XICOLOGY AND APPLIED PHARMACOLOGY 103, 77–89 (1990)

Perchloroethylene-Induced Rat Kidney Tumors: An Investigation of the Mechanisms Involved and Their Relevance to Humans

TREVOR GREEN, JENNY ODUM, JOHN A. NASH, AND JOHN R. FOSTER

Imperial Chemical Industries plc, Central Toxicology Laboratory, Alderley Park, Macclesfield, Cheshire, SK10 4TJ, United Kingdom

Received July 17, 1989; accepted October 23, 1989

Perchloroethylene-Induced Rat Kidney Tumors: An Investigation of the Mechanisms Involved and Their Relevance to Humans. GREEN, T., ODUM, J., NASH, J. A., AND FOSTER, J. R. (1990). *Toxicol. Appl. Pharmacol.* **103**, 77–89. Lifetime exposure to perchloroethylene by inhalation has been shown to cause a low incidence of renal tumors in male rats. The mechanisms responsible for the induction of these tumors have been investigated following exposure of rats to perchloroethylene by oral gavage (1500 mg/kg for up to 42 days) or by inhalation (400 ppm for 28 days). Comparisons have been made between rats and mice *in vivo* and between rats, mice, and humans *in vitro*. **High doses of perchloroethylene given by gavage were shown to be toxic to the rat kidney, causing [illegible] marked accumulation of protein droplets (α-2u-globulin) was seen in the P_2 segment of the kidney proximal tubules. This response were not seen after [illegible] perchloroethylene for 28 days and hence may not be associated with the tumors seen at this dose level.** Protein droplet formation was seen after exposure to 1000 ppm perchloroethylene, suggesting that 400 ppm is below the threshold dose required to induce this response. Perchloroethylene has been shown to be metabolized by glutathione conjugation in the liver, resulting in the formation of a mutagenic cysteine conjugate which is activated by the kidney enzyme β-lyase. Levels of the mercapturic acid of perchloroethylene have been compared in rat and mouse urine. The enzyme kinetics of hepatic glutathione conjugation and renal β-lyase activation have been compared in rat, mouse, and human tissues *in vitro*. **[illegible] with the rat being the species susceptible to kidney tumors. [illegible] to contain β-lyase, glutathione conjugation of perchloroethylene [illegible] liver. Perchloroethylene-induced male rat kidney tumors may [illegible] protein droplet nephropathy, and genotoxicity from the β-lyase pathway. These [illegible] appear to have little relevance to humans.** © 1990 Academic Press, Inc.

Perchloroethylene (tetrachloroethylene) is a colorless, volatile liquid of low acute toxicity which is used extensively as a dry cleaning agent and industrial degreaser. Two lifetime animal carcinogenicity studies have been reported in which perchloroethylene was administered to rats and mice by oral gavage (NCI, 1977) and by inhalation (NTP, 1985). Both studies reported a significant increase in hepatocellular carcinoma in male and female $B_6C_3F_1$ mice and in the inhalational study a low incidence of renal adenoma and adenocarcinoma in male F344 rats. Reduced survival of Osborne–Mendel rats during the oral study prevented any conclusions being drawn from that experiment. An increased incidence of mononuclear cell leukemia was also seen in F344 rats in the inhalation study.

A mechanism has been proposed for the development of liver tumors in mice exposed to perchloroethylene which is consistent with the absence of liver tumors in rats (Odum *et al.*, 1988). Mononuclear cell leukemias occur at very high rates in control F344 rats, mak-

0041-008X/90 $3.00

ing interpretation of this finding difficult. The incidence of adenomas and adenocarcinomas in the kidneys of male F344 rats was considered not to be statistically significant. However, kidney adenomas are rarely seen in F344 rats and adenocarcinomas have not been seen in historical controls at the testing laboratory (NTP, 1985). **Thus, the low incidence of kidney tumors in rats may be of toxicological if not statistical significance.**

Several studies have sought an explanation for the kidney tumors seen in male rats exposed to perchloroethylene. Mouse liver tumors are believed to be a result of perchloroethylene-induced hepatic peroxisome proliferation, but earlier studies have shown only marginal increases in peroxisomes in the rat kidney (Goldsworthy and Popp, 1987; Odum *et al.*, 1988). Male rats given high doses of perchloroethylene by oral gavage have been found to accumulate the protein α-2u-globulin in renal proximal tubular cells and to exhibit all the features of protein droplet nephropathy (Green *et al.*, 1986, Goldsworthy *et al.*, 1988). The strong correlation between this epigenetic effect and male rat-specific kidney cancer has led to suggestions that this is the mechanism responsible (Goldsworthy *et al.*, 1988).

Perchloroethylene is metabolized primarily by oxidation to trichloroacetic acid (Yllner, 1961; Daniel, 1963; Dekant *et al.*, 1985). Evidence of a second metabolic pathway for perchloroethylene, which may be related to the development of kidney tumors, has also been reported (Dekant *et al.*, 1986; Odum and Green, 1987). The presence of a second pathway, which is believed to be analogous to that known for hexachlorobutadiene (Nash *et al.*, 1984), was suggested following the identification of *N*-acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine in urine from rats dosed with perchloroethylene. The cysteine conjugate of perchloroethylene is known to be activated by the renal enzyme β-lyase to a nephrotoxic intermediate, which has also been shown to be mutagenic in a modified Ames bacterial mutation assay (Green and Odum, 1985).

Although these studies suggest several possible mechanisms for the renal cancer observed after inhalational exposure to perchloroethylene, the conclusions are drawn from a limited number of experiments conducted in male rats given high oral doses of perchloroethylene. It is not known whether these mechanisms are consistent with the observed species differences nor has the relevance of these mechanisms to humans been determined. In addition the quantitative extrapolation of results from gavage studies is difficult when the most appropriate route of exposure for humans, and the one used in the animal bioassay, is by inhalation.

In the present study the various mechanisms of perchloroethylene-induced renal cancer have been investigated in rats and mice of both sexes. Comparisons have been made between animals dosed by oral gavage and exposed by inhalation, and finally, *in vitro* techniques have been used to compare the metabolic pathways of perchloroethylene in rats and mice with those in humans.

METHODS

Materials

1,1,2,2-Tetrachloroethylene (Analar grade, 99.9%) was obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.). Biochemicals were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.).

1,1,2,2,-Tetrachloro[1,2-^{14}C]ethylene, specific activity 55 mCi/mmol was obtained from Imperial Chemical Industries plc, Physics and Radioisotope Services (Billingham, Cleveland, U.K.). The material had a chemical and radiochemical purity of greater than 98% as determined by radio-gas chromatography. *S*-(1,2,2-Trichlorovinyl)-L-cysteine (TCVC), *N*-acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine (NAc-TCVC), and *S*-(-1,2,2-trichlorovinyl)glutathione (TCVG) were synthesized as described by Moore and Green (1988).

Animals and Dosing

Male and female Fischer 344 rats (160–190 g) and male and female $B_6C_3F_1$ mice (22–25 g) were supplied by Charles River (Margate, Kent, U.K.). The animals were housed in temperature-controlled rooms fitted with a 12-

hr lighting cycle. Feed (PCD diet, Special Diets Services Ltd., Witham, Essex, U.K.) and water were provided *ad libitum*.

Gavage studies. A group of ten male rats each received single doses of perchloroethylene (1500 mg/kg) in corn oil (10 ml/kg) by gavage daily for 42 days. A control group of ten male rats received corn oil (10 ml/kg) alone.

Inhalation studies. Rats and mice of both sexes, five animals per group, were exposed to 400 ppm perchloroethylene, 6 hr/day, for 28 consecutive days. Exposures were whole body in stainless-steel chambers (Doe and Tinston, 1981) having an internal volume approximately 3.4 m^3. The chambers were air conditioned to have a nominal temperature of 22°C and a relative humidity of 40–60%. The air flow through the chambers was 300 liters/min. Atmospheres were generated by passing vaporized perchloroethylene into the input air of the chamber. Controls were exposed to air alone.

In a further study male rats, three per group, were exposed to 10 or 100 ppm perchloroethylene for 6 hr, or to 1000 ppm 6 hr/day for 1, 5, or 10 days.

Toxicity Studies

All of the animals were killed by overexposure to halothane. In the gavage study the rats were killed 24 hr after the final dose. During this period urine was collected. Blood samples were collected by cardiac puncture at termination and kidneys removed for histopathological examination. Rats exposed to 400 ppm perchloroethylene by inhalation were killed 18 hr after the end of the final 6-hr exposure and the kidneys removed for histopathology. Body weights were recorded during the experiments and organ weights at termination.

Blood and urine samples from the gavage study were analyzed for biochemical markers of both liver and kidney damage. Urine samples were combined to give a sufficiently large sample for the biochemical assays and the subsequent metabolism studies. Plasma urea, alkaline phosphatase (ALP), alanine aminotransferase (ALT), and urinary glucose, protein, ALP, and *N*-acetyl-β-D-glucosaminidase (NAG) were determined as described by Nash *et al.* (1984). Urine samples were analyzed for α-2u-globulin by the method of Stonard *et al.* (1987).

Slices of kidney were fixed in 10% neutral buffered formol saline, dehydrated through an ascending ethanol series, and embedded in paraffin wax. Sections (5 μm) were cut and stained with hematoxylin and eosin for light microscopic examination. Analysis of kidney sections for hyaline droplet formation was performed on the hematoxylin/eosin-stained sections under UV light on a Reichert Polyvar photomicroscope. Tissues were also fixed in 3% glutaraldehyde in 0.1 M sodium phosphate buffer, dehydrated, and embedded in epoxy resin. Sections (1 μm) were cut and stained with 1% toluidine blue in 1% borax for light microscopy. Suitable areas were then selected from the proximal tubules of the kidney for electron microscopy. Ultrathin sections of these areas were stained with uranyl acetate and lead citrate and viewed and photographed in a JEOL JEM 100CX electron microscope.

Metabolism and Pharmacokinetics

Gavage studies. Radiolabeled perchloroethylene (10–40 μCi per rat) was incorporated into the doses given on Days 1, 17, and 42 of the gavage study. After dosing the animals were transferred to metabolism cages for the separate collection of urine and feces for 24 hr. On Day 42 of the experiment two rats were fitted with biliary cannulas immediately before the final dose. Bile was collected for 24 hr.

Inhalation studies. Urine was collected from rats and mice on Days 1, 7, and 14 of exposure to 400 ppm perchloroethylene. The animals were placed in metabolism cages for the 18-hr period between exposures. Rats exposed to 10 or 100 ppm perchloroethylene for 6 hr and to 1000 ppm 6 hr/day for 1, 5, or 10 days were placed in metabolism cages at the end of the last exposure period in each case. Urine was collected for 18 hr.

Analysis of metabolites in urine and bile. Bile from rats dosed with perchloroethylene by gavage was initially analyzed for the presence of *S*-(1,2,2-trichlorovinyl)glutathione by thin-layer chromatography alongside an authentic standard. The samples were analyzed on silica gel GF plates with a developing solvent of ethyl acetate: methanol:acetic acid:water (60:15:15:10, v/v).

Bile (6.5 ml) was also incubated with γ-glutamyltranspeptidase (GGT, 50 units) at 37°C for 3 hr. Protein was precipitated with ice-cold ethanol (13 ml) and removed by centrifugation. The supernatant was concentrated under reduced pressure and chromatographed on preparative thin-layer plates using the same solvent system. Authentic TCVG, which had been incubated with GGT in the same way as the bile sample, was run as a standard on each plate. An area of each plate, 3 cm on either side of the R_F of the hydrolyzed TCVG (0.7) was removed and eluted with methanol (100 ml). The eluate was evaporated to low volume (1 ml) under reduced pressure and an excess of an ethereal solution of diazomethane added. When the reaction was completed the excess diazomethane was removed, and 10 ml of dichloromethane added followed by 2 ml of trifluoroacetic anhydride. The sample was allowed to stand at room temperature for 15 min, the dichloromethane evaporated off, and the residue dissolved in methanol (0.1 ml). The GGT-hydrolyzed TCVG standard was also eluted from the plate and derivatized in the same way. The samples were analyzed by gas chromatography–mass spectrometry using a VG 7070E instrument fitted with a methyl silicone column (15 m × 0.53 mm) operated at 100°C for 1 min followed by 10°C/min up to 200°C. The flow rate was 5 ml/min helium.

For comparison with the Day 42 urine samples used for the biochemical assays the urine samples taken at the other time points of the gavage study were similarly combined, as were the samples at each time point of the 400-ppm inhalation study. Samples (2 ml) were acidified with concentrated hydrochloric acid (0.1 ml) and extracted with ethyl acetate (8 ml). The solvent phase was separated, concentrated to 0.1 ml, and methylated with ethereal diazomethane solution as described previously. Excess diazomethane was removed and the residue dissolved in methanol (0.2 ml). Authentic NAc-TCVC was derivatized in the same way. The samples were analyzed by gas chromatography–mass spectrometry using the equipment described above. The column was operated from 130 to 215°C at a rate of 15°C/min after a 1-min delay. Under these conditions the methyl ester of NAc-TCVC had a retention time of 7.5 min.

Individual urine samples from rats exposed to 10, 100, and 1000 ppm perchloroethylene were analyzed for trichloroacetic acid and NAc-TCVC by gas chromatography and gas chromatography–mass spectrometry, respectively. Both metabolites were extracted into ethyl acetate at pH 1 and methylated with diazomethane as described above. Trichloroacetic acid was determined as described by Odum *et al* (1988) and NAc-TCVC as described above.

In Vitro Metabolism Studies

Renal metabolism. Activity of the renal enzyme β-lyase with TCVC as substrate was measured in cytosolic fractions from rat, mouse, and human kidney. Human kidney samples were obtained immediately following surgery from kidneys removed as a result of malignancy or renal failure. The tissues were in each case from parts of the kidney which were morphologically normal. Animal kidney samples were taken from animals killed by overexposure to halothane.

Cytosol fractions were prepared by homogenizing kidney samples in 4 vol of ice-cold 0.25 M sucrose/10 mM Tris–HCl buffer, pH 7.5, with a Teflon/glass homogenizer. The homogenates were centrifuged at 105,000*g* for 1 hr and the supernatant (cytosol) was removed and stored at −70°C until use. Protein was determined by the method of Lowry *et al.* (1951).

β-Lyase activity was measured using a method based on that of Stevens and Jakoby (1983). The reaction mixture contained, in a final volume of 1 ml, 50 mM Na/K phosphate buffer, pH 8.0, lactate dehydrogenase (0.1 unit), NADH (100 μM), approximately 1 mg of protein, and TCVC (0.2–7.0 mM). The reaction (37°C for 5 min) was started by the addition of TCVC and was monitored at 340 nm. Michaelis–Menten constants K_m and V_{max} were obtained from Lineweaver–Burk plots of s/v versus s.

Hepatic metabolism. Conjugation of perchloroethylene with glutathione was assayed in microsomal and cytosolic fractions of livers from rats, mice, and humans. Human liver samples were obtained from brain dead renal transplant donors after compliance with ethical and legal requirements. Animals were killed by overexposure to halothane and the livers removed immediately.

Livers from rats and mice were homogenized with a Teflon/glass homogenizer in sucrose (250 mM):EDTA (5.4 mM):Tris–HCl buffer, pH 7.5 at 4°C to give 25% (w/v) homogenates. These were centrifuged at 9000*g* for 20 min, and the supernatants were transferred to fresh tubes and centrifuged at 105,000*g* for a further 70 min. The supernatants (cytosol) were stored at −70°C and the pellets (microsomal fraction) were resuspended in the same buffer and centrifuged at 105,000*g* for 1 hr. The washed microsomal pellets were suspended in buffer and stored at −70°C. Human liver samples were homogenized in a Waring blender and cytosol and microsomal fractions prepared as for rats and mice.

Incubations contained, in a final volume of 3 ml, 0.1 M phosphate buffer, pH 7.4, glutathione (5 mM), glutathione reductase (5 units), and either 25 mg cytosolic or 15 mg microsomal protein. The reaction was started by the addition of [^{14}C]perchloroethylene (10 mM; 5 μCi) and the samples were incubated for up to 2 hr at 37°C. The [^{14}C]perchloroethylene used in these studies was purified immediately before use by preparative gas chromatography. The reaction was stopped by the addition of 50% trichloroacetic solution (0.1 ml) and the precipitated protein removed by centrifugation. Excess perchloroethylene was removed from the supernatant by extraction with diethyl ether (3 × 5 ml). The aqueous residue was then derivatized using 1-fluoro-2,4-dinitrobenzene as described by Reed *et al.* (1980) prior to analysis by high-pressure liquid chromatography.

Aliquots of the derivatized supernatant (50 μl) were analyzed using a 4.9-mm × 25-cm, 10-μm Lichrosorb NH_2 column eluted with a solvent gradient containing 3 M sodium acetate, pH 4.5, over 40 min. Fractions of 1 ml were collected and assayed for radioactivity by liquid scintillation counting. TCVG and TCVC were also derivatized and analyzed under the same conditions.

RESULTS

Male rats given a daily [redacted] mg/kg perchloroethylene for 42 days showed an increase in kidney/body weight ratios (1.3-fold) and evidence of kidney damage. Significant increases in urinary volume (+35%), glucose (+45%), ALP (+90%), and NAG (+57%) levels compared to controls indicated mild proximal tubular damage. Examination of the kidneys from these animals at the light microscope [redacted]

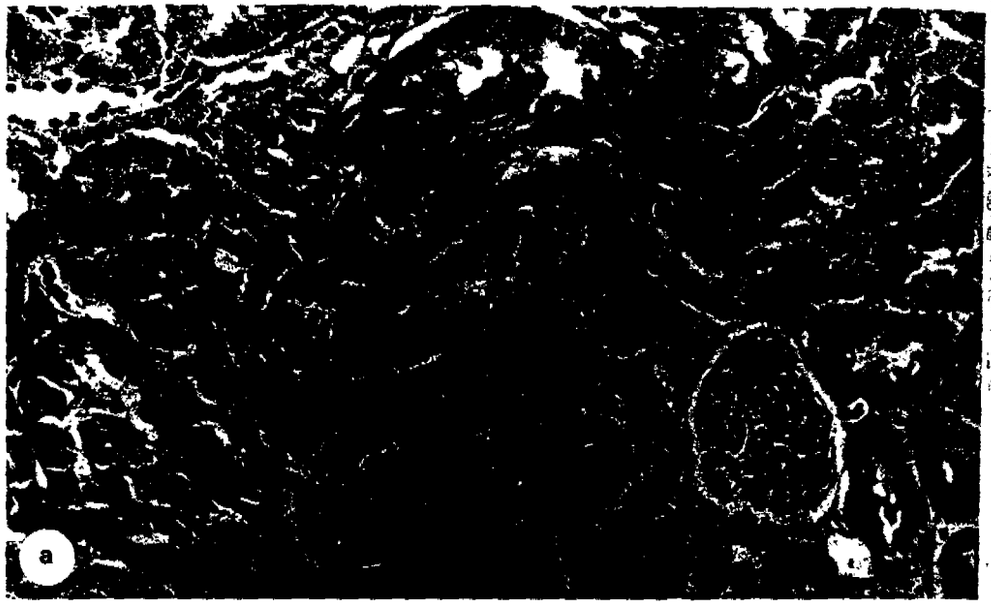
hyaline (protein) droplets in the S2 portion [redacted] tubule. The droplets were increased not only in number but also in size and shape, larger angular droplets being visualized (Fig. 1). In addition to the droplets, tubular casts were seen at the corticomedullary junction, with distortion and enlargement of the tubules containing them. Focal areas of basophilic proximal tubular regeneration were also apparent.

Rats and mice of both sexes were also exposed to perchloroethylene by inhalation at dose levels of up to 400 ppm for 28 days. In this experiment kidney/body weight ratios were unchanged, nor were there any compound-related changes in the kidneys of either species at the light or electron microscope levels. Increases in hyaline droplets were not seen in male rat kidneys, nor could any increase in α-2u-globulin be detected biochemically in the kidneys of these animals (data not shown). The kidneys taken from rats exposed by inhalation to 1000 ppm perchloroethylene for 10 days did show a clear increase in hyaline droplets in the proximal tubules. This was not accompanied by the cast formation or cellular regeneration that were seen after gavage dosing.

Bile collected from male rats for the 24 hr following the final dose of perchloroethylene on Day 42 of the gavage study was analyzed by thin-layer chromatography and found to contain radioactive material identical with authentic *S*-(1,2,2-trichlorovinyl)glutathione (R_f 0.3). The identity of this metabolite was confirmed following hydrolysis of the bile with γ-glutamyltranspeptidase in comparison with authentic *S*-(1,2,2-trichlorovinyl)-glutathione. Hydrolysis of the standard with GGT resulted in the formation of *S*-(1,2,2-trichlorovinyl)-L-cysteine rather than the expected *S*-(1,2,2-trichlorovinyl)cysteinylglycine, presumably as a result of contamination of the GGT with a peptidase which cleaved the cysteinylglycine bond. The mass spectrum of the derivatized product is shown in Fig. 2. The derivatization procedure resulted in two peaks, the second having a mass 14 units higher than the first, corresponding to the addition of a further methyl group, presumably at the nitrogen of cysteine. The derivatized extract of GGT-hydrolyzed bile was analyzed by GC/MS in the selective ion detection mode, focusing on ions *m/e* 360 and 362 from the first peak and *m/e* 374 and 376 from the second peak. The bile extract was found to contain all four ions in the correct ratios with retention times identical to those of the standard (9.5 and 10.5 min) (Fig. 2).

Urine from both the gavage and inhalation studies was analyzed for the presence of *N*-acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine. Gas chromatograms of methylated urine extracts were shown to contain a peak (7.8 min) with a mass spectrum identical to that of authentic *N*-acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine methyl ester (Fig. 3). Quantitation of the levels of *N*-acetyl-*S*-(1,2,2-trichlorovinyl)cysteine in [redacted] found the [redacted] considerably higher after gavage dosing than after inhalational exposure. [redacted] were also significantly higher in [redacted] mouse urine under the same conditions of exposure (Table 1). There was some evidence that the levels in mouse urine were increasing with increasing duration of exposure.

The activity of cysteine conjugate β-lyase with the cysteine conjugate of perchloroethylene as substrate was determined in kidney cytosol fractions from mouse, rat, and humans. Kinetic constants obtained from plots of s/v against s are shown in Table 2. The reaction was linear with protein concentration over the range used and had a pH optimum of 8.0. Addition of aminooxyacetic acid (0.1 mM) to those experiments reduced activity by 50%. The K_m was lower and the V_{max} higher for the rat compared to either mouse or human kidney β-lyase. A comparison of V_{max}/K_m for the three species gave similar values for mouse and human, whereas the value for the rat was up to 30-fold greater, with the value for the male rat being approximately twice that for the female (Table 2). The variation in β-lyase activity in human kidney sam-



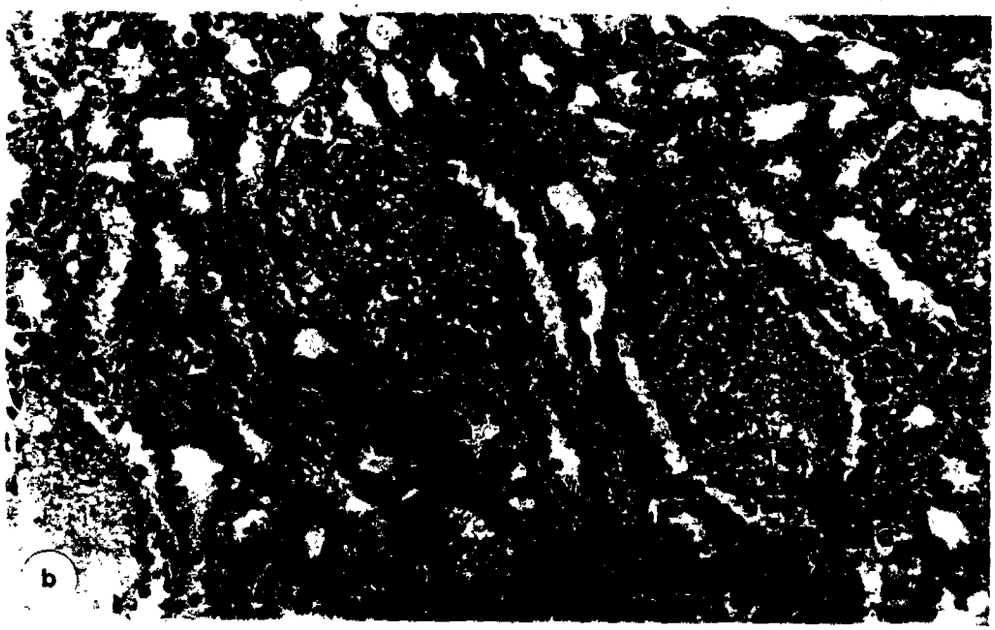
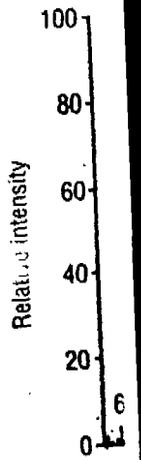


FIG. 1. Light micrographs taken from the kidney of a male rat given perchloroethylene, 1500 mg/kg body wt/day for 42 days, shows (a) the cortex where proximal tubules in the S_2 region exhibit an accumulation of hyaline droplets in the cytoplasm of the cells, and (b) the corticomedullary area of the same kidney. There is an accumulation of proteinaceous debris in the tubules in this region, which appear thin walled due to a compressed layer of epithelial cells and dilated due to the debris. H&E, ×400.



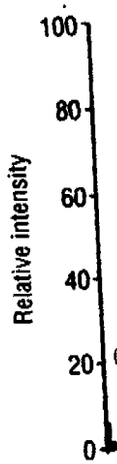


FIG.
deriva
from r
acetyl

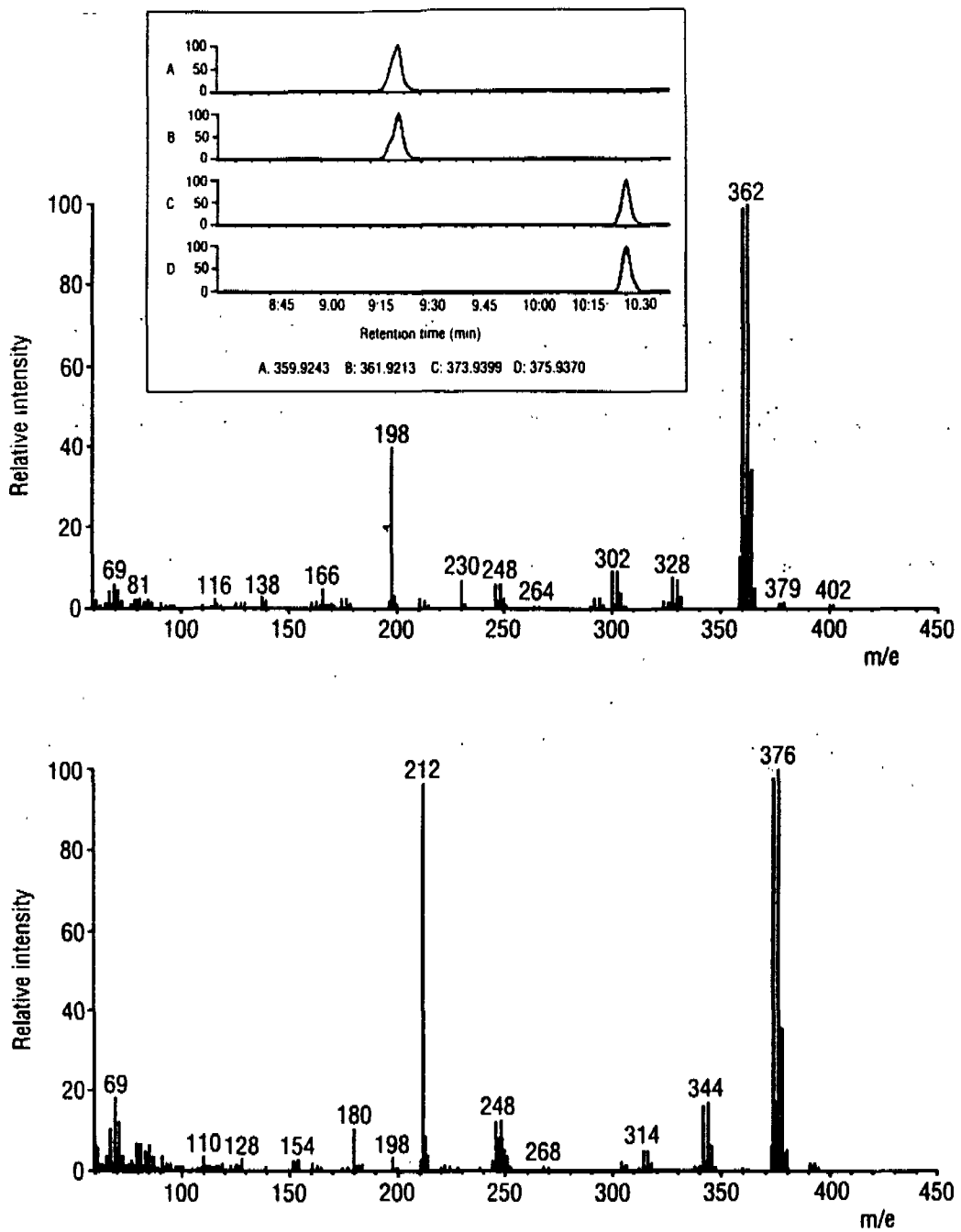


FIG. 2. Chemical ionization (isobutane) mass spectra obtained for the *N*-trifluoroacetyl methyl ester derivatives of *S*-(1,2,2-trichlorovinyl)-L-cysteine. Inset: Chromatogram of a GGT-hydrolyzed bile extract from male rats dosed orally with perchloroethylene (1500 mg/kg) showing the presence of the *N*-trifluoroacetyl methyl ester derivative of *S*-(1,2,2-trichlorovinyl)cysteine by selective ion detection.

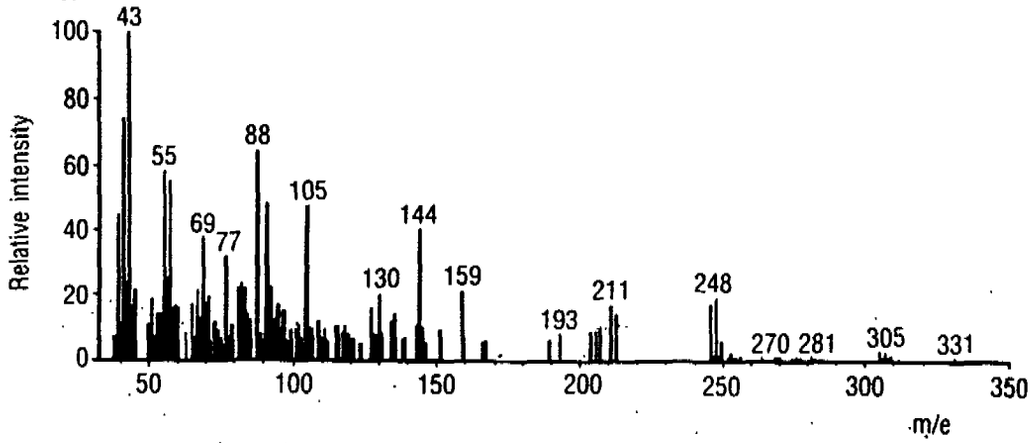


FIG. 3. Electron impact mass spectrum of the methyl ester of *N*-acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine extracted from the urine of male rats dosed orally with perchloroethylene (1500 mg/kg).

ples was remarkably small. In 11 individuals the rates determined for a single substrate concentration ranged from 0.1 to 0.56 nmol/min/mg protein. K_m and V_{max} values obtained for 7 of those individuals were also remarkably close (Table 2).

Hepatic conjugation of perchloroethylene with glutathione in rat liver fractions was found to occur primarily in the cytosolic fraction, the rate (18.2 pmol/min/mg protein) being threefold higher than that in microsomal fractions (6.4 pmol/min/mg protein). The rate of conjugation varied markedly between species, that in rat liver cytosol being five to six times greater than that in mouse liver cytosol (3.4 pmol/min/mg protein). In these experiments [illegible] human liver fractions, either microsomal or cytosolic. Based on the limit of detection of the assays used, any human rate would be at least

TABLE 1

N-ACETYL-*S*-(1,2,2-TRICHLOROVINYL)-L-CYSTEINE LEVELS IN RAT AND MOUSE URINE AFTER EXPOSURE TO PERCHLOROETHYLENE

Dose	Duration (days)	*N*-Acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine (μg/ml)			
		Rat		Mouse	
		Male	Female	Male	Female
Inhalation exposure, 400 ppm, 6 hr/day	1	0.75	1.30	0	0
	7	2.04	0.90	0.07	0
	14	0.55	1.04	0.20	0.15
Oral, 1500 mg/kg/day	1	23.00	—[a]	—	—
	17	41.11	—	—	—
	42	32.73	—	—	—

Note. Measurements were carried out on pooled urine samples from five animals (inhalation study) or three animals (oral study).

[a] —, Not determined.

TABLE 2

METABOLISM OF *S*-(1,2,2-TRICHLOROVINYL)-L-CYSTEINE BY KIDNEY CYTOSOLIC β-LYASE

Species[a]	Sex	K_m (mM)	V_{max} (nmol/min/mg)	V_{max}/K_m
Rat	M	0.68 ± 0.06	4.00 ± 0.11	5.88
Rat	F	1.26 ± 0.21	3.64 ± 0.41	2.88
Mouse	M	5.69 ± 2.22	1.15 ± 0.31	0.20
Mouse	F	4.43 ± 1.42	1.66 ± 0.27	0.37
Human (n = 3)	M	2.53 ± 0.09	0.49 ± 0.07	0.21
Human (n = 4)	F	2.67 ± 2.11	0.64 ± 0.54	0.24

Note. Values (means ± SD) were calculated from plots of s/v against s.
[a] n = 4 unless otherwise stated.

in order of magnitude less than that in the rat. The extremely low rates of glutathione conjugation found, even in the rat, prevented the accurate determination of K_m and V_{max} for this pathway, the data given being for a substrate concentration of 10 mM. Assay of the same cytosol fractions with 1-chloro-2,4-dinitrobenzene as a substrate for the glutathione-*S*-transferase enzymes gave considerably higher rates, rat cytosol (430 nmol/min/mg protein) being similar in activity to human cytosol (442 nmol/min/mg protein).

A comparison of the metabolism of perchloroethylene by the main cytochrome P450 pathway, based on urinary trichloroacetic acid levels, with that by the minor glutathione pathway, based on urinary *N*-acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine levels, is shown in Fig. 4. The data shown are the urinary levels of each metabolite following single exposures to dose levels of 10, 100, and 1000 ppm. Exposure to 1000 ppm perchloroethylene for 10 consecutive days had no significant effect on the levels of either metabolite compared to a single exposure (data not shown). It is clear from Fig. 4 that the glutathione pathway is minor at low dose levels but begins to increase significantly following saturation of the cytochrome P450 pathway.

DISCUSSION

Early investigations of the mechanism of perchloroethylene-induced cancer centered around the formation and reactivity of the epoxide believed to be formed during the oxidative metabolism of this chemical (Bonse *et al.*, 1975; Greim *et al.*, 1975; Henschler, 1977; Bolt *et al.*, 1982; Bartsch *et al.*, 1979). Against such a mechanism was a marked lack of detectable genotoxicity in short-term tests, suggesting that other nongenotoxic mechanisms may be important. This was found to be the case for the liver tumors seen in exposed mice. Hepatic peroxisome proliferation, mediated by the minor metabolites of

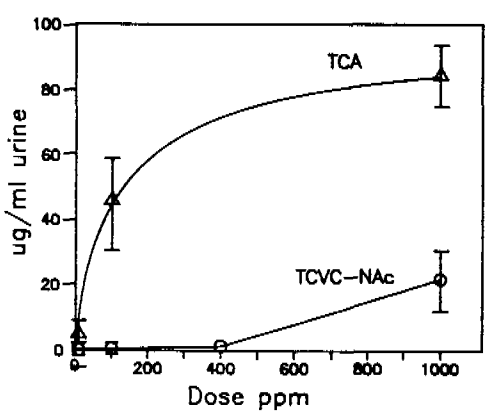


FIG. 4. Dose-dependent metabolism of perchloroethylene in the male rat. Rats were exposed to 10, 100, or 1000 ppm perchloroethylene for 6 hr. Urine was collected for the following 18 hr and analyzed for trichloroacetic acid (TCA) and *N*-acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine (TCVC-NAc). Each point represents the mean ± SD of three animals. The data point at 400 ppm (TCVC-NAc) is taken from Table 1.

perchloroethylene, trichloroacetic acid, provided an explanation both for the liver tumorigenicity in mice and the species differences between rats and mice (Odum *et al.*, 1988). From the present study it would appear that the low incidence of male rat kidney tumors is not related to epoxide formation or peroxisome proliferation (Odum *et al.*, 1988) but to a combination of insults including chronic toxicity, hyaline droplet formation, and the formation of mutagenic cysteine conjugates.

Prior to the NTP lifetime inhalation study (NTP, 1985) previous lifetime carcinogenicity studies in rats have been compromised by poor survival within the dosed groups (NCI, 1977; Rampy *et al.*, 1978). In these studies survival was reduced, in least in part, as a result of chemical-induced nephropathy which could be distinguished from the age-related nephropathy normally seen in F344 rats. Although mortality was slight in the latest NTP study there was evidence again of kidney damage characterized by tubular enlargement and hyperplasia. In the present short-term gavage studies kidney damage was apparent both histologically and from changes in plasma and urine markers of toxicity. Sustained cell damage or cytotoxicity has long been believed to either result in or promote the development of cancer, such a mechanism having been proposed for a number of chemicals (Reitz, 1987). Consequently, the possibility that the chronic nephropathy seen in the perchloroethylene cancer studies is causally related to the very low incidence of renal tumors in male rats cannot be ignored. At the same time the significance of this type of effect is very difficult to quantify.

Mature male rats given high oral doses of perchloroethylene were also shown to accumulate the protein droplets (hyaline droplets) in renal proximal tubular cells. This effect, which has been reported previously with perchloroethylene (Goldsworthy *et al.*, 1988), is seen in male rats but not in female rats nor in mice of either sex. Hyaline or protein droplet formation was accompanied by cast formation and proximal tubular cell regeneration (tubular basophilia), which is believed to be part of a cycle of necrosis and regeneration that results in cancer (Bruner, 1984; Charbonneau *et al.*, 1988; Trump *et al.*, 1984; Strasser *et al.*, 1988; Kanerva *et al.*, 1987). A significant number of apparently nongenotoxic male rat-specific renal carcinogens have been linked with this effect.

Hyaline droplet nephropathy, which is consistent with the male rat specificity observed in the perchloroethylene cancer bioassays, was first seen in the present studies following gavage dosing of perchloroethylene. The same response was not seen in male rats exposed to perchloroethylene by inhalation at the dose levels used in the NTP (1985) study, even after exposure to 400 ppm daily for up to 28 days. Thus, although there is a well-established link between hyaline droplet nephropathy and renal cancer in male rats, the lack of effect at the top dose level used in the NTP study questions the significance of this phenomenon in the development of tumors in that study. Hyaline droplet formation is frequently seen after a single dose of many of the agents that are known to cause this effect (Trump *et al.*, 1984). The present studies measuring hyaline droplet formation in sexually mature male rats exposed to 400 ppm perchloroethylene were continued for up to 28 days without effect. However, although droplets were not seen even over this larger time scale, the possibility that hyaline droplet formation is a contributory factor to the development of renal tumors in male rats over the lifetime of the animals cannot entirely be ruled out.

The difference in response between male rats dosed with perchloroethylene by gavage and those dosed by inhalational exposure appears to be due to differences in the magnitude of the dose. Although difficult to quantify exactly, 400 ppm by inhalation is a significantly lower dose than 1500 mg/kg by gavage. The belief that 400 ppm was below the threshold required to induce this response was confirmed when [redacted] were seen after exposure of rats to 1000 ppm per-

chloroethylene inhalation. This dose level would appear to approximate the threshold dose since hyaline droplets were seen but the later consequences of hyaline droplet formation, cast formation and cellular regeneration, were not developed at this dose level.

The possible mechanisms discussed so far are believed not to involve any direct interaction between perchloroethylene or its metabolites and DNA. A third mechanism appears to exist which does involve the formation of genotoxic metabolites in the kidney. Perchloroethylene has been found to be metabolized by a second minor pathway involving conjugation with glutathione (Fig. 5). The conjugate is metabolized by the mercapturic acid pathway and the *N*-acetylcysteine conjugate is excreted in urine (Dekant *et al.*; 1986; Odum and Green, 1987). In addition to being metabolized to the mercapturate, the cysteine conjugate of perchloroethylene has also been shown to be a substrate for the renal enzyme β-lyase and to be mutagenic in the Ames bacterial mutation assay (Green and Odum, 1985). As a result of the discovery of this pathway it must be assumed that there may be a genotoxic component to the development of tumors in the male rat kidney. The lack of response of perchloroethylene itself in the Ames assay is almost certainly due to a failure of the standard assay to replicate the number of steps involved in the formation and activation of this conjugate *in vivo* (Fig. 5).

In the present study the glutathione conjugate of perchloroethylene was detected in bile from orally dosed rats, and the *N*-acetylcysteine conjugate in urine from rats dosed orally and from rats and mice exposed to perchloroethylene by inhalation. *In vitro* measurement of the rates of hepatic glutathione conjugation of perchloroethylene, and of the activity of renal β-lyase with the cysteine conjugate, has enabled comparisons to be made between rats, mice, and humans. The results of these studies were consistent with the rat being the species susceptible to renal cancer by this mechanism. Rates of hepatic glutathione conjugation and renal β-lyase cleavage were both higher in rat tissues than mouse tissues. *In vivo* the levels of the *N*-acetylcysteine conjugate in rat urine were significantly higher than those in mouse urine (Table 2). The higher levels of *N*-acetylcysteine conjugate in urine from orally dosed rats compared to the levels in urine from rats exposed by inhalation are consistent with the differences in dose and behavior of the two pathways shown in Fig. 4. Glutathione conjugation increases markedly at higher dose levels following saturation of the cytochrome P450 pathway.

Three mechanisms have been identified which may cause or contribute to the development of kidney tumors in male rats exposed to perchloroethylene: sustained chronic toxicity, hyaline droplet nephropathy, and genotoxicity via the glutathione conjugation/β-lyase pathway. It seems feasible that at least two if not all three of these mechanisms contribute. The genotoxic glutathione pathway may constitute the initiating event which is then promoted by cell division induced by hyaline droplet nephropathy or chronic cytotoxicity. The glutathione/β-lyase mechanism alone may not be sufficient to induce tumors in the rat kidney since the levels of *N*-acetylcysteine conjugate in female rat urine and the activity of β-lyase in female rat kidney are not too dissimilar to those found in the male. Male rat-specific protein droplet nephropathy or increased sensitivity of the male rat to the cytotoxic effects of perchloroethylene may be essential factors in tumor development.

The mechanisms that occur in the rat seem to have little relevance to human risk assessment. Chemically induced nephropathy or cytoxicity and hyaline droplet nephropathy have both been shown to be threshold phenomena and as such have no relevance at occupational exposure levels. Furthermore hyaline droplet formation appears to be restricted to the male rat and is generally considered not to be relevant to humans. Glutathione conjugation of perchloroethylene also seems to be a high-dose phenomenon in the rat, increasing only after the major

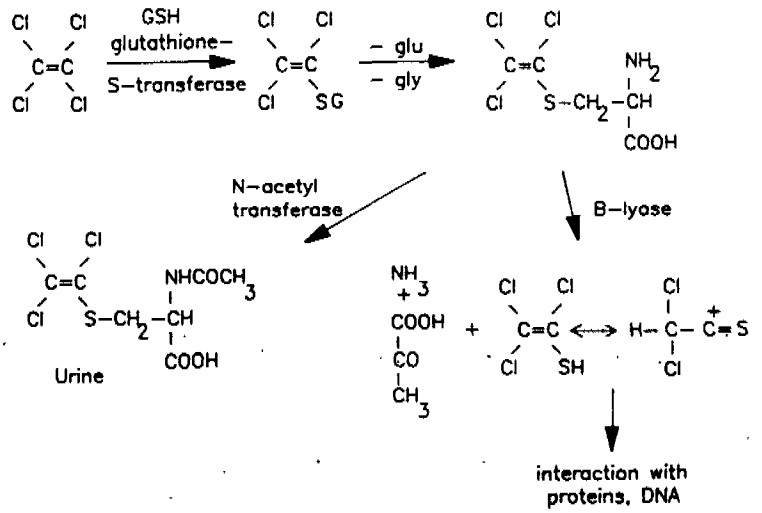


FIG. 5. Proposed metabolism of perchloroethylene by the glutathione conjugation pathway.

cytochrome P450 pathway has become saturated. Comparisons of the activity of the renal β-lyase did detect a rate in humans which was comparable to that in the mouse, but was an order of magnitude lower than that in the rat. However, the *in vitro* studies using human liver tissues failed to detect a rate for the conjugation of perchloroethylene with glutathione. Consequently the presence of renal β-lyase in human kidney is of no toxicological significance for perchloroethylene since current evidence suggests that the liver is the source of the substrate for this enzyme (Nash *et al.*, 1984). In conclusion, it would appear that the low incidence of cancer seen in the kidneys of male rats exposed to perchloroethylene arises from a combination of events which are largely unique to the male rat and appear to have little relevance to the determination of human risk from exposure to this chemical.

REFERENCES

BARTSCH, H., MALAVEILLE, C., BARBIN, A., AND PLANCHE, G. (1979). Mutagenic alkylating metabolites of halo-ethylenes, chlorobutadienes and dichlorobutenes produced by rodent or human liver tissues. Evidence for oxirane formation by P450-linked microsomal mono-oxygenases. *Arch Toxicol* **41**, 249–277.

BOLT, H. M., LAIB, R. J., AND FILSER, J. G. (1982). Reactive metabolites and carcinogenicity of halogenated ethylenes. *Biochem. Pharmacol.* **31**, 1–4.

BONSE, G., URBAN, T., REICHERT, D., AND HENSCHLER, D. (1975). Chemical reactivity, metabolic oxirane formation and biological reactivity of chlorinated ethylenes in the isolated perfused rat liver preparation. *Biochem. Pharmacol.* **24**, 1829–1834.

BRUNER, R. H. (1984). Pathological findings in laboratory animals exposed to hydrocarbon fuels of military interest. In *Renal Effects of Petroleum Hydrocarbons* (M. A. Mehlman, G. P. Hemstreet, J. J. Thorpe, and N. K. Weaver, Eds.), pp. 133–140. Princeton Scientific, Princeton, NJ.

CHARBONNEAU, M., STRASSER, J., LOCK, E. A., TURNER, M. J., AND SWENBERG, J. A. (1988). 1,4-Dichlorobenzene induced nephrotoxicity: Similarity with unleaded gasoline induced effects. In *Nephrotoxicity: Extrapolation from in Vitro to in Vivo and from Animal to Man* (P. A. Bach and E. A. Lock, Eds.). Plenum Press, New York.

DANIEL, J. W. (1963). The metabolism of ^{36}Cl-labelled trichloroethylene and tetrachloroethylene in the rat. *Biochem. Pharmacol.* **12**, 705–802.

DEKANT, W., HAUG, R., AND HENSCHLER, D. (1985). Absorption, elimination and metabolism of tetrachloroethylene. *Arch. Pharmacol.* **329**, Suppl. R24.

DEKANT, W., METZLER, M., AND HENSCHLER, D. (1986). Identification of *S*-1,2,2-trichlorovinyl-*N*-acetylcysteine as a urinary metabolite of tetrachloroethylene: Bioactivation through glutathione conjugation as

possible explanation of its nephrocarcinogenicity. *J. Biochem. Toxicol.* **1**, 57–72.

DOE, J. E., AND TINSTON, D. J. (1981). Novel chambers for long term inhalation studies. In *Proceedings of the Inhalation and Technology Symposium, Kalamazoo, Michigan, 1980* (B. J. K. Leong, Ed.). Ann Arbor Science, Ann Arbor, MI.

GOLDWORTHY, T. L., LYGHT, O., BURNETT, V. L., AND POPP, J. A. (1988). Potential role of α-2u-globulin, protein droplet accumulation and cell replication in the renal carcinogenicity of rats exposed to trichloroethylene, perchloroethylene and pentachloroethane. *Toxicol. Appl. Pharmacol.* **96**, 367–379.

GOLDWORTHY, T. L., AND POPP, J. A. (1987). Chlorinated hydrocarbon-induced peroxisomal enzyme activity in relation to species and organ carcinogenicity. *Toxicol. Appl. Pharmacol.* **88**, 225–233.

GREEN, T., AND ODUM, J. (1985). Structure/activity studies of the nephrotoxic and mutagenic action of cysteine conjugates of chloro and fluoroalkenes. *Chem.–Biol. Interact.* **54**, 15–31.

GREEN, T., ODUM, J., FOSTER, J. R., AND HEXT, P. M. (1986). Perchloroethylene induced hepatic peroxisome proliferation in mice and renal hyaline droplet formation in rats. *Toxicologist* **6**(1), 1262.

GREIM, H., BONSE, G., RADWAN, Z., REICHERT, D., AND HENSCHLER, D. (1975). Mutagenicity in vitro and potential carcinogenicity of chlorinated ethylenes as a function of metabolic oxirane formation. *Biochem. Pharmacol.* **24**, 2013–2017.

HENSCHLER, D. (1977). Metabolism and mutagenicity of halogenated olefins. A comparison of structure and activity. *Environ. Health Perspect.* **21**, 61–64.

KANERVA, R. L., RIDDER, G. M., LEFEVER, F. R., AND ALDEN, C. L. (1987). Comparison of short term renal effects due to oral administration of decalin or *d*-limonene in young adult Fischer-344 rats. *Food Chem. Toxicol.* **25**, 345–353.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265–275.

MOORE, R. B., AND GREEN, T. (1988). The synthesis of nephrotoxic conjugates of glutathione and cysteine. *Toxicol. Environ. Chem.* **17**, 153–162.

NASH, J. A., KING, L. J., LOCK, E. A., AND GREEN, T. (1984). The metabolism and disposition of hexachloro-1,3-butadiene in the rat and its relevance to nephrotoxicity. *Toxicol. Appl. Pharmacol.* **73**, 124–137.

National Cancer Institute (NCI). (1977). *Bioassay of Tetrachloroethylene for Possible Carcinogenicity.* Technical Report Series No. 13. U.S. Department of Health, Education and Welfare. Publication No. (NIH) 77-813 PB 272940.

National Toxicology Program (NTP). (1985). *Technical Report on the Toxicology and Carcinogenesis Studies of Tetrachloroethylene (Perchloroethylene) in F344/N Rats and B6C3F$_1$ Mice* (Inhalation Studies). DHSS-NIH Report 85-2657.

ODUM, J., AND GREEN, T. (1987). Perchloroethylene metabolism by the glutathione conjugation pathway. *Toxicologist* **7**, 1077.

ODUM, J., GREEN, T., FOSTER, J. R., AND HEXT, P. M. (1988). The role of trichloroacetic acid and peroxisome proliferation in the differences in carcinogenicity of perchloroethylene in the mouse and rat. *Toxicol. Appl. Pharmacol.* **92**, 103–112.

RAMPY, L. W., QUAST, J. F., LEONG, B. K. J., AND GEHRING, P. J. (1978). Results of long-term inhalation studies on rats of 1,1,1-trichloroethane and perchloroethylene formulations. In *Proceedings of the First International Congress on Toxicology* (G. L. Plaa and W. A. M. Duncan, Eds.), p. 562. Academic Press, New York.

REED, D. J., BATSON, J. R., BEATTY, P. W., BRODIE, A. E., ELLIS, W. W., AND POTTER, D. W. (1980). High performance liquid chromatography analysis of nanomole levels of glutathione, glutathione disulfide and related thiols and disulfides. *Anal. Biochem.* **106**, 55–62.

REITZ, R. H. (1987). Role of cytotoxicity in the carcinogenic process. In *Nongenotoxic Mechanism in Carcinogenesis* (B. E. Butterworth and T. J. Slaga, Eds.), Banbury Report 25. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

STEVENS, J., AND JAKOBY, W. B. (1983). Cysteine conjugate β-lyase. *Mol. Pharmacol.* **23**, 761–765.

STONARD, M. D., GORE, C. W., OLIVER, G. J. A., AND SMITH, I. K. (1987). Urinary enzymes and protein patterns as indicators of injury to different regions of the kidney. *Fundam. Appl. Toxicol.* **9**, 339.

STRASSER, J. R., CHARBONNEAU, M., BORGHOFF, S. J., TURNER, M. J., AND SWENBERG, J. A. (1988). Renal protein droplet formation in male Fischer 344 rats after isophorone treatment. *Toxicologist* **8**, 136.

TRUMP, B. F., LIPSKY, M. M., JONES, T. W., HEATFIELD, B. M., HIGGINSON, J., ENDICOTT, K., AND HESS, H. B. (1984). An evaluation of the significance of experimental hydrocarbon toxicity to man. In *Renal Effects of Petroleum Hydrocarbons* (M. A. Mehlman, G. P. Hemstreet, J. J. Thorpe, and N. K. Weaver, Eds.), pp. 273–288. Princeton Scientific, Princeton, NJ.

YLLNER, S. (1961). Urinary metabolites of ^{14}C-tetrachloroethylene in mice. *Nature (London)* **191**, 820.