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Investigations into the Biochemical Effects of Region-Specific Nephrotoxins

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SUMMARY

^{1}H NMR spectroscopy provides a useful initial biochemical screen with which to detect abnormal patterns of metabolites in urine collected from animals with different sites of nephrotoxic lesions. Male Fischer 344 rats were treated with nephrotoxic doses of sodium chromate (pars convoluta of proximal tubule), cisplatin, hexachlorobutadiene, mercury II chloride (pars recta of proximal tubule), propylene imine, and bromoethanamine (renal papilla) in order to induce damage in specific regions of the kidney. Urine was collected for up to 48 hr after dosing and was analyzed by ^{1}H NMR spectroscopy (400 MHz) and conventional biochemical methods to provide biochemical fingerprints of urine in various site-specific nephrotoxic states. Hexachlorobutadiene and $HgCl_2$ produced severe glycosuria and transient enzymuria. ^{1}H NMR urinalysis revealed aminoaciduria, glycosuria, and lactic aciduria after exposure to all proximal tubular toxins except cisplatin, whereas papillary insult resulted in early elevations in urinary trimethylamine *N*-oxide and dimethylamine, together with later elevations in urinary acetate, succinate, and *N,N*-dimethylglycine (after propylene imine). Trimethylamine *N*-oxide and dimethylamine are suggested as novel markers of site-specific renal papillary injury in the rat.

High resolution ^{1}H NMR spectroscopy has been used for the rapid multicomponent analysis of low MW compounds in plasma and urine (1, 2). We have recently explored the potential of this technique in the field of analytical toxicology in general, and nephrotoxicity in particular, with studies employing mercury II chloride (3) and *p*-aminophenol (4). The technique has also been successfully applied to the study of inborn errors of metabolism in humans (5).

In the present study we have applied ^{1}H NMR and other techniques to study the biochemical effects of six nephrotoxins, including mercury II chloride, in the male Fischer 344 rat. The compounds and doses were chosen for their known sites and severity of nephrotoxic action. Doses were selected to produce reproducible lesions. The following compounds were used: sodium chromate, which damages the pars convoluta of the proximal tubule (6); cisplatin, mercuric chloride, and HCBD, which all cause lesions in the pars recta (S3) of the proximal tubule but do so by different mechanisms (7–9); and PI and BEA, which are inner medullary toxins causing renal papillary necrosis (10, 11). All are acute nephrotoxins producing renal damage after a single injection. Because these toxins produce damage to a particular region within the kidney, the term 'region-specific' nephrotoxicity has been adopted in these experiments. An important objective of the present study was to use ^{1}H NMR and other techniques to map the biochemical abnormalities in the urine associated with region-specific nephronal damage and to detect novel urinary markers of this toxicity. Although numerous markers of renal tubular damage exist and are in current use in many toxicology and clinical laboratories, a specific and sensitive marker of renal papillary damage has so far eluded researchers.

Among the post promising candidates in recent years are urine profiles of NAG isoenzymes (10), the methodology for which requires time-consuming and labor-intensive sample preparation. However, no low MW compound has, as yet, been put forward as a possible urinary marker of papillary damage. We have therefore taken advantage of the nonselective but specific nature of NMR as an analytical technique to search for novel low MW markers of renal papillary damage.

Experimental Procedures

Animals and treatments. Forty male Fischer 344 rats were allocated to eight groups of five rats each. This strain of rat has been reported to be susceptible to certain nephrotoxins at doses that are

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[1] Unpublished observations.

ABBREVIATIONS: MW, molecular weight; HCBD, hexachloro-1,3-butadiene; BEA, 2-bromoethanamine hydrobromide; TSP, sodium 3-trimethyl-[2,2,3,3-^{2}H]-1-propionate; BUN, blood urea nitrogen; DMA, dimethylamine; DMG, *N,N*-dimethylglycine; NAG, *N*-acetyl-β-D-glucosaminidase; PI, propylene imine; TMAO, trimethylamine *N*-oxide; UFR, urine flow rate; CoA, coenzyme A.

comparable to those to which humans are exposed (12). Animals were housed individually in metabolism cages for a period of 6 days before treatment, to permit acclimatization. Food and tap water were allowed *ad libitum*. Urine was collected over ice for 24 hr before dosing and at times of 8, 24, and 48 hr after dosing. Plasma was sampled 48 hr after dosing. Table 1 outlines the treatments given and the concentrations of dosing solutions, route, and the vehicles used for the six nephrotoxins employed in the present study. Nephrotoxins were supplied by Aldrich Chemical Co. (Milwaukee, WI) (BEA and sodium chromate), BDH (Poole, Dorset, UK) (mercury II chloride), and Sigma Chemical Co. (St. Louis, MO) (cisplatin and PI).

Conventional biochemical procedures. NAG was measured in desalted urine by the method of Maruhn (13). A volume of 25 ml of urine was loaded onto a Sephadex G25-M minicolumn (Pharmacia Fine Chemicals, Piscataway, NJ). The first 2.5 ml was discarded and the protein-containing fraction eluted in 4 ml of 0.9% NaCl. This eluate was stored at 4° and used later for the determination of urinary enzymes. Urine was also tested for glucose by the hexokinase method (Sigma kit no. 16-UV). When elevations were seen by NMR, L-(+)-lactic acid and acetic acid were measured in perchloric acid extracts of urine using commercially available test kits [L-(+)-lactic acid, Sigma kit no. 826-UV: acetic acid, Boehringer kit no. 148261]. These methods (glucose, L-lactic acid, and acetic acid measurements) were all based on the appearance or disappearance of NADH at 340 nm. Urine osmolality was determined by freezing point depression on an Advanced Instruments 3W2 Osmometer, and plasma was tested for urea nitrogen using a Beckman diagnostic kit (based on the disappearance of NADH at 340 nm). Statistical analysis of data was carried out using unpaired Student's t test. A probability of $p < 0.05$ was taken as the level of significance.

^{1}H NMR urinalysis. Measurements were made on a Bruker 400 MHz spectrometer, operating at a field strength of 9.4 tesla (400 MHz ^{1}H frequency), at 25° using 0.45 ml of urine diluted with 0.05 ml of ^2H_2O (lock signal) in 5-mm tubes. Shifts were referenced to internal TSP (2 mM; $\delta = 0$ ppm).

No absolute NMR quantitation was attempted in this study because of the tendency of TSP to bind to protein (rats are physiologically proteinuric and the urine contains even more protein after nephrotoxic insult). Protein binding results in a broadening and a reduction in the intensity of the TSP resonance, thus making its use as an internal quantitation standard unreliable in this case. Urine was analyzed either without lyophilization or after a lyophilization step to correct for any increase in UFR caused by the toxin. Lyophilization of urine was exclusively used in these studies when dilute urine was produced. The volume to be lyophilized was calculated as follows: the UFR of the polyuric group was expressed as a ratio with that of the control group and a factor, F, was obtained. Multiplication of this factor by 0.45 [final volume (ml) in which the lyophilized sample was dissolved] gives the volume of urine to be lyophilized and reconstituted with ^2H_2O. Sixty-four free induction decays were collected into 16,384 computer points using 28° (3-μsec pulse) with an acquisition time of 1.7 sec. An additional delay of 3.0 sec between pulses was added to ensure that the spectra were fully T_1 relaxed (total scanning time was about 5 min per sample). A continuous secondary irradiation field at the resonance frequency of water was applied in order to suppress the intense water signal in nonlyophilized urine and to minimize spectrometer dynamic range problems. The detection limit of ^{1}H NMR spectroscopy at 9.4 tesla is approximately 50 μM. Metabolite identity was confirmed by measuring a combination of chemical shift, coupling constants and, ultimately, by standard addition.

^{1}H NMR analysis of acid-extracted renal tissue. Portions of separated renal cortex and papilla (inner medulla) were also examined by ^{1}H NMR spectroscopy. After homogenization of a known weight of tissue (500 mg of cortex; 50 mg of papilla) in 1.0 ml of ice-cold 0.9% NaCl and removal of protein by the addition of 6.5% trichloroacetic acid, the supernatants were titrated to pH 7, lyophilized, and redissolved in 0.50 ml of ^2H_2O containing 2 mM TSP, before analysis as outlined above.

Renal histopathology. Kidneys were removed from rats immediately after sacrifice. Portions of kidney tissue (100 mg) were placed in 2% Karnowsky's fixative solution (2% glutaraldehyde plus 2% paraformaldehyde in 100 mM cacodylate buffer) at 4° for histopathological assessment. After processing, wax (7 μm) sections were stained with hematoxylin and eosin and were examined by light microscopy.

TABLE 1

Doses, routes, and vehicles used for the administration of the six nephrotoxins

Control (S), saline; control (C), corn oil.

Treatment	Dose	Stock Solution	Route	Vehicle
Control (S)			IP[a]	0.9% NaCl
Control (C)			IP	Corn oil
Cisplatin	5 mg/kg	1 mg/ml	IP	0.9% NaCl
Sodium Chromate	20 mg/kg	10 mg/ml	SC[b]	0.9% NaCl
Mercuric Chloride	2 mg/kg	1 mg/ml	IP	0.9% NaCl
HCBD	200 mg/kg	100 mg/ml	IP	Corn oil
PI	20 μl/kg	1%	IP	0.9% NaCl
2-BEA/HBr	250 mg/kg	125 mg/ml	IP	0.9% NaCl

[a] IP, intraperitoneal.
[b] SC, subcutaneous.

Results and Discussion

^{1}H NMR analysis offers several advantages when compared with more conventional urinalysis methods. A high resolution ^{1}H NMR spectrum can be obtained from urine in a matter of minutes with only minimum sample pretreatment (1, 2). All molecules that have suitable NMR-detectable protons and are present in urine at near millimolar concentrations give signals, allowing many important intermediary metabolites, drug metabolites, and excretion products present in urine to be studied simultaneously (1, 2). NMR spectroscopy is nondestructive and so the sample may be analyzed further by conventional methods if required. In the present study, two general classes of nephrotoxin were used, those that produce damage to certain segments of the proximal tubule and others that produce damage to the inner medulla (renal papillary necrosis).

Proximal Tubular Toxins

Sodium chromate. Evan and Dail (6) have shown microscopically that the proximal convoluted tubule cells are the selective targets of sodium chromate intoxication. The functional observations reported here support their description. Subcutaneous administration of 20 mg/kg sodium chromate caused progressive increases in urinary enzyme and glucose excretion (Table 2). Using the same dose and route, Kirschbaum *et al.* (14) reported 2- and 3-fold elevations in urinary NAG at 2 and 4 hr, respectively, after dosing, followed by a 5-fold elevation at 12 hr. In the present study, a 2-fold increase in NAG excretion was witnessed at 8 hr, followed by 12- and 37-fold elevations at 8–24 and 24–48 hr, respectively. Viau *et al.* (15) observed a modest increase in NAG excretion after 10 mg/kg sodium chromate, whereas Maruhn *et al.* (16) reported a 4-fold increase after a dose of 30 mg/kg.

In the present study, after chromate dosing, considerable elevations in urine glucose were seen throughout the 24–48-hr period by both conventional (Table 2) and ^{1}H NMR urinalysis (Fig. 1), consistent with damage to the pars convoluta (S1) of the proximal tubule. Histological examination of kidneys at 48 hr revealed extensive necrosis in this segment of the proximal tubule (data not shown). Such increased levels of urine NAG and glucose indicate increased susceptibility of the F344 rat to

TABLE 2
Effect of six region-specific nephrotoxins on urine parameters in the male Fischer 344 rat
CDDP, *cis*-dichlorodiammine platinum II (cisplatin); GLC, glucose; OSM, urine osmolality.

Urine Parameters	0–8 hr				8–24 hr				24–48 hr			
	GLC	NAG	UFR	OSM	GLC	NAG	UFR	OSM	GLC	NAG	UFR	OSM
	μmol/hr/kg	mU/hr/kg	ml/hr	mOsm/kg of H_2O	μmol/hr/kg	mU/hr/kg	ml/hr	mOsm/kg of H_2O	μmol/hr/kg	mU/hr/kg	ml/hr	mOsm/kg of H_2O
Control (n = 5)	4.03 ± 1.30	84.79 ± 27.22	0.23 ± 0.03	2408 ± 116	8.37 ± 0.54	101.72 ± 5.20	0.19 ± 0.02	2570 ± 112	10.22 ± 1.34	93.63 ± 6.18	0.13 ± 0.004	2688 ± 184
Chromate (n = 5)	9.60 ± 0.78[a]	323.25 ± 20.29[a]	0.18 ± 0.01	2158 ± 113	32.50 ± 3.55[a]	2100.06 ± 256.21[a]	0.28 ± 0.04[a]	2156 ± 157	1420.25 ± 122.06[a]	6148.98 ± 588.30[a]	0.54 ± 0.08[a]	1490 ± 94[a]
$HgCl_2$ (n = 4)	25.42 ± 6.14[a]	649.50 ± 108.15[b]	0.33 ± 0.06	1380 ± 114[b]	123.48 ± 19.99[b]	9180.38 ± 1743.03[b]	0.33 ± 0.06	853 ± 50[c]	66.19 ± 25.55[a]	1079.26 ± 369.72[a]	0.59 ± 0.07[a]	633 ± 80[c]
CDDP (n = 5)	6.45 ± 1.41	144.18 ± 14.59	0.45 ± 0.08[a]	1324 ± 218[a]	13.28 ± 1.29[a]	202.55 ± 8.01	0.67 ± 0.05[b]	994 ± 175[b]	10.84 ± 1.06	217.82 ± 10.39[a]	0.53 ± 0.05[a]	1064 ± 119[b]
HCBD (n = 5)	8.62 ± 0.76[a]	264.21 ± 44.92[a]	0.48 ± 0.03[b]	1160 ± 72[b]	449.01 ± 46.10[c]	3127.77 ± 262.52[c]	1.64 ± 0.05[c]	530 ± 32[c]	399.44 ± 15.46[c]	552.05 ± 36.50[c]	0.77 ± 0.05[c]	874 ± 101[c]
PI (n = 5)	20.62 ± 6.15[a]	229.30 ± 92.74	0.90 ± 0.06[c]	962 ± 89[c]	35.47 ± 3.81[a]	1154.97 ± 108.50[c]	1.26 ± 0.10[c]	512 ± 13[c]	14.18 ± 3.72	428.15 ± 62.46[c]	1.21 ± 0.09[c]	526 ± 41[c]
BEA (n = 5)	10.16 ± 1.19[a]	262.86 ± 28.37[b]	1.54 ± 0.17[c]	680 ± 46[c]	16.12 ± 0.58	452.70 ± 203.42[c]	1.30 ± 0.12[c]	498 ± 25[c]	6.74 ± 3.68	257.26 ± 30.93[a]	1.44 ± 0.06[c]	522 ± 51[c]

[a] $p < 0.05$; [b] $p < 0.005$; [c] $p < 0.001$ when compared with control throughout the same time period.

Fig. 1. 400 MHz ^{1}H NMR spectra of urine from rats before (−24–0 hr) and after dosing with 20 mg/kg sodium chromate. See text for experimental conditions.

chromate-induced nephrotoxicity. The delay in the chromate-induced proximal tubular lesion has been observed previously (17). This latent period may reflect a genuine 'lag phase' before production of the lesion or, alternatively, may be the effect of slow release of toxin from the subcutaneous injection site. The latter explanation is unlikely, however, because changes in the ^{1}H NMR profile are apparent 8 hr after dosing. Further, intraperitoneal administration of sodium chromate (20 mg/kg) failed to cause significant elevation in urine NAG and only produced minor elevations in urinary glucose throughout 2 days after exposure (17), suggesting that this compound is more toxic when administered by other routes. Indeed, Tandon (18) stated that chromates in general damage proximal convoluted tubules more if they are injected subcutaneously or intravenously.

Mercury II chloride. The main site of action of mercury II salts such as $HgCl_2$, when systemically administered, is the kidneys, where the necrosis of the epithelial cell lining the pars recta of the proximal tubule is the principal pathological effect above a threshold dose of 1 mg/kg in rats (8). A dose of 2 mg/kg produced extensive necrosis of the pars recta of the proximal tubule at 24 hr (3). In the present study, histopathological examination of the kidneys at 48 hr showed complete necrosis of the pars recta with extensive necrosis of the pars convoluta (data not shown). This dose resulted in a considerable glyco-

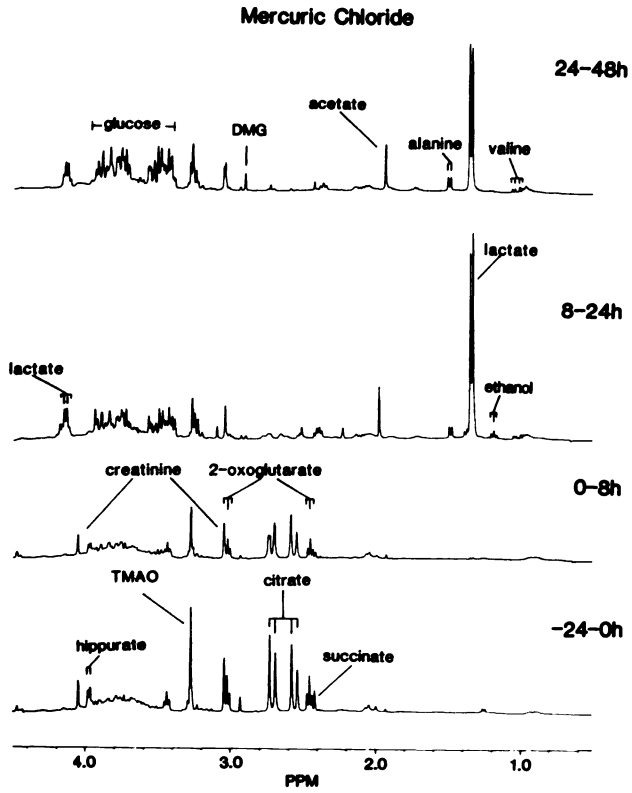

Fig. 2. 400 MHz ¹H NMR spectra of urine from rats before (–24–0 hr) and after dosing with 2 mg/kg $HgCl_2$. See text for experimental conditions.

TABLE 3

Effect of six nephrotoxins on plasma urea nitrogen levels 48 hr after dosing

Values are mean ± standard error. Control (S), saline; control (C), corn oil.

Treatment	Urea Nitrogen
	mg/100 ml of plasma
Control (S) ($n = 5$)	18.04 ± 0.54
Control (C) ($n = 5$)	16.01 ± 0.68
Cisplatin ($n = 5$)	29.73 ± 1.14[a]
Chromate ($n = 5$)	24.04 ± 0.88[b]
$HgCl_2$ ($n = 4$)	170.80 ± 13.62[a]
HCBD ($n = 5$)	34.98 ± 2.61[a]
PI ($n = 5$)	23.83 ± 2.10
BEA ($n = 5$)	31.14 ± 4.42[b]

[a] $p < 0.001$.
[b] $p < 0.05$.

suria and enzymuria together with a moderate elevation in UFR and a severe reduction in urine osmolality (Table 2). In addition, a considerable elevation in BUN was seen 48 hr after dosing (Table 3).

Whereas a similar reduction in osmolality was observed at 24 and 48 hr, a much more modest elevation in BUN (3-fold elevation) was noted 48 hr after 1.5 mg/kg $HgCl_2$ (8). Kyle *et al.* (19) reported a similar reduction in urine osmolality 8 hr after a dose of 1 mg/kg $HgCl_2$, which did not return to control levels until 3 days after dosing. Halman *et al.* (20) observed a peak in NAG excretion 4 days after 3 mg/kg $HgCl_2$, whereas Bomhard *et al.* (17) noted maximal excretion occurred 1 day after a dose of 0.75 mg/kg. The glycosuria seen in the present study after $HgCl_2$ (Table 2) has been observed previously (17, 20). Kyle *et al.* (19) also reported maximal glucose excretion throughout the period 8–24 hr after 1–3 mg/kg $HgCl_2$, whereas Bomhard *et al.* (17) observed peak excretion 3 days after a lower dose (0.75 mg/kg).

¹H NMR analysis of urine from $HgCl_2$-treated rats revealed a number of interesting features (Fig. 2). Glycosuria (confirmed by a conventional biochemical method) and aminoaciduria (alanine, glutamine, and valine) were observed. In addition, ¹H NMR urinalysis revealed lactic aciduria, which was also confirmed by a conventional biochemical method (Table 4). Lactic aciduria has previously been reported after nephrotoxic insult induced by $HgCl_2$ and *p*-aminophenol (3, 4, 21). Other changes in the ¹H NMR profile were the appearance of increased levels of acetate and ethanol together with a reduction in urinary citrate at 8–24 and 24–48 hr after dosing. The $HgCl_2$-induced hypocitraturia has been described previously and has been attributed to toxin-induced alterations in tubular acid-base status together with effects on Kreb's cycle (3).

In the SD rat, Nicholson *et al.* (3) observed, by ¹H NMR, increased urinary levels of glucose, acetate, ethanol, lactate, and amino acids (alanine, glutamine, and glycine) after exposure to $HgCl_2$. These results are in general agreement with those presented here except that Nicholson and co-workers found very considerable elevations in succinic and acetic acids after the highest dose (10 μmol/kg = 2 mg/kg) and no such elevations were seen here. In the earlier studies, rats were fasted throughout the experiment, which may explain in part the differences observed in the results because in fasting, a variety of alternative metabolic pathways are active that could affect the biochemical manifestation of the toxic lesions; these are under investigation.

HCBD. The kidney appears to be the major target organ in which HCBD produces necrosis of the pars recta of proximal tubules (9, 22). In the present study, histological examination of kidneys revealed complete necrosis of the pars recta at 48 hr (data not shown). The acute effects of HCBD on renal function have been examined previously using conventional methods for detecting nephrotoxicity (9, 22). In agreement with the results presented here, Berndt and Mehendale (22) reported an increase in UFR and a decrease in urine osmolality within 24 hr of exposure to HCBD. In addition, these effects were found to be dose related in the SD (22) and Wistar rat (9), respectively.

A 17-fold elevation in urinary NAG was seen in the present study throughout the period 8–24 hr after HCBD, although more modest elevations in urinary NAG have been reported (9). Lock and Ishmael (9) observed a 5-fold increase 24 hr after 200 mg/kg HCBD, with only an 11-fold increase after a 300 mg/kg dose. The greatest effect displayed by HCBD, however, was on urinary glucose excretion, with elevations as high as 54- and 39-fold seen 8–24 and 24–48 hr, respectively, after dosing

TABLE 4

Effect of HCBD and $HgCl_2$ on urinary lactic acid (measured by conventional biochemical method)

Values are mean ± standard error.

	Lactic Acid			
	–24–0 hr	0–8 hr	8–24 hr	24–48 hr
	μmol/hr/kg			
HCBD ($n = 5$)	5.60 ± 0.76	6.51 ± 0.56	66.67 ± 2.28[a]	44.35 ± 4.19[a]
$HgCl_2$ ($n = 4$)	4.39 ± 0.44	5.17 ± 0.60	39.24 ± 13.74[b]	48.64 ± 21.43

[a] $p < 0.001$.
[b] $p < 0.05$.

(Table 2). The disagreement in these results after the same dose of toxin may relate to the different rat strains employed in the studies. Lock and Ishmael used Alderley Park Wistar rats whereas F344 rats were used in the present study. Elevations in BUN similar to those seen here (Table 3) have been reported previously (9).

^{1}H NMR analysis of urine from rats receiving HCBD revealed a considerable glycosuria, together with aminoaciduria and lactic aciduria 8–24 hr after dosing (Fig. 3; Table 4). Elevations in the excretion of acetate and D-3-hydroxybutyrate were also seen.

Cisplatin. (*cis*-Dichlorodiammine platinum II) is an anticancer drug with nephrotoxic side effects (23), producing dose-dependent damage to the pars recta of the proximal tubule (7). No major changes were seen either by conventional methods or by ^{1}H NMR urinalysis after 5 mg/kg cisplatin (Table 2), although progressive reductions in both urinary TMAO and succinate were seen with ^{1}H NMR urinalysis (Fig. 4). Early changes were displayed in UFR and osmolality, with later elevations in urinary NAG and BUN at 48 hr (Table 3). The lack of any effect of cisplatin on urine glucose (and also on the urine ^{1}H NMR profile), combined with such late elevations in the above two parameters, suggests that the lesion induced by cisplatin may take longer than 48 hr to manifest itself. This long (so-called) initiation phase of cisplatin-induced nephrotoxic injury has been reported previously (24). In this instance, ^{1}H NMR urinalysis did not reveal any biochemical abnormalities before the establishment of structural lesions. Only minor histological changes were seen in the pars recta 48 hr after dosing (data not shown).

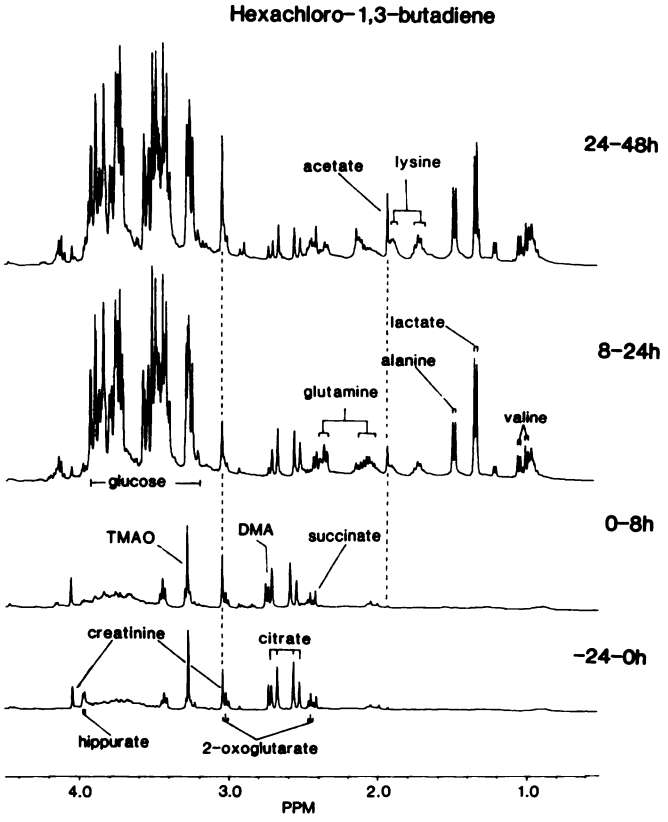

Fig. 3. 400 MHz ^{1}H NMR spectra of urine from rats before (−24–0 hr) and after dosing with 200 mg/kg HCBD. See text for experimental conditions.

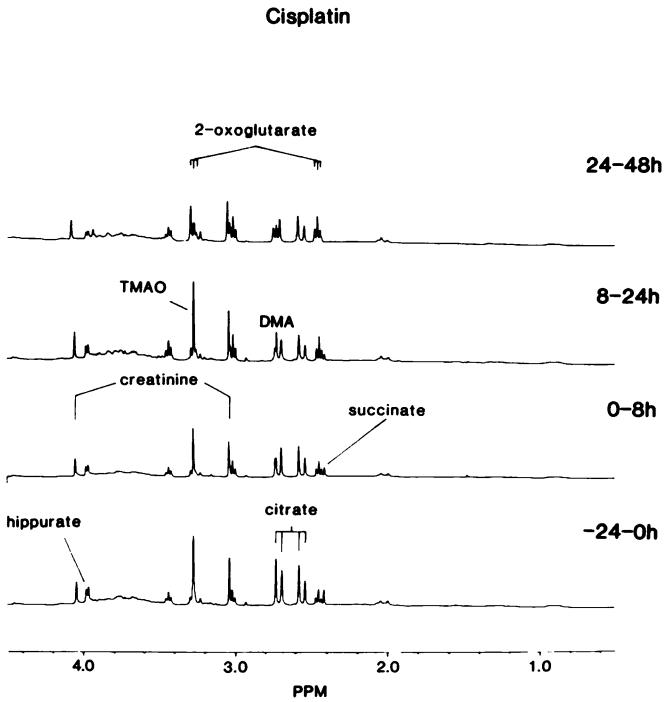

Fig. 4. 400 MHz ^{1}H NMR spectra of urine from rats before (−24–0 hr) and after dosing with 5 mg/kg cisplatin. See text for experimental conditions.

In agreement with the results presented here, a modest elevation in urinary NAG was reported 24 hr after 7.5 mg/kg cisplatin (25). In addition, this dose produced a 7-fold elevation in urine glucose on day 4 together with a progressive reduction in osmolality to a minimum on day 5 (25). This was combined with a significant elevation in BUN at 48 hr, which was seen in the present study after a dose of 5 mg/kg (Table 3). In another study, Halman and Price (10) administered 5 mg/kg cisplatin to rats and observed a small rise in urinary NAG on day 1, with the major peak of excretion appearing on day 4 of exposure. It is likely, therefore, that more dramatic changes would have been observed in the ^{1}H NMR profile (and also by conventional methods) had urine been collected at later time-points after exposure to cisplatin.

A reproducible and distinct pattern of proximal tubular damage has emerged from the results presented here and consists of considerable elevations in urine NAG and glucose, together with more minor elevations in urine lactate, amino acids, and acetate. Glucose and amino acids are well known to be reabsorbed throughout the proximal tubule (26, 27). Before the studies of Nicholson *et al.* (3) and Gartland *et al.* (4, 21), elevations in urinary lactate, acetate, and succinate after nephrotoxic insult were unknown. The lactic aciduria produced by *p*-aminophenol has been discussed previously (4). A number of possible explanations were put forward to explain the *p*-aminophenol-induced lactic aciduria, including decreased reabsorption by the proximal tubules, plasma overflow, and the effect of osmotic diuresis. In light of the evidence presented here, the most probable explanation for the lactic aciduria seen after proximal tubular insult is a decreased reabsorption in this segment, because lactic acid is known to be reabsorbed throughout the proximal tubule (28).

Renal Papillary Toxins

PI. This compound is an important chemical intermediate with a variety of applications in the production of polymers, coatings, adhesives, textiles, and paper finishes. It has been reported to be carcinogenic in rats (29) and to produce renal papillary necrosis after acute exposure (10). In the present study, complete necrosis of the renal papilla of rats was seen in rats receiving PI 48 hr after dosing (data not shown). Halman *et al.* (30) examined the effect of a range of doses of PI on a battery of urinary parameters and renal histology in the rat. In agreement with the results reported here (Table 2), Halman and co-workers described a rapid (within 24 hr) increase in urine volume and an equally rapid decrease in urine osmolality after 20 μl/kg PI. This group reported elevations in urine volume as high as 7-fold at the end of the experiment (16 days) and volume was still elevated 6 months after exposure to this papillary toxin.

Examining the effect of PI on urinary NAG, Halman *et al.* (30) noted a 3-fold increase in the urine activity of this enzyme 24 hr after the dose, followed by a drop in activity on day 2 and a further elevation on day 3, which remained high for several days. This initial trend is in agreement with the results of the present study (Table 2). PI has also been reported to bring about changes in the urine profile of NAG isoenzymes, seen as an increase in the intermediate (I) form (10). However, these changes were late in onset (7–9 days). This group later examined the effect of PI on the urinary excretion of protein, creatinine, and the electrolytes sodium and potassium (30). Immediately after PI administration, marked increases in urinary protein, sodium, and potassium were seen, together with a lesser elevation in urinary creatinine. Histopathological assessments showed that the administration of PI to rats resulted in a selective necrosis at the tip of the renal papilla, similar to that described in dogs after the injection of ethylene imine (30, 31).

A number of changes were seen in the ^{1}H NMR profile of urine after challenge with PI (Fig. 5). Urinary TMAO, elevated after PI, may prove to be a novel site-specific marker of papillary damage TMAO, betaine, L-α-glycerophosphorylcholine, and choline have been shown to be confined to the inner medulla of rats and rabbits and have been suggested to play a role in the maintenance of intracellular osmotic balance in this region (32, 33). ^{1}H NMR spectra of acid extracts of renal cortex and papilla (Fig. 7, *Inner Medulla*) clearly show that these tissues differ markedly in low MW composition.

Whereas the cortex contains predominantly amino acids and lactate, the papilla is high in the trimethylamines, inositol, and sorbitol, that is, osmolytes, in addition to DMG. This methylated amino acid may also be present as an osmolyte in the renal papilla because it is present in excess; approximately 10 times the concentration of DMG is present in the papilla, compared with the cortex (Fig. 7). Other changes highlighted by NMR were early increases in the excretion of DMA and a later increase in DMG after exposure to PI. The assignment of DMA was confirmed by solid phase extraction chromatographic separation monitored by NMR (34) and, by fast atom bombardment mass spectroscopic analysis of the compound purified from rat urine.[1]

The elevations in acetic acid seen by ^{1}H NMR urinalysis were later confirmed by a conventional biochemical method

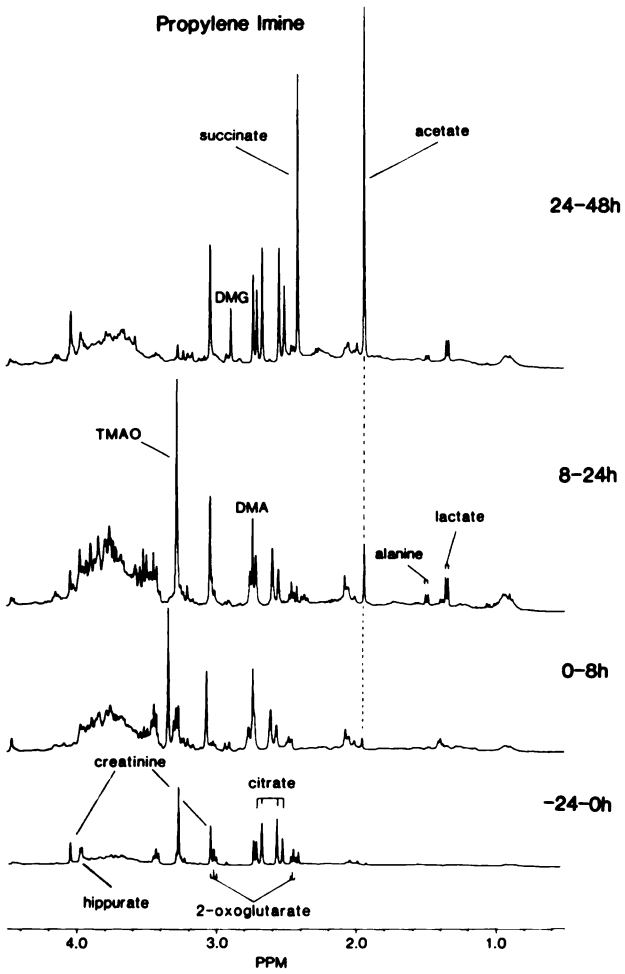

Fig. 5. 400 MHz ^{1}H NMR spectra of urine from rats before (−24–0 hr) and after dosing with 20 μl/kg PI. See text for experimental conditions.

TABLE 5

Effect of PI on urinary acetic acid (measured by conventional biochemical method)

Values are mean ± standard error.

	Acetic Acid			
	−24–0 hr	0–8 hr	8–24 hr	24–48 hr
	μmol/hr/kg			
PI (n = 5)	1.65 ± 0.17	3.12 ± 1.05	5.49 ± 1.43[a]	30.23 ± 4.99[b]

[a] $p < 0.05$.
[b] $p < 0.001$.

(Table 5). Any of these may prove to be novel site-specific markers of renal papillary necrosis.

BEA. Administration of BEA to rats results in the development of papillary necrosis in virtually 100% of animals (11). After a dose of 250 mg/kg BEA, all rats displayed renal papillary necrosis (with loss of papilla in some cases) at 48 hr (data not shown). BEA is a halogenated amine salt, $Br \cdot CH_2CH_2NH_2 \cdot HBr$, that is readily soluble in water and in which the strength of ionic bonds linking hydrobromide is so weakened that the salt is lost to solvent molecules. The rest of the molecule undergoes ring closure to form a three-membered ring (ethylene imine), which is stable in aqueous solution (11, 35).

Numerous studies have examined the effects of BEA on renal structure and function in a number of species and have been discussed in a recent review by Bach and Bridges (36). In the

male F344 rat, a single intraperitoneal dose of 250 mg/kg BEA produced an increase in UFR within 8 hr and a consequent decrease in urine osmolality (Table 2). This is in agreement with previous investigations (11, 37). Murray *et al.* (11) reported urine output to be considerably elevated within 12 hr after BEA (250 mg/kg, intravenously) together with reduced urine osmolality at 48 hr (urine osmolality was not measured before this time). In the present study, the greatly increased UFR was sustained until the end of the experiment (48 hr) whereas in a previous study this effect was shown to continue for at least 20 days after dosing (11). BEA produced a sustained increase in urinary NAG activity, the greatest elevation (2.5-fold) seen throughout the 8–24 hr period (Table 2). This is in agreement with the previous findings (38).

The ^{1}H NMR spectra of urine from rats after exposure to 250 mg/kg BEA showed a profile of metabolic disturbance that is considerably different from that observed after proximal tubular insult (Fig. 6). The severe glycosuria, lactic aciduria, and aminoaciduria seen after proximal tubular toxins like *p*-aminophenol or HCBD were absent from the urine profile after insult to the inner medulla. Instead, early elevations in TMAO and DMA, together with later increases in acetate and succinate, were seen. Other effects seen at later time points were reductions in the urine levels of both citrate and TMAO. However, minor elevations in lactate and alanine were seen at 8–24 and 24–48 hr after dosing and may herald secondary

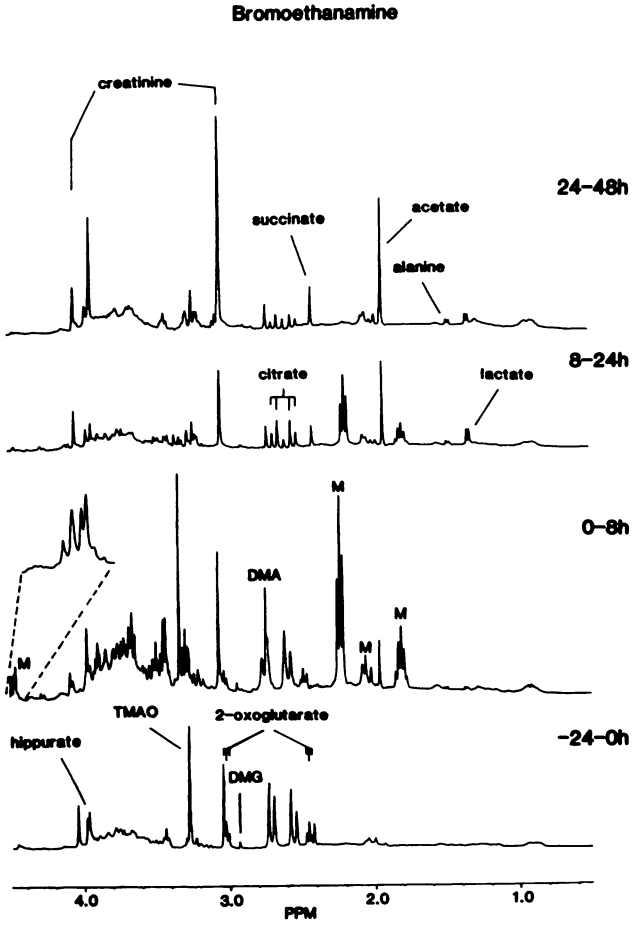

Fig. 6. 400 MHz ^{1}H NMR spectra of urine from rats before (–24–0 hr) and after dosing with 250 mg/kg BEA. See text for experimental conditions. *M*, BEA metabolites.

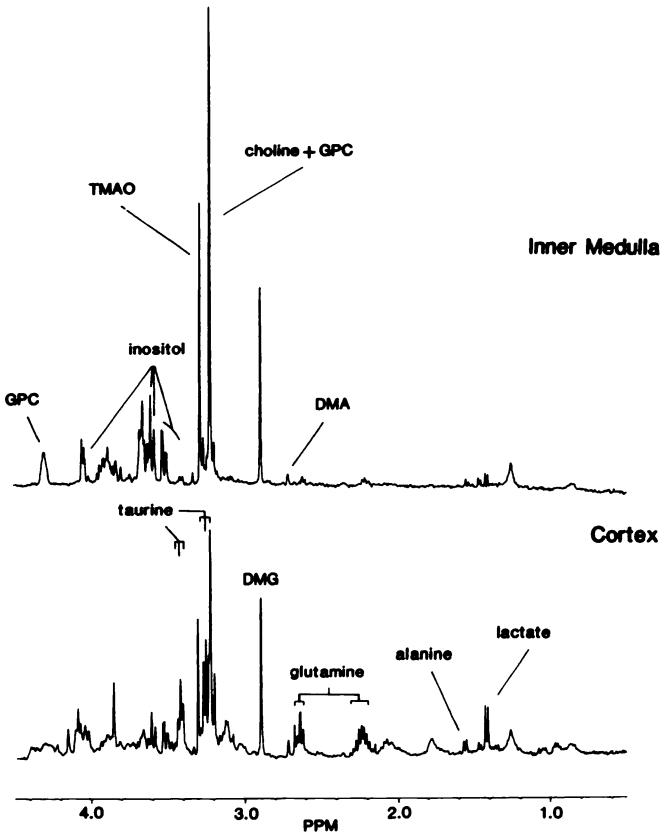

Fig. 7. 400 MHz ^{1}H NMR spectra of acid extracts of renal cortex and inner medulla obtained from control rats. See text for experimental conditions. *GPC*, L-α-glycerophosphorylcholine.

cortical injury induced by BEA. Such effects have been observed histologically in the proximal tubules of long looped nephrons after exposure to BEA (11).

Resonances from metabolites of BEA are visible in the ^{1}H NMR profile of urine collected 8 and 24 hr after treatment but are absent from urine collected at later time points. These metabolites are as yet unidentified.

The present study has shown that two nephrotoxins that cause injury to the renal papilla also cause late elevations in the excretion of acetic and succinic acids. The reason for the late elevations in these two organic acids after exposure to papillary toxins is unclear, although alterations to medullary fatty acid metabolism are probably implicated.

Acetate is an end product of fatty acid oxidation, a process that occurs throughout the kidney but principally within the cortex (39). Fatty acid oxidation is greatest within the cells of the proximal and distal convoluted tubules, as demonstrated by the distribution of activity of the mitochondrial enzyme 3-hydroxyacyl-CoA dehydrogenase (40). That urinary acetate and succinate levels are affected by the metabolic status of the rat was shown by Nicholson *et al.* (3), who reported considerable elevations in both organic acids after a 24-hr fasting period. In the same study, greater elevations were seen after nephrotoxic doses of $HgCl_2$, a toxin known to have an effect on the renal papilla (41). Further, Nicholson and co-workers described a strong correlation ($r = 0.97$) between succinate and acetate excretion in individual rats irrespective of mercury dose. Such findings of high levels of these organic acids after fasting alone would tend to exclude their use as reliable markers of papillary

injury. However, an effect of the toxins on fatty acid oxidation cannot be ruled out. The increased amount of urinary succinate could also arise as a result of increased fatty acid catabolism. β-Oxidation of fatty acids that have an odd number of carbon atoms leads to the formation of propionyl-CoA in addition to acetyl-CoA. The activated three-carbon unit in propionyl-CoA enters the citric acid cycle as succinyl-CoA via D- and L-methylmalonyl-CoA (42). In the model proposed above, toxin-induced increases in fatty acid oxidation would result in increased amounts of propionyl-CoA being generated. It is possible that the increased level of this CoA ester would lead to increased amounts of succinyl-CoA (and therefore succinate). Succinate would then overflow from the kidney into the urine, because any enzyme removing it would become saturated at such high substrate levels, as would any reabsorptive process. This hypothesis is supported by a previous study (43), in which normal rats that were fed 1 g of sodium propionate per day for 2 days showed a marked increase (5-fold) in the urinary excretion of succinate. Overflow from increased plasma levels, another possible cause of the organic aciduria, is unlikely because plasma levels remained within the control range (data not shown).

In summary, in the present study a reproducible pattern of proximal tubular damage has arisen. This pattern consists of enzymuria, glycosuria, aminoaciduria, and lactic aciduria, together with elevated acetate, and is seen after $HgCl_2$, HCBD, and sodium chromate administration. The pattern of low MW metabolites seen in urine after proximal tubular injury is distinct from that seen after medullary injury. Early elevations in TMAO and DMA are seen followed by later elevations in acetate and succinate, together with lactate and alanine. These latter two components may herald secondary cortical insult, in particular proximal tubular insult, because, as we have seen above, these parameters are associated with damage to this region.

^{1}H NMR fingerprint urinalysis in conjunction with conventional biochemical methods is a powerful analytical combination, in that not only can abnormal 'patterns' of low MW metabolites associated with region-specific nephrotoxicity be seen but novel markers of nephronal damage can also be discovered.

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