Transport-Dependent Anoxic Cell Injury in the Isolated Perfused Rat Kidney

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The hypothesis that decrease in energy demand may prevent anoxic cell damage has been examined in the medullary thick ascending limb of isolated perfused rat kidneys exposed to oxygen deprivation. The effects of decreasing active reabsorptive transport in the medullary thick ascending limb were observed on the extensive damage regularly induced by hypoxic perfusion (gassed with no oxygen) or potassium cyanide. Anoxic injury was consistently attenuated or abolished if reabsorptive transport was decreased with ouabain or furosemide or by halting the glomerular filtration rate with the use of a hyperoncotic medium (nonfiltering kidney). Comparison of the injury generated by warm ischemia for identical time periods showed that complete ischemia does not reproduce the severe lesions seen during hypoxic perfusion. These results suggest that transport activity is a determining factor of anoxic cell death in the thick ascending limb of Henle's loop. (Am J Pathol 1984, 116:327-341)

THE CONCEPT that a reduction in energy demand for cell activity can limit or prevent damage from O_2 deprivation has long been proposed in cardiac muscle[1,2] and has recently been addressed in a neuronal cell culture.[3] We have attempted to examine this question in an experimental model of anoxic injury to renal tubular cells.

Alcorn et al have described a striking histologic lesion found during isolated perfusion of the rat kidney.[4] The lesion consists of mitochondrial swelling, cytoplasmic disruption, and progressive nuclear pyknosis. This sequence of events is exclusively limited to the medullary thick ascending limb (mTAL) of Henle's loop, while adjacent structures remain unaffected. The damage to the mTAL is easily reproduced and quite consistent.[5-7] Transport activity appears to modify the damage, as indicated by the fact that it is prevented by furosemide, ouabain, or halting glomerular filtration.[7] We have recently shown that the mTAL lesion is markedly exaggerated by perfusion without oxygen and is prevented by the inclusion of RBC or hemoglobin in the perfusate.[8] Taken together, these results suggested that the hypoxic injury might be conditioned by cell work in the face of limited O_2. This proposition was examined more in depth in the present experiments, using hypoxic perfusion, cyanide, or ischemia. Both hypoxic perfusion and cyanide produced profound damage to the nephron, which was by far most severe in the mTAL segment. This injury was remarkably attenuated or abolished if reabsorptive transport was halted.

Materials and Methods

Male Sprague-Dawley rats, weighing 370–470 g, fed on Purina Rat Chow and allowed free access to water, were used for all experiments. Perfusion of the right kidney was performed according to the technique described by Ross et al.[9] In brief, a glass arterial cannula is inserted into the superior mesenteric artery and threaded across the aorta into the right renal artery. Perfusion is started while the cannula is still in the mesenteric artery to avoid any ischemia to the kidney during the isolation. The kidney is then

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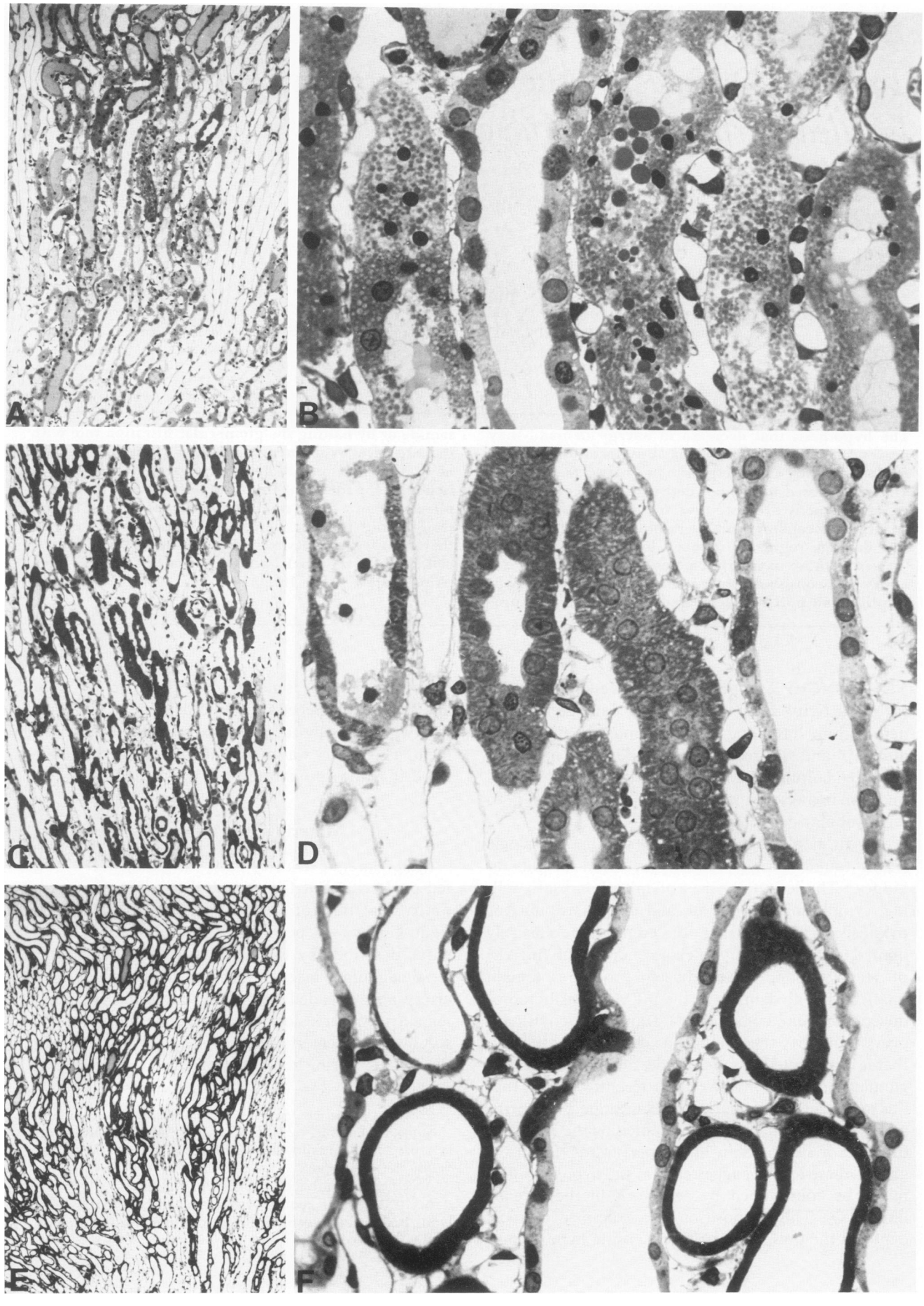
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placed in a constant-temperature cabinet, where the temperature is monitored and kept at 37 C by a thermostat. Perfusion medium is recirculated continuously with a pulsatile flow at a pressure of 85 mm Hg at the tip of the cannula. Regular perfusion medium, as used in controls, consisted of a Krebs–Ringer–Henseleit solution with bovine serum albumin at a concentration of 6.7 g/100 ml and glucose at 5 mM, gassed with 5% CO_2, 95% O_2, unless specified otherwise.

Experimental Groups

Effect of Decreasing Energy Utilization on the Hypoxic Perfusion Damage

Hypoxic Perfusion (n = 7)

Kidneys perfused for 90 minutes with a medium gassed with 95% N_2, 5% CO_2 served as a control group.

Ouabain (n = 6)

Ouabain (ICN Pharmaceuticals) was added to the perfusion medium at 10^{-3} to 10^{-2} M final concentration, and the kidneys were perfused for 90 minutes with no O_2.

The kidneys perfused with ouabain were usually given 20 minutes of equilibration at regular oxygenation (95% O_2, 5% CO_2) before being exposed to the 90 minutes of hypoxic perfusion.

Furosemide (n = 3)

Furosemide (Hoechst-Roussel Pharmaceuticals) was added to the perfusion medium at 10^{-3} M final concentration. The kidneys perfused with furosemide were equilibrated for 20 minutes at regular oxygenation before being exposed to 90 minutes of hypoxic perfusion.

Nonfiltration (n = 5)

Hyperoncotic medium was used (albumin concentration 11–12 g/dl) in order to prevent glomerular filtration. The kidneys were equilibrated for 30–60 minutes at regular oxygenation before being exposed to 90 minutes of hypoxic perfusion.

Effect of Decreasing Energy Utilization on Cyanide-Induced Damage

Potassium Cyanide (KCN) (n = 6)

The effect of KCN (Fisher Scientific Company) was examined, added to the perfusion medium at 10^{-4}–10^{-2} M final concentration, the kidneys being perfused for 90 minutes, gassed with 95% O_2, 5% CO_2. Damage to the mTAL equivalent to hypoxic perfusions was obtained at approximately 2.5 mM KCN.

Ouabain and KCN (n = 6)

After 20 minutes of perfusion with ouabain (10^{-2} M) at regular oxygenation, KCN was added at 1, 2.5, or 10 mM for an additional 90 minutes of perfusion.

Ouabain, KCN, and an Uncoupler of Mitochondrial Respiration (n = 3)

The effect of uncoupling mitochondrial respiration was examined by perfusing kidneys with ouabain (10^{-2} M) and KCN (2.5 mM) as in the preceding group in the presence of carbonyl cyanide m-chlorophenylhydrazone (mCCCP) (Sigma Chemical Company), at 10^{-4} M. mCCCP is a potent uncoupler of mitochondrial respiration.[10]

Comparison of Perfusion Hypoxic Damage at 15 and 90 Minutes With Warm Ischemia Damage of the Same Duration

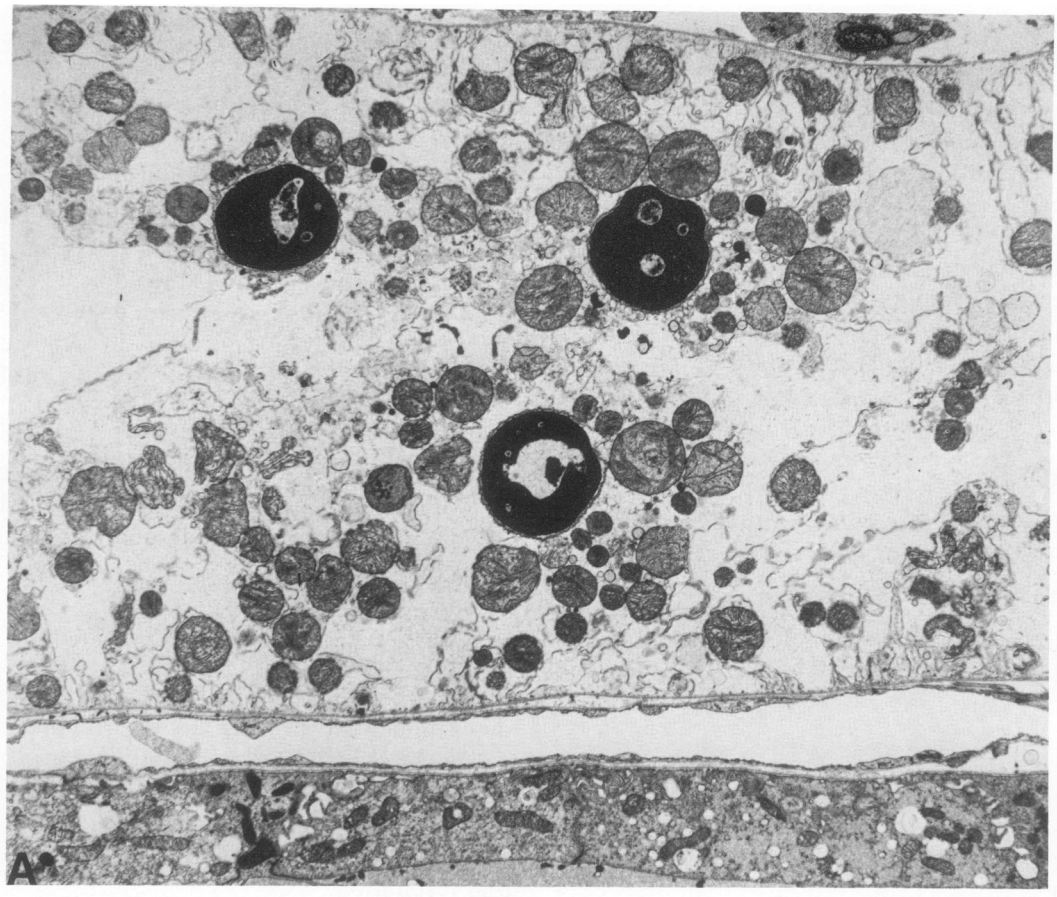
Perfusion (n = 30)

The damage to the outer medulla was examined after 15 and 90 minutes of perfusion with regular oxygenation (n = 7 and 8, respectively) or no oxygen (gassed with 5% CO_2, 95% N_2) (n = 7 and 8). Quantitation of damage to the mTAL under these conditions has been previously reported.[8]

Warm Ischemia (n = 13)

The morphology of the outer medulla was documented after 15 and 90 minutes of complete occlusion of the renal artery (n = 6 and 7). To avoid the potential protective effect of the reservoir of oxygen contained in the blood trapped inside the ischemic tissue,

Figure 1 – Ouabain protection against hypoxia. After 90 minutes of hypoxic perfusion, the nephron segment most extensively damaged is the mTAL. In low power (**A**) diffuse mTAL injury can be appreciated. High power (**B**) shows mTAL cell fragmentation, mitochondrial swelling, and nuclear pyknosis. In sharp contrast, the collecting duct is well maintained. If 1 mM ouabain is included in the perfusate, damage is much more limited and the consequent maintenance of tubular integrity can even be perceived at low power (**C**). High power (**D**) confirms the preservation of much of the mTAL epithelium; luminal membranes are intact, and only mild degrees of chromatin margination are present. Some tubules (*left*), however, still show severe injury. Preincubation with 10 mM ouabain or simultaneous inclusion of 10 mM ouabain in the perfusate under conditions of hypoxia will completely protect the mTAL from injury (**E** and **F**). The luminal dilatation associated with the extreme diuresis under these conditions results in organelle compression so that cellular detail cannot be appreciated by light microscopy. (**A**, ×100; **B**, ×640; **C**, ×100; **D**, ×640; **E**, ×40; **F**, ×640)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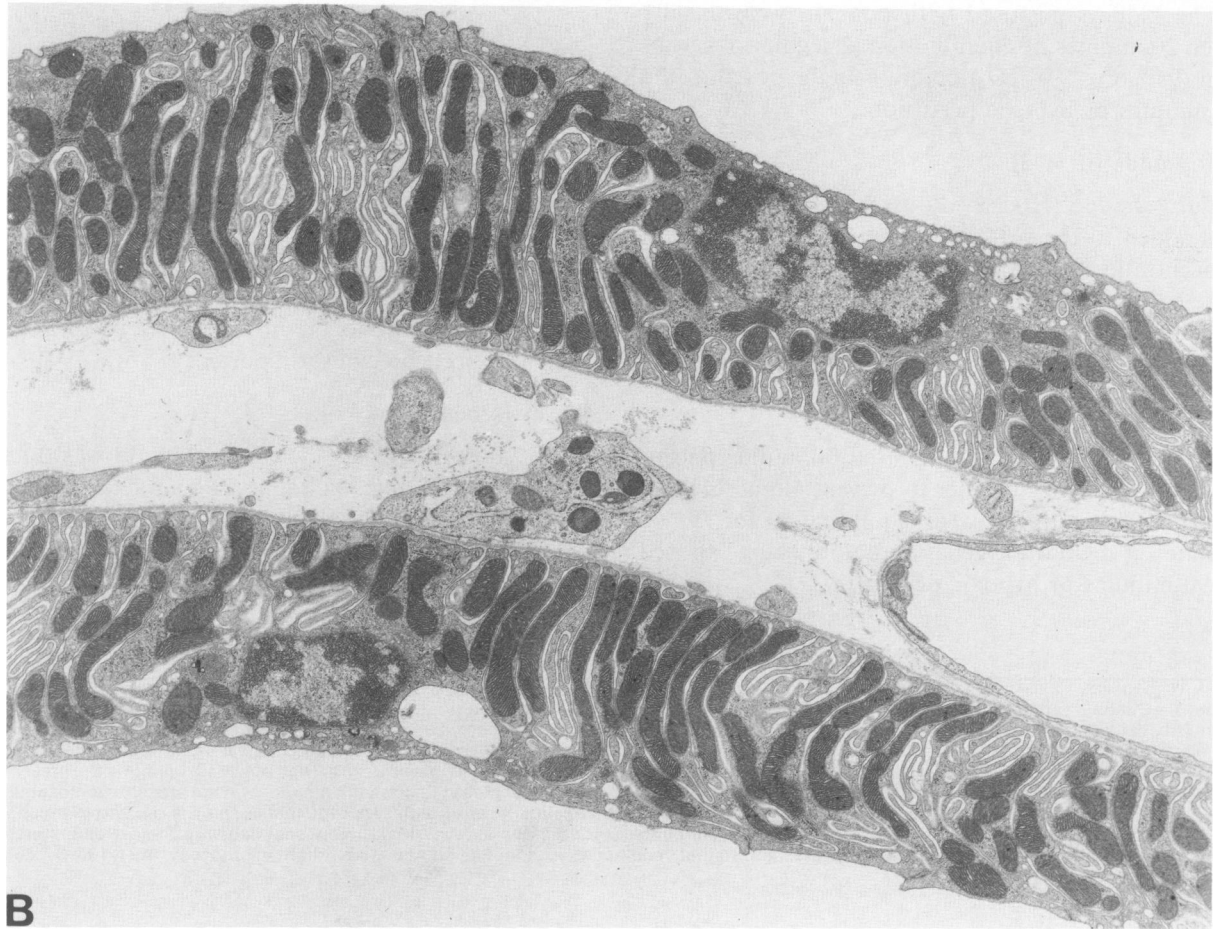
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two kidneys were initially flushed for 1 minute with a perfusate similar to the regular perfusion medium (except for its high albumin concentration [11–12 g/dl], designated to prevent glomerular filtration) and then exposed to 15 minutes of ischemia. Warm ischemia was insured by either leaving the kidney *in situ* (n = 3) with the animal still alive on a heating table (abdomen closed, rectal temperature at 37 C) or isolated and placed in the perfusion cabinet maintained at 37 C (n = 10) under conditions similar to those of perfused kidneys. Since ischemia may simply delay the morphologic expression of cell death (eg, by limiting the influx of calcium and water), three kidneys were perfused after 90 minutes of warm ischemia. The regular (oxygenated) perfusate used was supplemented with ouabain (10^{-2} M) for prevention of the damage produced to mTAL by control perfusion, and reflow was allowed for 15, 40, or 90 minutes before fixation.

Morphologic Techniques

A three-way stopcock was incorporated into the circuit 5 cm from the arterial cannula to allow perfusion with the fixation solution, at the same pressure applied during the functional study, for an additional 5–8 minutes. The fixative solution contained 1.25% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). A coronal cross-section (1–2 mm thick) was taken to include the entire inner strip of the outer medulla. This was subsequently divided into two pieces (each approximately 3×3 sq mm), postfixed in 2% OsO_4, dehydrated, and embedded in an Araldite–epon 812 mixture. Large 1-μ sections were cut to include the entire length of the mTAL. Selected blocks were examined by electron microscopy. The total of 76 kidneys from all experimental groups was evaluated for histologic features. Between 74 and 289 tubules (mean 180) were evaluated per kidney.

The histologic evaluation was completed by S.R. in a "blinded" fashion, that is, without knowledge of the way the kidneys had been perfused. Three zones of the inner stripe were analyzed:

A. Upper third—all mTALs intersecting a line immediately adjacent to outer strip (within approximately 0.2 mm).

B. Middle third—all mTALs intersecting a line drawn midway between the borders of the inner strip.

C. Lower third—all mTALs intersecting a line immediately adjacent to the inner medulla (within approximately 0.2 mm).

These points were chosen for analysis because they provided areas in which topographical landmarks were easily ascertained. A percentage score was used to indicate the fraction of tubules involved with minimal or mild (chromatin margination, minor degrees of mitochondrial swelling), moderate (overt mitochondrial swelling with limited nuclear pyknosis), and severe (overt mitochondrial swelling with extensive nuclear pyknosis and cell fragmentation) changes. The observations were limited here to the mTAL because of the selective vulnerability of this segment to hypoxia in this model.[8] As defined, severe changes in response to hypoxic perfusion were regularly much more extensive in the mTAL than in the proximal tubule.

The data are presented as mean ± (SEM) standard error of mean. For statistical analysis the Student *t* test was used.

Results

Effect of Decreasing Cellular Energy Utilization on Hypoxic Damage

Figure 1 (A and B) and Figure 2 (A) show the extensive, moderate to severe damage encompassing all mTALs after 90 minutes of low O_2 perfusion. Figures 1 (C to F), 2, 3, 4, and 5 illustrate partial to complete protection of mTAL injury with ouabain or furosemide supplementation of hyperoncotic perfusion. In Figure 6, quantitative analysis of the damage to the mTAL shows complete or partial protection with the different measures used to decrease active transport. Table 1 shows similarly low O_2 delivery, venous P_{O_2} and O_2 consumption for the whole kidney in all four groups.

Effect of KCN on mTAL Morphology and Effect of Decreasing Cellular Energy Utilization on KCN-Induced Damage

KCN reproduced an injury identical to hypoxia with extensive, moderate to severe damage to the mTAL. The lesion became progressively worse from 1 to 10 mM KCN, being equivalent to 90 minutes of hypoxic perfusion at 2–3 mM KCN. Ouabain (10^{-2} M) prevented this damage, as it did for the damage of oxygen deprivation. The protection of the mTAL was com-

Figure 2—Ouabain protection against hypoxia. Electron microscopy, after 90 minutes of hypoxic perfusion (**A**) reveals nuclear pyknosis, extensive membrane fragmentation, and severe mitochondrial swelling in the mTAL. The collecting duct (*lower*) shows comparatively slight changes. When 10 mM ouabain is included in the perfusate, electron microscopy (**B**) confirms the complete protection against hypoxia. Luminal and basolateral membranes are preserved; mitochondria and nuclei are essentially unremarkable. (**A**, ×6500, **B**, ×4000)

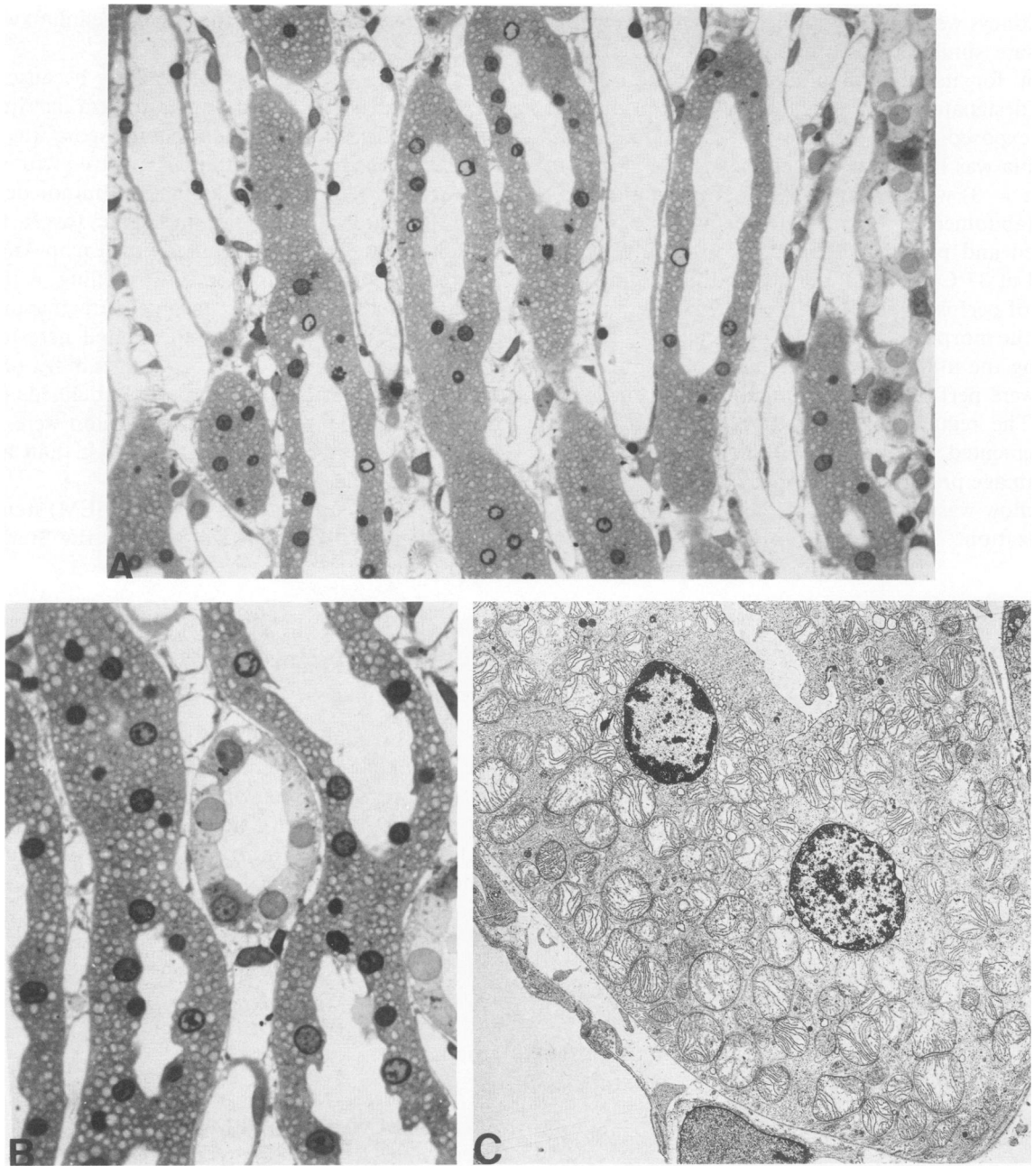

Figure 3 – Furosemide protection against hypoxia. If 1 mM furosemide is included in the perfusate (**A** and **B**), extensive mTAL mitochondrial swelling occurs and considerable chromatin margination is present, but luminal membranes are maintained, and cell fragmentation is minimal, as confirmed by electron microscopy (**C**). The nuclear pyknosis and cell fragmentation that characterizes severe injury are not present (compare with Figures 1 A and B and 2A). Thus furosemide confers limited but significant protection against hypoxia. (**A**, ×400; **B**, ×640; **C**, ×2400)

plete with KCN concentrations of 1.0 and 2.5 mM but incomplete with 10 mM (Figure 7). mCCCP led to focal reappearance of damage (Figures 8 and 9). Figure 9 summarizes the quantitative analysis of the damage to the mTAL with KCN alone, KCN and ouabain, and KCN, ouabain and mCCCP.

Comparison of the Hypoxic Injury of Perfusion With the Damage of Warm Ischemia at Equivalent Time Periods

Figures 10 and 11 show that complete ischemia caused only cytoplasmic edema, apical blebbing with

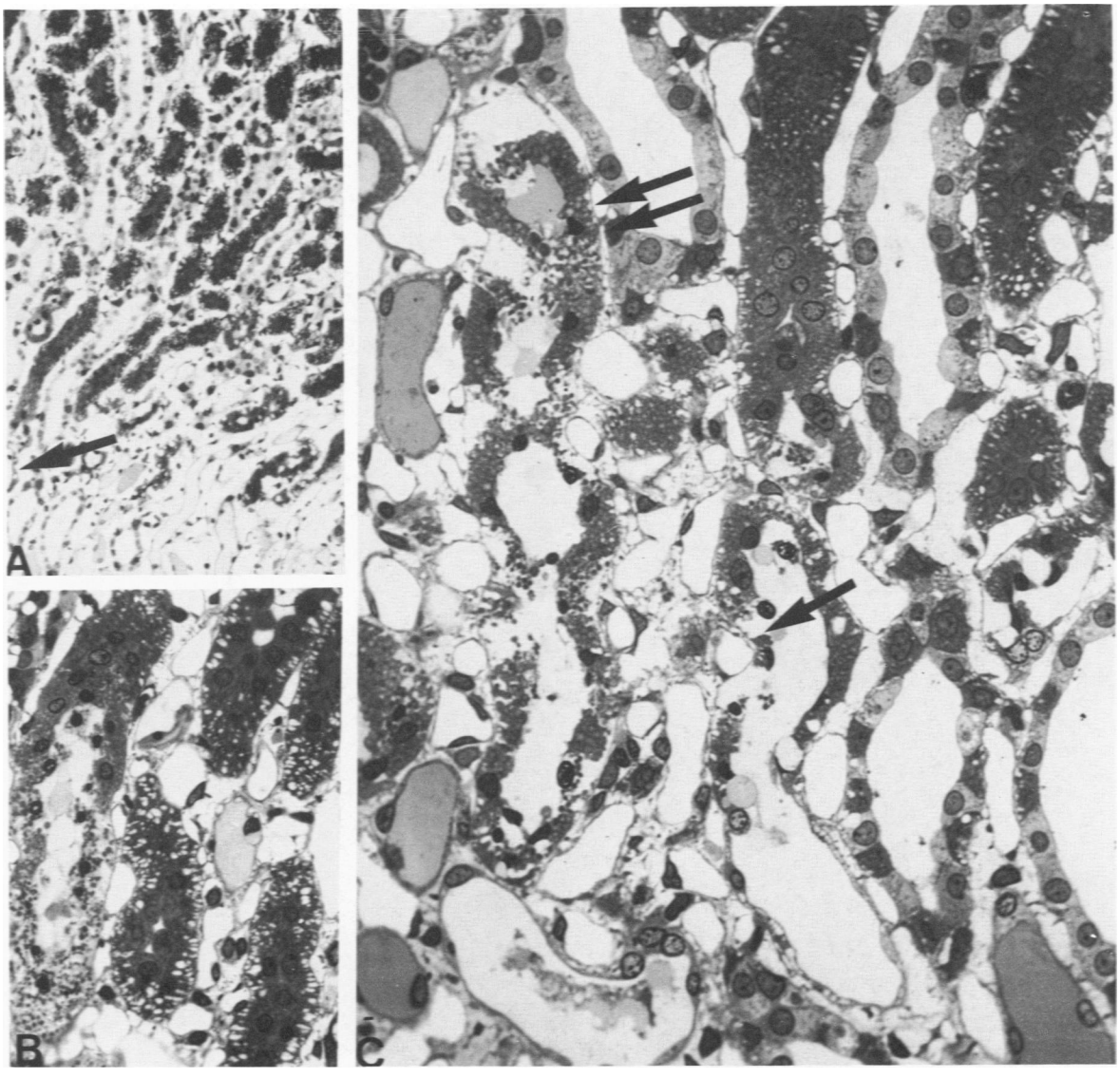

Figure 4 – Hyperoncotic medium protection against hypoxia. If the kidney is perfused with a hyperoncotic solution to prevent glomerular filtration, mTAL damage is sharply decreased. The extent of damage reduction is related to the length of incubation with hyperoncotic media prior to initiating the hypoxic perfusion. With a 60-minute preincubation, the mTAL injury is confined to a narrow zone of inner stripe just adjacent to the inner medulla. In the low power (**A**) micrograph luminal collapse is evident. At high magnification (**B**) basal vacuoles (separation of the lateral cellular interdigitations) are evident, but the epithelium is essentially preserved. The tubule on the left shows both maintenance of epithelium and the hypoxic injury. In **C**, higher-power micrograph of the area (*arrow*) in **A**, mTAL has been preserved except for a small zone (*arrow*) just adjacent to its transition to the thin limb. The adjacent mTAL (*double arrows*) shows severe injury. (**A**, × 100; **B**, × 370; **C**, × 460)

nuclear clearing and minimal mitochondrial swelling at 15 minutes or moderate mitochondrial swelling at 90 minutes. Severe damage with nuclear pyknosis and extensive cell fragmentation was not seen. The extent of damage was only minimally altered by prior flushing of the erythrocytes. These changes are similar to those reported in the literature.[11,17] In comparison with regular and hypoxic perfusion, the damage to the mTAL is remarkably reduced (compare Figures 1 A and B and 2 A with Figures 10 and 11). Perfusion (with ouabain) after ischemia was not associated with progression of injury, but in fact, striking reversal of the 90-minute ischemic changes was evident (Figure 12). Ischemia did not reproduce the severe lesion of hypoxic perfusion.

Discussion

Leichtweiss et al and Baumgartl et al[12,13] have previously shown that the partial pressure of O_2 in tissue,

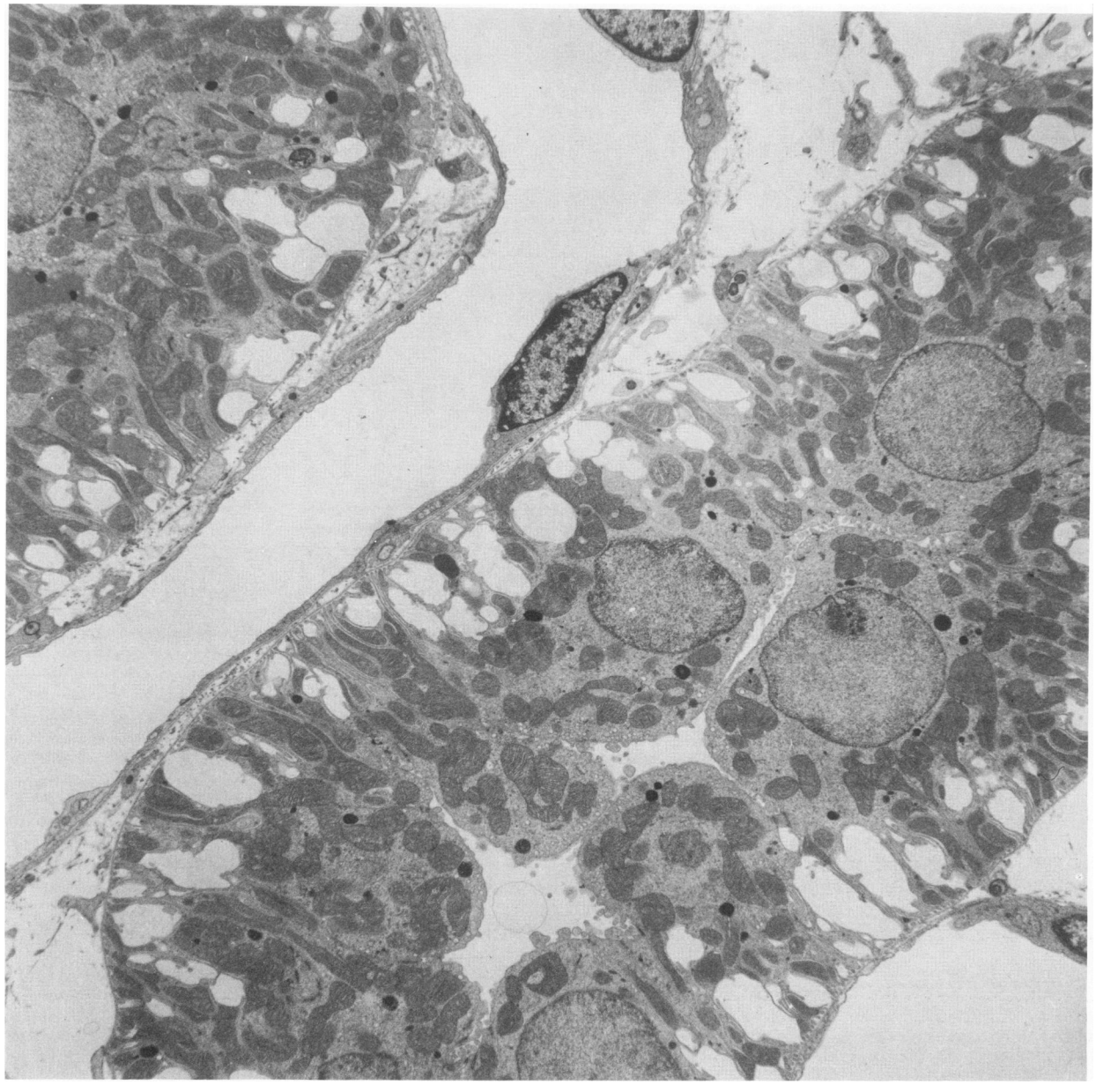

Figure 5—Hyperoncotic medium protection against hypoxia. This electron micrograph confirms maintenance of cell structure as determined in 1-μ plastic sections (see Figure 4). Although widening of the lateral cellular interspaces is observed, the basal membranes are intact. Mitochondria and nuclei show no injury. (×2800)

as measured by platinum microelectrodes inserted into the rat or dog kidney from cortex to medulla, drops steeply at the corticomedullary junction to values in the range of 10–20 mmHg in the medulla, both *in vivo* and during isolated perfusion.[12] Recent observations by Epstein et al, using organ spectrophotometry in whole isolated perfused rat kidney, have indicated that a significant fraction (20–40%) of cytochrome-a,a_3 as sensed by this technique is reduced but becomes more oxidized when mTAL transport is reduced by furosemide.[14] Cytochrome-a,a_3 should normally be 95–98% oxidized in the presence of enough oxygen to fulfill the needs of oxidative phosphorylation.[15] The authors concluded that substantial portions of the renal medulla may be operating on the verge of anoxia and therefore be predisposed to anoxic damage. This prediction has been supported by the recent demonstration that the basis for the selective damage of the mTAL seen consistently during isolated rat kidney perfusion is anoxia

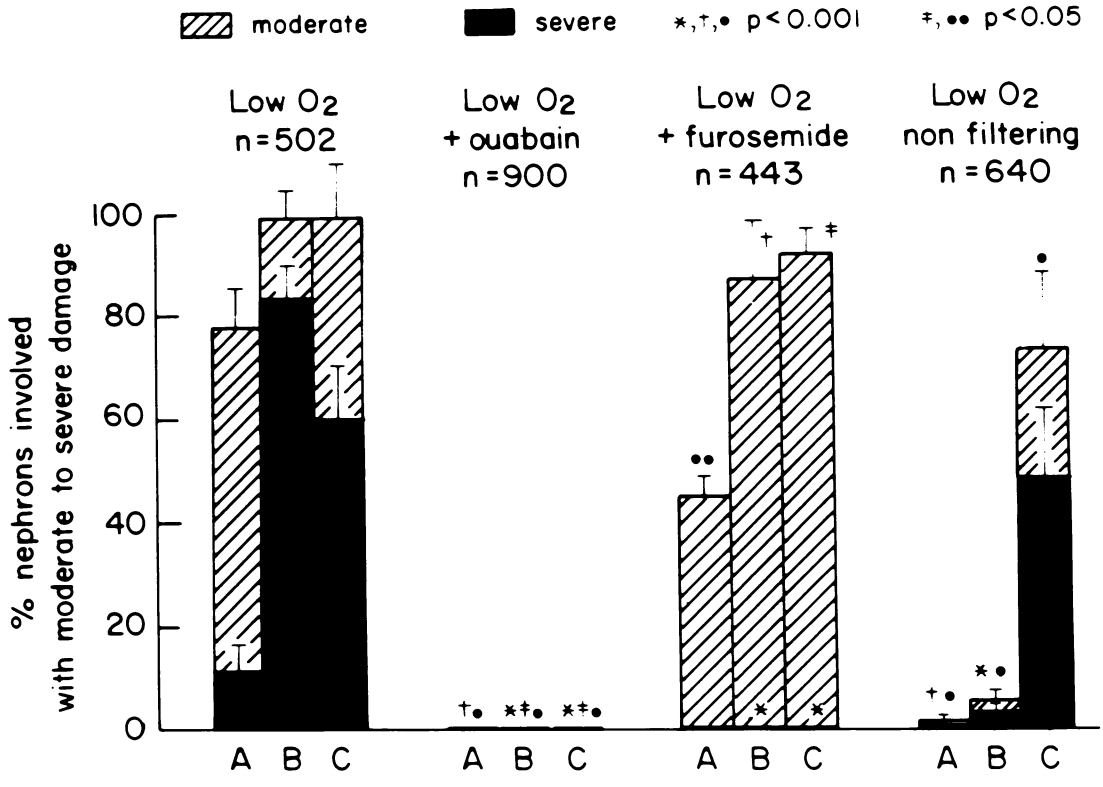

Figure 6—Quantitative analysis of the protection against hypoxia by ouabain, furosemide, and hyperoncotic perfusion (nonfiltering kidney). The extent of mTAL damage in the inner stripe of the outer medulla was evaluated in three regions: outer (**A**), mid (**B**), and inner (**C**) (see Materials and Methods). The extensive moderate and severe damage present after 90 minutes of hypoxic perfusion is essentially eliminated in the presence of ouabain (10^{-2} M). With furosemide (10^{-3} M), the severe damage is prevented. In nonfiltering kidneys, the frequency of moderate and severe injury is markedly diminished in regions **A** and **B**. In region **C**, although significantly reduced, damage is still present. *n*, total number of tubules evaluated in each group. Statistical comparisons are versus low O_2, for severe (*) or moderate (†, ‡) damage and extent of injury (moderate or severe) (•).

imposed by the medullary vascular system (cortico-medullary gradient of oxygen), the limited oxygen carrying capacity of the usual perfusate, and the high rate of metabolism mandated by active reabsorption of NaCl.[8] Diminution or abolition of the damage by increasing the O_2 content of the perfusate[8] or by decreasing transport work load of the mTAL[7] suggested that this injury could be directly related to the imbalance between O_2 demand and supply. Thus, ouabain, furosemide (to block active transport at the mTAL),

Table 1—Function and Oxygenation of the Whole Kidney Under Conditions of Hypoxic Perfusion

	GFR (ml/min)	Fr_{Na}* (%)	Renal perfusion flow (ml/min)	Arterial Po_2 (mmHg)	Venous Po_2 (mmHg)	O_2 delivery† (μmol/min)	O_2 consumption† (μmol/min)
Hypoxic perfusion (n = 7)	0.10 (±0.01)	41.2 (±2.1)	31.9 (±2.7)	36.5 (±1.6)	12.6 (±1.3)	1.44 (±0.13)	0.92 (±0.08)
Hypoxic perfusion with ouabain (n = 6)	0.23 (±0.08)	39.5 (±7.9)	34.4 (±3.2)	42.2 (±5.6)	17.2 (±2.6)	1.44 (±0.26)	0.85 (±0.13)
Hypoxic perfusion with furosemide (n = 3)	0.22 (±0.07)	46.9 (±10.2)	32.3 (±1.6)	43.3 (±3.8)	16.7 (±0.7)	1.86 (±0.16)	1.2 (±0.14)
Hypoxic perfusion with hyperoncotic medium (n = 5, nonfiltering kidneys)			33.7 (±0.4)	41.5 (±1.9)	19.5 (±1.3)	1.55 (±0.09)	0.9* (±0.10)

* Percent of filtered sodium that was reabsorbed (fractional sodium reabsorption).

† Oxygen delivery and oxygen consumption range from 16–28 and 3–6 μMol/min respectively for kidneys perfused with a normally oxygenated medium.

Values are mean ± SEM.

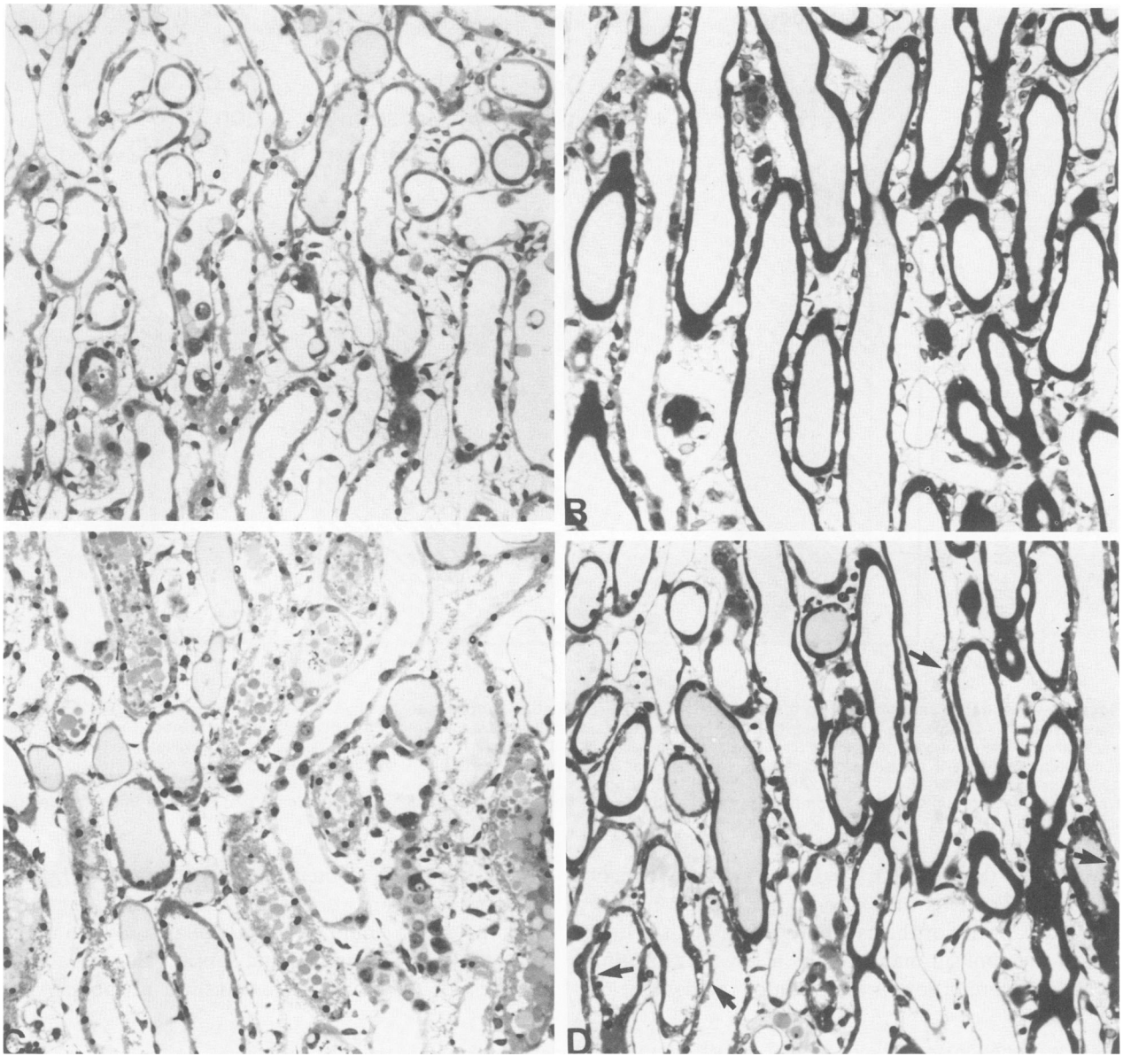

Figure 7—Ouabain protection against KCN. When KCN is included in the perfusate, changes identical to severe anoxic injury occur in the mTAL (**A** and **C**). Such changes can be completely prevented by preincubation with 10 mM ouabain at KCN concentrations of 1.0 and 2.5 mM (**B**). At 10 mM concentration of KCN (**D**), the damage can be markedly reduced but not eliminated. Cellular fragmentation can still be observed (*arrows*). (**A**, ×250; **B**, ×250; **C**, ×250; **D**, ×250)

or hyperoncotic albumin perfusion (to halt the glomerular filtration rate [GFR], "nonfiltering kidney") were found remarkably effective in preventing the hypoxic lesion regularly seen in the mTAL during isolated kidney perfusion using an acellular medium gassed with 95% O_2, 5% CO_2.[7] This protective effect might conceivably have been mediated simply by an increase in O_2 delivery to the outer medulla, resulting from the reduction in oxygen consumption by transporting tubules and the concomitant dissipation of the usual corticomedullary gradient of oxygen tension. Alternatively, the protective effect could have been at the cellular or mitochondrial level by restoring the balance between the demand for O_2 consumption and compromised O_2 delivery. To test this hypothesis further, in the present study the protective effect of the same maneuvers was examined against a background of more severe hypoxia produced by hypoxic perfusions (gassed with 0% O_2, 5% CO_2) and by cyanide, where decreasing the tubular requirement for energy would be unlikely to restore adequate O_2 or alter cyanide-induced cytotoxic anoxia.

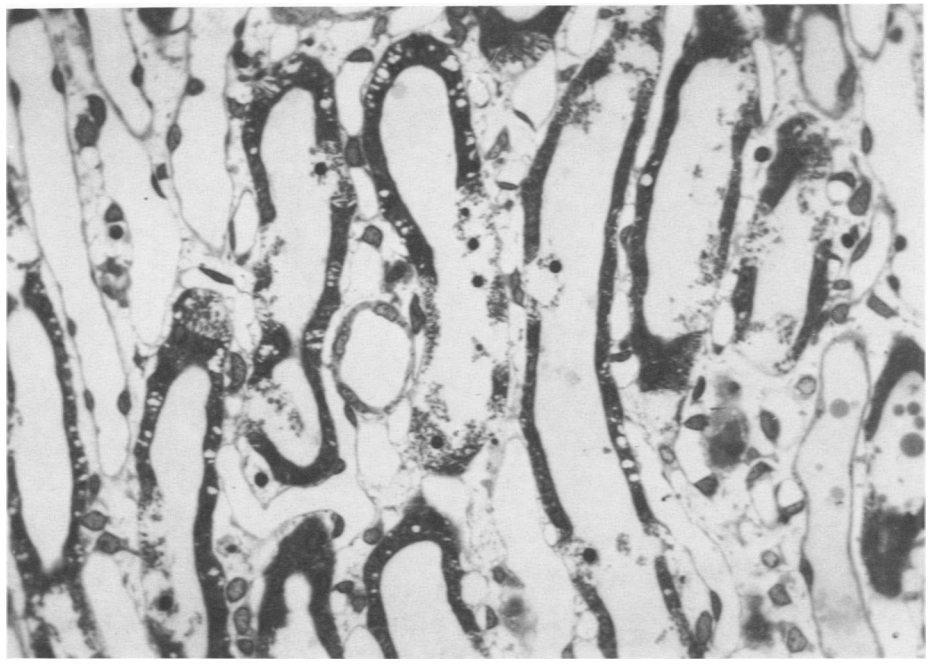

Figure 8 – Ouabain protection against both KCN and mCCCP. There is incomplete ouabain protection against 2.5 mM KCN if mCCCP is included in the perfusate. While much of the mTAL epithelium is preserved, focal epithelial destruction is evident. (×400)

The mTAL is an important medullary nephron segment which functions as the major driving force of the countercurrent mechanism for urinary concentration. The cells of the mTAL are regularly engaged in active chloride reabsorption coupled with a high rate of O_2 consumption.[16] Since this part of the nephron has a very low permeability to water, active solute reabsorption generates a gradient of osmolality and

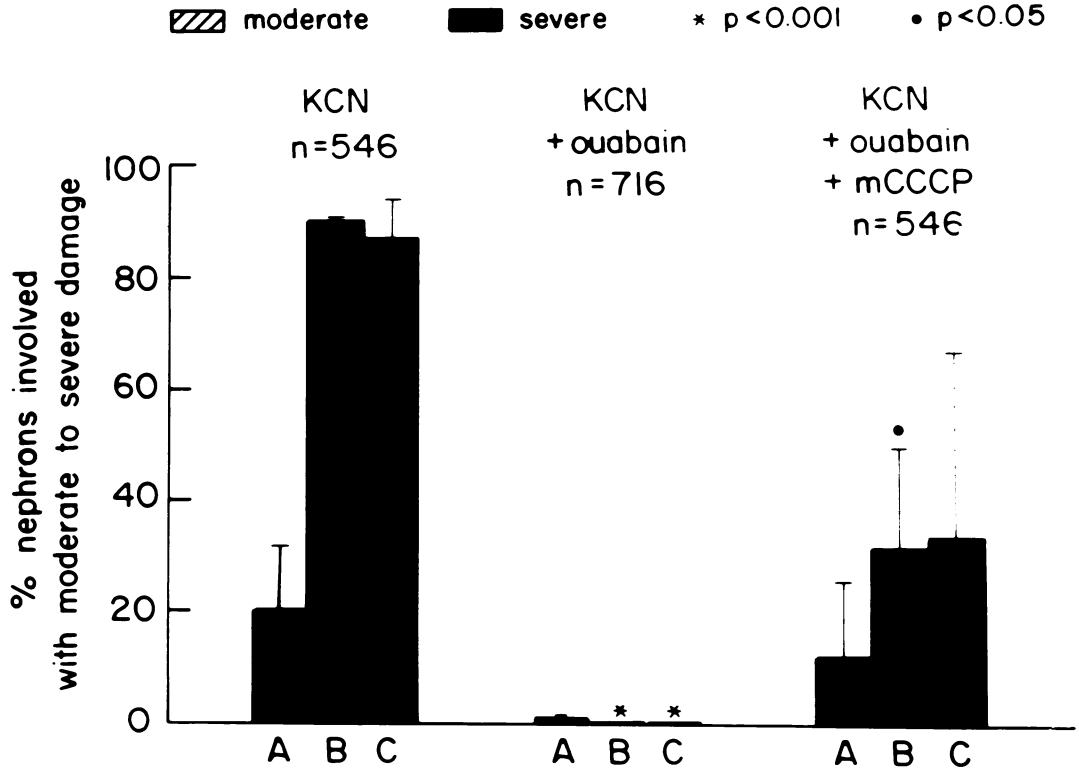

Figure 9 – Quantitative analysis of the protection against KCN by ouabain. The extent of damage to the mTAL was evaluated in three regions: outer (**A**), mid (**B**), and inner (**C**) (see Materials and Methods). KCN (2.5 mM) produced extensive severe damage and insignificant or no moderate damage. Ouabain (10 mM) essentially eliminated KCN-induced damage. In the presence of mCCCP, however, the protective effect of ouabain is partly lost. n, total number of tubule sections evaluated in each group. Statistical comparisons are versus KCN.

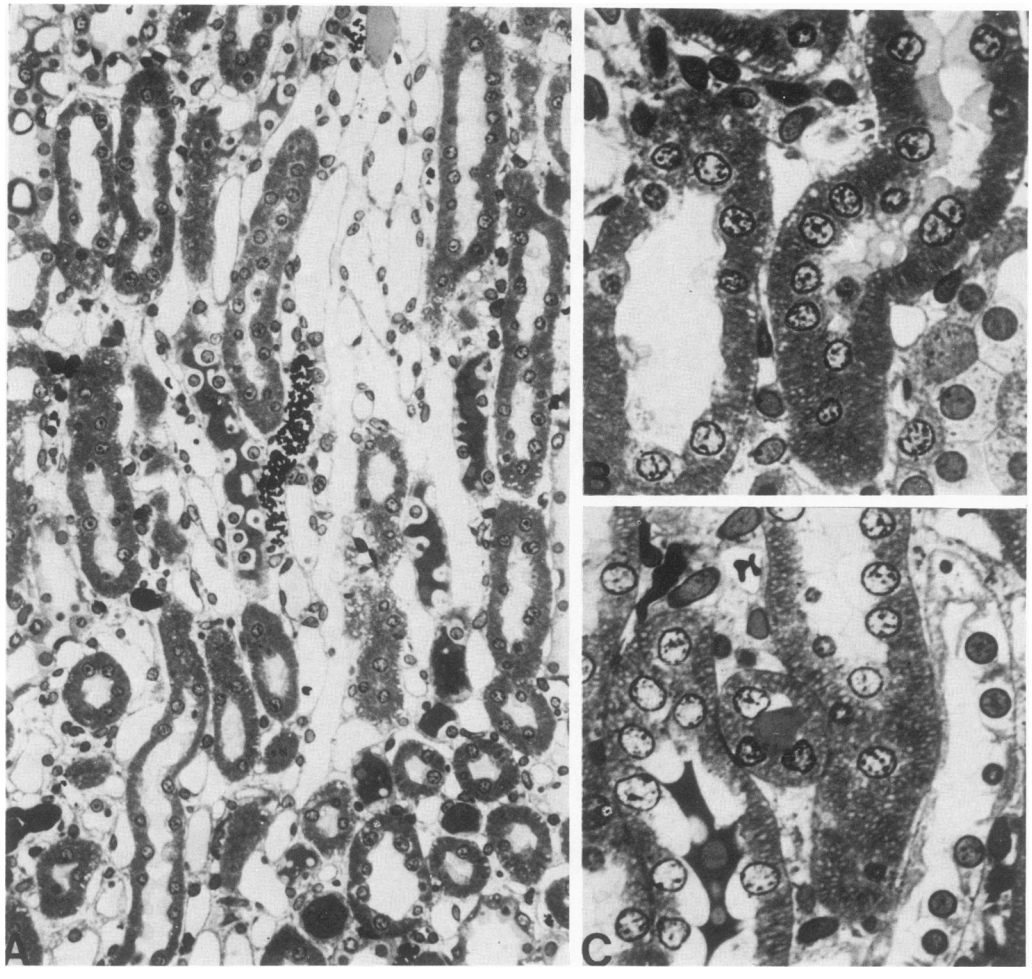

Figure 10—Ischemia (renal artery occlusion). After 15 minutes of ischemia (**A** and **B**) mTAL changes are those of apical blebbing and chromatin margination. Mitochondrial swelling is mild at this time point and can best be appreciated after 90 minutes of ischemia (**C**). (**A**, ×250; **B**, ×640; **C**, ×640)

thus becomes particularly energy-demanding. Furosemide and ouabain inhibit chloride transport by different mechanisms and produce a marked decrease in oxygen uptake by isolated cells of the mTAL. Therefore, both agents appear to be good probes with which to examine the effect of decreasing energy utilization on O_2 deprivation in this nephron segment.

Protection from the effect of 90 minutes of hypoxic perfusion by either ouabain or furosemide treatments or by the nonfiltering mode demonstrates that decreasing the tubular energy requirement can markedly attenuate or prevent the effects of anoxia. Table 1 shows that during low O_2 perfusion, O_2 delivery, O_2 consumption, and venous P_{O_2}, of the whole kidney, were initially very low, and were not depressed further by ouabain, by furosemide, or by halting GFR. Under normal oxygenation, these maneuvers can be shown to cause significant decreases in O_2 consumption.[16,17] With low O_2 perfusion, it appears from the low figures for fractional sodium reabsorption that the limited O_2 supply placed many cells below their critical P_{O_2} levels[12] and greatly restricted active sodium reabsorption, probably in all parts of the kidney. It is notable, though not surprising, that O_2 consumption was insignificantly affected by the addition of ouabain of furosemide or by halting GFR. Nevertheless, anoxic injury to mTAL cells was effectively prevented by these measures that inhibited active transport, presumably because the cellular demand for oxygen was modified. At the very low ambient P_{O_2} of these experiments, it seems unlikely that the primary mechanism of protection was to increase local P_{O_2}.

The prominent toxic effect of cyanide is considered to be inhibition of the mitochondrial respiratory chain. Cyanide binds to the cytochrome-a,a_3 (the terminal enzyme of the respiratory chain), prevents further transport of the electrons directly to oxygen, and thus mimics the effect of oxygen deprivation. In addition, inhibition of the respiratory chain by cyanide is not modified by variations in the local

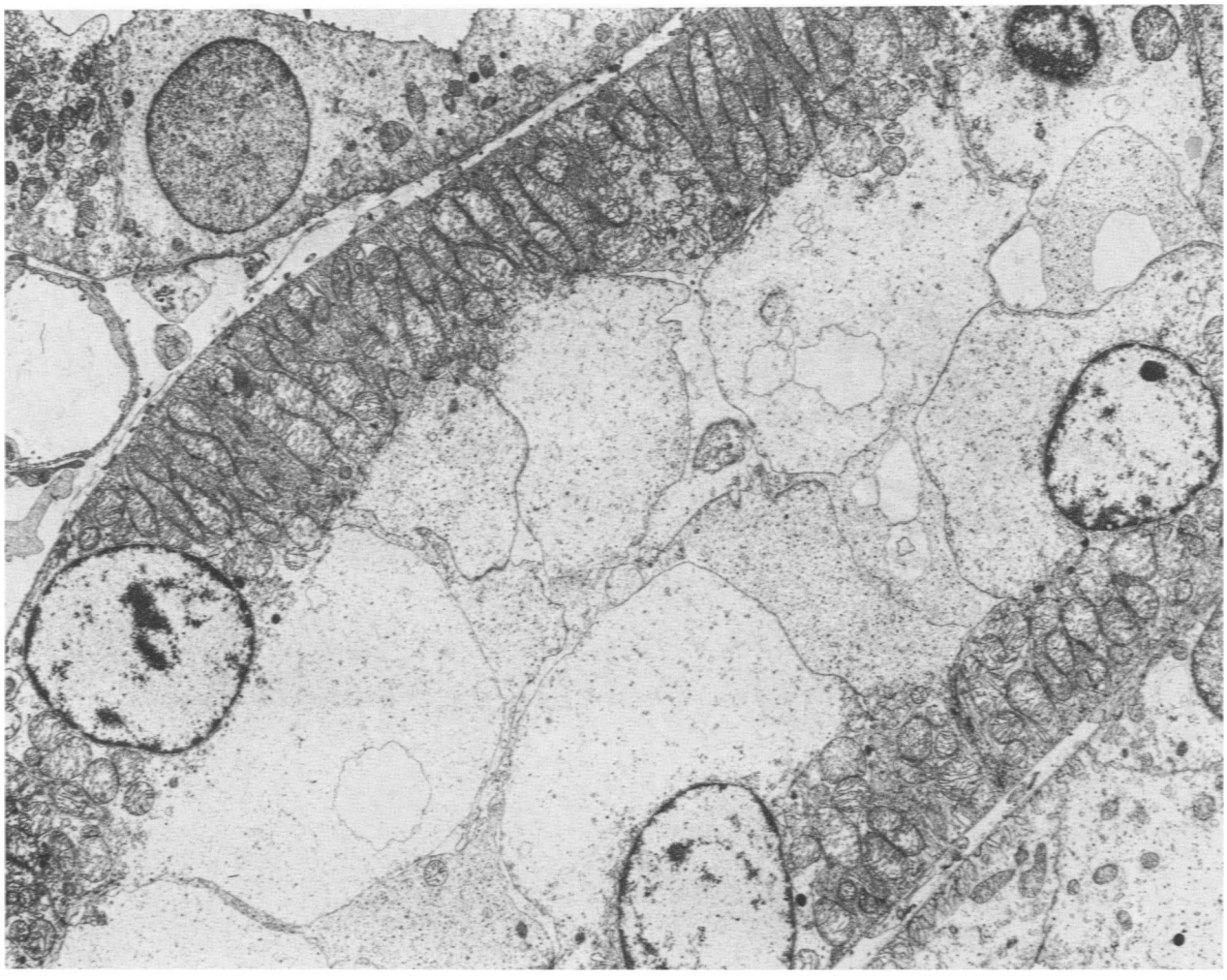

Figure 11 – Ischemia (renal artery occlusion). Electron microscopic study of mTAL at 90 minutes of ischemia shows cytoplasmic edema and blebbing of luminal membranes, but luminal and basolateral membranes are relatively preserved, chromatin margination is present, and mitochondrial swelling is evident. The adjacent collecting ducts (upper left and lower right) are relatively well maintained. (×4000)

availability of oxygen.[18] In the present experiments, reproduction of the severe damage by KCN at 2–3 mM supports the anoxic nature of the mTAL lesion during isolated perfusion.[8] Prevention or attenuation of KCN-induced damage at concentrations varying from 10^{-3} to 10^{-2} M by pretreatment with ouabain suggests again that the protection may not result from a simple increase in O_2 availability, since that would not be expected to affect cyanide-induced inhibition of mitochondrial electron transport. Taken together, these observations show that a reduction in cellular energy demand for active ion transport prevents cell necrosis in mTALs deprived oxygen.

The mTAL damage caused by hypoxic perfusions was compared with the changes seen after warm ischemia for identical time periods. Although in the absence of blood flow, oxygen deprivation is more severe and essentially absolute (tissue P_{O_2} is zero[12]) and the exogenous supply of glucose (which could support anaerobic glycolysis) is stopped, warm ischemia does not reproduce the severe mTAL damage seen during hypoxic perfusion. Comparison of ischemic and anoxic damage is complicated by multiple uncontrolled variables in ischemia such as acidosis, tissue osmolarity, or availability of calcium and water. Ischemic kidneys were reperfused to allow possible delayed morphologic expression of cell death. These experiments were performed in the presence of ouabain to prevent the production of new damage during the perfusion. Not only did the changes observed at the end of 90 minutes of ischemia not progress, but in fact, they were essentially reversed as early as 15 minutes and as long as 90 minutes after reflow. Although additional protective factors could be operative, these observations are compatible with the notion that during ischemia, the concomitant abolition of glomerular filtration and solute delivery to the mTAL (comparable to the situ-

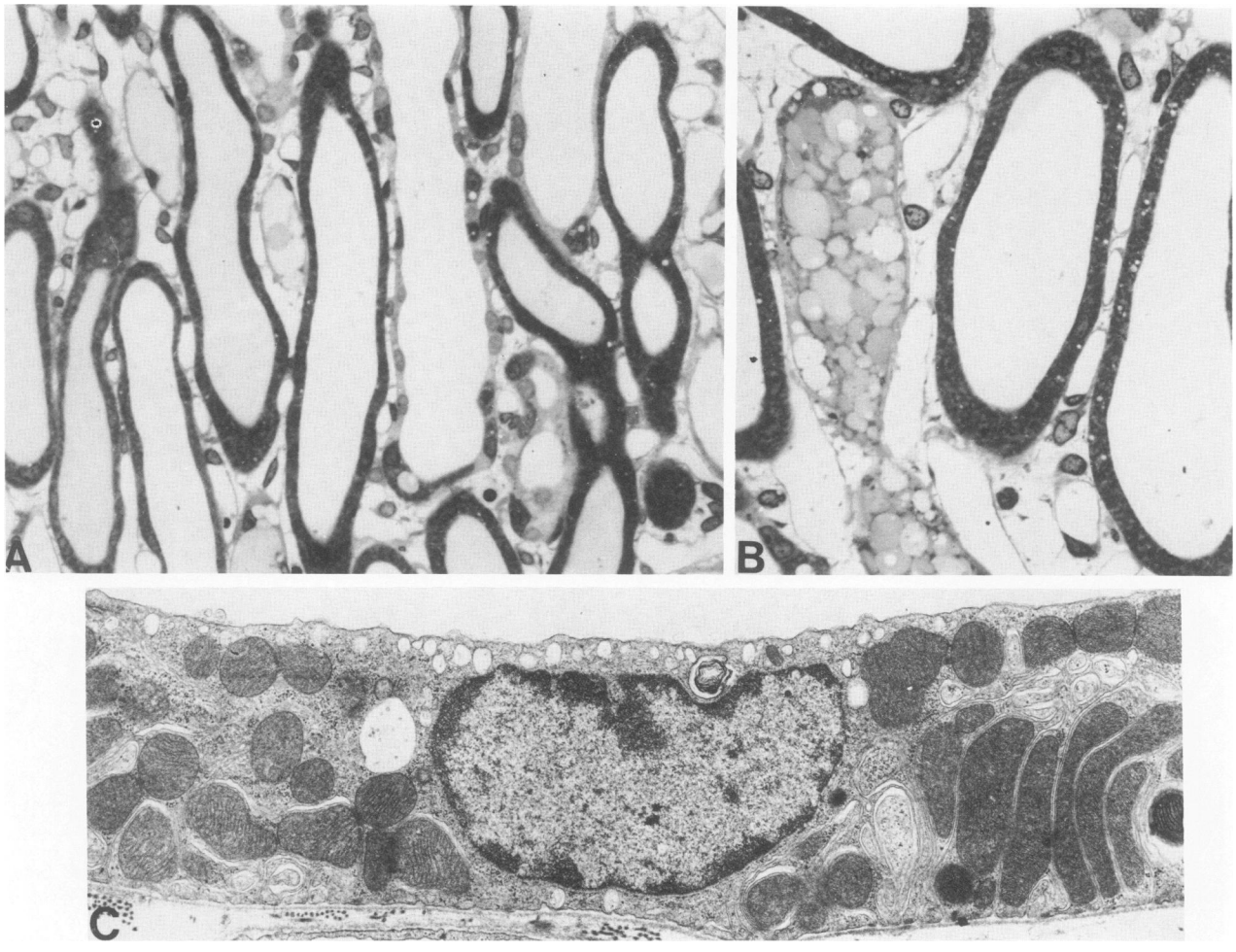

Figure 12—Ischemia with subsequent perfusion. Renal ischemia for 90 minutes was followed by perfusion for 90 minutes to allow delayed expression of damage. Ouabain (10^{-2} M) was included in the perfusate to prevent transport dependent injury. The mTAL changes observed after 90 minutes of ischemia (see Figure 11) have disappeared (**A** and **B**). The thin limb of Henle's loop (*right*, **B**) contains abundant cytoplasmic debris. Electron microscopy confirms the absence of cellular edema, the essentially unremarkable mitochondria and nuclei, and the preservation of luminal and basilar membranes. (**A**, ×240; **B**, ×600; **C**, ×12,000)

ation of the nonfiltering kidneys) may be protective by the reduction of tubular energy demand for active reabsorption.

The concept suggested by the present study is that continued glomerular filtration, with consequent solute delivery and demand for transport work imposed on the mTAL, increases the need for O_2 and exacerbates the mismatch between demand and supply, thereby accelerating anoxic injury. This may explain why the injury to the mTAL during isolated perfusion was not originally attributed to hypoxia,[4] since ischemic damage to the mTAL is known to be milder and to occur later than in the proximal tubules.[19] Our observations suggest that the severity of the anoxic damage depends on the *state of energy demand for transport work* at the time of limited O_2 supply. The greater the demand for energy, the worse will be the injury.*

The mechanism by which increasing energy utilization in the face of limited O_2 supply precipitates cell damage may involve adenosine triphosphate (ATP) depletion. Energy stores of the cell have been shown to decrease rapidly during anoxia, and ATP depletion has been thought to play an important role in the mediation of anoxic cell injury.[21] Continued cell work could conceivably accelerate the depletion of energy stores. ATP depletion, however, does not consistently reproduce anoxic cell injury (Farber[22]; our own unpublished observations). Alternatively, a high rate of ATP utilization stimulates mitochondrial activity and could therefore increase the production of oxygen-

* We have very recently shown that an increase in energy consumed in active transport induced by polyenes (such as amphotericin) produces anoxic-like damage which is prevented by ouabain.[20]

free radicals. Oxygen-free radicals have recently been suggested to play a role in ischemic injury in the heart,[23] the intestine,[24] and possibly the central nervous system.[25] Free radicals are a by-product of CN-insensitive respiration, which is a function of metabolic activity, and is accelerated by factors that enhance mitochondrial electron transport.[26] Diminution of cell work could be protective by decreasing the formation of these free radicals.

Whatever mechanism is operative, it seems conceivable that a general principle by which cell work would accelerate anoxic cell death may explain the relative vulnerability of certain tissues to anoxia such as the brain, the heart, and the outer medulla of the kidney. The latter may in fact represent an important, if not crucial, target in renal hypoperfusion[8] leading to acute renal failure by activation of tubuloglomerular feedback.[27] The protection of cells from anoxic cell death by preventing work could have important clinical applications in the kidney, as suggested by recent observations,[28,29] and may shed some light on the mechanisms of anoxic cell death in general.

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