Biochem. J. (1987) 246, 355–365 (Printed in Great Britain)

Human liver *N*-acetylglucosamine-6-sulphate sulphatase

Catalytic properties

Craig FREEMAN and John J. HOPWOOD*
Department of Chemical Pathology, Adelaide Children's Hospital, King William Road, North Adelaide, South Australia 5006, Australia

Kinetic parameters (K_m and $k_{cat.}$) of the two major forms (A and B) and a minor form (C) of human liver *N*-acetylglucosamine-6-sulphate sulphatase [Freeman, Clements & Hopwood (1987) Biochem. J. **246**, 347–354] were determined with a variety of substrates matching structural aspects of the physiological substrates *in vivo*, namely heparin, heparan sulphate and keratan sulphate. Enzyme activity is highly specific towards glucosamine 6-sulphate or glucose 6-sulphate residues. More structurally complex substrates, in which several aspects of the aglycone structure of the natural substrate were maintained, are hydrolysed with catalytic efficiencies up to 3900 times above that observed for the monosaccharide substrate *N*-acetylglucosamine 6-sulphate. Forms A and B both desulphate substrates derived from keratan sulphate and heparin. Aglycone structures that influence substrate binding and/or enzyme activity were penultimate-residue 6-carboxy and 2-sulphate ester groups for heparin-derived substrates and penultimate-residue 6-sulphate ester groups for keratan sulphate-derived substrates. The 4-hydroxy group of the *N*-acetylglucosamine 6-sulphate or the 2-sulphaminoglucosamine 6-sulphate under enzymic attack is involved in the catalytic mechanism. The presence of a 2-amino group in place of a 2-acetamido or a 2-sulphoamino group considerably decreases the catalytic efficiency of the sulphatase, particularly in the absence of a penultimate-aglycone-residue 6-carboxy group. Both forms A and B are exo-enzymes, since activity towards internal sulphate ester bonds was not observed. The effect of incubation pH on enzyme activity towards the variety of substrates evaluated was complex and dependent on substrate aglycone structure. The presence of aglycone 2-sulphate ester, 6-carboxy group and 6-sulphate ester groups on the glucosamine 6-sulphate residue under attack considerably affects the pH response. Sulphate and phosphate ions are potent inhibitors of enzyme activity.

INTRODUCTION

The sulphated glycosaminoglycans heparin and heparan sulphate are degraded from their non-reducing end by the sequential action of at least five lysosomal sulphatases and four other exo-enzyme activities [1,2]. The clinical expression of the Sanfilippo syndrome (mucopolysaccharidosis type III, MPS III) in humans may result from a deficiency of one of four enzyme activities leading to the accumulation of only heparan sulphate [3]. In these autosomal recessive disorders accumulating partially degraded heparan sulphate in the urine and tissues of patients results in progressive mental retardation and mild skeletal deformities [3]. The de-*O*-sulphation of α-linked *N*-acetylglucosamine 6-sulphate (GlcNAc6S) residues in heparan sulphate requires a specific sulphatase, *N*-acetylglucosamine-6-sulphate sulphatase (6SS), which has been shown to be deficient in cultured skin fibroblasts in Sanfilippo D (MPS IIID) patients [4]. We have recently reported the isolation of four forms of 6SS present in human liver that were active towards the heparin-derived disaccharide

Abbreviations used (for simplicity, IdOA has been used to abbreviate what is a mixture of reduced uronic acid species containing mostly IdOA [5]): GlcNAc6S, *N*-acetyl[1-^{14}C]glucosamine 6-sulphate; GlcNAc3S, *N*-acetyl[1-^{14}C]glucosamine 3-sulphate; Glc6S, [1-^{14}C]glucose 6-sulphate; ManNAc6S, *N*-acetyl[1-^{14}C]mannosamine 6-sulphate; GalNAc6S, *N*-acetyl[1-^{14}C]galactosamine 6-sulphate; GlcNAc6S-Ido, *O*-(α-*N*-acetylglucosamine 6-sulphate)-(1→4)-L-[6-^{3}H]idose; GlcNS6S-Ido, *O*-(α-2-sulphaminoglucosamine 6-sulphate)-(1→4)-L-[6-^{3}H]idose; GlcNH6S-Ido, *O*-(α-glucosamine 6-sulphate)-(1→4)-L-[6-^{3}H]idose; GlcNAc6S-IdOA, *O*-(α-*N*-acetylglucosamine 6-sulphate)-(1→3)-L-[6-^{3}H]idonic acid; GlcNS6S-IdOA, *O*-(α-2-sulphaminoglucosamine 6-sulphate)-(1→3)-L-[6-^{3}H]idonic acid; GlcNH6S-IdOA, *O*(-α-glucosamine 6-sulphate)-(1→3)-L-[6-^{3}H]idonic acid; GlcNAc6S-Ido2S, *O*-(α-*N*-acetylglucosamine 6-sulphate)-(1→4)-L-[6-^{3}H]idose 2-sulphate; GlcNS6S-Ido2S, *O*-(α-2-sulphaminoglucosamine 6-sulphate)-(1→4)-L-[6-^{3}H]idose 2-sulphate; GlcNAc6S-IdoA2S-anM6S, *O*-(α-*N*-acetylglucosamine 6-sulphate)-(1→4)-L-*O*-(α-iduronic acid 2-sulphate)-(1→4)-*O*-D-2,5-anhydro[1-^{3}H]mannitol 6-sulphate; GlcNS6S-IdoA2S-anM6S, *O*-(α-2-sulphaminoglucosamine 6-sulphate)-(1→4)-L-*O*-(α-iduronic acid 2-sulphate)-(1→4)-D-*O*-2,5-anhydro[1-^{3}H]mannitol 6-sulphate; GlcNH6S-IdoA2S-anM6S, *O*-(α-glucosamine 6-sulphate)-(1→4)-L-*O*-(α-iduronic acid 2-sulphate)-(1→4)-D-*O*-2,5-anhydro[1-^{3}H]mannitol 6-sulphate; IdoA2S-GlcNS6S-IdoA2S-anM6S, *O*-(α-iduronic acid 2-sulphate)-(1→4)-D-*O*-(α-2-sulphaminoglucosamine 6-sulphate)-(1→4)-L-*O*-(α-iduronic acid 2-sulphate)-(1→4)-D-*O*-2,5-anhydro[1-^{3}H]mannitol 6-sulphate; GlcNAc6S-Galitol, *O*-(β-*N*-acetylglucosamine 6-sulphate)-(1→3)-D-[1-^{3}H]galactitol; GlcNAc6S-Gal-GlcNAc6S-Galitol, *O*-(β-*N*-acetylglucosamine 6-sulphate)-(1→3)-D-*O*-(β-galactose)-(1→4)-D-*O*-(β-*N*-acetylglucosamine 6-sulphate)-(1→3)-D-[1-^{3}H]galactitol; GlcNAc6S-Gal6S-GlcNAc6S-Galitol, *O*-(β-*N*-acetylglucosamine 6-sulphate)-(1→3)-D-*O*-(β-galactose 6-sulphate)-(1→4)-D-*O*-(β-*N*-acetylglucosamine 6-sulphate)-(1→3)-D-[1-^{3}H]galactitol; GalNAc6S-GlcA-GalitolNAc6S, *O*-(β-*N*-acetylgalactosamine 6-sulphate)-(1→4)-D-*O*-(β-glucuronic acid)-(1→3)-D-*N*-acetyl[1-^{3}H]galactosaminitol 6-sulphate; GalNAc4S-GlcA-GalitolNAc4S, *O*-(β-*N*-acetylgalactosamine 4-sulphate)-(1→4)-D-*O*-(β-glucuronic acid)-(1→3)-D-*N*-acetyl[1-^{3}H]galactosaminitol 4-sulphate; GlcNAc6S-GlcA-anM, *O*-(α-*N*-acetylglucosamine 6-sulphate)-(1→4)-D-*O*-(β-glucuronic acid)-(1→4)-D-*O*-2,5-anhydro-[1-^{3}H]mannitol; 6SS, *N*-acetylglucosamine-6-sulphate sulphatase; MPS, mucopolysaccharidosis.

* To whom correspondence should be addressed.

GlcNAc6S-IdOA [5]. The two major forms of 6SS were purified more than 50000-fold to homogeneity in 78% yield. They both had a pI value greater than 9.5 and a native molecular mass of 75 kDa. However, assay by SDS/polyacrylamide-gel electrophoresis of the two forms after their separation by hydroxyapatite chromatography revealed that form A contained a polypeptide of molecular mass 78 kDa whereas form B contained two polypeptides, of molecular masses 48 and 32 kDa. Two low-abundance forms (C and D) had pI values of 5.8 and 5.4 respectively.

A single form of 6SS activity towards a heparan sulphate-derived trisaccharide (GlcNAc6S-GlcA-anM), purified in low yield from human urine, had a molecular mass of 95 kDa with pI values between 5.4 and 8.2. It was active towards the monosaccharides GlcNAc6S and Glc6S, but not GalNAc6S, ManNAc6S or Glc3S [6]. Evidence of a 6-sulphate sulphatase activity towards a heparin-derived substrate GlcNS6S-IdoA2S-anM6S as distinct from 6SS activity towards the *N*-acetylated derivative GlcNAc6S-IdoA2S-anM6S was reported to be present in bovine kidney extracts [7]. Habuchi *et al.* [8] has shown, after separation from other sulphatases, a 6-sulphate sulphatase activity in rat skin with molecular mass 70 kDa that was active towards both heparan sulphate and keratan sulphate as well as a keratan sulphate-derived tetrasaccharide GlcNAc6S-Gal6S-GlcNAc6S-Galitol. The de-*O*-sulphation of β-linked GlcNAc6S residues that occur in keratan sulphate results from the action of the same 6SS enzyme that acts upon heparan sulphate in cultured human skin fibroblasts [9]. However, MPS IIID patients excrete excessive amounts of heparan sulphate but not keratan sulphate in their urine [10–12]. The accumulation of keratan sulphate in these patients is prevented by the action of an alternative pathway for keratan sulphate degradation in which the A form of β-*N*-acetylhexosaminidase hydrolyses the non-reducing-end β-linked GlcNAc6S to produce the monosaccharide GlcNAc6S, which is found in increased above-normal amounts in the urine of MPS IIID patients [9–12]. We have shown that GlcNAc6S produced by β-*N*-acetylhexosaminidase A digestion of keratan sulphate-derived oligosaccharides is specifically desulphated by the 6SS in normal fibroblasts, which is deficient in MPS IIID skin fibroblasts [10,11]. Matalon *et al.* [13], however, presented details of a patient with heparansulphaturia and keratansulphaturia and diminished activity towards the keratan sulphate-derived disaccharide GlcNAc6S-Galitol in skin fibroblasts. The presence of excessive amounts of both heparan sulphate and keratan sulphate in the urine led to a proposal that two 6SS isoenzymes were present, one specific for heparan sulphate with enzyme deficiency resulting in heparansulphaturia (MPS IIID) and the other specific for both heparan sulphate and keratan sulphate with enzyme deficiency resulting in heparansulphaturia and keratansulphaturia [13].

In studies concerning the development of a variety of oligosaccharide structures for use as diagnostic substrates for lysosomal enzymes concerned with glycosaminoglycan degradation, we have reported a considerable effect of aglycone structure on expressed enzyme activity in incubations containing homogenate of cultured human skin fibroblasts, amniotic cells or leucocytes [14–26,29] and highly purified enzyme isolated from liver such as sulphamate sulphohydrolase [27] and α-L-iduronidase [28]. Substrates that possess as many as possible of the structural features of the natural substrate generally result in the greatest rate of hydrolysis and have been shown to be useful for the detection of residual enzyme activities in some patients. This has allowed correlation of residual activity with the degree of clinical severity, and the unequivocal determination of specific enzyme activity during a purification procedure of an enzyme from tissue homogenates. In the present paper we have focused attention on the structural requirements for catalysis of substrates by various forms of 6SS purified from human liver and clarified some ambiguities raised by other studies introduced above [6–13].

MATERIALS AND METHODS

Substrates

The radiolabelled monosaccharides GlcNAc6S, ManNAc6S, GalNAc6S and Glc6S and the disaccharides GlcNH6S-Ido, GlcNAc6S-Ido, GlcNAc6S-Ido2S, GlcNS6S-Ido and GlcNS6S-Ido2S were prepared as described previously [14,15,19,20]. GlcNH6S-IdOA was prepared from heparin [5] and either *N*-acetylated or *N*-sulphated [16] to give GlcNAc6S-IdOA or GlcNS6S-IdOA. GlcNAc6S-Galitol, GlcNAc6S-Gal-GlcNAc6S-Galitol and GlcNAc6S-Gal6S-GlcNAc6S-Galitol were prepared from bovine intervertebral-disc keratan sulphate by digestion with endo-β-galactosidase [9]. GlcNS6S-IdoA2S-anM6S was prepared by enzymic digestion (purified human liver sulphoiduronate sulphohydrolase and α-L-iduronidase, obtained from J. Bielicki and P. R. Clements of this Department) of a pentasulphated tetrasaccharide (IdoA2S-GlcNS6S-IdoA2S-anM6S) isolated from the nitrous acid degradation of heparin [15]. GlcNS6S-IdoA2S-anM6S was digested with purified human liver sulphamate sulphohydrolase [27] to produce GlcNH6S-IdoA2S-anM6S. This de-*N*-sulphated trisaccharide was *N*-acetylated [16] to produce GlcNAc6S-IdoA2S-anM6S. These trisaccharide substrates were purified by ion-exchange chromatography on Dowex 1 and then desalted on Sephadex G-10. GalNAc6S-GlcA-GalitolNAc6S and GalNAc4S-GlcA-GalitolNAc4S were prepared from chondroitin 6-sulphate and chondroitin 4-sulphate respectively [30]. GlcNAc3S was a gift from J. A. Bruce (University of Aberdeen, Aberdeen, U.K.). The structures of the substrates used in the present study are shown in Fig. 1.

N-Acetylglucosamine-6-sulphate sulphatase

Forms A, B, C and D were isolated from human liver as described in the preceding paper [5]. Form A and form B, the two major forms of 6SS present in liver, were separated by hydroxyapatite chromatography. Forms A, B and C enzyme preparations were free from other activities involved in heparan sulphate and keratan sulphate degradation: sulphamate sulphohydrolase, α-*N*-acetylglucosaminidase, sulphoiduronate sulphohydrolase, acetyl-CoA:glucosamine *N*-acetyltransferase, α-L-iduronidase, β-*N*-acetylhexosaminidase, galactose-6-sulphate sulphatase and β-D-glucuronidase [9,15,18,19,21,22,27,30]. Form D was only partially purified, and contained sulphamate sulphohydrolase,

β-N-acetylhexosaminidase and N-acetylgalactosamine-4-sulphate sulphatase activities. Protein concentration was determined by the method of Markwell *et al.* [31]. Sigma Chemical Co. (St. Louis, MO, U.S.A.) supplied crystalline globulin-free bovine serum albumin.

Kinetic experiments

Enzyme was dialysed overnight at 4 °C against 2 litres of 20 mM-Tris/HCl buffer, pH 7.4, containing 10% (v/v) glycerol, 0.5 M-NaCl and 0.1 mM-dithiothreitol.

The pH–activity profiles were obtained by using a standard 20 μl assay mixture containing substrate, 60 mM-sodium acetate buffer (pH as indicated), 0.1 mM-dithiothreitol and bovine serum albumin (50 μg/ml) (where appropriate). Reactions were initiated by the addition of 1 μl of dialysed enzyme (equivalent to 50 ng of protein) and incubation at 37 °C. Incubation times were optimized to give a percentage breakdown of substrate to product in the range 5–20%, which was linear with respect to time. Concentrations of each substrate and incubation time for the generation of pH–activity profiles were: GlcNAc6S, 6.8 μM and 8 h; Glc6S, 95 μM and 16 h; GlcNAc6S-Ido, 5 μM and 15.5 h; GlcNS6S-Ido, 5 μM and 9.5 h; GlcNAc6S-Ido2S, 10 μM and 8 h; GlcNS6S-Ido2S, 12.5 μM and 4 h; GlcNAc6S-IdOA, 15 μM and 0.5 h; GlcNS6S-IdOA, 15 μM and 0.5 h; GlcNH6S-IdOA, 7.5 μM and 3 h; GlcNAc6S-IdoA2S-anM6S, 1.6 μM and 5 min; GlcNS6S-IdoA2S-anM6S, 7.5 μM and 5 min; GlcNH6S-IdoA2S-anM6S, 1.6 μM and 1.5 h; GlcNAc6S-Galitol, 5 μM and 9.5 h; GlcNAc6S-Gal-GlcNAc6S-Galitol, 2.5 μM and 15.5 h; GlcNAc6S-Gal6S-GlcNAc6S-Galitol, 3.2 μM and 40 min. Reactions were terminated by plunging the reaction vials into a solid-CO_2/ethanol bath. The substrate was separated from the product for all substrates except the trisaccharides by high-voltage electrophoresis on Whatman 3MM chromatography paper in 1.74 M-formic acid, pH 1.7, at 45 V/cm for 40 min in a Shandon Southern model L-24 system (Shandon Southern Products, Runcorn, Cheshire, U.K.). The trisaccharide substrates GlcNS6S-IdoA2S-anM6S, GlcNH6S-IdoA2S-anM6S and GlcNAc6S-IdoA2S-anM6S were separated from their respective products (GlcNS-IdoA2S-anM6S, GlcNH-IdoA2S-anM6S and GlcNAc-IdoA2S-anM6S) by high-voltage electrophoresis on Whatman 3MM paper in 1.74 M-formic acid, pH 1.7, containing 0.10 M-NaCl at 23 V/cm for 1.5 h in the same equipment. The strips were scanned on a Packard model 7201 radiochromatogram scanner (Packard, Chicago, IL, U.S.A.). 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 Beckman EPS Ready-Solv (Gladesville, N.S.W., Australia) 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 the 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 profile. Substrate concentration ranged from 0.1 to 100 μM. Incubation time was adjusted to be within the linear range for substrate hydrolysis. All incubations were at the optimal pH for each substrate. K_m and $V_{max.}$ values were obtained from Lineweaver–Burk plots.

RESULTS

Effect of bovine serum albumin upon enzyme stability

Incubation of form A or B of 6SS in 60 mM-sodium acetate buffer, pH 5.0, with GlcNAc6S-IdOA at a concentration of 7.5 μM resulted in enzyme activity towards the substrate remaining linear with respect to time for only 20 min. Incubation times longer than 20 min resulted in a dramatic decrease in the rate of formation of product. The rate of product formation with respect to enzyme protein, for up to at least 60 ng of enzyme protein per incubation, was linear for both form A and form B over a 20 min period of incubation. The presence of bovine serum albumin at a concentration of 50 μg/ml resulted in a linear rate of product formation with respect to time for up to 90 min and with conversion of substrate into product up to 20%. The rate of conversion of substrate into product was higher in incubations containing bovine serum albumin. The concentration of bovine serum albumin to given maximal conversion of the substrates GlcNAc6S-IdOA and GlcNS6S-IdOA into their products was 50 μg/ml for both form A and form B (Fig. 2). Form A and form B activities towards GlcNAc6S-IdOA were increased by 57% and 105% respectively over enzyme activities in the absence of bovine serum albumin. Form A and form B activities towards GlcNS6S-IdOA were increased by 87% and 159% respectively in the presence of bovine serum albumin. The pH optima of enzyme activity towards the substrates were unaffected by the presence of bovine serum albumin (see below, Fig. 3).

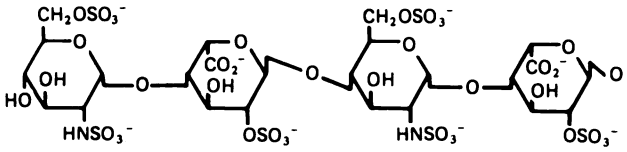
Incubation of form A or B with 7.5 μM-GlcNH6S-IdOA at pH 5.0 in the absence of bovine serum albumin resulted in the linearity of product formation with time for a period of 20 min. However, in the presence of bovine serum albumin (50 μg/ml) the rate of product formation was linear for 20 h, with 18% and 10% conversion of substrate into product for form A and form B respectively. Form A and form B incubations with 7.5 μM-GlcNAc6S at pH 5.7 in the presence of bovine serum albumin produced GlcNAc at a linear rate for at least 12 h. The mechanism by which albumin stimulates and stabilizes enzyme activity is unknown.

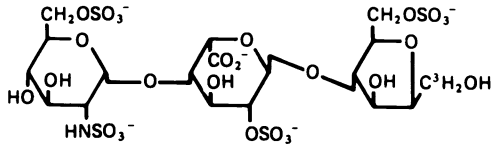
Effect of dithiothreitol upon 6SS activity

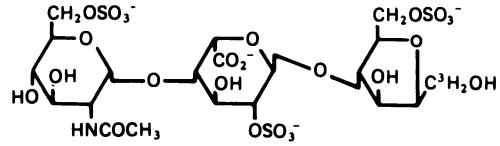
As form A and form B were dialysed in buffer containing 0.1 mM-dithiothreitol, the concentration routinely used during the enzyme purification steps [5], the initial concentration of dithiothreitol within the assay mixture was 5 μM. The addition of dithiothreitol to the assay mixture resulted in a maximum 6SS activity at a final concentration of 0.1 mM-dithiothreitol stimulating form A and form B activities towards GlcNAc6S-IdOA by 16% and 13% respectively.

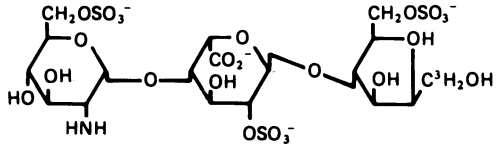
Effect of pH upon 6SS activity

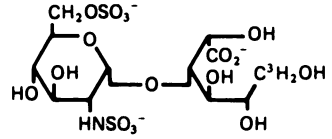
The pH optimum of form A was tested with each substrate and was shown to be dependent on the substrate structure (Fig. 3). The pH–activity profiles for substrates assessed with forms B and C, although generally having 30–50% lower enzyme activities than obtained with form A, were equivalent to the profiles obtained with form A. Although lower overall enzyme activities were obtained for both form A and form B, the pH–activity profiles for GlcNAc6S-IdOA and GlcNS6S-IdOA assessed in the absence of bovine serum albumin

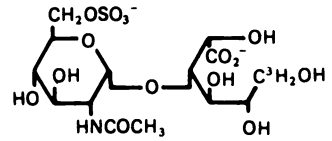

Heparan sulphate

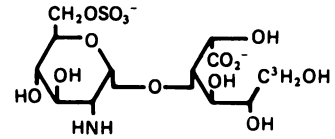

GlcNS6S-IdoA2S-anM6S

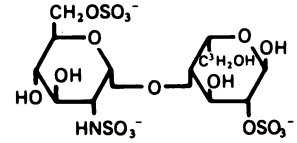

GlcNAc6S-IdoA2S-anM6S

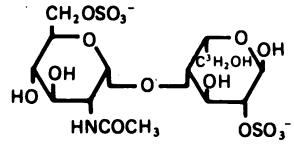

GlcNAc6S-IdoA2S-anM6S

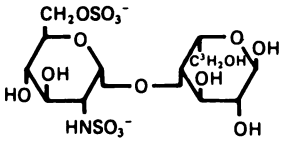

GlcNS6S-IdOA

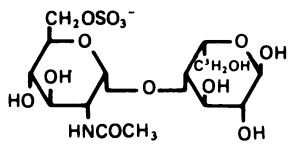

GlcNAc6S-IdOA

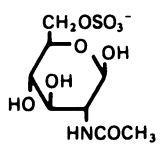

GlcNH6S-IdOA


GlcNS6S-Ido2S


GlcNAc6S-Ido2S


GlcNS6S-Ido


GlcNAc6S-Ido


GlcNAc6S

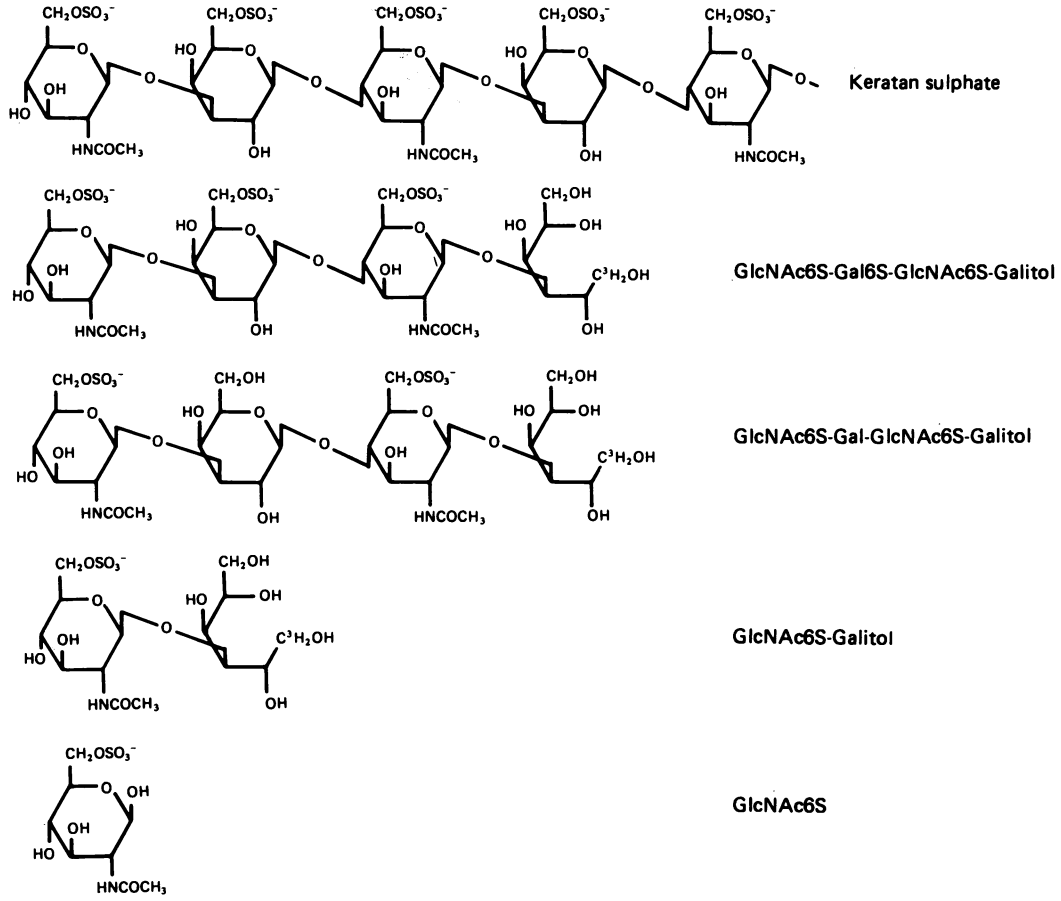


Fig. 1. Structures of substrates of 6SS used in this study, together with the natural substrates from which they were derived

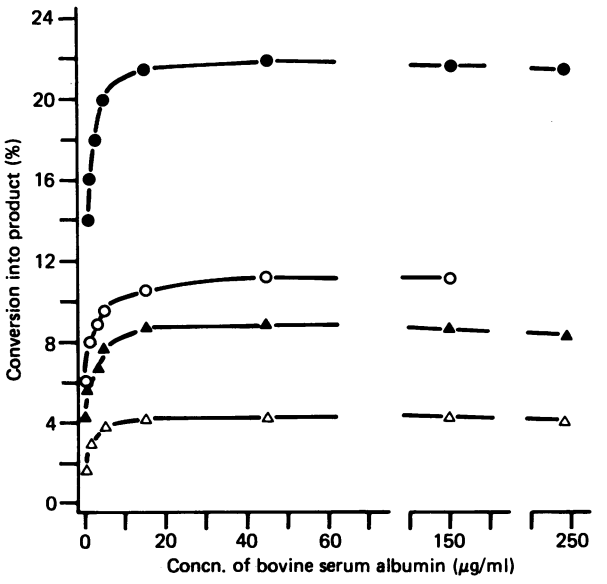


Fig. 2. Hydrolysis by 6SS of GlcNAc6S-IdOA and GlcNS6S-IdOA as a function of bovine serum albumin concentration

For experimental details see the Materials and methods section. The Figure shows the hydrolysis of GlcNAc6S-IdOA (●) and GlcNS6S-IdOA (▲) by form A and of GlcNAc6S-IdOA (○) and GlcNS6S-IdOA (△) by form B.

were similar to those obtained in the presence of bovine serum albumin.

Activity towards both the monosaccharides Glc6S and GlcNAc6S had a pH optimum of 5.7. The addition of an α-(1→4)-idose residue or a β-(1→3)-galactitol residue to GlcNAc6S to produce the disaccharide substrates GlcNAc6S-Ido or GlcNAc6S-Galitol respectively resulted in a shift of the pH optimum from 5.7 to 5.4 and to 5.0 respectively. The presence of a 6-carboxy group on the open-ring idose residue in the substrate GlcNAc6S-IdOA resulted in a shift in pH optimum from 5.4 to 5.0. In disaccharide substrates that contain idonic acid, variations at the 2-amino position of glucosamine, such as GlcNAc6S-IdOA, GlcNS6S-IdOA and GlcNH6S-IdOA, did not alter the pH optimum of hydrolysis. However, for disaccharide substrates that contain idose, similar structural variations GlcNAc6S-Ido, GlcNS6S-Ido and GlcNH6S-Ido resulted in pH optima of 5.4, 4.8 and no detectable activity (after 20 h at 37 °C at pH from 3 to 6.5) respectively. The addition of an idose 2-sulphate ester to GlcNAc6S-Ido and GlcNS6S-Ido lowered pH optima by 0.9 and 0.6 of a pH unit respectively. The trisaccharide substrates with the IdoA2S aglycone residues, particularly GlcNS6S-IdoA2S-anM6S, had the lowest pH optima for heparan sulphate-like structures. Unlike the optima obtained for substrate hydrolysis of structurally complex substrates with sulphamate sulphohydrolase [27], only single pH optima were observed for

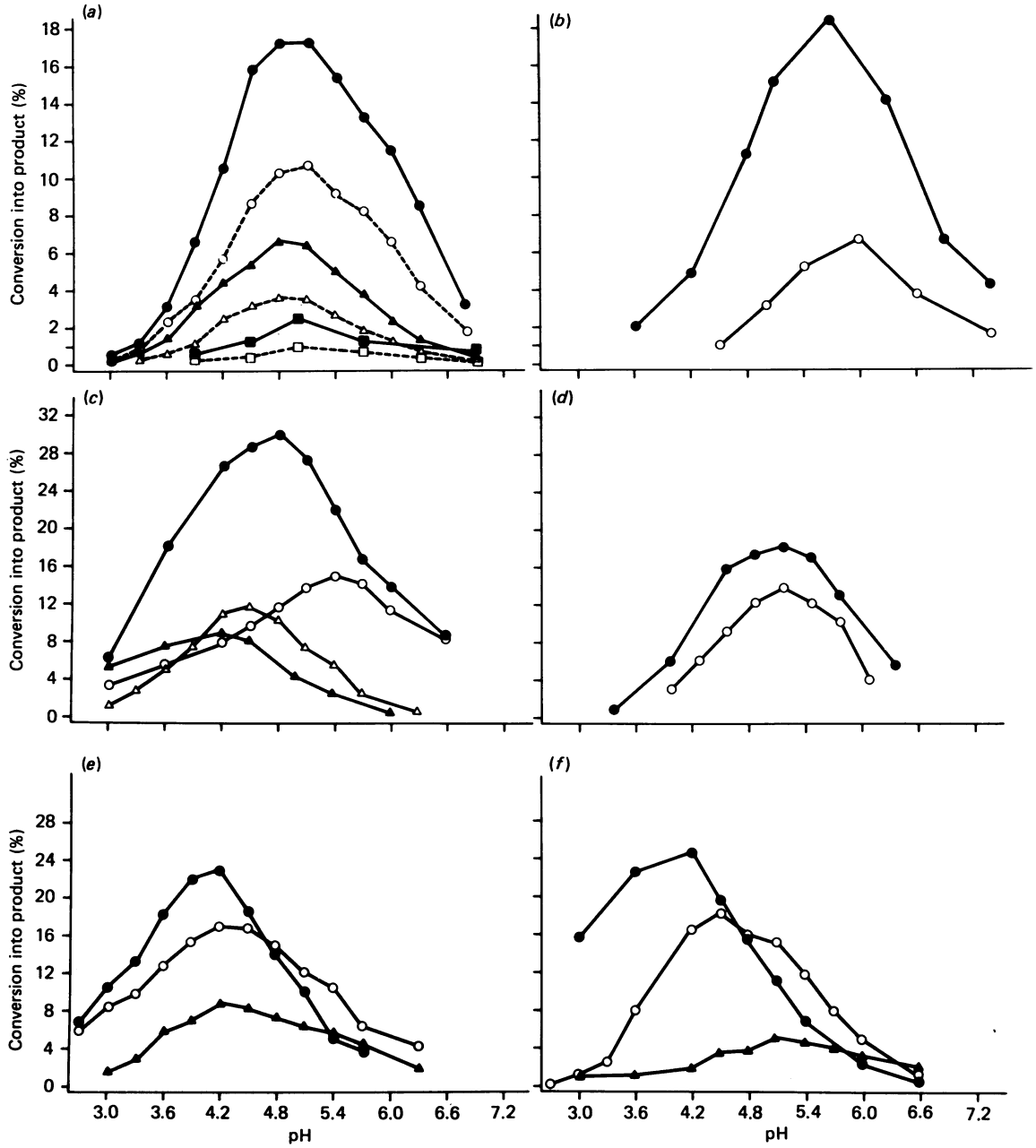


Fig. 3. Hydrolysis by 6SS of a variety of substrates as a function of incubation pH

For experimental details see the Materials and methods section. (*a*) Hydrolysis of GlcNAc6S-IdOA by form A (●) and form B (○), of GlcNS6S-IdOA by form A (▲) and form B (△), and of GlcNH6S-IdOA by form A (■) and form B (□). (*b*) Hydrolysis of GlcNAc6S (●) and Glc6S (○) by form A. (*c*) Hydrolysis of GlcNS6S-Ido (●), GlcNAc6S-Ido (○), GlcNS6S-Ido2S (▲) and GlcNAc6S-Ido2S (△) by form A. (*d*) Hydrolysis of GlcNAc6S-IdOA by form A with (●) and without (○) bovine serum albumin (50 μg/ml). (*e*) Hydrolysis of GlcNS6S-IdoA2S-anM6S (●), GlcNAc6S-IdoA2S-anM6S (○) and GlcNH6S-IdoA2S-anM6S (▲) by form A. (*f*) Hydrolysis of GlcNAc6S-Gal6S-GlcNAc6S-Galitol (●), GlcNAc6S-Gal-GlcNAc6S-Galitol (○) and GlcNAc6S-Galitol (▲) by form A.

6SS de-*O*-sulphation of similarly complex substrates (Fig. 3).

Extension of the disaccharide GlcNAc6S-Galitol to the tetrasaccharide GlcNAc6S-Gal-GlcNAc6S-Galitol, which contained both an internal and a non-reducing-end 6-sulphated *N*-acetylglucosamine residue, resulted in a shift in pH optimum from 5.0 to 4.5. The further addition of a 6-sulphate ester to give the tetrasaccharide GlcNAc6S-Gal6S-GlcNAc6S-Galitol resulted in a further decrease in pH optimum to 3.9.

The monosaccharides GalNAc6S, GlcNAc3S and ManNAc6S and 4- or 6-sulplated trisaccharides derived from chondroitin sulphates were not substrates for form A or B over the pH range tested (pH 3–6.5). However, GlcNAc3S at pH greater than 7.5 in 60 mM-Tris/acetate buffer was desulphated in the absence of enzyme. This is

Table 1. Human liver 6SS activity towards a variety of substrates

For experimental details see the Materials and methods section. Activity towards GalNAc6S, ManNAc6S, GlcNAc3S, GlcNH6S-Ido, IdoA2S-GlcNS6S-IdoA2S-anM6S, GalNAc6S-GlcA-GalitolNAc6S and GalNAc4S-GlcA-GalitolNAc4S was not detected. Values in parentheses refer to assays in the absence of bovine serum albumin.

	Form A					Form B		
Substrate	pH optimum	K_m (μM)	$k_{cat.}$ (turnover number) (mol/min per mol of enzyme)	$10^{-3} \times k_{cat.}/K_m$ (catalytic efficiency)	Relative catalytic efficiency*	K_m (μM)	$10^{-3} \times k_{cat.}/K_m$	Ratio of catalytic efficiencies: form A/ form B
Glc6S	5.7	62.5	0.585	9.4	0.4	62.5	7	1.3
GlcNAc6S	5.7	7.1	0.165	23.2	1.0	10.0	11	2.1
GlcNAc6S-Ido	5.4	8.0	0.105	13.1	0.6	8.0	7.5	1.8
GlcNS6S-Ido	4.8	10.8	0.518	47.9	2.1	9.2	25.3	1.9
GlcNAc6S-Ido2S	4.5	3.6	0.165	45.8	2.0	–	–	–
GlcNS6S-Ido2S	4.2	2.3	0.201	87.5	3.8	–	–	–
GlcNAc6S-IdOA	5.0	11.1	7.90	712	31	14.3	299	2.4
GlcNAc6S-IdOA	(5.0)	(10.0)	(6.79)	(679)	(29)	(13.2)	(241)	(2.8)
GlcNS6S-IdOA	5.0	8.0	2.46	307	13	8.3	139	2.2
GlcNS6S-IdOA	(5.0)	(7.6)	(1.725)	(227)	(9.8)	(6.6)	(91)	(2.5)
GlcNH6S-IdOA	5.0	12.5	0.068	5.5	0.2	14.3	2.6	2.1
GlcNS6S-IdoA2S-anM6S	4.1	0.25	22.69	90760	3912	–	–	–
GlcNAc6S-IdoA2S-anM6S	4.3	0.76	7.08	9315	402	–	–	–
GlcNH6S-IdoA2S-anM6S	4.3	0.35	0.11	314	14	–	–	–
GlcNAc6S-Galitol	5.0	2.2	0.017	7.8	0.3	2.8	4	2.0
GlcNAc6S-Gal-GlcNAc6S-Galitol	4.5	1.7	0.042	25	1.1	–	–	–
GlcNAc6S-Gal6S-GlcNAc6S-Galitol	3.9	1.0	0.473	473	20	–	–	–

* $k_{cat.}/K_m$ calculated relative to a value for GlcNAc6S = 1.

probably a result of the β-elimination of C-3 substituents known to occur with reducing sugars [32].

Kinetic properties of purified 6SS

Comparison of the binding and catalysis of various substrates can be made from the data shown in Table 1. K_m values were generally determined in the presence of 25 mM-NaCl and 50 μg of bovine serum albumin/ml as well as 0.1 M-dithiothreitol and should not be taken as absolute.

Neither form A nor form B was active towards GlcNAc3S, indicating specificity for the 6-sulphate ester. The monosaccharides GalNAc6S and ManNAc6S and the trisaccharides GalNAc6S-GlcA-GalitolNAc6S and GalNAc4S-GlcA-GalitolNAc4S also were not substrates, indicating the important influence on the expression of enzyme activity of the 4-hydroxy group, the position of the sulphate ester group and the positioning of the 2-amino ester groups of the target monosaccharide unit. The simplest substrate for 6SS was the monosaccharide Glc6S, for which both form A and form B had a K_m value of 62.5 μM. The catalytic efficiency of form B was 75% that of form A. All other substrates tested against both form A and form B generally had a $k_{cat.}$ for form B in the range 42–55% of the $k_{cat.}$ for form A. The addition of a 2-acetamido group to Glc6S to give GlcNAc6S resulted in 9- and 6-fold decreases in K_m for form A and form B respectively. The catalytic efficiencies of forms A and B towards GlcNAc6S were 2.5-fold and 1.6-fold higher respectively compared with that observed for Glc6S.

The extension of GlcNAc6S by linkage α-(1→4) with idose to give the disaccharide GlcNAc6S-Ido resulted in only a slight, perhaps insignificant, change in the K_m value for both form A and form B, and a 30% decrease in the turnover number and catalytic efficiency for form A whereas the turnover number was decreased 50% for form B. The extension of GlcNAc6S by β-(1→3) linkage with galactitol to give GlcNAc6S-Galitol resulted in K_m values 3.5-fold and 2.5-fold lower than GlcNAc6S-Ido for forms A and B respectively. The turnover numbers for GlcNAc6S-Ido by forms A and B were 5–6-fold higher than that found for GlcNAc6S-Galitol. The presence of a 2-sulphate group on GlcNAc6S-Ido to give GlcNAc6S-Ido2S resulted in the K_m of form A being more than halved from 8.0 to 3.6 μM, a turnover number increase by 50% and a 3.4-fold increase in catalytic efficiency. When the 2-*N*-acetyl group on GlcNAc6S-Ido was removed to give GlcNH6S-Ido, it was no longer a substrate for either form A or form B. Substitution of the 2-acetyl group on GlcNAc6S-Ido by a sulphate group to give GlcNS6S-Ido resulted in a slightly higher K_m value for both form A and form B and an increased (4–5-fold) turnover number for both form A and form B, resulting in an overall 3.5-fold increase in 6SS catalytic efficiency towards GlcNS6S-Ido compared with GlcNAc6S-Ido.

The addition of a 6-carboxy group to GlcNAc6S-Ido to give GlcNAc6S-IdOA resulted in an increase in the K_m value and 75-fold and 70-fold increases in the turnover number for forms A and B respectively. Compared with form A, form C hydrolysed GlcNAc6S-IdOA with a similar K_m value (11 μM) but at half the catalytic efficiency. Removal of the 2-*N*-acetyl group from GlcNAc6S-IdOA to give GlcNH6S-IdOA resulted in a slightly increased K_m value for form A and no change for form B. On the other hand, compared with GlcNAc6S-IdOA, turnover number and catalytic efficiency were decreased more than 100-fold for GlcNH6S-IdOA by both form A and form B. The influence of the 6-carboxy group was further emphasized since GlcNH6S-Ido was not a substrate, whereas GlcNH6S-IdOA was a substrate, albeit a poor one. An *N*-sulphate in place of the *N*-acetyl group on GlcNAc6S-IdOA to give GlcNS6S-IdOA resulted in a lowered K_m and turnover numbers leading to a halving of catalytic efficiency towards GlcNS6S-IdOA by forms A and B. These results contrast with those for GlcNAc6S-Ido compared with GlcNS6S-Ido, where there was a slight decrease in substrate-binding affinity, with a 3-fold increase in catalytic efficiency. As for forms A and B, form C at pH 5.0 hydrolysed GlcNS6S-IdOA at two-thirds the rate of hydrolysis of GlcNAc6S-IdOA.

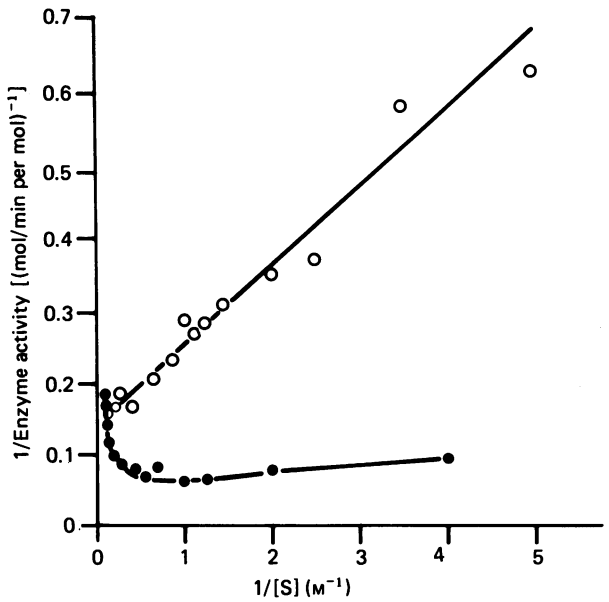


Fig. 4. Hydrolysis by form A 6SS of GlcNS6S6-IdoA2S-anM6S (●) and GlcNAc6S-IdoA2S-anM6S (○) as a function of substrate concentration

For experimental details see the Materials and methods section.

The presence of IdoA2S-aglycone residues (GlcNS6S-IdoA2S-anM6S, GlcNAc6S-IdoA2S-anM6S and GlcNH6S-IdoA2S-anM6S) resulted in a considerable decrease in form A K_m and an increase in $k_{cat.}$ for all three trisaccharide substrates compared with their disaccharide equivalents evaluated in Table 1. Form A 6SS de-*O*-sulphated the trisaccharide GlcNS6S-IdoA2S-anM6S with a 127-fold increase in catalytic efficiency compared with the most efficient disaccharide (GlcNAc6S-IdOA). Replacement of GlcNS6S in the trisaccharide substrate with GlcNAc6S or GlcNH6S residues resulted in a decrease in catalytic efficiency by 10-fold and 280-fold respectively. Form A was grossly inhibited by substrate GlcNS6S-IdoA2S-anM6S (at concentrations above 5 μM) but not by the other *N*-acetylated trisaccharide substrate (Fig. 4) or GlcNH6S-IdoA2S-anM6S (results not shown). A re-plot ($1/v$ versus [S]) of the data (Fig. 4) for GlcNS6S-IdoA2S-anM6S in accordance with Dixon & Webb [33] indicates that 6SS is able to bind two molecules of substrate, one with a K_m of 0.25 μM and the second with a K_m of 4.9 μM.

Incubation of forms A and B with IdoA2S-GlcNS6S-IdoA2S-anM6S under conditions (18 h, pH 4.0 and 5.0) that normally would de-*O*-sulphate the GlcNS6S residues in GlcNS6S-IdoA2S-anM6S more than 50 times over produced no detectable desulphated products.

Extension of the disaccharide GlcNAc6S-Galitol to the tetrasaccharide GlcNAc6S-Gal-GlcNAc6S-Galitol resulted in stronger binding of substrate to form A and a 3.7-fold increase in catalytic efficiency. The presence of a 6-sulphate ester on the galactose residue in the substrate GlcNAc6S-Gal6S-GlcNAc6S-Galitol further decreased the K_m value for form A and 18-fold increased the catalytic efficiency. Form B hydrolysed GlcNAc6S-Gal6S-GlcNAc6S-Galitol at half the rate of form A.

Effect of bovine serum albumin on kinetic properties

As well as its effect upon enzyme stability, bovine serum albumin had an effect upon the binding and hydrolysis of substrates. The K_m of form A towards GlcNAc6S-IdOA and GlcNS6S-IdOA was slightly, perhaps insignificantly, increased by 10% and 5% respectively and the turnover number increased by 17% and 42% respectively in the presence of albumin (Table 1). In the case of form B, the K_m towards GlcNAc6S-IdOA and GlcNS6S-IdOA was increased by 25% and 8% respectively and turnover number increased by 35% and 92% respectively in the presence of bovine serum albumin.

Effect of salts upon 6SS activity towards GlcNAc6S-IdOA

In a study similar to that carried out with leucocyte and fibroblast extracts [26] we examined the effect of increasing concentrations of NaCl, Na_2SO_4, Na_2HPO_4 and $CuCl_2$ upon enzyme activity towards the substrate GlcNAc6S-IdOA during incubation at its optimal pH of 5.0. Enzyme activity of both form A and form B was inhibited by NaCl concentrations greater than 25 mM, the initial concentration of NaCl within the incubation mixture (Fig. 5). There was no initial stimulation of enzyme activity similar to that observed with sulphamate sulphohydrolase [27]. Both Na_2SO_4 and Na_2HPO_4 were potent inhibitors of 6SS activity with 50% of form A activity occurring with concentrations of 30 μM-Na_2SO_4 and 6 μM-Na_2HPO_4 (Fig. 5). $CuCl_2$ inhibited form A activity by 30% at a concentration of 0.5 mM (Fig. 5). $CuCl_2$ inhibition of form A to 50% required a concentration of 10 mM.

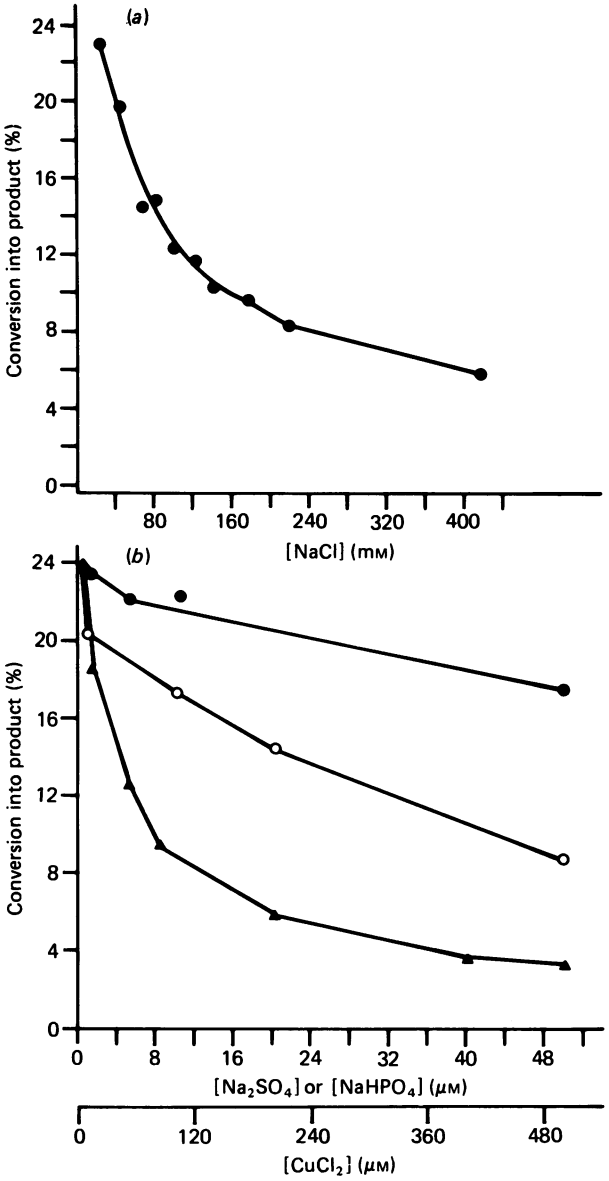


Fig. 5. Hydrolysis by form A 6SS of GlcNAc6S-IdOA as a function of (*a*) NaCl (●) and (*b*) $CuCl_2$ (●), Na_2SO_4 (○) and Na_2HPO_4 (▲) concentration at pH 5.0

For experimental details see the Materials and methods section.

DISCUSSION

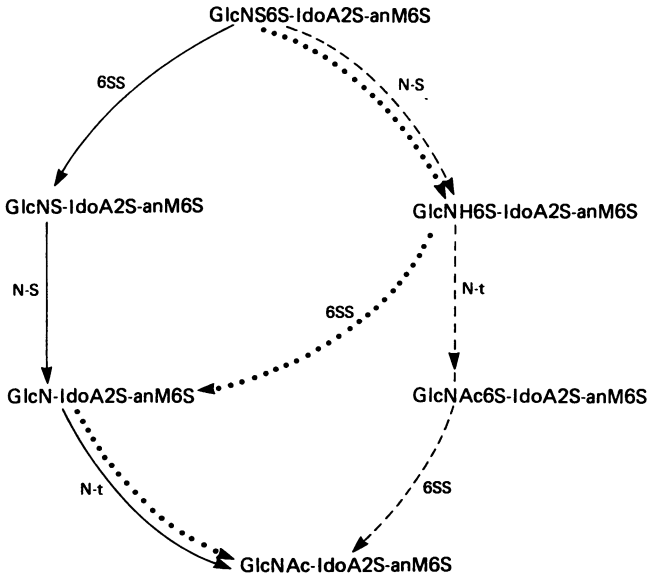
A major conclusion from the kinetic data summarized in Table 1 is that the aglycone structure of glucosamine 6-sulphate substrates considerably influences the catalytic efficiency of 6SS. An extreme example of this is the most complex of the chemically derived substrates, GlcNS6S-IdoA2S-anM6S, which is turned over by the purified form A of the enzyme more than 138-fold faster and bound 28-fold tighter than is the monosaccharide GlcNAc6S. That is, 6SS de-*O*-sulphates GlcNS6S-IdoA2S-anM6S 3900-fold more efficiently than it does the monosaccharide GlcNAc6S. The addition of a 6-carboxy group to a substrate (e.g. compare GlcNAc6S-Ido and GlcNAc6S-IdOA in Table 1) resulted in a 79-fold increase in enzyme turnover number for a single change in aglycone structure. The influence of the aglycone carboxy group on 6SS activity is apparent from the observation that GlcNH6S-IdOA is a substrate whereas GlcNH6S-Ido is not. The addition of a 2-sulphate ester group also resulted in a small but significant increase in catalytic efficiency for both form A and form B activities.

Substituents on the 2-amino group of the glucosamine 6-sulphate residue under attack clearly influence 6SS activity toward di- and tri-saccharide substrates. The extent of influence is dependent on aglycone structure. GlcNH6S-Ido is not a substrate for 6SS. GlcNH6S-IdOA is turned over 116-fold less than the *N*-acetylated (GlcNAc6S-IdOA) and 36-fold less than the *N*-sulphated (GlcNS6S-IdOA) disaccharide equivalent. The overall effects of *N*-acetylation and *N*-sulphation of the trisaccharide substrate (GlcNH6S-IdoA2S-anM6S) are to

increase 6SS turnover of substrate by 64- and 206-fold respectively (Table 1).

We have recently reported a similar study of substrate aglycone structure on sulphamate sulphohydrolase activity [27]. Like 6SS, the most complex substrate, GlcNS-IdoA2S-anM6S, was hydrolysed considerably (67000-fold) more efficiently than the monosaccharide 2-sulphaminoglucosamine (GlcNS) by purified human liver sulphamate sulphohydrolase [27]. Aglycone-residue 6-carboxy and 2-sulphate ester groups resulted in the largest increase in enzyme activity. We have also recently speculated that these structures favoured by the degradative enzymes are introduced through the substrate structure requirements of the enzymes involved in substrate biosynthesis [2,27]. For example, because the presence of GlcNS residues in precursor heparin chains favours the 5-epimerization of GlcA residues, the 2-sulphation of IdoA residues and the 6-sulphation of GlcNS residues are likely to result in many of the GlcNS residues in heparan sulphate having an IdoA2S-GlcNS6S aglycone sturcture [34,35]. A finding also consistent with observations that modification by maturation enzymes (i.e. those involved in 5-epimerization and *O*-sulphation) will occur preferentially or exclusively in the vicinity of GlcNS residues is that heparan sulphate has been shown to contain blocks of *N*-sulphated disaccharides (GlcNS6S-IdoA2S) interspersed with blocks of *N*-acetylated disaccharides (GlcNAc-GlcA) [1,36,37]. Of all of the aglycone structures evaluated, substrates containing features such as adjacent-residue 6-carboxy and 2-sulphate ester groups were most susceptible to hydrolysis by 6SS and sulphamate sulphohydrolase.

Our data reported above for substrate specificity and affinity for 6SS differ from those described [6,7,13] for 6SS isolated from other tissue sources. Although the kinetic data for urine 6SS towards GlcNAc6S-GlcA-anM, GlcNAc6S and Glc6S reported by Basner *et al.* [6] were not detailed, K_m values were about 100-fold higher and $V_{max.}$ values 100-fold lower than the trisaccharide/monosaccharide values reported in Table 1 for human liver 6SS. In contrast, the K_m values of crude 6SS from urine and cultured skin fibroblasts towards GlcNAc6S-IdOA were 27 and 6 μM respectively (C. Freeman, unpublished work). These values are similar to the K_m values reported for purified 6SS from liver (Table 1). We are unable to explain these different observations, except to suggest that substrate inhibition may have occurred under the incubation conditions used by Basner *et al.* [6]. Weissmann *et al.* [7] reported that there may be two different 6SS activities present in bovine kidney: one towards GlcNAc6S-IdoA2S-anM6S and the other towards GlcNS6S-IdoA2S-anM6S. Forms A, B, C and D exhibited 6SS activity towards both GlcNS6S-IdoA2S-anM6S and GlcNAc6S-IdoA2S-anM6S substrates. We were not able to detect, in the early stages of enzyme purification, 6SS activities towards GlcNS6S-IdOA that were not also observed with GlcNAc6S-IdOA. As shown in Table 1, Weissmann *et al.* [7] also reported that 6SS activity towards GlcNH6S-IdoA2S-anM6S was considerably lower than that observed with GlcNAc6S-IdoA2S-anM6S. We have been unable to detect any specific 6SS activity towards either keratan sulphate or heparan sulphate as reported by Matalon *et al.* [13]. Forms A, B, C and D were able to de-*O*-sulphate both α- and β-linked GlcNAc6S residues of substrates derived from heparan sulphate and keratan sulphate respectively. It is possible that the observations made by Matalon *et al.* [13], based on the production of uncharged product from the digestion of GlcNAc6S-Galitol, were complicated by the action of β-*N*-acetylhexosaminidase on this substrate to produce galactitol together with the much less efficient 6SS activity to yield GlcNAc-Galitol [9–11]. In conclusion, there is no evidence that forms A, B, C and D have different substrate specificities towards α- and β-linked GlcNAc6S or α-linked GlcNS6S and GlcNAc6S residues.



Scheme 1. Proposed pathways for exo-degradation of α-linked GlcNS6S residues of the trisaccharide substrate GlcNS6S-IdoA2S-anM6S derived from heparin

Abbreviations: N-S, sulphamate sulphohydrolase; N-t, acetyl-CoA:glucosamine *N*-acetyltransferase. —►, Pathway 1; – – ►, pathway 2; ••• ►, pathway 3.

The enzyme has been called an *N*-acetylglucosamine-6-sulphate sulphatase both in this paper and by other authors. It would appear, however, from substrate-specificity studies (Table 1) the enzyme should be called 2-amino-2-deoxy-D-glucose-6-sulphate sulphohydrolase, or briefly glucosamine-6-sulphate sulphatase.

There are three possible pathways for the turnover of non-reducing-end GlcNS6S residues in heparan sulphate and heparin (Scheme 1). 6SS is able to de-*O*-sulphate GlcNS6S, GlcNAc6S and GlcNH6S residues with IdoA2S aglycone groups (Table 1). It would seem that at low (less than 1 μM) concentrations GlcNS6S-IdoA2S-anM6S is by far the most catalytically efficient substrate for 6SS. However, as there is considerable substrate inhibition by the GlcNS6S-containing substrate which is not apparent for the GlcNAc6S- or GlcNH6S-containing substrate equivalent, it is possible that at high concentrations of substrate GlcNAc6S-IdoA2S-anM6S is more efficiently de-*O*-sulphated than is the GlcNS6S-containing substrate equivalent. In fact, the catalytic efficiency of 6SS de-*O*-sulphation of GlcNS6S-IdoA2S-anM6S at a concentration of 10 μM is about 20-fold lower than at a substrate concentration of 1 μM.

The relative contributions of pathways 1, 2 and 3 (Scheme 1) to the turnover of GlcNS6S residues *in vivo*

will depend on the relative activities of 6SS and sulphamate sulphohydrolase towards GlcNS6S-IdoA2S-anM6S to produce GlcNS-IdoA2S-anM6S and Glc-NH6S-IdoA2S-anM6S respectively. Preliminary studies with purified human liver sulphamate sulphohydrolase [27] towards GlcNS6S-IdoA2S-anM6S indicate that this substrate is about 10-fold more efficiently de-*N*-sulphated (K_m 77 nM, $k_{cat.}$ 72.6 mol/min per mol of enzyme; C. Freeman & J. J. Hopwood, unpublished work) than de-*O*-sulphated by 6SS. Thus, at this stage, it is possible that any one or all of the three pathways shown in Scheme 1 are involved in GlcNS6S-residue turnover. Further studies incorporating the three enzyme activities towards the substrate structures shown in Scheme 1 are needed to resolve questions regarding the order with which these enzymes act and the relative contribution of each pathway to heparan sulphate degradation.

Although speculation at present, we suggest that 6SS and sulphamate sulphohydrolase operate in close proximity of each other, possibly at an enzyme complex bound to the lysosomal membrane and the integral membrane protein acetyl-CoA:glucosamine *N*-acetyltransferase, the other enzyme in the degradative pathway of GlcNS6S residues. This co-operative action, by elimination of the need for 'intermediates' to diffuse from one enzyme to the next, might well provide a means of accelerating the hydrolysis of heparan sulphate substrates to even higher rates than we have observed for these enzymes in isolation.

The catalytic efficiency of 6SS towards the monosaccharide GlcNAc6S is similar to that observed for the enzyme towards the tetrasaccharide substrate GlcNAc6S-Gal-GlcNAc6S-Galitol but is 20-fold less efficient than towards GlcNAc6S-Gal6S-GlcNAc6S-Galitol. The tetrasaccharide containing Gal6S represents the most common sequence in both skeletal and corneal keratan sulphate [24]. We have already reported that the relative rate of hydrolysis of GlcNAc6S-Gal6S-GlcNAc6S-Galitol by the A form of β-*N*-acetylhexosaminidase to produce GlcNAc6S is about 50-fold higher than that observed for sulphate ester bond hydrolysis by the 6SS on the same substrate [9]. These observations provide evidence to suggest that the major pathway for degradation of keratan sulphate substrates, particularly those species with unsulphated galactose aglycone residues, is the action of β-*N*-acetylhexosaminidase to produce GlcNAc6S followed by the action of 6SS on that monosaccharide rather than the normally accepted order of sulphatase first and then glycosidase [1,2].

The work was supported by grants from the Research Trust of the Adelaide Children's Hospital and the National Health and Medical Research Council of Australia. We thank Dr. P. R. Clements (Department of Chemical Pathology, Adelaide Children's Hospital) for helpful discussions.

REFERENCES

1. Rodén, L. (1980) in The Biochemistry of Glycoproteins and Proteoglycans (Lennarz, W. J., ed.), pp. 267–371, Plenum Press, New York
2. Hopwood, J. J. (1987) in Heparin (Lane, D. & Lindahl, U., ed.), Edward Arnold, London, in the press
3. McKusick, V. & Neufeld, E. (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
4. Kresse, H., Paschke, E., von Figura, K., Gilberg, W. & Fuchs, W. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 6822–6826
5. Freeman, C., Clements, P. R. & Hopwood, J. J. (1987) Biochem. J. **246**, 347–354
6. Basner, R., Kresse, H. & von Figura, K. (1979) J. Biol. Chem. **254**, 1151–1158
7. Weissmann, B., Chao, H. & Chow, P. (1980) Biochem. Biophys. Res. Commun. **97**, 827–833
8. Habuchi, H., Tsuji, M., Nakanishi, Y. & Suzuki, S. (1979) J. Biol. Chem. **254**, 7570–7578
9. Hopwood, J. J. & Elliott, H. (1983) Biochem. Int. **6**, 141–148
10. Hopwood, J. J. & Elliott, H. (1983) Biochem. Int. **6**, 831–836
11. Hopwood, J. J. & Elliott, H. (1985) Biochem. J. **229**, 579–586
12. Fuchs, W., Beck, M. & Kresse, H. (1985) Eur. J. Biochem. **151**, 551–556
13. Matalon, R., Wappner, R., Deanching, M., Brandt, I. & Horwitz, A. (1982) Ann. Clin. Lab. Sci. **12**, 234–234
14. Hopwood, J. J., Muller, V., Smithson, A. & Baggett, N. (1979) Clin. Chim. Acta **92**, 257–265
15. Hopwood, J. J. (1979) Carbohydr. Res. **69**, 203–216
16. Hopwood, J. J. & Elliott, H. (1981) Carbohydr. Res. **91**, 165–190
17. Hopwood, J. J. & Elliott, H. (1981) Clin. Chim. Acta **112**, 55–66
18. Hopwood, J. J. & Elliott, H. (1981) Clin. Chim. Acta **112**, 67–75
19. Hopwood, J. J. & Elliott, H. (1982) Clin. Chim. Acta **120**, 77–86
20. Hopwood, J. J. & Elliott, H. (1982) Clin. Chim. Acta **123**, 241–250
21. Muller, V. J. & Hopwood, J. J. (1982) Clin. Chim. Acta **123**, 357–360
22. Hopwood, J. J. & Muller, V. (1982) Clin. Sci. **62**, 193–201
23. Hopwood, J. J. & Elliott, H. (1983) Clin. Sci. **65**, 325–331
24. Hopwood, J. J. & Elliott, H. (1983) Carbohydr. Res. **117**, 263–274
25. Hopwood, J. J. & Muller, V. (1983) Carbohydr. Res. **122**, 227–239
26. Elliott, H. & Hopwood, J. J. (1984) Anal. Biochem. **138**, 205–209
27. Freeman, C. & Hopwood, J. J. (1986) Biochem. J. **234**, 83–92
28. Clements, P. R., Muller, V. & Hopwood, J. J. (1985) Eur. J. Biochem. **152**, 29–34
29. Hopwood, J. J. & Elliott, H. (1981) Clin. Sci. **61**, 729–735
30. Hopwood, J. J., Elliott, H., Muller, V. J. & Saccone, G. T. P. (1986) Biochem. J. **234**, 507–514
31. Markwell, M. K., Haas, S. M., Bilber, L. L. & Tolbert, N. E. (1978) Anal. Biochem. **87**, 211–222
32. Hopwood, J. J. & Robinson, H. C. (1974) Biochem. J. **141**, 57–69
33. Dixon, M. & Webb, E. C. (1964) Enzymes, 2nd edn., pp. 75–80, Longman, Green and Co., London
34. Jacobsson, I. & Lindahl, U. (1980) J. Biol. Chem. **255**, 5094–5100
35. Jacobsson, I., Lindahl, U., Jensen, J. W., Rodén, L., Prihar, H. & Feingold, D. S. (1984) J. Biol. Chem. **259**, 1056–1063
36. Fransson, L. A., Sjöberg, I. & Havsmark, B. (1980) Eur. J. Biochem. **106**, 59–69
37. Gallagher, J. T. & Walker, A. (1985) Biochem. J. **230**, 665–674

Received 29 January 1987; accepted 12 May 1987