

## *In Vitro* Rat Hepatic and Intestinal Metabolism of the Organophosphate Pesticides Chlorpyrifos and Diazinon

T. S. Poet,<sup>1</sup> H. Wu, A. A. Kousba, and C. Timchalk

*Battelle, Pacific Northwest Division, Chemical Dosimetry, P.O. Box 999, Richland, Washington 99352*

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Chlorpyrifos (CPF) and diazinon (DZN) are thionophosphorus organophosphate (OP) insecticides; their toxicity is mediated through CYP metabolism to CPF-oxon and DZN-oxon, respectively. Conversely, CYPs also detoxify these OPs to trichloropyridinol (TCP) and 2-isopropyl-4-methyl-6-hydroxypyrimidine (IMHP), respectively. In addition, A-esterase (PON1) metabolism of CPF- and DZN-oxon also forms TCP and IMHP. This study evaluated the role intestinal and hepatic metabolism may play in both the activation and detoxification of CPF and DZN in Sprague-Dawley rats. Similar CYP- and PON1-mediated metabolic profiles were demonstrated in microsomes from liver or isolated intestinal enterocytes. The metabolic efficiency was estimated by calculating the pseudo-1st order rate constant from the metabolic constants by dividing  $V_{\max}/K_m$ . In enterocyte microsomes, the CYP metabolic efficiency for metabolism to the oxon metabolites was ~28-fold greater for CPF than DZN. Compared on a per nmol P450 basis, the  $V_{\max}$  for CPF in enterocytes was ~2–3 times higher than in liver microsomes for the production of CPF-oxon and TCP. The Michaelis-Menten rate constant ( $K_m$ ) for the metabolism of CPF to CPF-oxon was comparable in liver and enterocyte microsomes; however, the enterocyte  $K_m$  for TCP production was higher (indicating a lower affinity). The smaller volume of intestine, lower amount of CYP, and higher  $K_m$  for TCP in the enterocyte microsomes, resulted in a lower catalytic efficiency (2 and 62 times) than in liver for oxon and TCP. PON1-mediated metabolism of CPF- and DZN-oxon was also demonstrated in liver and enterocyte microsomes. Although PON1 affinity for the substrates was comparable in hepatic and enterocytic microsomes, the  $V_{\max}$  were 48- to 275-fold higher, in the liver. These results suggest that intestinal metabolism may impact the metabolism of CPF and DZN, especially following low-dose oral exposures.

**Key Words:** chlorpyrifos; diazinon; organophosphate insecticides; metabolism; liver; intestine.

The toxicity of broad-spectrum thionophosphorus organophosphate pesticides (OP), as with CPF and DZN, is associated

with the inhibition of acetylcholinesterase (AChE) in nerve tissue (Murphy, 1986; Sultatos, 1994). The chemical structure of CPF and DZN and their metabolic schemes are presented in Figure 1. The parent OPs are weak inhibitors of AChE, which undergo CYP-mediated oxidative desulfuration to CPF- and DZN-oxon, both potent inhibitors of AChE (Amitai, 1998; Chambers *et al.*, 1994; Sultatos and Murphy, 1983). CYPs also mediate the detoxification of CPF and DZN via dearylation, producing diethylphosphate, and TCP from CPF and IMHP from DZN (Ma *et al.*, 1994, 1995; Sams *et al.*, 2000). The balance between desulfuration and dearylation results in very different levels of AChE inhibition (Timchalk *et al.*, 2002b,a). Whereas B-esterases (B-est) such as AChE, butyrylcholinesterase (BuChE), and carboxylesterase (CaE) are stoichiometrically inhibited by the oxon (Chanda *et al.*, 1997; Clement, 1984), A-esterases (PON1) enzymatically hydrolyze OPs, but are not inhibited (Sultatos and Murphy, 1983). The PON1 metabolism of CPF-oxon and DZN-oxon results in the deactivation of the oxon to form TCP and IMHP, respectively. Although the overall detoxification of OP insecticides is mediated by multiple enzyme systems (CYP450, PON1, B-est), the capability of PON1 to protect against OP toxicity has been demonstrated in several studies in which exogenous administration of PON1 provided protection against acute OP poisoning in rodents (Costa *et al.*, 1990; Li *et al.*, 1993, 1995; Main, 1956).

Oral absorption of a chemical or drug can be altered by both intestinal and liver metabolism (Wacher *et al.*, 2001; Zhang and Benet, 2001). Although the blood flow and tissue volume of the intestine is slightly lower than the liver, the extensive microvilli structure results in a large surface area, ideally suited for absorption. Substances absorbed through the intestine are delivered to the liver via the portal vein, with over 75% of the total blood flow to the liver coming from the intestines (DeSesso and Jacobson, 2001). Since most absorption is thought to occur in the small intestine (Doherty *et al.*, 1997), intestinal first-pass metabolism may determine the balance between bioactivation/deactivation for orally administered xenobiotics (Wacher *et al.*, 2001).

A number of recent preclinical and clinical studies have demonstrated the importance of the intestines in first-pass metabolism, since both rodent and human intestinal epithelial

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<sup>1</sup> To whom correspondence should be addressed at Battelle, Pacific Northwest Division, Chemical Dosimetry, MSIN P7-59, P.O. Box 999, Richland, Washington 99352. Fax: (509) 376 9064. E-mail: torka.poet@pnl.gov.

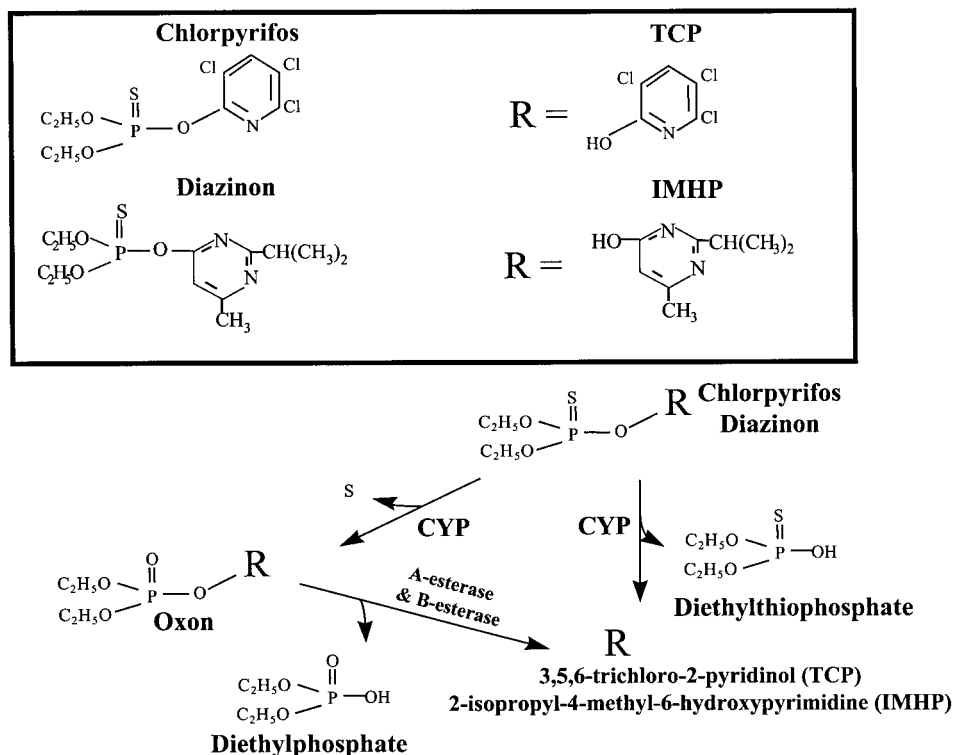


FIG. 1. Metabolic scheme for chlorpyrifos (CPF) and diazinon (DZN) and their major metabolites CPF- and DZN-oxon, TCP and IMHP, and diethyl- and diethylthiophosphate.

cells (enterocytes) contain enzymes capable of metabolizing a broad range of drugs and xenobiotics (Butler and Murray, 1997; Fabrizi *et al.*, 1999; Hall *et al.*, 1989; Obach *et al.*, 2001; Paine *et al.*, 1999; Sams *et al.*, 2000; Watkins, 1987; and Zhang *et al.*, 1999). In addition, P-glycoproteins (multidrug resistance proteins) on the apical borders of enterocytes are energy-dependent drug efflux pumps capable of lowering intracellular drug/chemical concentrations (Zhang and Benet, 2001). The role of intestinal metabolism and active extrusion of absorbed drugs are of current interest since collectively they may be a major determinant of oral drug bioavailability (Zhang and Benet, 2001). Although there has been a considerable focus on understanding the impact of intestinal metabolism on drug bioavailability, few studies have attempted to explore the influence of intestinal metabolism on the bioavailability of pesticides. In this regard, Lanning *et al.* (1996) investigated the interaction of CPF with P-glycoproteins and reported their upregulation in rat stomach, jejunum, and liver of rats following *in vivo* exposure to CPF. It was further reported that only the active metabolite of CPF, CPF-oxon, actively bound and activated P-glycoprotein, potentially contributing to the intestinal metabolism of this OP.

The metabolism of OPs to their oxon metabolites are likely mediated by CYP 3A4, 2B6 (human), 2B1/2 (rat), and 2D6 (human) (Ma and Chambers, 1995; Sams *et al.*, 2000; Tang *et al.*, 2001). Although the profile of enzymes in human intestine and liver are different, the common CYP in both the liver and small intestine is 3A4 (Obach *et al.*, 2001). The levels of 3A4

in the intestine may be as high as 90% of, or exceed the liver content; whereas, 2D6 and 2C are reported to be 10–20% of the hepatic levels (Watkins *et al.*, 1987; de Waziers *et al.*, 1990). CYP-mediated dearylation resulting in the deactivation of CPF to TCP is mediated by CYP 2C and 3A4 (Tang *et al.*, 2001). B-Esterase activity is similar in both intestine and liver, whereas, the activity of PON1 is approximately 100-fold lower in intestine (Pond *et al.*, 1995). Thus, the metabolizing enzymes responsible for both the bioactivation and detoxification are present in the small intestine at levels lower than the liver, but still significant.

In developing physiologically based pharmacokinetic (PBPK) models, hepatic metabolism is primarily considered, and the contribution of intestinal metabolism has been ignored and “lumped” within the liver compartment. This approach has provided reasonably good estimates of OP dosimetry (Timchalk *et al.*, 2002), since toxicity studies in animals are conducted at high doses, where gut metabolic capacity (Phases I and II) would be saturated, allowing levels of toxicant to pass to the portal circulation. However, at lower, “real world” exposure levels, intestinal metabolism could significantly impact systemic levels of these OPs, such that little if any parent compound or oxon are absorbed into the liver and distributed systemically. To assess this hypothesis, both hepatic and intestinal CYP- and PON1-mediated metabolism of CPF and DZN in microsomes prepared from liver and intestinal enterocytes of rats were evaluated. These data will subsequently be incorporated into the CPF (Timchalk *et al.*, 2002) and DZN (Poet

*et al.*, 2002) PBPK models to assess the role of intestinal metabolism, particularly at low environmentally relevant doses.

## MATERIALS AND METHODS

**Chemicals.** Chlorpyrifos and TCP were kindly provided by Dow Agro-Sciences (Indianapolis, IN), CPF-oxon (98% pure), DZN (98.5% pure), DZN-oxon (90% pure), IMHP (99% pure) and CPF-methyl (99.5% pure) were purchased from Chem Service, Inc. (West Chester, PA). Toluene (99.8% pure) was purchased from Burdick and Jackson (Muskegon, MI). Diazinon-oxon was stored at  $-80^{\circ}\text{C}$  until used, while all other test materials were stored at room temperature. The remaining chemicals used in this study were reagent grade or better and were purchased from Sigma (St. Louis, MO).

**Animals.** Adult male Sprague-Dawley rats (300–400 g) were obtained from Charles River, Inc. (Raleigh, NC). Prior to use, animals were housed in solid-bottom cages with hardwood chips, and were acclimated ( $\sim 1$  week) in a humidity- and temperature-controlled room with a 12-h light/dark cycle. Rodent feed (Purina rodent chow) and water were provided *ad libitum*.

**Microsomes.** The rats were killed by  $\text{CO}_2$  asphyxiation, and livers and small intestine excised. Rat liver microsomes were prepared from 6 individual animals. With regard to the intestinal metabolism, the majority of CYP activity resides in the enterocytes of the small intestine (Obach *et al.*, 2001; Wachter *et al.*, 2001). A section of the small intestine was excised from the base of the stomach (pyloric sphincter) to above the cecum. Enterocytes were eluted from the small intestine by the method of Fasco *et al.* (1993), and pooled from 3 animals for each microsomal sample. Microsomes were prepared from liver and enterocytes by differential ultracentrifugation by the method of van der Hoeven and Coon (1974). Protein concentration was determined using the BCA reagent (Pierce, Rockford, IL) with BSA as the standard, and microsomes were stored at  $-80^{\circ}\text{C}$  until used.

**CYP assay.** Total microsomal P450 content was determined from reduced CO difference spectra (Omura *et al.*, 1964). Preliminary studies to confirm linearity with respect to microsomal protein concentration and time were performed (data not shown). CPF was incubated in 1 ml of 50  $\mu\text{M}$  HEPES buffer containing, 15 mM  $\text{MgCl}_2$ , and 1 mM EDTA. Incubations with CPF contained 1 or 3 mg of microsomal protein for hepatic and enterocyte microsomes, respectively. For DZN, enterocyte microsomal incubations contained 4 mg of protein. The samples were incubated at  $37^{\circ}\text{C}$  for 3 min and 1 mM NADPH was added to initiate the reaction, which was terminated after 10 min by the addition of 200  $\mu\text{l}$  of 2.5 N acidic saturated salt solution (NaCl). Metabolism blanks with boiled microsomes were run for comparison for nonspecific breakdown of TCP, IMHP, or oxon. Preliminary studies verified that 1 mM EDTA was sufficient to block any PON1-mediated metabolism of the oxon (data not shown).

**PON1 assay.** Initial studies were conducted to verify the optimal incubation conditions for protein concentration and incubation period. Microsomes were prepared in buffer lacking EDTA. Oxon (CPF-oxon or DZN-oxon) was incubated in 1 ml of buffer (0.1 M Tri-HCl buffer, pH = 8.5, with 2 mM  $\text{CaCl}_2$ ) containing 0.1 mg or 0.5 mg of microsomal protein for hepatic and enterocyte microsomes, respectively. The samples were incubated at  $37^{\circ}\text{C}$  for 3 min, then 10  $\mu\text{l}$  of substrate in methanol was added to initiate the reaction (methanol was less than 0.1% of total volume). The reaction was terminated 2 min later by adding 200  $\mu\text{l}$  2.5 N HCl solution in concentrated salt (NaCl). Heat-inactivated microsomes were used to compare the nonspecific breakdown of the oxons.

**Chlorpyrifos, CPF-oxon, and TCP analysis.** Chlorpyrifos metabolism studies to date have measured the desulfuration reaction by monitoring the amount of inhibition of AChE from an exogenous source (Ma and Chambers, 1994). For this study, analytical methods were developed to directly measure the formation of oxon and TCP. Extraction of the metabolites was accomplished by adding 5  $\mu\text{g}/\text{ml}$  CPF-methyl internal standard solution in toluene with the same volume of the incubation system. The solutions were mixed well on a vortex mixer, layers were separated by centrifugation, and the organic

supernatant was removed. In order to place the response within the linear range of the calibration curve, the aliquot supernatant was diluted with toluene and quantitated on a Hewlett Packard 5890 gas chromatograph (GC) (Hewlett-Packard, Avondale, PA) equipped with an electron capture detector (ECD). Separation was achieved using an HP-1 capillary column (5 m  $\times$  0.53 mm i.d.  $\times$  2.65  $\mu\text{m}$  film thickness: Hewlett-Packard, Avondale, PA). Helium carrier gas was used with a head pressure of 2.5 psi. The GC oven temperature program included an initial hold at  $80^{\circ}\text{C}$  for 1 min followed by a  $25^{\circ}\text{C}/\text{min}$  ramp to  $220^{\circ}\text{C}$  and then a second ramp to  $270^{\circ}\text{C}$  at  $10^{\circ}\text{C}/\text{min}$ . The final temperature was held for 1 min. The injection port was  $275^{\circ}\text{C}$  and the detector was  $300^{\circ}\text{C}$ . The retention times for TCP, CPF-oxon and CPF were approximately 3.6, 5.6, and 5.0 min, respectively.

**Diazinon, DZN-oxon, and IMHP analysis.** An analytical method was developed to directly quantify the formation of DZN-oxon and IMHP. Chlorpyrifos was used as an internal standard and metabolites were extracted with toluene at a 1:1 ratio (v/v). The solutions were mixed well on a vortex mixer and layers were separated by centrifugation. A 1-ml aliquot of the extract was blown down under a gentle  $\text{N}_2$  stream and reconstituted in toluene (100  $\mu\text{l}$ –1 ml) to place the GC response within the linear range of the calibration curve. Samples were analyzed using a Hewlett-Packard 5890 GC equipped with nitrogen phosphorus (NPD) and flame ionization (FID) detectors. Separation was achieved using a Stabilwax-DB column (15 m  $\times$  0.53 mm i.d.  $\times$  0.25  $\mu\text{m}$  film thickness, Restek, Bellefonte, PA). Hydrogen was used as the carrier gas with a head pressure of 5 psi. Helium was the makeup gas for the NPD detector while  $\text{N}_2$  was the makeup gas for FID detector. A temperature program was used to ramp at a rate of  $15^{\circ}\text{C}/\text{min}$  from  $65^{\circ}\text{C}$  to  $165^{\circ}\text{C}$ , which was held for 2 min and then followed by a second ramp of  $50^{\circ}\text{C}/\text{min}$  to the final temperature of  $230^{\circ}\text{C}$ . The injection and detection temperatures were  $275^{\circ}\text{C}$  and  $300^{\circ}\text{C}$ , respectively. The retention times for DZN, DZN-oxon, and IMHP were approximately 6.0, 6.4, and 6.6 min, respectively.

**Data analysis.** All hepatic microsomal incubations were conducted with  $n = 6$  microsomal samples prepared from individual animals, and means and standard deviations were calculated. Enterocyte incubations were carried out in 2 microsomal samples, each prepared from pooled enterocytes from 3 animals. The metabolic rate constants ( $V_{\text{max}}$  and  $K_m$ ) for metabolite formation were calculated by fitting the Michaelis-Menten equation to the data with nonlinear regression using SlideWrite Plus (Advanced Graphics Software Inc., Encinitas, CA).

## RESULTS

### CYP-Mediated Metabolism

Both CPF and DZN were metabolized to their corresponding oxons and pyridinol/pyrimidinol metabolites in microsomes prepared from both liver and enterocytes. The comparative hepatic CYP metabolism of CPF and DZN to CPF-oxon/TCP and DZN-oxon/IMHP, respectively are presented in Figure 2 and Table 1. The CYP metabolism of DZN has a lower affinity and higher capacity than the metabolism of CPF. The hepatic  $V_{\text{max}}$  for DZN conversion to IMHP and DZN-oxon is  $\sim 2$ -fold higher than for the metabolism of CPF to TCP and CPF-oxon; whereas, the  $K_m$  is 23- and 47-fold higher for the metabolism of DZN to IMHP and DZN-oxon, respectively (Table 1). These parameters for CPF metabolism are similar to what has been reported previously. Ma and Chambers (1994) found  $K_m$  in the range of 1.1–3.2  $\mu\text{M}$  for CPF conversion to CPF-oxon and from 15–24  $\mu\text{M}$  for conversion of CPF to TCP. These researchers measured oxon production indirectly by determining inhibition of AChE activity and found  $V_{\text{max}}$  higher than reported here (3–6 nmol/min/mg).

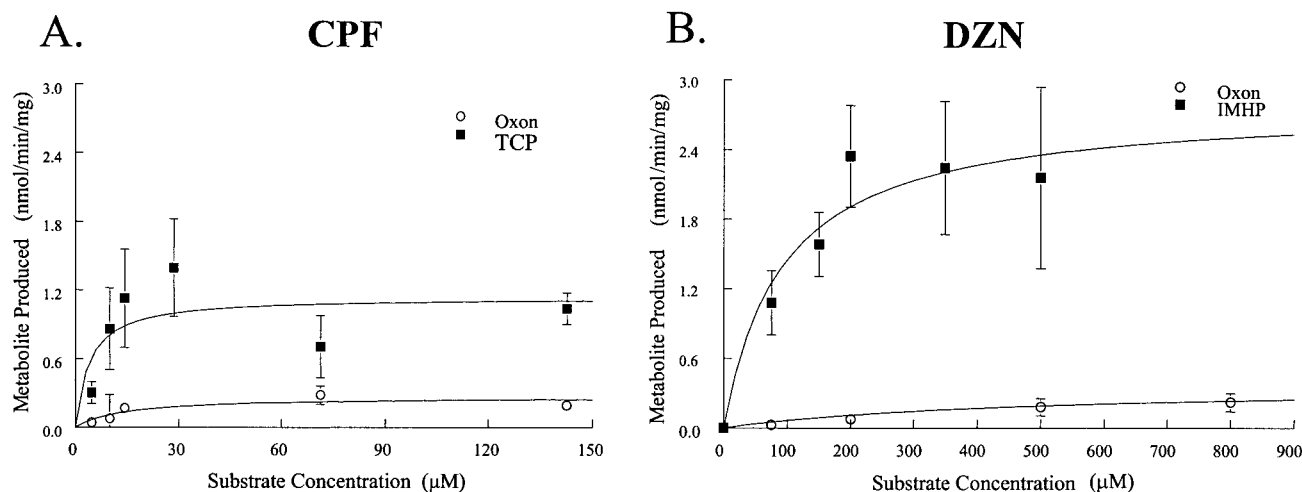


FIG. 2. The CYP-mediated metabolism of CPF (A) and DZN (B) measured in microsomes prepared from the livers of Sprague-Dawley rats. Values represent mean  $\pm$  SD for 6 determinations per substrate concentration. Note the different x-axis scales.

The comparative enterocyte CYP metabolism of CPF and DZN is presented in Figure 3 and Table 1. As with hepatic microsomes, the enterocyte  $K_m$  obtained for DZN metabolism to DZN-oxon is 30-fold higher than the corresponding  $K_m$  for enterocyte CYP metabolism of CPF to CPF-oxon. Once again, the  $V_{max}$  for IMHP production is  $\sim 2$ -fold higher than for TCP formation from CPF. However, the  $V_{max}$  is approximately the same for the formation of the oxons.

In microsomes, the CYP-mediated bioactivation of either CPF or DZN to their oxon metabolites is a less important pathway than the detoxification to the pyridinol/pyrimidinol (Figs. 2 and 3). In liver, the production of TCP is the high affinity, high capacity path for CPF. Whereas, in the entero-

cytes, the metabolism of CPF to TCP has a lower efficiency compared to the metabolism to CPF-oxon, although the capacity is still higher for the TCP pathway. For DZN, the CYP-mediated metabolism to IMHP in both the microsomes from liver and enterocytes is a higher capacity pathway than bioactivation to the DZN-oxon.

The  $K_m$  for the production of the oxon is similar in enterocytes and hepatic microsomes, possibly indicating that the same enzyme or enzymes are responsible for the metabolism to CPF-oxon and DZN-oxon, respectively in these two tissues. However, the  $K_m$  for the production of TCP from CPF is more than 10-fold higher in enterocyte microsomes, suggesting that the enzyme composition responsible for the metabolism of CPF to TCP may be different in enterocytes. In the case of DZN, the  $K_m$  for metabolism to either DZN-oxon or IMHP is reasonably comparable in both the liver and enterocytes.

To compare the CYP metabolic capacity of enterocytes relative to hepatic microsomes, the metabolism of CPF to TCP and CPF-oxon was normalized based on P450 content (see Fig. 4). Although the metabolism of CPF and DZN are similar in liver and enterocytes, the CYP content in intestine is lower than liver (Fasco *et al.*, 1993; Obach *et al.*, 2001). In this study, total reducible CO spectra of the CYP content in enterocyte microsomes used to evaluate CPF metabolism was 10-fold lower than in microsomes prepared from liver ( $0.793 \pm 0.140$  and  $0.0722$  nmol/mg, respectively). However, the  $V_{max}$  for enterocyte microsomal metabolism of CPF was only 4–5-fold lower on a per mg basis. DZN metabolism to the oxon is  $\sim 10$ -fold lower in enterocyte microsomes than liver microsomes on a per mg basis, and DZN deactivation to IMHP is  $\sim 5$ -fold lower. Thus, if metabolism is compared by first normalizing for CYP content, then the metabolic capacity of the enterocytes clearly exceeds that of the hepatic microsomes for CPF and is similar to or exceeds the capacity for DZN as well.

TABLE 1

Comparison of the Kinetic Parameters for CYP450 Metabolism of CPF and DZN in Hepatic and Enterocyte Microsomes

Sample	$K_m$	$V_{max}$	$V_{max}/K_m$
CPF			
Hepatic			
Oxon	10.6	0.268	18
TCP	4.08	1.14	279
Enterocyte			
Oxon	8.1	0.068	8.4
TCP	55.0	0.249	4.5
DZN			
Hepatic			
Oxon	498	0.375	0.75
IMHP	92.9	2.79	30
Enterocyte			
Oxon	235	0.030	0.13
IMHP	72.4	0.500	6.9

Note.  $K_m$  given as  $\mu M$ ;  $V_{max}$  given as nmol/min/mg;  $V_{max}/K_m$  given as pseudo 1<sup>st</sup> order rate: min<sup>-1</sup>.



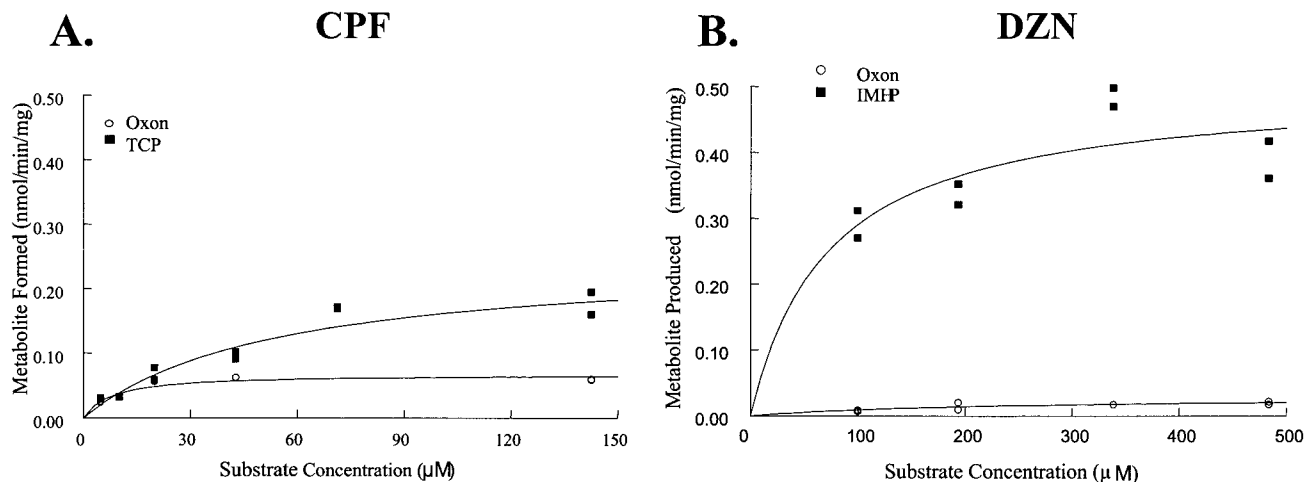


FIG. 3. The CYP-mediated metabolism of CPF (A) and DZN (B), measured in microsomes prepared from intestinal enterocytes of Sprague-Dawley rats. Data represent 2 individual determinations in microsomes prepared from enterocytes pooled from 3 rats per substrate concentration. Note the different x-axis scales.

#### PON1-Mediated Metabolism

The PON1-mediated metabolism of CPF-oxon and DZN-oxon to the inactive TCP and IMHP, respectively, was measured in microsomes prepared from liver and enterocytes and the results are presented in Figure 5 and Table 2. Incubations included calcium and no EDTA, since PON1 is calcium-dependent. The Michaelis-Menten constant ( $K_m$ ) for the metabolism of CPF-oxon and DZN-oxon were similar in both tissues, but the capacities ( $V_{max}$ ) were much greater (47 to 200-fold) in liver than enterocytes (Table 2), this is in agreement with the findings of Pond *et al.* (1995). Although the hepatic PON1 metabolism of CPF-oxon and DZN-oxon were fairly comparable, in the enterocytes, PON1-mediated detoxification of DZN-oxon had a much greater  $V_{max}$  than CPF-oxon, resulting in 5-fold increased metabolic efficiency for entero-

cyte DZN-oxon metabolism relative to CPF-oxon. Unlike CYP-mediated metabolism, the affinities for CPF-oxon and DZN-oxon are similar to each other (Table 2). This suggests that intestinal enterocytes may have a slightly greater capacity to detoxify DZN-oxon relative to CPF-oxon. The parameters for PON1-mediated metabolism of CPF in liver are similar to what has been reported previously by Mortensen *et al.* (1996), who determined a  $K_m$  and  $V_{max}$  of 240  $\mu\text{M}$  and 24  $\mu\text{mol/min/mg}$ , respectively.

#### DISCUSSION

Oral bioavailability of either drugs or chemicals (e.g., OP insecticide), defined as the amount of systemically available dose, is a function of the extent of intestinal absorption and associated first-pass metabolism (Timchalk, 2001). In light of the potential for ingesting low levels of pesticide residues on food, a better understanding of the role of intestinal and liver metabolism is particularly relevant for assessing risk. The toxic potency of OP insecticides is dependent upon a balance between delivered dose to the target site and the rates of bioactivation versus detoxification (Calabrese, 1991). Hence, there is a need to more fully assess the extent of oral bioavailability and first-pass metabolism of OP insecticides since it is a major component in determining the delivered dose.

A number of *in vivo* studies in both animals and humans suggest that the oral bioavailability of OP insecticides is fairly low. Braeckman *et al.* (1983) reported that only 1–29% of orally administered parathion was bioavailable in dogs, due to rapid first-pass metabolism. Wu *et al.* (1996) conducted similar bioavailability studies with DZN and suggested that only 35% of an 80 mg/kg oral dose was systemically bioavailable. In humans, the bioavailability of orally administered CPF, based upon recovery studies and PBPK/Pharmacodynamic model

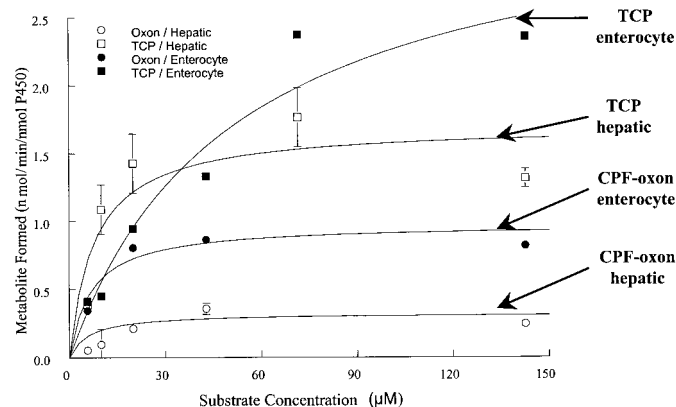


FIG. 4. The CYP-mediated metabolism of CPF measured in microsomes prepared from liver and intestinal enterocytes of Sprague-Dawley rats normalized for amount of CYP. Data represents mean  $\pm$  SD for 6 determinations per substrate concentration for liver microsomes and individual determinations (2) per substrate concentration for enterocytes.

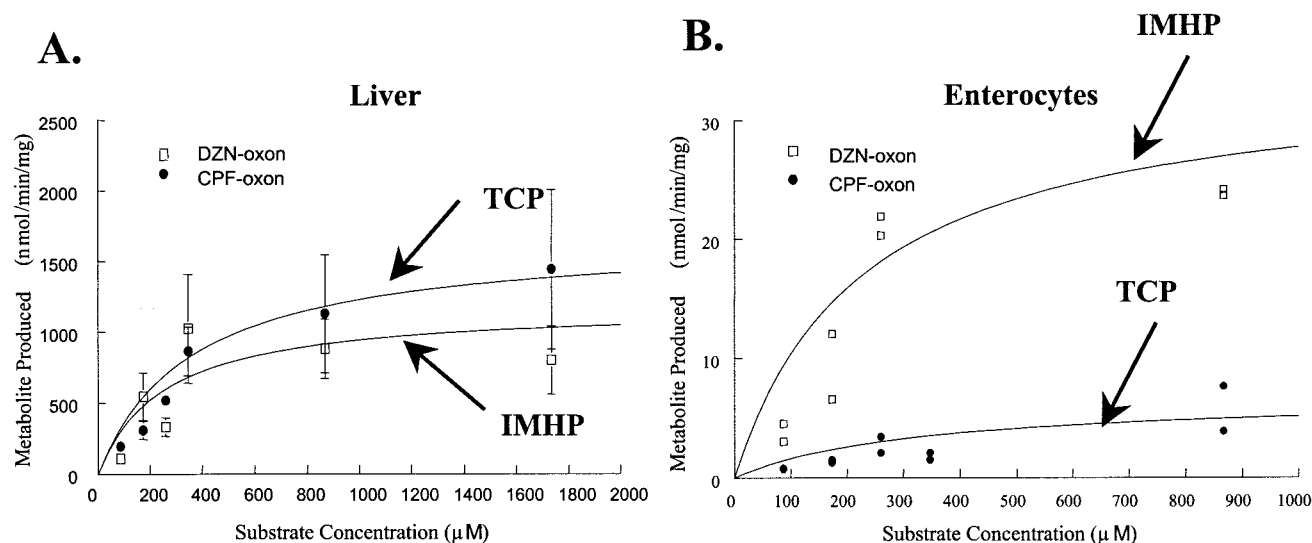


FIG. 5. The PON1-mediated metabolism of CPF-oxon and DZN-oxon in liver (A) and enterocyte (B) microsomes prepared from Sprague-Dawley rats. Data represent mean  $\pm$  SD for 6 determinations per substrate concentration for liver and individual determinations (2) per substrate concentration for enterocytes.

simulations, ranged from as low as 20–35% to 70% depending upon the physical form of the CPF when administered (Nolan *et al.*, 1984; Timchalk *et al.*, 2002).

The major metabolites formed from CYP-mediated hepatic metabolism for both CPF and DZN are the inactive pyridinol/pyrimidinol with the active oxon representing the minor metabolite (see Fig. 2). These results are consistent with previous reports in both rodents and humans (Ma and Chambers, 1994, 1995; Fabrizi *et al.*, 1999; Nolan *et al.*, 1984; Tang *et al.*, 2001; ). In addition, based upon the overall hepatic metabolic efficiency (pseudo-1<sup>st</sup> order rate constant:  $V_{max}/K_m$ ), CPF will undergo more hepatic metabolism *in vivo* than DZN.

Microsomes derived from intestinal enterocytes demonstrated a similar CYP-mediated metabolism profile involving both desulfuration and dearylation reactions, with dearylation to the pyridinol/pyrimidinol also representing the preferred pathway for metabolism (see Fig. 3). These are the first experimental results reported to characterize the CYP-mediated metabolism of CPF

and DZN in rodent enterocytes, and are consistent with the reported very high 3A subfamily activity in enterocytes (Obach *et al.*, 2001; Zhang and Benet, 2001), and the relative importance of 3A for both dearylation and desulfuration metabolism of CPF and DZN (Tang *et al.*, 2001). In the case of enterocyte CYP metabolism, the overall metabolic efficiency for the conversion of both CPF and DZN to their corresponding oxon metabolites is ~28-fold greater for CPF than DZN. This may suggest a different distribution of CYP forms within the enterocytes relative to that observed in the liver.

Although many tissues within the body are known to possess some CYP activity, the prevailing dogma is that the liver is the primary organ associated with CYP metabolism and is responsible for first-pass metabolism of chemicals and drugs absorbed from the gastrointestinal tract. It is clear from recent studies that enterocytes within the small intestine also possess CYP metabolic capacity, and in the case of some enzymes (i.e., 3A4) the concentration within the enterocytes is comparable to the liver (Obach *et al.*, 2001; Wachter *et al.*, 2001; Zhang and Benet, 2001). In comparing the CYP metabolism of CPF with DZN in the current study on a mg-protein basis, the hepatic microsomes are slightly more efficient at metabolizing CPF to CPF-oxon (2-fold) and clearly better at metabolizing to TCP (61-fold); however, if the metabolism is compared on a nmol P450 basis it is clear that the overall CYP metabolic rate for the enterocytes exceeds that of the liver microsomes (see Fig. 4). P-Glycoprotein efflux of the oxon metabolite may also contribute to lowering the bioavailability of these OP insecticides. Although the parent CPF apparently does not interact with P-glycoproteins, the oxon does (Lanning *et al.*, 1996; Yang *et al.*, 2001). Thus, a fraction of the oxon produced in the enterocytes may be transported back into the lumen of the small

TABLE 2

Comparison of the Kinetic Parameters for PON1 Metabolism of CPF-Oxon and DZN-Oxon in Hepatic and Enterocyte Microsomes

Sample	$K_m$	$V_{max}$	$V_{max}/K_m$
CPF			
Hepatic	345	1280	3700
Enterocytes	328	4.60	14
DZN			
Hepatic	268	1180	4400
Enterocytes	347	24.9	72

Note.  $K_m$  given as  $\mu M$ ;  $V_{max}$  given as nmol/min/mg;  $V_{max}/K_m$  given as pseudo 1<sup>st</sup> order rate:  $\min^{-1}$ .

intestine, thereby, never reaching the systemic circulation. Future studies are needed to evaluate this hypothesis and determine the potential role of P-glycoprotein efflux (cell to lumen) of oxon within intestinal enterocytes.

Since PON1 is an important detoxification pathway for the oxon (Main, 1956; Costa *et al.*, 1990; Li *et al.*, 1993; 1995), the PON1-mediated metabolism of CPF-oxon and DZN-oxon were evaluated in microsomes derived from intestinal enterocytes, the results clearly demonstrate that the enterocytes possess PON1 activity and, based on the comparable  $K_m$  values between the liver and enterocytes, they suggest that these are similar enzymes. Although the  $K_m$  values were comparable between the liver and enterocyte microsomes, it was clear that the  $V_{max}$  for the liver exceeded that of enterocytes, resulting in a much higher metabolic efficiency within the liver for CPF-oxon (255-fold), and DZN-oxon (69-fold) metabolism. Pond *et al.* (1995) compared the PON1 activity in numerous tissues including the small intestines of rats, using CPF-oxon as substrate. They reported ~113-fold greater activity in the liver versus the small intestines, which is consistent with the lower activity found in the current study. Although the intestines contain significantly less PON1 activity than the liver, the results still suggest that the oxon metabolites can be subjected to significant detoxification within the enterocytes. In addition, the intestines are reported to have high CaE activity (Pond *et al.*, 1995) that can stoichiometrically bind to and detoxify oxon. The importance of CaE detoxification of OP insecticides has been previously established (Chanda *et al.*, 1997; Clement, 1984; Fonnum *et al.*, 1985; Maxwell, 1992) and may be of particular importance when considering low-level environmental exposures.

Although the results of the current *in vitro* study clearly suggests that intestinal metabolism of CPF and DZN could modify *in vivo* bioavailability, there is still a question as to how important intestinal metabolism is relative to the contribution of the liver. In this regard, it has been demonstrated using *in vivo* animal studies and human clinical evaluations that the inhibition or induction of intestinal CYP metabolism can clearly modify the oral bioavailability of a number of drugs including, cyclosporine, tacrolimus, and digoxin (Floren *et al.*, 1997; Gomez *et al.*, 1995; Paine *et al.*, 1999; Salphati and Benet, 1998; Zhang and Benet, 2001). Hence, it is not unreasonable to anticipate that intestinal CYP metabolism would likewise impact OP bioavailability as a result of CYP-mediated metabolism (i.e., activation and detoxification). Second, although there was approximately a 10-fold lower total P450 per mg of protein in the intestinal microsomes prepared in this study than in liver microsomes, in rats the liver is ~4% of the total body volume whereas the small intestines comprise ~2.5% (Schoeffner *et al.*, 1999); hence the overall capacity of the small intestine for metabolism may be substantial. Third, the intestines, and specifically the enterocytes lining the gut walls, are designed to maximize surface area (numerous microvilli) to facilitate absorptive processes including the uptake of OP insecticides. Once the OPs are within the enterocytes,

based upon the *in vitro* metabolism results, it is reasonable to anticipate that they will be subjected to both CYP- and PON1-mediated metabolism prior to entering the liver via the portal circulation. Although the current *in vitro* studies support a working hypothesis that intestinal metabolism can contribute to the oral bioavailability of OP insecticides, additional *in vivo* kinetic studies are needed to substantiate the role of enterocyte metabolism in modifying the oral bioavailability of CPF and DZN, particularly at relevant low environmental exposure levels. In addition, the correlation between rat and human metabolism and intestinal absorption needs to be assessed.

In summary, the inhibition of AChE by CPF and DZN results in the accumulation of acetylcholine at the neuronal junctions, which is the primary toxic mode of action for these OPs. Although the parent OPs have some inhibitory potential, the primary toxicants are the oxon metabolites. Chlorpyrifos and DZN are bioactivated by CYP enzymes to the oxon metabolites and deactivated by both CYPs and PON1 to pyridinol and pyrimidinol, respectively (TCP from CPF and IMHP from DZN). The balance between bioactivation and detoxification will drive the toxicity from OP exposures. At low oral dose exposures, metabolism by CYPs and PON1 in intestine and liver may remove the active moiety from the circulation prior to systemic exposure. These results suggest that, in the rat, intestinal metabolism should be considered when evaluating biological response, particularly to low doses. The impact of intestinal metabolism may be further evaluated through appropriate *in vivo* studies and by incorporating an intestinal metabolic compartment into current PBPK models for both CPF and DZN (Poet *et al.*, 2002; Timchalk *et al.*, 2002).

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