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Proton NMR Spectra of Urine as Indicators of Renal Damage

Mercury-Induced Nephrotoxicity in Rats

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SUMMARY

Rats were injected intraperitoneally with $HgCl_2$ at doses of 2.5, 5, 7.5, and 10 μmol of Hg/kg. Urine was collected over a 24-hr period. At this time, plasma samples were taken and kidney damage was assessed by histological examination. Urinary γ-glutamyltransferase levels were significantly elevated at Hg^{2+} doses of 7.5 and 10 μmol/kg, consistent with the detection of acute tubular necrosis by light microscopy. Resonances for a large number of low molecular weight metabolites were assigned in high resolution ^1H NMR spectra of rat urine. Spectra from small volumes of urine (about 0.5 ml) were obtained in less than 5 min with no pretreatment. Significant Hg^{2+} dose-related decreases in the excretion of creatinine and citrate and increases of glucose, glycine, alanine, α-ketoglutarate, succinate, and acetate were detected. Elevated levels of lactate and creatinine in plasma of rats receiving the two highest doses were found by ^1H NMR. There was a good correspondence between the histopathology, enzyme excretion, and ^1H NMR urinary metabolite fingerprints in the assessment of Hg^{2+}-induced renal damage. ^1H NMR provided a sensitive measure of mercury-induced nephrotoxic lesions, and information on the molecular basis of mercury cytotoxicity was derived from the abnormal patterns of metabolite excretion. These suggested that primary metabolic effects of mercury were upon mitochondrial metabolism, in particular inhibition of certain citric acid cycle enzymes leading to decreased utilization of α-ketoglutarate and succinate by the renal tubular cells. The decrease in urinary citrate associated with Hg^{2+} dosing was attributed to intracellular, tubular acidosis with concomitant enhanced citrate reabsorption. The acidosis was assumed to arise from a combination of the inhibition of tubular carbonic anhydrase and a mild metabolic lactic acidosis due to increased activity of anaerobic pathways in the kidney. The possible extension of the ^1H NMR techniques to the investigation of the nephrotoxic potential of other compounds and drugs is discussed.

INTRODUCTION

High resolution proton NMR can be used for the rapid multicomponent analysis of low M_r compounds in small amounts (about 0.5 ml) of animal plasma and urine (1–3). The aim of this work was to assess its possible use in the early detection of biochemical changes associated with experimentally induced kidney dysfunction.

Mercuric chloride was chosen as the nephrotoxin since it is known to accumulate rapidly in the kidneys of experimental animals after injection, causing extensive damage to the proximal tubular epithelium and severe renal failure (4–7). The nephronal damage caused by Hg^{2+} is also well documented and acute exposure of experimental animals to this metal is also used as a model of acute renal failure (8, 9). Proximal tubular damage induced by Hg^{2+} is most severe in the pars recta (the straight section of the proximal tubule), but also affects the pars convoluta, and results in a general inhibition of solute reabsorption from the tubular lumen. The resultant Fanconi syndrome is characterized by increased excretion of amino acids, glucose, calcium, phosphate, bicarbonate, and some low M_r proteins (10).

Biochemical interest has centered on the uncoupling of oxidative phosphorylation and decreased respiratory control in mitochondria isolated from kidney cells of Hg^{2+}-treated animals. It has been suggested that this is the primary cause of Hg^{2+} toxicity in rats (11). A number of mitochondrial enzymes are known to be inhibited by Hg^{2+} (8) and mitochondrial degeneration is often accompanied by calcium deposition (5).

In this work, the ^1H NMR excretion profiles of a large number of low M_r metabolites in rat urine, including intermediates in the citric acid cycle, amino acids, and creatinine, are correlated with $HgCl_2$ dosage, kidney his-

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topathology, and excretion of the enzyme γ-glutamyltransferase.

EXPERIMENTAL PROCEDURES

Animal dosing and sample collection. Fifteen adult male Sprague-Dawley rats (weight range, 200 to 250 g) were divided into five groups of three animals each and were given 1 ml of water orally followed by single intraperitoneal injection of a 3.65 mM aqueous solution of $HgCl_2$ equivalent to a dose of 0, 2.5, 5.0, 7.5, or 10 μmol of Hg/kg body weight (0, 0.5, 1.0, 1.5, and 2.0 mg of Hg/kg). During the next 24 hr, the rats were kept in metabolic cages and deprived of food to eliminate contamination of urine with food particles, but water was provided *ad libitum*. The volumes and pH values of urine collected over the 24-hr period were recorded. Three additional rats provided with both food and water *ad libitum* acted as a second group of controls.

Rats were anesthetized with pentobarbital and tissues were removed for analysis (see below). Blood was removed from the abdominal aorta while the rats were under deep anesthesia and placed into heparinized tubes. The cells were separated immediately by centrifugation (5000 × *g*, 5 min) and the plasma was stored frozen at −20° prior to measurements.

Kidney histology. Kidneys were removed from the rats immediately after the withdrawal of blood and were rapidly weighed after the removal of adherent fat. Within 1 min of their removal from the abdominal cavity, the kidneys were bathed in 4% Karnowsky's fixative and transversely bisected. Small wedges (1 × 2 × 3 mm) of cortex and medulla were further fixed in the same medium (4 hr, ambient temperature). They were embedded in resin and 1-μm sections were cut and stained with toluidine blue for light microscopy. The remaining kidney was fixed in 2% Karnowsky's solution, dehydrated, and embedded in paraffin wax. Sections (7 μm thick) were cut from these blocks and stained using either the periodic acid-Schiff method or that with hematoxylin and eosin. Pathological changes were assessed by classifying lesions according to severity and type into cloudy swelling, vacuolar and hydropic degeneration, and necrosis. At least 200 profiles of proximal tubules were examined in three sections of the kidneys from each animal.

Creatinine and γ-glutamyltransferase. Creatinine levels in urine were determined on diluted but undialyzed samples by the alkaline-picrate, Jaffé method (12). These creatinine concentrations were used to standardize the NMR spectra.

The assay for γ-GT[1] was based on the procedure in Ref. 13. The urine was first dialyzed for 3 hr against distilled H_2O and diluted 50 times. Incubations with L-glutamyl-*p*-nitroanilide (substrate) and glycylglycine (acceptor, both purchased from Sigma) were carried out for 20 min at 37° in Tris-HCl buffer (0.1 M, pH 9). the *p*-nitroaniline released was diazotized and reacted with *N*-(1-naphthyl)ethylenediamine giving an azo dye with absorbance at 500 nm. One unit of enzyme activity corresponds to the release of 1 nmol of *p*-nitroaniline in 20 min at 37°.

^{1}H NMR. Measurements were made on Bruker WH400 (University of London Intercollegiate Research Service, Queen Mary College) and AM500 (Medical Research Council Biomedical NMR Centre, Mill Hill) spectrometers operating at 400 and 500 MHz, respectively, at 25° using 0.45 ml of urine or plasma diluted with 0.05 ml of ^2H_2O (lock signal) in 5-mm tubes. Shifts are referenced to sodium 3-(trimethylsilyl)-1-propanesulfonate (δ = 0 ppm) via internal acetate (δ = 1.933 ppm) or alanine (δ = 1.487 ppm).

For urine, 56 free induction decays were collected into 16,384 computer points using 28° (3-μs pulses), acquisition time of 1.7 sec. A further delay of 3.3 sec between pulses was added to ensure that the spectra were fully T_1-relaxed. The total accumulation time for each spectrum was therefore 280 sec. A continuous secondary irradiation field at the resonance frequency of water was applied in order to suppress the intense H_2O signal (about 100 M protons) and not to exceed the effective dynamic range of the instrument (3). The assignments of resonances were confirmed by consideration of a combination of standard additions, chemical shifts, coupling constants, and pH dependencies.

These considerations lead to relatively firm assignments for molecules with more than one set of proton resonances, e.g., citrate, creatinine, ethanol, glucose, hippurate, β-hydroxybutyrate, and α-ketoglutarate. Molecules with single, uncoupled resonances, e.g., acetate, formate, glycine, and sarcosine, were assigned on the basis of chemical shifts alone. This was aided by the wide dispersion of resonances at the high frequencies of observation used (400 or 500 MHz). This minimized overlap of signals increased the accuracy with which shifts could be measured (about ±0.002 ppm). Some resonances experience shifts with pH changes over the normal urinary range (3), and this can be used as a further assignment aid. The pH range of the urine samples studied here was narrow, about 7–8. In the future, it may become possible to use two-dimensional NMR methods (homonuclear ^{1}H-^{1}H correlations, ^{1}H-^{13}C cross-correlations) routinely to confirm further the assignments of resonances.

Spectra were obtained from plasma using the Hahn spin-echo pulse sequence (14) of (90°-τ-180°-τ-collect) repeated 96 times using 16,384 computer points, τ = 60 msec, 90° pulse = 9.5 μsec, acquisition time of 1.7 sec, and a further delay of 1 sec between cycles, together with continuous secondary irradiation of H_2O (1, 2). In this way, broad signals from plasma proteins were effectively suppressed and only signals from mobile, low M_r metabolites were observed. It should be noted that some low M_r metabolites such as cholesterol and its esters, present in plasma at millimolar concentrations, are immobilized through binding to macromolecular structures and are not amenable to direct NMR study either in single pulse or spin-echo spectra without sample pretreatment (1). With a value of 60 msec, doublets with *J* values of approximately 8 Hz are inverted whereas singlets and triplets remained upright (15).

The concentrations of metabolites in urine were determined by comparison of peak areas (or heights when line widths were constant) with those of creatinine (standardized chemically as above). For some samples, the concentration of creatinine was determined independently by NMR after a standard addition of valine (50 μl of a 200 mM solution). In all cases, there was good agreement (±10%) between NMR and conventional assays. For plasma, a standard addition of alanine was used to calibrate resonances.

Possible statistical relationships between the concentrations of different metabolites were tested by regression analysis, and mean 24-hr excretions were compared by Student's *t* test or Mann-Whitney tests according to procedures of Snedecor and Cochran (16).

RESULTS

NMR of rat urine. ^{1}H NMR spectra of normal rat urine have not been reported previously. In general, the types and concentrations of low M_r compounds detected by ^{1}H NMR were similar to those we have reported previously for normal human urine (3). A significant difference is the presence of allantoin in rat urine. This is the end product of purine metabolism in nonprimates and gives CH and NH peaks at 5.40 and 6.04 ppm, respectively. Fig. 1 shows a ^{1}H NMR spectrum of urine from a control rat which had been fed *ad libitum*. Rats for the Hg^{2+}-dosing experiment were deprived of food during the experiment to avoid any possible contamination of the urine samples. However, fasted rats excreted larger amounts of acetate (Table 1) than either normal rats or humans both fed and fasted. Small amounts of ethanol were also excreted by fasted rats, but not by fed rats. Although no specific steps were taken to exclude contam-

[1] The abbreviations used are: γ-GT, γ-glutamyltransferase; Sar, sarcosine; Me_2Gly, *N*,*N*-dimethylglycine; Cn, creatinine; RTA, renal tubular acidosis.

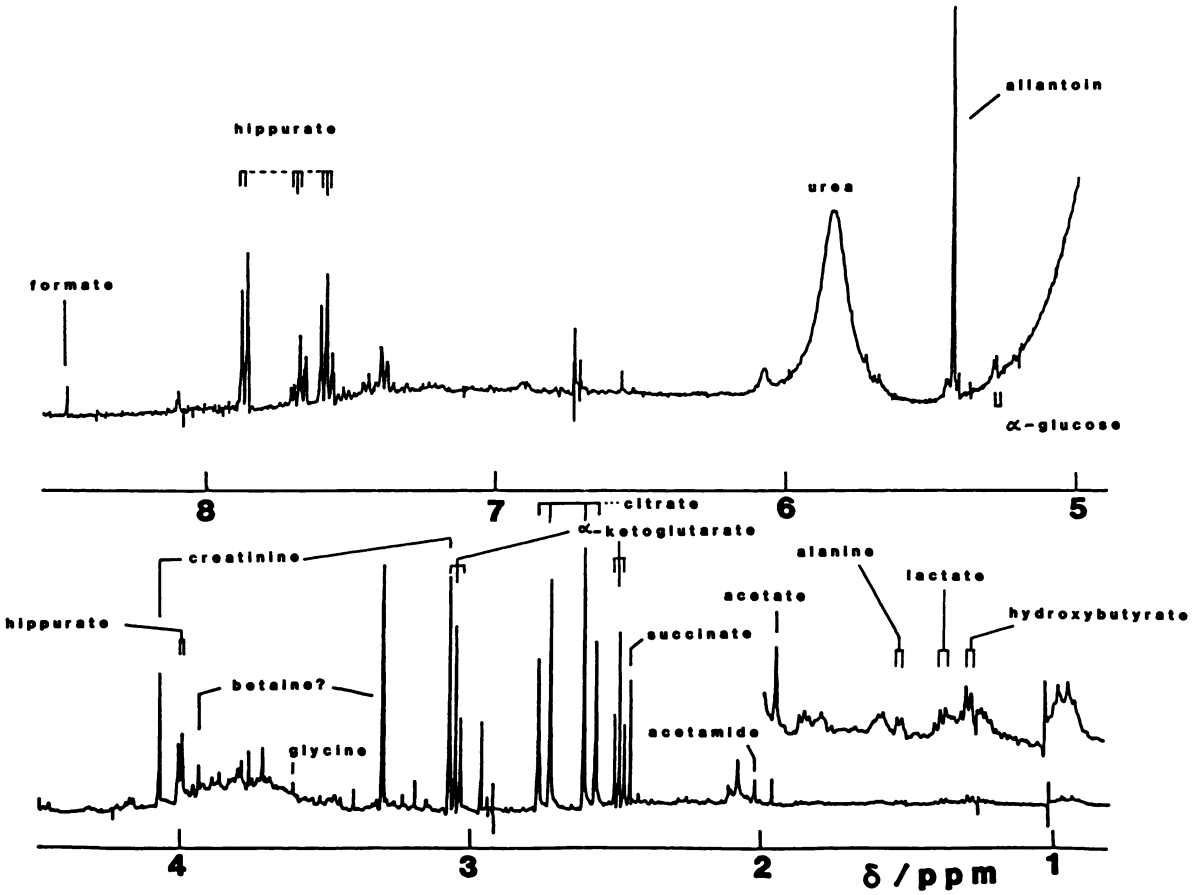

FIG. 1. *A 400-Hz ^{1}H NMR spectrum of urine collected over a 24-hr period from a control rat fed ad libitum*
The lower trace shows the aliphatic region, 0.8–4.6 ppm, and the upper trace is the aromatic region, 4.9–8.6 ppm.

ination of the urine samples by microorganisms which could account for the presence of ethanol, any significant growth of bacteria and/or yeasts was minimized by freezing the urine as it was collected. It seems likely that elevated concentrations of these compounds might have arisen from the absorption of the fermentation products of an altered gut flora during fasting. Previous reports of raised levels of plasma ethanol and acetate in alloxan-induced diabetic rats support this conclusion (17).

Hg^{2+}-dosed rats. Mercury treatment of rats caused marked alterations in the ^{1}H NMR spectral profiles of their urine (Fig. 2). From the intensities of the resonances, the 24-hr output of 21 metabolites was measured. Twelve of these compounds were excreted in quantities that were significantly altered after Hg^{2+} dosing of the animals (Table 1).

Resonances for citrate were readily detectable in urine spectra of control animals and those receiving the two lower Hg^{2+} doses of 2.5 and 5.0 μmol/kg. However, *no* resonances for citrate were detected at the two higher doses of 7.5 and 10 μmol/kg (see Fig. 2). Conversely, glucose was detected only in the urine of rats receiving the higher Hg^{2+} doses. The 24-hr excretion of the amino acids alanine and glycine was grossly elevated in Hg^{2+}-dosed animals (Table 1). Several other amino acids including tyrosine, valine, and threonine were detected sporadically but at levels too low to measure accurately and with no obvious association with Hg^{2+} dosing.

The urinary excretions of α-ketoglutarate, succinate, formate, acetate, lactate, and ethanol were elevated in Hg^{2+}-treated rats. Some of these were grossly abnormal in rats receiving the highest Hg^{2+} dose (Table 1). The 24-hr creatinine output declined with increasing Hg^{2+} dose, but only in rats receiving the highest doses was this statistically significantly less than that of the controls. The excretions of Sar and Me_2Gly closely paralleled that of Cn. Some strong statistical associations between 24-hr excretions were revealed by regression analyses: [Cn] = 0.44 [Sar] − 1.91 ($r = 0.86$, $n = 15$, $p < 0.001$); [Cn] = 0.25 [Me_2Gly] − 1.04 ($r = 0.77$, $n = 15$, $p < 0.01$); and [Sar] = 0.39 [Me_2Gly] + 2.84 ($r = 0.61$, $n = 15$, $p < 0.01$). The basis for this association is currently unclear, although the molecules are chemically related. There was also a strong correlation between succinate and acetate excretion in individual rats, irrespective of Hg^{2+} treatment ($r = 0.97$, $n = 15$, $p < 0.001$).

The 24-hr excretion of the ketone bodies, presumably related to fasting, was very variable but other compounds such as allantoin, dihydroxyacetone, and hippurate showed little variation and were not altered by Hg^{2+} dosing. No attempt was made to integrate the urea signal which may be affected by cross-relation from H_2O irradiation. The NH protons exchange chemically with solvent protons.

Plasma. Fewer well resolved signals were seen in spin-echo ^{1}H NMR spectra of rat plasma, as is the case with

TABLE 1

The urinary excretion of low M_r metabolites by control and Hg^{2+}-dosed fasted rats

ND, not detectable; AB, second order. δ shows chemical shifts and identity of peaks used for measurements.

Metabolite	δ	Metabolite excreted in 24 hr at Hg^{2+} dose (μmol/kg)				
		0	2.5	5.0	7.5	10.0
	ppm			*μmol*		
Acetamide (CH_3, s)	2.041	5.3 ± 1.9[a]	1.9 ± 0.4	1.3 ± 0.2	1.1 ± 0.9	4.0 ± 2.5
Acetate[b] (CH_3, s)	1.933	230 ± 54	134 ± 29	280 ± 118	326 ± 34	804 ± 208[c]
Acetoacetate (CH_3, s)	2.34	0.2 ± 0.1	3.0 ± 2.3	16.7 ± 9.4	10.5 ± 8.1	2.2 ± 2.0
Acetone [$(CH_3)_2$, s]	2.235	ND	1.0 ± 0.9	8.3 ± 4.5	5.4 ± 4.0	0.8 ± 0.7
Alanine (CH_3, d)	1.487	0.5 ± 0.2	0.6 ± 0.5	5.4 ± 1.1[c]	9.3 ± 1.0[c]	18.2 ± 2.4[c]
Allantoin (CH, s)	5.397	47 ± 9	55 ± 19	64 ± 20	49 ± 3	33 ± 10
Citrate [$(CH_2)_2$ AB]	2.639	48.5 ± 28.3	14.0 ± 3.8	19.8 ± 10.9	ND	ND
Creatinine (CH_3, s)	4.058	50.3 ± 4.8	44.3 ± 6.5	44.8 ± 15.7	25.7 ± 10.8	25.6 ± 9.3[c]
Di-Me-glycine[d] [$(CH_3)_2$, s]	2.894	9.8 ± 1.3	12.5 ± 5.8	9.5 ± 4.7	3.2 ± 1.1	8.2 ± 1.4
Di-OH-acetone[e] [$(CH_2)_2$, s]	4.475	2.7 ± 0.5	1.7 ± 0.7	3.6 ± 2.2	1.7 ± 0.8	1.6 ± 0.1
Ethanol[b] (CH_3, t)	1.195	9.9 ± 3.4	6.6 ± 2.6	29 ± 10	58 ± 38	129 ± 41[c]
Formate (CH, s)	8.467	17.7 ± 8.6	7.0 ± 5.5	12.6 ± 5.4	7.9 ± 2.4	156 ± 67[c]
Glucose[f] (CH, d)	5.242	ND	ND	18.8 ± 9.0	75 ± 55	48 ± 13
Glycine (CH_2, s)	3.567	20.3 ± 6.4	8.0 ± 1.0	25.6 ± 13.7	42.8 ± 6.4[c]	73.5 ± 8.5[c]
Hippurate (CH, d)	7.883	21 ± 15	14.3 ± 0.8	20 ± 11	8.4 ± 5.4	32 ± 21
β-OH-Butyrate (CH_3, d)	1.249	1.7 ± 0.3	8.6 ± 8.0	38 ± 18	56 ± 54	33 ± 30
α-Ketoglutarate (CH_3, t)	3.021	20.3 ± 1.9	6.4 ± 1.4[c]	44.0 ± 8.9[c]	55 ± 12[c]	62 ± 13[c]
Lactate (CH_3, d)	1.343	9.3 ± 4.2	2.2 ± 0.3	26.9 ± 2.0[c]	36 ± 14	202 ± 64[c]
Propionate (CH_3, t)	1.069	ND	1.4 ± 0.8	2.9 ± 2.8	6.9 ± 2.6	8.7 ± 2.9
Sarcosine (CH_3, s)	2.729	18.6 ± 1.9	21.9 ± 4.6	23.9 ± 10.7	8.7 ± 3.4	8.3 ± 3.6
Succinate[b] (CH_2, s)	2.415	66 ± 14	26.9 ± 6.5	99 ± 34	97 ± 32	254 ± 43[c]

[a] Mean ± SE.

[b] The concentrations of these metabolites are higher than in nonfasted control rats. For these metabolites, the mean levels of excretions were: acetate 58.5 ± 17.7; ethanol, not detectable, and succinate, 25.2 ± 7.1 μmol/24 hr ($n = 3$).

[c] $p < 0.05$, Student's t test.

[d] *N,N*-Dimethylglycine.

[e] Dihydroxyacetone.

[f] Glucose was estimated from the anomeric proton resonance, assuming the normal ratio of α to β anomers of 1:3; fed control rates excreted 12.5 ± 5.1 μmol of glucose/24 hr.

other animals. Lactate and creatinine levels in the plasmas of rats receiving the two higher doses of Hg^{2+} were significantly elevated ($p < 0.05$) (Table 2). Other metabolites such as alanine, valine, and glucose were detectable but concentrations were not affected by Hg^{2+} dosing.

Histopathology. The kidneys of rats receiving Hg^{2+} doses of 7.5 and 10.0 μmol/kg were paler than those of control animals and slightly swollen. In transverse section, there was a distinct pale band of necrotic tissue visible at the corticomedullary junction marking the location of the pars recta sections (S_3)[2] of the proximal tubules. The mean weight of the paired control kidneys was 1.7 g whereas those of Hg^{2+}-dosed (2.5, 5.0, 7.5, and 10 μmol/kg body weight) animals were 1.75, 2.08, 2.12, and 2.11 g, respectively, probably reflecting a degree of tissue edema at the higher doses.

An assessment of the observed structural damage to proximal tubules is shown in Table 3. At the lowest Hg^{2+} dose, 2.5 μmol/kg, damage was confined to the pars recta (S_3) whereas at 5.0 μmol/kg, degenerative changes and necrosis were much more pronounced and were also seen in the most proximal portions of the proximal tubules. Necrotic cells were observed in the terminal portion of the pars convoluta (S_2) as well as in S_3. A few tubular cell casts were seen in the distal tubules and medullary collecting ducts.

The pattern and severity of damage at the two higher doses were similar. The distal and collecting ducts of these animals were frequently seen to be obstructed with necrotic cellular debris derived from exfoliation and necrosis in the more proximal pars rectae (S_3). The distal and collecting duct cells themselves appeared normal by light microscopy. Nuclear pyknosis or karyorrhexis was frequently observed in S_3 cells. There was also widespread loss of microvilli. Many tubules were devoid of epithelial cells, leaving only the basic tubular architecture provided by the basal lamina. However, tubulorrhexis was not observed. No evidence for cellular regeneration or mitosis was found. Moderate to severe necrosis of the terminal pars convoluta (S_2) was observed in these animals together with occasional cell exfoliation. Hydropic, degenerative changes were severe in the S_2 cells which contained large fluid-filled vacuoles and numerous autophagolysosomes.

The detailed histopathology of the Hg^{2+}-induced proximal tubular lesions was generally similar to that described by earlier workers (4, 6, 18, 19). Glomerular

[2] The proximal tubular segment S_1 represents the initial periglomerular convolutions of the pars convoluta; S_2 is the terminal convolutions of the pars convoluta; S_3 is the straight terminal segment of the proximal tubule, the pars recta.

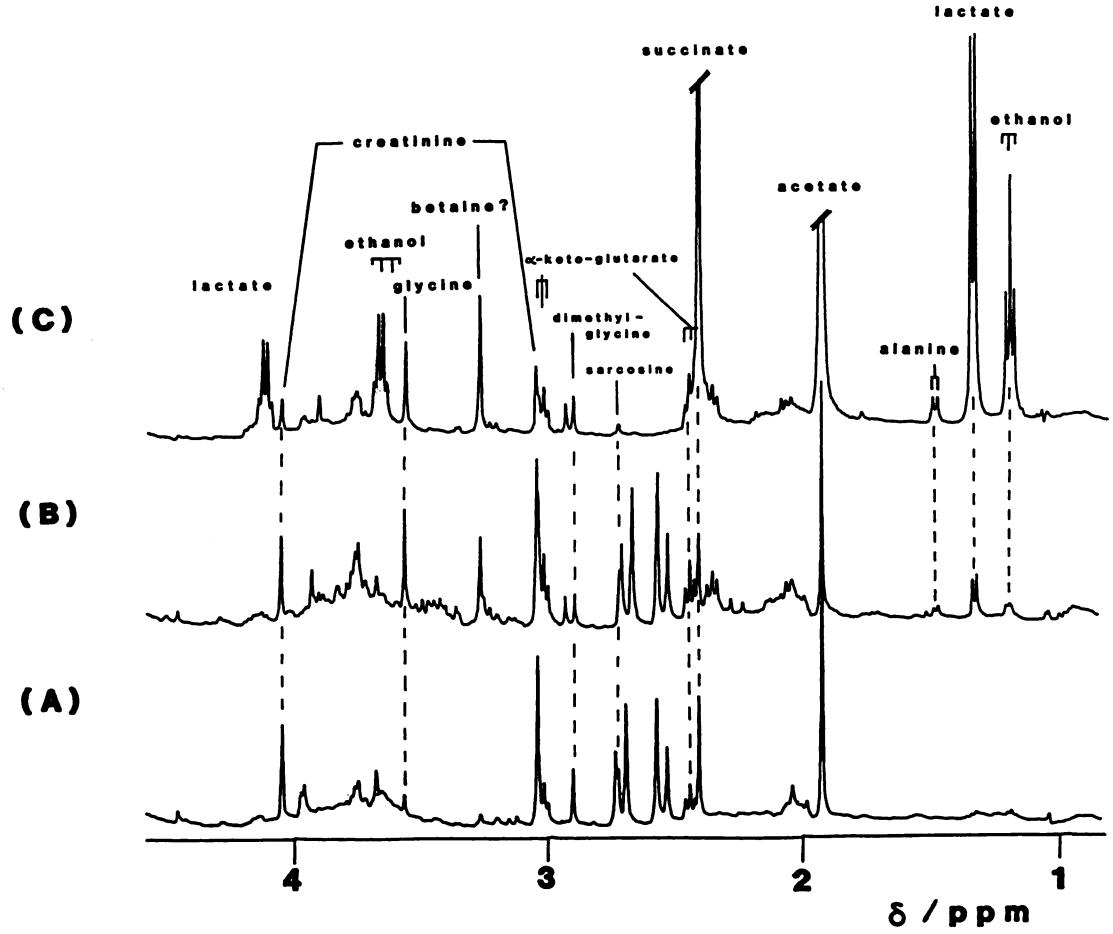

FIG. 2. *400-MHz ^1H NMR spectra of urine collected over 24-hr periods*
A, fasted control rat (lower trace); B, fasted rat which had received a dose of 5.0 μmol of Hg/kg body weight (middle trace); C, fasted rat which had received a dose of 10.0 μmol of Hg/kg body weight (upper trace).

TABLE 2
Lactate and creatinine concentrations in the plasmas of rats 24 hr after Hg^{2+} dosing
Values are means ± SE, $n = 3$.

Metabolite	Concentration at Hg^{2+} dose (μmol/kg)		
	0	7.5	10.0
	mM		
Lactate	1.03 ± 0.12	1.77 ± 0.22	1.90 ± 0.20
Creatinine	0.12 ± 0.02	0.28 ± 0.06	0.32 ± 0.06

capillaries and larger renal blood vessels appeared normal by light microscopy.

Excretion of γ-GT and urinary pH changes. There was a clear dose-response relationship between the excretion of this enzyme and Hg^{2+} dose (Table 3). At the two higher Hg^{2+} doses, γ-GT concentrations in urine were significantly elevated above those of controls ($p < 0.05$). A variety of nephrotoxins which cause acute tubular necrosis have previously been shown to cause this effect (20), especially mercurials. The elevations of urinary γ-GT observed in this study were consistent with the detection of tubular necrosis by light microscopy (Table 3).

The mean urinary pH values for rats dosed with 0, 2.5, 5, 7.5, and 10 μmol/kg were 7.1, 6.9, 6.7, 7.5, and 8.2, respectively. The increasing urinary alkalinity with increasing Hg^{2+} dose was probably an indication of failure to acidify the urine and concomitant renal tubular acidosis.

DISCUSSION

Administration of high doses of mercury (e.g., 10 μmol of Hg/kg) to experimental rats is known to result in very severe proximal tubular damage and possibly acute renal failure (8). The present study demonstrates, for the first time, that high resolution proton NMR can be used to assess nephrotoxicity and can reveal an informative pattern of associated biochemical changes. These could not have been detected simultaneously by any other single technique. The NMR method is rapid (a few minutes per sample) and there is no need to preselect metabolites for detection. All those molecules with suitable NMR-detectable protons and present in urine at near millimolar concentrations are likely to give resonances, allowing a large number of important intermediary metabolites present in urine to be studied (3).

Information derived from metabolite excretion patterns can be related to functional changes in the renal tubules after exposure to Hg^{2+}. A failure to reabsorb low

TABLE 3

Assessment of proximal tubular damage induced by Hg^{2+} dosing and the urinary excretion of γ-GT

−, little or no abnormality; +, few (< 10%) cells showing abnormalities; ++, moderate changes in >20% cells; +++, severe changes in >70% cells.

Hg^{2+} dose	γ-GT in urine	Cell pathology in proximal tubule segments								
		Cloudy swelling			Vacuolar/hydropic degeneration			Necrosis		
		S_1	S_2	S_3	S_1	S_2	S_3	S_1	S_2	S_3
μmol/kg	*units × 10^5*									
0	1.56 ± 0.47	−	−	−	−	−	−	−	−	−
2.5	[a]	+	+	++	−	+	++	−	+	+
5.0	10.4 ± 6.9	++	+++	+++	++	++	+++	+	++	+++[b]
7.5	25.9 ± 3.7[c]	++	++	(+)[d]	++	++	(+)[d]	+	++	+++[e]
10.0	27.3 ± 9.6[c]	++	++	(+)[d]	++	+++	(+)[d]	+	++	+++[e]

[a] Not determined.

[b] Occasional cell casts observed in medullary collecting ducts.

[c] Statistically significant difference from control, $p < 0.01$.

[d] Fewer cells were observed with mild degenerative changes in these tubular segments due to the great preponderance of severely damaged and necrotic cells.

[e] Complete stripping of tubular epithelium frequently observed, together with exfoliated necrotic debris obstructing the distal tubules and medullary collecting ducts.

molecular weight compounds such as amino acids and glucose is a typical manifestation of nonspecific proximal tubular damage. We observed by NMR a dose-related glycosuria and an apparently specific aminoaciduria. No glucose was detected in the urine or either control animals or those receiving 2.5 μmol of Hg/kg. The urine of all rats receiving higher Hg^{2+} doses contained glucose, although in quite variable quantities. Previous studies using conventional analytical methods have shown that urinary glucose was significantly elevated in fed rats 9–24 hr after receiving 2.5 μmol of Hg/kg and grossly elevated in rats receiving 5 μmol of Hg/kg (21). The absence of glucose in the urine at the lowest Hg^{2+} dose in our experiments can be attributed to fasting of the rats, causing a lowering in plasma glucose and therefore reducing the tendency towards urinary glucose overflow during proximal tubule dysfunction.

In the present study, the only amino acids with elevated levels in urine after Hg^{2+} dosing detected by ^{1}H NMR were alanine and glycine (Fig. 3). These appear to be good candidates as NMR markers for Hg^{2+}-induced proximal tubular lesions as they give well resolved ^{1}H NMR signals (Fig. 2, Table 1). In particular, the 24-hr excretion of alanine was significantly elevated in rats receiving 5 μmol of Hg/kg, making it as sensitive an indication of nephrotoxicity as urinary alkaline phosphatase (21) or γ-glutamyltransferase activity (Table 3).

The dose-related reduction in creatinine excretion (Table 1) may be related to lowered rates of glomerular filtration which might be expected due to perturbation of the renin-angiotension system, thereby causing a reduction in renal cortical blood flow (8). This, in turn, is probably related to the elevated levels of plasma creatinine observed in rats receiving the highest dose of Hg^{2+} (Table 2).

Marked, dose-dependent changes in the excretion of three Krebs cycle intermediates were observed: an elevation of succinate and α-ketoglutarate levels and a decrease in urinary citrate (Fig. 2, Table 1). At the two higher Hg^{2+} doses, no citrate ^{1}H NMR peaks from the urine were observed. We interpret this to mean the absence of citrate, although metal-binding reactions, especially those involving the formation of polymeric citrate complexes, could lead to severe broadening of citrate resonances (22). The addition of EDTA to urine samples that gave no citrate ^{1}H NMR signals did not result in the appearance of citrate resonances, although all the calcium and magnesium present in the urine became complexed with EDTA as evidenced by the appearance of sharp $[Ca\text{-}EDTA]^{2-}$ and $[Mg\text{-}EDTA]^{2-}$ ^{1}H resonances. This strongly suggested that citrate concentrations were indeed very low in the urine samples tested.

It has long been known that the urinary excretion of citrate is independent of the plasma citrate concentration (23), but is decreased during RTA (24). It has been established that the reabsorption of both citrate and α-ketoglutarate from the renal tubular fluid into the tubular cells is dependent on the intracellular pH and more particularly on the intracellular bicarbonate concentration (25, 26). This controls the rate of transport of citrate across the inner mitochondrial membrane, the activity of the citric acid cycle enzyme aconitase, and, therefore, the degree of utilization of citrate by the renal tubular cells (26). In the present study, urinary citrate levels were very low in rats receiving the highest Hg^{2+} doses and the urinary pH was high, indicative of RTA. There are probably two main effects of Hg^{2+} that could cause RTA. First, Hg^{2+} could be acting as a carbonic anhydrase inhibitor, causing a failure to reabsorb bicarbonate efficiently with a consequent lowering of the intracellular pH of the tubular cells. Both Hg^{2+} and Cd^{2+} are known to cause a substantial reduction in carbonic anhydrase activity in a variety of organs and tissues including the kidneys (27). Acetazolamide, a potent carbonic anhydrase inhibitor, is also known to reduce substantially urinary citrate excretion (25). In addition, Hg^{2+} could

cause a lactic acidosis due to other selective toxic effects on mitochondrial oxidative enzymes producing a consequent increase in activity of anaerobic metabolic pathways. This possibility is supported by the increase in the plasma lactate concentration in rats receiving the highest Hg^{2+} doses (Table 2) and also by the gross increase in urinary lactate excretion by these animals (Table 1). It should be noted that inhibition of renal carbonic anhydrase would produce both a type 1 (distal) and a type 2 (proximal) RTA as most of the enzyme is located in these tubules; lactic acidosis could also occur in both distal and proximal convoluted tubules, exacerbating the effect of carbonic anhydrase inhibition. It would be expected on the basis of the histopathological observations (Table 3) that the most severe Hg^{2+}-induced, intracellular metabolic disturbances would occur in the proximal tubules, which is correlated with the observed aminoaciduria and glycosuria at high Hg^{2+} doses.

The effects of Hg^{2+} on the urinary excretion of citric acid cycle intermediates cannot be explained simply in terms of RTA. We observed reduced citrate excretion together with increased excretion of α-ketoglutarate and succinate at the higher Hg^{2+} dose levels (Table 1). In simple RTA caused by KCl or acetazolamide injection, the excretion of both citrate and α-ketoglutarate is reduced, suggesting that similar factors and mechanisms control their reabsorption from the tubular fluid (25). It would appear, therefore, that despite RTA in the Hg^{2+}-treated rats, the utilization of α-ketoglutarate and succinate by the tubular cells is reduced, allowing these compounds to pass out in the urine in large quantities. Further release of metabolites into the urine may accompany cell necrosis and autolysis.

Our results may be explicable by specific inhibition of certain Krebs cycle enzymes. Mercury has a particularly high affinity for thiolates and sulfide as ligands (28). Mitochondrial malate dehydrogenase contains about 12 SH/mol (29) and succinate dehydrogenase contains an iron thiolate-sulfide cluster (30); it has previously been shown by histochemical studies that both of these enzymes are inhibited between 3 and 6 hr after administration of 20 μmol of Hg/kg to rats (8). In the present study, inhibition of these enzymes could explain the increased excretion of α-ketoglutarate and succinate as the renal tubular cells would be less able to utilize these substrates.

It has been proposed that dysfunction of kidney mitochondria is the primary cause of Hg^{2+} toxicity in rats and, moreover, mitochondria from the kidneys of Hg^{2+}-treated rats contained three times more calcium than normal. Crystalline deposits containing calcium are often seen in the damaged mitochondria of the proximal tubules of $HgCl_2$-treated rats (31). Since we have found that urinary output of citrate is severely disturbed, we propose that this has a direct effect on calcium metabolism. The affinity constant of citrate for Ca^{2+} is approximately 1.66×10^3 liters M^{-1} (32) and citrate has buffering capacity for Ca^{2+} both inside cells and in urine.[3] It has previously been suggested that citrate in the urine increases the solubility of calcium (33). Reduction in citrate excretion caused by Hg^{2+} will reduce the buffering capacity for Ca^{2+} in the urine and could lead to precipitation of Ca^{2+} salts and nephrocalcinosis. This would be exacerbated by the hypercalciuria which is expected to be associated with proximal tubular necrosis (10).

The elevation of lactate levels in both the plasma and urine of rats receiving the higher Hg^{2+} doses is consistent with reduced utilization of pyruvate in the citric acid cycle and an increase in anaerobic cell respiration. This may also account for the increased output of ethanol, although it is possible that Hg^{2+} inhibits liver alcohol dehydrogenase and so cause an increase in the circulating levels of ethanol similar to that which occurs after treatment with the enzyme's inhibitor, pyrazole (17). High lactate levels could also occur indirectly due to cortical ischemia at high Hg^{2+} doses, which might be expected due to the reduction of renal cortical blood flow following inappropriate stimulation of the renin-angiotensin system. It has been proposed that the resulting renal cortical hypoxia is an important cause of secondary tubular damage after acute Hg^{2+} exposure (9).

Conclusions based upon analysis of urinary metabolites must be regarded as tentative. The composition of urine will not only reflect the specific molecular target effects of mercury on the renal tubular cells but also a combination of systemic cellular effects which lead to abnormal levels of metabolites in the plasma and therefore possibly to urinary overflow. This is further complicated by impaired solute reabsorption from the tubular lumen caused by the reduced efficiency of the proximal tubular epithelium and indirect effects on tubular acid-base balance. The differential contributions of these effects are under investigation. Since in the present case little change in the ^1H NMR spectra of plasma was observed, except for lactate and creatinine, and as the kidney is the principal target organ in Hg^{2+} toxicity, the results suggest that the abnormal excretion profiles are largely a reflection of the effects of mercury on the kidney cortical cells.

We have demonstrated for the first time that analysis of ^1H NMR spectra of urine can provide new insights into the biochemical changes associated with nephrotoxicity as well as providing a means for its early detection. The method should be applicable to the study of a wide range of nephrotoxins and renal diseases in general. Metabolites at millimolar levels having suitable protons will usually appear in ^1H NMR spectra, and many of these are involved in important cellular biochemical processes. Much information on the biochemical mechanisms of nephrotoxicity can be derived from the changes in metabolite excretion patterns.

We have recently shown (34) that the major metabolites of the analgesic drug paracetamol can be detected and quantified by ^1H NMR. The possibility therefore emerges of investigating simultaneously the metabolism and excretion of drugs and their effect on renal function.

This approach may lend itself to large scale toxicological screening operations as well as drug metabolism studies. However, it should be emphasized that quantitative measurements by ^1H NMR are still at an early stage of development and further work on possible inter-

[3] J. R. Bales, J. K. Nicholson, and P. J. Sadler, unpublished.

ferences is still required before such utility can be established.

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