Renal glomerular proteoglycans

An investigation of their synthesis *in vivo* using a technique for fixation *in situ*

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Newly synthesized rat glomerular [³⁵S]proteoglycans were labelled *in vivo* after injecting Na₂[³⁵S]SO₄ intraperitoneally. At the end of the labelling period (7 h) the kidneys were perfused *in situ* with 0.01% (w/v) cetylpyridinium chloride. This fixed proteoglycans in the tissue and increased their recovery 2–3-fold during subsequent isolation of glomeruli from the renal cortex. The glomeruli were fractionated by a modified osmotic lysis and detergent extraction procedure [Meezan, Brendel, Hjelle & Carlson (1978) in *The Biology and Chemistry of Basement Membranes* (Kefalides, N. A., ed.), Academic Press, New York; Kanwar & Farquhar (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4493–4497] to obtain a basement membrane preparation. The proteoglycans released at each stage of the procedure were characterized using DEAE–Sephacel ion-exchange chromatography, chondroitinase ABC and HNO₂ digestion and Sepharose CL-4B gel-permeation chromatography. About 85% of the [³⁵S]proteoglycans synthesized were of the heparan sulphate variety, the remainder being chondroitin sulphate proteoglycans. Three sizes of heparan sulphate proteoglycans were identified. The largest (HS1, *K*_{av} 0.47) accounts for 44% of the total extractable heparan sulphates. About one third of HS1 were extracted from the glomerular basement-membrane fraction with 8 M-urea and 4 M-guanidine hydrochloride but the remainder were released from the glomerulus during preparation of the fraction. The two smaller molecules (HS2, *K*_{av} 0.56 and HS3, *K*_{av} 0.68) accounted for 27% and 28% of the extractable heparan sulphate respectively and were not associated with the basement membrane fraction. HS1, HS2 and HS3 were also isolated from non-fixed glomeruli labelled *in vivo* but with much lower recovery. In glomeruli labelled *in vitro*, heparan sulphate accounted for only 35% of the proteoglycans, the remainder being of the chondroitin sulphate type. Proteoglycans similar to HS1, HS2 and HS3 were present in glomeruli labelled *in vitro* but, in addition, a large, highly charged heparan sulphate (HS1a) was extracted from the glomerular basement-membrane fraction of these glomeruli. It accounted for 6% of the total heparan sulphate.

INTRODUCTION

The renal glomerulus consists of a tuft of capillaries surrounded by a capsule (Bowman's capsule). The lumen of each capillary is lined with a layer of fenestrated endothelial cells beneath which lies a specialized basement membrane. The foot processes of epithelial cells (podocytes) are attached to the abluminal side of the basement membrane. Blood filters through the capillaries, a protein-free filtrate collecting in the capsular space between the capillaries and Bowman's capsule, whilst plasma proteins and cellular elements are retained in the lumen. The mesangium, consisting of mesangial cells and an extracellular matrix, is located in the central core (axial region) of the glomerulus, in contact with the lumen of the capillaries where they branch out from the afferent arteriole through which arterial blood enters the unit (for a review of the structure, see ref. [1]).

Proteoglycans in the glomerular basement membrane form the main charge and steric exclusion barriers to the trans-capillary passage of plasma proteins [1,2]. Proteoglycans are also associated with the plasma membranes of the glomerular epithelial [3] and endothelial cells [2] and probably contribute to the charge barrier at these

locations [2]. There is a loss of proteoglycans from the basement membrane and other glomerular sites in several conditions associated with proteinuria. These include human diabetic nephropathy [4,5], human congenital nephrotic syndrome [6], experimentally induced diabetes [7] and experimental autologous immune-complex disease [8]. Knowledge of the biosynthesis, structure and metabolism of glomerular proteoglycans is therefore likely to be important in understanding the molecular basis underlying the development of proteinuria in these disorders.

There have been several recent reports on the biosynthesis of glomerular proteoglycans [9–13], mainly based on experiments carried out *in vitro*. We report below on the development of a reliable method for investigating glomerular [³⁵S]proteoglycan synthesis *in vivo*. By perfusing kidneys with cetylpyridinium chloride *in vivo*, newly synthesized glomerular proteoglycans were fixed in the tissue and subsequently recovered in yields 2–3-fold greater than from unfixed kidneys. Comparison of the proteoglycans isolated from glomeruli which had been labelled with [³⁵S]sulphate *in vivo* with those from glomeruli labelled *in vitro* showed several striking differences as well as some similarities.

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METHODS

³⁵S-Labeling of glomerular proteoglycans

Female Wistar rats (~180 g body wt.) were injected twice, intraperitoneally, with 0.5 mCi/100 g body wt. of Na₂[³⁵S]SO₄ (300–600 mCi/mmol, NEN Research Products, Stevenage, Herts., U.K.). The injections (0.5 ml) were given at 3.5 h intervals. The animals were anaesthetized 3.5 h after the second injection. The kidneys were perfused with 10 ml of phosphate-buffered saline (Dulbecco's formula without calcium and magnesium, pH 7.2), followed by 0.01% (w/v) cetylpyridinium chloride in phosphate-buffered saline (50 ml) (solution A) at room temperature and at a maximum pressure of 213 kPa. The retrograde perfusion was carried out via a cannula inserted into the aorta just below the renal arteries. After perfusion the kidneys were removed and placed in solution A containing the proteinase inhibitors phenylmethanesulphonyl fluoride (0.001 M), *N*-ethylmaleimide (0.001 M), disodium-EDTA (0.01 M), benzamide hydrochloride (0.005 M) and 6-aminohexanoic acid (0.1 M) (solution B). Glomeruli were isolated from the kidneys by passing the renal cortex [14] through graded sieves (106, 180 and 53 µm mesh sizes; Endecotte, London, U.K.) with solution B at room temperature. Glomeruli retained on the smallest sieve were washed three times and resuspended in the same solution. After adequate mixing 50 µl of the suspension was taken up into a calibrated glass capillary tube for counting glomeruli under the phase contrast microscope.

For labelling experiments carried out *in vitro*, glomeruli were isolated from 18 kidneys under sterile conditions and cultured in Dulbecco's modified Eagle's Medium supplemented with fetal-calf serum (10% v/v), L-glutamine (0.002 M), transferrin (5 µg/ml), insulin (0.66 i.u./ml), penicillin (100 units/ml) and streptomycin (100 µg/ml), at 37 °C in a 19:1 air/CO₂ atmosphere. After 2–3 h the medium was replaced with 6 ml of fresh medium containing 10% of the normal MgSO₄ concentration together with 150 µCi of Na₂[³⁵S]SO₄/ml, and supplements, as above. The incubation was continued for 24 h. The medium was decanted after centrifuging, proteinase inhibitors added to it (final concns. as above) and stored at –20 °C.

Preparation and extraction of glomerular basement membranes

A glomerular basement-membrane fraction was isolated by osmotic lysis and detergent extraction of glomeruli [15,16]. Proteinase inhibitors were added for the lysis (as above) during the DNAase digestion (as above but without EDTA) and for the detergent extraction (as above but without EDTA and phenylmethanesulphonyl fluoride and with leupeptin, 100 µg/ml). The method was also modified by carrying out the DNAase digestion step in 1.5 M-KCl/0.005 M-MgCl₂/0.005 M-CaCl₂ (pH 7.8). The high concentration of KCl was used to dissociate cetylpyridinium–proteoglycan complexes [17]; in control experiments, it did not inhibit DNAase digestion of calf-thymus DNA. The supernatants from the osmotic lysis, DNAase digestion and deoxycholate extraction steps [15] were retained. The final basement-membrane preparation was washed twice with distilled water and then with 0.15 M-NaCl before extraction at 4 °C for 18 h by tumbling in 8.0 M-urea/0.05 M-Tris/HCl buffer (pH 8.6) containing protease

inhibitors (as above) [18]. The residue was recovered by centrifuging (15000 g, 30 min, 4 °C) and re-extracted with the same solution for a further 2 h. The supernatants were pooled. The urea-insoluble residue was extracted overnight with 4.0 M-guanidine hydrochloride/0.05 M-sodium phosphate buffer (pH 7.0) containing proteinase inhibitors at room temperature. The residue was re-extracted for a further 4 h with the same solution and the supernatants pooled. Any remaining residue was digested with papain and assayed for radioactivity (see below).

Measurement of ³⁵S-radioactivity in macromolecules

Portions of the supernatants obtained above and extracts of the glomerular basement-membrane fraction were chromatographed on prepacked columns of Sephadex G-25 (PD-10 columns; Pharmacia) to measure ³⁵S-labelled macromolecules [19]. A portion of whole glomeruli was digested with papain [17], then treated with 0.5 M-NaOH for 1 h at room temperature, and the ³⁵S-labelled glycosaminoglycans released were assayed for radioactivity as above.

Characterization of glomerular [³⁵S]proteoglycans

Supernatants, extracts or culture media, containing [³⁵S]proteoglycans were dialysed extensively against distilled water and then against 8.0 M-urea/0.05 M-sodium acetate/0.1% (v/v) Triton X-100/0.075 M-NaCl (pH 6.0) before fractionation on a DEAE–Sephacel column (7.0 mm × 40.0 mm) equilibrated with the same buffer, but containing 0.5% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate instead of Triton X-100 (equilibration buffer). The column was eluted with equilibration buffer (5 ml), equilibration buffer containing 0.15 M-NaCl (5 ml) and then with equilibration buffer and an 0.15–0.75 M-NaCl gradient (46 ml) at a flow rate of 2.4 ml/h. Fractions (0.8 ml) were collected throughout and their ³⁵S-radioactivity and conductivity measured.

The supernatant fraction from the deoxycholate extract of glomeruli (see above) was treated differently. The fraction was mixed with an equal volume of 8.0 M-urea equilibration buffer and tumbled overnight at 4 °C with 2 ml of DEAE–Sephacel gel. The mixture was then poured into a column and eluted as for the other fractions.

³⁵S-Labelled peaks eluting from the DEAE column were pooled, dialysed into distilled water, divided into two parts and freeze-dried. Each was resuspended in a minimum volume of water, or in 50 mM-Tris/HCl/60 mM-sodium acetate/0.01 M-NaF (pH 8.0) [20,21]. The aqueous portion was degraded with freshly prepared ice-cold HNO₃ (4:1, v/v) to remove heparan sulphate chains [22–24]. The reaction was terminated by neutralizing with 1.0 M-Na₂CO₃. The buffered portion was digested overnight with chondroitinase ABC (0.1 unit, 37 °C; Miles Biochemical Inc.) to remove chondroitin sulphate chains [20]. Proteinase inhibitors were not added to the digest, but as large heparan sulphate proteoglycans were isolated following chondroitinase ABC digestion, the enzyme did not appear to contain significant proteinase activity against proteoglycans. The digests were then chromatographed on a Sepharose CL-4B column (0.006 m × 1.5 m; Pharmacia) and eluted with 4.0 M-guanidine hydrochloride/0.05 M sodium acetate/0.1% Triton X-100 at a flow rate of 2.4 ml/h. Fractions of 0.8 ml were collected and monitored for radioactivity.

³H-Labelled hyaluronate (which had been purified from the medium of [³H]glucosamine-labelled cultures of chick-embryo chondrocytes) and [³H]acetate were added to each sample before chromatography and marked the excluded volume (V_0) and the total volume (V_t) of the CL-4B column, respectively.

Peaks eluting from the Sepharose CL-4B column were pooled separately and concentrated to 0.5 ml using Centricon microconcentrators (Amicon). Each was treated with 0.05 M-NaOH containing 1.0 M-NaBH₄ for 18 h at 45 °C and then neutralized with glacial acetic acid [25]. [³⁵S]Glycosaminoglycans released in this reaction were chromatographed on a Sepharose CL-6B column (0.006 m × 1.5 m) and eluted under the same conditions as those described for Sepharose CL-4B chromatography.

RESULTS

Effect of cetylpyridinium chloride perfusion on recovery of glomeruli and glomerular proteoglycans

Recoveries of glomeruli from kidneys perfused with phosphate-buffered saline did not differ significantly from non-perfused kidneys (Table 1). However, the recovery of glomeruli from kidneys perfused *in situ* with 0.01 % cetylpyridinium chloride was 20 % lower than for non-perfused kidneys. This lower recovery is likely to be due to slight shrinkage of the glomeruli, allowing the smallest to pass through the 53 µm diameter sieve on which the majority were collected. Perfusion with higher concentrations of cetylpyridinium chloride (> 0.01 %) caused visible distortion of the shape, and shrinkage of the glomeruli, and a much reduced recovery from the sieving procedure (results not shown). The glomerular fraction obtained from kidneys perfused with 0.01 % cetylpyridinium chloride had a normal appearance under the phase-contrast microscope and was free of other contaminating tissue structures.

When examined under the electron microscope basement-membrane preparations from cetylpyridinium chloride-perfused kidneys contained some acellular glomerular 'ghosts', still surrounded by a Bowman's capsule, together with isolated loops of basement membrane. Basement membrane preparations from phosphate-buffered saline-perfused kidneys contained only isolated loops of membrane. Some mesangial matrix and chromatin-like material was present in basement-membrane preparations from both sources, as reported by others [26].

The effect of perfusion of kidneys *in situ* on the recovery of ³⁵S-labelled proteoglycans in glomeruli isolated by the sieving procedure was assessed. Ten animals were labelled with [³⁵S]sulphate *in vivo* for 7 h. Then the rats were anaesthetized and the left kidney perfused with 0.01 % (w/v) cetylpyridinium chloride. Both kidneys were removed and their respective glomeruli isolated, counted, digested with papain, and the [³⁵S]-glycosaminoglycans present were estimated by PD-10 column chromatography. Glomeruli from the kidney which had been perfused with cetylpyridinium chloride contained approx. 2.6 times more [³⁵S]glycosaminoglycan than those isolated from non-perfused kidneys (Table 2). Silver grain counts of autoradiographs of 0.5 µm thick sections of perfused and non-perfused glomeruli showed approx. twice as many

Table 1. Glomeruli recovered from the kidney cortex after organ perfusion *in situ* with cetylpyridinium chloride

Kidneys were perfused *in vivo*, either with an 0.01 % (w/v) solution of cetylpyridinium chloride in phosphate-buffered saline or with phosphate-buffered saline alone, or were not perfused, before isolation of glomeruli from the renal cortex of each by a selective sieving procedure. Experimental details are given in the Methods section. *n*, Number of kidneys examined for each procedure.

Perfusion protocol	Glomeruli recovered per kidney (mean ± S.D.)	<i>n</i>
No perfusion	12446 ± 2467	27
Cetylpyridinium chloride	9772 ± 1823	42
Phosphate-buffered saline	14273 ± 1145	5

Table 2. Effect of renal perfusion *in situ* with cetylpyridinium chloride on recovery of [³⁵S]glycosaminoglycans in isolated glomeruli

Rats were labelled *in vivo* with Na₂[³⁵S]SO₄ as described in the text. Then, under anaesthesia, the left kidney was perfused *in situ* with phosphate-buffered saline followed by 0.01 % (w/v) cetylpyridinium chloride in phosphate-buffered saline. Glomeruli were isolated from the renal cortex of the right and left kidney individually and the radioactivity due to [³⁵S]glycosaminoglycans measured for each. *n*, Number of rats examined. The effect of cetylpyridinium chloride perfusion on recovery of glomerular [³⁵S]glycosaminoglycan (d.p.m.) was analysed with a paired *t*-test and is significant (*t* = 4.29, *P* < 0.01). For experimental details, see the Methods section.

Kidney	Glycosaminoglycan ³⁵ S-radioactivity (mean d.p.m./1000 glomeruli ± S.D.)	<i>n</i>
Perfused	2640 ± 1200	10
Non-perfused	1000 ± 360	10

grains per unit area in the former than in the latter (results not shown). Thus, in the absence of cetylpyridinium chloride fixation *in situ*, about 50–60 % of the total glomerular [³⁵S]proteoglycans are lost during isolation of the glomeruli.

Characterization of glomerular proteoglycans

Glomeruli which had been labelled with [³⁵S]sulphate *in vivo* for 7 h were isolated from kidneys perfused with either phosphate-buffered saline followed by cetylpyridinium chloride, or with phosphate-buffered saline alone. For comparison, glomeruli were also isolated and labelled *in vitro* for 24 h. Subsequently the isolation of a basement-membrane preparation from each set of glomeruli involved, in sequence, hypotonic lysis, DNAase digestion and deoxycholate extraction. The basement-membrane preparations obtained after this

Table 3. Release of ^{35}S -labelled macromolecules from glomeruli during isolation of the basement-membrane fraction

Glomeruli were labelled either *in vivo* (7 h), or *in vitro* (24 h), as described in the text. In experiments carried out *in vivo* the kidneys were perfused at the end of the labelling period with either cetylpyridinium chloride (CPC) or phosphate-buffered saline (PBS). A basement-membrane fraction was prepared from the glomeruli using a modification of the Meezan fractionation scheme. ^{35}S -Labelled macromolecules released into the supernatant at each stage of the fractionation were measured by PD-10 column chromatography. ^{35}S -Labelled macromolecules in the basement membrane were extracted sequentially with 8 M-urea and 4 M-guanidine hydrochloride; the percentage shown is the sum for both extracts. Finally the residue was digested with papain. ^{35}S -Labelled macromolecules released into the incubation medium of the glomeruli labelled *in vitro* were also estimated, and accounted for 19% of the total synthesized.

Fractionation stage	^{35}S -macromolecules released at each stage (% of total)		
	Glomeruli labelled <i>in vivo</i>		Glomeruli labelled <i>in vitro</i>
	PBS-perfused	CPC-perfused	Not perfused
Hypotonic lysis	15	0	2
DNAase digestion	39	58	39
Deoxycholate extract	42	20	27
Basement-membrane extracts	3	5	10
Basement-membrane residue	1	17	3

procedure were extracted sequentially with 8 M-urea/4 M-guanidine hydrochloride and finally digested with papain. Most of the glomerular ^{35}S -labelled macromolecules were solubilized during the early stages of the procedure (Table 3). Although only small amounts of ^{35}S -macromolecules were solubilized during lysis of glomeruli labelled *in vitro*, about 20% of the total, presumably the most soluble fraction, had already been released into the culture medium during the incubation period with [^{35}S]sulphate. About 60% of the total ^{35}S -macromolecules in the cetylpyridinium chloride-perfused glomeruli labelled *in vivo* were released during the DNAase digestion. This correlates with the increased salt concentration (1.5 M-KCl) introduced at this stage to dissociate cetylpyridinium chloride-polyanion complexes, allowing proteoglycans to escape from the tissue. This result, together with the absence of ^{35}S -macromolecules in the preceding lysis stage, provide further evidence of the efficient fixation of [^{35}S]proteoglycans in the tissue following renal perfusion with cetylpyridinium chloride *in vivo*.

The combined yields of sequential extract with urea and guanidine hydrochloride solubilized 17%, 68% and 77% of the ^{35}S -macromolecules from the basement-membrane preparations of cetylpyridinium chloride-perfused, phosphate-buffered saline-perfused, glomeruli labelled *in vitro*, respectively. The combined extracts of the basement-membrane preparation and the supernatants from the glomerular fractionation (Table 3), were chromatographed on DEAE-Sephacel columns to isolate the [^{35}S]proteoglycans present. Representative chromatograms are shown for ^{35}S -molecules released into the DNAase supernatants (Figs. 1a and 2a). Portions of each ^{35}S -labelled peak eluted from the column were digested with chondroitinase ABC, or subjected to deaminative cleavage with HNO_3 , before dissociative gel-permeation chromatography on a Sepharose CL-4B column to determine the composition and hydrodynamic size (K_{av}) of the constituent proteoglycans (Figs. 1b, 1c,

2b and 2c). The procedure was repeated for all the glomerular fractions containing ^{35}S -macromolecules. From the results it was calculated that 85% of the extractable [^{35}S]proteoglycans synthesized *in vivo* were of the heparan sulphate type and 15% were the chondroitin sulphate type, whereas only 35% were heparan sulphate and 65% chondroitin sulphate *in vitro*.

Figs. 1 and 2 indicate the presence of several classes of ^{35}S -labelled heparan sulphate-containing molecules in glomeruli, distinguishable by differences in charge and hydrodynamic size. Combining results from all the fractionations, three classes of different size (HS1-HS3) were detected after labelling *in vivo* (Table 4). The HS1 variety are heterogeneous in charge. HS1 isolated from the basement membrane accounted for 31% of this class, and, therefore, by extrapolation, for 11.6% of the total extractable glomerular ^{35}S -labelled proteoglycans. HS2 and HS3 were extracted from the glomeruli during the DNAase digestion. The K_{av} of HS3 is compatible with it being a single heparan sulphate chain, but this was not confirmed because of the low amount of radioactivity available. The same three classes of heparan sulphate proteoglycans were isolated from glomeruli labelled *in vivo* which had been perfused with phosphate-buffered saline alone, but in a ratio of 50:37:13 (HS1/HS2/HS3). Recovery of HS1 and HS2 from unfixed glomeruli was decreased by 50% or more, whilst recovery of HS3 was even lower. In the absence of cetylpyridinium chloride perfusion only about 3% of the total extractable ^{35}S -labelled heparan sulphate proteoglycans were found associated with the glomerular basement-membrane fraction.

The heparan sulphate-containing molecules isolated from glomeruli labelled *in vitro* (Table 5) have both similarities with, and differences from, those detected *in vivo*. A large, highly charged molecule, HS1(a), extracted from the basement-membrane fraction (Table 5), was only detected in experiments carried out *in vitro*. HS1 and HS2, isolated from glomeruli labelled *in vitro*,

Fig. 1. Fractionation of [^{35}S]proteoglycans released from ^{35}S -labelled glomeruli *in vivo* during digestion with DNAase

A labelling experiment was carried out *in vivo* as described in the text. After perfusion-fixation of the kidneys with cetylpyridinium chloride, ^{35}S -labelled glomeruli were isolated and subjected to hypotonic lysis, DNAase digestion and detergent extraction, to obtain a basement-membrane preparation. ^{35}S -macromolecules released at each stage were analysed. The Figure shows (a) the DEAE-Sephacel ion-exchange chromatography profile of ^{35}S -labelled molecules released during the DNAase digestion. A linear NaCl gradient was used to elute the chromatography column (----). Fractions containing peaks I and II (Pk I and Pk II) were pooled as indicated by the bars (—). Molarities (M) refer to the concentrations of NaCl at which Pk I and Pk II were eluted. Pk I and Pk II were digested with chondroitinase ABC before chromatography on a dissociative Sepharose CL-4B column (panels b and c respectively). Hatched areas of the chromatograms correspond to the elution position of chondroitinase ABC digestion products. V_0 and V_t refer to the elution positions of [^3H]hyaluronate and [^3H]acetate included in each run, and mark the positions of the excluded and total volume of the column respectively. Numbers show the K_{av} value for each peak calculated using the V_0 and V_t values.

had properties which were indistinguishable from their counterparts in experiments carried out *in vivo*, but they were present in very different proportions in the latter (Tables 4 and 5). A small heparan sulphate was also isolated from glomeruli labelled *in vitro*. It was classified as HS3' to distinguish it from the small molecule found *in vivo* (HS3), which has a much lower charge (Tables 4 and 5).

The average size of heparan sulphate chains was investigated after alkaline-borohydride cleavage of HS2 and HS3(a) from the medium and DNAase extracts of glomeruli labelled *in vitro*. The K_{av} values (Sepharose CL-6B) were 0.60 for HS2 chains and 0.58 for HS3' chains (results not shown).

The amount of radioactivity incorporated into chondroitin sulphate proteoglycans *in vivo* was insufficient to

Fig. 2. Fractionation of [^{35}S]proteoglycans released from labelled glomeruli *in vitro* during digestion with DNAase

A labelling experiment was carried out *in vitro* as described in the text. The ^{35}S -labelled glomeruli were subjected to hypotonic lysis, DNAase digestion and detergent extraction to obtain a basement-membrane preparation. ^{35}S -macromolecules released at each stage were analysed. For other details see the legend to Fig. 1 and the text.

allow their further characterization, but this was possible for experiments carried out *in vitro* (Table 6). A variety of molecules were present, with one group, CS2, being the major group. However, this group is heterogeneous since the K_{av} (Sepharose CL-6B) of chondroitin sulphate chains cleaved from DNAase and deoxycholate-extracted CS2 (by alkaline-borohydride treatment) was 0.47, whilst that for medium-fraction CS2 chains, was 0.60. CS1 was the main chondroitin sulphate proteoglycan extracted from the basement-membrane fraction and has a large hydrodynamic size and high charge. A small amount of CS3, with quite different properties to CS1, was also extracted from the basement-membrane preparation as well as being present in other fractions (Table 6). Chains of both the medium and DNAase extract, CS3, had a K_{av} (Sepharose CL-6B) of 0.60 after alkaline-borohydride treatment. CS4 has the same charge as CS2, but is of much smaller size. Moreover, after alkaline elimination, CS4 chains had the same K_{av} (Sepharose 6B), 0.47, as those of DNAase- and deoxycholate-extracted CS2, suggesting that it may be derived from the

Table 4. Glomerular heparan sulphate proteoglycans detected after labelling *in vivo* and renal perfusion *in situ* with cetylpyridinium chloride

Rats were labelled *in vivo* (7 h) after which their kidneys were perfused *in situ* with cetylpyridinium chloride. Glomeruli were isolated from the renal cortex by sieving and subjected to hypotonic lysis, DNAase digestion and deoxycholate extraction, to obtain the basement-membrane fraction. The latter was extracted with urea and guanidine hydrochloride. The glomerular heparan [³⁵S]sulphate proteoglycans solubilized at each stage were isolated and characterized by DEAE-Sephacel chromatography, chondroitinase ABC digestion and dissociative Sepharose CL-4B chromatography. K_{av} refers to elution position from CL-4B. Charge refers to the NaCl concn. (M) required to displace the proteoglycan from DEAE-Sephacel. Abbreviation: GBM, glomerular basement membrane.

Proteoglycan	K_{av}	Charge	Fraction in which present	Distribution (%)	Glomerular [³⁵ S]heparan sulphate (% of total extractable)
HS1	0.47	0.42–0.51	DNAase	39	–
			Deoxycholate	29	44
			GBM extract	31	–
HS2	0.56	0.47	DNAase	100	27
HS3	0.68	0.39	DNAase	100	28

Table 5. Glomerular heparan sulphate proteoglycans detected after labelling *in vitro*

Isolated glomeruli were labelled *in vitro* (24 h) and subjected to hypotonic lysis, DNAase digestion and extraction with deoxycholate, to obtain the insoluble basement-membrane fraction. Medium refers to [³⁵S]proteoglycans secreted into the tissue-culture medium during the labelling period. For other details see the legend to Table 4.

Proteoglycan	K_{av}	Charge	Fraction in which present	Distribution (%)	Glomerular [³⁵ S]heparan sulphate (% of total extractable)
HS1(a)	0.33–0.40	0.71	GBM	100	6
HS1	0.48	0.45–0.55	Lysis	100	2
HS2	0.53–0.55	0.43	Medium	41	–
			Lysis	7	–
			DNAase	27	64
			Deoxycholate	15	–
			GBM extract	9	–
HS3'	0.61–0.67	0.58	Medium	33	28
			DNAase	67	–

Table 6. Glomerular chondroitin sulphate proteoglycans detected after labelling *in vitro*

The chondroitin sulphate proteoglycans were isolated by Sepharose CL-4B chromatography of HNO₂-treated peaks from DEAE-Sephacel columns. Other details are given in the legends to Tables 4 and 5 and in the text.

Proteoglycan	K_{av}	Charge	Fraction in which present	Distribution (%)	Glomerular [³⁵ S]chondroitin sulphate (% of total extractable)
CS1	0.3	0.71	GBM extract	100	8
CS2	0.3	0.56–0.60	Medium	48	–
			DNAase	31	56
			Deoxycholate	21	–
CS3	0.50–0.55	0.43	Medium	58	–
			Lysis	2	18
			DNAase	29	–
			GBM extract	11	–
CS4	0.60	0.58	DNAase	100	12
CS5	0.69	0.43	Deoxycholate	100	6

latter. Similarly, CS5 has the same charge as CS3 (Table 6) and both have the same chain size (K_{av} , Sepharose 6B, 0.60), suggesting that one is a product of the other.

DISCUSSION

Previous investigations on the biosynthesis of glomerular [^{35}S]proteoglycans have utilized techniques of labelling *in vivo* [9,10,13,27], organ perfusion *ex situ* with medium containing [^{35}S]sulphate [7,11,28], or incubation of isolated glomeruli with [^{35}S]sulphate *in vitro* [10,13,29]. Experiments based on labelling *in vivo* or organ perfusion *ex situ* involved subsequent isolation of glomeruli by the well-established sieving technique. Our studies show that if the glomerular proteoglycans are not fixed by perfusion of the kidney *in situ* following the labelling period, up to 60% of newly synthesized glomerular proteoglycans are lost during sieving. Previous characterization studies have therefore been based on only about half of the total population of ^{35}S -labelled glomerular proteoglycans.

Whilst investigations based on techniques of labelling *in vitro* do not suffer from recovery problems, it is clear from our results and from those of others [10,12,13], that the pattern of proteoglycan synthesis in isolated glomeruli maintained in tissue culture is very different from that *in vivo*. Isolated glomeruli synthesize predominantly chondroitin/dermatan sulphate-type proteoglycans (65% of the total in our experiments) whilst heparan sulphate proteoglycans account for 85% of total synthesis *in vivo*. Chondroitin/dermatan sulphate-type proteoglycans are either mainly, or exclusively, a product of mesangial cells [28] so the pattern of synthesis *in vitro* may reflect the preferential survival or activation of this cell-type in cultures of isolated glomeruli. Thus, whilst experiments carried out *in vitro* may be useful in special circumstances, we conclude that experiments carried out *in vivo* are required to assess the physiological pattern of glomerular synthesis and that these should be coupled with fixation *in situ* with cetylpyridinium chloride, or similar agent, to ensure complete recovery of labelled molecules.

Perfusion of the kidney *in situ* with Karnovsky's aldehyde fixative was used for quantitative electron-microscopic autoradiography of glomerular basement-membrane proteoglycans [30]. Cetylpyridinium chloride is a convenient fixative for chemical investigations since, as used here, it does not interfere with the isolation of intact glomeruli and can be readily dissociated from the proteoglycans subsequently [17]. Dissociation was achieved in our experiments by raising the concentration of KCl in the DNAase digestion buffer to 1.5 M [17], but it may be carried out at a later stage during the isolation of glomerular basement membrane if desired.

In our experiments carried out *in vivo* the labelling protocol was designed to achieve maximal labelling of proteoglycans within a relatively short period. Peak blood radioactivity was attained approx. 30 min after injecting [^{35}S]sulphate intraperitoneally, and fell to 50% of maximal levels, 3.5 h after the injection. Thus to achieve optimal blood levels of radioactivity each animal received two injections, 3.5 h apart, and was killed after a further 3.5 h. After this labelling period [^{35}S]HS1 proteoglycans (K_{av} , 0.47; Sepharose CL-4B/4 M-guanidine hydrochloride) are the major class found in the glomerulus, of which about 30% of those extracted were associated with the glomerular basement-membrane

fraction. Kanwar *et al.* [11] extracted a heparan [^{35}S]sulphate proteoglycan from rat glomerular basement membrane which had a K_{av} of 0.45 (Sepharose CL-4B/4 M-guanidine hydrochloride) after kidney perfusion *in vitro* with [^{35}S]sulphate for 4–5 h. There is agreement therefore that in experiments both *in vivo* and *in vitro*, a heparan sulphate proteoglycan of about this hydrodynamic size, with an estimated molecular mass of 130 000 Da using glycosaminoglycan standards [11], is the major glomerular basement-membrane proteoglycan synthesized in the rat. Parthasarathy & Spiro [31] characterized chemically the heparan sulphate proteoglycan extracted from bovine glomerular basement membrane and concluded that it consisted of a cluster of four heparan sulphate chains linked to one end of a core protein with an overall molecular mass of 200 000 Da. Whether the variation in size of the molecule between species is real, or due to methodological differences between laboratories, is unknown.

HS1 proteoglycans of the same size as those isolated from the basement-membrane fraction were also released during the DNAase digestion and the deoxycholate extraction of glomeruli (Table 4). They may be associated with the plasma membranes of glomerular cells or with the mesangial matrix. There is evidence indicating that the heparan sulphate proteoglycans of the glomerular basement membrane are immunologically distinct from those located in other parts of the glomerulus [3,32]. HS2 and HS3 may also be located at sites in the glomerulus distinct from the basement membrane. The present results do not indicate whether or not HS2 is derived from processing of one or other HS1 molecules, or whether HS3 is derived from HS1 or HS2.

The experiments carried out *in vitro* (Tables 5 and 6) demonstrate the different state of proteoglycan synthesis in cultured glomeruli compared with the state *in vivo*. Most of the proteoglycans synthesized in our experiments carried out *in vitro* have properties which are very similar to those described recently by Klein *et al.* [13] for cultures of isolated rat glomeruli. Thus our HS2 (glomerular), HS2 (culture medium), CS3 (glomerular) and CS2 (culture medium) have almost identical properties to proteoglycans HSt1A, HSm1, DSt1A and DSMII, reported by Klein *et al.* [13], when you compare (a) their elution positions from DEAE-Sephacel columns and (b) the K_{av} of their glycosaminoglycan chains on Sepharose CL-6B. However, these authors did not report the highly charged heparan sulphate (HS1a; Table 5) or chondroitin sulphate (CS1; Table 6) proteoglycans which were synthesized by glomeruli in our experiments carried out *in vitro*. Synthesis of a highly sulphated chondroitin sulphate by isolated glomeruli has been observed by Kobayashi *et al.* [33] and the molecule appears to be the product of mesangial cells [34]. Mesangial matrix is a contaminant of the glomerular basement-membrane fraction (see above) from which CS1 was extracted in our experiments. HS1(a) was also extracted from this fraction. Whether it is a component of the basement membrane, or a precursor of other basement-membrane heparan sulphate proteoglycans, is not known at present. Neither can we rule out the possibility that it is synthesized as an artefact of experimental conditions *in vitro*.

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