Urinary excretion of sulphated *N*-acetylhexosamines in patients with various mucopolysaccharidoses

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1. Sulphated *N*-acetylhexosamines have been isolated from human urine and tentatively identified as *N*-acetylglucosamine 6-sulphate (GlcNAc6S), *N*-acetylglactosamine 6-sulphate (GalNAc6S), *N*-acetylglactosamine 4-sulphate (GalNAc4S) and *N*-acetylglactosamine 4,6-disulphate (GalNAc4,6diS). 2. Urine from mucopolysaccharidosis-Type-IIID, -IVA and -VI patients compared with that from normal individuals contains elevated levels of GlcNAc6S (380-fold), GalNAc6S (180-fold) and GalNAc4S (420-fold) respectively. Urine from mucopolysaccharidosis-Type-VI patients also contain more than 600 times the normal level of GalNAc4,6diS. Urine from a mucopolysaccharidosis-Type-II and a multiple-sulphatase-deficient patient, and, in general, all mucopolysaccharidosis patients studied, contain at least 5–10-fold elevations of sulphated *N*-acetylhexosamines over the levels detected in urine from normal controls and a α -mannosidosis patient. Urine from patients with clinically mild phenotypes contains less sulphated *N*-acetylhexosamines than isolated from urine of clinically severe mucopolysaccharidosis patients. 3. The source of the four sulphated *N*-acetylhexosamines is not known. However, incubation of a series of oligosaccharide substrates, derived from keratan sulphate and chondroitin 6-sulphate and containing non-reducing-end β -linked 6-sulphated *N*-acetylhexosamine residues, with homogenates of cultured human skin fibroblasts has indirectly been shown to release GlcNAc6S and GalNAc6S respectively. Release of GalNAc4S could not be demonstrated in similar incubations of oligosaccharide substrates derived from chondroitin 4-sulphate and containing non-reducing-end β -linked GalNAc4S residues. 4. We propose that some, if not all, of the sulphated *N*-acetylhexosamine present in human urine is derived from the action of β -*N*-acetylhexosaminidase on sulphated GlcNAc or GalNAc residues at the non-reducing end of keratan sulphate, dermatan sulphate or chondroitin sulphate.

It is known that there are five different sulphatase activities involved in the degradation of the sulphated glycosaminoglycans heparan sulphate (HS), dermatan sulphate (DS) and keratan sulphate (KS). A deficiency in any one of these sulphatase activities in humans leads to the accumulation in tissues, and the excretion, of abnormal amounts of HS, DS and/or KS and the expression of a group of lysosomal storage disorders known as the mucopolysaccharidoses (McKusick & Neufeld, 1983). We have recently

described the excretion of abnormal amounts (> 500-fold increase over normal) of the monosaccharide *N*-acetylglucosamine 6-sulphate (GlcNAc6S) in the urine of a patient with mucopolysaccharidosis Type IIID (*N*-acetylglucosamine-6-sulphate sulphatase-deficient; MPS IIID; Sanfilippo D Syndrome) (Hopwood & Elliott, 1983b). This finding led us to speculate that this increased level of GlcNAc6S, which is not able to be further hydrolysed by these patients, may in part result from the action of the A form of β -*N*-acetylhexosaminidase on the β -linked GlcNAc6S residues at the non-reducing end of KS. It is not possible to connect directly the excessive excretion of GlcNAc6S in MPS IIID patients to KS catabolism, since several sulphated glycoproteins

Abbreviations used: GlcNAc6S, *N*-acetylglucosamine 6-sulphate; GalNAc6S, *N*-acetylglactosamine 6-sulphate; GalNAc4,6diS, *N*-acetylglactosamine 4,6-disulphate; HS, heparan sulphate; DS, dermatan sulphate; KS, keratan sulphate; MPS, mucopolysaccharidosis.

have been described that also contain β -linked GlcNAc6S residues and therefore may also be susceptible to hydrolysis by β -*N*-acetylhexosaminidase to produce GlcNAc6S.

The finding of elevated levels of GlcNAc6S in urine from MPS IIID patients with a deficiency of *N*-acetylglucosamine-6-sulphate sulphatase lead us to investigate the sulphated *N*-acetylhexosamine content of urine from other *N*-acetylhexosamine sulphate sulphatase-deficient patients such as mucopolysaccharidosis Type IVA (MPS IVA; *N*-acetylglucosamine-6-sulphate sulphatase-deficient; Morquio A Syndrome) and mucopolysaccharidosis Type VI (MPS VI; *N*-acetylglucosamine-4-sulphate sulphatase-deficient; Maroteaux-Lamy Syndrome). We now report the isolation of other *N*-acetylhexosamine sulphate esters [*N*-acetylglucosamine 4-sulphate (GalNAc4S), *N*-acetylglucosamine 6-sulphate (GalNAc6S), *N*-acetylglucosamine 4,6-disulphate (GalNAc4,6diS)] from the urine of normal controls and a variety of mucopolysaccharidoses and other lysosomal-storage-disorder patients.

Experimental

Materials

Radiolabelled GlcNAc6S (sp. radioactivity 60 Ci/mol) was prepared from [$1\text{-}^{14}\text{C}$]GlcNAc (The Radiochemical Centre, Amersham, Bucks., U.K.) by using a preparative method previously described (Hopwood & Elliott, 1981). Radiolabelled GalNAc6S (sp. radioactivity 52 Ci/mol) was prepared by *O*-sulphation from [$1\text{-}^{14}\text{C}$]galactosamine hydrochloride (The Radiochemical Centre) after *N*-acetylation by using preparative methods previously described (Hopwood & Elliott, 1981). Radiolabelled disulphated GlcNAc (GlcNAcdiS) was present in the GlcNAc sulphation mixture and separated from GlcNAc6S by descending paper chromatography in Solvent 1 [butan-1-ol/acetic acid/aq. NH_3 (1 mol/l), 2:3:1 (by vol.)] for 20 h at 25°C. Mobilities relative to GlcNAc were 0.66 for GlcNAc6S and 0.25 for the proposed disulphated GlcNAc component. Preparative high-voltage electrophoresis on Whatman 3MM paper in formic acid (1.75 mol/l, pH 1.7) at 45 V/cm for 40 min (Hopwood & Elliott, 1981) for the three major components separated in the above descending paper chromatography caused the proposed disulphated GlcNAc, GlcNAc6S and GlcNAc to migrate 19 cm, 9.5 cm and 0 cm from the origin respectively. The disulphated GlcNAc is probably GlcNAc 3,6-disulphate (Ishihara *et al.*, 1976a).

GalNAc4S and GalNAc6S standards were isolated from acid hydrolysates of the disaccharides (purchased from Miles Laboratories, Elkhart, IN, U.S.A.) 3-*O*- β -gluc-4-enuronosyl-*N*-acetyl-

galactosamine 4-*O*-sulphate and 3-*O*- β -gluc-4-enuronosyl-*N*-acetylglactosamine 6-*O*-sulphate respectively. Each disaccharide (3 mg) was maintained at 100°C for 65 min in 10 ml of formic acid (0.21 mol/l) and then rotary-evaporated to approx. 1 ml, mixed with 10000 d.p.m. of [$1\text{-}^{14}\text{C}$]GlcNAc6S (see below) and chromatographed on a column (1 cm $\times$ 200 cm) of Bio-Gel P2 in aq. 10% (v/v) ethanol. Fractions containing radioactivity were pooled, freeze-dried and dissolved in 100 μ l of water and subjected to preparative high-voltage electrophoresis in formic acid (1.75 mol/l) (as described above). The yields of GalNAc4S and GalNAc6S, based on recovered hexosamine (Hopwood & Robinson, 1974), were approx. 800 and 600 μ g respectively. Approx. 50 μ g of each monosaccharide was reduced by using NaB^3H_4 (Hopwood & Elliott, 1981) to [$1\text{-}^3\text{H}$]*N*-acetylglucosaminitol 4-sulphate and [$1\text{-}^3\text{H}$]*N*-acetylglucosaminitol 6-sulphate. These preparations were used as standards in descending paper chromatography. GalNAc4S and GalNAc6S were detected with alkaline AgNO_3 . The relative mobilities of these sulphated *N*-acetylhexosamines during descending paper chromatography in Solvent 1 were: GlcNAcdiS, 0.36; GlcNAc6S and GalNAc6S, 1.0; [$1\text{-}^3\text{H}$]*N*-acetylglucosaminitol 6-sulphate, 1.17; GalNAc4S, 1.25; [$1\text{-}^3\text{H}$]*N*-acetylglucosaminitol 4-sulphate, 1.33.

Preparation of radiolabelled substrates

KS tetrasaccharide β -GlcNAc6S- β -Gal6S- β -GlcNAc6S[$1\text{-}^3\text{H}$]galactitol (Substrate 1) was prepared with a specific radioactivity of 200 Ci/mol from KS by methods previously described (Hopwood & Elliott, 1983a). Chondroitin sulphate trisaccharide substrates β -GalNAc6S- β -GlcA-[$1\text{-}^3\text{H}$]*N*-acetylglucosaminitol-6S (Substrate 2) and β -GalNAc4S- β -GlcA-[$1\text{-}^3\text{H}$]*N*-acetylglucosaminitol-4S (Substrate 5) were prepared from chondroitin 6-sulphate and chondroitin 4-sulphate by the method previously described (Hopwood & Elliott, 1983c). These trisaccharides were partially desulphated by enzyme digestion with homogenates of human skin fibroblasts at pH 4.5 in the presence of 2-acetamido-2-deoxy-D-gluconolactone (10 mmol/litre) to produce the monosulphated trisaccharides β -GalNAc- β -GlcA-[$1\text{-}^3\text{H}$]*N*-acetylglucosaminitol-6S (Substrate 3) and β -GalNAc- β -GlcA-[$1\text{-}^3\text{H}$]*N*-acetylglucosaminitol-4S (Substrate 6). The specific radioactivity of the chondroitin 6-sulphate substrates was 250 Ci/mol and of the chondroitin 4-sulphate substrates, 90 Ci/mol. β -GalNAc6S- β -Glc-[$1\text{-}^3\text{H}$]*N*-acetylglucosaminitol-6S (Substrate 4) was prepared by carboxy group reduction of Substrate 2 by methods previously described (Hopwood & Elliott, 1983c).

Urine

Typing of each mucopolysaccharidosis patient was based on level and type of glycosaminoglycanuria (Hopwood & Harrison, 1982), clinical phenotype and nature of lysosomal enzyme deficiency present in leucocytes and/or cultured skin fibroblasts from each patient (Hopwood *et al.*, 1982). Random urine was collected from normal individuals, patients with mucopolysaccharidosis [MPS I (mild and severe phenotypes), II (mild and severe phenotypes), IIIA, IIIB, IIID, IVA and VI (mild and severe phenotypes)] and α -mannosidosis, mucopolipidosis Type II (Pollard *et al.*, 1980) and multiple sulphatase deficiencies. The samples were immediately frozen and stored at -20°C until used. Creatinine was determined as previously described (Hopwood & Harrison, 1982).

Determination of the level of N-acetylhexosamine sulphate monosaccharide in urine

[1- ^{14}C]GlcNAc6S (90000 d.p.m.) and/or [1- ^{14}C]GlcNAc6S (90000 d.p.m.) or [1- ^{14}C]GalNAc6S (90000 d.p.m.) was added to 20 ml of urine, rotary-evaporated at 25°C to approx. 5 ml and dialysed (12000- M_r cut-off dialysis tubing) for 16 h at 4°C against 100 ml of water. The diffusate was concentrated by rotary evaporation at 25°C to 10 ml, mixed with 50 μl of formic acid and applied to a column (1.5 cm $\times$ 7 cm) of Dowex 50 (H^+ form) equilibrated with formic acid (50 mmol/l), which was then washed with 30 ml of formic acid (50 mmol/l). The break-through fraction plus the washings were combined, rotary-evaporated at 25°C to 10 ml, applied to a column (1.5 cm $\times$ 7 cm) of Dowex 1 (Cl^- form) and batch-eluted with 25 ml each of LiCl (0.3 mol/l), LiCl (0.8 mol/l) and LiCl (3.0 mol/l). Fractions (5 ml) were collected and assayed for radioactivity. Approx. 70% of the initial radioactivity was eluted within the first 15 ml of LiCl (0.8 mol/l) for urine samples containing [1- ^{14}C]GlcNAc6S and within the first 15 ml of LiCl (3.0 mol/l) for urine 'spiked' with [1- ^{14}C]GlcNAc6S. Fractions containing radioactivity were pooled, rotary-evaporated at 25°C to approx. 1 ml, desalted on a column (1.6 cm $\times$ 100 cm) of Sephadex G-10 in aq. 10% (v/v) ethanol, freeze-dried, dissolved in 100 μl of water and subjected to preparative descending paper chromatography on Whatman 3MM paper with development in Solvent 1 at 25°C for 17 h. Radioactive material corresponding to the ^{14}C -labelled sulphated *N*-acetylhexosamine standards, located by using a Packard radiochromatogram scanner (Hopwood & Elliott, 1981) and/or GalNAc6S and GalNAc4S standards located by using alkaline AgNO_3 , were eluted with water and rotary-evaporated to a total volume of 100 μl . Recovery of radioactive material to this stage varied from 9 to 50% of the ^{14}C -

labelled sulphated *N*-acetylhexosamines added to the initial urine sample. Half (50 μl) was acid-hydrolysed in 2 ml of HCl (4 mol/l) at 100°C under N_2 for 6 h. The extent of de-*N*-acetylation and de-*O*-sulphation of the radiolabelled standard was monitored by high-voltage electrophoresis of 40 μl of hydrolysate at pH 1.7 in formic acid (1.75 mol/l) as described above.

The acid hydrolysate was rotary-evaporated to dryness, taken up in 100 μl of water and subjected to separation on a single-column Dionex amino acid analyser (Dionex Corp., Sunnyvale, CA, U.S.A.) with *o*-phthalaldehyde derivative detection. Norleucine was added as internal standard. Effluent fractions were collected and assayed for radioactivity (Hopwood & Elliott, 1983b). Where indicated below, the other half (50 μl) of the material eluted from the preparative-paper-chromatographic step was subjected to periodate oxidation (Liau & Horowitz, 1982; Hopwood & Elliott, 1983b) and then acid hydrolysis as described above. Sulphated *N*-acetylhexosamine levels were calculated from hexosamine values adjusted for recoveries of standards (glucosamine and galactosamine) from the amino acid analyser and recoveries of ^{14}C -labelled sulphated *N*-acetylhexosamine before the acid-hydrolysis step (see above).

Incubation of radiolabelled chondroitin sulphate and KS substrates with homogenates of cultured human skin fibroblasts

Incubation mixtures contained 15–30 μg of protein from cultured fibroblast homogenates in sodium formate buffer (50 mmol/l, pH 4.0, NaN_3 (4 mmol/l), D-saccharic acid 1,4-lactone (5 mmol/l) and approx. 500 pmol of substrate in a final volume of 20 μl in a sealed polypropylene tube (Hopwood & Elliott, 1981). Incubation time, at 37°C , was variable and dependent on the extent of hydrolysis; for example, fibroblast homogenates from normal individuals were incubated with Substrate 1 for 1 h to yield approx. 20% product, whereas fibroblasts from MPS IVA patients with a Substrate 2 required 18 h to yield approx. 1% product. At the end of the incubation period, radiolabelled products were separated from substrates by descending paper chromatography on Whatman 3MM paper in a solvent mixture of ethyl acetate/acetic acid/water (4:3:3, by vol.) or by high-voltage electrophoresis at pH 1.7 in formic acid (1.75 mol/l) as described above.

Results

Isolation and identification of sulphated N-acetylhexosamines from human urine

Sulphated *N*-acetylhexosamines have been isolated from human urine and tentatively identified

as GlcNAc6S, GalNAc6S, GalNAc4S and GalNAc4,6diS. Identification of these sulphated monosaccharides was based on their mobility in descending paper chromatography (Fig. 1) and high-voltage electrophoresis against standard compounds, their reaction with periodate (Table 1) and the identification and quantification of glucosamine or galactosamine by ion-exchange chromatography (Fig. 2).

As we have previously reported (Hopwood & Elliott, 1983b), the level of GlcNAc6S in urine from patients with a deficiency of *N*-acetylglucosamine 6-sulphate sulphatase (MPS IIID) shows a more-than-300-fold elevation (Table 1) over the level found in urine from normal individuals.

Urine from two (MPS IVA) patients with a deficiency of *N*-acetylgalactosamine-6-sulphate sulphatase and having a classical Morquio clinical phenotype had elevated levels of KS and chondroitin 6-sulphate and also excrete a more-than-180-fold excess of GalNAc6S (Table 1). Three patients, deficient in *N*-acetylgalactosamine-4-sulphate sulphatase and with severe clinical (Maroteaux-Lamy) phenotypes, compared with normal, excreted 420- and 510-fold-elevated levels of GalNAc4S and GalNAc4,6diS respectively (Table 1). One patient with a mild Maroteaux-Lamy phenotype, although showing approx. 200- and 80-fold elevation over normal levels of GalNAc4S and Gal-

NAc4,6diS respectively, excreted less of these compounds than did the more severely affected patients. Compared with normal, the urine from one MPS IH patient (Hurler Syndrome, severe clinical phenotype) contained 100-, 30- and 40-fold higher concentrations of GlcNAc6S, GalNAc4S and GalNAc4,6diS respectively, whereas the levels of these three sulphated monosaccharides in an MPS IS (Scheie Syndrome, mild clinical phenotype) urine were only increased 7-, 15- and 14-fold respectively. Similarly, a severely affected MPS II patient excreted higher levels of sulphated monosaccharides than did a mildly affected MPS II patient, a level that, in turn, was higher than that

Fig. 1. Preparative descending paper chromatography in Solvent 1 of sulphated *N*-acetylhexosamine fractions isolated from an MPS IVA urine (a) in the LiCl (0.8 mol/l) Dowex I eluate and (b) in the LiCl (3 mol/l) Dowex I eluate. The urine sample was spiked with [^{14}C]GlcNAc6S and [^{14}C]GlcNAc4S as described in the Experimental section. Arrows indicate peak positions of standards: a, GalNAc6S; b, [^3H]N-acetylgalactosaminitol 6-sulphate; c, GalNAc4S, d, [^3H]N-acetylgalactosaminitol 4-sulphate; e, [^{14}C]GlcNAc4S.

Fig. 2. Amino acid analysis of acid hydrolates of sulphated *N*-acetylhexosamine fractions isolated from urine (a) *N*-Acetylhexosamine 6-sulphate fractions (fraction 1, Fig. 1a) isolated from a normal control urine. (b) *N*-Acetylhexosamine 6-sulphate fractions (fraction 1, Fig. 1a) isolated from urine from an MPS IVA patient. The urine was spiked with [^{14}C]GalNAc6S. (c) *N*-Acetylhexosamine 4-sulphate fractions (fraction 2, Fig. 1a) isolated from urine from an MPS VI patient. (d) disulphated *N*-acetylhexosamine fractions (fraction 3, Fig. 1b) isolated from urine from an MPS VI patient. Arrows indicate peak position of standards: a, glucosamine; b, galactosamine; c, norleucine. Fractions indicated in (b) were collected every 2 min and assayed for radioactivity (histogram). The millimolar amount of norleucine is constant throughout (a), (b), (c) and (d).

Table 1. *Excretion of sulphated N-acetylhexosamines in urine from normal individuals, mucopolysaccharidoses and other lysosomal-storage-disorder patients*

Values shown were calculated from a single hexosamine assay of each fraction described in the Experimental section. Key to superscripts: ^a, severe clinical phenotype; ^b, mild clinical phenotype; ^c, periodate oxidation before acid hydrolysis. Abbreviations used: nd, none detected; —, not done.

Sample	Content ($\mu\text{mol}/\text{mmol}$ of creatinine)			
	GlcNAc6S	GalNAc6S	GalNAc4S	GalNAc4,6diS
Normal 1	0.03	nd	—	—
Normal 2	0.19	0.19	0.13	0.22
Normal 3	0.13	0.09	0.13	0.14
MPS IH ^a	12.66	nd	3.80	7.06
MPS IS ^b	0.82	0.25	1.96	2.43
MPS II(A) ^a	3.80	1.49	1.42	nd
MPS II(B) ^b	0.92	0.80	0.63	—
MPS IIIA	1.27	1.27	2.31	2.24
MPS IIIB	0.95	0.19	—	—
MPS IIID 1	53.80 (2.85) ^c	nd	3.16	—
MPS IIID 2	34.81	1.42	—	—
MPS IVA 1	0.32	17.41	—	—
MPS IVA 2	6.33	33.86 (nd) ^c	1.71	nd
MPS VI(A) ^a 1	2.12	2.02	60.13 (71.20) ^c	—
MPS VI(A) ^a 2	1.86	1.53	52.42	111.92
MPS VI(A) ^a 3	0.95	1.92	48.20	73.00 (34.06) ^c
MPS VI(B) ^b	0.38	0.83	25.95	14.36
Multiple sulphatase deficiency	8.86	9.49	2.22	1.95
Mucopolipidosis Type II	nd	0.35	0.29	3.89
α -Mannosidosis	0.09	0.12	0.12	nd

isolated from normal individuals. Although there appears to be a general (approx. 5–10-fold) elevation of *N*-acetylhexosamine sulphates in urine from all MPS patients, urine from some MPS patients show much higher (>200-fold) concentrations of specific sulphated monosaccharides, for example GlcNAc6S in MPS IIID, GalNAc6S in MPS IVA and GalNAc4S and GalNAc4,6diS in MPS VI.

Urine from a multiple-sulphatase-deficient patient compared with normal contained 76-, 68-, 17- and 10-fold-elevated levels of GlcNAc6S, GalNAc6S, GalNAc4S and GalNAc4,6diS respectively. A mucopolipidosis-Type-II patient excreted slightly increased levels of GalNAc6S and GalNAc4S and 20-fold higher amounts of GalNAc4,6diS, whereas urine from a clinically severe α -mannosidosis patient contained normal levels of all four sulphated *N*-acetylhexosamines (Table 1).

Incubations of fibroblast homogenates with sulphated oligosaccharide substrates

Hydrolysis of the non-reducing-end sulphated *N*-acetylhexosamine residues of oligosaccharide substrates by homogenates of cultured skin fibroblasts produced two product types (see Table 2). Product type 1 results from the action of a specific sulphatase, whereas product type 2 results from the action of β -*N*-acetylhexosaminidase, either direct-

ly on the β -glycosidic linkage of the sulphated *N*-acetylhexosamine residue or on the unsulphated *N*-acetylhexosamine residue (product 1) resulting from the action of the sulphatase (Scheme 1). We have previously shown that the addition of the β -*N*-acetylhexosaminidase inhibitor 2-acetoamido-2-deoxy-D-glucolactone and incubation in sodium acetate buffer (50 mmol/l) results in the complete inhibition of β -*N*-acetylhexosaminidase activity towards Substrate 1 and 2 and their partially desulphated equivalents (Hopwood & Elliott, 1983a). Further degradation of β -*N*-acetylhexosaminidase products (type 2) of the chondroitin sulphate-derived substrates was inhibited by the addition of the β -glucuronidase inhibitor D-saccharic acid 1,4-lactone to these incubation mixtures. Significant degradation of the β -*N*-acetylhexosaminidase product of KS-derived Substrate 1 by galactose-6-sulphate sulphatase is unlikely, since this enzyme is relatively inactive toward these substrates under the conditions studied (Hopwood & Elliott, 1983c).

Results recorded in Table 3 show that β -*N*-acetylhexosaminidase in fibroblast homogenates is most active towards Substrate 1, producing GlcNAc6S and radiolabelled β -Gal6S- β -GlcNAc6S-galactitol. In the presence of the β -*N*-acetylhexosaminidase inhibitor the relatively low level of sulphatase product (type 1) is not altered, but

Table 2. *Substrate structure, product (type) and method of separation*
Abbreviations used: p.c., paper chromatography; h.v.e., high-voltage electrophoresis.

Substrate	Radiolabelled product		Separation method
	Type 1	Type 2	
1	β -GlcNAc- β -Gal6S- β -GlcNAc6S-[1- 3 H]galactitol	β -Gal6S- β -GlcNAc6S-[1- 3 H]galactitol	H.v.e. P.c.
2	β -GalNAc- β -GlcA-[1- 3 H] <i>N</i> -acetylgalactosaminitol-6S	β -GlcA-[1- 3 H] <i>N</i> -acetylgalactosaminitol-6S	H.v.e. P.c.
3	Not applicable	β -GlcA-[1- 3 H] <i>N</i> -acetylgalactosaminitol-6S	H.v.e.
4	β -GalNAc- β -Glc-[1- 3 H] <i>N</i> -acetylgalactosaminitol-6S	β -Glc-[1- 3 H] <i>N</i> -acetylgalactosaminitol-6S	H.v.e.
5	β -GalNAc- β -GlcA-[1- 3 H] <i>N</i> -acetylgalactosaminitol-4S	β -GlcA-[1- 3 H] <i>N</i> -acetylgalactosaminitol-4S	H.v.e. P.c.
6	Not applicable	β -GlcA-[1- 3 H] <i>N</i> -acetylgalactosaminitol-4S	H.v.e.

Scheme 1. *Proposed pathways for exodegradation of β -linked sulphated *N*-acetylhexosamine residues of, for example, the trisaccharide (Substrate 2) derived from chondroitin 6-sulphate*

the level of β -*N*-acetylhexosaminidase product (type 2) is less than 1% of the inhibitor-free incubation mixtures. Normal levels of type 2 product in the absence of sulphatase product (type 1) were observed when Substrate 1 was incubated with homogenates from MPS IIID (*N*-acetylglucosamine-6-sulphate sulphatase-deficient) fibroblasts (Hopwood & Elliott, 1983a).

Type 1 and 2 products are also observed in normal control fibroblast homogenate incubations with Substrate 2 (Table 3). Under conditions where β -*N*-acetylhexosaminidase is inhibited, the level of type 1 product is increased and type 2 product considerably decreased. Fibroblasts from MPS IVA patients incubated with Substrate 2 produce detectable, but very low, levels of type 2 product in the absence of detectable levels of product 1. Products 1 and 2 were not detectable in incubations of MPS IVA fibroblasts with Sub-

strate 2 in the presence of the β -*N*-acetylhexosaminidase inhibitor. Except for a 4-fold increase in the level of type 2 product, a similar result was observed for Substrate 4, a carboxy-group-reduced equivalent of Substrate 2. These results suggest that the β -*N*-acetylhexosaminidase activity in cultured fibroblasts is able to cleave the glycosidic bond of the non-reducing-end GalNAc6S residues to yield, presumably, unlabelled GalNAc6S and labelled type 2 product without the sequential action of sulphatase to product 1, which is then hydrolysed by β -*N*-acetylhexosaminidase to product 2. The action of the *N*-acetylglucosamine-6-sulphate sulphatase activities of MPS VI and normal fibroblasts on Substrate 2 to yield its product seem to proceed at approximately the same rate as the action of β -*N*-acetylhexosaminidase on product 1 produced either *in situ* from Substrate 2 incubations or in separate incubations

Table 3. *Hydrolysis (pmol/min per mg of protein) of N-acetylhexosamine sulphate residues in a variety of oligosaccharide substrates by cultured skin fibroblasts*

Values represent the result of a single assay using incubation mixtures described below and in the Experimental section. Results represent single-point assays with no evidence that there is a linear release of products with time. Abbreviations used: nd, less than 25% of blank value; —, not done; nr, not relevant; MPS, mucopolysaccharidosis. All incubations contained β -glucuronidase inhibitor.

Substrate	β -Hex inhib.*	MPS IVA		MPS VI		Normal	
		1†	2†	1	2	1	2
1	—	—	105	—	76	—	83
	+	0.5	0.2	0.4	0.2	0.7	0.2
2	—	nd	0.14	23	20	52	21
	+	nd	nd	57	1.2	67	0.6
3	—	nr	46	nr	31	nr	38
	+	nr	0.2	nr	0.1	nr	0.1
4	—	nd	0.56	—	—	8	17
	+	nd	nd	—	—	21	0.8
5	—	3.1	1.4	nd	nd	2.1	4.3
	+	5.0	nd	—	—	7.4	nd
6	—	nr	6	nr	8	—	—

* + or — β -*N*-Acetylhexosaminidase inhibitor 2-acetoamido-2-deoxy-D-glucolactone (5 mmol/l).

† Type 2 product results from the action of either β -*N*-acetylhexosaminidase or a sequence of sulphatase and then β -*N*-acetylhexosaminidase.

‡ Type 1 product results from the action of sulphatase only.

with Substrate 3. However, the activity of β -*N*-acetylhexosaminidase in MPS IVA fibroblasts towards GalNAc6S glycosidic bonds in Substrate 2 is more than 300 times less than its activity towards the β -linked GalNAc residue in Substrate 3 (Table 3).

Substrate 5, the 4-sulphate ester equivalent to Substrate 2, was desulphated by fibroblasts from normal individuals and MPS IVA patients to a type 1 product at a rate approx. 10 times less than that observed for the 6-sulphated ester equivalent (Table 3). Hydrolysis of the type 1 product of Substrate 5 to a type 2 product by the action of β -*N*-acetylhexosaminidase was observed for incubations containing MPS IVA or normal fibroblast homogenates. Hydrolysis of Substrate 5 by either the action of sulphatase or β -*N*-acetylhexosaminidase to type 1 and/or 2 products was not observed in incubations using MPS VI fibroblast homogenates (Table 3). However, incubation of Substrate 6 (type 1 product of Substrate 5) with MPS IVA or VI fibroblast homogenates gave a type 2 product at a rate approx. 10 times less than that observed with Substrate 3.

Discussion

The source of the sulphated *N*-acetylhexosamines (GlcNAc6S, GalNAc6S, GalNAc4S and GalNAc4,6diS) in normal urine is not known. Sulphated *N*-acetylhexosamines have been identified as being integral parts of sulphated glycos-

aminoglycans, glycoproteins and sugar nucleotides. We have previously proposed that GlcNAc6S in urine may result from the action of β -*N*-acetylhexosaminidase on β -linked GlcNAc6S residues at non-reducing ends of KS chains and/or sulphated oligosaccharides of glycoproteins (Hopwood & Elliott, 1983a,b). We have previously reported that *N*-acetylglucosamine-6-sulphate sulphatase, which is deficient in MPS IIID fibroblasts, specifically desulphates both α - and β -linked GlcNAc6S residues at the non-reducing end of HS and KS respectively (Hopwood & Elliott, 1983a). Compared with normal individuals, MPS IIID patients excrete elevated levels of HS and the monosaccharide GlcNAc6S, but not KS (Hopwood & Elliott, 1983a,b). As KS is not present above normal levels in MPS IIID urine, it has been proposed that, in these patients, β -linked GlcNAc6S residues in KS are degraded by the action of β -*N*-acetylhexosaminidase (type A) to produce the monosaccharide GlcNAc6S, which will not de-*O*-sulphate and will therefore accumulate in tissues and be excreted in excess by these patients. Thus the GlcNAc6S monosaccharide present in MPS IIID urine may be considered as representing the block in KS degradation.

By similar logic, the excretion of large amounts of GalNAc6S in urine from MPS IVA patients and GalNAc4S and GalNAc4,6diS in the urine of MPS VI patients could be expected to result from the action of β -*N*-acetylhexosaminidase on chondroitin sulphate and DS, which accumulate in

these patients. Obviously, as 6-sulphated or 4-sulphated galactosaminoglycans accumulate in MPS IVA or MPS VI patients the efficiency of β -*N*-acetylhexosaminidase towards β -linked sulphated GalNAc residues must be much lower than that observed towards non-reducing-end β -linked GlcNAc6S residues in KS. Results reported in Table 3 show that the β -*N*-acetylhexosaminidase activity towards the non-reducing-end GalNAc6S glycosidic bond in Substrate 2 is approx. 750 times less than that towards β -linked GlcNAc6S in the KS Substrate 1. This difference is not totally due to the carboxyl group on the adjacent GlcA residue, since its reduction to Glc leads to only a 4-fold increased hydrolysis of the GalNAc6S glycosidic bond (Substrate 4, Table 3). The other differences, β -1,4-linked GalNAc components in chondroitin sulphate in place of β -1,3-linked GlcNAc residues adjacent to β -linked galactose residues (which may also be C-6-sulphated in KS), may provide the structural requirement for more efficient β -*N*-acetylhexosaminidase cleavage of sulphated *N*-acetylhexosamines. We have previously reported that β -linked GlcNAc6S residues in the tetrasaccharide Substrate 1 are hydrolysed by β -*N*-acetylhexosaminidase at a rate 400 times higher than that observed for a disaccharide, β -GlcNAc6S[1-³H]galactitol (Hopwood & Elliott, 1983a). This result suggests that β -*N*-acetylhexosaminidase hydrolysis of these substrates may be influenced by adjacent-residue C-6 sulphate esters, intact adjacent-residue galactose ring or a requirement for a second GlcNAc6S residue two residues removed from the bond under β -*N*-acetylhexosaminidase attack. Preliminary studies have shown that the activity of β -*N*-acetylhexosaminidase towards β -1,3 GlcNAc6S residues is increased 40-fold by the presence of C-6 sulphate esters on the adjacent galactose residue in KS tetrasaccharide structures of Substrate 1 type (Hopwood & Elliott, 1983d).

Cleavage of β -linked GalNAc4S residues was not observed from Substrate 5 under conditions similar to those used with Substrate 2 (Table 2).

Increased concentration of GalNAc4,6diS in urine from MPS VI and not MPS IVA patients suggests that the de-*O*-sulphation of this compound proceeds sequentially, first with the 4-sulphate sulphatase (deficient in MPS VI) to produce GalNAc6S, which is then de-*O*-sulphated by the 6-sulphate sulphatase (deficient in MPS IVA) to produce GalNAc. The finding of sulphated *N*-acetylhexosamines in urine suggests that these monosaccharide sulphates are probably, along with the relevant sulphated glycosaminoglycans, the substrates *in vivo* for their respective sulphatases.

It would appear that the data reported in Table 3 are consistent with the proposal that GlcNAc6S and GalNAc6S may, in part, result from the action of β -*N*-acetylhexosaminidase on KS and chondroitin 6-sulphate respectively. The presence of GalNAc4S, GalNAc6S and GalNAc4,6diS in rat urine has been connected indirectly to the synthesis and turnover of UDP-*N*-acetylgalactosamine 4,6-disulphate (Ishihara *et al.*, 1976a,b). It is also possible that sulphated *N*-acetylhexosamines may result from the action of β -*N*-acetylhexosaminidase towards the sulphated oligosaccharide moiety of glycoproteins that have been shown to contain GlcNAc6S or GalNAc6S (Liau & Horowitz, 1983). Proof of the source of the four sulphated *N*-acetylhexosamines identified in human urine (Table 1) will have to await further studies into the catabolism of sulphated glycosaminoglycans, glycoproteins and sugar nucleotides.

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