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PREPARATION FROM KERATAN SULFATE OF SUBSTRATES FOR THE MEASUREMENT OF 2-ACETAMIDO-2-DEOXY-D-GLUCOSE 6-SULFATE SULFATASE AND (1→3)-*N*-ACETYL-β-D-GLUCOSAMINIDASE*

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ABSTRACT

An extract of bacterial cells *Pseudomonas sp.* IFO-13309 grown on medium containing 0.1% bovine cornea keratan sulfate of low sulfate content degraded exhaustively bovine cornea keratan sulfate to give 2-acetamido-2-deoxy-β-D-glucopyranosyl 6-sulfate-(1→3)-D-galactose, isolated by gel filtration on Sephadex G-25 and purified by preparative paper chromatography. This was reduced with sodium borotritide to give 2-acetamido-2-deoxy-β-D-glucopyranosyl 6-sulfate-(1→3)-D-[1-^{3}H]galactitol, purified by gel filtration on Sephadex G-15, which was an excellent substrate for the measurement of 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase. The reduced, radioactive monosulfated disaccharide was desulfated with methanolic 70mM hydrogen chloride and purified by gel filtration on Sephadex G-15 to give *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→3)-D-[1-^{3}H]galactitol, which allowed the measurement of (1→3)-*N*-acetyl-β-D-glucosaminidase. This enzyme may participate in the normal degradation of keratan sulfate.

INTRODUCTION

Inadequate degradation of keratan sulfate and excessive keratan sulfaturia occur in various mucopolysaccharidoses, secondary to different enzyme defects. Thus, in Morquio disease type A (mucopolysaccharidosis type IV), the defective enzyme is a 6-sulfatase specific for 2-acetamido-2-deoxy-D-galactose 6-sulfate and presumably for D-galactose 6-sulfate[1,2]. In Morquio disease type B, the presence of a mutant β-D-galactosidase has been demonstrated[3,4], whereas in mucopolysaccharidosis type

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VIII, the defective enzyme is a 6-sulfatase specific for 2-acetamido-2-deoxy-D-glucose 6-sulfate[1,5,6]. However, a large number of patients with radiological signs of multiple osteochondrodysplasia and keratan sulfaturia have normal activities for those enzymes. This suggests that their disease may be secondary to the deficiency of additional enzymes involved in the degradation of keratan sulfate.

The accepted structure for this glycosaminoglycan indicates the presence of a β-D-(1→3)-2-acetamido-deoxyglucopyranosyl linkage between 2-acetamido-2-deoxy-D-glucose 6-sulfate and D-galactose. In the degradation of keratan sulfate, this linkage would presumably be cleaved by a specific β-D-glucosaminidase, which at the present time has not been measured nor isolated, possibly for lack of proper substrate. In this paper, we describe the preparation from bovine cornea keratan sulfate of two radioactive disaccharides suitable for the measurement of the 6-sulfatase active on 2-acetamido-2-deoxy-D-glucose 6-sulfate and of the glucosaminidase active on the (1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl linkage.

EXPERIMENTAL

Materials. — Fresh bovine corneas were obtained from Pel-Freeze Biological Inc. (Rogers, AR 72756), Pronase from Sigma Chemical Co. (St. Louis, MO 63178), bovine testicular hyaluronidase from Calbiochem-Behring Corp., (San Diego, CA 92112), Celite from Johns-Manville (Denver, CO 80217), Eastman chromogram sheets 6061 (without fluorescent indicator) from Eastman Organic Chemicals Div., Eastman Kodak (Rochester, NY, 14650), and Aquasol from New England Nuclear (Boston, MA 02118). The unsaturated disaccharide 6-sulfate, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose 6-sulfate, and the unsaturated, nonsulfated disaccharide, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose, obtained from Miles Laboratories Inc. (Elkhart, IN 46515), were convenient standards as the presence of the double bond did not affect their chromatographic behavior in the systems used. 2-Acetamido-2-deoxy-D-glucose 6-sulfate, 2-acetamido-2-deoxy-D-[1-^{3}H]glucitol, and D-[1-^{3}H]galactitol 6-sulfate were synthesized in this laboratory. The disulfated, saturated trisaccharide, *O*-(2-acetamido-2-deoxy-β-D-galactopyranosyl 6-sulfate)-(1→4)-*O*-(β-D-glucopyranosyluronic acid)-(1→3)-2-acetamido-2-deoxy-D-galactose 6-sulfate, was prepared from chondroitin 6-sulfate in this laboratory. *Pseudomonas* sp. IFO-13309 was purchased from the Institute for Fermentation (Osaka 532, Japan).

Methods. — Hexuronic acid was measured by the method of Bitter and Muir[7]; total hexosamines by the method of Gatt and Berman[8], after hydrolysis with 4M hydrochloric acid for 7 h at 100°; 2-amino-2-deoxy-D-glucose and D-galactose with an amino acid analyzer; neutral sugars with the anthrone reagent[9]; reducing sugars with the method of Park and Johnson[10]; sulfate esters with the benzidine method of Ginsberg and Di Ferrante[11]; and protein with the Bradford reagent[12].

Preparation of keratan sulfate. — Bovine corneas (705 g, wet weight) were incubated at 37° in 50mM Tris · HCl buffer (1000 mL, pH 7.0) containing 10mM

calcium chloride and Pronase (2 g) for 24 h. Thereafter, the pH was adjusted to 7.0 and additional Pronase (2 g) was added. After 48 h, additional Pronase was added (5 g), and the temperature raised to 56° for 7 h.

As the corneas disappeared, Celite (100 g) was added with stirring and the suspension filtered through a Celite pad. The filtrate was kept in boiling water for 5 min, filtered again through Celite, and evaporated under vacuum (to ~300 mL). After exhaustive dialysis, first against distilled water and then against 100mM acetate buffer (pH 5.3) containing 150mM sodium chloride, bovine testicular hyaluronidase (50 mg, 344 000 N.F. units) was added to the nondialyzable material and dialysis was continued at 37°, against frequent changes of acetate buffer, for 24 h. The nondialyzable material was concentrated to 200 mL, and ethanol was added to obtain 50 and 75% final concentrations. The two precipitates obtained at 4° were washed repeatedly with absolute ethanol and ether, dried *in vacuo* in the presence of phosphorus pentaoxide, and analyzed (Table I). The final ethanol supernatant was discarded.

The fraction of glycosaminoglycans precipitating at 50% ethanol concentration (3.3 g, see Table I for analyses) was added to the culture medium for the adaptive growth[13] of *Pseudomonas sp.* IFO-13309.

The fraction precipitating at 75% ethanol concentration (15.0 g) (see Table I) was further purified by chromatography on a column (35 × 2.5 cm) of Dowex AG 1-X2 (Cl^-, 200–400 mesh) anion-exchange resin, packed and eluted with distilled water (500 mL), followed by a solution of sodium chloride (500 mL) of increasing molarity (1.0, 2.0, and 3.0M).

Each eluate was concentrated, dialyzed, and precipitated with ethanol (4 vol.) in the presence of 5% calcium acetate. Each precipitate was dried with absolute ethanol and ether, and analyzed (Table II).

Culture of bacteria and preparation of crude adaptive enzymes. — The organism was grown at 25° in a medium containing 1.5% Bacto-Tryptone (Difco Laboratories, Detroit, MI 48232), meat extract Difco (0.45%), sodium chloride (0.15%), and bovine cornea keratan sulfate (fraction precipitable at 50% ethanol concentration, see previous preparation) (0.1%) in distilled water. The pH was adjusted to 7.0 and the medium was sterilized by filtration through a 0.22-mμ Millipore filter (Millipore Corp., Bedford, MA 01730). After 2–3 days in culture, the bacteria were collected by centrifugation, washed twice with 50mM Tris · HCl buffer (pH 7.4, 50 mL) containing 0.15M sodium chloride, and eventually suspended in the same buffer (5 mL). The suspension, sonicated at 20 KHz for 9 min, while cooled in an ice bath, was then centrifuged at 34 800*g* for 20 min at 4°. Solid ammonium sulfate was added to the clear supernatant (25% saturation); after 30 min, additional ammonium sulfate was added (50% saturation) to the supernatant solution obtained by centrifugation. The second precipitate obtained containing the desired enzyme activity could be conveniently stored at 4°. When needed, aliquots of the precipitate were dissolved in a small volume of 50mM Tris · HCl buffer (pH 7.4) and dialyzed against three changes of the same buffer (1 L each) at 4°.

Degradation of bovine cornea keratan sulfate. — The fraction of keratan sulfate precipitable by ethanol (75% concentration) and further eluted from a column of anion-exchange resin by sodium chloride (1.0 and 2.0M) was used for the preparation of the desired disaccharides.

Aliquots of the substrate (100 mg) were incubated at 37° with the fraction of *Pseudomonas* sonicate that precipitated with ammonium sulfate (50% saturation). Every 24 h, the incubation mixture was precipitated with abs. ethanol (3 vol.). The supernatant solution was removed by centrifugation and used for the isolation of the disaccharides, and the precipitate was dried with absolute ethanol and ether, redissolved in 50mM Tris · HCl buffer, and incubated again with *Pseudomonas* enzymes. This procedure was repeated until analysis of the ethanolic supernatant solution failed to reveal the presence of disaccharides.

Identification, isolation, and purification of the disaccharide monosulfate, O-(*2-acetamido-2-deoxy-β-*D-*glucopyranosyl 6-sulfate*)-(*1→3*)-D-*galactose.* — The supernatant solution obtained by ethanol precipitation of the incubation mixture was evaporated to dryness, and the oligosaccharides present were analyzed by descending paper chromatography on Whatman 3 MM paper (2:3:1, v/v, 1-butanol–acetic acid–M ammonium hydroxide) for 18 h. The dry chromatograms were stained with alkaline silver nitrate[14] (Fig. 1A). Larger aliquots of the same preparation of oligosaccharides were applied to a column (85 × 2.7 cm) of Sephadex G-25 fine, equilibrated and eluted with M sodium chloride, and previously calibrated with a saturated trisaccharide disulfate, an unsaturated disaccharide 6-sulfate, and 2-acetamido-2-deoxy-D-[i-^{3}H]-glucitol 6-sulfate. The column effluent was analyzed for reducing sugars (Fig. 1B), and the fractions eluted in the same position as that of the unsaturated disaccharide-6-sulfate were pooled and chromatographed on a column (105 × 2 cm) of Sephadex G-15 fine, packed and eluted with 10% ethanol. The effluent of the latter column was analyzed for reducing sugars (Fig. 1C). Aliquots (20 μL) of selected fractions between number 40 and 70 were analyzed firstly by analytical, and then by preparative, descending paper chromatography with the system described above. The material having a mobility similar to that of a standard of unsaturated disaccharide 6-sulfate was eluted with water, concentrated, chromatographed on a column (105 × 2 cm) of Sephadex G-15 fine, packed and eluted with water, and dried to give 7 mg of product. Aliquots of this material were analyzed and subjected to high-voltage paper electrophoresis (Fig. 2) on Whatman 3 MM paper (30 volts/cm, 90 min, in 50mM ammonium acetate–acetic acid buffer, pH 5.0).

Reduction of the purified disaccharide monosulfate with sodium borotritide. — To an aliquot of the purified disaccharide monosulfate (12 μmol) in 100mM borate buffer, pH 8.0 (1.0 mL), two portions of 0.6M sodium borotritide solution (20 μL each, 220 Ci/mol) were added at 1-h intervals. Three hours after the last addition, the excess of sodium borotritide was destroyed by addition of acetic acid to pH 5.0. Methanol was added and the solution evaporated several times. The reduced material was then purified by chromatography on Sephadex G-15 fine, eluted with water. Aliquots of the effluent (10 μL) were added to Aquasol (3 mL) and counted. One

large radioactive peak emerged after the void volume. This was collected, concentrated, and analyzed. High-voltage paper electrophoresis of an aliquot of this material showed the presence of a single, radioactive component having the same mobility as that of the nonreduced material, stained with silver nitrate (see Fig. 2).

An aliquot of the reduced disaccharide sulfate was hydrolyzed with 0.1M hydrochloric acid[15] for 2 h at 100°, and the products of hydrolysis were streaked on Whatman 3 MM paper and subjected again to high-voltage electrophoresis. Appropriate standard compounds were spotted at the origin. Half of the paper was cut into 1-cm wide segments perpendicularly to the direction of migration: they were eluted with water (1 mL), and the eluates were counted for radioactivity after addition of Aquasol (7 mL). The other half of the paper was stained with silver nitrate (Fig. 3).

Preparation of O-(*2-acetamido-2-deoxy-β-*D-*glucopyranosyl*)-(*1→3*)-D-[*1-*^{3}*H*] *galactitol.* — The radioactive, reduced disaccharide monosulfate was lyophilized and dried in the presence of phosphorus pentaoxide, and an aliquot (3.5 μmol) was dissolved in methanolic 0.07M hydrogen chloride[16]. After 24 h at 26°, the products were purified by chromatography on a column (5 × 1 cm) of Dowex 1-X8 (AcO^-, 200–400 mesh) anion-exchange resin, followed by gel filtration on Sephadex G-15 fine (Fig. 4A).

The final product was analyzed by high-voltage electrophoresis (Fig. 4B).

Enzyme assays. — Preparation of extracts from human skin fibroblasts was performed as described previously[1].

For the measurement of 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase, aliquots of the fibroblasts extracts (50 μL, containing 30–40 μg protein) were added to M sodium acetate–acetic acid buffer (10 μL, pH 5.7) containing bovine serum albumin (0.2%) and sodium azide (0.2%). Upon addition of ^{3}H-labeled, reduced disaccharide monosulfate substrate (8 nmol in 40 μL of distilled water, 10 000 c.p.m./nmol), incubation was performed for 7 h at 37°. Blank tubes contained buffer, substrate solution, and 0.15M sodium chloride (50 μL) instead of fibroblast extract. The incubation was terminated by heating the tubes for 2 min in boiling water: the supernatant solutions obtained by centrifugation were diluted to 1.0 mL with water and applied to columns (2 × 0.5 cm) of Dowex 1-X8 (AcO^-, 200–400 mesh) anion-exchange resin. The columns were washed with additional water (1.0 mL), and the effluents (2 mL) were collected in scintillation glass-vials and counted after addition of Aquasol (15 mL). The results are expressed as nmol of product formed/h/mg of protein.

For the assay of (1→3)-*N*-acetyl-β-D-glucosaminidase, the substrate *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→3)-D-[1-^{3}H]galactitol was separated from D-[1-^{3}H]galactitol by ascending t.l.c. on Eastman silica gel chromogram sheets (20 × 20 cm) with 16:8:1 (v/v) 1-butanol–acetic acid–water as eluent. When the solvent reached 0.5 cm from the upper edge of the sheets (6–7 h run), these were dried and cut into segments 2-cm wide and 0.5-cm high. Each segment was placed in a scintillation glass-vial with Aquasol (4 mL) and counted (Fig. 5A and B). The actual enzyme measurement used that fraction of a crude, human liver homogenate which could be precipitated with ammonium sulfate at 65% saturation. After dialysis

of the precipitate against phosphate buffer (0.01M, pH 7.2) containing 2mM mercaptoethanol, an aliquot of the retentate (15 μL = 100 μg of protein) was incubated with 2 nmol of substrate (10 μL, 10 000 c.p.m./nmol) and McIlvane buffer (pH 3.7, 10 μL) for 20 h at 37°. Blank tubes contained buffer, substrate, and 0.15M sodium chloride instead of liver extract. After heating the tubes for 2 min in boiling water, 25 μL of the supernatant solutions obtained by centrifugation were spotted on the t.l.c. plates for measurement of the quantity of product formed (Fig. 5C).

RESULTS

Table I shows analyses of the keratan sulfate fractions obtained by ethanol precipitation of a digest of bovine corneas. The fraction obtained at 50% ethanol concentration (3.3 g) still had measurable amounts of hexuronic acid, very little residual protein, a high concentration of neutral hexoses and hexosamine (essentially only 2-amino-2-deoxy-D-glucose), and a molar ratio of hexose to hexosamine to sulfate of 20 : 18 : 15. Because of its relative degree of purity, high solubility, and rather low sulfate content, this fraction was used for the adaptive growth of *Pseudomonas*, at a concentration of 0.1%.

The fraction obtained at 75% ethanol concentration (15 g) had higher protein and lower hexoses and hexosamine content, and a ratio 2-amino-2-deoxy-D-glucose to 2-amino-2-deoxy-D-galactose of 17. For this reason, it was not analyzed to a further extent, but rather purified by chromatography on anion-exchange resin, which was eluted with water and with increasing concentrations of sodium chloride. The analyses of the various fractions thus obtained are presented in Table II.

The fraction eluted with water and having a low content of neutral sugars and hexosamine (8.7 and 5.2%, dry weight) was discarded. The fractions eluted with 1.0 and 2.0M sodium chloride, even though rather crude, were combined (600 mg),

TABLE I

PARTIAL ANALYSES OF CRUDE KERATAN SULFATE FRACTIONS[a]

Components	*Fractions precipitated by ethanol concentration*	
	50% (yield 3.3 g)	*75% (yield 15 g)*
Hexoses (as galactose)	26.3 (1.00)	6.6 (1.00)
Hexuronate (as glucuronolactone)	0.66 (0.025)	less than 0.25
2-Amino-2-deoxyhexose	23.5 (0.90)	2.1 (0.32)
2-Amino-2-deoxy-glucose to -galactose ratio	essentially glucosamine	17.7 : 1
Sulfate	10.5 (0.75)	not performed
Protein	<0.1	2.6

[a]The fractions were obtained by digesting bovine corneas with Pronase and hyaluronidase, followed by ethanol precipitation. Results are as percent of dry weight, with molar ratios in parentheses.

TABLE II

PARTIAL ANALYSES OF THE FRACTIONS OBTAINED FROM THE 75% ETHANOL PRECIPITATE[a]

Components	*Fractions eluted with*		
	Water	*1.0 and 2.0M sodium chloride (yield 600 mg)*	*3.0M sodium chloride (yield 100 mg)*
Hexoses (as galactose)	8.7 (1.00)	22.4 (1.00)	33.5 (1.00)
Hexuronate (as glucuronolactone)	[b]	[b]	[b]
2-Amino-2-deoxyhexose (as 2-amino-2-deoxyglucose)	5.2 (0.61)	18.7 (0.84)	30.3 (0.91)
Sulfate	[c]	7.7 (0.64)	16.6 (0.93)
Protein	[c]	4.4	<0.1

[a]The fractions were obtained by chromatography on a column of Dowex 1-X2 anion-exchange resin. The results are expressed as described in footnote to Table I. [b]Not detectable. [c]Not performed.

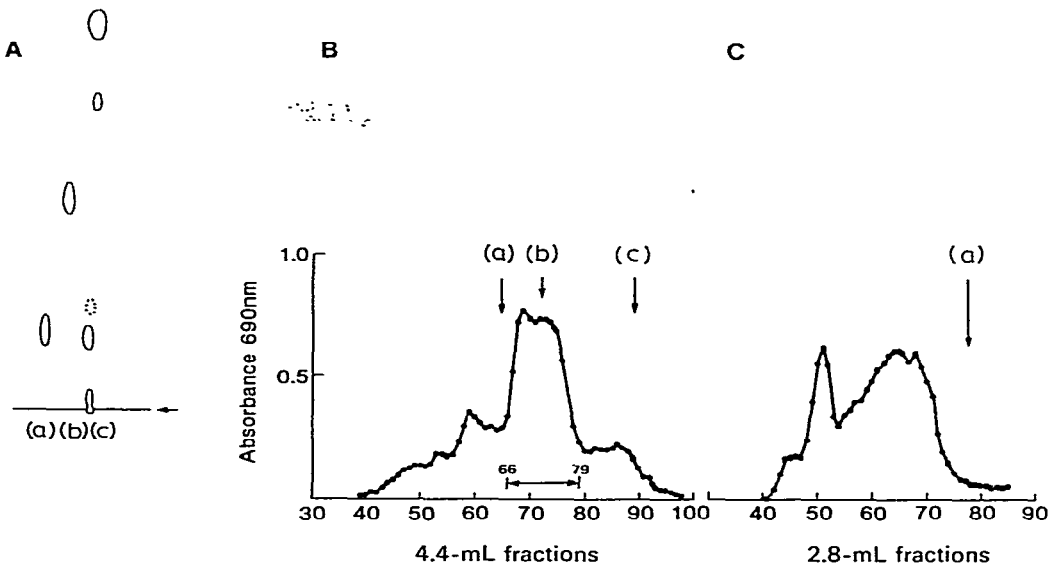

Fig. 1. (A) Descending paper chromatography (2:3:1, v/v, 1-butanol–acetic acid–M ammonium hydroxide) of standard sulfated and nonsulfated, unsaturated disaccharides, and of a *Pseudomonas* digest of bovine cornea keratan sulfate; the latter shows the presence of material migrating like an unsaturated disaccharide 6-sulfate: (a) unsaturated disaccharide 6-sulfate; (b) unsaturated, non-sulfated disaccharide; and (c) keratan sulfate digest (50 μL). (B) Gel filtration of a *Pseudomonas* digest of bovine cornea keratan sulfate on a column of Sephadex G-25 fine, equilibrated, and eluted with M sodium chloride. The eluate was analyzed for reducing sugars; the material eluted between tubes 66 and 79 was collected, dialyzed, and concentrated: (a) trisaccharide disulfate from chondroitin 6-sulfate; (b) unsaturated disaccharide 6-sulfate; and (c) 2-acetamido-2-deoxy-D-glucitol 6-sulfate. (C) The concentrated material was chromatographed and desalted on a column of Sephadex G-15 fine, equilibrated, and eluted with 10% ethanol: (a) 2-acetamido-2-deoxy-D-glucitol 6-sulfate.

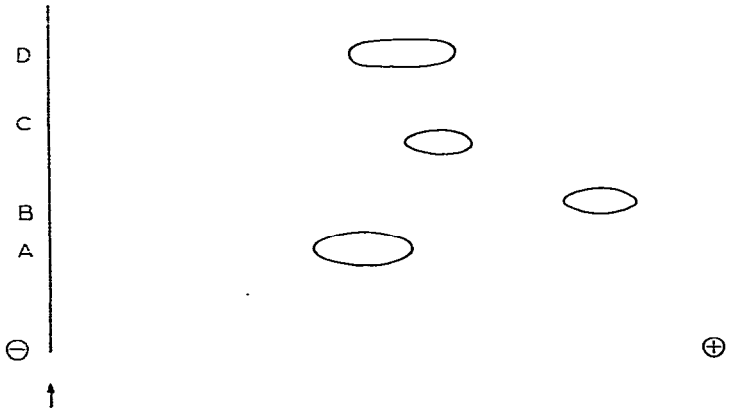

Fig. 2. High-voltage electrophoresis (30 V/cm, 90 min, 50mM ammonium acetate–acetic acid buffer, pH 5.0, alkaline silver nitrate staining) of a purified disaccharide monosulfate from a *Pseudomonas* digest of bovine cornea keratan sulfate (A). An unsaturated disaccharide 6-sulfate (B), 2-acetamido-2-deoxy-D-glucose 6-sulfate (C), and picric acid (D) were used as standards. The sodium borotritide-reduced, disaccharide monosulfate migrated like the nonreduced compound.

and aliquots were incubated as substrate with the adaptive enzymes obtained from *Pseudomonas*, in order to harvest the desired oligosaccharides.

The last fraction, eluted with 3.0M sodium chloride (100 mg), was also used for the preparation of oligosaccharides, but, because of its higher sulfate content, it gave a lower yield of them.

Extensive incubation of crude keratan sulfate with the adaptive enzymes of *Pseudomonas* gave two types of products: those soluble in ethanol (75% concentration), consisting of several reducing oligosaccharides, the major aliquot of them having the same chromatographic mobility as that of an unsaturated disaccharide 6-sulfate standard (Fig. 1A); and those insoluble in 75% ethanol, consisting essentially of a residual core that could not be degraded under the conditions used, did not migrate on paper chromatography, and had moderate reducing activity (Fig. 1A).

When large amounts of the oligosaccharide preparations were purified by gel filtration on Sephadex G-25 (Fig. 1B) and then desalted on Sephadex G-15 (Fig. 1C), large amounts of material were obtained which, on paper chromatography, migrated like the unsaturated disaccharide 6-sulfate standard. This material was eluted with water from the paper and emerged as a major peak, immediately after the void volume, from a column of Sephadex G-15. Analysis of this material gave a molar ratio of reducing groups (as galactose) to neutral sugars (as galactose) to 2-amino-2-deoxyhexose to sulfate of 100:80:89:114. High-voltage electrophoresis of this material demonstrated a single, strongly reducing component having $M_{GlcNAc\text{-}6\text{-}S}$ 0.81 (Fig. 2).

Reduction of an aliquot of this disaccharide sulfate with sodium borotritide produced a radioactive material, homogeneous on gel filtration on Sephadex G-15, and migrating on paper electrophoresis similarly to the reducing, nonradioactive precursor (Fig. 2). By analysis, it contained 2-amino-2-deoxyhexose but no anthrone-

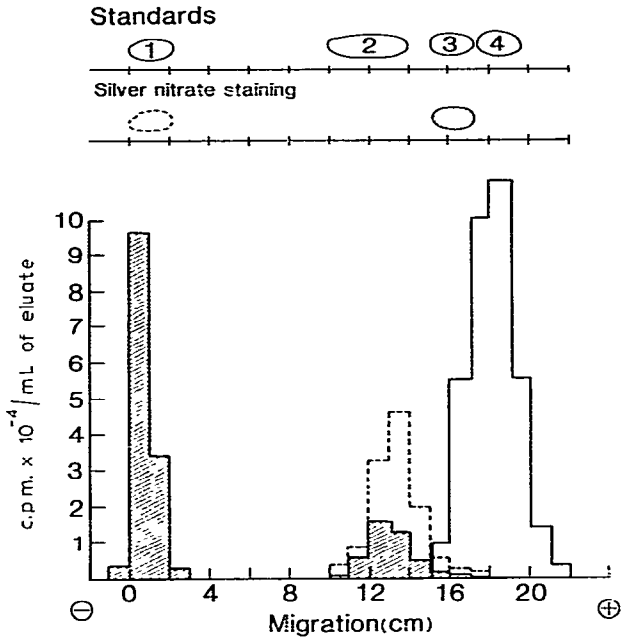

Fig. 3. High-voltage electrophoresis (see legend to Fig. 2 for conditions) of the sodium borotritide-reduced disaccharide monosulfate, before and after hydrolysis with 0.1M hydrochloric acid for 2 h at 100° Half of the paper was cut into 1-cm wide segments, which were eluted with water and the eluates counted for radioactivity. The other half of the paper containing the hydrolyzed sample was stained with alkaline silver nitrate. The standard compounds applied to the paper migrated as indicated: (1) D-galactose and D-[1-^{3}H]galactitol; (2) unsaturated disaccharide 6-sulfate; (3) 2-acetamido-2-deoxy-D-glucose 6-sulfate; and (4) D-galactose 6-sulfate and D-[1-^{3}H]galactitol 6-sulfate. Alkaline silver nitrate staining of the paper demonstrated that, after hydrolysis of the reduced disaccharide monosulfate, the strongly reducing compound migrates like 2-acetamido-2-deoxy-D-glucose 6-sulfate. A very faint reducing activity was demonstrated to migrate similarly to the D-galactose–D-galactitol standard. In the bottom part of the figure, the area designated with a broken line shows the migration of the sodium borotritide-reduced disaccharide monosulfate before hydrolysis; the shaded areas show the migration of the products of hydrolysis of the disaccharide monosulfate; 75% of the applied radioactivity remained at the origin, while 25% migrated similarly to the nonhydrolyzed material. The clear area designated with a continuous line shows where a standard of D-[1-^{3}H]galactitol 6-sulfate is expected to migrate. No radioactive products of the hydrolyzed disaccharide monosulfates were found in this area.

reacting material, and it was not reducing. Mild hydrolysis of the radioactive, reduced compound gave products that behaved as follows on paper electrophoresis: 75% of the applied radioactivity remained at the origin, while 25% moved similarly to the nonhydrolyzed material (Fig. 3). A nonradioactive, strongly reducing compound migrated with the same mobility as that of a standard of 2-acetamido-2-deoxy-D-glucose 6-sulfate, while a very weak reducing spot was found at the origin, where most of the applied radioactivity was located. No radioactivity was found to correspond to a standard of D-[1-^{3}H]galactitol 6-sulfate.

The radioactive, reduced compound was desulfated with methanolic 70mM hydrogen chloride. After purification by chromatography on Dowex 1-X8 anion-

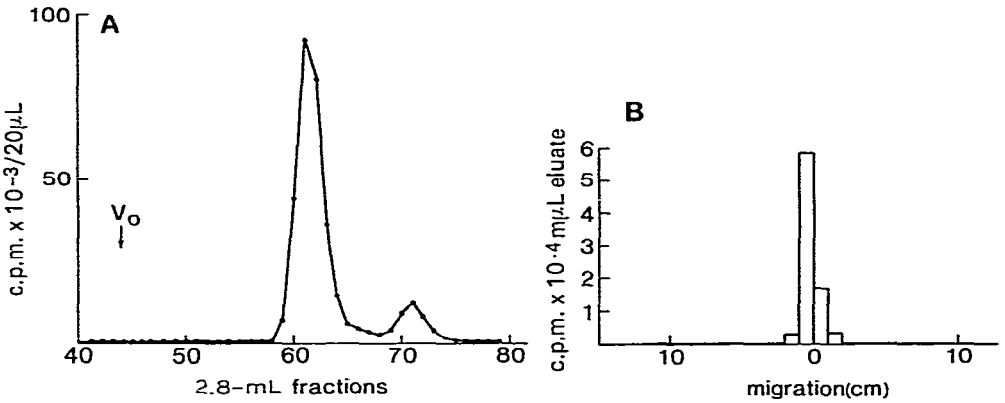

Fig. 4. (A) Gel filtration on Sephadex G-15 fine of the sodium borotritide-reduced, desulfated disaccharide. (B) The material obtained from the main peak shown in (A) did not migrate on high-voltage electrophoresis (see legend to Fig. 2 for conditions).

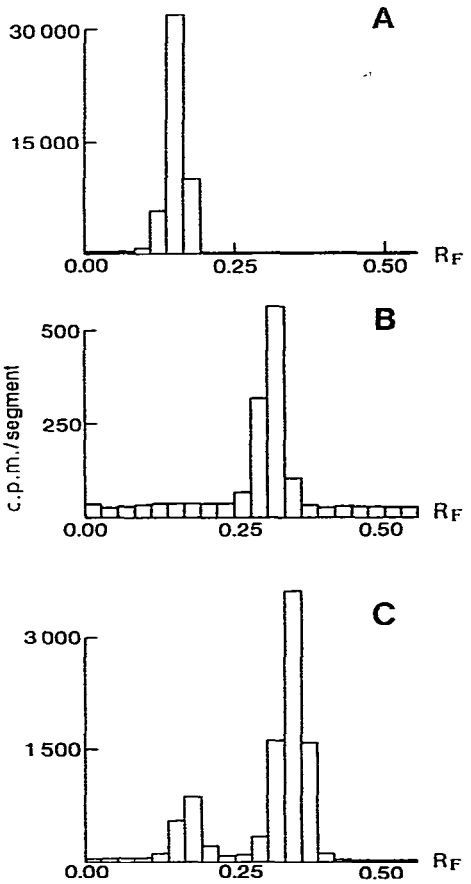

Fig. 5. (A) Migration of the sodium borotritide-reduced, desulfated disaccharide on t.l.c. (B) Migration of D-[1-^{3}H]galactitol in the same system. (C) Actual separation by t.l.c. of the remnant substrate and product formed upon incubation of 2 nmol of substrate (20 000 c.p.m.) with 100 μg (as protein) of a crude human liver extract, at pH 3.7, for 20 h at 37°.

exchange resin and by gel filtration on Sephadex G-15 (Fig. 4A). the product obtained did not migrate on high-voltage electrophoresis (Fig. 4B).

The measurement of the enzyme 2-acetamido-2-deoxy-D-glucose 6-sulfate sulfatase in fibroblast extracts by use of the disaccharide monosulfate described here gave values ranging between 0.21 and 0.44 nmol of product/h/mg of protein. At a pH optimum of 5.70, the apparent K_m of the crude enzyme preparation was found to be 0.74mM.

Separation of *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→3)-D-[1-^{3}H]-galactitol from D-[1-^{3}H]galactitol was readily achieved by t.l.c., as shown in Fig. 5. With this method of assay, it was found that a crude enzyme preparation from human liver has a pH optimum of 3.7.

DISCUSSION

Nakazawa *et al.*[13] have demonstrated that sequential degradation of keratan sulfate could be achieved with bacterial enzymes derived from *Pseudomonas* and *Actinobacillus* grown symbiotically on a medium containing keratan sulfate (1%) as a sole carbon source. They demonstrated the presence in the bacterial extracts of endoglycosidases, sulfatases, exo-β-D-galactosidase, and exo-*N*-acetyl-β-D-glucosaminidase, the combined effects of which lead to release of 2-acetamido-2-deoxy-D-glucose, D-galactose, and inorganic sulfate. The extent of degradation varied with keratan sulfates of different origin and structure, and fragments resistant to degradation were found. The structural reasons responsible for this resistance have not been elucidated[15].

Since our purpose was to isolate sulfated oligosaccharides of keratan sulfate, we have used only adaptive enzymes of *Pseudomonas*, because adaptive sulfatases were found essentially in extracts of *Actinobacillus*[13]. Moreover, our previous experiments indicated that when *Pseudomonas* was grown on a medium containing 0.1% rather than 1% keratan sulfate of low sulfate content, no adaptive sulfatases could be detected in the bacterial extracts.

The adaptive endoglycosidases thus induced, when incubated with hyposulfated keratan sulfate, produced small amounts of 2-acetamido-2-deoxy-D-glucose and D-galactose, a moderate amount of resistant fragments, and abundant oligosaccharides (Figs. 1 and 2). The presence of *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl 6-sulfate)-(1→3)-D-galactose among the ethanol-soluble products was monitored by paper chromatography (Fig. 1A), and the desired disaccharide was purified and isolated by gel filtration and preparative paper chromatography (Fig. 1B,C). Analyses of the material obtained suggested a disaccharide sulfate having equimolar amounts of neutral sugars, hexosamine, and sulfate. This disaccharide was reduced with sodium borotritide, and then hydrolyzed with 0.1M hydrogen chloride, under conditions which Nakazawa and Suzuki[15] have demonstrated to cleave the (1→3)-2-acetamido-2-deoxy-β-D-glucopyranosyl linkage to D-galactose, without removing any ester sulfate.

Even though only 75% of the material was hydrolyzed, the products analyzed by electrophoresis demonstrated the presence of a reducing, nonradioactive, negatively-charged sugar having the same mobility as that of authentic 2-acetamido-2-deoxy-D-glucose 6-sulfate, and of a radioactive, nonreducing compound remaining at the origin. No radioactivity was found corresponding to a standard of D-[1-^{3}H]-galactitol 6-sulfate. These results demonstrate that the disaccharide monosulfate isolated is *O*-(2-acetamido-2-deoxy-sulfo-β-D-glucopyranosyl 6-sulfate)-(1→3)-D-galactose and that its product of reduction is the corresponding D-galactitol. This radioactive disaccharide was the substrate for the measurement of the specific 6-sulfatase that uses the microcolumn-chromatographic method described previously[1,5]. The results of these measurements agree quite well with those of Basner *et al.*[17], which were obtained with a trisaccharide prepared from heparan sulfate and with an enzyme preparation derived from human urine. The availability of this disaccharide monosulfate substrate and of another one being prepared from heparin (bearing an *N*-sulfate rather than an *N*-acetyl group on the 2-amino-2-deoxy-D-glucose 6-sulfate residue) will allow an investigation of the role of the 2-amino substituents on the activity of the 6-sulfatase.

Desulfation of the reduced disaccharide monosulfate was achieved by treatment with methanolic hydrogen chloride[16], as indicated by the observation that it did not migrate any longer on paper electrophoresis and was not retained on an anion-exchange column. This disaccharide, *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)(1→3)-D-[1-^{3}H]galactitol, is now being used, in the t.l.c. method described herein, for the further purification of the specific (1→3)-β-D-glucosaminidase.

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NOTE ADDED IN PROOF

Recent results by Kresse[18] on the two 2-amino-2-deoxy-D-glucose sulfate sulfatases (one specific for heparan sulfate and the other for keratan sulfate) led us[19] to reconsider the enzymic defect of the patient described in refs. 1 and 5.

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