



## <sup>1</sup>H AND <sup>2</sup>H NMR SPECTROSCOPIC STUDIES ON THE METABOLISM AND BIOCHEMICAL EFFECTS OF 2-BROMOETHANAMINE IN THE RAT

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**Abstract**—Male Fischer 344 rats were dosed with 2-bromoethanamine hydrobromide (BEA, N = 6) or [1,1,2,2-<sup>2</sup>H<sub>4</sub>]-bromoethanamine hydrobromide (BEA-d<sub>4</sub>, N = 6) at 150 mg/kg i.p. and urine was collected –24 to 0 hr pre-dose and at 0–2 hr, 2–4 hr, 4–8 hr and 8–12 hr post-dose (p.d.). Urine samples were analysed directly using 500 and 600 MHz <sup>1</sup>H NMR and 92.1 MHz <sup>2</sup>H NMR spectroscopy. The major observed effect of BEA treatment was the induction of transient elevations in urinary glutaric acid (GTA) and adipic acid (ADA) excretion lasting up to 24 hr p.d. Most of the GTA was excreted in the 0–8 hr p.d. with maximal rates of 100–120 μM/hr for each rat occurring between 4 and 8 hr p.d. in animals treated with BEA or BEA-d<sub>4</sub>. GTA and ADA were shown to be of endogenous origin as there was no detectable incorporation of the <sup>2</sup>H label into either compound following treatment of rats with BEA-d<sub>4</sub>. Following BEA-treatment there was an initial decrease in the levels of urinary citrate, succinate, 2-oxoglutarate and trimethylamine-N-oxide. A subsequent recovery of citrate and succinate was noted following the onset of medullary nephropathy. The abnormal urinary metabolite profiles were similar to that observed in the urine of humans with glutaric aciduria type II (an inborn error of metabolism) caused by a lack of mitochondrial fatty acyl coenzyme A dehydrogenases indicating that BEA or its metabolites have similar metabolic consequences. The BEA metabolite aziridine was detected by <sup>1</sup>H and <sup>2</sup>H NMR spectroscopy of the urine 8 hr p.d. together with BEA itself and two novel metabolites 2-oxazolidone (OX) and 5-hydroxy-2-oxazolidone (HOX). The formation of OX requires the reaction of BEA with endogenous bicarbonate followed by a cyclisation reaction eliminating HBr. Dosing rats with authentic OX resulted in the excretion of HOX but did not cause glutaric or adipic aciduria indicating that either aziridine or BEA itself was responsible for the presumed defect in mitochondrial metabolism.

**Key words:** adipic acid; 2-bromoethanamine; carbamate formation; glutaric acid; 5-hydroxy-2-oxazolidone; <sup>1</sup>H and <sup>2</sup>H NMR spectroscopy; 2-oxazolidone; urinalysis

BEA¶ (I) is a potent nephrotoxin which causes RPN following a single dose of the compound [1, 2]. Since RPN is an undesired side effect of long term analgesic use [2], BEA has been used as an experimental

model of analgesic nephropathy for which various toxic mechanisms have been proposed including direct cellular injury, ischaemia, inhibition of prostaglandin synthesis and free radical formation [2, 3]. Although there have been a number of histological and clinical chemical investigations of BEA-toxicity in experimental animals [3–5], the metabolic fate and mechanisms of toxicity of BEA are poorly understood. The characteristic pattern of toxin-related events following the administration of BEA include an increased urinary output, an impairment in maximum urinary concentration and electrolyte wastage [6, 7]. Many of the pathological features of BEA-induced nephrotoxicity are similar to those observed in the nephropathy associated with long-term analgesic use in man. These include initial degenerative changes in the medullary interstitial cellular matrix, a denudation of the epithelial cells at the tip of the papilla and an extensive lesion centred on the collecting ducts and extending into the thin limbs of Henlé, the capillaries and the interstitial cells [3, 4, 8].

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¶ Abbreviations: BEA, 2-bromoethanamine hydrobromide; RPN, renal papillary necrosis; NMR, nuclear magnetic resonance; PR, pattern recognition; GTA, glutaric acid; SPEC, solid-phase extraction chromatography; TSP, 3-trimethylsilyl-[2, 2, 3, 3-<sup>2</sup>H<sub>4</sub>] propionic acid; FAB-MS, fast atom bombardment mass spectrometry; MS-MS, coupled mass spectrometry; OX, 2-oxazolidone; FT, Fourier transformed; ADA, adipic acid; HOX, 5-hydroxy-2-oxazolidone; MCPA, methylenecyclopropylacetic acid; 4-PA, 4-pentenoic acid; Ac, acetate; Ala, alanine; Cit, citrate; Cn, creatinine; Cr, creatine; DMA, dimethylamine; DMG, dimethylglycine; EtOH, ethanol; Hip, hippurate; Lac, lactate; MA, methylamine; N-ac, N-acetyl resonances; 2-OG, 2-oxoglutarate; Suc, succinate; Tau, taurine; TMAO, trimethylamine-N-oxide; Gly, glycine; AZ, aziridine; All, allantoin; ISB, isobutyrate; ISV, isovalerate; 3-OHB, 3-D-hydroxybutyrate.

In simple aqueous solution BEA (I) readily eliminates HBr to give aziridine (II), a powerful alkylating agent which also induces RPN [9]. However, no single aetiology for BEA nephrotoxicity has been conclusively proven and the evidence suggests that more than one toxic mechanism is involved in both BEA- and analgesic-induced RPN [2, 5]. The biochemical and toxicological relationships between RPN in man and the nephropathy induced by BEA in experimental animals are as yet unclear.

We have shown that high-resolution  $^1\text{H}$  NMR spectroscopy of biological fluids can give useful information on the metabolism and biochemical effects of a variety of drugs and xenobiotics, and in some cases can also provide information on mechanisms of toxicity [10–16]. Previous  $^1\text{H}$  NMR studies on BEA toxicity in rats have shown that there was a reduction in the concentrations of urinary trimethylamine-*N*-oxide, dimethylamine, citrate and 2-oxoglutarate from 8 hr p.d. followed by elevations in urinary succinate and acetate levels from 24 to 48 hr p.d. [1, 11]. Substantial creatinuria was also found to be a feature of BEA toxicity although this has been previously associated with testicular and liver damage [11]. Histopathological data showing secondary renal cortical damage following the medullary lesion induced by BEA in rats may also occur [8]. Biochemical observations on BEA-dosed rats included elevations in urinary lactate and alanine concentrations 8–24 hr p.d. which were also attributed to cortical (proximal tubular) damage secondary to the medullary lesion [11].

The effects of several toxins (including BEA) on the excretion of endogenous components of rat urine have previously been assessed by use of computer-based PR analysis of  $^1\text{H}$  NMR-generated data [17–19]. Subsequent study of BEA toxicity in the rat, in which urine was obtained over a nine day time-course following the administration of BEA, resulted in the generation of PR maps from the resonance intensities of 20 selected endogenous metabolites observed in the  $^1\text{H}$  NMR spectra. The maps described a distinct biochemical trajectory which could then be related to the onset, progression and recovery phases of the BEA-induced nephrotoxic episodes [19]. The main biomarkers observed during the BEA-induced metabolic perturbations, were increased levels of urinary acetate, lactate, creatine, dimethylamine, hippurate and methylamine and decreased urinary concentrations of 2-oxoglutarate and succinate, indicating that renal medullary damage may be detected by characteristic combinations of urinary changes in endogenous metabolites [19].

Three main sets of previously unassigned  $^1\text{H}$  NMR signals in the 0–8 hr urine of BEA-treated rats have previously been attributed to unknown BEA metabolites [20]. The major group of resonances had been tentatively assigned as GTA (1,5-pentandioic acid), but whether this metabolite was endogenous or a metabolite of BEA was unknown. In the present study the assignment of the GTA signals has been confirmed using a combination of  $^1\text{H}$  NMR spectroscopy, SPEC and mass spectrometry. The metabolic provenance of GTA, i.e. whether endogenous or exogenous, was tested by the

administration of BEA- $d_4$  to rats and the quantitative time-course of GTA excretion in relation to BEA treatment investigated by  $^1\text{H}$  and  $^2\text{H}$  NMR methods. The metabolism of BEA and one of its putative metabolites was also investigated using  $^1\text{H}$  NMR spectroscopy in order to interrogate the relationships between metabolism and toxicity.

#### METHODS AND MATERIALS

*Metabolism and effects of BEA.* Male Fischer 344 rats ( $N = 6$ ) were housed individually in metabolism cages and allowed free access to food and water throughout the study. The animals were subjected to regular light cycles (8:00 am to 8:00 pm). Each animal received a single i.p. dose of BEA (150 mg/kg in 0.5 ml of 0.9% saline) or 0.5 ml of saline (0.9%). Urine was collected over ice at the following time-points: –24 to 0 hr (control), 0–2 hr, 2–4 hr, 4–8 hr and 8–12 hr p.d. On collection, urinary volumes were recorded and urine was centrifuged at 3000 rpm for 10 min in order to remove particulate contaminants. Seventy microlitres of deuterium oxide ( $\text{D}_2\text{O}$ ) containing 1% of TSP was added as a reference compound ( $\delta$  0.0) to a subsample of each urine (630  $\mu\text{L}$ ) and placed in a 5 mm NMR tube prior to NMR analysis. A histopathological examination was carried out on the kidneys of each animal upon termination. An unidentified metabolite which appeared in large quantities in the urine at 4–8 hr p.d. was concentrated and isolated by SPEC by passing 2 mL of acidified BEA-dosed urine through a C18 Bond Elut<sup>TM</sup> column and eluting the various urinary components with a stepwise series of acid washes and methanol gradients (from 0 to 100% in 20% steps) prior to characterization by NMR and mass spectrometry using a previously developed SPEC-NMR procedure [21]. FAB-MS and MS-MS were performed on a VG Quatro triple quadrupole mass spectrometer (Fisons). A further unassigned metabolite predominant in the BEA-treated urine 8–12 hr p.d. was also isolated by SPEC-NMR using the same methodology.

*Metabolism of BEA- $d_4$ .* BEA- $d_4$  was synthesized from 1,2-dibromoethane- $d_4$  (> 99%) following the methodology employed by Philipossian [22].  $^1\text{H}$  and  $^2\text{H}$  NMR spectroscopy and FAB mass spectrometry were used to confirm the synthesized structure. Male Fischer 344 rats ( $N = 6$ ) were housed individually in metabolism cages and permitted free access to food and water. Each animal received a single dose of BEA- $d_4$  (150 mg/kg i.p. in 0.5 mL of 0.9% saline) or an equivalent volume of saline. Urine was collected over ice at –24–0 hr pre-dose and 0–4 hr, 4–8 hr, 8–12 hr, 12–24 hr and 24–48 hr p.d. and was handled as in the study of BEA. Urine (0.5 mL) from each sample was analysed by  $^2\text{H}$  NMR at 92.1 MHz (14.1 Tesla).  $\text{D}_2\text{O}$  (0.1 mL) containing 0.1% TSP was then added to each sample as a lock prior to reanalysis of the urine by  $^1\text{H}$  NMR spectroscopy.

*Metabolism and effects of OX.* In order to assess whether the BEA metabolite OX was responsible for the induction of GTA, groups of SD rats ( $N = 3$ ) were dosed with either 0.9% saline (control) or OX at 50 mg/kg or 100 mg/kg in 0.9% saline. Conditions

were matched to those from the BEA studies and urine collections were made at -24-0 hr pre-dose and 0-8 hr, 8-24 hr and 24-48 hr p.d. On collection samples were centrifuged and analysed by  $^1\text{H}$  NMR spectroscopy.

*NMR studies on the reactions of BEA with bicarbonate.* A model solution of a 100 mM BEA (0.07 mL) in  $\text{D}_2\text{O}$  was added to a 5 mm o.d. NMR tube containing 0.63 mL of an aqueous solution of 100 mM sodium hydrogen carbonate at pH\* 7.6 (\* indicating the meter reading, no account being taken of the effect of the deuterons on the pH measurement leading to an underestimate of about 0.4 pH units). The spontaneous formation of OX was monitored over a period of 3 hr by 500 MHz  $^1\text{H}$  NMR spectroscopy.

*$^1\text{H}$  NMR Analysis of urine from human subjects with glutaric aciduria type II.* Two urine samples from patients with glutaric aciduria type II were obtained from the Middlesex Hospital and analysed by 600 MHz  $^1\text{H}$  NMR spectroscopy in order to assess the biochemical similarity with the urine collected from rats with BEA-induced toxicity.

*$^1\text{H}$  NMR spectroscopy of urine.* NMR measurements were made either on a Bruker AMX 600 spectrometer operating at 600.13 MHz or on a JEOL GSX 500 spectrometer operating at 500.14 MHz  $^1\text{H}$  resonance frequency. For each sample 64 free induction decays were collected into 32 K time domain points at ambient probe temperature (303 K) using a secondary presaturation irradiation field at the water resonance frequency. Typical 600 MHz NMR parameters were as follows; a spectral width of 18518 Hz, a pulse width of  $90^\circ$  (9  $\mu\text{sec}$ ), an acquisition time of 3.54 sec and an additional delay of 2.00 sec. 500 MHz acquisition parameters were: a spectral width of 6002 Hz, a pulse width of  $45^\circ$  (3.2  $\mu\text{sec}$ ), an acquisition time of 2.73 sec and an additional delay of 2.27 sec. Spectra were FT after applying an exponential apodization function corresponding to a line broadening of 0.3 Hz. Assignments were made on the basis of chemical shifts, coupling constants, literature assignments [10, 23, 24] and ultimately, in the case of ADA, GTA and OX, by addition of the authentic compounds. Metabolites were quantified by integration of  $^1\text{H}$  NMR signals with respect to the singlet signal of TSP standard and expressed as the percentage recovered of the original dose.

A 500 MHz 2-D  $^1\text{H}$ - $^1\text{H}$  COSY 45 spectrum [25] was measured for a urine sample collected 4-8 hr following the administration of BEA. Parameters used were: an acquisition time of 0.15 sec, a pulse angle of  $45^\circ$  (4.3  $\mu\text{sec}$ ) with a relaxation delay of 1.4 sec, an initial delay of 20  $\mu\text{sec}$ , a spectral width of 7000 MHz in  $F_2$ , and 64 scans with 400 increments in  $F_1$ . The spectrum was FT having an application of a sine bell function to the data and the resulting spectra were symmetrised about the diagonal. A 600 MHz double quantum filtered phase sensitive 2-D COSY experiment [26] was performed on a typical urine sample collected 4-8 hr after dosing with BEA. Spectral conditions used were; acquisition time of 0.35 sec with a relaxation delay of 2.1 sec; 96 scans; spectral width of 5813 Hz; initial time delay of 30  $\mu\text{sec}$  incremented in 256 steps for  $F_1$ . The data

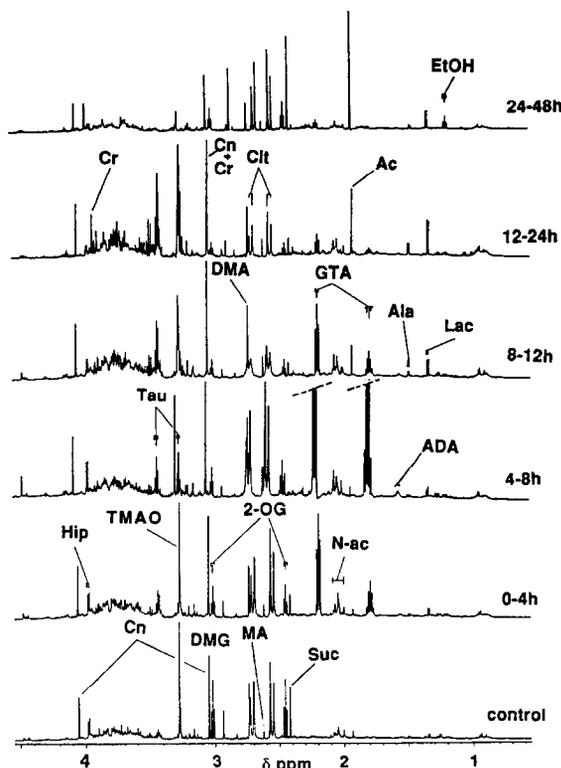


Fig. 1. 600 MHz  $^1\text{H}$  NMR spectra ( $\delta$  0.5- $\delta$  4.5) of urine obtained from a F344 rat and at various times post-administration of BEA- $\text{d}_4$  (150 mg/kg).

matrices were zero-filled in both dimensions (to 4 K points in the  $F_2$  dimension and 1 K points in the  $F_1$  dimension) and the sine bell squared function applied in both dimensions prior to two-dimensional FT.

*$^2\text{H}$  NMR spectroscopy of rat urine.* Samples were placed in a 5 mm o.d. tubes and spectra recorded on a Bruker AMX 600 spectrometer at ambient probe temperature (303 K).  $^2\text{H}$  spectra were measured at 92.13 MHz using an inverse detection 5 mm broadband probe in an unlocked mode. Typical parameters were: a spectral width of 1059 Hz, an acquisition time of 3.87 sec and a relaxation delay of 0.59 sec, a pulse width of 27  $\mu\text{sec}$  ( $90^\circ$ ), 64 scans were collected into 8 K data points and the spectrum referenced externally to BEA- $\text{d}_4$  relative to TSP. An apodization function corresponding to a line broadening factor of 0.3 Hz was applied prior to FT.

*Renal histopathology of rats treated with BEA and OX.* Animals were sacrificed by i.p. sodium pentobarbital overdose at 48 hr following the administration of BEA and OX. The kidneys were removed and the cortex and papilla separated by dissection. Papillae sections measuring approximately 1 mm  $\times$  3 mm were removed from each animal following termination and fixed in Karnovsky's fixative. Each tissue was immersed in a series of alcohols (25%, 50%, 70%, 95% and 99%) and embedded in Araldite<sup>TM</sup> resin. Semi-thin sections (0.5-1.0  $\mu\text{m}$ ) were cut, stained with toluidine blue or haematoxylin and eosin and viewed by light microscopy.

Table 1. List of major endogenous metabolite resonances observed in 500 and 600 MHz  $^1\text{H}$  NMR spectra of urine with associated NMR chemical shift and multiplicity data (pH 7)\*

Metabolite	$\delta/^1\text{H}$	Multiplicity of spin system	Assignment
Acetate	1.92	Singlet	$\text{CH}_3$
Adipic acid	1.60	Multiplet	$\text{CH}_2$
	2.22	Multiplet	$\text{CH}_2\text{COO}^-$
Alanine	1.48	Doublet	$\text{CH}_3$
	3.80	Quartet	CH
Citrate	2.54	AB system	$1/2 \text{CH}_2$
	2.66	AB system	$1/2 \text{CH}_2$
Creatine	3.04	Singlet	$\text{CH}_3$
	3.98	Singlet	$\text{CH}_2$
Creatinine	3.05	Singlet	$\text{CH}_3$
	4.06	Singlet	$\text{CH}_2$
Dimethylamine	2.72	Singlet	$(\text{CH}_3)_2$
Dimethylglycine	2.93	Singlet	$(\text{CH}_3)_2$
Glutaric acid	1.82	Quintet	$\text{CH}_2$
	2.23	Triplet	$2 \times \text{CH}_2\text{COO}^-$
Hippurate	3.97	Doublet*	$\text{CH}_2$
	7.55	Triplet*	<i>m</i> -CH
	7.64	Triplet*	<i>p</i> -CH
	7.84	Doublet*	<i>o</i> -CH
Lactate	1.37	Doublet	$\text{CH}_3$
	4.15	Quartet	$\text{CH}_2$
Methylamine	2.61	Singlet	$\text{CH}_3$
Succinate	2.41	Singlet	$(\text{CH}_2)_2$
Trimethylamine- <i>N</i> -oxide	3.27	Singlet	$(\text{CH}_3)_3$
Taurine	3.27	Triplet	$\text{CH}_2\text{SO}_3^-$
	3.43	Triplet	$\text{CH}_2\text{NH}_2^+$

\* Hippurate resonances comprise an AA'BB'C spin system which approximates to the multiplicities stated in the table.

## RESULTS

### $^1\text{H}$ NMR spectroscopy of rat urine following BEA and BEA- $d_4$ treatment

Typical 600 MHz  $^1\text{H}$  NMR spectra of urine obtained from one rat at various times following administration of BEA- $d_4$  are shown in Fig. 1. Strong resonances observed in  $^1\text{H}$  NMR spectra of control (pre-treatment) rat urine included creatinine, dimethylamine, dimethylglycine, hippurate, OX, succinate and trimethylamine-*N*-oxide, the NMR signals for these and other compounds having been assigned previously [10, 23, 24]. A list of the major endogenous metabolites with chemical shift and coupling constant data observed in  $^1\text{H}$  NMR spectra of control rat urine is given in Table 1. Polyuria was found to be characteristic for all the BEA-treated rats. The dominant BEA- $d_4$  induced alteration to the urinary  $^1\text{H}$  NMR spectra was the appearance of multiplets at  $\delta$  1.82 and 2.23 in urine samples obtained up to 12 hr p.d. It was possible to identify the multiplets as being from GTA using a combination of SPEC-NMR methodology [21] and FAB-MS of SPEC fractions. ADA was also identified as an abnormal metabolite in the urine of BEA-treated rats as described below.

The results of a typical SPEC-NMR procedure for the isolation and identification of GTA are shown in Fig. 2. GTA was poorly retained on the  $\text{C}_{18}$  column and eluted in the first and second acid washes. ADA was more strongly retained and was

eluted mainly in the 20% methanol fraction. The FAB MS-MS analysis of the second acid wash fraction showed that the main peak corresponded to an ion ( $M + \text{H}^+$ ) of 131 Da with a fragment of 87 Da ( $\text{CH}_2\text{CH}_2\text{CH}_2\text{-COOH}$ ), which, together with the  $^1\text{H}$  NMR spin-spin coupling data, chemical shifts and relative signal intensities was consistent with the structure of GTA. Assignment of both ADA and GTA was confirmed by standard addition of authentic standard compounds to the urine of BEA-treated rats. GTA and ADA are not usually detected in the  $^1\text{H}$  NMR spectra of control human or rat urine (indicating concentrations of  $< 0.1$  mM). However, in BEA dosed rats GTA reached maximum concentrations of 20–200 mM between 4–8 hr p.d. before subsiding to control levels (not detectable by NMR) by 48 hr p.d. Urinary GTA excretion was accompanied by elevated ADA excretion, although ADA was present in substantially lower concentrations ( $\sim 0.9$  mM). Since ADA signals were less well resolved in the  $^1\text{H}$  NMR spectra of whole urine than GTA, ADA concentrations were not quantified.

The spectra obtained from urine collected 0–4 hr p.d. showed a progressive elevation of taurine reaching a maximum concentration in the 12–24 hr p.d. samples falling back to control levels in the 24–48 hr p.d. samples (Fig. 1). A decrease in the level of OX was also observed from the first time-point (0–4 hr p.d.) with no subsequent recovery noted up to 48 hr p.d. By 8 hr p.d. a significant increase in

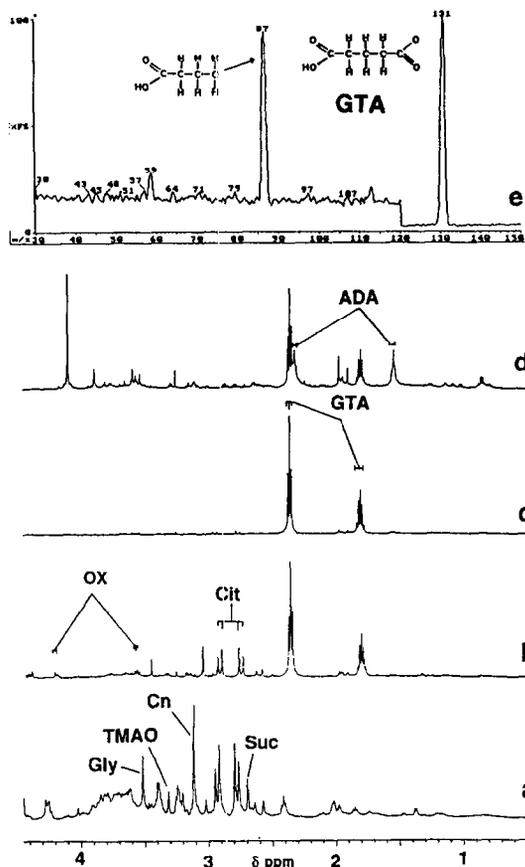


Fig. 2. 500 MHz  $^1\text{H}$  NMR spectra ( $\delta$  0.5– $\delta$  4.5) showing a series of acid and methanolic eluates from a SPEC-NMR procedure using 2 mL of urine from a BEA treated rat loaded onto a  $\text{C}_{18}$  Bond Elut<sup>TM</sup> column. (a) whole urine sample, (b) first acid wash, (c) second acid wash, (d) 20% methanol eluate, (e) FAB-MS spectrum of GTA isolated in the second acid wash (c).

urinary acetate, creatine, lactate and alanine concentrations were apparent, accompanied by mild glycosuria ( $\delta$  3.5–3.9). Citrate signals appeared broadened (probably due to the formation of calcium or magnesium complexes) and slightly diminished in intensity from 8 to 24 hr p.d. A sustained decrease in urinary hippurate concentration was noted from 8 hr p.d. Ethanol signals were observed in the  $^1\text{H}$  NMR spectra of BEA-treated rat urine (24–48 hr p.d.) and were confirmed by standard addition. A marked increase in dimethylglycine was observed between 24 and 48 hr p.d. accompanied by a slight initial increase and subsequent decrease in dimethylamine and methylamine levels (Fig. 1). Between 4 and 12 hr p.d. a decrease in urinary pH from  $7.8 \pm 0.3$  to  $6.5 \pm 0.4$  occurred (possibly attributable to the high concentrations of GTA) with consequent minor pH-induced changes in the chemical shifts of certain resonances, e.g. citrate, dimethylamine and trimethylamine-*N*-oxide. Concentration–time profiles of urinary GTA, trimethylamine-*N*-oxide and succinate excretion are shown in Fig. 3 indicating an initial decrease in both trimethylamine-*N*-oxide and succinate concen-

trations until 12 hr p.d. followed by an increase in succinate concentrations back towards control levels by 24 hr p.d. However, trimethylamine-*N*-oxide remained at low concentrations throughout the entire p.d. period. Conversely, urinary GTA concentrations were increased substantially as early as 4 hr p.d., reaching a maximum at 4–8 hr p.d. and then declining to control values by 48 hr p.d.

A two-dimensional  $^1\text{H}$ - $^1\text{H}$  COSY spectrum obtained 8 hr p.d. following the administration of BEA to a F344 rat shows the connectivities between spin-spin coupled protons on adjacent carbons, e.g. GTA, ADA, citrate, OX, 2-oxoglutarate and a previously unknown metabolite putatively assigned as 5-hydroxy-2-oxazolidone (HOX), see Fig. 4. From these spectra it was apparent that at least four major metabolites, not observed in control urine, were present in the urine collected from BEA-treated rats i.e. GTA, ADA, OX and HOX. The multiplets at  $\delta$  3.68 and 4.52 indicated the presence of OX (confirmed by standard addition), and HOX identified by three sets of resonances with the following chemical shifts ( $\delta$  4.27, 4.61 and 5.52) and spin-spin couplings, (9.89 Hz, 8.06 Hz and 3.3 Hz in an AA'X spin system). Coupling between the three sets of resonances was confirmed by a spin decoupling experiment. The nature of HOX was further investigated in the  $^2\text{H}$  NMR study of urine obtained from BEA- $\text{d}_4$ -treated rats. The proposed metabolic pathway of BEA is shown in Fig. 5. ADA proved to be more easily identifiable in the two-dimensional spectra as the coupling between the protons from the  $\text{CH}_2$  and the  $\text{CH}_2\text{COOH}$  groups could be observed whereas the  $\text{CH}_2\text{COOH}$  signal from ADA ( $\delta$  2.22) was overlapped in the one-dimensional spectra by the much stronger  $\text{CH}_2\text{COOH}$  resonance from GTA at  $\delta$  2.23 (see Table 2). ADA was, as described above, isolated in the 20% methanol eluate of urine 8 hr following BEA treatment and the addition of a standard solution of ADA to this fraction unambiguously confirmed the assignment.

#### $^2\text{H}$ NMR spectroscopy of urine following BEA- $\text{d}_4$ treatment

The  $^2\text{H}$  NMR spectra obtained over the entire time-course following the administration of BEA- $\text{d}_4$  is shown in Fig. 6. Between 0 and 4 hr p.d. both the parent compound, BEA- $\text{d}_4$ , and aziridine- $\text{d}_4$  (a spontaneous degradation product of BEA- $\text{d}_4$  in aqueous solution, data not shown) were observed accompanied by a pair of resonances corresponding to OX- $\text{d}_4$  (see Fig. 6). This metabolite was identified by reacting BEA- $\text{d}_4$  with excess bicarbonate solution and spiking some of the resulting solution containing OX- $\text{d}_4$  into a urine sample obtained 4–8 hr following the administration of BEA- $\text{d}_4$ . A further metabolite was found to be present in the urine spectra between 4 and 12 hr p.d., being most prominent between 8 and 12 hr p.d. This metabolite (HOX- $\text{d}_3$ ) is represented by the three singlets in the  $^2\text{H}$  NMR spectra (Fig. 6) which correspond to the three sets of resonances in the  $^1\text{H}$  spectrum (Fig. 4). No resonances from either GTA or ADA were observed in the  $^2\text{H}$  NMR spectra. A list of BEA metabolites and their relevant  $^1\text{H}$  and  $^2\text{H}$  chemical shifts is given in Table 2.

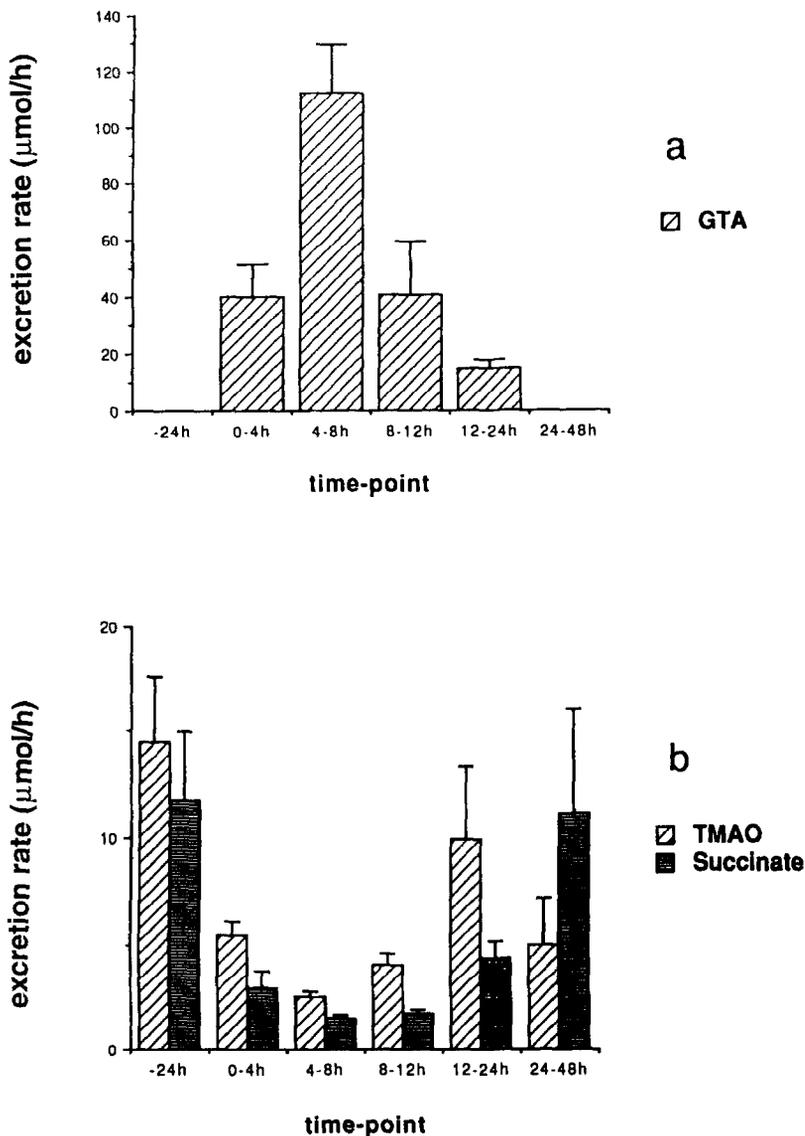


Fig. 3. Quantitative urinary excretion profiles of (a) GTA, and (b) TMAO and succinate in rat urine following the administration of BEA (150 mg/kg i.p., N = 60).

#### <sup>1</sup>H NMR spectroscopy of rat urine following OX treatment

In order to demonstrate that HOX developed from OX, and also to investigate the toxicity of this metabolite, OX was administered to SD rats at either 50 or 100 mg/kg. This resulted in the excretion of both OX ( $52 \pm 7\%$  of administered dose) and HOX ( $43 \pm 9\%$  of administered dose) 8 hr p.d. HOX was putatively assigned on the basis of the <sup>1</sup>H chemical shifts and J-coupling constants (Fig. 7). Further confirmation of structure is currently being undertaken. Although OX and HOX were found to be BEA metabolites, no sign of RPN was evident from the <sup>1</sup>H NMR urinary spectra of rats treated with OX at either dose level. GTA and ADA were also absent from the spectral profiles of OX-treated rats (see Fig. 7).

#### <sup>1</sup>H NMR spectroscopic study of the reaction of BEA with aqueous bicarbonate

Both aziridine and OX were formed spontaneously within 10 min following the addition of BEA (10 mM) to a solution of sodium hydrogen carbonate (100 mM) at pH\* 7.6, whereupon two multiplets of an AA'BB' spin system appeared in the <sup>1</sup>H NMR spectra at  $\delta$  3.69 and 4.52 (data not shown). This assignment was positively confirmed by the addition of authentic OX to the sample (Fig. 7).

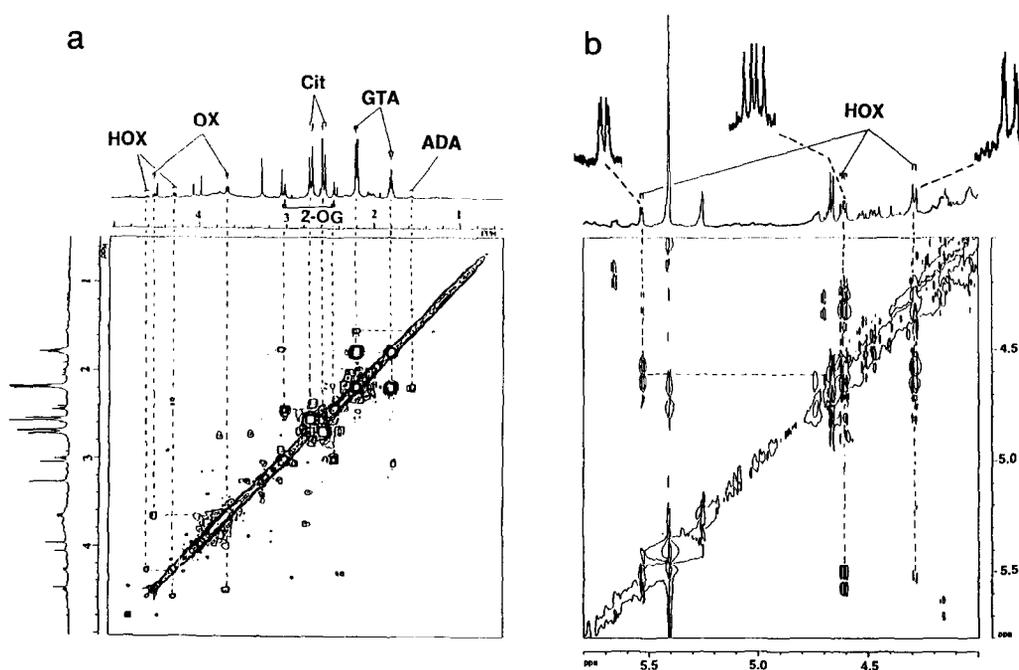
#### <sup>1</sup>H NMR spectroscopy of urine obtained from a human with glutaric aciduria type II

NMR studies were also performed on the urine obtained from two subjects with glutaric aciduria type II. These differed from control urine in that they contained substantial concentrations of GTA,

Table 2.  $^1\text{H}$  NMR chemical shift data (at pH 7.0) and NMR-derived urinary recovery data on BEA metabolites and metabonates in rats treated with 150 mg/kg BEA (N = 6)\*

Metabolite	$\delta$ $^1\text{H}$	Resonance multiplicity	% of BEA dose recovered in urine
2-Bromoethanamine (I)	3.49	Triplet	28 $\pm$ 9
2-Oxazolidone (IV)	3.69	Triplet	9 $\pm$ 10 <sup>†</sup>
Aziridine (II)	2.57	Singlet	31 $\pm$ 16
5-Hydroxy-2-oxazolidone (V)	5.52	Doublet of doublets*	7 $\pm$ 5
	4.61	Doublet of doublets*	
	4.27	Doublet of doublets*	

\* ABC spin system.

<sup>†</sup> Highly variable between animals.Fig. 4. (a) 500 MHz 2-D  $^1\text{H}$ - $^1\text{H}$  COSY 45 spectrum of rat urine ( $\delta$  1.0– $\delta$  5.0) 4–8 hr post-administration of BEA (150 mg/kg i.p.); (b) 600 MHz 2-D  $^1\text{H}$ - $^1\text{H}$  COSY spectrum, expanded region ( $\delta$  4.0– $\delta$  5.8) showing connectivities between the 5-hydroxy-2-oxazolidone protons.

ADA, lactate, isobutyrate and isovalerate (Fig. 8). Urinary concentrations of citrate and succinate were also depleted in comparison to the  $^1\text{H}$  profile of control urine. Abnormally high urinary concentrations of GTA and ADA and depleted levels of citrate and succinate were features of both BEA-treated rats and humans with glutaric aciduria type II. However, the urinary metabolite profiles were dissimilar in other respects, i.e. increased urinary levels of isobutyrate and isovalerate were characteristic of glutaric aciduria but not BEA nephropathy.

#### Renal histopathology of rats treated with BEA and OX

Histopathological examination of renal papillae from rats treated with BEA (150 mg/kg) showed a

severe degree of necrosis in all cellular elements at the tip of the papilla. In particular vacuolation of the interstitial and collecting duct cells was noted along with mitochondrial swelling and disruption of the basement membrane. Cellular debris was also observed in the lumens of the collecting ducts. No difference between papillae obtained from control rats and those treated with OX at either 50 or 100 mg/kg was noted which suggested that OX was not responsible for causing the RPN associated with BEA.

#### DISCUSSION

The treatment of rats with BEA resulted in a wide range of biochemical effects as indicated by changes

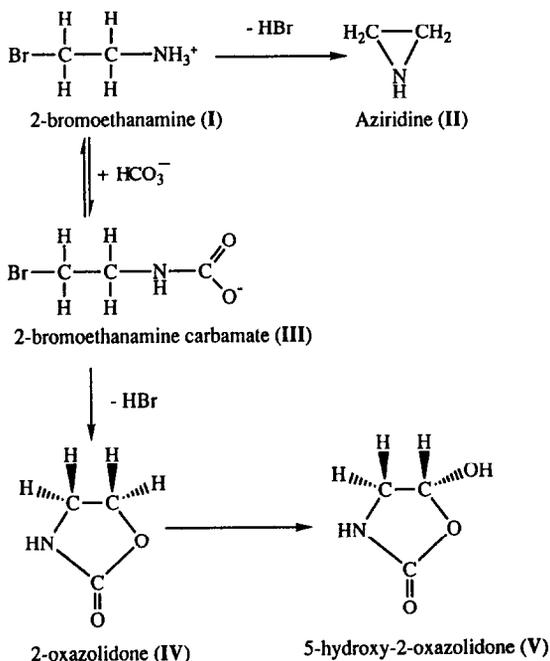


Fig. 5. Proposed metabolic pathways for BEA in the rat.

in the urinary low molecular weight metabolite profiles of urine. The major biochemical effect of BEA, now confirmed, was the induction of glutaric aciduria within the first 12 hr of dosing. GTA is an endogenous product of lysine, hydroxylysine and tryptophan metabolism and is present in normal human urine at trace concentrations ( $< 0.01 \mu\text{mol}$  per mg of creatinine) [27]. Although GTA resonances are not normally observed in the  $^1\text{H}$  NMR spectra of control urine, the high urinary GTA concentrations observed in this experiment 4–8 hr following the administration of BEA were derived from endogenous sources since  $^2\text{H}$  NMR signals for GTA were not present in the  $^2\text{H}$  NMR spectra of urine from rats treated with BEA- $d_4$ .

Substantial quantities of GTA have previously been noted in the urine of humans presenting with either a metabolic disorder known as glutaric aciduria type II [27] or Jamaican vomiting sickness [28]. Glutaric aciduria type II is essentially caused by an enzyme deficiency in the fatty acyl CoA dehydrogenases resulting in an accumulation and increased urinary excretion of GTA, ADA, isovaleryl glycine, 2-hydroxyglutaric acid, ethylmalonic acid and methyl succinic acid [27]. The  $^1\text{H}$  NMR spectrum obtained from urine of a human subject with glutaric aciduria type II is shown in Fig. 8. Although direct comparisons between rat and human urine are subject to limitations, the two spectra show similar proportions of GTA and ADA, suggesting that the BEA-induced lesions may have some biochemical connection with the defect in the human inborn error of metabolism. Thus, part of the mechanism of BEA-induced toxicity may result from an inhibition of fatty acid metabolism by disruption of

acyl CoA dehydrogenase enzyme systems [27]. Since the time course of BEA excretion is similar to that of GTA excretion, this suggests that BEA or its metabolites may have a direct but transient metabolic effect on the activity of acyl CoA dehydrogenases resulting in the excessive production of GTA and ADA similar to the human metabolic disorder. Low concentrations of citrate and succinate were features of urine collected from both BEA-treated rats and humans with glutaric aciduria type II, which would imply that mitochondrial perturbation was characteristic of both conditions. However, other metabolites observed in the spectrum of urine from the glutaric aciduria type II patient, i.e. isobutyrate and isovalerate, were absent from the rat urine collected post BEA administration, indicating a limited comparison with the human disease process.

Jamaican vomiting sickness, a toxic condition induced by the ingestion of hypoglycin A (a component of the unripe ackee fruit), is commonly identified by increased urinary levels of glutaric, adipic, sebacic, suberic, ethyl malonic and methyl succinic acids, *n*-butyryl glycine and *n*-hexanoyl glycine [28]. Hypoglycin A is metabolised to MCPA which has been shown to inhibit the transport of long-chain fatty acids into mitochondria, thus suppressing their oxidation, and was also observed to inhibit several acyl CoA dehydrogenases [28]. 4-PA, a synthetic analogue of MCPA, initiated the inhibition of the trans-mitochondrial transport of

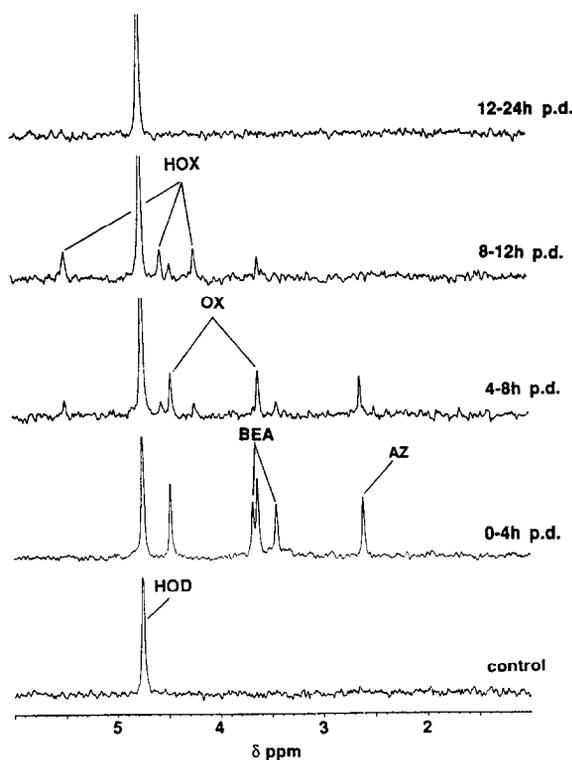


Fig. 6. 92.13 MHz  $^2\text{H}$  NMR spectra ( $\delta$  0.5– $\delta$  4.5) of urine at various time-points before (–24–0 hr) and post-administration of BEA- $d_4$  (150 mg/kg).

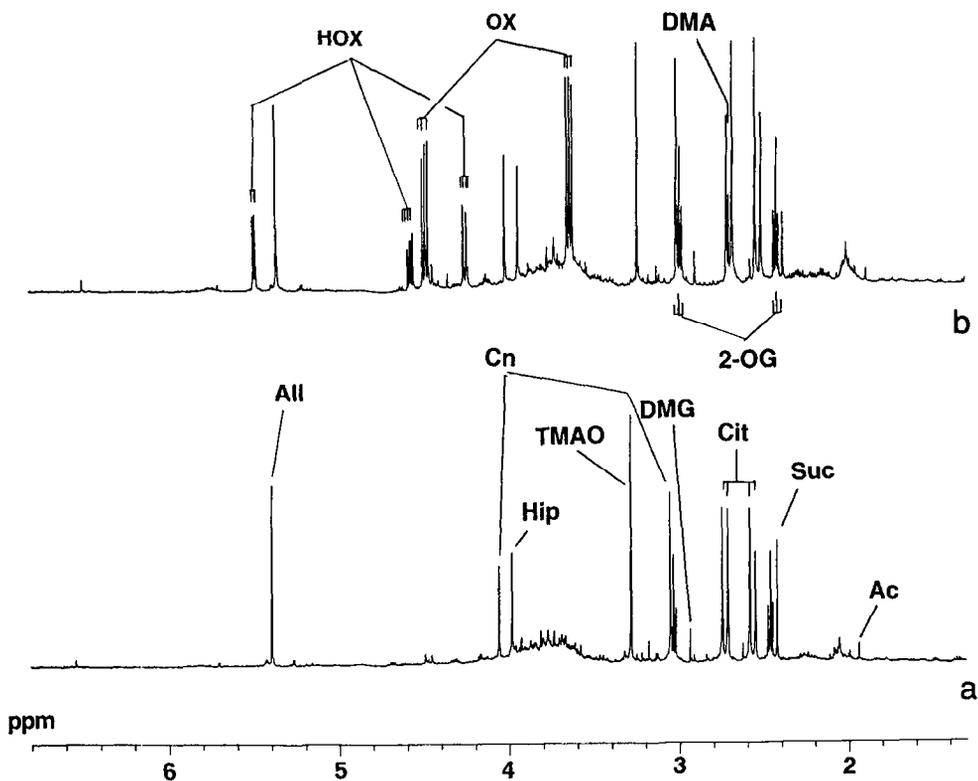


Fig. 7. 500 MHz  $^1\text{H}$  NMR urine spectra ( $\delta$  1.3–6.8) obtained from a F344 rat (a) –24 to 0 hr pre-dose, and (b) 0–8 hr post-administration of 2-oxazolidone (100 mg/kg).

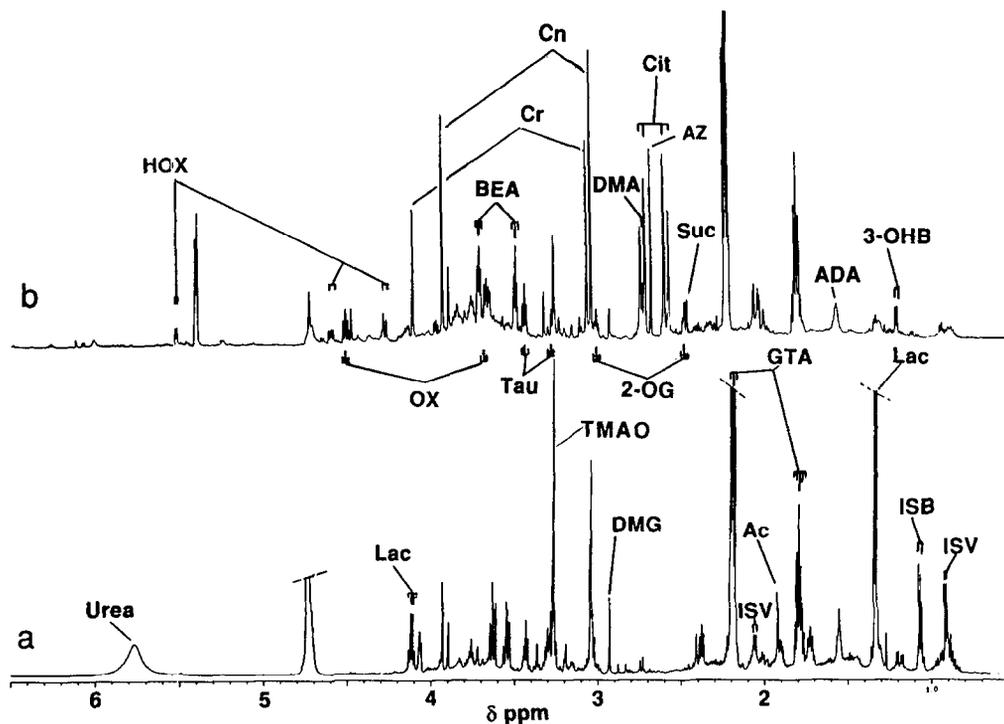


Fig. 8. 600 MHz  $^1\text{H}$  NMR spectrum of urine from a patient presenting with glutaric aciduria type II compared with the 4–8 hr p.d. spectrum of BEA-treated rat urine.

long-chain fatty acids causing elevation of adipic acid and long chain fatty acids in the urine [29]. However, unlike MCPA, acyl CoA dehydrogenase activity was not suppressed with 4-PA, therefore lysine and tryptophan metabolism was not inhibited. It was therefore suggested that MCPA exerted a dual metabolic effect, both on transmembrane transport of long-chain fatty acids and on the acyl CoA dehydrogenases [29]. A further inhibitory effect of 4-PA was expressed upon gluconeogenesis from pyruvate but not lactate, alanine or glycerol [29]. GTA is a known inhibitor of succinate dehydrogenase amongst other enzymes [30], therefore inhibition of this enzyme would result in a blockage of the tricarboxylic acid cycle at the point of conversion of succinate to malate. This phenomenon may account for the observed increase in succinate 24 hr following the administration of BEA as previously reported [1, 19]. The evidence drawn from these studies would suggest that BEA, like MCPA, exerts multiple toxic effects. The perturbation in tricarboxylic acid cycle intermediates indicates a disruption in mitochondrial function whilst the presence of high urinary concentrations of GTA and ADA would suggest suppression of fatty acyl CoA dehydrogenase activity.

BEA induced perturbations in the urinary levels of several renal osmolytes including trimethylamine-*N*-oxide, dimethylamine, dimethylglycine and methylamine. Since the cells of the renal inner medulla accumulate osmolytes in response to an increase in extracellular osmolality [31], a perturbation in their level may be important in the papillotoxic mechanism of BEA. Renal osmolytes play an important role in maintaining cell volume homeostasis, which helps to protect tissues against osmotic stress. The renal medulla is particularly susceptible to osmotic stress due to the presence of the countercurrent multiplier mechanism in the loops of Henlé. Markers of renal cortical toxicity (lactate, alanine and glucose) were also raised but at later time-points than the markers of papillary toxicity supporting the previously reported observations that cortical injury is secondary to BEA-induced papillary damage [1, 11]. Urinary acetate concentrations were grossly elevated in the urine of BEA-treated rat urine obtained 24–48 hr p.d., accompanied by increased amounts of lactate and ethanol. These metabolites have been noted in urine spectra obtained from fasting human subjects, lactate and ethanol being products of anaerobic glycolysis. Although ethanol is not usually observed in the  $^1\text{H}$  NMR urine profiles of control rats, it has been noted in the urine of rats treated with mercury (II) chloride and was attributed to an inhibition of liver alcohol dehydrogenase activity [32]. Pyruvate formed in glycolysis is decarboxylated to acetyl CoA in the mitochondrial matrix where it enters the tricarboxylic acid cycle. The initial depletion of tricarboxylic acid cycle intermediates, citrate, 2-oxoglutarate and succinate, following BEA-treatment may indicate an inhibition of pyruvate transport is occurring.

High resolution  $^1\text{H}$  and  $^2\text{H}$  NMR spectroscopy of urine has provided direct information on the toxicological effects of BEA. However, this also gave further insight into the previously undescribed

metabolism of BEA. OX (IV) appeared to be a facile reaction product of BEA with bicarbonate *in vivo*, and was shown to form spontaneously *in vitro* via the intermediate BEA carbamate (III) upon placing BEA in a solution of bicarbonate. Although several carbamates have been shown to affect the parasympathetic nervous system and to induce neurotoxic episodes [33], there has been no evidence of carbamate-induced nephrotoxicity. The production of the postulated novel BEA metabolite HOX (V) also occurred in rats dosed with OX. After dosing with OX, the absence of polyuria, GTA, ADA or any of the other metabolic changes associated with BEA treatment indicated that the mitochondrial and papillotoxic effects of BEA were due to either BEA itself or aziridine. Histopathological data showed no evidence of RPN in rats treated with OX supporting the hypothesis that OX and HOX were not nephrotoxic.

Autopsies on two patients with glutaric aciduria type II indicated cerebral oedema, patchy destruction of the cerebral white matter and reactive gliosis. Diffuse fatty liver was also a characteristic of these two patients [27]. Likewise, the primary histopathological effect following hypoglycine toxicity was noted to be fatty infiltrations in the liver with small lipid droplets being present in both the liver and the kidneys [34]. Depletion of liver glycogen was also found to be a feature of Jamaican vomiting sickness [34]. Since glutaric aciduria type II is associated with pathological lesions in the liver, it would be appropriate to obtain histopathological data on the livers of BEA-treated rats. Only renal histology was extensively performed in these studies but the effect of BEA on the liver and brain of rats is currently under investigation. Urinary concentrations of taurine were found to be elevated substantially in BEA-treated rats. Taurine has previously been observed in the urine profiles of rats treated with liver toxins and was attributed to being a marker of hepatotoxicity [35]. Elevation of urinary creatine levels have also been found to be associated with liver toxicity, particularly when taurine was also raised [36]. Therefore it is possible that BEA also exerts some toxic effects on the liver. Preliminary histopathological studies into the effect of BEA on the liver support this hypothesis.

These studies show that BEA produces a complex series of metabolic effects, many of which may be unrelated to the critical target lesion in the renal medulla. BEA clearly induces glutaric aciduria, but whether this compound also induces RPN characteristic of analgesics awaits further investigation. The discovery of BEA-induced glutaric aciduria is significant in that it implies that BEA causes a serious disruption of mitochondrial metabolism which may be partly implicated in the mechanism of toxic damage. However, the direct connection between the glutaric aciduria and renal medullary damage cannot yet be made and is currently under investigation.

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