Partial Characterization of Heparan and Dermatan Sulfate Proteoglycans Synthesized by Normal Rat Glomeruli*

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Rat glomerular heparan sulfate (HS) and dermatan sulfate (DS) proteoglycan synthesis was studied in vitro and in vivo. Incorporation of [35S]sulfate into macromolecules was linear over 16 h in vitro, and DS was the predominant glycosaminoglycan (GAG), while HS dominated in vivo incubations. Proteoglycans were found in the bottom ½ (high density) CsCl gradient fractions and eluted as two overlapping peaks from DEAE-Sepharose columns. The proportion of low density 35S-glycoproteins and 35S-proteoglycans increased with time.

Two high buoyant density HS proteoglycans were extracted from glomeruli and eluted in DEAE peak I. The first, HS-tIA, had an M₆ of 130 x 10⁶ with M₆, 12.5 x 10⁶ GAG chains. This proteoglycan was released from the tissue by trypsin and was partially displaced by heparin treatment. In addition, it was rapidly released into the medium of label-chase experiments after which it migrated slightly more rapidly than HS-tIA in gels, with HS chains similar in length to its tissue counterpart. The second, HS-tII, had an M₆ of 8.6 x 10⁶ with little or no attached protein. This proteoglycan was characterized as intracellular as it resisted release by trypsin treatment or heparin extraction in medium and was not detected in the medium of label-chase experiments.

Two tissue DS proteoglycans were characterized. The first, DS-tIA, co-purified with HS-tIA and was the predominant proteoglycan synthesized during 4-h in vitro incubations. Like HS-tIA, it was rapidly released into medium and displaced from cell surfaces or tissue "receptors" by heparin or trypsin treatments. A second, Sepharose CL-6B-excluded DS proteoglycan from DEAE peak II, DS-tIII, accumulated in tissue over 16 h in vitro. This proteoglycan was self-associating and contained clusters of iduronic acid residues along its M₆, 26 x 10⁶ DS chains. It resisted extraction from the tissue with heparin, trypsin, and detergent. No DS-tII was detected in the incubation medium. Instead, medium proteoglycans eluted as single Sepharose CL-6B-included peaks. DS chains from medium proteoglycans were shorter (M₆, 18 x 10⁶) and had more regularly spaced iduronic acid residues than GAGs from DS-tIII.

The length and sulfation patterns of DS-mII GAG were similar to GAG from DS-tIA.

Thus, glomeruli rapidly synthesized and released Sepharose CL-6B-included heparin-displaceable DS and HS proteoglycans while retaining a Sepharose CL-6B-excluded self-associating DS proteoglycan and an intracellular HS.

Heparan sulfate (HS) proteoglycan is an integral component of basement membranes. This proteoglycan has been extracted from glomerular basement membrane (GBM) where it appears in a lattice-like pattern along the lamina rara externa and interna (1, 2) and helps maintain the glomerular capillary barrier to protein leak. Evidence for this role includes observations that in situ heparitinase treatment results in leakage of 125I-albumin and native ferritin into the urinary space (3, 4) and that there is a diminution in the number of HS-related charge sites along the lamina rara externa of GBM from humans with a condition associated with proteinuria (5). In addition to its role in filtration, HS proteoglycan may anchor cells to their substratum, as suggested by its presence as an integral membrane macromolecule (6, 7) and as a component of fibroblast adhesion sites in other tissues (8-11). This function may be partially fulfilled through interaction with specific peptide domains of fibronectin and laminin (12-16).

Despite their potentially great functional importance, GBM proteoglycans account for only a small proportion (3-5%) of total glomerular proteoglycan (17). Their study has been complicated by frequent contamination of GBM preparations with mesangial components (18). Previous studies have focused on biochemical characterization of the principal M₆, 13 x 10⁶ HS proteoglycans from these GBM preparations (2, 19). However, DS is the dominant proteoglycan synthesized by intact isolated glomeruli after in vitro [35S]sulfate labeling (20). DS proteoglycan has been localized to the glomerular mesangium (21) where it may maintain tissue integrity as suggested by its ability to be cross-linked to fibronectin at fibroblast cell surfaces (22) and to mediate enhanced binding of fibronectin to collagen in other tissues (23). Since the HS proteoglycan content of isolated glomeruli is greater than that which can be accounted for by that found in GBM (20, 24), this proteoglycan must, like DS proteoglycan, be synthesized and incorporated into non-GBM glomerular components such as the mesangium and cell surfaces.

1 The abbreviations used are: HS, heparan sulfate; DS, dermatan sulfate; GAG, glycosaminoglycan; GBM, glomerular basement membrane; CHAPS, 3-(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.
2 Since the original submission of this manuscript, another publication has appeared in which the partial characterization of several glomerular proteochondroitin sulfate proteoglycans is described (51).
This study describes the biochemical characteristics and metabolic processing of proteoglycans from normal rat glomeruli. Emphasis is placed on descriptions of DS proteoglycan synthesized during shorter labeling the tissue. A large high buoyant density self-associating DS proteoglycan accumulated in the tissue over 16 h without being released intact into the incubation medium. A lower molecular weight DS was the predominant proteoglycan synthesized during shorter labeling experiments. This proteoglycan and an HS proteoglycan with similar biochemical characteristics were rapidly released from the tissue. These proteoglycans were displaced from cell surfaces or tissue GAG receptors by treatment with heparin or trypsin, while the larger DS proteoglycan resisted these extractions. A second HS with little or no protein core resisted heparin extractions and was, therefore, thought to be intracellular. Glomeruli isolated after in vivo [35S]sulfate labeling contained different proportions of proteoglycans which had similar properties to those synthesized in vitro.

**EXPERIMENTAL PROCEDURES**

**Materials**

The following chemicals were obtained from the indicated sources: RPMI 1640 sulfate-free medium with MgCl2 substituted for MgSO4, Gibco Laboratories; [35S]sulfate, carrier free, International Chemical Nuclear; CHAPS, Behring Diagnostics; Sepharose CL-4B and CL-6B and Sephadex G-50, Pharmacia Fine Chemicals; agarose (low EEO), porcine intestinal mucosal heparin, bovine pancreatic trypsin type III-S, and DEAE-Sephacel, Sigma; chondroitinase ABC from Proteus vulgaris, chondroitinase AC II from Arthrobacter aurescens, unsaturated chondroitin 4-sulfate and chondroitin 6-sulfate disaccharide standards, Miles Laboratories; heparitinase was purified from Flavobacterium heparinum as previously described (25); Aquasol II and ENHANCE, New England Nuclear; CsCl, Kawecki Berylco; ultrapure guanidine HCl, Schwarz/Mann; papain, Cooper Biomedical (Worthington). All reagents are of commercial grade or of the best available quality and were used without further purification.

**Methods**

**Glomerular Isolation and [35S]Sulfate Labeling**

Glomeruli were isolated from normal 250-g male Sprague-Dawley rat kidneys (Sprague-Dawley Co., Madison, WI) by serial sieving through wire meshes (26). Intact glomeruli were retained on 150 (100 mm2) and 200 (74 mm2) mesh screens and contained less than 5% tubular elements when examined by phase contrast microscopy. Glomerular cells excluded trypan blue both before and after in vitro incubations. Glomeruli from 10 rat kidneys were gently rocked (80 rpm on a junior orbit shaker) and then placed in 10 ml of RPMI medium containing 0.1 mM Na2SO4 and 250 μM/mL Na3SO4 at 37 °C in a humidified mixture of 95% air and 5% CO2. The incubation media were separated by gentle centrifugation (200 × g for 5 min) and, in the case of in vitro label-chase experiments, glomeruli reuspended in RPMI medium containing 0.5 mM Na2SO4 without Na2S2O4 after two washes in nonradioactive medium.

For in vivo experiments, groups of 10 rats were injected intraperitoneally either with 1 mg Na2S2O4 per animal 4 h prior to sacrifice or with 0.5 mCi Na235S2O4 per animal 4 h and were sacrificed 16 h after the initial injection.

**Proteoglycan Extraction and Purification**

4 M Guanidine HCl Extractions—Tissue 35S-proteoglycans were extracted by shaking glomeruli at 60 rpm in 8 volumes/volume wet glomeruli of an extraction buffer containing 4 M guanidine HCl, 0.05 M sodium acetate, 1 mM iodoacetamide, 5 mM benzamidine HCl, 10 mM sodium EDTA, and 0.1 M N-acetylmalonylamic acid, pH 5.8, for 24 h (27). When the residual tissue was re-extracted with the 4 M guanidine HCl-protease inhibitor extraction mixture without iodoacetamide but containing 10 mM dithiothreitol and the reaction terminated after 24 h with iodoacetamide (final concentration, 30 mM) and an additional 20% of the total incorporated radioactivity was released. Thus, the majority of glomerular 35S-proteoglycans are not disulfide bonded to the guanidine HCl-insoluble glomerular matrix. 35S-GAGs in the tissue residue were released from the twice-extracted tissue by papain digestion (17). The incubation media were diluted 1:1 with 8 M guanidine HCl extraction buffer containing 2% protease inhibitors.

**Heparin Extractions of Radiolabeled Glomeruli**—35S-Proteoglycans were displaced from cell surface or intercellular matrix "GAG binding sites" by two different heparin treatment protocols. In one experiment performed in duplicate, glomerular proteoglycans were released from the tissue fraction by a 30-min treatment with a 10 mM Na2S2O4, 3 M guanidine HCl extraction buffer. The residual tissue 35S-macromolecules were extracted with 0.05 M Tris and 0.35 M NaCl (high salt buffer), followed by a third extraction in high salt buffer containing 0.2% CHAPS each at pH 8.0 and 4 °C for 1 h in the presence of protease inhibitors. Tissue 35S-macromolecules remaining after these three treatments were extracted with the 4 M guanidine HCl extraction buffer described above at 4 °C.

**CcI Gradient Ultracentrifugation**—4 M guanidine-extracted 35S-proteoglycans were separated by CcI density gradient ultracentrifugation (0.44 g of CcI/g of extract) at 40,000 rpm and 4 °C for 60 h using a Ti-70 rotor and a Beckman model L5-65 ultracentrifuge. The gradients were cut into bottom 5% (high buoyant density), middle 7.0, and top 5% (low buoyant density) fractions using a Beckman tube slicer, and the fractions were dialyzed against at least five 6-liter changes of 0.5 M sodium acetate and 0.1 M sodium sulfate in the presence of protease inhibitors (27). In preparation for DEAE-Sephacel chromatography, the dialysis solutions containing 0.05 M Tris, 0.1% CHAPS, 0.1 M NaCl, and protease inhibitors, pH 7.0 (DEAE buffer). Aliquots of each fraction were counted in a Beckman LS-5900 scintillation counter using Aquasol 2 as the fluorophore.

**DEAE-Sephacel Chromatography**—DEAE-Sephacel chromatographic columns (2-m total bed volume) were equilibrated in DEAE buffer (see above) and preconditioned by loading with 2 mg each of chondroitin sulfate proteoglycan from the Swara rat chondrosarcoma (30), heparin, and bovine serum albumin. The columns were then washed with 10 volumes of DEAE buffer and stripped with the same buffer containing 2 M NaCl. After re-equilibration with DEAE buffer, samples were loaded and the columns washed with 10 column volumes of starting buffer. A 100-ml linear gradient from 0.1 to 1.0 M NaCl in DEAE buffer was then applied and 2-ml fractions collected. Fractions from early (peak I) and late (peak II) elution peaks were separately pooled, dialyzed against two 4-liter changes of distilled water, and lyophilized. The purity at each peak eluted was estimated by comparing conductivities measured using a Radiometer conductivity meter with standards of 0.1-1.0 M NaCl in DEAE buffer. Recovery of radioactivity from DEAE-Sephacel columns was 85-96%.

**Analytical Methods**

**Gel Filtration Chromatography**—Gel filtration was carried out on 0.9 × 110-em columns of Sepharose CL-4B and CL-6B equilibrated with 0.5 M sodium acetate at 4 °C, 0.5% sodium acetate containing 0.2% CHAPS, pH 7.0, at 3.0-4.0 ml/h with 1.0-1.2-ml fractions collected (associative conditions). Rat chondrosarcoma chondroitin-sulfate proteoglycan (30) in association with 4% hyaluronic acid and glucuronolactone were used to mark the column void (Vo) and total (Vt) volumes, respectively (31). To dissociate proteoglycan aggregates, samples were either eluted from Sepharose columns run in the presence of 4 M guanidine HCl (associative conditions), or columns were run under associative conditions after reducing 35S-proteoglycan samples overnight with 10 mM dithiothreitol in 4 M guanidine HCl, pH 7.4, at 25 °C under nitrogen followed by dialysis with 40 mM iodoacetamide in the dark at 25 °C for 16 h (32). Recovery of radioactivity from Sepharose columns varied from 80 to 95%. Proteoglycan and GAG size were estimated using the data of Heinegard and Hascall.
Proteoglycans Synthesized by Normal Rat Glomeruli

GAG Cleavage from Intact Proteoglycan—$^{35}$S-GAGs were released from the proteoglycan core protein by alkaline $\beta$-elimination in 0.05 N NaOH and 1 M NaBH$_4$ for 48-60 h at 45 °C. The reaction mixture was neutralized by the dropwise addition of acetic acid and desalted on Sephadex G-50 columns (17, 27, 35). $^{35}$S-GAGs were recovered from these columns by lyophilization.

Determination of HS and DS $^{35}$GAG Content and Chain Size—$^{35}$S-GAGs were subject to nitrous acid denaminative cleavage by methods modified from Conrad et al. (17, 36). Insensitive (V$_4$) $^{35}$S-GAGs were digested with chondroitinase ABC or AC. When present, $^{35}$S-macromolecules excluded from Sephadex G-50 columns after both nitrous acid and chondroitinase ABC treatments were further digested with highly purified heparitinase (25) to cleave N-acetylated regions of HS. Equivalent results were obtained starting with either alkalireleased $^{35}$S-GAG or intact $^{35}$S-proteoglycans and were independent of the sequence of digestion. HS proteoglycan content was determined by percent sensitivity to nitrous acid and heparitinase while DS proteoglycan was that portion susceptible to chondroitinase ABC digestion. Material excluded from or partially included in Sephadex G-50 columns ($K_m < 0.1$) after the degradative sequence including heparitinase treatment was designated "sulfated glycoprotein" (35). This material has been previously shown to be insensitive to keratinase (17).

$^{35}$S-GAGs present in the Sephadex G-50 V$_4$ after nitrous acid treatment were used for determining DS chain size on Sepharose CL-6B columns, while HS size was characterized using the chondroitinase ABC-insensitive $^{35}$S-GAG.

Agarose-Polyacrylamide Gel Electrophoresis—Electrophoresis in 0.6% agarose-1.8% polyacrylamide gels was performed according to a method modified from the work of McDevitt and Muir (27, 37). Rat chondrosarcoma chondroitin sulfate proteoglycan (M, 2.6 X $10^6$ (30)) and chondroitin 4-sulfate (M, 100,000) were used as markers. Labeled proteoglycans were detected by fluorography after impregnating gels with ENHANCE. $R_a$ was the ratio of the distance from the origin to an autoradiographic band as compared to the distance migrated by the chondroitin sulfate proteoglycan marker. The distance migrated by a proteoglycan varied between gels, but its position relative to markers ($R_a$) showed little variation.

Chondroitin 4.6 Sulfate Ratios—The sulfation pattern of DS GAG was determined by paper chromatography after chondroitinase ABC or AC digestion and expressed as chondroitin 4 to 6 sulfate ratios (38).

Periodate Oxidation and Alkaline Cleavage—4'-Iduronic acid residues lacking 2-O-sulfate substitution within DS chains were selectively cleaved by oxidation in 20 mM sodium metaperiodate and 50 mM sodium citrate, pH 3.0, at 4°C in the dark for 24 h using 2 mg/ml pig skin DS as carrier (39, 40). Addition of 20 mM sodium perchlorate to the reaction mixture during the oxidation step did not change the Sephadex G-50 elution profile (results not shown). The reaction was terminsted by addition of a 20-fold molar excess of sodium citrate (M, 200,000) as a reducing agent. After dilution against 2 changes of 2 liters of distilled water, the oxidized chains were cleaved at room temperature by adjusting the pH to 12 with 1 N NaOH. The reaction was stopped after 30 min by neutralization with 1 M acetic acid. The reaction products were applied to a 0.9 X 110-cm Sephadex G-50 column, and 1-ml fractions were collected. Oligosaccharides obtained after testicular hyaluronidase or chondroitinase AC digestion of rat chondrosarcoma chondroitin sulfate were used to calibrate the column. Periodate oxidation-alkaline cleavage products were detected in the Sephadex G-50 column void volume by eluting these oligosaccharides from Sepharose CL-6B columns.

RESULTS

Total Glomerular $^{35}$S-Sulfate Uptake in Vitro

Glomeruli isolated from normal rats were incubated in serum-free medium for either 4 or 16 h. Total incorporation of Na$_2^{35}$SO$_4$ into glomerular and incubation medium macromolecules was linear over this time (Table I). $^{35}$S-Macromolecules appeared progressively in the incubation medium. This fraction contained 41 ± 3.1% of the total $^{35}$S-sulfate incorporated after 4 h and 58 ± 7% after 16 h. Extraction with 4 M guanidine HCl buffer containing protease inhibitors released at least 95% of tissue $^{35}$S-macromolecules.

Proteoglycans were separated from the majority of glomerular proteins and glycoproteins by CsCl gradient ultracentrifugation. $^{35}$S-Glycoproteins were found predominantly in the top 2/3 CsCl gradient fractions (Table I). In contrast, $^{35}$S-proteoglycans were found mostly in high buoyant density CsCl gradient fractions. This gradient fraction contained 95% of total glomerular $^{35}$S-proteoglycans after 4 h and 84% after 16 h in vitro. Characterization of these high density $^{35}$S-proteoglycans, especially a previously uncharacterized tissue DS proteoglycan, was emphasized in these studies. The biochemical characteristics of high density glomerular proteoglycans described in detail below are summarized in Table II.

Glomerular $^{35}$S-Macromolecules Synthesized Over 16 h in Vitro

Tissue Fraction $^{35}$S-Macromolecules

Tissue Extraction with 4 M Guanidine HCl and Protease Inhibitors—The majority (66 ± 9%) of $^{35}$S-macromolecules synthesized by glomeruli over 16 h in vitro and retained in the tissue were recovered from the bottom 1/3 fraction of CsCl gradients (Table I). This gradient fraction contained a mixture of HS and DS, with only 6% of this gradient fraction being $^{35}$S-glycoprotein. Before DEAE-Sephal chromatographic purification, these high density $^{35}$S-proteoglycans eluted from Sepharose CL-6B columns as a partially excluded and a diffuse included peak (Fig. 1A). In contrast, when Stow et al. (20) incubated isolated rabbit glomeruli for 16 h in serum-containing medium, no Sepharose CL-6B-excluded $^{35}$S-proteoglycans were detected. We, therefore, performed additional 16-h isolated glomerular incubations in medium containing either 10% fetal calf serum or 5 ng/ml insulin (41). Total $^{35}$S-sulfate incorporation was not altered under these conditions, and Sepharose CL-6B-excluded $^{35}$S-proteoglycans were still present in high density CCl gradient fractions (data not shown).

High density $^{35}$S-proteoglycans were partially resolved by DEAE-Sephal chromatography into two peaks eluting at 0.44 M NaCl (peak I) and 0.56 M NaCl (peak II) (Fig. 2A). Two HS proteoglycans (defined by sensitivity to nitrous acid cleavage or heparitinase digestion (see "Methods") were present in DEAE peak I. HS-tIA eluted from Sepharose CL-6B columns within the $K_v$ 0.24 peak (M, 13 X $10^4$), and HS-tIB eluted at $K_v$ 0.68 (M, 8.6 X $10^4$) (Fig. 3A). Proteoglycans with similar elution volumes ($K_v$ 0.30 and 0.68, respectively) were observed when high density glomerular $^{35}$S-macromolecules (prior to DEAE purification) were chromatographed after chondroitinase ABC digestion (Fig. 1B). After enzyme digestion, pooled radioactivity from Sepharose CL-6B-included peaks contained exclusively HS, while DS oligosaccharides appeared in the included peak V$_4$.

HS-tIA migrated as a single $R_a$ 2.3 band in autoradiographs of 0.6% agarose-1.8% polyacrylamide gels both before (Fig. 3A, inset, lane a) and after reduction with $\beta$-mercaptoethanol.

4 Newly synthesized [H]leucine- or [H]serine-labeled glomerular proteins were found in the top 1/3 CsCl gradient fractions (98% of [H]), with insufficient high density [H]-labeled material for further analysis (unsuitable observations by authors).

5 Proteoglycan nomenclature was based on: (a) their GAG component, either HS or DS; (b) whether the $^{35}$S-proteoglycan was extracted from the glomerular tissue (t) or was released into the incubation medium (m); (c) their elution position from DEAE-Sephal columns (with DEAE peak I or peak II); and (d) their relative hydrodynamic size analyzed by Sepharose CL-6B chromatography (A or B).
TABLE I
Incorporation of [35S]sulfate into macromolecules by isolated glomeruli in vitro and in vivo

|                      | Total incorporation | CsCl distribution | HS 6.4 | DS 14.4 | 35S-GP a |
|----------------------|---------------------|-------------------|--------|---------|----------|
|                      | dpm × 10^6/mg protein | %                | %      | %      | %        |
| I. In vitro a         |                     |                   |        |         |          |
| A. 4 h (2)           | 16.45 ± 0.8         | B 92              | 40     | 54      | 6        |
| 1. Incubation medium  |                     | C 95              | 31     | 61      | 8        |
| (41 ± 3.2%) b         |                     | T 12              | 72     | 8       | 7        |
| 2. Glomerular extract |                     | T 18 ± 2          | 28     | 22      | 45       |
| (59 ± 3.2%)           |                     | 55.4 ± 9          |        |         |          |
| B. 16 h (4)          | 5.2 ± 0.8           | B 72 ± 2          | 34     | 65      | 2        |
| 1. Incubation medium  |                     | T 18 ± 2          | 72     | 8       | 7        |
| (61 ± 5%)             |                     | T 28 ± 8          | 26     | 22      | 45       |
| (39 ± 5%)             |                     | 7.5 ± 9           |        |         |          |
| II. In vivo glomerular extracts a | |                   |        |         |          |
| A. 4 h (2)           | 4.2                 | B 82              | 60     | 30      | 10       |
|                       |                     | T 17              | 37     | 25      | 22       |
| B. 16 h (4)          | 11.9 ± 5.0          | B 39 ± 12         | 68     | 21      | 11       |
|                       |                     | T 62 ± 0.6         |        |         |          |

a Glomerular and incubation medium 35S-proteoglycans were separated by CsCl gradient ultracentrifugation for 60 h at 40,000 rpm at 4 °C and cut into bottom (B) 5 and top (T) 5% fractions. Since no separation of low and high density 35S-proteoglycans occurred with samples from 4-h in vitro experiments, CsCl gradients were used in only two experiments. Therefore, standard deviation of CsCl distribution was not calculated for this time period.

b The percent heparan-35SO, (HS) and dermatan-35SO, (DS) GAG in each CsCl gradient fraction was determined by serial HNO3 and chondroitinase ABC digestions (see "Experimental Procedures").

c-35S-Glycoproteins (GP) are defined as 35S-macromolecules insensitive to serial HNO3, chondroitinase ABC, as well as heparitinase digestions.

d Glomeruli isolated from normal rats were incubated in RPMI 1640 medium containing 0.1 mM Na2SO4 and 250 µg/ml Na2SO4 for 4 or 16 h. After separation of the medium by gentle centrifugation, glomerular proteoglycans were extracted with 4 M guanidine HCl in the presence of protease inhibitors (see "Experimental Procedures").

e The number of experiments performed at each time period and used in determining the mean and standard deviation for total [35S]sulfate incorporation is indicated in parentheses.

f The proportion of radioactivity in incubation medium and extracted from glomeruli is indicated under that particular fraction.

g In vivo experiments, glomeruli were isolated and 35S-proteoglycans extracted with 4 M guanidine HCl in protease inhibitors either 4 h after a single intraperitoneal injection of 1 mCi of Na235SO4/animal (A) or 16 h after the first of four 0.5 mCi Na235SO4 injections (total dose, 2 mCi/animal, B).

TABLE II
Characterization of glomerular proteoglycans

|                      | HS-tIA a | HS-tIB | DS-tIA | DS-tIB | HS-mI | DS-mI |
|----------------------|----------|--------|--------|--------|-------|-------|
| 1. % Total 35S-proteoglycan |          | 10     | 4      | 5      | 29    | 21    |
| 2. Sepharose CL-6B |          |        |        |        |       |       |
| R g                | 0.24     | 0.68   | 0.24   | 0.24   | 0.30  | 0.14  |
| 3. GAG chain length (M, = 106) | 130     | 8.6    | 150    | 150    | 500   | 100   |
| 4. Gel R g         | 12.5     | 8.6    | 18     | 26     | 12.5  | 18    |
| 5. C4:6 SO4, ratio | 2.3      | 4.6    | 2.3    | 2.3    | 1.7   | 2.8/3.8 |
| 6. Tissue localization b | RB/ECM  | Intraocular | RB/ECM | ECM   |       |
| 7. Iduronic acid spacing |         |        |        |       |       | Clustered |
|                      |          |        |        |       |       | Even   |

a Dermatan sulfate (DS) and heparan sulfate (HS) proteoglycans were synthesized in vitro by glomeruli isolated from normal rats. Bottom 5 CsCl gradient fraction proteoglycans from glomerular extracts (g) and incubation media (m) were further purified by DEAE-Phenylacetyl chromatography. 35S-Macromolecules eluting in early (I) and late (II) portions of the linear salt gradient were pooled separately. Intact proteoglycan (2) and GAGs (3) were characterized by elution from Sepharose CL-6B columns (tissue fraction DEAE peak 1 contained two peaks, A and B) and by electrophoresis in 0.6% agarose-1.5% acrylamide gels (4). Dermatan sulfate chains were analyzed for the ratio of 4:6 sulfates after chondroitinase ABC digestion (6) and for the spacing of iduronic groups by susceptibility to periodate cleavage (pH 3.0, 4 °C) and alkaline elimination (6).

b These proteoglycans eluted predominantly in the front half of the Sepharose CL-6B peak.

c Average molecular weight estimated from Sepharose CL-6B profile.

d Migration in 0.6% agarose-1.5% acrylamide gels relative to an M, 2.6 × 106 chondroitin-sulfate proteoglycan.

e A fast migrating band was apparent after gel electrophoresis of DEAE- and Sepharose-purified 35S-proteoglycans.

f RB, cell surface, heparin receptor bound; ECM, extracellular matrix.

(results not shown), while HS-tIB appeared in gels as a chondroitinase ABC-resistant band in a position similar to that of the chondroitin sulfate marker (Fig. 5A, inset, lanes c and d). HS-GAG chains from HS-tIA had an M, of 12.5 × 106 (Sepharose CL-6B R g, 0.58, Fig. 4A). HS-tIB contained no detectable protein since the elution volume did not change after alkaline borohydride treatment (results not shown). The hydrodynamic size of glomerular HS-tIA proteoglycan was similar to previously described rat GBM or rabbit glomerular HS proteoglycan (2, 20), while HS-tIB resembled it in its size and resistance to alkaline borohydride treatment intracellular heparan sulfate from rat granulosa cells (42).
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A large DS proteoglycan (DS-tII) was present in DEAE peak I as determined from the partial sensitivity (46%) of alkaline borohydride-released $^{35}$S-GAGs from this peak to chondroitinase ABC digestion (Fig. 3B, inset, lane a). When applied to Sepharose CL-6B columns run under associative conditions (see "Experimental Procedures"), DS-tII had a larger apparent hydrodynamic size (Fig. 5A, $K_v$ 0.29) than when eluted from a similar column in 4 M guanidine HCl (Fig. 5B, $K_v$ 0.48). The latter conditions would dissociate proteoglycans aggregated on a hyaluronic acid backbone (32) or those associated through interaction of GAG chains (43). To distinguish between these possibilities, DS-tII from the dissociative Sepharose CL-6B $K_v$ 0.48 peak was pooled, exhaustively dialyzed against distilled water, and lyophilized. The retentate was reduced and alkylated in the presence of 4 M guanidine HCl, conditions which denatured the hyaluronic acid binding region of cartilage proteoglycan protein core (32). The reduced and alkylated products eluted from an associative Sepharose CL-4B column in a position similar to untreated DS-tII (Fig. 5C, $K_v$ 0.23), indicating that reassociation of $^{35}$S-proteoglycans had occurred. Therefore, DS-tII proteoglycans may form
aggregates through GAG chain-chain interaction or through interactions of the protein core not dependent for structural integrity on disulfide bond formation.

DS chains were released from the DS-tII proteoglycan protein core by alkaline β-elimination, eluting with the $K_v$ of Sephadex G-50 columns after nitrous acid treatment (see "Methods"). These chains had an apparent molecular weight of $26 \times 10^3$ on Sepharose CL-6B ($K_v = 0.43$, Fig. 4B) and were sulfated primarily at the 4 position of galactosamine, with a C4 to 6 sulfate ratio of 1.95:1 (Table II). The presence of iduronic acid within DS-tII 35S-GAG was evident from an 11% difference in sensitivity to chondroitinase ABC and chondroitinase AC digestions. This small difference was similar to that previously reported for DS from isolated rat glomeruli (24).

The presence of iduronic acid residues within DS-tII GAG was confirmed by oxidation of iduronic acid residues lacking 2-O-sulfate groups with periodate at pH 3.0 and 4 °C followed by alkaline cleavage (39, 40). After this treatment, a significant proportion (33%) of the reaction products was included in Sephadex G-50 columns (Fig. 6A). DS chains remaining in the column void volume were thought to be derived from unmodified regions of the DS chains. When rechromatographed on Sepharose CL-6B, these periodate-treated and alkaline-cleaved DS chains were more retarded ($K_v = 0.64$, Fig. 6C) by the column than the original 35S-GAGs ($K_v = 0.43$, Fig. 4B). When these periodate-resistant 35S-GAGs were treated with chondroitinase AC to confirm their derivation from unmodified chondroitin sulfate-rich regions, 10–20% of the reaction products were included in Sepharose CL-6B columns, eluting at $K_v = 0.73$ (Fig. 6D). The presence of 2-O-sulfated iduronic acid residues in DS-tII may account for this partial resistance to chondroitinase AC digestion after periodate oxidation and alkaline cleavage.
Fig. 4. Sepharose CL-6B chromatography of glomerular 35S-GAGs. 35S-GAGs were released by alkali under reductive conditions (see "Methods"). HS-tIA 35S-GAG was chromatographed on Sepharose CL-6B after digestion of glomerular extract DEAE peak I, Sepharose CL-6B peak A (Fig. 3A) with chondroitinase ABC (A). Oligosaccharides in the column V, after this treatment demonstrate that DEAE peak I contained a mixture of HS and DS proteoglycans. In B, 35S-GAGs from DS-tII (Fig. 3B, peak A) were chromatographed on Sepharose CL-6B, while C shows the elution profile of DS-tIA 35S-GAG. The latter 35S-GAG was isolated by treatment of DEAE peak I, Sepharose CL-6B peak A (35S-proteoglycans (Fig. 3A, peak A) with nitrous acid, and desalting on Sephadex G-50 (see "Methods"). DS-mII GAGs were purified from 35S-proteoglycans in Fig. 3D, peak A, in a similar manner, and their elution profile is illustrated in D.

DS-tII GAG was further characterized by Sephadex G-50 chromatography after chondroitinase AC digestion. Disaccharides accounted for 59% of the chondroitinase AC digestion products of DS-tII GAG chains (Fig. 6B). Iduronic acid-rich sequences resistant to chondroitinase AC digestion eluted predominantly as tetrasaccharides (27%). Tetrasaccharides may result from digestion of regions along DS chains containing regularly spaced iduronic acid residues (occurring approximately every third repeat disaccharide unit). Longer chondroitinase AC-insensitive products, which eluted prior to the tetrasaccharide peak from Sephadex G-50 columns and constituted 14% of the total column radioactivity, would originate from regions containing more closely spaced iduronic acid moieties.

Partial Localization of Glomerular Proteoglycans by Heparin and Trypsin Treatment—35S-Proteoglycans noncovalently bound to tissue GAG binding sites were released using two different previously described heparin treatment protocols (6, 28). Glomeruli were treated either with 2 mg/ml heparin in RPMI 1640 medium for 1 h at 37 °C or with 100 μg/ml heparin in 0.05 M Tris buffer, pH 8.0, at 4 °C followed by detergent extraction. Residual glomerular 35S-macromolecules were released with 4 M guanidine HCl. 35S-Proteoglycans in each of these extracts were compared to those extracted with 4 M guanidine HCl directly (total tissue extracts).

Each heparin extraction protocol released similar proportions of total tissue-associated radioactivity (33% with heparin in medium and 36% with heparin in Tris buffer, Table III). This proportion was greater than that released upon exposure to medium alone (21%). A greater proportion of total glomerular heparan 35S sulfate proteoglycan was released by heparin in medium (47%) than by medium without heparin.
were pooled and chromatographed under associative conditions after reduction and alkylation (C). The column profiles shown in are of columns eluted with 0.5 M sodium acetate, pH 7.0, and 0.2% guanidine HCl. The contents of the $K_{av}$ 0.29 peak shown in A were pooled and chromatographed under associative conditions after treatment with medium alone (19%). The hydrodynamic size of the major heparin-releasable DS proteoglycan as well as its $35S$-GAG chains ($K_{av}$ 0.2 and 0.5, respectively) were similar to DS-tIA. DS-tII proteoglycan, on the other hand, was only extracted with 4 M guanidine HCl after heparin and detergent treatments (Fig. 7D). Like the molecule released in the total guanidine HCl extract, this proteoglycan was partially excluded from Sepharose CL-6B columns and migrated as a diffuse $R_{r}$ 1.8 chondroitinase ABC-sensitive autoradiographic band in agarose-polyacrylamide gels (Fig. 8, lanes g and h). Since most DS-tII proteoglycan resisted heparin and detergent treatments, it may be more tightly bound within the glomerular matrix. Thus, heparin did not preferentially release either HS or DS proteoglycan but displaced a specific subtype of each proteoglycan, HS-tIA and DS-tIA proteoglycans, from glomerular cell surfaces or intercellular matrix GAG binding sites.

Trypsin treatment without prior heparin extraction released 46% of total tissue-associated radioactivity (Table III). These $35S$-proteoglycans eluted from DEAE-Sephacl columns as two separate peaks at 0.34 and 0.47 M NaCl in DEAE buffer. Most (71%) tissue-associated heparan-$35SO_4$ proteoglycan was released by trypsin, accounting for all extracellular HS proteoglycan. Trypsin-released HS proteoglycan in DEAE peak I eluted later than its heparin-extracted counterpart from Sepharose CL-6B columns ($K_{av}$ 0.4, Fig. 7E). As expected, HS-GAG from this peak was similar in length to that released by heparin ($K_{av}$ 0.58, not shown). Thus, there are trypsin-sensitive sites within the protein core of extracellular HS proteoglycan. HS-tIB was not released from the tissue by trypsin treatment but was detected in the guanidine HCl-extracted residual tissue ($K_{av}$ 0.7, Fig. 7G). Tissue DS proteoglycan fragments released by trypsin eluted from DEAE-Sephacl in peak II and from subsequent Sepharose CL-6B columns at $K_{av}$ values of 0.2 and 0.4 (Fig. 7F), with both $35S$-proteoglycans having GAGs similar in length to DS-tIA ($K_{av}$ 0.5, not shown). On the other hand, DS-GAG from DEAE peak 11 $35S$-proteoglycans remaining in the tissue after trypsin treatment (Fig. 7H) was similar in length to DS-tII GAG

![Diagram](https://via.placeholder.com/150)
FIG. 6. Dermatan-^{35}SO_4, GAG chain modification. DS-tII and DS-mII ^{35}S-GAGs were chromatographed on Sephadex G-50 columns after oxidation with periodate and alkaline cleavage (see "Methods") as shown in A and B, respectively. Similar columns run after chondroitinase AC digestion of these ^{35}S-GAGs are shown in C and D for DS-tII and DS-mII GAG, respectively. ^{35}S-GAGs apparently insensitive to periodate oxidation and alkaline
(Sepharose CL-6B for, 0.43, not shown). Therefore, as occurred with heparin treatment, DS-tIA and HS-tIA were accessible to trypsin, while intracellular HS-tIB and tissue DS-tII resisted these treatments.

Partial Characterization of Low Buoyant Density 35S-Proteoglycan—Twenty-four percent of total tissue fraction 35S-macromolecules and 16% of total tissue 35S-proteoglycans were in the top ½ CsCl gradient fraction after 16 h in vitro incubations (Table I). 35S-Glycoproteins in this fraction did not bind to DEAE-Sephacel columns (Fig. 9A), while 35S-proteoglycans eluted from the columns at 0.18 (peak I) and 0.55 M NaCl (peak II). Peak I 35S-proteoglycans migrated in agarose-polyacrylamide gels as a doublet with $R_g$ values of 2.3 and 2.6 which was insensitive to chondroitinase ABC digestion (Fig. 9A, inset, lanes a and b). Peak II 35S-proteoglycans migrated to an $R_g$ of 1.3 in gels (Fig. 9A, inset, lane c) and were partially sensitive to chondroitinase ABC digestion (Fig. 9A, inset, lane d). Similar low density 35S-proteoglycans were released into the medium (Fig. 9B). Insufficient radioactivity was present in top gradient fractions for further analysis.

Incubation Medium 35S-Macromolecules

The medium fraction was examined for the presence of proteoglycan species different from those found in tissue fractions. The majority (72 ± 2%) of 35S-macromolecules synthesized by glomeruli over 16 h in vitro and released into the incubation medium were recovered from the bottom ½ CsCl gradient fractions (Table I). This fraction contained both HS and DS proteoglycans which eluted from DEAE-Sephacel columns as two partially resolved peaks. These peaks eluted at 0.44 M (29%, peak I) and at 0.55 M NaCl (71%, peak II, Fig. 2B) with no 35S-glycoprotein detected in the column wash. DEAE peak I 35S-proteoglycans eluted from Sepharose CL-6B columns as a single peak ($K_v$ 0.22, Fig. 3C) which contained both HS (40%) and DS (60%) proteoglycans, while DEAE peak II 35S-proteoglycans eluted earlier from these columns ($K_v$ 0.14, Fig. 3D) and contained mainly (86%) DS proteoglycan.

Two HS proteoglycans were present in medium fractions. These DEAE peak I proteoglycans migrated faster in agarose-polyacrylamide gels ($R_g$ values of 2.8 and 3.8. Fig. 3C, inset, lanes a and c) than those from tissue (Fig. 3A, inset, lane a) and were partially resolved from each other by pooling the front or back halves of the $K_v$ 0.22 Sepharose CL-6B peak prior to gel electrophoresis (Fig. 3C). There was also a small amount of HS proteoglycan in DEAE peak II which formed a shoulder following the major $K_v$ 0.14 DS proteoglycan peak (see below). HS chains from medium DEAE peak I proteoglycans had an $M_r$ of 12.5 × 10^6 (Sepharose CL-6B $K_v$ 0.58, not shown). This GAG chain length was similar to that of HS from extracellular tissue fraction proteoglycan.

Medium DS proteoglycan from DEAE peak II (DS-mII), after purification by Sepharose CL-6B chromatography, migrated in gels as a single band at $R_g$ 2.8 (Fig. 3D), a position similar to that of medium HS proteoglycan. No small single chain DS was detected. DS-mII GAG chains had an $M_r$ of 18 × 10^6 (Sepharose CL-6B $K_v$ 0.53, Fig. 4D) and were sulfated primarily at the 4 position of galactosamine, with a C4 to 6 sulfate ratio of 2.32:1 (Table II). These properties were similar to those described above for DS-tIA (see "Tissue Extraction with 4 M Guanidine HCl and Protease Inhibitors"). Like tissue fraction DS-tII, these GAGs were 11% less sensitive to chondroitinase AC than to chondroitinase ABC digestion, suggesting the presence of iduronic acid residues. DS-mII GAG was less sensitive (26%) to periodate oxidation/alkaline cleavage than DS-tII (Fig. 6F) and had a lower disaccharide to tetrasaccharide product ratio (0.9:1) than DS-tII GAG (2:1) after chondroitinase AC digestion (Fig. 6E), suggesting that DS-mII had more regularly spaced iduronic acid residues than DS-tII GAG.

### Table III

| Heparin extraction of glomerular proteoglycans |
|-----------------------------------------------|
| Isolated rat glomeruli were extracted by three different treatment protocols (see "Experimental Procedures"): a, sequentially with heparin (Hep, 100 µg/ml) and 0.1 M NaCl, then 0.3 M NaCl, then 0.3 M NaCl and 0.2% CHAPS, and finally 4 M guanidine HCl (GdnCl), each in 0.05 M Tris and protease inhibitors, pH 8.0, each for 1 h at 4 °C; b, heparin (Hep, 2 mg/ml) in RPMI 1640 medium for ½ h at 37 °C, then 4 M guanidine HCl in protease inhibitors, or c, trypsin (50 µg/ml) in RPMI 1640 medium for 10 min at 37 °C, stopped with 50 mM FMPS, then 4 M guanidine HCl in protease inhibitors. |
|-----------------------------------------------|

| 1. Percent | Hep | NaCl | CHAPS | GdnCl | Trypsin | Gdn* |
|------------|-----|------|-------|-------|---------|------|
| a          | 36  | 12   | 30    | 21    | 21      | 21   |
| b          | 33  |      |       | 67    |         |      |
| c          |     |      | 54    |       |         | 46   |

2. Resolution on DEAE-Sephacel and Sepharose CL-6B chromatography (%):a

| DEAE peak | I   | II  | I   | II  | I   | II  | I   | II  |
|-----------|-----|-----|-----|-----|-----|-----|-----|-----|
| 1         | 60  | 50  | 78  | 25  | 39  | 34  | 55  |
| II        | 29  | 50  | 11  | 58  | 50  | 63  |

* Isolated glomeruli were placed directly in 4 M guanidine HCl extraction buffer in the presence of protease inhibitors at 4 °C for 24 h (see "Experimental Procedures"). 35S-Proteoglycans extracted from glomerular tissue fractions as described for protocol a were partially resolved by DEAE-Sephacel chromatography into two peaks (I and II). The contents of these peaks were eluted from Sepharose CL-6B columns ($K_v$ 0.27 and $K_v$ 0.69).
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Heparin Extraction

FIG. 7. Heparin and trypsin treatment of glomeruli. Glomerular tissue macromolecules [35S]sulfate labeled over 16 h in vitro were extracted with medium containing 2 mg/ml heparin for 30 min or with 50 μg/ml trypsin for 10 min each at 37 °C (see "Methods"). The residual tissues were extracted with 4 M guanidine HCl in the presence of protease inhibitors. 35S-Proteoglycans released by each treatment were purified by DEAE-Sephasel chromatography, eluting as two partially separated peaks (I and II, see Table III). Sepharose CL-6B chromatographic profiles of 35S-proteoglycans released by heparin in medium are shown in A (DEAE peak I) and B (DEAE peak II), while 35S-proteoglycans released by guanidine HCl extraction of the heparin-treated tissues are illustrated in C (DEAE peak I) and D (DEAE peak II). Trypsin treatment released 35S-proteoglycans with Sepharose CL-6B elution profiles illustrated in E (DEAE peak I) and F (DEAE peak II). 35S-Proteoglycans remaining in the tissue after this treatment eluted from Sepharose CL-6B as shown in G (DEAE peak I) and H (DEAE peak II).
Glomerular Proteoglycans Synthesized during 4-h In Vitro Incubations

Since proteoglycans may be synthesized and degraded at different rates, the effect which time of incubation had on the type and percent of proteoglycan species present was next determined. After 4-h in vitro incubations, 95% of [\(^{35}\)S]sulfate-labeled glomerular macromolecules were found in the bottom 1/5 CsCl gradient fractions. These proteoglycans eluted as two peaks from Sepharose CL-6B columns with \(K_{av}\) values of 0.21 which contained both HS and DS proteoglycans (85% of the total eluted radioactivity) and 0.63 (15%) without prior DEAE-Sephacel purification (Fig. 1OA). HS proteoglycan accounted for 30% of high density tissue proteoglycan after 4 h in vitro (the remainder was DS proteoglycan) and eluted mainly with the second half of the \(K_{av}\) 0.21 Sepharose CL-6B peak (38% of the pooled contents of this portion of the peak were sensitive to nitrous acid, while only 12% of earlier eluting proteoglycans were HS). Like HS-tIA, this proteoglycan eluted in DEAE peak I (0.3 M NaCl, Fig. 11A), migrated to an \(R_g\) of 2.3 in agarose-polyacrylamide gels (Fig. 11A, inset, lane a), and had GAG chains with an \(M_r\) of 12.5 \(\times\) 10^5 (Sepharose CL-6B \(K_{av}\) 0.59).

No Sepharose CL-6B-excluded tissue DS proteoglycan similar to DS-III from 16-h in vitro incubations was detectable after 4 h in vitro. DS proteoglycan eluted from DEAE-Sephacel columns with peak II (Fig. 11A) and in the front half of the \(K_{av}\) 0.21 Sepharose CL-6B peak. It migrated as two chondroitinase ABC-sensitive bands with \(R_g\) values of 2.1 and 2.8 in agarose-polyacrylamide gels (Fig. 11A, inset, lane b). DS chains from 4-h tissue fraction proteoglycans had an average molecular weight of 18 \(\times\) 10^5 (Sepharose CL-6B \(K_{av}\) 0.52) and a C4 to C6 sulfate ratio of 2.3:1 (Table II). After chondroitinase ABC digestion, a minor disaccharide component (15%) migrated more slowly than chondroitin 6-sulfate standards on paper chromatography, possibly representing chondroitin 4,6-sulfate or DS sulfated at the 2 position of iduronic acid (44). Tissue DS proteoglycan from 4-h in vitro incubations contained iduronic acid-rich regions as demonstrated by an 11% difference in sensitivity to chondroitinase ABC and AC digestions. It, therefore, resembled DS-tIA and DS-mII proteoglycans in hydrodynamic size. GAG chain length, iduronic acid content, as well as in C4 to C6 sulfate ratio. DS-tIA may be rapidly synthesized and released into medium, while DS-mII accumulates slowly in tissue, becoming detectable if the rapidly synthesized molecules are chased from the tissue. This hypothesis was tested in label-chase experiments described below.

Label-Chase Experiments

Glomerular macromolecules were \([^{35}\text{S}]\)sulfate labeled over 4 h in vitro and then chased for 2 h into medium containing unlabeled 0.5 mM Na\(_2\)SO\(_4\). In three separate chase experiments (each run in duplicate) 31 \(\pm\) 1.6% of radioactivity originally incorporated into glomerular tissue was released into the medium. The Sepharose CL-6B elution profiles of proteoglycans from the pulse-labeled glomeruli and from the chase medium showed similar \(K_{av}\) 0.21 chromatographic peaks (compare Fig. 10, A and C). HS-tIIB, the \(K_{av}\) 0.63 intracellular proteoglycan, was not detected in the medium. \[^{35}\text{S}\)-Proteoglycans remaining in glomeruli after the chase eluted earlier (\(K_{av}\) of 0.12, Fig. 10B) than those from the pulse-labeled tissue fraction. Thus, lower molecular weight proteoglycans were rapidly cleared from the glomerulus, allowing the detection of a population of higher molecular weight tissue proteoglycans. In order to determine whether artifacts were introduced by in vitro incubation, in vivo \([^{35}\text{S}]\)sulfate-labeled proteoglycans were isolated. Glomeruli synthesized similar \[^{35}\text{S}\)-proteoglycans in vivo and in vitro. Maximal in vivo \([^{35}\text{S}]\)sulfate incorporation into proteoglycan occurs 4 h after single intraperitoneal Na\(_2\)SO\(_4\) injections (17). Glomeruli isolated after this time period contained \[^{35}\text{S}\)-macromolecules whose CsCl gradient distribution was similar to that seen after 4-h in vitro incubations (Table I). As previously reported for the case of isolated GBM (17), glomeruli \([^{35}\text{S}]\)sulfate labeled in vivo synthesized and retained a larger proportion of HS than of DS proteoglycan (Table I).

When rats received multiple intraperitoneal injections of Na\(_2\)SO\(_4\) over 16 h (see "Methods"), a greater proportion (62 \(\pm\) 0.5%) of \[^{35}\text{S}\)-macromolecules were present in the top \(1/5\) (less buoyantly dense) CsCl gradient fractions than 4 h after a single injection (17%, Table I), with \[^{35}\text{S}\)-glycoproteins accounting for 11% of the latter gradient fraction (Fig. 12B, excluded peak). This change to less buoyantly dense CsCl gradient fractions after longer exposures to \([^{35}\text{S}]\)sulfate was similar to that seen after corresponding in vitro labeling periods (compare 4- and 16-h CsCl gradient distributions in Table I).

By far the major high density proteoglycan was HS-tIA,
which eluted from DEAE-Sepharose columns at 0.4 M NaCl (Fig. 12A, DEAE peak I) and migrated as a chondroitinase ABC-resistant band to an $R_c$ of 2.3 in subsequent agarose-polyacrylamide gels (Fig. 12A, inset, lanes a and b). DEAE-Sepharose-purified high density proteoglycans synthesized over 16 h in vitro eluted as a single peak $K_r$ of 0.26 peak from Sepharose CL-6B columns which was composed largely (70%) of HS proteoglycan (Fig. 13A). HS-tIA (Fig. 13A, inset, lanes a and b) as well as HS-tIB (Fig. 13A, inset, lanes c and d) proteoglycans were detected in this peak. A minor slower migrating HS proteoglycan ($R_c$ 1.1) not detected in vitro was also present in this gradient fraction (Fig. 12A, inset, lanes a and b).

Several large HS proteoglycans were present in the top 50% CsCl gradient fractions. These proteoglycans eluted from DEAE-Sepharose CL-6B columns as two included peaks at 0.11 and 0.35 M NaCl (Fig. 12B, peak I). After DEAE purification, these proteoglycans eluted from Sepharose CL-6B columns with the void volume and as diffuse included peaks (Fig. 13, B and C). $^{35}$S-Proteoglycans eluting at 0.11 M NaCl from DEAE-Sepharose CL-6B columns were partially excluded from Sepharose CL-6B (Fig. 13B) and migrated as a chondroitinase ABC-resistant band slightly slower than rat chondroitin sulfate proteoglycan standard in agarose-polyacrylamide gels (Fig. 13B, inset, lanes a and b). $^{35}$S-Proteoglycans eluting at 0.35 M NaCl migrated as two chondroitinase ABC-resistant bands in gels run both before ($R_c$ 2.2 and 2.8, Fig. 12B, inset, lanes a and b) and after digestion with chondroitinase ABC.

Sepharose CL-6B chromatography (Fig. 13C, inset). Further characterization of HS proteoglycans was limited by low uptake of radioactivity in vivo.

The DS proteoglycan DS-tII was detected in the bottom 50% CsCl gradient fraction, eluting as a minor 0.47 M NaCl peak from a DEAE column (Fig. 12A, peak II). This $^{35}$S-proteoglycan co-migrated with DS-tII from 16-h in vitro incubations in gels (compare Fig. 12A, inset, lane c with lane e) and was sensitive to prior chondroitinase ABC digestion (Fig. 12A, inset, lane f). DS chains cleaved from this proteoglycan had an average molecular weight similar to those synthesized in vitro ($K_r, 0.42, M, 26,000$). Low density DS proteoglycan synthesized in vivo also eluted from a DEAE column at 0.47 M NaCl (Fig. 12B, DEAE peak II) and migrated slightly slower than high density DS-tII proteoglycan in agarose-polyacrylamide gels (compare Fig. 12B, inset, lanes c and e). Thus, less DS-tII was isolated from glomeruli $^{35}$S-sulfate.

**Fig. 8.** Gel electrophoresis of DEAE-Sepharose and Sepharose CL-6B purified $^{35}$S-macromolecules from serial heparin, detergent, and guanidine HCl extracts. Glomerular tissue macromolecules $^{35}$S-sulfate labeled over 16 h in vitro were serially extracted with heparin (100 μg/ml) and then detergent (0.2% CHAPS, 0.35 M NaCl) each in 0.05 M Tris buffer, pH 8.0, followed by guanidine HCl as described under “Methods” and purified by DEAE-Sepharose chromatography (Table III). Sepharose CL-6B chromatographic profiles of $^{35}$S-proteoglycans from each pooled DEAE peak were similar to their counterparts illustrated in Fig. 3. Equivalent quantities of radioactivity from each pooled Sepharose CL-6B chromatographic peak were subject to electrophoresis in 0.6% agarose-1.8% polyacrylamide gels both before (lanes a, c, e, and g) and after (lanes b, d, f, and h) chondroitinase ABC digestion. Heparin-extracted HS-tIA was electrophoresed in lanes a and b. HS-tIB (lanes c and d) was also extracted by heparin under these conditions. Additional HS-tIA proteoglycan was released from the tissue by detergent treatment (lanes e and f), while DS-tII (lanes g and h) was released only after the heparin- and detergent-treated tissue was extracted with guanidine HCl.

**Fig. 9.** DEAE-Sepharose chromatography of top 50% CsCl gradient fraction $^{35}$S-proteoglycans after a 16-h labeling period in vitro. Top 50% CsCl gradient fraction glomerular extract (A) and incubation medium (B) $^{35}$S-macromolecules were eluted from DEAE-Sepharose columns with a linear salt gradient from 0.1 to 1.0 M NaCl (see “Methods”). Fractions indicated by bars were pooled separately as peaks I and II. Insets, equivalent aliquots from DEAE peak I (lanes a and b) and II (lanes c and d) were electrophoresed in 0.6% agarose-1.8% acrylamide gels before (lanes a and c) and after (lanes b and d) digestion with chondroitinase ABC. --- $^{35}$S-sulfate; ---, molarity. The migration in gels of chondroitin sulfate proteoglycan (Mr 2.6×10$^6$) is designated by the top arrow, while the bottom arrow represents the position to which chondroitin sulfate GAG migrated.
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FIG. 10. Sepharose CL-6B chromatography of bottom 1/3 CsCl gradient fraction $^{35}$S-proteoglycans from a label-chase experiment. Isolated glomeruli were incubated in medium containing $[^{35}S]$sulfate for 4 h in vitro followed by a 2-h chase in medium containing 0.5 mM Na$_2$SO$_4$ and no radioactivity (see "Methods"). Sepharose CL-6B chromatographic profiles of all $^{35}$S-proteoglycans extracted from glomeruli before and after the chase are shown in A and B, respectively. $^{35}$S-Proteoglycans released into the medium during the chase eluted from the column as illustrated in C.

labeled in vivo than was extracted from glomeruli isolated after in vitro labeling, but they had similar biochemical properties.

DISCUSSION

Rat glomeruli synthesized a mixture of HS and DS proteoglycans. A high density $M$, $13 \times 10^4$ HS proteoglycan (HS-tIA) with $M$, $12.5 \times 10^3$ GAG chains was extracted from glomeruli $[^{35}S]$sulfate labeled both in vivo and in vitro. This proteoglycan was similar in hydrodynamic size to that previously reported in extracts of rat and rabbit glomeruli as well as isolated rat, rabbit, and bovine GBM (3, 20, 24, 45). However, GAG chain length estimates varied in these studies with those from in vitro $[^{35}S]$sulfate-labeled isolated rabbit glomeruli and GBM (20) as well as from bovine GBM (19, 45) having lengths by Sephacryl S-200 chromatography using globular protein standards. HS from rat GBM labeled ex situ had an $M$, of 26,000 (3).

Glomerular HS proteoglycan has been localized to the GBM by immunofluorescence microscopy and electron microscopic autoradiography (21, 25, 46). Antibody to intact rat glomerular HS proteoglycan failed to stain the mesangium or cell surfaces. This anti-HS proteoglycan core protein antibody also stained rough endoplasmic reticulum and Golgi apparatus within glomerular epithelial cells, suggesting that this cell was a source of GBM HS proteoglycan. However, antibody to bovine aorta HS proteoglycan core protein stained both the epithelial and endothelial aspects of GBM (25), and one high density HS proteoglycan synthesized by aortic endothelial cells in culture had an $M$, ($13 \times 10^4$) similar to that from isolated glomeruli (47). Therefore, endothelial cells cannot be eliminated as a source for GBM HS proteoglycan. A mesangial location for HS proteoglycan was suggested by studies which showed that total GBM HS could not account for the total glomerular HS content (17). In addition, anti-aorta HS proteoglycan antibody-stained mesangium (25) and heparitinase but not chondroitinase ABC treatment of tissue prior to electron microscopic autoradiography resulted in a partial loss of mesangial grains (21).
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Studies reported here describe the release of HS-tIA proteoglycan from tissue by heparin in medium, with an M, 8,600 HS (HS-tIB) having little or no protein core extracted from the remaining tissue by guanidine HCl. HS-tIB had properties similar to the intracellular HS described by Yanagishita and Hascall (42) in granulosa cells and may represent a product of proteoglycan endocytosis and degradation. However, HS-tIB was released by heparin extractions carried out in "Tris buffer, indicating that this treatment, which releases liver cell surface heparan sulfate proteoglycan (6), may disrupt glomerular cell integrity. A possible cell surface location for heparin-released proteoglycans was suggested by studies describing staining of glomerular epithelial and endothelial cell surfaces with anti-hepatocyte cell membrane HS proteoglycan antibody (48). Heparin-displaceable HS proteoglycan may bind to cell surfaces and/or tissue "GAG receptors" through interaction with specific domains of other extracellular matrix macromolecules such as fibronectin, type IV collagen, or laminin. Mild trypsin treatment released all extracellular HS proteoglycan, with only HS-tIB remaining in the tissue. Trypsin-released HS proteoglycan fragments were smaller (Seph- arose CL-6B Ks, 0.42, M, 46,000) than HS-tIA but had similarly sized GAGs. Since more HS proteoglycan was released by trypsin than heparin, it is possible that a portion of glomerular HS proteoglycans is intercalated in cell membranes or bound to tissue receptors inaccessible to heparin.

Although HS was the predominant proteoglycan extracted from glomeruli [35S]sulfate labeled in vivo and from GBM (17), DS accounted for 60% of high density glomerular macromolecules synthesized over 4 or 16 h in vitro (Table I). The proportion of glomerular [35S]macromolecules which were DS proteoglycan has varied between studies depending on incubation conditions. Glomeruli perfused ex situ with Na2[35SO4] synthesized a greater proportion of HS (85%) (3), while DS was the predominant proteoglycan extracted from in vitro [35SO4]labeled isolated glomeruli (75% (20) and 38% (24)). Newly synthesized DS proteoglycans from in vivo and ex situ studies may be preferentially lost during the glomerular isolation process, while proteoglycans [35S]sulfate labeled in vitro were not subject to the serial sieving process prior to extraction. However, autoradiographic studies identified few, mostly mesangial, grains attributable to DS proteoglycan (21). It is, therefore, possible that glomeruli incubated in vitro may alter their proteoglycan phenotype as a reaction to incubation conditions or based on the preferential survival of cell populations responsible for DS proteoglycan synthesis.

Two major DS proteoglycans were synthesized by isolated glomeruli in vitro. A small DS proteoglycan (DS-tIa) copurified with HS-tIa by CsCl density gradient ultracentrifugation, DEAE-Sepahcel and Sepharose CL-6B chromatography, as well as by agarose-polyacrylamide gel electrophoresis (DS-tIa was detected at each purification step by partial sensitivity to chondroitinase ABC digestion). However, DS-tIa may be slightly larger than HS-tIA since it eluted predominantly with the front half of the Ks, 0.21 Sepharose CL-6B peak after 4-h in vitro incubations (Fig. 10A), and, after chondroitinase ABC treatment, HS-tIa eluted slightly later from Sepharose CL-6B than the proteoglycan mixture (Fig. 2B). DS-tIa contained longer GAG chains (M, 18 x 10^6) than HS-tIA. These GAGs had a chain length and chondroitin 4:6 sulfite ratio similar to medium DS proteoglycans (DS-mII) from 16-h in vitro incubations (Table II). The possibility that HS-tIa and DS-tIa GAGs were attached to the same protein core could not be completely eliminated since protein core size and composition were not studied.

Sepharose CL-6B-included HS and DS proteoglycans were rapidly released from the tissue, with 31% of these proteoglycans found in the medium after a 2-h chase of 4-h in vitro [35SO4]-labeled glomeruli. The proportion of glomerular proteoglycans released into medium was comparable to the half-life reported for granulosa cell surface proteoglycans (4 h) and HS proteoglycan from adhesion sites (42, 49). Whereas heparin treatment of liver cell membranes released only HS proteoglycan (6), this treatment as well as limited trypsin digestion released both HS and DS proteoglycans from isolated glomeruli and from granulosa cell surfaces (28). Thus, DS-tIa as well as HS-tIa proteoglycans may be rapidly released from cell surface or extracellular matrix "GAG receptors" and appear in medium.

A second DS proteoglycan (DS-tII) accumulated in tissue fractions over time, without being found intact in the incubation medium. This proteoglycan eluted later than DS-tIa and HS proteoglycans from DEAE-Sepahcel columns. Intact DS-tII proteoglycan (partially excluded from Sepharose CL-
6B) as well as its GAG chains ($M_w \times 10^6$) were larger than other glomerular proteoglycans. That this proteoglycan may be “self-associating” was evident from its differential elution from CL-4B columns in the presence and absence of 4 M guanidine HCl without a change in elution volume when samples were reduced and alkylated. The latter procedure dissociated proteoglycan-hyaluronate aggregates (32). DS proteoglycan with similar migration by gel electrophoresis and with similarly sized GAGs was detected in vivo as a minor late-eluting DEAE-Sepahcel peak in both high and low density CsCl gradient fractions. Thus, DS-tII was confirmed as a normal component of glomeruli.

DS-tII GAG chains contained iduronic acid residues as determined by a difference in sensitivity to chondroitinase ABC and AC digestions as well as by the appearance of short Sephadex G-50-included oligosaccharides after periodate oxidation and alkaline cleavage. A greater proportion of oligosaccharides (33%) was included in these columns than previously reported for the “self-associating” bovine scleral DS (50). In addition to a region of closely spaced iduronic acid residues, the presence of a large unmodified span within DS-tII GAG was suggested by Sepharose CL-6B chromatography of Sephadex G-50-excluded periodate-treated GAGs. Although reduced in size when compared to intact DS-tII GAG ($M_w 26,000$), periodate treatment and alkaline cleavage left a chain which had an $M_r$ of approximately 9,000 ($K_{av} 0.64$). The presence of long glucuronic acid-containing regions was confirmed by the generation of a large proportion (%) of disaccharides by chondroitinase AC digestion of DS-tII GAG. These studies, therefore, suggest that iduronic acid residues are clustered along DS-tII GAG.

Whether DS-tIA and DS-tII are distinct proteoglycans or part of a continuum of DS proteoglycans with varying numbers of GAG chains of polydisperse length/proteoglycan protein core was not resolved by these experiments. That these were distinct proteoglycans was suggested by their partial separation by DEAE-Sepahcel and by Sepharose CL-6B chromatography. In addition, DS-tIA was preferentially released from tissue by heparin, detergent, or trypsin treatments, while the majority of DS-tII proteoglycan was detected in 4 M guanidine HCl extracts of the residual tissue. $^{35}$S-Proteoglycans remaining in tissue fractions after a 2-h chase of 4-h pulse-labeled glomeruli eluted earlier from Sepharose CL-6B columns than those found in pulse-labeled glomeruli suggesting that larger proteoglycan accumulated in tissue over time, while the smaller ones were released into the medium. From the above results one might speculate that DS-tII represents a small but real component of the glomerulus which has a slow turnover rate, while HS-tIA and DS-tIA, the major glomerular proteoglycans, are rapidly synthesized and transported to cell surfaces or tissue “GAG receptors” from which they are rapidly released into medium.

In addition, the Sepharose CL-2B elution volume ($K_{av}$ 0.62) of glomerular proteoglycans failed to change after incubation with 2% hyaluronic acid (unpublished observation by authors).

Fig. 13. Sepharose CL-6B chromatography of glomerular $^{35}$S-sacromolecules synthesized over 16 h in vivo. Glomeruli isolated from rats each given 0.5 mCi of intraperitoneal Na$_2$SO$_4$, four times over 16 h were extracted with 4 M guanidine HCl in the presence of protease inhibitors (see Methods). A, bottom 5% CsCl gradient fraction $^{35}$S-proteoglycans purified by DEAE-Sepahcel chromatography were eluted from Sepharose CL-6B columns and pooled as two separate fractions indicated by bars A and B. Equivalent quantities of each sample were electrophoresed in 0.6% agarose-1.8% polyacrylamide gels before (inset, lanes a and c) and after (inset, lanes b and d) chondroitinase ABC digestion. B and C, top 5% CsCl gradient fraction DEAE-Sepahcel peaks $^{35}$S-sacromolecules eluted as two partially separable peaks (Fig. 12, peak 1), and each was chromatographed on Sepharose CL-6B. Autoradiographs of gels run with samples pooled from Sepharose CL-6B peaks as indicated by bars (A, inset, lanes a and b; B, inset, lanes c and d) before (lanes a and c) and after (lanes b and d) chondroitinase ABC digestion.
These studies have characterized a previously described extracellular glomerular HS proteoglycan as being displaced from glomerular cell surfaces and/or tissue binding sites by heparin. A previously uncharacterized smaller intracellular HS proteoglycan with little or no protein core was also described. A heparin-displaceable DS proteoglycan was rapidly released from the tissue, leaving behind a large self-associating DS proteoglycan which contained GAGs with clusters of iduronic acid residues.

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