Biosynthesis of Chondroitin Sulfate. Assembly of Chondroitin on Microsomal Primers†

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ABSTRACT: A microsomal preparation from chick embryo epiphyseal cartilage has previously been shown to synthesize chondroitin when incubated with UDP-[^{14}C]glucuronic acid and UDP-*N*-[^{3}H]acetylgalactosamine. The "primer" for this synthesis has now been shown to be similar to the "acceptor" for sulfate, glucuronic acid, or *N*-acetylgalactosamine described in the previous paper. The final product of polymerization on primer was found to consist of two distinct types of glycosaminoglycan. (1) Approximately 25–50% of the newly formed glycosaminoglycan consisted of nonsulfated chondroitin chains with an average final mol wt of ~35,000 relative to chondroitin sulfate standards, representing the formation of large nonsulfated chondroitin on a small primer estimated to be less than mol wt 8000. (2) The remaining 50–75% of the final product consisted of chondroitin sulfate primer (average mol wt ~25,000) plus the addition of newly formed chondroitin (mol wt ~5000–15,000). There was no indication of primers intermediate in size between the small and large types, nor were there indications of endogenous nonsulfated chondroitin chains in the microsomal preparation. Individual polysaccharide chains were formed rapidly with no accumulation of small sized products. After short incubation periods, the chain lengths of the newly formed products were approximately 50% that of the final heteropolysaccharides formed after longer incubations, indicating that the bulk of increase in incorporation with longer incubation times chiefly represented formation of more chondroitin chains rather than a time-related elongation of chondroitin chains. Thus, a rapid, organized, preferential formation of individual chondroitin chains is indicated, rather than random addition of sugars to all the primer sites available for further chain assembly. Essentially all of the radioactive products were linked to protein by alkali labile bonds, suggesting that the microsomal glycosaminoglycan contains a protein–polysaccharide linkage similar to that known to occur in the proteoglycan of extracellular matrix.

Cell-free biosynthesis of various portions of heteropolysaccharides has been previously demonstrated with several animal systems, but the sequence and interrelationship of steps in assembly of these glycosaminoglycans have not been delineated completely.

It has been established with microsomal preparations from chick embryo epiphyses that appropriate single sugars can be added individually to the nonreducing ends of exogenous oligosaccharides derived from chondroitin sulfate (Telser *et al.*, 1966). However, these oligosaccharides do not act as effective "primers" for polymerization to form long chondroitin chains. (The term "primer" will hereafter be utilized to define any substance upon which an oligosaccharide or polysaccharide chain can be formed as opposed to an "acceptor" of a single sugar or sulfate.) In contrast, the polymerization of polysaccharide has been demonstrated utilizing the endogenous primer of a microsomal preparation from chick embryo epiphyses (Silbert, 1964; Perlman *et al.*, 1964). Previous work in this laboratory demonstrated that these microsomal preparations could act as primers to accept equimolar amounts of glucuronic acid and *N*-acetylgalactosamine from UDP-glucuronic acid and UDP-*N*-acetylgalactosamine to form chains consisting of *N*-acetylchondrosine repeating units (Silbert, 1964). The chain length of the final product was not determined, other than to show that it was larger than a standard of chondroitin 4-sulfate. The size of the primer relative to the newly synthesized portion of the final product was also not determined. The final product was insoluble, but could be solubilized by proteolytic digestion (pancreatin) indicating that it (and, therefore, presumably the primer) was linked to protein.

In the previous paper, the presence of alkali labile sulfate-accepting and sugar-accepting microsomal chondroitin sulfates of varying molecular size was reported (Richmond *et al.*, 1973). It would seem likely that this endogenous acceptor would also be representative of the primer for additional polysaccharide chain synthesis. Further identification of the primer for polymerization was therefore undertaken to determine its alkali lability, to ascertain its size, and to compare it with the microsomal chondroitin sulfate previously described. The process of heteropolysaccharide assembly on the microsomal primer was investigated.

Experimental Procedures

UDP-[^{14}C]glucuronic acid, UDP-*N*-acetylgalactosamine, UDP-*N*-[^{3}H]acetylgalactosamine, 3′-phosphoadenosine 5′-phosphosulfate, and ^{35}S-labeled pAdo-5′-phosphosulfate[1] were prepared as previously described (Silbert, 1962, 1964; DeLuca and Silbert, 1968). UDP-*N*-acetyl[^{14}C]galactosamine was purchased from New England Nuclear Corp. (Boston, Mass.). UDP-glucuronic acid was purchased from Sigma Chemical Co. (St. Louis, Mo.). Glycosaminoglycans, glycosaminoglycan-degrading enzymes, and disaccharide standards were obtained as described in the previous paper (Richmond *et al.*,

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[1] Abbreviation used is: pAdo, 3′-phosphoadenosine.

TABLE I: Incorporation of Sugars into Glycosaminoglycan.[a]

Nucleotides Added	Glycosaminoglycan Isolated[b] Total Radioactivity (cpm) ^{14}C	^{3}H	Precursor Incorp as nmol of ^{14}C	^{3}H
1. UDP-[^{14}C]GlcUA	150		0.01	
2. UDP-[^{14}C]GlcUA, UDP-GalNAc	3,600		0.24	
3. UDP-GalN[^{3}H]Ac		280		0.01
4. UDP-GalN[^{3}H]Ac, UDP-GlcUA		5700		0.23
5. UDP-[^{14}C]Gal-NAc	2,160		0.03	
6. UDP-[^{14}C]Gal-NAc, UDP-GlcUA	26,000		0.41	

[a] Reaction mixtures contained 0.05 M 2-(*N*-morpholino)-ethanesulfonic acid (pH 6.5), 0.01 M $MnCl_2$, and 20 μl of microsomal preparation in a total volume of 50 μl. In addition, the indicated reaction mixtures contained UDP-[^{14}C]glucuronic acid (UDP-[^{14}C]GlcUA), 10 nmol (1.5×10^5 cpm); UDP-glucuronic acid, 10 nmol; UDP-*N*-[^{3}H]acetylgalactosamine (UDP-GalN[^{3}H]Ac), 10 nmol (2.5×10^5 cpm); UDP-*N*-acetylgalactosamine, 160 nmol; UDP-*N*-acetyl[^{14}C]-galactosamine, 10 nmol (6.3×10^5 cpm). Incubations were conducted for 3 hr at 37°. [b] Each number represents the average of duplicate or triplicate incubations. Reaction mixtures 1 and 3 were run in triplicate.

1973). Microsomal preparations of chick embryo epiphyseal cartilage were prepared as previously described (Silbert, 1964, 1966), and radioactively labeled glycosaminoglycans were extracted from origins of chromatographed reaction mixtures (Silbert, 1964, 1966). Pancreatin or alkali was used as previously described (Silbert, 1966; Richmond *et al.*, 1973).

Various combinations of unlabeled or labeled UDP-glucuronic acid and UDP-*N*-acetylgalactosamine were incubated with microsomal preparations under varying conditions. Reactions were carried out with UDP-[^{14}C]glucuronic acid in the presence or absence of UDP-*N*-acetylgalactosamine; other reactions were carried out with UDP-*N*-[^{3}H]acetylgalactosamine or UDP-*N*-acetyl[^{14}C]galactosamine in the presence or absence of UDP-glucuronic acid. Microsomal preparations were also preincubated with 3′-phosphoadenosine 5′-phosphosulfate, ^{35}S-labeled pAdo-5′-phosphosulfate, UDP-[^{14}C]-glucuronic acid, or UDP-*N*-[^{3}H]acetylgalactosamine prior to formation of heteropolysaccharide by using UDP-glucuronic acid and UDP-*N*-acetylgalactosamine together. Incubation mixtures were similar to those described in the preceding paper (Richmond *et al.*, 1973).

Samples of isolated [^{14}C]glycosaminoglycan, [^{3}H]glycosaminoglycan, and [^{35}S]glycosaminoglycan together with standards of hyaluronic acid and chondroitin 4-sulfate were chromatographed on a DEAE-cellulose column as described in the previous paper (Richmond *et al.*, 1973). Aliquots of each fraction were assayed for radioactivity and for the glycosaminoglycan standards. Selected fractions from the column were pooled, dialyzed against water or desalted by Sephadex G-15 to remove LiCl, and lyophilized.

Samples of labeled glycosaminoglycan from DEAE-cellulose column fractions were chromatographed on a Sepharose 4B column together with a standard of chondroitin 6-sulfate.

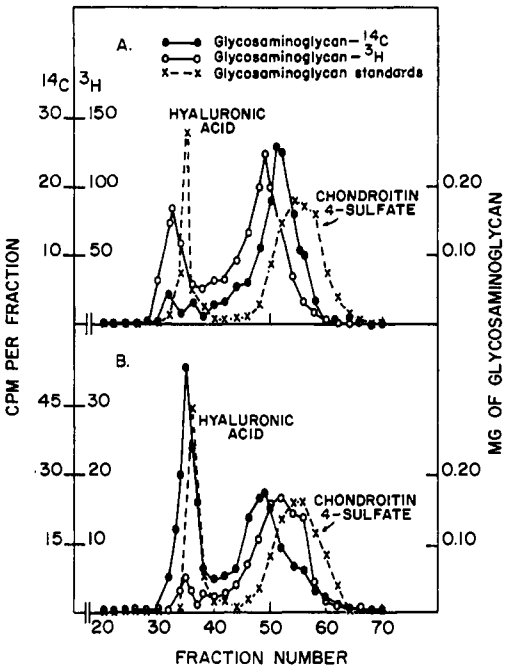

FIGURE 1: DEAE-cellulose chromatography of glycosaminoglycans labeled in the presence of one or both sugar nucleotides. Glycosaminoglycans from four separate reaction mixtures, as described in Table I, were chromatographed on a column of DEAE-cellulose (1×5 cm) with standards of hyaluronic acid (1 mg) and chondroitin 4-sulfate (5 mg). A logarithmic gradient of LiCl was utilized for elution; 125 ml of water was in the mixing flask and 1 M LiCl was in the reservoir flask. Fractions of 2.5 ml were collected and assayed for radioactivity and glycosaminoglycan standards. Part A represents the simultaneous chromatography on a single column of samples from reaction mixtures 1 and 4 of Table I. Part B represents the simultaneous chromatography on a single column of samples from reaction mixtures 2 and 3 of Table I. The size of the aliquots of the glycosaminoglycans obtained from reaction mixtures 1–4 (Table I) was selected to give convenient ratios of ^{3}H to ^{14}C in order to facilitate assay. Thus, most of the [^{14}C]glycosaminoglycan and [^{3}H]glycosaminoglycan from the triplicate reaction mixtures of reactions 1 and 3 were utilized, while only small aliquots from reactions 2 and 4 were utilized.

Fractions were assayed for radioactivity and the glycosaminoglycan standard.

Chondroitinase digestions were performed as described in the preceding paper (Richmond *et al.*, 1973). Uronic acid containing material was assayed by the Bitter and Muir (1962) carbazole method. Radioactivity was determined with a low-background (0.5 cpm) gas flow counter or with a liquid scintillation counter.

Results

Incorporation of Sugars. Results of sugar incorporations in a representative experiment are shown in Table I. Incorporation of either ^{14}C or ^{3}H into glycosaminoglycan in the presence of a complete reaction mixture containing UDP-*N*-acetylgalactosamine and UDP-glucuronic acid was approximately 20–25 times as great as the incorporation in reaction mixtures containing a single labeled sugar nucleotide. However, incorporation with a single labeled sugar nucleotide was low, so that this proportion can only be considered to be an estimate. Similar experiments using slightly different incubation conditions have been reported previously (Silbert, 1964).

Characterization of Products by Charge and Charge Density. Chromatography of these glycosaminoglycans on DEAE-cellulose is shown in Figures 1A and 1B. [^{3}H]Glycosamino-

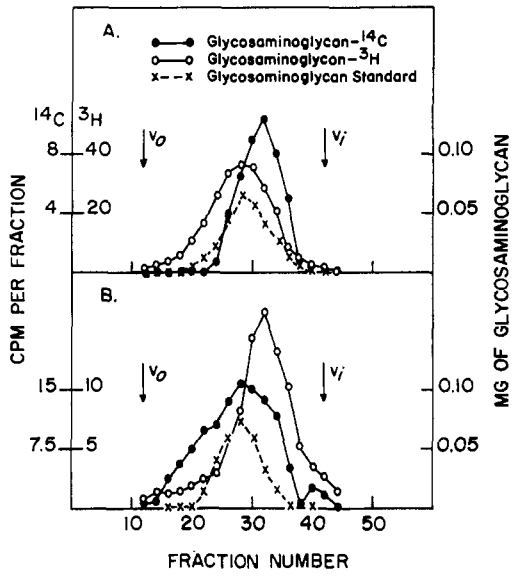

FIGURE 2: Size determination of [^{3}H]glycosaminoglycan and [^{14}C]-glycosaminoglycan by Sepharose 4B column chromatography. Fractions 42–60, representing the peak II region from the DEAE-cellulose columns of Figures 1A and 1B, were pooled separately, dialyzed to remove LiCl, and lyophilized. Aliquots were chromatographed on a Sepharose 4B column (1 × 60 cm) together with Blue Dextran (V_0), Phenol Red (V_i), and a standard of chondroitin 6-sulfate (1 mg). The eluent utilized was 0.1 M LiCl and a flow rate of 4 ml/hr was maintained. Fractions of 1 ml were collected and assayed for radioactivity and the chondroitin 6-sulfate standard. Part A represents peak II from Figure 1A (reaction mixtures 1 and 4 of Table I) and B represents peak II from Figure 1B (reaction mixtures 2 and 3 of Table I).

glycan (Figure 1A) or [^{14}C]glycosaminoglycan (Figure 1B) formed in the presence of both sugar nucleotides was found in two discrete peaks on DEAE-cellulose chromatography. The first of these peaks (peak I) appeared just before and overlapping standard hyaluronic acid, thus representing material of relatively low charge or charge density. The second (peak II) appeared before but overlapping standard chondroitin 4-sulfate and thus represents material of a higher charge than does peak I. A larger aliquot of the radioactive glycosaminoglycan obtained from reaction mixtures that contained only one of the sugar nucleotides (shown also in the previous paper (Richmond *et al.*, 1973)) was cochromatographed with the material formed with both sugar nucleotides present in order to show the precise relationship. The peak I material formed in the presence of a single sugar nucleotide was minimal in proportion to the peak II material. Furthermore, the peak II material was eluted from DEAE-cellulose later, closer to the area of standard chondroitin 4-sulfate, than was the glycosaminoglycan formed in the presence of both sugar nucleotides. This would indicate a higher charge density than the peak II material formed with both sugar nucleotides in the reaction mixture. All the labeled material of peaks I and II was degradable by chondroitinase.

There were variable small amounts of labeled material that were eluted from DEAE-cellulose before peak I, as described in the previous paper (Richmond *et al.*, 1973). This material was generally resistant to chondroitinase and was not sufficient for further identification. Data are not included in the graphs or tables.

Characterization of Products by Size. In order to investigate the size of the newly formed chains, peaks I and II materials from Figure 1 were examined by chromatography on Sepharose 4B. Peak II glycosaminoglycan (Figure 2) labeled with [^{14}C]glucuronic acid or *N*-[^{3}H]acetylgalactosamine formed in the presence of both sugar nucleotides was estimated to have mol wt ~35,000–40,000 relative to standard chondroitin 6-sulfate (mol wt 41,000). Material formed in the presence of only one sugar nucleotide (UDP-[^{14}C]glucuronic acid, UDP-*N*-[^{3}H]acetylgalactosamine, or UDP-*N*-acetyl-[^{14}C]galactosamine) was estimated to have mol wt ~20,000–25,000 relative to the standard.

The size (larger) and charge density (lower) of the peak II material formed when both sugar nucleotides were present (relative to the peak II material formed when a single sugar nucleotide was present) confirm that it represents the addition of a nonsulfated oligosaccharide chain onto the endogenous chondroitin sulfate primer. The approximate size of this newly formed chain could be estimated by gel chromatography to be ~20–30 disaccharide units.

Peak I formed in the presence of a single sugar nucleotide was insufficient for characterization. The average size of peak I formed with both sugar nucleotides present was mol wt ~35,000 relative to standard chondroitin 6-sulfate (mol wt 41,000). The size and charge density (by location on DEAE-cellulose chromatography) of the peak I material formed with both sugar nucleotides would thus indicate that it represents a large nonsulfated molecule. Details concerning this material are presented later in the paper (see Figures 6 and 7).

Characterization of Primers. The data described here and in the previous paper indicate that the final peak I product after polymerization represents the addition of a large polysaccharide chain to a small primer. However, the possibility can be considered that some peak I formed with both sugar nucleotides in the reaction mixture could represent addition of a small oligosaccharide chain to a large nonsulfated primer. This was ruled out by the following experiment. A microsomal preparation was incubated under the usual conditions with relatively high concentrations (0.001 M) of pAdo-5′-phosphosulfate. (Under these conditions a large percentage of newly synthesized chondroitin can be sulfated, resulting in a marked shift of the elution pattern on DEAE-cellulose chromatography (Silbert and DeLuca, 1969; DeLuca *et al.*, 1973).) Following this, labeled sugar nucleotides were added and the glycosaminoglycan products isolated and chromatographed on DEAE-cellulose in the usual fashion. No changes in the chromatographic pattern were noted. Should nonsulfated large molecular weight material have been a significant primer for peak I polymerization, this primer would have been sulfated (as much as 80%) and the resulting product would no longer have been found in the peak I area.

From the above data it would seem apparent that the endogenous primer for polymerization of sugars is similar in structure to the glycosaminoglycan sulfate acceptor or sugar acceptor as described in the previous paper (Richmond *et al.*, 1973). In order to examine this directly, microsomal preparations were prelabeled by preincubation with pAdo-5′-phospho[^{35}S]sulfate, UDP-[^{14}C]glucuronic acid, or UDP-*N*-[^{3}H]acetylgalactosamine followed by polysaccharide polymerization in the same reaction mixture.

The reaction mixture for prelabeling with [^{35}S]sulfate is given in Figure 3 which shows the DEAE-cellulose chromatogram of the resulting products. The chromatographic pattern of [^{35}S]glycosaminoglycan appeared to be altered slightly when the [^{35}S]glycosaminoglycan had been subsequently incubated with sugar nucleotides to form additional glycosaminoglycan. The differences were not striking, however. The

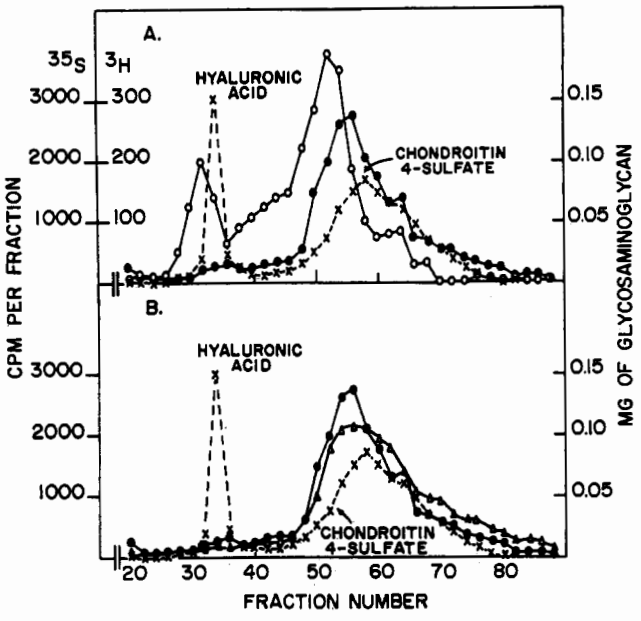

FIGURE 3: DEAE-cellulose chromatography of [^{35}S]glycosaminoglycan with and without subsequent polymer formation. The reaction mixture contained 0.05 M 2-(*N*-morpholino)ethanesulfonic acid (Mes) (pH 6.5), 0.01 M $MnCl_2$, 3′-phosphoadenosine 5′-phospho[^{35}S]sulfate (7 nmol (11.5×10^6 cpm)), and 20 μl of microsomal preparation in a total volume of 50 μl. After 1 hr, 25 μl was removed; UDP-glucuronic acid (25 nmol) and UDP-*N*-[^{3}H]-acetylgalactosamine (13.5 nmol (4.4×10^5 cpm)) were added to the remaining 25 μl. Incubation was then continued for an additional 4 hr. Aliquots of reaction mixtures were chromatographed on DEAE-cellulose as described in Figure 1 and assayed for radioactivity and glycosaminoglycan standards. (A) Chromatography of [^{35}S]glycosaminoglycan (●—●) and [^{3}H]glycosaminoglycan (○—○) formed in the incubation mixture to which sugar nucleotides were added; glycosaminoglycan standards are shown (×- - -×). (B) Chromatography of [^{35}S]glycosaminoglycan (△—△) removed from the reaction mixture prior to the addition of sugar nucleotides; the [^{35}S]glycosaminoglycan of A (●—●) is shown superimposed for purposes of comparison.

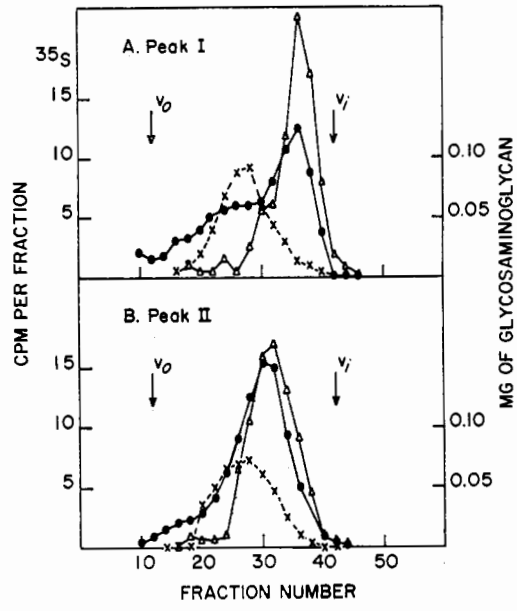

FIGURE 4: Sepharose 4B column chromatography of [^{35}S]glycosaminoglycan fractions from DEAE-cellulose columns of Figure 3. Representative fractions from peak I (fractions 31–33) and peak II (fractions 50–53) from DEAE-cellulose column chromatograms of Figure 3 were chromatographed on Sepharose 4B. Conditions of chromatography were identical with those described in Figure 2. (A) Chromatography of peak I [^{35}S]glycosaminoglycan (●—●) formed in the incubation mixtures to which sugar nucleotides were added, and [^{35}S]glycosaminoglycan (△—△) formed in the incubation mixture to which nucleotides were not added. Each of these was chromatographed separately, but results are shown superimposed for purposes of comparison. Glycosaminoglycan standards are shown (×- - -×). (B) Chromatography of peak II [^{35}S]glycosaminoglycan from the same reaction mixtures as described in A.

pattern of [^{3}H]glycosaminoglycan in this experiment was similar to that shown in Figure 1 for material that was not preincubated with pAdo-5′-phospho[^{35}S]sulfate.

Figure 4 shows the chromatographic pattern on Sepharose 4B of [^{35}S]glycosaminoglycan from peak I and peak II areas from the DEAE-cellulose chromatograms of Figure 3. It is readily apparent that following the addition of sugar nucleotides to the reaction mixture some of the [^{35}S]glycosaminoglycan from the peak I area was found to be of larger molecular size, while some was found to remain small in molecular size. The pattern on Sepharose 4B was markedly different from the pattern of the same fractions from DEAE-cellulose columns of [^{35}S]glycosaminoglycan formed without subsequent additions of sugars (see also Figure 3 of the previous paper (Richmond *et al.*, 1973)). The results directly indicated that small molecular size sulfated material had acted as a primer for polymerization to form chondroitin of mol wt ~35,000 (as compared to the standard chondroitin 6-sulfate). Similarly, following the addition of sugar nucleotides to the reaction mixture, some of the [^{35}S]glycosaminoglycan from the peak II area was found to be larger in size. The amount of shifted material was much less, however, than that seen with the peak I [^{35}S]glycosaminoglycan.

The preincubation experiments carried out with UDP-[^{14}C]glucuronic acid and UDP-*N*-[^{3}H]acetylgalactosamine were similar to those with pAdo-5′-phospho[^{35}S]sulfate. UDP-[^{14}C]glucuronic acid was incubated with a microsomal preparation as described in the previous paper (Richmond *et al.*, 1973). After 2 hr, a 100-fold excess of cold UDP-glucuronic acid plus UDP-*N*-acetylgalactosamine were added. Incubation was continued for an additional 2 hr. A similar experiment was conducted using UDP-*N*-[^{3}H]acetylgalactosamine for prelabeling, with subsequent addition of cold UDP-*N*-acetylgalactosamine and UDP-glucuronic acid. Following the incubations, the labeled glycosaminoglycans were isolated and chromatographed on DEAE-cellulose. Insufficient labeled material was found in the peak I area to provide meaningful information. Peak II material (both before and after polymerization) was characterized by Sepharose 4B in the same fashion as sulfate labeled material shown in Figure 4B. Results were comparable to Figure 4B, indicating that a proportion of the prelabeled glycosaminoglycan acceptor (labeled with either *N*-[^{3}H]acetylgalactosamine or [^{14}C]glucuronic acid) chromatographed as a larger glycosaminoglycan when both sugar nucleotides were added to the incubation mixtures. This therefore indicated that some, but not all, of the glycosaminoglycan that had accepted a single sugar or sulfate had acted as a primer for further polymerization.

Time Course for Polymerization. Polymerization of a polysaccharide in a given time interval could be envisioned as a random slow additional growth of polysaccharide chains on most or all of the available primer molecules; alternatively, polymerization in this same time interval could represent preferential rapid formation of individual chondroitin chains on a portion of the available primer. Since the experiments described above demonstrated that only a small proportion of the single sugar-labeled or sulfate-labeled acceptor had acted

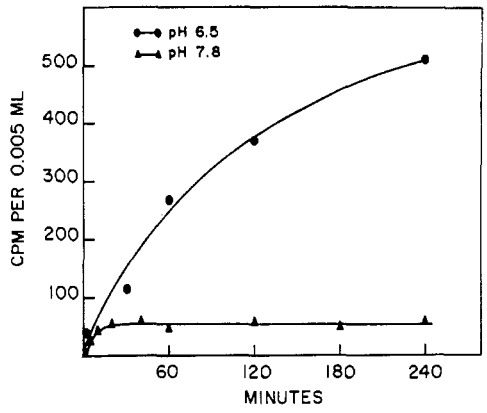

FIGURE 5: Timed incorporation of radioactivity into glycosaminoglycan. Reaction mixtures in Mes (pH 6.5) and Tris (pH 7.8) were incubated as described under Experimental Procedures. Aliquots were removed as indicated and chromatographed as described under Experimental Procedures. Origins of the chromatograms were assayed for radioactivity with a low background counter.

as primer, this latter mechanism might be indicated. In order to examine this further, timed incorporation studies were conducted.

In previous experiments (Silbert, 1964), incubation of microsomal preparations for the biosynthesis of chondroitin sulfate were conducted at pH 7.8. Subsequently it became apparent that biosynthesis was considerably greater at pH 6.5. This suggested either a difference in enzyme stability, product size, or available substrates at the two pH levels. Incorporation of radioactive sugars into microsomal glycosaminoglycan with time at pH 6.5 and 7.8 is shown in Figure 5. Incorporation at pH 6.5 continued for more than 4 hr with a gradually diminishing rate. In contrast, incorporation at pH 7.8 was much less, and reached a maximum before 1 hr (as previously reported; Silbert, 1964). The difference in incorporation at the two pH levels appeared to be due to a difference in availability of endogenous primer. This was indicated by the following results: when further microsomal preparation or boiled microsomal preparation was added to an incubation mixture, additional incorporation was noted. Moreover, preincubation of the microsomal preparation at 37° for 2 hr prior to the addition of sugar nucleotides resulted in no loss in incorporating activities, so that cessation of incorporation did not appear to be due to loss of enzyme activity. As will be described in detail later in the paper, product size was similar at both pH 7.8 and 6.5.

Figure 6 compares the patterns of elution from DEAE-cellulose of [^{14}C]glycosaminoglycan formed in 2 min and 4 hr at pH 6.5. Some radioactive material was found in fractions appearing before the peak I area. The amount of this material did not appear to be time dependent, so that much more was present in proportion to the peaks I and II glycosaminoglycan after 2 min of incubation than after 4 hr of incubation. The bulk of this material was not degradable by chondroitinase, so it does not appear that this material is related to chondroitin sulfate. Amounts were not sufficient for further characterization. Glycosaminoglycan formed at pH 7.8 (2 min and 4 hr) had elution profiles similar to those of glycosaminoglycan formed at pH 6.5. (Earlier experiments with chondroitin formation at pH 7.8 showed a larger peak I relative to peak II (Silbert, 1964; Silbert and DeLuca, 1969). The reason for the difference between the earlier experiments and the present experiments is not apparent, but may be related to differences in the source of chick embryos.)

TABLE II: Tabulation of Results from DEAE-Cellulose Chromatograms of [^{14}C]Glycosaminoglycan.

	Cpm of ^{14}C Incorp.[a]			% of Total Incorp.	
Time	Total	Peak I (Fractions 30–41)	Peak II (Fractions 42–60)	Peak I	Peak II
2 min	1,725	415	655	24	38
4 hours	20,600	4550	13,600	22	66

[a] The actual number of counts recovered in the 4-hr incubation was one-fifth that shown. The numbers were multiplied by five to allow for direct comparison with the 2-min sample, since the 2-min sample consisted of 75 μl of the reaction mixture while the 4-hr sample was only 15 μl. The numbers shown for the DEAE-cellulose chromatogram fractions additionally have been adjusted to represent total samples rather than the aliquots chromatographed.

Table II details the radioactivity of materials shown in Figure 6. In the experiment shown here, it appeared as though the ratio of peak I material to peak II material at 2 min was larger than the ratio at 4 hr. However, this was not considered to be a significant finding, since proportions of peak I to peak II varied considerably from one experiment to another.

Size of Products Relative to Time Course of Polymerization. Gel chromatography of [^{14}C]glycosaminoglycan formed at pH 6.5 is shown in Figure 7. The average molecular size of peak I and peak II glycosaminoglycan formed in 4 hr and peak II glycosaminoglycan formed after 2 min was slightly smaller than that of standard chondroitin 6-sulfate of mol wt 41,000. Peak I glycosaminoglycan obtained after 2-min incubations was somewhat smaller than the glycosaminoglycan that had been formed after 4-hr incubations, appearing to be slightly smaller in size than standard chondroitin 4-sulfate of mol wt 26,000 but larger than a standard of heparin. (The first paper of this series (Richmond *et al.*, 1973) details the chromatography of glycosaminoglycan standards on Sepharose 4B.) From comparison with the standards, the molecular size of the 2-min peak I glycosaminoglycan was estimated to be approximately 20,000. Peak I and peak II material obtained from DEAE-cellulose chromatograms of [^{14}C]glycosaminoglycan formed in incubations at pH 7.8 had patterns on Sepharose 4B similar to those of the material formed at pH 6.5.

The results indicate that individual glycosaminoglycan chains of peak I material formed in 2 min were large (mol wt 20,000) even though the total peak I material represented less than 10% of that finally formed during longer incubations. After the first 2 min, sugars continued to be added, but even after 4 hr of incubation the average chains only reached approximately 35,000. Thus, formation of glycosaminoglycan occurred rapidly to form relatively large glycosaminoglycan chains, and the increased amount incorporated into peak I glycosaminoglycan with time mainly represented formation of more chains, rather than growth of these chains throughout the entire period. Incorporation at pH 6.5 continued long after incubation at pH 7.8 had come to an end, even though the final products were the same molecular size. Thus, the continued incorporation was apparently due to a greater availability of primer at pH 6.5.

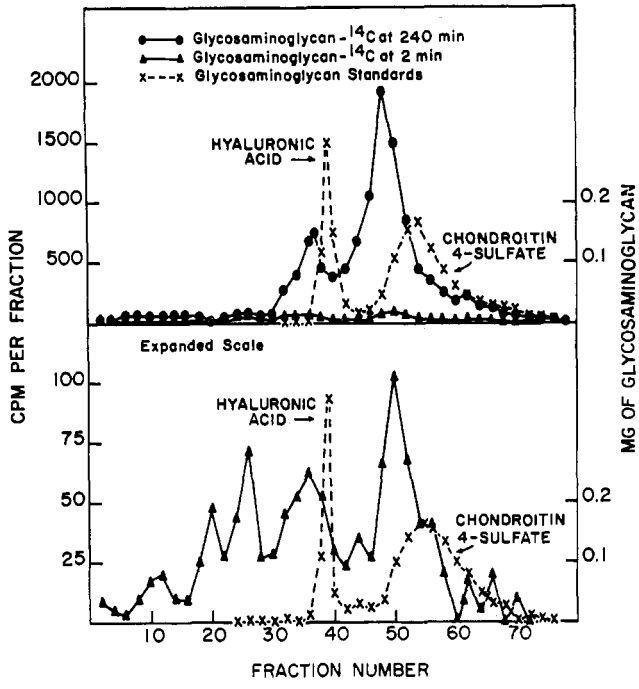

FIGURE 6: Chromatography on DEAE-cellulose of [^{14}C]glycosaminoglycan formed in 2-min and 4-hr incubations. The reaction mixture was as described for Mes (pH 6.5) under Experimental Procedures. After 2-min incubation, 75 μl was removed and the [^{14}C]glycosaminoglycan was isolated. Incubation was continued for an additional 4 hr, 15 μl was removed, and the [^{14}C]glycosaminoglycan isolated. Aliquots of the isolated [^{14}C]glycosaminoglycan were chromatographed on DEAE-cellulose columns (1 × 5 cm) together with standards of hyaluronic acid (1 mg) and chondroitin 4-sulfate (5 mg). The columns were eluted with a logarithmic gradient of LiCl. There were 125 ml of water in the mixing flask and 1 M LiCl in the reservoir. Fractions of 2.5 ml were collected. Fractions were assayed for radioactivity and standard glycosaminoglycans. The top figure shows the elution profiles of [^{14}C]glycosaminoglycan obtained from 2-min and 4-hr incubations. These chromatograms were run separately but are shown superimposed for purposes of comparison. Since the aliquot removed at 2 min (75 μl) was five times the aliquot removed at 4 hr (15 μl), the results of the 4-hr chromatogram were multiplied by five for representation on the graph. The lower figure shows the elution pattern of the 2-min [^{14}C]glycosaminoglycan on an expanded scale.

The molecular size of peak II glycosaminoglycan was only slightly larger after 4-hr incubation than after 2-min incubation (despite the incorporation of 21 times as much labeled sugar at 4 hr as at 2 min). The increased incorporation here with time also appeared to represent addition to more primer molecules, rather than uniform chain growth throughout the entire period.

Alkali Extractability of Products. Alkali extractability of radioactive glycosaminoglycan is shown in Table III. More than 90% of the glycosaminoglycan formed with both sugar nucleotides in the reaction mixture was extractable by alkali, while the remainder required pancreatin for extraction. A somewhat smaller percentage (72%) of the material labeled with only UDP-*N*-[^{3}H]acetylgalactosamine in the reaction mixture was alkali labile in this experiment. Chromatography of the alkali labile glycosaminoglycans on DEAE-cellulose showed patterns similar to those of whole glycosaminoglycan samples extracted with pancreatin.

Discussion

The first paper in this series described the relative size and charge density of endogenous microsomal chondroitin sulfate

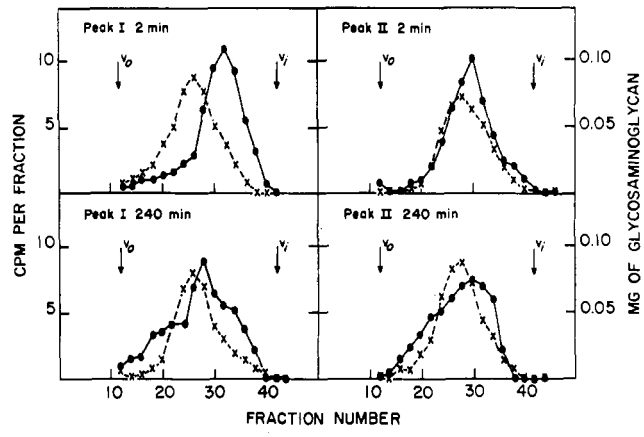

FIGURE 7: Sepharose 4B chromatography of radioactive glycosaminoglycan obtained from DEAE-cellulose chromatograms. Peak I and peak II fractions from the DEAE-cellulose chromatograms of Figure 6 were pooled, dialyzed, and lyophilized. Aliquots (representing equal amounts of [^{14}C]glycosaminoglycan to facilitate comparison) were chromatographed together with Blue Dextran (V_0), Phenol Red (V_i), and chondroitin 6-sulfate (1 mg) on a Sepharose 4B column (1 × 60 cm). The column was eluted with 0.1 M LiCl and 1-ml fractions were collected. The flow rate was 4 ml/hr. [^{14}C]Glycosaminoglycan (●—●) was determined with a low background gas flow counter and standard chondroitin 6-sulfate (×---×) by uronic acid determinations.

from chick embryo cartilage that acted as an acceptor for the addition of sulfate, glucuronic acid, and *N*-acetylgalactosamine.

In the present paper it was demonstrated that endogenous material with similar properties also served as a primer for polysaccharide polymerization. Polymerization was found to occur on two types of primer resulting in two distinct types of glycosaminoglycan. The first type (peak I on DEAE-cellulose chromatography) appeared to be nonsulfated chondroitin with an average molecular size of approximately 35,000 relative to a chondroitin 6-sulfate standard. This glycosaminoglycan appeared to represent new polymerization of a long chondroitin chain on a small primer. The second type of glycosaminoglycan (peak II on DEAE-cellulose chromatography) appeared to consist of a primer of chondroitin sulfate (average molecular size approximately 25,000) to which a nonsulfated chondroitin chain of approximately 5000–15,000 molecular size was added. The peak I and peak II products were discretely separated on DEAE-cellulose chromatography indicating that there was little significant primer intermediate in

TABLE III: Alkali Extractability of Glycosaminoglycans.

Nucleotides Included in Reaction Mixture	% of Glycosaminoglycan Extractable: 0.1 N NaOH	Remainder after Alkali: Extractable with Pancreatin	Remainder after Alkali: Not Extractable with Pancreatin
1. UDP-[^{14}C]GlcUA	93	7	<0.1
2. UDP-[^{14}C]GlcUA, UDP-GalNAc	96	4	<0.1
3. UDP-GalN[^{3}H]Ac	72	28	<0.1
4. UDP-GalN[^{3}H]Ac, UDP-GlcUA	88	12	<0.1

size between that for peak I and peak II. Had there been such an intermediate size primer, the DEAE-cellulose chromatogram of labeled glycosaminoglycan would have shown a continuum rather than the two discrete peaks.

The data also supported the previous conclusion that little, if any, large molecular weight, nonsulfated chondroitin is present in the microsomal preparations. This absence of nonsulfated polysaccharide contributes to the conclusion that sulfation and polymerization *in vivo* probably occur together or with only a short time interval between polymerization and sulfation.

Products formed at short incubation intervals indicated that polymerization took place rapidly on primer with no significant accumulation of small sized products. Thus, glycosaminoglycan (peak I) formed at 4 hr was less than twice the size of glycosaminoglycan (peak I) formed at 2 min even though the total glycosaminoglycan formed at 4 hr was more than 10 times the amount of that formed in 2 min. This leads to the conclusion that most of the increased synthesis in a given time inverval represents the formation of new chains or portion of chains on a primer rather than a slow chain growth by the gradual addition of sugars to all the primers. This would mean that in a given time interval, only a portion of the endogenous primer is utilized as a substrate for new chondroitin chain formation. This mechanism is consistent with the results of the experiments in which acceptor was prelabeled before polymerization. In those experiments only a fraction of the prelabeled acceptor was found to serve as primer in a given time interval.

Recently, the incorporation of sugars into alkaki stable as well as alkali labile macromolecular material has been described (Derge and Davidson, 1972). Microsomal systems similar to those used here were utilized. It was reported that the majority of the product did not represent extensive chain elongation. This is not in agreement with the present work, nor with work previously reported (Silbert, 1964; Silbert and DeLuca, 1969), since essentially all of the incorporation into macromolecular material reported here was into glycosaminoglycan chains that have been identified as chondroitin. Furthermore, 90–95% of this material was linked to protein by alkali labile bonds.

Previously, addition of individual sugars had been demonstrated utilizing oligosaccharide acceptors (Telser *et al.*, 1966), but this mechanism of chain growth had not been previously demonstrated for true polysaccharide formation in animal systems, since primers for polymerization had not been characterized. Formation of peak II (growth of a relatively large heteroglycan on a glycosaminoglycan primer) demonstrates that biosynthesis of glycosaminoglycan can occur by addition of sugars to the nonreducing end of a proteoglycan, chondroitin sulfate.

It might be suggested that formation of peak I glycosaminoglycan is truly representative of *in vivo* heteropolysaccharide formation, while formation of peak II is an artifact of the *in vitro* system representing addition to chondroitin sulfate that ordinarily would be complete in an *in vivo* system.

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