Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 7108–7111, November 1984
Cell Biology

De novo cellular synthesis of sulfated proteoglycans of the developing renal glomerulus *in vivo*

(glomerular basement membrane/autoradiography/developing capillaries)

YASHPAL S. KANWAR*, MICHAEL L. JAKUBOWSKI*, LIONEL J. ROSENZWEIG†, AND JOHN T. GIBBONS*

*Department of Pathology, Northwestern University Medical School, Chicago, IL 60611; and †Department of Veterinary Biology, University of Minnesota, St. Paul, MN 55108

Communicated by Carl W. Gottschalk, July 11, 1984

ABSTRACT **The site of cellular synthesis of glomerular proteoglycans was investigated in developing glomeruli of 4- to 5-day-old rats. [^{35}S]Sulfate was administered intravenously and animals were sacrificed 15 min to 12 hr later. The outermost layers of the kidney cortices were utilized for characterization of proteoglycans and electron microscopic autoradiography. Sepharose CL-6B chromatography and cellulose acetate electrophoresis revealed that most (≈96%) of the radioactivity was associated with heparan sulfate-proteoglycan synthesized during maturation of glomerular capillaries. Tissue autoradiography revealed the following: (*i*) during the S-shaped body stage, there is rapid incorporation of [^{35}S]sulfate by mesenchymal cells into the cleft region (site for development of future glomerular extracellular matrices); (*ii*) during the precapillary stage, mesenchyme-derived cells showed higher incorporation of radioisotope than did epithelial cells; and (*iii*) during the mature capillary stage, all glomerular cell types (mesangial, endothelial, and epithelial) incorporated [^{35}S]sulfate, incorporation by mesangial cells being the greatest. Radiolabeling was also higher in the mesangial matrix than in the glomerular basement membrane of peripheral capillary loops. Synthesis of a single major species of sulfated glycosaminoglycan by cells of different embryologic origin may be unique to glomerular capillaries.**

The renal glomerulus is an intricate sieve of the mammalian vasculature that goes through a series of developmental stages—vesicle, S-shaped body, precapillary and capillary—during its maturation (1–4). All these stages are represented in the renal cortex during the first few days of life when, presumably, active synthesis of extracellular matrices (ECM) [glomerular basement (GB) membrane and mesangial matrix (MM)] occurs (1–4). Important constituents of ECM are the anionic sites (5–8) rich in heparan sulfate-proteoglycan (9–11), which apparently influence the charge-selective properties of glomerular capillaries (5, 12, 13). The site of cellular synthesis of glomerular proteoglycans (PGs) of ECM is unknown. This report describes the synthesis of PGs of ECM by epithelial, endothelial, and mesangial cells of the glomerulus during development.

METHODS

Radiolabeling of Cells and ECM. Twenty-one 4- to 5-day-old rats (10 g each) were injected via the inferior vena cava with [^{35}S]sulfate (0.25 mCi/g of body weight; specific activity >1200 Ci/mmol, 1 Ci = 37 GBq; Amersham). Animals (three per time point) were sacrificed 15 min, 30 min, and 1, 2, 4, 6, and 12 hr later. The animals were anesthetized before they were killed, and the kidneys were flushed with 0.15 M NaCl via the intracardiac route and fixed by perfusion with Karnovsky's aldehyde fixative. Cortical pieces of the radiolabeled kidneys were processed for electron microscopic autoradiography (14–16).

Morphometric Analysis. Four time points (1, 2, 6, and 12 hr) at which sufficient radioactivity had been incorporated were chosen for analysis. The analysis was carried out on these developmental stages of the glomerulus: S-shaped, precapillary, and capillary. The vesicle stage was not analyzed because it could not always be identified. By electron microscopy, each stage was identified and stages lying within the frame of grid mesh uninterrupted by the grid bar were photographed. Five such samples of each stage per time point were photographed at ×1400 magnification. Multiple photographs of various segments encompassing the whole stage were printed at a final magnification of ×15,000. The following compartments were designated: for the S-shaped body stage—(*i*) mesenchymal cells, (*ii*) epithelial cells, and (*iii*) cleft (the region where the future development of the ECM will take place); for the precapillary stage—(*i*) endothelial and mesangial cells, (*ii*) epithelial cells, and (*iii*) ECM (GB membrane and MM); and for the capillary stage—(*i*) epithelial cells, (*ii*) endothelial cells, (*iii*) mesangial cells, (*iv*) GB membrane, and (*v*) MM. Precapillary stage mesangial and endothelial cells were sometimes difficult to delineate; therefore, they were grouped together for morphometric analysis. The same was true for GB membrane and MM. The mean and SD of the grain density (concentration of radioactivity) of the various compartments were then determined (14–16).

Calculation of Relative Volume of Developing Glomeruli and Tubules in the Subcapsular Cortex. One hundred 0.5-μm sections from 25 Epon-embedded kidneys of 4- to 5-day-old rats were prepared. An ≈1-mm^2 (0.5 × 2.0 mm) subcapsular-cortical area of each section was photographed and printed at a final magnification of ×300. Five compartments were designated: (*i*) vesicle stage, (*ii*) S-shaped body stage, (*iii*) precapillary stage, (*iv*) capillary stage, and (*v*) tubules and interstitium. A transparent overlay with a two-dimensional array of points spaced 1 cm apart was placed over each micrograph and the total number of points over each compartment was determined. This number is proportional to the area of a given compartment (14–16). The relative volume of each compartment was computed as a percentage of the total volume.

Clearance Studies. Six 4- to 5-day-old rats were given [^{35}S]sulfate (5 μCi/g of body mass) intravenously. Blood samples were taken from each animal during the next 12 hr, and the radioactivity in each sample was measured.

Characterization of Renal Cortical Proteoglycans (PGs) and Glycosaminoglycans. Twelve 4- to 5-day-old rats were injected intravenously with [^{35}S]sulfate (0.25 mCi/g of body mass) and sacrificed 12 hr later. The 12-hr labeling period was cho-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ECM, extracellular matrices; GB membrane, glomerular basement membrane; MM, mesangial matrix; PG, proteoglycan.

sen because the clearance studies indicated that by this time the isotope is completely cleared from the circulation and maximum labeling of ECM of developing glomeruli has been achieved. The radiolabeled kidneys were perfused with Krebs–Ringer bicarbonate buffer and cortices were dissected free. Only the outermost portion of renal cortices (0.5–1.0 mm subcapsular region) were used since in the newborn rat this region is almost completely occupied by the developing glomeruli. The relative proportions of the various glomerular stages in this region were determined as described above: 10.69 ± 2.98% vesicle stage, 60.55 ± 5.06% S-shaped body stage, 12.06 ± 2.41% precapillary stage, 7.08 ± 1.55% capillary stage, and 9.46 ± 2.13% interstitium and tubules. The renal cortices were pooled and homogenized, and the PGs were extracted with 4 M guanidine·HCl (17, 18). All procedures were carried out at 4°C and protease inhibitors were used as described (17, 18). The radiolabeled PGs were characterized by Sepharose CL-6B chromatography before and after treatment with chondroitin ABC lyase (EC 4.2.2.4, degrades chondroitin 4- and 6-sulfates) and either nitrous acid or heparitinase (EC 4.2.2.8) (both degrade heparan sulfate) as described (17, 18). The radiolabeled glycosaminoglycans obtained from PG fractions by alkaline borohydride treatment (17) were characterized either by Sepharose CL-6B chromatography or by cellulose acetate electrophoresis and fluorography (10, 14, 15).

RESULTS

Radiolabeling of Cells and ECM in Various Stages of Glomerular Development. The various developmental stages of the glomerulus have been described in detail by Reeves *et al.* (1, 2). A rapid incorporation of [^{35}S]sulfate into the cleft of the S-shaped body stage was observed (Fig. 1*a* and Table 1). Incorporation seemed to be much greater in the mesenchymal cells than in the epithelial cells. The mean grain densities in the cleft region, mesenchymal cells, and epithelial cells were 0.976 ± 0.009, 0.380 ± 0.032, and 0.108 ± 0.004, respectively (Table 1), after 6 hr radiolabeling. During the precapillary stage a relatively slow incorporation of [^{35}S]sulfate into the ECM (GB membrane and MM) was observed (Fig. 1*b* and Table 1). The mesenchyme-derived cells (mesangial and endothelial) showed a more rapid incorporation than the epithelial cells. At 6 hr, the mean grain densities in the ECM, mesenchyme-derived cells, and epithelial cells were 0.583 ± 0.042, 0.253 ± 0.019, and 0.120 ± 0.005,

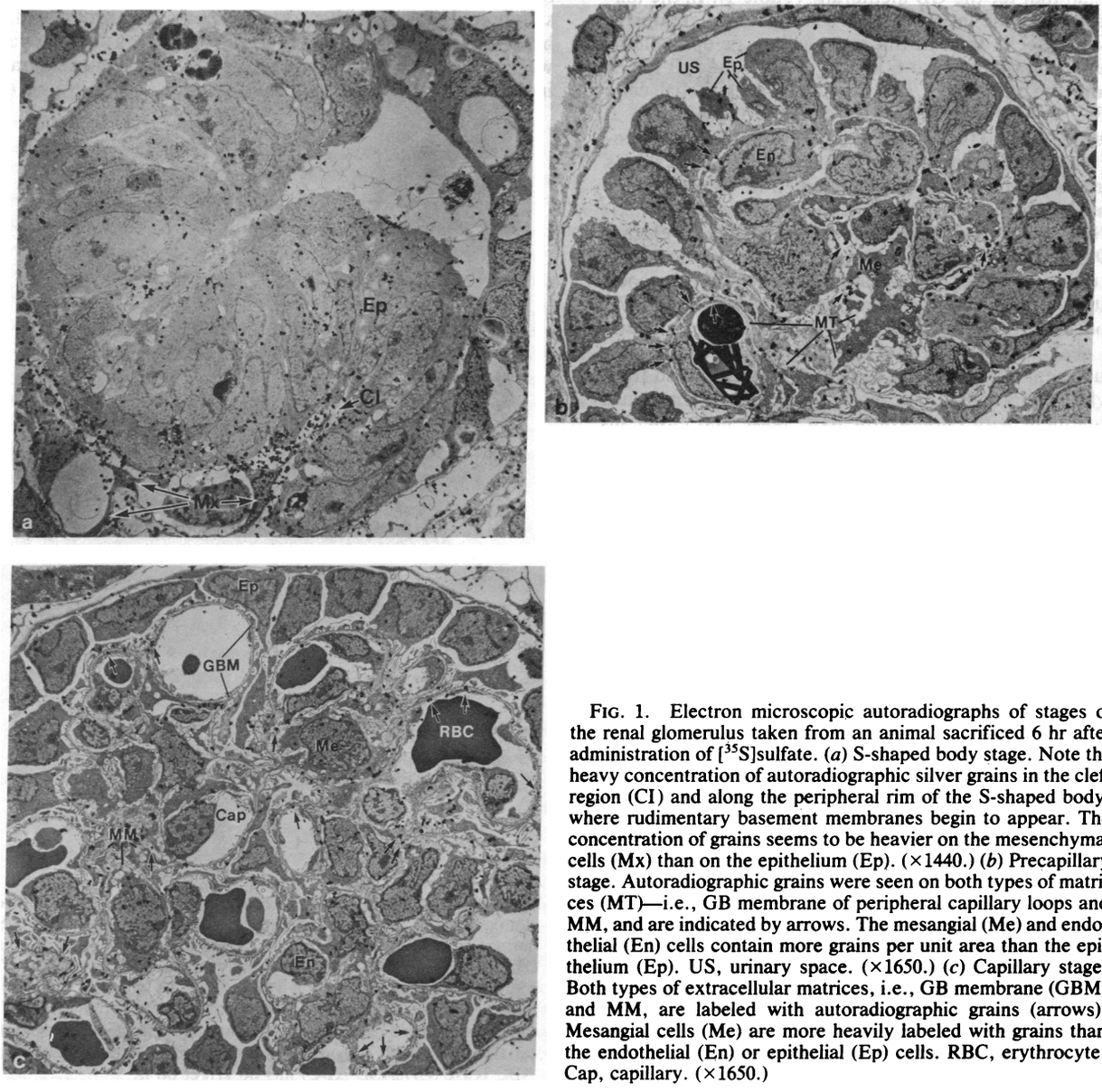

FIG. 1. Electron microscopic autoradiographs of stages of the renal glomerulus taken from an animal sacrificed 6 hr after administration of [^{35}S]sulfate. (*a*) S-shaped body stage. Note the heavy concentration of autoradiographic silver grains in the cleft region (CI) and along the peripheral rim of the S-shaped body, where rudimentary basement membranes begin to appear. The concentration of grains seems to be heavier on the mesenchymal cells (Mx) than on the epithelium (Ep). (×1440.) (*b*) Precapillary stage. Autoradiographic grains were seen on both types of matrices (MT)—i.e., GB membrane of peripheral capillary loops and MM, and are indicated by arrows. The mesangial (Me) and endothelial (En) cells contain more grains per unit area than the epithelium (Ep). US, urinary space. (×1650.) (*c*) Capillary stage. Both types of extracellular matrices, i.e., GB membrane (GBM) and MM, are labeled with autoradiographic grains (arrows). Mesangial cells (Me) are more heavily labeled with grains than the endothelial (En) or epithelial (Ep) cells. RBC, erythrocyte; Cap, capillary. (×1650.)

Table 1. Incorporation of [^{35}S]sulfate (autoradiographic grain density) into compartments of various developmental stages of the glomerulus

Stage	Compartment	Autoradiographic Grain Density			
		1 hr	2 hr	6 hr	12 hr
S-Shaped body	Epithelial cell	0.089 ± 0.006	0.102 ± 0.003	0.108 ± 0.004	0.051 ± 0.002
	Mesenchymal cell	0.218 ± 0.007	0.379 ± 0.008	0.380 ± 0.032	0.094 ± 0.006
	Cleft region	0.254 ± 0.008	0.691 ± 0.009	0.976 ± 0.009	1.781 ± 0.051
Precapillary	Epithelial cell	0.068 ± 0.003	0.084 ± 0.001	0.120 ± 0.005	0.051 ± 0.001
	Mesenchymal cell	0.099 ± 0.069	0.114 ± 0.001	0.253 ± 0.019	0.103 ± 0.003
	ECM (GB membrane + MM)	0.229 ± 0.007	0.355 ± 0.023	0.583 ± 0.042	0.732 ± 0.016
Capillary	Epithelial cell	0.052 ± 0.002	0.065 ± 0.003	0.099 ± 0.007	0.041 ± 0.001
	Endothelial cell	0.052 ± 0.003	0.086 ± 0.006	0.109 ± 0.005	0.075 ± 0.004
	Mesangial cell	0.061 ± 0.003	0.103 ± 0.003	0.198 ± 0.006	0.089 ± 0.003
	GB membrane	0.073 ± 0.003	0.166 ± 0.007	0.276 ± 0.016	0.542 ± 0.021
	MM	0.160 ± 0.007	0.262 ± 0.009	0.591 ± 0.175	1.046 ± 0.042

Rats were sacrificed at the indicated times after injection of [^{35}S]sulfate. Grain densities are given as the mean and SD of five different samples.

respectively (Table 1). During the capillary stage, relatively slow [^{35}S]sulfate incorporations into the ECM and the cells were observed (Fig. 1*c*). The incorporation into the MM was greater than that for the GB membrane (Table 1). In the mature glomerulus, the incorporation seemed to be greater in the mesangial cells than in the endothelium or epithelium. The mean grain densities at 6 hr in the MM, the GB membrane, and the mesangial, endothelial, and epithelial cells were 0.591 ± 0.175, 0.276 ± 0.016, 0.198 ± 0.006, 0.109 ± 0.005, and 0.099 ± 0.007, respectively. The mean grain densities of the various compartments of the glomerular stages after various radiolabeling times are given in Table 1.

Characterization of PGs and Glycosaminoglycans. Approximately 96% of the total radioactivity (efficiency of extraction ≈ 95%) was recovered in the PG fraction. The estimations of efficiency of extraction and the radioactivity associated with the PG fraction were made as described (17). Sepharose CL-6B elution profiles of the PG fraction showed a broad peak with a K_{av} value of 0.25 ($M_r \approx 120{,}000$). The K_{av} value seems to be similar to that of PGs extracted from either whole glomeruli (18) or purified basement membranes (17). Treatment with chondroitin ABC lyase and either nitrous acid or heparitinase revealed that practically all the radioactivity (>95%) was associated with the heparan sulfate-PG fraction (Fig. 2). Sepharose CL-6B chromatograms of glycosaminoglycan chains revealed a K_{av} value of 0.45 ($M_r \approx 25{,}000$) (data not shown). An examination of glycosaminoglycan chains by cellulose acetate electrophoresis and fluorography revealed a single spot that comigrated with heparan sulfate and was sensitive to treatment with nitrous acid or heparitinase (Fig. 3). No other sulfated glycosaminoglycan was indicated on the fluorogram of the electrophoretogram, indicating that all the autoradiographic silver grains ob-

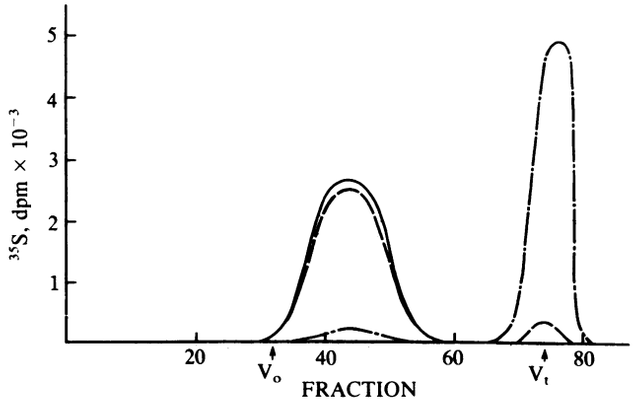

FIG. 2. Sepharose CL-6B chromatograms of radiolabeled proteoglycans extracted from renal cortices of 5-day-old rats. The intact proteoglycan (—) eluted as a single broad peak with a K_{av} value of 0.25 ($M_r \approx 120{,}000$). Treatment with chondroitin ABC lyase (---) and either nitrous acid or heparitinase (···) released <5% and >95%, respectively, of the radioactivity into the V_t fraction, indicating that practically all of the radioactivity was associated with heparan sulfate-PG.

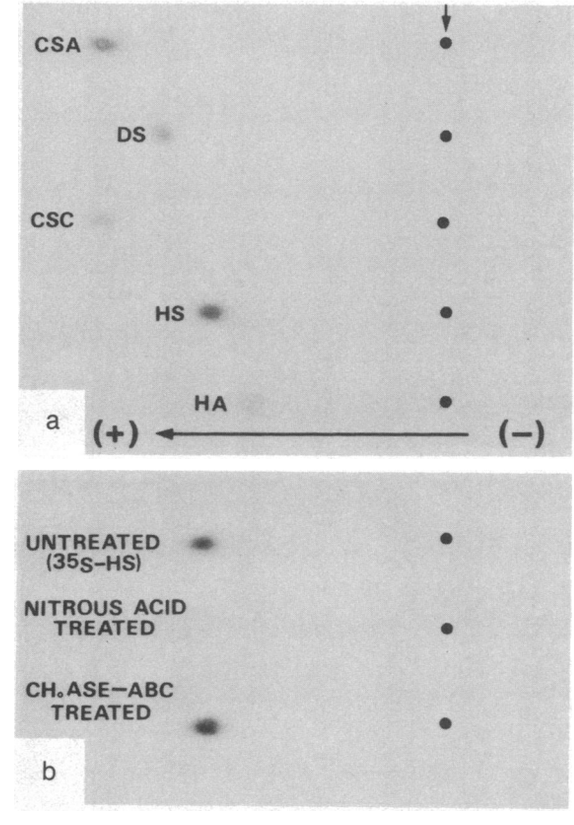

FIG. 3. Cellulose acetate electrophoretograms of (*a*) unlabeled standards, which include chondroitin sulfates A (CSA) and C (CSC), dermatan sulfate (DS), heparan sulfate (HS), and hyaluronic acid (HA) (*b*) radiolabeled glycosaminoglycan chains (detected by fluorography) obtained from the PG fraction by alkaline borohydride treatment. The untreated radiolabeled glycosaminoglycan spot comigrates with the heparan sulfate standard. The spot disappears after treatment with nitrous acid or heparitinase, while chondroitin ABC lyase (CH$_o$ASE-ABC) has no effect on it.

served by electron microscopy represent heparan sulfate residues.

DISCUSSION

The combined biochemical and tissue autoradiographic data indicate that (*i*) heparan sulfate-PG is the major ECM sulfated PG synthesized *de novo* in developing glomerular capillaries, (*ii*) the molecular weights of intact PG and its glycosaminoglycan chains seem to be similar to those of the mature glomerular species (17, 18), (*iii*) [^{35}S]sulfate incorporation into the ECM is greater in the initial stages of glomerular development, (*iv*) in the S-shaped body and precapillary stage, the mesenchymal cells synthesize relatively more PGs than do epithelial cells (*v*) [^{35}S]sulfate incorporation into ECM by the glomerular cells decreases as the capillaries mature, and (*vi*) in the mature glomerulus (capillary stage), [^{35}S]sulfate incorporation is greater in the MM than in the GB membrane of peripheral capillary loops.

The differential [^{35}S]sulfate incorporation into the two types of ECM of glomerulus, i.e., MM and GB membrane, may not be unique to the developing capillaries; differential incorporation has also been observed in mature glomeruli from adult animals (14, 15). However, why such a differential incorporation or synthesis of PGs occurs is unknown. It may be related to the inherent ready deposition of nocuous circulating colloidal or noncolloidal substances (possibly cations or immune complexes) into the MM (19). Rapid turnover/synthesis of PGs would result in clearing the substances more efficiently, thereby maintaining the functional integrity of the MM.

The synthesis/turnover of PGs can even vary within the same species of ECM in different locations such as maturing lobules of mammary and submandibular gland (20, 21). Furthermore, the synthesis/turnover of PGs could vary in different contiguous segments of the same lobule depending on the stage of development (20, 21). It is possible that PGs of ECM regulate the morphogenetic development of organs (22) or lobulation of apocrine (20) and exocrine glands (23, 24). Chondroitin and heparan sulfates are among the various types of sulfated proteoglycans that have been shown to regulate morphogenesis. They are usually present as a mixture; relative proportions vary during different stages of development (22–24). It seems rather unusual that here a single species of sulfated PG, i.e., heparan sulfate-PG, is synthesized during the entire course of maturation or vascularization of the glomerulus and is regulating the morphogenetic development of glomerular capillaries. Although a regulatory role for heparan sulfate-PG in the development of glomerular capillaries is speculative, it is conceivable since heparan sulfate-PG has also been found to be the exclusive proteoglycan associated with extraglomerular capillaries (25, 26).

Finally, the most distinctive observation of this investigation is that a single major type of sulfated PG (heparan sulfate-PG) is synthesized during glomerular capillary development by a mixture of cells of dissimilar embryologic origin.

We greatly appreciate the secretarial assistance provided by Ms. Mary Giles. We are grateful to Drs. Alfred Linker and Martin Mathews for supplying us with purified heparitinase and glycosaminoglycan standards, respectively. This research was supported by National Institutes of Health Grant AM 28492. Y.S.K. is a recipient of a Research Career Development Award (AM 01018).

1. Reeves, W. H., Caulfield, J. P. & Farquhar, M. G. (1978) *Lab. Invest.* **39,** 90–100.
2. Reeves, W. H., Kanwar, Y. S. & Farquhar, M. G. (1980) *J. Cell Biol.* **85,** 735–753.
3. Larsson, L. & Maunsbach, A. B. (1980) *J. Ultrastruct. Res.* **72,** 392–406.
4. Ekblom, P. (1981) *J. Cell Biol.* **91,** 1–10.
5. Rennke, H. G., Cotran, R. A. & Venkatachalam, M. A. (1975) *J. Cell Biol.* **67,** 638–646.
6. Caulfield, J. P. & Farquhar, M. G. (1976) *Proc, Natl. Acad. Sci. USA* **73,** 1646–1650.
7. Kanwar, Y. S. & Farquhar, M. G. (1979) *J. Cell Biol.* **81,** 137–153.
8. Gallo, G. R., Glaser, T. C. & Lamm, M. E. (1981) *J. Clin. Invest.* **67,** 1305–1313.
9. Kanwar, Y. S. & Farquhar, M. G. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 1303–1307.
10. Kanwar, Y. S. & Farquhar, M. G. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4493–4497.
11. Linker, A., Hovingh, P., Kanwar, Y. S. & Farquhar, M. G. (1981) *Lab. Invest.* **44,** 560–565.
12. Rennke, H. G. & Venkatachalam, M. A. (1978) *Kidney Int.* **13,** 278–288.
13. Kanwar, Y. S., Linker, A. & Farquhar, M. G. (1980) *J. Cell Biol.* **86,** 688–693.
14. Kanwar, Y. S., Rosenzweig, L. J., Linker, A. & Jakubowski, M. L. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 2272–2275.
15. Kanwar, Y. S., Rosenzweig, L. J. & Jakubowski, M. L. (1983) *Lab. Invest.* **49,** 216–225.
16. Kanwar, Y. S., Jakubowski, M. L. & Rosenzweig, L. J. (1983) *Eur. J. Cell Biol.* **31,** 290–295.
17. Kanwar, Y. S., Hascall, V. C. & Farquhar, M. G. (1981) *J. Cell Biol.* **90,** 527–532.
18. Kanwar, Y. S., Veis, A., Kimura, J. H. & Jakubowski, M. L. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 762–766.
19. Michael, A. F., Keane, W. F., Raij, L., Vernier, R. L. & Maver, S. M. (1980) *Kidney Int.* **17,** 141–154.
20. Silberstein, G. B. & Daniel, C. W. (1982) *Dev. Biol.* **91,** 215–222.
21. Bernfield, M. & Banerjee, S. D. (1982) *Dev. Biol.* **90,** 291–305.
22. Trelstad, R. L., Kimiko, H. & Toole, B. P. (1974) *J. Cell Biol.* **62,** 815–830.
23. Banerjee, S. D., Cohn, R. H. & Bernfield, M. R. (1977) *J. Cell Biol.* **73,** 445–463.
24. Thompson, H. A. & Spooner, B. S. (1983) *J. Cell Biol.* **96,** 1443–1450.
25. Vaccaro, C. A. & Brody, J. S. (1981) *J. Cell Biol.* **91,** 427–437.
26. Simionescu, M., Simionescu, N. & Palade, G. E. (1982) *J. Cell Biol.* **95,** 425–434.