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Hybrid Glycosaminoglycans Synthesized by Monolayers of Chick Embryo Arterial Fibroblasts†

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ABSTRACT: Monolayer cultures of arterial fibroblasts from 13-day chick embryonic aorta incorporated $^{35}SO_4^{2-}$ into glycosaminoglycans containing both glucuronic and iduronic acids. Bacterial chondroitinase ABC converted more than 98% of the $^{35}SO_4$-labeled polymer to mono- or disaccharides, including (1) *N*-acetyl-D-galactosamine 4-sulfate, (2) $\Delta^{4,5}$-glucuronic acid 2- or 3-sulfate→*N*-acetylgalactosamine 6-sulfate, and (3) the unsaturated disaccharides normally obtained from chondroitin 4-sulfate and chondroitin 6-sulfate sequences. Chondroitinase AC converted only 77% of the $^{35}SO_4$-labeled polymer to the same mono- and disaccharides and yielded, in addition, the following oligosaccharide products: (1) $\Delta^{4,5}$-glucuronic acid→*N*-acetylgalactosamine 4- or 6-sulfate→iduronic acid→*N*-acetylgalactosamine 6- or 4-sulfate; (2) *N*-acetylgalactosamine 4-sulfate→iduronic acid 2- or 3-sulfate→*N*-acetylgalactosamine 6-sulfate; (3) $\Delta^{4,5}$-glucuronic acid→*N*-acetylgalactosamine 4-sulfate→(iduronic acid→*N*-acetylgalactosamine 4-sulfate)$_2$; (4) $\Delta^{4,5}$-glucuronic acid→*N*-acetylgalactosamine 4- or 6-sulfate→(iduronic acid→*N*-acetylgalactosamine 6- or 4-sulfate)$_2$; (5) higher oligosaccharides containing iduronic acid and *N*-acetylgalactosamine 4-sulfate.

Three types of polymeric structures were distinguished in early studies on the sulfated glycosaminoglycans of connective tissues: chondroitin 4-sulfate, composed of repeating GlcUA→GalNAc-4-SO_4 disaccharides (A units),[1] chondroitin 6-sulfate, composed of repeating GlcUA→GalNAc-6-SO_4 disaccharides (C units), and dermatan sulfate, composed of repeating IdUA→GalNAc-4-SO_4 disaccharides (B units) (Jeanloz, 1970; Mathews, 1975). More recently, evidence has been presented suggesting that chondroitin sulfate is a copolymer of A and C disaccharide units (Habuchi et al., 1973; Kimata et al., 1974; Seno et al., 1974, 1975), and it has also been recognized that dermatan sulfate is a hybrid structure composed predominantly of B units but also containing A and C units (Fransson & Malmström, 1971; Habuchi et al., 1973; Cöster et al., 1975; Malmström et al., 1975a). All of these polymers occur as protein–polysaccharides in which the repeating disaccharide units are linked to a core protein through a unique galactosyl–galactosyl–xylose linkage region (Rodén & Horowitz, 1978).

Present evidence indicates a close biosynthetic relationship among the three disaccharide types found in these connective tissue polysaccharides (Rodén & Horowitz, 1978). Chondroitin, the initial biosynthetic product, is made up of repeating GlcUA→GalNAc disaccharide units and is attached to the core protein through the usual linkage region. It serves as the unsulfated polymeric precursor for copolymers of A, B, and C units. Specific sulfotransferases catalyze the transfer of

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[1] Abbreviations used: GlcUA, D-glucuronic acid; IdUA, L-iduronic acid; GalNAc, *N*-acetyl-D-galactosamine; unit A, GlcUA→GalNAc-4-SO_4; unit B, IdUA→GalNAc-4-SO_4; unit C, GlcUA→GalNAc-6-SO_4; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-β-D-*gluco*-hex-4-enopyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-β-D-*gluco*-hex-4-enopyranosyluronic acid)-6-*O*-sulfo-D-galactose.

0006-2960/79/0418-5475$01.00/0

sulfate from phosphoadenosine phosphosulfate to chondroitin to generate A and C units (Rodén & Schwartz, 1975). B units are formed by C_5 epimerization of the D-glucuronic acid residues of chondroitin, followed by a specific sulfation reaction (Malmström et al., 1975b). Copolymers of A, B, and C units apparently result from divergent metabolic fates of the disaccharide units of chondroitin. A body of data concerning the distribution of A, B, and C units in these hybrid structures is accumulating, largely from the laboratories of Fransson (Fransson & Malmström, 1971; Cöster et al., 1975; Malmström et al., 1975a) and Suzuki (Habuchi et al., 1973), but the mechanisms that regulate the maturation of chondroitin are not understood.

The most active systems for the biosynthesis of sulfated glycosaminoglycans are found in the connective tissues of young organisms (Buddecke et al., 1973). Chondrocyte cultures prepared from chick embryo cartilage incorporate $^{35}SO_4^{2-}$ into chondroitin sulfate at high rates (Nevo & Dorfman, 1972; Schwartz et al., 1974; Schwartz & Dorfman, 1975). These polymers vary in their ratios of A to C units depending on the source of the cartilage cells and the conditions of culture (Kim & Conrad, 1976, 1977). However, chondrocytes have a very low capacity for synthesis of polymers containing B disaccharide units (Kimata et al., 1978). Arterial tissues, on the other hand, synthesize polymers containing A, B, and C units, and the relative proportions of these three units in the products are reflected in the structures and yields of copolymeric oligosaccharides that can be generated from the biosynthetic products (Buddecke & Kresse, 1973). The present report describes the structural characterization of the hybrid glycosaminoglycans synthesized by chick embryo aorta fibroblast cultures.

Experimental Procedures

Cell Culture. Arterial cell cultures were prepared from the left and right innominate arteries, the systemic aorta, the ductus arteriosis, and the left pulmonary artery of 13-day chick embryos (stage 39). Sections of these tissues (3–5 mm) proximal to the heart were placed in Dulbecco's pH 7.4 Tris–saline containing $CaCl_2$ (0.26 g/L) and $MgSO_4 \cdot 7H_2O$ (0.2 g/L) and were freed of adhering pericardial tissue. The arterial tissue was minced with scissors and shaken in 5 mL of a 0.25% solution of 2× crystallized trypsin in Tris–saline G (10 mL of 1 M Tris-HCl, pH 7.0, plus 8.5 g of NaCl plus 100 mL of glycerol plus 890 mL of H_2O) for 1 h at 37 °C. The trypsin solution was removed and the tissue was shaken with 5 mL of a solution containing 28 units of collagenase (Sigma Chemical Co., Type I, 280 units/mg) and 50 units of elastase (Sigma Chemical Co., Type II, 60 units/mg) for 18 min at 37 °C. The partially dissociated tissue was drawn up in a sterile Pasteur pipette 4–5 times, and, after incubation for an additional 2 min, the single cell suspension was separated from undissociated tissue fragments and diluted with 5 mL of culture medium (below). The cells were pelleted and resuspended for culture in Dulbecco's modified Eagle's medium, containing 2 g of D-glucose, 50 mg of streptomycin sulfate and 100 000 units of penicillin per L, and 10% fetal calf serum (Kim & Conrad, 1976). Cells were plated at 1×10^6 cells/5 mL of medium in 60-mm Falcon plastic tissue culture dishes and cultured at 35 °C in 7% CO_2–93% air. Medium was replaced on day 3 and at 2-day intervals thereafter. Dye exclusion tests showed that 94% of the freshly dissociated cells were viable. The cultures contained fibroblastic cells with uniform morphology and grew exponentially with a cell doubling time of approximately 24 h. At confluence the cells continued to grow to a density of $(10–12) \times 10^6$ cells/dish, yielding cultures containing 2 to 3 layers of cells.

Preparation of $^{35}SO_4$-Labeled Oligosaccharides from Arterial Glycosaminoglycans. Preliminary studies demonstrated that the arterial fibroblast cultures incorporated $^{35}SO_4^{2-}$ into chondroitinase-sensitive glycosaminoglycans at 0.15 nmol/(h 10^6 cells) and that this rate was stimulated 3–4-fold by the addition of *p*-nitrophenyl β-D-xyloside to the cultures. Furthermore, the β-xyloside preferentially stimulated the synthesis of hybrid sequences but did not alter the qualitative profiles of the hybrid sequences (see Results). Therefore, the $^{35}SO_4$-labeled glycosaminoglycans used here for structural characterizations of the hybrids were prepared by using cultures containing the β-xyloside. The medium from day 5 cultures was removed and replaced with 5 mL of culture medium containing 1 mM *p*-nitrophenyl β-D-xyloside and 100 μCi/mL of $H_2{}^{35}SO_4$ (New England Nuclear). After incubation for 22 h at 35 °C, the culture medium, which contained more than 90% of the total $^{35}SO_4$-labeled glycosaminoglycan product, was removed, dialyzed exhaustively against 0.1 M aqueous Na_2SO_4 and then water, and finally dried by lyophilization. The dried preparation was redissolved in one-fifth of its original volume of water.

Aliquots of the $^{35}SO_4$-labeled glycosaminoglycan solution were digested with thermolysin and chondroitinase AC or ABC as described previously (Kim & Conrad, 1974, 1976). Briefly, a 200-μL aliquot of sample was adjusted to pH 7.3 with 2 μL of enriched Tris buffer (Yamagata et al., 1968; Saito et al., 1968) and digested with 20 μg of thermolysin (Sigma, Type X, 83 units/mg) at 37 °C for 20 h. Thermolysin action was blocked by addition of 10 μL of 0.1 M EDTA, pH 7.0, to chelate Ca^{2+}, and 0.2 unit of chondroitinase ABC or chondroitinase AC in 20 μL of a 1:10 dilution of pH 7.3 enriched Tris buffer was added. The chondroitinase digestion was allowed to proceed for 4 h at 37 °C. Preliminary experiments showed that the yields of oligosaccharides generated under these conditions did not change after 2 h of incubation.

Chromatography and Electrophoresis of $^{35}SO_4$-Labeled Oligosaccharides. The chondroitinase digest was spotted 2.5 in. from one end of a 1 × 22.5 in. strip of Whatman No. 3 chromatography paper, and the oligosaccharides were resolved by development of the chromatogram for two 24-h descents in solvent 1 (below). After the first descent the chromatogram was dried and scanned for radioactive peaks by using a strip scanner. The end of the strip was then cut off between the major peak, ΔDi-6S, and ΔDi-4S, the next most prominent peak, which moved ahead of the ΔDi-6S peak. A new 1 × 10 in. segment of Whatman No. 3 paper was sewed onto the bottom of the original chromatogram prior to the second descent. The excised strip and the original chromatogram were cut into 0.5-in. segments which were counted in a scintillation counter (Kim & Conrad, 1976, 1977).

Solvents used for paper chromatography were as follows: solvent 1, 1-butanol, glacial acetic acid, and 1 N ammonium hydroxide (2:3:1.5); solvent 2, 1-butanol, glacial acetic acid, and 1 N ammonium hydroxide (2:3:1).

Samples were electrophoresed on 1 × 22.5 in. strips of Whatman No. 3 paper in pyridine, glacial acetic acid, and water (1:5:400). Samples were spotted 4.5 in. from one end of the strip and electrophoresed toward the anode for 1–2 h at 25 V/cm. Dried strips were cut into 0.5-in. segments which were counted as described above.

Oligosaccharides used for structural characterization studies were purified to homogeneity by preparative paper chromatography and electrophoresis as described above. The preparative strips were cut into segments containing the purified

Table I: Products of Chondroitinase Digestion of Aorta Fibroblast Mucopolysaccharides

oligosaccharide		$R_{\Delta Di\text{-}6S}$[a] chromatography			oligosaccharide yield			
					% of total $^{35}SO_4$		mol/mol of D[d]	
designation[b]	assigned structure[c]	solvent 1	solvent 2	electrophoresis	AC digest	ABC digest	AC	ABC
origin[d]	$(IdUA\rightarrow GalNAc\text{-}4S)_n$	0.0			10.8	1.8		
A_I		0.2			4.0	0	0.5	0
A_{Ix}	$\Delta UA\rightarrow GalNAc\text{-}4(6)S\rightarrow[IdUA\rightarrow GalNAc\text{-}6(4)S]_2$[e]			1.0				
A_{Iz}	$\Delta UA\rightarrow GalNAc\text{-}4S\rightarrow(IdUA\rightarrow GalNAc\text{-}4S)_2$[e]			1.0				
A_{II}	$GalNAc\text{-}4S\rightarrow IdUA\text{-}2(3)S\rightarrow GalNAc\text{-}6S$	0.4		1.3	2.4	0	0.3	0
B	$\Delta UA\rightarrow GalNAc\text{-}4(6)S\rightarrow IdUA\rightarrow GalNAc\text{-}6(4)S$	0.6	0.3	1.0	6.5	0	1.2	0
C	$\Delta UA\text{-}2(3)S\rightarrow GalNAc\text{-}6S$	0.7	0.5	1.3	4.0	5.4	0.7	1.0
ΔDi-6S	$\Delta UA\rightarrow GalNAc\text{-}6S$	1.0	1.0	1.0	50.8	53.9	18.8	20.0
ΔDi-4S	$\Delta UA\rightarrow GalNAc\text{-}4S$		1.7	1.0	19.2	35.0	7.1	13.0
D	$GalNAc\text{-}4S$	1.7	2.8	0.8	2.7	4.0	1.0	1.5
standards								
ΔDi-4S	$\Delta UA\rightarrow GalNAc\text{-}4S$		1.7	1.0				
GalNAc-6S	$GalNAc\text{-}6S$		1.8	0.8				
GalNAc-4S	$GalNAc\text{-}4S$		2.8	0.8				

[a] Ratio of distance traveled to distance traveled by standard ΔDi-6S. [b] See Figure 1. [c] Abbreviations used: ΔUA, 4-deoxy-D-*gluco*-4-hexenopyranosyluronic acid; GalNAc, *N*-acetyl-D-galactosamine; IdUA, L-iduronic acid; S, sulfate (number preceding S designates position of sulfate). [d] Percent of total $^{35}SO_4$ divided by (percent of total $^{35}SO_4$ in peak D of chondroitinase AC digest plus moles of $^{35}SO_4$ per mole of oligosaccharide). [e] Tentative.

components, and the labeled oligosaccharides were eluted from the segments with water by using spin thimbles (Reeve Angel Co.). Eluates were concentrated under reduced pressure and rechromatographed and/or reelectrophoresed if further purification was required. All products used for structural studies gave single, symmetrical peaks of radioactivity on both chromatograms and electrophoretograms.

The oligosaccharides were characterized by their rates of chromatographic or electrophoretic migration relative to standard ^3H-labeled ΔDi-6S, run on the same strip of Whatman No. 3 paper. The [^3H]ΔDi-6S was prepared from [^3H]-chondroitin sulfate synthesized by incubation of chick embryo epiphyseal chondrocytes with [^3H]glucosamine as described earlier (Kim & Conrad, 1976, 1977). The [^3H]ΔDi-6S was isolated from chondroitinase digests of the [^3H]chondroitin sulfate by preparative paper chromatography. [^3H]ΔDi-4S, isolated from the same chromatograms, was used to prepare [^3H]GalNAc-4-SO_4 by partial acid hydrolysis in 0.1 N HCl at 100 °C for 60 min (Suzuki, 1960).

Periodate Oxidation. Samples were dissolved in 30 μL of 0.02 M $NaIO_4$ in 0.033 M sodium acetate, pH 3.8, and incubated in the dark for 60 min at 20 °C. Excess periodate was destroyed by addition of 3 μmol of ethylene glycol.

Sulfatase Digestion. Susceptibility of oligosaccharides to desulfation by chondrosulfatases (Yamagata et al., 1968) was tested by using chondro-6-sulfatase and chondro-4-sulfatase from Miles Laboratories. Substrates were incubated with 40 μL of a solution containing 0.025 unit of sulfatase in enriched Tris buffer, pH 7.5 (Yamagata et al., 1968). At the end of 1 h the entire incubation mixture was spotted on a strip of Whatman No. 3 paper for chromatography or electrophoresis.

Results

Oligosaccharides from $^{35}SO_4$-Labeled Mucopolysaccharides. Figure 1 shows the paper chromatographic profiles of $^{35}SO_4$-labeled oligosaccharides obtained by digestion of aorta fibroblast mucopolysaccharides with chondroitinases AC and ABC. Because of the multiple development procedure required to separate the slower moving oligosaccharides (see Experimental Procedures), ΔDi-4S appears in two peaks in these profiles. Equal aliquots of digested mucopolysaccharide are shown for the chondroitinase AC (panel a) and chondroitinase ABC (panel b) treatments. In both digests the major peaks are ΔDi-6S and ΔDi-4S. The chondroitinase AC digest contains, in addition, peaks designated A_I, A_{II}, B, C, and D, as well as undigested material at the origin of the chromatogram. In the chondroitinase ABC profile, the origin material is much reduced, peaks A_I, A_{II}, and B are absent, and the ΔDi-4S and peaks C and D are larger than the corresponding peaks in the chondroitinase AC digest. Each of the unidentified components was purified to electrophoretic and chromatographic homogeneity as described under Experimental Procedures, and the pure compounds were characterized according to their rates of paper chromatographic and paper electrophoretic migrations (relative to ΔDi-6S) and yields, as shown

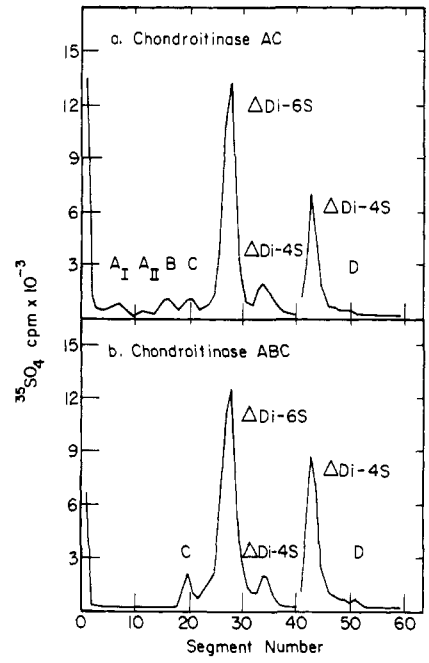

FIGURE 1: Duplicate aliquots of $^{35}SO_4$-labeled biosynthetic product were treated with thermolysin and then chondroitinase AC (panel a) or chondroitinase ABC (panel b). The digests were paper chromatographed on Whatman No. 3 paper in solvent 1, and the segments of the chromatograms were counted as described under Experimental Procedures.

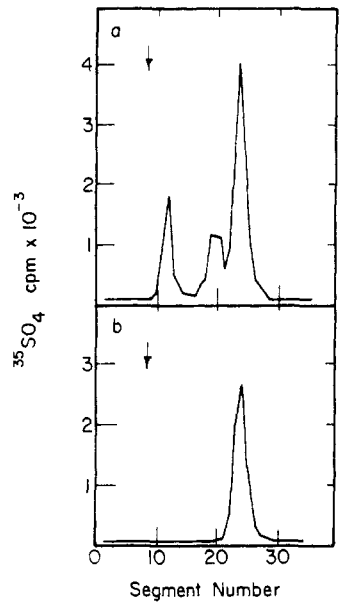

FIGURE 2: Characterization of peak D. Panel a shows a partial acid hydrolysate (0.1 N HCl; 100 °C; 60 min) of standard $^{35}SO_4$-labeled ΔDi-4S paper chromatographed for 24 h in solvent 1. The fastest moving peak is GalNAc-4-SO_4, the intermediate peak is unhydrolyzed ΔDi-4S, and the slowest peak is free SO_4^{2-}. Panel b shows purified peak D chromatographed under identical conditions. The arrows indicate the position of standard ΔDi-6S.

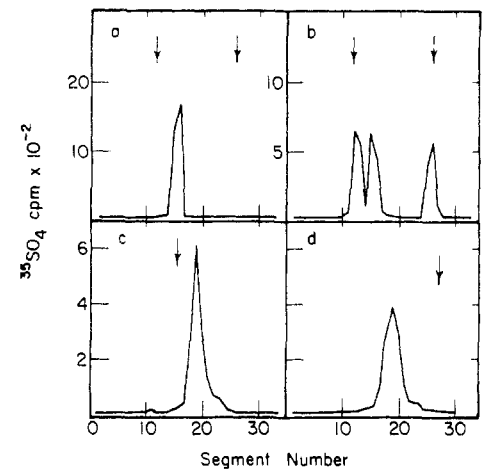

FIGURE 3: Characterization of peak C. Panels a and b show the paper electrophoretic migration of products obtained by treatment of purified peak C with chondro-4-sulfatase and chondro-6-sulfatase, respectively. The peak of radioactivity at segment 15 is unreacted peak C. The solid and dashed arrows show the positions of migration of standard ΔDi-6S and SO_4^{2-}, respectively. The slow moving peak in panel b was eluted, and its paper chromatographic migration was compared with that of standards. Panel c shows the migration (solvent 1) of the slow peak from panel b compared to that of standard ΔDi-6S (arrow). Panel d shows the migration (solvent 2) of the slow peak from panel b compared to that of standard ΔDi-4S (arrow).

in Table I. All of the products contain bound $^{35}SO_4$. The final structural assignments and molar ratios are also shown in Table I. The data supporting these assignments are presented below.

Characterization of Peak D. The yield of peak D is maximized by exhaustive digestion of the $^{35}SO_4$-labeled mucopolysaccharide preparation with chondroitinase ABC. On paper chromatograms, peak D migrates ahead of ΔDi-4S, the most rapidly migrating sulfated disaccharide normally found in chondroitinase digests of mucopolysaccharides. This suggests that peak D is a sulfated monosaccharide. For the purpose of obtaining monosaccharide standards for comparison with peak D, ΔDi-6S and ΔDi-4S were subjected to a limited acid hydrolysis (Suzuki, 1960) to release GalNAc-6-SO_4 and GalNAc-4-SO_4, respectively. Figure 2a shows a paper chromatographic profile of a partial hydrolysate of ΔDi-4S. The slowest moving peak in the profile is identified as free $^{35}SO_4^{2-}$ by chromatographic and electrophoretic comparison with standard $^{35}SO_4^{2-}$. The intermediate peak is unhydrolyzed ΔDi-4S. GalNAc-4-SO_4, which peaks at segment 23 of the chromatogram, comigrates with the purified peak D. Under the same chromatographic conditions GalNAc-6-SO_4 obtained in ΔDi-6S hydrolysates comigrates with ΔDi-4S and is clearly resolved from peak D (Glaser & Conrad, 1979a). The paper electrophoretic migration rate of peak D is also identical with that of the sulfated GalNAc's (Table I) and is slower than expected for a sulfated uronic acid (see below) which is doubly charged. On the basis of these data, peak D is identified as GalNAc-4-SO_4.

Characterization of Peak C. Peak C was obtained in good yield from chondroitinase AC digests, but the yield was even higher in chondroitinase ABC digests. The high electrophoretic mobility of peak C relative to that of standard ΔDi-6S and the somewhat lower chromatographic mobility (Table I) are properties observed previously for a disulfated uronic acid containing disaccharide (Shively & Conrad, 1976). The nature of the sulfation of peak C was partially elucidated by determining its susceptibility to chondrosulfatases. Panels a and b of Figure 3 show the electrophoretic profiles of peak C after its treatment with chondro-4-sulfatase and chondro-6-sulfatase, respectively. The mobility of peak C is not altered by chondro-4-sulfatase treatment, but chondro-6-sulfatase converts peak C into equimolar amounts of free $^{35}SO_4^{2-}$ and a slower moving peak with a mobility equal to that of the ΔDi-6S standard. The slower moving product of chondro-6-sulfatase treatment was eluted from the paper electrophoretogram segments, and its paper chromatographic behavior was compared with those of standard ΔDi-6S and ΔDi-4S as shown in panels c and d of Figure 3. The data show that the product of chondro-6-sulfatase treatment of peak C is neither ΔDi-6S nor ΔDi-4S but a structure that migrates between the two standards. Since previous studies have demonstrated the strict specificity of chondro-6-sulfatase for disaccharide substrates (Seno et al., 1974), and since the product of chondro-6-sulfatase treatment of peak C migrates on electrophoretograms and chromatograms in the monosulfated disaccharide region and is not further cleaved by chondroitinase ABC, it is concluded that peak C is a disulfated disaccharide in which one of the SO_4 substituents is at C_6 of the GalNAc residue and the second is at C_2 or C_3 of the nonreducing terminal $\Delta^{4,5}$-uronic acid, a disaccharide identified previously by Suzuki et al. (1968). For further substantiation of this structural assignment, peak C was treated with sodium periodate under conditions that destroyed the ΔDi-6S standard. Figure 4 shows that the rates of chromatographic and electrophoretic migration of peak C are unaltered by the periodate treatment. Under the same oxidation conditions standard ΔDi-6S is converted to multiple products. The resistance of peak C to periodate is consistent with the structure proposed. Further evidence for the unsaturation of the uronic acid moiety is presented in the characterization of peak A_{II}.

Characterization of Peak A_{II}. The presence of peak A_{II} in chondroitinase AC digests but not in chondroitinase ABC digests indicates that it contains an internal, chondroitinase ABC susceptible iduronic acid residue. Like peak C, peak A_{II} exhibits a higher electrophoretic mobility than the ΔDi-6S standard (Table I). Digestion of peak A_{II} with chondroitinase

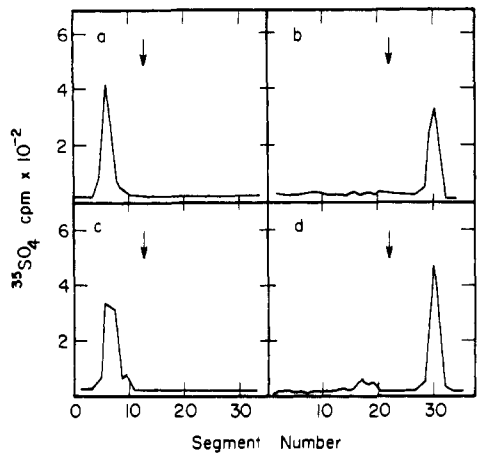

FIGURE 4: Effect of periodate treatment on the migration of peak C. A sample of purified peak C was treated with $NaIO_4$ as described under Experimental Procedures. Panels a and c compare the paper chromatographic migrations of periodate-treated (panel a) and untreated (panel c) peak C in solvent 2 (40 h). Panels b and d compare the paper electrophoretic migrations of periodate-treated (panel b) and untreated (panel d) peak C. The arrows show the positions of migration of standard ΔDi-6S.

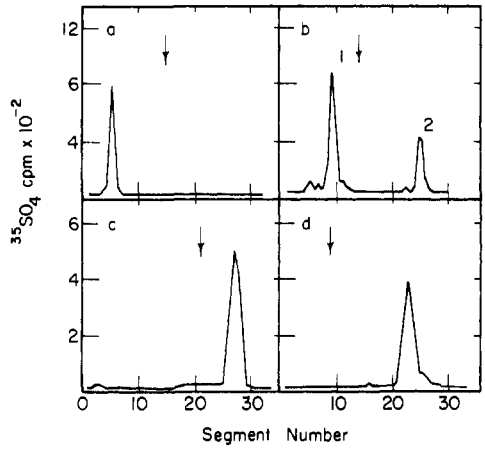

FIGURE 5: Characterization of peak A_{II}. Purified peak A_{II}, before (panel a) and after (panel b) chondroitinase ABC digestion, was paper chromatographed in solvent 1 for 48 h. Products 1 and 2 from the chondroitinase-treated sample were eluted from the paper segments and examined further. Panel c shows the paper electrophoretic migration (2 h) of product 1 from panel b. Panel d shows the paper chromatographic migration of product 2 from panel b in solvent 2 (24 h). The arrows indicate the positions of migration of standard ΔDi-6S.

ABC yielded two new peaks (Figure 5) which were identified by their chromatographic and electrophoretic migrations as the previously characterized peaks C and D, as shown in Table II. The total number of $^{35}SO_4$ counts per minute in the peak C formed from A_{II} is approximately twice the number of $^{35}SO_4$ counts per minute recovered in peak D, as expected from the identifications of peak C as a disulfated disaccharide and peak D as a monosulfated monosaccharide. These data lead to the structural assignment for peak A_{II} shown in Table I. The finding that peak C can be formed by chondroitinase ABC digestion of peak A_{II} confirms the conclusion above that peak C has an unsaturated uronic acid at its nonreducing terminal.

Characterization of Peak B. The presence of peak B in chondroitinase AC digests and its absence in chondroitinase ABC digests indicate that peak B contains an internal, chondroitinase ABC susceptible iduronic acid residue. Its electrophoretic migration at a rate identical with that of ΔDi-6S (Table I) suggests that it contains, on the average, one SO_4 substituent per disaccharide unit. Its chromatographic behavior and its chondroitinase ABC susceptibility indicate that it contains more than a single disaccharide unit. β-Glucuronidase does not act on peak B. However, chondroitinase ABC converts peak B to two products which migrate on paper chromatograms in positions corresponding to ΔDi-6S and ΔDi-4S, as shown in Figure 6 and Table II. The total number of counts per minute in each of the products was identical. Free $^{35}SO_4^{2-}$ was released from product 1, eluted from the ΔDi-6S position, by chondro-6-sulfatase (Figure 6c) but not by chondro-4-sulfatase; similarly, chondro-4-sulfatase, but not chondro-6-sulfatase, released free $^{35}SO_4^{2-}$ from product 2, eluted from the ΔDi-4S position (Figure 6d). These observations lead to the conclusion that peak B is an oligosaccharide containing equimolar amounts of the 6-sulfated and 4-sulfated disaccharides and that the internal uronic acid is an iduronic acid residue. Since the oligosaccharide is generated by chondroitinase AC but is resistant to β-glucuronidase, the nonreducing terminal uronic acid is unsaturated. Although the data could fit either an octasaccharide or a tetrasaccharide, only the latter possibility is consistent with the rates of migration

Table II: Characterization of Products Formed by Chondroitinase ABC Digestion of Peaks A_{II} and B

saccharide	$^{35}SO_4$ cpm[a]	$R_{\Delta Di\text{-}6S}$[b] chromatography solvent 1	chromatography solvent 2	electrophoresis
standards				
ΔDi-4S			1.7	1.0
peak C		0.7		1.3
peak D		1.7	2.8	
peak A_{II} products[c]				
product 1	2284	0.7		1.3
product 2	984	1.7	2.8	
peak B products[d]				
product 1	3090	1.0	1.0	1.0
product 2	3030		1.7	1.0

[a] Total $^{35}SO_4$ counts per minute in each product peak (Figures 5b and 6b). [b] Ratio of distance moved to distance moved by standard ΔDi-6S. [c] From Figure 5b. [d] From Figure 6b.

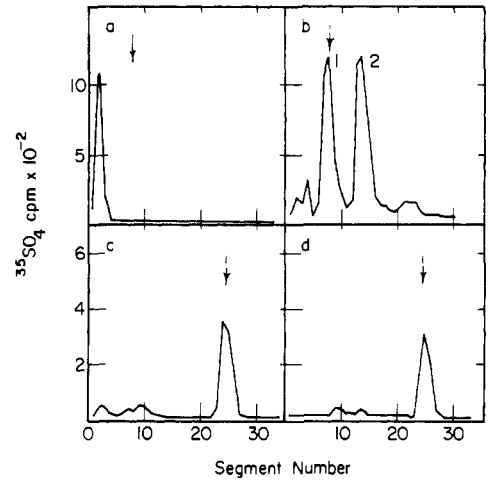

FIGURE 6: Characterization of peak B. Purified peak B, before (panel a) and after (panel b) digestion with chondroitinase ABC, was paper chromatographed in solvent 2 for 21 h. Products 1 and 2 from the chondroitinase-treated sample were eluted from the paper segments and examined further. Panel c shows the paper electrophoretic profile of chondro-6-sulfatase-treated product 1 from panel b. Panel d shows the paper electrophoretic profile of chondro-4-sulfatase-treated product 2 from panel b. The solid arrows indicate the positions of migration of standard ΔDi-6S; the dashed arrows show the positions of $^{35}SO_4^{2-}$.

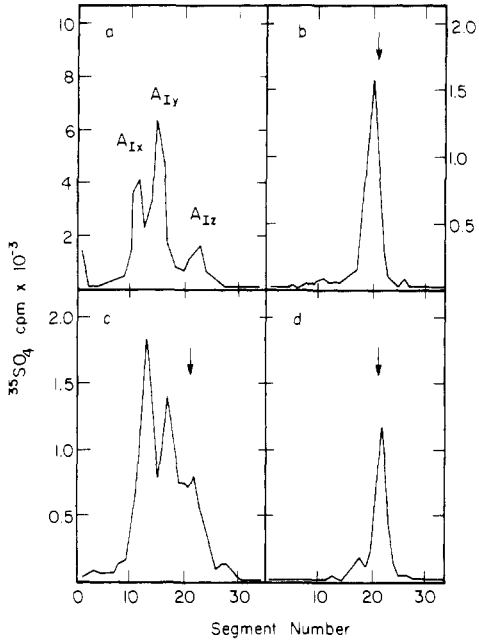

FIGURE 7: Purification of the components of peak A_I. Peak A_I was eluted from chromatograms of chondroitinase AC digests (Figure 1a) and rechromatographed on Whatman No. 3 paper for 6 days in solvent 1. The separation profile is shown in panel a. Peaks A_{Ix}, A_{Iy}, and A_{Iz} were eluted separately and paper electrophoresed for 2 h, as shown in panels b, c, and d, respectively. The arrows indicate the positions of migration of standard ΔDi-6S.

of peak B in solvents 1 and 2 (Glaser & Conrad, 1979b). The data do not define the sequence of the two disaccharides in the tetrasaccharide.

Characterization of Peak A_I. When peak A_I was eluted from the initial chromatogram of the chondroitinase AC digestion products (Figure 1a) and rechromatographed for a longer development time, it separated into three peaks, designated A_{Ix}, A_{Iy}, and A_{Iz} in Figure 7a. Elution of the three peaks from the Figure 7a chromatogram gave A_{Ix} and A_{Iz}, both of which were electrophoretically (panels b and c of Figure 7) and chromatographically (not shown) pure and both of which electrophoresed at the same rate as standard ΔDi-6S. A_{Iy}, however, contained a mixture of components, most of which were distinguishable from A_{Ix} and A_{Iz} by their electrophoretic behavior (Figure 7c). Figure 8 shows the chromatographic profiles of the products obtained when A_{Ix} (panel a) and A_{Iz} (panel b) were digested with chondroitinase ABC. A_{Iz} yields a single peak with a chromatographic mobility of ΔDi-4S. The major products obtained from A_{Ix} appear to be a mixture of ΔDi-4S and ΔDi-6S in a 2:1 molar ratio. The amounts of the A_I components obtained were insufficient for a more detailed structural characterization. The available information suggests tentative identifications of A_{Ix} and A_{Iz} as hexasaccharides in which all of the internal uronic acids are iduronic acid residues. A_{Ix} appears to contain two ΔDi-4S and one ΔDi-6S disaccharides, while A_{Iz} appears to contain three ΔDi-4S disaccharides.

Discussion

Arterial tissue was chosen for the present study because it had previously been reported to accumulate dermatan sulfate–chondroitin sulfate copolymers (Buddecke & Kresse, 1973). Mature arterial tissue proximal to the heart consists of three concentric cell layers: an inner tunica intima, a tunica media, and an outer tunica adventitia (Ham, 1969). However, in developing chick embryos (days 5–18) the tunica media predominates (Karrar, 1960), and this tissue thus provides a

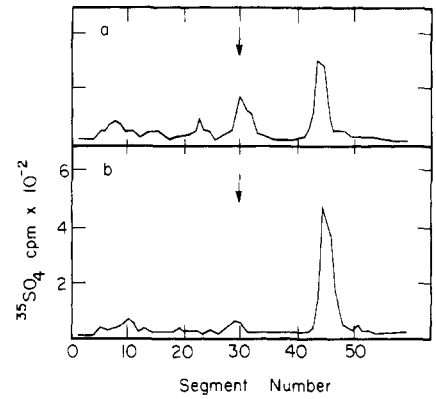

FIGURE 8: Characterization of peaks A_{Ix} and A_{Iz} from panels b and d of Figure 7, respectively. Purified peaks A_{Ix} (panel a) and A_{Iz} (panel b) were digested with chondroitinase ABC and paper chromatographed for 48 h in solvent 1. The arrows indicate the positions of migration of standard ΔDi-6S.

relatively homogeneous population of fibroblastic cells. The aorta fibroblast cultures described here incorporate $^{35}SO_4^{2-}$ from the culture medium into mucopolysaccharides at approximately one-twentieth the rate reported for chick embryo chondrocytes (Kim & Conrad, 1976, 1977; Glaser & Conrad, 1979a). More than 98% of the incorporated $^{35}SO_4$ is converted to mono- or disaccharides by chondroitinase ABC (Table I), indicating that chondroitin sulfate–dermatan sulfate polymers are the primary $^{35}SO_4$-containing products synthesized by these cells. Chondroitinase AC converts only 77% of the incorporated $^{35}SO_4$ to mono- and disaccharides. Thus, approximately 20% of the total chondroitinase ABC sensitive material produced by these cells is present in hybrid sequences which contain both D-glucuronic and L-iduronic acid residues. These hybrid sequences have been the focus of this work. A number of structural features of the oligosaccharides which are generated by the bacterial chondroitinases can be inferred from the known specificities of these enzymes (Yamagata et al., 1968; Saito et al., 1968). Fragments formed from internal regions of the polymers have 4-deoxy-β-D-*gluco*-hex-4-enopyranosyluronic acid (structurally identical 4-deoxy-α-L-*ido*-hex-4-enopyranosyluronic acid) residues at their nonreducing terminals and $GalNAcSO_4$ residues at their reducing terminals. Furthermore, since chondroitinase AC is specific for linkages in which the uronic acid is D-glucuronic acid while chondroitinase ABC cleaves bonds in which the uronic acid is either D-glucuronic or L-iduronic acid, the release of an oligosaccharide by one or both of these enzymes may indicate (1) the residue to which the reducing terminal of the released oligosaccharide was linked in the original polymer and (2) the uronic acid (glucuronic or iduronic) from which the nonreducing terminal unsaturated uronic acid was derived. The only fragments formed by chondroitinase digestion which are not terminated by an unsaturated uronic acid are those derived from the nonreducing terminals of the original substrates. A substrate with a nonreducing terminal uronic acid yields a disaccharide containing a saturated uronic acid from its nonreducing end. If a $GalNAcSO_4$ residue is at the nonreducing end of the substrate, it will be released as a free, sulfated monosaccharide (Glaser & Conrad, 1979a). Thus, only monosaccharides or saturated disaccharides, both of which are readily separated from the unsaturated disaccharides by paper chromatography (Glaser & Conrad, 1979a), are derived from the nonreducing terminals of the original polymers.

Since all of the oligosaccharides described here are recognized and quantitated by their $^{35}SO_4$ labels, any unsulfated sequences in these polymers would not be observed. However,

unsulfated GalNAc residues generally represent only a small percentage of the total GalNAc residues in these polymers (Kimata et al., 1974; Kim & Conrad, 1974), and the present data indicate that there were no unsulfated GalNAc residues in the oligosaccharides described. Among the products of chondroitinase digestion of the aorta mucopolysaccharides, only one disulfated disaccharide was found, namely, 4-deoxy-*gluco*-hex-4-enouronic acid 2- or 3-sulfate→GalNAc-6-SO_4 (peak C). In chondroitinase AC digests this disaccharide is found both as a free peak C disaccharide and as a latent product in a trisaccharide (peak A_{II}) from which peak C is formed by chondroitinase ABC treatment. The chondroitinase AC specificity dictates that the free peak C in the chondroitinase AC digest must have been generated from a [GlcUA-2(3)-SO_4→GalNAc-6-SO_4]→GlcUA→ sequence, while the chondroitinase AC resistant A_{II} was generated from a GalNAc-4-SO_4→[IdUA-2(3)-SO_4→GalNAc-6-SO_4]→GlcUA→ sequence (peak C precursor is indicated in brackets). As can be seen from the molar ratios given in Table I, the yields of peak C and GalNAc-4-SO_4 (peak D) in the chondroitinase ABC digest are elevated over their yields in the chondroitinase AC digest by amounts that can be accounted for solely by the conversion of peak A_{II} to peaks C and D; i.e., the additional peak C and peak D in the chondroitinase ABC digest do not appear to be derived from the higher molecular weight material at the origin of the chromatogram of the chondroitinase AC digest.

Sulfated glucuronic acid has not been reported in these polymers previously, but sulfated iduronic acid residues have been identified in pig skin dermatan sulfate (Fransson et al., 1974a,b; Cöster et al., 1975) and shark cartilage (Suzuki et al., 1968). In pig skin the iduronic acid sulfate is linked to an unsulfated GalNAc residue (Cöster et al., 1975), a sequence not found in the present work. In the study of shark cartilage mucopolysaccharides, chondroitinase ABC digests yielded 2-acetamido-2-deoxy-3-*O*-(4-deoxy-β-D-*gluco*-hex-4-enopyranosyluronic acid 2- or 3-sulfate)-6-*O*-sulfo-D-galactose (Suzuki et al., 1968), identical with peak C described here. However, it was not possible to state whether the original sulfated uronic acid in the shark cartilage product was a D-glucuronic or an L-iduronic acid residue because chondroitinase ABC destroyed the asymmetry at the C_5 of the uronic acid. Sequences containing sulfated uronic acids linked to a 4-sulfated GalNAc residue, found in bovine lung (Suzuki et al., 1968), were not observed here nor were sequences containing iduronic acid linked to disulfated GalNAc residues.

The only nonreducing terminal found among the chondroitinase digestion products of aorta fibroblast mucopolysaccharides is GalNAc-4-SO_4, which constitutes 4% of the bound SO_4 in the starting material. Calculations using the chondroitinase ABC data in Table I show that the average degree of polymerization of the chondroitinase-sensitive aorta mucopolysaccharides is 47 monosaccharide residues per GalNAc-4-SO_4 equivalent to a molecular weight of 11 800. Approximately 75% of the total GalNAc-4-SO_4 was released as the monosaccharide by chondroitinase AC; thus, 75% of the nonreducing terminal GalNAc-4-SO_4 in the original polymer mixture was linked to a glucuronic acid residue. The remaining GalNAc-4-SO_4 terminal in chondroitinase AC digests is recovered in peak A_{II} where it is linked to an IdUA-2(3)-SO_4→GalNAc-6-SO_4. Thus, the nonreducing terminal sequence of the remaining 25% of the original mucopolysaccharide chains is the GalNAc-4-SO_4→IdUA-2(3)-SO_4→GalNAc-6-SO_4→GlcUA→ sequence referred to above. Sjöberg et al. (1973) have presented evidence that the normal nonreducing terminal residue in dermatan sulfate produced by human skin fibroblasts is GalNAcSO_4. In the latter work the position of the sulfate substituent on the GalNAc was not determined.

The most prominent differences in the products of chondroitinase AC and ABC digestion of the arterial glycosaminoglycans are the higher levels of ΔDi-4S and ΔDi-6S in the chondroitinase ABC products. All of the additional ΔDi-6S and some of the ΔDi-4S in the chondroitinase ABC digests are derived from the cleavage of peaks B and A_I, but at least 70% of the additional ΔDi-4S must be derived from the higher oligosaccharides that remain at the origin of the chromatograms of the chondroitinase AC digest. The degree of polymerization of the origin fragments has not been determined, but it is clear from their resistance to chondroitinase AC and their conversion to ΔDi-4S by chondroitinase ABC that a relatively large fraction of the iduronic acid containing disaccharide units in these polymers is found in those sequences which contain 4 or more disaccharide units and therefore do not migrate on chromatograms (the oligosaccharides which contain 3 disaccharide units migrate as peak A_I) and that the GalNAc residues in these iduronic acid rich sequences are 4-sulfated. This is the most common sequence found in fully matured dermatan sulfate higher animals (Suzuki et al., 1968; Habuchi et al., 1973). This material is only 10% of the total sulfated mucopolysaccharides synthesized by the arterial cells. This may indicate that a fully matured dermatan sulfate [e.g., one in which more than 90% of the uronic acid residues in the polymer have been biosynthetically converted to L-iduronic acid residues (Malmström et al., 1975b)] represents 10% of the total sulfated mucopolysaccharides; alternatively, it may indicate that most of the sulfated polymers synthesized by these cells contain 10% L-iduronic acid and 90% D-glucuronic acid. Further data will be required to distinguish between these two extremes or the intermediate possibilities. Hybrid sequences containing both D-glucuronic acid and L-iduronic acid, represented here by peaks A_{Ix} and B, have been found previously by Habuchi et al. (1973) in the meniscus of the human knee joint and by Fransson et al. (1974b) in pig skin.

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Protein Synthesis Kinetics with Ribosomes from Temperature-Sensitive Mutants of *Escherichia coli*†

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ABSTRACT: The kinetics of MS2 ribonucleic acid (RNA) directed protein synthesis have been investigated at seven temperatures between 30 and 47 °C by using ribosomes isolated from a wild type strain and seven temperature-sensitive mutants of *Escherichia coli*. The amount of MS2 coat protein formed at each temperature was determined by gel electrophoresis of the products formed with control ribosomes. With ribosomes from each of the mutant strains, the activation energy required to drive protein synthesis below the maximum temperature (up to 40 °C) was increased relative to the control (wild type) activity. Preincubation of the ribosomes at 44 °C revealed the kinetics of thermal inactivation, with ribosomes from each of the mutants having a half-life for inactivation less than that of the control ribosomes. A good correlation was observed between the relative activity of the different ribosomes at 44 °C and their relative rate of thermal inactivation. Mixing assays allowed the identification of a temperature-sensitive ribosomal subunit for each of the mutants. Defects in one or more of three specific steps in protein synthesis (messenger RNA binding, transfer RNA binding, and subunit reassociation) were identified for the ribosomes from each mutant. The relationship between temperature sensitivity and protein synthesis in these strains is discussed.

The biosynthesis of protein in cells involves a complex series of reactions beginning with free amino acids and ending with a structurally complete and functionally active macromolecule. The intermediate steps between these two states have been systematically investigated by a large number of workers [see the review by Weissbach & Pestka (1977)]. Although protein synthesis is a very complex activity, involving numerous cooperative elements, in vitro it can be analyzed kinetically and thermodynamically as a simple two-state process, going from free amino acids to polypeptide (Spirin, 1978). A critical component for this transition is the ribosome. Its structural and functional contributions to the process of protein synthesis have been extensively examined by a variety of biochemical techniques and assays (Nomura et al., 1974). A recent approach to the study of the involvement of the multiple macromolecular components of the ribosome in protein formation has been the isolation of a collection of temperature-sensitive mutants of *Escherichia coli*, defective in protein synthesis at 44 °C (Kushner et al., 1977; Champney, 1979). For some of these mutants, the ribosome has been identified as the source of the temperature-sensitive protein synthesis activity.

In order to quantitate the effect of temperature on the ribosomal activity and to identify the ribosomal component responsible for the temperature effect, we have conducted a

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0006-2960/79/0418-5482$01.00/0