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THE EFFECT OF PENULTIMATE *N*-ACETYLGALACTOSAMINE 4-SULFATE ON CHONDROITIN CHAIN ELONGATION*

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ABSTRACT

Previous work has shown that odd-numbered oligosaccharides containing nonreducing terminal, non-sulfated *N*-acetylgalactosamine (GalNAc) or 6-sulfated GalNAc are excellent acceptors for enzymic addition of glucuronic acid (GlcA). However, the presence of a 4-sulfated GalNAc group blocks further addition. We have now used even-numbered oligosaccharides (a mixture of 4-sulfated, 6-sulfated, and non-sulfated) as acceptors of [^{3}H]GalNAc to investigate the effect of sulfate residues on the GalNAc in the penultimate position. ^{3}H-Labeled oligosaccharides were partially degraded with chondroitin AC lyase. The labeled trisaccharides, consisting of the added [^{3}H]GalNAc and the nonreducing terminal disaccharides of the oligosaccharide acceptors, were then characterized. Both non-sulfated and mono-sulfated ^{3}H-trisaccharides were observed. However, the ^{3}H-trisaccharides were shown by chromatography with prepared standards to be non-sulfated or to be sulfated only at the 6-position. Thus the oligosaccharides containing a 4-sulfated, penultimate GalNAc at the nonreducing end did not serve as acceptors for [^{3}H]GalNAc. Microsomes from mastocytoma cells, which make only chondroitin 4-sulfate, exhibited the same substrate specificity for exogenous oligosaccharide acceptors as did microsomes from chick cartilage which makes chondroitin 6-sulfate.

INTRODUCTION

Odd-numbered oligosaccharides derived from non-sulfated or 6-sulfated chondroitin have been shown to act as excellent acceptors for glucuronic acid (GlcA) when incubated with UDP-GlcA and a microsomal enzyme preparation from chick embryo cartilage[1–3]. However, it was suggested that oligosaccharides containing a 4-sulfate on the nonreducing terminal *N*-acetylgalactosamine (GalNAc) would not serve as a substrate[4]. These conclusions were firmly established

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by use of chondroitin lyases, which yielded disaccharides containing a [^{14}C]GlcA (incorporated from UDP-[^{14}C]GlcA) plus the terminal GalNAc or GalNAc sulfate to which the [^{14}C]GlcA had been added[3]. Nonsulfated and 6-sulfated ^{14}C-disaccharides were found, but there were no 4-sulfated ^{14}C-disaccharides.

Similar experiments have also been conducted with microsomal preparations from neoplastic mast cells[5], which produce chondroitin 4-sulfate but not chondroitin 6-sulfate[6,7]. The substrate requirements were identical to those of the chick cartilage system; GlcA could not be added to a terminal GalNAc sulfated on the 4 position. Thus it is apparent that 4-sulfated GalNAc at the nonreducing terminal of chondroitin oligosaccharides completely blocks further chain polymerization.

Similarly, even-numbered oligosaccharides have been utilized to study incorporation of GalNAc from UDP-[^{3}H]GalNAc[1,3]. In contrast to the odd-numbered oligosaccharides, it was originally stated that oligosaccharides 4-sulfated on the penultimate GalNAc at the non-reducing end do act as acceptors[4]. More recently, however, it has been indicated that these oligosaccharides are poor acceptors[8], and that the slight activity found might be related to contamination with 6-sulfated oligosaccharides. In neither report was there a description of the use of chondroitinases or identification of the products.

We have now examined the addition of [^{3}H]GalNAc to even-numbered oligosaccharides. The substitution on the penultimate GalNAc was definitively established by isolation and identification of ^{3}H-trisaccharides from the nonreducing end, obtained by partial degradation with chondroitin lyase. Our results indicated that there was no addition of [^{3}H]GalNAc when the penultimate GalNAc at the nonreducing end of the oligosaccharide acceptor was sulfated in the 4-position. Identical results were obtained with both a chick embryo microsomal system and a mast cell microsomal system.

EXPERIMENTAL

Materials. — UDP-[^{3}H]GalNAc was purchased from New England Nuclear (Boston, MA). Chondroitin sulfate A (from shark), chondroitin sulfate C (from whale), bovine testicular hyaluronidase, and bovine liver β-glucuronidase were purchased from Sigma Chemical Co. (St. Louis, MO). Chondroitin ABC lyase, chondroitin AC lyase, chondro-4-sulfatase, chondro-6-sulfatase, and various disaccharides (ΔDi-OS, ΔDi-4S, and ΔDi-6S*) produced by degradation with chondroitin lyases were purchased from Miles Laboratories, Inc. (Naperville, IL). Mixed tetrasaccharides from chondroitin sulfate were prepared by testicular

*The abbreviations used are: ΔDi-OS, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose; and Tri-4S, α-(2-acetamido-2-deoxy-β-D-galactopyranosyl)-(1→4)-*O*-(β-D-glucopyranosyluronic acid)-(1→3)-2-acetamido-2-deoxy-D-galactose-4-sulfate.

hyaluronidase digestion, followed by gel filtration[4] on Sephadex G-25. Hexasaccharides were prepared similarly. The oligosaccharides from whale cartilage were found to contain approximately 75–80% 4-sulfated, 15–20% 6-sulfated, and 2–5% non-sulfated residues. The oligosaccharides from shark cartilage were found to contain approximately 10–15% 4-sulfated, 80–85% 6-sulfated, and 2–5% non-sulfated residues.

Chick embryos (14 day) were purchased from SPAFAS Inc. (Norwich, CT). Twice-washed microsomal preparations sedimenting between 10,000 and 105,000*g* were prepared as previously described from chick embryo epiphyses[9], and from cultured P815Y mastocytoma cells[7].

Methods. — Use of chondroitin AC lyase, chondroitin ABC lyase, chondro-4-sulfatase, and chondro-6-sulfatase in enriched Tris buffer followed by paper chromatography (Whatman 1) of products with 2:3:1 butanol–acetic acid–M ammonium hydroxide was according to standard techniques[10].

Standards of [^{3}H]trisaccharides having a single 4-sulfate were prepared from [^{3}H]chondroitin 4-sulfate formed by cultures of P815Y mastocytoma cells as follows: Cells were cultured with [^{3}H]glucosamine in sulfate-depleted medium (Fischer's with 0.1mM magnesium sulfate and no serum) in order to produce incompletely sulfated glycosaminoglycans. Under these conditions, the mast cells synthesized [^{3}H]chondroitin 4-sulfate having ~30% non-sulfated [^{3}H]GalNAc residues. The mast cells were treated with 0.5M sodium hydroxide at room temperature overnight and then the base was neutralized with glacial acetic acid. After centrifugation at 15,000*g* for 5 min, the supernatant solution containing the [^{3}H]chondroitin sulfate was eluted from a column (1.8 × 46 cm) of Sephadex G-50 with 0.5M ammonium hydrogencarbonate. The excluded peak was pooled, lyophilized, and treated with testicular hyaluronidase. Following chromatography on a column (1.5 × 168 cm) of Sephadex G-25, the tetrasaccharide peak consisting of a mixture of mono- and di-sulfated tetrasaccharide was recovered, treated with β-glucuronidase[4], and rechromatographed on the same column. The resulting ^{3}H-trisaccharide peak was pooled, lyophilized, and chromatographed on a column (0.7 × 13 cm) of DEAE-cellulose to separate monosulfated ^{3}H-trisaccharides from disulfated ^{3}H-trisaccharides. A logarithmic gradient of 0–0.2M lithium chloride in 0.01M sodium acetate, pH 5.5 was used. The monosulfated ^{3}H-trisaccharide peak was desalted on Sephadex G-10, and then chromatographed on Whatman 1 paper in 2:3:1 butanol–acetic acid–M ammonium hydroxide, yielding two spots. Upon further treatment with chondroitin AC lyase, one yielded [^{3}H]GalNAc 4-sulfate plus [^{3}H]ΔDi-OS and the other yielded non-sulfated [^{3}H]GalNAc plus [^{3}H]ΔDi-4S identified by paper chromatography as before with appropriate standards and by use of appropriate chondrosulfatases. This latter trisaccharide (Tri-4S*), constituting trisaccharide 4-monosulfate with non-sulfated GalNAc at the nonreducing end, was utilized as a standard for identification. It was found to co-chromatograph with ΔDi-4S in the foregoing system.

Incubations of UDP-[^{3}H]GalNAc together with microsomal preparations

were carried out in the presence and absence of tetrasaccharides containing various mixtures of non-sulfated, 4-sulfated, and 6-sulfated residues. Reaction mixtures consisted of 0.4mM UDP-[^{3}H]GalNAc (1.07 Ci/mmol), 1.8mM tetrasaccharide, 0.05M 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6.5), 0.015M $MnCl_2$, and 5 μL of microsomes. These were incubated in total reaction volumes of 25 μL for 3.5 h at 37°. Following centrifugation at 15,000*g* for 5 min, supernatant solutions were chromatographed on Sephadex G-25 (0.7 × 68 cm) to separate the resulting ^{3}H-pentasaccharides from the remaining UDP-[^{3}H]GalNAc and degradation products. Incubation mixtures containing microsomes from mast cells or chick cartilage were equally effective in promoting incorporation of [^{3}H]GalNAc. Tetrasaccharide mixtures from whale cartilage (mostly 4-sulfated) and shark cartilage (mostly 6-sulfated) were equally effective (~3%) as acceptors. Hexasaccharides were also used as acceptors, but for ease of product identification, tetrasaccharide substrates were used for further experiments.

The carbazole method of Bitter and Muir[11] was used to assay uronic acid-containing materials. Radioactivity was determined with a liquid-scintillation spectrometer, using Aquassure liquid scintillant (New England Nuclear, Boston, MA). Disaccharide standards on the paper chromatograms were located with an ultraviolet lamp.

RESULTS AND DISCUSSION

The ^{3}H-pentasaccharide products obtained as described in the Experimental section were degraded partially with 0.05 units of chondroitin AC lyase, and 25 μg each of chondroitin sulfate A and chondroitin sulfate C as carrier. Reactions were stopped by heating for 5 min at 100° when they had reached ~40% completion as monitored by the appearance of A_{232}. This took 25 min under the foregoing con-

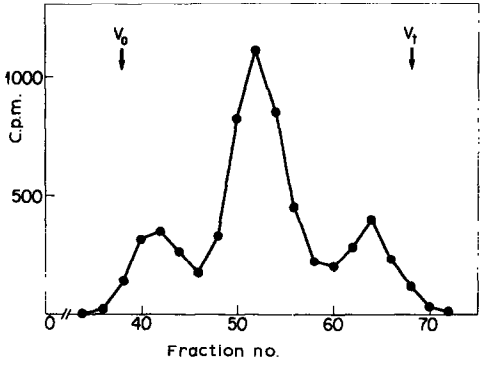

Fig. 1. Sephadex G-25 chromatogram of partially degraded pentasaccharides. ^{3}H-Pentasaccharides were degraded partially with chondroitin AC lyase, applied to a Sephadex G-25 column (0.7 × 68 cm), and eluted with 0.5M ammonium hydrogencarbonate. Fractions of 0.2 mL were collected. An acetylchondrosine standard was found with a peak at fraction 58.

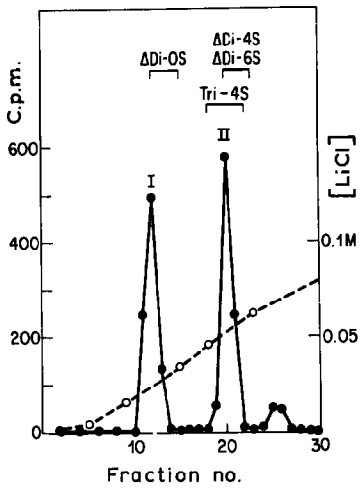

Fig.2. DEAE-cellulose chromatogram of trisaccharides. ^{3}H-Trisaccharides recovered after partial chondroitin AC lyase degradation of ^{3}H-pentasaccharide were applied to a DEAE-cellulose column, (0.7 × 13 cm) and eluted with a 0–0.2M logarithmic gradient of LiCl, buffered with 0.01M sodium acetate, pH 5.5. Fractions of 1 mL were collected. The elution positions of a Tri-4S standard and disaccharide standards are indicated.

ditions. The resulting mixtures of ^{3}H-monosaccharide, ^{3}H-trisaccharides, and ^{3}H-pentasaccharides were chromatographed on Sephadex G-25 (Fig. 1). The ^{3}H-trisaccharide peak (fractions 47–58) was isolated and chromatographed on DEAE-cellulose as described in the Experimental section. Two major peaks with approximately equal amounts were observed (Fig. 2), the first corresponding to non-sulfated trisaccharide and the second corresponding to mono-sulfated trisaccharides. This indicated that there had been addition of [^{3}H]GalNAc onto oligosaccharides containing non-sulfated penultimate GalNAc at the nonreducing end and onto oligosaccharides containing sulfated penultimate GalNAc respectively.

The ^{3}H-trisaccharides were chromatographed on Whatman 1 paper alongside standards of ΔDi-6S, ΔDi-4S, ΔDi-OS, and [^{3}H]Tri-4S (prepared as described in the Experimental section). The non-sulfated ^{3}H-trisaccharide migrated near ΔDi-OS (Fig. 3,I), while all of the sulfated ^{3}H-trisaccharide migrated near the standard ΔDi-6S (Fig. 3,II). No ^{3}H-trisaccharide appeared in the position of the prepared standard of [^{3}H]Tri-4S.

These results demonstrate that there was no addition of [^{3}H]GalNAc onto an oligosaccharide that contained 4-sulfate on the penultimate GalNAc at the non-reducing end. Therefore, in order for polymerization of chondroitin 4-sulfate to proceed, there must be a lag of sulfation that includes pre-terminal GalNAc as well as the terminal GalNAc as previously described. These results are contrary to previous statements[4] that tetrasaccharide or hexasaccharide with 4-sulfated penultimate GalNAc will serve as an acceptor for the addition of GalNAc. As has recently been suggested[8], it is likely that small amounts of non-sulfated or 6-sulfated contaminants were the acceptors in this previous work.

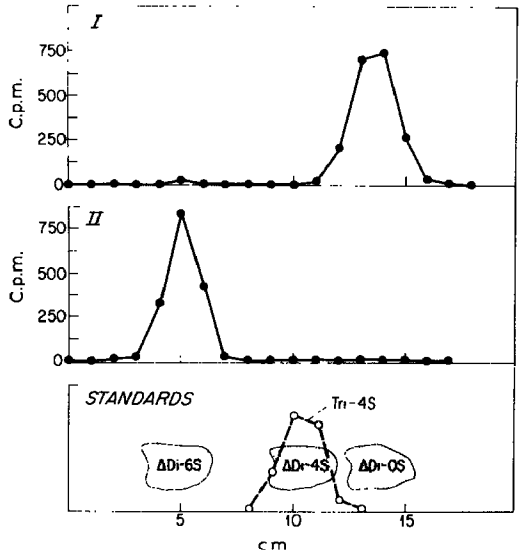

Fig. 3. Paper chromatogram of trisaccharides. Peaks I and II from the DEAE chromatogram (Fig. 2) were desalted, spotted on Whatman 1 paper, and eluted with 2:3:1 butanol–acetic acid–M ammonium hydroxide. The migrations of the Tri-4S standard and disaccharide standards are indicated.

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