

Distribution of anionic sites in glomerular basement membranes: Their possible role in filtration and attachment

(lysozyme binding site/epithelial attachment/glomerular filtration)

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ABSTRACT Lysozyme ($pI = 11$) has been used to identify anionic sites in the glomerular capillary wall. A solution of 1-3% lysozyme was perfused into the left kidney at varying rates. After perfusion, the kidney was fixed *in situ* and processed for electron microscopy. Lysozyme was seen as an electron-dense deposit which was not present when succinylated lysozyme ($pI = 4.5$) or myoglobin ($pI = 6.9$) was perfused instead of native lysozyme. First, the epithelial cell plasma membrane was outlined by a 300-400 Å electron-dense layer. Second, there were discrete dense deposits in the subepithelial portions (lamina rara externa) of the basement membrane which, in normal section, extended from the epithelial cell membrane to the lamina densa and, in grazing section, formed a continuous reticular pattern. The discrete binding sites in the lamina rara externa were also found in glomeruli that had been prefixed and then perfused with lysozyme and in isolated glomeruli incubated with lysozyme. Third, the lamina densa itself showed a homogeneous increase in density. Fourth, similar discrete dense areas were seen in the subendothelial light layer of the basement membrane, between Bowman's capsule and the parietal epithelium, and between the endothelium of peritubular capillaries and its basement membrane. The experiments show that, in addition to their location on the epithelial surface, anionic sites are present throughout the basement membrane and are distributed in a discrete reticular pattern in the lamina rara externa.

This paper is an outgrowth of work published previously (1, 2) in which dextrans were used as tracers to investigate glomerular capillary permeability. Initially dextran was injected into rats *in vivo*. Later on these studies were extended to include *in vitro* perfusions of dextran in protein solutions. The first protein used for this purpose was lysozyme ($pI = 11$; molecular weight = 14,000). When lysozyme was present in such perfusates, a dense layer—presumed to consist of bound lysozyme—was observed on the outer surface of the plasma membrane of the glomerular epithelial cell, and, in addition, patchy dense areas were found in the glomerular basement membrane (3). This distribution was so striking that it was decided to investigate the presumptive lysozyme binding further on the assumption that it may reveal anionic sites in the glomerular capillary wall.

MATERIALS AND METHODS

Dextran T-70 (molecular weight = 70,000) was obtained from Pharmacia Fine Chemicals, Piscataway, N.J. Egg white lysozyme (Grade I) and equine skeletal muscle myoglobin were obtained from Sigma Chemical Co., St. Louis, Mo. Succinylated lysozyme was prepared using a modification of the procedure of Habeeb and Atassi (4). The isoelectric point (pI) of the succinylated lysozyme was determined on an LKB-Ampholine column using standard techniques (5).

Lysozyme Perfusion Experiments. The basic design of these experiments was to isolate the vasculature of the left kidney from the systemic circulation with clamps as previously described (6) and then to perfuse the kidney retrograde through the aorta with buffer followed by the lysozyme test solution.

Ten Sprague-Dawley rats weighing between 140 and 300 g were used, and perfusion was carried out with a Harvard Infusion pump at rates varying from 0.97 to 5.0 ml/min. Rats were perfused with 2-3 ml of Krebs-Ringer's-bicarbonate, pH 7.4, followed by 20 ml of a solution consisting of 1-3% lysozyme and 4% Dextran T-70 in Krebs-Ringer's-carbonate. In addition, the following controls or variations of this procedure were carried out: (i) 3% myoglobin was substituted for lysozyme; (ii) 2% succinylated lysozyme was substituted for lysozyme; (iii) 15 ml of Karnovsky's aldehyde fixative (7) was perfused before the lysozyme-dextran solution; and (iv) 10 ml of Krebs-Ringer's-bicarbonate was perfused after the lysozyme-dextran solution.

Experiments with Isolated Glomeruli. Glomeruli were isolated from three rats weighing 180-365 g using a variation of the technique of Greenspon and Krakower (8). Glomeruli were separated from minced renal cortex by passing them through a 100 mesh stainless steel screen; they were subsequently collected on a 150 or 200 mesh screen. The isolated glomeruli were incubated in 2 ml of 2% lysozyme in Krebs-Ringer's-bicarbonate for 30 min at 25°. In two experiments the glomeruli were fixed immediately after incubation with lysozyme, and in two other experiments they were washed with Krebs-Ringer's-bicarbonate before fixation.

Tissue Processing. Most kidneys perfused with lysozyme-dextran or myoglobin-dextran were fixed by injection *in situ* as previously described (1) using the fixative mixture of Simionescu *et al.* (9) (which contains 1.5% formaldehyde, 2.5% glutaraldehyde, 0.66% OsO₄, and 2-3% lead citrate in 0.1 M arsenate buffer, pH 7.4) required for the demonstration of dextrans. Blocks of these tissues were dehydrated and embedded in Epon without further treatment (1). In a few cases, e.g., those perfused with lysozyme-dextran and those prefixed by aldehyde perfusion before lysozyme, kidneys were fixed *in situ* with Karnovsky's aldehyde fixative (7) (consisting of 1% formaldehyde and 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, with 4.4 mM CaCl₂). Blocks from these kidneys were postfixated in 1% OsO₄ in acetate-veronal buffer (pH 7.2) and stained in block with uranyl acetate (10) before dehydration. Isolated glomeruli were fixed in suspension in aldehyde fixative (7), pelleted, postfixated in OsO₄, and stained with uranyl in block. Techniques for sectioning and microscopy were the same as given previously (1).

RESULTS

The normal glomerular capillary wall is composed of three layers: (i) a fenestrated endothelium facing the capillary lumen, (ii) a continuous basement membrane, and (iii) an elaborate epithelial layer that covers the basement membrane with interdigitating projections known as foot processes which are separated by 250 Å gaps—the so-called filtration slits. The

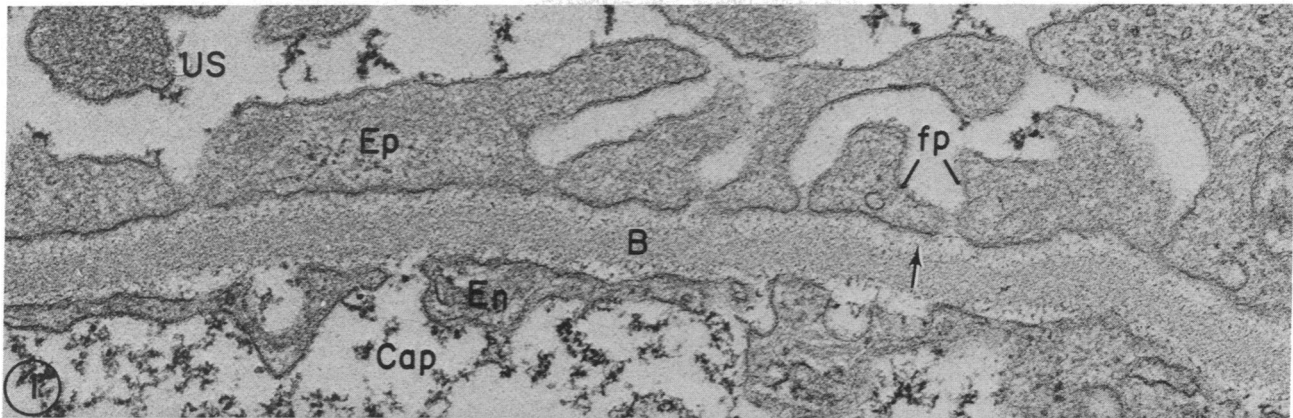


FIG. 1. Glomerular capillary wall from a rat perfused with succinylated lysozyme. The epithelium (Ep) with its foot processes (fp) is seen above the basement membrane (B). The latter consists of three layers: (i) a thin light layer, the lamina rara externa, adjoining the epithelium (arrow); (ii) a central broad layer of greater density, the lamina densa (B); and (iii) another thin light layer, the lamina rara interna, adjoining the endothelium (En). The capillary lumen (Cap) and urinary space (US) contain dextran particles, but otherwise the glomerular structure appears normal. Tissue fixed *in situ* with special fixative to demonstrate dextrans (9). $\times 43,000$.

basement membrane itself consists of three distinct layers of differing electron density—a thin layer of low density adjoining the endothelium (lamina rara interna), a thicker, central dense layer (lamina densa), and a second thin layer of low density adjoining the epithelium (lamina rara externa) (Fig. 1).

Lysozyme experiments

The glomerular capillary wall from animals perfused with native lysozyme ($pI = 11$) and dextran either before or after fixation differed from the normal in the occurrence of dense deposits in several places (Figs. 2–4). Since they were not seen in animals perfused with dextran alone or with succinylated lysozyme or myoglobin (see below), these deposits were presumed to be due to the binding of lysozyme to components of the capillary wall. First, the entire epithelial cell membrane—both those portions surrounding the foot processes and those portions surrounding the epithelial cell bodies—appeared outlined by a uniform 300–400 Å dense-staining layer (Figs. 2 and 3). The only exception was at the base of the foot processes, where the layer was noticeably thinner (Fig. 2). At the

level of the slits the two opposing layers were thick enough to completely fill the slits. Second, in the lamina rara externa there were dense deposits consisting of discrete areas of increased electron density which sometimes exhibited a regular pattern, particularly in partially flushed glomeruli. In normal sections through the capillary wall (Figs. 2 and 4), these deposits appeared to extend from the lamina densa to the epithelial cell membrane. They were typically located at the base of the foot processes and were absent along the filtration slits. When cut in grazing section, the densities were seen to form an apparently continuous reticular pattern (Fig. 3). Third, the lamina densa showed a slight but definite homogeneous increase in electron density (Fig. 3). Some deposits were seen in other components of the capillary wall, but in these locations the binding of lysozyme was less striking. Areas of increased density were also seen in the lamina rara interna and mesangium, but the pattern was less regular (Figs. 2 and 4). The luminal surface of the endothelium was also covered by a continuous layer in some capillary loops but not in others.

Outside the glomerulus similar discrete dense areas due to

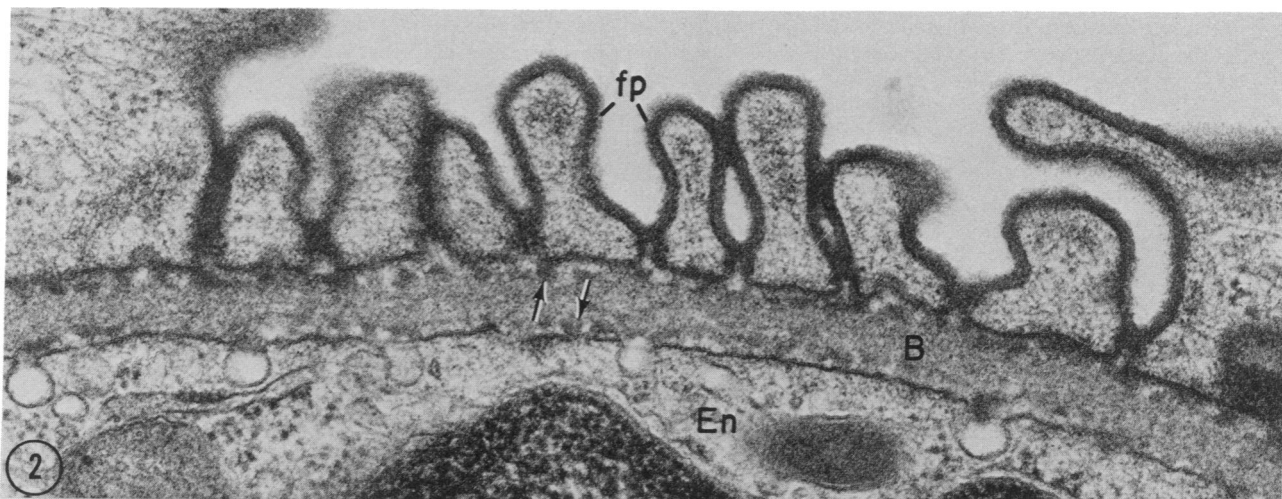


FIG. 2. Capillary wall from a rat perfused with native lysozyme. Note the binding of the lysozyme to the cell coat of the foot processes (fp) where it forms a continuous layer 300–400 Å thick except at the base of the foot processes where it is considerably less. Discrete electron densities are also seen in both the lamina rara externa and interna (arrows). The lamina densa (B) also shows a slight but definite general increase in density. En = endothelial cell. Tissue fixed *in situ* with formaldehyde-glutaraldehyde (7), postfixated in OsO_4 , and stained in block with uranyl acetate. $\times 43,000$.

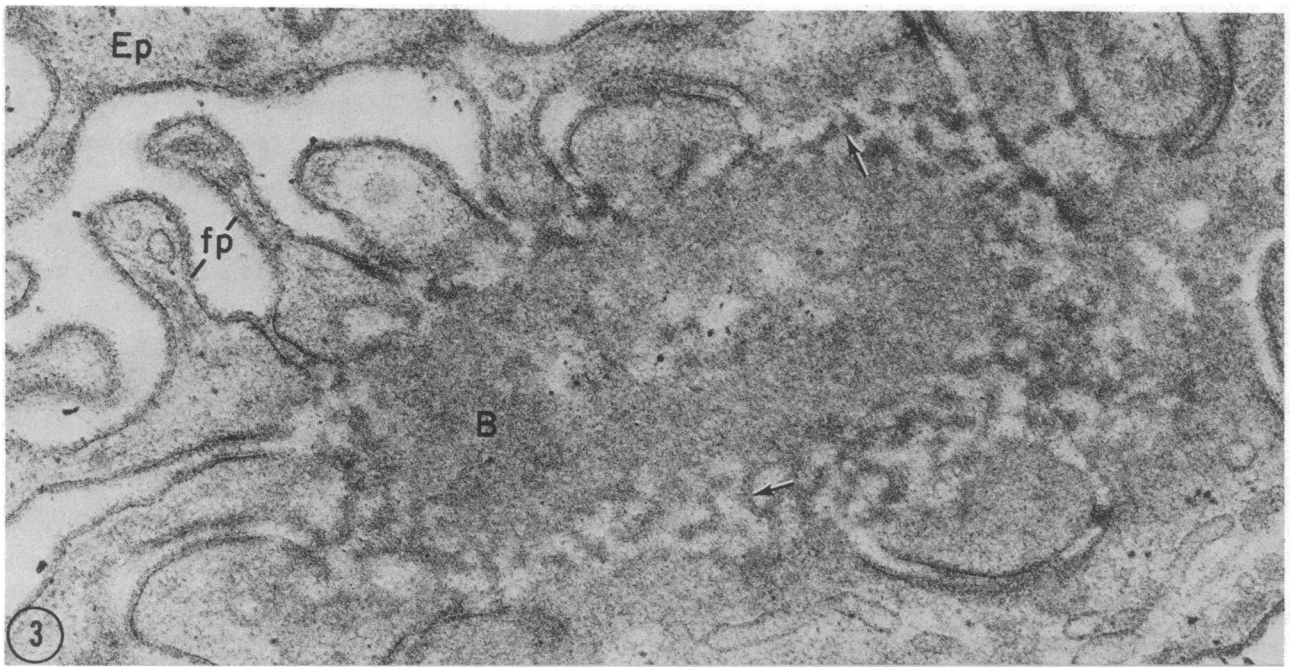


FIG. 3. Grazing section of capillary wall from a rat perfused with native lysozyme and flushed with Krebs-Ringer's-bicarbonate, which shows the regular reticular pattern of discrete densities in the lamina rara externa (arrows). Note also the binding of lysozyme to the foot processes (fp) and to the lamina densa (B) which shows increased density. Tissue preparation as in Fig. 1. $\times 57,000$.

lysozyme binding were seen between the tubular epithelium and its basement membrane, between Bowman's capsule and the parietal epithelium, and between the endothelium of peritubular capillaries and its basement membrane. A continuous dense layer was also seen along the luminal plasma membrane of parts of the tubule epithelium in the cortex and on the blood front of arterial and capillary endothelia. Lysozyme also bound to collagen fibrils in a periodic pattern.

In isolated glomeruli incubated in lysozyme, binding was seen in the lamina rara externa as described in perfused glomeruli, but little or no binding was seen on the cell coat.

Controls

When myoglobin ($pI = 6.9$; molecular weight = 17,000) (11) or succinylated lysozyme ($pI = 4.5$) was substituted for native lysozyme in the perfusate, dextran was seen in both the capillary lumen and the urinary spaces, indicating that the perfusate had

reached and penetrated the capillary wall; however, no areas of increased density, indicating binding of lysozyme, were seen anywhere in the glomerulus or elsewhere in the examined regions of the kidney, and all the elements of the glomerular capillary wall in these animals had the usual or "normal" appearance (Fig. 1).

DISCUSSION

The results of these experiments leave little doubt that the cationic protein, lysozyme, binds to components of the glomerular capillary by interaction with anionic groups. That lysozyme binds because of its high, positive net charge ($pI = 11$) is indicated by the failure of myoglobin ($pI = 6.9$) to bind as well as by the fact no lysozyme binding occurred when the net charge on the molecule was made negative ($pI = 4.5$) by succinylation. Apparently the lysozyme is acting as a "stain" for anionic groups, and what is demonstrated by this binding is the

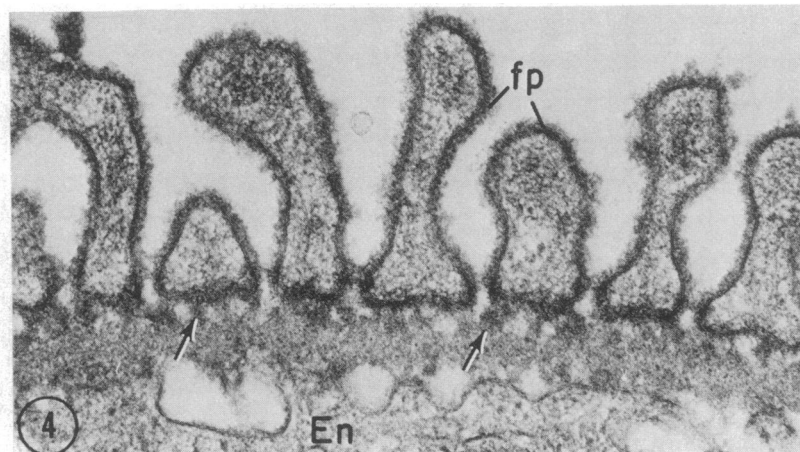


FIG. 4. Capillary wall from kidney perfused first with fixative and then with lysozyme. Note the thick coat of lysozyme on the foot processes (fp) and the discrete densities (arrows) between the lamina densa and the foot processes and the endothelium (En). The densities extend from the basement membrane to the base of the foot processes and are mainly absent along the filtration slits. Tissue preparation as in Fig. 2. $\times 57,000$.

presence of anionic sites located throughout all the layers of the basement membrane, particularly in the lamina rara externa where they assume a striking reticular pattern. It is clear that this binding pattern is not an artifact, i.e., aggregates of lysozyme (and other residual proteins) produced during perfusion, since it is seen in isolated glomeruli. It is also clear that the pattern is not the result of lysozyme aggregating mobile sites in the basement membrane, since it is seen in prefixed glomeruli. In addition to the binding to the basement membrane, binding to anionic sites on the outer surfaces of cell membranes was demonstrated which was particularly pronounced in the glomerular epithelium but was also seen on membranes of other cell types (glomerular endothelium, endothelium of peritubular capillaries, and luminal surface of proximal tubule epithelium). Anionic sites on membranes or "cell coats" have been seen by others. Therefore, what is particularly striking in the present work is (a) the demonstration of anionic sites throughout all the layers of the basement membrane, and (b) the delineation of the reticular pattern of anionic sites in the lamina rara externa.

The anionic sites on the membrane of the glomerular epithelial cell (as well as on other cell membranes) are most probably due to the local presence of sialoglycoproteins (i.e., cell coats or intrinsic membrane proteins). The anionic sites detected in the basement membrane could be carboxyl groups of the collagenous or noncollagenous glycoproteins, sialyl groups of glycoproteins, or sulfated groups of glycosaminoglycans. The presence of the first two has been demonstrated in isolated glomerular basement membranes by biochemical analysis, but sulfated glycosaminoglycans have not been detected (12-14).

Physiologic data clearly indicate that the glomerular filter selects by both size and charge (15-17), and there is now growing agreement among workers in the field (see refs. 3, 18, and 19) that the basement membrane acts as the size barrier. Recently it has been surmised that the epithelial slits (17, 21) are the charge barrier because of the high concentration of anionic sites provided by the close apposition of epithelial cell membranes at this level, as demonstrated repeatedly by the colloidal iron (20-23), alcian blue (24, 25), and ruthenium red (21, 25) techniques. Our new findings indicate, however, that anionic sites are also present in the basement membrane throughout all its layers, and thus they open the possibility that the basement membrane is the barrier for both size and charge. The recent work of Rennke *et al.* (18) demonstrates, in fact, that the basement membrane does select molecules of the same dimensions according to charge (ferritin versus cationized ferritin). It is worthwhile pointing out that a common size and charge barrier would represent a practical and efficient bioengineering solution for the filtration problem. Our hypothesis does not necessarily leave the anionic sites in the epithelial slits without an important function: the work of Seiler *et al.* (26) suggests that they may play a role in maintaining the epithelial slits open for passage of the glomerular filtrate.

The specialized sites in the lamina rara externa may play a further role not necessarily related to filtration, because similar discrete binding sites for cations were seen between many epithelia and endothelia and their basement membranes. The exact function of such anionic sites is unknown, but their location and morphology suggest that they might be related to the attachment of the cells in question to their basement membranes, or in more molecular terms, the binding of membrane sialoglycoproteins to collagens. Perhaps the binding sites are more prominent between the visceral epithelial cell and the basement membrane because of the high hydraulic pressure in glomerular capillaries and the need for particularly strong attachment in this location.

The assumption of a dual role in filtration and attachment for anionic sites in the glomerular basement membrane is supported by observations made on aminonucleoside-nephrotic rats in which loss or absence of anionic sites [as determined by loss of colloidal iron (23) and lysozyme binding (J. P. Caulfield and M. G. Farquhar, unpublished findings)] is associated with proteinuria as well as a progressive loosening of the epithelial cell-basement membrane attachment (6).

Finally, the existence of fixed negative charges in the basement membrane raises the question of what their role is in the trapping of antigen-antibody complexes which occurs in immune complex disease. In this regard it is of interest to note that in certain types of immune complex-induced glomerulonephritis, occurring in man or experimental animals, which are characterized by the deposition of soluble immune complexes in glomeruli, the deposits typically occur in the lamina rara externa (27-30).

Note Added in Proof. Since submitting this manuscript we have found that lysozyme that had been catalytically inactivated with 2-methoxy-5-nitrobenzyl bromide according to the technique of Reiss and Lukton [(1975) *Bio-Org. Chem.* 4, 1-25] binds to glomerular components in the same manner as native lysozyme. This shows that the catalytic site is not involved in lysozyme binding.

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1. Caulfield, J. P. & Farquhar, M. G. (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.
2. Caulfield, J. P. & Farquhar, M. G. (1975) "The permeability of glomerular capillaries of aminonucleoside nephrotic rats to graded dextrans," *J. Exp. Med.* 142, 61-83.
3. Farquhar, M. G. (1975) "The primary glomerular filtration barrier-basement membrane or epithelial slits? *Kidney Int.* 8, 197-211.
4. Habeeb, A. F. S. A. & Atassi, M. Z. (1971) "Enzymatic and immunochemical properties of lysozyme. V. Derivatives modified at lysine residues by guanidination, acetylation, succinylation or maleylation," *Immunochemistry* 8, 1047-1059.
5. LKB-Produkter AB. Bromma, Sweden, (1973) *Instruction Manual, LKB 8100 Amopholine.*
6. Caulfield, J. P., Reid, J. A. & Farquhar, M. G. (1976) "Alterations of the glomerular epithelium in aminonucleoside nephrosis. Evidence for formation of occluding junctions and epithelial cell detachment," *Lab. Invest.* 34, 43-59.
7. Karnovsky, M. J. (1965) "A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy," (abstr.) *J. Cell Biol.* 27, (2): 137A.
8. Greenspon, S. A. & Krakower, C. A. (1950) "Direct evidence for the antigenicity of the glomeruli in the production of the nephrotoxic serums," *Arch. Pathol.* 49, 291-297.
9. Simionescu, N., Simionescu, M. & Palade, G. E. (1972) "Permeability of intestinal capillaries. Pathway followed by dextrans and glycolgens," *J. Cell Biol.* 53, 365-392.
10. Farquhar, M. G. & Palade, G. E. (1965) "Cell junctions in amphibian skin," *J. Cell Biol.* 26, 263-291.
11. Mahler, H. R. & Cordes, E. H. (1971) in *Biological Chemistry* (Harper and Row, New York), 2nd ed., p. 96.
12. Kefalides, N. A. & Winzler, R. J. (1966) "The chemistry of glomerular basement membrane and its relation to collagen," *Biochemistry* 5, 702-713.
13. Spiro, R. G. (1967) "Studies on the renal glomerular basement membrane," *J. Biol. Chem.* 242, 1915-1922.
14. Westberg, N. G. & Michael, A. G. (1970) "Human glomerular basement membrane. Preparation and composition," *Biochemistry* 9, 3837-3846.
15. Wallenius, G. (1954) "Renal clearance of dextran as a measure

- of glomerular permeability," *Acta. Soc. Med. Ups.* **59**, 1-91.
16. Renkin, E. M. & Gilmore, J. P. (1973) "Glomerular filtration," in *Handbook of Physiology*, eds. Orloff, J. & Berliner, R. W. (Amer. Physiological Society, Washington, D.C.) Vol. 8, *Renal Physiology*, pp. 185-248.
 17. Chang, R. L. S., Deen, W. M., Robertson, C. R. & Brenner, B. M. (1975) "Permeability of the glomerular capillary wall: III. Restricted transport of polyanions," *Kidney Int.* **8**, 212-218.
 18. Rennke, H. G., Cotran, R. S. & Venkatachalam, M. A. (1975) "Role of molecular charge in glomerular permeability. Tracer studies with cationized ferritin," *J. Cell Biol.* **67**, 638-646.
 19. Ryan, G. B. & Karnovsky, M. J. (1976) "Distribution of endogenous albumin in the rat glomerulus: Role of hemodynamic factors in glomerular barrier function," *Kidney Int.* **9**, 36-45.
 20. Jones, D. B. (1969) "Mucous substances of the glomerulus," *Lab. Invest.* **21**, 119-125.
 21. Latta, H., Johnston, W. H. & Stanley, T. M. (1975) "Sialoglycoproteins and filtration barriers in the glomerular capillary wall," *J. Ultrastruct. Res.* **51**, 354-376.
 22. Mohos, S. C. & Skoza, L. (1970) "Histochemical demonstration and localization of sialoproteins in the glomerulus," *Exp. Mol. Pathol.* **12**, 316-323.
 23. Michael, A. F., Blau, E. & Vernier, R. L. (1970) "Glomerular polyanion: Alteration in aminonucleoside nephrosis," *Lab. Invest.* **23**, 649-657.
 24. Behnke, O. & Zelandar, T. (1970) "Preservation of intercellular substances by the cationic dye alcian blue in preparative procedures for electron microscopy," *J. Ultrastruct. Res.* **31**, 424-438.
 25. Shea, S. M. (1971) "Lanthanum staining of the surface coat of cells. Its enhancement by the use of fixatives containing alcian blue or cetylpyridinium chloride," *J. Cell Biol.* **51**, 611-620.
 26. Seiler, M. W., Venkatachalam, M. A. & Cotran, R. S. (1975) "Glomerular epithelium: Structural alterations induced by polyanions," *Science* **189**, 390-393.
 27. Cochrane, C. G. & Koffler, D. (1973) "Immune complex disease in experimental animals and man," *Adv. Immunol.* **16**, 185-264.
 28. Wilson, C. B. & Dixon, F. J. (1974) "Immunopathology and glomerulonephritis," *Annu. Rev. Med.* **25**, 83-98.
 29. Davies, D. R. & Clarke, A. E. (1975) "Demonstration of immunoglobulin containing deposits in glomerular basement membrane in experimental chronic serum sickness using horseradish peroxidase labelled antiserum," *Br. J. Exp. Pathol.* **56**, 28-33.
 30. Feenstra, K., Lee, R., Greben, H. A., Arends, A. & Hoedemaeker, Ph. J. (1975) "Experimental glomerulonephritis in the rat induced by antibodies directed against tubular antigens," *Lab. Invest.* **32**, 235-242.