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INTRALUMINAL NUCLEI AND OTHER INCLUSIONS AS AGONAL ARTIFACTS OF THE RENAL PROXIMAL TUBULES

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A considerable body of older literature is devoted to the significance of inclusions which have frequently been seen in the lumens of the renal and other secretory tubules. These inclusions have generally been described as vesicular, and are sometimes seen to be in continuity with the tubule epithelium. Nuclei have been mentioned in association with them on occasion, or have been so figured without comment. The formation of these vesicles has often been interpreted as evidence of a normal process of secretion in progress in these tubules, but others have contended that they were artifactual, representing merely poor fixation. Von Möllendorff [1] reviewed the subject in some detail, and its minute consideration is not required here. In relation to the kidney these reports are little attended to now because they are obviously not pertinent to modern ideas of the mode of urine formation; however, the occurrence of the vesicles still awaits an explanation. Bell [2] considered them artifact, but believed they did not arise from the tubule epithelium. He reported them to be more numerous in diseased kidneys. Decreased interest in, and hence decreased familiarity with these objects, has led more recently to frequent references to them as pathologic.[3-5]

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Our attention was drawn to these matters some years ago when we observed large numbers of nuclei in the tubule lumens of rat kidneys rapidly frozen within a few seconds of removal for processing by the method of freezing-dehydration. This investigation reports studies of this apparent paradox, the discovery of the relation of this phenomenon to the inclusions of the older literature, and our conclusion that changes of this general nature arise from agonal disruption of the tubule epithelial cells through continued physiologic reabsorption of urine.

METHODS

All experimental procedures were carried out on male Sprague-Dawley or Holtzman rats weighing 200 to 250 gm. Incidental observations have been made on kidneys of mice and other species.

Our original observations were made on frozen-dried material. Animals were anesthetized with Nembutal®, their kidneys removed and transverse median slices quickly cut, placed on a small square of aluminum foil, then plunged into isopentane chilled to $-160°$ C. with liquid nitrogen. Dehydration and paraffin embedding were completed by a previously described technique.[6] Sections cut at 6 μ were stained for alkaline phosphatase to demonstrate the brush border; naphthol AS-MX phosphate was used as substrate.[7]

The relation of conventional modes of fixation to nuclear displacement was studied by removing kidneys, hemisecting them, and placing portions for appropriate times in a wide variety of chemical fixatives. Since the results obtained indicated that the nuclear effect was independent of fixative, it is sufficient to say here that 21 different mixtures were used, representing all the principal fixing agents and their usual combinations. Each mixture was used at room temperature for the conventionally prescribed time or at 4° C. for twice this time. Routine methods of paraffin embedding and sectioning were used. Periodic acid-Schiff staining with a hematoxylin counterstain facilitated the study of relations between the brush border, nuclei and other structures.

As a means of reducing to a minimum the interval between cessation of normal function and immobilization of cellular elements, the method of Swann, Sinclair and Parker [8] was used. In this, liquid nitrogen was poured into the open abdomen of an anesthetized animal, freezing the functioning kidneys *in situ* almost instantly. The frozen kidneys were then removed and processed further by freeze-drying. Removal after freezing can be facilitated by freeing the kidneys of all tissue connections except through the renal pedicle before pouring on the liquid nitrogen.

The following procedures were employed in attempts to modify the postmortem redistribution of water between the tubular, cellular and vascular compartments:

1. Kidneys from untreated rats were fixed in 10 per cent unbuffered formalin with 1.5 per cent or 3.0 per cent NaCl, or 9 per cent or 18 per cent glucose added. Fixation in 10 per cent unbuffered formalin provided controls. Routine procedures followed by periodic acid Schiff-hematoxylin staining were used in section preparation.

2. Thirty cc. of either 3 per cent NaCl or 18 per cent glucose were administered intravenously to Nembutal-anesthetized animals within approximately 10 minutes, and the kidneys then removed and fixed in either 10 per cent unbuffered formalin, or in the same fluid with NaCl to 1.5 per cent added in the animals given NaCl injections, or with glucose to 9 per cent in the case of glucose injection.

3. Conditions essentially those well known from stop-flow studies were induced by ligating the ureters of experimental animals and waiting an appropriate length of time. In animals made osmotically diuretic by the intravenous administration of 10 cc. of 20 per cent glucose, the interval allowed was 6 minutes; in untreated animals,

6 hours. At the end of the period of ureteral ligation the renal pedicle was tied off firmly, the kidney removed with its ligature in place, and placed intact in 10 per cent formalin, or 10 per cent formalin containing glucose to 10 per cent, or NaCl to 5 per cent. From other kidneys similarly handled up to removal from the animal, median slices were cut and fixed in the same fixatives, or in 10 per cent formalin with sodium acetate added up to 10 per cent.

In the interest of improving cytologic fixation, trial was also made of a 10 per cent formalin, 6 per cent mercuric chloride, 2 per cent sodium acetate fixative with stop-flow kidneys.

Results

Occurrence of Intraluminal Nuclei and Vesicles

Primary Observations. Figure 1 shows a typical cluster of intraluminal nuclei in conventionally handled frozen-dried material. It will be noted that these nuclei appear still to be surrounded by a matrix resembling the cytoplasm of the tubule epithelial cells. The brush border appears to be of a single thickness here, and the geometric possibility that these nuclei are actually still in the epithelium appears to be ruled out. We confirmed this conclusion from the study of serial sections.

Intraluminal Nuclei Independent of Fixation. It was our impression at first that the nuclei in tubule lumens were associated with the technical procedure involved. However, inspection of chemically fixed kidneys that we had previously regarded as well-preserved showed that similarly displaced nuclei were readily found. Failure to be aware of them before was evidently due to unconscious suppression of this detail.

Kidneys fixed by 21 different methods were examined to assess the role of fixation in this effect. Fixation at 4° C. seemed to be of some value in reducing the numbers seen, but intraluminal nuclei were found whatever the procedure used.

Origin of Intraluminal Nuclei. From examination of chemically fixed kidneys, two significant observations were made. Most important was that in addition to those nuclei frankly within the tubule lumen, certain other nuclei occupied positions at intermediate levels between this and the presumably normal location of the nucleus in the basal portion of the tubule epithelium. Associated with the intermediate nuclei, according to their level, were certain characteristic alterations in the adjacent brush border. Nuclei in the subapical portions of the cells were generally associated with a doming of the overlying brush border; thinning of the brush border as if it were being stretched could also be observed. The apical cytoplasm in such cells would often appear attenuated (Fig. 2). Nuclei at higher levels were within a defect in the brush border, and the intraluminally displaced free edges of this interruption suggested unmistakably that these nuclei, along with more or less cytoplasm, were being ejected into the tubule lumen (Fig. 3). Examination of a large

number of intraluminal nuclei showed that in many cases in the nearby brush border, breaks existed through which the nuclei might have left the epithelial cells (Fig. 4). Re-examination of serial sections of frozen-dried kidneys showed that wherever there was an intraluminal nucleus, there was almost always a corresponding break in the brush border, even though it was frequently not apparent in the same section as that in which the associated nucleus occurred.

Intraluminal Vesicles. The second significant observation made on chemically fixed preparations was that in certain cases large numbers of intraluminal vesicles occurred (Fig. 5). On close inspection it appeared that these vesicles arose through a process related to that by which the nuclei were ejected. With careful searching, examples could be discovered in which the walls of the vesicles were continuous with the free surface of tubule epithelium (Fig. 6).

The characteristic of the tubule epithelium that makes it susceptible to damage of this sort belongs almost exclusively to the convoluted portion of the proximal tubule. The straight portion of the proximal tubule rarely showed a modest extrusion of an isolated sphere of cytoplasm. In these instances the extruded sphere maintained its integrity very precisely and might remain attached through a thin cytoplasmic extension to its point of origin (Fig. 7). Nuclear ejection was not seen in the straight segment, and neither change was encountered in any other segment of the renal tubule.

Kidneys Frozen in Situ. Freeze-dry sections from kidneys frozen while actually functioning showed open tubule lumens, even, intact brush borders, and no intraluminal nuclei or vesicles.

Mechanism of Nuclear Ejection and Vesicle Formation

From considerations noted in the discussion and the foregoing, the hypothesis was formed that nuclear ejection and vesicle formation were consequences of continued postmortem reabsorption of fluid by the epithelium of the proximal tubule. The results in this section were obtained in experiments designed to counter such behavior.

Fixation in Hypertonic Fixatives. Kidneys from untreated animals, fixed in 10 per cent formalin solution made hypertonic with glucose (9 and 18 per cent), showed, in comparison with the same fixative without glucose, a suggestive but not conclusive reduction in the number of intraluminal nuclei. No corresponding effect was noted with fixatives made hypertonic to the same extent with NaCl.

Elevation of Tonicity of Blood. In animals receiving hypertonic solutions of NaCl or glucose intravenously before their kidneys were fixed in solutions similar to those above, full osmotic diuresis was in progress

before the completion of the infusion. In all kidneys from these animals, regardless of the fixative, tubule lumens were found to be widely dilated. Displacement of epithelial nuclei was much reduced, particularly in the salt-infused animals (Fig. 8), but intraluminal nuclei could be found occasionally, and doming of the brush border, sometimes with complete disruption, was commonplace. The nuclei appeared shrunken.

Stop-flow Conditions. In those kidneys fixed whole, nuclei were not seen in the tubule lumens. The quality of fixation varied considerably among the several fixatives, 10 per cent formalin with 5 per cent added NaCl being clearly superior from the point of view of cytologic preservation of the tubule epithelium (Figs. 9 and 10). In the kidneys from which slices were cut for fixation, nuclear displacement remained at a low level, but cytologic preservation was rather poor. In all of these kidneys, unless fixed whole in simple 10 per cent formalin, vesicle formation in the straight segment of the proximal tubule was noteworthy, and in those fixatives with glucose added to 10 per cent, this was also noted in the convoluted part of the proximal tubule. These vesicles were of the "granuloid" variety[2] (Fig. 11). Subsequently, improved but not optimal cytologic preservation was obtained by the use of a 10 per cent formalin, 6 per cent mercuric chloride, 2 per cent sodium acetate fixative.

Discussion

One of the important advantages of the freeze-dry method for microscopic examination is the rapidity with which tissue and cell constituents are immobilized. The appearance of intraluminal nuclei in preparations that should be outstanding for their lifelike preservation constitutes a striking paradox, and raises questions both of how and when they get there.

The element of time has been quite well fixed by the results of the freezing-drying procedures. In kidneys frozen while actually functioning, no intraluminal nuclei were found. This was apparent in lantern slides of such a kidney shown by Swann and his colleagues at the 1958 Federation meetings, and was confirmed by us. In kidneys frozen within 5 to 10 seconds after removal, many were found. This was therefore the interval within which the displaced nuclei proceeded from their normal basal position into the tubule lumen.

In so short an interval it is to be expected that the departure of the nucleus from the cell will be violent. The resemblance of cells such as that in Figure 4 to instantaneous photographs of projectiles piercing armor plate is appropriate, if misleading. Actually, it can be seen from studying a wider range of cells that the disruption of the brush border is quite independent of the movement of the nucleus. The actual se-

quence of events seems to be that the brush border is disrupted by some force acting on it from within the cell and that the cytoplasm then streams out through the resulting gap, carrying the nucleus with it. In addition to nuclear ejection, it appears that vesicle formation might also be a part of this process if the conditions are appropriate for the maintenance of the interface between the naked cytoplasm and the intratubule fluid. It is difficult otherwise to see how nuclei could become enclosed in intraluminal vesicles, as they occasionally do (Fig. 5). It is also apparent that vesicles can arise by a more delicate extrusion of cytoplasm through the brush border not only in the more stable straight segment of the proximal tubule (Fig. 7), but also in the convoluted segment under appropriate conditions (Fig. 11). It should be noted that the lability of the convoluted portion of the proximal tubule and the relative stability of the straight portion reflect again the fundamental differences that have been shown to exist between these segments on a number of other grounds.[9,10]

We have now described some agonal events that take place in the proximal tubular cells, but we have not explained them. In the absence of any other mechanism to account for these events, it seemed possible that the cells were simply swelling and bursting. Since the process is not a pathologic one but occurs in normal kidneys, the mechanism will also necessarily be one normally operating. This suggests the normal resorptive activity of these cells.

How might removal of a kidney interfere with the smooth working of the normal resorptive process in a way likely to produce bursting and discharge of cell contents into the tubular lumen? The factor most obviously and immediately interfered with at the moment of extirpation would be the blood supply. At that moment, according to Swann, Railey and Carmignani,[11] 24 per cent of the volume of the kidney would be comprised by intratubular urine, a considerable portion of which must necessarily be in the proximal tubule. There is no apparent reason that resorption of this intratubular urine should not proceed normally so long as the supply lasts. If the cessation of the flow of blood could in any way interfere with the normal transfer of the resorbate from cell to blood vessels, the effects observed might be accounted for.

Here the possibility has to be considered that resorption may be effected in two ways. Water may move in response to osmotic gradients created by active transport of a solute out of the cell at the vascular pole, or into the cell at the lumen pole. Assuming other transport to be passive, in the first case no problem seems to arise for the cell in maintaining its volume, since the passive entry of the solute at the lumen pole of the cell cannot proceed more rapidly than it is pumped out at the

other. In the second case, however, a solute actively transported into the cell, though it will be free to diffuse through both the cell and the interstitial and vascular spaces, will increase in concentration within the cell, and its accompanying water will cause the cell to swell.

Other sets of plausible if more complicated circumstances contributing to this effect could be proposed, but need not be, since it is sufficient for present purposes to have one reasonable hypothesis on which to base further experiment. It should be noted, however, that sodium, the most plentiful cation in the tubular urine, is generally transported out of cells. In the kidney of Necturus, in fact, Giebisch[12] has presented evidence that the proximal reabsorption of sodium, and hence of water, is by virtue of the active transport of this ion out of the cell at the vascular pole. If the suggested mechanism has any validity, it may be questioned whether sodium reabsorption plays any role in the effect.

Little need be said about the mechanism of extrusion of cytoplasm and nucleus once the brush border is disrupted, since we have no experimental evidence bearing on this. One would expect, however, that elastic contraction of the stretched cell walls, compression of the broken cell by any residual tissue pressure within the tubule, and continued imbibition of water and solutes by the naked cytoplasm by virtue of its protein content might all contribute.

Are our experimental results compatible with the hypothesis proposed? We think they are. Attempts to suppress the disruption of tubule epithelium by raising the tonicity of their environment with hypertonic fixatives or by the intravenous injection of hypertonic solutions were at least partially successful. The difference in effectiveness of glucose and salt under these two conditions is interesting, and is perhaps related to differences in the permeability of cells by, and the diffusibility of, the two solutes. NaCl would be expected to diffuse into a tissue more rapidly, but to enter cells less rapidly than glucose, and therefore to be less effective osmotically in a fixative and more so in an injectate. This agrees with our results.

Under stop-flow conditions, essentially an equilibrium replaces the normal steady state in the urine-cell-blood system of the kidney. No disturbance of the osmotic balance between the 3 compartments is to be expected on interruption of the blood flow. Fixation under these conditions might be expected to occur without any osmotic disruption of tubular cells. The complete suppression of nuclear ejection in such kidneys lends strong support to our hypothesis.

Such conditions impose certain limitations. Unless the kidney is kept intact and the pedicle ligated, fluid movements are permitted and the equilibrium is to some extent upset. Fixation of the deeper parts of the

intact kidney does not occur rapidly, and deterioration of tubule structure unrelated to normal activities of the cells supervenes. Therefore, while the results obtained with stop-flow kidneys are compatible with our hypothesis, further work remains to be done to obtain ideal fixation.

These observations and conclusions need to be considered in relation to recent studies by others. Hanssen[13] has clearly recognized the importance of continued tubular reabsorption in postmortem renal changes. His study, however, is concerned mainly with the resorbed tubule urine as a source of "diluting fluid,"[14] and his methods were such that tubule urine reabsorbed but not returned to the extratubular spaces did not enter his consideration. Although his work was published in 1960, it did not come to our notice until the appearance of the 1962 abstract of Herman and Hanssen,[15] and so did not play any part in the development of the work or ideas presented here.

Novikoff[4] has apparently described and illustrated the nuclear ejection phenomenon as seen with the electron microscope while entertaining entirely different ideas of its nature than we do. His Figures 4, 5, and 7 are the direct counterparts of our Figures 2, 3, and 4. He interpreted these changes as the initial stages in loss of alkaline phosphatase in hydronephrotic kidneys. As we have shown, these changes have no pathologic significance whatsoever, but the question arises why this effect should have been prominent in kidneys in which the experimental procedure was the same that we used to protect them from this type of damage. No certain explanation can be given, but failure to establish or maintain a balance between active resorptive and passive osmotic forces is indicated.

Finally, Stone, Bencosme, Latta and Madden[5] referred to "apical swelling" in their electron microscope study of uranium injury in the kidney of the rat, again an obvious reference to the early stages of the process we have described. These authors suggested that this phenomenon might have a resorptive basis. Since they were not aware of the particular conditions under which it occurred, they could only suppose that, since it had been observed in a number of conditions, it might be "a common labile reaction of the tubule cells." They were thus very close to recognizing it as a normal finding of conventionally prepared tissue sections.

Summary

Disruption of the epithelium in the convoluted segment of the proximal tubule, frequently with ejection of nuclei into the tubule lumen, has been described and identified as an agonal artifact. The effect occurs very rapidly on sudden interruption of the renal blood supply and is

the result to be expected from the continuing accumulation under such conditions of material actively transported into the cell from the intratubular urine. It is of no pathologic significance, nor can it, because of the rapidity with which it takes place, be ascribed to poor fixation in the usual sense.

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[*Illustrations follow*]

Legends for Figures

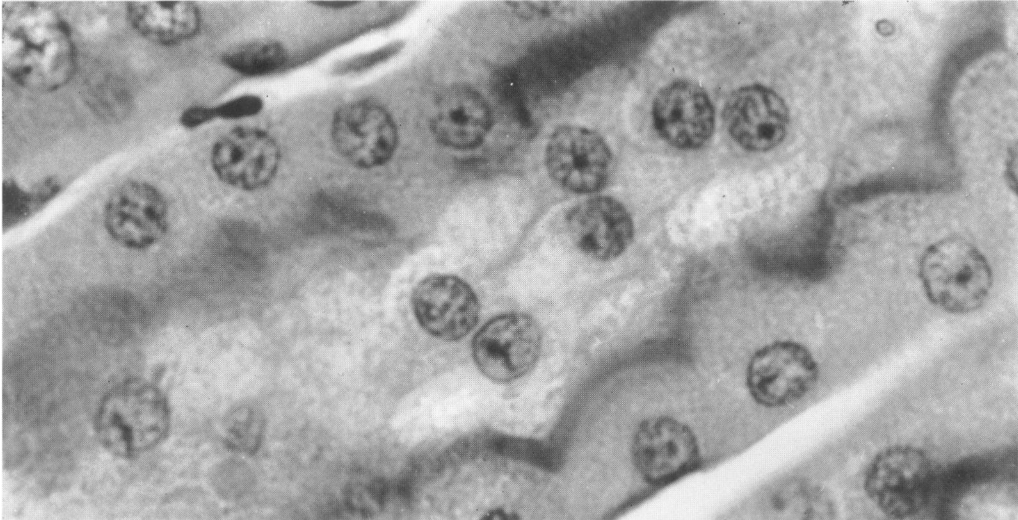
Fig. 1. Intraluminal nuclei in the convoluted portion of a proximal tubule. Frozen-dried kidney of rat. The brush border is demonstrated by alkaline phosphatase activity. Naphthol AS-MX phosphate substrate, red violet LB coupler. Hematoxylin counterstain. × 1,200.

Fig. 2. The apical cytoplasm shows swelling with thinning of the overlying brush border. Convoluted segment, proximal tubule. Fixed in calcium acetate, 2 per cent, in 10 per cent aqueous formalin. Periodic acid-Schiff (PAS) reaction, hematoxylin counterstain. × 1,200.

Fig. 3. Disruption of the brush border over nuclei in 3 cells appears in the convoluted segment of a proximal tubule. Eversion of the edges of the defect is seen over nuclei at the right, discharge of cytoplasm into the lumen is visible in all 3. Some upward displacement of these nuclei is apparent (cf. Fig. 10). Fixed in calcium acetate, 2 per cent in formalin, 10 per cent in absolute alcohol. PAS reaction, hematoxylin counterstain. × 1,200.

Fig. 4. Two intraluminal nuclei, the upper lying next to a defect in the brush border through which it appears to have been ejected. The route of progress of the other nucleus lies out of the plane of section. Convoluted segment of a proximal tubule, from the same section shown in Figure 3. × 1,200.

Fig. 5. Intraluminal vesicles in the convoluted segment, proximal tubule. Note a nucleus slightly to the left of the center, apparently about to be extruded into the lumen. Two vesicles to the right contain nuclei. Schaudinn's mercuric chloride alcohol fixation. PAS reaction, hematoxylin counterstain. × 1,200.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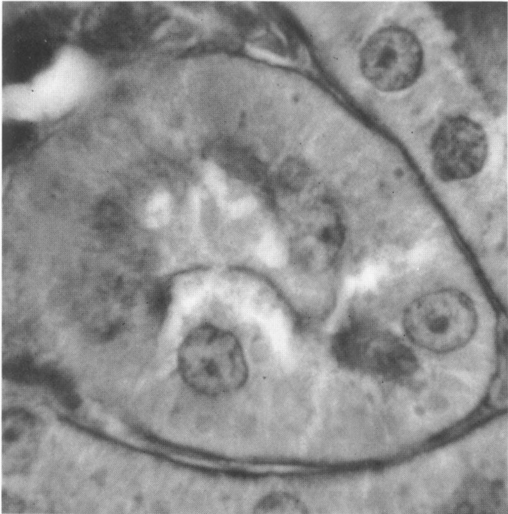
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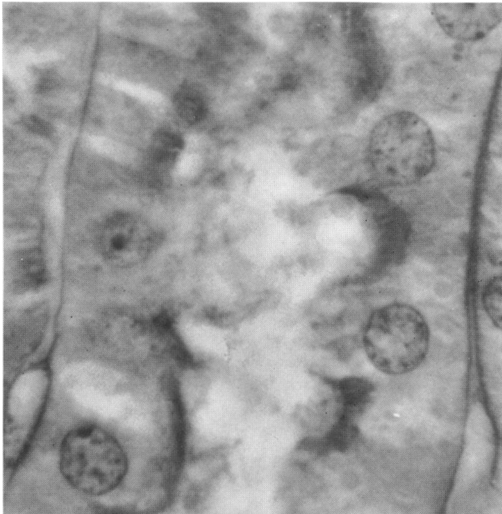
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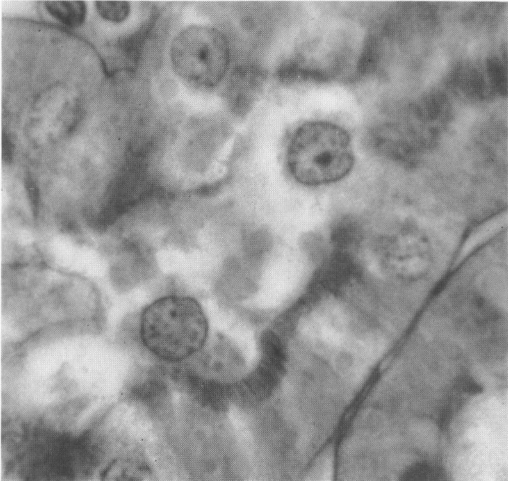
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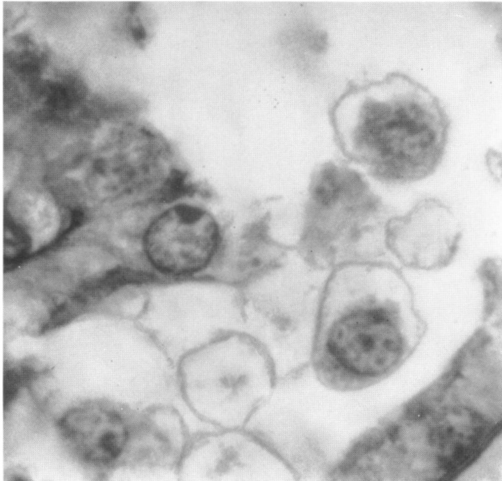
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FIG. 6. Vesicle formation is evident in the convoluted segment of a proximal tubule. Note particularly the disruption of the brush border over a nucleus at the lower right and continuity of everted edges with an intraluminal vesicle. Aqueous acetic mercuric chloride formalin fixation. PAS reaction, hematoxylin counterstain. × 1,200.

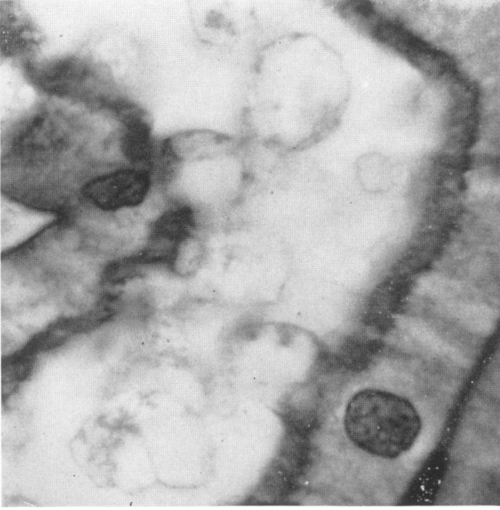
FIG. 7. Extrusion of a granuloid vesicle has occurred into the lumen of a straight segment of the proximal tubule. Disturbance of the brush border is absent, though the integrity of the convoluted segments of proximal tubules in this same section is poor. Nuclei lie in close contact with the basement membrane. Bouin fixation. PAS reaction, hematoxylin counterstain. × 1,200.

FIG. 8. Kidney of a rat infused with 30 cc. of 3 per cent NaCl for 10 minutes preceding death, fixed in NaCl, 1.5 per cent, in unbuffered 10 per cent formalin. Nuclei of proximal tubules are largely in place and the lumens are clear, though brush borders present a disordered appearance. A nucleus nearly through the brush border is seen to the right of the center at the lower edge of the figure. PAS reaction, hematoxylin counterstain. × 250.

FIG. 9. Kidney of a rat fixed whole in NaCl, 5 per cent, in 10 per cent formalin, renal pedicle ligated after 6 minutes of ureteral ligation during osmotic diuresis. Contours of tubules as preserved are well rounded and, in the proximal tubule, show minimal signs of disturbance of the brush border. Nuclei of the proximal tubules tend to lie close to the basement membrane. Hematoxylin and eosin stain. × 250.

FIG. 10. Convoluted segment of the proximal tubule, same kidney shown in Figure 9. Brush border exhibits some irregularity but is excellently preserved by comparison with Figures 1 to 6. Nuclei are essentially in place and the lumen is clear. PAS reaction, hematoxylin counterstain. × 1,000.

FIG. 11. "Granuloid" vesicles lie in the convoluted segment of a proximal tubule. Stop-flow rat kidney, fixed whole in glucose, 10 per cent, in 10 per cent formalin. Nuclei remain in place. Hematoxylin and eosin stain. × 500.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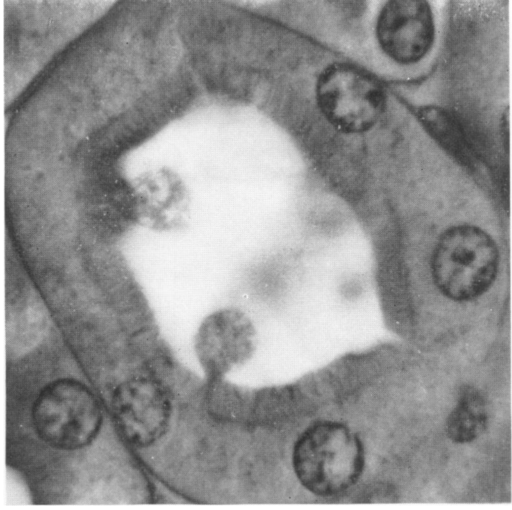
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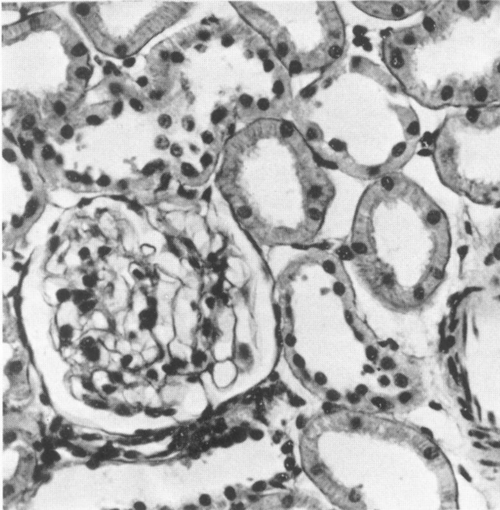
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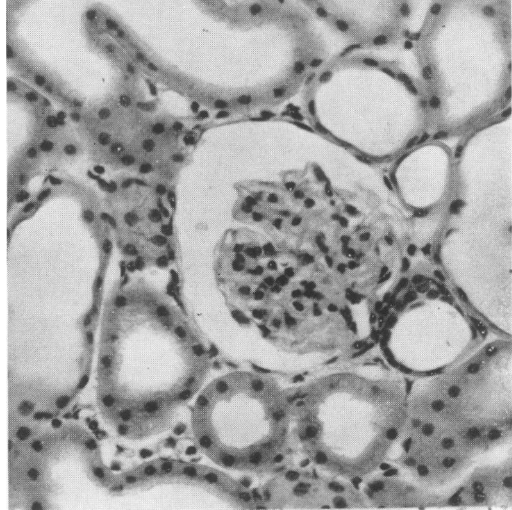
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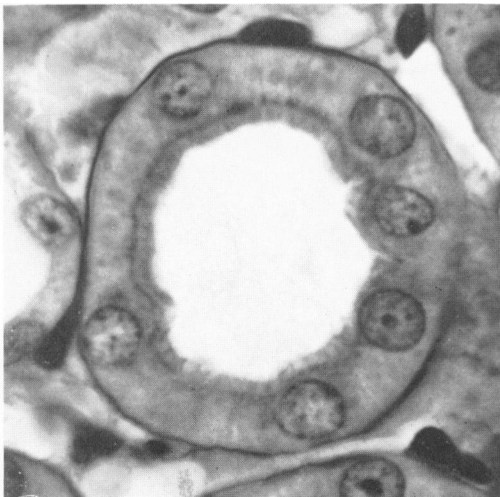
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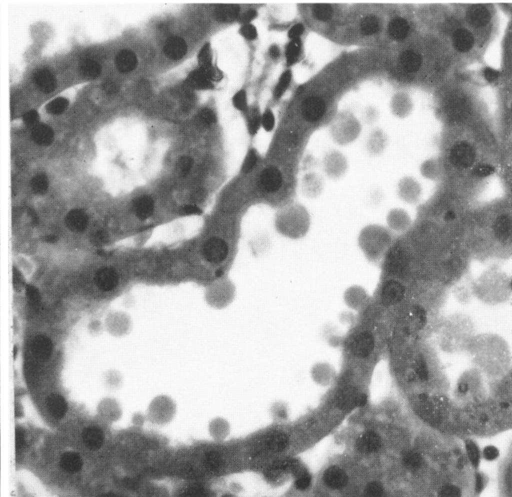
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