BIOCHEMICAL MEDICINE AND METABOLIC BIOLOGY 39, 319–329 (1988)

Adenine Nucleotide Metabolism by Isolated Kidney Tubules during Oxygen Deprivation

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Received September 28, 1987

Degradation of cell nucleotides during ischemic tissue injury is well documented and has been characterized in some detail for several tissues (1–11). This process may contribute to cell injury by limiting the availability of nucleotide precursors during recovery (7–9) and as a result of deleterious effects of reactive oxygen metabolites generated during metabolism of hypoxanthine and xanthine by xanthine oxidase (12,13). Published data for the kidney have been collected during *in vivo* models of renal ischemia (1–6). *In vivo* studies, however, do not allow assessment of the individual contributions of the various different cell types within the kidney to the observed changes and generally sample from a rather heterogenous pattern of injury. The recent development of models of oxygen deprivation injury to isolated rabbit proximal tubules (14,15) has provided the opportunity to assess tubule purine nucleotide metabolism during oxygen deprivation in a particularly well-controlled setting which allows direct measurements in tubule cells during both reversible and irreversible forms of cell injury. This manuscript details the patterns of purine degradation produced by oxygen deprivation injury to the tubule preparation.

MATERIALS AND METHODS

Animals. New Zealand White rabbits weighing an average of 2.5 kg were used for these studies.

Tubule isolation. Suspensions enriched in proximal tubules were isolated from renal cortex using collagenase to separate the tubules then Percoll (Pharmacia, Piscataway, NJ) to enrich a proximal tubule fraction as detailed in recent publications (14,15).

General procedures for tubule incubation. The final tubule pellet at the end of the isolation procedure was resuspended to a concentration of 5–7.5 mg tubule protein/ml in a 95% O_2/5% CO_2 gassed ice-cold solution containing 105 mM NaCl, 2.1 mM KCl, 25 mM $NaHCO_3$, 2.4 mM KH_2PO_4, 1.2 mM $CaCl_2$, 2.5 mM

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0885-4505/8 $3.00
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$MgSO_4$, 0.6% dialyzed dextran (Pharmacia T40), 5 mM glucose, 4 mM lactate, 1 mM alanine and 10 mM sodium butyrate. Five-milliliter aliquots of this tubule suspension were placed in siliconized 25-ml Erlenmeyer flasks which were then gassed with 95% O_2/5% CO_2, sealed, and kept on ice until use. As they were needed for experiments flasks were placed in a shaking water bath at 37°C and were gently shaken for the desired periods of warm incubation.

Oxygen deprivation of tubule suspensions. To oxygen-deprive the preparation, flasks were gassed with 95% N_2/5% CO_2 for 5 min. This reduces the medium O_2 concentration to less than 10 μM which results in a high and consistent degree of oxygen deprivation-induced injury (14,15). The nature and degree of oxygen deprivation were further modulated by controlling the incubation conditions during oxygen deprivation to produce either hypoxic conditions at pH 7.4 or 6.6 or a high tubule density simulating more of the conditions present during ischemia *in vivo* (14, 15). For hypoxia studies, tubules were kept in the metabolic shaker for the entire duration of oxygen deprivation. In studies where a pH of 6.6 during hypoxia was used, hydrochloric acid was added to adjust the pH at the beginning of hypoxia, and, where a reoxygenation period was assessed, $NaHCO_3$ was added to return the pH to 7.4 at the end of hypoxia.

To study tubules oxygen-deprived under high density pelleted conditions, the contents of a flask were gassed with 95% N_2/5% CO_2 for 5 min, then were rapidly transferred to a 15-ml centrifuge tube which was gassed with 95% N_2/5% CO_2, sealed, and centrifuged in a table-top centrifuge for 15–30 sec, a time sufficient to lightly pellet all tubules at the bottom of the centrifuge tube. The tube was then incubated without shaking at 37°C for the desired duration of oxygen deprivation. At the end of the oxygen deprivation period these tubules were resuspended in the full volume of incubation medium by gently inverting the tube several times and either sampled or regassed with 95% O_2/5% CO_2 for continued incubation under oxygenated conditions.

Measurement of purine levels. To measure total suspension purine levels, 0.4 ml of tubule suspension was added to an equal volume of 12% TCA in a 1.5-ml microcentrifuge tube which was vigorously mixed and then centrifuged. The supernatant was removed from the protein pellet and was neutralized and extracted by mixing with an equal volume of 0.5 M trioctylamine (Aldrich) in Freon-113 (Matheson, East Rutherford, NJ). Separation of the Freon and aqueous layers was facilitated by a brief centrifugation. Then the aqueous layer was removed, filtered through 0.45-μm nitrocellulose filters, and frozen.

To measure medium nucleotides the tubule suspension was centrifuged for 30 sec at 12,000*g* in a microcentrifuge. Then a sample of the supernatant was immediately added to an equal volume of 12% TCA. Subsequent steps were identical to those described above for measurement of total suspension nucleotide levels.

In most of the studies in this manuscript adenine nucleotides were quantified by HPLC at an absorbance of 254 nm with a Whatman Partisil 10 SAX strong anion exchange column and a gradient elution system utilizing 5 mM $NH_4H_2PO_4$, pH 3.0 (Buffer A), and 0.5 M $NH_4H_2PO_4$, pH 4.50 (Buffer B), at a flow rate of 1.5 ml/min. Samples were initially eluted isocratically with Buffer A for 6 min

to elute AMP. The percentage of B was then increased over 30 min to 50% to elute ADP. The percentage of B was then further increased over 5 min to 100% which was continued between 35 and 45 min to elute ATP. The column was then reequilibrated with Buffer A for 15 min prior to injection of the next sample.

In several of the experiments nucleotides were quantified on 20-μl aliquots of sample by HPLC at an absorbance of 254 nm with a Beckman (Fullerton, CA) C_{18} ion pairing reversed phase column using an isocratic mobile phase consisting of 19% acetonitrile, 40 mM KH_2PO_4, 10 mM tetrabutylammonium dihydrogen phosphate, pH 3.5, at 24°C and a flow rate of 1 ml/min. There was good agreement when nucleotides were measured on the same samples by both methods.

Intracellular nucleotide levels were estimated by subtracting the nucleotide levels measured in the tubule suspending medium after removal of tubules by high speed centrifugation from the total suspension levels. Measured nucleotides were factored by milligrams of total protein (16) present in the tubule suspension prior to separation of tubules by centrifugation.

Nucleosides, bases, and inosine monophosphate (IMP) were determined on 5-μl aliquots of sample with the same C_{18} column as for nucleotide measurements using an isocratic mobile phase consisting of 5% acetonitrile, 20 mM KH_2PO_4. 20 mM tetrabutyl dihydrogen ammonium phosphate, pH 2.7, at 25°C and a flow rate of 1 ml/min. This procedure allows rapid, highly reproducible measurements but cannot be used for sample volumes in excess of 10 μl due to loss of definition of the peaks for adenosine, hypoxanthine, and xanthine. Levels of intracellular nucleosides are too low to resolve with this method but medium levels after oxygen deprivation-induced breakdown of cell nucleotides can be readily followed. For both the nucleotide and nucleoside assays standard curves were generated using purine solutions of known composition in medium used and processed as for tubule samples.

Measurement of protein recovery. The proportion of total suspension protein recovered when tubules are rapidly separated from their medium by centrifugation through bromododecane is a useful index of the extent to which tubules have maintained their structural integrity (14,15). To assess this 0.4 ml of 12% trichloroacetic acid in a 1.5-ml microcentrifuge tube was overlaid with 0.7 ml of bromododecane. Tubule suspension (0.4 ml) was gently layered on the bromododecane and was immediately centrifuged at 12,000*g* for 30 sec. Total suspension protein and protein recovered in the trichloroacetic acid layer were measured by the Lowry assay using bovine serum albumin as a standard (16).

Reagents. All reagents were of the highest grade commercially available. Biochemial reagents were obtained from Sigma (St. Louis, MO). Allopurinol was used as a 13.33-mM stock solution prepared fresh daily in 15 mM NaOH. Allopurinol was present throughout tubule incubation at 37°C including the 15 min prior to oxygen deprivation, oxygen deprivation and, where studied, reoxygenation.

Statistics. Most studies involved multigroup comparisons and repeated-measures designs and were, therefore, initially assessed by analysis of variance using models accounting for repeated measures (BMDP Statistical Software, Los Angeles, CA) (17). Individual group comparisons in these multigroup studies were then

TABLE 1
Protein Recovery after Centrifugation through Bromododecane of Tubules Subjected to Oxygen Deprivation[a]

	Oxygen deprivation	Oxygen deprivation + reoxygenation
	Percentage recovery[b]	
Group I		
Hypoxia, pH 7.4	40.7 ± 7.0	33.6 ± 4.1
Hypoxia, pH 6.6	97.8 ± 3.4*	92.6 ± 5.6*
High density	95.5 ± 4.5*	97.5 ± 2.5*
Group II		
30 min	95.3 ± 5.4	85.0 ± 5.1
30 min, 200 μM allopurinol	87.3 ± 0.8	88.8 ± 5.2
60 min	73.3 ± 1.7	70.2 ± 2.1
60 min, 200 μM allopurinol	72.5 ± 1.8	71.3 ± 6.0

[a] Protein recoveries of paired control oxygenated tubules corresponding to these experimental groups ranged from 90.0 to 97.8%. Group I tubules were oxygen-deprived for 30 min under the indicated conditons. Group II tubules were oxygen-deprived under high density conditions for the indicated durations.

[b] Values are means ± SE for experiments on 4–6 separate preparation.

* $P < 0.01$ vs. corresponding pH 7.4 group.

made using the Neuman-Keuls test (18). Where only two groups were being studied, paired or unpaired *t* tests were used as appropriate.

RESULTS

Table 1 summarizes data on the percentage of tubules sufficiently intact to be recovered by centrifugation through bromododecane under each of the three major oxygen deprivation conditions tested, hypoxia at pH 7.4, hypoxia at pH 6.6, and high density incubation conditions. This parameter correlates well with other indices of cell injury (14,15) and serves as a rapid way of quantitating the extent of injury in this type of preparation. The majority of tubules subjected to oxygen deprivation under low density hypoxic conditions at pH 7.4 sustained severe irreversible injury and the percentage of intact tubules dropped markedly. In contrast, tubules handled identically except for the presence of a medium pH of 6.6 during the period of oxygen deprivation maintained their structural integrity. A similarly decreased susceptibility to irreversible injury during 30 min of oxygen deprivation was seen in the tubules incubated at high density as a pellet during oxygen deprivation. This incubation condition is accompanied by a decrease of pH within the tubule pellet to approximately 7.0 (14).

Figure 1 summarizes the changes of intracellular AMP and ATP which occurred during each type of oxygen deprivation. As seen *in vivo* (1–6), ATP fell during oxygen deprivation and recovered with reoxygenation while AMP increased during oxygen deprivation and fell during recovery. Cell ATP and AMP levels at the end of oxygen deprivation were no higher in hypoxic tubules at pH 6.6 than in those kept at pH 7.4; however, both nucleotides were slightly but significantly greater in the tubules incubated at high density than in the pH 7.4 hypoxic

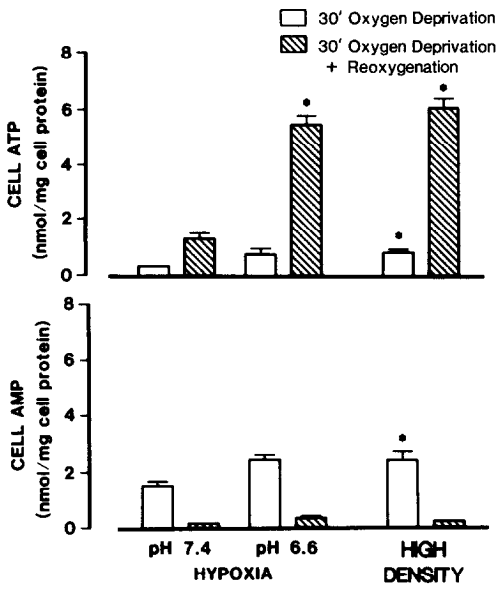

FIG. 1. Cell AMP and ATP levels during oxygen deprivation under hypoxic conditions at pH 7.4 or 6.6 or under high density pelleted conditions and following oxygen deprivation and 60 min reoxygenation under each of the same conditions. Values are means ± SE for experiments on four to five separate tubule preparations. $^*P < 0.05$ vs corresponding pH 7.4 hypoxia group. Control oxygenated preparations had ATP and AMP levels averaging 7.2 ± 0.27 and 0.34 ± 0.07 nmole/mg tubule cell protein, respectively.

tubules. After 60 min reoxygenation cell ATP levels recovered best during the conditions which allowed maintenance of tubule cell integrity, hypoxia at pH 6.6, and high density oxygen deprivation. The ATP levels measured after recovery from hypoxia at pH 7.4 were substantially less than those seen in control oxygenated tubules and in the pH 6.6 and high density groups, even when adjusted for differences in the number of intact cells present.

The metabolic fate of the lost intracellular nucleotides is shown in Fig. 2 which summarizes changes in medium purine levels. AMP, inosine, and hypoxanthine were detected in substantial quantities. The largest increases during oxygen deprivation were of hypoxanthine. Its level contined to rise when tubules were reoxygenated after hypoxia at pH 7.4. Lower hypoxanthinc levels were seen after hypoxia at pH 6.6 and high density oxygen deprivation and these did not decrease during reoxygenation.

Neither adenosine nor xanthine was detected in quantities greater than the 0.3 nmole/mg protein resolution of the assay system for those compounds under any of the conditions tested. IMP levels at the end of 30 min oxygen deprivation were 0.47 ± 0.11 and 0.45 ± 0.13 nmole/mg cell protein in the hypoxia, pH 7.4, and high density groups, respectively. They decreased to less than 0.30 nmole/mg cell protein during reoxygenation, a level similar to that seen in oxygenated control tubules.

Figure 3 summarizes the changes of all adenine nucleotides, including ADP,

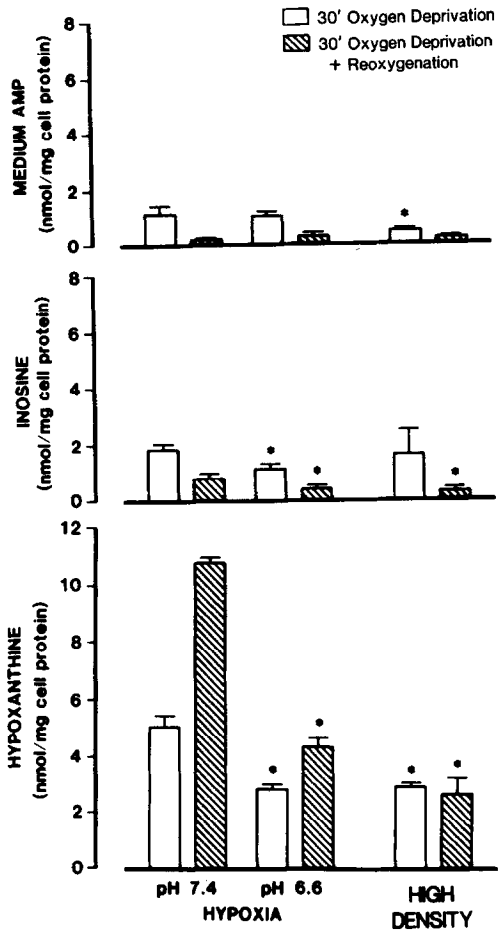

FIG. 2. Medium AMP, inosine, and hypoxanthine levels for the same experiments detailed in Fig. 1. Medium AMP levels of control oxygenated preparations were 0.21 ± 0.07 nmole/mg tubule cell protein. Control medium inosine and hypoxanthine levels were less than 0.3 and 1.2 nmole/mg protein, respectively. Labels and conditions are otherwise the same as detailed for Fig. 1.

and their nucleoside and base degradation products which occurred in each of the models. Presentation of the data in this format emphasizes that most recovery of cell ATP during reoxygenation was from the remaining cell nucleotides rather than from the nucleosides and bases.

The xanthine oxidase inhibitor, allopurinol, has been reported to have beneficial effects on the outcome of ischemic acute renal failure *in vivo* in a number of species, including the rabbit (19–21). The minimal metabolism of hypoxanthine and its minimal apparent reutilization (Figs. 2 and 3) make it less likely that inhibition of xanthine oxidase in tubule cells can account for these actions of allopurinol. To directly test the effects of allopurinol, purine levels were assessed during 30 and 60 min of high density oxygen deprivation. Protein recovery after centrifugation through bromododecane for each of these experimental conditions

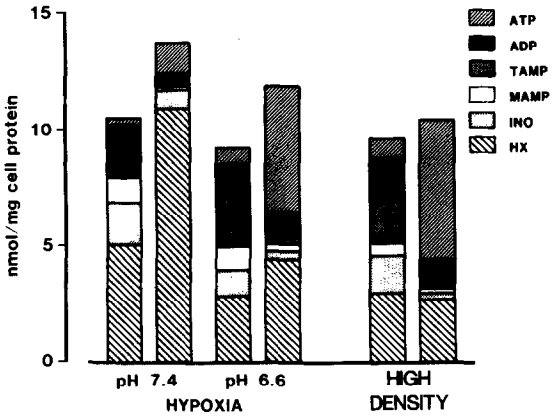

FIG. 3. Overall summary of tubule cell ATP, ADP, and AMP (TAMP) and medium AMP (MAMP), inosine (INO), and hypoxanthine (HX) for the experiments detailed in Fig. 1.

is summarized in Table 1 (Group II experiments). A moderate decrease was seen as the period of oxygen deprivation was extended from 30 to 60 min. Allopurinol (200 μM) did not alter the percentage protein recovery or any of several other parameters of cell injury.

Concentrations of the major purine metabolites after treatment with allopurinol are illustrated in Fig. 4. No effects of allopurinol on the pattern of alterations produced by oxygen deprivation and reoxygenation were detected. Of interest, however, our HPLC separation procedure used to measure nucleoside levels clearly separated the allopurinol. Its concentration decreased during incubation with the appearance of a new peak having exactly the same retention time as pure samples of the allopurinol metabolite, alloxanthine (data not shown).

To further test the potency of allopurinol in our experimental system, oxygenated tubules were incubated with 250 μM exogenous ATP (approximately 50 nmole ATP/mg tubule protein) with or without added xanthine oxidase. In the absence of xanthine oxidase, the exogenous ATP was degraded to hypoxanthine without substantial further metabolism. Xanthine oxidase metabolized virtually all of the hypoxanthine. Allopurinol did not significantly alter the metabolism of the added ATP in the absence of xanthine oxidase but it almost completely inhibited degradation of hypoxanthine by the xanthine oxidase (Fig. 5).

DISCUSSION

Purine nucleotide degradation during oxygen deprivation-induced cell injury has been detailed for a variety of tissues including the kidney (1–11), but relatively little information has been available with regard to the contribution of renal tubule cells themselves as opposed to other components of the intact kidney including vascular endothelial cells, interstitial cells, and intravascular cells.

The importance of such an analysis is highlighted by the extensive work on myocytes showing that purine degradation in those cells does not proceed substantially beyond inosine. The hypoxanthine and uric acid formed during tissue

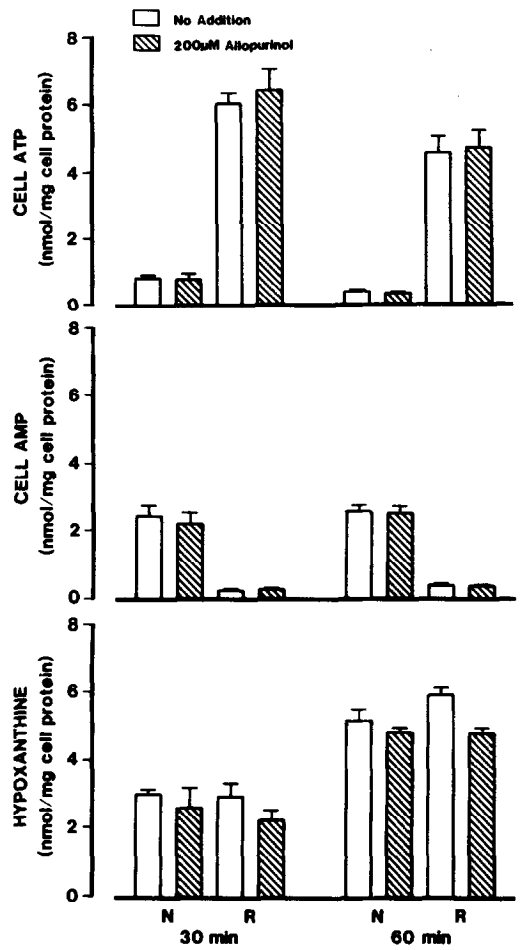

FIG. 4. Cell ATP, cell AMP, and medium hypoxanthine levels of tubule preparations subjected to 30 or 60 min oxygen deprivation under high density conditions (N) or oxygen deprivation plus reoxygenation (R) with and without 200 μM allopurinol. Values are means $\pm$SE of four to six experiments on separate tubule preparations.

ischemia are largely derived from metabolism within endothelial cells (7,8). These are important distinctions when considering both the availability of metabolites for reincorporation into cell nucleotides as well as the contribution of purine metabolism to injurious processes such as reactive oxygen metabolite formation during metabolism of hypoxanthine by xanthine oxidase (12,13). Metabolites generated outside of the major parenchymal cell of the tissue are less available to participate in either of these processes.

The bulk of the renal cortex is composed of proximal tubule cells; they are highly dependent on oxidative metabolism as their energy source (22) and injury to both outer medullary and cortical proximal tubule segments is prominent in experiment models of acute renal failure (23). The tubule preparation used in this manuscript allows for study of the metabolic consequences of oxygen dep-

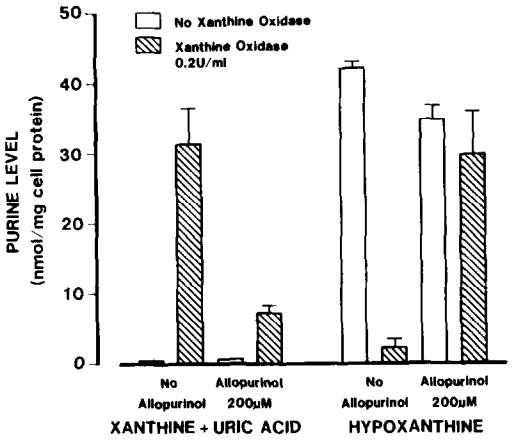

FIG. 5. Effects allopurinol on the activity of exogenous exanthine oxidase added to the tubule preparation. Oxygenated tubules were treated with 250 μM exogenous ATP and various combinations of 0.2 U/ml xanthine oxidase (Sigma Grade III) and 200 μM allopurinol. Purines were measured after 75 min incubation. Values of means $\pm$SE for experiments on four separate tubule preparations.

rivation directly on tubule cells without contributions from other cell types, permits controlled assessment of both reversible and irreversible forms of injury, and allows for good identification and quantification of these stages of injury.

The results of the present studies are similar to those reported previously (14,15) in that severity of injury produced by oxygen deprivation was importantly conditioned by pH and density. Incubating tubules at pH 6.6 or at high density as pellets did not substantially ameliorate the ATP depletion produced by oxygen deprivation but prevented the loss of structural integrity which impaired recovery during reoxygenation.

In severely injured tubule preparations the predominant purine present was hypoxanthine indicating the capacity of tubules themselves to metabolize purines that far. Hypoxanthine was not converted to xanthine or uric acid, suggesting little or no effective xanthine oxidase activity in the tubules. The absence of allopurinol effects on purine degradation is consistent with this. Studies of *in vivo* ischemia have also failed to document substantial metabolism of hypoxanthine in the rabbit (4,20). In contrast, this process appears to be more prominent in rat (6) and dog (24) kidney. Whether xanthine oxidase is totally absent in the rabbit kidney is not clear from the present studies. The fact that allopurinol itself was apparently metabolized to alloxanthine (25) suggests that some enzyme was present but its activity toward its physiological substrates was low under the experimental conditions used.

Not addressed in prior *in vivo* studies, but readily evident from the data in this manuscript, is the fact that hypoxanthine was not salvaged to any appreciable extent. Even though ATP levels did recover when hypoxic pH 7.4 tubules were reoxygenated, this recovery appeared to be entirely from the remaining intracellular nucleotides. The inability of the tubules to use hypoxanthine is similar to that observed when oxygenated tubules were treated with exogenous hypoxanthine (15). Myocytes also salvage hypoxanthine poorly, an effect which has been

attributed to low activity of adenylosuccinate synthetase (26). Whether this is also the case for tubule cells remains to be determined, but the absence of substantial IMP accumulation in the tubules argues against this mechanism.

The cumulative values in Fig. 3 make it appear as if there was a moderate increase of the total purine pool during reoxygenation of hypoxic tubules. Rather than de novo synthesis this likely represents the contribution of continued guanine nucleotide degradation to the hypoxanthine levels measured.

The less injured preparations, hypoxic tubules at pH 6.6 and tubules incubated at high density, showed much less breakdown of their adenine nucleotides to hypoxanthine. This suggests that the latter process is most prominent when cell integrity is lost. As seen in the tubules made hypoxic at pH 7.4, however, there was no further metabolism of the hypoxanthine and recovery of ATP levels was from the remaining cell nucleotides.

Degradation of AMP may be via deamination to IMP or dephosphorylation to adenosine (8). There is evidence for both processes in the kidney (27–29). The data in this manuscript do not indicate which one predominated since neither adenosine nor IMP accumulated preferentially. Further studies with adenosine deaminase inhibitors will be required to resolve this issue.

Inhibition of xanthine oxidase by allopurinol has been proposed as a measure useful for ameliorating oxygen deprivation-induced cell injury. Initial interest focused on the potential for allopurinol to preserve the purine pool (19) while more recent studies have emphasized its ability to prevent generation of reactive oxygen metabolites (2,13,21). The data in this paper provide little evidence to support either effect of allopurinol in the rabbit kidney. This is somewhat surprising in view of protective effects against *in vivo* renal ischemia which have been described in the rabbit (20). It is of interest, however, that the latter study failed to clearly document hypoxanthine metabolism to xanthine. This suggests that protective effects of allopurinol at the whole tissue level may involve other mechanisms. A similar conclusion was recently reached in studies of the effects of allopurinol on ischemic injury to rabbit heart (30).

SUMMARY

Suspensions enriched in isolated rabbit proximal tubules were subjected to varying degrees of oxygen deprivation-induced injury by incubating them under hypoxic conditions at pH 7.4 or pH 6.6 or under high density pelleted conditions and adenine nucleotide degradation was characterized. The major metabolite was hypoxanthine. Its levels increased with the extent of irreversible injury. It was not further degraded or salvaged. Recovery of cell ATP during reoxygenation was predominantly from the remaining cell nucleotides. Allopurinol did not alter the pattern of purine metabolism or the extent of cell injury. These observations provide information on the intrinsic purine metabolic capacity of renal tubule cells during oxygen deprivation which is relevant to understanding both the salvage mechanisms available in these cells as well as the contribution of purine metabolism to the pathogenesis of oxygen deprivation-induced tubule cell injury.

ACKNOWLEDGMENTS

These studies were supported by NIH Grant DK-34275 and the Veterans Administration. Dr. Weinberg is the recipient of NIH RCDA DK-01337. The technical assistance of Anne Lawton was invaluable. Some of the data were reported in abstract form in *Kidney Int.* **29,** 296 (1986).

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