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Trichloroethylene-induced formic aciduria: effect of dose, sex and strain of rat.

Noreen Yaqoob, Andrew R Evans and Edward A Lock

School of Pharmacy and Biomolecular Sciences
Liverpool John Moores University
Byrom Street,
Liverpool, L3 3AF, UK

Running title: Trichloroethylene-induced formic aciduria

Footnote: Initial studies on TCE-induced formic aciduria were presented as a poster at a British Toxicology Society meeting (Yaqoob *et al.*, 2009).

Correspondence to : Edward A Lock at address above

Telephone UK 0151 231 2187

Fax UK 0151 231 2170

e-mail e.lock@ljmu.ac.uk

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Abstract

The industrial solvent trichloroethylene (TCE) has been reported to increase the excretion of formic acid in the urine of male Fischer 344 (F-344) rats following large oral doses. We have examined the dose-response relationship for formic aciduria in male and female Fischer 344 rats, the effect of some known metabolites of TCE and examined the response in male Wistar rats to help understand its relevance to renal toxicity. We report that doses of TCE as low as 8mg/kg for 3 days to both male and female F344 rats produced formic aciduria. The formic aciduria was time-dependent being more marked after 3 doses compared to one dose in male F344 rats and to a lesser extent in female F344 rats. TCE administration to male Wistar rats produced less formic aciduria than in male F344 rats, indicating a strain difference in response. As TCE is primarily metabolized by cytochrome P450 2E1, Wistar rats were administered inducers of cytochrome P450 2E1 followed by TCE, this increased formic acid excretion to a concentration similar to that observed in male F344 rats, indicating a role for P450. Administration of the major metabolites of TCE, trichloroethanol and trichloroacetic acid to male F344 rats also produced a marked and sustained formic aciduria, while the metabolite of TCE formed via glutathione conjugation had no effect on formic acid excretion. The mechanism whereby this response occurs is currently not understood, but the formic acid excreted is not a metabolite of TCE, but appears to be due to interference with the metabolic utilization of formate by a down stream metabolite of TCE. Over the three days of the studies no histopathological evidence of kidney toxicity was observed in F344 rats given TCE, indicating that the perturbation of formate metabolism does not lead to acute renal injury.

Introduction

Trichloroethylene (TCE) has been used commercially for over 85 years, primarily as a non-flammable solvent for degreasing metal parts but also as a general purpose solvent (Bakke *et al.*, 2007). From the 1970's onward TCE became of environmental concern, due to evaporation while handling, chemical processing and leakage from chemical waste sites, leading to its contamination of ground water and air (Wu and Schaum, 2000; Ellis and Rivett, 2007; Fan *et al.*, 2009). As a consequence, TCE has been detected in the blood of a large number of non-occupationally exposed adults (Churchill *et al.*, 2001; Blount *et al.*, 2006). Concern was raised about possible long term health effects of TCE and a number of epidemiology studies has suggested links between TCE exposure and an increased incidence of tumours at a number of sites (Moore *et al.*, 2010; Scott and Chiu, 2006; Scott and Jinot, 2011; Wartenberg *et al.*, 2000). In general, these studies have not shown a clear causal relationship between TCE exposure and an increased incidence of tumours in humans, although genetic variability in genes involved in reductive metabolism of TCE appears to increase the risk of renal cancer (Moore *et al.*, 2010). Extensive studies with TCE in experimental animals, has shown that the primary target organs for toxicity are the liver and lungs in mice and liver and kidneys in rats. Long term studies have shown an increase in tumour incidence in these tissues, see reviews by Bull, (2000); Lock and Reed, (2006) and Jollow *et al.*, (2009).

TCE is primarily metabolised in the liver via cytochrome P450 2E1 (Kim and Ghanayem, (2006), and to a lesser extent by other cytochromes P450 (Nakajima *et al.*, 1990), to form an unstable epoxide which re-arranges to form chloral. Chloral is then converted to trichloroethanol and its glucuronide and trichloroacetic acid (TCA) which are the major metabolites in the urine of experimental animals and man (Bloemen *et al.*, 2001; Lash *et al.*, 2000; Lock and Reed, 2006). A minor pathway of TCE metabolism which seems to operate when the oxidative pathway become saturated, is via glutathione conjugation producing the cysteine conjugate S-(1,2-dichlorovinyl)-L-cysteine (DCVC) which is converted to a reactive metabolite by the enzyme cysteine conjugate β -lyase in the kidney (Green *et al.*, 1997; Anders, 2005; Lock and Reed, 2006). Liver tumours have been shown to be related to peroxisome proliferation following TCE and TCA administration in rats and mice (Bull, 2000), while metabolism via DCVC may explain the low incidence of renal tumours in male rats only. Whether enough DCVC is formed from TCE in male rats to cause renal injury has been questioned as the amount formed is very

small <0.01% of the administered dose (Birner *et al.*, 1993; Bloemen *et al.*, 2001; Green *et al.*, 1997). More recently it has been proposed that a marked and sustained formic aciduria produced by TCE could lead to renal tubule necrosis and regeneration, which may account for the small increase in renal cancer (Green *et al.*, 1998). Acute exposure to formic acid has been reported to cause renal injury in humans and rats (Jacques, 1982; Liesivuori and Savolainen, 1987).

Our hypothesis was to examine whether there is indeed a link between formic aciduria and renal injury. The work presented in this paper explores the dose-response relationship for TCE-induced formic aciduria in rats, following acute administration, and the presence of renal injury. It also examines whether there is a sex difference in TCE-induced formic aciduria and response to TCE metabolites. During the course of these studies we moved from F344 rats to Wistar rats, our in-house strain, and discovered they were less responsive with regard to formic acid excretion following TCE administration. We have explored this response in limited detail.

Materials and Methods

Materials: Deuterium oxide (D₂O, 99.9%) from Cambridge Isotope Laboratories, Inc. Andover, Massachusetts, USA. Trichloroethylene (TCE), reagent grade, 98%, inhibited with about 1% 1-2-epoxybutane from Sigma Aldrich, Poole, UK. Pure corn oil, low in saturates and high in polyunsaturates from Tesco Supermarket, Liverpool, UK. Protein assay kit, Bio-Rad Detergent Compatible from Bio-Rad Laboratories, UK. Li-Heparin, 10ml tubes from Sarstedt, Leicester, UK. Quantichrome assay kits for plasma urea and creatinine from Bioassay Systems, Hayward, USA. All other chemicals were purchased from Sigma Aldrich, Poole, UK.

Animals: All animal procedures were performed in accordance with a license issued under the UK, Scientific Procedures Act, 1986. Fischer-344 (F344) rats and Wistar-derived rats were bred in the Life Science Support Unit at Liverpool John Moores University. The rats were housed in plastic cages in a room maintained at 20°C ± 2°C and a relative humidity of 50% ± 5% with a 12h light-dark cycle starting at 04.00h. Rats were allowed food (rat expanded diet), and water *ad libitum*. F344 male rats (190-240g) and female F344 rats (135-165g) between 7-9 weeks of age and Wistar-derived male rats (205-253g) were used for these studies, with a minimum of 3 rats /group with some studies being done in duplicate or triplicate.

Preparation of dosing solutions: Stock solutions of TCE at 200mg/ml or 3.125mg/ml were freshly prepared in corn oil and serially diluted in corn oil to cover doses of 1000 to 32mg/kg

and 16 to 2mg/kg respectively. Trichloroethanol (TCE-OH) 3.125mg/ml; perchloroethylene (PCE) 3.125mg/ml; carbon tetrachloride (CCl₄) 3.125mg/ml and 16mg/ml or chloroform (CHCl₃) 20mg/ml were prepared in corn oil. Trichloroacetic acid (TCA) was prepared at 3.125mg/ml in 2mM phosphate buffer neutralised to pH 7.4 and then diluted in buffer to give 1.56 and 0.78 mg/ml. S-(1,2-dichlorovinyl)-L-cysteine (DCVC) was prepared at 0.4mg/ml in 2mM phosphate buffer, pH 7.4. All solutions were stored in tightly sealed glass containers, wrapped in aluminium foil, at 4°C. For the studies with P 450 inducers, stock solutions of pyridine at 100mg/ml; isoniazid at 100mg/ml and phenobarbitone at 80 mg/ml were prepared in isotonic saline prior to use.

Treatments: Rats were placed in metabolic cages for 24h to acclimatise prior to dosing and urine collected (Day 0). Urine was collected at 4°C in containers to which was added 0.1ml of 10% sodium azide to prevent bacterial growth. The major metabolites of TCE, TCA, TCE-OH and its glucuronide and formic acid are stable under these conditions (Green *et al.*, 1998). Rats were dosed daily by oral gavage with TCE in corn oil at 5ml/kg/day for 3 days over the dosing range of 2-1000mg/kg/day, while controls received corn oil alone at 5ml/kg/day for 3 days (Day 1, 2 & 3).

For one study rats were given one dose of TCE at 1000mg/kg (Day 1) followed by corn oil for 2 days (Day 2 & 3). The rats were returned to the metabolic cages after each dose and urine collected over 24h for 3 days (Day 1, 2 & 3) as described above.

For the studies with TCE metabolites and other halogenated chemicals the rats were dosed by oral gavage for 3 days (Day 1, 2 & 3) as follows; TCE-OH, 16mg/kg/day; PCE, 16mg/kg/day; CCl₄, 16 and 80mg/kg/day; CHCl₃, 100mg/kg/day; TCA, 4, 8 and 16mg/kg/day and DCVC, 2mg/kg/day. Control rats received either corn oil alone or 2mM phosphate buffer, pH 7.4 at 5ml/kg/day.

For the studies with cytochrome P450 inducers, male Wistar-derived rats were pretreated with either pyridine at 100mg/kg/day ip for 3 days (Kim *et al.*, 1992) or isoniazid at 100mg/kg/day po for 4 days (Lake *et al.*, 1998) which induces P450 2E1 or phenobarbitone at 80 mg/kg/day, ip for 3 days (Taylor *et al.*, 1985) which induces P450 2B1/2. The rats were placed in metabolic cages for 24h after the last dose of inducer (day 0) for urine collection and then dosed with TCE at 1000mg/kg/day for 3 days or corn oil alone for 3 days at 5ml/kg/day. The rats were returned to the metabolic cages after each dose and urine collected over 24h (Day 1, 2 & 3).

Urine analysis & termination: Urine was collected daily for 4 days, kept ice-cold and urine volume and pH measured. A sample of urine was taken for $^1\text{H-NMR}$ analysis and the remainder was frozen at -80°C . After the final urine collection the rats were killed by a rising concentration of carbon dioxide and blood collected from the heart into heparinised tubes. The kidneys and liver were removed, weighed and a cross section of the kidney, showing the cortex, medulla and papilla was fixed in buffered formal saline for histological examination. The remaining kidney tissue and liver was stored at -80°C for subsequent analysis. The blood was centrifuged at 330g for 10 min at 4°C for separation of plasma. The plasma was carefully removed and stored frozen at -80°C for subsequent analysis for creatinine and urea using commercially available assay kits.

Preparation of microsomes and cytochrome P450 assays: Livers from male F344 or male Wistar rats dosed with corn oil or the various inducing agents, in the presence or absence of TCE were perfused *in situ* with ice-cold 0.9% saline, rapidly excised, blotted dry, weighed and then homogenized (1:4 w/v) in ice-cold buffer (0.154 M KCl, 50mM Tris-HCl, pH 7.4). The microsomal fraction was then prepared as described by Taylor *et al.*, (1985) and re-suspended in ice-cold storage buffer (0.154M KCl, 10mM HEPES, 1mM EDTA, 20 % (w/v) glycerol, pH 7.6) and stored in 500ul aliquots at -80°C . 4-Nitrophenol hydroxylase activity indicative of P450 2E1 was measured in liver microsomes by monitoring the increase in the formation of 4-nitrocatechol from 4-nitrophenol as described by Reinke and Moyer, (1985). Results are expressed as nmol 4-nitrocatechol formed $\text{min}^{-1} \text{mg protein}^{-1}$. Protein was measured using a Bio-Rad protein assay kit using bovine serum albumin as a standard.

Analysis of urine and plasma using $^1\text{H-NMR}$ spectroscopy: Aliquots of urine or plasma (500 μl) were mixed with phosphate buffer (250 μl ; 0.2M pH 7.4 in D_2O). Urine samples contained sodium-3(trimethylsilyl) propionate-2,2,3,3- d_4 (TSP; 0.5mg TSP/ml buffer) and plasma samples maleic acid disodium hydrate (0.5mg/ml buffer) as an internal standard. Samples were centrifuged at 14,000g for 10 min at 4°C and supernatant (600 μl) placed into 5mm NMR tubes and $^1\text{H-NMR}$ spectra acquired using a Bruker 300MHz instrument (Bruker Analytik GmbH, Germany). The standard 'noesypr1d' pulse sequence was utilised for data acquisition on urine, which efficiently suppresses the large water signal while the standard pulse sequence 'cpmgpr1d' was utilised for data acquisition on plasma samples. All spectra were baseline corrected using Mestrec software and normalised to TSP or maleic acid. Urinary metabolites were quantified using the intensity of the resonance with reference to the TSP resonance that was set at 0ppm, while plasma metabolites were quantified in the same way with reference to

the maleic acid signal at 6ppm. The accuracy of the determination of formic acid was measured by spiked addition and found to be 98%. The detection of formic acid using the 3σ method (Miller and Miller, 1993) gave an LOD of 0.004mg/ml and using the 10σ method an LOQ of 0.04mg/ml.

Histopathology: This was only performed on F344 male rats exposed to 1 or 3 daily doses of TCE at 1000mg/kg and 3 daily doses of TCE at 16mg/kg. The portion of kidney fixed in buffered formal saline was then dehydrated through a series of alcohols (0-100%v/v) and then placed in xylene. The tissue was then embedded in paraffin wax and tissue sections (5 μ m) cut, processed and stained with haematoxylin and eosin. Microscopic examination of the kidney was then performed blinded to the treatment by experienced pathologists

Statistical Analysis: The data are expressed as mean \pm standard error of the mean (SEM). Significant differences were determined between control and treated groups at each dose or time using one-way ANOVA with Bonferroni correction. For the induction studies differences between control and treated were determined using a two tailed *t*-test. Statistical analysis was conducted using InStat Graphpad with a p-value < 0.05 considered statistically significant.

Results

Urinary excretion of formic acid by male F344 and Wistar rats following 1 or 3 daily oral doses of trichloroethylene at 1000mg/kg/day.

We have examined the concentration of formic acid in urine 1 day before dosing (Day 0) and following exposure to 1 or 3 doses of TCE at 1000mg/kg over 3 days (Day 1, 2 & 3).

Administration of TCE to male rats, of either strain, as a single dose (Day1) followed by corn oil alone (Day 2, 3) or 3 daily doses had no effect on body weight, liver or kidney weights or urine volume (data not shown). $^1\text{H-NMR}$ analysis of urine showed that the most marked difference in the spectra between control (corn oil treated) and TCE-treated rats was the appearance of a resonance at 8.44ppm which was attributed to formic acid (compare Figure 1A and Figure 1D). No statistical difference was seen between untreated rats and corn oil treated rats with regard to formic acid excretion.

Following a single dose of TCE, the excretion of formic acid by F344 rats (Figure 2A) when compared to the Wistar rats (Figure 2B), at each time point was not statistical different. With F344 and Wistar rats given a single dose of TCE formic acid excretion peaked at 2 days and

then gradually declined (Figure 2A& 2B). However, after daily dosing of TCE to F344 rats the formic acid concentration increased after each dose and was statistically significant increased ($p < 0.017$ ANOVA) (Figure 2A), the rats excreting about 60mg over the 3 days. After 3 doses of TCE to Wistar rats the formic acid concentration did not increase with time remaining fairly constant over the 3 day period (Figure 2B). The major strain difference was related to the total amount of formic acid excreted following 3 daily doses of TCE, which in the Wistar rats totalled about 10mg, i.e. about 16% of the response seen in F344 rats. In agreement with Green *et al.*, (1998), a statistically significant acidification of urine was seen each day after dosing F344 rats with daily doses of TCE at 1000mg/kg/day (Figure 2C). After a single dose of TCE at 1000mg/kg to F344 rats followed by 2 daily doses of corn oil, urinary pH was not significantly reduced after dosing TCE (Figure 2C). Acidification of the urine was only seen in Wistar rats after 3 doses of TCE (Figure 2D).

The only other change of note in the $^1\text{H-NMR}$ spectra was the appearance of a TCE metabolite, TCE-O-glucuronide in the range 3.5-4.6ppm after 1000mg/kg/day which could still be detected after 62.5mg/kg/day (Figure 1C&D) but not after 16mg/kg/day (Figure 1B). Small changes in individual animals were seen for α -ketoglutarate, citrate and succinate however the mean of all animals showed these findings were not statistically significant from corn oil alone treated rats. Histopathological examination of the kidneys from F344 rats given TCE at 16mg/kg or 1000mg/kg daily for 3 days showed no compound related morphological changes.

The effect of induction of cytochrome P450 on trichloroethylene-induced formic aciduria in male Wistar rats

One possible explanation for the marked strain difference in formic aciduria produced after TCE administration may be related a strain difference in the activity of hepatic cytochrome(s) P450, particularly P450 2E1 (Kim and Ghanayem, 2006), which is the first step in the metabolism of TCE. We therefore treated Wistar rats with agents known to induce P450 2E1 (pyridine and isoniazid) and P450 2B1/B2 (phenobarbitone) which is also known to metabolise TCE (Nakajima *et al.*, 1990) to see if the formic aciduria could be increased to a similar level found in F344 rats. Treatment with these various inducers alone, or following TCE, did not increase liver to body weight ratio (Table 1). The un-induced hepatic activity of 4-nitrophenol hydroxylase (P450 2E1) was slightly higher in F344 rats compared to Wistar-derived rats (Table 1). Prior treatment with pyridine or isoniazid increased hepatic activity of 4-nitrophenol hydroxylase (Table 1), and increased formic acid excretion in the urine following TCE administration, with isoniazid producing the most marked increase (Table 1). In contrast, prior

treatment with phenobarbitone followed by TCE did not increase formic acid excretion when compared to TCE administration to naïve rats (Table 1). This indicates that induction of hepatic cytochrome P450 2E1 in Wistar-derived rats can increase formic acid excretion following TCE exposure. However, our primary objective was to examine the dose-response relationship for TCE-induced formic aciduria so for all subsequent studies we used F344 rats.

Dose-response relationship for trichloroethylene-induced formic aciduria in male and female F344 rats and plasma levels of formic acid.

We have examined extent of formic aciduria following TCE administration to female F344 rats and looked at much lower doses than previously reported in male F334 rats. To our surprise the increase in formic acid in urine in both male and female rats occurred at low doses of TCE, down as low as 8mg/kg/day (Figures 3). Thus a 125 -fold decrease in the dose of TCE still produced a response, which is well below doses of TCE that are thought to be toxic. In general, there was a lot of individual animal variability in the extent of formic acid excreted, some rats responding better than others, hence the large standard errors, such that day1 was not statistically significant compared to day 0 (Figure 3A & B). However, after 3 doses of TCE urinary formic acid excretion of statistically significantly increased compared to day 0 and in many cases to day 1 (Figure 3A & 3B). There was no sex difference with regard to the dose of TCE producing formic aciduria, both sexes responding at 8mg/kg/day, however male F344 rats tended to excrete slightly more formic acid than female F344 rats (Figure 3). Although in female F344 rats the response was more marked than in Wistar male rats (compare Figure 3B and Figure 2B). Urine pH was generally decreased at doses of 16mg/kg/day for 3 days and above in male and female rats (data not shown). Evidence of absorption and metabolism was seen by the presence of a TCE metabolite, TCE-O-glucuronide in the ¹H-NMR spectra of urine at 1000mg/kg TCE (Figure 1D) but was below the limits of detection by ¹H-NMR at 32.5mg/kg TCE. Plasma samples taken 24h after the last dose of TCE had elevated concentrations of formic acid compared to controls at doses of 8mg/kg/day TCE, and above, in male and female rats (Figure 4A & B).

Do trichloroethylene metabolites, perchloroethylene and carbon tetrachloride produce formic aciduria in male F344 rats?

The two major cytochrome P450 dependant metabolites of TCE, trichloroethanol (TCE-OH) and trichloroacetic acid (TCA) were able to increase the excretion of formic acid in urine, when given at doses likely to be formed from TCE *in vivo* (Figure 5). Both TCE-OH and TCA at

16mg/kg/day after either 1 or 3 doses increased urinary formic acid excretion, while DCVC a minor metabolite formed via glutathione conjugation, given at a concentration higher than thought to be formed *in vivo* 2mg/kg/day for 3 days had no effect on formic acid excretion (Figure 5). The response with TCA at 16mg/kg/day was very marked being higher than that seen after TCE, however lower doses of 4 and 8 mg/kg/day TCA for 3 days showed little or no response (Figure 5) suggesting a steep dose response curve. PCE and carbon tetrachloride at 16mg/kg/day for 3 days did not increase formic acid excretion in the urine. However, increasing the dose of carbon tetrachloride to 80mg/kg/day produced a marked increase in formic acid in urine to the levels seen with TCE (Figure 5). Chloroform at a dose of 100mg/kg/day also produced a small statistically significant formic aciduria after the third dose (Figure 5).

Discussion

We have shown for the first time that formic acid excretion following TCE administration is not just a high dose phenomenon but occurs at a dose about 125-fold times lower than the top dose used in the cancer bioassay (1000mg/kg/day). Green *et al.*, (1998) reported that treatment of male F344 rats with the large doses of TCE produced a marked and sustained formic aciduria which is associated with acidification of the urine. Urinary pH falls after TCE treatment by about half a pH unit, but no further, this is presumably due to buffering of the urinary pH by increased ammonium ion excretion (Liesivuori and Savolainen, 1987; Green *et al.*, 1988). Most importantly Green and co-worker's (1998) showed that the formic acid excreted in the urine of TCE-treated rats is not a metabolite of TCE, indicating that it occurs as a result of perturbation of endogenous cellular metabolism. Following a single dose of TCE the formic aciduria was still present 72h after dosing, which is in agreement with Green *et al.*, (1998) who reported it reached a maximum by 48h with a half-life of 4-5 days. The consequence of this is that following multiple dosing formic aciduria reaches a steady state concentration which is maintained for as long as TCE is administered. Green *et al.*, (1998) proposed that a marked and sustained excretion of formic acid following TCE administration could lead to renal tubule cell injury and repair which could explain the low incidence of renal tumours seen at 500 & 1000mg/kg/day by gavage and following exposure at 600ppm for 6h/day in male F344 and Sprague-Dawley rats (Maltoni *et al.*, 1988; NTP, 1988; 1990). Our findings show that the same amount of formic acid is excreted by an oral dose of 16mg/kg/day TCE as by 1000mg/kg/day and hence the lower dose would have the same potential to cause renal tubule injury, however it unlikely that this is a carcinogenic dose to the male rat kidney, although a bioassay has not been

conducted at such low doses. To the best of our knowledge this is the first time a biological change has been detected in rats following TCE exposure at such low doses.

With a number of chlorinated solvents, including TCE, there is often a sex difference observed with toxicological responses, male rats being more sensitive than female rats (Lash *et al.*, 2001, Pohl *et al.*, 1984, Werner *et al.*, 1995) so it seemed prudent to examine the response of female F344 rats to TCE. Similar to male rats, female rats showed a marked and sustained formic aciduria which was observed down at doses as low as 8mg/kg/day, the only sex difference was related to the amount of formic acid excreted which generally was lower in females, which may reflect a lower rate of metabolism in this sex of rat. Associated with the increase in formic acid there was an acidification of the urine which was statistically significant at doses of 62.5mg/kg/day and above. Female rats do not develop renal tumours after exposure two years exposure to TCE (NTP,1988), suggesting that a marked and sustained formic aciduria does not cause renal tubule cell injury and repair in female rats, although it is accepted that the amount of formic acid excreted is lower compared to male rats. We found no evidence of renal tubule cell injury after three doses of 1000mg/kg/day TCE to male F344 rats, and even after 28 or 90 days dosing no renal tubule injury was observed (Green *et al.*, 1998; Lock, unpublished observation) clearly any affect of formic acid on the kidney is a result of chronic exposure to a sustained high concentration.

The absorption, metabolism and excretion of TCE in rats have been extensively studied. Prout *et al.*, (1985) and Green and Prout, (1985) reported studies in two strains of rat at doses of 10, 500 and 1000mg/kg when given TCE orally in corn oil. Both strains of rat Osborn-Mendel and Wistar handled TCE in a very similar manner, at the high doses a large amount of TCE is exhaled unchanged in expired air typically about 50% of the dose (Prout *et al.*, 1985) with 2-4% exhaled as CO₂. At 10mg/kg about 1% of the dose was exhaled as unchanged TCE while about 10% was exhaled as CO₂, indicating that metabolism was saturated at the higher doses. The main route of excretion of absorbed TCE is via the urine where it is present as TCE-O-glucuronide and TCA, with 89-91% excreted as TCE-O-glucuronide and 6-8% as TCA over the first 24h at 10, 500 and 1000mg/kg (Green and Prout, 1985). In our studies, we would expect a similar pattern of absorption, metabolism and excretion and at the higher doses have detected TCE-O-glucuronide in the urine. We have not isolated and purified the glucuronide from urine and the ¹H-NMR spectra is not available in the literature, our identification is based on our experience that the resonance pattern is typical of a glucuronide conjugate. At low oral doses of TCE, (2-16mg/kg) TCE is rapidly absorbed into the blood stream, Lee *et al.*, (2000) reported peak blood levels of 0.5µg/ml, 2h after dosing 2mg/kg. In our studies low doses of TCE would

be completely absorbed and quite extensively metabolised over 24h to TCA and TCE-O-glucuronide. Lee and co-workers used an emulsifying solvent to administer TCE which enables rapid absorption, while we used corn oil, as in the cancer bioassays, which tends to slow and prolong the extent of absorption. Our findings at low doses of TCE may have relevance to TCE heavily contaminated drinking water. The only report we are aware that has measured urinary formic acid excretion in workers occupationally exposed to TCE is Green *et al.*, (2004). They reported that in 70 workers exposed to 25ppm (0.5 to 250ppm) TCE over 4.1 years had a statistically significant increase in urinary formic acid excretion: Controls, 5.55 ± 3.0 mg formate/g creatinine (54); compared to exposed workers at 9.45 ± 4.78 mg formate/g creatinine (70) $p < 0.01$, suggesting that this response can be observed in humans. We have shown that TCE in solution in water given orally to male F344 rats at 8mg/kg/day for 3 days produced a marked increase in formic aciduria: controls excreting 1.5mg formic acid/24h while TCE-treated excreted 10mg excreted/24h. It seems that doses lower than 8mg.kg/day TCE only produces a small formic aciduria in F344 rats.

We discovered by chance, wanting to use our inbred strain of Wistar rats that they excreted less formic acid in their urine after TCE exposure than F344 rats. As TCE is primarily metabolised by cytochrome P450 2E1 (Kim and Ghanayem, 2006) we examined the hepatic activity of P450 2E1, using 4-nitrophenol as substrate and showed that the activity with this substrate was only slightly higher in the F344 strain. We examined the effect of inducing P450 2E1 in male Wistar rats by pre-treating them with known inducers showed that this did indeed increase the extent of formic aciduria after TCE administration to concentrations seen in F344 rats given TCE, supporting the view that oxidative metabolism of TCE is required to cause the formic aciduria. In contrast, induction of P450 2B1/2 also reported to metabolise TCE did not increase formic acid excretion. We are aware that pyridine also increases P450 3A1 and P450 3A2, in addition to P450 2E1 (Kim, *et al.*, 2001), so we cannot exclude a role for P450 3A1/A2 in this response. However, it was not our aim to understand in detail the basis for the strain difference in response to TCE, but our findings indicate that metabolism of TCE by cytochrome P450 is an essential step in the production of formic aciduria.

We also established that the major metabolites of TCE; TCE-OH and TCA both produce a marked and sustained increase in formic acid in the urine of male F344 rats at dose likely to be formed *in vivo* from TCE when given at 1000mg/kg (Figure 5). Choral an intermediate metabolite following P450 metabolism did not produce a significant increase in formic aciduria (Dow and Green, 2000), while the metabolite of the minor glutathione conjugation pathway

DCVC, also did not produce this response. A limited dose-response study with TCA showed a very marked response at 16mg/kg/day, much greater than that seen with TCE, with only a small response at 8mg/kg/day suggesting a very steep dose response curve (Figure 5). So at this stage we cannot rule out that a strain difference in the production of TCE-OH and or TCA could explain the strain difference in formic aciduria. Interestingly, PCE which like TCE undergoes metabolism to generate TCA (Odum *et al.*, 1988) showed little evidence of formic aciduria after 3 daily doses 16mg/kg (Figure 5). Other chlorinated solvents at higher doses also caused formic aciduria, namely carbon tetrachloride and chloroform (Figure 5) and bromdichloromethane (Lock *et al.*, 2004).

The mechanism whereby TCE and halomethanes perturb formate metabolism is not understood and has not been studied in this manuscript. Currently there are 2 main routes whereby TCE could form reactive intermediates. TCE oxide is a reactive electrophile formed during TCE oxidation which rearranges to acylating intermediates (Miller and Guengerich, 1982). Mice treated with TCE have N(6)-dichloroacetylLys residues in P4502E1 as detected by immunochemical methods Cai and Guengerich (2001). Furthermore, Lys adducts were detected in the reaction of TCE oxide with both P450 2E1 and NADPH-P450 reductase, with a large amount of N(6)-formylLys observed (Cai and Guengerich, 2001). So it is possible that these reactive intermediates could disrupt enzymes in pathways utilising formate. Another possibility is that carbon tetrachloride is able to produce formic aciduria and is known to undergo reductive metabolism by cytochrome P450 to form a trichloromethyl radical (Weber *et. al.*, 2003), similarly chloroform can undergo activation to free radical intermediates *in vitro* and *in vivo* (Tomasi *et al.*, 1985). While TCA has been reported *in vitro* to form a dichloromethyl radical in the presence of rat and mouse liver microsomes (Merdink *et al.*, 2000). Thus there are two possible routes of metabolism that could lead to reactive metabolites which could perturb biological enzyme activities and potentially lead to formic aciduria. Further studies are needed to understand the basis for the perturbation of formate metabolism by TCE metabolites.

In summary, we have shown that TCE produces an increase in the urinary excretion of formic acid in both male and female F344 rats and most importantly that this is not a high dose phenomenon, being seen at doses as low as 8mg/kg/day for 3 days. Both TCE-OH and TCA the primary metabolites also produce formic aciduria, suggesting it is a cytochrome P450-mediated TCE metabolite and not the parent compound that produces the increased formate excretion in urine. There is also a marked rat strain difference in TCE-induced formic aciduria, Wistar rats

being low responders and F344 rats being higher responders, which could also be related to cytochrome P450 metabolism.

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Figure legends

Figure 1. ¹H-NMR spectra of male F344 rat urine after dosing with 16, 62.5 or 1000mg/kg trichloroethylene/day for 3 days.

Male F344 rats were placed in metabolism cages and urine collected for 24h (Day 0) then dosed orally with 16, 62.5 or 1000mg/kg/day TCE for 3 days with 24h urine collected on Day 1, 2 and 3. The urine was then analysed using ¹H-NMR spectroscopy with TSP (0.5mg/ml) as reference signal at 0ppm. Formic acid appears at 8.44ppm. A, B, C and D show typical spectra from one rat in each group on day 3.

Figure 2. Urinary formic acid concentration and pH in male F344 and Wistar rats given daily doses of trichloroethylene (1000mg/kg) for one or three days.

Three groups of male F344 rats (A, C) and male Wistar rats (B, D) were dosed orally with either a single dose of TCE (1000mg/kg)(Day1) followed by daily doses of corn oil for 2 days (Days 2 & 3) or daily doses of TCE (1000mg/kg) for three days (Day1,2 &3). Controls received corn oil alone. Urine was collected for 24h before dosing Day 0 and 24h after dosing on Day 1, 2 & 3 the urine pH measured and the samples analysed using ¹H-NMR spectroscopy. Values are mean ± SEM, n =3

* P<0.05 statistically significantly different from corn oil control (Day 0).

** P<0.01 statistically significantly different from corn oil control (Day 0).

Figure 3. Dose-response relationship for the excretion of formic acid in urine by male (A) and female (B) F344 rats following trichloroethylene.

Male and female F344 rats were placed in metabolism cages and urine collected for 24h (Day 0) then dosed orally with either 2, 4, 8, 12, 16, 31, 62.5, 125, 250, 500 or 1000mg/kg/day TCE for 3 days (Days 1,2 & 3), controls received corn oil alone. Urine was collected on days 1 &3 and analysed for formic acid using ¹H-NMR spectroscopy. Values are mean ± SEM, n=3-5 except for corn oil where n= 8.

* P<0.05 statistically significantly different from corn oil control (Day 0).

** P<0.01 statistically significantly different from corn oil control (Day 0).

*** P<0.001 statistically significantly different from corn oil control (Day0).

† P<0.05 statistically significantly different from TCE on Day 1.

††† P<0.001 statistically significantly different from TCE on Day 1.

Figure 4. Concentration of formic acid in the plasma of male and female F344 rats following 3 daily doses of trichloroethylene.

Male F344 rats (A) were given oral doses of TCE at 2, 4, 8, 16, 31, 62.5, 125, 250, 500 or 1000mg/kg/day for three days and female F344 rats (B) daily oral doses of TCE at 8, 16 or 31mg/kg/day for 3 days. Controls received corn oil alone. All rats were killed 24h after the last dose, plasma collected and analysed for formic acid using ¹H-NMR spectroscopy. Values are mean ± SEM, n=3-5.

* P<0.05 statistically significantly different from corn oil control

Figure 5. The ability of various halogenated chemicals to produce formic aciduria in male F344 rats.

Male F344 rats were orally dosed daily for 3 days with of corn oil, trichloroethylene (TCE, 16mg/kg), trichloroethanol (TCE-OH 16mg/kg), trichloroacetic acid (TCA, 4, 8 or 16 mg/kg), S-(1,2,-dichlorovinyl)-L-cysteine, (DCVC, 2mg/kg), perchloroethylene (PCE,16mg/kg), carbon tetrachloride (CCl₄, 16 or 80mg/kg) or chloroform (CHCl₃,100mg/kg). Urine was collected 24h before dosing day 0 and after dosing on days 1 and 3. Urine samples were analysed for formic acid using ¹H-NMR spectroscopy. Values are mean ± SEM, n=3-5.

* P<0.05 statistically significantly different from corn oil control.

** P<0.01 statistically significantly different from corn oil control.

*** P<0.001 statistically significantly different from corn oil control.