Copolymers of Chondroitin 4-Sulfate and Chondroitin 6-Sulfate in Chick Embryo Epiphyses and Other Cartilage*

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Tetrasaccharides were prepared from chondroitin sulfate by means of a limited degradation with chondroitinase ABC. Tetrasaccharides containing one sulfate per disaccharide unit were isolated and found to be of three types: a tetrasaccharide with two 4-sulfated disaccharide units, a tetrasaccharide with two 4-sulfated disaccharide units, and a tetrasaccharide with one 4-sulfated disaccharide unit and one 6-sulfated disaccharide unit. Samples of each of these three types of tetrasaccharides were obtained from chick embryo epiphyseal cartilage and from a mixture of bovine tracheal cartilage and shark cartilage. The presence of both a 4-sulfated disaccharide unit and a 6-sulfated disaccharide unit in the same tetrasaccharide molecule indicates the existence of mixed 4 and 6 sulfation on the same chondroitin sulfate chain.

Galactosamine-containing glycosaminoglycans have been classified into three main categories: chondroitin 4-sulfate, chondroitin 6-sulfate, and dermatan sulfate (the latter containing primarily iduronic acid rather than glucuronic acid). It has generally been considered that an individual polysaccharide chain contains only one of these types of glycosaminoglycan. However, chondroitin 6-sulfate and dermatan sulfate segments have been shown to exist as copolymers on the same chain in bovine tracheal cartilage, shark cartilage, and whale cartilage (2-4). Recent studies have also been reports of chondroitin 4-sulfate and chondroitin 6-sulfate segments on the same chain in bovine tracheal cartilage, shark cartilage, and whale cartilage (2-4).

Studies of the biosynthesis of chondroitin sulfate conducted with a microsomal preparation from chick embryo epiphyseal cartilage have suggested that the structure of an endogenous primer might be involved with the location of subsequent sulfation in other cartilage, experiments were undertaken to examine the possibility of mixed sulfation in chondroitin sulfate from the chick embryo cartilage. In addition, a commercial preparation of chondroitin sulfate from shark cartilage and bovine tracheal cartilage was examined to confirm the previously reported work. In order to look at mixed sulfation, chondroitinases, which do not have transglycosylase activity, were utilized to prepare tetrasaccharides. These were then separated according to sulfation pattern. A tetrasaccharide composed of one 4-sulfated disaccharide unit and one 6-sulfated disaccharide unit was isolated from chick embryo epiphyseal cartilage and from the commercial chondroitin sulfate, verifying the existence of mixed sulfation on the same chondroitin sulfate chain.

EXPERIMENTAL PROCEDURES

Materials—Chondroitinase ABC, chondroitinase AC, chondro-4-sulfatase, and chondro-6-sulfatase were purchased from Miles Laboratories, Inc., Kankakee, Ill. Chondroitin sulfate (a mixture from bovine tracheal cartilage and shark cartilage) was purchased from Nutritional Biochemicals Co., Cleveland, Ohio. Crude chondroitin sulfate was prepared as follows from the epiphyses of 200 12-day-old chick embryos, obtained from Spafas, Inc., Norwich, Conn. Tibial and femoral epiphyses were dissected and homogenized in 4 parts of 0.25 M sucrose followed by centrifugation at 100,000 x g. The 100,000 x g pellet was used for unrelated experiments. The 100,000 x g supernatant was incubated overnight with 0.5 M NaOH at 20°C, neutralized, and subjected to gel filtration on a Sephadex G-50 column (2.5 x 89 cm). The uronic acid-containing fractions at the void volume were used as the crude chondroitin sulfate preparation. Approximately 10 mg of chondroitin sulfate was obtained from 200 embryos.

Methods—Gel filtration to separate oligosaccharides of varying sizes was performed on a Sephadex G-25 column (2.6 x 116 cm) with 1 M LiCl as eluant. Desalting was accomplished on a Sephadex G-10 column (1.5 x 61 cm). Ion exchange chromatography to separate undersulfated and over-sulfated oligosaccharides from oligosaccharides containing one sulfate per disaccharide unit was conducted on a DEAE-cellulose (DE52) column (1 x 13 cm). Elution was accomplished with a linear gradient of 0 to 0.5 M LiCl in 50 mM Tris, pH 7.4.

Two descending paper chromatographic systems were utilized. (A) Isopropyl alcohol:acetic acid:1-butanol:water:1% aqueous acetic acid (6:3:3:1:1, v/v, solvent system A) and (B) 1-butanol:acetic acid:isopropanol:water:1% aqueous acetic acid (9:1:3:1:1, v/v, solvent system B) were used to separate the different types of sulfated tetrasaccharides. Chromatography was conducted on Whatman No. 3MM for 40 h. (B) 1-butanol:acetic acid:isopropanol:water:1% aqueous acetic acid (9:1:3:1:1, v/v, solvent system B) was used to separate the different types of disaccharides following complete degradation with chondroitinase ABC (7). Chromatography was conducted on Whatman No. 1 along with disaccharide standards for 16 to 18 h. After chromatography, the sample was either visualized with the aid of an ultraviolet viewbox or segments of the chromatogram were washed with 95% ethanol and eluted with water for analysis, or both.

Uronic acid containing material was assayed by the modified carbazole method of Bitter and Muir (8), using glucuronolactone as the standard. Terminal N-acetylgalactosamine was assayed by the method of Reissig et al. (9), using N-acetylgalactosamine as the standard. Total hexosamine was determined by hydrolyzing the sample in 2 M HCl for 3 h at 100°C followed by evaporation of the HCl. Hexosamine was then assayed by the methods of Levey and McAdam (10), using N-acetylgalactosamine as the standard. Absorbance at 232 nm was used as an indication of Δ,5 double bond formation during chondroitinase degradation and as a measure of the concentration of the isolated degradaton products. Millimolar absorption coefficients of 5.7 for ΔDi-OS, 5.5 for ΔDi-DS, and 5.1 for ΔDi-4S were utilized (11).

The abbreviations used are: ΔDi-OS, 2-acetamido-2-deoxy-3-O-[(β-D-galactopyranosyluronic acid)-N-acetylgalactosamine]; ΔDi-4S, 2-acetamido-2-deoxy-3-O-[(β-D-galactopyranosyluronic acid)-N-sulfido-D-galactose]; ΔDi-6S, 2-acetamido-2-deoxy-3-O-[(β-D-galactopyranosyluronic acid)-N-sulfido-D-galactose].
RESULTS

A sample of commercial chondroitin sulfate prepared from a mixture of bovine tracheal cartilage and shark cartilage yielded only disaccharides following either chondroitinase AC or ABC treatment, thus demonstrating that no iduronic acid moieties as found in dermatan sulfate were present.

Commercial chondroitin sulfate (1 g) was incubated at 37°C with 12 mm enriched Tris buffer (pH 8.0) (7) and 4 units of chondroitinase ABC. When the reaction had proceeded to 50% of its maximum degradation, as measured by absorbance at 232 nm, it was stopped by boiling for 10 min. After centrifugation, the supernatant, which contained the oligosaccharides, was chromatographed on Sephadex G-25. Four oligosaccharide peaks were obtained which were identified as disaccharides, tetrasaccharides, hexasaccharides, and a peak composed of octasaccharides and larger oligomers. Identification of oligosaccharide size was made by the molar ratio of uronic acid (as determined by the carbazole assay) to double bond presence (as determined by absorbance at 232 nm).

Disaccharides were chromatographed on a DE52 column to separate nonsulfated and oversulfated molecules from molecules containing one sulfate per disaccharide unit. Each peak was desalted on a Sephadex G-10 column, concentrated, spotted on Whatman No. 1, and chromatographed in System B. The first peak from the DE52 column, representing less than 1% of the total disaccharides, co-chromatographed with the ADi-0S standard, indicating very limited nonsulfated regions in the chondroitin sulfate. The second peak co-chromatographed with the ADi-4S and ADi-6S standards with a ratio of approximately 60% ADi-4S to 40% ADi-6S. Disaccharides from the third peak representing 8% of the total disaccharides appeared as a homogeneous spot moving somewhat slower than the ADi-0S standard. Based on its chromatographic behavior on DE52 and paper, its susceptibility to chondroitinase ABC and chromatographed in Solvent B was desalted on a Sephadex G-10 column, concentrated, spot chromatographed on Whatman No. 3MM in Solvent A. Three distinct spots of approximately 60% ADi-4S to 40% ADi-6S. Disaccharides from the third peak representing 8% of the total disaccharides appeared as a homogeneous spot moving somewhat slower than the ADi-0S standard. It was shown that 75% were B-sulfated and 25% were 4-sulfated. No nonsulfated regions were present here as well.

Similar methods were then applied to chondroitin sulfate from chick embryo epiphyseal cartilage as well as chondroitin sulfate and disaccharide standards were similarly chromatographed. ADi-4S and ADi-6S standards run off the end of the paper under these conditions. The dotted spot indicates less material and the nonshaded spot still less.

Mixed Sulfation of Chondroitin Sulfate in Cartilage

SHARK AND BOVINE

CHICK

STANDARDS

![Fig. 1. Paper chromatography of tetrasaccharides. The major peak of shark and bovine cartilage tetrasaccharides from the DE52 column was chromatographed on Whatman No. 3MM in Solvent A as described under "Experimental Procedures." Tetrasaccharides from chick embryo epiphysal cartilage as well as chondroitin sulfate and disaccharide standards were similarly chromatographed. ADi-4S and ADi-6S standards run off the end of the paper under these conditions. The dotted spot indicates less material and the nonshaded spot still less.](http://www.jbc.org/content/250/22/7647/F1)

![Fig. 2. Chondroitinase degradation products of shark and bovine cartilage tetrasaccharides. Shark and bovine tetrasaccharides from the chromatogram of Fig. 1 were each incubated with 0.5 unit of chondroitinase ABC for 1% h at 37°C in a total volume of 50 μl containing 15 μl of enriched Tris buffer (pH 8.0) (7). Total reaction mixtures along with disaccharide standards and untreated tetrasaccharides were chromatographed in System B as described under "Experimental Procedures."](http://www.jbc.org/content/250/22/7647/F2)
confirmed the above identification.

Following chondroitinase ABC treatment of each of these spots of its position on chromatography. The disaccharides obtained approximate. Analysis of Spot III produced values too low for one 4-sulfated disaccharide unit and one 6-sulfated disaccharide unit. The molar ratio for Spot mine was 1.0:0.49:0.47, indicating a tetrasaccharide composed of two 6-sulfated disaccharide units. The molar ratio for Spot I was cut into 1-cm strips and eluted with water. Analyses of the fractions are shown in Fig. 3. For Spot I the molar ratio of uronic acid:absorbance at 232 nm (O—O), and terminal N-acetylated hexosamine (●). O.D., optical density.

Tetrasaccharides were separated directly by paper chromatography in System A. Three spots were observed under UV illumination (Fig. 1) with positions identical with the three spots found upon chromatography of the tetrasaccharides from the shark and bovine chondroitin sulfate mixture. Spot I exhibited strong absorbance, Spot II absorbed moderately, whereas Spot III was barely visible. The chromatogram was cut into 1-cm strips and eluted with water. Analyses of the fractions are shown in Fig. 3. For Spot I the molar ratio of uronic acid:absorbance at 232 nm:terminal N-acetylated hexosamine was 1.0:0.46:0.47, indicating a tetrasaccharide composed of two 6-sulfated disaccharide units. The molar ratio for Spot II was 1.0:0.52:0.14, suggesting a tetrasaccharide composed of one 4-sulfated disaccharide unit and one 6-sulfated disaccharide unit. However, due to the very small amount of material, the terminal N-acetylated hexosamine assay for this spot was only approximate. Analysis of Spot III produced values too low for significant calculations, but this tetrasaccharide was considered to consist of two 4-sulfated disaccharide subunits because of its position on chromatography. The disaccharides obtained following chondroitinase ABC treatment of each of these spots confirmed the above identification.

**DISCUSSION**

Isolation of a tetrasaccharide consisting of one 4-sulfated disaccharide unit and one 6-sulfated disaccharide unit from bovine tracheal cartilage and shark cartilage was first reported by Seno et al. (2, 3). However, testicular hyaluronidase, which has known transglycosylase activity (13, 14), was utilized as the degradative enzyme to obtain this tetrasaccharide. The transglycosylase activity would give rise to mixed tetrasaccharides even if a mixture of pure chondroitin 4-sulfate and pure chondroitin 6-sulfate were used as the substrate. A calculation was made in an attempt to correct for this, but the conclusion that these chondroitin sulfate preparations contained mixed 4 and 6 sulfation on the same chondroitin sulfate chain is somewhat equivocal.

Recently, chondroitin sulfate preparations from shark and from whale cartilage have been examined by the use of chondroitinase C, which degrades chondroitin 6-sulfate, but not chondroitin 4-sulfate (15). Although no separation techniques were used that would distinguish between different sulfation patterns of the resulting tetrasaccharides, a tetrasaccharide was obtained which was resistant to chondroitinase C. When treated with chondroitinase AC, this yielded equal amounts of ΔDI-4S and ΔDI-6S, indicating that a true mixed tetrasccharide existed.

We have now verified these findings utilizing different methods with the readily available eliminase, chondroitinase ABC. Polysaccharidases of this type have no transglycosylase activity (13). The resulting mixed tetrasccharide has been completely separated from the 6,6-sulfated and 4,4-sulfated tetrasccharides, thus confirming mixed sulfation on the same chondroitin sulfate chain in shark or bovine tracheal cartilage, or both.

We have also found mixed sulfation in the chondroitin sulfate from chick embryo epiphysial cartilage. Approximately 40% of the tetrasccharides isolated consisted of one 4-sulfated disaccharide unit and one 6-sulfated disaccharide unit, while no more than 7% of the tetrasccharides could have contained two 4-sulfated disaccharide units. Thus, most of the 4-sulfated disaccharide units were adjacent to a 6-sulfated, rather than another 4-sulfated disaccharide unit. This indicates that the 4-sulfated disaccharides were scattered along the chains, rather than concentrated in one particular region and also demonstrates that little or no pure chondroitin 4-sulfate exists in this cartilage.

The mixed sulfation in the chondroitin sulfate from chick embryo epiphysial cartilage was not anticipated. Studies of sulfate incorporation into chondroitin sulfate using a microsomal preparation from this source were previously conducted with 3'-phosphoadenosine 5'-phosphosulfate as the sulfate donor (5, 6, 16, 17) When 3'-phosphoadenosine 5'-phospho[35S]sulfate was added to the microsomal preparation, 30 to 40% of the newly incorporated [35S]sulfate went into position 3 while 90 to 70% went into position 6 of endogenous chondroitin sulfate (6). However, when UDP-glucuronic acid and UDP-N-acetylgalactosamine were added to the microsomal preparation in order to synthesize new chondroitin on endogenous primer, sulfation of the newly formed chondroitin by 3'-phosphoadenosine 5'-phosphosulfate was exclusively in position 6 (6). Thus, even though the enzymes for 4-sulfation were present, no sulfation on position 4 of newly formed chondroitin occurred. This suggested that the endogenous primer might determine the sulfation pattern of the newly synthesized chondroitin, directing only 4 or 6 sulfation on an individual polysaccharide chain. For this reason, mixed sulfation was not expected. The high degree of interspersion of 4-sulfate among predominantly 6-sulfate in chick embryo epiphysial cartilage shown here indicates that a particular primer for chondroitin sulfate synthesis is probably not programmed to yield only one type of sulfation, as was previously thought. Our previous observation of only 6-sulfation on newly formed chondroitin (6) must be reinterpreted in light of this new information. Mixed 4 and 6 sulfation on the same chondroitin sulfate chain in chick embryo epiphysial cartilage must be considered in any proposed biosynthetic scheme for this system.

**REFERENCES**

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