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Biosynthesis of Chondroitin Sulfate. Microsomal Acceptors of Sulfate, Glucuronic Acid, and *N*-Acetylgalactosamine†

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ABSTRACT: A microsomal preparation from chick embryo epiphyseal cartilage has previously been shown to accept [^{35}S]sulfate, [^{14}C]glucuronic acid, and *N*-[^{3}H]acetylgalactosamine into endogenous glycosaminoglycan. The endogenous glycosaminoglycan has now been characterized further. Incubations of microsomal preparations at pH 6.5 with 3′-phosphoadenosine 5′-phosphosulfate resulted in incorporation of sulfate into endogenous chondroitin 6-sulfate (60–70%) and chondroitin 4-sulfate (30–40%) while incubations at pH 7.8 resulted in incorporation into chondroitin 6-sulfate exclusively. Incorporation appeared to be into occasional nonsulfated galactosamine units in predominantly sulfated molecules with no "oversulfation" to form 4,6-disulfated hexosamine units. Endogenous polysaccharide molecules of low sulfate content were not found. Gel filtration demonstrated that the endogenous sulfate-accepting chondroitin sulfate ranged in size from mol wt ~2000 to ~40,000 relative to chondroitin sulfate standards. Of the total, approximately 90% was of mol wt 16,000 or larger. Incubation of microsomal preparations with UDP-[^{14}C]glucuronic acid or UDP-*N*-[^{3}H]-acetylgalactosamine also resulted in incorporation of the appropriate sugar into similar endogenous chondroitin sulfate. Essentially all the acceptor glycosaminoglycan was linked to protein by alkali labile bonds.

Considerable information is now available on the intermediates involved in the synthesis of the heteropolysaccharide (glycosaminoglycan) portion of the proteoglycan, chondroitin sulfate. Uridine sugar nucleotides have been shown to be the precursors for the sugars of the chondroitin chains (Silbert, 1964; Perlman *et al.*, 1964) and significant sulfation of newly synthesized chondroitin (Silbert and DeLuca, 1969) has been demonstrated with 3′-phosphoadenosine 5′-phosphosulfate as sulfate donor. It is also well established that at least some of the chondroitin sulfate heteropolysaccharide found in extracellular matrix is linked to serine units of protein by an alkali-labile xylosyl-serine bond which is part of a glucuronosyl-galactosyl-galactosyl-xylosyl-serine protein–polysaccharide "bridge" (Rodén and Smith, 1966; Lindahl and Rodén, 1966; Helting and Rodén, 1968). Uridine sugar nucleotides have been shown to be intermediates in the synthesis of this "bridge" (Helting and Rodén, 1969a,b; Brandt *et al.*, 1969; Baker *et al.*, 1972). Alternatively, it has been suggested that some of the polysaccharide chains may be attached to protein by an as yet unidentified bond which is alkali stable (Katsura and Davidson, 1966; Lyons and Singer, 1971). This could represent a different type of separately formed glycosaminoglycan or could be an intermediate in the formation of the glycosaminoglycans attached by the alkali labile bond.

Studies on the structure of proteoglycans have mainly involved the examination of end products of synthesis, such as are found in connective tissue matrix. The structure of intracellular proteolycans at the time of synthesis has not been investigated to any great extent, for the most part because presently available sources do not contain enough intracellular polysaccharide for chemical characterizations. Incorporation of radioactive sugars or [^{35}S]sulfate into this material provides an alternative method of characterization by looking at intracellular glycosaminoglycans as "acceptors." ("Acceptor" will hereafter be the term used to describe material into which sulfate or a single sugar can be incorporated.)

Addition of sulfate to chondroitin or chondroitin sulfate has been reported from many laboratories where soluble (105,000*g* supernatant) enzymes were utilized with either exogenous or extracellular endogenous acceptors (D'Abramo and Lipmann, 1957; Adams, 1960; Suzuki and Strominger, 1960a; Perlman *et al.*, 1964; Meezan and Davidson, 1967; Robinson, 1969). The addition of sulfate has been low relative to the large amount of acceptor present. In contrast, when microsomal preparations have been utilized to demonstrate sulfate incorporation into intracellular endogenous particulate material, a much greater efficiency in sulfation has been observed (DeLuca and Silbert, 1968). Products were found to be protein-linked macromolecules that were identified as chondroitin sulfate.

Certain questions, however, remained unanswered by this

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work. It was not known whether these materials represented essentially completely formed intracellular chondroitin sulfate destined for transport to extracellular matrix, or whether portions of it were incompletely formed molecules which could serve as primers for both sulfation and polysaccharide polymerization. It was not determined whether the incorporation of sulfate was onto occasional nonsulfated loci in a mainly sulfated molecule, or whether some of the incorporation was into relatively nonsulfated polysaccharides. The attachments to protein were not investigated, so it was not known if the linkages were similar to those found in proteoglycans obtained from extracellular material.

Incorporation of single sugars (either glucuronic acid or *N*-acetylgalactosamine) has been demonstrated with microsomal fractions from chick embryo cartilage using both endogenous (Silbert, 1964) and exogenous acceptors (Telser *et al.*, 1966). Products utilizing some exogenous oligosaccharide acceptors have been well characterized (Telser *et al.*, 1966) but characterization of the products utilizing endogenous acceptors has not been described.

The first of these reports describes a better characterization of the endogenous intracellular sulfate acceptor and sugar acceptor. These materials may in part represent partially or completely synthesized chondroitin sulfate at the intracellular site of biosynthesis. Further reports deal with the identification of the primers for and the process of hetereopolysaccharide polymerization (Richmond *et al.*, 1973) and finally the sulfation of this newly polymerized polysaccharide (DeLuca *et al.*, 1973). Although these reports deal specifically with chondroitin sulfate formation, it is likely that the results are representative of the mode of synthesis of other animal heteropolysaccharides as well.

Experimental Procedures

The 3′-phosphoadenosine 5′-phosphosulfate and the ^{35}S-labeled pAdo-phosphosulfate[1] were synthesized with yeast enzyme by the method of Robbins (1962), chromatographed on Dowex 1-X8 according to the method of Wilson *et al.* (1961), and finally obtained as previously described (Silbert, 1967). UDP-[^{14}C]glucuronic acid and UDP-*N*-acetylgalactosamine were prepared as previously described (Silbert, 1962, 1964). UDP-*N*-[^{3}H]acetylgalactosamine was prepared as previously described (Silbert, 1964) or alternatively by the method of Maley (1970).

Chondroitin 4-sulfate and chondroitin 6-sulfate were obtained from Seikagaku Kogyo, Co., Ltd. (Tokyo, Japan). The chondroitin 4-sulfate (lot 4200 A, super-special grade) was from whale cartilage and the chondroitin 6-sulfate (lot 4201 C, super-special grade) was from shark cartilage. Analyses of these compounds as supplied by Seikagaku Kogyo indicated a molar ratio of 4-sulfate to 6-sulfate of 80.1:19.9 and 8.8:91.2, respectively. Average molecular weights were given as 26,000 for the chondroitin 4-sulfate and 41,000 for the chondroitin 6-sulfate. Tetrasaccharide disulfate was prepared by testicular hyaluronidase degradation of chondroitin 6-sulfate.

Hyaluronic acid (umbilical cord) and heparin were purchased from Calbiochem (Los Angeles, Calif.). Chondroitinase AC, chondroitinase ABC, chondro-4-sulfatase, chondro-6-sulfatase, and the disaccharide standards, 2-acetamido-2-deoxy-3-*O*-(β-D-gluc-4-enepyranosyluronic acid)-D-galactose[1] (ΔDi-0S), 2-acetamido-2-deoxy-3-*O*-(β-D-gluc-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose (ΔDi-4S), and 2-acetamido-2-deoxy-3-*O*-(β-D-gluc-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose (ΔDi-6S), were purchased from Miles Laboratories, Inc. (Kanakee, Ill.).

Frozen 14-day chick embryos were purchased from Pel-Freez Biologicals (Rogers, Ark.). Twice washed microsomal preparations sedimenting between 10,000*g* and 105,000*g* were prepared from chick embryo epiphyses as previously described (Silbert, 1964, 1966). Epiphyseal cartilage from 250 embryos yielded approximately 0.5 ml of a microsomal preparation.

In order to sulfate endogenous "acceptor" material, microsomal preparations were incubated at 37° with 3′-phosphoadenosine 5′-phospho[^{35}S]sulfate. Two types of incubation mixtures were used, patterned on previous work (pH 7.8) and on more recent observations that incorporation is greater at pH 6.5: (1) 0.05 M Mes (2-(*N*-morpholino)ethanesulfonic acid) (pH 6.5), 0.01 M $MnCl_2$, 3′-phosphoadenosine 5′-phospho[^{35}S]sulfate (3.5 nmol (5.8×10^6 cpm)), and 15 μl of microsomal preparation in a total volume of 25 μl; (2) identical with mixture 1 except for the substitution of 0.05 M Tris (pH 7.8) for the 0.05 M Mes.

When aliquots were removed at varying time intervals to assay for 3′-phosphoadenosine 5′-phospho[^{35}S]sulfate, it was noted that degradation occurred to varying degrees depending upon the starting concentration of pAdo-phosphosulfate. At the concentrations given above, almost all pAdo-phosphosulfate was degraded by 10 min in the Mes system and was degraded even more rapidly in the Tris system. Similar experiments in the Mes system were conducted with higher concentrations of pAdo-phosphosulfate (0.001 M) with lower specific activities (80×10^6 cpm/μmol). In these experiments about half the pAdo-phosphosulfate was still present after 1 hr and only a trace remained after 2 hr of incubation.

Addition of single sugars to endogenous "acceptor" material was accomplished by incubating microsomal preparations with the appropriate sugar nucleotide. Incubation mixtures consisted of 0.05 M Mes (pH 6.5), 0.01 M $MnCl_2$, UDP-[^{14}C]glucuronic acid (10 nmol (1.5×10^6 cpm)) or UDP-*N*-[^{3}H]acetylgalactosamine (10 nmol (1.8×10^6 cpm)), and 100 μl of microsomal preparation in a total volume of 200 μl. Reaction mixtures were kept at 37° for 2 hr.

The resulting labeled glycosaminoglycans were isolated from reaction mixtures, as previously described (Silbert, 1964, 1966) by chromatography on Whatman No. 1 paper in ethanol–1 M ammonium acetate (pH 7.8) (5:2). Degradation of radioactive substrates could be noted by scanning the chromatograms for radioactivity. In this chromatographic system 3′-phosphoadenosine 5′-phospho[^{35}S]sulfate and [^{35}S]sulfate are widely separated and easily identified. Degradation products of UDP-[^{14}C]glucuronic acid and UDP-*N*-[^{3}H]acetylgalactosamine are also widely separated from the sugar nucleotides. Chromatogram origins (containing all of the glycosaminoglycans) were then treated in the following ways. (1) Origins were incubated overnight at 37° with 2 ml of 1% pancreatin in 0.05 M Tris (pH 8.5). The suspensions were boiled and centrifuged, and the pellets were washed with 1 M NaCl as previously described (DeLuca and Silbert, 1968). The 1 M NaCl washing was added to the initial supernatant from the boiled pancreatin incubation and dialyzed overnight against several changes of water. The material

[1] Abbreviations used are: ΔDi-0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluc-4-enepyranosyluronic acid)-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluc-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluc-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; Mes, 2-(*N*-morpholino)ethanesulfonic acid; pAdo-phosphosulfate, 3′-phosphoadenosine 5′-phosphosulfate.

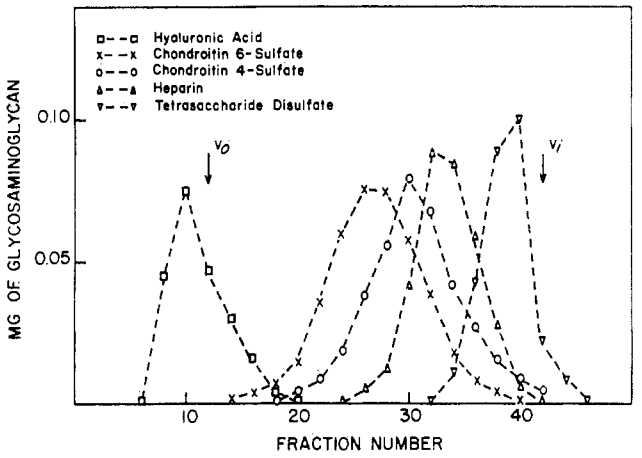

FIGURE 1: Chromatography of standards on Sepharose 4B. Glycosaminoglycan and oligosaccharide standards were chromatographed separately with Blue Dextran and Phenol Red as indicators on a column of Sepharose 4B (1 × 60 cm). Columns were eluted with 0.1 M LiCl at a flow rate of 4 ml/hr and 1-ml fractions were collected. Fractions were analyzed for the presence of uronic acid by the carbazole method, and V_0 and V_i were located by peak color intensity of the Blue Dextran and Phenol Red. Results of separate chromatograms are shown superimposed.

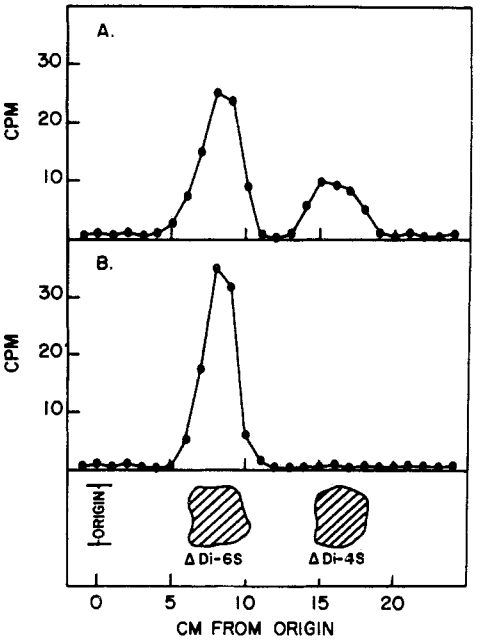

FIGURE 2: Products of chondroitinase digestion of [^{35}S]glycosaminoglycan. Samples of [^{35}S]glycosaminoglycan formed at pH 6.5 and 7.8 as described under Experimental Procedures were each incubated for 2 hr at 37° in a reaction mixture containing 20 μl of enriched Tris buffer (pH 8.0) (Saito *et al.*, 1968), 0.12 mg of chondroitin 4-sulfate, 0.12 mg of chondroitin 6-sulfate, and 0.5 unit of chondroitinase ABC in a total volume of 75 μl. Total reaction mixtures were chromatographed and assayed as described under Experimental Procedures: (A) the degradation products of [^{35}S]-glycosaminoglycan formed at pH 6.5; (B) the degradation products of [^{35}S]glycosaminoglycan formed at pH 7.8.

remaining with the dialysis tubing was assayed for radioactivity. The labeled material that had been at the origins of the chromatograms was generally recovered quantitatively within the dialysis tubing, although there were occasional losses of small amounts of radioactivity (less than 5% of the total). (2) An alternative method involved solubilization by alkali treatment. Origins were sequentially extracted for 18-hr periods with water (at room temperature), 0.1 N NaOH (0–2°) and 0.5 N NaOH (0–2°). Following alkali extraction the strips were treated with pancreatin as above (37°). Any unextractable material remaining on the paper after pancreatin treatment was assayed directly with a gas flow counter.

Samples of [^{35}S]glycosaminoglycan, [^{14}C]glycosaminoglycan, or [^{3}H]glycosaminoglycan were each placed together with standards of hyaluronic acid, chondroitin 4-sulfate, and tetrasaccharide disulfate on a DEAE-cellulose column and eluted with a logarithmic gradient of LiCl. (Elution was similar to the method of Hurlbert *et al.* (1954).) Aliquots of each fraction were assayed for radioactivity and for the standard glycosaminoglycans. Selected fractions from the column were pooled, desalted by gel filtration or dialysis, and lyophilized.

For reference purposes the relative molecular sizes of glycosaminoglycan standards and tetrasaccharide disulfate were determined by chromatography on a Sepharose 4B column (Figure 1) in a manner similar to that utilized by Wasteson (1971) in identification of the size of chondroitin sulfate chains. The standards obtained from Seikagaku Kogyo were also compared with standards from Dr. M. Mathews.

For estimation of molecular size, most samples of labeled glycosaminoglycan from lyophilized fractions of the DEAE-cellulose column were chromatographed on Sepharose 4B together with standard chondroitin 6-sulfate and markers of Blue Dextran (V_0)[2] and Phenol Red (V_i).[3] The sizes of the smallest molecular weight samples were estimated by chromatography on Sephadex G-15.

Samples of labeled glycosaminoglycan were incubated with chondroitinase ABC or AC, together with carrier chondroitin 4-sulfate and chondroitin 6-sulfate. After incubation, reaction mixtures were spotted on Whatman No. 1 paper and chromatographed overnight in 1-butanol–acetic acid–1 N NH_4OH (2:3:1) (Saito *et al.*, 1968). Disaccharide degradation products of the carrier chondroitin 4-sulfate and chondroitin 6-sulfate (ΔDi-4S and ΔDi-6S) were located with ultraviolet light. Strips of the chromatograms were eluted with water and the eluates assayed for radioactivity. The identity of the radioactive disaccharide products was confirmed by treatment with chondro-4-sulfatase and chondro-6-sulfatase.

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 into Endogenous Acceptors. [^{35}S]Sulfate, [^{14}C]-glucuronic acid, and *N*-[^{3}H]acetylgalactosamine were incorporated into the microsomal fraction. Since the number of counts per minute of [^{35}S]sulfate incorporated was much greater than the counts per minute of ^{14}C- or ^{3}H-labeled sugar incorporated (due to the much higher specific activity of the [^{35}S]sulfate), identification of the ^{35}S-labeled products could be more detailed.

Incorporation of [^{35}S]sulfate into microsomal acceptor glycosaminoglycan at pH 6.5 was somewhat faster and greater than that previously reported for incubation at pH 7.8 (De-

[2] Even though the Blue Dextran is partially included in relation to hyaluronic acid (Figure 1), it was used to represent V_0 for purposes of convenience. Molecular size estimations are not affected since these estimations are based on size relative to the various glycosaminoglycan standards and not relative to V_0.

[3] Phenol Red was found in the same position as [^{35}S]sulfate and is considered to be an accurate representation of V_i.

TABLE I: Characterization of Fractions from a DEAE-Cellulose Chromatogram of [^{35}S]Glycosaminoglycan.

Fraction No.	[^{35}S]Sulfate Incorporated		Approx Av. Mol Wt	Products after Chondroitinase Digestion	
	cpm	% of total		% as ΔDi-4S	% as ΔDi-6S
(A) 20–27	580	1.3	2,000	40	60
(B) 28–33	1,300	2.8	5,000	37	63
(C) 34–36	535	1.2		37	63
(D) 37–40	845	1.8	8,000	32	68
(E) 41–44	1,650	3.6		36	64
(F) 45–48	4,175	9.1	16,000	27	73
(G) 49–54	13,000	28.1	21,000	41	59
(H) 55–64	14,700	31.9	28,000	29	71
(I) 65–90	9,300	20.2	38,000	29	71
Total	46,085				

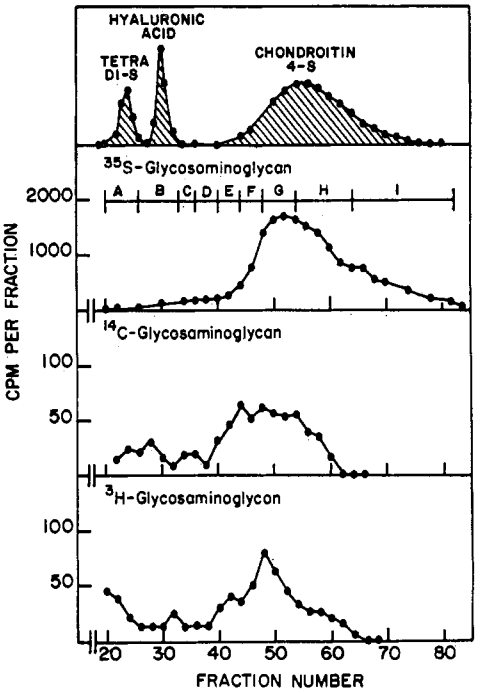

FIGURE 3: Gradient elution of [^{35}S]glycosaminoglycan, [^{14}C]glycosaminoglycan, and [^{3}H]glycosaminoglycan from a DEAE-cellulose column. Labeled glycosaminoglycan, formed as described under Experimental Procedures, together with tetrasaccharide disulfate (1 mg), hyaluronic acid (1 mg), and chondroitin 4-sulfate (6 mg), were eluted from a column of DEAE-cellulose (1 × 5 cm) with a LiCl logarithmic gradient. There were 125 ml of water in the mixing flask and 1 M LiCl in the reservoir. Fractions of 2.5 ml were collected and assayed for radioactivity. Standards were located by uronic acid determinations.

Luca and Silbert, 1968). This may in part be due to an observed greater stability of 3′-phosphoadenosine 5′-phosphosulfate at the lower pH. There was also a difference in the amount of incorporation of [^{35}S]sulfate into chondroitin 4-sulfate. Chondroitinase (either AC or ABC) treatment of the product formed in incubation at pH 6.5 demonstrated that 30–40% of the [^{35}S]sulfate incorporated was into the 4 position with the remainder incorporated into the 6 position, while at pH 7.8, [^{35}S]sulfate was incorporated only into the 6 position. This is shown in Figure 2. There were no disulfated disaccharides such as ΔDi-4,6S found after chondroitinase digestion of any of the chondroitin [^{35}S]sulfate preparations. (Chondroitin sulfate containing galactosamine 4,6-disulfate has been found in other tissues (Suzuki and Strominger, 1960b; Mathews and Decker, 1968; Suzuki *et al.*, 1968).) This indicates that the incorporation of sulfate into endogenous material was entirely onto nonsulfated galactosamine in the endogenous glycosaminoglycan. The structures of the sulfated disaccharides were confirmed by treating each with the appropriate chondrosulfatase. Chondro-4-sulfatase degraded the [^{35}S]ΔDi-4S, while chondro-6-sulfatase degraded the [^{35}S]ΔDi-6S.

Incorporation of [^{14}C]glucuronic acid and *N*-[^{3}H]acetylgalactosamine into microsomal acceptor glycosaminoglycan was examined at pH 6.5 and 7.8. Insufficient material was obtained with incubation at pH 7.8, so that all further work involved the products obtained by incubation at pH 6.5.

Characterization of Acceptors by Charge and Charge Density. Figure 3 shows the pattern of elution from DEAE-cellulose of [^{35}S]glycosaminoglycan, [^{14}C]glycosaminoglycan, and [^{3}H]glycosaminoglycan. Aliquots of fractions 40–64 from the DEAE-cellulose chromatograms of [^{14}C]glycosaminoglycan and [^{3}H]glycosaminoglycan were quantitatively degraded by chondroitinase, thus identifying this material as glycosaminoglycan. The small amounts of radioactive material appearing before fraction 30 were not modified by incubation with chondroitinase, so this material did not appear to be related to chondroitin sulfate and was not examined further. Results of the DEAE-cellulose chromatography of [^{35}S]glycosaminoglycan are summarized in Table I. [^{35}S]Glycosaminoglycan formed by longer incubations with 3′-phosphoadenosine 5′-phospho[^{35}S]sulfate of higher concentration and lower specific activity showed an identical pattern.

Characterization of Acceptors by Size. Various fractions from the DEAE-cellulose column chromatogram of [^{35}S]-glycosaminoglycan were examined by gel filtration to establish approximate molecular size (Figure 4). It can readily be seen that there was a progressive increase in size of the [^{35}S]-glycosaminoglycan directly related to the position of elution from the DEAE-cellulose column. Approximate molecular sizes were determined by the positions of elution of the [^{35}S]-glycosaminoglycan relative to the standards. This information is shown in Table I. Size estimation relative to the position on DEAE-cellulose chromatography was consistent with the results described by Hallén (1972) for chromatography of various chondroitin sulfate preparations on DEAE-cellulose.

The endogenous sulfate acceptor appeared to be predominantly slightly smaller than the chondroitin 4-sulfate standard whose average mol wt was given as 26,000. Thus, the largest portion of this endogenous acceptor material could be estimated to range in mol wt from ~15,000 to 30,000. There appeared to be some material of larger size and less material of smaller size; the smaller material was estimated by Sepharose filtration to range in mol wt from 2000 to 8000.

Since the relative position of [^{35}S]glycosaminoglycan elution from the DEAE-cellulose column was shown to be directly related to the size of the polysaccharide chain, it would therefore indicate that there were no significant differences in the final degree of sulfation (per galactosamine) of the chondroitin [^{35}S]sulfate. In particular, there was no indication of the presence of larger [^{35}S]glycosaminoglycan of low sulfate content, even after short incubations with pAdo-phospho[^{35}S]sulfate of

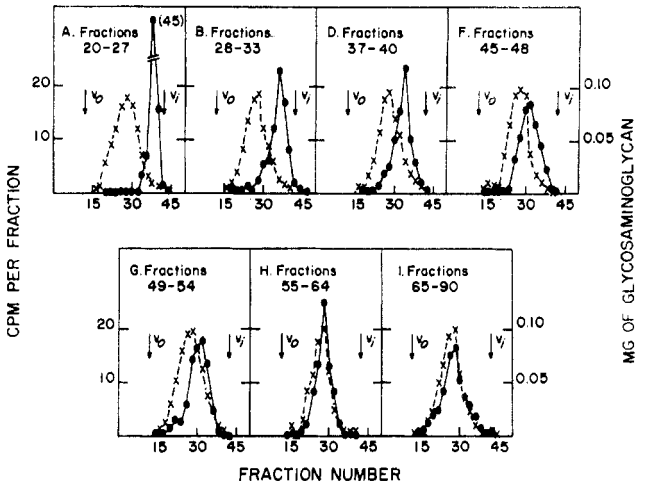

FIGURE 4: Chromatography of [^{35}S]glycosaminoglycan fractions on Sepharose 4B. Aliquots of fractions from the DEAE-cellulose column chromatogram (Figure 3, [^{35}S]glycosaminoglycan) together with standards of chondroitin 6-sulfate (1 mg), Blue Dextran, and Phenol Red were chromatographed on a column of Sepharose 4B (1 × 60 cm). For convenience of comparison, each aliquot taken for chromatography contained the same amount of radioactivity (150 cpm). Conditions of elution were identical with those described in Figure 1. Fractions were assayed for radioactivity (●—●) on a low background gas flow counter, and for standard chondroitin 6-sulfate (×- - -×) by carbazole determination.

high specific activity. If low sulfated high molecular weight material had been present, it would have been eluted from the DEAE-cellulose column before the standard chondroitin 4-sulfate. However, all the material eluted before the chondroitin 4-sulfate standard was of smaller molecular size as shown by Sepharose gel filtration.

Sepharose chromatography studies were performed on pooled fractions 45–64 obtained from the DEAE-cellulose chromatograms of [^{14}C]glycosaminoglycan and [^{3}H]glycosaminoglycan. Results were similar to those found with fractions 45–64 from the [^{35}S]glycosaminoglycan chromatogram.

Position of Sulfate. Fractions from the DEAE-cellulose column of [^{35}S]glycosaminoglycan were analyzed by chondroitinase digestion to determine the position of the sulfate groups. The proportions of chondroitin 4-[^{35}S]sulfate to chondroitin 6-[^{35}S]sulfate remained relatively constant through all the fractions from the DEAE-cellulose column ranging from 27 to 41% for chondroitin 4-sulfate and from 59 to 73% for chondroitin 6-sulfate (Table I). Thus, there was no difference in size between 4-[^{35}S]sulfate acceptors and 6-[^{35}S]sulfate acceptors, since the proportions of the two labeled chondroitin sulfates remained essentially constant for all sizes of [^{35}S]glycosaminoglycan analyzed.

Alkali Extractability of Acceptors. Results of a representative alkali extraction of [^{35}S]glycosaminoglycan from the origin of a paper chromatogram of an incubation mixture are shown in Table II. Almost all of the sulfated material could be separated from the microsomal preparations by 0.1 N NaOH. The small amount of the [^{35}S]glycosaminoglycan remaining on the paper after treatment with 0.1 N NaOH could not be extracted with 0.5 N NaOH, but was extractable with pancreatin. Further identification of the material which could not be extracted with alkali was not attempted due to insufficient amounts. In this experiment, >90% of the [^{14}C]glycosaminoglycan and [^{3}H]glycosaminoglycan was extractable with 0.1 N NaOH.

TABLE II: Extractability of [^{35}S]Glycosaminoglycan.

Method of Extraction	[^{35}S]Glycosaminoglycan Extracted	
	cpm	% of Total
H_2O	30	4.5
0.1 N NaOH	600	87.5
0.5 N NaOH	5	0.7
Pancreatin	45	6.6
Remainder after extractions	5	0.7

Samples of [^{35}S]glycosaminoglycan, [^{14}C]glycosaminoglycan, and [^{3}H]glycosaminoglycan solubilized by alkali rather than pancreatin were also chromatographed on DEAE-cellulose and Sepharose 4B. Results were similar to the chromatograms of pancreatin-solubilized glycoaminoglycans. Furthermore, aliquots of several fractions from the DEAE-cellulose column of pancreatin-solubilized [^{35}S]glycosaminoglycan were treated with alkali and chromatographed on Sepharose 4B. Results indicated that the size was not affected by this treatment.

Discussion

The DEAE-cellulose and Sepharose chromatography data presented in this paper indicate that there is an absence of nonsulfated polysaccharide chains in the microsomal preparation. This would indicate that the observed sulfate incorporation (both 4-sulfate and 6-sulfate) was onto an occasional nonsulfated galactosamine in a predominantly sulfated chain. Since some of these sulfated chains were shown to be of relatively short length and presumably are incompletely polymerized chondroitin sulfate, it would suggest that sulfation might take place during chain elongation with an occasional galactosamine being missed. This mechanism would require a similar intracellular location for polymerization and sulfation.

The amount of endogenous [^{35}S]glycosaminoglycan of various molecular sizes is not necessarily a true indication of the relative proportions of different sized endogenous polysaccharide in the microsomal preparation. Completely sulfated chains would not be seen since [^{35}S]sulfate could only be incorporated where the sulfation of the acceptor had been incomplete. However, it would appear from the data on the incorporation of [^{14}C]glucuronic acid and *N*-[^{3}H]acetylgalactosamine into the same or similar acceptors that the amount of small sized polysaccharide chains in the microsomal preparation (including those that might already be fully sulfated prior to incubation with pAdo-phospho[^{35}S]sulfate) is small in proportion to the amount of larger sized material.

Most of the microsomally bound radioactive glycosaminoglycan was found to be solubilized by alkali as well as by pancreatin, suggesting that the predominant linkage to protein is probably similar to the xylosyl-serine linkage found in chondroitin sulfate obtained from extracellular connective tissue. Although it remains to be proven, it seems reasonable to suggest that this linkage is identical with the extracellular proteoglycan link and the endogenous acceptors are indeed linked to protein by means of a galactosyl-galactosyl-xylosyl-serine "bridge." The linkage of the small amount of microsomal material that was not extractable with alkali is unknown, as is the relationship of this material to alkali stable

extracellular chondromucoproteins that have been found in other cartilage preparations (Lyons and Singer, 1971).

Acknowledgment

The authors wish to express their appreciation to Dr. L. Rodén for advice in the preparation of tetrasaccharides and for the supply of various oligosaccharides that were utilized in the identification of tetrasaccharide preparations.

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