SUBLETHAL EFFECTS AND TOXICOKINETICS OF INHALED 1,3-DICHLOROPROPENE IN RATS

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Hui-Min Yang

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Name: HUI-MIN YANG

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Examining Committee:

Chairman: Dr. J.M. Webster, Professor

Dr. F.C.P. Law, Professor, Senior Supervisor, Dept. of Biological Sciences, SFU

Dr. A. Farrell, Associate Professor, Dept. of Biological Sciences, SFU

Dr. J.E. Rahe, Professor, Dept. of Biological Sciences, SFU, Public Examiner

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Sublethal effects and toxicokinetics of inhaled 1,3-dichloropropene in rats

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ABSTRACT

1,3-Dichloropropene (1,3-DCP) is widely used as a preplant soil fumigant for the control of parasitic nematodes in cotton, vegetables, fruit trees and tobacco. Commercial formulations of 1,3-DCP are comprised of cis- and trans-isomers and have been shown to be carcinogenic and mutagenic in laboratory animals. 1,3-DCP is absorbed by animals following dermal, oral and inhalation routes of administration. Although inhalation is the main route of occupational exposure, little is known of the sublethal effects and toxicokinetics of 1,3-DCP in animals following vapor inhalation.

The purposes of this study were (1) to examine the absorption and elimination of inhaled 1,3-DCP in rats which were untreated or pretreated with chemicals, (2) to develop a toxicokinetic model which describes the absorption and clearance of inhaled 1,3-DCP, and (3) to study the sublethal effects of 1,3-DCP in the rats.

Both isomers of 1,3-DCP were absorbed systemically by the rat following vapor inhalation. The blood concentration-time profiles could be described adequately by a 2-compartment open toxicokinetic model with zero-order absorption and saturable clearance processes. Pretreatment

of rats with a mixed-function oxidase inhibitor (piperonyl butoxide) and a glutathione-depleting agent (diethyl maleate) changed the blood concentration-time profiles of the 1,3-DCP isomers. However, pretreatment of rats with SKF-525A (another mixed-function oxidases inhibitor) failed to alter the metabolic disposition of 1,3-DCP in the rat. 1,3-DCP in the blood was removed rapidly by the various tissues of the rat but unchanged 1,3-DCP was detected only in the adipose tissue. This finding is consistent with the rapid conversion of 1,3-DCP to reactive and conjugated metabolites by the rat. Results from both *in vivo* and *in vitro* studies strongly indicated that a reactive metabolite(s) of 1,3-DCP destroyed the hepatic cytochrome P-450 of rats exposed to 1,3-DCP vapor.

To my father

ACKNOWLEDGEMENT

I would like to express my sincere gratitude to my supervisor, Dr. F.C.P. Law, for his encouragement, support and guidance in the experimental work and the preparation of my thesis. My thanks also go to Drs. T. Farrell and M. Davis (West Virginia University) for their helpful suggestions.

I would also like to thank Mr. Dick Jol and Ms. Loekie Van der Wal of the Animal Care Facility and Ms. Ann Erdman of the Radiochemical Laboratory for their assistance.

My gratitude to my husband is far beyond words.

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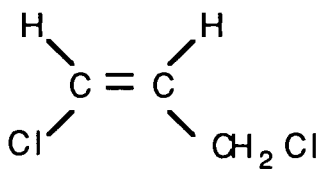
§1. INTRODUCTION

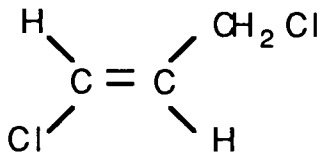
1,3-Dichloropropene (1,3-DCP) was introduced in 1956 as a preplant soil fumigant for the control of parasitic nematodes in cotton, vegetables, fruit trees and tobacco. Agricultural uses of 1,3-DCP have increased markedly in recent years. In 1979, over 5.4 million kilograms were used in California alone (California Department of Food and Agriculture, 1979). This represents almost a fourfold increase over 1977.

The commercial formulation of 1,3-DCP is an admixture of 1,2-dibromoethane (e.g. Dorlone®) or 1,2-dichloropropane (e.g. Telone®, Dow Chemical Co., USA; D-D® Soil Fumigant, Shell Chemical Co., USA). The major active constituents of these products are cis- and trans- 1,3-DCP. However, the relative amount of 1,3-DCP in these fumigants has been changed over the years, e.g., 55% in D-D® mixture, 78% in Telone, 85% in Telone C and 92-98% in Telone II. Other chlorinated hydrocarbons also may be present as impurities in the products including chloropicrin, isomers of dichloropropane and dichloropropene. Epichlorohydrin is often added to stabilize the material and prevent corrosion. Some commercial products do not contain epichlorohydrin any more. Instead, epoxidized soybean oil is used to stabilize the preparation (Dow Chemical Co., 1975).

Cis-1,3-DCP (I) and trans-1,3-DCP (II) has the formula $C_3H_4Cl_2$ and a molecular weight 110.98.



(I)



(II)

It is a clear, light straw-colored liquid with a sharp, sweet and chloroform-like odor. The density and vapor pressure at 25°C are 1.2 and 28 mm Hg, respectively. The boiling points of cis- 1,3-DCP and trans- 1,3-DCP are 104.3°C and 112.0°C, respectively. The flash point, determined by the tag closed cup method, is 21°C. The explosive limits are of the order of 5 to 14.5% by volume. It is soluble in several organic solvents including acetone and toluene. Its solubility in water at 20°C is 1000 ppm (Martin et al., 1974). Spontaneous decomposition of the material will not occur under normal conditions if it is placed in a suitable container. However, polymerization may occur. 1,3-DCP reacts readily with aluminum, aluminum alloys, other active metals and some metal salts and halogens (Torkelson et al., 1977, Hayes, 1982).

Single-dose oral LD_{50} of 1,3-DCP in both male and female JCL:ICR mice is 640 mg/kg. In male and female Wistar rats the oral LD_{50} is 560 and 510 mg/kg, respectively (Toyoshima et al., 1978). For male Sprague-Dawley rats, the oral LD_{50} is 325 mg/kg, the intraperitoneal LD_{50} is 175 mg/kg (Jeffrey et al., 1985).

Little information is available on the acute inhalation toxicity of 1,3-DCP. The single 4 hrs LC_{50} is 729 ppm for rats (Shell Chemical Company, 1977). A single exposure to 400 ppm of 1,3-DCP for 7 hrs was lethal to guinea pig and severely injurious to rats.

The dermal LD_{50} in rabbits is 504 mg/kg (Torkelson et al., 1977). After application to the shaved back of rabbits, dermal irritation was observed, with redness, edema and eventual necrosis.

After 1,3-DCP was instilled into the eyes of rabbits, severe conjunctiva irritation and slight to moderate corneal injury were seen. The injury gradually healed by the 8th day (Torkelson et al., 1977).

Early experiments showed that rats and guinea pigs exposed to 1,3-DCP vapor at either 50 or 11 ppm for 7 hrs/day (5 days/week for 1 month) produced liver and kidney damage (Torkelson et al., 1977). In contrast, more recent data

showed no gross or microscopic changes in the liver and kidneys of mice or rats exposed to up to 93 ppm 1,3-DCP for 6 hrs/day (5 days/week for 13 weeks). The only 1,3-DCP related effects were slight reduction in body weight gain and slight epithelial changes in the nasal turbinates and septum in mice (Torkelson et al., 1981). Degeneration of the nasal olfactory epithelium and/or hyperplasia of the respiratory epithelium were also observed in rats and mice exposed to 90, or 150 ppm Telone II 6 hrs/day (5 days/week for 13 weeks) (Stott et al., 1985).

The effects observed after Fischer 344 rats and CD-1 mice were exposed to D-D® vapor for 6 hrs/day, 5 days/week for 6 or 12 weeks were slight increases in mean ratios of liver/body weight and kidney/body weight of female rats at the 50 ppm exposure level. The only compound-related histopathologic changes were slight to moderate diffuse hepatocytic enlargement of the male mice after exposure to 50 ppm D-D® vapor for 12 weeks.

In separate experiments, rats, guinea pigs, rabbits and dogs were exposed repeatedly to 1,3-DCP mixture vapor at either 3 or 1 ppm for 7 hrs/day (5 days/week for 6 months) (Torkelson et al., 1977). No gross adverse effects were detected in the animals. The hematocrit, WBC counts, hemoglobin and differential count of WBC of the animals

remained unchanged. Only rats exposed to 3 ppm of 1,3-DCP showed a slight, reversible change in the kidneys.

The mutagenicity of 1,3-DCP has been studied in detail by several laboratories. Cis- and trans- 1,3-DCP showed mutagenicity in *Salmonella typhimurium* strains TA 1535 and TA 100 (De Lorenzo et al., 1977, Neudecker et al., 1977, 1980, Stolzenberg et al., 1980, Moryia et al., 1983, Creedy, 1984). Mutation in these strains is an indication of base-pair substitution mutation.

Conflicting results were reported on the mutagenicity of 1,3-DCP in the presence of liver microsomes. Inclusion of rat liver post-mitochondrial fraction (S9) in the Ames test enhanced the mutagenicity of 1,3-DCP (Moryia, 1983). In contrast, De Lorenzo et al. (1977) showed that the liver microsomal fraction made little or no difference on the results of the test. However, the addition of S9 to the test system caused a 3-fold decrease in the mutagenicity of 1,3-DCP in TA 100 (Neudecker et al., 1977, and 1980). Recently, the mutagenicity of 1,3-DCP has been reinvestigated by Talcott (1984) using test strain TA 100. Four commercially available preparations of 1,3-DCP were examined for mutagenicity before and after they were purified by silica acid chromatography. Although all of these 1,3-DCP preparations showed mutagenic activity before chromatographic purification, none of them retained their mutagenic effects

after chromatography. The specific mutagenic activities of the unpurified 1,3-DCP preparations appeared to be caused by trace amounts of polar impurities. The two tentatively identified impurities, epichlorhydrin and 1,3-dichloro-2-propanol, are known mutagens. Watson et al. (1987) have confirmed that the impurities of 1,3-DCP are responsible for the direct mutagenicity previously observed in TA 100. The impurities have been identified as the autoxidation products of 1,3-DCP, cis- and trans-2-chloro-3 (chloromethyl) oxiranes. However, even purified cis-1,3-DCP can cause the bacterial mutation in the presence of rat hepatic S9 fraction or washed microsomes.

1,3-DCP also induced a significant increase in sister chromatic exchange of Chinese hamster ovary cells, which occurred with and without metabolic activation (Tomkins et al., 1980).

The mutagenic activity of 1,3-DCP was not affected by SKF-525A (SKF) or 1,1,1-trichlorprope-2,3-oxide (TCPO), but it was clearly enhanced in the presence of cyanamide (CA) (Neudecker et al., 1986). SKF and TCPO are known inhibitors of microsomal oxygenase and epoxide hydrolase, respectively, whereas CA is an inhibitor of aldehyde dehydrogenase.

1,3-DCP is a weak alkylation agent which binds linearly with DNA (Eder et al., 1982). The distribution of covalently

bound 1,3-DCP to macromolecules was investigated by Dietz (1984a). Male Fischer 344 rats and B6C3F1 mice were given single oral doses of 0, 1, 50, or 100 mg/kg of ^{14}C-1,3-DCP and the forestomach (FS), glandular stomaches (GS), livers, kidneys and bladders were removed at 2 hrs post-dosing for analysis. Binding of 1,3-DCP to the forestomachs and glandular stomachs was dose-dependent. Limited binding was also noted in the livers, kidneys and urinary bladders.

There were no deleterious effects on the libido, fertility, or the reproductive tract morphology of the male and female Wistar rats after inhaling 0, 10, 30, or 90 ppm (v/v) D-D® (cis- and trans- 1,3-DCP were the major continents) 6 hrs/day, 5 days/week for 10 weeks. No treatment-related dominant lethal effect was observed in the male rats (Linnett et al., 1988). No evidence of teratogenic or embryotoxic response was observed in pregnant Fischer 344 rats and New Zealand white rabbits after inhaling 0, 20, 60, or 120 ppm of 1,3-DCP for 6 hrs/day during the gestation period. Slight fetal toxicity was observed only at the level which produced significant maternal toxicity (Hanley et al., 1987).

Van Durren et al. (1979) have demonstrated that cis-1,3-DCP caused fibrosarcomas after it was injected subcutaneously to male ICR:Ha Swiss mice. The carcinogenicity of 1,3-DCP was confirmed by a recent study of

the National Toxicology Program (NTP, 1985). Technical-grade 1,3-DCP (Telone II, with 1% epichlorohydrin as a stabilizer) increased the incidence of tumors in the forestomach and liver in male F344/N rats and in the forestomach in female F344/N rats. An increase in the incidence of forestomach, lung and urinary bladder tumors also was observed in the male B6C3F1 and an increase in the incidence of urinary bladder tumors was observed in the female mice. In contrast, Lomax et al. (1989) reported that 1,3-DCP did not have carcinogenic effects on rats and mice exposed to 5, 20, or 60 ppm technical-grade 1,3-DCP (Telone II, with soybean oil as a stabilizer) for 1-yr or 2-yr.

1,3-DCP has been shown to be absorbed by the skin, the respiratory system and the gastrointestinal tract (Hayes, Jr., 1978). Stott (1986) reported that the uptake rates of 1,3-DCP vapor by rats decreased with increasing 1,3-DCP exposure level. The results were explained by a 1,3-DCP depression in the ventilation frequency of rats. The data obtained from the surgically isolated upper respiratory tract (URT) and lower respiratory tract (LRT) of rats showed that about 20 to 30% of the inhaled vapor was absorbed by the upper respiratory tract. The majority of the inhaled 1,3-DCP was absorbed by the lower respiratory tract (Stott et al., 1986).

1,3-DCP is eliminated mainly in the urine of laboratory animals. About 51-61% and 63-79% of an orally administered dose was excreted within two days in the urine of rats and mice, respectively (Dietz et al., 1984b). Feces and expired CO_2 eliminated roughly 18% and 6% of the administered dose in rats and 15% and 14% of the administered dose in mice, respectively. At the end of 48 hrs, only 2-6% of the original dose remained in the carcase. Similar results were seen in the male and female Carworth Farm E rats; 80%-90% of the administered radioactivity was found in the urine within 24 hrs after a single oral dose of ^{14}C-cis-1,3-DCP or ^{14}C trans-1,3-DCP (Hutson et al., 1971).

After exposing rats to 30, 90, 300, or 900 ppm of 1,3-DCP vapor for 3 hrs, both cis- and trans- isomers were found to be eliminated rapidly from the blood in a biphasic manner (Stott et al., 1986). The half-lives of elimination for cis-1,3-DCP and trans- 1,3-DCP in the first elimination phase were 5 min and 6.3 min, respectively, in rats exposed to 300 ppm for 3 hrs and were increased to over 14 min and 27 min, respectively, in rats exposed to 900 ppm. The half-lives of both isomers for the following second elimination phase were 25 to 43 min. It should be emphasized that plateau blood levels of cis- and/or trans-1,3-DCP were not attained at the conclusion of a 3-hr exposure period.

There were marked difference between the metabolism and toxicity of the cis- and trans- isomers of 1,3-DCP in rats. About 3.9% and 83% of the cis- isomer were recovered as ^{14}C-labelled carbon dioxide and radioactivity in the urine, respectively, from rats dosed with ^{14}C-cis-1,3-DCP. In contrast, about 23.6% of the trans- isomer appeared as ^{14}C-labelled carbon dioxide and about 55.6-60.4% of the administered radioactivity was recovered in the urine from rats dosed with ^{14}C-trans-1,3-DCP (Hutson et al., 1971). Cis-1,3-DCP was twice as mutagenic as the trans- isomer (Neudecker et al., 1977). Alkylation of DNA by cis-1,3-DCP was two fold higher than by trans-1,3-DCP (Eder, 1982). Similarly, glutathione-dependent detoxification of cis-isomer was 4-5 fold higher than that of the trans- isomer (Climie et al., 1979).

Glutathione plays a key role in the deactivation of 1,3-DCP. When cis-1,3-DCP was incubated with glutathione and rat liver cytosol (containing glutathione S-alkyl transferase), a very rapid loss of cis- 1,3-DCP was observed (Climie et al., 1979). 1,3-DCP was excreted primarily as its derivatives of mercapturic acid, N-acetyl-(3-chloroprop-2-enyl)cysteine and its sulfoxide or sulfone, in the urine (Watson et al., 1987, Dietz et al., 1984b, Creedy et al., 1984). N-acetyl-(3-chloroprop-2-enyl)cysteine was also found in the urine of exposed workers. The metabolite levels correlated well with

exposure level and duration of exposure (Osterloh et al., 1984).

A decrease in the contents of hepatic and renal GSH was observed in rats receiving 40 mg/kg or 200 mg/kg of 1,3-DCP ip. However, the GSH level was either back to or above the control values in 24 hrs (Jeffrey et al., 1985). Dietz et al. (1984a) also found that tissue non-protein sulfhydryl contents in the forestomach of rats and mice were depleted after oral administration of ^{14}C-1,3-DCP. The contents of non-protein sulfhydryl were depleted up to 80% in a dose-dependent manner. The forestomach of rats had been identified as a target tissue for 1,3-DCP upon prolonged oral administration (NTP, 1985). The non-protein sulfhydryl contents of kidney (31%) and liver (41%) in rats exposed to 90 ppm 1,3-DCP vapor for 3 hrs also were found to decrease (Stott et al., 1986). However, there was no change in the non-protein sulfhydryl of the lung which was the primary portal-of-entry tissue of 1,3-DCP vapor.

Because of the extensive use of 1,3-DCP and its relative high vapor pressure (Maddy et al., 1976), inhalation is the most important route for occupational exposure. However, little information is available on the metabolic disposition of inhaled 1,3-DCP in animals. Technical grade 1,3-DCP contains a mixture of cis- and trans-1,3-DCP isomers. Therefore it is important to clarify the metabolic

disposition of these isomers as a mixture. The purposes of this thesis were to study the sublethal effects of inhaled 1,3-DCP, to study exposure concentration and treatment-related changes in absorption and clearance and to develop a toxicokinetic model for inhaled 1,3-DCP in rats. The following studies were undertaken to achieve the objectives:

1) to study the tissue distribution of cis- and trans-1,3-DCP in rats after inhaling 1,3-DCP vapor,

2) to investigate the dose-dependent kinetics of cis- and trans-1,3-DCP in rats after inhaling different concentrations of 1,3-DCP vapor and the effects of pretreating rats with inhibitors of MFO or with a glutathione-depleting agent on the toxicokinetics of cis- and trans- 1,3-DCP,

3) to examine the effects of inhaled 1,3-DCP on the mixed-function oxidase system of rats, and

4) to determine the formation of 1,3-DCP reactive metabolites by rats liver microsomes *in vitro*.

§2. MATERIAL AND METHODS

I. Animals.

Male Wistar rats were purchased from the Animal Care Center of the University of British Columbia (Vancouver, B.C., Canada) and acclimated to the laboratory environment for at least 7 days prior to use. Rats were housed in the stainless steel cages (three or four per cage) with tap water and Rodent Laboratory Rat Chow (Purina Mills, Inc., St. Louis, MO) available *ad libitum*. Animals were housed in a room with a temperature of 20 ± 5°C and a relative humidity of 40-60%. The rats were maintained on a 12-hr light:12-hr dark photoperiod.

II. Chemicals

1,3-(^{14}C-2)-dichloropropene (specific activity 5.7 mCi/mmole) was purchased from Amersham International Inc., Amersham, UK. The radiochemical purity of the 1,3-(^{14}C)-DCP was 93.3% as determined by thin-layer chromatography (TLC) on silica-gel plates using methylene chloride as the developing solvent. Unlabeled 1,3-dichloropropene (1,3-DCP) was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Unlabeled 1,3-DCP was distilled under vacuum before use and its purity was 96.7% as shown by gas chromatographic

analysis. Chlorocyclohexane (an internal standard for gas chromatographic analysis of 1,3-DCP) was purchased from Matheson Coleman & Bell, Norwood, Ohio.

SKF-525A was obtained from the Medical Department, Smith Kline & French Inc., Montreal, Quebec. Piperonyl butoxide (80%) was obtained from INC Pharmaceuticals Inc., Life Sciences Group, Plainview, NY. Diethyl maleate was purchased from Aldrich Chemical Company, Inc., Milwaukee Wis.

Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, NADP, HEPES, benzo(α)pyrene, 7-ethoxycoumarin, 7-ethoxyresorufin, EDTA and butylated hydroxytoluene were purchased from Sigma Chemical Company, St. Louis, Missouri. Aminopyrene was obtained from Aldrich Chemical Company (Canada) Ltd., Montreal, Quebec. [^{3}H]-benzo(α)pyrene, Protosol and Biofluor were obtained from New England Nuclear, Lachine, Quebec.

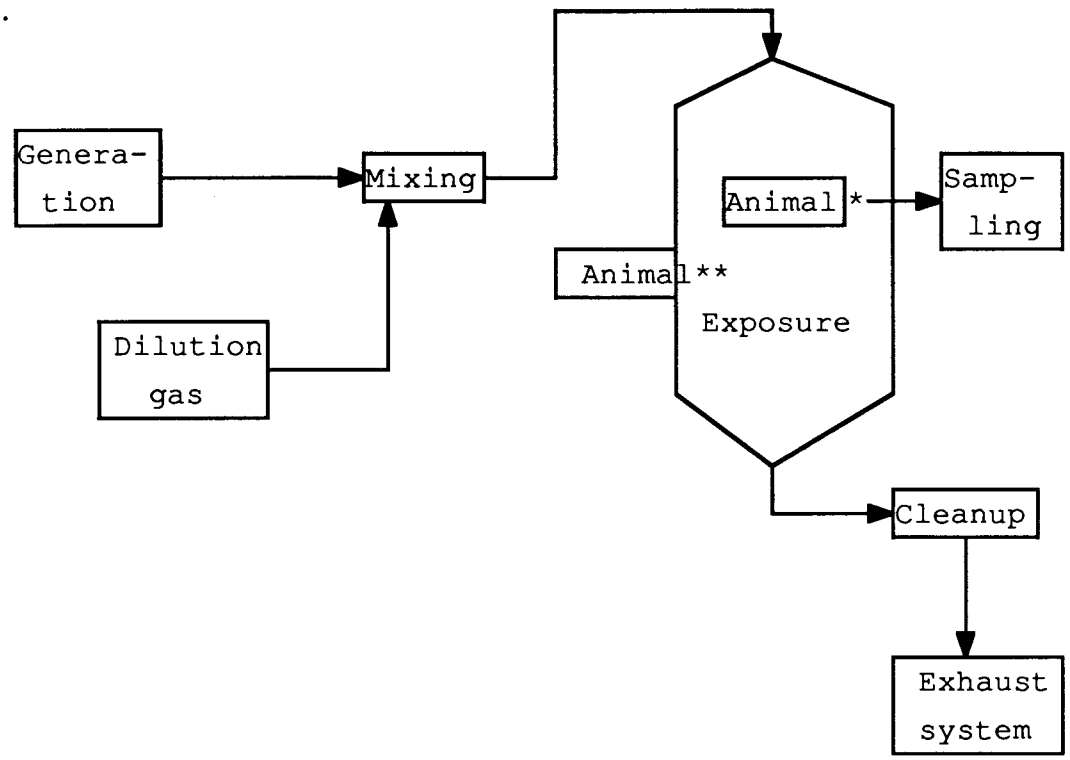
All other chemicals were obtained at the highest purity commercially available.

III. Animal chambers.

Two types of inhalation chambers were used for the study: whole-body and nose-only exposure chambers. Fig. M-1 shows the basic exposure system.

Fig. M-1

Basic inhalation exposure system components.



* Whole-body exposure

** Nose-only exposure

1. Whole-body inhalation chamber.

Fig. M-2 shows a 14 ft^3 ($\approx$ 400 l) dynamic whole-body inhalation chamber. The pyramidal top of the chamber provides a dispersion space required for uniform flow throughout the entire chamber. Room air is filtered through a glove box filter before entering the chamber and is exhausted through a drainage at the bottom of the chamber. The chamber is equipped with an inlet orifice plate (diameter 27 cm) and a butterfly damper in the supply pipe. The pressure difference between the two sides of the orifice plate was calibrated by an air velocity meter (Model 441; KURZ Instruments Inc. Carmel Valley CA) and used to monitor the rate of air flow into the chamber. A negative static pressure (0.3 inch of water) was maintained inside the chamber during its operation.

1,3-DCP vapor was generated by a J-tube system (Miller, et al, 1980). The generation system is shown in Fig. M-3. The J-tube is filled with 5-mm glass beads to provide a large surface area for vaporization. 1,3-DCP was metered into the J-tube via a positive displacement piston pump (FMI, Fluid Metering Inc., Oyster Bay, NY). Preheated compressed N_2 gas (10 l/min) passed through the J-tube and carried the 1,3-DCP vapor into the chamber. A heating tape was used to help maintaining the temperature of the J-tube. 1,3-DCP vapor was diluted with filtered air (130 l/min) at the chamber inlet.

Fig M-2

Whole body inhalation chamber.

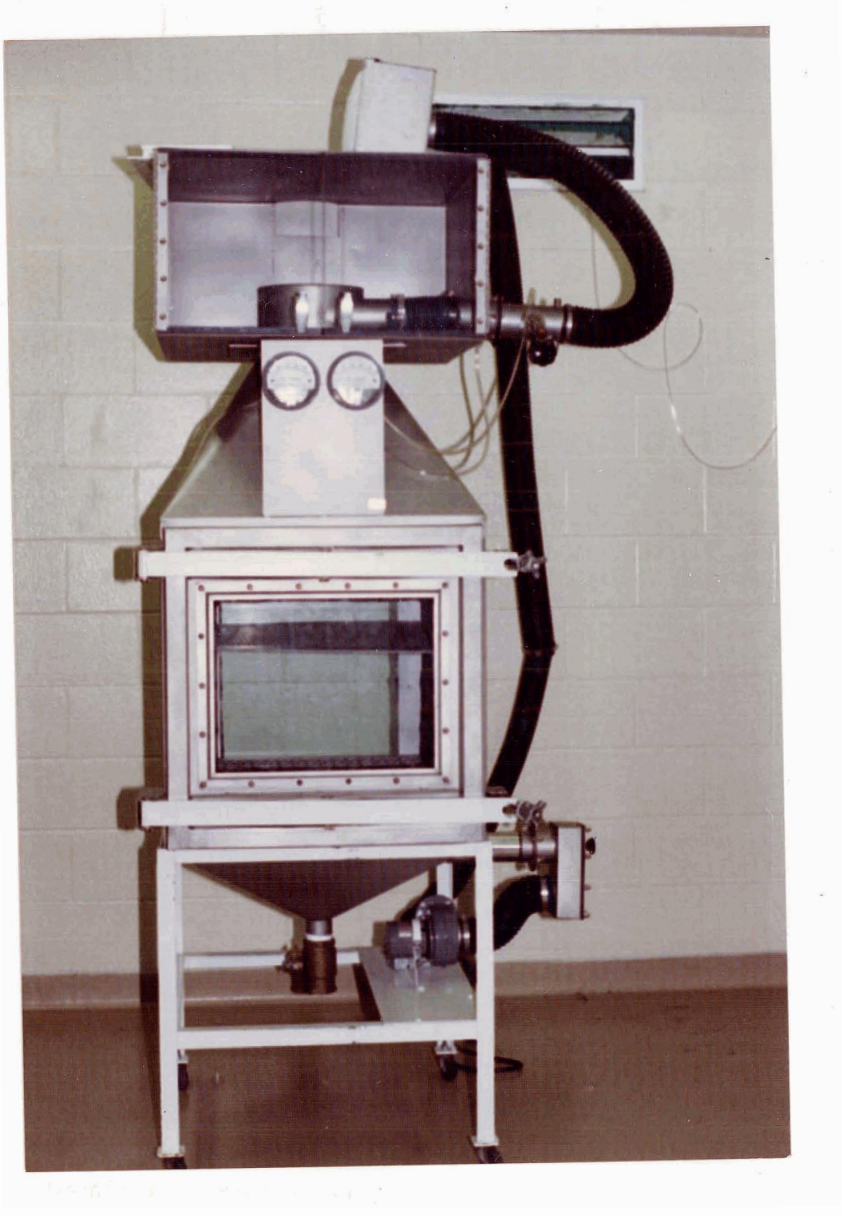
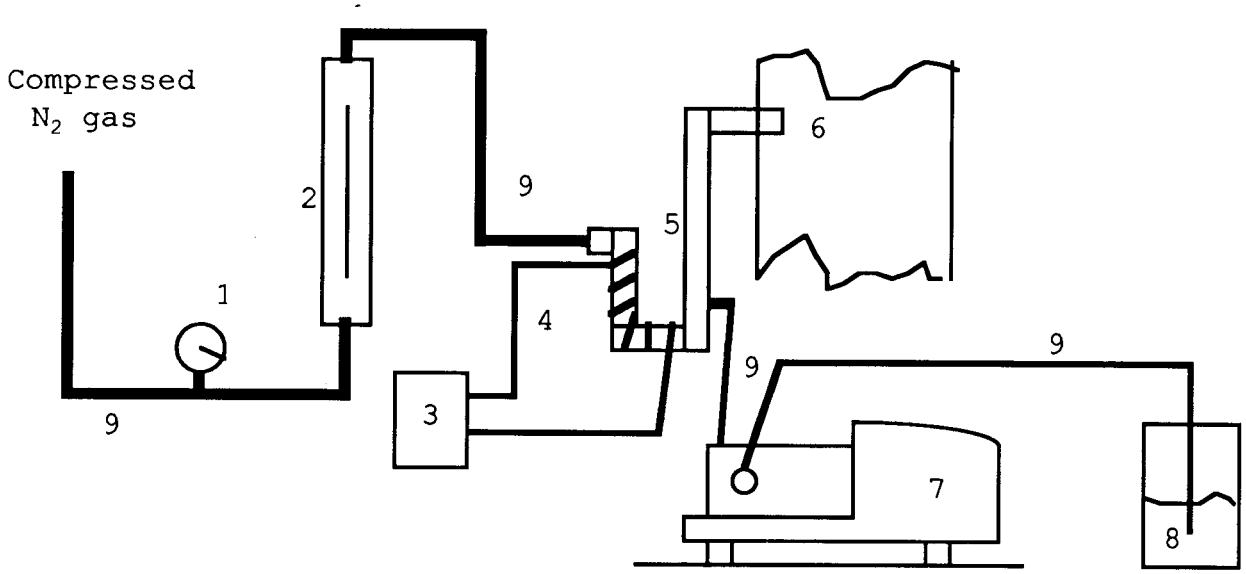


Fig. M-3

J-Tube vaporization system for generating 1,3-DCP.



1. Air pressure regulator,
2. rotameter,
3. electrical transformer,
4. heating tape,
5. J-Tube assembly,
6. chamber,
7. FMI Pump,
8. chemical reservoir,
9. connecting tubes

Air exhausted from the chamber was cleansed by charcoal filters before being released into the atmosphere.

1,3-DCP concentration in the whole-body chamber was monitored continuously with a Miran-IA gas analyzer (Foxboro-Wilks, Norwald, Conn). The closed-loop system, which was connected with the gas analyzer and another pump, was used for calibration (Fig M-4): 1,3-DCP was injected into the loop through the septum, vaporized and circulated through the cell and the absorption were recorded. The following equation was used for the determination of 1,3-DCP concentration in a gaseous phase resulting from injection of a known volume of liquid 1,3-DCP into the system,

$$C_{(ppm)} = \frac{\rho V}{M} \times \frac{(RT)}{(P)} \times \frac{10^3}{5.64},$$

where: V = sample volume (μl). Total volume of cell and calibration system is 5.64 liter,

p = 1,3-DCP density (g/cm^3) (1.18),

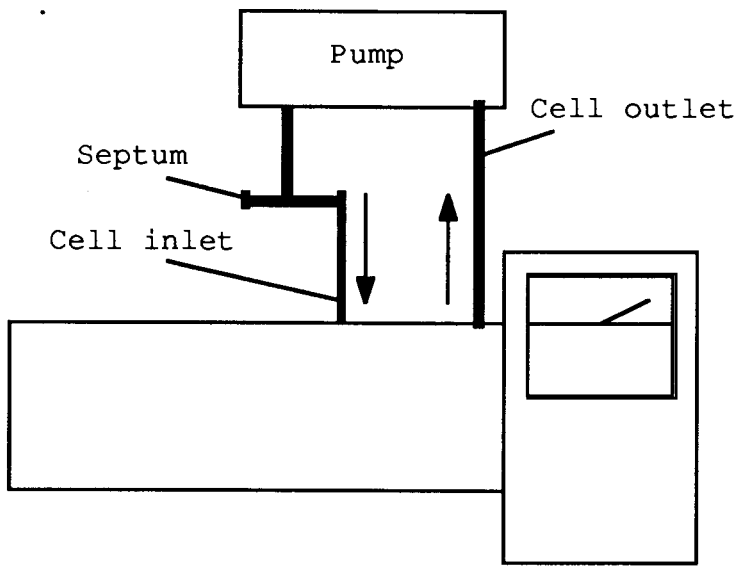
M = molecular weight of 1,3-DCP (110.98),

$\frac{(RT)}{(P)}$ = molar volume of gas (24.4 liter at standard condition).

Monitoring wavelength was 13 μm. The calibration range of 1,3-DCP concentration ranged from 5-45 ppm and 80-500 ppm.

Fig. M-4

Schematic diagram of closed loop calibration system for MIRAN Gas Analyzer.



The calibration curves for 1,3-DCP with the MIRAN gas analyzer are shown in Figs. M-5 and M-6. The curves were rechecked during the exposure periods.

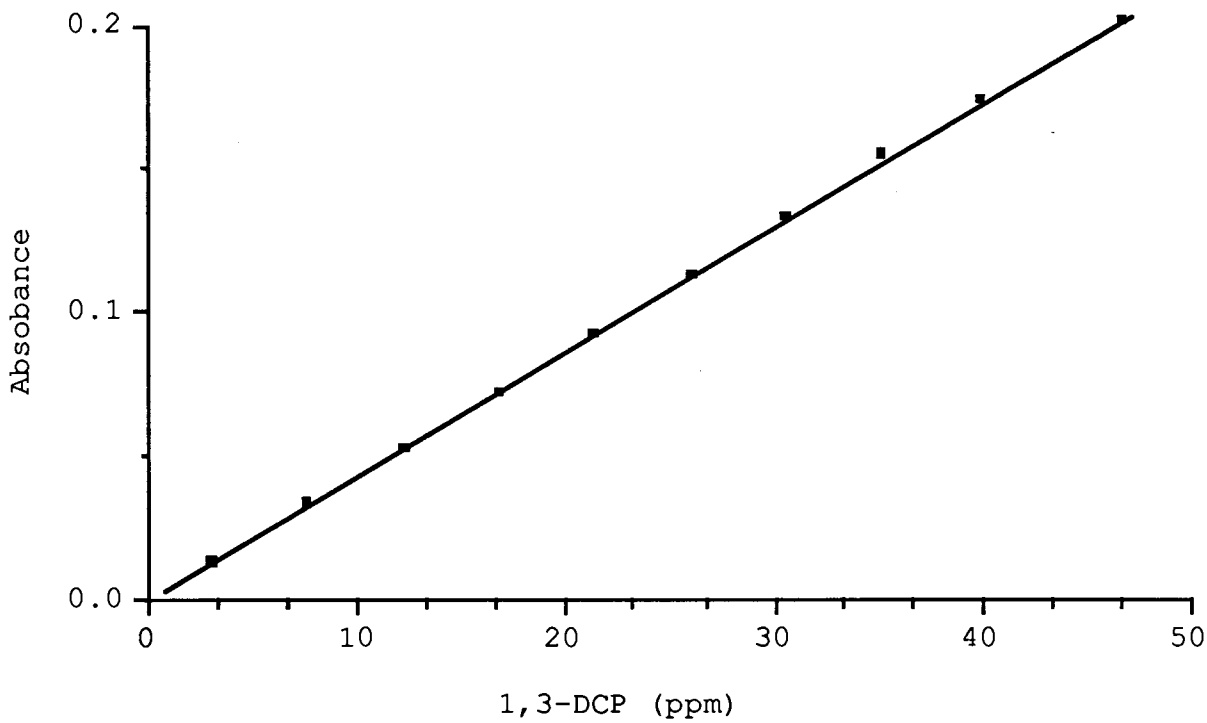
1,3-DCP concentration in the chamber was also analyzed by gas chromatography every half-hour during the experiments. An air sample (0.2-1.5 ml) was withdrawn from the chamber with a 0.5-ml or 2.0-ml gas-tight syringe (Hamilton Company, Reno, NV) and analyzed by a Hewlett-Packard 5750 gas chromatograph equipped with an electron capture detector. A 6 ft x 3/8 in i.d. glass column packed with 5% SE-30 Q (100-120) was used to determine 1,3-DCP concentration in the sample. The operating conditions of the gas chromatograph were: column temperature, 40°C; injection port temperature, 160°C; detector temperature, 250°C; carrier gas (5% methanol-argon) flow 30 ml/min. The amount of 1,3-DCP in the sample was estimated by the area of the chemical peak according to the following equation:

$$\text{Conc}_{\text{test}} = \frac{\text{Conc}_{\text{std.}} \times \text{Area}_{\text{test}}}{\text{Area}_{\text{std.}}}$$

Different amounts of 1,3-DCP were injected into several airtight glass containers of known volumes and mixed well. An air sample was withdrawn from the container and analyzed

Fig.M-5

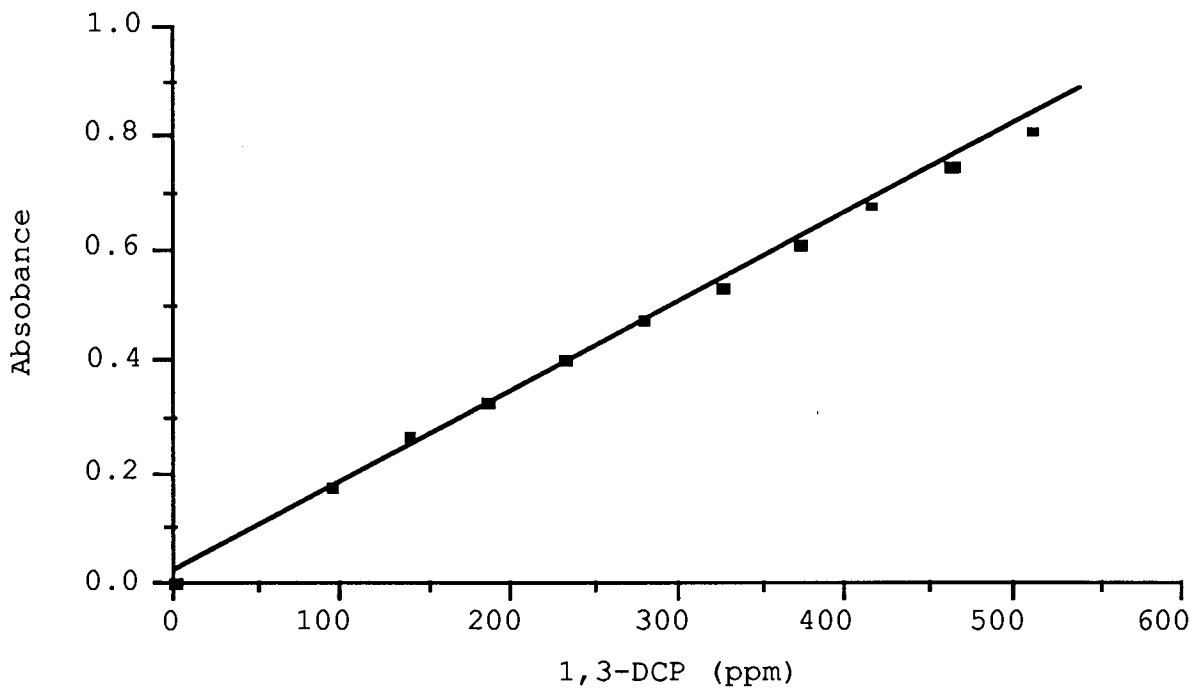
Calibration curve of MIRAN Gas Analyzer for 1,3-DCP.*



* The condition for analysis was wavelength 13 μm, pathlength 15.75 m, slit width 1 mm and range 0 - 0.25 A.

Fig. M-6

Calibration curve of MIRAN Gas Analyzer for 1,3-DCP.*



* The condition for analysis was wavelength 13 μm, pathlength 6.75 m, slit width 1 mm and range 0 - 1 A.

for 1,3-DCP by gas chromatography. The equation for calibrating the standard concentration is,

$$\text{Conc}_{\text{std.}}(\text{ ppm}) = \frac{\frac{\text{wt of agent}}{\text{MW of agent}} \times 10^6}{V/v}$$

Where: wt of agent = gram of 1,3-DCP.

MW of agent = 110.98.

V = volume of container (liter).

v = molar volume of gas (24.4 liter at standard condition).

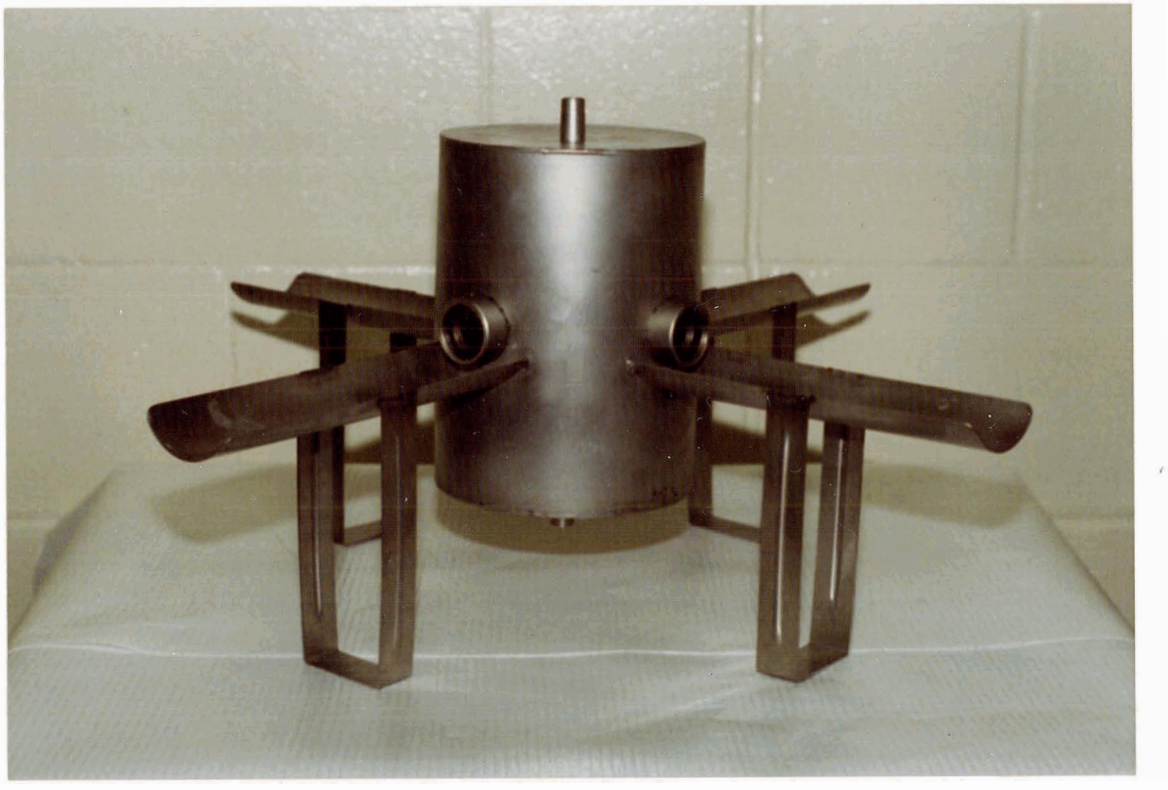
2. Nose-only inhalation chamber.

A small (3.6 liter) nose-only dynamic chamber was used in the experiment (Fig. M-7). The chamber wall has four holes to which are connected the rat exposure tubes. Three to four rats were exposed to the chemical in each experiment. The rats were held individually in a plastic rat exposure tube with only their noses exposed to 1,3-DCP vapor. The chamber was connected to a vapor generating and exhausting system.

1,3-DCP vapor was generated from an impinge bottle. A magnetic stirring bar was placed on the bottom of container to facilitate complete vaporization. Compressed medical air was bubbled through the 1,3-DCP in the bottle and carried the

Fig. M-7

Nose-only inhalation chamber.



1,3-DCP vapor to the chamber. Total chamber air flow was maintained at 2 l/min by a calibrated gas flow meter (rotameter; Matheson 603). Experimental air was exhausted through a charcoal adsorption filter. The whole system was put in a fume-hood to avoid contamination of the working area.

Chamber concentration of 1,3-DCP was monitored by gas chromatography during the experiment. An air sample was withdrawn with an air-tight syringe and analyzed by gas chromatography as previously described.

IV. Toxicokinetics of inhaled 1,3-DCP in rats.

1. Dose-dependent toxicokinetics of inhaled 1,3-DCP in rats.

Male Wistar rats (250-300 g) were exposed to nominal concentrations of 100, 300, 500 ppm of 1,3-DCP mixture vapor in a nose-only chamber for 4 hrs. Cis- and trans-1,3-DCP concentration in the whole blood of rats during and after exposure were monitored as a function of time.

At least 36 hrs before exposure, the rat was implanted surgically with a cannula in the right jugular vein for taking repetitive blood samples, essentially by the methods of Harms and Ojeda (1974). Briefly, after the rat was put under general halothane anesthesia, a longitudinal skin

incision of about 2-cm was made over the area where the right external jugular vein passed under the pectorals major muscle. Another incision of approximately 1-cm was made at the dorsal midline about 3-cm behind the ears. A tube (Silastic Medical Grade Tubing, Dow Corning Co., Midland Michigan; i.d. 0.02", o.d. 0.037") was inserted into the jugular vein and advanced down the vein towards the heart. The free end of cannula was then forced through the connective tissues underneath the skin to the dorsal incision. The incisions were sutured by Autoclips (Clay Adams, Parsippary, NJ). The cannula was filled with heparinized saline (88 I.U./ml), plugged and attached firmly to the back of the rat. The whole procedure took approximately 20 min.

A blood sample (0.25 ml) was taken from the rats via the jugular vein cannula with an 1-ml glass syringe at 2, 3, 3.5, and 4 hr during 1,3-DCP exposure as well as at 2, 5, 10, 15, 20, 30, 45, 60, 75, 90, and 120 min after the exposure. Each time after a blood sample was withdrawn, about 0.3 ml of heparinized saline (44 I.U./ml) was injected to flush the cannula and replace the blood. An aliquot of the blood sample (0.2 ml) was transferred to an ice-cold centrifuge tube containing 0.4 ml of 1.0 N H_2SO_4. The mixture was vortexed vigorously to precipitate blood proteins before being stored at -20°C until analysis. Depending on the concentration of 1,3-DCP in the blood, 0.5 ml to 2.0 ml of

hexanes, containing chlorocyclohexane as an internal standard, was used to extract a sample. The mixture was shaken in an Eberbach reciprocating shaker (Eberbach Corporation, Ann Arbor, Michigan) for 15 min and centrifuged at 1,000 X g for 10 min. The extract was passed through a Florisil® (60-100 mesh) column and an aliquot of the elute (1 µl) was injected into the gas chromatograph.

Cis- and trans-1,3-DCP in the hexane extract were determined by a Hewlett-Packard Model 5880A gas chromatography which was equipped with an electron capture detector and a 30 m x 0.32 mm i.d. fused silica capillary column coated with DB-1 (J & W Scientific, Folsom, CA). Analysis was performed using the following GC conditions: helium (1.5 ml/min) was the carrier gas and nitrogen (30 ml/min) was employed as an ECD makeup gas. Split injection was used at oven temperature 40°C; the injector and detector temperatures were maintained at 160°C and 300°C, respectively. The signal from the ECD was recorded on a HP 5880A series terminal. Under these conditions the retention times of cis-1,3-DCP and trans-1,3-DCP were 2.4 min and 2.7 min, respectively.

Calibration curve was prepared by adding increasing quantities of 1,3-DCP liquid to 0.2 ml blood samples collected previously from untreated rats. 1,3-DCP in the blood was extracted with hexanes containing chlorocyclohexane

as an internal standard and analyzed by gas chromatography. The retention time of chlorocyclohexane was 5.74 min. The calibration range of cis- and trans-1,3-DCP mixture concentration was 0.025-3 μg/ml (Figs. M-8 and M-9).

The efficiency of recovery of 1,3-DCP from blood was determined by spiking chemical (100-400 ng) to the blood (0.2 ml) which collected from untreated rats and extracting with various volumes (0.5-2.0 ml) of hexanes. The samples were shaken and centrifuged. The hexane extract was passed through a Florisil® column and the contents of cis- and trans- 1,3-DCP in the elute were analyzed by gas chromatograph.

The post-exposure blood concentration-time data of cis- and trans-1,3-DCP from the blood were fitted initially to the following exponential equation to determine the number of compartments to best describe the data:

$$C_B = \sum_{i=1}^{n} A_i e^{-X_i t},$$

where C_B is the blood concentration of 1,3-DCP at time t and A_i and X_i are the coefficient and constant of the exponential components, respectively. The parameters of the equation were determined by nonlinear, least-square regression analysis. Curve fitting was performed with NONLIN (Metzler et al, 1974). The best number of compartments to describe

Fig. M-8

Blood calibration curve of cis-1,3-DCP.

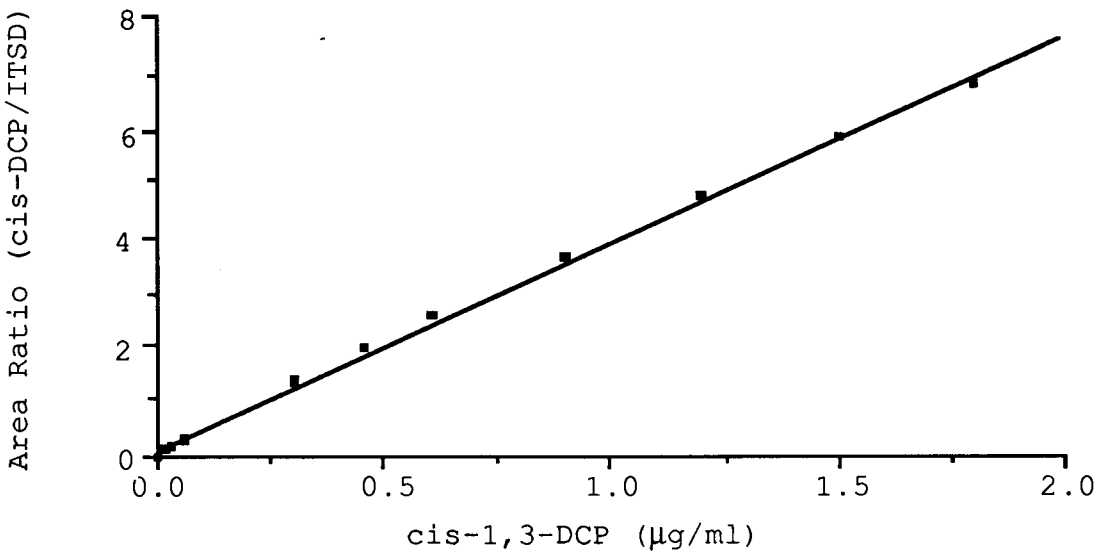
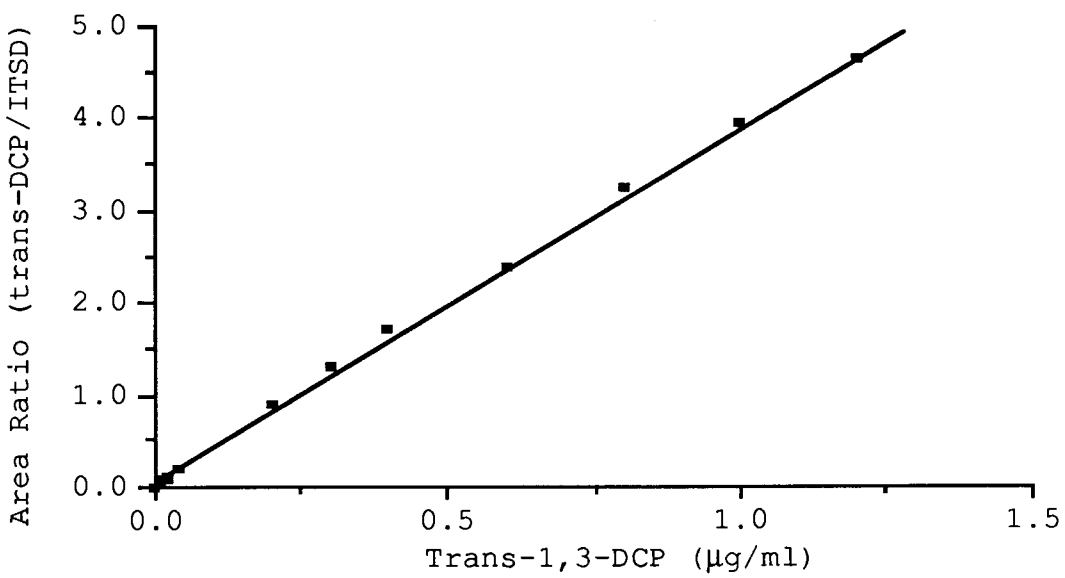


Fig. M-9

Blood calibration curve of trans-1,3-DCP.



the post-exposure blood data was obtained by the method of Akaike's Information Criterion (AIC) (Yamaoka et al, 1978).

The combined exposure and post-exposure blood data was then fitted to a two-compartment open toxicokinetic model (see Results) with NONLIN (Metzler et al., 1974) to obtain the toxicokinetic parameters for the model. Computer simulation was carried out with the optimized toxicokinetic parameters using CSMP (IBM, 1972). This technique was described in detail by Ramsey et al (1980).

2. The effects of chemical pretreatments on the toxicokinetics of 1,3-DCP.

The toxicokinetics of 1,3-DCP in the rats also were studied after pretreating the animals with inhibitors of mixed-function oxidase (MFO) or with a hepatic glutathione-depleting agent. SKF-525A (SKF), dissolved in saline (50 mg/kg) or piperonyl butoxide (PIP, 1 ml/kg), was administered ip to rats. Diethyl maleate (DEM, 0.7 ml/kg) was given to rats ip. Control animals received saline ip. The injection volume was adjusted to about 0.2-0.3 ml. One hour after chemical pretreatment, the rats were put individually into the rat exposure tubes and exposed to a nominal 300 ppm concentration of 1,3-DCP vapor for 4 hrs in a nose-only exposure chamber. The time course of cis- and trans-1,3-DCP concentrations in the whole blood of rats pretreated with

chemical was obtained by procedure similar to that used for the untreated rats (see IV. 1.).

3. Tissue distribution of 1,3-DCP in rats.

a. Exposure of rats to ^{14}C-1,3-DCP.

Twenty rats in groups of 4-5 were used in this study. Rats were exposed to a nominal concentration of 100 ppm ^{14}C-1,3-DCP (specific activity 0.0128 μCi/μmol) mixture vapor for 3 hrs in a nose-only exposure chamber. They were sacrificed by CO_2 at various times after exposure (0, 3, 6, 12 and 24 hr). The following tissues were removed for ^{14}C analysis: liver, brain, spleen, kidney, heart, lung, trachea, stomach (empty of contents), small intestine sample (empty of contents), large intestine sample (empty of contents), urinary bladder (empty of contents), testes, epididymis, subcutaneous fat sample, perirenal fat sample and muscle . The removed tissues were rinsed with saline (0.9% NaCl), dried with tissue paper and stored at -20°C until analysis. Radioactivity in the tissues was determined according to the method described by Law (1981). Briefly, the tissues were minced and divided into portions suitable for combustion (200-300 mg) in a Packard Tri-Carb Biological Material Oxidizer. Tissue ^{14}C was converted to $^{14}CO_2$ which was trapped in Carbo-Sorb® and mixed with toluene based Permafluor® scintillation cocktail (Canberra Packard, Mississauge, Ont.).

The radioactivity in the sample was determined in a Beckman LS 8000 liquid scintillation counter (Beckman Instrument Co., Chicago, IL) using the automatic external standard method.

After exposure, the rats were put individually in a metabolic cage for collection of urine and feces. Urine was collected at 3, 6, 12, and 24 hrs after exposure. Aliquots of urine sample (0.5 ml) were added directly to a glass liquid scintillation vial which contained 10 ml of Bioflour cocktail for ^{14}C determination. Feces were weighed and added to capped vials that contained 5 ml of 1% Triton X-100. The vials were allowed to stand at room temperature overnight. Duplicate samples (< 300 mg) of the solubilized feces were combusted to $^{14}CO_2$ in a Packard Tri-Carb Biological Material Oxidizer as described above.

Total radioactivity in the blood was determined by liquid scintillation counting. A blood sample (0.2 ml) was pipetted into a glass scintillation vial that contained 1 ml of a Protosol and ethanol mixture (1:1 v/v). The vial was capped and left at room temperature overnight for digestion. After the addition of conc. HCl (0.3 ml) and 30% H_2O_2 solution (0.3 ml) to the sample for neutralizing and decolorizing, the radioactivity in the blood was determined by a scintillation counter.

b. Exposure of rats to unlabeled 1,3-DCP.

Twenty-six male Sprague-Dawley rats (≈ 300 g) purchased from Charles River Canada, Inc., St. Constant, Quebec were used in this experiment. Rats were exposed to a nominal 300 ppm concentration of 1,3-DCP mixture vapor for 4 hrs in the whole-body inhalation chamber. Three or four rats were removed from the chamber at selected time intervals (0, 0.5, 1, 2, 3, 6, 12, 18 hrs) after exposure and immediately sacrificed by CO_2. Blood samples were obtained by heart puncture and stored in individual capped vials which contained a pinch of heparin. The following tissues were removed for 1,3-DCP analysis from each rat: liver, kidneys, brain, lungs, stomach (empty of contents), small intestines (empty of contents), perirenal fat samples and muscles. The removed tissues were rinsed with saline, dried with tissue paper and stored in a -20°C freezer until analysis.

Approximately 0.5-g of tissue was weighed and transferred to a glass homogenizer tube. The tissue was homogenized in 2 ml H_2O at low speed. A solution containing 2 ml of 1N H_2SO_4 and 5 ml ether and 25 µg/ml chlorocyclohexane as an internal standard were added to the tissue homogenate. The mixture was shaken in a reciprocating shaker for 10 min and centrifuged at 1,000 X g for 10 min. The ether extract was passed through a Florisil® column before being analyzed by gas chromatography, using conditions as described before.

Recovery of 1,3-DCP from tissues was estimated by introducing a known amount of the chemical directly into tissue samples collected from untreated rats and extracting with ether as described above.

V. Effects of 1,3-DCP on the MFO system.

1. Effects of inhaled 1,3-DCP on the hepatic and renal MFO systems of the rat.

Twenty-two male Wistar rats (≈ 300 g) in groups of four (4-6 rats/group) were exposed to different nominal concentrations of 1,3-DCP vapor (0, 35, 150, 300 ppm) 6 hrs/day for 3 days in a whole-body exposure chamber. The rats were housed individually in stainless-steel wire mesh cages located on the same level of the whole-body chamber. Exposure began at 0630 to 0700 every day. After a 6 hrs exposure each day, the rats were removed from the inhalation chamber and kept in a separate room. Purina rat chow and water were provided *ad libitum* except during the exposure periods.

The rats were sacrificed by decapitation on the third day immediately after the 6-hr period exposure. The livers and kidneys were quickly excised. The samples were rinsed with ice-cold 1.15% KCl-0.05 M HEPES buffer (pH 7.3) and stored separately in cooled beakers.

The livers and kidneys were dissected free of any pieces of connective tissue, gently blotted dry with tissue paper and weighed. The samples were suspended (1:4 w/v) in 1.15% KCl-0.05 M HEPES buffer (pH 7.3) and homogenized with a motor-driven Teflon-glass homogenizer (4 strokes) as described by Bend et al (1972). The homogenate was centrifuged for 30 min at 10,000 X g in a Sorvall RC-5B refrigerated superspeed centrifuge (DuPont Company, Newtown, CT). The post-mitochondrial supernatant was transferred to another centrifuge tube and centrifuged at 105,000 X g for 60 min in a Sorvall preparative Ultracentrifuge OTD 75B (DuPont Company, Newtown CT). The microsomal pellet was resuspended in HEPES-KCl buffer and recentrifuged at 105,000 X g for 60 min. The washed microsomes were finally suspended in HEPES-KCl buffer (pH 7.3) at a concentration of 6 mg protein/ml. The entire procedure was carried out at 0°-4°C. Protein concentrations was determined by the method of Lowry et al (1951), using bovine serum albumin as the standard. Microsomal cytochrome P-450 and cytochrome b_5 contents were determined by the method described by Omura and Sato (1964).

A typical incubation mixture for MFO enzyme assay consisted of microsomal protein (1.5 mg), NADP (2.8 mM), glucose-6-phosphate (8 mM), glucose-6-phosphate dehydrogenase (2 units), 0.25 ml 1.0 M HEPES buffer (pH 7.4 - 7.8) and substrate as indicated. The final volume was adjusted to 2

ml with distilled water. Incubation was carried out at 37°C in a Dubnoff metabolic incubator (GCA/Precision Scientific). The incubation mixture was pre-incubated for 5 min to generate NADPH before the addition of substrate. Incubation time was either 10 min or 15 min. A blank sample containing no NADPH-generating system also was included in the assay. Substrate concentrations were aminopyrene, 5 μM; 7-ethoxycoumarin, 0.2 μM; 7-ethoxyresorufin, 0.1 mM; benzo(α)pyrene, 0.16 μM. Aminopyrene N-demethylase activity was measured by the formation of formaldehyde (Sladek and Mannering 1969). The activity of 7-ethoxycoumarin O-deethylase was assayed fluorimetrically by determining the production of 7-hydroxycoumarin (Creaven et al., 1965). The activity of 7-ethoxyresorufin O-deethylase by microsomes was measured fluorimetrically by determination of the amount of resorufin produced using the method developed by Pohl and Fouts (1980). Benzo(α)pyrene hydroxylase was determined by the formation of water soluble, radioactive metabolites (Depierre et al., 1975).

2. *In vitro* destruction of cytochrome P-450.

Male Wistar rats (≈ 300 g) were used in these studies. The rats were pretreated ip with 80 mg/kg of phenobarbital (PB) (dissolved in saline) daily for 3 days to induce cytochrome P-450. Control animals were treated ip with saline only. The rats were killed at 24 hr after the last

pretreatment. Liver microsomes were prepared as mentioned before.

The incubation mixture contained microsomal protein (3 mg), NADP (2.8 mM), glucose-6-phosphate (8 mM), glucose-6-phosphate dehydrogenase (2 units), 0.25 ml 1.0 M HEPES buffer (pH 7.8), $MgCl_2$ (15 mM), butylated hydroxytoluene (20 µM, dissolved in 0.01 ml ethanol), EDTA (1.5 mM) and 10 mM or 1 mM 1,3-DCP in a final volume of 2.0 ml. The sample was preincubated at 37°C for 5 min to generate NADPH and the reaction was initiated by the addition of 0.01 ml of 1,3-DCP (dissolved in ethanol). At the conclusion of a 10 min incubation, the mixture was diluted with 4 ml 1.15% KCl-0.05 M HEPES buffer (pH 7.3) and kept in ice. The content of cytochrome P-450 was measured as described before.

3. *In vitro* metabolism and irreversible binding of ^{14}C-1,3-DCP in rat microsomal protein.

Liver microsomes were prepared from untreated male Wistar rats as described before. A typical incubation contained microsomal protein, HEPES buffer (1.0 M, pH 7.4), glucose-6-phosphate (8 mM), glucose-6-phosphate dehydrogenase (2 units), NADP (2.8 mM) and test substances as indicated. The final volume was adjusted with distilled water to 2 ml. The mixture was pre-incubated for 5 min at 37°C to allow NADPH generation. The reaction was started by the addition of ^{14}C-

1,3-DCP (0.12 nmole; 19 nCi in 20 µl ethanol) and stopped by adding 1 ml of ethanol. Boiled microsomes were used as controls. After the addition of trichloroacetic acid (TCA, 30%), the mixture was poured into a 12-ml centrifuge tube and centrifuged at 1,000 X g for 15 min. The supernatant was discarded, precipitated proteins were extracted repeatedly with methanol according to the procedure described previously by Jollow et al (1973). Briefly, the precipitated proteins were resuspended in 3 ml 80% methanol and vortexed thoroughly for 5 min in a centrifuge tube. A narrow stainless-steel spatula was inserted in the centrifuge tube to facilitate mixing. The mixture was centrifuged at 1,000 X g for 10 min, and the supernatant fraction was discarded. Another 3 ml fresh 80% methanol was added into the centrifuge tube to repeat the extraction. This procedure was repeated seven times. No radioactivity was found in the methanol extract of the last extraction, indicating that all the 1,3-DCP and metabolites that were not bound covalently with the hepatic microsomal protein had been removed completely.

The protein precipitates then were digested in 1 ml of Protosol and ethanol mixture (1:1 v/v) and transferred into a 20-ml liquid scintillation vial which contained 10 ml Biofluor. The radioactivity in the sample was measured in a Beckman LS 8000 liquid scintillation counter and represented the amount of ^{14}C-1,3-DCP-derived radioactivity bound irreversibly to microsomal protein.

4. Statistical analysis.

Mean and standard deviations were calculated for each experimental group. The statistical significance of difference among the means of treated and control groups of rats was determined using one-way anova test with $p < 0.05$ accepted as significant.

§3. RESULTS

I. Pharmacokinetics of inhaled 1,3-DCP in rats.

1. Dose-dependent pharmacokinetics of 1,3-DCP.

1,3-DCP concentration in the exposure chamber was stable during the exposure period. The nominal (actual mean ± SD) concentrations were 100 ppm (91.1 ± 11.0 ppm); 300 ppm (294.3 ± 32.9 ppm); and 500 ppm (487.1 ± 44.4 ppm). The actual concentration was determined from the average of 10 different gas chromatographic determinations at 30-min intervals during the study.

The extraction procedure removed > 85% of the 1,3-DCP added to the blood (Table R-1).

Concentrations of unchanged cis- and trans-1,3-DCP in the blood of rats exposed to various 1,3-DCP mixture vapor concentrations are shown in Fig. R-1 and Fig. R-2, respectively. Blood concentrations of both 1,3-DCP isomers increased with exposure time and reached a plateau level in about 2-2.5 hrs when rats were exposed to 100 ppm 1,3-DCP. The exposure time required by the isomers to reach a plateau in the blood was extended to 3-3.5 hr in rats exposed to 300 ppm or 500 ppm 1,3-DCP.

Table R-1

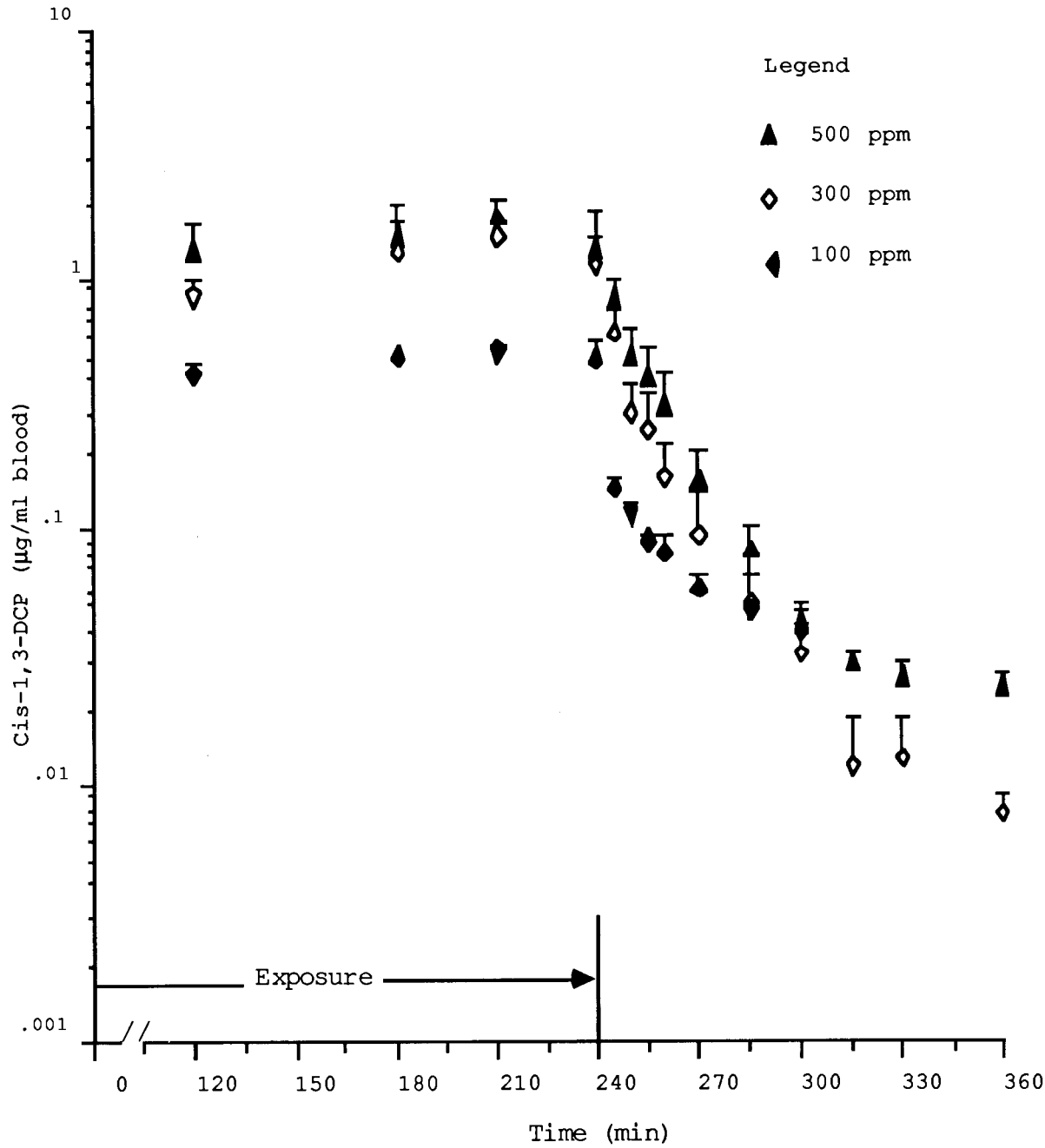
Recovery (%) of cis- and trans-1,3-DCP from whole blood.*

Solution (Hexane) extraction volume (ml)	cis-DCP	trans-DCP
0.5	85.87 ± 1.09	86.55 ± 0.74
1.0	95.66 ± 1.01	95.70 ± 0.52
2.0	94.92 ± 5.99	95.02 ± 6.07

* Values represent mean ± S.D. from 3 determinations.

Fig. R-1

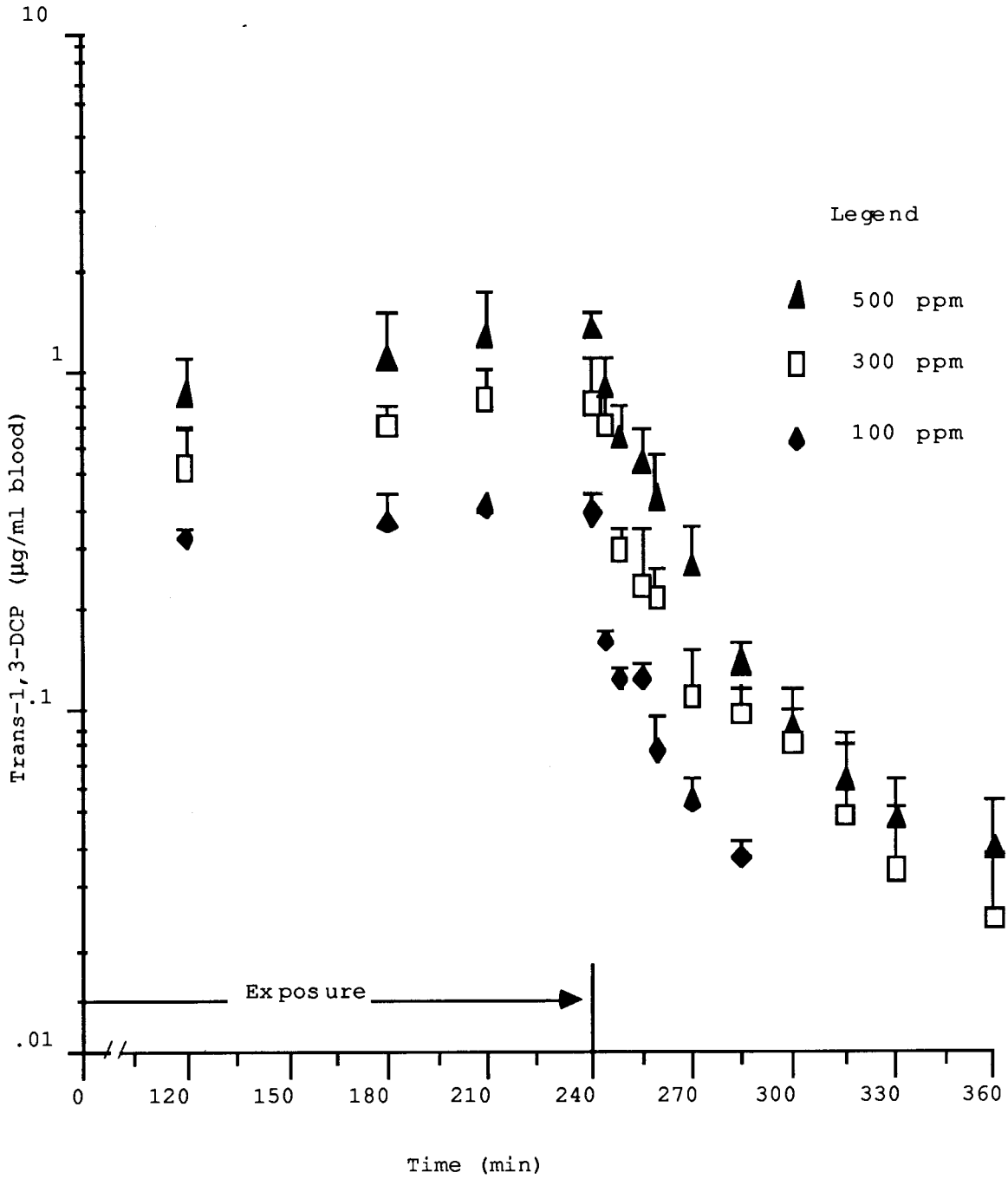
Blood kinetic data for cis-1,3-DCP in rats exposed to 100, 300 and 500 ppm 1,3-DCP.



* Data are mean ± SD of 3 rats.

Fig. R-2

Blood kinetic data for trans-1,3-DCP and in rats exposed to 100, 300 and 500 ppm 1,3-DCP.



* Data are mean ± SD of 3 rats.

The average end-exposure blood levels of cis- and trans-1,3-DCP were 0.49 ± 0.10 μg/ml and 0.38 ± 0.06 μg/ml, respectively for the 100 ppm rats; 1.13 ± 0.32 μg/ml and 0.75 ± 0.26 μg/ml, respectively for the 300 ppm rats; and 1.66 ± 0.17 μg/ml and 1.34 ±0.19 μg/ml, respectively for the 500 ppm rats.

At the conclusion of a 4-hr exposure, blood concentration of cis- and trans-1,3-DCP in rats declined rapidly. The post-exposure data were analyzed by nonlinear, regression analysis to determine the number of compartments (or exponentials) which best described the blood elimination curve.

Tables R-2 and R-3 show the mean of the weighed sum of square deviation (WSSR) and Akaike's information criterion (AIC) of the postexposure data. The WSSR (WSSR = $\sum_{i=1}^{N} W_i(\hat{Y}_i - Y_i)^2$) measures the deviation between the observed points and predicted values from the fitted curve in minimized regression analysis (Balant and Garrett, 1983). Akaike's information criterion (AIC) (Yamaoka et al, 1978) is defined as, AIC = n x ln WSSR + 2p, where n is the number of experimental data points and p is the variable parameters in an estimated model. The fit with a smaller AIC value is

Table R-2

Akaike's information criterion (AIC) for cis-1,3-DCP.

	one-compartment		two-compartment		three-compartment	
	WSSR	AIC	WSSR	AIC	WSSR	AIC
100 ppm (n=10)	0.74×10^{-2}	-44.98	0.44×10^{-3}	-69.15	nd*	
300 ppm (n=12)	0.43×10^{-2}	-61.38	0.30×10^{-3}	-89.22	0.29×10^{-3}	-85.62
500 ppm (n=11)	0.90×10^{-2}	-47.82	0.23×10^{-2}	-58.91	0.41×10^{-2}	-73.68

* nd: not determined

Table R-3

Akaike's information criterion (AIC) for trans-1,3-DCP.

	one-compartment		two-compartment		three-compartment	
	WSSR	AIC	WSSR	AIC	WSSR	AIC
100 ppm (n=10)	0.51×10^{-2}	-48.72	0.14×10^{-2}	-57.22	nd*	
300 ppm (n=12)	0.49×10^{-2}	-59.72	0.86×10^{-3}	-76.60	0.87×10^{-3}	-72.57
500 ppm (n=11)	0.83×10^{-2}	-48.74	0.69×10^{-3}	-72.03	0.56×10^{-3}	-70.28

* nd: not determined.

considered to be best for the experimental data. As shown in Tables R-2 and R-3, a bi-exponential decline was the best to describe the time course of the cis- and trans- isomers in the blood after rats were exposed to 1,3-DCP mixture vapor in a nose-only exposure chamber. The half-lives of the post-exposure terminal phase of cis- and trans-1,3-DCP were 34.4 min and 26.5 min, respectively, in rats exposed to 100 ppm 1,3-DCP; 54.5 min and 66.0 min, respectively in rats exposed to 300 ppm 1,3-DCP; and 49.8 min and 52.9 min, respectively in rats exposed to 500 ppm 1,3-DCP.

Since the average blood levels of the 1,3-DCP isomers increased disproportionately to the exposure concentration and the calculated half-lives of elimination were not constant, the disappearance of the 1,3-DCP isomers from the blood was well described as a zero-order process rather than first-order process. Therefore, the exposure and post-exposure time course of 1,3-DCP isomers in the blood could be described by a two-compartment open toxicokinetic model with zero-order absorption and saturable elimination processes. The differential equations describing the dynamics of this models are given in equations (1), (2) and (3):

$$\frac{dA_1(t)}{dt} = \left\{ \begin{matrix} k^\circ, & 0 < t \leq t_b \\ 0, & t > t_b \end{matrix} \right\} + k_{21}A_2(t) - \left[k_{12} + \frac{V_{max}V_1}{K_mV_1 + A_1(t)} \right] A_1(t) \quad (1)$$

$$\frac{dA_2(t)}{dt} = k_{12}A_1(t) - k_{21}A_2(t) \quad (2)$$

$$C_1(t) = \frac{A_1(t)}{V_1} \quad (3)$$

Where,

k° = Zero-order input rate constant ($\mu g/min$).

k_{12}, k_{21} = Apparent first-order mass transfer coefficients (min^{-1}).

K_m = Michaelis constant ($\mu g/ml$).

V_{max} = Enzymic activity at saturation ($\mu g/g \cdot min$).

$A_1(t)$ = Amount of a 1,3-DCP isomer in compartment 1 at time t (μg).

$A_2(t)$ = Amount of a 1,3-DCP isomer in compartment 2 at time t (μg).

t = Elapsed time (min).

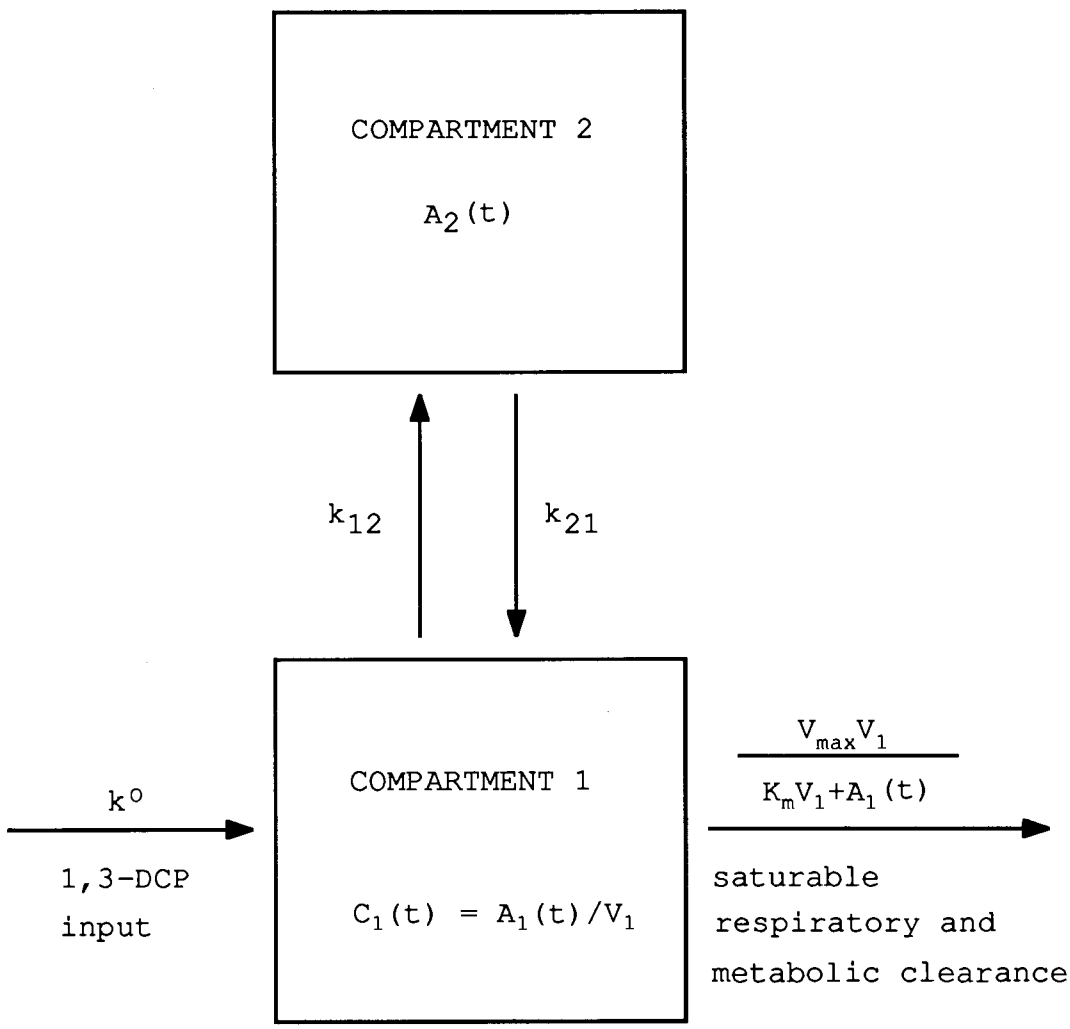
t_b = Time at end of exposure period (min).

V_1 = Apparent volume of distribution of compartment 1 (ml).

The model (Fig. R-3) assumed that a constant value (k°) of the inspired 1,3-DCP vapor was absorbed into compartment 1 throughout the exposure period. This value varied with the exposure concentration since Stott and Kastl (1986) reported that the respiratory ventilation frequency of rats exposed to $\geq$ 90 ppm 1,3-DCP decreased with increasing exposure concentration. The k° for the proposed model was estimated

Fig. R-3

Two-compartment toxicokinetic model with zero-order (constant rate) input and saturable clearance describing the kinetics of inhaled 1,3-DCP vapour in rats.



from the uptake rate reported by Stott and Kastl (1986) and the ratio of cis/trans 1,3-DCP in the present study. Therefore, the uptake rates of 1,3-DCP for the 100 ppm rats and 300 ppm rats were 307 nmol/min and 880 nmol/min, respectively. For the 500ppm rats, the uptake rate was 1345 nmol/min which was the average uptake rate of 300 ppm and 900 ppm rats reported by Stott and Kastl (1986). The k° were calculated from these uptake rates after correcting for the cis/trans isomer ratio (1.43). Therefore, the k° of cis-1,3-DCP (trans-1,3-DCP) were 19.4 µg/min (13.6 µg/min), 55.7 µg/min (39.1 µg/min) and 85.1 µg/min (59.7 µg/min) for 100 ppm, 300 ppm and 500 ppm 1,3-DCP, respectively, in the present study. Since the empirical values for K_m, V_{max}, k_{12}, k_{21} and V_1 were not available, I determined values for these parameters which produced the best fits with the blood data. Tables R-4 and R-5 show the toxicokinetic parameters which produced the best fits with the empirical data. Computer simulated blood concentration-time curves generated from these parameters are shown in Figs. R4 - R9. The model-predicted 1,3-DCP concentrations in the blood agreed with the experimentally obtained concentrations only before the 315 min time point. Total clearance, Cl_{total}, of absorbed 1,3-DCP from the rat is given by equation (4),

$$Cl_{total} = \frac{2\,V_{max} \cdot V_1^{\,2}}{D + 2\,K_m \cdot V_1} \qquad (4)$$

where, D = Total dose absorbed (µg),

Table R-4

Toxicokinetic parameters describing the uptake and clearance of cis-1,3-DCP in the blood of rats exposed to different 1,3-DCP vapor concentration for 4 hrs.*

	Exposure concentration (ppm)		
	100	300	500
k° (μg/min)	19.4	55.7	85.1
k_{12} (min^{-1})	0.157	0.225	0.120
k_{21} (min^{-1})	0.024	0.046	0.051
V_{max} (μg/g.min)	0.212	0.292	0.279
K_m (μg/ml)	0.293	0.198	0.610
V_1 (ml)	139	208	416
Cl_{total} (ml/min)	1.73	1.88	4.61

* Values are mean of 3-4 rats

Table R-5

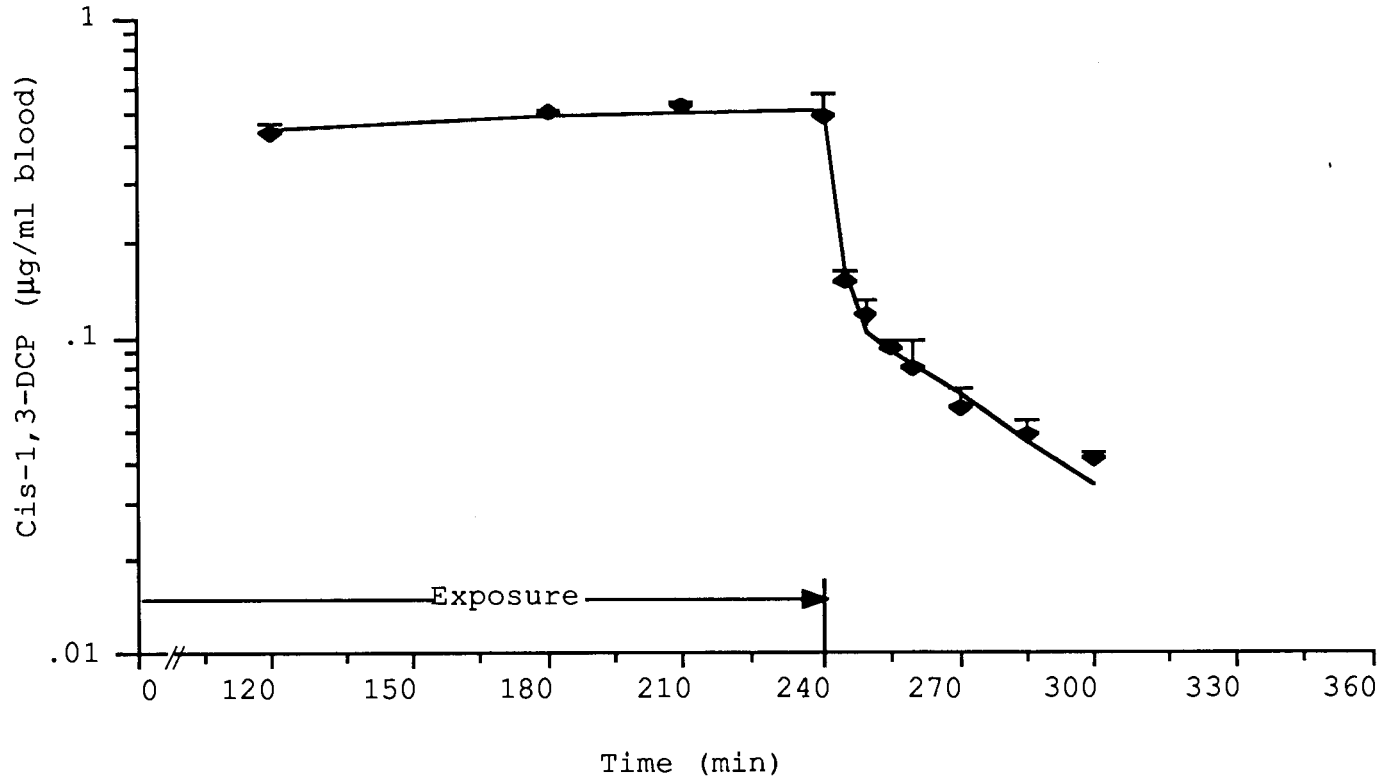
Toxicokinetic parameters describing the uptake and clearance of trans-1,3-DCP in the blood of rats exposed to different 1,3-DCP vapor concentration for 4 hr.*

	Exposure concentration (ppm)		
	100	300	500
k° (μg/min)	13.6	39.1	59.7
k_{12} (min^{-1})	0.212	0.063	0.160
k_{21} (min^{-1})	0.038	0.012	0.034
V_{max} (μg/g.min)	0.111	0.057	0.133
K_m (μg/ml)	0.160	0.098	0.129
V_1 (ml)	165	607	418
Cl_{total} (ml/min)	1.82	4.42	3.22

* Values are mean of 3-4 rats.

Fig. R-4

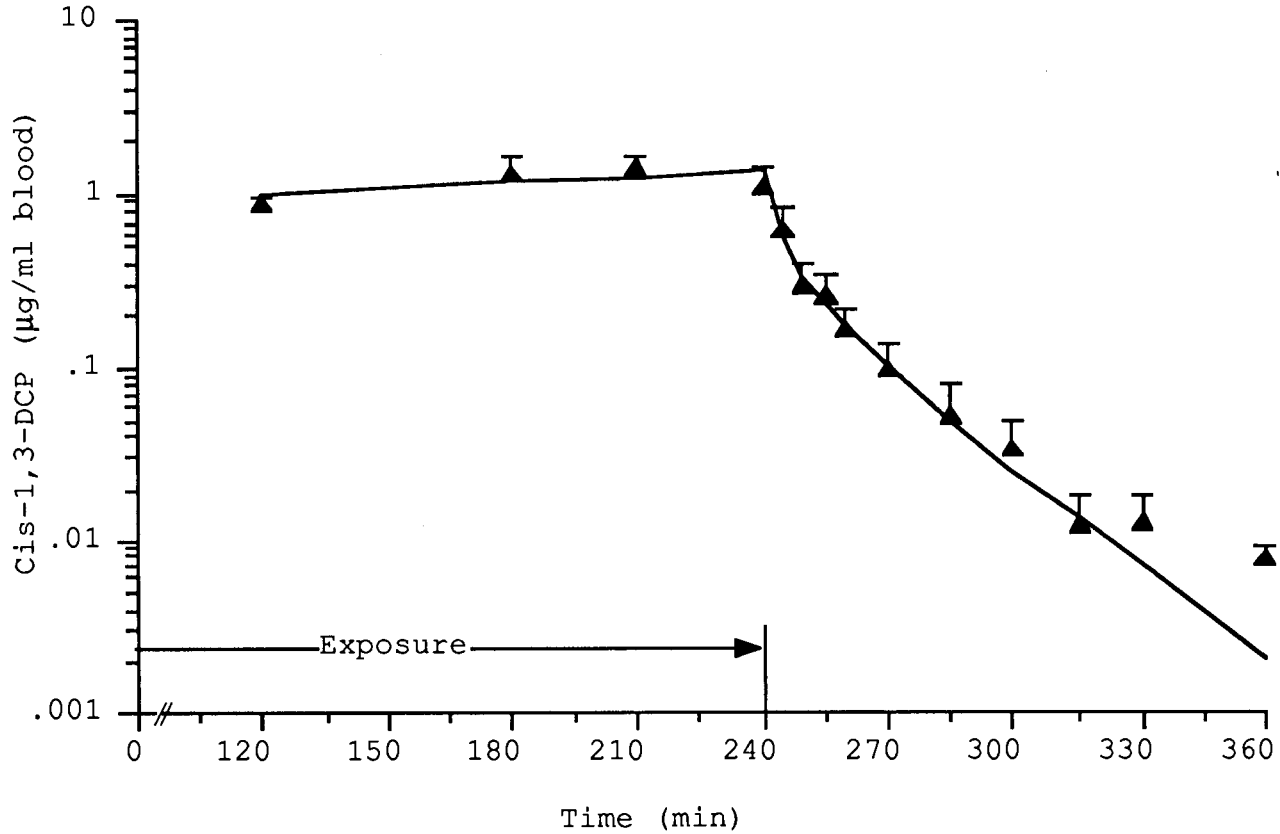
Blood kinetic data for cis-1,3-DCP in rats exposed to 100 ppm 1,3-DCP.*



* Data are mean ± SD of 3 rats. The solid line represents the predicted blood levels based on computer simulation of the optimized toxicokinetic parameter values.

Fig. R-5

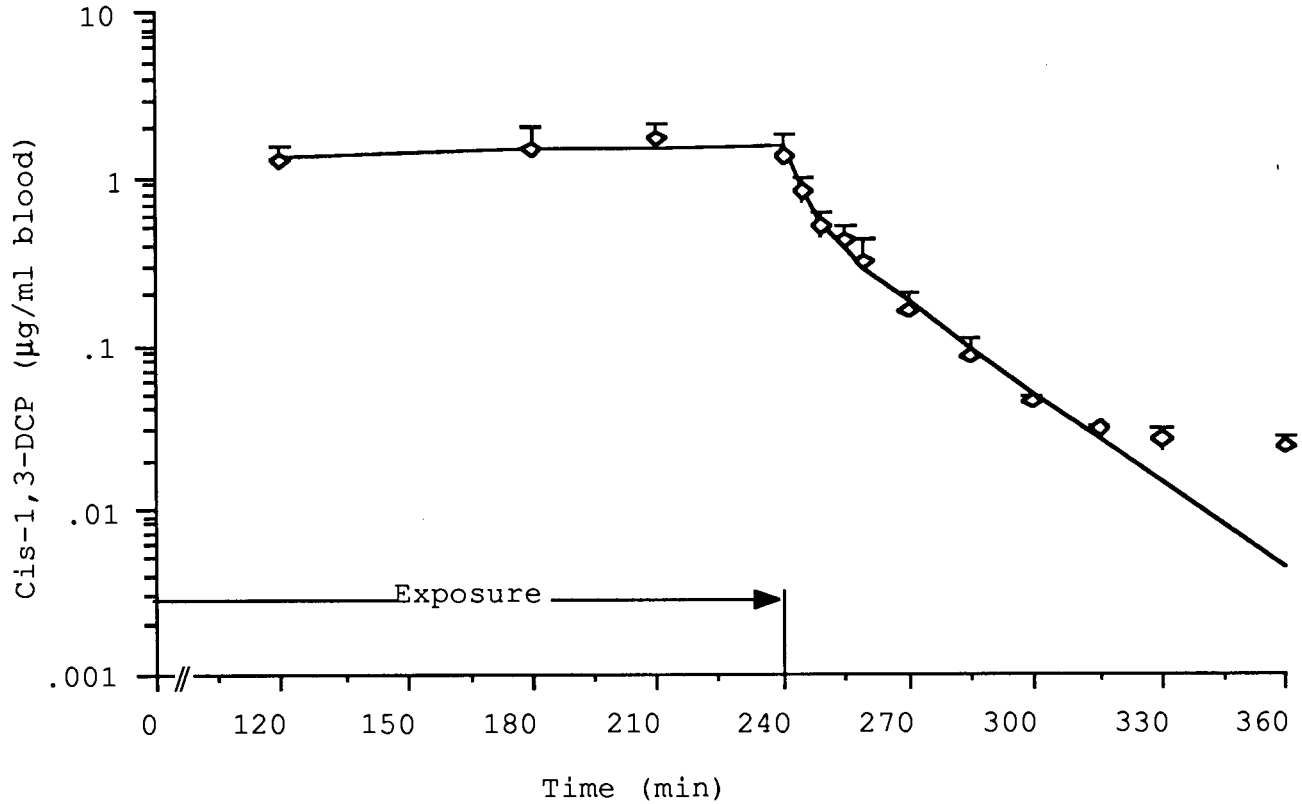
Blood kinetic data for cis-1,3-DCP in rats exposed to 300 ppm 1,3-DCP.*



* Data are mean ± SD of 3 rats. The solid line represents the predicted blood levels based on computer simulation of the optimized toxicokinetic parameter values

Fig. R-6

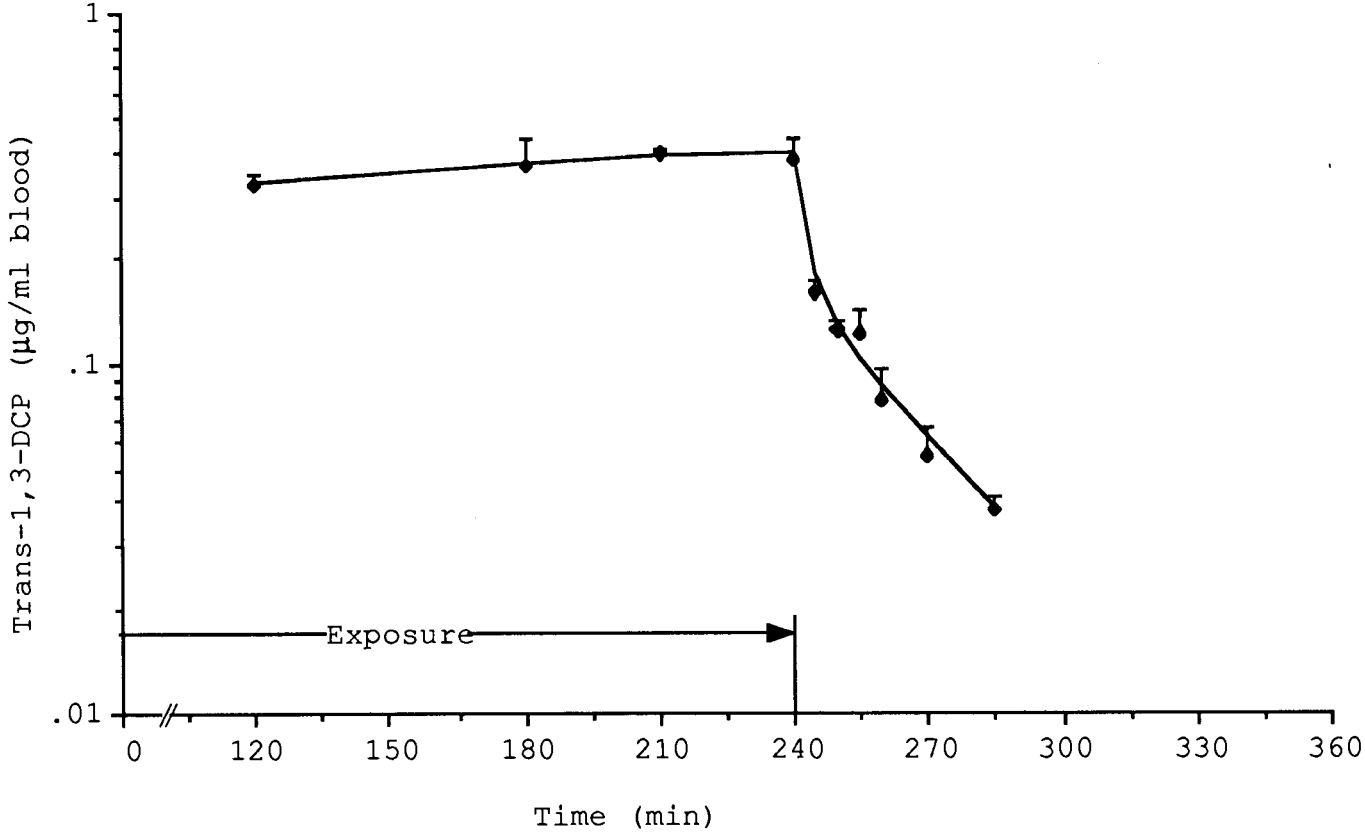
Blood kinetic data for cis-1,3-DCP in rats exposed to 500 ppm 1,3-DCP.*



* Data are mean ± SD of 3 rats. The solid line represents the predicted blood levels based on computer simulation of the optimized toxicokinetic parameter value.

Fig. R-7

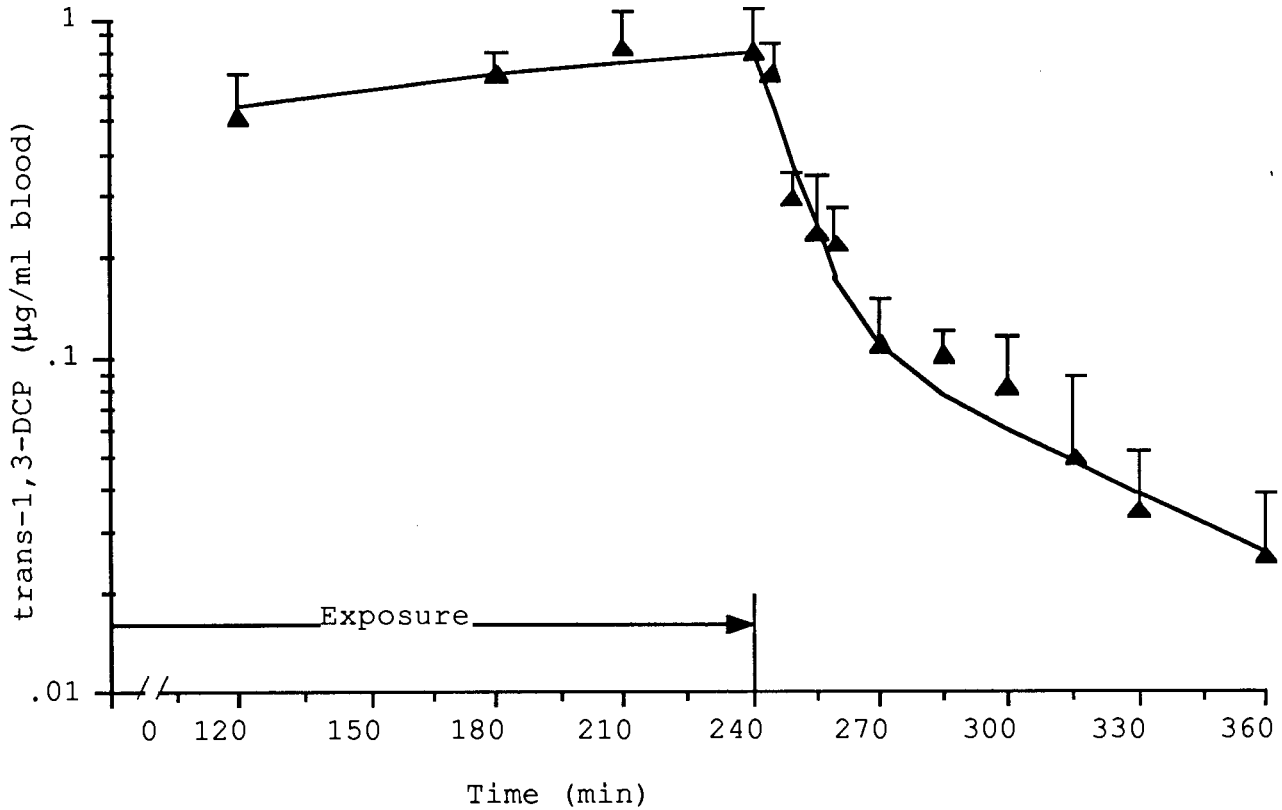
Blood kinetic data for trans-1,3-DCP in rats exposed to 100 ppm 1,3-DCP.*



* Data are mean ± SD of 3 rats. The solid line represents the predicted blood levels based on computer simulation of the optimized toxicokinetic parameter values.

Fig. R-8

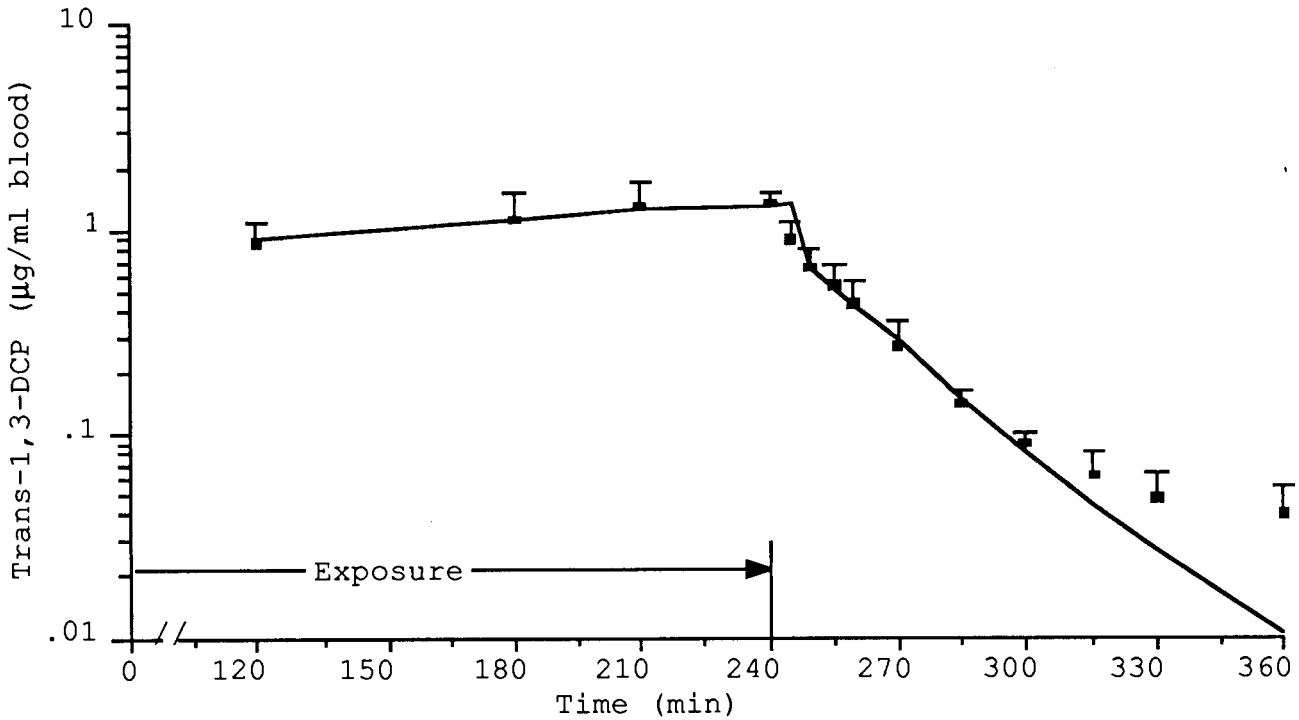
Blood kinetic data for trans-1,3-DCP in rats exposed to 300 ppm 1,3-DCP.*



* Data are mean ± SD of 3 rats. The solid line represents the predicted blood levels based on computer simulation of the optimized toxicokinetic parameter values.

Fig. R-9

Blood kinetic data for trans-1,3-DCP in rats exposed to 500 ppm 1,3-DCP.*



* Data are mean ± SD of 3 rats. The solid line represents the predicted blood levels based on computer simulation of the optimized toxicokinetic parameter values.

K_m = Michaelis constant (μg/ml),

V_{max} = Enzymic activity ar saturation (μg/g.min),

V_1 = Apparent volume of distribution of compartment 1 (ml).

Clearance is defined as the volume of blood cleared of a toxicant per unit of time and it is dose-dependent. The calculated Cl_{total} of cis-1,3-DCP (trans-1,3-DCP) are 1.73 ml/min (1.82 ml/min), 1.88 ml/min (4.42 ml/min) and 4.61 ml/min (3.22 ml/min) for rats exposed to 100 ppm, 300 ppm and 500 ppm 1,3-DCP, respectively.

To further substantiate that 1,3-DCP was elimination from the blood by a zero order process, attempts were also made to fit the blood data to the same model with a first order elimination process. However, the results showed that the first order elimination didn't describe adequately the experimental data.

2. Effects of pretreatment of rats with MFO inhibitors or glutathione depleting agent on the toxicokinetics of 1,3-DCP.

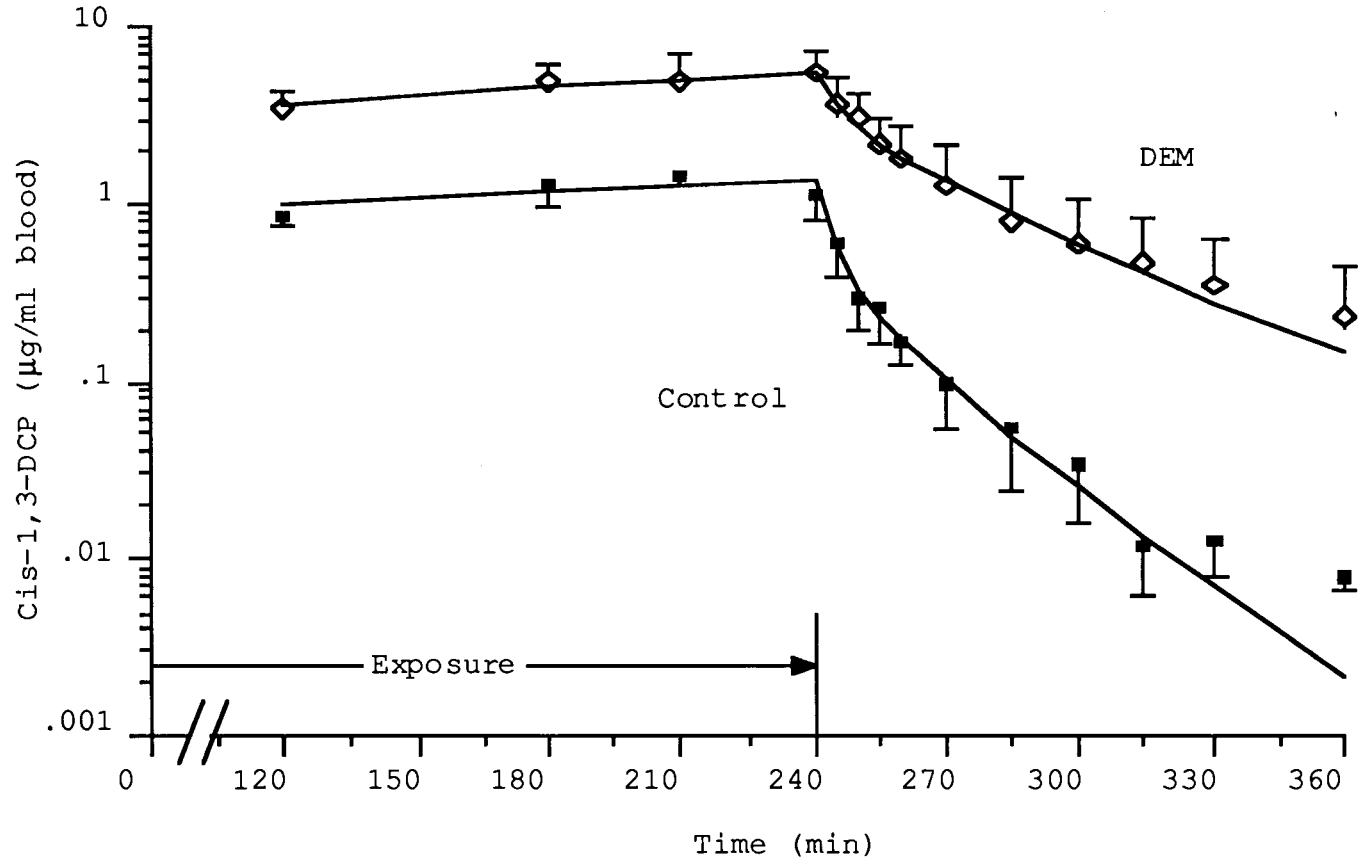
The actual concentration of 1,3-DCP in the exposure chamber for this study was 298.8 ± 36.1 (n = 40). A steady-state blood concentration of cis- or trans-1,3-DCP could not be achieved in rats pretreated with diethyl maleate and piperonyl butoxide after they were exposed to 1,3-DCP for 4

hrs (Fig. R-10, R-11, R-12, and R-13). In contrast, the blood concentrations of both 1,3-DCP isomers in rats pretreated with SKF-525A were similar to those of the control rats (Fig. R-14 and R-15). Both isomers reached their steady-state blood concentrations after approximately 3.5 hrs of exposure. The average end-exposure blood levels for cis- and trans-1,3-DCP were: 1.13 ± 0.32 μg/ml and 0.75 ± 0.26 μg/ml, respectively in control rats; 1.01 ± 0.20 μg/ml and 0.75 ± 0.18 μg/ml, respectively, in rats pretreated with SKF-525A; 2.42 ±1.24 μg/ml and 2.46 ± 1.20 μg/ml, respectively, in rats pretreated with piperonyl butoxide; and 5.47 ± 1.80 μg/ml and 2.74 ± 0.71 μg/ml, respectively, in rats pretreated with diethyl maleate.

The model also was used to simulate the time course of cis- and trans-1,3-DCP concentration in the blood of rats pretreated with MFO inhibitors and diethyl maleate. The best estimates of the toxicokinetic parameters are shown in Tables R-6 and R-7. Blood concentration-time curves generated from these parameters are shown in Figs. R-10 to R-15. As with the control rats ip saline, the model-predicted 1,3-DCP concentrations in the blood agreed with the experimental data only during the period before the 315 min time point. The calculated Cl_{total} of cis-1,3-DCP (trans-1,3-DCP) were 1.88 ml/min (4.42 ml/min), 5.42 ml/min (3.85 ml/min), 1.09 ml/min (2.56 ml/min), and 1.05 ml/min (1.83 ml/min), for saline-,

Fig. R-10

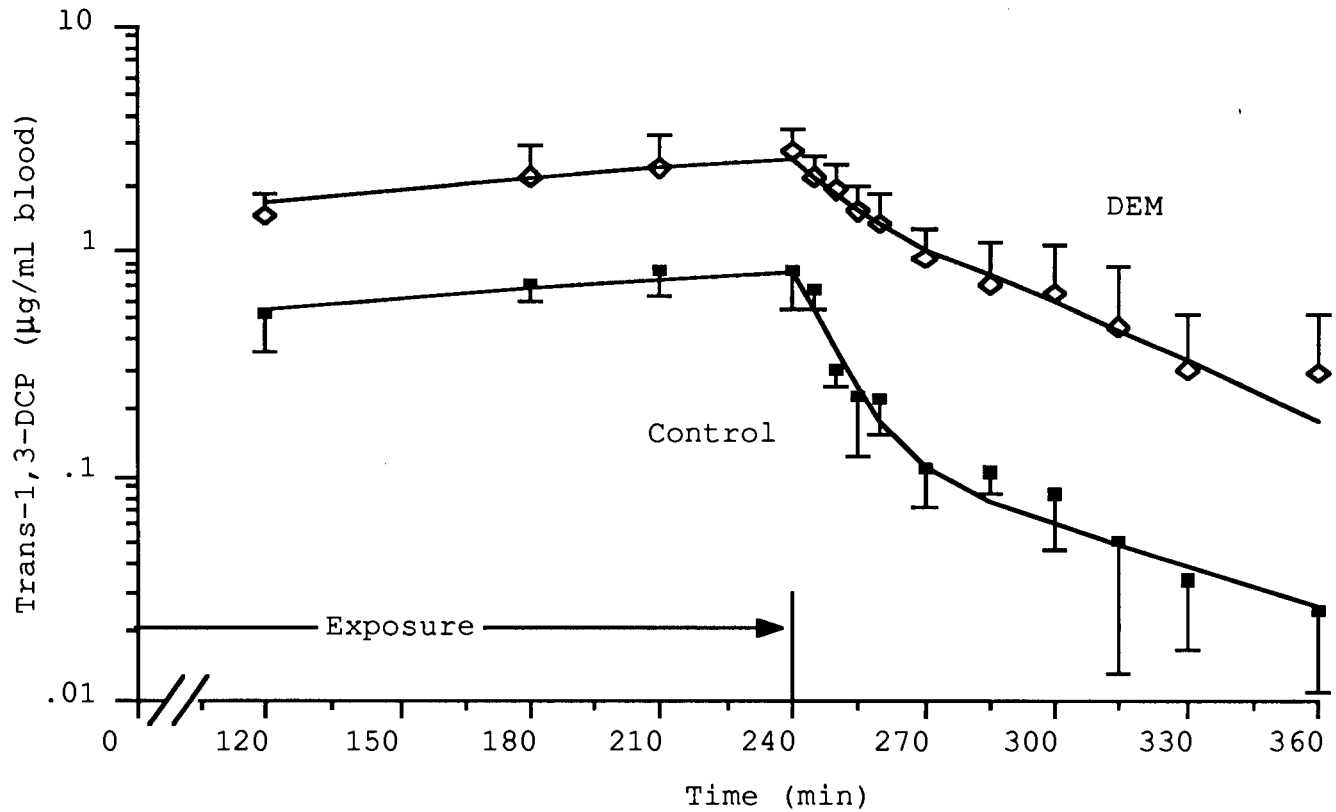
Time-course of cis-1,3-DCP in the blood of rats treated with saline (control) or diethyl maleate prior to exposure of 300 ppm 1,3-DCP vapor for 4 hours.*



* Data are mean ± SD of 3-4 rats. The solid line represents the predicted blood level based on computer simulation of the optimized toxicokinetic parameter values.

Fig. R-11

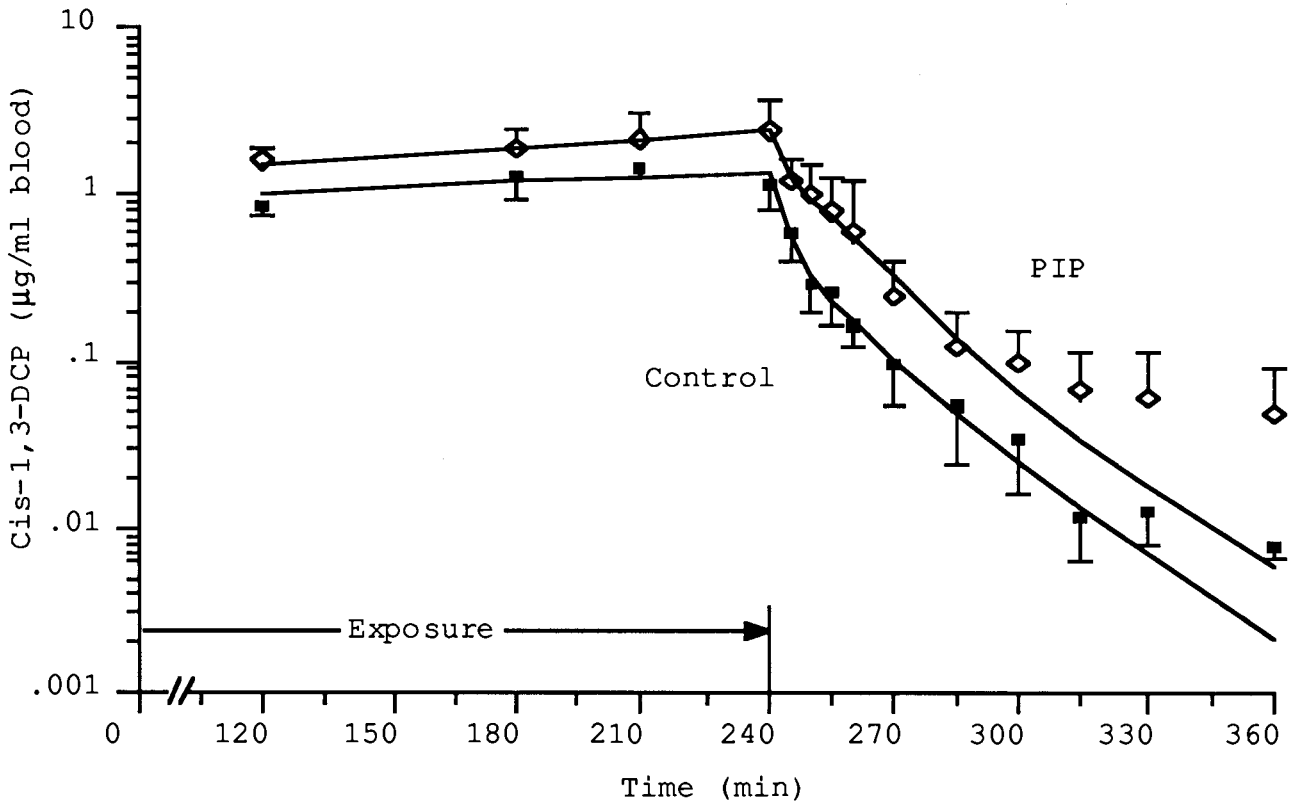
Time-course of trans-1,3-DCP in the blood of rats treated with saline (control) or diethyl maleate prior to exposure of 300 ppm 1,3-DCP vapor for 4 hours.*



* Data are mean ± SD of 3-4 rats. The solid line represents the predicted blood level based on computer simulation of the optimized toxicokinetic parameter values.

Fig. R-12

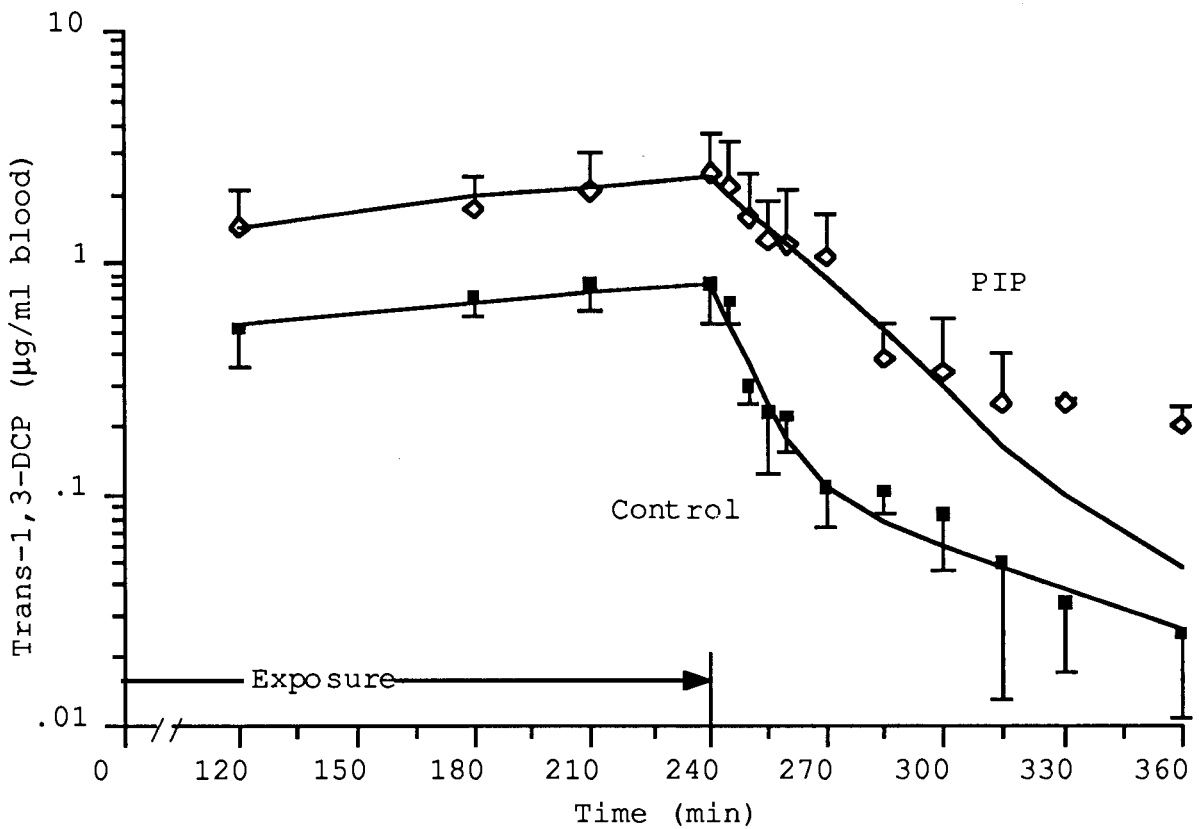
Time-course of cis-1,3-DCP in the blood of rats treated with saline (control) or piperonyl butoxide prior to exposure of 300 ppm 1,3-DCP vapor for 4 hours.*



* Data are mean ± SD of 3-4 rats. The solid line represents the predicted blood level based on computer simulation of the optimized toxicokinetic parameter values.

Fig. R-13

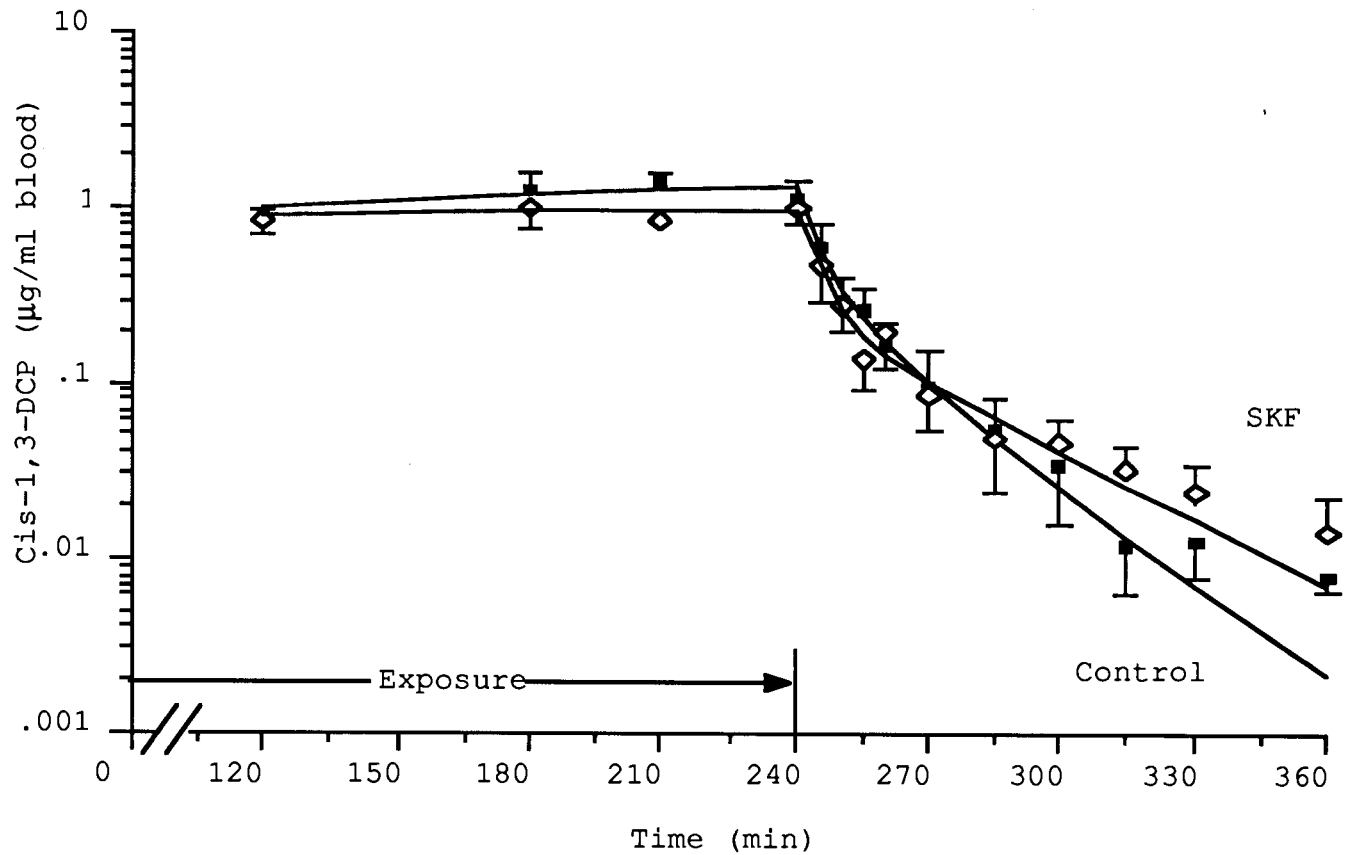
Time-course of trans-1,3-DCP in the blood of rats treated with saline (control) or piperonyl butoxide prior to exposure of 300 ppm 1,3-DCP vapor for 4 hours.*



* Data are mean ± SD of 3-4 rats. The solid line represents the predicted blood level based on computer simulation of the optimized toxicokinetic parameter values.

Fig. R-14

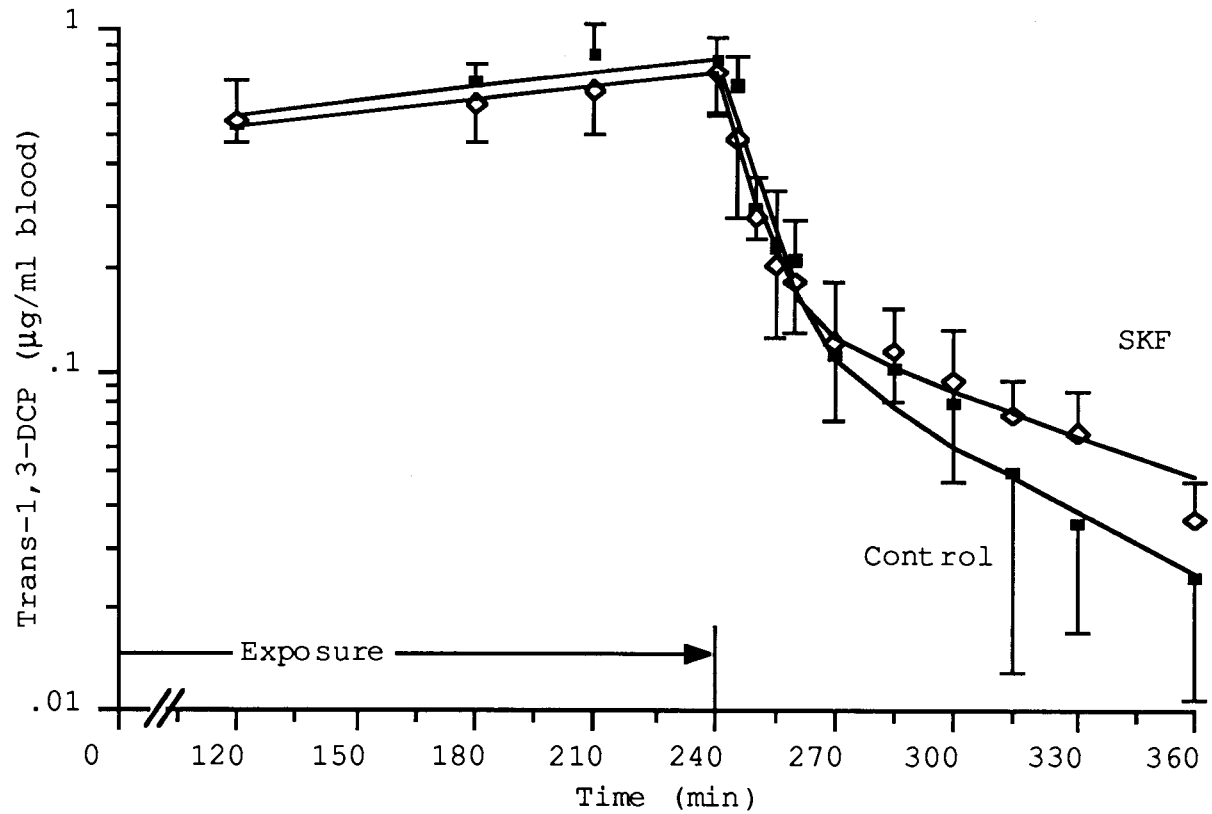
Time-course of cis-1,3-DCP in the blood of rats treated with saline (control) or SKF-525A prior to exposure of 300 ppm 1,3-DCP vapor for 4 hours.*



* Data are mean ± SD of 3-4 rats. The solid line represents the predicted blood level based on computer simulation of the optimized toxicokinetic parameter values.

Fig. R-15

Time-course of trans-1,3-DCP in the blood of rats treated with saline (control) or SKF-525A prior to exposure of 300 ppm 1,3-DCP vapor for 4 hours.*



* Data are mean ± SD of 3-4 rats. The solid line represents the predicted blood level based on computer simulation of the optimized toxicokinetic parameter values.

Table R-6

Toxicokinetic parameters describing the uptake and clearance of cis-1,3-DCP in the blood of rats pretreated with MFO inhibitors or a glutathione depleting agent.[a]

	Pretreatment[b]			
	SKF	PIP	DEM	Saline
k° (μg/min)	55.7	55.7	55.7	55.7
k_{12} (min^{-1})	0.070	0.265	0.096	0.225
k_{21} (min^{-1})	0.035	0.041	0.025	0.046
V_{max} (μg/g.min)	0.222	0.368	0.436	0.292
K_m (μg/ml)	0.640	0.170	0.985	0.198
V_1 (ml)	412	141	128	208
Cl_{total} (ml/min)	5.42	1.09	1.05	1.88

a. Rats were pretreated with one of the chemicals as described in the Methods before being exposed to 300 ppm 1,3-DCP for 4 hrs in a nose-only inhalation chamber. Values are mean of 3-4 rats.

b. SKF, PIP, and DEM are the abbreviations of SKF-525A, piperonyl butoxide and diethyl maleate, respectively. The rats pretreated with saline are as control.

Table R-7

Toxicokinetic parameters describing the uptake and clearance of trans-1,3-DCP in the blood of rats pretreated with MFO inhibitors or a glutathione depleting agent.[a]

	Pretreatment[b]			
	SKF	PIP	DEM	Saline
k° (μg/min)	39.1	39.1	39.1	39.1
k_{12} (min^{-1})	0.081	0.040	0.058	0.063
k_{21} (min^{-1})	0.009	0.015	0.013	0.012
V_{max} (μg/g.min)	0.056	0.069	0.078	0.057
K_m (μg/ml)	0.113	0.104	0.145	0.098
V_1 (ml)	572	419	333	607
Cl_{total} (ml/min)	3.85	2.56	1.83	4.42

a. Rats were pretreated with one of the chemicals as described in the Methods before being exposed to 300 ppm 1,3-DCP for 4 hr in a nose-only inhalation chamber.

b. SKF, PIP, and DEM are abbreviations of SKF-525A, piperonyl butoxide and diethyl maleate, respectively. The rats pretreated with saline are as control.

SKF 525-A-, piperonyl butoxide- and diethyl maleate-pretreated rats, respectively.

3. Tissue distribution of 1,3-DCP in rats.

a. Tissue distribution of ^{14}C after exposing rats to ^{14}C-1,3-DCP in a nose-only exposure chamber.

The actual exposure concentration in the chamber was 97.35 ± 7.73 ppm (n =30). ^{14}C was distributed widely in the rat tissues following a 3-hr exposure to 1,3-(^{14}C)-DCP. Table R-8 shows radioactivity distribution in various tissues High ^{14}C levels were found in the following organs: the urinary bladder (40.14 µg/g), kidney (21.48 µg/g), small intestine (14.10 µg/g) and liver (7.92 µg/g). They were 2 to 20 fold higher than those in the muscle, heart, testes, brain and fat.

Cumulative excretion of ^{14}C in the urine and feces of rats is shown in Fig R-16. The urine was the major elimination route of 1,3-DCP. Nearly all 1,3-DCP absorbed was excreted within 24 hrs.

The concentration of ^{14}C in the blood increased with time during the 3 hrs of exposure (Fig R-17). Peak concentration of ^{14}C in the blood was about 5 µg/ml. ^{14}C was eliminated

Table R-8
Radioactivity concentration in tissue (μg 1,3-(^{14}C)-DCP equivalent/g) of rats exposed to 100 ppm ^{14}C-1,3-DCP* for 3 hrs.

	Time after exposure (hr)				
	0	3	6	12	24
Urinary bladder	40.14 ± 22.93	211.9 ± 171.5	50.92 ± 12.46	5.55 ± 1.75	3.65 ± 0.38
Kidney	21.48 ± 3.97	17.62 ± 10.33	3.48 ± 0.61	2.22 ± 0.35	2.20 ± 0.28
Small Intestine	14.10 ± 3.85	5.63 ± 2.86	2.07 ± 0.33	2.57 ± 1.59	1.04 ± 0.14
Liver	7.92 ± 1.36	3.20 ± 1.72	1.39 ± 0.50	1.53 ± 0.38	1.56 ± 0.26
Large Intestine	4.96 ± 0.32	4.53 ± 1.58	3.74 ± 1.07	4.73 ± 3.21	3.31 ± 1.46
Epididymis	4.70 ± 0.95	3.18 ± 2.54	0.95 ± 0.14	1.86 ± 1.00	0.74 ± 0.10
Trachea	3.95 ± 0.27	2.90 ± 0.30	1.77 ± 0.70	2.03 ± 0.65	1.31 ± 0.22
Subcutaneous fat	3.80 ± 0.13	5.14 ± 0.23	1.05 ± 0.40	0.64 ± 0.45	0.66 ± 0.16
Lung	3.77 ± 0.45	2.83 ± 1.43	1.47 ± 1.08	1.84 ± 0.35	1.21 ± 0.15
Brain	3.63 ± 0.39	1.43 ± 0.66	0.85 ± 0.19	0.72 ± 0.20	0.68 ± 0.02
Stomach	3.32 ± 0.67	3.88 ± 1.58	1.25 ± 0.14	0.88 ± 0.20	0.72 ± 0.05
Spleen	3.22 ± 0.32	2.32 ± 0.65	1.47 ± 0.17	1.50 ± 0.37	1.23 ± 0.18
Testes	3.10 ± 0.24	1.40 ± 0.75	0.52 ± 0.10	0.46 ± 0.02	0.36 ± 0.02
perirenal fats	3.03 ± 0.83	10.16 ± 1.60	0.61 ± 0.27	0.31 ± 0.14	0.32 ± 0.05
Heart	2.88 ± 0.44	1.98 ± 0.92	0.97 ± 0.18	0.87 ± 0.20	0.89 ± 0.15
Muscles	1.72 ± 0.38	1.83 ± 1.25	0.68 ± 0.11	0.63 ± 0.20	0.58 ± 0.04

* Specific activity of ^{14}C-1,3-DCP was 0.0128 μCi/μmole. Each value represents mean ± SD of 3 or 4 rats.

Fig. R-16

Cumulative excretion (1,3-DCP) of radioactivity derived from 1,3-(2-^{14}C)-DCP in rats exposed to 100 ppm ^{14}C-1,3-DCP (specific activity 0.01283 μCi/μmole) for 3 hrs (each point represents mean ± SD of 3 or 4 rats).

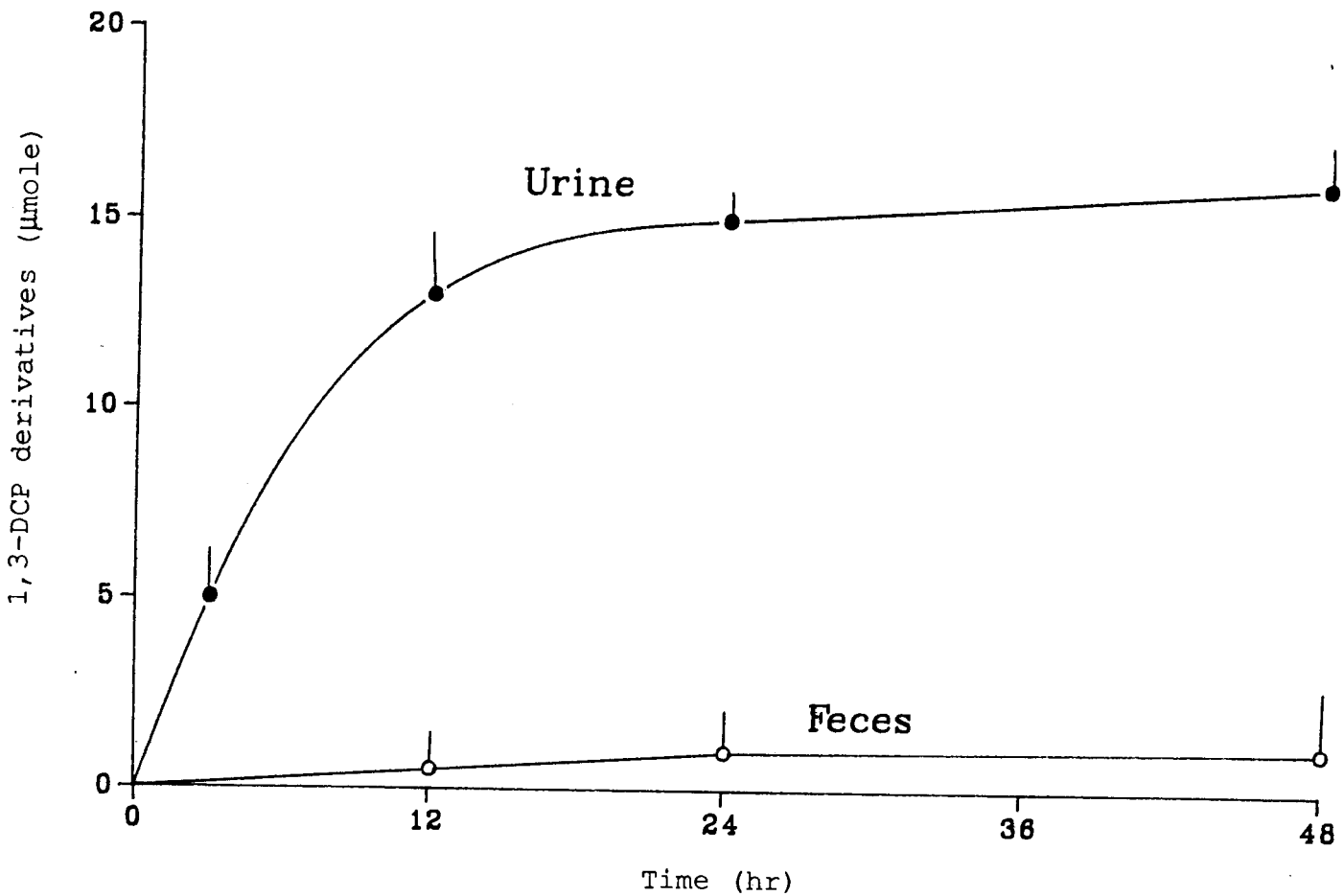
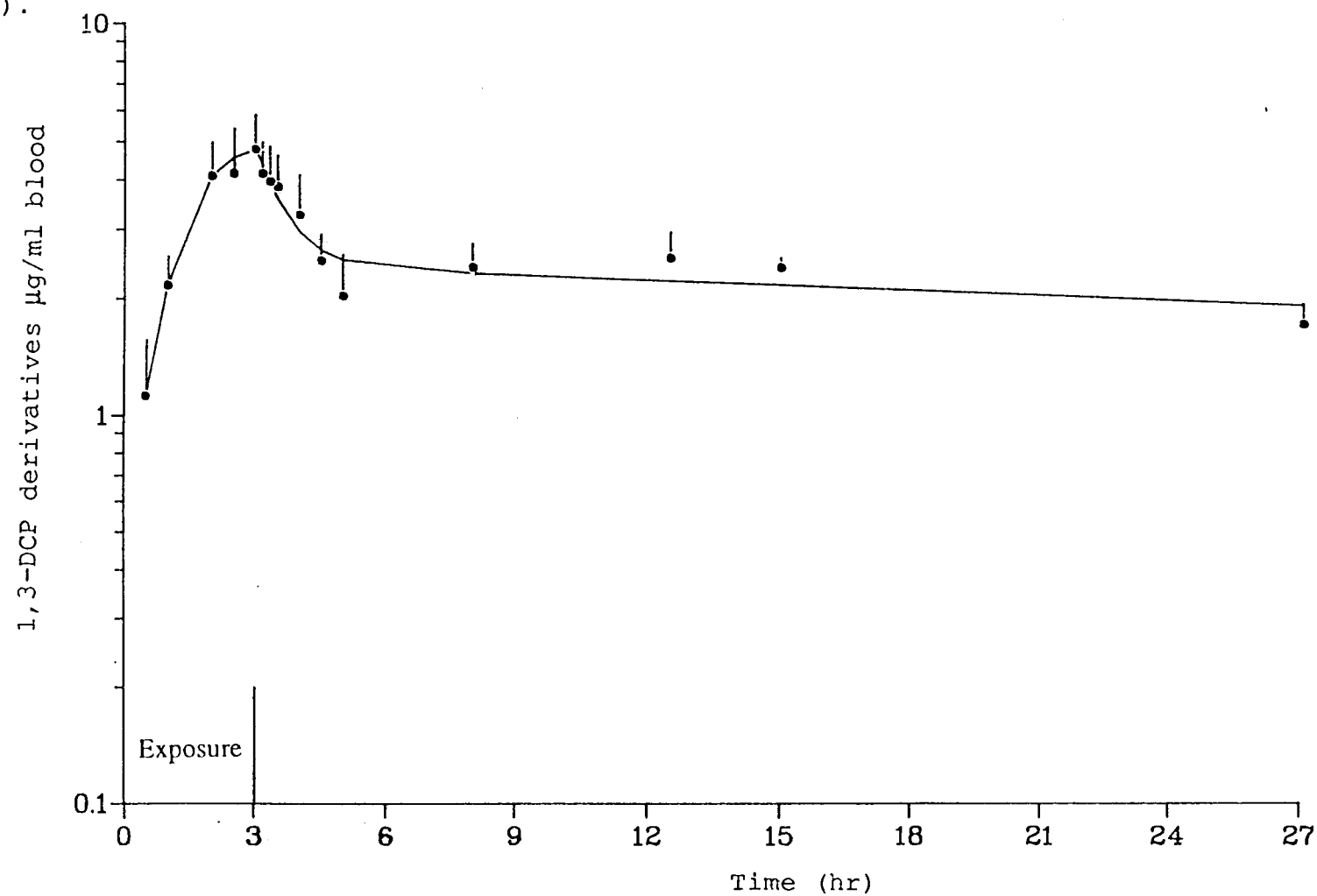


Fig. R-17

Appearance and disappearance of ^{14}C in blood from rats exposed to 100 ppm 1,3-(2-^{14}C)-DCP (specific activity 0.01283 µCi/µmol) for 3 hrs (each point represents mean ± SD of 3 or 4 rats).



from the blood in a biphasic, exponential manner (Fig R-17). The equation for the best fit of the data was

$$F(t) = 2.499\ e^{-0.025t} + 2.420\ e^{-0.000167t}$$

where F(t) is the fraction remaining to be excreted at time t (min). The estimated elimination half-lives of fast and slow phases were 27.72 min (0.46 hr) and 4149.7 min (69.16 hr), respectively.

b. Tissue distribution of unchanged 1,3-DCP after exposing rats to unlabeled 1,3-DCP in a whole-body chamber.

In this experiment, the actual 1,3-DCP concentration in the whole-body chamber was 280.3 ± 17.7 ppm (n = 8). Table R-9 shows the percentage recoveries of cis- and trans-1,3-DCP by ether from various tissues. Ether extraction recoved 98.0% and 91.5%, respectively, of added cis- and trans-1,3-DCP to tissues.

Among the tissues examined, cis- and trans-1,3-DCP were found in the stomach and the adipose tissue of rats following 1,3-DCP vapor exposure. About 2.19 mg/g and 4.35 mg/g of the cis- and trans-isomer, respectively, were found in the fat immediately after 1,3-DCP exposure (Table R-10). Twelve hours after exposure, there was no unchanged 1,3-DCP in the fat. Trans-1,3-DCP concentration in the fat was 2-3 times higher than that of cis-1,3-DCP, although the ratio of cis:trans isomers in the exposure mixture was 1.3:1. About

Table R-9

Recovery (%) of cis- and trans-1,3-DCP from rats tissues.*

	cis-1,3-DCP	trans-1,3-DCP
Liver	100.68 ± 10.65	93.28 ± 10.02
Kidney	94.44 ± 6.39	85.80 ± 8.48
Brain	96.54 ± 8.74	87.87 ± 5.84
Lung	97.30 ± 19.81	96.79 ± 1.28
Stomach	101.01 ± 17.47	95.06 ± 10.96
Small intestine	94.48 ± 11.39	91.55 ± 6.94
Muscle	94.90 ± 13.12	88.18 ± 8.24
Perirenal fat	104.98 ± 21.05	93.78 ± 6.34

* Values are represented as recovery (%) ± standard deviation from 4-5 determinations. The 1,3-DCP concentration range was 50 ng/ml to 500 ng/ml. The ratio of these two isomers was 1.3:1 (cis:trans).

Table R-10

Time course of cis- and trans-1,3-DCP concentration in the fat (μg/g) of rats exposed to 300 ppm of 1,3-DCP vapor for 4 hours.

Postexposure Time(hr)	cis-1,3-DCP*	trans-1.3-DCP
0	2192.5 ± 1253.1	4350.6 ± 1140.7
0.5	676.5 ± 420.1	1716.4 ± 603.0
1	374.1 ± 74.4	1102.7 ± 122.1
2	213.8 ± 179.1	572.1 ± 511.6
3	268.9[a]	487.5[a]
6	nd	102.6[a]
12	nd	nd
18	nd	nd

*: The values are presented as mean ± SD of 3 or 4 rats.

a: The values are presented as one single rat value or an average value of two rats.

nd: not detectable.

53.1 μg/g trans-DCP could be detected in the stomach immediately after exposure(data not shown), but it disappeared in 1 hr after exposure. No cis-1,3-DCP could be detected in the stomach at any time after exposure.

III. Biotransformation of 1,3-DCP by rats.

1. Effects of inhaled 1,3-DCP on rats MFO systems.

As indicated by an on-line MIRAN infrared spectrophotometer and the gas chromatography, chamber concentration of 1,3-DCP was very stable. The nominal (actual as mean ± SD) concentrations of the chamber were: 35 ppm, (35.3 ± 2.8 ppm); 150 ppm, (140.7 ± 14.4 ppm); and 300 ppm, (295.9 ± 28.0 ppm). The actual concentrations were calculated from 10 different gas chromatographic determinations of each exposure.

After 1,3-DCP exposure, body weight loss was detected in rats at all exposure levels (Table R-11). However, there were no changes of liver to body weight and lung to body weight ratios between the treated and control rats. In contrast, kidney to body weight ratio of the treated rats was higher than that of the control, especially for rats exposed to 35 ppm and 300 ppm 1,3-DCP (Table R-11).

Table R-11

Effects of 1,3-DCP mixture vapor inhalation on the body and organ weights of rats.[a]

	control	35 ppm	150 ppm	300 ppm
1st day body wt (g.)	284.2 ± 8.2	290.5 ± 22.9	291.0 ± 15.7	300.16 ± 7.3
final body wt (g.)	261.2 ± 5.3	239.7 ± 15.6*	241.1 ± 14.0*	237.6 ± 5.3**
weight lost (g.)	23.0 ± 4.2	50.7 ± 7.9**	49.8 ± 4.6**	62.5 ± 6.3**
% liver wt of body wt	3.39 ± 0.15	3.56 ± 0.14	3.59 ± 0.17	3.53 ± 0.28
% lung wt of body wt	0.51 ± 0.12	0.48 ± 0.09	0.52 ± 0.04	0.42 ± 0.04
% kidney wt of body wt	0.84 ± 0.04	0.92 ± 0.05*	0.90 ± 0.06	0.94 ± 0.03**

a. Rats were exposed to 1,3-DCP 6 hr/day for 3 days.

* Significantly different ($p < 0.05$) from the control values.

** Significantly different ($p < 0.01$) from the control values.

Values are mean ± SD (n = 4 - 6).

Table R-12 summarizes the effects of 1,3-DCP on hepatic MFO activities and cytochrome P-450 and cytochrome b_5 contents after exposing rats to 35, 150, and 300 ppm 1,3-DCP vapor for 3 days. Microsomal cytochrome P-450 and cytochrome b_5 contents were significantly decreased. Hepatic microsomal MFO enzyme activities such as aminopyrene N-demethylase, 7-ethoxycoumarin O-deethylase, 7-ethoxyresorufin O-deethylase, benzo(α)pyrene hydroxylase also were significantly decreased in the rats exposed to 150 ppm and 300 ppm 1,3-DCP. In contrast, no significant change in the renal microsomal cytochrome P-450 content and 7-ethoxycoumarin O-deethylase activity was detected in the 1,3-DCP-treated rats (Table R-13).

2. *In vitro* destruction of cytochrome P-450.

Cytochrome P-450 content in liver microsomes of rats was lowered after incubation with 1,3-DCP (Table R-14). In the absence of 1,3-DCP or a NADPH generating system, cytochrome P-450 was not destroyed. Cytochrome P-450 destruction also was observed in microsomes prepared from untreated and phenobarbital-treated rats. A greater loss of cytochrome P-450 was found in the phenobarbital-treated rats than in the untreated rats.

3. *In vitro* metabolism and irreversible binding of ^{14}C-1,3-DCP to rat liver microsomal protein.

Table R-12

Effects of 1,3-DCP mixture vapor inhalation on the hepatic mixed-function oxidases of rats.[a]

	control	35 ppm	150 ppm	300 ppm
Cytochrome P-450 (pmol/mg microsomal protein)	0.35 ± 0.20	0.30 ± 0.14**	0.19 ± 0.03**	0.23 ± 0.05**
Cytochrome b_5 (pmol/mg microsomal protein)	307 ± 63	94.6 ± 12.7*	98.5 ± 26.0**	138 ± 32**
7-Ethoxycoumarin O-deethylase (pmol coumarin formed/mg microsomal protein/min)	13.9 ± 1.1	13.8 ± 1.2	14.1 ± 4.2	10.3 ± 0.7**
7-Ethoxyresorufin O-deethylase (pmol resorufin formed/mg microsomal protein/min)	0.80 ± 0.17	0.67 ± 0.08	0.50 ± 0.06*	0.53 ± 0.13*
Aminopyrene N-demethylase (nmol HCHO formed/mg microsomal protein/min)	3.95 ± 0.59	3.65 ± 1.02	2.23 ± 0.36**	2.24 ± 0.43**
Benzo(a)pyrene hydroxylase (nmol products formed/mg microsomal protein/min)	0.83 ± 0.19	0.82 ± 0.10	0.38 ± 0.06**	0.45 ± 0.14**

a. Rats were exposed to 1,3-DCP, 6 hr/day for 3 days.

* significantly different ($p < 0.05$) from the control values.

** significantly different ($p < 0.01$) from the control values.

Values are mean ± S.D. (n = 5 or 6).

Table R-13

Effects of 1,3-DCP mixture vapor inhalation on the renal mixed-function oxidases of rats.[a]

	control	35 ppm	150 ppm	300 ppm
Cytochrome P-450 (pmol/mg microsomal protein)	85.0 ± 41.0	78.6 ± 1.1	66.0 ± 3.0	62.0 ± 3.0
7-Ethoxycoumarin 0-deethylase (pmol coumarin formed/ mg microsomal protein/min)	32.5 ± 3.8	49.3 ± 11.4	51.1 ± 13.0	27.5 ± 2.14
Aminopyrene N-demethylase (pmol HCHO formed/ mg microsomal protein/min)	265 ± 94	109 ± 64	489 ± 26	n.d.

a. Rats were exposed to 1,3-DCP 6 hr/day for 3 days.

n.d.: not detectable data.

Values are mean ± SD (n = 3).

Table R-14

1,3-DCP-mediated destruction of cytochrome P-450 in rat liver microsomes.

Incubation substrate	Animal treatment	Contents of P-450 (nmole/mg protein) +NADPH	-NADPH
10 mM 1,3-DCP	None	0.35 ± 0.03	0.59 ± 0.09
	Phenobarbital	0.63 ± 0.12	1.32 ± 0.03*
1 mM 1,3-DCP	None	0.42 ± 0.22	0.67 ± 0.15*
	Phenobarbital	0.66 ± 0.18	1.24 ± 0.20*

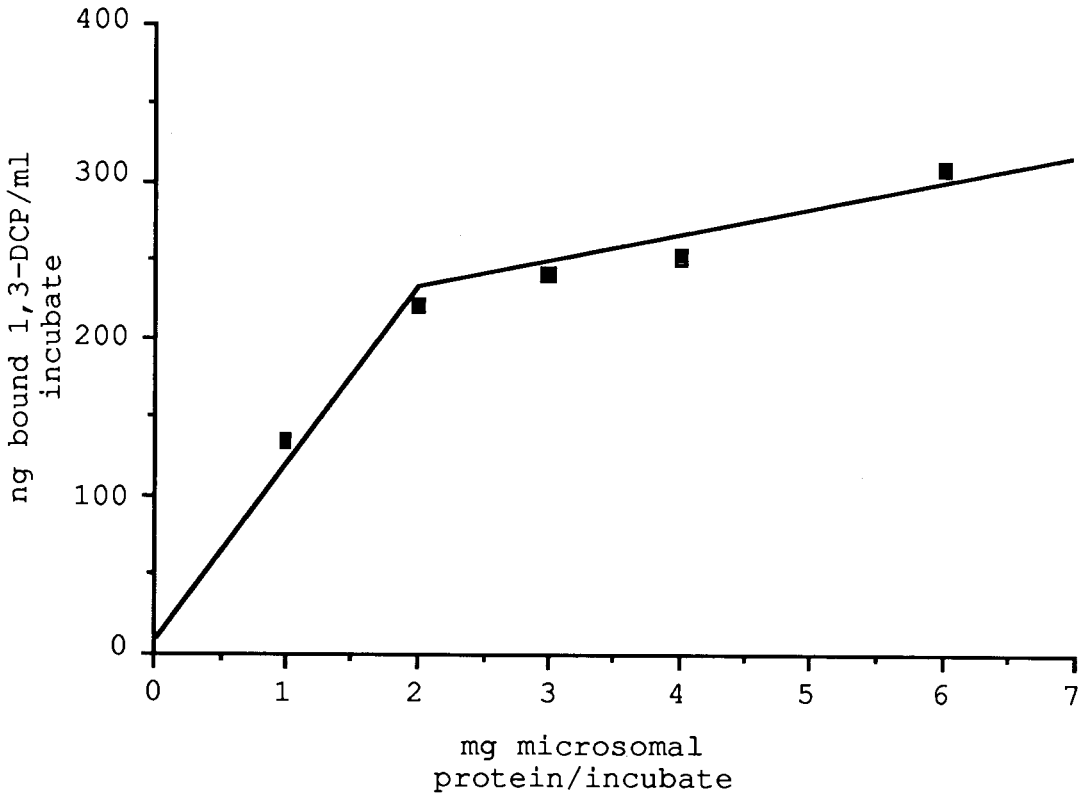
* Significantly different ($P < 0.01$); results are expressed as mean ± S.D. (n = 3-4).

^{14}C-1,3-DCP was incubated for varying lengths of time with washed liver microsomes in the presence of a NADPH-generating system. Figs. R-18 and R-19 show that linearity of binding to rat liver microsomal protein is a function of both time and microsomal protein concentrations. The binding of ^{14}C-1,3-DCP to microsomal protein was linear for about 10 min incubation time and up to 2 mg/ml microsomal protein per incubation.

The effects of NADPH, anaerobic (N_2 gas atmosphere), carbon monoxide atmosphere (90% CO, 10% O_2), piperonyl butoxide (1 mM) and SKF-525A (1 mM) on the extent of binding of ^{14}C-1,3-DCP derivatives to microsomal protein are given in Table R-15. No binding occurred in the absence of NADPH. But O_2 and CO did not significantly affect binding. Addition of SKF-525A (1 mM) did not change the binding of 1,3-DCP to microsomes. Addition of piperonyl butoxide, a methylenedioxyphenyl MFO inhibitor, to the incubation at 1 mM significantly decreased ^{14}C-1,3-DCP bound to the microsomal proteins.

Fig. R-18

Linearity of irreversible binding of 1,3-DCP to rats liver microsomes.

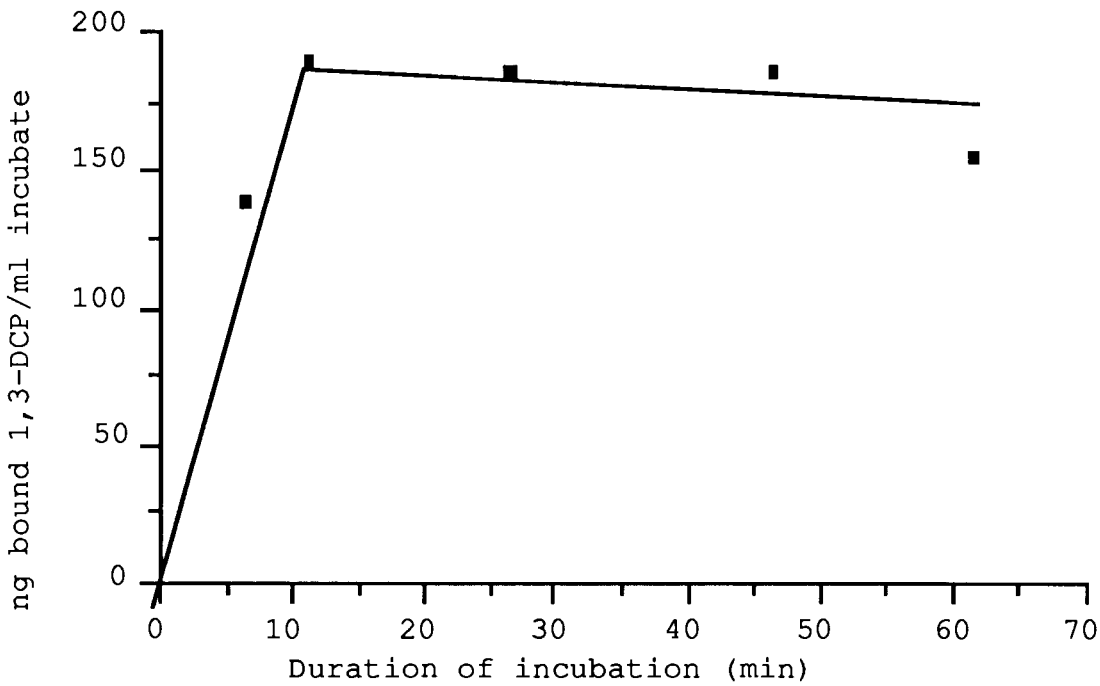


* 1,3-(^{14}C-2)-DCP were incubated with different amount of rats microsomes for 10 min at 37°C in the presence of a NADPH-generating system.

Values are mean of four determinations.

Fig. R-19

In vitro irreversible binding of 1,3-DCP to rats liver microsomes.



* Rats liver microsomal protein (2 mg/ml) were incubated with ^{14}C-1,3-DCP for varying lengths of time in the presence of a NADPH-generating system.

Values are mean of four determinations.

Table R-15

Conditions for irreversible protein binding of ^{14}C-1,3-DCP to rats liver microsomal protein.

Reaction Mixture	^{14}C-1,3-DCP bound (nmole/incubation)[b]
	Liver microsomes
Complete[a]	2.350 ± 0.314
-NADPH	0.564 ± 0.058*
$+N_2$	2.477 ± 0.162
+CO	2.112 ± 0.181
Boiling microsomes	0.806 ± 0.058*
SKF-525A (1 mM)	2.627 ± 0.258
Piperonyl butoxide (1 mM)	0.601 ± 0.147*

a. Incubations were carried out with ^{14}C-1,3-DCP (0.056mM, specific activity of ^{14}C-1,3-DCP was 0.156 nCi/nmole) and a NADPH-generating system, except as noted.

b. Incubation time was 10 min and microsomal protein concentration was 2 mg/incubate.

* significantly different ($p < 0.05$) from complete reaction mixture.

§4. DISCUSSION

When a whole-body inhalation chamber is operated normally under dynamic conditions, the flow rate (F) through the chamber generally falls in the range of 0.1-1.0 V (where: V = volume of chamber in liter, MacFarland, H.N., 1983).

The equation of the concentration-time curve in a dynamic inhalation chamber is described by Silver (1946).

$$C_t = \frac{f}{F}\left(1 - e^{-\frac{F}{V}t}\right)$$

where: C_t = the concentrations after t min of operation,

f = total flow of agent through chamber(liter),

F = total flow through chamber during operation(liter),

V = volume of chamber (liter).

This equation assumes that f and F remain constant and that there is perfect mixing in the chamber. Because of the exponential form of the equation, the chamber theoretically never attains equilibrium. Therefore, the time required to attain a given percentage of the equilibrium concentration in the chamber is given by the equation,

$$t_x = K \cdot \frac{V}{F}$$

where: t_x = time required to attain x% of the equilibrium concentration,

K = a "constant" whose value is determined by the value of x, e.g., t_{99}, the time required to reach 99% of the equilibrium concentrations, K=4.605,

F = total flow through chamber (liter),

V = volume of chamber (liter).

The duration of exposure time is defined as the interval from the agent begin to flow to the chamber to the time when the agent flow is stopped. It is suggested the duration of exposure should be at least 13 times t_{99} (MacFarland, H.N., 1976).

In the present study, the total flow rate through the whole-body chamber (F) was about 140 l/min which is equivalent to 35% of the chamber volume (≈ 400 liters) and is within the normal F range required for a dynamic inhalation chamber. Under the experimental conditions, the calculated t_{99} was 13 min and the exposure periods should be more than 2.5 hrs. Since rats were exposed to 1,3-DCP in the whole-body chamber for more than 4 hours in the present study, the experimental design has met all the exposure requirement of a good inhalation study.

The results indicate that uptake of both cis-1,3-DCP and trans-1,3-DCP by rats is rapid. During a 4-hr 1,3-DCP exposure, the isomers reach a steady concentration in the blood, which does not increase proportionately with increasing exposure concentrations (Figs. R-1 and R-2). The concentration of cis-isomer in blood was higher than that of trans-isomer in the blood. This reflects the cis/trans ratios (1.3-1.4) of the 1,3-DCP used in the study. In contrast, the trans-isomer concentration was higher than that of the cis-isomer in the post-exposure period. This is consistent with previous reports that cis-1,3-DCP is metabolized by glutathione conjugation 4-5 times faster than trans-isomer (Climie et al., 1979).

The elimination of both 1,3-DCP isomers from the blood of rats following inhalation exposure is related to time in a bi-exponential manner (Figs. R-1 and R-2). During the course of my work, Stott and Kastl (1986) reported similar biphasic elimination curves in the blood of rats exposed to various concentrations of technical grade 1,3-DCP in a nose-only chamber. These authors also showed that the uptake of 1,3-DCP by rats did not increase proportionately with increasing exposure concentration due to an exposure level-related decrease in the respiratory ventilation frequency of rats exposed to >90 ppm 1,3-DCP and the saturation of 1,3-DCP metabolism in rats exposed to >300 ppm 1,3-DCP. My results

on the end-exposure blood levels and the half-lives of the post-exposure terminal phase of the elimination are consistent with their findings.

Previous studies suggested that metabolic clearance is the major route by which 1,3-DCP is eliminated from the rat (Dietz et al., 1984: Hutson et al., 1971). Therefore, about 50-80% of the ^{14}C-labeled 1,3-DCP administered orally was excreted in the urine by rats and mice. Another 14-17% of the oral dose was eliminated as $^{14}CO_2$. Only < 0.6% of the administered 1,3-DCP was exhaled as a volatile organic material (Dietz et al., 1984). Hutson et al. (1971) also showed that rats which received 10 mg/kg of the ^{14}C-labeled cis- or trans-1,3-DCP excreted about 81 and 57% of the 1,3-DCP-derived radiolabel, respectively, in the urine within 24 hr. Another 4% of the cis- isomer and 24% of the trans-isomer were excreted as CO_2 and less than 4% of both isomers were exhaled. A majority of the absorbed 1,3-DCP is conjugated with glutathione in rats since N-acetyl-S-(3-chloroprop-2-enyl)cysteine has been identified as the primary urinary excretion product (Climie et al., 1979: Dietz et a., 1984: Fisher and Kilgore, 1988). The mercapturic acid conjugate of cis-1,3-DCP also has been identified in the urine of fumigant applicators exposed to the vapor of 1,3-DCP (Osterloh et al., 1984). In a recent study, Fisher and Kilgore (1989) showed that glutathione conjugation of 1,3-DCP

in the rat may become saturated at an exposure level as low as 78 ppm 1,3-DCP.

Total clearance (Cl_{total}) represents the volume of plasma cleared of 1,3-DCP, by all elimination mechanisms, per unit time. The calculated Cl_{total} of cis-1,3-DCP (trans-1,3-DCP) are 1.73 ml/min (1.82 ml/min), 1.88 ml/min (4.42 ml/min), and 4.61 ml/min (3.22 ml/min) for rats exposed to 100 ppm, 300 ppm, and 500 ppm vapor mixture of 1,3-DCP, respectively. The Cl_{total} of both isomers in the 100 ppm 1,3-DCP exposed rats are very similar since they are cleared by a linear process. The Cl_{total} of trans-1,3-DCP is higher than that of cis-1,3-DCP in the 300 ppm exposed rats since trans-1,3-DCP is cleared by a saturated (nonlinear) process and cis-1,3-DCP still cleared by a linear process. The Cl_{total} of cis-1,3-DCP is higher than that of the trans- isomer in the 500 ppm rats since both isomers are metabolized or cleared by the maximal rates and cis-1,3-DCP has been shown to be metabolized at a faster rate than trans-1,3-DCP (Climie et al., 1979).

The results presented in Table R-14 provide evidence that microsomal cytochrome P-450 content is lowered by a metabolite of 1,3-DCP, since the loss of cytochrome p-450 occurs only in incubation with 1,3-DCP and in the presence of a NADPH-generating system. Cytochrome P-450 destruction did not result from lipid peroxidation, since both EDTA and BHT (strong inhibitors of lipid peroxidation) did not prevent the

loss of P-450 when added to the incubation mixture. Destruction of cytochrome P-450 by chemicals containing a terminal olefin group has previously been reported by Ortiz de Montellano and Correia (1983). Results of the present study (Table R-14) are similar to those reported for other olefins, which show a similar degree of cytochrome P-450 destruction regardless of concentration used (Ortiz de Montellano and Mico, 1980). Ethylene and other terminal olefins were shown to destroy cytochrome P-450 by prosthetic heme alkylation (Ortiz de Montellano and Correia, 1983). Several olefinic compounds, such as 2,2-diethyl-4-pentenamide and secobarbital, inactivate cytochrome P-450 by heme alkylation and also disrupt heme biosynthesis (Ortiz de Montellano and Correia, 1983). It is unknown whether these mechanisms are involved in the destruction of cytochrome P-450 by 1,3-DCP. Nevertheless, inactivation of cytochrome P-450 by 1,3-DCP may account for the *in vivo* loss of liver microsomal cytochrome P-450 contents and decrease of its metabolic activities observed in rats exposed to 1,3-DCP vapor for 3 days (Table R-12).

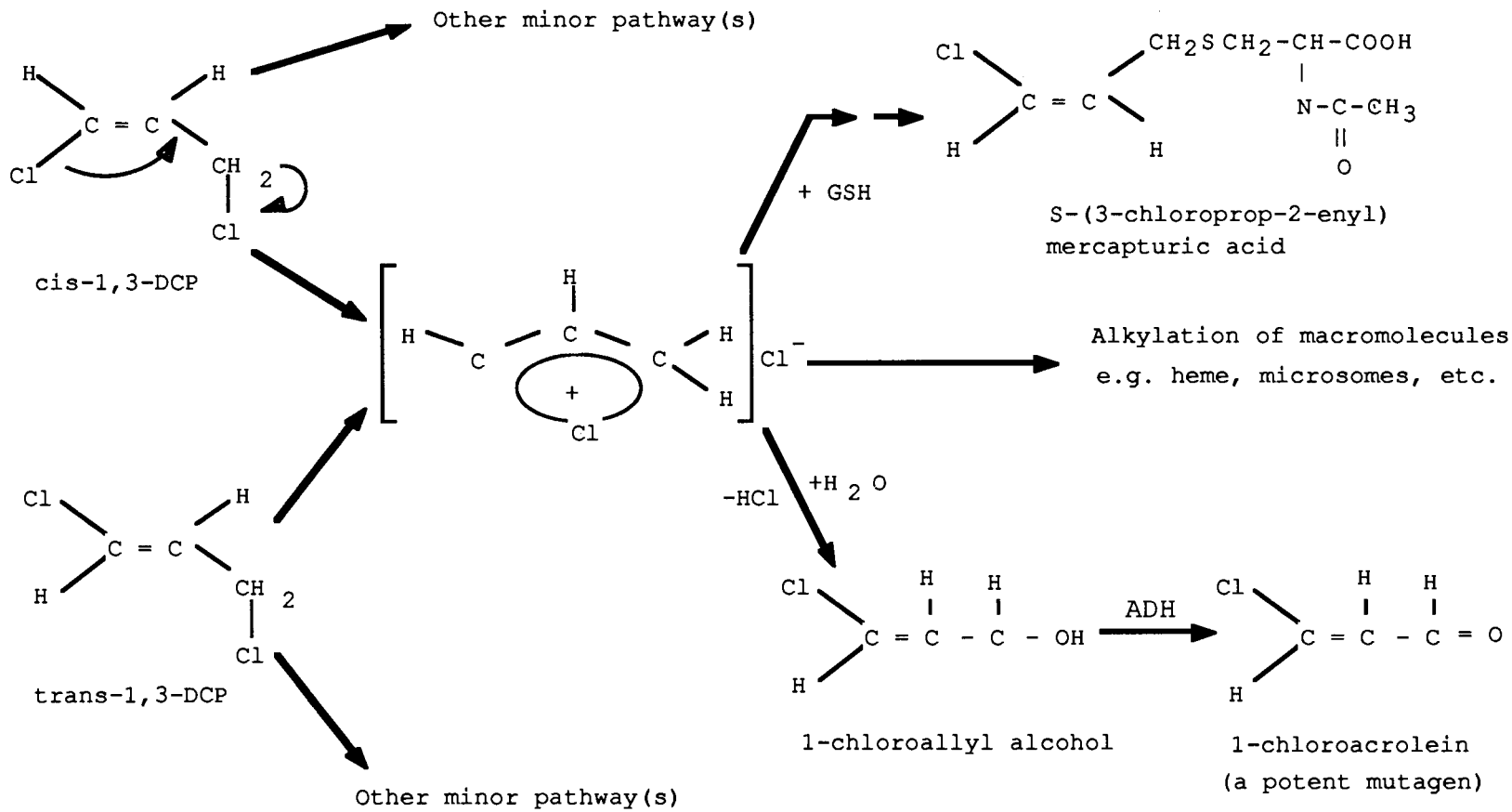
The chemical identity of the 1,3-DCP metabolite responsible for cytochrome P-450 destruction remains unknown, but is probably an epoxide or a cation of 1,3-DCP. Van Durren et al. (1979) have postulated that halogenated olefinic hydrocarbons are metabolized by microsomal oxygenates to reactive epoxides which could react with

macromolecules and initiate carcinogenic or mutagenic processes. More recently, Neudecker and Henschler (1986) have shown that 1,3-DCP is metabolized *in vitro* by rat liver post-mitochondrial supernatant via a hydrolytic-oxidative pathway which includes cleavage of the Cl-leaving group in the allylic position and the oxidation of allylic alcohol by alcohol dehydrogenase to 1-chloro-acrolein. This metabolic pathway is not affected by SKF-525A since the mutagenic activity of 1,3-DCP is not changed by the inclusion of SKF-525A in the *Salmonella*/rat microsome assay. In Fig. D-1, the metabolic pathways of 1,3-DCP presented. These metabolic pathways are consistent with the report that cis-1,3-DCP is more active than trans-1,3-DCP as an alkylating compound since the cis- isomer favors cation stabilization due to steric hindrance and neighboring effects of the chlorine atoms (Neudecker et al., 1980). The formation of a cation intermediate, instead of a 1,3-DCP oxide as a reactive metabolite of 1,3-DCP, also is consistent with my findings that 1,3-DCP is metabolized to an alkylating agent which binds irreversibly to rat liver microsomes and that SKF-525A has no effect on the binding or the formation of the metabolite (Table R-15).

Toxicokinetics is the study of the time course of a toxicant or its metabolite levels in different fluids, tissues, and excreta of the body. A toxicokinetic model reduces complicated biological processes into simple

Fig. D-1

Metabolic pathways of 1,3-DCP.



differential equations which could be used to describe the biological fates of a toxicant. The following information has been incorporated into the 1,3-DCP model: (a) the model is a two compartment model since 1,3-DCP is eliminated from the blood in a biphasic manner, (b) a decrease in the respiratory ventilation frequency of rats exposed to >90 ppm 1,3-DCP, and (c) 1,3-DCP absorbed by the rat is metabolized by glutathione conjugation, the enzymic activity of which is saturable at an exposure concentration of 78 ppm 1,3-DCP. This information appears to be essential for the successful development of a 1,3-DCP model. I have attempted unsuccessfully to use a two-compartment toxicokinetic model with zero-order (constant rate) input and first-order (linear) elimination to describe the time course of the blood data; although such a model describes adequately the blood data of rats exposed to 100 ppm or 300 ppm 1,3-DCP it does not fit the data of rats exposed to 500 ppm 1,3-DCP (data not shown).

The proposed toxicokinetic model fails to predict the blood concentration of 1,3-DCP in the rats beyond the 315 min time point (Figs. R-5 to R-15). This most likely results from 1,3-DCP-induced hepatic cytochrome P-450 destruction which begins to show its effects on the kinetics of 1,3-DCP in the blood at about 315 min after the initiation of the experiment. Since the proposed kinetic model does not include cytochrome P-450 destruction, the model is valid only

during the period before the 315 min time point. Several lines of evidence offer additional support to this hypothesis: (1) The discrepancy between the model-predicted and experimental blood concentration data after the 315 min experimental period exacerbates with increasing 1,3-DCP exposure concentrations. This is expected since 1,3-DCP is metabolized by rats at rates approaching V_{max} when they are exposed to high 1,3-DCP vapor concentrations. In other words, cytochrome P-450 destruction is more extensive in the 500 ppm rats than the 100 ppm rats. Therefore, the difference between the model-predicted and experimentally obtained blood concentration is larger in the 500 ppm rats than in the 100 ppm rats. (2) The discrepancy between the model-predicted and experimental data is more evident in the cis- isomer blood curve than the trans- isomer blood curve, since the cis- isomer blood curve is more sensitive to cytochrome P-450 destruction than that of the trans- isomer. (3) The discrepancy between the model-predicted and experimental results is most evident in rats pretreated with piperonyl butoxide (Figs. R-12 and R-13). Since piperonyl butoxide inhibits hepatic MFO activities, it has a similar effect on the kinetics of 1,3-DCP as a chemical which destroys cytochrome P-450.

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