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INTERACTIONS OF DIETHYLPHENYLPHOSPHINE WITH PURIFIED, RECONSTITUTED MOUSE LIVER CYTOCHROME P-450 MONOOXYGENASE SYSTEMS

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Abstract—Purified mouse liver cytochrome P-450 reconstituted with purified NADPH–cytochrome P-450 reductase and phosphatidylcholine metabolized diethylphenylphosphine to diethylphenylphosphine oxide. NADPH was required for the reaction and the amount of oxide formed was time and cytochrome P-450 dependent. Purified phenobarbital-induced cytochrome P-450 produced more oxide per nmole enzyme than any of the purified uninduced cytochrome P-450s. The phosphine oxide was also formed in lesser amounts in incubation mixtures containing only NADPH–cytochrome P-450 reductase and NADPH. Diethylphenylphosphine bound to oxidized purified phenobarbital-induced cytochrome P-450 and uninduced cytochrome P-450 with K_s values of 16 μM and 11–18 μM respectively. Diethylphenylphosphine was also a competitive inhibitor of *p*-nitroanisole O-demethylation catalyzed by a reconstituted phenobarbital-induced cytochrome P-450-dependent monooxygenase system, with a K_i value of 5 μM. The phosphine oxide produced no observable optical difference spectrum with oxidized phenobarbital-induced cytochrome P-450 and caused no inhibition of *p*-nitroanisole O-demethylation.

Microsomal oxidations of xenobiotics are catalyzed by the cytochrome P-450-dependent monooxygenase system and the FAD-containing monooxygenase. The substrate specificities of these two enzymes toward nitrogen- and sulfur-containing compounds overlap considerably [1–3], while their relative effectiveness as phosphorus oxidases has not yet been determined. The FAD-containing monooxygenase does not metabolize the thiono moiety of phosphorodithioates, but it will catalyze oxidative desulfuration of phosphonodithioates such as the insecticide fonofos [4].

Metabolism of diphenylmethylphosphine and 3-dimethylaminopropyldiphenylphosphine by rat liver microsomes was reported by Wiley and coworkers [5], with oxide formation attributed to cytochrome P-450. More recent studies [6] have shown that diethylphenylphosphine is an excellent substrate for purified pig liver microsomal FAD-containing monooxygenase with an apparent K_m value of less than 2.5 μM. This led us to reinvestigate the contributions of cytochrome P-450 to diethylphenylphosphine oxidation using purified cytochrome P-450 fractions from control and phenobarbital (PB)-induced mice.

Diethylphenylphosphine is known to produce unusual optical difference spectra with phenobarbital-induced rat liver microsomes and the results of these ligand binding studies further suggested that PB-induced microsomes contain heterogeneous cytochrome P-450 [7]. Beumel and coworkers [8] showed that piperonyl butoxide and several isocyanides give rise to different spectral interactions with different purified reconstituted cytochrome P-450 fractions. Furthermore, not all the isozymes from uninduced mouse liver produce the optical difference spectra characteristic of microsomal interaction with these same ligands. This led us to study spectral interactions of diethylphenylphosphine with purified uninduced and PB-induced cytochrome P-450 isozymes.

Mansuy and coworkers [7] found that diethylphenylphosphine is a competitive inhibitor of O-deethylation of 7-ethoxycoumarin in PB-induced rat liver microsomes. The inhibition (of *p*-nitroanisole demethylation) by purified reconstituted cytochrome P-450-dependent monooxygenase system has also been studied.

MATERIALS AND METHODS

Materials. Diethylphenylphosphine was purchased from the Pressure Chemical Co. (Pittsburgh, PA). The oxide was prepared by the method of Smyser and Hodgson [6]. NADPH and dilauroylphosphatidylcholine were purchased from the Sigma Chemical Co. (St. Louis, MO) and Sep-pak C_{18} cartridges from Waters Associates (Milford, MA).

Enzyme purification. Cytochrome P-450 isozymes were purified from the livers of uninduced and PB-induced male Dub:ICR mice (Dominion Laboratories, Dublin, VA) by a slight modification of the method of Levi and Hodgson [9]. The A_0 fraction consists of cytochrome P-450 which does not bind to the DE-52 column under the conditions used. Cytochrome P-450 reductase was purified by the method of Yasukochi and Masters [10] with the following modifications: the final column wash and elution buffer contained 0.1% sodium cholate and no Emulgen. The reductase was then dialyzed extensively to remove detergent.

Metabolite production. Incubation mixtures con-

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tained 100 μM diethylphenylphosphine, 0.4 nmole cytochrome P-450, 2400 units cytochrome P-450 reductase, 50 μg of dilauroylphosphatidylcholine (DPC), 50 mM potassium phosphate, pH 7.6, and an NADPH-regenerating system [11] in a total volume of 2 ml, unless otherwise specified. DPC, cytochrome P-450 reductase, and cytochrome P-450 were combined and preincubated at 37° for 1 min. Buffer and substrate were then added, and the reaction was initiated with the NADPH-regenerating system. Unless otherwise specified, samples were incubated for 10 min at 37°.

Isolation and analysis of diethylphenylphosphine oxide. Incubation mixtures were applied to Sep-pak C_{18}-cartridges, washed with 2 ml of high performance liquid chromatography (HPLC)-grade water and then eluted with 2 ml of HPLC-grade methanol. Methanol samples containing the oxide were analyzed by reverse phase HPLC according to the method of Smyser and Hodgson [6].

Optical difference spectra. Spectra were recorded on an SLM/Aminco DW-2C UV/VIS spectrophotometer. Sample and reference cuvettes contained 50 mM potassium phosphate buffer, pH 7.6, and 0.39 nmole cytochrome P-450 in a final volume of 1 ml. The phosphine, in acetone, was added to the sample cuvette while an equal volume of acetone was added to the reference cuvette. The volume of added acetone was always less than 15 μl.

Inhibition of p-*nitroanisole demethylation.* *p*-Nitrophenol formation was monitored at 405 nm and 37°. Assay mixtures of three replicates contained 0.2 nmole cytochrome P-450, 1200 units cytochrome P-450 reductase, 25 μg DPC, NADPH-regenerating system [11], and 50 mM potassium phosphate buffer, pH 7.6, in a total volume of 1 ml. Diethylphenylphosphine in acetone or diethylphenylphosphine oxide in water was added to final concentrations of 5 and 10 μM or 10 and 100 μM respectively. *p*-Nitroanisole in acetone was added to give final concentrations between 10 and 1000 μM. DPC, cytochrome P-450 reductase and cytochrome P-450 were preincubated at 37° for 1 min. Buffer, substrate, and inhibitor were added, and the reaction was initiated by adding the NADPH-regenerating system. Kinetic constants were obtained using the KINFIT program of Knack and Rohm [12].

RESULTS AND DISCUSSION

Initial studies were conducted to determine the conditions for maximum diethylphenylphosphine conversion to diethylphenylphosphine oxide by purified reconstituted mouse liver cytochrome P-450. A time course (Fig. 1) shows that, under these experimental conditions, the reaction was complete in approximately 20 min. Oxide formation was also dependent upon cytochrome P-450 content up to a concentration of 0.3 nmole/ml (Fig. 2). The time course and cytochrome P-450 content studies indicate that conversion of diethylphenylphosphine to the oxide is an enzymatic process, although significant nonenzymatic oxide formation also occurred. Furthermore, NADPH was required for metabolism (Table 1).

Attempts were made to identify the factors affecting nonenzymatic phosphine oxidation (Table 1). Diethylphenylphosphine oxide was produced under all the experimental conditions, even in buffer alone. These results were not unexpected since phosphines are known to spontaneously produce oxides and other products [13]. Wiley and coworkers [5] reported that diphenylmethylphosphine also degrades slowly in the presence of buffer alone but that the degradation rate increases in the presence of NADPH and microsomes. Incubation with NADPH and cytochrome P-450 reductase produced more oxide than buffer alone or the NADPH regenerating system alone. The reductase is known to form H_2O_2, a factor which may account for the additional oxide formation.

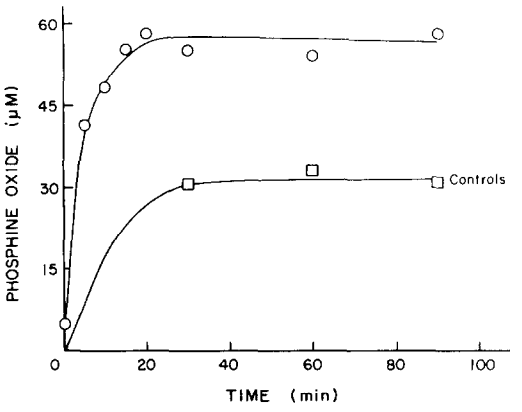

Fig. 1. Time course of diethylphenylphosphine oxide production by reconstituted cytochrome P-450 monooxygenase (A_0 fraction). Controls contained phospholipid, buffer, NADPH-regenerating system and 100 μM diethylphenylphosphine. Experimental samples also contained 0.3 nmole P-450 and 1700 units reductase. Both were incubated as described in Materials and Methods.

Diethylphenylphosphine was incubated with various reconstituted purified cytochrome P-450 fractions from PB-induced and uninduced mouse liver

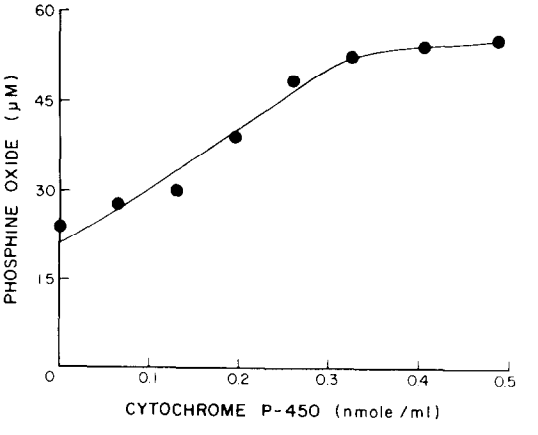

Fig. 2. Effect of cytochrome P-450 concentration on diethylphenylphosphine oxide production by reconstituted cytochrome P-450 monooxygenase (A_0 fraction). Samples containing 6000 units reductase/nmole P-450 were incubated as described in Materials and Methods.

Table 1. Enzymatic and nonenzymatic diethylphenylphosphine oxide formation

Incubation mixture	Oxide produced (μM)
Complete*	73.3 ± 7.1†
−Reductase	20.0 ± 1.7
−Cytochrome P-450	28.7 ± 2.9
−NADPH-regenerating system	21.0 ± 2.6
NADPH-regenerating system only	16.3 ± 4.0
Buffer only	14.2 ± 2.4

* Complete incubation mixtures contained PB-induced cytochrome P-450 (0.4 nmole), cytochrome P-450 reductase (2400 units), DPC, and an NADPH-regenerating system. An incubation time of 10 min was used, under conditions described in Materials and Methods.

† Each value represents the mean ± S.D. of three experiments.

microsomes (Table 2). The PB-induced cytochrome P-450 was the cytochrome best able to catalyze conversion of diethylphenylphosphine to the phosphine oxide. Previously we had shown [6] that diethylphenylphosphine is an excellent substrate, with a K_m value of less than 2.5 μM, for the liver microsomal FAD-containing monooxygenase. Furthermore, with the FAD-containing enzyme, the reaction goes to over 70% completion with similar substrate concentrations to those used in the experiments reported above with cytochrome P-450. Thus, it appears that the contribution of the FAD-containing monooxygenase to diethylphenylphosphine oxide formation in uninduced microsomes is more significant than that of cytochrome P-450.

As seen in the optical difference spectra, diethylphenylphosphine bound to oxidized forms of both PB-induced and uninduced cytochrome P-450s (Fig. 3). With purified PB-induced P-450, two major absorption bands occurred at 371 and 454 nm and a major trough at 413 nm. This difference spectrum is similar to that formed with PB-induced rat liver microsomes [7]. The peaks and trough shifted to 373, 458 and 415 nm, respectively, with the A_0 fraction of purified uninduced cytochrome P-450. The spectrum of the A_1 fraction was similar to that of the A_0

Table 2. Diethylphenylphosphine oxide formation by reconstituted purified cytochrome P-450 fractions

Cytochrome P-450 fraction	Oxide detected (μM)
PB-induced	66.3 ± 5.0
Uninduced	
A_0	45.0 ± 3.6
A_1	47.7 ± 2.1
B_1	49.3 ± 2.5
B_2	42.0 ± 4.6
B_3	45.0 ± 2.6
Control 1 (−P-450)	34.0 ± 0.0
Control 2 (−P-450, −reductase)	15.7 ± 0.6

All samples contained a final cytochrome P-450 content of 0.2 nmole/ml. An incubation time of 10 min was used under conditions described in Materials and Methods. Each value represents the mean ± S.D. of three experiments.

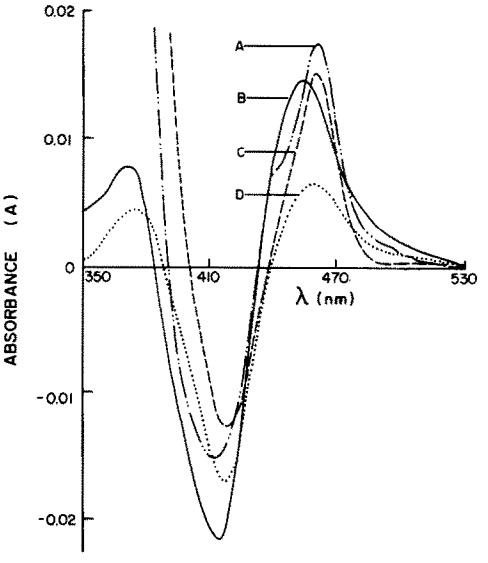

Fig. 3. Optical difference spectra of 68 μM diethylphenylphosphine with uninduced and induced purified cytochrome P-450. Key: (A) PB-induced (reduced); (B), PB-induced (oxidized); (C) uninduced A_0 (reduced); and (D) uninduced A_0 (oxidized).

fraction. A shoulder on the 459 nm peak of the dithionite-reduced PB-induced cytochrome P-450 was not present in spectra with dithionite-reduced uninduced A_0 or A_1 cytochrome P-450. This shoulder was also observed with PB-induced rat liver microsomes [7]. Diethylphenylphosphine oxide produced no observable optical difference change with oxidized PB-induced cytochrome P-450.

To determine the affinity of diethylphenylphosphine binding to cytochrome P-450, optical difference spectra were recorded with increasing concentrations of the phosphine (Fig. 4). Spectral dissociation constants for diethylphenylphosphine binding to uninduced A_0 cytochrome P-450 fraction and PB-induced cytochrome P-450 were 11 and

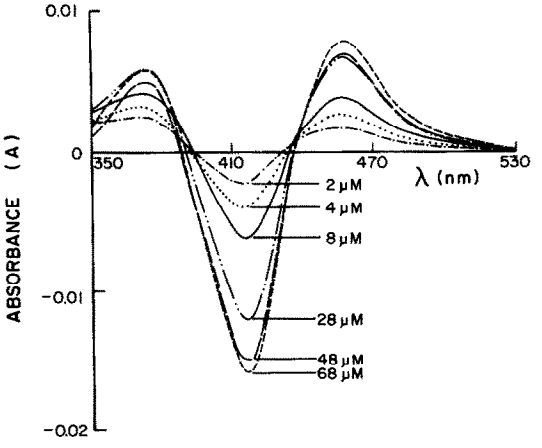

Fig. 4. Effect of diethylphenylphosphine concentration on the difference spectrum of purified uninduced cytochrome P-450 (A_1 fraction).

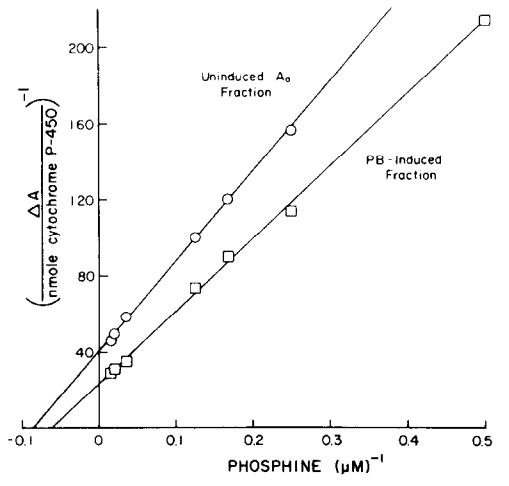

Fig. 5. Spectral binding of diethylphenylphosphine to purified, oxidized, uninduced and PB-induced cytochrome P-450 monooxygenase. ΔA is the difference between peak at 454 nm for PB-induced cytochrome P-450 or 458 nm for uninduced cytochrome P-450 and baseline at 530 nm.

16 μM respectively (Fig. 5). The K_s value for phosphine binding to uninduced A_1 cytochrome P-450 fraction was 18 μM (results not shown).

It is apparent that diethylphenylphosphine does bind to the oxidized forms of both PB-induced and uninduced P-450 with high affinity. The differences in binding affinity do not appear sufficiently significant to explain the differences in phosphine oxide formation between uninduced and PB-induced cytochrome P-450.

Interactions between the phosphine and cytochrome P-450 may inhibit other reactions catalyzed by cytochrome P-450. The effects of two concentrations of diethylphenylphosphine (5 and 10 μM) on *p*-nitroanisole demethylation by purified PB-induced cytochrome P-450 are shown in Fig. 6. At the concentrations used, diethylphenylphosphine was an excellent competitive inhibitor with a K_i value of 5–

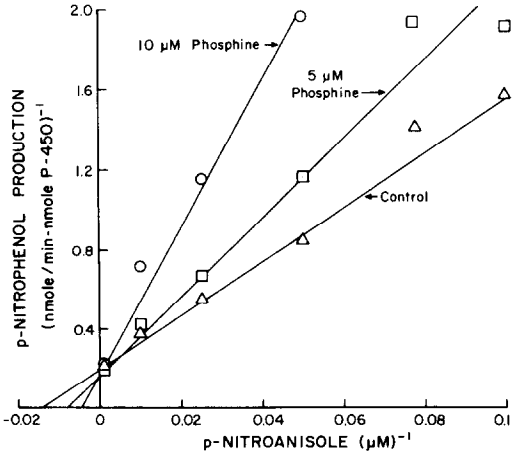

Fig. 6. Inhibition of *p*-nitroanisole demethylation by diethylphenylphosphine. Samples were incubated as described in Materials and Methods.

10 μM. The phosphine oxide, at concentrations of 10 and 100 μM, had no effect on *p*-nitroanisole demethylation catalyzed by purified PB-induced cytochrome P-450 (results not shown). Diethylphenylphosphine at a concentration of 100 μM was shown previously to be a competitive inhibitor of 7-ethoxycoumarin O-deethylation in PB-induced rat liver microsomes [7] with a K_i value of 2 μM.

This study confirms that purified reconstituted cytochrome P-450 monooxygenase systems catalyze oxidation of diethylphenylphosphine to its oxide. It is important to use a purified system for this study since phosphines can spontaneously react with other substances present in incubation mixtures, and heat treatment of microsomes at 50° for 1 min, which should inactivate the FAD-containing monooxygenase [14], may leave slight residual activity. Since diethylphenylphosphine is an excellent substrate for microsomal FAD-containing monooxygenase with a K_m value of less than 2.5 μM [6], any residual FAD-containing monooxygenase activity in heat-treated microsomes could be attributed erroneously to cytochrome P-450. Since purified reconstituted cytochrome P-450-dependent monooxygenase systems have no FAD-containing monooxygenase, this problem does not occur. This study and previous work [6] confirm that both cytochrome P-450 and the FAD-containing monooxygenase have phosphorus oxidase activity.

It is apparent that, while diethylphenylphosphine interacts readily with cytochrome P-450 and is an effective inhibitor of at least one cytochrome P-450 mediated reaction, it is not readily metabolized to the oxide. Based on this and previous studies it must be concluded that, *in situ*, the FAD-containing monooxygenase is much more important than cytochrome P-450 for the oxidation of phosphines. At the same time, it is an effective inhibitor, thus raising the possibility of complex interactions between these two enzymes during the course of phosphine oxidation.

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