# Oxidation of Trichloroethylene by Liver Microsomal Cytochrome P-450: Evidence for Chlorine Migration in a Transition State Not Involving Trichloroethylene Oxide†

Randall E. Miller and F. Peter Guengerich*

ABSTRACT: Trichloroethylene (TCE) was metabolized by cytochrome P-450 containing mixed-function oxidase systems to chloral (2,2,2-trichloroacetaldehyde), glyoxylic acid, formic acid, CO, and TCE oxide. TCE oxide was synthesized, and its breakdown products were analyzed. Under acidic aqueous conditions the primary products were glyoxylic acid and dichloroacetic acid. The primary compounds formed under neutral or basic aqueous conditions were formic acid and CO. TCE oxide did not form chloral in any of these or other aqueous systems, even when iron salts, ferriprotoporphyrin IX, or purified cytochrome P-450 was present. Ferric iron salts catalyzed the rearrangement of TCE oxide to chloral only in $CH_2Cl_2$ or $CH_3CN$. A 500-fold excess of iron was required for complete conversion. A kinetic model involving the zero-order oxidation of TCE to TCE oxide by cytochrome P-450 and the first-order degradation of the epoxide was used to test the hypothesis that TCE oxide is an obligate intermediate in the conversion of TCE to other metabolites. Kinetic constants for the breakdown of TCE oxide and for the oxidative metabolism of TCE to stable metabolites were used to predict epoxide concentrations required to support the obligate intermediacy of TCE oxide. The maximum levels of TCE oxide detected in systems using microsomal fractions and purified cytochrome P-450 were 5–28-fold lower than those predicted from the model. The kinetic data and the discrepancies between the observed metabolites and TCE oxide breakdown products support the view that the epoxide is not an obligate intermediate in the formation of chloral, and an alternative model is presented in which chlorine migration occurs in an oxygenated TCE–cytochrome P-450 transition state.

The microsomal mixed-function oxidase system containing cytochrome P-450[1] is capable of converting a wide variety of olefins and aromatic compounds to epoxides, as well as catalyzing other oxidative reactions (Wislocki et al., 1980). In some cases epoxides can be isolated, while in other situations the epoxide rearranges, hydrolyzes, or is attacked by nucleophilic groups on macromolecules to form adducts which may be responsible for initiation of toxic and carcinogenic phenomena (Miller & Miller, 1981).

TCE is an industrial chemical which is suspected as a carcinogenic agent. Certain evidence suggests that the action of P-450 converts TCE to compounds which become irreversibly bound to proteins (Van Duuren & Banerjee, 1976) and nucleic acids (Banerjee & Van Duuren, 1978), and TCE oxide has been postulated to be the metabolite responsible for such binding. Leibman (1965) reported that TCE is converted to chloral by liver microsomes and provided evidence that the reaction was catalyzed by P-450. Chloral, trichloroethanol, and trichloroacetic acid are the major metabolites of TCE detected under in vivo conditions (Defalque, 1961; Kimmerle & Eben, 1973). TCE is also converted to CO in microsomal incubations (Traylor et al., 1976). Attempts to demonstrate TCE oxide as a metabolite of TCE have not been successful to date (Bartsch et al., 1979). Henschler et al. (1979) synthesized TCE oxide and reported that the compound rearranged to dichloroacetic acid, CO, and formic acid but not to significant amounts of chloral or glyoxylic acid. The lack of rearrangement to chloral is not consistent with the view that TCE is metabolized to chloral exclusively via TCE oxide (Powell, 1945), and the hypothesis has been presented that the iron in the heme of P-450 acts as a Lewis acid to catalyze the rearrangement (Henschler et al., 1979).

We have reexamined the rearrangement of TCE oxide under a variety of conditions. The data indicate that TCE oxide does not rearrange to chloral in the presence of P-450. TCE oxide was identified as a metabolite of TCE in this work, but kinetic studies are inconsistent with the view that formation of TCE oxide is an obligate step in the metabolism of TCE to the observed enzymatic products. Alternatively, we propose that chlorine migration occurs within an oxygenated TCE–P-450 transition state leading to chloral formation.

## Experimental Procedures

*Chemicals.* TCE oxide was synthesized by using a modification of a method previously described (Kline & Van Duuren, 1977). A 600-mL solution of distilled TCE containing 3.4 mL of 70% *tert*-butyl hydroperoxide was heated at 60 °C with oxygen bubbled through the solution at a rate of 300 mL $min^{-1}$ for 30 h. The temperature was increased to 85 °C for an additional 30 h. The vessel was fitted with a dry ice condenser. $^1H$ NMR analysis indicated that the reaction mixture contained 82% TCE, 8% 2,2-dichloroacetyl chloride, and 10% TCE oxide. The mixture was distilled with the use of a spinning band column at a vacuum of 130 mmHg. The distillate was collected when the head temperature was 30 °C and the pot temperature was 45–46 °C. The recovered dis-

† From the Departments of Pharmacology (R.E.M.) and Biochemistry (F.P.G.) and Center in Environmental Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232. *Received August 13, 1981.* This work was supported in part by Grants ES 02205 and ES 00267 from the U.S. Public Health Service. R.E.M. was supported by U.S. Public Health Service Training Grant GM 07628, and this work constitutes a portion of the material submitted for his doctoral thesis. F.P.G. is the recipient of U.S. Public Health Service Research Career Development Award ES 00041.

[1] Abbreviations: P-450, liver microsomal cytochrome P-450; TCE, 1,1,2-trichloroethylene; chloral, 2,2,2-trichloroacetaldehyde; PNBP, (*p*-nitrobenzyl)pyridine; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; GC, gas chromatography; HPLC, high-pressure liquid chromatography.

0006-2960/82/0421-1090$01.25/0 

tillate was washed 3 times with ice-cold 6 N NaOH to remove 2,2-dichloroacetyl chloride and dried over anhydrous $MgSO_4$. The $^1H$ NMR spectrum (100 MHz) showed only two singlets at $\delta$ 6.52 and 5.31 corresponding to TCE and TCE oxide, respectively (Kline & Van Duuren, 1977). The TCE oxide accounted for 35% of the mixture. The $^{13}C$ NMR spectrum showed singlets for TCE oxide at 73.7 (CHCl) and 85.7 ppm ($CCl_2$) in addition to the two singlets for TCE at 116.6 (CHCl) and 123.9 ppm ($CCl_2$). The epoxide reacted with PNBP reagent without heating (Barbin et al., 1975) to produce a characteristic adduct with an absorption maximum at 560 nm.

[1,2-$^{14}C_2$]TCE (3.4 mCi/mmol) was purchased from New England Nuclear, Boston, MA, and radiochemical purity was judged to be >99% as determined by counting fractions recovered from GC using a Tenax column at 160 and 220 °C.

$FeBr_3$ was prepared by bromination of iron powder as described by Gregory & Thackrey (1950).

TCE, 2,2-dichloroacetic acid, 2,2-dichloroacetyl chloride, tetrachloroethylene, PNBP, 2,2,2-trichloroethanol, and glyoxylic acid hydrate were purchased from the Aldrich Chemical Co., Milwaukee, WI. Chloral hydrate was purchased from Mallinckrodt, Inc., St. Louis, MO.

*Enzyme Preparations.* Male Sprague-Dawley rats (200–250 g) and male ICR Swiss mice (20–25 g) were obtained from Harlan Industries, Indianapolis, IN. Rats were induced with phenobarbital administered in drinking water (Guengerich & Martin, 1980) while mice were given intraperitoneal injections of 80 mg/kg body weight daily for 5 days. Liver microsomes were prepared by differential centrifugation and stored at −70 °C. Rat liver cytochrome P-450 (fraction $B_2$), NADPH–P-450 reductase, and epoxide hydrolase were prepared from livers of phenobarbital-induced rats as described elsewhere (Guengerich & Martin, 1980).

*Assays.* The decomposition of TCE oxide was monitored by reacting aliquots of incubation mixtures with PNBP reagent (Guengerich et al., 1979).

Incubations involving mixed-function oxidative activities of microsomes or purified P-450 included 0.1 M potassium phosphate buffer (pH 7.7) and an NADPH-generating system composed of 10 mM glucose 6-phosphate, 1.0 IU of yeast glucose-6-phosphate dehydrogenase $mL^{-1}$, and 0.5 mM NADPH. In some cases the NADPH system was replaced with 5 mM iodosobenzene. TCE was dissolved in acetone and added to each vial to give a final concentration of 25 mM [the final concentration of acetone was ≤1% (v/v)]. In general, incubations were stopped by the addition of $ZnSO_4$ to 30 mM, centrifuged at 2000*g* for 10 min to precipitate protein, acidified with $H_2SO_4$, and extracted with ether.

Each ether phase was concentrated under $N_2$ and analyzed directly for chloral and 2,2,2-trichloroethanol by using GC (Tenax, 140 °C) with an electron capture detector. The ether phase was analyzed for dichloroacetic acid and trichloroacetic acid by using GC (Tenax, 170 °C) following derivatization with diazomethane. The aqueous phase was analyzed for glyoxylic acid by isocratic reverse-phase HPLC [Partisil PXS 10/25 ODS column (Whatman, Inc., Clifton, NJ); 80% $H_2O$/20% $CH_3CN$ (v/v)] after derivatization with 2,4-dinitrophenylhydrazine (Fieser & Fieser, 1967). The 2,4-dinitrophenylhydrazone was monitored at 360 nm. Separate incubations were conducted for formic acid. Acetonitrile was added to the ether extract, and most of the ether was evaporated under $N_2$. Following derivatization with *p*-nitrophenacyl bromide (Morozowich & Douglas, 1975), the formate ester was analyzed by isocratic reverse-phase HPLC [Partisil PXS 10/25 ODS column; 55% $H_2O$/45% $CH_3CN$ (v/v)] with monitoring at 260 nm.

Table I: Decomposition Products of TCE Oxide in Aqueous Media[a]

| incubation medium | products formed (per μmol of TCE oxide) | | | |
|---|---|---|---|---|
| | glyoxylic acid (nmol) | dichloro-acetic acid (nmol) | formic acid (nmol) | CO (nmol) |
| 0.1 N HCl | 678 ± 47 | 178 ± 25 | 136 ± 19 | 95 ± 9 |
| $H_2O$ | 624 ± 26 | 30 ± 0 | 146 ± 27 | 290 ± 21 |
| 20 mM Tris-HCl, pH 7.6 | 70 ± 17 | 40 ± 2 | 429 ± 29 | 604 ± 74 |
| 20 mM potassium phosphate, pH 7.7 | 80 ± 10 | 24 ± 5 | 528 ± 63 | 52 ± 5 |
| 0.1 N NaOH | 9 ± 3 | 39 ± 3 | 514 ± 51 | 394 ± 28 |

[a] All incubations were carried out for 30 min at 37 °C. TCE oxide was added to the medium with mixing to give an initial concentration of 1.0 mM. Results are presented as means ± SD of determinations of three individual incubations.

Unlabeled CO was determined spectrally as the carboxyhemoglobin adduct by using freshly prepared rabbit erythrocytes (Tsukamoto et al., 1980). Following a 30-min incubation with TCE oxide, the solution was adjusted to neutral pH, and the erythrocytes were added. After 45 min, the carboxyhemoglobin content was determined (Commins & Lawther, 1965). $^{14}CO$ was analyzed by a modification of a method previously described (Ahr et al., 1980). Incubations (5 mL) were stopped with $ZnSO_4$ (to 30 mM), and 2 mL of 1.5 M $K_3Fe_3(CN)_6$ and 1 mL of carrier CO were injected into the sealed flasks. Vacuum was applied to each flask in order to remove volatile products; CO was oxidized to $CO_2$ by passage through a 15-cm hopcalite column at 95 °C and trapped in 30 mL of an ethanolamine/2-methoxyethanol solution (2:1 v/v). After 90 min, 2.5-mL aliquots of the trapping solution were added to 10 mL of scintillation cocktail, and the radioactivity was determined.

Enzymatic TCE oxide formation was determined in the following manner: Incubations (1.0 mL) were stopped with 1.0 mL of benzene and centrifuged for 10 min at 2000*g*. Each benzene extract was added to 0.5 mL of 50 mM PNBP in ethylene glycol/acetone (4:1 v/v) with mixing. After 10 min, 1.0 mL of a 1:1 (v/v) triethylamine/acetone solution was added, and the absorbance was measured at 520 nm. A standard curve was prepared with authentic TCE oxide.

## Results

*Synthesis and Decomposition of TCE Oxide.* TCE oxide was synthesized as described, and its identity was verified by $^1H$ and $^{13}C$ NMR and facile reaction with PNBP. The reaction with PNBP was conducted at 23 °C for 5 min. Under these conditions related compounds other than halo epoxides did not react with PNBP to yield adducts having absorbance in the region of 500–560 nm. Compounds that did not react with PNBP include chloral, chloroacetyl chloride, dichloroacetyl chloride, glyoxylic acid, and TCE. The $t_{1/2}$ of TCE oxide was determined under various conditions using PNBP to monitor the epoxide concentrations (Guengerich et al., 1979). The $t_{1/2}$ of TCE oxide at 37 °C was 12 s in 0.1 M potassium phosphate buffer (pH 7.7), 13 s in 0.1 N HCl, 13 s in 0.1 N NaOH, 17 s in 0.1 M potassium phosphate buffer containing mouse liver microsomes (5 mg $mL^{-1}$) or rat liver microsomes (10 mg $mL^{-1}$), and 9 s in 0.1 M potassium phosphate buffer containing purified rat liver epoxide hydrolase at a concentration of 3 mg $mL^{-1}$ (data not shown). These results indicate that TCE oxide is probably a poor substrate for epoxide hydrolase.

The degradation products of TCE oxide were determined under various conditions (Table I). At low pH, apparent

Table II: Rearrangement of TCE Oxide to Chloral[a]

| incubation conditions for nonaqueous systems[b] | | chloral formation (%) |
|---|---|---|
| solvent | catalyst | |
| $CH_3CN$ | | <0.1 |
| | 13 mM $FeCl_2$ | 3.5 ± 0.1[c] |
| | 58 mM $FeCl_3$ | 6.6 ± 1.3[c] |
| | $FeBr_3$ | <0.1 |
| | $Fe^{3+}$ PP IX | <0.1 |
| $CH_2Cl_2$ | | 4.1 ± 0.9 |
| | $FeCl_2$ | 4.8 ± 1.4[d] |
| | 49 mM $FeCl_3$ | 143.6 ± 2.1[c] |
| | 2 mM $FeCl_3$ | 8.5 ± 0.8[c] |
| | 0.1 mM $FeCl_3$ | 7.2 ± 1.0[e] |
| | $FeBr_3$ | 85.9 ± 13.4[c] |
| | $Fe^{3+}$ PP IX | 4.4 ± 0.9[d] |
| hexane | | 4.6 ± 0.3 |
| | $FeCl_2$ | 4.9 ± 0.1[d] |
| | $FeCl_3$ | 5.2 ± 0.1[d] |
| | $Fe^{3+}$ PP IX | 4.4 ± 0.1[d] |

[a] TCE oxide (100 μM) was incubated in each nonaqueous media for 30 min at 37 °C. Results are presented as means ± SD of determinations of three individual incubations. [b] TCE oxide was incubated in a solution containing either anhydrous $FeCl_3$, $FeCl_2$, $FeBr_3$, or ferriprotoporphyrin IX ($Fe^{3+}$ PP IX). Solutions were saturated when concentrations are not indicated. [c] Significantly greater than control ($p < 0.005$). [d] Not significantly greater than control ($p > 0.10$). [e] Significantly greater than control ($p < 0.01$).

hydrolysis of the epoxide predominates to form glyoxylic and dichloroacetic acids. The incubations in water also represent an acidic medium since the final pH after 30-min incubation was 3.5. Formic acid and CO were the major products formed at neutral or high pH. However, in phosphate buffer the level of CO was greatly reduced. One possibility that was considered was formation of formyl phosphate. The hydroxamic acid assay of Lipmann & Tuttle (1945) suggested that TCE oxide formed labile esters in *N*-(2-acetamido)iminodiacetic acid, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid, and Tris buffers (approximately 200 nmol/μmol of TCE oxide). This value was doubled in the presence of phosphate buffer. The identity of these presumed esters was not pursued.

*Conversion of TCE Oxide to Chloral.* So that the hypothesis that the iron of the heme group of P-450 catalyzes the rearrangement of TCE oxide to chloral via chlorine migration could be tested, TCE oxide was incubated in aqueous and nonaqueous solvents in the presence of $Fe^{2+}$ or $Fe^{3+}$ salts or various hemoproteins including P-450 (Table II). In the aqueous systems no chloral was detected under any of the incubation conditions.[2] Rearrangement of the epoxide to chloral did occur in some of the nonaqueous systems examined; $Fe^{3+}$ salts catalyzed the rearrangement to the greatest extent. Essentially complete rearrangement of TCE oxide occurred with a 500-fold excess of $FeCl_3$ or $FeBr_3$, but only a small amount of rearrangement occurred with a 20-fold excess. $FeCl_2$ was only slightly effective in catalyzing the rearrangement of the epoxides to chloral, while ferriprotoporphyrin IX was ineffective. The low level of chloral detected in the absence of any iron was probably produced by thermal rearrangement (Mekhryushev & Poluektov, 1973) of the epoxide during GC analysis, as residual epoxide was detected after 30-min incubations in $CH_2Cl_2$ and hexane but not in $CH_3CN$.

[2] TCE oxide (100 μM) was added to either bovine serum albumin, ferriprotoporphyrin IX, bovine liver catalase, equine hemoglobin, or rat liver P-450 (all at 100 μM) or rat liver microsomes prepared from phenobarbital-treated rats (10 mg of protein $mL^{-1}$) in 0.1 M potassium phosphate buffer (pH 7.4). The extent of conversion of TCE oxide to chloral was less than 0.1% in all cases and in a system devoid of proteins or ferriprotoporphyrin IX.

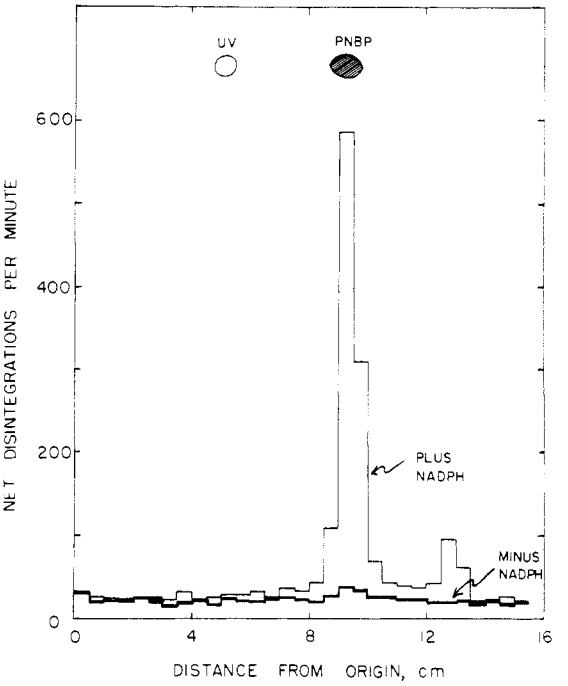


FIGURE 1: Thin-layer chromatography of TCE oxide–PNBP adduct. Rat liver microsomes (10 mg of protein $mL^{-1}$) were incubated with or without an NADPH-generating system in the presence of 100 mM potassium phosphate buffer (pH 7.7) and 11 mM [1,2-$^{14}C_2$]TCE (9 mCi $mmol^{-1}$) for 60 s at 37 °C. One milliliter of each incubate was extracted with an equal volume of benzene, and then 200 nmol of unlabeled TCE oxide was added. One-half milliliter of a solution of 50 mM PNBP in acetone was added to each benzene extract. After reaction for 5 min at 23 °C, adducts were extracted from benzene into equal volumes of $H_2O$. The aqueous extracts were concentrated to dryness by lyophilization and dissolved in minimal amounts of $CH_3OH$. Aliquots were chromatographed on Whatman $KC_{18}$ reverse-phase plates by using the solvent system $CH_3OH$–$H_2O$ (80:20 v/v). Residual PNBP reagent was located by using UV light (254 nm) and migrated along with a standard (zone denoted "UV" on the graph). Plates were sprayed with a 50% solution of triethylamine in acetone to visualize PNBP adducts, and only one major purple adduct was found (zone denoted "PNBP" on the graph). One-half-centimeter zones were scraped from the plates into scintillation vials. One milliliter of $CH_3OH$ was added to each vial to elute radioactivity, and then 5 mL of scintillation cocktail was added. The counting efficiency was approximately 55% for all samples of interest. Background radioactivity was not subtracted in the graph.

*Enzymatic Formation of TCE Oxide.* Studies on the distillation of TCE oxide (see Experimental Procedures) suggested that the boiling point at atmospheric pressure is approximately 100 °C. Bartsch et al. (1979) trapped volatile materials swept from mouse liver microsomal incubations and were not able to detect TCE oxide as the PNBP adduct. The high boiling point of the epoxide may have precluded its detection in this system. An alternative means of separating the epoxide involves extraction into benzene from aqueous enzyme incubations and reaction of the benzene layer with PNBP reagent. When such a procedure was used, adducts having visible absorption spectra identical with those of the TCE oxide–PNBP adduct were isolated from the incubations of TCE with (a) rat liver microsomes and NADPH, (b) mouse liver microsomes and NADPH, (c) purified rat liver P-450 and iodosobenzene, and (d) a reconstituted enzyme system containing purified rat liver P-450, NADPH–P-450 reductase, phospholipid, and NADPH. The point should be reemphasized that chloral and acyl chlorides do not react with PNBP under the conditions used (see above).

Rat liver microsomes were incubated with [1,2-$^{14}C_2$]TCE

Table III: Rate Constants for Metabolism of TCE to Various Products[a]

| system[b] | product [nmol min$^{-1}$ (nmol of P-450)$^{-1}$] | | | |
|---|---|---|---|---|
| | CO | chloral | glyoxylic acid | trichloroacetic acid |
| rat liver microsomes, NADPH | <0.1 | 3.3 ± 0.4 | 0.9 ± 0.2 | <0.1 |
| mouse liver microsomes, NADPH | 0.5 ± 0.2 | 7.8 ± 1.7 | 4.9 ± 1.4 | <0.1 |
| P-450, iodosobenzene | 4.8 ± 0.4 | 53.8 ± 5.6 | 13.0 ± 3.0 | 12.2 ± 1.4 |
| P-450, NADPH-P-450 reductase, NADPH | 2.7 ± 0.5 | 20.1 ± 2.8 | 3.0 ± 1.4 | <0.1 |

[a] Results are presented as means ± SD of determinations of three individual incubations. [b] The incubation conditions for these systems are described under Figures 2–5.

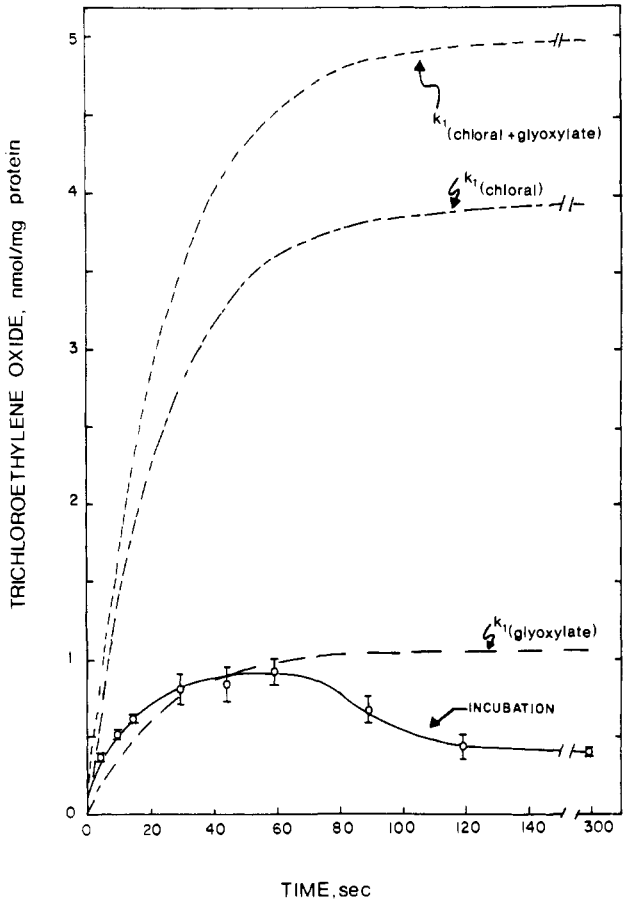


FIGURE 2: Time course of TCE oxide formation in rat liver microsomes. The first-order rate constants, $k_1$, for epoxide formation were calculated from the concentrations of chloral and glyoxylic acid at 1 min by using the expression $k_1 = [C][P\text{-}450]^{-1}(t + k_2^{-1}e^{-k_2t} - k_2^{-1})^{-1}$ and an experimentally determined $k_2$ value of 2.5 min$^{-1}$ under these conditions. [C] indicates the concentration of chloral or glyoxylic acid. The theoretical time course for epoxide formation was calculated by using [TCE oxide] = $k_1k_2^{-1}$[P-450]$(1 - e^{-k_2t})$, with $k_1$ calculated for each of the final products. Data are shown for actual epoxide levels from a rat liver microsomal incubation [10 mg of protein mL$^{-1}$ (O)], predicted epoxide levels assuming only a glyoxylic acid contribution to $k_1$ (– –), predicted epoxide levels assuming only a chloral contribution to $k_1$ (– – –), and predicted epoxide levels assuming a contribution to $k_1$ from both chloral and glyoxylic acid (– · –). TCE oxide levels were determined as described under Experimental Procedures; results are expressed as means ± SD of determinations from three incubations.

and NADPH, and the incubation was extracted with benzene as before. Authentic TCE oxide was added to the organic phase prior to PNBP. The TCE oxide–PNBP adduct was extracted into water and lyophilized, and an aliquot was chromatographed by using a reverse-phase thin-layer plate. Visualization of the plate with base showed a purple spot at $R_f$ 0.6 (Figure 1). More than 85% of the radioactivity migrated with the PNBP adduct.

*Kinetic Analysis of the Role of TCE Oxide in Enzymatic*

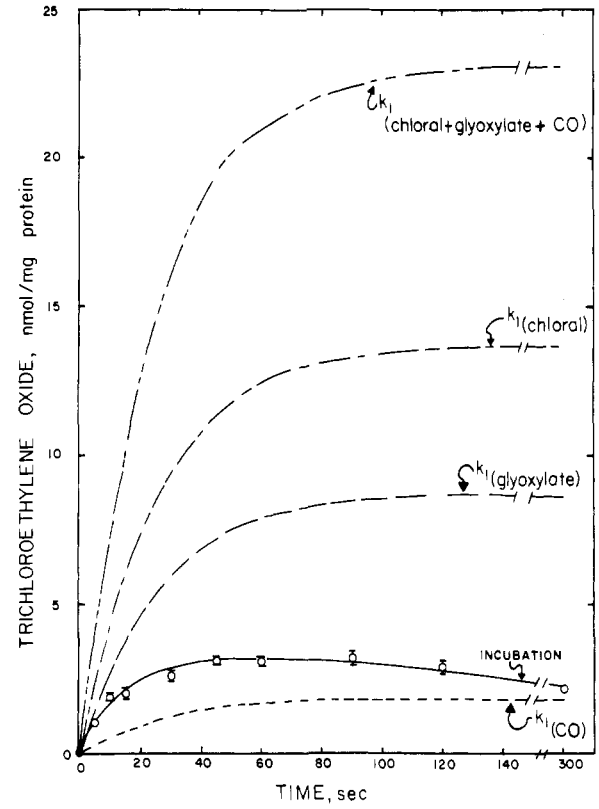


FIGURE 3: Time course of TCE oxide formation in mouse liver microsomes. The first-order rate constants, $k_1$, for epoxide formation were calculated as described under Figure 2. The observed rate $k_2$ was found to be 2.5 min$^{-1}$ under these conditions. Data are shown for actual epoxide levels from a mouse liver microsomal incubation [2 mg of protein mL$^{-1}$ (O)], predicted epoxide levels assuming only a CO contribution to $k_1$ (– – –), predicted epoxide levels assuming only a glyoxylic acid contribution to $k_1$ (– – –), predicted epoxide levels assuming only a chloral contribution to $k_1$ (– – –), and predicted epoxide levels assuming a contribution to $k_1$ from chloral, CO, and glyoxylic acid (– · · –). TCE oxide levels were determined as described under Experimental Procedures; the results are expressed as means ± SD of determinations from three incubations.

*TCE Oxidation.* For assessment of the role of TCE oxide in TCE metabolism, a kinetic model involving two steps was developed on the basis of an obligatory epoxide intermediate:

$$\text{TCE} \xrightarrow[k_1]{\text{P-450}} \text{TCE oxide}$$

$$\text{TCE oxide} \xrightarrow{k_2} \text{breakdown products}$$

The rate constant $k_2$ describes the first-order decomposition of TCE oxide, and $k_1$ measures the zero-order formation of TCE oxide. Values for $k_1$ can be estimated by the overall apparent zero-order rate of conversion of TCE to products. Incubations were carried out with saturating concentrations of TCE (25 mM) to ensure zero-order kinetics of epoxide formation. (Steady-state kinetics indicate biphasic behavior for the oxidation of TCE to chloral by rat liver microsomes

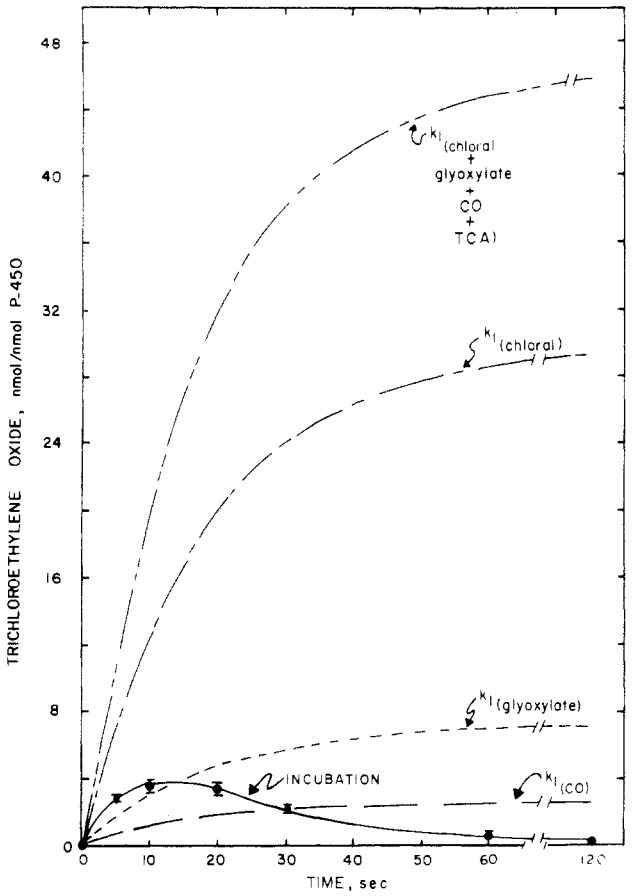


FIGURE 4: Time course of TCE oxide formation in incubations of P-450 with iodosobenzene. The first-order rate constants, $k_1$, for epoxide formation were calculated from the concentrations of chloral, CO, glyoxylic acid, and trichloroacetic acid at 30 s as described under Figure 2. Under these conditions $k_2$ was 3.5 $min^{-1}$. Data are shown for actual epoxide levels from an incubation containing 5 $\mu$M P-450 and 5 mM iodosobenzene (●), predicted epoxide levels assuming only a CO contribution to $k_1$ (−−), predicted epoxide levels assuming only a glyoxylic acid contribution to $k_1$ (---), predicted epoxide levels assuming only a chloral contribution to $k_1$ (−·−), and predicted epoxide levels assuming a contribution to $k_1$ from chloral, CO, glyoxylic acid, and trichloroacetic acid (TCA) (−···−). TCE oxide levels were determined as described under Experimental Procedures; results are presented as means ± SD of determinations of three incubations.

with apparent $K_m$ values of 0.3 and 2.2 mM.) Values for $k_2$ were determined experimentally (see below), and at any time $t$ the concentration of a metabolite [C] is given by the expression (Hess & Wurster, 1970)

$$[C] = k_1[\text{P-450}](t + k_2^{-1}e^{-k_2t} - k_2^{-1})$$

Thus, quantitation of TCE metabolites as a function of time gives $k_1$ when $k_2$ is known (Table III).

The P-450/iodosobenzene system had the highest rate of TCE metabolism. In addition, this was the only system producing trichloroacetic acid. Trichloroethanol was not produced in any of the systems investigated. The mouse microsomal system had a considerably higher rate of metabolism than did the rat microsomal system. [The microsomal incubations were conducted in 0.1 M potassium phosphate buffer (pH 7.7) which suppressed the level of CO formed, as noted previously in the epoxide decomposition studies. When the phosphate buffer was replaced with 0.1 M Tris-HCl at pH 7.6, the level of CO production was greatly increased in both systems.] With the exception of trichloroacetic acid in the P-450/iodosobenzene system, the product distribution in all four systems was similar.

The expected concentration of TCE oxide at any time $t$ is

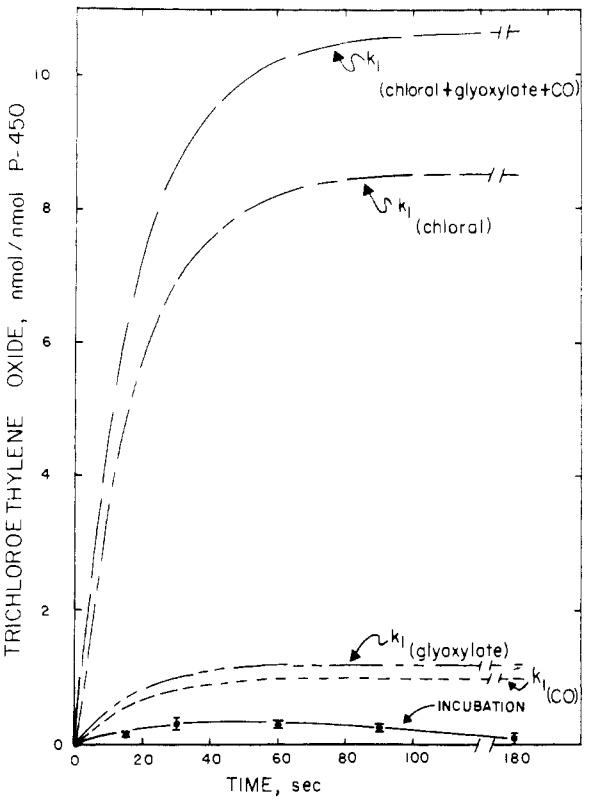


FIGURE 5: Time course of TCE oxide formation using a reconstituted P-450 system. The first-order rate constants, $k_1$, for epoxide formation were calculated from the concentrations of chloral, CO, and glyoxylic acid at 90 s as described under Figure 2. Under these conditions $k_2$ was 3.5 $min^{-1}$. Data are shown for actual epoxide levels from an incubation containing 2 $\mu$M P-450, 2 $\mu$M NADPH–P-450 reductase, and 50 $\mu$M L-$\alpha$-dilauroylglyceryl-3-phosphorylcholine (●), predicted epoxide levels assuming only a CO contribution to $k_1$ (---), predicted epoxide levels assuming only a glyoxylic acid contribution to $k_1$ (−···−), predicted epoxide levels assuming only a chloral contribution to $k_1$ (−·−), and predicted epoxide levels assuming a contribution to $k_1$ from chloral, CO, and glyoxylic acid (−−). TCE oxide levels were determined as described under Experimental Procedures; the results are expressed as means ± SD of determinations from three separate incubations.

given by the expression (Hess & Wurster, 1970)

$$[\text{TCE oxide}] = (k_1k_2^{-1})[\text{P-450}](1 - e^{-k_2t})$$

Theoretical kinetic courses of epoxide formation based on the preceding equation are shown for rat liver microsomes (Figure 2), mouse liver microsomes (Figure 3), P-450/iodosobenzene (Figure 4), and the reconstituted system containing P-450 and NADPH–P-450 reductase (Figure 5). In all cases the experimentally determined concentration of TCE oxide was maximal between 10 and 60 s. The microsomal systems reached a steady-state epoxide level after approximately 2–3 min. Theoretical concentrations of TCE oxide are also shown on the basis of the rates of formation of each of the various TCE metabolites. In all systems the concentrations of TCE oxide were 5–28-fold lower than those predicted on the basis of total metabolites. On the basis of $k_1$ values derived only from rates of chloral formation, theoretical levels of epoxide formed were 4–23-fold higher than the experimentally determined TCE oxide levels.

## Discussion

The data with the epoxide trapping reagent PNBP provide evidence that TCE oxide is indeed a product of TCE metabolism. Under the conditions used, none of the compounds examined other than the epoxide reacted with PNBP to pro-

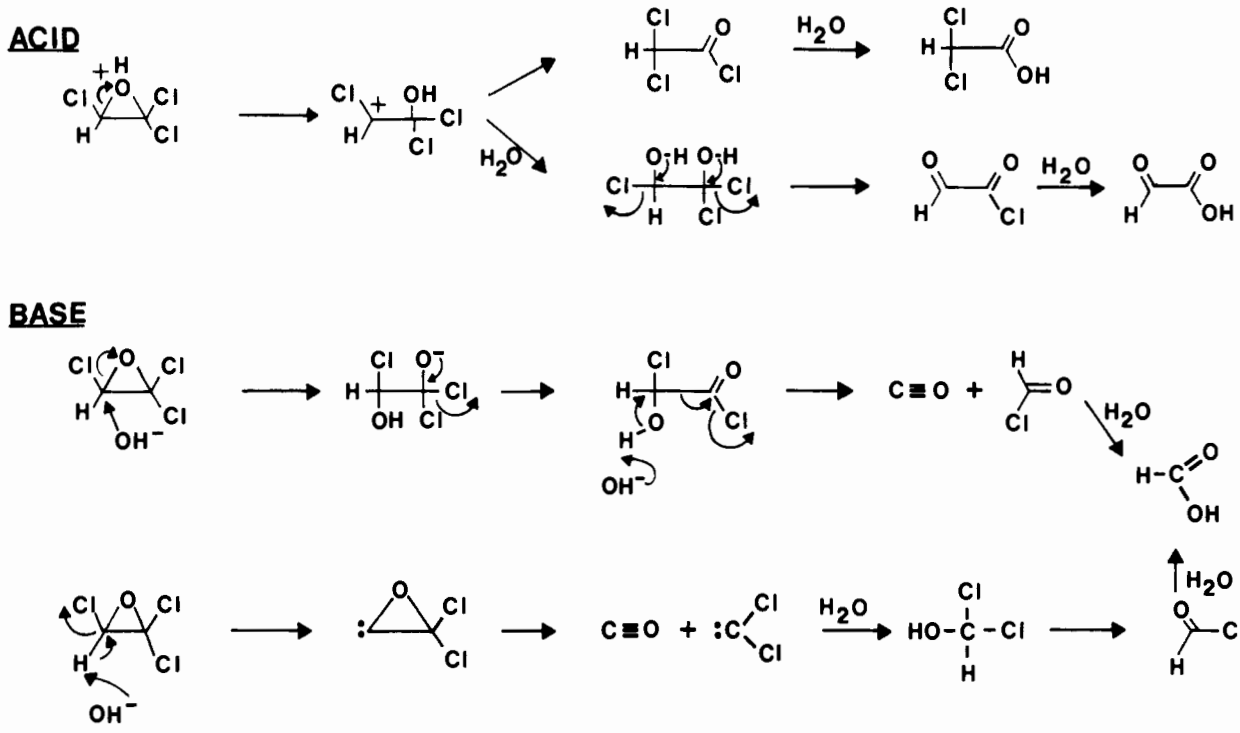


FIGURE 6: Postulated mechanisms for hydrolysis and rearrangement of TCE oxide under acidic and basic conditions. See text for discussion.

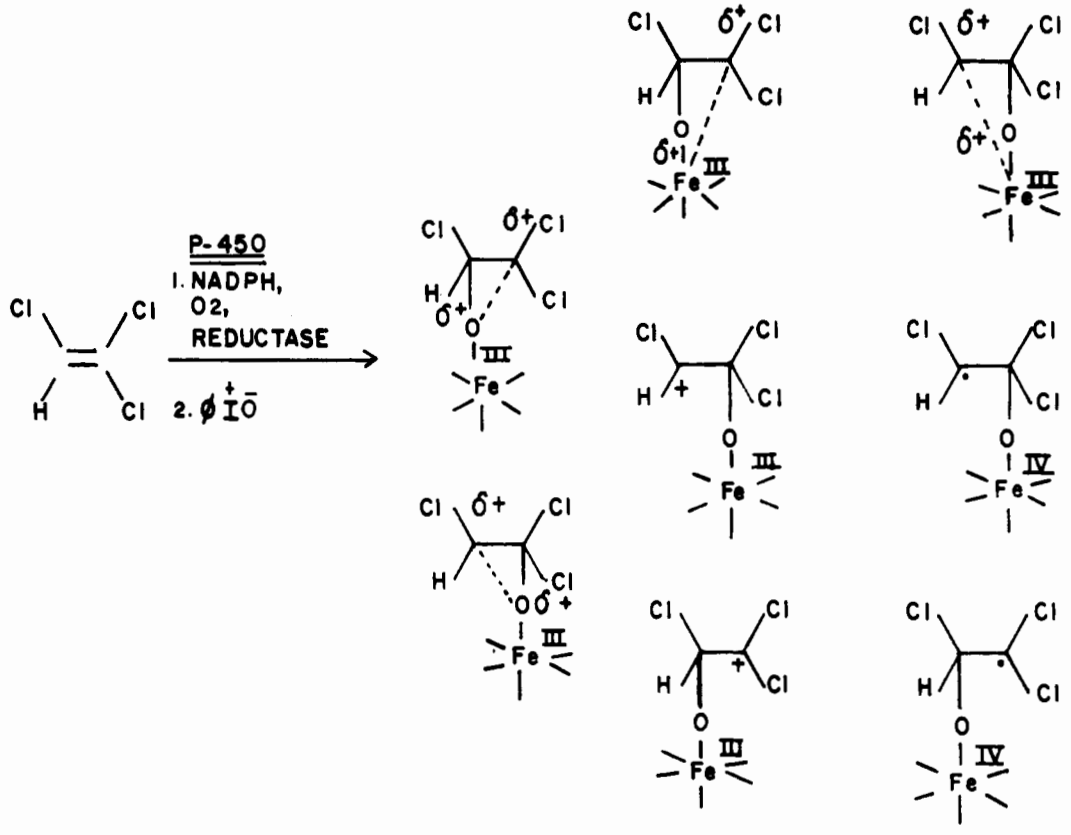


FIGURE 7: Postulated structures contributing to the TCE-oxygenated P-450 transition state.

duce a colored adduct. The epoxide was produced in both microsomal and reconstituted systems. Previously Bartsch et al. (1979) reported that TCE was not metabolized by mouse liver microsomes to a volatile alkylating metabolite (i.e., TCE oxide) that could be trapped by reaction with PNBP. However, vinyl chloride and 2-chlorobutadiene were metabolized to volatile alkylating metabolites that reacted with PNBP, indicative of epoxides. This particular method requires that the epoxide be volatile enough to be removed from the microsomal system as a vapor and trapped by PNBP. The method we have developed does not depend on the volatility of TCE oxide.

The decomposition products of TCE oxide were pH dependent (Table I). The scheme in Figure 6 is presented to account for the observed results. Under acidic conditions the oxygen would be protonated, resulting in the cleavage of the C–O bond to produce the carbonium ion intermediate shown. This intermediate could either undergo chlorine migration to

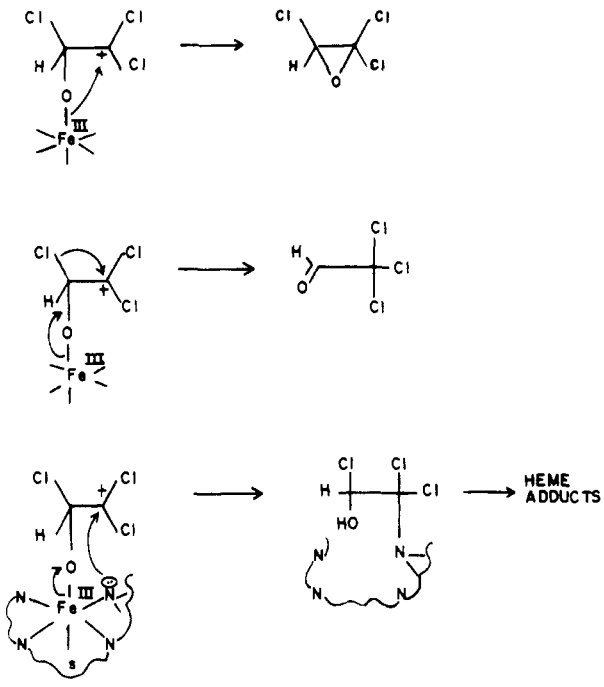


FIGURE 8: Possible rearrangements of one form of the postulated TCE-oxygenated P-450 transition state. See text for discussion.

form dichloroacetyl chloride or hydrolysis to form a glycol. The glycol would be unstable and dehydrohalogenate to form glyoxyl chloride, which could be hydrolyzed to form glyoxylic acid. Two base-catalyzed mechanisms are proposed to account for CO and formic acid, which were formed in nearly equal amounts (Table I). The first mechanism involves nucleophilic attack by hydroxide ion and opening of the oxirane ring. C–C bond fission of a subsequent *gem*-halohydrin acyl halide would produce CO and formyl chloride, which would be hydrolyzed to formic acid. Alternatively proton abstraction and chlorine loss could produce a carbene which might rearrange to CO and the dichlorocarbene, which could also react with $H_2O$ to produce formic acid. The mechanisms explain the equal amounts of CO and formic acid produced, in nonphosphate buffers, but would predict a decrease in formic acid and not CO in the presence of phosphate (Table I) if formyl chloride reacts with phosphate. However, a definitive mechanism can only be elucidated by using TCE oxide with individual carbons labeled or with TCE oxide analogues.

Comparison of the observed levels of TCE oxide produced in the incubation systems with the concentrations predicted from kinetic considerations indicates that the epoxide cannot be an *obligatory* intermediate in the oxidation of TCE to chloral (by P-450). The results presented for microsomes and reconstituted P-450 systems suggest the existence of a pre-epoxide transition state involving the binding of TCE to the activated oxygen of P-450 (Figure 7). This postulated transition state could involve either a cation or a free radical species. Groves et al. (1980) have suggested that P-450 forms such transition states in the epoxidation of olefins on the basis of studies carried out with manganoporphyrins as models. Radicals were favored over carbonium ions as intermediates in that work. The actual electronic distribution is best described by a mixture of localization such as presented in Figure 7 and would be expected to be a function of the substrate under consideration.

The postulated transition state could undergo different types of rearrangement to produce known products of TCE metabolism (Figure 8). This figure illustrates the rearrangement of one form of the transition state to produce TCE oxide,

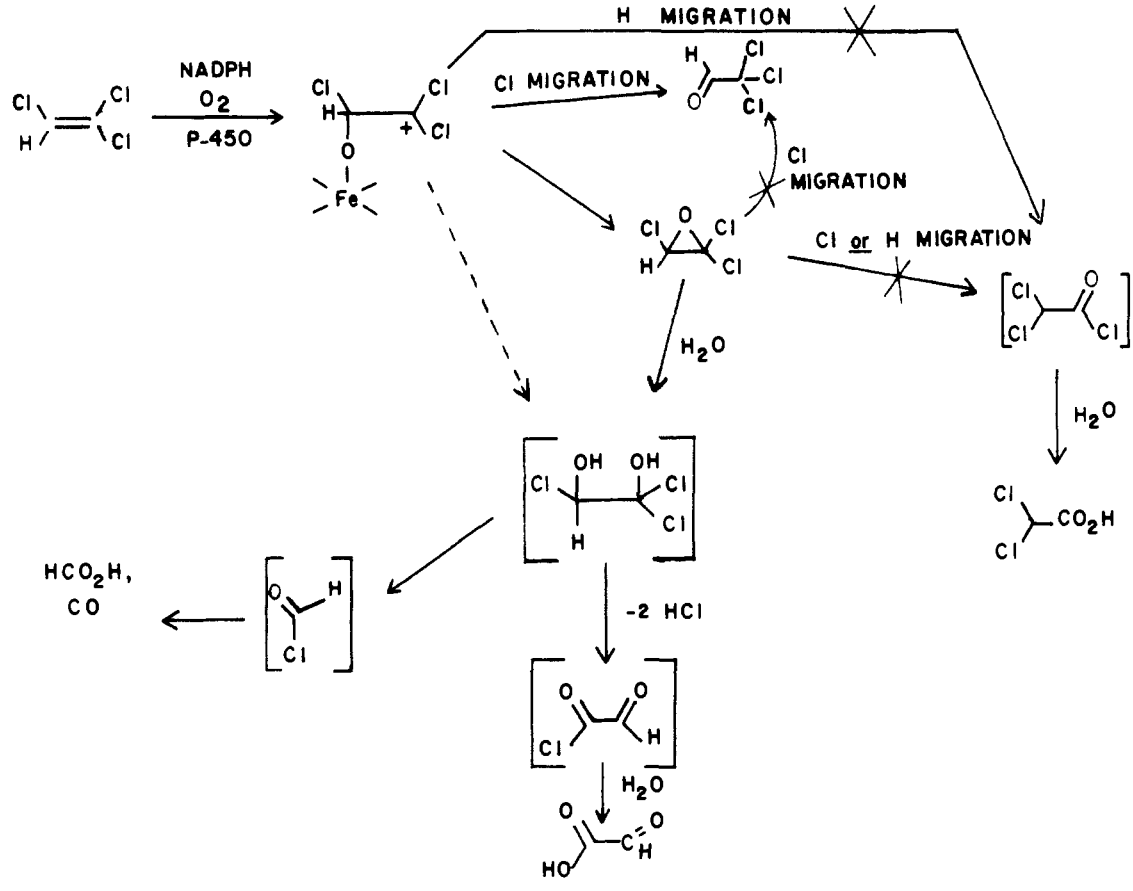


FIGURE 9: Postulated scheme for the metabolism of TCE. See text for discussion.

chloral, or heme adducts. In other studies which are not presented, the heme of P-450 was destroyed during oxidative metabolism of TCE, as in the case with vinyl chloride (Guengerich & Strickland, 1977) and a variety of other olefins and acetylenes (Ortiz de Montellano et al., 1981). Epoxides have been shown not to be the agents responsible for heme destruction in the cases of vinyl chloride (Guengerich & Strickland, 1977) and methyl allylisopropylacetate (Ortiz de Montellano et al., 1979). Moreover, TCE oxide did not destroy the heme of purified P-450 under the conditions presented in Table II. Ortiz de Montellano et al. (1981) have postulated attack of a porphyrin pyrrolic nitrogen on a carbonium ion in the destruction of P-450 heme during ethylene metabolism, as we also suggest here. Such a mechanism is consistent with the structure of the isolated porphyrin adduct (Ortiz de Montellano et al., 1981).

Some of the kinetic data (Figures 2 and 4) are consistent with the view that the minor metabolites of TCE (glyoxylic acid, CO, and formic acid) are formed from the decomposition of TCE oxide. However, in some cases the levels of these metabolites were higher than can be explained with the epoxide as an intermediate (Figures 3 and 5), and we hypothesize that hydration or C–C bond fission of the transition state can occur to some extent.

The evidence for group migration in a P-450 oxygenated transition state is also of interest in interpretation of other mechanistic studies, particularly with aromatic compounds. Application of the mechanism shown in Figure 8 to an aromatic system would predict migration from the carbon at the point of electrophilic attack to an adjacent carbon. Such results have been observed with tritium, deuterium, and halide shifts in the conversion of monocyclic aromatic compounds to phenols and collectively termed the "NIH shift" (Daly et al., 1972). In the same sense, the oxidation of TCE to chloral involves an "NIH shift" in an acyclic system, although the evidence here argues against the necessary intermediacy of an epoxide. Thus, aromatic 1,2 shifts can be explained by the mechanism in Figure 8 without the need to invoke epoxides. The principal evidence that epoxides are formed in such cases is the isolation of dihydrodiols (which have been dealt with primarily in polycyclic ring systems) and not the 1,2 shifts (Daly et al., 1972).

A working scheme for the metabolism of TCE is presented in Figure 9. In this scheme TCE oxide is not an obligatory intermediate in the formation of any of the metabolites. While rearrangement of TCE oxide to chloral occurred in the presence of excess ferric iron salts and $CH_2Cl_2$, we have excluded such a reaction from the scheme. Such rearrangement did not occur with lower concentrations of iron, and complexation of iron in a porphyrin structure would reduce its ability to act as a Lewis acid. Moreover, the incubation of TCE oxide with a stoichiometric amount of purified P-450 did not result in rearrangement to chloral (Table II). Dichloroacetic acid was identified as a decomposition product of TCE oxide (Table I) but was not detected as a metabolite in any of the enzymatic incubations. The possible formation of some of the glyoxylic acid, CO, and formic acid without the intermediacy of TCE oxide is also presented. Possible reactive metabolites that might bind irreversibly to protein and nucleic acids are chloral, TCE oxide, dichloroacetyl chloride, and formyl chloride (Figure 9). Further effort will be required to elucidate the roles of these compounds in such binding and the significance of the adducts formed.

Acknowledgments

We are indebted to Drs. T. L. Macdonald and L. T. Burka for helpful discussions and for critical review of the manuscript. We also thank Drs. V. Ullrich and J. A. Groves for their suggestions.

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