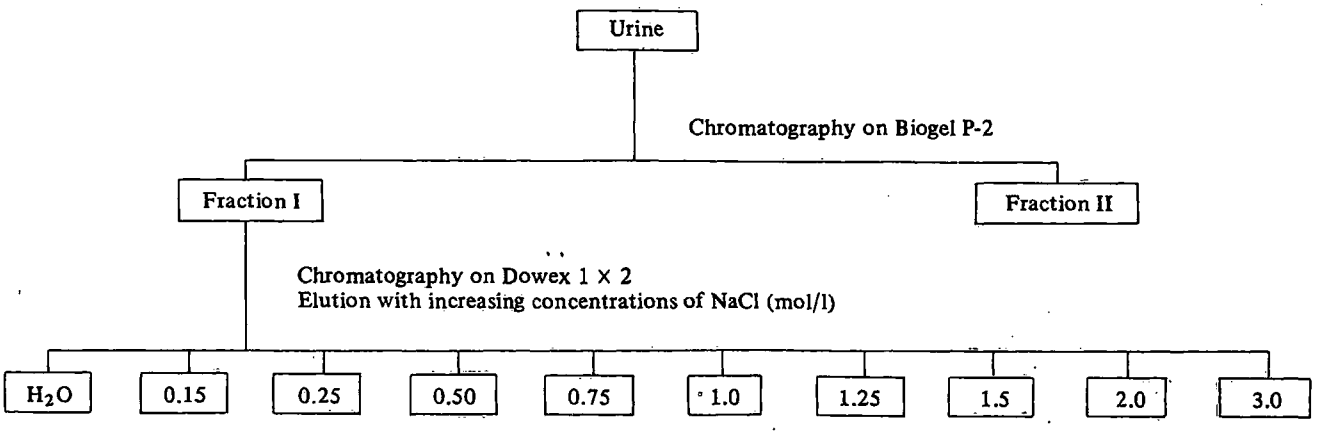

Fig. 1. Separation and isolation of urinary glycosaminoglycans.

Tab. 1. Enzyme activities (U/g protein) in fibroblasts of 6 normal children and 2 patients with fucosidosis.

	Normal children mean value (min.-max. values)	Patient M. C.	Patient M. S.
α-*L*-Fucosidase (EC 3.2.1.51)	0.897 (0.317–1.32)	0.072	0.068
β-*D*-Galactosidase (EC 3.2.1.23)	7.17 (5.60–9.53)	8.32	14.9
β-*D*-Glucuronidase (EC 3.2.1.31)	3.48 (1.80–6.05)	2.87	4.85
β-*D*-Glucosidase (EC 3.2.1.21)	0.571 (0.322–1.08)	1.22	1.06
α-*D*-Galactosidase (EC 3.2.1.22)	1.92 (1.23–2.37)	1.62	2.06
α-N-Acetyl-*D*-glucosaminidase (EC 3.2.1.50)	0.132 (0.073–0.190)	0.102	0.149
β-N-Acetyl-*D*-glucosaminidase (EC 3.2.1.30)	137 (104–211)	124	184
α-*D*-Mannosidase (EC 3.2.1.24)	1.20 (0.691–1.62)	1.47	1.29
Arylsulfatase A (EC 3.1.6.1)	8.42 (4.22–12.0)	12.1	18.1
Acid phosphatase (EC 3.1.3.2)	12.5 (6.96–15.3)	21.8	24.3

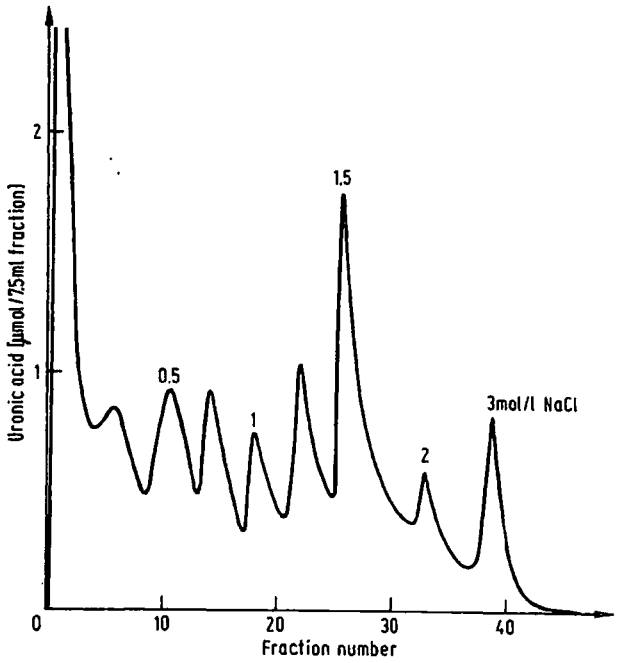

Fig. 2. Chromatography of the urinary glycosaminoglycans from a fucosidosis patient on Dowex 1 × 2. The column was eluted with NaCl of increasing molarity.

galactosidase, were somewhat elevated. These data clearly confirmed the diagnosis of fucosidosis in these two patients.

The urinary glycosaminoglycans were isolated by first subjecting the urine concentrates to chromatography on Bio Gel P-2. The glycosaminoglycans, which appeared in the excluded volume of the column, were thus separated from low molecular weight uronides, e. g. phenolic glucuronides, which were retarded on the gel. The purified glycosaminoglycans were subsequently fractionated on a column of Dowex 1 × 2 using NaCl solutions of increasing molarity (fig. 2). As indicated by the analysis of the glycosaminoglycan constituents (tab. 2) and by electrophoretic analysis using authentic glycosaminoglycan standards (not shown), most of the chondroitin sulfates, as well as dermatan sulfate and heparan sulfate, were found in the 1.25 and 1.5 mol/l NaCl fractions. The 2.0 and 3.0 mol/l NaCl fractions, however, contained keratan sulfate as a copolymer with variable amounts of chondroitin-4- and -6-sulfates, and with dermatan sulfate.

In 7 healthy children from 6 to 12 years of age, the sum of the keratan sulfate contents of the 2.0 and 3.0 mol/l NaCl fractions ranged from 0.23 to 1.18 µmol glucosamine equivalents per day (mean value 0.77 ± 0.29), as compared to values of 6.7 µmol/day in fucosidosis patient M. C., and 3.5 µmol/day in patient M. S., as shown in table 2 and figure 3. Also in the 2.0 and 3.0 mol/l NaCl fractions there was an increase in the galactosaminoglycans chondroitin-4- and -6-sulfates, and in dermatan sulfate, which are thought to be linked to the same peptide core as keratan sulfate. Thus, there were 6.4 µmol galactosamine equivalents per day in patient M. C. and 3.1 µmol galactosamine equivalents per day in patient M. S., as compared to a value of 1.26 ± 0.91 µmol/day (range 0.23–2.08) in healthy children (Table 2, Fig. 3).

Tab. 2. Analyses of constituents of glycosaminoglycan fractions obtained by Dowex chromatography of urines from 2 fucosidosis patients and 2 controls. The "3.0 mol/l" fraction represents the sum of the 2.0 and 3.0 mol/l NaCl fractions. Values are given as µmol per day; n. d. = not determined.

	Patient M. C.			Patient M. S.			normal children		
	1.25 mol/l	1.5 mol/l	3.0 mol/l		1.5 mol/l	3.0 mol/l		1.5 mol/l	3.0 mol/l
Glucosamine	4.0	2.7	6.7	1.6	2.0	3.5	1.0	2.2	0.82
Galactosamine	3.5	14.4	6.4	3.1	7.3	3.1	7.7	20.5	0.22
Uronic acid	6.3	17.1	6.4	2.6	6.7	2.2	9.4	24.3	0.28
Galactose	1.9	3.8	n. d.	1.5	2.1	3.5	2.1	5.0	n. d.
Sulfate	4.6	12.3	12.8	4.2	8.4	7.3	8.9	27.2	1.2

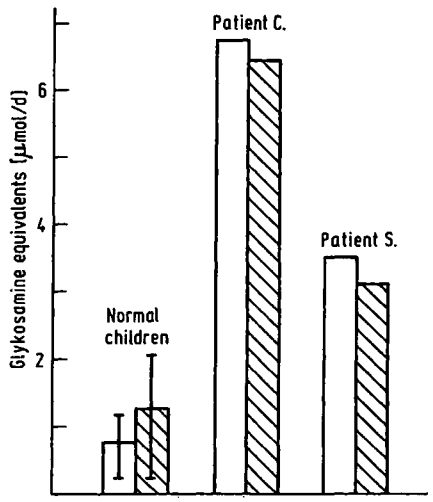

Fig. 3. Keratan sulfate (□) and chondroitin sulfate (▨) contents of the combined 2.0 and 3.0 mol/l NaCl fractions obtained by Dowex chromatography from the urines of 7 healthy children and two patients with fucosidosis.

Therefore, keratan sulfate excretion in the two fucosidosis patients amounted to 8.7 and 4.5 times that of the mean value of the control group; the galactosaminoglycan excretion was also markedly higher than the upper limit of the controls.

Cellulose acetate electrophoresis of the 2.0 and 3.0 mol/l NaCl keratan sulfate fractions showed them to be homogeneous, with a mobility identical to that of chondroitin sulfate-keratan sulfate copolymers from tracheal cartilage. In patient M. C., the fucose and mannose contents of these fractions were 0.6 and 0.8 µmol/day, respectively. The major amino acids in the 2.0 and 3.0 mol/l NaCl fractions were aspartic acid, glutamic acid, threonine, and serine.

Discussion

Previous investigations of urinary keratan sulfate excretion have frequently been incomplete, as chemical characterization of this compound was not attempted. In addition, such studies were hampered by methodological problems. Thus, the commonly used cetylpyridinium chloride procedure for precipitating the urinary glycosaminoglycans may lead to erroneous results due to the coprecipitation of sialic acid-containing glycoproteins. Furthermore, the use of this procedure may lead to an incomplete precipitation of keratan sulfate at higher electrolyte concentrations, or to a loss of short-chain keratan sulfate. Another source of error involves dialysis of the sample, leading to a

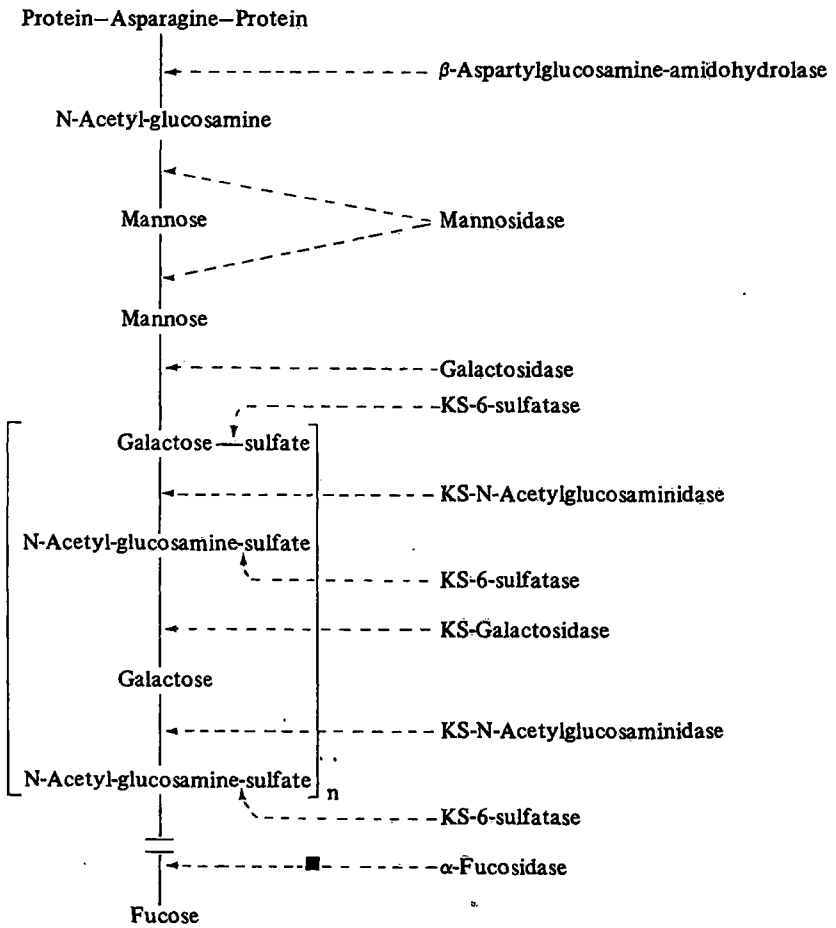

Fig. 4. Postulated structure of the polysaccharide chains of proteokeratan sulfate, and the catabolic block in fucosidosis (■). KS = keratan sulfate

loss of short-chain polysaccharides. It was therefore desirable to develop a procedure for the quantitation of keratan sulfate which would be free of these disadvantages.

By using Bio Gel P-2 chromatography, short-chain keratan sulfates are quantitatively recovered, yet completely separated from low molecular weight glucuronides. The subsequent chromatography on Dowex 1 × 2 then allows a further fractionation of the glycosaminoglycans. Thus, 0.5 mol/l NaCl elutes hyaluronate and chondroitin, or chondroitin sulfate with a low degree of sulfation. The fractions from 0.75 to 1.5 mol/l NaCl encompass the overlapping elution profiles, in that order, of heparan sulfate, chondroitin-4- and -6-sulfates, and dermatan sulfate. The last steps, using 2.0 and 3.0 mol/l NaCl, elute keratan sulfate-chondroitin sulfate complexes, whose polysaccharide chains have a common peptide core. Whereas in the 2.0 mol/l NaCl fraction the copolymers contain more chondroitin sulfate than keratan sulfate, the reverse is found in the 3.0 mol/l NaCl fraction. Both fractions also contain the neutral sugars typical for keratan sulfate: mannose, which is localized in the linkage region between polysaccharide chain and protein core, and fucose, which forms a substantial part of the non-reducing terminus of the keratan sulfate chains (6, 7, 8). The amino acids aspartic acid, glutamic acid, threonine, and serine, which dominate in these two fractions, point to the presence of the peptide core typical of keratan sulfate, the chondroitin sulfates, and dermatan sulfate.

At present, we are lacking detailed studies concerning the role of α-*L*-fucosidase in the degradation of keratan sulfate. It is likely, however, that keratan sulfate is catabolized sequentially by exoglycosidases and sulfatases. Using lysosomal enzymes from rabbit kidney, we were able to achieve such a degradation of the keratan sulfate polysaccharide chain (19). The complete enzymatic degradation of keratan sulfate by a multi-enzyme system from *Charonia Lampas* has recently been demonstrated (20).

Glycoproteins with a terminal fucose on their carbohydrate chains have been described by several authors. *Thomas & Winzler* isolated a glycopeptide from erythrocyte membranes exhibiting close structural resemblance to keratan sulfate (21). The oligosaccharide chains of this glycopeptide were linked to the peptide moiety via aspartyl-N-acetylglucosamine and three mannosyl residues, as in the keratan sulfate of the cornea. The sequential digestion of this glycopeptide with purified neuraminidase, α-*L*-fucosidase, β-*D*-galactosidase, and β-N-acetyl-*D*-glucosaminidase led to its complete degradation and structural elucidation, one of the oligosaccharide chains having the sequence Fuc-Gal-GlcNAc. *Tsay* and colleagues isolated a decasaccharide from the urine of a fucosidosis patient with the structure Fuc (α 1 → 2) Gal (β 1 → 4) GlcNAc (β 1 → 2) Man [Fuc(α 1 → 2) Gal (β 1 → 4) GlcNAc (β 1 → 2) Man] (α 1 → 3/6) Man (β 1 → 4) GlcNAc, which closely resembles the structural elements of keratan sulfate (22). From the liver of a patient with G_{M1}-gangliosidosis type I, *Callahan* et al. isolated a polysaccharide containing galactose, hexosamine and fucose, which was chemically similar to undersulfated keratan sulfate of human cartilage (23). Its accumulation in the patient's liver was thought to be due to the genetic deficiency of β-galactosidase.

A raised keratan sulfate excretion was also reported in some cases of achondroplasia, rheumatoid arthritis and dermatomyositis (24). An excessive keratan sulfaturia is found in *Morquio* disease (mucopolysaccharidosis type IV). As discussed by *Ginsberg* et al. (25), the increased keratan sulfate excretion in *Morquio* disease may be caused by the following enzymatic defects:

1. N-acetylgalactosamine-6-sulfate sulfatase, classical *Morquio*, mucopolysaccharidosis type IVA;
2. β-galactosidase, mild *Morquio*, mucopolysaccharidosis type IVB;
3. N-acetylglucosamine-6-sulfate sulfatase, *Morquio-Sanfilippo* intermediate, which might occupy the vacant position V of *McKusick's* classification.

Our results suggest that the increased excretion of keratan sulfate in fucosidosis is due to the mechanism depicted in figure 4. The α-fucosidase deficiency leads to a block in the degradation of those keratan sulfate chains which contain a terminal fucose residue. It is unlikely, however, that the catabolism of all of the keratan sulfate chains is similarly impaired, as part of the chains terminate in neuraminic acid, instead of fucose (6). Our finding that the keratan sulfate excreted in fucosidosis appears as a copolymer with chondroitin and dermatan sulfates is surprising, as these latter polysaccharides are not known to contain fucosyl residues and might therefore be expected to be degraded independently of the keratan sulfate chains. Further work is needed to clarify this point.

By analogy with the genetic mucopolysaccharidoses, it is tempting to speculate that the skeletal abnormalities found in patients with fucosidosis are caused by an impaired keratan sulfate catabolism.

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