

# ANIONIC SITES IN THE GLOMERULAR BASEMENT MEMBRANE

## In Vivo and In Vitro Localization to the Laminae Rarae by Cationic Probes

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### ABSTRACT

Cationized ferritin (CF) of narrow pI range (7.3–7.5) and the basic dye ruthenium red (RR) have been used as cationic probes to partially characterize anionic sites previously demonstrated in the glomerular basement membrane (GBM). When CF was given i.v. to normal rats and the left kidney was fixed by perfusion 15 min thereafter, clusters of CF molecules were found throughout the lamina rara interna (LRI), lamina rara externa (LRE), and mesangial matrix distributed at regular (~60 nm) intervals. When kidneys were perfused with aldehyde fixative containing RR, small (20 nm) RR-stained particles were seen in the same locations distributed with the same 60 nm repeating pattern, forming a quasi-regular, lattice-like arrangement. Fine (~3 nm) filaments connected the sites and extended between them and the membranes of adjoining endothelial and epithelial cells. When CF was given i.v. followed by perfusion with RR *in situ*, both probes localized to the same sites. CF remained firmly bound after prolonged perfusion with 0.1–0.2 M KCl or NaCl. It was displaced by perfusion with buffers of high ionic strength (0.4–0.5 M KCl) or pH (<3.0 or >10.0). CF also bound (clustered at ~60 nm intervals) to isolated GBM's, and binding was lost when such isolated GBM's were treated with buffers of high ionic strength or pH. These experiments demonstrate the existence of a quasi-regular, lattice-like network of anionic sites in the LRI and LRE and the mesangial matrix. The sites are demonstrable *in vivo* (by CF binding), in fixed kidneys (by RR staining), and in isolated GBM's (by CF binding). The results obtained with CF show that the binding of CF (and probably also RR) to the laminae rarae is electrostatic in nature since it is displaced by treatment with buffers of high ionic strength or pH. With RR the sites resemble in morphology and staining properties the proteoglycan particles found in connective tissue matrices and in association with basement membranes in several other locations.

**KEY WORDS** cationized ferritin · ruthenium  
red · proteoglycans · ionic  
interaction · isolated GBM's

Recent studies have shown that the glomerular capillary wall contains fixed negatively charged sites which may be important in maintaining nor-

mal glomerular functions in filtration of blood plasma and especially in the retention of the anionic plasma proteins in the circulation. Clearance studies (2, 38) have established that anionic macromolecules are more restricted in their transglomerular passage than neutral molecules of the same size and configuration whereas the passage of cationic macromolecules is actually facilitated, indicating that the capillary wall acts as a charge as well as a size barrier in the filtration of macromolecules. The maintenance of this charge barrier function appears to be critical for normal filtration function since loss of charge selectivity appears to be responsible for the enhanced filtration of anionic plasma proteins seen in several experimental glomerular diseases associated with proteinuria (2, 3). Electron microscope studies have demonstrated fixed negatively charged sites in association with all three components of the peripheral portions of the glomerular capillary wall: they occur along the surfaces of the endothelium (6, 23) and epithelium (1, 6, 17, 23, 24, 31-33) (associated with the highly anionic cell coat material) and within the glomerular basement membrane (GBM)<sup>1</sup> (6, 7, 42). The evidence currently available suggests that the GBM normally functions as both the size and the charge barrier to filtration of macromolecules (5, 10, 11, 36, 39) whereas the sites on the endothelium and epithelium may have other functions (e.g., maintenance of the normal foot process and slit arrangement [43]). Thus, it is of considerable interest for renal physiology to establish the nature and arrangement of anionic sites in the GBM about which relatively little is known at present.

Using lysozyme as a cationic "stain," anionic sites were previously demonstrated in the GBM (6, 7). Particularly striking was the concentration of sites in the inner and outer light layers (lamina rara interna and externa) of the GBM in which they were found distributed in a regular reticular pattern. In this paper we report our findings obtained with two additional cationic probes—the basic dye, ruthenium red (RR), and cationized ferritin (CF)—which we have used to further study

<sup>1</sup> Abbreviations used in this paper: CF, cationized ferritin; DMPA, N,N-dimethyl-1,3 propanediamine; EDC, carbodiimide hydrochloride; GBM, glomerular basement membrane; HBSS, Hank's balanced salt solution; LRE, lamina rara externa; LRI, lamina rara interna; RR, ruthenium red.

and characterize the anionic sites in the laminae rarae of the GBM.<sup>2</sup>

## MATERIALS AND METHODS

### *Materials*

Male Charles River CD<sup>®</sup> rats weighing 100-150 g were used in these experiments. Horse-spleen ferritin, twice crystallized (cadmium free) was obtained from Calbiochem, San Diego, Calif.; N,N-dimethyl-1,3-propanediamine (DMPA) from Eastman Kodak, Rochester, N.Y.; 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) from Story Chemical Co., Muskegon, Mich.; ampholines (pH range 3.5-10 and 6-8) from LKB-Instruments Inc., Rockville, Md.; and ruthenium red from Ventron Corp., Danvers, Mass.

### *Preparation and Characterization of Cationized Ferritin (CF)*

**PREPARATION:** The cationization reaction was carried out according to the method of Danon et al. (8) except that, instead of varying the pH of the reaction, the degree of modification of the protein was controlled with varying amounts of carboxyl group activator (EDC) while keeping the pH and concentration of nucleophile (DMPA) constant as follows: 0.5 ml of native ferritin (100 mg/ml) was mixed with 2.0 ml of 2 M DMPA (pH 7.0), EDC in varying amounts (12.5-100 mg) was added, and the reaction was carried out for ~2 h. With this procedure, CF's with isoelectric points of narrow range close to neutrality (pI = 6.5-7.8) could be obtained, and the results were highly reproducible. After modification, the ferritin was extensively dialyzed against 0.15 M NaCl (8-10 changes, 1 liter each) over a period of 48 h at 4°C. The ferritin solutions were concentrated by filtration in a diaflow unit (Amicon Corp., Scientific Sys. Div., Lexington, Mass.) provided with a PM-10 membrane, and then centrifuged at 15,000 g for 30 min to remove aggregates. Finally, the preparations were sterilized by passing them through a Millipore filter (Millipore Corp., Bedford, Mass.) (0.22 μm pore size) and stored at 4°C.

**CHARACTERIZATION:** A drop of the CF solution was placed on a carbon-coated grid, negatively stained with phosphotungstic acid, and examined by electron microscopy. The size, shape, and state of dispersion of CF molecules was identical to that of the native (unmodified) ferritin.

The isoelectric point (pI) of CF preparations was determined with a LKB-8100 Ampholine Electrofocusing unit using standard techniques in a sucrose gradient column (27).

<sup>2</sup> A preliminary report of these findings was published previously (18).

A 5% suspension of washed human erythrocytes prepared in 0.15 M NaCl was incubated with ferritins of varying pI, and the amount of agglutination introduced was determined by visual inspection and by light microscopy.

CF with a pI of 6.9–7.2 caused little or no detectable agglutination. CF, pI 7.3–7.5, produced minimal microscopic agglutination, while CF with a pI of 7.6–7.9 caused visual agglutination.

### *Preparation of Ruthenium Red (RR) Perfusate*

A 0.2% solution of RR was prepared in Karnovsky's aldehyde fixative (1% formaldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, with 4.4 mM CaCl<sub>2</sub>) as described by Luft (28). The dye-fixative solution was passed through a millipore filter (0.22 μm pore size) before use to remove large dye particles.

### *Technique for Kidney Perfusion*

Rats were anesthetized with ether, and the abdominal cavity was exposed by a midline incision. The abdominal aorta was catheterized with polyethylene tubing, and two ligatures were placed on it, one above and the other below the origin of the left renal artery, thus isolating the left kidney from the systemic circulation. A small opening was made in the left renal vein and retrograde aortic perfusion was started. Initially, oxygenated Hanks' balanced salt solution (HBSS) was perfused at a pressure of 120–150 mm of Hg with a flow rate of 4–5 ml/min to wash out the blood. This was followed by perfusion with appropriate solutions as described in individual experiments. The perfusion pressure and flow rate were kept constant in all the experiments. All perfused solutions were kept at room temperature (~25°C).

### *Preparation of Isolated GBM's*

Glomeruli were isolated by the technique of Krawker and Greenspon (22), and the basement membrane fractions were prepared therefrom by the method of Meezan et al. (30).

### *Labeling of Anionic Sites in Intact Kidneys*

**EXPERIMENTS WITH CF:** CF (50 mg/ml) was introduced via the saphenous vein (0.25 mg/g body weight) over a period of 2 min. 15–20 min after introduction of the tracer, the animals were anesthetized and the left kidney was perfused with HBSS for 1 min, followed by perfusion fixation with Karnovsky's aldehyde fixative for 2–3 min. Small (1 mm<sup>3</sup>) pieces of cortex were prepared and immersed in aldehyde fixative for an additional 2 h, after which the tissue blocks were

postfixed at 4°C in 1% OsO<sub>4</sub> in acetate-veronal buffer (pH 7.2) for 2–3 h.

**EXPERIMENTS WITH RR:** The left kidney was exposed and flushed with HBSS for 1 min (by perfusion as described above) followed by perfusion with 20 ml of 1% formaldehyde in 0.1 M cacodylate buffer, pH 7.4, and perfusion with 25 ml of RR fixative solution. Small (1 mm<sup>3</sup>) pieces of kidney cortex were excised and fixation was continued by immersion of the blocks in the dye-fixative solution for 3 h at 25°C. They were then placed for 1 h in 0.15 M cacodylate buffer (pH 7.4) containing 0.1% RR, and postfixed in 1% OsO<sub>4</sub> (pH 7.2) containing 0.05% RR for 3 h at room temperature.

**COMBINED CF AND RR EXPERIMENTS:** For these combined experiments, CF (pI 7.3–7.5) was introduced i.v. as described above, but the amount given was reduced to 0.1 mg/g body weight, and it was allowed to circulate for a shorter time interval (8–10 min). The left kidney was flushed, fixed, perfused with RR, and processed exactly as described for the experiments with RR alone.

**EFFECTS OF VARIOUS TREATMENTS (BUFFERS OF HIGH IONIC STRENGTH, OR VARYING PH) ON CF BINDING:** CF (0.25 mg/g body weight) was injected i.v. as described above. 15 min thereafter, the left kidney was flushed with 0.15 M NaCl by perfusion, and then perfused for 5 min with one of the following solutions: (a) 50 mM Tris-HCl buffer, pH 7.4, containing KCl (0.025–0.5 M); (b) the same buffer containing NaCl (0.3, 0.4, 0.5 M) or MgCl<sub>2</sub> or CaCl<sub>2</sub> (0.1, 0.135, 0.17 M); or (c) a variety of buffers of varying pH—i.e., 0.1 M citrate buffer (pH 2.0–5.0), 0.1 M citrate-phosphate buffer (pH 5.0–6.5), 0.1 M phosphate buffer (pH 6.5–7.5), 0.1 M Tris buffer (pH 7.5–9.0), 0.1 M glycine-NaOH buffer (pH 9.0–10.0), and 0.1 M carbonate buffer (pH 9.5–10.5). Perfusion with these solutions was carried out for 5 min, followed by flushing with 0.15 M NaCl for 1 min, and Karnovsky's aldehyde fixative for 2–3 min. Additional fixation and processing was done as described earlier for the CF experiments.

### *Experiments on Isolated GBM's*

**LABELING OF ANIONIC SITES WITH CF:** Isolated GBM's were suspended in 0.15 M NaCl and incubated with CF (pI 7.3–7.5, 50–100 μg/ml) for 15 min at 37°C. They were washed by sedimentation and resuspension (first, in 0.15 M NaCl, and then in 0.1 M cacodylate buffer), placed in 0.4 ml polyethylene tubes, pelleted by centrifugation at 10,000 g for 5 min in a Beckman Microfuge, (Beckman Instruments, Spinco Div., Palo Alto, Calif.), followed by fixation in Karnovsky's fixative for 3 h at room temperature. Postfixation was the same as for blocks of kidney tissue.

**TREATMENT WITH BUFFERS OF HIGH IONIC STRENGTH AND VARYING PH AFTER CF LABELING:** After labeling the isolated GBM's with CF, they were washed in 0.15 M

NaCl followed by incubation for 15 min at 37°C in Tris-HCl buffer containing various salts of different ionic strength and pH as done in the case of the experiments performed on perfused kidneys. Subsequently, they were washed with 0.1 M cacodylate buffer and processed as described above.

**PREPARATION AND LABELING OF SONICATED GBM'S:** Isolated GBM's were suspended in 0.15 M NaCl and then sonicated using a Branson sonifier, Model S125 (Branson Ultrasonic Corp., Stamford, Conn.) 20 Kc/s at the No. 6 power setting as follows: preparations were subjected to several (2-20) 30-s bursts over a period of 3-30 min, and the suspension was allowed to cool after each 30-s burst. Sonicated GBM's were incubated with CF (pI 7.3-7.5) and processed as described above for unsonicated GBM's.

**PREPARATION AND LABELING OF FIXED GBM'S:** Isolated GBM's were fixed in Karnovsky's fixative for 20-30 min, washed with 0.1 M cacodylate buffer, and incubated with CF under the same conditions as unfixed GBM's.

### *Tissue Processing*

After postfixation in OsO<sub>4</sub>, specimens were dehydrated in graded series of ethanol, transferred to propylene oxide, and embedded in Epon. Some specimens were stained in block in uranyl acetate before dehydration. Thin sections (40-60 nm) were prepared and mounted on carbon-coated grids, stained with uranyl acetate and lead citrate, and examined with a Siemens Elmiskop 102 or a Philips 301 electron microscope operating at 80 kV.

## RESULTS

### *In Vivo Experiments with CF*

In accord with the findings of Rennke et al. (36, 37), the distribution and localization of CF of different pI's differed. When CF with a pI of 6.9-7.2 was given i.v. and the kidney was flushed with saline, relatively few CF molecules bound to the basement membrane. Those present were primarily scattered diffusely in the LRI and LRE. Occasionally, particles were also seen distributed randomly in the lamina densa and mesangial matrix.

After administration of CF with a pI of 7.3-7.5 followed by flushing with saline, many more molecules were seen binding to the basement membrane where they were characteristically grouped into clusters in the laminae rarae, distributed in equal numbers in the LRI and LRE. In cross-sections through the peripheral capillary loops, these clusters were seen to be regularly spaced at intervals of ~60 nm (Fig. 1) in both

the LRI and LRE, with each cluster containing ~5-15 ferritin molecules. No binding of CF was seen on the endothelial or epithelial cell surfaces. In grazing sections through the capillary loops, the clusters of CF molecules in the laminae rarae were seen to be disposed in a network with a reticular pattern (Fig. 2) which was similar to the pattern of binding seen previously with lysozyme (6, 7). This pattern was particularly striking in the LRE, but it was also characteristically present in the LRI where it was most readily detected in mesangial regions (see Fig. 17).

When CF with a pI of 7.6-7.9 was given *in vivo* followed by flushing of the kidney with saline, numerous CF molecules were found sticking to the endothelial cell membrane where they were often aggregated into large clumps. Relatively few particles were found binding to sites in the LRE. Occasionally, a thrombus (consisting of platelets and fibrin) was found in the lumen of a glomerular capillary. When CF with a pI of >7.9 was given, frequent thrombi and large aggregates of CF were found in the lumen of glomerular capillaries. Aggregates of CF molecules were also seen plugging the endothelial fenestrae.

These results indicated that CF with a pI of 7.3-7.5 is optimal as a cationic probe for labeling anionic sites in the GBM since (a) it binds to and labels equally well specific sites in both the LRI and LRE and in the mesangial matrix, and (b) it does not bind to the endothelial and epithelial cell surfaces. CF with a pI of <7.2 is not suitable since it does not bind to the sites in the laminae rarae under the conditions tested. CF with a pI >7.5 is also not suitable for labeling the GBM sites because it binds to anionic sites on the cell surfaces of erythrocytes, endothelium, and platelets, resulting, respectively, in agglutination of erythrocytes *in vitro*, and thrombus formation and plugging of endothelial fenestrae when given *in vivo*. Hence, preparations with a pI 7.3-7.5, were used in the remaining experiments with CF.

### *Experiments with RR*

In cross-sections through the periphery of glomerular capillaries stained with RR, both the LRE and LRI contained rows of small (~20 nm) RR-positive particles arranged at intervals of ~60 nm (Fig. 4). Sometimes these particles appeared to be staggered in such a way that alternate granules were located in different planes (Fig. 9).

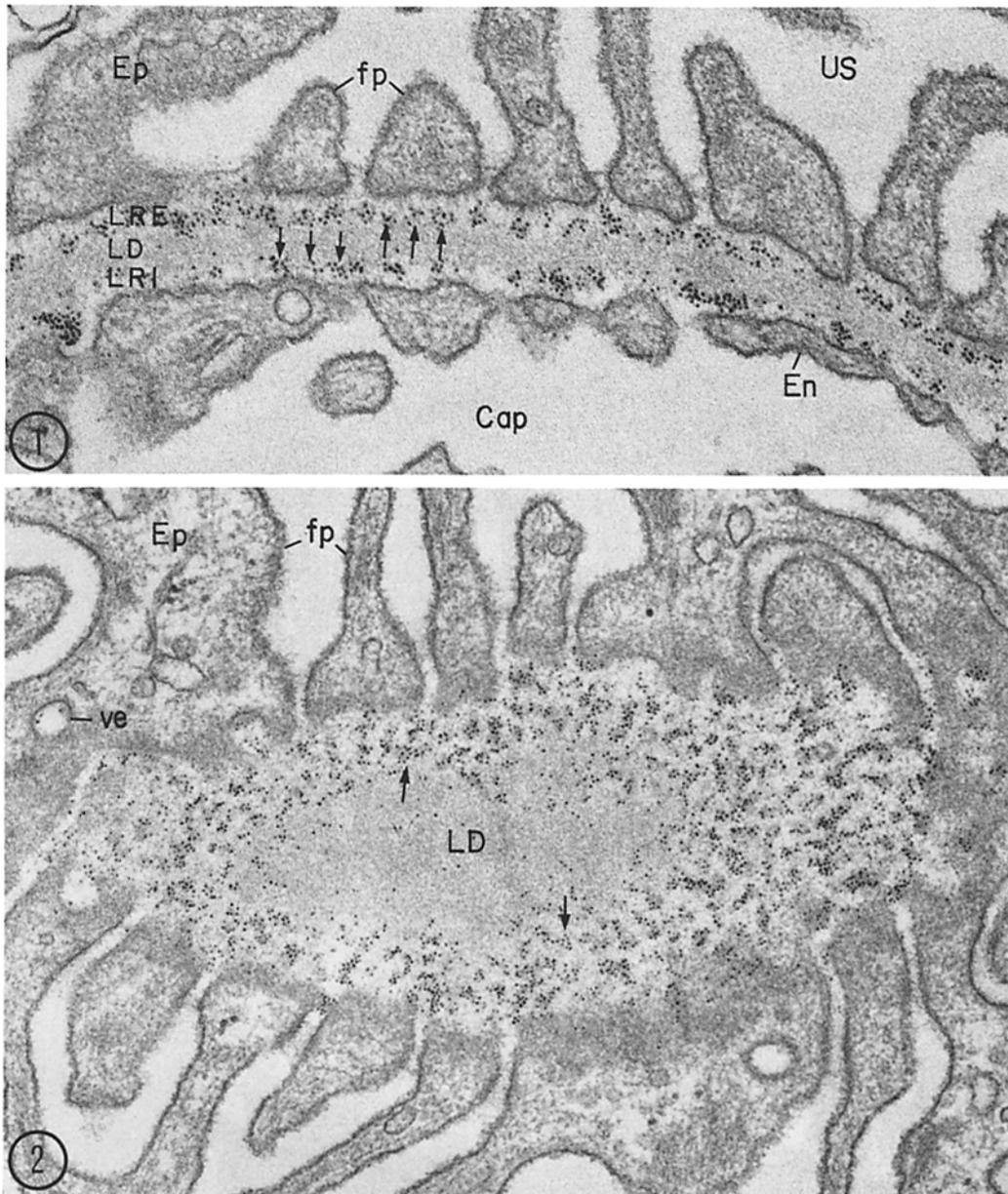
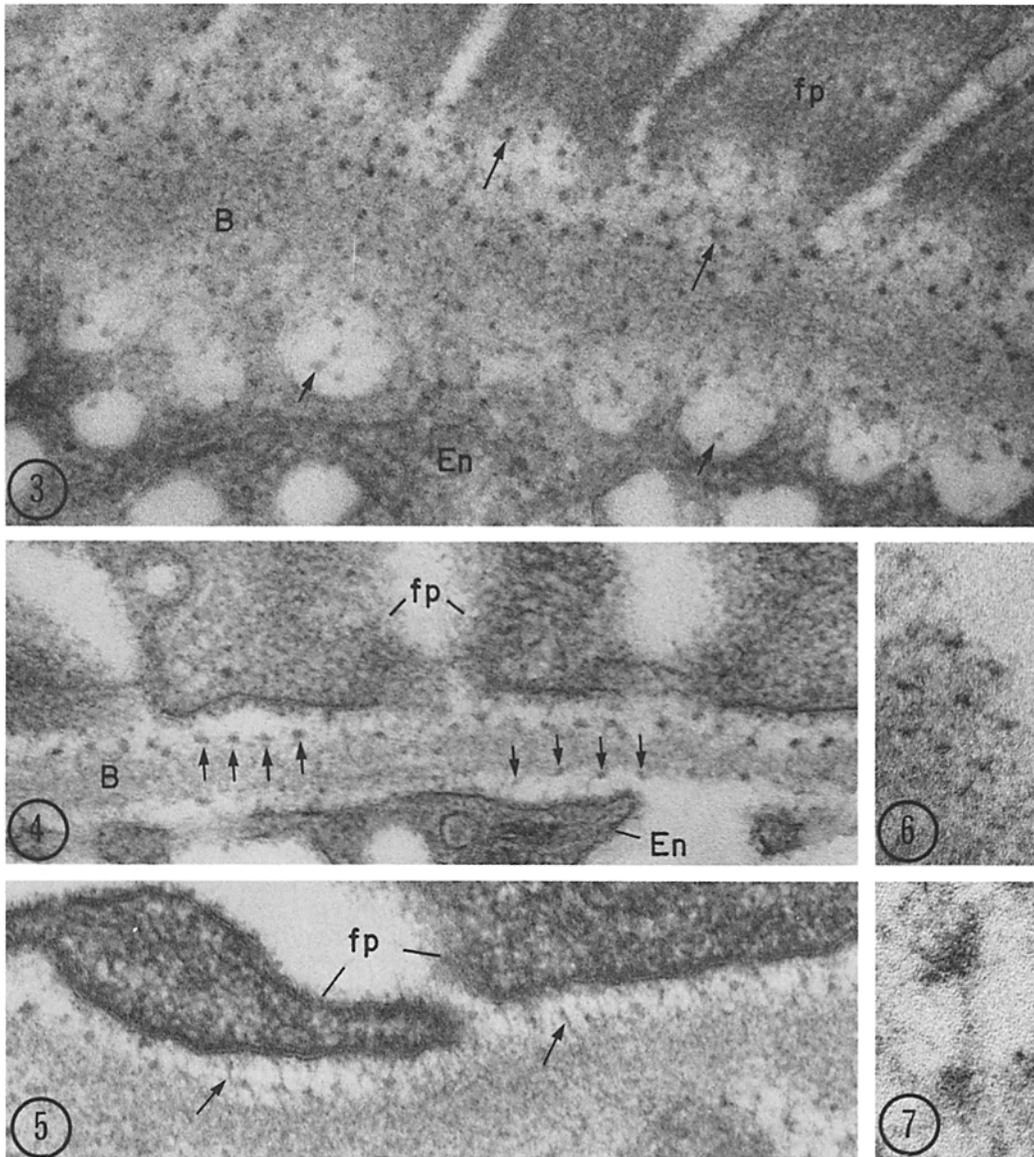


FIGURE 1 Portion of a peripheral region of a glomerular capillary from a rat given cationized ferritin (pI = 7.3-7.5) by i.v. injection, after which the kidney was briefly flushed with HBSS and fixed by aldehyde perfusion. The distribution of CF is restricted primarily to the lamina rara interna (LRI) and externa (LRE) of the GBM where it occurs in discrete clusters located at regular ~60 nm intervals (arrows). A few molecules are seen scattered in the lamina densa (LD). No CF binding to the endothelium (En) or epithelium (Ep) is seen. fp, foot processes; US, urinary space; Cap, capillary lumen.  $\times 80,000$ .

FIGURE 2 Grazing section of the same glomerulus as that in Fig. 1, showing the reticular pattern formed by clusters of CF molecules in the lamina rara externa (arrows). Very few CF molecules are present in the lamina densa (LD), and no binding to the epithelium (Ep) is seen. Several CF molecules are present in a pinocytotic vesicle (ve) in the epithelium. No regular relationship between the clusters of CF molecules and the epithelial slits between the foot processes (fp) is evident: a few slits contain CF aggregates (lower left), a few are free of CF, and a few contain CF at low concentration.  $\times 60,000$ .



FIGURES 3-7 Portions of glomerular capillaries from a kidney perfused with aldehyde fixative containing RR. Fig. 3 shows a partially grazing section, and Fig. 4 a cross section, illustrating the presence of polygonal, RR-stained particles in the laminae rarae of the GBM. In Fig. 4, the particles are seen to occur at regular intervals ( $\sim 60$  nm) in both the lamina rara interna ( $\downarrow$ ) and externa ( $\uparrow$ ) of the GBM (B). In Fig. 3, the rows of particles are seen in the LRE (long arrows) at the base of the foot processes (fp) and superimposed on the endothelial fenestrae (short arrows). Fig. 5 demonstrates fine filaments (arrows) emanating from the tips of the angular profiles of RR-stained granules connecting them to the epithelial cell membranes. Fig. 6 is a higher magnification view of the LRE showing a quasi-regular, lattice-like arrangement of RR-stained particles. The particles occur at a regular distance from one another with a center to center spacing of  $\sim 60$  nm (ave). Fine ( $\sim 3$  nm) filaments interconnect the particles. Fig. 7 depicts two RR-stained particles with their angular (often triangular) profiles and the fine ( $\sim 3$  nm) filaments which connect them. The latter extend from the tips of the particles and radiate in several directions giving them a stellate configuration. En, endothelium. Fig. 3,  $\times 100,000$ ; Fig. 4,  $\times 110,000$ ; Figs. 5 and 6,  $\times 150,000$ ; Fig. 7,  $\times 350,000$ .

It was more difficult to demonstrate the regularity of the particles in the LRI than in the LRE in cross sections through the capillaries. However, in grazing sections the RR-positive sites could be regularly recognized in the LRI either directly (Fig. 8) or viewed through the circular openings of the endothelial fenestrae (Fig. 3). In places in particularly favorable sections, these sites appeared to form a quasi-regular lattice, with the points of the lattice located at intervals of ~60 nm from each other (Figs. 6, 8, and 10). This relatively ordered distribution of RR-positive sites was detectable in both laminae rarae (LRI and LRE) and was observed in both the peripheral and axial (mesangial) regions of the capillary loops. Fine (~3 nm) filaments emanated in several directions from the RR-stained particles. Some of these connected one site to another (Fig. 7), and others extended to insert either into the lamina densa or the membranes of adjoining epithelial (Fig. 5) and endothelial cells.

The sites demonstrated in the laminae rarae either with RR or with CF had no detectable regular relationship with the filtration slits, the epithelial foot processes, or the endothelial fenestrae.

RR-positive particles of similar morphology were also observed in association with other basement membranes: those of Bowman's capsule, the kidney tubules, the endothelium of peritubular capillaries, and the renal arterioles.

RR also stained the electron-dense cell coats covering the endothelium, and epithelium (Figs. 3, 5, and 9).

#### *Combined CF and RR Experiments*

Since the distribution of CF binding sites and RR-stained particles in the laminae rarae was so similar, it was of interest to determine whether or not these two cationic probes were interacting with the same anionic sites. When CF was given in vivo followed by perfusion with RR-containing fixative, the sites of binding of the CF molecules coincided in distribution with those of the RR-stained particles (Figs. 11 and 12). Thus, both tracers apparently bind to the same sites.

#### *Effects of Buffers of Varying Ionic Strength and pH on CF Binding*

When CF was given in vivo and the kidney was perfused with Tris buffer (pH 7.4) containing 0.025–0.2 M KCl or NaCl, the periodic distribu-

tion of ferritin in the LRI, the LRE (Figs. 13 and 14) and the mesangium (Fig. 17) was maintained. However, when buffers containing 0.3 M KCl or NaCl were perfused, the CF was partially displaced: fewer molecules were present, and the regular pattern of CF binding was no longer seen (Fig. 15). At 0.4–0.5 M KCl or NaCl, CF binding was largely lost: the tracer was displaced from its binding to the anionic sites (Figs. 16 and 18). No clusters were present and only occasional molecules could be seen in the LRI, the LRE, or the mesangial matrix (Fig. 18). It is of interest that although a few CF molecules were seen beneath the slit diaphragm, between it and the GBM (Fig. 16), there was no accumulation of CF against the slit diaphragm. No structural alterations of the slit diaphragm and epithelial foot processes were evident.

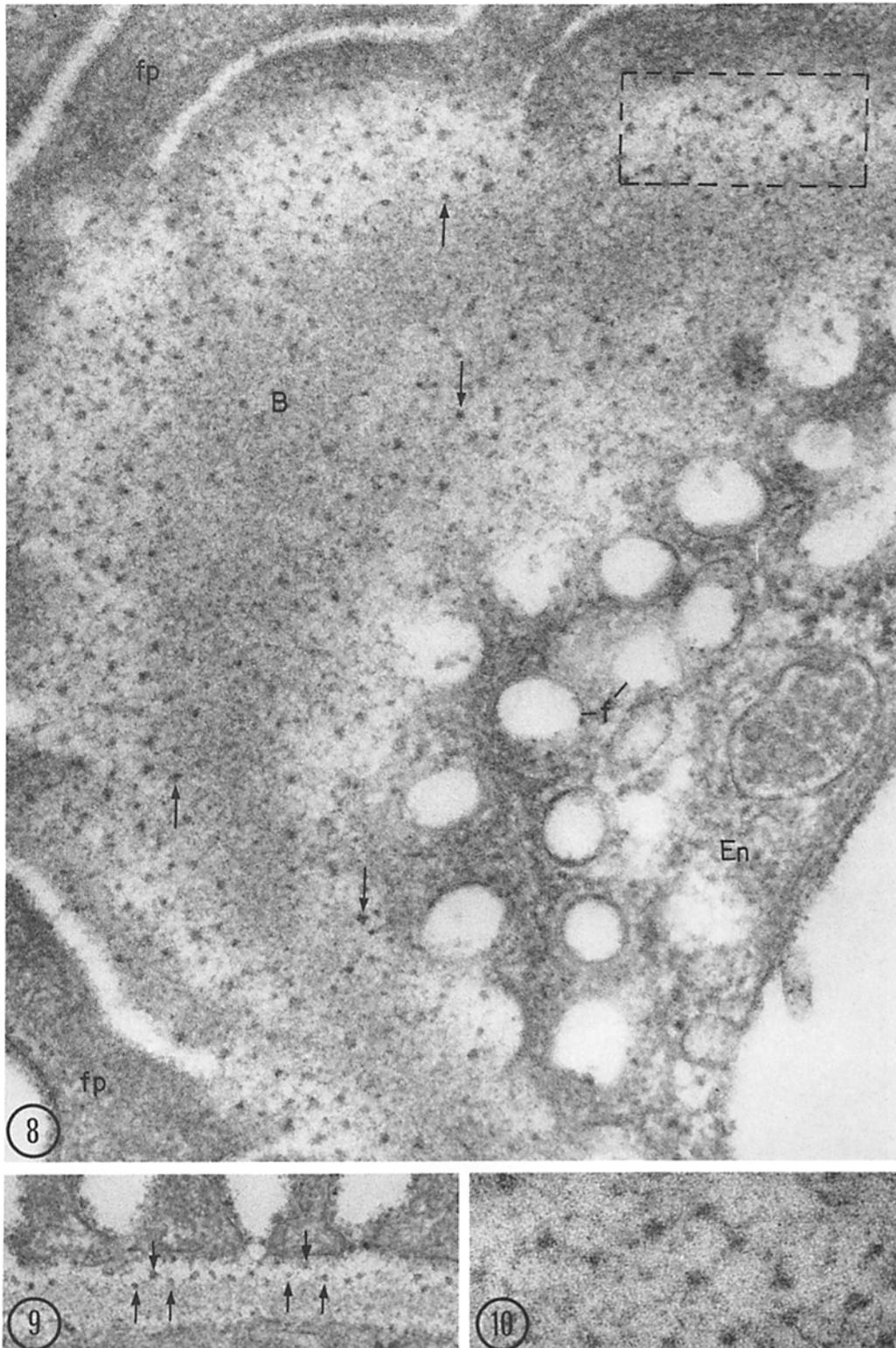
A similar loss of binding of CF molecules to the anionic sites was observed when kidneys were perfused with divalent cations ( $MgCl_2$  or  $CaCl_2$ ) of equivalent ionic strength (0.1–0.17 M).

CF binding to sites in the laminae rarae and mesangium was not disturbed by buffers of a wide pH range (from 3.5–10.0); however, perfusion with carbonate-bicarbonate buffer, pH 10.5, completely dislodged the CF bound to these sites. Glomerular morphology remained remarkably unaltered under these conditions. When 0.1 M citrate buffer, pH 3.0, was perfused, some displacement of CF molecules was observed; relatively few clusters of ferritin particles remained in the LRE and LRI. This acidic solution caused a distinct deterioration in fine structure, so it was decided not to try buffers below pH 3.0.

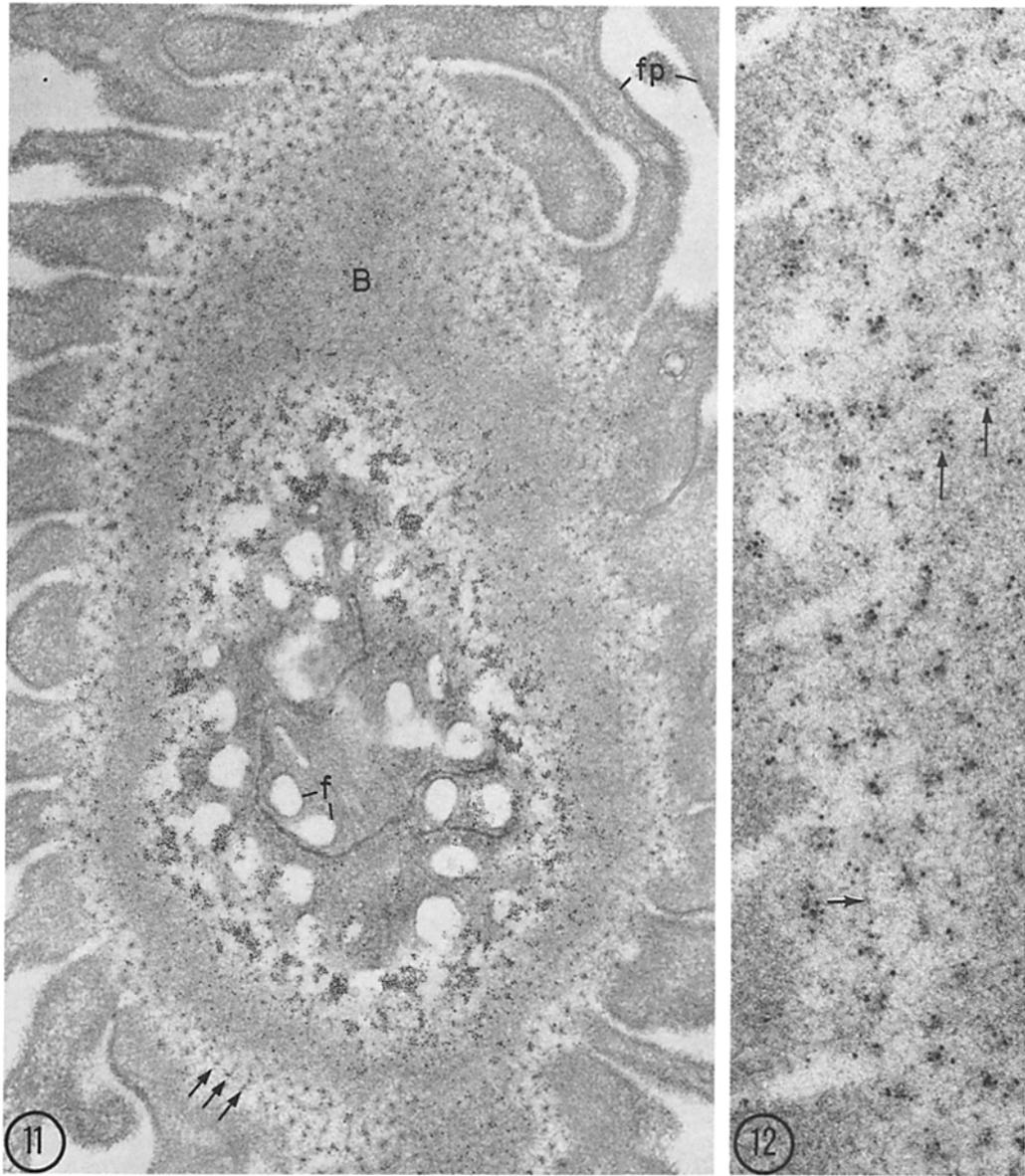
#### *Experiments on Isolated GBM's*

**MORPHOLOGY OF ISOLATED GBM'S:** GBM's isolated by the method of Meezan et al. (30) consist of intact tubes of basement membranes devoid of attached cellular elements (4). Since they consist of closed basement membrane loops, CF has access to the outside of the GBM corresponding to the LRE, but it does not have access to the LRI on the inside of the closed tubes (Fig. 19).

**CF BINDING TO ISOLATED GBM'S:** When fresh isolated GBM's were incubated with CF, the distribution of molecules in the exposed LRE was exactly the same as in the intact kidney. It occurred in clusters at intervals of 60 nm (Fig. 19), and, in places where GBM's were cut in grazing



FIGURES 8-10 Portions of glomerular capillaries stained with RR. Fig. 8 is a grazing section which cuts broadly through the GBM (*B*) and shows the regular distribution of the polygonal granules in both the lamina rara interna ( $\downarrow$ ) and externa ( $\uparrow$ ). Fig. 10 is an enlargement of part of the LRE showing the quasi-regular lattice-like arrangement of RR-stained sites. Fig. 9 shows a region of the GBM where the row of RR-stained particles in the LRE appear to be staggered with alternate particles (arrows) located at a greater distance from the lamina densa. *fp*, foot processes; *En*, endothelium; *f*, endothelial fenestrae. Fig. 8,  $\times 104,000$ ; Fig. 9,  $\times 80,000$ ; Fig. 10,  $\times 208,000$ .

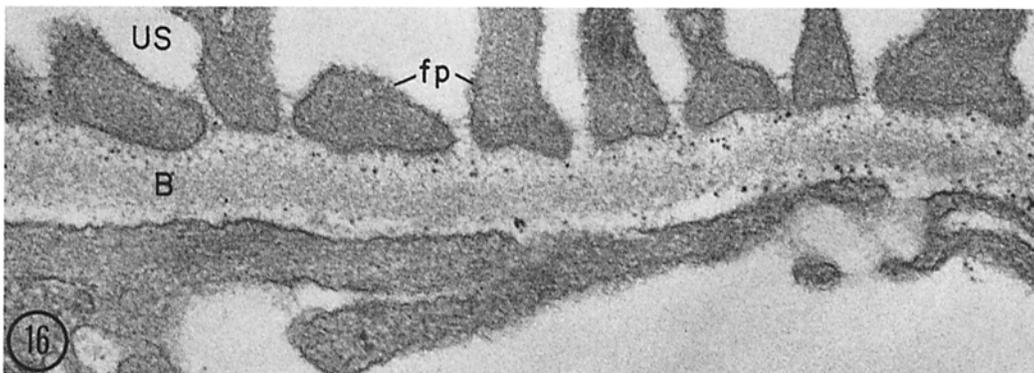
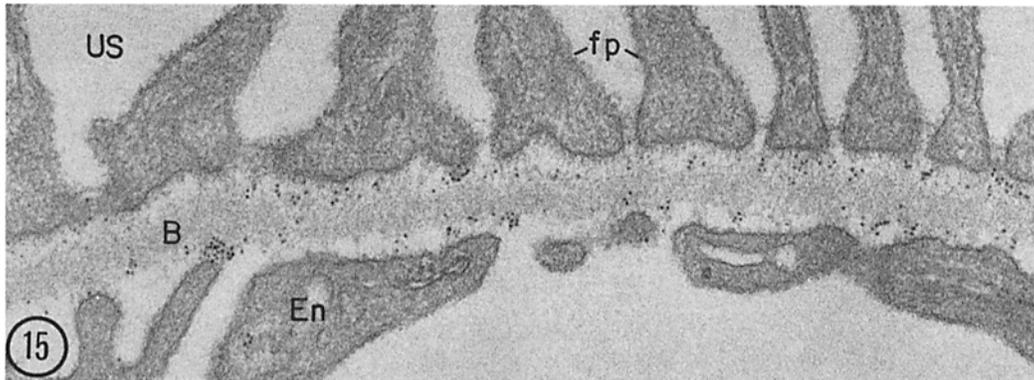
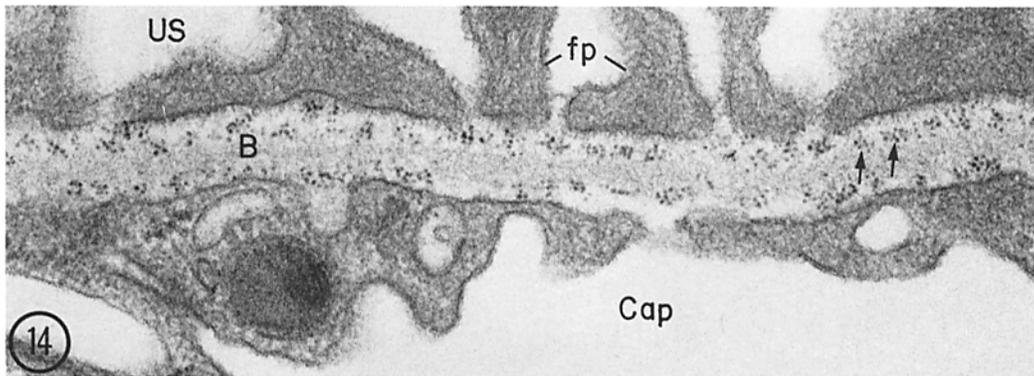
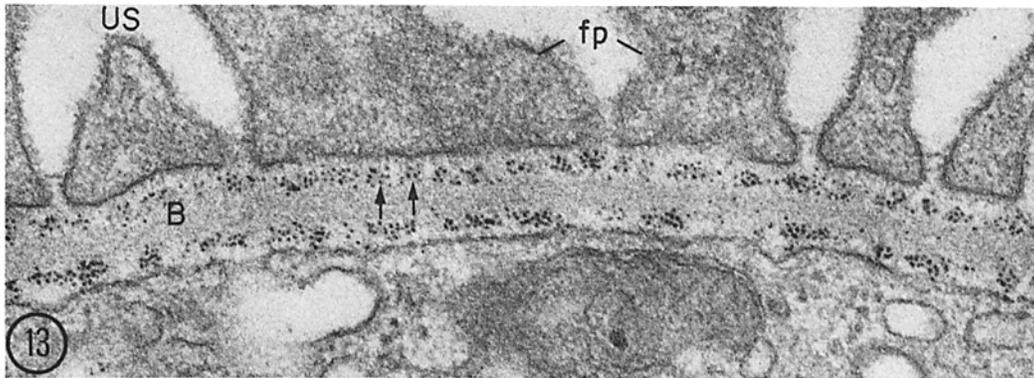


FIGURES 11 and 12 Grazing section of a glomerular capillary from the kidney of a rat which had been given CF ( $pI = 7.3-7.5$ ) *in vivo* followed by kidney perfusion with RR. Note that the distribution of the clusters of CF molecules and the RR-stained positive sites in the LRE coincide (arrows). Fig. 12 is an enlargement of a portion of the LRE showing CF molecules clustered on the RR-stained particles (long arrows). RR-stained filaments can be seen connecting the particles (short arrow). *B*, lamina densa; *fp*, foot processes; *f*, endothelial fenestrae. Fig. 11,  $\times 38,000$ ; Fig. 12,  $\times 105,000$ .

section, regular clusters of CF particles could be discerned (Fig. 20). No CF label was seen on the inside surface of the GBM loops corresponding to the LRI, indicating that the tracer does not pene-

trate the intact GBM under these conditions.

EFFECTS OF BUFFER OF VARYING PH AND IONIC STRENGTH ON CF BINDING: Treatment of isolated GBM's with buffers of different ionic



FIGURES 13-16 Portions of glomerular capillaries from the kidneys of rats which were injected i.v. with CF ( $pI = 7.3-7.5$ ) followed by perfusion of the kidney with 0.05 M Tris buffer (pH 7.4) containing varying concentrations of KCl. After perfusion with 0.1 M (Fig. 13) or 0.2 M (Fig. 14) KCl, most of the CF molecules remain bound to the sites in the LRI and LRE (arrows). However, binding is reduced and largely lost after perfusion with 0.3 M (Fig. 15) or 0.4 M (Fig. 16) KCl. *B*, lamina densa; *fp*, foot processes; *US*, urinary space; *En*, endothelium; *Cap*, capillary lumen.  $\times 80,000$ .

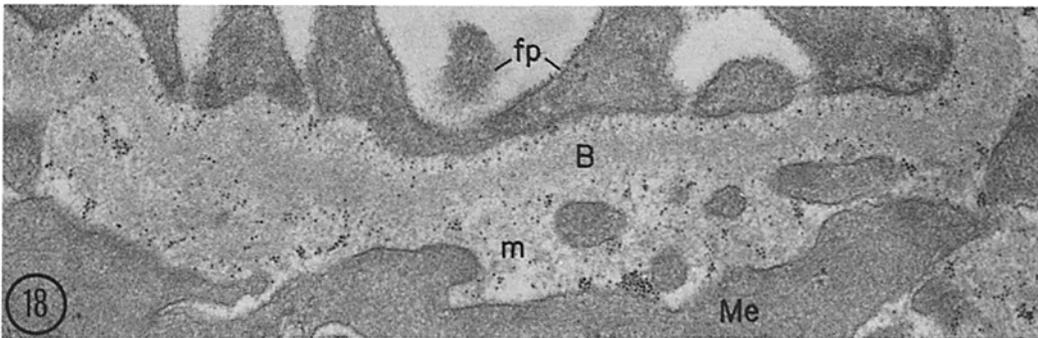
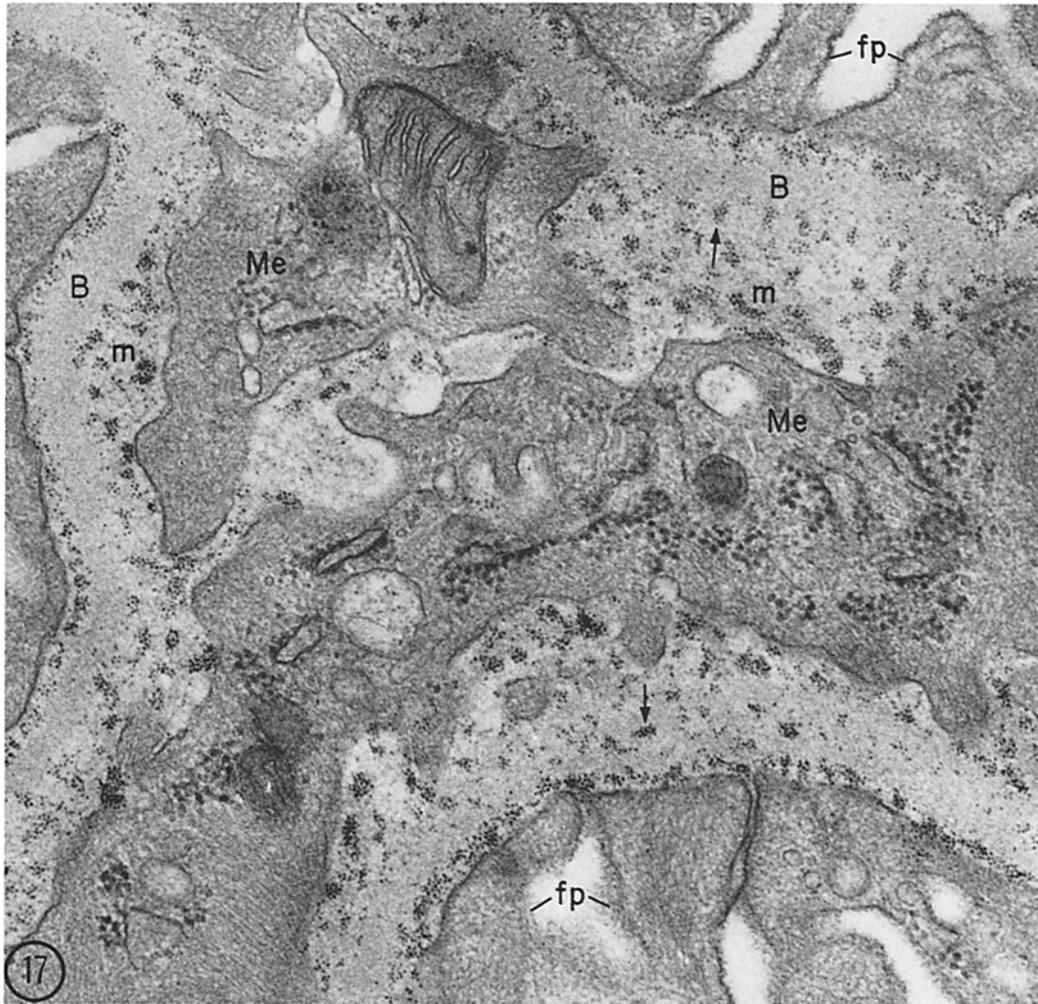
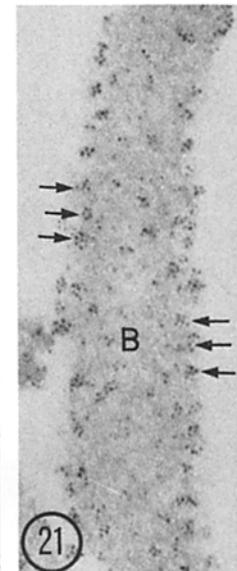
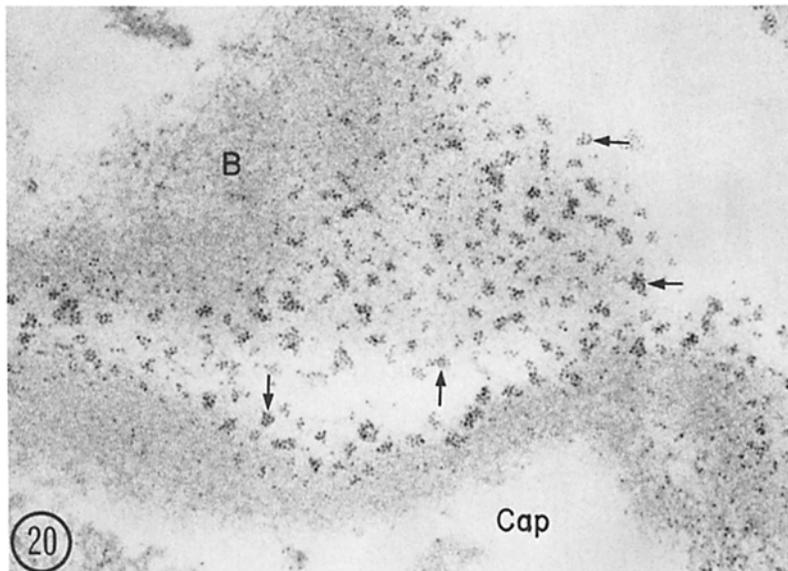
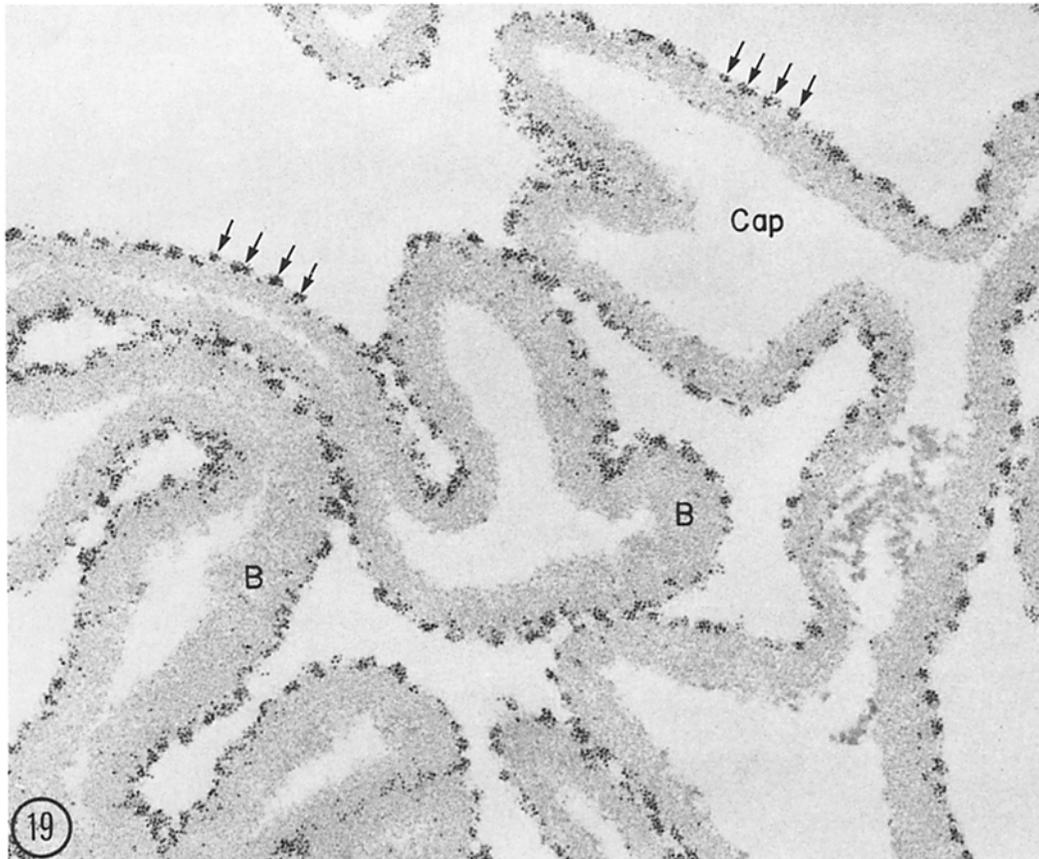


FIGURE 17 Axial region of a glomerulus from a rat which had been given CF (pI 7.3-7.5) by i.v. injection to show the binding of CF (arrows) to sites in the mesangial matrix (*m*) between the mesangial cells (*Me*) and the basement membrane (*B*) as well as to sites in the laminae rarae of the GBM.  $\times 60,000$ .

FIGURE 18 Field similar to that in Fig. 17 showing part of a glomerulus from a rat given CF followed by kidney perfusion with 0.3 M KCl (as in Fig. 15). Note the reduction in CF binding to anionic sites in the mesangial matrix (*m*) between the mesangial cell (*Me*) and the GBM (*B*) as well as from the laminae rarae after perfusion with buffer of high ionic strength.  $\times 60,000$ .



FIGURES 19-21 Portions of isolated GBM's incubated with CF ( $pI = 7.3-7.5$ ). Fig. 19 shows several loops of intact, isolated GBM (*B*), demonstrating the binding of CF to the LRE in the same regular pattern as in the intact glomerulus (see Fig. 1). CF molecules are located in clusters at regular ( $\sim 60$  nm) intervals on the outer GBM surface (arrows). CF is not seen binding to the LRI on the inside of the basement membrane presumably because it does not penetrate the intact loops. Fig. 20 is a grazing section through an intact isolated GBM loop showing the regular pattern of distribution of CF molecules (arrows) which occur in clusters at a distance of  $\sim 60$  nm (ave) from one another. Fig. 21 shows a piece of isolated GBM from a preparation which had been subjected to sonication before incubation with CF to disrupt the intact loops into short segments. Clusters of CF molecules (arrows) can be seen on both sides of the GBM (*B*) corresponding to the lamina rara interna and externa. *Cap*, former capillary lumen. Figs. 19 and 20,  $\times 60,000$ ; Fig. 21,  $\times 80,000$ .

strength or pH yielded exactly the same results as observed with the intact perfused kidneys: CF binding was lost when the labeled membranes were incubated with Tris buffer (pH 7.4) containing either 0.3 M KCl or NaCl, or 0.1 CaCl<sub>2</sub> or MgCl<sub>2</sub>; 0.1 M carbonate buffer, pH 10.5; or 0.1 M citrate buffer, pH 3.0.

**RELABELING OF ANIONIC SITES AFTER TREATMENT WITH BUFFERS OF HIGH IONIC STRENGTH:** Since the CF bound to the isolated GBM's was displaced by treatment with buffers of high ionic strength, a question arose as to whether or not the sites themselves remained intact after this treatment. When the GBM's treated with high salt buffer were reincubated in CF under the same conditions as the initial labeling, CF bound to the anionic sites in the same distribution as that observed in freshly isolated GBM's, indicating that the binding sites remained intact after high salt treatment.

**EFFECT OF SONICATION ON THE ISOLATED GBM'S:** Isolated GBM's were subjected to sonication with two purposes in mind: (a) to open up the intact loops of GBM so that CF could gain access to the LRI on its inner surface, and (b) to see whether or not the sites were affected by this treatment. After brief sonication the GBM was disrupted into short segments with open ends so that CF gained access to both sides of the GBM. Under these conditions, the ferritin localized at regular intervals of ~60 nm on both sides (LRE and LRI) of the isolated GBM's (Fig. 21). If sonication was prolonged to 15-30 min no CF binding was seen, suggesting that the binding sites are disrupted by prolonged sonication.

**EFFECT OF FIXATIVE ON CF BINDING:** When isolated GBM's were fixed before incubation with CF, CF was found binding all along the outside of the membranes in a uniform layer instead of in the clustered pattern seen with fresh, unfixed membranes.

## DISCUSSION

In this investigation, we have used RR and CF as cationic probes to further characterize the anionic sites demonstrated previously in the GBM. The results obtained with these probes have revealed that the sites are distributed in a lattice-like arrangement throughout the lamina rara interna and externa of the GBM, and the mesangial matrix. In all these locations, the sites have a similar, periodic distribution with a quasi-regular (~60

nm, ave) center-to-center spacing. It is clear that RR and CF reveal the same sites, because if both procedures are carried out on the same kidney the two coincide: CF molecules are concentrated at the RR-stained sites. It is also clear (cf. reference 41) that the binding of cationic probes to the sites is ionic in nature because CF binding is lost after treatment with buffers of high ionic strength or pH. The fact that the sites can be demonstrated in isolated GBM's (from which the cells have been removed by detergent treatment) indicates that the sites are true constituents of the GBM and not part of the cell coats of the adjoining endothelium and epithelium. The fact that the sites can be demonstrated with RR (or lysozyme [6]) in prefixed glomeruli indicates that the pattern observed does not result from aggregation of mobile sites in the GBM by cationic probes.

The detailed organization of the sites is particularly well demonstrated after staining with RR. In such preparations, they are seen as ~20-nm polygonal particles with fine (3 nm) filaments radiating out from their tips connecting them with one another and with the adjoining endothelial and epithelial cell membranes as diagrammed in Fig. 22.

Anionic sites with a regular repeating pattern, which took on a reticular rather than a particulate appearance in grazing sections, were previously demonstrated in the laminae rarae (especially the LRE) using lysozyme (6, 7). A similar reticular pattern is seen with CF if the kidney is fixed directly without a rigorous saline flush. However, in specimens in which saline perfusion was prolonged, a particulate pattern was evident, suggesting that loosely bound CF was washed out by prolonged flushing. Thus, the reticular pattern could be explained by binding of lysozyme and CF to the connecting fibrils as well as the punctate sites. The tracer data are compatible with the assumption that CF (and lysozyme) binds avidly to the punctate anionic sites from which it can be removed only by high salt treatment and binds more loosely to the connecting filaments from which it is displaced by flushing with saline.

The presence of RR-stained particles in the laminae rarae (especially the LRE) has been noted (13) or illustrated without comment (14) by others, but their general occurrence and details of their distribution were not appreciated. Similar particles were also described in the laminae rarae recently by Schurer et al. (42) after injection of

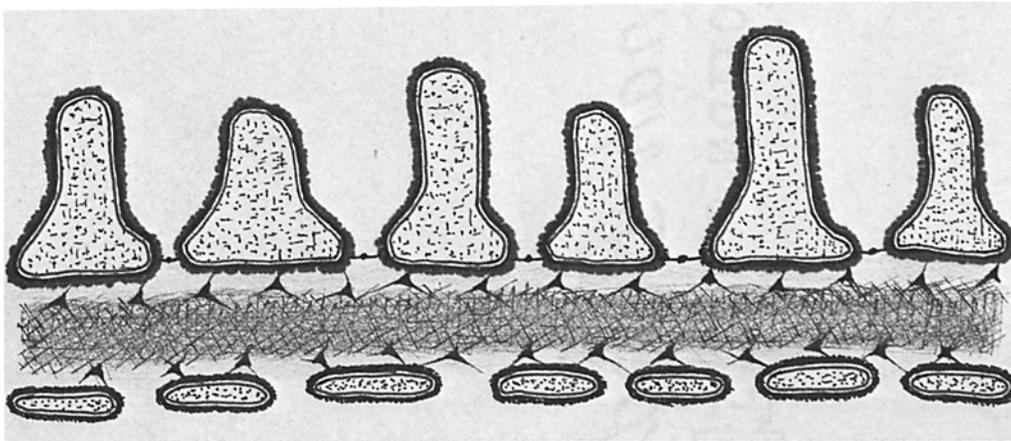


FIGURE 22 Diagrammatic representation of the proposed distribution of anionic sites in the lamina rara interna and externa of the GBM based on results of labeling with cationic probes. The sites are visualized as a network of angular particles (~20 nm) with fine filaments (~3 nm) extending from their points to connect one particle with another, with the membranes of adjoining endothelial and epithelial cells, and with the lamina densa.

another cationic probe – polyethylenimine, a cationic polymer (mol wt = 30,000–40,000).<sup>3</sup>

#### *Nature of the Anionic Sites in the GBM*

At the time of the initial detection of the anionic sites in the GBM, it was pointed out (6) that of the known constituents of GBM the most likely candidates for constituents of the anionic sites are the carboxyl groups of the collagenous or noncollagenous glycopeptides and the sialyl groups of heteropolysaccharides. Both have been demonstrated in isolated GBM's by biochemical analysis (4, 21, 45, 46, 49). It was also pointed out that another possibility that should be considered is sulfate groups of sulfated glycosaminoglycans (GAG's). Sulfated GAG's have not yet been detected in glomerular basement membranes (45), but glucuronic acid (9, 46) and small amounts of sulfate esters (46) have been detected in the lens capsule which is a well-studied basement membrane consisting, like the GBM, of Type IV collagen. Moreover, proteoglycan parti-

cles consisting in part of chondroitin sulfate have been identified (based on RR staining and enzyme digestion) in association with basement membranes in a number of other locations—i.e., the embryonic cornea (48), various other embryonic tissues (15, 16) and smooth muscle cells in the aorta (50).

Sulfated GAG's are readily extracted from tissues by the usual fixation and dehydration methods; however, they can be retained in connective tissue when cationic dyes or reagents (such as cetyl pyridinium chloride [29], RR [28], alcian blue [1, 47], or safranin O [44]) are added to the fixative. RR has been shown to bind to, to precipitate and to stain GAG's in a variety of connective tissues (15, 16, 34, 48, 50), where they occur as proteoglycan particles (20–50 nm in diameter) often closely associated with collagen fibrils (16, 34, 47, 48, 50).

The polygonal anionic sites with interconnecting fibrils which we have demonstrated with RR in the laminae rarae and mesangial matrix of the GBM bear a striking resemblance to the particles found in association with basement membranes in other locations, and, except for their smaller size, they also resemble the proteoglycan particles found in connective tissue matrices of cartilage (47), aorta (50), and various embryonic tissues (15, 48). This similarity in morphology and staining properties suggests that the particles in the GBM consist at least in part of proteoglycans.

<sup>3</sup> Diffuse staining of the laminae rarae has also been described after immersion of kidney slices or blocks in various cationic stains (colloidal iron [17, 35], alcian blue [1], and ruthenium red [23]). Most of these studies, with the exception of those by Jones (17) and Latta (23), have interpreted the staining as localization of anionic groups in the closely apposed endothelial and epithelial cell membranes.

Enzyme digestion studies reported elsewhere (19, 20) indicate that this is indeed the case since (a) the particles are not removed by neuraminidase treatment (under conditions in which the sialoglycoprotein cell coat of the epithelium is removed), but (b) they are susceptible to removal with glycosaminoglycan-degrading enzymes. Specifically, the GBM sites appear to consist in large part of heparan sulfate, since they can no longer be demonstrated after digestion with purified heparitinase or after nitrous acid oxidation. In contrast to RR-stained sites found in association with other basement membranes (15, 48, 50), they do not appear to consist of chondroitin sulfate since they are unaffected by treatment with chondroitinase ABC and testicular hyaluronidase.

Our results indicate that the binding sites in the laminae rarae are disrupted or removed by sonication. The failure to detect GAG's by biochemical analysis in isolated GBM's could be because all such previous analyses were carried out on GBM's prepared by methods involving extensive sonication. This point should be checked by repeating the analysis on unsonicated GBM's.

#### *Possible Functions of the Anionic Sites*

We have already pointed out in the Introduction that the existence of fixed negative charges in the glomerular capillary wall is essential for maintenance of normal glomerular filtration functions, and for the retention of the plasma proteins (most of which are polyanionic in nature) in the circulation (2, 38). Loss of such fixed negative charges has also been shown to be associated with proteinuria in several experimental glomerular diseases (2, 3, 7, 31, 32). Although anionic sites occur on the surfaces of both endothelium and epithelium (6, 17, 23, 24, 31-33, 43), the available evidence indicates that the GBM serves as both the charge and the size barrier in the filtration of macromolecules since all anionic proteins or neutral particles used as tracers (anionic ferritin [12, 37], endogenous albumin [39], catalase [40], and neutral dextrans of varying size [55-78 Å] (5, 10)) do not penetrate beyond the LRI under normal flow conditions. The role of the GBM as the charge barrier was demonstrated directly by the experiments of Rennke et al. (36, 37) which showed that ferritin penetrates the GBM in increasing amounts the greater the isoelectric point. It is tempting to suggest that the meshwork formed by the clusters of anionic sites and their fibrillar

connections in the LRI could provide the charge barrier which normally retards entry of anionic macromolecules into the lamina densa. This idea is attractive because the presence of polysaccharide polymers of proteoglycans has already been shown to limit the transport of macromolecules through connective tissue matrices according to both size and charge (25, 26). However, at the moment, it has not been established whether the anionic groups in the LRI or those in the lamina densa (sialoproteins? collagenous peptides? others?) are more important in creation of the charge barrier.

The present results obtained with CF of narrow pI range do show that the charge density of the proteoglycan particles in the laminae rarae is greater than that of the sialoglycoproteins on the adjoining cell membranes since the CF fractions used (pI 7.3-7.5) bound to the sites in the GBM and not to the membranes of the endothelium and epithelium.

The fact that similar and even more highly developed polyanionic networks exist in the LRE of the GBM and in the laminae rarae of other basement membranes (Bowman's capsule, arteriolar and capillary endothelium, kidney tubule) suggests that they have several functions. It was previously suggested (6) that another possible function that the sites may subservise is attachment of cells to their basement membranes. The present observations demonstrate that the fine filaments radiating from the sites connect or attach the sites to the adjoining membranes and to the lamina densa. Therefore, the filament-particle meshwork could function in attachment, and disruption of either the fibrils or the sites could lead to loosening of that attachment.

In connective tissue matrices, the GAG's not only serve to limit penetration by macromolecules but also confer a high degree of hydration and hence turgidity to the ground substance along with sufficient plasticity to absorb and dissipate stress. In the laminae rarae and mesangium, the existence of a turgid, highly hydrated layer of ground substance could serve to prevent direct or intimate contact between the basement membrane and endothelium on one side and the epithelium on the other and thereby assure the availability of a large surface area for filtration. Evidently all these proposals are just suggestions which remain to be tested.

In summary, the main new findings in the present paper are (a) the demonstration of the

lattice-like arrangement of the anionic sites in the GBM; (b) the demonstration of their general occurrence throughout the lamina rara interna, lamina rara externa, and mesangial matrix; (c) the demonstration that the interaction of CF (and undoubtedly also RR) is ionic in nature; (d) the demonstration of the presence of anionic sites both *in situ* and in isolated GBM's; and (e) the demonstration of the similarity in morphology of the sites after RR staining to proteoglycan particles in connective tissue matrices.

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## REFERENCES

- BEHNKE, O., and T. ZELANDER. 1970. Preservation of intercellular substances by the cationic dye alcian blue in preparative procedures for electron microscopy. *J. Ultrastruct. Res.* **31**:424-438.
- BRENNER, B. M., M. P. BOHRER, C. BAYLIS, and W. M. DEEN. 1977. Determinants of glomerular permselectivity: insights derived from observations *in vivo*. *Kidney Int.* **12**:229-237.
- BRENNER, B. M., T. H. HOSTETTER, and H. D. HUMES. 1978. Molecular basis of proteinuria of glomerular origin. *N. Engl. J. Med.* **298**:826-833.
- CARLSON, E. C., K. BRENDL, J. T. HJELLE, and E. MEEZAN. 1978. Ultrastructural and biochemical analyses of isolated basement membranes from kidney glomeruli and tubules and brain and retinal microvessels. *J. Ultrastruct. Res.* **62**:26-53.
- CAULFIELD, J. P., and M. G. FARQUHAR. 1974. The permeability of glomerular capillaries to graded dextrans. Identification of the basement membrane as the primary filtration barrier. *J. Cell Biol.* **63**:883-903.
- 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. U. S. A.* **73**:1646-1650.
- CAULFIELD, J. P., and M. G. FARQUHAR. 1978. Loss of anionic sites from the glomerular basement membrane in aminonucleoside nephrosis. *Lab. Invest.* **39**:505-512.
- DANON, D., T. GOLDSTEIN, Y. MARIKOVSKY, and E. SKUTELSKY. 1972. Use of cationized ferritin as a label of negative charges on cell surfaces. *J. Ultrastruct. Res.* **38**:500-510.
- DISCHE, Z. 1964. The glycans of the mammalian lens capsule. A model of basement membranes. In *Small Blood Vessel Involvement in Diabetes Mellitus*. M. D. Siperstein, A. R. Colwell, Sr., and K. Meyers, editors. American Institute of Biological Sciences, Washington, D. C. 201-213.
- FARQUHAR, M. G. 1975. The primary glomerular filtration barrier—basement membrane or epithelial slits? *Kidney Int.* **8**:197-211.
- FARQUHAR, M. G. 1978. Structure and function in glomerular capillaries. Role of the basement membrane in glomerular filtration. In *Biology and Chemistry of Basement Membranes*. N. Kefalides, editor. Academic Press, Inc., New York. 43-80.
- FARQUHAR, M. G., S. L. WISSIG, and G. E. PALADE. 1961. Glomerular permeability. I. Ferritin transfer across the normal capillary wall. *J. Exp. Med.* **113**:47-66.
- FOWLER, B. A. 1978. Ruthenium red staining of rat glomerulus. Perfusion of ruthenium red into normal and nephrotic rat kidney. *Histochemie*. **22**:155-162.
- GRONIEWSKI, J., W. BICZYSKOWA, and M. WALSKI. 1969. Electron microscope studies on the surface coat of the nephron. *J. Cell Biol.* **40**:585-601.
- HAY, E. D., and S. MEIER. 1974. Glycosaminoglycan synthesis by embryonic inductors: neural tube, notochord and lens. *J. Cell Biol.* **62**:889-898.
- HAY, E. D., D. L. HASTY, and K. L. KIEHNAU. 1978. C. Morphological investigation of fibers derived from various types. Fine structure of collagen and their relation to glucosaminoglycans (GAG). In *Collagen-Platelet Interaction*. H. Gastpar, K. Kuhn, and R. Marx, editors. F. K. Schattauer Verlag, Stuttgart-New York. 129-151.
- JONES, D. B. 1969. Mucosubstances of the glomerulus. *Lab. Invest.* **21**:119-125.
- KANWAR, Y. S., and M. G. FARQUHAR. 1978. Partial characterization of anionic sites in the glomerular basement membrane. *J. Cell Biol.* **79**(2, Pt. 2): 150a. (Abstr.).
- KANWAR, Y. S., and M. G. FARQUHAR. 1978. Characterization of anionic sites in the glomerular basement membrane (GBM). *Kidney Int.* **14**:713. (Abstr.).
- KANWAR, Y. S., and M. G. FARQUHAR. 1979. Presence of heparan sulfate in the glomerular basement membrane. *Proc. Natl. Acad. Sci. U. S. A.* In press.
- KEFALIDES, N. A. 1973. Structure and biosynthesis of basement membranes. *Int. Rev. Connect. Tissue Res.* **6**:63-104.
- KRAKOWER, C. A., and S. A. GREENSPON. 1951. Localization of the nephrotoxic antigen within the isolated renal glomerulus. *Arch. Pathol.* **51**:629-639.
- LATTA, H., and W. H. JOHNSTON. 1976. The glycoprotein inner layer of glomerular capillary basement membrane as a filtration barrier. *J. Ultrastruct. Res.* **57**:65-67.
- LATTA, H., W. H. JOHNSTON, and T. M. STANLEY. 1975. Sialoglycoproteins and filtration barriers in the glomerular capillary wall. *J. Ultrastruct. Res.* **51**:354-376.
- LAURENT, T. C. 1966. *In vitro* studies on the transport of macromolecules through the connective tissue. *Fed. Proc.* **25**:1128-1133.
- LAURENT, T. C. 1977. Interaction between proteins and glycosaminoglycans. *Fed. Proc.* **36**:24-27.
- LKB-PRODUKTER AB. 1973. Instruction Manual, LKB 8100 Ampholine. Broma, Sweden.
- LUFT, J. H. 1971. Ruthenium red and violet. II. Fine structural localization in animal tissues. *Anat. Rec.* **171**:369-416.
- MATUKAS, V. J., B. J. PANNER, and J. L. ORBISON. 1967. Studies on ultrastructural identification and distribution of protein-polysaccharide in cartilage matrix. *J. Cell Biol.* **32**:365-377.
- MEEZAN, E., J. T. HJELLE, K. BRENDL, and E. C. CARLSON. 1975. A simple, versatile, nondisruptive method for the isolation of morphologically and chemically pure basement membranes from several tissues. *Life Sci.* **17**:1721-1732.
- MICHAEL, A. G., E. BLAU, and R. L. VERNIER. 1970. Glomerular polyarion. Alteration in aminonucleoside nephrosis. *Lab. Invest.* **23**:649-657.
- MOHOS, S. C., and L. SKOZA. 1969. Glomerular sialoprotein. *Science (Wash. D. C.)*. **164**:1519-1521.
- MOHOS, S. C., and L. SKOZA. 1970. Histochemical demonstration and localization of sialoproteins in the glomerulus. *Exp. Mol. Pathol.* **12**:316-323.
- MYERS, D. B., T. C. HIGHTON, and D. G. RAYNS. 1973. Ruthenium-red positive filaments interconnecting collagen fibrils. *J. Ultrastruct. Res.* **42**:87-92.
- NOLTE, A., and M. OHKUMA. 1969. Elektronenmikroskopisch-histochemische untersuchungen zur darstellung saurer mucopolysaccharide und sialinsäurehaltiger glycoproteine in nierentrinde und innerem nierenmark der ratte. *Histochemie*. **17**:170-180.
- RENNKE, H. G., R. S. COTRAN, and M. A. VENKATACHALAM. 1975. Role of molecular charge in glomerular permeability. Tracer studies with cationized ferritins. *J. Cell Biol.* **67**:638-646.
- RENNKE, H. G., and M. A. VENKATACHALAM. 1977. Glomerular permeability: *in vivo* tracer studies with polyanionic and polycationic ferritins. *Kidney Int.* **11**:44-53.
- RENNKE, H. G., Y. PATEL, and M. A. VENKATACHALAM. 1978. Glomerular filtration of proteins: clearance of anionic, neutral, and cationic horseradish peroxidase in the rat. *Kidney Int.* **13**:278-288.
- RYAN, G. B., and M. J. KARNOVSKY. 1976. Distribution of endogenous albumin in the rat glomerulus: role of hemodynamic factors in glomerular barrier function. *Kidney Int.* **9**:36-45.
- RYAN, G. B., S. J. HEIN, and M. J. KARNOVSKY. 1976. Glomerular permeability to proteins. Effects of hemodynamic factors on the distribution of endogenous immunoglobulin G and exogenous catalase in the rat glomerulus. *Lab. Invest.* **34**:415-427.
- SCHODT, K. P., R. A. GELMAN, and J. BLACKWELL. 1976. The effect of changes in salt concentration and pH on the interaction between glycosaminoglycans and cationic polypeptides. *Biopolymers*. **15**:1965-1977.
- SCHURER, J. E., D. KALICHARAN, Ph. J. HOEDEMAEKER, and I. MOLENAAR. 1978. The use of polyethyleneimine for demonstration of anionic sites in basement membranes and collagen fibrils. *J. Histochem. Cytochem.* **26**:688-689.

43. SEILER, M. W., M. A. VENKATACHALAM, and R. S. COTRAN. 1975. Glomerular epithelium: structural alterations induced by polycations. *Science (Wash. D. C.)* **189**:390-393.
44. SHEPARD, N., and N. MITCHELL. 1976. The localization of proteoglycan by light and electron microscopy using safranin O. A study of epiphyseal cartilage. *J. Ultrastruct. Res.* **54**:451-460.
45. SPIRO, R. G. 1967. Studies on the renal glomerular basement membrane. Preparation and composition. *J. Biol. Chem.* **242**:1915-1922.
46. SPIRO, R. G. 1972. Basement membranes and collagens. In *Glycoproteins: Their Composition, Structure and Function*. A. Gottschalk, editor. Elsevier Publishing Co., Amsterdam. 964-999.
47. THYBERG, J., LOHMANDER, S., and U. FRIBERG. 1973. Electron microscopic demonstration of proteoglycans in guinea pig epiphyseal cartilage. *J. Ultrastruct. Res.* **45**:407-427.
48. TRELSTAD, R. L., K. HAYASHI, and B. P. TOOLE. 1974. Epithelial collagens and glycosaminoglycans in the embryonic cornea. Macromolecular order and morphogenesis in the basement membrane. *J. Cell Biol.* **62**:815-830.
49. WESTBERG, N. G., and A. F. MICHAEL. 1970. Human glomerular basement membrane. Preparation and composition. *Biochemistry*. **9**: 3837-38'6.
50. WIGHT, T. N., and R. ROSS. 1975. Proteoglycans in primate arteries. I. Ultrastructural localization and distribution in the intima. *J. Cell Biol.* **67**:660-674.