Biosynthesis of Chondroitin Sulfate

CHAIN TERMINATION*

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Incubation of chick embryo epiphyses microsomal preparations with either UDP-\(^{14}C\)GlcUA or UDP-\(^{14}C\)-GalNAc plus exogenous chondroitin 6-sulfate resulted in the incorporation of either a single \(^{14}C\)GlcUA or a \(^{14}C\)GalNAc onto the nonreducing ends of the exogenous glycosaminoglycan. Degradation by chondroitinase ABC yielded the terminal products \(^{14}C\)Di-6S, \(^{14}C\)Di-8S, and \(^{14}C\)GalNAc. Incubations of the microsomal preparations with either UDP-\(^{14}C\)GlcUA or UDP-GalN[H]Ac without exogenous chondroitin 6-sulfate resulted in the addition of a single sugar onto the nonreducing end of endogenous chondroitin sulfate. Degradation by chondroitinase ABC yielded the terminal products \(^{14}C\)Di-6S, \(^{14}C\)Di-8S, and GalN[H]Ac in a molar ratio of approximately 1:1:3.5. Incubations of the microsomal preparations with both UDP-\(^{14}C\)-GlcUA and UDP-GalN[H]Ac together resulted in formation of \(^{14}C\)Hchondroitin chains added to the endogenous chondroitin sulfate. Degradation by chondroitinase ABC resulted in products with a molar ratio of \(^{14}C\)HDi-6S to GalN[H]Ac varying from approximately 1:1.5 to 1:3.

The results of these experiments indicate that chondroitin 6-sulfate terminates at its nonreducing end in a mixture of GlcUA and GalNAc (some sulfated). GalNAc is somewhat more frequent as the terminal sugar and adds more readily to endogenous acceptors.

Despite the attention that has been given to the elongation of the glycosaminoglycan portion of chondroitin sulfate, the nonreducing ends of endogenous or newly formed chondroitin have not been investigated in detail. For this reason the nature of glycosaminoglycan chain termination is not known. Single sugars have been added to endogenous chondroitin sulfate by utilizing either UDP-glucuronic acid or UDP-N-acetylglalactosamine with the microsomal enzyme from chick embryo epiphyses (4). This implies that at least some of the endogenous chondroitin sulfate glycosaminoglycan terminates in glucuronic acid and some in N-acetylgalactosamine or N-acetylgalactosamine sulfate at its nonreducing end. However, this does not necessarily reflect the relative proportions of these end groups in the microsomal preparation, since only those molecules that are accepting sugars would be seen in this method.

Newly synthesized chondroitin glycosaminoglycan, endogenous chondroitin sulfate, and exogenous chondroitin sulfate were therefore examined in detail in order to characterize chain termination. In all cases the chains were shown to be a mixture having nonreducing ends of glucuronic acid and N-acetylgalactosamine or N-acetylgalactosamine 6-sulfate.

**EXPERIMENTAL PROCEDURES**

* The abbreviations used are: ADi-OS, 2-acetamido-2-deoxy-3-O-(\(\beta\)-D-glucopyranosyluronic acid)-n-galactose; ADi-4S, 2-acetamido-2-deoxy-3-O-(\(\beta\)-D-glucopyranosyluronic acid)-4-O-sulfono-D-galactose; ADi-8S, 2-acetamido-2-deoxy-3-O-(\(\beta\)-D-glucopyranosyluronic acid)-6-O-sulfono-D-galactose; Di-4S, 2-acetamido-2-deoxy-3-O-(\(\beta\)-D-glucopyranosyluronic acid)-4-O-sulfo-D-galactose; Di-6S, 2-acetamido-2-deoxy-3-O-(\(\beta\)-D-glucopyranosyluronic acid)-6-O-sulfo-D-galactose; N-acetylcchondrosine (Di-8S) was prepared by acetylation of chondrosine (Miles Laboratory, Inc.) with acetic anhydride and then isolated after degradation of chondroitin ABC, Hexaasaccharide, pentasaccharide, and tetrasaccharide from chondroitin 6-sulfate or chondroitin 4-sulfate were prepared as previously described (6, 7). N-Acetylcchondrosine 6-sulfate was prepared by isolation of the disaccharide product after degradation of chondroitin ABC.

Materials—UDP-\(^{14}C\)Glucuronic acid and UDP-N-acetyl\(^{14}C\)Glucosamine were purchased from New England Nuclear. UDP-N[H]acetylgalactosamine and nonradioactive UDP-N-acetylgalactosamine were synthesized as described previously (1). UDP-glucuronic acid was purchased from Sigma Chemical Co. Chondroitinase ABC, chondroitin 6-sulfate, chondroitin 4-sulfate, and various disaccharides produced by degradation with chondroitinase ABC (Di-4S, ADi-4S, ADi-8S) were purchased from Miles Laboratory, Inc. The chondroitin 6-sulfate has approximately 10% chondroitin 4-sulfate. Chondroitin was prepared by repeated treatment of chondroitin 4-sulfate with acetic chloride in methanol (8).

Degradation of the resulting chondroitin by chondroitinase ABC yielded Di-8S as the only disaccharide. N-Acetylcchondrosine (Di-8S) was prepared by acetylation of chondrosine (Miles Laboratory, Inc.) with acetic anhydride and also by isolation after degradation of chondroitin tetrasaccharide with chondroitinase ABC. Hexaasaccharide, pentasaccharide, and tetrasaccharide from chondroitin 6-sulfate or chondroitin 4-sulfate were prepared as previously described (6, 7). N-Acetylcchondrosine 6-sulfate was prepared by isolation of the disaccharide product after degradation of chondroitin ABC.
4-sulfate tetrasaccharide and chondroitin 6-sulfate tetrasaccharide by chondroitinase ABC. Structure of the Di-4S and Di-6S were confirmed by their susceptibility to degradation by chondroitinase ABC and chondroitinase 6-B (Miles).

Frozen 14-day chick embryos were purchased from Pel-Freez Biologicals. Twice washed microsomal preparations sedimenting between 10,000 x g and 105,000 x g were prepared from chick embryo epiphyses as previously described (1, 9). Epiphysial cartilage from 200 embryos yielded a microsomal pellet of 0.5 ml which was suspended in an additional 0.5 ml of 0.25M sucrose.

**Methods**—Typical incubations with microsomal preparations contained 0.5 m Mes (pH 6.5), 0.01 m MnCl2, 0.0006 m UDP-['4C]glucuronic acid, or 0.0007 m UDP-N-acetyl['%]galactosamine, or both, or 0.0007 m UDP-N-acetyl['%]galactosamine with or without oligosaccharides, chondroitin or chondroitin 6-sulfate, plus 0.001 to 0.01 ml of microsomal enzyme in a total volume of 0.025 ml. Incubations were conducted for several hours or longer at 37°C. Different combinations and amounts of the above materials were utilized as described later. Following the incubations, labeled products were isolated by paper chromatography. Total reaction mixtures were spotted on Whatman No. 1 paper and chromatographed with ethanol, 1 m ammonium acetate (pH 7.8) (5:2), as previously described (1, 9). In this system polysaccharides remain at the origin while sugar nucleotides and their degradation products move varying degrees down the paper completely separated from the material at the origin. After chromatography, origins were washed with water which removes exogenous labeled glycosaminoglycans or oligosaccharides while endogenous labeled glycosaminoglycans linked to protein remain on the paper (1, 6). Radioactive endogenous glycosaminoglycans were then quantitatively eluted from the origins with 0.5 m NaOH or with pancreatin (1, 9).

Aliquots of the labeled glycosaminoglycans were chromatographed on a column of DEAE-cellulose (1 x 5 cm) with a logarithmic gradient of LiCl (10). Gel filtration was performed on a column (2.7 x 120 cm) of Sephadex G-25 with 0.1 m LiCl as eluant. Labeled glycosaminoglycans were degraded with chondroitinase ABC (11), and reaction mixtures chromatographed on Whatman No. 1 paper in butanol, acetic acid, 1 m NH4OH (2:3:1) together with disaccharide standards. Isolated products were incubated with chondro-4-sulfatase and chondro-6-sulfatase (11) and chromatographed in the same system for further identification.

Uronic acid-containing material was assayed by the modified carbazole method of Bitter and Muir (12). Radioactivity was determined with a Lowbeta II (Beckman) low background planchette counter or with a Beckman liquid scintillation spectrometer.

**RESULTS**

**Nonreducing Terminal Sugars of Exogenous Chondroitin and Chondroitin Sulfate**—Microsomal preparations were incubated together with UDP-['4C]glucuronic acid plus exogenous pentasaccharide, or chondroitin, or chondroitin 6-sulfate in order to incorporate ['4C]glucuronic acid into exogenous acceptors. Separately N-acetyl['4C]galactosamine was incorporated into exogenous hexasaccharide or chondroitin, or chondroitin 6-sulfate by incubation together with microsomal preparations and UDP-N-acetyl['4C]galactosamine. Concentrations of the chondroitin or chondroitin sulfate were chosen so that the molarity of nonreducing ends was comparable to the molarity of the pentasaccharide and hexasaccharide. Results are shown in Table I.

As was previously shown (3, 6, 7), ['4C]glucuronic acid or N-acetyl['4C]galactosamine was readily incorporated into appropriate oligosaccharide acceptors. Incorporation into glycosaminoglycans was less than 6% of the incorporation into oligosaccharides. Chondroitin 6-sulfate was a better acceptor than chondroitin, and both were capable of accepting slightly more ['4C]glucuronic acid than N-acetyl['4C]galactosamine. After isolation of the labeled chondroitin 6-sulfate or chondroitin, samples were separately chromatographed on Sepharose 6B. Results are shown in Fig. 1, A and B. Incorporation of ['4C]glucuronic acid or N-acetyl['4C]galactosamine was selectively into smaller species of the chondroitin or chondroitin 6-sulfate. Thus, the chondroitin and the commercial preparation of chondroitin 6-sulfate contained acceptor molecules that ended in glucuronic acid and in N-acetylgalactosamine or N-acetylgalactosamine 6-sulfate.

The samples from the Sepharose 6B column of chondroitin 6-sulfate (Fig. 1B, Fractions 25 to 60) were pooled, dialyzed against water, lyophilized, and subjected to degradation with chondroitinase ABC.

Use of chondroitinase ABC provides a characterization of the nonreducing end of an acceptor molecule (6). Thus the addition of ['4C]glucuronic acid to chondroitin sulfate termi-
nating in N-acetylgalactosamine would yield N-acetyl[14C]-
chondrosine (Di-OS) upon degradation with chondroitinase
ABC. Addition of [14C]glucuronic acid to chondroitin sulfate
terminating in N-acetylgalactosamine 4-sulfate or 6-sulfate
would yield N-acetyl[14C]chondrosine 4-sulfate (Di-4S) or N-
acetyl[14C]chondrosine 6-sulfate (Di-6S), respectively. The addi-
tion of N-acetyl[14C]galactosamine to chondroitin sulfate
terminating in glucuronic acid would yield free N-acetyl-
[14C]galactosamine upon degradation with chondroitinase
ABC.

Paper chromatography of the chondroitinase degradation
products is shown in Fig. 2. All of the N-acetyl[14C]galacto-
amine-labeled material was degraded to free N-acetyl[14C]-
galactosamine; approximately 90% of the [14C]glucuronic acid-
labeled products chromatographed in the area of Di-6S while
approximately 10% chromatographed in the area of Di-4S. Thus,
the chondroitin 6-sulfate contained acceptor glycosa-
minglysans ending in glucuronic acid (capable of accepting
N-acetylgalactosamine) and in N-acetylgalactosamine or N-
acetylgalactosamine 6-sulfate (capable of accepting glucuronic acid).
Although there is chondroitin 4-sulfate (approximately
10%) in the commercial chondroitin sulfate, there was no
Di-4S found, consistent with previous reports that oligosac-
charides ending in N-acetylgalactosamine 4-sulfate will not
accept a glucuronic acid (7). Alternatively, the possibility that
all the chondroitin 4-sulfate terminates in glucuronic acid
cannot be excluded.

Nonreducing Terminal Sugars of Endogenous Chondro-
tin Sulfate—Microsomal preparations were incubated with
UDP-[14C]glucuronic acid (240 × 10⁶ cpm/µmol) in order to
incorporate [14C]glucuronic acid into endogenous acceptors.
Separately N-[3H]acetylgalactosamine was incorporated into
different acceptors by incorporation of microsomal prepara-
tions with UDP-N-[3H]acetylgalactosamine (800 × 10⁶
µmol/µmol). Total incorporation into glycosaminoglycans after
an 18-h incubation with 0.020 ml of microsomal preparation
was 0.04 nmol of [14C]glucuronic acid and 0.07 nmol of N-
[3H]acetylgalactosamine. After isolation of the [14C]glycosa-
minglycan and the [3H]glycosaminoglycan, these were com-
bined and chromatographed on a column of DEAE-cellulose.
Results are shown in Fig. 3. Fractions 60 to 68, representing
the addition of a single sugar residue to endogenous chondro-
itin sulfate (4, 5), were pooled, dialyzed against water, lyoph-
ilized, and aliquots degraded with chondroitinase ABC.

A chromatogram of the chondroitinase degradation prod-
ucts is shown in Fig. 4. All of the incorporated [3H]acetylga-
lactosamine chromatographed as free N-[3H]acetylgalacto-
amine after chondroitinase degradation, while [14C]glucuronic
acid incorporation resulted in degradation products evenly
distributed between [14C]Di-6S and [14C]Di-4S. Thus, some of
the endogenous chondroitin sulfate has glucuronic acid at its
nonreducing end and can accept N-[3H]acetylgalactosamine,
while some endogenous chondroitin sulfate ends in N-acetyl-
galactosamine or N-acetylgalactosamine 6-sulfate and can
accept [14C]glucuronic acid. Based on specific activity and the
number of counts incorporated, the molar ratio of endogenous
acceptor chondroitin sulfate ending in N-acetylgalacto-
amine:N-acetylgalactosamine 6-sulfate:glucuronic acid was
1:13.5. No acceptor chondroitin sulfate ended in N-acetyl-
galactosaminoglycan.
Nonreducing Terminal Sugars of Newly Synthesized Chondroitin—Microsomal preparations were incubated together with UDP-[14C]glucuronic acid (240 x 10^6 cpm/µmol) plus UDP-N-[3H]acetylglucosamine (800 x 10^6 cpm/µmol) together in the same incubation mixture. Total formation of [14C,3H]glycosaminoglycan after an 18-h incubation with 0.020 ml of microsomal preparation represented 0.83 nmol of [14C]glucuronic acid and 0.83 nmol of N-[3H]acetylglucosamine. After isolation of the resulting [14C,3H]glycosaminoglycan, a sample was chromatographed on DEAE-cellulose (Fig. 5). As described in previous work (1, 5), [14C]glucuronic acid-labeled and N-[3H]acetylglucosamine-labeled glycosaminoglycan co-chromatographed throughout the DEAE-cellulose chromatogram. Fractions from this column were pooled (Table II) into Peak I, II, IIA, IID, dialyzed against water, and lyophilized. Aliquots of these peaks were subjected to degradation with chondroitinase ABC and products chromatographed on Whatman No. 1 paper. Results for Peak IIC are shown in Fig. 6. Free N-[3H]acetylglucosamine was determined directly from this chromatogram. However, the [14C,3H]Di-OS peak representing nonreducing ends was partly hidden in the large amount of [14C,3H]ADi-OS that resulted from the chondroitinase ABC degradation products of newly formed [14C,3H]chondroitin. An aliquot of newly formed [14C,3H]chondroitin sulfate was chromatographed on DEAE-cellulose (Fig. 5). As described in previous work (1, 5), [14C]glucuronic acid-containing products (M) and N-[3H]acetylgalactosamine as acceptors. As shown in Fig. 1, A and B, the larger glycosaminoglycans are much poorer acceptors than the smaller glycosaminoglycans. Glycosaminoglycans of all sizes are in turn poorer acceptors than the oligosaccharides (Table I). This could be due to the polymerizing enzymes having decreased affinity for acceptor substrate when the chains get beyond a certain size, or it could be due to the nature of chondroitin chain termination has been subject to considerable speculation. We and others have shown that chondroitin sulfate glycosaminoglycans from chick embryo epiphyseal cartilage microsomal preparations and from matrix have a considerable degree of size heterogeneity (4, 13, 14). Thus it would appear that the size for chain termination in this tissue is not rigidly controlled. What has not been clear is why the chains stop growing or whether they end with a specific sugar.

Previous experiments have indicated that up to heptasaccharide the larger oligosaccharides are better acceptors than the smaller oligosaccharides (7). These findings have now been extended to include the use of chondroitin and chondroitin sulfate as acceptors. As shown in Fig. 1, A and B, the larger glycosaminoglycans are much poorer acceptors than the smaller glycosaminoglycans. Glycosaminoglycans of all sizes are in turn poorer acceptors than oligosaccharides (Table I). This could be due to the polymerizing enzymes having decreased affinity for acceptor substrate when the chains get beyond a certain size, or it could be due to the nature of chondroitin chain termination has been subject to considerable speculation. We and others have shown that chondroitin sulfate glycosaminoglycans from chick embryo epiphyseal cartilage microsomal preparations and from matrix have a considerable degree of size heterogeneity (4, 13, 14). Thus it would appear that the size for chain termination in this tissue is not rigidly controlled. What has not been clear is why the chains stop growing or whether they end with a specific sugar.

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DISCUSSION

The nature of chondroitin chain termination has been subject to considerable speculation. We and others have shown that chondroitin sulfate glycosaminoglycans from chick embryo epiphyseal cartilage microsomal preparations and from matrix have a considerable degree of size heterogeneity (4, 13, 14). Thus it would appear that the size for chain termination in this tissue is not rigidly controlled. What has not been clear is why the chains stop growing or whether they end with a specific sugar.

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![Fig. 5. DEAE-cellulose chromatography of newly synthesized [14C,3H]chondroitin. Aliquots of newly synthesized chondroitin consisting of [14C]glucuronic acid (—) and N-[3H]acetylglucosamine (—–) were chromatographed on a column of DEAE-cellulose (1 x 5 cm) with standards of hyaluronic acid and chondroitin 4-sulfate (— —). A logarithmic gradient of LiCl was utilized for elution; 100 ml of 0.1 M LiCl was initially in the constant volume mixing flask and 1 M LiCl was in the reservoir flask. Fractions of 2 ml were collected and assayed for radioactivity and for the glycosaminoglycan standards.](image1)

![Fig. 6. Chondroitinase ABC degradation products of newly formed [14C,3H]chondroitin. An aliquot of newly formed [14C,3H]chondroitin from Fractions 61 to 64 of Fig. 5 was degraded with chondroitinase ABC, chromatographed, and assayed as newly formed in Fig. 2. [14C]Glucuronic acid-containing products (●) and N-[3H]acetylgalactosamine-containing products (— —) are shown.](image2)
architecture of the microsomal membranes relative to the large proteoglycan or glycosaminoglycan substrate. In this second case, larger exogenous molecules might have more difficulty getting to the site of the enzyme. These two possibilities have not been resolved, since the polymerizing enzymes have not been solubilized in such a fashion as to permit the study of chondroitin chain polymerization separate from the microsomal system.

Substantial chain elongation on endogenous chondroitin sulfate has previously been shown with these microsomal preparations, indicating that when chondroitin sulfate chains of considerable size are part of the microsomal preparation they can act as highly effective acceptors (5). These endogenous large acceptors have been shown to compete favorably with exogenous oligosaccharide acceptors, since very high concentrations of oligosaccharide were required to inhibit elongation of chondroitin on endogenous chondroitin sulfate (6). This suggests that the architectural arrangement of enzyme and proteoglycan acceptors may be of considerable importance. It is possible that the disruption occurring in preparation of the microsomal enzyme distorts the membrane architecture to permit further chain extension.

The sugars at the nonreducing end of the chondroitin sulfate have also been a subject for speculation. It is clear that either glucuronic acid or N-acetylgalactosamine can be added to appropriate oligosaccharide acceptors (3, 6, 7). Furthermore, it has been shown recently that there is no coupled addition of sugars and therefore no disaccharide intermediate and no necessary interaction between the glucuronyltransferase and the N-acetylgalactosaminyltransferase at the time of addition of glucuronic acid and N-acetylgalactosamine (6). Thus the chain termination could be due to the relative activities of each enzyme at the chain end, or could be due to some modification of the terminal sugar to prevent further elongation. This latter has been suggested as a mechanism, since oligosaccharides ending in N-acetylgalactosamine 4-sulfate are not capable of accepting a glucuronic acid (7), so that 4-sulfation of a terminal N-acetylgalactosamine would stop the polymerization. Although this could be a mechanism for termination of chondroitin 4-sulfate, it could not serve the same function for chondroitin 6-sulfate since an oligosaccharide with a terminal nonreducing N-acetylgalactosamine 6-sulfate accepts glucuronic acid readily (3, 6, 7).

We have now shown that exogenous chondroitin or chondroitin 6-sulfate is capable of accepting glucuronic acid and N-acetylgalactosamine (Figs. 1 and 2). We have also shown that endogenous chondroitin sulfate similarly can accept both glucuronic acid and N-acetylgalactosamine (Fig. 3 and 4). This means that some of the exogenous and endogenous chondroitin sulfate chains end in glucuronic acid, some in N-acetylgalactosamine, and some in N-acetylgalactosamine 6-sulfate. The relative proportions of these termini in the entire exogenous or endogenous sample cannot be determined however, since only the small number of molecules accepting the additional sugar are seen.

Newly synthesized chondroitin gives direct information regarding the proportion of chains ending in each of the sugars (Figs. 5 and 6; Table II). This indicates that the chondroitin consists of a mixture of chains with some terminating in glucuronic acid and 1.5 to 3 times as many terminating in N-acetylgalactosamine. These results are fairly close to those found with endogenous chondroitin sulfate.

From these data, chain termination of chondroitin sulfate would appear to be controlled by a mixture of architectural considerations and relative activities of glucuronyltransferase and N-acetylgalactosaminyltransferase resulting in a mixture of sizes and a mixture of nonreducing ends.

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