Acetylene, a mammalian metabolite of 1,1,1-trichloroethane*

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1,1,1-Trichloroethane (TCE) is a widely used industrial solvent of low acute toxicity. It is slowly oxidized to trichloroethanol and trichloroacetic acid by cytochrome *P*-450-dependent mono-oxygenases. Increased inhalative uptake by rats under hypoxia and spin-trapping experiments indicate that TCE is also reductively metabolized to a radical intermediate. Acetylene is formed as a metabolite, suggesting transfer of an additional electron to form the corresponding carbene. Hypoxia and induction of mixed-function mono-oxygenases accelerate the formation of acetylene. Experiments performed *in vitro* with rat liver microsomal fractions yield analogous results.

INTRODUCTION

1,1,1-Trichloroethane (TCE), also known as methyl chloroform, is widely used as an industrial solvent for degreasing and dry cleaning (Aviado *et al.*, 1976; IARC, 1979); it is a ubiquitous water and air pollutant (Westerick *et al.*, 1984; Frank *et al.*, 1991). TCE is slowly metabolized to trichloroethanol by hepatic cytochrome *P*-450-dependent mono-oxygenases and is further oxidized to trichloroacetic acid (Hake *et al.*, 1960; Aviado *et al.*, 1976; Kawai *et al.*, 1991). Mammalian toxicity of TCE is low, but long exposure leads to loss of cytochrome *P*-450 chromophore, to a similar extent to that produced by the acutely toxic tetrachloromethane (Vainio *et al.*, 1976; Savolainen *et al.*, 1977; Dürk *et al.*, 1987).

Inhalation experiments with male Sprague–Dawley rats in a closed all-glass exposure chamber (Frank & Dürk, 1983) were performed to determine the rate of TCE uptake and the possibility of lipid peroxidation. Inhalative uptake was strongly accelerated during hypoxia, indicative of reductive metabolism (Dürk & Frank, 1984). Spin-trapping experiments with α -phenyl *N*-t-butyl nitron proved that radical intermediates were formed. Alkanes were also monitored (Dumelin & Tappel, 1978; Frank *et al.*, 1980) for assessment of lipid peroxidation: when rats were exposed to TCE, ethane exhalation was increased. In addition, acetylene was exhaled which was identified by gas chromatography, mass spectrometry and copper acetylide formation. Microsomal experiments with [¹³C]TCE indicated that acetylene is a metabolite of the chlorocarbon.

EXPERIMENTAL

Experiments *in vivo*

Exposure of animals. Male Sprague–Dawley rats were purchased at a weight of 160 g and maintained for a week on standard lab chow diet (Altromin), with free access to tap water. For induction of hepatic mono-oxygenases, four rats were pretreated with phenobarbital (Remmer *et al.*, 1967). The animals were exposed to freshly distilled TCE (Merck, Darmstadt, Germany) at an initial vapour concentration of 2000 p.p.m. in an exposure chamber with recirculating atmosphere; for gas-chromatographic determination of TCE and exhaled hydrocarbons, air samples were withdrawn at intervals of 30 min (Frank & Dürk, 1983). In one group of animals, the oxygen

partial pressure was lowered to 8 kPa by replacement of consumed oxygen with nitrogen 4 h after the start of exposure. TCE uptake and hydrocarbon exhalation were monitored for a total period of 8 h. For peak assignment of the individual exhaled hydrocarbons, mass spectra were recorded as described below.

E.s.r. spectroscopy. For spin-trap experiments (Poyer *et al.*, 1987), each of the animals received orally an emulsion of 25 mg of α -phenyl *N*-t-butyl nitron in 1 ml of corn oil and 1 ml of potassium phosphate buffer (50 mmol/l), pH 7.4. In experiments with the mono-oxygenase inhibitor metyrapone, the drug was administered intraperitoneally (250 mg/kg body weight) 1 h before exposure to the solvent at an initial concentration of 5000 p.p.m. After 2 h, the rats were decapitated and exsanguinated. The livers were homogenized and extracted with chloroform/methanol as described by Folch *et al.* (1957); for e.s.r. examination the chloroform extract was concentrated *in vacuo* to a volume of 1 ml. Spectra were recorded on an e.s.r. instrument (model Varian E-9) at the following settings: time constant 10 s; scan range 10 mT; modulation amplitude 0.1 mT; field set 323 mT; scan time 30 min; modulation frequency 100 kHz; microwave power 25 mW; microwave frequency 9.102 GHz; gain 2.5×10^4 and 5×10^5 (see Fig. 3).

Experiments *in vitro*

Preparation of [¹³C]TCE. [¹³C]TCE was prepared by Grignard synthesis similar to the method published by Vaillieras (1967) for unlabelled halocarbons. Magnesium shavings, (0.37 g; 15 mmol) in 10 ml of dry tetrahydrofuran and 1.4 g (17.8 mmol) of isopropyl chloride in 10 ml of dry tetrahydrofuran were used to prepare isopropyl magnesium chloride. The latter was converted into trichloromethyl magnesium chloride by reaction with 1.8 g (11.7 mmol) of tetrachloromethane. Finally, 2.7 g (18.8 mmol) of iodo[¹³C]methane was added. For isolation of the product, the reaction mixture was diluted with 200 ml of water and extracted six times with 5 ml of pentane. The combined extracts were washed three times with 30 ml of water; for removal of residual tetrahydrofuran and iodine, the solution was passed through a column (45 cm $\times$ 1.6 cm) of (from top to bottom) 25 ml of sodium thiosulphate/silica gel (1:9, v/v), 10 ml of silica gel/H₂SO₄ (27:23, v/v), 10 ml of silica gel and a 1 cm layer of sodium sulphate. [¹³C]TCE was eluted with pentane. The fractions were checked by gas chromatography, and those containing [¹³C]TCE

Abbreviation used: TCE, 1,1,1-trichloroethane.

* Dedicated to E. Bayer on the occasion of his 65th birthday.

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were collected. The product was further purified by distillation over a 20 cm distillation column. Traces of iodine were removed with a few crystals of sodium thiosulphate. The chemical purity was ascertained by gas chromatography and the isotopic purity by gas chromatography/mass spectrometry. The yield was 60%, the isotopic purity 99%.

Microsomal incubations. Microsomal fractions were prepared from phenobarbital-treated animals by a standard procedure (Remmer *et al.*, 1967). They were diluted to a final concentration of 2 mg of microsomal protein/ml with 20 ml of buffer (50 mM-Tris, 120 mM-KCl, 5 mM-MgCl₂) adjusted to pH 7.4 with HCl. The incubation was performed in a three-necked round-bottomed flask with stopper, septum adaptor and gas inlet tube. NADP⁺ (1 mM), sodium isocitrate (8 mM) and 50 µg of isocitrate dehydrogenase/ml were added. The flask was flushed with argon, and 10 µmol of [¹³C]TCE were added directly into the suspension with a 5 µl syringe; the mixture was gently stirred with a magnetic stirrer at 37 °C. Control incubations were performed in the absence of either TCE or NADP⁺, and flushing the flask with CO/O₂ (19:1, v/v) instead of argon.

Gas chromatography/mass spectrometry of volatile metabolites. Head-space samples (5 ml) were withdrawn with a gas-tight syringe 10, 60 and 90 min after the experiment had been started. Acetylene was separated from ethane and ethene by gas chromatography on a fused-silica GSQ PLOT-column (30 m × 0.53 mm; J & W, Folsom, CA, U.S.A.), coupled to a quadrupole mass spectrometer (Finnigan 4021) with a silanized fused-silica capillary (70 cm × 0.1 mm) as interface (J & W). The samples were injected in the splitless mode, while the gas chromatography oven was cooled to -60 °C with liquid nitrogen. At 3 min after the injection, the oven temperature was raised and maintained at 60 °C. The mass spectrometer was operated with an electron energy of 70 eV, ion source temperature 250 °C, cathode current 200 µA and SEV acceleration voltage 2 kV. The ions *m/z* 24, 25, 26 (C₂H₂) and 27 (¹³CCH₂) were monitored at a rate of 2 scans/s. Calibration was performed with a synthetic commercial gas standard (Messer-Griesheim, Düsseldorf, Germany) containing 1.74 p.p.m. of acetylene in nitrogen.

RESULTS

Male Sprague-Dawley rats were exposed to TCE by inhalation at an initial concentration of 2000 p.p.m. Equilibrium was

Fig. 1. Inhalative uptake of TCE (■) by a rat exposed at an initial concentration of 2000 p.p.m. under normoxia and 5 h later under hypoxia

Ethane (+), acetylene (△) and pentane (◇) are the main volatile hydrocarbons exhaled by the animal. The arrow indicates the time at which the oxygen partial pressure was lowered to 8 kPa.

reached at a concentration of 300 p.p.m. within 2.5 h. Later on, zero-order elimination of TCE from the recirculating atmosphere was observed (Fig. 1). TCE uptake in a normoxic atmosphere was 17 µmol/kg per h for both phenobarbital-treated and untreated animals (Table 1). On decreasing the oxygen partial pressure to 8 kPa, uptake of TCE was accelerated to 30 µmol/kg per h. Exhalation of alkanes was monitored as an indication of lipid peroxidation; ethane exhalation was increased 3–5-fold (Table 1), but pentane exhalation was not increased above control rates. A peak with a gas-chromatographic retention time not observed previously (Riely *et al.*, 1974; Frank *et al.*, 1980; Dürk & Frank, 1984) was now present (Fig. 2); retention time, absorption by aqueous ammonia and the mass spectrum of the peak proved it to be acetylene. The exhalation rate of the latter was 5 nmol/kg per h in phenobarbital-treated and untreated animals, but during hypoxia it was increased sixfold in phenobarbital-treated animals (Table 1); no such increase was observed for untreated animals. In control experiments without TCE no acetylene exhalation was found.

Table 1. TCE uptake and acetylene and ethane exhalation in male rats under normal or decreased oxygen partial pressure

The data are means ± s.d. (*n* = 4). Pentane was not increased above control values (range: 0–2 nmol/kg per h).

O ₂ (kPa)	Pheno- barbital pretreat- ment	TCE	<i>v</i> _{TCE} (µmol/kg per h)	<i>v</i> _{acetylene} (nmol/kg per h)	<i>v</i> _{ethane} (nmol/kg per h)
8/20	+/-	-	-	0/0	2 ± 1
20	-	+	18 ± 5	5 ± 2	4 ± 2
8	-	+	30 ± 7	5 ± 3	10 ± 1
20	+	+	17 ± 7	6 ± 3	4 ± 2
8	+	+	30 ± 6	29 ± 5	11 ± 1

Fig. 2. Gas-chromatographic separation of the volatile hydrocarbons in an air sample from an exposure experiment of a rat to TCE

The main peaks are methane/air (a), ethane (b), ethene (c), propane (d), acetylene (e) and pentane (f).

Fig. 3. E.s.r. spectrum of a spin adduct extracted from the liver tissue of a rat pretreated with the spin-trapping α -phenyl *N*-t-butyl nitron and exposed to TCE by inhalation for 2 h (upper trace, gain: 2.5×10^4) and of a rat treated with metyrapone 1 h before exposure to TCE (lower trace, gain 5×10^5)

Table 2. Metabolism *in vitro* of TCE by microsomal fractions from phenobarbital-treated animals (20 ml, 2 mg of microsomal protein/ml): release of acetylene, ethane and pentane within the first 10 min

Results are based on four experiments.

Incubation mixture			Amount released (pmol)		
TCE (0.5 mM)	NADP (1 mM)	CO/O ₂ (19:1, v/v)	Acetylene	Ethane	Pentane
—	—	—	0	10	0
+	—	—	0	11	8
—	+	—	0	15	50
+	+	—	100	60	60
+	+	+	0	80	100

Fig. 4. Mass fragmentogram (m/z 27) of a sample of gas phase above a microsomal incubation mixture with $[^{13}\text{C}]\text{TCE}$

The conditions are described in the Experimental section. Mass spectra of acetylene exhaled by a rat exposed to unlabelled TCE (a) and acetylene from an experiment carried out *in vitro* with $[^{13}\text{C}]\text{TCE}$ (b) are shown.

In order to investigate whether radical intermediates are involved in the metabolism of TCE, some animals received the spin-trapping agent α -phenyl *N*-t-butyl nitron and were exposed to the xenobiotic as described before. The e.s.r. spectrum obtained

from an extract of hepatic lipids is shown in Fig. 3 (upper trace), supporting the involvement of a radical intermediate. Pretreatment with metyrapone, a cytochrome *P*-450 inhibitor, inhibited radical formation by more than 80 % (Fig. 3, lower trace); no evidence of radical formation was found in e.s.r. spectra from control animals.

Experiments *in vitro* with microsomal fractions from phenobarbital-pretreated animals with $[^{13}\text{C}]\text{TCE}$ under anaerobic conditions suggest that acetylene is a metabolite of the xenobiotic (Table 2). It was only formed in the complete incubation mixture; in the absence of the chlorocarbon or NADPH, and in the presence of CO/O₂ (19:1, v/v), no acetylene was found. Mass fragmentography of the ion m/z 27 (Fig. 4) and the mass spectrum of the acetylene peak demonstrated a shift of the molecular ion by one mass unit (Fig. 4, insert *b*) compared with unlabelled acetylene (Fig. 4, insert *a*). In ambient air samples, no acetylene was found.

DISCUSSION

TCE is virtually non-toxic, but prolonged exposure results in loss of the cytochrome *P*-450 chromophore (Vainio *et al.*, 1976; Savolainen *et al.*, 1977). Thus it seems to be a suicide substrate of cytochrome *P*-450. This may be due to metabolic formation of reactive intermediates via reductive metabolism. The acceleration of inhalative uptake by rats during hypoxia and the detection of a strong spin adduct suggest reductive metabolism to radical intermediates, in agreement with the findings of Salmon *et al.* (1981), who showed that in a microsomal system reductive dehalogenation takes place at a maximum rate of 200 pmol/min per mg of microsomal protein. A similar value has been reported by Thompson *et al.* (1984, 1985), who identified 1,1-dichloroethane as a metabolite.

Reductive metabolism of TCE may proceed further in the transfer of a second electron to form the respective carbene intermediate (Ahr *et al.*, 1980, 1982; Salmon *et al.*, 1981), although to a much smaller extent than the first electron transfer. Acetylene may arise from such a twofold reduction of TCE, as shown in Scheme 1. The yield of acetylene relative to TCE metabolized is only 0.1 %, for which there may be several reasons: owing to a low steady-state concentration of the primary radical, transfer of a second electron may be relatively slow; the resulting carbanion or carbene may bind to the porphyrin ring of cytochrome *P*-450 or other components of the microsomal membrane; acetylene is metabolized to ketene which may also covalently bind to nucleophilic sites in hepatic mono-oxygenases.

Scheme 1. Suggested pathway of reductive metabolism of TCE to acetylene

Alkynes are known suicide substrates of cytochrome *P*-450 (Ortiz de Montellano & Carreia, 1983; Hyman & Arp, 1988).

Attempts to identify vinyl chloride as an intermediate have been unsuccessful. Therefore it is likely that the intermediates in Scheme 1 do not escape the reactive site of the mono-oxygenase enzyme. After transfer of the second electron, the dichloroethyl carbanion seems to collapse under elimination of Cl^- and HCl all the way to acetylene.

The fact that acetylene exhalation increases during hypoxia in phenobarbital-induced animals but not in control animals suggests that two different isoenzymes are involved in reductive metabolism of TCE, i.e. a constitutive form with high affinity for the xenobiotic, and an inducible form which catalyses reductive metabolism of TCE only at low oxygen partial pressure. Phenobarbital induction accelerates destruction of cytochrome *P*-450 by TCE *in vitro* (Fiorucci *et al.*, 1988). Metyrapone inhibits the phenobarbital-inducible form of cytochrome *P*-450 (Luu-The *et al.*, 1980) suggesting that the same isoenzyme is involved in reductive metabolism of TCE as well as of tetrachloromethane (Noguchi *et al.*, 1982).

The metabolic formation of the radical intermediate may be one of the reasons for the moderate increase in ethane during TCE exposure under hypoxia. However, ethane is not a selective indicator of lipid peroxidation. Pentane, another alkane potentially arising from lipid peroxidation, is not increased; the latter is metabolized more rapidly (Frank *et al.*, 1980) than ethane, owing to its intrinsically higher reactivity (Frommer *et al.*, 1970) and its larger lipid/air partition coefficient. Moderate amounts of ethane and pentane may arise from sources other than lipid peroxidation, such as microbial metabolism in the gut.

The formation of a triple bond by reductive metabolism of mono-oxygenases has hitherto not been reported. The occurrence of alkynes in animal metabolism is a rare case; for example, the metabolic origin of histrionicotoxins in amphibia is not yet understood (Daly *et al.*, 1987). There is evidence that cytochrome *P*-450 isoenzymes (Ortiz de Montellano, 1989; Guengerich & MacDonald, 1990) also catalyse the oxidative desaturation of some substrates, such as sterol derivatives (Nagata *et al.*, 1986) or valproic acid (Rettie *et al.*, 1987).

Obviously, the metabolic formation of radicals may always be expected for polyhalogenated alkanes (van Dyke & Gandolfi, 1975; Bunyan *et al.*, 1966). However, this does not necessarily entail overt hepatotoxicity if the rate is slow. The suggestion of the formation of carbenes by a second electron transfer comes mainly from experiments carried out *in vitro* (Ahr *et al.*, 1980, 1982) but exhalation of acetylene proves that it also occurs *in vivo*, although only to less than 1%. Acetylene is known as a suicide substrate of microsomal mono-oxygenases (Ortiz de Montellano & Carreia, 1983), but it is relatively slowly oxidized and therefore non-toxic; once it was even suggested as an inhalation anaesthetic (correspondence of the Nobel Committee, 1926; B. Holmstedt, personal communication).

The present study suggests that the relative participation of the main pathway of oxidative metabolism of TCE versus reduction to reactive intermediates may have an influence on its toxicity. Under normal conditions favouring the oxidative pathway, TCE is largely non-toxic. It is conceivable that, on pathological restriction of the oxygen supply to the liver, an amplification of the reductive pathway may have an influence on the hepatotoxicity of polyhalogenated alkanes.

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