Diagnosis of Maroteaux–Lamy syndrome by the use of radiolabelled oligosaccharides as substrates for the determination of arylsulphatase B activity

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1. The kinetic parameters (K_m and V) of human arylsulphatase B (4-sulpho-*N*-acetylgalactosamine sulphatase) activity in cultured skin fibroblasts were determined with a variety of substrates matching structural aspects of the physiological substrates *in vivo* chondroitin 4-sulphate and dermatan sulphate. More structurally complex substrates, in which several aspects of the aglycone structure of the natural substrate were maintained, were desulphated up to 4400 times faster than the minimum arylsulphatase-B-specific substrate, namely the monosaccharide *N*-acetylgalactosamine 4-sulphate. Aglycone structures that influence substrate binding and/or enzyme activity were an adjacent-residue C-6 carboxy group and a second but internal *N*-acetylgalactosamine 4-sulphate residue. 2. Arylsulphatase B activity in fibroblast homogenates assayed with *O*-(β -*N*-acetylgalactosamine 4-sulphate)-(1 $\rightarrow$ 4)-*O*-D-(β -glucuronic acid)-(1 $\rightarrow$ 3)-*O*-D-*N*-acetyl[1- 3 H]galactosaminitol 4-sulphate derived from chondroitin 4-sulphate as substrate clearly distinguished Maroteaux–Lamy-syndrome patients from normal controls and other mucopolysaccharidosis patients. We recommend the use of the above trisaccharide substrate for both postnatal and prenatal diagnosis of Maroteaux–Lamy syndrome.

INTRODUCTION

Maroteaux–Lamy syndrome (MPS VI) is an autosomal recessive disorder of mucopolysaccharide catabolism that in its severe form results in dwarfism, gross skeletal changes and cloudy corneas without mental deterioration (McKusick & Neufeld, 1983). The variability of MPS VI clinical phenotypes (mild to severe) causes considerable difficulty in its clinical recognition and diagnosis. The basis of this heterogeneity of clinical phenotype is unknown, but, as suggested for other mucopolysaccharidoses, may result from different allelic mutations. The known enzyme deficiency responsible for both severe and mild forms of MPS VI is the lysosomal enzyme 4-sulpho-*N*-acetylgalactosamine sulphatase [first termed arylsulphatase B (aryl B) before the natural substrate *in vivo* was discovered]. This enzyme is involved in the degradation of dermatan sulphate and chondroitin 4-sulphate. MPS VI patients therefore store and excrete excessive amounts of dermatan sulphate and chondroitin 4-sulphate. We have reported that, compared with the normal, urine from MPS VI patients contains increased (> 450-fold) amounts of the monosaccharides *N*-acetylgalactosamine 4-sulphate (GalNAc4S) and *N*-acetylgalactosamine 4,6-disulphate (Hopwood & Elliott, 1985). These observations suggest that 4-sulphated *N*-acetylgalactosamines are also substrates *in vivo* for human aryl B.

The exact diagnosis of MPS VI is based on the demonstration of a gross deficiency of aryl B in, for example, the patient's cultured skin fibroblasts or leucocytes. The importance of the correct diagnosis lies in the hereditary nature of these disorders, and their early recognition allows appropriate family counselling and prenatal diagnosis. The most favoured procedures for assay of aryl B activity utilize one of two readily available synthetic substrates (nitrocatechol sulphate or 4-methylumbelliferyl sulphate) by using either a combination of inhibitors of other arylsulphatase (mainly type A) activities that also desulphate these substrates (Baum *et al.*, 1959) or fractionation of the various arylsulphatase activities by ion-exchange chromatography or electrophoresis (Ratazzi *et al.*, 1973; Fluharty *et al.*, 1974; Schwartz *et al.*, 1980; Hopwood *et al.*, 1982).

There are now several described variations of other lysosomal enzyme deficiencies in which enzyme activity against commonly used substrates (such as 4-methylumbelliferyl derivatives) does not correspond to activity against substrates that closely conform to substrates *in vivo*, and errors in diagnosis have thereby arisen (Tallman *et al.*, 1974; Owada *et al.*, 1977; O'Brien *et al.*, 1977). For the greatest reliability it is therefore important (as in prenatal diagnosis) to use substrates that match as closely as possible the structures of the substrates *in vivo*. We have previously shown that the structure of the residue adjacent to the group under enzymic attack plays an

Abbreviations used: GalNAc4S, *N*-acetylgalactosamine 4-[35 S]sulphate; GalitolNAc4S, *N*-acetyl[1- 3 H]galactosaminitol 4-sulphate; GalNAc6S, *N*-acetyl[1- 14 C]galactosamine 6-sulphate; GalNAc4S-Ido, *O*-(β -*N*-acetylgalactosamine 4-sulphate)-(1 $\rightarrow$ 4)-L-[6- 3 H]idose; GalNAc4S-GlcA-GalitolNAc4S, *O*-(β -*N*-acetylgalactosamine 4-sulphate)-(1 $\rightarrow$ 4)-*O*-D-(β -glucuronic acid)-(1 $\rightarrow$ 3)-*O*-D-*N*-acetyl[1- 3 H]galactosaminitol 4-sulphate; GalNAc4S-Glc-GalitolNAc4S, *O*-(β -*N*-acetylgalactosamine 4-sulphate)-(1 $\rightarrow$ 4)-*O*-D-(β -glucose)-(1 $\rightarrow$ 3)-*O*-D-*N*-acetyl[1- 3 H]galactosaminitol 4-sulphate; GalNAc4S-GlcA-GalNAc4S-GlcA-GalitolNAc4S, *O*-(β -*N*-acetylgalactosamine 4-sulphate)-(1 $\rightarrow$ 4)-*O*-D-(β -glucuronic acid)-(1 $\rightarrow$ 3)-*O*-D-(β -*N*-acetylgalactosamine 4-sulphate)-(1 $\rightarrow$ 4)-*O*-D-(β -glucuronic acid)-(1 $\rightarrow$ 3)-*O*-D-*N*-acetyl[1- 3 H]galactosaminitol 4-sulphate; MPS VI, mucopolysaccharidosis type VI (Maroteaux–Lamy syndrome); aryl B, arylsulphatase B, or 4-sulpho-*N*-acetylgalactosamine sulphatase.

Fig. 1. Structures of aryl B substrates used in this study

important role in the enzymic degradation of mucopolysaccharides (Hopwood & Muller, 1979, 1982; Muller & Hopwood, 1984; Clements *et al.*, 1985; Freeman & Hopwood, 1986). The detection and assessment of residual mutant enzyme activity in many mucopolysaccharidoses is likely to be successful if the diagnostic substrates match the structure of the substrates used *in vivo* (Hopwood & Muller, 1979; Muller & Hopwood, 1984).

Oligosaccharides prepared from chondroitin 4-sulphate have been used to assay specifically aryl B activity in cultured skin fibroblasts (Matalon *et al.*, 1974; Habuchi *et al.*, 1979; Fluharty, 1981; Hopwood & Elliott, 1985). We have extended these studies by evaluating a series of oligosaccharides, derived from chondroitin 4-sulphate and dermatan sulphate, and the monosaccharide GalNAc4S as substrates for the assessment of aryl B activity in cultured human skin fibroblasts. We also

report the use of a radiolabelled trisaccharide isolated from chondroitin 4-sulphate as a diagnostic substrate for the prenatal and postnatal detection of Maroteaux-Lamy syndrome.

MATERIALS AND METHODS

Preparation of radiolabelled substrates

GalNAc4[^{35}S]S was prepared by mild acid hydrolysis of UDP-GalNAc4[^{35}S]S prepared from UDP-GalNAc and 3'-phosphoadenylyl [^{35}S]sulphate by the procedure of Tsuji *et al.* (1970). Radiolabelled *N*-acetylgalactosaminitol 4-sulphate, GalNAc4S-GlcA-GalitolNAc4S, GalNAc4S-Glc-GalitolNAc4S (Fig. 1) and GalNAc6S were prepared as previously described (Hopwood & Elliott, 1983, 1985; Elliott & Hopwood, 1984). GalNAc4S-GlcA-GalitolNAc4S was prepared by β -glucuronidase digestion of the hexasaccharide fraction produced in the same preparation used to yield GalNAc4S-GlcA-GalitolNAc4S from the tetrasaccharide (Hopwood & Elliott, 1983, 1985). The pentasaccharide was subjected to the same preparative descending paper chromatography and high-voltage electrophoresis as described for the trisaccharide above. Particular care was taken with the descending-chromatography step described below, which was included to separate GalNAc4S and GalNAc6S trisaccharide species. The single peak of radioactivity obtained in this step was cut into eight segments and the radiolabelled material eluted from each was evaluated as substrates in standard (see 'Enzyme assays' below) incubations containing homogenates of MPS VI fibroblasts. Fractions with very low (less than 0.1 pmol/min per mg of cell protein) activity were pooled and used as a substrate for the specific assessment of aryl B activity. It was not possible to lower aryl B activity assessed with the pentasaccharide substrate to below 1 pmol/min per mg of cell protein with homogenates of MPS VI fibroblasts.

GalNAc4S-Ido (Fig. 1) was prepared from dermatan sulphate (Hopwood & Muller, 1983). Carboxy reduction of dermatan sulphate (10 mg) was performed by mixing with 30 mg of 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide hydrochloride in 1.7 ml of water at 25 °C maintained at pH 4.75 with 0.1 M-HCl by a Radiometer Tritrator II. The reaction consumed approx. 0.2 ml of 0.1 M-HCl during 45 min; the end point was indicated by no further consumption of acid. The reaction mixture was dialysed at 4 °C against two changes of water (2 litres) over a 3 h period and rotary-evaporated to dryness at 30 °C, and the total volume was adjusted to 0.1 ml with water. $NaBH_4$ (7 mg) and 100 mCi of NaB^3H_4 (9.8 Ci/mmol) were added, and the mixture was heated at 50 °C for 90 min, cooled to 25 °C and acidified with acetic acid in a fume-hood to release tritium gas. The reaction mixture was dialysed successively against four changes of water (2 litres) and three changes of 0.2 M-NaCl over 3 days at 4 °C, then applied to a column (1 cm $\times$ 100 cm) of Sephadex G-10, and eluted with 10% (v/v) ethanol at 25 °C. As we have reported for similarly treated heparin, the uronic acid-containing fractions and radiolabelled material, all of which was eluted in the void volume, were combined and freeze-dried, and the residue hydrolysed with 0.1 M-HCl at 100 °C for 1 h (Hopwood & Elliott, 1981a). The hydrolysate was neutralized with 1 M-NaOH, applied to a column (1 cm $\times$ 200 cm) of

Scheme 1. Proposed pathways for exo-degradation of β -linked 4-sulphated *N*-acetylgalactosamine residues of the trisaccharide substrate GalNAc4S-GlcA-GalitolNAc4S derived from chondroitin 4-sulphate

Sephadex G-25 and eluted with 0.2 M-NaCl in 0.05 M-sodium acetate buffer, pH 5.3, as previously described (Hopwood & Elliott, 1981a). Radiolabelled material eluted in the same position as a disaccharide standard (iduronosylanhidromannitol 6-sulphate; Muller & Hopwood, 1984) was freeze-dried, dissolved in water (100 μ l) and subjected to preparative high-voltage electrophoresis at pH 1.7 (Hopwood & Elliott, 1983). A single peak of radioactive material was present, with the same mobility as iduronosylanhidromannitol 6-sulphate. The extremely acid-labile 2-*O*-sulpho-*L*-idopyranosidic bonds (Shively & Conrad, 1976; Hopwood & Elliott, 1981a), compared with the relative stability of other glycosidic bonds, dictates the production of disaccharides from the repeating sequence of 2-*O*-sulpho-*L*-idopyranosyl-disaccharide in carboxy-group-reduced dermatan sulphate. It is likely that the 2-*O*-sulphate ester is also hydrolysed under these conditions to yield a mixture of GalNAc4S-idose and GalNAc4S-(1 $\rightarrow$ 6)anhydroidose disaccharides. We have not attempted to identify the relative proportions of these two components in the disaccharide fraction, and have tentatively labelled this disaccharide fraction as GalNAc4S-Ido (Fig. 1). We were unable to detect uronic acid (Hopwood & Elliott, 1981a) in this disaccharide fraction.

The specific radioactivities of the radiolabelled substrates GalNAc4S and GalNAc4S-Ido were approx. 2 and 450 Ci/mol of hexosamine (Hopwood & Elliott, 1985) respectively. The penta- and tri-saccharide substrates derived from chondroitin 4-sulphate all had a specific radioactivity of 90 Ci/mol.

Enzyme preparation

Fibroblast cultures were established from skin biopsies taken in or provided to this hospital (Hopwood *et al.*, 1982). Patients classified as having MPS VI excreted excessive amounts of dermatan sulphate in their urine (Hopwood & Harrison, 1982), had normal activities of other mucopolysaccharidosis enzymes and were deficient in aryl B activity when cultured fibroblasts were assayed with GalNAc4S-GlcA-GalitolNAc4S or after electrophoretic separation of arylsulphatase isoenzymes by the

method described by Ratazzi *et al.* (1973). Typing of other mucopolysaccharidosis patients was based on the extent and type of glycosaminoglycanuria, clinical phenotype and nature of lysosomal-enzyme deficiency (Hopwood & Harrison, 1982; Hopwood *et al.*, 1982).

Cultured amniotic cells and peripheral-blood leucocytes were isolated as previously described (Carey & Pollard, 1977). All cells were suspended in aqueous Triton X-100 (1 g/l) to a concentration of 5–10 g of protein/l and were disrupted by rapid freezing and thawing six times in a solid- CO_2 /ethanol mixture.

Enzyme assays

Incubation mixtures contained 8–15 μ g of protein from cultured fibroblast homogenates in 50 mM-sodium formate buffer, pH 4.0, containing 4 mM- NaN_3 ,

Fig. 2. Separation by electrophoresis of an incubation mixture containing normal fibroblast homogenate and GalNAc4S-GlcA-GalitolNAc4S incubated in 50 mM-sodium formate buffer, pH 4.0 (—), or 50 mM-sodium acetate buffer, pH 4.0, containing 2-acetamido-2-deoxy-D-gluconolactone (5 mM) (----)

Table 1. Aryl B activity (towards the substrates shown in Fig. 1) in cultured-skin-fibroblast homogenates from MPS-IVA (*N*-acetylglactosamine 6-sulphate sulphatase-deficient) and MPS-VI (severe clinical phenotype) patients and an unrelated normal person

Key: n.d., none detected; —, not done.

<i>β</i> - <i>N</i> -Acetylhexosaminidase inhibitor*...	Aryl B activity (pmol/min per mg of protein)					
	MPS IVA		MPS VI		Normal	
	—	+	—	+	—	+
GalNAc4S-GlcA-GalNAc4S-GlcA-GalitolNAc4S	15.1	13.3	1.10	0.64	12.8	9.8
GalNAc4S-GlcA-GalitolNAc4S	4.1	5.0	0.03	n.d.	3.6	3.4
GalNAc4S-Glc-GalitolNAc4S	1.7	1.6	0.05	n.d.	1.2	1.4
GalNAc4S-Ido	—	—	0.004	n.d.	0.009	0.006
GalNAc4S	0.015	—	n.d.	—	0.012	0.008
GalNAc6S	n.d.	—	0.08	—	0.06	—

* 2-Acetoamido-2-deoxy-D-gluconolactone (5 mM).

Table 2. K_m and V values for aryl B activity in cultured-skin-fibroblast homogenates towards oligosaccharides and a monosaccharide substrate

All incubations were at pH 4.0 in 50 mM-sodium acetate buffer containing *β*-*N*-acetylhexosaminidase inhibitor (5 mM).

Substrate	K_m (μ M)	V (pmol/min per mg of protein)
GalNAc4S-GlcA-GalNAc4S-GlcA-GalitolNAc4S	86	134
GalNAc4S-GlcA-GalitolNAc4S	100	30
GalNAc4S-Glc-GalitolNAc4S	48	7
GalNAc4S-Ido	23	0.05
GalNAc4S	11	0.03

5 mM-D-saccharic acid 1,4-lactone and approx. 500 pmol of substrate in a final volume of 20 μ l in a sealed polypropylene tube (Hopwood & Elliott, 1981a). Incubation time, at 37 °C (or 45 °C where indicated), was variable and dependent on extent of hydrolysis; for example, fibroblast homogenates from normal individuals were incubated with GalNAc4S-GlcA-GalitolNAc4S for 8 h to yield approx. 10% product, whereas incubation with GalNAc4S for 24 h was required to yield 0.3% product. At the end of the incubation period, products and residual substrate were separated by high-voltage electrophoresis at pH 1.7 as described below. Incubation mixtures not immediately subjected to electrophoresis were stored at -20 °C. Sulphatase activity was expressed as pmol of product (Product 1+2+3, Scheme 1 and Fig. 2) produced/min per mg of protein. This assumes that Product 2 (Scheme 1) is not directly produced by the action of *β*-*N*-acetylhexosaminidase (see the Discussion section).

General methods

High-voltage electrophoresis to separate reaction products from substrates in all reaction mixtures was performed on Whatman 3MM chromatography paper in 1.74 M-formic acid, pH 1.7, at 42 V/cm for 60 min in a Shandon Southern model L-24 System (Shandon Southern Products, Runcorn, Cheshire, U.K.). Descending chromatograms were run on Whatman 3MM paper

in a solvent mixture of ethyl acetate/acetic acid/water (4:3:3, by vol.). Each sample strip was either cut into 1 cm segments or scanned on a Packard model 7201 radiochromatogram scanner, and the radioactivities of the located product and substrate regions were counted in an ISOCAP 300 liquid-scintillation system (Nuclear-Chicago) as described previously (Hopwood, 1979). Protein was determined by the Folin method of Lowry *et al.* (1951), with bovine serum albumin as standard.

RESULTS

Fibroblast aryl B activity was measured at pH 4.0 with the substrates shown in Fig. 1. Desulphation of these substrates by aryl B yields unlabelled sulphate (except for GalNAc4S) and the corresponding tritiated oligosaccharide with decreased mobility on high-voltage electrophoresis. Hydrolysis of GalNAc4S-GlcA-GalitolNAc4S produced three products (Fig. 2). Product 1 results from the action of aryl B. Product 2 results from the action of *β*-*N*-acetylhexosaminidase either directly on the β -glycosidic linkage of the GalNAc4S residue or on the GalNAc residue in the product of aryl B activity (Scheme 1). We have previously shown that the addition of *β*-*N*-acetylhexosaminidase inhibitor (2-acetoamido-2-deoxy-D-gluconolactone) and incubation in 50 mM-sodium acetate buffer, pH 4.0, results in the complete inhibition of *β*-*N*-acetylhexosaminidase activity towards

Fig. 3. Hydrolysis by aryl B activity in fibroblast homogenate of GalNAc4S-GlcA-GalitolNAc4S as a function of incubation pH

Incubations were for 5 h at 37 °C and the incubation media contained 15 μ M substrate and a total of 20 μ g of cell protein. Percentage conversions into Product 2 (Scheme 1) in 50 mM-sodium formate ($\blacktriangle$), 50 mM-sodium acetate ($\triangle$) and 50 mM-sodium dimethylglutarate ($\blacksquare$) buffers and into Products 1 and 2 (Scheme 1) in 50 mM-sodium formate ($\bullet$), 50 mM-sodium acetate ($\circ$) and 50 mM-sodium dimethylglutarate ($\square$) buffers are shown.

these types of substrates (Hopwood & Elliott, 1983, 1985). Further degradation of β -N-acetylhexosaminidase Product 2 (i.e. GlcA-GalitolNAc4S) to Product 3 (i.e. N-acetylgalactosaminitol-sulphate 4) was inhibited by the addition (5 mM) of the β -glucuronidase inhibitors D-saccharic acid 1,4-lactone to these incubation mixtures (Scheme 1 and Fig. 2). Results recorded in Table 1 suggest that GalNAc4S-GlcA-GalitolNAc4S, GalNAc4S-GlcA-GalitolNAc4S and GalNAc4S-Glc-GalitolNAc4S are desulphated by the same enzyme at a considerably higher rate than observed for GalNAc4S-Ido and GalNAc4S. MPS-VI-fibroblast homogenates incubated in the presence of an inhibitor of β -N-acetylhexosaminidase with all substrates except the pentasaccharide were unable to produce detectable aryl B activity. In the absence of β -N-acetylhexosaminidase inhibitor, small amounts of Product 2 were produced from incubations of MPS-VI-fibroblast homogenates containing GalNAc4S-GlcA-GalitolNAc4S and GalNAc4S-Glc-GalitolNAc4S. Although the amount of hydrolysis was low, it appeared that the amounts of product from incubations of GalNAc4S-Ido with normal and MPS-VI-fibroblast homogenates were significantly decreased when incubation mixtures contained the β -N-acetylhexosaminidase inhibitor. The monosaccharide GalNAc6S was desulphated after incubation with MPS-VI-fibroblast or normal-fibroblast homogenates. Hydrolysis of GalNAc6S was not detected in incubations with MPS-IVA (N-acetylgalactosamine 6-sulphate sulphatase-deficient)-fibroblast homogenates.

Apparent K_m and V values measured in normal-fibroblast homogenates and recorded in Table 2 were obtained from Lineweaver-Burk plots. Some comparison of binding and catalysis of various substrates can be made from the data. Desulphation of GalitolNAc4S by fibroblast homogenates was not detected. The most

effective substrate with the highest V value was the pentasaccharide. However, as separation of products from substrate was difficult and high residual activity in MPS-VI-fibroblast homogenates was obtained, the trisaccharide GalNAc4S-GlcA-GalitolNAc4S was evaluated as a diagnostic substrate for the detection of MPS-VI patients. The effect of incubation pH on fibroblast hydrolysis of GalNAc4S-GlcA-GalitolNAc4S is complex and is recorded in Fig. 3. The pH optimum of aryl B activity was 3.5–4.0 in formate buffer. Hydrolysis of the trisaccharide substrate in formate buffer produced two products at pH 4.0, but virtually only one at pH 3.5. Incubation in acetate buffer at pH 4.0 caused almost complete inhibition of β -N-acetylhexosaminidase activity towards this substrate and diminished the overall aryl B activity at this incubation pH. It was possible to decrease β -N-acetylhexosaminidase activity to very low values without a large decrease in aryl B activity by incubation of the trisaccharide substrate in sodium formate buffer, pH 3.5 (Fig. 3). Aryl B activity in normal-fibroblast homogenates increased linearly with time up to 24 h at 37 °C at low protein concentrations (approx. 5 μ g total in an incubation volume of 20 μ l), as shown by the linear increase in Product 1 as well as Product 2 (Fig. 4). Activity was not linear with protein over the concentration range studied, mainly owing to the non-linearity with time of the formation of Product 1 (Fig. 5). Incubations at 45 °C gave higher aryl B activities than were observed at 37 °C, but the conversion into product was not linear with time (Fig. 4). Dialysis of fibroblast homogenates against 50 mM-sodium formate buffer, pH 4.0, for 16 h at 4 °C resulted in an up to 2-fold increase in aryl B activity. Sulphatase activity in cell homogenates measured with GalNAc4S-GlcA-GalitolNAc4S was slightly stimulated by the addition of NaCl up to 20 mM. At higher NaCl concentrations enzyme activity was inhibited (Fig. 6). Enzyme activity was inhibited by 40% and 50% by 1 mM- Na_2SO_4 and -phosphate respectively.

Fig. 4. Hydrolysis of 20 μ M-GalNAc4S-GlcA-GalitolNAc4S by cultured-skin-fibroblast homogenate as a function of incubation time in 50 mM-sodium formate buffer, pH 4.0

Percentage conversions into Product 1 ($\circ$) and Product 2 ($\triangle$) at an incubation temperature of 37 °C and into Product 1 ($\bullet$) and Product 2 ($\blacktriangle$) at an incubation temperature of 45 °C are shown. Products 1 and 2 are shown in Scheme 1.

Fig. 5. Hydrolysis of 20 μ M-GalNAc4S-GlcA-GalitolNAc4S by cultured-skin-fibroblast homogenates as a function of protein concentration

Percentage conversions into Products 1 and 2 (●), Product 1 (○) and Product 2 (△) in 50 mM-sodium formate buffer, pH 4.0, after 8 h at 37 °C are shown. Percentage conversion into Product 1 in 50 mM-sodium acetate buffer, pH 4.0, containing 2-acetoamido-2-deoxy-D-gluconolactone (5 mM) after 8 h at 37 °C (▲) is also shown.

Fig. 6. Hydrolysis by cultured-skin-fibroblast homogenates of GalNAc4S-GlcA-GalitolNAc4S as a function of NaCl concentration

Results are expressed as percentage conversions into Product 1 (○) and Product 2 (△).

The activity of aryl B (assessed by the method described under 'Enzyme assays') towards GalNAc4S-GlcA-GalitolNAc4S in cells from a group of individuals at risk for arylsulphatase B deficiency is shown in Table 3. These results demonstrate that the defect in MPS VI, previously defined by combination of clinical phenotype, dermatan sulphaturia and an absence of aryl B activity (Ratazzi *et al.*, 1973), is a deficiency of a single enzyme activity towards the chondroitin 4-sulphate-derived

Table 3. Aryl B activity of leucocyte and cultured-skin-fibroblast homogenates from MPS-VI patients, obligate heterozygotes and other mucopolysaccharidosis types and unrelated normal subjects towards GalNAc4S-GlcA-GalitolNAc4S

Key: n, number of patients; n.d., not detected; —, not done.

	Aryl B activity (pmol/min per mg of protein)	
	Leucocytes	Fibroblasts
MPS VI		
Range	n.d.	n.d.-0.12 (n = 6)
Median value		0.05
Obligate heterozygotes		
Range	—	0.3-5.6 (n = 10)
Median value		1.3
Normal subjects		
Range	0.4-0.9 (n = 6)	1.3-6.9 (n = 13)
Median value	0.6	2.8
Other mucopolysaccharidoses		
MPS I	0.8	5.3
MPS II	—	4.0
MPS IIIA	—	2.6
MPS IIID	—	1.9
MPS IVA	—	3.2

substrate. Fibroblasts from ten MPS-VI obligate heterozygotes gave values generally below normal values, although several heterozygote values were clearly in the normal range. Fibroblasts from patients with other mucopolysaccharidosis disorders gave values within the normal range, whereas enzyme activities for fibroblast homogenates from six clinically severe patients were all well below the lowest heterozygote value observed. Fibroblasts from a clinically mild MPS-VI patient were assayed for aryl B activity against GalNAc4S-GlcA-GalitolNAc4S under non-standard conditions in 50 mM-sodium acetate buffer, pH 4.0, containing 5 mM- β -N-acetylhexosaminidase inhibitor. Aryl B activities in fibroblasts from a normal control, an MPS-VI severe patient and an MPS-VI mild-phenotype patient were 9.2, none detected and 0.16 pmol/min per mg of protein respectively. Thus under these assay conditions this substrate demonstrated significant residual activity in this MPS-VI mild case compared with the undetectable activity in the MPS-VI severe case.

Five prenatal assessments of aryl B activity were carried out for two families with the standard assay with GalNAc4S-GlcA-GalitolNAc4S as substrate (Table 4). Fibroblasts cultured from the two index cases gave values between 0.02 and 0.16 pmol/min per mg of protein and, except for prenatal assessment 2, the test amniotic cells gave higher values that were clearly within the range obtained from the heterozygote or normal controls. Electrophoretic separation and fluorogenic identification of arylsulphatase activities in these preparations by the method of Ratazzi *et al.* (1973) gave results consistent with the values reported in Table 4. On the basis of these results pregnancies 1, 3, 4 and 5 were allowed to go to

Table 4. Prenatal assessment of aryl B activity in pregnancies at risk for MPS VI

Aryl B activities shown are averages for at least three determinations. Normal cell controls were different cell lines for each prenatal assessment.

Pregnancy no. ...	Family A		Family B		
	1	2	3	4	5
Skin fibroblasts					
Mother	5.6	0.86	0.38	0.39	0.70
Father	3.9	2.6	0.36	0.22	0.67
Propositus	0.04	0.16	0.02	0.02	0.09
Normal control 1	5.8	2.1	2.1	1.69	3.6
Normal control 2	7.3	2.7	1.7	1.00	1.7
Amniotic cells					
Foetus at risk	0.76	0.06	0.35	0.29	0.63
Normal control 1	2.2	2.9	3.4	1.26	0.65
Normal control 2	2.2	3.6	2.7	1.65	0.70

term and pregnancy 2 was terminated. All prenatal assessments were confirmed on follow-up samples of cord blood, urine or skin fibroblasts from pregnancies 1 and 3–5 and cultured skin fibroblasts from the abortus of pregnancy 2.

DISCUSSION

A major conclusion from the kinetic data (Table 2) is that the aglycone structure attached to GalNAc4S considerably influences the catalytic efficiency of fibroblast homogenate aryl B. The observed V value increases with increasing chain length of substrate, and this is consistent with the results reported by Habuchi *et al.* (1979) for rat skin aryl B. The GalNAc4S residue in the pentasaccharide was desulphated more than 4000-fold faster than was the monosaccharide GalNAc4S.

Similar findings have been reported for the different sulphatase (*N*-acetylgalactosamine 6-sulphate sulphatase) involved in the desulphation of oligosaccharides and a monosaccharide derived from chondroitin 6-sulphate (Elliott & Hopwood, 1984; Hopwood & Elliott, 1985). The non-reducing end sulphate ester in GalNAc6S-GlcA-GalitolNAc6S was hydrolysed approx. 1000-fold faster than was the monosaccharide GalNAc6S.

The presence of an adjacent-residue C-6 carboxy group and a sulphate ester group two residues removed from the GalNAc4S appears to be responsible for this increased aryl B activity. Substrates without the second GalNAc4S residue have lower K_m values, suggesting tighter binding to the enzyme of these structures, compared with the trisaccharides. The lack of detectable activity for GalitolNAc4S suggests that an intact monosaccharide ring structure is required for aryl B activity. Activity towards GalNAc6S by MPS-VI fibroblasts, but not by MPS-IVA fibroblasts, suggests that aryl B activity specifically attacks the 4-sulphate ester bond.

We have previously reported similar findings for other lysosomal-enzyme activities towards substrates derived from sulphated glycosaminoglycans. Sulphamate sulphohydrolase activity is favoured by adjacent monosaccharide residues with a C-6 carboxy group and a post-penultimate monosaccharide sulphamate group (Hopwood & Elliott,

1981b; Freeman & Hopwood, 1986). Adjacent-residue C-6 carboxy and sulphamate groups in oligosaccharides appear, compared with the monosaccharide substrate, to decrease considerably the affinity of substrate to enzyme although at the same time increasing catalytic efficiency many thousandfold. Similarly, the presence of a C-6 carboxy group on the monosaccharide adjacent to the one under enzymic attack increases *N*-acetylglucosamine 6-sulphate sulphatase, *N*-acetylgalactosamine 6-sulphate sulphatase and α -*N*-acetylglucosaminidase activities towards their substrates (Hopwood & Elliott, 1981a, 1982, 1983; Elliott & Hopwood, 1984). Thus the aglycone structure of substrates for aryl B, as for other lysosomal enzymes involved in the hydrolysis of sulphated glycosaminoglycans, has considerable influence on the catalytic efficiency. This observation is important if studies of the residual or mutant enzyme activity that may be present in cultured fibroblasts from some patients are to allow biochemical discrimination between the many different clinical phenotypes that have been observed for MPS VI.

The structure present in aryl B substrates that appears to confer specificity of attack is GalNAc4S. All structures that contain this minimum structural requirement are specifically desulphated by aryl B. This is shown by the absence of detectable sulphatase or considerably decreased activity toward these substrates in MPS-VI-fibroblast homogenates shown by other means to be deficient in aryl B activity. Synthetic substrates such as 4-methylumbelliferyl sulphate and nitrocatechol sulphate are hydrolysed by other sulphatases as well as aryl B, and even under conditions designed to decrease or inhibit hydrolysis by other sulphatases MPS-VI fibroblasts show up to 20% of normal control activities (Fluharty, 1981).

There are two major problems associated with using GalNAc4S substrates to measure residual aryl B activity that may be present in MPS-VI patients. Under the conditions used to assess aryl B activity towards GalNAc4S-GlcA-GalitolNAc4S, *N*-acetylgalactosamine 6-sulphate sulphatase activity (which is normal in MPS-VI fibroblasts) is 10-fold more efficient at desulphation of GalNAc6S-GlcA-GalitolNAc6S. Thus small amounts of non-reducing-end GalNAc6S residues in the diagnostic substrate would be completely desulphated under the extended conditions used to detect residual aryl B. We suspect that the differences in aryl B activity observed in fibroblasts from the propositus in family A as determined in assessments 1 and 2 (Table 4) may have resulted from the presence of trace amounts of GalNAc6S residues in the substrate preparation used in assessment 2. A second source of apparent residual activity may result from the action of β -*N*-acetylhexosaminidase on the β -glucosidic bond of the GalNAc4S residue to yield the radiolabelled GlcA-GalitolNAc4S. This product may mistakenly be considered to have resulted from the sequential action of aryl B and β -*N*-acetylhexosaminidase activities (Hopwood & Elliott, 1985). Any apparent contribution to residual aryl B activity via this pathway is probably eliminated by incubation under conditions that inhibit β -*N*-acetylhexosaminidase activity towards these substrates (Table 1; Hopwood & Elliott, 1985).

We conclude that, under the conditions defined in the study, radiolabelled GalNAc4S-GlcA-GalitolNAc4S isolated from chondroitin 4-sulphate is an effective and highly specific substrate for aryl B and may be used to diagnose MPS VI. Care to remove small amounts of

trisaccharides containing GalNAc6S residues from substrate preparations and choice of incubation conditions to inhibit β -N-acetylhexosaminidase activity make GalNAc4S-GlcA-GalitolNAc4S an important substrate for the prenatal and postnatal detection of aryl B deficiencies. Further work is required to investigate the possibility that some MPS-VI clinical variants may produce significant, but very low, residual activity with the trisaccharide substrate and thereby provide a means of biochemical classification of different MPS-VI phenotypes, as we have already reported for a group of MPS-I patients (Hopwood & Muller, 1979; Muller & Hopwood, 1984).

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