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METABOLISM-DEPENDENT BINDING OF THE CHLORINATED INSECTICIDE DDT AND ITS METABOLITE, DDD, TO MICROSOMAL PROTEIN AND LIPIDS*

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Abstract—Dichlorodi[U-^{14}C]phenyltrichloroethane ([^{14}C]DDT), incubated with rat hepatic microsomes and NADPH, produced reactive intermediates which covalently bound to microsomal protein and lipids. In atmospheric oxygen, DDT bound to microsomal protein; however, binding was increased up to approximately 70% by oxygen depletion. Low levels of [^{14}C]DDT binding to microsomal lipids occurred under atmospheric oxygen but, in contrast to protein binding, DDT-phospholipid binding was increased up to 20-fold by oxygen depletion. Dichlorodiphenyldichloroethane (DDD) was rapidly formed from DDT under anaerobic conditions, although when DDD was utilized as substrate, binding to microsomal protein occurred only in the presence of oxygen. Sodium dithionite, added to microsomes, produced [^{14}C]DDT phospholipid and protein binding, and DDD formation, but failed to support DDD metabolism or binding. The data are consistent with the reductive formation of a DDT free-radical intermediate that led to the formation of DDD and that was bound preferentially to microsomal lipids.

The metabolic formation of reactive metabolites and their subsequent binding to cellular macromolecules is a mechanism thought to be involved in the toxicity of many biologically inert compounds. Numerous halogenated hydrocarbons exhibit cellular toxicity, and microsomal cytochrome P-450, primarily of the liver, has been shown to metabolize and activate these compounds through either oxidative or reductive mechanisms.

For halothane [1–3] and carbon tetrachloride (CCl_4) [4, 5], it has been demonstrated that metabolic pathways which lead to dehalogenation are enhanced under low oxygen conditions in microsomal incubations, and the binding of these compounds to microsomal protein and particularly microsomal lipids is also increased when incubations are depleted of oxygen. The hepatotoxic effects associated with exposure to CCl_4 and halothane are thought to be related to the occurrence of free-radical intermediates resulting from reductive metabolism, which bind to cellular components and/or initiate lipid peroxidation [6–8]. Other less halogenated alkanes, such as 1,2-dichloroethane, are activated by hepatic micrsomes but the presence of oxygen is required for binding to occur [9].

The chlorinated insecticide dichlorodiphenyltrichloroethane (DDT) is dechlorinated to dichlorodiphenyldichloroethane (DDD) under anaerobic conditions in microsomes [10, 11]. However, the ability of this compound to covalently bind to microsomal components has not been reported. *In vivo*, total DDT metabolism involves both reductive and oxidative pathways [12], and although hepatotoxicity has not been related to DDT exposure, long-term exposure in mice has shown that DDT as well as its dechlorinated metabolites, DDD and DDE [1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethane], are carcinogenic [13, 14].

In view of the ability of DDT to be dechlorinated under reductive conditions, and the structural similarity of DDT to CCl_4, this study was undertaken in order to clarify the ability of DDT and its metabolite, DDD, to bind to microsomal components during metabolism. The results show that the reductive metabolism of DDT enhances the production of intermediates capable of binding to proteins and lipids, whereas DDD is metabolized to species which bind to protein only under aerobic conditions.

MATERIALS AND DISCUSSION

Animals. Male Sprague–Dawley rats weighing 200–250 g were purchased from Hilltop Farms (Scottsdale, PA) and fed a standard laboratory chow diet. For 4 days prior to killing the animals by decapitation, rats were allowed access to drinking water containing 0.2% sodium phenobarbital. Microsomes were prepared [2] and stored in 0.1 M Tris–HCl (pH 7.5) at −70° until use.

Chemicals. Dichlorodi[U-^{14}C]phenyltrichloroethane [^{14}C]DDT) was purchased from the Amersham Corp. Arlington Heights, IL. Analysis of [^{14}C]DDT (63 mCi/mmole) by high pressure liquid chromatography (HPLC) indicated a radiochemical purity greater than 98% as purchased. [^{14}C]DDT, therefore, was not further purified. Dichlorodi[U-^{14}C]phenyldichloroethane ([^{14}C]DDD) was extracted

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from microsomal mixtures following anaerobic incubation with [^{14}C]DDT using sodium dithionite as reducing agent. The extracted [^{14}C]DDD was chromatographed by HPLC as described below. The [^{14}C]DDD-containing fractions eluted by HPLC were collected, dried under nitrogen, and diluted with appropriate amounts of nonradiolabeled DDD in acetone. HPLC analysis indicated [^{14}C]DDD purity greater than 99%. Non-radioactive DDT was obtained from Applied Science Laboratories, State College, PA, and non-radioactive DDD and metyrapone were purchased from the Aldrich Chemical Co., Milwaukee, WI. Butylated hydroxytoluene (BHT) was obtained from the Calbiochem Co., La Jolla, CA.

Incubations. Incubations were carried out in 10-ml hypo-vials sealed with hycar septa (Pierce Chemical Co., Rockford, IL). Microsomal incubations contained (in a total volume of 3 ml) 0.5 to 6.0 mg microsomal protein in 0.1 M Tris–HCl (pH 7.4), [^{14}C]DDT or [^{14}C]DDD, and an NADPH-generating system consisting of 0.8 units glucose-6-phosphate dehydrogenase, 4.6 μmoles glucose-6-phosphate and 1.8 μmoles NADP. Some incubations contained 3–5 mg crystalline sodium dithionite. The reductant (NADPH or sodium dithionite) was omitted in the blank reactions.

Incubations were prepared by placing the reaction vials on ice and adding all components of the reaction mixture. The vials were sealed and repeatedly evacuated and purged with 100% prepurified nitrogen, or known mixtures of nitrogen and oxygen, using a Firestone valve apparatus (Pierce Chemical Co.). The gases containing various ratios of nitrogen and oxygen were mixed in an anesthesia machine, and oxygen concentrations were monitored with a Bio-Marine Industries 202R oxygen analyzer. Some aerobic incubations utilized atmospheric oxygen and were not sealed. Following purging of the vials, incubations were initiated by placing the vials in a Dubnoff metabolic shaker maintained at 37° and stopped after an incubation period of 15 min by placing the vials on ice.

Metabolite analysis. Radiolabeled compounds were extracted from the incubation mixtures three times with 2.5 vol. of diethyl ether. Extracted metabolites in ether were dried under a stream of nitrogen and dissolved in hexane for HPLC analysis. Greater than 97% of radioactive DDT could be recovered from the microsomal incubations by this procedure. DDT and DDD were separated with a Beckman 340 HPLC system equipped with a 5 μm Altex Ultrasphere ODS column (4.6 mm × 15 cm). Compounds were eluted isocratically with 85% acetronitrile in water at a flow rate of 1 ml/min. Fractions were collected, and Safety-Solve (Research Products International Corp., Mt. Prospect, IL), was added for scintillation counting. Compound elution was monitored by u.v. absorbance at 210 nm, and radiolabeled compounds were identified by comparison of their retention times to those of authentic standards.

Binding assays. Lipids were recovered from the incubation mixtures by extraction with 3 vol. of chloroform–methanol (2/1, v/v). The lipid extract was dried under nitrogen, and 5–7 mg silica gel H was added. The phospholipids bound to the silica gel were washed with 5 ml of acetone and centrifuged at 500 *g* for 10 min. The washing procedure was performed three times to ensure removal of unbound radiolabeled compounds. Lipids were redissolved in 2 ml of methanol, and aliquots were taken for scintillation counting. Phosphate was determined by the method of Lowry *et al.* [15]. Greater than 85% of the phospholipid phosphate initially extracted was recovered following the washing procedure.

Following lipid extraction the microsomal protein was solubilized in 1% sodium dodecyl sulfate (SDS) and precipitated with 5 vol. of acetone. The precipitated protein was centrifuged (500 *g*) and resolubilized in 1% SDS. The solubilization/precipitation procedure was repeated two additional times to remove all unbound radioactive compounds. Aliquots of the final protein solutions were taken for scintillation counting. Protein was determined by the method of Lowry *et al.* [16]. Treatment of the protein with trichloroacetic acid (10%) had no effect on the amount of bound material; therefore, protein acidification was not routinely performed.

Liquid scintillation spectrometry. Radioactive samples were counted on a Beckman LS-7500 liquid scintillation counting system. Disintegrations per minute were determined from the system's H-number method of external standardization and quantities of radiolabeled compounds reported on a molar basis.

RESULTS

Preliminary studies demonstrated that hepatic microsomes from phenobarbital-treated rats exhibited a greater capacity to activate DDT to species which bind to microsomal components than microsomes from untreated rats. Therefore, microsomes from phenobarbital-treated rats were used in this study. Cytochrome P-450 levels were 1.9 nmoles/mg microsomal protein. The binding of DDT to microsomal proteins and lipids under nitrogen and air as a function of microsomal protein concentration is shown in Fig. 1. Binding was approximately linear up to a microsomal protein concentration of 5 mg/ml, and binding to both microsomal protein and lipids was enhanced under anaerobic conditions. The reactive intermediate formed anaerobically preferentially bound to lipids. Binding as a function of DDT concentration (Fig. 2) demonstrated binding nearing saturation under either air or nitrogen (2 mg microsomal protein/ml) at 100 μM DDT during the 15-min incubation period.

Analysis of DDT metabolite formation under anaerobic incubation conditions revealed that DDT was rapidly converted to DDD and that DDD accounted for greater than 95% of the anaerobic metabolites. Essentially no DDE was formed under anaerobic conditions. Figure 3 shows metabolite binding and DDD formation as a function of decreasing oxygen tensions and indicates that lipid binding and DDD formation were inversely dependent upon oxygen tension. Little reductive activation of DDT occurred at atmospheric oxygen levels.

As shown in Table 1, binding of DDT to microsomal components required NADPH and was com-

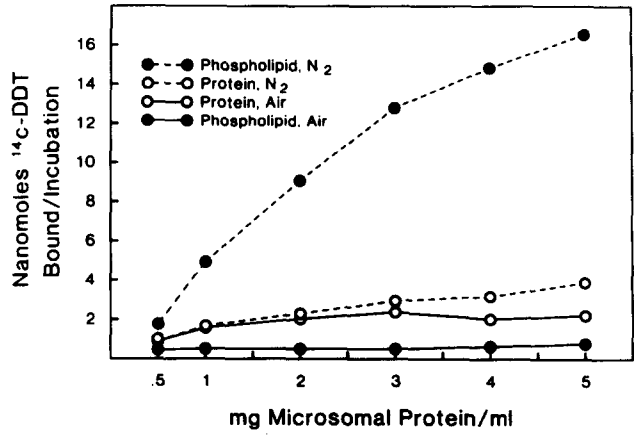

Fig. 1. Binding of [^{14}C]DDT to microsomal lipids and proteins under air and nitrogen with increasing amounts of microsomal protein. Incubations contained 75 μM [^{14}C]DDT (0.1 μCi) and were carried out for 15 min. Values represent total binding per incubation and are the average of duplicate determinations.

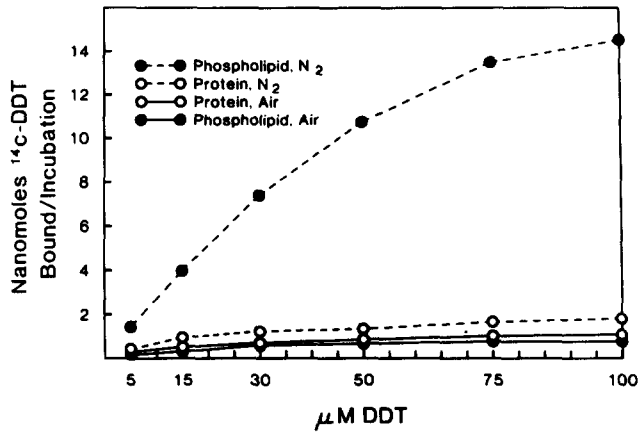

Fig. 2. Binding of [^{14}C]DDT to microsomal lipids and proteins under air and nitrogen with increasing amounts of [^{14}C]DDT (0.1 μCi). Incubations contained 2 mg microsomal protein/ml (3 ml total) and were incubated for 15 min. Values represent total binding per incubation and are the averages of duplicate determinations.

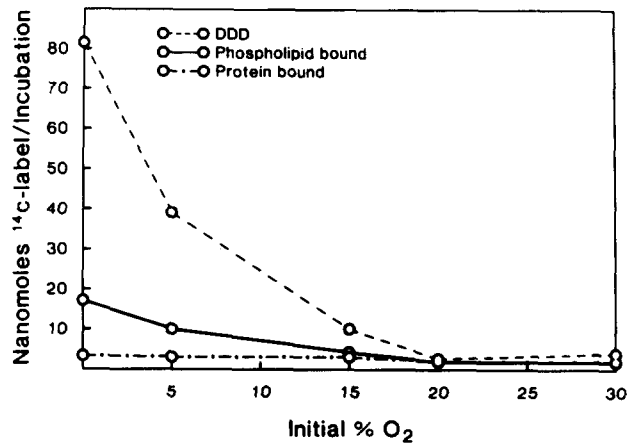

Fig. 3. [^{14}C]DDT protein and phospholipid binding, and DDD formation in the presence of various oxygen levels. "Initial $\%O_2$" represents the percent of oxygen in nitrogen of the gas phase at the initiation of the incubation. Incubations contained (in a total volume of 3 ml) 6 mg microsomal protein, 75 μM [^{14}C]DDT (0.1 μCi), and an NADPH-generating system. Incubation time was 15 min. Values reported represent the average of duplicate determinations and are the total binding and DDD formation in each incubation.

Table 1. Effects of various incubation conditions on [^{14}C]DDT protein and phospholipid binding, and [^{14}C]DDD formation in hepatic microsomes*

	[^{14}C]DDT			
	Protein-bound (pmoles)	Phospholipid-bound (pmoles)	DDD (total nmoles recovered)	DDT
Nitrogen				
Blank (−NADPH)	78 ± 2	240 ± 15	1.32 ± 0.44	210.06 ± 18.71
NADPH	594 ± 1	19,000 ± 80	73.20 ± 2.50	104.73 ± 16.34
NADPH + BHT (1 mM)	372 ± 1	5,170 ± 15	25.43 ± 0.70	126.70 ± 5.93
NADPH + metyrapone (1 mM)	72 ± 1	447 ± 33	2.71 ± 0.31	227.38 ± 3.49
Sodium dithionite	1,032 ± 14	12,010 ± 22	150.90 ± 15.31	17.48 ± 0.50
Air				
Blank (−NADPH)	48 ± 1	580 ± 27	1.69 ± 1.05	216.43 ± 5.48
NADPH	408 ± 2	4.070 ± 32	3.25 ± 0.19	210.13 ± 10.84
NADPH + BHT (1 mM)	84 ± 0.2	710 ± 10	2.97 ± 0.47	226.72 ± 3.61
NADPH + metyrapone (1 mM)	54 ± 0.7	567 ± 6	1.34 ± 0.23	186.29 ± 4.74

* Values represent the means ± standard errors of triplicate determinations. "Protein-bound" represents [^{14}C]DDT bound to total protein in each incubation and "Phospholipid-bound" represents [^{14}C]DDT bound to total recovered phospholipid per incubation. Incubation mixtures contained (in a total volume of 3 ml) 6 mg microsomal protein, 225 nmoles [^{14}C]DDT (0.1 μCi), and an NADPH-generating system or sodium dithionite. Incubations were carried out for 15 min.

pletely inhibited by metyrapone under both aerobic and anaerobic conditions. DDD was formed anaerobically where binding was enhanced and sodium dithionite reduced cytochrome P-450 readily converted DDT to DDD. Both protein and lipid binding were likewise supported by cytochrome P-450 reduced with dithionite. BHT partially inhibited DDT binding and DDD formation anaerobically; however, aerobically BHT was a more effective inhibitor of binding. DDT incubated in the presence of only sodium dithionite was not converted to DDD (data not shown).

DDD binding to microsomal protein is shown in Table 2. Unlike DDT, DDD bound to microsomes to a greater degree in microsomal incubations containing oxygen. BHT and metyrapone, as well as a nitrogen atmosphere, inhibited this binding. In contrast to DDT binding in microsomal incubations containing sodium dithionite, this artificial electron donor was ineffective in supporting DDD binding or in producing any DDD metabolites. Small quantities of radioactivity were associated with the phospholipid fraction in DDD-containing incubations carried out with NADPH under aerobic conditions; however, no reductive binding to lipids could be detected.

DISCUSSION

The pattern of reactivity of DDT during its microsomal metabolism indicates that this apparently non-hepatotoxic compound was reduced under low oxygen conditions to produce large quantities of reactive intermediates. These reactive intermediates bound readily to microsomal lipids. In the presence of oxygen, binding of DDT to microsomal protein occurred, but at low rates. Although low levels of reductive metabolites other than DDD were detected in this study, the anaerobic formation of DDD with lipid binding suggests that a reductive pathway for DDT reactive metabolite formation involves a monodechlorination step leading to the formation of DDD. DDD itself was not reduced. Similar to the formation of $CHCl_3$ from CCl_4 [4] and of CF_3CHCl from halothane [17] during the reductive activation of these compounds, it is proposed that DDT is reduced to a reactive free-radical intermediate by a one-electron transfer to DDT from cyto-

Table 2. Metabolism-dependent binding of [^{14}C]DDD to microsomal protein*

[^{14}C]DDD (nmoles bound/6 mg protein)			
Air		Nitrogen	
Blank (−NADPH)	1.33 ± 0.03	Blank (−NADPH)	1.58 ± 0.10
NADPH	5.42 ± 0.23	NADPH)	3.03 ± 0.24
NADPH + BHT (1 mM)	2.52 ± 0.45	Sodium dithionite	1.59 ± 0.18
NADPH + metyrapone (1 mM)	1.31 ± 0.06		

* Values represent the means ± standard errors of triplicate determinations of [^{14}C]DDD bound to total protein in each incubation. Incubations contained (in a total volume of 3 ml) 6 mg microsomal protein, 252 nmoles [^{14}C]DDD (0.01 μCi), and an NADPH-generating system or sodium dithionite. Incubations were carried out for 15 min.

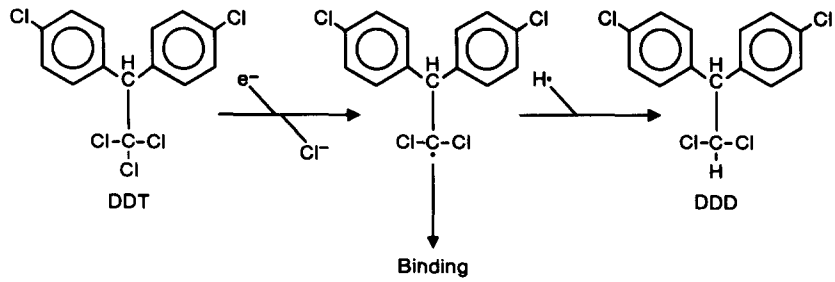

Fig. 4. Proposed pathway for the reductive formation of DDD and a DDT reactive intermediate by cytochrome P-450.

chrome P-450 (Fig. 4). This reduction would release a chloride ion. The free-radical thus formed could either bind to unsaturated bonds of fatty acids or to a lesser extent, protein, or add a hydrogen atom to form DDD.

The involvement of cytochrome P-450 in both the oxidative and reductive metabolism of DDT is confirmed in this study by the requirement for NADPH and the ability of metyrapone, a compound which binds to reduced cytochrome P-450 [18], to inhibit metabolism. Likewise, other studies have shown that the anaerobic formation of DDD from DDT in microsomes requires NADPH, is enhanced in microsomes from phenobarbital-treated rats compared to untreated rats, and is inhibited by carbon monoxide [10].

The ability of oxygen, which binds to reduced cytochrome P-450 heme iron [19], to inhibit reduction suggests that during the reductive process DDT directly interacts with the reduced heme iron of cytochrome P-450 to accept electrons. Such an interaction would not be expected to be related to the ability of DDT to form a Type I binding spectrum in oxidized microsomes. Type I binding is distinct from the binding of ligands to the sixth coordination site of cytochrome P-450 heme iron, but it is associated with cytochrome P-450 substrate oxidation [19]. Accordingly, BHT, which exhibits a Type I binding spectrum [20] and is oxidatively metabolized [21], has a limited ability to inhibit DDT reduction. BHT does, however, effectively inhibit the oxidation of DDT, as well as DDD, either by competitively inhibiting oxidation or by scavenging activated oxygen species [20]. Similarly, it has been reported that the Type I compound, 2-hydroxyestradiol-17β, inhibits microsomal CCl_4 oxidative activation but not reductive CCl_4 activation [22] and, for tertiary amine N-oxides, Type I compounds have no effect on reduction whereas Type II compounds, which coordinate to cytochrome P-450 heme iron [19], significantly inhibit N-oxide reduction [23]. The enhanced rate of DDD formation in microsomes when excess sodium dithionite was used further supports the fact that reductive metabolism proceeds with cytochrome P-450 maintained in the reduced state.

Even though DDT is dechlorinated by active cytochrome P-450, evidence suggesting that DDT interacts with reduced cytochrome P-450 heme, but in a nonenzymatic manner, has been demonstrated in studies which show that reduced heme in solution can also catalyze the formation of DDD from DDT [24]. Other halogenated compounds such as halothane [25] and a variety of tetrahalogenated methanes [26], which are also reductively dehalogenated by cytochrome P-450 [27], likewise release halide ions by interacting with reduced heme. Dehalogenation of these compounds capable of undergoing similar type reductions may only require the reduced heme moiety of cytochrome P-450 in the absence of oxygen or any other agent which competitively binds to reduced heme iron.

The ability of chlorinated ethanes to be reduced has been shown to be related to the degree of halogenation of both those carbons undergoing reduction and adjacent carbons [28]. For DDT, dechlorination by either reduced cytochrome P-450 or heme apparently relates to both the trichlorinated carbon and the total influence of the para-chlorphenyl substituents of the molecule. DDD is not activated under anaerobic conditions in microsomes but the fact that methoxychlor [2,2-bis(*p*-methoxyphenol)-1,1,1-trichloroethane] binds covalently to microsomes only under conditions of oxidative metabolism [29] and 1,1,1-trichlorethane is not reductively dechlorinated [28] shows that the trichlorinated carbon of DDT in itself is not responsible for dechlorination. The more chlorinated ethanes such as hexachloroethane and pentachloroethane [28], in addition to DDT, release chloride ions under reductive conditions, indicating that the overall degree of chlorination contributes to the instability of DDT and other ethanes to reduction.

In conclusion, this study demonstrates that DDT was metabolized and activated to species that bind preferentially to microsomal lipids under low oxygen conditions in a manner similar to the hepatotoxic compounds CCl_4 and halothane. In view of the fact (1) that DDD is formed *in vivo* in rat livers [30], (2) that only slightly lowered oxygen levels stimulate DDD formation in microsomes, and (3) that oxygen tensions in the rat liver average approximately 25 torr and can be as low as 5 torr [31], the reduction and reactive intermediate formation of DDT most likely occur to a significant extent *in vivo*. The lack of hepatotoxicity exhibited by DDT may indicate that such pathways and protein or lipid binding, *per se*, may not be critical to hepatotoxicity. The activation of DDT and DDD by microsomes under aerobic conditions through such intermediates as epoxides [32] or acyl chlorides may be of conse-

quence in the carcinogenicity associated with long-term animal exposure to these compounds.

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