The Copolymeric Structure of Dermatan Sulphate Produced by Cultured Human Fibroblasts

DIFFERENT DISTRIBUTION OF IDURONIC ACID- AND GLUCURONIC ACID-CONTAINING UNITS IN SOLUBLE AND CELL-ASSOCIATED GLYCANS

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The structure of dermatan [^{35}S]sulphate-chondroitin [^{35}S]sulphate copolymers synthesized and secreted by fibroblasts in culture was studied. ^{35}S -labelled glycosaminoglycans were isolated from the medium, a trypsin digest of the cells and the cell residue after 72 h of $^{35}\text{SO}_4^{2-}$ incorporation. The galactosaminoglycan component (dermatan sulphate-chondroitin sulphate copolymers) was isolated and subjected to various degradation procedures including digestion with testicular hyaluronidase, chondroitinase-AC and -ABC and periodate oxidation followed by alkaline elimination. The galactosaminoglycans from the various sources displayed significant structural differences with regard to the distribution of various repeating units, i.e. IdUA-GalNAc-SO₄ (L-iduronic acid-N-acetyl-galactosamine sulphate), GlcUA-GalNAc-SO₄ (D-glucuronic acid-N-acetyl-galactosamine sulphate) and IdUA-(SO₄)-GalNAc (L-iduronosulphate-N-acetyl-galactosamine). The galactosaminoglycans of the cell residue contained larger amounts of IdUA-GalNAc-SO₄ than did those isolated from the medium or those released by trypsin. In contrast, the glycans from the latter two sources contained large proportions of periodate-resistant repeat periods [GlcUA-GalNAc-SO₄ and IdUA-(SO₄)-GalNAc]. Periods containing L-iduronic acid sulphate were particularly prominent in copolymers found in the medium. Kinetic studies indicated that the ^{35}S -labelled glycosaminoglycan of the cell residue accumulated radioactivity more slowly than did the glycans of other fractions, indicating that the material remaining with the cells was not exclusively a precursor of the secreted polymers. The presence of copolymers rich in glucuronic acid or iduronic acid sulphate residues in the soluble fractions may be the result of selective secretion from the cells. Alternatively, extracellular, polymer-level modifications such as C-5 inversion of L-iduronic acid to D-glucuronic acid, or sulphate rearrangements, would yield similar results.

Fibrous connective tissue is the major source of the acid galactosaminoglycan dermatan sulphate (Meyer *et al.*, 1956). Dermatan sulphate is composed largely of the repeating unit L-iduronic acid-N-acetyl-D-galactosamine 4-sulphate (Fransson, 1970). Variable quantities of D-glucuronic acid and L-iduronic acid O-sulphate are also present in the polymer (Fransson *et al.*, 1974a,b). In the tissue the dermatan sulphate-chondroitin sulphate copolymeric chains are covalently bound to a protein core to form a proteoglycan (see, e.g., Fransson, 1970). The polysaccharide chains can be isolated after proteolytic digestion of the proteoglycan.

Connective-tissue cells synthesize and secrete these macromolecules. Both dermatan sulphate and chondroitin sulphate have been isolated from fibroblasts grown in culture (Suzuki *et al.*, 1968; Saito & Uzman, 1971; Bates & Levene, 1971; Goggins *et al.*, 1972; Wasteson *et al.*, 1973). In the course of studies on

dermatan sulphate-storage diseases (Hurler's and Hunter's syndromes) partial chemical characterization of intracellular as well as extracellular dermatan sulphate from normal cells has been performed (Matalon & Dorfman, 1966; Schafer *et al.*, 1968; DiFerrante *et al.*, 1971). Moreover, the kinetics of intracellular accumulation and secretion into the medium has been documented (Fratantoni *et al.*, 1968; DiFerrante *et al.*, 1971). The present study is concerned with a more detailed chemical characterization of dermatan sulphate-chondroitin sulphate copolymers obtained from cultured human fibroblasts. The medium, a trypsin digest of the cells in monolayer and the cell residue were studied separately. The galactosaminoglycans of the various sources displayed distinct differences with regard to their copolymeric structure. This has encouraged speculations with regard to the possibility of polymer-level modifications of the macromolecules.

Experimental

Materials

Dermatan sulphate and hyaluronic acid were prepared from pig skin (Fransson & Rodén, 1967a). Chondroitin 4-sulphate was obtained from horse nasal septum and chondroitin 6-sulphate from shark cartilage (Antonopoulos *et al.*, 1961). A mixture of polysaccharides (including hyaluronate, dermatan sulphate, chondroitin sulphate, heparan sulphate, heparin and sulphated glycopeptides) was isolated from pig intestine (I. Carlstedt & L.-Å. Fransson, unpublished work). *N*-Acetylchondrosine was the same preparation as that described previously (Fransson & Malmström, 1971). Chondroitin sulphate di-, tetra- and hexa-saccharides were isolated after digestion with testicular hyaluronidase (Fransson & Malmström, 1971). 4-Sulphated or 6-sulphated unsaturated disaccharides were prepared by chondroitinase-AC digestion of the respective polysaccharides (Yamagata *et al.*, 1968). Bovine serum albumin and twice-crystallized trypsin from bovine pancreas (type III) were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Carrier-free $\text{Na}_2^{35}\text{SO}_4$ (92 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Chondroitinase-AC and -ABC and chondro 4- and 6-sulphatase were obtained from Miles Laboratories, Elkhart, Ind., U.S.A. Papain was prepared by the method of Kimmel & Smith (1954). Testicular hyaluronidase (20000 units/mg) was a product of AB Leo, Helsingborg, Sweden. Earle's minimal essential medium was obtained from Flow Laboratories, Irvine, Ayrshire, U.K. Penicillin and streptomycin sulphate were purchased from AB KABI, Stockholm, Sweden. Calf serum was a product of the Statens Bakteriologiska Laboratorium, Stockholm, Sweden. Sephadex G-25 and G-50 (superfine grade) and G-150, as well as Blue Dextran, were obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Microgranular DEAE-cellulose (Whatman type DE-32) was used for ion-exchange chromatography. Other chemicals were of analytical grade.

Analytical and chromatographic methods

Uronic acid was determined by an automated version of the carbazole-borate method (Heinegård, 1973). Protein was determined as described by Lowry *et al.* (1951) with bovine serum albumin as standard. Radioactivity was measured with a Packard Tri-Carb liquid-scintillation counter. The scintillation mixtures used were Instagel (3 ml of liquid mixed with 2 ml of sample) and Omnifluor in toluene (4 g/litre). The latter scintillator was used for counting strips of paper chromatograms (10 ml/strip of 4 cm²).

Electrophoresis of glycosaminoglycans was carried out on strips of cellulose acetate in 0.1 M-HCl (1.9 V/

cm for 6 h) as described by Wessler (1971). High-voltage paper electrophoresis of oligo- and mono-saccharides was performed on Whatman 3 MM paper in buffer A (0.2 M-acetic acid brought to pH 5.0 by the addition of pyridine) (40 V/cm; 45 min or 1.5 h). The shorter time was used when split products obtained after chondrosulphatase digestion were separated. Papers were stained with aniline hydrogen phthalate (Partridge, 1949).

Ion-exchange chromatography (stepwise elution) was carried out on columns (3 mm × 60 mm) of DE-32 DEAE-cellulose equilibrated with 0.25 M-pyridine acetate, pH 5.0. The columns were eluted stepwise with 2 ml each of 0.25, 0.50, 0.75 and 3.0 M-pyridine acetate, pH 5.0 (0.25, 0.50, 0.75 and 3.0 M-acetic acid brought to pH 5.0 by the addition of pyridine). Gradient elution was performed on columns (6 mm × 140 mm) of the same resin. The ion exchanger was precycled according to the recommendation of the manufacturers and finally equilibrated with 0.10 M-sodium acetate buffer, pH 5.0 (starting buffer). The samples (with 0.5 mg of pig intestinal polysaccharide as carrier) were dissolved in the starting buffer and applied to the column. Elution was performed with a linear gradient (0.10–2.50 M-sodium acetate, pH 5.0; total elution volume, 100 ml) at a rate of 3 ml/h. The recovery of radioactivity in this procedure was 85–90%.

Degradative methods

Sodium salts of poly- or oligo-saccharides were digested by chondroitinase-AC or -ABC in 0.2 M-Tris-HCl, pH 8.0, at 37°C overnight. Digestion mixtures contained (per ml) 0.02 unit of enzyme, 0.1 mg of serum albumin and 1 mg of carrier (dermatan sulphate or chondroitin sulphate hexasaccharide). Hydrolysis with chondro 4- and 6-sulphatases was performed as described by Yamagata *et al.* (1968). Digestion with testicular hyaluronidase was carried out as described by Fransson & Rodén (1967b). Poly- and oligo-saccharides (radioactive material plus carrier) were oxidized by sodium periodate at pH 3.0 and 4°C for 24 h as described by Fransson (1974). The oxidized polysaccharides were subsequently cleaved by alkaline elimination at pH 12 and room temperature for 30 min (Fransson & Carlstedt, 1974). Oligosaccharides so obtained [general carbohydrate structure, $\text{GalNAc}-(\text{UA-GalNAc})_n\text{-R}^*$

* Abbreviations: IdUA, L-iduronic acid; IdUA-SO₄, L-iduronic acid O-sulphate; UA, uronic acid; R, fragment derived from an oxidized and degraded iduronic acid

(Fransson *et al.*, 1974a,b)] were resolved by gel chromatography on Sephadex G-50.

Incorporation of $^{35}\text{SO}_4^{2-}$ into glycosaminoglycans by cultured fibroblasts

Fibroblast cultures were established from skin specimens of human foetuses which were obtained surgically (age, 15 weeks). The cells were grown as monolayers in glass flasks at 37°C. They were fed twice a week with Earle's minimal essential medium supplemented with 10% (w/v) calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml). Confluent cultures were maintained in Roux flasks (220 cm²) containing 55 ml of sulphate-poor Earle's medium (two changes of medium in which MgCl_2 was substituted for MgSO_4). At this stage $\text{Na}_2^{35}\text{SO}_4$ (5 µCi/ml) was administered (final concentration of sulphate, 0.05 mM) and the cells were left to incorporate the radioactive isotope for 72 h. After the incorporation period the medium was collected and the monolayer was washed twice with Earle's balanced salt solution. The washings were combined with the medium. The cells were brought into suspension by treatment with 55 ml of prewarmed 0.01% (w/v) trypsin in Earle's balanced salt solution for 35 min at 37°C with occasional gentle shaking (Kraemer, 1971a). The cells were recovered by centrifugation at 600g for 10 min and washed once with Earle's balanced salt solution. The washing was combined with the trypsin digest. After this treatment 90–95% of the cells were viable as determined by the Trypan Blue staining method (Paul, 1965). Finally, the trypsin digests as well as the media were centrifuged at 10 000g for 10 min to ensure removal of cellular debris.

The trypsin digests and the media (together with 0.5 mg of carrier dermatan sulphate) were dialysed against 0.1 M-(NH_4)₂ SO_4 for 6 h and then against water for 2 days. ^{35}S -labelled macromolecules were recovered from the retentates by freeze-drying. After digestion with papain (three batches each of 0.6 mg/ml added at 0, 3 and 6 h) in 5 ml of 1.0 M-NaCl–0.05 M-EDTA (disodium salt)–0.01 M-cysteine–HCl–0.05 M-sodium phosphate buffer, pH 6.9, for 24 h at 65°C the material was dialysed against water for 2 days. The cells were subjected to three freeze-thaw cycles (together with 0.5 mg of carrier dermatan sulphate) in 5 ml of the above-mentioned buffer and similarly digested with papain followed by dialysis. Final purification of ^{35}S -labelled glycosaminoglycans was achieved by ion-exchange chromatography (gradient elution) was used for large-scale experiments; see above). A small and variable amount of radioactivity was not retained by the column. Since this material did not coincide with free $^{35}\text{SO}_4^{2-}$, it is probably peptide-bound. Radioactive, anionic material was pooled, dialysed and recovered by freeze-drying.

^{35}S -labelled galactosaminoglycan was isolated from the ^{35}S -labelled glycosaminoglycans of the cells, the trypsin digest and the medium, by the following procedure. Material (approx. 0.5 mg) was dissolved in 1.5 ml of 0.24 M- NaNO_2 –1.8 M-acetic acid and kept at room temperature for 80 min (Lagunoff & Warren, 1962). By this treatment glucosaminidic linkages of non-acetylated or *N*-sulphated residues in heparan sulphate are cleaved. Galactosaminoglycans which contain solely *N*-acetylated residues resist this treatment (Lindahl *et al.*, 1973). Excess of HNO_2 was evaporated with methanol (Lindahl *et al.*, 1973), the residue was dissolved in buffer A, and the material was subjected to gel chromatography on a column (8 mm × 1400 mm) of Sephadex G-50 (eluent, buffer A; elution rate, 9 ml/h). ^{35}S -labelled galactosaminoglycans were recovered from the void-volume fractions by freeze-drying.

Heparan [^{35}S]sulphate was isolated from the same materials after exhaustive digestion with chondroitinase-ABC (this enzyme degrades the galactosaminoglycan component) followed by gel chromatography on the same column.

Results

Synthesis and secretion of ^{35}S -labelled glycosaminoglycans

The rate of accumulation of ^{35}S -labelled glycosaminoglycans was followed by incorporation of $^{35}\text{SO}_4^{2-}$ into macromolecular anionic products obtained from the cells, the trypsin digest and the medium (Fig. 1). The amount of radioisotope incorporated into the polysaccharides of the cell fraction reached a plateau after 48 h (Fig. 1a). Trypsin-released ^{35}S -labelled glycosaminoglycans accumulated rapidly and constituted 60% of the total radioactivity after 3 h (Fig. 1b). This pool also reached a plateau after 48 h. Accumulation of ^{35}S -labelled glycosaminoglycans in the medium progressed linearly with time for the entire length of the experiment. After 72 h of incubation ^{35}S -labelled glycosaminoglycans were distributed approximately as follows: 10% in the cells, 30% in the trypsin digest and 60% in the medium.

The ^{35}S -labelled glycosaminoglycans obtained from the three sources after 72 h of incubation were subjected to HNO_2 degradation and digestion with chondroitinase-ABC to quantify the amounts of ^{35}S -labelled galactosaminoglycan and heparan [^{35}S]sulphate respectively. In all three fractions galactosaminoglycans accounted for more than 80% of the total radioactivity. The approximate amounts of heparan [^{35}S]sulphate were 10% in the cells, 20% in the trypsin digest and 10% in the medium. The yields of material resistant to chondroitinase-ABC and material susceptible to HNO_2 were in good agreement.

Fig. 1. Incorporation of $^{35}\text{SO}_4^{2-}$ into glycosaminoglycans isolated from the cells (●), the trypsin digest (○) and the medium (Δ)

Fibroblasts were grown to confluence in glass flasks (45 cm^2) containing 12 ml of minimal essential medium. After two changes of medium containing MgCl_2 instead of MgSO_4 , $\text{Na}_2^{35}\text{SO}_4$ ($5\text{ }\mu\text{Ci/ml}$) was introduced. The cells were left to incorporate radioisotope for the indicated periods of time. Medium, trypsin digest and cells were recovered as described in the Experimental section. After papain digestion and dialysis, ^{35}S -labelled glycosaminoglycans were purified by ion-exchange chromatography (stepwise elution). Free and peptide-bound radioactive sulphate was eluted with 0.5 M -pyridine acetate, whereas ^{35}S -labelled glycosaminoglycans were recovered from the last fraction (3.0 M -pyridine acetate). Appropriate portions of this fraction were assayed for radioactivity. An expanded plot of the first 12 h in (a) is shown in (b).

The molecular size distribution of the two ^{35}S -labelled glycosaminoglycans was studied by gel chromatography on Sephadex G-150. Fig. 2 shows that the ^{35}S -labelled glycosaminoglycans from the various source were very polydisperse. However, all ^{35}S -labelled galactosaminoglycan molecules appeared to be within the same range of molecular size. The heparan [^{35}S]sulphate obtained from the trypsin digest (Fig. 2b) was significantly larger than that found in the medium (Fig. 2c).

To investigate the charge polydispersity of the ^{35}S -labelled glycosaminoglycans, ion-exchange chromatography (gradient elution) was performed. As Fig. 3 shows, the heparan [^{35}S]sulphate and ^{35}S -labelled galactosaminoglycan components were poorly resolved by this technique, suggesting a relatively high sulphate content in the heparan sulphate molecules. The trypsin digest contained a significant heparan sulphate component (Fig. 3b) confirming the estimations presented above. The ^{35}S -labelled galactosaminoglycans obtained from the three sources were eluted in the position of standard dermatan sulphate. The same ^{35}S -labelled polysaccharides were also subjected to cellulose acetate electrophoresis in 0.1 M -HCl. The results indicated that the various ^{35}S -labelled galactosaminoglycans had the same sulphate content as standard dermatan sulphate.

Structure of ^{35}S -labelled galactosaminoglycans

The galactosaminoglycan dermatan sulphate is composed of three principal types of repeat periods, i.e. those containing IdUA, IdUA- SO_4 or GlcUA (Fransson *et al.*, 1974b). The IdUA and GlcUA residues are usually combined with GalNAc- SO_4 to form monosulphated repeat periods. The IdUA- SO_4 residues may be combined with either GalNAc or GalNAc- SO_4 to form mono- or di-sulphated repeats (Cöster *et al.*, 1975). All of these repeat periods may be released as disaccharides by chondroitinase-ABC.

The ^{35}S -labelled galactosaminoglycans obtained after HNO_2 degradation of ^{35}S -labelled glycosaminoglycan were completely degraded by chondroitinase-ABC. The split products were resolved into disaccharides and linkage-region fragments by gel chromatography. High-voltage paper electrophoresis of the disaccharides gave only monosulphated species from the ^{35}S -labelled galactosaminoglycan of the cell fraction. Small amounts of disulphated species were obtained from the ^{35}S -labelled galactosaminoglycans of the trypsin digest (2%) and the medium (4%). Monosulphated disaccharides obtained by chondroitinase-ABC digestion could be derived from three different repeating units, i.e. (a) IdUA-GalNAc- SO_4 , (b) GlcUA-GalNAc- SO_4 and (c) IdUA- (SO_4) -

GalNAc (Fransson *et al.*, 1974b; Cöster *et al.*, 1975). Two of these disaccharides (*a* and *b*) are susceptible to chondrosulphatase (Suzuki *et al.*, 1968). The disaccharides derived from the various ^{35}S -labelled galactosaminoglycans were digested exhaustively with a mixture of chondro 4- and 6-sulphatase followed by electrophoresis to separate free and disaccharide-bound sulphate. The yield of sulphatase-resistant disaccharide, corresponding mainly to IdUA($-\text{SO}_4$)-GalNAc, was 11, 18 and 35% from the ^{35}S -labelled galactosaminoglycans obtained from the cells, the trypsin digest and the medium respectively.

The distribution of the different repeat periods and thus the character of the copolymeric sequence may be assessed after various chemical and enzymic degradations (Scheme 1). The ^{35}S -labelled galactosaminoglycans obtained from the cells, the trypsin digest and the medium were separately subjected to periodate oxidation-alkaline elimination. Fig. 4 shows that selective oxidation and scission of non-sulphated IdUA residues produced distinctly different degradation patterns for the various species. The ^{35}S -labelled galactosaminoglycan of the cell fraction (Fig. 4a) was degraded to a larger extent than were those of the trypsin digest and the medium (Figs. 4b and 4c).

Fig. 2. Gel chromatography on Sephadex G-150 of ^{35}S -labelled glycosaminoglycans (—), ^{35}S -labelled galactosaminoglycan (····) and heparan [^{35}S]sulphate (---) obtained from the cells (*a*), the trypsin digest (*b*) and the medium (*c*) after 72 h of $^{35}\text{SO}_4^{2-}$ incorporation

^{35}S -labelled glycosaminoglycans were isolated from the various compartments as described in the Experimental section. ^{35}S -labelled galactosaminoglycan and heparan [^{35}S]sulphate refer to ^{35}S -labelled polymers recovered after HNO_2 degradation and chondroitinase-ABC digestion respectively. Column size, 8 mm $\times$ 1500 mm; eluent, 0.2M-pyridine acetate, pH 5.0; elution rate, 3 ml/h; V_0 , elution volume of Blue Dextran. In an attempt to reconstitute the elution pattern of the total ^{35}S -labelled glycosaminoglycans the chromatograms of the galactosaminoglycan and heparan sulphate components were combined in the appropriate ratios (4:1 and 9:1, w/w, for the material derived from the trypsin digest and the medium respectively). A comparison between the elution pattern for the total material derived from the trypsin digest (—, in *b*) and the reconstituted curve showed that the mixture of the two polysaccharides was eluted before the individual polysaccharides. This might be due to degradation of galactosaminoglycans during the HNO_2 treatment. However, galactosaminoglycans from the cells were unaffected (*a*). Although the presence of free amino groups or *N*-sulphate groups in galactosaminoglycans has never been observed, it is possible that structural differences between intra- and extra-cellular molecules may result in partial susceptibility to HNO_2 . Alternatively, an interaction between the two polysaccharides may account for the observation.

Almost equal proportions of the various oligosaccharides ($n = 1-7$) were obtained from the cell material, whereas the polymers of the other two fractions preferentially gave rise to longer oligosaccharide fragments ($n = 5-7$). However, gel chromatography on Sephadex G-150 of periodate-resistant fragments from the ^{35}S -labelled galactosaminoglycan of the trypsin digest suggested the absence of completely periodate-resistant polysaccharides. The results of periodate oxidation-alkaline elimination (Figs. 4a-4c) were used to calculate the amounts of IdUA-GalNAc-SO₄ periods in the various polymers (Table 1). The polysaccharides of the trypsin digest and the medium contained fewer IdUA-GalNAc-SO₄ periods than did those of the cell fraction. It may thus be concluded that both the amount and the distribution of periodate-resistant periods (containing GlcUA or IdUA-SO₄) differed between galactosaminoglycans retained by the cells and those solubilized by trypsin or present in the medium.

To estimate the proportions of GlcUA-GalNAc-SO₄ and IdUA(-SO₄)-GalNAc in the periodate-resistant material eluted in the void volume, digestions with chondroitinase-AC were performed (Fig. 5). From the yield of disaccharide it may be inferred that GlcUA-GalNAc-SO₄ periods are more prominent in fragments derived from the ^{35}S -labelled galactosaminoglycan of the trypsin digest (Fig. 5a) than in those of the polysaccharide in the medium (Fig. 5b). Similar results were obtained when periodate-resistant oligosaccharides were digested with the same enzyme ($n = 5-6$ and $5-7$ in Figs. 4b and 4c respectively). Since the chondroitinase-AC-resistant fragments were eluted primarily in the void volume (Figs. 5a and 5b) it may be concluded that IdUA(-SO₄)-GalNAc periods preferentially occur in clusters.

To assess the distribution of GlcUA-containing periods the three ^{35}S -labelled galactosaminoglycan

Fig. 3. Ion-exchange chromatography on DE-32 DEAE-cellulose (gradient elution) of ^{35}S -labelled glycosaminoglycans (—) and ^{35}S -labelled galactosaminoglycans (·····) from the cells (a), the trypsin digest (b) and the medium (c)

The ^{35}S -labelled polysaccharides were isolated as described in the Experimental section (see also Fig. 2). The shape of the gradient was determined by conductivity measurements (---). The points of elution of hyaluronic acid and dermatan sulphate are indicated by arrows (1) and (2) respectively. The isolated heparan sulphate from the trypsin digest (b) chromatographed ahead ($V_e = 55-60$ ml) of the presumed heparan sulphate component ($V_e = 60-65$ ml) of the mixture (—). It seems less likely that this effect is due to degradation of heparan sulphate by chondroitinase-ABC. However, interaction between the two polysaccharides is a plausible explanation (see also Fig. 2).

Scheme 1. Scheme of degradation of copolymeric dermatan sulphate-chondroitin sulphate chains

Copolymeric chains were degraded by two different methods. Digestion with testicular hyaluronidase cleaves hexosaminidic bonds to D-glucuronic acid residues when two or more GlcUA-GalNAc-SO₄ periods are located in clusters (*p* and *s* ≥ 2). Periodate at low pH and temperature selectively oxidizes nonsulphated L-iduronic acid residues (▲). The oxidized residues are cleaved by alkali and the resulting fragments contain GlcUA and/or IdUA-SO₄ as uronic acid components. Further degradation of the fragments obtained by the two methods can be accomplished by chondroitinase-AC (↑).

fractions were digested with testicular hyaluronidase. As shown in Figs. 4(d)–4(f) the polysaccharides of the cell fraction contained fewer hyaluronidase-susceptible sites (Fig. 4d) than did those obtained from the trypsin digest and the medium. The polymers from the trypsin digest contained the largest number of hyaluronidase-susceptible sites. The polymeric fragments (void volume fractions in Figs. 4d–4f) obtained after hyaluronidase digestion were examined for the distribution of periodate-resistant hexuronic acid residues (GlcUA or IdUA-SO₄) (Fig. 6). The results showed that the yield of periodate-resistant oligosaccharides varied considerably between the three species. Fragments derived from polymers of the cell fraction (Fig. 6a) contained fewer GlcUA or

IdUA-SO₄ residues than did those derived from polymers of the other two fractions (Figs. 6b and 6c). Periodate-resistant hexuronic acid residues were most abundant in fragments of the polysaccharides obtained from the medium (Fig. 6c). Since the above oligosaccharides were obtained after hyaluronidase digestion IdUA(-SO₄)-GalNAc and single GlcUA-GalNAc-SO₄ periods account for the periodate-resistant structures (see also Scheme 1). Digestion of the various oligosaccharides (*V*_e = 32–57 ml in Fig. 6) by chondroitinase-AC showed that IdUA(-SO₄)-GalNAc periods comprised the major portion of these structures. These results are in good agreement with estimations based on chondrosulphatase digestion (see above).

Fig. 4. Gel chromatography on Sephadex G-50 of ^{35}S -labelled galactosaminoglycans after various types of degradation

The polysaccharides were degraded by periodate oxidation-alkaline elimination (*a-c*) or by digestion with testicular hyaluronidase (*d-f*). The ^{35}S -labelled galactosaminoglycans were derived from the cells (*a* and *d*), the trypsin digest (*b* and *e*) and the medium (*c* and *f*). The elution volumes of various oligosaccharide standards (di-, tetra- and hexa-saccharides from chondroitin sulphate) are indicated by arrows. The symbol n denotes the number of hexuronic acid residues in the oligosaccharide fragments. The fragments obtained after periodate oxidation-alkaline elimination (*a-c*) have the general carbohydrate structure $\text{GalNAc}(\text{UA-GalNAc})_n\text{-R}$, where UA denotes GlcUA or IdUA- SO_4 . After digestion with testicular hyaluronidase the oligosaccharides (*d-f*) should have the general carbohydrate sequence $\text{GlcUA-GalNAc}(\text{IdUA-GalNAc})_{n-2}\text{-GlcUA-GalNAc}$. The various oligosaccharide fractions were pooled as indicated by vertical dotted lines. In order to calculate the radioactivity associated with each oligosaccharide species, fractions were subdivided as indicated by vertical dashed lines. Column size, 8 mm $\times$ 1400 mm; eluent, 0.2 M-pyridine acetate, pH 5.0; elution rate, 9 ml/h. V_0 , Elution volume of Blue Dextran.

The galactosaminoglycans obtained from the trypsin digest and the medium contained approx. 70% of periodate-resistant hexuronic acid residues (IdUA- SO_4 and GlcUA) (see Table 1). Whereas GlcUA-GalNAc- SO_4 periods were the dominating units in the galactosaminoglycans derived from the trypsin

digest, almost equal proportions of IdUA(SO_4)-GalNAc and GlcUA-GalNAc- SO_4 periods were present in the polysaccharides in the medium. Further, the results of hyaluronidase degradation indicated that two-thirds of the GlcUA-GalNAc- SO_4 units of the polymer released by trypsin were arranged in

Table 1. Content of various disaccharide repeating units in galactosaminoglycans from various sources

After periodate oxidation-alkaline elimination of galactosaminoglycans oligosaccharides of the general structure GalNAc-(UA-GalNAc)_n-R were obtained. Since R is derived from an oxidized IdUA residue, the relative amount of

IdUA-GalNAc-SO₄ periods (x) was calculated as:

$$x = \left(\sum_{n=0}^{n=8} \frac{a_n}{n+1} \right) / \left(\sum_{n=0}^{n=8} a_n \right) \times 100 (\%)$$

where a_n represents the total radioactivity in oligosaccharides $n = 0, 1, 2, \dots$ etc. shown in Figs. 4(a)–4(c); the void volume fraction is referred to as $n = 8$. Unsaturated ³⁵S-labelled disaccharides were obtained by chondroitinase-ABC digestion of the galactosaminoglycans. After chondrosulphatase digestion of these disaccharides, remaining disaccharide-bound radioactivity (y , % of total) corresponds to IdUA-SO₄-containing units. The relative amount (%) of GlcUA-GalNAc-SO₄ periods was calculated as $100 - (x + y)$. The presence of two or more GlcUA-containing units in clusters constitute a hyaluronidase-susceptible site. After digestion with this enzyme, oligosaccharides ($n = 2-7$) of the following general carbohydrate sequence should be obtained, GlcUA-GalNAc-(IdUA-GalNAc)_{n-2}-GlcUA-GalNAc. The amount of GlcUA-containing units arranged in clusters may be calculated as:

$$\left(\sum_{n=2}^{n=7} \frac{2a_n}{n} \right) / \left(\sum_{n=1}^{n=8} a_n \right) \times 100$$

(symbols as above; $n = 8$ is the void volume fraction in Figs. 4d–4f). Values are expressed as the percentage of total radioactivity.

³⁵ S-labelled galactosaminoglycan from	Disaccharide repeating units		
	IdUA-GalNAc	IdUA-GalNAc*	GlcUA-GalNAc†
	SO ₄ x (%)	SO ₄ (SO ₄) y (%)	SO ₄ 100 - (x + y) (%)
Cells	40	11	49 (21)
Trypsin digest	27	18	55 (36)
Medium	30	35	35 (32)

* Disulphated as well as monosulphated species are included in these results. It should be noted that the former disaccharides account for less than 5% of the total radioactivity.

† Values in parentheses represent GlcUA-containing units arranged in clusters.

clusters, whereas the remainder occurred as single units (Table 1). The galactosaminoglycan isolated from the medium contained very few single GlcUA-GalNAc-SO₄ units.

Hyaluronidase degradation of the ³⁵S-labelled galactosaminoglycans of the trypsin digest and the medium yielded a series of oligosaccharides indicating that clusters of GlcUA-GalNAc-SO₄ periods were located at several positions along the chain (Figs. 4e and 4f). Very few of these clusters contained more than two repeating units, as shown by the low yield of tetrasaccharide and disaccharide from this material (Fig. 4e; $n = 1$ and 2). Single GlcUA-GalNAc-SO₄ periods do not constitute hyaluronidase-susceptible sites (Scheme 1). However, such units may be detected after chondroitinase-AC digestion. The octasaccharide fractions obtained after hyaluronidase degradation of ³⁵S-labelled galactosaminoglycan from the trypsin digest and the medium respectively (Figs. 4e and 4f; $n = 4$) gave markedly different degradation patterns after treatment with chondroitinase-AC

(Fig. 7). Whereas the former octasaccharide was largely degraded to tetrasaccharides, the latter octasaccharide was almost entirely resistant to chondroitinase-AC digestion. These results indicate that the major portion of the octasaccharide derived from the trypsin-released ³⁵S-labelled galactosaminoglycan had the carbohydrate sequence GlcUA-GalNAc-IdUA-GalNAc-GlcUA-GalNAc-IdUA-GalNAc. In the octasaccharides derived from the medium internal single GlcUA-GalNAc-SO₄ periods were very rare.

Discussion

In the conventional nomenclature dermatan sulphate and chondroitin sulphate connote polysaccharides composed of IdUA-GalNAc-SO₄ and GlcUA-GalNAc-SO₄ repeating units respectively. No polysaccharide chain made up solely of IdUA-(SO₄)-GalNAc repeating units has been described. The galactosaminoglycans under study are copolymers

Fig. 5. Chondroitinase-AC digestion of ^{35}S -labelled polymeric fragments obtained after periodate oxidation-alkaline elimination

The ^{35}S -labelled polymeric fragments were obtained as void-volume fractions after periodate oxidation-alkaline elimination of ^{35}S -labelled galactosaminoglycans from the trypsin digest and the medium respectively (see Figs. 4b and 4c). These fragments were digested with chondroitinase-AC and subjected to gel chromatography on Sephadex G-50. The graphs refer to material derived from the trypsin digest (a) and the medium (b) respectively. For technical details see Fig. 4.

containing variable proportions of all three repeating units (Table 1). Similar copolymeric variants have been isolated from various tissues (Fransson & Havsmark, 1970; Fransson *et al.*, 1974b), indicating that the cell culture conditions have not led to the production of unusual polymers. However, the copolymeric variants showed markedly different distribution between the three sources studied.

The results of $^{35}\text{SO}_4^{2-}$ incorporation into ^{35}S -labelled glycosaminoglycans from the various sources showed that the trypsin-released polymers as well as the polymers found in the medium accumulated radioactivity much more rapidly than did those of the cell fraction (Fig. 1). This suggests that the intracellular synthesis pool is very small. Therefore a considerable

Fig. 6. Periodate oxidation-alkaline elimination of various ^{35}S -labelled polymeric fragments obtained after hyaluronidase digestion

The ^{35}S -labelled polymeric fragments were obtained as void-volume fractions after hyaluronidase digestion of ^{35}S -labelled galactosaminoglycan from cells, trypsin digest and medium respectively (see Figs. 4d and 4f). These fragments were oxidized with periodate, treated with alkali and subjected to gel chromatography on Sephadex G-50. The various graphs refer to material derived from the cells (a), the trypsin digest (b) and the medium (c). For technical details see Fig. 4.

Fig. 7. Chondroitinase-AC degradation of octasaccharides derived from hyaluronidase digests of ³⁵S-labelled galactosaminoglycan

The octasaccharides were derived from ³⁵S-labelled galactosaminoglycans which were isolated from the trypsin digest and the medium respectively (Figs. 4e and 4f; *n* = 4). After digestion with chondroitinase-AC the split products were subjected to gel chromatography on Sephadex G-25. The graphs refer to material derived from the trypsin digest (a) and the medium (b) respectively. Column size, 0.9mm × 1100mm; eluent, 0.2M-pyridine acetate, pH 5.0; elution rate, 9 ml/h. For further details see Fig. 4.

proportion of the cell material may not be a precursor of the secreted material. This agrees with the results of Fratanoni *et al.* (1968), who proposed the existence of an intracellular storage pool destined for degrada-

tion. However, trypsin may not release all glycosaminoglycans from the cell surface. This may be due to steric effects or to the nature of the protein core of the proteoglycan(s). Thus residual extracellular material might contribute to the cell fraction. Although it has been claimed that treatment with trypsin under the present conditions does not cause leakage of intracellular material into the medium (Kraemer, 1971a), the origin of the trypsin-released copolymers cannot be determined with certainty. Presumably a substantial portion of the trypsin-released material is derived from the cell surface and/or the intercellular space. This is supported by the finding that heparan [³⁵S]sulphate, which is a common cell-surface component (Kraemer, 1971a,b), was enriched with the trypsin-released material. However, the change in the microenvironment of the fibroblast caused by digestion with trypsin might stimulate the secretion of newly synthesized copolymers. Thus intracellular material might be added to the trypsin digest. Contributions from the medium by entrapment must also be considered.

A portion of the copolymers of the cell fraction had sequences in common with copolymers from the trypsin digest and the medium. This might represent reingested lysosomal material or residual cell-surface material. However, the remainder of the cellular material was largely composed of IdUA-GalNAc-SO₄ periods (Figs. 4d and 6a). It is noteworthy in this context that fully sulphated segments of a galactosaminoglycan synthesized by a particulate fraction of fibroblasts contained mostly IdUA-GalNAc-SO₄ periods (Malmström *et al.*, 1975). These periods were formed by the concomitant C-5 inversion of GlcUA residues and 4-sulphation of GalNAc residues. Sulphation without inversion was directed towards both the 4- and 6-positions. Although it is tempting to speculate on the possibility that the newly synthesized galactosaminoglycan chains contain exclusively IdUA-GalNAc-SO₄ periods, formation of copolymeric molecules containing, in addition, GlcUA-GalNAc-SO₄ periods cannot be excluded.

The structural differences between the copolymer of the cell fraction and those of the trypsin digest and the medium may be the result of selective secretion of molecules with a certain structure. Alternatively, polymer-level modifications of the newly synthesized chains during or after secretion from the cell would give the same result. The latter possibility seems to be favoured by the demonstration of a so-called 'reverse epimerase' in fibroblast secretions (Fransson *et al.*, 1973). It was observed that the addition of unlabelled, L-iduronic acid-rich dermatan sulphate chains to the cell culture markedly decreased the incorporation of ³⁵SO₄²⁻ into D-glucuronic acid-rich, extracellular copolymeric molecules. This suggests a precursor-product relationship between iduronic acid-rich and glucuronic acid-rich copolymeric molecules. It is

noteworthy that the copolymeric structure of polysaccharides derived from the trypsin digest differed from that of the medium polysaccharides. The former material contained considerable quantities of GlcUA-GalNAc-SO₄ periods both arranged in clusters and as single units often in an alternating fashion (carbohydrate sequence -GlcUA-GalNAc-IdUA-GalNAc-GlcUA-GalNAc-). It is possible that copolymers with the characteristic features mentioned above become specifically entrapped in the matrix surrounding the cells owing to interaction with other macromolecules.

The copolymers obtained after trypsin digestion contained almost the same amount of periodate-resistant periods as the material found in the medium (Table 1). However, the latter material contained very few single GlcUA-GalNAc-SO₄ periods. Instead, a corresponding increase in the number of IdUA(-SO₄)-GalNAc periods was observed. Thus it seems unlikely that the copolymeric chains of the trypsin digest are the only precursors of chains found in the medium. Instead, the characteristic features of the polymers in the medium may be the result of an alternative polymer-level modification of chains which contain large amounts of IdUA-GalNAc-SO₄ periods. It was noticed that the copolymers of the medium contained 30–35% of IdUA(-SO₄)-GalNAc periods (Table 1). If these residues were formed by direct sulphation of IdUA during or after secretion a corresponding amount of non-sulphated repeating units should be present in the newly synthesized chains. This possibility appears less likely from the fact that all copolymers had similar charge densities and appeared to be fully sulphated. It should also be pointed out that no IdUA-SO₄ residues could be detected in the galactosaminoglycan product formed by a particulate enzyme preparation from fibroblasts (Malmström *et al.*, 1975). Moreover, formation of IdUA requires concomitant 4-sulphation of GalNAc. Since most of the GalNAc residues in intracellular copolymers should be sulphated, the origin of the IdUA(-SO₄)-GalNAc periods is obscure. A transfer of sulphate groups from GalNAc moieties to adjacent IdUA residues may account for the observed result. Two competing polymer-level modifications of IdUA-GalNAc-SO₄-containing chains are postulated:

Reaction (1) is a C-5 inversion of single IdUA residues per GlcUA, catalysed by the so-called 'reverse epimerase', whereas reaction (2) is a sulphate rearrangement. The absence of GlcUA(-SO₄) residues

in this type of polysaccharide suggests that these are alternative reactions.

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