MOLECULAR PHARMACOLOGY, 25:318–321

Reductive-Oxygenation Mechanism of Metabolism of Carbon Tetrachloride to Phosgene by Cytochrome P-450

LANCE R. POHL,[1] RICHARD D. SCHULICK,[1] ROBERT J. HIGHET,[2] AND JOHN W. GEORGE[1]

Laboratory of Chemical Pharmacology and Laboratory of Chemistry, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205

Received September 19, 1983; Accepted November 23, 1983

SUMMARY

The mechanism of metabolism of carbon tetrachloride (CCl_4) to phosgene ($COCl_2$) in rat liver microsomes was investigated. When the oxygen dependency of the reaction was studied, it was found that the rate of the reaction increased as the oxygen concentration in the reaction atmosphere was decreased from 100% to 5%. Decreasing the oxygen concentration below 5% caused a decrease in the rate of the reaction. The reaction was not inhibited by superoxide dismutase or catalase nor was it supported by cumene hydroperoxide. A reconstituted form of cytochrome P-450 from phenobarbital-pretreated rats metabolized CCl_4 to $COCl_2$. These results are consistent with a mechanism we call reductive oxygenation. The first step of the reaction is the cytochrome P-450-dependent reductive dechlorination of CCl_4 to trichloromethyl radical ($CCl_3\cdot$). This intermediate is trapped by oxygen to form trichloromethylperoxyl radical ($CCl_3OO\cdot$), which decomposes to $COCl_2$ and possibly an electrophilic form of chlorine.

INTRODUCTION

The results of several studies have indicated that the hepatotoxicity produced by carbon tetrachloride (CCl_4) is due to a reactive and toxic metabolite (1–6). For this reason, there has been considerable interest in determining the pathways of metabolism of CCl_4. Most investigations of the mechanism of metabolism of CCl_4 have been done at low concentrations of oxygen (7–10). Under these conditions it appears that rat liver cytochrome P-450 reduces CCl_4 by one electron to form an unstable anion radical intermediate ($CCl_4^{\cdot-}$), which spontaneously dechlorinates to produce trichloromethyl radical ($CCl_3\cdot$) and chloride ion. Trichloromethyl radical can abstract a hydrogen atom from some component of the medium to produce chloroform ($CHCl_3$), or possibly become further reduced by cytochrome P-450 to form dichlorocarbene ($:CCl_2$). Both $CCl_3\cdot$ and $:CCl_2$ may also bind irreversibly to various tissue components, and these reactions have been suggested to be responsible, at least in part, for the hepatotoxicity produced by CCl_4.

Recently it has been shown that rat liver microsomes also metabolize CCl_4 to phosgene ($COCl_2$) (11–13) and an unidentified electrophilic form of chlorine (E-Cl),[3] which was trapped with 2,6-dimethylphenol to form 4-chloro-2,6-dimethylphenol (14, 15). The formation of both of these reactive products, however, was oxygen-dependent (12, 14), and $CHCl_3$ did not appear to be a precursor of either metabolite (11, 14).

In the present investigation we have studied the mechanism of metabolism of CCl_4 to $COCl_2$ in greater detail and have compared the results with those of previous investigations on the metabolism of CCl_4 to E-Cl. We have concluded that both $COCl_2$ and E-Cl may be formed from cytochrome P-450 through a common pathway which we call reductive oxygenation.

EXPERIMENTAL PROCEDURES

Materials. Chemicals were obtained from the following sources: Cumene hydroperoxide from ICN Pharmaceuticals (Plainview, N. J.); homocysteine and cysteine from Aldrich Chemical Company (Milwaukee, Wisc.); NADPH, catalase, and dilauroyl L-α-phosphatidylcholine from Sigma Biochemicals (St. Louis, Mo.); superoxide dismutase from Calbiochem-Behring (La Jolla, Calif.); and tetrabutylammonium phosphate (0.5 M, pH 7.5) from Regis Chemical Company (Morton Grove, Ill.). OTZ was synthesized from cysteine and phosgene by the method of Koneko *et al.* (16).

Preparation of microsomes. Male Sprague-Dawley rats (180–200 g), were obtained from Taconic Farms (Germantown, N. Y.) and maintained on Purina rat chow and water ad libitum. Animals were treated daily with sodium phenobarbital (80 mg/kg in normal saline, ip) for 3 days. Rats were killed 24 hr after treatment with phenobarbital, and liver microsomes were prepared as described elsewhere (13). Protein content was determined by the method of Lowry *et al.* (17), with bovine serum albumin as a standard. Cytochrome P-450 was measured by the method of Omura and Sato (18), and NADPH-dependent cytochrome *c* reductase by the method of Omura and Takesue (19).

[1] Laboratory of Chemical Pharmacology.

[2] Laboratory of Chemistry.

[3] The abbreviations used are: E-Cl, electrophilic chlorine; OTZ, 2-oxothiazolidine-4-carboxylic acid; POTZ, perhydro-2-oxo-1,3-thiazine-4-carboxylic acid; SDS/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; HPLC, high-pressure liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EIMS, electron-ionization mass spectrometry.

0026-895X/84/020318–04$02.00/0

Purification of cytochrome P-450 and cytochrome P-450 reductase. A major form of cytochrome P-450 was purified from microsomes of phenobarbital-treated rats by the method of West *et al.* (20) to a specific activity of 12 nmoles/mg of protein. Cytochrome P-450 reductase was purified from microsomes of phenobarbital-treated rats by the method of Yasukochi and Masters (21) to a specific activity of 45,400 nmoles/min/mg of protein. SDS/PAGE analysis of the enzyme preparations by the method of Laemmli (22), as modified by Guengerich (23), indicated that the enzymes were apparently homogeneous.

HPLC. HPLC analyses were performed with a Waters Associates Instrument equipped with a Model 6000A pump, a Model U6-K universal injector, a Model 450 variable-wavelength detector (set at 220 nm), and a Whatman Partisil 5 ODS-3 RAC column (9.4 mm × 10 cm). The solvent system consisted of 5 mM tetrabutylammonium phosphate in water (pH 7.5), and the rate of flow was 3 ml/min.

NMR spectrometry. ^{13}C-NMR analysis was performed with a JEOL Model FX-60 instrument. POTZ (50 mg) was dissolved in deuterated methanol (0.8 ml) and was analyzed at 15 MHz. Fourier transform (8K) yielded spectra with chemical shifts precise to 0.1 ppm.

EIMS. EIMS was performed with a V.G. Micromass 16F mass spectrometer at an accelerating voltage of 4 kV, an electron energy of 70 eV, an ionizing current of 500 μamp, and a source temperature of 225°. The same was introduced into the source on an unheated direct insertion probe.

Synthesis of POTZ. Homocysteine (1 g, 7.4 mmole) was dissolved in 83 ml of water, and the solution was made basic with 8.3 ml of an aqueous solution of 40% potassium hydroxide. The mixture was cooled to approximately −5° (ice-salt bath) in an atmosphere of nitrogen and phosgene (1.17 g, 13.4 mmole, dissolved in 5 ml of toluene) was added dropwise over a period of 3 min, with vigorous stirring. After 30 min, concentrated hydrochloric acid was added to adjust the pH from approximately pH 10 to pH 7.5. The reaction mixture was washed with ethyl acetate (300 ml, two times) and made acidic to pH 2 with concentrated hydrochloric acid. It was then extracted with ethyl acetate (300 ml, four times) and the extracts were combined and dried over anhydrous magnesium sulfate. The dried organic extract was filtered by gravity, and the ethyl acetate was removed by rotary evaporation under vacuum to give 460 mg of a white solid. The product was crystallized from water to yield 157 mg (13% yield) of a pale white crystalline solid, m.p. 182–183° (uncorrected), which was characterized as POTZ from its chemical analysis, EIMS, and ^{13}C-NMR spectrum.

$C_5H_7NO_3S$

Calculated: C 37.25, H 4.39, N 8.69, S 19.89
Found: C 37.44, H 4.37, N 8.69, S 20.07

EIMS: m/z ≥ 80 amu and relative intensity ≥ 5% of base peak were as follows: m/z (relative intensity) 161 (21, M^+), 116 (16, M^+, —COOH), 102 (11), 88 (100), 87 (26). ^{13}C-NMR chemical shifts in parts per million downfield from tetramethylsilane were as follows: 173.8 and 169.8 (—SCONH— or COOH), 56.0 (—CH—), and 26.7 and 26.3 (—SCH_2— or —CH_2—).

Purification of cumene hydroperoxide. Cumene hydroperoxide (pale yellow, 10 ml) was added dropwise to a solution of 25% sodium hydroxide (30 ml). The temperature of the reaction mixture was maintained below 30° in a water bath. The resulting pale pink dispersion was washed with petroleum ether (40 ml, three times), acidified to pH 7.5 with concentrated hydrochloric acid, and extracted with hexane (60 ml, two times). The hexane extracts were combined, dried over anhydrous sodium sulfate, and filtered, and the solvent was removed by rotary evaporation under vacuum to give a pale yellow oil, which was distilled under vacuum to yield 4 g of a colorless oil, b.p. 45–47° at 0.12 mm [lit. 60° at 0.2 mm (24)].

Metabolism of CCl_4 to $COCl_2$. The incubations were conducted in sealed vials (rubber septum) at 37° and were stopped by cooling in an ice bath. An internal standard of POTZ (1 μg in 10 μl of water) was added, and the reaction mixtures were transferred to conical centrifuge tubes. The aqueous reaction mixtures were washed with ethyl acetate (6 ml) and made acidic to approximately pH 2 with concentrated hydrochloric acid. The acidic reaction mixtures were then extracted with ethyl acetate (6 ml), and the organic extracts were evaporated to dryness under a stream of nitrogen. The residues were mixed with water, (500 μl), and an aliquot (100 μl) was analyzed for OTZ and POTZ by HPLC. The retention time of POTZ was typically 1–2 min longer than that of OTZ. When the HPLC column was new, the retention time of POTZ was approximately 15 min and that of OTZ was 13 min. As the column was used, the retention times of both POTZ and OTZ slowly became shorter. The amount of OTZ in the samples was determined by comparing the ratio of peak areas of OTZ/POTZ to a standard curve, which was made by adding various amounts of OTZ (0.10, 0.25, 0.50, 1.0, and 5.0 μg) and a constant amount of POTZ (1 μg) to microsomal reaction mixtures, followed by the extraction procedure and HPLC analysis. The standard curve of the ratios of peak areas of OTZ/POTZ versus OTZ concentrations was linear, and the limits of detection of the assay were approximately 0.04 nmole of OTZ/nmole of P-450/min.

RESULTS

When the oxygen dependency of the metabolism of CCl_4 to $COCl_2$ by rat liver microsomes was investigated in greater detail, it was found that the rate of the reaction increased as the O_2 concentration of the reaction mixture atmosphere was decreased from 100% to 5% (Fig. 1). Under an atmosphere of nitrogen, in which the oxygen tension was considerably below 5%, no $COCl_2$ was detected.

The metabolism of CCl_4 to $COCl_2$ by rat liver microsomes was not inhibited by superoxide dismutase (1000 units/ml) or catalase (1300 units/ml), nor was it supported by 2 mM cumene hydroperoxide (results not shown). In contrast, the metabolism of $CHCl_3$ (5 mM) to $COCl_2$ was supported more effectively by 2 mM cumene hydroperoxide (3.17 ± 0.27 nmoles/nmole of P-450/min, mean ± SE, $N = 4$) than by 1 mM NADPH (1.11 ± 0.02 nmoles/nmole of P-450/min, mean ± SE, $N = 4$).

When CCl_4 (5 μmoles) was incubated with a major form of cytochrome P-450 (1 nmole) induced by pheno-

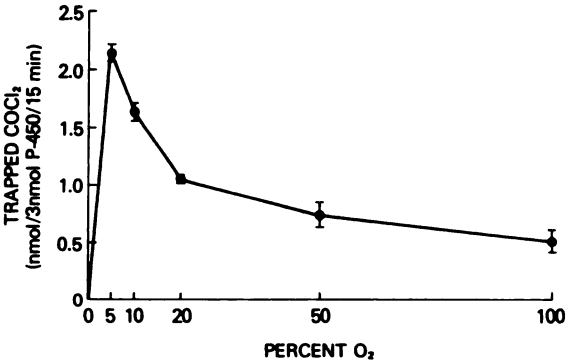

FIG. 1. *Effect of oxygen concentration on the metabolism of CCl_4 to $COCl_2$ by rat liver microsomes*

CCl_4 (5 μmoles) was incubated with 3 nmoles of microsomal cytochrome P-450, cysteine (10 μmoles), and NADPH (1 μmole) in 1 ml of 20 mM Hepes (pH 7.5) for 15 min. Phosgene was measured as the cysteine derivative, OTZ, by HPLC as described under Experimental Procedures. The oxygen concentrations were regulated as described elsewhere (25). The results represent the means ± standard error of the mean of three determinations.

barbital, cytochrome P-450 reductase (8000 units), dilauroyl L-α-phosphatidylcholine (100 μg), cysteine (10 μmoles), NADPH (1 μmole), and Hepes buffer (18 μmoles, pH 7.5) in a total volume of 1 ml for 30 min, 0.23 ± 0.02 nmole of $COCl_2$ was trapped as OTZ/nmole of P-450/min (mean ± SE, $N = 3$). The reaction required NADPH and cytochrome P-450 reductase. Cytochrome P-450 reductase did not catalyze the reaction in the absence of cytochrome P-450.

DISCUSSION

We previously suggested that cytochrome P-450 in rat liver microsomes might metabolize CCl_4 to $COCl_2$ and E-Cl by at least three different mechanisms (14). The preponderance of evidence from recent studies on the mechanism of formation of E-Cl (15, 25, 26) and the results of the present study, however, indicate that the major, if not the only, pathway for this route of metabolism is by a reductive-oxygenation mechanism. The first step of the reaction is the cytochrome P-450-dependent reductive-dechlorination of CCl_4 to $CCl_3\cdot$, whereas the second step is the reaction of $CCl_3\cdot$ with O_2 to form trichloromethylperoxyl radical ($CCl_3OO\cdot$). This species then decomposes to $COCl_2$ and E-Cl (15, 25).

The most suggestive evidence for this mechanism is derived from the studies of the oxygen dependency of the metabolism of CCl_4 to $COCl_2$ and E-Cl. For example, the rates of metabolism of CCl_4 to $COCl_2$ (Fig. 1) and E-Cl (15, 25) by rat liver microsomes increased in nearly identical manner as the atmospheric O_2 concentration was decreased from 100% to 5%; this behavior was similar to that observed during the reductive dechlorination of hexachloroethane ($Cl_3C\text{-}CCl_3$) to pentachloroethane ($Cl_3C\text{-}CHCl_2$) in which the pentachloroethyl radical ($Cl_3C\text{-}CCl_2\cdot$) is believed to be an intermediate (15, 27). Under an atmosphere of nitrogen, in which the O_2 concentration was considerably below 5%, the rates of formation of $COCl_2$ (Fig. 1) and E-Cl (15, 25) from CCl_4 did not further increase as was observed with hexachloroethane (15, 27). At these low concentrations of O_2, it is believed that the trapping of $CCl_3\cdot$ by O_2 became rate-determining, and alternate reactions of $CCl_3\cdot$ became kinetically important. In this regard, it appears that only at low concentrations of O_2 does $CCl_3\cdot$ (a) abstract a hydrogen atom from some component of the medium to produce $CHCl_3$ (8, 11), (b) become further reduced by cytochrome P-450 to form $:CCl_2$ (7), or (c) irreversibly react with microsomal lipid (28).

Pathways involving superoxide anion radical or hydrogen peroxide do not appear to contribute significantly to the metabolism of CCl_4 to $COCl_2$ and E-Cl because superoxide dismutase and catalase did not inhibit the formation of either of these products (25). The oxygenation of CCl_4 to trichloromethyl hypochlorite (CCl_3OCl) by cytochrome P-450 also does not appear to be involved in the metabolism of CCl_4 to $COCl_2$ and E-Cl. This conclusion is based upon the unique O_2 dependency of the metabolism of CCl_4 to $COCl_2$ (Fig. 1) and E-Cl (15, 25) and the finding that cumene hydroperoxide did not support the metabolism of CCl_4 to detectable levels of $COCl_2$ or E-Cl (25), but did support the oxidation of the carbon-hydrogen bond of $CHCl_3$ and other compounds (29).

In conclusion, it has become apparent that the metabolism of CCl_4 is very complex. After its initial reductive dechlorination to $CCl_3\cdot$ by cytochrome P-450, several additional metabolites may be produced (Fig. 2). At low oxygen tension, $CCl_3\cdot$ may abstract a hydrogen atom from a cellular component to form $CHCl_3$, react with unsaturated lipids, or be further reduced by cytochrome

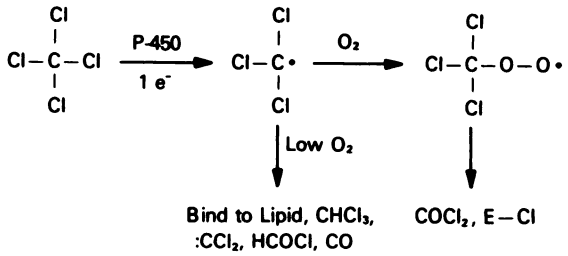

FIG. 2. *Possible metabolites of CCl_4 produced by cytochrome P-450*

P-450 to produce $:CCl_2$; this intermediate may react with water to form formyl chloride (HCOCl). Decomposition of HCOCl would yield CO, another known metabolite of CCl_4 (7). If the oxygen concentration of the environment is high enough, $CCl_3\cdot$ can be trapped by O_2 to form $CCl_3OO\cdot$, which can lead to the formation of $COCl_2$ and E-Cl. Therefore, at least eight potentially toxic products may be formed during the metabolism of CCl_4.

ACKNOWLEDGMENTS

We thank Dr. James R. Gillette for critically reviewing the manuscript, and Patricia L. Morris for her assistance in synthesizing POTZ.

REFERENCES

1. Butler, T. C. Reduction of carbon tetrachloride *in vivo* and reduction of carbon tetrachloride and chloroform *in vitro* by tissues and tissue constituents. *J. Pharmacol. Exp. Ther.* **134**:311–319 (1961).
2. Reynolds, E. S. Liver parenchymal cell injury. IV. Pattern of incorporation of carbon and chlorine from carbon tetrachloride into chemical constituents of liver *in vivo*. *J. Pharmacol. Exp. Ther.* **155**:117–126 (1967).
3. Gordis, E. Lipid metabolites of carbon tetrachloride. *J. Clin. Invest.* **48**:203–209 (1969).
4. Burdino, E., E. Gravela, G. Ugazio, V. Vannini, and A. Calligaro. Initiation of free radical reactions and hepatotoxicity in rats poisoned with carbon tetrachloride or bromotrichloromethane. *Agents Action* **4**:244–253 (1973).
5. Sipes, I. G., G. Krishna, and J. R. Gillette. Bioactivation of carbon tetrachloride, chloroform and bromotrichloromethane: role of cytochrome P-450. *Life Sci.* **20**:1541–1548 (1977).
6. Recknagel, R. O., E. A. Glende, and A. M. Hruszkewycz. Chemical mechanisms in carbon tetrachloride toxicity, in *Free Radicals in Biology* (W. A. Pryor, ed.), Vol. 3. Academic Press, New York, 97–132 (1977).
7. Ahr, H. J., L. J. King, W. Nastainczyk, and V. Ullrich. The mechanism of chloroform and carbon monoxide formation from carbon tetrachloride by microsomal cytochrome P-450. *Biochem. Pharmacol.* **29**:2855–2861 (1980).
8. Wolf, C. R., W. G. Harrelson, W. M. Nastainczyk, R. M. Philpot, B. Kalyararaman, and R. P. Mason. Metabolism of carbon tetrachloride in hepatic microsomes and reconstituted monooxygenase systems and its relationship to lipid peroxidation. *Mol. Pharmacol.* **18**:553–558 (1980).
9. Kubic, V. L., and M. W. Anders. Mechanism of the microsomal reduction of carbon tetrachloride and halothane. *Chem. Biol. Interact.* **34**:201–207 (1981).
10. Trudell, J. R., B. Bosterling, and A. J. Trevor. Reductive metabolism of carbon tetrachloride by human cytochromes P-450 reconstituted in phospholipid vesicles: mass spectral identification of trichloromethyl radical bound to dioleoyl phosphatidylcholine. *Proc. Natl. Acad. Sci. U. S. A.* **79**:2678–2682 (1982).
11. Shah, H., S. P. Hartman, and S. Weinhouse. Formation of carbonyl chloride in carbon tetrachloride metabolism by rat liver *in vitro*. *Cancer Res.* **39**:3942–3947 (1979).
12. Kubic, V. L., and M. W. Anders. Metabolism of carbon tetrachloride to phosgene. *Life Sci.* **26**:2151–2155 (1980).

13. Pohl, L. R., R. V. Branchflower, R. J. Highet, J. L. Martin, D. S. Nunn, T. J. Monks, J. W. George, and J. A. Hinson. The formation of diglutathionyl dithiocarbonate as a metabolite of chloroform, bromotrichloromethane, and carbon tetrachloride. *Drug Metab. Dispos.* **9**:334–339 (1981).
14. Mico, B. A., R.V. Branchflower, L. R. Pohl, A. T. Pudzianowski, and G. H. Loew. Oxidation of carbon tetrachloride, bromotrichloromethane and carbon tetrabromide by rat liver microsomes to electrophilic halogens. *Life Sci.* **30**:131–137 (1982).
15. Pohl, L. R., and B. A. Mico. Electrophilic halogens as potentially toxic metabolites of halogenated compounds. *Trends Pharmacol. Sci.*, in press.
16. Koneko, T., T. Shimokobe, Y. Ota, E. Toyokawa, T. Inui, and T. Shiba. Synthèses and properties of 2-oxothiazolidine-4-carboxylic acid and its derivatives. *Bull. Chem. Soc. Jpn.* **37**:242–244 (1964).
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–273 (1951).
18. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2370–2378 (1964).
19. Omura, T., and S. Takesue. A new method for simultaneous purification of cytochrome b_5 and NADPH cytochrome *c* reductase from rat liver microsomes. *J. Biochem.* **67**:249–257 (1970).
20. West, S. B., M. T. Huang, G. T. Miwa, and A. Y. H. Lu. A simple and rapid procedure for the purification of phenobarbital-inducible cytochrome P-450 from rat liver microsomes. *Arch. Biochem. Biophys.* **193**:42–50 (1979).
21. Yasukochi, Y., and B. S. S. Masters. Some properties of a detergent-solubilized NADPH cytochrome *c* (cytochrome P-450) reductase purified by biospecific affinity chromatography. *J. Biol. Chem.* **251**:5337–5344 (1976).
22. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (*Lond.*) **227**:680–685 (1970).
23. Guengerich, F. P. Separation and purification of multiple forms of microsomal cytochrome P-450. Partial characterization of three apparent homogeneous cytochromes P-450 prepared from livers of phenobarbital and 3-methylcholanthrene-treated rats. *J. Biol. Chem.* **253**:7931–7939 (1978).
24. Armstrong, G. P., R. H. Hall, and D. C. Quin. The autoxidation of isopropylbenzene. *J. Chem. Soc.* 666–670 (1950).
25. Mico, B. A., and L. R. Pohl. Reductive-oxygenation of carbon tetrachloride: trichloromethylperoxyl radical as a possible intermediate in the conversion of carbon tetrachloride to electrophilic chlorine. *Arch. Biochem. Biophys.* **225**:596–609 (1983).
26. Mico, B. A., R. V. Branchflower, and L. R. Pohl. Formation of electrophilic chlorine from carbon tetrachloride: involvement of cytochrome P-450. *Biochem. Pharmacol.* **32**:2357–2359 (1983).
27. Nastainczyk, W., H. J. Ahr, and V. Ullrich. The reductive metabolism of halogenated alkanes by liver microsomal cytochrome P-450. *Biochem. Pharmacol.* **31**:391–396 (1982).
28. Kieczka, H., H. Remmer, and H. Kappus. Influence of oxygen on the inhibition of liver microsomal activation of carbon tetrachloride by the catechol 2-hydroxyestradiol-17β. *Biochem. Pharmacol.* **30**:319–324 (1981).
29. White, R. E., and M. J. Coon. Oxygen activation by cytochrome P-450. *Annu. Rev. Biochem.* **49**:315–356 (1980).

Send reprint requests to: Dr. Lance R. Pohl, Laboratory of Chemical Pharmacology, National Heart, Lung, and Blood Institute, Building 10, Room 8N110, National Institutes of Health, Bethesda, Md. 20205.