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AN ASSAY FOR IDURONATE SULFATASE (HUNTER CORRECTIVE FACTOR)*

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ABSTRACT

A substrate for α-L-idopyranosyluronic acid 2-sulfate 2-sulfohydrolase (iduronate sulfatase), the enzyme deficient in the Hunter syndrome, was prepared by deaminative cleavage of heparin and subsequent reduction of the disulfated disaccharide fragment with sodium borotritide, to give *O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1 → 4)-2,5-anhydro-D-[^{3}H-1]mannitol 6-sulfate. Incubation of this compound with enzyme gave a monosulfated radioactive product, which can be separated from the substrate by paper chromatography or electrophoresis. The reaction follows Michaelis–Menten kinetics, with a K_M of 3mM. The phosphate ion is a potent inhibitor.

INTRODUCTION

The defect in the Hunter syndrome (an X-linked recessive disorder characterized by lysosomal storage and urinary excretion of dermatan sulfate and heparan sulfate)[1] is a profound deficiency of iduronate sulfatase[2–4] (α-L-idopyranosyluronic acid 2-sulfate sulfohydrolase, EC 3.1.6.–).

Before the enzymic defect was elucidated, it was known that fibroblasts derived from the skin of patients suffering from Hunter disease degrade endogenous glycosaminoglycan [^{35}S]sulfates at a reduced rate, and that this metabolic abnormality can be corrected by adding to the culture medium a protein designated as "Hunter corrective factor"[5]. It is this factor which was eventually identified as the enzyme, iduronate sulfatase[2]. Correction of Hunter fibroblasts in culture is now thought to occur through capture of the enzyme by the cells, followed by its sequestration into lysosomes[6,7]. Normal catabolic function would thereby be restored, since the lysosomes contain not only stored glycosaminoglycans but also all the other enzymes necessary for the degradation of these polymers.

Two methods have been used to identify the enzymic activity of the factor[2]: (a) measurement of [^{35}S]sulfate ions released by the Hunter corrective factor from "oversulfated" areas of dermatan [^{35}S]sulfate that had been extracted from Hunter

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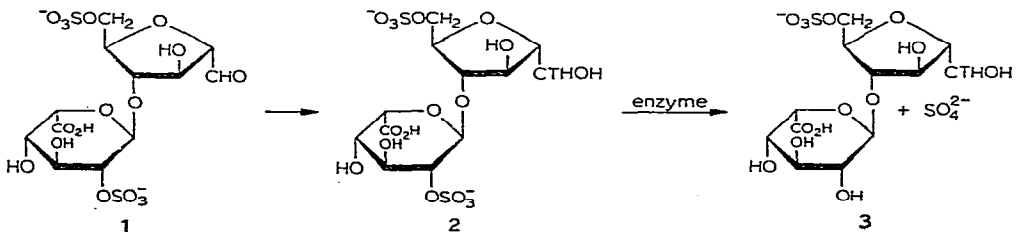
fibroblasts (such areas presumably containing sulfated iduronic acid residues)[8,9]; and (b) measurement by gas–liquid chromatography[9] of L-iduronic acid released through the coupled action of Hunter factor and α-L-iduronidase (EC 3.2.1.76) on the disaccharide *O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1 → 4)-2,5-anhydro-D-mannose 6-sulfate (**1**). The first of these methods is too imprecise and the second too laborious to qualify as routine assays for the study of the enzyme. Although the enzyme can be assayed by measuring the release of sulfate ions from **1**, the carcinogenic properties of benzidine[10] (used to precipitate sulfate ions)[11] discouraged this approach.

We present here an assay based on the use of disaccharide **1** after reduction with sodium borotritide into *O*-(α-L-idopyranosyluronic acid 2-sulfate)-(1 → 4)-2,5-anhydro-D-[^{3}H-1]mannitol 6-sulfate (**2**). Compound **2** is converted by iduronate



1 2 3

sulfatase into the idosyluronicanhydromannitol 6-sulfate **3**. Product and residual substrate are separated by paper chromatography or electrophoresis, and determined quantitatively by measurement of radioactivity.

EXPERIMENTAL

Reagents. — Barium L-iduronate prepared by synthesis[12] was a gift from Dr. Gilbert Ashwell; [^{35}S]sulfuric acid (carrier free), sodium borotritide (25 mCi per 3.9 mg) and Liquifluor were purchased from New England Nuclear (Boston, Mass. 02118); Bio-Solv BBS-3 from Beckman Instruments Inc. (Fullerton, Calif. 92634), Cellex-E (ECTEOLA-cellulose) from Bio-Rad Laboratories (Richmond, Calif. 94804); and heparin (170 units/mg) from Sigma Chemical Co. (St. Louis, Mo. 63178).

Enzyme. — The preparation of Hunter corrective factor (pools 1 and 3) purified by Cantz *et al.*[5] was used in present experiments. The phosphate buffer in which the enzyme was stored was removed by dialysis against 0.15M sodium chloride after addition of bovine serum albumin to a concentration of 1 mg/ml.

Analytical methods. — Uronic acid content was determined by the carbazole–borate method of Bitter and Muir[13] with L-iduronic acid as standard, and 2,5-anhydro-D-mannose by the indole method of Dische and Borenfreund[14], without prior deamination (although the deamination step had to be included for the standard, D-glucosamine). The benzidine test for inorganic sulfate was performed by the method of Antonopoulos[11], modified as follows to increase reproductibility: Tubes

were prewashed in nitric acid; the benzidine sulfate precipitate was allowed to form for 2 h at 0°, and was collected by centrifugation at 12,000 *g* at low temperature. A tracer amount of [^{35}S]sulfate was added to the test mixture before precipitation, and recovery of the radioactivity after redissolving the precipitate in M hydrochloric acid was used to correct for any loss of benzidine sulfate that might have occurred during the vigorous washing procedure. *Benzidine must be handled with caution*[10].

Descending paper chromatography was performed in 4:1 (v/v) 95% ethanol–M ammonium acetate (Solvent A), or in 2:3:1 (v/v) 1-butanol–acetic acid–0.1M ammonium hydroxide (Solvent B). Paper electrophoresis was performed in 1.6M formic acid at 33 V/cm. The paper was prewashed in the solvents to be used, or preferably in M acetic acid. Sugars were detected with the silver nitrate reagent[15].

Radioactivity was measured in a liquid scintillation spectrometer, in 9.5 ml of scintillation fluid composed of 1:2.7:24 (v/v) Liquifluor, Bio-Solv BBS-3, and toluene either with 0.5 ml of water (for radioactive solutions) or without water (for paper strips). Counting efficiency was 37% and 4%, respectively, for tritium-labeled compounds in solution and on paper.

Preparation of substrate. — Hog-mucosa heparin was degraded with butyl nitrite at −15° by the procedure of Cifonelli and King[16]. Compound **1** was isolated from the products of this deamination cleavage by gel filtration on Sephadex G-15 resin, followed by ion-exchange chromatography on Cellex-E in a linear gradient of 0.02 to M ammonium hydrogen carbonate and lyophilization, essentially as recommended by Höök *et al.*[17]. From 11.9 g of heparin, 2.25 g of the ammonium salt of **1** was obtained. Colorimetric analysis showed the ratio of sulfate (released by hydrolysis in 4M hydrochloric acid at 100° for 6 h)/uronic acid/anhydromannose to be 2:0.9:1.2. This ratio includes a correction for the contribution of anhydromannose to the absorbance at 530 nm in the carbazole test for uronic acid. Gas–liquid chromatography by the procedure of Eisenberg[2,18] showed L-iduronic acid to be the only uronic acid component; a 2% contamination by D-glucuronic acid would have been easily detected. The disulfated disaccharide migrated chromatographically and electrophoretically with the same mobility as a sample of **1** given to us by Dr. U. Lindahl. Although chromatography revealed some silver nitrate-reactive material streaking from the origin to the major spot, **1** was reduced without further purification.

The radioactive-labeled substrate **2** was prepared by dissolving **1** (10 mg) in 0.5M sodium borate buffer (0.5 ml), pH 7.0, and adding twice a freshly prepared 1% solution of sodium borotritide (0.2 ml) (25 mCi/ml), at an interval of 30 min. The reaction was followed by determination of residual **1**; when absorbance in the indole test was reduced to 1% of the initial value, the solution was mixed with 1 g of Dowex-50 (H^+) ion-exchange resin and the suspension filtered. The pH of the filtrate (plus washes) was adjusted to 5 with 0.1M ammonium hydroxide, and the solution was evaporated to dryness. Excess of borate ions was removed by two cycles of methanol addition and evaporation. The residue was chromatographed on Whatman 3MM paper for 18 h in Solvent A. The radioactive area was cut out and eluted with water after extensive washing with absolute ethanol.

Unlabeled substrate **2** was prepared by reducing larger batches (up to 50 mg) of **1** with sodium borohydride. After removal of borate ions, **2** was purified on a small column of Cellex-E resin in the same manner as **1** had been, except that some marker radioactive disaccharide was added to help locate **2** as it emerged from the column. Appropriate fractions were pooled and lyophilized. Paper chromatography of an aliquot showed but one spot, in contrast to **1** .Colorimetric analysis gave a ratio of sulfate group to uronic acid residues of 2.2:1. All of the uronic acid was found to be iduronic acid by g.l.c.[2,18]. For subsequent work, substrate of the desired specific activity was obtained by mixing labeled and unlabeled **2**.

Assignment of the two sulfate groups to C-2 and C-6 of the L-iduronic and 2,5-anhydro-D-mannitol residues, respectively, is based on the structural determinations of Lindahl and Axelsson[9] and of Wolfrom *et al.*[19].

RESULTS AND DISCUSSION

Unless otherwise indicated, reactions are performed in sealed capillary tubes holding 25 μl of reaction mixture, composed of 0.1M sodium acetate–acetic acid buffer, pH 4.0, 5mM sodium azide, 0.3mM substrate (0.05 μCi), iduronosulfatase, and 12.5 μg additional bovine serum albumin. After 4 h at 37°, the mixtures are spotted on Whatman 1 paper and subjected to chromatography in Solvent B or to high voltage electrophoresis (Fig. 1). Paper strips in the substrate and product regions are counted

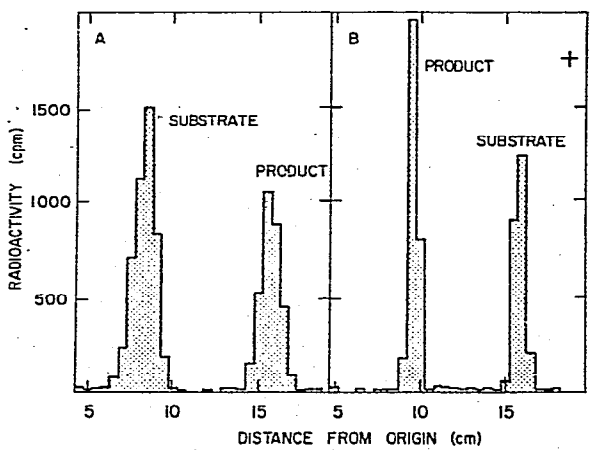


Fig. 1. Separation of **3** from **2** by paper chromatography for 24 h in Solvent B (left panel) and by electrophoresis for 1 h (right panel).

(preliminary experiments showed no radioactivity in any other position), and the conversion of substrate to product is calculated in percent. A correction is applied for any counts appearing in the product region (~2% of the substrate) in a control incubation containing all reagents except the enzyme. The molar yield of product can be calculated from the amount of substrate used and the percent of the conversion.

In this assay system, the reaction is a linear function of enzyme concentration up to 50% conversion (Fig. 2).

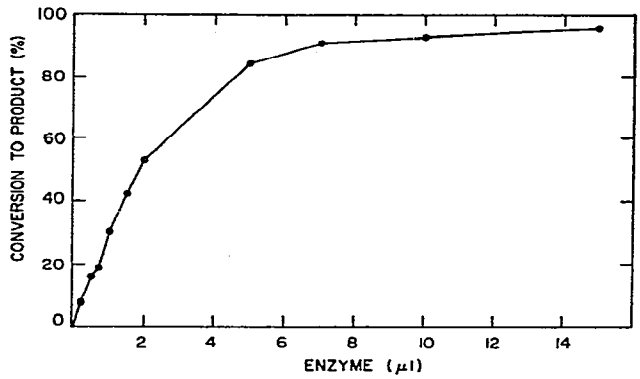


Fig. 2. Conversion of substrate **2** into product **3** as a function of enzyme concentration.

A 4-h incubation was selected for convenience in routine assays. The deviation from linearity after 5 h (Fig. 3) may be due to inactivation of the enzyme, or substrate depletion, or both.

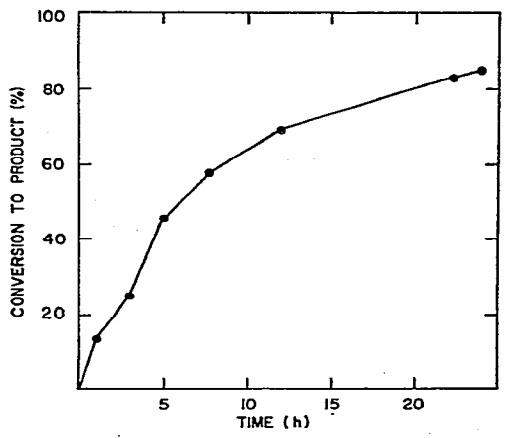


Fig. 3. Conversion of substrate **2** into product **3** as a function of time; 2 μl of enzyme (see Fig. 2) was used.

The reaction shows a typical Michaelis–Menten saturation curve, with K_M around 3mM (Fig. 4). At low substrate concentration, the formation of product is directly proportional to the amount of substrate added. With a radioactive assay in which both product and residual substrate must be measured, it is advantageous to perform the reaction at very low substrate concentration, since the *percent* conversion is then independent of the amount of substrate used.

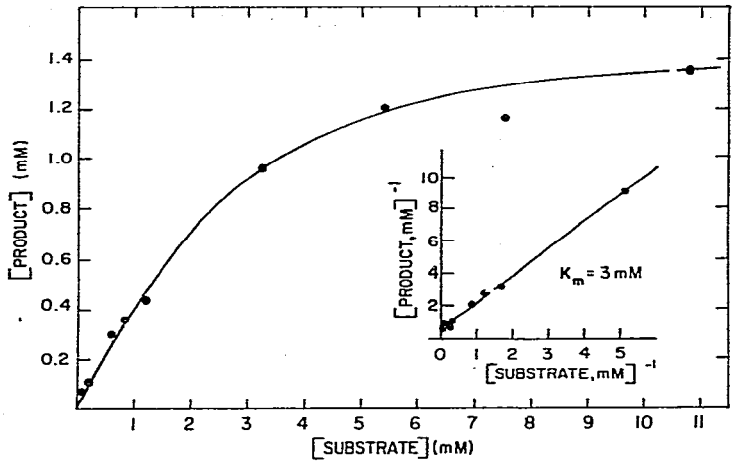


Fig. 4. Conversion of substrate **2** into product **3** as a function of substrate concentration.

The reaction is markedly inhibited by inorganic phosphate ions. The kinetics of the inhibition have yet to be established, but preliminary experiments suggest that they are complicated. Assay mixtures containing mM phosphate showed as little as one-sixth of the activity of similar mixture to be free of phosphate ions. Surprisingly, sulfate ions do not inhibit, even at a concentration 0.02M.

The product of the enzymic reaction was shown to have structure **3** in the following manner. Compound **2** having a specific activity of 4.0×10^5 c.p.m./μmole uronic acid was converted to **3**, the specific activity of which was determined to be 5.0×10^5 c.p.m./μmole uronic acid. This indicates that the disaccharide backbone remains intact. Colorimetric analysis of **3** gave a ratio for sulfate groups (released by hydrolysis in 2M trifluoracetic acid at 100° for 5 h) to uronic acid residues of 1.2. This experiment was performed with 1 μmole of **2**, other reagents in the usual concentration, and sufficient enzyme to effect a 63% conversion to **3** in 29 h at 37°, in a total volume of 130 μl. Before analysis, **3** was purified by both chromatography in Solvent B and by electrophoresis on paper that had been extensively prewashed to remove benzidine-precipitable material.

The two products of the reaction, inorganic sulfate and **3** were formed in equivalent amounts, *i.e.* 36% and 35%, respectively. For this determination, the standard assay mixture, modified to include 7mM substrate, was incubated for 28 h at 37°. An aliquot was assayed for inorganic sulfate ions by the benzidine test and the remainder subjected to paper electrophoresis and anlyzed for radioactive **2** and **3** in the usual way.

Removal of the sulfate group from the L-idosyluronic acid residue of **1** was shown previously by release of L-iduronic acid in the presence of α-L-iduronidase[2]. It is assumed that reduction of **1** to **2** does not change the nature of this reaction. Thus, all evidence is consistent with the reaction proceeding as described.

The assay method described here is applicable to the monitoring of the purification of enzyme from urine, providing the effect of other hydrolases that might be present in early stages of purification is taken into account. This means that paper chromatograms must be examined for 2,5-anhydro-D-[^{3}H]mannitol sulfate and 2,5-anhydro-D-[^{3}H]mannitol as well as for **3**. We are investigating the potential of the present assay for cell extracts and for diagnostic purposes.

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