AN ULTRASTRUCTURAL STUDY OF GLOMERULAR PERMEABILITY USING CATALASE AND PEROXIDASE AS TRACER PROTEINS*,‡

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The renal glomerulus effectively restricts plasma proteins and other macromolecules from entering the urinary space (1–6). Even though this barrier is not absolute, minimal amounts of protein normally leak into Bowman's space (7). Clearance of a particular macromolecule varies inversely with its size (1–6), as evidenced by studies on the glomerular clearance of proteins and dextrans (5–9). In general, molecules smaller than mol wt 68,000 can pass into the urine, whereas those larger are effectively restricted.

While physiological studies indicate that the glomerular filter behaves as if it were an isoporous membrane (10), pores conforming to the predicted dimensions have not so far been found in morphological studies of the glomerulus. Ultrastructural studies using particulate tracers, such as saccharated iron oxide (11) and ferritin (mol wt 500,000) (12) have indicated the presence of a filtration barrier at the level of the basement membrane. Graham and Karnovsky (13), using enzyme tracers of different molecular weights (horseradish peroxidase, myeloperoxidase), have suggested that a basement membrane barrier alone cannot explain the glomerular mechanism to prevent protein loss in the filtrate. This was indicated by the behavior of myeloperoxidase (mol wt 170,000) which crossed the basement membrane but was held back at the epithelial slit pore. On the other hand, the smaller molecule horseradish peroxidase (mol wt 40,000) permeated the basement membrane and passed through the slit pore into the urinary space. These observations have tended to confirm the hypothesis proposed by Hall (14) that the epithelial slit pore represents the filtration

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barrier in the glomerulus. Taken together, the findings with ferritin and the peroxidases seem to indicate that the basement membrane is a coarse prefilter and that the epithelial filtration slit is the final, definitive barrier (13). The current experiments employ another enzyme tracer, beef liver catalase (mol wt 240,000), to investigate this problem. Catalase has peroxidatic activity, and can be demonstrated ultrastructurally (15), using a modification of Graham and Karnovsky's method for peroxidase (16). Being of intermediate size between myeloperoxidase (13) and ferritin (12), it appears to be useful in further clarifying the glomerular barriers to macromolecules. In addition, Graham and Karnovsky's experiments with horseradish peroxidase (13) were repeated, using very small doses of enzyme, in an attempt to reveal possible gradients of diffusion across the glomerular wall to this tracer. The results of these experiments suggest the presence of a relative barrier to both tracers at the basement membrane, and a further barrier to catalase at the level of the slit pore, providing evidence for the presence of two filtration barriers in the glomerulus.

Materials and Methods

Catalase Experiments.—Mice from Charles River Breeding Laboratories, North Wilmington, Mass. weighing 25–30 g and maintained on a standard laboratory chow, were used. Beef liver catalase was obtained as a twice crystallized aqueous suspension with 0.1% thymol as preservative (Type C-100, Sigma Chemical Co., St. Louis, Mo.). Various batches contained between 20–27 mg/ml. Before use, the bottle was shaken with a rotatory motion to avoid frothing, and a uniform suspension of all sedimented crystals was obtained. The amount of catalase needed was then pipetted off and warmed in a water bath at 37–40°C until it became clear. A few minutes of ultrasonic vibration in a "Maxomatic" ultrasonic vibrator (L and R Manufacturing Co., Kearny, N. J.) at the same temperature was sometimes necessary to effect solution. Prolonged heating was avoided to prevent denaturation of catalase. In some experiments NaCl was then added to the catalase solution to achieve a concentration of 0.85%. The experimental findings were the same whether or not the injected solution contained isotonic NaCl. The osmotic effect of the catalase itself, was considered negligible. After centrifugation to remove a faint turbidity that sometimes formed after addition of NaCl, the solution was injected into the tail vein. 0.5–0.7 ml of solution containing a standard dose of 13.5 mg was injected slowly over a period of 5 min. A few animals were given higher doses, from 20 to 27 mg. Mice were sacrificed 1, 5, 30, 60, 90 min and 3, 6, and 12 hr after injection. The kidneys were removed under ether anesthesia and thin slices were fixed by immersion for 3 hr at room temperature in 2% paraformaldehyde:2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, containing 0.05% $CaCl_2$ (17). Tissues were then washed overnight in cold 0.1 M cacodylate buffer, pH 7.4, and sections 40 μ thick were cut on a TC-2 Smith-Farquhar tissue chopper (Ivan Sorvall, Inc., Norwalk, Conn.). The sections were collected in 0.05 M tris (hydroxymethyl)aminomethane (Tris)-HCl buffer at pH 8.5, and incubated at 37°C for 2 hr in a medium consisting of 10 mg 3, 3′ diaminobenzidine tetrahydrochloride (Sigma Chemical Co., St. Louis, Mo.) dissolved in 10 ml of Tris-HCl buffer at pH 8.5. A dialysis bag containing 25 mg of BaO_2 in 1 ml of Tris-HCl buffer was used as the peroxide source (15). After incubation, sections were washed twice in buffer, postfixed for 90 min in 1.3% OsO_4 in 0.2 M s-collidine buffer at pH 7.4, and embedded in Epon after rapid dehydration. Thick sections were cut on glass knives and viewed unstained. Thin sections, cut on glass or diamond knives, were examined unstained, or lightly stained with lead citrate, in a Philips

EM 200 electron microscope. Controls consisted of kidneys from animals not injected with catalase, but reacted in the complete cytochemical medium. The controls for the specificity of the cytochemical reaction itself were as described previously (15). These included inhibition of the reaction by the catalase inhibitor 3-amino-1, 2, 4-triazole (15).

In order to assess possible toxic effects of catalase on the kidney, 12 mice were injected with catalase as described above, and sacrificed at 3, 6, and 12 hr after injection. Kidneys from these animals were fixed in neutral formalin and processed for light microscopy after hematoxylin and eosin staining. Kidneys from some animals injected with catalase were also processed for routine electron microscopy without the reaction for catalase.

Peroxidase Experiments.—Two mice were injected with 0.5 and 1 mg respectively of horseradish peroxidase (Type II, Sigma Chemical Co.) in 0.2 ml of saline, and the kidneys were removed under ether anesthesia at exactly 2 min after injection. Slices of cortex were fixed and chopped into sections 40 μ thick as described above and were reacted in the complete Graham and Karnovsky medium for peroxidase (16) for 15 min, after 30 minutes of preincubation in the medium without added H_2O_2. Sections were then processed for electron microscopy.

RESULTS

General.—None of the mice injected with catalase showed visible signs of toxicity up to 24 hr after injection. Kidneys from the group of 12 animals processed for routine light microscopy were histologically normal. Kidneys from catalase-injected animals were without abnormalities when examined by electron microscopy.

Tracer Studies, Catalase Experiments, Light Microscopy.—Up to 3 hr after injection,[1] catalase was seen as a brown reaction product in glomerular and peritubular capillaries. Between 5 min and 3 hr, glomerular and peritubular basement membranes also stained positively (Fig. 1). This staining was light 5 min and intense 30 min after injection. A few glomeruli showed light staining of basement membranes or failed to stain completely even at later time intervals. When higher doses of catalase were injected (up to 27 mg), basement membrane staining appeared even at the earliest time interval, i.e., 1 min after injection. At 6 hr, catalase staining in the glomeruli and peritubular capillaries decreased considerably, and at 12 hr it was totally absent. At all time intervals prominent, brown staining of endogenous catalase in proximal tubular cell microbodies (18), endogenous peroxidase in red cells (hemoglobin), and leukocyte granules was present. Tubular lumina were uniformly empty of reaction product at all time intervals.

Electron Microscopy.—In the ensuing description the presence of reaction product of variable electron density is interpreted as representing catalase. The reaction was considered positive when the electron density obviously exceeded that seen in tissues not injected with catalase but exposed to the cytochemical procedure (Fig. 2). That the reaction product represented injected catalase was also indicated by its inhibition when 3-amino-1,2,4-triazole, a catalase inhibitor, was included in the incubation medium (15).

[1] All time intervals given are after the completion of the injection.

1 min after injection the tracer was visualized as an electron-opaque reaction product in most glomerular capillary lumina. Basement membranes were light gray and were only slightly denser than those of noninjected controls (Fig. 3). They were always less densely stained than the capillary lumina (Fig. 3). 5 min after injection the density of reaction product in the basement membranes

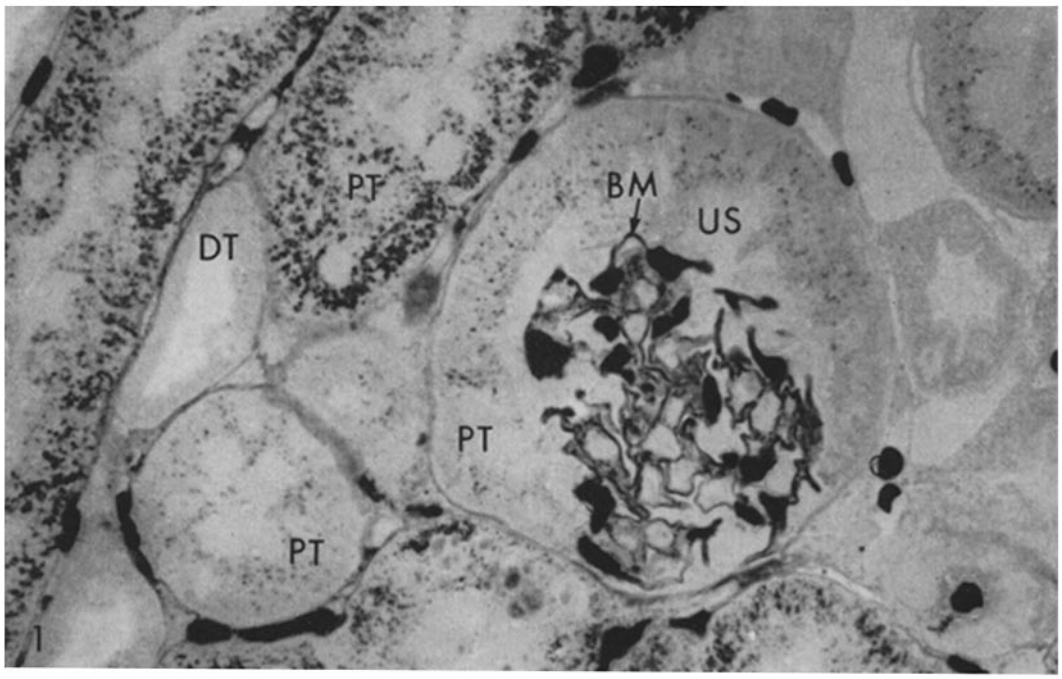


FIG. 1. Light micrograph of kidney from mouse sacrificed 30 min after injection of catalase. Reaction product is present in the glomerular basement membranes (BM) but not in the urinary spaces (US). Black staining in capillary lumina represents staining of both red blood cells and tracer. Proximal and distal tubular lumina (PT, DT), are empty of reaction product. Proximal tubular cell microbodies (dark granules) stain positively for endogenous catalase. 2 μ Epon embedded section. Not counterstained. × 750.

increased considerably (Fig. 4). Occasional concentrations of density could be seen on the luminal side of the basement membrane, opposite the endothelial fenestrae (Fig. 4). The images obtained at 30, 60, 90, and 180 min were similar, and are consequently described together. In most glomeruli, the density of reaction product was usually less in the basement membrane than in the lumen (Figs. 5–7), although occasionally it approached that of the lumen. Along the basement membrane there was variation in the density of staining (Fig. 8). The whole thickness of the membrane stained, but there was often a gradient of

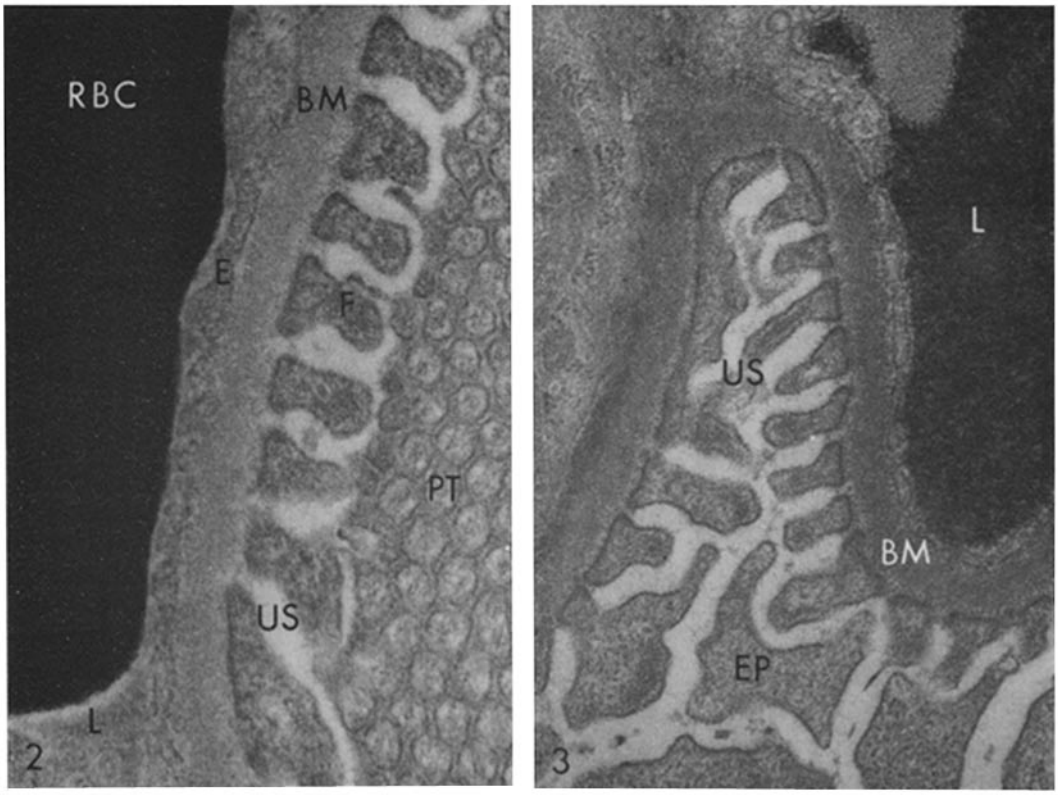


FIG. 2. Electron micrograph of glomerulus from kidney of mouse not injected with catalase but subjected to the entire cytochemical procedure. Reaction product is absent from the capillary lumen (L), basement membrane (BM), and urinary space (US). A red blood cell (RBC) is stained positively. F, foot process of epithelial cell; PT, brush border of capsular cell. In the mouse, Bowman's capsular cells are represented by proximal tubular cells. E, endothelium. Section counterstained with lead citrate. × 51,000.

FIG. 3. Electron micrograph from mouse sacrificed 1 min after injection of catalase. Reaction product is present in capillary lumen (L). Basement membrane (BM) appears slightly more dense than in Fig. 2. EP, epithelium; US, urinary space. Lightly counterstained with lead citrate. × 32,500.

staining intensity across the basement membrane, a greater concentration of enzyme was present in the inner (subendothelial) layers of the membrane than the outer (subepithelial) layers (Figs. 5–8). Occasionally (Fig. 8) small jets of the tracer could be seen penetrating into the basement membrane from the endothelial fenestrae and fanning out as areas of decreasing density. Catalase

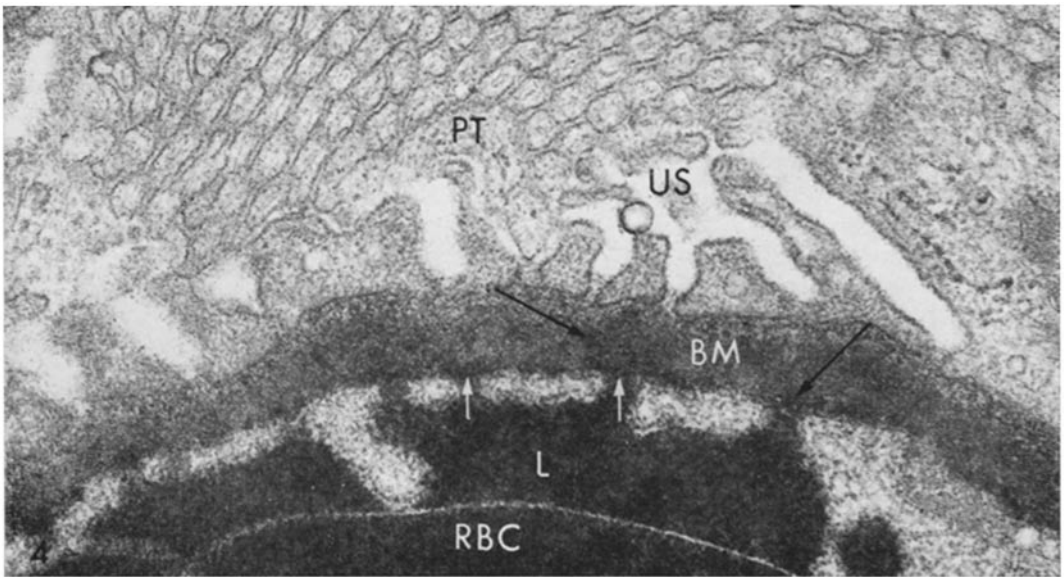


FIG. 4. Glomerulus from mouse sacrificed 5 min after catalase injection. Reaction product is visible in the capillary lumen (L) and in the basement membrane (BM). Density of staining in basement membrane is greater than at 1 min (cf. Fig. 3). Focal areas of increased density (black arrows) are also seen in the subendothelial (inner) layer of the basement membrane, especially opposite endothelial fenestrae (white arrows). Urinary space (US) is free of reaction product. PT, brush border of capsular cell; RBC, red blood cell. Lightly stained with lead citrate. × 43,000.

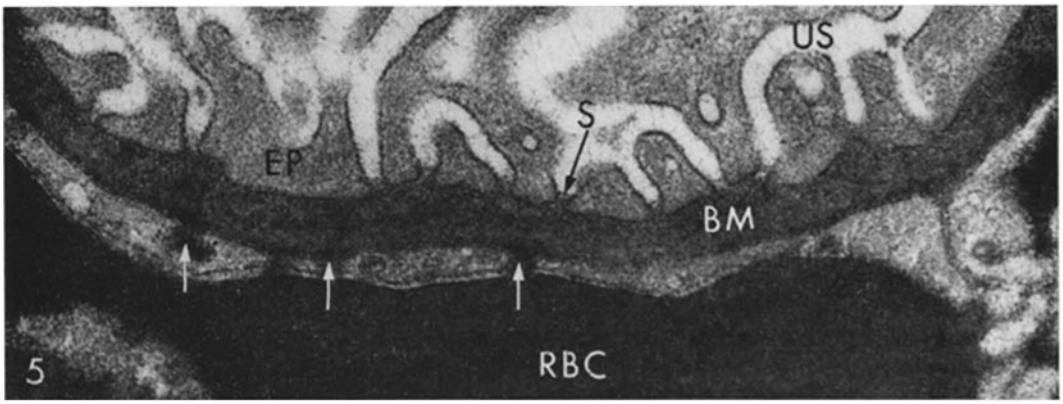


FIG. 5. Glomerulus from mouse sacrificed 90 min after catalase injection. Reaction product is present in endothelial fenestrae (white arrows) and basement membrane (BM), extending into a slit pore (S), and stopping abruptly at the level of the slit diaphragm. In other slit pores density decreases gradually towards the urinary space (US). The subendothelial layer of the basement membrane has areas of greater density, mostly near the fenestrae. The enzyme is not present in the urinary space. RBC, red blood cell, EP, epithelium. Lead citrate. × 46,000

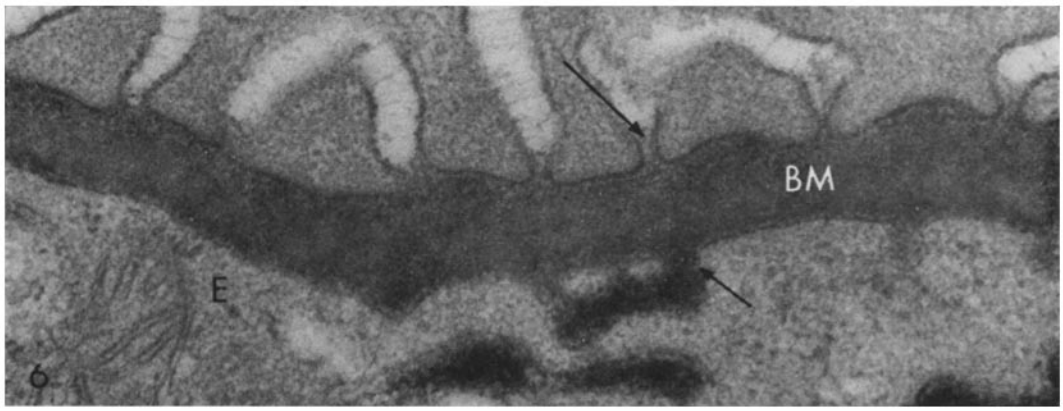


FIG. 6. Catalase-injected mouse sacrificed 90 min after injection. Reaction product density is greater in endothelial fenestra (small arrow) and in the subendothelial layer opposite the fenestra. The density of staining is seen to decrease from the basement membrane (BM) into the slit pores (long arrow). E, endothelium. Lead citrate. × 69,000.

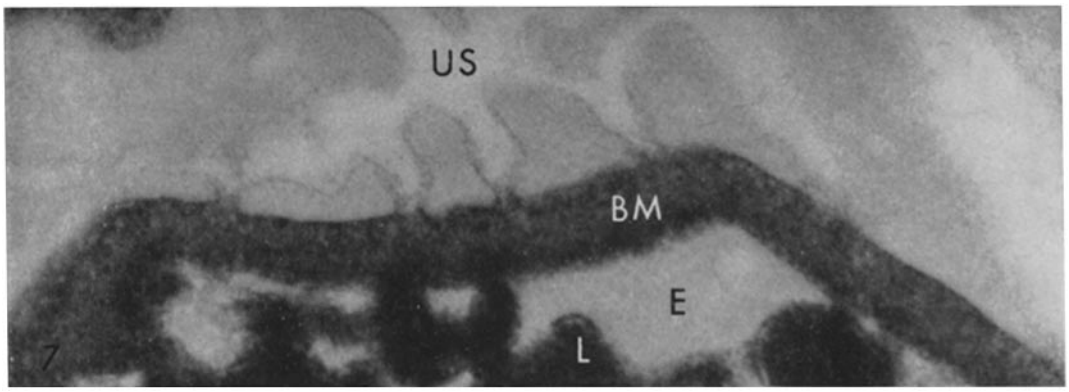


FIG. 7. Glomerulus from mouse killed 30 min after catalase injection. A concentration gradient of reaction product from the inner (subendothelial) layer of the basement membrane (BM) to the outer is demonstrated. Urinary space (US) is devoid of reaction product. E, endothelium; L, Capillary lumen. Section not counterstained. × 64,000.

staining trailed off in intensity towards the urinary space with variable extensions into the slit pores (Figs. 5 and 6), but with no clear-cut demarcations in most slit pores (Fig. 6). Uncommonly, there appeared to be an abrupt cessation of staining at the level of the slit diaphragm (Fig. 5, arrow). 6 hr after injection the capillary lumina showed considerably decreased amounts of tracer, and at

12 hr the glomeruli and other vessels appeared similar to those of noninjected animals. When larger doses of catalase were injected, the basement membranes showed a greater concentration of enzyme at the earliest time period than when smaller doses were employed.

The tubular lumina and absorption vacuoles were empty of reaction product in the vast majority of cases (Fig. 9). On the two occasions that tracer was present in tubules or Bowman's space, the finding was confined to an isolated nephron. Occasional dense droplets containing reaction product were present in the cytoplasm of glomerular epithelial cells.

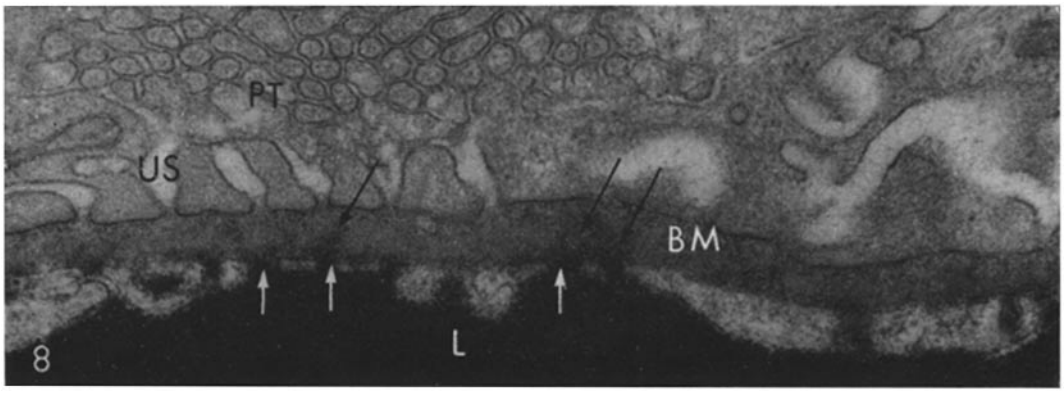


FIG. 8. Same glomerulus as in Figs. 5 and 6, demonstrating the variability of penetration of the tracer into the basement membrane (BM) in different areas. Enzyme is present in capillary lumen (L); endothelial fenestrae (white arrows) and small "jets" (black arrows) of tracer are seen to fan out into the basement membrane. US, urinary space; PT, brush border of capsular cell. Lead citrate. × 39,000.

Other Findings and Controls.—In both catalase-injected and control noninjected mice, prominent staining of proximal tubular cell microbodies was seen (Figs. 1 and 9). Both groups also showed reaction product in the red cells, leukocyte granules, occasional lysosomal dense bodies, and mitochondrial cristae (probably due to cytochrome C or cytochrome oxidase) (19, 20). In noninjected animals, capillary lumina, basement membranes, and other tissue structures were unstained (Fig. 2).

Peroxidase Experiments.—2 min after injection of 0.5 mg of horseradish peroxidase all capillary lumina contained reaction product (Figs. 10, 11). The basement membranes in general stained less intensely than the capillary luminal contents, and in addition areas of increased density were visualized in the inner third of the membrane, especially opposite the endothelial fenestrae (Figs. 10, 11). Peroxidase was also present throughout the thickness of the basement

membrane, the slit pores, the urinary spaces, and the tubular lumina, as described previously (13).

DISCUSSION

The above experiments indicate that the glomerulus effectively restricts beef liver catalase (mol wt 240,000) (21) from entering the urinary space and strongly suggest that such restriction is effected by barriers at the level of (*a*) the base-

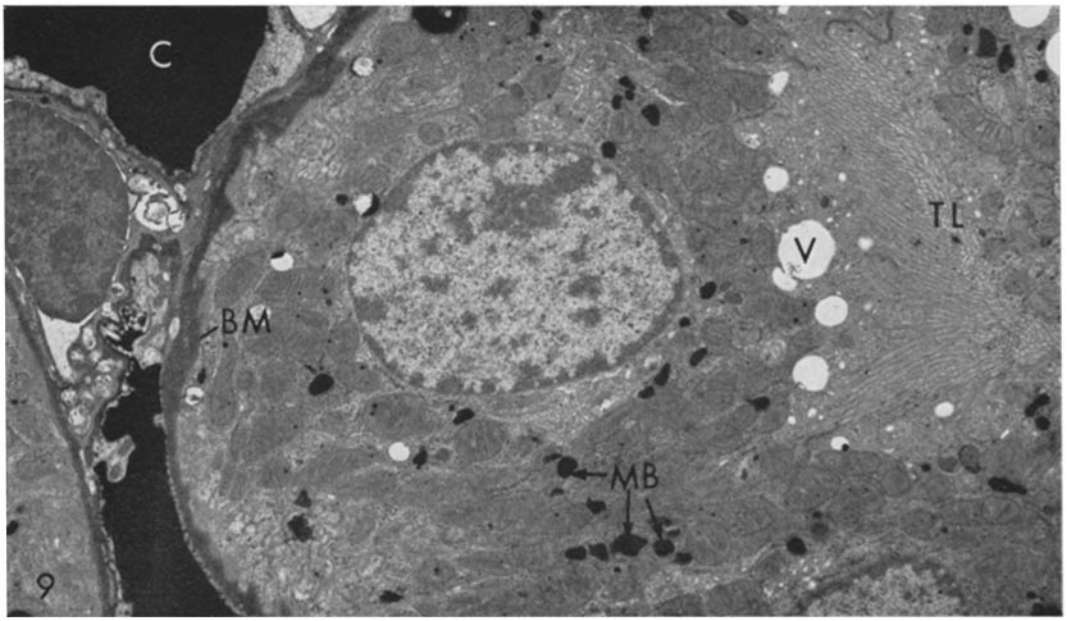
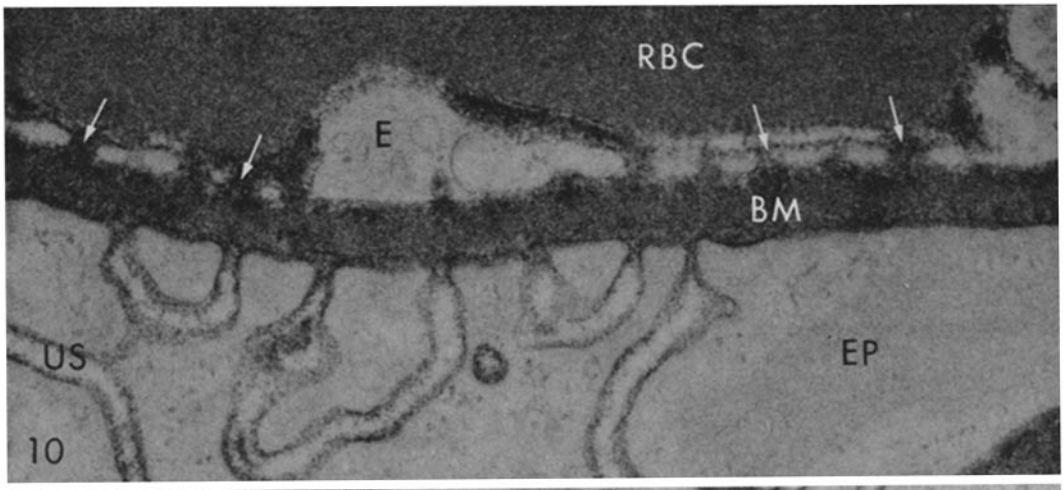


FIG. 9. Proximal tubule from mouse, 30 min after catalase injection. Reaction product is present in peritubular capillary (C), basement membrane (BM), and microbodies (MB), but is absent in tubular lumen (TL) and absorption vacuoles (V). Lead citrate. × 8000.

ment membrane and (*b*) the epithelial slit pores. That the basement membrane, particularly the lamina densa, impedes the diffusion of catalase is evidenced by (*a*) the difference in enzyme content of the basement membrane (as estimated by density of reaction product) at sequential time intervals (Figs. 3–7) and (*b*) the images of basement membrane showing gradients of reaction-product density from the inner layer across the lamina densa to the outer (as in Figs. 4–7). Variations in staining density at different levels of the basement membrane are here interpreted as indicative of differences in enzyme concentration. It can be argued alternatively that such differences could be explained on the basis of differences in the state of hydration of the various layers of the base-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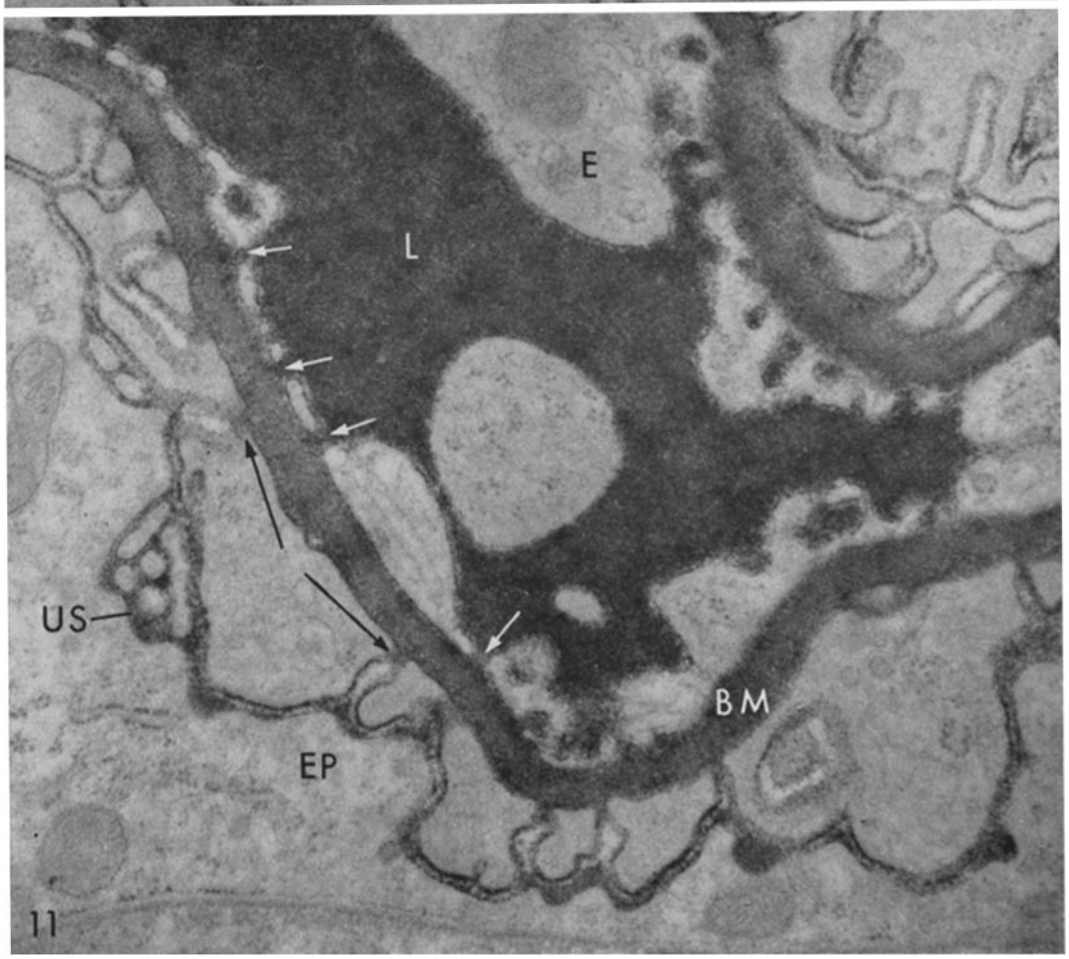
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ment membrane. If the proximal layer of the membrane is conceived as a hydrated gel, it would have less dense packing of structural components than the more compact lamina densa; thus it could hold more enzyme in its interstices and give rise to density gradients in the electron microscopic images. However, even if the latter argument is true, the existence in the lamina densa of smaller "pores" than the more loosely packed proximal layer could also be interpreted as evidence that these "pores" constitute a sieve restraining macromolecules to varying degrees according to their molecular sizes. Thus molecules would encounter increasing difficulty in passage as their sizes approach that of the pores.

In regard to the basement membrane, the experiments with catalase support prior studies with ferritin (mol wt 500,000) which indicate that passage of large molecules is restricted by the basement membrane (12). Clearance data for exogenous and endogenous macromolecules indicate that a partial glomerular barrier (molecular sieve) exists for molecules with a mol wt of 40,000 (1, 2, 5). Our experiments with small doses of horseradish peroxidase (see Figs. 10, 11) suggest that partial restriction to such a relatively small molecule also occurs in the basement membrane, but we cannot determine whether significant sieving occurs in addition at the epithelial slit pore. The need to use small amounts of horseradish peroxidase to reveal a concentration gradient can be explained by its intense peroxidatic acitivity (22) and consequent difficulty in distinguishing differences in enzyme concentration by the cytochemical method when large amounts are present at various sites.

The restriction by the basement membrane is not an absolute one, since catalase reached the slit pore in quantities sufficient to give fairly strong staining. Since it was only rarely seen in tubular lumina or urinary spaces, it might be inferred that there is a further barrier. Farquhar et al. (12) reported that the few molecules of ferritin that eventually crossed the basement membrane were taken up by the epithelial cells and were thus excluded from the urinary spaces. In the present experiments, uptake of catalase by podocytes was rarely observed. Rather, the electron microscopic images (Figs. 5, 6) suggested that the final barrier was at the level of the slit pore.

The nature of the barrier at the slit pore remains unclear. Calculations based on physiological data (10) suggest that the glomerulus behaves as if it were a membrane with cylindrical water-filled pores having a diameter range of 70–82

FIGS. 10 and 11. Glomeruli from mouse injected with 0.5 mg of horseradish peroxidase and killed 2 min later. Reaction product is visible in capillary lumen (L), endothelial fenestrae (white arrows), basement membrane (BM), slit pores (black arrows), and urinary space (US). However, staining of basement membrane is considerably less than that of the lumen. In addition, areas of increased density of tracer are seen opposite the endothelial fenestrae and in the subendothelial layer of the basement membrane. Lightly stained with lead citrate. E, endothelium; EP, epithelium; RBC, red blood cell. × 54,000, × 36,000.

A. Measurements of the width of the slit pore at its narrowest point in various species, including the mouse (23), have shown that it is too wide (200–300 A) to account for molecular sieving. However, material comparable in density to the outer layer of the basement membrane can sometimes be seen in the slit pore proximal to the slit diaphragm (24), and the presence of such material may influence the permeability properties of the slit. Alternately, it has been suggested (25) that the sialoprotein cell coat of the podocyte may serve to trap macromolecules that cross the basement membrane and thus prepare them for pinocytosis by the epithelial cells. This sialoprotein coat may be demonstrated by using colloidal iron (25, 26), ruthenium red, (27) or colloidal thorium (28), and exists as a 400–700 A thick layer (25) on the urinary surface of the podocyte extending to the level of the slit diaphragm and filling the space of the slit pore. Mohos and Skoza (26) proposed that the strongly anionic cell coat may set up an electrostatic field at the slit pore which would repel like-charged molecules and prevent their entry into the urinary space. Lastly, the 60 A thick slit diaphragm might itself represent a filtration barrier. Graham and Karnovsky showed that when myeloperoxidase is used as a tracer (13) it is restricted by the glomerulus at the slit pore, and favorable electron microscopic images reveal it to be concentrated or pooled in this area as if it were being held back. Such an appearance, i.e. pooling of tracer at the slit pore, was not seen in the present experiments with catalase. Rather, the density of the enzyme usually decreased from the basement membrane towards the slit. The reasons for this are unclear. Differences in electrical charges between the two proteins could conceivably account for this phenomenon, since catalase has an isoelectric point of pH 5.7 (29) while that of myeloperoxidase is greater than pH 10 (30).

An additional protective mechanism to prevent diffusion of proteins into the urinary space could be the narrowing of the slit pores themselves, consequent to blunting of the foot processes. Such a phenomenon, leading in time to foot-process fusion, was observed to occur when rats were infused with large doses of albumin (31, 32). Finally, epithelial reabsorption of leaking protein as suggested by Farquhar et al. (12) may act as a "monitor" to control proteinuria, but its role in the present experiments appears limited in view of the uncommon occurrence of catalase-positive reabsorption droplets in epithelial cells.

In summary, the evidence gathered from ultrastructural studies using graded tracer macromolecules suggests strongly that there are two barriers to the filtration of proteins in the glomerulus. The basement membrane acts as a molecular sieve serving as a relative barrier to molecules over a wide size range but presenting increased resistance to large molecules. Since the basement membrane filter is not absolute, it alone cannot explain the relatively small amounts of protein present in the glomerular filtrate. The experimental evidence to date suggests that the second filter is at the level of the slit pore and that it practically excludes molecules approximately the size of albumin or above from

entering the urinary space. The nature of this second barrier is not clear but it may be represented by structural components in the slit pore, namely basement membrane substance, the slit diaphragm, and the anionic cell coat of the podocyte foot processes. Finally, the small amounts of protein that do escape into the urinary space are reabsorbed either by glomerular or proximal tubular epithelial cells.

SUMMARY

Mice were injected intravenously with beef liver catalase (mol wt 240,000) and very small doses of horseradish peroxidase (mol wt 40,000) and the site of localization of these enzymes in the kidney was studied by ultrastructural cytochemistry. 1 min after injection, catalase was present in glomerular capillary lumina and there was minimal permeation of the basement membrane. After 5–180 min, staining of the basement membrane increased progressively but was usually less than that in capillary lumina. At all time intervals the inner (subendothelial) layer of the basement membrane contained more reaction product than the lamina densa and the outer (subepithelial) layer. Catalase permeated the entire thickness of the basement membrane and extended up to the slit pore but not beyond the level of the slit diaphragm and was not seen in the urinary space or tubular lumina. Horseradish peroxidase permeated the whole thickness of the basement membrane within 2 min after injection; however, gradients of staining from the inner to outer layers of the basement membrane were frequently seen.

The findings with both enzymes indicate that (*a*) the basement membrane restricts the passage of proteins over a wide range of molecular size with increasing impediment for larger molecules and (*b*) the slit pore functions as an additional barrier for molecules that cross the basement membrane.

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