Tetrachloroethylene Oxide: Hydrolytic Products and Reactions with Phosphate and Lysine

Tadao Yoshioka, Joel A. Krauser, and F. Peter Guengerich*

Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0146

Received April 8, 2002

Tetrachloroethylene, or perchloroethylene (PCE), has considerable industrial use and is of toxicological interest because of a variety of effects. Most of the existing literature presents PCE oxide as a critical intermediate in the oxidative metabolism of PCE to Cl_3CCO_2H, oxalic acid, and products covalently bound to proteins, including trichloroacetyl derivatives of lysine. PCE oxide was synthesized by photochemical oxidation of PCE and characterized. Decomposition at neutral pH ($t_{1/2}$ = 7.9 min at 0 °C, 5.8 min at 23 °C, 2.6 min at 37 °C) yielded only trace (∼1%) Cl_3CCO_2H; the major products identified were CO (73% yield) and CO_2 (63% yield). In phosphate buffer (0.10 M) a major product was identified as oxalyl phosphate. Oxalyl chloride also reacted to form CO and CO_2 in aqueous solution and to form oxalyl phosphate in neutral phosphate buffer. Oxalyl phosphate decomposed to oxalic acid ($t_{1/2}$ = 53 min at 37 °C) but did not react with lysine. Reaction of PCE oxide with free lysine yielded the oxalic acid amide derivatives of lysine plus lysine dimers in which cross-linking of the amino groups involved oxalo linkage. The reaction of PCE oxide with albumin yielded mainly N^6-oxalolysine and some (<5%) N^6-trichloroacetyllysine. We propose a reaction pathway for PCE oxide based on our previous studies with trichloroethylene oxide, in which C−C bond scission is a major product of reaction in aqueous buffer and yields CO and CO_2. Oxalyl species are proposed as intermediates and prominent acylating species formed in the reactions of the epoxide. The formation of Cl_3CCO_2H in cytochrome P450 reactions is postulated to result from intramolecular migration within an enzyme intermediate.

Introduction

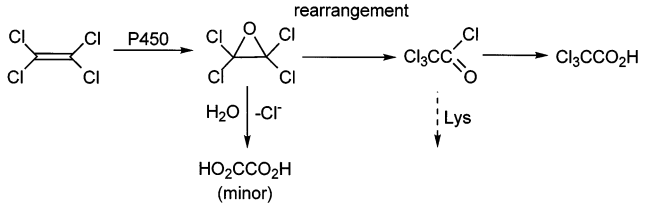
Tetrachloroethylene, or perchloroethylene (PCE),[1] is produced to the extent of about 10^8 kg annually in the United States alone (*1*, *2*). This compound is used extensively in the dry cleaning industry, is employed as a solvent in degreasing applications, and is a common contaminant in waste dump sites (*1*, *2*).

A number of toxicities have been associated with PCE administration in experimental animals, primarily involving the liver as the target organ (*2*). Effects include fatty liver degeneration, necrosis, liver enlargement, abnormal liver function, decreased ATP levels, and increased serum transaminases (*2*). The effects in humans are less well-defined beyond general narcosis, but some of the same liver effects seen in animal models have been documented following high exposure (*1*). PCE has been shown to cause hepatocellular carcinomas in mice but not rats (*3*, *4*). The mechanism of tumorigenesis is unclear; PCE is not very mutagenic in standard test systems, even in the presence of activating enzymes (*5*).

Metabolic activation is generally considered to be involved in all PCE toxicity, except general central nervous system depression (*2*). Two pathways are operative. One involves enzymatic conjugation with GSH to form a conjugate that can be processed to reactive products by the action of cysteine conjugate β-lyase (*6*, *7*). The reaction product is mutagenic (*7*). The other pathway involves oxidation by P450 (*8*). Human P450s 1A2, 2B6, and 2C8 have been reported to activate PCE to a product causing micronucleus formation in lymphoblastoma cells (*9*). Evidence for both the GSH conjugation and oxidative pathways has been found in rats (*6*).

Cl_3CCO_2H and oxalic acid are the two major oxidation products identified in vivo or in liver microsomal incubations (*6*, *8*, *10*, *11*). The pathway leading to these products is usually presented with PCE oxide as an obligate intermediate (Scheme 1) (*1*, *5*, *6*, *8*, *10*, *12*−*14*). Evidence

Scheme 1. Literature Scheme for Formation and Reactions of PCE Oxide (*2*)



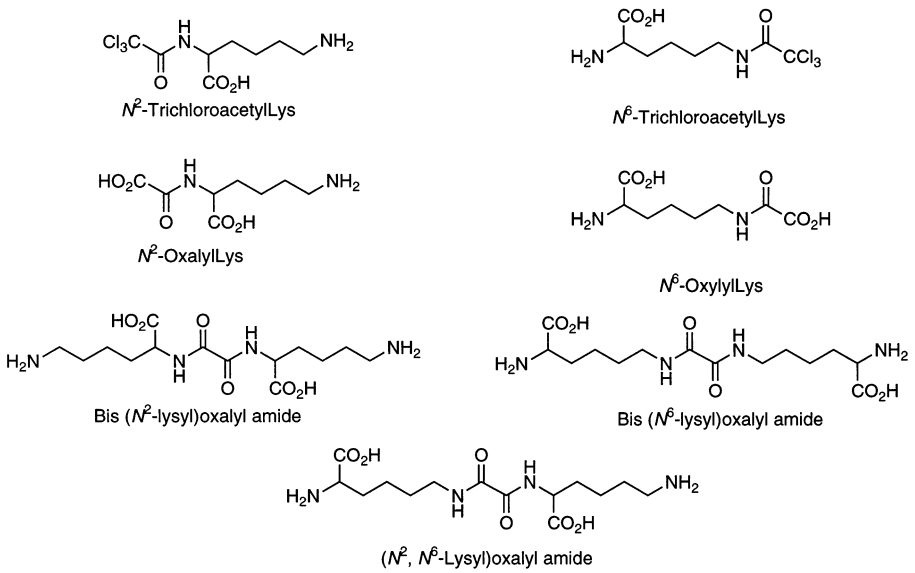
has been presented for the formation of N^6-trichloroacetylLys in proteins following microsomal oxidation of PCE, and the pathway involving PCE oxide (and its hydrolytic opening to Cl_3CCO_2H) is the basis of assessments of risk estimated from physiologically based pharmacokinetics and other modeling (*15*, *16*). However, in the literature (a meeting proceedings account) PCE oxide was reported to be hydrolyzed to Cl_3CCO_2H rather

* To whom correspondence should be addressed. Telephone: (615) 322-2261. Fax: (615) 322-3141. E-mail: guengerich@toxicology.mc.vanderbilt.edu.

[1] Abbreviations: PCE, perchloroethylene (tetrachloroethylene); TCE, trichloroethylene; MOPS, 3-(*N*-morpholino)propanesulfonate; ESI, electrospray ionization; CBZ, carbobenzyloxy; Orn, ornithine.

10.1021/tx020028j CCC: $22.00
Published on Web 07/23/2002

Scheme 2. Structures of Lys Adducts



inefficiently (3−4% yield) and did not form oxalic acid at neutral pH (*14*).

Studies in this laboratory with the related trichloroethylene (TCE) oxide have shown that this epoxide is not the source of the major enzymatic oxidation product, chloral hydrate (*17*). Chloral hydrate results from a chloride ion migration within a P450 enzyme intermediate, which yields TCE oxide as a minor product (*17*). The hydrolysis of TCE oxide is dominated by C−C bond scission, yielding CO and HCO_2H (*17*, *18*). This scission appears to occur following opening of the epoxide to an acyl halide, as judged by ^{18}O and ^{2}H labeling studies (*18*). Isotopic labeling studies also indicated that Lys does not react directly with TCE oxide but rather with an acyl halide product (glyoxal chloride) (*18*). The hydrolysis of the very unstable epoxide of vinylidene chloride seems to follow a course similar to that of TCE oxide, with chloride and hydride ion migration in an enzyme intermediate (*19*), although labeling studies have not been done to characterize the mechanism of epoxide hydrolysis in detail.

In light of the findings on the mechanisms of hydrolysis and reaction of TCE oxide (*17*, *18*, *20*−*22*), the reported low yields of Cl_3CCO_2H and oxalic acid (*14*), and the general industrial and toxicological significance of PCE, we reinvestigated the mechanism of hydrolysis of PCE oxide, the role of the epoxide in PCE oxidation, and reactions of the epoxide with free and protein-bound Lys (Scheme 2). Our results indicate that, as in the case of TCE oxide, only very limited Cl^- migration occurs and the hydrolysis is characterized by facile C−C bond scission to CO and CO_2. The enzymatic formation of trichloroacetyl chloride and oxalic acid is probably explained by an enzyme intermediate that forms trichloroacetyl chloride and oxalyl chloride.

Experimental Procedures

Caution! *PCE oxide and the related acyl chlorides are reactive acylating agents and should be handled carefully. Gloves and other protective clothing should be used appropriately.*

Spectroscopy. Unless otherwise noted, ^{1}H, ^{13}C, and ^{31}P NMR spectra were recorded on a Bruker DPX 300 AVANCE spectrometer operating at 300.1, 75.5, and 121.4 MHz, respectively (Bruker, Billerica, MA). Samples were prepared in either D_2O or $CDCl_3$. ^{13}C shifts are reported relative to CH_3OH or 1,4-dioxane (*23*), and ^{31}P shifts are reported relative to H_3PO_4. Mass spectrometry was carried out with a Finnagan-MAT TSQ7000 series mass spectrometer (Finnigan, Sunnyvale, CA). For LC/MS analysis, a Waters 2690 separation module was used (Alliance, Waters, Milford, MA).

Synthesis of PCE Oxide (*24*). PCE (180 mL, distilled over P_2O_5) was added to a photochemical reaction vessel and warmed in an 80 °C water bath. O_2 was bubbled through the sample for 10 min and the system was illuminated (Hanovia medium-pressure 450 W mercury arc lamp filtered by a sleeve of Pyrex) to start the reaction under a constant flow of O_2 (∼300 mL min^{-1}). After 7 h the lamp was turned off, and the reaction mixture was cooled in an ice bath and then washed with a cold 1 N NaOH solution to remove trichloroacetyl chloride. The residual PCE mixture was dried over Na_2SO_4, and PCE oxide was obtained by distillation (yield 5% based on PCE; bp 35−38 °C at 65 mmHg).

Quantitative analysis was done using proton-decoupled ^{13}C NMR with an inverse gated decoupling method and delay time of 20 s between successive transmitter pulses. The spectrum showed three singlets at 90.6, 105.2, and 120.4 ppm, corresponding to PCE oxide (61%, w/w), hexachloroethane (2%, w/w), and residual PCE (37%, w/w), respectively (concentrations were estimated based on the integration of the spectrum measured in the addition of a known amount of CCl_4). The signal at 90.6 ppm is in agreement with the assignment of Campbell and Vogl (*24*).

Synthesis of Oxalyl Phosphate (*25*). Oxalyl chloride (1.0 mL, 12 mmol) was added dropwise to a stirred solution of 2.0 M K_2HPO_4 (10 mL, 20 mmol) in an ice bath. After 20 min, the reaction mixture was diluted with H_2O (400 mL) and applied to a Dowex 1X8 column (Cl^- form, 2.2 × 12 cm) at a flow rate of 1.2 mL min^{-1} (4 °C). Elution was performed as described previously (*26*): 100 mL of 50 mM $MgCl_2$, followed by 100 mL of H_2O and then a 500 mL linear gradient of 0.1 to 1.0 M KCl. The flow rate was 1.5 mL min^{-1}, and 9 mL fractions were collected. Fractions were analyzed for oxalic acid and oxalyl phosphate using HPLC (vide infra), and the fractions containing oxalyl phosphate were pooled and stored at −80 °C. To determine the concentration of oxalyl phosphate, 0.2 mL of 1 N HCl was added to 2.0 mL of each pooled fraction and the samples were heated at 37 °C for 30 min. By quantifying oxalic acid by HPLC (vide infra) and inorganic phosphate (*27*) formed from oxalyl phosphate, the concentration of oxalyl phosphate in the stock solution was determined to be 21 mM. ^{31}P and ^{13}C NMR spectra of oxalyl phosphate were measured after adjusting the pH to 8 and adding D_2O [final 10% (v/v)], using a Bruker DRX

500 NMR spectrometer at 202.5 and 125.7 MHz, respectively. ^{31}P NMR showed a peak at −0.50 ppm (using 85% H_3PO_4 as an external standard) and ^{13}C NMR showed peaks at 161.0 (J_{CP} = 10.3 Hz) and 164.4 ppm (J_{CP} = 7.3 Hz) (using dioxane as an external standard).

Synthesis of Lys Derivatives. (1) N^6-Trichloroacetyl-L-Lys. To 5.0 mmol of L-Lys in 2.5 mL of 2 N NaOH (5.0 mmol), 1.0 mL (1.5 equiv) of ethyl trichloroacetate was added in one portion and the reaction was stirred for 2 h at room temperature. The solid that was formed was collected, washed with CH_3CN, and then dried to give the product (23% yield, fine needles from aqueous C_2H_5OH): mp 204 °C (decomp.); ^{1}H NMR (D_2O) δ 1.51 (m, 2H, γ-H), 1.72 (m, 2H, β-H), 1.91 (m, 2H, δ-H), 3.41 (t, 2H, J = 6.9 Hz, ϵ-H), 3.76 (t, 1H, J = 6.3 Hz, α-H); ^{13}C NMR (D_2O) 22.4 ppm (γ), 28.2 ppm (δ), 30.8 ppm (β), 41.4 ppm (ϵ), 55.3 ppm (α), 92.3 ppm ($COCCl_3$), 165.1 ppm ($COCl_3$), 175.4 ppm (CO_2H); MS, m/z 291/293/295/297 (MH^+).

(2) N^2-Trichloroacetyl-L-Lys. N^6-Carbobenzyloxy (CBZ)-L-Lys benzyl ester·HCl was prepared (*28*) by heating N^6-CBZ-L-Lys (9.81 g, 35.0 mmol) and *p*-toluenesulfonic acid (6.7 g, 35 mmol) in a mixture of 100 mL of toluene and 66 mL of benzyl alcohol for 20 h, using a Dean−Stark trap. The yield was 65%. To 1.5 mmol of N^6-CBZ-L-Lys benzyl ester in 8 mL of CH_2Cl_2 and 0.8 mL of $(C_2H_5)_3N$, trichloroacetyl chloride (0.22 mL, 1.3 equiv) was added; the reaction was stirred at room temperature for 50 min. The reaction mixture was diluted with CH_2Cl_2 and washed sequentially with 1 N HCl, 1 N NaOH, and H_2O and dried over Na_2SO_4. Evaporation of the organic solvent gave a pale yellow syrup (92%). The compound was then hydrolyzed with 1 NaOH in aqueous CH_3OH (200 mL, room temperature, 1 h) to the corresponding carboxylic acid derivative (80%), which was treated with 4 mL of HBr in CH_3CO_2H (30%, w/v) for 1 h, followed by the addition of $(C_2H_5)_2O$ to give N^2-trichloroacetylLys·HBr (91%, overall yield 67%). Neutralization with $NaHCO_3$ followed by purification using a Sep-Pak Vac 35 cm^3 cartridge (C18, 10 g, Waters) gave free base (fine powder from aqueous C_2H_5OH): mp 191 °C, (decomp); ^{1}H NMR (D_2O) δ 1.42 (m, 2H, γ-H), 1.66 (m, 2H, δ-H), 1.83 (m, 1H, β-H1), 1.96 (m, 1H, β-H2), 2.98 (t, 2H, J = 7.6 Hz, ϵ-H), 4.20 (dd, 1H, J = 5.1 and 8.1 Hz, α-H); ^{13}C NMR (D_2O) δ 22.7 (γ), 27.0 (δ), 31.2 (β), 39.9 (ϵ), 57.2 (α), 92.2 (CCl_3), 164.2 ($COCCl_3$), 178.0 (CO_2H); MS *m/z* 291/293/295/297 (MH^+).

(3) N^6-Oxalyl-L-Lys. The compound was synthesized according to a literature procedure (*29*) (yield 45%, fine needles from aqueous 2-propanol): mp 182 °C (decomp.); ^{1}H NMR (D_2O) δ 1.42 (m, 2H, γ-H), 1.58 (m, 2H, β-H), 1.90 (m, 2H, δ-H), 3.24 (t, 2H, J = 6.7 Hz, ϵ-H), 3.92 (t, 1H, J = 6.5 Hz, α-H); ^{13}C NMR (D_2O) 22.2 ppm (γ), 28.4 ppm (β), 30.2 ppm (δ), 39.6 ppm (ϵ), 54.1 ppm (α), 163.8 ppm ($COCO_2H$), 165.5 ppm ($COCO_2H$), 173.8 ppm (CO_2H); MS *m/z* 219 (MH^+).

(4) N^2-Oxalyl-L-Lys. Ethyl chlorooxoacetate (0.27 mL, 1.2 equiv) was added to 2.0 mmol of N^6-CBZ-L-Lys benzyl ester HCl (vide supra) dissolved in 8 mL of CH_2Cl_2 [and 1 mL of $(C_2H_5)_3N$] and stirred at 0 °C for 1.5 h. The reaction mixture was diluted with CH_2Cl_2 and washed with 1 N HCl, 1 N NaOH, and then H_2O and dried with Na_2SO_4. Evaporation of the organic solvent gave a pale yellow syrup (91%). The compound was then hydrolyzed with 0.7 g of NaOH in a mixture of 5 mL of $(CH_3)_2$-NCHO and 10 mL (1 h, room temperature) to give the corresponding dicarboxylic acid derivative (82%), which was then treated with 6 mL of HBr in CH_3CO_2H (30%, w/v) for 1.5 h followed by addition of $(C_2H_5)_2O$ to give the HBr salt of the title compound in quantitative yield (overall yield 75%). Neutralization with $NaHCO_3$ yielded the free base (prisms from aqueous C_2H_5OH) mp 225 °C (decomp.); ^{1}H NMR (D_2O) δ 1.43 (m, 2H, γ-H), 1.67 (m, 2H, δ-H), 1.85 (m, 1H, β-H_1), 1.96 (m, 1H, β-H_2), 2.97 (t, 2H, J = 7.4 Hz, ϵ-H), 4.36 (dd, 1H, J = 4.9 and 8.8 Hz, α-H); ^{13}C NMR (D_2O) δ 22.7 (γ), 26.8 (δ), 30.8 (β), 39.8 (ϵ), 53.6 (α), 165.0 ($COCO_2H$), 165.9 ($COCO_2H$), 176.4 (CO_2H); MS *m/z* 219 (MH^+).

(5) Bis(N^2-L-lysyl)oxalyl Amide. Oxalyl chloride (0.13 mL, 0.5 eq) was added in portions to 3.0 mmol of *N*-CBZ-L-Lys methyl ester·HCl (vide supra) in 6 mL of pyridine, and stirred at room temperature for 2.5 h. The reaction mixture was concentrated in vacuo; the residue was dissolved in CH_2Cl_2 and washed with 1 N HCl and then H_2O and dried over Na_2SO_4. Evaporation of the organic solvent gave a solid (91%), which was purified by recrystallization from a CH_3OH/H_2O mixture. A 6 mL solution of HBr in CH_3CO_2H (30%, w/v) was added to the compound (700 mg) and the reaction proceeded at room temperature for 40 min, followed by addition of $(C_2H_5)_2O$ to give a precipitate (97% yield). The compound (560 mg) was then hydrolyzed with 1 N NaOH in 5 mL aqueous CH_3OH to give the title compound, which was neutralized by addition of 1 N HCl, followed by purification by using AG1X8 ion-exchange resin (acetate form; 1.0 × 15 cm) with elution with H_2O. After lyophilization, the solid (76% yield, overall yield 67%) was obtained as a fine powder (recrystallized from aqueous C_2H_5-OH): mp 124 °C (decomp.); ^{1}H NMR (D_2O) δ 1.37 (m, 2H, γ-H), 1.67 (m, 2H, δ-H), 1.81 (m, 1H, β -H1), 1.89 (m, 1H, β-H2), 2.97 (t, 2H, J = 7.5 Hz, ϵ-H), 4.18 (dd, 1H, J = 5.2 and 7.8 Hz, α-H); ^{13}C NMR (D_2O) δ 22.7 (γ), 27.0 (δ), 31.5 (β), 39.8 (ϵ), 55.9 (α), 161.0 (*C*ONH), 178.5 (CO_2H); MS, *m/z* 347 (MH^+).

(6) Bis(N^6-L-lysyl)oxalyl Amide. To 3.0 mmol of L-Lys in 3.0 mL of 1 N NaOH (3.0 mmol), 190 μL (0.5 eq) of diethyl oxalate was added. The reaction was stirred for 15 min in an ice−water bath and then at room temperature. After 1.5 h at room temperature, 2.5 mL of 1 N HCl was added; the solid was collected and dried to give the product (40% yield): mp 280 °C (decomp.); ^{1}H NMR (D_2O-DCl) δ 1.50 (m, 2H, γ-H), 1.61 (m, 2H, β-H), 1.99 (m, 2H, δ-H), 3.31 (t, 2H, J = 6.7 Hz, ϵ-H), 4.13 (t, 1H, J = 6.3 Hz, α-H); ^{13}C NMR (D_2O-DCl) 22.1 ppm (γ), 28.2 ppm (β), 29.8 ppm (δ), 39.5 ppm (ϵ), 53.2 ppm (α), 161.4 ppm (*C*ONH), 172.3 ppm (CO_2H); MS, *m/z* 347 (MH^+).

(7) (N^2,N^6-L-Lysyl)oxalyl Amide. N^2-Ethoxycarbonylcarbonyl-N^6-CBZ-L-Lys methyl ester was prepared in the following procedure: ethyl chlorooxoacetate (0.27 mL, 1.2 equiv) was added to N^6-CBZ-L-Lys methyl ester·HCl (2.0 mmol) in a mixture of 8 mL of CH_2Cl_2 and 1.0 mL $(C_2H_5)_3N$ at 0 °C, and the reaction was stirred for 1.5 h, yielding a pale yellow syrup after concentration in vacuo (0.86 g, 91% yield). N^2-CBZ-L-Lys methyl ester (2.3 mmol) (*28*, *30*) was added to 1.6 mmol of N^2-ethoxycarbonylcarbonyl-N^6-CBZ-L-Lys methyl ester and the reaction stirred at room temperature for 2 days. The reaction mixture was evaporated and the residue was dissolved in $CHCl_3$ and washed with 0.5 N HCl and dried over Na_2SO_4. Concentration in vacuo and purification using silica gel flash chromatography give the corresponding oxalyl amide (57%). To 500 mg of the compound, 4 mL of HBr in CH_3CO_2H (30%, w/v) was added and the reaction kept at room temperature for 1 h followed by addition of $(C_2H_5)_2O$. After removal of the $(C_2H_5)_2O$ by decantation, the precipitate was extracted with H_2O and NaOH was added (to pH ∼10) and the reaction was allowed to stand at room temperature to give sodium salt of the title compound. Neutralization followed by purification by using a Sep-Pak Vac 35 cm^3 cartridge (C18, 10 g, Waters) gave the product in 66% yield (overall yield 38%) (fine powder from aqueous C_2H_5OH): mp 242 °C (decomp.); ^{1}H NMR (D_2O) δ 1.43 (m, 4H, γ-H), 1.68 (m, 4H, δ-H), 1.89 (m, 4H, β-H), 3.01 (t, 1H, J = 7.4 Hz, ϵ-H), 3.33 (dt, 1H, J = 2.4, 6.8 Hz, ϵ'-H), 3.74 (t, 1H, J = 5.7 Hz, α'-H), 4.22 (dd, 1H, J = 5.0 and 8.0 Hz, α-H); ^{13}C NMR (D_2O): 22.3 and 22.7 (γ), 26.7 and 28.4 (δ), 30.7 and 31.5 (β), 39.8 and 39.9 (ϵ), 55.3 and 55.8 (α), 161.1 and 161.5 (*C*ONH), 175.4 and 178.6 (CO_2H); MS, *m/z* 347 (MH^+).

Synthesis of Ornithine (Orn) Derivatives. (1) N^5-Trichloroacetyl-L-Orn. To 5.0 mmol of l-Orn·HCl in 5.0 mL of 2 N NaOH (10 mmol), 1.0 mL (1.5 equiv) of ethyl trichloroacetate was added in one portion and stirred for 1.5 h at room temperature. After the reaction mixture was neutralized by adding 3 N HCl, the reaction mixture was concentrated in vacuo to give a white solid. After cooling, the solid was collected and then dried to give the product (36% yield, fine needles from aqueous C_2H_5OH): mp 202 °C (decomp.); ^{1}H NMR (D_2O) δ 1.67 (m, 2H, γ-H), 1.88 (m, 2H, β-H), 3.37 (t, 2H, J = 6.8 Hz, δ-H),

3.73 (t, 1H, J = 5.9 Hz, α-H); ^{13}C NMR (D_2O) δ 24.5 (γ), 28.4 (β), 41.2 (δ), 55.0 (α), 92.2 ($COCCl_3$), 165.2 ($COCl_3$), 175.0 (CO_2H); MS, m/z 277/279/281/283 (MH^+).

(2) N^5-Oxalyl-L-Orn. The compound was synthesized according to a literature procedure (*29*) [yield 45%, crystals from aqueous $(CH_3)_2CO$]: mp 158 °C (decomp.); ^{1}H NMR (D_2O-DCl) δ 1.74 (m, 2H, γ-H), 1.98 (m, 2H, β-H), 3.36 (t, 2H, J = 6.8 Hz, δ-H), 4.16 (t, 1H, J = 6.2 Hz, α-H); ^{13}C NMR (D_2O-DCl) δ 24.3 (γ), 27.6 (β), 39.4 (δ), 53.0 (α), 159.8 ($COCO_2H$), 162.3 ($COCO_2H$), 172.1 (CO_2H); MS m/z 205 (MH^+).

(3) Quantitation of PCE Oxide. PCE oxide solutions were diluted into dry CH_3CN, and the concentration was estimated (at room temperature) using a colorimetric assay with 4-(4-nitrobenzyl)pyridine reagent (*31*)(50 mM in ethylene glycol-acetone (43:7, v/v)), using ϵ_{524} = 880 M^{-1} cm^{-1}, which was calculated with the use of a sample in which the concentration of PCE oxide was quantified using decoupled ^{13}C NMR (vide supra).

Measurement of $t_{1/2}$ of PCE Oxide. (1) In 3-(*N*-Morpholino)propanesulfonate (MOPS) Buffer. To each test tube (on ice) containing 100 μL of 100 mM potassium MOPS (pH 7.4), 10 μL of 0.1 M PCE oxide in CH_3CN was added (final concentration of the oxide was ~10 mM). At appropriate times, 500 μL of the 4-(4-nitrobenzyl)pyridine reagent was added and the samples were kept at room temperature for 5 min. At that time, 500 μL of $(C_2H_5)_3N$-acetone (1:1, v/v) was added and the absorbance was measured at 535 nm.

(2) In CH_3CN. A solution of 100 mM PCE oxide in CH_3CN was kept in a Teflon-sealed vial at room temperature (23 ± 1 °C). At appropriate times, 10 μL of the solution was dissolved in 1.0 mL of 100 mM potassium MOPS buffer (pH 7.4). The mixtures were kept in an ice bath for 15 min and then the amount of Cl_3CCO_2H was analyzed by HPLC (vide infra).

Measurement of Potential Products of PCE Oxide. (1) CO. Determination of CO was done with an electrochemical sensor (Draeger Pac III, Draeger, Pittsburgh, PA) (*18*). To each Teflon-sealed vial containing 990 μL of 100 mM sodium phosphate or potassium MOPS (pH 7.4) buffer, 10 μL of 80 mM PCE oxide solution (in CH_3CN) was added (final concentration 0.80 mM), and the mixture was stirred for 90 min in an ice bath. At appropriate times, the vial was placed in a circuit containing the detector and a peristaltic pump and CO was determined.

(2) CO_2. Determination of CO_2 was done with an electrochemical sensor (pHoenix CO_2 electrode attached to an Orion model 920A meter). A solution of 100 mM sodium phosphate or potassium MOPS (pH 7.4) buffer (30 mL), containing oxalyl chloride (2 mM) or PCE oxide (10 mM) was kept in an ice bath for 10 min (or 90 min in the case of PCE oxide). After the reaction, a 1 M citrate buffer (pH 4.5) and 1 N HCl were added to adjust the pH to ~5.1, just before measurements were made. Calibration curves were prepared according to the manufacturer's instructions by using $NaHCO_3$ in both MOPS and phosphate buffers containing 1% (v/v) CH_3CN (at 0 °C).

(3) HCO_2H. HCO_2H was analyzed by HPLC as described previously (*18*).

(4) HCHO. HCHO was estimated colorimetrically using the Nash procedure (*32*).

(5) Oxalic Acid. To 990 μL of MOPS or phosphate buffer (0 °C), 10 μL of 100 mM PCE oxide solution in CH_3CN was added (final concentration of PCE oxide 1.0 mM), and the mixture was stirred for 90 min. At appropriate times, 30 μL of the reaction mixture was directly injected onto HPLC (vide infra) for measurement of oxalic acid. The determination of oxalic acid by HPLC was also done with a reaction mixture consisting of 960 μL of the each buffer and 40 μL of 250 mM PCE oxide in CH_3CN (final concentration of oxide 10 mM) in an ice bath for 90 min.

(6) Cl_3CCO_2H. To 6 mL of MOPS or phosphate buffer kept in an ice bath, 60 μL of 100 mM PCE oxide in CH_3CN (final concentration PCE oxide 1.0 mM) was added. At appropriate times, aliquots of the reaction mixture (1.0 mL) were transferred to test tubes and washed with $(C_2H_5)_2O$ (3 × 1 mL) to remove residual PCE and PCE oxide. Twenty microliters of concentrated H_2SO_4 was added to the aqueous layer, and Cl_3CCO_2H was extracted with $(C_2H_5)_2O$ (3 × 1 mL). The combined layers were evaporated under a stream of N_2 to dryness, and the residue was dissolved in 100 μL of 100 mM potassium MOPS buffer (pH 7.4) and analyzed by HPLC (vide infra). The extraction efficiencies of Cl_3CCO_2H from the MOPS and phosphate buffers were 90 and 84%, respectively.

Reactions of Electrophiles with Lys. The indicated amounts of PCE, PCE oxide, oxalyl chloride, or trichloroacetyl chloride (solutions in dry CH_3CN) were reacted with Lys (final concentration of 10 mM; unless otherwise indicated) in 100 mM potassium MOPS or sodium phosphate buffer (pH 7.4) or H_2O in an ice bath for 90 min. In control experiments, PCE oxide was reacted in H_2O for 90 min in an ice bath, followed by the addition of Lys to the reaction mixture, and allowed to stand for more 90 min. Adducts with Lys were analyzed using HPLC and LC/MS (vide infra).

Reactions with bovine serum albumin were done as described previously (*20*) and, following digestion with proteinase K, the amino acids were derivatized with phenylisothiocyanate. In the analysis of the albumin Lys adducts, the Orn derivatives (also phenylthiocarbamates) were utilized to generate internal standard curves (*20*).

HPLC and MS. Determination of Cl_3CCO_2H, oxalic acid, and oxalyl phosphate was performed by using an octadecylsilane column (Beckman Ultrasphere ODS, 4.6 × 250 mm, Beckman, San Ramon, CA) with the solvent 10 mM tetra-*n*-butylammonium hydroxide (adjusted pH to 7.0 with H_3PO_4): CH_3CN (4:1, v/v) at a flow rate of 1.0 mL min^{-1} and detection at 210 nm (*18*). Determination of adducts with Lys was performed by using the same column with 10 mM $NH_4CH_3CO_2$ (pH 6.5) at a flow rate of 0.7 mL min^{-1} and detection at 210 nm.

For HPLC/MS (electropray) (ESI) analysis, the HPLC conditions were same as those for the determination of the adducts ($NH_4CH_3CO_2$), and 200 μL effluent min^{-1} was introduced into the mass spectrometer (*18*, *20*).

Direct inlet ESI MS (positive ion mole) was used to establish molecular masses of chemicals synthesized in this work.

Microsomal Incubations. Liver microsomes were prepared from phenobarbital-treated rats (*33*) and incubated (5 μM P450) with 1.0 mM PCE and an NADPH-generating system (*34*) in 100 mM potassium MOPS buffer (pH 7.4) at 37 °C. Reactions were terminated by the addition of 50 μL of concentrated H_2SO_4. Samples were extracted three times with 1.0 mL of ethyl acetate in the analysis of oxalic acid or with $(C_2H_5)_2O$ for Cl_3CCO_2H, and the combined organic phase was concentrated to dryness under an N_2 stream. The residue was dissolved in a solution of 20% CH_3CN (v/v) [for oxalic acid, or 25% CH_3CN (v/v) for Cl_3CCO_2H] in 10 mM tetra-*n*-butylammonium hydroxide (buffer to pH 6.5) with H_3PO_4, and 30 μL aliquots were injected into a 4.6 × 250 mm Beckman octadecylsilane HPLC column equilibrated with the injection buffer. A_{210} was monitored for the estimation of Cl_3CCO_2H and oxalic acid, utilizing external standard curves. Recoveries for Cl_3CCO_2H and oxalic acid were 81 and 17%, respectively.

Results

Reactivity of PCE Oxide. In CH_3CN solution, PCE oxide rearranged quantitatively to trichloroacetyl chloride with first-order kinetics. Because trichloroacetyl chloride was hydrolyzed quantitatively to the corresponding acid when buffer was added, the amount of trichloroacetyl chloride was determined as Cl_3CCO_2H. The rate constant was 0.0183 ± 0.0009 h^{-1} ($t_{1/2}$ = 39 h at 23 ± 1 °C). At −20 °C, rearrangement to trichloroacetyl chloride was not observed over the course of 100 h.

The decomposition of PCE oxide in 100 mM potassium MOPS (pH 7.4) at 0 °C was analyzed by measuring the residual amount of the oxide with 4-(4-nitrobenzyl)-

Table 1. Decomposition Products of PCE Oxide[a]

	products formed (nmol/μmol of PCE oxide)				
buffer	CCl_3CO_2H	oxalic acid	CO	CO_2	oxalyl phosphate
potassium MOPS (0.10 M)	12	<3	730	630	<2
sodium phosphate (0.10 M)	10	<3	60	50	180

[a] Incubations were at 0 °C for 90 min except for measurement of oxalyl phosphate (120 min). Initial concentrations of PCE oxide were 0.80 mM for the measurement of CO and oxalyl phosphate, 10 mM for oxalic acid, and 1.0 mM for the other measurements.

Table 2. Decomposition Products of Oxalyl Chloride[a]

	products formed (nmol/μmol of oxalyl chloride)			
buffer	CO	CO_2	oxalic acid	oxalyl phosphate
potassium MOPS (0.10 M)	970	910	<3	<2

[a] Incubations were for 5 min at 0 °C. Initial concentrations of PCE oxide were 1.0 mM for measurement of CO, CO_2, and oxalyl phosphate and 10 mM for measurement of oxalic acid.

pyridine reagent. The reagent did not give the characteristic purple pigment (ϵ_{535}) with trichloroacetyl chloride, Cl_3CCO_2H, oxalyl chloride, or oxalic acid, which are potential products formed from PCE oxide. The kinetics were pseudo-first-order, and the rate constant of the decomposition of PCE oxide was 0.088 ± 0.006 min^{-1} ($t_{1/2}$ = 7.9 min). At 23 °C, the rate constant was 0.12 ± 0.02 min^{-1} and at 37 °C the rate constant was 0.26 ± 0.16 min^{-1}. The rate constants were the same in both MOPS and phosphate buffers (0.10 M each).

Decomposition Products of PCE Oxide. Decomposition products of PCE oxide were measured in both 100 mM potassium MOPS and sodium phosphate buffers (pH 7.4) at 0 °C. PCE itself was stable and yielded none of the decomposition products under the same conditions (results not shown). Cl_3CCO_2H was formed from PCE oxide in both the buffers, although in very low amounts (Table 1). Although oxalyl chloride has been considered to be one of the active intermediates formed from PCE oxide, oxalic acid was not detected in either of the buffers. CO and CO_2 were the major products (73 and 63 mol %) in MOPS buffer. However, in the phosphate buffer, the amounts of CO and CO_2 formed were much less. In the phosphate buffer, formation of oxalyl phosphate (18 mol %) was observed.

Neither HCO_2H nor HCHO was detected as a product (limit of detection 0.1% yield).

Decomposition Products of Oxalyl Chloride. Although oxalic acid was not detected as a decomposition product of PCE oxide (vide supra), oxalyl chloride has been considered as reactive product resulting from the decomposition of PCE oxide. The decomposition products of oxalyl chloride, therefore, were investigated under the same conditions used for the reactions of PCE oxide. CO and CO_2 were the major products formed in MOPS buffer (Table 2) and oxalic acid was not detected. The formation of oxalyl phosphate was also observed in the phosphate buffer.

Formation and Reactions of Oxalyl Phosphate. Because oxalyl phosphate was formed from PCE oxide (Table 1) and oxalyl chloride in phosphate buffer, kinetic

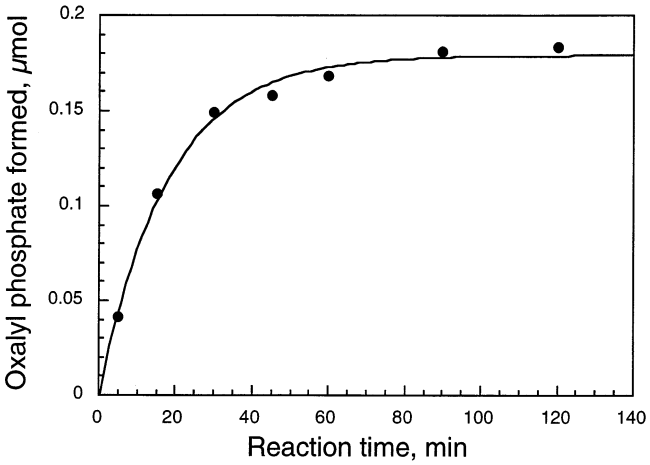


Figure 1. Time dependence of formation of oxalyl phosphate from PCE oxide in 100 mM sodium phosphate buffer (pH 7.4) at 0 °C. The initial concentration of PCE oxide was 1.0 mM. The curve is a fit to a single exponential with a rate constant of 0.056 min^{-1} (±0.004 min^{-1}).

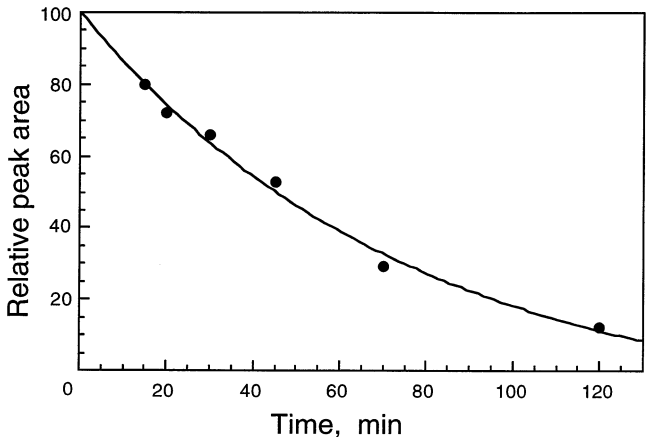


Figure 2. Hydrolysis of oxalyl phosphate in 100 mM sodium phosphate buffer (pH 7.4) at 37 °C. The relative peak area refers to the integral of the HPLC peak. The curve is a fit to a single exponential with a rate constant of 0.013 min^{-1} (±0.004 min^{-1}).

experiments on the formation of and hydrolysis of oxalyl phosphate were done. The reaction of oxalyl chloride with sodium phosphate buffer to give oxalyl phosphate at 0 °C was rapid (within 5 min) and the amount of oxalyl phosphate formed was a linear function of the concentration of phosphate from 50 to 250 mM at pH 7.4 (results not shown). In the reaction of PCE oxide in 100 mM sodium phosphate buffer (pH 7.4) at 0 °C, oxalyl phosphate was formed more slowly and the yield was 18 mol %, based on PCE oxide (Figure 1). The observed first-order rate constant was 0.056 ± 0.004 min^{-1}, which is slightly less than the rate of decomposition of PCE oxide (note no effect of 0.10 M phosphate on rate of PCE oxide decomposition, vide supra).

Oxalyl phosphate was not stable in the phosphate buffer (especially at higher temperature) and decomposed slowly with first-order kinetics, with concomitant formation of oxalic acid. The rate constant of decomposition (hydrolysis to oxalic acid and inorganic phosphate) in 100 mM sodium phosphate at 37 °C was 0.013 ± 0.004 min^{-1} ($t_{1/2}$ = 53 min) (Figure 2). At 0 °C, hydrolysis of the phosphate was not observed during the course of 120 min. No CO formation was observed in the decomposition of oxalyl phosphate (results not shown).

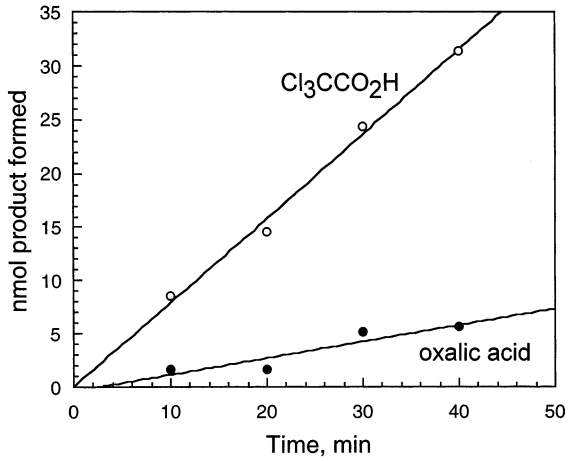


Figure 3. Formation of Cl_3CCO_2H and oxalic acid in NADPH-fortified rat liver microsomes. The rate of formation of Cl_3CCO_2H (○) and oxalic acid (●) were 0.32 and 0.06 nmol min^{-1} (mg protein)$^{-1}$, respectively.

Microsomal Oxidation of PCE. P450-catalyzed oxidation of PCE has been reported to yield Cl_3CCO_2H and oxalic acid, with Cl_3CCO_2H predominating (*6*, *8*). We carried out oxidation of PCE with liver microsomes prepared from phenobarbital-treated rats and observed the same pattern (Figure 3), with respective rates of 0.32 nmol of Cl_3CCO_2H and 0.06 nmol of oxalic acid formed min^{-1} (mg microsomal protein)$^{-1}$.

Products of Reactions with Free Lys. Reaction of PCE oxide with Lys was investigated to gain insight into the reactive electrophile(s) formed from the oxide. Reactions of trichloroacetyl chloride, oxalyl chloride, and oxalyl phosphate with Lys were also performed in H_2O or 100 mM potassium MOPS or 100 mM sodium phosphate buffer (pH 7.4) at 0 °C. Trichloroacetyl chloride (final 10 mM) reacted with Lys (final 10 mM) to yield trace amounts of the *N*-trichloroacetyl derivatives in all three solutions. Oxalyl chloride (10 mM) reacted with Lys (10 mM) to give an adduct that corresponded to the major adduct formed from PCE oxide and Lys (vide infra), although in low yield. Oxalyl phosphate (2.1 mM) did not react with Lys (2 mM) in H_2O. PCE (10 mM) was also unreactive toward Lys (10 mM) in H_2O, as expected. PCE oxide (10 mM) gave several adducts with Lys (10 mM) in the three solutions. The reaction product of the oxide with Lys in H_2O (90 min) was analyzed using LC/MS, with five products at t_R 3.40, 6.28, 9.15, 12.3, and 19.0 min (Figure 4). The small peak at t_R = 3.82 min was unreacted Lys. By comparison with the authentic compounds, the compounds with t_R = 6.28, 9.15, and 12.3 min were assigned to bis(N^2-lysyl)oxalyl amide, (N^2,N^6-lysyl)oxalylamide, and bis(N^6-lysyl)oxalyl amide, respectively. Bis(N^2-lysyl)oxalyl amide was the major adduct and was also formed from the reaction of oxalyl chloride with Lys in H_2O, as described above. These oxalyl amides showed MH^+ ions at *m*/*z* 347 (Figures 5 and 6, panels B−D). The peak at t_R 3.40 min (*m*/*z* 219, Figure 6A) was shown to be N^2-oxalylLys but not N^6-oxalylLys by comparison of t_R values. The identity of the compound eluting at t_R 19.0 min (*m*/*z* 218, Figure 6E, cf. Figure 4B) remains unknown.[2] Control experiments indicated no formation of these Lys adducts when the oxide hydrolysis preceded the addition of Lys under the same conditions.

[2] The molecular ion would correspond to an NH_4^+ adduct of an internally cyclized oxalyl Lys adduct, but attempts to synthesize such a standard were unsuccessful.

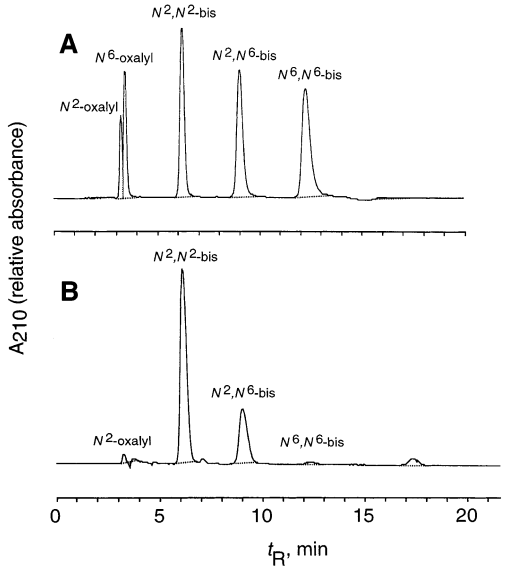


Figure 4. HPLC of products of reaction of PCE oxide and Lys. (A) Reference standards. (B) The reaction contained 10 mM of each reactant (in H_2O). The identities of the peaks were established by t_R values and subsequently by HPLC/MS (Figure 5). N^2-Oxalyl = N^2-oxalylLys; N^6-oxalyl = N^6-oxalylLys; N^2,N^2-bis = bis (N^2-lysyl)oxalyl amide; N^2,N^6-bis = (N^2,N^6-lysyl)oxalyl amide; N^6,N^6-bis = bis (N^6-lysyl)oxalyl amide.

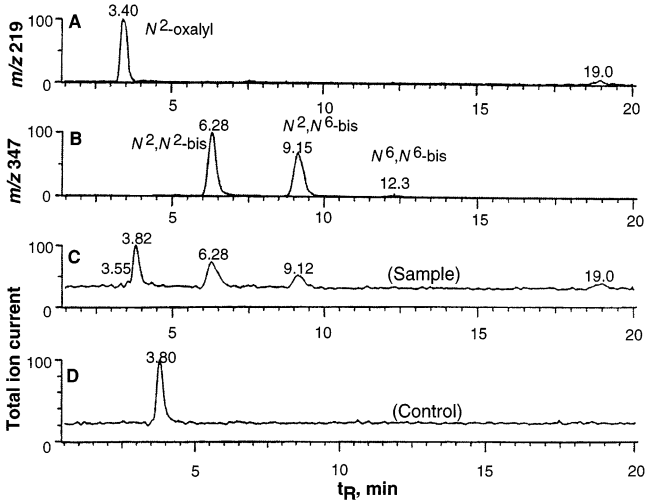


Figure 5. HPLC/MS analysis of products of reaction of PCE oxide with Lys. The reaction proceeded in H_2O at 0 °C for 90 min. N^2-Oxalyl = N^2-oxalylLys; N^2,N^2-bis = bis (N^2-lysyl)oxalyl amide; N^2,N^6-bis = (N^2,N^6-lysyl)oxalyl amide; N^6,N^6-bis = bis (N^6-lysyl)oxalyl amide.

The amounts of the bis Lys oxalyl amides increased with increasing concentrations of PCE oxide (Figure 7).

Products of Reaction of PCE Oxide with Albumin. Bovine serum albumin was reacted with varying amounts of PCE oxide and the Lys adducts were separated and quantified by HPLC/MS after derivatization, with the use of appropriate Orn derivatives as internal standards. In this system, the formation of bis adducts should be rare, and only the N^6-adducts will be formed. The major product was the N^6-oxalylLys derivative, and the N^6-trichloroacetylLys adduct accounted for <5% of the total (Figure 8).

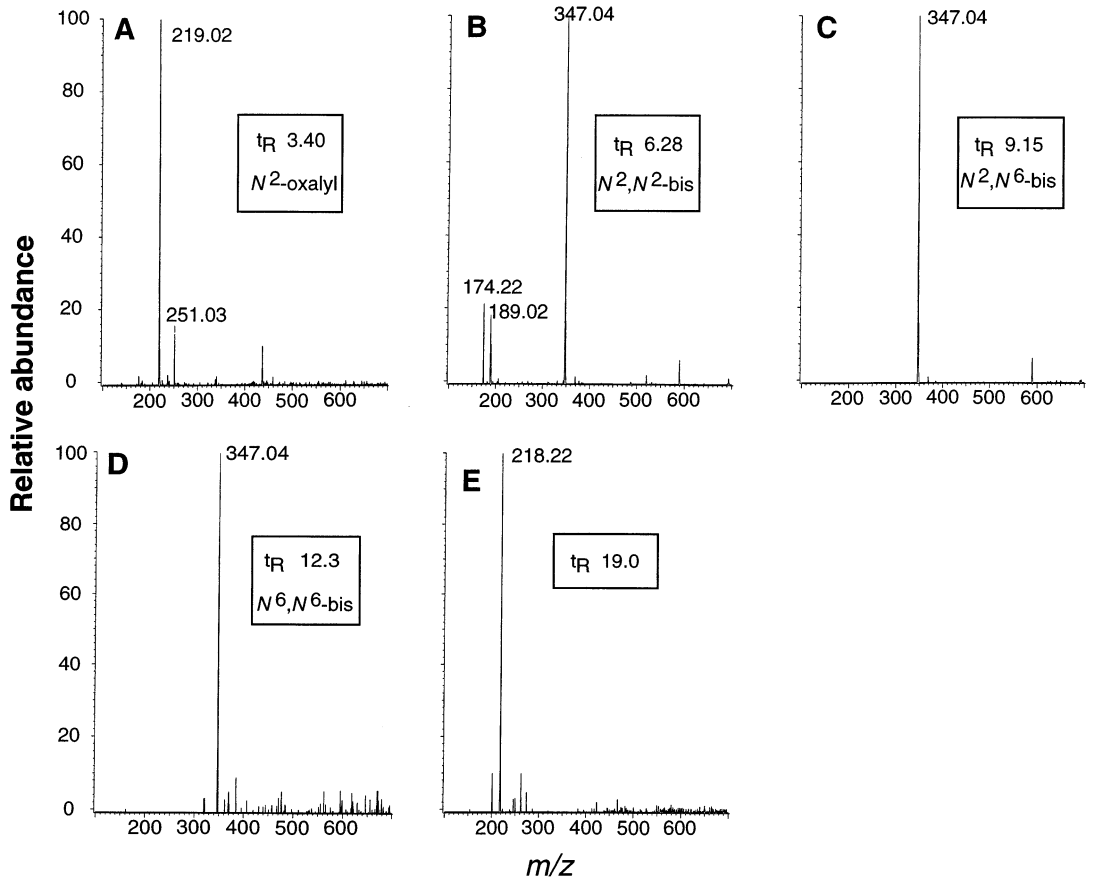


Figure 6. Mass spectra of products of reaction of PCE oxide with Lys. Chromatograms are shown in Figure 5. N^2-Oxalyl = N^2-oxalylLys; N^2,N^2-bis = bis (N^2-lysyl)oxalyl amide; N^2,N^6-bis = (N^2,N^6-lysyl)oxalyl amide; N^6,N^6-bis = bis (N^6-lysyl)oxalyl amide.

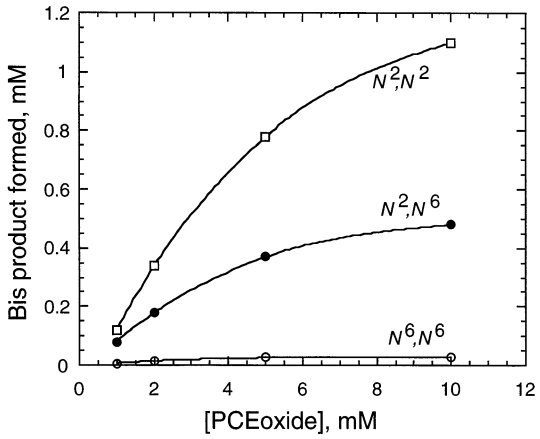


Figure 7. Formation of Lys adducts from PCE oxide as a function of PCE oxide concentration. Lys (1.0 mM) was incubated with varying concentrations of PCE oxide in H_2O (0 °C, 90 min) and the products were analyzed by HPLC/UV as described.

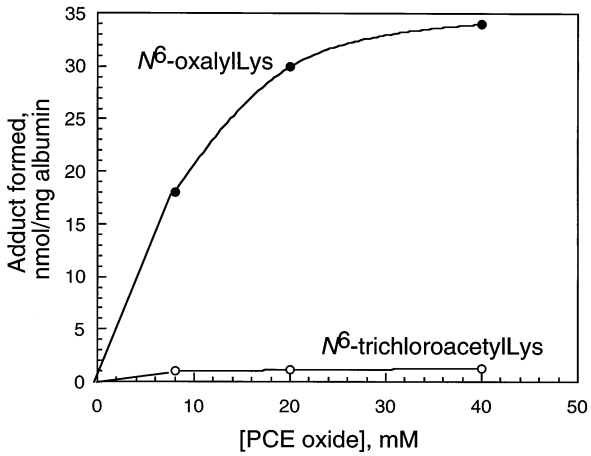


Figure 8. Formation of Lys adducts in albumin treated with PCE oxide. Bovine serum albumin (1.0 mg mL^{-1}) was incubated with varying concentrations of PCE oxide for 90 min at 0 °C and the Lys adducts were quantified by HPLC/MS following derivatization with phenylisothiocyanate.

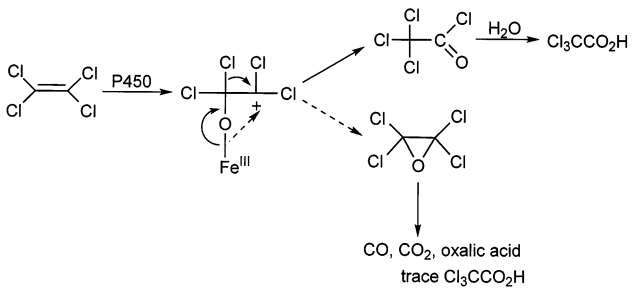
Discussion

In light of the attention given to PCE oxide as an oxidation product of PCE (*1*, *2*, *5*, *6*, *8*, *10*, *12*−*14*, *35*), we prepared this epoxide and characterized some of its properties and its behavior in several reactions. At 37 °C, the $t_{1/2}$ in aqueous buffer (pH 7.4) was 2.6 min, compared to the $t_{1/2}$ of 11.6 min reported by Kline et al. (*36*).[3] Analysis of the hydrolysis products indicated the prominence of C−C bond scission and the reaction of oxalyl equivalents with nucleophiles. The limited formation of oxalic acid and Cl_3CCO_2H is not consistent with the products of microsomal oxidation of PCE, suggesting that the epoxide is not an obligatory intermediate in the P450 reaction (Scheme 3).

The prominence of C−C bond scission was not noted in previous hydrolytic studies with PCE oxide, except in one report by Henschler et al. (*14*), in which the reported yields of CO and CO_2 were ∼30%. The yields of CO and CO_2 in our study were 73 and 63%, respectively (Table 1). Henschler et al. (*14*) reported the formation of CO_2 in an amount equivalent to CO, although the method of

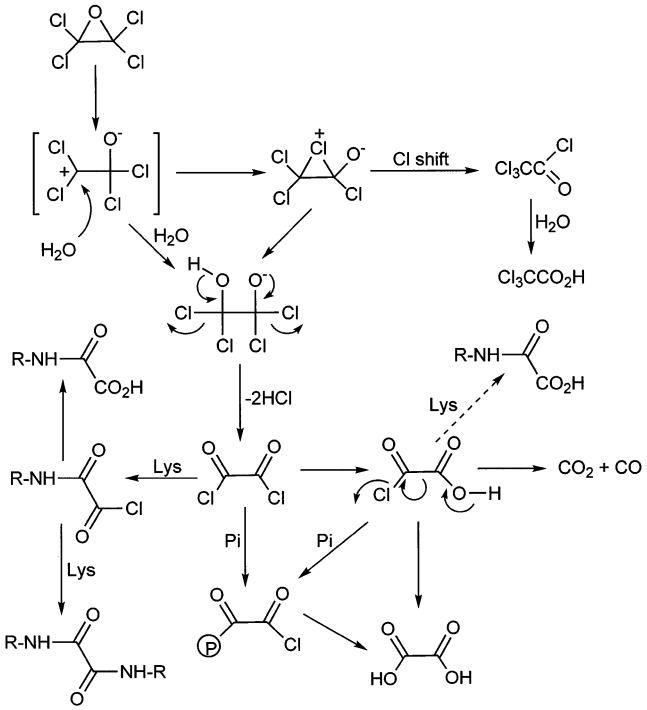
[3] The discrepancy may be due in part to the fact that the rate was determined in a 33% acetone solution in the work of Kline et al. (*36*).

Scheme 3. Postulated Oxidation of PCE by P450[a]



[a] In the Fe−O− intermediate, intermediate, the broken arrow indicates that epoxide formation competes with acid chloride formation.

Scheme 4. Postulated Mechanisms of Decomposition and Reactions of PCE Oxide



analysis was unspecified. We estimated CO_2 formation from PCE oxide using a selective electrode.[4] The results support the view that C−C bond scission yields CO and CO_2 (Scheme 4). No HCHO or HCO_2H was detected, and the formation of CH_3OH seems highly unlikely. Oxalyl chloride also decomposes to CO and CO_2 (Table 2). A mechanism for C−C bond cleavage is presented in Scheme 4, based on the more extensive studies with TCE oxide (*18*). Alternate possibilities can be considered, such as a scission to generate free phosgene which could go on to hydrolyze. However, no evidence for Lys adducts generated from phosgene was apparent (Figures 4 and 5).

In previous work with TCE oxide, we found that the yields of several hydrolysis products were lower in the presence of phosphate buffer (*17*). The same pattern was observed in this work with PCE oxide (Table 1), where much less CO and CO_2 were recovered. The results suggested that oxalyl phosphate might be formed, and HPLC analysis of the PCE oxide hydrolysis reaction yielded a peak subsequently identified as oxalyl phosphate by co-chromatography with authentic material and its demonstrated conversion to oxalic acid. Oxalyl phosphate underwent first-order hydrolysis to oxalic acid, with $t_{1/2}$ = 59 min at 37 °C (Figure 3). Thus, under the conditions used in Table 1, no oxalic acid would be expected in the time frame of the experiment. The patterns of PCE oxide products obtained in the reactions done in the absence and presence of phosphate differ (Table 1), but as indicated, the kinetics of PCE oxide decomposition were identical. Thus, the epoxide must be hydrolyzed, and then a product reacts rapidly with phosphate (Scheme 4), rather than have a nucleophilic attck of phosphate on PCE oxide itself. The phosphate appears to be a poor leaving group, as judged by the stability and the lack of reactivity of oxalyl phosphate with Lys. The formation of such a phospho-adduct may be a general issue in reactions that generate potential acylating agents and possibly alkylating agents, in that phosphate ions may block other reactions. Some of the microsomal studies reported with PCE had been done in 0.1 M phosphate buffers (*6*, *35*). Whether such reactions (of reactive products of PCE oxide) occur with DNA phosphates in unknown.

The reaction of PCE oxide with free Lys yielded primarily oxalyl amides, consistent with other reactions such as the formation of oxalyl phosphate. Thus, reaction of the oxalyl species with a nucleophile appears to trap it, in a reaction that competes with C−C bond scission (Scheme 4). The mono-oxalyl amides were formed at both the N2 and N6 atoms, with N2 predominant due to its lower pK_a (*37*). Cross-linked products containing two Lys groups bound with an oxalo species were formed. The Lys-N^2 adducts are, in a sense, artifacts in the sense that they are only found in the models and would not be formed in protein reactions. We detected only trace amounts of trichloroacetyl derivatives in the reactions of PCE oxide with free Lys (Figures 4 and 5). Reaction of PCE oxide with albumin yielded predominately oxalo Lys derivatives as opposed to trichloroacetyl Lys derivatives (Figure 8). This pattern is similar to that observed with TCE oxide (*20*), in that the amount of (di/tri)-chloroacetyl Lys is low compared to another amide. However, the major product in the TCE oxide reacton was N^6-formylLys. Here with PCE oxide, an adduct formed from the 2-carbon entity is dominant.

The course of products formed from PCE oxide can be compared with products of microsomal oxidation of PCE. The literature indicates the production of Cl_3CCO_2H > oxalic acid (*6*, *8*), reflecting the in vivo products, and the same pattern was observed here (Figure 3). This pattern contrasts with the products of the hydrolysis of PCE oxide (Table 1). Further, Dekant and his associates have shown that the major protein recovered from a microsomal PCE oxidation system is N^6-trichloroacetylLys and apparently not an oxalyl or other derivative (*38*). Why is there a difference in products between P450 oxidation of PCE and PCE oxide hydrolysis? We propose that the apparent discrepancy can be explained with proposals we have provided for TCE (Scheme 3) (*17*) and vinylidene chloride (*19*). The P450 intermediate is a tetrahedral FeO−PCE entity that can collapse via Cl^- migration to yield trichloroacetyl chloride or via formation of PCE oxide, with the former being favored. Also, the enzyme (P450)

[4] A number of other methods were considered and all were unsatisfactory. Gas production was not appropriate because of the known production of CO. Ba^{2+} precipitation proved unreliable. Acid−base titrations of HCO_3^- were not quantitative because of the formation of HCl.

may favor formation of oxalic acid from the intermediate.

In conclusion, the general features of PCE oxide reactions resemble those demonstrated with other halogenated epoxides. Hydrolysis is dominated by C−C bond scission (Table 1) (*17*−*19*). PCE oxide is more stable than the unsymmetrical epoxides, as previously shown by others (*5*, *13*, *14*). Some Cl^- migration does occur, even in the absence of Lewis acids (*39*). Reaction of PCE oxide with Lys generates primarily amides arising from acyl halides (or their equivalents). As documented with vinylidene chloride oxide (*40*) and TCE oxide (*18*), the electrophile reacting with the nucleophile (Lys) is not the epoxide itself (which would not yield a stable adduct) but a reaction hydrolysis product.

The analysis of protein adducts with PCE oxide has been limited to Lys adducts in this study, which are stable. However, in previous work with TCE oxide, we found that the majority of protein adducts were unstable and were released (nonenzymatically) from proteins with a $t_{1/2}$ of ∼1 h (*21*, *22*), and similar behavior might be expected with the PCE oxide adducts. This aspect of the work is being pursued at this time. Finally, the relevance of any protein adducts to biological functions and toxicity remains to be considered.

Acknowledgment. This work was supported by U.S. Public Health Service Grants R01 ES10546 and P30 ES00267. We thank M. Voehler for his assistance with some of the NMR experiments and M. L. Manier for assistance with the MS work.

References

(1) U. S. Departament of Health and Human Services (1993) *Toxicological profile for tetrachloroethylene*, Public Health Service, Agency for Toxic Substances and Disease Registry, Atlanta.

(2) Reichert, D. (1983) Biological actions and interactions of tetrachloroethylene. *Mutat. Res.* **123**, 411−429.

(3) National Cancer Inst. (1977) Bioassay of tetrachloroethylene for possible carcinogenicity. *National Toxicology Program Technical Report*, 77-813.

(4) National Cancer Inst. (1986) Carcinogenesis bioassay of tetrachloroethylene. *National Toxicology Program Technical Report*, 232.

(5) Henschler, D. (1977) Metabolism and mutagenicity of halogenated olefins—a comparison of structure and activity. *Environ. Health Perspect. 21*, 61-64.

(6) Birner, G., Rutkowska, A., and Dekant, W. (1996) *N*-Acetyl-*S*-(1,2,2-trichlorovinyl)-L-cysteine and 2,2,2-trichloroethanol. Two novel metabolites of tetrachloroethene in humans after occupational exposure. *Drug Metab. Dispos.* **24**, 41−48.

(7) Volkel, W., Friedewald, M., Lederer, E., Pahler, A., Parker, J., and Dekant, W. (1998) Biotransformation of perchloroethylene: Dose-dependent excretion of trichloroacetic acid, dichloroacetic acid, and *N*-acetyl-*S*-(trichlorovinyl)-L-cysteine in rats and humans after inhalation. *Toxicol. Appl. Pharmacol.* **153**, 20-27.

(8) Costa, A. K., and Ivanetich, K. M. (1980) Tetrachloroethylene metabolism by the hepatic microsomal cytochrome P-450 system. *Biochem. Pharmacol.* **29**, 2863−2869.

(9) White, I. N. H., Razvi, N., Gibbs, A. H., Davies, A. M., Manno, M., Zaccaro, C., De Matteis, F., Pahler, A., and Dekant, W. (2001) Neoantigen formation and clastogenic action of HCFC-123 perchloroethylene in human MCL-5 cells. *Toxicol. Lett.* **123**, 129-138.

(10) Daniel, J. W. (1963) The metabolism of ^{36}Cl-labeled trichloroethylene and tetrachloroethylene in the rat. *Biochem. Pharmacol.* **12**, 795−802.

(11) Ikeda, M., Ohtsuji, H., Imamura, T., and Komoike, Y. (1972) Urinary excretion of total trichloro-compounds, trichloroethanol, and trichloroacetic acid as a measure of exposure to trichloroethylene and tetrachloroethylene. *Brit. J. Industr. Med.* **29**, 328-333.

(12) Powell, J. F. (1945) Trichloroethylene: absorption, elimination, and metabolism. *Brit. J. Industr. Med.* **2**, 142−145.

(13) Henschler, D., and Bonse, G. (1977) Metabolic activation of chlorinated ethylenes: dependence of mutagenic effect on electrophilic reactivity of the metabolically formed epoxides. *Arch. Toxicol.* **39**, 7-12.

(14) Henschler, D., and Hoos, R. (1982) Metabolic activation and deactivation mechanisms of di-, tri-, and tetrachloroethylenes. *Adv. Exp. Med. Biol.* **136A**, 659−666.

(15) Ruden, C. (2002) The use of mechanistic data and the handling of scientific uncertainty in carcinogen risk assessments. *Regul. Toxicol. Pharmacol.* **35**, 80−94.

(16) Loizou, G. D. (2001) The application of physiologically based pharmacokinetic modelling in the analysis of occupational exposure to perchloroethylene. *Toxicol. Lett.* **123**, 59−69.

(17) Miller, R. E., and Guengerich, F. P. (1982) Oxidation of trichloroethylene by liver microsomal cytochrome P-450: evidence for chlorine migration in a transition state not involving trichloroethylene oxide. *Biochemistry* **21**, 1090−1097.

(18) Cai, H., and Guengerich, F. P. (1999) Mechanism of aqueous decomposition of trichloroethylene oxide. *J. Am. Chem. Soc.* **121**, 11656−11663.

(19) Liebler, D. C., and Guengerich, F. P. (1983) Olefin oxidation by cytochrome P-450: evidence for group migration in catalytic intermediates formed with vinylidene chloride and *trans*-1-phenyl-1-butene. *Biochemistry* **22**, 5482-5489.

(20) Cai, H., and Guengerich, F. P. (2000) Acylation of protein lysines by trichloroethylene oxide. *Chem. Res. Toxicol.* **13**, 327−335.

(21) Cai, H., and Guengerich, F. P. (2001) Reaction of trichloroethylene oxide with proteins and DNA: instability of adducts and modulation of functions. *Chem. Res. Toxicol.* **14**, 54-61.

(22) Cai, H., and Guengerich, F. P. (2001) Reaction of trichloroethylene and trichloroethylene oxide with cytochrome P450 enzymes: inactivation and site of modification. *Chem. Res. Toxicol.* **14**, 451−458.

(23) Gottlieb, H. E., Kotlyar, V., and Nudelman, A. (1997) NMR chemical shifts of common laboratory solvents as trace impurities. *J. Org. Chem.* **62**, 7512−7515.

(24) Campbell, R. W., and Vogl, O. (1977) A practical synthesis of tetrachloroethylene oxide. *J. Macromol. Sci.-Chem.* **A11**, 515−534.

(25) Kofron, J. L., and Reed, G. H. (1990) Synthesis of oxalyl phosphate and processing of the acyl phosphate by phosphoenolpyruvate-dependent enzymes. *Arch. Biochem. Biophys.* **280**, 40−44.

(26) Kim, J., and Duraway-Mariano, D. (1996) Phosphoenolpyruvate mutase catalysis of phosphoryl transfer in phosphoenolpyruvate; kinetics and mechanism of phosphorus-carbon bond formation. *Biochemistry* **35**, 4628−4635.

(27) Ames, B. (1966) Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol.* **8**, 115-118.

(28) Coughlin, D. J., and Wood, R. (1994) Preparation of glycyltyrosyl-(N-diethylenetriaminepentaacetic acid)lysine hydrochloride (gyk-dtpa), U. S. Patent Appl. 92−890519, cited as *Statutory Invent. Regist.*

(29) Nunn, P. B., Partridge, L. Z., and Euerby, M. R. (1989) Synthesis and characterization of ω-N-oxalyl derivatives of some L- and D-diamino acids. *J. Chem. Res.* **M**, 2401-2415.

(30) Balajthy, Z. (1991) An improved preparation of N-benzyloxycarbonyl-L-lysine methyl ester hydrochloride. *Org. Prep. Proced. Int.* **23**, 375−376.

(31) Epstein, J., Rosenthal, R. W., and Ess, R. J. (1955) Use of *p*-(4-nitrobenzyl)pyridine as analytical reagent for ethylenimines and alkylating agents. *Anal. Chem.* **27**, 1435-1439.

(32) Nash, T. (1953) The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* **55**, 416−421.

(33) Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., and Kaminsky, L. S. (1982) Purification and characterization of liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. *Biochemistry* **21**, 6019−6030.

(34) Guengerich, F. P. (2001) Analysis and characterization of enzymes and nucleic acids. In *Principles and Methods of Toxicology* (Hayes, A. W.) 4th ed., pp 1625−1687, Taylor & Francis, New York.

(35) Greim, H., Bonse, G., Radwan, Z., Reichert, D., and Henschler, D. (1975) Mutagenicity in vitro and potential carcinogenicity of chlorinated ethylenes as a function of metabolic oxirane formation. *Biochem. Pharmacol.* **24**, 2013−2017.

(36) Kline, S. A., Solomon, J. J., and Van Duuren, B. L. (1978) Synthesis and reactions of chloroalkene epoxides. *J. Org. Chem.* **43**, 3596−3600.

(37) Mahler, H. R., and Cordes, E. H. (1971) *Biological Chemistry*, 2nd ed., Harper & Row, New York.

(38) Birner, G., Richling, C., Henschler, D., Anders, M. W., and Dekant, W. (1994) Metabolism of tetrachloroethene in rats:

identification of N^{ϵ}-(dichloroacetyl)-L-lysine and N^{ϵ}-(trichloroacetyl)-L-lysine as protein adducts. *Chem. Res. Toxicol.* **7**, 724−732.

(39) McDonald, R. N. (1969) Rearrangements of α-halo epoxides and related α-substituted epoxides. In *Mechanisms of Molecular Migrations* (Thyagarajan, B. S.) pp 67−107, Wiley, New York.

(40) Liebler, D. C., Meredith, M. J., and Guengerich, F. P. (1985) Formation of glutathione conjugates by reactive metabolites of vinylidene chloride in microsomes and isolated hepatocytes. *Cancer Res.* **45**, 186−193.

TX020028J