Multiple Forms of 2-Deoxy-D-glucoside-2-sulphamate Sulphohydrolase from Human Placenta

By Eduard PASCHKE* and Hans KRESSE*

Medizinisch-Chemisches Institut und Pregl-Laboratorium der Universität Graz, Harrachgasse 21, A-8010 Graz, Austria

(Received 19 January 1979)

2-Deoxyglucoside-2-sulphamate sulphohydrolase was purified about 10000-fold from the soluble extract of human placenta by using as substrate [*N*-sulpho-³⁵S]heparin. Differently charged enzyme forms were observed on chromatography on DEAE-cellulose, all of which had an apparent mol.wt. of 110000 as determined by gel filtration. By using immobilized heparan sulphate as affinity matrix the sulphamate sulphohydrolase could be separated into two forms, a minor one with low and a major one with high affinity for the adsorbent. When tested with [*N*-sulpho-³⁵S]heparan sulphate the low-affinity form had a K_m of 0.2 mM, and the high-affinity form a K_m of 0.03 mM. Both forms exhibited the same K_m of 10 μ M towards [*N*-sulpho-³⁵S]heparin and were equally well adsorbed to immobilized heparin. The two forms could be distinguished by their pH-optima and by the influence of KCl on heparan sulphate sulphohydrolase activity.

The occurrence of *N*-desulphation of [³⁵S]heparin has been known since Lloyd *et al.* (1966) and Lemaire *et al.* (1967) found inorganic [³⁵S]sulphate in the urine after intravenous administration of [*N*-sulpho-³⁵S]heparin to rats. Lloyd *et al.* (1968), Dietrich (1970) and Friedman & Arsenis (1972) described sulphamate sulphohydrolase activities participating in the degradation of heparin in the soluble extract from various mammalian tissues. It was concluded that the enzyme liberated sulphate only from sulphamate groups at the non-reducing end of the polymer. The lysosomal arylsulphatases A and B could clearly be resolved from the sulphamate sulphohydrolase protein (Friedman & Arsenis, 1972), which is also located intralysosomally (Friedman & Arsenis, 1974).

There is some confusion about the terminology of the enzyme. Its recommended name is sulphoglucosamine sulphamidase (EC 3.10.1.1). The mammalian enzyme, however, does not act on sulphoglucosamine. Furthermore, it is not working on sulphamide, and there is only one amino group involved. In accordance with the chemical nature of substrates and of the reaction product it is therefore suggested to name the enzyme 2-deoxy-D-glucoside-2-sulphamate sulphohydrolase or, briefly, sulphamate sulphohydrolase.

Studies on the properties of human sulphamate sulphohydrolase are of interest because an inherited disorder, Sanfilippo's disease type A or mucopolysaccharidosis III A, is caused by the inactivity of that enzyme (Kresse & Neufeld, 1972; Kresse, 1973, Matalon & Dorfman, 1974). Patients suffering from

this metabolic disorder accumulate in their tissues and excrete into their urine large amounts of partially degraded heparan sulphate. Heparan sulphate shares with heparin the presence of sulphamate groups. Though the enzyme deficiency has been measured by using heparin as substrate instead of the storage material, i.e. heparan sulphate, it seemed reasonable to assume that the same enzyme might attack both polysaccharides. On the other hand, the finding of an unusually high residual activity of sulphamate sulphohydrolase activity towards heparin in the brain of a patient with Sanfilippo's disease type A (Witting *et al.*, 1975) could be explained by the existence of multiple forms of sulphamate sulphohydrolase differing in their ability to attack the two substrates containing sulphoamino groups.

We therefore carried out a purification of sulphamate sulphohydrolase from human placenta and tested simultaneously for enzyme activity towards heparin and heparan sulphate. By affinity chromatography on immobilized heparan sulphate multiple enzyme forms can be separated that are distinguishable by Michaelis constant and pH-optimum with respect to the two polymers.

Materials and Methods

Materials

Human placentae were obtained about 1 h after delivery from a nearby maternity home. The liver of a patient suffering from Sanfilippo's disease type A (Cain *et al.*, 1977) was obtained at autopsy and stored frozen at -20°C until use. [*N*-sulpho-³⁵S]-Heparin (sp. radioactivity 25 mCi/g) and trimethyl-

* Present address: Physiologisch-Chemisches Institut der Universität Münster, Waldeyerstrasse 15, D-4400 Münster, Federal Republic of Germany.

amine- N -[^{35}S]sulphonate (sp. radioactivity 4.3 Ci/mol) were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Aminoethyl-Bio-Gel P-150 was obtained from Bio-Rad Laboratories, Munich, Germany. Sephadex gels, Sephacryl S-200 and concanavalin A-Sepharose were from Pharmacia, Uppsala, Sweden. Chondroitin ABC lyase, methyl α -mannoside and dithiothreitol were obtained from Sigma, St. Louis, MO, U.S.A., DE-52 DEAE-cellulose was from Whatman, Maidstone, Kent, U.K., and heparin and Dowex 1 (X2; 200–400 mesh) were purchased from Serva, Heidelberg, Germany. All other chemicals used were of analytical grade.

Preparation of [N -sulpho- ^{35}S]heparan sulphate

Minced liver from a patient with Sanfilippo's disease type A was dried in acetone and treated with papain (Kresse & Buddecke, 1970). After digestion for 24 h at 65°C glycosaminoglycans were precipitated by addition of 3 vol. of ethanol containing 13 g of potassium acetate/litre. After centrifugation the precipitate was washed in methanol, dissolved in 0.15 M-NaOH and kept for 4 h at 37°C before neutralization with acetic acid. Further purification procedures included fractionation of the glycosaminoglycan-hexadecylpyridinium complexes, ion-exchange chromatography on Dowex 1 (X2) and digestion with chondroitin ABC lyase (Kindler *et al.*, 1977). The heparan sulphate preparation had the following composition: hexosamine, 1.42 $\mu\text{mol}/\text{mg}$; uronic acid, 1.29 $\mu\text{mol}/\text{mg}$; total sulphate, 1.29 $\mu\text{mol}/\text{mg}$; and sulphoamino groups, 0.68 $\mu\text{mol}/\text{mg}$. Probably all heparan sulphate chains contained a sulphoamino group at the non-reducing terminus of the polymer.

For hydrolytic removal of N -sulphate groups 125 mg of heparan sulphate was dissolved in 125 ml of 0.04 M-HCl and kept under an N_2 atmosphere for 90 min at 100°C. After neutralization with KOH the material was recovered by ethanol precipitation as described above. Resulphation was performed by the method of Lloyd *et al.* (1971). The N -desulphated heparan sulphate was dissolved in 2.5 ml of water and treated with 125 mg of trimethylamine- N -[^{35}S]sulphonate and 105 mg of Na_2CO_3 . The pH was adjusted to pH 9.6 by addition of 1 M-NaOH. The solution was stirred at 52–56°C for 24 h, cooled to room temperature (21°C) and treated with 1 M-HCl to pH 7.5. After dialysis for 3 days against three changes (5 litres each) of water the material was again precipitated with ethanol and potassium acetate (see above), then dissolved in 3 ml of pyridine/acetic acid buffer (0.2 M with respect to pyridine), pH 5.5, and applied to a Sephadex G-25 column (2 cm $\times$ 190 cm) equilibrated and eluted with the same buffer. Radioactive material appearing in the exclusion volume of the column was pooled, concentrated to 3 ml under

reduced pressure and dialysed for 3 days against several changes of 0.15 M-KCl. The final preparation had initially a specific radioactivity of 3.9 Ci/mol of sulphoamino groups and contained 2.6 μmol of sulphoamino groups/ml.

[N -sulpho- ^{35}S]Heparan sulphate was chromatographed on a Sephadex G-200 column (1 cm $\times$ 96 cm) equilibrated and eluted with 1 M-NaCl. It was eluted with a K_{av} value of 0.8, which corresponds to a mol.wt. of 3000 according to the calibration curve of Wåsteson (1969).

Assay of sulphamate sulphohydrolase activity

Sulphamate sulphohydrolase activity was determined by using either [N -sulpho- ^{35}S]heparin or [N -sulpho- ^{35}S]heparan sulphate as substrate. [^{35}S]Heparin was dialysed extensively against 0.15 M-KCl and diluted with unlabelled heparin before use (Schmidt *et al.*, 1977). The solution had the following composition: hexosamine, 0.74 $\mu\text{mol}/\text{ml}$; uronic acid, 0.72 $\mu\text{mol}/\text{ml}$; total sulphate, 1.87 $\mu\text{mol}/\text{ml}$; and sulphoamino groups, 0.69 $\mu\text{mol}/\text{ml}$. The specific radioactivity was initially 7.6 Ci/mol of sulphoamino groups.

For heparin the incubation mixture contained 30 μl of a suitable diluted enzyme preparation, 20 μl of [N -sulpho- ^{35}S]heparin solution corresponding to 13.4 nmol of N -sulphonate groups and 10 μl of 1 M-sodium acetate buffer, pH 5.0, containing 0.15 M-KCl, 2 mM-dithiothreitol, 15 mM- NaN_3 and 2 g of bovine serum albumin/litre. For controls a solution of 1 g of bovine serum albumin/litre was used instead of enzyme. In the assays with heparan sulphate 12.8 nmol of N -sulphonate groups were used, and the pH of the acetate buffer was decreased to pH 4.5. After incubation at 37°C for specified periods of time the amount of liberated inorganic [^{35}S]sulphate was determined after paper electrophoresis in a hexadecylpyridinium chloride-containing barbital buffer (method 1; Kresse, 1973). In the controls for both substrates about 0.05% of the total radioactivity migrated like inorganic sulphate.

During chromatographic purification sulphamate sulphohydrolase activity was measured by a more rapid but less accurate procedure for the separation of inorganic sulphate and the polymer (method 2). The incubation was stopped by the addition of 190 μl of human serum and 750 μl of ethanol. After mixing and standing at room temperature for 10 min the samples were centrifuged. Sulphate release was measured by counting a 500 μl portion of the supernatant.

Under the assay conditions the enzyme was not saturated with either substrate. Sulphate release was linear with time and proportional to the amount of enzyme provided that not more than 1.2% in the case of heparin, or 6% in the case of heparan sul-

phate, of the total radioactivity was converted into inorganic sulphate.

Coupling of heparan sulphate and heparin to aminoethyl-Bio-Gel P-150

The coupling procedure followed the method of Baues & Gray (1977) for the purification of lectins. Aminoethyl-Bio-Gel P-150 was swollen in 0.2M-potassium phosphate buffer, pH 9.0, according to the manufacturer's recommendation. An excess of buffer amounting to one-eighth of the volume of the settled gel was allowed to remain. Heparan sulphate or heparin was added (56mg/ml of gel suspension in each case) together with 10.4mg of NaCNBH₃. The mixture was stirred for 6h and left for 45 days at room temperature. For acetylation of remaining free amino groups the gel was washed with 0.2M-sodium acetate followed by dropwise addition of 0.2ml of acetic anhydride/ml of gel suspension. After 30min at 0°C addition of acetic anhydride was repeated and the reaction allowed to proceed at room temperature. Finally the gel was washed sequentially with water, 0.1M-NaOH, water and 0.1M-sodium acetate buffer, pH 5.0, containing 0.15M-KCl, 2mM-2-mercaptoethanol and 3mM-Na₂N₃ and then stored at 4°C until use.

The gels contained 5.6μmol of hexosamine/ml after coupling with heparan sulphate and 6.2μmol/ml after treatment with heparin.

Purification of sulphamate sulphohydrolase

Crude enzyme preparation. Three fresh placentae were freed from adhering blood coagula and foetal membranes and cut in about 1cm³ cubes. After addition of 4ml of 0.05M-Tris/HCl buffer, pH 7.0, containing 0.15M-KCl and 2mM-2-mercaptoethanol, per g of tissue, homogenization with an Ultra-Turrax (Jahnke and Kunkel, Freiburg, Germany) homogenizer was performed in 15 bursts of 15s with intervals of 20s while the mixing vessel was kept in a methanol bath at -20°C. All further steps were done at 4°C except when stated otherwise. The homogenate was centrifuged at 11300g for 1h and the supernatant used for further purification procedures.

(NH₄)₂SO₄ precipitation (step I). The supernatant was made 70% saturated with (NH₄)₂SO₄. After standing overnight the precipitate was collected by centrifugation for 30min at 80000g and suspended in 0.05M-Tris/HCl buffer, pH 7.0, containing 0.15M-KCl and 2mM concentrations of MgCl₂, MnCl₂ and CaCl₂ (buffer A). The suspension (1320ml) was dialysed against three changes (5 litres each) of the same buffer for 24h. The dialysis residue was then centrifuged at 11300g for 30min to remove insoluble material.

Chromatography on concanavalin A-Sepharose (step II). The clear dialysis residue from step I

(1300ml, A₂₈₀ 79.3) was applied to a concanavalin A-Sepharose column (1.8cm × 24cm) as described previously (Glössl *et al.*, 1979). The column was then washed with buffer A until the A₂₈₀ was below 0.2. Desorption was performed with 835ml of buffer A containing additionally 0.5M-methyl α-mannoside. The eluate was dialysed for 24h against three changes (5 litres each) of 0.01M-Tris/HCl buffer, pH 7.2, containing 0.15M-KCl and 2mM-2-mercaptoethanol (buffer B), treated with (NH₄)₂SO₄ and centrifuged as described above. The sediment was dissolved in 250ml of 0.15M-Tris/HCl buffer, pH 8.0, containing 0.15M-KCl and 10mM-dithiothreitol. After standing at room temperature for 4h the solution was dialysed against 0.01M-Tris/HCl buffer, pH 8.2, containing 0.025M-KCl and 2mM-2-mercaptoethanol (buffer C) until buffer and dialysis residue exhibited the same conductivity.

DEAE-cellulose chromatography (step III). The enzyme preparation of step II (265ml) was applied at a flow rate of 15ml/h to a DE-52 DEAE-cellulose column (2.5cm × 24cm) equilibrated with buffer C. The column was washed with 480ml of that buffer and developed with a linear KCl gradient consisting of 1200ml of buffer C and 1200ml of that buffer containing 0.2M-KCl. Sulphamate sulphohydrolase-containing fractions were pooled, treated with (NH₄)₂SO₄ and centrifuged as described in step I.

Gel filtration on Sephacryl S-200 (step IV). The precipitate obtained after step III was suspended in 13ml of 0.01M-Tris/HCl buffer, pH 8.0, containing 0.15M-KCl and 10mM-dithiothreitol. After standing at room temperature for 4h the material was dialysed for 16h against two changes (5 litres each) of buffer B and chromatographed on a column of Sephacryl S-200 as described previously (Glössl *et al.*, 1979), except that NaCl was substituted by KCl. Fractions containing sulphamate sulphohydrolase activity were pooled, treated with (NH₄)₂SO₄ and centrifuged as described in step I.

Affinity chromatography on immobilized heparan sulphate (step V). The precipitate was dissolved in 5ml of 0.1M-sodium acetate buffer, pH 5.0, containing 0.15M-KCl and 2mM-2-mercaptoethanol (buffer D) and dialysed for 24h against 3 × 5 litres of that buffer. The dialysis residue was applied to a column (1.2cm × 22cm) of heparan sulphate coupled to aminoethyl-Bio-Gel P-150 at a flow rate of 2ml/h. After washing the column with buffer D (68ml) desorption was performed by applying buffer D, the KCl concentration of which was increased to 1.0M. Sulphamate sulphohydrolase-containing fractions were combined and dialysed extensively against buffer B.

Other methods

Analyses of uronic acids (Bitter & Muir, 1962), hexosamine (Boas, 1953), total sulphate (Greiling *et al.*, 1964) and sulphoamino groups (Lagunoff &

Warren, 1962) were performed as described. Protein was monitored by reading the A_{280} . Radioactivity was measured in a liquid-scintillation spectrometer (Beckman, model LS 8 100) with 2.1% 2,5-diphenyloxazole and 0.026% 1,4-bis-(5-phenyloxazol-2-yl)-benzene in toluene/ethylene glycol monomethyl ether (1:1, v/v).

Results and Discussion

Purification of sulphamate sulphohydrolase

The main form of sulphamate sulphohydrolase was purified about 10000-fold from a crude extract of human placenta (Table 1). The most efficient purification step was chromatography on immobilized concanavalin A. Almost all of the applied enzyme activity was retained by the affinity matrix. The enzyme was desorbed on elution in the presence of 0.5M-methyl α -mannoside, suggesting that as other

lysosomal enzymes sulphamate sulphohydrolase too is a glycoprotein. From Table 1 it might be deduced that chromatography on DEAE-cellulose removed an inhibitor of the sulphamate sulphohydrolase. No attempt was made to substantiate that assumption.

Tests to achieve further purification failed due to the lability of the enzyme under various conditions, e.g. low ionic strength and low pH at low ionic strength. Considerable loss of enzyme activity was observed after isoelectric focusing, polyacrylamide-gel electrophoresis at pH8.5 and cation-exchange chromatography at pH4.2. Thus, placental sulphamate sulphohydrolase seemed to be more labile than the enzyme from human urine (Kresse & Neufeld, 1972).

Charge heterogeneity of sulphamate sulphohydrolase

On anion-exchange chromatography on DEAE-cellulose at pH8.2 sulphamate sulphohydrolase

Table 1. Summary of purification of sulphamate sulphohydrolase from human placenta [*N*-sulpho- 35 S]Heparin was used as substrate throughout the purification procedure.

Step	Total protein (A_{280})	Total activity (nmol/h)	Specific activity (nmol/h per A_{280})	Purification (-fold)	Yield (%)
Crude extract	134000	836	0.006	1	100
(NH ₄) ₂ SO ₄ -precipitation	103000	376	0.003	0.5	50
Concanavalin A-Sepharose	742	608	0.823	137	73
DEAE-cellulose	238	788	3.26	543	94
Sephacryl S-200	28	363	13.06	2200	44
Immobilized heparan sulphate					
'Low-affinity enzyme'	8.9	38	4.2	700	4.5
'High-affinity enzyme'	6.3	381	60.6	10100	45.5

Fig. 1. Chromatography of sulphamate sulphohydrolase on DEAE-cellulose

Enzyme solution (560ml) obtained after chromatography on concanavalin A-Sepharose (A_{280} 0.678) was loaded on a column (2.5cm $\times$ 24cm) of DEAE-cellulose. Fractions of 11.5ml were collected; for further details see the Materials and Methods section. Measurement of liberated [35 S]sulphate was performed by method 2. The horizontal bars mark the fractions combined as pools 1, 2 and 3 respectively. $\bullet$, A_{280} ; $\square$, enzyme activity (pmol of sulphate released/h per ml); ----, [KCl].

regularly exhibited charge heterogeneity. As shown in Fig. 1 three major not-well-separated peaks of enzyme activity were observed. The peak fractions were desorbed at KCl molarities of 0.03 M, 0.052 M and 0.065 M respectively. In some enzyme preparations small additional activity peaks occurred at 0.025 M and 0.090 M-KCl, the activities being too low for further examination.

The three major enzyme forms were pooled as indicated in Fig. 1 and chromatographed separately on a calibrated Sephacryl S-200 column. Reference proteins used were ferritin (mol.wt. 480000), aldolase (mol.wt. 147000), bovine serum albumin (mol.wt. 67000), ovalbumin (mol.wt. 45000), chymotrypsinogen (mol.wt. 25000) and cytochrome *c* (mol.wt.

12400). In each case most of sulphamate sulphohydrolase activity emerged as a sharp peak with a K_{av} value of 0.21 (Fig. 2), corresponding to an apparent mol.wt. of 110000.

Affinity to immobilized heparin and heparan sulphate

After gel filtration sulphamate sulphohydrolase-containing fractions from all the three differently charged enzyme forms were combined and subjected to affinity chromatography on immobilized heparin and/or heparan sulphate. First, the sample was dialysed against 0.01 M-Tris/HCl buffer, pH 7.0, containing 0.15 M-KCl and 2 mM-mercaptoethanol and was then loaded on a column of heparin covalently coupled to aminoethyl-Bio-Gel P-150 and equilibrated with the same buffer. No binding of sulphamate sulphohydrolase to the affinity matrix occurred under these conditions (result not shown). Secondly, when sample and affinity column were equilibrated with 0.1 M-sodium acetate buffer, pH 5.0, containing 0.15 M-KCl and 2 mM-2-mercaptoethanol the enzyme was completely adsorbed (Fig. 3). Desorption occurred by increasing the KCl concentration to 1.0 M in the buffer mentioned above.

In a third set of experiments the enzyme obtained after gel filtration (4.8 ml, A_{280} 3.25) was applied at pH 5.0 (see above) to a column (1.2 cm $\times$ 22 cm) of heparan sulphate coupled to aminoethyl-Bio-Gel

Fig. 2. Gel chromatography on Sephacryl S-200 of sulphamate sulphohydrolase pool 1 (a), pool 2 (b) and pool 3 (c) obtained after DEAE-cellulose chromatography

The pools were obtained as shown in Fig. 1. Fractions of 6.8 ml were collected; for details see the Materials and Methods section. Measurement of liberated [35 S]sulphate was performed by method 2. ●, A_{280} ; □, enzyme activity (pmol of sulphate released/h per ml).

Fig. 3. Chromatography of sulphamate sulphohydrolase on immobilized heparin

Enzyme obtained after chromatography on Sephacryl S-200 (0.9 ml, A_{280} 3.2) was chromatographed on a column (0.9 cm $\times$ 9.5 cm) of aminoethyl-Bio-Gel P-150 coupled with heparin. Fractions of 0.6 ml were collected; see the text for further details. The arrow indicates the position where the KCl concentration of the buffer was increased to 1.0 M. Measurement of liberated [35 S]sulphate was performed by method 2. ●, A_{280} ; □, enzyme activity measured towards [*N*-sulpho- 35 S]heparin (nmol of sulphate released/h per ml); ▲, enzyme activity measured towards [*N*-sulpho- 35 S]heparan sulphate (nmol of sulphate released/h per ml).

P-150. With the starting buffer a component of sulphamate sulphohydrolase was eluted that was slightly retarded by the gel when compared with the bulk of protein (Fig. 4). The major part of the enzyme however, was not desorbed until the KCl concentration of the elution buffer was increased to 1.0M. Sulphamate sulphohydrolase-containing fractions were pooled as indicated in Fig. 4 and dialysed against 0.01M-Tris/HCl buffer, pH7.2, containing 0.15M-KCl and 2mM-2-mercaptoethanol. When tested against [*N*-sulpho-³⁵S]heparin, 9% of the recovered activity was found in the low-affinity pool (pool 1) and 89% in the high-affinity pool (pool 2). Fractions between the two pools contained the remaining 2% of recovered activity. Pool 1 was rechromatographed on an affinity column of the same size and under conditions exactly as during the first run. Of the sulphamate sulphohydrolase activity measured towards heparin 90% could not be adsorbed to the affinity matrix, thus indicating that the column had not been overloaded during the previous step.

Portions from the low- and from the high-affinity pool were tested on analytical-scale columns of heparin coupled to aminoethyl-Bio-Gel. As expected, in both cases adsorption to the affinity matrix occurred at pH5.0.

Fig. 4. Chromatography of sulphamate sulphohydrolase on immobilized heparan sulphate

Enzyme obtained after chromatography on Sephacryl S-200 was chromatographed on immobilized heparan sulphate as described in the text. Fractions of 1.8ml were collected. The horizontal bars mark the fractions combined as pool 1 ('low-affinity enzyme') and pool 2 ('high-affinity enzyme') respectively. The arrow indicates the position where the KCl concentration of the buffer was increased to 1.0M. Measurement of liberated [³⁵S]sulphate was performed by method 2. ●, *A*₂₈₀; □, enzyme activity measured towards [*N*-sulpho-³⁵S]heparin (nmol of sulphate released/h per ml); ▲, enzyme activity measured towards [*N*-sulpho-³⁵S]heparan sulphate (nmol of sulphate released/h per ml).

Kinetic properties of different sulphamate sulphohydrolase forms towards heparin and heparan sulphate

[*N*-sulpho-³⁵S]Heparin and [*N*-sulpho-³⁵S]heparan sulphate were used as substrates of the two sulphamate sulphohydrolase forms separated by affinity chromatography. Enzyme from pool 1 was used after rechromatography as described in the preceding paragraph.

Both enzyme forms were able to act on the two substrates. Sulphamate sulphohydrolase from the 'low-affinity pool' exhibited a single pH-optimum at pH4.6 towards both substrates, whereas the 'high-affinity enzyme' worked best on heparan sulphate at pH4.4 and on heparin between pH4.8 and 5.0 (Fig. 5).

Fig. 5. Effect of pH on sulphamate sulphohydrolase activity

Enzyme from pool 1 (a) and pool 2 (b) obtained after affinity chromatography on immobilized heparan sulphate was tested either towards [*N*-sulpho-³⁵S]-heparin (□) or towards [*N*-sulpho-³⁵S]heparan sulphate (●). Incubation was carried out in 0.167M-sodium acetate buffer of the respective pH as described in the Materials and Methods section. Liberated [³⁵S]sulphate was measured by method 1.

From the results of gel-filtration experiments the labelled heparan sulphate had a mean mol.wt. of 3000, whereas that of heparin was 8000. On incubation of the two enzyme forms in the presence of 30.5–488 μmol of heparan sulphate/litre and 6.6–155 μmol of heparin/litre respectively, in each case the plot of $1/v$ against $1/[S]$ revealed a straight line relationship (Fig. 6), thus allowing the calculation of Michaelis constants. As shown in Table 2 both enzyme forms had the same affinity towards heparin, whereas the 'low-affinity enzyme' had a Michaelis constant towards heparan sulphate one order of magnitude higher than the 'high-affinity enzyme'. In each case half-maximal hydrolysis of heparan sulphate occurred at higher substrate concentrations

when compared with heparin. Smaller differences were observed when the specific activities of the two enzyme forms, calculated from Lineweaver–Burk plots, were compared by using either heparan sulphate or heparin as substrates. Towards heparan sulphate the 'high-affinity enzyme' was 16 times more active than the 'low-affinity enzyme', whereas towards heparan sulphate the ratio was 9.

The finding of similar activity ratios for the two pools towards heparan sulphate and heparin (Table 2) seems incompatible with the elution profiles of sulphamate sulphohydrolase activity from the affinity column (Fig. 4), where the ratio of heparan sulphate-degrading activity to heparin-degrading activity of the 'high-affinity enzyme' was much greater than that of the 'low-affinity enzyme'. Since the activities in Fig. 4 were obtained before dialysis, differences in the influence of K^+ ions on the enzyme activity could exist. Sulphamate sulphohydrolase activity was therefore tested at various KCl concentrations (Fig. 7). In each case the highest activities were measured at up to 0.2M-KCl. Towards heparan sulphate, however, the enzyme from the 'high-affinity pool' showed a second maximum at a final KCl concentration of 0.3M.

In conclusion, the results described in the present paper give evidence for the existence of multiple sulphamate sulphohydrolase forms. These forms cannot be distinguished by their affinity towards heparin, but differ in their affinity towards heparan sulphate. They can additionally be characterized by their pH-activity curves and by the influence of K^+ ions on the enzyme activity. The genetic relationship between these two forms has not yet been elucidated. It might be that they are coded for by different genes

Fig. 6. Relationship between sulphamate sulphohydrolase activity and the concentrations of either $[N\text{-sulpho-}^{35}\text{S}]\text{-heparin}$ (a) or $[N\text{-sulpho-}^{35}\text{S}]\text{heparan sulphate}$ (b). Sulphamate sulphohydrolase from pool 1 (■) and pool 2 (●) obtained after affinity chromatography on immobilized heparan sulphate was incubated with the respective substrates. $[^{35}\text{S}]\text{Sulphate}$ release was measured by method 1.

Table 2. Kinetic properties of the different sulphamate sulphohydrolase forms obtained after chromatography on immobilized heparan sulphate

Details are given in the text. Release of $[^{35}\text{S}]\text{sulphate}$ was measured by method 1.

	$[N\text{-sulpho-}^{35}\text{S}]\text{-Heparin}$		$[N\text{-sulpho-}^{35}\text{S}]\text{-Heparan sulphate}$	
	K_m (μM)	$V_{\max.}$ (nmol/h per A_{280})	K_m (μM)	$V_{\max.}$ (nmol/h per A_{280})
Pool 1 ('low-affinity enzyme')	10	8.1	220	42
Pool 2 ('high-affinity enzyme')	10	132	32	370

Fig. 7. Effect of KCl concentration on sulphamate sulphohydrolase activity

Enzyme from pool 1 (a) and pool 2 (b) obtained after affinity chromatography on immobilized heparan sulphate was tested either towards $[N\text{-sulpho-}^{35}\text{S}]\text{-heparin}$ (□) or towards $[N\text{-sulpho-}^{35}\text{S}]\text{heparan sulphate}$ (●). $[^{35}\text{S}]\text{Sulphate}$ release was measured by method 1.

and that the 'low-affinity form' remains active in Sanfilippo's disease type A. On this basis the high residual activity of heparin-degrading sulphamate sulphohydrolase in some organs of patients with that disease (Witting *et al.*, 1975) could be accounted for.

We thank Professor H. Cain, Katharinen-Hospital, Stuttgart, Germany, for his help in obtaining autopsy material and Miss R. Schuligoi for technical assistance. The helpful suggestions concerning the nomenclature of the enzyme by Professor J. E. Scott, Department of Medical Biochemistry, University of Manchester Medical School, Manchester, U.K., are gratefully acknowledged. This work was supported in part by the Fonds zur Förderung der Wissenschaftlichen Forschung in Österreich.

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