N-Acetylgalactosamine-6-Sulfate Sulfatase in Man

ABSENCE OF THE ENZYME IN MORQUIO DISEASE

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ABSTRACT Human *N*-acetylgalactosamine-6-sulfate sulfatase (6-sulfatase) activity is measured by using as a substrate a sulfated tetrasaccharide obtained by digesting purified chondroitin-6-sulfate (C-6-S) with testicular hyaluronidase. The amount of inorganic sulfate released is measured turbidimetrically. The enzyme from human kidney has a pH optimum of 4.8; its activity is augmented by low levels of NaCl and inhibited by phosphate and high levels of NaCl. Free glucuronate, acetylgalactosamine, inorganic sulfate, polymeric C-6-S, or tetrasaccharide obtained from chondroitin-4-sulfate do not affect the enzyme activity. The method may be used for the diagnosis of Morquio disease since extracts of Morquio fibroblasts are devoid of 6-sulfatase activity.

INTRODUCTION

Matalon et al. (1) demonstrated the presence of chondroitin sulfate *N*-acetylgalactosamine-6-sulfate sulfatase (6-sulfatase) [1] in extracts of cultured human skin fibroblasts and have provided evidence that its deficiency is responsible for the pathogenesis of Morquio disease (mucopolysaccharidosis IV). For the assay, Matalon et al. (1) used biologically labeled polymeric chondroitin-4/6-sulfate extracted from femural and tibial epiphyses of chick embryos or oligosaccharides derived from it. The required purification of the substrate, the short half-life of the $^{35}SO_4$ label, the need for electrophoretic separation of the released inorganic $^{35}SO_4$ before its measurement, and the need to ascertain whether the sulfate released was in position 4 or 6 of the galactosamine moieties make the method (1) rather laborious and not ideal for the routine assay of the enzyme activity. Using a 6-sulfated tetrasaccharide as a substrate, we have developed a simple method for the measurement of 6-sulfatase activity. This report describes the preparation of the substrate, the optimal conditions of the enzyme assay as established with a crude enzyme preparation from human kidney, and the applicability of the method for the diagnosis of Morquio disease.

METHODS

Unsaturated 6-sulfated (Δ-Di-6S) and nonsulfated (Δ-Di-OS) disaccharides, shark cartilage chondroitin-6-sulfate (C-6-S), and whale cartilage chondroitin-4-sulfate were from Miles Laboratories, Inc., Miles Research Div., Elkhart, Ind.; bovine testicular hyaluronidase (3,000 USP U/mg) from Worthington Biochemical Corp., Freehold, N. J.; gelation from ICN Nutritional Biochemicals Div., International Chemical & Nuclear Corp., Cleveland, Ohio; AG 1x2 (200–400 mesh, Cl^- form) and Bio-Gel A-0.5 m (100–200 mesh) from Bio-Rad Laboratories, Richmond, Calif. Dermatan sulfate from pig intestinal mucosa was a gift of Dr. J. A. Cifonelli of University of Chicago. Tritiated heparin fractions were prepared as described (2).

Hexuronate (3), hexosamine (4), chloride (5), and reducing groups (6) were measured as previously published. Inorganic sulfate was assayed as described under *Assay of kidney enzyme* by a modification of Dodgson's method (7). The sulfate content of the mucopolysaccharides was assayed by Dodgson and Price's method (8) after hydrolysis in 1 N HCl for 5 h at 110°C. When polymeric C-6-S was tested as a possible substrate or inhibitor of the enzyme activity, or when enzyme assays were performed with urine concentrates or extracts of leukocytes or fibroblasts, sulfate was determined according to Saito et al. (9) (see *Assay of fibroblast enzyme*); in this method (9), polymeric glycosaminoglycans are precipitated with cetylpyridinium chloride before addition of the gelatin-$BaCl_2$ reagent.

Substrate preparation. 400 mg of shark cartilage C-6-S dissolved in 8 ml of a pH 4.8, 0.125 M Tris-acetate buffer

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[1] *Abbreviations used in this paper:* C-6-S, chondroitin-6-sulfate; 6-sulfatase, *N*-acetylgalactosamine-6-sulfate sulfatase.

containing 1.6 μmol of NaN_3 were incubated at 37°C with 12,000 USP units of testicular hyaluronidase for 48 h, and its depolymerization was monitored turbidimetrically (10). Upon completion of incubation, the digest was boiled for 20 min to inactivate hyaluronidase and was clarified by centrifugation. The supernate, which is the substrate, was diluted with a pH 4.8, 0.125 M Tris-acetate buffer to give 32 μmol hexuronate/ml, and stored frozen in portions until used. A portion of the degraded C-6-S was chromatographed on an AG 1 × 2 column (20 × 0.9 cm) packed with water and eluted with a linear gradient of 0–3.0 M NaCl in water (Fig. 1). The molecular weight of the degraded C-6-S was determined by gel filtration (Fig. 2) on a Bio-Gel A-0.5 m column (98 × 1 cm), packed and eluted with 0.025 M NaCl, as previously described (11–13). The column was calibrated with various carbohydrates of known molecular weight (11) (Fig. 3) and its void volume was assessed with blue dextran 2000.

Enzyme preparation. Whole normal human kidney was homogenized at 5°C in a Waring blendor (Waring Products Div., Dynamics Corp. of America, New Hartford, Conn.) with 1.2 ml of cold (5°C) 0.15 M NaCl/g of wet tissue. After centrifugation at 600 *g* for 30 min at 0°C, the supernate (50 ml) was dialyzed for 24 h at 5°C against four changes of 0.15 M NaCl (1 liter each). The retentate was centrifuged at 20,200 *g* for 30 min at 0°C and the supernate (which had 16.5 mg protein/ml [14]) was stored frozen until used.

Human skin fibroblasts from normal individuals and from Morquio patients were cultured (11) and homogenized (15) as described previously. Urinary concentrates were prepared by precipitation of fresh urine with $(NH_4)_2SO_4$ at 70% saturation (11). Leukocytes, harvested from whole blood according to Fallon et al. (16), were homogenized by the same method used for fibroblasts. Before enzyme assay, all extracts and homogenates were dialyzed against 0.15 M NaCl, as described for the preparation of kidney extract.

Assay of kidney enzyme. Assays were performed in glass tubes (100 × 12 mm) prewashed in concentrated HNO_3. The standard reaction mixture (total volume, 0.6 ml) contained: 0.3 ml of water containing 0.31 μmol of NaN_3, 0.15 ml of pH 4.8, 0.6 M Tris-acetate buffer, 0.05 ml of substrate solution (containing 1.6 μmol of hexuronate), and 0.1 ml (1.65 mg protein) of kidney extract. Control tubes contained only the substrate or all of the other components minus substrate. After incubation at 37°C for 8 h, the released inorganic sulfate was measured as follows. To each tube (except for the one containing substrate alone) was added 1.6 ml of 4% trichloroacetic acid to precipitate proteins. At this time, the substrate control was mixed with the reaction components of the other control tube. The precipitate in each tube was removed by centrifugation at 685 *g* for 25 min at 5°C and the supernates were recovered. To a 1.4-ml portion of each supernate, 0.5 ml of gelatin-$BaCl_2$ reagent was added. After 15 min at room temperature, the turbidity produced was measured at 360 nm against water. Net absorbance values were obtained by subtracting the value of the control from that of corresponding complete reaction mixture. 1 unit of enzyme activity is defined as that amount releasing 1 nmol inorganic sulfate/h at 37°C. Specific activity is units per milligram of protein.

Optimal conditions for the kidney enzyme (pH, length of incubation, amount of enzyme needed) and the effect upon it of various ions were determined with minor modifications of the described technique (see Figs. 4–7 and their legends).

Assay of fibroblast enzyme was performed under conditions given in the first paragraph of the preceding section but with an incubation period of 16 h. 0.1 ml of fibroblast extract usually had 0.4 mg protein. After incubation, to each

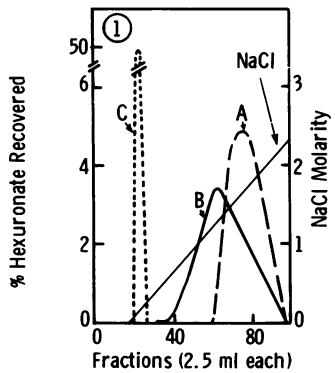

FIGURE 1 Elution patterns of polymeric C-6-S (A), degraded C-6-S (B), and D-glucuronic acid (C) from AG 1 × 2 column. NaCl concentration as measured in the fractions is shown. Each sample was chromatographed separately.

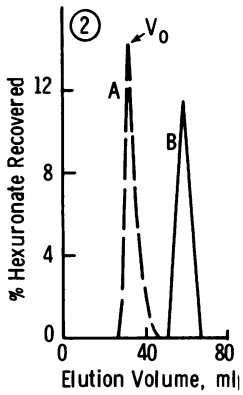

FIGURE 2 Elution patterns of polymeric C-6-S (A) and degraded C-6-S (B) from a calibrated Bio-Gel A-0.5 m column. V_0, void volume.

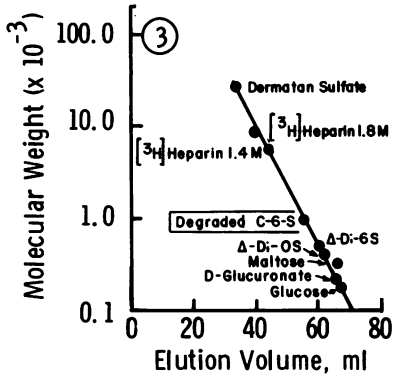

FIGURE 3 Calibration of Bio-Gel A-0.5 m column with carbohydrates of known molecular weight. The elution position of degraded C-6-S as obtained from Fig. 2 has been marked and its mol wt is 980±8%. Δ-Di-6S, unsaturated 6-sulfated disaccharides; Δ-Di-OS, unsaturated nonsulfated disaccharides.

TABLE I

Partial Analysis of Polymeric C-6-S and Hyaluronidase-Degraded C-6-S

Sample	Composition		Molar ratios, hexosamine = 1.0	Molar ratio, hexuronate/ reducing groups (as glucose)
		% dry wt		
Polymeric C-6-S	Hexosamine	34.05	1.0	
	Hexuronate	36.82	0.99	
	Sulfate	18.26	1.0	
				1.8:1
		mg/ml solution		
Hyaluronidase-degraded C-6-S	Hexosamine	5.74	1.0	
	Hexuronate	6.22	0.99	
	Sulfate	3.08	1.0	

tube (except for the one containing substrate alone) was added 0.6 ml of 0.17% cetylpyridinium chloride in 0.3 M HCl (9), to precipitate protein and polymeric glycosaminoglycans. At this time the substrate control was mixed with the reaction components of the other control tube. After 10 min at 37°C, the tubes were centrifuged at 685 *g* and to 0.6-ml aliquots of each supernate was added 1.4 ml of gelatin-$BaCl_2$ reagent in 0.15 M HCl (9). Turbidities were measured and net absorbance values obtained as described.

RESULTS

The C-6-S used for the preparation of the substrate had a hexuronate/hexosamine/sulfate molar ratio of 1:1:1 (Table I). Its molecular weight is reported (17) to be between 40,000 and 80,000 and it has a galactosamine-6-sulfate/galactosamine-4-sulfate molar ratio of 90/10 (17). When chromatographed on AG 1×2, it was quantitatively eluted as a single peak with maximum at 1.7 M NaCl (Fig. 1). From a Bio-Gel A-0.5 m column it eluted as a single peak with the void volume (Fig. 2). Its products of hyaluronidase degradation had a hexuronate/hexosamine/sulfate molar ratio of 1:1:1 and a hexuronate/reducing group molar ratio of 1.8:1 (Table I). This material was eluted quantitatively from the AG 1×2 column (Fig. 1) as a broad peak, with maximum at 1.3 M NaCl. Analyses of the various fractions for sulfate or hexosamine revealed patterns of elution similar to that of hexuronate, thus excluding the presence of free hexuronate or free inorganic sulfate (Fig. 1). Hyaluronidase-degraded C-6-S was eluted from Bio-Gel A-0.5 m column as a single peak (Fig. 2) and its molecular weight, as determined from the calibration shown in Fig. 3, was 980±8%. On the basis of its composition and molecular weight, the degraded C-6-S appears to be essentially a sulfated tetrasaccharide. In fact, its digestion with bovine liver

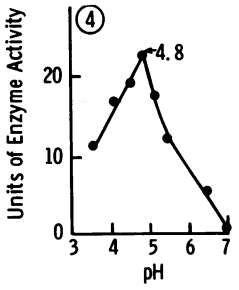

FIGURE 4 The pH optimum of the reaction was determined with 0.16 M Tris-acetate buffer of various pH and under the conditions described under *Assay of kidney enzyme*. 0.1 ml of kidney extract (1.65 mg protein) was employed and incubation was performed for 8 h at 37°C. Results are expressed as units of enzyme activity per milligram protein; 1 U corresponds to 1 nmol of sulfate released at 37°C. Kidney 6-sulfatase exhibits maximal activity at pH 4.8.

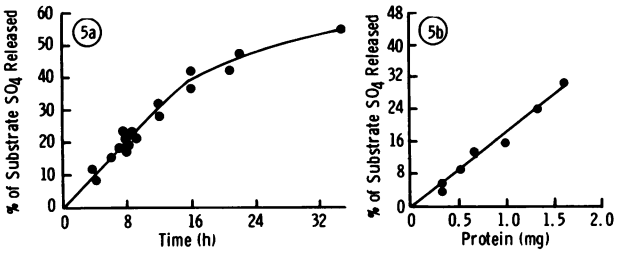

FIGURE 5 The release of sulfate as a function of time of incubation (*a*) or protein concentration (*b*). In *a*, the experiment was performed exactly as described under *Assay of kidney enzyme*, with 0.1 ml (1.65 mg protein) of kidney extract, but the incubation period was varied. The scale on the ordinate of the figure represents the substrate sulfate released per 1.65 mg of extract protein. The figure shows that after incubation for 8, 12, and 16 h at 37°C, 20, 30, and 41% of substrate sulfate was released, respectively, per 1.65 mg of protein. The experimental condition for 5*b* were the same as those in 5*a*, except that the incubation period was 12 h and various levels of kidney extract protein were employed. The figure shows that 30% of substrate sulfate was released per 1.65 mg of protein in 12 h of incubation at 37°C.

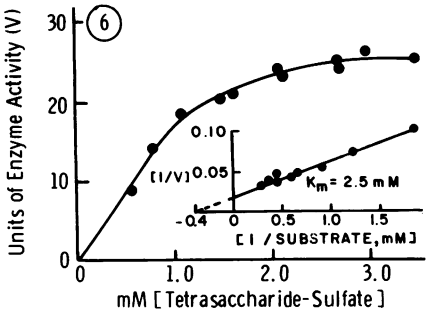

FIGURE 6 The enzyme activity as a function of substrate concentration. The inset shows the Lineweaver-Burk plot of the data. The enzyme activity has been expressed in units per milligram protein.

β-glucuronidase produced free hexuronate (accounting for 50% of the hexuronate of the tetrasaccharide) and a trisaccharide (with mol wt 800±7%), containing hexuronate/hexosamine/sulfate in a molar ratio of 1:2:2.

With the 6-sulfated tetrasaccharide as substrate and human kidney extract as enzyme source, optimum conditions were established for 6-sulfatase measurement (Figs. 4–7). Though the substrate contained equimolar levels of sulfate, hexosamine, and hexuronate, usually its concentration has been represented in terms of hexuronate. Since low levels of NaCl ($\leq$ 0.05 M) (Fig. 7) augmented the enzyme activity, a NaCl concentration of 25 mM was always maintained in routine assays. Under these conditions the enzyme showed maximum activity at pH 4.8 (Fig. 4). Enzyme activity was linear with time (Fig. 5*a*) for 16 h, during which 41% of the sulfate present on the substrate was hydrolyzed; the deviation from linearity thereafter might be due to substrate depletion and/or inactivation of the enzyme. Essentially all of the sulfate present in the substrate was hydrolyzed by extended incubation, to suggest that with oligosaccharides the sulfatase might either hydrolyze "internal" sulfate or that it might continue to act after the concomitant action of β-*N*-acetylhexosaminidase and

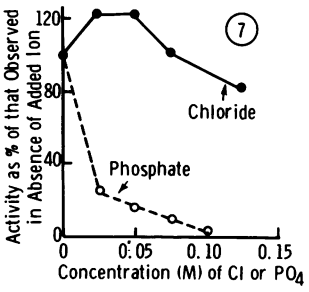

FIGURE 7 The effect of chloride and phosphate ions on the enzyme activity. Enzyme preparations previously dialyzed to remove chloride ions and progressive additions of NaCl to the reaction mixture were employed. Alternatively, a pH 4.8, 0.6 M sodium phosphate buffer was used to add increasing amount of phosphate ions to the reaction mixture.

TABLE II

6-Sulfatase Activity in Extracts of Cultured Skin Fibroblasts

Subject		Enzyme activity*	
		U/mg protein	
Normal	(E.)	35.4	
	(P.)	70.2	
	(N.)	79.8	71.7±36 %‡
	(L.)	66.9	
	(M.)	106.4	
Morquio	(G.)	ND§	
	(R.)	ND	

* 1 U activity = 1 nmol of inorganic sulfate released at 37°C/h.

‡ Average, and the value after the ± sign is coefficient of variation in percent.

§ ND, not detectable, and less than 1.5 U.

β-glucuronidase present in the crude enzyme preparation. In view of the demonstration (1) that extracts of fibroblasts lacking β-*N*-acetylhexosaminidase release as much 6-sulfate as extracts of normal fibroblasts from oligosaccharides (1) (the total amount being more than the sulfate present at the nonreducing terminus), it seems that, at least with low-molecular-weight substrates, the concomitant action of exoglycosidases might not be required. The kidney 6-sulfatase activity increased linearly with protein level (Fig. 5*b*) and the enzyme showed typical Michaelis-Menten kinetics (Fig. 6), with an apparent K_m of 2.5 mM. The concentration of the Tris-acetate buffer (pH 4.8) could be varied from 0.11 to 0.31 M without affecting the enzyme activity. Phosphate and high levels of NaCl (over 75 mM) inhibited the enzyme activity (Fig. 7), which, however, was not affected by the addition to the incubation mixture of *N*-acetyl-D-galactosamine ($\leq$ 1.6 μmol), D-glucuronate ($\leq$ 1.6 μmol), inorganic sulfate ($\leq$ 0.625 μmol), polymeric C-6-S ($\leq$ 1.6 μmol as hexuronate), or 4-sulfated tetrasaccharides ($\leq$ 1 μmol as galactosamine-4-sulfate). The latter ones were obtained from whale cartilage C-4-S by digestion with testicular hyaluronidase,[2] according to the method given for preparing degraded C-6-S.

The specific activity of the kidney 6-sulfatase, measured under standard assay conditions, was 24; and the precision of the methods, computed in terms of coefficient of variation, is ±6%. Its activity is totally destroyed by boiling for 30 min.

While the trisaccharide produced by digesting the tetrasaccharide (degraded C-6-S) with β-glucuronidase is an equally good substrate, Δ-Di-6S or the polymeric C-6-S are not. These observations and the pH optimum

[2] Singh, J. Unpublished data.

of 4.8 show that the enzyme has properties different from those of the bacterial chondro-6-sulfatase (18), which uses unsaturated 6-sulfated disaccharide as its substrate and shows maximum activity in an alkaline pH range.

DISCUSSION

The proposed assay of 6-sulfatase activity employs a natural substrate of simple preparation, which contains not more than 10% of galactosamine-4-sulfate. Since human kidney extract, as well as other crude enzyme preparations, contain[2] *N*-acetylgalactosamine-4-sulfate sulfatase (4-sulfatase or arylsulfatase B), some of the sulfate hydrolyzed from the substrate does not reflect the activity of 6-sulfatase. Although there are methods available (19, 20) for the removal of 4-sulfatase activity from enzyme preparation, its contribution may be measured by incubating a given enzyme preparation with the same amount of 4-sulfated tetrasaccharide present in the 6-sulfated tetrasaccharide substrate. By incubating the kidney extract with 0.16 μmol (as hexuronate) of 4-sulfated tetrasaccharides under the specified conditions, we have estimated that only 2.4% of the sulfate hydrolyzed may be due to the activity of 4-sulfatase on the 4-sulfated tetrasaccharides present in our substrate. The values reported in this paper have been corrected accordingly.

The suitability of the method for the diagnosis of Morquio disease from extracts of cultured skin fibroblasts is indicated by the data in Table II. The absence of measurable activity in Morquio fibroblasts is consistent with the finding of Matalon et al. (1).

Concentrates of normal human urine and extracts of normal leukocytes were also assayed for 6-sulfatase with this method and the activities found were 37 and 47 units/mg protein, respectively.

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