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Tubular leakage and obstruction after renal ischemia: Structural-functional correlations

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Tubular leakage and obstruction after renal ischemia: Structural-functional correlations. These ischemic sequelae were evaluated in rats following relief of 60, 25, and 15 min of renal artery occlusion. Light/electron microscopy after 60 min's ischemia showed necrosis of isolated cells in both proximal convoluted (PCT) and proximal straight tubules (PST); necrosis was present only in PST with 25 min's ischemia. Thirty-five percent of ^{14}C-inulin microinjected into ischemic PCT was recovered in contralateral urine after 60 min of ischemia; 11% was recovered after 25 min. Backleak was insignificant after 15 min of ischemia. Horseradish peroxidase (mol wt, 40,000), injected into PCT passed through the cytoplasm of tubular cells into the interstitium. Intravenous administration of peroxidase demonstrated that loss of selective permeability of tubular cells to large molecules was not an artefact of tubular injection techniques. Intrarenal inulin sequestration was used as an approximate index of obstruction. After 60 min of ischemia, 28% of microinjected inulin was not recovered in urine from either kidney; after 25 min of ischemia, non-recovery was 12%. About three-quarters of this missing inulin was recovered from the ischemic kidney itself. Recovery in urine was substantially complete in control and 15 min's-ischemic animals. Impacted swollen blebs of brush border were seen in > 75% of PST following 60 min of ischemia, whereas this phenomenon was much less frequent and evanescent after 25 min. We conclude that increasing duration of renal ischemia causes incremental tubular leakiness to large molecules, which diffuse through damaged cells. After 60 min of ischemia, most PST appear filled with impacted brush border, which may cause obstruction.

Défaut d'étanchéité tubulaire et obstruction après ischémie rénale: Corrélations structuro-fonctionnelles. Les séquelles de l'ischémie ont été évaluées chez le rat après la levée, à la 60ème, 25ème ou 15ème minutes d'une occlusion de l'artère rénale. La microscopie photonique et électronique après 60 minutes d'ischémie montre des nécroses de cellules isolées à la fois dans les tubes contournés proximaux (PCT) et dans les tubes droits proximaux (PST); la nécrose n'apparaît que dans PST après 25 minutes d'ischémie. 35% de l'inuline-^{14}C injectée dans PCT est récupérée dans l'urine controlatérale après 60 minutes d'ischémie, 11% après 25 minutes. Cette quantité est négligeable après 15 minutes d'ischémie. La peroxydase de raifort (PM 40.000), injectée soit dans PCT soit par voie intraveineuse, passe à travers le cytoplasme des cellules tubulaires vers l'interstitium. L'administration par voie intraveineuse montre que la fuite tubulaire des grosses molécules n'est pas un artefact lié à l'injection tubulaire. La séquestration d'inuline dans le rein a été utilisée comme un index approximatif de l'obstruction. Après 60 minutes d'ischémie, 28% de l'inuline n'est pas récupérée dans les urines des deux reins; après 25 minutes d'ischémie cette valeur est de 12%. Les trois quarts environ de l'inuline manquante sont trouvés dans le rein lui-même. La récupération de l'inuline est pratiquement totale chez les animaux contrôles ou après 15 minutes d'ischémie. Des fragments boursouffiés de bordure en brosse sont encastrés dans 75% des PST après 60 minutes d'ischémie alors que cet aspect est beaucoup plus rare après 25 minutes d'ischémie. Nous concluons que l'allongement de la durée de l'ischémie rénale détermine une augmentation de la fuite des grosses molécules à travers les cellules lésées de la paroi tubulaire. Après 60 minutes d'ischémie, la plupart des PST sont remplis de fragments encastrés de bordure en brosse, ce qui détermine l'obstruction.

An extensive literature exists concerning experimental acute renal failure. Many types of insult have been used to produce renal damage, and perhaps this is one reason why unanimity of opinion concerning the pathogenesis and maintenance of renal failure has yet to emerge [1]. Arendshorst, Finn, and Gottschalk [2] have formulated a comprehensive schema to explain the reduced function which follows one hour of complete renal arterial occlusion. These authors assign primacy to proximal tubular obstruction in the pathophysiology of acute renal failure, with tubular leakage of filtrate and intrinsic changes in glomerular hemodynamics playing secondary roles. The presence of extensive tubular obstruction by intraluminal casts in this model has been convincingly demonstrated by Tanner and Steinhausen [3]. We have used a combined morphological and functional approach to evaluate the sites of tubular leakage of filtrate and to try to determine the nature of the obstructing material after ischemia. We found evidence of tubular leakiness to large molecules which correlated with specific, localized lesions in damaged epithelium. The casts seen in proximal straight tubules appear to be composed of swollen blebs of brush border lost from tubular epithelium. Impaction of this material may cause the obstruction described by others [2, 3] as a key feature of the model. In support of this concept, we have found that some of the inulin microinjected into a tubule remains sequestered in the ischemic kidney.

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Methods

Functional studies. Experiments were performed in male Sprague-Dawley rats (200 to 300 g) maintained on a standard rat chow diet and allowed water *ad lib*. They were anesthetized with Inactin (100 mg/kg of body wt, i.p.). Rats were placed on a thermostatically-controlled, heated micropuncture table, a tracheostomy was performed, and the left internal juglar vein was cannulated. The left kidney was exposed through a flank incision and mounted in a Lucite holder. The renal capsule was gently removed, and the kidney surface was bathed by constant dripping of light mineral oil at 37° C. The left ureter was cannulated with PE-50 tubing as was the urinary bladder. During surgery, the rat was given a constant infusion of 5% mannitol in water at 0.05 ml/min to promote a modest diuresis. At the start of ischemia, the infusion was changed to isotonic saline at 0.028 ml/min. Surgical fluid losses were not replaced. After preparative surgery and prior to ischemia, proximal transit time was measured with lissamine green (0.05 ml of a 5% solution). Rats whose transit time exceeded 12 sec were discarded.

The technique of microinjection was similar to that described by others [4–7]. Using a constant volume quartz pipette, identical samples, approximately 7 nl in volume, of isotonic saline containing ^{14}C-inulin (colored by the addition of FD&C [no. 3] dye) were transferred to a Petri dish under oil. One or two of these droplets were then transferred by micropipette directly into a scintillation vial containing a control urine collection and were used as 100% reference standards for the ensuing microinjection. Another droplet, to be used for the microinjection, was drawn into a micropipette which already contained a small quantity of water-equilibrated mineral oil. Care was taken that all of the droplet was aspirated into the micropipette, after which a small drop of mineral oil was drawn into the tip. Tip diameters of micropipettes were 10 to 12μ for proximal, and 7 to 9μ for distal tubular microinjections. An identical microinjection technique was followed for control and ischemia-damaged nephrons. Random late proximal and distal tubular convolutions were selected by observation of lissamine green dye transit. The pipette was slowly advanced into the tubular lumen. By gentle suction, tubular fluid was aspirated back into the micropipette. The small oil droplet at the tip was seen to float up through the isotope solution to coalesce with the mineral oil behind the injectate. Gentle manual pressure was then applied to the system so that the colored injectate was seen to enter the lumen of the selected nephron. Injections in which the blue colored injectate leaked around the injection site, where there was retrograde injectate flow or where oil entered the lumen, were discarded. Each microinjection lasted for a minimum of 3 min. Urine was collected from each kidney in the control period preceding microinjection and for 40 to 60 min after the injection and was put directly into scintillation vials containing 10 ml of Aquasol (New England Nuclear, Boston, Mass.). Before terminating a collection, a 5- to 10-min urine sample was collected to insure that urine counts had returned to background levels.

Radioactivity was measured on a Beckman Scintillation Counter. Net counts for each sample were derived by substracting as background the radioactivity of a control urine sample. Counts which appeared on the noninjected (right) side were expressed as a percentage of total counts given and were used as a minimum estimate to tubular backleak. Counts which failed to appear on either side (non-recovered counts) were expressed as a percentage of total counts given and were used as an approximate index of intrarenal obstruction.

The following groups were studied. *1) Control:* In 17 rats, 20 proximal microinjections were performed, and in 4 rats, 12 distal microinjections were made. *2) Complete ischemia:* The left renal artery was carefully dissected away from the renal vein and then selectively occluded, for periods of 15 min (4 rats/8 proximal microinjections), 25 min (17 rats/20 proximal microinjections), and 60 min (22 rats/26 proximal and 8 rats/15 distal microinjections). Occlusion was produced by a small modified Blalock clamp, the jaws of which were covered by polyethylene tubing. The kidney surface was observed to ensure complete blanching, and occasional microscopic checks were made to confirm the absence of red blood cell movement in the capillaries. Microinjections were performed after one hour of blood reflow. Repeat microinjections were made only if interim urine radioactivity had returned to background. Less than 10% of rats subjected to 60 min of ischemia became anuric and were not studied. In post-ischemic kidneys, tubules were selected for microinjection randomly; no conscious attempt was made to select nephrons according to apparent degree of injury.

An additional series of experiments was done to try to account for nonrecovered isotope. Eight rats underwent 60 min of complete left renal ischemia with intratubular microinjection of ^{14}C-inulin, as described in detail above. Urine collections were obtained in the standard fashion and nonrecovered counts were calculated as described. Both kidneys were then excised, and the radioactivity of each was

determined. Kidneys were individually dissolved in Protosol (New England Nuclear, Boston, Mass.) at 37° C for 7 to 10 days, after which 10 ml of Aquasol were added to yield a clear solution for counting. A background correction was determined from 12 kidneys of 6 normal rats; each kidney was dissolved and counted separately, and a mean correction was calculated. In addition, recovery experiments were done in which varying known amounts of ^{14}C-inulin were added to control kidneys at the outset of the dissolution to determine quenching of isotope by kidney tissue.

Morphologic studies. Both kidneys from rats subjected to left renal artery occlusion for 60, 25, or 15 min were studied by light and electron microscopy after intravascular perfusion of fixative. In the 60-min ischemia group, 6 rats each were examined after 30 min and 2 hr of blood reflow, and 2 rats after 24 hr of reflow. In the 25-min ischemia group, 6 rats were examined at 30 min, 3 at 2 hr, and 4 rats at 24 hr after release of occlusion. In the 15-min-ischemia group, 2 rats were studied at 30 min and 2 rats at 24 hr following renal ischemia. The perfusion-fixation procedure was modified after Griffith, Bulger, and Trump [8]. 1.25% glutaraldehyde fixative in 0.1 M sodium cacodylate buffer (325 mOsm/kg, pH 7.4) was perfused at 120 mm of Hg for 10 min through an aortic PE-160 polyethylene cannula immediately following aortic cross clamping above the renal arteries. Following perfusion fixation, the kidneys were immersed in fixative for 3 hr. Samples of renal tissue were processed for embedding in paraffin as well as in Epon 812 after postfixation in osmium tetroxide for 90 min. Paraffin sections were stained with hematoxylin-eosin. Epon sections (1- to 2-μ thick) were stained with 1% aqueous toluidine blue in 1% borax for light microscopy. Thin sections, 500 to 1,000 Å, were stained with uranyl acetate and lead citrate and were viewed in a Phillips 201 electron microscope.

Tracer experiments with horseradish peroxidase. To further study tubular integrity, we used horseradish peroxidase (HRP) of 40,000 daltons mol wt (Einstein-Stokes radius, a_e = 30Å), a probe molecule that can be cytochemically demonstrated in cells or tissues by light and electron microscopy [9]. HRP (Type II, Sigma Chemical Company, St. Louis, Mo.) was dissolved in normal saline (1 mg/ml), and 7 nl of this solution was microinjected over a 3- to 5-min period into both control (N = 3) and 60-min ischemia-damaged nephrons (N = 6), exactly as described for ^{14}C-inulin above. The pipette was then changed to one containing 1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer (325 mOsm/kg, pH 7.4, colored with lissamine green), which was then gently and continuously microperfused manually for 10 min. Thereafter, the kidney was excised and trimmed to a small block of tissue containing the perfused nephron. This block was immersed in the same fixative for 3 more hours. After an overnight rinse in 0.1 M sodium cacodylate buffer, pH 7.4, at 4° C, 40- to 60-μ sections of the fixed tissue were cut with a tissue chopper (Smith-Farquhar TC-2, Ivan Sorvall, Inc., Norwalk, Conn.), washed in 0.05 M tris-hydrochloric acid buffer (pH, 7.56), and reacted for 30 min at room temperature in a medium containing 5 mg of 3,3′ diaminobenzidine tetrahydrochloride, 10 ml of 0.05 M tris-hydrochloric acid buffer (pH, 7.56), and 0.01% hydrogen peroxide [9]. Following incubation, the sections were rinsed thrice in buffer, postfixed in 1% aqueous osmium tetroxide for 90 min, dehydrated in graded alcohols, and embedded in Epon 812. By light microscopy of 2-μ Epon sections, peroxidase was visualized as a brown reaction product. By electron microscopy, it was seen as a black, electron dense material.

To circumvent the criticism that microinjection *per se,* by raising intratubular pressure, may artefactually cause transepithelial leakage of tracer molecules, the following experiments were performed. Horseradish peroxidase in normal saline (0.8–1 ml of 3 mg/ml of solution) was injected i.v. (1 ml HRP/100 g of body wt) into 3 rats 1 to 2 hr after 60 min of left renal ischemia and into 2 rats 1 to 2 hr after 25 min of ischemia. A control rat was similarly injected. Within 5 min of injection, kidneys were fixed by intravascular perfusion of 1.25% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH, 7.4) for 10 min. Cortical slices of excised kidneys were immersed for 3 more hours in the same fixative, washed overnight in cold 0.1 M sodium cacodylate buffer, and reacted for peroxidase as described above.

Statistical analyses. Results are given as the mean ± 1 SEM, with the number of observations given in parentheses. Statistical comparisons were made by unpaired Student's t tests, with significance assigned to a P value of less than 0.05.

Results

After release of 15 or 25 min of arterial occlusion, the left kidney surface promptly regained a normal appearance. Within 1 to 2 min of blood reflow, the impression was one of reactive hyperemia. All tubules visualized were normal in appearance and diameter. After 25 min of ischemia, urine flow was 10.2 ± 4.6 μl/min from the experimental kidney and 12.9 ± 3.9 μl/min from the control kidney (NS).

Following 60 min of ischemia, the left kidney was slow to recover a normal appearance and did so in a

patchy fashion. Within 5–10 min, however, surface tubules showed homogeneity of diameter, which appeared increased. Tubules appeared more translucent than normal. Urine flow was 9.0 ± 0.4 μl/min from the experimental kidney, and 30.7 ± 5.7 μl/min from the control side ($P < 0.01$).

Functional studies. Data on backleak of inulin are summarized in Figure 1 and Table 1. Urinary inulin recoveries from the microinjected side are also presented in Table 1. After microinjection into tubules of control animals, insignificant numbers of counts were recovered in urine from the contralateral kidney, ipsilateral recovery being virtually complete. After 15, 25, and 60 min of ischemia, increasing fractions of counts injected into proximal tubules were recovered from urine of the contralateral kidney. Mean backleak at 25 and 60 min were significantly different from each other and from control ($P < 0.001$ for each comparison). Mean backleak at 15 min was not significantly different from control or 25-min ischemia. Distal injections were performed only in controls and after 60 min of ischemia. Backleak from distal injections was significantly less than from proximal injections in the ischemic group ($P < 0.01$).

Not all counts administered in the ischemic kidneys were recovered during the one-hour urine collection following microinjection, even though in urine collected during the subsequent 5 to 10 min, counts had invariably returned to background. Values for nonrecovered counts in all groups are given in Figure 2 and Table 1. The fraction of counts not recovered in urine increased with longer ischemic periods. The percentages of nonrecoveries at 25 and 60 min were significantly different from each other and from control ($P < 0.01$ for each comparison). Mean nonrecovery at 15 min was not different from

Table 1. Mean values for radioactivity recovered following ^{14}C-inulin microinjection from urine of contralateral kidney (backlead, %), ipsilateral kidney, and for isotope which failed to appear in either urine (non-recovery, %) (comparisons not connected by arrows are not significantly different)

	Proximal microinjections			Distal microinjections	
Control ($N = 20$)	15 min ($N = 8$)	25 min ($N = 20$)	60 min ($N = 26$)	Control ($N = 12$)	60 min ($N = 15$)
Contralateral recovery (backleak), %					
0.9 ± 0.4	2.8 ± 2.2	10.8 ± 2.3	34.9 ± 5.5	0.8 ± 0.8	9.0 ± 2.5
	← $P < 0.001$ →	← →	→	← $P < 0.01$ →	
		← $P < 0.01$ →		← $P < 0.01$ →	
Ipsilateral recovery, %					
97.6 ± 1.3	93.0 ± 4.7	78.6 ± 4.7	38.5 ± 7.0	94.3 ± 2.1	85.7 ± 5.7
	← $P < 0.001$ →	← $P < 0.001$ →	← $P < 0.001$ →		
		← $P < .001$ →			
Nonrecovery, %					
2.8 ± 1.1	7.8 ± 4.3	11.6 ± 2.7	27.5 ± 4.5	5.3 ± 1.8	8.0 ± 3.3
	← $P < 0.01$ →	← $P < 0.01$ →	← $P < 0.01$ →		
		← $P < 0.05$ →			

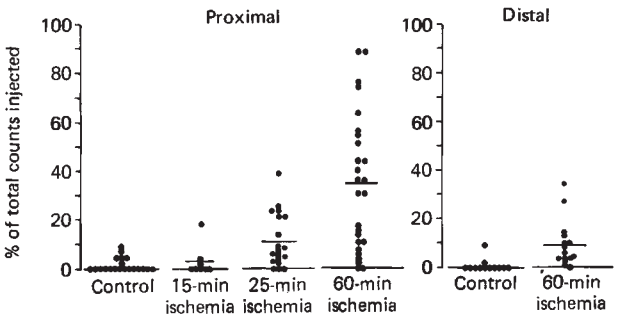


Fig. 1. *Percent recovery of radioactivity in urine from contralateral kidney after injection of ^{14}C-inulin into proximal and distal tubules of control or ischemic kidneys ("backleak").*

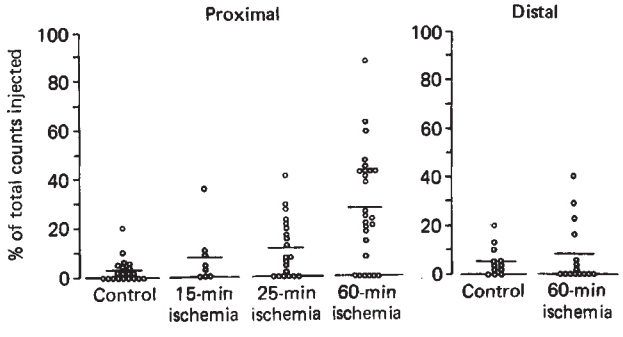


Fig. 2. *Percent nonrecovery of radioactivity in urine from either kidney after injection of ^{14}C-inulin into proximal and distal tubules of control or ischemic kidneys.*

control or 25-min ischemia. Nonrecovery after distal injection was significantly less than with proximal injection ($P < 0.01$) following ischemia.

Missing counts were sought in kidney tissue of 8 rats, using techniques described in the section on Methods. Digests of contralateral, noninjected kidneys all counted at background rates. The ischemic kidney contained 72.6 ± 13.6% of the "missing" counts which had not appeared in the urine from either kidney in these rats.

Morphological studies. By light and electron microscopy, all right kidneys exhibited normal features. Proximal convoluted tubules are illustrated in Figures 3a, 3c, 5a; proximal straight tubules are shown in Figure 3e. Fig. 7a shows outer medullary collecting ducts, ascending thick limbs and descending thin limbs of Henle.

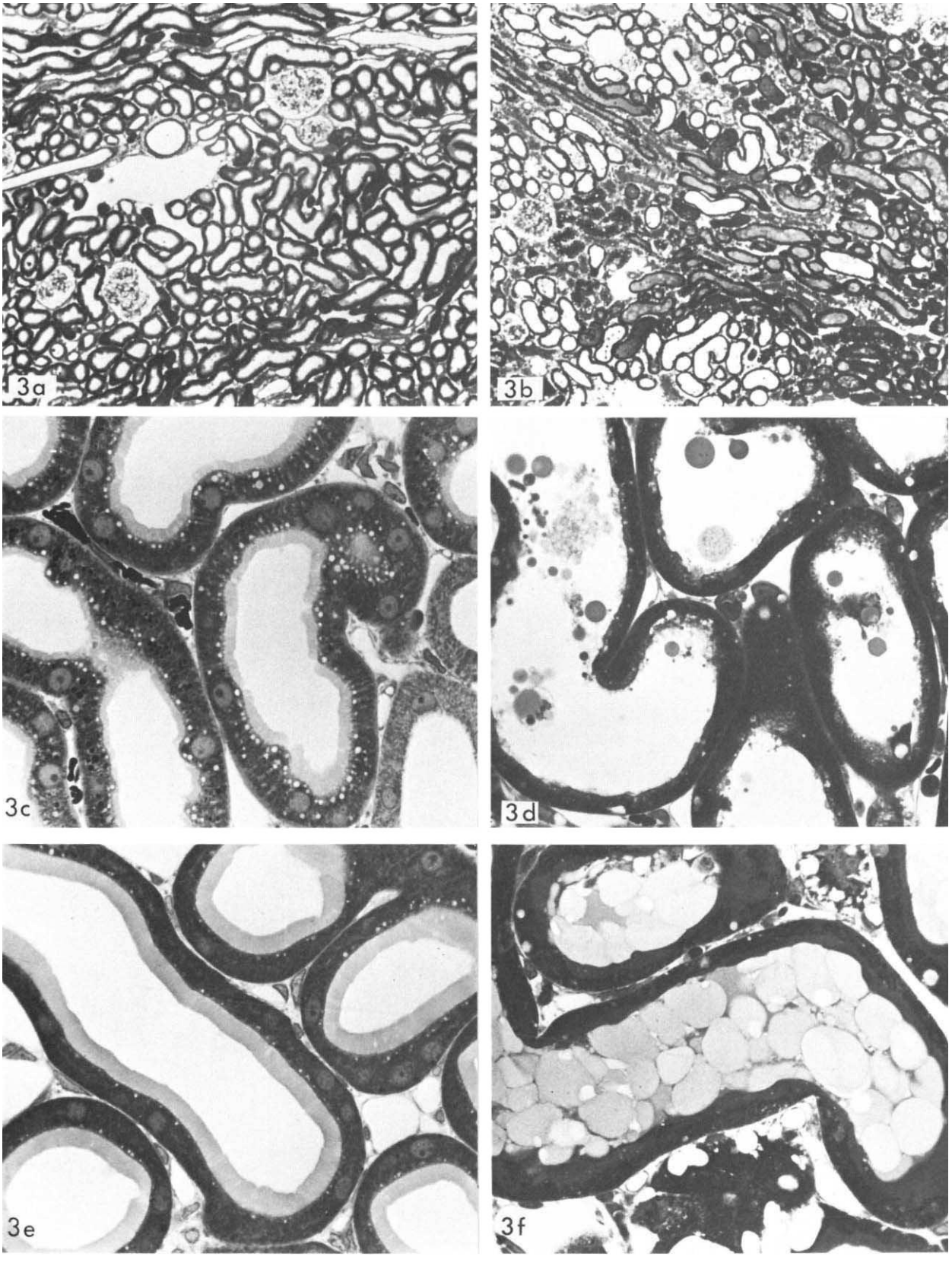
Proximal convoluted tubules are present only in the cortex; straight segments occur in the outer stripe of the outer medulla, as well as in the medullary rays of the cortex. The pathological changes in proximal tubules described below will be in reference to this anatomic segmentation only and not the more precise divisions characterized by distinctive cell types [10]. Documentation of differential proximal tubule (PT) cell response to a 25-min ischemic injury with respect to cell type reported previously in abstract form [11] will be the subject of a separate communication.

60-min ischemia. At 30 min and 2 hr after reflow of blood, left kidneys showed alterations indicative of tubular obstruction, as well as disruption of epithelial integrity. At 30 min, almost all proximal straight tubules were occluded by faintly staining, foamy material, whereas the bulk of proximal convoluted tubules had open dilated lumina (Fig. 3b). At higher magnification, proximal convoluted tubules were largely devoid of an intact brush border and their lumina contained numerous, dense-staining free-floating spherical bodies, and clumps of amorphous debris (Fig. 3d). Such alterations of brush border morphology were present in proximal straight tubules also, but their lumina were densely packed with spherical or ovoid blebs (Fig. 3f), much larger in size than those encountered floating free in convoluted segments.

Electron microscopy revealed that pathological changes in brush border microvilli were responsible for the formation of both free-floating and impacted blebs in PT lumina (Fig. 4, a, b, and c). There was extensive effacement of microvilli, due to disintegration and shedding of brush border fragments into tubular lumina (Fig. 4b), as well as incorporation of microvilli into the luminal cytoplasm of PT cells. Incorporated brush border membranes could be visualized in PT cytoplasm in the form of a labyrinthine system of vesicles and membranous formations (Figs. 4, b, and c). Extrusion of cytoplasmic material into PT lumina in the form of spherical or ovoid droplets was sometimes observed (Fig. 4a). Impacted material in the lumina of straight PT consisted of numerous plasma membrane-bound blebs (Fig. 4c). The cytoplasmic origin of these blebs was revealed by finding within them ribosomes, and rarely, within either cytoplasmic organelles such as rough endoplasmic reticulum or mitochondria (not illustrated). This type of nephron obstruction was present at both 30 min and at 2 hr following blood reflow; 24 hr later, impacted membrane blebs in straight PT had disappeared, presumably by breakdown into amorphous debris. At this latter time period, thin limbs of Henle, distal tubules, and collecting ducts contained densely staining amorphous and hyaline casts, as reported previously by Tanner and Sophasan [12].

The cellular manifestations of ischemic injury were in general less severe in convoluted than straight PT. Changes other than in the brush border included cytoplasmic and nuclear condensation, vesiculation of the apical cytoplasm, disarray of organelles, and, in cells showing evidence of irreversible injury, striking high amplitude swelling and distortion of mitochondria with amorphous densities in the matrix (Fig. 5b). These morphological changes of both reversible and irreversible PT cell injury have been previously described [13–15] and were less severe at 30 min than at 2 hr after blood reflow.

Fig. 3. *Light micrographs from toluidine blue-stained 2-μ thick Epon sections of control right and ischemic left kidneys from rats subjected to one hour of left renal ischemia and 30 min of blood reflow after the ischemic period (tissue fixed by intravascular perfusion).* **Panel a** is deep cortex and part of outer stripe of outer medulla from control right kidney showing convoluted proximal tubules (PT) to the left, and straight PT at the top and bottom in medullary rays and in the outer stripe towards the right. All PT show open lumina. (Magnification, × 50). **Panel b** is the corresponding area to that shown in panel a from ischemic left kidney. Almost all straight PT in the medullary rays and in the outer stripe are obstructed by lightly stained foamy material. The lumina of convoluted PT are dilated compared to panel a (×50). **Panel c** is convoluted PT from control right kidney, showing open lumina and prominent brush border (×600). **Panel d** is convoluted PT from ischemic left kidney showing dilated lumina, thinning of epithelium and striking loss of the brush border. The tubular lumina contain amorphous debris as well as many spherical densely staining blebs of different sizes (×600). **Panel e** is straight PT from control right kidney, showing open lumina and prominent brush border (×600). **Panel f** is straight PT from ischemic left kidney, showing luminal impaction by numerous spherical and ovoid blebs. The blebs are much larger than those present free-floating in convoluted PT lumina in panel d (×600).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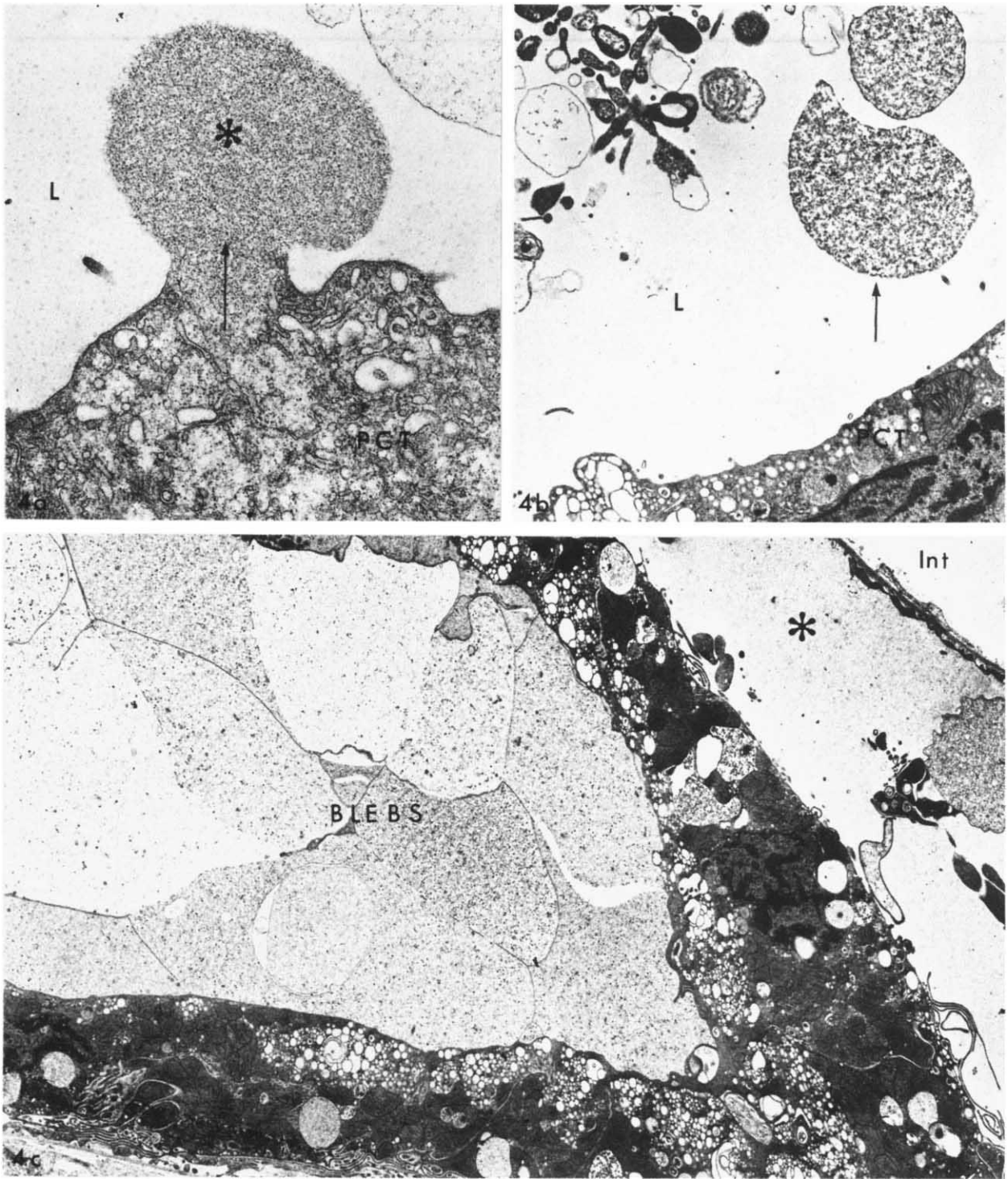


Fig. 4. *Electron micrographs of proximal tubules from ischemic left kidneys obtained within 30 min of blood reflow.* Tissue was fixed by intravascular perfusion of glutaraldehyde. Secondary fixation was in osmium tetroxide. Tissue was stained with uranyl acetate and lead citrate. **Panel a** is cytoplasm of a proximal convoluted tubule (*PCT*) cell, seen being extruded (*arrow*) into the tubular lumen (*L*) in the form of a spherical mass (*asterisk*). The cytoplasmic mass appears not to be bound by a plasma membrane. How a limiting membrane is acquired to form a bleb is not known. Brush border microvilli are denuded. At top right is shown a portion of a luminal bleb (×19,000). **Panel b** is proximal convoluted tubule (*PCT*), showing total denudation of brush border microvilli. The lumen (*L*) contains fragmented brush border microvilli at the top left and swollen membrane bound blebs (*arrow*). The apical cytoplasm of the cell contains vesicular membrane profiles derived from interiorized brush border microvilli (×8000). **Panel c** is proximal straight tubule, showing luminal impaction by numerous membrane-bound *blebs*. Brush border microvilli are absent. Apical cytoplasm of epithelial cells contains vesicular membrane profiles derived from interiorized brush border microvilli. Towards the right the epithelium is sheared away from the underlying tubular basement membrane. The intervening space (*asterisk*) is occupied by granular material as well as irregular cytoplasmic fragments. *Int* denotes interstitium (×4,000).

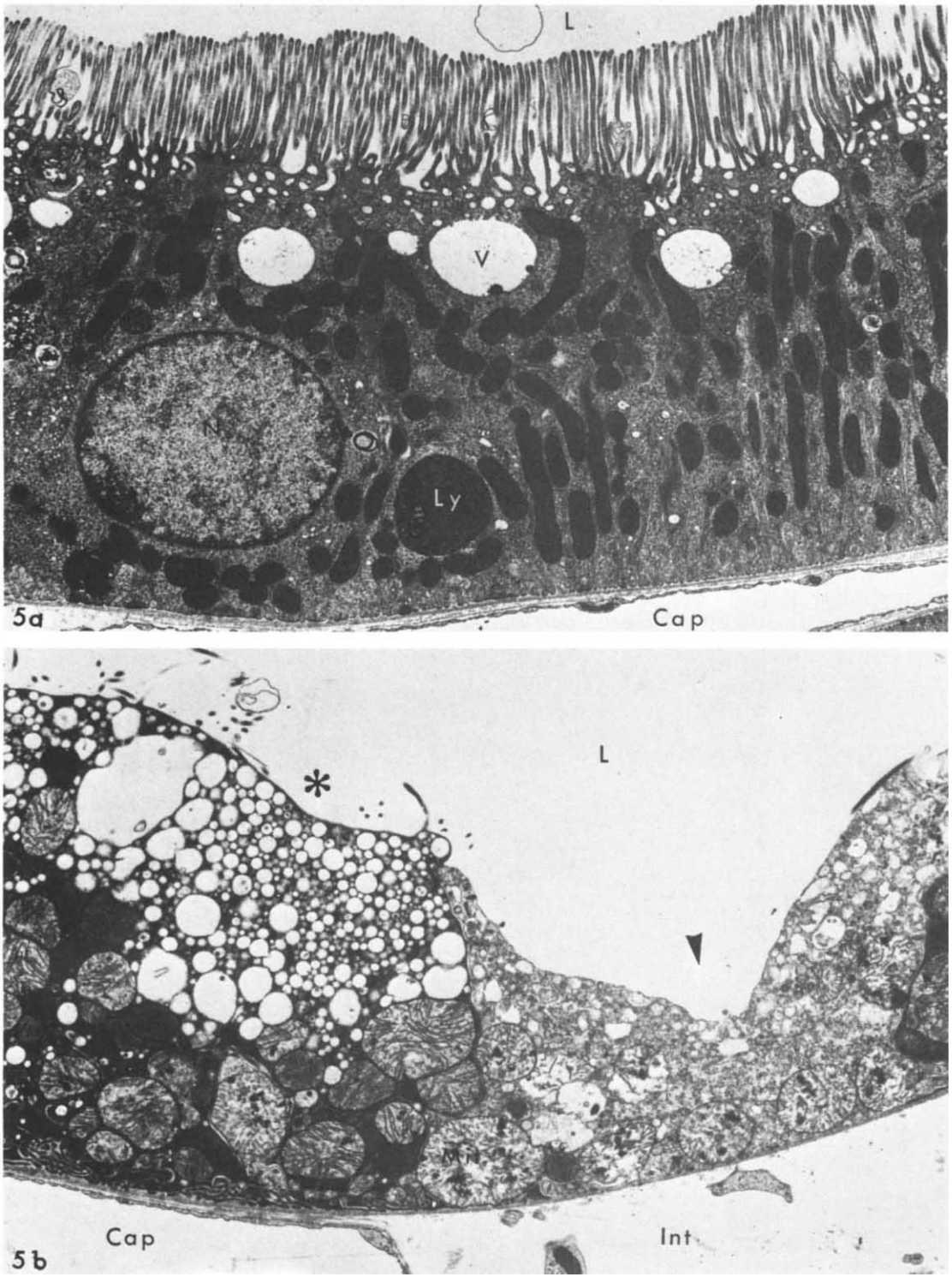


Fig. 5. *Electron micrograph of a proximal convoluted tubule.* **Panel a** is from a control right kidney. The ultrastructural appearance is normal, with intact brush border microvilli, nucleus (*N*), and cytoplasmic organelles. *Ly* denotes lysosome; *V* denotes reabsorption vacuole; *L* denotes lumen; and *Cap* denotes capillary. Fixation was by intravascular perfusion. Secondary fixation was in osmium tetroxide. Tissue was stained with uranyl acetate and lead citrate (×8,000). **Panel b** is from left kidney subjected to one hour of ischemia and two hours of blood reflow. The epithelial cell shown to the left (*asterisk*) is in stage 3 of irreversible cell injury. Note the high amplitude swelling of mitochondria with dense material in their matrix and numerous apical cytoplasmic vacuoles, derived from interiorized brush border microvilli. The cell towards the right (*arrowhead*) is in stage 4 of irreversible injury with complete dissolution of cellular architecture, focal disruption of plasma membranes, and marked mitochondrial swelling (*Mit*) with matrical densities. *Cap* denotes capillary; *Int* denotes interstitium; and *L* denotes lumen. Fixation was by intravascular perfusion. Secondary fixation was in osmium tetroxide. Tissue was stained with uranyl acetate and lead citrate (×8,000).

Occasional isolated cells in both PT segments, however, showed signs of obvious necrosis even after 30 min of reflow, including dissolution of organelles, plasma membrane rupture and disintegration, lack of affinity for stains and karyolysis, designated as stage 4 of irreversible injury by Reimer, Ganote, and Jennings [13]. After 2 hr, the number of frankly necrotic cells was still small, compared to the majority which could be characterized as showing either reversible damage or stages 2 and 3 irreversible cellular injury. The ultrastructure of stage 3 and 4 necrotic epithelium is illustrated in Fig. 5b (compared to control PT cell in Fig. 5a). Stage 4 necrotic, convoluted PT cells at the 2-hr period are also illustrated in Fig. 8a by light microscopy. Exfoliation of necrotic cells into PT lumina was also observed, with consequent exposure of denuded epithelial basement membranes to the tubular luminal fluid (Fig. 6).

Ischemia-induced degenerative changes were observed also in other segments of the nephron (Fig. 7b). Thus, thin and thick limbs of Henle and collecting ducts showed variable degrees of mitochondrial swelling as well as cytoplasmic swelling and vacuolization. Milder, but qualitatively similar changes, were seen in distal tubules also.

Twenty-four hours following the ischemic insult, all straight PT and at least 50% of profiles of convoluted PT exhibited epithelial necrosis. Ascending thick limbs of Henle also showed focal necrosis, but distal tubules and collecting tubules, though obstructed by casts, appeared viable.

25-min ischemia. During the early reflow periods

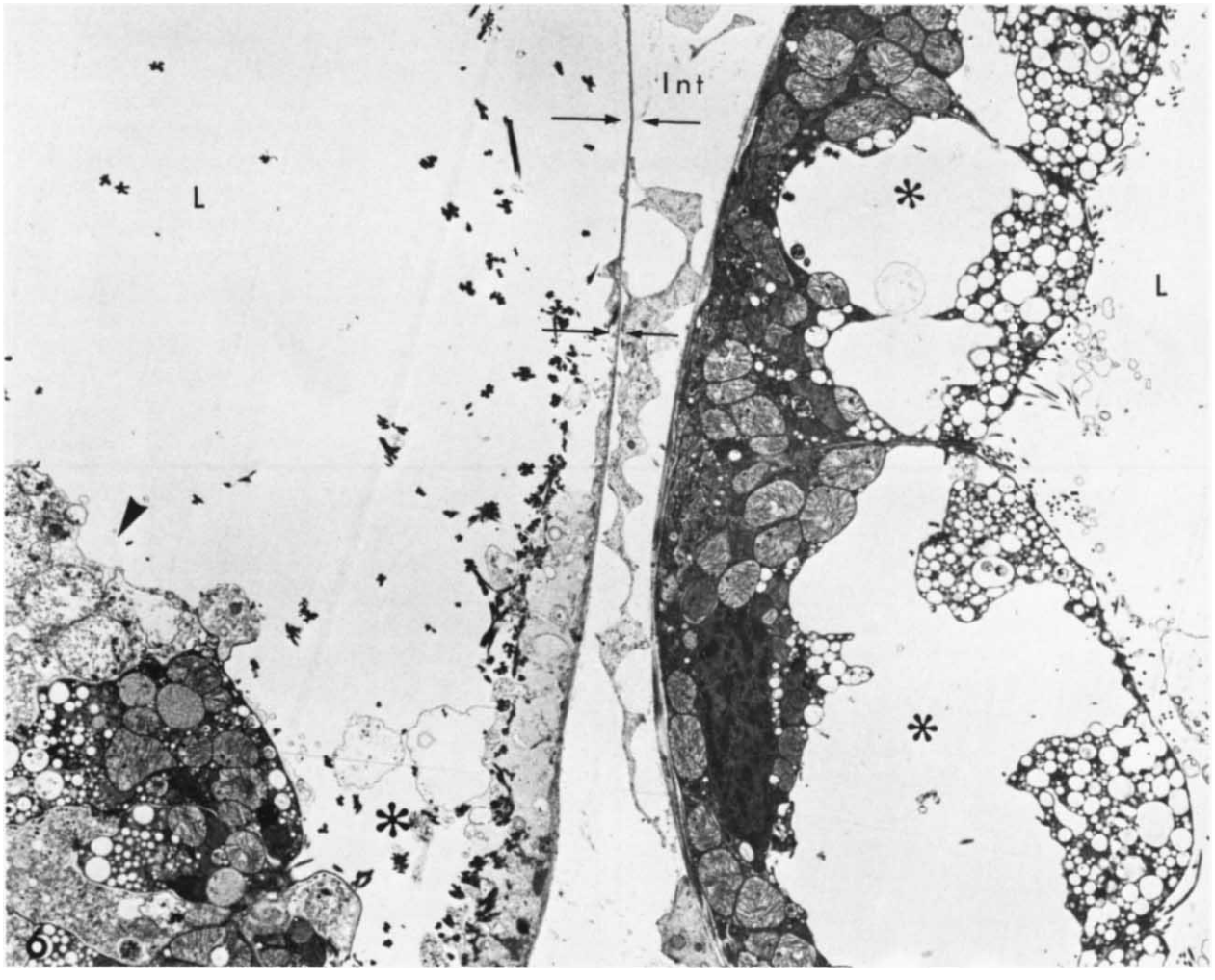


Fig. 6. *Electron micrograph of proximal convoluted tubules from a left kidney subjected to one hour of ischemia and two hours of blood reflow.* The cells to the *right* show stage 3 of irreversible injury. Apical portions of the cytoplasm are seen to be breaking away from the cells (*asterisks*). In the tubule shown at the *left*, a large expanse of tubular basement membrane (*double set of arrows*) is totally denuded of epithelium and directly exposed to the tubular lumen (*L*). Towards the *bottom left*, necrotic cells (*arrowhead*) are seen in the process of being sheared away (*asterisk*) from the tubular basement membrane. *L* denotes lumen; *Int* denotes interstitium. Fixation was by intravascular perfusion. Secondary fixation was in osmium tetroxide. Tissue was stained with uranyl acetate and lead citrate (×4,000).

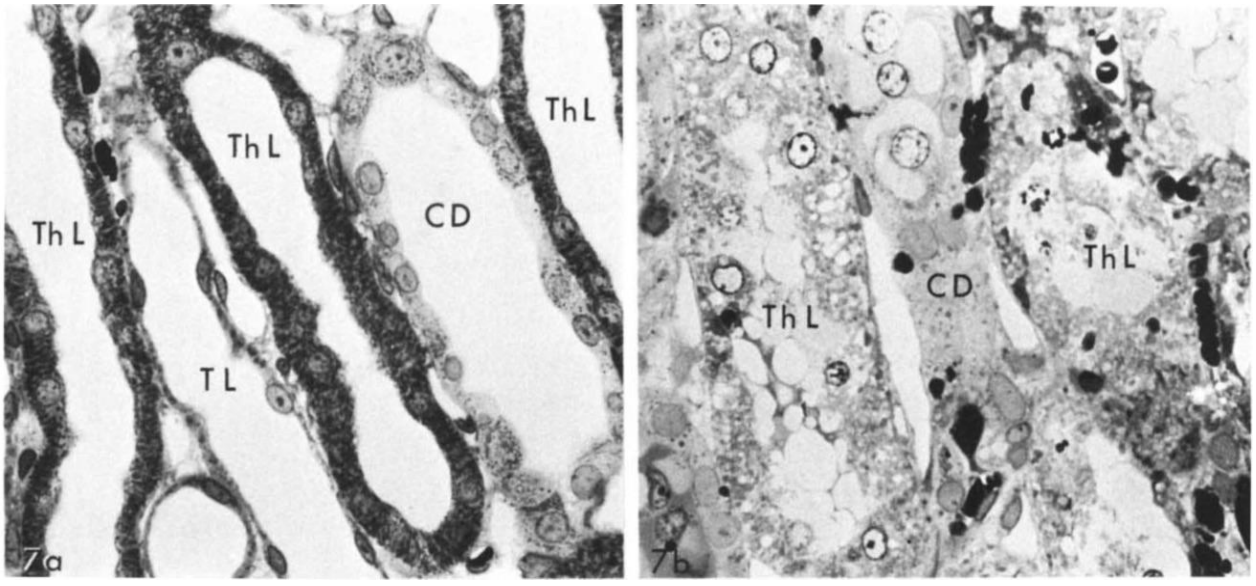


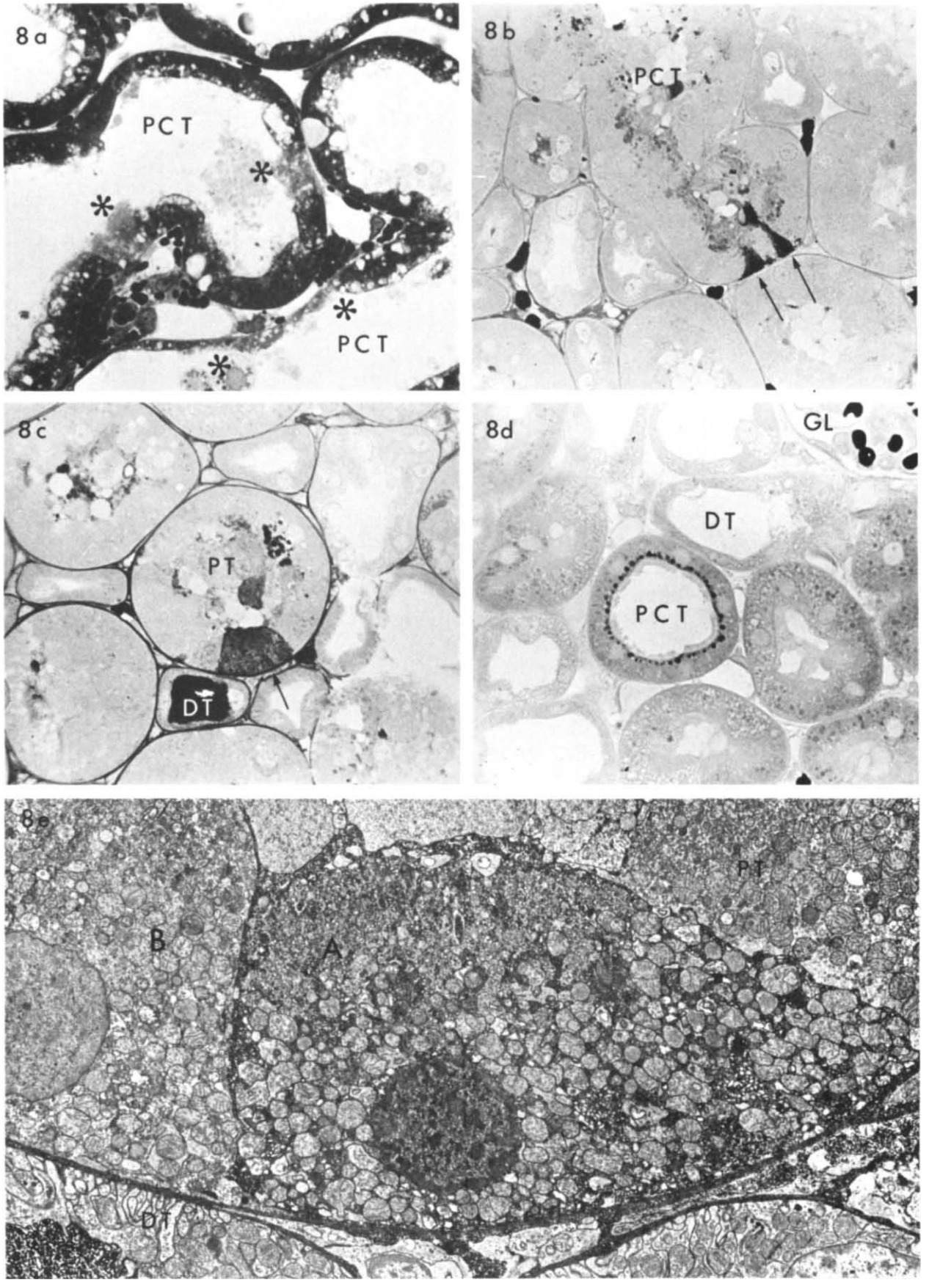
Fig. 7. *Light micrographs of renal tubules from the inner stripe of the outer medulla from control right kidney (panel a) and left kidney (panel b) subjected to one hour of ischemia and two hours of blood reflow.* Tissues were fixed by intravascular perfusion of glutaraldehyde. Secondary fixation was in osmium tetroxide. Epon sections, 2-μ thick, were stained with toluidine blue. **Panel a** is control right kidney, showing normal appearance of ascending thick limbs of Henle (*ThL*), descending thin limbs of Henle (*TL*), and collecting duct (*CD*) (×600). **Panel b** is ischemic left kidney, showing marked cytoplasmic swelling and degenerative changes in ascending thick limbs of Henle (*ThL*) and collecting duct (*CD*) (×600).

(30 min and 2 hr), convoluted PT exhibited pathological changes involving brush border microvilli, indicative of reversible cellular injury. Briefly, these consisted of interiorization of microvilli into epithelial cytoplasm and subsequent restitution of normal architecture. Similar alterations in brush border morphology were observed in straight PT also, but numerous cells in this segment of the nephron exhibited, in addition, signs of irreversible injury at the 2-hr period, and subsequently showed signs of overt necrosis. Occasional straight PT exhibited luminal obstruction by cytoplasmic and membrane blebs derived from shed brush border microvilli after 30 min of reflow, but this obstructive lesion was transient and had disappeard by 2 hr. Other segments of the nephron appeared to be normal. At 24 hr, exfoliation of necrotic straight PT cells into tubular lumina was accompanied by signs of regeneration of survivor cells to reconstitute the epithelium.

15-min ischemia. This duration of ischemia resulted only in minimal focal alterations in the morphology of PT cells, as observed at 30 min following blood reflow. Twenth-four hours later, the histology of these kidneys was entirely normal.

Tracer studies with horseradish peroxidase. Figure 8a is a light micrograph of proximal convoluted tubules subjected to 60 min of ischemia and 2 hr of blood reflow. In 4 of 6 similarly damaged nephrons, horseradish peroxidase microinjected into tubular lumina penetrated diffusely through the cytoplasm of isolated cells and out into the interstitium and blood vessels (Fig. 8, b, and c). In contrast, there was no evidence of leakage in 3 control tubules (Fig. 8d). In both non-leaky cells in ischemic nephrons, and in control tubules, entry of tracer into the tubular epithelium was restricted to pinocytotic uptake, and incorporation into phagolysosomes (Fig. 8d).

Transcellular diffusion of peroxidase in microinjected PT from ischemic kidneys could also be demonstrated by electron microscopy. In Figure 8e, peroxidase is visible as an electron dense reaction product throughout the cytoplasmic and nuclear matrix, but not in the mitochrondria or other membrane-bound organelles of cell A. HRP does not penetrate the adjacent cell B in this manner. That transcellular diffusion of the tracer molecule through epithelial cells was not a pressure artefact of microinjection was indicated by the entry of peroxidase into the cytoplasm of similarly distributed, isolated cells of ischemically damaged nephrons from animals injected i.v. with the tracer. In control PT, and in convoluted PT subjected to 25 min of ischemia, epithelial cells excluded peroxidase from their cytoplasmic matrix, the internal distribution of the tracer being confined to phagolysosomes (Fig. 9a). However, isolated cells or clusters of adjacent cells in straight PT from the 25-min ischemia group (Fig. 9b), and both convoluted and straight PT from the 60-min


8a
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ischemia group exhibited intracellular diffusion of the tracer (Fig. 9, c, and d). These cells were sharply demarcated from adjacent, apparently less injured PT cells.

Discussion

The significance of our findings may be summarized as follows: *1)* The structural-functional studies demonstrate that large molecules can pass through damaged epithelial cells and thus support the view that tubular backleak of glomerular filtrate is a significant feature of postischemic renal failure. *2)* They provide a structural basis for the development of tubular obstruction, described by others [2, 3, 12] as a key feature of the pathophysiology immediately following one hour of complete renal ischemia. Alterations in proximal tubular microvilli, leading to impaction of shed fragments in the proximal straight tubules (PST), appear to be an important pathogenetic factor in this early obstructive process.

Tubular backleak of glomerular filtrate was first suggested as a pathogenetic mechanism in acute renal failure by Richards in 1929 [18] and later in the studies by Oliver [19, 20]. Backleak has more recently been confirmed by studies employing direct tubular microinjection of normally impermeable inulin [5–7, 21, 22]. Each investigator who used this technique to study tubular permeability after complete arterial occlusion has been able to demonstrate tubular inulin leakage [5, 6]. It is of interest that Daugharty et al [25], who induced ischemic damage by prolonged (three hours) but *partial* arterial occlusion, found no evidence of backleak of inulin. In our experiments, 35% of inulin injected intratubularly in kidneys subjected to one hour of complete ischemia appeared in urine from the contralateral kidney. This value probably represents a minimum estimate of tubular leakiness. In the studies of Tanner, Sloan, and Sophasan [5], a known amount of ^{14}C-inulin was injected i.v. into animals who had undergone one hour of left renal artery occlusion. Recovery of isotope in urine from the contralateral, untouched kidney was 55.3%. Applying this figure to our findings would indicate that (34.9/55.3) × 100 or about 63% of microinjected inulin leaked from the tubule. There was substantial variation in leakiness among nephrons; some tubules demonstrated little loss of inulin, whereas others were extremely permeable. This variability has been noted by others [5, 6] and may reflect differential degrees of tubular damage. After 25 min of ischemia, the leakage, though significant, was less marked.

Our morphological findings corresponded well with the functional estimates of backleak. Following 15 min of ischemia, backleak was insignificant, and tubular cells suffered only minimal reversible injury, without progression to necrosis. After 25 min of ischemia and two hours of blood reflow, functional evidence for modest backleak was accompanied by progression of cell injury to frank necrosis in isolated cells of straight but not convoluted proximal tubules. Within two hours of reflow after 60 min of ischemia, however, both convoluted and straight proximal tubular cells exhibited evidence of irreversible cell injury, including the presence of frankly necrotic cells and occasional areas of tubular basement membrane denuded of epithelium. Correspondingly, backleak was severe in these animals. Degenerative changes in distal tubules and collecting ducts likewise correlated with functional evidence for backleak in the distal nephron.

The possible channels for tubular backleak were outlined by intratubular injection of horseradish peroxidase (HRP), a molecule much larger than inulin.

Fig. 8. *Light micrograph of proximal convoluted tubules (PCT).* **Panel a** is from left kidney subjected to one hour of ischemia and two hours of blood reflow. Necrotic tubular epithelial cells, occurring singly or in clusters (*asterisks*), are sharply demarcated from adjacent cells, which although injured, have not yet proceeded on to necrosis or stage 4 of irreversible injury. Fixation was by intravascular perfusion or glutaraldehyde. Secondary fixation was in osmium tetroxide. The Epon section, 2-μ thick, was stained with toluidine blue (× 600). **Panels b through d** are light micrographs of proximal tubules microinjected with 7 nl of horseradish peroxidase in saline (1 mg/ml) and fixed by microperfusion with glutaraldehyde. Sites of peroxidase localization were revealed by cytochemical reaction with diaminobenzidine and hydrogen peroxide. Secondary fixation was in osmium tetroxide (2-μ Epon sections). Peroxidase was revealed as brown reaction product, shown here in *black*. **Panel b** is proximal convoluted tubule (*PCT*) from left kidney subjected to ischemia and microinjected within two hours of blood reflow. Peroxidase is seen to be present in collapsed tubular lumen, diffusely in the cytoplasm of two tubular epithelial cells (*arrows*), and the tubular basement membranes, interstitium, and capillaries. Red blood cells, in peritubular capillaries show endogenous peroxidase activity of hemoglobin (×550). **Panel c** is proximal tubules (*PT*) from left kidney subjected to one hour of ischemia and microinjected within two hours of reflow. Peroxidase is present in phagolysosomes (droplets) in some epithelial cells, diffusely in the cytoplasm of one epithelial cell (*arrow*) demarcated sharply from the others, and in tubular basement membranes and interstitium. The lumen of one distal tubule (*DT*) also contains the tracer, either because it is the distal segment of this proximally injected nephron or due to backdiffusion of peroxidase from the interstitium through leaky epithelium not in the plane of section (×600). **Panel d** is proximal convoluted tubule (*PCT*) from kidney not subjected to renal ischemia. Peroxidase is present only in phagolysosomes (droplets) in the apical cytoplasm of epithelial cells. Unlike ischemically injured tubules, diffusion of tracer into the peritubular interstitium and capillaries has not occurred. Red blood cells in glomerulus (*GL*) show endogenous peroxidase activity of hemoglobin. *DT* denotes distal tubule (×500). **Panel e** is of thin section of proximal tubule (*PT*) cut adjacent to the area shown in panel c. Peroxidase is present diffusely in the cytoplasmic and nuclear sap of cell A, in the peritubular interstitium and basement membranes, and in the distal tubular lumen (*DT*). Epithelial cell B excludes peroxidase from its interior. Tissue was counterstained with lead citrate (×5,000).

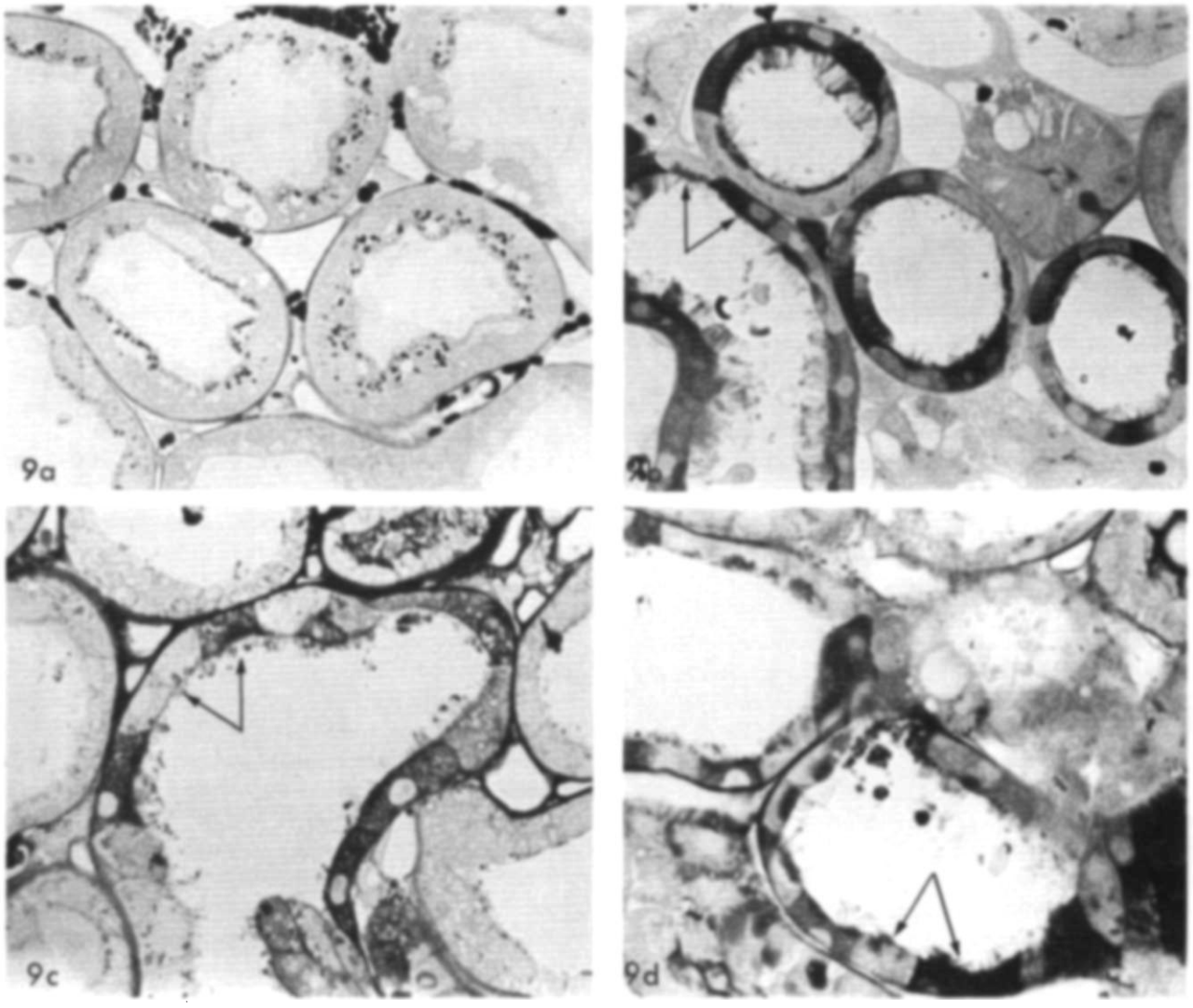

Fig. 9. *Light micrographs of 2-μ thick Epon sections from left kidney subjected to 25 min of ischemia (panel a and b) or 60 min of ischemia (panel c and d) and two hours of blood reflow from rats injected i.v. with horseradish peroxidase.* Fixation was by intravascular perfusion of glutaraldehyde within 5 min of injection. Sites of peroxidase localization were revealed by cytochemical reaction with diaminobenzidine. Secondary fixation was in osmium tetroxide (2-μ thick Epon sections. Peroxidase is visualized as brown reaction product, shown here in *black*. **Panel a** is proximal convoluted tubules from 25-min ischemic kidney. Peroxidase is present in phagolysosomes in the apical cytoplasm of epithelial cells (reabsorbed from glomerular filtrate) and in the peritubular interstitium (derived from peritubular capillary plasma) (×550). **Panel b** is proximal straight tubules from 24-min ischemic kidney. In addition to the sites of localization in panel a, peroxidase is present diffusely in the cytoplasm of some single or clustered tubular epithelial cells, sharply contrasted with adjacent cells that have excluded the tracer (*arrows*) (×550). **Panels c and d** are proximal convoluted tubules from 60-min ischemic kidneys, showing peroxidase localization in the tubular basement membranes, as well as diffusely in the cytoplasm of single or clustered epithelial cells sharply demarcated from adjacent cells that have excluded the tracer (*arrows*) (×660).

Participation of injured cells in the backleak process was indicated by diffusion of the tracer through their plasma membranes and cytoplasm into the peritubular interstitium and capillaries. That such a large protein can so readily travel from lumen to interstitium indicates that these cells had lost their normal selective permeability. Cells permeable to HRP were immediately adjacent in the same nephron to cells with normal impermeability to this protein, indicating that functional damage may be of varying severity among cells of a given nephron, as well as among different nephrons in the same kidney (Fig. 1). Some investigators have argued that backleak is, in effect, an artefact of the microinjection technique [1, 23]. It is reasoned that cells damaged by ischemia may be especially sensitive to disruption by increased intratubular pressure during microinjection of markers. To the extent that tubular obstruction occurs, however, initial intratubular pressure may be already elevated. Moreover, in recent studies, other investi-

gators have monitored intratubular pressure continuously during microinjection of inulin [5, 6] and have shown that backleak occurs in ischemic tubules even if pressure is maintained at normal levels. While we did not monitor intratubular pressure, we used the same slow injection method employed by these investigators. The most definitive evidence that the diffusion of horseradish peroxidase through plasma membranes and cytoplasm of ischemic proximal tubular cells was not related to a pressure artefact is our demonstration of similar abnormal permeability after i.v. injection of the marker. These experiments clearly prove that some proximal tubular cells had lost the ability to maintain their internal milieu. It is therefore reasonable to assume that PT cells damaged by ischemia permit the diffusion of smaller molecules including inulin, electrolytes, and other solutes across their walls and that backleak may be functionally important in postischemic renal failure.

Functional studies by others have established that tubular obstruction develops early, and persists up to 24 hr following one hour of renal ischemia [2, 3, 12]. Our data support this view by showing that inulin injected into tubules may remain within the kidney for prolonged periods, a finding consistent with retention within obstructed tubules.[a] In most cases, half or less of the injected marker remained *in situ;* most of the remainder was recovered from the urine of the contralateral kidney, suggesting obstruction and backleak in the same nephron. Our experiments have localized the early obstructive lesion to straight segments of proximal tubules, the lumina of which are impacted by swollen membrane-bound cytoplasmic blebs derived from disrupted brush border microvilli, and extruded apical cytoplasm of epithelial cells. The mechanisms whereby membrane fragments derived from microvilli adopt a spherical configuration to form blebs, and undergo swelling during their transit in the filtrate are not clear (compare the sizes of floating blebs in convoluted tubules [Fig. 3d] with the impacted blebs in straight tubules [Fig. 3f]). Plasma membrane blebs may be induced in other cell types by toxic injury or prolonged anoxia [16], but again, the causative mechanisms are unknown.

In contrast to the early reflow period, the primary site of tubular casts at 24 hr after ischemia was in the distal nephron, in agreement with the findings of Tanner and Sophasan [12]. Disappearance of the luminal blebs in straight PT during this late reflow period was presumably due to autolytic processes accompanying extensive epithelial necrosis. The precise composition of the distal tubular casts is not known, but it is presumed by other workers that they consist largely of cell debris and glycoproteins, particularly Tamm-Horsfall protein [17].

Other investigators have commented upon the roles played by tubular obstruction and backleak in acute ischemic renal failure [2, 5, 6, 12]. Tanner et al demonstrated both obstruction and backleak early after release of occlusion, and they believe that both alterations contributed towards the development of renal failure [5]. Arendshorst et al believe that obstruction is of greater importance in renal failure early after ischemia, but that a combination of primary reduction of glomerular filtration, obstruction, and backleak is responsible for the functional abnormality 24 hr following the ischemic insult [2]. Our studies do not offer quantitative information regarding the extent to which tubular backleak actually contributes towards renal failure after ischemia. The data show such striking changes in cellular permeability and integrity, however, that it seems reasonable to propose that this factor is of some importance in ischemia. It is possible that this phenomenon may also be important in other models of acute renal failure characterized by tubular necrosis and disruption [7, 24]. Whether backleak could occur in the absence of obstruction is not known and deserves further investigation. In any case, our data are consistent with the presence of obstruction and confirm that backleak is present in postischemic renal failure: they demonstrate clearly the anatomical correlates of each pathophysiological process.

[a] Although we favor intratubular sequestration of these missing counts, we cannot prove such a location. It could be argued that at least some isotope leaked from tubules into an interstitial area devoid of blood flow. In this fashion, the isotope might not gain access to the circulation to appear in contralateral urine, thus underestimating backleak and overestimating obstruction. In other studies [11], however, we have seen that following a 25-min ischemic insult and a similar reflow interval, renal plasma flow reaches frankly hyperemic levels (141%) compared to control values. Although we did not measure renal plasma flow following 60-min ischemia, it appears unlikely to us that substantial areas of avascular interstitium, sufficient to harbor significant fractions of leaked isotope, persist throughout the two hours allowed for blood reflow and urine collection. We did not document again the elevated tubular hydrostatic pressures found by others in this model. Any level of pressure, however, is consistent with the coincidental presence of both tubular obstruction *and* backleak.

Acknowledgments

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