Human glucosamine-6-sulphatase deficiency

Diagnostic enzymology towards heparin-derived trisaccharide substrates

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Glucosamine-6-sulphatase (6S) activity towards a series of radiolabelled heparin-derived trisaccharide substrates was determined in cultured human skin fibroblast and leucocyte homogenates, and in urine supernatants of normal individuals and patients affected with 6S deficiency [Sanfilippo D syndrome; mucopolysaccharidosis (MPS) type IIID]. The *N*-sulphated and *N*-acetylated derivatives of the trisaccharide substrate *O*-(α -glucosamine 6-sulphate)-(1 $\rightarrow$ 4)-*L*-*O*-(α -iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-*D*-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate (GlcNH6S-IdoA2S-anM6S) were prepared by enzymic digestion of a pentasulphated tetrasaccharide isolated following the HNO₂ deamination of heparin. Purified lysosomal enzymes and MPS-patient skin fibroblasts were used along with chemical degradation to confirm the structure of each of the substrates that were utilized to study the interaction of the enzyme activities required to degrade the highly sulphated regions of heparan sulphate. Human liver, skin fibroblast and urine 6S activities were separated by chromatofocusing into at least four and possibly up to six individual activities. 6S activities present in each of the tissues generally had similar catalytic properties, including *K_m* values, pH optima and inhibition with NaCl, Na₂SO₄ and NaH₂PO₄. Leucocyte and skin fibroblast 6S activities towards GlcNAc6S-IdoA2S-anM6S were maximal at pH 4.1 and 3.9 respectively, with *K_m* values of 2.8 μ M and 0.9–1.7 μ M respectively. Urine 6S activity towards GlcNAc6S-IdoA2S-anM6S was stimulated 30-fold by BSA at pH 3.9, which shifted the pH optimum from 5.1 to 4.2 and decreased the *K_m* value at pH 4.2 from 4.0 μ M to 0.5 μ M. Residual 6S activity present in the skin fibroblast homogenates from MPS IIID patients was characterized for activity towards GlcNAc6S-IdoA2S-anM6S and observed to have similar pH optima and *K_m* values to normal skin fibroblast 6S activities, although the residual 6S activity was less than 1 % of the normal control range.

INTRODUCTION

The sulphated glycosaminoglycans heparin and heparan sulphate are degraded from their non-reducing terminals by the sequential action of at least five lysosomal sulphatases and four other exo-enzyme activities [1,2]. A deficiency in humans of any one of the four enzyme activities that act upon the non-reducing terminal glucosamine residue of heparan sulphate may lead to the tissue accumulation and urine excretion of partially degraded heparan sulphate and may result in progressive mental retardation and mild skeletal deformities, which presents as the Sanfilippo syndrome [mucopolysaccharidosis type III (MPS III)] [3]. The de-*O*-sulphation of α -linked glucosamine 6-sulphate residues in heparan sulphate requires a specific sulphatase,

glucosamine-6-sulphatase (6S), which has been shown to be deficient in cultured skin fibroblast and leucocyte homogenates in Sanfilippo D (MPS IIID) patients [4]. We recently reported the purification and characterization of the major forms of 6S present in human liver that were active towards a heparin-derived disaccharide substrate GlcNAc6S-IdoA [5]. By the use of oligonucleotides directed against peptide sequence data generated from pure 6S, cDNA clones coding for 6S have been isolated [6] and the 6S gene has been localized to human chromosome 12q14 [7].

In studies concerning the development of a variety of oligosaccharide structures for use as diagnostic substrates for lysosomal enzymes concerned with glycosaminoglycan degradation, we have reported a considerable effect of aglycone structure

Abbreviations used: 6S, glucosamine-6-sulphatase; MPS, mucopolysaccharidosis; GlcNAc6S, *N*-acetyl[1-¹⁴C]glucosamine 6-sulphate; GlcNAc6S-IdoA, *O*-(α -*N*-acetylglucosamine 6-sulphate)-(1 $\rightarrow$ 3)-*L*-[6-³H]idonic acid; IdoA2S-GlcNS6S-IdoA2S-anM6S, *O*-(α -*L*-iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-*D*-*O*-(α -2-sulphaminoglucosamine 6-sulphate)-(1 $\rightarrow$ 4)-*L*-*O*-(α -iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-*D*-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate; IdoA-GlcNS6S-IdoA2S-anM6S, *O*-(α -*L*-iduronic acid)-(1 $\rightarrow$ 4)-*D*-*O*-(α -2-sulphaminoglucosamine 6-sulphate)-(1 $\rightarrow$ 4)-*L*-*O*-(α -iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-*D*-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate; GlcNAc6S-IdoA2S-anM6S, *O*-(α -*N*-acetylglucosamine 6-sulphate)-(1 $\rightarrow$ 4)-*L*-*O*-(α -iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-*D*-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate; GlcNS6S-IdoA2S-anM6S, *O*-(α -2-sulphaminoglucosamine 6-sulphate)-(1 $\rightarrow$ 4)-*L*-*O*-(α -iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-*D*-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate; GlcNH6S-IdoA2S-anM6S, *O*-(α -glucosamine 6-sulphate)-(1 $\rightarrow$ 4)-*L*-*O*-(α -iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-*D*-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate; GlcNH6S-IdoA2S-anM6S, *O*-(α -2-sulphaminoglucosamine)-(1 $\rightarrow$ 4)-*L*-*O*-(α -iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-*D*-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate; GlcNH6S-IdoA2S-anM6S, *O*-(α -glucosamine)-(1 $\rightarrow$ 4)-*L*-*O*-(α -iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-*D*-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate; GlcNAc-IdoA2S-anM6S, *O*-(α -*N*-acetylglucosamine)-(1 $\rightarrow$ 4)-*L*-*O*-(α -iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-*D*-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate; GlcNAc6S-GlcA-anM, *O*-(α -*N*-acetylglucosamine 6-sulphate)-(1 $\rightarrow$ 4)-*D*-*O*-(β -glucuronic acid)-(1 $\rightarrow$ 4)-*D*-*O*-2,5-anhydro[1-³H]mannitol; GlcNS-UA-GlcNS-UOA, *O*-(α -2-sulphaminoglucosamine)-(1 $\rightarrow$ 4)-*O*-(α -*L*-iduronic acid or β -*D*-glucuronic acid)-(1 $\rightarrow$ 4)-*O*-(α -2-sulphaminoglucosamine)-(1 $\rightarrow$ 3)-[6-³H]idonic acid or gulonic acid; GalNAc4S-GlcA-GalitolNAc4S, *O*-(β -*N*-acetyl-galactosamine 4-sulphate)-(1 $\rightarrow$ 4)-*O*-*D*-(β -glucuronic acid)-*O*-*D*-*N*-acetyl[1-³H]galactosaminitol 4-sulphate; GalNAc4S-GlcA-GalNAc4S-GlcA-GalitolNAc4S, *O*-(β -*N*-acetyl-galactosamine 4-sulphate)-(1 $\rightarrow$ 4)-*O*-*D*-(β -glucuronic acid)-(1 $\rightarrow$ 3)-*O*-*D*-(β -*N*-acetyl-galactosamine 4-sulphate)-(1 $\rightarrow$ 4)-*O*-*D*-(β -glucuronic acid)-(1 $\rightarrow$ 3)-*O*-*D*-*N*-acetyl[1-³H]galactosaminitol 4-sulphate; Gal6S-GlcNAc6S-Gal6S-anM6S, *O*-(β -galactose 6-sulphate)-(1 $\rightarrow$ 4)-*D*-*O*-(β -*N*-acetylglucosamine 6-sulphate)-(1 $\rightarrow$ 3)-*D*-*O*-(β -galactose 6-sulphate)-(1 $\rightarrow$ 4)-*D*-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate; IdoA2S-anM6S, *O*-(α -*L*-iduronic acid 2-sulphate)-(1 $\rightarrow$ 4)-*D*-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate; IdoA-anM6S, *O*-(α -*L*-iduronic acid)-(1 $\rightarrow$ 4)-*D*-*O*-2,5-anhydro[1-³H]mannitol 6-sulphate.

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upon expressed enzyme activity in incubations containing homogenates of cultured human skin fibroblasts, amniotic cells or leucocytes and purified human liver enzymes, for example sulphamate sulphohydrolase, 6S, iduronate-2-sulphatase and α -L-iduronidase [2,8–11]. Substrates that possess structural features of the natural substrate generally result in the greatest rate of hydrolysis and have been shown to be useful for the detection of residual enzyme activity in MPS patients. The biochemical characterization of MPS I (α -L-iduronidase-deficient) fibroblast activity towards IdoA-anM6S has allowed a correlation of residual α -L-iduronidase activity with the degree of clinical severity [12]. A study of the influence of aglycone structure upon the catalytic properties of purified human liver 6S towards heparin-derived substrates indicated that the disaccharide GlcNAc6S-IdOA and the trisaccharide substrates GlcNAc6S-IdoA2S-anM6S and GlcNS6S-IdoA2S-anM6S were acted upon by 6S 30-fold, 400-fold and 3900-fold more efficiently than the monosaccharide substrate GlcNAc6S [9], which had been used for the routine diagnosis of MPS IIID patients [13]. GlcNAc6S-IdOA subsequently was evaluated for use as a convenient and specific substrate for the routine diagnosis of MPS IIID patients with the use of cultured skin fibroblast and leucocyte homogenates for enzyme activity; however, skin fibroblast homogenates from three MPS IIID patients had no significant 6S activity towards GlcNAc6S-IdOA [14].

We now report the preparation and the evaluation of a series of heparin-derived trisaccharide substrates for the determination of 6S activity in leucocyte and cultured human skin fibroblast homogenates and urine supernatants. One of these substrates, GlcNAc6S-IdoA2S-anM6S, was used as a specialist substrate for the detection and characterization of residual 6S activity in cultured skin fibroblasts from MPS IIID patients.

MATERIALS AND METHODS

Materials

Heparin (prepared from bovine lung), BSA (globulin-free), glucosamine *N*-sulphate and *N*-acetylmannosamine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Pharmacia Fine Chemicals (Uppsala, Sweden) supplied concanavalin A–Sephadex 4B, Sephadex G-10 and Sephadex G-25 (superfine grade). Dowex AG-1 X4 (100–200 mesh; Cl^- form) was supplied by Bio-Rad Laboratories (Richmond, CA, U.S.A.). NaB^3H_4 (10 Ci/mmol) and *N*-acetyl[4,5,6,7,8,9- ^{14}C]neuraminic acid were obtained from Amersham International (Sydney, N.S.W., Australia). Basal modified Eagle's medium and foetal-calf serum were supplied by Flow Laboratories (Irvine, Ayrshire, Scotland, U.K.). Heat-inactivated foetal-calf serum was prepared by heating at 60 °C for 30 min. MCDB 104 medium was supplied by KC Biological (Lenexa, KS, U.S.A.).

Fibroblast cultures were established from skin biopsies taken in or provided to this hospital. MPS IIID fibroblasts were obtained from patients with heparansulphaturia and with deficient 6S activity in leucocyte and cultured skin fibroblast homogenates [13,14]. Homogenates of cultured human skin fibroblasts and chorionic villus cells, homogenates of peripheral-blood leucocytes and urine supernatants were prepared as described previously [15,16] and dialysed for 16 h at 4 °C against 150 mM-NaCl. Protein was determined by the method of Lowry *et al.* [17], with BSA as a standard.

Uronic acid was determined by the carbazole method of Bitter & Muir [18]. Descending paper chromatography was on Whatman 3MM chromatography paper developed in butan-1-ol/acetic acid/7 M- NH_3 (2:2:1, by vol.) for 72 h [19]. High-voltage electrophoresis was performed on Whatman 3MM

chromatography paper in 0.7 M-formic acid, pH 1.7, containing 0.1 M-NaCl at 22.5 V/cm for 2 h for trisaccharide substrates and at 19 V/cm for 3 h for tetrasaccharide substrates with a Shandon Southern model L-24 HVE system (Shandon Southern Products, Runcorn, Cheshire, U.K.). Areas of radioactivity were detected with a Packard model 7201 radiochromatogram scanner (Packard Instruments, Chicago, IL, U.S.A.). Preparative high-voltage electrophoresis was performed as described above in 0.7 M-formic acid, pH 1.7, at 45 V/cm for 60 min. Areas of radioactivity were eluted from the strip with 10% (v/v) ethanol.

6S and sulphamate sulphohydrolase were purified from human liver as described previously [5,8]. Purified human liver α -L-iduronidase [11] and iduronate-2-sulphatase [10] were generously given by Dr. P. R. Clements and Ms. J. Bielicki respectively, both from this Department. 6S activity was assayed with GlcNAc6S-IdOA [5], sulphamate sulphohydrolase with GlcNS-IdoA2S-anM6S [8], α -L-iduronidase with IdoA-anM6S [11] and iduronate-2-sulphatase with IdoA2S-anM6S [10]. One enzyme activity unit corresponds to a substrate turnover of 1 $\mu\text{mol}/\text{min}$.

Preparation of radiolabelled substrates

IdoA2S-GlcNAc6S-IdoA2S-anM6S was prepared by the limited HNO_2 degradation of heparin. A 1 ml solution of heparin (200 mg) was treated with HNO_2 (10 ml) at pH 1.5 [20] for 30 s at 22 °C. The reaction mixture was neutralized with 2 M- Na_2CO_3 , rotary-evaporated at 30 °C to approx. 2 ml and applied to a column (1.2 cm $\times$ 200 cm) of Sephadex G-25, and developed in 50 mM-sodium acetate buffer, pH 5.3, containing 0.2 M-NaCl. The uronic acid-containing fractions corresponding to the tetrasaccharide pool (Fig. 1) were desalted on a Sephadex G-10 column (1 cm $\times$ 100 cm) developed in 10% (v/v) ethanol and radiolabelled by reduction with NaB^3H_4 (3 Ci/mmol) by the method described previously [21]. The radiolabelled tetrasaccharide fraction (26.7 μmol) was applied to a 10 ml Dowex 1 (Cl^- form) column equilibrated in 50 mM-sodium acetate buffer, pH 5.5 (buffer A). The column was washed with 50 ml of the same buffer and eluted by a linear gradient generated from buffer A and buffer A containing 3 M-LiCl by using an LKB 11300 Ultragrad gradient mixer on a 24 h program at a flow rate of 37 ml/h. Fractions (6 ml) were collected and assayed for radioactivity (Fig. 2). The most highly sulphated tetrasaccharide eluted by 1.6 M-LiCl was concentrated and desalted on a Sephadex G-10 column (1 cm $\times$ 100 cm) developed in 100 mM-ammonium acetate buffer, pH 6.7, to yield 5.3 μmol of the tetrasaccharide IdoA2S-GlcNS6S-IdoA2S-anM6S, which was freeze-dried and stored in 10% (v/v) ethanol at –20 °C.

Preparative enzymic digestion of the substrates occurred in 2 ml of 60 μM -sodium acetate buffer, pH 3.9, containing 50 mM-NaCl and 100 μg of BSA/ml (buffer B) with the appropriate purified enzyme at 37 °C for 24 h. IdoA-GlcNS6S-IdoA2S-anM6S was isolated in 42% yield following the digestion of IdoA2S-GlcNS6S-IdoA2S-anM6S (1 μmol) with iduronate-2-sulphatase (70 munits). GlcNS6S-IdoA2S-anM6S was isolated in 86% yield following the digestion of IdoA-GlcNS6S-IdoA2S-anM6S (0.4 μmol) with α -L-iduronidase (30 munits). GlcNH6S-IdoA2S-anM6S was recovered in 68% yield following the digestion of GlcNS6S-IdoA2S-anM6S (0.2 μmol) with sulphamate sulphohydrolase (15 munits). GlcNH6S-IdoA2S-anM6S was prepared directly in 42% yield by the incubation of IdoA2S-GlcNS6S-IdoA2S-anM6S (1.5 μmol) concurrently with iduronate-2-sulphatase (70 munits), α -L-iduronidase (70 munits) and sulphamate sulphohydrolase (70 munits). Dowex 1 chromatography as described above was used to separate the substrate from the products: IdoA-GlcNS6S-IdoA2S-anM6S was eluted by 1.4 M-LiCl, GlcNS6S-IdoA2S-anM6S by 1.2 M-LiCl and

GlcNH6S-IdoA2S-anM6S by 0.8 M-LiCl. The substrates were desalted and stored in 10% (v/v) ethanol at -20°C .

GlcNH-IdoA2S-anM6S was prepared from the complete digestion of GlcNH6S-IdoA2S-anM6S (50 nmol) by glucosamine-6-sulphatase (5 munits) in 0.5 ml of buffer B at 37°C for 24 h. The mixture was desalted and the product purified by preparative high-voltage electrophoresis. GlcNH-IdoA2S-anM6S and GlcNH6S-IdoA2S-anM6S were each quantitatively *N*-acetylated [22] to yield GlcNAc-IdoA2S-anM6S and GlcNAc6S-IdoA2S-anM6S respectively, or *N*-sulphated [22] to yield GlcNS-IdoA2S-anM6S and GlcNAc6S-IdoA2S-anM6S respectively. The trisaccharide substrates were further purified by preparative high-voltage electrophoresis.

Analytical digestion of substrates involved the incubation of $1\text{ }\mu\text{l}$ of purified enzyme (0.1 munit) or $5\text{ }\mu\text{g}$ of skin-fibroblast homogenate protein in an assay mixture containing $4\text{ }\mu\text{M}$ substrate, 60 mM-sodium acetate buffer, pH 3.9, and $100\text{ }\mu\text{g}$ of BSA/ml in a total volume of $10\text{ }\mu\text{l}$ at 37°C for 2–24 h. Separation of products from the substrates was by high-voltage electrophoresis. Analytical HNO_2 degradation of the radiolabelled substrates at pH 1.5 and at pH 4.0 following de-*N*-acetylation with hydrazine [23] was as described previously [20,24]. Products were analysed by descending paper chromatography or by high-voltage electrophoresis and compared with a standard, IdoA2S-anM6S [19].

6S enzyme assay

The standard enzyme assay mixture contained $4\text{ }\mu\text{M}$ -GlcNAc6S-IdoA2S-anM6S in 60 mM-sodium acetate buffer, pH 3.9 (fibroblast homogenate), pH 4.1 (leucocyte homogenate) or pH 4.2 (urine supernatant), $1\text{ }\mu\text{l}$ (1–3 μg of protein) of dialysed enzyme and 5 mM-*N*-acetylmannosamine in a total volume of $10\text{ }\mu\text{l}$ and was incubated at 37°C for 30 min (fibroblast and leucocyte homogenates), 12 h (MPS-IIID fibroblast homogenate) or 4 h (urine supernatant). Reactions were stopped by freezing in an ethanol/solid CO_2 bath, and the substrate was separated from the product (GlcNAc-IdoA2S-anM6S) by high-voltage electrophoresis. Areas of radioactivity were cut from the strip and placed in 20 ml glass scintillation vials along with 5 ml of water and 10 ml of scintillation cocktail, and their radioactivities were measured in a Searle model 6868 Isocap ambient-temperature liquid-scintillation counter (Searle Analytic, Des Plaines, IL, U.S.A.). Enzyme activity was determined from the percentage breakdown of substrate to product and expressed as pmol/min per mg of protein. The pH-enzyme activity profile was determined by using the standard assay mixture in 60 mM-sodium acetate at various pH values from 3.3 to 5.6. Kinetic data (K_m and V_{max}) values were obtained from Lineweaver-Burk plots with a substrate concentration of 0.5–20 μM . Skin-fibroblast homogenate activity towards $4\text{ }\mu\text{M}$ -GlcNS6S-IdoA2S-anM6S and $4\text{ }\mu\text{M}$ -GlcNH6S-IdoA2S-anM6S were determined in 60 mM-sodium acetate buffer, pH 3.9, in a total volume of $10\text{ }\mu\text{l}$ at 37°C for 10 min (GlcNS6S-IdoA2S-anM6S) or 4 h (GlcNH6S-IdoA2S-anM6S), and the products were separated from the reactants by high-voltage electrophoresis.

Chromatofocusing of 6S activity

A 1 litre volume of morning void urine from a normal healthy adult male was concentrated to 50 ml in an Amicon ultrafiltration stirred cell containing a YM10 membrane and dialysed for 16 h at 4°C against 2 litres of 15 mM-sodium dimethylglutarate buffer, pH 6.0, containing 500 mM-NaCl and 0.1 mM-dithioerythritol (buffer C). After centrifugation at 4000 *g* for 10 min, the supernatant was applied to a 25 ml column of concanavalin A-Sepharose equilibrated in buffer C. The column was washed

with 200 ml of buffer C and eluted with 200 ml of buffer C containing 15% (w/v) methyl α -mannoside. The eluate was concentrated as described above to 30 ml and dialysed for 16 h at 4°C against 1 litre of 20 mM-Tris/HCl buffer, pH 7.5, containing 10% (v/v) glycerol and 0.1 mM-dithioerythritol (buffer D). The dialysed enzyme solution was applied to a chromatofocusing PBE 94 column (0.9 cm $\times$ 15 cm) equilibrated in buffer D. The column was washed with 45 ml of buffer D before the application of 160 ml of 1:8 dilution of Polybuffer 74/HCl, pH 4.0, followed by 50 ml of the same buffer containing 500 mM-NaCl. Urine 6S activity was detected by activity towards GlcNAc6S-IdoA [5]. Human liver (50 g) was homogenized in 3 vol. of buffer C, centrifuged at 25000 *g* for 30 min as previously described [25] and processed as described above. Cultured normal human skin fibroblasts from four 50 ml culture bottles were harvested at confluence as previously described [15,16], freeze-thawed six times in $500\text{ }\mu\text{l}$ of buffer C and dialysed for 16 h at 4°C against 200 ml of buffer D. The suspension was centrifuged at 4000 *g* for 10 min and the supernatant was applied to a 3 ml PBE 94 column equilibrated in buffer D. The column was washed with 15 ml of buffer D before the application of 30 ml of a 1:8 dilution of Polybuffer 74/HCl, pH 4.0, followed by 6 ml of the same buffer containing 500 mM-NaCl. Skin fibroblast 6S activity was detected by activity towards GlcNAc6S-IdoA2S-anM6S and GlcNAc6S-IdoA.

RESULTS

Preparation of substrates

Limited HNO_2 degradation of bovine lung heparin at pH 1.5 resulted in the production of a disaccharide fraction and higher-molecular-mass oligosaccharides, which were separated by Sephadex G-25 chromatography (Fig. 1). The tetrasaccharide fraction was separated by Dowex 1 ion-exchange chromatography into fractions according to their degree of *O*- and *N*-sulphation (Fig. 2). The most highly sulphated fraction, proposed to be the pentasulphated tetrasaccharide IdoA2S-GlcNS6S-IdoA2S-anM6S, migrated further towards the anode upon high-voltage electrophoresis analysis compared with a previously described tetrasulphated tetrasaccharide Gal6S-GlcNAc6S-Gal6S-anM6S [28]. Each of the other Dowex 1 fractions contained a number of tetrasaccharides differing in their position of *O*-sulphation and internal glucosamine *N*-sulphation or *N*-acetylation and were not characterized further. Deamination of IdoA2S-GlcNS6S-IdoA2S-anM6S by HNO_2 at pH 1.5 gave only IdoA2S-anM6S, as determined by high-voltage electrophoresis and by paper chromatography. The pentasulphated tetrasaccharide was resistant to treatment with hydrazine used to de-*N*-acetylate *N*-acetylglucosamine residues and to HNO_2 degradation at pH 4.0 under conditions specific for non-substituted glucosamine residues, which indicated that the internal glucosamine residue was *N*-sulphated. The pentasulphated tetrasaccharide was also resistant to heating at 80°C for 30 min in 20 mM- H_2SO_4 reported to hydrolyse internal 2-deoxy-2-*C*-formyl-D-pentofuranosyl residues that result from an alternate ring contraction and deamination [20].

The availability of highly purified heparan sulphate degradation enzymes and MPS skin-fibroblast cell lines enabled the controlled stepwise degradation of the IdoA2S-GlcNS6S-IdoA2S-anM6S to proceed as described in Scheme 1, which also provided strong evidence of the substrate structures. On an analytical scale, the conversion of substrate into product following incubation with purified enzymes was more than 95% complete and was monitored by high-voltage electrophoresis at pH 1.7 (Fig. 3). IdoA2S-GlcNS6S-IdoA2S-anM6S was degraded

Scheme 1. Proposed pathway for the exo-degradation of IdoA2S-GlcNS6S-IdoA2S-anM6S

Abbreviations: Id2S, iduronate-2-sulphatase; Id, α -L-iduronidase; 6S, glucosamine-6-sulphatase; NS, sulphamate sulphonylhydrolase; NAT, acetyl-CoA:glucosamine *N*-acetyltransferase; NAG, α -N-acetylglucosaminidase; MPS I, MPS II and MPS IIIA–IIID, mucopolysaccharidosis disorders resulting from blockage in the pathway caused by the enzyme deficiency.

by purified iduronate-2-sulphatase to yield IdoA-GlcNS6S-IdoA2S-anM6S; however, MPS II (iduronate-2-sulphatase-deficient) fibroblasts were unable to degrade the substrate unless the incubation time was prolonged by at least 10-fold compared with incubation with normal control fibroblasts. Whereas purified α -L-iduronidase degraded IdoA-GlcNS6S-IdoA2S-anM6S to yield the tetrasulphated trisaccharide GlcNS6S-IdoA2S-anM6S, MPS I (α -L-iduronidase-deficient) fibroblasts were unable to digest the tetrasulphated tetrasaccharide, providing evidence that the non-reducing terminal residue of the pentasulphated tetrasaccharide was IdoA2S. GlcNS6S-IdoA2S-anM6S was desulphated either by purified sulphamate sulphonylhydrolase or 6S to yield GlcNH6S-IdoA2S-anM6S or GlcNS-IdoA2S-anM6S respectively. Both trisaccharides were subsequently desulphated either by 6S or by sulphamate sulphonylhydrolase to yield GlcNH-IdoA2S-anM6S. Prolonged (24 h)

Fig. 1. Gel chromatography on Sephadex G-25 of products derived from heparin after treatment with HNO_2

For full experimental details see the Materials and methods section. Fractions (approx. 1.8 ml) were collected, assayed for uronic acid-containing material and pooled as indicated (—). Arrows indicate the positions of V_0 (void volume) and the following oligosaccharides: a, heparin-derived octasaccharide; b, GalNAc4S-GlcA-GalNAc4S-GlcA-GalitolNAc4S [26]; c, GlcNS-UA-GlcNS-UA [27]; d, GalNAc4S-GlcA-GalitolNAc4S [26]; e, IdoA2S-anM6S [19]; f, IdoA-anM6S [19]; g, *N*-acetyl[4,5,6,7,8,9- ^{14}C]neuraminic acid.

Fig. 2. Ion-exchange chromatography on Dowex 1 of tetrasaccharide fraction from Fig. 1 after radiolabelling with NaB^3H_4

For full experimental details see the Materials and methods section. Fractions were assayed for radioactivity (●) and pooled as indicated (—). ----, Concentration of LiCl.

incubation of IdoA2S-GlcNS6S-IdoA2S-anM6S with a normal control skin-fibroblast homogenate produced a single product, GlcNH-IdoA2S-anM6S. Analysis of the incubation mixture during the period of incubation showed the production and subsequent degradation of each of the intermediate products (results not shown). GlcNH-IdoA2S-anM6S, but not GlcNH6S-IdoA2S-anM6S, was *N*-acetylated (albeit slowly compared with *N*-acetylation of radiolabelled glucosamine [29]) by a control skin-fibroblast homogenate following the addition of 5 mM-acetyl-CoA and incubation overnight at pH 5.7. GlcNH6S-IdoA2S-anM6S and GlcNH-IdoA2S-anM6S were *N*-acetylated with acetic anhydride to yield GlcNAc6S-IdoA2S-anM6S and GlcNAc-IdoA2S-anM6S respectively. GlcNS6S-IdoA2S-anM6S was desulphated by purified human liver 6S to yield GlcNAc-

Fig. 3. Separation of heparin-derived oligosaccharides by high-voltage electrophoresis

For full experimental details see the Materials and methods section. (a) Tetrasaccharides (1, GlcNS-UA-GlcNS-UOA; 2, IdoA2S-GlcNH6S-IdoA2S-anM6S; 3, IdoA2S-GlcNAc6S-IdoA2S-anM6S; 4, IdoA2S-GlcNS6S-IdoA2S-anM6S) and (b) trisaccharides and disaccharides (5, GlcNH-IdoA2S-anM6S; 6, IdoA-anM6S; 7, GlcNH6S-IdoA2S-anM6S; 8, IdoA2S-anM6S; 9, GlcNAc6S-IdoA2S-anM6S; 10, GlcNS6S-IdoA2S-anM6S) were separated by high-voltage electrophoresis in 0.7 M-formic acid, pH 1.7, containing 0.1 M-NaCl at 19 V/cm for 3 h. GlcNAc-IdoA2S-anM6S and GlcNS-IdoA2S-anM6S had similar mobilities towards the anode to those of trisaccharides 7 and 9 respectively. (c) The substrate GlcNS6S-IdoA2S-anM6S was separated by high-voltage electrophoresis in 0.7 M-formic acid, pH 1.7, containing 0.1 M-NaCl at 22.5 V/cm for 2 h from the products (1, GlcNH-IdoA2S-anM6S; 2, GlcNH6S-IdoA2S-anM6S; 3, GlcNS-IdoA2S-anM6S) following incubation with a normal fibroblast homogenate. (d) The substrate GlcNAc6S-IdoA2S-anM6S was separated by high-voltage electrophoresis in 0.7 M-formic acid, pH 1.7, containing 0.1 M-NaCl at 22.5 V/cm for 2 h from the products (1, IdoA-anM6S; 2, GlcNAc-IdoA2S-anM6S; 3, IdoA2S-anM6S) following incubation with a normal fibroblast homogenate in the presence (—) or in the absence (---) of 5 mM-*N*-acetylmannosamine.

IdoA2S-anM6S. α -*N*-Acetylglucosaminidase activity present in MPS II skin fibroblasts degraded GlcNAc-IdoA2S-anM6S to yield IdoA2S-anM6S. Degradation did not occur in the presence of 5 mM-*N*-acetylmannosamine (an inhibitor of α -*N*-acetylglucosaminidase [30]) or following the incubation of GlcNAc-IdoA2S-anM6S with MPS IIIB (α -*N*-acetylglucosaminidase-deficient) fibroblasts. Prolonged incubations, with a normal control fibroblast homogenate, completely degraded GlcNAc6S-IdoA2S-anM6S and GlcNAc-IdoA2S-anM6S to the monosaccharide anM6S. The degradation of IdoA2S-GlcNS6S-IdoA2S-anM6S to yield anM6S following overnight incubation with a control skin-fibroblast homogenate in the presence of acetyl-CoA was not observed because of inhibition of each of the sulphatases by acetyl-CoA.

The stepwise degradation of IdoA-GlcNS6S-IdoA2S-anM6S to yield GlcNH6S-IdoA2S-anM6S on a preparative scale resulted in from 42 to 86% conversion of each substrate to product. IdoA2S-GlcNS6S-IdoA2S-anM6S was degraded more efficiently to GlcNH6S-IdoA2S-anM6S in 42% overall yield by the simultaneous hydrolysis by purified iduronate-2-sulphatase, α -L-iduronidase and sulphamate sulphohydrolase activities. GlcNH6S-IdoA2S-anM6S was chemically *N*-sulphated and *N*-acetylated to yield GlcNS6S-IdoA2S-anM6S and GlcNAc6S-IdoA2S-anM6S respectively. GlcNAc6S-IdoA2S-anM6S and GlcNAc-IdoA2S-anM6S were degraded by HNO₂ at pH 4.0, but only following de-*N*-acetylation with hydrazine. GlcNH6S-

IdoA2S-anM6S and GlcNH-IdoA2S-anM6S were degraded by HNO₂ at pH 4.0, and IdoA-GlcNS6S-IdoA2S-anM6S, GlcNS6S-IdoA2S-anM6S and GlcNS-IdoA2S-anM6S were degraded by HNO₂ at pH 1.5. In each case HNO₂ degradation gave IdoA2S-anM6S as the only radiolabelled product detected by high-voltage electrophoresis and paper chromatography.

Determination of fibroblast 6S activity

High-voltage electrophoresis of the whole incubation mixture was used to assess the level of desulphation by the enzyme preparations (Fig. 3). The addition of 0.1 M-NaCl to the electrophoresis buffer (0.7 M-formic acid, pH 1.7) substantially improved the resolution of the product(s) from the substrate. Skin fibroblast activity towards GlcNS6S-IdoA2S-anM6S produced three products, GlcNS-IdoA2S-anM6S and GlcNH6S-IdoA2S-anM6S, resulting from both 6S and sulphamate sulphohydrolase activity towards the original substrate, and GlcNH-IdoA2S-anM6S, which resulted from subsequent sulphatase activity towards both of these products (Fig. 3c and Scheme 1). Skin-fibroblast homogenate sulphamate sulphohydrolase acted towards GlcNS6S-IdoA2S-anM6S with from 1.1-fold to 8.1-fold the rate of 6S-activity as determined by the amounts of the respective products formed (Table 1). Attempts to inhibit the competing sulphamate sulphohydrolase activity with CuCl₂ [8] and Na₂SO₃ [31] were unsuccessful. Fig. 3(d) shows the electrophoretic separation of a reaction mixture following the incubation of a

Table 1. Hydrolysis of trisaccharide substrates (GlcNX6S-IdoA2S-anM6S, X = S, Ac and H) by normal control and MPS IIIA fibroblast homogenates

For full experimental details see the Materials and methods section. Abbreviations: 6S, glucosamine-6-sulphatase; NS, sulphamate sulphohydrolase.

Substrate	Fibroblast homogenate	6S activity (pmol/min per mg of protein)	NS activity (pmol/min per mg of protein)
GlcNS6S-IdoA2S-anM6S	Control 1	98.2	106.7
	Control 2	74.5	149.9
	Control 3	63.2	410.8
	Control 4	81.9	658.4
	Control 5	121.2	131.7
	MPS IIIA	261.9	0.0
GlcNAc6S-IdoA2S-anM6S	Control 1	95.9	
	MPS IIIA	235.3	
GlcNH6S-IdoA2S-anM6S	Control 1	7.5	
	MPS IIIA	14.1	

normal fibroblast homogenate with GlcNAc6S-IdoA2S-anM6S, which gave up to three products following 6S activity, namely GlcNAc-IdoA2S-anM6S as well as IdoA2S-anM6S and IdoA-anM6S, which result from subsequent α -N-acetylglucosaminidase and iduronate-2-sulphatase activity. N-Acetylmannosamine (5 mM) completely inhibited fibroblast α -N-acetylglucosaminidase activity towards GlcNAc-IdoA2S-anM6S at pH 3.9, but did not affect 6S activity towards GlcNAc6S-IdoA2S-anM6S, and was included in subsequent incubations to result in the formation of a single product (Fig. 3d). Whereas purified human liver 6S acted towards GlcNAc6S-IdoA2S-anM6S 10-fold more efficiently than towards GlcNAc6S-IdoA2S-anM6S [9], 6S activity present in fibroblasts from a normal control and a sulphamate sulphohydrolase-deficient (MPS IIIA)

patient acted towards both substrates with similar activity (Table 1). GlcNH6S-IdoA2S-anM6S was hydrolysed 13-fold to 18-fold more slowly than either of the other two trisaccharide substrates. GlcNAc6S-IdoA2S-anM6S was therefore evaluated as a substrate for the detection and characterization of 6S activity in normal and MPS IIID fibroblast homogenates.

6S activity towards GlcNAc6S-IdoA2S-anM6S

Maximal sulphatase activity towards GlcNAc6S-IdoA2S-anM6S by homogenates of cultured control skin fibroblasts was observed in 60 mM-sodium acetate buffer at pH 3.9 (Fig. 4a). 6S activity assayed in 60 mM-sodium formate or 15 mM-sodium dimethylglutarate buffer, pH 3.9, was decreased by 52% and 73% respectively. Skin-fibroblast 6S activity from MPS IIIB and MPS IIIC patients both exhibited a similar pH optima (results not shown). Control leucocyte sulphatase activity was maximal at pH 4.1 (Fig. 4a). 6S activity was linear for incubations at 37 °C containing 0.3–3 μ g of homogenate protein for up to 19 h, and for up to 30% conversion of substrate into product for both control fibroblast and leucocyte homogenates. BSA at a concentration of 100 μ g/ml did not significantly affect control skin-fibroblast 6S activity; however, activity towards GlcNAc6S-IdoA2S-anM6S was inhibited 47% by the addition of 1 mg of BSA/ml. Control fibroblast 6S activity towards GlcNAc6S-IdoA2S-anM6S was not influenced by the addition of 5 mM-CuCl₂, but was inhibited 50% by 75 mM-NaCl, 40 μ M-Na₂SO₄ and 100 μ M-NaH₂PO₄.

6S activity in homogenates of cultured human skin fibroblasts and leucocytes from normal control, MPS IIID and other MPS patients is shown in Fig. 5. The range of normal control skin-fibroblast 6S activity towards 4 μ M-GlcNAc6S-IdoA2S-anM6S was 64–121 pmol/min per mg of protein, and MPS I, MPS II and MPS IIIA fibroblast 6S activities were elevated above the normal control range. 6S activity towards 20 μ M-GlcNAc6S-IdoA2S-anM6S ranged from 86 to 220 pmol/min per mg of protein (results not shown); however, accurate determination of 6S activity towards the higher substrate concentration was difficult owing to the poor separation of the product from the substrate by high-voltage electrophoresis. Leucocyte homogenate activity towards 4 μ M-GlcNAc6S-IdoA2S-anM6S from normal

Fig. 4. Hydrolysis of GlcNAc6S-IdoA2S-anM6S as a function of pH by (a) a normal leucocyte homogenate incubated in 60 mM-sodium acetate buffer (■), and by a normal fibroblast homogenate incubated in 60 mM-sodium formate buffer (●) or 60 mM-sodium formate buffer (○), and (b) a normal urine supernatant incubated in 60 mM-sodium acetate buffer (●) or 60 mM-sodium acetate buffer containing 100 μ g of BSA/ml (○) and by a different urine supernatant incubated in 60 mM-sodium acetate buffer (▲)

Each buffer contained 5 mM-N-acetylmannosamine. For full experimental details see the Materials and methods section.

Fig. 5. 6S activity towards GlcNAc6S-IdoA2S-anM6S in a variety of cell types

For full experimental details see the Materials and methods section. Lane 1, cultured skin fibroblasts from normal controls (●) and MPS IIID obligate heterozygotes (○). Lane 2, skin fibroblasts from a variety of MPS patients and an I-cell (ML III) patient. Lane 3, leucocytes from normal controls (●) and MPS IIID obligate heterozygotes (○). Lane 4, leucocytes from a variety of MPS patients.

controls and MPS patients ranged from 66.5 to 127.0 and from 37 to 81 pmol/min per mg of protein respectively. Previously fibroblast 6S activity towards GlcNAc6S-IdoA and β -hexosaminidase activity in all MPS-patient cell lines other than MPS IIID were shown to be elevated above the normal range, whereas MPS-patient leucocyte sulphatase activity was lower than normal [14]. Sulphatase activity towards GlcNAc6S-IdoA2S-anM6S in both leucocyte and fibroblast homogenates from MPS IIID obligate heterozygotes lay between the normal and MPS IIID values (Fig. 5). 6S fibroblast activity from an ML III (I-cell) patient lay below the normal range. 6S activity was detected in chorionic villus tissue from a 12-week pregnancy with a substrate turnover of 85 pmol/min per mg of protein, which indicates the trisaccharide substrate may be useful for prenatal diagnosis for 6S deficiency.

Control fibroblast sulphatase activity towards GlcNAc6S-IdoA2S-anM6S had apparent K_m values of 0.8–1.7 μM ($n = 4$), with corresponding V_{max} values of 140.8–222.2 pmol/min per mg of protein, whereas leucocyte 6S activity towards the trisaccharide substrate had an apparent K_m value of 2.8 μM with a corresponding V_{max} value of 105.3 pmol/min per mg of protein (Tables 2 and 3). Skin fibroblast and leucocyte activities towards GlcNAc6S-IdoA2S-anM6S each had K_m values 5.5-fold lower

Table 2. Skin fibroblast, leucocyte and urine 6S activities towards GlcNAc6S-IdoA2S-anM6S and GlcNAc6S-IdoA

For full experimental details see the Materials and methods section.

Tissue	Substrate	pH	K_m (μM)	V_{max} (pmol/min per mg of protein)
Fibroblast	GlcNAc6S-IdoA	4.2	7.2	20.8
	GlcNAc6S-IdoA2S-anM6S	3.9	1.3	140.8
Leucocyte	GlcNAc6S-IdoA	4.5	15.3	18.5
	GlcNAc6S-IdoA2S-anM6S	4.2	2.8	105.3
Urine 1	GlcNAc6S-IdoA	5.1	27.0	35.7
	GlcNAc6S-IdoA2S-anM6S	4.6	22.3	476.0
Urine 2	GlcNAc6S-IdoA2S-anM6S	5.1	4.2	40.2
	+ BSA (100 $\mu\text{g}/\text{ml}$)	5.1	4.0	94.7
	GlcNAc6S-IdoA2S-anM6S	4.2	4.2	41.1
	+ BSA (100 $\mu\text{g}/\text{ml}$)	4.2	0.5	95.8

and V_{max} values up to 6.7-fold higher than for their respective activities towards the disaccharide substrate GlcNAc6S-IdoA (Table 2).

6S activity towards GlcNAc6S-IdoA2S-anM6S in one urine supernatant was maximal at pH 4.6 (Fig. 4b) and had a K_m value 1.2-fold lower and a V_{max} value 13.3-fold higher than for activity towards GlcNAc6S-IdoA (Table 2). 6S activity towards GlcNAc6S-IdoA2S-anM6S in a second urine supernatant had maximal activity at pH 5.1, whereas in the presence of 100 μg of BSA/ml activity was increased by 2-fold at pH 5.1 and by 30-fold at pH 3.9 to result in an apparent shift in the pH optimum from 5.1 to 4.2 (Fig. 4b). Sulphatase activity at both pH 4.2 and pH 5.1 was linear for incubation times up to 12 h, both in the presence and in the absence of BSA. Fibroblast homogenate 6S activity towards GlcNAc6S-IdoA2S-anM6S at pH 3.9 was not inhibited by the addition of urine supernatant, and therefore it is unlikely that BSA was removing the influence of an endogenous inhibitor of urine 6S activity. In the absence of BSA urine 6S activities towards GlcNAc6S-IdoA2S-anM6S at pH 4.2 and pH 5.1 had similar K_m and V_{max} values (Table 2). At pH 5.1 the addition of BSA resulted in a 2.3-fold increase in the V_{max} value with little effect upon the respective K_m value, whereas at pH 4.2 the addition of BSA increased the V_{max} value by 2.3-fold while the K_m value was decreased by 8.4-fold (Table 2). When the urine sample was concentrated 5-fold the pH optimum for activity towards GlcNAc6S-IdoA2S-anM6S, determined in the absence of BSA, shifted from pH 5.1 to 4.5, similarly to the pH optimum for the first urine sample.

Chromatofocusing of human liver, urine and fibroblast 6S activity

Since the observed fibroblast and urine 6S activity towards GlcNAc6S-IdoA2S-anM6S would be the summation of individual forms of activities present in a particular enzyme preparation, we compared the different forms of 6S activity that were present in human liver, skin-fibroblast homogenates and urine supernatants. The chromatofocusing profile for human liver 6S activity was similar to that observed for 6S activity following elution from the combined concanavalin A–Sephrose/Blue A–agarose step [5], and the Blue-A–agarose column did not remove any forms of 6S activity. Some 90% of the recovered 6S activity had a pI value greater than 7.5 (forms A and B) and two minor forms with pI values of 5.8 (form C) and 5.4 (form D) representing 7% and 3% of the recovered enzyme activity

Table 3. MPS IIID patients' (1 to 3) fibroblast 6S activities towards 4 μ M-GlcNAc6S-IdoA2S-anM6S

For full experimental details see the Materials and methods section.

Fibroblast homogenate	6S activity (pmol/min per mg of protein)	K_m (μ M)	V_{max} (pmol/min per mg of protein)
1	0.73	2.6	1.10
2	0.52	3.3	0.77
3	0.63	2.6	0.91
Control range	64–121 ($n = 14$)	0.8–1.7 ($n = 4$)	140.3–222.2 ($n = 4$)

(results not shown). Chromatofocusing of skin-fibroblast 6S activity separated five fractions with activity towards GlcNAc6S-IdoA2S-anM6S (Fig. 6a). Although close to the limits of detection, a similarly shaped profile was obtained for 6S activity towards GlcNAc6S-IdoA. A 41% fraction of the recovered fibroblast 6S activity did not bind to the chromatofocusing matrix and may therefore be equivalent to either one or both of the major forms A or B of human liver 6S. Two other forms of fibroblast 6S activity had pI values of 5.7 (38% of activity) and 5.4 (12% of activity), identical in pI value with human liver forms C and D, whereas two other forms of fibroblast 6S activity had pI values of 4.8 (2% activity) and 6% of the recovered activity required the addition of salt to the elution buffer, which indicated a pI value less than 4.0. Human urine 6S activity towards GlcNAc6S-IdoA (assayed in the presence of BSA) was separated into five forms with pI values similar to those forms found in skin fibroblasts. The major forms had pI values of greater than 7.5 and 5.9 and represented 21% and 42% of the recovered activity. The minor forms had pI values of 5.3, 4.8 and less than 4.0 and represented 14%, 15% and 8% of the recovered activity.

MPS-IIID-fibroblast 6S activity towards GlcNAc6S-IdoA2S-anM6S

Significant 6S activity towards 4 μ M-GlcNAc6S-IdoA2S-anM6S was detected in the fibroblast homogenates from six

severely affected MPS IIID patients following incubation for a period of 12 h. The pH optimum for 6S activity in each of the MPS-IIID-patient fibroblasts was pH 3.9, the assay was linear with respect to time for over 16 h incubation, and the addition of 100 μ g of BSA/ml had no effect upon 6S activity. 6S activities in MPS IIID fibroblasts ranged from 0.16 to 0.73 pmol/min per mg of protein, less than 1% of the normal range. No significant 6S activity was observed in MPS IIID fibroblasts towards 20 μ M-GlcNAc6S-IdoA2S-anM6S. Skin-fibroblast 6S activity towards GlcNAc6S-IdoA2S-anM6S from three of these severely affected MPS IIID patients had apparent K_m values of 2.6 to 3.3 μ M with V_{max} values of 0.77 to 1.1 pmol/min per mg of protein, approx. 0.7% of the control values (Table 3). No significant 6S activity was detected in either a urine supernatant (in the presence of BSA) or leucocyte homogenate from one of the MPS IIID patients. To demonstrate that 6S activity detected in the MPS IIID fibroblasts was unlikely to be the result of uptake of 6S from the cell culture medium, which itself contained endogenous 6S activity towards GlcNAc6S-IdoA2S-anM6S of 1.2 pmol/min per ml of medium, MPS IIID fibroblasts were cultured in either MCDB 104 medium or basal modified Eagle's medium supplemented with heat-inactivated foetal-calf serum. Although neither of the culture media contained detectable 6S activity, MPS IIID 6S activity from fibroblasts grown in either of these media was similar to that observed for fibroblasts grown in basal modified Eagle's medium containing foetal-calf serum.

DISCUSSION

HNO₂ degradation of heparin has been employed for the analysis of the heparin structure [20,23,32] and to product simple disaccharide substrates for enzymes that normally act towards more complex oligosaccharides, namely iduronate-2-sulphatase [19], α -L-iduronidase [12,19], glucuronate-2-sulphatase [33,34] and β -D-glucuronidase [19,35]. In this study we have shown that limited HNO₂ degradation of bovine lung heparin produced a pentasulphated tetrasaccharide, IdoA2S-GlcNS6S-IdoA2S-anM6S, which resulted from the incomplete degradation of the internal glucosamine N-sulphate residues. The degradation of IdoA2S-GlcNS6S-IdoA2S-anM6S with purified enzymes on a preparative scale resulted in poorer yields compared with the quantitative yields obtained on analytical scale. We have pre-

Fig. 6. Chromatofocusing of 6S activity from (a) human urine and (b) cultured skin fibroblasts

For full experimental details see the Materials and methods section. Arrow 1 indicates the start of Polybuffer elution and arrow 2 elution by Polybuffer containing 500 mM-NaCl. Fractions were assayed for protein (○), pH (-----), 6S activity towards GlcNAc6S-IdoA (●) and towards GlcNAc6S-IdoA2S-anM6S (■).

viously observed that many of the heparan sulphate-degrading enzymes are susceptible to both substrate and product (including sulphate) inhibition [2,8–10]. IdoA2S-GlcNS6S-IdoA2S-anM6S is identical with the tetrasaccharide 11-4NS prepared by Weissmann & Chao [36] by an alternative procedure. Degradation of tetrasaccharide 11-4NS by partially purified bovine liver hydrolases gave the trisaccharide GlcNH6S-IdoA2S-anM6S, which was chemically *N*-acetylated or *N*-sulphated to yield GlcNAc6S-IdoA2S-anM6S and GlcNS6S-IdoA2S-anM6S respectively [31]. Bovine kidney 6S acted towards GlcNS6S-IdoA2S-anM6S with a K_m value of 0.5 mM [37], whereas human liver 6S acted towards the substrate with a K_m value of 0.25 μ M at pH 4.1 [9].

The more efficient turnover of the complex trisaccharide substrates GlcNS6S-IdoA2S-anM6S and GlcNAc6S-IdoA2S-anM6S compared with the structurally simpler substrate GlcNAc6S-IdoA currently used for the detection of MPS IIID patients [14] confirms the influence of aglycone substrate structure upon enzyme activity that we have reported for purified human liver 6S [9] and sulphamate sulphonylhydrolase [2,8]. Whereas purified human liver 6S acted towards GlcNS6S-IdoA2S-anM6S 10-fold more efficiently than towards GlcNAc6S-IdoA2S-anM6S [9], fibroblast 6S activity towards both substrates was observed to be similar. We have previously shown that there is considerable substrate inhibition of purified human liver 6S activity towards GlcNS6S-IdoA2S-anM6S, particularly at substrate concentrations above 5 μ M, which was not observed for activity towards GlcNAc6S-IdoA2S-anM6S [9]. Other heparan sulphate-degrading enzymes present in the fibroblast homogenate may also compete for the binding of the substrates, since it has been shown many enzyme activities including 6S are inhibited by oligosaccharide analogues that are substrates for other heparin-degrading enzyme activities [2,8–11,33].

Both of the urine and fibroblast 6S activities were shown by chromatofocusing to be the summation of many individual forms of activity. Although it is likely that the forms of 6S present in various tissues may have different kinetic parameters, 6S activities towards GlcNAc6S-IdoA2S-anM6S in fibroblasts, leucocytes and urine (when assayed in the presence of BSA) were shown to have similar K_m values and pH optima, and were inhibited by NaCl, Na₂SO₄ and NaH₂PO₄ at concentrations similar to that reported for human liver form A [9]. Each form of fibroblast 6S activity separated by chromatofocusing had similar relative activities towards both GlcNAc6S-IdoA2S-anM6S and GlcNAc6S-IdoA and was also present in liver and/or urine. Human liver form A, form B and form C activities towards each of several heparin-derived disaccharide substrates had similar K_m values; however, the respective k_{cat} values for forms B and C were approximately half that observed for form A [9]. Fibroblast 6S was shown to be composed of two polypeptides with molecular masses of 82 kDa and 78 kDa, which were derived by different carbohydrate processing of the same polypeptide [38]. Human liver form A had a single 78 kDa polypeptide, whereas forms B and C contained 48 kDa and 32 kDa polypeptides. Purified human urine 6S had a single polypeptide of molecular mass 99 kDa [39]. Proteolytic and/or oligosaccharide processing may account for the changes in pI values of the forms separated by chromatofocusing and represent different stages of maturation of the mature enzyme. Processing appears to influence the catalytic event rather than the binding of the substrate to the enzyme (K_m).

We have previously reported both the enhancement of activity and stability of purified human liver 6S, sulphamate sulphonylhydrolase, glucuronate-2-sulphatase and iduronate-2-sulphatase following the addition of BSA to the incubation mixture [2,5,8–10,33]. Human urine 6S activity towards GlcNAc6S-

IdoA2S-anM6S in the absence of BSA was maximal at pH 5.1, which was similar to that reported for human urine 6S activity towards GlcNAc6S-IdoA [14] and GlcNAc6S-GlcA-anM [39]. In the absence of BSA urine 6S activity bound both the disaccharide and trisaccharide substrates with similar affinities, which suggests that at the higher pH the influence of the aglycone IdoA2S residues may be diminished compared with its effect at low pH. Whereas BSA enhanced the V_{max} value of urine 6S activity towards GlcNAc6S-IdoA2S-anM6S at pH 5.1 without alteration of the K_m value, as was observed for purified liver 6S activity towards GlcNAc6S-IdoA at pH 5.0 [9], BSA stimulated urine 6S activity at pH 4.2 by both decreasing the apparent K_m value and increasing the V_{max} value (Table 2). It is possible that BSA may be preferentially stimulating a low- K_m form of urine 6S activity at pH 4.2. Purified rat preputial-gland and partially purified chick-embryo liver β -D-glucuronidases were shown to exist both in a low- K_m form at pH 3.0, which was stimulated by BSA, and a high- K_m form at pH 5.5 [40,41]. BSA may therefore be replacing some unknown activator *in vivo* that may be diluted with the urine or be purified away from the liver enzyme.

To date, of six MPS IIID patients described in the literature, only one has a mild phenotype [42–44]. Although fibroblasts from one patient had no detectable 6S activity towards GlcNAc6S-GlcA-anM, each of the other MPS IIID fibroblasts tested from both the mild and the severely affected patients had similar residual activity of 0.33–0.38 pmol/min/mg protein [42–44]. No significant 6S activity towards GlcNAc6S-IdoA was detected in MPS-IIID-fibroblast homogenates [14], although normal fibroblast 6S activity towards GlcNAc6S-GlcA-anM and GlcNAc6S-IdoA were reported to be similar [14,39]. Each of the MPS IIID cell lines tested had residual 6S activity towards GlcNAc6S-IdoA2S-anM6S and had a pH optimum and K_m values for activity towards GlcNAc6S-IdoA2S-anM6S similar to those of the normal controls, although the V_{max} values were less than 1% of the normal range. Skin fibroblasts from an MPS IIID patient were shown to have normal size and quantity of mRNA encoding for 6S; however, there was a deficiency of 6S-protein-cross-reactive material, which suggests a defective translation or a post-translational instability of the enzyme, which is consistent with the substantially decreased 6S activity in the patient's fibroblasts [38]. However, from each of the studies reported, there does not appear to be any correlation between the clinical phenotype and residual enzyme activity measured in cultured skin-fibroblast homogenates for 6S activity [38,42–44].

In conclusion, we have shown that the series of heparin-derived substrates described in this paper are useful as model oligosaccharides for studying the activities and interactions of the enzymes that degrade heparan sulphate. GlcNAc6S-IdoA2S-anM6S was particularly useful for the detection and characterization of specific 6S residual enzyme in MPS IIID patients' tissues. When used in conjunction with recombinant DNA procedures for the analysis of 6S gene defects, the determination of residual 6S activity may be invaluable for the prediction of the clinical phenotype of MPS IIID patients [45].

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