AN ULTRASTRUCTURAL STUDY OF GLOMERULAR PERMEABILITY IN AMINONUCLEOSIDE NEPHROSIS USING CATALASE AS A TRACER PROTEIN*,†

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Physiological studies (1–4) have indicated that increased glomerular permeability is the major cause of proteinuria in human and experimental nephrosis, but the ultrastructural basis for such protein leakage is poorly understood. Previous morphological studies using ferritin (mol wt 500,000) (5, 6) have shown that the glomerular basement membrane becomes more permeable to this particle in experimental nephrosis induced by the aminonucleoside of puromycin (7), and it has been suggested that a basement membrane lesion is the primary cause of proteinuria (6).

However, the most striking ultrastructural changes in experimental aminonucleoside nephrosis and human lipoid nephrosis occur not in the basement membrane but in the epithelial cells of the glomerulus. These epithelial alterations, which include fusion of foot processes and vacuolization, have been considered to be secondary to the proteinuria (6). In a previous communication (8) we have reviewed these changes and reported that the enzyme tracer horse-radish peroxidase (HRP) (mol wt 40,000) is transported across altered epithelial cells into the urinary space through a system of vacuoles. The contribution of such a vacuolar pathway to the pathogenesis of proteinuria could not be clearly assessed from the peroxidase experiments since this enzyme also diffused easily through normal basement membranes and along intercellular junctions of fused epithelial cells in nephrotic glomeruli.

We report here on the passage of beef liver catalase across the glomerular capillary wall into the urinary space in aminonucleoside nephrosis. This enzyme,
which can be demonstrated ultrastructurally by a cytochemical technique (9), has a larger molecular weight (240,000) than peroxidase and is restricted by the normal glomerulus from entering the glomerular filtrate (9, 10). In a separate paper (11) we have suggested that there are two barriers to diffusion of catalase across the glomerular capillary wall in normal mice, one in the basement membrane and one at the level of the epithelial slit. The results of the present experiments indicate that this is true in normal rats also, and further suggest that catalase gains access to the urinary space only through the vacuolar pathway in aminonucleoside nephrosis. These observations lead us to believe that this mechanism may play an important role in the pathogenesis of proteinuria in this experimental model. In addition the present experiments support previous work (6) which suggests increased permeability of the basement membrane.

**Materials and Methods**

Beef liver catalase was obtained as a twice-crystallized aqueous suspension containing 20–27 mg/ml with 0.1% thymol as a preservative (type C-100, Sigma Chemical Co., St. Louis, Mo.). To avoid injection of large volumes of tracer solution, a few batches of catalase were concentrated using the following technique: the suspension was placed at 4°C for 1–2 wk to allow maximum crystallization to take place, centrifuged at 12,000 g and the sediment dissolved at 35–40°C in a small volume of distilled water to a final concentration of 50–60 mg/ml. Ultrasonic vibration of the solution for 5–30 min in a Maxomatic ultrasonic vibrator (L & R Manufacturing Co., Kearny, N. J.) at the same temperature was often necessary to dissolve the sediment completely. The solution was then centrifuged at 48,000 g for 15 min to remove amorphous impurity and either used immediately or stored at 4°C with 0.1% thymol. Alternatively, once crystallized catalase (Preparation C-30, Sigma Chemical Co.) was recrystallized, as described by Tauber and Petit (12), by dissolving the crystalline sediment from a 50 ml suspension containing 1 g in the least possible volume of 0.05 M phosphate buffer (10–20 ml), pH 7.3, at 40°C. The solution was centrifuged to remove amorphous impurity, and allowed to crystallize over 2–4 wk at 4°C. The crystals were then scraped off the sides of the test tubes, centrifuged, and redisolved at 40°C in distilled water to a final concentration of 50–60 mg/ml.

The preparations of catalase used were finally brought into solution by warming to 40°C in a water bath (sometimes with ultrasonic vibration) and enough NaCl was added to achieve a concentration of 0.85%. After centrifugation at 48,000 g to remove a faint turbidity that formed after the addition of NaCl, the solution was ready for use.

Male Charles River rats (Charles River Breeding Laboratories, North Wilmington, Mass.), weighing 80–120 g and fed on a standard chow, were used. Rats were made nephrotic by daily subcutaneous administration of 1.67 mg/100 g body weight of the aminonucleoside of puromycin for 10 days (6). Proteinuria was confirmed by the quantitative sulfosalicylic acid method (13).

Control and nephrotic rats were injected with 1.5–7 ml of solution containing a total of between 75 and 140 mg of catalase and sacrificed at serial time intervals. The duration of injection was usually 15 min, but in a separate experiment 140 mg of catalase in 2.5 ml was injected over a 3 min period and the kidneys removed immediately. A number of animals had to be excluded from the experiments due to technical difficulties including inadequate localization of catalase when lower doses of enzyme were injected, and toxic manifestations when larger doses were given. Two nephrotic rats sacrificed after the injection of tracer at 3, 15, and 30 min respectively, and one rat each sacrificed at 60 min and 12 hr after injection, were con-
sidered satisfactory and were evaluated. Normal rats were sacrificed at 3, 15, 30, and 60 min after injection; these animals served as controls. Kidneys were removed under ether anesthesia and thin slices fixed by immersion for 3 hr, at room temperature, in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 with 0.05% CaCl₂ (14). After overnight washing in cold 0.1 M cacodylate buffer, tissues were processed for the catalase histochemical reaction and electron microscopy, as detailed elsewhere (9, 11).

Cytochemical controls consisted of kidneys from normal and nephrotic animals that did not receive injections of catalase but were reacted for catalase under the same conditions as were the test kidneys. Additional controls for the specificity of the cytochemical reaction itself were as those described previously (9), and included incubation of sections in media containing 3-amino-1, 2, 4-triazole, a known catalase inhibitor (9).

RESULTS

General.—Rats receiving aminonucleoside became nephrotic by the 10th day and had marked proteinuria as compared with normal rats (above 100 mg in 24 hr; normal, less than 15 mg in 24 hr). Glomeruli from these animals appeared normal by light microscopy but showed striking alterations by electron microscopy. These changes included extensive fusion of the foot processes of podocytes and vacuolization of epithelial cytoplasm. The few slit pores that remained were nearly obliterated by contiguous masses of podocyte cytoplasm and appeared as “close” junctions (8). Basement membranes were without obvious abnormalities. A few epithelial vacuoles, large and small, were shown by serial sectioning to be continuous on one side with the extracellular space overlying the basement membrane through wide gaps, and some, with the urinary space on the other side. These changes occurred without respect to whether exogenous tracers had been injected or not, and have been reviewed in detail previously (8). In addition many vacuoles were shown to be totally intracytoplasmic.

When given in smaller doses (75–100 mg), normal and nephrotic rats tolerated injections of catalase well and showed no toxic effects. Kidneys from normal animals showed no histological abnormalities by light or electron microscopy, and kidneys from nephrotic animals showed only the characteristic changes of aminonucleoside nephrosis, and no additional changes attributable to the injection of catalase. When doses between 120 and 140 mg were given, catalase crystals and intracapillary thrombi were seen by light and electron microscopy in the glomeruli of some animals, both normal and nephrotic. Such animals often showed hemoglobinuria and were excluded from the experiment.

Localization of Catalase in Normal Rats.—Studies were limited to 3, 15, 30, and 60 min after injection of tracer; findings at these intervals were practically similar and are described together. A detailed time sequence study of the localization of catalase in normal glomeruli as was done with our study on normal mice, was not attempted (11). Localization of catalase in kidneys of rats was in general less satisfactory than in mice, because comparable density of reaction product in the glomerular basement membranes was difficult to obtain unless high, sometimes toxic doses of catalase were employed.
Fig. 1. Electron micrograph from glomerulus of normal rat sacrificed 30 min after injection of 140 mg of catalase. Tracer is present in the capillary lumen (L) and the basement membrane (BM), but not in the slit pore (arrow) nor the urinary space (US). Staining for catalase is less in the basement membrane than in the lumen and there is a concentration gradient from the endothelial surface across the basement membrane towards the slit pore. EP, epithelium; E, endothelium. X 26,800.

Fig. 2. Same rat as in Fig. 1. Glomerular capillary showing catalase in endothelial fenestrae (arrows) and in basement membrane (BM). Catalase staining extends up to the slit pores but not beyond, and is not present in the urinary space (US). RBC, red cell; EP, epithelium. X 30,000.

By light microscopy, catalase was seen as a faint, light brown reaction product in capillary lumina. The basement membranes were focally stained in some glomeruli. All tubular lumina were empty of reaction product.

By electron microscopy, catalase was seen as a gray to black, granular, sometimes clumpy reaction product in capillary lumina, endothelial fenestrae, and
to varying degrees, in the basement membranes of the glomeruli (Figs. 1, 2). Density of reaction product in basement membranes was always less than that in the capillary lumina. The density of staining in the various layers of the basement membrane indicated a concentration gradient across the structure (Fig. 1), as described in mice (11). Rarely, in rats given higher doses of the enzyme, did the full thickness of the basement membrane stain, and in such areas the tracer appeared to extend up to the opening of the filtration slit but not through the slit pore into the urinary space (Fig. 2). The tracer often permeated the mesangial matrix more prominently than the basement membrane itself, and was taken up in vacuoles by mesangial cells. It was not seen in tubular lumina or Bowman's space and was only rarely found in dense droplets in the podocyte cytoplasm.

*Localization of Catalase in Nephrotic Rats.*—By light microscopy the enzyme was visible in many tubular lumina as early as 3 min after injection. These included proximal tubules, distal tubules, and collecting ducts.

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**Fig. 3.** Nephrotic glomerulus 15 min after injection of 70 mg of catalase. Reaction product is seen in the form of flakes and clumps in the capillary lumen (L) and in the epithelial vacuoles (V). It is also seen in the lamina densa of the basement membrane (BM). EP, epithelium. × 14,300.
Fig. 4. Nephrotic rat injected with 140 mg of catalase and sacrificed within 3 min. Tracer is seen in capillary lumen (L) and in epithelial vacuole (V). An epithelial vacuole containing catalase is seen to open out over the basement membrane as a basal pocket (P). Also seen is an epithelial vacuole (V₁) that is free of catalase. EP, epithelium; BM, basement membrane. × 16,500.

Fig. 5. Same rat as in Fig. 4. Clumps of catalase (arrows) lying free in urinary space, presumably having been discharged from an epithelial vacuole. EP, epithelium; V, vacuoles. × 19,800.
Fig. 6. Nephrotic rat 30 min after catalase injection. Catalase is seen in capillary lumen (L), basement membrane (BM), and epithelial vacuoles (V). In addition a number of large and small vacuoles contain densely packed reaction product (arrows). EP, epithelium. X 19,800.

Fig. 7. Nephrotic rat 30 min after injection of catalase. Reaction product is present in mesangial area (MES), and vacuoles (V), but not in the narrowed slit pore between masses of blunted epithelium (arrow), nor in the urinary space (US). EP, epithelium. X 40,000.
Fig. 8. Nephrotic rat 30 min after catalase injection. Reaction product is present in the lumen (L) of proximal tubule (PT) and in the invaginations in between microvillar processes (arrows). Uptake of catalase in tubular cell could not be evaluated due to presence of reaction product in tubular microbodies (MB). Tubules from animals not injected with catalase were devoid of reaction product except in microbodies. X 16,900.
At all time intervals except 12 hr after injection, catalase was seen by electron microscopy in glomerular capillary lumina, in the basement membranes, in the basal pockets, and in intracytoplasmic vacuoles of epithelial cells (Fig. 3). Clumps of reaction product were also seen in Bowman’s space. As early as 3 min after injection catalase was present in basal pockets overlying basement membrane (Fig. 4). Although serial sectioning was not attempted in this study, we have shown by this technique that pockets overlying the basement mem-

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**Fig. 9.** Glomerulus of nephrotic rat not injected with catalase, but reacted in the complete cytochemical medium. Note that the capillary lumen (L), basement membrane (BM), mesangium (MES), and vacuole (V) are free of reaction product. Part of red blood cell (RBC) stains positively. X 28,200.

brane are, in some cases, continuous with intracytoplasmic vacuoles. Similarly, some apparently intracytoplasmic vacuoles open to the urinary space (8). Both of these instances were uncommon, and most intracytoplasmic vacuoles do not appear to be connected to either cell surface.

The density of staining in the basement membranes themselves was quite faint at all time intervals. Clumps of catalase were also seen in the Bowman’s space, opposite irregular concavities of epithelial cell cytoplasm as early as 3 min after injection (Fig. 5). 30–60 min after injection of tracer, many epithelial vacuoles containing catalase were seen to be smaller, condensed, and with no
interspersed clear or amorphous material (Fig. 6). 12 hr after injection only very rare dense bodies could still be seen in epithelial cells.

Close junctions between adjacent epithelial cells or fused foot processes (Fig. 7) and the few persistent slit pores were unstained. Many proximal and distal tubules and collecting ducts contained reaction product. In proximal tubules it appeared in tubular lumina, as well as in the surface invaginations in between microvilli (Fig. 8). The staining in glomeruli and tubular lumina was abolished by the catalase inhibitor 3-aminotriazole, as reported previously (9). Uptake of catalase into tubular cytoplasm could not be evaluated due to simultaneous staining of endogenous catalase in proximal tubular cell microbodies (15) (Fig. 8).

Control animals not injected with catalase did not show staining in capillary lumina, basement membranes, or other sites (Fig. 9) except for endogenous peroxidase activity in red cells, leukocyte granules, and renal microbodies.

**DISCUSSION**

The present experiments indicate that beef liver catalase, an enzyme with a molecular weight of 240,000, passes across glomerular epithelial cells into the urinary spaces of rats with aminonucleoside nephrosis. In contrast, the glomerular filtration barrier is adequate to prevent its passage across the glomerular wall in normal rats, and also in mice (11). These and prior studies using myeloperoxidase (16) suggest that the slit pore as well as the basement membrane constitute the normal filtration barrier.

The presence of tracer in basal pockets overlying the basement membrane, in intracytoplasmic vacuoles and as clumps in the Bowman's space, strongly suggests that the enzyme passes into the glomerular filtrate through a vacuolar pathway in nephrotic glomeruli. In a previous study on aminonucleoside nephrosis (8) we have shown that a smaller tracer, horseradish peroxidase, traverses glomerular epithelial cells through close junctions and residual slit pores in addition to a vacuolar pathway. In contrast, catalase was not observed to permeate through close junctions or residual slit pores in the present experiments. Farquhar and Palade (6) showed that the tracer ferritin also does not traverse close junctions or residual slit pores but enters epithelial vacuoles.

Blunting of foot processes, reduction in the width of slit pores, and foot process fusion have been shown to occur in response to massive infusions of homologous protein or albumin (17, 18). Such changes were considered to be the result of increased presentation of protein that had crossed the basement membrane. In these experiments, actual loss of protein into the urine was quite small, thus the epithelial changes appeared to be adequate to prevent massive protein leakage across the glomerulus. In aminonucleoside nephrosis, increased presentation of protein to the glomerular epithelial cells is probably caused by increased permeability of the basement membrane. This was shown by the
ferritin experiments of Farquhar et al. (5, 6) and is suggested in our experiments by the appearance of catalase within epithelial cell vacuoles and the Bowman's space within 3 min of injection. Since the basement membrane appears to restrict larger molecules more effectively (e.g., ferritin and catalase) (5, 11), increase in its permeability in the nephrotic animal would allow enhanced numbers of larger molecules to reach the slit pore. However, the restriction of passage of catalase by close junctions and residual slit pores in nephrotic glomeruli implicates basal pockets and vacuoles as an abnormal epithelial pathway for passage of this tracer into the glomerular filtrate. Thus in aminonucleoside nephrosis increased permeability of the epithelial barrier as well as that of the basement membrane would appear to be responsible for protein leakage.

Epithelial and basement membrane changes may be interrelated since it has been suggested that the podocyte manufactures a major proportion of basement membrane material (19). Thus the effect of puromycin aminonucleoside on the podocyte might include synthesis of defective basement membrane material, as has been suggested (6), in addition to formation of basal pockets and vacuoles. Biochemical studies suggest that the glomerular basement membrane becomes altered at the molecular level in anti-kidney serum nephrosis (20-22) but such evidence is not available for experimental aminonucleoside nephrosis and human lipoid nephrosis, diseases which have no clear-cut immunological basis. More recently Blau and Michael (23) found decreases in the sialic acid content of epithelial cell membranes in aminonucleoside-treated rats, whereas glomerular basement membrane preparations from normal and nephrotic rats were without significant chemical differences. However, information regarding the structural organization of basement membrane substance in aminonucleoside nephrosis is not available at present.

The mechanism of formation of basal pockets and epithelial vacuoles is as yet unclear. However, it is suggested that they might represent defective, widened slit pores covered over by fused epithelial cytoplasm. While our experiments with peroxidase (8) and catalase indicate that some epithelial vacuoles serve to condense and conserve the engulfed protein, this does not appear to be their major role, as has been suggested by the ferritin experiments (6). Taken together, these experiments with tracers of progressively increasing molecular sizes would seem to indicate that the podocyte differentially reabsorbs greater amounts of protein of large molecular size.

To summarize, all available evidence seems to suggest that the glomerular pathological changes responsible for proteinuria in aminonucleoside nephrosis include both epithelial cell and basement membrane abnormalities. Basement membrane changes are probably secondary to an effect of puromycin aminonucleoside upon the protein synthetic machinery of the podocyte. Since the epithelial slit pore appears to be the final barrier for proteins in normal glo-
meruli, it appears that an abnormality of the epithelial cell must precede, or occur concomitantly with basement membrane changes for proteinuria to occur. It is suggested that this abnormality is represented in aminonucleoside nephrosis, at least in part, by a system of vacuoles serving as the route of transfer of proteins from the basement membrane to the urinary space.

Epithelial vacuoles have been demonstrated in some types of human nephrosis but have not been emphasized in others. Although it is possible that an entirely different mechanism for transfer of proteins across the epithelium operates in different types of human or experimental nephrosis, attention should be paid to vacuolization of the epithelium as a possible mechanism in some forms.

SUMMARY

Beef liver catalase (mol wt 240,000) was injected intravenously into normal rats and rats made nephrotic with aminonucleoside of puromycin. The localization of the tracer in the kidneys was then studied by ultrastructural cytochemistry, 3 min–12 hr after injection. Passage of catalase into the urinary space in normal rats was restricted by the basement membrane and by the epithelial slit pore. Nephrotic glomeruli showed extensive fusion of foot processes and formation of pockets and vacuoles in the fused epithelium; within 3 min after injection, catalase appeared in basal pockets, epithelial vacuoles, and the urinary space. Residual slit pores and close junctions in fused epithelium were impermeable to catalase. These studies indicate that alteration of the epithelial cells and basement membrane is responsible for protein leakage in aminonucleoside nephrosis.

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