The Defect in the Hunter Syndrome: Deficiency of Sulfoiduronate Sulfatase

(skin fibroblasts/mucopolysaccharide degradation/mucopolysaccharidosis)

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Communicated by C. B. Anfinsen, May 1, 1978

ABSTRACT Skin fibroblasts cultured from patients affected with the Hunter syndrome are deficient in the activity of a protein, named the “Hunter corrective factor,” that is required for degradation of dermatan and heparan sulfates. We now show that this factor, purified from human urine, removes about 2% of the sulfate residues from [3H]mucopolysaccharide accumulated within Hunter fibroblasts; these groups are derived from “oversulfated” regions of the polymer. Acetone-powder extracts of fibroblasts derived from patients with the Hunter syndrome are deficient in this sulfatase, in contrast to similar extracts from fibroblasts of individuals of other genotype. Hunter corrective factor coupled to α-L-iduronidase (or alternatively, mixed extracts from Hurler and Hunter fibroblasts) release iduronic acid from 4-O-α-L-sulfoiduronosyl-D-sulfoanhydromannose. We conclude that the Hunter corrective factor is a sulfatase for sulfated iduronic acid residues.

The Hunter syndrome is a genetic disorder associated with failure to degrade dermatan sulfate and heparan sulfate; lysosomal storage of these polymers leads to numerous clinical problems, including skeletal abnormalities, limitation of joint motion, hepatosplenomegaly, deafness, and cardiovascular disease (1, 2). Of the known mucopolysaccharidoses, the Hunter syndrome is the only one transmitted as an X-linked recessive trait.

Fibroblasts cultured from the skin of Hunter patients do not adequately degrade sulfated mucopolysaccharide because of a deficiency of a specific protein that is present in cell secretions, cells, and urine of individuals who do not have the Hunter syndrome (3–5). Because this protein, when added exogenously to Hunter cells, accelerates the degradation of sulfated mucopolysaccharide, it has been named the “Hunter corrective factor,” and abbreviated simply as “Hunter factor.” Purified Hunter factor has no effect on the mucopolysaccharide metabolism of cells derived from normal individuals or from patients with mucopolysaccharide storage disorders other than the Hunter syndrome (5).

Several analogous corrective factors have recently been identified as the “missing enzyme” in the corresponding disorder. Thus, the Hurler corrective factor has been identified as the enzyme α-L-iduronidase, and the Hurler and Scheie syndromes as α-L-iduronidase deficiency diseases (6, 7); the Sanfilippo A corrective factor has been identified as heparan sulfate sulfatase (probably an N-sulfatase) (8), and the Sanfilippo B factor as N-acetyl-α-galactosaminidase (9, 10). In a mucopolysaccharidosis due to β-galactosidase (EC 3.2.1.31) deficiency, β-galactosidase serves as corrective factor (11).

We have now identified the Hunter corrective factor as a sulfatase for sulfated iduronic acid residues, which occur in several mucopolysaccharides (12–15). A preliminary report of this work has been presented (16).

MATERIALS AND METHODS

Reagents. H2[35SO4], D-[6-3H]glucosamine, and D-[1-3H]-galactose were purchased from New England Nuclear Corp.; Sephadex G-200 and DEAE-Sephadex A-50, from Pharmacia; Biogel P-2 from Bio-Rad; chondroitinase ABC, and the reference unsaturated disaccharides Δ Di-4S, ΔDi-6S, and Δ Di-OS from Miles Laboratories; chondroitin 4-sulfate from Seikagaku Kogyo Co. The unsaturated disulfated disaccharides, Δ Didi6P and Δ Didi6S, were gifts from Dr. Sakaru Suzuki, and 4-O-α-L-sulfoiduronosyl-D-sulfoanhydromannose, from Dr. UlF Lindahl.

Cell Culture. Fibroblasts derived from skin of normal or affected individuals were maintained as described (17); acetone powders of the cells were made by the procedure of Hall et al. (11).

Corrective Factors. Pools 1–5 of the Hunter corrective factor preparation of Cantz et al. (5) were used in the present experiments. Their corrective activity, assayed as described (5, 17), had diminished by no more than one half after storage at −15° for 18 months. The α-L-iduronidase (Hurler corrective factor) was a fraction eluted from hydroxyapatite (18).

Preparation of Radioactive Mucopolysaccharide. Very heavy cultures of Hunter fibroblasts (5) were harvested immediately after transplantation, to a density of 5 mg of protein per 75-cm² Falcon flask) were labeled for 6 days with [35SO4] (20 μCi per flasks of medium containing 3 mCi, 9 μmol of inorganic sulfate). The cells were then harvested by trypsinization, washed twice with 0.9% NaCl, suspended in 1.0 ml of 0.9% NaCl, and disrupted by 10 cycles of freezing and thawing. The insoluble debris was removed by centrifugation at 10,000 x g for 20 min. The supernatant fluid, which contained 90% of the cell-associated radioactivity, was dialyzed against 2 liters of 0.05 M (NH4)SO4 followed by four changes, 2 liters each, of water. This dialyzed solution was used as a substrate for the Hunter factor without further purification, unless otherwise indicated.

Incorporation of [3H]glucosamine or [3H]galactose, which label the hexosamine or uronic-acid residues of mucopolysaccharide, respectively, was performed in fibroblasts grown to confluence but not later than 2 weeks after transplantation. The usual medium was modified to contain only 2 mg of glucose and 1 mCi of either radioactive precursor per 10 ml. After 3 days, cells were harvested and mucopolysaccharide was extracted as above.
Labeled mucopolysaccharides were separated on Sephadex G-200. A major peak, appearing immediately after the void volume, contained 90% dermatan sulfate, as previously documented (5), whereas a minor, greatly retarded peak contained 75% heparan sulfate and 25% dermatan sulfate. Further purification was achieved by adsorption to DEAE-Sephadex A-50 in 5 mM phosphate buffer (pH 7.0) and elution by discontinuous increments of NaCl in the same buffer. The heparan sulfate was eluted with 0.6 M NaCl, and the dermatan sulfate with 0.8–1.0 M NaCl.

Radioactivity. Except for corrective factor assays, for which a scintillation fluid has been stipulated (17), samples were counted in a mixture of Liquifluor (New England Nuclear Corp.), Bio-Solv BBS-3 (Beckman Instrument Co.), and toluene, 1:2.7:24 (v/v).

Digestion with Chondroitinase ABC. [35S]Mucopolysaccharide was treated with chondroitinase ABC by the procedure of Saito et al. (19); presumably, only the dermatan sulfate component was digested. Mucopolysaccharide (about 100,000 cpm) was mixed with 10 μl of “enriched Tris buffer” (19) and 0.05 units of chondroitinase ABC in a total volume of 0.05 ml. After 45 min at 37°, another 0.05 unit of enzyme in 5 μl was added, and the incubation was continued for 45 min longer; the reaction was stopped by immersing the tubes in a boiling-water bath for 2 min. An aliquot was applied to Whatman 1 and subjected to descending chromatography in n-butanol-acetic acid–0.1 N NH₄OH (2:3:1, v/v) for 22 hr. The paper was cut into 3-mm strips, each of which was counted in 0.5 ml H₂O and 9.5 ml of scintillation fluid.

Paper Electrophoresis. To identify inorganic sulfate, electrophoresis was performed in 0.2 M ammonium acetate buffer (pH 5.0) at 25 V/cm. Authentic [35SO₄] was applied in the same salt mixture as the unknown sample, to correct for the interference of chloride with the migration of sulfate ions.

Gas-Liquid Chromatography of Iduronic Acid. Iduronic acid was measured by gas-liquid chromatography as 1,4-idonolactone butaneboronate by the procedure of Eisenberg (20) and manuscript in preparation. A tube containing a known amount of barium iduronate was prepared as standard, and to this tube as well as to each unknown sample a constant amount of mannitol was added as internal standard. The samples were reduced with sodium borohydride, freed of boric acid, lactonized, and derivatized. They were then analyzed with a Beckman GC65 gas chromatograph on a column of OV17 on GasChrom Q. From the ratio of peak areas of idonolactone butaneboronate to mannitol butaneboronate, the amount of iduronic acid in each unknown sample was calculated.

RESULTS

Sulfatase activity of Hunter corrective factor

Incubation of the Hunter corrective factor with [35S]mucopolysaccharide isolated from Hunter fibroblasts resulted in the release of a small amount of material of low molecular weight (Fig. 1). About 1.5% of the radioactivity was released from the fraction that contained primarily dermatan sulfate and 4.5% from the fraction that contained primarily heparan sulfate. In both cases, the radioactive product was shown to correspond to inorganic sulfate by chromatography on BioGel P-2.

Similar experiments were done with analogous dermatan sulfate and heparan sulfate fractions that had been labeled with H in the hexosamine or uronic acid moieties. In contrast to results obtained with [35S]mucopolysaccharide, there was no factor-dependent release of low molecular weight material from the tritium-labeled polymers.

A routine assay for the sulfatase activity of the Hunter corrective factor was developed, taking advantage of the solubility of sulfate and insolubility of residual mucopolysaccharide in 80% ethanol. Unfractionated [35S]mucopolysaccharide

§In the experiments with tritium-labeled substrates, a small amount of radioactive material was found in the fractions fully retarded on Sephadex G-200; this amount was identical in incubations with and without Hunter factor.
extracted from Hunter fibroblasts (about 100,000 cpm) is incubated with Hunter corrective factor, usually 5–20 units, in 0.12 M sodium acetate (pH 4.0) and 5 mM NaCl. The incubation mixture, 0.07–0.11 ml, contains, in addition, NaCl (<0.07 M) and sodium phosphate (pH 6.0) (<5 mM) that are introduced with the Hunter factor. After 20 hr at 37°C, 0.1 ml of chondroitin 4-sulfate (10 mg/ml in 1 M NaHCO₃) and 4 volumes of absolute ethanol are added. The mixture is thoroughly agitated on a Vortex mixer, chilled in ice for 30 min; and centrifuged at 10,000 × g for 20 min at 2°C. The supernatant solution is collected and again centrifuged. An aliquot of the supernatant solution and of the precipitate (redissolved in water) are counted. A control incubation without Hunter factor is carried through the same steps, and all values are corrected for this blank (usually about 300 cpm). The assay is based on preliminary experiments that showed an optimal pH at 4.0, with half maximal activity at pH 3.5 and 4.7. Citrate–phosphate buffer is inhibitory. The rate of release of ethanol-soluble material is linear for 10 hr but diminishes markedly thereafter.

**Table 1.** Sulfate released from [³⁵S]mucopolysaccharide by acetone-powder extracts of fibroblasts

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Number of cell lines</th>
<th>Ethanol-soluble released (cpm/mg of protein)</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hunter</td>
<td>6</td>
<td>1,400</td>
<td>200–3,000</td>
<td></td>
</tr>
<tr>
<td>Hurler</td>
<td>3</td>
<td>25,000</td>
<td>15,000–31,000</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>2</td>
<td>15,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maroteaux-Lamy</td>
<td>1</td>
<td>33,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sanfilippo A</td>
<td>1</td>
<td>20,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Glucuronidase deficiency</td>
<td>1</td>
<td>24,000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-cell</td>
<td>1</td>
<td>6,000</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sulfatase activity of acetone-powder extracts was assayed as described in Methods, with 10⁶ cpm of [³⁵S]mucopolysaccharide substrate, about 0.1 mg of protein for extracts of Hunter fibroblasts, and about 0.05 mg of protein for extracts of fibroblasts of other genotype.

**Table 2. Absence of inhibitor in extracts from Hunter fibroblasts**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Ethanol-soluble released (cpm per sample)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Extract, normal fibroblasts</td>
<td>2800</td>
</tr>
<tr>
<td>2. Extract, Hunter fibroblasts</td>
<td>140</td>
</tr>
<tr>
<td>3. Mixture, 1 + 2</td>
<td>2200</td>
</tr>
<tr>
<td>4. Hunter factor</td>
<td>2600</td>
</tr>
<tr>
<td>5. Mixture, 2 + 4</td>
<td>2700</td>
</tr>
</tbody>
</table>

Sulfatase activity was assayed as described in Methods, with 2 × 10⁶ cpm of [³⁵S]mucopolysaccharide, and amounts of Hunter factor or normal acetone-powder extract selected to be in the linear range. Extract from Hunter fibroblasts contained 0.1 mg of protein; higher amounts gave an apparent inhibition, perhaps because of dilution of the radioactive substrate with endogenous mucopolysaccharide.

The ethanol-soluble reaction product behaves as inorganic sulfate by chromatography on Bio gel P-2 and by paper electrophoresis at pH 5.0 (Rₚicrate = 3.0).

Up to a point, there is a linear relationship between the amount of Hunter factor added and the amount of [³⁵SO₄]− released (Fig. 2). Maximal release corresponds to about 2% of the added radioactivity (the amount varies somewhat between different preparations of [³⁵S]mucopolysaccharide). This means that only 2% of the sulfate residues of the mucopolysaccharide substrate are susceptible to the action of the Hunter corrective factor.

No sulfate was released at all when the [³⁵S]mucopolysaccharide derived from Hunter fibroblasts was replaced by an analogous preparation derived from Hurler fibroblasts.

**Table 3. Decrease of “oversulfated” areas of [³⁵S]mucopolysaccharide upon incubation with Hunter factor**

<table>
<thead>
<tr>
<th>Fraction*</th>
<th>Units of corrective activity</th>
<th>ΔDidiS (%)†</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (3 samples)</td>
<td>7.6</td>
<td></td>
</tr>
<tr>
<td>Pool 1</td>
<td>60</td>
<td>5.1</td>
</tr>
<tr>
<td>Pool 2</td>
<td>50</td>
<td>5.3</td>
</tr>
<tr>
<td>Pool 3</td>
<td>46</td>
<td>5.4</td>
</tr>
<tr>
<td>Pool 4</td>
<td>16</td>
<td>6.1</td>
</tr>
<tr>
<td>Pool 5</td>
<td>10</td>
<td>6.0</td>
</tr>
</tbody>
</table>

* The fractions “pools 1–5” are described in Figs. 2 and 3 of ref. 5.
† The % of ΔDidiS is calculated on the basis of moles of disulfated disaccharide obtained relative to total disaccharide released by chondroitinase ABC. Because the specific activity of disulfated disaccharide is twice that of monosulfated, the percentage is calculated as follows:

\[
\% = \frac{0.5 \text{ (cpm, ΔDidiS)} \times 100}{0.5 \text{ (cpm, ΔDidiS)} + (\text{cpm, ΔDi-4S}) + (\text{cpm, ΔDi-6S})}
\]

Less than half of the mucopolysaccharide was converted to unsaturated disaccharides by chondroitinase ABC. The proportion degraded was the same in samples that had been incubated with the Hunter factor and controls.
The complete incubation mixture, no. 4, contained 0.2 μmol of disaccharide; Hunter factor, 60 corrective units, in 0.25 ml of 0.9% NaCl-0.01 M Na phosphate (pH 6.0) enriched with 0.25 mg of albumin; a final concentration of 0.12 M sodium acetate buffer (pH 4.0) and 5 mM Na₂HPO₄ in a total volume of 0.5 ml. After 24 hr at 37°C, α-L-iduronidase was added [sufficient to hydrolyze 0.3 μmol of phenyliduronide per 17 hr under standardized conditions (6)] in phosphate-NaCl-albumin buffer as above. The buffer was adjusted to remain as 0.12 M sodium acetate (pH 4.0) and 5 mM Na₂HPO₄. After another 24 hr at 37°C, iduronate released was measured by gas-liquid chromatography as described in Methods. Samples 1–3, from which one or both enzymes were omitted, were incubated with the equivalent amount of albumin in buffered saline. Sample 5 differed in that both Hunter factor and α-L-iduronidase were incubated together for 48 hr.

**Sulfatase activity of acetone powders of fibroblasts**

Acetone-powder extracts prepared from Hunter fibroblasts are profoundly deficient in the sulfatase associated with Hunter corrective factor, when compared to similar extracts of fibroblasts from normal individuals or from patients with other mucopolysaccharidoses (Table 1). The defect is not due to the presence of inhibitors in extracts of Hunter fibroblasts, as shown by mixing experiments (Table 2). The Hunter genotype, shown to be associated with deficiency of Hunter factor, is now correlated with a deficiency of sulfatase activity.

An interesting exception to that correlation is the marked deficiency of sulfatase in extracts of fibroblasts from I-cell disease patients (Table 1). These cells have markedly reduced activity of several lysosomal enzymes (e.g., ref. 21) and are unable to correct the defect of Hunter fibroblasts (4).

Homogenates prepared by freeze-thawing of fibroblasts, were found unsatisfactory for studying the sulfatase because of the presence of apparent inhibitors, particularly in homogenates of normal cells. The interfering substances are presumably removed in the preparation of acetone powders.

**Identification of Hunter corrective factor as a sulfodionuronolate sulfatase**

Action of the Hunter factor on [³⁸S]mucopolysaccharide resulted in the disappearance of some “oversulfated” areas—i.e., areas with two sulfate groups per disaccharide consisting of uronic acid and hexosamine. This was determined by digesting with chondroitinase ABC the substrate and the product of Hunter factor activity. The bacterial enzyme degraded a portion of the [³⁸S]mucopolysaccharide used as substrate (presumably, the dermatan sulfate component only) into unsaturated disaccharides of which 7.5% were disulfated. After treatment of the [³⁸S]mucopolysaccharide with Hunter factor, the proportion of disulfated disaccharides produced by chondroitinase ABC was reduced to a limiting value of about 5% (Table 3). Apparently, one-third of the “oversulfated” areas of mucopolysaccharide isolated from Hunter fibroblasts was removed by the Hunter factor. By contrast, mucopolysaccharide isolated from Hurler fibroblasts and similarly treated with chondroitinase ABC, showed the presence of about 4% oversulfated areas, none of which was removed by the Hunter factor (4.1 or 4.5%, for control sample or sample treated with Hunter factor, respectively).

These results are consistent with the hypothesis that the Hunter corrective factor is a sulfatase for oversulfated areas of the mucopolysaccharide, provided these are suitably exposed. Since the sulfate residue that is unique to such areas is that linked to L-iduronate, the results further suggest that the Hunter factor is a sulfatase for sulfated iduronic acid. A direct demonstration thereof was obtained by using for substrate the disaccharide 4-0-α-L-sulfodionuronol-b-sulfanohydromannose, and measuring iduronic acid released by the combined action of Hunter factor and α-L-iduronidase. Whereas either enzyme alone released only traces of iduronic acid, as determined by gas-liquid chromatography, the two enzymes together released as much as 40% of the available iduronic acid in the best experiment presented in Table 4.

Release of iduronic acid from 4-0-α-L-sulfodionuronol-b-sulfanohydromannose was also catalyzed by acetone-powder extracts of normal fibroblasts. Extracts of Hurler fibroblasts (which have Hunter factor but not α-L-iduronidase) or of...
Hunter fibroblasts (which have α-L-iduronidase but not Hunter factor) had only slight activity, whereas a mixture of the two caused the release of as much iduronic acid as did the normal extract (Table 5).

**DISCUSSION**

The results demonstrate that the Hunter corrective factor has sulfodiuronate sulfatase activity. It is unlikely that the sulfatase is a chance contaminant of the admittedly impure factor preparation, since it is specifically absent from extracts of Hunter fibroblasts.

We were surprised to find that acetone-powder extracts of normal fibroblasts did not release more $^{35}$SO$_4$$^-$ from [35$^S$]-mucopolysaccharides than did the purified Hunter factor. These extracts contain several of the enzymes necessary for mucopolysaccharide degradation, such as α-L-iduronidase and β-glucuronidase (6, 11), as well as the sulfodiuronate sulfatase. Presumably, one or more of the unknown degradative enzymes (e.g., hydrolases for the O-sulfated N-acetylgalactosamine residues of dermatan sulfate) is inactivated during preparation or assay of the acetone-powder extract.

Hunter patients store and secrete both dermalan sulfate and heparan sulfate. Sulfodiuronate residues have been well characterized in mammalian dermalan sulfate and heparin (12–15). They have not been reported in heparan sulfate. That, however, is probably because heparan sulfate is poorly studied; it is generally assumed to be similar to heparin (22, 23), the difference being primarily in molecular size and shape (larger and more branched molecule in heparan sulfate) and ratio of N-acetylated to N-sulfated glucosamine (higher in heparan sulfate$^1$).

Not all sulfated iduronic-acid residues are available to the sulfatase. We speculate that the unavailable ones are buried within the molecule. This hypothesis is consistent with the finding of a disulfated disaccharide at the nonreducing end of dermalan sulfate isolated from Hunter fibroblasts but not from Hurler fibroblasts (Sjöberg, I., Fransson, L. Å., Matalon, R. & Dorfman, A., manuscript in preparation).

A disulfated disaccharide containing iduronic acid has been found in the urine of a Hunter patient (27). Such a fragment could result in vivo from the action of an endoglycosidase (e.g., hyaluronidase) on dermalan sulfate.

The clinical consequences of sulfodiuronate sulfatase deficiency are, on the whole, similar to those of α-L-iduronidase deficiency. The severe and mild forms of the Hunter syndrome have as their counterpart the Hurler and Scheie syndromes, respectively (1, 28). There is, however, one consistent phenotypic difference between the two enzyme deficiencies. α-L-iduronidase deficiency is always associated with cloudy corneas, whereas corneas remain clear in the Hunter syndrome. One must presume that corneal mucopolysaccharides do not require Hunter factor for degradation, and, therefore, that they are devoid of sulfodiuronate residues.

We thank Dr. Ulf Lindahl (University of Uppsala, Sweden) and Dr. Sakaru Suzuki (University of Nagoya, Japan) for generous gifts of disulfated disaccharides. This work was supported in part by a grant from the Deutsche Forschungsgemeinschaft.

1 Because of the large proportion of sulfated iduronic-acid residues in heparin, it is puzzling that this polymer should not be stored and excreted in the Hunter syndrome. Although “heparin-like” fractions have been reported among the mucopolysaccharides of Hurler and Sanfilippo patients these are not considered “true” heparin but rather degradation products of heparan sulfate (24, 25). Perhaps man, in contrast to other mammals, does not synthesize heparin. Experimental data on the subject are sparse (e.g., ref. 26).