Identification of the Cytochrome P450 Isoenzymes Involved in the Metabolism of Diazinon in the Rat Liver

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ABSTRACT: The metabolism of diazinon, an organophosphorothionate pesticide, to diazoxon and pyrimidinol has been studied in incubations with hepatic microsomes from control Sprague–Dawley (SD) rats or SD rats treated with different P450-specific inducers (phenobarbital, dexamethasone, β-napthoflavone, and pyrazole).

Results obtained indicate an involvement of CYP2C11, CYP3A2, and CYP2B1/2, whereas CYP2E1 and CYP1A1 do not contribute to the pesticide oxidative metabolism. Indeed, diazinon was metabolized by microsomes from control rats; among the inducers, phenobarbital and dexamethasone only increased the production of either metabolites, although to different extents. The production of the two metabolites is self-limiting, due to P450 inactivation; therefore, the inhibition of CYP-specific monooxygenase activities after diazinon preincubation has been used to selectively identify the competent CYPs in diazinon metabolism. Results indicate that, after diazinon preincubation, CYP3A2-catalyzed reactions (2β- and 6β-testosterone hydroxylation) are very efficiently inhibited; CYP2C11- and CYP2B1/2-catalyzed reactions (2α- and 16β-testosterone hydroxylation, respectively) are weakly inhibited, while CYP2E1-, CYP2A1/2-, and CYP1A1/2-related activities were unaffected. Results obtained by using chemical inhibitors or antibodies selectively active against specific CYPs provide a direct evidence for the involvement of CYP2C11, CYP3A2, and CYP2B1/2, indicating that each of them contributed about 40–50% of the diazinon metabolism, in hepatic microsomes from untreated, phenobarbital-, and dexamethasone-treated rats, respectively.

The higher diazoxon/pyrimidinol ratio observed after phenobarbital-treatment together with the significantly more effective inhibition toward diazoxon production exerted by metyrapone in microsomes from phenobarbital-treated rats supports the conclusion that CYP2B1/2 catalyze preferentially the production of diazoxon. © 1998 John Wiley & Sons, Inc. J Biochem Toxicol 13: 53–61, 1999

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INTRODUCTION

Organophosphorus pesticides (OP) are widely used in agriculture. They show several interesting features for environmental safety, such as limited environmental persistence and selective toxicity. In spite of their selectivity features, these pesticides are often highly toxic to humans and are responsible for the majority of accidental intoxication among agricultural and pesticide industry workers [1].

A key metabolic step in the acute toxicity of phosphorothionates (OPT) is their sulfoxidation by cytochrome P450 [2] to give the oxons (phosphate triesters), which are about three orders of magnitude more toxic than the corresponding OPT [3]. In this process, activated sulfur atoms are produced, which attack the very cytochrome P450 molecule catalyzing it, with consequent enzyme loss and reduction of the associated monooxygenase activities [4–7]. This process (desulfuration) proceeds through a phosphooxythiiran intermediate that can alternatively degrade by dearylation to less toxic products. Such reactions are depicted in Figure 1 for the case of diazinon. The relative proportions between the oxon (through desulfuration) and the aryl alcohol (through dearylation) depend on the isoenzyme catalyzing the reaction, as well as on the OPT structure [8–10]. Phosphate triesters, the target of which is brain acetylcholinesterase (AChE), may be hydrolyzed by plasma oxonases or "sequestered" by B-esterases, exerting in this way an effective detoxication [11–15]. Conjugation of OPT with GSH has been shown *in vitro* [16,17], but it might not be relevant *in vivo* [16].

The quantitative levels of activation and detoxication processes result in dramatic differences in toxicity. The different tolerance to OP within mammals
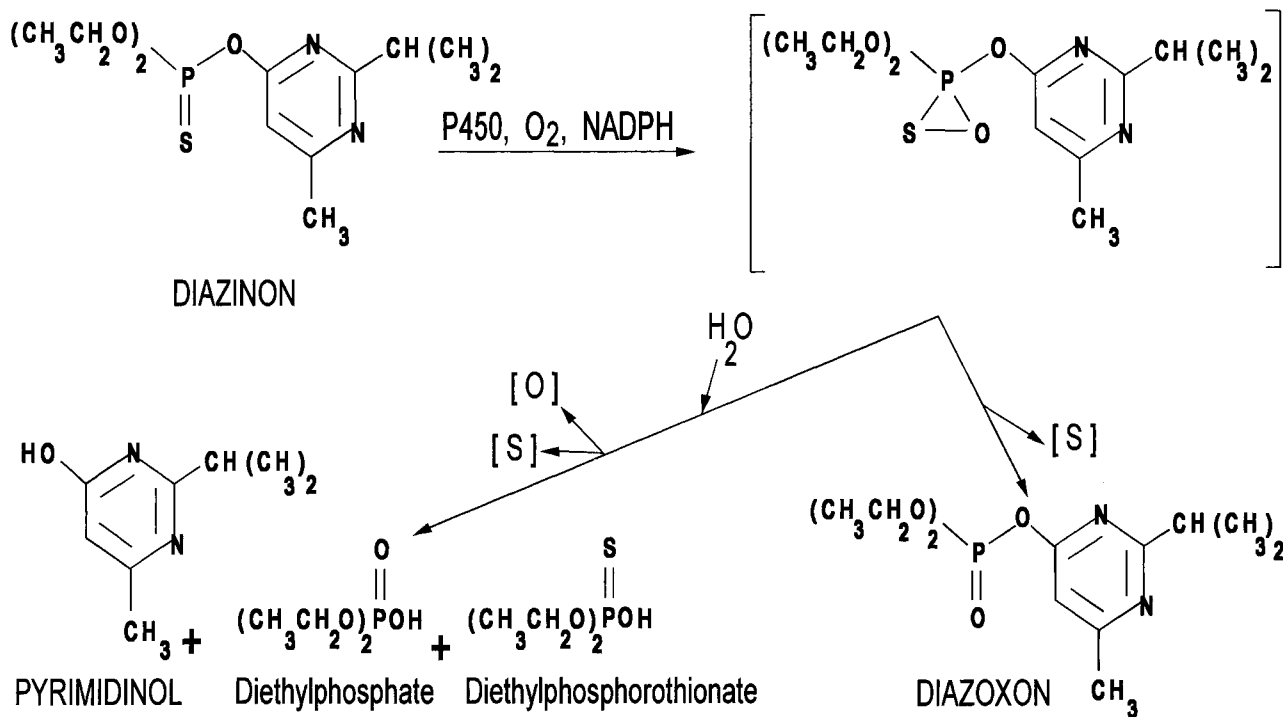
FIGURE 1. P450-dependent metabolism of diazinon.

[21,22] and between mammals, birds, and insects [18–20] is primarily caused by distinct levels of oxonases. *In vitro* data have been published with contrasting evidence about a role for hepatic sulfoxidation in the differential toxicity of OPT in mammals [8]. However, it was shown that the selectivity of action of OPT among fish species [23] depends on different levels of sulfoxidation [4], together with differences in oxonase levels and in the affinity of the corresponding oxons for AChE [17]. With reference to humans, an association between serum oxonase levels and individual susceptibility to OP pesticides has been suggested [24–27]. The relevance of hepatic P450-supported sulfoxidation in OPT toxicity is not yet clear but may be critical in view of the wide interindividual differences observed *in vitro* for such activity [9].

Parathion sulfoxidation in the rat liver is catalyzed by the constitutive CYP2C11, CYP3A2 [5], and the phenobarbital-inducible CYP2B1 [6,7,28]. Furthermore, it has been recently shown that CYP3A4, the human CYP orthologous to rat CYP3A2, and CYP2B6, orthologous of rat CYP2B1, catalyze the sulfoxidation of parathion much more efficiently and with much higher production of paraoxon than other hepatic CYPs [9]. In this article, we identify the hepatic CYPs responsible for the

diazinon oxidative metabolism in the rat. Gathering information on the CYPs involved in the metabolism of different OPT may allow a better use of the animal model to assess the relevance of sulfoxidation in the human individual susceptibility to the whole OPT class or of groups of OPT with similar enzymological dependence. Moreover, this information may be useful in the prediction of possible toxicological interactions occurring at metabolic level, between different OPT or between OPT and other chemicals.

MATERIALS AND METHODS

Products

Diazinon (purity 99%) was obtained from Riedel-de Haën (Germany). Diazoxon (purity 99%) and pyrimidinol (purity 99%) were kindly gifted by Ciba Geigy (Basel, Switzerland). NADP, glucose-6-phosphate (G6P), and G6P-dehydrogenase (G6PDH) were supplied by Boheringer Mannheim GmBh (Mannheim, Germany). Defatted bovine serum albumin (BSA) was from Serva, Feinbiochemica (Heidelberg, Germany). Aniline was from BDH Chemicals Ltd. (Poole, En-

gland). Sodium phenobarbital (PB) was from FLUKA AG (Buchs, Switzerland). β-Napthoflavone (BNF), pyrazole (PYR), dexamethasone (DEX), α-napthoflavone (ANF), 4-methylpyrazole (4MPYR), metyrapone (MET), triacetyloleandomycin (TAO), resorufin, pentoxyresorufin, ethoxyresorufin, testosterone, corticosterone, 4-androsten-3,17-dione (17OT), and 2α-, 16α-, 6β-, 16β-hydroxy-testosterone (2α, 16α, 6β, 16β) were purchased from Sigma Chemicals Co. (St. Louis, MO). 2β-, 6α-, 7α-hydroxytestosterone (2β, 6α, 7α) were supplied by Steroids Reference Collection (D.N. Kick, Department of Chemistry, Queen Mary College, London, England).

Rabbit antirat CYP2C11, antirat CYP3A2, and antirat CYP2B1 polyclonal antibodies (Ab) for immunoinhibition studies were purchased from Gentest Corp. (Woburn, MA).

All other analytical or HPLC grade chemicals were obtained from commercial sources.

Animals and Microsomal Preparations

Male SD rats (180–200 g b.w.) were obtained from Harlan Nossan (Correzzana, Italy). They were maintained on a 12 hour light cycle and provided with food (contaminant-free pellet diet) and water *ad libitum* for 1 week, before starting the experiment. Rats were treated with either (1) daily i.p. injection of PB (100 mg/Kg b.w.) in saline for 3 days, (2) single i.p. injection of BNF (80 mg/Kg b.w.) in corn oil 48 hours before sacrifice, (3) daily i.p. injection of PYR (200 mg/Kg b.w.) in saline for 3 days, or (4) daily i.p. injection of DEX (40 mg/Kg b.w.) in corn oil for 3 days. Liver microsomes (NT, PB, BNF, PYR, and DEX microsomes) were prepared from untreated, PB-, BNF-, PYR-, or DEX-treated animals, respectively, after a 24 hour fasting period as previously described [29]. Microsomal protein content was determined according to Oyama and Eagle [30], using BSA as a standard. Cytochrome P450 (P450) levels were measured by the method of Omura and Sato [31]. The microsomal protein mean content of the preparations was 17 ± 3, 28 ± 2, 18 ± 2, 20 ± 1, and 21 ± 2 (mg/g tissue) in NT, PB, DEX, BNF, and PYR microsomes, respectively. The P450 mean content was 1.2 ± 0.2, 2.5 ± 0.4, 1.7 ± 0.1, 1.4 ± 0.2, and 1.2 ± 0.1 (nmol/mg protein) in NT, PB, DEX, BNF, and PYR microsomes, respectively.

In Vitro Metabolism of Diazinon

Enzymatic Incubation

The standard incubation mixture (0.5 mL) contained 2 mg/mL microsomal protein, 2 mM G6P, 2 mM MgCl$_2$, 2–4 U/mL G6PDH, and 50 or 500 μM diazinon in 50 mM Tris-HCl, 1 mM EDTA, pH 7.4. The mixtures were kept at 37°C for 3 minutes before starting the reaction. The enzymatic incubation was started with the addition of NADP (1 mM final concentration), carried out at 37°C under shaking (130 cycles/min) and stopped after 5 minutes, unless otherwise indicated, by adding 3 mL cold CH$_2$Cl$_2$. Blanks differed from samples only because NADP was added after the addition of cold CH$_2$Cl$_2$. When required, the following compounds (final concentrations: 0.05 mM for TAO and 0.1 mM for the other inhibitors) were also added to the incubation mixture: MET and 4MPYR as aqueous solution, and ANF and TAO as methanol solution (methanol was never more than 5% of the incubation volume). In the experiments with TAO, the mixtures were preincubated at 37°C for 15 minutes, before adding the substrate. All incubations were carried out in duplicate.

Extraction of Diazinon and Its Metabolites from Incubation Mixture

After stopping the reaction, samples were vigorously shaken for 10 minutes and centrifuged at 3000 rpm for 10 minutes. The resulting organic layer was withdrawn, and the remaining aqueous phase was extracted again with 2 mL CH$_2$Cl$_2$. The pooled organic phases were stored at -20°C; they were dried under nitrogen and resuspended with 250 μL of methanol (HPLC grade) immediately before HPLC analysis.

HPLC Analysis

Quantitative analysis of diazinon and its metabolites was carried out according to the procedure described by Keizer *et al.* [32] with minor modifications. Briefly, a Perkin Elmer Series 200 Liquid Chromatograph equipped with a Perkin Elmer diode array LC 235 detector was used, with a Supelco LC$_{18}$-DB ($L = 25$ cm; diam $= 4.6$ mm) reversed-phase column. The mobile phase (1 mL/min flow rate) consisted of mixtures of methanol:water 75:25 (v/v) to assay diazinon and diazoxon, and methanol:water 45:55 (v/v) to assay pyrimidinol; the injection volume was 6 μL. The absorption of the eluate was measured continuously at 245 or 225 nm for diazinon and diazoxon or pyrimidinol analysis, respectively. The three compounds were identified by comparing their retention times with pure analytical standards; their amounts were determined referring to a calibration straight line (average correlation coefficient $R^2 = 0.999$, 0.996, and 0.999 for diazinon, diazoxon, and pyrimidinol, respectively) prepared with known amounts of the analytical stan-

TABLE 1 Inhibition of Regio- and Stereo-Selective Testosterone Hydroxylation after Diazinon Preincubation with Liver Microsomes from Untreated, PB-, or DEX-Treated SD Rats

	$IC_{50}(\mu M)$							
	2α	16α	$17OT$	16β	2β	6β	7α	6α
NT microsomes	128	133	216	210	131	90	>500	>500
	(2.20 ± 0.22)	(2.46 ± 0.25)	(1.22 ± 0.10)	(0.06 ± 0.01)	(0.17 ± 0.02)	(0.95 ± 0.12)	(0.37 ± 0.04)	(0.07 ± 0.03)
PB microsomes	169	293	350	117	30	73	>500	—
	(0.23 ± 0.05)	(2.23 ± 0.16)	(2.08 ± 0.13)	(1.86 ± 0.20)	(0.75 ± 0.10)	(1.1 ± 0.08)	(0.22 ± 0.06)	(0.03 ± 0.01)
DEX microsomes	79	89	122	>500	39	47	287	—
	(1.24 ± 0.44)	(1.56 ± 0.52)	(1.07 ± 0.20)	(0.18 ± 0.16)	(1.34 ± 0.28)	(4.57 ± 1.40)	(0.37 ± 0.15)	(n.d.)

n.d. = not detectable.

Data in parentheses represent the activities of testosterone hydroxylation obtained with microsomes preincubated without diazinon and are expressed as nmoles (mg protein)$^{-1}$ (min)$^{-1}$. Values represent means $\pm$ S.E. calculated on at least three different microsomal preparations.

dards (range 3–500 μM for the parent compound and 1.5–100 μM for the two metabolites). The extraction efficiencies of diazinon, diazoxon, and pyrimidinol were 77 $\pm$ 2, 93 $\pm$ 3, and 78 $\pm$ 3 (%, means $\pm$ deviations), respectively.

Assays of Monooxygenase Inhibition and Cytochrome P450 Loss

After a standard incubation at 37°C for 15 minutes with different amounts of diazinon (10–500 μM), aliquots of the mixture were diluted as required and immediately used in the assays of P450 and of monooxygenase activities. Ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-O-depenthylase (PROD) were assayed by the continuous spectrofluorimetric procedure of Burke *et al.* [33]. Aniline hydroxylase (AnOH) was determined as described by Ko *et al.* [34]. Regioselective testosterone hydroxylation was measured following the previously described method [35]. Control reactions, which differed from samples only for the absence of diazinon, were concurrently carried out.

Immunoinhibition Assays

Antirat liver CYP3A2 and CYP2C11 antibodies were used to inhibit diazinon metabolism. Liver microsomes (500 μg protein) were suspended in 50 mM Tris-HCl, 1 mM EDTA, pH 7.4, mixed with different amounts of antiserum and preincubated at room temperature under mild agitation for 30 minutes (final volume 500 μL). Then the mixtures were transferred to a water bath at 37°C and added with the NADPH-generating system and diazinon (final concentrations: 2 mM G6P, 2 mM $MgCl_2$, 2–4 U/mL G6PDH, and 50 or 500 μM diazinon); the reaction was started by injecting NADP (1 mM final concentration) and stopped after 5 minutes by adding 5 mL cold CH_2Cl_2. The mixtures were then processed for the assay of diazinon metabolism as described previously. The amounts of antiserum used were expected to produce maximal inhibi-

tion of the specific enzymatic activity: 25 μL anti-CYP2C11 antiserum (more than 90% inhibition of the 16α-hydroxylation of testosterone), 50 μL anti-CYP3A2 antiserum (more than 90% inhibition of the 6β-hydroxylation of testosterone). Control reactions, in which normal goat serum replaced the anti-CYP specific antiserum, were also concurrently carried out.

RESULTS

Effects of Different Pretreatments on Diazinon Metabolism in Rat Liver Microsomes

Diazinon was oxidatively metabolized by NT microsomes. Among a number of substances used to induce diazinon metabolism (Figures 2A and 2B), DEX and PB were effective in increasing both diazoxon and pyrimidinol production, whereas PYR and BNF treatment failed to affect significantly the hepatic microsomal production of either metabolite. In any case, metabolite formation increased at maximum by a factor of about 3 with respect to microsomes from control animals. Furthermore, when expressed per nmole P450 (Figures 2C and 2D), DEX treatment increased the production of pyrimidinol, whereas PB increased that of diazoxon; PYR and BNF treatment were ineffective.

P450 Loss and Inhibition of Different CYPs during In Vitro Diazinon Metabolism by Rat Liver Microsomes

The production of diazinon metabolites by NT and PB microsomes was not linear with time, indicating its metabolism-dependent inhibition (Figures 3A and 3B). A substantial decrease in the metabolite production rate was already apparent within 15 minutes of incubation.

Preincubation of microsomes from differently induced animals with diazinon inhibited testosterone hydroxylation at selected substrate positions, indicat-
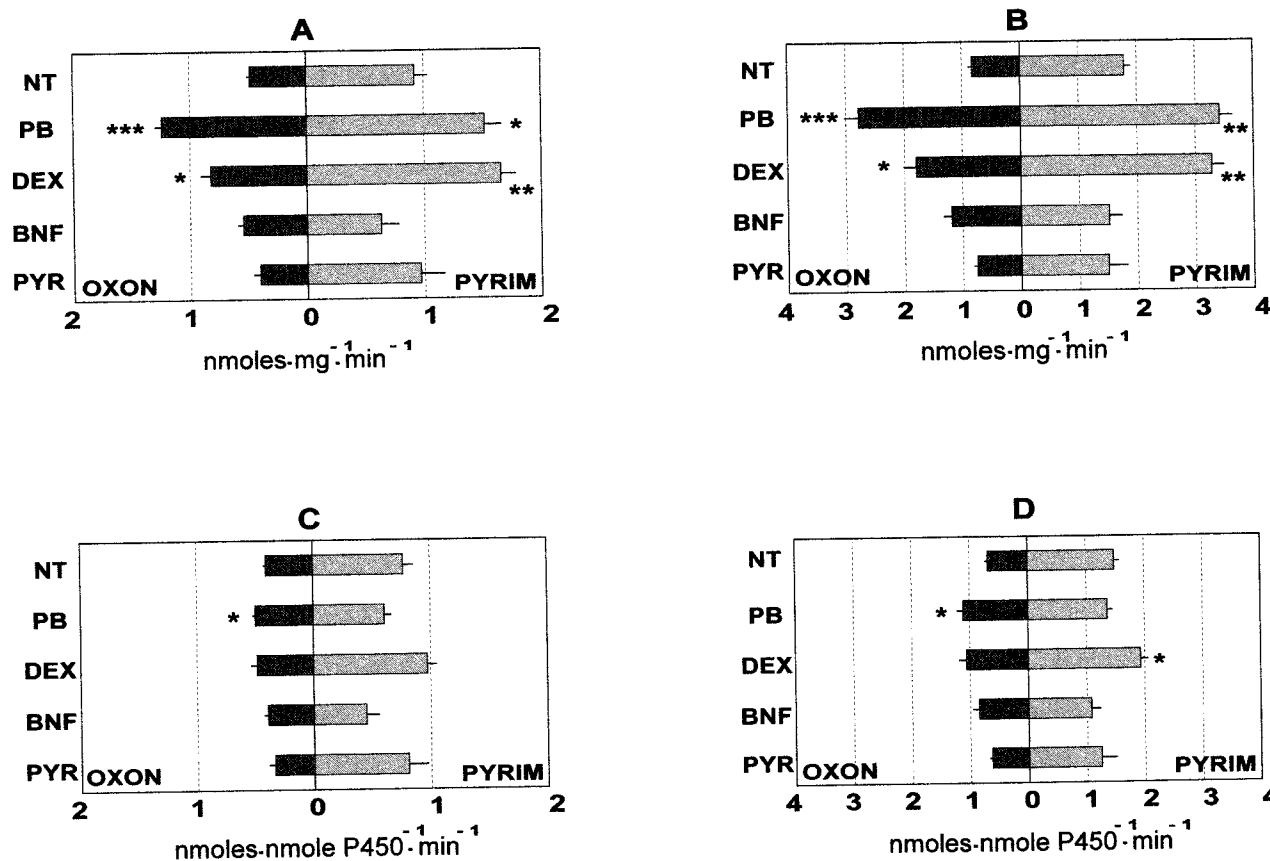
FIGURE 2. Formation of diazoxon (oxon) and pyrimidinol (pyrim) in incubations of 50 μM (A and C) or 500 μM (B and D) diazinon with hepatic microsomes from untreated SD rats (NT) or from SD rats treated with phenobarbital (PB), dexamethasone (DEX), β-napthoflavone (BNF), and pyrazole (PYR). Results are expressed as nmoles (mg protein)$^{-1}$ (min)$^{-1}$ (A and B) or as nmoles (nmol P450)$^{-1}$ (min)$^{-1}$ (C and D). Bars represent means $\pm$ standard errors (S.E.), calculated on at least three different microsomal preparations. Results marked with three, two, and one stars significantly differed from results obtained with NT microsomes at $p \leq 0.001, 0.01$, and 0.05, respectively. Data were compared using Student's t-test.

TABLE 2. Monooxygenases Inhibition after Diazinon Pre-incubation with Liver Microsomes from Untreated, PB-, BNF-, or PYR-Treated SD Rats

	$IC_{50}(\mu M)$		
	PROD	EROD	AnOH
NT microsomes	46	29	>500
	(0.019 ± 0.004)	(0.071 ± 0.008)	(0.92 ± 0.03)
PB microsomes	290		
	(1.8 ± 0.4)		
BNF microsomes		>500	
		(7.1 ± 0.2)	
PYR microsomes			>500
			(1.5 ± 0.1)

   Data in parentheses represent the monooxygenase activities obtained with microsomes preincubated without diazinon and are expressed as nmoles (mg protein)$^{-1}$ (min)$^{-1}$. Values represent means $\pm$ S.E. calculated on at least three different microsomal preparations.

ing selective inhibition of CYPs (Table 1). The most effective diazinon inhibition occurred on 2β- and 6β-hydroxylations (mainly catalyzed by CYP3A2) [35,36], with an IC_{50} of about 40–50 μM, as resulting with DEX microsomes. 2α-Hydroxylation (catalyzed by CYP2C11) as well as 16β-hydroxylation (catalyzed by CYP2B1/2) [35] was inhibited with an IC_{50} of about 120–130 μM (as measured in NT and PB microsomes, respectively); 17OT- and 16α-hydroxylation (each one catalyzed mainly by the CYP2C family and by CYP2B1/2) [35] were weakly inhibited by diazinon preincubation in all the samples, whereas 6α- and 7α-hydroxylations (catalyzed by both CYP2A1 and CYP2A2) [37] were not inhibited. PROD and EROD were inhibited after diazinon preincubation with NT

microsomes (Table 2); in microsomes from PB-pre-treated animals, PROD was only weakly inhibited, whereas no inhibition of EROD was observed in BNF microsomes (Table 2). Aniline hydroxylase was inhibited neither in NT nor in PYR microsomes (Table 2). Cytochrome P450 decreased significantly in DEX microsomes only, reaching a loss of about 30%.

Inhibition of Diazinon Metabolism by Selective Chemical Inhibitors and Anti-CYP Antibodies

Among inhibitors selectively active against specific CYPs, only metyrapone produced an effective inhibition (about 30% in the total production of metabolites) of diazinon metabolism in NT microsomes (Table 3). In the same microsomes, anti-CYP3A2 Ab did not significantly affect diazinon metabolism, whereas anti-CYP2C11 Ab inhibited the formation of both diazoxon and pyrimidinol by about 40%. With DEX microsomes, anti-CYP3A2 Ab and TAO inhibited the metabolism of 50 μM diazinon by about 40%; both Ab and TAO seemed a little less effective at 500 μM diazinon concentration. Finally, in incubations containing PB microsomes and 50 μM diazinon, metyrapone inhibited to a similar extent (60–70%) the formation of each metabolite while different inhibition (40% and 70% for pyrimidinol and diazoxon production, respectively) could be observed at high (500 μM) diazinon concentration.

DISCUSSION

In this article, we have used three different techniques to identify the CYPs responsible for the metabolism of diazinon. The inhibition of enzyme activities supported by specific CYPs, which is observed after preincubation with diazinon, can be used to selectively identify the metabolism-competent CYPs, because it is caused by a mechanism-based process [38,39]. The inhibition observed in the present work may partly be caused also by reversible substrate competition; however, we chose preincubation times long enough to allow a substantial enzyme inactivation by diazinon metabolism (Figure 2). Furthermore, the complete agreement between the results obtained with this and the other techniques indicates that metabolism-unrelated competitive inhibition of the tested CYPs did not affect the qualitative identification of competent CYPs.

The results obtained indicated that CYP3A2, CYP2B1, and CYP2C11 support diazinon oxidation, whereas CYP2E1 and CYP1A1/2 do not. Indeed, diazinon metabolism was present in NT microsomes, in-
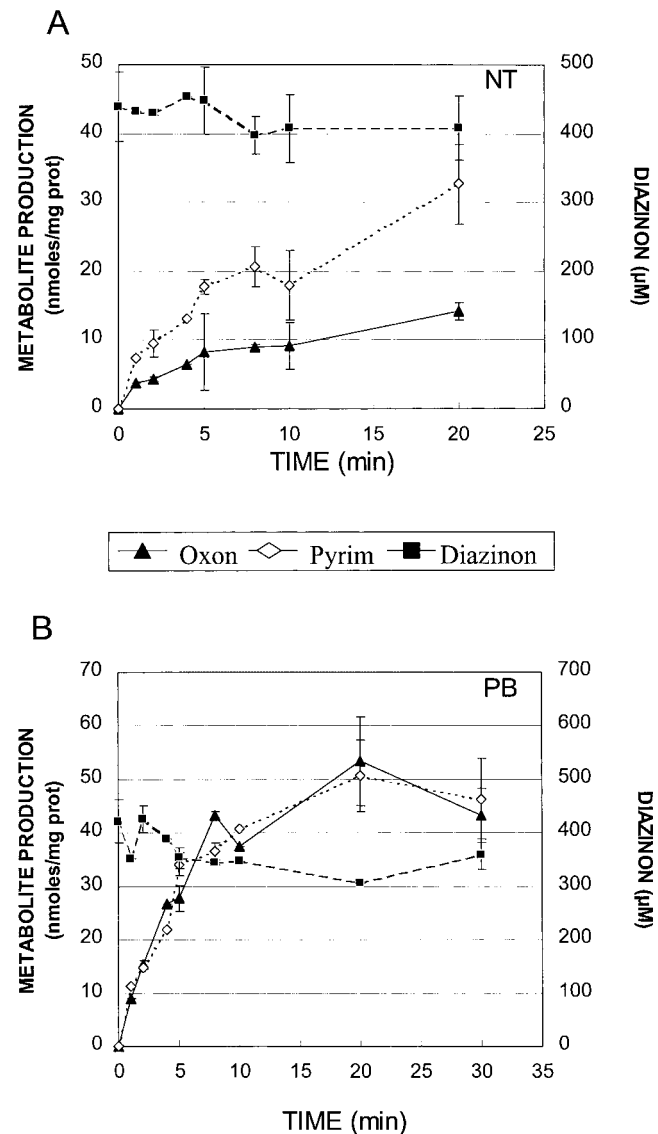

FIGURE 3. Time dependence of the formation of diazoxon (oxon) and pyrimidinol (pyrim) in incubations of 500 μM diazinon with hepatic microsomes from untreated (A) or PB-treated (B) SD rats. Data represent means ± S.E. calculated on 3–4 different microsomal preparations.

dicating the involvement of constitutive CYPs (among which CYP2C11 is the most abundant); moreover, diazinon metabolism increased following pretreatment with DEX, a selective inducer of CYP3A2 [40,41], and with PB, an inducer of several CYPs, including CYP2B1/2 and CYP3A2 [40,41]. DEX and PB, however, differed in their effects. Indeed, only PB altered the proportions between diazoxon and pyrimidinol, reaching a diazoxon/pyrimidinol ratio of 0.8, compared with the value of 0.5 measured in NT and DEX microsomes (Figures 2C and 2D). This result indicates that

TABLE 3. Chemical and Immunoinhibition of Diazinon Metabolism in Liver Microsomes from Untreated, PB-, or DEX-Treated SD Rats

Diazinon Concentration	Inhibitor	Metabolite Production (% of control)					
		NT Microsomes		PB Microsomes		DEX Microsomes	
		Oxon	Pyrim	Oxon	Pyrim	Oxon	Pyrim
50 μM	—	100 (0.41 ± 0.06)	100 (1.33 ± 0.25)	100 (1.1 ± 0.2)	100 (1.27 ± 0.02)	100 (0.7 ± 0.1)	100 (1.6 ± 0.3)
	MET	54 ± 13	75 ± 2	31 ± 3	35 ± 3		
	TAO	124 ± 26	92 ± 7			43 ± 3	56 ± 15
	ANF	118	98				
	4MPYR	91	102				
	control serum	100 (0.22)	100 (1.83)			100 (0.42)	100 (2.38)
	antirat CYP3A2 Ab	91	84			48	65
500 μM	—	100 (0.69 ± 0.07)	100 (2.03 ± 0.2)	100 (2.2 ± 0.2)	100 (2.5 ± 0.1)	100 (2.1 ± 0.5)	100 (3.8 ± 0.5)
	MET	74 ± 13	80 ± 2	32 ± 1*	59 ± 1*		
	TAO	99 ± 7	101 ± 4			76 ± 13	86 ± 10
	ANF	93 ± 16	82 ± 20				
	4MPYR	93	92				
	control serum	100 (0.48 ± 0.12)	100 (2.73 ± 0.57)			100 (0.52)	100 (4.43)
	antirat CYP2C11 Ab	60	60				
	antirat CYP3A2 Ab	122	74			77	86

Values are expressed as percentage of diazoxon (oxon) and pyrimidinol (pyrim) produced in standard incubations carried out without inhibitors or with non-immune goat serum (control serum). Data in parentheses refer to the activities of controls, expressed in nmoles (mg protein)$^{-1}$ (min)$^{-1}$.

Values represent means ± deviation, resulting from 2 to 4 different microsomal preparations. When the deviation is not indicated, data represent the mean of a duplicate experiment. Differential inhibition on oxon and pyrim production was compared using Student's t-test. Asterisks indicate values differing significantly from each other at $p < 0.01$.

PB-inducible isoenzymes (mainly CYP2B1/2) are less active in the production of pyrimidinol with respect to constitutive CYPs. Moreover, P450 loss due to diazinon metabolism was apparent in DEX microsomes only, indicating that, following induction, the CYPs competent in diazinon metabolism represent a significant fraction of the total P450 in DEX microsomes and not in PB microsomes. This is in line with the well-known different specificity of the two inducers. Treatments with BNF or PYR were ineffective in diazinon metabolism induction, indicating no support from CYP1A1/2 and CYP2E1, respectively.

More precise information could be derived from the inhibition of model activities by diazinon metabolism. Testosterone hydroxylation at selected, CYP-specific positions indicated that CYP3A2-supported activities were effectively inhibited; CYP2C11- and CYP2B1/2-catalyzed activities were weakly inhibited, whereas CYP2A1/2-supported activities were unaffected. PROD inhibition in PB microsomes was concordant in indicating the involvement of CYP2B1/2 in diazinon metabolism: indeed, this activity in hepatic microsomes from PB-induced rats is considered a selective marker of CYP2B1 [33]. Lack of inhibition of

EROD in BNF microsomes indicated that CYP1A1/2 did not contribute to diazinon metabolism. The effective inhibition of PROD and EROD observed in NT microsomes could be considered indicative of the involvement of constitutive CYPs. Indeed, due to the low content of CYP2B1 and CYP1A1/2 in NT microsomes, the limited PROD and EROD activities measured may not be exclusively associated with these CYPs. Finally, lack of inhibition of aniline hydroxylase ruled out the involvement of CYP2E1.

The use of selective chemical inhibitors or antibodies similarly pointed at the involvement of CYP2C11, CYP3A2, and CYP2B1 and indicated that each of them contributed about 40–50% of the diazinon oxidation observed in NT, DEX, and PB microsomes, respectively. These contributions were almost independent of the diazinon concentration used. Although evident at high diazinon concentration only, the inhibition exerted by MET on diazinon metabolism in PB microsomes was significantly more effective toward diazoxon production. This observation, together with the high diazoxon/pyrimidinol ratio observed in PB microsomes, supports the conclusion that CYP2B1/2 catalyzes preferentially the production of diazoxon.

A previous article showed the involvement of CYP3A2 and CYP2C11 in parathion metabolism by microsomes from untreated rat liver [5]. Rat liver CYP2B1/2 also catalyzes parathion oxidation [6,7]; however, it may not be relevant *in vivo,* due to its low level in adult untreated rats [42]. All these enzymes were inactivated during parathion metabolism [5–7]. Together with such reports, our results indicate that both parathion and diazinon are metabolized in the rat liver by the same CYPs, with consequent enzyme inactivation. The preferential production of the oxon metabolite shown by CYP2B1/2 with diazinon is a feature shared by PB-inducible CYPs from mouse liver and human CYP2B6 in the metabolism of other OP [9,10]. Since CYP3A2 and CYP2B1/2 catalyze diazinon metabolism with a different diazoxon to pyrimidinol ratio, changes in their relative contributions to diazinon metabolism may have different toxicological consequences. The oxidative transformation of parathion in human liver is almost completely dependent on CYP3A4 [9], while CYP2B6, also catalyzing this reaction, is normally of minor importance because of its limited amount ($<2\%$) in liver of healthy humans [43]. The rat, possibly with experimentally altered levels of its CYP3A2 and CYP2B1/2, may therefore represent a suitable model, mimicking some aspects of the human toxicology of OP. These may encompass the relevance of either different levels of sulfoxidation in the susceptibility of humans to OPT or toxicological interactions that can take place in case of exposures involving both parathion and diazinon or drugs acting on the same CYPs. It was shown in fish that a pretreatment with sublethal doses of diazinon caused acquired resistance to normally lethal doses of the same compound, because of monooxygenase inhibition [4]. It seems a reasonable hypothesis that a low-dose diazinon exposure can result in antagonistic effects on subsequent paraoxon intoxication and *vice versa.*

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