Olefin Oxidation by Cytochrome P-450: Evidence for Group Migration in Catalytic Intermediates Formed with Vinylidene Chloride and *trans*-1-Phenyl-1-butene†

Daniel C. Liebler and F. Peter Guengerich*

ABSTRACT: Oxidation of the carcinogen vinylidene chloride (VDC) by rat liver cytochrome P-450 (P-450) in microsomal and purified enzyme systems produced both $ClCH_2CO_2H$ and Cl_2CHCHO with concomitant suicide inactivation of three of the eight P-450 isozymes examined. The proposed intermediary role of VDC oxide in $ClCH_2CO_2H$ and Cl_2CHCHO production was evaluated by using chemical and kinetic studies. Aqueous decomposition of authentic VDC oxide, prepared by *m*-chloroperoxybenzoic acid oxidation of VDC and characterized by nuclear magnetic resonance (NMR) and mass spectrometry, failed to produce Cl_2CHCHO and yielded $ClCH_2CO_2H$ only at pH <2. Moreover, kinetic studies of VDC oxide production in the iodosobenzene-supported oxidation of VDC by P-450 did not support its proposed role as an obligate intermediate in the formation of $ClCH_2CO_2H$ and Cl_2CHCHO. [2,2-$^2H_2$]VDC was synthesized and found to be oxidized to $Cl_2C^2HCO_2H$ by microsomes supplemented with aldehyde dehydrogenase and NAD^+, indicating transfer of deuterium in the formation of the precursor $Cl_2C^2HC^2HO$. To test the hypothesis that the heme Fe(III) of P-450 acts as a Lewis acid in catalyzing the rearrangement of a transient epoxide intermediate to Cl_2CHCHO, the decomposition of VDC oxide in the presence of Fe(III) was studied. While $FeBr_3$-saturated $CHCl_3$ effected approximately 50% rearrangement of epoxide to Cl_2CHCHO, neither an equivalent concentration of (*meso*-tetraphenylporphyrinato)iron(III) chloride in $CHCl_3$ nor highly purified cytochrome P-450 in aqueous buffer produced Cl_2CHCHO from VDC oxide. Parallel studies using *trans*-1-phenylbutene 1,2-oxide, a stable model epoxide, indicated that, although binding of epoxide to P-450 did occur, ferric P-450 did not catalyze epoxide degradation. Oxidation of the parent olefin, *trans*-1-phenyl-1-butene, by purified P-450 yielded 1-phenyl-1-butanone and 1-phenyl-2-butanone, in addition to *trans*-1-phenyl-1-butene 1,2-oxide, although these two ketones were not produced via epoxide degradation during the experiment. The data collectively demonstrate that group migration occurs in catalytic intermediates leading to formation of carbonyl products. The intermediates may collapse to epoxides, although epoxides are not obligate precursors to carbonyl products.

The view that epoxides are the primary products of cytochrome P-450[1] mediated olefin biotransformation has gained widespread acceptance (Wislocki et al., 1980), and epoxides have been postulated as obligate intermediates in the oxidative biotransformation of several carcinogenic vinyl halide monomers (Bonse & Henschler, 1976). The oxidation of VDC to Cl_2CHCHO and $ClCH_2CO_2H$ via the intermediate VDC oxide had been proposed (Bartsch et al., 1975; Bonse et al., 1975; Costa & Ivanetich, 1982) although no direct evidence for the intermediacy of an epoxide has been presented. Attempts to synthesize this presumably unstable epoxide have been unsuccessful (Greim et al., 1975), and its chemistry and role in VDC oxidation have remained speculatory. Synthesis of trichloroethylene oxide (Kline & Van Duuren, 1977), a more stable homologue of VDC oxide, has permitted a less ambiguous evaluation of its role in trichloroethylene oxidation. Trichloroethylene oxide was found to be neither a chemically nor catalytically competent precursor to chloral (2,2,2-trichloroacetaldehyde), the principal P-450-derived metabolite of trichloroethylene (Miller & Guengerich, 1982). In an attempt to reconcile the formation of chloral with the observed hydrolytic decomposition of trichloroethylene oxide, Henschler et al. (1979) suggested that ferric P-450, acting as a Lewis acid, catalyzed the rearrangement of a transient epoxide intermediate to chloral. Analogous rearrangement of VDC oxide would be expected to yield Cl_2CHCHO. Ortiz de Montellano et al. (1982) studied the suicide inactivation of microsomal P-450 by several olefins and suggested that the alkylation of heme leading to inactivation took place following the stepwise formation of a catalytic intermediate. They proposed that such an intermediate could alkylate heme or produce epoxides depending on chemical characteristics of both the intermediate and the enzyme active site. An intermediate partitioning between product and inactivation could also conceivably partition between individual products.

In this paper we describe the synthesis of VDC oxide and its decomposition in aqueous and nonaqueous systems and in the presence of Lewis acids. We further report specificity among various P-450 isozymes toward production of Cl_2CHCHO and $ClCH_2CO_2H$ from VDC and toward suicide inactivation during VDC turnover. We also present evidence that TPB is oxidized directly to carbonyl-containing products that correspond formally to TPB oxide rearrangement products yet are not derived from TPB oxide. The data indicate that similar

† From the Departments of Pharmacology (D.C.L.) and Biochemistry (F.P.G.) and Center in Environmental Toxicology (D.C.L. and F.P.G.), Vanderbilt University School of Medicine, Nashville, Tennessee 37232. *Received May 13, 1983.* This work was supported in part by U.S. Public Health Service Grants ES 02205 and ES 00267. D.C.L. was supported in part by U.S. Public Health Service Training Grant GM 07628, and this work constitutes a portion of the material submitted for his doctoral thesis. F.P.G. is the recipient of U.S. Public Health Service Research Career Development Award ES 00041 and a Burroughs Wellcome Scholar in Toxicology.

[1] Abbreviations: P-450, rat liver microsomal cytochrome P-450; VDC, vinylidene chloride (1,1-dichloroethylene); TPB, *trans*-1-phenyl-1-butene; Fe^{III}TPPCl, (*meso*-tetraphenylporphyrinato)iron(III) chloride; HLPC, high-performance liquid chromatography; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; Tris–acetate, tris(hydroxymethyl)aminomethane acetate; EDTA, ethylenediaminetetraacetic acid; NMR, nuclear magnetic resonance. The description of and rationale for the nomenclature of individual forms of P-450 have been described previously (Guengerich et al., 1982).

0006-2960/83/0422-5482$01.50/0

catalytic intermediates serve as precursors to both carbonyl and epoxide products of VDC and TPB oxidation by P-450.

Experimental Procedures

Chemicals. VDC oxide was prepared fresh daily: 400 mg of *m*-chloroperoxybenzoic acid and 100 μL of VDC were heated in 2 mL of $CDCl_3$ for 3 h at 60 °C. The mixture was then distilled in vacuo (20 mmHg) into a dry ice condenser to yield 1–2 mL of a solution containing 25–35 mM VDC oxide in $CDCl_3$. The average yield was ~5% on the basis of the amount of peroxyacid used. The ^1H NMR spectrum of this solution showed a singlet at 3.2 ppm, in close agreement with previously reported shifts for methylene protons of α-halo epoxides (Miller & Guengerich, 1982; Walling & Fredricks, 1962). The signal decayed over several hours at 23 °C with the concomitant appearance of another singlet at 4.5 ppm, which corresponds to 2-chloroacetyl chloride. A 75-MHz ^{13}C NMR spectrum of the epoxide recorded at −50 °C showed a signal at 58.32 ppm, corresponding to the methylene carbon and in agreement with previously reported values for α-halo epoxides. Attempts to detect a ^{13}C resonance for the dichloromethylene carbon at high field were unsuccessful, perhaps due to the expected lack of efficient spin–lattice relaxation available to that nucleus and to the relatively low concentrations of VDC oxide formed in this synthesis. The electron-impact mass spectrum (direct probe analysis; 70 eV) exhibited an $M^+ - 1$ base peak at m/z 111; $M^+ - 1$ peaks are commonly observed with cyclic ethers not bearing alkyl substituents (Budzikiewicz et al., 1967).

Cl_2CHCHO was synthesized by treating dichloroacetyl chloride with 2 equiv of diethylamine in CH_2Cl_2 at 0 °C to afford the corresponding *N*,*N*-diethylamide. The resulting solid was treated with 1 equiv of diisobutylaluminum hydride in dry tetrahydrofuran at −78 °C, the excess reducing agent was quenched with CH_3OH, and the mixture was acidified with dilute H_2SO_4 at 0 °C. The mixture was extracted with three portions of ether, and the extracts were washed with brine and dried over anhydrous $MgSO_4$. After removal of the ether in vacuo, the oily residue was distilled to yield Cl_2CHCHO (bp 90–91 °C, 760 mmHg). The identity of the product was confirmed via ^1H NMR (5.97 ppm, d, 1 H; 9.41 ppm, d, 1 H), GC–MS [m/z 84, 100% ($M^+ - 28$); m/z 112, 45% (M^+)], and positive reaction with 2,4-dinitrophenylhydrazine.

Synthesis of [2,2-$^2H_2$]VDC involved $LiAl^2H_4$ reduction of dichloroacetyl chloride according to the procedure of Sroog & Woodburn (1963) to yield the intermediate 2,2-dichloro-[1,1-$^2H_2$]ethanol. The corresponding tosylate was prepared with 1.1 equiv of *p*-toluenesulfonyl chloride (Fieser & Fieser, 1967) and isolated as a pale yellow oil. Treatment of the tosylate with 1 equiv of diazabicyclo[5.4.0]undec-7-ene in toluene at 25 °C followed by distillation of the mixture afforded [2,2-$^2H_2$]VDC (bp 32 °C) of >99.9% isotopic purity as determined by ^1H NMR.

$Fe^{III}TPPCl$ was prepared according to Fleischer et al. (1971), and [1-^{3}H]TPB oxide (*trans*-8-ethyl[7-^{3}H]styrene 7,8-oxide) was prepared by Dr. Philip Wang (Wang et al., 1982).

Assays. VDC oxide was routinely quantitated by using the colorimetric reagent 4-(*p*-nitrobenzyl)pyridine (ϵ_{560} = 19.1 mM^{-1} cm^{-1}) (Guengerich et al., 1979). Steady-state levels of VDC oxide in reconstituted P-450 systems were measured by using the fluorometric assay of Nelis & Sinsheimer (1981). For VDC oxide decomposition studies, 300–600 nmol of VDC oxide was added to 1 mL of aqueous medium and mixed. After 5 min, the mixture was analyzed for products. Cl_2CHCHO and $ClCH_2CO_2H$ were quantitated by using electron capture GC (Tenax, 150 °C) of ether extracts of the incubation mixtures. $ClCH_2CO_2H$ was chromatographed as methyl chloroacetate following treatment of the ether extracts with CH_2N_2. Glycolic acid was quantitated colorimetrically following lyophilization of the incubation mixture (Calkins, 1943). HCHO was estimated colorimetrically (Nash, 1953; Cochin & Axelrod, 1959). HCO_2H was measured by HPLC as described elsewhere (Miller & Guengerich, 1982). CO was quantitated via head-space analysis of sealed incubation mixtures: following separation from other gases by GC (5A Molecular Sieve, 100 °C), CO was detected as CH_4 (flame ionization) following reduction on an in-series nickel-catalyst column (Porter & Vollman, 1962). Prior to electron-capture GC analysis of nonaqueous incubations in which VDC oxide was decomposed in $CHCl_3$, 1 mL of H_2O and 2 mL of hexane were added, and after being mixed, the organic layer was evaporated under a gentle stream of nitrogen to remove $CHCl_3$. Cl_2CHCHO, $ClCH_2CO_2H$, and Cl_2CHCO_2H formed from VDC in microsomal and reconstituted systems were quantitated on a Varian Model 3700 capillary gas chromatograph (Varian Associates, Walnut Creek, CA) operated in the splitless mode, equipped with a 50-m Carbowax 20 M capillary GC column and electron-capture detector.

Ferric iron dependent TPB oxide decomposition was monitored in incubations containing 16 nmol of [1-^{3}H]TPB oxide (4.2 mCi $mmol^{-1}$) and either 300 μM $P\text{-}450_{PB\text{-}B}$ (in a total volume of 50 μL of aqueous buffer) or varying concentrations of $FeBr_3$ or $Fe^{III}TPPCl$ (in 100 μL of $CHCl_3$). Aqueous incubations were extracted with 200 μL of hexane, and 10-μL aliquots were then analyzed by HPLC. Nonaqueous incubations were terminated by dilution with hexane, and aliquots were analyzed by HPLC. Unlabeled TPB oxide was added to all samples prior to HPLC on a Supelcosil LC-Si silica column eluted with hexane/tetrahydrofuran (99:1) at a flow rate of 1.0 mL min^{-1}. The eluate passed through a UV monitor (254 nm) and a Flo-One Model HP radioactive flow detector (Radiomatic Instruments, Tampa, FL) in Liposolve scintillation cocktail (Radiomatic Instruments) at a volume ratio of 1:1. The fraction of radioactivity remaining as TPB oxide was compared to that calculated for control incubations without iron.

Microsomal incubations involving [2,2-$^2H_2$]VDC contained 10 mg of liver microsomal protein (prepared from phenobarbital-induced rats) mL^{-1}, 50 mM [2,2-$^2H_2$]VDC, 1 mM $NADP^+$, 100 mM glucose 6-phosphate, 1 IU of glucose-6-phosphate dehydrogenase mL^{-1}, 1 mM NAD^+, and 100 mM potassium phosphate (pH 7.7) in a total volume of 2.0 mL at 37 °C. Incubations were terminated after 30 min by adding $ZnSO_4$ to a final concentration of 1% (w/v) and centrifuged at 3500 rpm for 5 min. The supernatants were acidified with H_2SO_4 and extracted with ether, and the extracts were analyzed by combined GC–MS. The instrument was equipped with a 1.6-m Tenax (60/80 mesh) column and operated in the chemical-ionization mode with CH_4 as the ionizing gas. Mass spectra were recorded with a Ribermag R10-10B GC–MS equipped with a Ribermag 1000 DS data system (Nermag, Inc., Santa Clara, CA).

Conversion of TPB to monooxygenated products was monitored following 60-min incubations of 10 mM TPB with 1 μM $P\text{-}450_{PB\text{-}B}$, 1 μM NADPH–cytochrome P-450 reductase, 74 μM L-α-dilauroyl-*sn*-glycero-3-phosphocholine, 0.5 mM $NADP^+$, 1 IU of glucose-6-phosphate dehydrogenase mL^{-1}, 100 mM glucose 6-phosphate, and 100 mM potassium phosphate (pH 7.7) in a total volume of 0.75 mL at 37 °C. Incubations were terminated with $ZnSO_4$ as described above and

Table I: Product Formation and Suicide Inactivation in VDC Oxidation by Purified Cytochrome P-450[a]

product [nmol min^{-1} (nmol of P-450)$^{-1}$]	P-450 isozyme							
	UT-A	PB-B	βNF-B	PB-C	PB-D	PB/PCN-E	UT-F	βNF/ISF-G
$ClCH_2COOH$	0.030	0.030	0.030	0.010	0.010	0.010	0.010	0.010
Cl_2CHCHO	0.170	0.030	<0.010	<0.010	<0.010	<0.010	<0.010	0.021
inactivation[b]	0.004	0.019	<0.002	<0.002	<0.002	<0.002	0.003	<0.002

[a] Incubations were carried out for 30 min at 37 °C. Incubations contained 0.72 μM P-450, 1 μM NADPH-cytochrome P-450 reductase, 74 μM L-α-dilauroyl-*sn*-glycero-3-phosphocholine, 5 mM VDC, 50 mM $NADP^+$, 1 unit of glucose-6-phosphate dehydrogenase mL^{-1}, 10 mM glucose 6-phosphate, and 100 mM potassium phosphate (pH 7.7) in a total volume of 0.75 mL. [b] P-450 destruction was monitored as described previously (Guengerich & Strickland, 1977).

Table II: Aqueous Decomposition of VDC Oxide[a]

medium	product [nmol (μmol of VDC oxide)$^{-1}$] [b]				
	$ClCH_2COOH$	$HOCH_2COOH$	HCHO	HCOOH	CO
2 N HCl	1130 ± 90	<25	<10	<25	<25
0.1 N HCl	645 ± 135	226 ± 19	<10	317 ± 65	<25
H_2O	<25	382 ± 48	64 ± 50	268 ± 33	<25
20 mM potassium phosphate, pH 7.7	<25	392 ± 37	235 ± 53	197 ± 73	458 ± 81
0.1 N NaOH	<25	73 ± 5	916 ± 37	235 ± 32	766 ± 33

[a] Incubations were carried out at 25 °C as described under Experimental Procedures. [b] Results are presented as means ± SD of three to five individual experiments.

centrifuged; the supernatants were extracted with ether at neutral pH. The extracts were analyzed by capillary GC–MS in a 50-m SE-30 WCOT capillary column with the spectrometer operating in the electron-impact mode at an ionizing voltage of 70 eV.

Rat liver microsomes, P-450s, and NADPH-P-450 reductase were prepared as described elsewhere (Guengerich et al., 1982). Protein concentrations were estimated as described by Lowry et al. (1951). P-450 concentrations were estimated by usng ferrous CO vs. ferrous difference spectra (Omura & Sato, 1964).

Results

Oxidation of VDC by Purified P-450s. Oxidation of VDC by P-450 isozymes purified in this laboratory (Guengerich et al., 1982) yielded $ClCH_2CO_2H$ and Cl_2CHCHO with concomitant suicide inactivation of three isozymes (Table I). All of the isozymes produced $ClCH_2CO_2H$ with relatively small variations in rate, whereas measurable levels of Cl_2CHCHO were produced by only three of the isozymes, P-$450_{UT\text{-}A}$, P-$450_{PB\text{-}B}$, and P-$450_{\beta NF/ISF\text{-}G}$. Of these P-$450_{UT\text{-}A}$ exhibited the greatest product selectivity, as its rate of Cl_2CHCHO formation was approximately 6 times that for $ClCH_2CO_2H$.

Of the eight isozymes, only P-$450_{UT\text{-}A}$, P-$450_{PB\text{-}B}$, and P-$450_{UT\text{-}F}$ underwent suicide inactivation at a detectable rate. P-$450_{PB\text{-}B}$ was inactivated at the highest rate, approximately 5 times faster than P-$450_{UT\text{-}A}$. P-$450_{UT\text{-}A}$ formed products (largely Cl_2CHCHO) at a rate roughly 50-fold greater than it underwent inactivation, while P-$450_{PB\text{-}B}$ formed considerably less Cl_2CHCHO and was inactivated more rapidly.

Aqueous Decomposition of VDC Oxide. VDC oxide was synthesized as described and characterized by ^1H and ^{13}C NMR, by mass spectrometry, and by reaction with the colorimetric reagent 4-(*p*-nitrobenzyl)pyridine. Neither VDC, its metabolites, nor any VDC oxide decomposition product interfered with detection of VDC oxide by this method.

Both the rate of VDC oxide decomposition and the resulting product distribution were sensitive to changes in pH. VDC oxide decomposed rapidly in neutral or basic solutions with a half-life of approximately 2 s; its decomposition rate in dilute HCl was roughly doubled. In acid, VDC oxide produced $ClCH_2CO_2H$ and glycolic acid with lower pH favoring the rearrangement product (Table II). No $ClCH_2CO_2H$ was produced at pH >3. In neutral and basic solutions, the formation of glycolic acid and the one-carbon products, formaldehyde, formate, and CO were favored. The pH dependence of formation of these products suggests that they arise via the hydration of VDC oxide and fragmentation of the resulting *vic*-diol (Miller & Guengerich, 1982). Cl_2CHCHO was not formed in any aqueous system tested.

Kinetic Evaluation of VDC Oxide as a Precursor to $ClCH_2CO_2H$ and Cl_2CHCHO. To test the hypothesis that VDC oxide is an obligate intermediate in the oxidation of VDC to stable metabolites, we applied the following two-step kinetic model used previously in this laboratory (Miller & Guengerich, 1982):

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

$$\text{VDC oxide} \xrightarrow{k_2} \text{metabolite(s)}$$

In this model, k_1 describes the zero-order formation of VDC oxide from saturating VDC concentrations, and k_2 describes the pseudo-first-order decomposition of VDC oxide to more stable metabolites. Half-life studies of VDC oxide decomposition yield estimates of k_2, while k_1 can be calculated from the following equation (Hess & Wurster, 1970):

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

where levels of metabolites are measured vs. time. This information can then be used to predict the levels of VDC oxide that, at any time, would be required to account for observed metabolite levels from the expression (Hess & Wurster, 1970):

$$[\text{VDC oxide}] = (k_1 k_2^{-1})[\text{P-450}](1 - e^{-k_2 t})$$

This expression was used to predict VDC oxide levels in the iodosobenzene-supported oxidation of VDC by purified P-$450_{PB\text{-}B}$. If VDC oxidation proceeded through an epoxide intermediate as predicted in the above model, then the time course of measured epoxide levels should match that calculated from observed metabolite levels.

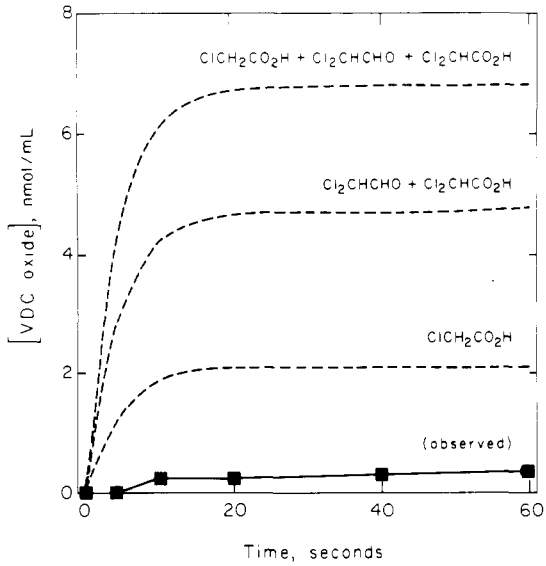

FIGURE 1: Time course of VDC oxide formation in the iodosobenzene-supported oxidation of VDC by P-450. 20 μM P-450$_{PB-B}$ was incubated with 50 mM VDC, 37 μM L-α-dilauroyl-*sn*-glycero-3-phosphocholine, 100 mM potassium phosphate (pH 7.7), and 5 mM iodosobenzene at 37 °C. VDC oxide levels (■) were measured as described under Experimental Procedures. Zero-order rate constants, k_1, describing VDC oxide formation were calculated by using k_1 = [metabolite][P-450]$^{-1}$ $(t + k_2^{-1}e^{-k_2t} - k_2^{-1})^{-1}$ from concentrations of $ClCH_2CO_2H$ or (Cl_2CHCHO plus Cl_2CHCO_2H) measured at 10 s and from a k_2 value of 0.231 s^{-1} determined under experimental conditions. Theoretical levels of VDC oxide vs. time were calculated by using [VDC oxide] = $k_1k_2^{-1}$[P-450]$(1 - e^{-k_2t})$ assuming contributions to k_1 by $ClCH_2CO_2H$, Cl_2CHCHO plus Cl_2CHCO_2H, or $ClCH_2CO_2H$ plus Cl_2CHCHO plus Cl_2CHCO_2H.

Measured levels of VDC oxide are depicted together with VDC oxide levels calculated from quantitation of metabolites vs. time in Figure 1. Separate curves are calculated on the basis of two major products, $ClCH_2CO_2H$ and Cl_2CHCHO, and for combined levels of both metabolites. A significant fraction of the Cl_2CHCHO produced in this system was oxidized nonenzymatically to Cl_2CHCO_2H in the presence of iodosobenzene; thus, the levels of Cl_2CHCHO are expressed as Cl_2CHCHO plus Cl_2CHCO_2H. Observed epoxide levels were 5-fold lower than those predicted from $ClCH_2CO_2H$, 20-fold lower than predicted from Cl_2CHCHO, and 30-fold lower than predicted from measured levels of both products. The kinetics of VDC oxide formation were not studied in the NADPH-supported system because the pyridine nucleotide interfered with the quantitation of low levels of VDC oxide by the assay used.

Deuterium Transfer in the Oxidation of [2,2-$^2H_2$]VDC. To confirm transfer of hydrogen in the microsomal oxidation of VDC to Cl_2CHCHO, we synthesized [2,2-$^2H_2$]VDC and incubated it with NADPH-fortified rat liver microsomes. Because the expected deuterated product, $Cl_2C^2HC^2HO$, undergoes rapid proton exchange with water, aldehyde dehydrogenase and NAD^+ were also added to the incubations to afford $Cl_2C^2HCO_2H$. Ether extracts of these incubations were treated with CH_2N_2, and the esters were analyzed by GC–MS. Chemical-ionization mass spectra of standard $Cl_2CHCO_2CH_3$ and the [2,2-$^2H_2$]VDC incubation product are shown in Figure 2. Both compounds had identical retention times, and both exhibit the characteristic 10:6:1 cluster of peaks at $M^+ + 1$, $M^+ + 3$, and $M^+ + 5$ due to the presence of ^{35}Cl and ^{37}Cl (Benyon, 1960). The [2,2-$^2H_2$]VDC incubation product peaks were shifted one mass unit higher than those of the standard, indicating the incorporation of deuterium at carbon 2 and confirming that hydrogen transfer occurs in

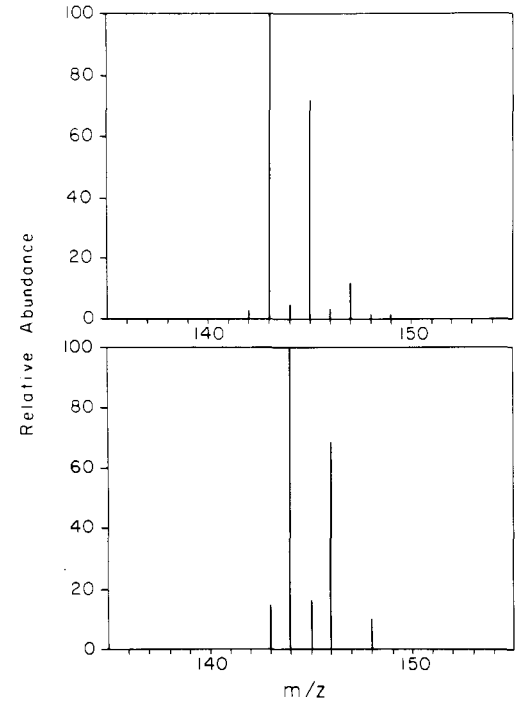

FIGURE 2: Chemical-ionization mass spectra of authentic $Cl_2CHC-O_2CH_3$ (upper) and CH_2N_2-treated $Cl_2C^2HCO_2H$ recovered from microsomal incubations containing [2,2-$^2H_2$]VDC and aldehyde dehydrogenase plus NAD^+ (lower). Incubation conditions are described under Experimental Procedures.

Table III: Rearrangement of VDC Oxide to Cl_2CHCHO in the Presence of Fe(III)[a]

medium	VDC oxide conversion to Cl_2CHCHO (%)
53 mM $FeBr_3/CHCl_3$ (saturated)	46
26 mM $FeBr_3/CHCl_3$	9
2.6 mM $FeBr_3/CHCl_3$	<5
76 mM $Fe^{III}TPPCl/CHCl_3$ (saturated)	<5
300 μM P-450/buffer	<5

[a] VDC oxide (72 μM) was incubated in each system for 24 h at 25 °C. *Dichloroacetaldehyde* was assayed as described under Experimental Procedures.

the oxidation of VDC to Cl_2CHCHO by P-450. Cl_2CHCO_2H recovered from incubations with [2,2-$^2H_2$]VDC contained deuterium in 85% isotopic excess. The k_H/k_D ratio for the microsomal oxidation of VDC and [2,2-$^2H_2$]VDC was 1.4 ± 0.3, indicating that hydrogen transfer in the formation of Cl_2CHCHO was not rate limiting.

Effects of Fe(III) on VDC Oxide Decomposition. We examined the hypothesis that the ferric heme iron of P-450 acts as a Lewis acid in catalyzing the rearrangement of a transient VDC oxide intermediate to Cl_2CHCHO within the hydrophobic active site of the enzyme. By a comparison of the activity of Fe(III) salts with that of a synthetic Fe(III) porphyrin complex in an organic solvent and with purified P-450 in buffer, the effect of complexation of Fe(III) on its ability to act as a Lewis acid was deduced.

When VDC oxide was incubated with a 700-fold molar excess of $FeBr_3$ (53 mM) in $CHCl_3$, approximately half of the epoxide was converted to Cl_2CHCHO (Table III). If the $FeBr_3$ concentration was reduced by half, only 9% conversion was detected. A further 10-fold reduction of the $FeBr_3$ concentration abolished any detectable conversion. Incubation of a 1000-fold molar excess of $Fe^{III}TPPCl$ with VDC oxide failed to effect a detectable conversion to Cl_2CHCHO, as did

Table IV: Degradation of [1-^{3}H]TPB Oxide in the Presence of Fe(III)[a]

solvent	catalyst	percent radioactivity remaining as TPB oxide
$CHCl_3$		77
	0.027 mM $FeBr_3$	30
	0.27 mM $FeBr_3$	25
	27 mM $FeBr_3$	4
	76 mM $Fe^{III}TPPCl$	81
10 mM Tris-acetate (pH 7.4), 1 mM EDTA, 20% glycerol[b]		74
	300 μM P-450$_{PB-B}$	87

[a] [1-^{3}H]TPB oxide (16 nmol) was incubated in 300 μL of the indicated media for 24 h at 25 °C. After each incubation, the remaining labeled epoxide was quantitated by HPLC as described under Experimental Procedures. [b] Aqueous incubations were terminated after 30 min.

a 4-fold excess of purified P-450 in aqueous buffer. These data strongly suggest that complexation of Fe(III) in a porphyrin ring significantly diminishes its ability to act as a Lewis acid relative to uncomplexed Fe(III).

Effects of Fe(III) on Decomposition of TPB Oxide. In order to further assess the ability of heme Fe(III) in P-450 to catalyze epoxide degradation, we studied the effect of Fe(III) on the decomposition of TPB oxide. This relatively water-stable epoxide bound to microsomal P-450 as judged by perturbation of the Soret spectrum: in difference spectroscopy, a peak was formed at 389 nm and a trough at 422 nm; the binding constant determined by this method was 2.0 mM. Addition of $FeBr_3$ to [1-^{3}H]TPB oxide in $CHCl_3$ accelerated the decomposition of the epoxide. Incubation with 27 mM $FeBr_3$ catalyzed almost complete degradation within 24 h (Table IV). Substitution of 76 mM $Fe^{III}TPPCl$ abolished this effect; there was essentially no epoxide degradation in the presence of the ferric porphyrin complex. When [1-^{3}H]TPB oxide was incubated for 30 min in the presence of 300 μM P-450$_{PB-B}$, there was no loss of epoxide compared with parallel incubations containing buffer only. Similar results were obtained for 24-h incubations although approximately 70% of the epoxide had hydrolyzed during those incubations.

Rearrangement of [1-^{3}H]TPB oxide in the Fe(III)/$CHCl_3$ systems produced 1-phenyl[1-^{3}H]butan-2-one and 1-phenyl-[2-^{3}H]butan-1-one, which eluted close to [1-^{3}H]TPB oxide in the HPLC system used.[2] As other data suggest the oxidation of VDC directly to $ClCH_2CO_2H$ and Cl_2CHCHO via a non-epoxide pathway, we sought to determine whether the two ketone rearrangement products of TPB oxide were formed directly from TPB by P-450. Direct formation of these products would lend greater validity to the use of TPB oxide as a model for evaluating Fe(III)/heme–epoxide interaction in systems where direct formation of carbonyl metabolites from olefins is considered.

We incubated TPB with purified P-450, NADPH–P-450 reductase, phospholipid, O_2, and NADPH. Ether extracts of these incubations were analyzed by capillary GC–MS with selected ion monitoring to detect monooxygenated products.

[2] The [^{3}H]TPB oxide used in these experiments contained approximately 20% 1-phenyl[1-^{3}H]butan-2-one and 1-phenyl[2-^{3}H]butan-1-one, which accumulated slowly during storage. Thus, under conditions where [^{3}H]TPB oxide was not degraded, approximately 80% of the radiolabel eluted as [^{3}H]TPB oxide.

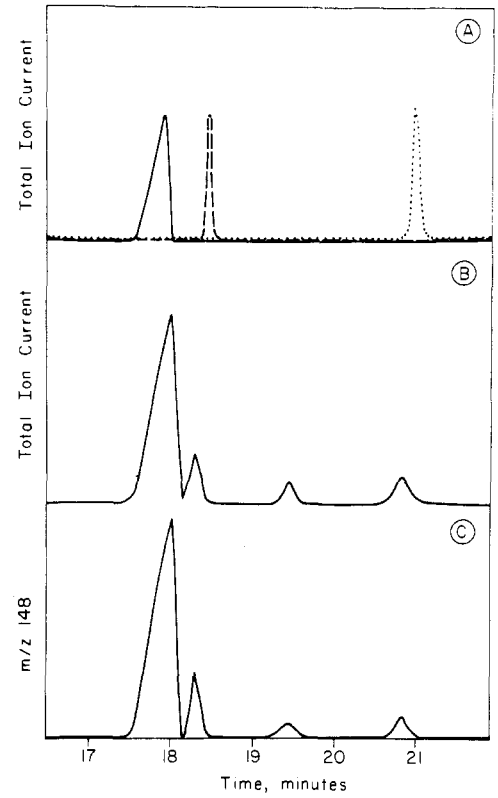

FIGURE 3: Capillary GC–MS separation of monooxygenated TPB metabolites. TPB was incubated with P-450, NADPH–P-450 reductase, phospholipid, NADPH, and O_2 and prepared for GC–MS analysis as described under Experimental Procedures. Total ion current for authentic TPB oxide (—), 1-phenyl-2-butanone (– –), and 1-phenyl-1-butanone (···) (A) is compared with that for TPB metabolites (B) and ion-current at m/z 148 for TPB metabolites (C). The region between 16.5 and 22.0 min is shown for each chromatogram.

TPB was converted to several monooxygenated products in the reconstituted system (Figure 3). Although TPB oxide was the major product formed, monooxygenated products with retention times and mass spectra identical with those of 1-phenyl-1-butanone and 1-phenyl-2-butanone were also produced (Figures 3 and 4). The two ketones were formed in nearly equal amounts, approximately 10 to 15% of epoxide levels. TPB oxide underwent no significant degradation in the presence of P-450 (vide supra). Further GC–MS analysis of the incubation extracts indicated the formation of at least two alcohols in TPB oxidation. Two of these products were tentatively identified as 1-phenyl-2-buten-1-ol [m/z 148 (2%), 120 (10%), 107 (100%), 91 (50%), 79 (88%), 77 (40%)] and 4-phenyl-3-buten-2-ol [m/z 148 (70%), 133 (38%), 105 (100%), 91 (79%), 77 (48%)].

Discussion

Progress in the study of olefin oxidation by P-450 has been hampered by a lack of information regarding the chemistry of many of the presumed intermediate epoxides, especially VDC oxide. Authentic VDC oxide, synthesized as described in this paper and characterized by both NMR and mass spectrometry, is a highly labile compound that is particularly sensitive to hydrolytic decomposition. Although rearrangement of VDC oxide to $ClCH_2CO_2H$ took place readily in organic solvents, hydrolysis was the only significant pathway for its degradation at physiological pH (Table II). The predominance of two-carbon products in acid vs. one-carbon products in neutral and basic solutions suggests two general pathways for the decomposition of VDC oxide. In acid, protonation and ring opening are rapid and may be followed by chloride mi-

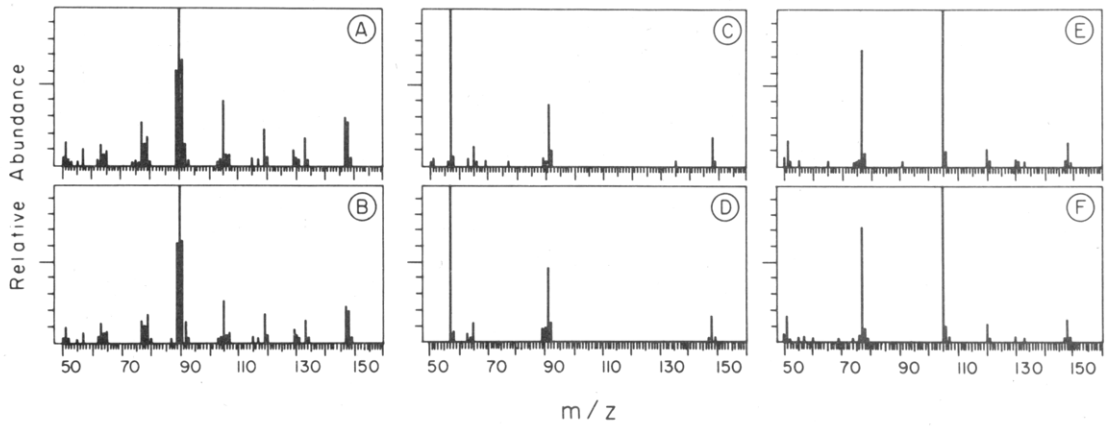

FIGURE 4: Mass spectra of monooxygenated TPB metabolites. Mass spectra of (A) authentic TPB oxide and (B) TPB metabolite eluting at 17.8 min in Figure 3; (C) authentic 1-phenyl-2-butanone and (D) TPB metabolite eluting at 18.3 min; (E) authentic 1-phenyl-1-butanone and (F) TPB metabolite eluting at 20.8 min.

gration or hydrolysis of the resulting cationic intermediate. Alternatively, nucleophilic attack by water in neutral or basic solutions may yield a *vic*-diol that fragments to one-carbon products. No $ClCH_2CO_2H$ was produced by VDC oxide above pH 2, suggesting that the rate of ring opening and rearrangement is significantly slower than that of hydrolysis under milder conditions.

Low levels of VDC oxide were formed in the iodosobenzene-supported oxidation of VDC by P-450 (Figure 1), and accumulation of two of its hydrolysis products, formaldehyde and CO, was monitored during VDC oxidation by microsomal preparations, suggesting that VDC oxide was also formed in the NADPH-supported system (data not shown). The iodosobenzene-supported system produced observed epoxide levels that were at least an order of magnitude below those predicted from a kinetic model in which the epoxide is an obligate precursor to $ClCH_2CO_2H$ and Cl_2CHCHO. These data indicate that VDC oxide is not a catalytically competent intermediate in the oxidation of VDC to these products and suggest that they are formed via a pathway not requiring an epoxide intermediate.

Although VDC oxide undergoes rapid hydrolysis in an aqueous environment, we examined the hypothesis that rearrangement of a transiently formed epoxide intermediate within the hydrophobic active site of P-450 yields Cl_2CHCHO before hydrolysis of the epoxide can take place. The proposal that ferric P-450 acts as a Lewis acid in catalyzing the rearrangement of epoxides is based not on studies of P-450 heme iron but, rather, on the observation that high concentrations of free Fe(III) in an organic solvent effected a partial conversion of trichloroethylene oxide to chloral (Henschler et al., 1979). We also observed 50% conversion of VDC oxide to Cl_2CHCHO under similar conditions (Table III). However, even higher concentrations of a porphyrin-liganded Fe(III) complex failed to afford Cl_2CHCHO from VDC oxide, as did a 4-fold molar excess of purified P-450.

The conclusion that P-450 did not catalyze rearrangement in the latter experiment is made somewhat equivocal by the rapid hydrolysis of VDC oxide under the conditions used. Although release of epoxide into a lipophilic environment may retard its hydrolysis and thus facilitate its interaction with heme, the half-life of the epoxide is only slightly greater in microsomes (data not shown). To more directly evaluate the ability of P-450 heme Fe(III) to catalyze rearrangement, we selected TPB oxide for use as a model epoxide. This stable epoxide was used previously in this laboratory in the characterization of a cytosolic epoxide hydrolase (Wang et al., 1982). TPB oxide binds to P-450, eliciting a Soret difference spectrum, and undergoes very slow hydrolysis to form the corresponding glycol. Free Fe(III) catalyzed the degradation of [1-^{3}H]TPB oxide in $CHCl_3$ (Table IV), but neither Fe^{III}TPPCl nor purified P-450 degraded the epoxide.

The relative ability of Fe(III) to act as a formal electron-pair acceptor will dictate its ability to catalyze rearrangement. Ligation of iron by four basic nitrogens and an axial chloride in Fe^{III}TPPCl stabilizes Fe(III) through electron donation into vacant d orbitals, resulting in significantly decreased electrophilicity of iron. Incorporation of Fe(III) into P-450 heme would be expected to further reduce its tendency to function as an electron-pair acceptor due to added axial thiolate ligation. Sono & Dawson (1982) estimated that thiolate ligation in P-450-cam raised the K_D for a sixth ligand by up to 4 orders of magnitude compared to that of myoglobin, in which histidine functions as the fifth ligand. Moreover, half-wave potentials for reduction of ferric porphyrins in dimethylformamide were made more negative as electron-donating substituents were incorporated into the porphyrin π-system (Kadish & Larson, 1977), suggesting a decreased electron-accepting tendency for Fe(III). Our data clearly demonstrate that free and porphyrin-liganded Fe(III) differed significantly in their ability to act as Lewis acids and that predictions concerning P-450 heme catalyzed rearrangements on the basis of observations in free Fe(III) systems are inappropriate.

Selectivity between purified P-450 isozymes in the production of Cl_2CHCHO and suicide inactivation, but not $ClCH_2CO_2H$ production (Table I), implies that these products or processes do not require the intermediacy of VDC oxide. This observation, coupled with the results of the chemical studies of VDC oxide decomposition, and kinetic studies of epoxide formation provide compelling evidence that VDC oxide is neither a chemically nor a catalytically competent precursor to $ClCH_2CO_2H$ and Cl_2CHCHO in P-450-catalyzed VDC oxidation. Our results are consistent, however, with a stepwise oxidation of VDC as depicted in Figure 5. The initial abstraction of an electron from the π-bond of VDC by oxoiron (formally Fe^V=O) P-450 (White & Coon, 1980) to produce a radical intermediate may be followed by a second electron transfer and formation of a cationic intermediate. These intermediates may then partition between ring closure to form an epoxide, group transfer (Cl or H migration) with release of 2-chloroacetyl chloride or Cl_2CHCHO, or heme alkylation. The stereoelectronic topography of the enzyme active site may be expected to influence the partitioning of the intermediate, as individual isozymes vary in their ability to produce Cl_2-CHCHO or undergo inactivation (Table I). The recovery of $Cl_2C^2HCO_2H$ from aldehyde dehydrogenase supplemented

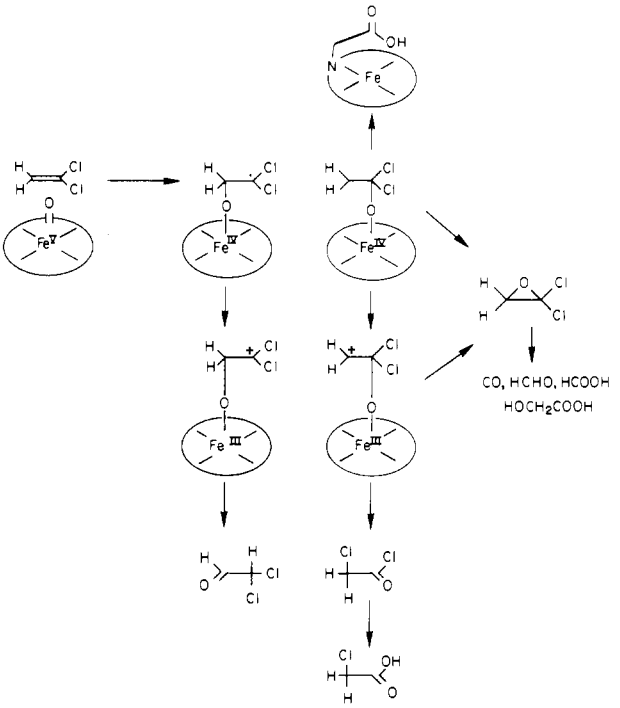

FIGURE 5: Stepwise oxidation of VDC by P-450. See text for discussion.

incubation of [2,2-$^2H_2$]VDC with microsomes clearly demonstrated that deuterium from C-2 of the substrate was transferred to the adjacent carbon in the formation of the product aldehyde. The inability of ferric P-450 to convert VDC oxide to Cl_2CHCHO effectively excludes the possibility that [2,2-$^2H_2$]VDC oxide was the precursor to the deuterium-labeled aldehyde but is consistent with a stepwise oxidation of [2,2-^2H]VDC with accompanying deuterium migration.

Structure determination of heme adducts formed during suicide inactivation of P-450 by vinylidene fluoride and other vinyl halides has indicated that the least substituted carbon of the olefin alkylates heme (Ortiz de Montellano et al., 1982). The intermediate giving rise to heme alkylation in Figure 5 would also produce $ClCH_2CO_2H$. Interestingly, P-$450_{UT\text{-}A}$ has a very high rate of product formation relative to that of heme destruction (Table I) and also produces a high ratio of Cl_2CHCHO to $ClCH_2CO_2H$. The intermediate orientation giving rise to Cl_2CHCHO is opposite that which gives rise to the identified heme adducts, although the possibility that the Cl_2CHCHO-producing orientation leads to heme alkylation cannot be discounted (Ortiz de Montellano et al., 1982).

The stepwise oxidation scheme depicted in Figure 5 is consistent with similar mechanisms proposed for oxidation of trichloroethylene (Miller & Guengerich, 1982) and for iodosobenzene-supported olefin oxidation by model metalloporphyrin complexes related to P-450 (Groves et al., 1979, 1980a). However, with the exception of the allylic cyclohexen-3-ol, epoxides were the only olefin oxidation products reported in the model systems (Groves et al., 1980b). Similar results have been reported for the oxidation of cyclohexene by purified rabbit liver P-$450_{LM\text{-}2}$ (White et al., 1979). As our data confirm that hydride migration occurred in the oxidation of VDC to Cl_2CHCHO, it was of interest to determine whether similar group transfer occurred in the oxidation of TPB. In addition to the major expected product, TPB oxide, oxidation of TPB also yielded significant amounts of 1-phenyl-1-butanone and 1-phenyl-2-butanone under conditions where essentially no TPB oxide was degraded. These results are consistent with stepwise oxidation of TPB to TPB oxide,

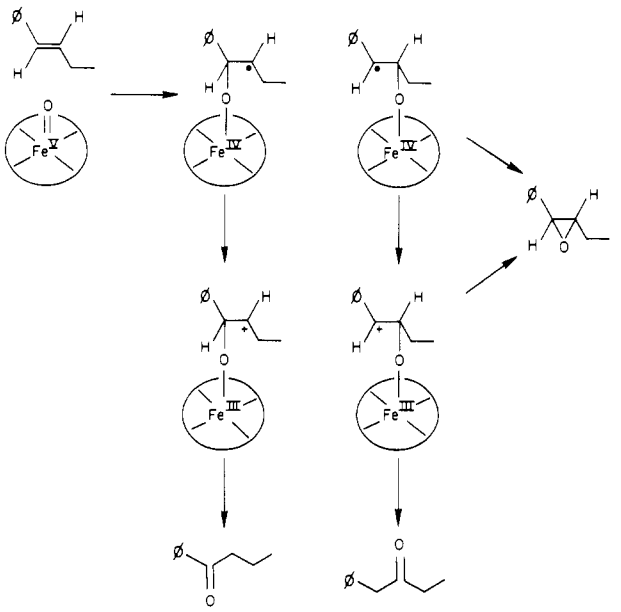

FIGURE 6: Stepwise oxidation of TPB by P-450. See text for discussion.

1-phenyl-1-butanone, and 1-phenyl-2-butanone according to the scheme depicted in Figure 6. As with VDC (Figure 5), sequential abstraction of electrons from the olefin π-bond would yield either of the two intermediates depicted. Both intermediates could then either migrate hydride to release a ketone or undergo ring closure to form an epoxide. We were unable to detect TPB-mediated suicide inactivation of P-450 (data not shown).

A number of examples of substituent migration associated with microsomal oxidation of aromatic ring systems to phenols have been reported and collectively termed the "NIH shift" (Daly et al., 1972). These migration products formally derive from rearrangements of the corresponding epoxides, as do the non-epoxide products of VDC and TPB. The data reported here provide compelling evidence that some group migrations that correspond formally to epoxidation/rearrangement are due instead to direct, stepwise oxidation of unsaturated carbon centers. This possibility was also addressed in a recent study of warfarin hydroxylation (Bush & Trager, 1982). Microsomal P-450 oxidized [7-^2H]warfarin to 7-hydroxywarfarin with a high degree of deuterium retention, prompting the authors to suggest that this formal meta-hydroxylation was neither a direct oxygen insertion nor the result of an arene oxide rearrangement. They concluded instead that a stepwise addition–rearrangement mechanism best explained their data. The results presented here lend considerable support to that suggestion. Stepwise oxidation involving sequential electron transfer and substituent migration may explain some heretofore baffling aspects of P-450-catalyzed oxidation of aromatic compounds as well as olefins.

The results presented argue strongly that group migraton occurs during the oxidation of unsaturated compounds, within an enzyme intermediate. The exact electron distribution of the intermediate is not precisely known. We have presented intermediates in which radicals and carbocations are localized (Figures 5 and 6). We prefer a homolytic cleavage of the olefin π-bond to form a radical intermediate as a first step. However, the intermediate may shift electrons to form a carbocation-type intermediate in some cases. Thus, transfer of hydride or halide ions would occur more readily than the corresponding radicals. However, we cannot presently determine the exact nature of the intermediate with certainty,

and resonance forms of the intermediate with C–O–C and Fe–C bond character may also contribute (Miller & Guengerich, 1982).

Acknowledgments

We thank Dr. T. L. Macdonald and Dr. R. E. Miller for their suggestions and their criticism of the manuscript. We also thank A. Slaughter for his assistance with mass spectral measurements and Dr. C. M. Watkins of the University of Alabama (Birmingham) for access to the 75-MHz NMR spectrometer.

Registry No. VDC, 75-35-4; P-450, 9035-51-2; TPB, 1005-64-7; 1-phenyl-1-butanone, 495-40-9; 1-phenyl-2-butanone, 1007-32-5; *trans*-1-phenyl-1-butene 1,2-oxide, 69140-50-7; dichloroacetyl chloride, 79-36-7; [2,2-$^2H_2$]VDC, 22280-73-5; 2,2-dichloro[1,1-$^2H_2$]ethanol, 55289-93-5; VDC oxide, 68226-83-5; glycolic acid, 79-14-1; $ClCH_2CO_2H$, 79-11-8; Cl_2CHCHO, 79-02-7.

References

Bartsch, H., Malaveille, C., Montesano, R., & Tomatis, L. (1975) *Nature (London) 255*, 641–643.

Benyon, J. H. (1960) in *Mass Spectroscopy and Its Applications to Organic Chemistry*, p 298, Elsevier, Amsterdam.

Bonse, G., & Henschler, D. (1976) *CRC Crit. Rev. Toxicol. 4*, 395.

Bonse, G., Urban, T., Reichert, D., & Henschler, D. (1975) *Biochem. Pharmacol. 24*, 1829–1834.

Budzikiewicz, H., Djerassi, C., & Williams, D. (1967) in *Mass Spectrometry of Organic Compounds*, p 251, Holden-Day, San Francisco.

Bush, E. D., & Trager, W. F. (1982) *Biochem. Biophys. Res. Commun. 104*, 626–632.

Calkins, V. P. (1943) *Anal. Chem. 15*, 762.

Cochin, J., & Axelrod, J. (1959) *J. Pharmacol. Exp. Ther. 125*, 105–110.

Costa, A. K., & Ivanetich, K. M. (1982) *Biochem. Pharmacol. 31*, 2083–2092.

Daly, J. W., Jerina, D. M., & Witkop, B. (1972) *Experientia 28*, 1129–1149.

Fieser, L. F., & Fieser, M. (1967) in *Reagents for Organic Synthesis*, p 1180, Wiley, New York.

Fleischer, E. B., Palmer, J. M., Srivastava, T. S., & Chatterjee, A. (1971) *J. Am. Chem. Soc. 93*, 3162–3167.

Greim, H., Bonse, G., Radwan, Z., Reichert, D., & Henschler, D. (1975) *Biochem. Pharmacol. 24*, 2013–2017.

Groves, J. T., Nemo, T. E., & Myers, R. S. (1979) *J. Am. Chem. Soc. 101*, 1032–1033.

Groves, J. T., Kruper, W. J., & Haushalter, R. C. (1980a) *J. Am. Chem. Soc. 102*, 6377–6380.

Groves, J. T., Kruper, W. J., Nemo, T. E., & Myers, R. S. (1980b) *J. Mol. Catal. 7*, 169–177.

Guengerich, F. P., & Strickland, T. W. (1977) *Mol. Pharmacol. 13*, 993–1004.

Guengerich, F. P., Crawford, W. M., & Watanabe, P. G. (1979) *Biochemistry 18*, 5177–5182.

Guengerich, F. P., Dannan, G. A., Wright, S. T., Martin, M. V., & Kaminky, L. S. (1982) *Biochemistry 21*, 6019–6030.

Henschler, D., Hoos, W., Fetz, H., Dallmeier, E., & Metzler, M. (1979) *Biochem. Pharmacol. 28*, 543–548.

Hess, B., & Wurster, B. (1970) *FEBS Lett. 9*, 73–77.

Kadish, K. M., & Larson, G. (1977) *Bioinorg. Chem. 7*, 95.

Kline, S. A., & Van Duuren, B. L. (1977) *J. Heterocycl. Chem. 14*, 455–458.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Miller, R. E., & Guengerich, F. P. (1982) *Biochemistry 21*, 1090–1097.

Nash, T. (1953) *Biochem. J. 55*, 416–421.

Nelis, H. J. C. F., & Sinsheimer, J. E. (1981) *Anal. Biochem. 115*, 151–157.

Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2370–2378.

Ortiz de Montellano, P. R., Kunze, K. L., Beilan, H. S., & Wheeler, C. (1982) *Biochemistry 21*, 1331–1339.

Porter, K., & Vollman, D. H. (1962) *Anal. Chem. 34*, 748–749.

Sono, M., & Dawson, J. H. (1982) *J. Biol. Chem. 257*, 5496–5502.

Sroog, C., & Woodburn, H. (1963) *Org. Synth. 4*, 271–272.

Walling, C., & Fredricks, P. (1962) *J. Am. Chem. Soc. 84*, 3326–3331.

Wang, P., Meijer, J., & Guengerich, F. P. (1982) *Biochemistry 21*, 5769–5776.

White, R. E., & Coon, M. J. (1980) *Annu. Rev. Biochem. 49*, 315–356.

White, R. E., Groves, J. T., & McClusky, G. A. (1979) *Acta Biol. Med. Ger. 38*, 475–482.

Wislocki, P. G., Miwa, G. T., & Lu, A. Y. H. (1980) *Enzymatic Basis of Detoxication*, Vol. 1, pp 135–182, Academic Press, New York.