Sequential Degradation of Keratan Sulfate by Bacterial Enzymes and Purification of a Sulfatase in the Enzymatic System*

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SUMMARY

Pseudomonas sp. IFO-13309 and Actinobacillus sp. IFO-13310, bacteria which exhibit a symbiotic growth in a medium containing keratan sulfate as a sole carbon source, were isolated from soil.

Extracts of these organisms were shown to contain an endoglycosidase, a sulfatase, an exo-β-D-galactosidase, and an exo-β-D-N-acetylglucosaminidase which, together, catalyze an extensive cleavage of corneal keratan sulfate. The Pseudomonas extract was particularly rich in the endoglycosidase activity and poor in the exoglycosidase activities. The Actinobacillus extract, in sharp contrast, contained principally the exoglycosidases.

The sulfatase activity did not show this marked difference in distribution. A sulfatase was purified from the crude extract of Actinobacillus. The purified sulfatase reacted little or not at all with keratan sulfate, but acted on 2-acetamido-2-deoxy-6-O-sulfo-D-glucose, 2-acetamido-2-deoxy-6-O-sulfo-β-D-galactosyl-(1→3)-D-galactose, and a tetrasaccharide trisulfate having 2-acetamido-2-deoxy-6-O-sulfo-D-glucose at the nonreducing end (prepared from keratan sulfate with an endogalactosidase). The enzyme removed one sulfite group from the tetrasaccharide trisulfate, producing an oligosaccharide which, unlike the parent oligosaccharide, was susceptible to hydrolysis with exo-β-D-N-acetylglucosaminidase. The data suggest that the nonreducing end is the only site at which enzymatic desulfation is carried out.

Previously studies in this laboratory centered around the purification of depolymerases and sulfatases which preferentially attack chondroitin sulfate, dermatan sulfate, and related substances (e.g. chondroitinase-ABC, chondroitinase-AC, chondroitinase-4-sulfatase, chondroitinase-6-sulfatase, and Δ-glucuronide hydrolase)

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β-D-galactoside and phenyl 2-acetamido-2-deoxy-β-D-glucoside from Dr. H. Kushida, Kyoto General Medical Chemical Laboratory, Kyoto; 2-acetamido-2-deoxy-4-O-sulfouronic acid-glucoside; 2-acetamido-2-deoxy-6-O-sulfon-β-D-galactoside (17), and 2-acetamido-2-deoxy-6-O-sulfon-β-D-glucoside from Dr. Y. Nakashima in this laboratory. Keratan sulfates of bovine cornea and shark cartilage were prepared according to Meyer et al. (18) and Furuhashi (19), respectively, with the exception that, in each case, further digestion with chondroitinase-ABC (1) was carried out after the ethanol fractionation. The resulting digests were deproteinized with 5% trichloroacetic acid, dialyzed against running tap water, and mixed with 2 volumes of ethanol containing 1% potassium acetate. The precipitates were washed successively with ethanol and ether and dried over P$_2$O$_5$ in a vacuum. The yields were 1.5 g from 100 g (dry weight) of cornea and 700 g from 20 kg (dry weight) of cartilage.1 The following analyses were obtained and calculated on a dry weight basis. Cornea sample: hexose (as galactose), 30.4%; hexosamine (as glucosamine), 32.3%; sulfate, 17.4%; 6-deoxyhexose (as fucose), 0.6%; sialic acid (as N-acetyleneuraminic acid), 1.2%; uronic acid (as glucuronic acid), 2.2%; and protein (as Lowry protein), 2.5%. Cartilage sample: hexose (as galactose), 26.5%; hexosamine (as glucosamine), 25.7%; sulfate, 23.0%; 6-deoxyhexose (as fucose), 0.9%; sialic acid (as N-acetyleneuraminic acid), 1.8%; uronic acid (as glucuronic acid), 2.5%; and protein (as Lowry protein), 4.0%.

Keratan sulfate oligosaccharide (substrate for the assay of sulfatase, see below) was prepared from 30 mg of corneal keratan sulfate with 30 units of a purified endo-galactosidase preparation as described in the accompanying paper (12). The digestion was carried out in 3 ml of 0.05 M Tris-HCl, pH 7.2, at 37° for 20 hours. The resulting mixture was heated for 5 min in a boiling water bath and then centrifuged. The supernatant solution so obtained was used for the sulfatase assay.

The isolation and properties of a disaccharide monosulfate and tetrasaccharide trisulfate from corneal keratan sulfate are described in the accompanying paper (12). Available evidence has indicated that this disaccharide monosulfate is 2-acetamido-2-deoxy-6-O-sulfouronic acid-glucosyl(1→3)-β-D-galactosyl (15) and that the tetrasaccharide trisulfate is 2-acetamido-2-deoxy-6-O-sulfouronic acid-glucosyl(1→3)-β-D-galactosyl(1→3)-6-O-sulfouronic acid-galactosyl(1→4)-2-acetamido-2-deoxy-6-O-sulfouronic acid-glucosyl(1→3)-β-D-galactosyl (15). 2-Acetamido-2-deoxy-6-O-sulfouronic acid-glucosyl(1→3)-β-D-galactosyl (15) was crystallized from water in colorless needles (m.p. 90°). 2-Acetamido-2-deoxy-6-O-sulfouronic acid-glucosyl(1→3)-β-D-galactosyl (15) was crystallized from water in colorless needles (m.p. 90°). The following commercial materials were used: CM-cellulose from the Brown Co.,Keene, N. H.; Sephadex G-200 from Pharmacia, Uppsala; potassium p-nitrophenyl sulfate from Sigma, St. Louis, Mo.

Methods

Analytical Procedures

The following compounds were determined by the indicated methods: phenol released enzymatically from phenyl glycosides by the method of Hockenbuhl et al. (20); p-nitrophenol released enzymatically from p-nitrophenyl sulfate by the method of Dodgson and Spencer (21); sulfate by the method of Dodgson (22) as modified by Kawai et al. (23); reducing activity of sugar by the method of Park and Johnson (24); uronic acid by the method of Bitter and Muir (25); protein by the method of Lowry et al. (26); hexose by the method of Trevellian and Harrison (27); hexosamine by the method of Zamenoff and Shettles (29); and sialic acid by the method of Werner and Odin (30). For the determination of ester sulfate and hexosamine in keratan sulfate, samples were hydrolyzed with 0.1 N HCl at 100° for 7 hours.

1 We wish to thank Dr. T. Fujiiwa, Seikagaku Kogyo Company, for assistance in preparing the keratan sulfates.

2 The abbreviations used are: 6-sulfo-Gal, β-D-galactose 6-sulfate; 6-sulfo-GlcNs, 2-acetamido-2-deoxy-6-O-sulfouronic acid-glucosamine; Δ4 GlcUA(1→3)4-sulfo-GalNAc and Δ4 GlcUA(1→3)4-sulfo-GalNAc, 2-acetamido-2-deoxy-3-O-(β-D-gluc-4-enepyranosyluronic acid)-4-O-sulfo-β-D-galactose and 2-acetamido-2-deoxy-3-O-(β-D-gluc-4-enepyranosyluronic acid)-6-O-sulfo-β-D-galactose, respectively (i.e. unsaturated disaccharides derived from chondroitin sulfates by lyase reaction).

Culture Conditions

The organisms were grown at 30° with vigorous aeration in the following medium: Medium A (for screening and stock culture): 1.0% shark cartilage keratan sulfate, 0.2% NH$_4$NO$_3$, 0.1% K$_2$HPO$_4$, 0.0001% FeCl$_3$·6H$_2$O (adjusted to pH 7.0); Medium B (for adaptive growth of Pseudomonas): 1.0% shark cartilage keratan sulfate, 1.5% peptone, 0.45% yeast extract, and 0.15% NaCl (adjusted to pH 7.0); and Medium C (for adaptive growth of Actinobacillus): 1.0% shark cartilage keratan sulfate, 0.1% glucose, 0.1% Casamino acid, 0.01% yeast extract, 0.3% NaCl, 0.1% K$_2$HPO$_4$, 0.05% KCl, 0.05% MgCl$_2$·6H$_2$O, and 0.001% FeCl$_3$·6H$_2$O (adjusted to pH 7.0).

Enzyme Assay

Assay I—This assay measured the liberation of reducing substances from corneal keratan sulfate. Routine incubation mixture (100 μl) contained 5 μmol of Tris-HCl, pH 7.2, 0.1 μmol (as galactose) of keratan sulfate, and enzyme. Controls contained heat-inactivated enzyme. The reaction mixture was incubated at 37° for 3 to 180 min, depending on the enzyme activity present, and then heated in a boiling water bath for 2 min. The resulting precipitate was removed by centrifugation and aliquots of the supernatant solution were assayed for reducing activity. This assay obviously does not indicate whether the reducing material is monosaccharide or oligosaccharide. If, however, the assay is made with an enzyme preparation which contains little exoglycosidase activity (e.g. the extract of Pseudomonas), the value may actually represent an “endoglycosidase” activity. One unit of enzyme was defined as the quantity that catalyzed the release of 1 μmol (as galactose) of product per hour.

Assay II—This assay measured the liberation of SO$_4$ from keratan sulfate oligosaccharides (for the preparation of the oligosaccharides see above). Routine incubation mixture (200 μl) contained 10 μmol of Tris-HCl, pH 7.2, 0.65 μmol (as galactose) of oligosaccharides, and enzyme. Controls contained heat-inactivated enzyme. The reaction mixture was incubated at 37° for 1 to 2 hours, depending on the enzyme activity present. At the end of the incubation, 200 μl of a solution containing 1% cetylpyridinium chloride in 0.3 N HCl were added to the mixture. The resulting precipitate was removed by centrifugation and aliquots of the supernatant solution were assayed for SO$_4$ as one unit of enzyme was defined as the quantity that catalyzed the release of 1 μmol of SO$_4$·H$_2$O per hour.

Assay III—This assay measured the liberation of phenol from phenyl 2-acetamido-2-deoxy-β-D-glucoside or phenyl β-D-galactoside. Routine incubation mixture (300 μl) contained 5 μmol of phenyl 2-acetamido-2-deoxy-β-D-glucoside or phenyl 2-acetamido-2-deoxy-β-D-galactoside and 0.001% FeCl$_3$·6H$_2$O (for galactoside) or 0.001% FeCl$_3$·6H$_2$O (for glucoside) in Tris-HCl, pH 7.2 (for acetylglucosaminidase), 0.1 μmol of phenyl glycoside, and enzyme. Controls contained heat-inactivated enzyme. The reaction mixture was incubated for 1 to 3 hours, depending on the enzyme activity present, and then heated in a boiling water bath for 2 min. The resulting precipitate was removed by centrifugation and aliquots of the supernatant solution were assayed for phenol. One unit of enzyme was defined as the quantity that catalyzed the release of 1 μmol of phenol per hour.

RESULTS

Isolation of Bacteria

In an attempt to find a source of enzymes which would decompose keratan sulfate, a number of soil bacteria were examined and it was found that Pseudomonas sp. IFO-13309 and Actinobacillus sp. IFO-13310 could grow interdependently in a liquid medium containing shark keratan sulfate as a sole source of carbon (Medium A, see “Methods”). Thus, when the organisms were grown together in a single bottle they reached a density of about 1.5 (expressed as A$_{660}$) within 14 hours. However, when they were grown separately, it was obvious that the growth rates of Pseudomonas and Actinobacillus were at best 10% and 50%, respectively, of that attained by the mixed culture. This phenomenon could be interpreted as suggesting that some enzymes required for keratan sulfate utilization are distributed between the two organisms in a complementary manner (see below).
### Table I

**Characteristics of bacteria**

<table>
<thead>
<tr>
<th>Test</th>
<th>Pseudomonas sp. IFO-13309</th>
<th>Actinobacillus sp. IFO-13310</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gram stain</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Shape</td>
<td>Rods, 0.2 to 0.3 by 1.2 to 1.5 micrometers, occurring singly</td>
<td>Rods, 0.6 by 2 to 3 by 1.5 micrometers, occurring singly, in pairs, in groups, and sometimes as filaments</td>
</tr>
<tr>
<td>Motility</td>
<td>Motile by means of a polar flagellum</td>
<td>Non-motile</td>
</tr>
<tr>
<td>O2-requirement</td>
<td>Aerobic, slightly oxidative</td>
<td>Aerobic, fermentative</td>
</tr>
<tr>
<td>Acid formation from glucose</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Catalase</td>
<td>Positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Oxidase</td>
<td>Weakly positive</td>
<td>Positive</td>
</tr>
<tr>
<td>Gelatin</td>
<td>Yellow, circular, viscid colonies; slow liquefaction</td>
<td>Pale brown, circular colonies; slow liquefaction</td>
</tr>
<tr>
<td>Agar</td>
<td>Yellow colonies</td>
<td>Grayish white colonies (later become yellowish brown)</td>
</tr>
<tr>
<td>Broth</td>
<td>Turbid with pellicle and yellow sediment</td>
<td>Turbid with pellicle and gray (viscid) sediment</td>
</tr>
<tr>
<td>Litmus milk</td>
<td>Coagulation occurs; casein is not hydrolyzed; litmus is reduced</td>
<td>Coagulation occurs; casein is not hydrolyzed; litmus is reduced</td>
</tr>
<tr>
<td>Potato</td>
<td>Scent, yellow growth</td>
<td>Spreading slimy growth; luxuriant yellow colonies; potato is darkened and decomposed</td>
</tr>
<tr>
<td>Glucose mineral salts</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>7% NaCl broth</td>
<td>No growth</td>
<td>No growth</td>
</tr>
<tr>
<td>Indole production</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Nitrate reduction</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>Starch hydrolysis</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Methyl red reduction</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Optimum temperature</td>
<td>30-37°C</td>
<td>20-37°C</td>
</tr>
</tbody>
</table>

The classification of the organisms was based on their morphological, cultural, and physiological characteristics which are summarized in Table I.

Reactions Involved in Keratan Sulfate Degradation

*Pseudomonas* and *Actinobacillus* were grown in Mediums B and C, respectively, at 30°C with aeration. The cells were harvested by centrifugation at 3°C at the end of the logarithmic phase (usually between 9 and 13 hours), and were washed twice with cold water. Approximately 13 g (wet weight) of *Pseudomonas* cells or 10 g (wet weight) of *Actinobacillus* cells were obtained from 1-liter cultivation. The cells were suspended in 3 volumes of 0.05 M Tris-HCl, pH 7.2, and treated at 0°C in a 10-kHz sonic oscillator for 9 min. The supernatant solutions, obtained after centrifugation at 17,000 X g for 30 min, were tested for their capacity to decompose keratan sulfate. Fig. 1 shows the time course of the degradation of keratan sulfate by the extracts. Samples were withdrawn at suitable time intervals and assayed for release of reducing substance. It can be seen that the release of reducing substance proceeded more rapidly with the *Pseudomonas* extract than with the equivalent amount of the *Actinobacillus* extract.

Aliquots of the 5-hour digests were chromatographed on paper with butyric acid-0.5 N ammonia (5:3). In all cases control experiments were made in which enzyme preparation and substrate were incubated separately and chromatographed under the same condition. The results are shown in Fig. 2. The profiles of the two systems differ markedly from each other. Thus, it will be noted that the chromatogram of the *Actinobacillus* digest shows the presence of two major components corresponding to galactose...
Pseudomonas extract (Curve A) than with the Actinobacillus extract. The release of SO$_4^{2-}$ proceeded more rapidly with the former (Fig. 1), there was a distinct lag in the appearance of SO$_4^{2-}$ in each extract when crude extracts were examined (Fig. 3). Enzyme preparations were used at 100 μg (as protein)/200 μl, and the increase in SO$_4^{2-}$ with time was measured. It can be seen from Fig. 3 that, whereas liberation of reducing sugars was examined with the silver nitrate reagent (31). A, 5-hour digest with the Pseudomonas extract; B, 5-hour digest with the Actinobacillus extract. The latter extract, when incubated without keratan sulfate, gave a faint spot between the 80% and 2-0 acetamido-2-deoxyglucose, respectively, whereas the Pseudomonas digest shows five or more spots with relatively slow mobilities. Evidence will be presented in the accompanying paper (12) to indicate that the slower moving components of the Pseudomonas digest represent sulfated oligosaccharides with varying molecular size. Therefore, the results of paper chromatography may reflect a difference in relative composition of endoglycosidase and exoglycosidase in the two organisms; i.e. it is supposed that the ratio of endoglycosidase to exoglycosidase activity is much higher in the Pseudomonas extract than in the Actinobacillus extract. This difference in enzyme composition would account for the symbiotic growth of the two organisms in Medium A (see above).

The appearance of galactose and 2-0 acetamido-2-deoxyglucose in the chromatogram (Fig. 2) suggests the participation of a sulfatase system in the degradation of keratan sulfate. In order to obtain more information on the type and nature of such a sulfatase system, the time-activity curves of the crude extracts were examined (Fig. 3). Enzyme preparations were used at 100 μg (as protein)/200 μl, and the increase in SO$_4^{2-}$ with time was measured. It can be seen from Fig. 3 that, whereas liberation of reducing substance as described under "Methods Assay 1."

The adaptive growth of the two organisms in Medium A (see above).

Further support for this view comes from experiments in which the glycosidase and sulfatase activities were assayed separately by using polymerized keratan sulfate, phenyl 2-0 acetamido-2-deoxy-β-glucoside, phenyl β-glucoside, and keratan sulfate oligosaccharides as substrates. It can be seen in Table II that the Actinobacillus extract is rich in β-D-N-acetylgalactosaminidase and relatively poor in an endoglycosidase activity. The Pseudomonas extract, in sharp contrast, has a low β-D-N-acetylgalactosaminidase activity and a distinctly high endoglycosidase activity. β-D-Galactosidase activity is very low in both extracts, but it shows the same trend of distribution as that shown by β-D-N-acetylgalactosaminidase. In regard to sulfatase activity, it appears that the Actinobacillus extract is about as active on a protein basis as the Pseudomonas extract, provided the activities were measured using the oligosaccharide mixture as a substrate.

Enzyme Induction

When crude extracts from the cells grown in the presence of 1% keratan sulfate and from control cells grown in the absence of keratan sulfate were tested for endoglycosidase, sulfatase, and exoglycosidase (measured by Assays 1, 2, and 3, respectively), it was noticed that the endoglycosidase and sulfatase activities, but not the exoglycosidase activities, were greatly enhanced by the adaptive growth. Thus, the extract of unadapted Pseudomonas cells was shown to contain only one-thirtieth the endoglycosidase activity of the adapted cell extract on a protein basis. Moreover, neither this extract of unadapted Pseudomonas cells nor the extract of unadapted Actinobacillus cells could catalyze any significant release of SO$_4^{2-}$ from the oligosaccharide mixture under the standard assay conditions.
Purification of Sulfatase from Actinobacillus

The results described above suggested the existence of an inducible sulfatase or sulfatases which preferentially attacks the oligosaccharides from keratan sulfate. In order to characterize this sulfatase activity, purification experiments were carried out with Actinobacillus cells grown in Medium C.4

All operations were conducted between 0° and 4°. All centrifugations were at 17,000 × g for 30 min.

**Step 1: Preparation of Extracts**—Approximately 120 g (wet weight) of cells were suspended in 4 volumes of 0.05 M Tris-HCl, pH 7.2, and treated at 0° in a 10-kHz sonic oscillator for 9 min. The suspension resulting from this treatment was centrifuged. The supernatant fluid thus obtained could be stored in an ice bath for at least 1 month without significant loss of activity.

**Step 2: Streptomycin and Ammonium Sulfate Precipitation**—To 447 ml of the crude extract were added, with stirring, 112 ml of a 5% streptomycin sulfate solution in 0.05 M Tris-HCl, pH 7.2. The resultant precipitate was removed by centrifugation. Solid ammonium sulfate was added to the supernatant fraction to attain 50% saturation and the precipitate formed was removed by centrifugation. The concentration of ammonium sulfate was then raised to 90% saturation and the precipitate formed was collected by centrifugation. The precipitate was dissolved in 20 ml of 0.05 M Tris-HCl, pH 7.2, and dialyzed for 24 hours against four 2-liter changes of the same buffer.

**Step 3: CM-cellulose Chromatography**—A column (1.8 × 59 cm) of CM-cellulose was prepared and equilibrated with 0.05 M Tris-HCl, pH 7.2. The dialyzed ammonium sulfate fraction was applied to the column and washed with 260 ml of 0.05 M Tris-HCl, pH 7.2. The column was developed by linear gradient elution with 600 ml of 0.05 M Tris-HCl, pH 7.2. The enzyme was eluted with the same salt solution. The flow rate was 13 ml per hour and fractions of 5 ml were collected. Although a small portion of the sulfatase activity appeared in the fractions (for which there is no explanation at this moment), most of the sulfatase activity was found in the fractions from tubes 120 to 160 (Fig. 4). The sulfatase activity was well separated

![Fig. 4. Purification of sulfatase by CM-cellulose chromatography.](http://www.jbc.org/)

**Note:** Fractions were obtained as described in the text and checked for sulfatase activity (○—○) (by Assay 2), glycosidase activity (●—●) (by Assay 1), and ultraviolet (280 nm)-absorbing material (······). Solid bars above curve indicate fractions containing the indicated exoglycosidase activities measured by Assay 3. Dashed line refers to the gradient of NaCl.

**Fig. 5. Step 4 of the purification procedure.** Sephadex G-200 chromatography of the sulfatase peak from CM-cellulose (see Fig. 4) is shown. Fractions were obtained as described in the text and checked for sulfatase activity (○—○) (by Assay 2), glycosidase activity (●—●), and β-d-N-acetylgalactosaminidase activity (Δ—Δ), from β-d-galactosidase, but it was accompanied by some β-d-N-acetylgalactosaminidase and keratan sulfate-depolymerizing activity. Fractions 124 to 160 were pooled and concentrated to 7 ml on a Diaflo PM-10 (membrane filter).

**Step 4: Sephadex G-200 Chromatography**—The CM-cellulose fraction was applied to a Sephadex G-200 column (1.7 × 114 cm) previously equilibrated with 0.1 M NaCl in 0.05 M Tris-HCl, pH 7.2. The enzyme was eluted with the same salt solution. The flow rate was 5 ml per hour and fractions of 4 ml were collected. A single peak was observed (Fig. 5). The fractions (tubes 46 to 52) which were devoid of the glycosidase activities were pooled, concentrated to 5 ml on the membrane filter, and dialyzed for 4 hours against two 2-liter changes of 0.05 M Tris-HCl, pH 7.2.

A summary of the purification procedure is shown in Table III. Although a large loss in activity has been observed during the Sephadex G-200 step, this procedure appears to be essential to remove the glycosidase present in the CM-cellulose fraction. The absence of reducing material after extensive treatment (8 hours) of keratan sulfate (0.1 μmol as sulfate) by the Sephadex G-200 fraction (2.0 μg as protein) indicated that this enzyme preparation is free from endo- and exoglycosidase activities. It also showed no activity on phenyl 2-acetamido-2-deoxy-β-d-glucoside and phenyl β-d-galactoside.

**Table II**

**Enzyme activities involved in degradation of keratan sulfate**

The standard assay systems described under "Methods" were used.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activities</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoglycosidase</td>
<td>1.80±a</td>
<td>(0.11)a</td>
</tr>
<tr>
<td>Sulfatase</td>
<td>0.22</td>
<td>0.22</td>
</tr>
<tr>
<td>β-d-N-Acetylgalactosidase</td>
<td>0.30</td>
<td>3.01</td>
</tr>
<tr>
<td>β-d-Galactosidase</td>
<td>≤0.001</td>
<td>0.22</td>
</tr>
</tbody>
</table>

* These values are based on the reducing activities of products released from keratan sulfate (Assay 1, see "Methods"). Essentially all of the reducing substances produced by the Pseudomonas extract are in oligosaccharide forms (see Fig. 2), indicating that the reducing value may actually represent the amount of oligosaccharides released by an endoglycosidase action. However, it should be noted that most of the products of the Actinobacillus system are in monosaccharide forms (see Fig. 2) which may be formed by a combined action of endo- and exoglycosidases.

* Preliminary tests had shown that the cells tend to be higher in specific activity than the Actinobacillus or Pseudomonas cells grown in Medium R with respect to the sulfatase.

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**Fig. 5.** Step 4 of the purification procedure. Sephadex G-200 chromatography of the sulfatase peak from CM-cellulose (see Fig. 4) is shown. Fractions were obtained as described in the text and checked for sulfatase activity (○—○) (by Assay 2), glycosidase activity (●—●) (by Assay 1), and ultraviolet (280 nm)-absorbing material (······). Solid bars above curve indicate fractions containing the indicated exoglycosidase activities measured by Assay 3. Dashed line refers to the gradient of NaCl.
Table III

Purification of sulfatase

The standard assay systems described under "Methods" were used.

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein</th>
<th>Total activity</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml</td>
<td>mg</td>
<td>nmoles</td>
<td>units/mg protein</td>
</tr>
<tr>
<td>1. Crude extract</td>
<td>450</td>
<td>3100</td>
<td>1797</td>
<td>0.58</td>
</tr>
<tr>
<td>2. Ammonium sulfate</td>
<td>45</td>
<td>587</td>
<td>1383</td>
<td>2.36</td>
</tr>
<tr>
<td>3. CM-cellulose</td>
<td>7</td>
<td>11.4</td>
<td>565</td>
<td>49.5</td>
</tr>
<tr>
<td>4. Sephadex G-200</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tubes 38-45</td>
<td>10</td>
<td>2.76</td>
<td>347</td>
<td>126</td>
</tr>
<tr>
<td>Tubes 46-52</td>
<td>8</td>
<td>4.11</td>
<td>109</td>
<td>26.5</td>
</tr>
</tbody>
</table>

Specificity of Purified Sulfatase

The purified enzyme exhibited a pH optimum of about 7.2 (in 0.05 M Tris-HCl).

The enzyme was tested for its ability to remove the sulfates of various synthetic and naturally occurring sulfate esters; i.e. p-nitrophenyl sulfate, galactose 3-sulfate, galactose 6-sulfate, 2-acetamido-2-deoxy-6-O-sulfo-D-glucose, 2-acetamido-2-deoxy-4-O-sulfo-D-galactose, 2-acetamido-2-deoxy-6-O-sulfo-D-galactose, Δ1-GlcUA(1→3)-4-sulfo-GalNAc, Δ1-GlcUA(1→3)-6-sulfo-GalNAc, 6-sulfo-GalNAc(1→3)Gal, 6-sulfo-GalNAc(1→3)-6-sulfo-GalNAc(1→4)-6-sulfo-GalNAc(1→3)Gal, corneal keratan sulfate, chondroitin 4-sulfate, chondroitin 6-sulfate, dermatan sulfate, heparan sulfate, and heparin. In these tests, 0.28 unit of the enzyme was incubated with each sulfate ester (an amount equivalent to 0.4 μmol of sulfate) in 200 μl of 0.05 M Tris-HCl, pH 7.2, for 15 hours. The reactions were followed by the measurement of SO₄²⁻ (Assay 2), except that the reaction with p-nitrophenyl sulfate was followed by the measurement of free p-nitrophenol.

Among the sulfate esters, only 2-acetamido-2-deoxy-6-O-sulfo-D-glucose, 6-sulfo-GalNAc(1→3)Gal, and 6-sulfo-GalNAc(1→3)-6-sulfo-GalNAc(1→4)-6-sulfo-GalNAc(1→3)Gal gave positive results. Thus, the monosaccharide and disaccharide were quantitatively cleaved by the enzyme to give rise to SO₄²⁻. In the case of the tetrascarries, however, the liberation of SO₄²⁻ could not proceed beyond a maximum which is equivalent to one-third of the total sulfate present in the added substrate. Subsequent treatment (20 hours, 37°C) of the resulting mixture with Actinobacillus β-d-N-acetylglucosaminidase (the Sephadex G-200 fraction) tubes 38 to 45, 0.24 unit) yielded free 2-acetamido-2-deoxyglucose to the extent of 50% of the total hexosamine present in the tetrascarrie (see the accompanying paper, Ref. 12, for details). The confirmation of the sulfatase activity in the absence of the total hexosamine in the tetrascarries would be a significant demonstration of a second sulfatase fraction which may be present in other tissues. The sulfatase fractions were retained on CM-cellulose (cf. Fig. 4), this activity, unlike the activity on 2-acetamido-2-deoxy-6-O-sulfo-D-glucose, was recovered almost exclusively in Fractions 15 to 40 with an average specific activity of 0.1 μmol of SO₄²⁻ released per hour per mg of protein. Although it is not possible at this stage to define the function of this second sulfatase, it is logical to assume that the enzyme may attack the nonreducing galactose 6-sulfate terminal, thereby exposing the terminal group for further hydrolysis by the exo-β-D-galactosidase present in the Actinobacillus extract.

Nishida-Fukuda and Egami (15) have reported the occurrence in the marine gastropod liver of a multi-enzyme system capable of degrading keratan sulfate to yield galactose, 2-acetamido-2-deoxyglucose, and sulfate. They have purified an exo-β-D-galactosidase, an exo-β-D-N-acetylglucosaminidase, and two sulfatase fractions catalyzing a desulfation of polymerized keratan sulfate. No endoglycosidase activity has been encountered in this tissue. Thus, the liver system appears to differ from the bacterial system in that the desulfation occurs at the polysaccharide level prior to the action of the glycosidases. In any event, our bacterial system which has the capacity of degrading keratan sulfate to great extent should be an excellent source from which sulfatases and glycosidases of defined specificity may be obtained.

The term "glycosulfatase" has been used to describe a group of enzymes capable of hydrolyzing ester-sulfate linkages in a variety of sugars. Although earlier studies demonstrated a number of enzymes of this type in many organisms, the results of much of the earlier studies should be accepted with reserve in view of the scarcity of reliable information on the specificities. In more recent years, it has become increasingly evident that there are many different types of glycosulfatases, each being characterized by a remarkably high specificity toward the sugar part of the substrate molecule. Some examples of such enzymes related to hexosamine or mucopolysaccharide metabolism are chondroitin-4-sulfate (EC 3.1.6.9) (1), chondroitin-6-sulfate (EC 3.1.6.10) (1), disulfoglucosamine 6-sulfate (EC 3.1.6.11) (32), disulfoglucosamine N-sulfate (32), UDP-N-acetylglucosamine 4-
sulfatase (33), sulfiduronate sulfatase (34), a sulfatase active toward an oligosaccharide derived from chondroitin sulfate (35), and a sulfatase catalyzing the desulfation of polymerized chondroitin 4-sulfate (36). The present demonstration of the sulfatase active toward the nonreducing terminal 2-acetamido-2-deoxy-6-O-sulfoglucose will add a further interest to the problems concerning the role occupied in mucopolysaccharide metabolism of the sulfatases with such high, bewildering specificities.

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REFERENCES

Sequential degradation of keratan sulfate by bacterial enzymes and purification of a sulfatase in the enzymatic system.
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