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Alterations in Renal Cortex Cation Homeostasis during Mercuric Chloride and Gentamicin Nephrotoxicity

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To help better understand the role of changes in cellular cation homeostasis in the pathogenesis of renal tubular cell injury, the alterations in cation content of renal cortex and isolated renal cortical mitochondria occurring during models of nephrotoxicity secondary to gentamicin and $HgCl_2$ were determined both during a developing phase of injury prior to the appearance of cell necrosis and after advanced injury when cell necrosis was present. At 3 hr after 5 mg/kg $HgCl_2$ or after 4 daily doses of 100 mg/kg gentamicin, tubular cell integrity was still intact but mitochondrial functional changes were present. There were no alterations of renal cortex tissue electrolytes at this stage in the $HgCl_2$ model but tissue K^+, and more prominently, tissue Mg^{2+} were decreased in the gentamicin model. K^+ and Mg^{2+} contents of isolated mitochondria were slightly reduced after $HgCl_2$. Only K^+ content was slightly reduced after gentamicin. No evidence for tissue or mitochondrial Ca^{2+} overload was present in either model. At 12 hr after 5 mg/kg $HgCl_2$ or after 10 daily 100 mg/kg doses of gentamicin, widespread areas of tubular cell necrosis were present and the function of isolated mitochondria was severely compromised. Tissue electrolytes at this stage of injury in both models were characterized predominantly by a twofold increase in Na^+ content and five- to sixfold increases in Ca^{2+}. Isolated mitochondria showed marked decreases in K^+ content and marked increases in content of Na^+ and Ca^{2+}. These data suggest that neither cellular and mitochondrial Ca^{2+} overload nor substantial changes in cellular Na^+ and K^+ homeostasis can be implicated in the early stages of renal tubular cell injury produced by gentamicin and $HgCl_2$.

INTRODUCTION

Alterations in the integrity of cellular and subcellular membranes have been proposed to play critical roles in the pathogenesis of cellular injury in various tissues, including the kidney (Trump *et al.*, 1976; Jennings and Reimer, 1981; Farber *et al.*, 1981; Farber, 1982; Humes and Weinberg, 1983). The resulting loss of the major intracellular cations, K^+ and Mg^{2+}, and intracellular overload with the major extracellular cations, Na^+ and, particularly, Ca^{2+}, have the potential to contribute to the disruption of multiple intracellular processes and, thereby, to the pathogenesis of irreversible cell injury. When studied *in vitro,* many nephrotoxins alter the permeability properties of both the plasma membrane as well as subcellular organellar membranes (Humes and Weinberg, 1982; Humes and Weinberg, 1983a). However, little is known about the extent to which such interactions may contribute to pertubations in cellular cation homeostasis during *in vivo* toxicity. This complex process is dependent, not only on the nature of the direct interaction between membrane and toxin, but also on the availability of the toxin *in vivo* to interact at various sites and the intactness of compensatory mechanisms. The present study was designed to assess the importance of altered cellular cation homeostasis in the pathogenesis of two well-studied models of nephrotoxic

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renal tubular cell injury, acute renal failure (ARF) secondary to gentamicin and $HgCl_2$. For each model, effects during the early developing phase of injury were contrasted with those present during advanced injury. Since mitochondrial function is altered during the early developing phases of renal tubular injury secondary to gentamicin (Simmons *et al.*, 1980) and $HgCl_2$ (Weinberg *et al.*, 1982a; Weinberg *et al.* 1982b) and since marked alterations in mitochondrial cation homeostasis occur after *in vitro* exposure to these nephrotoxins (Weinberg *et al.*, 1982a; Weinberg and Humes, 1980; Weinberg *et al.*, 1980) cation levels of isolated renal cortical mitochondrial were measured in addition to whole renal cortex cation content.

METHODS

Animals

Male Sprague–Dawley rats weighing 275–325 g and allowed free access to food and water were used for all studies.

Nephrotoxin Treatment Procedures

(a) $HgCl_2$ was administered as a single subcutaneous injection of 5 mg $HgCl_2$/kg body wt in a volume of approximately 0.4 ml of an aqueous solution. Studies were done at 3 and 12 hr after $HgCl_2$.

(b) Gentamicin was administered as a single daily subcutaneous injection of 100 mg gentamicin/kg body wt in a volume of approximately 0.7 ml of an aqueous solution of gentamicin sulfate. Studies were done at 24 hr after the last of 1, 2, 4, and 10 daily doses of gentamicin.

Control groups were always run simultaneously with each experimental group and, for the purpose of statistical analysis, data from experimental groups are compared only to data from the corresponding control groups.

Procedures for Obtaining Tissue and Mitochondrial Samples

Rats were stunned, decapitated, and both kidneys removed and placed in ice-cold 0.275 *M* sucrose for 1–2 min. The kidneys were then gently blotted dry, and their capsules removed. The renal cortices were dissected, and, as required for specific experiments, tissue samples were taken for determination of wet and dry weights and isolation of renal cortical mitochondria. In studies where samples of muscle were required they were obtained from the left thigh and were carefully stripped of connective tissue.

To isolate renal cortical mitochondria, the dissected cortex was minced on an ice-cold tile plate, then placed in 14 ml of an ice-cold solution of 0.275 *M* sucrose, 1 m*M* EGTA,[2] 5 m*M* Tris–HCl, pH 7.4, in a Potter Elvehjem homogenizer (Thomas size BB) and homogenized with a motor-driven Teflon pestle. A sample of this homogenate was saved for determination of protein and electrolyte content in a dry plastic tube previously rinsed with dilute HCl and mitochondria were isolated from the remainder by differential centrifugation as previously detailed (Weinberg *et al.*, 1982a). The final mitochondrial resuspension was in 0.275 *M* sucrose and was stored in a dry plastic tube previously rinsed with dilute HCl.

[2] Abbreviations used: EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetate; Tris, tris(hydroxymethyl)aminomethane.

Measurements of Electrolyte Levels

Dried tissue samples. Samples of renal cortex were weighed to obtain the wet tissue weight in tared glass tubes, then dried for 24 hr at 180°C and reweighed to obtain the dry weight. Tissues were then digested and dissolved in concentrated HNO_3 (approximately 1 ml HNO_3/200 mg dry tissue) (Humes and Weinberg, 1983a).

Na^+ and K^+ levels in the HNO_3 tissue digests were measured on a Beckman Klina flame photometer. Mg^{2+} levels were measured on aliquots of the HNO_3 digests diluted 1:400 with 1% La_2O_3 on a Perkin Elmer model 306 atomic absorption spectrophotometer (AAS). Ca^{2+} levels were similarly measured using the AAS on 1:50 dilutions. Concentrated HNO_3 incubated in glass tubes without tissue served as the blank. Electrolyte levels are reported as nanomoles per milligram dry tissue weight.

Tissue homogenates and mitochondrial suspensions. Both preparations were handled similarly. The samples were frozen and thawed at least twice before determination of electrolytes and all dilutions were done in 0.0125% Acationox (Scientific Products), a detergent with extremely low cation content (Aithal and Toback, 1978). All electrolyte measurements were done on a Perkin Elmer model 306 AAS.

Na^+ was determined on 1:150 and 1:7.5 dilutions of homogenates and mitochondria, respectively. K^+ was determined on 1:300 and 1:75 dilutions of homogenates and mitochondria, respectively. The diluting solution used in the AAS determinations of Na^+ and K^+ was prepared as described in Willard *et al.* (1974) and contained 247 mg Li_2CO_3, 1.5 ml concentrated HCl, and deionized, glass-distilled water to a volume of 1000 ml. Acationox was added to a concentration of 0.0125%.

Mg^{2+} was determined on 1:50 and 1:25 dilutions of homogenates and mitochondria respectively. Ca^{2+} was determined on 1:10 and 1:5 dilutions of homogenates and mitochondria, respectively. For Mg^{2+} and Ca^{2+} determinations all samples and standards were diluted in a solution made by adding 11.7 grams La_2O_3 and 50 ml concentrated HCl to deionized, glass-distilled water to a volume of 1000 ml. Acationox was added to a concentration of 0.0125%.

All dilutions were done in dry plastic tubes previously rinsed with dilute HCl.

Solutions containing either 0.275 *M* sucrose or 0.275 *M* sucrose, 1 m*M* EGTA, 5 m*M* Tris–HCl, pH 7.4, were diluted exactly as the samples and served as blanks for mitochondria and homogenates, respectively.

Electrolyte levels obtained are reported as nmol/mg homogenate or mitochondrial protein.

The validity of these tissue-handling procedures for measuring the total electrolyte content of the homogenate and mitochondrial samples was established by several procedures.

(1) Addition of known amounts of electrolytes to the samples before their dilution resulted in quantitative recovery.

(2) In comparing renal cortex samples dried and then digested in nitric acid with those which were homogenized, the electrolyte levels determined on the nitric acid digests were the same as those determined on the homogenates when the ratio of mg tissue protein/mg tissue dry weight was accounted for.

(3) Mitochondrial and homogenate samples spanning a range of Ca^{2+} levels from 10 to 600 nmol/mg protein gave the same values when measured by the

procedures used in the present paper as when measured using a recently reported Butanol:HCl–lanthanum extraction method (Tew *et al.*, 1981).

Serum samples. Mg^{2+} was measured by AAS on 1:75 dilutions of serum in the same lanthanum solution used for mitochondrial samples. Serum K^+ levels were measured on a Beckman Klina flame photometer.

Measurement of Mitochondrial Respiration

Well-standardized procedures were used exactly as described previously (Weinberg *et al.*, 1982a).

Morphologic Studies

Rats were anesthetized with intraperitoneal sodium pentobarbital and the kidneys were perfused *in situ* for 8 min via a catheter in the lower abdominal aorta with a solution containing 100 m*M* sodium cacodylate pH 7.4 and 2% gluteraldehyde at 37°C and a perfusion pressure of 150 mm Hg. Then the kidneys were removed and hemisected. A 3-mm-thick slice of one hemisection was placed in 4% formaldehyde, 1% gluteraldehyde, 84 m*M* sodium phosphate, pH 7.2, for at least 24 hr, then mounted in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). One-millimeter-thick slices of the other hemisections were saved in the same fixative and samples from about one-half of the rats in each group were subsequently processed by postfixation in osmium, staining with uranyl acetate, sequential dehydration in increasing concentrations of ethanol, and embedding in Epon. Semithin sections were stained with toluidine blue and examined by light microscopy and areas of these were further processed for electron microscopy as required to clarify details of fine structure.

Quantitative assessment for the presence of advanced renal tubular cell injury was done with light microscopy of the H&E-stained hemisections. For each section we graded in a blinded fashion the amount of injury present in 20 proximal tubules in each of three areas: (1) outer cortex just beneath the capsule (early sections of proximal convoluted tubule), (2) medullary ray (late part of proximal convoluted tubule, early sections of proximal straight tubule), and (3) outer medulla just beneath corticomedullary junction (proximal straight tubules).

The scale used for grading was

0—Completely normal tubule with full extent of fine detail of entire brush border clearly discernible.

1—All cells completely intact but various focal brush border abnormalities—smudging, focal loss, decrease in height, brush border debris in lumen—were present. Any such abnormality, no matter how minor, was sufficient to place a tubule in this category, accounting for its frequent occurrence in control kidneys as detailed in Table I.

2—Abnormal large vacuoles present within tubule cells. Projections of tubule cell cytoplasm into lumen. These findings were almost always accompanied by moderate to extensive loss of brush borders.

3—Loss or obvious detachment from basement membrane of no more than 1–2 individual tubular cells per tubule. Tubules with this finding always had extensive loss of brush borders in remaining cells and frequently but not always had grade 2 cellular vacuolization.

4—Obvious necrosis of majority of cells within tubule with loss and/or detach-

TABLE I
Morphology of Models of Injury[a]

	Stage of injury					
	0	1	2	3	4	5
Control (N = 10)						
OC	36 ± 13	64 ± 13	0	0	0	0
MR	55 ± 10	45 ± 10	0	0	0	0
OM	60 ± 14	40 ± 14	0	0	0	0
4-Dose gentamicin (N = 5)						
OC	30 ± 18	70 ± 18	0	0	0	0
MR	20 ± 11	80 ± 11	0	0	0	0
OM	26 ± 15	74 ± 15	0	0	0	0
10-Dose gentamicin (N = 6)						
OC	0	10 ± 9	3 ± 3	8 ± 5	6 ± 5	73 ± 16
MR	0	12 ± 11	30 ± 11	33 ± 7	15 ± 8	10 ± 6
OM	22 ± 9	60 ± 6	18 ± 7	0	0	0
5 mg/kg $HgCl_2$, 3 hr (N = 7)						
OC	28 ± 9	72 ± 9	0	0	0	0
MR	18 ± 8	82 ± 8	0	0	0	0
OM	48 ± 17	52 ± 17	0	0	0	0
5 mg/kg $HgCl_2$, 12 hr (N = 4)						
OC	0	32 ± 15	55 ± 10	13 ± 5	0	0
MR	0	0	0	28 ± 9	60 ± 5	12 ± 11
OM	0	0	0	0	78 ± 20	22 ± 20

[a] Each value is the mean percent of tubules (±SE) falling into the indicated morphologic stage. OC—outer cortex, MR—medullary ray, OM—outer medulla at corticomedullary junction. N indicates number of animals studied.

ment of cells from basement membrane, extensive vacuolization, and loss of normal cell contour.

5—Maximum loss of normal tubular architecture with extensive bare and sometimes disrupted areas of basement membrane and/or extensive debris, no longer bearing any resemblance to normal mature tubular cells.

Results are reported as mean percent of tubules graded falling into each category.

BUN levels were determined as previously described (Weinberg *et al.*, 1982b) on serum obtained at the time of sacrifice.

Proteins were determined by the Lowry method (Lowry *et al.*, 1951) with bovine serum albumin (Sigma A7511) as standard.

All reagents were of the highest purity commercially available. Organic compounds, including gentamicin sulfate, were obtained from Sigma Chemical Company. All water used was deionized and glass-distilled.

Unpaired t tests were used for statistical analyses with $P < 0.05$ two-tailed considered to be statistically significant.

TABLE II
BUN Levels after Gentamicin and $HgCl_2$ Treatment[a]

	Number of daily 100 mg/kg doses of gentamicin		
	Control	4	10
N	11	14	5
BUN (mg/dl)	16 ±1	18 ±1	121*** ±14
	Hours after 5 mg/kg $HgCl_2$		
	Control	3	12
N	17	18	8
BUN (mg/dl)	19 ±1	22 ±1	46*** ±4

[a] *N* = number of experiments. Relative to control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

RESULTS

Renal Function and Morphologic Effects

The alterations in BUN resulting from the treatment protocols used are summarized in Table II. Representative sections of renal cortex under each of the experimental conditions studied are illustrated in Figs. 1–4 and results of quantitative morphologic studies are summarized in Table I. Twenty-four hours after

FIG. 1. Representative semithin section of outer cortex stained with toluidine blue (×680) from rat treated with 100 mg/kg gentamicin for 4 days.

the last of four 100 mg/kg doses of gentamicin, gross tubule structure remained intact; however, large numbers of myeloid bodies and focal loss of brush border in S_1 and S_2 proximal tubule segments as previously reported for similar treatment protocols were present (Fig. 1) (Simmons *et al.*, 1980; Soberon *et al.*, 1979). After 10 doses of gentamicin, there was widespread necrosis in outer cortex with complete denudation of tubular basement membrane in some areas and replacement of the normal tubular epithelium with a lining of flat poorly differentiated cells in other areas (Fig. 2). Tubule structure was better preserved in inner cortex and outer medulla areas where S_3 proximal tubule segments are prominent but large casts filled in lumens of these tubules. An interstitial nephritis was also present. By light microscopy, tubular structure was well preserved 3 hr after $HgCl_2$ but ultrastructural studies showed the presence, mainly within S_1 and S_2 segments, of increased numbers of small vesicles, dense bodies, and vacuoles (Fig. 3). At 12 hr after $HgCl_2$, extensive necrosis was present in S_3 segments and to a lesser extent in S_2 segments with good preservation of tubular cell integrity in S_1 segments as previously reported by others (Fig. 4) (Gritzka and Trump, 1968; Barnes *et al.*, 1980). These data indicate that the points chosen for study in these models spanned a range of injury from the developing phase prior to loss of renal tubular cell integrity (3 hr for $HgCl_2$, 4 or fewer doses for gentamicin) well into the period during which advanced cellular injury with necrosis was widespread (12 hr for $HgCl_2$, 10 doses for gentamicin) and severe sustained loss of renal excretory function had occurred.

Functional Changes of Isolated Renal Cortical Mitochondria

Basic mitochondrial respiratory parameters are summarized in Table III. Mild, but significant inhibitions of State 3 respiratory rates and 2,4-dinitrophenol (DNP)

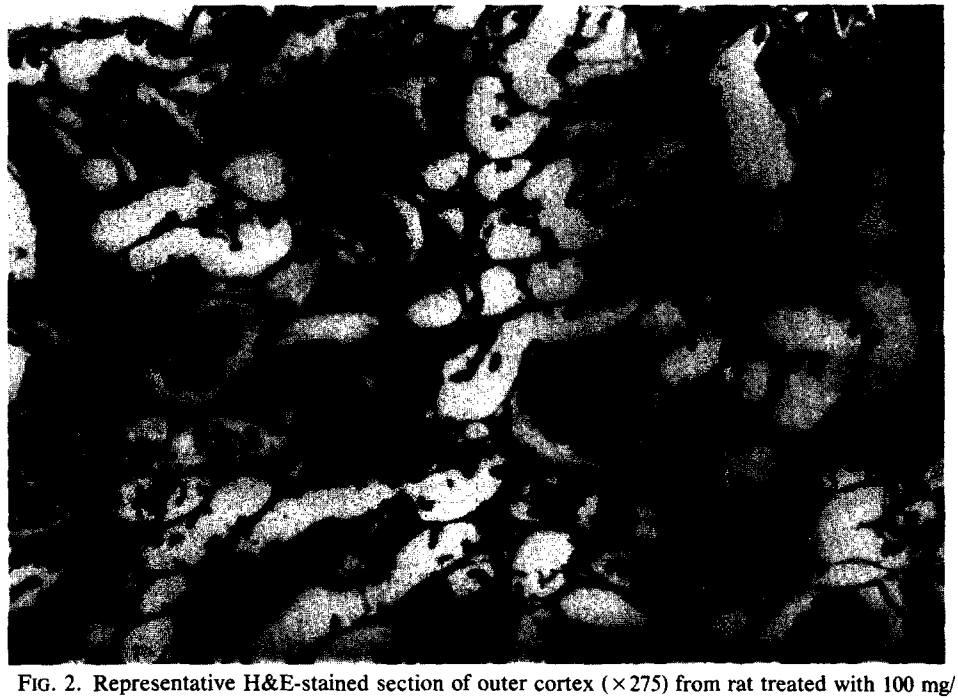

FIG. 2. Representative H&E-stained section of outer cortex (×275) from rat treated with 100 mg/kg gentamicin for 10 days.

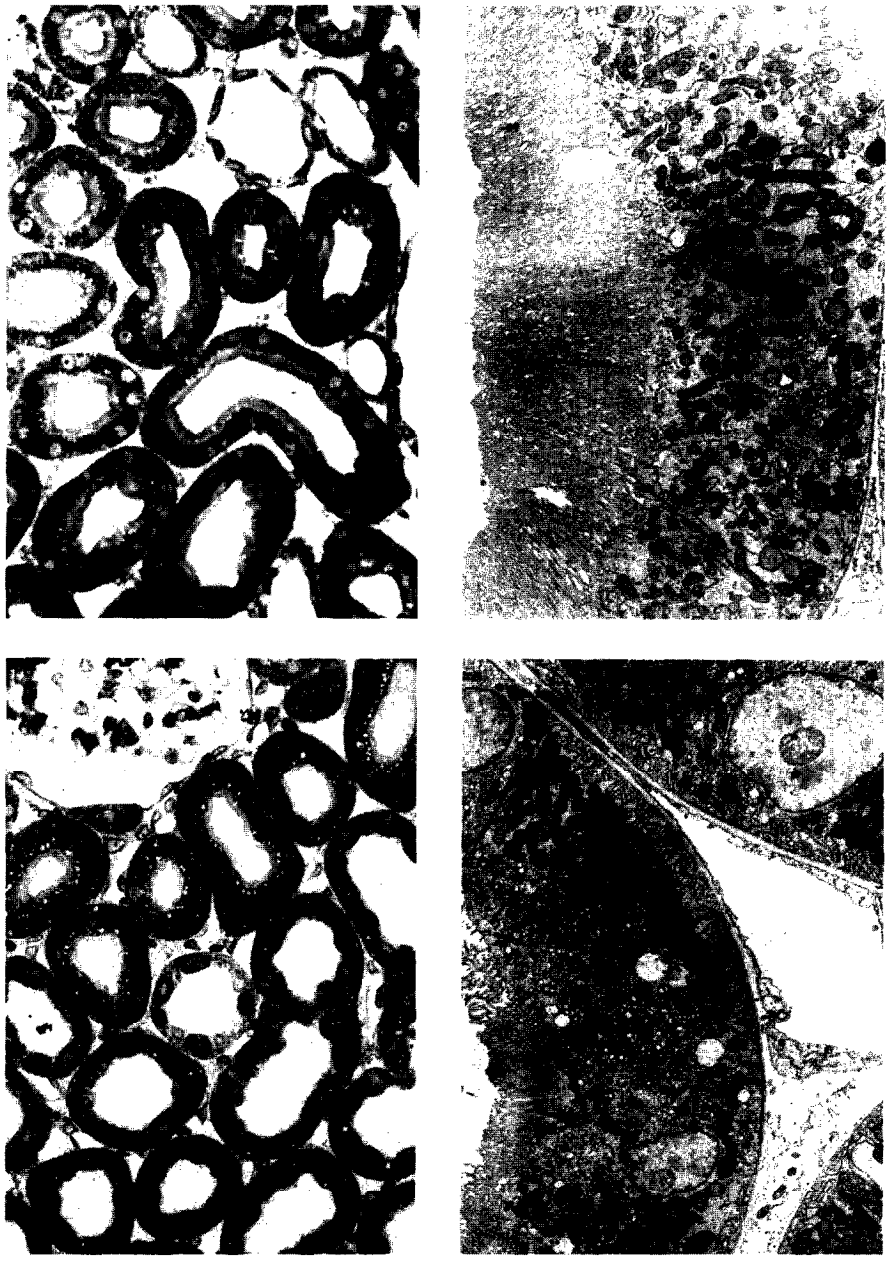

FIG. 3. Morphology of rat kidney 3 hr after 5 mg/kg $HgCl_2$. Frames on the left are toluidine blue-stained semithin sections of outer cortex (bottom) and outer medulla (top) (×200). Frames on the right are electron micrographs of S_2 (bottom) and S_3 (top) proximal tubule segments (×3600).

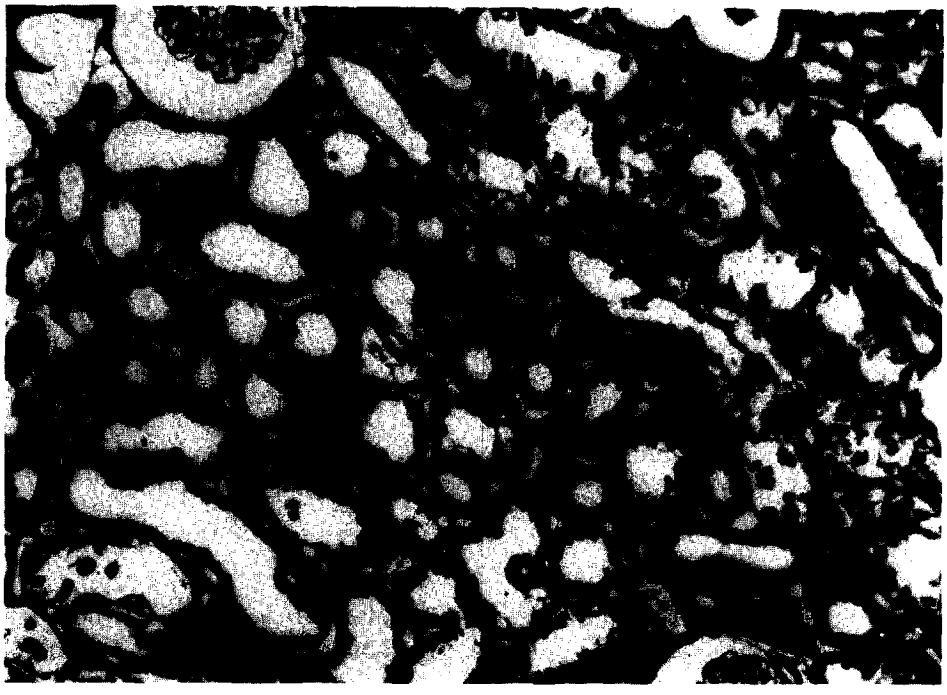

FIG. 4. Representative H&E-stained section of outer cortex (×275) 12 hr after treatment with 5 mg/kg $HgCl_2$.

uncoupled rates were present at 3 hr after $HgCl_2$ and after 4 doses of gentamicin. For both $HgCl_2$ (as previously reported in Weinberg *et al.*, 1982b) and gentamicin (data in Table III) the inhibitory effects appeared to be more marked with sodium pyruvate–sodium malate than with sodium succinate as substrate.

TABLE III
Respiratory Function of Isolated Mitochondria[a]

		Respiratory rate (natom eq O/mg protein/min)		
	N	State 4	State 3	DNP uncoupled
Control[b]	10	107 ± 2	380 ± 6	400 ± 8
3-hr Hg^{2+}	12	103 ± 3	321 ± 8***	362 ± 9**
12-hr Hg^{2+}	8	94 ± 9	178 ± 35***	224 ± 41***
Control[b]	11	107 ± 2	390 ± 8	409 ± 5
4-dose gentamicin	14	99 ± 3	350 ± 10**	366 ± 9**
Control[c]	11	44 ± 2	175 ± 6	107 ± 3
4-dose gentamicin	14	39 ± 2	135 ± 6***	88 ± 4**
Control[b]	4	103 ± 4	407 ± 12	428 ± 14
10-dose gentamicin	5	84 ± 7	160 ± 20***	189 ± 24***

[a] Data for each experimental group are given with data from the concurrent control group. *N* = number of experiments. Relative to corresponding control ** $P < 0.01$, *** $P < 0.001$.

[b] Respiratory substrate for this control group and its corresponding experimental groups was 10 m*M* sodium succinate.

[c] Respiratory substrate for this control group and its corresponding experimental group was 5 m*M* sodium pyruvate–5 m*M* sodium malate.

TABLE IV
Renal Cortex Water and Protein Content[a]

	N	Mean ratios ± SE	
		Dry wt/wet wt	Tissue protein/dry wt
Control	9	0.222 ± 0.001	0.72 ± 0.01
3-hr Hg^{2+}	5	0.214 ± 0.002	0.72 ± 0.01
12-hr Hg^{2+}	6	0.168 ± 0.004***	0.73 ± 0.01
Control	8	0.242 ± 0.001	0.72 ± 0.01
4-dose gentamicin	8	0.230 ± 0.005	0.73 ± 0.01
10-dose gentamicin	3	0.143 ± 0.003***	0.72 ± 0.04

[a] Dry and wet weights of renal cortex were determined as under Methods on renal cortex obtained from the right kidney. Renal cortex from the left kidney was carefully weighed prior to homogenization as described under Methods, then protein content was determined on a sample of homogenate. The dry weight of the left renal cortex sample was estimated by multiplying its wet weight by the dry/wet weight ratio obtained from the corresponding right kidney. N = number of experiments.
*** $P < 0.001$ relative to control.

In the setting of advanced tissue injury, 12 hr after $HgCl_2$ and after 10 doses of gentamicin, severe dysfunction of isolated mitochondria characterized by marked inhibitions of State 3- and DNP-uncoupled rates was present.

Effects on Tissue Water and Protein Content

These parameters, as summarized in Table IV, were determined in an untreated control group, at 3 and 12 hr after $HgCl_2$ and after 4 and 10 doses of gentamicin. Of these groups, tissue water was significantly increased (decreased dry to wet weight ratio) in the 12-hr $HgCl_2$ and 10-dose gentamicin groups, concomitant with the presence of advanced injury. The tissue protein/dry weight ratios, however, were not altered under any of the conditions tested, indicating that relative changes in tissue electrolyte levels factored for either value should be equivalent.

Renal Cortex Cation Levels

In studies done on homogenates of renal cortex, no changes had occurred in any cation at 3 hr after $HgCl_2$. At 12 hr after $HgCl_2$, tissue Na^+ and Ca^{2+} were markedly elevated and Mg^{2+} was slightly increased. K^+ was not significantly affected, but was more variable than in the control or 3-hr groups.

In studies done on homogenates of renal cortex, tissue Mg^{2+} was substantially decreased while K^+ was slightly but significantly decreased after 4 doses of gentamicin. Small but significant decreases in Na^+ and Ca^{2+} were also detected in this group of experiments.

After 10 doses of gentamicin, marked increases in tissue Na^+ and Ca^{2+} were seen while Mg^{2+} was moderately increased. These changes are similar to those seen 12 hr after $HgCl_2$. Tissue K^+ was significantly increased in the 10-dose gentamicin group (Table V).

In general, the results of the studies done on dried renal cortex digested with concentrated HNO_3 were similar to those obtained on the tissue homogenates. No alterations in cation content were present 3 hr after $HgCl_2$. Twelve hours after $HgCl_2$, marked increases in Na^+ and Ca^{2+} were present. After 4 doses of genta-

TABLE V
Cation Content of Whole Renal Cortex

	N	K^+	Na^+	Ca^{2+}	Mg^{2+}
A. Measurements made on homogenates of renal cortex (nmol/mg protein ± SE)					
Control	17	439 ± 5	313 ± 5	11.9 ± 0.4	44.8 ± 0.8
3-hr Hg^{2+}	18	425 ± 5	322 ± 5	12.2 ± 0.4	44.8 ± 1.0
12-hr Hg^{2+}	8	422 ± 21	609 ± 35***	65.6 ± 12.0***	47.8 ± 1.2*
Control	11	424 ± 11	359 ± 13	8.9 ± 0.2	50.8 ± 1.3
4-dose gentamicin	14	393 ± 8*	304 ± 10**	8.1 ± 0.3*	43.1 ± 1.1***
Control	4	394 ± 14	336 ± 9	9.7 ± 0.4	49.2 ± 1.2
10-dose gentamicin	5	529 ± 24**	669 ± 23***	46.0 ± 8.3*	58.2 ± 2.5*
B. Measurements made on dried renal cortex dissolved in nitric acid (nmol/mg dry weight ± SE)					
Control	6	303 ± 5	207 ± 4	9.3 ± 0.6	37.5 ± 0.6
3-hr Hg^{2+}	5	308 ± 4	227 ± 10	10.5 ± 0.4	38.3 ± 0.4
Control	8	307 ± 3	235 ± 3	7.2 ± 0.2	34.9 ± 0.5
12-hr Hg^{2+}	3	297 ± 1	330 ± 20***	24.8 ± 0.5***	37.9 ± 0.4*
Control	16	302 ± 2	225 ± 2	7.4 ± 0.1	35.2 ± 0.2
4-dose gentamicin	8	285 ± 4***	232 ± 13	8.5 ± 1.0	30.7 ± 0.6***

Note. N = number of experiments. Relative to corresponding control group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

micin, tissue Mg^{2+} was substantially decreased while tissue K^+ was slightly decreased. Unlike the studies done on tissue homogenates, there were no differences in Na^+ and Ca^{2+} between the control and 4-dose gentamicin groups in the experiments done for the determination of electrolytes on dried tissue samples digested in HNO_3.

Mitochondrial Cation Levels

Three hours after $HgCl_2$, isolated mitochondria showed small but significant decreases in K^+ and Mg^{2+} content relative to simultaneously studied controls. However, it should be noted that the actual levels of K^+ and Mg^{2+} in the mitochondria from the $HgCl_2$-treated rats were still within the range of normal values recorded for control mitochondrial preparations studied at other times for different experiments. Twelve hours after $HgCl_2$, mitochondria had markedly increased content of Ca^{2+}, moderately increased Na^+, and markedly decreased K^+.

Mitochondria isolated after 4 doses of gentamicin showed significantly decreased levels of K^+ but no significant changes in other cations. When isolated at the time of advanced injury, after 10 doses, the mitochondria showed marked increases in Ca^{2+} and Na^+, a small increase in Mg^{2+}, and markedly decreased K^+ (Table VI).

Influence of Mitochondrial Isolation Conditions on Their Ca^{2+} Content

Due to the obligate mixing of the intra- and extracellular contents which occurs during tissue homogenization, mitochondria are transiently exposed to higher concentrations of Ca^{2+} during their isolation than they are exposed to *in situ* and Ca^{2+} uptake may occur with resulting changes in both mitochondrial Ca^{2+} content and subsequent function (Peng *et al.*, 1977; Van Rossum *et al.*, 1976). For this reason, a calcium chelator, EGTA, is routinely included in the tissue-homo-

TABLE VI
Cation Content of Isolated Renal Cortical Mitochondria[a]

	N	Content (nmol/mg protein ± SE)			
		K^+	Na^+	Ca^{2+}	Mg^{2+}
Control	17	117 ± 2	12.2 ± 1.0	11.0 ± 0.4	33.0 ± 0.5
3-hr Hg^{2+}	18	109 ± 2**	11.7 ± 0.6	11.8 ± 0.5	31.1 ± 0.7*
12-hr Hg^{2+}	8	62 ± 8***	18.6 ± 1.4***	41.9 ± 10.2***	33.6 ± 1.5
Control	11	107 ± 2	12.0 ± 0.9	9.8 ± 0.5	32.1 ± 0.8
4-dose gentamicin	14	96 ± 3**	10.2 ± 0.5	11.3 ± 0.5	30.8 ± 0.5
Control	4	101 ± 3	12.2 ± 1.5	10.5 ± 0.6	31.4 ± 0.4
10-dose gentamicin	5	67 ± 7**	36.8 ± 4.7**	64.4 ± 10.3**	37.2 ± 1.2**

[a] Relative to corresponding control group * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. N = number of experiments.

genizing medium. It is possible, however, that EGTA may act to remove a portion of mitochondrial Ca^{2+} which was present *in situ* and may, thereby, serve to obscure a small early difference in mitochondrial Ca^{2+} levels between control and nephrotoxin groups. To assess this issue, renal cortical mitochondria were isolated from control kidney cortex and 3 hr after $HgCl_2$ in either the standard EGTA-containing homogenizing medium or a medium containing 0.275 *M* sucrose, 5 m*M* Tris–HCl, pH 7.4, or a medium containing 20 μ*M* (approximately 1.3 nmole/mg protein) ruthenium red, a potent specific inhibitor of mitochondrial Ca^{2+} uptake which is not a Ca^{2+} chelator (Reed and Bygrave, 1974). As shown in Fig. 5, the mitochondria isolated in 0.275 *M* sucrose, 5 m*M* Tris–HCl had the highest Ca^{2+} levels. The lowest Ca^{2+} levels were in the mitochondria isolated with EGTA, while those isolated with ruthenium red were intermediate. Of note,

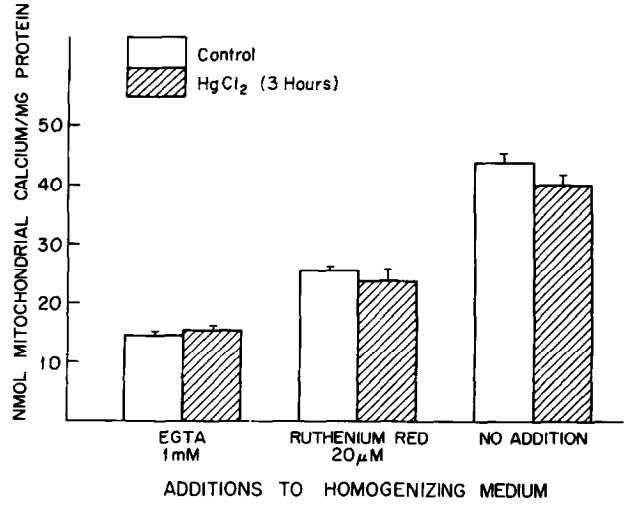

FIG. 5. Effect of the composition of the medium used to homogenize the renal cortex on the Ca^{2+} content of the mitochondrial fraction as finally isolated. The homogenizing medium contained 0.275 *M* sucrose, 5 m*M* Tris–HCl, pH 7.4, and the indicated additions. The $HgCl_2$ group received 5 mg/kg 3 hr prior to sacrifice. All control groups, $N = 6$, $HgCl_2$, 1 m*M* EGTA, $N = 3$, $HgCl_2$, ruthenium red, and no addition groups, $N = 5$.

TABLE VII
Sequential Changes in Renal Cortex and Muscle K^+ and Mg^{2+} Content during Early Gentamicin Nephrotoxicity

	Number of gentamicin doses			
	Control (N = 16)	1 (N = 8)	2 (N = 8)	4 (N = 8)
	(nmol/mg dry wt ± SE)			
K^+				
Renal cortex	302 ± 2	308 ± 3	304 ± 3	285 ± 4***
Muscle	445 ± 4	449 ± 2	444 ± 1	431 ± 6*
Mg^{2+}				
Renal cortex	35.2 ± 0.2	34.2 ± 0.7	33.7 ± 0.5**	30.7 ± 0.6***
Muscle	42.6 ± 0.4	40.7 ± 0.6	41.2 ± 1.0	43.3 ± 0.6

[a] N = number of rats studied.
Relative to control, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

however, Ca^{2+} contents of mitochondria isolated from rats treated 3 hr previously with $HgCl_2$ did not differ from those of controls with any of the isolation methods.

These studies suggest that EGTA in addition to preventing Ca^{2+} uptake (as ruthenium red alone does) also removes a component of bound Ca^{2+} but that this effect is not serving to obscure any difference between Hg^{2+}-treated and control mitochondria.

Sequential Changes in K^+ and Mg^{2+} during Developing Gentamicin Nephrotoxicity

To further assess the renal cortex Mg^{2+} and K^+ depletion seen after 4 doses of gentamicin, additional studies were done using dried samples of renal cortex as well as samples of muscle obtained after 1, 2, and 4 doses of gentamicin. As summarized in Table VII, Mg^{2+} depletion was detected in kidney after as few as two doses of gentamicin and was specific for kidney since at no point were changes in muscle levels seen. In contrast, tissue K^+ depletion appeared in kidney only after the 4th dose and, at that time, was accompanied by a similar decrease in muscle K^+. Since it has been suggested that muscle Mg^{2+} may be less sensitive as an indicator of total body Mg^{2+} depletion than serum Mg^{2+} (Dunn and Wasler, 1966), serum Mg^{2+} levels were also determined in the same groups (Table VIII). They were not decreased at any point.

TABLE VIII
Serum Mg^{2+} Levels during Developing Gentamicin Nephrotoxicity[a]

	Number of doses of gentamicin			
	Control	1	2	4
Serum Mg^{2+} (meq/l)	1.66 ± 0.02	1.72 ± 0.04	1.68 ± 0.06	1.92*** ± 0.04

*** $P < 0.001$, relative to control.

DISCUSSION

Previous studies have provided valuable but somewhat fragmentary information on cellular electrolyte shifts during renal injury. Renal slices subjected to either anoxia or hypothermia show marked cellular electrolyte shifts acutely predominated by loss of intracellular K^+ and uptake of Na^+ and water (Mudge, 1951; MacKnight, 1968; Whittembury and Proverbio, 1970; Trump *et al.*, 1974). Divalent cation metabolism has not been extensively studied but available data suggest there also occurs, under these conditions, an increase in intracellular Ca^{2+} but little change in Mg^{2+} (MacKnight, 1968). Of note, these acute changes occur in the absence of any alteration in the extracellular space as measured with inulin so the alterations are reflected in whole-tissue values as well as those corrected for extracellular volume (Whittembury and Proverbio, 1970). A recent electron microprobe study has demonstrated very similar alterations in cellular Na^+ and K^+ metabolism during *in vivo* ischemia (Mason *et al.*, 1981). If slices are reoxygenated (or rewarmed and provided with substrate) or the whole kidney is reperfused *in situ* (Mudge, 1951; MacKnight, 1968; Whittembury and Proverbio, 1970; Mason *et al.*, 1981) recovery of Na^+ and K^+ levels towards normal occurs but little is known of how cells destined to go on to necrosis differ from those destined to recover. Hours after the ischemic episode and reflow, when necrosis has occurred, kidney tissue levels of Na^+ and Ca^{2+} are high, K^+ is depressed, and Mg^{2+} is variably altered (Keeler, 1968), a pattern similar to that seen in cardiac (Whalen *et al.*, 1974) and hepatic injury (Gallagher *et al.*, 1956; Thiers *et al.*, 1960).

Studies of nephrotoxic renal injury are more limited. Carafoli *et al.* (1971) studied changes in electrolyte content of mitochondria isolated during uranyl nitrate toxicity. They showed progressive increases in Ca^{2+} and Na^+ and loss of K^+ but close correlations between the time course of these events, kidney function, anatomical changes, and mitochondrial function were not reported. Cronin *et al.* (1982), in a dog model of gentamicin nephrotoxicity, found an early decrease in tissue K^+ and Mg^{2+} after gentamicin treatment which was not, however, predictive of degree of injury. When creatinine was substantially elevated, tissue Na^+ and Ca^{2+} were increased. Renal cortical Na–K-ATPase activity was also decreased at this late interval (Cronin *et al.*, 1982).

The data on tissue and mitochondrial cation levels obtained in the present study must be interpreted in the context of the degree of tissue injury present. In the control groups and at the early stages of injury studied, i.e., 3 hr after $HgCl_2$ and 4 or fewer doses of gentamicin, when morphologic integrity is still preserved and tissue dry/wet weight ratios are not altered, the measurements of tissue K^+ and Mg^{2+} largely reflect intracellular levels. Though not measured in the present study, the extracellular space of the kidneys, as isolated, is likely intermediate between the value of 26% of wet weight measured with inulin in incubated slices (Whittembury and Proverbio, 1970) and 39% of tissue volume measured by planimetry in perfusion-fixed tissues (Mason *et al.*, 1981). Based on the serum and tissue electrolyte levels measured in the present study it can be calculated using these estimates of the extracellular space that 98–98.7% of the K^+ and 95.7–97.1% of the Mg^{2+} are intracellular. On the other hand, less than 35% of the Na^+ and 65% of the Ca^{2+} are intracellular. Thus, changes in tissue K^+ and Mg^{2+} are good indicators of changes in intracellular levels under these conditions while

changes in Na^+ and Ca^{2+} are much more subject to pertubations in extracellular volume and are, therefore, less precise and reproducible indicators of cellular levels. This is illustrated by the results of the 4-dose gentamicin experiments. In the first set of studies, done to obtain tissue homogenate (and mitochondrial) cation levels, the gentamicin-treated group had decreases in homogenate levels of all four cations measured. When the experiment was repeated a subsequent time on separate groups of rats to measure cation levels on dried tissue samples, only K^+ and Mg^{2+} levels were low in the treated rats while Na^+ and Ca^{2+} no longer differed from controls.

When necrosis has occurred, no tissue electrolyte value can be interpreted as an unequivocal indicator of intracellular levels because of the imprecise and variable separation of the intra- and extracellular spaces at this time. However, the pattern of electrolyte changes and their severity may provide clues to the factors involved in promoting injury at that point and may be useful in grading the degree of injury present.

Mitochondrial electrolyte levels are of value in assessing degree of mitochondrial injury and in interpreting the changes occurring in the whole-tissue electrolyte values. The normal mitochondrial membrane has a low passive permeability to cations (Humes and Weinberg, 1983; Brierley and Jung, 1980) and, in fact, is dependent on this property for its normal integrated function (Humes and Weinberg, 1983). The fact that mitochondria after being washed during their isolation with sucrose solutions free of added cations retain large amounts of K^+ and Mg^{2+} reflects this relative impermeability of the inner mitochondrial membrane. Any factor altering the normal integrity of the membrane may serve to decrease the K^+ and Mg^{2+} content of the mitochondria as finally isolated. The stage at which loss occurred i.e., *in situ* or during isolation, cannot be specified. The presence of a high mitochondrial level of Na^+ and Ca^{2+} after the standard mitochondrial isolation procedures, on the other hand, suggests that those changes occurred *in situ* since the isolation procedures done in cation-free solution containing a Ca^{2+} chelator should serve only to lower, not to raise mitochondrial Na^+ and Ca^{2+} levels. The presence of mitochondrial Ca^{2+} overload, concomitant with increased tissue Ca^{2+}, therefore, supports the notion that the increase in tissue Ca^{2+} was not limited to the extracellular space *in situ*, but was available to interact with mitochondria intracellularly.

Taking these considerations into account, the data reported in the present paper provide substantial insights into the timing and nature of the changes in cellular cation homeostasis occurring during nephrotoxic renal injury. In the model of $HgCl_2$ nephrotoxicity utilized, previous detailed studies (Weinberg *et al.*, 1982b) have established that functional alterations of isolated mitochondria became detectable 1–3 hr after treatment with the toxin, the time when intracellular Hg^{2+} levels are reaching their peak (Weinberg *et al.*, 1982b; Kirschbaum *et al.*, 1980). The present study demonstrates that (1) these changes in mitochondrial functional integrity are not secondary to cellular mitochondrial Ca^{2+} overload since none has occurred, (2) they are not secondary to other changes in tissue cation homeostasis since none have occurred, (3) neither these mitochondrial effects nor effects of Hg^{2+} at other cell sites have substantially disrupted overall cellular cation homeostasis during this interval, (4) the selective decreases in mitochondrial K^+ and Mg^{2+} are compatible with the functional data demonstrating impaired functional integrity of the inner mitochondrial membrane, and (5) when tissue

necrosis is present, cellular and mitochondrial Ca^{2+} overload occur as well documented in other states of advanced injury (Keeler, 1968; Whalen *et al.*, 1975; Thiers *et al.*, 1960; Gallagher *et al.*, 1956; Mittnacht and Farber, 1981) and are accompanied by severe changes in mitochondrial function and electrolyte content.

The changes in cation homeostasis occurring during gentamicin toxicity differ from those seen in $HgCl_2$ toxicity in several ways. (1) A prominent loss of tissue Mg^{2+} is seen early during the course of injury and, based on the absence of any concomitant effects on serum or muscle Mg^{2+}, this appears to be specific for kidney. The Mg^{2+} depletion is a feature of early injury rather than late injury in which tissue Mg^{2+} is increased. The early substantial decrease in kidney Mg^{2+} is similar to that reported in the dog model of gentamicin nephrotoxicity (Cronin *et al.*, 1982) and may be related to the competitive interactions between gentamicin and Mg^{2+} described in several renal membrane systems (Weinberg and Humes, 1980; Weinberg *et al.*, 1980; Humes *et al.*, 1982). (2) In contrast to data obtained with the dog (Cronin *et al.*, 1982), the early depletion of kidney K^+ was delayed relative to that of Mg^{2+}, was mild, and was accompanied by a similar loss of muscle K^+. (3) As was the case for $HgCl_2$ toxicity, no evidence for early cellular or mitochondrial Ca^{2+} overload was obtained. The early mitochondrial functional defects occurred prior to any changes in Ca^{2+}. They were accompanied by a decrease in mitochondrial K^+ but, interestingly, not in mitochondrial Mg^{2+} despite the marked concomitant change in tissue levels. (4) Late injury, like that seen with $HgCl_2$, was characterized by tissue and mitochondrial Ca^{2+} overload.

In summary, cellular Ca^{2+} overload cannot be implicated as a progressive ongoing process in the early stages of either toxic lesion, nor can substantial changes in cellular Na^+ and K^+ homeostasis. The early mitochondrial functional changes are accompanied by mild losses of K^+ and, with Hg^{2+}, Mg^{2+} from the mitochondria as isolated but are not accompanied by any changes in mitochondrial Ca^{2+} levels. Ca^{2+} overload is a common late feature of injury with both toxins. Selective kidney Mg^{2+} depletion is an early feature of gentamicin toxicity deserving further study as to its role in the pathogenesis of the renal tubular cell injury produced by gentamicin.

Recent data from J. L. Farber and his collaborators, although still controversial (Smith *et al.*, 1981), have implicated cellular Ca^{2+} overload as a critical component in the pathogenesis of the transition from reversible to irreversible hepatic cell injury secondary to both ischemia (Farber *et al.*, 1981; Mittnacht and Farber, 1981; Farber, 1982) and toxins, such as galactosamine (Farber, 1982). The results of the present study suggest that, if Ca^{2+} overload is the critical mediator of the transition from reversible to irreversible injury in the types of nephrotoxic renal tubular cell injury studied, it is a relatively abrupt and late process in the sequence of toxin-induced injurious subcellular events. If alterations of intracellular Ca^{2+} metabolism are involved in the early events of cell injury induced by these toxins, they are likely to involve changes of the intracellular regulation and sites of action of Ca^{2+} rather than a major net influx of the ion from the extracellular space.

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