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# Characterization of heparan sulfate-proteoglycan of glomerular basement membranes

(sulfated proteoglycans/autoradiography/sedimentation velocity centrifugation)

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**ABSTRACT** **Native and *de novo* synthesized heparan sulfate-proteoglycan (HS-PG) of basement membranes from isolated whole glomeruli were characterized. Sepharose CL-6B chromatograms of [$^{35}$S]sulfate-labeled *de novo* synthesized HS-PG extracted from whole glomeruli indicated identical molecular weight characteristics to that isolated from purified basement membranes ($M_r$ of intact HS-PG ≈ 130,000; $M_r$ of chains ≈ 25,000). Electron microscopic autoradiography showed that almost all radioactive grains were localized to the basement membranes proper. The estimated $M_r$ of core protein ≈ 18,000. The sedimentation coefficient of native intact HS-PG was 5.56 S, corresponding to a $M_r$ between 150,000–250,000, a value in accord with gel filtration data on newly synthesized HS-PG. Physicochemical characteristics of HS-PG of native functional basement membranes differed remarkably from that isolated from the basement membrane-producing tumor, Engelbreth-Holm–Swarm sarcoma.**

Recently, heparan sulfate (HS)—a regulator of glomerular permselectivity (1–3)—has been shown to be an integral component of basement membranes[§] (4–6), and initial biosynthetic studies indicate that the *de novo* synthesized HS exists in a macromolecular form (7). In this communication we describe the characterization of newly synthesized and existing HS-proteoglycan (HS-PG) of the basement membranes of the glomerulus. The results of this study indicate that both the *de novo* synthesized and native HS-PG of basement membranes are present in macromolecular forms, and, further, that the molecular characteristics of the intact PG, its chains, and the core protein significantly differ from HS-PG isolated from the basement membrane-producing tumor, Engelbreth-Holm–Swarm (EHS) sarcoma (8).

## METHODS

**Radiolabeling, Isolation, and Characterization of Glomerular PGs.** Radiolabeling of GBMs with [$^{35}$S]sulfate (Amersham) was carried out under sterile conditions in an *ex situ* organ perfusion apparatus (7). Satisfactory labeling was achieved by constantly recirculating for 7–8 hr a chemically defined medium (7) containing [$^{35}$S]sulfate (500 μCi/ml; 1 Ci = 37 GBq) of high specific activity (>1,300 Ci/mmol) through isolated rat kidneys via their renal arteries. The labeled kidneys were processed as follows: (*i*) for biochemical characterization of PGs, the kidneys were perfused with Krebs–Ringer bicarbonate buffer containing 1% bovine serum albumin and protease inhibitors (7), after which glomeruli were isolated and utilized for extraction of PGs as described (7); and (*ii*) for *in situ* localization and characterization of HS-PG, the kidneys were perfused either with purified heparitinase (gift from A. Linker) or chondroitinase ABC (Miles) or the appropriate buffer controls under the conditions previously used (4), and the tissue was processed for electron microscopic autoradiography (9) to delineate the source of radiation.

Radiolabeled PGs were extracted from whole glomeruli to increase the yield of the PGs and also to ascertain whether PGs so extracted have the same biochemical characteristics as those obtained from purified basement membranes. This was necessary because extensive breakdown of PGs, as evidenced by variable chromatographic elution profiles, was observed during detergent or sonication treatment—the two traditional methods (10, 11) used in purification of basement membranes.

Radiolabeled PGs and their D1–D5 fractions, obtained from CsCl gradient centrifugation (7), were treated either with nitrous acid or chondroitinase ABC and characterized by chromatographing them on Sepharose CL-6B and eluting with a solution containing 4 M guanidine·HCl, 0.1 M Tris·HCl, 0.1 M $Na_2SO_4$, and 0.5% (vol/vol) Triton X-100 at pH 7.0 (7).

**Isolation and Characterization of Nonradiolabeled Glomerular PGs.** Male Charles River Breeding Laboratories CD rats weighing 150–200 g were used. Under ether anesthesia, both the kidneys were perfused with cold Krebs–Ringer buffer containing 1% bovine serum albumin and protease inhibitors through the retrograde aortic route (12). The cortices were dissected free, and glomeruli were isolated in the presence of protease inhibitors by the method of Krakower and Greenspon (11). PGs were extracted from glomeruli with 4 M guanidine·HCl containing a mixture of protease inhibitors (7). Approximately 100 kidneys were needed to obtain a sufficient amount of PGs for these studies. All procedures were carried out at 0–4°C. Extracted PGs were fractionated by centrifugation (35,000 rpm for 72 hr at 8°C) on a CsCl gradient with an initial density of 1.42 g/ml (7). Five equal fractions (D1–D5) were collected and extensively dialyzed against distilled water at 4°C. The D1 fraction containing most of the HS-PG was utilized for amino acid analysis and molecular weight determination of both the intact PG and its core protein.

Material for determination of the molecular weight of core protein was prepared from the aliquots of the D1 fraction treated either with nitrous acid or purified heparitinase to remove the chains. Subsequently, intact PG and the core-protein preparations were chromatographed on Sepharose CL-6B. In addition, they were also subjected to $NaDodSO_4$/polyacrylamide gel electrophoresis either on 5% or 10%

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Abbreviations: HS, heparan sulfate; HS-PG, HS–proteoglycan; GBM, glomerular basement membrane; EHS sarcoma, Engelbreth-Holm–Swarm sarcoma.

[§]For simplicity's sake we have used the terminology basement membranes to include both the basement membrane proper glomerular basement membrane (GBM) and the basement membrane-like material—i.e., mesangial matrix of the glomerulus.

polyacrylamide slab gels with Tris glycine buffer, pH 8.2 (13). Slab gels were stained with Coomassie brilliant blue and destained with acetic acid/methanol solution.

Preliminary physicochemical characterization of the intact native HS-PG was carried out by measuring the sedimentation velocity and viscosity in 4 M guanidine·HCl at a low concentration of HS-PG. The objective was to estimate the order of magnitude of these parameters and the molecular weight, and to compare with the value for the *de novo* synthesized HS-PG derived from chromatographic analysis. A definitive concentration dependence study has yet to be carried out.

A portion of D1 fraction (HS-PG) in CsCl/4 M guanidine·HCl was dialyzed directly against 4 M guanidine·HCl. The dialyzed fraction (≈4.0 mg/ml) was loaded into a double-sector ultracentrifuge cell with the dialysate in the solvent sector. Sedimentation was then followed with an AnH rotor in a Spinco model E ultracentrifuge at 56,000 rpm at 20°C (14). A slowly migrating single hypersharp boundary was observed. Boundary positions were measured, with a Nikon microcomparator, from schlieren photographs taken during a 2-hr run.

Viscosity measurements were carried out with a Cannon–Ubblehode suspended level viscometer, with a flow time of ≈140 sec for 4 M guanidine·HCl. The viscosity was measured at several concentrations from 0.1 to 2.0 mg/ml. The reduced viscosity showed little concentration dependence. Because of the low value of the viscosity, a number of independent measurements at 2.0 mg/ml were made and averaged.

## RESULTS

**Characterization of Radiolabeled Glomerular PGs.** PGs extracted from the radiolabeled glomeruli had a yield ≈50 times (≈2 × $10^6$ dpm per kidney; efficiency of extraction, ≈94%) greater than that from purified basement membranes (≈4 × $10^4$ dpm per kidney; ref. 7). Sepharose CL-6B chromatograms revealed that the molecular characteristics (Fig. 1*A*) of the intact PGs ($K_{avg}$, 0.24; $M_r$, ≈130,000) and their chains ($K_{avg}$, 0.45; $M_r$, ≈25,000) were identical to those previously described (7), indicating that the HS-PG extracted from the whole glomeruli has an identical macromolecular form as that extracted from isolated GMBs. Furthermore, autoradiographic data indicated that almost all of the reduced silver grains (source of radiation) were associated with basement membranes (Fig. 2*A*). The technical details for the delineation of the source of radiation have been reported previously (9).

Relative levels of *de novo* synthesized PGs extracted from the whole glomeruli indicated that there was an enrichment of HS-PG because after nitrous acid treatment, ≈95% of the radioactivity was released into the $V_t$ fraction (Fig. 1*B*) as compared to ≈85% obtained previously (7). Further enrichment of HS-PG was observed in the D1 fraction of CsCl gradient, where ≈67% of the HS-PG was recovered (Table 1). Similarly, tissue autoradiography revealed that treatment with purified heparitinase removed almost all of the grains (≈95%) from the basement membranes (Fig. 2*B*) and that residual grains could be eliminated by further treatment with chondroitinase ABC (not shown). The data obtained validate the fact that PGs extracted from the whole glomeruli represent integral components of the basement membranes and are enriched in the D1 fraction of the CsCl gradient. Therefore, a preparative D1 fraction enriched with HS-PG can be obtained from a large-scale preparation of nonradiolabeled glomeruli and utilized for further characterization of HS-PG.

**Characterization of D1 Fraction PGs Extracted from Nonradiolabeled Glomeruli.** Amino acid analysis of the D1 fraction (Table 2) revealed a high content of lysine and alanine and a low content of threonine, serine, glutamic acid, proline, and valine when compared to the BM-1 fraction of PG isolated from EHS sarcoma. Only a trace level content (less than one residue per molecule) of cysteine (measured as cystine plus cysteic acid in our system) was detected in the HS-PG. Together with the high efficiency of ionic extraction (≈94%), this indicates that the HS-PG is not crosslinked through disulfide bridges to other GPs or type IV collagen. Glucosamine was present in significant amounts, and galactosamine was not detected in the D1 fraction.

Sepharose CL-6B chromatograms of D1 fraction revealed a $K_{avg}$ value of ≈0.23, which indicated that intact native and *de novo* synthesized PGs have similar molecular weights (Fig. 3). After nitrous acid treatment, the $K_{avg}$ value increased to ≈0.47, meaning that the core protein has a low molecular weight (Fig. 3).

$NaDodSO_4$/polyacrylamide gel electrophoresis of D1 fraction revealed a broad band with a $M_r$ of 130,000–150,000 (Fig. 4). After heparitinase digestion of the D1 fraction, a single band with a $M_r$ ≈18,000 was observed (Fig. 5). Similarly, a single band with a $M_r$ ≈18,000 was seen when the nitrous acid-treated D1 fraction was subjected to electrophoresis (Fig. 5). The estimated $M_r$ of the core protein of HS-PG isolated from EHS sarcoma is ≈250,000 (8). Taken together, the amino acid composition and the molecular weights suggest that these two proteins are quite dissimilar.

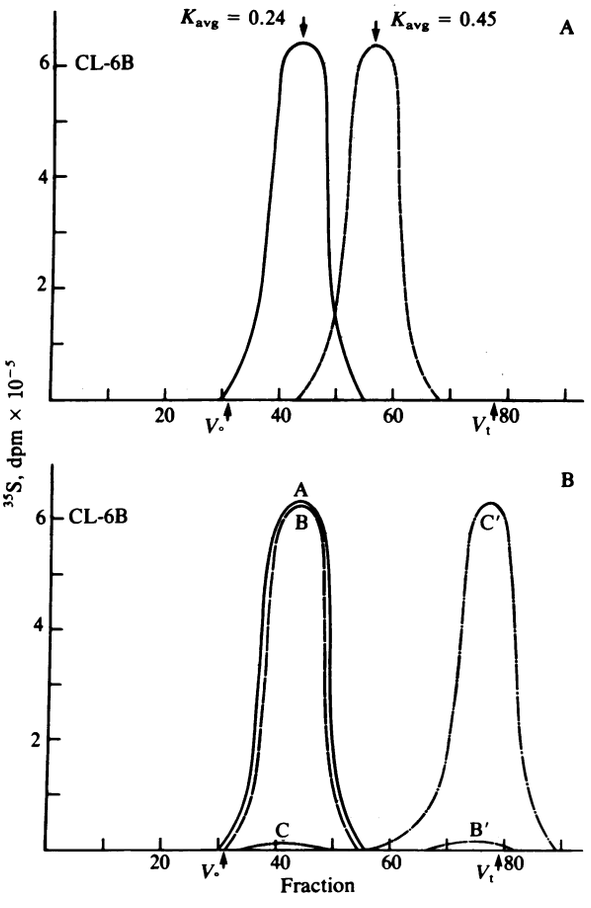
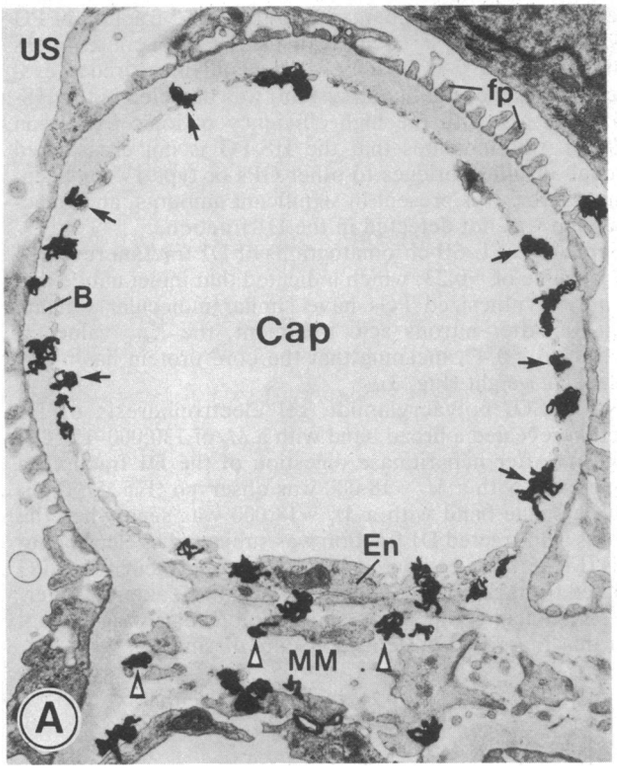



FIG. 1. (*A*) Sepharose CL-6B chromatograms of radiolabeled intact PGs (—) and glycosaminoglycan chains (---) released after alkaline treatment. With the alkaline treatment, the $K_{avg}$ increased from 0.24 ($M_r$ ≈130,000) to 0.45 ($M_r$ ≈25,000), indicating that the *de novo* synthesized HS-PG has 4–5 glycosaminoglycan chains attached to its core protein. (*B*) Sepharose CL-6B chromatograms of *de novo* synthesized PG either intact (*A*) or after treatment with chondroitinase ABC (*B*) or nitrous acid (*C*). Most of the newly synthesized PG is HS-PG because the majority of the counts are released into the $V_t$ fraction after nitrous acid treatment.



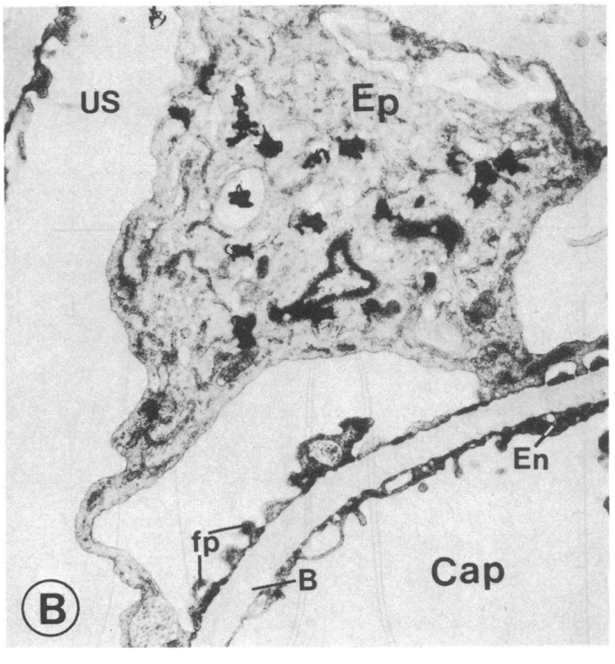




FIG. 2. Electron microscopic autoradiograms of the glomerular capillary loops (Cap) from the kidneys, treated (*B*) as well as untreated (*A*), perfused with [$^{35}$S]sulfate to label the *de novo* synthesized PGs. (*A*) Almost all of the grains are associated either with the basement membrane (B) or basement membrane-like material—i.e., mesangial matrix (MM). (*B*) After treatment with heparitinase, all the grains associated with basement membranes are lost, indicating that the majority of the [$^{35}$S]sulfate was probably incorporated into HS-PG. The grains present in the cytoplasm of the visceral epithelium (Ep) are unaffected by the heparitinase treatment because the enzyme does not have access to the intracellular compartment. US, urinary space; fp, foot process; En, endothelium. (×14,250.)

**Physical Characterization of D1 Fraction PGs.** The small quantity of PG available thus far precluded an extensive physicochemical study. Nevertheless, a reasonable approximation of the molecular weight of the intact HS-PG could be made. In 4 M guanidine·HCl, the intact PG sedimented as a single hypersharp boundry (Fig. 6) with a sedimentation coefficient, $s_{20,0.4\%}$, of 5.56 S. This is a considerably lower value when compared with those obtained for the bovine nasal septum cartilage PG subunit: 21.3 S for $M_r$ = 2,300,000 (14) and 28 S for $M_r$ = 2,990,000 (15) in 4 M guanidine·HCl.

The average reduced viscosity, as determined from three independent measurements was 50 ± 8 ml/g, also much lower than the values reported for the cartilage PG subunit in 4 M guanidine·HCl: 141 ml/g (14) and 131 ml/g (15).

Pasternak *et al.* (14) and Hascall and Sajdera (15) examined the concentration dependence of reduced viscosity for the $M_r \approx 2{,}500{,}000$ cartilage PGs and found that, in 4 M guanidine·HCl, a value for reduced viscosity at 2 mg/ml was not more than 10% higher than the intrinsic viscosity. Thus, the value is in error on the high side. Similarly, the concentration dependence of the sedimentation coefficient for cartilage PG subunit in 4 M guanidine·HCl is small, but $s$ increases with dilution, hence the value reported for $s$ at 4 mg/ml would be too small. By using the relationship of Creeth and Knight (16) with $K_s$ = 1.6 (15), where $K_s$ is the concentration-dependence coefficient and the reciprocal sedimentation coefficient is

$$1/s = (1/s^\circ)(1 + K_sC),$$

then $s^\circ$ would be on the order of 7.3 S for the HS-PG. Hascall and Sajdera (15) demonstrated that the generalized Scheraga–Mandelkern equation relating molecular weight, intrinsic viscosity, and sedimentation coefficient was valid as an approximation of average molecular weight. In this equation:

$$\frac{M^{2/3}}{s^\circ[\eta]^{1/3}} = \frac{N\eta_\circ}{\beta(1 - \bar{v}\rho)}$$

$N$ is Avogadro's number; $\eta_\circ$, the solvent viscosity in poises; $\beta$, a universal constant ($2.12 \times 10^6$); $\bar{v}$, the partial specific volume (≈0.55 ml/g); and $\rho$, the density of solvent. The term $[\eta]$ is the intrinsic viscosity in dl/g and $s^\circ$ is the sedimentation coefficient at infinite dilution. By applying the appropriate values, $M^{2/3} = 6.31 \times 10^{15}\, s^\circ[\eta]^{1/3}$. By inserting the estimate $s^\circ$ = 7.3, $[\eta]$ = 50 ml/g, the estimated molecular weight

Table 1. Distribution of *de novo* synthesized radiolabeled intact glomerular PGs in a dissociative gradient

| Fraction | Density, g/ml | % of total dpm | HS-PG, % of total dpm | CS-PG, % of total dpm | $K_{avg}$* |
|---|---|---|---|---|---|
| D1 | 1.56 | 67 | 67 | 0 | 0.24 |
| D2 | 1.48 | 13 | 13 | 0 | 0.24 |
| D3 | 1.40 | 7 | 7 | 0 | 0.25 |
| D4 | 1.33 | 5 | 4 | 1 | 0.26 |
| D5 | 1.28 | 8 | 6 | 2 | 0.26 |

Glomeruli from [$^{35}$S]sulfate-labeled kidneys were isolated and extracted with 4 M guanidine·HCl. The extract was fractionated by centrifugation (35,000 rpm for 72 hr) on a CsCl gradient with an initial density of 1.42 g/ml. Five equal fractions (D1–D5) were collected and subjected to treatment with nitrous acid to identify HS-PG and with chondroitinase ABC to identify chondroitin sulfate-PG (CS-PG). A total of ≈2 × $10^6$ dpm were obtained from the extract of the glomeruli isolated from one kidney with an efficiency of extraction of ≈94%.

*From Sepharose CL-6B chromatograms.

Table 2. Amino acid composition of HS-PG*

| Amino acid | D1, GBM | EHS sarcoma† |
|---|---|---|
| Lysine | 99 | 29 |
| Histidine | 24 | 32 |
| Arginine | 68 | 54 |
| Aspartic acid | 92 | 81 |
| Threonine | 47 | 68 |
| Serine | 64 | 84 |
| Glutamic acid | 115 | 154 |
| Proline | 56 | 81 |
| Glycine | 101 | 106 |
| Alanine | 97 | 70 |
| Valine | 37 | 63 |
| Methionine | 11 | 13 |
| Isoleucine | 21 | 26 |
| Leucine | 80 | 81 |
| Tyrosine | 28 | 24 |
| Phenylalanine | 31 | 32 |

*Residues per 1,000 amino acids.
†From ref. 8.

has the approximate value of 200,000. Considering all of the approximations, a range from 150,000–250,000 would be a conservative estimate of the size of HS-PG, far smaller than the value for cartilage PGs and less than one-third of the EHS sarcoma PGs.

## DISCUSSION

The results of this investigation indicate that: (*i*) *de novo* synthesized HS-PG extracted from the whole glomeruli is identical to that of the purified basement membranes (7), (*ii*) both newly synthesized and native HS-PG exist in macromolecular forms, (*iii*) both types of HS-PG have low molecular weights ($M_r \approx 130,000$) in contrast to the high molecular weight of HS-PG isolated from EHS sarcoma (8), and (*iv*) the HS-PG differs from the high molecular weight fraction enriched with hexuronic acid and containing disulfide bonds bridged to type IV collagen as described by Levine and Spiro (17).

HS-PG isolated from EHS sarcoma exhibited molecular weights for intact, core protein, and chains as ≈750,000, ≈250,000, and ≈70,000, respectively. These values differ significantly from HS-PG of native basement membranes, which were ≈130,000–150,000, ≈18,000, and ≈25,000, respectively. This low value for the molecular weight of core protein cannot be due to a proteoglytic fragment of a presumably larger size PG because procedures of isolation and characterization were carried out at 4°C and in the presence of protease inhibitors. Further, proteolytic degradation,

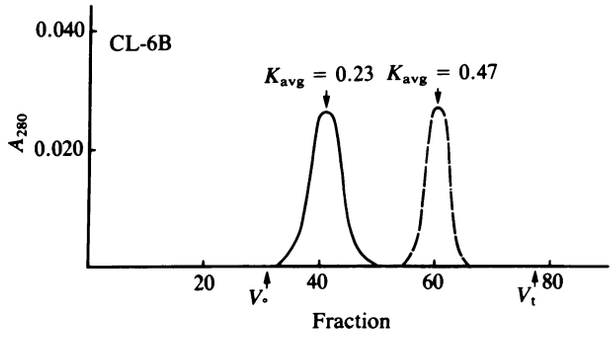



FIG. 3. Sepharose CL-6B chromatograms of nonradiolabeled intact PG (—) and core protein (---) obtained after nitrous acid treatment. The intact PG has a $K_{avg}$ value of ≈0.23, indicating that its molecular weight is similar to that of *de novo* synthesized PGs. The core protein hs a $K_{avg}$ value of ≈0.47, meaning that it has a low molecular weight.

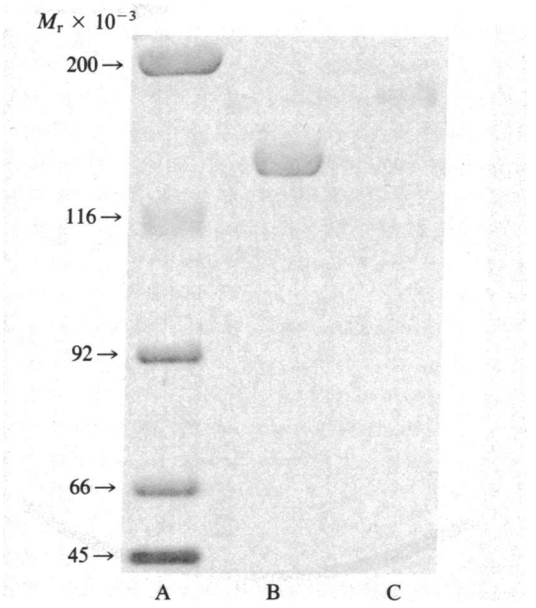



FIG. 4. NaDodSO$_4$/polyacrylamide gel (5%) electrophoresis. Lanes: A, molecular weight standards; B, intact PG; and C, heparitinase control. In lane B, a broad band of $M_r$ = 130,000–150,000 for the intact PG is observed.

which usually occurs during preparation of purified basement membranes, was circumvented by isolating the HS-PG from whole glomeruli.

It is conceivable that the low molecular weight characteristics of HS-PG isolated from GBM are shared by native basement membranes throughout the mammalian kingdom. Therefore, the high molecular weight HS-PG isolated from the basement membrane-producing tumor, EHS sarcoma, may not be truly representative of PGs present in native basement membranes. Although there is an immunologic crossreactivity between the HS-PG of EHS sarcoma and connective tissue basement membranes, it only ensures similarities for part of this large molecule, and it would seem inappropriate to extrapolate such data to basement membranes of normal tissues without considerable caution.

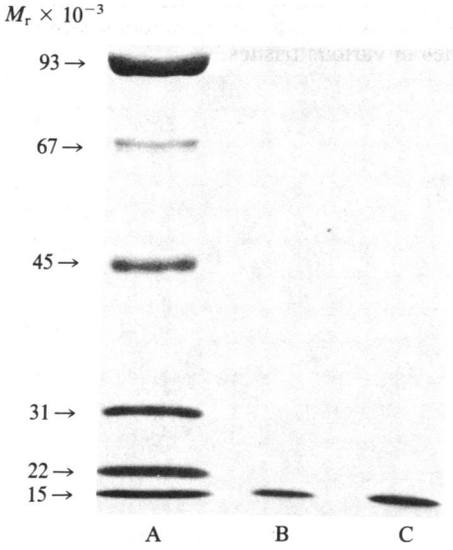



FIG. 5. NaDodSO$_4$/polyacrylamide gel (10%) electrophoresis. Lanes: A, molecular weight markers; B, D1 fraction treated with heparitinase; and C, D1 fraction treated with nitrous acid. A single band of $M_r \approx 18,000$ is observed.

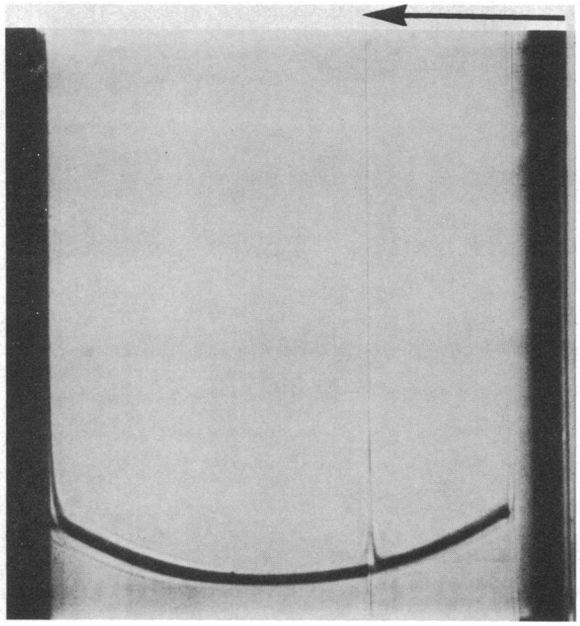


FIG. 6. Velocity sedimentation behavior of intact glomerular PG subunit in 4 M guanidine·HCl at 20°C. PGs at a concentration of ≈4.0 mg/ml were centrifuged for 64 min at 56,000 rpm in a Spinco AnH rotor.

The HS-PG of glomerular extracellular matrices also differs from detergent-extracted HS-PG isolated from cellular tissues such as hepatocytes (18). The latter has a lower molecular weight and fewer, relatively smaller, polysaccharide chains. However, the core protein seems to be of comparable size, yet it has a lower content of lysine and arginine and contains a larger number of serine residues. In addition, the core protein of the hepatocyte HS-PG seems to be intercalated into the plasma membrane, indicating that its hydrophobic region is embedded in the lipid bilayer (19). A similar situation where HS-PG is intercalated into the plasmalemma is true for mouse mammary epithelial cells (20). *In vitro*, the cellular HS-PG associates with lipid vesicles, while extracellular HS-PG fails to intercalate into the vesicles (20). Therefore, the fundamental difference between the cellular and extracellular HS-PG seems to be that the former has hydrophobic and the latter has ionic interactions with vicinal macromolecules in various tissues.

Finally, the results of this study emphasize the importance of isolating GPs or antigens from appropriate natural basement membranes in order to characterize them and study their biological properties.

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