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Studies of the Metabolism of Diethyl *p*-Nitrophenyl Phosphorothionate (Parathion) and Benzphetamine Using an Apparently Homogeneous Preparation of Rat Liver Cytochrome P-450: Effect of a Cytochrome P-450 Antibody Preparation

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SUMMARY

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A procedure is described for simultaneous purification of cytochrome P-450 and partial purification of NADPH-cytochrome *c* reductase from the livers of phenobarbital-treated rats. Using a reconstituted system containing these enzymes, we have examined the metabolism of benzphetamine and parathion. Both dilauroylphosphatidylcholine and deoxycholate must be present in the reconstituted system for maximal metabolism of these two substrates. Excluding deoxycholate from an otherwise complete reconstituted system decreases the rate of metabolism of benzphetamine by about 80%, and of parathion by approximately 40%. The maximal rates of metabolism of benzphetamine and parathion using this rat liver reconstituted system resemble those observed using reconstituted systems containing cytochrome P-450 and NADPH-cytochrome *c* reductase similarly prepared from the livers of phenobarbital-treated rabbits. An antibody (or antibodies) to the rat liver cytochrome P-450 has been prepared by injecting this enzyme into rabbits. Using Ouchterlony double-diffusion analysis, this antiserum exhibits good reactivity against rat liver cytochrome P-450 but poor reactivity with apparently homogeneous cytochrome P-450 purified from the livers of phenobarbital-treated rabbits. In contrast, using quantitative immunoprecipitation, some affinity of the rat liver cytochrome P-450 antibody for rabbit liver cytochrome P-450 can be demonstrated. The antibody preparation also inhibits the ability of a rabbit liver reconstituted system to metabolize parathion. However, in all cases, the antibody preparation is much more active against rat liver cytochrome P-450.

INTRODUCTION

Using a minor modification of the method developed by Imai and Sato for purification of rabbit liver cytochrome P-450 (1, 2), we have obtained an apparently homogeneous preparation of cytochrome

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P-450 from the livers of phenobarbital-treated rats.

Using a reconstituted mixed-function oxidase system containing this apparently homogeneous rat liver cytochrome P-450, we have examined the metabolism of the substrates benzphetamine and parathion (diethyl *p*-nitrophenyl phosphorothionate). Benzphetamine metabolism was examined because its use as a substrate for reconstituted mixed-function oxidase systems has been frequently reported (3, 4) and is a convenient means of comparison of the activity of reconstituted systems containing cytochrome P-450 and NADPH–cytochrome *c* reductase prepared from different species and from the same species by different procedures. The metabolism of parathion by mammalian liver homogenates and microsomes has been extensively studied (5–11). This commercially important organophosphate insecticide is metabolized *in vitro* by mammalian hepatic mixed-function oxidase systems to diethyl *p*-nitrophenyl phosphate (paraoxon) (5–9, 12), diethylphosphorothioic acid (7–9), diethylphosphoric acid (12), and *p*-nitrophenol (7, 8). Diethylphosphoric acid and *p*-nitrophenol are also formed by hydrolysis of the metabolite paraoxon by an esterase(s) present in various tissues (13, 14) and particulate fractions of tissues, including the hepatic endoplasmic reticulum (14). A study of parathion metabolism using a reconstituted mixed-function oxidase system containing an apparently homogeneous preparation of cytochrome P-450 isolated from rabbit liver has recently been reported (12). The results of this study indicated that parathion was metabolized to paraoxon, diethylphosphorothioic acid, and diethylphosphoric acid by a single species of cytochrome P-450. It was of interest to determine whether the metabolism of parathion by a reconstituted system containing an apparently homogeneous preparation of rat liver cytochrome P-450 would be similar to that seen with rat liver microsomes (7–10) and the rabbit liver reconstituted system (12).

Antiserum to rat liver cytochrome P-450 has also been prepared by injection of the purified enzyme into rabbits. The ability of this partially purified antibody preparation to cross-react with purified rabbit liver cytochrome P-450 has been examined. The ability of this antibody preparation to inhibit benzphetamine and parathion metabolism by reconstituted systems containing the purified rabbit and rat liver cytochrome P-450 has also been studied.

MATERIALS AND METHODS

Isolation of microsomes. Male Sprague-Dawley rats weighing 150–200 g were given sodium phenobarbital, 0.1% in their drinking water, for 3 days. Liver microsomes were prepared as described previously (15), except that they were washed once by resuspension in 1.15% potassium chloride solution containing 1 mM EDTA and resedimentation at 105,000 × *g* for 1 hr.

Purification of cytochrome P-450 from phenobarbital-treated rabbits. The cytochrome P-450 from phenobarbital-treated rabbits was purified as described previously (12). The method used was a minor modification of the method of Imai and Sato (1, 2).

Purification of cytochrome P-450 and NADPH–cytochrome c reductase from livers of phenobarbital-treated rats. Cytochrome P-450 was purified from the livers of phenobarbital-treated rats essentially by the method described by Imai and Sato for isolation of this enzyme from rabbits (2), except that 0.8% rather than 0.6% (w/v) sodium cholate (recrystallized twice from hot 50% ethanol) was included in the reagent used for equilibrating the ω-amino-*n*-octyl-Sepharose 4B affinity column. In addition, 100 mM rather than 80 mM potassium phosphate was used for washing the hydroxylapatite column, and the eluate from the column was diluted 5-fold with 20% glycerol containing 0.2% Emulgen 913. Finally, the solubilized microsomes were centrifuged at 105,000 × *g* for 1 hr.

Cytochrome P-450 was also prepared by a procedure which allowed for the simultaneous purification of this enzyme and NADPH–cytochrome *c* reductase. This procedure is a modification of that previously described for the simultaneous prep-

aration of these two enzymes from rabbit liver (12). The rat liver cytochrome P-450 was eluted from the ω-amino-*n*-octyl-Sepharose 4B affinity column as described by Imai and Sato (2) and further purified as described above. In contrast to the previous procedure using rabbit liver (12), the affinity column was next washed with an additional 1500 ml of 50 mM potassium phosphate buffer (pH 7.25) containing 0.3% sodium cholate, 0.2% sodium deoxycholate, and 20% glycerol. About 15–20% of the total NADPH-cytochrome *c* reductase activity placed on the column was eluted after 700–1000 ml of this buffer. The specific activity of these reductase fractions was considerably higher than those which eluted together with cytochrome P-450. Only the reductase eluted subsequent to cytochrome P-450 was used in further purification steps involving this enzyme. Those reductase fractions having an activity greater than 0.5 unit/ml were pooled and concentrated to about 5 ml using a Diaflo ultrafiltration apparatus equipped with a PM-30 membrane (Amicon). The concentrated sample was diluted 10-fold with 50 mM Tris-Cl buffer (pH 7.7) containing 0.2% Emulgen 913 and 20% glycerol, and solid potassium chloride was added to a final concentration of 150 mM. The sample was then applied to a DEAE-Sephadex A-50 column (1.5 × 11 cm) which had been equilibrated with the same buffer. The column was washed with 100 ml of 50 mM Tris-Cl buffer (pH 7.7) containing 175 mM potassium chloride, 0.2% Emulgen 913, and 20% glycerol. The reductase was eluted from the column using 50 mM Tris-Cl (pH 7.7) containing 300 mM potassium chloride, 0.2% Emulgen 913, and 20% glycerol. The fractions containing reductase activity were pooled, and to them was added 0.25 volume of 100 mM potassium phosphate (pH 7.25) containing 20% glycerol. The sample was then applied to a hydroxylapatite column (2.5 × 7.5 cm) which had been equilibrated with 20 mM potassium phosphate (pH 7.25) containing 0.2% Emulgen 913 and 20% glycerol. The column was washed first with 100 ml of 30 mM potassium phosphate (pH 7.25) containing 0.20% Emulgen 913 and 20% glycerol, followed successively by 100 ml of 30 mM and 100 ml of 75 mM potassium phosphate (pH 7.25) containing 20% glycerol. The reductase was then eluted by washing the column with 150 mM potassium phosphate (pH 7.25) containing 20% glycerol.

The specific content of different preparations of cytochrome P-450 prepared by this method ranged from 15.0 to 16.4 nmoles/mg of protein. These preparations were free of cytochrome b_5 and NADPH-cytochrome *c* reductase. An occasional sample had a very weak activity of NADH-cytochrome b_5 reductase as judged from NADH-ferricyanide reductase activity. The specific activity of NADPH-cytochrome *c* reductase prepared as described above ranged from 20.5 to 28.3 units/mg of protein among different preparations. The enzyme was free of NADH-cytochrome b_5 reductase, cytochrome b_5, and cytochrome P-450. The purity of NADPH-cytochrome *c* reductase ranged from 42% to 58%, based on the specific activity (48.8 units/mg of protein) of homogeneous rat liver NADPH-cytochrome *c* reductase reported previously (16). Only cytochrome P-450 prepared by the method for simultaneous purification of this enzyme and NADPH-cytochrome *c* reductase was used in the experiments described here.

A typical preparation of cytochrome P-450 and reductase retained 0.33 and 0.50 mg of Emulgen 913 per milligram of protein, respectively.

SDS[2]-polyacrylamide gel electrophoresis of the cytochrome P-450 and NADPH-cytochrome *c* reductase purified as described above was carried out using 7.5% gels and a buffer system consisting of 0.2 M Tris-acetate (pH 6.0) containing 0.1% (w/v) SDS. Prior to application to the gels, the enzymes were treated for 5 min at 100° with 20 mM Tris-acetate (pH 6.0) containing 2 M urea, 1% SDS, 0.002% bromphenol blue, and 1 mM EDTA. The cytochrome P-450 preparation (16.4 nmoles/mg of protein) showed only a single band on staining with Coomassie blue. The NADPH-cytochrome *c* reductase preparation (28.3

[2] The abbreviation used is: SDS, sodium dodecyl sulfate.

units/mg of protein) showed one major band and several minor bands with higher molecular weights. Using the appropriate protein standards, the average molecular weights of the cytochrome P-450 and NADPH-cytochrome *c* reductase were estimated to be 48,500 (seven separate determinations) and 79,000 (four separate determinations), respectively. As judged by their ability to metabolize benzphetamine, both cytochrome P-450 and NADPH-cytochrome *c* reductase prepared as described above were stable for at least 6 months when stored under nitrogen at −70°.

Preparation of antibody(ies) to rat liver cytochrome P-450. Adult female New Zealand rabbits were immunized according to the following schedule. On days 1 and 8, rabbits were injected at each of four intramuscular sites and in each footpad with 100 μg of cytochrome P-450 (specific content, 16.4 nmoles/mg of protein) in Freund's complete adjuvant. On day 15, the same amount of cytochrome P-450 in Freund's adjuvant was injected at four intramuscular sites along the flank. The rabbits were boosted by intravenous injection of 100 μg of cytochrome P-450 in NaCl on the 28th day. Blood was collected from an ear vein 20 days after this last injection. Because the serum from preimmune rabbits strongly inhibited benzphetamine demethylation when it was added to the reconstituted system, the sera from both immune and preimmune animals were further purified by ammonium sulfate precipitation (20–30% saturation). These ammonium sulfate fractions were further purified on a DEAE-cellulose column (5 × 10 cm) using 20 mM sodium phosphate, pH 8.0, as the eluting buffer. The column fractions containing antibody (or antibodies) to rat liver cytochrome P-450 were determined using Ouchterlony double-diffusion analysis. These column fractions were pooled and are referred to as partially purified antibody to rat liver cytochrome P-450 (immune antibody). The corresponding fractions from the DEAE-cellulose column of preimmune serum were also pooled (preimmune antibody). Ouchterlony double-diffusion analysis was performed using commercially available Immuno-Plates (Hyland Division of Travenol Laboratories) that contained 2% agar, 1% NaCl, and 2.1% NaN_3 and were buffered to pH 7.0–7.2 using 7.5% glycine. There was a clear immunoprecipitation band between cytochrome P-450 purified from phenobarbital-treated rats and the partially purified antibody (Fig. 1). Under the same experimental conditions, the partially purified antibody did not show any cross-reactivity with cytochrome P-450 (0.1 nmole) purified from phenobarbital-treated rabbits or with NADPH-cytochrome *c* reductase (2 μg, 28.3 units/mg of protein) from phenobarbital-treated rats (data not shown). However, a faint precipitin band appeared with purified rabbit liver cytochrome P-450 when whole rather than partially purified rabbit antiserum was used.

Analytical methods. The demethylation of benzphetamine by the rat liver reconstituted mixed-function oxidase system was measured at 25° in a Gilford recording spectrophotometer as described previously (12), except that 0.1 M EDTA was also included in the incubation and the NADPH concentration was 0.1 rather than 0.2 mM.

The rate of parathion metabolism to its various products was determined as described previously (10). Unless otherwise indicated, the 0.5-ml reaction mixture contained 150 μg of dilauroyl-L-3-phosphatidylcholine, 0.5 nmole of cytochrome P-450, 0.25 unit of NADPH-cytochrome *c* reductase, 50 μg of sodium doexycholate, 0.05 M *N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid buffer (pH 7.5), 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.1 mM [*ethyl*-^{14}C]parathion (specific activity, approximately 1 μCi/μmole), and 0.1 mM NADPH. The incubation was carried out at 37° with shaking. The reaction was started by addition of NADPH after 3 min and terminated 5 min later by the addition of 25 μl of 1 N HCl. A lower ratio of NADPH-cytochrome *c* reductase to cytochrome P-450 was used for assay of parathion metabolism than for benzphetamine demethylation, because the metabolism of parathion departs rapidly from linearity when more than a 2-fold ratio of reductase to P-450 (units per nanomole) is used. This is most likely due to

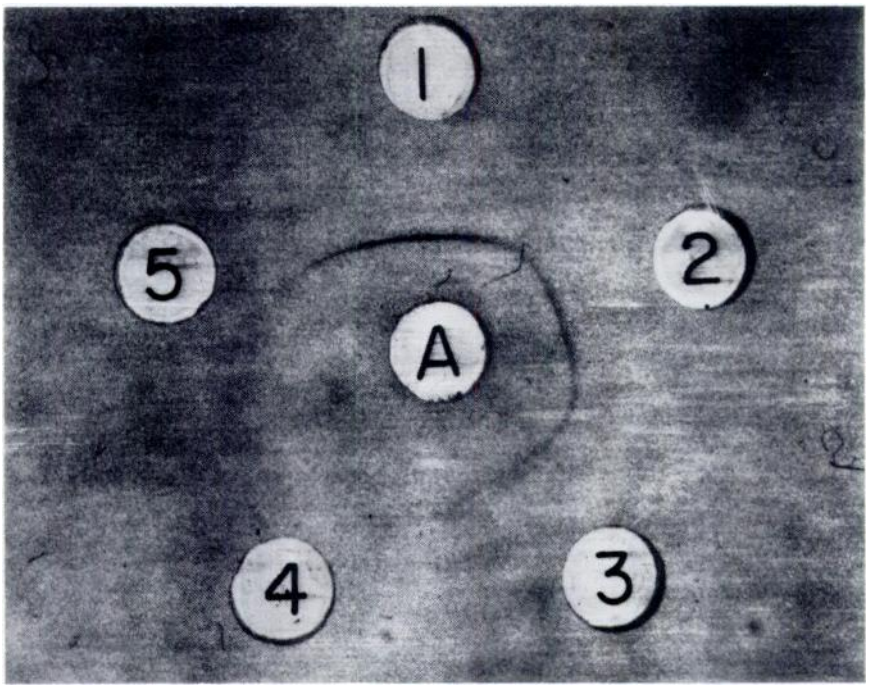

FIG. 1. *Ouchterlony double-diffusion in agar gels of rat liver cytochrome P-450 and rabbit antisera*
The center well (*A*) contained cytochrome P-450 (0.1 nmole, 15.4 nmoles/mg of protein) isolated from the livers of phenobarbital-treated rats. Other wells contained partially purified antibody: *1*, 65 μg of protein; *2*, 32 μg; *3*, 16 μg; *4*, 8 μg; *5*, 4 μg. See MATERIALS AND METHODS for additional details.

inhibition of parathion metabolism as a result of covalent binding of the sulfur atom released in the metabolism of parathion to paraoxon (10).

When the partially purified antibody was present in the incubation mixtures used to measure benzphetamine or parathion metabolism, the reaction, containing all components except NADPH, was first incubated at room temperature for 30 min before further incubation as described above.

Protein was determined by the method of Lowry *et al.* (17). Cytochrome P-450 was determined by the method of Omura and Sato (18) in the presence of 20% glycerol and 0.2% Emulgen 913 (2). NADH-cytochrome b_5 reductase activity was assayed by NADH-dependent reduction of ferricyanide (19). Cytochrome b_5 was determined either by measuring the difference spectrum of NADH-reduced minus air-saturated cytochrome b_5 in the presence of excess NADH-cytochrome b_5 reductase (1) or by measuring the reduction of cytochrome *c* by NADH (1). NADPH-cytochrome *c* reductase was assayed by measuring the NADPH-dependent reduction of cytochrome *c* (20). These spectrophotometric measurements were carried out at room temperature using a Cary 15 spectrophotometer. Statistical comparisons were made using Student's *t*-test.

Materials. NADPH and horse heart cytochrome *c* were products of Boehringer/Mannheim. Cyanogen bromide was obtained from Eastman Kodak, and 1,8-diaminooctane, from Aldrich. Sepharose 4B, CM-Sephadex (C-50), and DEAE-Sephadex (A-50) were products of Pharmacia. DEAE-cellulose (DE-22) was obtained from Whatman, and hydroxylapatite was purchased from Bio-Rad. Cholic acid (Eastman Organic Chemicals) was purified as previously described (12). Emulgen 913, a non-ionic detergent, was kindly supplied by Kao-Atlas Company, Japan. The ω-amino-*n*-octyl derivative of Sepharose

was prepared from cyanogen bromide-activated Sepharose 4B and 1,8-diaminooctane by a method described by Cuatrecasas (21). [*ethyl*-^{14}C]Parathion (99% pure) was purchased from Amersham/Searle.

RESULTS

The ability of the purified cytochrome P-450, in the presence of the necessary cofactors, to metabolize benzphetamine was examined first (Table 1). Cytochrome P-450, NADPH-cytochrome *c* reductase, and dilauroylphosphatidylcholine were essential for demethylation activity. In addition, deoxycholate had a considerable effect on the activity, even in the presence of dilauroylphosphatidylcholine. An NADPH-cytochrome *c* reductase to cytochrome P-450 ratio of about 5:1 units/nmole was used in these experiments. Preliminary studies indicated that the maximal rate of benzphetamine metabolism by this reconstituted system was obtained at a reductase to P-450 ratio of approximately 3.5 units/nmole or higher.

TABLE 1

Requirements for benzphetamine demethylation by rat liver reconstituted mixed-function oxidase system

Benzphetamine (1 mM) was incubated with the rat liver reconstituted enzyme system as described in MATERIALS AND METHODS. The NADPH-cytochrome *c* reductase had a specific activity of 20.5 units/mg of protein. The specific content of cytochrome P-450 was 15.4 nmoles/mg of protein. The blank contained all the components for each experiment except benzphetamine.

Conditions	Benzphetamine demethylation		
	Experimental (+ benzphetamine)	Blank (− benzphetamine)	Experimental − blank
	nmoles NADPH oxidized/nmole P-450/min		
Complete system	75.0	24.0	51.0
−Cytochrome P-450	4.0	3.5	0.5
−NADPH-cytochrome *c* reductase	0	0	0
−Dilauroylphosphatidylcholine	8.5	11.5	−3.0
−Deoxycholate	25.5	16.0	9.5
−Mg^{++}	59.0	14.5	44.5

Considerable NADPH oxidation was seen in the absence of benzphetamine. At reductase to P-450 ratios of less than 3.5 units/nmole, the substrate-independent oxidation of NADPH increased in proportion to the concentration of the reductase at a constant concentration of cytochrome P-450. This substrate-independent NADPH oxidation probably was not due to lipid peroxidation mediated by NADPH-cytochrome *c* reductase, since the concentration of EDTA (0.1 mM) in the reaction was in excess of the amount required to inhibit microsomal lipid peroxidation (22). In addition, no appreciable amounts of ferrous ions, which are required for lipid peroxidation by purified NADPH-cytochrome *c* reductase (23), were present in these incubations.

The requirements for the metabolism of parathion by the reconstituted system are shown in Table 2. All three mixed-function oxidase-catalyzed metabolites of parathion—namely, paraoxon, diethylphosphorothioic acid, and diethylphosphoric acid—were formed by the reconstituted system. The requirements for parathion metabolism were similar to those seen for benzphetamine demethylation. However, in the case of parathion metabolism, there was some activity in the absence of dilauroylphosphatidylcholine. In addition, less deoxycholate was required for parathion than for benzphetamine metabolism.

The ability of the partially purified antibody to rat liver cytochrome P-450 to precipitate purified rat and rabbit liver cytochromes P-450 from solution was examined as described by Thomas *et al.* (24). To solutions containing 3 nmoles of rat or rabbit liver cytochrome P-450, various amounts of antibody were added, and the cytochrome P-450 remaining in the solution after incubation and centrifugation was determined (Fig. 2). As can be seen, 3 mg of this preparation of partially purified antibody precipitated about 85% of the rat liver cytochrome P-450 (1 mg of antibody per nanomole of cytochrome P-450). This same ratio of antibody to cytochrome P-450 precipitated about 30% of the rabbit enzyme. Preimmune antibody did not precipitate rat liver cytochrome P-450 from solution. Because of a limited supply, the abil-

TABLE 2

Requirements for parathion metabolism by rat liver reconstituted mixed-function oxidase system

Parathion (0.1 mM) was incubated with the rat liver reconstituted enzyme system as described in MATERIALS AND METHODS. The NADPH-cytochrome *c* reductase had a specific activity of 28.3 units/mg of protein. The specific content of cytochrome P-450 was 15.4 nmoles/mg of protein. The values are the means ± standard deviations of duplicate determinations.

Conditions	Product formation		
	Paraoxon	Diethylphosphorothioic acid	Diethylphosphoric acid
	nmoles/nmole P-450/5 min		
Complete system	4.22 ± 0.33	1.34 ± 0.18	0.38 ± 0.13
−Cytochrome P-450	0.08 ± 0.04	0.12 ± 0.04	0.04 ± 0.01
−Reductase	0.11 ± 0.03	0.05 ± 0.01	0.04 ± 0.03
−NADPH	0	0	0.01 ± 0
−Dilauroylphosphatidylcholine	1.39 ± 0.04	0.46 ± 0.03	0.23 ± 0.01
−Deoxycholate	2.25 ± 0.26	0.85 ± 0.08	0.28 ± 0.06
$-Mg^{++}$	4.04 ± 0.13	1.09 ± 0.33	0.33 ± 0.09

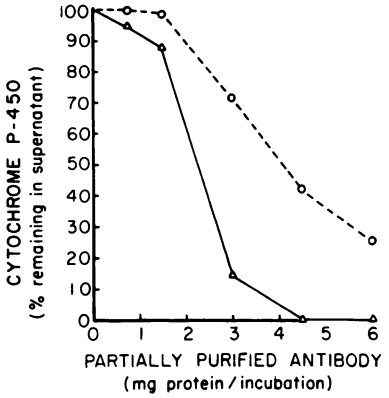

FIG. 2. *Quantitative immunoprecipitation analysis of purified rat and rabbit liver cytochromes P-450, using partially purified antibody against rat liver cytochrome P-450*

Rat (Δ— —Δ) and rabbit (O- - -O) liver cytochromes P-450 and partially purified antibody to rat liver cytochrome P-450 were dissolved in 50 mM potassium phosphate, pH 7.25, containing 0.85% sodium chloride. Each incubation contained 3 nmoles of cytochrome P-450 and various amounts of partially purified antibody, as shown. The total volume of the incubations was 1.5 ml. The mixture was incubated for 16 hr at 4° and centrifuged at 2000 rpm for 20 min, and the cytochrome P-450 remaining in the supernatant was determined as described in MATERIALS AND METHODS. The rabbit and rat liver cytochromes P-450 used in these experiments had specific contents of 16.9 and 15.0 nmoles/mg of protein, respectively.

ity of the preimmune antibody to precipitate rabbit liver cytochrome P-450 from solution was not examined. However, as shown in Table 4, the preimmune antibody, in contrast to the immune antibody, had no effect on the ability of the rabbit liver reconstituted system to metabolize parathion.

The ability of the partially purified antibody to inhibit benzphetamine demethylation by the rat liver reconstituted system was examined using 0.1 nmole of cytochrome P-450 and various amounts of a partially purified antibody preparation (Fig. 3). Maximal inhibition (approximately 90%) was obtained when about 0.1 mg of the antibody protein had been added (1 mg of antibody protein per nanomole of cytochrome P-450). The partially purified preimmune antibody preparation (rabbits not injected with cytochrome P-450) caused slight activation at low concentrations and slight inhibition at higher concentrations.

The effect of the partially purified antibody preparation on parathion metabolism by the rat liver reconstituted enzyme system was also examined (Table 3). These incubations contained 0.5 nmole of cytochrome P-450. The rates of formation of paraoxon, diethylphosphorothioic acid, and diethylphosphoric acid in the presence of 0.5 mg of immune antibody (1 mg of antibody per nanomole of P-450) were 77%, 72%, and 78% slower, respectively, than the rates of formation of these metabolites in the presence of the same amount of preimmune antibody. These inhibition values are somewhat less than that seen

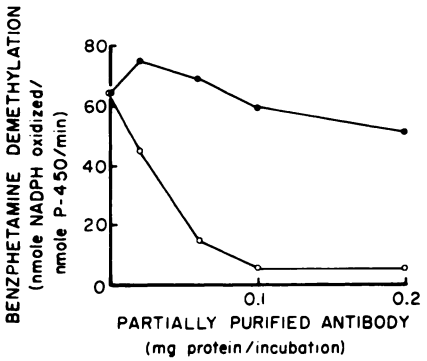

FIG. 3. *Inhibition of benzphetamine demethylation by partially purified antibody to rat liver cytochrome P-450*

Benzphetamine (1 mM) was incubated with the reconstituted enzyme system in the absence and presence of various amounts of partially purified immune (O– –O) and preimmune (●– –●) antibody preparations. The composition of the reconstituted enzyme system is described in MATERIALS AND METHODS. The antibody preparations were incubated wtih the complete incubation system except NADPH for 30 min. NADPH was then added, and the oxidation of NADPH was followed for approximately 5 min. The cytochrome P-450 used in these experiments had a specific content of 15.4 nmoles/mg of protein. The NADPH-cytochrome *c* reductase had a specific activity of 20.5 units/mg of protein.

with benzphetamine (92%) at the same antibody to cytochrome P-450 ratio.

The ability of the antibody to rat liver cytochrome P-450 to inhibit the metabolism of parathion by a rabbit liver reconstituted system was also examined (Table 4). Each incubation contained 0.25 nmole of cytochrome P-450 purified from the livers of phenobarbital-treated rabbits (12). An NADPH-cytochrome *c* reductase to cytochrome P-450 ratio of 0.1 was used in these experiments. This lower reductase to P-450 ratio accounts for the slower rate of metabolism of parathion seen in Table 4 as compared with Table 3. Under the conditions of this experiment, the rate of formation of diethylphosphoric acid was so small that it could not be accurately measured. Consequently the formation of this metabolite was not determined. An immune antibody to cytochrome P-450 ratio of 0.4 mg of protein per nanomole of P-450, which inhibited parathion metabolism by the rat liver reconstituted system by about 50% (Table 3), had no significant effect on the rate of metabolism of parathion by the rabbit liver reconstituted system (0.1 mg of antibody protein per 0.25 nmole of P-450). However, an immune antibody to rabbit liver cytochrome P-450 ratio of 0.8 (0.2 mg of antibody protein per 0.25 nmole of P-450) significantly inhibited the metabolism of parathion to paraoxon and diethylphosphorothioic acid as compared with the control incubations or those containing a similar ratio of preimmune antibody to cytochrome P-450. The degree of inhibition in this case was about 25%. A slightly higher ratio of 1.0 mg of antibody protein per nanomole of P-450 inhibited the metabolism of parathion by the rat liver reconstituted system by about 75%. The preimmune antibody had no effect on the metabolism of parathion by the rabbit liver reconstituted system.

DISCUSSION

The specific content of cytochrome P-450 purified as described in this report ranged from 15.0 to 16.4 nmoles/mg of protein determined according to Lowry *et al.* (17). Using a different purification procedure, Ryan *et al.* (3) obtained an apparently homogeneous preparation of cytochrome P-450 from the livers of phenobarbital-treated rats with a specific content of 12–13 nmoles/mg of protein based on the Lowry method. This difference in the specific content of apparently homogeneous cytochrome P-450 from the livers of phenobarbital-treated rats may be due to a variation in the degree of dissociation of the heme group from the holoenzyme using these two different purification procedures.

The degree of stimulation of the rate of metabolism of benzphetamine by dilauroylphosphatidylcholine and deoxycholate (Table 1) was much greater than for parathion (Table 2). Whereas there was an absolute requirement for dilauroylphosphatidylcholine for metabolism of benzphetamine by the reconstituted system, in the absence of this phospholipid the rate of metabolism of parathion was still about one-third of that in its presence. Similarly, deletion of deoxycholate from the reconsti-

TABLE 3

Inhibition of parathion metabolism by partially purified antibody to rat liver cytochrome P-450

Parathion (0.1 mM) was incubated with the reconstituted enzyme system in the absence and presence of the immune and preimmune antibody preparations and in the absence and presence of NADPH. The composition of the reconstituted enzyme system is described in MATERIALS AND METHODS. The antibody was incubated with the complete incubation system except NADPH for 30 min. NADPH was then added, where noted, and the incubation was carried out for 5 min. The results are expressed as the means ± standard deviations of duplicate determinations under each experimental condition. The difference between the mean of the amount of metabolite formed in the presence of NADPH (experimental) and that formed in the absence of NADPH (blank) is also shown.

Additions	Product formation		
	Paraoxon	Diethylphosphorothioic acid	Diethylphosphoric acid
	nmoles/nmole P-450/5 min		
None (control)			
+NADPH (A)	4.90 ± 0.66	1.79 ± 0.30	0.38 ± 0.05
−NADPH (B)	0.15 ± 0	0.16 ± 0.02	0.09 ± 0.01
A − B	4.75	1.63	0.29
Preimmune antibody (0.2 mg)			
+NADPH (A)	5.10 ± 0.43	1.79 ± 0.06	0.49 ± 0.18
−NADPH (B)	0.17 ± 0.02	0.21 ± 0.02	0.05 ± 0.07
A − B	4.93	1.58	0.44
Preimmune antibody (0.5 mg)			
+NADPH (A)	4.74 ± 0.22	1.63 ± 0.18	0.57 ± 0.11
−NADPH (B)	0.09 ± 0.01	0.14 ± 0.03	0.08 ± 0
A − B	4.65	1.49	0.49
Immune antibody (0.2 mg)			
+NADPH (A)	2.87 ± 0.51[a]	1.19 ± 0.37[a]	0.33 ± 0.02[a]
−NADPH (B)	0.11 ± 0.02	0.18 ± 0.02	0.07 ± 0.01
A − B	2.76	1.01	0.26
Immune antibody (0.5 mg)			
+NADPH (A)	1.17 ± 0.10[a]	0.59 ± 0.05[a]	0.18 ± 0.04[a]
−NADPH (B)	0.10 ± 0.01	0.17 ± 0.02	0.07 ± 0.01
A − B	1.07	0.42	0.11

[a] Significantly different from the control and corresponding preimmune antibody groups ($P < 0.05$).

tuted system inhibited the metabolism of benzphetamine by about 80%, while the metabolism of parathion was less inhibited (approximately 40%). Additional experiments, using different preparations of rat liver cytochrome P-450 and NADPH-cytochrome *c* reductase, showed results similar to those shown in Table 1. The metabolism of benzphetamine, as measured by formaldehyde formation (25), was also inhibited approximately 80% by the deletion of deoxycholate.

The reason for the difference in requirements of benzphetamine and parathion metabolism for dilauroylphosphatidylcholine is not known at this time. It has been reported that this phospholipid plays a role in binding both the reductase and substrate to cytochrome P-450 (26). Because parathion is much more lipophilic than benzphetamine, the binding of parathion to the reductase–cytochrome P-450 complex may require less phospholipid than is the case for benzphetamine.

The reason why deoxycholate is required for maximal rates of benzphetamine and parathion metabolism by the rat liver reconstituted system is not known.

TABLE 4

Effect of antibody to rat liver cytochrome P-450 on metabolism of parathion by rabbit liver reconstituted system

Parathion (0.1 mM) was incubated with a rabbit liver reconstituted system in the presence of the immune and preimmune antibody preparations. The composition of the reconstituted system was the same as described in MATERIALS AND METHODS, except that 0.25 nmole of rabbit liver cytochrome P-450 (16.1 nmoles/mg of protein) and 0.025 unit of rabbit liver NADPH-cytochrome *c* reductase (13.2 units/mg of protein) were used. An incubation containing all components of the reconstituted system except NADPH was carried out, and the amounts of paraoxon and diethylphosphorothioic acid formed were subtracted from the amounts of these metabolites formed in each incubation. The results are the means ± standard deviations of four separate incubations under each experimental condition.

Additions	Product formation	
	Paraoxon	Diethylphosphorothioic acid
	nmoles/nmole P-450/5 min	
None (controls)	1.68 ± 0.33	0.95 ± 0.15
Preimmune antibody (0.1 mg)	1.51 ± 0.12	0.83 ± 0.10
Preimmune antibody (0.2 mg)	1.76 ± 0.25	0.99 ± 0.13
Immune antibody (0.1 mg)	1.52 ± 0.12	0.82 ± 0.13
Immune antibody (0.2 mg)	1.29 ± 0.16[a]	0.70 ± 0.20[a]

[a] Significantly different from the control and preimmune antibody (0.2 mg) groups ($p < 0.05$).

The requirement for both dilauroylphosphatidylcholine and deoxycholate for maximal activity has also been reported for a reconstituted system isolated from the livers of phenobarbital-treated rabbits (27). However, the requirement for dexoycholate for maximal activity appeared to vary somewhat from preparation to preparation (27). Recent studies in our laboratory using rabbit liver reconstituted systems showed only a slight stimulatory effect of deoxycholate on benzphetamine and parathion metabolism in the presence of dilauroylphosphatidylcholine (12). In contrast, both deoxycholate and dilauroylphosphatidylcholine were consistently required for maximal activity of rat liver reconstituted systems containing reductase and cytochrome P-450 prepared as described in MATERIALS AND METHODS. The ability of deoxycholate to stimulate the rate of metabolism of benzphetamine by a rat liver reconstituted system containing a partially purified preparation of cytochrome P-450 from phenobarbital-treated rats has been reported previously (28). However, these experiments with deoxycholate were carried out in the absence of any exogenous lipid. The ability of deoxycholate to activate the rat liver reconstituted system in the absence of phospholipid was thought to be related to the ability of this detergent to facilitate the interaction between NADPH-cytochrome *c* reductase and cytochrome P-450 (28). The doexycholate may also, like phospholipid (26), facilitate the interaction between the substrate and cytochrome P-450. Because of the greater lipid solubility of parathion, this might account for the greater stimulation by deoxycholate of benzphetamine compared with parathion metabolism.

The rate of metabolism of benzphetamine using a reconstituted system containing reductase and cytochrome P-450 prepared from the livers of phenobarbital-treated rabbits (12) is quite similar to that described here for the rat liver reconstituted system (Table 1). With both systems the turnover number was approximately 50 nmoles of benzphetamine metabolized per minute per nanomole of cytochrome P-450. The quantitative and qualitative aspects of parathion metabolism using the two reconstituted systems were also quite similar. In both cases three products—paraoxon, diethylphosphorothioic acid, and diethylphosphoric acid—were isolated from the incubations. As noted previously (12), these three products probably result from the nonenzymatic breakdown of a common oxygenated intermediate of parathion by different chemical pathways. This common intermediate is thought to be a sulfine derivative of parathion formed in the transfer of an oxygen atom to one of the unshared electron paris on the thiono sulfur group of parathion (12). The finding that the antibody to rat liver cytochrome P-450 equally inhibited the rates of forma-

tion of all three metabolites (Table 3) also suggests that these products are formed by breakdown of a common intermediate. However, the existence of multiple forms of cytochrome P-450 with similar molecular weights, which catalyze the formation of different products on reaction with parathion, cannot be excluded by these data. The rates of formation of paraoxon (nanomoles of product formed per nanomole of P-450 per 5 min) were not significantly different using the rat (Table 2) and rabbit (12) reconstituted systems. However, the rate of formation of diethylphosphorothioic acid and, occasionally, diethylphosphoric acid was slightly but significantly slower using the rat (Table 2) compared with the rabbit liver reconstituted system (12). Water is required for the breakdown of the hypothetical sulfine derivative of parathion to diethylphosphorothioic acid (11) and to diethylphosphoric acid (12). The slower rate of formation of these two metabolites in the rat than in the rabbit liver reconstituted system may mean that, under the conditions of these experiments, the active site of rat liver cytochrome P-450 is less accessible to water than that of rabbit liver cytochrome P-450.

As determined by SDS–polyacrylamide gel electrophoresis, the molecular weight of cytochrome P-450 isolated from rat liver (48,500) is quite similar to that isolated from rabbit liver (49,700). In addition, these enzymes catalyze quantitatively similar reactions with the structurally diverse substrates benzphetamine and parathion. Ouchterlony double-diffusion analysis demonstrated poor cross-reactivity between the antibody to phenobarbital-induced rat liver cytochrome P-450 and cytochrome P-450 from the livers of rabbits treated with phenobarbital. However, quantitative immunoprecipitation and an examination of the ability of the antibody to inhibit the metabolism of parathion by a rabbit liver reconstituted system clearly demonstrated an affinity of the antibody for phenobarbital-induced rabbit liver cytochrome P-450. The antibody data suggest that the cytochromes P-450 induced in rabbits and rats by phenobarbital are not identical proteins. However, the finding that they do have similar molecular weights, exhibit some immunological cross-reactivity, and catalyze quantitatively and qualitatively similar reactions with the limited number of substrates that have so far been examined indicates that there are structural similarities between these two enzymes, especially as concerns the active site.

A previous study using Ouchterlony double-diffusion analysis (29) also showed poor reactivity of rabbit liver cytochrome P-448 with a partially purified antibody produced in rabbits against rat liver cytochrome P-448. However, in contrast to the results obtained in this study using cytochrome P-450, there were marked differences in the rates of metabolism of various substrates by the cytochrome P-448 enzymes isolated from these two species. Rat liver cytochrome P-448 was much more active in catalyzing benzphetamine *N*-demethylation and benzo[*a*]pyrene hydroxylation. On the other hand, rabbit liver cytochrome P-448 was mildly active in coumarin hydroxylation while the rat liver cytochrome P-448 was inactive (29).

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