ELECTRON MICROSCOPIC STUDIES OF EXPERIMENTAL NEPHRITIS WITH FERRITIN-CONJUGATED ANTIBODY*

The Basement Membranes and Cisternae of Visceral Epithelial Cells in Nephritic Rat Glomeruli

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The acute nephritis that rapidly follows the injection of antikidney serum is the result of a specific reaction between antigens localized in the glomeruli and antibodies contained in the nephrotoxic sera. The antibodies, demonstrated by means of the fluorescence technique, were found in greatest concentration apparently in or on the glomerular basement membrane of nephritic animals and were noted to remain there for many months (1). The precise localization of nephrotoxic globulin in the glomeruli, however, was difficult to establish owing in part to the limited resolution of the ultraviolet light microscope and in part to the relatively thick sections employed.

This paper reports the use of ferritin-conjugated antibody globulin (2) to identify the sites of antigen-antibody interaction by electron microscopy. This technique has been applied to the localization of viral and bacterial antigens (3–6). Preliminary studies from this laboratory on the application of ferritin-conjugated antibody to the study of experimental glomerulonephritis have been described elsewhere (7).

Materials and Methods

The sera used to produce nephritis were the same rabbit antisera to rat kidney employed in groups 1 and 3 referred to in reference 1. Long-Evans rats, varying in age from 2 to 3 months, were injected intravenously on 2 or 3 successive days with a high titered antikidney serum which produced proteinuria and renal lesions within 24 hours. The animals were killed 1 week later.

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Duck antibodies to rabbit globulin were conjugated with ferritin as previously described (2, 3, 6). Before use the conjugated globulins were adsorbed successively with acetone-extracted, rat and mouse liver powders.

The effective use of a ferritin-conjugated antibody for detecting sites of immunologic reaction within animal tissue by electron microscopy requires a fixative which preserves fine structure without diminishing the capacity of the antigen to react with antibody. To determine the effect of fixation, small blocks of nephritic kidney were frozen and cut at a thickness of 4 microns in a cryostat. The sections were then immersed in acetone, buffered osmium tetroxide, or different concentrations of calcium or phosphate formalin at 4°C. At intervals the sections were washed and stained with fluorescein-labeled duck antibody to rabbit globulin. It was found that osmium tetroxide almost immediately prevented specific binding of the duck antibody and that acetone (after 10 to 20 minutes) decreased the amount of tagging. The best results were obtained by fixing the tissue for brief periods of time at 4°C in 5 per cent formalin buffered at pH 7.2 with phosphates.[1]

On the basis of these experiments the following technique was adopted. Thin slices of renal cortex were fixed for periods up to 1 hour at 4°C in 5 per cent formalin phosphate buffered at pH 7.2. To facilitate penetration of ferritin the fixed tissue was cut by hand with a razor blade into small fragments under a dissecting microscope in the cold. The smallest pieces were collected in a watch glass, immersed for 30 minutes at room temperature in the ferritin-conjugated antibody, washed three times, for 10 minutes each time, in cold Tyrode's solution, and centrifuged. The resulting pellets were fixed in osmium tetroxide, dehydrated in graded dilutions of ethanol, and embedded in methacrylate. Sections 0.25 microns thick were cut on a Porter-Blum type microtome and fragments of glomeruli were identified in a phase contrast microscope. The block was then trimmed so the glomeruli could be sectioned for electron microscopy.

In addition, slices of renal cortex from normal rats were fixed for 1 hour in the cold with 1 per cent osmium tetroxide buffered with veronal acetate at pH 7.4 containing 0.14 M sucrose (8). The tissue was then transferred directly to 70 per cent ethanol, dehydrated in graded dilutions of ethyl alcohol, and embedded in a 1:1 mixture of epon 812 + DDSA[2] (62:100) and epon 812 + MNA[3] (100:89) according to the method of Luft (9). The thin sections were stained with lead hydroxide.

RESULTS

When a frozen section of kidney from a rat injected with rabbit antikidney serum is cut in a cryostat and stained with fluorescein-labeled duck antibody to rabbit globulin the sites of binding of the rabbit antibodies which produce nephritis are seen to fluoresce in ultraviolet light. This is illustrated in Fig. 1 where the presence of the nephrotoxic globulin is indicated by the brightly fluorescing central portion of the capillary wall. The cytoplasm of the epithelial (arrow) and endothelial (insert) cells exhibits distinct, though lesser, fluorescence. At the top of the picture there are two faintly fluorescent tubular basement membranes.

[1] It is interesting that methacrylate-embedding of tissue which was previously treated with the specific fluorescein-labeled antibody greatly decreased or eradicated the fluorescence. Also, sections of formalin-fixed nephritic kidney first embedded in methacrylate could not be subsequently stained with fluorescent antibody even after removal of the plastic with amyl acetate or acetone.

[2] DDSA, dodecenyl succinic anhydride.

[3] MNA, methyl nadic anhydride.

A problem encountered in the use of ferritin-conjugated antibody is to obtain pieces of tissue sufficiently thin so that the conjugate can penetrate. Fig. 2 illustrates such a thin section of nephritic kidney fixed in formalin and cut by hand. It has been immersed in fluorescein-labeled duck antibody to rabbit globulin with consequent staining of the tissue but superimposition prevents accurate localization of the fluorescein. Fig. 3 shows a fragment of the same kidney, which, after exposure to the ferritin-conjugated antibody, was embedded in methacrylate, cut at 0.25 microns in a Porter-Blum type microtome, and viewed by a phase contrast microscope. It demonstrates the fine fragmentation of the tissue necessary for the penetration of the ferritin-conjugated antibody. Parts of two glomerular tufts are in the center and upper left, and tubules are visible at the periphery of the field.

The following three figures illustrate the results obtained by electron microscopy of the kidneys of nephritic rats which have been treated with ferritin-conjugated antibody to rabbit globulin. Fig. 4 shows a thin section through one of the glomerular parts seen in Fig. 3. The basement membrane (*B*) is clearly defined and is seen to contain numerous ferritin granules. Some ferritin is also evident in the epithelial foot processes (*p*) and in the endothelial cytoplasm (*EN*). Fig. 5 illustrates an oblique section through the capillary wall of another glomerulus. Three layers are evident: the epithelial foot processes (*p*), the basement membrane proper (*B*), and the endothelial cytoplasm (*EN*). Ferritin granules bound to antibody are most numerous in the basement membrane but are also present in the epithelial foot processes and endothelial cytoplasm. Fig. 6 shows a tangential section of a glomerular capillary wall. The greater concentration of ferritin-conjugated antibody in the basement membrane (*B*) as compared to the foot processes (*p*) is clearly visible.

The next two figures illustrate a normal rat kidney fixed in osmium tetroxide and embedded in epon. In Fig. 7, the nucleus (*N*) is at the top, the cytoplasm in the center of the field, and the foot processes (*p*) and the basement membrane (*B*) of a capillary loop are evident in the lower left. In the cytoplasm a large cisterna contains finely granular and filamentous material closely resembling, as Farquhar, Wissig, and Palade (10) have pointed out, the ground substance of the basement membrane. This material is concentrated on the periphery forming two ring-like profiles. The cisterna is enclosed by a membrane of the endoplasmic reticulum with attached RNA granules. Fig. 8, illustrating the left portion of Fig. 7 at higher magnification, reveals that communications between the distended cisterna and the endoplasmic reticulum form a lacunar system which appears to extend into the foot processes.

The possibility that an antigenic relationship exists between material in the basement membrane and the cisterna is supported by the evidence presented in the following four figures which illustrate nephritic rat kidneys treated with ferritin-conjugated antibody to rabbit globulin. Fig. 9 shows a section of the same kidney illustrated in Fig. 4. A distended cisterna contains basement membrane-like material which is concentrated in a central and peripheral zone. Numerous granules of ferritin conjugated with duck antibody to rabbit

globulin are evident within this cisterna. Only a few, scattered, ferritin granules are present in the surrounding epithelial cytoplasm. Fig. 10 shows the epithelial cytoplasm, the foot processes and the basement membrane from a glomerulus of the same rat seen in Fig. 6. In the epithelial cytoplasm a distended cisterna with a triloculated mass of basement membrane–like material contains numerous ferritin granules mainly localized at the periphery. The capillary basement membrane is also tagged with ferritin. Fig. 11 illustrates the localization of ferritin-conjugated antibody in the basement membrane of a capillary wall. Ferritin granules are also present in what may be an epithelial cisterna close to the basement membrane (arrow). In Fig. 12, showing the kidney from another nephritic rat, a cisterna contains bands of dense ferritin-tagged material.

In no instances have significant amounts of ferritin been encountered in the nuclei of glomerular epithelial or endothelial cells or in the tubular epithelial cells; but tubular basement membranes were observed to contain ferritin granules.

Controls for the foregoing experiments were carried out by handling duplicate fragments of renal cortex from the nephritic rats as follows:

(*a*) Immersion for 30 minutes in duck antibodies to rabbit globulin which had not been conjugated with ferritin. The tissue was then washed and treated, as described above, with ferritin-conjugated antibody. The purpose of this control was to determine if the untagged antirabbit globulin blocked subsequent binding in the glomeruli of the conjugated antiserum.

(*b*) Treatment with ferritin-conjugated non-specific antibody globulins (anti-influenza virus globulin or anti-Pneumococcus type II globulin).

(*c*) Exposure to purified ferritin alone.

In addition, fragments of renal cortex of normal rats and of rats injected with normal rabbit serum were treated with ferritin-conjugated duck antibody to rabbit globulin.

In each case there was a striking decrease in concentration of ferritin in sections from the control as compared to the experimental animals. Figs. 13, 14, and 15 illustrate the result of two of the foregoing controls. In Fig. 13 a glomerular capillary wall formed by the epithelial foot processes (*p*), the basement membrane (*B*), and the endothelial cytoplasm (*EN*) is visible. The kidney fragment was treated with unconjugated duck antibody to rabbit globulin prior to application of the ferritin-conjugated antibody. Only an occasional ferritin granule is visible. Fig. 14 shows a glomerular capillary wall from a normal rat kidney treated with ferritin-conjugated duck antibody to rabbit globulin. A few, scattered ferritin granules may be identified. In Fig. 15, illustrating a section of normal kidney treated in the same manner, neither the basement membrane nor the epithelial cisterna contains ferritin.

DISCUSSION

The data obtained by these electron microscopic studies of rat kidney tissue treated with ferritin-conjugated duck antibody to rabbit globulin support the opinion that nephrotoxic antibody is mainly localized in the glomerular basement membranes, and, to a lesser extent, in the contiguous epithelial and endothelial cytoplasm.[4] The significance of a smaller amount of ferritin-conjugated antibody randomly distributed in the epithelial and endothelial cytoplasm is unclear. The possibility that this distribution of ferritin reflects the presence of nephrotoxic globulin is suggested by the blocking experiments. However, the paucity of the ferritin granules and their lack of relation to specific cytoplasmic structures makes interpretation difficult. The tubular basement membranes appeared to contain the nephrotoxic globulin. The presence of antigen in this latter structure was not readily demonstrated by the use of fluorescein-labeled antibody to rabbit globulin. This appears to illustrate the sensitivity of ferritin-conjugated antibody in detecting small amounts of antigen.

Regarding the origin of the glomerular basement membrane there has been some controversy. Hall and Roth (12) thought that the developing glomerular basement membrane in rats was intimately associated with the endothelium, whereas Benedetti and Marinozzi (13), from a study of rat kidneys, and Kurtz (14), from a study of human embryonic kidneys, proposed that glomerular basement membrane was a product of both endothelial and visceral epithelial cells, since it appeared to receive a contribution from each, in the form of a double membrane. Vernier (15), however, in examining human fetal kidneys, observed that the epithelial cytoplasm contained material which resembled the lamina densa of the glomerular basement membrane, both morphologically and in its reaction to silver impregnation. He interpreted these data as supporting the concept that the epithelium contributed to the formation and maintenance of the basement membrane. Moreover, the results of electron microscopic examination of kidney biopsies from patients with nephrosis demonstrated deposition of basement membrane–like material in the epithelial cytoplasm, as well as thickening of the glomerular basement membrane on the epithelial side, suggesting that secretions from the epithelial cells provide material for the basement membrane (16–18).

The present studies with ferritin-conjugated antibody show that both the glomerular basement membrane and the basement membrane–like material collected in the cisternae of the glomerular epithelial cells in nephritic rats are tagged by ferritin-conjugated antibody to rabbit globulin. This observation is consistent with the presence of similar antigens in the cisternae and basement membranes, thus lending support to the hypothesis advanced by Farquhar,

[4] These structures have been shown to constitute the "glomerular basement membranes" as seen in ordinary sections (11).

Wissig, and Palade (10) that the cisternae of the epithelial endoplasmic reticulum contain components of the basement membrane and "may be concerned with synthesis, intracellular transport, and storage of these components."

It should be emphasized that the foregoing experiments were carried out with nephrotoxic sera which contained an unknown number of antibodies to renal tissue. Therefore one cannot be certain whether the antigens which bound the nephrotoxic sera, and hence the ferritin-conjugated antibodies, in the glomerular basement membranes are single or multiple. In order to solve this problem it will be necessary to employ a nephrotoxic antibody prepared against purified antigens (19).

SUMMARY

Ferritin-conjugated antibody has been used to identify by electron microscopy the sites at which nephrotoxic globulins localize in rat kidney during acute experimental glomerulonephritis. Antibody was concentrated in the glomerular basement membrane and in basement membrane-like material contained in distended cisternae of the endoplasmic reticulum. These data confirm and amplify, at the ultrastructural level, the results of studies obtained with the fluorescent antibody technique, and are consistent with the hypothesis that the cisternae and capillary basement membrane possess common proteins.

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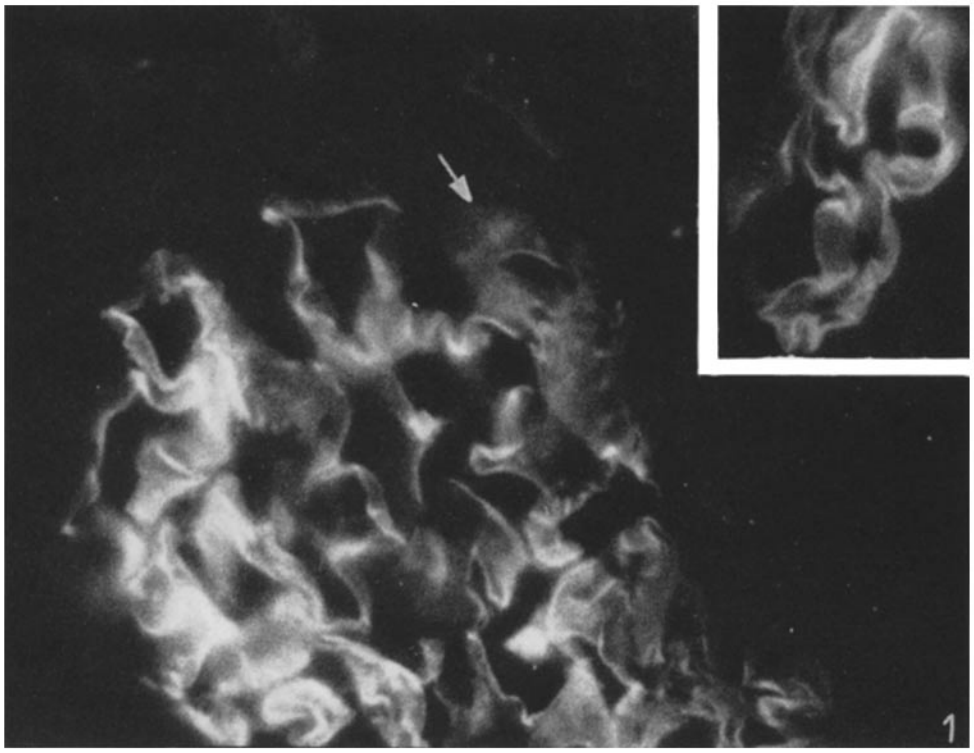
EXPLANATION OF PLATES

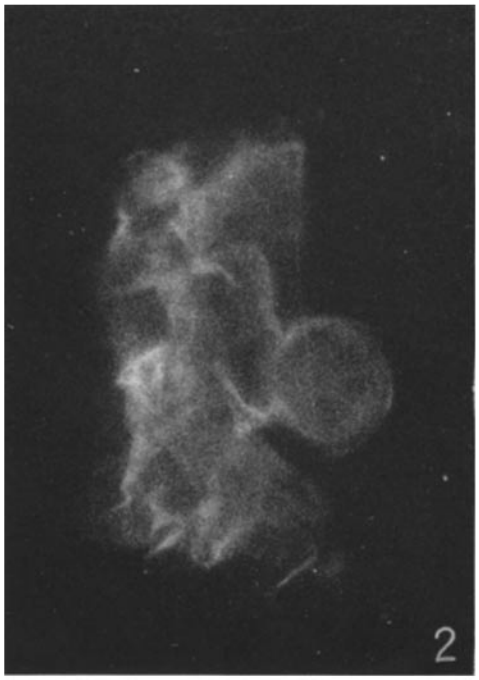
PLATE 92

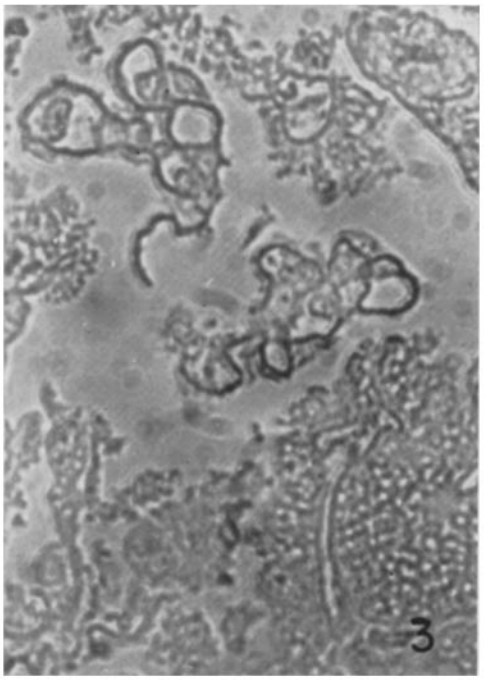
FIG. 1. A glomerulus of a nephritic rat kidney rapidly frozen in a carbon dioxide–butyl alcohol bath, sectioned in a cryostat at a thickness of 4 microns, stained with fluorescein-labeled duck antibody to rabbit globulin, and photographed by ultraviolet light microscopy. The fluorescent globulin is apparent in the basement membrane as well as in the epithelium (arrow). In the upper part of the picture are two faintly fluorescent portions of tubular basement membranes. Magnification × 1,000. The insert illustrates fluorescence of the endothelial cytoplasm. × 1,000.

FIG. 2. A small fragment from another part of the same renal cortex illustrated in Fig. 1. The specimen was fixed at 4°C in 5 per cent formalin buffered with phosphates at pH 7.2, cut by hand under a dissecting microscope, and stained with fluorescein-labeled duck antibody to rabbit globulin. The fluorescence is evident. Magnification × 950.

FIG. 3. A phase contrast photograph of methacrylate-embedded renal tissue from the same rat illustrated in Figs. 1 and 2, but treated with ferritin-conjugated antibody. Two glomerular fragments, formed by 5 to 6 capillary loops, are situated in the center and in the upper left corner of the picture. Parts of tubules are at the periphery. This block was further trimmed and sectioned for electron microscopy (see Fig. 4). Magnification × 850.







(Andres *et al.*: Experimental nephritis)

PLATE 93

FIG. 4. Electron micrograph of a cross-sectioned capillary formed by the epithelial foot processes (*p*), the basement membrane proper (*B*), and the endothelial cytoplasm (*EN*) from a portion of the glomerulus illustrated in Fig. 3. There is evident tagging of the basement membrane with ferritin-conjugated duck antibody to rabbit globulin. Magnification × 60,000.

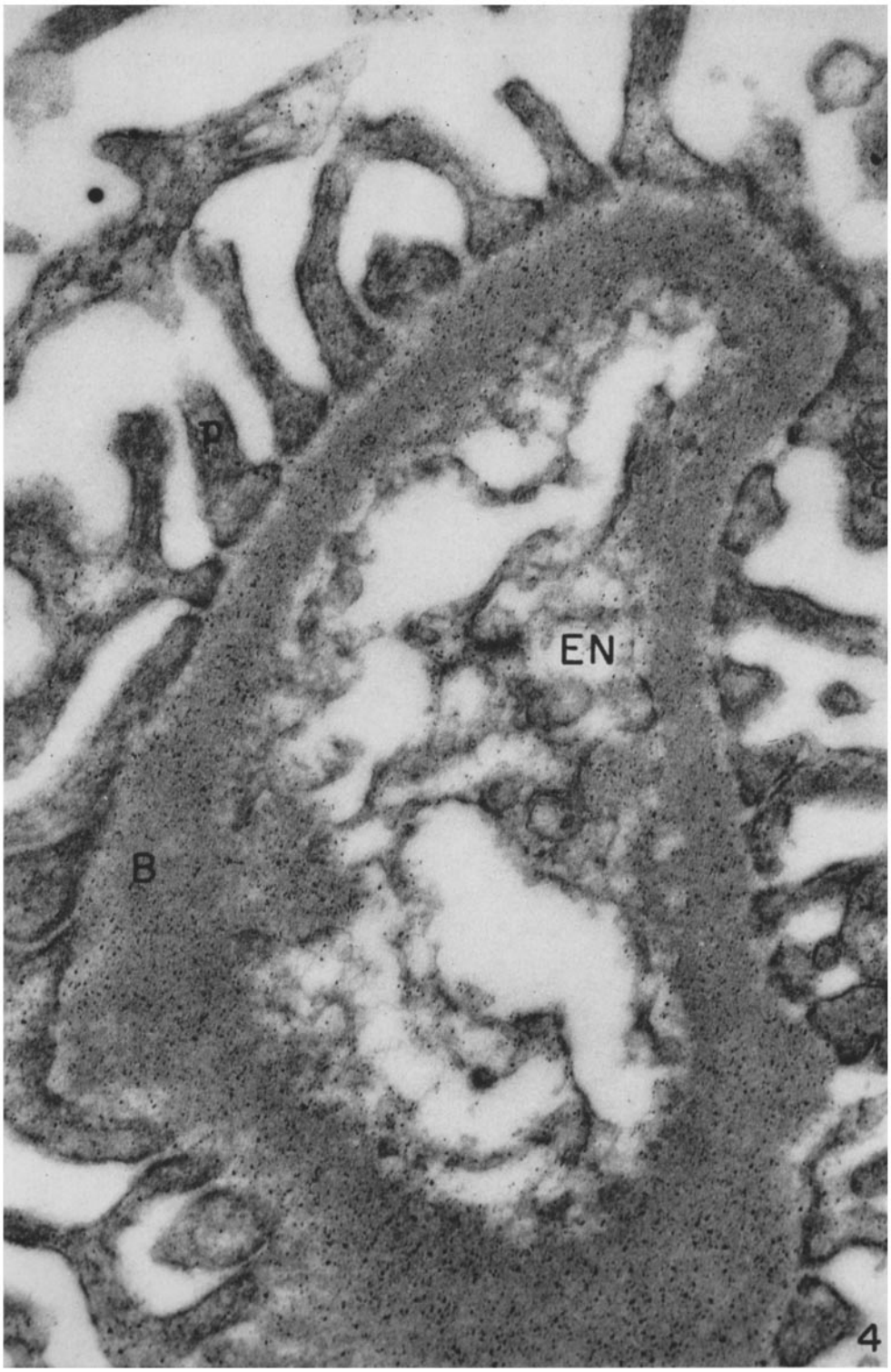


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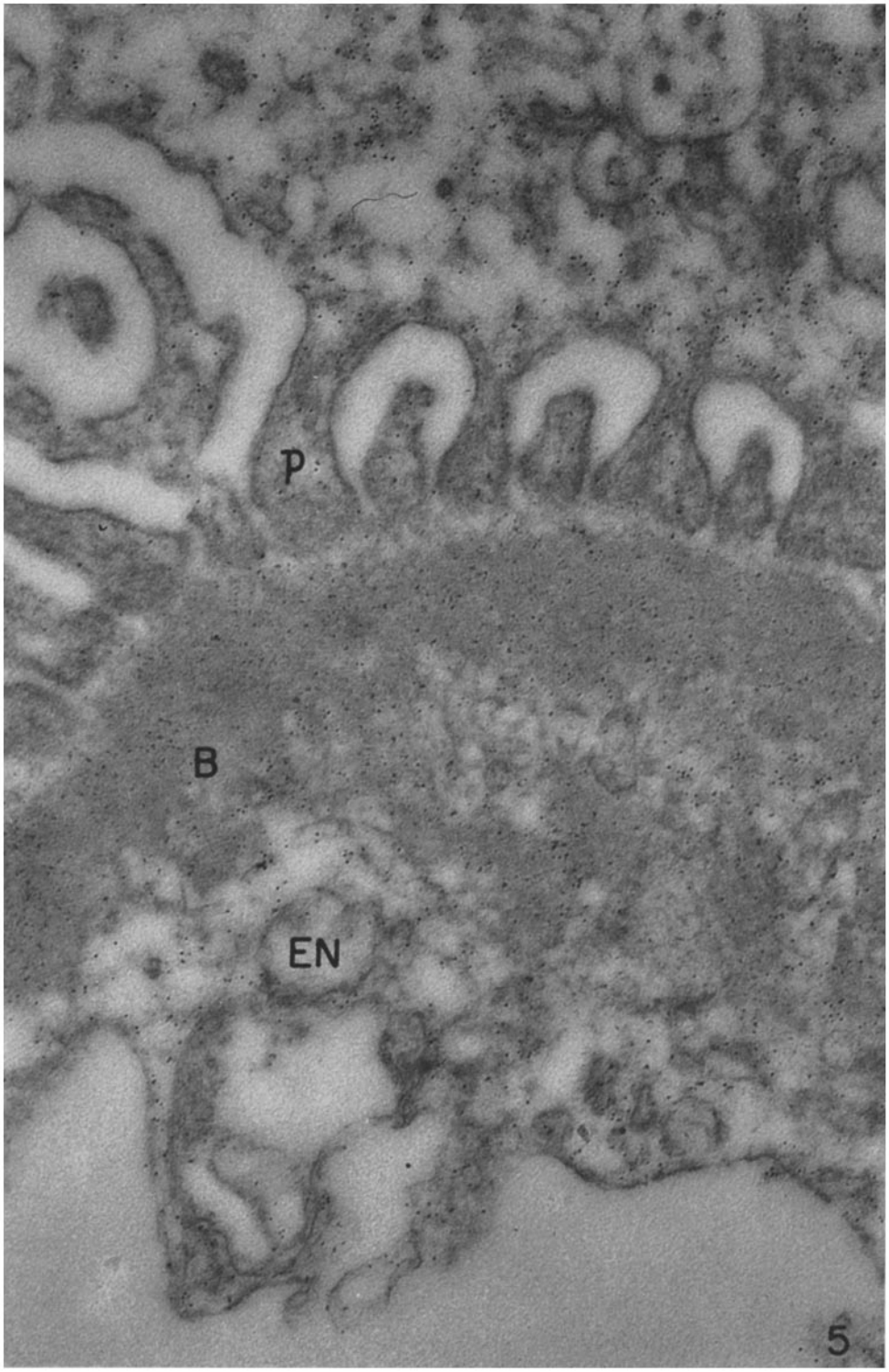
PLATE 94

FIG. 5. Oblique section of a capillary wall of another glomerulus. The epithelial foot processes (*p*), the basement membrane proper (*B*), and the endothelium (*EN*) are visible. Antibody globulin, tagged with ferritin, is concentrated in the basement membrane. Whether the scattered ferritin granules, also present in the foot processes and in the endothelium, result from specific tagging is uncertain. Magnification × 62,000.



(Andres *et al.*: Experimental nephritis)

PLATE 95

FIG. 6. Tangential section of a glomerular capillary wall from another nephritic rat. The basement membrane is heavily tagged with ferritin. Magnification × 72,000.

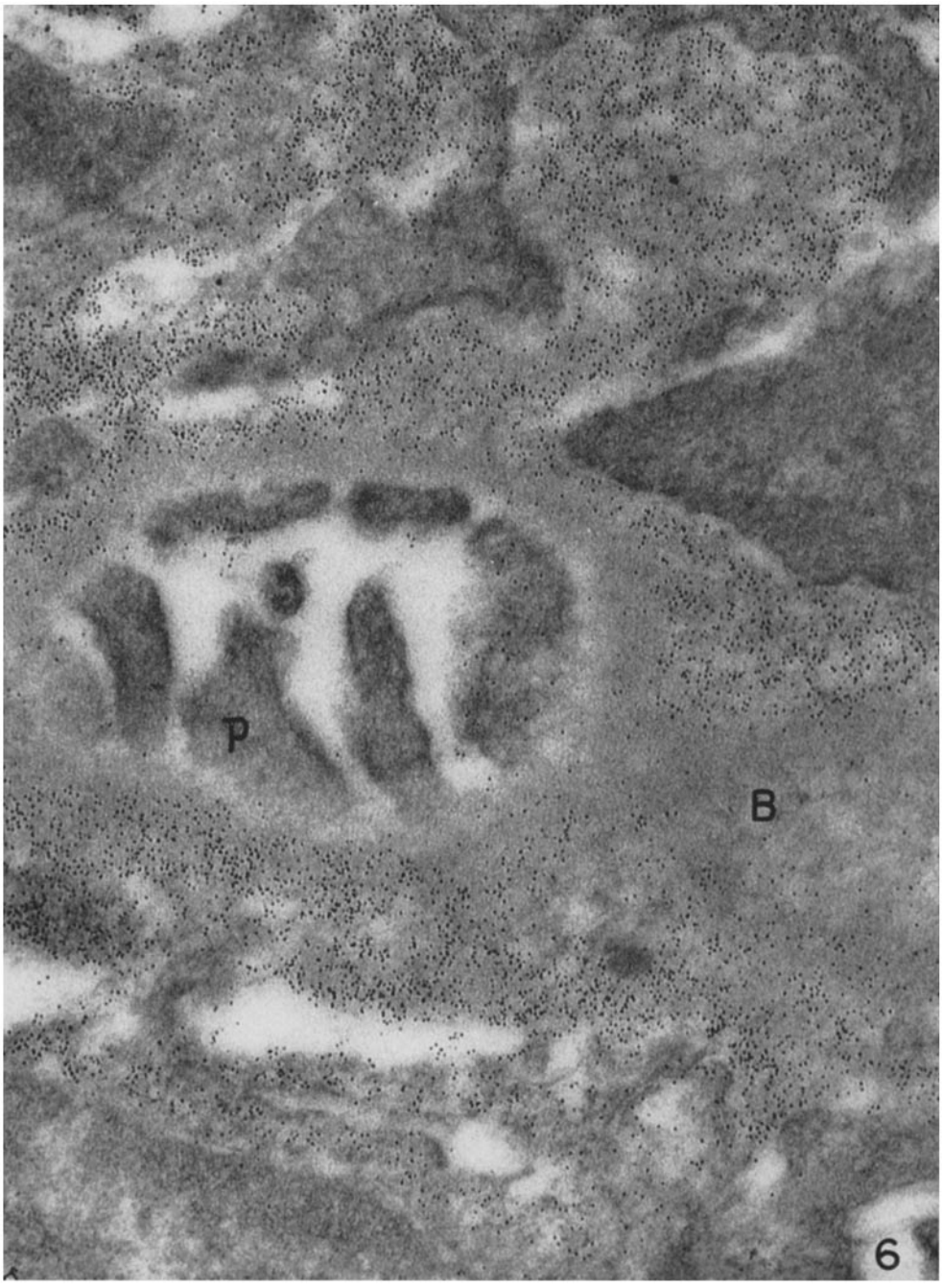


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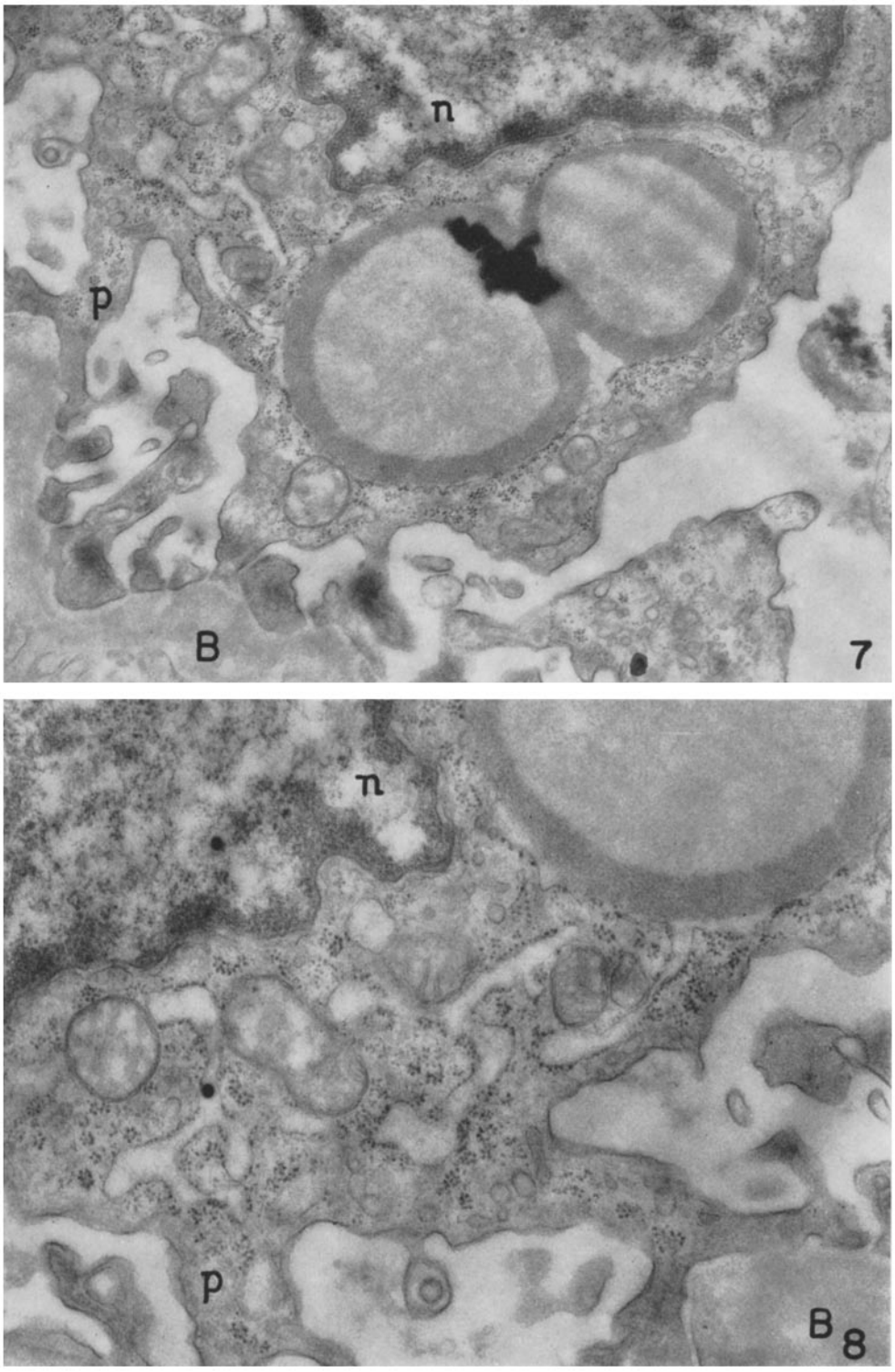
PLATE 96

FIG. 7. A visceral epithelial cell of a normal rat glomerulus fixed in osmium tetroxide and embedded in epon. The nucleus (*n*) is seen at the top; the basement membrane (*B*) and a row of foot processes (*p*) are present below and on the left. Near the center of the micrograph is a large cisterna, limited by a membrane of the endoplasmic reticulum, with attached RNA granules. Two ring profiles of dense, finely filamentous and granular material are contained in this cisterna. In the center of the field there is a precipitate resulting from lead staining. Magnification × 17,000.

FIG. 8. The left portion of Fig. 7, rotated 90°. In the upper right corner the distended cisterna of the endoplasmic reticulum contains material resembling in density and texture the ground substance of glomerular basement membrane. This material is concentrated at the periphery. There is direct continuity of the cisterna with an elongated profile of the endoplasmic reticulum which forms a lacunar system presumably extending to the end of the foot processes (*p*). Magnification × 31,000.



(Andres *et al.*: Experimental nephritis)

PLATE 97

FIG. 9. A micrograph illustrating a nephritic rat kidney treated with ferritin-conjugated duck antibody to rabbit globulin. In the cytoplasm of this epithelial cell a distended cisterna contains basement membrane–like material which is concentrated in a central and a peripheral zone. Numerous ferritin granules are present in this area whereas only few are evident in the remaining epithelial cytoplasm (*EP*). Magnification × 57,000.

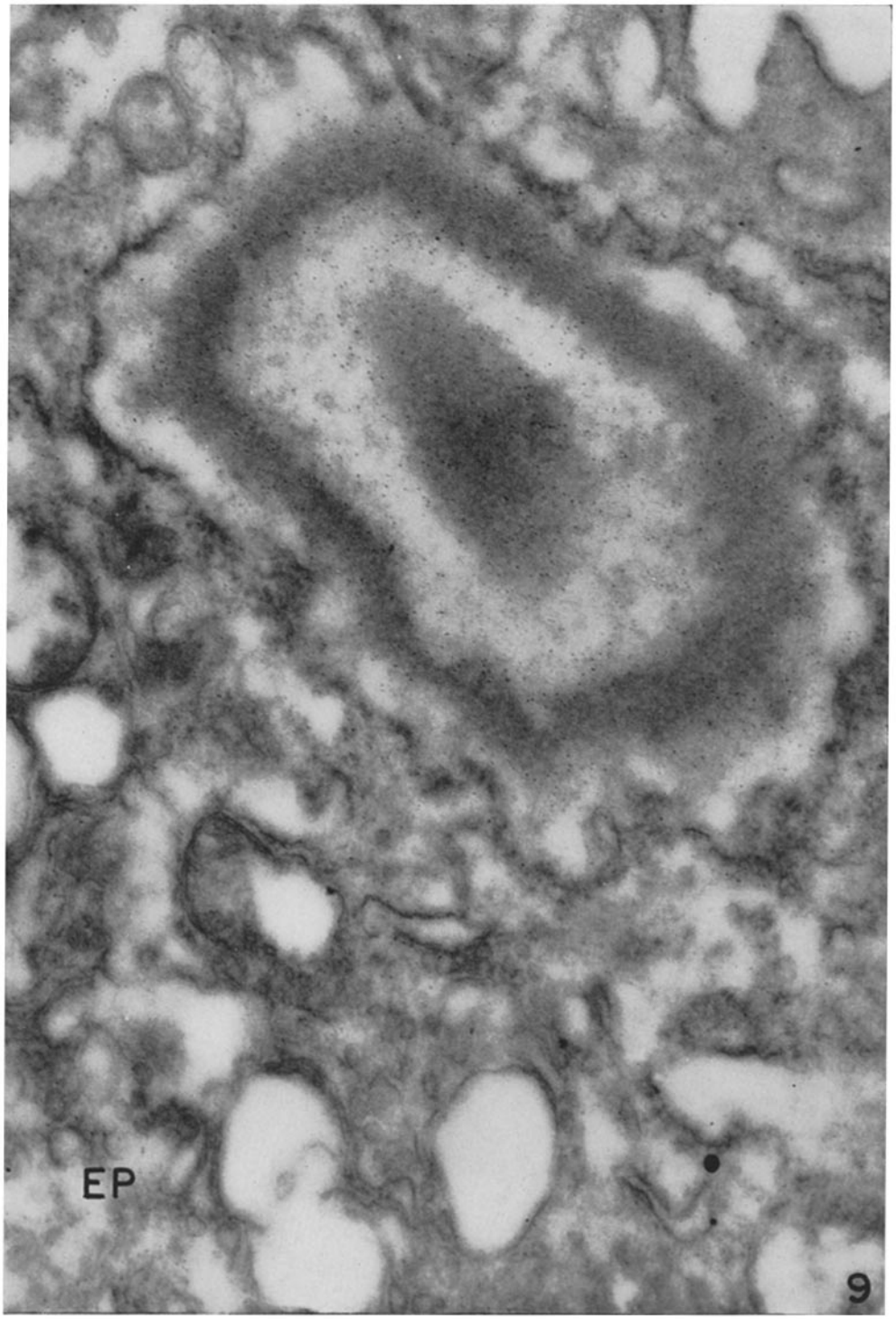


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PLATE 98

FIG. 10. Another nephritic kidney treated with the ferritin-conjugated antibody. The epithelial cytoplasm (*EP*), the foot processes (*p*), and the capillary basement membrane (*B*) are visible. In the epithelial cytoplasm a triloculated mass of basement membrane–like material contains ferritin granules mainly localized at the periphery. Ferritin is also present in the capillary basement membrane. Magnification × 70,400.

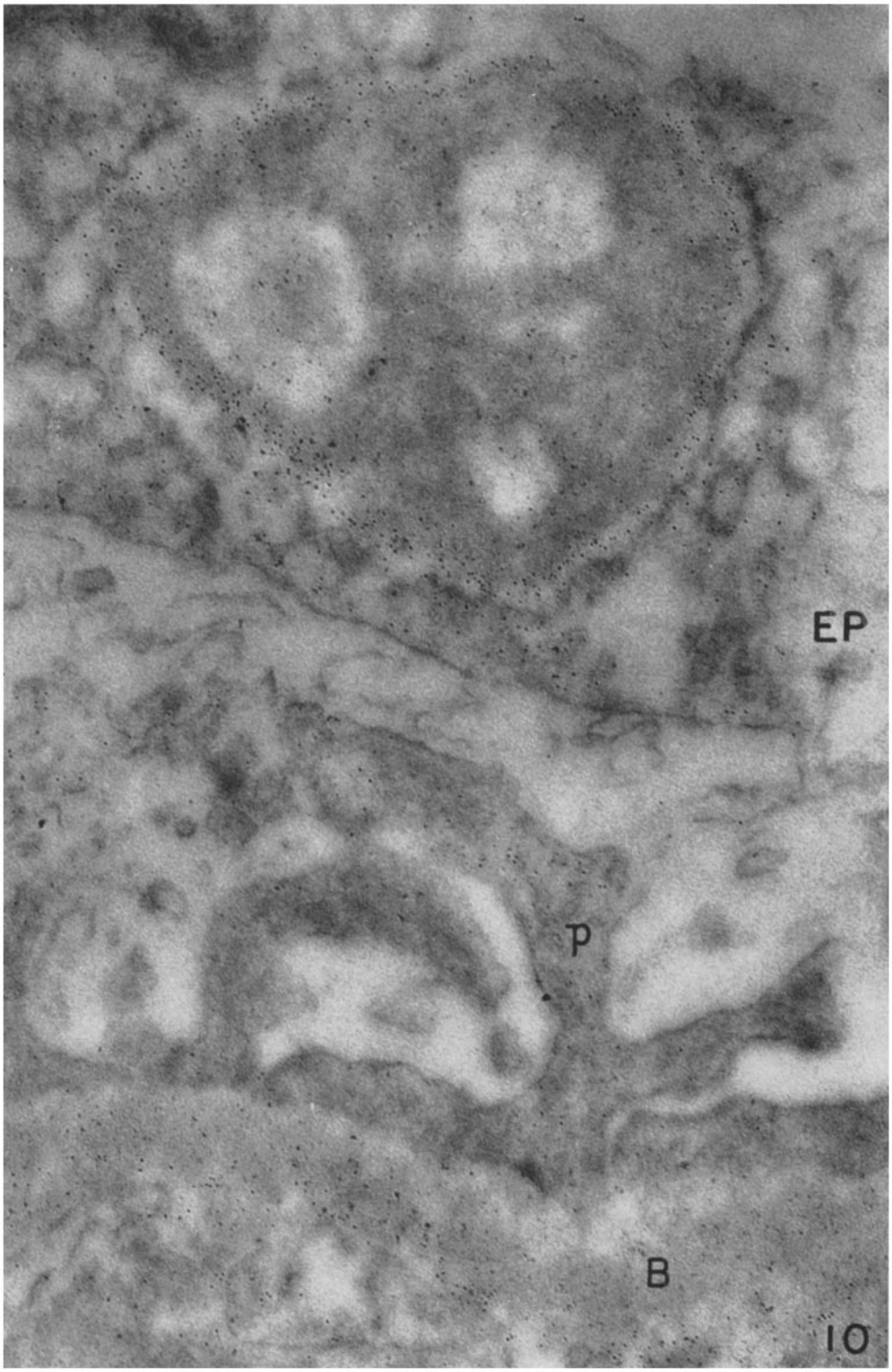

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PLATE 99

FIG. 11. A cross-section of a glomerular capillary wall from the same kidney illustrated in Fig. 10. Ferritin granules have tagged the basement membrane (*B*). It is difficult to be absolutely certain whether the structure indicated by the arrow is an epithelial cisterna or a section of contiguous glomerular basement membrane. Magnification × 43,000.

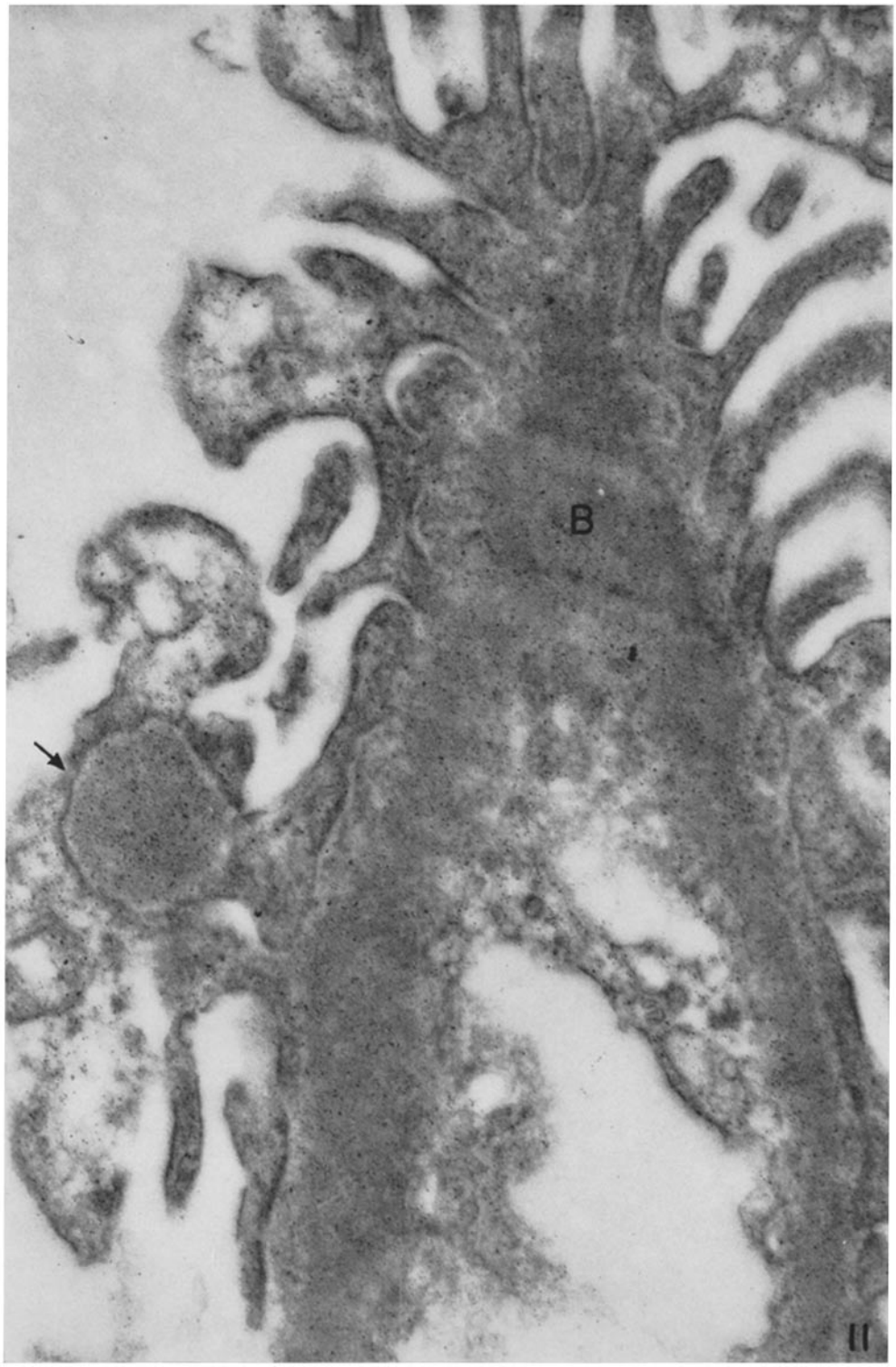

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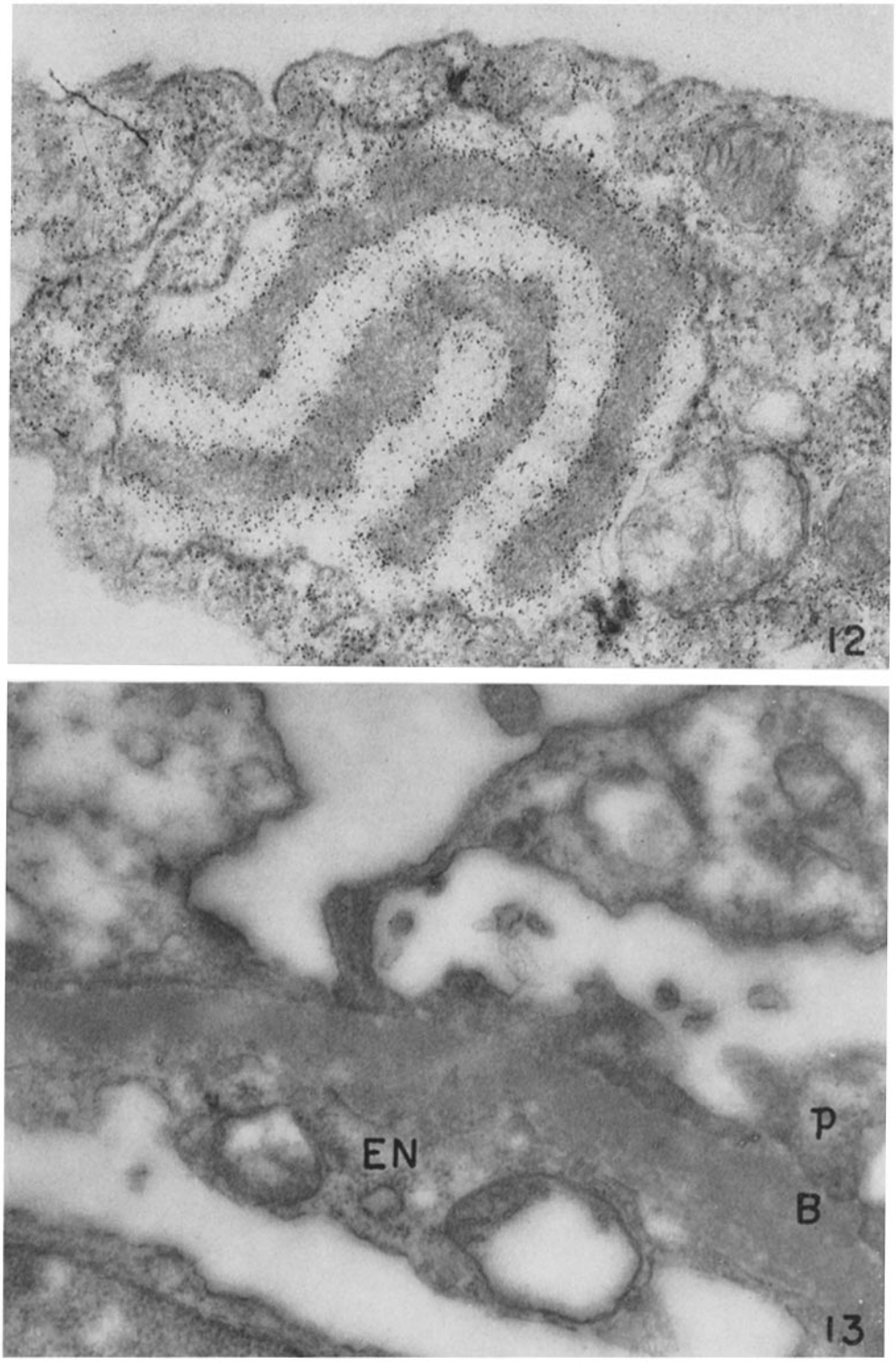
PLATE 100

FIG. 12. Section of a kidney of a nephritic rat showing a cisterna containing bands of dense material which have been tagged with ferritin granules. Magnification × 59,000.

FIG. 13. A glomerular capillary wall from a nephritic kidney treated with unconjugated duck antibody to rabbit globulin prior to immersion in ferritin-conjugated antibody. Very few ferritin granules are seen. Magnification × 47,000.

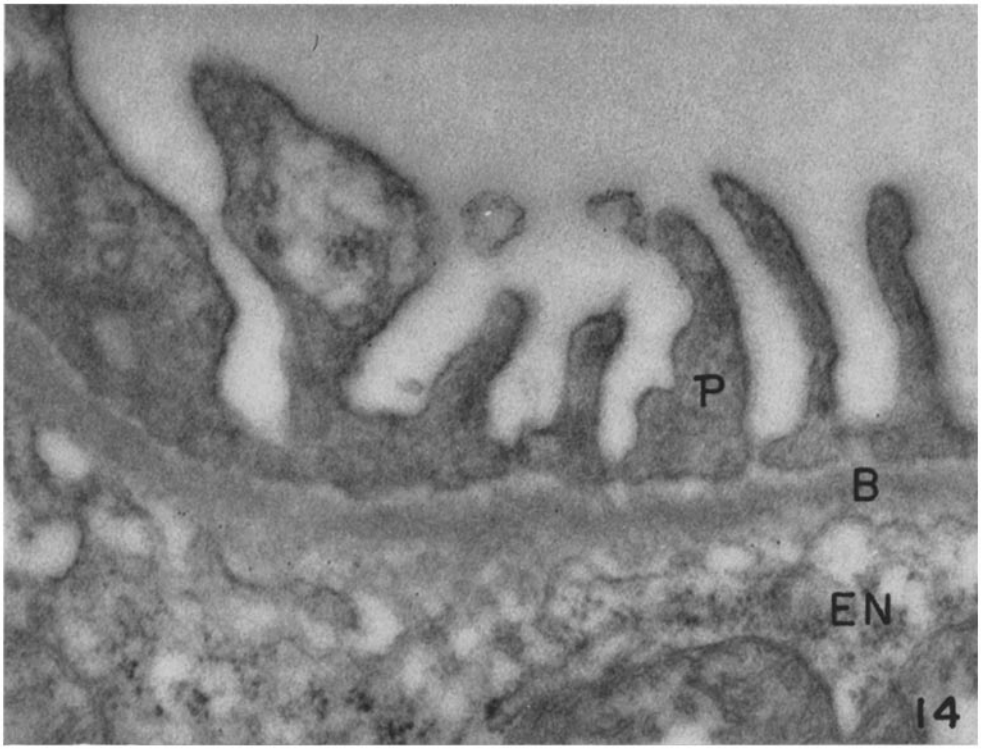


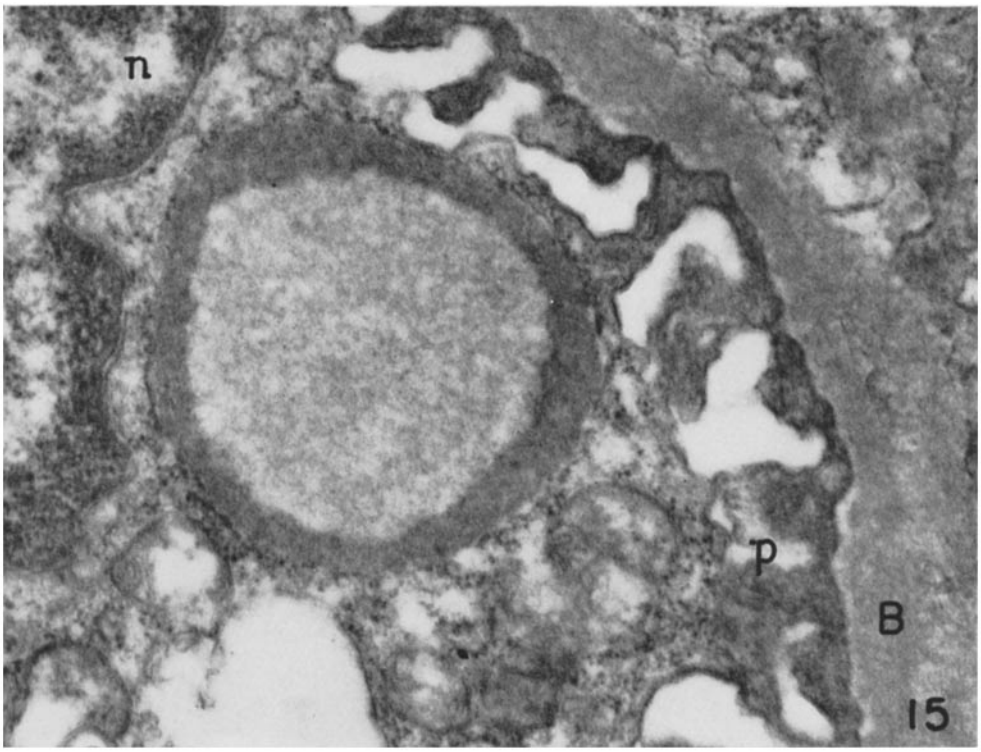
(Andres *et al.*: Experimental nephritis)

PLATE 101

FIG. 14. A glomerular capillary wall from a normal rat kidney treated with ferritin-conjugated duck antibody to rabbit globulin. It is evident that no tagging has resulted. Magnification × 63,000.

FIG. 15. A normal rat kidney, treated in a similar manner to that illustrated in Fig. 14. The nucleus (*n*) is seen on the left; near the center of the micrograph is a dilated cisterna of the epithelial endoplasmic reticulum containing basement membrane–like material, at the right a row of foot processes (*p*) and the capillary basement membrane (*B*). No ferritin is present in the basement membrane or in the cisterna. Magnification × 45,000.





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