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Biosynthesis of Heparan Sulfate

Formation of a Glycosaminoglycan Precursor

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Microsomal preparations from Englebreth-Holm-Swarm mouse sarcoma were incubated with UDP-*N*-acetyl[^{3}H]glucosamine and UDP-[^{14}C]glucuronic acid to form proteoglycan containing [^{3}H, ^{14}C]glycosaminoglycan with equimolar amounts of [^{3}H]glucosamine and [^{14}C]glucuronic acid. The labelled glycosaminoglycan was totally resistant to degradation by testicular hyaluronidase, but could be degraded readily by a crude *Flavobacter heparinum* enzyme preparation which is capable of degrading heparin and heparan sulfate. Chromatography of the [^{3}H, ^{14}C]glycosaminoglycan on DEAE-cellulose provided a pattern with three peaks: the first appearing before hyaluronic acid, the second and largest appearing at the site of hyaluronic acid, and a third appearing slightly beyond hyaluronic acid but before a standard of chondroitin sulfate. When 3′ -phosphoadenosine 5′ -phosphosulfate was also included in the reaction mixture, a change appeared in the [^{3}H, ^{14}C]glycosaminoglycan so that chromatography on DEAE-cellulose presented a pattern with a significant amount of material which co-chromatographed in the area where heparan sulfate would be found. There was no material that co-chromatographed with the more highly sulfated substance, heparin. This indicates that the microsomal preparation from the Englebreth-Holm-Swarm sarcoma is capable of producing a heparan sulfate-like molecule and is controlled in its sulfation of precursors so that heparin is not formed.

Many, if not all cells synthesize heparan sulfate, which appears as a component of the cell surface [1-5], and may be released to become a component of the extracellular matrix. The structure of heparan sulfate consists of glucosamine residues alternating with uronic acid residues, some of which are iduronic acid and some of which are glucuronic acid. The glucosamine is partially *N*-acetylated and partially *N*-sulfated, and in addition there are other ester sulfates on the iduronic acid or glucosamine. The structure thus bears many similarities to heparin in that it contains *N*-sulfate and the same repeat-

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ing sugars that heparin contains. It is distinguished from heparin by the presence of considerable amounts of *N*-acetyl and lesser amounts of *O*-sulfate, *N*-sulfate and iduronic acid. Both heparin and heparan sulfate appear to be linked to protein as proteoglycans [6, 7], but the proteoglycan structures appear to be different.

Since the glycosaminoglycan structure of heparan sulfate bears so many similarities to that of heparin, it would be most likely that synthesis would proceed in a manner similar to that of heparin. Furthermore the synthesis of heparin proceeds through intermediates which are closely related to heparan sulfate in structure [8-11]. The biosynthesis of heparin has been studied extensively with microsomal systems from mouse mast cell tumors where synthesis has been shown to proceed stepwise by initial incorporation of equimolar amounts of glucuronic acid and *N*-acetylglucosamine [11] followed by partial deacetylation [10], *N*-sulfation [8-10, 12], epimerization of glucuronic acid to iduronic acid [13], 2-*O*-sulfation of iduronic acid [14] and 6-*O*-sulfation of glucosamine [14]. There have also been reports of the use of a microsomal preparation from aorta to demonstrate the formation of heparan sulfate-like material in a similar manner [15, 16]. However, these structures were not identified in regard to charge or size and the formation was examined in a system which was producing other glycosaminoglycans as well, so that definition from heparin was not clear. More recently, micrososmal fractions from rat liver cells have been used for similar synthesis [17]. The products were closer in structure to heparin than to heparan sulfate, suggesting that cell disruption had disturbed the control of sulfation, allowing it to proceed beyond that of heparan sulfate formation.

Recently, the Englebreth-Holm-Swarm sarcoma has been shown to contain substantial amounts of heparan sulfate [18] and can supply a ready source of tissue for investigation of biosynthesis. We have used a microsomal preparation from this tumor to demonstrate the synthesis of a glucuronic acid-containing and glucosamine-containing glycosaminoglycan from UDP-glucuronic acid and UDP-*N*-acetylglucosamine precursors and have used 3′ -phosphoadenosine 5′ -phosphosulfate to show sulfation of a portion of this material to form a heparan sulfate-like glycosaminoglycan. No heparin-like material was formed.

Materials and Methods

UDP-[^{14}C]Glucuronic acid and UDP-*N*-acetyl[^{3}H]glucosamine were purchased from New England Nuclear Corporation (USA). UDP-*N*-Acetylgalactosamine was prepared as previously described [19]. 3′ -Phosphoadenosine 5′ -phosphosulfate and heparin were obtained from Sigma Chemical Company (USA). Chondroitin ABC lyase, chondroitin 6-sulfate and chondroitin 4-sulfate were obtained from Miles Laboratories (USA). Testicular hyaluronidase was obtained from Calbiochem (USA), crude *Flavobacter heparinum* enzyme was prepared as previously described [20] and a hexasaccharide from chondroitin sulfate was prepared with testicular hyaluronidase [21].

Frozen Englebreth-Holm-Swarm mouse sarcoma was a generous gift of Dr. G. Martin, Bethesda MD (USA). Pieces of approximately 3-5 g were homogenized in 0.25 M sucrose with a Polytron homogenizer. Homogenates were centrifuged at 10 000 × g for 10 min and the supernatant was then centrifuged at 105 000 × g for 30 min. The glassy pellet was resuspended in 0.25 M sucrose and centrifuged at 105 000 × g. This washed micro-

somal pellet, containing approximately 0.2 ml packed volume, was resuspended in an additional 0.2 ml of 0.25 M sucrose. The microsomal preparation made in this fashion maintained activity after storage for at least several months at -20°C and was still active after several freezing and thawing procedures.

For synthesis experiments, appropriate substrates were incubated with 0.005-0.025 ml of microsomal preparation for periods ranging up to 6 h at 37°C in 0.05 M 2-(*N*-morpholino)ethane-sulfonic acid (MES), pH 6.5 and 0.01 M $MnCl_2$. Following incubations, the entire reaction mixtures were spotted on Whatman No. 1 chromatography paper which was then developed with a solvent of ethanol: 1 M ammonium acetate, pH 7.8 (5:2). As previously described [19, 22], in this system glycosaminoglycans and proteoglycans remain at the origin of the paper chromatograms while sugar nucleotides and degradation products move down the paper completely separated from the radioactively-labelled glycosaminoglycans. The radioactively-labelled glycosaminoglycans were eluted from the paper with 0.5 M NaOH overnight at room temperature or by incubation with 1% Pancreatin (Viobin, USA) in 0.05 M Tris, pH 8.5, overnight at 37°C.

Radioactively-labelled glycosaminoglycans were incubated with hyaluronidase together with standard hyaluronic acid at 37°C for 1 h. Other fractions of radioactively-labelled glycosaminoglycan were incubated with crude *F. heparinum* enzyme or with chondroitin ABC lyase [23]. Following incubations, the radioactively-labelled material was chromatographed on a 1 × 5 cm column of DEAE-cellulose with a logarithmic gradient of 0 - 1.0 M LiCl in 10 mM NaAc, pH 5.6 as previously described [11]. Standards of hyaluronic acid and chondroitin 4-sulfate were co-chromatographed. Column fractions containing radioactively-labelled material were pooled, dialyzed, lyophilized and chromatographed on a column of Sepharose 4B with 0.5 M NaAc, pH 5.6, as eluant.

Fractions from the Sepharose 4B column were pooled, dialyzed, lyophilized and small sized material was rechromatographed on Sephadex G-100, both before and after treatment with crude *F. heparinum* enzyme.

Results

When incubations contained UDP-[^{14}C]glucuronic acid as the only sugar nucleotide, there was minimal incorporation of [^{14}C]glucuronic acid into microsomal material. The presence of UDP-*N*-acetylglucosamine increased incorporation of [^{14}C]glucuronic acid five- to tenfold, while the presence of UDP-*N*-acetylgalactosamine did not result in substantial additional incorporation, indicating that it could not substitute for UDP-*N*-acetylglucosamine.

UDP-[^{14}C]Glucuronic acid, 0.3 mM (550 × 10^6 cpm/μmol) and 0.4 mM UDP-*N*-acetylglucosamine were incubated together with 0.015 ml of enzyme in a total volume of 0.025 ml to form [^{14}C]glycosaminoglycan. Susceptibility of the [^{14}C]glycosaminoglycan to degradation by hyaluronidase and *F. heparinum* enzyme is shown in Fig. 1. There was no effect of testicular hyaluronidase on the radioactively-labelled glycosaminoglycan, whereas standard hyaluronic acid was readily degraded. Crude *F. heparinum* enzyme degraded all the radioactively-labelled glycosaminoglycan. Incubation with chondroitin ABC lyase and a chondroitin sulfate standard demonstrated the same pattern as shown in Fig. 1 for hyaluronidase.

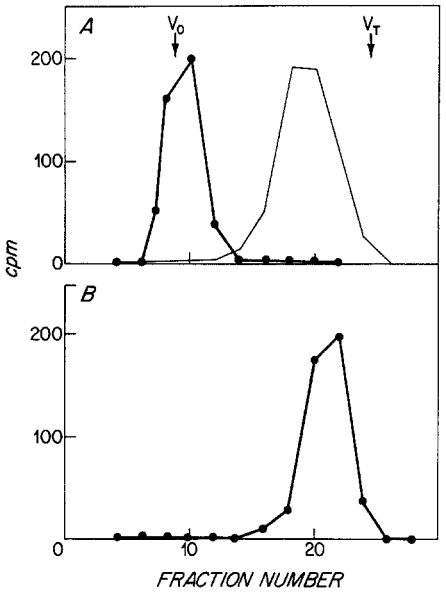

Figure 1. Degradation of [^{14}C]glycosaminoglycan. [^{14}C]Glycosaminoglycan together with carrier hyaluronic acid was incubated with A) hyaluronidase or B) *F. heparinum* enzyme, and then chromatographed on a 1 × 35 cm column of Sephadex G-50, with 0.5 M NaAc, pH 5.6 as eluant. Fractions of 1 ml were assayed for radioactivity (●) and uronic acid (—).

In another experiment, 0.15 mM UDP-[^{14}C]glucuronic acid (550 × 10^6cpm/μmol), alone or with 0.09 mM UDP-*N*-acetyl[^{3}H]glucosamine (5 100 × 10^6 cpm/μmol) was incubated for 4 h with 0.015 ml of enzyme in a total volume of 0.025 ml. When UDP-*N*-acetyl[^{3}H]glucosamine was utilized as the sole sugar nucleotide, there was considerable incorporation into material which was later demonstrated to be mainly non-glycosaminoglycan in nature. In the presence of UDP-glucuronic acid the incorporation of [^{3}H]glucuronic was enhanced. Because of the incorporation of [^{3}H]glucosamine into non-glycosaminoglycan material, ratios of incorporation of [^{3}H]glucosamine and [^{14}C]glucuronic acid could not be calculated until the labelled glycosaminoglycan could be separated from the non-glycosaminoglycan material. The total amount of [^{14}C]glucuronic acid incorporated in these experiments was 2 400 cpm.

Chromatography of the [^{3}H, ^{14}C]glycosaminoglycan on DEAE-cellulose is shown in Fig. 2. A pattern of three major peaks was seen, the first appearing in fractions 10-14, before standard hyaluronic acid, the second in fractions 15-20 at about the position of hyaluronic acid and a broad third peak appearing mainly in fractions 22-34 half way between the standards of hyaluronic acid and chondroitin 4-sulfate. In the second and third peak there appeared to be a nearly 1:1 molar ratio of [^{14}C]glucuronic acid to [^{3}H] glucosamine incorporated. The first peak, however, contained an excess of [^{3}H]glucosamine. There was additional radioactivity appearing in fractions 2-6, but this was shown to be small material unrelated to glycosaminoglycan formation and was not characterized further.

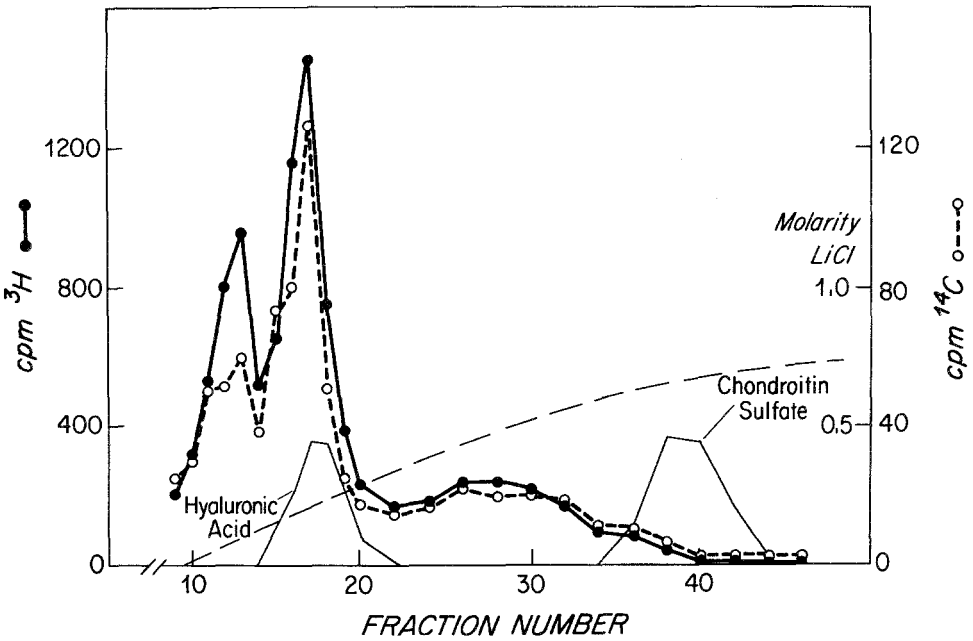

Figure 2. Chromatography on DEAE-cellulose of [^{3}H, ^{14}C]glycosaminoglycan. An aliquot of [^{3}H, ^{14}C]glycosaminoglycan was chromatographed together with standards of hyaluronic acid and chondroitin sulfate on a column of DEAE-cellulose (1 × 5 cm). The column was eluted with a logarithmic gradient of 0 - 1 M LiCl in 0.01 M NaAc, pH 5.6, with 200 ml in the mixing flask. Fractions of 4.3 ml were assayed for ^{3}H (●), ^{14}C (○), and uronic acid (—).

The glycosaminoglycan-containing peaks were pooled, dialyzed, lyophilized and aliquots chromatographed on a 1 × 100 cm column of Sepharose 4B (Fig. 3). Only ^{3}H was counted since there were insufficient amounts of ^{14}C. Approximately one third of the [^{3}H]-labelled material from the first peak (Fig. 2, fractions 10-14) of the DEAE-cellulose column chromatographed on Sepharose 4B as broad heterogeneous material, the same size and larger than the standard of chondroitin 6-sulfate. Two thirds chromatographed as a smaller sized substance that appeared later (Fig. 3A, fractions 40-48). This later-appearing material was pooled, dialyzed, lyophilized and chromatographed on Sephadex G-100, together with a hexasaccharide standard derived from chondroitin sulfate. Chromatography demonstrated a substance which was somewhat larger than the hexasaccharide. This material was pooled, concentrated and desalted on a Sephadex G-10 column and incubated with crude *F. heparinum* enzyme. When it was then rechromatographed on the Sephadex G-100 column, it was found to be unaffected by the *F. heparinum* enzyme, indicating that it was not a glycosaminoglycan or a portion of glycosaminoglycan. It was not examined further. The second DEAE-cellulose peak from Fig. 2 (fractions 15-20) appeared on Sepharose 4B chromatography (Fig. 3B) as heterogeneous material similar to the larger material of the first DEAE-cellulose peak. The third DEAE-cellulose peak was similar to the second DEAE-cellulose peak upon chromatography on Sepharose 4B and is not shown. All these materials were degradable by *F. heparinum* enzyme.

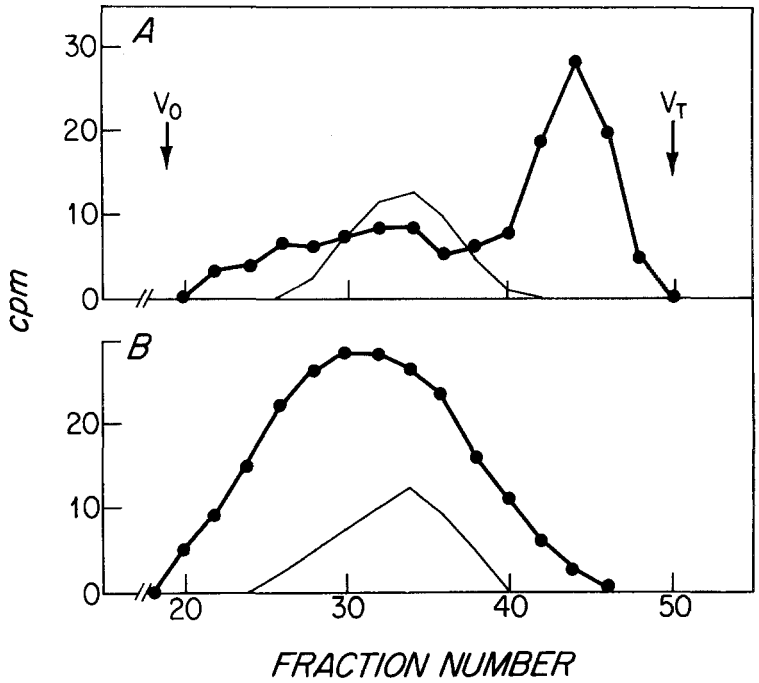

Figure 3. Chromatography of [^{3}H]glycosaminoglycan on Sepharose 4B. [^{3}H]Glycosaminoglycan from Fig. 2, A) fractions 10-14 and B) fractions 15-20 were chromatographed on a 1 × 100 cm column of Sepharose 4B with 0.5 M NaAc, pH 5.6, as eluant. Fractions of 1 ml were assayed for radioactivity (●) and uronic acid (—).

[^{3}H, ^{14}C]Glycosaminoglycan was also formed in a similar incubation mixture as above, but with the presence of 2 mM 3′-phosphoadenosine 5′-phosphosulfate. Chromatography on DEAE-cellulose is shown in Fig. 4. The pattern of glycosaminoglycan was markedly changed from the non-sulfated [^{3}H, ^{14}C]glycosaminoglycan that had been formed without 3′-phosphoadenosine 5′-phosphosulfate (Fig. 2). Instead of 3 peaks, two were found: the first at fractions 14-20 like the second peak of Fig. 2 and the second occurring later at fractions 30-38 just before the standard of chondroitin sulfate. Thus, the first and third peaks of Fig. 2 were reduced in size and a new peak was found, indicating a more anionic substance. This new peak contained approximately 60% of the [^{3}H, ^{14}C]glycosaminoglycan. No additional material was found when the column was subsequently washed with 2 M LiCl.

Each of the above analyses was conducted on glycosaminoglycan that had been eluted from paper by incubation with 0.5 M NaOH or with 1% pancreatin. There were no noticeable differences in chromatographic patterns between glycosaminoglycans obtained by the two methods.

Discussion

Previous work on the biosynthesis of heparin has indicated that when 3′-phosphoadenosine 5′-phosphosulfate was absent from a heparin-synthesizing murine mast cell microsomal system, a glycosaminoglycan containing equal amounts of *N*-acetylglucosamine and glucuronic acid appeared upon DEAE-cellulose chromatography in fractions where hyaluronic acid appeared [11]. Some material has also been found to be deacetylated and appeared earlier [10]. Upon addition of 3′-phosphoadenosine 5′-phosphosulfate a change occurred, with a shift of some [^{3}H, ^{14}C]glycosaminoglycan to fractions chromatographing near a heparin standard [8, 9, 12]. Thus extensive sulfation had taken place. It has been demonstrated that the sulfated material consisted of two types of glycosaminoglycan [12]. The first of these contained *N*-sulfate, but little *O*-sulfate, while the second contained both *N*-sulfate and *O*-sulfate. The glycosaminoglycan appeared with or later than chondroitin sulfate standards upon DEAE-cellulose chromatography. There was minimal sulfated material appearing before or with the earlier portion of chondroitin sulfate where heparan sulfate would be found [2].

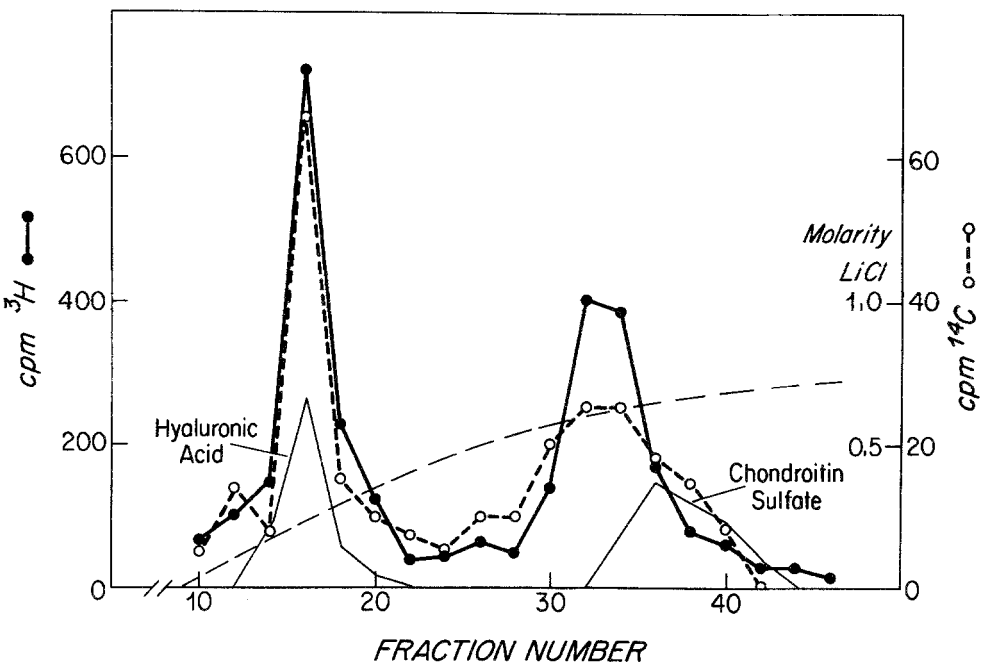

Figure 4. Chromatography on DEAE-cellulose of [^{3}H, ^{14}C]glycosaminoglycan formed in the presence of 3′-phosphoadenosine 5′-phosphosulfate. An aliquot of [^{3}H, ^{14}C]glycosaminoglycan formed in the presence of 3′-phosphoadenosine 5′-phosphosulfate was chromatographed in a similar fashion to that described in Fig. 2. Fractions were assayed for ^{3}H (●), ^{14}C (○), and uronic acid (—).

We have now performed similar experiments with a microsomal preparation from heparan sulfate-producing mouse sarcoma. All the [^{3}H, ^{14}C]glycosaminoglycan formed with UDP-[^{14}C]glucuronic acid and UDP-*N*-acetyl[^{3}H]glucosamine as substrates was resistant to hyaluronidase but susceptible to *F. heparinum* enzyme (Fig. 1). Thus no hyaluronic acid was formed, indicating that the glycosaminoglycan was similar to heparin or heparan sulfate in its basic glycosaminoglycan structure. When sugar nucleotides were present in the absence of 3' -phosphoadenosine 5' -phosphosulfate, the glycosaminoglycan formed was similar upon DEAE-cellulose chromatography (Fig. 2) to that formed during incubation of these substrates with a microsomal preparation from mouse mast cell tumor [8, 11]. All the [^{3}H, ^{14}C]glycosaminoglycan was as large or larger than a chondroitin sulfate standard by chromatography on Sepharose 2B (Fig. 3). When 3' -phosphoadenosine 5' -phosphosulfate was present, there was a shift of some material to fractions where heparan sulfate would be found (Fig. 4). There was no formation of material as highly sulfated as heparin.

Since heparan sulfate contains both *N*-sulfate and *O*-sulfate, but less of each than heparin contains, the mechanism of heparan sulfate formation must include a means of limiting the extent of sulfation. The microsomal synthesizing system from the Englebreth-Holm-Swarm sarcoma appears to be capable of this limitation in sulfation.

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