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Synthesis of Glycosaminoglycans by Human Embryonic Lung Fibroblasts

DIFFERENT DISTRIBUTION OF HEPARAN SULPHATE, CHONDROITIN SULPHATE AND DERMATAN SULPHATE IN VARIOUS FRACTIONS OF THE CELL CULTURE

By INGRID SJÖBERG and LARS-ÅKE FRANSSON
Department of Physiological Chemistry 2, University of Lund, S-22007 Lund 7, Sweden

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Foetal human lung fibroblasts, grown in monolayer, were allowed to incorporate $^{35}SO_4^{2-}$ for various periods of time. ^{35}S-labelled macromolecular anionic products were isolated from the medium, a trypsin digest of the cells in monolayer and the cell residue. The various radioactive polysaccharides were identified as heparan sulphate and a galactosaminoglycan population (chondroitin sulphate and dermatan sulphate) by ion-exchange chromatography and by differential degradations with HNO_2 and chondroitinase ABC. Most of the heparan sulphate was found in the trypsin digest, whereas the galactosaminoglycan components were largely confined to the medium. Electrophoretic studies on the various ^{35}S-labelled galactosaminoglycans suggested the presence of a separate chondroitin sulphate component (i.e. a glucuronic acid-rich galactosaminoglycan). The ^{35}S-labelled galactosaminoglycans were subjected to periodate oxidation of L-iduronic acid residues followed by scission in alkali. A periodate-resistant polymer fraction was obtained, which could be degraded to disaccharides by chondroitinase AC. However, most of the ^{35}S-labelled galactosaminoglycans were extensively degraded by periodate oxidation–alkaline elimination. The oligosaccharides obtained were essentially resistant to chondroitinase AC, indicating that the iduronic acid-rich galactosaminoglycans (i.e. dermatan sulphate) were composed largely of repeating units containing sulphated or non-sulphated L-iduronic acid residues. The L-iduronic acid residues present in dermatan sulphate derived from the medium and the trypsin digest contained twice as much ester sulphate as did material associated with the cells. The content of D-glucuronic acid was low and similar in all three fractions. The relative distribution of glycosaminoglycans among the various fractions obtained from cultured lung fibroblasts was distinctly different from that of skin fibroblasts [Malmström, Carlstedt, Åberg & Fransson (1975) *Biochem. J.* **151**, 477–489]. Moreover, subtle differences in co-polymeric structure of dermatan sulphate isolated from the two cell types could be detected.

In recent years the synthesis and secretion of proteoglycans (glycosaminoglycans) by cultured cells of different origin have been studied in a number of laboratories. Established mammalian cell lines of lymphoid, epithelial and fibroblastic origin produce and secrete a variety of glycosaminoglycans into the medium (Saito & Uzman, 1971). However, fibroblasts generally secrete larger quantities.

Cultured fibroblasts obtained from different tissues seem to retain a specific pattern of secretion. Thus fibroblast-like cells from bovine aorta secrete a mixture of glycosaminoglycans (hyaluronate, heparan sulphate, chondroitin sulphate and dermatan sulphate), the distribution of which closely resembles that found in the intact tissue (Kresse *et al.*, 1975). Cultured smooth-muscle cells of primate arteries synthesize principally dermatan sulphate (60–80% of total) and little or no hyaluronic acid (Wight & Ross, 1975). In contrast, skin fibroblasts of the same species secrete primarily hyaluronic acid (50–60% of total), with lesser amounts of the galactosaminoglycans.

Previous studies in our laboratory have been concerned with the structural features of galactosaminoglycans synthesized by cultured human skin fibroblasts (Malmström *et al.*, 1975*a*). It was shown that galactosaminoglycans isolated from the medium, a trypsin digest of the cells, and the cell residue differ

with respect to the distribution of IdUA-* and GlcUA-containing units. In the present study the structure of galactosaminoglycans produced by fibroblasts from human foetal lung has been investigated. The co-polymeric structure of the galactosaminoglycans synthesized by these cells was distinctly different from that of glycans secreted by skin fibroblasts.

Experimental

Materials

Glycosaminoglycan standards (hyaluronate, pig skin dermatan sulphate, chondroitin 4-sulphate and chondroitin 6-sulphate) and chondroitin sulphate oligosaccharides (hyaluronidase type) were obtained from sources listed previously (Malmström *et al.*, 1975*a*). Cartilage proteoglycan was a gift from Dr. Sven Inerot of this Department. Twice-crystallized trypsin from bovine pancreas (type III), twice-crystallized papain and bovine serum albumin, were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Chondroitinase AC and ABC EC 3.2.1.35) were obtained from Miles Laboratories, Elkhart, IN, U.S.A. Carrier-free $Na_2{}^{35}SO_4$ (124mCi/mmol) was a product of The Radiochemical Centre, Amersham, Bucks., U.K. Materials used for cell cultures (Earle's minimal essential medium, calf serum and antibiotics) were obtained from sources listed previously (Malmström *et al.*, 1975*a*). Scintillation materials were supplied by Packard A.B., Bandhagen, Sweden. Sephadex and Sepharose gels were purchased 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 separatory 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 2450 liquid-scintillation counter. The scintillation mixtures used were Insta-gel (3ml of liquid-mixed with 2ml of sample) and Omnifluor in toluene (4g/litre).

Electrophoresis of ^{35}S-labelled glycosaminoglycans was performed on strips of cellulose acetate in 0.1M-barium acetate (5V/cm for 2.5h) as described by Wessler (1968). The strips were cut, and radioactivity was counted in Omnifluor/toluene (10ml per 2mm×10mm strip). High-voltage paper electrophoresis of oligosaccharides was performed on Whatman 3MM paper in 0.1M-acetic acid adjusted to pH5.0 by the addition of pyridine (40V/cm; 1h). Papers were stained with aniline hydrogen phthalate (Partridge, 1949).

Ion-exchange chromatography was carried out on columns (6mm×140mm) of DE-32 DEAE-cellulose (equilibrated with 0.1M-sodium acetate, pH5.0) that were eluted with a linear gradient (0.1–2.5M-sodium acetate, pH5.0; total elution volume, 100ml) at a rate of 3ml/h (Malmström *et al.*, 1975*a*). Material was recovered by dialysis and freeze-drying. Gel chromatography was used for separation of products obtained after chemical and enzymic degradations (see the legends to the Figures).

Degradative methods

Poly- and oligo-saccharides were digested by chondroitinases AC or ABC in 0.5M-Tris–HCl, pH8.0, at 37°C overnight. The reaction mixtures contained (per ml) 0.1 unit of enzyme, 0.1mg of bovine serum albumin, radioactive substrate and 0.5mg of carrier dermatan sulphate. Oxidations with periodate were performed in the presence of 0.02M-$NaIO_4$ at pH3.0 and 4°C for 24h (Fransson, 1974). The oxidized products were subsequently cleaved by alkaline elmination at pH12.0 for 30min at room temperature (Fransson & Carlstedt, 1974). Deaminative cleavage of heparan sulphate was performed by the method of Lagunoff & Warren (1962) as used by Lindahl *et al.* (1973).

Isolation of ^{35}S-labelled glycosaminoglycans from fibroblast cultures

Fibroblast cultures were established from pieces of human embryonic lung (age 15 weeks). The fibroblasts were grown in monolayers as described earlier (Malmström *et al.*, 1975*a*). Confluent monolayers obtained after six to eight passages were used in the following way. Three changes of medium, where the two last ones contained $MgCl_2$ instead of $MgSO_4$, were made before $Na_2{}^{35}SO_4$ was added (5μCi/ml; final concn. of sulphate 0.05mM). The cells were allowed to incorporate radioactivity for 3, 12, 24 and 72h. The following fractions were obtained initially: medium, a trypsin digest of the cells, and the cell residue. Culture medium plus two washings of the cell layer with Earle's balanced salt solution were combined in the first fraction. The trypsin digest consisted of material solubilized by treatment of the monolayer with a prewarmed solution (40ml) of trypsin (0.1mg/ml) in Earle's balanced salt solution for 30min at 37°C followed by centrifugation (600*g* for 10min at 4°C), resuspension of the cells in the same salt solution and re-centrifugation (Kraemer, 1971). As shown by Kraemer (1971), no irreversible cell damage occurs under these conditions. Moreover, the cells used in the present study excluded Trypan Blue (>95%) and could be satisfactorily re-plated.

* Abbreviations: IdUA, L-iduronic acid; GlcUA, D-glucuronic acid; ΔUA, 4,5-unsaturated glycuronic acid.

The cell residue (containing material not solubilized by trypsin) was suspended in water and extracted with trichloroacetic acid (final concn. 7%, w/v) for 30min in an ice bath (Kraemer, 1971). After centrifugation (10000*g* for 20min) a precipitate and a supernatant solution were obtained. The latter fraction was extracted with diethyl ether. Both fractions were then freeze-dried. The trichloroacetic acid extract (soluble material) has been shown to contain cell-sap heparan sulphate (Kraemer, 1971). The medium and the trypsin digest (plus 1mg of carrier dermatan sulphate) were dialysed against 0.1M-$(NH_4)_2SO_4$ for 6h, followed by water for 3 days. After addition of carrier to the two cell fractions, all fractions (medium, trypsin digest, soluble and insoluble cellular material) were digested with papain (0.4mg/ml) in 1.0M-NaCl/0.05M-EDTA (disodium salt)/0.01M-cysteine hydrochloride/0.05M-sodium phosphate buffer, pH7.0, for 24h at 65°C. The digestion was carried out in the presence of 1.0M-NaCl to minimize precipitation of ^{35}S-labelled polyanions by basic proteins and peptides (Scott, 1960). Dialysis against water for 2 days was followed by centrifugation (10000*g* for 10min) and freeze-drying of the supernatants. The ^{35}S-labelled glycosaminoglycans of the various fractions were finally purified and partially fractionated by ion-exchange chromatography (see above).

The heparan sulphate and the galactosaminoglycan content of the various fractions (or subfractions) were determined as follows. One portion of the ^{35}S-labelled glycosaminoglycans was subjected to digestion with chondroitinase ABC, followed by gel chromatography on Sephadex G-50 (Malmström *et al.*, 1975*a*). Heparan sulphate emerges with the void volume, whereas the degradation products of the galactosaminoglycans are retarded. Another portion of the material was treated with HNO_2, which depolymerizes heparan sulphate (see Lindahl *et al.*, 1973), but does not attack galactosaminoglycans. In the ensuing gel chromatography, galactosaminoglycans are excluded from the gel, whereas the degradation products of heparan sulphate are included. The same procedure was also used for the isolation of ^{35}S-labelled galactosaminoglycans on a preparative scale (Malmström *et al.*, 1975*a*).

Results

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

The rate of accumulation of ^{35}S-labelled glycosaminoglycans was followed by incorporation of $^{35}SO_4^{2-}$ into macromolecular anionic products obtained from the soluble and insoluble cell fraction, the trypsin digest and the medium respectively (Fig. 1*a*). During the first day the highest rate of accumulation was observed for the trypsin-released material (●——●). During the following days the rate of accumulation into this fraction declined. Secretion of radioactive glycosaminoglycans into the medium (▲——▲) was essentially linear with respect to time during the course of the experiment. The cellular fractions contained small quantities of

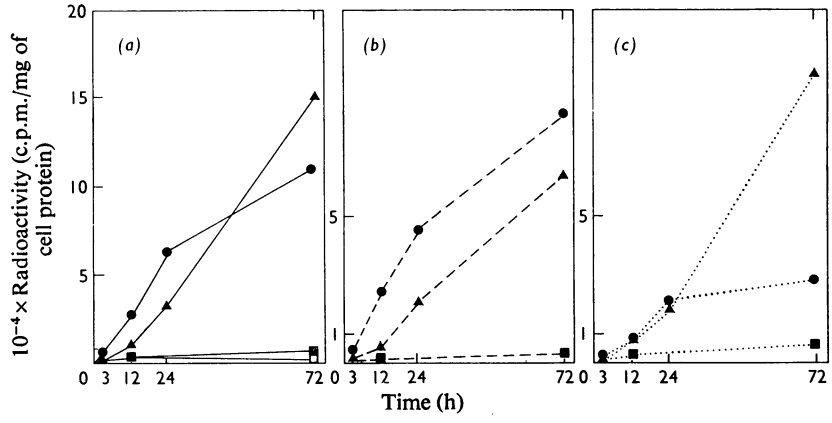

Fig. 1. *Incorporation of $^{35}SO_4^{2-}$ into total glycosaminoglycan* (——), *heparan sulphate* (– – – –) *and galactosaminoglycan* (· · · ·) *isolated from the medium* (▲), *a trypsin digest of the cells* (●), *a trichloroacetic acid extract of the cells* (□) *and the cell residue* (■)

Fibroblasts in monolayer were allowed to incorporate radioisotope for the indicated periods of time. Medium, trypsin digest and one soluble and one insoluble cell fraction were recovered as described in the Experimental section. After papain digestion and dialysis, ^{35}S-labelled glycosaminoglycans were isolated by ion-exchange chromatography. The amounts of total glycosaminoglycan recovered are shown in (*a*). The heparan sulphate content (*b*) and the total galactosaminoglycan content (*c*) were measured after differential degradation as described in the Experimental section.

Table 1. *Distribution of ^{35}S-labelled glycosaminoglycans after 72h of incorporation of radioisotope*

For details concerning heparan sulphate and total galactosaminoglycans, see the legend to Fig. 1 and the Experimental section. GlcUA-rich galactosaminoglycan corresponds to material released as disaccharide by chondroitinase-AC ($n = 1$ in Fig. 4). Similar results are obtained when material excluded from Sephadex G-50 after periodate oxidation–alkaline elimination is considered to be GlcUA-rich (Fig. 5). Values are expressed as the percentages of total radioactivity.

^{35}S-labelled material from:	Total glycosamino-glycan (%)	Heparan sulphate (%)	Total galactosamino-glycan (%)	GlcUA-rich galactosamino-glycan (%)	IdUA-rich galactosamino-glycan (%)
Insoluble cellular pool*	5	1–2	2–3	0–1	2–3
Trypsin-liberated pool	40	30	10	6	4
Medium pool	55	22	33	7	26

* Small quantities were found in the soluble cellular pool (~1%). Most of this material consisted of low-molecular-weight heparan sulphate (as determined by gel chromatography).

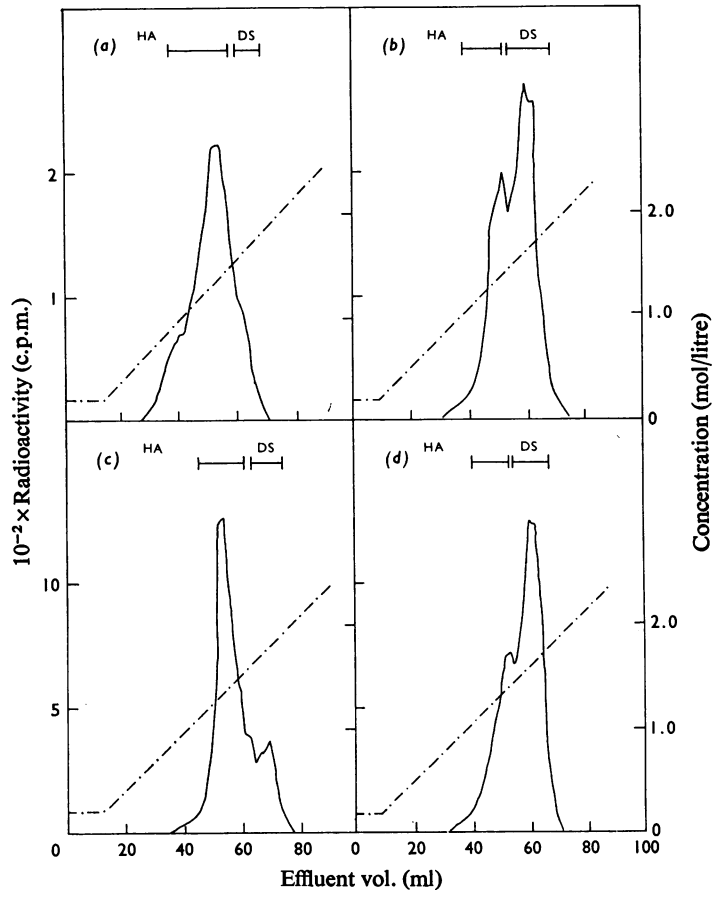

Fig. 2. *Ion-exchange chromatography on DE-32 DEAE-cellulose of ^{35}S-labelled glycosaminoglycans from the soluble cell fraction (a), the insoluble cell fraction (b), the trypsin digest (c) and the medium (d) after 72h of $^{35}SO_4^{2-}$ incorporation*

The ^{35}S-labelled polysaccharides were isolated as described in the Experimental section. The shape of the gradient (–·–·) was determined by conductivity measurements. The points of elution of hyaluroicate (HA) and pig skin dermatan sulphate (DS) are indicated on the top of the graphs. Material was pooled as indicated by horizontal bars, dialysed against water and freeze-dried. The heparan sulphate component was identified by its susceptibility to degradations with HNO_2. The galactosaminoglycan component was further purified by treatment with HNO_2 followed by gel chromatography as described in the Experimental section.

^{35}S-labelled material. Their rate of accumulation appeared to level off at 12h (□——□, ■——■).

The ^{35}S-labelled glycosaminoglycans obtained from the various fractions were subjected to differential degradations to determine their heparan sulphate and galactosaminoglycan contents. Fig. 1(*b*) shows that most of the heparan sulphate was found in the trypsin digest (●----●). This material continued to accumulate during the experiment. Heparan sulphate was also secreted into the medium (▲----▲). The galactosaminoglycan component (Fig. 1*c*) was largely confined to the medium (▲····▲), where a high rate of accumulation was observed throughout the experiment. The amount of ^{35}S-labelled galactosaminoglycan in the trypsin digest (●····●) reached a plateau after 24h. After 72h of $^{35}SO_4^{2-}$ incorporation, 95% of the total ^{35}S-labelled glycosaminoglycans were found in the medium and the trypsin digest (Table 1). Heparan sulphate was the main component of the trypsin digest, whereas most of the galactosaminoglycans were found in the medium.

The distribution of ^{35}S-labelled glycosaminoglycans after 72h of incorporation was also examined by ion-exchange chromatography (Fig. 2). The heparan sulphate and galactosaminoglycan components were poorly resolved, suggesting a relatively high sulphate content in the heparan sulphate molecules. However, the relative distributions of the two components were in agreement with the estimations presented above.

The galactosaminoglycan components of the various fractions were subjected to cellulose acetate electrophoresis in the presence of barium acetate, which separates chondroitin sulphate from dermatan sulphate. As Fig. 3 shows, the ^{35}S-labelled galactosaminoglycans from the insoluble cell fraction (*a*) gave a broad peak overlapping both the pig skin dermatan sulphate and the chondroitin sulphate standards. Most of the ^{35}S-labelled galactosaminoglycans of the trypsin digest (*b*) migrated as chondroitin sulphate, whereas the material from the medium (*c*) appeared to separate into one dermatan sulphate and one chondroitin sulphate component.

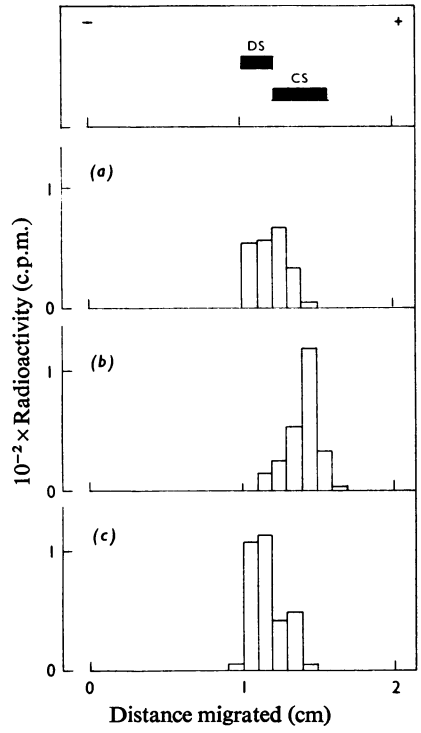

Fig. 3. *Cellulose acetate electrophoresis of ^{35}S-labelled galactosaminoglycans from the insoluble cell fraction (a), the trypsin digest (b) and the medium (c)*

^{35}S-labelled galactosaminoglycans isolated by ion-exchange chromatography, followed by HNO_2 treatment and gel chromatography, were subjected to electrophoresis in 0.1M-barium acetate. The positions of pig skin dermatan sulphate (DS) and chondroitin sulphate (CS) are indicated at the top.

Structure of ^{35}S-labelled galactosaminoglycans

Galactosaminoglycans are usually classified as chondroitin sulphate or dermatan sulphate. Chondroitin sulphate is composed exclusively of GlcUA-containing repeating units, whereas dermatan sulphate always contains IdUA (Cöster *et al.*, 1975). Dermatan sulphate may contain three types of repeating units: (I) GlcUA-GalNAc-SO_4, (II) IdUA-GalNAc-SO_4 and (III) IdUA(-SO_4)-GalNAc(-SO_4). Completely non-sulphated repeating units are very scarce. During biosynthesis of this molecule, chondroitin-like chains (or portions of chains) are subjected to inversion of configuration at C-5 of GlcUA residues (to form IdUA), followed by sulphation of GalNAc moieties to yield the final product (Malmström *et al.*, 1975*b*). When $^{35}SO_4^{2-}$ is incorporated into the molecule, the various repeating units should attain similar specific radioactivities throughout the chain.

To study the occurrence and distribution of the various repeating units (I–III) in ^{35}S-labelled galactosaminoglycans from the various cell-culture fractions, several degradation experiments were performed. Degradations with chondroitinase-AC (which cleaves only GalNAc–GlcUA bonds) followed by gel chromatography gave the results shown in Fig. 4. The release of radioactivity (representing the disaccharide ΔUA-GalNAc-SO_4, corresponding to repeating unit I) from the galactosaminoglycans of the insoluble cell fraction (*a*) and the medium (*c*) was rather small, whereas the glycan of the trypsin digest (*b*) yielded larger quantities. The yield of oligosaccharides ($n = 2$–5 in Fig. 4) was very low.

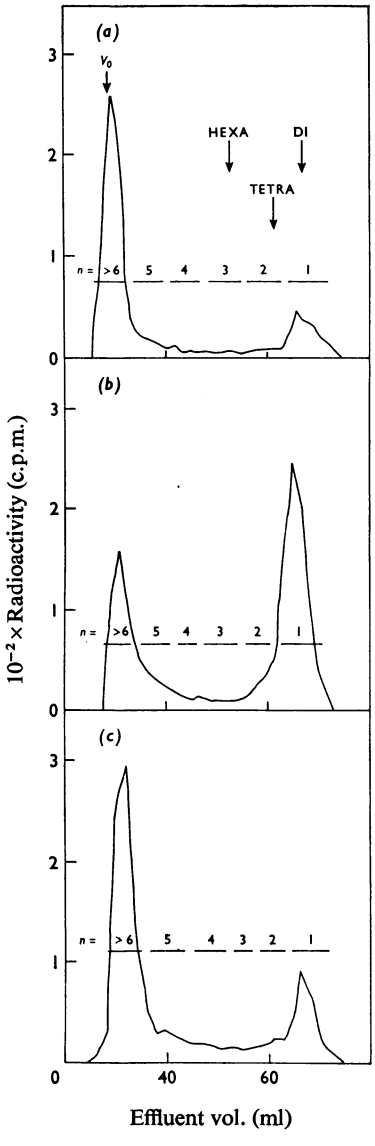

Fig. 4. *Gel chromatography on Sephadex G-50 of* ^{35}*S-labelled galactosaminoglycans after digestion with chondroitinase AC*

^{35}S-labelled material from the insoluble cell fraction (*a*), the trypsin digest (*b*) and the medium (*c*) were digested with chondroitinase AC. The elution volumes of various chondroitin sulphate oligosaccharides are indicated in the upper graph. DI, TETRA and HEXA refer to disaccharide, tetrasaccharide and hexasaccharide respectively. *n* is the number of repeating units in the various oligosaccharide fractions of the general carbohydrate structure $(GlcUA\text{-}GalNAc)_n$. To calculate the radioactivity associated with each oligosaccharide, material was subdivided as indicated by the horizontal bars. The column size was 8mm×1500mm; the eluent was 0.2M-pyridine acetate, pH5.0; the elution rate was 6ml/h, and V_0 is the elution volume of Blue Dextran.

These results show that the repeating units of the various galactosaminoglycan chains are largely assembled in 'blocks', i.e. either clusters of GlcUA-containing units (type I) or clusters of IdUA-containing units (types II and III). Alternating segments (type I and II) seem to be very rare.

Selective periodate oxidation of IdUA residues followed by alkaline elimination to achieve fragmentation was also applied to the various ^{35}S-labelled galactosaminoglycans. As Fig. 5 shows, the glycans of the insoluble cell fraction (*a*) and the medium (*c*) were degraded to a greater extent than were those of the trypsin digest (*b*). Thus units containing IdUA (type II) were more prevalent in the glycans of the two former fractions than in the trypsin-released material. Conversely, in the latter material the galactosaminoglycans contained a large proportion of periodate-resistant uronic acid residues (present in repeating units I and III). It should also be noted that the degradation products from the cell material (*a*) were generally smaller than those derived from the medium (*c*).

All three glycan fractions gave a distinct void-volume peak on gel chromatography (Fig. 5) after periodate oxidation–alkaline elimination. This material was further examined by gel chromatography on Sepharose CL6B. As Fig. 6 shows, the respective ^{35}S-labelled galactosaminoglycans were first chromatographed directly (——). Then material excluded from Sephadex G-50 after periodate oxidation–alkaline elimination was rechromatographed on Sepharose CL6B (----). The ^{35}S-labelled galactosaminoglycans isolated after periodate oxidation–alkaline elimination of material derived from the trypsin digest (*b*) and the medium (*c*) appeared within the same range of molecular sizes as did the starting material. However, the fragments obtained after degradation of the cellular material (----) in (*a*) were of a smaller size compared with the starting material (——). [It should also be pointed out that the ^{35}S-labelled galactosaminoglycans (——) of the cellular fraction were larger than were those of the trypsin digest and the medium.]

The ^{35}S-labelled periodate-resistant galactosaminoglycans derived from the three cell fractions (Fig. 5; void-volume fractions) were subjected to digestion by chondroitinase AC. As Fig. 7 shows, all glycan fractions yielded disaccharide on digestion. Material derived from the trypsin digest (*b*) was mostly degraded to disaccharide, and the other fractions (*a* and *c*) also contained material not

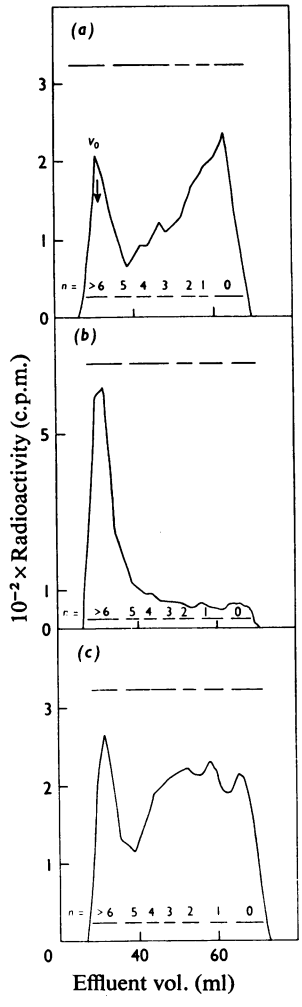

Fig. 5. *Gel chromatography on Sephadex G-50 of* ^{35}*S-labelled galactosaminoglycans after periodate oxidation–alkaline elimination*

The polysaccharides were degraded by periodate-oxidation–alkaline-elimination as described in the Experimental section. The ^{35}S-labelled material was derived from the insoluble cell fraction (*a*), the trypsin digest (*b*) and the medium (*c*). The fragments obtained after this type of degradation have the carbohydrate structure GalNAc-(UA-GalNAc)$_n$-R, where UA is either GlcUA or IdUA-SO_4 and R is a C_4 fragment derived from an oxidized and degraded IdUA (Fransson & Carlstedt, 1974). Thus the symbol *n* denotes the number of hexuronic acid residues in the oligosaccharide ($n = 0$ corresponds to the compound GalNAc-R). To calculate the radioactivity associated with each oligosaccharide, material was subdivided as indicated by the lower horizontal bars. Fractions were also pooled for further degradations; this is indicated by the upper horizontal bars; the fractions were $n \geqslant 6$, $n = 3$–5, $n = 2$, $n = 1$ and $n = 0$. For experimental details, see the legend to Fig. 4.

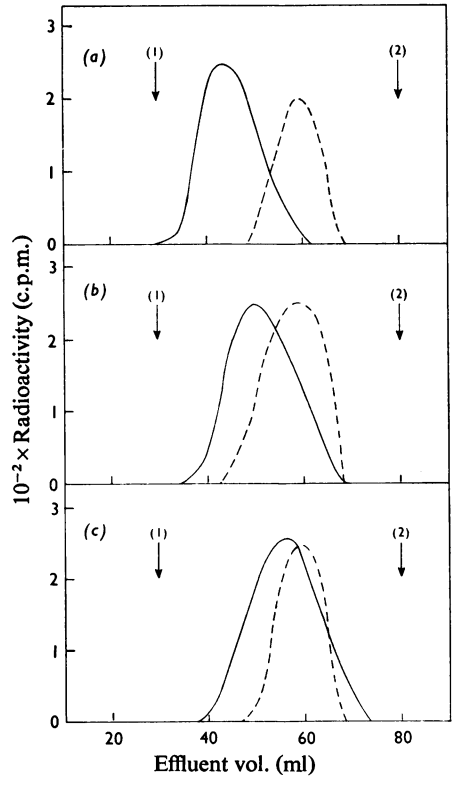

Fig. 6. *Gel chromatography on Sepharose CL6B of* ^{35}*S-labelled galactosaminoglycans* (——) *and* ^{35}*S-labelled glycans recovered after periodate-oxidation–alkaline-elimination of the former material* (----)

^{35}S-labelled galactosaminoglycans were isolated from the insoluble cell fraction (*a*), the trypsin digest (*b*) and the medium (*c*) as described in the Experimental section. One portion of this material was chromatographed directly (——). Another portion was subjected to periodate oxidation–alkaline elimination followed by gel chromatography as shown in Fig. 5. Material excluded from this gel ($n \geqslant 6$) was re-chromatographed on Sepharose CL6B (----). The column size was 10mm × 1400mm; the eluent was 0.5M-guanidine hydrochloride, pH7.0; the elution rate was 3ml/h. The elution volumes of cartilage proteoglycan (1) and D-glucuronolactone (2) are indicated.

susceptible to this enzyme. The latter fragments should contain IdUA(-SO_4)-GalNAc(-SO_4) repeating units (type III). The above results (Figs. 3–7) suggest that the galactosaminoglycan fractions may be composed of one IdUA-rich and one GlcUA-rich population. The latter population was distinguished by its electrophoretic mobility (Figs. 3*b* and 3*c*) and by its susceptibility to chondroitinase AC (Figs. 4

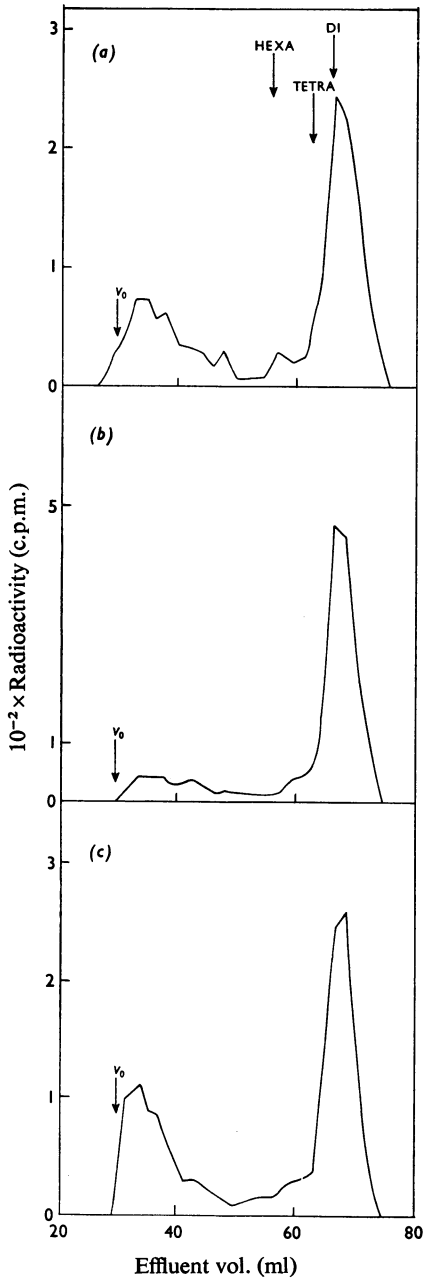

Fig. 7. *Chondroitinase-AC digestion of ^{35}S-labelled polymeric fragments obtained after periodate oxidation–alkaline elimination*

The various ^{35}S-labelled degradation products were derived from the galactosaminoglycans of the insoluble cell fraction (*a*), the trypsin digest (*b*) and the medium (*c*). The various ^{35}S-labelled galactosaminoglycans were first degraded by periodate oxidation–alkaline elimination followed by gel chromatography as shown in Fig. 4. Polymeric fragments corresponding to material eluted in the void volume ($n \geq 6$) were subsequently digested with chondroitinase AC. For further experimental details, see the legend to Fig. 4.

and 7). Moreover, it was recovered as a polymeric fraction after periodate oxidation–alkaline elimination (Figs. 5 and 6). The data of Fig. 4 were used to calculate the amount of GlcUA-rich galactosaminoglycan present in the various fractions. The disaccharides released by chondroitinase AC were assumed to be derived almost entirely from this component. As Table 1 shows, GlcUA-rich variants accounted for a minor proportion (one-third) of the total galactosaminoglycans. However, they were equally distributed between the trypsin digest and the medium.

The oligosaccharide fragments $n = 1$–5 in Fig. 5 obtained after periodate oxidation–alkaline elimination of the various ^{35}S-labelled galactosaminoglycans may contain either IdUA-SO_4 or GlcUA, or both. To assess their uronic acid composition, degradations with chondroitinase AC were performed. All the oligosaccharides ($n = 1$–5) were almost completely resistant to this enzyme, indicating that the uronic acid component was IdUA-SO_4.

An attempt was made to estimate the content of various repeating disaccharide units in the IdUA-rich galactosaminoglycan (dermatan sulphate) from the medium, the trypsin digest and the insoluble cell fraction. Since periodate oxidation–alkaline elimination cleaves the chain at the position of IdUA, the results shown in Fig. 5 may be used to calculate the relative amounts (x) of IdUA-containing units. The formula

$$x = \left(\sum_{n=0}^{n=5} \frac{a_n}{n+1} \Big/ \sum_{n=0}^{n=5} a_n \right) \times 100\ (\%)$$

as described elsewhere (Malmström *et al.*, 1975*a*) was used. The symbol a_n represents the total radioactivity in oligosaccharides $n = 0, 1, 2, \ldots$ etc. The relative amount (y) of GlcUA-containing units was calculated as:

$$y = \left(\sum_{n=2}^{n=5} \frac{a_n}{n} \Big/ \sum_{n=2}^{n=6} a_n \right) 100\ (\%)$$

from the data shown in Fig. 4. The IdUA-SO_4 content was calculated as $100-(x+y)$ (%). As Table 2 shows, glycans associated with the cells contained larger quantities of IdUA-GalNAc sequences compared with the other two glycan fractions. A rather low and constant amount of GlcUA-GalNAc units were found in all three

Table 2. *Content of various repeating disaccharide units in the IdUA-rich galactosaminoglycans obtained from the various sources after 72h of incorporation of radioisotope*

The relative amounts of IdUA-, GlcUA- and IdUA-SO_4-containing repeating units were calculated as described elsewhere (Malmström *et al.*, 1975*a*). See also the Results section. In the results marked *, disulphated as well as monosulphated species are included. The former disaccharide species account for less than 5% of the total radioactivity in the galactosaminoglycans. In results marked †, oligosaccharides ($n = 2$–5 in Fig. 4) released by chondroitinase-AC digestion were considered to be derived from a co-polymer composed of GlcUA- and IdUA-containing units. It should be recalled that the yield of disaccharides ($n = 1$) was used to estimate the quantity of GlcUA-rich variants (see Table 1).

^{35}S-labelled galactosaminoglycan from	Disaccharide repeating units containing IdUA (%)	IdUA-SO_4 (%)*	GlcUA (%)†
Insoluble cellular pool	63	20	17
Trypsin-liberated pool	38	41	21
Medium pool	44	36	20

co-polymeric dermatan sulphate fractions. Repeating units containing IdUA-SO_4 were more prevalent in glycans isolated from the medium and the trypsin digest compared with those of the cellular material.

Discussion

Cultures of fibroblast-like cells established from human embryonic lung were allowed to incorporate $^{35}SO_4^{2-}$ into glycosaminoglycans for various periods of time. The glycans were isolated from the medium, a trypsin digest of the cells and one soluble and one insoluble cell fraction. The specific radioactivity of the various glycans has not been recorded, mainly for two reasons. First, the methods available for determination of glycosaminoglycans and components thereof (e.g. sulphate) are too insensitive to permit detection of the amounts produced by fibroblasts in culture. Secondly, in the present work, specific degradations have been performed and the occurrence and distribution of periodate- and chondroitinase-AC-susceptible sites in the molecules have been assessed. As long as the specific radioactivity of the various repeating units is similar throughout the chain, the conclusions reached must be valid. Moreover, glycans produced by cells from different sources may well attain different specific radioactivities depending on the amount of unlabelled material present at the time of $^{35}SO_4^{2-}$ administration. However, comparisons between the various newly synthesized glycans with regard to their co-polymeric structure can still be made.

After incorporation of $^{35}SO_4^{2-}$ by monolayer cultures of fibroblasts, ^{35}S-labelled glycosaminoglycans are found both in the medium and in the cell layer. Part of the latter material may be released by brief trypsin digestion. The origin of the trypsin-released material is difficult to define. One portion of the material may be bound to the cell surface or laid down in the intercellular matrix and another portion may represent material passing through this pool *en route* to the medium. If trypsin treatment results in incomplete release of these glycans, part of this material might be recovered with the insoluble cell fraction. Moreover, if trypsin treatment stimulates secretion of intracellular material, this might be added to the trypsin digest. The soluble cell fraction contained small amounts of ^{35}S-labelled glycosaminoglycans, mostly heparan sulphate of low molecular weight. It seems reasonable to suggest that the soluble material was derived from a degradation pool (see also Kraemer, 1971).

The ^{35}S-labelled glycosaminoglycans were mainly found in the trypsin digest and the medium. Heparan sulphate and GlcUA-rich galactosaminoglycans were prominent components of the trypsin digest, whereas IdUA-rich galactosaminoglycans (dermatan sulphate) were largely confined to the medium (Table 1). This distribution is different from that obtained previously with human skin fibroblasts (Malmström *et al.*, 1975*a*). In the latter case, galactosaminoglycans containing both IdUA and GlcUA dominated in both the trypsin digest and the medium. Moreover, a separate chondroitin sulphate-like population could not be detected in cultures of skin fibroblasts.

Gel chromatography of ^{35}S-labelled galactosaminoglycans from the three sources indicated the following order of molecular sizes: cellular > trypsin-released material > secreted material. Similar results were obtained with skin fibroblasts (Malmström *et al.*, 1975*a*). Partial degradation by glycosidases secreted by the cells or present in the medium is a possible explanation.

As Table 2 shows, the ^{35}S-labelled IdUA-rich galactosaminoglycans from the insoluble cell fraction, the trypsin digest and the medium contained similar quantities of GlcUA-GalNAc-SO_4 repeat sequences. However, in the glycan from the cellular fraction, one IdUA residue out of four carried a sulphate group, whereas one residue out of two was sulphated in the material derived from the trypsin digest and the medium. In previous work the structural features of ^{35}S-labelled co-polymeric dermatan sulphate produced by skin fibroblasts were also examined (Malmström *et al.*, 1975*a*). Comparisons with the results of the present study showed both similarities and dissimilarities. In both cell cultures,

dermatan sulphate from the trypsin digest and the medium contained fewer IdUA residues than did the cell-associated glycans. Conversely, excreted chains contained larger quantities of IdUA-SO_4 residues than did the cellular material. However, dermatan sulphate secreted by skin fibroblasts also contained larger amounts of GlcUA than did cellular glycans. All co-polymeric chains isolated from lung fibroblasts had a similar GlcUA content (Table 2). Moreover, dermatan sulphate produced by skin fibroblasts and recovered from the trypsin digest contained alternating sequences (-GlcUA-GalNAc-IdUA-GalNAc-GlcUA-GalNAc-) (Malmström *et al.*, 1975*a*). Similar sequences could not be detected in corresponding material from lung fibroblasts.

The composition of the intercellular matrix (material released by trypsin) laid down by cultured fibroblasts from skin and lung was distinctly different. Whereas the matrix surrounding the cells from skin contained both dermatan sulphate and heparan sulphate, that of the lung cells contained large amounts of heparan sulphate, very little dermatan sulphate, and, in addition, a separate GlcUA-rich galactosaminoglycan component. In the previous paper (Malmström *et al.*, 1975*a*) it was proposed that co-polymeric dermatan sulphate chains containing alternating sequences were entrapped in the intercellular matrix owing to interactions with other macromolecules. It has since been demonstrated that co-polymeric chains with these features may interact *in vitro* to form aggregates (Fransson, 1976). It is possible that the relatively low content of co-polymeric glycans in the matrix produced by lung fibroblasts was because these cells did not synthesize aggregating species. It is not known whether IdUA-rich and GlcUA-rich chains are part of the same proteoglycan or if separate proteoglycan species exist. Therefore the significance of a relative enrichment of chondroitin sulphate-like chains in the trypsin digest cannot be evaluated. Although heparan sulphate is considered to be a general cell-surface component (Kraemer, 1971), its biological function remains unknown. Therefore it is difficult to ascertain whether the relatively high content of heparan sulphate in the trypsin digest of lung fibroblasts serves a specific purpose.

The dermatan sulphate produced and secreted by lung fibroblasts was mainly confined to the medium (Table 1). The IdUA residues of the secreted material were sulphated to a larger extent (approx. 50%) than were those of the cell-associated glycans (approx. 25%). This twofold increase in IdUA-SO_4 content might 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 would give the same result. In the previous paper (Malmström *et al.*, 1975*a*) two competing polymer-level reactions were proposed, i.e. IdUA $\rightarrow$ GlcUA and IdUA $\rightarrow$ IdUA-SO_4. Only the latter reaction should be considered in the present case. Finally, the present studies confirm that fibroblasts from different sources retain a specific pattern of glycosaminoglycan synthesis in cell culture. It is also apparent that even subtle differences in co-polymeric structure can be detected.

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