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REVIEW

Trichloroethylene: Mechanisms of Renal Toxicity and Renal Cancer and Relevance to Risk Assessment

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1,1,2-Trichloroethylene (TCE) is an important solvent that is widespread in the environment. We have reviewed carcinogenicity data from seven bioassays with regard to renal injury and renal tumors. We report a consistent but low incidence of renal tubule carcinoma in male rats. Epidemiology studies on workers exposed to TCE (and other chlorinated solvents) indicate a weak association between high-level exposure and renal cancer. There appears to be a threshold below which no renal injury or carcinogenicity is expected to arise. TCE is not acutely nephrotoxic to rats or mice, but subchronic exposure to rats produces a small increase in urinary markers of renal injury. Following chronic exposure, pathological changes (toxic nephrosis and a high incidence of cytomegaly and karyomegaly) were observed. The basis for the chronic renal injury probably involves bioactivation of TCE. Based on the classification by E. A. Lock and G. C. Hard (2004, *Crit. Rev. Toxicol.* 34, 211–299) of chemicals that induce renal tubule tumors, we found no clear evidence to place TCE in category 1 or 2 (chemicals that directly or indirectly interact with renal DNA), category 4 (direct cytotoxicity and sustained tubule cell regeneration), category 5 (indirect cytotoxicity and sustained tubule cell regeneration associated with α_{2u}-globulin accumulation), or category 6 (exacerbation of spontaneous chronic progressive nephropathy). TCE is best placed in category 3, chemicals that undergo conjugation with GSH and subsequent enzymatic activation to a reactive species. The implication for human risk assessment is that TCE should not automatically be judged by linear default methods; benchmark methodology could be used.

Key Words: **trichloroethylene; renal toxicity; renal cancer; mechanisms of toxicity; risk assessment.**

1,1,2-Trichloroethylene (TCE) is a widespread chemical that has a number of uses. Starting in the 1930's it was used as a general anesthetic, although this has been discontinued. TCE has important industrial uses as a non-flammable solvent for degreasing metal parts, and as a general purpose solvent. In addition to being found in the workplace, TCE has been detected in a variety of urban and rural areas of the U.S. and other regions of the world. TCE is not formed by natural sources, but has been found in measurable amounts in the food chain and in drinking water. During production, storage, and use TCE evaporates into the atmosphere and has been identified as a common contaminant of air. In addition, leakage from chemical waste sites has lead to its contamination of ground water. It was estimated 10 years ago that TCE was present in 34% of the U.S.'s drinking water; this figure is likely to be much higher at the present time.

The detection of TCE in air, soil, and water has frequently meant that compliance with certain environmental laws such as the Comprehensive Environmental Response, Compensation and Liability Act has been difficult. This has triggered huge clean up bills running into billions of dollars to protect public health. One of the consequences has been a focus over the last decade on re-evaluation of the safety assessment of TCE with particular regard to exposure levels and safety limits in drinking water. Currently the Environmental Protection Agency (EPA) has set a maximum level in drinking water for TCE of 5 ppb (5 μg/l). The Occupational Safety and Health Administration has set a permissible exposure limit of 100 ppm for an 8 h day and 40 h week, with a level of 200 ppm for a 15 min average exposure limit in air during the working day.

In 2003 the EPA issued draft guidelines on TCE risk assessment which followed its guidelines for carcinogen risk assessment. A major change in these guidelines from the EPA's previous cancer policy was to place increased emphasis on the "weight of scientific evidence," notably on dose response relationships, mechanisms or modes of action, and metabolic and toxicokinetic processes. Debate and discussion continue regarding the safety assessment of TCE and currently a National Academy of Science panel is reviewing the toxicology and safety assessment aspects.

This article will focus on our current knowledge of the mechanisms of nephrotoxicity of TCE, which may explain the low incidence of renal tubule tumors seen in rats. The toxicology of TCE has been reviewed by others (Bruning and

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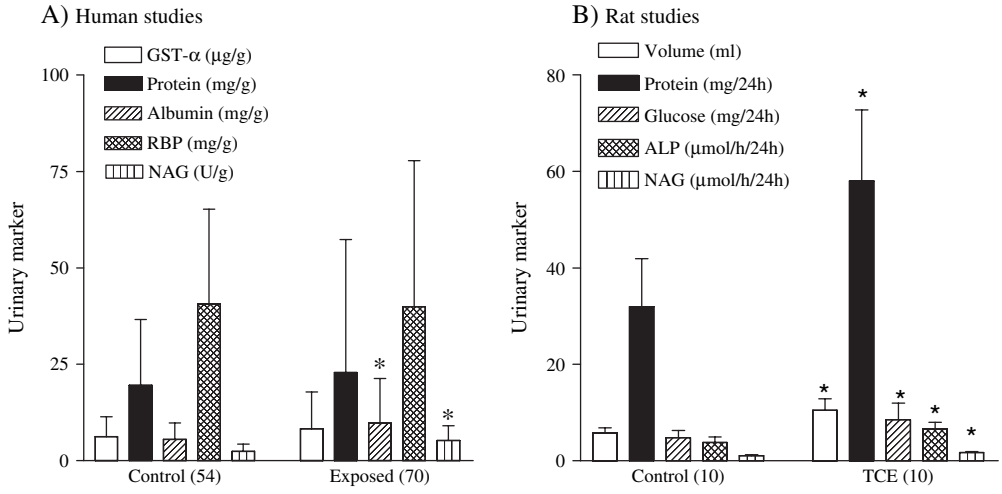

FIG. 1. Urinary markers of renal function in (A) workers exposed to trichloroethylene at 32 ppm for an average of 4.1 years and (B) rats exposed to daily oral doses of trichloroethylene at 2000 mg/kg/day for 42 days. The numbers in brackets represent the number of workers or rats exposed or not exposed (controls) to trichloroethylene. Data adapted from Green *et al.*, (1997, 2004). *Statistically significantly different from control $p < 0.05$.

Bolt, 2000; Davidson and Beliles, 1991; Goeptar *et al.*, 1995; Lash *et al.*, 2000a,b); this review builds on these previous reviews and adds new information generated over the last five years. The findings in experimental animals will be discussed with regard to their relevance to humans.

Toxicity of TCE

Effects of Acute and Chronic Exposure to TCE in Humans

Deaths in workers resulting from TCE exposure are rare. Mild anesthesia and other types of central nervous system depression have been reported occasionally; these effects are concentration and duration dependent, and may be due to sensitization of the myocardium to adrenaline. Hepatic toxicity is not a common finding in humans and was reported to be rare when TCE was used as an anesthetic. Changes in clinical chemistry markers of liver injury have been observed, along with jaundice, hepatomegaly, and reduced liver function. In most cases these effects have been shown to be reversible (Davidson and Beliles, 1991).

Similarly, acute renal injury is not a common finding in humans. There is limited evidence that some workers exposed to TCE have renal tubule injury as judged by an increase in the excretion of proteins and enzymes in the urine. Bruning and coworkers conducted a retrospective study in Arnsberg, Germany on 39 workers exposed to ''high levels'' of TCE from 1956 to 1975. They reported that the urinary excretion of GSH transferase α, a marker of proximal tubule cell damage, was elevated in the urine of TCE-exposed workers but not in the control group (see Bruning and Bolt, 2000). It should be noted that total urinary protein levels, as well as serum and urine creatinine, and serum urea, were unchanged compared to the control group indicating that the injury was mild. The levels of TCE to which these workers were exposed over 30 years ago is not known, although 19 narcotic episodes were reported, which is indicative of peak exposure >500 ppm.

Recently Green *et al.* (2004) reported a small increase in the urinary excretion of proteins in a group of 70 workers currently exposed to TCE compared to an age and sex matched control population. The mean exposure to TCE, estimated from urinary trichloroacetic acid (TCA) concentration, was 32 ppm (range 0.5–252 ppm) with an average exposure of 4.1 years (range 1–20 years). There were small, but statistically significant, differences between the exposed and control populations for the nephrotoxicity markers α_1-microglobulin, N-acetylglucosaminidase (NAG), and albumin (Fig. 1). However, neither NAG nor albumin showed a significant correlation with either the magnitude or duration of exposure. Urinary GSH transferase α showed a dose-dependent increase but this was not statistically significant compared to controls. Overall this study found no evidence of renal toxicity under conditions of TCE exposure that were in accordance with the current occupational exposure limits. The likely explanation for the differences between these two studies is the exposure level of TCE. This provides a strong argument in favor of a threshold for human nephrotoxicity of >250 ppm as indicated by Green *et al.* (2004).

Vamvakas and coworkers (1998) identified an increase in renal cancer among the workers in Arnsberg, Germany exposed to TCE from 1956–1975. Initially Henschler and coworkers (1995a) reported 5 cases of renal cancer (4 renal cell carcinoma) compared to none in the control population. This cohort study found an increased incidence of renal cell carcinoma with a standardized incidence ratio of 7.97, 95% confidence interval of 2.59–18.59. There was much controversy regarding this finding, it was considered likely to be biased by the priori presence of a cluster of renal tubule tumors and to have serious methodological limitations (Bloemen and Tomenson, 1995;

Henschler *et al.*, 1995b; Swaen, 1995). In a later study 58 patients with renal cell cancer, 19 of which had a history of high exposure to TCE were identified. Following adjustment for age, smoking, and obesity factors known to influence renal cancer rates, the study demonstrated an association of renal cancer with long-term exposure to TCE with an odds ratio of 10.8, 95% confidence interval of 3.36–34.75. Again there was much debate regarding the study, particularly its design and the selection of controls (see Green and Lash, 2000; Vamvakas *et al.*, 2000; Mandel, 2001). Other reports in 99 renal cancer patients, including the 39 workers with previous ''high'' exposure to TCE, showed a small increase in urinary excretion of proteins such as α_1-microglobulin compared to non-cancer controls (Bolt *et al.*, 2004).

A number of epidemiology studies have been conducted to examine the relationship between TCE (and TCE with halogenated solvents) exposure and renal cell cancer mortality or incidence. Wartenberg and coworkers (2000) reviewed over 80 published papers and letters (including the studies from Arnsberg) and found a Relative Risk for kidney cancer of 1.7, with a 95% confidence interval of 1.1–2.7. However, these authors caution that few studies isolate TCE exposure per se, and that the findings are likely to be confounded by exposure to other solvents, and by other risk factors in general.

Overall, the data indicates there may be a weak association between high-level exposure to TCE and renal cancer in humans. There also appears to be a threshold below which no renal injury and hence renal carcinogenicity is expected to arise.

Effects of Acute and Chronic Exposure to TCE in Rats and Mice

Acute and chronic exposure to TCE causes toxicity in a variety of organs in experimental animals. This review will consider only renal effects. TCE is not acutely toxic to the kidney in experimental animals. In several studies daily high doses of TCE have been administered to rats or mice by either oral gavage or inhalation. No morphological changes in the kidneys were observed in these studies, and only small increases in urinary excretion of known markers of renal injury were detected (Fig. 1; Green *et al.*, 1997).

Carcinogenicity bioassays with TCE have shown clear evidence of non-neoplastic lesions in the kidney. These have been identified as renal tubule cytomegaly, karyomegaly, and toxic nephrosis/nephropathy, and occur in both male and female rats and male and female mice. Cytomegaly was first observed in the inner cortex and outer stripe of the outer medulla of rats that died early in the studies, and was seen microscopically as frank enlargement of the nucleus and cytoplasm of scattered, individual tubule cells. The epithelial cells of the *pars recta* of the proximal tubule were often enlarged and contained nuclei several times their normal size (karyomegaly). The enlarged nuclei were often hyperchromatic and had an irregular or oblong shape. Other lesions observed in severely affected kidneys were described as toxic nephropathy in order to distinguish them from the common spontaneous nephropathy of aging rats (see later). Toxic nephropathy occurred only in rats administered TCE, and consisted of dilated tubules lined with elongated and flattened epithelial cells. The degree of flattening was often severe and was roughly proportional to the degree of dilation. The lumens of the affected tubules were empty or contained wisps of eosinophilic material (NTP 273). In mice, the pathology of cytomegaly was similar to that found in TCE dosed rats, but was generally less severe and did not develop as extensive loss of epithelial cells and tubule dilation (NTP 243).

TCE has been evaluated for carcinogenicity in a number of studies using six different strains of rat and four different strains of mouse exposed by either inhalation or oral gavage to formulations of TCE of varying purity and with different stabilizers. These studies were conducted on three different continents and published between 1976 and 1990. During this time some changes in the nomenclature and classification of preneoplastic and neoplastic renal lesions have occurred and therefore the reader is referred to the original papers or technical reports for detailed information. Criteria for the classification of proliferative renal lesions (hyperplasia, adenoma, and carcinoma) have been reported by various independent groups and by the Society of Toxicologic Pathology (STP) (Hard *et al.*, 1995). While the NTP criteria differ in descriptive detail from those of the STP (Hard *et al.*, 1995), in practice, the actual diagnoses of atypical (focal) tubule hyperplasia, adenoma, and carcinoma are usually in accord.

The findings in each of the bioassays will now be discussed in detail. In the first study (NCI TR 2) in Osborne-Mendel rats and $B6C3F_1$ mice, industrial grade TCE was given by oral gavage five days a week for 78 weeks and the animals killed at 110 weeks (rats) or 90 weeks (mice). There were 20 animals/group for controls and 50 animals/group for TCE treatment. The TCE used in this bioassay contained certain contaminants, particularly epichlorohydrin (0.09%), which is a potent mutagen reported to produce local sarcomas following subcutaneous injection in mice and nasal carcinomas in rats after inhalation exposure (NTP TR 243). In rats, the initial doses were 650 and 1300 mg/kg body weight; the doses were reduced to 549 and 1097 mg/kg during the course of the study based on survival and body weight data. In mice, the initial doses were 1000 and 2000 mg/kg for males and 700 and 1400 mg/kg for females. During the course of the study, the dose was increased to give ''time weighted average'' doses of 1169 and 2339 mg/kg for male mice and 869 and 1739 mg/kg for female mice. In rats administered TCE there was no increase in the incidence of primary tumors in either sex. One renal tubule adenoma and one renal hamartoma were reported in the low-dose males, while in the controls, one renal hamartoma and two malignant mixed renal tumors were diagnosed. Mixed malignant tumors are synonymous with liposarcomas, which have never been shown to be induced by chemicals. TCE produced

a clear, compound-related chronic nephropathy in both male and female rats at both dose levels; this may have been recorded as toxic nephrosis in subsequent studies. In the B6C3F1 mice, one renal tubule adenoma was seen in a top dose male mouse, which was within the historical control range. TCE also produced a compound-related increase in chronic nephropathy in mice of both sexes. The survival of the rats was compromised by the dosage regimen and IARC considered this bioassay to be an ''inadequate study of carcinogenic activity.''

The second study (NTP TR 243) used epichlorohydrin-free, highly purified TCE (stabilized with 8 ppm di-isopropylamine) administered by gavage in corn oil to F344 rats and $B6C3F_1$ mice five days a week for 103 weeks with 50 animals/control or treatment groups. Doses were 500 and 1000 mg/kg for rats, and 1000 mg/kg for mice. In the male F344 rats there was a statistically significant increase in the incidence of renal carcinoma in animals killed at the end of the experiment (control, 0/33; 500 mg/kg, 0/20; 1000 mg/kg, 3/16; $p < 0.05$). One high-dose female also had a renal carcinoma, and two adenomas were detected in low-dose males. However, the combined incidence of renal tubule adenomas and carcinomas was not statistically significant. Additional tumors found in the kidneys of male rats included a transitional cell carcinoma of the renal pelvis in a low dose animal and a carcinoma of the renal pelvis in a high dose animal. A transitional cell papilloma of the renal pelvis was reported in an untreated control, currently this would be classified as transitional cell hyperplasia (Hard *et al.*, 1995). TCE caused no increase in renal tubule hyperplasia and the incidence of chronic progressive nephropathy (CPN) was reduced in the TCE treated male and female rats compared to controls. Cytomegaly was observed with a high incidence in both male (98%) and female (100%) treated rats, with severity being more marked in the males. In the mice, one renal tubule adenoma was seen in a control male, and one renal tubule carcinoma in a treated male. Toxic nephropathy was observed in 90% of the TCE-dosed male mice and in 98% of the dosed female mice compared to none in the controls (NTP TR 243). There was a high incidence of toxic nephropathy in both sexes of both species resulting in a poor survival rate. Thus this bioassay was judged by the NTP to be an ''inadequate study of carcinogenic activity.''

In the third study, TCE (stabilized with 8 ppm di-isopropylamine) was evaluated in four strains of rat (ACI, August, Marshall, and Osborne-Mendel) (NTP TR 273). Males and females of each strain were given TCE by oral gavage in corn oil at 0, 500, or 1000 mg/kg, five days a week for 103 weeks with 50 animals per control or treatment groups. TCE administration was associated with a small increased incidence of renal tubule adenoma and carcinoma in all four strains of rat. However, the number of renal tubule tumors was low and not consistently dose-related. In male Osborne-Mendel rats the incidence was 12% at 500 mg/kg and 4% at 1000 mg/kg compared to 0% in the control group, which is statistically significant at the 500 mg/kg dose. Due to the extent of chemically induced toxicity, reduced survival and incomplete documentation of experimental data, this bioassay was considered by the NTP to be an ''inadequate study of carcinogenic activity'' of TCE.

Henschler and coworkers (1980) conducted an inhalation bioassay with highly purified TCE (stabilized with 0.0015% triethanolamine) in both sexes of Han:Wistar rats, Han:NMRI mice and hamsters. The animals were exposed to 0, 100, or 500ppm TCE for 6 h/day for five days/week for 78 weeks with 30 animals per control and treatment groups. No increase in the incidence of renal tubule tumors was seen in either sex of any species. Renal tubule adenoma and renal tubule cystadenoma were recorded separately in this study, both are benign tumors and for this review they have been combined under the term adenoma. In male rats, one adenoma was seen at 100 ppm and one at 500 ppm with one carcinoma at 500 ppm, while in male mice two adenomas were seen at 100 ppm and one at 500 ppm. No renal tumors were reported in female rats or mice.

Henschler *et al.* (1984) also conducted a study with both highly purified TCE (stabilized with 0.0015% triethanolamine) and industrial grade TCE (99.4%) in Swiss mice in the presence and absence of 1,2-epoxybutane (0.8%) and/or the epoxide stabilizer epichlorohydrin (0.8%). Mice were dosed orally with TCE in corn oil at 2400 mg/kg (males) or 1800 mg/kg (females) for 78 weeks with 50 animals per control and treatment groups and then terminated at 106 weeks. There was no increase in incidence of renal tumors, with one adenoma being detected in control males, one adenoma in the purified TCE treated group and two in the TCE + 1,2-epoxybutane group. In female mice, four adenomas were detected in the highly purified TCE treated group with none in the controls or other groups. No renal tubule tumors were reported with the epichlorohydrin stabilized TCE.

Fukuda *et al.* (1983) exposed female Sprague-Dawley rats and female ICR mice to industrial grade TCE, containing 0.019% epichlorohydrin, by inhalation exposure at 0, 50, 150, and 450 ppm for 7 h/day for 5 days/week for 104 weeks with 50 animals per control and treatment groups. No renal tubule tumors were seen in mice; one renal tubule carcinoma was observed in a rat exposed to 450 ppm TCE.

Lastly, Maltoni *et al.* (1988) conducted a large inhalation study with purified TCE (stabilized with 20 ppm butylhydroxytoluene) in Sprague-Dawley rats, and Swiss and B6C3F1 mice with 130 rats and 90 mice per control and treatment group. Male and female rats and mice were exposed to 100, 300, or 600 ppm TCE for 7 h/day, for 5 days/week, up to 104 weeks and then observed until they died. In rats, renal tubule cytomegaly and karyomegaly were observed in males at the two top doses with an incidence of 78 and 17% respectively. Five renal tubule carcinomas (four in males; incidence 3.1%) and one in a female (incidence 0.7%) were observed at an exposure level of 600 ppm TCE. There was no increase in the incidence of renal tubule tumors in mice of either sex.

TABLE 1
Combined Renal Tubule Tumor Incidence from Bioassays Conducted in Male Rats of Seven Different Strains Exposed to Trichloroethylene by Either Inhalation or Oral Gavage

Studies	Dose	Total number of animals treated	Adenoma[a] (% incidence)	Carcinoma[a] (% incidence)	Adenoma and carcinoma
Inhalation[b]					
Maltoni *et al.*, 1988;	0 ppm	160	2 (1.3)	0 (0)	2
Henschler *et al.*, 1980	100 ppm	160	1 (0.6)	0 (0)	1
	300 ppm	130	0 (0)	0 (0)	0
	500 ppm	30	1 (3.3)	1 (3.3)	2
	600 ppm	130	0 (0)	4 (3.1)*	4
Oral gavage[c]					
NCI 2, 1976;	0 mg/kg	520	2 (0.4)	0 (0)	2
NTP 243, 1990[d];	500 mg/kg	250	10 (4.0)*	3 (1.2)	13*
NTP 273, 1988[d]	650 mg/kg	50	0 (0)	1 (2.0)	1
	1000 mg/kg	250	2 (0.8)	5 (2.0)*	7
	1300 mg/kg	50	0 (0)	0 (0)	0

[a]Combined tumor incidence for the studies and strains shown.
[b]Han-Wistar and Sprague-Dawley strains.
[c]Osborne-Mendel, Fischer 344, ACI, August, and Marshal strains.
[d]Two control groups were used: untreated (50 animals) and corn oil treated (50 animals).
*Statistically significantly different from control and untreated rats, Kruskal-Wallis test followed by Dunn's multiple comparisons test $p < 0.05$.

Thus, taking into consideration the data from all the studies there is evidence that exposure of male and to a lesser extent female rats, but not mice, to TCE by either gavage or inhalation produces a small increase in the incidence of renal tubule tumors (Tables 1–3). This is supported by statistical analysis which shows a clear increase in carcinoma incidence in male rats, but not in female rats or mice of either sex. In addition, these findings are generally higher than historical control values. For example, in two studies in male F344 rats, renal tubule tumors were observed in 1 out of 649 animals; 0.15% incidence and 3 out of 748 animals; 0.4% incidence (NTP 243), while renal tubule carcinoma in control

TABLE 2
Combined Renal Tubule Tumor Incidence for Bioassays Conducted in Female Rats of Seven Different Strains Exposed to Trichloroethylene by Either Inhalation or Oral Gavage

Studies	Dose	Total number of animals treated	Adenoma[a] (% incidence)	Carcinoma[a] (% incidence)	Adenoma and carcinoma
Inhalation[b]					
Maltoni *et al.*, 1988;	0 ppm	210	0 (0)	0 (0)	0
Fukuda *et al.*, 1983;	50 ppm	50	0 (0)	0 (0)	0
Henschler *et al.*, 1980	100 ppm	160	0 (0)	0 (0)	0
	150 ppm	50	0 (0)	0 (0)	0
	300 ppm	130	0 (0)	0 (0)	0
	450 ppm	50	0 (0)	1 (2.0)	1
	500 ppm	30	1 (3.3)	0 (0)	1
	600 ppm	130	0 (0)	1 (0.8)	1
Oral gavage[c]					
NCI 2, 1976;	0 mg/kg	515	4 (0.8)	0 (0)	4
NTP 243, 1990[d];	500 mg/kg	293	5 (1.7)	1 (0.3)	6
NTP 273, 1988[d]	650 mg/kg	50	0 (0)	0 (0)	0
	1000 mg/kg	286	1 (0.3)	3 (1.0)	4
	1300 mg/kg	50	0 (0)	0 (0)	0

[a]Combined tumor incidence for the studies and strains shown.
[b]Han-Wistar and Sprague-Dawley strains.
[c]Osborne-Mendel, Fischer 344, ACI, August, and Marshal strains.
[d]Two control groups were used: untreated (50 animals) and corn oil treated (50 animals).

TABLE 3
Combined Renal Tubule Tumor Incidence from Bioassays Conducted in Male Mice of Three Different Strains Exposed to Trichloroethylene by Either Inhalation or Oral Gavage

Studies	Dose	Total number of animals treated	Adenoma[a] (% incidence)	Carcinoma[a] (% incidence)	Adenoma and carcinoma
Inhalation[b]					
Maltoni *et al.*, 1988;	0 ppm	210	4 (1.9)	0 (0)	4
Henschler *et al.*, 1980;	100 ppm	210	2 (0.95)	0 (0)	2
Fukuda *et al.*, 1983	300 ppm	180	0 (0)	0 (0)	0
	500 ppm	30	1 (3.3)	0 (0)	1
	600 ppm	180	0 (0)	0 (0)	0
Oral gavage[c]					
NCI 2, 1976;	0 mg/kg	120	2 (1.7)	0 (0)	2
NTP 243, 1990;	1000 mg/kg	100	0 (0)	1 (1.0)	1
Henschler *et al.*, 1984	2000 mg/kg	50	1 (2.0)	0 (0)	1
	2400 mg/kg	100	1 (1.2)	1 (1.0)	2

[a]Combined tumor incidence for studies and strains shown.
[b]Han-NMRI, ICR, Swiss, and $B_6C_3F_1$ strains.
[c]Swiss and $B_6C_3F_1$ strains.

female rats is very rare. Thus reports of renal tubule tumors in seven different strains of male rat exposed to TCE in different laboratories by both gavage and inhalation supports the view that the tumors although very small in number are probably TCE related.

Metabolism and Excretion of TCE

TCE is an uncharged, nonpolar, and highly lipophilic compound which can readily cross membranes by diffusion. The primary routes of entry into the body are via the lungs and the gastrointestinal tract, and TCE is rapidly and extensively absorbed following either inhalation or oral exposure. Dermal absorption may also occur if direct contact is made with the liquid. As TCE is lipophilic, it undergoes considerable distribution into lipid in body tissues.

In Vivo *Studies in Humans*

Humans come into contact with TCE in the workplace and as a contaminant of air and drinking water. Several inhalation studies have been conducted examining the rates of uptake and elimination of TCE (see Davidson and Beliles, 1991), and absorption through the gastrointestinal tract is extensive, as documented by the numerous cases of poisoning by oral ingestion. TCE can be eliminated unchanged in expired air, being cleared rapidly from tissues with a rich blood supply, however exhalation may continue for several hours, depending on the length of exposure, due to a slower clearance from adipose tissue. However, the majority of absorbed TCE undergoes metabolism within the body.

TCE is primarily metabolized by cytochromes P450 to chloral, and then to trichloroethanol (TCOH) and its glucuronide, and TCA (Fig. 2). These metabolites have been identified in human urine and used extensively as quantitative indices of exposure and body burden. TCOH and TCOH-glucuronide have a half-life of renal elimination of about 10 h (range 7 to 14 h). In contrast, TCA has a half-life of elimination of about 52 h (range 35–70 h). In the body, TCA is very tightly and extensively bound to plasma proteins, although some is transported into hepatic and renal cells, possibly by a monocarboxylic transporter or a renal organic anion transporter. TCA is reabsorbed following filtration at the glomerulus and this may well account for its prolonged half-life in the body. Estimates of the extent of metabolism in humans, as a percentage of retained TCE, have been made although many of the older studies were hampered by a lack of sensitive detection methods. More recent inhalation studies (single or repeat doses of TCE at 50 to 350 ppm) showed an average of 11% of the retained TCE was eliminated unchanged via the lungs, a further 2% was exhaled as TCOH, and 58% was excreted in urine as metabolites. Nearly 30% of the dose was unaccounted for (see Davidson and Beliles, 1991); part of this will be excreted in the feces, some exhaled as carbon dioxide and some will most likely be retained in body fat.

Studies have shown that TCE can also undergo conjugation with glutathione (GSH) with some excretion in the urine as the mercapturic acid (N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine) (NAcDCVC) (e.g., Bernauer *et al.*, 1996) (Fig. 3). Both regioisomers of the mercapturate were present in identical, but very small, concentrations in all human urine samples (Bernauer *et al.*, 1996). Bloemen *et al.* (2001) exposed volunteers to TCE using repeated 15 min inhalation exposures at 50 and 100 ppm and were unable to detect GSH metabolites in the urine, the amounts produced being below the limits of detection (40 pmol/ml). This group also examined the urine of workers exposed to 0.4 and 21 ppm TCE over an 8 h time-weighted average exposure, collecting urine overnight at the end of the fourth working day, again they were unable to

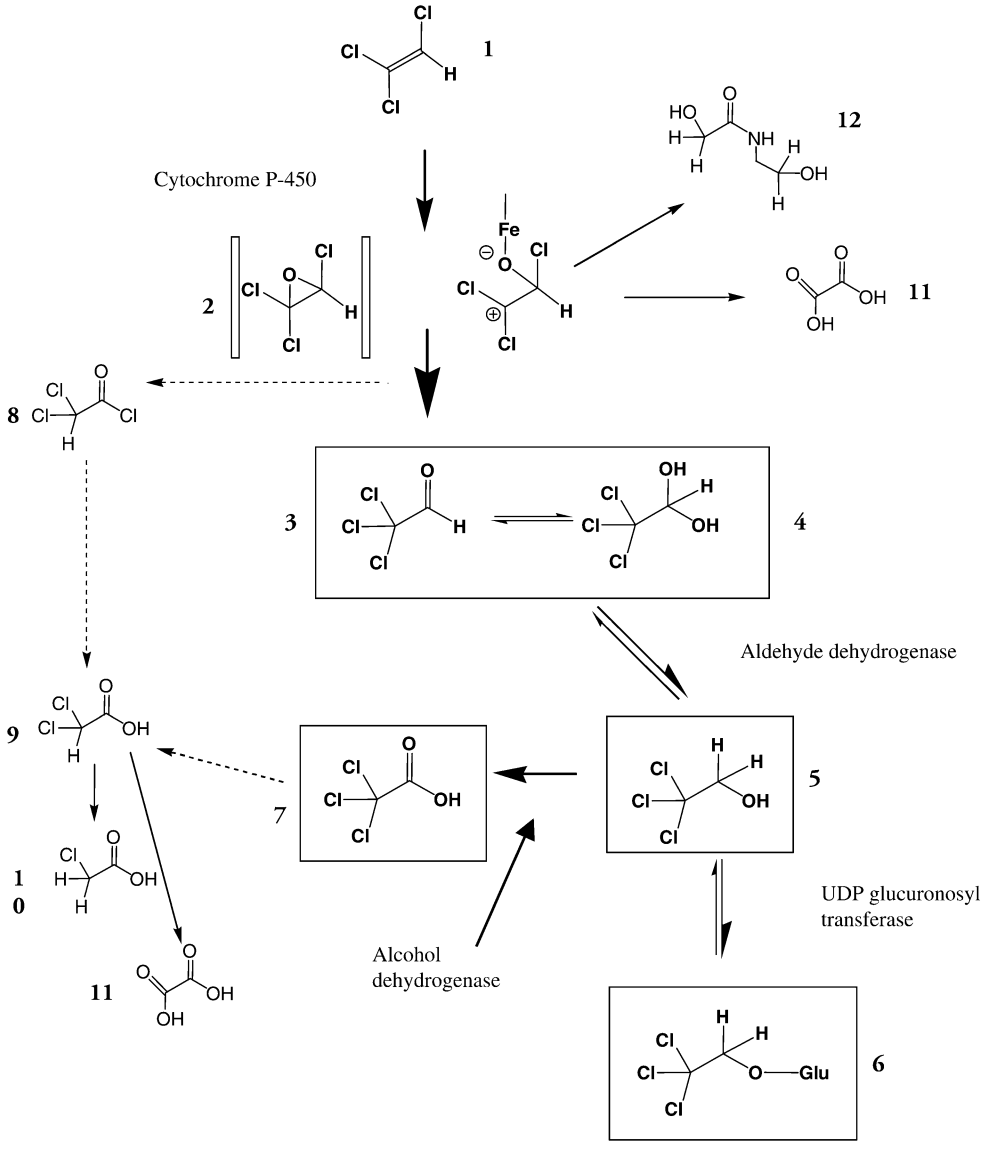

FIG. 2. Cytochrome P-450 dependent bioactivation and metabolism of trichloroethylene. Trichloroethylene (1); Trichloroethylene oxide (2); chloral (3); chloral hydrate (4); Trichloroethanol (5); Trichloroethanol glucuronide (6); Trichloroacetic acid (7); Trichloroacetyl chloride (8); dichloroacetic acid (9); monochloroacetic acid (10); oxalic acid (11); N-hydroxyacetyl-aminoethanol (12).

detect NAcDCVC. Lash *et al.* (1999b) also examined the urine of volunteers exposed to 100 ppm TCE by inhalation for 4 h and did not detect any NAcDCVC (limit of detection 400 pmol/ml). In contrast, Lash and coworkers did however identify S-(1,2-dichlorovinyl) glutathione (DCVG) in the blood of volunteers exposed to 100 ppm TCE by inhalation. They reported peak blood levels of 46 nmol/ml in males 2 h after exposure and 13 nmol/ml in females 4 h after exposure. These values are high relative to the amounts reported to be formed *in vitro* with human liver (see later). These later findings need to be confirmed, but if correct imply that the DCVG formed is not excreted as the mercapturate, suggesting it may have undergone metabolic activation by renal cysteine conjugate β-lyase (β-lyase). Overall, these findings indicate that GSH-mediated metabolism is a minor pathway in humans exposed to TCE, probably <0.01% of the dose.

In conclusion, these studies in humans indicate that at least 60% and perhaps as high as 90% of the retained TCE is metabolized in the body, with the bulk of the metabolites excreted in the urine. The major route for metabolism of TCE is via oxidation to chloral hydrate with GSH conjugation being a minor route.

In Vivo *Studies in Rats and Mice*

In experimental animals, the metabolism of TCE is qualitatively similar to that of humans, but the use of radiolabelled TCE has enabled more accurate assessment of both routes

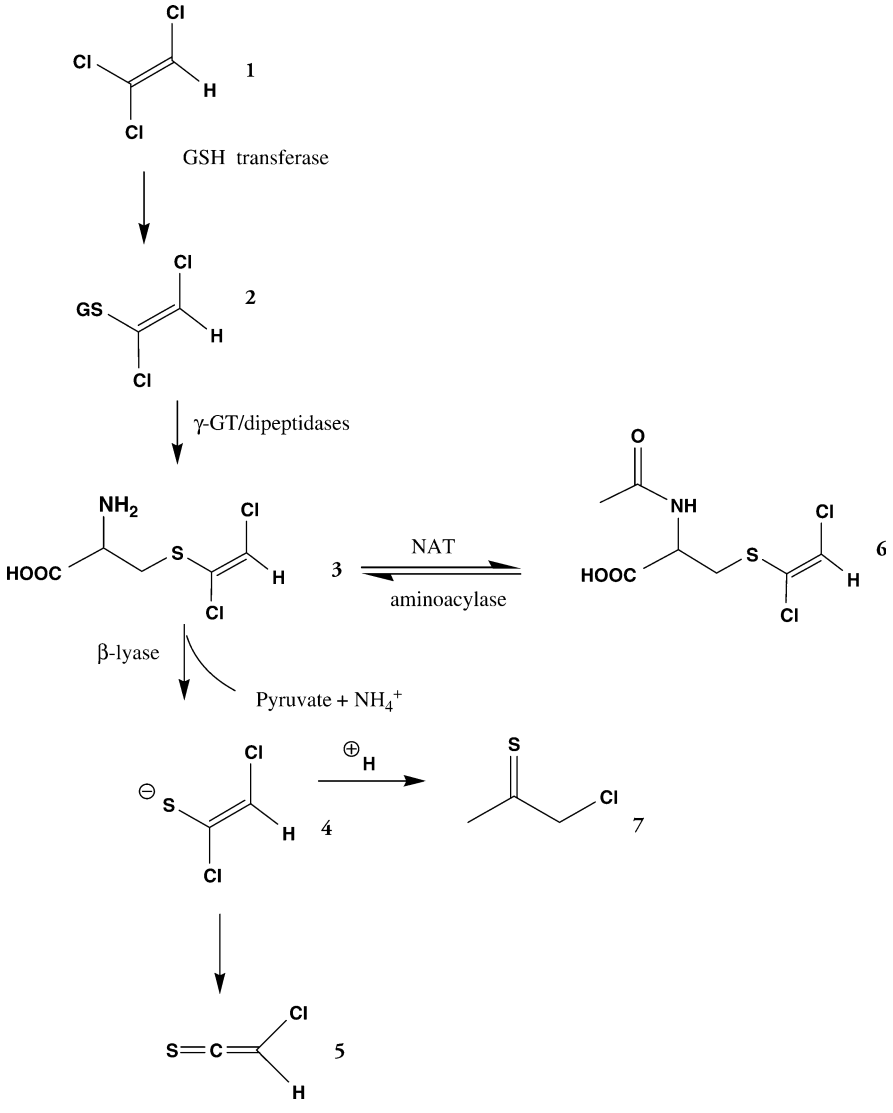

FIG. 3. Glutathione S-transferase and cysteine conjugate β-lyase-dependent bioactivation of trichloroethylene. Trichloroethylene (1); *S*-(1,2-dichlorovinyl)-glutathione (2); *S*-(1,2-dichlorovinyl)-L-cysteine (3); 1,2-dichloroethenethiolate (4); chlorothioketene (5); *S*-(1,2-dichlorovinyl)-N-acetyl-L-cysteine (6); chlorothionoacetyl chloride (7). GSH, glutathione; γ-GT, γ-glutamyltransferase; NAT, N-acetyltransferase.

and rates of excretion, and determination of dose-response relationships. Some of the salient points will now be discussed, for more detail the reader is referred to reviews and the original articles (Davidson and Beliles, 1991; Goeptar *et al.*, 1995; Lash *et al.*, 2000a).

Two oral studies in rats recovered between 90–95% of the dose of radiolabelled TCE in expired air and urine, suggesting almost complete absorption by this route. Prout *et al.* (1985) studied the pharmacokinetics of a single oral dose of TCE in the NCI strains of rat (Osborne-Mendel) and mouse (B6C3F1) over a dose range of 10–2000 mg/kg. In rats and mice given 10 mg/kg the routes and rates of excretion of TCE were very similar; 1–4% was exhaled unchanged, 10–12% was exhaled as CO_2, 8–17% was excreted in the feces, and the remainder was found in urine. After three days the animals were killed and about 5% of the dose was found in the carcass. At the higher doses, 500, 1000, and 2000 mg/kg, there was a marked change in the route of elimination of radioactivity in the rat, with a switch from urinary elimination to exhalation of unchanged TCE and traces of TCOH. This switch in the route of excretion was much less marked in the mouse where even at 2000 mg/kg only 14% of the dose was exhaled as TCE relative to 78% of that dose in the rat. Thus in the rat metabolism saturates at 1000 mg/kg, while in the mouse it is still linear at 2000 mg/kg (Fig. 4). Prout and coworkers also examined the blood levels of TCE and its major metabolites in rats and mice given 1000 mg/kg. In the mouse, peak blood concentrations of chloral and TCOH were seen about 1–3 h after dosing, and both chloral and TCOH were rapidly cleared with half-lives of 1–2 h. In contrast, TCA was maintained in the blood stream for up to

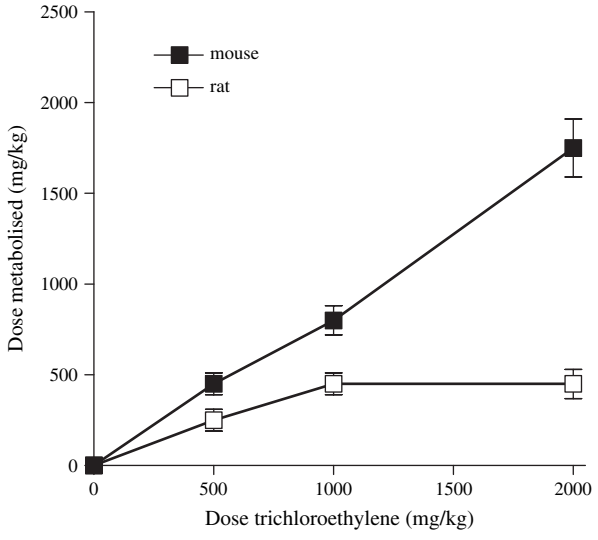

FIG. 4. Dose dependent metabolism of trichloroethylene in the rat and mouse. Data adapted from Prout *et al.* (1985).

30 h at a level about six fold higher than that seen in the rat. In the rat, blood concentrations of chloral, TCOH, and TCA were lower than in the mouse.

Examination of the metabolites excreted in urine and bile demonstrated the formation of TCOH-glucuronide, a small amount of free TCOH and TCA, trace quantities of mono and dichloroacetic acid, oxalic acid, and N-hydroxyacetyl-aminoethanol in urine (see Fig. 2 and Lash *et al.*, 2000a) Additional studies with trichloro[2-^{14}C]acetic acid given orally to rats at 75 mg/kg found 7% of the dose exhaled as $^{14}CO_2$ in 24 h, 59% excreted in urine and 1% in feces. In subsequent studies, rats and mice administered 10 mg/kg radiolabelled TCA exhaled 12% and 15% of the dose as $^{14}CO_2$ respectively, in the first 24 h. Urinary excretion of radioactivity after an intravenous dose was similar for rats and mice at 35% of the dose.

Thus there is a marked species difference in the rate of metabolism of TCE between rats and mice. The mouse metabolizes four times more TCE per kg body weight than the rat and consequently is exposed to a four-fold higher dose of TCE metabolites. This is due primarily to a higher metabolic rate in the mouse and extensive first-pass metabolism. The more extensive oxidative metabolism in the mouse may make this species more susceptible to tissue injury and carcinogenesis produced by TCE metabolites. Humans have a lower metabolic rate relative to rodents being closer to the rat than the mouse, suggesting that the kidneys of the rat and human would see less oxidative metabolites of TCE than the kidneys of mice. This lends support to the notion that oxidative metabolites of TCE may not be responsible for the renal carcinogenesis.

Dichloroacetic acid (DCA) is a minor metabolite of TCE which can be formed by rodent gastrointestinal microflora. It is unclear whether DCA is formed in tissues. In the presence of strong acids some TCA that is blood can undergo non-enzymatic conversion to DCA (Ketcha *et al.*, 1996). Thus the reports of the presence of DCA in rat, mouse, and dog tissues have been reassessed due to these artifacts. The position with regard to human tissue is not clear. Recently Delinsky *et al.* (2005) have reported studies using hydrophilic interaction liquid chromatography with tandem mass spectrometry to determine DCA concentrations in biological samples. They were able to detect DCA in blood and kidney 2 h after a single oral dose of 2 g/kg to rats. Thus, future work using this method should help resolve the amount of DCA in tissue to enable a clearer view on its role, if any, in TCE-induced nephrotoxicity.

Dekant and coworkers first reported the conjugation of TCE with GSH, identifying NAcDCVC, albeit in very small amounts, in the urine of rats given a single oral dose of 400 mg/kg TCE. A more detailed study confirmed the presence of a low concentration of NAcDCVC in rat urine and demonstrated the presence of DCVG in the bile of rats given TCE (Dekant *et al.*, 1990). Both regioisomers of the mercapturic acid (1,2-NAcDCVC and 2,2-NAcDCVC) were present in rat urine, with twice the amount of 1,2-NAcDCVC formed compared to the 2,2-isomer (see Goeptar *et al.*, 1995). Green and coworkers (1997) also confirmed the presence of NAcDCVC in rat urine following oral administration of TCE at either 500 or 2000 mg/kg per day for up to ten days. Amounts in the range of 1.5 to 33 μg/24 h urine sample were observed, increasing with both time and dose. However, NAcDCVC only accounted for 0.001 to 0.008% of the administered dose of TCE and was present at levels at least 1000-fold lower than those of TCA. Bernauer *et al.* (1996) also reported that NAcDCVC only accounted for about 0.001% of a 200mg/kg dose given to rats. Taken together these findings suggest that some TCE undergoes GSH conjugation *in vivo*, followed by processing to DCVC and then NAcDCVC which is excreted in urine, however this is a very minor route of metabolism.

DCVC is a substrate for the renal enzyme β-lyase which forms a reactive thiol moiety that is toxic to renal cells (see later). DCVC can also undergo metabolism by a flavin monooxygenase to produce DCVC sulphoxide (Krause *et al.*, 2003), which is also a substrate for renal β-lyase (Anderson and Schultze, 1965). However, recent studies have shown that inhibition of renal β-lyase with aminooxyacetic acid does not afford protection again the renal injury, leading the authors to suggest that the sulphoxide metabolite is reactive in it own right (Lash *et al.*, 1994, 2003). Quantitative data on the formation of the sulphoxide is not available but it is likely to be a minor route.

In Vitro *Metabolism Studies Using Human Tissue*

All of the pathways of metabolism of TCE detected *in vivo* have also been observed *in vitro* using either cells in culture, tissue slices, whole tissue homogenates, or subcellular fractions. The findings support the concept that oxidative

metabolism is quantitatively the major pathway and demonstrate that liver is the major organ responsible. Conjugation of TCE with GSH can be detected, but this is a minor route.

The first step in the oxidation pathway is catalyzed by cytochromes P450, and leads to the formation of chloral which readily equilibrates with chloral hydrate, via chlorine migration. Human liver microsomes readily metabolize TCE to chloral hydrate and TCOH. CYP2E1 appears to be the major isoform responsible, although other isoforms such as CYP2C11/8, CYP1A1/2, and CYP2B1/2 can also metabolize TCE (see Lash *et al.*, 2000a). There is little or no CYP2E1 in human kidney, hence studies with human renal microsomes failed to detect chloral hydrate formation in three out of four samples, and in the fourth only a very small amount was observed at the highest substrate concentration (Cummings and Lash, 2000). Human kidney does, however, contain the enzymes alcohol dehydrogenase and aldehyde dehydrogenase which can metabolize chloral hydrate to TCOH and TCA (Lock *et al.*, 2005). Thus any chloral hydrate present in the blood stream and delivered to the kidney would undergo metabolism, although the liver plays the major role in the formation of these metabolites. Thus, exposure of the kidneys to oxidative metabolites of TCE is largely due to extra-renal metabolism. This contrasts with rodent kidney that possess CYP2E1 and can metabolize TCE to TCOH (see next section).

There has been considerable focus on the GSH conjugation pathway of TCE, although few studies have used human renal tissue. Green and coworkers (1997) reported rates of formation of DCVG of 0.2 pmol/min/mg protein (range 0.05 to 0.38) in four different human liver cytosol fractions following incubation with 1.9 mM TCE and 5 mM GSH, and using radiolabelled TCE and HPLC separation. In contrast, Lash *et al.* (1999a) reported rates of metabolism of 2.8 nmol/min/mg protein (range 1.74–4.18) in cytosol fractions from twenty human livers following incubation with 2 mM TCE and 5 mM GSH, and using a chemical derivatization method and HPLC. These latter values are >30,000 times higher than those reported by Green and coworkers.

Studies of DCVG formation either *in vivo* or *in vitro* are difficult due to the low amount formed and interference by impurities in TCE. Green *et al.* (1997) found that samples of [^{14}C] TCE contained trace amounts of [^{14}C] dichloroacetylene which reacts rapidly with GSH to form DCVG. Green and coworkers cleaned their radiolabelled TCE with GSH followed by vacuum distillation but were still able to detect background levels of DCVG in incubations containing no tissue. Thus caution is needed in interpreting some of the findings reported in this area. TCE is not available with a purity of >99% and the consequence of interference from even this low level of impurities is considerable when the metabolic conversion represents <0.01% of the administered material. Lash and coworkers (1999a) discuss possible reasons for their much higher levels of DCVG, but currently the precise basis for the discrepancy is not clear.

Human liver microsomes also possess a GSH transferase that is able to conjugate TCE at a rate similar to that found in the cytosol. Human renal microsomes and cytosol are also able to form DCVG but at a rate approximately 10% of that of the liver. Thus, human liver and kidney both have the ability to form DCVG *in vitro*.

The next step in the processing of DCVG is cleavage of the glutamate and glycine residues to form DCVC. Human kidney possesses abundant γ-glutamyltransferase and cysteinyl glycinase and thus is readily able to form DCVC. DCVC may then be metabolized via the enzyme β-lyase to form a reactive thiol moiety, pyruvate and ammonia. This enzyme, which is localized in both the cytosol and mitochondria, has been isolated from human kidney and studied in considerable detail (see review by Cooper *et al.*, 2002). In general, β-lyase activity with DCVC is much lower in human kidney compared to rat kidney (Green *et al.*, 1997). DCVC may also undergo metabolism to NAcDCVC by a competing pathway catalyzed by N-acetyltransferase. Kinetic studies on the relative clearance of DCVC by these two pathways have shown that the flux through the N-acetyltransferase pathway is two orders of magnitude higher than via the β-lyase pathway in rodents, and about 27-fold higher in humans. Human kidney can also metabolize DCVC via flavin monooxygenase 3 to form DCVC sulphoxide (Krause *et al.*, 2003). The amount of this enzyme in human kidney has been determined in 26 samples and showed a 5–6-fold variation, however the authors did not report the activity with DCVC as substrate therefore it is difficult to estimate the likely contribution of this pathway.

Overall, these *in vitro* metabolism studies show that human renal tissue is able to form DCVG which can be further processed to DCVC; however, the flux via this pathway appears to be very small.

In Vitro *Metabolism Studies Using Animal Tissue*

There have been numerous *in vitro* studies on the metabolism of TCE in rat and mouse tissues; only key points relevant to the above discussion will be presented, for additional details see reviews by Goeptar *et al.* (1995) and Lash *et al.* (2000a).

TCE can undergo metabolism by cytochrome P450 in rat, but not human, kidney to form chloral hydrate (Cummings *et al.*, 2001) and via glutathione S-transferase to form DCVG (Cummings *et al.*, 2000; Green *et al.*, 1997). Rodent kidneys contain high concentrations of the enzymes that process GSH conjugates including β-lyase. The metabolic clearance of DCVC (V_{max}/K_m) through the β-lyase pathway is about 10-fold greater in rat kidney than in human kidney (Green *et al.*, 1997). As discussed earlier DCVC can also undergo N-acetylation and in rodents the metabolic clearance through this pathway is two orders greater than through the β-lyase pathway and about 60-fold greater in the rat versus the human kidney (Green *et al.*, 1997). Overall, these *in vitro* kinetic measurements, although small in number, tend to indicate that

DCVC is more likely to be diverted down the mercapturate pathway rather than the β-lyase pathway in both rodents and humans. However, all the rat and human studies indicate that very little mercapturate appears in urine after exposure to TCE, indicating metabolism via this pathway is either very small or that *in vivo* some DCVC has been metabolized via the β-lyase pathway.

Mode of Action Studies with TCE: Relevance to Renal Cancer

Recently the NTP/NCI database has been reviewed with regard to renal tubule carcinogens, which were divided into categories based on mechanistic information (Lock and Hard, 2004). TCE will now be discussed in detail with reference to these categories.

Category 1: Direct Interaction of the Parent Compound or a Metabolite with Renal DNA

Category 1 carcinogens induce renal tumors through direct interaction of the parent compound or a metabolite with renal DNA. Genotoxic chemicals normally produce tumors in mice as well as rats, with both sexes being susceptible. The tumor incidence is often high, with a short latency period and, with the most potent direct acting carcinogens, metastases to other organs are sometimes seen. Renal tumors produced by these genotoxic chemicals are associated with a clear background of preneoplastic lesions (atypical hyperplasia), which is not linked to the presence of chronic progressive nephropathy (CPN).

Exposure to TCE via either inhalation or oral administration produced a low incidence of renal tubule tumors in three out of six bioassays conducted in male and female rats (Tables 1 and 2). The incidence of renal tumors was not consistently dose related the tumors were only detected after two years or at the death of the rats, and only a single metastasis was reported (Maltoni *et al.*, 1988). Five bioassays have been conducted with TCE in male (Table 3) and female mice; no increase in the incidence of renal tubule tumors was observed in either sex, nor was there any obvious increase in renal tubule hyperplasia. Thus there is no basis for placing TCE in category 1.

This is consistent with the following data:

1. Genetic toxicology studies with TCE have shown it to be non-genotoxic in various strains of *S. typhimurium* in the presence or absence of a rat liver metabolic activation system (NTP TR 273). The sister chromatid exchange assay, however, was considered positive (Bruning and Bolt, 2000).

2. Chloral hydrate, the major oxidative metabolite of TCE, has been evaluated in the standard battery of genotoxicity assays including genetic alterations in rodent germ cells. The findings reveal a number of positive and negative findings *in vivo* so it is difficult to make a clear judgment. However the concentration required to elicit a response *in vitro* is >0.5 mg/ml (Moore and Harrington-Brock, 2000), and it is unlikely that such a concentration would accumulate in the kidneys as chloral hydrate as it is readily converted to TCOH and TCA by renal alcohol and aldehyde dehydrogenases. TCOH was negative in the Ames test and although TCA has been found to be weakly genotoxic this may be due to its acidity. Studies with neutralized TCA found no evidence of chromosomal damage in human lymphocytes *in vitro* or in the bone marrow micronucleus assay. DCA has weak mutagenic activity in a number of *in vitro* and *in vivo* assays (see Moore and Harrington-Brock, 2000), but it is questionable whether it is formed to a significant extent *in vivo* (see previous section: *In Vivo* Studies in Rats and Mice).

3. Neither TCE nor its metabolites appear to bind to DNA. Early studies reported covalent binding of ''reactive metabolites'' of [^{14}C]-labeled TCE to macromolecules in both the liver and kidney. Some of this can be accounted for by metabolic incorporation from the C1 pool (Eyre *et al.*, 1995b). No good evidence for formation of TCE-DNA adducts has been found in the liver. Although radiolabel from [^{14}C]-TCE has been shown to bind to cytosolic and microsomal proteins, the amount is very small. Metabolism of TCE to its oxide yields an electrophile that will react with lysine residues in proteins (Cai and Guengerich, 2001a) resulting in inactivation of certain enzymes. TCE-oxide will also react with other amino acids in proteins, but these are generally unstable with a collective half-life of about 1 h (Cai and Guengerich, 2001b). TCE-oxide will also react with 2′-deoxyguanosine *in vitro*, but not with the other three nucleosides found in DNA, the adduct was however transient having a half-life of about 30 min. Thus adducts formed via oxidation of TCE are likely to be very unstable and hence are unlikely to produce a stable enough mutation to produce a genotoxic response.

However, both DCVG and DCVC are capable of inducing point mutations, as indicated by their weak mutagenicity in several strains of *S. typhimurium* (see Moore and Harrington-Brock, 2000). The mutagenicity of DCVC was increased in the presence of rat renal S9 fraction, was dependent on bacterial β-lyase and ameloriated by aminooxyacetic acid, a β-lyase inhibitor. The 1,2-isomers of DCVC and NAcDCVC were shown to be stronger mutagens than the 2,2-isomers, which is consistent with the higher β-lyase activity with 1,2-DCVC as substrate. Little genotoxicity information is available on the conjugates in other recommended test systems. DCVC has been reported to induce a weak DNA repair response, but no micronuclei formation, in Syrian hamster embryo fibroblasts and to increase unscheduled DNA synthesis to a small extent in LLC-PK1 cells. DCVC has also been reported to produce DNA strand breaks in rabbit renal tubule DNA after doses of 5–100 mg/kg.

These studies suggest that DCVC is metabolized by β-lyase to generate a reactive electrophile that interacts with bacterial

DNA *in vitro*. However, this metabolite will also readily react with protein and GSH, and the amount likely to reach the DNA may well be very small in intact renal tubules. Furthermore, Volkel and Dekant (1998) studied the reactivity of the chlorothioketene formed from DCVC with DNA bases in both organic and aqueous solvents. They found evidence for a covalent interaction with the base cytosine but not adenine, guanine, or thymidine in organic solvent. In aqueous solution DNA adducts with chlorothioketene were not detected, demonstrating that under physiological conditions DNA adduct formation is not likely to arise. The studies in *S. typhimurium* demonstrate the potential for mutagenicity in systems with limited membrane barriers, reduced protective enzymes and higher than normal metabolic capacity. Since there is no definitive data on mammalian cells from either *in vitro* or *in vivo* studies, the genotoxic potential of DCVC and DCVG remains unclear.

Overall, at the present time, there is no good evidence to support a direct interaction of TCE or a metabolite with rat renal DNA, or to suggest that TCE is a direct acting renal carcinogen.

Category 2: Indirect DNA Reactivity Mediated by Oxidative Stress

Certain transition metals such as ferric and copper ions, as well as potassium bromate, can induce renal tubule tumors in both rats and mice by indirectly producing DNA damage through the production of reactive oxygen species (see Lock and Hard, 2004). 8-Hydroxy-2′-deoxyguanosine (8-OHdG) is most commonly used as a marker of oxidative damage produced by reactive species such as singlet oxygen or hydroxyl radical. The 8-OHdG adduct can cause misreading of the DNA sequence during replication associated with G to T base transversions and is therefore likely to be involved in mutagenesis and carcinogenesis. Oxidative injury is also usually associated with lipid peroxidation, and the aldehydic product 4-hydroxy-2-nonenal is frequently used as a marker of this response.

There have been a number of reports of oxidative stress following treatment with TCE or its metabolites, but few have examined rat kidney. An increase in ethane exhalation in male NMRI mice administered TCE has been reported, but only under hypoxic conditions. DCVC, when given to mice at nephrotoxic doses, also increased ethane exhalation and malondialdehyde production in the renal cortex, while prior treatment of the mice with aminooxyacetic acid to inhibit β-lyase reduced the lipid peroxidation and nephrotoxicity. In contrast, in the liver there is substantial *in vivo* evidence for TCE-induced lipid peroxidation. For instance, Channel *et al.* (1998) demonstrated a 2-fold increase in thiobarbituric acid-reactive substances and 8-OHdG in the livers of mice dosed with TCE. In F344 rats, a single dose of TCE resulted in an increase in thiobarbituric acid-reactive substances, and the 8-OHdG/dG ratio in the liver and an increased excretion of 8-OHdG and 8-epiprostaglandin F2alpha in the urine (Toraason *et al.*, 1999). Finally, Von Tungeln *et al.* (2002) demonstrated that neonatal mice dosed with CH or TCA had higher levels of the modified deoxyguanosine adducts M_1G (3-(2-deoxy-β-D-erthyro-pentofuranosyl-pyrimidol[1,2-α]purin-10(3H)-one) and 8-OHdG in the liver compared to controls.

Many studies have been conducted *in vitro* in isolated rat or rabbit renal tubule cells or in renal cell lines. Treatment with DCVC resulted in increases in lipid peroxidation and other indicators of oxidative stress, and these could be blocked by aminooxyacetic acid and a number of antioxidant scavengers. Although several of these antioxidants can reduce the extent of oxidative injury they do not prevent cell death. It seems likely that oxidative stress plays some role in DCVC-induced nephrotoxicity but is probably secondary to mitochondrial damage.

TCE-induced oxidative stress in the liver is almost certainly related to hepatic peroxisome proliferation following metabolism of TCE to TCA (Laughter *et al.*, 2004; Nakajima *et al.*, 2000). DCA can also cause peroxisome proliferation but little of this metabolite appears to be formed from TCE (see previous section: *In Vivo* Studies in Rats and Mice). The role of chemicals that are agonists of peroxisome proliferator activated receptor alpha (PPARα) has recently been reviewed, together with its link to carcinogenesis in mouse and rat liver, rat pancreas and testis, and its relevance to humans (Klaunig *et al.*, 2003 and references therein). The kidney possesses peroxisomes that can be induced by peroxisome proliferating agents; however the response in the rat kidney is typically 5–10-fold lower that that observed in the liver. Only one study has examined peroxisome proliferation in rat kidney after exposure to TCE or TCA. Goldsworthy and Popp (1987) determined cyanide-insensitive palmitoyl CoA oxidation as a measure of peroxisome proliferation, and reported an increase in rat and mouse liver and kidney. These responses were, however, small compared to those seen with WY-14643, the positive control. If the oxidative stress is due to peroxisome proliferation, then the relevance of these findings in rodents to humans is questionable (Klaunig *et al.*, 2003). There appears to be little or no relevance of peroxisome proliferation to renal carcinogenesis; several carcinogenicity studies with potent peroxisome proliferators have been conducted with no increase in renal tubule tumors reported.

In summary, there is limited evidence that TCE and TCA can cause a small increase in enzymes associated with peroxisome proliferation in the rat kidney but this is not considered sufficient to lead to tumor formation where a marked and sustained response is needed. Thus it is unlikely that TCE falls into this category of carcinogens.

Category 3: Conjugation with GSH and Subsequent Enzymatic Activation to a Reactive Species

A number of halogenated alkenes and aromatic compounds have been shown to undergo, to varying degrees, metabolism

via conjugation with GSH (Anders, 2004, 2005). The conjugates formed accumulate within the kidney where they undergo activation to a chemically reactive species which can acylate proteins leading to renal tubule necrosis and compensatory cell regeneration. Some of the GSH-derived metabolites also have the potential to interact with DNA. These chemicals tend to produce a relatively low incidence of renal tubule adenomas and carcinomas in 2-year bioassays, but it is not clear whether it is the induced cytotoxicity/regeneration caused by the reactive species or DNA interaction that is the driving force for renal tumor development.

As discussed earlier, TCE can undergo metabolism via a minor pathway of GSH conjugation to form DCVG which is then processed to DCVC. This cysteine conjugate can then either be a substrate for renal β-lyase, or may be N-acetylated to produce the mercapturate which is excreted in urine. DCVC can also undergo metabolism by a flavin monooxygenase to produce DCVC sulphoxide. There is considerable evidence suggesting that the cytotoxicity of TCE is mediated via conjugation with GSH and further processing of DCVG. The potential role of each of the above pathways in the toxicity of TCE will now be considered.

DCVC was discovered nearly fifty years ago when soybean meal was extracted with TCE and then used as cattle feed. Young calves developed aplastic anemia and renal injury; the causative agent was identified as DCVC and subsequent studies showed DCVC was nephrotoxic in a number of species of laboratory animals. Administration of synthetic DCVG to rats showed not only that it was nephrotoxic, but also that it was converted to DCVC before exerting its renal toxicity.

Both DCVG and DCVC are acutely toxic to human renal proximal tubules in culture, 500 μM of either compound causing >90% cytotoxicity following 48 h exposure (Lash *et al.*, 2001; McGoldrick *et al.,* 2003). Exposure of human renal tubule cells to DCVG at doses as low as 5 μM for 24 h causes no toxicity, however daily dosing for 3 days results in about 35% cytotoxicity and for 10 days about 55% cytotoxicity. Thus repeat exposure to very low doses of DCVG can also result in toxicity (Lock *et al.*, 2005). A role for the further metabolism of the GSH conjugate is supported by studies showing that both acivicin (an inhibitor of γ-glutamyltransferase) and aminooxyacetic acid (an inhibitor of β-lyase) can block its toxicity. The β-lyase catalysed β-elimination reaction results in the formation of 1,2-dichloroethenethiolate, pyruvate and ammonia. The thiolate may lose chloride to give chlorothioketene, or may tautomerize to give chlorothionoacyl chloride (Fig. 3). The formation of 1,2-dichloroethenethiolate and chlorothioketene has been demonstrated by Fourier-transform ion cyclotron resonance mass spectrometry. Both the thioketene and the thionoacyl chloride may contribute to the toxicity of DCVC, but the finding that the thioketene is highly unstable in aqueous environments favors a role for the thionoacyl chloride. 1,2-DCVC is a better substrate for β-lyase than its regioisomer 2,2-DCVC, and is more cytotoxic to rat renal tubule cells. β-Lyase is located in both the cytosol and mitochondria in rat kidney (see Cooper *et al.*, 2002) and human renal cortex. It seems likely that bioactivation of DCVC by the renal mitochondrial enzyme is probably responsible for cell injury; in cytosol the reactive metabolite will readily be scavenged by GSH and other cytosolic proteins and therefore is unlikely to reach its mitochondrial target. We cannot however exclude that inhibition of cytosolic protein targets may be a contributory factor in the cytotoxicity.

N-Acetylation of DCVC to the mercapturic acid is a detoxification pathway, however it represents only a very small percentage of the dose of TCE, with <0.01% appearing in the urine as a mercapturate (Bernauer *et al.*, 1996; Green *et al.*, 1997).

DCVC sulphoxide is nephrotoxic when administered to rats and partial protection against DCVC cytotoxicity can be afforded by prior treatment of human renal cells with methimazole, a flavin monooxygenase inhibitor. Thus this pathway of metabolism may also be relevant to renal injury following exposure to TCE.

In vivo, delivery of NAcDCVC or DCVC to the kidney, and the balance between the activation and detoxification pathways are critical factors in determining toxicity. Accumulation of DCVC and NAcDCVC into renal tubule cells occurs via zwitterion (brush border membrane) and organic anion transporters (OATs, basolateral membrane) respectively, in rat and mouse kidney. DCVC was reported to be a substrate for human OAT1 and rabbit Oat1 expressed in CHO and Cos cells (Groves *et al.*, 2003). Blocking DCVC renal accumulation with probenecid, an organic anion transport inhibitor, prevents the renal toxicity.

Once inside the renal tubule cell the relative rates of β-lyase bioactivation, and N-acetylation and deacetylation of NAcDCVC will influence the extent of cellular injury. Human renal β-lyase can metabolize DCVC and there is about a five-fold inter-individual variation in the cytosolic enzyme activity with DCVC (Green *et al.*, 1997) and S-[2-(fluoromethoxy)-1,3,3,3,-tetrafluoro-1-propenyl]-L-cysteine as substrates (Altuntas and Kharasch, 2002). The activity of the human mitochondrial enzyme with DCVC as substrate has not been reported. The N-acetyltransferases are known to be polymorphic, and with one haloalkene (S-[2-(fluoromethoxy)-1,3,3,3,-tetrafluoro-1-propenyl]-L-cysteine; FDVE-cysteine) both the human renal cytosolic and microsomal enzymes exhibit a 60–70 fold inter-individual variation in activity (Altuntas and Kharasch, 2002). N-acetyltransferase activity with DCVC as substrate has been measured in human renal cytosol; the kinetic data suggests a greater affinity for N-acetylation compared to β-lyase activity (Green *et al.*, 1997). There is about a 7-fold inter-individual variation in the activity of human renal cytosolic N-deacetylase with FDVE-cysteine as substrate (Altuntas and Kharasch, 2002). Importantly the balance of N-acetylation versus N-deacetylation appears to lie in favor of deacetylation (2–50-fold). Thus the greatest risk for renal injury would exist in those individuals with

a high N-deacetylation/N-acetylation ratio in combination with a high β-lyase/N-acetylation ratio. Working with 20 human kidney samples and FDVE-cysteine as substrate, Altuntas and Kharasch (2002) estimated that there could be up to a 50-fold individual variability in the rates of activation versus detoxification. Data with DCVC as substrate is not available, but the inter-individual variability is likely to be similar.

The mechanism of cell death following exposure of human proximal tubule cells to DCVC or DCVC sulphoxide appears to involve necrosis and apoptosis, the apoptosis appearing at earlier times and lower doses (Lash *et al.*, 2001, 2003, 2005). The reactive metabolite produced by bioactivation of DCVC by renal mitochondrial β-lyase binds to mitochondrial proteins and inhibits mitochondrial respiration. Associated with this impairment are changes in Ca^{2+} homeostasis and mitochondrial membrane potential collapse, leading to oxidative stress (see Anders, 2004; Cooper *et al.*, 2002; Lash *et al.*, 2000b). In agreement with this, several proteins that regulate apoptosis and stress responses are increased, in addition to proteins associated with cellular growth and differentiation at early times after exposure of human proximal tubule cells to DCVC (Lash *et al.*, 2005).

The activity of β-lyase in rat renal cytosol is about 10-fold higher than that in the mouse. However, the mouse is about 10-fold more sensitive to DCVC than the rat (Eyre *et al.*, 1995a; Green *et al.*, 1997). This difference between *in vitro* and *in vivo* may be due to the dose delivered to the kidney or to differences in renal processing between rats and mice. It appears not to be related to N-acetyltransferase activity, which is similar in both species (Green *et al.*, 1997). The activity of β-lyase in human renal cytosol is about 3 to 11-fold less than in rat renal cytosol, and overall β-lyase activation would appear to be a less important pathway leading to nephrotoxicity in humans than in the rat (Goeptar *et al.*, 1995; Green *et al.*, 1997; Lash *et al.*, 2000b).

DCVC is nephrotoxic to the rat and, to a greater extent, the mouse kidney (Eyre *et al.*, 1995a; Green *et al.*, 1997; Vaidya *et al.*, 2003a). However, DCVC has never been tested for carcinogenicity in either species in a standard bioassay. Thus, although conjugation of TCE with GSH and the further processing of the resultant conjugate are of paramount importance with regard to the nephrotoxicity of TCE, its relationship to carcinogenicity is still unclear. At this time many of the findings support putting TCE in this category.

Category 4: Direct Cytotoxicity and Sustained Tubule Cell Regeneration

Many chemicals that are not either directly or indirectly DNA reactive are able to induce renal tumors in rodents via pathways that are commonly referred to as ''epigenetic mechanisms.'' One of these pathways involves sustained stimulation of cell proliferation as a regenerative response to chemically induced renal cell injury. Non-DNA reactive chemicals that damage the rodent renal tubule, thereby stimulating repair and regenerative cell proliferation, can be grouped into two separate categories based on the pathophysiological nature of the response. In the first case (this category), the regenerative response is to direct cytotoxicity elicited by the chemical or its metabolite(s). In the second, the regeneration is in response to indirect cytotoxicity caused by cellular lysosomal overload, and this latter response will be discussed under category 5. Chemicals acting via either of these mechanisms tend to produce a low incidence of renal tubule tumors, with a long latency, the tumors most often being observed at scheduled termination of the two-year bioassays, and usually only the male rat is affected. Tumor metastasis with these chemicals is uncommon. A moderate level of karyomegaly in the region that is affected by the tubule cell injury/regeneration is a usual accompaniment at the chronic timepoints.

The precise molecular mechanism whereby continual cell turnover leads to emergence of neoplastic cells has not been defined. With enhanced cell proliferation an increased number of cells will be in S-phase of the cell cycle, which is a very vulnerable stage of DNA replication. Thus, an increase in the spontaneous error rate of DNA replication would result in a greater opportunity for the fixation of a mutational event. Prolonged cell proliferation might also facilitate the clonal expansion of already initiated renal tubule cells, which would not normally undergo division. Non-mutagenic DNA modification may also be facilitated during the time of increased cell turnover, possibly resulting in altered gene expression that could be relevant to the carcinogenic process.

Administration of TCE to rats or mice does not readily cause acute renal failure, and even subchronic exposure produces only mild renal injury (Goldsworthy *et al.*, 1988; Green *et al.*, 1997; Mensing *et al.*, 2002) as evidenced by a marked incidence of cytomegaly, and toxic nephrosis of the tubules in the inner cortex (NTP TR 2, 243, and 273). Renal tubule cell karyomegaly is seen in some kidneys after TCE exposure. These responses are distinguishable from CPN pathology, and the incidence of CPN was much lower in TCE treated rats than in the controls (NTP TR 2, 243, and 273), indicating that exacerbation of CPN is not involved.

Oral administration of TCE (1000 mg/kg/day for 10 days) resulted in no increase in renal tubule cell replication in either male or female F334 rats, which is consistent with the lack of obvious renal pathology over this time scale (Goldsworthy *et al.*, 1988). Similarly, three days after a single oral dose of TCE (1000 mg/kg) or 3 weeks after 1100 mg/kg/day for 5 days/week there was no evidence of renal tubule cell proliferation in male rats (Eyre *et al.*, 1995a and references therein). Eyre and coworkers did however report a 2–3-fold increase in cell turnover in the renal cortex and outer stripe of the outer medulla of male B6C3F1 mice. Others reported no change after three weeks exposure to 2400 mg/kg/day for 5 days/week. Surprisingly there appears to be no data on cell turnover in rats

or mice administered TCE sub-chronically or chronically, therefore it is not possible, at this time, to know if renal tubule repair and regeneration is increased under these conditions. It is likely that it is, and this may be an important contributory factor to the small increase in renal tubule tumors observed at two years in rats.

There is ample evidence that administration of DCVC to rats and mice causes acute renal failure followed by renal tubule repair and regeneration, an important response which enables the animals to survive (Eyre *et al.*, 1995a; Vaidya *et al.*, 2003a,b,c). Renal cell regeneration has also been observed in isolated renal cells in culture exposed to DCVC (Kays and Schnellmann, 1995; Lash *et al.*, 2001; Nony and Schnellman, 2003; Nowak *et al.*, 1999). However, the relevance of these findings to TCE induced renal carcinogenesis is uncertain due to the small amount of DCVC that appears to be formed *in vivo*.

Male F344 rats exposed to TCE excrete large quantities of formic acid in their urine following either oral or inhalation exposure for 28 days. The formate is not derived from TCE, suggesting that perturbation of formate metabolism by either the chemical or a metabolite occurs resulting in acidification of the urine. Subsequent studies have shown that TCOH and TCA, but not DCVC, also increase formate excretion in male F344 rats (Dow and Green, 2000). Chronic exposure of male F334 rats to TCOH in the drinking water (0.5 g/l or 1 g/l for 52 weeks) lead to a large increase in the urinary excretion of formic acid which reached a maximum at 12 weeks and then declined to reach a steady state level of about 15–20 mg/24 h/rat. Control rats treated in a similar way excrete <1 mg/24 h/rat of formic acid in their urine. Renal cell replication was measured at 29 and 40 weeks after exposure, there was a small increase at 29 weeks in the 1 g/l dose group, but not at the 0.5 g/l dose nor at later times at either dose. Thus there was some indication of increased cell turnover although it did not appear to be sustained. Renal tubule degeneration was observed at 40 weeks, but was no longer present at 52 weeks (Green *et al.*, 2003). Thus, at the present there is limited evidence to suggest that chronic renal injury may occur as the result of sustained exposure of the kidney to formic acid following administration of TCOH in male F344 rats.

Associated with the increase in formic acid there was an increase in urinary methylmalonic acid and plasma N-methyltetrahydrofolate, indicating a sustained acidosis, vitamin B_{12} deficiency and impaired folate metabolism respectively (Green *et al.*, 2003). Dietary supplementation with folic acid partially ameliorated the excretion of formic acid produced by both TCOH and TCA, leading the authors to suggest that these TCE metabolites may interact with vitamin B_{12}, probably by a chloromethyl radical mechanism, inhibiting both the methylmalonyl CoA and methionine salvage pathways (Fig. 5; Dow and Green, 2000). Inhibition of the latter pathway causes a secondary folate deficiency to develop due to the "methyl folate trap," and leads to a major impairment in formate metabolism and the excretion of large amounts of formate in the urine.

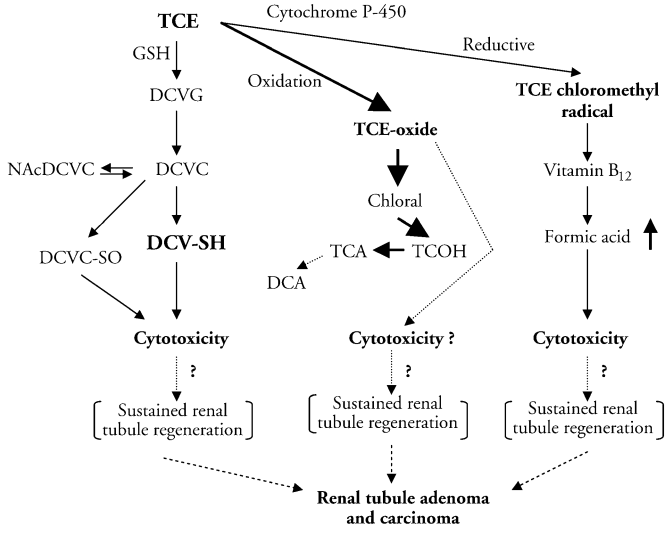

FIG. 5. Schematic for the metabolism of trichloroethylene and its relevance to renal tubule injury, renal tubule regeneration and renal carcinogenicity in the rat. Three possible routes for trichloroethylene bioactivation are shown, one metabolism via GSH conjugation leading to a reactive thiol that can acylate proteins and cause mutagenicity, however flux via this pathway is small and it is still unclear if sufficient DCVC is formed from trichloroethylene to cause chronic renal injury and repair. A second major route of metabolism is oxidation by cytochrome P-450 leading to chloral and its subsequent metabolites that are not nephrotoxic, there is some evidence for minor metabolism to the epoxide which can acylate proteins but this is considered unlikely to contribute to renal injury and repair. The third route involves metabolism to form a chloromethyl radical which can perturb vitamin B_{12} and folate metabolism, leading to excessive excretion of formic acid and the potential for chronic renal injury and repair. These routes of metabolism are not mutually exclusive and it may be a combination of these bioactivation mechanisms that can lead to renal injury. TCE, Trichloroethylene; GSH, glutathione; DCVG, *S*-(1,2-dichlorovinyl)glutathione; DCVC, *S*-(1,2-dichlorovinyl)-L-cysteine; DCV-SH, 1,2-dichloroethenethiolate; NAcDCVC, *S*-(1,2-dichlorovinyl)-N-acetyl-L-cysteine; DCVC-SO, *S*-(1,2-dichlorovinyl)-L-cysteine sulphoxide; TCE-oxide, Trichloroethylene oxide; TCOH, Trichloroethanol; TCA, Trichloroacetic acid, DCA, Dichloroacetic acid. The bold arrows represent a major route of metabolism while the dotted arrows are putative routes. The sustained renal tubule regeneration is placed in brackets to indicate only weak evidence to support this notion following TCE administration. It is clear that DCVC can case renal injury and repair in the rat and mouse; however, it is unclear if enough is produced from TCE to cause renal damage following either oral or inhalation exposure.

Formic acid and methylmalonic acid (markers of folate and vitamin B_{12} deficiency respectively) have been measured in workers occupationally exposed to TCE (mean exposure 32 ppm [0.5–252 ppm] with an average duration of exposure of 4.1 years). Small statistically significant increases in some markers of renal injury (urinary albumin and N-acetylglucosaminidase activity) were seen in the TCE exposed group compared to the control group. There was also a small statistically significant increase in formate excretion which showed a statistically significant correlation with exposure, as judged by urinary TCA excretion (Green *et al.*, 2004).

The relevance of the increased formate excretion in male F344 rats to renal tubule cell cytomegaly and toxic nephrosis following chronic exposure to TCE awaits further investigation, although it is known that sustained excretion of formate can lead to acidosis and chronic renal failure. Further studies in rats and mice of both sexes examining cell proliferation over an 18 month period is needed to understand the contribution of the toxic nephrosis to renal cancer. At the present time there is insufficient data, particularly with regard to sustained repair and regeneration, to put TCE into this category.

Category 5: Indirect Cytotoxicity and Sustained Tubule Cell Regeneration Associated with α_{2u}-globulin Accumulation

Conventional male, but not female, rats are physiologically proteinuric because of the high urinary excretion of a low molecular weight protein, alpha-2u-globulin (α_{2u}-globulin). This protein is synthesized in the liver of the male rat, freely filtered at the glomerulus into the tubule lumen, and about 40% excreted in the urine. The remainder is endocytosed by cells in the P2 segment of the proximal tubule, where it undergoes catabolism within cellular phagolysosomes (see Lehmann-McKeeman, 1997).

A number of chemicals and/or their metabolites have been shown to bind non-covalently to α_{2u}-globulin, interfere with its intrarenal lysosomal processing, and prolong its half-life. The result is accumulation of the protein-chemical complex as hyaline droplets within the P2 segment, followed by an increase in granular casts at the junction of the inner and outer stripes of the medulla, papillary mineralization and finally renal tubule hyperplasia. There is also an accelerated onset of cortical changes typical of CPN. The link between hyaline droplet nephropathy, renal tubule cell proliferation and renal tumors is well substantiated, and provides a mechanistic basis for the production of male-rat specific renal tumors by a non-genotoxic mechanism that has no relevance to humans (see Lehmann-McKeeman, 1997).

The tumor incidence in rats exposed to TCE is not male rat specific and there is no evidence of an increase in hyaline droplets or α_{2u}-globulin in the kidneys of male rats administered TCE (Goldsworthy *et al.*, 1988). Recently Green *et al.* (2003) reported an increase in hyaline droplets and immunostaining for α_{2u}-globulin in the kidneys of male rats exposed to TCOH in the drinking water for 52 weeks. However, the increase in α_{2u}-globulin was transient, being observed after 28 and 40, but not 52, weeks of exposure. Although there may be changes in α_{2u}-globulin in male rats it is clear that TCE does not induce tumors via this mechanism.

Category 6: Exacerbation of Spontaneous Chronic Progressive Nephropathy

CPN is a confounding factor, particularly in male rats, with respect to renal tubule tumor formation (Hard, 2002). CPN can be exacerbated by chemical exposure leading to an increase in the incidence and average severity of the spontaneous disease, and slight or marginal increases in renal tubule hyperplasia and/or adenoma. There is no evidence that chronic TCE exposure enhances CPN, on the contrary one NTP bioassay showed a decrease in the extent of CPN in the TCE treated animals compared to controls (NTP 243). Thus TCE does not fall into this category of carcinogens. It should also be noted that, as there is no strict counterpart of rat CPN in man, renal tumors arising as a result of chemical exacerbation of CPN should be regarded as having no relevance for human hazard assessment.

Summary

TCE administration by inhalation or gavage to male and female rats produces a small increase in renal tubule tumors, both adenoma and carcinoma. The carcinomas are more significant as none were seen in the controls in the six studies reported in seven different strains of rat. Three of the bioassays where TCE was given by gavage have been considered inadequate studies of carcinogenic potential due to early deaths and technical issues; one study used a carcinogenic stabilizing agent. However, the consistent picture over all the studies, conducted in four different countries, is a small increase in renal tubule carcinoma in rats exposed to TCE. In contrast, in mice of either sex there was no obvious increase in renal tubule tumors. It is important to note that TCE produces chronic renal tubule injury in the kidneys of both sexes of rats and mice; while only in the rat does it progress in a small number of animals to tumors. In humans, extensive epidemiology studies have been conducted and the overall picture is one of weak evidence linking renal tubule tumors in humans to TCE and other halogenated solvent exposure. A population of workers exposed to ''high'' concentrations of TCE several decades ago in Arnsberg, Germany has shown a high incidence of renal tubule tumors, but this work has been challenged. Recent studies with this group of people have focused on looking for mutations of the von Hippel-Lindau (VHL) tumor suppressor gene. Some mutations in the VHL gene have been observed; however, the same issues as raised with the earlier findings are relevant to these studies plus additional technical problems with regard to DNA quality and quantity (Brauch *et al.*, 2004). Further work on this latter approach is needed before any conclusions can be drawn as mutations in the VHL gene are a common finding in human renal tubule carcinoma.

The mechanism of the renal tubule injury in experimental animals is not clear. The injury does not occur acutely, only being observed after many weeks/months of exposure, as mild renal tubule damage associated with a high incidence of cytomegaly and in some cases karyomegaly at termination of the studies. Renal tubule injury occurs in nearly all the animals but only in rats does it appear to progress to adenoma and carcinoma. The renal injury in the mouse does not seem to

resemble that seen after chronic exposure to DCVC (Jaffe *et al.*, 1984), nor is it clear if it is related to chronic exposure to formic acid. Future studies need to establish the basis for the chronic renal injury and whether a low and sustained chronic renal tubule repair is occurring to support a case for classification in category 4. TCE undergoes extensive oxidative metabolism to chloral hydrate and then TCOH and TCA. Metabolism via this pathway can afford an electrophile that will react with renal proteins but there is no evidence to suggest stable adducts with DNA (Fig. 5). Recent studies have suggested that a chloromethyl radical formed from TCE, TCOH and TCA can perturb vitamin B_{12} and folate metabolism in rats and this may be a contributory factor to the renal injury observed on a chronic basis (Fig. 5). Further studies are required to explore the relationship between perturbation of this pathway and its effect on chronic renal tubule cell regeneration and repair in both sexes of rats and mice. A minor pathway of metabolism involves GSH conjugation; this pathway affords DCVC which is a known nephrotoxicant in experimental animals. DCVC undergoes bioactivation by β-lyase forming an electrophile that can react with proteins and DNA and produce a mutagenic response *in vitro* in bacterial systems (Fig. 5). The quantitative aspects of this pathway are important with regard to the balance between activation to form the electrophile versus detoxification to form the mercapturic acid, as are the rates of these reactions in animals and humans. Current information indicates that β-lyase activity is lower in human kidney than in rat kidney, however the extent of inter-individual variations in enzyme activity in the human population is currently not known.

We have examined the published data on TCE and its metabolites and find no evidence for a stable DNA adduct to classify TCE as a direct acting genotoxin (category 1) nor evidence to support an indirect affect on DNA via oxidative stress (category 2). Likewise we could find no evidence to classify TCE as acting by perturbation of α_{2u}-globulin processing in the kidney (category 5) nor via enhancement of chronic progressive nephropathy (category 6), both mechanisms having no relevance to humans. At this time there is insufficient evidence to place TCE in a category of direct cytotoxicity followed by sustained regeneration (category 4), although intuitively this may be the case. Whether this is due to perturbation of folate metabolism or via a reactive thiol mediated toxicity remains to be determined as does the extent of any chronically induced renal tubule repair and regeneration. Thus we have placed TCE in a group of chemicals that undergo conjugation with GSH followed by further metabolism in the kidney, to afford a reactive electrophile (category 3). Although DCVC has been shown to be mutagenic *in vitro*, there is no good evidence in the whole animal to show that this metabolite is sufficiently stable to enter the nucleus and react with DNA. Thus, at this time the weight of evidence points to a mechanism of toxicity via formation of a GSH conjugate of TCE. The implications for human risk assessment by placing TCE in category 3 is that it should not automatically be judged by linear default methods, since there is some evidence for cytotoxicity/regeneration a bench mark methodology could be used.

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