Purification and Properties of \( N \)-Acetylgalactosamine 6-Sulphate Sulphatase from Human Placenta

By Josef GLÖSSL, Wolfgang TRUPPE and Hans KRESSE
Medizinisch-Chemisches Institut und Pregl-Laboratorium der Universität Graz, Harrachgasse 21, A-8010 Graz, Austria

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1. \( N \)-Acetylgalactosamine 6-sulphate sulphatase was purified about 20000-fold from the soluble extract of human placenta with \( N \)-acetylgalactosamine 6-sulphate–glucuronic acid–\( N \)-acetyl[\( ^{3} \)H]galactosaminitol 6-sulphate as substrate in the activity assay. The enzyme appears to be a glycoprotein with a mol. wt. of about 100000 as determined by gel filtration. On gel electrophoresis in the presence of sodium dodecyl sulphate the major protein band had a mol. wt. of 78000. Variable charge heterogeneity was observed in several enzyme preparations. 2. The purified enzyme released up to one sulphate molecule from the disulphated trisaccharide. It was active towards \( N \)-acetylgalactosamine 6-sulphate and exhibited no measurable \( N \)-acytelyglucosamine 6-sulphate sulphatase or any other known lysosomal sulphatase activity. Hydrolysis of \([1-{^3}\text{H}]\)galactitol 6-sulphate was achieved by incubation neither with a crude nor with a purified enzyme preparation. Chondroitin 6-sulphate and keratan sulphate, as well as heparin and heparan sulphate, served as competitive inhibitors of the enzyme. 3. Purified \( N \)-acetylgalactosamine 6-sulphate sulphatase activity was optimal at pH 4.9 and 4.4 when assayed in 0.02M-sodium acetate buffer and at pH 4.2 and 5.2 in 0.1M-sodium acetate buffer. A single pH-optimum at pH 4.8 was observed for the crude enzyme and for the purified enzyme after mild periodate treatment. The sulphatase activity was inhibited by a variety of anions and cations and activated by thiol-specific and thiol reagents.

Intralysosomal degradation of sulphated glycosaminoglycans requires the sequential removal of sulphate groups and sugar residues from the non-reducing terminal of the polysaccharide chain (for reviews see Neufeld et al., 1975; Dorfman & Matalon, 1976). Deficiency of any of the known exoglycosidases or sulphatases results in the intralysosomal accumulation of partially degraded glycosaminoglycan fragments. In the Morquio syndrome (mucopolysaccharidosis IV) the affected patients usually excrete in the urine excessive amounts of keratan sulphate and chondroitin 6-sulphate and accumulate both polysaccharides in cartilage (Pedini et al., 1962). As with the Sanfilippo syndrome the Morquio syndrome appears to be biochemically heterogeneous. In a few patients the primary defect has been attributed to the inactivity of a \( \beta \)-galactosidase (Arbisser et al., 1978; K. von Figura, personal communication). With a variety of sulphated oligo- and mono-saccharides as potential substrates, it became evident that the enzymic deficiency of the more frequent type of the Morquio syndrome was \( N \)-acetylgalactosamine 6-sulphate sulphatase (EC 3.1.6.?), specific for the galactose configuration (Matalon et al., 1974; Singh et al., 1976; DiFerrante et al., 1978; Horwitz & Dorfman, 1978; Glössl & Kresse, 1978). On the basis of the available genetic evidence this sulphatase is considered to attack \( N \)-acetylgalactosamine 6-sulphate linkages in chondroitin 6-sulphate and galactose 6-sulphate bonds in keratan sulphate (DiFerrante et al., 1978). Studies on the purified enzyme, however, have not yet been performed.

In the present paper methods for obtaining highly purified \( N \)-acetylgalactosamine 6-sulphate sulphatase from a human source and some properties of the enzyme are reported for the first time.

Experimental

Materials

Human placentae were obtained about 1h after delivery from a nearby maternity home. NaB\(^{3}\)H\(_{4}\) (sp. radioactivity, 278mCi/mmol) was obtained from New England Nuclear Corp., Dreieichenhain, Germany. Concanavalin A–Sepharose and Sephacryl S-200 (Pharmacia) were purchased from Albrecth's Wtw., Vienna, Austria. DE-52 DEAE-cellulose (Whatman) was from Comesa, Vienna, Austria. Dowex 1 (X2; 200–400mesh), reference proteins for molecular-weight determinations (Kollektion MS-II), acrylamide, and \( NN' \)-methylenebisacrylamide.
were from Serva, Heidelberg, Germany. α-Methyl-mannoside, p-nitrophenyl glycosides, p-nitroacetochol sulphate and chondroitin 6-sulphate from shark cartilage were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. All other chemicals used were of analytical grade.

Chondroitin 4-sulphate from calf nasal cartilage and heparan sulphate from bovine aorta were prepared as described previously (Kresse & Buddecke, 1970). Heparin was from Serva, Heidelberg, Germany. Keratan sulphate from human rib cartilage and iduronic acid 2-sulphate-[1-3H]anhydromannitol 6-sulphate and N-acetylglucosamine 6-sulphate-glucuronic acid-[1-3H]anhydromannitol prepared from heparan sulphate were kindly provided by Dr. A. Schmidt, Dr. U. Klein and Dr. R. Basner respectively of the University of Münster, Münster, Germany. N-Acetylgalactosamine 6-sulphate was a gift from Dr. A. Olavesen, University College, Cardiff, Wales, U.K. UDP-N-acetylgalactosamine 4-[35S]sulphate was provided by Dr. W. Mlekosch of this Institute.

Preparation of substrates of N-acetylgalactosamine 6-sulphate sulphatase

A reduced trisaccharide, N-acetylgalactosamine 6-sulphate-glucuronic acid-N-acetyl[1-3H]galactosaminitol 6-sulphate, was obtained from chondroitin 6-sulphate by sequential treatment of the appropriate saccharides with hyaluronidase, β-glucuronidase and NaB₃H₄ as described in detail previously (Glössl & Kresse, 1978). The final product contained 92.7 nmol of hexuronate and 184 nmol of sulphate/ml of water (sp. radioactivity, 27 Ci/mol of hexuronate).

Galactose 6-sulphate was prepared by the direct sulphation procedure I of Lloyd (1962). The potassium salt was obtained by ion-exchange chromatography on Dowex 50W (X8; 200–400 mesh, H⁺ form), and subsequent neutralization of the effluent with KOH. The product was purified by descending paper chromatography on Whatman 3MM paper in butan-1-ol/1M-NH₃/acetic acid (2:1:3, by vol.). Material migrating with Rf values between 0.40 and 0.44 was eluted with water, concentrated and analysed by i.r. spectroscopy.

The preparation showed a major band at 1235 cm⁻¹ (S–O stretching) and minor bands at 775, 815, and 995 cm⁻¹, attributable to a sulphate group in the 6-position. Bands at 725, 855, and 925 cm⁻¹ (attributable to a sulphate group in the 4-position) were not observed. Reduction of galactose 6-sulphate with NaB₃H₄ was performed by the method of Ögren & Lindahl (1975). The specific radioactivity of the final product was 0.73 Ci/mol of sulphate.

Enzyme-activity determinations

The standard reaction mixture for the assay of N-acetylgalactosamine 6-sulphate sulphatase activity contained 0.46 nmol of trisaccharide (about 9000 c.p.m.), 0.02 M-sodium acetate buffer, pH 4.8, 0.002 M-mercaptoethanol, and up to 15 µl of a suitably diluted enzyme solution in a final volume of 60 µl. After incubation for 0.5 to 6 h at 37°C the reaction was stopped by boiling. The rate of formation of monosulphated ³H-labelled trisaccharide was determined by the following micro-column procedure. The reaction mixture was diluted to 0.5 ml with water and loaded on a 0.5 ml column of Dowex 1 (X2; 200–400 mesh, Cl⁻ form) prepared in a Pasteur pipette. Monosulphated material was desorbed directly into a scintillation vial by elution with 2 × 1.0 ml of 0.4 M-NaCl, and remaining substrate by applying 2 × 1.0 ml of 0.9 M-NaCl. Radioactivity of both fractions was assayed after addition of 12 ml of Unisolve 1 (Zinsser, Frankfurt, Germany). In controls with boiled enzyme solutions about 0.4% of the total ³H radioactivity applied behaved like monosulphated material. Galactitol 6-sulphate sulphatase activity was measured exactly as described (DiFerrante et al., 1978), except that after incubation the assay mixture was subjected to high-voltage electrophoresis at pH 3.9 (see below). The paper was cut in 1 cm segments, placed in scintillation vials and eluted with 1 ml of water before the addition of 2 ml of Unisolve 1.

N-Acetylglucosamine 6-sulphate sulphatase activity was determined as described in detail elsewhere (Basner et al., 1979). N-Acetylglucosamine 6-sulphate–glucuronic acid–[1-3H]anhydromannitol, prepared from heparan sulphate, was used as substrate.

Activities of arylsulphatases A and B (Baum et al., 1959), UDP-N-acetylgalactosamine 4-sulphate sulphatase (Fluharty et al., 1975), L-iduridine 2-sulphate sulphatase (Lim et al., 1974), 2-sulpho-aminoglucosamine sulphamidase (Kresse, 1973), β-glucuronidase (von Figura, 1977) and β-N-acetylglucosaminidase (von Figura, 1977) were determined as described previously. For all enzymes 1 unit of activity was defined as the amount of enzyme catalysing the hydrolysis of 1 µmol of substrate per min under the condition of saturation with substrate.

Purification of N-acetylgalactosamine 6-sulphate sulphatase

Crude enzyme preparation. Two placenta, each about 350 g, were obtained within 2 h of delivery and cut in about 1 cm³ cubes. Then 4 ml of 0.075 M-NaCl was added/g of tissue before homogenization with an Ultraturrax homogenizer (Jahnke and Kunkel, Freiburg, Germany) for 15 × 15 s at −20°C. All further steps were performed at +4°C, except where otherwise stated. The homogenate was centrifuged at 11 300 g for 1 h. The supernatant was used for further purification procedures.

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Step I [(NH₄)₂SO₄ precipitation]. The supernatant was saturated to 70% with solid (NH₄)₂SO₄, left overnight, and the precipitate was obtained by centrifugation for 30 min at 8000g. The precipitate was dissolved in 0.05 M-Tris/HCl buffer, pH 7.0, containing 0.075 M-NaCl and 2 mM-2-mercaptoethanol (buffer A), final volume 750 ml, dialysed for 14 h against two changes of 5 litres each of buffer A, and then for 6 h against 5 litres of buffer A containing additionally 2 mM each of MnCl₂, MgCl₂, and CaCl₂ (buffer B).

Step II (chromatography on concanavalin A-Sepharose). The non-diffusible material from step I (900 ml) was loaded at room temperature (22°C) on a concanavalin A-Sepharose column (1.8 cm × 17 cm), equilibrated with buffer B, at a flow rate of 40 ml/h, the effluent being collected at +4°C. The column was then washed at a flow rate of 150 ml/h with 350 ml of buffer B until the A₂₈₀ was below 0.1. Adsorbed material was eluted at the same flow rate with 500 ml of buffer B containing 0.5 mM-methyl α-mannoside. The proteins that were desorbed by methyl α-mannoside were dialysed for 2 days against four changes of 5 litres each of 0.01 M-Tris/HCl buffer, pH 7.8, containing 0.04 M-NaCl and 2 mM-2-mercaptoethanol (buffer C).

Step III (DEAE-cellulose chromatography). The enzyme preparation of step II (550 ml) was applied at a flow rate of 25 ml/h to a DE-52 DEAE-cellulose column (2.6 cm × 30 cm) pre-equilibrated with buffer C. The column was eluted with 200 ml of the same buffer and developed with a linear NaCl concentration gradient consisting of 800 ml of buffer C and 800 ml of buffer C containing 0.25 M-NaCl at the final concentration. Enzyme-containing fractions were pooled and subjected to (NH₄)₂SO₄ precipitation and centrifugation as described in step I. The precipitate was dissolved in 8 ml of 0.15 M-Tris/HCl, pH 8.0, containing 0.15 M-NaCl and 0.01 M-dithiothreitol. The solution was left at room temperature for 2 h and then dialysed for 24 h against two changes of 5 litres each of 0.01 M-Tris/HCl buffer, pH 7.2, containing 0.15 M-NaCl and 2 mM-2-mercaptoethanol (buffer D).

Step IV (gel filtration on a Sephacryl S-200 column). The material obtained after step III (8.9 ml) was applied to a column (2.4 cm × 143 cm) of Sephacryl S-200, pre-equilibrated with buffer D, and eluted at a flow rate of 20 ml/h (fraction volume, 6.4 ml). The void volume of the column was 274 ml as shown with 35S-labelled proteoglycans. N-Acetylgalactosamine 6-sulphate sulphatase-containing fractions were first treated with (NH₄)₂SO₄ and then with 2 ml of dithiothreitol-containing buffer as described in step III. At the end of the reaction period the material was dialysed overnight against 5 litres of 0.04 M-β-alanine/0.01 M-acetic acid/0.04 M-NaCl, pH 4.6, and then for 4 h against 2 × 2.5 litres of the same buffer without NaCl.

Step V (preparative polyacrylamide-gel electrophoresis). Preparative polyacrylamide-gel electrophoresis was performed by the method of Rodbard & Chrombach (1971). System 35 of Jovin et al. (1970) was chosen with an operative pH at 0°C of 5.0 in the concentration gel and of pH 3.5 in the separation gel. The composition of the buffers was as follows: upper buffer, 0.04 M-β-alanine/0.01 M-acetic acid; lower buffer, 0.05 M-KOH/0.062 M-acetic acid; concentration-gel buffer, 0.049 M-pyridine/0.019 M-acetic acid; separation-gel buffer, 0.038 M-KOH/0.289 M-acetic acid. Polymerization was initiated for the concentration gel by 0.055 mM-K₂S₂O₈/0.014 mM-riboflavin/3.3 mM-NNN'-tetramethylethlenediamine, and for the separation gel by 0.092 mM-K₂S₂O₈/0.014 mM-riboflavin/16.5 mM-NNN'-tetramethylethlenediamine. Concentration-gel solution (4 ml) and 12 ml of separation-gel solution [total acrylamide concentration of the separation gel, 7% (w/v); cross-linking, 2%] in tubes 17 mm in diameter were employed for 1 ml of enzyme preparation of step IV. At the end of the run the gel was frozen at −20°C, cut transversely, and the slices (1.6 mm) were homogenized in 3 ml of 0.01 M-Tris/HCl/0.01 M-glycine buffer, pH 7.2, containing 0.04 M-NaCl and 2 mM-2-mercaptoethanol, centrifuged at 82 000 g for 40 min before enzyme-activity determination in the supernatant. The peak fractions, two slices from each gel, were combined and stored at 4°C.

Protein determination

Protein was monitored by measuring A₂₈₀, except after polyacrylamide-gel electrophoresis, where protein was quantified by staining with Amido Black (Kaltwasser et al., 1967).

Polyacrylamide-gel electrophoresis

Analytical gel disc electrophoresis was performed in System 35 (see above) of Jovin et al. (1970) and in the anodic system no. 1 of Maurer (1968) which has an operative pH of 9.0 in the separation gel. The gels were stained for protein with Coomassie Brilliant Blue R 250 (Rodbard & Chrombach, 1971) or sliced and extracted with 1.5 ml of 0.01 M-Tris/HCl/0.01 M-glycine buffer, pH 7.2, containing 0.04 M-NaCl and 2 mM-2-mercaptoethanol, as described above.

For sodium dodecyl sulphate/polyacrylamide-gel electrophoresis the protein solution was first dialysed against 6 M-guanidinium chloride in 0.5 M-Tris/HCl buffer, pH 8.1, containing 2 mM-EDTA. Reduction was then performed with an at least 50-fold molar excess of dithiothreitol at 50°C for 4 h. After cooling to room temperature the sample was treated for 1.5 h with a 30% excess of iodoacetamide over...
dithiothreitol, dialysed against water and freeze-dried (Pitt-Rivers & Ambesi Impimbato, 1968). For gel electrophoresis the procedure of Weber & Osborn (1969) in 7% gels was employed. At the end of the runs the gels were first soaked with methanol/ acetic acid/water (5:1:4, by vol.) followed by 12.5% (w/v) trichloroacetic acid and then stained with Coomassie Brilliant Blue R 250 as described above.

**High-voltage electrophoresis**

Electrophoresis on Whatman 3MM paper was carried out in 1.9M-formic acid (pH1.7) at 40V/cm for 45 min or in 0.06M-pyridine/acetic acid, pH3.9, at 60V/cm for 20 min.

**Paper chromatography**

For descending paper chromatography Schleicher and Schüll paper no. 2043 a was used and butan-1-ol/1M-NH$_3$/glacial acetic acid (2:1:3, by vol.) was the solvent.

**Analytical methods**

Analyses of uronic acids (Bitter & Muir, 1962) and sulphate (Stuhlsatz, 1963) were performed as described. Radioactivity was determined in a Beckman LS 8100 liquid-scintillation spectrometer with Unisol 1 (Zinsser, Frankfurt, Germany) as scintillation 'cocktail'.

**Results**

**Purification of N-acetylgalactosamine 6-sulphate sulphatase**

N-Acetylgalactosamine 6-sulphate sulphatase was purified about 20000-fold from the soluble extract of human placenta as summarized in Table 1. N-Acetylgalactosamine 6-sulphate–glucuronic acid–N-acetyl[1-$^3$H]galactosaminitol 6-sulphate was used as substrate throughout the purification procedure.

After (NH$_4$)$_2$SO$_4$ precipitation (step 1) most of the concomitant proteins were removed by the second step, affinity chromatography on concanavalin A-Sepharose. Almost 99% of the applied protein, but only 6% of the enzyme, was not bound by the affinity matrix. On elution in the presence of 0.5m-methyl α-mannoside the enzyme was desorbed at about constant specific activity. Subsequent chromatography on DE-52 DEAE-cellulose at pH7.8 revealed a charge heterogeneity of N-acetylgalactosamine 6-sulphate sulphatase (Fig. 1). Regularly, most of the enzyme activity emerged at an ionic strength of $I=0.129$, and a second peak appeared at $I=0.112$. In one out of four different purifications an additional peak was observed at $I=0.082$ and $I=0.164$ respectively. For further purification only the major enzyme peak was used. Further 16-fold purification was achieved by gel chromatography on a calibrated Sephacryl S-200 column (Fig. 2). Most of the protein impurities were eluted in the void volume of the column, whereas the sulphatase appeared as a sharp peak with a $K_s$ (the quotient of the differences between elution volume and void volume).

**Table 1. Summary of purification of N-acetylgalactosamine 6-sulphate sulphatase from human placenta**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total protein $(A_{280}/cm)$</th>
<th>Total activity (nmol/h)</th>
<th>Specific activity (nmol/h per $A_{280}$)</th>
<th>Purification (-fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude enzyme solution</td>
<td>52300</td>
<td>3250</td>
<td>0.062</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>(NH$_4$)$_2$SO$_4$ precipitation</td>
<td>47100</td>
<td>3330</td>
<td>0.071</td>
<td>1.14</td>
<td>102</td>
</tr>
<tr>
<td>Concanavalin A-Sepharose</td>
<td>617</td>
<td>1410</td>
<td>2.28</td>
<td>36.8</td>
<td>43.2</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pool 1</td>
<td>53</td>
<td>71</td>
<td>1.34</td>
<td>21.6</td>
<td>2.2</td>
</tr>
<tr>
<td>Pool 2</td>
<td>206</td>
<td>356</td>
<td>1.73</td>
<td>27.9</td>
<td>10.9</td>
</tr>
<tr>
<td>Sephacryl S-200 (pool 2)</td>
<td>15</td>
<td>648</td>
<td>43.8</td>
<td>706</td>
<td>19.9</td>
</tr>
<tr>
<td>Polyacrylamide-gel electrophoresis</td>
<td>0.3*</td>
<td>361</td>
<td>1200</td>
<td>19400</td>
<td>11.1</td>
</tr>
</tbody>
</table>

* Weight (mg), as quantified by staining with Amido Black.
volume and between total volume and void volume) of 0.23. In a separate experiment the enzyme eluting at ionic strength \( I = 0.112 \) from the DEAE-cellulose column was also subjected to gel chromatography. It had exactly the same elution behaviour as the main fraction of the enzyme.

The last purification step, preparative polyacrylamide-gel electrophoresis in a discontinuous cathodic buffer system, resulted in a remarkable increase in specific activity of the enzyme. At least seven different protein bands were removed, as judged by analytical polyacrylamide-gel electrophoresis under the same operative conditions.

Properties

Purity of the enzyme preparation. On analytical gel electrophoresis in the cathodic System 35 and in an anodic system operative at pH 9.0 a single protein band was observed at total polyacrylamide-gel concentrations of 5 and 8% respectively. As determined in parallel runs, N-acetylgalactosamine 6-sulphate sulphatase activity co-migrated with that protein band (Fig. 3). Owing to the limited amount of protein used in these experiments, each impurity constituting about 15% or less of the 6-sulphatase protein would have escaped detection.

Protein (10\( \mu \)g) was subjected to polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. One major and two minor faster migrating protein bands were observed (Fig. 3). The latter ones, which were considered as impurities, comprised about 30% of the applied protein.

On incubation for 24h with appropriate substrates the enzyme preparation was free of iduronide 2-sulphate sulphatase, heparin sulphamidase, arylsulphatase A and arylsulphatase B as measured with \( p \)-nitrocatechol sulphate and UDP-N-acetylgalactosamine 4-sulphate as substrates, and of \( \beta \)-glucuronidase. Of special importance was the finding of an unmeasurably low \( N \)-acetylgalactosamine 6-sulphate sulphatase activity. On the other hand, \( \beta \)-\( N \)-acetylgalactosaminidase activity was still present with a specific activity of 144 m-units/mg of protein. This corresponds to a 10-fold purification over the crude soluble extract with a yield of about 0.002%.

Estimation of molecular weight. A calibrated Sephacryl S-200 column (\( V_I = 608 \text{ ml as determined with } [\text{35S}]\text{sulphate} \)) equilibrated and eluted with 0.01M-Tris/HC1 buffer, pH 7.2, in 0.15M-\( \text{NaCl} \) was used to determine the molecular weight of the enzyme. The void volume of the column was 274 ml, as determined

**Fig. 2. Analysis of step IV (gel chromatography on Sephacryl S-200)**

Fractions (6.4ml) were collected; for further details see the Experimental section. The horizontal bar marks the fractions pooled for further purification. •, \( A_{280} \); ○, enzyme activity (nmol of sulphate released/h per ml).

**Fig. 3. Tracings of polyacrylamide-gel electrophoretograms**

(a) Electrophoretogram of proteins obtained after step IV in the cathodic system, operative at pH 3.5. Total polyacrylamide-gel concentration, 7% (w/v). (b) Electrophoretogram after step V in the cathodic system operative at pH 3.5. Left trace, gel concentration 5%; right trace, gel concentration 8%. (c) Electrophoretogram after step V in the anodic system, operative at pH 9.0. Left trace, gel concentration 5%; right trace, gel concentration 8%. (d) Sodium dodecyl sulphate/polyacrylamide-gel electrophoretogram of proteins obtained after step V. The small arrows indicate the position of the tracking dye and the broad arrows indicate the peak fraction of \( N \)-acetylgalactosamine 6-sulphate sulphatase activity as determined in parallel runs.
with $^{35}$S-labelled proteoglycans. The enzyme was eluted with a $K_v$ of 0.23 (Fig. 4) corresponding to an apparent mol.wt. of 100000.

Molecular-weight determinations of the protein bands visible after polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate gave a value of 78000 for the major protein band and values of 71000 and 45000 for the two minor protein bands.

**Substrate specificity and kinetic parameters.** Beside its activity towards the disulphated $^3$H-labelled trisaccharide, N-acetylgalactosamine 6-sulphate sulphatase released sulphate from N-acetylgalactosamine 6-sulphate. On incubation of 8 µg of enzyme protein with 1875 nmol of sulphated monosaccharide 1.76 nmol of inorganic sulphate was formed/h. Shortage of material precluded kinetic studies with this substrate. When the N-acetylgalactosamine 6-sulphate sulphatase preparation was incubated in the presence of 7.7—186 µmol of disulphated trisaccharide/litre the plot 1/v against 1/[S] showed a straight-line relationship (Fig. 5). A Michaelis constant of 0.2 mm was found.

$[1-^3$H]Galactitol 6-sulphate was incubated with an amount of enzyme hydrolysing 0.5 nmol of sulphate/h from the $^3$H-labelled trisaccharide under conditions identical with those described by DiFerrante et al. (1978). On high-voltage electrophoresis of the digest we could not detect unsulphated $^3$Hgalactitol. The same negative result was obtained when a crude enzyme preparation (after chromatography on concanavalin A-Sepharose) was used. Prolongation of the time of incubation for up to 72 h and increasing the substrate concentration up to 43 mmol/litre did not give rise to measurable enzyme activities. The formation of 1.5 nmol of $^3$Hgalactitol would have easily been detected.

Sulphate release was also not observed on incubation with the trisaccharide N-acetylgalactosamine 6-sulphate—glucuronic acid—[1-3H]hydromannitol or the disaccharide iduronic acid 6-sulphate—[1-3H]hydromannitol 6-sulphate.

On incubation of 4.6 nmol of disulphated trisaccharide for 48 h with an amount of enzyme hydrolysing 180 pmol of sulphate/h complete degradation was not achieved (Fig. 6). In the digest no unsulphated saccharides could be detected in several separation systems, indicating that the enzyme attacks only one sulphate ester bond per molecule.

**Influence of sulphated glycosaminoglycans.** Keratan sulphate from human rib cartilage (mol.wt. 10000), chondroitin 6-sulphate from shark cartilage (mol.wt. 20000), chondroitin 4-sulphate from calf nasal cartilage (mol.wt. 20000), heparan sulphate from bovine aorta (mol.wt. 40000) and commercial heparin (mol.wt. 15000) were tested at 0.02 to 55 µM concentrations on the influence of N-acetylgalactosamine 6-sulphate sulphatase activity with the $^3$H-labelled trisaccharide as substrate (Fig. 5). All
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Fig. 6. Paper electrophoretograms and paper chromatogram of the $^3$H-labelled trisaccharide before and after digestion with purified N-acetylgalactosamine 6-sulphate sulphatase
(a)(i)–(a)(iii), Undigested substrate; (b)(i)–(b)(iii), digested material. (i) High-voltage electrophoresis at pH 1.7; (ii) high-voltage electrophoresis at pH 3.9; (iii) descending paper chromatography in butan-1-ol/1M-NH$_3$/acetic acid (2:1:3, by vol.).

Table 2. Influence of sulphated glycosaminoglycans on N-acetylgalactosamine 6-sulphate sulphatase activity

<table>
<thead>
<tr>
<th>Glycosaminoglycan added</th>
<th>Competitive inhibition</th>
<th>Inhibition constant ($\mu$m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitin 6-sulphate</td>
<td>+</td>
<td>40</td>
</tr>
<tr>
<td>Chondroitin 4-sulphate</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Keratan sulphate</td>
<td>+</td>
<td>40</td>
</tr>
<tr>
<td>Heparan sulphate</td>
<td>+</td>
<td>0.05</td>
</tr>
<tr>
<td>Heparin</td>
<td>+</td>
<td>1</td>
</tr>
</tbody>
</table>

Highly purified enzyme (0.08$\mu$g) was incubated with 1.38–11.0 nmol of $^3$H-labelled trisaccharide in 20mM-sodium acetate buffer, pH 4.8, containing 2mM-2-mercaptoethanol without further additions or in the presence of 33 or 66$\mu$g of chondroitin 6-sulphate, 66$\mu$g of chondroitin 4-sulphate, 17 or 33$\mu$g of keratan sulphate, 0.5 or 1$\mu$g of heparan sulphate and 2.0 or 4.95$\mu$g of heparin respectively; final volume, 60$\mu$l. The reaction was terminated after 1h at 37°C.

sulphated glycosaminoglycans bearing sulphate ester groups on C-6 of the hexosamine moiety served as competitive inhibitors of the enzyme, regardless of whether glucosamine or galactosamine was the constituent of the polymer. Surprisingly, heparan sulphate exhibited a $K_i$ of 50nm, the lowest inhibition constant (Table 2), though it contained only 0.75 mol of sulphate/mol of glucosamine. It seems less likely that the polysaccharides were working through nonspecific polyanion interaction, since the same inhibition constants were obtained when different concentrations of each glycosaminoglycan were tested. Furthermore, chondroitin 4-sulphate produced no measurable effect.

pH optimum. Purified N-acetylgalactosamine 6-sulphate sulphatase from human placenta had two pH optima that were dependent on the buffer concentration. In 0.02M-sodium acetate buffer the enzyme was most active between pH 4.8 and 5.0. A second pH optimum was observed at pH 4.4 (Fig. 7). A similar behaviour, but with lower absolute activities, was found when the incubation was carried out in 0.02M-barbituric acid/sodium barbiturate buffer. In 0.1M-sodium acetate buffer the highest activity was measured at pH 4.2. The second optimum occurred between pH 5.0 and 5.2. These differences were best observed at the buffer concentrations mentioned above. Nevertheless, at up to 0.05M-acetic acid/acetate concentrations, the enzyme was regularly more active at pH 5.0 than at pH 4.2. The converse was true for buffer concentrations between 0.1 and 0.5M (Fig. 8). Such double-peaked pH optima were also observed for the less pure enzyme obtained after
Fig. 7. Effect of pH and buffer concentration on N-acetylgalactosamine 6-sulphate sulphatase activity
(a) Crude enzyme obtained after step II; (b) purified enzyme obtained after step V; (c) purified enzyme (step V) after mild periodate treatment. The assays were either performed in 0.02 M-sodium acetate buffer (●) or in 0.1 M-sodium acetate buffer (○), all the buffers containing 2 mM-2-mercaptoethanol. For each enzyme source the maximal activity was taken as 100%. Details are given in the Experimental section.

Table 3. Influence of anions, cations, chelators, thiol-specific compounds and thiol reagents on N-acetylgalactosamine 6-sulphate sulphatase activity

<table>
<thead>
<tr>
<th>Added substance</th>
<th>Concentration (mm)</th>
<th>Activity (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anions (as sodium salts)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloride</td>
<td>17</td>
<td>87</td>
</tr>
<tr>
<td>33</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Sulphate</td>
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</tr>
<tr>
<td>50</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Sulphite</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Thiosulphate</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
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<td>44</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Nitrate</td>
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<td>1</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Cyanide</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>17</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Cations (as chlorides)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fe^{2+}</td>
<td>17</td>
<td>1</td>
</tr>
<tr>
<td>17</td>
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<td></td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>17</td>
<td>43</td>
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<tr>
<td>17</td>
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<td>Zn^{2+}</td>
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<tr>
<td>17</td>
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</tr>
<tr>
<td>Mn^{2+}</td>
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<td>54</td>
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<tr>
<td>17</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Hg^{2+}</td>
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<td>28</td>
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<tr>
<td>2-Mercaptoethanol</td>
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<td>2</td>
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<tr>
<td>Dithiothreitol</td>
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<td>121</td>
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<tr>
<td>2</td>
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<td>EDTA</td>
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</tr>
<tr>
<td>N-Ethylmaleimide</td>
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</tr>
</tbody>
</table>

Stability. No loss of activity occurred after storage of the enzyme solution at 4°C for 10 weeks; thereafter the activity declined rapidly. Freezing to −20°C and immediate thawing decreased the activity by

gel chromatography on Sephacryl S-200. A crude enzyme preparation (after chromatography on concanavalin A-Sepharose), however, had a single pH optimum at pH 4.8. A similar situation (Fig. 7) was found after mild periodate treatment of the purified enzyme (0.01 M-sodium periodate in 0.01 M-sodium phosphate buffer, pH 6.0, containing 0.15 M-NaCl; 8 h at 4°C in the dark) by the method of Hickman et al. (1974).

Stability. No loss of activity occurred after storage of the enzyme solution at 4°C for 10 weeks; thereafter the activity declined rapidly. Freezing to −20°C and immediate thawing decreased the activity by
HUMAN N-ACETYLGALACTOSAMINE 6-SULPHATE Sulphatase

20%. Half of the activity was destroyed after keeping the enzyme solution for 30 min at 50°C and for 10 min at 55°C respectively. An almost complete loss of activity occurred after freeze-drying of an enzyme solution containing 0.01 M-Tris/HCl buffer, pH 7.2, and 0.04 M-NaCl. Furthermore, the enzyme was inactivated by isoelectric focusing in polyacrylamide gel.

Effect of anions, cations, chelators, thiol-specific compounds and thiol reagents. At concentrations of 17 mm various anions and cations were inhibitors of N-acetylgalactosamine 6-sulphate sulphatase (Table 3). For inhibition by NaCl, which ion was responsible for inhibition could not be determined. KCl exhibited a more profound inhibitory effect than NaCl. Thiol-specific compounds as well as thiol reagents led to an activation of the enzyme. This suggests that a thiol group not involved in the catalytic site is required for a proper conformation of the enzyme.

Discussion

This is the first report on purification and partial characterization of a mammalian N-acetylgalactosamine 6-sulphate sulphatase. The enzyme seems to be identical neither with the barely-characterized chondroitin sulphatase [EC 3.1.6.4; Pincus (1950); Dodgson & Spencer (1954); Dodgson & Lloyd (1958)], which is claimed to hydrolyse 6-sulphate groups, nor with the bacterial chondro-6-sulphatase (EC 3.1.6.10; Yamagata et al., 1968).

A disulphated trisaccharide derived from chondroitin 6-sulphate was used as substrate throughout the purification procedure. Exclusively 6-sulphated chondroitin sulphate as the source for substrate preparation could not be obtained. It was therefore important to determine the proportion of 4-sulphated galactosamine residues. Highly purified N-acetylgalactosamine 4-sulphate sulphatase, kindly provided by Dr. W. Mlekuš from this Institute, converted up to 7% of the substrate into monosulphated trisaccharide. The Michaelis constant of the 4-sulphatase with its natural substrate is one order of magnitude higher than that of N-acetylgalactosamine 6-sulphate sulphatase. The conclusion seems therefore justified that the hydrolysis of 4-sulphated ester bonds did not contribute significantly to the formation of monosulphated trisaccharide.

The final enzyme preparation gave only one single protein band in an anodic and a cathodic polyacrylamide-gel-electrophoresis system. However, the preparation was not considered to be pure, since β-N-acetylxehosaminidase activity could be detected and different protein bands were observed on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. It seems less likely that N-acetylgalactosamine 6-sulphate sulphatase is composed of three different subunits, which would not be present in a simple molar ratio on the basis of the staining intensity.

N-Acetylgalactosamine 6-sulphate sulphatase exhibited several remarkable features. 1. In partial accordance with the findings of DiFerrante et al. (1978), the enzyme was active towards N-acetylgalactosamine 6-sulphate. Of other mammalian sulphatases involved in the degradation of sulphated glycosaminoglycans only N-acetylgalactosamine 6-sulphate sulphatase (Basner et al., 1979) and arylsulphatase B (Farooqui, 1976) have been shown to attack sulphated monosaccharides. Previous investigations failed to detect such activities (Dodgson & Lloyd, 1958). On the other hand, all attempts to use [1-3H]galactitol 6-sulphate as a substrate were unsuccessful, thus preventing identification of the enzyme as a keratan sulphate-degrading galactose 6-sulphate sulphatase as well as a chondroitin 6-sulphate-attacking N-acetylgalactosamine 6-sulphate sulphatase. 2. N-Acetylgalactosamine 6-sulphate sulphatase has high specificity for the position of the sulphate group and does not act on a 6-sulphated glucosamine derivative. For arylsulphatase B, which acts physiologically as an N-acetylgalactosamine 4-sulphate sulphatase, the nature of the hexosamine moiety has been shown to be less important (Farooqui, 1976). 3. Despite the structural requirements outlined above, all sulphated glycosaminoglycans containing 6-sulphated hexose derivatives served as competitive inhibitors of the enzyme, regardless of whether the glucose or galactose configuration at C-4 was present. 4. The purified enzyme removed only one sulphate group from the disulphated trisaccharide, suggesting that the enzyme acts as an exosulphatase. This observation conflicts with findings of Matalon et al. (1974) and Singh et al. (1976) on crude enzyme preparations. 5. When tested after the last two purification steps the enzyme exhibited two pH optima. In contrast with the behaviour of arylsulphatase A (Baum et al., 1958; Yang & Srivastava, 1976), the pH optima were independent of the time of incubation, but varied with the buffer concentration. A single rather broad pH optimum was found for the crude enzyme, the purified enzyme after mild periodate treatment, and for crude enzymes from human skin fibroblasts (Glössl & Kresse, 1978) and human kidney (Singh et al., 1976). More experiments are required to confirm the suggestion that the activity peak at the more acidic pH may result from enzyme aggregation.

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References

J. Glössl, W. Truppe and H. Kresse

1979