

Effect of β -D-Xyloside on the Glomerular Proteoglycans.

I. Biochemical Studies

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ABSTRACT The effect of *p*-nitrophenyl- β -D-xylopyranoside on glomerular extracellular matrices (glomerular basement membrane and mesangial matrix) proteoglycans was studied. The proteoglycans of rat kidneys were labeled with [35 S]sulfate in the presence or absence of β -xyloside (2.5 mM) by using an isolated organ perfusion system. The proteoglycans from the glomeruli and perfusion medium were isolated and characterized by Sepharose CL-6B chromatography and by their behavior in CsCl density gradients. With xyloside treatment there was a twofold decrease in 35 S-labeled macromolecules in the tissues but a twofold increase in those recovered in the medium as compared with the control. The labeled proteoglycans extracted from control kidneys eluted as a single peak with $K_{av} = 0.25$ ($M_r = \sim 130,000$), and $\sim 95\%$ of the radioactivity was associated with heparan sulfate proteoglycan (HS-PG), remainder with chondroitin (or dermatan) sulfate proteoglycan (CS-PG). In the xyloside-treated kidneys, the proteoglycans extracted from the tissue eluted as two peaks, $K_{av} = 0.25$ ($M_r = \sim 130,000$) and 0.41 ($M_r = \sim 46,000$), which contained ~ 40 and $\sim 60\%$ of the total radioactivity, respectively. The first peak contained mostly the HS-PG ($\sim 90\%$) while the second peak had a mixture of HS-PG ($\sim 70\%$) and CS-PG ($\sim 30\%$). In controls, $\sim 90\%$ of the radioactivity, mostly HS-PG, was confined to high density fractions of a CsCl density gradient. In contrast, in xyloside experiments, both HS-PG and CS-PG were distributed in variable proportions throughout the gradient. The incorporated 35 S activity in the medium of xyloside-treated kidneys was twice that of the controls and had three to four times the amount of free chondroitin (or dermatan) sulfate glycosaminoglycan chains. The data suggest that β -xyloside inhibits the addition of de novo synthesized glycosaminoglycan chains onto the core protein of proteoglycans and at the same time stimulates the synthesis of chondroitin or dermatan sulfate chains which are mainly discharged into the perfusion medium.

Glomerular extracellular matrices (GEMs)¹ contain a network of anionic sites (1, 10, 30) which are largely made up of heparan sulfate (11, 12) and small amounts of chondroitin sulfate glycosaminoglycans (GAGs) (13, 23). Glomerular basement membranes (GBMs) contain predominantly heparan sulfate, while chondroitin sulfate is enriched in the mesangial matrix of GEMs (14, 17). Heparan sulfate GAGs of

GEMs are covalently bound by O-glycosidic linkage to the core (13, 19) protein via galactosyl-galactosyl-xylosyl-serine. GAG chains are added onto the core protein stepwise and are initiated by UDP-D-xylose:core protein xylosyltransferase followed by other transferases for completion of these chains (5, 6, 33).

In cultures of cartilage and other tissues, it has been shown that D-xylose or β -xylosides added to the medium stimulates the synthesis of free GAG chains by acting as an initiator of chain formation, thus bypassing the requirement for the core protein and the xylosyl transferase (24, 29, 31, 37). The xyloside-initiated chains are not retained in the extracellular

¹ Abbreviations used in this paper: CS-PG, chondroitin sulfate proteoglycan; GAG, glycosaminoglycan; GBM, glomerular basement membranes; GEM, glomerular extracellular matrix; HS-PG, heparan sulfate proteoglycan.

matrices and are discharged rapidly into the medium. At the same time, the net synthesis of chondroitin sulfate on the core protein is inhibited. The studies so far reported concerning xylosides pertain primarily to the synthesis of proteoglycans in cartilage (26, 36, 37), chick limb bud mesenchymal cell cultures (25), embryonic salivary glands (43, 44), mast cells (32, 39, 40), bone marrow-derived cells (38), 3T3 cells (7), hepatocytes (42), or murine embryonal carcinoma cell lines (22). Data concerning the synthesis of heparan sulfate proteoglycan of native mammalian basement membranes in the presence of xylosides is limited. The purpose of the present study was to investigate the effect of β -D-xyloside on the de novo synthesized proteoglycans of GEMs.

MATERIALS AND METHODS

Materials: Male Charles River CD® rats weighing 300–350 g were used in these experiments. Sepharose CL-6B, Sephadex G-50, and DEAE-Sephacel were purchased from Pharmacia Fine Chemicals (Piscataway, NJ); *p*-nitrophenyl- β -D-xylopyranoside from Research Product International Corp. (Elk Grove, IL); chondroitinase-ABC from Miles Laboratories, Inc. (Elkhart, IN); [³H]serine (15 Ci/mmol) and [³⁵S]sulfate (~900 Ci/mmol) from Amersham Corp. (Arlington Hts., IL). [³H]Serine-labeled cartilage proteoglycan monomer (22) was a gift of Dr. James H. Kimura (Rush Medical College, Chicago, IL).

Radiolabeling of Glomerular Proteoglycans: Radiolabeling of glomerular proteoglycans was carried out in an ex situ organ perfusion apparatus (17). The ex situ organ perfusion technique is much more effective in labeling the proteoglycans than are isolated glomerular culture systems; in the latter the viability of the various cell types of the glomerulus has been seriously questioned (28). Furthermore, the ex situ organ perfusion technique almost mimics in vivo conditions while obviating many of the problems encountered when labeling in vivo, including: (a) the rapid clearance of sulfate from blood, thereby making maintenance of constant levels of [³⁵S]sulfate difficult, and (b) the presence of high levels of inorganic sulfate in the plasma (0.6 mg/dl) which competitively reduces the uptake of [³⁵S]sulfate by the kidney.

The entire ex situ organ perfusion procedure was carried out under sterile conditions. Animals were anesthetized by injection of inactin (10 mg/100 g body wt). The right kidney was isolated, and its renal artery was cannulated and flushed with oxygenated Krebs-Ringer bicarbonate, pH 7.4. The cannulated kidney was removed from the animal and attached to a recirculating ex situ perfusion system. The perfusate consisted of modified Krebs-Ringer bicarbonate containing 7.5% BSA, basal medium Eagle amino acid (5 ml of amino acid solution/100 ml of perfusate), insulin (240 μ U/ml), glucose (1.8 mg/ml), [³⁵S]sulfate (100 μ Ci/ml), and inorganic sulfate (30 μ M). The kidneys were perfused at 8–10 ml/min and labeled for 1, 2, 3, and 4 h under normothermic and well-oxygenated conditions. Subsequently, the kidneys were perfused with a chase medium similar to the labeling perfusate, but lacking [³⁵S]sulfate and containing 3.0 mM of inorganic sulfate. For the xyloside experiments, 2.5 mM of *p*-nitrophenyl- β -D-xylopyranoside was included in the perfusate. Four kidneys were used for each time point per variable. A small biopsy was taken from the kidneys from the last time point (4 h) and processed for electron microscopy in order to ascertain any cytotoxic effect of the xyloside on the tissues. At the end of the labeling period, the perfusate was saved for isolation and characterization of proteoglycans and free GAG chains in the medium.

Isolation of Glomeruli and Extraction of Proteoglycans: Radiolabeled kidneys were perfused with Krebs-Ringer bicarbonate containing 1% BSA and protease inhibitors (1 mM of phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate), pH 7.0. The kidneys were bisected and cortices were dissected free and frozen at –20°C. After 12–24 h, the cortices were thawed at 4°C and glomeruli were isolated by the sieving technique (21). The proteoglycans were extracted from isolated glomeruli with 4 M guanidine-HCl containing 0.01 M sodium EDTA, 0.01 M sodium acetate, pH 5.8, and a mixture of protease inhibitors (0.1 M 6-aminohexanoic acid, 0.005 M benzamidine-HCl, and 0.001 M phenylmethylsulfonyl fluoride; references 13 and 19). The extraction was carried out for 48 h at 4°C with continuous stirring. The unextracted residue was pelleted by centrifugation at 10,000 rpm in a Beckman microfuge (Beckman Instruments Inc., Palo Alto, CA). The residue was extracted with 0.5 N NaOH at 60°C for 3 h to solubilize remaining GAGs. The extracts were dialyzed against distilled water containing 1 mM of phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate at 4°C. Aliquots from both the guanidine-HCl and the NaOH extracts were saved for monitoring radioactivities. Aliquots from the guanidine-HCl extracts were assayed for protein content (27). The remaining samples were frozen and lyophilized before further characterization.

Characterization of Extracted Glomerular Proteoglycans: Samples from untreated and xyloside-treated kidneys were eluted under dissociative conditions on a Sepharose CL-6B column (100 \times 0.9 cm). Each sample was reconstituted with 100–200 μ l of 4 M guanidine-HCl before chromatographic analysis. The column was eluted with 4 M guanidine-HCl, 100 mM Tris-HCl, 0.1 M sodium sulfate, 0.5% (vol/vol) Triton X-100, pH 7.0, at a flow rate of ~2.4 ml/h, and fractions of ~0.6 ml were collected. We determined the void (V_0) and total (V_t) volumes of columns by eluting [³⁵S]serine-labeled cartilage monomer proteoglycan (for V_0) and [³H]serine (for V_t) with the same buffer. The radioactivity in each of the effluent fractions was determined in a Beckman LS-9000 scintillation counter.

Aliquots of intact glomerular proteoglycans were treated either with chondroitinase-ABC (0.25 U/ml) after reconstitution in 0.5 ml of 0.1 M Na acetate, 0.1 M Tris-HCl buffer, pH 7.3, for 2 h at 37°C (35) or with nitrous acid (prepared by mixing equal volumes of 20% [wt/vol] butyl nitrite and 1 N HCl) for 4 h at room temperature (3). After the treatments, the samples were lyophilized to dryness and reconstituted with 4 M guanidine-HCl. Degraded as well as undegraded samples were chromatographed on Sepharose CL-6B. The molecular weights of glomerular proteoglycans from xyloside-treated and control kidneys were estimated by interpolation of K_{av} values for peak elution positions with the data obtained from sized tryptic-chymotryptic fragments of cartilage proteoglycan (4).

Some of the lyophilized aliquots of the extracted proteoglycans were pooled, reconstituted with 4 M guanidine-HCl, adjusted to an initial density of 1.42 g/ml by addition of 0.45 g of CsCl/g solution, and centrifuged at 35,000 rpm in a SW Ti 55 rotor for 72 h at 10°C (13, 19). Five equal fractions, D₁ to D₅ from bottom to top, were collected. The density of each fraction was determined with a Bausch & Lomb Abbe-3L refractometer (Bausch & Lomb Inc., Rochester, NY). An aliquot of each of the fractions was saved for monitoring radioactivity, and the remainder was chromatographed before and after treatment with chondroitinase-ABC or with nitrous acid, as described above.

Characterization of Glomerular GAGs: Extracted glomerular proteoglycans from xyloside-treated and control kidneys were treated with 0.5 N NaOH at 60°C for 3 h in order to liberate the GAG chains. The hydrolyzates were dialyzed against distilled water, lyophilized, and chromatographed on Sepharose CL-6B before and after treatment with nitrous acid. The molecular weights of the chains were estimated by comparison of peak elution positions with values obtained by Wasteson for chondroitin sulfate (45). Portions were also reconstituted with 4 M guanidine-HCl and subjected to CsCl density gradient centrifugation as described above. D₁ to D₅ fractions were collected and a small aliquot of each fraction was used for monitoring radioactivity. The D₁ fractions from the xyloside and control experiments were chromatographed on Sepharose CL-6B before and after treatment with chondroitinase-ABC or nitrous acid.

In xyloside experiments, the effluent fractions from Sepharose CL-6B corresponding to elution peaks of intact proteoglycans (peak 1, fractions 35–45, with K_{av} = 0.25; and peak 2, fractions 46–56, with K_{av} = 0.41; see Fig. 3) were recovered. The peaks were separately pooled, dialyzed against distilled water, and lyophilized. Samples were then treated with 0.5 N NaOH at 60°C for 3 h, redialyzed against distilled water, lyophilized, and chromatographed before and after treatment with chondroitinase-ABC or nitrous acid.

Isolation and Purification of Proteoglycans and GAGs from the Perfusion Medium: Disposable columns of Sephadex G-50 (fine) were prepared in plastic serological pipettes to give a 10-ml bed volume. The gels were equilibrated with 8 M urea, 0.15 M NaCl, 0.05 M sodium acetate, pH 6.0, and 0.5% (vol/vol) Triton X-100 (2). Aliquots of 1.0 ml from the perfusion media (xyloside and control) were applied to the individual columns and eluted with the same solution. The excluded fractions were collected, dialyzed against distilled water, lyophilized, and reconstituted to 1 ml with 0.1 M NaCl.

The reconstituted fractions were then applied to a 5.0-ml column of DEAE-Sephacel equilibrated with 0.1 M NaCl. After loading, columns were washed with 10 ml of 0.1 M NaCl followed by elution with a continuous 0.1–1.0 M gradient using a total volume of 40 ml. Fractions of 1.0 ml were collected at a flow rate of 4.0 ml/h, and aliquots (100 μ l) were counted for radioactivity.

Characterization of Medium Proteoglycans and GAGs: The fractions with incorporated radioactivity obtained from DEAE-Sephacel chromatography were pooled, dialyzed against distilled water at 4°C, and lyophilized. Characterization of these samples was done as described above for the glomerular proteoglycans.

RESULTS

Morphological Features

After 4 h of perfusion, the overall architecture of the glomeruli was well preserved. In preliminary experiments, the

effect of xyloside concentrations (from 1–5 mM) on the glomeruli was tested. At concentrations up to 2.5 mM in the perfusion medium, no deterioration in the glomerular morphology was observed (compare Fig. 1B with 1A). The epithelial and endothelial cells remained adherent to the basement membranes. The mesangial cells did not show any cytopathic effects. At 5 mM concentration of xyloside in the medium, mild swelling of the Golgi complexes was observed. Therefore, subsequent experiments were carried out with 2.5 mM xyloside in the perfusate.

Extraction of Proteoglycans

The efficiency of extraction of radiolabeled proteoglycan

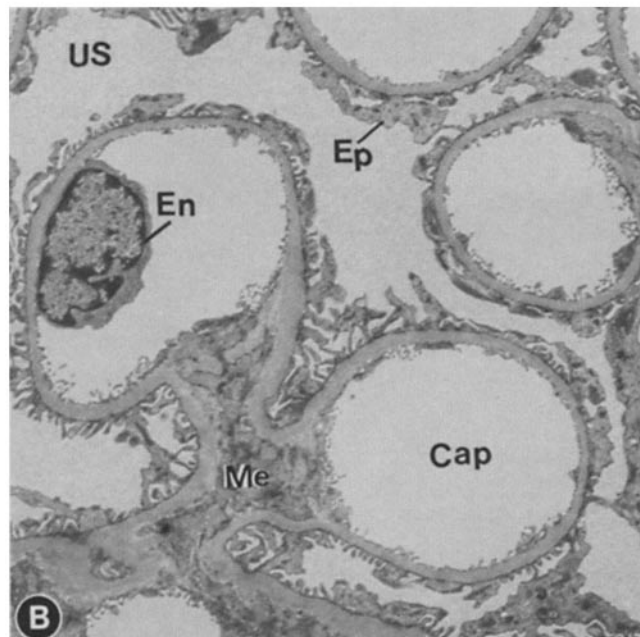
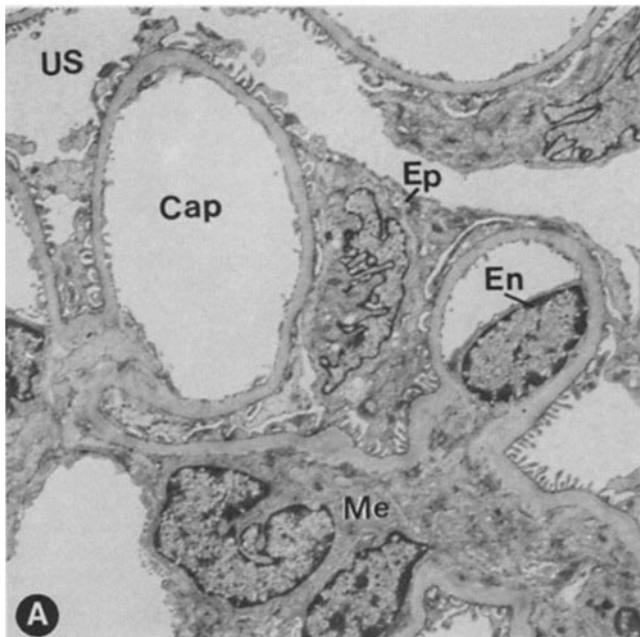


FIGURE 1 Portions of glomerular capillaries from kidneys of control (A) and xyloside (B) experiments. No discernible changes in the epithelial (Ep), endothelial (En), and mesangial (Me) cells are observed in the xyloside-treated kidney (2.5 mM, 4 h) as compared with the control. US, urinary space; Cap, capillary lumen. $\times 9,000$.

from whole glomeruli was $\sim 96\%$. In controls, under the present experimental conditions, the [^{35}S]sulfate incorporation was $\sim 0.85 \times 10^6$ dpm/kidney per 4 h. Under identical labeling conditions, in the presence of 2.5 mM β -xyloside, the [^{35}S]sulfate incorporation decreased substantially to $\sim 0.2 \times 10^6$ dpm/kidney per 4 h (Table I). The [^{35}S] incorporation into the glomerular proteoglycans in both the control and xyloside-treated kidneys remained linear up to 4 h (Fig. 2). The incorporated radioactivity normalized against micrograms of glomerular protein from four kidneys per time point was two to three times less in xyloside-treated kidneys than in the control (Fig. 2).

Characterization of Extracted Glomerular Proteoglycans

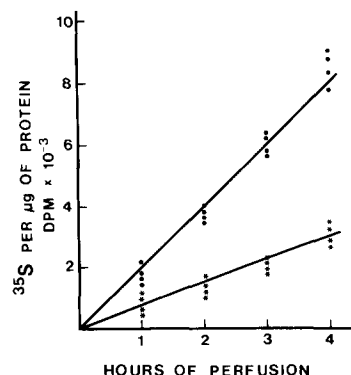
The proteoglycans extracted from glomeruli of control kidneys eluted on Sepharose CL-6B as a single peak of radioactivity with a K_{av} value of 0.25 (Fig. 3A). This value is similar to that obtained for labeled proteoglycans isolated from purified basement membranes prepared from similarly perfused kidneys which have M_r in the range of 130–150,000. After nitrous acid oxidation to degrade N-sulfated heparan sulfate, $\sim 95\%$ of the radioactivity was released from the proteoglycan peak into the V_1 fraction, while after chondroitinase-ABC digestion to degrade chondroitin 4,6 sulfate and dermatan sulfate, only $\sim 5\%$ of the radioactivity was recovered in the V_1 fraction (Fig. 3A). The positions of the elution peaks resistant to the above treatments remained unchanged.

The glomerular proteoglycans extracted from xyloside-treated kidneys when chromatographed on Sepharose CL-6B eluted as two peaks (peaks 1 and 2) of radioactivity with K_{av} values of 0.25 and 0.41 indicating that in addition to the high molecular weight proteoglycan (peak 1), a smaller molecular weight proteoglycan with lower K_{av} value (0.41, $M_r = \sim 46,000$; peak 2) (45) was present in the extract (Fig. 3B). Approxi-

TABLE I
Total and Percent Radioactivity in Glomerular and Medium Fractions

	Control	Xyloside
Glomeruli		
Total	$\sim 0.85 \times 10^6$ dpm	$\sim 0.20 \times 10^6$ dpm
Peak 1	100%	$\sim 40\%$
Peak 2	—	$\sim 60\%$
Media		
Total	$\sim 8.56 \times 10^6$ dpm	$\sim 16.88 \times 10^6$ dpm
Peak A	$\sim 30\%$	$\sim 15\%$
Peak B	$\sim 70\%$	$\sim 85\%$

Peaks 1 and 2 are from Fig. 3; peaks A and B are from Fig. 8.



short half-life of the radioisotope.

FIGURE 2 Incorporation of [^{35}S]sulfate into glomerular macromolecules of ex situ perfused normal (\bullet) and xyloside ($*$) treated kidneys. Each point represents labeled macromolecules in glomeruli isolated from a single perfused kidney. All the experiments were performed with a single batch of [^{35}S]sulfate and within 2–3 d to eliminate any variability due to

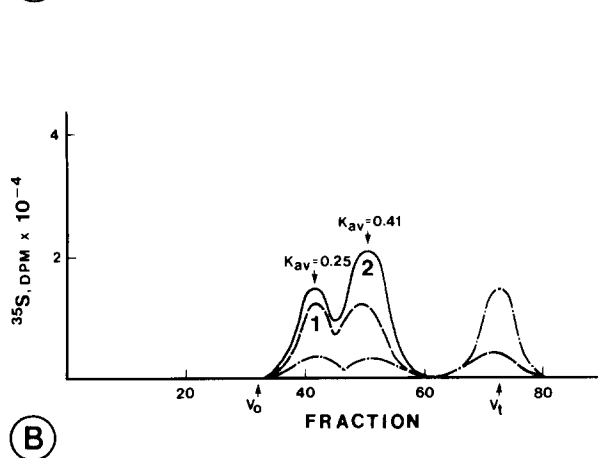
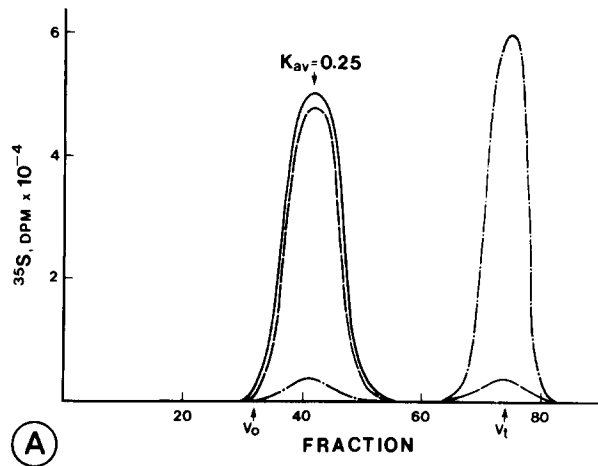


FIGURE 3 Sephadex CL-6B chromatograms of aliquots of glomerular extracts from normal (A) and xyloside-treated (B) kidneys; untreated (—) and after treatment with chondroitinase-ABC (---) or nitrous acid (- - -).

mately 40 and 60% of the radioactivity was associated with peaks 1 and 2, respectively. After treatment with chondroitinase-ABC, ~10 and ~30% of the radioactivity from peaks 1 and 2 was released into the V_t fractions (Fig. 3B). With nitrous acid oxidation, ~90 and ~70% of the radioactivity was released into the V_t fraction (Fig. 3B). The results of these treatments suggest that peak 2 contains relatively more of the chondroitinase-ABC sensitive proteoglycan than does peak 1.

Characterization of GAG Chains of Extracted Glomerular Proteoglycans

The GAG chains of glomerular proteoglycans obtained from control kidney eluted as a single peak of radioactivity with a K_{av} value of 0.47; $M_r = \sim 25,000$ (45). Treatment with nitrous acid or chondroitinase-ABC resulted in the release of ~95 or ~5% of the radioactivity, respectively, into the V_t fractions (Fig. 4A). No significant change in the elution profiles of the resistant GAG peaks was observed.

The GAG chains of glomerular proteoglycans obtained from xyloside-treated kidney eluted as a single peak of radioactivity with K_{av} value of 0.47, $M_r = \sim 25,000$ (45). Treatment with nitrous acid or chondroitinase-ABC resulted in the release of ~65 or ~35%, respectively, of the radioactivity into the V_t fractions (Fig. 4B). Successive treatments of chondroitinase-ABC and nitrous acid released all the counts into the V_t fraction, indicating that the xyloside treatment did not

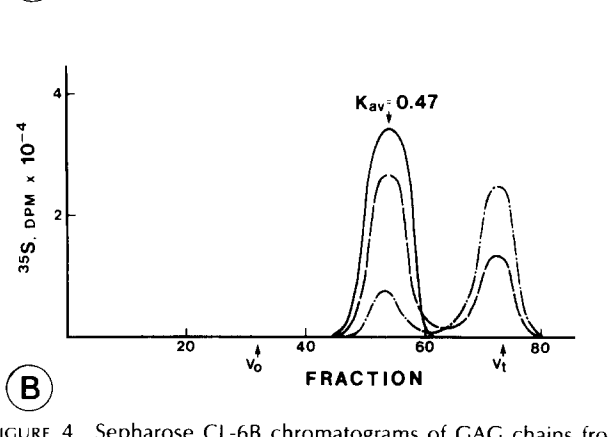
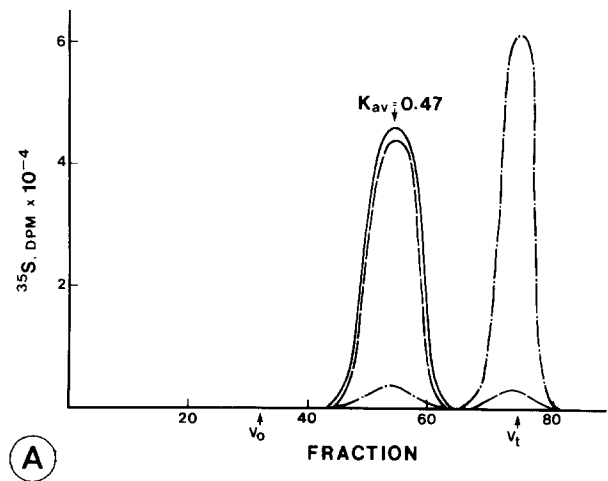


FIGURE 4 Sephadex CL-6B chromatograms of GAG chains from glomeruli of control (A) and xyloside-treated (B) kidneys; intact chains (—) and after treatment with chondroitinase-ABC (---) or nitrous acid (- - -).

result in the de novo synthesis of a new class of proteoglycans or GAGs.

In addition, the GAG chains, obtained from elution peaks 1 (fractions 35–45) and 2 (fractions 46–56) of Sephadex CL-6B chromatograms (Fig. 3B) of proteoglycans extracted from xyloside-treated kidneys, were characterized. Aliquots of each peak were treated with 0.5 N NaOH to release GAG chains. The treated samples were then dialyzed, lyophilized, and chromatographed before and after treatment with chondroitinase-ABC or nitrous acid. Chondroitinase-ABC and nitrous acid treatments released ~10 and ~90%, respectively, of the radioactivity into the V_t fractions from the GAG chains ($K_{av} = 0.47$, $M_r = \sim 25,000$) obtained from peak 1 (Fig. 5A). Similar treatments of GAG chains ($K_{av} = 0.47$, $M_r = \sim 25,000$) from peak 2 released ~45 and ~55% of the radioactivity into the V_t fraction (Fig. 5B).

The data so far obtained from characterization of glomerular proteoglycan and GAGs indicate that with xyloside treatment, there is an overall decreased synthesis of proteoglycan and a relative increase in the synthesis of chondroitin sulfate compared with heparan sulfate. The increase in the synthesis of chondroitin sulfate varied from 30–40%, as determined from various elution profiles from different experiments.

Behavior of Glomerular Proteoglycans and GAGs in Dissociative CsCl Gradients

When the extracted glomerular proteoglycans from control kidneys were fractionated in a dissociative CsCl density gra-

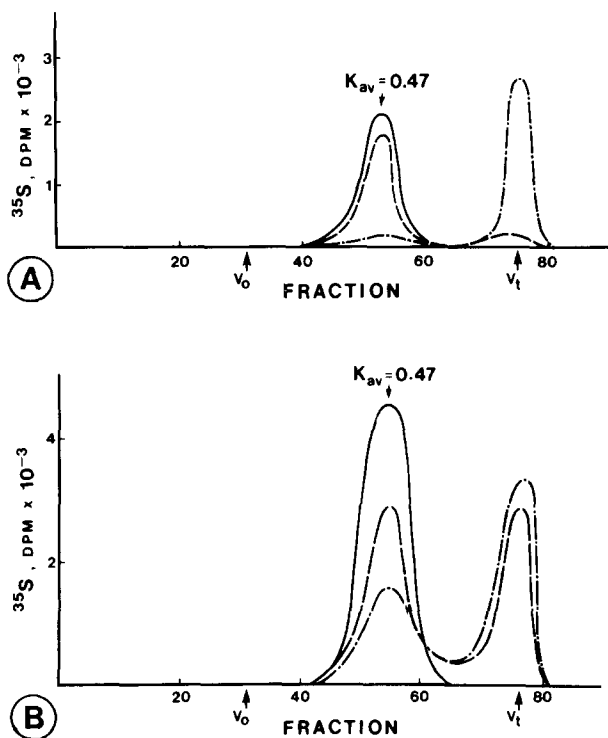


FIGURE 5 Sepharose CL-6B chromatograms of alkaline-treated fractions corresponding to peak 1 (A) and peak 2 (B) of Fig. 3B. Profiles for intact chains (—) and for samples treated with chondroitinase-ABC (---) or nitrous acid (···) are shown.

dent, a reasonable separation of the two types of proteoglycans was achieved. Nitrous acid-sensitive proteoglycan, i.e., heparan sulfate-proteoglycan (HS-PG) was exclusively present in the denser D₁ and D₂ fractions of the gradient, with the D₁ fraction containing ~70% of the total radioactivity of the gradient (Fig. 6A, left-hand bar graph). The chondroitin sulfate proteoglycan (CS-PG) was present in small amounts along with HS-PG in the lighter D₄ and D₅ fractions of the gradient. Aliquots of the D₁–D₅ fractions of the CsCl gradient each eluted as a single peak on Sepharose CL-6B chromatography, and no significant change in the K_{av} values (~0.25) was observed (figure not shown).

When glomerular proteoglycans from xyloside-treated kidneys were fractionated in the dissociative CsCl density gradient, only a partial separation of the two types of proteoglycans could be achieved (Fig. 6A, right-hand bar graph). Almost 95% of the D₁ fraction was nitrous acid sensitive, and the proteoglycan in this fraction eluted as a single peak with K_{av} value ~0.25, indicating that the proteoglycans in peak 1, Fig. 3B, are very similar to those in the control. Except for the D₁ fraction of the gradient, the other fractions eluted as two peaks (K_{av} = ~0.25 and ~0.41) similar to those shown in Fig. 3B. The decrease in the buoyant density of the CsCl gradient fractions (D₂–D₅) corresponded to an increase in the presence of chondroitinase-ABC-sensitive material, with the D₅ fraction containing almost equal proportions of CS and HS. In addition, in the lighter D₄ and D₅ fractions, relatively more CS was consistently present in the second peak of the Sepharose CL-6B chromatograms than in the first, similar to the results shown in Fig. 3B. Therefore, the proteoglycans in peak 2, Fig. 3B, have lower average buoyant densities than peak 1 proteoglycans.

The sedimentation behavior of released GAG chains was

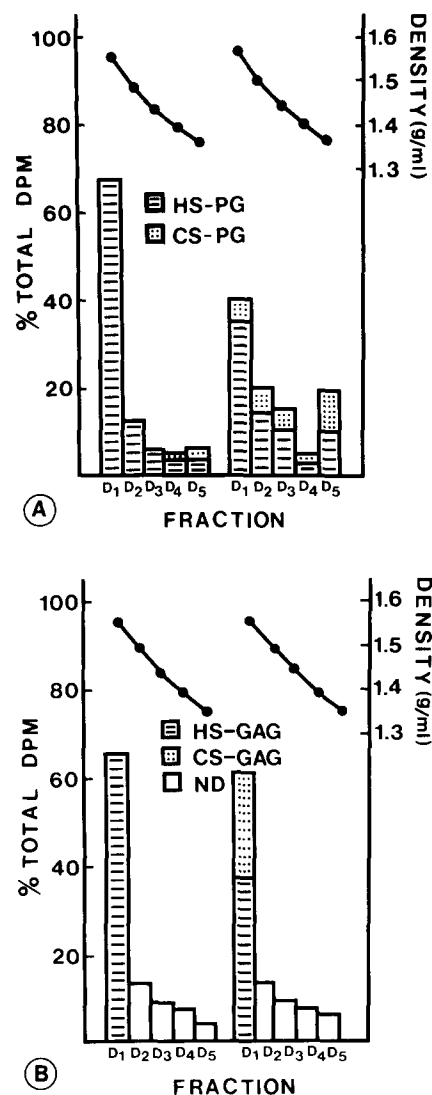


FIGURE 6 Sedimentation behavior of glomerular proteoglycans (A) and GAGs (B) of control (left-hand bar graphs in A and B) and xyloside-treated (right-hand bar graphs in A and B) kidneys in CsCl gradient. In controls, the HS-PG sedimented in the denser D₁ and D₂ fractions (A, left-hand bar graph) whereas in xyloside-treated kidneys, HS-PG as well as CS-PG were distributed throughout the gradient fractions (A, right-hand bar graph). The GAG chains, in control (B, left-hand bar graph) sedimented in the D₁ fraction (~65% of the total radioactivity) and contained exclusively heparan sulfate. The GAG chains from xyloside-treated glomeruli also sedimented mainly in the D₁ fraction (~60% of the total radioactivity); however, it contained both the chondroitin sulfate (~35%) and heparan sulfate (~65%). ND, not determined.

also determined in dissociative CsCl gradients. The GAG chains from control (Fig. 6B, left-hand bar graph) and xyloside-treated samples (Fig. 6B, right-hand bar graph) sedimented mainly in the D₁ fractions which contained ~65 and ~60% of the total radioactivity, respectively. The D₁ fractions were chromatographed on Sepharose CL-6B before and after treatment with chondroitinase-ABC or nitrous acid. The D₁ fraction of the GAG chains from glomerular proteoglycans of normal kidney contained almost exclusively heparan sulfate, while the D₁ fraction of the GAG chains from glomerular proteoglycans of xyloside-treated kidney contained ~35 and ~65% of chondroitin and heparan sulfates, respectively. The other fractions, D₂–D₅, were not analyzed further. The fact

that the released GAG chains exhibited higher density than the intact molecules further indicates that the peak 2 (Fig. 3B) molecules are proteoglycans and not free GAG chains.

Characterization of Medium Proteoglycan and GAGs

Aliquots of labeling medium were eluted on Sephadex G-50, and the excluded fractions were collected, pooled, and lyophilized. They were then dissolved and applied to a DEAE-Sephacel column eluted with a 0.1–1.0 M NaCl continuous gradient. In the medium of control kidneys, the incorporated [³⁵S]sulfate eluted as a single sharp peak of radioactivity at ~0.25 M NaCl (Fig. 7A). With the medium from xyloside-treated kidneys, the incorporated [³⁵S]sulfate eluted as a somewhat broader peak but at the same salt concentration (Fig. 7B). The total incorporated ³⁵S activity in the whole-medium fractions (total volume 80 ml) of the control and of xyloside-treated kidneys was ~8.6 × 10⁶ and ~16.9 × 10⁶ dpm, respectively (Table I). These data indicated that there were substantial amounts of de novo synthesized proteoglycans and GAGs which were discharged into the perfusion medium compartment for both samples. Furthermore, with the xyloside treatment, about twice as many de novo synthesized molecules were released into the medium.

The characterization of medium proteoglycan and GAGs was carried out by Sepharose CL-6B chromatography. The medium proteoglycan and GAGs from control kidneys eluted as two peaks of radioactivity, A and B, with corresponding *K_{av}* values of ~0.25 and ~0.47, respectively (Fig. 8A). This indicated that medium contains both the macromolecular form of proteoglycan (*K_{av}* = ~0.25) and free GAG chains (*K_{av}* = ~0.47). The incorporated ³⁵S activity was relatively more

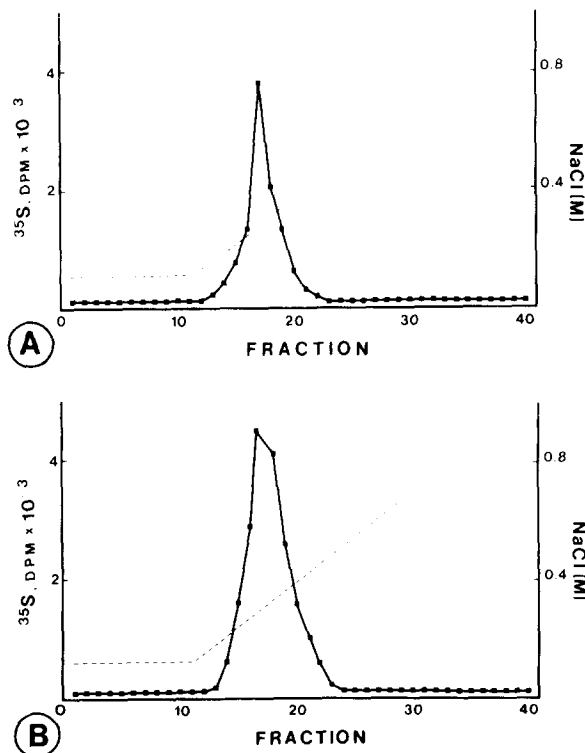


FIGURE 7 Elution profiles of incorporated [³⁵S]sulfate of media from control (A) and xyloside (B) experiments on DEAE-Sephacel. The samples applied to DEAE-Sephacel were obtained from void fractions from Sephadex G-50 chromatography.

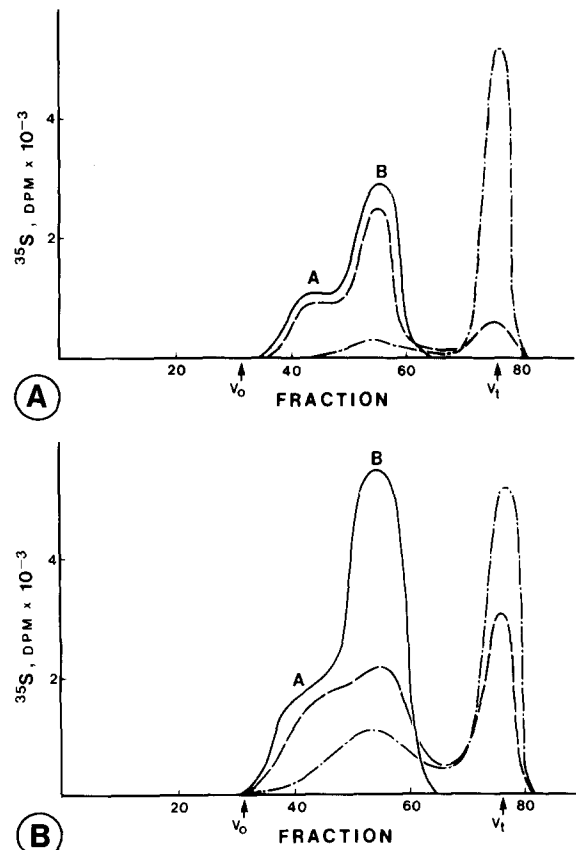


FIGURE 8 Sepharose CL-6B chromatograms of media fractions obtained after DEAE-Sephacel chromatography from control (A) and xyloside (B) experiments. In control, two peaks, A and B (—), are observed. With chondroitinase ABC (---) or nitrous acid (- - -) treatments, ~10% or ~90%, respectively, of the radioactivity was released into the *V_i* fractions. In xyloside experiments, again two peaks (—) are observed, with most of the radioactivity associated with peak B. The treatments with chondroitinase-ABC (---) and nitrous acid (- - -) indicate that peak B had almost equal proportions of heparan and chondroitin sulfate.

associated with free GAG chains in the medium. Treatment with nitrous acid or chondroitinase-ABC indicated that ~90% of the radioactivity in the medium was associated with heparan GAGs (Fig. 8A).

The incorporated ³⁵S activity in the medium of xyloside-treated kidneys also eluted as two peaks with *K_{av}* values corresponding to a macromolecular form of proteoglycan (*K_{av}* = ~0.25, peak A) and free GAG chains (*K_{av}* = ~0.47, peak B) (Fig. 8B). Most of the radioactivity was associated with peak B indicating the predominant presence of GAG chains. The data indicate that with xyloside treatment, a larger fraction of labeled free GAG chains was released into the medium. The results of the treatment with chondroitinase-ABC and nitrous acid indicated that peak A contained mostly the HS-PG, while peak B was enriched with chondroitin sulfate, ~60% of the total (Fig. 8B). These data on the characterization of medium proteoglycan/GAG suggest that with xyloside treatment, there is a twofold increase in the de novo synthesis of free GAG chains, predominantly composed of chondroitin sulfate.

DISCUSSION

The organ perfusion system employed in this investigation provided an effective means of determining the effect of β-D-

xyloside on the de novo synthesized glomerular proteoglycans. The results obtained establish that xyloside competes with the native acceptor for the initiation of synthesis of heparan and chondroitin sulfate chains. The net synthesis of glomerular proteoglycan was markedly reduced in the presence of xyloside. The effect of xyloside did not seem to be due to its cytotoxicity since no discernible changes in the morphology of the glomerular cells were observed during the time frame of the labeling period. The Golgi complexes appeared normal up to the concentration of 2.5 mM of xyloside in the perfusion medium. The glomerular matrices also remained unaltered. Thus, in the absence of recognizable morphological changes in the glomerulus, the decreased ^{35}S incorporation in the matrices appears to be due to the reduced addition of GAG chains onto the core protein rather than to the toxicity of xyloside. This phenomenon has also been observed in other culture systems as well, where the effect of xyloside has been investigated (7, 22, 25, 26, 29, 32, 36–40, 42–44).

Previously, the effects of xyloside have been studied most extensively in systems that synthesize cartilage-specific (i.e., chondroitin sulfate) proteoglycans (25, 26, 29, 36, 37). In such systems, xylosides have been demonstrated to compete with the core protein for initiation of chondroitin sulfate synthesis. The data presented here indicate that the xyloside can also influence the synthesis of HS-PG on the core protein of HS-PG of extracellular matrices. The effect of xyloside on the synthesis of core protein of glomerular proteoglycans is extremely difficult to assess because of the technical limitation of labeling the peptide chain in the perfusion system. However, in view of the results obtained in past experiments with embryonic chick chondrocytes (36) and chick limb bud mesenchymal cells (25), the core protein synthesis may not be affected by the xyloside treatment. Consequently, the inhibition of HS-PG synthesis in the presence of β -xyloside results from competition of xyloside with the acceptor of heparan sulfate on the core protein for initiation of heparan sulfate synthesis rather than any change in core protein synthesis itself.

The proteoglycans synthesized in the presence of xyloside were of two types as indicated by two elution peaks (peaks 1 and 2) on Sepharose CL-6B chromatograms. The larger molecular weight proteoglycan (peak 1, Fig. 3B) contained mostly (~90%) heparan sulfate GAG chains while the smaller molecular weight proteoglycan (peak 2, Fig. 3B) contained a mixture of heparan sulfate (~70%) and chondroitin sulfate (~30%) GAGs. The presence of larger amounts of de novo synthesized chondroitin (or dermatan) sulfate in the smaller molecular weight proteoglycan indicates that xyloside must have some stimulatory effect on the net synthesis of chondroitin sulfate including synthesis on core proteins. This stimulatory effect is further substantiated by the fact that a three- to fourfold increase in incorporated ^{35}S activity associated with chondroitin sulfate GAG chains, presumably synthesized on xyloside primer, was observed in the perfusion medium. Similar effects have also been observed in other culture systems (7, 22, 25, 26, 29, 32, 36–40, 42–44), but whether such effects are indeed operative under in vivo conditions is unknown. The xyloside perturbed synthesis of GEM proteoglycans was also reflected in some of their physicochemical properties. In controls in a CsCl gradient, the HS-PG was concentrated in the denser bottom D_1 fraction. However, the peak 2 HS-PG and CS-PG synthesized under the influence of xyloside, distributed throughout the gradient in

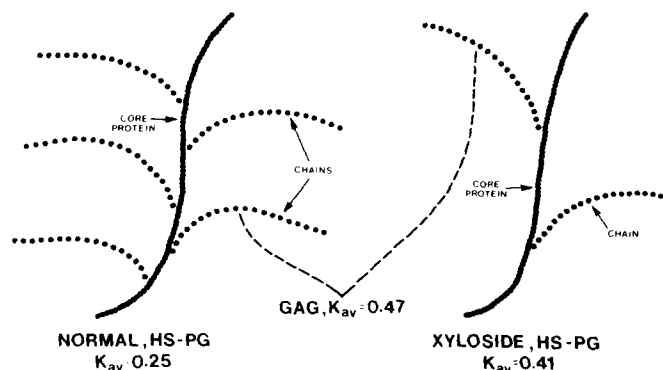


FIGURE 9 Schematic drawing of the glomerular HS-PG before and after treatment with xyloside.

variable proportions. This is probably reflective of the change in the buoyant density of the proteoglycans which is directly related to fewer chains present on the core protein. A schematic drawing of the presumptive change that occurs under the influence of xyloside is given in Fig. 9 and is derived in a manner similar to the results obtained by Lohmander et al. (25) on the CS-PG in chick limb bud mesenchymal cell experiments. The fact that under the influence of xyloside two types of proteoglycans are synthesized, i.e., a larger one (peak 1, Fig. 3B) that behaves like normal HS-PG and a smaller one (peak 2, Fig. 3B) with fewer GAG chains, and that both species of proteoglycans contain the same size GAGs, indicate that this is a direct consequence of xyloside competition with the endogenous core for both heparan and chondroitin (or dermatan) sulfate synthesis. The free GAG chains (including xyl-GAG) would concentrate in the D_1 fraction (Fig. 6B) and are absent in the glomerular extract of xyloside-treated kidneys indicate that they are retained on the core protein of the proteoglycans and that the core protein is essential for their retention in the GEMs. The net decrease in incorporated activity for xyloside-treated kidneys is then primarily the result of decreased numbers of chains on the core as represented by peak 2 (Fig. 3B). Why the proportion of chondroitin (or dermatan) to heparan sulfate increases under the influence of xyloside is not known.

The fact that the β -D-xyloside can selectively perturb the synthesis of HS-PG may allow one to further explore certain aspects of the mechanisms by which HS-PG determines the permselectivity properties of the GBM in in vivo states. The HS-PG of the GBM has been shown to regulate the transglomerular passage of macromolecules like ferritin (15) and albumin (34). In addition, HS-PG has been implicated in the protection of the GBM from "clogging" by the circulating plasma proteins by virtue of its high degree of electronegativity and hydrophilicity (16). These observations concerning permselectivity and clogging have been made from experiments carried out under in situ perfusion conditions in which certain determinants of permeability, e.g., renal blood flow and transcapillary hydraulic pressure difference, are not accurately maintained. Therefore, with the knowledge that β -D-xyloside inhibits the synthesis of HS-PG of GBM, it is plausible that the experiments can be reproduced in in vivo states where the hemodynamic conditions of the glomerulus are well regulated. Furthermore, it is conceivable that long-term in vivo treatment with β -D-xyloside may give some insights into the influence of HS-PG on the process of assemblage of basement membranes. To this end, interestingly enough, an associated loss of HS-PG with thickening of GBM in experimentally

induced diabetic nephropathy (18) and lamination and splitting of tubular basement membranes in drug-induced renal polycystic disease (9) have been observed.

Finally, the present experiments do not shed any light on the effect of xyloside on the inhibition/stimulation of a particular cell type/types of the glomerulus. To obtain such information, one has first to determine the normal cellular biosynthesis of GEMs, which at present is unknown. The glomerulus is made up of three cell types (i.e., epithelium, endothelium, and mesangial cell), and it is presumed by most workers in this field that perhaps all the cells participate in the synthesis of proteoglycans of GEMs (8). The data from glomerular cell culture lines indicate that the mesangial cells synthesize CS-PG to a large extent while epithelial and endothelial cells predominantly synthesize HS-PG (41). It is conceivable that the xyloside could selectively stimulate the mesangial cell (mesenchymal in origin) for increased synthesis of chondroitin sulfate. However, whether this actually occurs under *in vivo* conditions remains to be determined. Quantitative electron microscopic autoradiographic studies are in progress to answer these questions about the effect of xyloside on cellular biosynthesis.

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REFERENCES

1. Caulfield, J. P., and M. G. Farquhar. 1976. Distribution of anionic sites in glomerular basement membranes. Their possible role in filtration and attachment. *Proc. Natl. Acad. Sci. USA.* 73:1646-1650.
2. Chang, Y., M. Yanagishita, V. C. Hascall, and T. N. Wight. 1983. Proteoglycan synthesized by smooth muscle cells derived from monkey (*Macaca memestrina*) aorta. *J. Biol. Chem.* 258:5679-5688.
3. Cifonelli, J. A., and J. King. 1972. The distribution of 2-acetamido-2-deoxy-D-glucose residues in mammalian heparins. *Carbohydr. Res.* 21:173-186.
4. Heinegård, D., and V. C. Hascall. 1974. Characterization of chondroitin sulfate isolated from trypsin-chymotrypsin digests of cartilage proteoglycans. *Arch. Biochem. Biophys.* 16:427-441.
5. Helting, T., and L. Rodén. 1969. Biosynthesis of chondroitin sulfate. I. Galactosyl transfer on the formation of the carbohydrate-protein linkage region. *J. Biol. Chem.* 244:2790-2798.
6. Helting, T., and L. Rodén. 1969. Biosynthesis of chondroitin sulfate. II. Glucuronosyl transfer on the formation of carbohydrate-protein linkage region. *J. Biol. Chem.* 244:2799-2805.
7. Johnston, L. S., and J. M. Keller. 1979. The effect of β -xyloside on heparan sulfate synthesis by SV-40 transformed Swiss mouse 3T3 cells. *J. Biol. Chem.* 254:2575-2578.
8. Kanwar, Y. S. 1984. Biophysiology of glomerular filtration and proteinuria. *Lab. Invest.* 51:1-15.
9. Kanwar, Y. S., and F. A. Carone. 1984. Reversible tubular cell and basement membrane changes in drug induced renal cystic disease. *Kidney Int.* 26:1-15.
10. Kanwar, Y. S., and M. G. Farquhar. 1979. Anionic sites in the glomerular basement membranes. *In vivo* and *in vitro* localization to laminae rarae by cationic probes. *J. Cell Biol.* 81:137-153.
11. Kanwar, Y. S., and M. G. Farquhar. 1979. Presence of heparan sulfate in the glomerular basement membranes. *Proc. Natl. Acad. Sci. USA.* 76:1303-1307.
12. Kanwar, Y. S., and M. G. Farquhar. 1979. Isolation of glycosaminoglycans (heparan sulfate) from glomerular basement membranes. *Proc. Natl. Acad. Sci. USA.* 76:4493-4497.
13. Kanwar, Y. S., V. C. Hascall, and M. G. Farquhar. 1981. Partial characterization of newly synthesized proteoglycans isolated from the glomerular basement membranes. *J. Cell Biol.* 90:527-532.

14. Kanwar, Y. S., M. L. Jakubowski, and L. J. Rosenzweig. 1983. Distribution of sulfated glycosaminoglycans in the glomerular basement membrane and mesangial matrix. *Eur. J. Cell Biol.* 31:290-295.
15. Kanwar, Y. S., A. Linker, and M. G. Farquhar. 1980. Increased permeability of glomerular basement membrane to ferritin after removal of glycosaminoglycans (heparan sulfate) by enzyme digestion. *J. Cell Biol.* 86:688-693.
16. Kanwar, Y. S., and L. J. Rosenzweig. 1982. Clogging of glomerular basement membrane. *J. Cell Biol.* 93:489-494.
17. Kanwar, Y. S., L. J. Rosenzweig, and M. L. Jakubowski. 1983. Distribution of *de novo* synthesized sulfated glycosaminoglycans in glomerular basement membrane and mesangial matrix. *Lab. Invest.* 49:216-225.
18. Kanwar, Y. S., L. J. Rosenzweig, A. Linker, and M. L. Jakubowski. 1983. Decreased *de novo* synthesis of glomerular proteoglycans in diabetes: biochemical and autoradiographic evidence. *Proc. Natl. Acad. Sci. USA.* 80:2272-2275.
19. Kanwar, Y. S., A. Veis, J. H. Kimura, and M. L. Jakubowski. 1984. Characterization of heparan sulfate-proteoglycan of glomerular basement membranes. *Proc. Natl. Acad. Sci. USA.* 81:762-766.
20. Kimura, J. H., T. E. Hardingham, and V. C. Hascall. 1980. Assembly of newly synthesized proteoglycan and link protein into aggregates in cultures of chondrosarcoma chondrocytes. *J. Biol. Chem.* 255:7134-7143.
21. Krakower, C. A., and S. A. Greenspan. 1951. Localization of nephrotoxic antigen within the isolated renal glomerulus. *Arch. Pathol.* 51:629-639.
22. Krentz, L. J. L., and J. M. Keller. 1983. Multiple heparan sulfate proteoglycans synthesized by a basement membrane producing embryonic carcinoma cell line. *Biochemistry.* 22:4412-4419.
23. Lemkin, M. C., and M. G. Farquhar. 1981. Sulfated and non-sulfated glycosaminoglycans are synthesized by kidney *in vivo* and incorporated into glomerular basement membranes. *Proc. Natl. Acad. Sci. USA.* 78:1726-1730.
24. Levitt, D., and A. Dorfman. 1973. Control of chondrogenesis in limb-bud cell cultures by bromodeoxyuridine. *Proc. Natl. Acad. Sci. USA.* 70:2201-2205.
25. Lohmander, L. S., V. C. Hascall, and A. I. Caplan. 1979. Effect of 4-methyl-umbelliferyl- β -D-xylopyranoside on chondrogenesis and proteoglycan synthesis in chick limb bud mesenchymal cultures. *J. Biol. Chem.* 254:10551-10561.
26. Lohmander, L. S., K. Madsen, and A. Minek. 1979. Secretion of proteoglycans by chondrocytes: influence of colchicine, cytochalasin B and β -D-xyloside. *Arch. Biochem. Biophys.* 192:148-157.
27. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* 193:265-275.
28. Norgård, J. O. M. 1978. Retraction of epithelial foot processes during culture of isolated glomeruli. *Lab. Invest.* 38:320-329.
29. Okayama, M., K. Kimata, and S. Suzuki. 1973. The influence of *p*-nitrophenyl β -D-xyloside on synthesis of proteochondroitin sulfate by slices of embryonic chick slices. *J. Biochem.* 74:1069-1073.
30. Rennke, H., R. S. Cotran, and M. A. Venkatchalam. 1975. Role of molecular charge in glomerular permeability: tracer studies with cationized ferritins. *J. Cell Biol.* 67:638-646.
31. Robinson, H. C., M. J. Brett, P. J. Traloggan, D. A. Lowther, and M. Okayama. 1975. The effect of β -D-xylosides and β -D-galactosides on chondroitin sulfate biosynthesis in embryonic chick cartilage. *Biochem. J.* 148:25-34.
32. Robinson, H. C., and U. Lindahl. 1981. Effect of cycloheximide, β -D-xyloside and β -D-galactosides on heparin biosynthesis in mouse mastocytoma. *Biochem. J.* 194:575-586.
33. Robinson, H. C., A. Telsler, and A. Dorfman. 1966. Studies on biosynthesis of linkage region of chondroitin sulfate-protein complex. *Proc. Natl. Acad. Sci. USA.* 56:1859-1866.
34. Rosenzweig, L. J., and Y. S. Kanwar. 1982. Removal of sulfated (heparan sulfate) or nonsulfated (hyaluronic acid) glycosaminoglycans results in increased permeability of the glomerular basement membrane to 125 I-bovine serum albumin. *Lab. Invest.* 47:177-184.
35. Saito, H., T. Yamagata, and S. Suzuki. 1968. Enzymatic methods for determination of small quantities of isomeric chondroitin sulfates. *J. Biol. Chem.* 243:1536-1542.
36. Schwartz, N. B. 1977. Regulation of chondroitin sulfate synthesis: effect of β -xylosides on the synthesis of chondroitin sulfate proteoglycan, chondroitin sulfate chains, and core protein. *J. Biol. Chem.* 252:6316-6321.
37. Schwartz, N. B., L. Galligani, P. Ho., and A. Dorfman. 1974. Stimulation of synthesis of free chondroitin sulfate chains by β -D-xyloside on cultured cells. *Proc. Natl. Acad. Sci. USA.* 71:4047-4051.
38. Spooncer, E., J. T. Gallagher, F. Krizsa, and T. M. Dexter. 1983. Regulation of hemopoiesis in long term bone marrow cultures. IV. Glycosaminoglycan synthesis and stimulation of hemopoiesis by β -D-xyloside. *J. Cell Biol.* 96:510-514.
39. Stevens, R. L., and F. K. Austen. 1982. Effect of *p*-nitrophenyl- β -D-xyloside on proteoglycan and glycosaminoglycan synthesis in rat serosal mast cell cultures. *J. Biol. Chem.* 257:253-259.
40. Stevens, R. L., E. Razin, F. K. Austen, A. Hein, J. P. Caulfield, N. Seno, K. Schmid, and F. Akiyama. 1983. Synthesis of chondroitin sulfate E glycosaminoglycan onto *p*-nitrophenyl- β -D-xyloside and its localization to secretory granules of rat serosal mast cells and mouse bone marrow-derived mast cells. *J. Biol. Chem.* 258:5977-5984.
41. Striker, G. E., P. D. Killen, and F. M. Farin. 1980. Human glomerular cells *in vitro*: isolation and characterization. *Transplant. Proc.* 12:88-99.
42. Sudhakaran, P. R., W. Sinn, and K. von Figura. 1981. Initiation of altered heparan sulfate on β -D-xyloside in rat hepatocytes. *Hoppe-Seyler's Z. Physiol. Chem.* 362:39-46.
43. Thompson, H. A., and B. S. Spooner. 1982. Inhibition of branching morphogenesis and alteration of glycosaminoglycans biosynthesis on salivary glands treated with β -D-xyloside. *Dev. Biol.* 89:417-424.
44. Thompson, H. A., and B. S. Spooner. 1983. Proteoglycan and glycosaminoglycan synthesis in embryonic mouse salivary glands: effects of β -D-xyloside on inhibition of branching morphogenesis. *J. Cell Biol.* 96:1443-1450.
45. Wasteson, Å. 1971. A method for the determination of molecular weight and molecular weight distribution of chondroitin sulfate. *J. Chromatogr.* 59:87-97.