PATHOGENESIS OF EXPERIMENTAL NEPHROSIS ELECTRON MICROSCOPIC OBSERVATIONS

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A nephrotic syndrome can be induced in rats by the administration of an aminonucleoside, 6-dimethylamino purine, 3-amino-*d*-ribose. During the early stages of the disorder, no detectable glomerular lesions are seen by light microscopy, although tubular alterations which parallel the progression of proteinuria, hypoalbuminemia, hyperlipemia and edema do occur. The microscopic changes resemble those of pure nephrosis in man.[1,2] Farquhar, Vernier, Good and Brunson[3-6] illustrated glomerular epithelial lesions in the nephrotic syndrome of man by electron microscopy where none were seen by light microscopy. Among the limitations imposed by biopsy in a study of disease are an inability to procure such specimens at the earliest stages of a disorder when clinical manifestations are not yet evident, and an insufficient number of specimens obtained consecutively to provide a sequence illustrating the changing morphologic features of the condition.

We feel that observations on the experimental model of aminonucleoside-induced nephrosis may help elucidate the sequential structural and functional changes in this form of renal injury.

METHODS

Male Sprague-Dawley rats, weighing 120 gm., were caged individually in metabolic units. Water and a diet of Purina Rat Chow were provided *ad libitum*. Eight rats received daily subcutaneous injections of 6-dimethylamino purine, 3-amino-*d*-ribose (supplied by the Lederle Laboratories), 0.3 ml. of a 0.5 per cent solution per 100 gm. of body weight. Tissues from these rats and from 4 untreated control animals were examined by electron microscopy. Experimental animals were sacrificed in pairs after 1, 5, 8, and 14 days of treatment. Twenty other rats received smaller daily doses, 0.3 ml. of a 0.25 per cent solution per 100 gm. of body weight for 60 days. Ten animals served as untreated controls

Supported in part by Grants C-2836 and A-587 from the United States Public Health Service.

Presented in part at the Fifty-fifth Annual Meeting of the American Association of Pathologists and Bacteriologists, Cleveland, Ohio, April 24, 1958.

Received for publication, June 30, 1959.

for this group. One experimental and one control rat from the 60-day group were examined by electron microscopy.

Animals were weighed daily. Daily urine specimens were collected for measurement of protein content. Total and fractional estimations of serum proteins, serum cholesterol, and nonprotein nitrogen were determined by methods previously described [7] from blood collected at the time of decapitation.

Tissue sections from all of the animals were examined by conventional light microscopic techniques. For this purpose, renal tissue was fixed in cobalt-formalin. Paraffin sections were stained with hematoxylin and eosin and by the periodic acid-Schiff (PAS) method. Frozen sections were stained with oil red O for lipid. For electron microscopy, fragments of kidney, 1 to 2 mm. in diameter, were selected from the cortex, corticomedullary junction, and medulla and promptly immersed in Dalton's 1 per cent osmic acid and bichromate solution buffered to pH 7.6. Tissues were fixed for 45 minutes to 1 hour at room temperature.[8] The methods of dehydration and embedding used were those previously described.[9] Polymerization was carried out at 60° C. Thin sections were cut with glass knives on a Servall Porter-Blum microtome, placed on collodion-covered copper grids, and examined, without removing the plastic, in an EMU-3B RCA electron microscope.

Results

Normal Kidney

The ultrastructure of adult mammalian kidney has been defined by a number of investigators.[10–25] In the glomerulus the thin endothelial cytoplasm is interrupted by numerous small spaces or pores. Between the plasma membrane of the endothelial cell and the limiting membrane of the glomerular epithelium lie 3 zones. The central zone is electron dense; although relatively distinct as an entity, this zone or membrane does not possess the distinct sharpness characteristic of plasma membranes or nuclear membranes. The electron-dense membrane varies somewhat in thickness; it has a granular character, and at times its granules are arranged in linear manner, thereby suggesting cross sections of platelike structures. On either side of the electron-dense membrane are zones of low density. The term basement membrane as used in electron microscopy is limited to the electron-dense structure. Each glomerular epithelial cell is plicated over the basement membrane with a number of relatively uniform foot processes. The tip of each foot process is characterized by an area that is granular and electron dense. No cells other than those of the capillary endothelium and the visceral and parietal glomerular epithelium were observed in the glomerulus.

The ultrastructural characteristics of the tubular epithelium have not been the cause of as much controversy as have the features of the glomerulus.[20,26-29] Rhodin has recently summarized the features of the different tubular cells.[29] Common to all of them is a complex infolding of the plasma membrane at the basilar part of the cell. This feature is most striking in the distal convoluted tubule. The proximal convoluted tubular epithelium has numerous projections of the apical part of the plasma membrane; these microvilli correspond to the brush border observed by light microscopy.

Kidney in Aminonucleoside-treated Rats

Appearance of the Kidney Prior to Proteinuria. With the electron microscope, normal glomerular epithelial foot processes were uniform in appearance. Only occasional processes were noted to be large or fused. After 24 hours of treatment with aminonucleoside, large and fused foot processes were seen in much larger numbers (Fig. 1). Four to 6 such abnormal processes were observed surrounding a capillary in cross section, whereas in the control animal enlarged processes of this nature were rarely encountered. Thus it seemed that this glomerular alteration was quantitative, in that the number of large processes was increased; it was not qualitative, in that the processes were similar to those seen occasionally in controls.

By the fifth day of aminonucleoside treatment, prior to proteinuria, glomerular lesions were dramatic as viewed by electron microscopy, in striking contrast to the absence of alterations detectable by conventional microscopy. Progressive merging of fused foot processes into cytoplasmic masses adjacent to the basement membrane constituted the principal change (Fig. 2). Not all foot processes were affected; approximately three fourths remained unfused. The main mass of the glomerular epithelial cytoplasm contained smooth-surfaced profiles outlining foci with approximately the same electron density as the remainder of the cytoplasm (Fig. 3). In some glomerular epithelium there were collections of granular electron-dense material arranged in irregular lines; these were of approximately the same size as mitochondria but did not have outlining membranes. In other epithelial cells there were increased numbers of membrane-lined vacuoles, some of which contained electron-dense structures.

Appearance of the Kidney Following the Development of Proteinuria. Specimens from rats that had received aminonucleoside for 8 days were characterized by rather dramatic electron microscopic changes in the glomeruli and tubules. This was in sharp contrast to the lack of demonstrable alteration in glomeruli and the equivocal lesions in the tubules as observed by conventional microscopy.

More of the glomerular foot processes were fused than at the fifth day stage. A prominent, granular, electron-dense zone was present within the glomerular epithelial cytoplasm, adjacent to the basement membrane, and there were cytoplasmic vacuoles. Within the epithelium, and free in Bowman's space were discrete, electron-dense bodies measuring approximately 1 μ in diameter. There was evidence that these bodies corresponded to PAS-positive granules identified by light microscopy. No alterations in the endothelium or basement membrane of the glomerulus were identified.

Electron-dense bodies, similar to those in the glomerulus, were found in the lumens of the proximal convoluted tubules and also in the cytoplasm of some of the proximal tubular epithelial cells (Fig. 5). These were accompanied by numerous altered and distorted mitochondria in both locations. Cytoplasmic vacuoles were also present in the epithelium of the distal convoluted tubules, but they were smaller and less frequent than those in the proximal segment.

Tubular lesions, as viewed by conventional means, became more prominent with continuing treatment, and during the second month glomerular alterations also became manifest. In the chronic phase of the disease, at 60 days, the glomeruli rather uniformly contained vacuolated epithelium in both the visceral and parietal layers, and there was formation of adhesions and epithelial crescents. It was thought that there was a thickening of the glomerular basement membrane.

Electron microscopy likewise demonstrated more advanced lesions (Figs. 6 to 9). These appeared principally in the visceral and parietal epithelium. In some areas the basement membrane was tortuous and irregular. Although on occasion the electron-dense zone appeared to be thickened, segments with this degree of thickness also were observed, though less frequently, in control animals at sites where the membrane was twisted or turned.

Such focal knobs or thickenings were almost always directed toward the endothelial side (Fig. 6). In one instance (Fig. 8) a subendothelial deposit of granular material, almost certainly lipid, was found between the endothelium and the basement membrane. The glomerular epithelium was practically devoid of foot processes. Dense deposits of electron-dense substance were found beneath the plasma membrane, both on the surface adjacent to the basement membrane and also on the side facing Bowman's space. Large cytoplasmic vacuoles were prominent. In some of the cells, accumulations of material arranged in irregular lamellas suggested the "myelin figures" [30, 31] identified in a variety of other tissues (Fig. 6). Parietal epithelium was also vacuolated, and there were adhesions between the visceral and parietal layers (Fig. 7).

All types of tubular epithelium were altered, even those in the collecting ducts. Occasional epithelial elements were considerably distorted whereas their neighbors were relatively undisturbed (Fig. 9). The tubular lesions described at 8 days of treatment were more severe after 14 days and had attained an advanced stage in the animals which had received aminonucleoside for 60 days.

Discussion

The nephrotic syndrome in man presents challenging questions concerning its cause, the site of injury, and the correlation of structural with physiologic abnormalities. Hence, the investigation of an experimental renal disease which mimics a human disorder provides a rare and important opportunity. In this paper, electron microscopic examination of the renal lesions induced in rats by 6-dimethylamino purine, 3-amino-*d*-ribose have been described. Initially, the aminonucleoside produced a nephrotic syndrome characterized by proteinuria and hyperlipemia. With continuation of treatment, edema and hyperlipemia, having reached a maximum, diminished after 30 days although proteinuria persisted. The disease subsequently became chronic in character, resembling the "dry" phase of chronic glomerulonephritis.[32] This clear-cut sequential division of the syndrome into pre-proteinuric, early post-proteinuric, and chronic phases permitted the definition of the primary lesion in nephrosis by electron microscopy. The experimental observations assume considerable significance in view of the remarkable histologic similarities between aminonucleoside injury and nephrosis in man.[3, 6]

When kidney sections from aminonucleoside-treated rats were examined by light microscopy, no glomerular lesions were identified in the early and reversible stages of injury. At this stage, swelling of tubular epithelium and intracellular lipid deposits in the proximal convoluted tubules were seen. These observations were identical to those described in "pure" or "lipoid" nephrosis in children.[1, 2]

In the advanced stage the light microscopic features of the experimental lesion were strikingly similar to those designated the Ellis type II glomerulonephritis.[33] The experimental lesions were characterized by involvement of all glomeruli, proliferation of glomerular epithelium, apparent thickening of the glomerular basement membrane, proliferation of glomerular parietal epithelium with the formation of crescents, dilatation of cast-filled renal tubules, and the intracytoplasmic deposition of lipid and "hyaline" droplets in the proximal convoluted tubules. A failure to demonstrate hyalinized glomeruli probably indicates that the experimental animals were not observed for a sufficient period of time.

The investigations of Oliver,[34] Davies,[35] and others[36] have suggested

that protein commonly escapes the blood stream at the glomerulus and passes into the lumen of the tubules. This occurs despite a failure to detect any alteration in the glomerulus by light microscopy. Part of the protein is believed to be resorbed in the proximal convoluted tubule where it may be identified as intracellular PAS-positive granules or "hyaline" droplets. These were thought by earlier microscopists to represent degeneration of tubular cells. Apparently cytoplasmic enzymes of the tubular epithelium undergo a form of alteration or "exhaustion" when continually "insulted" by protein and other products.[37] Obviously, that part of the protein in the glomerular filtrate that is not resorbed is lost in the urine.

Examination of the sequential changes in experimental nephrosis by electron microscopy indicates that the glomeruli are altered and leak protein although this is not demonstrable by conventional microscopy. The glomerular epithelial alteration was encountered not only at the time when proteinuria first occurred, but also during the period immediately preceding its development. These observations, along with those of other investigators, serve to redefine the morphologic parallel of the nephrotic syndrome in man and in the experimental animal; the entity is probably now better designated as a primary glomerulopathy.[3-6, 21, 38-46]

In a review of the observations of others and our own, two features are manifest: (1) In both clinical and experimental states in which proteinuria occurs, ultrastructural alteration of the glomerular epithelial cell has been found consistently. (2) Many if not all of the tubular epithelial lesions found in a number of different types of renal damage are similar. The latter appears to be the case by electron microscopy as well as by enzyme studies, at least in some instances,[37] and may well merely reflect accommodation to an abnormal glomerular filtrate.

Glomerular Lesions and Proteinuria

At the present stage of knowledge concerning ultrastructural pathologic alterations, observations are limited and frequently isolated. Proteinuria in the absence of overt renal abnormality occurs in the newborn animal.[47, 48] At birth the glomerular epithelial foot processes are poorly formed, and there is a prominent subendothelial space. We have been unable as yet to correlate a functional change with the prominent subendothelial space, although this space is thought to be the site of deposition of electron-dense material in lupus erythematosus and diabetic glomerulosclerosis in the adult.[3, 49, 50] In the newborn, the basement membrane is not thickened, and we have been unable to identify any discontinuities in it. Kurtz[51] examined the kidney of the human newborn by electron microscopy and also noted an absence of glomerular epithe-

lial foot processes. Since his specimens were obtained at necropsy and there was considerable autolysis, it might seem that delayed fixation had caused alteration of the processes. However, we have allowed pieces of renal tissue to be incubated at 37° C. for a period of one hour before fixation and have examined renal tissues removed at necropsy; the glomerular epithelial foot processes appear to be among the structures most resistant to change.

In pure nephrosis and in familial nephrosis,[3, 5, 43] fusion of the foot processes has been the only glomerular lesion reported. Similarly, in the experimental proteinuria produced by saccharated iron, the glomerular lesion appears to be confined to the epithelium.[52] In these conditions there appears to be a specific correlation of structure and function. In the electron microscopic studies of chronic glomerulonephritis, lupus erythematosus, and diabetic glomerulosclerosis, alterations have been reported in all of the glomerular components; viz., endothelium, basement membrane, and epithelium.

There has not yet been sufficient investigation of nephrotoxic nephritis, serum sickness, and acute glomerulonephritis to permit an appraisal of the various changes; it would appear, however, that these are similar, in that both glomerular endothelium and epithelium are altered, although the degree of change in the two cell types varies in the different diseases.[53–57] On the other hand, it appears that in amyloidosis the primary alteration is characterized by thickening of the basement membranes, not only in the glomeruli but in other sites as well.

Our own investigations[38, 47] and those of others[39, 41, 42, 45, 46, 52, 53] appear to indicate that fusion of glomerular epithelial foot processes and a loss of the spaces between them parallel the development of proteinuria. Conversely, when foot processes emerge from a solid cytoplasmic mass either in the course of normal maturation or during recovery from disease, then proteinuria ceases. Such observations warrant a re-evaluation of the hypothesis that the spaces between the foot processes represent the sites for the passage of the glomerular filtrate. However, assumptions regarding the passage of nonprotein substances into the glomerular filtrate cannot be made on the basis of the present data. It would appear likely that protein is able to pass through the basement membrane and enter the epithelial cytoplasm when the foot processes are fused. The protein then moves or is moved through the epithelium into the glomerular filtrate.

In aminonucleoside nephrosis, when proteinuria existed for 3 days or more, it was possible to demonstrate round or oval electron-dense structures within the cytoplasm of the glomerular epithelium (Fig. 4). By a comparison with sections examined by light microscopy, it appeared that these structures corresponded to PAS-positive droplets. Similar

structures were identified in some of the partly formed glomeruli of the newborn mouse with proteinuria and also in rats (Fig. 10) with elevated serum albumin and proteinuria for several days following the intraperitoneal injection of dilute bovine albumin.[59]

In aminonucleoside nephrosis, intracytoplasmic, electron-dense structures that appeared to correlate with PAS-positive droplets also were found, after the onset of proteinuria, in the proximal segment of the convoluted tubule. Such structures could not be distinguished by electron microscopy from similar bodies seen in proximal convoluted tubules of rats with proteinuria associated with rabbit anti-rat kidney serum nephritis (Fig. 11).[60]

Tubular Structural Alterations

The electron microscopic observations of renal tubular damage have been fewer than those of glomerular changes. Since the tubules of the newborn are not completely developed, they constitute a special case. Thus it is not surprising that their appearance differs somewhat from that found in experimental nephrosis, although in both situations proteinuria occurs.[61] The tubular injury induced by sucrose is also unlike that caused by aminonucleoside.[62] In preliminary observations in the nephrotoxic nephritis of rats, we observed electron-dense bodies, swollen mitochondria and distorted proximal convoluted tubular epithelium not unlike the lesion seen in the aminonucleoside nephrosis.[63] Fisher and Gruhn[37] compared the tubular lesions in the latter with nephrotoxic nephritis by histochemical methods and concluded that the tubular lesion was similar in the two conditions and that it seemed to increase in severity with progression of proteinuria.

Although we have not yet excluded the possibility of direct injury to the tubules in either aminonucleoside nephrosis or in nephrotoxic nephritis, we believe that the tubular damage is probably secondary to the abnormal glomerular filtrate. The chronologic sequence of the alterations following aminonucleoside injury tends to support this concept; i.e., first, glomerular epithelial cell injury; second, proteinuria; third, prompt evolution of the tubular lesion.

The exact site of action of aminonucleoside within the glomerular epithelium is still unknown. Since some of the abnormal organelles in these cells are similar in size and configuration to mitochondria but lack outlining membranes, it is suggested that an enzyme associated with these mitochondria might be the site of action of the drug.

Summary

Sequential electron microscopic observations of the kidneys were made in rats with the nephrotic syndrome induced by injections of an

aminonucleoside, 6-dimethylamino purine, 3-amino-*d*-ribose. The initial lesion, which developed prior to the occurrence of proteinuria, was characterized by alterations in glomerular epithelium, with fusion of foot processes, cytoplasmic vacuolation, and accumulations of granular electron-dense material. The latter was thought possibly to represent degenerating mitochondria.

At 8 days, shortly after the onset of proteinuria, glomerular lesions were more advanced although glomerular alterations were not demonstrable by conventional microscopy at this stage. With continuing proteinuria, the tubular epithelium, especially in the proximal segment, exhibited cytoplasmic vacuolation, swelling of mitochondria, and the appearance of electron-dense bodies. In the chronic stage of nephrosis, the lesions were more advanced in both the glomerular and tubular epithelium. In contrast, only minor abnormalities of the glomerular endothelium and basement membrane developed.

From the evidence it was tentatively concluded that the ultrastructural parallel of proteinuria appeared to be fusion of glomerular epithelial foot processes. Many of the tubular lesions encountered in the nephrotic syndrome were thought to be secondary to resorption of an abnormal glomerular filtrate. The term "primary glomerulopathy" was proposed to characterize the nephrotic syndrome.

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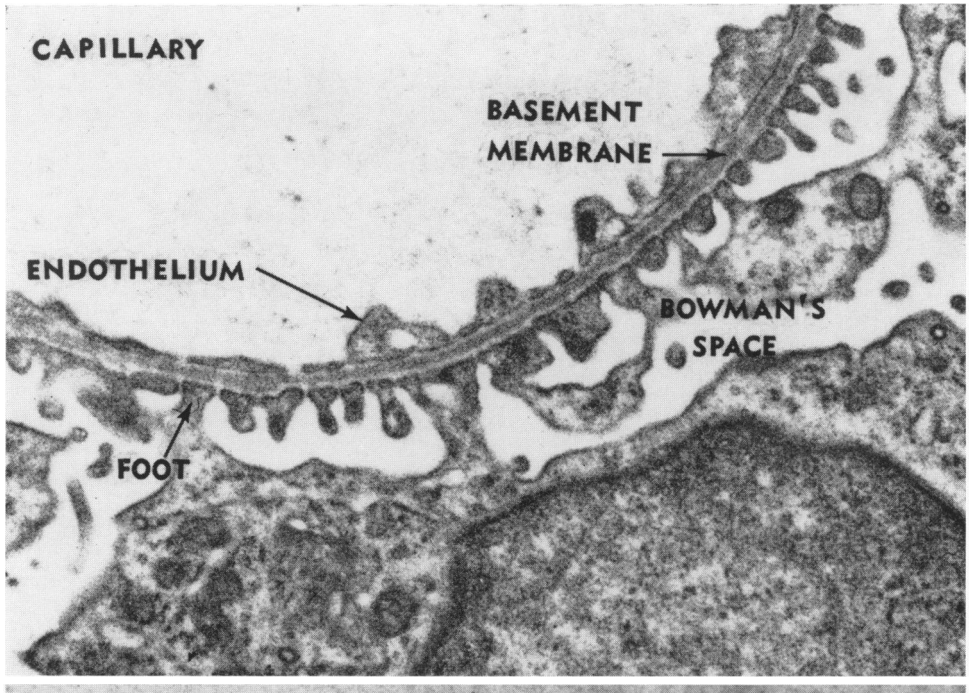
[*Illustrations follow*]

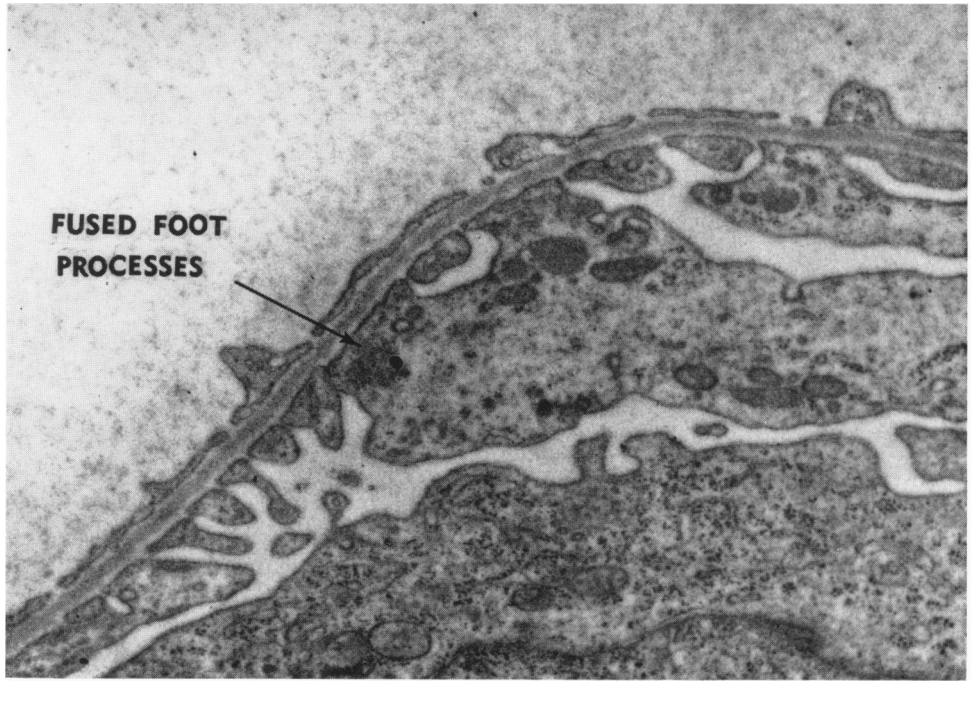
LEGENDS FOR FIGURES

FIG. 1. Electron micrograph of a rat glomerulus after one day of treatment with aminonucleoside. This resembles the glomerulus of the untreated rat. Although the majority of the epithelial foot processes are uniform in appearance, the 3 in the center of the illustration are slightly enlarged. After 24 hours of treatment, the number of enlarged foot processes is greater than in control animals. The glomerular epithelial nucleus is in the lower right corner. Approximately × 20,000.

FIG. 2. A rat glomerulus 5 days after the inception of daily treatment with aminonucleoside. The orientation of the structures is as in Figure 1. Several enlarged foot processes are seen. Within the glomerular epithelial cytoplasm are several mitochondria, measuring 0.5 to 1 cm. in diameter in the reproduction. In at least one of these an outlining membrane cannot be defined. × 25,000.

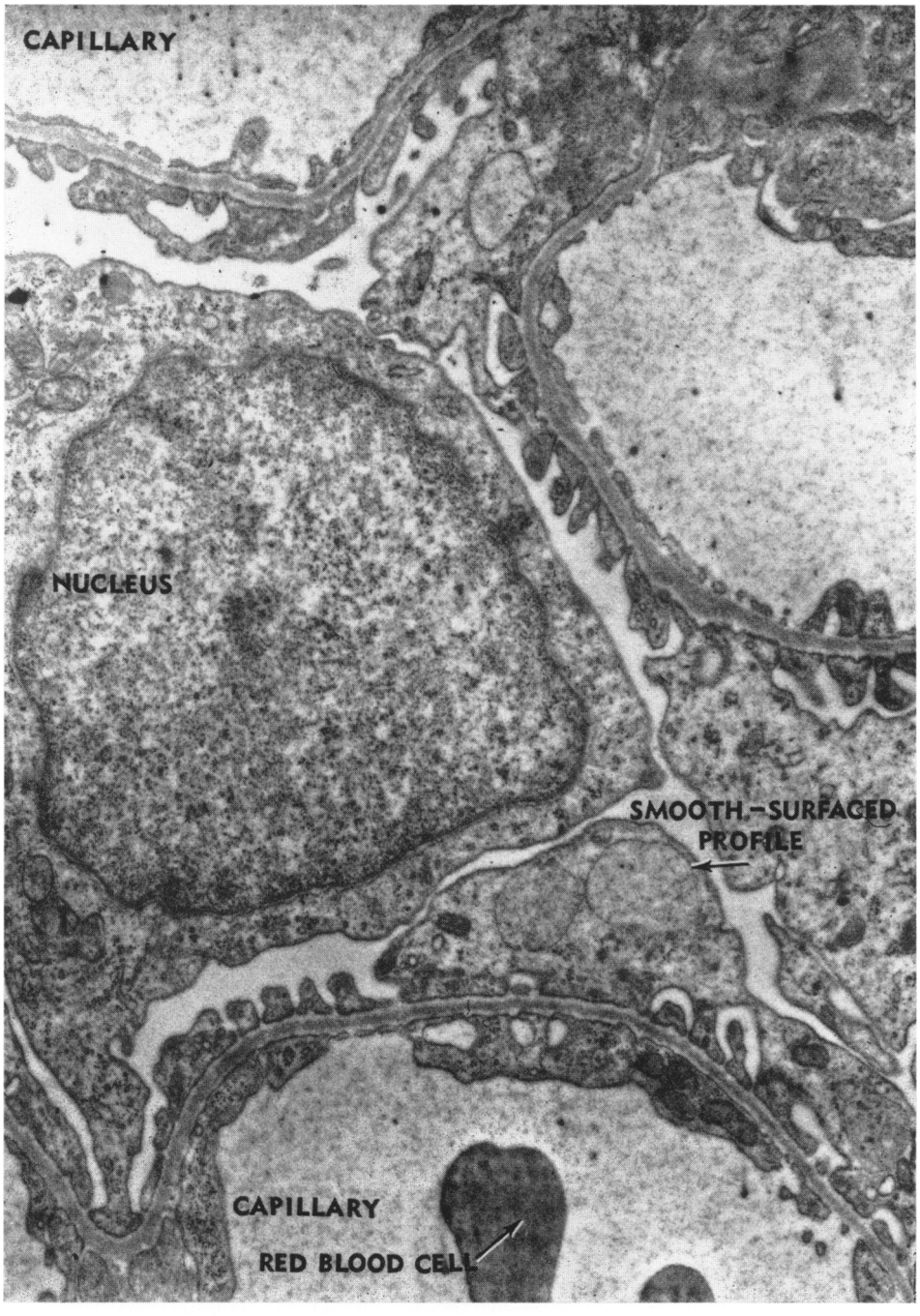

CAPILLARY
BASEMENT MEMBRANE
ENDOTHELIUM
BOWMAN'S SPACE
FOOT

1


FUSED FOOT PROCESSES

2

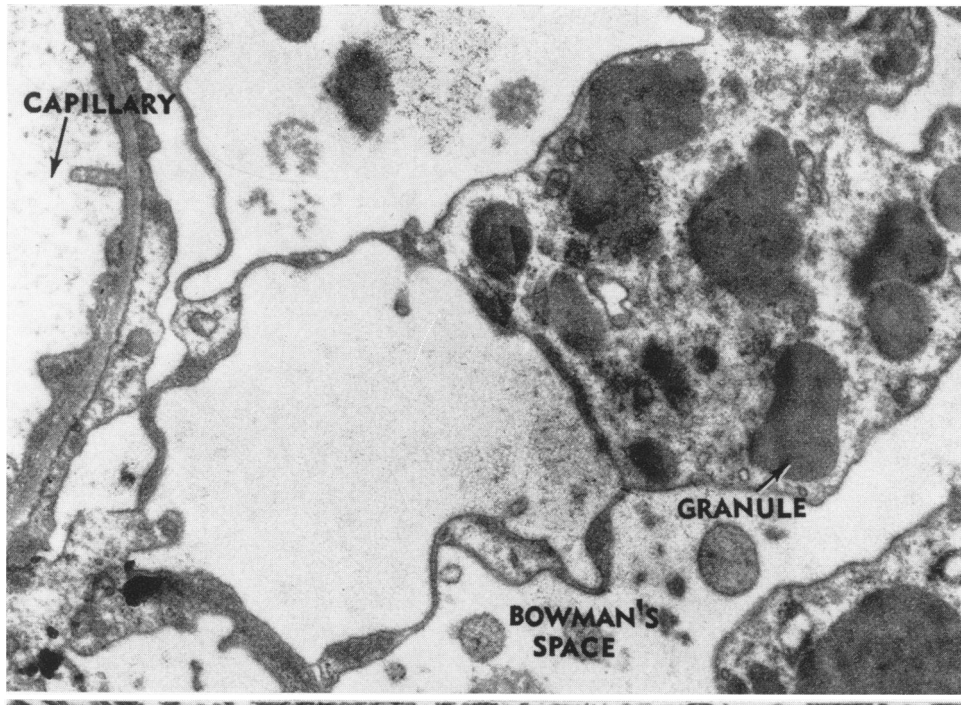
FIG. 3. A portion of a rat glomerulus; treatment with aminonucleoside for 5 days. Two smooth-surfaced profiles lie adjacent to each other within the epithelial cytoplasm. The electron-dense material is manifest at the tips of the foot processes, both those of normal appearance and those that are fused. × 20,000.


CAPILLARY
NUCLEUS
SMOOTH-SURFACED PROFILE
CAPILLARY
RED BLOOD CELL

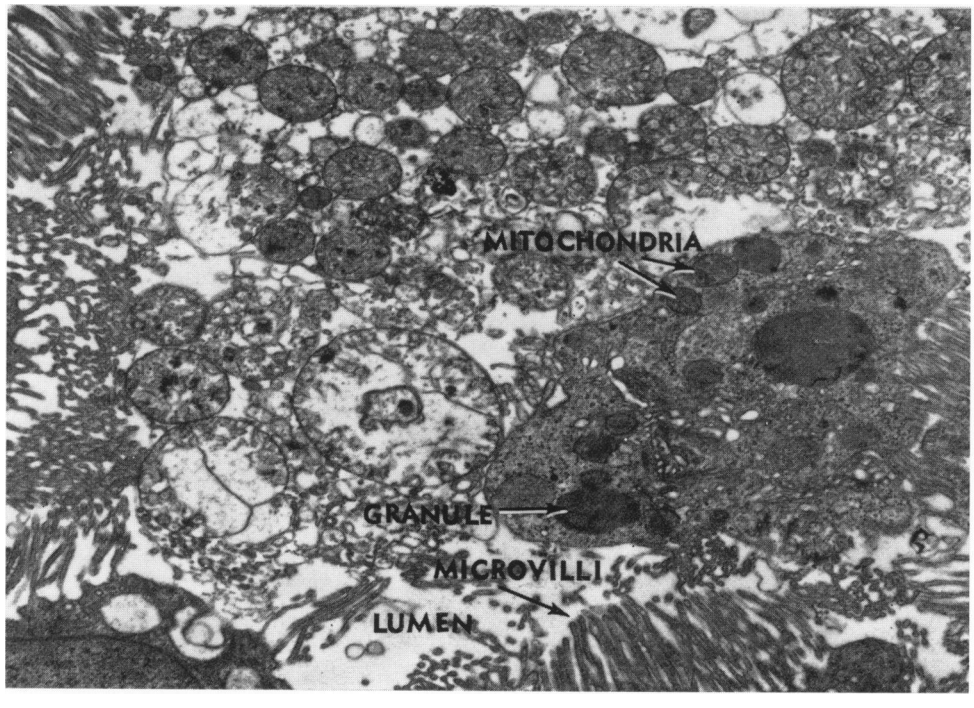
3

FIG. 4. Rat glomerulus; animal treated with aminonucleoside for 8 days. Proteinuria had been present for one day. No foot processes are present in this particular illustration. There is, however, a continuous mass of epithelial cytoplasm abutting against the basement membrane. Several electron-dense structures are shown. It was thought that these corresponded to PAS-positive granules identified by light microscopy. × 20,000.

FIG. 5. A cross section of the proximal convoluted tubule in a rat treated with aminonucleoside for 8 days. One of the electron-dense structures is labeled. These granules are thought to correspond to the PAS-positive granules identified by light microscopy. Mitochondria differ in size and structure from these granules. However, an internal lamellar structure can be seen in some of them. Many irregular bodies are present in the lumen. Some of these are thought to be altered mitochondria. × 9,000.

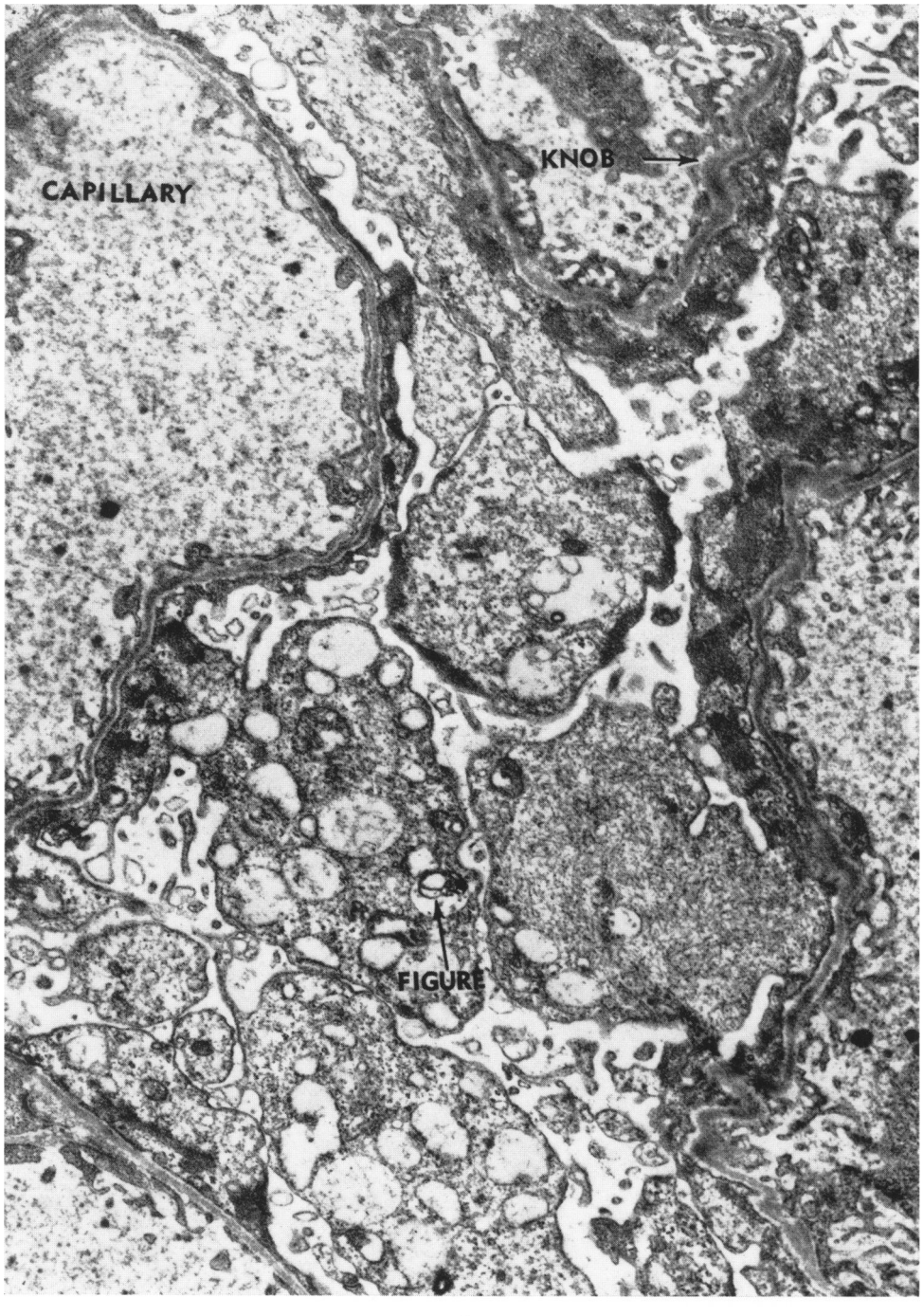

CAPILLARY
GRANULE
BOWMAN'S SPACE

4


MITOCHONDRIA
GRANULE
MICROVILLI
LUMEN

5

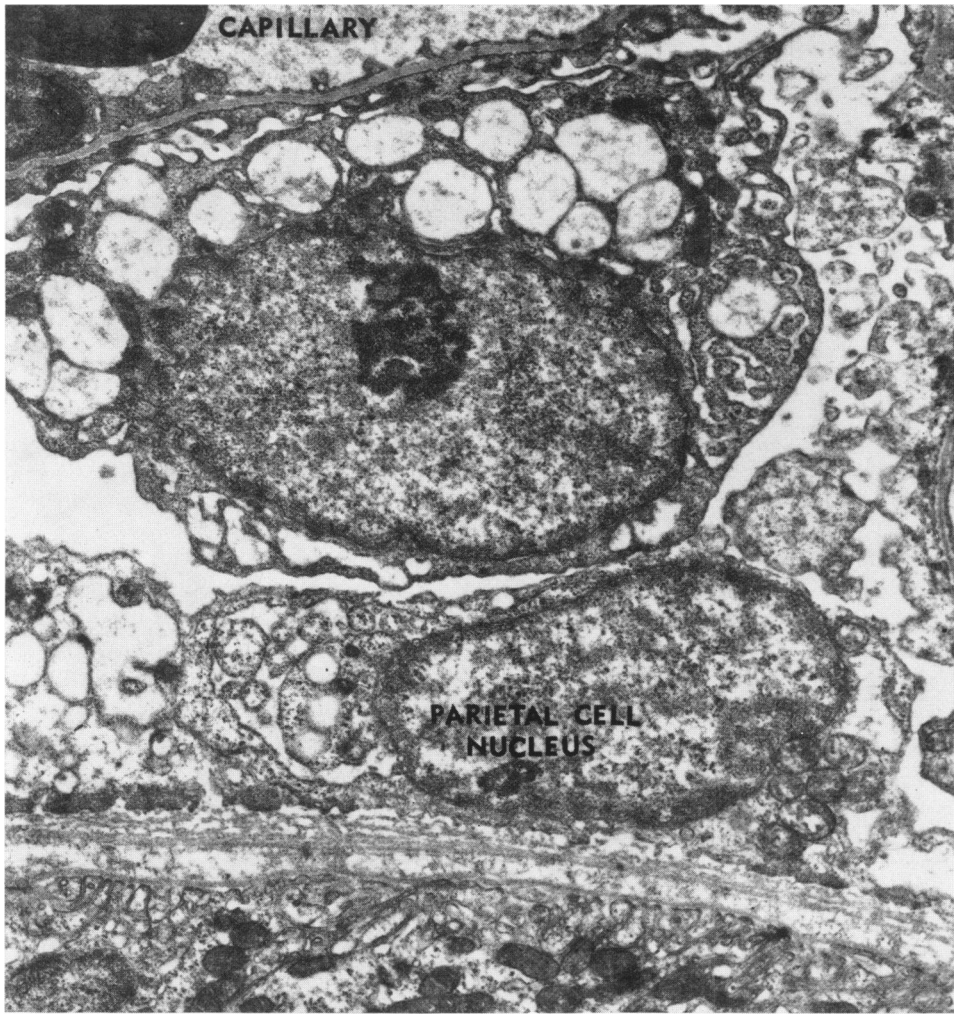
FIG. 6. Portion of a rat glomerulus; daily injections of aminonucleoside for 60 days. In many areas the endothelium and basement membrane appear unaltered. The epithelium is vacuolated, and few mitochondria are identified. A slight distortion of the basement membrane is labeled "knob." An irregular lamellar structure similar to some types of "myelin figures" is labeled "figure." × 12,000.



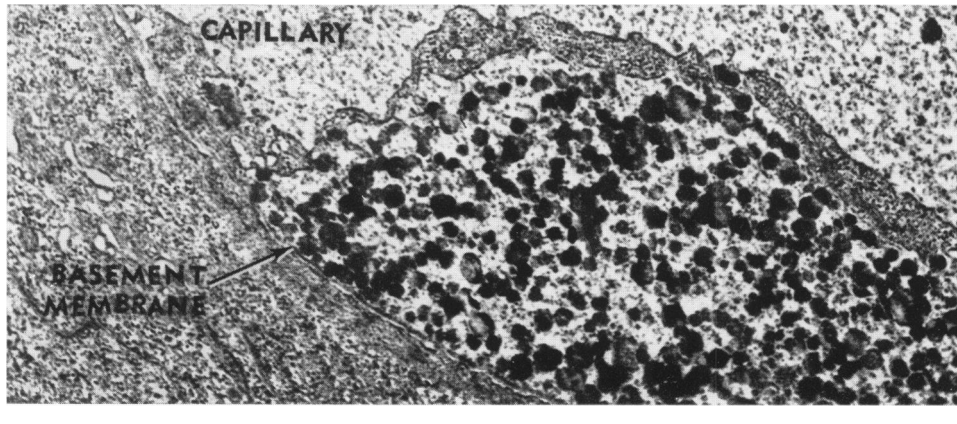
6

FIG. 7. Portion of the rat glomerulus and adjacent tubular epithelium; treated with aminonucleoside 60 days. Below a nucleus of the parietal glomerular epithelium is a laminated basement membrane that surrounds the glomerulus. At the bottom of the illustration is part of the tubular epithelial cell. Both the parietal and the adjacent visceral epithelium above it are vacuolated. By light microscopy the glomerulus was the seat of cellular proliferation, adhesions, and crescent formation. × 18,000.

FIG. 8. Portion of the wall of a glomerular capillary; rat treated 60 days with aminonucleoside. At the left the epithelial cell has no discrete foot processes. The basement membrane does not appear thickened. An endothelial cell projects into the capillary lumen and overlies numerous electron-dense structures, probably lipid. These lie in the zone between the electron-dense basement membrane and the plasma membrane of the endothelial cell. × 18,000.


CAPILLARY
PARIETAL CELL NUCLEUS

7


CAPILLARY
BASEMENT MEMBRANE

8

FIG. 9. Two cells in the proximal convoluted tubule of a rat treated 60 days with aminonucleoside. The microvilli, Palade's granules of the endoplasmic reticulum, and mitochondria in the cell to the left are more like those of control animals than are the distorted structures of the cell to the right and top of the photograph. A cytoplasmic lipid inclusion is labeled. × 12,000.

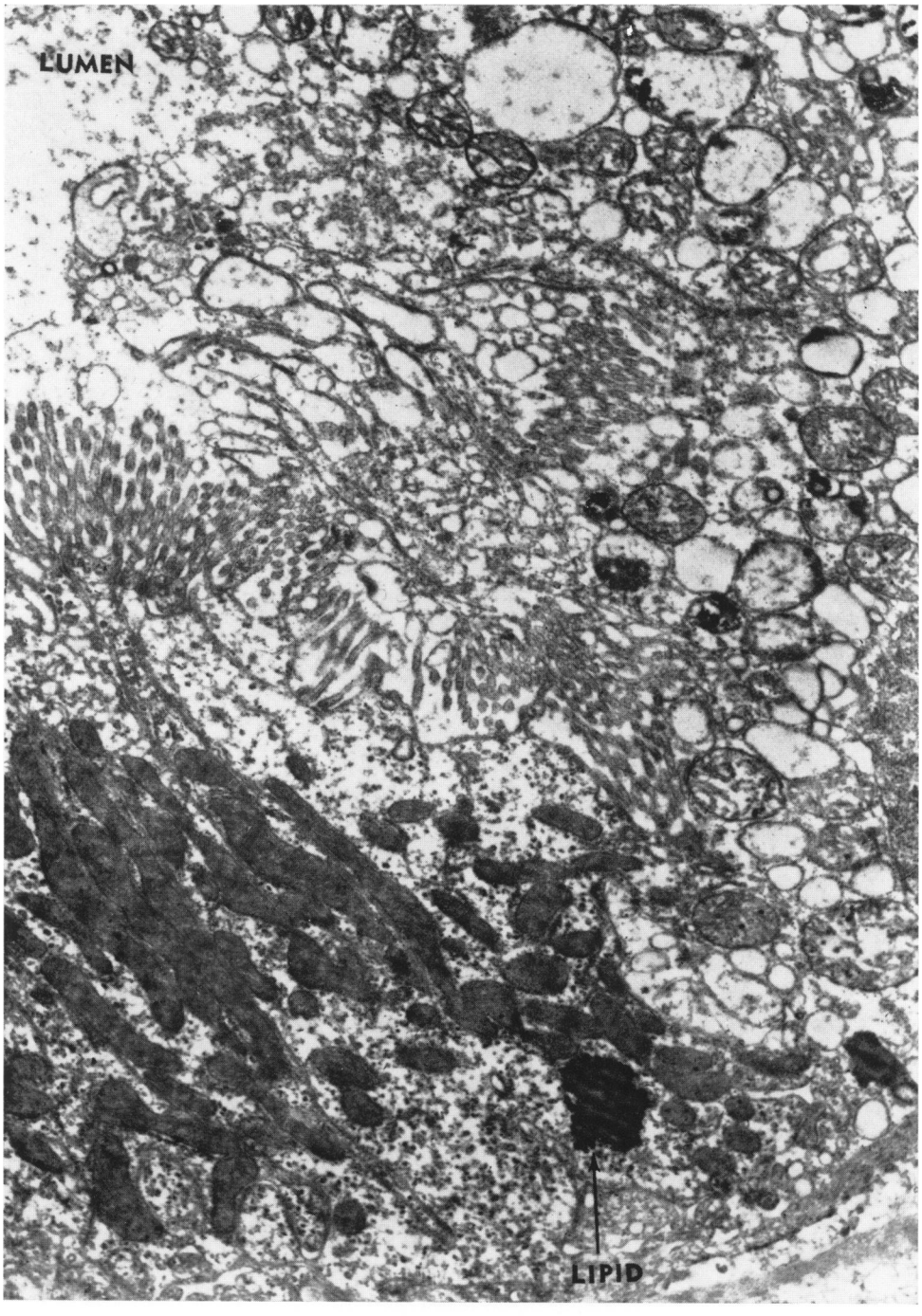
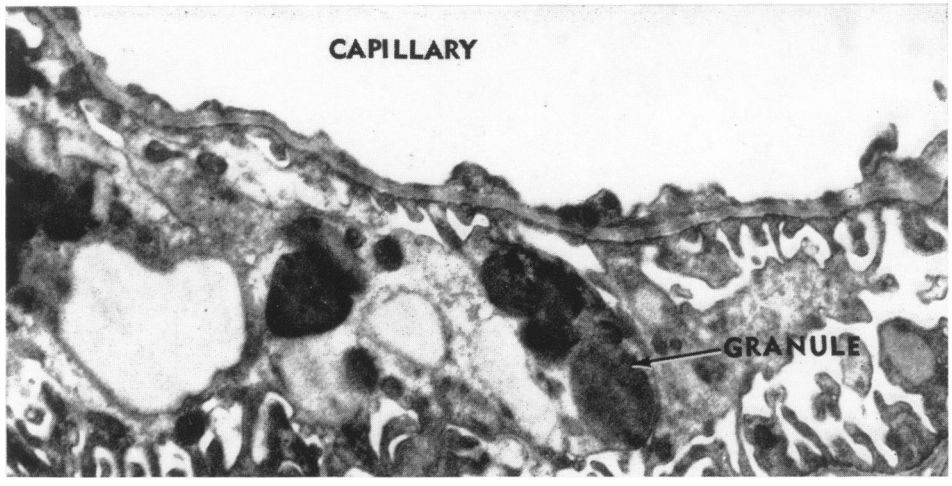
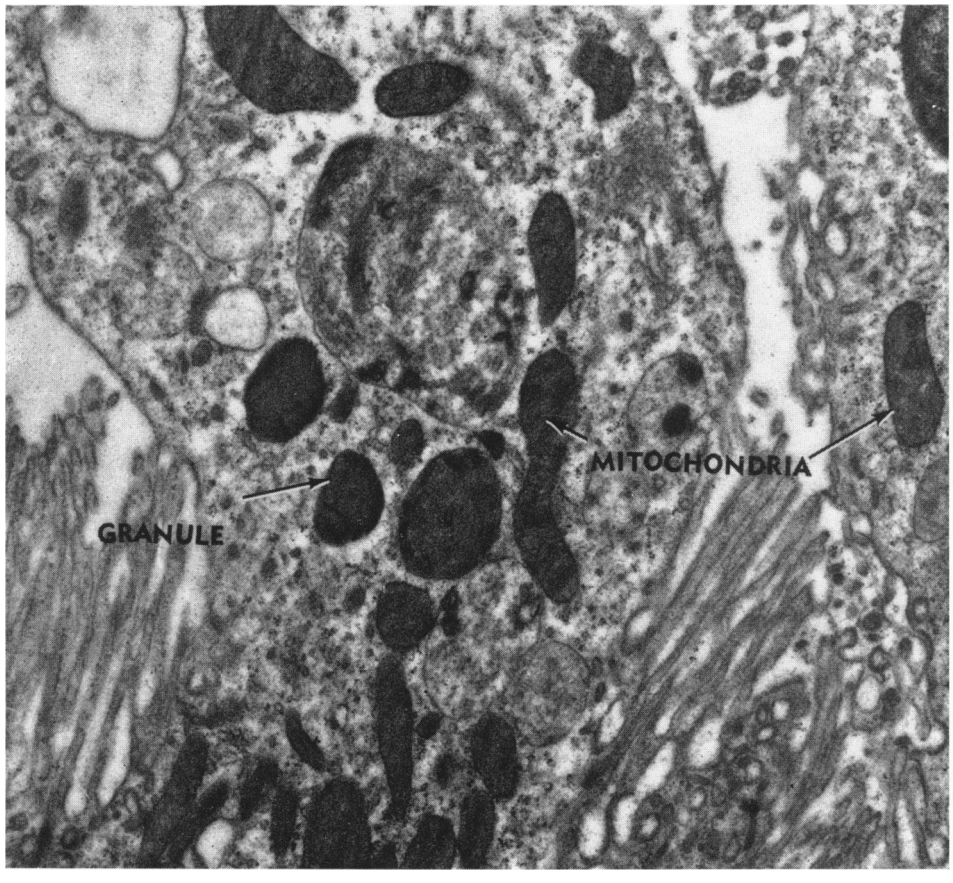

LUMEN
LIPID

FIG. 10. Portion of a glomerulus in a rat with proteinuria; animal had received daily intraperitoneal injections of dilute bovine albumin. Some of the foot processes are apparently fused. Note particularly the electron-dense granules within the epithelial cytoplasm. Approximately × 14,000.

FIG. 11. Portion of a proximal convoluted tubular cell; rat with nephritis and proteinuria induced by injections of rabbit anti-rat-kidney serum. Within the cytoplasm are electron-dense structures that are not recognizable as mitochondria. These appear to correspond to PAS-positive droplets seen by light microscopy. Note the similarity between this illustration and Figure 5. Approximately × 18,000.



10



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