# ROLE OF MOLECULAR CHARGE

# IN GLOMERULAR PERMEABILITY

## Tracer Studies with Cationized Ferritins

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## ABSTRACT

Mouse kidneys were perfused with Krebs-Ringer bicarbonate buffer (KRB) containing native, anionic horse spleen ferritin or various cationized derivatives, and the glomerular localization of the probe molecules determined by electron microscopy. Ferritins cationic with respect to the medium (KRB, pH 7.45) accumulated in the subendothelial layers of the glomerular basement membrane (GBM) in amounts far exceeding those observed with anionic ferritins, the degree being greater for the more cationized derivatives. Strongly cationized ferritins, in addition, permeated the full thickness of the GBM in considerable amounts, but appeared to be retarded from entry into the urinary spaces at the level of the filtration slits. Very strongly cationized derivatives adhered to glomerular endothelium and GBM and formed aggregates in the outer layers of the latter. The results suggest that intrinsic negative charges are present in the GBM and endothelium, and that the barrier function of the glomerular capillary wall may be ascribed in part to its electrophysical properties.

The primary event in urine formation is ultrafiltration of plasma across the glomerular capillary wall, a complex membrane with high hydraulic conductivity, but which, by its action as a molecular sieve (20, 36), excludes albumin and other large plasma proteins from the urinary space. Tracer studies suggest that either the glomerular basement membrane (GBM), the epithelial filtration slits, or both, may be ascribed major roles in the filtration function (4, 7, 10, 18, 21, 31, 35). The mechanisms responsible are at present unknown, but are fundamental to an understanding of the pathogenesis of proteinuria in disease states (nephrotic syndrome).

Glomerular epithelial cells are endowed with a thick anionic cell coat, rich in sialic acid residues and stainable with colloidal iron (11, 14, 27, 28). This sialoprotein extends into the filtration slits and has been postulated on theoretical grounds to pose an electrostatic barrier to the filtration of negatively charged plasma proteins (27, 28). Sialic acid residues have also been detected in GBM preparations (19, 26, 34, 37) but their functional role, if any, is unknown. We now report ultrastructural tracer experiments that indicate that the sieving properties of the GBM may be regulated by fixed anionic groups in the capillary wall, including the GBM.
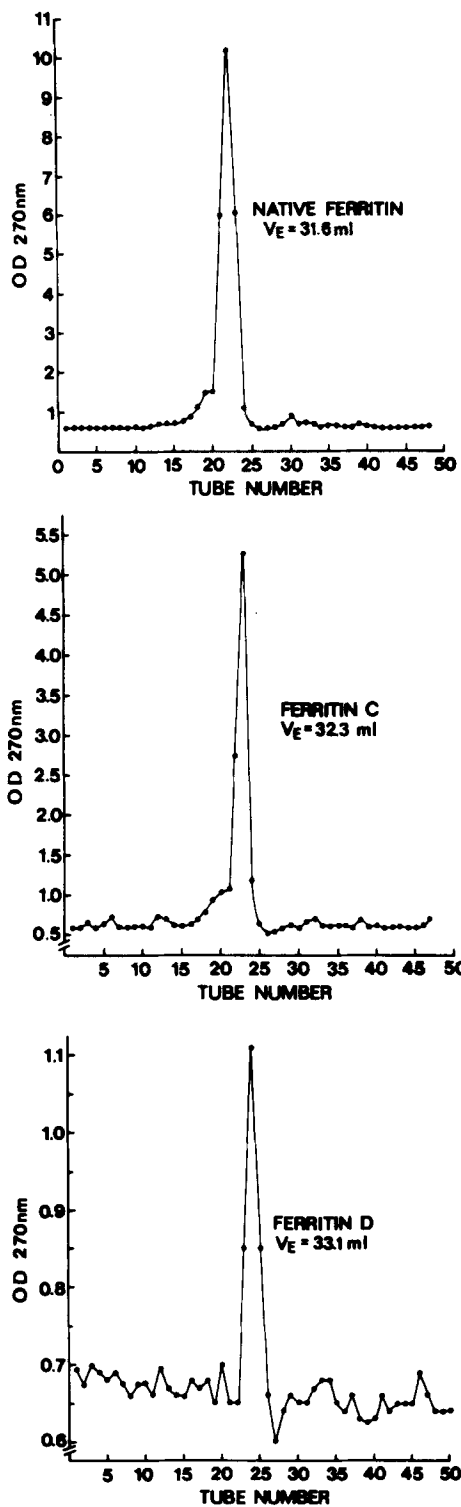
## MATERIALS AND METHODS

### Ferritin and Cationized Derivatives

The probe molecules used were horse spleen ferritin (Sigma Chemical Co., St. Louis, Mo.) and various cationized ferritin derivatives (5). These were originally prepared to specifically label anionic sites on cell membranes (5, 29). The native ferritin molecule consists of a spherical apoferritin shell with an external diameter of 122 Å (9, 12), a mol wt ≈ 480,000, and an internal core of ferric hydroxide phosphate micelles varying from 0 to 40% of its dry weight (8); the isoelectric point (pI) is 4.1–4.6 (6). The cationization reaction (13) was carried out as described previously (5) at various constant pH levels, using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride as activator and 1,6-hexanediamine as nucleophile to replace carboxyl groups. The dialyzed end product was concentrated by ultrafiltration, and ferritin was determined at 270 nm on a Gilford spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio) using a value of $E_{270}^{1\%} = 79.9$ (5).

### Characterization of Tracer Molecules

The region of isoelectric precipitation was established in distilled water by titration. Isoelectric focusing was carried out on a MRA – M 137A analytical unit (MRA Corp., Boston, Mass.) with acrylamide and ampholyte solutions of 4% and 2%, respectively. Estimation of the region of focusing was done as described by Righetti and Drysdale (reference 30; see Table I). Samples of tracer molecules were negatively stained with uranyl oxalate on Formvar-carbon coated grids and examined by electron microscopy to confirm that the preparative procedure did not significantly change the molecular size or shape. In order to confirm further a monomolecular dispersion and similarity in molecular size, three of the tracers used were subjected to gel filtration on a 1.12 cm × 50 cm Ultrogel AcA22 (LKB-Produckter AB Stockholm, Sweden) column with a bed volume ($V_t$) = 50.5 ml and void volume ($V_o$) = 17.1 ml (determined by elution of blue dextran). Samples of 1 ml were eluted with phosphate-buffered saline pH 7.2, containing 1 mg/ml bovine serum albumin

Figure 1  Elution profiles for native ferritin, ferritin C (pI 8–9) and ferritin D (pI >8.8) on a 1.12 × 50 cm Ultrogel AcA22 column. Average fraction volume = 1.46 ml. Fractionation range given by the manufacturer is 60,000–1,000,000 daltons. Dimers of ferritin and other aggregates if present in significant amounts should have eluted from the column shortly after the void volume (tube no. 12). Differences in concentration of ferritin in the 1-ml sample applied to the column and some adsorption to the gel bed by ferritin D account for the difference in the OD scale.

| | Native ferritin | Modified ferritins | | | |
| --- | --- | --- | --- | --- | --- |
| | | A | B | C | D |
| By isoelectric precipitation | 3.9–5.1 | 4.5–5.5 | 6.8–8.8 | 7.5–9 | 9.3–11.3 |
| By isoelectric focusing | 4.6 | 4.7–5.6 | 7.4–8.4 | 8–9 | >8.8 |
| Kidneys perfused ca. 4 ml/min/kidney | 6 | 2 | 4 | 2 | 6 |

to minimize adsorption of the positively charged molecules to the gel bed. Fractions with an average of 1.46 ml were collected and analyzed spectrophotometrically at 270 nm for their protein content. Elution profiles with elution volumes ($V_e$) are given in Fig. 1; the distribution coefficients were: $K_{av}$ = 0.434 for native ferritin, $K_{av}$ = 0.455 for ferritin C, and $K_{av}$ = 0.479 for ferritin D. Electron microscopic examination of ferritin molecules from the original samples and from several of the collected fractions supported on Epon and positively stained with alkaline bismuth (1) failed to reveal appreciable aggregation of molecules.

## Organ Perfusion

The method for in vitro perfusion experiments previously described by us (33) was modified for the mouse kidney. The general procedure was as follows: (a) Perfusion of the right kidney with Krebs-Ringer bicarbonate buffer (KRB) pH 7.45 at 120 mm of Hg for 1 min; followed by (b) 6–8 min of perfusion with 3 mg/ml of ferritin in KRB (two animals in each group; see Table I) at the same pressure, at the end of which a small fragment of cortex was excised and fixed by immersion in 2% formaldehyde-2.5% glutaraldehyde in 0.1 M cacodylate buffer (15); and (c) perfusion of the remainder of the kidney with the same fixative for 10 min. The results obtained by both methods of fixation did not differ. In addition, data were obtained from kidneys perfused for shorter (1-4 min) and longer (10 min) time periods (included in Table I); the results were similar and reproducible, irrespective of the duration of perfusion. After 3 h in the fixative, tissue was prepared for electron microscopy by standard procedures. Accentuation of membrane structure was achieved by postfixation in

ferro-osmium (17), or through *en bloc* uranyl acetate staining (16). Thin sections cut with diamond knives were stained with alkaline bismuth for ferritin (1) and examined in a Philips 201 electron microscope (Philips Electronic Instruments, Mt. Vernon, N.Y.) without counterstain. The pI ranges of native and modified ferritins as determined by titration and isoelectric focusing and the number of animals perfused with each of the tracers are shown in Table I.

## RESULTS

By electron microscopy, native ferritin localized in sparse amounts in the lamina rara interna (LRI, subendothelial layer) of the GBM and occasionally in the lamina densa (LD) (Fig. 2). Localization of modified, but still anionic, ferritin (ferritin A, pI 4.7–5.6) was similar to that of native ferritin. Use of ferritins of progressively higher pIs changed the distribution dramatically (Fig.s 3–7). Ferritin B (pI 7.4–8.4) accumulated in the LRI in amounts far exceeding those observed with anionic ferritin (Fig. 3). The accumulation was more marked opposite endothelial fenestrae. With ferritin C (pI 8–9), accumulation of the protein in the LRI and endothelial fenestrae was more intense. In addition, numerous particles were localized in the lamina rara externa (LRE, subepithelial layer) of the GBM, sometimes appearing as aggregates behind the filtration slits (Fig. 4). Localization of ferritins with pI greater than 8.8 (ferritin D) is shown in Figs. 5–7. Concentration of the tracer in

FIGURES 2–5  Mouse glomerular capillary wall, 6–8 min after perfusion of 3 mg/ml of tracer in Krebs-Ringer bicarbonate solution. With native ferritin, pI 4.6 (Fig. 2), only occasional molecules are seen in the lamina rara interna. Cationized ferritin B, pI 7.4–8.4 (Fig. 3), accumulates in the lamina rara interna but is largely confined to this layer. Ferritin C with pI 8–9 (Fig. 4) penetrates the glomerular filter to a greater extent with numerous particles reaching the lamina rara externa. some of them accumulating proximal to the filtration slits. This phenomenon is accentuated with cationized ferritin D with pI 8.8 and higher (Fig. 5). The tracer adheres to the endothelial cell surface, accumulates in the lamina rara interna, and reaches the lamina rara externa, where it forms aggregates in the filtration slits. Figs. 2–5 postfixed in ferro-osmium and stained with alkaline bismuth. (× 55,000 approx.).
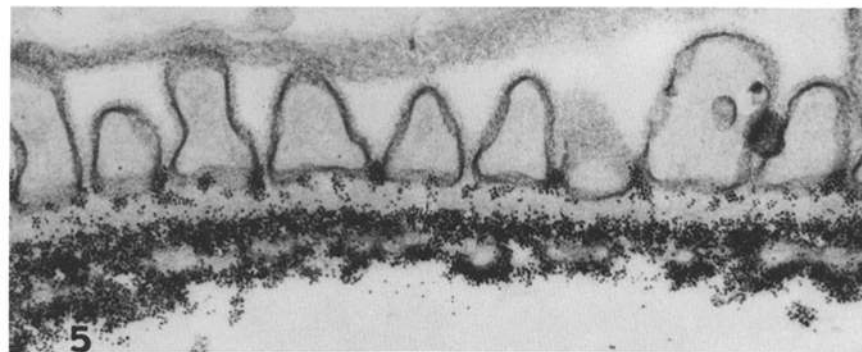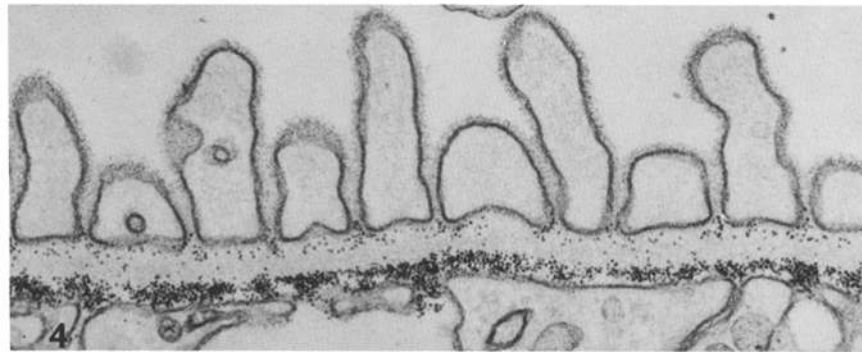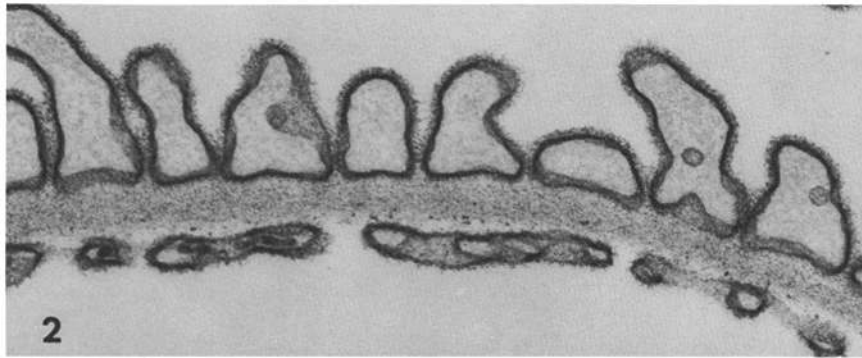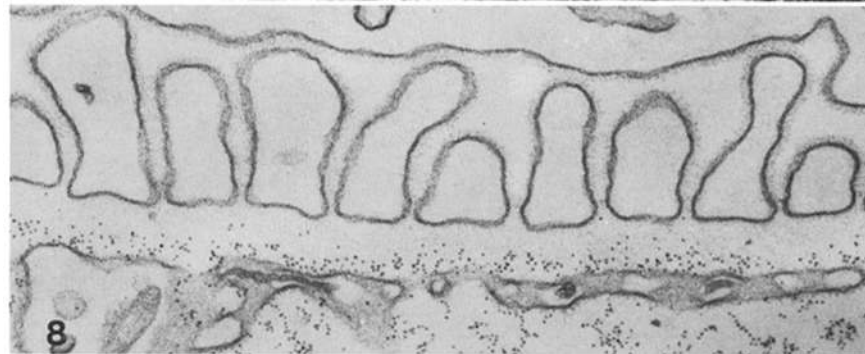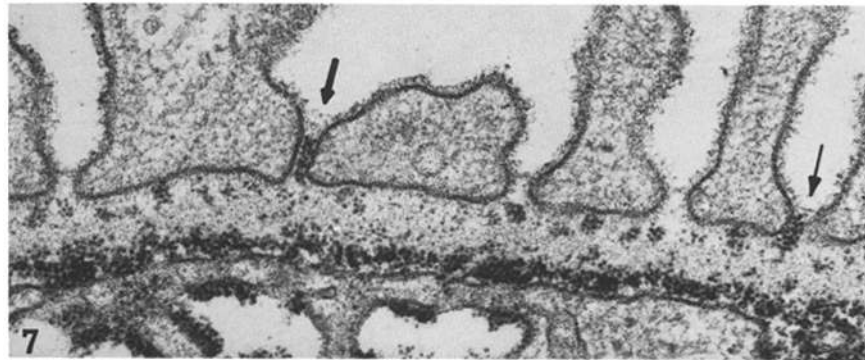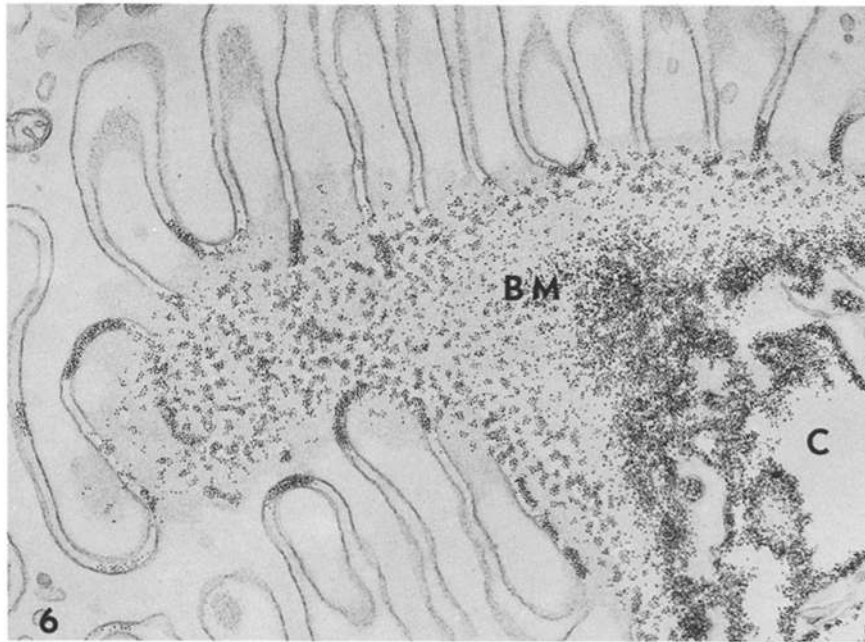
the GBM was noticeably greater, with proportionately larger amounts appearing in each layer. Thus, a gradient of concentration across the LD was observed, as with the weaker cationized ferritins. Marked accumulation of cationic ferritin occurred proximal to the level of the filtration slit diaphragms (Figs. 5, 6; thin arrow, Fig. 7). In occasional slits ferritin aggregates apparently encroached upon the level of these limiting structures (thick arrow, Fig. 7). In addition, this strongly cationized ferritin showed marked affinity or adherence to the cell membranes of capillary endothelial cells and both LRI and LRE where they were localized in the form of clumps or aggregates. Prolonged (15 min) subsequent reperfusion with KRB before fixation failed to alter the distribution of adherent particles; whereas particles of anionic ferritin could be easily removed from the areas of localization by such treatment. In no case, however, with either anionic or cationic ferritins were tracer molecules localized in the urinary spaces. Likewise, uptake of tracer particles by glomerular epithelial cells were not observed in these experiments.

In a separate experiment, native ferritin was perfused in a concentration of 50 mg/ml in an effort to achieve distribution patterns in the GBM similar to that seen with lower doses (3 mg/ml) of cationized ferritins. Fig. 8 illustrates that this could not be accomplished; although greater numbers of ferritin molecules localized in the GBM, the distribution pattern remained the same with only occasional particles encroaching upon the lamina densa, as previously described for this tracer (7).

## DISCUSSION

Our experiments show that the localization and distribution of ferritin molecules of similar sizes within the glomerular capillary wall can be altered by changing the charge of the tracer. The results suggest: (a) that the GBM and endothelial cell membranes are negatively charged structures; (b) that the GBM, along with the endothelium, constitutes the primary filter for large anionic proteins; the properties of the filter are largely determined by electrophysical characteristics; and (c) the filtration slit diaphragm constitutes a further potential barrier distal to the GBM.

The first inference is based on the finding of aggregation of cationic ferritin (pI 8.8 and higher) in the lamina rara interna and externa of the GBM, adherence of the tracer to the endothelium, and failure to dislodge the tracer particles by KRB reperfusion. During the course of previous work from this laboratory (33), it was observed that perfusion with cationic macromolecules (poly-L-lysine, poly-L-arginine, protamine sulfate, and lysozyme), but not with neutral or anionic proteins (myoglobin, poly-DL-alanine, heparin, polyglutamic acid, and ovalbumin), resulted in the formation of electron dense aggregates in the GBM, particularly in the lamina rara externa. Similar patterns have also been observed with the cationic reagents colloidal iron and ruthenium red (11, 14). Since studies on isolated GBM suggest the presence of significant amounts of sialic acid (19, 26, 34, 37), the above findings are consistent with a model of the GBM composed of a central fibrous protein-polysaccharide lattice (the lamina densa),

FIGURE 6   Slightly oblique cut through a glomerular capillary loop (C) from kidney perfused with ferritin D (pI 8.8 and higher). The basement membrane (BM) shows irregularly distributed ferritin particles that tend to occur in aggregates. Many epithelial foot-processes (to the left) are sectioned coplanar with the basement membrane which shows accumulation of the tracer in the filtration slits. Same technique as Figs. 2-5. (× 38,000 approx.).

FIGURE 7   Glomerulus from kidney perfused with 3 mg/ml cationized ferritin D (pI 8.8 and higher) for 8 min en bloc stained with uranyl acetate, counterstained with alkaline bismuth and lead citrate. Numerous tracer particles are seen in the lamina rara externa in form of aggregates, frequently in relation to the slit diaphragm, which can be observed distal to the site of ferritin accumulation (thin arrow). Occasionally this thin structure is· no longer visible (thick arrow) and it appears as if the tracer had penetrated beyond this level. (× 63,000 approx.).

FIGURE 8   Mouse glomerular capillary wall 8 min after perfusion of native ferritin 50 mg/ml in Krebs-Ringer bicarbonate solution. Discrete particles are observed at the level of the lamina rara interna in concentrations similar to that reached in the capillary lumen. Occasional particles are seen beyond this layer. Postfixed in ferro-osmium, stained with alkaline bismuth. (× 55,000 approx.).

interposed between two less dense but negatively charged layers (lamina rara interna and externa).

Our findings with anionic ferritins, in agreement with the original study by Farquhar et al. (7), indicate that restriction of these large anionic proteins occurs in the proximal layers of the glomerular filter. However, the mechanisms and structures responsible for this restriction need to be defined. Theoretical considerations suggest that the "skin" of the glomerular ultrafilter should be located in close contact with the capillary lumen containing the solution to be filtered (plasma) as in artificial ultrafiltration systems (23). If the limiting structure were to be placed distal to the LRI and endothelial fenestrae (such as for example, the lamina densa), continuous and progressive accumulation of tracer molecules in the relatively unstirred subendothelial layers may be expected to occur as a function of ultrafiltration flux. In our present experiments with negatively charged ferritins, very few molecules localized in the LRI. However, with increasing pI, cationized derivatives did accumulate proximal to the lamina densa in amounts out of proportion to the concentration of tracer in the perfusate (see Figs. 2-5) and further penetrated the more distal layers of the GBM. Even when the perfusate concentration of anionic ferritin was increased by a factor of more than 15 (see Fig. 8), the amount of this negatively charged tracer in the GBM did not exceed that observed with low concentrations of the cationized derivatives.

The observed differences in the localization of various ferritins cannot be due to steric hindrance alone (pore theory) since (a) the diameter of the endothelial fenestrae exceeds that of anionic as well as cationic ferritins by a factor of up to 9; and (b) the disparity in the degree of penetration of tracer into the GBM between differently charged ferritins was not dependent on variations in molecular size or shape. The differences may be explained if one considers that the character of the primary limiting barrier to anionic macromolecules is partly determined by the fixed negative charges in the gel that constitutes the LRI and the glycocalyx lining the endothelial fenestrae. The result of the interaction between these innermost layers of the capillary wall and anionic ferritins can be compared to the exclusion of certain macromolecules from naturally occurring and artificial charged gels (22, 24). These electrophysical phenomena would represent an important factor that influences the movement of macromolecules towards the glomerular filtering membrane in

addition to (a) convectional flux or solvent drag and diffusion; and (b) fluid shear parallel to the membrane surface, accomplished by the laminar flow. The electrostatic forces generated by the anionic sites are repulsive for negatively charged ferritins, act synergistically with the fluid shear, and tend to prevent these molecules from entering the GBM to any important extent. For cationized ferritin, the electrostatic forces result in attraction which acts in conjunction with the convectional flux, dragging the tracer into the basement membrane. With increasing pI of the tracer, the ability of the glomerular filter to exclude these molecules from the GBM and retain them in the perfusing solution is progressively decreased (Fig. 2-5).

Previous experiments (4, 7, 35) and our present studies suggest that the lamina densa constitutes an additional limiting barrier to the filtration of macromolecules. Thus, concentration gradients across this structure were observed irrespective of the pI of the tracer molecules, the absolute concentration reached in the LRI, and the actual number of molecules traversing it. The mechanism by which this component of the glomerular capillary wall exerts its function is at present not known. Whether its action is determined by mechanical "pores" of fixed dimensions or by pathways subject to change by electrostatic interaction, as in artificial gel systems (22, 24), remains to be determined.

Our observations are also consistent with more recent studies which suggest that the primary filtering mechanism in the glomerulus is located in the proximal layers of the glomerular capillary wall (32). They may also be relevant to the study of Westberg and Michael (37) who showed that sonicated, washed human GBM contains bound fibrinogen, IgG, and albumin roughly in the ratio of 8.6:2.4:1. Considering the relatively low plasma content of fibrinogen and IgG compared with that of albumin, this would indicate preferential localization of the first two of these molecules in the GBM. The accumulation of fibrinogen may be due to trapping of these filamentous molecules within the lattice structure of the GBM (37). Our results may explain the preferential localization of the basic IgG molecules (pI 7.3 8.2) in the GBM as being due to charge effects. Whether GBM-bound fibrinogen and IgG have any role to play in the glomerular restrictive function is at present unknown.

Accumulation of cationized ferritin, proximal to the level of the filtration slit diaphragms without actual entry of tracer into the urinary spaces, may

indicate that these diaphanous membranes (40 Å thick, with repeating subunits and isoporous structure [18, 31]) represent an additional mechanical barrier operating in tandem with the endothelium and GBM (10, 35). However, the localization of the primary barrier to anionic and neutral molecules in the proximal layers of the glomerular filter (4, 7, 32, 35) would suggest that the restrictive function of the slit diaphragms in vivo is secondary.

Prior studies have suggested a role for altered or diminished glomerular polyanion in proteinuric states (2, 3, 25). Alterations in glomerular sialic acid metabolism, and diminished stainability of anionic sites have been shown to occur in experimental nephrosis and coincide with the onset of proteinuria (2, 3, 25). We previously showed that neutralization of glomerular anionic sites rapidly leads to epithelial changes that mimic those found in the nephrotic syndrome (33).

These observations and the results of our present study suggest a role for endothelial, GBM, and epithelial anionic sites in the maintenance of the normal glomerular filtration function and architecture and point out the need for investigation of the role played by them in the pathogenesis of proteinuria.

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