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Chloroethylene Mixtures: Pharmacokinetic Modeling and *in Vitro* Metabolism of Vinyl Chloride, Trichloroethylene, and *trans*-1,2-Dichloroethylene in Rat[1]

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Environmental and occupational exposures are typically to mixtures of chemicals, although most toxicity information is for individual compounds. Interactions between chemicals may involve pharmacokinetic and/or pharmacodynamic effects resulting in modulation of toxicity. Therefore, physiologically based pharmacokinetic modeling has been used to analyze data describing the metabolism of vinyl chloride (VC) and trichloroethylene (TCE) mixtures in rats. A single saturable pathway was modeled, representing cytochrome P450 2E1. This was partially validated using preexposure to *trans*-1,2-dichloroethylene (*t*DCE) which virtually eliminated *in vivo* metabolism of both VC and TCE at low concentrations. Microsomes from *t*DCE-exposed animals showed inhibition of metabolism of P450 2E1 substrates (chlorzoxazone, *p*-nitrophenol, and TCE) and no effect on 7-ethoxycoumarin deethylation. Studies with liver microsomes from VC-exposed animals found that neither suicide inhibition nor induction occurred during 6-hr exposures to high concentrations. Therefore, these effects were not modeled. Modeling of mixtures of VC and TCE was successful only using competitive inhibition, as might be predicted for cytochrome P450 2E1 substrates, and not uncompetitive or noncompetitive inhibition. These results were further confirmed by determining the depletion of glutathione due to VC metabolism. The validation of a detailed model for the inhibition kinetics of metabolism of these two compounds permits better understanding of the implications of coexposures for toxicity. It is notable that competitive inhibition only becomes significant at relatively high concentrations (tens of ppm), while at typical low environmental concentrations (ppb), absorption is perfusion limited and enzyme is in excess so that the chemicals will be metabolized independently.

Interactions between chemicals affecting their toxicity may result from alterations in pharmacokinetics (absorption, distribution, metabolism, or elimination) and/or pharmacodynamics (quantitative descriptions of the mechanisms of toxicity). Pharmacokinetic modeling is currently more completely developed than modeling of toxicity processes. Therefore, pharmacokinetically important interactions are more readily characterized quantitatively.

Complex mixtures of organics and metals are typically present in contaminated surface and ground water (Germolec *et al.*, 1989). These mixtures arise from releases of the individual chemicals, environmental weathering including biodegradation, and formation of by-products, for instance, during chlorination. All three of these processes create mixtures of chloroethylenes. Chloroethylenes are particularly common contaminants due to their widespread usage as industrial chemicals. Trichloroethylene (TCE)[4] and tetrachloroethylene are known to undergo anaerobic dechlorination in field and laboratory studies (Freedman and Gossett, 1989). This process results in the formation of TCE, dichloroethylene isomers, vinyl chloride (VC), and ethylene, although the extent of formation of each species is site-specific and variable.

Most animal studies of toxicity utilize individual pure chemicals while human exposures and consequently epidemiological data mostly involve mixtures. The single-chemical approach results from a scientific need to identify which chemicals in a mixture are of greatest concern. The problem of "reconstituting" mixtures is complicated by their

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[4] Abbreviations used: VC, vinyl chloride; TCE, trichloroethylene; *t*DCE, *trans*-1,2-dichloroethylene; PBPK, physiologically based pharmacokinetic; NPSH, nonprotein sulfhydryl; V_{max_c}, V_{max} normalized to 1 kg body weight in PBPK modeling; P450, liver microsomal cytochrome P450.

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infinite variation and the potential for complex interactions ranging from synergism and potentiation to antagonism (U.S. EPA, 1986). The difficulties, particularly in the area of pharmacodynamics, are also increased greatly when the concern is chronic toxicity, such as cancer.

VC is a known human carcinogen which causes cancer, notably hepatoangiosarcomas, in every animal species tested (ATSDR, 1989). VC is a classic mutagenic chemical due to the formation of an electrophilic epoxide metabolite via metabolism by cytochrome P450 (P450), particularly P450 2E1 (Guengerich *et al.*, 1991; Gwinner *et al.*, 1983; Guengerich and Strickland, 1977). Chloroethylene oxide can adduct DNA forming 7-(2-oxoethyl)guanine and three minor cyclic ethenoadducts (Fedtke *et al.*, 1990).

Chloroethylene oxide rearranges spontaneously to form chloroacetaldehyde (Fedtke *et al.*, 1990). Chloroacetaldehyde is responsible for the majority of adduction of proteins. *In vitro* and *in vivo* studies have found P450 itself to be a target resulting in suicide inhibition (Ivanetich *et al.*, 1977; Pessayre *et al.*, 1979; Reynolds *et al.*, 1975; Watanabe *et al.*, 1976).

GSH depletion has been demonstrated *in vivo* and linked to chloroacetaldehyde formation (Plugge and Safe, 1977). The extent of depletion is dose dependent. Metabolism of the GSH conjugates results in several stable metabolites identified in rat urine.

TCE, in contrast, causes cancer in two animal species but the results are highly species dependent (Bruckner *et al.*, 1989). Oral exposure resulted in hepatocellular carcinomas in B6C3F1 mice and some kidney tumors in male rats. Lung tumors were also seen in mice exposed by inhalation.

P450 2E1 is the major enzyme responsible for the initial metabolism of TCE, although other P450s can also be involved (Miller and Guengerich, 1983; Nakajima *et al.*, 1990). Direct GSH conjugation, mediated by transferases, also occurs at very low rates (Bruckner *et al.*, 1989). The extent of epoxide formation appears limited, apparently due to rearrangement occurring in the enzyme-active site (Miller and Guengerich, 1983). Neither significant destruction of P450 nor GSH depletion have been reported for TCE. These results contrast strongly with those for VC but are similar to findings with tetrachloroethylene.

The major products of TCE metabolism include the unstable product chloral hydrate and the chemically stable forms trichloroethanol and trichloroacetate. Dichloroacetate is a minor product. Oral exposure to chloral hydrate, trichloroacetate, or dichloroacetate causes B6C3F1 mouse liver tumors (Herren-Freund *et al.*, 1987; Bull *et al.*, 1990; DeAngelo *et al.*, 1991) demonstrating that formation of a genotoxic epoxide intermediate from TCE is not obligatory to its induction of cancer.

The studies reported here utilize *in vitro* measurements of enzyme activities and cofactor levels to validate the structure of a physiologically based pharmacokinetic (PBPK) model. A model previously developed for mixtures of TCE and 1,1-dichloroethylene (vinylidene chloride) was used with TCE and VC (Andersen *et al.*, 1987). Competitive, noncompetitive, and uncompetitive inhibition of oxidative P450 metabolism were all considered as possible interactions between these chemicals. Differences in VC-induced GSH depletion assisted in better understanding of the results of coexposures to TCE and VC. Finally, the implications of the results for risk assessment of mixtures were considered.

EXPERIMENTAL PROCEDURES

Animals. Male Sprague–Dawley rats were purchased from Charles River Breeding Laboratories (Raleigh, NC). Animals were quarantined for 2 weeks for verification of health status by pathological, fecal parisitological, and serum chemistry examinations. Water and food (Purina Formulab 5008) were provided *ad libitum*. On the day of experiments, body weights ranged from 250 to 400 g. A few experiments were repeated using animals outside this weight range. The animals used in this study were handled in accordance with the principles stated in the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animal Resources (National Research Council, Department of Health and Human Services, National Institute of Health, Publication 86-23, 1985) and the Animal Welfare Act of 1966, as amended.

Chemicals. Chemicals were obtained from manufacturers with the specified purity: VC (Fluka, New York, NY, 99.5%); *t*-1,2-dichloroethylene (*t*DCE) (Aldrich, Milwaukee, WI, 98%) and TCE (Aldrich, 99.9%). Purities were confirmed by GC–MS. 6-Hydroxychlorzoxazone was a generous gift of Dr. John Lipscomb (Toxicology Div., WPAFB). All other chemicals were purchased from standard sources. Vinyl chloride is a known human carcinogen and appropriate precautions should be taken to limit exposure of personnel to this gas. Trichloroethylene is a neurotoxin at high concentrations so exposure to the liquid or its vapor needs to be limited.

Gas uptake exposures. Three male Sprague–Dawley rats were exposed to VC, TCE, *t*DCE, or a mixture using a closed recirculating system similar to that described by (Gargas *et al.*, 1986). This method starts with a fixed amount of chemical in the atmosphere. The concentration decreases as the chemical is absorbed and metabolized. Chamber concentrations were monitored using a gas-sampling valve connected to a gas chromatograph. Chromatography was performed on a 12 ft × $\frac{1}{8}$ in. stainless steel column packed with 10% SE30 on 80/100 mesh Chromosorb WHP. The GC was equipped with a hydrogen flame ionization detector with the temperature set at 250°C, injection temperature at 175°C, constant oven temperature of 120°C, and nitrogen carrier at 21 cc/min. Maximum exposure time was 6 hr.

Partition coefficient determinations. A modification of the vial equilibration method was used to determine partition coefficients (Gearhart *et al.*, 1993). Animals were sacrificed by CO_2 asphyxiation. Blood was obtained from the posterior vena cava. Liver, fat, muscle (slow), and kidney (rapid) were collected for analysis. Tissues (except blood) were homogenized without diluent. Blood (1 ml) was added or tissues (1 g) were smeared on the side of a 21.9-ml vial. All samples were incubated for 3 hr with VC (114 ppm) or TCE (600 ppm) before determining headspace concentration. Chromatographic conditions were the same as those for gas uptake.

Nonprotein sulfhydryl. Nonprotein sulfhydryl (NPSH) was measured as an estimate of reduced GSH levels on livers following selected exposures (Ellman, 1959; Baker *et al.*, 1990). Animals were euthanized using CO_2. Livers were not perfused for NPSH analysis, but as much blood as possible was drained before mincing. Minced tissue was homogenized in 5 vol 5% sulfosalicylic acid. Supernatant (100 μl) from a 1500*g* spin (10 min) was

added to phosphate/EDTA (80/0.8 mM final) and 5,5'-dithiobis-2-nitrobenzoic acid (0.15 mM final). Absorption at 412 nm was compared to GSH standard and quantitated as moles GSH per milligram wet weight liver. The NPSH data (nmol GSH/mg protein) were analyzed using a one-factorial analysis of variance with Tukey multiple comparisons (Rosner, 1990). The factor was exposure levels.

Induction of P450s and preparation of microsomes. Induction of rats was carried out by intraperitoneal injection of pyridine (200 mg/kg) for 4 days or by addition of 10% ethanol or 0.1% phenobarbital to drinking water for 7 days. Livers from control and exposed rats were removed and perfused with cold 0.15 M Tris–KCl (pH 7.4) with added heparin (2 U/ml). The liver was minced with 4 vol of Tris–KCl and homogenized with a Tri-R-Stir-R tissue homogenizer Model K41 (Tri R Instruments, Rockville Centre, NY). The homogenate was centrifuged at 500*g* for 10 min at 4°C and the supernatant was recentrifuged at 9000*g* for 10 min. The resulting supernatant was further centrifuged at 105,000*g* for 60 min. Microsomal pellets were suspended in 0.15 M Tris–KCl (pH 7.4) and then stored at −80°C until use.

Assays for enzymatic activity. *In vitro* metabolism was measured by the formation of 6-hydroxychlorzoxazone using a modification of the procedure of Peter *et al.* (1990). Samples were injected onto a C18 reversed-phase column (4.6 × 150 mm) using a 25% acetonitrile/75% phosphoric acid (0.5%) mobile phase and a 15-min run time. 7-Ethoxycoumarin O-deethylation was measured using the procedure of Greenlee *et al.* (1978) modified to use a plate reader (CytoFluor 2300, Millipore Inc., Bedford, MA) with a fixed emission filter wavelength of 460 nm and excitation of 380 nm.

TCE metabolism in rat microsomes was assessed by the disappearance of TCE from the headspace using a modification of the method of Nakajima *et al.* (1990). The protein content was 1–2 mg in 1 ml of 100 mM potassium phosphate (pH 7.4). Incubation time was 40 min at 37°C after a 20-min preincubation time with TCE and no regenerating system. The regenerating system contained 0.66 mM NADP, 13.7 mM glucose 6-phosphate, 2.8 U/ml glucose-6-phosphate dehydrogenase, and 100 mM potassium phosphate (pH 7.4). Experiments showed that the reaction was linear over this time period and the NADPH regeneration was optimal. The TCE was added as a vapor. Headspace disappearance was measured by gas chromatographic analysis on a Hewlett–Packard 5880 or 5890 equipped with a flame ionization detector. A 10% SE 30 80/100 Chromasorb WHP 12 ft × $\frac{1}{8}$ in. stainless steel column was used under the following conditions: oven temperature, 125°C; detector temperature, 250°C; injection temperature, 175°C; carrier flow, 21 cc/min nitrogen. The amount of TCE metabolized was determined using the calculations of Sato and Nakajima (1979).

Attempts to measure microsomal metabolism of VC *in vitro* by monitoring the disappearance of parent from the vial headspace were unsuccessful, whereas good results were obtained with TCE. This made it impossible to carry out experiments with VC/TCE mixtures to parallel the *in vivo* experiments.

PBPK modeling. A modified version of a model for competitive, noncompetitive, and uncompetitive inhibition of enzyme metabolism by two substrates was used (Andersen *et al.*, 1987). The model was run using SIMUSOLV (Dow Chemical Co, Midland, MI). This model consists of four physiological compartments: liver, fat, rapidly perfused (e.g., kidney), and slowly perfused (e.g., muscle) tissues. Metabolism was modeled as occurring entirely in the liver.

Parameter values other than chemical specific ones were: Q_{PC} (alveolar ventilation, $L\ hr^{-1}\ kg$) = 14, Q_{CC} (cardiac output, $L\ hr^{-1}\ kg$) = 14; tissue blood flows as percentage of cardiac output, Q_{LC} (liver) = 0.25, Q_{FC} (fat) = 0.09, Q_{SC} (slow) = 0.15, Q_{RC} (rapid) = 0.51; tissue volumes as a fraction of body weight, V_{LC} (liver) = 0.04, V_{FC} (fat) = 0.07, V_{SC} (slow) = 0.75, V_{RC} (rapid) = 0.05 (U.S. EPA, 1988; Gargas *et al.*, 1986). $Q_{PC} = Q_{CC} = 14$ has been widely used in PBPK models for several strains of rats (Andersen *et al.*, 1987 and U.S. EPA, 1988). Measurements of cardiac output in Sprague–Dawley rats have recently been published from which $Q_{CC} = 16$ was calculated (Delp *et al.*, 1991). This small difference in estimates for Q_{CC} did not affect the modeling significantly.

In this model, the Michaelis–Menten equation for metabolism of each compound was adapted as follows:

1. $(V_{MAX1} \times C_{VL1})/(K_{M1} \times T_1 + C_{VL1} \times T_2)$
2. $T_1 = 1 + C_{VL2}/K_{MI12} + (C_{VL2})^2/(K_{MI21} \times K_{M22})$
3. $T_2 = 1 + C_{VL2}/K_{M21} + C_{VL1}/K_{MI11}$

V_{MAX1} and K_{M1} represent the apparent maximal rate and concentration producing half-maximal activity for chemical 1. C_{VL1} and C_{VL2} are the venous concentrations of chemicals 1 and 2 in blood leaving the liver, the only metabolizing organ in the model. K_{MI12} is the apparent constant for inhibition of metabolism of chemical 1 by chemical 2. Similarly, K_{MI21}, K_{M21}, K_{MI11}, and K_{M22} are apparent inhibition constants where the first number indicates the chemical being metabolized and the second number is the chemical acting as inhibitor.

When the appropriate inhibitory constants are set to a large number (10^6), these equations reduce to the standard equations for the three types of inhibition. Values for competitive inhibition were $K_{MI12} = K_{M1}$, $K_{MI21} = K_{M2}$, and $K_{M12} = K_{M21} = K_{MI11} = K_{M22} = 1{,}000{,}000$. Values for noncompetitive inhibition were $K_{MI12} = 0.65$, $K_{MI21} = 2.2$, $K_{M12} = 0.65$, $K_{M21} = 2.2$, and $K_{MI1} = K_{M22} = 1{,}000{,}000$. Values for uncompetitive inhibition were $K_{M12} = 3.0$, $K_{M21} = 3.0$, and $K_{MI12} = K_{MI21} = K_{MI11} = K_{M22} = 1{,}000{,}000$.

RESULTS

Estimating metabolic parameters for vinyl chloride with a single saturable oxidative pathway. Modeling VC pharmacokinetics requires chemical specific parameters describing its partitioning into tissues and its metabolism. At a minimum, a single saturable pathway representing P450 oxidation is required. More complex metabolic models could be required if it is necessary to describe modulation of P450 levels, multiple isoforms, or multiple metabolites. The initial hypothesis used was to model a single saturable pathway presumed to represent formation of chloroethylene oxide by P450. This would represent the obligatory step prior to rearrangement to chloroacetaldehyde, conjugation to GSH, or macromolecular binding. *In vitro* studies to support this choice of a single saturable pathway were then undertaken.

VC partition coefficients were measured *in vitro* for blood, liver, fat, muscle, and kidney (Table 1). This PBPK model consists of four compartments requiring partition coefficients: liver, fat, slowly perfused (muscle), and rapidly perfused (kidney). These partitions are similar to previous measurements, although the blood/air is somewhat higher resulting in the tissue/blood partitions being generally lower (Clement International, 1990).

Kinetic data for metabolism of VC were collected by measuring the disappearance of parent compound from a recirculating closed chamber system. Initial concentrations in the gas uptake studies ranged from 95 to 10,000 ppm (Fig. 1). Optimization of individual runs ($n = 18$) gave apparent kinetic constants of $V_{max_c} = 3.6 \pm 2.3$ mg kg^{-1} hr^{-1} (58 μmol kg^{-1} hr^{-1}) and $K_m = 0.08 \pm 0.09$ mg/liter (1 μM). Optimization of all runs estimated $V_{max_c} = 3.0$ mg kg^{-1} hr^{-1}

TABLE 1
Partition Coefficients for Vinyl Chloride and Trichloroethylene

Vinyl Chloride		
Blood/air (PB1)	2.4 ± 0.5	$n = 7$
Fat/blood (PF1)	10.0 ± 3.0	$n = 5$
Muscle/blood (PS1)	0.4 ± 0.2	$n = 4$
Liver/blood (PL1)	0.7 ± 0.3	$n = 5$
Kidney/blood (PR1)	0.7 ± 0.4	$n = 6$
Trichloroethylene		
Blood/air (PB2)	20.5 ± 1.4	$n = 4$
Fat/blood (PF2)	26 ± 5	$n = 3$
Muscle/blood (PS2)	0.6 ± 0.1	$n = 8$
Liver/blood (PL2)	1.3 ± 0.1	$n = 6$
Kidney/blood (PR2)	1.0 ± 0.2	$n = 5$

while K_m was estimated at 0.01, which was the lower bound present for optimization of this parameter.

Liver nonprotein sulfhydryl after VC exposure. Depletion of NPSH, largely glutathione, occurs during exposure to VC (Bruckner *et al.*, 1989). Therefore, this biological effect of VC metabolism was used to validate some aspects of the model and demonstrate, by correlation, that altered VC metabolism was occurring as predicted by the model (Tables 2–4). Because these exposures are not done at constant concentrations, the results are somewhat different (particularly at low concentrations) than those reported elsewhere (Bruckner *et al.*, 1989) but the extent of depletion at high concentrations is very consistent.

NPSH depletion increased with dose and time (6 hr versus 3 hr) resulting in a maximum of about 44% depletion during a 6-hr exposure starting at 5000 ppm VC (Tables 2 and 3). NPSH depletion was actually greater at 3 hr than at 6 hr, 18% versus 5%, with a starting concentration of approximately 600 ppm. This result likely is explained by the decrease in the total rate of VC metabolism that occurs as the exposure concentration decreased. Glutathione is complexly regulated by the body in an attempt to maintain normal levels so resynthesis is expected to occur (D'Souza *et al.*, 1988).

Modulation of P450 levels potentially requiring modeling. Alterations in enzyme levels may occur through several mechanisms including suicide inhibition, altered gene expression (induction or repression), and a variety of post-transcriptional alterations such as reduced turnover due to stabilization of protein. All three of these effects are known to occur for P450 2E1 (Yang *et al.*, 1990) and other P450 isoforms.

Enzymatic activities using several substrates were measured in liver microsomes from control and VC-exposed animals. The animals were exposed to a starting concentration of 10,000 ppm (1%) or 5000 ppm VC for a 6-hr period during which time chamber atmosphere was monitored. At these high (saturating) concentrations, the exposures more closely approximate constant concentration exposures.

Chlorzoxazone (25 μM) hydroxylation was measured as an indicator of P450 2E1 activity (Table 5). No difference in activity was seen between animals treated with high VC concentrations and untreated controls. Microsomes from pyridine and ethanol-treated animals were used as positive controls and showed about a five-fold increase in activity as expected. Comparison of control and 10,000 ppm VC-treated animals at high chlorzoxazone concentrations (1.25 mM) also found no difference (data not shown).

TCE metabolism was also measured *in vitro* (Table 6). Again, microsomes from animals exposed to high VC concentrations for 6 hr had identical activity compared to controls. Good induction of activity was seen with the pyridine-treated animals. Deethylation of 7-ethoxycoumarin was also apparently unaffected by 6-hr exposure to a high concentration of VC (Table 7). By contrast, a significant increase in activity was seen with phenobarbital induction.

Together, these results show no significant effect of high VC treatment on selected P450 activities, suggesting that no effects would occur at low VC concentrations either. Thus, protein stabilization, enzyme induction, and suicide inhibition were not modeled. The possibility that any of these effects might occur during longer or repeated exposures

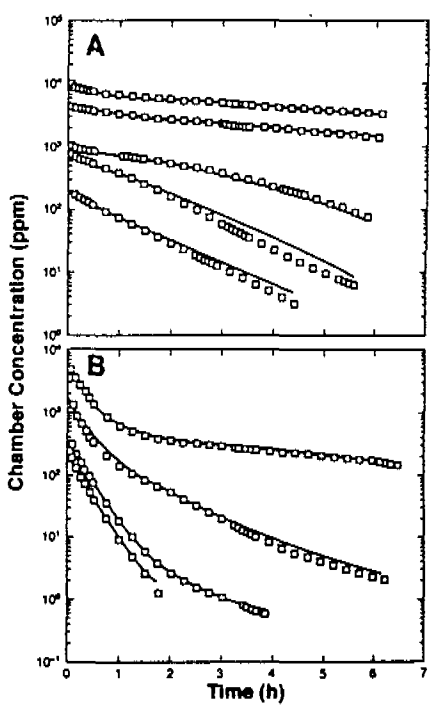

FIG. 1. Metabolism of individual chemicals. (A) Vinyl chloride (□) chamber concentrations decrease as it is metabolized by rats. Solid lines represent model simulations of data. (B) Trichloroethylene (□) concentrations measured in the chamber. Solid lines represent model simulations of data.

TABLE 2
Nonprotein Sulfhydryl Content in Livers of Rats Exposed for 6 hr to VC, TCE, or Mixtures

Exposures (6 hr)	Initial concentration (ppm)	NPSH (nmol/mg)	NPSH depletion (%)	Replicates:rats
Untreated	—	5.5 ± 0.6	—	30:6
Vinyl chloride	200	5.8 ± 0.3	0	15:3
	600	5.2 ± 0.5	5	14:3
	1000	4.1 ± 0.6[a,b,c]	25	45:9
	5000	3.0 ± 0.5[a,b,c,d]	44	24:6
Trichloroethylene	600	5.5 ± 0.8	0	15:3
	1000	5.7 ± 0.7	0	15:3
	5000	5.4 ± 0.9	0	15:3
VC/TCE mixtures	5000:600	3.6 ± 0.4[a]	35	15:3
	5000:5000	4.4 ± 0.4[a]	20	30:6
	1000:1000	4.3 ± 0.3[a]	22	29:6
	600:5000	5.2 ± 0.3	5	15:3
	35:1000	5.9 ± 0.4	0	15:3

[a] Significantly different from 0-ppm control (6 hr) at $p < 0.01$.
[b] Significantly different from 200 ppm (6 hr) at $p < 0.01$.
[c] Significantly different from 600 ppm (6 hr) at $p < 0.01$.
[d] Significantly different from 1000 ppm (6 hr) at $p < 0.01$.

might need to be considered further for purposes of modeling chronic bioassay data.

In vivo demonstration of P450 2E1 metabolism of VC. The involvement of P450 2E1 in VC metabolism *in vivo* was demonstrated using *trans*-1,2-dichloroethylene. *t*DCE has been suggested to be a suicide inhibitor based upon limited *in vitro* studies (Costa and Ivanetich, 1982) and modeling of gas uptake results (Gargas *et al.*, 1990).

There was no direct information available on the isoform specificity of *t*DCE metabolism, so studies were carried out with microsomes prepared from *in vivo t*DCE treated animals. *In vitro* chlorzoxazone hydroxylation was reduced 58% after 1.5- and 4.5-hr *t*DCE exposure (40 ppm initial concentration) (Table 5). Similarly, metabolism of *p*-nitrophenol dropped approximately 50% following *t*DCE treatment (data not shown). *t*DCE blocked the metabolism of TCE *in vitro* between 61 and 84% (Table 6). Deethylation of 7-ethoxycoumarin, in contrast, showed a small increase (Table 7) indicating that P450 2E1-catalyzed reactivity was reduced with some specificity.

Animals were exposed to *t*DCE for 90 min (40 ppm starting concentration) prior to the addition of VC. The VC was added directly into the chamber so both chemicals were present for the remainder of the exposure. Metabolism of VC (200 ppm starting concentration) for 3 hr was decreased approximately 85% (Fig. 2). Liver NPSH was unaffected by exposure to *t*DCE alone or followed by 200 ppm VC (Table 3). Microsomes prepared from these animals showed a 90% decrease for *in vitro* TCE metabolizing capability, in good agreement with the *in vivo* results.

TABLE 3
Nonprotein Sulfhydryl in Rats Exposed to VC and *t*DCE

Exposure	Initial concentration (ppm)	NPSH (nmol/mg)	NPSH depletion (%)	Replicates:rats
Untreated	—	6.0 ± 1.0*	—	43:17
Vinyl chloride (3 hr)	600	4.9 ± 0.5**	18	16:6
	5000	4.0 ± 0.9**	34	4:2
*t*DCE (4.5 hr)	40	6.4 ± 0.8	0	6:3
*t*DCE:VC (1.5:3 hr)	40:200	5.9 ± 0.4	0	10:5
	40:5000	5.5 ± 0.6	0	16:8

* Note significantly different from 0 ppm control (6 hr) at $p < 0.01$.
** Significantly different from 0 ppm control (3 hr) at $p < 0.01$.

TABLE 4
Correlation of Modeled VC Metabolism with Measured NPSH Depletion

Starting concentration (ppm)	NPSH depletion measured after 6 hr (%)	Modeled VC metabolized (μmol)
600 VC	5[a]	55
1000 VC	25	88
5000 VC	44	114
5000 VC/600 TCE	35	91
5000 VC/5000 VC	20	42

[a] This value represents partial recovery since depletion after 3 hr was 18%.

TABLE 6
In Vitro Trichloroethylene Metabolism by Rat Liver Microsomes

Treatment	Rate (nmol/min/mg protein) ± SD (% of control value)	Replicates:rats
Unexposed controls	0.70 ± 0.20	62:12
10000 ppm VC (6 hr)[a]	0.74 ± 0.22	8:3
5000 ppm VC (6 hr)[a]	0.72 ± 0.18	15:5
40 ppm *t*DCE (1.5 hr)[a]	0.21 ± 0.07[b] (39%)	9:3
40 ppm *t*DCE (4.5 hr)[a]	0.11 ± 0.05[b] (16%)	7:3
40 ppm *t*DCE (1.5 hr) and 200 ppm VC (3 hr)[a]	0.07 ± 0.02[b] (10%)	12:3
Pyridine-induced	1.7 ± 0.1[b] (243%)	5:3

[a] Nominal starting concentrations in closed-chamber experiments.
[b] Statistically different from controls at $p < 0.01$.

Estimating metabolic parameters for trichloroethylene. Partition coefficients were determined *in vitro* for blood, liver, fat, kidney, and muscle from male Sprague–Dawley rats (Table 1). These values were used in the PBPK model to fit gas uptake data for TCE and estimate an apparent K_m and V_{max_c} (Fig. 1). Optimization of six individual gas uptake exposures (200–6500 ppm) estimated $K_m = 0.09 \pm 0.11$ mg/liter (approximately 1 μM) and $V_{max_c} = 11.4 \pm 4.2$ mg kg^{-1} hr^{-1} (87 μmol kg^{-1} hr^{-1}).

Liver nonprotein sulfhydryl after TCE. Depletion of NPSH, largely glutathione, does not occur to any significant extent following trichloroethylene metabolism. This result was confirmed by analysis of livers of rats exposed to high TCE concentrations (600 to 5000 ppm initial concentration; Table 2).

Effect of tDCE preexposure on TCE metabolism. Preexposure of rats to approximately 40 ppm *t*DCE largely inhibited trichloroethylene metabolism at initial concentrations, although there was some apparent concentration dependence. Exposure to an initial concentration of 200 ppm showed 95% inhibition (V_{max_c} = 0.5 mg kg^{-1} hr^{-1}). Exposures starting at 500 and 2000 ppm showed 71 and 76% inhibition, respectively (V_{max_c} = 2.6 and 2.1 mg kg^{-1} hr^{-1}). These data support the involvement of P450 2E1 as the major form of P450 involved in TCE metabolism even at relatively high concentrations, although some involvement of other isoforms, as suggested by *in vitro* data (Nakajima *et al.*, 1993), cannot be excluded.

Gas uptake with mixtures of VC and TCE. Gas uptake exposures were performed using several different combinations of VC and TCE. These initial results were used in the PBPK model to estimate the various inhibitory constants required for competitive, noncompetitive, and uncompetitive inhibition. Using these parameters, the model was run to simulate potential results given different exposure concentrations. This gave estimates of concentrations that would likely distinguish between the three forms of inhibition.

Metabolic parameters were estimated for 15 mixtures using the competitive inhibition model. Apparent kinetic constants for VC are estimated to be $K_m = 0.10 \pm 0.03$

TABLE 5
Chlorzoxazone (25 μM) Metabolism by Rat Liver Microsomes

Treatment	Rate (nmol/min/mg protein) ± SD (percentage of control value)	Replicates:Rats
Unexposed controls	0.060 ± 0.018	29:10
10000 ppm VC[a]	0.054 ± 0.016	20:3
5000 ppm VC[a]	0.055 ± 0.012	12:5
40 ppm *t*DCE (1.5 h)[a]	0.025 ± 0.012[b] (42%)	6:3
40 ppm *t*DCE (4.5 h)[a]	0.025 ± 0.011[b] (42%)	6:3
Pyridine-induced	0.37 ± 0.08[b] (617%)	6:3
Ethanol-induced	0.27 ± 0.03[b] (450%)	3:3

[a] Nominal starting concentrations in closed-chamber experiments.
[b] Statistically different from controls at $p < 0.01$.

TABLE 7
7-Ethoxycoumarin Deethylation by Rat Liver Microsomes

Treatment	Rate (nmol/min/mg protein) ± SD (% of control value)	Replicates:rats
Unexposed controls	1.3 ± 0.4	57:11
10000 ppm VC (6 hr)[a]	1.1 ± 0.2	15:3
40 ppm *t*DCE (1.5 hr)[a]	1.7 ± 0.1* (133%)	3:2
40 ppm *t*DCE (4.5 hr)[a]	1.7 ± 0.1* (132%)	8:3
Phenobarbital-induced	5.1 ± 0.5* (392%)	3:3

[a] Nominal starting concentrations in closed-chamber experiments.
* Statistically different from controls at $p < 0.01$.

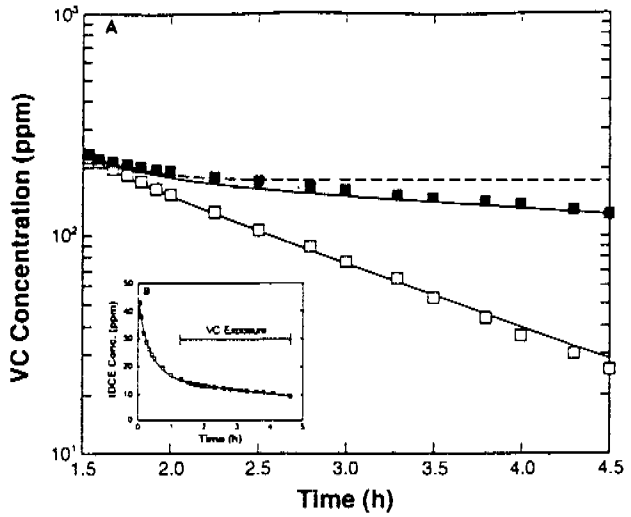

FIG. 2. Metabolism of VC in the presence and absence of *t*DCE. (A) The decrease in VC chamber concentration is much greater in the absence (□) than in the presence (■) of *t*DCE. Solid lines represent model simulations. The dashed line is modeled VC concentrations with no metabolism ($V'_{max_c} = 0$). (B) The *t*DCE exposure started 1.5 hr prior to the addition of VC. The effects of *t*DCE cannot be accounted for by competitive inhibition alone.

mg/liter and $V_{max_c} = 3.1 \pm 1.0$ mg kg^{-1} hr^{-1}. Constants for TCE are estimated to be $K_m = 0.21 \pm 0.08$ mg/liter and $V_{max} = 9.7 \pm 2.2$ mg kg^{-1} hr^{-1}. These values are consistent with those estimated for the individual chemicals.

Results from a representative gas uptake exposure to a mixture and the simulated results are illustrated in Fig. 3. These data were used to distinguish between competitive, noncompetitive, and uncompetitive inhibitory kinetics. It should be noted that mixtures of high concentrations of both chemicals (e.g., 5000 ppm of both VC and TCE) were uninformative for distinguishing kinetic mechanisms.

Figure 3 illustrates attempts to simulate the mixture of 700 ppm VC and 1000 ppm TCE with competitive, noncompetitive, and uncompetitive inhibition using parameters that reasonably simulated several other data sets. Noncompetitive inhibition was easily excluded (Fig. 3B). Optimization of each individual mixture for the inhibition constants of the noncompetitive model resulted in estimates of K_{M12} and K_{M21} varying 100-fold. Thus, it was impossible to use a single set of parameters for noncompetitive inhibition to consistently fit all mixtures data.

Uncompetitive inhibition was more difficult to rule out. For many of the mixtures, a reasonable simulation of gas uptake data was obtained using a single set of constants describing uncompetitive inhibition (e.g., Fig. 3C). Two mixtures (25:1000 and 85:1000) were predicted by the model as not being consistent with a single set of parameters and the data were subsequently collected verifying this (Fig. 4A). As shown in Fig. 4B, it was possible to adjust the parameters for uncompetitive inhibition to simulate the mixture of 25:1000. However, these alternate parameters were unsuccessful at simulating the 700:1000 mixture (Fig. 4C). The mixtures of 85:1000 gave similar results. Thus, uncompetitive inhibition does not provide satisfactory simulation of all the mixtures using a single consistent set of parameters.

Validation of mixtures modeling with NPSH measurements. Livers of animals exposed to mixtures of TCE and VC were removed and analyzed for NPSH. As previously described, increasing VC resulted in lower NPSH level (i.e., more depletion) (Table 2). Increasing the concentration of TCE (600 and 5000 ppm starting concentration) present simultaneously with 5000 ppm VC resulted in less NPSH depletion, 35 and 20% respectively, (Table 4) than in the absence of TCE, 44%.

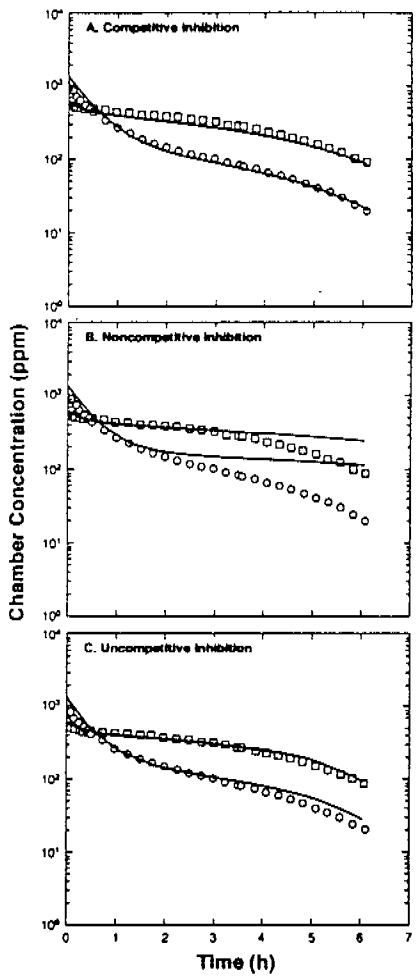

FIG. 3. A mixture with initial concentrations of 700 ppm VC (□) and 1000 ppm TCE (○). Data (○, □) and simulation (solid line). (A) Competitive inhibition; (B) noncompetitive inhibition; (C) uncompetitive inhibition. Reasonable fits are obtained with competitive or uncompetitive inhibition but not noncompetitive using parameters listed under Methods.

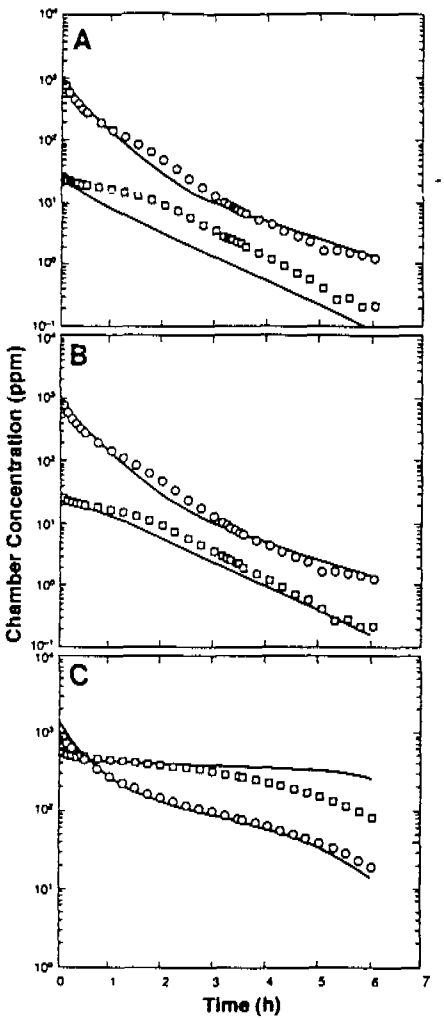

FIG. 4. Demonstration that a single set of values of parameters for uncompetitive inhibition will not fit all the data. (A) Failure to model the mixture 25 ppm VC and 1000 ppm TCE using $K_{M12} = K_{M21} = 3.0$ (as in Fig. 3). (B) Modeling the mixture of 25:1000 using $K_{M12} = 3.0$ and $K_{M21} = 0.20$ provides a good fit. (C) The parameter values in (B) do not provide a good simulation for 700 ppm VC and 1000 ppm TCE.

Modeling competitive inhibition during constant concentration exposures. Human exposures of interest for risk assessment may approximate constant concentration exposures. Occupational exposures are typically of 8-hr duration and environmental exposure is 24 hr. Therefore, exposures of rats to mixtures of VC and TCE were simulated for 8- and 24-hr periods. It should be noted that near steady state is achieved rapidly at low concentrations for both chemicals. Within a half hour, the venous concentration of VC is near steady state and TCE is about 75% steady state. The metabolism of VC and TCE over 8 and 24 hr is approximately a linear function of time, due to the rapid approach to steady state. Therefore, only the 24-hr results are presented here (Table 8; Fig. 5).

TCE is a more effective inhibitor of VC metabolism than vice versa. This is due to the greater blood–air partition coefficient for TCE which results in a higher concentration in blood at the same exposure concentration. At concentrations below 30 ppm of each chemical there is little effect on the metabolism of the other chemical. As the concentrations of either chemical increases, inhibition of metabolism becomes increasingly significant (Fig. 5; Table 8).

DISCUSSION

Modeling was begun with the assumption that a single saturable pathway representing P450 2E1 in the liver compartment might be adequate to describe initial oxidative metabolism of VC and TCE. However, suicide inhibition via formation of a heme adduct by VC or related vinyl halogens has previously been demonstrated (Watanabe *et al.*, 1976; Ortiz de Montellano *et al.*, 1982). P450 2E1 is also frequently "induced" by exposure to its substrates, although the mechanisms may not be transcriptional (Yang *et al.*, 1990). Finally, limited *in vitro* data suggested that VC is metabolized by several P450 isoforms (Reynolds *et al.*, 1975) while extensive studies show this to be true for TCE (Miller and Guengerich, 1983). Modeling mixtures requires having good models for the individual chemicals, so these aspects were investigated further.

Suicide inhibition of P450 by VC has been demonstrated both *in vitro* and *in vivo* at high concentrations (about 5% or 50,000 ppm) using untreated and phenobarbital-induced animals (Ivanetich *et al.*, 1977; Reynolds *et al.*, 1975). To determine if this effect needed to be modeled, metabolism of several P450 substrates was measured in liver microsomes from rats exposed to several thousand ppm of VC. No differences were found between control and VC-treated rats in metabolism of chlorzoxazone, TCE, or 7-ethoxycoumarin. Because suicide inhibition is a stochastic occurrence, these data indicate that it occurs too rarely to be apparent *in vivo* at concentrations used in chronic studies ($\leq$10,000 ppm) with VC. No evidence exists for such effects with TCE (Pessayre *et al.*, 1979). Therefore, suicide inhibition was not modeled for either compound.

These studies also provide no evidence for increased P450 activity due to exposure to VC or TCE for 6 hr at high concentrations. P450 2E1 activity is increased by both transcriptional and post-transcriptional regulation including protein stabilization by substrate (Yang *et al.*, 1990). No such effects were apparent using *in vitro* assays and microsomes from VC-exposed animals. The *in vivo* data for both VC and TCE were consistent with a constant V_{max}. Whether changes might occur during chronic exposures such as during cancer studies has not been determined.

The issue of modeling P450 isoforms was considered. This issue is important for extrapolation from high to low

TABLE 8
Estimated Values Obtained by Simulating Constant Concentration Exposures to VC/TCE Mixtures for Rats

VC (ppm)	TCE (ppm)	Inhibition (%)[a]	A_{M1}[b] (μmol)	A_{M2}[b] (μmol)	C_{VL1}[c] (μM)	C_{VL2}[c] (μM)	C_{V1}[d] (μM)	C_{V2}[d] (μM)
0	10	0	0	61	0	0.2	0	1.2
1	10	0	3	61	0.01	0.2	0.05	1.2
10	10	0	25	60	0.1	0.3	0.5	1.2
100	10	5	231	58	1.7	0.5	5.5	1.4
300	10	21	423	48	15	2	22	2.7
1,000	10	59	476	25	82	5	89	5.3
3,000	10	83	495	11	277	7	285	7.1
10,000	10	94	502	4	966	8	978	8.0

[a] Percentage inhibition of TCE metabolism due to increasing VC.
[b] A_M, amount metabolized of chemical 1 (VC) and chemical 2 (TCE).
[c] C_{VL}, venous concentration leaving liver.
[d] C_V, venous blood concentration.

doses and across species using PBPK models as is required for cancer risk assessment.

Substrates for P450 are frequently metabolized by multiple isoforms, often in a concentration-dependent manner. Descriptions of this complexity might be required, for instance, if the metabolite profile changed with concentration or across species. Such modeling would require estimates of the kinetic parameters (e.g., V_{max}, K_m) and concentration for each isoform. Alternatively, it may be satisfactory to simply model an "averaged" P450 enzyme where the apparent V_{max} and K_m describe the metabolism without describing the activity of each isoform.

Based upon existing literature one would expect P450 2E1 to metabolism both TCE and VC (Guengerich *et al.*, 1991; Miller and Guengerich, 1983). However, monoclonal antibodies to P450 2E1 only inhibited 60% of the trichloroethylene metabolism in control microsomes (Nakajima *et al.*, 1993). P450 2B1 also metabolizes TCE, but is present at very low levels in untreated rats so its involvement would be expected to be minimal. TCE is also metabolized by the male rat-specific isoform P450 2C11 *in vitro*.

*t*DCE exposure at moderate concentrations was a highly effective inhibitor of the metabolism of the other chlorinated ethylenes. Quantitatively, the inhibition of TCE metabolism was similar *in vitro* and *in vivo*. *In vitro* assays of enzyme activity with microsomes from the *t*DCE-treated rats clearly show large decreases in other activities associated with P450 2E1 (chlorzoxazone, *p*-nitrophenol). Essentially no change was seen in 7-ethoxycoumarin activity by comparison.

These results show that P450 2E1 activity accounts for much of the TCE and VC metabolism *in vivo* with untreated animals, except perhaps at high concentrations. *t*DCE was somewhat less effective at inhibiting the metabolism of TCE at 500 and 2000 ppm than at 200 ppm and lower (71–76% versus 95%). The involvement of a single isoform may simplify the difficulties of interspecies extrapolation. Measurements of P450 2E1 activity using readily available *in vitro* systems could permit scaling of metabolic terms or direct use of the values determined *in vitro*. If the values for K_m are similar across species, one may be able to use the relative activity of P450 2E1 across species determined with any substrate. This would be particularly useful with humans where *in vivo* estimates are unavailable for VC.

Coexposure to VC and TCE are successfully described as competitive inhibition using a fixed single saturable pathway for oxidative metabolism. A wide range of mixtures was used in gas uptake studies in order to distinguish between kinetic inhibition models. Competitive inhibition consistently simulated the data using a single set of parame-

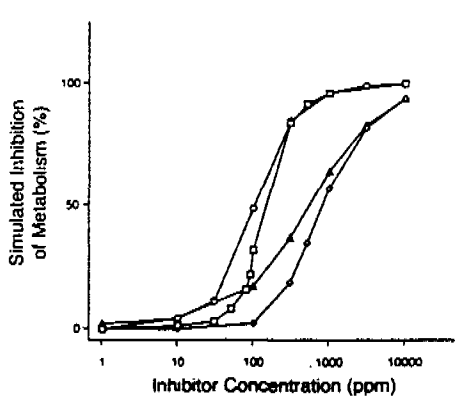

FIG. 5. Prediction of inhibition of metabolism VC or TCE during a 24-hr exposure simulated for constant concentration exposures of rats to mixtures of VC and TCE. Indicated points represent modeling output with lines connecting them to approximate the shape of the curve. Inhibition by TCE of VC metabolism (VC at (○) 1 or 10 ppm; (□) 100 ppm) and metabolism and inhibition by VC of TCE (TCE at (△) 1 or 10 ppm; (◇) 100 ppm). TCE is a more effective inhibitor of VC metabolism than vice versa.

ters. Furthermore, the kinetic parameter values calculated from the competitive inhibition model are essentially identical to those obtained from studies with individual chemicals. By contrast, noncompetitive inhibition would fit very few data sets with a single set of parameter values. Uncompetitive inhibition tended to more readily simulate multiple data sets except for several key mixtures that were only fit with widely varying parameter values.

The results of the mixtures simulations were further validated using glutathione depletion. This endpoint was chosen because it was dependent upon metabolism of VC. Glutathione conjugation is an important pathway for detoxification of VC metabolites. This is apparently only an acute effect since GSH levels return to control values as exposures continue over a period of days (Du *et al.*, 1979). The potential implications of this aspect for modeling VC carcinogenicity remain to be explored.

Finally, the results of this study are of interest from the perspective of risk assessment for mixtures. Exposure of humans to chlorinated ethylene mixtures may occur occupationally, generally by inhalation. Environmental exposure may be oral or by inhalation. Exposure to water may involve both oral and inhalation pathways, the latter due to water uses such as washing or showering. The inhalation studies reported here may readily be extrapolated to the oral route using the PBPK model.

Although one might expect P450 2E1 substrates to be competitive inhibitors in mixtures, such effects are highly concentration dependent. The inhibition effects observed here only occur at relatively high concentrations, as has also been reported for competitive inhibition between toluene and xylene (Tardif *et al.*, 1993). This arises from several factors. The perfusion limitation at low concentrations is one factor. Metabolism of the inhibitor, the parent compound, at low concentrations diminishes its effectiveness.

The degree of inhibition is not simply predictable from the metabolism equation due to the dependence of the steady-state venous concentration upon the rate of metabolism and vice versa. Competitive inhibition may be described as resulting from an alteration of the K_m for the substrate (e.g., $v = (V_{max} * S)/[K_m(1 + I/K_i) + S]$: v is velocity, S is substrate concentration, and I is inhibitor concentration). Thus, as the concentration of inhibitor increases the effective K_m would increase and metabolism would decrease. However, as metabolism decreases, the steady-state venous concentration of the substrate increases. These two effects counterbalance up to a point.

For instance, the predicted steady-state concentration of VC in venous rat blood at an exposure concentration of 10 ppm is 0.5 μM. However, as metabolism decreases to zero, the venous concentration increases to 1 μM. Similarly, TCE (10 ppm) is predicted to give a venous concentration of 1.2 μM increasing to 8 μM in the absence of metabolism (see Table 8). This effect loses importance as the inhibitor venous concentration increases dramatically (orders of magnitude) as exposure concentration increases similarly.

These discussions are all based upon experimental data and simulations for rats. However, similar results are obtained for simulations of humans. Simulations were run scaling the rat model to a 70-kg human using values in the literature (Chen and Blancato, 1989; Fisher and Allen, 1993). Although the values for VC, particularly, must be considered limited estimates due to a lack of much data, the results for the mixture were essentially the same. That is, no inhibition was seen for either chemical at concentrations below approximately 10 ppm.

Occupational exposures to these chlorinated hydrocarbons may be as high as tens of ppm, but environmental exposures are more typically low ppb levels. These latter are clearly in a range where the metabolism becomes perfusion limited and enzyme is saturating, so no inhibition would be predicted to occur. Under these conditions the pharmacokinetics of the compounds would be independent. This is consistent with the standard U.S. EPA approach of assuming additivity. However, it remains to be shown that the pharmacodynamics of the two chemicals are also independent. Toxicity, and thus, chemical risk assessment, ultimately depends upon both pharmacokinetics and pharmacodynamics.

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