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On the Molecular Pathology of Ischemic Renal Cell Death

Reversible and Irreversible Cellular and Mitochondrial Metabolic Alterations

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CELL DEATH is one of the commonest reactions of tissues seen in disease, and ischemia is perhaps the most important among its protean etiologic agents. Despite the frequency of this cellular response and the many studies concerned with its pathogenesis, the underlying molecular events are still poorly understood. Since many instances of ischemic necrosis follow only temporary interruption of the local circulation, it is possible theoretically to prevent the irreversibility of the process by appropriate therapy, if and when the sites and mechanism(s) of irreversible damage are identified at the molecular level. Such information would not only have important practical application but would also give new insight into the integration and balance of cellular metabolic processes and might lead to the development of more sensitive indexes of cell death than are now available.

Two general ideas have gradually emerged from the many studies on ischemic necrosis: [1-10] The first states that a major factor in the initiation of cell death in the ischemic tissue is the progressive accumulation

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of acid, especially lactic acid, with the concomitant denaturation of protein and interference with those essential metabolic reactions sensitive to a decrease in pH. The second general hypothesis suggests that the irreversible damage to the cell is a consequence of the marked lowering of the available energy supply. The over-all purpose of this study was to begin to test each of these hypotheses.

Since mitochondria are the organelles most concerned with the major energy supply in aerobic cells and are among the first organelles to show ultrastructural changes during ischemia, it was decided to concentrate first upon the functional alterations of kidney cortex mitochondria during the reversible and irreversible stages of kidney ischemia. In addition, certain experiments were carried out to test whether the accumulation of acid plays a major role in the genesis of this lesion. The results of these studies are the subject of this communication.

Materials and Methods

White male rats of the Wistar strain (Carworth Farms) weighing 200–300 gm. and maintained on Wayne Laboratory Blox were used. The rats were deprived of food overnight and then anesthetized with Nembutal (0.6 mg./gm. body weight). The abdominal wall was opened along the midline and the renal pedicles were dissected free from connective and adipose tissue. A small rubber-covered bulldog clamp was used to occlude the left renal pedicle close to the kidney. The animals were wrapped in moistened gauze-padding around the area of incision to minimize loss of body heat. After varying periods of ischemia, the kidneys were either removed from the animal and studied, or the clamp was removed and the left kidney allowed to recover for varying periods of time. The right kidney was used as a control in the histologic studies. In all chemical and metabolic studies, only the cortex was used. The cortex was rapidly separated in the cold from the medulla. This dissection was facilitated by the difference in color between the two major zones. The left kidneys from sham-operated rats were used as the controls.

Histologic Studies

The kidneys were removed, cut into 3 pieces and fixed in Stieve's solution for 18–24 hr. After 2 washes in 95% alcohol, the tissue was embedded, and sections were stained with hematoxylin and eosin.

Chemical Determination

For ATP determination, the kidney cortex was rapidly cut away from the medulla and a known weight was homogenized in 3.2% (w/v) $HClO_4$. ATP was determined by the luciferin-luciferase reaction.[11] Lactic acid was determined by the method of Barker and Summerson,[12] using kidney cortex homogenized in 3.2% (w/v) $HClO_4$. Protein was determined with the biuret method as modified by Gornall *et al.*,[13] using trichloracetic acid (TCA) precipitates of tissue or tissue fractions.

Preparation of Mitochondria

Approximately 1 gm. of renal cortex (from 3–4 kidneys) was homogenized in a Teflon-glass homogenizer using enough 0.3 M sucrose to give a 10% homogenate.

This homogenate was spun at 650 × g for 10 min. to remove the nuclei and debris. The supernatant was spun at 7600 × g for 15 min. The sedimented mitochondria were then washed with an equal volume of sucrose, centrifuged, and sedimented again. Finally, the mitochondria were resuspended in about 6 ml. 0.3 M sucrose containing 1 mg. bovine serum albumin per milliliter. In the polarographic experiments, the mitochondria were prepared in 0.25 M mannitol, and the time of each centrifugation was reduced by 25%. Occasional examination of the final mitochondrial suspension with the light and electron microscope showed the preparations to be free from nuclear contamination and to be only slightly and irregularly contaminated with other cytoplasmic components.

Manometric Experiments [14]

The incubation medium contained KH_2PO_4, 50 μmoles; sucrose, 6 μmoles; $MgCl_2$, 42 μmoles; Na_2EDTA, 1.5 μmoles; glycylglycine, 50 μmoles; Na-ATP, 3.5 μmoles; Na-AMP, 3.5 μmoles; Na pyruvate, 30 μmoles; Na fumarate, 3 μmoles; and 0.3 ml. mitochondrial suspension in a total volume of 2.8 ml., pH 7.0. The center well contained 0.2 ml. 20% KOH with a piece of folded filter paper, and the side arm contained 180 μmoles glucose and 0.5 mg. Type III Sigma yeast hexokinase in 0.2 ml. water. This hexokinase contained 12–16 enzyme units per milligram protein where 1 U. of activity is defined as that which will catalyze the phosphorylation of 1 μmole of glucose per min. at pH 8.5 at 25° C. The flasks were allowed to equilibrate at 37° C. for 5–10 min. After tipping, the oxygen uptake was followed for 20 min. The flasks were then removed, plunged into ice, and 0.5 ml. cold 50% trichloracetic acid was added to stop the reaction. The trichloracetic acid supernatant was analyzed for inorganic phosphate using the elon method.[15] The respiratory quotient (QO_2) is expressed as microliters of oxygen uptake per hour per milligram of protein. The P:O ratio is the moles of PO_4 esterified per atom O_2 consumed.

Experiments with the Oxygen Electrode

An oxygraph from Gilson Medical Electronics was employed. The mitochondria were prepared in 0.25 M mannitol as described above. The incubation medium was that described by Hagihara.[16] The total volume was 1.8 ml., and the temperature was 25° C.

Mitochondrial ATPase Experiments

The incubation medium contained 50 mM Tris, 5 mM Na-ATP, 1 mM Na_2-EDTA in 0.3 M sucrose pH 7.4, and 0.3 ml. mitochondria. The final volume was 3 ml. In these experiments, the mitochondria from 1 gm. kidney cortex were suspended in 10 ml. sucrose before use in the enzyme assay. Five millimolar $MgCl_2$ and/or 0.1 mM DNP were included in the incubation medium as indicated in the tables of results. All the reagents except the mitochondria were preincubated at 37° C. Mitochondria were added and after 10 min. the enzyme reaction was stopped by cooling and by the addition of 0.7 ml. cold 50% TCA. The elon method [15] was used to estimate the inorganic phosphate in the supernatant.

Swelling Experiments

Mitochondria were prepared as before in 0.3 M sucrose. The mitochondria from 1 gm. cortex were suspended finally in 2 ml. 0.3 M sucrose. Swelling was studied by adding 0.05 ml. mitochondrial suspension to 2.95 ml. 0.15 M KCl-0.02 M Tris

medium, pH 7.4,[17] and recording the changes in optical density at 520 mμ using a Zeiss spectrophotometer.

Results

Morphologic Changes

A prerequisite for this study was the demonstration of a reasonably well-delineated point of irreversibility of the system. The criterion for making this decision was the presence or absence of unequivocal necrosis. In order to establish this, the left renal pedicle was clamped for periods of time varying between 10 min. and 2 hr. The blood supply was then restored and the kidneys were examined after 24-, 48-, or 72-hr. periods of recovery. Examination of all 3 periods gave identical results, indicating that necrosis, if present, was evident at 24 hr.

With periods of 10 or 20 min. of ischemia, virtually no differences were found between the ischemic left kidney and the control right kidney or the kidneys from normal animals. At 24, 48, or 72 hr., essentially all portions of the nephron were normal (Fig. 1). The only alteration seen was occasional vacuolation of scattered cells in the proximal convoluted tubule and some eosinophilic amorphous material in some distal convoluted tubules. However, it was impossible to distinguish with certainty sections from ischemic or from control kidneys.

In contrast, the left kidney from all animals subjected to temporary ischemia for 30 min., 1 hr., or 2 hr. showed gross and microscopic changes characteristic of extensive necrosis, confined almost entirely to the distal portion of the proximal convoluted tubule (Fig. 2 and 3). The rat kidney, unlike that of many other mammals, such as the dog and man, does not have a straight terminal segment of the proximal convoluted tubule but, rather, has a convoluted proximal tubule throughout its length until it becomes the thin descending limb of the loop of Henle. This convoluted terminal segment is located between the outer glomeruli-containing zone of the cortex and the medulla.[18]

Grossly, the left ischemic kidney of all animals in the groups subjected to periods of ischemia of 30 min. or longer was found to be swollen and much paler than the right one at 24 hr. On section, the inner zone of the cortex [18] was grayish white (necrotic), while the medulla, including the papilla, was red and hemorrhagic. Microscopically, the distal segment of the proximal convoluted tubule showed extensive coagulation necrosis with pyknosis, karyorrhexis, or karyolysis (Fig. 2 and 3). The tubular epithelium of other portions of the nephron showed little damage. However, many of the lumens of the thin limbs and distal convoluted tubules were filled with eosinophilic granular material.

The vasa recta were often dilated and filled with red blood cells. In many sections at 72 hr., evidence of regeneration of tubular epithelium in the proximal convoluted tubules was present. The control kidney (right kidney) showed no evidence of necrosis but did contain occasional eosinophilic granular material in the proximal convoluted tubules. Their lining-cells, although essentially normal, did show a rare mitotic figure.

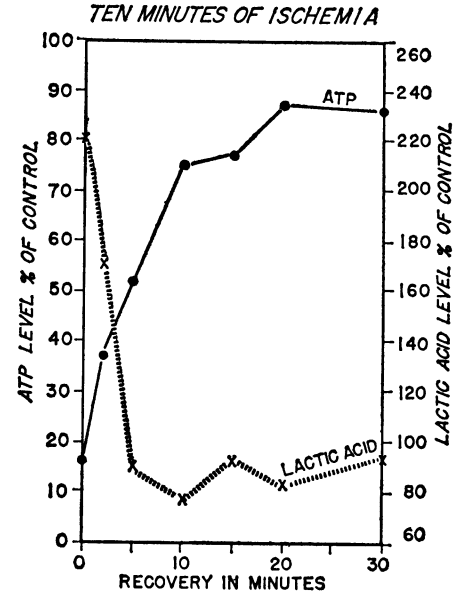
On the basis of this initial phase of the study, it was evident that the point of irreversibility was somewhere between 20 and 30 min. of temporary ischemia. In order to delineate this more accurately, an additional group of animals was subjected to a 25 min. period of ischemia. When examined at 24 hr. or thereafter, the left kidneys of these animals clearly showed that this time period was close to the critical point between reversibility and irreversibility. Every section examined contained many distal segments of the proximal convoluted tubules in which 1 or 2 cells were necrotic, while the remainder were intact and normal appearing. Although an occasional tubule showed uniform necrosis, this was distinctly uncommon.

From this phase of the study it became evident that the kidney of the rat can recover completely from a 20-min. period of clamping of its pedicle, but not from a 30-min. period; and that both reversible and irreversible alterations in the same tubule are present following a 25-min. period of interruption of blood flow.

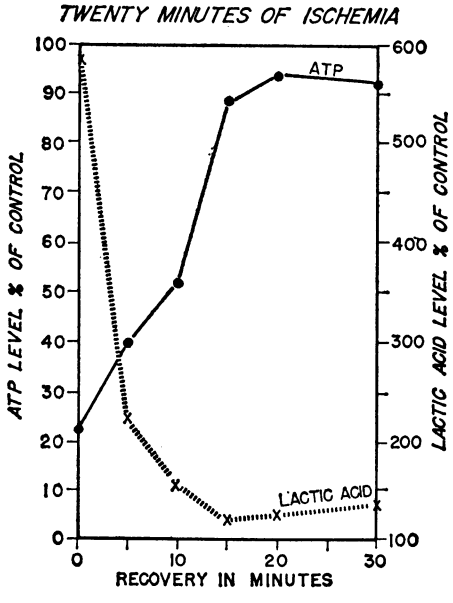
Biochemical Changes

ATP and Lactic Acid Levels. In 1940, Emmel [6] showed that ischemia of the kidney produced a rapid fall in tissue pH. It is now known that this fall in pH is due principally to the accumulation of lactic acid—the end product of glycolysis. Busch *et al.*[7] reported that within 3 hr. of ischemia of the liver, the ATP level fell to 6% of the control value, and the lactic acid concentration rose 20-fold, both effects being reversible.

As recorded in Text-fig. 1–5, the concentration of ATP in the kidney cortex decreases rapidly to approximately 15–20% of the control level, but shows no further decrease below this concentration, even after 2 hr. of ischemia. In contrast, the lactic acid concentration shows a progressive increase with the period of ischemia from 10 min. to 2 hr., the levels rising from approximately 2 times the control value at 10 min. to about 12 times the control value at 2 hr. These data suggest that sufficient glucose or glucose precursors are available in the tissue (cells plus extracellular fluid plus blood) to provide for a continuous



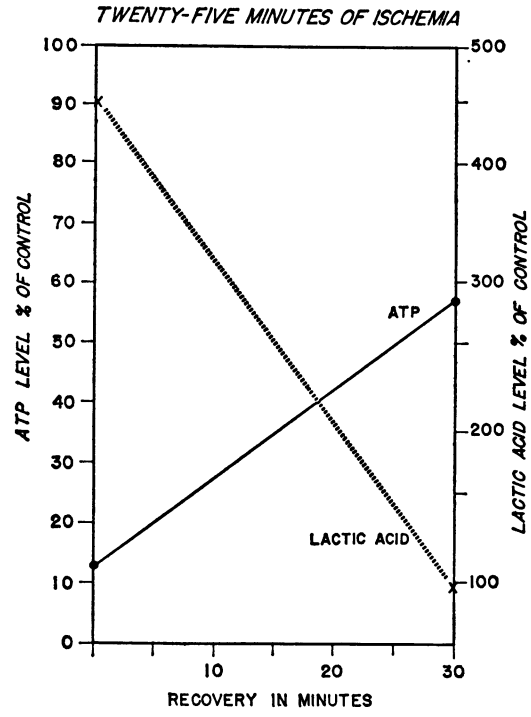
TEXT-FIG. 1. Renal cortical ATP and lactic acid concentrations (% of control values) as a function of time after establishing the circulation following 10 min. of ischemia.



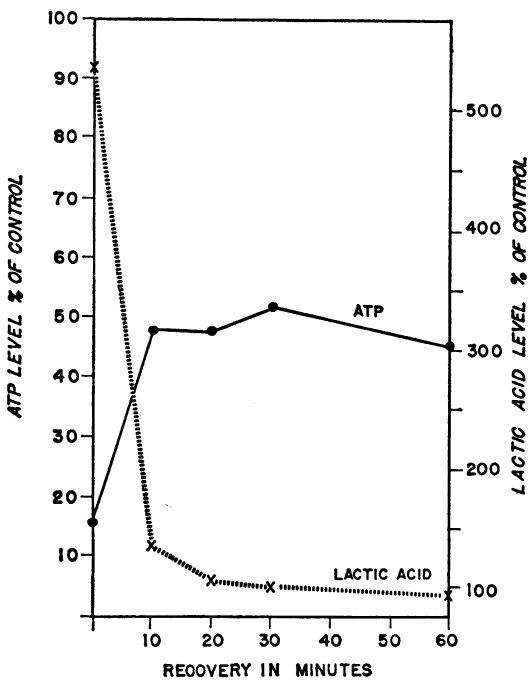
TEXT-FIG. 2. Renal cortical ATP and lactic acid concentrations (% of control values) as a function of time after reestablishing the circulation following 20 min. of ischemia.

production of lactic acid via glycolysis for at least 2 hr. The uninterrupted glycolytic activity is probably a major factor in generating ATP at a rate sufficient to maintain its concentration at about 20% of the control value.

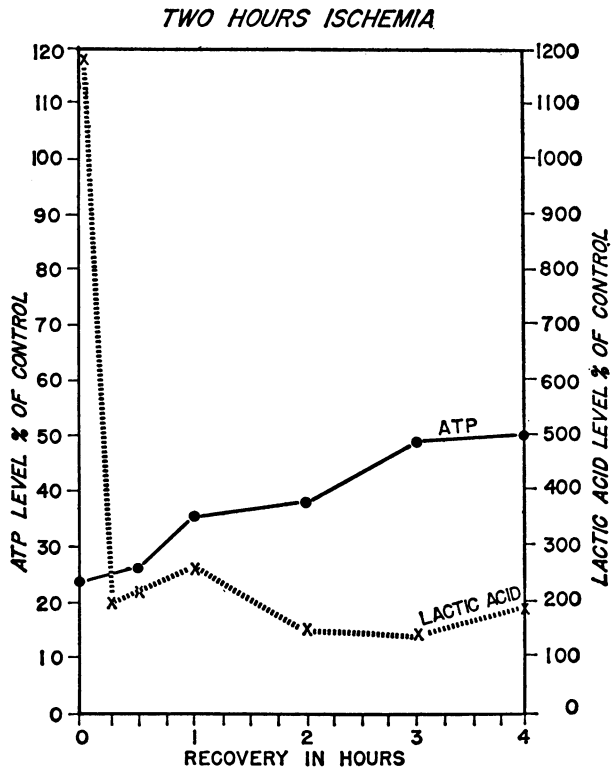
The changes in ATP and lactic acid concentrations in the kidney in which active circulation has been reestablished after periods of ischemia



TEXT-FIG. 3. Renal cortical ATP and lactic acid concentrations (% of control values) as a function of time after reestablishing the circulation following 25 min. of ischemia.



TEXT-FIG. 4. Renal cortical ATP and lactic acid concentrations (% of control values) as a function of time after reestablishing the circulation following 30 min. of ischemia.



TEXT-FIG. 5. Renal cortical ATP and lactic acid concentrations (% of control values) as a function of time after re-establishing the circulation following 2 hr. of ischemia.

of 10, 20, 25, or 30 min., or 2 hr. are also presented in Text-fig. 1–5. After 10 or 20 min. (Text-fig. 1 and 2), the kidney is able to recover very rapidly so that the lactic acid level is restored to the control levels within 5–10 min., and ATP levels within 10–20 min. However, after periods of ischemia of 25 or 30 min., even though the lactic acid level is rapidly reduced to control values, the ATP concentration remains at about 50% of the control value. Surprisingly, the *rate* of return of the ATP level is not a valid index of its eventual level, since the recovery is just as rapid after 30 min. of ischemia as after 10 min. In the 2-hr. experiments, the lactic acid level falls very rapidly but does not return to the control level even after 4 hr. of recovery. Also, with this period of ischemia, the rate of recovery of ATP is very much slower than after 30 min. of ischemia, although it does eventually reach the 50% level by about 3 hr.

These results with lactic acid are consistent with the hypothesis relating necrosis to a decrease in tissue pH. If the increasing lactic acid were truly the major factor in the genesis of cell death, it should be possible to prevent this irreversible damage by inhibiting the main

source of lactic acid, glycolysis. Two glycolytic inhibitors, acting at different sites in the pathway, were used for this purpose, iodoacetate and 2-deoxyglucose. Iodoacetate inhibits several SH-enzymes, including glyceraldehyde phosphate (triose phosphate) dehydrogenase,[19] and 2-deoxyglucose competes with glucose for phosphorylation via ATP and hexokinase; the resulting 2-deoxyglucose-6-phosphate inhibits cellular glucose utilization via glycolysis (cf. Ref. 20). Each inhibitor was injected intravenously into groups of rats prior to the clamping of the left renal pedicle, as detailed in Table 1. In the animals pretreated with

Table 1. The Effect of Pretreatment with Iodoacetate or 2-Deoxyglucose on the ATP and Lactic Acid Levels of Kidney Cortex Tissue after a 30 Min. Period of Ischemia

Group*	No. of rats	ATP (μg./100 mg. protein)†	Lactic acid (μg./100 mg. protein)†
Control + water	5	349 ± 15.8	47 ± 2.8
30 min. ischemia + water	6	39 ± 2.6	415 ± 6.5
30 min. ischemia + IAc	6	7 ± 3.4	133 ± 8.0
Control + glucose	4	386 ± 13.0	39 ± 8.2
30 min. ischemia + glucose	5	52 ± 5.4	458 ± 12.9
30 min. ischemia + water	5	41 ± 2.2	338 ± 11.5
30 min. ischemia + 2 DG	7	29 ± 2.8	207 ± 13.4

* Na iodoacetate (IAc) (56 mg./ml.) was injected intravenously in a dose of 1 mg./kg. body weight. The left renal pedicle was clamped 30 min. after the injection. Deoxyglucose (2DG) (0.5 ml.) was injected intravenously over a period of 30 sec. The 2-deoxyglucose solution contained 416 mg./ml. and the glucose solution contained an equimolar amount of glucose. The left renal pedicle was clamped 30 sec. after the injection.

† Mean ± S. E.

2-deoxyglucose, the lactic acid levels reached after a 30-min. ischemic period were considerably less than in animals not receiving this glucose analogue. The concentration, 207 μg./100 mg. protein, is that normally found after 10 min. of ischemia. The decrease in ATP concentration was considerably greater in the deoxyglucose pretreated animals than in the corresponding controls. The iodoacetate injection prevented much of the increase in lactic acid concentration and exaggerated the decrease in ATP, following the 30-min. period of interruption of circulation. Histologic examination revealed that neither the injection of iodoacetate nor deoxyglucose (Fig. 4) had any protective effect upon the degree of necrosis induced by the 30-min. period of temporary ischemia, even though each compound diminished to a considerable degree the rise in lactic acid concentration. The injection of the inhibitors themselves did not produce renal necrosis.

These data suggest that the degree of elevation of lactic acid production and therefore the drop in tissue pH is not the major or only factor in the genesis of ischemic cell death in the distal portion of the proximal convoluted tubule. However, since the *duration* of exposure to lowered

pH was not controlled, it is still possible that this may play a role. Also, it should be pointed out that this type of experiment is not wholly satisfactory, since the ATP as well as the lactic acid concentrations were reduced by each agent. The further lowering of the ATP concentration below that seen with ischemia alone may favor cell damage under these circumstances and thereby counteract any protective effect exerted by the decrease in lactic acid production.

Mitochondrial Metabolism—Manometric Experiments. The results shown in Text-fig. 1–5 suggested the presence of a correlation between the ability of the kidney cortex to restore its ATP concentration to normal levels and the genesis of cell death, since ATP recovery was complete following a period of ischemia of 20 min. or less, but not after one of 25 min. or longer. In view of the key role mitochondria play in ATP generation through oxidative phosphorylation, it became important to study their metabolism in considerable detail in relation to varying periods of ischemia and recovery.

The QO_2 of the mitochondria from ischemic kidney cortex fell as the length of the period of ischemia increased (Table 2). After 30 min.

Table 2. The Effect of Varying Periods of Ischemia and Recovery on the Mitochondrial Metabolism of the Kidney Cortex*

Conditions	No. of exper.	QO_2†		P:O†	
		+ATP‡	—ATP§	+ATP‡	—ATP§
10 min. of ischemia	7	77.8	64.4	94.5	51.7
20 min. of ischemia	6	80.7	47.3	72.5	19.1
20 min. of ischemia + 20 min. of recovery	4	95.0	65.2	108.0	65.5
30 min. of ischemia	5	49.1	34.2	89.0	20.2
30 min. of ischemia + 30 min. of recovery	3	85.5	70.8	90.4	92.0
1 hr. of ischemia	4	39.9	30.0	92.0	44.8
1 hr. of ischemia + 20 hr. of recovery	3	59.4	23.5	88.2	38.1
2 hr. of ischemia	4	23.9	14.1	0.0	0.0
2 hr. of ischemia + 24 hr. of recovery	3	0.0	0.0	0.0	0.0

* The results were obtained by use of the Warburg manometric technique with sodium pyruvate and fumarate as substrates.

† Expressed as percentage of control value. The respiratory quotient, QO_2, is expressed as microliters of oxygen uptake per hour per milligram of protein. The P:O is the moles PO_4 esterified per atom O_2 uptake. Since there was considerable day-to-day variation in the absolute values, all results are expressed as a percentage of the control values in any 1 experiment. A typical set of control values were as follows: QO_2(+ATP), 137; QO_2(—ATP), 112; P:O(+ATP), 2.31; P:O(—ATP), 2.15.

‡ The incubation medium contained both ATP (3.5 μmoles) and AMP (3.5 μmoles).

§ The incubation medium contained AMP (3.5 μmoles) but no ATP.

of ischemia, the QO_2 in the presence of ATP was about one-half that in the control mitochondria. The restoration of circulation was followed by a remarkable return of the QO_2 toward control values, even under conditions (e.g., 30 min. ischemia) leading to cell death. The inhibition of O_2 uptake, after any period of ischemia, was much greater in

the absence than in the presence of ATP. Kielley and Kielley [21] have shown that ATP or ADP stabilize the oxidative ability of mitochondria. Thus, it is possible that the mitochondria from the ischemic kidneys are slightly damaged and that ATP present during the preincubation period restores their functional integrity, perhaps by affecting water or ion extrusion from these organelles.

As seen in Table 2, periods of ischemia up to 1 hr. have little effect on the P:O ratio of the kidney cortex mitochondria when the incubation medium contains both ATP and AMP. If, however, ATP is excluded from the medium, even a 10-min. period of ischemia causes some changes in the mitochondria that indicate some uncoupling of phosphorylation from respiration. No oxidative phosphorylation could be detected by this method in mitochondria after a 2-hr. period of ischemia, even though the cells do not show much histologic evidence of irreversible damage at this time.

Experiments Using the Oxygraph. P:O ratios were also measured polarigraphically with the oxygraph. The results using the oxygraph, with succinate as substrate, are recorded in Table 3. In general, there is good agreement between these results and those obtained monometrically. It should be pointed out that since the reaction time is short with the oxygraph, only the phosphorylation associated with the reaction succinate-fumarate is measured—i.e., there is no phosphorylation associated with the oxidation of fumarate under these conditions.

Little effect of ischemia was observed in State 4 respiration (respiration when ADP is absent or used up),[22,23] even when the period of absent circulation was as long as 2 hr. The ADP:O ratio showed little change from the control after 30 min. of ischemia with or without a period of recovery. However, the mitochondria from the 2-hr. experiment again showed no oxidative phosphorylation. State 3 respiration (respiration in presence of ADP) was progressively decreased as the period of ischemia increased. For example, the QO_2 was decreased 40% following a 30-min. period of ischemia. However, this change is not irreversible, since these mitochondria recovered to a considerable degree by 30 min. after reestablishment of the circulation. On comparing the QO_2 in State 4 with that in the presence of 2,4-dinitrophenol (DNP), it is also apparent that mitochondria, even after 30 min. of ischemia, are still tightly coupled and can be stimulated, to an almost normal degree, to increase their O_2 consumption by an uncoupling agent such as DNP. The degree of stimulation of O_2 consumption is larger with the addition of ADP than of DNP both in controls and after 30 min. of ischemia. Again, the mitochondria after 2 hr. of ischemia show

Table 3. The Effects of Varying Periods of Ischemia and Recovery on the Mitochondrial Metabolism of the Kidney Cortex

Condition	No. of exper.	QO_2 State 4	QO_2 State 3	QO_2 DNP*	RC†	ADP:O
Control	12	42.4 ± 4.7	169.0 ± 5.5	147.0 ± 2.8(4)	4.0	1.51 ± 0.060
10 min. ischemia	4	38.2 ± 2.18	148.6 ± 15.8	—	3.89	1.34 ± 0.047
20 min. ischemia	4	38.9 ± 3.4	123.8 ± 6.6	—	3.17	1.37 ± 0.056
30 min. ischemia	6	42.7 ± 3.6	90.5 ± 4.95	111.5‡ ± 19.5(3)	2.12	1.53 ± 0.15
30 min. ischemia + 30 min. recovery	6	32.3 ± 8.8	147.2 ± 8.65	150.0 ± 10.6(3)	4.56	1.57 ± 0.14
2 hr. ischemia	5	40.6 ± 3.6	55.5 ± 3.2	66.0 ± 4.6(3)	1.37	Not obtainable
2. hr. ischemia + 24 hr. recovery	3	31.7	61.0	61.0 ± 12.4(3)	1.93	Not obtainable

These results were obtained by the use of an oxygraph using potassium succinate as a substrate. The respiratory quotient, QO_2, is expressed as microliters of oxygen uptake per hour per milligram of protein.

* DNP (2,4-dinitrophenol), final concentration 1.6×10^{-5} M; determinations were made in only some of the experiments, as indicated by figures in parentheses.

† RC, respiratory control ratio: $\frac{QO_2 \text{ State 3}}{QO_2 \text{ State 4}} = \frac{QO_2 \text{ with ADP}}{QO_2 \text{ without ADP}}$

‡ Calculations showed that there was no significant difference between this result and that of the control value.

almost no capacity to respond either to ADP (State 3) or DNP. Thus, it is evident that mitochondria have sustained little measurable irreversible damage by 30 min. after interruption of circulation, even though many of the cells are destined to die.

ATPase Experiments. Since the activity of DNP-stimulated ATPase correlates with the level of respiration of mitochondria,[24] it became of interest to measure the ATPase activities in the kidney cortex mitochondria after exposure to ischemia. The results of these experiments are recorded in Tables 4 and 5. Both 30-min. and 2-hr. periods of

Table 4. The Effect of 30 Min. of Ischemia Followed by Varying Periods of Recovery on the Mitochondrial ATPase Activity of the Kidney Cortex

	Control (9)*	30 min. of ischemia (10)	30 min. of ischemia + 10 min. of recovery (8)	30 min. of ischemia + 30 min. of recovery (6)
No additions†	4.06 ± 0.45	2.61 ± 0.29	3.92 ± 0.37	4.17 ± 0.34
plus Mg^{++}	13.50 ± 0.62	11.59 ± 0.48	14.04 ± 0.56	13.25 ± 0.39
plus Mg^{++} and Ca^{++}	13.59 ± 1.34	10.02 ± 1.17	11.61 ± 1.82	14.25 ± 1.01
plus DNP	17.14 ± 1.39	5.48 ± 0.55	14.20 ± 0.55	12.74 ± 0.91
plus DNP and Mg^{++}	20.50 ± 1.64	13.94 ± 1.07	23.10 ± 2.58	19.09 ± 2.40

† Additions made where indicated were: $MgCl_2$, 5 mM; $CaCl_2$, 26 mM; DNP, 0.1 mM (final concentrations).
* ATPase activity is expressed as micromoles P_i liberated per milligram mitochondrial protein per hour.
Mean ± S. E. The number of experiments is in parentheses.

Table 5. The Effect of 2 Hr. of Ischemia Followed by Varying Periods of Recovery on the Mitochondrial ATPase Activity of the Kidney Cortex

	Control (5)*	2 hr. of ischemia (7)	2 hr. of ischemia plus 2 hr. recovery (4)	2 hr. of ischemia plus 4 hr. recovery (6)
No additions†	4.44 ± 0.65	4.46 ± 0.65	3.33 ± 0.25	—
plus Mg^{++}	16.3 ± 1.72	15.36 ± 1.05	14.13 ± 0.52	16.42 ± 0.95
plus Mg^{++} and Ca^{++}	18.99 ± 1.13	7.69 ± 0.51	15.72 ± 0.83	17.33 ± 0.61
plus DNP	13.38 ± 2.17	5.92 ± 0.71	11.53 ± 1.06	16.21 ± 1.13
plus Mg^{++} and DNP	13.56 ± 1.38	9.70 ± 0.44	10.99 ± 0.42	13.79 ± 0.72

† Additions where indicated were final concentrations: $MgCl_2$ = 5 mM; $CaCl_2$ = 26 mM and DNP = 0.1 mM.
* ATPase activity is expressed as micromoles P_i per milligram of mitochondrial protein per hour. Mean ± standard error of the mean. The number of experiments in parentheses.

ischemia induced a considerable decrease in the levels of ATPase in the presence of DNP. However, this effect is completely reversed by 10 min. or 30 min. of recirculation of blood after the 30-min. period of interruption and almost completely reversed after 2 hr. of ischemia. Since ATP is known to have ameliorating effects on damaged mitochondria,[17,21] mitochondria were preincubated at 37° C. with ATP in solutions containing either KCl or NaCl before the assay for ATPase was carried out. As seen in Table 6, preincubation with a KCl-ATP

Table 6. The Effect of Preincubation in Various Media upon the Mitochondrial ATPase Activity of the Cortex of Control and Ischemic Kidneys

Conditions (2 exper. each)	Control				30 min. ischemia			
	No additions	plus Mg^{++}	Plus DNP	Plus Mg^{++} DNP	No additions	Plus Mg^{++}	Plus DNP	Plus Mg^{++} DNP
No preincubation	5.9*	15.2	25.1	13.0	3.3	9.6	7.9	8.9
Percentage†	100	100	100	100	100	100	100	100
Preincubated in KCl solution‡ for 15 min. at 37° C.	7.9	12.5	20.3	10.0	5.2	13.7	17.2	10.9
Percentage†	132	82	81	76.5	160	144	214	123
Preincubated in NaCl solution§ for 15 min. at 37° C.	4.9	10.7	5.9	9.4	2.8	8.5	5.4	7.3
Percentage†	82	70	24	72	80	92	64	81

* Absolute values of ATPase activity.
† Results expressed as percentage of the measured ATPase activity after no preincubation period.
‡ KCl solution: 0.15 M KCl, 0.021 M $MgCl_2$, 0.00125 M K_3-EDTA, 0.0043 M Na-ATP, 0.025 M glycylglycine pH 7.4.
§ NaCl solution: 0.15 M NaCl, 0.021 M $MgCl_2$, 0.00125 M K_3-EDTA, 0.0043 M Na-ATP, 0.025 M glycylglycine pH 7.4.

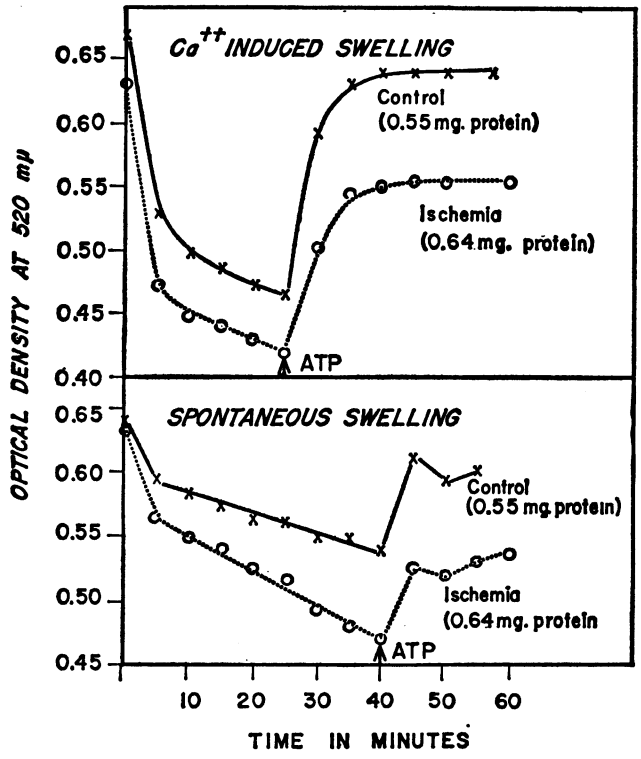
solution, but not with an NaCl-ATP solution, caused a striking increase in the DNP-stimulated ATPase activity of mitochondria from kidneys subjected to a 30-min. period of ischemia. This suggests that the K^+ might somehow be involved in the regulation of ATPase activity in the mitochondria. The basis for this effect is not clear, since it has been shown [25] that mitochondria do not appear to contain a K^+ stimulated ATPase.

Swelling Experiments. Mitochondria are most susceptible to swelling during respiration in the absence of ATP or ADP,[26] but not under anaerobic conditions. This active swelling is stimulated by several compounds such as free fatty acids, thyroxine, P_i and Ca^{++}. The swelling caused by Ca^{++} may be related to its ready accumulation by mitochondria and to its uncoupling property. The addition of ATP to swollen mitochondria causes the extrusion of water providing the medium contains little or no sucrose or mannitol.[27]

In view of these considerations, it became of interest to observe the response of mitochondria from ischemic or control kidneys to conditions causing swelling (spontaneous and Ca^{++}) or contraction (ATP). The results of a typical experiment are portrayed graphically in Text-fig. 6. It is apparent that the mitochondria from the kidney cortex after 30 min. of ischemia respond to Ca^{++}-induced or spontaneous swelling in a manner similar to those from controls, and that ATP is equally effective in causing contraction in mitochondria from both types of kidneys. This suggests that the osmotic properties of these mitochondria have not been seriously affected by a 30-min. period of ischemia.

Discussion

It is evident from this study that there exists in the rat kidney conditions under which there is a sharp division between reversible and irreversible effects of temporary occlusion of the renal circulation. The "point of no return" [28] is about 25 min. The cells of the nephron show no obvious death, as evidenced by necrosis, when exposed to 20 min. or less of ischemia, while extensive necrosis is evident with periods of ischemia of 30 min. or more. With 25 min. of circulatory interruption, one sees patchy and spotty necrosis of susceptible tubules. The distal portion of the proximal convoluted tubule which is segregated into a special zone in the kidney of the rat [18] is the segment of the nephron most sensitive to ischemia. Extensive necrosis can be induced in this region without any obvious irreversible cell damage to other portions of the nephron. These observations agree well with those of previous investigators using rat or other species.[29–40]



TEXT-FIG. 6. Spontaneous and Ca^{++} induced swelling of mitochondria from control or ischemic renal cortex. Period of ischemia was 30 min. without any recovery period thereafter. Arrow indicates time at which ATP was added to medium.

The kidney also shows a highly reproducible metabolic pattern of reaction to ischemia. The concentration of ATP falls rapidly to a level of approximately 15–20% that of the control, while the lactic acid increases rapidly. Thereafter, the amount of ATP in the kidney cortex remains quite steady for periods up to 2 hr. following interruption of the renal circulation. However, the lactic acid levels increase with time during the ischemic period. Thus, one may tentatively conclude that the glycolytic breakdown of glucose or a derivative continues for a considerable period of time. This metabolic utilization of carbohydrate is probably responsible for the maintenance of the ATP level at a reasonably constant level. This conclusion is supported by the findings with the glycolytic inhibitors, 2-deoxyglucose and iodoacetate, each of which caused a significant decrease in the ischemic level of renal cortical ATP. The cell is thus able to carry on at least some of its complex energy-generating systems despite the unavailability of a continuing oxygen and food supply. This pattern has been found in previous studies in the kidney as well as in the heart, brain, and liver (see Ref. 7 for review of the literature).

Since lactic acid continues to accumulate with time, it is possible that it may play a major role in causing irreversible cell damage. The progressive decrease in tissue pH with increasing lactic acid concentration may be responsible for inducing irreversible alterations in protein configuration leading to inability of the cell to function. In other words, according to this formulation, the metabolic cellular alterations leading to a loss of viability are the result primarily of the persistence of a high level of glycolysis unbalanced by respiration. If this were so, one should be able to prevent irreversible cell death induced by ischemia by inhibiting glycolysis. Both series of experiments utilizing 2 different types of inhibitors, 2-deoxyglucose and iodoacetic acid, failed to support this hypothesis. Even though the lactic acid production was considerably decreased, no protective effort against cell death was evident. However, it should be pointed out that such experiments are complicated by the drop in ATP concentration accompanying the decreased rate of glycolysis. Thus, it is possible but not probable (see below) that the decrease in ATP counteracted the beneficial effect of lowered lactic acid levels.

Despite the low levels of ATP in the ischemic cortex, the cells recover the ability to generate and maintain this nucleotide at normal levels, so long as they have not been irreversibly damaged, but fail to do so normally after periods of ischemia leading to cell death. It is thus evident that there is a correlation between ability to regenerate ATP to control levels and the ability of the cells to recover or maintain viability. A similar correlation between the level of restoration of ATP and cell viability has been found in the liver.[7] Busch *et al.*[7] found that the liver was able to reestablish almost control levels of ATP and ADP after periods of ischemia shorter than those leading to irreversible cell damage, but failed to do so with longer periods of ischemia. These authors suggest that there is a definite relationship between cell death and lack of ability to recover normal ATP levels. In our experiments, the kidney cortex, after a lethal period of ischemia, reestablished its ATP concentration at a level of approximately 50% of the control value. This 50% recovery phenomenon may well be a reflection of the fact that about one-half of the cortex is irreversibly damaged.

In contrast, the *rate* of recovery does not correlate well with reversibility of cell damage. For example, after 30 min. of ischemia, the rate of return of ATP to the 50% control level is the same as after 10 or 20 min. of ischemia, but the biologic fate of the cortical cells under these conditions is quite different. After a 2-hr. period of ischemia, a comparable level of ATP is obtained only after 2–3 hr.

The study of the metabolism of isolated mitochondria showed many reproducible alterations induced by ischemia but failed to provide any evidence that any one type of disturbance has any relevance to cell integrity and cell viability. Thus, isolated renal mitochondria, like cardiac muscle sarcosomes [41,42] and brain mitochondria,[43] show varying degrees of loss of respiratory control, respiratory rate, phosphorylation rate, and 2,4-dinitrophenol-activated ATPase activity following either reversible or irreversible ischemic damage; and yet, mitochondria from either type of renal cortex showed essentially full recovery of these metabolic properties after reestablishment of the circulation. Thus, it is evident that the sites of irreversible injury cannot reside in those mitochondrial loci concerned with these fundamental properties. Although major emphasis has been placed upon these loci to explain ischemic cell death (cf. Ref. 44), the evidence for this in the past has been derived for most part from studies of mitochondria isolated at the end of a period of ischemia without a subsequent period of recirculation. Our results suggest that the mitochondria, despite their obvious importance in the generation of utilizable energy through oxidative processes, may not be the major target by which ischemia destroys cell viability.[44]

Recently, Ozawa *et al.*[45] have postulated that brain ischemia causes the activation of some extra-mitochondrial lipolytic enzymes. These enzymes may then act on the phospholipid in the mitochondrial membranes to liberate a fatty acid-like substance. The endogenous uncoupler can then interact with the mitochondria and cause decreased mitochondrial function. Although such a mechanism could explain the alterations in mitochondrial metabolism *during* ischemia, it is difficult to reconcile this pathogenetic sequence with the recovery of mitochondria following exposure of the cells to periods of ischemia long enough to lead to cell death. Since the mitochondria regain the metabolic properties affected, presumably the endogenous uncoupler is somehow destroyed or otherwise inactivated after the circulation is reestablished, even though the cells are destined to lose their vitality.

On the basis of this study and that of Busch *et al.*,[7] the only metabolic alteration so far observed that correlates with the induction of ischemic cell death is the failure of the irreversibly altered cell to restore its ATP level to the previous control level. Since the mitochondria from such damaged cells recover their respiratory activity and oxidative phosphorylation, as well as their respiratory control, as measured by current techniques, during the time period when the intact cell is unable to restore in vivo its ATP to control values, it must be concluded that either present methodology for the study of mitochondria is not sensitive enough

to detect this type of irreversible damage, or the metabolic alteration responsible for the change in ATP level is extramitochondrial. The concentration of ATP at any one time is the resultant of the rates of its generation, utilization, and destruction. Possibly, the major defect lies in excessive destruction rather than in underproduction. Perhaps pertinent to this discussion are the findings of Gerlach *et al.*[46] that renal ischemia is accompanied by a net decrease not only in the concentration of ATP but of all adenine nucleotides and that the resynthesis of adenine nucleotides is a relatively slow process. Conceivably, the extent of loss of such nucleotides during a period of 25 min. or longer is such that ischemic cells are unable to generate their essential adenine nucleotides at a rate sufficiently rapid to prevent permanent damage.

These considerations about cellular ATP presuppose that a lowered level of cellular ATP is a potent cause of irreversible cell death. This is certainly not the case in the liver where ATP levels of 20% of the control values can be present for periods of 24–48 hr. after the administration of ethionine without inducing cell death.[47] Although the liver can resist ischemia for periods considerably longer than can the kidney,[48] it is still subject to ischemic necrosis. Thus, unless the metabolism of the distal segment of the proximal convoluted tubule is radically different from that of the liver, it is unlikely that it cannot tolerate a 30-min. period of ATP concentration at a level of 20% of the control value followed by a prolonged period at 50% of the control level, while the liver can tolerate a level of 20% of the control value for 24–48 hr. However, this point will have to be clarified before such a suggestion can be accepted.

Since it is now evident that most cells carry on innumerable chemical reactions, and since most metabolic activities will cease eventually in any cell following the initiation of the process of cell deaths,[49–56] it becomes virtually impossible to identify, by a straightforward or "head on" systematic analysis, the significant molecular event or events which trigger irreversible cell damage. The major dependence in such an approach is upon a time-sequence analysis in which the unwarranted assumption is made that what happens first is necessarily more significant than what follows. In this area as well as in so many others in biology, *post hoc, ergo propter hoc* is a dangerous guidepost.

The results obtained from this study clearly support the importance of the need for caution in assigning pathogenetic significance to one or more metabolic alterations. The use of the reversible-irreversible model has shown that profound metabolic changes in cells are compatible with cell integrity and vitality.

An additional cautionary note concerning cell sampling is also indicated. Although the reversible-irreversible renal ischemia model is a convenient one, it likewise has serious drawbacks, the major one being nonuniformity of cell response. The high degree of susceptibility to ischemic damage of the distal segment of the proximal convoluted tubule, coupled with the unusual resistance of many other segments of the nephron to the same environment, poses a serious complication in the interpretation of experimental results with the renal model, even if efforts are made, as was the case in this study, to confine the biochemical analysis to certain zones. The difficulty of interpreting molecular events using different cell populations is obvious, especially when it is becoming more evident that many cells have the ability to adjust and adapt rapidly to altered environmental conditions.

Finally, it is evident that the known alterations in mitochondrial metabolism do not seem to be the primary determinants of the death of cells of the proximal convoluted tubule of the kidney following ischemia, and that the key loci in these cells responsible for their sensitivity to interruption of the blood circulation must be sought elsewhere. Hopefully, the use of model systems which enable some selection between those biochemical alterations relevant to the problem of cell viability, and those irrelevant, may give significant clues to the molecular pathogenesis of ischemic cell death.

Summary

Various metabolic properties of renal cortical mitochondria and ATP and lactic acid levels were studied in rat kidney subjected to varying periods (10 min. to 2 hr.) of temporary interruption of all circulation followed by periods of different lengths of recirculation. The viability of the cell of the distal portion of the proximal convoluted tubules is the most sensitive to ischemia, the point of irreversibility under the conditions used being approximately 25 min. Renal cortical ATP concentration rapidly falls to about 20% of the control values after interruption of the circulation and remains at that level for at least 2 hr. The levels return to control values within 10–20 min. after return of the circulation, following periods of ischemia up to 20 min. However, the recovery level is approximately only 50% of the control values after ischemic periods longer than 25 min. The concentration of lactic acid in the ischemic renal cortex is proportional to the duration of ischemia up to at least 2 hr.

Mitochondria of the kidney cortex show a rapid change in respiration, oxidative phosphorylation, respiratory control and DNP-activated

ATPase following clamping of the renal pedicle. However, mitochondria recover these metabolic properties even after periods of ischemia which lead to cell death.

No evidence for lactic acid accumulation or mitochondrial metabolic damage as bases for loss of cell viability was obtained. The only correlation so far found between chemical events and cell death in the model examined is the level to which ATP returns following restoration of circulation. However, the molecular basis for this correlation remains obscure and does not appear to reside in known metabolic properties of mitochondria.

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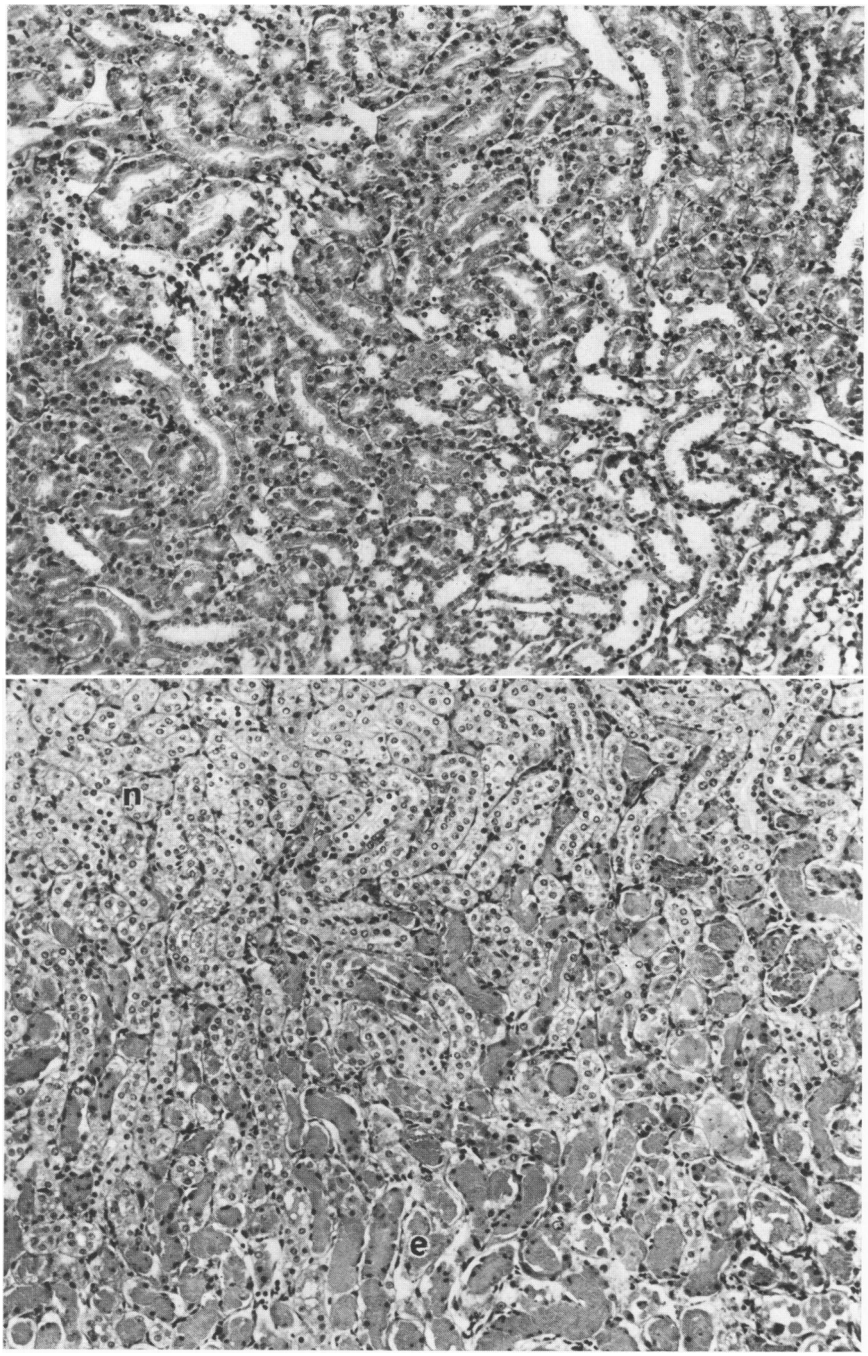
Legends for Figures

Figures 1–4 are of sections stained with hematoxylin and eosin; magnification on all is × 120.

Fig. 1. Section of control kidney.

Fig. 2. Thirty minutes of ischemia followed by 24 hr. of recovery. Many tubules are filled with eosinophilic amorphous material (e) containing a few pyknotic nuclei, while others are essentially normal (*n*). Necrosis is confined essentially to distal segment of proximal convoluted tubule.



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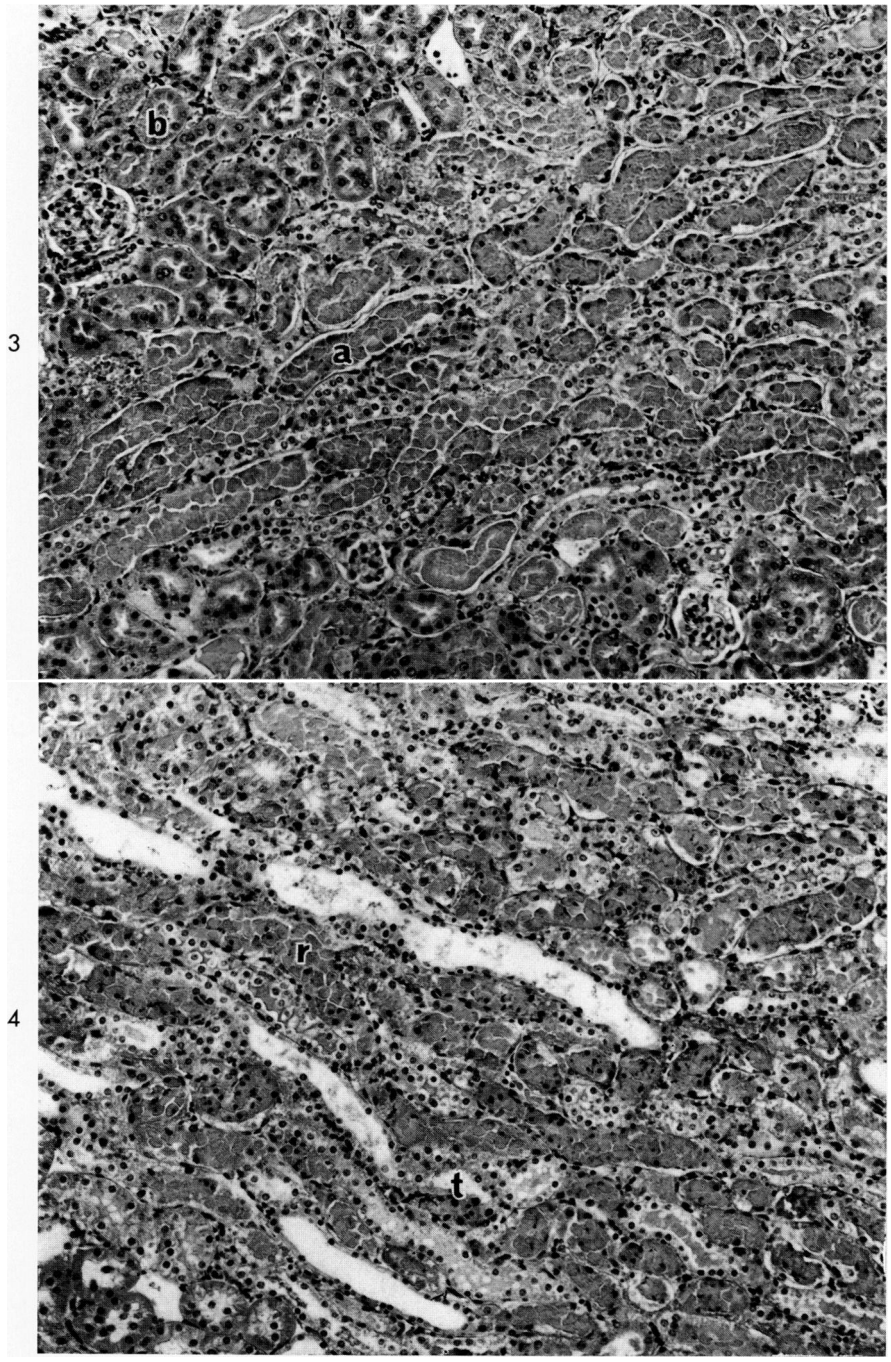

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Fig. 3. One hour of ischemia followed by 72 hr. of recovery. Distal segments of the proximal convoluted tubules show extensive necrosis with their lumens filled with eosinophilic amorphous material (*a*). The majority of the proximal portions of the proximal convoluted tubules (*b*) and glomeruli are intact and show no obvious damage. **Fig. 4.** 2-Deoxyglucose (2DG) plus 30 min. of ischemia followed by 24 hr. of recovery. The 2-DG was injected intravenously before clamping renal pedicle. Necrosis of distal segments of proximal convoluted tubules is evident (*r*). Intact collecting and distal convoluted tubules (*t*) can be seen alongside the necrotic tubules. Microscopic appearance is same as that of kidneys following 30 min. of ischemia and 24 hr. recovery without administration of 2-DG.