An Ultrastructural Study of Glomerular Basement Membrane Synthesis

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THE SITE OF SYNTHESIS, rate of turnover, and mode of degradation of the glomerular basement membrane (GBM) is not known. Electron microscopic study of glomerular development suggested that both epithelial and endothelial cells contributed to GBM synthesis in the rat,[1] but in vivo labeling of the GBM by $AgNO_3$ administration [2,3] suggested that the epithelial cell was the site of normal GBM synthesis. This concept was based on the observation that a subepithelial zone of GBM free of granules appeared after $AgNO_3$ administration was terminated. Interpretation of the results of these studies is complicated by the observation that even with continuous $AgNO_3$ administration the dense particles occupied a subendothelial position.[4] These findings can be interpreted to mean that: (1) GBM, formed by the epithelial cell, varies with age, and the silver labels only the initial material, or (2) continuous feeding of $AgNO_3$ results in formation of an altered GBM which no longer accumulates granules after in vivo silver administration, or (3) changes occur in silver transport affecting its exposure to the GBM.

We set about to study $AgNO_3$ localization in basement membrane, both during the time of physiologic growth and following induced growth (contralateral nephrectomy). Newborn and adult rats were given drinking water containing 15 mM $AgNO_3$ for various periods to examine the location of silver granules as a function of age, length of exposure, and distribution after cessation of silver imbibition. Animals subjected to unilateral nephrectomy were included to test the effects of a potent physiologic growth stimulus on granule distribution and density. The results show that in all instances studied, GBM staining patterns are similar. Granules are initially randomly dispersed. A new layer of GBM, free of densities, becomes visible beneath the epithelial cell after the tenth week and gradually thickens, even when $AgNO_3$ imbibition continues.

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Methods

Weanling rats

Twelve weanling Sprague-Dawley rats were divided into groups of 4. $AgNO_3$ (15 mM) was substituted for tap water in 2 groups. One group was maintained on drinking water containing 15 mM $AgNO_3$ for 10 weeks and switched to tap water and 1 group remained on tap water for the duration of the experiment. Two animals from each group were sacrificed at 10 and 26 weeks.

An additional group of 4 rats was maintained on drinking water containing 15 mM $AgNO_3$ for 12 months.

Adult rats

Sixteen 200-g Sprague-Dawley rats were placed on 15 mM $AgNO_3$ drinking water for 10 and 20 weeks. Four control and 4 experimental animals were sacrificed at 10 and 20 weeks.

Adult rats, unilateral nephrectomy

Sixteen adult Sprague-Dawley rats were divided into 2 groups of 8. One group was placed on drinking water containing 15 mM $AgNO_3$. At 10 weeks one-half of each group underwent unilateral nephrectomy. Two from each subgroup (ie—nephrectomy or intact) were then placed on tap water and the other two on drinking water containing 15 mM $AgNO_3$. All were sacrificed 7 weeks later.

Histologic techniques

At sacrifice the renal arteries were segregated from the circulation by a technique previously described [5] and the kidneys were perfused with one-half strength Karnovsky's solution.[6] The tissue was fixed for an additional 4 hours in the above fixative, washed in 0.1 M sodium cacodylate buffer, postfixed in 1% OsO_4 buffered with s-collidine (pH 7.4), dehydrated in a graded series of alcohol, and embedded in Epon. Thin sections were examined in an RCA-3G or AEI electron microscope.

Results

Controls

The renal architecture of the control animals did not differ significantly from descriptions previously published.[2]

10 weeks 15 mM $AgNO_3$ feeding, weanling rats

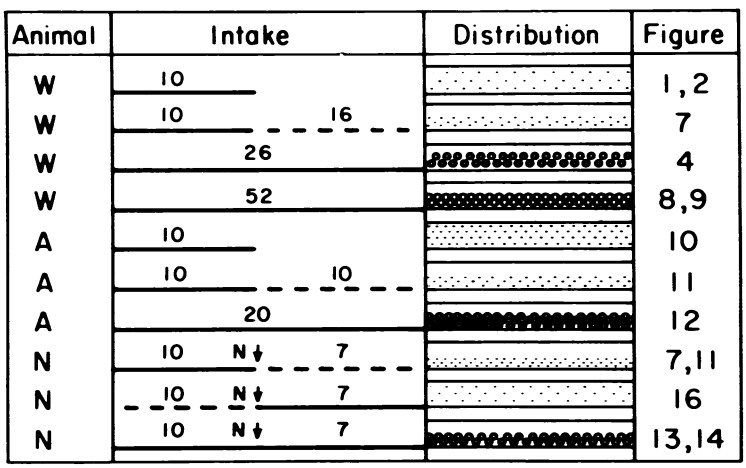
All four animals examined after 10 weeks of imbibition of 15 mM $AgNO_3$ were similar histologically. The lamina densa of GBMs were uniformly stippled with irregular, electron-dense deposits, which corresponded to that previously seen in similarly treated rats [3,4] (Fig 1 and 2, and Text-fig 1). There appeared to be no predilection for any aspect of the GBM, although few grains were seen in the lamina rara interna and externa. Nearly all loops had a similar density of granules, but a few had none. The basement membrane adjacent to

mesangial areas appeared similar to that of peripheral capillary loops, although occasionally there was an apparent increase in density in these regions.

The mesangial matrix was lightly stippled in a uniform fashion. The granules showed no discernible relation to fibrils in the mesangial matrix; however, the small number of particles made this assessment difficult (Fig 2). The number of granules present in the GBM decreased at the vascular poles (Fig 5) and none were seen in the basal lamina of the afferent or efferent anteriole. Tubular, capsular or vascular basal lamina did not contain granules. The only other cellular changes noted were in the proximal tubules where large crystalline masses were seen in single-membrane-bound bodies presumed to be cytosomes (Fig 3).

10 weeks 15 mM $AgNO_3$ feeding followed by 16 weeks tap water, weanling rats

The number and size of the dense granules in the peripheral capillary loops were not noticeably different from that seen at 10 weeks; however, they were confined to an area of the lamina densa near the capillary space (Fig 7). A thick zone of lamina densa lay external to this layer, which contained no granules. There was otherwise no morphologic difference between these two zones, and they were similar in appearance to that of the controls. Occasionally, near a mesangial



TEXT-FIG 1. Granule distribution in GBM. Weanling rats (W) were placed on 15 mM $AgNO_3$ drinking water (—) for 10, 26, or 52 weeks before sacrifice. One group was maintained on tap water (– – –) for 16 weeks following $AgNO_3$ imbibition. Adult rats (A) were given $AgNO_3$ for 10 and 20 weeks before sacrifice. A third group had equal exposure to $AgNO_3$ and tap water. (N), adult rats subjected to unilateral nephrectomy (N↓) 10 weeks after experiment began and sacrificed 7 weeks later. Localization of granular distribution is shown in far right column.

area, the granules appeared to lie on or near a dense line in the lamina densa with a clear zone of GBM on each side (Fig 7).

Few dense granules were seen in the GBM adjacent to mesangial cells and only occasionally were granules still present in the mesangial matrix. The tubular cells were normal in appearance.

26 weeks 15 mM $AgNO_3$ feeding, weanling rats

There were striking differences at 26 weeks between this group and those placed on tap water after the initial 10-week exposure. In this group the size and density of silver granules in the GBM were markedly increased but they, too, remained in a zone of the lamina densa near the capillary side (Fig 4). A zone of lamina densa containing no dense granules was regularly present on the outer (epithelial) aspect of the GBM. The GBM was otherwise unaltered, although thickness was not accurately assessed. The mesangial matrix contained large numbers of granules, many of which were in clusters (Fig 6). A few of the smaller aggregates appeared to be arranged along or on fibrils. Granules were also present within single-membrane-bound structures of mesangial cells (Fig 9) but were not seen in glomerular epithelial cells of any group.

Other basement membranes contained no granules or only a few. No cellular changes were seen other than the previously noted proximal tubular cytosomes filled with dense crystalline aggregates (Fig 3).

12 months 15 mM $AgNO_3$ feeding, weanling rats

Four rats were maintained on 15 mM $AgNO_3$ drinking water for 12 months after weaning. The cortex was grey-black in color and glomeruli were easily visible on inspection of the cut surface. There was a second dark zone noted on sectioning which appeared to be in the area of the inner stripe of the outer zone of the medulla. Electron microscopy revealed large, irregular aggregates of dense particles on the endothelial aspect of the GBM (Fig 8). Similar collections were noted in the mesangial matrix and mesangial cells. A zone of GBM free of dense granules was present subepithelially in peripheral GBM, as well as in that covering the mesangium (Fig 9).

A small number of particles was present in Bowman's membrane on its outer aspect. Preliminary findings indicate that the particles in the inner stripe of the outer medulla lie within cytosomes of interstitial cells. Further details will be the subject of a future communication.

10 weeks 15 mM $AgNO_3$ feeding, adult rats

Rats weighing 200 g were placed on drinking water containing 15 mM $AgNO_3$ and sacrificed 10 weeks later. The GBM and mesangial matrix were uniformly strippled with particles. There appeared to be no polarization to one surface or the other, although the lamina rara interna and externa contained few dense bodies (Fig 10). The size and density of the deposits were similar to that noted in weanling rats after 10 weeks of $AgNO_3$ imbibition (Fig 1 and 2). They were not seen in other renal structures.

10 weeks 15 mM $AgNO_3$ feeding followed by 10 weeks tap water, adult rats

As was seen in similarly exposed weanling rats, a subepithelial zone free of dense particles appeared (Fig 11). The mesangial matrix and GBM covering the mesangium contained few particles. As was noted previously (Fig 7) near mesangial regions, the granules occasionally were concentrated on or near a dense line which was surrounded by granule-free GBM. The tubular and vascular basement membranes contained no particles.

20 weeks 15 mM $AgNO_3$ feeding, adult rats

The position and density of granules was identical to that seen in similarly treated weanling rats. The particles were largely restricted to the subendothelial zone and were larger and more numerous than those seen at 10 weeks (Fig 12). The mesangial matrix was densely studded with similar structures. Mesangial cells contained aggregates of these particles in single-membrane-bound bodies. Occasional granules were present in the basement membrane at the vascular pole and in the outer aspect of Bowman's membrane near this region. Proximal tubular cells contained lysosomal material similar to that noted in the correspondingly treated weanling rats (Fig 3).

15 mM $AgNO_3$ feeding 10 weeks prenephrectomy followed by tap water for 7 weeks.

The distribution of dense particles in the peripheral GBM was similar to that in the kidneys of animals not subjected to unilateral nephrectomy (Fig 7 and 11). The zone of dense particles occupied a subendothelial position. Near the mesangium, zones of GBM free of particles on either side of the granule-laden layer were occasionally seen (Fig 7 and 11). Smaller numbers of particles were seen in mesangial matrix and the GBM covering the mesangium.

15 mM $AgNO_3$ feeding for 10 weeks, prenephrectomy and 7 weeks postnephrectomy.

The granules occupied a subendothelial position (Fig 13). Their

number and size increased compared to any group in this series. No structural abnormalities other than the granules were detected. Focal collections of small granules were seen on the outer aspect of Bowman's membrane. They were also present in the basal lamella of the intima and smooth muscle cells of small arteries and arterioles (Fig 14). The cortical tubular basement membranes contained only rare granules. In the inner stripe of the outer medulla granules were seen in the interstitial cells.

Tap water prenephrectomy, 15 mM $AgNO_3$ feeding for 7 weeks postnephrectomy.

In this group granules were present throughout the lamina densa but there was considerable variation in the density and some variation in distribution between glomeruli in the same kidney, as well as between those from kidneys of different animals. The predominant pattern was a light scattering of grains throughout the lamina densa and mesangial matrix (Fig 15). Where many granules were present, they were often more concentrated near the lamina rara interna (Fig 16). Endothelial cells not infrequently contained dense granular material in membrane-bound cytoplasmic organelles (Fig 16). No other structural abnormalities were noted.

Body and kidney weight

With one exception, intact animals maintained on 15 mM $AgNO_3$ drinking water during any part of the experiment had lower body weights than controls (Table 1). In the group subjected to unilateral nephrectomy, animals exposed to 15 mM $AgNO_3$ drinking water either pre- or postnephrectomy weighed less than the corresponding control at sacrifice. The apparent increase in kidney to body weight ratio reflects the relatively poor growth of the experimental group.

Discussion

Our findings confirm the observation [2,3,4] that short-term (10 weeks or less) $AgNO_3$ imbibition results in uniform deposition of dense granules in the GBM and mesangial matrix. If the silver nitrate solution is replaced by tap water after 10–12 weeks, a subepithelial zone of GBM free of granules appears and widens with time. It has been suggested that the granule-free zone represents new formation of normal GBM. The observation that continued $AgNO_3$ administration is also associated with a granule-free subepithelial zone of GBM in both weanling and adult rats confirms and extends the observations of Olcott and Richter.[4] This selective metal deposition during a

period of new membrane formation suggests that newly formed GBM is not labeled. The GBM thickens with age[9] and at all ages has a measurable turnover.[10] In the present study it was shown that with either normal maturation or a physiologic stimulus to growth (unilateral nephrectomy), granule localization was primarily related to length of silver exposure, initially diffuse and subsequently localized, rather than to new membrane formation. This suggests that the site of granule deposition is not solely related to the presence of newly formed GBM.

The absence of uniform staining may be based on the fact that silver does not have access to the subepithelial, granule-free zone. This seems unlikely, since silver continues to be excreted in significant amounts in the urine and deposition in a peritubular area where divalent metal ion absorption is maximal, gradually increases with continued exposure. Since silver does seem to have access to this zone, then there must be an alteration in the silver-protein relationship following 10–12 weeks of exposure. This suggests that the portion of the newly formed GBM responsible for silver aggregation, the protein and/or mucopolysaccharide components formed during the silver nitrate administration, differs from normal GBM. Aside from the altered metal distribution there is no direct evidence for altered membrane or membrane formation. $AgNO_3$ is toxic when given in large

Table 1. Body and Kidney Weight of Adult Rats given 15 mM $AgNO_3$ Drinking Water*

Group	No. of Animals	Water intake: HOH → HOH†		HOH → Ag^+		Ag^+ → HOH		Ag^+ → Ag^+	
		BW‡	KW§	BW	KW	BW	KW	BW	KW
Unilateral Nephrectomy	1	537	0.475	419	0.440	460	0.400	499	0.376
	2	554	0.472	433	0.487	423	0.624	435	0.455
Intact	1	540	0.290	476	0.337	563	0.332	450	0.346
	2	546	0.331	432	0.348	508	0.339	413	0.340

* Sixteen adult rats were divided into two groups of 8. One group was placed on 15 mM $AgNO_3$ drinking water and the other on tap water. At 10 weeks one-half of each group (ref. 4) was nephrectomized. Two from each subgroup (animals No. 1 and 2 above) were then placed on water (HOH) or 15 mM $AgNO_3$ (Ag^+) drinking water.

† Composition of drinking water for first 10 weeks and for the subsequent 7 weeks (eg–HOH → Ag^+, water for first 10 weeks and 15 mM $AgNO_3$ for the subsequent 7 weeks).

‡ Body weight (g) at sacrifice.

§ Kidney weight (g/100 g body weight).

doses[7] and may provide a mechanism for formation of an anomalous product. It is also possible that the mechanism for aggregation is lost. Since this process is not understood, changes in the mechanism must remain speculative.

No marked changes are seen in the granule density among the peripheral capillary loops within 4 months after cessation of $AgNO_3$ imbibition. The number of dense granules seen in the mesangial matrix and GBM over the mesangium decreases strikingly. Since the nature of the silver-basement-membrane interaction is unknown, it is not possible to suggest a mechanism for the observed loss of metal. These findings suggest that a selective removal of silver or a silver-protein complex may occur. Whether the mesangial cells participate in breakdown of GBM and/or removal of the silver complex has not been demonstrated. However, it has been suggested that the mesangial cell participates in removal of material trapped in the glomerulus.[1,8] The presence of dense particles in their cytosomes may therefore result from phagocytosis of circulating silver-protein complexes or from degradation of the surrounding matrix and the GBM containing the bound metal.

The in vivo method of silver staining may not be a valid test of the site of normal GBM synthesis. However, these findings do not exclude the epithelial cell as a contributor to, or the sole site of, synthesis of the GBM, but they do raise the question of whether silver staining is a measure of normal GBM synthesis.

Summary

Weanling rats were given 15 mM $AgNO_3$ in their drinking water for 10 weeks and divided into 2 groups. At this time the GBM was uniformly stippled with granules. One group was continued on 15 mM $AgNO_3$ drinking water and the second was placed on tap water. Animals sacrificed 10 and 26 weeks later from both groups had a subepithelial zone of lamina densa free of dense granules. The number and density of granules was greater in the group receiving continuous $AgNO_3$ in their drinking water. Similar findings were noted in adult rats and in rats subjected to unilateral neprectomy. These findings suggest that the newly formed GBM in $AgNO_3$-treated rats is abnormal and reopens the question of the cellular site of synthesis of the GBM.

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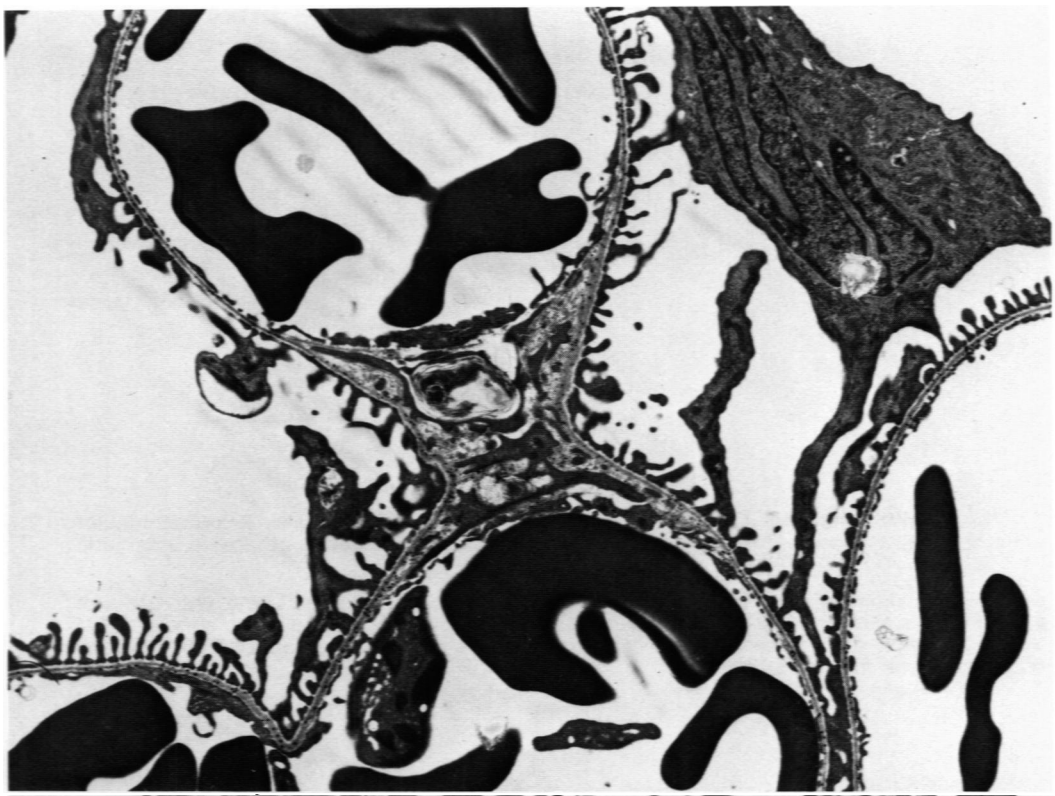
[*Illustrations follow*]

Legends for Figures

Fig 1. Weanling rats, 15 mM $AgNO_3$ drinking water for 10 weeks. Granules are distributed uniformly throughout lamina densa and mesangial matrix. × 3120.

Fig 2. Weanling rats, 15 mM $AgNO_3$ drinking water for 10 weeks. Mesangial matrix contains many granules but none are seen in mesangial cells. Basement membrane covering mesangial region is uniformly stippled. × 6120.



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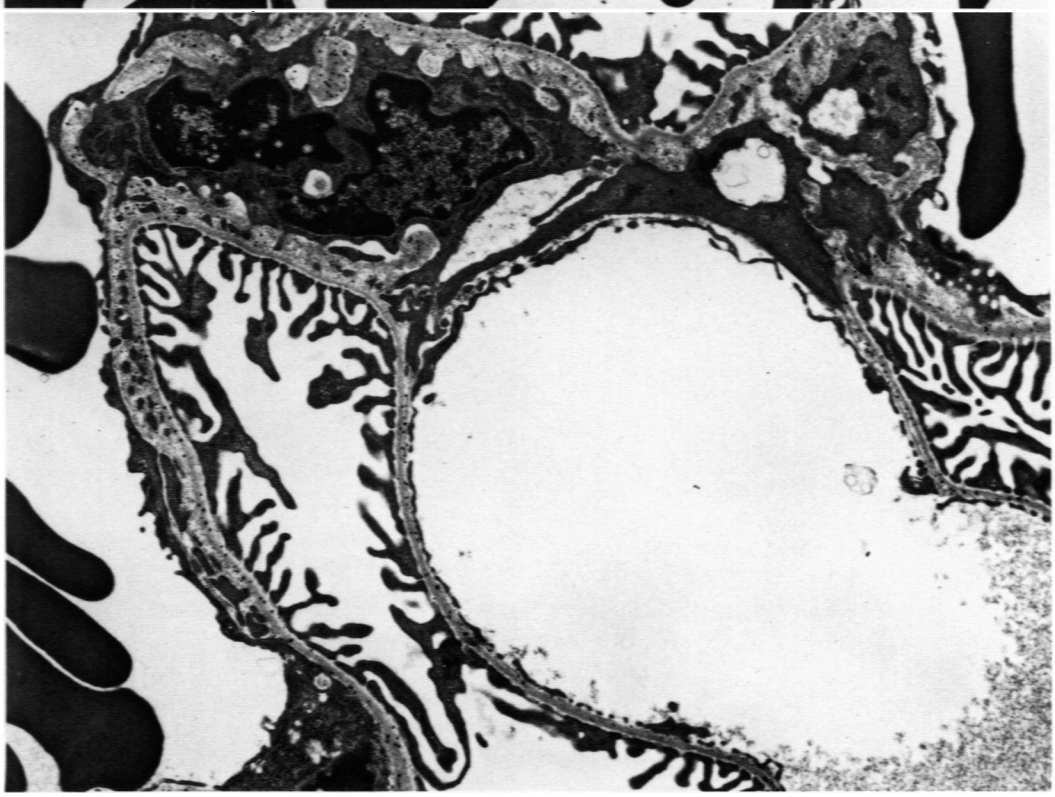

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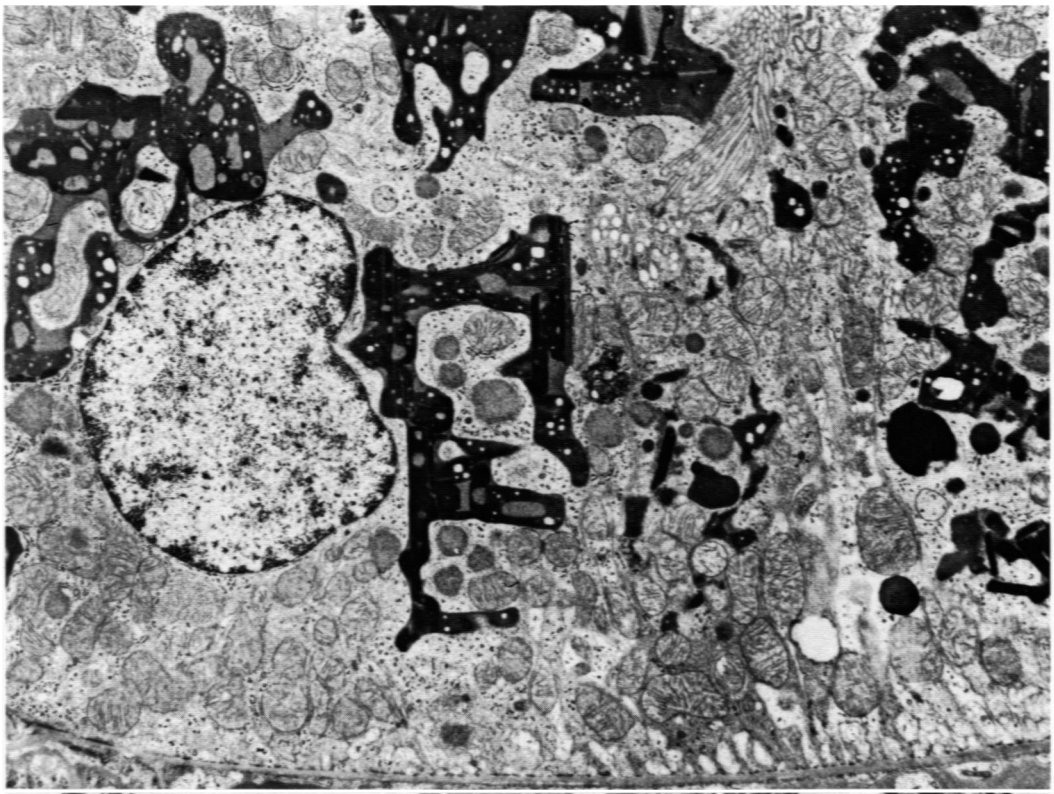
Fig 3. Weanling rats, 15 mM $AgNO_3$ drinking water for 10 weeks. Proximal tubular cell with cytosomes containing dense, angular inclusions. Cell is otherwise unremarkable. × 6410.

Fig 4. Weanling rats, 15 mM $AgNO_3$ drinking water for 26 weeks. Large aggregates of dense particles lie on endothelial aspect of lamina densa. Granule-free zone of GBM is present on epithelial side of lamina densa, as well as that part of GBM covering mesangial region. × 8030.



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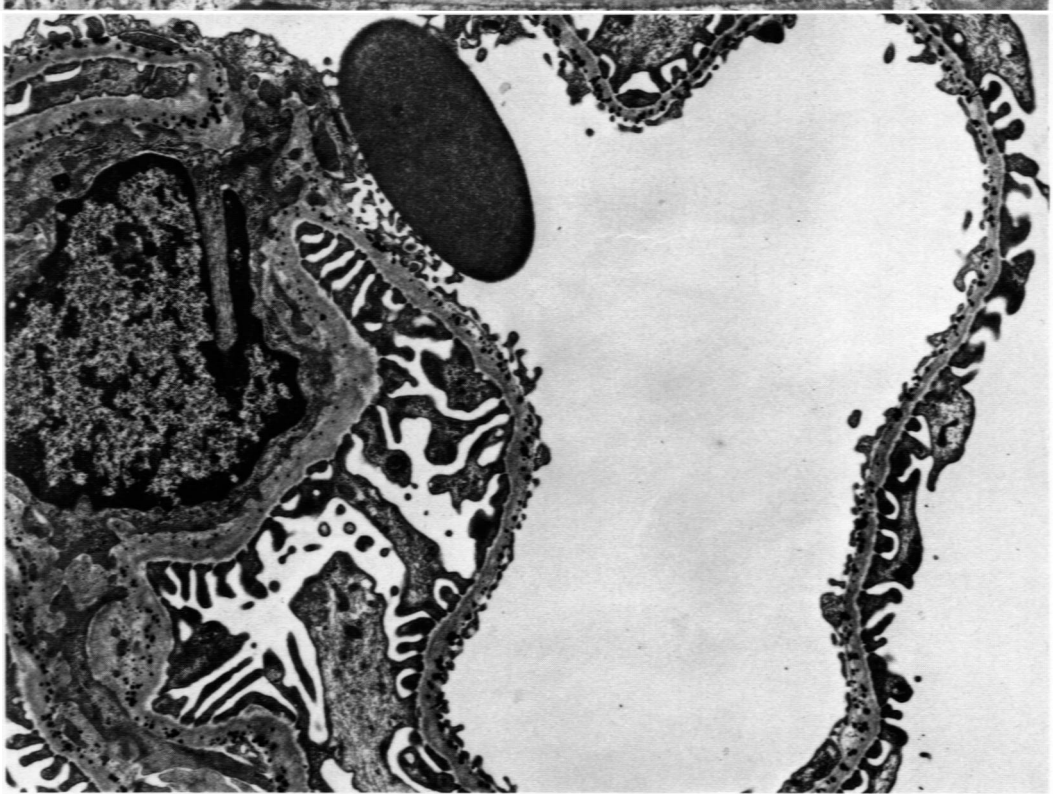

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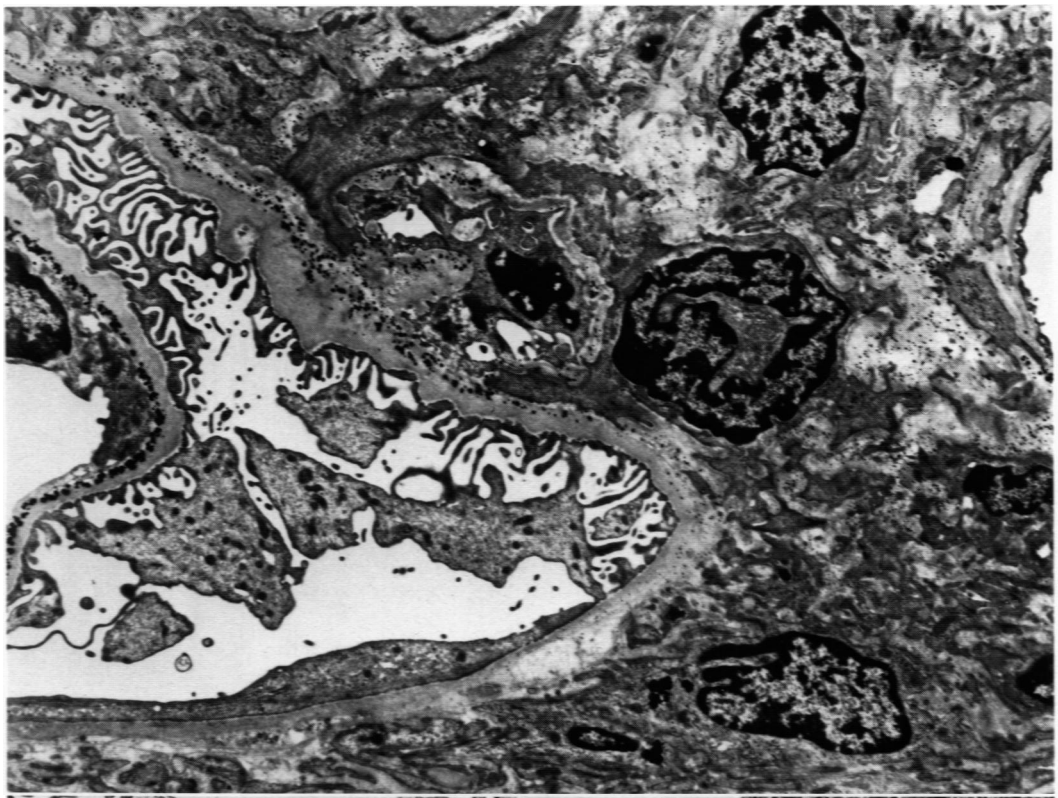
Fig 5. Weanling rats, 15 mM $AgNO_3$ drinking water for 26 weeks. Number of dense particles decreases near vascular pole and few were present in Bowman's membrane. × 3260.

Fig 6. Weanling rats, 15 mM $AgNO_3$ drinking water for 26 weeks. Mesangial matrix contains large aggregates of particles but is otherwise unremarkable. × 6220.



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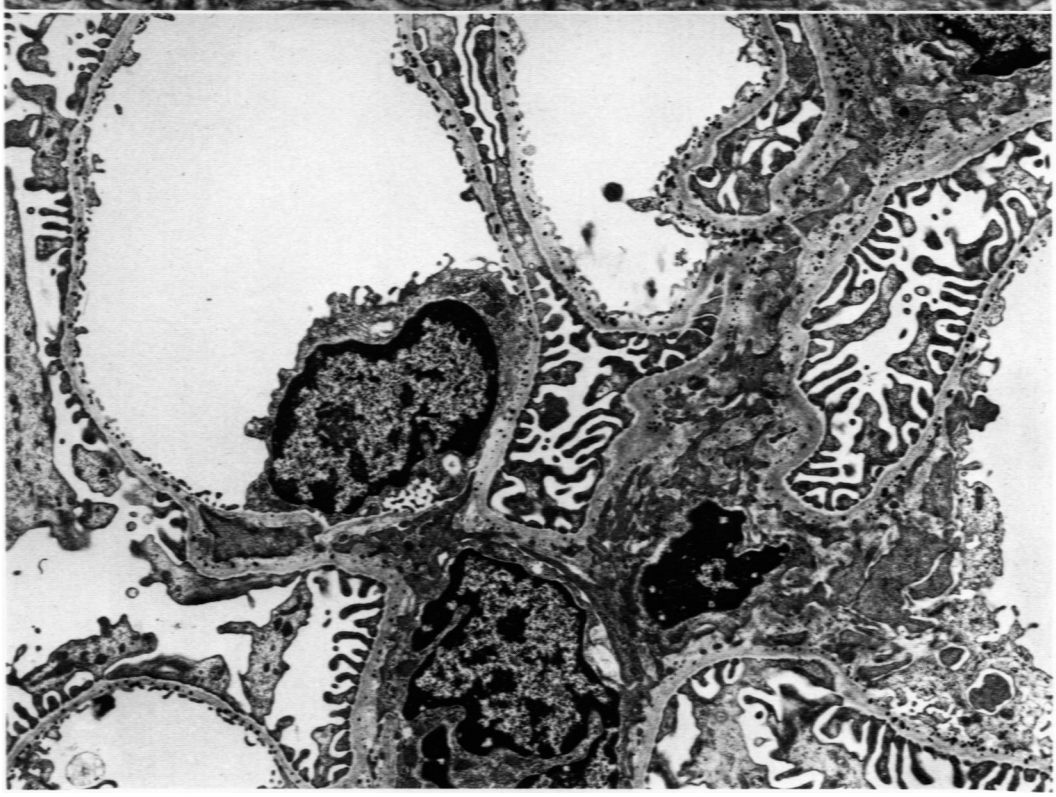

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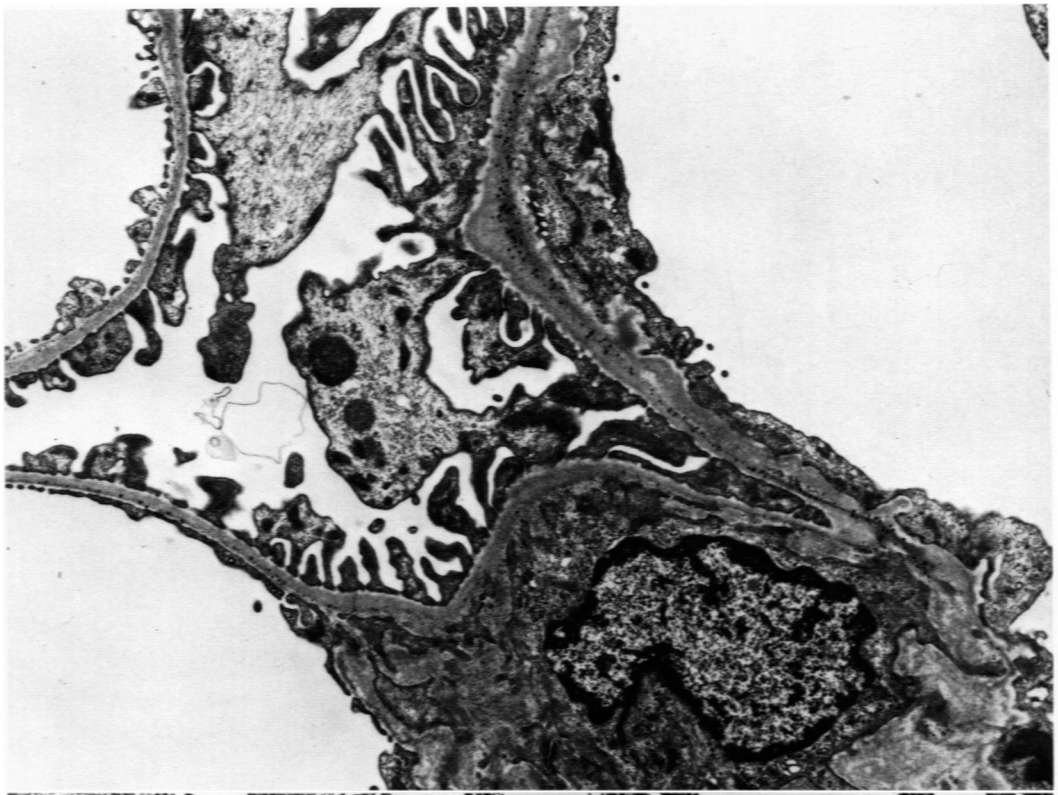
Fig 7. Weanling rats, 15 mM $AgNO_3$ drinking water for 10 weeks, followed by tap water for 16 weeks. Granules are less numerous in mesangial matrix and GBM in this region. In rare areas the particles appear to lie on a dense line within the lamina densa near mesangial regions. Elsewhere they are seen in subendothelial position. × 7630.

Fig 8. Weanling rats, 15 mM $AgNO_3$ drinking water for 12 months. Number and size of particles is accentuated but there is a uniform zone of GBM beneath epithelial cells which is granule-free. × 2690.



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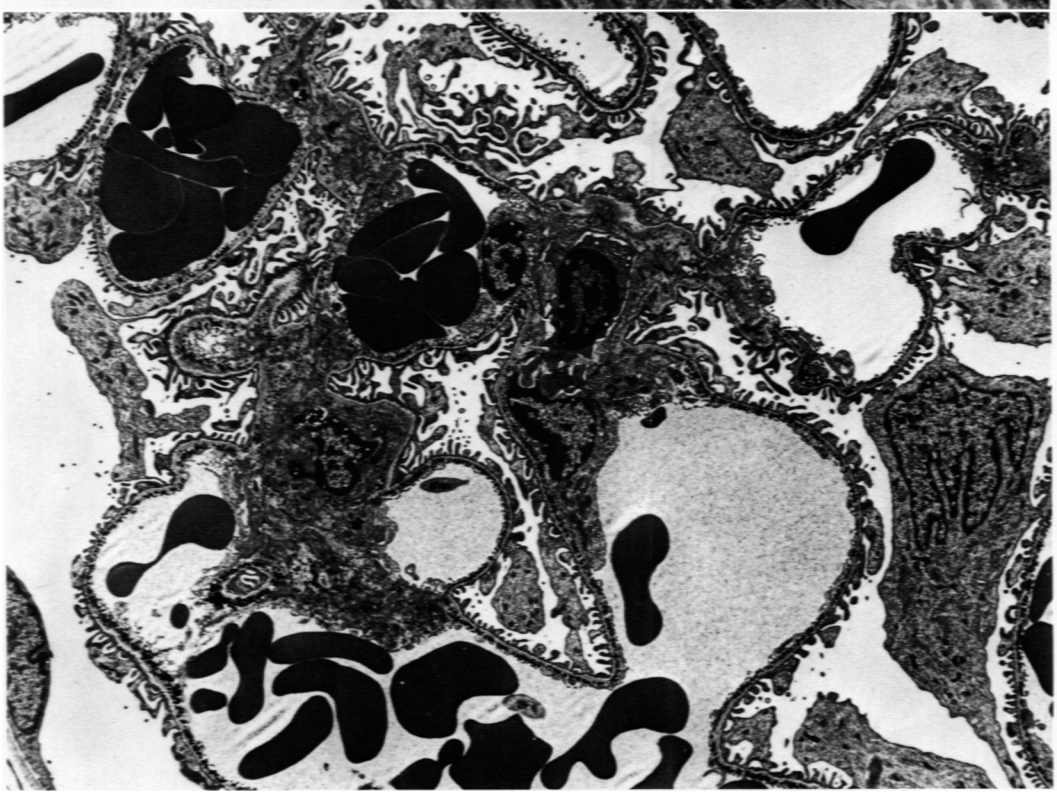

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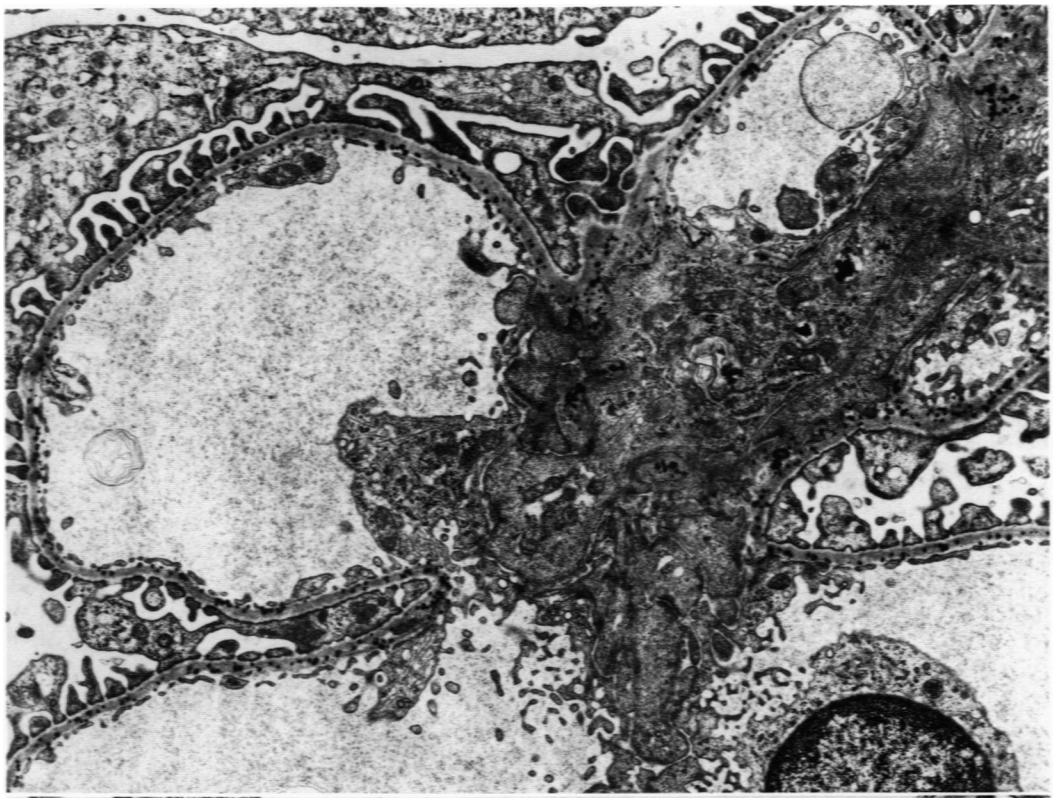
Fig 9. Weanling rats, 15 mM $AgNO_3$ drinking water for 12 months. Mesangial cells contain large aggregates of particles within cytosomes. Mesangial matrix is diffusely studded with large, dense granules. × 8500.

Fig 10. Adult rats, 15 mM $AgNO_3$ drinking water for 10 weeks. Lamina densa contains randomly scattered dense granules. × 13,130.



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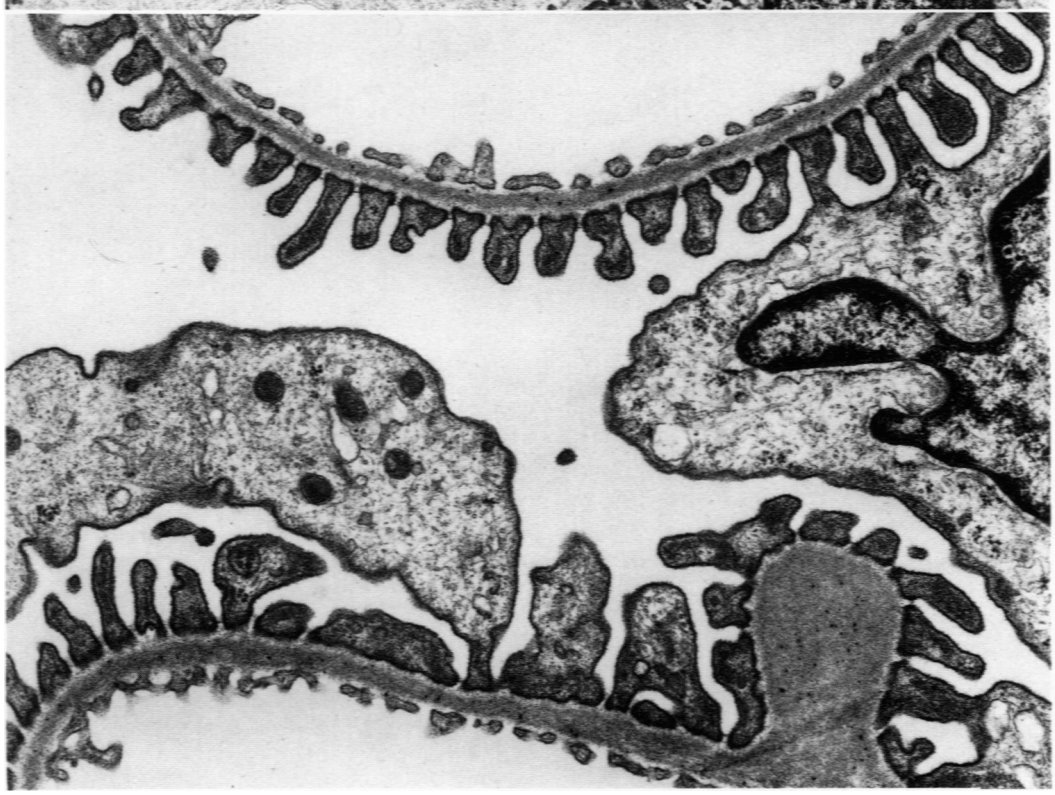

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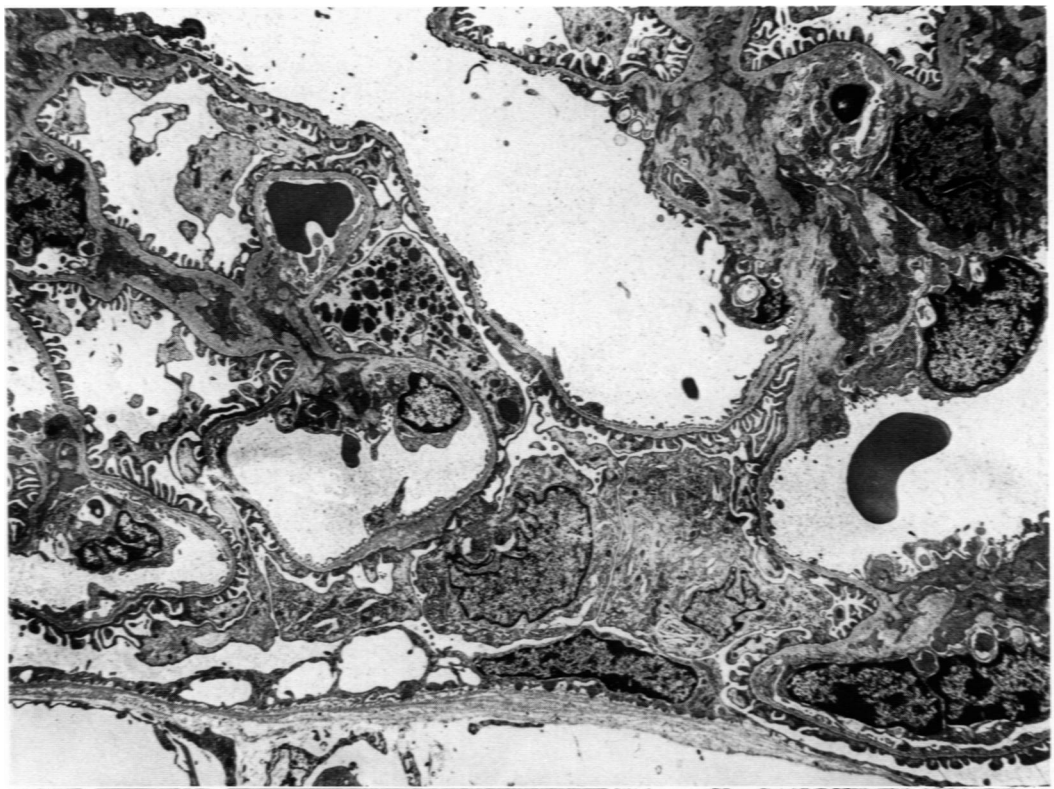
Fig 11. Adult rats, 15 mM $AgNO_3$ drinking water for 10 weeks, followed by tap water for 10 weeks. Dense particles again are seen on endothelial aspect of GBM. Mesangial matrix and GBM covering this region contain few granules. × 1850.

Fig 12. Adult rats, 15 mM $AgNO_3$ drinking water for 20 weeks. Dense particles are present in subendothelial region of peripheral GBM and that covering mesangium. Mesangial matrix is diffusely studded with granules. × 7750.



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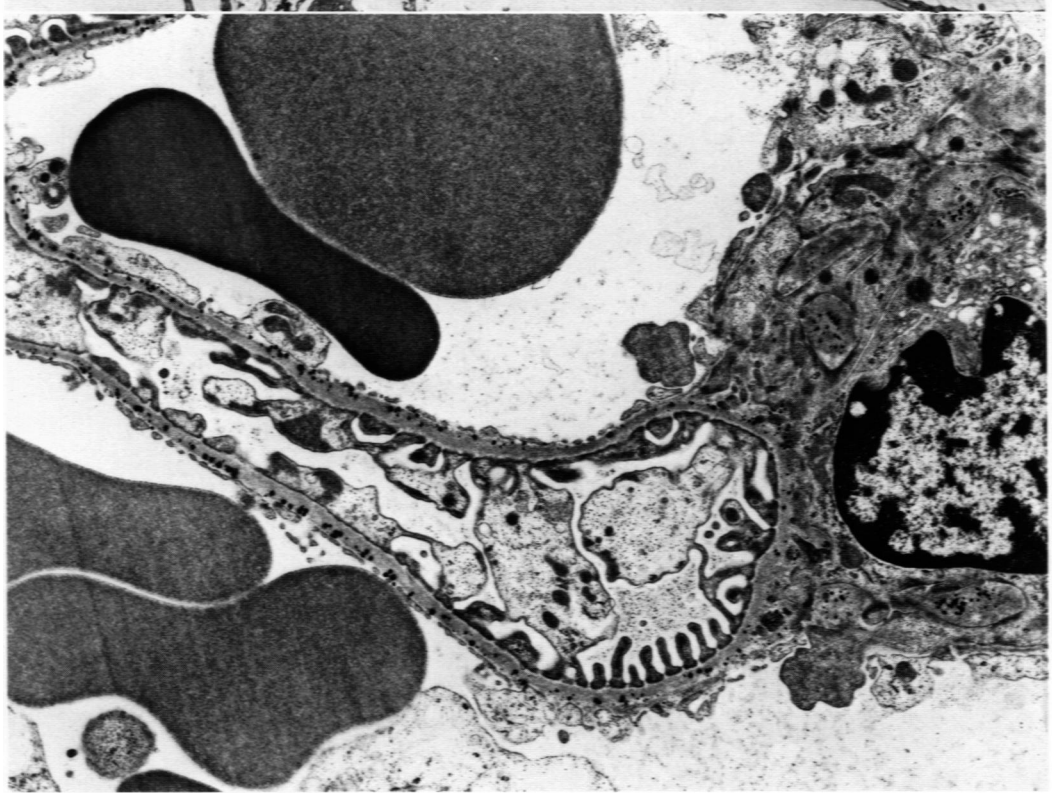

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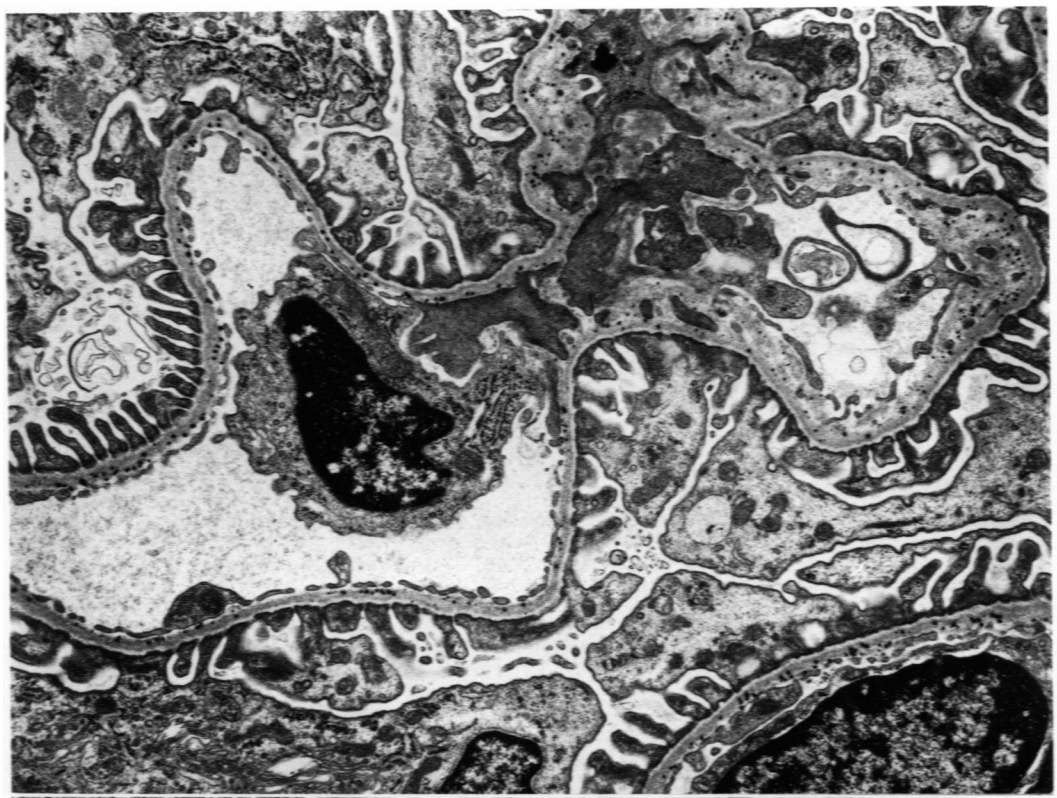
Fig 13. Adult rats, unilateral nephrectomy, 15 mM $AgNO_3$ drinking water for 10 weeks prenephrectomy and 7 weeks postnephrectomy. Distribution of particles is similar to intact rats (Fig 12). × 5400.

Fig. 14. Adult rats, unilateral nephrectomy, 15 mM $AgNO_3$ drinking water for 10 weeks prenephrectomy and 7 weeks postnephrectomy. Granules are seen in region of internal elastic lamina and external lamina of smooth muscle in this small artery. × 9500.



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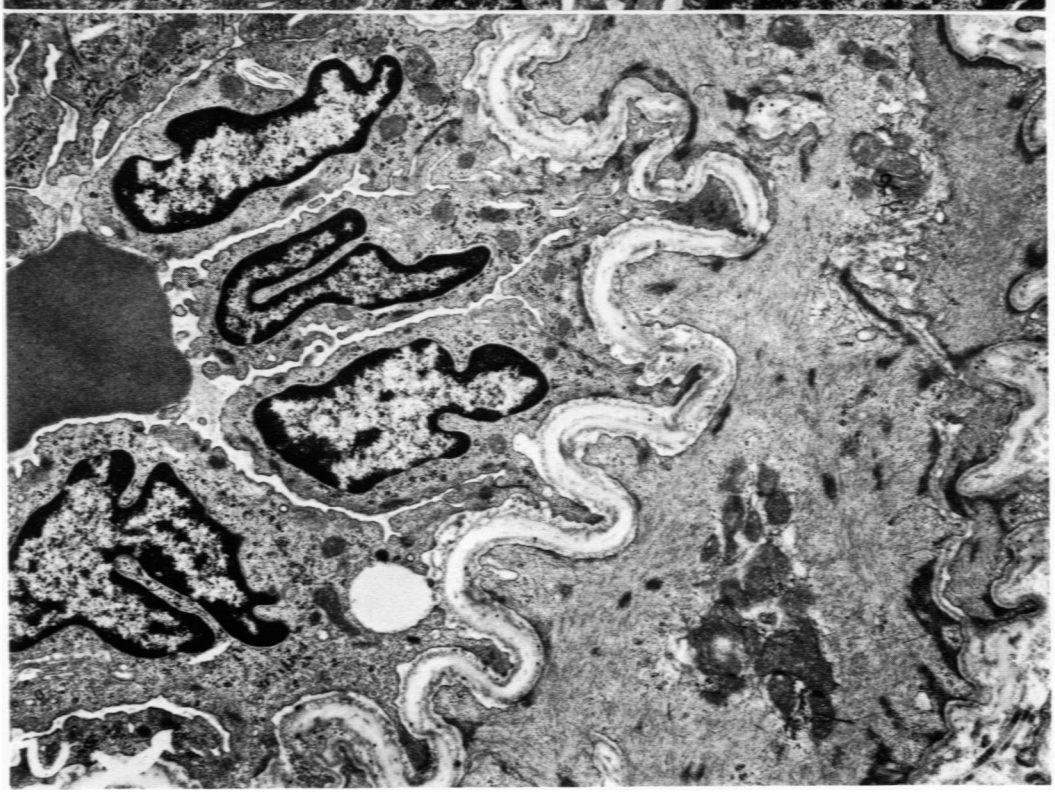

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Fig. 15. Adult rats, unilateral nephrectomy, tap water for 10 weeks prenephrectomy and 15 mM $AgNO_3$ drinking water for 7 weeks postnephrectomy. Mesangial matrix contains randomly scattered particles, as does GBM covering this region. Peripheral GBM also displays randomly dispersed granules (inset × 4200). ×10,500.

Fig. 16. Adult rats, unilateral nephrectomy, 15 mM $AgNO_3$ drinking water for 10 weeks prenephrectomy and 7 weeks postnephrectomy. Endothelial cells frequently have dense particles in cytosomes. × 19,000.

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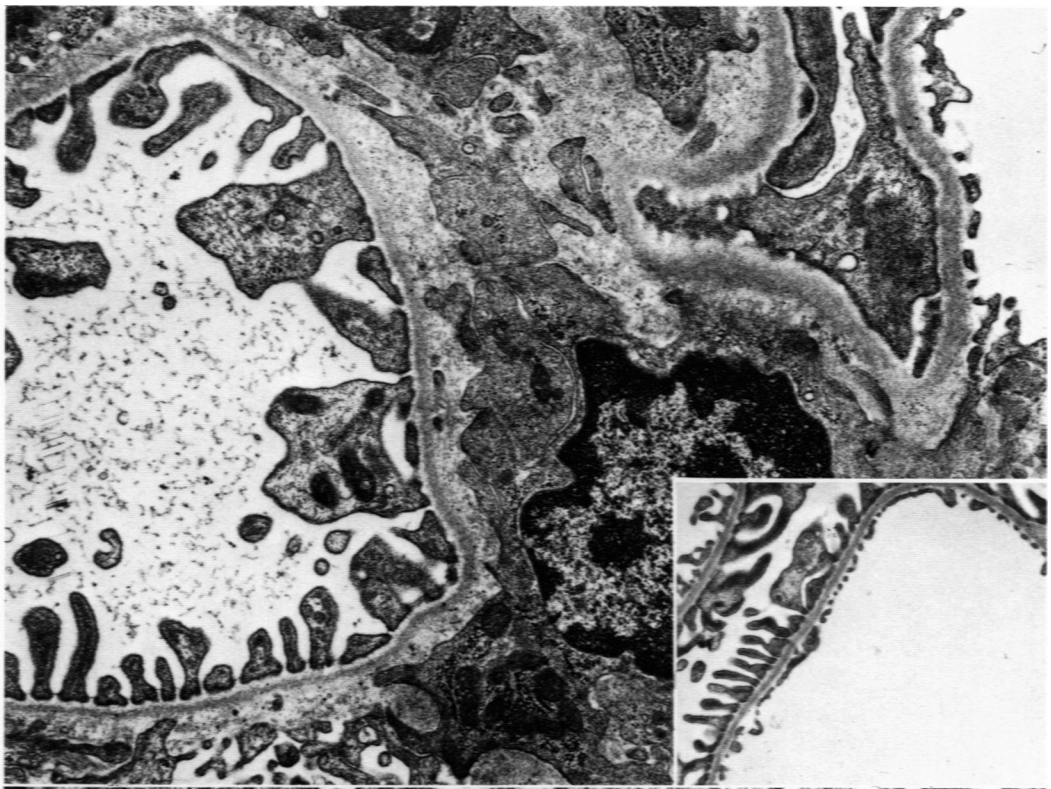

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