Biosynthesis of Chondroitin Sulfate

INDEPENDENT ADDITION OF GLUCURONIC ACID AND N-ACETYLGALACTOSAMINE TO OLIGOSACCHARIDES*

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Pentasaccharide 6-sulfate and hexasaccharide 6-sulfate were prepared from chondroitin 6-sulfate. Each oligosaccharide was incubated with a chick cartilage microsomal enzyme preparation and UDP-[1$^4$C]glucuronic acid and/or UDP-N-[3H]acetylgalactosamine. As previously reported by other investigators, a single sugar was added from UDP-[1$^4$C]glucuronic acid to the nonreducing end of pentasaccharide 6-sulfate and from UDP-N-[3H]acetylgalactosamine to the nonreducing end of hexasaccharide 6-sulfate. The labeled oligosaccharides were characterized by gel chromatography and degradation by chondroitinase ABC followed by identification of products. The oligosaccharides in concentrations above their $K_m$ inhibited chondroitin synthesis on endogenous primers, reinforcing the assumption that the enzymes involved in the additions to exogenous oligosaccharides are the same as those involved in chondroitin polymerization.

When either the pentasaccharide 6-sulfate or hexasaccharide 6-sulfate was incubated in reaction mixtures containing both of the sugar nucleotides there was generally growth of oligosaccharide by two or three sugars. With longer incubation under conditions of limiting oligosaccharide concentration, as many as 14 to 16 sugars could be added but no further chondroitin polymerization took place. Addition of each sugar was shown to depend upon the concentration of appropriate acceptor but was otherwise independent of the addition of the alternate sugar. No paired addition of sugars was noted. It was concluded that two specific enzymes are involved in alternate additions of sugars to the oligosaccharides and that the two enzymes have no apparent interaction with one another. It is suggested that the rapid polymerization to form large chondroitin chains which previously has been shown to take place on endogenous primers is facilitated by interaction of the two enzymes with a component of the endogenous primer. This component is not present in the exogenous oligosaccharides since they do not serve in the same fashion as primers for polymerization.

It is now well established that the sugar nucleotides UDP-glucuronic acid and UDP-N-acetylgalactosamine are involved in the synthesis of the polysaccharide (glycosaminoglycan) portion of chondroitin sulfate proteoglycan (1–3). A microsomal enzyme preparation from chick embryo epiphyses has been used in these studies. Present evidence indicates that the polymerization of chondroitin takes place with microsomal enzymes on previously formed microsomal proteoglycan primer to yield a growth of glycosaminoglycan chains of substantial size (10 to 40 disaccharide units) (3). After a 2-min incubation these chains grow to substantial size so that further increase in incorporation over several hours chiefly represents incorporation onto additional chondroitin primer rather than a time-related elongation on all the primer. The sugar additions are to the nonreducing ends of glycosaminoglycan primes of varying length which have been shown to be attached to protein in the microsomal preparation by alkali-labile bonds. Thus the structure of this intracellular proteoglycan primer appears to be similar in these respects to the structure of chondroitin sulfate proteoglycan found in extracellular matrix. The primers will also act as acceptors of single sugars (N-acetylgalactosamine or glucuronic acid) from their respective sugar nucleotides when a single sugar nucleotide is incubated with the microsomal enzyme and primer system (4).

Soluble chondroitin sulfate-derived oligosaccharides have also been used as acceptors for both N-acetylgalactosamine and glucuronic acid (5, 6) with the chick cartilage enzyme system. A single glucuronic acid can be transferred from UDP-glucuronic acid to an N-acetylgalactosamine residue at
the nonreducing end of an odd numbered oligosaccharide, and a single N-acetylgalactosamine can similarly be transferred from UDP-N-acetylgalactosamine to the glucuronic acid at the nonreducing end of an even numbered oligosaccharide. It has been presumed that the microsomal enzymes involved in addition to the oligosaccharides are the same enzymes as those involved in polymerization but this has not been examined. Investigation of polymerization (as opposed to the addition of a single sugar) with the oligosaccharides as primers has not been described. N-Acetylgalactosaminyl- and glucuronyltransferase activities have not been separated from one another suggesting that (a) a single enzyme with two enzymatic sites might be involved, (b) that two interrelated enzymes might be bound together to promote placement of the two repeating sugars in a paired fashion; (c) alternatively it is possible that there are two enzymes that act independently of one another and that the incorporation is solely dependent upon the relative amounts of each enzyme, each sugar nucleotide, and suitable primer ending in the appropriate sugar.

The above three possibilities have been examined with the use of the chick cartilage microsomal enzyme and primer system, chondroitin sulfate-derived oligosaccharides, and radiologically labeled sugar nucleotides.

**EXPERIMENTAL PROCEDURE**

*Materials—UDP-["C"]glucuronic acid was 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. Chondroitin 6-sulfate and various disaccharides produced by degradation with bacterial enzyme (ΔDi-6S, ΔDi-4S, ΔDi-6S) were purchased from Miles Laboratories, Inc. Tetra and hexasaccharides from chondroitin 6-sulfate were prepared by degradation with testicular hyaluronidase followed by chromatography on Sephadex G-25 (6) and DEAE-cellulose. N-Acetyllactosamine was prepared by acetylation of chondrose (Miles Laboratories, Inc.) with acetic anhydride and by isolation after degradation of chondroitin tetrasaccharide with chondroitinase ABC (Miles Laboratories, Inc.). Pentasaccharide 6-sulfate was prepared by treatment of the hexasaccharide 6-sulfate with β-glucuronidase and separation of the products on Sephadex G-25 (6). Chondroitinase ABC, chondro-4-sulfatase, and chondro-6-sulfatase were purchased from Miles Laboratories, Inc.; testicular hyaluronidase was purchased from Sigma. Glucuronyltransferase was prepared from rat liver (7).

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, 8). Epiphysial cartilage from 200 embryos yielded approximately 0.5 ml of a microsomal preparation which was suspended in an additional 0.5 ml of 0.25 M sucrose.

**Methods—**Incubations of one or both sugar nucleotides together with microsomal preparations were carried out in the presence and absence of oligosaccharides. A typical incubation mixture contained 0.05 M Mes (pH 6.5), 1.0 M MnCl₂, 0.0005 M UDP-["C"]glucuronic acid and/or 0.0006 M UDP-N-["H"]acetylgalactosamine with or without 0.005 M pentasaccharide 6-sulfate or hexasaccharide 6-sulfate, and 0.001 M of microsomal enzyme in a total volume of 0.025 ml. Incubations were conducted for several hours at 37°. Different combinations, permutations, and amounts of the above materials were utilized as described later.

Following incubations, labeled products were isolated by paper chromatography or by gel filtration. In order to measure the formation of polysaccharides on endogenous primer, reaction mixtures were spotted (5) on Whatman No. 4 paper and chromatographed with butanol/acetone acid/1 M NH₄OH (2:3:1) (9). In this system polysaccharides and 6-sulfated oligosaccharides (tetra or larger) remain at the origin, well separated from sugar nucleotides and degradation products which move down the paper. Origins were eluted with water and washed. Chromatography in both systems was generally conducted for 18 hours.

Gel filtration was performed on a column (2.7 x 120 cm) of Sephadex G-25 with 1 M LiCl as eluant.

Following isolation of labeled oligosaccharides or polysaccharides, aliquots were degraded with chondroitinase ABC (9), and reaction mixtures chromatographed on Whatman No. 1 in butanol/acetone acid/1 M NH₄OH (2:3:1) together with disaccharide standards. Isolated products were incubated with chondro-6-sulfatase and chondro-4-sulfatase (9) and chromatographed in the same system for further identification.

Liver glucuronyltransferase was incubated at 37° with pentasaccharide 6-sulfate as follows: 0.05 M Mes (pH 6.5), 1.0 M MnCl₂, 0.0015 M pentasaccharide 6-sulfate, 0.0001 M UDP-["C"]glucuronic acid (3.6 x 10⁴ cpm/mmol), and 0.01 ml of enzyme were contained in a total volume of 0.05 ml. Aliquots were taken at 30 min, 1 h, 2 h, and 3 h and analyzed for incorporation of ["C"]glucuronic acid into oligosaccharides. The same incubation with cartilage enzyme was run as a control. The liver enzyme preparation and the cartilage enzyme preparation were also assayed for activity in transferring glucuronic acid to phenolphthalein. Incubations at 37° were as follows: 0.05 M Mes (pH 6.5), 0.01 M MnCl₂, 0.0015 M UDP-glucuronic acid, 0.00015 M phenolphthalein, and 0.005 ml of enzyme were contained in a total volume of 0.5 ml. At zero time and at the above time intervals aliquots were diluted to 1 ml with 0.5 M glycine (pH 10.5) and read at 555 nm in a spectrophotometer to determine loss of color as the phenolphthalein glucuronide was formed (10).

Uronic acid-containing material was assayed by the modified carbazole method of Bitter and Muir (11). Reducing N-acetylgalactosamine was assayed by the method of Reissig et al. (12). Radioactivity was determined with a liquid scintillation spectrometer.

**RESULTS**

Chick embryo microsomal enzyme was incubated with UDP-glucuronic acid and phenolphthalein, a nonspecific acceptor of glucuronic acid, to see if nonspecific glucuronyltransferase was present. Liver glucuronyltransferase (also microsomal) was incubated with pentasaccharide to see if it contained the transferase activity of the chick embryo enzyme. Results are shown in Table 1. Incubations were linear over the 3-h time period for all the active reactions. Chick embryo enzyme was inactive in incorporating glucuronic acid into phenolphthalein, and liver glucuronyltransferase was inactive in the 3-h time period for all the active reactions. Chick embryo enzyme was inactive in incorporating glucuronic acid into phenolphthalein, and liver glucuronyltransferase was inactive in the 3-h time period for all the active reactions.

**Table I**

<table>
<thead>
<tr>
<th>Enzyme Source</th>
<th>Glucuronic acid incorporated per hour into</th>
<th>Phenolphthalein</th>
<th>Pentasaccharide</th>
</tr>
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<tr>
<td>Liver</td>
<td>0.002</td>
<td>0</td>
<td>0.005</td>
</tr>
<tr>
<td>Cartilage</td>
<td>0</td>
<td>0</td>
<td>0.035</td>
</tr>
<tr>
<td>Liver plus cartilage</td>
<td>0.028</td>
<td>0</td>
<td>0.035</td>
</tr>
</tbody>
</table>

1The abbreviations used are: ΔDi-6S, 2-acetamido-2-deoxy-3-O-(β-D-gluc-4-enepyransyluronic acid)-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-O-(β-D-gluc-4-enepyransyluronic acid)-4-O-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-O-(β-D-gluc-4-enepyransyluronic acid)-6-O-sulfo-D-galactose; ΔDi-9S, 2-acetamido-2-deoxy-3-O-(β-D-gluc-4-enepyransyluronic acid)-6-O-sulfo-D-galactose; N-acetyllactosamine; ΔDi-8S, 2-acetamido-2-deoxy-3-O-(β-D-gluc-4-enepyransyluronic acid)-6-O-sulfo-D-galactose; N-acetyllactosamine 6-sulfate; Mes, 5-(N-morpholino)pentanesulfonic acid.
in incorporating glucuronic acid into chondroitin-derived oligosaccharides. Neither enzyme preparation inhibited the activity of the other. The liver glucuronyltransferase lost all its activity upon freezing while the chick embryo enzyme retained its activity after being stored frozen for at least 2 years. Thus it is apparent that the addition of glucuronic acid to pentasaccharide 6-sulfate is not related to nonspecific glucuronyltransferase activity similar to that found in liver.

Effects of pentasaccharide 6-sulfate and hexasaccharide 6-sulfate upon polymerization of chondroitin on endogenous primers is shown in Table II. With concentrations of oligosaccharide higher than their $K_m$ (see below) there was a diminution of chondroitin synthesis on the endogenous material. Although the nature of the inhibition seen at higher concentrations of oligosaccharides cannot be determined with any certainty in this crude enzyme system, the results suggest that the enzymes involved in addition of sugars to exogenous oligosaccharide acceptors are the same as those involved in polymerization on the endogenous primer.

Incorporation of [14C]glucuronic acid and N-[3HJacetilgalactosamine into hexasaccharide 6-sulfate with time is shown in Fig. 1A. Incorporation proceeded in a linear fashion for 2 to 3 hours until the amount of sugar nucleotide substrate remaining became limiting. When higher concentrations of sugar nucleotides relative to the acceptor oligosaccharides were used, incorporation proceeded for at least 18 h.

Incorporation of [14C]glucuronic acid and N-[3HJacetilgalactosamine with varying concentrations of appropriate oligosaccharides indicated a $K_m$ for pentasaccharide 6-sulfate of $7 \times 10^{-4}$ and a $K_m$ for hexasaccharide 6-sulfate of $1.2 \times 10^{-4}$. This latter is in close agreement with previously published results (5). In both instances the amount of enzyme utilized was selected to ensure that the oligosaccharide substrates were not significantly depleted in the course of the incubation.

Incorporation of [14C]glucuronic acid and N-[3HJacetilgalactosamine into oligosaccharides when both sugar nucleotide precursors were together in the incubation mixtures is shown in Fig. 1, B and C. With pentasaccharide 6-sulfate as the acceptor (Fig. 1B) there was substantially more [14C]glucuronic acid added than N-[3HJacetilgalactosamine, and there was a time lag in the addition of the N-[3HJacetilgalactosamine. There was little stimulation of [14C]glucuronic acid addition by the presence of UDP-N-[3HJacetilgalactosamine. Similar results but with the reverse sugars were seen when the added acceptor was the hexasaccharide 6-sulfate (Fig. 1C) instead of the pentasaccharide. It would appear from these results that little if any true polymerization took place on the oligosaccharides, in contrast to the simultaneously occurring polymerization on endogenous primers.

Gel filtration of the oligosaccharides labeled with [14C]glucuronic acid and N-[3HJacetilgalactosamine is shown in Fig. 2 with nonradioactive hexasaccharide 6-sulfate as an internal standard. When pentasaccharide 6-sulfate was used as an acceptor in incubations with UDP-[14C]glucuronic acid, the [14C]-labeled product cochromatographed exactly with the standard, indicating the formation of [14C]hexasaccharide 6-sulfate. When hexasaccharide 6-sulfate was used as the acceptor in incubations with UDP-N-[3HJacetilgalactosamine, the [3H]-labeled product chromatographed where a heptasaccharide would be expected. These results are similar to those previously reported by other investigators (5). When both UDP-[14C]glucuronic acid and UDP-N-[3HJacetilgalactosamine were present in the incubation mixtures, the labeled products were skewed slightly toward the areas of larger oligosaccharides. The label (either [14C]glucuronic acid or N-[3HJacetilgalactosamine) that would represent the second sugar added was found in fractions indicating larger oligosaccharides. Under these conditions there was no true polymerization on the oligosaccharide acceptors.

The above results were confirmed by degradation of the

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**Table II**

<table>
<thead>
<tr>
<th>Molarity of oligosaccharide a</th>
<th>Incorporated into endogenous primer</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>[14C]Glucuronic acid</td>
</tr>
<tr>
<td>cpm/h</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>385</td>
</tr>
<tr>
<td>$4 \times 10^{-4}$</td>
<td>385</td>
</tr>
<tr>
<td>$1.2 \times 10^{-4}$</td>
<td>405</td>
</tr>
<tr>
<td>$3.2 \times 10^{-4}$</td>
<td>385</td>
</tr>
<tr>
<td>$1.2 \times 10^{-4}$</td>
<td>355</td>
</tr>
<tr>
<td>$3 \times 10^{-4}$</td>
<td>275</td>
</tr>
<tr>
<td>$6.8 \times 10^{-4}$</td>
<td>200</td>
</tr>
</tbody>
</table>

a Incubation mixtures of 0.025 ml contained 0.05 M Mes (pH 6.5), 0.01 M MnCl₂, and 0.005 ml of enzyme plus (a) 0.0012 M UDP-[14C]glucuronic acid (22 x 10⁶ cpm/μmol), 0.00016 M UDP-N-acetylglactosamine, and pentasaccharide 6-sulfate as shown; (b) 0.0011 M UDP-N-[3HJacetilgalactosamine (20 x 10⁶ cpm/μmol), 0.002 M UDP-glucuronic acid, and hexasaccharide 6-sulfate as shown.

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Fig. 1. Incorporation of [14C]glucuronic acid and N-[3HJacetilgalactosamine into pentasaccharide 6-sulfate and hexasaccharide 6-sulfate. A, reaction mixtures (incubated at 37°C) contained 0.05 M Mes (pH 6.5), 0.01 M MnCl₂, 0.010 ml of cartilage enzyme, and either 0.00044 M UDP-[14C]glucuronic acid (32 x 10⁶ cpm/μmol) and 0.003 M pentasaccharide 6-sulfate, or 0.0006 M UDP-N-[3HJacetilgalactosamine (30 x 10⁶ cpm/μmol) and 0.003 M hexasaccharide 6-sulfate in a total volume of 0.05 ml. B, reaction mixtures were as described in A but contained pentasaccharide 6-sulfate and both sugar nucleotides. C, reaction mixture was as described in A but contained hexasaccharide 6-sulfate and both sugar nucleotides. Aliquots of 0.002 ml were removed from each incubation at indicated time intervals and the amount of labeled oligosaccharide determined as described under "Experimental Procedure." Incorporation of [14C]glucuronic acid (O—O) and N-[3HJacetilgalactosamine (X—X) are indicated.
labeled oligosaccharides with chondroitinase ABC and identification of products. The products to be expected are shown in the scheme in Fig. 3. The chromatogram of products from chondroitinase digestion of one of the preparations is shown in Fig. 4. Table III lists the results of chondroitinase ABC degradation of all four labeled oligosaccharide mixtures previously shown in Fig. 2. Products were further identified by use of chondro-6-sulfatase and chondro-4-sulfatase. Addition of [14C]glucuronic acid alone to pentasaccharide resulted in an oligosaccharide yielding the expected [14C]Di-6S as its only labeled product upon chondroitinase digestion while addition of N-[3H]acetylgalactosamine alone to hexasaccharide yielded only the expected free N-[3H]acetylgalactosamine after chondroitinase treatment. The pattern of degradation products from the [14C, 3H]-doubly labeled oligosaccharides formed on pentasaccharide 6-sulfate acceptor after a 2-h incubation indicated a mixture of hexasaccharide and heptasaccharide while the degradation products of the oligosaccharide formed with a 5-h incubation indicated hexa, hepta, and a small amount of octasaccharide (yielding some [14C, 3H]Di-OS). No [14C, 3H]ADi-0S was found, indicating that there was no measurable growth to an oligosaccharide of nine sugars.

The chondroitinase degradation products of the oligosaccharide formed when both UDP-[14C]glucuronic acid and UDP-N-[3H]acetylgalactosamine were incubated with hexasaccharide 6-sulfate as acceptor yielded similar findings. Only a small fraction of the products was found as [14C, 3H]ADi-0S, indicating a small amount of nine membered oligosaccharide formed.

Additional incubations were conducted with higher concentrations of sugar nucleotides (0.004 M) and lower concentrations of pentasaccharide (6 x 10^-5, 4 x 10^-5; 1.5 x 10^-5, 4 x 10^-5 M) to make the oligosaccharide concentration limiting and thus optimize the possibility of further polymerization on this exogenous acceptor. A maximum of 6.5 ADi-0S to each ADi-6S was achieved after chondroitinase treatment of the products of

CHONDROITINASE DEGRADATION OF OLIGOSACCHARIDES

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Fig. 2. Gel filtration of [14C]glucuronic acid-labeled and N-[3H]acetylgalactosamine-labeled oligosaccharides. Labeled oligosaccharides prepared as in Fig. 1 by 2-h incubations with sugar nucleotides were chromatographed on a column of Sephadex G-25 (2.7 x 120 cm). LiCl (1 M) was used as eluant, and 5-ml fractions were collected. A, product formed when UDP-[14C]glucuronic acid was incubated with pentasaccharide 6-sulfate. B, product formed when UDP-N-[3H]acetylgalactosamine was incubated with hexasaccharide 6-sulfate. C, product formed when pentasaccharide 6-sulfate was incubated with both sugar nucleotides. D, product formed when hexasaccharide 6-sulfate was incubated with both sugar nucleotides. Oligosaccharides labeled with [14C]glucuronic acid (••••••••••) and N-[3H]acetylgalactosamine (O--O) are shown. Standard hexasaccharide 6-sulfate (O---O) assayed by carbazole determinations of uronic acid is also shown.

Fig. 3. Products of chondroitinase degradation. The products of chondroitinase digestion of oligosaccharides labeled with one or more radioactive sugars (italics) on an odd numbered or even numbered acceptor are shown.

Fig. 4. Chondroitinase degradation products of a labeled oligosaccharide preparation. An aliquot of [14C, 3H]-labeled oligosaccharide from Fig. 2D (hexasaccharide 6-sulfate as acceptor incubated with both sugar nucleotides) was incubated with chondroitinase ABC as described under "Experimental Procedure." After chromatography as described, 1-cm strips were eluted from the paper and assayed for [14C] (••••••••••) and [3H] (O--O). Location of standards was determined by ultraviolet absorption, assay for uronic acid, and assay for reducing N-acetylgalactosamine.

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shown in Fig. 6. Results show that more than half (56%) of the UDP-N-acetylgalactosamine and UDP-[14C]glucuronic acid is preincubation, then 2 h after addition of nonradioactive labeled octasaccharide. Chondroitinase degradation products would yield a mixture of 14C singly labeled and 14C,3H-doubly labeled mainly with 14C and with little 3H, while addition of [3H]glucuronic acid at the same time as N-acetylgalactosamine was added to preformed [3H]heptasaccharide and how much was added to heptasaccharide formed after the addition of the galactosamine is shown in Fig. 5. Products were identified at varying time intervals to see how much [14C]glucuronic acid and N-[3H]acetylgalactosamine if one enzyme with two activities were involved or if two enzymes were working in conjunction with one another.

In order to examine this point further, heptasaccharide was used as an acceptor of N-[3H]acetylgalactosamine from UDP-N-[3H]acetylglactosamine. After 2 h, nonradioactive UDP-N-acetylglactosamine was added in excess together with UDP-14C]glucuronic acid, and incubation continued an additional 4 hours. Incorporation of [14C]glucuronic acid and N-[3H]acetylgalactosamine is shown in Fig. 5. Products were identified at varying time intervals to see how much [14C]glucuronic acid was added to preformed [3H]heptasaccharide and how much was added to heptasaccharide formed after the addition of the relatively nonlabeled N-acetyl-galactosamine. Preferential addition of [14C]glucuronic acid at the same time as N-acetylgalactosamine would result in an octasaccharide fraction mainly with 14C and with little 3H, while addition of [14C]glucuronic acid to the entire pool of heptasaccharide would yield a mixture of 14C singly labeled and 14C,3H-doubly labeled octasaccharide. Chondroitinase degradation products of the labeled oligosaccharides after a 4-h incubation (2-h preincubation, then 2 h after addition of nonradioactive UDP-N-acetylglactosamine and UDP-[14C]glucuronic acid) is shown in Fig. 6. Results show that more than half (56%) of the degradates isolated (ΔDi-OS and Di-OS) was labeled with N-[3H]acetylgalactosamine as well as [14C]glucuronic acid. Material isolated at the end of the 6-h incubation showed a similar pattern with slightly less doubly labeled disaccharides (44%). There was also more ΔDi-OS than after 4 h, indicating a continued growth of the oligosaccharides during the last 2 h of incubation. Since incorporation of N-acetyl-galactosamine into heptasaccharide was linear for at least 4 h, an independent further addition of [14C]glucuronic acid would result in oligosaccharides yielding less than 50% 14C-singly labeled disaccharide and at least 50% 14C,3H-doubly labeled disaccharide. This latter was found, indicating independent addition of each sugar, requiring only the appropriate acceptor.

The independence of each enzyme activity cannot easily be resolved with the previous observations (3) of true rapid polymerization that takes place on endogenous microsomal primers. One explanation might be a permanent attachment of both enzymes to the microsomal membrane in juxtaposition with the microsomal primer so that concentrations of primer substrate in the immediate area of the enzymes would always remain high. This explanation was excluded by addition of

<table>
<thead>
<tr>
<th>Oligosaccharide formed with</th>
<th>Percentage of total degradation products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[14C]</td>
</tr>
<tr>
<td>Pentasaccharide</td>
<td></td>
</tr>
<tr>
<td>+ UDP-[14C]GlcUA</td>
<td>100</td>
</tr>
<tr>
<td>+ UDP-[14C]GlcUA</td>
<td>35</td>
</tr>
<tr>
<td>+ UDP-GalN[3H]Ac (2 h)</td>
<td>28</td>
</tr>
<tr>
<td>Hexasaccharide</td>
<td></td>
</tr>
<tr>
<td>+ UDP-GalN[3H]Ac</td>
<td>100</td>
</tr>
<tr>
<td>+ UDP-[14C]GlcUA (2 h)</td>
<td>50</td>
</tr>
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</table>

**TABLE III**

Degradation of labeled oligosaccharides by chondroitinase

**Fig. 5.** Addition of [14C]glucuronic acid during and after formation of heptasaccharide. The initial reaction mixture was identical with that shown in Fig. 1A with hexasaccharide 6-sulfate and UDP-N-[3H]acetylgalactosamine. After 2 h, nonradioactive UDP-N-[3H]acetylglactosamine (to 0.0016 M) and UDP-[14C]glucuronic acid (to 0.0004 M, 32 x 10^6 cpm/μmol) were added to 0.025 ml of the reaction mixture. Aliquots of 0.002 ml were removed at indicated times and assayed for 

**Fig. 6.** Chondroitinase degradation products of the oligosaccharide shown in Fig. 5. An aliquot of the [14C, 3H]-labeled oligosaccharide from Fig. 5 (4 h) was incubated with chondroitinase ABC as described under "Experimental Procedure." After chromatography as described, 1-cm strips were eluted from the paper and assayed for [14C] (0-0) and [3H] (O-O). Location of standards was determined by ultraviolet absorption, assay for uronic acid, and assay for reducing N-acetylgalactosamine.
heat-inactivated microsomal material to the polysaccharide-synthesizing system. Results are shown in Table IV. The addition of heat-inactivated material in 2-fold excess over non-heat-inactivated microsomal enzyme substantially increased chondroitin synthesis. Thus enzymes attached to a membrane particle are capable of producing polymerization on primer attached to another particle; the enzyme need not be in permanent juxtaposition with the primer. This also indicates that in these experiments the availability of endogenous primer is the limiting factor in the amount of chondroitin synthesized.

**DISCUSSION**

It would seem apparent that at least two enzymes are involved in the addition of glucuronic acid and N-acetylgalactosamine during chondroitin synthesis. Furthermore our experiments indicate that the addition of single sugars to appropriate exogenous oligosaccharide acceptors involves the same specific enzymes that are involved in true polymerization on endogenous primers. The one difference of significance between the addition to exogenous oligosaccharides and to endogenous primers rests in the ease of rapid polymerization on the latter.

The addition of sugars to exogenous oligosaccharides or endogenous primers could take place in several ways.

1. Each enzyme could form a suitable enzyme-substrate complex with the appropriate oligosaccharide (or endogenous primer) and sugar nucleotide; a single sugar would then be transferred. This would result in the independent addition of sugars directly dependent upon the relative concentration of the appropriate acceptors. We have shown that the addition of sugars to oligosaccharides fits this scheme.

2. The two enzymes could interact with one another and then form enzyme-substrate complexes with an appropriate oligosaccharide (or endogenous primer) and both sugar nucleotides; two sugars would then be transferred. We have shown that there is no interaction of the two enzymes in this fashion since there is no paired addition of sugars to oligosaccharides.

3. The two enzymes could interact with an appropriate oligosaccharide (or endogenous primer) in such a way as to provide for them to remain attached while polymerization continued.

The third mechanism would appear to fit the pattern of polysaccharide polymerization that we have previously demonstrated on endogenous primers, since polymerization occurs rapidly and preferentially on an individual primer molecule (3). This polymerization does not occur through interaction with the polysaccharide chain alone, since the oligosaccharide acceptors do not function in the same manner; another portion of the primer must be involved.

Our findings do not exclude the possibility of other single sugar intermediates (lipid sugar, etc.) in the synthesis. However, the possibility of a disaccharide (or larger) intermediate would not be consistent with our data.

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Biosynthesis of chondroitin sulfate. Independent addition of glucuronic acid and N-acetylgalactosamine to oligosaccharides.
J E Silbert and A C Reppucci, Jr


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