Isolation and characterization of dermatan sulphate and heparan sulphate proteoglycans from fibroblast culture

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$^{35}\text{SO}_4^{2-}$ and $[^3\text{H}]$ leucine-labelled proteoglycans were isolated from the medium and cell layer of human skin fibroblast cultures. Measures were taken to avoid proteolytic modifications during isolation by adding guanidinium chloride and proteolysis inhibitors immediately after harvest. The proteoglycans were purified and fractionated by density-gradient centrifugation, followed by gel and ion-exchange chromatography. Our procedure permitted the isolation of two major proteoglycan fractions from the medium, one large, containing glucuronic acid-rich dermatan sulphate chains, and one small, containing iduronic acid-rich ones. The protein core of the latter proteoglycan had an apparent molecular weight of 47 000 as determined by polyacrylamide-gel electrophoresis, whereas the protein core of the former was considerably larger. The major dermatan sulphate proteoglycan of the cell layer was similar to the large proteoglycan of the medium. Only small amounts of the iduronic acid-rich dermatan sulphate proteoglycan could be isolated from the cell layer. Instead most of the iduronic acid-rich glycans appeared as free chains. The heparan sulphate proteoglycans found in the cell culture were largely confined to the cell layer. This proteoglycan was of rather low buoyant density and seemed to contain a high proportion of protein. The major part of the heparan sulphate proteoglycan from the medium had a higher buoyant density and contained a smaller amount of protein.

Although the structure and metabolism of glycosaminoglycans produced by cells in cultures has been studied for many years, relatively little is known about the structure of the parent proteoglycans. Kresse *et al.* (1975) reported the presence of proteoglycans of different sizes in a fibroblast culture medium and in an EDTA extract of the cell monolayer, but no further characterization was performed. A chondroitin sulphate proteoglycan was isolated from the medium of a glial-cell culture (Norling *et al.*, 1978). These proteoglycans, like the cartilage monomers, can bind to hyaluronic acid.

In previous work (Cöster *et al.*, 1979) proteoglycans were isolated from the medium, an EDTA extract of the fibroblast monolayer, a dithiothreitol extract of the dispersed cells and a 4 M-guanidinium chloride extract of the cell residue. In all extracts a mixture of proteoglycans containing dermatan sulphate and heparan sulphate was found. A glucuronic acid-rich proteodermatan sulphate was obtained free from proteoheparan sulphate and idu-

ronic acid-rich dermatan sulphate proteoglycans. In the present paper a modified procedure is described that permits the isolation of several different proteoglycan species, i.e. one glucuronic acid-rich and one iduronic acid-rich proteodermatan sulphate and two different proteoheparan sulphates.

Experimental

Materials

Papain (EC 3.4.22.2, twice crystallized) and guanidinium chloride (technical grade) were purchased from Sigma. Stock solutions of guanidinium chloride were treated with charcoal to decrease the $A_{280}^{1\text{cm}}$ (water blank) to below 0.1. Solutions of urea were passed through a mixed anion-cation exchanger to remove cyanate. Chondroitinase AC (EC 4.2.2.5) and ABC (EC 4.2.2.4) and dithiothreitol were obtained from Miles Laboratories. $\text{Na}_2^{35}\text{SO}_4$ (carrier-free) and L-[4,5- ^3H]leucine (40–60 Ci/mmol) were products of The Radiochemical Centre. Earle's minimal essential medium, calf serum and antibiotics were obtained from sources listed pre-

Abbreviation used: SDS, sodium dodecyl sulphate.

viously (Malmström *et al.*, 1975). Instagel was bought from Packard. Sepharose and Sephadex gels as well as calibration kits for electrophoresis were obtained from Pharmacia Fine Chemicals, and microgranular ion-exchange DEAE-cellulose (DE-52) was purchased from Whatman. Ultrafiltration membranes (PM-10 and -30) were manufactured by Amicon. Polyacrylamide (enzyme grade) was bought from Eastman Kodak and *NN'*-diallyltartardiamide (Electrophoresis grade) from Bio-Rad.

Analytical and separation methods

Radioactivity was determined in a Packard 2650 scintillation counter with automatic quench correction. Gel chromatography was performed on columns of Sepharose CL-2B, CL-4B and CL-6B as described in the legends to the appropriate Figures.

Ion-exchange chromatography was performed at 4°C on columns (6 mm × 140 mm) of DE-52 DEAE-cellulose (acetate form). The samples were eluted at a rate of 6 ml/h with an acetate gradient in 6 M-urea [mixing vessel, 75 ml of 0.05 M-sodium acetate/6 M-urea/bovine serum albumin (5 µg/ml); reservoir, 75 ml of 1.5 M-acetate/6 M-urea/albumin (5 µg/ml)]. Before chromatography, 10 mg of albumin was applied to the column and washed through with 3 bed volumes of starting buffer. The proteoglycan fractions were supplemented with 10 mg of albumin and then dialysed against starting buffer before chromatography. The pretreatment of the columns with albumin and the presence of the protein in the buffers significantly increased the recovery of radiolabelled proteoglycans.

Paper chromatography was carried out on Whatman 3MM paper in the solvent isobutyric acid/0.05 M-ammonia (5:3, v/v) for 48 h (Suzuki *et al.*, 1968).

Polyacrylamide-gel electrophoresis was conducted in gels, 8 × 2.5 and 6 × 5 according to the

notation of Hjertén (1962), in 0.1% (w/v) SDS as described by Neville (1971). Before electrophoresis the various proteoglycan fractions were supplemented with 100 µg of albumin, dialysed against water and freeze-dried. Chondroitinase-ABC digestion was carried out in 100 µl of 0.01 M-Tris/0.01 M-acetate, pH 7.3, with 0.01 unit of enzyme at 37°C for 4 h. The samples were diluted 1:1 with concentrated upper reservoir buffer containing 0.01 M-EDTA/10% (v/v) 2-mercaptoethanol/3.8% (w/v) SDS and the solutions were heated for 3 min at 100°C. Gels containing radioactive samples were frozen immediately after electrophoresis, cut serially and solubilized. Polyacrylamide cross-linked by bisacrylamide was treated with 500 µl of 35% (w/w) H₂O₂ for 3 h at 80°C, and polyacrylamide cross-linked by *NN'*-diallyltartardiamide was brought into solution by oxidation with 500 µl of 2% (w/v) HIO₄ overnight at 37°C.

Density-gradient centrifugation in CsCl and 4 M-guanidinium chloride was performed as described by Cöster *et al.* (1979). For details, see also the legend to Fig. 1.

Incorporation of ³⁵SO₄²⁻ and [³H]leucine into proteoglycans by cultured fibroblasts

Fibroblast cultures were established from human embryonic skin and were grown in monolayer as described by Malmström *et al.* (1975). The cells were incubated with the radioactive precursors (5 µCi of ³⁵SO₄²⁻ and 5 µCi of [³H]leucine/ml) for 72 h as described by Cöster *et al.* (1979).

Isolation of proteoglycans (Scheme 1)

The culture medium (50 ml) was decanted and the cell layer was carefully washed with 10 ml of phosphate-buffered saline (137 mM-NaCl/3 mM-KCl/8 mM-NaHPO₄/1 mM-KH₂PO₄, pH 7.4). The medium and the washings were combined and

Scheme 1. Flow diagram for the isolation and fractionation of proteoglycans from fibroblast cultures

supplemented with 0.1 M-di-isopropyl phosphorofluoridate in propanol to a final concentration of 1 mM. The solution was then centrifuged at 10000 g for 30 min. The supernatant was diluted (1:1) with 7.5 M-guanidinium chloride/50 mM-sodium acetate/16 mM-EDTA/4 mM-*N*-ethylmaleimide, pH 5.8. The mixture was concentrated 10-fold in a Diaflo ultrafiltration cell over a PM-30 membrane and dialysed against 4 M-guanidinium chloride/50 mM-sodium acetate/2 mM-*N*-ethylmaleimide/8 mM-EDTA, pH 5.8. The non-diffusible material was subjected to density-gradient centrifugation as described above.

The cell layer was extracted for 6 h with 25 ml of 4 M-guanidinium chloride/50 mM-sodium acetate/2 mM-*N*-ethylmaleimide/16 mM-EDTA, pH 5.8. The extract was cleared of cell debris by centrifugation at 30000 g for 30 min. The pellet was re-extracted with 5 ml of the above extractant. The combined supernatants were concentrated as described for the medium and subjected to density-gradient centrifugation. The final pellet was discarded. Less than 1% of the total ^{35}S -labelled material is obtained in this fraction (Cöster *et al.*, 1979).

Isolation and identification of glycosaminoglycans

The samples (with 0.5 mg each of dermatan sulphate and heparan sulphate as carrier) were digested with papain (Cöster *et al.*, 1979) and chromatographed on columns (10 mm $\times$ 20 mm) of DE-52 DEAE-cellulose. The ^{35}S -labelled polysaccharides were recovered as described by Sjöberg *et al.* (1979), treated with HNO_2 (Lagunoff & Warren, 1962) and subjected to gel chromatography on Sephadex G-50 to determine the relative amounts of heparan sulphate and galactosaminoglycans. The latter were eluted with the void volume and subjected to periodate oxidation/alkaline elimination followed by gel chromatography on Sephadex G-50 to assess the distribution of L-iduronic acid in the polymers (Malmström *et al.*, 1975). The galactosaminoglycans were also subjected to chondroitinase-AC digestion (Suzuki *et al.*, 1968) followed by separation on Sephadex G-50 of the split products to determine the ratio of L-iduronic acid to D-glucuronic acid. The distribution of 4- and 6-sulphated saccharides in the galactosaminoglycans was estimated after chondroitinase-ABC digestion followed by paper chromatography (see above).

Results

After density-gradient centrifugation (Fig. 1) most of the ^{35}S -labelled proteoglycans (75%) were obtained in fractions with densities higher than 1.39 g/ml, whereas most of the protein was found in the top fractions. However, significant amounts of ^{35}S -

Fig. 1. *CsCl*-density-gradient centrifugation of proteoglycans from the medium (a) and the cell layer (b)

The proteoglycans were extracted from the cell culture as described in the Experimental section and *CsCl* was added to a density of 1.35 g/ml. After centrifugation (60 h at 34000 rev./min), 2 ml fractions were collected from the bottom and fractions 1–2, 3–6 and 7–8 were pooled, and designated G12, G36 and G78 respectively. Solid bars, $^{35}\text{SO}_4^{2-}$ (d.p.m.); dashed bars, ^3H (d.p.m.); —, density (g/ml).

labelled material were also found in fractions with intermediate densities.

The fraction PGM G12 from the medium was separated into two major populations on Sepharose CL-2B, as shown in Fig. 2(a). The larger one (PGM G12-I) was a pure proteogalactosaminoglycan (Table 1). Most of the uronosyl residues were D-glucuronic acid, and approximately two-thirds of the *N*-acetylgalactosamine residues carried 4-sulphate groups. The smaller proteoglycan (PGM G12-II) contained 38% heparan sulphate (Table 1). The galactosaminoglycan component of this fraction was an iduronic acid-rich dermatan sulphate. The *N*-acetylgalactosamine residues were exclusively sulphated in the 4-position.

The fraction PGM G36 was also separated into two fractions on Sepharose CL-2B (Fig. 2b). Both populations contained small amounts of heparan sulphate (Table 1). The galactosaminoglycan components of the two fractions were similar to those obtained at a higher density.

The proteoglycans from the cell layer were recovered in one heavy (G12) and one lighter fraction (G36). Both were resolved into two frac-

Fig. 2. Gel chromatography on Sepharose CL-2B of fractions obtained after CsCl-density-gradient centrifugation. Subfractions of PGM, fractions G12 (a) and G36 (b), and PGC, fractions G12 (c) and G36 (d), were chromatographed on a column of Sepharose CL-2B. Column size, 1.8 cm $\times$ 145 cm; elution rate, 6 ml/h; fraction size, 3.4 ml; eluent, 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8; —, $^{35}\text{SO}_4^{2-}$ (d.p.m.); ----, ^3H (d.p.m.). Abbreviations: V_0 , void volume; V_t , total volume. The fractions were pooled as indicated by the upper bars and designated as shown in the Figure as I and II. However, in one experiment the material obtained between the major proteoglycan fractions was pooled separately (H, lower bars). In this case the major fractions were designated Ia and IIa.

tions by gel chromatography (Figs. 2c and 2d). The smaller species (PGC G12-II and PGC G36-II) chromatographed in a more retarded position than the smaller proteoglycans from the cell medium (PGM G12-II and PGM G36-II). In both the former fractions heparan sulphate was a minor component. The side chains of the larger proteoglycans were similar to those of the corresponding fraction of the medium, whereas the smaller proteoglycan contained more non-sulphated iduronic acid residues (Table 1).

The large proteoglycan of low buoyant density that was derived from the cells (PGC G36-I) on chromatography was slightly more retarded than the large proteoglycans of the other fractions (cf. I in Fig. 2d with I in Figs. 2a, 2b and 2c). Furthermore,

the $^{35}\text{S}/^3\text{H}$ ratio was considerably lower than that of the other fractions. The side chains were completely depolymerized by HNO_2 treatment, and no material was liberated by chondroitinase-ABC treatment, which shows that this proteoglycan was a proteoheparan sulphate. Since this proteoglycan chromatographed between the two major proteoglycans on Sepharose CL-2B (Fig. 2), fractions between these peaks were pooled separately in a second preparation (Ia, H and IIa in Fig. 2). The amounts of heparan sulphate in these fractions are given in Table 2. This procedure afforded an enrichment of the heparan sulphate of the medium into fraction PGM G12-H (44% of the heparan sulphate of the medium as calculated from Table 2). Similarly, the cell-derived heparan sulphate contained in the heavy

Table 1. *Structure and distribution of ³⁵S-labelled glycosaminoglycans in various proteoglycan fractions (preparation I)*

The quantity and the relative distribution of ³⁵S-labelled glycosaminoglycans was determined as described in the Experimental section and are given as percentages of total glycosaminoglycan. The amount of non-sulphated L-iduronic acid was estimated after periodate oxidation/alkaline elimination (Malmström *et al.*, 1975). By this method an overestimation of non-sulphated L-iduronic acid residues is obtained, since, if the procedure is used on chondroitin sulphate, 11% of the uronosyl residues will be accounted for as L-iduronic acid. The proportion of D-glucuronic acid in the galactosaminoglycans was determined after treatment by chondroitinase AC (Malmström *et al.*, 1975). The amount of sulphated iduronic acid was estimated as total uronate minus the sum of glucuronic acid and non-sulphated iduronic acid. Similar results were obtained after periodate oxidation (37°C) of all non-sulphated uronic acid residues followed by alkaline elimination. The proportion of 4- and 6-sulphated disaccharides was determined by paper chromatography after chondroitinase-ABC digestion. Results are given as percentages of total unsaturated and sulphate disaccharides; n.d., not determined. Abbreviations: GalNAc, *N*-acetylgalactosamine; IdA, iduronic acid; GlcA, glucuronic acid, OseA, 4,5-unsaturated glycuronic acid.

Proteoglycan fraction	Distribution of ³⁵ S-labelled glycosaminoglycan (%)	Proportion of heparan sulphate (%)	Proportion of galactosaminoglycans (%)	Structure of galactosaminoglycans (%)			
				IdA-GalNAc	GlcA-GalNAc	IdA-GalNAc SO ₄	OseA-GalNAc-4-SO ₄
PGM G12-I	27	0	100	19	73	8	64
PGM G12-II	19	38	62	41	39	20	100
PGM G36-I	5	18	82	24	69	7	60
PGM G36-II	10	14	86	41	28	31	100
PGC G12-I	20	6	94	22	62	16	56
PGC G12-II	10	5	95	51	27	22	100
PGC G36-I	7	100	0	n.d.	n.d.	n.d.	n.d.
PGC G36-II	3	0	100	54	26	20	100

Table 2. *Distribution of heparan sulphate in the various proteoglycan fractions (preparation II)*

The quantity of ³⁵S-labelled glycosaminoglycan was determined as described in the Experimental section. The various fractions were obtained as shown in Fig. 2 (lower bars). The amount of heparan sulphate was determined after HNO₂ degradation followed by gel chromatography, and the results are given as the percentage of total glycosaminoglycan in the fraction.

	Distribution of ³⁵ S-labelled glycosaminoglycan in the culture (%)	Proportion of heparan sulphate of total ³⁵ S-labelled glycosaminoglycan in the fraction (%)	Distribution of ³⁵ S-labelled heparan sulphate in the culture (%)
PGM G12-Ia	30	0	0
PGM G12-H	5	80	22
PGM G12-IIa	16	20	16
PGM G36-Ia	4	15	4
PGM G36-H	1	35	2
PGM G36-IIa	11	12	6
PGC G12-Ia	6	2	0
PGC G12-H	2	57	5
PGC G12-IIa	8	2	1
PGC G36-I	8	100	43
PGC G36-II	9	0	0

fraction (PGC G12) was enriched in this middle fraction (H).

When subjected to ion-exchange chromatography in 6 M-urea (Fig. 3), some ³H-labelled material was removed from the proteoglycan in all fractions. The glucuronic acid-rich proteoglycans from the medium (PGM G12-Ia and PGM G36-Ia) and the cells (PGC G12-Ia) (Figs. 3a, 3b and 3c) were eluted at a somewhat higher ionic strength than was needed

to elute the smaller proteodermatan sulphates (Figs. 3d, 3e, 3f and 3g). Although the smaller proteodermatan sulphates of the cell layer (PGC G12-IIa and G36-II, Figs. 3e and 3f) contained a higher ratio of ³⁵S to ³H than did the corresponding fractions from the medium (PGM G12-IIa, Fig. 3d), they chromatographed similarly on DEAE-cellulose DE-52. The two fractions containing heparan sulphate (PGM G12-H and PGC G36-I, Figs. 3h

Fig. 3. Ion-exchange chromatography on DE-52 DEAE-cellulose of various proteoglycan subfractions of PGM and PGC obtained by gel chromatography

Subfractions of PGM and PGC were dialysed against 6 M-urea/0.05 M-sodium acetate, pH 5.8, and chromatographed on a column of DE-52 DEAE-cellulose. The column size was 6 mm $\times$ 140 mm and the elution rate 3 ml/h. The samples were eluted with a salt gradient as described in the Experimental section (fraction size 2 ml). —, $^{35}\text{SO}_4^{2-}$ (d.p.m.); ---, ^3H (d.p.m.); ···, conductivity (S). The fractions were pooled as indicated by the bar. (a) Fraction PGM G12-Ia, (b) PGM G36-Ia, (c) PGC G36-Ia, (d) PGM G12-IIa, (e) PGC G12-IIa, (f) PGC G36-IIa, (g) PGM G36-I, (h) PGC G36-I and (i) PGM G12-H. The breakthrough fraction, which is not shown in the Figures, contained ^3H -labelled material in all cases.

Fig. 4. Gel chromatography on Sepharose CL-6B of proteoglycan fractions before and after papain digestion. Proteoglycan fractions were subjected to chromatography on Sepharose CL-6B before (a, c and e) and after (b, d and f) papain digestion. Column size, 6 mm $\times$ 140 cm; eluent, 4 M-guanidinium chloride/albumin (5 $\mu\text{g}/\text{ml}$)/0.05 M-sodium acetate, pH 5.8; fraction size, 0.7 ml; elution rate, 1 ml/h. —, $^{35}\text{SO}_4^{2-}$ (d.p.m.); ---, ^3H (d.p.m.). The samples were: (a) and (b), PGM G12-I; (c) and (d), PGM G12-II; (e) and (f), PGC G12-II.

and 3i) had very different $^{35}\text{S}/^3\text{H}$ ratios as well as elution positions.

Further characterization of the major proteogalactosaminoglycans

To ascertain the proteoglycan nature of the macromolecules, they were subjected to gel chromatography on Sepharose CL-6B before and after papain digestion. The large glucuronic acid-rich proteoglycan was excluded from the gel before, but included after, digestion (Figs. 4*a* and 4*b*). Papain treatment of proteodermatan sulphate (PGM G12-II) also resulted in a shift in the elution position, albeit to a smaller extent (Figs. 4*c* and 4*d*). It should be noted that the polysaccharide chains from the two proteoglycans had an identical elution position (Figs. 4*b* and 4*d*). The dermatan sulphate-containing fraction from the cell layer had a much lower $^3\text{H}/^{35}\text{S}$ ratio and was eluted later from Sepharose CL-6B before and after papain digestion (Figs. 4*e* and 4*f*). Before digestion, this fraction (PGC G12-II) was eluted partly at the position of free chains (Fig. 4*e*). It is concluded that this fraction contained dermatan sulphate peptides of relatively low molecular weight.

The major proteogalactosaminoglycans of the medium and the cell layer were examined by SDS/polyacrylamide-gel electrophoresis before and after chondroitinase-ABC digestion. Before digestion the large proteochondroitin sulphate (PGM G12-Ia) did not penetrate the 5%-acrylamide gel, whereas the smaller proteodermatan sulphate (PGM G12-Ia) did (Figs. 5*a* and 5*c*). After digestion of PGM G12-Ia a large protein component entered the gel (Fig. 5*b*). The apparent molecular weight of this component was over 400 000. In contrast, the dominant protein band obtained after chondroitinase-ABC digestion of the smaller proteoglycan (PGM G12-IIa) had an apparent molecular weight of 47 000 on an 8%-acrylamide gel (Fig. 5*d*). The major dermatan sulphate fraction of the cell layer (PGC G12-IIa) also yielded one dominant ^3H -labelled band after chondroitinase-ABC digestion (results not shown), which had mobility identical with the core of the proteodermatan sulphate from the medium.

Further characterization of heparan sulphate proteoglycans

The cell-derived proteheparan sulphate (PGC G36-I) was eluted close to the void volume when chromatographed on Sepharose CL-4B (Fig. 6*a*), whereas that of the medium was more retarded (Fig. 6*b*). The former proteoglycan chromatographed in the void volume on Sepharose CL-6B before, but was well included after, papain digestion (results not shown). Papain digestion of the medium-derived proteheparan sulphate caused a smaller shift in

Fig. 5. SDS/polyacrylamide gel electrophoresis of PGM G12-Ia and PGM G12-IIa before (a and c) and after (b and d) digestion by chondroitinase ABC

Proteoglycans were dialysed against water, freeze-dried and redissolved in buffer and digested with chondroitinase ABC as described in the Experimental section. The samples were PGM G12-Ia before (a) and after (b) digestion and PGM G12 before (c) and after (d) digestion. Gels (a), (b) and (c) are $6 \times 5\%$ gels with NN' -diallyltartaramide as cross-linker. PGM G12-IIa after digestion (d) was applied to an $8 \times 2.5\%$ gel with bisacrylamide as cross-linker. The doubly labelled component at S in (c) and (d) is likely to be proteoglycan sulphate. The gels were sliced and assayed for radioactivity as described in the Experimental section. —, $^{35}\text{SO}_4^{2-}$ (d.p.m.); ----, ^3H (d.p.m.); S, start of lower gel; M, tracking dye.

Fig. 6. Gel chromatography on Sepharose CL-4B of the proteoglycan sulphate fractions PGC G36-I (a) and PGM G12-H (b)

After ion-exchange chromatography (Figs. 3h and 3i) the proteoglycans were pooled, dialysed against 4 M-guanidinium chloride/0.05 M-sodium acetate, pH 5.8, and subjected to gel chromatography on a column (0.6 cm $\times$ 145 cm) of Sepharose CL-4B; eluent, 4 M-guanidinium chloride/0.05 M-sodium acetate (pH 5.8)/albumin (5 μ g/ml); fraction size, 0.7 ml; elution rate, 2 ml/h. —, $^{35}\text{SO}_4^{2-}$ (d.p.m.); ----, ^3H (d.p.m.).

elution position. The heparan sulphate chains of the two proteoglycans were of the same size (results not shown).

To lessen the likelihood that the cell-derived proteoglycan sulphate was aggregated by hydrophobic proteins from the cell membrane, it was subjected to dialysis from 4 M-guanidinium chloride into 6 M-urea and finally against 1% (w/v) SDS/0.05 M- NaHCO_3 . No liberation of ^3H radioactivity and no change in elution position was observed when results of chromatography (Sepharose CL-2B) in 4 M-guanidinium chloride and SDS were compared (results not shown). The results also indicate that the heparan sulphate proteoglycan is heterogeneous.

Discussion

The present procedure for the purification and subfractionation of proteoglycans from fibroblast

cultures is an improvement of our earlier scheme (Cöster *et al.*, 1979). In addition to the 'proteoglycan sulphate' that was obtained in an apparently pure state with the earlier technique, one proteoglycan and two different proteoglycan sulphates can be resolved by the present procedure. The proteoglycans have furthermore been purified to a state that makes studies of the protein core possible, as all non-proteoglycan proteins have been removed. The risk of proteolytic modification is minimized by the addition of proteinase inhibitors and high concentrations of guanidinium chloride. As shown earlier (Cöster *et al.*, 1979), two different galactosaminoglycan-containing proteoglycans can be resolved by gel chromatography of the culture medium. The relative amount obtained of these is the same with the two procedures. However, the present one affords a more distinct separation, owing to the presence of guanidinium chloride in the eluting buffer and to the fact that the bulk of protein was removed in the density-gradient-centrifugation step. Both proteoglycans are dermatan sulphate proteoglycans and will be referred to as the glucuronic acid-rich (the large one) and the iduronic acid-rich dermatan sulphate proteoglycan (the small one), respectively.

The protein cores of these two proteoglycans show marked differences. The iduronic acid-rich proteoglycan has a protein core with an apparent molecular weight of 47 000, which is in agreement with that obtained from the small scleral proteoglycan described by Cöster & Fransson (1981). However, the apparent molecular weight of the protein core from the glucuronic acid-rich proteoglycan was estimated to be over 400 000. This is larger than a native proteoglycan from bovine sclera (Cöster & Fransson, 1981), which chromatographs close to the native glucuronic acid-rich proteoglycan on Sepharose CL-2B. As chondroitinase-ABC digestion removes nearly all of the ^{35}S -radioactivity, a much lower molecular weight would be expected. The anomalous migration might be explained by the presence of oligosaccharides (Segrest *et al.*, 1971). Oligosaccharide substitution is well established for cartilage proteoglycans (Lohmander *et al.*, 1980), for dermatan sulphate proteoglycans from follicular fluid (Yanagishita *et al.*, 1979) and from bovine sclera (Cöster & Fransson, 1981).

Also in the cell layer components can be found that seem to correspond to the glucuronic acid-rich and the iduronic acid-rich proteoglycans. However, the latter macromolecules derived from the cell layer contain only little protein (low $^3\text{H}/^{35}\text{S}$ ratio). Furthermore, this fraction contained only a small amount of the 47 000-mol.wt. protein characteristic of the iduronic acid-rich proteoglycan from the medium. Thus this fraction contains mainly free

dermatan sulphate chains, probably part of a degradation pool. The dominant proteogalactosaminoglycan in the cell layer is thus the glucuronic acid-rich one, as the major amount of the iduronic acid-rich components appears as free chains. This is in agreement with the fact that trypsin releases glycans that are rich in glucuronic acid (Malmström *et al.*, 1975). It is likely that this proteoglycan is identical with or at least closely similar to the glucuronic acid-rich proteoglycan from the culture medium, since the hydrodynamic volumes of the native proteoglycans, the size and behaviour of the protein cores as well as the structure of the polysaccharide chains are very similar. These proteoglycans interact specifically with hyaluronic acid (Cöster *et al.*, 1979) (L. Cöster, unpublished work), as does a closely similar proteoglycan species from glial cells (Norling *et al.*, 1978). Thus hyaluronic acid present in the pericellular environment (Sjöberg *et al.*, 1979) might bind and selectively retain the glucuronic acid-rich proteoglycan. The iduronic acid-rich one, however, has no affinity for hyaluronic acid and will therefore appear preferentially in the medium.

The proteoheparan sulphate from the cell layer has a much higher $^3\text{H}/^{35}\text{S}$ ratio than the heparan sulphate proteoglycan from the medium. The former must therefore be either rich in protein or carry heparan sulphate side chains that are sparsely sulphated. However, earlier data show that heparan sulphate from the cell surface is rather highly sulphated (Malmström *et al.*, 1975). ^3H could not be removed from the proteoglycans, and the macromolecules did not change their hydrodynamic volume when treated with SDS, which strongly suggest that all the protein is covalently linked to the proteoglycan and that the proteoglycans themselves are not in an aggregated form. A speculative but very attractive idea consistent with the high $^3\text{H}/^{35}\text{S}$ ratio is that this proteoglycan is attached to the cell membrane bilayer by a hydrophobic anchor that is rich in hydrophobic amino acids such as leucine. Finally, this proteoglycan has a slightly larger K_{av} on Sepharose CL 2B than a dermatan sulphate proteoglycan with a molecular weight of about 400 000. This is similar to that of a heparan sulphate proteoglycan isolated by Rollins & Culp (1979), and it is considerably larger than a heparan sulphate proteoglycan obtained from liver plasma membranes (Oldberg *et al.*, 1979).

The heparan sulphate proteoglycan from the culture medium is smaller than that of the cell layer and contains in addition much less protein, as indicated by the lower $^3\text{H}/^{35}\text{S}$ ratio. It is, however, a 'true' proteoglycan, as papain digestion removes protein (^3H label) from the macromolecules. The polysaccharides from the cell-layer-derived heparan

sulphate proteoglycan have the same size as those derived from the medium. The heparan sulphate proteoglycan from the medium might either be derived from the cell-surface-associated ones by limited proteolytic cleavage or constitute a separate secretion product. The former alternative would then be principally different from the export of the galactosaminoglycan-containing proteoglycans and would represent an event that is rather correlated with cell division and turnover than with the secretion of macromolecules to the connective-tissue matrix.

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