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# TRICHLOROETHYLENE OXIDATION BY TOLUENE DIOXYGENASE

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**SUMMARY:** Trichloroethylene was oxidized by purified toluene dioxygenase obtained from recombinant *E. coli* strains. The major oxidation products were formic acid and glyoxylic acid. Other potential products, dichloroacetic acid, chloral, phosgene, carbon monoxide, and carbon dioxide, were not detected. [$^{14}$C]trichloroethylene became covalently attached to protein components and NADPH suggesting non-specific alkylation by reactive products. Oxidation of deuterated trichloroethylene yielded 50.2% deuterated formate. Oxidation of trichloroethylene in $D_2O$ yielded 43.7% deuterated formate. These data indicate that both carbon atoms are giving rise to formic acid. The results are consistent with a mechanism of TCE oxygenation not involving epoxide, dioxetane, or dihydroxy intermediates and indicate significant differences from those previously proposed for cytochrome P-450 (Miller, R.E. & Guengerich, F.P. (1982) *Biochemistry* 21, 1090-1097) or methane monooxygenase (Fox, B.G., Borneman, B.G., Wackett, L.P., & Lipscomb, J.D. (1990) *Biochemistry* 29, 6419-6227). 

Trichloroethylene (TCE) is biodegraded by various bacteria biosynthesizing catabolic oxygenases which gratuitously oxidize (cooxidize) chlorinated ethylenes including TCE (1). Specific enzymes implicated in TCE oxidation are monooxygenases physiologically active with methane (2), propane (3), isoprene (4), ammonia (5), and toluene (6). Only one dioxygenase, toluene dioxygenase, is known to oxidize TCE (7, 8). TCE oxidation by *Pseudomonas putida* F1 results in cytotoxicity and loss of toluene dioxygenase activity (9) suggesting that highly reactive products are generated.

Previously, the products of TCE oxidation by soluble methane monooxygenase (sMMO) were elucidated using purified enzyme components (2). The immediate reaction products released from the enzyme were identified as trichloroethylene epoxide and 2,2,2-trichloracetaldehyde (chloral). The formation of chloral, which arises via atomic migration in the oxygenation reaction (10), implies the intermediacy of a monoatomic, highly electrophile oxygen species which is generated by sMMO to oxidize TCE and other

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Abbreviations: TCE, trichloroethylene; sMMO, soluble methane monooxygenase; $Fd_{TOL}$, ferredoxin component of toluene dioxygenase; $ISP_{TOL}$, iron-sulfur protein component of toluene dioxygenase; HPLC, high pressure liquid chromatography; FPLC, fast protein liquid chromatography; GC, gas chromatography; MS, mass spectrometry; UV, ultraviolet.

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substrates (2). The major enzymatic product is TCE-epoxide. Synthetic TCE-epoxide undergoes spontaneous decomposition in aqueous buffers via isomerization and hydrolysis to yield dichloroacetate, glyoxylate, formate, and carbon monoxide (11). These same decomposition products were detected in similar proportions in sMMO reaction mixtures.

Stable, identifiable products of TCE oxidation by toluene dioxygenase would be expected to arise from highly reactive intermediates. Thus, the observation of signature products requires a minimalistic reaction system of substrates, cofactors, and purified toluene dioxygenase enzyme components. The three proteins comprising this multi-component enzyme system are unstable and difficult to purify (12). In this study, we have utilized two recombinant overproducing *E. coli* strains to obtain purified toluene dioxygenase components in high yield (13, 14). Reactions were conducted with purified enzyme components to determine the products and develop further insights into product stoichiometries and enzyme modification reactions *in vitro*. These data have importance in further understanding the reaction mechanisms of aromatic hydrocarbon dioxygenases. Additionally, the safe use of toluene dioxygenase-containing bacteria in TCE bioremediation necessitates a knowledge of the products of enzymatic TCE decomposition.

## MATERIALS AND METHODS

*Chemicals:* [1,2-$^{14}$C] TCE (4.1 mCi/mmol) was obtained from Sigma Radiochemicals, St. Louis, MO. *d*-TCE was obtained from Cambridge Isotope Laboratories, Cambridge, MA. 4-Carboxythiazolidin-2-one was synthesized as detailed by Mansuy, et al (15). All other chemicals were of the highest purity available and were used without further purification.

*Enzymes:* Ferridoxin-NADP$^+$ reductase from spinach leaves was purchased from Sigma Chemical Co., St. Louis. *E. coli* JM109 (pDTG 614) and *E. coli* JM 109 (pDTG 601a) were provided by David T. Gibson, University of Iowa. The growth of the bacteria and the purification of $ISP_{TOL}$ from *E. coli* JM109 (pDTG 601A) and $ferredoxin_{TOL}$ from *E. coli* JM109 (pDTG614) were conducted as described previously (13,14).

*Enzyme Reaction Conditions:* Except where noted, 10 $\mu$l (33 nmol) [$^{14}$C]TCE in methanol was added to a Teflon-sealed 2 ml glass reaction vial containing 12 $\mu$g spinach reductase, 3.8 $\mu$g $ferredoxin_{TOL}$ and 29 $\mu$g $ISP_{TOL}$ in 0.4 ml 50 mM Tris buffer, pH 7.5. The reaction was initiated by the addition of 240 nmol of NADPH. In larger scale experiments for product elucidation, 25 $\mu$l (83 nmol) [$^{14}$C]TCE in methanol was added to a Teflon sealed 10 ml reaction vial containing 75 $\mu$g spinach reductase, 20 $\mu$g $ferredoxin_{TOL}$ and 70 $\mu$g $ISP_{TOL}$ in 2.0 ml 50 mM Tris buffer, pH 7.5. The reaction was initiated by the addition of 1.2 $\mu$mol of NADPH.

To estimate TCE oxidation activity, reaction mixtures of 0.4 ml in 2 ml vial were shaken at 23 °C for 5 min. Aliquots of 10 $\mu$l were withdrawn and spotted onto 1 x 1 cm$^2$ Kodak (Rochester, NY) chromagram sheets coated with silica gel. In 30 min, the unreacted volatile TCE was evaporated and the radioactivity retained on the sheets was determined using a Beckman LS 6800 scintillation counter.

*Determination of Acidic Products:* Acidic products from the enzyme oxidation of [$^{14}$C]TCE were separated by high pressure liquid chromatography (HPLC) fitted with a Bio-Rad Aminex HPX-87H column (isocratic solvent elution: 5% acetonitrile, 95% 0.01 N $H_2SO_4$, flow rate 0.6 ml/min) via UV absorbance detection at 210 nm. The radioactivity from column fractions collected at 10 seconds interval was measured by scintillation counting. The products were identified by comparison with authentic standards and quantified by the radioactivity associated with each observed peak.

*Determination of Volatile Products:* Chloral was analyzed on a Hewlett Packard 5890A gas chromatograph (GC) fitted with an ECD detector and a RSL-160

polydimethylsiloxane column (Alltech Assoc., 30 m by 0.53 micro I.D. capillary column, $H_2$ carrier gas, flow rate 7 ml/min, injector temperature 150 °C, detector temperature 250 °C, column temperature 30 °C). Chloral was also determined by HPLC (see above). CO was quantified by measuring the shift in the Soret maximum upon binding to reduced hemoglobin (16). In experiments to determine CO, 1.8 mM TCE was added and the reactions were initiated by adding a limiting concentration of NADPH in a 2.0 ml enzyme reaction mixture to prevent potential CO oxidation to $CO_2$. $CO_2$ was determined as described (10).

*Determination of Reaction Stoichiometry:* [$^{14}$C]TCE (9.2 nmol) in 1.5 $\mu$l methanol was added to a 10 ml reaction vial containing 220 $\mu$g spinach reductase, 60 $\mu$g $Fd_{TOL}$, and 465 $\mu$g $ISP_{TOL}$ in 2 ml 50 mM Tris pH 7.5. The reaction was initiated by the addition of 1.2 $\mu$mole NADPH. The disappearance of TCE was determined by headspace GC as previously described (17). The oxidation of NADPH was quantitatively determined by the decrease in absorbance at 340 nm using a Beckman DU7400 spectrophotometer. The acidic products was determined as described above.

*Covalent Labeling of Enzyme Components:* A 12 ml enzyme reaction mixture set up as described for the stoichiometry analysis was concentrated to 1 ml by ultrafiltration. The protein components were separated from small molecules by Sephadex G-25 (1.5 x 30 cm) gel filtration (buffer, 50 mM Tris PH 7.5, flow rate 1 ml/min). The protein peak was determined via the absorbance at 280 nm. The radioactivity was determined by analyzing 0.7 ml of each fraction by scintillation counting. The remaining protein was pooled, concentrated by ultrafiltration, and fractionated by Fast Protein Liquid Chromatography (FPLC) using a Superose 12 HR 10/30 prepacked column from Pharmacia (buffer: 50 mM Tris, pH 7.5, flow rate, 0.5 ml/min). Eluting protein was determined via absorbance at 280 nm and radioactivity was measured by scintillation counting. The elution volumes of individual proteins were determined by parallel chromatography of purified enzyme components.

*Determination of d-formate from d-TCE oxidation:* Deuterated -TCE (10 nmol) was added to a Teflon-sealed 10 ml reaction vial containing 100 $\mu$g spinach reductase, 40 $\mu$g $ferredoxin_{TOL}$ and 320 $\mu$g $ISP_{TOL}$ in 2 ml 50 mM Tris pH 7.5. Non-deuterated TCE was added to a comparable enzyme reaction mixture in $D_2O$. The reaction was initiated by the addition of 1.2 $\mu$mol NADPH to each vial. The reaction vials were shaken at 23 °C for 30 min and concentrated to 0.2 ml using a Speed-Vac Concentrator (Savant Instruments, Inc.). The contents were analyzed by GC/MS using a DB-wax column (J & W Scientific, California, 30 m, 0.5$\mu$m) operated with a temperature gradient of 80 °C to 140 °C, 10 °C/min, 140 °C for 5 min coupled to a Kratos MS-25 mass spectrometer. The ratio of parent ion peaks at 47:46 was linear to the ratio of *d*-formate: *h*-formate as demonstrated with standards.

## RESULTS

*Requirements for TCE oxidation by toluene dioxygenase:* Toluene dioxygenase enzyme components, singly and in combination, were incubated with [$^{14}$C]TCE and NADPH. Only a complete three component enzyme system and reduced pyridine nucleotide showed detectable TCE oxidation. The specific activity, with $ISP_{TOL}$ as the limiting component, was 12 nmol/min per mg $ISP_{TOL}$. The specific activity of a complete reaction mixture containing catalase and superoxide dismutase was 11 nmol/min per mg $ISP_{TOL}$.

*Reaction products and stoichiometries:* The standard assay for toluene dioxygenase uses [$^{14}$C]toluene for the radiometric detection of the non-volatile polar $^{14}$C product, *cis*-toluene dihydrodiol (19). A comparable assay was used here for the quantitative determination of non-volatile products of TCE oxidation. In five independent determinations, non-volatile $^{14}$C products accounted for 70 ± 6% of the TCE disappearance.

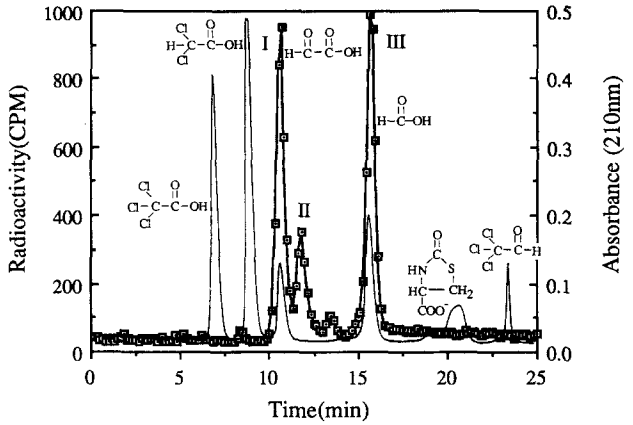



**Figure 1.** Acidic products of TCE oxidation determined by HPLC. Standard organic acid and aldehyde products monitored by absorbance at 210 nm (—). [$^{14}$C] oxidation products quantified by scintillation counting (⊡—⊡).

Non-enzyme controls indicated that <5% of the apparent TCE consumption was due to the loss of volatile TCE during the process of sampling for head-space GC analysis. Theoretically, one mole of reduced pyridine nucleotide is consumed during the oxidation of one mole of substrate in many multi-component oxygenase systems, including toluene dioxygenase. The oxidation of 7.8 mol of NADPH per mol of TCE consumed indicated that NADPH disappearance was not a reliable stoichiometric indicator of TCE oxidation. Older enzyme preparation typically gave higher NADPH:TCE stoichiometries, consistent with previous studies showing significant uncoupling during electron transfer by toluene dioxygenase components *in vitro* (Subramanian and Gibson, personal communication).

Organic acid products were anticipated based on previous studies on TCE oxidation by monooxygenases (2, 20, 21). An HPLC was fitted with an organic acids column and calibrated with standard compounds via UV absorbance detection at 210 nm (Figure 1). A [$^{14}$C]TCE reaction mixture was analyzed using the same HPLC conditions. Peaks corresponding to trichloroacetate, dichloroacetate, and chloral were not observed. GC analysis also failed to detect chloral with a detection limit of 2 pmol/ml. Separate reaction mixtures containing L-cysteine did not yield 4-carboxythiazolidin-2-one, indicating phosgene was not a product (15).

In the standard enzyme reaction mixture, three major radioactive product peaks were detected (Figure 1). The minor peak of radioactivity at 13 min was also observed in non-enzyme controls and was not considered further. The major product, compound III, comigrated at 15.4 min with authentic formic acid. GC-MS confirmed the identity of compound III as formic acid (see next section). The second most abundant radioactive product, compound I, eluted at 10.6 min, coincident with authentic glyoxylic acid. Reaction mixtures containing 2 mM cysteine showed a decrease in the compound I peak and the appearance of new peaks at 14.3 min and 15.3 min. A synthetic mixture containing the cysteine hemiacetal of glyoxylic acid gave a peak at 15.3 min. The 14.3 min peak is likely

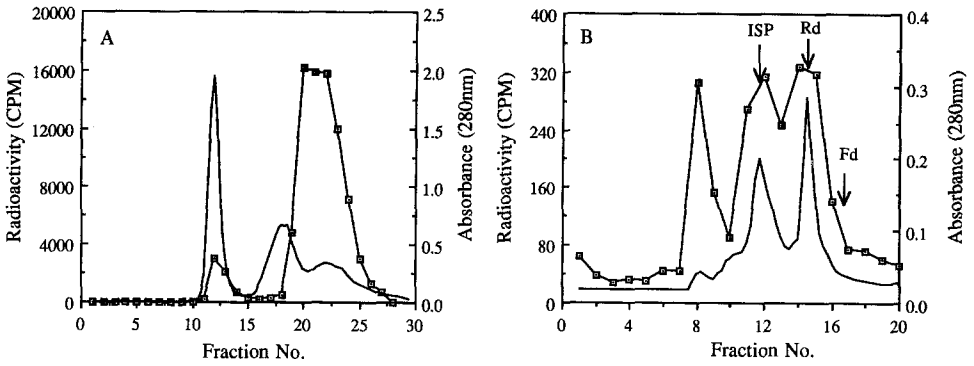



**Figure 2.** Gel filtration chromatography of [$^{14}$C]TCE reaction mixtures. (A) Sephadex G-25 chromatography and (B) Superose 12 chromatography of fractions 12-14 from the Sephadex G-25 column. Radioactivity (▫—▫) and absorbance at 280 nm (—) were monitored.

the thioester product from cysteine trapping of a transient glyoxyl chloride intermediate. Treatment of the reaction mixture with acid led to the disappearance of the peaks at 14.3 min and 15.3 min and the reappearance of glyoxylic acid at 10.6 min. The minor peak at 12.0 min, compound II, did not comigrate with any of the standards analyzed by HPLC. Unlike the other products, compound II showed significant long wavelength UV absorbance with bands at 260 nm and 340 nm resembling the absorption spectrum of NADPH. Treatment of compound II with acid and cyanide caused spectral shifts characteristic of pyridine nucleotides (22, 23). These data suggested that compound II is an NADPH-adduct formed by an alkylation reaction with an acyl chloride intermediate. Quantitative determination of the organic acid products indicated that formate, glyoxylate, and the putative NADPH-adduct account for 47, 17 and 6% of the TCE oxidized, respectively. These data are consistent with the formation of 70% non-volatile products from TCE.

Volatile and enzyme-bound products were looked for. Methods previously successful in CO trapping from methane monooxygenase reaction mixtures (2) failed to yield detectable CO. Carbon dioxide, which could arise from further oxidation of CO, was also not detected. The possibility that radioactivity was being incorporated into the protein fraction via enzyme alkylation was addressed. A [$^{14}$C]TCE-containing reaction mixture was fractionated into high and low molecular weight materials by Sephadex G-25 gel filtration chromatography (Figure 2A). The incorporation of $^{14}$C into the protein fraction accounted for 1.4% of the TCE consumed in the reaction mixture. The protein fractions were further subjected to gel filtration on a Superose 12 column to resolve individual proteins (Figure 2B). Radioactivity was detected in fractions corresponding to $Fd_{TOL}$, $ISP_{TOL}$, the flavoprotein reductase, and a contaminating protein that constituted a minor component of the incubation mixture. These data indicated a significant degree of non-specificity in $^{14}$C incorporation into protein.

*Oxidation of deuterated trichloroethylene and trichloroethylene in deuterated water:* Deuterated trichloroethylene was incubated with toluene dioxygenase. Formic acid was analyzed for deuterium content by GC-MS. In three independent experiments, 50.2 ± 1.4% of the formic acid contained deuterium. A complementary experiment was conducted with non-deuterated TCE in the presence of $D_2O$. Following correction for the small amount of $H_2O$ in the reaction mixture, the deuterium content in the formic acid was 43.7±0.4%. These data indicate that both carbon atoms are giving rise to formic acid.

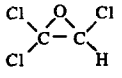
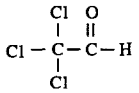
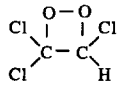
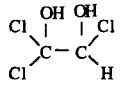
## DISCUSSION

There is currently great interest in using bacteria containing catabolic oxygenases to bioremediate chlorinated solvent wastes. The development of effective biotreatment systems will require a knowledge of the breakdown products and their possible toxic or inhibitory effects on sustained solvent metabolism. Previous studies have shown that toluene dioxygenase is inactivated *in vivo* during TCE oxidation (Householder and Wackett, unpublished data). The data presented here suggest that the reaction products are diffusible and modify proteins and reduced pyridine nucleotide in solution. These results are consistent with *in vivo* experiments in which protein, nucleic acid, and lipid components are covalently modified during TCE oxidation by *Pseudomonas putida* F1 (9).

It is likely that toluene dioxygenase processes TCE differently than does methane monooxygenase. As was the case with methane monoxygenase, information on the toluene dioxygenase reaction mechanism could be gained from elucidating the products of the reaction with TCE. For example, toluene dioxygenase oxidizes toluene enantiospecifically to (+)-(1S, 2R)-dihydroxy-3-methylcyclohexa-3,5-diene(*cis*-toluene dihydrodiol) (24, 25). Both hydroxyl groups in dioxygenated products are indicated by $^{18}O_2$ studies to be derived from the same molecule of molecular oxygen (26, 27). A dioxetane has been proposed as a possible enzyme-bound intermediate following initial dioxygen attack on aromatic hydrocarbons (28), but direct evidence for this is currently lacking. With the substrate indan, toluene dioxygenase has been shown to act exclusively as a monooxygenase yielding (-) - (1R) - indanol as the product (27). In this context, TCE is a valuable substrate for mechanistic studies. Oxygenation via the differential pathways noted above would yield different and diagnostic products (Table 1).

Studies on the product stoichiometries with toluene dioxygenase yielded unexpected results. The absence of detectable dichloroacetate and chloral suggested that TCE monooxygenation is not occuring to any significant extent. The absence of detectable phosgene and carbon dioxide indicates a TCE dioxetane is not released from the enzyme surface, although it is a viable reaction intermediate which could be further processed by the enzyme. Formate and glyoxylate, expected decomposition products of a dihydroxylated

**Table 1.** Possible initial and stable products via oxygenase-catalyzed reaction with TCE

| | *Monooxygenation* | | *Dioxygenation* | |
|---|---|---|---|---|
| **Initial Products** |  TCE-epoxide |  2,2,2-Trichloacetaldehyde (Chloral) |  TCE-dioxetane |  1,2-Dihydroxy-TCE |
| **Stable products** | Dichloroacetate<br>Glyoxylate<br>Formate<br>Carbon monoxide | Chloral is stable at pH 7.0 | Phosgene (carbon dioxide)<br>Formyl chloride (formate) | Glyoxylate<br>Formate<br>Carbon monoxide |

intermediate, were detected. However, carbon monoxide was not detected at significant levels, nor was carbon dioxide, a possible oxidation product of carbon monoxide. In contrast, carbon monoxide is the major detectable product of TCE oxidation by methane monooxygenase and it is proposed to arise from hydration of TCE-epoxide forming dihydroxy-TCE. The high yield of formate, the lack of carbon monoxide, and the results of deuterium labelling experiments support subtly different TCE oxidation mechanisms than any of those described previously. The mechanism proposed by Miller and Guengerich (10) for the rearrangement of 1,2-dihydroxy-TCE to formate would yield 100% *d*-formate from *d*-TCE. The present data indicated that both carbon atoms in TCE are giving rise to formate. This mitigates against a free dihydroxy-TCE reaction product. In one possible reaction pathway, an iron bound dioxygenated intermediate might rearrange on the enzyme surface to yield formate from both carbon atoms as shown in Figure 3. A similar iron dioxygen species has been proposed in the reaction catalyzed by phthalate dioxygenase

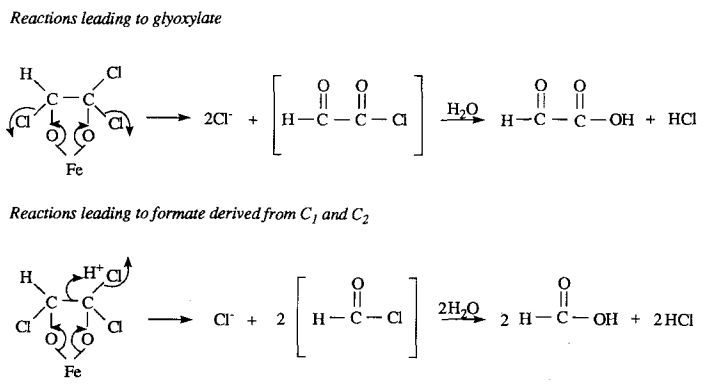



**Figure 3.** Proposed mechanism of TCE oxidation by toluene dioxygenase.

(29). Further studies with TCE and other non-physiological substrates may contribute to a greater knowledge of aromatic hydrocarbon dioxygenase reaction mechanisms.

## ACKNOWLEDGMENTS

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