# Microsomal Oxidation of Tribromoethylene and Reactions of Tribromoethylene Oxide

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*Received June 4, 2002*

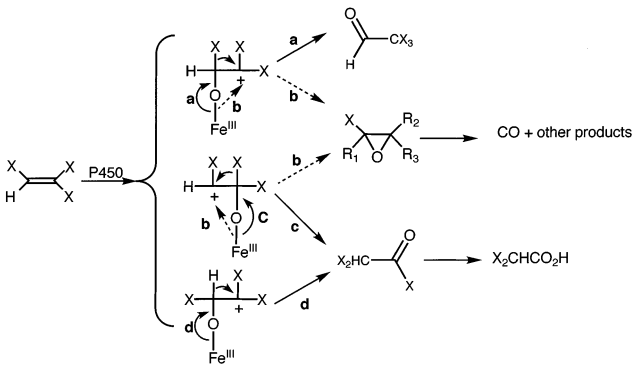
Halogenated olefins are of interest because of their widespread use in industry and their potential toxicity to humans. Epoxides are among the enzymatic oxidation products and have been studied in regard to their toxicity. Most of the attention has been given to chlorinated epoxides, and we have previously studied the reactions of the mono-, di-, tri-, and tetrachloroethylene oxides. To further test some hypotheses concerning the reactivity of these compounds, we prepared tribromoethylene (TBE) oxide and compared it to trichloroethylene (TCE) oxide and other chlorinated epoxides. TBE oxide reacted with $H_2O$ about 3 times faster than did TCE oxide. Several hydrolysis products of TBE oxide were the same as formed from TCE oxide, i.e., glyoxylic acid, CO, and $HCO_2H$. $Br_2CHCO_2H$ was formed from TBE oxide; the yield was higher than for $Cl_2CHCO_2H$ formed in the hydrolysis of TCE oxide. The yield of tribromoacetaldehyde was < 0.4% in aqueous buffer (pH 7.4). In rat liver microsomal incubations containing TBE and NADPH, $Br_2CHCO_2H$ was a major product, and tribromoacetaldehyde was a minor product. These results are consistent with schemes previously developed for halogenated epoxides, with migration of bromine being more favorable than for chlorine. Reaction of TBE oxide with lysine yielded relatively more *N*-dihaloacetyllysine and less *N*-formyllysine than in the case of TCE oxide. This same pattern was observed in the products of the reaction of TBE oxide with the lysine residues in bovine serum albumin. We conclude that the proposed scheme of hydrolysis of halogenated epoxides follows the expected halide order and that this can be used to rationalize patterns of hydrolysis and reactivity of other halogenated epoxides.

## Introduction

Halogenated olefins have had many uses as solvents and as vinyl monomers in the plastics industry. Many of these chemicals are used in large volumes, e.g., perchloroethylene (PCE)[1] at $10^8$ kg year$^{-1}$ in the United States (*1*). These chemicals can all be toxic at high doses, and the study of their toxicities has been a matter of considerable interest. Vinyl chloride (VC) is clearly a rodent and human carcinogen (*2, 3*). The carcinogenicity of trichloroethylene (TCE) and perchloroethylene (PCE, tetrachloroethylene) is more controversial (*1, 4, 5*), but these compounds can have other detrimental effects (*6*). Vinylidene chloride (VDC) is relatively toxic in liver and kidney (*7, 8*) but probably not carcinogenic (*9*−*11*).

Although GSH conjugation can contribute to bioactivation of some of the more highly substituted vinyl halides (*12, 13*), much of the existing literature is centered on the formation and roles of the epoxides (*14, 15*). The chlorooxirane derivatives of the above compounds are all relatively unstable, with $t_{1/2}$ of ∼2 s to 2.6 min under physiological conditions (*16*−*21*). Some of the early studies on epoxides yielded ambiguous results in terms of what the products were or whether the epoxide was the principal electrophile that reacts with macromolecules (*22*). Recent work in this laboratory has led to the hypothesis that a major feature of the reaction of halooxiranes with nucleophiles is the intermediacy of acid halide hydrolytic products, which behave as acylating agents (*20, 21, 23*). However, the situation is complicated by the possibility that P450 enzymes can generate acyl halides in 1,2-migration reactions exclusive of epoxide products (Scheme 1) (*18, 19, 21*). Thus, modification of macromolecules may be difficult to understand in the context of a single reactive species.

**Scheme 1. Proposed Mechanism of P450-Catalyzed Oxidation of Vinyl Halides**[a]




[a] X = halogen.

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[1] Abbreviations: TBE, tribromoethylene; TCE, trichloroethylene; VC, vinyl chloride; VDC, vinylidene chloride (1,1-dichloroethylene); PCE, perchloroethylene (tetrachloroethylene); VB, vinyl bromide; CBZ, carbobenzyloxy; MOPS, 3-(*N*-morpholino)propanesulfonate; Orn, ornithine.

10.1021/tx020047t CCC: $22.00 
Published on Web 10/10/2002

Brominated olefins are used less commonly than the chlorinated analogues in industry and have been studied less. Vinyl bromide (VB) has been studied in several contexts and exhibits behavior generally similar to VC, in regard to both carcinogenicity (*24*) and mechanism of bioactivation (*25*−*29*). Despite the limited industrial use of bromine compounds, they can be highly useful in studying mechanisms of reactions of halooxiranes through insights in the halide order. A key model for our research on vinyl halides has been TCE and the reactions of TCE oxide (*19*, *20*, *23*, *30*). We extended this research project to tribromoethylene (TBE) in order to address some general hypotheses about vinyl halides and their epoxides. The literature contains some studies on the chemistry of peracid oxidation of TBE (*31*) and the use of TBE as a model substrate for bacterial methane monooxygenase (*32*). Interestingly, TBE has also been demonstrated to be a volatile natural product produced by algae (*33*) and is a metabolite of 1,1,2,2-tetrabromoethane in rats (*34*). TBE has also been shown to be toxic to algae, crustaceans, and fish (*35*).

We synthesized TBE oxide and compared it with TCE oxide in terms of rates of hydrolysis, hydrolytic products, and reactions with free and protein Lys. With this information about TBE oxide and previous results with VC oxide, VDC oxide, TCE oxide, and PCE oxide, we postulate general mechanisms for the formation of halogenated epoxides and their reactivity.

## Experimental Procedures

***Caution:*** TBE oxide and the acyl halides used here are reactive acylating agents and should be handled carefully. Gloves and other protective clothing should be used appropriately.

**Materials.** TBE was purchased from TCI America (Portland, OR) and distilled under reduced pressure (bp 48−52 °C, 10 Torr). TCE oxide was prepared as described previously (*20*). $Br_3CCHO$ and $Br_2CHCO_2H$ were purchased from Aldrich Chemical Co. (Milwaukee, WI).

**HPLC and Spectroscopy.** HPLC analysis was done using a Hitachi 7100 or a Spectra-Physics 8700 pumping system. Mass spectra were measured with Finnigan TSQ7000 instruments, using either direct injection (synthetic chemicals) or HPLC introduction (albumin products), in an electrospray/positive ion mode. NMR spectra were collected on Bruker 300 and 400 MHz instruments in the Vanderbilt facility. The HOD signal was adjusted to $\delta$ 4.79 in $D_2O$ solutions. $CH_3OH$ (49.5 ppm) was used as an internal reference for $^{13}C$ NMR spectra ($D_2O$ solutions). $(CH_3)_4Si$ was used as an internal standard in $CDCl_3$ solutions.

**Synthesis of TBE Oxide.** Distilled TBE was oxidized with *m*-chloroperbenzoic acid using the same procedure described for TCE oxide (*20*, *36*), for 100 min. Analysis of the reaction for TBE oxide using 4-(4-nitrobenzyl)pyridine reaction (*37*) indicated that TBE oxide formation had peaked at this time. The product was distilled in vacuo (bp 60−66 °C, 15 Torr) to yield a colorless liquid, in residual TBE (the most concentrated fraction of TBE oxide was obtained at 60−66 °C). The product was stable in $CDCl_3$ and could be washed (quickly) with cold 6 N NaOH to remove acid and acyl bromide impurities. The $^1H$ NMR spectrum ($CDCl_3$) showed a proton singlet at $\delta$ 5.42 (along with the TBE singlet at $\delta$ 7.00) [cf. $\delta$ 5.31 for TCE oxide (*19*)]. The $^{13}C$ NMR spectrum (in $CDCl_3$) yielded peaks at 55.5 and 64.7 ppm, which are assigned to the CHBr and $CBr_2$ carbons of TBE oxide, respectively (with the CHBr and $CBr_2$ carbon signals of TBE found at 92.8 and 110.4 ppm, respectively) [cf. TCE and TCE oxide shifts in ref (*19*)]. The concentration of the TBE oxide in the NMR tube was calculated with the addition of a known amount of TCE ($\delta$ 6.46 singlet), with integration of the proton signals. From these measurements, the concentration of TBE oxide in the TBE was 4.9% (v/v) in the sample used, and the $\epsilon_{560}$ of the 4-(4-nitrobenzyl)pyridine complex (*37*) was calculated to be 19 800 $M^{-1}$ $cm^{-1}$ under these conditions (*20*, *21*). This extinction coefficient was subsequently used in other assays. The residual TBE did not interfere with any of the subsequent measurements, and it did not yield any of the analyzed products of TBE oxide. TBE oxide was stored in the presence of desiccant at −80 °C.

**Synthesis of $N^6$-DibromoacetylLys.** Ethyl dibromoacetate (0.88 g, 1.2 equiv) was added to 3.0 mmol of L-Lys·HCl in 1.5 mL of 2 N NaOH (3.0 mmol), and the reaction was stirred at room temperature (2 h). The addition of 1 mL of 1 N HCl yielded a white precipitate, which was collected by suction filtration, washed with 50% (v/v) aqueous $C_2H_5OH$, and dried in vacuo over $P_2O_5$ to give the product (203 mg, 20%). Recrystallization from hot aqueous $C_2H_5OH$ gave 170 mg of crystalline material (mp 190 °C, decomp): $^1H$ NMR ($D_2O$) $\delta$ 1.40 (m, 2H, $\gamma$-H), 1.58 (m, 2H, $\beta$-H), 1.87 (m, 2H, $\delta$-H), 3.25 (*t*, 2H, $J$ = 6.7 Hz, $\epsilon$-H), 3.69 (*t*, 1H, $J$ = 6.3 Hz, α-H), 6.17 (s, 1H, -COC*H*$Br_2$); $^{13}C$ NMR ($D_2O$) $\delta$ 22.3 ($\gamma$), 28.3 ($\delta$), 30.7 ($\beta$) 37.0 (−$CHBr_2$), 40.5 ($\epsilon$), 53.3 (α), 168.2 (-*C*O$CHBr_2$), 175.4 (*C*$O_2$H); MS *m*/*z* 345, 347, 349 ($MH^+$) (plus $MNa^+$ ions at 367, 369, 371).

**Synthesis of $N^2$-DibromoacetylLys.** $Br_2CHCO_2H$ (5.6 g, 26 mmol) was dissolved in 5.0 mL of $SOCl_2$ and heated at reflux for 2 h. Excess $SOCl_2$ was removed in vacuo. A portion of the resulting dibromoacetyl chloride (0.19 mL, 1.2 equiv) was added to 0.81 g (2.0 mmol) of $N^6$-CBZ-L-Lys benzyl ester·HCl (*21*, *38*) in a mixture of 8 mL of ethyl acetate/1 mL of $(C_2H_5)_3N$ at 0 °C, and the mixture was stirred for 2 h, yielding a pale yellow solid (0.86 g, 90% yield): $^1H$ NMR ($CDCl_3$) $\delta$ 1.24−1.48 (m, 4H, $\beta$- and $\gamma$-H), 1.77−201 (m, 2H, $\beta$-H), 3.14 (m, 2H, $\epsilon$-H), 4.58 (m, 1H, α-H), 4.73 (br s, 1H, NH-), 5.09 (s, 2H, PhC$H_2$-), 5.17 and 5.24 (each d, total 2H, $J$ = 12.1 Hz, PhC$H_2$-), 5.81 (s, 1H, −C*H*$Br_2$), 7.08 (app d, 1H, $J$ = 6.6 Hz, NH), 7.26−7.36 (m, 10 H, aromatic).

The above compound (740 mg) was hydrolyzed with 1 N NaOH (2 mL) in $CH_3OH$ (4 mL) for 1.5 h at room temperature. The mixture was acidified with 1 N HCl, and the resulting carboxylic acid was extracted into ethyl acetate (3×). The solvent was removed in vacuo, and the residue was treated with 3 mL of HBr in $CH_3CO_2H$ (30% w/v) at room temperature for 1 h, followed by the addition of 200 mL of $(C_2H_5)_2O$ to give $N^2$-dibromoacetylLys·HBr. The free base was obtained by neutralization with NaOH, followed by decolorization with activated charcoal and concentration in vacuo [yield 210 mg (47%); mp 172 °C (from $C_2H_5OH$, decomp)]: $^1H$ NMR ($D_2O$) $\delta$ 1.43 (m, 2H, $\gamma$-H), 1.63−1.82 (m, 3H, $\beta$-$H_1$ and $\delta$-H), 1.90 (m, 1H, $\beta$-$H_2$), 2.99 (t, 2H, $J$ = 7.5 Hz, $\epsilon$-H), 4.19 (dd, 1H, $J$ = 5.0 and 8.1 Hz, α-H), 6.28 (s, 1H, -C*H*$Br_2$); $^{13}C$ NMR ($D_2O$) $\delta$ 22.6 ($\gamma$), 26.9 ($\delta$), 31.5 ($\beta$), 37.0 (-*C*$HBr_2$), 39.9 ($\epsilon$), 56.2 (α), 167.3 (-*C*O$CHBr_2$), 178.4 (-*C*$O_2$H); MS *m*/*z* 345, 347, 349 ($MH^+$).

**Synthesis of $N^5$-DibromoacetylOrn.** (This compound was used as an internal standard in the analysis of Lys residues in albumin modified with TBE oxide.) The procedure used was the same as for preparations of $N^6$-dibromoacetylLys (vide supra). L-Orn·HCl (5.5 nmol) was stirred with 2.1 equiv of NaOH and 1.3 equiv of $Br_2CHCOCl$ in 3.4 mL of $H_2O$ for 1.6 h at room temperature. The reaction was neutralized with 1 N HCl and concentrated in vacuo. $C_2H_5OH$ was added to the residue. Cooling at 4 °C yielded a precipitate, which was collected and dried over $P_2O_5$ (320 mg, 18% yield): mp 164 °C (decomp); $^1H$ NMR ($D_2O$) $\delta$ 1.66 (m, 2H, $\gamma$-H), 1.88 (m, 2H, $\beta$-H), 3.32 (t, 2H, $J$ = 6.6 Hz, $\delta$-H), 3.75 (t, 1H, $J$ = 6.0 Hz, α-H), 6.16 (s, 1H, -C*H*$Br_2$); $^{13}C$ NMR ($D_2O$) $\delta$ 24.5 ($\gamma$), 28.4 ($\beta$), 36.9 (-*C*$HBr_2$), 40.2 ($\delta$), 55.0 (α), 168.2 (-*C*O$CHBr_2$), 175.0 (-*C*$O_2$H); MS *m*/*z* 331, 333, 335 ($MH^+$), 353, 355, 357 ($MNa^+$).

**Analysis of TBE and TCE Oxide Hydrolysis Kinetics and Products.** The general methods were as previously described (*20*, *21*).

The kinetics of hydrolysis of TBE oxide were examined using the following procedure. To 1.0 mL of 100 mM potassium MOPS

(pH 7.4) in an ice bath was added 80 $\mu$L of TBE oxide (in dry $CH_3CN$), with stirring (final concentration of TBE oxide ~0.8 mM). At varying times, 40 $\mu$L aliquots of the solution were added to 500 $\mu$L of a solution of 4-(4-nitrobenzyl)pyridine reagent (*16*, *17*, *19*, *20*). The mixture was reacted at room temperature for 5 min, and then 500 $\mu$L of a mixture of $(C_2H_5)_3N$/acetone (1:1, v/v) was added. $A_{560}$ measurements were used to determine the remaining TBE oxide ($\epsilon_{560}$ = 19 800 $M^{-1}$ $cm^{-1}$), fitting the data to a plot for (*pseudo*) first-order decomposition.

In the analysis of hydrolysis products, 40 $\mu$L of a $CH_3CN$ stock solution of TBE or TCE oxide (1.8 $\mu$mol) was added to 1.0 mL of potassium 3-(*N*-morpholino)propanesulfonate (MOPS) buffer at 0 °C. TBE was removed by extraction of the solution with 1.0 mL of $(C_2H_5)_2O$, and 30 $\mu$L of the aqueous phase was injected onto a 4.6 × 250 mm octadecylsilane column (5 $\mu$m, Beckman, San Ramon, CA) equilibrated with 10 mM tetra-*n*-butylammonium sulfate (pH 6.0) (for analysis of $HCO_2H$ and glyoxylic acid) or the same buffer plus 20% $CH_3CN$ (v/v) for the analysis of $Br_2CHCO_2H$ (flow rate 1.2 mL $min^{-1}$, $A_{210}$ detection in all cases).

$Br_3CCHO$ (tribromoacetaldehyde, bromal) was analyzed by treating 1.0 mL aliquots of the hydrolyzed samples with 2,4-dinitrophenylhydrazine and extraction as described for HCHO previously (*39*). The derivatized extracts, including a set of standards of known concentration, were analyzed by HPLC [6.2 × 80 mm Zorbax octadecylsilane column, 3 $\mu$m (MacMod, Chadds Ford, PA), 54% aqueous $CH_3CN$ (v/v), flow rate 2.5 mL $min^{-1}$, $A_{360}$].

CO was analyzed in a closed gas-phase circuit using an electrochemical sensor (ERMF-0503, Draeger, Pittsburgh, PA), as described earlier (*20*), with calibration using standardized amounts of CO.

**Microsomal Incubations.** Liver microsomes prepared from phenobarbital-treated rats (4.6 mg of protein $mL^{-1}$) (*40*) were incubated with an NADPH-generating system (*40*) and 10 mM TBE (added without organic solvent) in 0.10 M potassium MOPS buffer (pH 7.4). The flask was shaken in air at 37 °C, and 1.0 mL aliquots were withdrawn at various time points, quenched with the addition of 0.15 mL of 25% $HClO_4$, and chilled. Insoluble protein was removed by centrifugation at 3000*g* for 10 min, and 1.0 mL of the supernatant was analyzed for $Br_3CCHO$, using 2,4-dinitrophenylhydrazone as described above.

In separate incubations, 5.0 mL aliquots of a similar incubation were withdrawn after varying incubation times and quenched by the addition of $H_2SO_4$ to 2% (w/v). Precipitated protein was removed by centrifugation at 3000*g* for 10 min, and the supernatants (pH <2) were extracted 5× with 5 mL of $(CH_3CH_2)_2O$. The pooled organic extracts were concentrated to dryness under an $N_2$ stream. Direct analysis of $Br_2CHCO_2H$ by ion-pair HPLC ($A_{210}$, vide supra) was unsuccessful because of interfering peaks. $Br_2CHCO_2H$ in the extracts was derivatized using 4-nitrophenacyl bromide (Aldrich) according to Morozowich and Douglas (*19*, *41*). A 0.10 M solution of 4-nitrophenacyl bromide (200 $\mu$L) and 1 $\mu$L of *N*,*N*-diisopropyl-*N*-ethylamine was added to each sample, and the *p*-nitrophenacyl ester product was analyzed after 15 min at room temperature, using HPLC [6.2 × 80 mm Zorbax octadecylsilane column, 3 $\mu$m, linear gradient of 18−45% $CH_3CN$ in $H_2O$, $A_{260}$].

The analyses of $Br_3CCHO$ and $Br_2CHCO_2H$ were done using comparisons with standards prepared using the same procedure.

Benzene extracts of NADPH-fortified microsomal incubations were analyzed for TBE oxide using 4-(4-nitrobenzyl)pyridine as described elsewhere (*19*).

**Reaction of TBE Oxide with Lys.** In the reaction with Lys, 100 $\mu$L of 100 mM TBE oxide (in dry $CH_3CN$) was added to 900 $\mu$L of an aqueous solution of (10 mM) L-Lys (free base) on ice, and reaction was allowed to occur for 30 min. The samples were washed with an equal volume of $(C_2H_5)_2O$, and residual $(C_2H_5)_2O$ was removed under an $N_2$ stream. The aqueous samples were diluted 5-fold with 10 mM $NH_4CH_3CO_2$ buffer (pH 6.5) and analyzed using a 4.6 × 250 mm Beckman octadecylsilane HPLC column (5 $\mu$m), with an (isocratic) buffer of 10 mM $NH_4CH_3CO_2$ (pH 6.5) at a flow rate of 0.7 mL $min^{-1}$ ($A_{210}$).

**Table 1. Decomposition Products of TCE and TBE Oxides**[a]

| | yield ($\mu$mol/$\mu$mol of epoxide) | |
|---|---|---|
| product | TCE oxide | TBE oxide |
| $CHX_2CO_2H$[b] | 0.18 | 0.53 |
| $OHCCO_2H$ | 0.08 | 0.08 |
| $HCO_2H$ | 0.38[2] | 0.16 |
| CO | 0.42[2] | 0.37 |
| $CX_3CHO$[b] | <0.001 | <0.004 |
| rate of hydrolysis[c] | 0.0042 $s^{-1}$ [d] ±0.0002 | 0.0114 $s^{-1}$ ±0.0018 |

[a] Incubations were done at 0 °C for 10 min in 0.10 M potassium MOPS buffer (pH 7.4). [b] X = Cl for TCE oxide, X = Br for TBE oxide. [c] Estimated using 4-(4-nitrobenzyl)pyridine reagent. [d] At 0 °C. Compare with 0.0069 $s^{-1}$ reported previously (*20*).

**Reaction of TBE Oxide with Albumin.** The indicated amounts of TBE oxide, diluted to 50 $\mu$L in dry $CH_3CN$ (to give final concentrations of 8 and 20 mM TBE oxide), were added to a solution of bovine serum albumin (1.0 mg in 200 $\mu$L of 250 mM potassium MOPS buffer, pH 7.4). The samples were incubated at 0 °C for 30 min and then evaporated to dryness under an $N_2$ stream. The residue was dissolved in 250 $\mu$L of potassium MOPS buffer (pH 7.4). To the mixture was added 50 $\mu$L of a solution containing both 4 mM $N^5$-formylOrn (*23*) and 4 mM $N^5$-dibromoacetylOrn (vide supra). The albumin was digested with proteinase K overnight at 37 °C under Ar, and the digest was derivatized with phenylisothiocyanate as described elsewhere (*23*).

Aliquots of the modified amino acid digest were analyzed by combined HPLC/electrospray MS in the general procedure described for TCE oxide products (*23*). Internal standard calibration curves were prepared using varying amounts of $N^6$-formylLys and $N^6$-dibromoacetylLys, carried through the derivatization and HPLC/MS steps with fixed amounts of $N^5$-formylOrn and $N^5$-dibromoacetylOrn. The HPLC and MS conditions were identical to those described for PCE (*21*), using a Zorbax octylsilane column (4.6 × 250 mm) connected to a Finnegan TSQ7000 instrument operating in the electrospray mode, with single ion monitoring ($MH^+$ ion in each case). A 10 mM $NH_4CH_3CO_2$ buffer (pH 6.5) was used with an increasing $CH_3OH$ gradient. The appropriate *m*/*z* peaks were integrated, and the internal standard curves (based on Orn derivatives) were used to do calculations.

## Results

**Hydrolysis of TBE Oxide.** TBE oxide was prepared as described, using a general approach used previously for VDC and TCE oxides (*18*, *20*, *36*). We were unable to obtain a mass spectrum due to the instability of the compound, but the $^1H$ and $^{13}C$ NMR spectra and reactivity are consistent with the nature of the product.

The rate of hydrolysis (at 0 °C and pH 7.4) was estimated using 4-(4-nitrobenzyl)pyridine reagent and was 0.0114 (±0.0018) $s^{-1}$ under these conditions (Table 1) (*20*). This rate is about 3 times faster than that of TCE oxide (measured in a parallel experiment) (Table 1). The rate corresponds to a $t_{1/2}$ of 61 s (pseudo-first-order reaction at 0 °C) and would be considerably faster at higher temperatures (estimated ~5 s at 23 °C, based on behavior of TCE oxide) (*19*, *20*).

The products were identified and quantified by HPLC with the exception of CO, which was measured with a gas-selective electrode (Table 1). The products of TCE oxide hydrolysis have been measured previously (*19*, *20*) and were analyzed again here in parallel experiments (Table 1). More than 95% of the TBE oxide products were accounted for, except for the low recovery of $HCO_2H$. Only

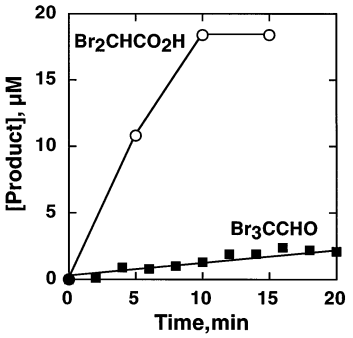



**Figure 1.** Products of P450-catalyzed oxidation of TBE. Liver microsomes prepared from phenobarbital-treated rats (4.6 mg of protein $mL^{-1}$) were incubated with TBE (10 mM) and an NADPH-generating system in 0.10 M potassium MOPS buffer (pH 7.4) at 37 °C; the products were analyzed as described under Experimental Procedures. (■) $Br_3CCHO$; (○) $Br_2CHCO_2H$.

trace $Br_3CCHO$ was detected (<0.4%), and the possibility that this had been formed slowly in the TBE oxide by rearrangement prior to hydrolysis cannot be excluded. The yield of glyoxylic acid from TBE oxide is very similar as from TCE oxide. CO and $HCO_2H$ were both formed, as in the case with TCE oxide. In the case of TCE oxide, the yield of $HCO_2H$ was nearly equivalent to that of CO in this (Table 1) and previous work. However, here we recovered only 50% $HCO_2H$ relative to CO.[2] The yield of $Br_2CHCO_2H$ was clearly higher than that of $Cl_2CHCO_2H$ recovered here in the parallel experiment or in any of the previous studies in this laboratory (*19, 20*).

**Microsomal Oxidation of TBE.** Liver microsomes (prepared from rats treated with phenobarbital) were used as a source of P450 and incubated with TBE (Figure 1). $Br_2CHCO_2H$, detected as the *p*-nitrophenacyl ester, was the major product detected (Figure 1). $Br_3CCHO$ was formed, detected, and measured as the 2,4-dinitrophenylhydrazone. The rates of formation of these products are much lower than for formation of $Cl_3CCHO$ (major and other products, $Cl_2CHCO_2H$, not detected) from TCE measured under similar conditions (*19*) and less than that of $Cl_3CCO_2H$ formed from PCE (*21*).

A benzene extract of an incubation containing a higher concentration of microsomal protein yielded a 4-(4-nitrobenzyl)pyridine adduct, as judged by the characteristic $\lambda_{max}$ of the purple adduct observed in the presence of $(C_2H_5)_3N$ under nonaqueous conditions (*19*). However, under the conditions used in the assays of Figure 1, the concentration of TBE oxide formed (measured over 15−150 s) was <1 $\mu$M (corresponding to <0.01 $A_{520}$ in the assays).

**Reaction of TBE Oxide with Lys.** The reactions of TBE oxide with free Lys in solution yielded a mixture of acylated Lys derivatives (Figure 2)(plus $Br_2CHCO_2H$ resulting from hydrolysis of TBE oxide). The major Lys products were the N2 derivatives, consistent with the lower p$K_a$ of the Lys N2 nitrogen relative to the N6 atom (*42*). The amount of the dibromoacetyl product was ~2× greater than that of the formyl.

**Reaction of TBE Oxide with Albumin.** TBE oxide was reacted with bovine serum albumin, and the Lys adducts were analyzed (HPLC/MS) after proteinase K digestion and derivatization with phenylisothiocyanate (Figure 3). In the protein, only the N6 amino groups can be modified (barring the possibility of an N-terminal Lys N2 amino group in a protein). The amount of $N^6$-dibromoacetylLys was 2−3× greater than that of $N^6$-formylLys.

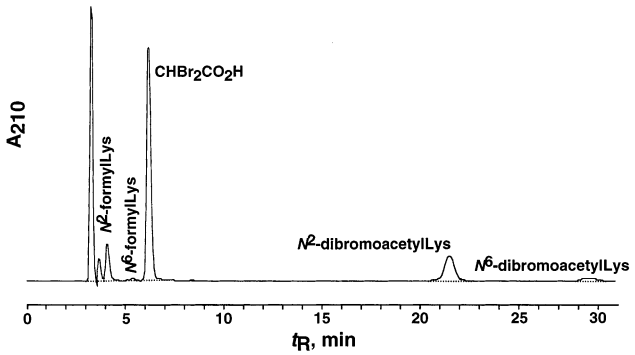



**Figure 2.** Products of reaction of TBE oxide with Lys. TBE oxide and Lys (100 mM each) were mixed in $H_2O$, and the reaction proceeded at 0 °C for 30 min. An aliquot of the reaction was diluted with the HPLC solvent and analyzed by HPLC as described under Experimental Procedures. The identities of the peaks were identified by co-chromatography with standard reference materials and HPLC/MS in a similar chromatography system. The first two peaks (near void volume) are unidentified.

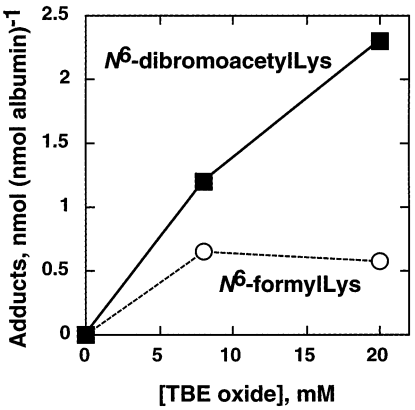



**Figure 3.** Formation of Lys adducts in bovine serum albumin modified with TBE oxide. Reactions were done at 0 °C for 30 min in 250 mM potassium MOPS buffer (pH 7.4) with bovine serum albumin (4 mg $mL^{-1}$) and the indicated concentrations of TBE oxide. Lys adducts were quantified (followed proteinase K digestion and derivatization with phenylisothiocyanate) using HPLC/MS. $N^5$-FormylOrn and $N^5$-dibromoacetylOrn were used to generate internal standard curves for $N^6$-formylLys and $N^6$-dibromoacetylLys. Results are shown for the formation of $N^6$-dibromoacetylLys (■) and $N^6$-formylLys (○).

## Discussion

A central focus of the work described in this report was the examination of the halide order in reactions of polyhalooxiranes. Specifically, reactions involving TBE oxide were compared to those with TCE oxide. The results, along with previous work, support some relatively general hypotheses regarding the reactions of chlorinated and brominated epoxides.

The results obtained with NADPH-fortified microsomal incubations of TBE contrast with those with TCE, with $Br_3CCHO$ as a minor product and $Br_2CHCO_2H$ as a major one (Figure 1). The rate of oxidation of TBE was much less than that of TCE (in liver microsomes prepared from

[2] An error of ±5% is estimated to be associated with the precision of measurement of both CO and $HCO_2H$. However, accurate comparison of each relative to each other is probably more difficult due to the inherent error in the calibration of each assay. In the present experiments with TCE oxide (Table 1), the CO:$HCO_2H$ ratio was nearly unity. However, in previous work, the ratio has varied from 1.4 to 0.6 (*19, 20*).

phenobarbital-treated rats) (*19*), as judged by the rates of formation of the characterized products. It is of interest to note that the rate of oxidation of TBE to $Br_3CCHO$ (and $HCO_2H$) was reported to be ~1% that of TCE for the bacterial enzyme methane monooxygenase (*32*). A possible explanation is that the bulkier bromine atoms restrict access of the (FeO complex of the) oxygenases to the olefin. In the case of TCE (*19*), we were able to measure steady-state levels of the epoxide in microsomal incubations and found that these were kinetically inconsistent with an obligatory role of TCE epoxide in the formation of $Cl_3CCHO$ (*19*). With TBE such an argument cannot be presented because of the lower rates of formation of products (Figure 1) plus the higher rate of hydrolysis of TBE oxide (Table 1). Thus, although we were only able to provide qualitative evidence for the microsomal oxidation of TBE to TBE oxide, we could not quantify it (below a level of 1 $\mu$M), and the possibility that it is an intermediate in the formation of $Br_2CHCO_2H$ cannot be dismissed. However, TBE oxide did not form $Br_3CCHO$, and the enzymatic oxidation of TBE to $Br_3CCHO$ is attributed to pathway **a** in Scheme 1. $Br_2CHCO_2H$ could be formed by pathway **c** or **d** or by hydrolysis of the epoxide.

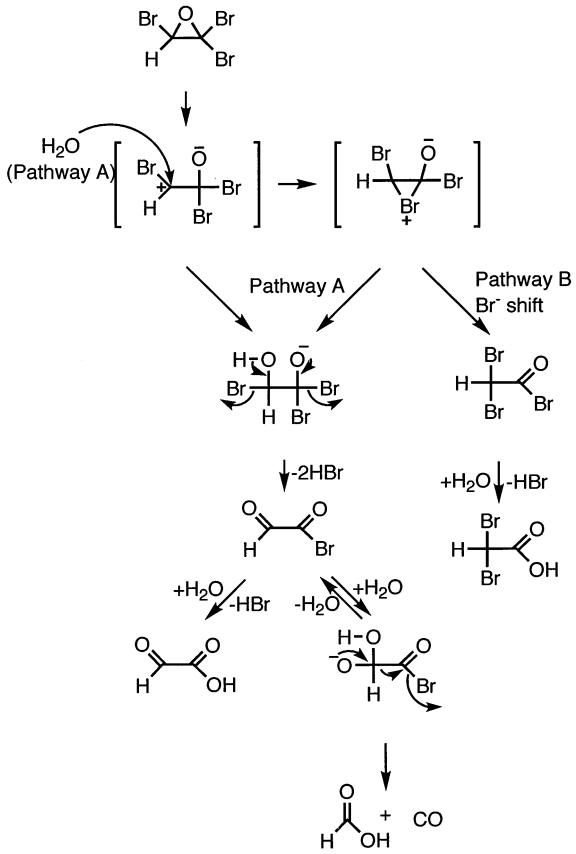
TBE oxide was synthesized in this work, apparently for the first time. The same method we employed for the synthesis of TCE oxide (*20*, *36*) was used, and the spectral and other properties are in order with those expected for such a halooxirane. TBE oxide hydrolyzed ~3× faster in $H_2O$ than did TCE oxide (Table 1). The effect of substitution of bromine for chlorine was similar to that seen with VC and VB (*17*, *29*). The pattern of hydrolysis products seen with TBE oxide was shifted from 1-carbon products to $Br_2CHCO_2H$, relative to TCE oxide, as judged by a direct comparison to work done either with TCE oxide in parallel experiments or with previous analyses in this laboratory (*19*, *20*).[2] The results reflect some error in the difficulty of making several difficult measurements. The fraction of the total products recovered as $X_2CHCO_2H$ (X = halogen) was 27% in the case of TCE oxide and 62% in the case of TBE oxide, with normalization for the number of carbons (Table 1). The $HCO_2H$ production was decreased relative to CO, although the difference may reflect some error in the CO measurements.[2]

The shift from 1-carbon products to $Br_2CHCO_2H$ associated with substitution of bromine for chlorine appears to be consistent with the scheme developed earlier (Scheme 2) for TCE oxide on the basis of $^{18}O$ and $^{2}H$ labeling patterns (*20*). Pathway B of Scheme 2 may be facilitated due to the properties of bromide as a leaving group, yielding more $Br_2CHCO_2H$.

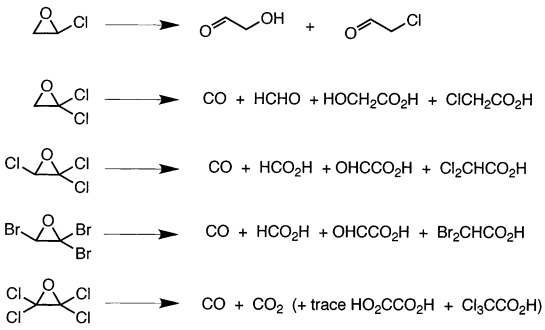
The patterns of Lys adducts are also shifted to a predominance of dibromoacetyl adducts relative to TCE oxide (Figures 2 and 3). The change is most clearly seen in the case of albumin, where the $N^6$-dihaloacetylLys:$N^6$-formylLys ratio is shifted from the value of ~0.1 observed for TCE oxide (*23*) to 2−3 (Figure 3).

This pathway can be generalized for other halooxiranes, at least for the *gem*-substituted ones. At very low pH values, hydrolysis of TCE and VDC oxides is dominated by formation of 2-carbon carboxylic acids (*18*, *19*). However, throughout most of the pH range, the composition of TCE oxide products is relatively invariant, when phosphate trapping is avoided (*20*, *21*). At neutral to high

**Scheme 2. Proposed Mechanism of Hydrolysis of TBE Oxide (*20*)**



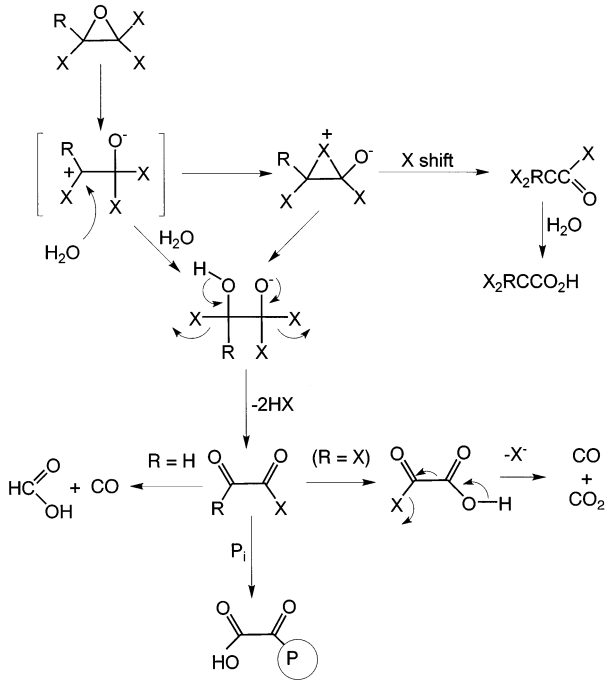

**Scheme 3. Hydrolysis Products of Halooxiranes**




pH, C−C bond scission is a dominant reaction. The major 1-carbon products obtained with the haloxiranes are shown in Scheme 3. CO is a product in all cases and can be rationalized with Scheme 4, generalized to accommodate the observations with tri- and tetrahalooxiranes (*18*−*21*). Thus we propose that the *gem*-dihalo-substituted carbon yields CO and that the valence of the second carbon dictates the course of the other product and determines its oxidation state, yielding HCHO, $HCO_2H$, or $CO_2$. The leaving group ability of the halogen can alter the balance between C−C scission and halide migration (e.g., see Table 1).

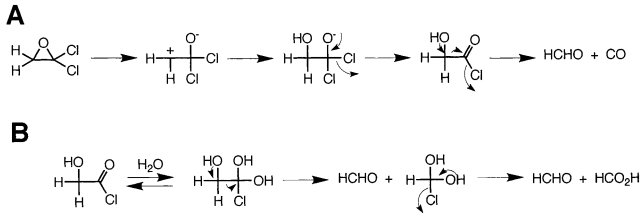
VDC oxide requires some extra explanation because both HCHO and $HCO_2H$ are produced, along with CO (*18*). In terms of oxidation state, CO and $HCO_2H$ are equivalent and in one sense differ only in their hydration. We previously proposed formyl chloride as a precursor of $HCO_2H$ (*19*) and subsequently rejected it as an intermediate (*20*). Formyl chloride is generally dehydro-

**Scheme 4. Proposed General Mechanism of Hydrolysis of Tri- and Tetra-halooxiranes[a]**




[a] $R_1$ = H or X (halogen); $P_i$ = phosphate ion. See (*20*, *21*).

**Scheme 5. Schemes for Hydrolysis of VDC Oxide To Explain Observed Products (*18*): (A) HCHO + CO; (B) HCHO + $HCO_2H$**

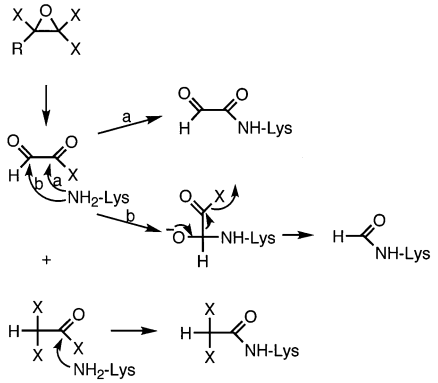



halogenated to CO except at high pH (>13) (*43*). Further, the isotopic labeling patterns we found with TCE oxide rejected this possibility (*20*). An explanation for the hydrolysis of VDC oxide is presented in Scheme 5. In part A, the formation of CO and HCHO is explained by the general mechanism presented in Schemes 2−4. A reversible hydration step is proposed in part B, yielding HCHO and $HCO_2H$.

The above considerations apply to the *gem*-dihalooxiranes discussed here and should apply to others. In the case of monohalogenated oxiranes (from VC and VB), the principles do not appear to be operative. CO has not been detected as a product, and hydrolysis appears to involve simple halide migration (*17*). In the context of the enzymatic oxidations, we have no proof that a 1,2-shift occurs with a P450−VC (or −VB) intermediate but cannot rule this out. Our current view is that the paradigm of Schemes 2 and 4 will only apply to *gem*-dihalooxiranes such as VDC, TCE, TBE, and PCE.

The reactions of TBE oxide with Lys are assumed to be similar to those previously defined by isotopic labeling with TCE oxide, yielding $N^6$-formyl- and $N^6$-dihaloacetylLys. The increased fraction of dihaloacetyl adducts with TBE oxide (relative to *N*-formyl adducts, more abundant with TCE oxide) is postulated to reflect the concentrations of the individual acylhalides rather than

**Scheme 6. Proposed General Mechanism of Reaction of Halogenated Epoxides with Lys (*20*)**




their reactivity (Scheme 6) (*20*). One issue that first surfaced in our first work with TCE (*19*) is the reaction of intermediates with phosphate (Scheme 5). The presence of phosphate in aqueous systems had the effect of diminishing the production of some products of TCE oxide (*19*), VDC oxide (*18*), and PCE oxide (*21*). The effect was explained in the case of PCE oxide, where oxalyl phosphate was identified in 0.1 M phosphate reactions (*21*). Oxalyl phosphate is not stable indefinitely but slowly hydrolyzes to oxalic acid, without reacting with nucleophiles such as Lys (*21*). We have not identified any organic phosphates generated from VDC oxide or TCE oxide but presume that the reactions are similar; i.e., one might expect to find products such as glyoxyl phosphate from TCE oxide or TBE oxide (Schemes 2 and 4). These reactions with phosphate should be considered experimental artifacts, and phosphate and other potential nucleophilic buffers should be avoided in experiments with these halooxiranes. The relevance of the phosphate reaction to reactions with the phosphate groups of nucleic acids is not clear. In the case of TCE, we have shown that unstable products are formed in the reaction of TCE oxide with oligonucleotides, but the possibility that the reaction is with phosphates instead of the nucleic acid bases has not been addressed (*30*).

In conclusion, we have extended studies of halooxiranes to TBE oxide, an apparent oxidation product of TBE. We considered the halide order in the context of several previous studies with chlorooxiranes and interpret reactivity in the context of a general mechanism first proposed for TCE oxide (*20*). The results are generally consistent with a set of general paradigms (Schemes 2 and 4) for *gem*-substituted dihalooxiranes but probably do not apply to monohalooxiranes. This paradigm leads to general predictions about the reactions of some vinyl halides. However, the point should be emphasized that generation of acyl halides in P450 enzymes in paths independent of halooxirane formation (Scheme 1) will alter the balance of reactive products, and therefore the halooxiranes cannot be considered the only factors in the prediction of reactivity of vinyl halides.

**Acknowledgment.** This work was supported in part by U.S. Public Health Service Grants R01 ES10546 and P30 ES00267. J.A.K. was supported in part by U.S. Public Health Service Training Grant T32 ES07028. We thank M. Voehler and L. Manier for technical assistance with several aspects of NMR and mass spectrometry, respectively.

## References


(1) United States Department of Health and Human Services (1993) Toxicological profile for tetrachloroethylene. Public Health Service, Agency for Toxic Substances and Disease Registry.

(2) Apfeldorf, R., and Infante, P. F. (1981) Review of epidemiologic study results of vinyl chloride-related compounds. *Environ. Health Perspect.* **41**, 221−226.

(3) Spirtas, R., and Kaminski, R. (1978) Angiosarcoma of the liver in vinyl chloride/poly(vinyl chloride) workers: 1977 update of the NIOSH register. *J. Occup. Med.* **20**, 427−429.

(4) National Cancer Institute (1977) Bioassay of tetrachloroethylene for possible carcinogenicity. *Natl. Toxicol. Program Tech. Rep.*, 77−813.

(5) National Cancer Institute (1986) Carcinogenesis bioassay of tetrachloroethylene. *Natl. Toxicol. Program Tech. Rep.* **232**.

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

(7) Haley, T. J. (1975) Vinylidene chloride: a review of the literature. *Clin. Toxicol.* **8**, 633−643.

(8) Szabo, S., Jaeger, R. J., Moslen, M. T., and Reynolds, E. S. (1977) Modification of 1,1-dichloroethylene hepatotoxicity by hypothyroidism. *Toxicol. Appl. Pharmacol.* **42**, 367−376.

(9) Quast, J. F., Humiston, C. G., Wade, C. E., Ballard, J., Beyer, J. E., Schwetz, R. W., and Norris, J. M. (1983) A chronic toxicity and oncogenicity study in rats and subchronic toxicity study in dogs on ingested vinylidene chloride. *Fundam. Appl. Toxicol.* **3**, 55−62.

(10) Jones, R. B., and Mackrodt, W. C. (1983) Structure−genotoxicity relationship for aliphatic epoxides. *Biochem. Pharmacol.* **32**, 2359−2362.

(11) Jacobson-Kram, D. (1986) The reproductive Effects Assessment Group's review of the mutagenicity of vinylidene chloride. *Environ. Mutagen.* **8**, 161−169.

(12) Dekant, W., Vamvakas, S., Koob, M., Köchling, A., Kanhai, W., Müller, D., and Henschler, D. (1990) A mechanism of haloalkene-induced renal carcinogenesis. *Environ. Health Perspect.* **88**, 107−110.

(13) Anders, M. W., Dekant, W., and Vamvakas, S. (1992) Glutathione-dependent toxicity. *Xenobiotica* **22**, 1135−1145.

(14) 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.

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

(16) Guengerich, F. P., and Watanabe, P. G. (1979) Metabolism of [$^{14}$C]- and [$^{36}$Cl]-labeled vinyl chloride in vivo and in vitro. *Biochem. Pharmacol.* **28**, 589−596.

(17) Guengerich, F. P., Crawford, W. M., Jr., and Watanabe, P. G. (1979) Activation of vinyl chloride to covalently bound metabolites: roles of 2-chloroethylene oxide and 2-chloroacetaldehyde. *Biochemistry* **18**, 5177−5182.

(18) 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.

(19) 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.

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

(21) Yoshioka, T., Krauser, J. A., and Guengerich, F. P. (2002) Tetrachloroethylene oxide: hydrolytic products and reactions with phosphate and lysine. *Chem. Res. Toxicol.* **15**, 1096−1105.

(22) Miller, R. E., and Guengerich, F. P. (1983) Metabolism of trichloroethylene in isolated hepatocytes, microsomes, and reconstituted enzyme systems containing cytochrome P-450. *Cancer Res.* **43**, 1145−1152.

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

(24) Benya, T. J., Busey, W. M., Dorato, M. A., and Berteau, P. E. (1982) Inhalation carcinogenicity bioassay of vinyl bromide in rats. *Toxicol. Appl. Pharmacol.* **64**, 367−379.

(25) Ottenwälder, H., Laib, R. J., and Bolt, H. M. (1979) Alkylation of RNA by vinyl bromide metabolites in vitro and in vivo. *Arch. Toxicol.* **41**, 279−286.

(26) Ottenwälder, H., and Bolt, H. M. (1980) Metabolic activation of vinyl chloride and vinyl bromide by isolated hepatocytes and hepatic sinusoidal cells. *J. Environ. Pathol. Toxicol.* **4**, 411−417.

(27) Bolt, H. M., Filser, J. G., and Hinderer, R. K. (1978) Rat liver microsomal uptake and irreversible protein binding of [1,2-$^{14}$C]-vinyl bromide. *Toxicol. Appl. Pharmacol.* **44**, 481−489.

(28) Barbin, A., Brésil, H., Croisy, A., Jacquignon, P., Malaveille, C., Montesano, R., and Bartsch, H. (1975) Liver-microsome-mediated formation of alkylating agents from vinyl bromide and vinyl chloride. *Biochem. Biophys. Res. Commun.* **67**, 596−603.

(29) Guengerich, F. P., Mason, P. S., Stott, W. T., Fox, T. R., and Watanabe, P. G. (1981) Roles of 2-haloethylene oxides and 2-haloacetaldehydes derived from vinyl bromide and vinyl chloride in irreversible binding to protein and DNA. *Cancer Res.* **41**, 4391−4398.

(30) 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.

(31) Shakhnazaryan, G. M. (1974) Molecular rearrangements. XI. Factors influencing the oxidation of tribromoethylene to dibromoacetic acid during the oxidation of peracetic acid. *Arm. Khim. Zh.* **27**, 177−181.

(32) Fox, B. G., Borneman, J. G., Wackett, L. P., and Lipscomb, J. D. (1990) Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. *Biochemistry* **29**, 6419−6427.

(33) Marshall, R. A., Harper, D. B., McRoberts, W. C., and Dring, M. J. (1999) Volatile bromocarbons produced by Falkenbergia stages of *Asparagopsis* spp. (Rhodophyta). *Limnol. Oceanogr.* **44**, 1348−1352.

(34) Kennedy, C. H., Cohen, K. B., Bechtold, W. E., Chang, I. Y., Eidson, A. F., Dahl, A. R., and Henderson, R. F. (1993) Effect of dose on the metabolism of 1,1,2,2-tetrabromoethane in F344/N rats after gavage administration. *Toxicol. Appl. Pharmacol.* **119**, 23−33.

(35) Canton, J. H., and Wegman, R. C. C. (1983) Studies on the toxicity of tribromoethene, cyclohexene and bromocyclohexane to different freshwater organisms. *Water Res.* **17**, 743−747.

(36) Iyer, R. S., and Harris, T. M. (1993) Preparation of aflatoxin $B_1$ 8,9-epoxide using *m*-chloroperbenzoic acid. *Chem. Res. Toxicol.* **6**, 313−316.

(37) 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.

(38) 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*.

(39) Miller, G. P., and Guengerich, F. P. (2001) Binding and oxidation of alkyl 4-nitrophenyl ethers by rabbit cytochrome P450 1A2: evidence for two binding sites. *Biochemistry* **40**, 7262−7272.

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

(41) Morozowich, W., and Douglas, S. L. (1975) Resolution of prostaglandin *p*-nitrophenacyl esters by liquid chromatography and conditions for rapid, quantitative *p*-nitrophenzylation. *Prostaglandins* **10**, 19−33.

(42) Mahler, H. R., and Cordes, E. H. (1971) *Biological Chemistry*, 2nd ed., 45, Harper & Row, New York.

(43) Dowideit, P., Mertens, R., and von Sonntag, C. (1996) Nonhydrolytic decay of formyl chloride into CO and HCl in aqueous solution. *J. Am. Chem. Soc.* **118**, 11288−11292.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