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Note

^{35}S-Labeled dermatan sulfate. Preparation of a substrate for enzymic degradation studies

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Five clinically distinct types of mucopolysaccharidoses share a common feature, namely the accumulation of dermatan sulfate. The defective enzyme in each of these disorders has now been identified[1]. Concurrently, many of the details of the fine structure of dermatan sulfate have been elucidated[2]. The catabolic pathway for dermatan sulfate has been inferred from the chemical and genetic information to proceed in a stepwise manner from the nonreducing terminus. Sequential removal of each terminal sulfate or glycosyl residue is implicit in the scheme, so the absence of any single enzyme, as in the mucopolysaccharidoses, prevents further breakdown. There has been no experimental confirmation of this pathway, due in part to the lack of suitable substrates. The materials accumulated in the mucopolysaccharidoses can be considered as "limit dermatan sulfates" in that they are derived from proteoglycans from which protein and chondroitin sulfate regions have been removed by proteases and endoglycosidases, such as hyaluronidase. On the premise that similar material, preferably radiolabeled, should serve as substrate for documenting the stepwise catabolism, ^{35}S-labeled limit dermatan sulfate was prepared by enzymic degradation.

EXPERIMENTAL

Materials. — Weaned male Sprague-Dawley rats (45–50 g) were obtained from a local breeder; carrier-free [^{35}S]sulfuric acid (40–50 mCi in 0.5–1.0 mL of water) and [^{14}C]acetic anhydride (10 Ci/mol, 20% solution in benzene) from New England Nuclear (Boston, MA 02118); papain (crude, type 1, 0.31 U/mg), ribonuclease A (Type 1-A, 5 times recrystallized), deoxyribonuclease II (from porcine spleen, 1950 U/mg), chondroitinase ABC, chondroitinase AC, and dermatan sulfate (chondroitin

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sulfate B, from pig skin) from Sigma Chemical Co. (St. Louis, MO 63178); hyaluronidase (B grade, from bovine testes, 400 USP U/mg), Pronase (B grade, 45 000 PUK/g), and cetylpyridinium chloride from Calbiochem. Behring Corp. (San Diego, CA 92112); chondroitin 6-sulfate (chondroitin sulfate C) from Seikagaku Kogyo Co. (Nagoya, Japan); Dowex 1 (AG 1-X2, 200–400 mesh) and phosphocellulose (Cellex P) from Bio-Rad Laboratories (Richmond, CA 94804); and Sephadex G-200 from Pharmacia Fine Chemicals (Piscataway, NJ 08854). Glycosaminoglycuronan standards were provided by Dr. M. B. Mathews.

Preparation of heparinase. — Heparinase from heparin-induced *Flavobacterium heparinum* (ATCC 13 125) was prepared by an adaptation of different steps of the procedures of Linker and Hovingh[3,4]. The procedure used involved streptomycin treatment, ammonium sulfate precipitation, Sephadex G-200 gel-filtration, and phosphocellulose chromatography. Gel filtration yielded two fractions having enzyme activity. The first, eluted with the void volume, contained chondroitinase B activity, and was unsuitable for the present purposes. Only the second, retarded fraction was processed further. The specific activity of the final preparation was 0.11 units/mg of protein, a unit being defined as 1 μmol (measured spectrophotometrically[3]) of α,β-unsaturated uronide formed from heparin per hour.

Analytical methods. — The procedure for the isolation of dermatan sulfate was monitored by electrophoresis on cellulose acetate strips in the barium acetate buffer system of Wessler[5], with a Beckman Microzone apparatus. The glycosaminoglycuronan bands were detected with the Alcian Blue reagent of Hata and Nagai[6]. Radioautographs were prepared by placing the strips in contact with film in X-ray cassettes for several weeks. Alternatively, stained strips were cut into narrow segments for counting by liquid scintillation.

The uronic acid content was determined by the modified carbazole method of Bitter and Muir[7] with D-glucurono-6,3-lactone as the standard. Carbazole-to-orcinol ratios were derived from the carbazole determination of Dische[8] and the orcinol determination of Mejbaum[9]. Analysis of hexosamine and sulfate content, was carried out, after hydrolysis in 6M hydrochloric acid in sealed ampoules for 8 h at 100°, by the Elson–Morgan[10] and sodium rhodizonate[11] procedures, respectively. Susceptibility to chondroitinases AC and ABC was tested as described by Suzuki *et al.*[12,13]. Hexosamine was identified by *N*-[^{14}C]acetylation of hydrolyzed material by the method of Carlson[14].

Preparation of ^{35}S-labeled limit dermatan sulfate. — *Acetone powder.* Four rats were injected subcutaneously, each with 10 mCi of carrier-free $H_2{}^{35}SO_4$. They were sacrificed 4 days later under ether, and much of the hair was removed with the aid of a depilitant. After being washed, the carcasses were homogenized in a Waring Blendor for 2 min with acetone (200 mL). The residue recovered by filtration on a Büchner funnel was re-homogenized for 2 min with acetone (150 mL). The resulting residue was stirred for 30 min in 1 : 1 (v/v) acetone–ether (150 mL), filtered off, washed on the funnel with two 100-mL portions of the same solvent mixture, and then with three 100-mL portions of ether. The yield of air-dried powder was 60 g.

Protease treatment. Acetone powder (31.5 g) containing 3.3×10^8 c.p.m. was suspended in 50mM sodium acetate (pH 6.5) containing 10mM cysteine and 10mM EDTA (300 mL). Papain (3 g) and 10% sodium azide (0.6 mL) were added, and the mixture was incubated for 20 h at 65°. The papain was inactivated by heating at 100° for 10 min. Upon cooling, calcium chloride was added to a concentration of 20mM, followed by Pronase (200 mg). The mixture was incubated for 24 h at 55°, with an addition of Pronase (100 mg) at 18 h. The enzyme was inactivated by heating to 100° for 10 min, and the mixture was centrifuged at 4000*g* for 15 min. Approximately 83% of the radioactivity of the acetone powder was solubilized by the sequential proteolysis. The supernatant solution was dialyzed against 50mM potassium phosphate buffer (10 vol., pH 7.0) overnight at 4° with one buffer change. About 8% of the radioactivity appeared in the dialyzate.

Nuclease treatment. Ribonuclease (50 mg) and 10% sodium azide (1 mL) were added to the dialyzed solution (550 mL), and the mixture was incubated for 22 h at 37°. Deoxyribonuclease (27.5 mg) and magnesium chloride to a concentration of 5mM were added, and the incubation was continued for an additional 7 h at 37°. The enzymes were inactivated by heating at 100° for 10 min, and the mixture was centrifuged at 4000*g* for 30 min. Glycosaminoglycuronans in the supernatant fluid were precipitated by adding 5% cetylpyridinium chloride (40 mL) to the solution preheated to 37°. After maintaining this temperature for 30 min, the precipitate was allowed to aggregate overnight at room temperature and the precipitated material was recovered by centrifugation at 4000*g* for 30 min. Cetylpyridinium chloride was removed by suspending the precipitate in 10% (w/v) potassium acetate in ethanol (450 mL), cooling the mixture to 4° for 1 h, and recovering the precipitate by centrifugation. The insoluble material was subjected to the potassium acetate–ethanol washing three more times, and the final precipitate was dried under a stream of nitrogen. The dried material was dissolved in 0.1M sodium acetate (pH 5.0) containing 0.1M sodium chloride (45 mL).

Hyaluronidase treatment. Hyaluronidase (61 mg) and 10% sodium azide (0.1 mL) were added and the mixture was incubated for 22 h at 37°, with an addition of hyaluronidase (5 mg) at 17 h. The enzyme was inactivated by heating at 100° for 10 min. Undegraded glycosaminoglycuronans were recovered by adding ethanol to 80%, heating at 79° for 5 min, cooling to 4° for 1 h, and centrifuging at 4000*g* for 5 min. The precipitate was dried under a stream of nitrogen and dissolved in 50mM potassium phosphate buffer, pH 6.8 (25 mL). A small amount of insoluble debris was removed by centrifugation. Cellulose acetate electrophoresis indicated that about 14% of the radioactivity was in heparin and heparan sulfate-like materials.

Heparinase treatment. Calcium acetate was added to a concentration of mM, and 10% sodium azide (0.1 mL) and 2 units of heparinase were added. The mixture was incubated for 70 h at 30°, with an addition of heparinase (0.6 unit) at 20 h. The solution was then adjusted to 10mM calcium chloride and treated with Pronase (1.2 mg) for 1 h at 55°. Pronase was inactivated by heating at 100° for 10 min, and undegraded glycosaminoglycuronans were recovered by ethanol precipitation as

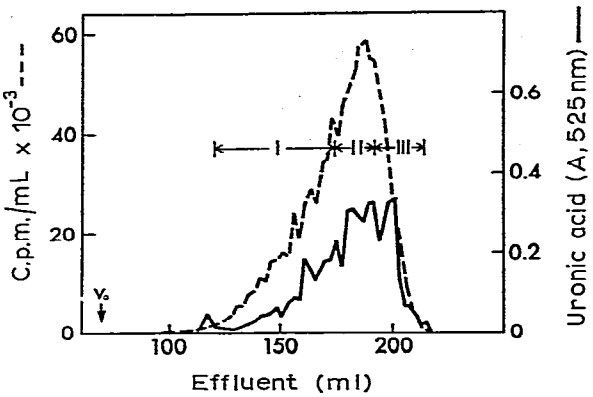

Fig. 1. Gel filtration on Sephadex G-200 of dermatan sulfate sample obtained from Dowex-1 chromatography. Fractions from the column were arbitrarily pooled in three size-groups and labeled Fraction I, II, and III.

described earlier. The precipitated material was dissolved in water (17 mL), and insoluble debris were removed by centrifugation. Heparan sulfate-like materials were no longer detected by electrophoresis.

Dowex-1 chromatography. The solution was applied to a column (1.7 × 10 cm) of Dowex-1 (Cl^-), and the column was washed with water (60 mL), followed by 0.5M sodium chloride (60 mL). A linear gradient from 0.5 to 3.0M sodium chloride (450 mL) was applied, and 5-mL fractions were collected. The bulk of the radioactivity was eluted before the dermatan sulfate fraction. Keratan sulfate, which has strong affinity for Dowex-1, was presumed to have remained adsorbed on the resin. Fractions eluted between 1.35 and 1.8M sodium chloride were pooled, dialyzed against water (4 L), and concentrated to 2.1 mL by rotary evaporation.

Sephadex G-200 gel filtration. The material from the Dowex-1 chromatography was subjected to gel filtration on Sephadex G-200. A column (1.7 × 81 cm) of the gel in M sodium chloride that had a V_o of 68.5 mL and V_t of 205.5 mL at a flow rate of 18 mL/h at room temperature was used. Three-mL fractions were collected and analyzed for radioactivity and uronic acids. Rough size estimation of the mol. wt. was made by the formula of Wasteson[15]. The elution profile showed a continuous asymmetric peak with good correspondence between radioactivity and uronic acids (Fig. 1). A wide range of molecular size was indicated, so the eluate was arbitrarily divided into three fractions. Each fraction was dialyzed against water and concentrated to less than 2 mL.

RESULTS AND DISCUSSION

^{35}S-Labeled limit dermatan sulfate was obtained in a yield of 8.0 mg (as uronic acid) and 7.3 × 10^6 c.p.m. from 31.5 g of acetone powder (Table I), equivalent to 3.8 mg of uronic acid and 3.5 × 10^6 c.p.m. per weanling rat. The polydisperse

TABLE I

PREPARATION OF ^{35}S-LABELED LIMIT DERMATAN SULFATE

Step	*Yield (mg of uronic acid)*	*Radioactivity (c.p.m. × 10^{-6})*
Acetone powder (31.5 g)		330
Protease		250
Nuclease; cetylpyridinium chloride precipitation	145	160
Hyaluronidase; ethanol precipitation	45	120
Heparinase; ethanol precipitation	14	70
Dowex-1	9	14
Sephadex G-200		
I	2.56	2.8
II	3.77	3.2
III	1.68	1.3

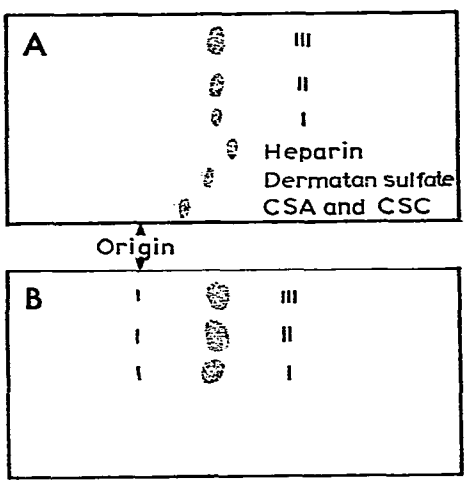

Fig. 2. Electrophoresis of dermatan sulfate fractions on cellulose acetate. A composite drawing of Alcian Blue-stained sample and standard electrophoretograms (A) is compared with a corresponding autoradiographic pattern (B). The following amounts of material were applied: standard glycosaminoglycuronans 0.25 μg; Fraction I, 1320 c.p.m., 0.24 μg of uronic acid; Fraction II, 1490 c.p.m., 0.28 μg of uronic acid; Fraction III, 1120 c.p.m., 0.16 μg of uronic acid. For autoradiography, X-ray film was exposed to the dried strip for 5 weeks in an X-ray cassette before development. The electrophoretic conditions are given in the Experimental section. Abbreviations: CSA, chondroitin 4-sulfate; CSC, chondroitin 6-sulfate.

material was arbitrarily divided into three fractions by size. Fraction I constituted about 40% of the total and was estimated to have an average of ~20 repeating disaccharide units per fragment. Fraction II made up ~45% of the material and contained fragments of intermediate mol.wt. corresponding to ~5–10 disaccharide units. Fraction III, which was smaller, accounted for less than 20% of the total.

TABLE II

ANALYSIS OF LIMIT DERMATAN SULFATE FRACTIONS

Fraction	*Carbazole-to-orcinol ratio*	*Molar ratio of*		
		Uronic acid	*Hexosamine*	*Sulfate*
I	0.40:1	1.0	1.35	0.95
II	0.40:1	1.0	0.89	0.98
III	0.68:1	1.0	0.87	1.22
Standard dermatan sulfate	0.39:1	1.0	0.95	0.95

Each fraction was subjected to electrophoresis on cellulose acetate and examined by Alcian Blue staining and radioautography (Fig. 2). Staining indicated that each fraction contained only glycosaminoglycuronans that migrated at the same speed as reference dermatan sulfate. Radio-autography showed that the radioactivity was coincident with the Alcian Blue-staining material. A trace of radioactivity was present at the origin, but this is believed to be an artifact; all radioactive glycosaminoglycuronans that we have examined by this technique leave similar traces at the origin[16]. Thus, the material in each of the fractions was electrophoretically identical to reference dermatan sulfate.

Chemical analyses of Fractions I, II, and III are summarized in Table II. The ratio of uronic acid to hexosamine to sulfate was near unity for each fraction, as in standard pig-skin dermatan sulfate. The ratios of carbazole-to-orcinol reaction of Fractions I and II were 0.40:1, very close to the value of 0.39:1 for the standard, suggesting that the majority of uronic acid residues was L-iduronic acid. Fraction III had a carbazole-to-orcinol ratio of 0.68:1, indicating a higher percentage of D-glucuronic acid. The hexosamine in all fractions was identified as galactosamine with no detectable glucosamine.

No unsaturated disaccharides were produced on treatment of any of the materials with chondroitinase AC, implying the absence of any extensive chondroitin sulfate-like segments. This does not rule out isolated D-glucuronic acid-containing residues, which could be susceptible to the bacterial enzyme. However, all of the radioactivity was recovered by ethanol precipitation, suggesting that no extensive degradation resulting in nonprecipitable oligosaccharides had occurred. In contrast, treatment with chondroitinase ABC provided a high yield of unsaturated disaccharides, and radioactivity could no longer be precipitated by ethanol. Therefore, the larger oligosaccharide preparations had chemical and physical properties consistent with their being limit dermatan sulfate fragments with regard to mammalian endoglycosidase susceptibility. Because of its low yield, small size, and poor analytical properties, Fraction III was not considered useful as a substrate for degradative studies.

The designation "dermatan sulfate" is somewhat ambiguous in that it refers to a group of related glycosaminoglycuronans of varying degrees of complexity[17]. In the usual context, the term implies native dermatan sulfates that are derived from

proteolysis of certain proteoglycans. They contain numerous *O*-α-L-idopyranosyluronic acid-(1→3)-2-acetamido-2-deoxy-D-galactopyranosyl disaccharide units (with various proportions of sulfate groups), but hybrid species that contain regions of chondroitin 4- and 6-sulfate structure are also present. An amino acid or oligopeptide may be linked to the reducing end. The molecular weight varies from 20 000 to 100 000. In contrast, ideal dermatan sulfate can be construed as a poly(oligo)saccharide from which all extraneous disaccharide units have been excised and from which residual amino acid or peptide fragments have also been removed. Regions of ideal structure in various native dermatan sulfates may range from only a few to 20 or more disaccharide units[2].

The limit dermatan sulfates described in the present report are thought to be intermediate between native and ideal structures. Although all extended chondroitin sulfate regions were excised by hyaluronidase, single disaccharide units containing D-glucuronic acid residues undoubtedly remain, which would be susceptible to cleavage by chondroitinase AC, but do not give rise to disaccharide products. Therefore, the hyaluronidase-treated material is expected to be somewhat larger and more complex than dermatan sulfate of ideal structure. These materials will closely resemble the dermatan sulfate fragments that accumulate in tissues of mucopolysaccharidosis patients. As such, they should be ideal substrates for documenting the stepwise catabolic pathway of such polymers and for investigating the malfunctions that occur in a number of genetic diseases.

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