

ALTERED FUNCTIONAL PROPERTIES OF THE RENAL
GLOMERULUS IN AUTOLOGOUS IMMUNE
COMPLEX NEPHRITIS

AN ULTRASTRUCTURAL TRACER STUDY*

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The present study was undertaken in order to investigate the nature of the glomerular damage in a form of experimental immune complex nephritis. The model of autologous immune complex nephritis (AIC)¹ (Heymann's nephritis) (1) was chosen for several reasons. First, the model bears a remarkable resemblance, in terms of ultrastructural and immunofluorescence features, to an important renal disease in man, membranous glomerulonephritis. Furthermore, the model is highly reproducible. In addition, the basic pathogenetic mechanism has been thoroughly documented (2-4). Briefly, the disease is produced in rats by immunization with homologous kidney preparations, which results in the formation of autoantibodies against a renal antigen, normally found in the brush border of proximal tubules. This antigen is presumed to enter the circulation in small amounts and combine with autoantibody to form complexes which deposit in glomeruli.² The complexes are detected as granular deposits of immunoglobulin and complement along the epithelial side of the glomerular basement membrane. The disease, like its human counterpart, is manifested by proteinuria, which is often heavy.

Our approach was to use the enzymatic tracers horseradish peroxidase (HRP) (40,000 daltons) and catalase (240,000 daltons), and the electron-opaque tracer ferritin (ca. 500,000 daltons) as probe molecules with which to assess the altered functional properties of the glomerular capillary wall. These ultrastructural tracers can be directly visualized, and their location within the glomerular capillary wall can be followed at sequential intervals after intravenous injection. Such protein tracers have been extensively used to identify

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¹*Abbreviations used in this paper:* AIC, autologous immune complex; CFA, complete Freund's adjuvant; CL, capillary lumen; DAB, 3,3' diaminobenzidine-tetra-HCl; GBM, glomerular basement membrane; HRP, horseradish peroxidase; US, urinary space.

²It is of interest that a related pathogenetic mechanism may operate in man. It has recently been reported that a tubular-derived antigen may be present in the glomerular immune deposits in some cases of human membranous glomerulonephritis (5).

not only the protein filtration barrier(s) in the normal glomerulus (6-10), but to investigate the abnormally permeable glomerulus in aminonucleoside nephrosis (11-13).

Materials and Methods

Animals.—Female Lewis strain rats weighing between 120-150 g (Microbiological Associates, Bethesda, Md.) were used in all experiments. Tubular antigen was obtained from the kidneys of normal Sprague-Dawley rats of both sexes (Charles River Breeding Laboratories, Wilmington, Mass.).

Immunization.—The renal cortex was fractionated into tubular and glomerular-rich fractions according to the method of Krakower and Greenspon (14), and the tubular-rich fraction was used for immunization.

40 Lewis rats were immunized. For immunization, complete Freund's adjuvant (CFA) (Difco Laboratories, Detroit, Mich.) supplemented with *Mycobacterium tuberculosis* H37RA (Difco) to a total of 5 mg/ml was combined with an equal volume of 50% tubular suspension to form a stable emulsion. Each rat received 0.4 ml of this emulsion divided equally between the two hind foot pads. In addition, 0.1 ml of *B. pertussis* vaccine (a gift of Dr. B. Devlin, Park, Davis and Co., Detroit, Mich.) at a concentration of 2.0×10^{11} organisms/ml was administered subcutaneously to the dorsum of each hind paw (15). 50% of the animals had not developed significant proteinuria by 3 mos after immunization. These animals were boosted by the intramuscular injection of 0.4 ml tubular antigen in CFA in two separate sites, followed by subcutaneous injection of 0.2 ml pertussis vaccine. 20 sham control animals were similarly injected with pertussis vaccine and CFA, but no tubular antigen. An equal number of uninjected littermates served as normal controls.

Estimation of Proteinuria.—Urines were collected over a 24-h period, their protein content estimated with Albutix (Miles Research Div., Miles Laboratories, Inc., Elkhart, Ind.) and quantitated using a modification of the trichloroacetic acid turbidometric method of Henry et al. (16). Using this method normal female Lewis rats excreted less than 10 mg/24 h.

Immunofluorescence.—Fluorescein-labeled antisera to rat gamma globulin and rat complement (zymosan) were purchased from Cappel Laboratories, Downingtown, Pa. The anti-gamma globulin conjugate gave no staining with normal rat kidney, whereas the anticomplement conjugate reacted with some tubular basement membranes. Sections were examined using a dark field fluorescence microscope with a Kp 490 excitation filter and a 410 nm barrier filter.

Tracers.—HRP (type II, Sigma Chemical Co., St. Louis, Mo.) was administered intravenously in doses ranging from 2 to 20 mg/100 g body weight. The enzyme was injected in 0.2 ml saline over a 1-min period. Animals were sacrificed from 0 to 30 min and 24-72 h after injection. A total of eight experimental, two sham control, and six normal control animals were used in HRP experiments.

Catalase (type C-100, Sigma) was prepared as described by Venkatachalam et al. (17). 3-4 ml containing a total of 74-100 mg catalase were injected intravenously over a 10-min period. Rats were killed 0, 5, 10 min and 24 h after the end of injection. Five experimental, one sham control, and four normal control rats were injected with catalase.

Ferritin (twice crystallized horse spleen ferritin, Nutritional Biochemical Corp., Cleveland, Ohio, or Miles Laboratories, Inc., Kankakee, Ill.) solution containing 100 mg/ml was used. To reduce its cadmium content, ferritin was dialyzed against 0.1 M EDTA in 0.07 M phosphate buffer pH 7.2 for 48 h, followed by dialysis against 0.1 M phosphate buffer pH 7.2 for 24-48 h (6).³ Immediately before injection, ferritin was passed through a 0.45 μ m Millipore

³ The cadmium content of the twice crystallized protein solution was determined by atomic absorption spectrophotometry, and found to be between 3.4-3.6 μ g/ml. After dialysis, the cadmium content was reduced to approximately 0.45 μ g/ml. These determinations were generously provided by Mr. L. Kopito.

filter, and administered intravenously over a 3–5 min period in doses ranging from 75 to 100 mg/100 g body weight. The animals were sacrificed 0, 5, 30, 60 min and 24–72 h after the end of injection. In experiments using ferritin, eleven experimental, four sham control, and seven normal control animals were used.

In all experiments, the animal was anesthetized with ether, and the left kidney removed by cutting the renal pedicle. Care was taken not to obstruct the renal vein. Thin slices of kidney were immersed in fixative at 4°C for 1–3 h. A piece of cortex from the right kidney was frozen on a cryostat chuck with Tissue Tek (Miles Research Div.), Frozen tissue was stored at –20°C for no more than 6 mos until sectioned for immunofluorescence. Frozen sections for immunofluorescent study were air dried and washed three times for 30 min in phosphate-buffered saline. The tissue was stained for 30 min in a moist chamber at room temperature. Sections were then washed and mounted in buffered glycerol and examined (18).

Cytochemistry and Electron Microscopy.—Kidneys from animals injected with HRP or catalase were fixed for 1 h in 2.5% purified glutaraldehyde (Polysciences, Inc., Warrington, Pa.) in 0.1 M cacodylate buffer, pH 7.3 at 4°C. They were washed overnight at 4°C in 0.15 M cacodylate buffer, pH 7.3.

Renal cortical tissue from animals injected with horseradish peroxidase was cut into 20- μ thick sections with a Smith-Farquhar tissue chopper (Ivan Sorvall, Newton, Conn.). The sections were incubated in a medium containing 0.05% 3,3'-diaminobenzidine-tetra-HCL (DAB) (Sigma) and 0.01% H₂O₂ in 0.05 M Tris-HCl buffer, pH 7.1 for 1 h at room temperature (19). They were then washed three times in distilled water.

Sections (20- μ thick) of kidneys from animals injected with catalase were preincubated for 1 h at 37° C in 0.1% DAB in the "universal" buffer of Theorell and Stenhagen (20), pH 10.5 or 0.05 M glycine buffer pH 10.5. Tissue sections were then transferred to fresh medium composed of 0.1% DAB, 0.01% H₂O₂ in either universal or glycine buffer, pH 10.5, and incubated for 1 h at 37°C (21). The sections were then washed three times in distilled water.

Kidneys from uninjected experimental and control animals, and animals injected with ferritin were handled differently. They were fixed for 3 h in formaldehyde-glutaraldehyde, diluted 1:1 with 0.1 M cacodylate buffer, pH 7.3 for 3 h at 4°C (22), and washed overnight in 0.15 M cacodylate buffer pH 7.3.

Almost all kidneys were postfixed in potassium ferrocyanide reduced 2% OsO₄ for 1 h at 4°C (23). However, additional portions of uninjected experimental and control kidneys, as well as kidneys from animals injected with ferritin, were postfixed in OsO₄ in s-collidine buffer pH 7.2. The tissue was dehydrated in graded ethanol, embedded in Epon. Thin sections were cut with a diamond knife (E. I. DuPont de Nemours & Co., Wilmington, Del.) on an LKB Ultratome III (LKB Instruments, Inc., Bromma, Sweden) and picked up on carbon-coated grids. Sections containing ferritin were stained with bismuth (24) alone, or with bismuth followed by lead citrate. All other sections were counterstained with lead citrate. They were examined in a Philips 300 electron microscope operated at 60 kV and equipped with an anti-contamination device and a 30 μ objective aperture.

RESULTS

General.—With one exception, animals with significant proteinuria were selected for study. Even the animal with no measurable proteinuria showed glomerular immune complex deposits by immunofluorescence. In most animals, proteinuria tended to increase with time. Urinary protein excretion ranged from 50 mg/24 h to more than 500 mg/24 h.

Light Microscopy.—Early in the disease, glomeruli appeared normal in toluidine blue-stained, 1- μ thick sections. With advanced disease, the glomerular capillary wall appeared to be somewhat thickened, but there was no detectable increase in the number of mesangial cells or leukocyte infiltration.

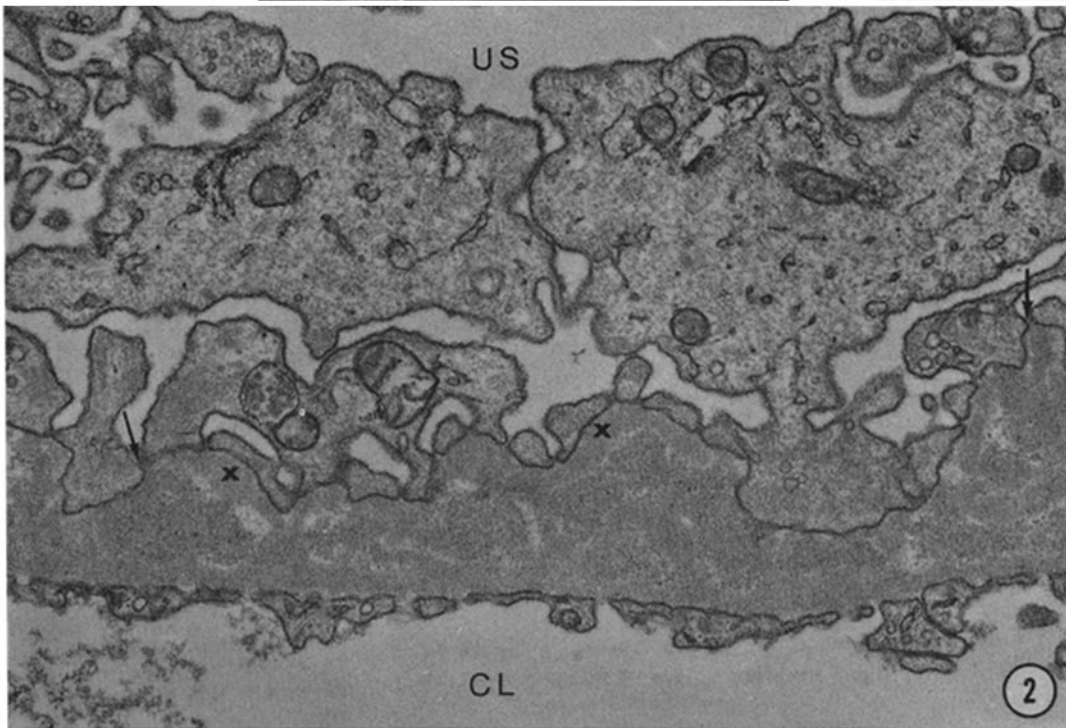
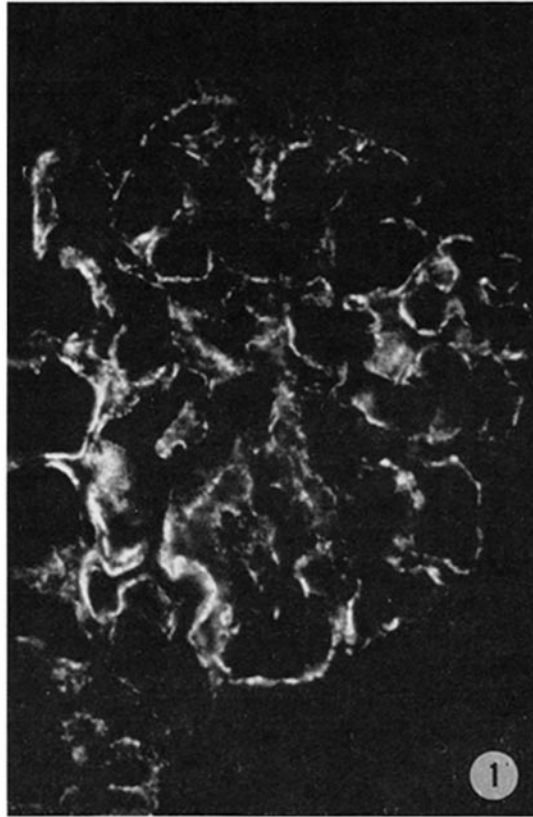
Immunofluorescence.—Granular staining along the peripheral glomerular basement membrane (GBM) with both anti-IgG and anticomplement was observed in all experimental animals examined (Fig. 1).

Electron Microscopy.—In animals with mild disease, widely-spaced irregular deposits of finely granular, moderately electron-dense material were present in the subepithelial portions of the GBM. Although most of the deposits appeared to underlie swollen epithelial foot processes, many of them extended up to the level of the slit pore membrane between foot processes (Fig. 2). The deposits were often surrounded by an irregular electronlucent rim which demarcated the edge of the deposit from adjacent GBM. In tissue fixed with partially reduced osmium, and stained with bismuth, the deposits also contained small, electron-dense aggregates ranging in diameter from 140 Å to 300 Å, and composed of two or more oblong or ringlike structures (Fig. 12). Particles of similar structure, surrounded by fixed plasma proteins were seen in glomerular capillary lumens of both uninjected experimental and control animals fixed in the same way. The nature of these particles is not known. Swollen foot processes were almost always associated with underlying deposits, and in those areas of the GBM where no deposits were visible, the foot processes were often similar in appearance to those of control kidneys. Aggregation of microfilaments was prominent in swollen podocytes. Except for focal foot process swelling, no further abnormalities of epithelial cells were observed. Similarly, endothelial cells appeared to be normal. Although mesangial cells were not extensively examined, these observed showed no ultrastructural abnormalities.

In animals with well-established disease, usually 6–8 months after immunization, the deposits were larger, in many areas confluent, and few uninvolved areas of GBM remained. Many foot processes were swollen and with swelling of foot processes the number of slit pore complexes per unit length of GBM was moderately reduced. The overall thickness of the GBM was considerably increased, and its epithelial surface highly irregular in outline. In many areas a layer of finely fibrillar GBM separated the deposits from the overlying epithelial cells (Fig. 2). Although the immune complex deposits markedly distorted the geometrical relationship of adjacent foot processes, the narrow space between foot processes, at the site of the slit pore membrane, was main-

FIG. 1. Glomerulus from a rat with AIC nephritis stained with fluorescein-labeled anti-serum to rat gamma globulin. Granular deposits are present along the GBM of peripheral capillary loops. $\times 1,700$.

FIG. 2. Glomerular capillary wall of a rat immunized 8 mo before sacrifice. Irregular, finely granular, immune complex deposits are present within the basement membrane. Some of the deposits extend to the level of the slit pore complex (arrows) while many others are separated from the epithelium by a layer of basement membrane material (x). Note the swelling of foot processes immediately adjacent to deposits, and their more normal appearance when GBM separates them from deposits. CL, capillary lumen; US, urinary space. $\times 21,300$.



tained and measured approximately 400 Å in width. A few "close" junctions between podocytes were observed in controls, and their number appeared to be slightly increased in experimental animals. Variable numbers of vacuoles containing amorphous, slightly electron-dense material were observed in some epithelial cells. As in animals with mild disease, neither endothelial nor mesangial cells showed morphological abnormalities.

Tracer Studies.—Almost all our observations were based on tissue postfixed in partially reduced osmium (23). With this fixative the immune complex deposits were much less electron dense than with conventional fixation, permitting the more ready identification of ferritin. In addition, partially reduced osmium has the advantage of rendering the reaction product, resulting from the oxidation of DAB, more electron opaque, thereby facilitating the visualization of the peroxidatic tracers (23).

The behavior of the tracers in sham control animals immunized with CFA and pertussis but no tubular antigen was indistinguishable from that in normal control littermates. An experimental and a control animal were injected in each tracer experiment.

Horse radish Peroxidase.—1 min after injecting a high dose of HRP (20 mg/100 g body weight) into an experimental animal, dense, black reaction product uniformly stained the GBM and the deposits. Reaction product also stained the glycocalyx of epithelial cells, and was present within the urinary space between foot processes. A few small pinocytotic vesicles attached to the epithelial plasma membrane contained HRP. Similarly, pinocytotic uptake of the tracer was observed in mesangial and proximal convoluted tubular cells. Except for the absence of deposits the distribution of the tracer in control kidneys was the same.

In contrast, sections obtained 1 min after injecting a low dose of HRP into an experimental animal (2–5 mg/100 g body weight), showed that the tracer selectively accumulated in deposits, staining them black, while the adjacent GBM remained less intensely stained (Fig. 3). In slit pore complexes overlying normal GBM a clear zone, free of reaction product, separated basement membrane from the slit diaphragm. In those areas of GBM containing deposits, the space up to the level of the slit diaphragm was filled with reaction product concentrated within deposits (Fig. 4). An intact slit diaphragm covered by reaction product was evident in each slit pore complex in sections cut perpendicular to the plane of the basement membrane (Fig. 4). Even at this low dose, pinocytosis within mesangial, glomerular epithelial, and tubular epithelial cells was evident. Except for the absence of immune complex deposits, the distribution of HRP at low doses was essentially the same, in control animals.

At 30 min after injection of a high dose of HRP into an experimental animal, there was no significant difference in the distribution of the tracer from that seen 1 min after injection, except that pinocytotic uptake of the tracer by mesangial cells was more pronounced; pinocytotic activity of epithelial cells

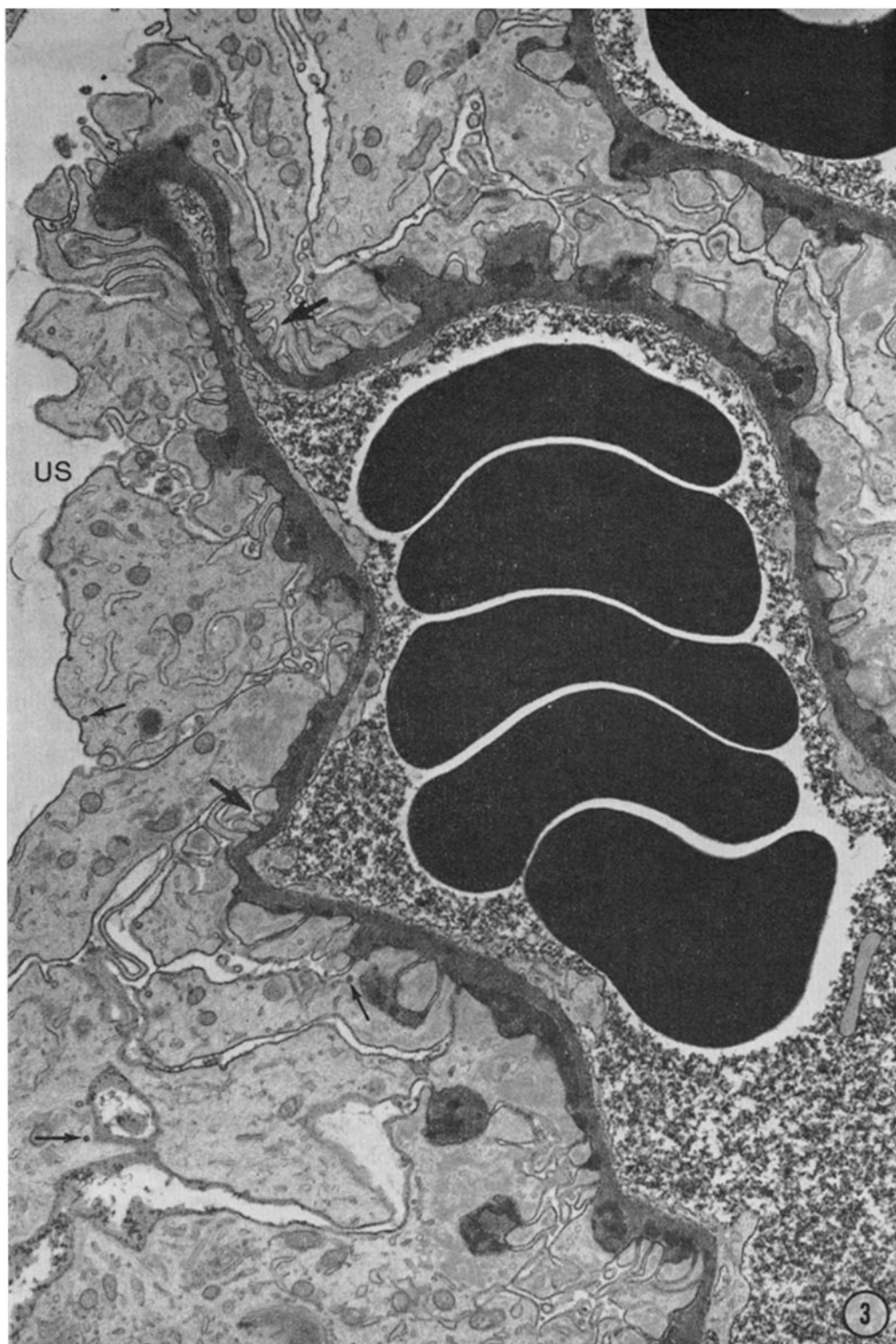


FIG. 3. Glomerulus from an experimental animal injected with a low dose (2 mg/100 g body weight) of HRP and killed 1 min after injection. There is selective concentration of the tracer in deposits, staining them more intensely than the adjacent GBM. Groups of normal appearing foot processes (large arrows) overlie areas of GBM which contain no deposits. A few pinocytotic vesicles near epithelial cell membranes contain HRP (small arrows). $\times 11,200$.

remained minimal. The distribution of the tracer in mesangial matrix and the GBM of the control animal 30 min after injection was no different than after 1 min. Similarly, no difference in the degree of pinocytosis between experimental and control animals was observed.

24 h after injection of a high dose of HRP no circulating tracer could be demonstrated cytochemically. The GBM in all animals, as well as the immune complex deposits in experimental animals, contained no demonstrable HRP. However, a few vacuoles in glomerular epithelial cells, and many vacuoles in proximal convoluted tubular cells, contained reaction product. This is somewhat surprising since lysosomes in tubular cells contain hydrolytic enzymes capable of inactivating peroxidase (25). It is possible that the observed persistence was due to the large dose of the tracer given (26). We were unable to detect any difference in the number of HRP containing tubular cell vacuoles between experimental and control animals.

Catalase.—Catalase as a tracer in the rat is of limited use, since in order to administer adequate amounts of this poorly soluble enzyme, large volumes must be injected intravenously (13). Animals were studied immediately and 24 h after injection.

In experimental animals killed immediately after the end of a 10-min injection of catalase, the tracer selectively accumulated within immune complex deposits, staining them moderately intensely while the adjacent basement membrane remained lightly stained (Fig. 5). Rarely, irregular deposits stained with catalase projected into subepithelial spaces filled with amorphous material (Fig. 6). Because of their infrequent presence, it was not possible to perform serial sections in order to determine whether these subepithelial spaces were continuous with the urinary space, or represented subepithelial pockets. Substantial amounts of reaction product were observed in the urinary space, as well as some in the lumen of proximal convoluted tubules. In control animals, immediately following a 10-min injection of catalase, the tracer was present in highest concentration in the loose, subendothelial layer of GBM with small jets of reaction product extending up to the lamina densa (Fig. 7). The remainder of the GBM was lightly stained with reaction product. In some control animals a very small amount of reaction product was present in the urinary space as well as in a few proximal convoluted tubular lumens.

24 h after injection of catalase, the kidneys of both experimental and control animals were free of the tracer. The only positively-stained organelles were endogenous catalase containing peroxisomes which are normally present in glomerular epithelial cells and proximal convoluted tubular cells (27). There was no difference in the number and appearance of these organelles between experimental and control animals.

Ferritin.—The distribution of ferritin in the GBM of experimental animals was nonuniform. 3 min after injection, the tracer was present in high concentration in some deposits (Fig. 8 a), but was partially excluded from others. The

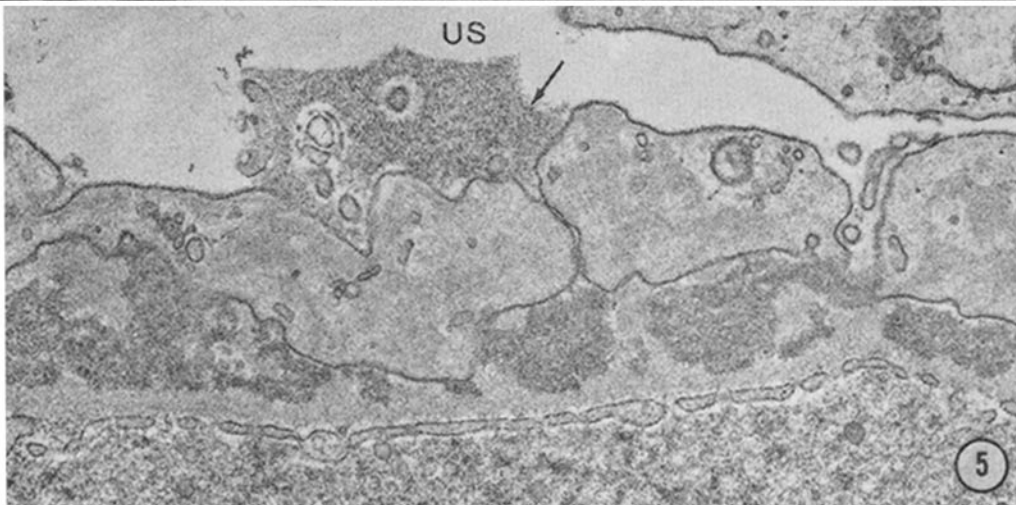
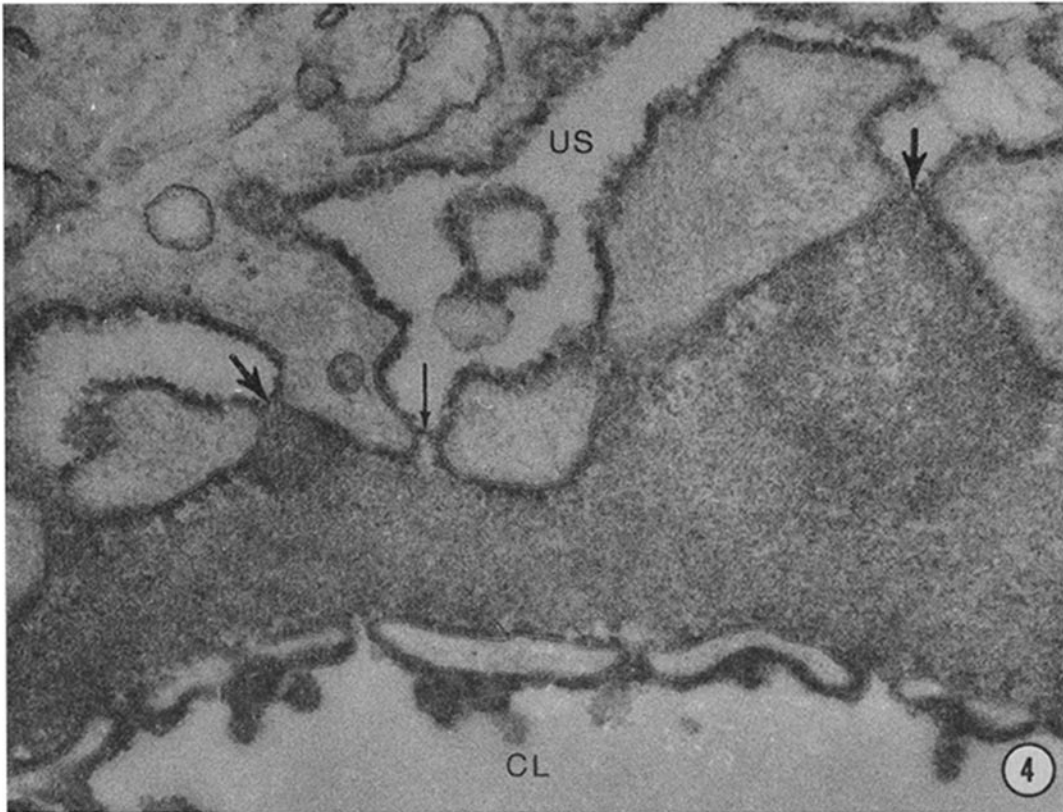


FIG. 4. Glomerular capillary wall of an experimental animal injected with HRP 1 min before sacrifice. In areas free of deposits, the space between GBM and the slit diaphragm is virtually free of reaction product (small arrow). Where deposits extend to the slit pore complex, reaction product is present up to the level of the slit diaphragm (large arrows). $\times 82,500$.

FIG. 5. Immediately following a 10-min injection of catalase, reaction product is selectively concentrated within immune complex deposits. A large aggregate of reaction product (arrow) is present in the urinary space. $\times 25,000$.

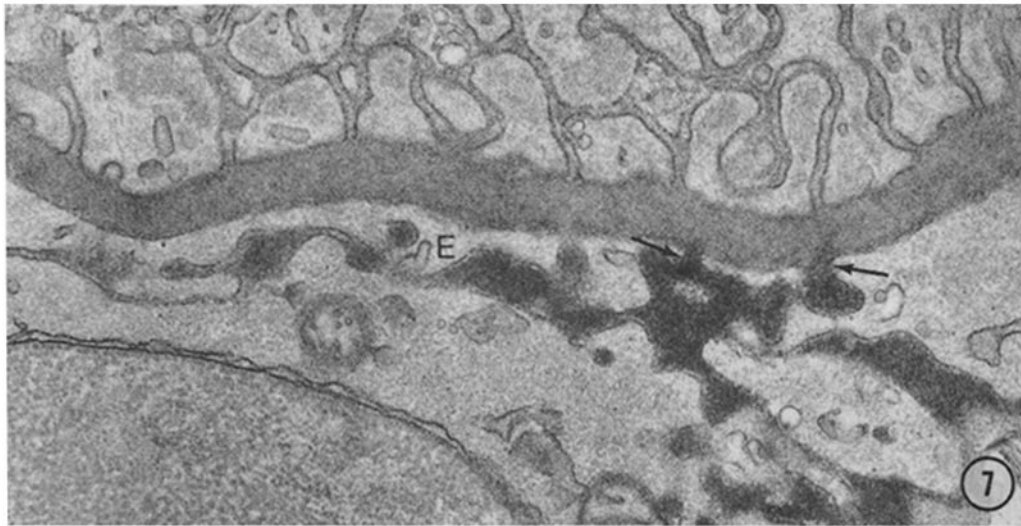
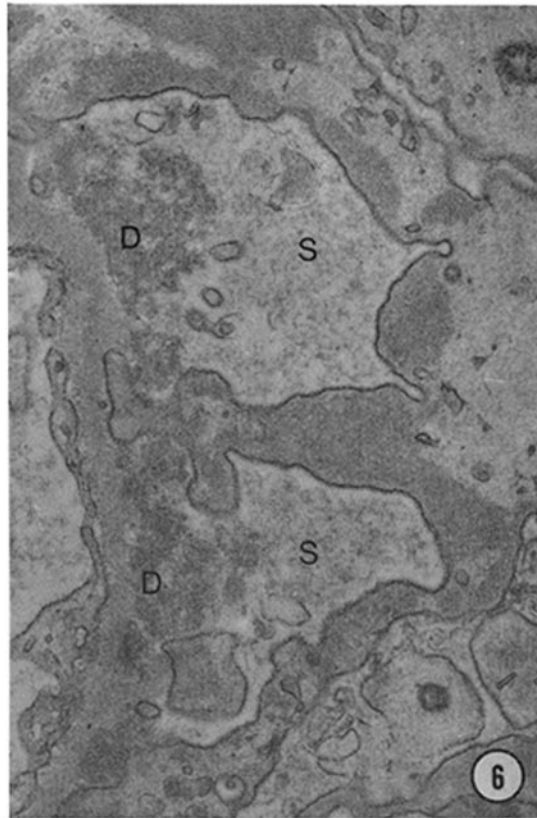


FIG. 6. Kidney from the same experimental animal injected with catalase as in Fig. 5. Note the two vacuolar spaces (*S*) formed underneath the epithelium. Immune complex deposits (*D*) project into these spaces. $\times 15,000$.

FIG. 7. Kidney from a control rat fixed immediately following a 10-min injection of catalase. The highest concentration of reaction product is seen in the lamina rara interna, with small jets of reaction product extending through fenestrae (arrows) of endothelial cells (*E*) up to the level of the lamina densa. $\times 32,500$.

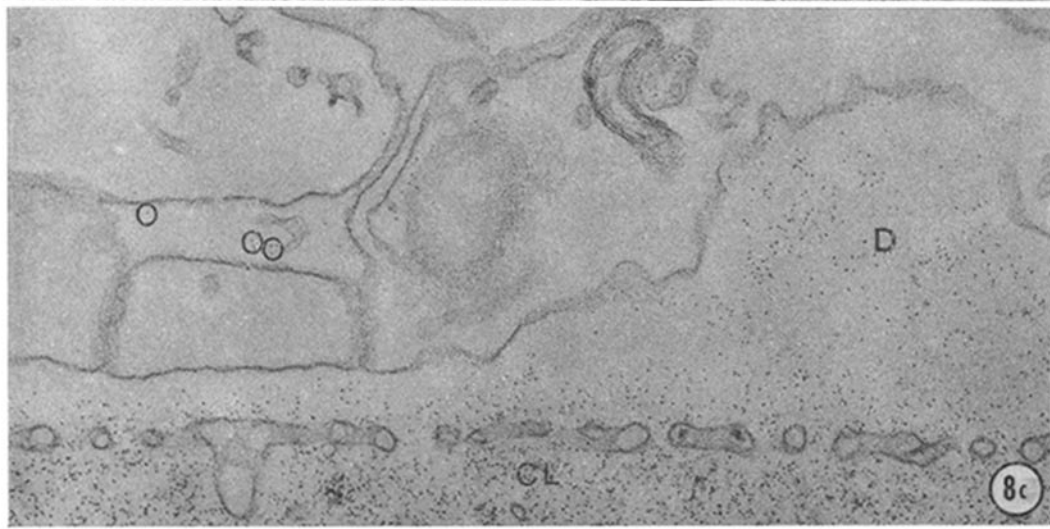
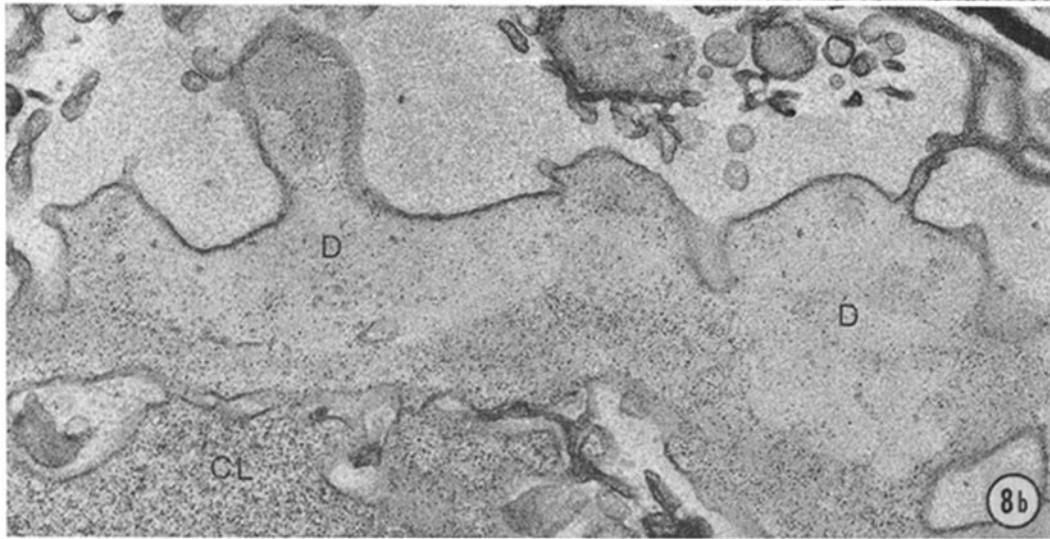
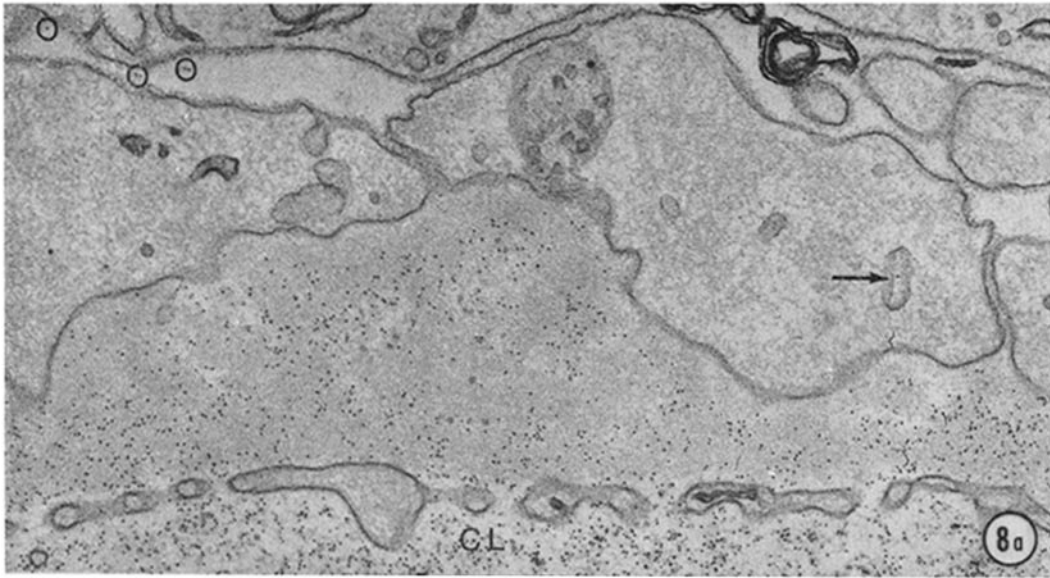
tracer was present, however, in high concentration in those areas of GBM immediately adjacent to deposits, whether or not they contained ferritin. In areas of GBM free of deposits, ferritin was limited to the subendothelial layer, in a distribution similar to that of control animals (Fig. 8 *c*). In animals in which there were numerous deposits with few areas of GBM remaining uninvolved, ferritin was present throughout almost the entire thickness of the GBM. However, as previously described, a layer of apparently newly formed basement membrane material sometimes separated the deposits from overlying podocytes. Although ferritin became aligned at the outer rim of deposits, very few particles penetrated this subepithelial layer of basement membrane (Fig. 9). At this time, a small amount of ferritin was present within epithelial pinocytotic vesicles, multivesicular bodies, free in the urinary space, and rarely in the slits between podocytes (Fig. 10). In control animals there were virtually no particles in vesicles and only a rare particle in the urinary space. In both experimental and control animals a few vesicles containing a small amount of ferritin were present in mesangial cells.

Between 10 and 60 min after injection, the distribution of ferritin in deposits and GBM of experimental animals was similar to that after 3 min. Control animals continued to have a subendothelial distribution of ferritin within the GBM, and few ferritin particles were seen in the urinary space and epithelial cell vesicles. In experimental animals at these later times many more particles were present in epithelial vesicles, in the urinary space, and a few were present in the slits between podocytes. Subepithelial spaces, located between swollen foot processes and GBM, were observed in rare instances and were similar in appearance to those seen in catalase injected experimental animals (Fig. 11). Many free ferritin particles were present within such epithelial spaces.

24–48 h after injecting 100 mg/100 g body weight ferritin, some particles were still seen in capillary lumens. However, 72 h after injection of ferritin, when the tracer was no longer detectable in the circulation, many ferritin particles remained lodged in the immune complex deposits, while the adjacent uninvolved GBM was free of the tracer (Fig. 12). Large membrane-bound vacuoles containing a high concentration of ferritin were present in mesangial and glomerular epithelial cells as well as in the basal portion of the proximal convoluted tubular cells. At these late time periods many ferritin particles were present free within the cytoplasm of these cells, an observation reported by others as well (6). The intracellular distribution of ferritin was essentially similar in control animals but the concentration of ferritin in glomerular and tubular epithelial cell vacuoles appeared to be less. No ferritin remained within the GBM of control animals.

DISCUSSION

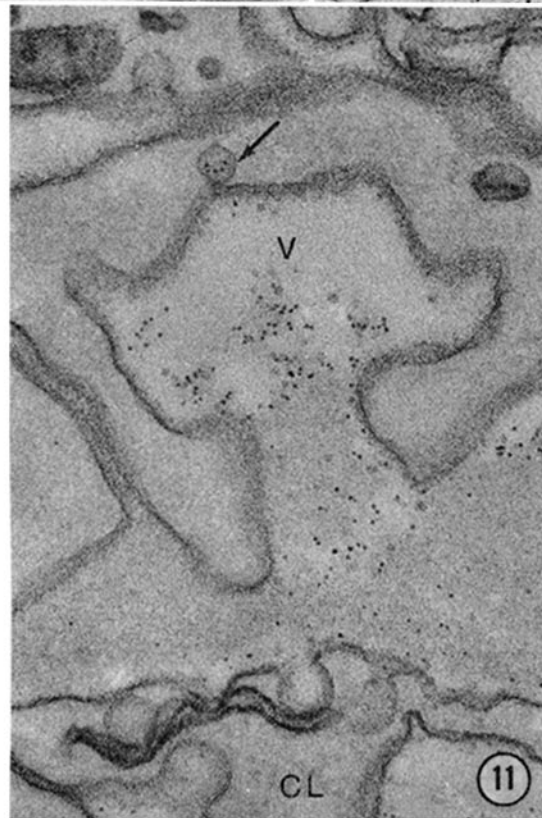
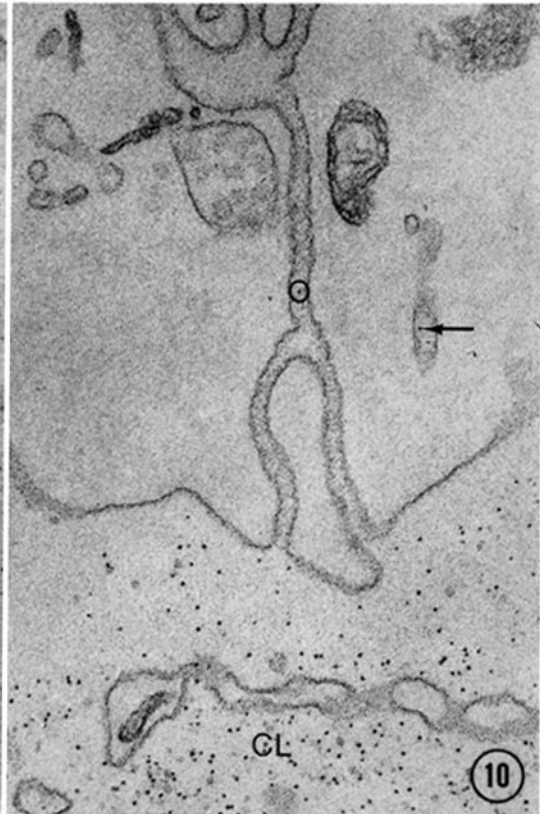
Current Concepts of Normal Glomerular Filtration Barriers.—The results of the present study can best be discussed in light of current knowledge concerning



the ultrastructural basis for protein filtration in the normal glomerulus. This knowledge has been based largely on experiments using tracers which can be visualized at the ultrastructural level (28, 29). The early studies of Farquhar et al. (6), showed that the lamina densa of the GBM behaved as a filtration barrier for large protein molecules, such as ferritin; later studies using peroxidatic enzymes of mol wt ranging from 12,000–240,000 indicated that a second filtration barrier resides in the slit pore complex (7, 9, 10, 28). Thus, Karnovsky and co-workers have suggested that the glomerulus contains two filters in series: (a) the GBM, which acts as a coarse filter restricting large proteins, and (b) the slit pore complex which acts as a fine filter restricting small proteins (28). Recent observations made in Karnovsky's laboratory indicate that the slit diaphragm, which continuously spans the space between foot processes, in fact has a highly ordered, isoporous substructure (30). Its central filament and adjacent cross bridges form rectangular pores measuring $50 \times 150 \text{ \AA}$. The dimensions of these pores are consistent with those calculated from physiological data (31–33), and suggest that the slit diaphragm may indeed represent the fine filter of the glomerulus.

Glomerular Alterations in Autologous Immune Complex Nephritis.—In the present study, several findings concerning the nature of the glomerular injury emerged. First, direct evidence was obtained for increased permeability of the glomerular capillary wall to large proteins; thus ferritin and catalase, which in normal animals largely failed to traverse the glomerular capillary wall, were readily detectable in the urinary space shortly after injection. Furthermore, the results showed that the GBM itself was abnormally permeable. Of particular interest was the finding that this increased permeability was present only in those regions where deposits were present. It is not clear how the deposition of immune complexes brought about this change. Leukocytes did not appear to be involved, since in none of the animals studied were they present in glomeruli. This is in contrast to nephrotoxic serum nephritis (34) and certain forms of tissue damage produced by immune complexes, notably the Arthus reaction (35), where neutrophil-dependent mechanisms have been shown to operate. It is possible that the passage and accumulation of immune complexes within the basement membrane in some way alters the spacing of the macro-

FIG. 8. (a) Glomerulus of an experimental animal injected with ferritin 3 min before sacrifice. Tissue was fixed in partially reduced OsO_4 resulting in greater electron lucency of deposits than with conventional fixation. Ferritin particles are present not only in the full thickness of GBM but also in epithelial vesicles (arrow) and in the urinary space (encircled). Bismuth stain followed by lead citrate. (b) Renal tissue from same animal and fixed as in Fig. 8 a. The deposits (D) appear as electron lucent areas from which ferritin is partially excluded. Bismuth stain, followed by lead citrate. (c) Glomerulus from the same animal as in Fig. 8 a and 8 b. In the area of the deposit (D) ferritin particles are present throughout its thickness, while in the adjacent uninvolved GBM to the left of the deposit, ferritin is present in a subendothelial position. Free ferritin (encircled) is present in the urinary space. Bismuth stain. $\times 50,000$.



molecular components of the GBM surrounding the immune complexes, however, this cannot be resolved with present preparative methods for electron microscopy.

Alteration of the permeability of the GBM is not sufficient by itself to explain the passage of large proteins into the urinary space. For this to occur, either the slit pore complex must be altered or bypassed. Although the present study does not clearly define which of these two alternatives is operative, there is indirect evidence that the slit pore diaphragm may be abnormally permeable. As previously mentioned, recent evidence suggests that the highly ordered substructure of the slit pore membrane may represent the fine filter of the glomerulus (30). In the normal glomerulus a small electron lucent space, usually containing few of the fibrils normally seen in the lamina rara externa, separates the slit diaphragm from the underlying GBM. By contrast, in experimental animals, immune complex deposits extended up to and were in contact with the slit diaphragm. It is possible that such deposits directly alter the porosity of the diaphragm, although this remains to be proved.

If large proteins escape into the urinary space by bypassing the slit pore membrane, the most likely mechanism would be vesicular transport. However, ferritin particles were numerous in the urinary space within minutes of injection. Such a rapid rate of appearance of ferritin in the urinary space would argue against a vesicular transport system. Even though with time there was considerably more pinocytotic uptake of ferritin in experimental than control kidneys, this may be attributable to the presentation of more tracer to epithelial cells secondary to increased permeability of the altered GBM. No convincing evidence was obtained that ferritin was released from pinocytotic vesicles into the urinary space. Further evidence against significant vesicular transport was obtained from experiments with catalase, an enzyme which is not readily taken up by pinocytotic vesicles (36, 37). In these experiments, substantial amounts of reaction product were present in the urinary space, while there was little evidence of pinocytotic vesicle labeling. In addition, experiments with HRP, a tracer which readily traverses even the normal GBM, also argue against excessive vesicular transport. In experimental animals given HRP, labeling of pinocytotic vesicles was no greater than in normal controls, and similar to that observed by others in normal mouse glomeruli (7). Thus, the failure to

FIG. 9. Glomerulus from an experimental animal injected with ferritin 3 min before sacrifice. Ferritin particles are aligned at the outer rim of the deposit and have not penetrated the peripheral layer of GBM. $\times 77,000$.

FIG. 10. Glomerulus from an experimental animal injected with ferritin 8 min before sacrifice. Ferritin particles are present throughout the thickness of the basement membrane. A single particle (encircled) is seen in the slit between podocytes and another particle is present in a pinocytotic vesicle (arrow). $\times 67,500$.

FIG. 11. A subepithelial vacuolar space (V) in the glomerular capillary wall of an experimental animal injected with ferritin 1 h before sacrifice. Many ferritin particles are present free within the space as well as in an epithelial pinocytotic vesicle. $\times 70,200$.

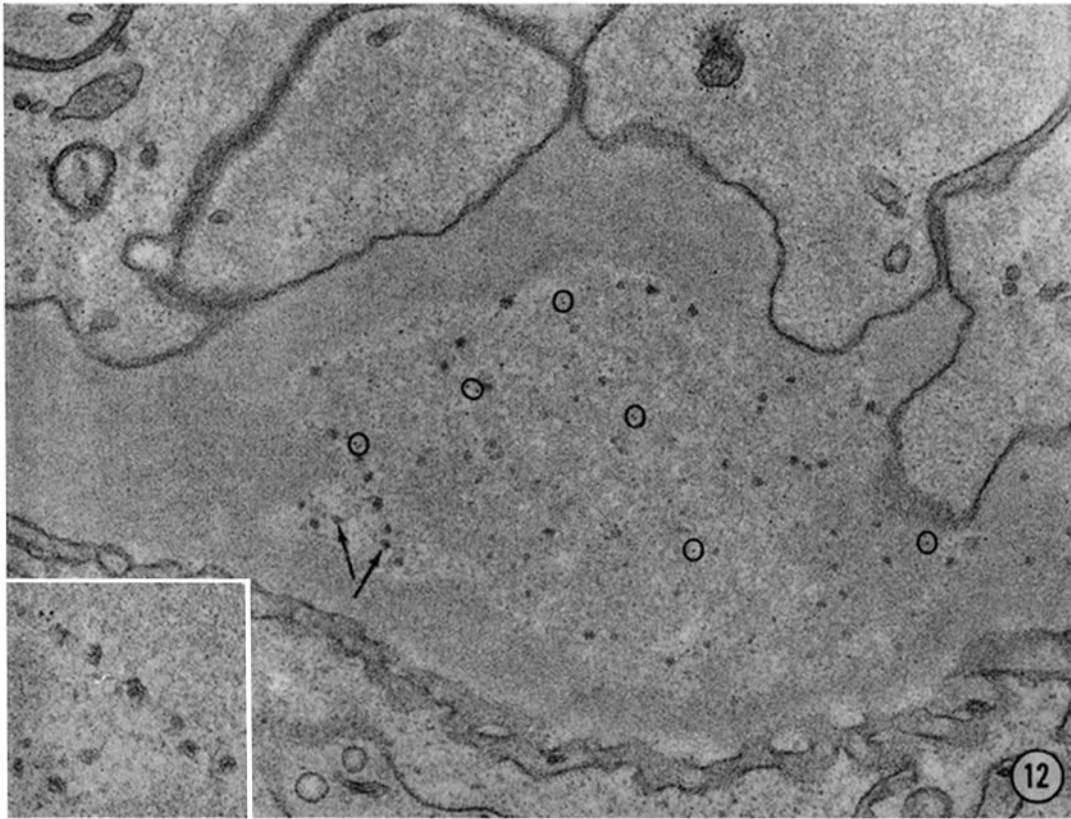


FIG. 12. AIC deposit in an animal injected with ferritin 72 h before sacrifice. Many ferritin particles remain lodged within the deposit (some are encircled) while the adjacent GBM is virtually free of ferritin. Aggregates of material (arrows) similar to those seen in the capillary lumen are also present in the deposit. These aggregates appear to be composed of tubular and ringlike structures (*insert*). Note the many ferritin particles free within epithelial cell cytoplasm. $\times 47,000$. Insert $\times 88,000$.

demonstrate significant pinocytotic transport of any of the tracer molecules across epithelial cells suggests that large proteins may pass through an altered slit pore membrane.

Comparison to Other Experimental Models Characterized by Proteinuria.—Structurally and functionally AIC nephritis differs from other models in which glomerular permeability has been studied with tracers. In aminonucleoside nephrosis, evidence has been obtained indicating transport of tracers by large vacuoles which originate on the GBM side and which open into the urinary space (12, 13). In AIC nephritis, in contrast, only rare subepithelial pockets containing tracer were observed. In addition there was less foot process swelling and "close" junction formation in AIC nephritis than in aminonucleoside nephro-

sis, even in those animals with severe proteinuria. Functional studies have shown that differential protein clearance is more selective in aminonucleoside nephrosis than in AIC nephritis (38). These functional data cannot be explained on the basis of presently available morphological observations (12, 13).

Recent studies using tracers have been applied to chronic serum sickness in rabbits.⁴ Surprisingly, although these animals had heavy proteinuria, they exhibited a lower HRP clearance than controls. It was suggested that this paradoxical finding could be explained on the basis of decreased availability of slit pore complexes which are presumably the barrier to filtration of small proteins. We did not obtain data from our experiments with HRP bearing on this point.

The studies in rabbits with chronic serum sickness also revealed that immune complex deposits may have different properties in different diseases. This may be related to the density of packing of antigen-antibody complexes. Thus, in chronic serum sickness ferritin was largely excluded from deposits, while in AIC nephritis penetration of ferritin into deposits was substantial, and only some deposits showed partial exclusion of this tracer.

A third experimental model characterized by proteinuria, nephrotoxic serum nephritis, was studied by Gang et al. using lanthanum salts (34). Evidence was obtained for focal increased permeability of the lamina densa and was attributed to a lytic effect of neutrophils on the GBM. As previously mentioned in AIC nephritis, there is no evidence that neutrophils induced increased glomerular permeability.

Possible Mechanisms in the Perpetuation and Healing of AIC Disease.—The fact that three tracers of widely differing molecular weights and isoelectric points are selectively trapped in these deposits suggests that this may provide a mechanism for modification of immune complex diseases. Trapping of circulating molecules could result in an effective increase in local concentration of antigen and/or antibody, thus favoring their interaction, and this could lead to growth or dissolution of deposits (39–41).

In later stages of AIC nephritis, a layer of basement membrane material often separated deposits from overlying epithelial cells. Interestingly this layer was poorly permeable to ferritin and behaved more like normal basement membrane. It is of interest that similar morphological changes have been observed in kidneys from patients with membranous glomerulonephritis (42). It is conceivable that eventually such formation of basement membrane could seal off the immune complexes and perhaps render the glomerulus more normally permeable.

SUMMARY

The altered functional properties of the glomerular capillary wall in a model of autologous immune complex disease (Heymann's nephritis) was studied

⁴ Brentjens, J., G. Andres, and D. O'Connell. 1973, personal communication.

by electron microscopy using intravenously injected protein tracers of varying molecular weight. There was an increase in the permeability of the glomerular basement membrane (GBM) itself to large molecules; this change was focal and was found in those areas where the GBM contained immune complex deposits. Both ferritin and catalase, tracers normally restricted from passing the glomerular filter, were present in the urinary space within minutes of injection. No evidence was obtained for increased glomerular epithelial transport in this disease. Foot process swelling and "close" junction formation was moderate, even in animals with marked degrees of proteinuria. Indirect evidence, therefore, makes an alteration in the slit pore complex likely. In addition, there was immediate and selective concentration of all tracers within deposits, though ferritin was partially excluded from some deposits. This phenomenon may be of significance in the perpetuation of the disease.

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BIBLIOGRAPHY

1. Heymann, W., D. B. Hackel, S. Harwood, S. T. Wilson and J. L. P. Hunter. 1959. Production of nephrotic syndrome in rats by Freund's adjuvant and rat kidney suspension. *Proc. Soc. Exp. Biol. Med.* **100**:660.
2. Glasscock, R. J., T. S. Edgington, J. I. Watson, and F. J. Dixon. 1968. Autologous immune complex nephritis induced with renal tubular antigen. II. The pathogenetic mechanism. *J. Exp. Med.* **127**:573.
3. Grupe, W. E., and M. H. Kaplan. 1969. Demonstration of an antibody to proximal tubular antigen in the pathogenesis of experimental autoimmune nephrosis in rats. *J. Lab. Clinl Med.* **74**:400.
4. Sugisaki, T., J. Klassen, G. A. Andres, F. Milgrom, and R. T. McCluskey. 1973. Passive transfer of Heymann nephritis with serum. *Kidney Internat.* **3**:66.
5. Naruse, T., K. Kitamura, Y. Miyakawa, and S. Shibata. 1973. Deposition of renal tubular epithelial antigen along the glomerular capillary walls of patients with membranous glomerulonephritis. *J. Immunol.* **110**:1163.
6. Farquhar, M. G., S. L. Wissig, and G. E. Palade. 1961. Glomerular permeability. I. Ferritin transfer across the normal glomerular capillary wall. *J. Exp. Med.* **113**:47.
7. Graham, R. C., and M. J. Karnovsky. 1966. Glomerular permeability. Ultrastructural cytochemical studies using peroxidase as protein tracers. *J. Exp. Med.* **124**:1123.
8. Menefee, M. G., L. B. Mueller, A. L. Bell, and J. K. Meyers. 1964. Transport of globin by the renal glomerulus. *J. Exp. Med.* **120**:1129.
9. Oliver, D., and E. Essner. 1972. Protein transport in mouse kidney utilizing tyrosinase as an ultrastructural tracer. *J. Exp. Med.* **136**:291.
10. Venkatachalam, M. A., M. J. Karnovsky, H. D. Fahimi, and R. S. Cotran. 1970. An ultrastructural study of glomerular permeability using catalase and peroxidase as tracer molecules. *J. Exp. Med.* **132**:1153.
11. Farguhar, M. G., and G. E. Palade. 1961. Glomerular permeability. II. Ferritin

- transfer across the glomerular capillary wall in nephrotic rats. *J. Exp. Med.* **114**:699.
12. Venkatachalam, M. A., M. J. Karnovsky, and R. S. Cotran. 1969. Glomerular permeability. Ultrastructural studies in experimental nephrosis using horseradish peroxidase. *J. Exp. Med.* **130**:381.
 13. Venkatachalam, M. A., R. S. Cotran, and M. J. Karnovsky. 1970. An ultrastructural study of glomerular permeability in aminonucleoside nephrosis using catalase as a tracer protein. *J. Exp. Med.* **132**:1168.
 14. Krakower, C. A., and S. A. Greenspon. 1951. Localization of the nephrotoxic antigen within the isolated renal glomerulus. *Arch. Pathol.* **51**:629.
 15. Klassen, J., T. Sugisaki, F. Milgrom, and R. T. McCluskey. 1971. Studies on multiple renal lesions in Heymann's nephritis. *Lab. Invest.* **25**:577.
 16. Henry, R. J. 1964. *Clinical Chemistry: Principles and Techniques*. Harper and Row, New York. 188.
 17. Venkatachalam, M. A., and H. D. Fahimi. 1969. The use of beef liver catalase as a protein tracer for electron microscopy. *J. Cell Biol.* **42**:480.
 18. Goldman, M. 1968. *Fluorescent Antibody Methods*. Academic Press, New York. 101 (Method 2a).
 19. Graham, R. C., and M. J. Karnovsky. 1966. The early stage of absorption of injected horseradish peroxidase in the proximal tubule of the mouse kidney. Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14**:291.
 20. Theorell, T., and E. Stenhagen. 1938. Ein Universalpuffer für den pH-Bereich 2.0 bis 12.0. *Biochem. Z.* **299**:416.
 21. Herzog, V., and H. D. Fahimi. 1973. An improved cytochemical method for demonstration of the preoxidatic activity of beef liver catalase (BLC). *J. Histochem. Cytochem.* **21**:412.
 22. Karnovsky, M. J. 1965. Formaldehyde-glutaraldehyde fixation of high osmolality for use in electronmicroscopy. *J. Cell Biol.* **27**:137 (Abstr.).
 23. Karnovsky, M. J. 1971. Use of ferrocyanide reduced osmium tetroxide in electron microscopy. Abstr. No. 284, Eleventh Annual Meeting of the American Society for Cell Biology, New Orleans, La.
 24. Ainsworth, S. K., and M. J. Karnovsky. 1972. An ultrastructural staining method for enhancing the size and electron opacity of ferritin in thin sections. *J. Histochem. Cytochem.* **20**:225.
 25. Straus, W. 1959. Concentration of acid phosphatase, ribonuclease, desoxyribonuclease, beta glucuronidase and cathepsin in "droplets" isolated from kidney cells of normal rats. *J. Biophys. Biochem. Cytol.* **2**:513.
 26. Straus, W. 1971. Comparative analysis of the concentration of injected horseradish peroxidase in cytoplasmic granules of the kidney cortex, in the blood, urine and liver. *J. Cell. Biol.* **48**:62.
 27. Hruban, Z., E. L. Vigil, A. Slesers, and E. Hopkins. 1972. Microbodies. Constituent organelles of animal cells. *Lab. Invest.* **27**:184.
 28. Karnovsky, M. J., and S. K. Ainsworth. 1972. The structural basis of glomerular filtration. *Advances in Nephrology from the Necker Hospital*. Year Book Medical Publishers, Inc., Chicago, Ill. **2**:35.

29. Schneeberger, E. E. Glomerular permeability to protein molecules—its possible structural basis. *Nephron*. In press.
30. Rodewald, R., and M. J. Karnovsky. 1973. Isoporous structure of the glomerular slit diaphragm. Abstr. no. 576, Thirteenth Meeting of the American Society for Cell Biology, Miami, Fla.
31. Landis, E. M. and J. R. Pappenheimer. 1963. Exchange of substances through the capillary walls. Handbook of Physiology. W. F. Hamilton and P. Dow, editors. Section II, Circulation Vol. II. The William and Wilkins Co., Baltimore, Md.
32. Pappenheimer, J. R., E. M. Renkin, and L. M. Borrero. 1951. Filtration, diffusion and molecular sieving through peripheral capillary membrane. *Am. J. Physiol.* **167**:13.
33. Pappenheimer, J. R. 1965. Über die Permeabilität der Glomerulummembranen in der Niere. *Klin. Wochenschr.* **33**:362.
34. Gang, N. F., W. Mautner, and N. Kalant. 1970. Nephrotoxic serum nephritis. II. Chemical, morphological and functional correlates of glomerular basement membrane at the onset of proteinuria. *Lab Invest.* **23**:150.
35. Leber, P. D., and R. T. McCluskey. 1974. Immune complex diseases. In *The Inflammatory Process*. B. W. Zweifach, L. Grant, and R. T. McCluskey, editors. Academic Press, Inc., New York. 2nd edition. In press.
36. Schneeberger, E. E., and M. J. Karnovsky. 1971. The influence of intravascular fluid volume on the permeability of newborn and adult mouse lungs to ultrastructural protein tracers. *J. Cell Biol.* **43**:319.
37. Raviola, E., and M. J. Karnovsky. 1972. Evidence for a blood-thymus barrier using electron opaque tracers. *J. Exp. Med.* **136**:466.
38. Lanigan, R., A. Barabas, K. Peters, and J. Hardwicke. 1969. Some experimental models of the nephrotic syndrome. In *Methods and Achievement in Experimental Pathology*. E. Bajusz, G. Jasmin, Editors. **4**:141.
39. Valdes, A. J., L. B. Senterfit, A. L. Pollack, and F. G. Germuth. 1969. The effect of an antigen excess on chronic immune complex glomerulonephritis. *J. Hopkins Med. J.* **124**:9.
40. Wilson, C. B., and F. J. Dixon. 1970. Antigen quantitation in experimental immune complex glomerulonephritis. I. Acute serum sickness. *J. Immunol.* **105**:279.
41. Lambert, P. H., and P. A. Miescher, 1972. Dynamics of immune complex nephritis during antibody excess. *Fed. Proc.* **31**:791.
42. Gluck, M. C., G. Gallo, J. L. Lowenstein, and D. S. Baldwin. Membranous glomerulonephritis. Evolution of clinical and pathological features. *Ann. Int. Med.* **78**:1, 1973.