Biochemical Pharmacology, Vol. 35, No. 11, pp. 1805–1807, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00

THIOL STIMULATION OF THE CYTOCHROME P-450-DEPENDENT REDUCTION OF 1,1,1-TRICHLORO-2,2-BIS(*p*-CHLOROPHENYL)ETHANE (DDT) TO 1,1-DICHLORO-2,2-BIS(*p*-CHLOROPHENYL)ETHANE (DDD)

MICHAEL J. KELNER,* JOHN C. MCLENITHAN and M. W. ANDERS†
Department of Pharmacology, University of Rochester, Rochester, NY 14642, U.S.A.

(*Received* 19 *January* 1985; *accepted* 8 *November* 1985)

Abstract—The enzymatic reduction of 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane (DDT) to 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane (DDD) was studied with rat hepatic microsomal fractions. The reaction required NADPH and was inhibited by dioxygen and carbon monoxide, which shows that the reaction is catalyzed by cytochromes P-450. Moreover, when the reaction was studied in the presence of deuterium oxide, no deuterium was incorporated into the DDD, which suggests that a one-electron reduction of DDT to the 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethyl radical followed by hydrogen atom abstraction accounts for the formation of DDD. The microsomal reduction of DDT to DDD was stimulated markedly by several thiols, including glutathione. The stimulatory effect of thiols was concentration dependent and was not due to conservation of cytochrome P-450, because nonthiol antioxidants failed to stimulate the reaction. The mechanism of the stimulation is not understood, but thiols do not promote the formation of DDD by preventing the alkylation of microsomal lipids and, thereby, stimulating the formation of a reduced alkane. Finally, these results show that soluble, low-molecular weight compounds may enhance the activity of membrane-bound enzymes.

The cytochrome P-450-dependent enzymatic reduction of chlorinated alkanes is well known; examples include the metabolism of carbon tetrachloride to chloroform [1–3], of halothane to 2-chloro-1,1,1-trifluorethane [4, 5], and of hexachloroethane to pentachloroethane [6]. The metabolic reduction of DDT‡ to DDD [7, 8] and to DDE [9] has also been reported.

During the course of investigations on the enzymology of the dehydrochlorination of DDT to DDE, it was observed that DDD was formed, confirming earlier observations [7, 8], and that the reduction of DDT to DDD was stimulated markedly by thiols. The experiments reported herein were designed to characterize this stimulation.

MATERIALS AND METHODS

Male Long–Evans rats (Blue Spruce Farms, Altamont, NY) weighing about 200 g were used. Hepatic microsomal fractions were isolated as described previously [10], and the microsomal protein content was measured by the method of Lowry *et al.* [11]. The reaction mixtures contained, unless noted otherwise, 3 nmoles of DDT (added in 10 μl of ethanol), 50 μmoles of phosphate buffer (pH 7.4), 15 μmoles of magnesium chloride, 10 μmoles of sodium isocitrate, 1 μmole of $NADP^+$, 1 E.U. of isocitrate dehydrogenase (Sigma), 6 μmoles of thiol (adjusted to pH 7.4), and 3–6 mg of microsomal protein in a final volume of 3.0 ml. The reactions were started by the addition of substrate and were usually incubated for 15 min at 37° in capped flasks in the presence of a reduced dioxygen tension, which was produced by evacuating and flushing the flasks with nitrogen five times. The reaction was stopped by placing the flasks in ice and injecting 1.0 ml of 0.09% heptachlor epoxide, which served as an internal standard for gas chromatographic analysis, in heptane.

The contents of the reaction flasks were transferred to test tubes, the reaction flasks were rinsed with 1.0 ml of 0.09% heptachlor epoxide in heptane, the heptane rinses were added to the test tubes, and the mixtures were shaken for 1 min and then centrifuged. The heptane phase was transferred to another test tube. The aqueous layer from the first extraction was extracted a second time with 1.0 ml of the internal standard solution, and the heptane phases were pooled.

A 1.0-μl sample of the pooled organic layers was injected into a Hewlett–Packard 6730 gas chromatograph equipped with a ^{63}Ni electron capture detector. The column was a 6 ft × 3.0 mm i.d. glass tube packed with 1.5% OV-17 and 1.95% OV-110 on Chromosorb WHP (100–120 mesh). The column, injector, and detector temperatures were 215, 250, and 250° respectively. A Hewlett–Packard 3390A recorder-integrator was used for quantification.

Incorporation of deuterium into DDD was measured with a Hewlett–Packard 5992A gas chromatograph/mass spectrometer. The gas chromatographic conditions were as described above.

* Present address: Department of Pathology, University of California, San Diego, CA 92103.
† Address all correspondence to: M. W. Anders, Department of Pharmacology, University of Rochester, 601 Elmwood Ave., Rochester, NY 14642.

‡ Abbreviations: DDT, 1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane; DDD, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane; and DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethene.

Deuterium incorporation during the metabolic conversion of DDT to DDD was analyzed by a selected ion monitoring program with simultaneous monitoring of m/z 82, 83, and 84. Mass spectral analysis of DDD showed a major peak at m/z 83 ($HC^{35}Cl^{35}Cl$), and incorporation of deuterium would yield a fragment at m/z 84 ($^2HC^{35}Cl^{35}Cl$).

The binding of [^{14}C]DDT (Amersham, Arlington Heights, IL; 85 mCi/mmole) to microsomal phospholipids was determined by the method of Baker and Van Dyke [8] with 76 μM DDT as the substrate.

RESULTS

The objective of the first series of experiments was to characterize the reduction of DDT to DDD in the presence of 5 mM glutathione. DDD formation was detected when DDT was incubated with NADPH-fortified hepatic 9000 g supernatant or microsomal fractions in the absence of dioxygen. No DDD formation was observed when the reactions were carried out in the presence of dioxygen, when NADPH was omitted from the reaction mixture, or when heat-inactivated microsomal fractions were employed (data not shown). The average rates of DDD formation were 10 and 30 pmoles DDD/mg protein/min in the absence and presence of 5 mM glutathione respectively. The rate of microsomal metabolism of DDT to DDD was linear with time for up to 15 min, with DDT concentrations up to 5 μM, and increased with increasing protein concentrations up to 2.5 mg protein/ml (data not shown). Kinetic parameters were established for the microsomal metabolism of DDT to DDD in the presence of glutathione; the Michaelis constant (K_m) for the reaction was 55 μM, and the maximal velocity (V_{max}) was 150 pmoles DDD/mg protein/min.

The effects of several thiols and methionine on DDT reduction were studied (Table 1); all of the compounds tested stimulated DDT reduction. The concentration dependency of the stimulatory effect of glutathione on the metabolism of DDT to DDD is shown in Fig. 1. The reduction of DDT to DDD was maximal at 1.0 mM glutathione.

Low concentrations of carbon monoxide inhibited the conversion of DDT to DDD (Fig. 2). As little as 1.5 torr (0.2%) of carbon monoxide in nitrogen decreased the reaction rate by approximately 50%,

Table 1. Effects of thiols on the microsomal metabolism of DDT to DDD

Thiol	DDD formation (pmoles/mg protein/min)
Control	8 ± 0
Glutathione	23 ± 1
L-Cysteine	14 ± 0
Cysteamine	22 ± 1
L-Methionine	23 ± 1
D-Penicillamine	22 ± 1

The reaction mixtures were prepared and incubated as described in Materials and Methods; the thiol concentration was 2 mM. DDD formation was measured by gas chromatography. The values are mean ± S.D. ($N \geq 3$).

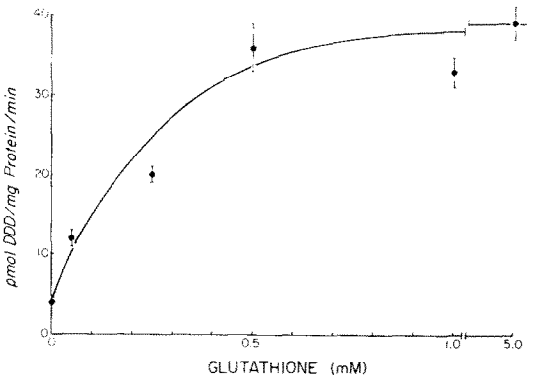

Fig. 1. Concentration-dependence of the stimulation of DDT reduction to DDD by glutathione. Reaction mixtures were prepared and incubated and product formation was measured as described in Materials and Methods. The values are the mean ± S.D. (N = 4).

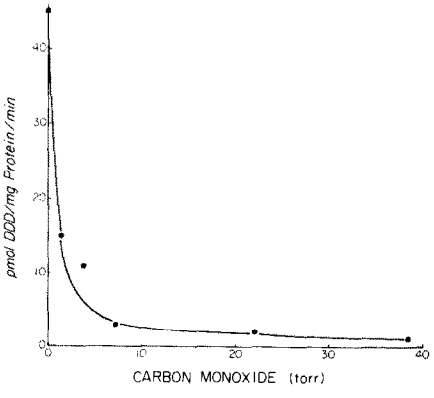

Fig. 2. Inhibition of the microsomal metabolism of DDT to DDD by carbon monoxide. Reaction mixtures were prepared and incubated and product formation was measured as described in Materials and Methods. The values are the mean ± S.D. (N = 4).

and 20 torr (2.5%) of carbon monoxide in nitrogen completely inhibited the reaction.

The effects of inhibitors (EDTA, 1 mM; Mn^{2+}, 0.5 mM; butylated hydroxytoluene, 0.5 mM) of lipid peroxidation on the metabolism of DDT were studied. No acceleration of the conversion of DDT to DDD was found with any of the inhibitors tested (data not shown).

The effect of glutathione on the binding of [^{14}C]-DDT metabolites to microsomal phospholipids was studied. DDT binding in the absence and presence of 5 mM glutathione was 200 ± 21 and 155 ± 6 pmoles DDT bound per mmole phospholipid respectively (mean ± S.D., N = 3; $P < 0.05$).

No measurable incorporation of deuterium into DDD during the reductive metabolism of DDT was observed when the reaction was carried out in the presence of deuterium oxide (data not shown).

DISCUSSION

The formation of DDD from DDT is catalyzed by hepatic microsomal enzymes. The subcellular location, the requirement for NADPH, and the inhi-

bition by carbon monoxide show that the reaction is catalyzed by cytochromes P-450; these findings confirm previous reports [7, 8].

The mechanism of DDT reduction to DDD by cytochromes P-450 may be explained by either a one- or two-electron reduction. The initial cytochrome P-450-catalyzed reductive step may yield the 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethyl radical, which may abstract a hydrogen atom from microsomal lipids to give DDD. This is the mechanism of the cytochrome P-450-dependent reduction of carbon tetrachloride to chloroform [2, 3] and of halothane to 2-chloro-1,1,1-trifluorethane [3, 5]. The intermediate radical may undergo a second one-electron reduction to yield the 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethyl carbanion, which may undergo protonation to yield DDD. The finding that no deuterium was incorporated into DDD favors a one-electron reduction followed by hydrogen atom abstraction. Such a mechanism is consistent with the observation that DDT, like carbon tetrachloride, becomes covalently bound to microsomal lipids [8].

This suggested mechanism is supported by chemical studies. For example, iron(II)deuterophorphyrin is rapidly oxidized by alkyl halides, including DDT, and DDD has been identified as a product [12]. DDT showed third-order reaction kinetics: rate $= k_3(Fe^{II}Porp)^2(RX)$ and $k_3 = 8 \times 10^3$ liter^2/moles^2 sec. In the present study, with an average value of 1.0 nmole cytochrome P-450/mg protein, the kinetic constants for the enzymatic reaction were calculated and found to be remarkably constant over various incubation times and changing DDT concentrations: $k_3 = 1.5 \times 10^7$ liter^2/mole^2 sec for various DDT concentrations and 2.0×10^7 liter^2/moles^2 sec for various times; one standard deviation $= 0.2 \times 10^7$ liter^2/mole^2 sec. These values for the enzymatic system are about 100-fold higher than the values reported by Wade and Castro [12] for their chemical system.

The observation that thiols and methionine enhance markedly the enzymatic reduction of DDT to DDD is novel, although the mechanism is unknown. The finding that thiols of diverse structure all stimulate the reduction of DDT suggests a chemical, rather than an enzymatic, mechanism.

Previous studies showed that inhibitors of lipid peroxidation enhance the rate of aldrin epoxidation, presumably by protecting cytochromes P-450 from destruction by NADPH-initiated lipid peroxidation [13]. This is not the mechanism of thiol stimulation in the present study, because nonthiol antioxidants failed to alter the reduction of DDT.

Thiols may stimulate DDT reduction by altering the fate of the 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethyl radical, which may undergo reduction to the corresponding alkane (DDD) or may alkylate microsomal lipids [8]. Reduction of the radical by thiols would prevent the alkylation of lipids and, thereby, stimulate DDD formation. This hypothesis was tested in the present study by examining the effect of glutathione on the binding of DDT metabolites to microsomal phospholipids. Glutathione produced a modest decrease in the binding of DDT metabolites to phospholipids, but the magnitude of the effect is insufficient to account for the marked stimulation in the metabolism of DDT to DDD caused by thiols, including glutathione.

Divergent results have been reported when the effects of thiols on the metabolism of polychlorinated alkanes have been studied. Glutathione has been reported to stimulate the reduction of carbon tetrachloride to chloroform and to inhibit slightly the covalent binding of carbon tetrachloride-derived metabolites to microsomal proteins [2, 14]; others have reported that cysteine fails to alter the reduction of carbon tetrachloride to chloroform or the covalent binding of carbon tetrachloride-derived metabolites to microsomal lipids or proteins [15]. The microsomal reduction of halothane to 2-chloro-1,1,1-trifluoroethane is inhibited slightly by glutathione [5]; in contrast, the metabolism of hexachloroethane to pentachloroethane is stimulated markedly by cysteine and penicillamine, but glutathione has no effect [6]. These divergent findings do not allow the description of a mechanism for the effect of thiols on the reduction of polyhalogenated alkanes that can be generalized to a wide range of compounds, and it is possible that several mechanisms are involved, but to different extents, with different substrates. The results presented here, along with observations from other laboratories, show that soluble, low-molecular weight compounds enhance the activity of membrane-bound enzymes. The mechanism of this effect warrants further investigation.

Acknowledgement—This research was supported by National Institute of Environmental Health Sciences Grant ES03126.

REFERENCES

1. T. C. Butler, *J. Pharmac. exp. Ther.* **134**, 311 (1961).
2. H. J. Ahr, L. J. King, W. Nastainczyk and V. Ullrich, *Biochem. Pharmac.* **29**, 2855 (1980).
3. V. L. Kubic and M. W. Anders, *Chem. Biol. Interact.* **34**, 201 (1980).
4. J. H. Sharp, J. R. Trudell and E. N. Cohen, *Anesthesiology* **50**, 2 (1979).
5. H. J. Ahr, L. J. King, W. Nastainczyk and V. Ullrich, *Biochem. Pharmac.* **31**, 383 (1982).
6. W. Nastainczyk, H. J. Ahr and V. Ullrich, *Biochem. Pharmac.* **31**, 391 (1982).
7. E. G. Esaac and F. Matsumura, *Pharmac. Ther.* **9**, 1 (1980).
8. M. T. Baker and R. A. Van Dyke, *Biochem. Pharmac.* **33**, 255 (1984).
9. B. Gold and G. Brunk, *Chem. Biol. Interact.* **41** 327 (1982).
10. M. W. Anders, *Biochem. Pharmac.* **27**, 1098 (1978).
11. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
12. R. S. Wade and C. E. Castro, *J. Am. chem. Soc.* **95**, 226 (1973).
13. S. E. Lewis, C. F. Wilkinson and J. W. Ray, *Biochem. Pharmac.* **16**, 1195 (1967).
14. H. Uehleke, K. H. Hellmer and S. Tabarelli, *Xenobiotica* **3**, 1 (1973).
15. R. L. Waller and R. O. Recknagel, *Toxic. appl. Pharmac.* **66**, 172 (1982).