N-Acetylglucosamine-6-sulfate Sulfatase from Human Urine

(Received for publication, January 17, 1978, and in revised form, May 17, 1978)

Reinhard Basner,‡ Hans Kresse,§ and Kurt von Figura+¶

From the ‡Institute of Physiological Chemistry, University of Münster, D 4400 Münster, West Germany and the §Institute of Medical Chemistry, University of Graz, A 8010 Graz, Austria

N-Acetylglucosamine-6-sulfate sulfatase, which liberates sulfate from the N-acetylglucosamine 6-sulfate residue at the nonreducing terminus of a 3H-labeled trisaccharide prepared from heparan sulfate, was purified 138-fold from human urine. The final N-acetylglucosamine-6-sulfate sulfatase preparation was free of all lysosomal sulfatases known to act on sulfated polysaccharides and gave a single band in polyacrylamide gel electrophoresis. The enzyme appears to be a glycoprotein with a molecular weight of around 97,000 and displays considerable charge heterogeneity. Multiple forms with pI values between 5.4 and 8.3 with a maximum at pH 7.7 were detected. The enzyme acts on the 3H-trisaccharide with a pH optimum at 5.5 and is active towards the sulfated monosaccharides N-acetylglucosamine-6-sulfate and glucose 6-sulfate. Although predominantly an exosulfatase, the enzyme catalyzes hydrolysis of sulfate from internal N-acetylglucosamine 6-sulfate moieties at a low rate. The Km for the 3H-trisaccharide, N-acetylglucosamine 6-sulfate, and glucose 6-sulfate were 0.15, 1.5, and 7.1 mM, respectively. The enzyme is inhibited by albumin, H+3+, PO43-, SO42-, and CN-. Enzyme activity was highest in kidney and cultured fibroblasts but could be demonstrated in all human tissues tested.

Intralysosomal degradation of sulfated glycosaminoglycans involves the sequential removal of sulfate groups and sugar residues starting from the nonreducing terminal sugar of the polysaccharide chain (1, 2). Endoglycosidases, such as hyaluronidase and endoglucuronidase, may support the degradation accomplished by exoglycosidases and sulfatases (3, 4). Deficiency of any of the known exoglycosidases and sulfatases results in the intralysosomal accumulation of partially degraded glycosaminoglycan fragments (1, 2). N-Acetylglucosamine 6-sulfate residues are known to occur in heparan sulfate, heparin, and keratan sulfate (5, 6). Heparan sulfate and heparin contain in addition N-sulfated glucosamine residues and iduronate sulfate residues, whereas keratan sulfate contains additional galactose 6-sulfate residues. In recent years, the elucidation of the enzymatic defects in mucopolysaccharidoses provided evidence for the existence of sulfatases specific for N-sulfated glucosamine residues (7), for iduronate sulfate residues (8), and for galactose 6-sulfate residues (9). Evidence that a N-acetylglucosamine-6-sulfate sulfatase is involved in the degradation of heparan sulfate and keratan sulfate was provided by the recent report of a mucopolysaccharidoses with increased excretion of these two polysaccharides in a patient whose fibroblasts lacked N-acetylglucosamine-6-sulfate sulfatase activity (9).

Prior reports of failure to demonstrate N-acetylglucosamine-6-sulfate sulfatase activity in mammalian tissues using N-acetylglucosamine 6-sulfate as substrate (10) led us to prepare a monosulfated 3H-labeled trisaccharide from heparan sulfate bearing a N-acetylglucosamine 6-sulfate residue at its nonreducing terminus. In this report, we demonstrate the existence of an N-acetylglucosamine-6-sulfate sulfatase which acts on this substrate in different human tissues and describe the purification and characterization of this enzyme from human urine.

**Experimental Procedures**

**Materials**

NaH3H2O (specific activity, 271 Ci/mol) and heparin-[N-33S]SO4 (specific activity, 37 Ci/g) were obtained from Amersham Buchler (Braunschweig). Sephadex, concanavalin A-Sepharose 4B, and Sephaeryl gels were from Pharmacia (Uppsala), Dowex 1-X2 was from Serva (Heidelberg), and DE52-cellulose and CM52-cellulose were from Whatman (Maidstone). Protein calibration kit size I was obtained from Boehringer Mannheim (Mannheim). All chemicals used were of analytical grade.

The p nitrophenylglycosides were from Koch-Light (Colnbrook); p-nitroacetochol sulfate was from Sigma (St. Louis). N-acetylglucosamine 6-sulfate, N-acetylgalactosamine 6-sulfate, glucose 6-sulfate, and glucose-6-sulfate were a gift of Dr. Olavesen (Cardiff).1 IdoUA-[1H3]aMan-ol(6S) and GlcUA-[1H3]aMan-ol(6S) were a gift from Dr. M. Höök (Uppsala); IdoUA(2S)-[1H1]aMan-ol(6S), GalNAC(4S)-GlcUA-[1H]N-ac-galactosaminitol(4S), heparan-[35S]sulfate from bovine aorta, heparan-[35S]sulfate oligosaccharides obtained after nitrous acid degradation, keratan-[35S]sulfate from human rib cartilage, and [U-14C]GlcUA-Xyl were provided by Dr. U. Klein and Dr. R. Niemann, this institute; UDP-[35S]GalNAc(4S) and GalNAc(6S)-GlcUA-[1H]N-ac-galactosaminitol(6S) were gifts from Dr. W. Mlekusch and J. Gössel (Graz). Human placenta a-N-acetylgalactosaminidase and rat liver b-glucuronidase, both enzymes free of other lysosomal hydrolases, were provided by W. Röhrborn and R. Niemann, this institute. Di-4-aminoacyclopropyl-p-aminophenyl-1-thio-b-N-acetylgalactosamine-Sepharose 4B was prepared as described (11).

**General Methods**

**Preparation of 3H-labeled Monosulfated Trisaccharide with GlcNAc(6S) Residue at Nonreducing Terminator from Heparan Sulfate—Heparan sulfate, 1.22 g, isolated from bovine aorta (12), was dissolved in 10 ml of H2O and treated for 15 min at −5°C with 48 ml of nitrous acid prepared from a mixture of H2SO4 and Ba(NO3)2 (13). After quenching the reaction by addition of 17 ml of 1 M Na2CO3, the reaction mixture was loaded on a Sephadex G-25 superfine column (5 × 220 cm), equilibrated, and eluted with 1 M NaCl. The tetrasaccharide fraction eluting between 2490 and 2970 ml and comprising 20.7% of the total uronic acid was desalted on a Sephadex G-25.

1 All sugars were of D configuration unless otherwise stated. The position of carbon to which sulfate groups are linked is marked in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom reprint requests should be addressed.

---

* This work was supported by Grant SFB 104 from the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom reprint requests should be addressed.

1 The abbreviation used is: aMan-ol, 2,5-anhydro-n-mannitol.
medium column (3.5 x 230 cm) in water. An aliquot of the tetrasaccharide fraction (corresponding to 39.8 µmol of anhydromannose and 87.6 µmol of uronic acid) was treated in 2.1 ml of 0.1 M sodium borate, pH 8.0, with 0.3 mmol of NaBH₄ (14). After 7 h, 0.9 mmol of NaBH₄ was added and, 2 h later, the reaction mixture was brought to pH 5 with acetic acid and loaded on a Sephadex G-15 column (3.6 x 240 cm), eluted with 0.025% (w/v) bovine serum albumin in 15 mM NaCl and then desalted as described above. Monosulfated and disulfated H-labeled tetrasaccharides were isolated from an aliquot of the H₂-tetrasaccharide fraction by paper electrophoresis at pH 1.7. For preparation of H-labeled trisaccharides with a N-acetylglucosamine-6-sulfate residue at the nonreducing terminus, the H₂-tetrasaccharides were digested with 180 milliunits of bovine liver β-glucuronidase (Mann, New York; specific activity, 2.5 milliunits/mg, containing α-L-iduridinase and iduronide sulfatase as impurities) and 10 milliunits of purified α-N-acetylglucosaminidase from human placenta (specific activity, 2200 milliunits/mg) for 100 h in 0.15 M sodium acetate (pH 4.8), 0.05 M NaCl, and 0.06% NaN₃ (w/v) in a final volume of 6 ml. The digest was subjected to five rechromatographies on a Sephadex G-25 fine column (3.6 x 240 cm) in 1 M NaCl and then desalted as described above. The H₂-tetrasaccharide fraction was used as substrate for enzyme assays.

Paper Electrophoresis—Electrophoresis on Whatman No. 3MM paper was carried out in 1.9 M formic acid (pH 1.7) at 40 V/cm for 45 to 60 min or in pyridine/acetate, pH 5.3 (0.08 M with regard to pyridine), at 60 V/cm for 30 to 45 min.

Assay of N-Acetylglucosamine-6-sulfate Sulfatase—The standard incubation mixture in 1 ml of plastic tubes contained 220 µmol of monosulfated H₂-tetrasaccharide (about 11,000 cpm) in 0.16 M sodium acetate (pH 5.5), 0.017% (w/v) bovine serum albumin, 0.03% (w/v) NaN₃, and up to 5 µl of enzyme solution in a final volume of 6 ml. Paraffin oil, 5 µl, was layered on the incubation mixture. After incubation for 0.5 to 48 h at room temperature, the formation of unsulfated H₂-labeled trisaccharide was determined either by paper electrophoresis at pH 1.7 or by the following microcolumn procedure. The reaction was stopped by addition of 0.5 ml of 10 mM HCl and the mixture was loaded on a column (0.5 x 2.5 cm) of Dowex 1-X2, 200 to 400 mesh, equilibrated with 10 mM HCl. The unsulfated H₂-labeled trisaccharide was eluted with 4.5 µl of 0.5 M NaCl and the monosulfated H₂-tetrasaccharide was eluted with 5 µl of 500 mM NaCl in 10 mM HCl. Both fractions were assayed for radioactivity after mixing with an equal volume of Unisolve 1 (Zinsser, Frankfurt). In control assays incubated with boiled enzyme, about 2 to 2.5% of the total H radioactivity was recovered with 40 µg of NaCl in 10 mM HCl, whereas on paper electrophoresis about 0.4 to 0.8% of the total H radioactivity behaved like unsulfated H₂-labeled material.

When sulfated monosaccharides were used as substrates, the incubation mixture consisted of 20 mM substrate in 0.16 M sodium acetate (pH 5.5), 0.017% (w/v) bovine serum albumin, 0.03% (w/v) NaN₃, and up to 20 µl of enzyme solution in a final volume of 0.1 ml. After incubation for 16 h at 37°C the released sulfate was determined. Then 0.2 ml of 2% Luviskol K 90 (Bayer, Leverkusen) containing 1% BSA, was added and the mixture was shaken vigorously. After 60 min at room temperature, absorbance was read at 566 nm.

Assays of Other Sulfatases and Glycosidases—Arylsulfatase A was determined with p-nitrocatechol sulfate (16) and N-acetylgalactosamine-4-sulfate sulfatase with UDP-[3H]GalNAc ([45] (17). The incubation mixture for the determination of iduronide sulfatase activity contained 0.5 µmol of 1-IdoUA ([3H]Man-NS) in 0.16 M sodium acetate (pH 4.1), 0.017% (w/v) bovine serum albumin, 0.03% (w/v) NaN₃, and 5 µl of enzyme solution in a final volume of 6 µl. Paraffin oil, 5 µl, was layered on the assay mixture and after incubation at 37°C for up to 24 h the product formation was assayed by paper electrophoresis at pH 1.7. Sulfamidase was assayed as described (7). Activities of iduronide sulfatase and sulfamidase were expressed as released sulfate per h in per cent of added substrate.

All other glycosidases were assayed with 5 mM concentrations of their p-nitrophenylglycosides in 50 mM sodium citrate (pH 4.6), 0.1% (w/v) bovine serum albumin, 0.02% (w/v) NaNO₃, and 0.05% of enzyme solution in a final volume of 0.1 ml. After incubation for up to 24 h at 37°C the reaction was stopped by addition of 0.5 ml of 0.4 M NaOH buffer. After addition of 0.5 ml of 4 M H₂SO₄, the absorbance was read at 405 nm. For all enzymes 1 unit was defined as that amount of enzyme catalyzing the hydrolysis of 1 µmol of substrate/min unless otherwise stated.

Preparation of Urinary Proteins, Tissue Extracts, and Fibroblast Suspensions for N-Acetylglucosamine-6-sulfate Sulfatase Activity Determination—Urinary proteins were precipitated from fresh human morning urine by addition of 20 g of ammonium sulfate/100 ml of urine. The precipitate was suspended in 0.15 M NaCl (2 ml/liter urine), stirred for 1 h at 4°C and centrifuged at 8000 x g for 30 min. The supernatant was dialyzed against 0.15 M NaCl for 48 h and used for enzyme determination.

Tissues were homogenized in 100 mM NaCl, 0.02% (w/v) NaNO₃, in 10 mM Tris/HCl buffer, pH 7.5 (40% w/w). The homogenates were subjected to three cycles of freezing and thawing, centrifuged at 30,000 x g for 30 min. The supernatant was made 70% saturated with ammonium sulfate. The precipitate was suspended in 100 mM NaCl, 0.02% (w/v) NaNO₃ in 10 mM Tris/HCl buffer, pH 7.5 (0.4 to 2.7 g wet weight), dialyzed against the same buffer for 40 h, and centrifuged at 30,000 x g for 30 min.

Suspensions of human skin fibroblasts were prepared as described elsewhere (7).

Isoelectric focusing was done in a 110-ml column (LKB 8101, LKB Produkter AB) with a mixture of Ampholine, pH 5 to 7 and 7 to 9 (1:1; final concentration, 0.75% w/v) according to the producer's manual. Polyacrylamide gel electrophoresis was performed according to Rodbard and Chrambach in the anodic system 35.7 of Ref. 18 with an operative pH of 5.0 in the stacking gel and 4.0 in the separation gel. The gels were stained for proteins with Coomassie brilliant blue R 250 (19) or for carbohydrates (20) or were frozen for 24 h at -20°C. The frozen gels were sliced and the activity was extracted by homogenization of the slices in either 10 mM NaCl, 0.017% (w/v) bovine serum albumin in 10 mM Tris/HCl buffer, pH 7.2, or 150 mM NaCl, 1% (w/v) glycine in 10 mM Tris/HCl buffer, pH 7.2. The enzyme activity was recovered after centrifugation at 8000 x g for 20 min in the supernatant. Polyacrylamide gel electrophoresis in the presence of 0.1% (w/v) sodium dodecyl sulfate was done according to Weber and Osborn (21) in 10% gels.

Analytical Methods—Analyses of hexosamines (22), uronic acids (23), ester sulfate (24), anhydromannose (25), and protein (26) were done as described. Radioactivity measurements were taken as follows. Liquid samples were mixed with an equal volume of Unisolve 1 (Zinsser, Frankfurt) in a Packard liquid scintillation spectrometer Tri-Carb B 2450. Electrophoresis strips were cut into segments (1 to 2 x 1.5 cm), eluted with 1 ml of water in scintillation vials, and counted after addition of 2 ml of Unisolve.

RESULTS—Preparation of H₂-labeled Monosulfated Trisaccharide with N-Acetylglucosamine-6-Sulfate Residue at Nonreducing Terminus—Heparan sulfate isolated from bovine aorta was treated with nitrous acid under conditions that release all N-sulfate groups and convert the generated glucosamine residues into anhydromannose (13). After chromatography on Sephadex G-25, 20.7% of the total uronic acid was recovered in the position of the tetrasaccharides (Fig. 1). NaB₃H₄ reduction yield H₂-tetrasaccharides with a specific activity of 68.8 Ci/mol. The H₂-tetrasaccharides were treated with nitrous acid under conditions converting glucosamine residues into anhydromannose (15). The product was rechromatographed twice on Sephadex G-25 and once on Sephadex G-15. The H₂-tetrasaccharides thus obtained contained 1 N-acetylglucosamine residue/molecule in penultimate position. An unknown part of these N-acetylglucosamine residues bears sulfate ester groups at C6. To obtain trisaccharides exposing at the nonreducing terminus...
Human N-Acetylglucosamine-6-sulfate Sulfatase

FIG. 1. Gel chromatography on Sephadex G-25 of heparan sulfate after treatment with nitrous acid. Effluent fractions were analyzed for uronic acid (C—O) and pooled as indicated. The pooled Fractions I to VI were assayed for uronic acid, hexosamine, and anhydromannoside. The obtained data were in agreement with di-, tetra-, and hexasaccharide structure of Fractions VI, V, and IV, respectively, whereas Fraction III contains mainly octasaccharide and Fractions II and I contain mixtures of larger fragments.

exclusively N-acetylglucosamine 6-sulfate residues, the \(^3\)H-tetrasaccharide fraction containing 33% of material subjected to sodium borohydride reduction was exhaustively digested with \(\beta\)-glucuronidase and \(\alpha\)-N-acetylglucosaminidase. The \(\beta\)-glucuronidase preparation from bovine liver contained as impurities \(\alpha\)-L-iduronidase and traces of iduronide sulfatase activity. Then 8.2% of starting tetrasaccharide fraction was recovered as \(^3\)H-trisaccharides after five recrmatographies on Sephadex G-15 that removed undigested \(^3\)H-tetrasaccharides, \(^3\)H-disaccharides, and \([1-\^3\)H]anhydromannitol. High voltage electrophoresis separated the \(^3\)H-trisaccharides into 39% unsulfated, 56% monosulfated, and 5% disulfated \(^3\)H-trisaccharides (Fig. 2). The presence of unsulfated \(^3\)H-trisaccharides suggested that the digestion with \(\alpha\)-N-acetylglucosaminidase was incomplete. A second exhaustive digestion of the monosulfated \(^3\)H-trisaccharide with \(\alpha\)-N-acetylglucosaminidase liberated 3% of the radioactivity as monosulfated \(^3\)H-disaccharides. The fraction of the monosulfated \(^3\)H-trisaccharide with the structure GlcNAc(6S)-UA-[1-\(^3\)H]aMan-ol was used for further studies.

Demonstration of N-Acetylglucosamine-6-sulfate Sulfatase Activity in Human Urine and Different Human Tissues

Incubation of crude urinary proteins with the monosulfated \(^3\)H-trisaccharide for 19.5 h under the standard conditions for determination of N-acetylglucosamine-6-sulfate sulfatase activity converted 86.4% of the substrate into unsulfated \(^3\)H-labeled material. The release of unsulfated \(^3\)H-labeled material was linear with time for up to 24 h and proportional to the concentration of urinary proteins (Fig. 3), provided that less than 12% of the substrate was split.

The N-acetylglucosamine-6-sulfate sulfatase activity in three urine samples varied between 15 and 21 microunits/mg of urinary protein. Tissue extracts of liver, spleen, heart, kidney, and cerebrum obtained from a female autopsy case (age, 55 years), of a placenta, and of suspensions of 21 different human skin fibroblasts cell lines were assayed for N-acetylglucosamine 6-sulfate sulfatase activity. N-Acetylglucosamine-6-sulfate sulfatase activity was detectable in all tissues (Table I). The specific activity varied from 0.03 microunits/mg in liver up to 2.99 microunits/mg in human skin fibroblasts. The high

![Figure 2](http://www.jbc.org/)

**Fig. 2.** High voltage electrophoresis of the \(^3\)H-trisaccharides for 60 min at pH 1.7. The standards shown are: A, L-IdoUA-[1-\(^3\)H]aMan-ol(6S); B, [1-\(^3\)H]aMan-ol(6S); C, L-IdoUA(2S)-[1-\(^3\)H]aMan-ol(6S). I to IV correspond to unsulfated (I), monosulfated (II), disulfated (III), and trisulfated (IV) \(^3\)H-labeled trisaccharides.

![Figure 3](http://www.jbc.org/)

**Fig. 3.** Time-dependent formation of unsulfated \(^3\)H-labeled material by urinary proteins. Monosulfated \(^3\)H-trisaccharide was incubated under standard assay conditions with 145 \(\mu\)g of urinary proteins for up to 48 h. The release of unsulfated \(^3\)H-labeled material was assayed with the microcolumn procedure. All values represent the mean of triplicates.

**Table I**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Activity</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>microunits (\times) mg protein(^{-1})</td>
<td>microunits (\times) g wet weight(^{-1})</td>
</tr>
<tr>
<td>Liver</td>
<td>0.033</td>
<td>0.62</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.24</td>
<td>12.36</td>
</tr>
<tr>
<td>Heart</td>
<td>0.19</td>
<td>2.77</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.01</td>
<td>13.49</td>
</tr>
<tr>
<td>Cerebrum</td>
<td>0.25</td>
<td>0.30</td>
</tr>
<tr>
<td>Placenta</td>
<td>0.60</td>
<td>2.13</td>
</tr>
<tr>
<td>Skin fibroblasts</td>
<td>2.99 ± 1.74 (1-s range)</td>
<td>((n = 21))</td>
</tr>
</tbody>
</table>
specific activity of N-acetylglucosamine-6-sulfate sulfatase in human urine led us to choose human urine as source for enzyme purification.

**Purification of N-Acetylglucosamine-6-sulfate Sulfatase**

Unless otherwise indicated, all following operations were done at 4°C.

**Step 1: Ammonium Sulfate Precipitation**—First morning urine (86 liters) from healthy young men was collected on a pellet of 3 M Tris/HCl, pH 7.5. The Tris/HCl buffer was diluted by the urine to approximately 5 to 15 mM concentrations. Within 2 h after voiding, 500 g of ammonium sulfate were added per liter of urine. After 3 to 5 days, the supernatant was sucked off and the precipitate was collected by centrifugation at 10,000 × g for 30 min. The precipitate was suspended in 590 ml of 0.15 M NaCl, stirred for 1 h, and centrifuged at 10,000 × g for 30 min. The extraction of the precipitate was repeated three times with 270, 170, and 210 ml of 0.15 M NaCl. The first extract containing 53% of extractable protein and 62% of the extractable N-acetylglucosamine-6-sulfatase activity was made 70% saturated with ammonium sulfate (500 g/liter of solution) again.

**Step 2: Sephacryl S-200 Chromatography**—The precipitate was collected by centrifugation at 10,000 × g for 30 min and suspended in 40 ml of 0.5 M NaCl in 10 mM Tris/HCl buffer, pH 7.5. After dialysis for 72 h against 6 × 5 liters of the same Tris/HCl buffer, the dialyzed solution was centrifuged at 176,000 × g for 30 min and the clear brown supernatant was diluted with the Tris/HCl buffer to 99 ml and applied to a downward flowing column of Sephacryl S-200 superfine (7.5 × 120 cm). The column was eluted at 25 ml/h in fractions of 15.7 ml (Fig. 4). Fractions comprising about 90% of the eluted activity were pooled and made 70% saturated with ammonium sulfate.

**Step 3: DE-cellulose**—The ammonium sulfate precipitate was dialyzed for 48 h against 4 × 5 liters of 10 mM NaCl in 10 mM Tris/HCl buffer, pH 7.2. The dialyzed solution (36 ml) was loaded on a DE52 cellulose column (2.6 × 41 cm) equilibrated with the same Tris/HCl buffer. The dialyzed solution was centrifuged at 8000 × g for 6 min and the supernatant was used for further purification

**Step 4: CM-cellulose**—The ammonium sulfate precipitate was dialyzed for 16 h against 5 liters of 10 mM NaCl in 10 mM sodium acetate, pH 4.6. The dialyzed solution (3.6 ml) was centrifuged at 8000 × g for 8 min and the supernatant was loaded on a CM52 cellulose column (1.2 × 4.6 cm), equilibrated with the sodium acetate buffer. The column was washed with 45 ml of this buffer and then eluted with 50 to 500 mM NaCl in 10 mM sodium acetate, pH 4.6. Two fractions of 15 ml were collected of each salt concentration. The fractions indicated by the horizontal bar (Fig. 6) were pooled and made 70% saturated with ammonium sulfate.

**Step 5: Polyacrylamide Gel Electrophoresis**—The ammonium sulfate precipitate was dialyzed for 16 h against 10 mM NaCl in 10 mM Tris/HCl buffer, pH 7.2 (final volume, 0.6 ml), and separated by polyacrylamide gel electrophoresis (separation gel, 1.5 × 6 cm, with 10% total gel concentration) for 8 h at 150 V. The frozen gel was sliced and activity was extracted with 150 mM NaCl in 10 mM Tris/HCl buffer, pH 7.2, containing 1% glycine. After this step only 1% of the starting activity was left. A summary of the purification procedure is given in Table II.

Native heparan sulfate contains sulfamingo groups and sulfate groups at C2 of iduronic acid and C6 of N-acetylglucosamine. Since nitrous acid treatment removes all sulfamingo groups and the exhaustive digestion of the monosulfated 3H-trisaccharide with α-N-acetylglucosaminidase removes all monosulfated 3H-trisaccharides that contain an internal iduronic 2-sulfate residue, it was unlikely that either sulfamingo or iduronic sulfatase activities interfered with the N-acetylglucosamine 6-sulphate sulfatase determination using the monosulfated 3H-trisaccharide as substrate. This assumption was confirmed by the complete separation of the three heparan sulfate-degrading sulfatases during the purification. Whereas

**FIG. 4. Gel chromatography of N-acetylglucosamine-6-sulfate sulfatase on Sephacryl S-200 superfine. Absorbance at 280 nm (----), N-acetylglucosamine-6-sulfate sulfatase activity (C---O), iduronide sulfatase activity (A--A), and sulfamingo activity (D---O) were recorded.**

**FIG. 5. Chromatography of N-acetylglucosamine-6-sulfate sulfatase on DE52 cellulose. Absorbance at 280 nm (---), sodium chloride concentration (---), sodium chloride concentration (---), N-acetylglucosamine-6-sulfate sulfatase activity (O---O), iduronide sulfatase activity (O---O), and sulfamingo activity (C---O) were recorded.**

**FIG. 6. Chromatography of N-acetylglucosamine-6-sulfate sulfatase on CM52 cellulose. A, sodium chloride concentration (---), N-acetylglucosamine-6-sulfate sulfatase activity measured either with the monosulfated 3H-trisaccharide (O---O) or with N-acetylglucosamine 6-sulfate (C---D), and glucose 6-sulfate sulfatase activity (A--A) were recorded. B, absorbance at 280 nm (---), sodium chloride concentration (---), iduronide sulfatase activity (O---O), and sulfamingo activity (C---O) were recorded. The bar indicates the fractions that were pooled for further purification.**

**TABLE II. Purification of N-Acetylglucosamine-6-sulfate Sulfatase**

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ammonium Sulfate Precipitation</td>
</tr>
<tr>
<td>2</td>
<td>Sephacryl S-200 Chromatography</td>
</tr>
<tr>
<td>3</td>
<td>DE-cellulose</td>
</tr>
<tr>
<td>4</td>
<td>CM-cellulose</td>
</tr>
<tr>
<td>5</td>
<td>Polyacrylamide Gel Electrophoresis</td>
</tr>
</tbody>
</table>
chromatography on Sephacryl S-200 did not separate the heparan sulfate-degrading sulfatases (Fig. 4), chromatography on DE- and CM-cellulose separated N-acetylgalactosamine-6-sulfate sulfatase almost completely from the other two sulfatases (Figs. 5 and 6). After polyacrylamide gel electrophoresis, the N-acetylgalactosamine-6-sulfate sulfatase preparation was free of sulfamidase and iduronide sulfatase.

Properties of N-Acetylglucosamine-6-sulfate Sulfatase

Purity—The final N-acetylgalactosamine-6-sulfate sulfatase preparation gave a single protein band in polyacrylamide gel electrophoresis with a $R_f$ value between 0.21 and 0.31 (Fig. 7) that co-migrated with the enzyme activity. In the presence of sodium dodecyl sulfate, a major protein band and two minor, slightly faster migrating protein bands were observed (Fig. 7).

The enzyme preparation was free from the four sulfatases known to act on sulfated glycosaminoglycans: sulfamidase, iduronide sulfatase, N-acetylgalactosamine-4-sulfate sulfatase, and N-acetylgalactosamine-6-sulfate sulfatase. The final preparation was furthermore assayed for the presence of the following lysosomal enzymes: arylsulfatase A, $\alpha$-N-acetylgalactosaminidase, $\alpha$-N-acetylgalactosaminidase, $\beta$-N-acetylgalactosaminidase, $\alpha$-l-fucosidase, $\alpha$-mannosidase, $\alpha$-glucosidase, $\beta$-glucosidase, $\alpha$-galactosidase, $\beta$-galactosidase, and $\beta$-glucuronidase. Only a low $\beta$-N-acetylgalactosaminidase activity was detectable. Compared to the ratio of N-acetylgalactosamine-6-sulfate sulfatase and $\beta$-N-acetylgalactosaminidase in the starting material, the N-acetylgalactosamine-6-sulfate sulfatase was purified 935-fold over $\beta$-N-acetylgalactosaminidase. The residual $\beta$-N-acetylgalactosaminidase activity was completely removed by passage over a di-6-amino-caproyl-p-aminophenyl-1-thio-$\beta$-D-N-acetylgalactosaminidase-Sepharose 4B column equilibrated and eluted with 0.15 M NaCl in 10 mM Tris/HCl, pH 7.5.

Molecular Weight by Sephadex G-200 Chromatography—The molecular weight of N-acetylgalactosamine-6-sulfate sulfatase (preparation obtained after Step 2) was determined on a calibrated column of Sephadex G-200 (Fig. 8). The apparent molecular weight of the N-acetylgalactosamine-6-sulfate sulfatase was 97,000 (95% confidence limits: 75,000 and 125,000). Molecular weight determination of the protein bands visible after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate gave $M_r = 99,000$ for the major protein band and $M_r = 80,000$ and 65,000 for the two minor protein bands.

Glycoprotein Nature of N-Acetylglucosamine-6-sulfate Sulfatase—N-Acetylglucosamine-6-sulfate sulfatase bound to concanavalin A-Sepharose 4B equilibrated with 150 mM NaCl in 10 mM Tris/HCl buffer, pH 7.5. In the Tris/HCl buffer, 1 M methyl-$\alpha$-glucoside eluted 32% of the enzyme activity. Further purification of the enzyme was not attempted.

Fig. 7. Polyacrylamide gel electrophoresis of N-acetylgalactosamine-6-sulfate sulfatase in the absence (A) and presence (B) of sodium dodecyl sulfate at 15% total gel concentration. The arrows mark the position of the dye front. The gels were scanned at 570 nm.

Fig. 8. Molecular weight determination of N-acetylgalactosamine-6-sulfate sulfatase by gel chromatography. A Sephadex G-200 column (1.2 x 97 cm), running in 10 mM Tris/HCl buffer, pH 7.5, containing 0.5 M NaCl and 0.02% NaN$_3$ was calibrated with cytochrome c ($M_r = 12,500$), chymotrypsinogen A ($M_r = 25,000$), ovalbumin ($M_r = 45,000$), bovine serum albumin ($M_r = 67,000$), aldolase ($M_r = 158,000$), and catalase ($M_r = 240,000$). $V_v$ and total volume were determined with ferritin and T$_2$O, respectively.
ther evidence for the glycoprotein nature of the N-acetylglucosamine-6-sulfate sulfatase was provided by the positive staining for carbohydrates of the protein band observed after polyacrylamide gel electrophoresis and of the major protein band obtained after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

**Determination of pH**—In a pH gradient, N-acetylglucosamine-6-sulfate sulfatase distributed between pH 5.4 and 8.3. The maximum of the enzyme activity was recovered at pH 7.7 (Fig. 9). After isoelectric focusing, 120% of the applied enzyme activity was recovered.

**Substrate Specificity of the N-Acetylglucosamine-6-sulfate Sulfatase**—N-Acetylglucosamine 6-sulfate ester linkages are present in heparin, heparan sulfate, and keratan sulfate. Exhaustive digestion of heparin [N-35S]0₄, heparan [35S]sulfate, heparan [35S]sulfate fragments obtained after nitrous acid degradation and of keratan [35S]sulfate with N-acetylglucosamine-6-sulfate sulfatase did not release 35SO₄²⁻. The rate of sulfate release from 3H-tetrasaccharides bearing either one or two sulfate ester groups in unknown position was 6 and 3%, respectively, of that observed with the monosulfated 3H-trisaccharide (Fig. 10). None of the following tri- and disaccharides proved to be a substrate for N-acetylglucosamine-6-sulfate sulfatase: 3H-trisaccharides prepared from chondroitin 4-sulfate and chondroitin 6-sulfate with either N-acetylgalactosamine 4-sulfate or N-acetylgalactosamine 6-sulfate residues at their nonreducing terminus, L-IdoUA(2S)-[1-3H]aMan-ol(6S) and GlcUA(1-3H)Man-ol(6S) (Table III).

**Effect of Substrate Concentration on Activity of N-Acetylglucosamine-6-sulfate Sulfatase**—N-Acetylglucosamine-6-sulfate sulfatase was incubated in the presence of 37 to 210 μm monosulfated 3H-trisaccharide and of 625 μm to 20 nm concentrations of either N-acetylgalactosamine 6-sulfate or glucose 6-sulfate. The plot of 1/V versus 1/S showed a straight line relationship. Apparent Kᵣ values of 0.19 to 11.56 pm, respectively, and Vₘₐₙ values of 9.9 to 98.0 nm/min/mg of protein, respectively, were found (Table IV).

**pH Optimum, pH Stability, and Thermal Stability**—The pH activity profile determined with the monosulfated 3H-trisaccharide indicates a broad pH optimum with optimal...
activity at pH 5.5. The effect of the pH on the stability of the enzyme activity was tested by incubating a N-acetylglucosamine-6-sulfate sulfatase preparation obtained after purification Step 3 with 36 mM concentrations of buffers with pH values between 3.1 and 10.4 for 17 h at 4°C. The enzyme activity was stable between pH 4.5 and 7.5. A crude enzyme preparation (after Step 1, protein concentration 7 mg/ml) in 0.5 M NaCl in 10 mM Tris/HCl (pH 7.5), 0.02% NaN₃ was kept at 70°C. Within 3 min more than 90% of the activity were destroyed.

Effect of Metals, Chelators, Detergent, -SH-protective Compounds, and -SH reagents—The effect of 17 mM Cu²⁺, Zn²⁺, Ba²⁺, Co²⁺, Fe³⁺, Mn²⁺, Hg²⁺, Mg²⁺ (all as chlorides), S₂O₅²⁻, CN⁻, SO₃²⁻, SO₄²⁻, PO₄³⁻, NO₃⁻, I⁻ (all as sodium salts), EDTA, Triton X-100 (0.17 to 1.7% v/v), and 10 mM glutathione-reduced, cysteine, dithiothreitol, and N-ethylmaleimide on N-acetylglucosamine-6-sulfate sulfatase activity were tested. Hg²⁺, S₂O₅²⁻, SO₃²⁻, SO₄²⁻, CN⁻, and PO₄³⁻ inhibited almost completely the enzyme activity (0 to 5% residual activity). In the presence of the other compounds the activity was in the range of 80 to 120% of that of the control.

Effect of Bovine Serum Albumin—The N-acetylglucosamine-6-sulfate sulfatase activity was assayed in the presence of up to 0.5% (w/v) bovine serum albumin. Concentrations above 0.1% inhibited the enzyme activity. At 0.5% bovine serum albumin, the activity was decreased to 22% of that of the albumin free control.

Enzymatic Characterization of the Monosulfated ³H-Trisaccharide

Monosulfated ³H-trisaccharide (I), 2.3 nmol, was digested with N-acetylglucosamine-6-sulfate sulfatase. Eighty-eight per cent of the ³H radioactivity was released as unsulfated material (II), which co-migrated on high voltage electrophoresis at pH 5.3 with an unsulfated ³H-trisaccharide standard (Fig. 11A).

Exhaustive degradation with purified α-N-acetylglucosaminidase converted II quantitatively into a ³H-labeled product (III) that had the same Kᵥ on high voltage electrophoresis at pH 5.3 as L-IdoUA-[α-³H]aMan-ol and had a slightly lower Kᵥ value on Sephadex G-15 than L-IdoUA-[α-³H]aMan-ol and [U-¹⁴C]GlcUA-Xyl (Fig. 11B). Digestion of Product III with purified rat liver β-glucuronidase converted Product III quantitatively to the ³H-labeled Product IV that behaved as uncharged ³H-labeled material in high voltage electrophoresis and had the same Kᵥ on Sephadex G-15 as anhydromannose (Fig. 11C). By this sequential enzymatic degradation 88% of the monosulfated ³H-trisaccharide could be characterized as GlcNAc(6S)-α-1-4GlcUA-β-1-[³H]aMan-ol.

DISCUSSION

The preparation of a monosulfated ³H-trisaccharide with a N-acetylglucosamine-6-sulfate residue at the nonreducing terminus provided a natural substrate with which N-acetylglucosamine-6-sulfate sulfatase activity in a variety of human tissues and human urine could be demonstrated. Together with α-N-acetylglucosaminidase, which itself is inactive towards terminal N-acetylglucosamine 6-sulfate residues (27), N-acetylglucosamine-6-sulfate sulfatase is responsible for the removal of N-acetylglucosamine-6-sulfate residues in heparan sulfate as shown by the sequential degradation of the trisaccharide GlcNAc(6S)-GlcUA-α-1-4[³H]aMan-ol. The enzyme may

![Figure 11](http://www.jbc.org/)

Fig. 11. Characterization of the monosulfated ³H-trisaccharide by sequential enzymatic degradation. A, paper electrophoresis for 30 min at 60 V/cm at pH 5.3 of monosulfated ³H-trisaccharide (I) after digestion for 141 h with 0.42 µg of purified N-acetylglucosamine-6-sulfate sulfatase; B, paper electrophoresis for 45 min at 60 V/cm at pH 5.3 of Compound II after digestion with 0.01 milliunits of α-N-acetylglucosaminidase for 162 h; C, paper electrophoresis for 45 min at 60 V/cm at pH 5.3 of Compound III after digestion with 8.6 milliunits of rat liver β-glucuronidase for 240 h; D, elution pattern of Compounds II to IV on a Sephadex G-15 column. The Sephadex G-15 column (1 x 240 cm) was equilibrated and eluted with 0.02 M HCl in 0.1 M NaCl. The following reference compounds were used: a) unsulfated ³H-trisaccharide; b) L-IdoUA-[α-³H]aMan-ol; c) monosulfated ³H-trisaccharide; d) GlcUA-GalNAc(4S)-GlcUA-GalNAc(4S); e) GlcNAc(4S)-GlcUA-[α-³H]aMan-ol(4S); f) GalNAc(4S)-GlcUA; g) [U-¹⁴C]GlcUA-Xyl; h, anhydromannose. Void volume (Vₒ) and total volume (Vₜ) were determined with dextran blue and Tₐ₂O, respectively.
also be involved in the degradation of 6-sulfate groups of the glucosamine moieties in heparin and keratan sulfate. The glycoprotein enzyme exhibits typical features of a lysosomal hydrolase, including acid pH optimum and extensive charge heterogeneity. Like several other sulfatases, N-acetylglucosamine-6-sulfate sulfatase is inhibited by PO₄³⁻ and SO₄²⁻ (28–31).

A remarkable feature of this sulfatase is its activity towards the sulfated monosaccharides N-acetylglucosamine 6-sulfate and glucose 6-sulfate. Both sulfated monosaccharides are split with a 10-fold higher velocity than the monosulfated ³H-trisaccharide. The affinity of the enzyme towards the sulfated monosaccharides, however, is 10- to 50-fold lower than towards the trisaccharide. The N-acetylglucosamine-6-sulfate sulfatase and the N-acetylgalactosamine-6-sulfate sulfatase (9) have in common the recognition of the sulfate group at C-6 and the conformation at C-4. However, C-2 can either bear a hydroxyl or a N-acetyl group. Activity of other mammalian polysaccharide-degrading sulfatases towards monosaccharides has not been investigated extensively. Only N-acetylgalactosamine-4-sulfate sulfatase has been assayed so far only with polysaccharides (7, 8, 28, 34, 35), oligosaccharides (8, 31), and glycocides (17). Earlier attempts to detect sulfatas in mammalian tissues that were active towards a variety of sulfated monosaccharides including N-acetylglucosamine 6-sulfate, were unsuccessful (10).

The purification procedure outlined in Table II produced an enzyme enriched 136-fold in specific activity, with only 1% yield of initial enzyme activity. The large loss in activity raised the question that the enzyme was being separated from an activator on purification. However, we were unable to find any activator. The glucosamine-6-sulfate sulfatase preparation was free of other lysosomal enzyme activities and at least 90% pure as judged by two polyacrylamide gel electrophoresis procedures.

The enzyme may provide a tool for enzymatic characterization of oligosaccharides derived from heparan sulfate, heparin, and keratan sulfate. The N-acetylglucosamine-6-sulfate sulfatase seems to act almost exclusively as an exosulfatase. Low activity was observed towards tetrasaccharides bearing N-acetylglucosamine 6-sulfate residues in penultimate position. The failure to detect sulfatase activity towards polysaccharides known to contain N-acetylglucosamine 6-sulfate residues may indicate that the activity of N-acetylglucosamine-6-sulfate sulfatase is restricted to the disaccharide unit at the nonreducing terminus.

The enzymatically characterized monosulfated ³H-trisaccharide should have arisen from the following pentasaccharide (trisaccharide in italics): -GlcN(2S or 2,6DiS)-UA-GlcN(2S or 2,6DiS)-UA-GlcN(2S or 2,6DiS). - According to the current concept of heparin (and heparan sulfate) biosynthesis, the formation of N-sulfate groups precedes the epimerization of the D-glucuronic acid to L-iduronic acid in the nascent polysaccharide chain. O-Sulfatation goes either parallel or follows the epimerization (for review see Ref. 36). It is noteworthy that in the monosulfated ³H-trisaccharide all N-acetylglucosamine 6-sulfate residues are linked to glucuronic acid residues, which in turn are linked to N-sulfated glucosamine residues. Sequential enzymatic degradation of additional heparin and heparan sulfate oligosaccharides will be valuable in determining the structure of these polysaccharides and testing hypotheses concerning their biosynthesis.

REFERENCES


Downloaded from http://www.jbc.org/ by guest on August 15, 2017
N-Acetylglucosamine-6-sulfate sulfatase from human urine.
R Basner, H Kresse and K von Figura


Access the most updated version of this article at http://www.jbc.org/content/254/4/1151

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/254/4/1151.full.html#ref-list-1