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Inhibition and Inactivation of Constitutive Cytochromes P450 in Rat Liver by Parathion

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SUMMARY

Phosphorothioate pesticides, such as parathion (*O,O*-diethyl-*O*-4-nitrophenyl phosphorothioate), undergo enzymic oxidation to the active insecticidal agents that are the analogous organophosphorus compounds. In hepatic microsomal fractions, the NADPH-mediated conversion of parathion to paraoxon occurs with concomitant loss of cytochrome P450 (P450) and associated activities. In this study, the capacity of parathion to inactivate specific P450 enzymes was studied in rat hepatic microsomes. Parathion was a potent inhibitor of P450 3A2- and 2C11-mediated androst-4-ene-3,17-dione (androstenedione) 6β- and 16α-hydroxylation (K_i values of 13 ± 2 and 2.3 ± 0.1 μM, respectively, and K_m/K_i ratios of 1.4 ± 0.2 and 11 ± 1, respectively). After a 10-min preincubation between parathion and NADPH-supplemented microsomes, to inactivate P450 before androstenedione hydroxylation was carried out, the corresponding K_m/K_i ratios were increased to 3.5 ± 0.4 and 35 ± 6, reflecting 2.5- and 3.2-fold enhancement of inhibition of P450 3A2- and 2C11-dependent activities. In contrast to these findings, P450 2A1/2-mediated androstenedione 7α-hydroxylation was refractory to inhibition and P450 2C6-mediated progesterone 21-hydroxylation was inhibited but not inactivated by the pesticide. Further studies established that androstenedione 6β- and 16α-hydroxylation pathways were inactivated with maximal half-times of 2.59 min and 1.72 min, respectively. Although the incubation of parathion (50 μM) with rat liver microsomes for 10 min led to a 16% decrease in P450 estimated spectrophotometrically, immunoblot analysis revealed no change in the microsomal content of P450 2C11 apoprotein. Finally, NADPH-mediated metabolism of parathion to paraoxon (by desulfuration) and 4-nitrophenol (by oxidative cleavage of the phosphorothioate ester) occurred efficiently in microsomes (4.32 and 4.35 nmol/min/mg of protein, respectively). P450 loss was estimated under the same incubation conditions and, thus, 210 parathion molecules were oxidized for each molecule of holo-P450 lost. These findings establish that parathion is a potent inhibitor and inactivator of the principal constitutive P450s, 3A2 and 2C11, in rat liver, whereas the P450s 2A1 and 2A2 are refractory to either inhibition or inactivation. Another major constitutive enzyme, P450 2C6, is inhibited effectively by parathion but does not appear to be subject to inactivation.

Hepatic P450 hemoproteins are important in a wide range of drug, steroid, pesticide, and carcinogen hydroxylations. The multiplicity of the P450 system is responsible for its unusually low substrate specificity; each P450 exhibits a characteristic profile of activities toward a range of lipophilic substrates. However, over the last decade it has emerged that C_{19}- and C_{21}-steroids such as androstenedione and progesterone are subject to a number of positional hydroxylations catalyzed extensively by individual P450s (1–4). Thus, the major enzymes involved in androstenedione 6β-, 7α-, and 16α-hydroxylation in male rat liver are P450s 3A2, 2A1/2, and 2C11, respectively (1–3). Similarly, progesterone 2α-, 6β-, 16α-, and 21-hydroxylations appear to be catalytic indicators for the P450s 2C11, 3A2, 2C11, and 2C6, respectively (4). Quantitatively minor P450s may participate in the formation of other hydroxysteroid metabolites (5, 6).

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Several phosphorothioates, including chlorpyrifos and to a lesser extent parathion, are in widespread use in agriculture, and there have been numerous reports of undesirable human exposure resulting in toxicity (7–9). In many cases, drug therapy has been attempted for the treatment of symptoms of phosphorothioate pesticide exposure in humans. The commercially important phosphorothioate pesticides undergo P450-mediated activation to the oxon, or phosphate ester (Fig. 1). The oxon is widely held to be the mediator of acute organophosphorus pesticide toxicity, due to its anticholinesterase action. During phosphorothioate activation, an additional metabolic process has been reported in which the thionosulfur atom becomes covalently attached to the P450 apoprotein, resulting in deactivation of the enzyme (10, 11). Prolonged inhibition of P450 activity by phosphorothioates has been reported in studies with experimental animals *in vivo* and *in vitro* (12, 13). Indeed, for individuals who received drug treatment for phosphorothioate poisoning there have been reports of toxicity from the supportive therapy (14). That is, the toxic

ABBREVIATIONS: P450, cytochrome P450; androstenedione, androst-4-ene-3,17-dione; HPLC, high performance liquid chromatography.

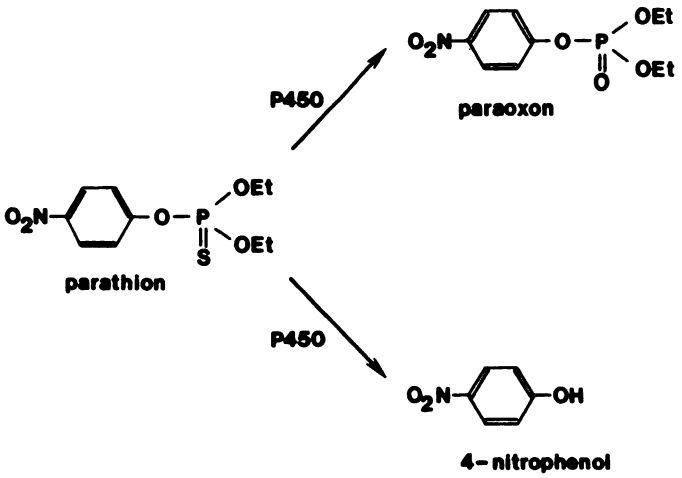

Fig. 1. P450-mediated oxidation of parathion to paraoxon and 4-nitrophenol. *Et*, ethyl.

effects are attributed to impaired elimination of the drugs intended to ameliorate the symptoms of pesticide intoxication.

The addition of parathion to NADPH-supplemented hepatic microsomes results in oxidation of the pesticide as well as inhibition of P450 reactions. The present study was undertaken to provide a more detailed account of the differential inhibition and inactivation of constitutive P450s during parathion metabolism, to facilitate the interpretation of adverse interactions in patients receiving drug therapy after phosphorothioate poisoning. The K_m/K_i ratios for a series of P450-specific reactions were determined, as well as parameters of phosphorothioate-mediated P450 inactivation (the time for loss of 50% enzyme activity, $t_{1/2}$), to assess the relative susceptibility of different P450s to inactivation.

Materials and Methods

Chemicals. Parathion was generously provided by Rhône Poulenc (Brisbane, Australia). Paraoxon and biochemicals were purchased from Sigma Chemical Co. (St. Louis, MO). [^{14}C]Androstenedione (specific activity, 59 mCi/mmol), [^{14}C]parathion (specific activity, 21 mCi/mmol), ACS II scintillant, and Hyperfilm-MP were obtained from Amersham Australia, Sydney. [^{14}C]Progesterone (specific activity, 60 mCi/mmol) was from New England Nuclear (Sydney, Australia). Hydroxytestosterone standards were purchased from Sigma, Steraloids (Wilton, NH), or the Medical Research Council Steroid Reference Collection (Queen Mary's College, London, England). Thin layer chromatography plates (silica gel 60 F254 type) were purchased from E. Merck (Darmstadt, Germany). Analytical grade solvents and miscellaneous chemicals were purchased from Ajax Chemicals (Sydney, Australia).

Animals and preparation of microsomal fraction. Male Wistar rats (approximately 250 g) were used in these experiments. Animals were anesthetized and killed, and washed hepatic microsomes were isolated by standard methods (15); microsomes were resuspended in 50 mM potassium phosphate, pH 7.4, containing 20% glycerol and 1 mM EDTA, frozen in liquid nitrogen, and stored at −70°. Protein was determined according to the method of Lowry *et al.* (16).

Androstenedione and progesterone hydroxylation assays. Assays (0.4-ml reaction volumes) of microsomal androstenedione and progesterone hydroxylation were performed at 37° in potassium phosphate buffer (0.1 M, pH 7.4, containing 1 mM EDTA), contained 0.15 mg of microsomal protein, and were initiated with 1 mM NADPH (17). In the derivation of kinetic parameters, the [^{14}C]androstenedione concentration was varied over the range of 5 to 200 μM. In inactivation experiments, androstenedione and progesterone concentrations were 50 μM (0.18 μCi/0.4 ml). After 2.5 min, the reactions were transferred to ice and extracted with 5 ml of chloroform. The organic phase was separated, reduced to dryness (under N_2), applied to thin layer chromatography plates (activated at 100° for 15 min before use), and developed in the solvent systems described previously for the separation of androstenedione (2) and progesterone (18) metabolites. Radioactive regions were identified by autoradiography (Hyperfilm-MP; Amersham) for approximately 60 hr. Metabolite formation was quantitated by scintillation counting.

Kinetics of the inhibition and inactivation by parathion of androstenedione hydroxylation. Direct inhibition was assessed under the incubation conditions described above, using varying concentrations of androstenedione (5–200 μM) and parathion (0–100 μM). In parallel experiments, parathion (0–25 μM) was preincubated with microsomes and NADPH for 10 min and the components of the reaction were transferred to tubes containing substrate (5–200 μM). Therefore, in these experiments P450 deactivation during parathion oxidation occurred before addition of the steroid substrate. The experiments were performed at least in triplicate.

The data were subjected to graphical analysis according to the Hanes-Woolf (i.e., S/V versus S), for determination of the K_m (Michaelis constant) and V_{max} (maximal reaction velocity), Lineweaver-Burk (i.e., $1/V$ versus $1/S$), and Dixon (i.e., $1/V$ versus I) methods. Replots of the slopes of the Dixon plots at each substrate concentration were constructed as a function of I and were used to define the mode of inhibition. K_i (the equilibrium dissociation constant for the enzyme-inhibitor complex) values were determined from the slope of the line of best fit in this replot (19). In competitive inhibition the line intercepts the origin, but in other types of inhibition the y-intercept is a function of the K_i and V_{max}. Kinetic parameters were determined individually from each experiment, and the K_m/K_i ratio was used as a measure of relative inhibition potency.

Finally, varying concentrations of parathion (5, 10, 25, or 50 μM) were preincubated with microsomes and NADPH for 0–3 min before transfer to vials containing androstenedione or progesterone. Thus, time-dependent inactivation of steroid hydroxylation was assessed.

Purification of microsomal P450 2C11 and preparation of anti-P450 antiserum. The enzyme P450 2C11 was isolated from sodium cholate-solubilized hepatic microsomes from untreated adult male rats essentially as described elsewhere (20, 21). The final preparation was apparently homogeneous by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Anti-P450 IgG was isolated from serum of rabbits that had been immunized with P450 2C11 and was rendered monospecific by cross-adsorption, as outlined previously (20). The IgG preferentially inhibited androstenedione 16α-hydroxylation and recognized an antigen (~50 kDa) present in male but not female rat hepatic microsomes (20).

Hepatic microsomal metabolism of parathion. The metabolism of parathion was investigated in hepatic microsomal fractions from untreated male rats. In these experiments the concentrations of microsomal protein and parathion were 0.5 mg/ml and 250 μM, respectively. A typical NADPH-generating system was used in these reactions (comprising 1 mM NADP, 5 mM glucose 6-phosphate, and 1 unit of glucose 6-phosphate dehydrogenase). The incubations (0.4 ml) were conducted for 2 min and were terminated by rapid freezing. Preliminary experiments were undertaken with [^{14}C]parathion as substrate. The substrate and its ^{14}C-labeled metabolites paraoxon and 4-nitrophenol were collected as eluate fractions from the HPLC separation described below and were then subjected individually to the extraction procedure. Each compound was extracted quantitatively. All of the radiolabel was recovered in either the parathion, paraoxon, or 4-nitrophenol fractions. In subsequent experiments on the microsomal metabolism of unlabeled parathion, 4,4′-dihydroxybiphenyl was used as an internal standard for quantitiation.

Separation of parathion, paraoxon, and 4-nitrophenol by HPLC. Parathion and its two metabolites, paraoxon and 4-nitrophenol, were applied to an Ultrasphere-Si column (5 μm, 4.6 mm i.d × 25 cm; Beckman, San Ramon, CA) attached to a Waters Associates HPLC system and were eluted with a mobile phase of dichloromethane/acetonitrile/acetic acid (93:7:0.02) (22); the detection wavelength was 254 nm. Retention times of the compounds were as follows: 4-nitrophenol, 4.3 min; 4,4'-dihydroxybiphenyl, 6.5 min; paraoxon, 8.7 min; and parathion, 3.3 min. HPLC metabolite peak areas were calculated on a Waters 730 data module, and product formation was determined from standard curves prepared using known quantities of the authentic metabolites.

Optical difference spectroscopy. Optical difference spectra elicited by parathion in rat hepatic microsomes (1-ml aliquots of 1–2 mg of protein/ml in 0.1 M potassium phosphate buffer, pH 7.4) were recorded on a Cary 2300 spectrophotometer at 37°, using 1-cm cuvettes. Parathion was added to the sample cuvette in microliter volumes of ethanol; solvent was added to the reference cuvette. The resultant difference spectra were monitored between the wavelengths 380 and 500 nm. Double-reciprocal plots of the data were constructed (ΔA between the absorbance maximum and minimum versus parathion concentration). From these plots the dissociation constant and maximal extent of the binding of parathion to microsomal P450 were determined (23). Microsomal P450 content was measured by the procedure of Omura and Sato (24).

Results

Binding of parathion to P450 in rat hepatic microsomes. Parathion produced a type I optical difference spectrum (λ_{max} and λ_{min} near 390 and 420 nm, respectively) when added to oxidized microsomes from untreated rat liver (data not shown). From double-reciprocal analysis of the optical titration, values of 8.7 μM and 0.0293 absorbance units/nmol of P450 were obtained for the spectral dissociation constant (K_s) and maximal absorbance change (ΔA_{max}), respectively. Application of the extinction coefficient 110 cm^{-1} M^{-1} (25) to this ΔA_{max} value yielded a value of approximately 27% of the total microsomal P450 being involved in the parathion binding interaction.

Inactivation of microsomal P450 by parathion. Preincubation of parathion (50 μM) with hepatic microsomes for 10 min led to a 16% decrease in spectrally apparent P450 content in control microsomes. In additional experiments, P450 destruction by parathion was monitored after a 2-min preincubation (because this facilitated direct comparison with studies of the effects of parathion on P450 catalysis). Concentrations of parathion up to 25 μM were not associated with the loss of spectrally apparent P450, but higher concentrations (50, 100, and 150 μM) were similarly effective in decreasing holo-P450 (13–17%; data not shown).

Inhibition and inactivation by parathion of microsomal steroid hydroxylation reactions. The capacity of parathion to inhibit and inactivate important constitutive P450-mediated steroid hydroxylations was investigated. The 6β-, 7α-, and 16α-hydroxy metabolites of androstenedione represented the three principal pathways of oxidation of the steroid in untreated rat liver. Michaelis constants for the three pathways were obtained from Hanes-Woolf plots and were 17 ± 1, 2.2 ± 1.1, and 24 ± 3 μM (mean ± standard error), respectively, whereas maximal reaction velocities were 2.98 ± 0.21, 0.29 ± 0.02, and 4.80 ± 0.20 nmol/min/mg of protein, respectively.

Parathion produced quite effective inhibition of androstenedione 6β- and 16α-hydroxylation (K_i values of 13 ± 2 and 2.3 ± 0.1 μM, respectively; Table 1), but the 7α-hydroxylation pathway was refractory to inhibition. Thus, values of 1.4 ± 0.2 and 11 ± 1 were calculated for the K_m/K_i ratios for androstenedione 6β- and 16α-hydroxylation. A typical kinetic analysis of the inhibition of androstenedione 16α-hydroxylation by parathion is shown in Fig. 2. The nature of the kinetic plots, including the intersection of the Dixon plot slope replot with the origin (Fig. 2C), suggested that the mode of inhibition was competitive.

Preincubation of parathion with NADPH and untreated rat liver microsomes for 10 min before measurement of androstenedione hydroxylation revealed significant increases in inhibition potency against the 6β- and 16α-hydroxylation pathways (reflected by increases in the K_m/K_i ratios from 1.4 ± 0.2 to 3.5 ± 0.4 and from 11 ± 1 to 35 ± 6, respectively; Table 1). Thus, after the 10-min preincubation step the inhibition of androstenedione 6β- and 16α-hydroxylation (kinetics of inhibition of 16α-hydroxylation are shown in Fig. 3) was enhanced 2.5- and 3.2-fold, respectively. The kinetics of inhibition observed after the preincubation step could best be described as apparent linear noncompetitive (mixed) (Fig. 3). Thus, after the preincubation step a clear difference in the apparent mode of inhibition was noted.

After the kinetic analysis from preincubation studies the process of enhanced inhibition was investigated further in time course experiments. The data presented in Fig. 4 and Table 2 were obtained from experiments in which parathion, NADPH, and microsomes from untreated rat liver were preincubated for periods of up to 3 min before transfer to tubes containing substrate. Androstenedione hydroxylation was then estimated as usual. Pseudo-first-order plots (logarithm of percent activity remaining as a function of preincubation time) were constructed (Fig. 4) and the first-order kinetic rate constants and times for loss of 50% of initial enzyme activity were calculated

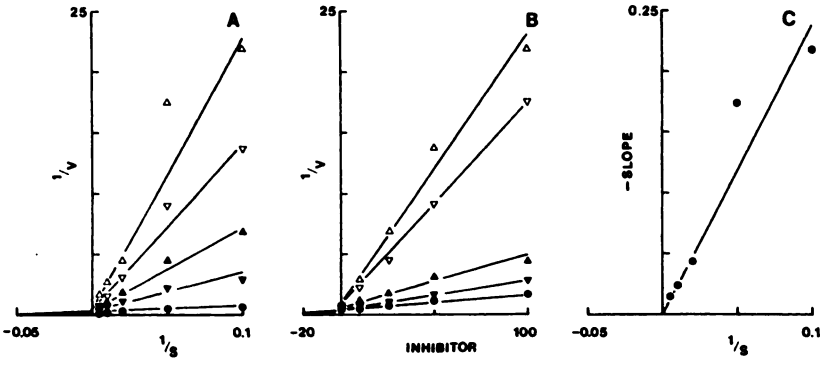

Fig. 2. Kinetics of inhibition by parathion of microsomal P450 2C11-mediated androstenedione 16α-hydroxylation (no preincubation step). A, Lineweaver-Burk plots at the following parathion concentrations: ●, 0 μM; ▼, 10 μM; ▲, 25 μM; ▽, 50 μM; △, 100 μM. B, Dixon plots at the following androstenedione concentrations: ●, 10 μM; ▼, 20 μM; ▲, 50 μM; ▽, 100 μM; △, 200 μM. C, Primary replot of the slopes of the Dixon plots versus reciprocal androstenedione concentration. Units are as follows: substrate and inhibitor concentrations, μM; V, nmol/min/mg of protein.

TABLE 1

Kinetic parameters of the inhibition by parathion of androstenedione hydroxylation pathways in untreated rat liver microsomes

Data are means ± standard errors of estimates obtained from separate microsomal suspensions [three (no preincubation) or four (preincubation)].

Androstenedione hydroxylation pathway	No preincubation				Preincubation			Increase in potency
	K_m	V_{max}	K_i	K_m/K_i	K_m	K_i	K_m/K_i	
	μM	nmol/min/mg of protein	μM		μM	μM		fold
6β	17 ± 1	2.98 ± 0.21	13 ± 2	1.4 ± 0.2	12 ± 1	3.6 ± 0.4	3.5 ± 0.4	2.5
7α	2.2 ± 1.1	0.29 ± 0.02			3.8 ± 1.0			
16α	24 ± 3	4.80 ± 0.20	2.3 ± 0.1	11 ± 1	14 ± 1	0.42 ± 0.06	35 ± 6	3.2

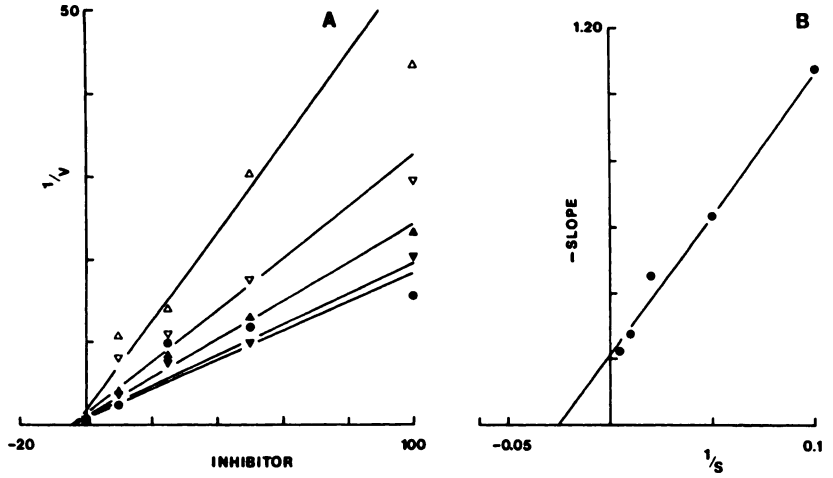

Fig. 3. Kinetics of inhibition by parathion of microsomal P450 2C11-mediated androstenedione 16α-hydroxylation (after a 10-min preincubation between NADPH, microsomes, and the pesticide). A, Dixon plots at the following androstenedione concentrations: ●, 10 μM; ▼, 20 μM; ▲, 50 μM; ▽, 100 μM; △, 200 μM. B, Primary replot of the slopes of the Dixon plots versus reciprocal androstenedione concentration. Units are as follows: substrate and inhibitor concentration, μM; V, nmol/min/mg of protein.

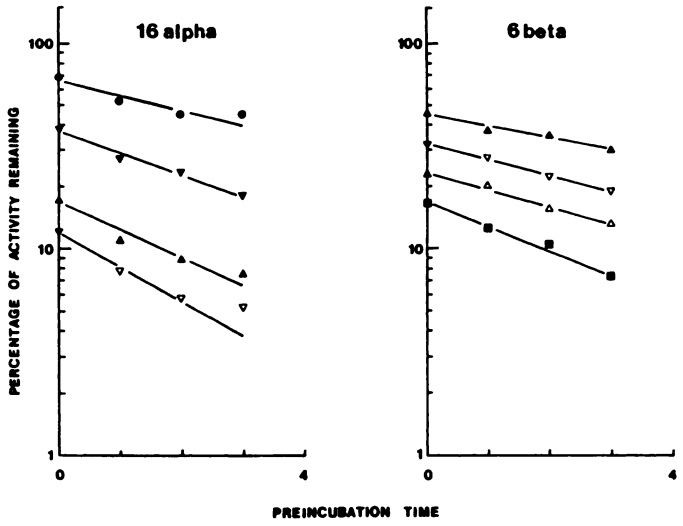

Fig. 4. Semilogarithmic plots of the effect of preincubation between parathion and NADPH-fortified rat liver microsomes on remaining androstenedione 16α-hydroxylation (*left*) and 6β-hydroxylation (*right*) activities. Parathion concentrations were as follows: ●, 5 μM; ▼, 10 μM; ▲, 25 μM; ▽, 50 μM; △, 100 μM; ■, 200 μM. Unit of preincubation time, min.

TABLE 2

Inactivation by parathion of microsomal androstenedione hydroxylases in male rat liver

Formation of hydroxyandrostenedione metabolites was measured after either 0, 1, 2, or 3 min of preincubation between parathion and NADPH-supplemented microsomal fractions before transfer to flasks containing the steroid substrate. Each experiment was performed in duplicate, and values were determined from the linear portions of plots of log (percent remaining activity) versus time.

Parathion concentration	Time for loss of 50% of activity (pseudo-first-order rate constant) for hydroxyandrostenedione metabolite formation[a]		
	6β	7α	16α
μM	min (min^{-1})		
5	NI[b]	NI	3.24 (0.214)
10	NI	NI	2.75 (0.252)
25	5.02 (0.138)	NI	1.92 (0.360)
50	3.76 (0.184)	NI	1.87 (0.370)
100	3.56 (0.194)	NI	ND[c]
150	2.71 (0.255)	NI	ND

[a] Time (min) required for a 50% decrease in metabolite formation and pseudo-first-order rate constant (min^{-1}).
[b] NI, no inactivation observed at this concentration of parathion.
[c] ND, inactivation not determined at this concentration of parathion because the zero-time activity (direct inhibition) was too extensive (<10% activity uninhibited).

from the slopes of the linear portions of the plots (Table 2). Thus, the time required for loss of 50% of the zero-time activity was 3.24 min with 5 μM parathion and was 1.87 min with 50 μM parathion. Inactivation of the enzymes of androstenedione 6β- and 7α-hydroxylation was not observed with 5 μM parathion. However, 6β-hydroxylase activity was inactivated by 25 or 50 μM parathion ($t_{1/2}$ of 5.02 and 3.76 min, respectively; Table 2).

The relationships between $t_{1/2}$ values and reciprocal inhibitor concentrations were established for the inactivation of androstenedione 6β- and 16α-hydroxylation by parathion (Fig. 5). The *x*- and *y*-intercepts from these plots provided estimates of the dissociation constant for the enzyme-inactivator complex (K_{inact}) and the maximal rate of enzyme inactivation, respectively. Thus, in the case of P450 2C11-mediated androstenedione 16α-hydroxylation, the K_{inact} was 4.6 μM and the maximal $t_{1/2}$ was 1.72 min, whereas for P450 3A2-mediated androstenedione 6β-hydroxylation the K_{inact} was 24 μM and the maximal $t_{1/2}$ was 2.59 min. The K_{inact} values for parathion against androstenedione 16α- and 6β-hydroxylation of 4.6 and 24 μM, re-

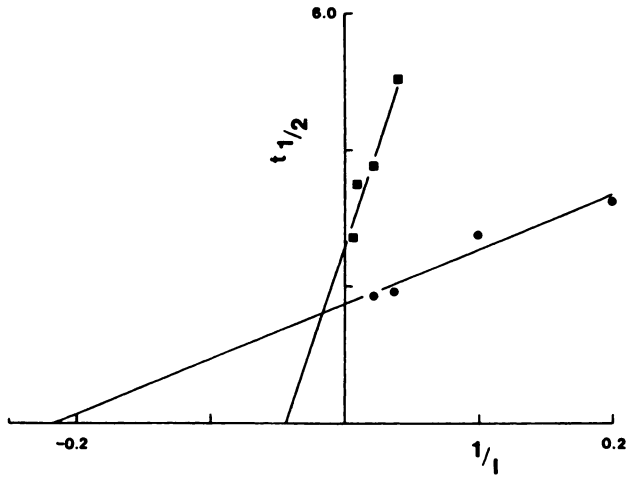

Fig. 5. Replots of the times for 50% loss of androstenedione 16α-hydroxylation (●) and 6β-hydroxylation (■) activities (from the lines in Fig. 4) versus reciprocal parathion concentration. The *y*-intercept provides estimates for the maximal inactivation rates of the activities. The units are as follows: parathion concentration, μM; $t_{1/2}$, min.

spectively, may be contrasted with the corresponding K_i values of 0.42 ± 0.06 μM and 3.6 ± 0.4 μM determined from the preincubation studies summarized in Table 1. The difference in the estimates for these constants is presumably related to the finding that parathion is not a pure inactivator of P450 steroid hydroxylases but also elicits quite potent reversible inhibition (reflected by the K_i values in the absence of preincubation). From these experiments it emerged that the major constitutive P450s, 2C11 and 3A2, in male rat liver are preferentially susceptible to inhibition and inactivation by parathion, whereas the P450s 2A1 and 2A2 are apparently refractory to inhibition.

Further studies assessed the effect of the phosphorothioate on regioselective progesterone hydroxylation, because 21-hydroxylation of the steroid is considered to be mediated extensively by P450 2C6, another quantitatively significant enzyme in rat liver (4, 26). Progesterone 21-hydroxylation was inhibited extensively at low concentrations of parathion (1 and 5 μM), but evidence of inactivation was not obtained (data not shown). Higher concentrations of parathion (>10 μM) inhibited the activity almost completely. Thus, it was not possible to use this activity to determine whether inactivation of P450 2C6 occurred at higher concentrations of the pesticide. In keeping with the observed effects of the phosphorothioate on P450s 2C11 and 3A2, the 2α- and 16α-hydroxylations of progesterone (P450 2C11-mediated) were inactivated by concentrations of 5 μM (but not 1 μM) parathion; at least 25 μM parathion appeared necessary for inactivation of progesterone 6β-hydroxylation (P450 3A2 mediated).

Immunochemical analysis of P450 2C11 content of microsomes during parathion metabolism. It was considered important to assess whether the microsomal content of P450 2C11 apoprotein was altered during NADPH-supported metabolism of parathion. Incubation of parathion with microsomes from untreated rat liver and NADPH for periods of up to 30 min did not result in any alteration in microsomal P450 2C11 in these fractions (Fig. 6). The experiment was performed under conditions of linearity between microsomal protein and apparent P450 2C11 content. Therefore, the present data support the notion that P450 inactivation by parathion occurs

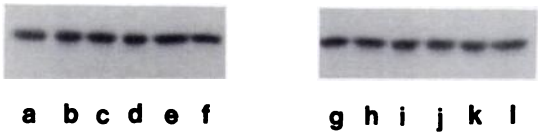

Fig. 6. Immunoblots of time-dependent changes in P450 2C11 content in hepatic microsomes during incubation. *Lanes a–f*, blots of P450 2C11 0, 2, 5, 10, 20, and 30 min, respectively, after the addition of NADPH to male rat hepatic microsomes (parathion excluded). *Lanes g–l*, blots of P450 2C11 0, 2, 5, 10, 20, and 30 min, respectively, after the addition of NADPH to microsomes containing 50 μM parathion. Lanes contained 18.8 μg of microsomal protein and were processed as described in Materials and Methods. The complete experiment was performed in duplicate, with similar results being obtained on both occasions.

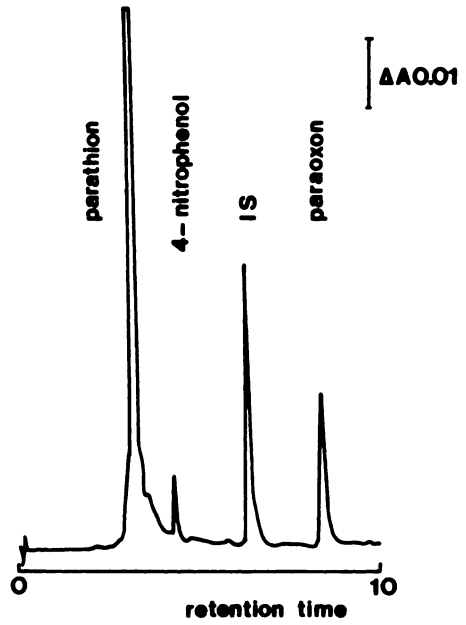

Fig. 7. Typical HPLC separation of parathion and its metabolites produced in a standard incubation, as described in Materials and Methods. The internal standard (*IS*) is 4,4′-dihydroxybiphenyl.

along with heme loss from the holoenzyme. However, it is possible that P450 2C11 apoprotein modification occurs without significant alteration of either the apparent molecular weight of the enzyme or the epitope recognized by the anti-P450 2C11 IgG.

Metabolism of parathion in microsomes from untreated rat liver. Apart from eliciting P450 inactivation, parathion also undergoes oxidative metabolism to paraoxon and 4-nitrophenol (Fig. 1). As part of the present study, parathion metabolism in rat liver microsomes was estimated, to determine the number of catalytic events that lead to P450 inactivation. A typical HPLC profile of metabolites of parathion produced in rat liver microsomes is presented in Fig. 7. Both principal metabolites were formed at similar rates in microsomal incubations (0.5 mg of microsomal protein, 2-min reaction time, and 0.4-ml reaction volume) when the parathion concentration was 250 μM. Thus, in separate incubations with three different microsomal fractions, paraoxon formation was 4.32 ± 0.57 nmol/min/mg of protein (mean ± standard error) and 4-nitrophenol formation was 4.35 ± 0.39 nmol/min/mg of protein. P450 loss under the same conditions was 12 ± 2%. Thus, 8.67 ± 0.52 nmol of parathion was consumed while 0.042 ± 0.007 nmol of holo-P450 was lost.

Discussion

Studies with experimental animals have described the capacity of parathion and analogues to impair the function of hepatic P450 enzymes and to decrease microsomal drug oxidation (10–14). The process of inactivation is considered to involve the transfer of atomic sulfur to P450 from the phosphorothioate during its oxidation to paraoxon and 4-nitrophenol (10, 11, 27, 28). Exposure of humans to phosphorothioate pesticides such as parathion raises the concern that the metabolic capacity of the individual may be compromised and that untoward effects from subsequent drug therapy to alleviate the anticholinergic effects of intoxication may be experienced.

The nature of the covalent binding between P450 and the sulfur atom of agents such as parathion and carbon disulfide has been examined in microsomes (13) and in reconstituted systems incorporating purified P450 (27). It appears clear that P450 is the primary microsomal target of the sulfur atom, because most of the radiolabel derived from [^{35}S]parathion that was covalently attached to the membrane was precipitated by an antibody to the cytochrome (9). About half of the incorporated radiolabel was released as thiocyanate from the membrane after treatment with cyanide ions (29, 30), and 75% was released after treatment with dithiothreitol (27). Halpert *et al.* (27) suggested that atomic sulfur from parathion became bound covalently to cysteine and at least three other amino acids of P450 2B1, and they demonstrated that the release of bound sulfur occurred without restoration of P450 activity. More recently, evidence has been presented that oxidation of parathion by P450 also leads to the formation of high molecular weight aggregates (27) that could be associated with heme-protein adduction (31). The present finding that approximately 210 nmol of parathion were metabolized (to paraoxon and 4-nitrophenol) for each 1 nmol of holo-P450 that was lost is of interest regarding the overall significance of the coordination of atomic sulfur in the P450 inactivation process. It has been suggested from extensive mechanistic work that initial oxidation of the parathion molecule by P450 occurs at the thionosulfur atom, giving rise to an *S*-oxide (32). However, a precise explanation for the stoichiometric discrepancy is not available at this time. It is also conceivable that holo-P450 loss may underestimate the extent of P450 deactivation, which may also proceed by heme-protein adduction. However, high molecular weight aggregates that were immunoreactive with the anti-P450 2C11 IgG used in the present study were not observed.

The relationship between parathion metabolism and holo-P450 loss in microsomes from untreated rat liver is similar to that estimated for allylisopropylacetamide in phenobarbital-induced fractions (33, 34). Decker *et al.* (35), in contrast, estimated that 20–30 molecules of spironolactone were oxidized by P450 for each molecule of the hemoprotein that was deactivated, and Guengerich (36) found a ratio of 9 for the inactivation of human P450 3A4 by gestodene. It is noteworthy that this group of P450 inactivators exert their effects by at least two distinct mechanisms; allylisopropylacetamide and gestodene contain olefinic functionalities that generate abnormal porphyrins after undergoing microsomal oxidation, whereas spironolactone and parathion reportedly transfer sulfur to the P450 apoprotein. Both classes of agents generate heme-protein adducts (31, 37).

The primary emphasis of the present study was to investigate the relative susceptibilities of different P450s in untreated rat liver to inhibition and inactivation by parathion. Previous studies have not addressed this aspect because the multiplicity of the microsomal P450 system in mammalian liver has been established only relatively recently. Because different P450s are now known to participate to varying extents in microsomal oxidation pathways, knowledge of the relative susceptibilities of different P450s to inhibition and inactivation may provide useful information on the consequences of exposure to the pesticides. It emerges from the data that inhibition and inactivation do not occur uniformly with each of the principal rat hepatic P450s. Thus, the male-specific P450 2C11 was particularly susceptible to inhibition and inactivation, whereas P450 3A2 was inactivated to a slightly lesser degree than P450 2C11. P450 2C6, in contrast, was inhibited efficiently by parathion but was not subject to inactivation, whereas the P450s 2A1/2 appeared to be completely refractory to inhibition. It is therefore possible that the observed loss of spectrophotometrically determined P450 may be related most closely to inactivation of the quantitatively important P450s 2C11 and 3A2. Interestingly, Decker *et al.* (35) called into question the precise relationship between heme destruction and the covalent binding of spironolactone. A similar question could be raised regarding the findings of the present study. The loss of P450 2C11-dependent activities occurred efficiently at concentrations of parathion that were 5–10-fold lower than those necessary for P450 heme loss. Thus, heme degradation would not appear to be an obligatory requirement for P450 inactivation during parathion metabolism.

The *in vivo* consequences of inhibition and inactivation are probably short term and longer term impairment of drug metabolism, respectively. An increasing number of metabolites of drugs and other xenobiotics elicit potent inhibition of P450, even though the parent compounds themselves may be relatively inert. In many cases, inhibition may be ascribed to a reactive metabolite that interacts directly with the P450 and leads to inactivation of the enzyme. Unlike metabolite intermediate complexation, the inactivated P450 may not generally be restored. In the case of allylisopropylacetamide, it appears that reactivation with exogenous heme may be partially effective in the restoration of lost P450 (38). It is becoming increasingly apparent, however, that heme loss is not the sole mechanism of P450 inactivation. Apoprotein modification has been reported and a variation, heme-protein adduction, may be a relatively widespread phenomenon (31, 37). In view of the present findings that indicate the nonuniform effects of parathion in the inactivation of major constitutive P450s in rat liver, it may now be appropriate to consider the question of selectivity in other inactivation processes. Certainly, the studies of Halpert *et al.* (39) have indicated that chloramphenicol is a potent inactivator of some, but not all, microsomal P450s in rat liver. Subsequent studies identified the dichloromethyl moiety as the portion of the chloramphenicol molecule that undergoes P450 oxidation to an oxamyl species that inactives the enzyme (40, 41). More recent work has utilized this knowledge to facilitate the design of molecules with the structural features to promote inhibition of specific P450s (42). It should be added, however, that it appears difficult to achieve potent inactivation while retaining specificity for a particular P450 (43).

Studies of the present type are useful in defining the consequences of inactivation of drug-metabolizing enzymes on sub-

sequent drug therapy. There have been reports that drug administration for the control of adverse effects in individuals who have been exposed to phosphorothioate pesticides can lead to additional toxic effects. Thus, the phenothiazine tranquilizer promazine produced hypotension, convulsions, apnea, and then death in an agricultural worker poisoned with parathion (14). Because these are symptoms of promazine overdosage and not of parathion intoxication, it seems that phosphorothioate exposure can, in some instances, produce a significant decrease in the metabolic capacity of certain human hepatic P450s.

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