Biochem. J. (1990) 271, 75–86 (Printed in Great Britain)

Human liver iduronate-2-sulphatase

Purification, characterization and catalytic properties

Julie BIELICKI, Craig FREEMAN, Peter R. CLEMENTS and John J. HOPWOOD*

Lysosomal Diseases Research Unit, Department of Chemical Pathology, Adelaide Medical Centre for Women and Children, King William Road, North Adelaide, South Australia 5006, Australia

Human iduronate-2-sulphatase (EC 3.1.6.13), which is involved in the lysosomal degradation of the glycosaminoglycans heparan sulphate and dermatan sulphate, was purified more than 500000-fold in 5% yield from liver with a six-step column procedure, which consisted of a concanavalin A–Sepharose–Blue A–agarose coupled step, chromatofocusing, gel filtration on TSK HW 50S-Fractogel, hydrophobic separation on phenyl-Sepharose CL-4B and size separation on TSK G3000SW Ultrapac. Two major forms were identified. Form A and form B, with pI values of 4.5 and < 4.0 respectively, separated at the chromatofocusing step in approximately equal amounts of recovered enzyme activity. By gel-filtration methods form A had a native molecular mass in the range 42–65 kDa. When analysed by SDS/PAGE, dithioerythritol-reduced and non-reduced form A and form B consistently contained polypeptides of molecular masses 42 kDa and 14 kDa. Iduronate-2-sulphatase was purified from human kidney, placenta and lung, and form A was shown to have similar native molecular mass and subunit components to those observed for liver enzyme. Both forms of liver iduronate-2-sulphatase were active towards a variety of substrates derived from heparin and dermatan sulphate. Kinetic parameters (K_m and $k_{cat.}$) of form A were determined with a variety of substrates matching structural aspects of the physiological substrates *in vivo*, namely heparan sulphate, heparin and dermatan sulphate. Substrates with 6-sulphate esters on the aglycone residue adjacent to the iduronic acid 2-sulphate residue being attacked were hydrolysed with catalytic efficiencies up to 200 times above that observed for the simplest disaccharide substrate without a 6-sulphated aglycone residue. The effect of incubation pH on enzyme activity towards the variety of substrates evaluated was complex and dependent on substrate aglycone structure, substrate concentration, buffer type and the presence of other proteins. Sulphate and phosphate ions and a number of substrate and product analogues were potent inhibitors of form A and form B enzyme activities.

INTRODUCTION

The sulphated glycosaminoglycans heparan sulphate and dermatan sulphate are degraded from their non-reducing termini by the sequential action of highly specific lysosomal sulphatases and other exoenzyme activities (McKusick & Neufeld, 1983; Neufeld & Muenzer, 1989; Hopwood, 1989). In the group of disorders referred to as the mucopolysaccharidoses, the Hunter syndrome (mucopolysaccharidosis type II) is characterized by a deficiency of one of these enzymes, iduronate-2-sulphatase (McKusick & Neufeld, 1983). The clinical symptoms of patients with this disorder, which is inherited as an X-chromosome-linked recessive trait, range from mild to severe and include skeletal dysmorphism, coarse facies, hepatosplenomegaly, cardiovascular problems and frequently, though not always, mental retardation. These symptoms are secondary to the primary defect, which is the deficiency of iduronate-2-sulphatase, and are the result of accumulation in various tissues of excessive amounts of partially degraded heparan sulphate and dermatan sulphate fragments. Attempts at purifying this enzyme from various human tissues and bodily fluids have been only partially successful (Cantz *et al.*, 1972; Di Natale & Ronsisvalle, 1981; Archer *et al.*, 1982; Yutaka *et al.*, 1982; Wasteson & Neufeld, 1982; Lissens *et al.*, 1984). The most recent purification has been from human placenta (Di Natale & Daniele, 1985), where the enzyme was reported purified 35000-fold to homogeneity. In the present paper we describe the 500000-fold purification of iduronate-2-sulphatase from human liver, lung, kidney and placenta, together with some of the enzyme's kinetic and physical properties.

MATERIALS AND METHODS

Materials

Human livers, lungs and kidneys obtained from autopsies of normal adults with times *post mortem* ranging from 6 to 72 h, and human placentas obtained from normal live births, were stored at −20 °C. Concanavalin A–Sepharose, PBE 94 chromatofocusing medium, Polybuffer 74, phenyl-Sepharose CL-4B, Sephacryl S-300 and Superose 12, as well as the molecular-mass standard kits for SDS/PAGE and gel chromatography, were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden). E. Merck (Darmstadt, Germany) supplied TSK HW

Abbreviations used: IdoA2S-anM6S, L-*O*-(α-iduronic acid 2-sulphate)-(1→4)-D-*O*-2,5-anhydro[1-^{3}H]mannitol 6-sulphate; IdoA2S-anM, L-*O*-(α-iduronic acid 2-sulphate)-(1→4)-D-*O*-2,5-anhydro[1-^{3}H]mannitol; IdoA2S-anT4S, L-*O*-(α-iduronic acid 2-sulphate)-(1→3)-D-*O*-2,5-anhydro[1-^{3}H]-talitol 4-sulphate; Ido2S-anM6S, L-*O*-(α-idose 2-sulphate)-(1→4)-D-*O*-2,5-anhydro[1-^{3}H]mannitol 6-sulphate; GlcA2S-anM6S, D-*O*-(β-glucuronic acid 2-sulphate)-(1→4)-D-*O*-2,5-anhydro[1-^{3}H]mannitol 6-sulphate; IdoA2S-GlcNS-UA-GlcNAc-GlcOA, L-*O*-(α-iduronic acid 2-sulphate)-(1→4)-D-*O*-(α-2-sulphaminoglucosamine)-(1→4)-*O*-(β-D-glucuronic or α-L-iduronic acid)-(1→4)-D-*O*-(α-*N*-acetylglucosamine)-(1→3)-D-*O*-[6-^{3}H]gulonic acid; IdoA2S-GlcNS6S-IdoA2S-anM6S, L-*O*-(α-iduronic acid 2-sulphate)-(1→4)-D-*O*-(α-2-sulphaminoglucosamine 6-sulphate)-L-*O*-(α-iduronic acid 2-sulphate)-(1→4)-D-*O*-2,5-anhydro[1-^{3}H]mannitol 6-sulphate; IdoA2S-GlcNAc6S-IdoA2S-anM6S, L-*O*-(α-iduronic acid 2-sulphate)-(1→4)-D-*O*-(α-*N*-acetylglucosamine 6-sulphate)-(1→4)-L-*O*-(α-iduronic acid 2-sulphate)-D-*O*-2,5-anhydro[1-^{3}H]mannitol 6-sulphate; IdoA2S-GlcNH6S-IdoA2S-anM6S, L-*O*-(α-iduronic acid 2-sulphate)-D-*O*-(α-glucosamine 6-sulphate)-(1→4)-L-*O*-(α-iduronic acid 2-sulphate)-(1→4)-D-*O*-anhydro[1-^{3}H]mannitol 6-sulphate; GlcNAc6S, *N*-acetylglucosamine 6-sulphate; GlcNS, sulphaminoglucosamine; anM6S, 2,5-anhydromannitol 6-sulphate; anM, 2,5-anhydromannitol; IdoA-anM6S, L-*O*-(α-iduronic acid)-(1→4)-D-*O*-2,5-anhydro[1-^{3}H]mannitol 6-sulphate.

* To whom correspondence should be addressed.

50S-Fractogel and ethylene glycol, and LKB (Bromma, Sweden) supplied Ultrogel AcA 34 and TSK G3000SW Ultrapac. Blue A Matrex agarose gel, the DC-2 hollow-fibre concentrator having 10 kDa-cut-off hollow fibres, the ultrafiltration stirred cells (model 8200) and the Diaflo ultrafiltration membrane YM10 were obtained from Amicon (Danvers, MA, U.S.A.). Protein assay reagent and silver staining kit for PAGE were obtained from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Dialysis membrane tubing with a 10–12 kDa cut-off was obtained from Union Carbide Corp. (Chicago, IL, U.S.A.), and Beckman Instruments (Palo Alto, CA, U.S.A.) supplied EP ReadySolv scintillant. BSA (crystalline grade) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Iduronate-2-sulphatase enzyme assay

Iduronate-2-sulphatase was routinely assayed as previously described (Hopwood, 1979) with the substrate L-*O*-(α-iduronic acid 2-sulphate)-(1→4)-D-*O*-2,5-anhydro[1-^{3}H]mannitol 6-sulphate (IdoA2S-anM6S) but modified as detailed below. The total assay volume of 12 μl included 1 μl of 340 μM-IdoA2S-anM6S (specific radioactivity 2582 c.p.m./pmol), 3 μl of 0.2 M-sodium acetate buffer, pH 4.0, containing 2 mg of BSA/ml, 10 mM-$CuCl_2$ and 3 mM-NaN_3 and up to 8 μl of enzyme sample either diluted in 50 mM-sodium acetate buffer, pH 4.0, or dialysed against 50 mM-sodium acetate buffer, pH 4.0, containing 10% (v/v) glycerol and 0.1 mM-dithioerythritol. In the kinetic studies of purified enzyme $CuCl_2$ was omitted, as its presence was required only to inhibit α-L-iduronidase, which may be present in the sample being assayed. Assay tubes were incubated at 37 °C for 1–2 h to give less than 30% breakdown of substrate to product. The reaction was stopped by freezing the assay mixture, and substrate and product were separated on Whatman 3MM chromatography paper in 0.75 M-formic acid, pH 1.7, by using high-voltage electrophoresis at 45 V/cm for 30 min on a Shandon Southern model L-24 system (Shandon Southern Products, Runcorn, Cheshire, U.K.). The chromatography paper was air-dried and then scanned on a Packard model 7201 radiochromatogram (Packard, Chicago, IL, U.S.A.). Areas of radioactivity (9–10 cm from the origin for the product and 14–15 cm for the substrate) were cut from the strip, then placed in 5 ml plastic vials and eluted in 1.5 ml of water, which was followed by the addition of 3.0 ml Beckman EP ReadySolv scintillant, and their radioactivities were determined in an LKB Rackbeta II liquid-scintillation counter. Enzyme activity was determined from the percentage conversion of substrate into product and was expressed as pmol of product/min per μl.

Other lysosomal enzyme activities

α-L-Iduronidase, with the fluorogenic substrate 4-methylumbelliferyl α-L-iduronide (Clements *et al.*, 1985*b*), sulphamate sulphohydrolase (Hopwood & Elliott, 1982), *N*-acetylglucosamine-6-sulphatase (Freeman *et al.*, 1987), *N*-acetylgalactosamine-4-sulphatase (Hopwood *et al.*, 1986) and glucuronate-2-sulphatase, with GlcA2S-anM6S (Freeman & Hopwood, 1989), were assayed by previously reported procedures.

Purification of iduronate-2-sulphatase

All procedures were carried out at 4 °C unless otherwise stated.

Step 1. Liver extraction. Approx. 900 g of liver was homogenized in 3 vol. of 15 mM-sodium dimethylglutarate buffer, pH 6.0, containing 0.5 M-NaCl and 0.1 mM-dithioerythritol (buffer A), and delipidated with carbon tetrachloride and centrifuged further at 27000 *g* for 30 min (Clements *et al.*, 1983; Mahuran *et al.*, 1983*b*).

Step 2. Concanavalin A–Sepharose–Blue A–agarose chromatography. The step 1 clear supernatant (1.5–2 litres) was applied to a 300 ml concanavalin A–Sepharose column equilibrated in buffer A containing 1 mM-$MnCl_2$ and 1 mM-$CaCl_2$ and washed extensively with 6 litres of buffer A. By using a method described elsewhere, the enzyme was eluted from concanavalin A–Sepharose with 12% (w/v) methyl α-D-mannoside in buffer A directly on to a 300 ml Blue A-agarose column and the methyl α-D-mannoside solution (1 litre) was recycled over both columns at 2 ml/min for 24 h (Clements *et al.*, 1983; Mahuran *et al.*, 1983*b*), after which time unbound proteins were displaced with 2 litres of buffer A. This fraction (2–2.5 litres), from now on referred to as the concanavalin A recycling eluate (CARE), was concentrated to 250–300 ml on an Amicon hollow-fibre concentrator. Two or three such CARE fractions were combined and concentrated to approx. 70–80 ml in an Amicon stirred cell fitted with a YM10 membrane filter and then dialysed for 16 h against 5 litres of 30 mM-Tris/HCl buffer, pH 7.2, containing 10% (v/v) glycerol, 0.1 mM-dithioerythritol and 3 mM-NaN_3 (buffer B). No significant loss of enzyme activity occurred from this processing and subsequent centrifugation in step 3.

Step 3. Chromatofocusing chromatography (Fig. 1). The dialysed fraction from step 2 was centrifuged at 1800 *g* for 10 min, to remove precipitate that developed during dialysis, and the supernatant was loaded on to a PBE 94 column (45 cm × 1.5 cm) equilibrated in buffer B (flow rate 1 ml/min) and washed with 200–300 ml of buffer B until absorption at 280 nm approached baseline level. Bound proteins were eluted with 1 litre of Polybuffer 74 that had been diluted 1 to 18 in water, the pH was adjusted to 4.0 with HCl and the solution was made 10% (v/v) in glycerol, 0.1 mM-dithioerythritol and 3 mM-NaN_3 (buffer C). The column was then eluted with 300 ml of buffer C containing 0.25 M-NaCl and then 300 ml of buffer C containing 1.0 M-NaCl. Fractions containing iduronate-2-sulphatase activity were pooled and designated form A, form B and form C (Fig. 1). NaCl was added to form A to a final concentration of 0.25 M and the pH was adjusted to 5.0 with 0.12 M-sodium dimethylglutarate

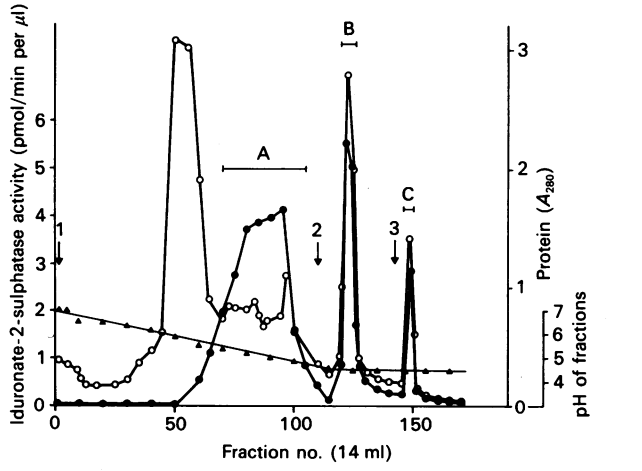

Fig. 1. Chromatofocusing chromatography of step 2 fraction (Table 1)

For experimental details see the Materials and methods section. The arrow 1 indicates the start of Polybuffer elution. Fractions indicated were assayed for iduronate-2-sulphatase activity (●), protein (A_{280}, ○) and pH (▲). Elution at arrow 2 was with Polybuffer that contained an additional 0.25 M-NaCl. Elution at arrow 3 was with Polybuffer that contained an additional 1.0 M-NaCl. Fractions were pooled as indicated A, B or C (horizontal bars).

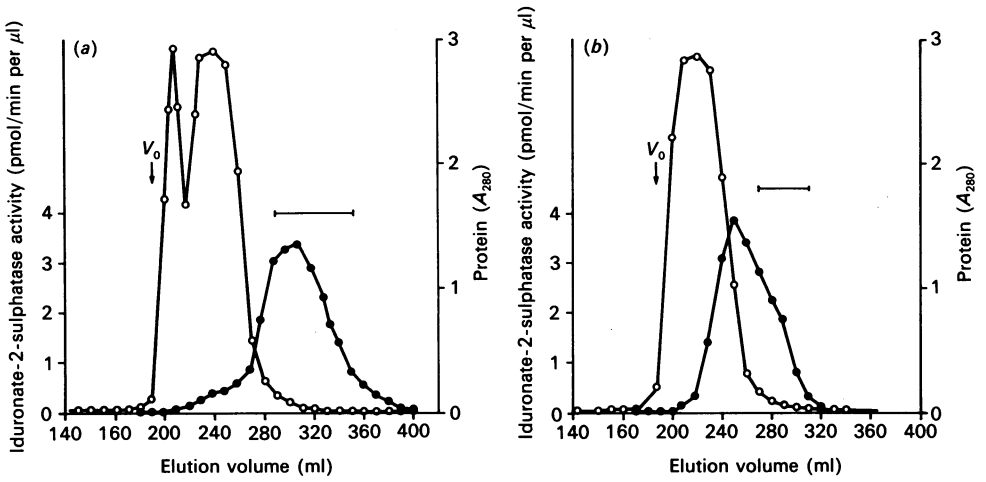

Fig. 2. TSK HW 50S chromatography of iduronate-2-sulphatase activity (*a*) form A and (*b*) form B

For experimental details see the Materials and methods section. Fractions were assayed for iduronate-2-sulphatase activity (●) and protein (A_{280}, ○). Column void volume (V_0) is arrowed. Pooled fractions are indicated as horizontal bars.

buffer, pH 6.8. Form A and form B were processed identically for the remaining procedures.

Step 4. TSK HW 50S-Fractogel chromatography (Fig. 2). Step 3 form A and form B fractions were each concentrated to 2.0 ml in an Amicon ultrafiltration stirred cell fitted with a YM10 membrane and the buffer was changed to 15 mM-sodium dimethylglutarate buffer, pH 6.0, containing 0.5 M-NaCl, 10% (v/v) glycerol, 0.1 mM-dithioerythritol and 3 mM-NaN_3 (buffer D). The samples were centrifuged at 1800 *g* for 10 min, and the supernatant was loaded on to the TSK HW 50S-Fractogel column (110 cm × 2.5 cm) equilibrated in buffer D and having a flow rate of 0.5 ml/min. Fractions eluted with buffer D containing enzyme activity were pooled (see Fig. 2) and loaded directly on to the next column.

Step 5. Phenyl-Sepharose CL-4B chromatography (Fig. 3). This hydrophobic column (3 cm × 1.0 cm) was equilibrated in buffer D and run at room temperature (20–25 °C). The sample from step 4 (usually 60–70 ml) was loaded and washed on with three 10 ml portions of buffer D. The column was eluted with three 10 ml portions of buffer D (without glycerol) for each concentration of ethylene glycol starting at 25% (v/v) and increasing to 30%, 45% and 50% (v/v). The eluate was collected into tubes standing in ice. Fractions containing enzyme activity were pooled as shown in Fig. 3.

Step 6. TSK G3000SW Ultrapac chromatography (Fig. 4). The pooled fractions containing form A or form B enzyme activities were separately concentrated in an Amicon ultrafiltration stirred cell to 1.0 ml and separately applied at room temperature to an

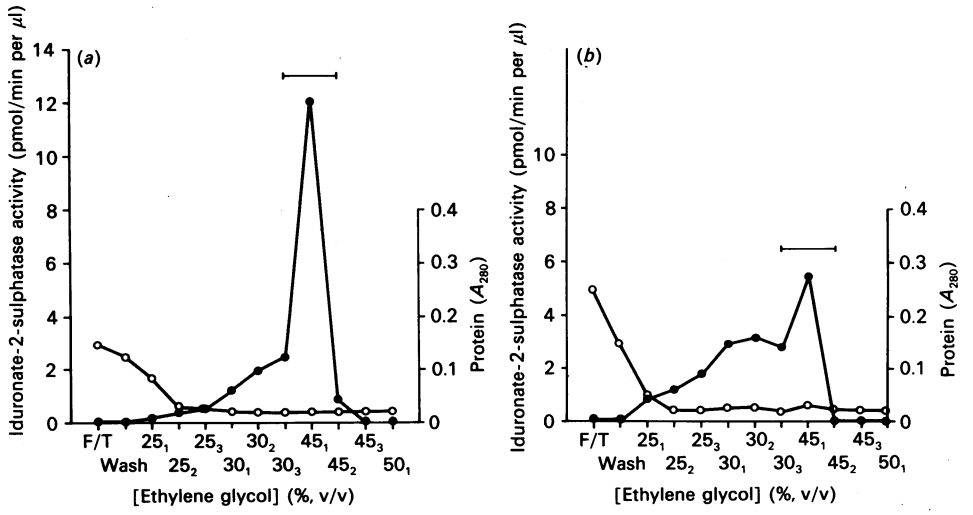

Fig. 3. Phenyl-Sepharose CL-4B chromatography of step 4 liver iduronate-2-sulphatase activity (*a*) form A and (*b*) form B

For experimental details see the Materials and methods section. Fractions were assayed for iduronate-2-sulphatase activity (●) and protein (A_{280}, ○). Fractions indicate: flow through (F/T); wash; 25_1, first 25% (v/v) ethylene glycol; 25_2, second 25% (v/v) ethylene glycol; 30_1, first 30% (v/v) ethylene glycol; etc. Pooled fractions are indicated as horizontal bars.

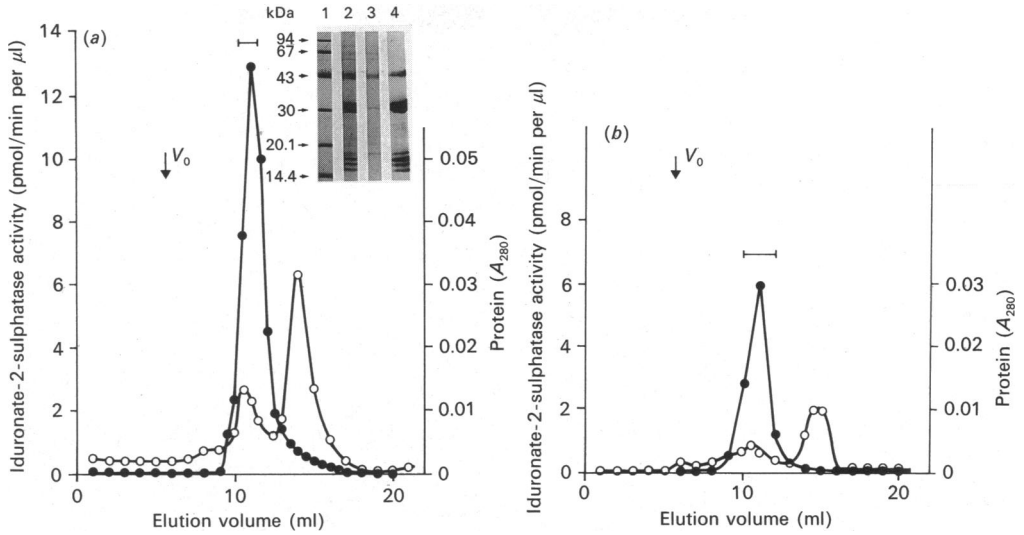

Fig. 4. TSK G3000SW chromatography of step 5 liver iduronate-2-sulphatase activity (*a*) form A and (*b*) form B

For experimental details see the Material and methods section. Fractions were assayed for iduronate-2-sulphatase activity (●) and protein (A_{280}, ○) and pooled as shown by the horizontal bars. Column void volume (V_0) is arrowed. The inset shows SDS/PAGE of: lane 1, molecular-mass standards; lane 2, pooled fractions from phenyl-Sepharose (Fig. 3*a*); lane 3, the iduronate-2-sulphatase peak (Fig. 4*a*); lane 4, the major protein peak (Fig. 4*a*). Lanes 1–4 were stained with Coomassie Brilliant Blue.

LKB Ultrachrom GTi f.p.l.c. system with a TSK G3000SW Ultrapac column (30 cm × 0.8 cm), equilibrated and eluted in buffer D at a flow rate of 0.5 ml/min and pressure of 150 kPa. Fractions containing iduronate-2-sulphatase activity were pooled as shown in Fig. 4.

Other human tissues

Human kidney, lung and placenta were also processed as described above for the isolation of iduronate-2-sulphatase from liver. Generally 1600 g of kidney, 800 g of lung or 860 g of placenta were used in each batch.

Gel-permeation chromatography

Estimations of native protein molecular mass were attempted with the use of different gel-permeation supports. These included TSK HW 50S-Fractogel (110 cm × 2.5 cm), Ultrogel AcA 34 (155 cm × 2.5 cm), Sephacryl S300 (850 cm × 2.5 cm) and finally the f.p.l.c. system with a TSK G3000SW Ultrapac column (30 cm × 0.8 cm) and Superose 12 (30 cm × 1 cm). All columns were calibrated in buffer D, with as protein standards thyroglobulin (660 kDa), ferritin (440 kDa), catalase (232 kDa), fructose-bisphosphate aldolase (158 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), chymotrypsinogen A (25 kDa) and RNAase (13.7 kDa).

SDS/PAGE

Samples for electrophoresis were prepared by precipitation with deoxycholate/trichloroacetic acid as described by Mahuran *et al.* (1983*a*). Discontinuous SDS/polyacrylamide slab gels using 12% acrylamide were run according to the method of Laemmli (1970). All samples were denatured before electrophoresis by incubation for 2 min at 100 °C in the presence or in the absence (where indicated) of 0.1 mM-dithioerythritol. Gels were stained with either Coomassie Brilliant Blue R250 or GradiPure Colloidal Electrophoresis Gel Stain and, when necessary, silver-stained by using the method of Merril *et al.* (1981). Pharmacia molecular-mass standards were used for gel calibration and included the following proteins: phosphorylase *b* (94 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soya-bean trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa).

Kinetic experiments

Enzyme solution containing 500 μg of BSA/ml was dialysed overnight at 4 °C against 2 litres of 5 mM-sodium acetate buffer, pH 4.0, containing 10% (v/v) glycerol, 0.1 mM-dithioerythritol and 3 mM-NaN_3. The pH–activity profiles were obtained with a 12 μl assay mixture containing substrate, 50 mM-sodium acetate or 10 mM-sodium dimethylglutarate buffer (pH as indicated), 0.1 mM-dithioerythritol and BSA (100 or 500 μg/ml, where indicated). Reactions were initiated by the addition of 1 μl of dialysed enzyme (equivalent to 8–20 ng of protein) and incubated at 37 °C. Incubation times were optimized to give a percentage breakdown of substrate to product in the range 5–25%, which was linear with respect to time. Concentrations of each substrate and incubation time for generation of pH–activity profiles were: IdoA2S-anM, 28 μM, 6 h; IdoA2S-anM6S, 28 μM, 20 min; IdoA2S-anT4S, 1 μM, 5 min; IdoA2S-GlcNS-UA-GlcNAc-GlcOA, 42 μM, 15 min; IdoA2S-GlcNS6S-IdoA2S-anM6S, 10 μM, 15 min; IdoA2S-GlcNAc6S-IdoA2S-anM6S, 10 μM, 15 min; IdoA2S-GlcNH6S-IdoA2S-anM6S, 10 μM, 15 min. Reactions were terminated by plunging the reaction vials into an ethanol/solid CO_2 bath. The substrate was separated from the product (for all substrates except the tetrasaccharides) by high-voltage electrophoresis as described above. The tetrasaccharide substrates IdoA2S-GlcNS6S-IdoA2S-anM6S, IdoA2S-GlcNAc6S-IdoA2S-anM6S and IdoA2S-GlcNH6S-IdoA2S-anM6S were separated from their respective products by high-voltage electrophoresis on Whatman 3MM paper in 0.75 M-formic acid, pH 1.7, containing 0.10 M-NaCl at 22 V/cm for 2.5 h in the same equipment. The strips were scanned as described above, and areas of radioactivity were cut from the strip, placed in 20 ml glass scintillation vials along with 5 ml of water and 10 ml of scintillant and their radioactivities measured in a Searle model 6868 Isocap ambient-temperature liquid-scintillation counter (Searle Analytic, Des Plaines, IL, U.S.A.) at 19% counting efficiency. Enzyme activity was determined from percentage breakdown of each substrate to product.

Kinetic data (K_m and $k_{cat.}$) were obtained with assay conditions similar to those for the pH–activity profiles. Substrate concentrations ranged from 0.1 to 100 μM. Incubation time was adjusted within the linear range for substrate hydrolysis. Most incubations were at the optimum pH for each substrate. K_m and $V_{max.}$ values were obtained from Lineweaver–Burk plots. K_i data were obtained from Dixon plots.

Substrates, products and substrate analogues

IdoA2S-anM6S, IdoA-anM6S and IdoA2S-anM were isolated from the disaccharide fraction prepared by deaminative cleavage of heparin from pig intestinal mucosa as described previously (Hopwood, 1979). IdoA2S-anT4S was prepared from dermatan sulphate (Hopwood & Muller, 1983). Ido2S-anM6S was obtained from IdoA2S-anM6S by carboxy reduction (Karamanos *et al.*, 1988). GlcA2S-anM6S was prepared from IdoA2S-anM6S by heating in hydrazine (Shaklee *et al.*, 1985; Freeman & Hopwood, 1989). IdoA2S-GlcNS-UA-GlcNAc-GlcOA, isolated from the urine of a patient with mucopolysaccharidosis type II, was a gift from Isoko Takazono (Kurume University, Japan) while working in the Department of Chemical Pathology, Adelaide. IdoA2S-GlcNS6S-IdoA2S-anM6S was isolated from the tetrasaccharide fraction prepared by nitrous acid degradation of heparin (Hopwood, 1979). This pentasulphated tetrasaccharide was subjected to mild acid hydrolysis (50 mM-HCl for 30 min at 80 °C) to de-*N*-sulphate the GlcNS6S residue and produce IdoA2S-GlcNH6S-IdoA2S-anM6S, which was *N*-acetylated (Hopwood & Elliott, 1981) to produce IdoA2S-GlcNAc6S-IdoA2S-anM6S.

RESULTS

Purification of iduronate-2-sulphatase

Extraction of iduronate-2-sulphatase from human liver under the conditions outlined above has not been optimized solely for this enzyme, as the procedure is used to extract several lysosomal enzymes under study in this laboratory (Mahuran *et al.*, 1983*b*; Clements *et al.*, 1985*a*; Freeman & Hopwood, 1986; Freeman *et al.*, 1987; Gibson *et al.*, 1987; Clements *et al.*, 1989; Freeman & Hopwood, 1989). Approx. 90% of the iduronate-2-sulphatase activity in the step 1 high-speed supernatant was recovered in the CARE fraction. The remaining 10% enzyme activity was bound to Blue A–agarose and was eluted at 0.5 M-NaCl buffered at pH 7.5 in 30 mM-Tris/HCl, but was not further characterized. To obtain sufficient enzyme at a concentration at which it was relatively stable, two or three liver extracts were combined at the CARE stage for the subsequent steps. Iduronate-2-sulphatase was completely bound to the chromatofocusing gel PBE 94, and the enzyme activity was recovered in 92% yield with approx. 65% removal of protein at this step (Table 1). Two major peaks of iduronate-2-sulphatase activity were eluted from the chromatofocusing gel in approximately equal abundance. Form A was eluted with Polybuffer at pH 4.5–4.8, whereas form B was more acidic and required 0.25 M-NaCl for elution. Another, though less abundant, form C was more tightly bound and required 1 M-NaCl for elution (Fig. 1 and Table 1). Form A and form B accounted for approx. 85% of the total enzyme loaded; form C comprised about 5–10% of the total activity and was not further characterized. Re-application of form A or form B to the chromatofocusing gel and eluting as before resulted in each form being eluted in its original position.

A significant loss of enzyme activity (approx. 50%) occurred during the concentration process of form A and form B from step 3. Attempts to overcome these losses by addition of NaCl or detergents were unsuccessful. Step 4, TSK HW 50S chromatography, was valuable in removing a large (approx. 98%) amount of protein. Form A enzyme appeared to interact with the matrix since, from molecular-mass calibration data, iduronate-2-sulphatase activity was eluted at an apparent molecular mass of 14 kDa (Fig. 2*a*). Recovery of the form B enzyme on this column, compared with form A, was not as good, as most of the enzyme activity, which was eluted with an apparent molecular mass of 45 kDa, was co-eluted with the major protein peak, which was not included in the pooled fractions (Fig. 2*b*). Re-application separately of form A and

Table 1. Purification of iduronate-2-sulphatase from 2400 g of human liver

For full experimental details see the Materials and methods section.

Step	Total activity (μmol/h)	Total protein (mg)	Specific activity (μmol/h per mg)	Purification (fold) (total)	Recovery (%)
1. Homogenate supernatant	200	150000	0.0013	1	
2. Concanavalin A–Sepharose–Blue A–agarose					
CARE fraction	152	982	0.154	119	76
Blue A–agarose eluate	12	65	0.185	142	6
3. Chromatofocusing of CARE fraction					
pI 4.5 (form A)	80	257	0.311	239	40
pI < 4.0 (form B)	50	85	0.588	452	25
pI < 4.0 (form C)	10	8	1.25	961	5
4. TSK HW 50S chromatography					
Form A	20	2.3	8.70	6692	10
Form B	20	1.0	20.00	15385	10
5. Phenyl-Sepharose chromatography					
Form A	14	0.22	63.6	48923	7
Form B	6	0.08	75.0	57692	3
6. TSK G3000SW chromatography					
Form A	10	0.014	714	549450	5
Form B	4	0.015	266	205128	2

Table 2. Purification of iduronate-2-sulphatase from human liver, lung, kidney and placenta: summary of yield, native and subunit molecular-mass data

The yield was calculated assuming the step 2 CARE fraction to be 100% (see Table 1). Native molecular mass was determined by chromatography on TSK G3000SW. Subunit molecular masses refer to the polypeptide components at approx. 42 kDa (1) and 18 kDa (2) on SDS/PAGE (Fig. 5); the 18 kDa polypeptide was sized as 14.4 kDa under optimum conditions (Merle & Kadenbach, 1980). *n* represents the number of different preparations in each case.

Tissue	Form	Yield (%)	10^{-3} × Native molecular mass (kDa)	10^{-3} × Subunit molecular mass (kDa)	
				(1)	(2)
Liver	A	1.8–6.0	41–44 ($n = 18$)	42.0–45 ($n = 13$)	17–18.5 ($n = 17$)
Liver	B	0.9–2.0	43–44 ($n = 5$)	40.0–41 ($n = 3$)	17–17.5 ($n = 3$)
Lung	A	1.5–2.4	39 ($n = 1$)	40.5–42 ($n = 3$)	17–18.0 ($n = 3$)
Kidney	A	2.3–3.6	42–44 ($n = 6$)	42.0–43 ($n = 6$)	17–18.0 ($n = 6$)
Placenta	A	1.3–1.9	43, 44 ($n = 2$)	44, 46 ($n = 2$)	17–17.5 ($n = 2$)

form B enzyme that was co-eluted with the main protein peak on TKS HW 50S did not separate iduronate-2-sulphatase activity from a significant amount of the contaminating protein.

Form A enzyme activity was eluted from the hydrophobic column with 45% ethylene glycol, whereas most of the protein associated with the form A enzyme passed directly through or was eluted with 25–30% ethylene glycol (Fig. 3*a*). Elution of form B enzyme activity required 30–45% ethylene glycol (Fig. 3*b*). SDS/PAGE of form A showed major polypeptide species of molecular masses 43 kDa and 30 kDa with several minor species below 20 kDa (see Fig. 4*a* inset), whereas SDS/PAGE of form B enzyme from the 45% ethylene glycol wash contained a major polypeptide component with a molecular mass of 42 kDa (result not shown).

Table 1 shows that after the hydrophobic step the form A enzyme was purified approx. 49000-fold with 7% yield and that the form B enzyme was purified approx. 58000-fold with 3% yield. The latter yield was an underestimate, since form B enzyme was eluted broadly from this column (Fig. 3*b*) and only the pooled fractions were taken into consideration. Other fractions, which had more than half of the total iduronate-2-sulphatase activity but also a number of contaminating proteins that were difficult to remove in subsequent purification steps, were not studied further. Most of the smaller-molecular-mass polypeptides seen in form A enzyme were not directly associated with iduronate-2-sulphatase activity, since they were removed by the TSK G3000SW chromatography step (Fig. 4*a*). Form A was purified more than 500000-fold over the total iduronate-2-sulphatase found in the liver homogenate with a 5% recovery, and form B was purified 205000-fold with 2% recovery (Table 1). The final purification step for form A gave an 11-fold purification, achieved mostly by the removal of a 30 kDa polypeptide (Fig. 4*a*).

Native molecular mass and subunit size

As already mentioned, form A enzyme had an apparent native molecular mass of 14 kDa on TSK HW 50S, whereas that of form B enzyme was 45 kDa under identical conditions (buffer D). On Sephacryl S300 under buffer D conditions the molecular mass of form A enzyme was 53 kDa, whereas on Ultrogel AcA 34 it was 65 kDa, on TSK G3000SW it was 42 KDa and on Superose 12 it was 24 kDa. Form B enzyme under similar conditions (buffer D) on Ultrogel AcA 34 had a molecular mass of 100 kDa and on TSK G3000SW it was 42 kDa. The native molecular masses of form A and form B, determined by chromatography on TSK G3000SW, were consistently found to be 43 kDa for many different preparations from liver and for form A from other tissues (Table 2). Form A in a lower-ionic-strength buffer [10 mM-Tris/HCl buffer, pH 7.6, containing 10 mM-NaCl, 10% (v/v) glycerol and 0.1 mM-dithioerythritol] was eluted with a higher molecular mass of 90–130 kDa, which may reflect protein aggregation under conditions of low salt and neutral pH.

SDS/PAGE revealed that reduced form A from liver contained mostly a 42 kDa polypeptide, with minor amounts of 30 kDa, 20 kDa and 18 kDa polypeptides also present (Fig. 5, lane 2). Silver staining, beside detection of extra bands (due to contaminants from dithioerythritol; Tasheva & Dessev, 1983; Clements *et al.*, 1985*a*) also resulted in a change, relative to Coomassie Brilliant Blue staining of relative band intensities (Fig. 5, compare lanes 2 and 7). The 42 kDa and 18 kDa polypeptides were unchanged in non-reduced form A, but, however, the 30 kDa and 20 kDa polypeptides disappeared, and a new polypeptide appeared at 48 kDa (Fig. 5, lanes 7 and 8). Form A from liver was subjected to SDS/PAGE under optimum conditions for polypeptides with molecular masses of less than 20 kDa (Merle & Kadenbach, 1980). The small polypeptide (18 kDa), in both reduced and non-reduced iduronate-2-sulphatase preparations, had a molecular mass of 14.4 kDa. To prevent

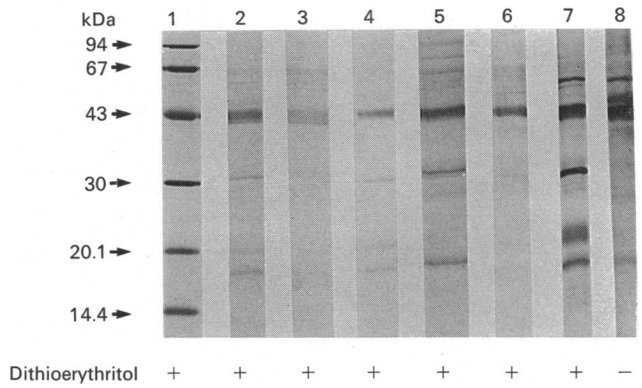

Fig. 5. SDS/PAGE of iduronate-2-sulphatase from liver, kidney, lung and placenta

All samples, except lane 8, were reduced with dithioerythritol and electrophoresed according to details in the Materials and methods section. Except for lanes 1, 2 and 3 all were on separate electrophoresis runs. Lane 1, molecular-mass standards; lanes 2 and 3, liver form A and form B respectively; lane 4, form A from kidney; lanes 5 and 6, form A from lung and placenta respectively; lane 7, form A from liver denatured in the presence of 0.1 mM-dithioerythritol; lane 8, the same enzyme preparation used in lane 7 except that denaturation was in the absence of dithioerythritol. Lanes 1–6 were stained with Coomassie Brilliant Blue, and lanes 7 and 8 were stained with silver.

confusion in the text, the 18 kDa component, identified in all preparations of iduronate-2-sulphatase, will be called the 14 kDa polypeptide. Form B from liver contained a major 42 kDa polypeptide, with smaller amounts of 30 kDa and 14 kDa polypeptides also present (Fig. 5). Lung, kidney and placenta form A preparations all contained the major 42 kDa polypeptide, with minor amounts of 30 kDa, 20 kDa and 14 kDa polypeptides also present (Fig. 5 and Table 2).

Effect of pH on iduronate-2-sulphatase activity

The pH optimum for the activity of iduronate-2-sulphatase form A towards each substrate was shown to be dependent upon the substrate structure (Fig. 6). Because NaCl was a potent inhibitor of sulphatase activity (see below), enzyme activity was determined in the absence of NaCl, but in the presence of BSA since the enzyme was unstable for incubations in the absence of added BSA. The shape of the pH–activity profile towards IdoA2S-anM6S was clearly influenced by the buffer composition, the concentration of the substrate and the amount of added BSA (Figs. 6*a*, 6*c* and 6*e* and Table 3). Form A activity towards 34 μM-IdoA2S-anM6S in the presence of 100 μg of BSA/ml in sodium acetate buffer and in sodium dimethylglutarate buffer resulted in a broad pH–activity profile with maximal activity at pH 4.5 and 5.4 respectively; however, activity in sodium acetate buffer was twice that observed in the presence of sodium dimethylglutarate buffer (Fig. 6*e*). The incubation of form A in sodium acetate buffer containing 500 μg of BSA/ml resulted in a sharper pH–activity profile by apparently stimulating iduronate-2-sulphatase activity, particularly in the range pH 3.9–5.1, with maximal activity at pH 4.5 (Fig. 6*e* and Table 3). Maximum activity towards 10 μM-IdoA2S-anM6S in the presence of 100 μg of BSA/ml in sodium dimethylglutarate buffer was observed at

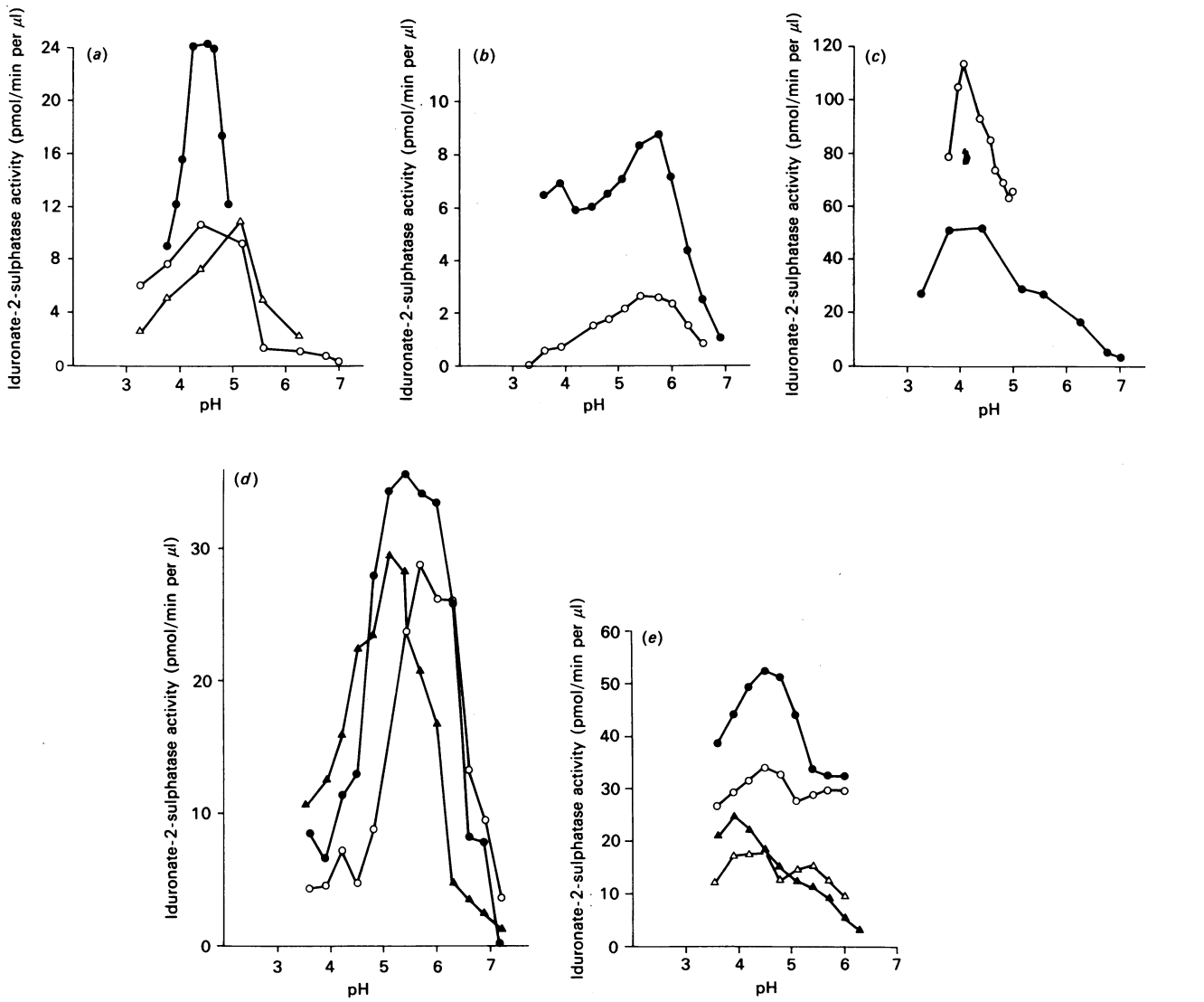

Fig. 6. Hydrolysis by liver iduronate-2-sulphatase form A of a variety of substrates as a function of incubation pH

(*a*) IdoA2S-anM6S in 10 mM-sodium dimethylglutarate buffer (○), IdoA2S-anM6S in 50 mM-sodium acetate buffer (●) and IdoA2S-anT4S in 10 mM-sodium dimethylglutarate buffer (△), all containing 500 μg of BSA/ml and a substrate concentration of 1 μM. (*b*) 42 μM-IdoA2S-GlcNS-UA-GlcNAc-GlcOA (●) and 34 μM-IdoA2S-anM (○) in 10 mM-sodium dimethylglutarate buffer, both containing 100 μg of BSA/ml. (*c*) 28 μM-IdoA2S-anM6S in 50 mM-sodium acetate buffer (○) and 10 mM-sodium dimethylglutarate buffer (●), both containing 500 μg of BSA/ml. (*d*) IdoA2S-GlcNS6S-IdoA2S-anM6S (○), IdoA2S-GlcNAc6S-IdoA2S-anM6S (●) and IdoA2S-GlcNH6S-IdoA2S-anM6S (▲) at a concentration of 10 μM in 10 mM-sodium dimethylglutarate buffer, all containing 100 μg of BSA/ml. (*e*) 34 μM-IdoA2S-anM6S in 50 mM-sodium acetate buffer containing 500 μg of BSA/ml (●), 50 mM-sodium acetate buffer containing 100 μg of BSA/ml (○), 10 mM-sodium dimethylglutarate buffer containing 100 μg of BSA/ml (△) and 10 μM-IdoA2S-anM6S in 10 mM-sodium dimethylglutarate buffer containing 100 μg of BSA/ml (▲).

Table 3. Effect of buffer type and concentration of BSA on human liver iduronate-2-sulphatase (form A) activity towards IdoA2S-anM6S

Refer to Fig. 6(*e*) for the pH–activity profile obtained for IdoA2S-anM6S under these various condition of pH, BSA concentrations and buffer type and concentration.

Buffer	pH	Concn. of BSA (μg/ml)	K_m (μM)	$k_{cat.}$ (mol/min per mol of enzyme)	$10^{-6} \times k_{cat.}/k_m$ (catalytic efficiency) ($M^{-1} \cdot min^{-1}$)
50 mM-Sodium acetate	4.5	500	5.7	2457	436
	4.5	100	3.6	1386	385
	5.4	100	6.1	1607	263
10 mM-Sodium dimethylglutarate	3.9	100	4.4*	1040	236
	4.5	100	2.3	882	284
	5.4	100	10.0	788	79

* Substrate inhibition above 17 μM-IdoA2S-anM6S.

pH 3.9. Sulphatase activity towards 10 μM-IdoA2S-anM6S at pH 3.9 (Fig. 6*e*) was 50% higher than activity towards 34 μM-IdoA2S-anM6S owing to substrate inhibition at low pH (Fig. 6*e* and Table 3). The pH–activity profile for form A enzyme towards IdoA2S-anT4S was different from that observed for IdoA2S-anM6S (Fig. 6*a*) with the pH optimum at 5.0. Form B activity towards IdoA2S-anM6S and IdoA2S-anT4S had a similar pH–activity profile to that seen for form A (results not shown).

Because of observed relatively high pH optima towards IdoA2S-anM and the tetrasaccharide and pentasaccharide substrates, iduronate-2-sulphatase was assayed in sodium dimethylglutarate buffer in the presence of 100 μg of BSA/ml. Maximal activity towards 34 μM-IdoA2S-anM was at pH 5.5, and activity towards 42 μM-IdoA2S-GlcNS-UA-GlcNAc-GlcOA was maximal at pH 5.7 (Fig. 6*b*). Similar pH optima were observed for activity towards both substrates when assayed at 10 μM concentration (results not shown). Enzyme activity towards tetrasaccharide substrates as a function of incubation pH is shown in Fig. 6*d*. Sulphatase activity towards the tetrasaccharide substrates was similar in either sodium acetate or sodium dimethylglutarate buffer in the pH range 3.6–5.7. The addition of 500 μg of BSA/ml inhibited form A activity towards IdoA2S-GlcNAc6S-IdoA2S-anM6S by 85% at pH 3.9 and by 15% at pH 5.4 compared with activity in the presence of 100 μg of BSA/ml. Maximal activities towards the highly sulphated tetrasaccharide substrates IdoA2S-GlcNAc6S-IdoA2S-anM6S, IdoA2S-GlcNS6S-IdoA2S-anM6S and IdoA2S-GlcNH6S-IdoA2S-anM6S (at 10 μM concentration) were at pH 5.5, 5.7 and 5.1 respectively (Fig. 6*d*). The pH profile for activity towards IdoA2S-GlcNAc6S-IdoA2S-anM6S was broad, with 50% of the maximal activity observed between pH 4.6 and 6.5, whereas 50% maximal activity towards IdoA2S-GlcNS6S-IdoA2S-anM6S and IdoA2S-GlcNH6S-IdoA2S-anM6S were in the ranges pH 5.0–6.5 and pH 4.2–6.0 respectively (Fig. 6*d*). At pH 6.3 IdoA2S-GlcNAc6S-IdoA2S-anM6S, IdoA2S-GlcNS6S-IdoA2S-anM6S and IdoA2S-GlcNH6S-IdoA2S-anM6S have approx. 80%, 90% and 12% respectively of their maximal activities. Iduronate-2-sulphatase activity towards each of the tetrasaccharide substrates removed only the non-reducing terminal sulphate ester, since prolonged incubation at pH 3.9, 4.5 and 5.4, which would desulphate IdoA2S-anM6S more than 100 times over, failed to desulphate the internal IdoA2S residue.

Kinetic properties of purified iduronate-2-sulphatase

The apparent K_m and $k_{cat.}$ values for form A activity towards IdoA2S-anM6S were dependent upon the buffer composition

Table 4. Human liver iduronate-2-sulphatase (form A) activity towards a variety of substrates

For experimental details see the Materials and methods section. All incubations contained 10 mM-sodium dimethylglutarate buffer and 500 μg of BSA/ml for disaccharide substrates and 100 μg of BSA/ml for tetrasaccharide and pentasaccharide substrates. Activity towards Ido2S-anM6S and GlcA2S-anM6S was not detected. Relative catalytic efficiency is $k_{cat.}/K_m$ calculated relative to a value for IdoA2S-anM = 1.

Substrate	pH optimum	K_m (μM)	$k_{cat.}$ (turnover no.) (mol/min per mol of enzyme)	$10^{-6} \times k_{cat.}/K_m$ (catalytic efficiency) ($M^{-1} \cdot min^{-1}$)	Relative catalytic efficiency
IdoA2S-anM	5.4	19.2	161	8.4	1.0
IdoA2S-anM6S	4.0	4.0	2114	529	63.0
IdoA2S-anM6S	5.0*	2.5	905	362	43.1
IdoA2S-anT4S	4.0*	1.1	270	246	29.3
IdoA2S-anT4S	5.0	0.7	507	724	86.2
IdoA2S-GlcNS6S-IdoA2S-anM6S	5.7	1.4	2177	1568	186.7
IdoA2S-GlcNAc6S-IdoA2S-anM6S	5.7	3.1	4858	1568	186.7
IdoA2S-GlcNH6S-IdoA2S-anM6S	5.4	2.5	1925	770	91.7
IdoA2S-GlcNS-UA-GlcNAc-GlcOA	5.4	1.9	756	399	47.5

* Not optimum pH.

and the amount of added BSA (Table 3). In sodium dimethylglutarate buffer containing 100 μg of BSA/ml the affinity towards IdoA2S-anM6S was strongest at pH 4.5 with an apparent K_m value of 2.3 μM, compared with 4.4 and 10.0 μM at pH 3.9 and 5.4 respectively. However, the maximal turnover rate ($k_{cat.}$) was slightly higher at pH 3.9 compared with the enzyme at pH 4.5 (Table 3). Substrate inhibition was observed at pH 3.9 at concentrations greater than 17 μM IdoA2S-anM6S, but was not observed with 68 μM-IdoA2S-anM6S at pH 4.5 or 5.4. Form A catalytic activity towards IdoA2S-anM6S was 3 times more efficient at pH 4.5 compared with pH 5.4. Sulphatase activity observed in sodium acetate buffer containing 100 μg of BSA/ml resulted in an increase in both the apparent K_m value (from 2.3 to 3.6 μM) and the turnover number (from 882 to 1386 mol/min per mol of enzyme) at pH 4.5, whereas at pH 5.4 the K_m value decreased from 10.0 to 6.1 μM while the corresponding $k_{cat.}$ value doubled (Table 3). Incubation in sodium acetate buffer at pH 4.5 in the presence of 500 μg of BSA/ml compared with 100 μg of BSA/ml resulted in an increased K_m from 3.6 to 5.7 μM and a doubling of $k_{cat.}$ (Table 3).

Because of the relatively high pH optima for the tetrasaccharide substrates and the inhibition of desulphation by higher concentrations of BSA (see above) kinetic data were compared for all substrates at each substrate's pH optimum in sodium dimethylglutarate buffer in the presence of 100 μg of BSA/ml (Table 4). The simplest substrate tested for iduronate-2-sulphatase activity was the disaccharide IdoA2S-anM. The addition of a 6-sulphate ester group, to produce the disaccharide IdoA2S-anM6S, gave the largest, a 63-fold, increase in catalytic efficiency, resulting from 5-fold and 13-fold increases in binding affinity and turnover number respectively. IdoA2S-anT4S at pH 5.0 had a 3.6-fold higher affinity for enzyme but was turned over less efficiently, such that its relative catalytic efficiency was only twice that seen for IdoA2S-anM6S at pH 5.0 (Table 4). The influence on iduronate-2-sulphatase activity of aglycone anM or GlcN 6-sulphate ester group and of aglycone uronic acid (2-sulphate) residues further from the site of catalysis is demonstrated by the data given in Table 4. Activities towards the tetrasaccharide substrates that differ only in their glucosamine substituent enabled a comparison of the effect of aglycone GlcNS, GlcNAc and GlcNH residues. The effect of the GlcNS substituent was to increase the binding affinity by up to 2-fold compared with GlcNAc and GlcNH, and with anM6S in IdoA2S-anM6S. However, whereas a GlcNAc substituent in the tetrasaccharide increased the $k_{cat.}$ value by up to 2.3-fold compared with IdoA2S-anM6S, GlcNS and GlcNH substituents had marginal effect upon the turnover number compared with that observed for IdoA2S-anM6S, resulting in only a 3-fold increase in catalytic efficiency for GlcNS and GlcNAc and a similar catalytic efficiency for GlcNH-substituted tetrasaccharide substrates (Table 4). The pentasaccharide substrate, IdoA2S-GlcNS-UA-GlcNAc-GlcOA, which did not possess a 6-sulphate ester on the adjacent residue to the IdoA2S under attack, had a slight increase in binding affinity over IdoA2S-anM6S but a 10-fold higher affinity than IdoA2S-anM. However, the $k_{cat.}$ values of IdoA2S-anM6S and IdoA2S-anM, compared with the pentasaccharide, were 3-fold higher and 4.7-fold lower respectively.

Neither form A nor form B was active towards Ido2S-anM6S, the carboxy-reduced derivative of IdoA2S-anM6S, or the C-6 epimer GlcA2S-anM6S, which indicated the important influence upon enzyme specificity of the presence and configuration of the 6-carboxy group. However, both GlcA2S-anM6S and Ido2S-anM6S were potent competitive inhibitors of sulphatase activity towards IdoA2S-anM6S with K_i values, which suggest that they bind with greater efficiency than the disaccharide substrate IdoA2S-anM6S (Table 5). IdoA-anM6S, the product of sulphatase activity towards IdoA2S-anM6S, along

Table 5. Human liver (form A) iduronate-2-sulphatase activity towards IdoA2S-anM6S in the presence of substrate analogues

The enzyme preparation was from a different batch of livers compared with the preparation used to generate the data recorded in Tables 3 and 4. All incubations contained 50 mM-sodium acetate buffer and 500 μg of BSA/ml. For full experimental details see Materials and methods section.

Substrate or analogue	pH	K_i (μM)	K_m (μM)
IdoA2S-anM6S	4.5		5.0
IdoA2S-anM	5.5		14.3
IdoA-anM6S	4.5	1.7	
IdoA-anM	4.5	11.7	
anM6S	4.5	0.25	
anM	4.5	No inhibition	
GlcNAc	4.5	No inhibition	
GlcNS	4.5	No inhibition	
GlcNAc6S	4.5	No inhibition	
SO_4^{2-}	4.5	64	
Ido2S-anM6S	4.5	1.0	
GlcA2S-anM6S	4.5	1.0	

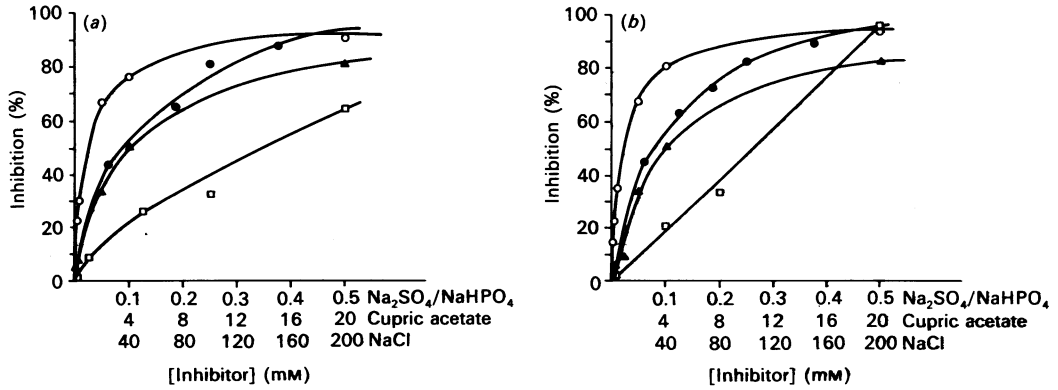

Fig. 7. Hydrolysis of IdoA2S-anM6S by liver (*a*) form A (*b*) form B iduronate-2-sulphatase as a function of NaCl (●), Na_2HPO_4 (○), Na_2SO_4 (▲) and cupric acetate (□) concentration at pH 4.5 in 50 mM-sodium acetate buffer containing 500 μg of BSA/ml

For experimental details see the Materials and methods section.

with anM6S, the product of α-L-iduronidase activity towards IdoA-anM6S, were both strongly binding competitive inhibitors; however, the monosaccharides GlcNAc, GlcNS, GlcNAc6S and anM did not inhibit sulphatase activity towards IdoA2S-anM6S (Table 5).

Effect of salts upon iduronate-2-sulphatase activity towards IdoA2S-anM6S

We examined the effect of increasing concentrations of NaCl, Na_2SO_4, NaH_2PO_4 and cupric acetate upon enzyme activity towards IdoA2S-anM6S at pH 4.5. Both form A and form B activities were inhibited 50% by 30 mM-NaCl (Fig. 7). Phosphate and sulphate were potent inhibitors of both forms in that 50% inhibition was observed in the 25–100 μM range (Fig. 7). $CaCl_2$ or $MgCl_2$ at concentrations of 0.5, 1, 2 and 5 mM had no detectable effect on form A or form B sulphatase activity. $MnCl_2$ at an optimum concentration of 2 mM increased enzyme activity of both forms by 20% over control values (results not shown). Cupric acetate inhibited both forms of the enzyme to the extent that 10–14 mM gave 50% inhibition (Fig. 7).

DISCUSSION

We have purified iduronate-2-sulphatase from human liver by more than 500000-fold with a net recovery of 7% activity of two enzyme forms. The purification procedure was a six-step six-column process consisting of a concanavalin A–Sepharose–Blue A–agarose coupled step, chromatofocusing, two gel-filtration steps on different gel supports and hydrophobic separation on phenyl-Sepharose. The procedure has the advantage that other lysosomal enzymes involved in glycosaminoglycan degradation can be conveniently obtained from the same liver (Mahuran *et al.*, 1983*b*; Clements *et al.*, 1985*a*; Freeman & Hopwood, 1986, 1989; Freeman *et al.*, 1987; Gibson *et al.*, 1987). An important purification step in the procedure was that of chromatography on TSK HW 50S, as there appeared to be a combination of gel filtration and some interaction of the enzyme with this matrix. Chromatofocusing led to the separation of two major forms (A and B) from all four tissues used. Other minor forms were also observed, but these were not studied.

Over the past 5 years, more than 100 livers have been processed through to purified iduronate-2-sulphatase and used to generate monoclonal antibodies and amino acid sequence information for this extremely low-abundance protein. The processed livers were collected with times *post mortem* ranging from 6 h to 72 h from donors aged from 1 year to 78 years old. Although care was taken to minimize exposure of iduronate-2-sulphatase to proteolytic enzymes, doubtless extracted from the liver during homogenization, and to minimize the time of liver storage, we cannot eliminate the possibility that the species characterized has undergone some enzyme processing either during extraction and purification or *in vitro* during the interval *post mortem*. However, the consistent properties and molecular-mass values (Table 2) of the component subunits of the enzyme isolated from a large number of different tissues and donors suggest that proteolysis of the enzyme *in vitro* was not significant. That is, as all form A preparations contained the 42 kDa polypeptide as the major species, together with lesser amounts of 30 kDa, 20 kDa and 14 kDa polypeptides, it is unlikely that this polypeptide pattern is a result of donor age, liver storage or conditions used during the purification process. Consistent multi-polypeptide band patterns were also observed for other lysosomal enzymes, α-L-iduronidase (Clements *et al.*, 1989), *N*-acetylglucosamine-6-sulphatase (Freeman *et al.*, 1987), glucuronate-2-sulphatase (Freeman & Hopwood, 1987) and *N*-acetylgalactosamine-4-sulphatase (Gibson *et al.*, 1987), purified to homogeneity from these same livers.

Form A and form B were separated at the chromatofocusing step on the basis of different pI values. Form A was seen to interact with the TSK HW 50S matrix such that it was eluted with an apparent molecular mass of 14 kDa, whereas form B was eluted with a molecular mass of 45 kDa. Both forms were shown consistently to have similar native molecular mass of 45 kDa and contain polypeptides of 42 kDa and 14 kDa that were unaltered by reduction (Table 2 and Fig. 5). The maximum native molecular mass for form A was 65 kDa (Ultrogel AcA 34). These findings suggest that the native form A enzyme contains at least a polypeptide of 42–45 kDa. The *N*-terminal amino acid sequence obtained from the 42 kDa polypeptide in form A was used to construct an oligonucleotide probe and isolate cDNA fragments that mapped to the human X-chromosome and were used to demonstrate gross DNA deletions in the iduronate-2-sulphatase-deficient mucopolysaccharidosis type II patients (Wilson *et al.*, 1990). These findings strongly support the proposal that the 42 kDa polypeptide present in purified enzyme preparations is encoded by the iduronate-2-sulphatase gene. Both form A and B also contain a 14 kDa polypeptide, which we propose interacts with the 42 kDa polypeptide to give active enzyme. This proposal is supported by the observation that the 30 kDa and 20 kDa polypeptides, present in all form A preparations, associate under non-reducing conditions to give a 48 kDa component on SDS/PAGE and therefore do not associate with the 42 kDa polypeptide (Fig. 5, lanes 7 and 8). Further support came from the *N*-terminal amino acid sequence of the 14 kDa polypeptide in form A being present in the cDNA fragment isolated with the 42 kDa *N*-terminal amino acid sequence (Wilson *et al.*, 1990). This cDNA clone was shown to contain the 42 kDa polypeptide *N*-terminal to the 14 kDa polypeptide. As judged from the yield of amino acids obtained during *N*-terminal sequencing, the 42 kDa and 14 kDa polypeptides were present in approximately equal molar amounts in form A iduronate-2-sulphatase from liver.

Iduronate-2-sulphatase isolated from kidney, lung and placenta consistently contained the 42 kDa and 14 kDa polypeptides. However, these different tissue preparations also contained other polypeptides and therefore it is not possible, at this time, to be sure that these two polypeptides are the only components required for iduronate-2-sulphatase activity. It is possible that the 30 kDa and 20 kDa polypeptides consistently found in form A may represent a further form of iduronate-2-sulphatase. Final resolution of these questions may occur from maturation studies of this enzyme, in, for example, cultured skin fibroblasts.

Other investigators have reported purification of iduronate-2-sulphatase from various human sources. Wasteson & Neufeld (1982) described the isolation (50000-fold) of the enzyme from human plasma with a native molecular mass of 110 kDa containing a single polypeptide of 80 kDa. Lissens *et al.* (1984) purified (up to 155-fold) two forms of iduronate-2-sulphatase from human urine. One form was reported to have a native molecular mass of 96 kDa and contain a single polypeptide that under reducing conditions had a molecular mass of 66 kDa. Di Natale & Daniele (1985) reported the presence of two forms of iduronate-2-sulphatase and went on to purify (30000-fold) only the more acidic form from human placenta in 6.5% yield. Their preparation had a native molecular mass of 80 kDa, which was reported to contain a single polypeptide of 80–90 kDa on SDS/PAGE run under reducing conditions. Western-blot analysis of various iduronate-2-sulphatase preparations demonstrated subunit molecular masses of 72 kDa for placenta, 60 kDa for fibroblasts and two polypeptides in serum of 75 and 83 kDa (Daniele & Di Natale, 1987).

Values for the native molecular mass of partially purified

iduronate-2-sulphatase, from a variety of human tissues, have been reported variously by many investigators as 30–50 kDa, 70–90 kDa, 110 kDa, 80–115 kDa, 96 kDa, 114 kDa and 170–190 kDa[Yutaka *et al.* (1982), Di Natale & Daniele (1985), Wasteson & Neufeld (1982), Di Natale & Ronsisvalle (1981), Lissens *et al.* (1984), Cantz *et al.* (1972) and Di Natale & Ronsisvalle (1981) respectively]. These values should be compared with those reported in the present paper varying from 14 kDa to 130 kDa for human liver iduronate-2-sulphatase, where the behaviour of the enzyme on gel-permeation chromatography was dependent upon the nature of the gel matrix and the pH and ionic strength of the eluting buffer. Under conditions of low ionic strength and neutral pH, iduronate-2-sulphatase was eluted with higher molecular-mass values, suggesting aggregation of the enzyme. This property was also observed with purified human liver α-L-iduronidase (Clements *et al.*, 1985*a*). A further complication with estimation of the native molecular mass of this enzyme has been its interaction with some gel-matrix materials. For instance, TSK HW 50S gave a molecular mass of 14 kDa compared with values of 45 and 65 kDa for the same enzyme preparation subjected to TSK G3000SW and Ultrogel AcA 34 chromatography respectively. Thus it would be unwise to compare findings or make comparisons between investigators reporting the native size of iduronate-2-sulphatase prepared from different sources using different analytical conditions and methods. A further difficulty in comparing reported values for the native molecular mass of this extremely low-abundance enzyme is the very low recovery that most investigators have achieved, and therefore there may be selective removal of some forms of iduronate-2-sulphatase.

A major conclusion from the kinetic data (summarized in Tables 3, 4 and 5) is that the aglycone structure adjacent to the non-reducing-end iduronate-2-sulphate residue considerably influences the catalytic efficiency of iduronate-2-sulphatase. The extreme example, which comes from the addition of 6-sulphate ester to IdoA2S-anM to give IdoA2S-anM6S, resulted in a 63-fold increase in catalytic efficiency and a significant change in the pH optimum for the reaction. The change in pH optimum is consistent with the involvement of an ionizable group in enzyme–substrate interaction. The more complex substrates, compared with IdoA2S-anM6S, which have increased chain length and *N*-sulphated glucosaminide residues, gave only a further 3-fold increase in catalytic efficiency. This relatively small and constant increase in catalytic efficiency with variation of aglycone structure from -anM6S to -GlcNAc6S and -GlcNS6S was unexpected when compared with results for other heparan sulphate hydrolases (Clements *et al.*, 1985*b*; Freeman & Hopwood, 1986, 1987). We have previously described the importance of the aglycone structure to the catalytic efficiency of enzymes involved in heparan sulphate degradation. Purified human liver α-L-iduronidase, the enzyme that follows iduronate-2-sulphatase by action on its product from degradation of both dermatan sulphate and heparan sulphate, was stimulated 6-fold by the presence of a 6-sulphate ester on the disaccharide substrate (compare IdoA-anM with IdoA-anM6S in Clements *et al.*, 1985*b*). The next two enzymes, sulphamate sulphohydrolase and glucosamine-6-sulphatase, in the heparan sulphate-degradative pathway have also been purified from human liver, and their interaction with a variety of substrate structures has been studied (Freeman & Hopwood, 1986, 1987). Purified sulphamate sulphohydrolase turned over substrates in which aspects of the aglycone structure of the natural substrate were present (particularly IdoA2S residues) 370000-fold faster than the simpler substrates (Freeman & Hopwood, 1986). Purified glucosamine-6-sulphatase hydrolysed complex substrates with aglycone IdoA2S residues with catalytic efficiencies up to 3900 times above that observed for monomeric substrates (Freeman & Hopwood, 1987). These heparan sulphate-degrading enzymes are inhibited by their products and by each other's substrate. We have interpreted these observations, which demonstrate the involvement of similar aglycone structures in binding and catalysis, to suggest that all of these enzymes operate in close proximity to each other, possibly as an enzyme complex bound to the lysosomal membrane (Clements *et al.*, 1985*b*; Freeman & Hopwood, 1986, 1987, 1989; Hopwood, 1989). This association would lead to co-operative action by the elimination of the need for 'intermediates' to diffuse from one enzyme to the next, and might well provide a mechanism for acceleration of heparan sulphate degradation to even higher rates than those observed for these enzymes in isolation.

This work was supported by grants from the National Health and Medical Research Council of Australia and the Adelaide Children's Hospital Research Foundation. Mr. Ross James (South Australian Forensic Science Centre) and Dr. Anthony Bourne (Department of Histopathology, Adelaide Medical Centre for Women and Children) supplied livers, kidneys and lungs from autopsy material. We thank Julie Hagedorn, Elaine Ravenscroft and Larissa Cox for expert technical assistance, and Dr. Gary Gibson for helpful discussion.

REFERENCES

Archer, I. M., Harper, P. S. & Wusteman, F. S. (1982) Biochim. Biophys. Acta **708**, 134–140
Cantz, M., Chrambach, A., Bach, G. & Neufeld, E. F. (1972) J. Biol. Chem. **247**, 5456–5462
Clements, P. R., Mahuran, D. & Hopwood, J. J. (1983) J. Chromatogr. **261**, 77–82
Clements, P. R., Brooks, D. A., Saccone, G. T. P. & Hopwood, J. J. (1985*a*) Eur. J. Biochem. **152**, 21–28
Clements, P. R., Muller, V. & Hopwood, J. J. (1985*b*) Eur. J. Biochem. **152**, 29–34
Clements, P. R., Brooks, D. A., McCourt, P. A. G. & Hopwood, J. J. (1989) Biochem. J. **259**, 199–208
Daniele, A. & Di Natale, P. (1987) Hum. Genet. **75**, 234–238
Di Natale, P. & Daniele, A. (1985) Biochim. Biophys. Acta **839**, 258–261
Di Natale, P. & Ronsisvalle, L. (1981) Biochim. Biophys. Acta **661**, 106–111
Freeman, C. & Hopwood, J. J. (1986) Biochem. J. **234**, 83–92
Freeman, C. & Hopwood, J. J. (1987) Biochem. J. **246**, 355–365
Freeman, C. & Hopwood, J. J. (1989) Biochem. J. **259**, 209–216
Freeman, C., Clements, P. R. & Hopwood, J. J. (1987) Biochem. J. **246**, 347–354
Gibson, G. J., Saccone, G. T. P., Brooks, D. A., Clements, P. R. & Hopwood, J. J. (1987) Biochem. J. **248**, 755–764
Hopwood, J. J. (1979) Carbohydr. Res. **69**, 203–216
Hopwood, J. J. (1989) in Heparin: Chemical and Biological Properties: Clinical Applications (Lane, D. & Lindahl, U., eds.), pp. 191–227, Edward Arnold, London
Hopwood, J. J. & Elliott, H. (1981) Carbohydr. Res. **91**, 165–190
Hopwood, J. J. & Elliott, H. (1982) Clin. Chim. Acta **123**, 241–250
Hopwood, J. J. & Muller, V. (1983) Carbohydr. Res. **122**, 227–239
Hopwood, J. J., Elliott, H., Muller, V. & Saccone, G. T. P. (1986) Biochem. J. **234**, 507–514
Karamanos, N. K., Hjerpe, A., Tsegenidis, T., Engfeldt, B. & Antonopoulos, C. A. (1988) Anal. Biochem. **172**, 410–419
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Lissens, W., Zenati, A. & Liebaers, I. (1984) Biochim. Biophys. Acta **801**, 365–371
Mahuran, D., Clements, P. R., Carella, M. & Strasberg, P. M. (1983*a*) Anal. Biochem. **129**, 513–516
Mahuran, D., Clements, P. & Hopwood, J. (1983*b*) Biochim. Biophys. Acta **757**, 359–365
McKusick, V. A. & Neufeld, E. F. (1983) in The Metabolic Basis of Inherited Disease (Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L. & Brown, M. S., eds.), 5th edn., pp. 751–771, McGraw-Hill, New York
Merle, P. & Kadenbach, B. (1980) Eur. J. Biochem. **105**, 499–507

Merril, C. R., Goldman, D., Sedman, S. A. & Ebert, M. H. (1981) Science **211**, 1437–1438
Neufeld, E. F. & Muenzer, J. (1989) in The Metabolic Basis of Inherited Disease (Scriver, C. R., Beaudet, A. L., Sly, W. S. & Valle, D., eds.), 6th edn., pp. 1565–1587, McGraw-Hill, New York
Shaklee, P. N., Glaser, J. H. & Conrad, H. E. (1985) J. Biol. Chem. **260**, 9146–9149
Tasheva, B. & Dessev, G. (1983) Anal. Biochem. **129**, 98–102
Wasteson, Å. & Neufeld, E. F. (1982) Methods Enzymol. **83**, 573–578
Wilson, P. J., Morris, C. P., Anson, D. S., Occhiodoro, T., Bielicki, J., Clements, P. R. & Hopwood, J. J. (1990) Proc. Natl. Acad. Sci. U.S.A., in the press
Yutaka, T., Fluharty, A. L., Stevens, R. L. & Kihara, H. (1982) J. Biochem. (Tokyo) **91**, 433–441

Received 14 February 1990/20 April 1990; accepted 1 May 1990