Appendix A: Metabolism

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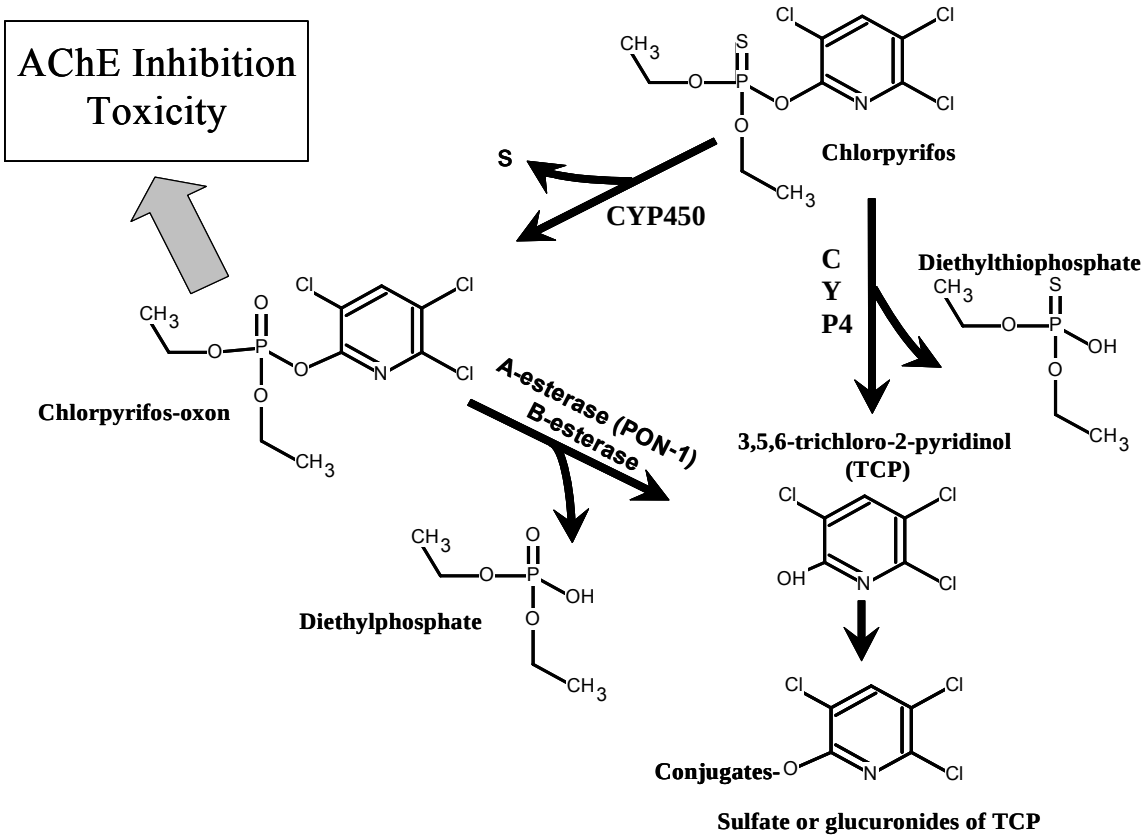
1. Introduction

The metabolism and pharmackokinetics of chlorpyrifos has been extensively studied in animals and man as well as in *in vitro* systems. Early studies conducted in the 1960's and 1970's focused on the identification of primary metabolic pathways, and later studies using the newer instrumentation of identification and metabolite characterizations and enzymology provided a more comprehensive understanding of its metabolic transformations. The focus of this issue paper is on the effects of chlorpyrifos on children and during pregnancy. As such, the focus of this summary is on metabolism and toxicokinetic studies that evaluated effects in juvenile animals and pregnant rats. Studies in humans and in adult animals provide support and complementary information. This chapter provides review of the most recent investigations on the metabolism of chlorpyrifos *in vivo* (rats and humans) and i*n vitro*.

There are several enzymes that play a role in the metabolism and toxicity of chlorpyrifos (Figure 1). The toxicity of chlorpyrifos has historically been attributed to the chlorpyrifos oxon. The oxon targets acetylcholinesterase (AChE) by binding to the active site and inactivating the ChE enzyme. The chlorpyrifos oxon also binds to other serine esterases such as butyrlycholinesterase.

The cytochrome P450 family of microsomal enzymes (CYPs) are responsible for the metabolic activation (producing chlorpyrifos oxon) and deactivation (producing 3,5,6-trichloro-2-pyridinol: TCP) of chlorpyrifos. Another group of important enzymes in the detoxification of chlorpyrifos is the A-esterases, also known as paraxonase or PON1. These are calcium activated enzymes and are distributed in various tissues including the liver, brain and blood. A-esterases hydrolyze chlorpyrifos oxon. It has also been demonstrated that chlorpyrifos oxon binds irreversibly to other non-target serine esterases, namely carboxylesterase (CarbE). Carboxylesterase is considered as a scavenger of the chlorpyrifos oxon removing it from reaching the target AChE enzyme. This binding has been described to be stoichometric. CarbEs are distributed among different tissues (liver, blood (not human), lung, brain) with highest abundance in the liver. The glutathione dependent enzymes may also play an important role in the secondary metabolism of chlorpyrifos producing water soluble metabolites that are readily excreted into the urine.

Figure 1: Major metabolic pathways of chlorpyrifos metabolism (Reproduced from Timchalk et al 2006).



Abundant studies have established that chlorpyrifos is readily absorbed following oral exposures by laboratory animals or humans and undergoes metabolic transformations mainly by the liver enzymes. Although lipophillic, chorpyrifos' extensive metabolism into water soluble metabolites does not lead to any accumulation of the parent material or its metabolites in the body tissues (Tables 1 and 2). The initial metabolic attack on the chlorpyrifos molecule is its desulfuration, bioactivation it to the more toxic and potent cholinesterase inhibitor, the oxon form. Chlorpyrifos oxon is highly reactive and either binds to a serine esterase or is rapidly deactivated through hydrolytic cleavage by a process called dearylation releasing TCP. Simultaneously during the desulfuration process, dearylation will be acting not only on the chlorpyrifos oxon metabolite, but also on the parent chlorpyrifos, leading to the release of TCP. TCP is further conjugated to form glycine or glucuronide conjugates and eliminated into the urine.

The metabolism of chlorpyrifos in humans is well understood through documented incidental poisoning cases as well as in human exposure laboratory studies. Three distinct biotransformation routes of chlorpyrifos were proposed in

humans: (1) cleavage reactions at the aromatic phosphoester bond producing TCP, (2) cleavage reactions at the alkyl phosphoester bonds, and (3) glutathione (GSH) dependent nucleophilic substitution of the 6-chlorine at the aromatic moiety, chiefly cysteine *S*-conjugates of mono-dechlorinated chlorpyrifos, chlorpyrifos oxon, mono-*O*-deethyl chlorpyrifos, and mono-*O*-deethyl chlorpyrifos oxon as well as the 6-mercapturic acid conjugate of 3,5-dichloro-2-pyridinol. These routes yielded 15 metabolites excreted into the urine. Even at very high incidental ingestion of a chlorpyrifos dose, neither the parent material nor the oxon were found in the urine indicating their rapid metabolism and transformations.

Blood chlorpyrifos concentrations following oral and dermal doses in adult humans were extremely low (<30 ng/mL), and no chlorpryifos was detected in the urine indicating the complete metabolic transformation. TCP kinetic data showed a 1-2 hour delay in the absorption of the oral dose. The average blood TCP concentration reached a maximum value 6 hours after the dose ingestion. Following the dermal dose, the highest mean concentration was observed after 24 hours of the dose application. Approximately 72% of the ingested dose and 1%-3% of the dermal dose were absorbed. In another human study, chlorpyrifos was detected in the blood at low concentrations (1.1-18 ng/g), and only terminal metabolites were detected in the urine. Chlorpyrifos oxon was not detected in the blood.

This chapter will first examine the metabolism of chlorpyrifos in adult animals, followed by review of *in vitro* investigations and the various enzymes that act on it. The metabolism and toxicokinetic studies of chlorpyrifos in pregnant dams, fetuses and post-natal pups will be explored in this chapter. Its toxicokinetics and metabolic pathways in humans will be discussed. Finally, a brief discussion of the metabolic changes during human pregnancy and the ontogeny of metabolic enzymes during human development will be briefly discussed.

2. Metabolism in Adult Animals

Early research by Smith *et al* (1967), using ^{36}Cl- chlorpyrifos (single oral dose of 50 mg/kg bw) in male rats indicated its rapid absorption and elimination. Its biological half-life in fat was the longest (62 h) for all the tissues studied, with liver, kidney and muscle showing half-lives for radioactivity of 10, 12 and 16 h, respectively and most of the radioactivity was eliminated in the urine (90%) and faeces (10%). Urinary metabolites identified were 3,5,6-trichloro-2-pyridinol (TCP ; 20-25%) and TCP phosphate (75-80% of the recovered radiolabel, indicating desethylation). This study suggested that the desethyalation is a major pathway in the metabolic degradation of chlorpyrifos. However, such pathway was found in later studies to be of minor importance. This study relied on the non-reliable technique of paper chromatography in the identification of this metabolite. Trace amounts of chlorpyrifos were reported.

Studies by Dow (Nolan *et al* 1987: MRID 40458901) demonstrated that an orally administered ^{14}C-chlorpyrifos dose in rats was readily absorbed from the GI tract and rapidly excreted in the urine (>84%) and feces (>5%) with no detection of the parent in

the excreted radioactivity. Residual ^{14}C-radioactivity in tissues (including blood) and carcass were less than 0.3% at animal sacrifice time (72 hours for males and 144 hours for females) (Table 1). Its elimination half life was estimated to be 8-9 hours in males and females following a single low oral dose of 0.5 mg/kg, but was longer following a larger single oral dose of 25 mg/kg (12.4 hours for males and 23.2 for females) indicating slower absorption and elimination with increased dose. There was no difference in its excretion half life if administered as a single dose or multiple daily doses (0.5 mg/kg/day for 15 days). The urinary metabolites were investigated in this study by HPLC and MS analysis. Urinary metabolites were either the 3,5,6-TCP (hydrolysis product of chlorpyrifos) or its glucuronide (mainly) and the sulfate conjugates.

Table 1: Distribution of radioactivity (% of dose) recovered 72 hours (males) or 144 hr (females) after Fisher rats were given oral doses of 25 or 0.5 mg 14C-CPF/kg Bw (Nolan *et al* 1987)*

	25 mg/kg	0.5 mg/kg	Multiple 0.5 mg/kg
Males			
Urine	88.73±2.53	85.23±3.40	91.71±4.74
Feces	7.49±3.48	9.76±2.34	5.75±3.99
Tissues & carcass	0.20±0.16	<0.01	<0.01
TOTAL including cage washes and traps	98.36±1.93	96.88±1.25	98.46±0.99
Females			
Urine	87.99±10.53	83.94±13.04	90.65±2.62
Feces	8.35±4.65	11.42±3.12	5.59±2.49
Tissues & carcass	0.19±0.13	<0.01	<0.01
TOTAL (including cage wahes and traps)	97.05±8.28	97.19±13.33	96.99±1.34

* >50% of the radioactivity was excreted in the urine during the first 12 hours.

In an acute pharmacokinetic study by Dow completed in 1998 (Mendrala & Brzak, 1998: MRID 44648102), groups of 24 Fischer 344 male ratsks were given a single gavage dose of 0.5, 1, 5, 10, 50, or 100 mg/kg chlorpyrifos (99.8% a.i.) in corn oil. Four rats from each group were killed 10 and 20 minutes and 1, 3, 6, and 12 hours after treatment. Cholinesterase (ChE) activity was measured in the brain and plasma at each time point, as well as the plasma concentration of the test material and its oxon metabolite. In a separate portion of the study, four male rats were given a single gavage dose of ^{14}C- chlorpyrifos labeled at ring positions 2 and 6 (89.4% a.i. (impurities: 6.1% TCP, 3.5% chlorpyrifos oxon, 1% unidentified) at a concentration of 5 or 100 mg/kg and were sacrificed three hours later. Blood was collected from the animals at sacrifice and the concentration of the test material and its metabolites TCP and chlorpyrifos oxon determined.

Peak chlorpyrifos blood concentrations occurred within three hours of treatment in all but the lowest dose group where chlorpyrifos was not detected at all intervals. The area under the curve (AUC) was calculated as 0.4, 1.1, 5.0, and 12.5 µmole hr L^{-1} for the 5.0, 10.0, 50.0, and 100 mg/kg groups, respectively and yielded calculated blood half-lives of chlorpyrifos of 2.7,1.5, 2.1, and 7.3 hours for the 5.0, 10.0, 50.0, and 100.0 mg/kg dose groups, respectively. Regardless of dose, the highest concentration of the oxon detected was 2.5 ng/g in the blood of rats treated with 50 mg/kg test material one hour post-treatment. Following treatment with 5 or 100 mg/kg labeled test material, ≥98% of the activity detected in the blood was identified as TCP metabolite with the remaining attributed to the parent compound. Since the oxon is an intermediate in the formation of TCP and none of the chlorpyrifos oxon was detected, these studies support that the half-life of the oxon metabolite is short (*in vitro* half life in rat blood of 10 seconds and 55 seconds in human blood (Brzak *et al,* 1998) .

Plasma ChE activity decreased in a time- and dose-dependent manner. The plasma ChE activities of rats treated with 0.5, 1, 5 or 10 mg/kg were maximally decreased 3-6 hours after treatment, coinciding with the peak concentration of chlorpyrifos in the blood and likely reflecting the desulfuration and dearylation metabolic processes on chlorpyrifos, with both the decrease and recovery of activity being dose-dependent. Plasma ChE activity was not significantly inhibited in the 0.5 mg/kg group (chlorpyrifos below limit of detection at all time points). In the 1 mg/kg dose group, plasma ChE activity was significantly inhibited approximately 28% and 40% relative to controls at 3 and 6 hours post exposure, respectively (chlorpyrifos detected at 3 hours only). By 12 hours post-exposure, plasma ChE activity was still significantly inhibited about 16% for the 1 mg/kg group (chlorpyrifos still detected). The decrease in plasma ChE activity of rats treated with 50 or 100 mg/kg began within 10 minutes of treatment (chlorpyrifos detected within 30 minutes). By 12 hours after treatment, plasma ChE activity in both groups was approximately 11% of the control group and had not shown signs of recovery (chlorpyrifos concentration remained high). Brain ChE activity was not affected as dramatically by test material treatment as plasma activity with only the 10, 50, and 100 mg/kg dose groups showing significant effects (only dose levels where chlorpyrifos concentrations reached >100 ng/g and oxon was detected). The brain ChE activity of rats treated with 10 mg/kg test material began to decline within three hours of treatment and was significantly decreased by six hours after treatment. The brain ChE activity in the 50 or 100 mg/kg dose groups decreased significantly within one hour of treatments; and by 12 hours, it was approximately 30% and 20%, respectively, of control. In none of the affected groups did brain ChE show signs of recovery (chlorpyrifos still detected at 12 hours in all affected groups).

Sunaga *et al* 1989 investigated urinary metabolites and biological half-life of chlorpyrifos following a single ip injection of 0.2 mmol chlorpyrifos /kg body weight (equivalent to 70 mg/kg body weight) to male Wistar rats weighing 200 g. Both chlorpyrifos and TCP levels in blood showed maximum values at 5 h post-injection (0.66 µmol/L for chlorpyrifos and 38 µmol/L for TCP), and then decreased rapidly (0.02 µmol/L for chlorpyrifos and 10 µmol/L for TCP after 48 hours of injection). Biological half-lives of the blood chlorpyrifos and TCP were estimated to 8.15 and 24.66 h,

respectively. Urine was collected for 96 h post-injection and hydrolyzed with 4 N HCl or beta-glucuronidase with sulfatase to release the conjugated TCP. Urinary excretion levels of the acid hydrolysis-released TCP and the enzyme hydrolysis-released TCP accounted for 86 and 54% of chlorpyrifos administered, respectively. Urinary excretion levels of diethylthiophosphate (DETP) and diethylphosphate (DEP) accounted for 45 and 15% of chlorpyrifos administered, respectively. These results indicate that 1) about half of the chlorpyrifos administered was directly hydrolyzed to DETP and TCP, 2) 10 to 20% was hydrolyzed to DEP and TCP after the oxidation to chlorpyrifos oxon, and 3) about 30% was dealkylated to TCP-phosphate after the oxidation.

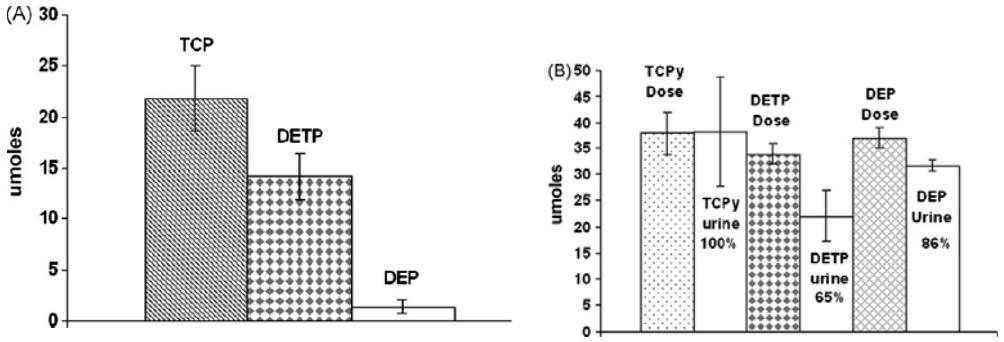
The toxicokinetics (absorption, metabolism and elimination) of DEP, TCP and DETP following *in vivo* oral exposure in the rat were evaluated and compared to the *in vivo* pharmacokinetics of chlorpyrifos following oral exposure to an equal molar dose (Timchalk *et al* 2007a). Groups of male Sprague–Dawley rats were orally administered chlorpyrifos, DEP, TCPy or DETP at doses of 140 µmol/kg body weight dissolved in a corn oil matrix, and the time-courses of the metabolites were evaluated in blood and urine. The chlorpyrifos dose was equivalent to ~50 mg/kg of body weight, which did produce some signs of acute toxicity in the rat (chlorpyrifos LD_{50} ~92 mg/kg). The selection of this high dose was done to optimize the quantitative measurement of all metabolites in both the blood and urine. Following oral administration, all three metabolites were well absorbed with peak blood concentrations being attained between 1 and 3 h post-dosing. Peak blood DEP concentrations of 0.31±0.14 µg/mL were attained within 1 h post-dosing, and the DEP was quantifiable through 24 h post-dosing. The DEP blood pharmacokinetics were biphasic with estimated half-lives ($t_{1/2}$) for the alpha (α, initial) and beta (β, terminal) phases of 0.2 and 52 h, respectively. Peak amounts of DEP in urine (3921±2226 µg) were attained within 12 h post-dosing and the urinary elimination was adequately described by a one-phase exponential decay with an estimated $t_{1/2}$ of ~13 h (Table 3). Following the chlorpyrifos oral dose (group I), DEP was likewise rapidly detected in the blood with peak levels of 0.85±0.34 µg/mL attained by 3 h post-dosing (Table 2). The DEP was detectable in the blood through 48 h post-dosing and the biphasic blood time-course had an estimated $t_{1/2}$ of 0.2 and 118 h for the α and β phase, respectively (Table 3). Peak urinary DEP (107±57µg) was achieved by 12 h post-dosing, with an estimated $t_{1/2}$ of 14 h (one-phase exponential decay) (Table 2). In comparing the DEP blood concentrations and the amount excreted in the urine following equal molar doses of chlorpyrifos (group I) or DEP (group II) it is clear that animals dosed with DEP had lower blood concentrations and substantially greater amounts of DEP excreted in the urine than in those animals dosed with chlorpyrifos. The shapes of the blood time-courses were fairly similar although the DEP from the chlorpyrifos dose group had a doubling of the β phase (118 h) relative to the DEP dose group (52 h). Of importance to note, in comparing the DEP versus the chlorpyrifos dose groups, the total amount of DEP excreted in the urine (Table 2) was substantially different (5940 µg versus 210 µg, respectively) and a comparison of the urinary AUC (2725µmol h^{-1} and 61,183 µmol h^{-1}, respectively), indicates that the amount of DEP excreted in the urine for the chlorpyrifos dose was only 4% of the amount detected following an equal molar dose of DEP. Yet the $t_{1/2}$ for the urinary excretion of DEP was very comparable (13–14 h) for both groups (Table 3).

Following oral administration of TCP (group III) peak blood TCP concentrations of 18±2.8 µg/mL were rapidly attained within 1 h post-dosing, and the TCP was quantifiable through 72 h post-dosing. The TCP blood pharmacokinetics had a terminal half-life ($t_{1/2\beta}$) estimate of 5.1 h. Peak amounts of TCP in urine (3048±1230 µg) were attained within 12 h post-dosing. The average total recovery of TCP in the urine through 72 h post-dosing was 38±10 µmol, which was very comparable to the 38±4 µmol of TCP that was orally administered to the rats. This suggests that TCP was well absorbed orally and undergoes negligible, if any, metabolism. Following the chlorpyrifos oral dose (group I), TCP was likewise rapidly detected in the blood with peak levels of 18±17µg/mL attained by 3 h post-dosing. In contrast to the group III results, the TCP was detectable in the blood only through 48 h post-dosing and the blood time-course had estimated terminal $t_{1/2\beta}$ of 8.5 h. Near peak urinary TCP concentrations were attained by 12 h (710±325 µg) and were maintained at elevated level through 48 h post-dosing (1948±693µg). In comparing the TCP blood concentrations and the amount excreted in the urine following equal molar doses of TCP (group III) or chlorpyrifos (group I) it is clear that the peak concentrations of TCP in the blood were very comparable, although the TCP dosed blood concentrations decreased faster than the chlorpyrifos dose group. Consistent with this observation was the finding that the urinary TCP time-course and cumulative concentration for the TCP group were slightly higher than the urinary TCP concentrations following the chlorpyrifos dose group. Urinary TCP (22±3 µmol) accounted for ~63% of the orally administered chlorpyrifos dose (35±2 µmol). In this regard, no attempt was made to quantify the extent of TCP conjugation (i.e. sulfation and glucuronidation) following either the chlorpyrifos or TCP doses. Although the overall recovery of TCP was different following the chlorpyrifos or TCP doses; in general, the shapes of the blood and urinary time-courses were fairly similar with comparable *t*1/2 for the blood β phase (5–9 h) and urinary excretion rate (17–21 h).

Following oral administration of DETP (group IV) peak blood DETP concentrations of 2.0±0.1 µg/mL were rapidly attained within 1 h post-dosing, and the DETP was quantifiable through 24 h post-dosing (Table 2). The DETP blood pharmacokinetics had a terminal phase half-life ($t_{1/2}$) of 4.2 h (Table 3). Peak amounts of DETP in urine (2105±1481µg) were attained within 12 h post-dosing and the estimated urinary $t_{1/2}$ was ~8 h. The average total recovery of DETP in the urine through 48 h post-dosing was 22±5 µmol of DETP, which was ~65% of the 34±2 µmol of DETP that was orally administered to the rats (Figure 2B). The incomplete recovery of DETP in the urine may suggest that the DETP was not completely absorbed, and/or the DETP was eliminated from the body by other pathways (i.e. additional metabolism or alternative excretion route). Following the chlorpyrifos oral dose (group I), DETP was likewise rapidly detected in the blood with peak levels of 1.4±0.3 µg/mL attained by 3 h post-dosing (Table 2). The DETP was detectable in the blood through 24 h post-dosing and the blood time-course had an estimated $t_{1/2\ \beta}$ of 31 h (Table 3). Peak urinary DETP concentrations following the chlorpyrifos dose were attained by 12 h (616±68 µg) and the urinary elimination $t_{1/2}$ was ~22 h. A comparison of the DETP blood concentrations and urinary excretion rates following equal molar doses of DETP (group IV) or chlorpyrifos (group I) indicates that the peak blood DETP concentrations, were very

comparable (Table 2.4). However, differences were noted in the terminal blood time-course (4 h versus 31 h), urinary excretion rates (8 h versus 21 h) and the cumulative amounts of DETP (3743 μg versus 1783 μg) quantified in the urine when comparing the DETP versus chlorpyrifos dose groups. The total amount of metabolites recovered as TCP, DETP and DEP in the urine following oral administration of chlorpyrifos or individual metabolites are presented in Figure 2. In the current study the overall average dose of chlorpyrifos administered to all rats was 36±2.5 μmol/rat, and of the recovered metabolites TCP was the highest at 22±3 μmol (62%); whereas, the metabolites DETP and DEP accounted for 14±2 μmol (40%) and 1.4±1 μmol (4%), respectively. The overall average oral doses of TCP, DETP and DEP were 38±4, 34±2, and 37±2 μmol/rat, respectively. All of the orally administered TCP (38±10 μmol) was recovered in the urine; whereas ~65% (22±5 μmol) of the DETP dose and ~86% (32±1 μmol) of the DEP dose were recovered (Figure 2B).

Figure 2: (A) Mass balance (μmol) of the chlorpyrifos metabolites TCP, DETP, and DEP measured in urine at 72 h post-dosing



The chlorpyrifos was orally administered to groups of rats at a dose of 140μmol/kg of body weight. (B) Mass balance (μmol) for the TCP, DETP and DEP doses recovered in the urine following oral administration of each metabolite at a dose of 140μmol/kg of body weight. The data represents the mean±S.D. of three animals. The % value represents the relative amount of the orally administered analyte quantified in the urine (Timchalk *et al*, 2007a).

In the case of DEP and TCP virtually all the administered dose was recovered in the urine by 72 h post-dosing, suggesting negligible, if any, metabolism; whereas with DETP, ~50% of the orally administered dose was recovered in the urine. The chlorpyrifos oral dose was likewise rapidly absorbed and metabolized to DEP, TCP and DETP, with the distribution of metabolites in the urine followed the order: TCP (22±3 μmol) > DETP (14±2 μmol) > DEP (1.4±0.7μmol). Based upon the total amount of TCP detected in the urine a minimum of 63% of the oral chlorpyrifos dose was absorbed. These studies support the hypotheses that DEP, DETP and TCP present in the environment can be readily absorbed and eliminated in the urine of rats and potentially humans.

Table 2: Concentration of chlorpyrifos (Group I), DEP, TCPy and DETP in blood (µg/mL) or the metabolite amounts in urine following oral administration of either DEP (group II), TCPy (group III), or DETP (group IV) to male Sprague–Dawley rats (Timchalk *et al* 2007a)

Time post-dosing (h)	Blood metabolites (µg/mL) (mean±S.D.)			Urine metabolites (µg) (mean±S.D.)		
	DEP	TCPy	DETP	DEP	TCPy	DETP
Group I [a]						
1	0.52±0.26	7.5±5.2	1.08±0.26	-	-	-
3	0.85±0.34	18±17	1.37±0.33	-	-	-
6	0.61±0.10	16±6.0	0.97±0.16	-	-	-
12	0.26±0.05	9.9±6.2	-	107 ± 57	710 ± 325	616±68
24	0.21±0.03	3.1±2.1	0.21±0.04*	47 ± 26	772 ± 462	269±102
48	0.27±0.07	1.1±1.3	ND	47 ± 25	1948 ± 693	775±225
72	ND	ND	ND	9.0 ± 3.1	156 ± 56	123±34
Group II, III, IV						
1	0.31±0.14	18±2.8	1.99 ±0.13	-	-	-
3	0.20 ± 0.07	9.4 ± 1.7	1.26 ± 0.14	-	-	-
6	0.08 ± 0.01	9.9 ±0.8	1.23 ± 0.21	-	-	-
12	0.06 ± 0.02*	9.4 ± 8.5	0.37 ± 0.18	3921 ± 2226*	3048 ± 1230	2105 ± 1481
18	-	0.62 ± 0.13*	0/06 ± 0.03	-	-	-
24	0.03 ± 0.01	0.35 ± 0.18*	0.05 ± 0.01	1256 ± 241	2723 ± 647	1428 ± 1451
48	ND	0.11 ± 0.00*	ND	492 ± 392	1131 ± 196	210 ± 155
72	ND	0.11 ± 0.01	ND	271 ± 96*	697 ± 100	ND

Values are mean±S.D. for three animals per time-point, * indicates *n* = 2 due to a sample loss. ND, non-detected; (–) no sample.
[a] chlorpyrifos (~140µmol/kg body weight, ~50 mg/kg body weight). Overall average dose: chlorpyrifos 36.0±2.5µmol/rat (*n* = 21 animals).
[b] DEP, TCPy, and DETP (140µmol/kg). Overall average dose: DEP, 37.0±1.9µmol/rat (*n* = 21 animals); TCPy, 38.0±4.0µmol/rat (*n*=24 animals); DETP, 34.3±2.0µmol/rat (*n* = 24 animals).

Table 3: Calculated area-under-the-concentration (AUC) for DEP, DETPand TCP in male Sprague–Dawley rats orally administered chlorpyrifos (chlorpyrifos; group I), DEP (group II), TCP (group III) or DETP (group IV) at equal molar doses (Timchalk *et al* 2007a)

Metab-olites	AUC (blood: μmolmL^{-1} h^{-1} or urine: μmol h−1)[a]							
	Group I (Chlorpyrifos)		Group II (DEP)		Group III (TCP)		Group IV (DEPT)	
	Blood	Urine	Blood	Urine	Blood	Urine	Blood	Urine
DEP	14.7 $t_{1/2\alpha}$, 0.2 h; $t_{1/2\beta}$, 118 h	2725 $t_{1/2}$, 13.9 h	1.88 $t_{1/2\alpha}$, 0.2 h $t_{1/2\beta}$, 52 h	61,183 $t_{1/2\alpha}$, 12.6 h	N/A	N/A	N/A	N/A
DETP	16.7 $t_{1/2\alpha}$, 5.4 h $t_{1/2\beta}$, 31 h	28614 $t_{1/2}$, 21.7 h	N/A	N/A	N/A	N/A	13.4 $t_{1/2}$, 4.2 h	40,861 $t_{1/2}$, 8h
TCP	280 $t_{1/2\alpha}$, 0.3 h $t_{1/2\beta}$, 8.5 h	66,784 $t_{1/2}$, 20.6 h	N/A	N/A	153 $t_{1/2\alpha}$, 0.3 h $t_{1/2\beta}$ 5.1 h	102,806 $t_{1/2}$, 17.3 h	N/A	N/A

N/A, not applicable.
[a] AUC calculations did not extrapolate to infinity.

3. *In Vitro* Metabolism of Chlorpyrifos

Esterases which interact with OPs have been characterized according to their interaction with paraoxon: (1) A-esterases (also called paraoxonase) which hydrolyze paraoxon and are not inhibited by it and (2) B-esterases [such as the aliesterases and cholinesterases] which are inhibited by l0^{-8} M paraoxon and will not hydrolyze it (Aldrige 1953 a & 1953b as cited in Pond *et al* 1995). The A-esterases are calcium activated and detoxify OPs by enzymatic hydrolysis to their constituent acids and alcohols. AliEs, also called carboxylesterases (CarbEs), are phosphorylated by OPs, and thereby stoichiometrically degrade them (*i.e*., one molecule of esterase binds one molecule of OP). A-Esterase activity appears to provide protection against the adverse effects of OP exposure. Literature cited by Pond *et al* 1995 indicates that mammals have the highest levels of A-esterase activity while birds, which are more susceptible to OP toxicity, have little to no activity. Paraoxon and chlorpyrifos oxon are detoxified via A-esterases and aliesterases. These enzyme activities were measured in various tissues of Sprague-Dawley rats by Pond *et al* 1995. High A-esterase activities were detected in liver, serum and liver mitochondria/microsoma1 fractions. Low or no A-esterase activities were detected in other tissues (lung, spleen, kidney, brain, small intestine and skeletal muscle). Highest aliesterase activities were observed in the small intestine and liver with moderate activity in kidney, serum and lungs. Low activities were noted in brain, spleen and skeletal muscle.

3.1. Cytochrome P450 (The CYP Enzymes)

The liver has been identified as the site of chlopyrifos metabolism. It is thought that the majority of the conversion of chlorpyrifos to chlorpyrifos oxon (the active

metabolite) occurs in the liver via cytochrome P450-dependent metabolism. Ma and Chambers (1994) using rat hepatic microsomes investigated the kinetics of this activation (desulfuration) and dearlyation (oxidative ester cleavage, known also as detoxification) reactions. The oxidative desulfuration was assessed by monitoring the amount of inhibition produced in an exogenous source of AChE. Among two OPs investigated, it was found that in rat hepatocytes, the dearlyation reaction tended to predominate in chlorpyrifos. Hepatic microsomes from males were found to have higher capabilities of these reactions than the females as Table 4 illustrates.

Table 4: Metabolism of chlopyrifos by rat hepatic microsomes (umole product formed/min/g wet wt equivalent of liver microsomes at 10 uM substrate concentration) (Ma and Chambers, 1994)

Insecticide	Sex	Desulfuration	Dearlyation
Chlorpyrifos	Male	5.55±0.62	17.3±2.5
	Female	2.11±0.78*	13.8±3.8*

*Significantly different (P<0.05) from the value for microsomes from male rats within each compound.

This pattern of hepatic metabolism is similar to what was seen earlier by Sultatos and Murphy (1983). The cytochrome P-450 monooxygenase system was implicated in these reactions as evidenced by the requirement for NADPH and inhibition by carbon monoxide. The kinetic constants are shown in Table 5.

Table 5: Kinetic constants for activation and detoxification of chlorpyrifos by mouse hepatic microsomes (Sultatos and Murphy, 1983)

Substrate	Product	appKm (uM)	appVmax (nmoles/100 mg liver/min)
Chlorpyrifos	CP-Oxon	20.90±3.24	3.91±0.16
Chlorpyrifos	3,5,6-TCP	16.12±3.40	8.13±0.29

It has been found that in addition to the liver microsomes, other tissues such as brain mitochondria and brain microsomes have limited desulfuration activity for the organophosorothionate compounds (Chambers & Chambers 1989). For chlorpyrifos, the male rat liver microsomal desulfuration activity was 4132 fold higher than either the brain mitochondria or brain microsomes and the female rat microsomal deslufuration activity was 1186 fold higher than either the brain mitochondria or brain microsomes. Liver microsomes desulfuration activity from males was 3-4 times higher than in the females.

Poet *et al* (2003) explored the *in vitro* rat hepatic and intestinal metabolism of chlorpyrifos. Using enterocytes of the small intestine and liver micosomes from male Sprague Dawley rats, the formation of chlorpyrifos oxon (measured analytically) was demonstrated, as well as detoxification products. The enterocytes were very effective in

these metabolic reactions; however their impact on the metabolism of chlopyrifos is not well understood. The kinetics parameters for the CYP450 metabolism of chlorpyrifos in hepatic and enterocyte microsomes is presented in Table 6. This study demonstrated that the CYP-mediated bioactivation of either chlorpyrifos to the oxon metabolite by liver microsomes is a less important pathway than the detoxification to the pyridinol. In liver, the production of TCP is the high affinity, high capacity path for chlorpyrifos. Whereas, in the enterocytes, the metabolism of chlorpyrifos to TCP has a lower efficiency compared to the metabolism to chlorpyrifos oxon, although the capacity is still higher for the TCP pathway.

Table 6: Comparison of the Kinetic Parameters for CYP450 Metabolism of chlorpyrifos and diazinon in Hepatic and Enterocyte Microsomes (Poet *et al*, 2003)

Sample	K_m	V_{max}	V_{max}/k_m
Chlorpyrifos			
Hepatic			
Oxon	10.6	0.268	8
TCP	4.08	1.14	279
Enterocyte			
Oxon	8.1	0.068	8.4
TCP	55.0	0.249	4.5

These studies have demonstrated the role of the cytochrome P450 microsomal enzymes in the biotransormations of chlorpyrifos. Further investigations (Tang et al 2001, Buratti *et al* 2003, 2006, Foxenberg *et al* 2007, Sams *et al* 2004 and others) identified the individual CYP enzymes involved in the activation and deactivation of chlorpyrifos. Sams *et al* 2004 investigated the kinetics of chlopyrifos biotransormations using human liver microsomes obtained from individual donors to characterize the CYP forms involved in these reactions. Pooled HLM from 21 donors of mixed gender and microsomes prepared from baculovirus-infected insect cells expressing recombinant human CYPs 1A2, 2A6, 2B6, 2C*91 (Arg_{144}), 2C19, 2D6*1, 2E1, and 3A4 (Supersomes™) were used. Standard incubations procedures were used (Tang *et al* 2001). Chemical inhibitors were used to investigate the role of the individual CYPs in catalyzing chlopyrifos biotransformation. The oxon and the TCP metabolites were measured analytically. Chlorpyrifos, underwent desulfuration in human liver microsomes with mean K_m = 30 µM and V_{max} = 353 pmol $min^{-1}mg^{-1}$. Dearalyation of these compounds proceeded with K_m = 12 µM and V_{max} = 653 pmol $min^{-1}mg^{-1}$. The apparent intrinsic clearance (V_{max}/ K_m) of dearylation was 4.5x greater than desulfuration for chlorpyrifos. Among the various CYPs, the highest desulfuration activity occurred by CYP2B6, and the highest dearylation by CYP2C19 for chlorpyrifos. Chlorpyrifos was more readily detoxified (dearylated) than bioactivated (desulfurated) in all HLM preparations (Table 7). Hydrolysis of chlorpyrifos oxon in HLM exhibited Michelis-Menten kinetics with a mean K_m of 2.2 mM and V_{max} of 233 nmole $min^{-1}mg^{-1}$.

Table 7: Desulfuration/dearylation ratios for individual CYPs toward chlorpyrifos (Sams *et al*, 2004)

CYP isoform	Chlorpyrifos
CYP1A2	>10
CYP2A6	>1
CYP3B6	>10
CYP2C9	3.8
CYP2C19	0.14
CYP 2D6	>1
CYP3A4	>1

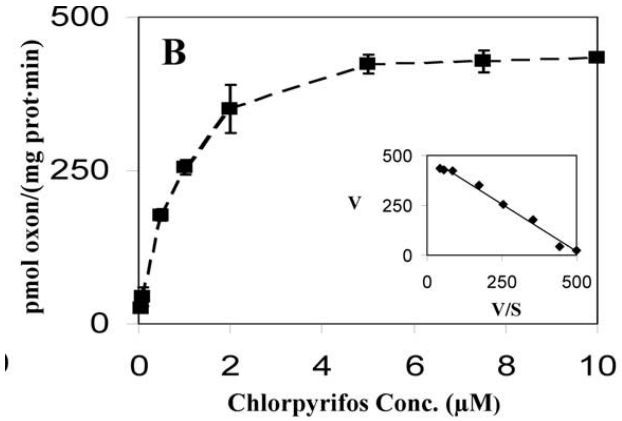
The CYP3A4 has been described as the most abundant isoform of cytochrome P450 in the adult human liver and is responsible for metabolism of approximately half the currently used drugs (Dai *et al 2001*). CYP3A4 varies 40-fold in individual human livers, and metabolism of CYP3A4 substrates varies at least 10 fold *in vivo.* CYP3A4 variants exist in the human population. Using direct sequencing of genomic DNA in 72 individuals from three different ethnic groups, including Caucasians, Blacks (African-Americans and African pygmies), and Asians, single nucleotide polymorphisms (SNPs) in *CYP3A4* were identified. Racial variability was observed for the frequency of individual SNPs. CYP3A R162Q was identified only in Black populations with an allelic frequency of 4%. CYP3A4 F189S and CYP3A4 M445T were identified in Caucasians with allelic frequencies 2% and 4%, respectively. L293P and P467S were only observed in Asians at allelic frequencies of 2%. The catalytic activities of the most common CYP3A4 allele (CYP3A4*1) and its allelic variants were assessed using their action on testosterone and chlorpyrifos biotransformation to the oxon and TCP. CYP3A4 F189S exhibited lower turnover numbers for testosterone and chlorpyrifos, while CYP3A4 L293P had higher turnover numbers for both substrates. The turnover numbers of the CYP3A4 M445T and P467S alleles to metabolize these compounds were not significantly different from those of wild-type CYP3A4. CYP3A4 activates the chlorpyrifos into oxon and it inactivates chlorpyrifos into TCP. The relative rates of activation and inactivation are critical to the toxicity of the compound. The L293P allele could possibly increase the toxicity of chlorpyrifos and other OP insecticides to individuals carrying this allele. Interestingly, the F189S allele decreased both activation and inactivation of chlorpyrifos and could also potentially affect toxicity after exposure to OP insecticides. Potentially, these alleles may contribute to the known variability in metabolism of clinically used drugs and environmental compounds that are CYP3A4 substrates.

Foxenberg *et al* (2007) investigated the human hepatic cytochrome P450 family of enzymes in the metabolism of chlorpyrifos. They found that the metabolism kinetics varied considerably among individual donors and that pooled human liver microsomes do not estimate the inter-individual variability that occurs within a population. P450 specific enzyme activities also showed variation among different individuals. Separate

studies were conducted with individual recombinant human P450s to identify specific P450s that biotransform chlorpyrifos. The most metabolically active P450s for chlorpyrifos were CYP2B6, 2C19, and 3A4 while CYP1A2, 3A5, and 3A7 had limited metabolism of chlorpyrifos. CYP2B6 also showed a higher affinity and activity for chlorpyrifos metabolism to chlorpyrifos-oxon, with a K_m value of 0.81 µM and V_{max} of 12,544 pmol/min/nmol P450. In contrast, CYP2C19 has a high affinity and activity for TCP formation (dearylation), with a *K*m of 1.63 µM and a V_{max} of 13,128 pmol/min/nmol P450. CYP3A4 has a high V_{max} for chlorpyrifos metabolism, but also a high K_m (>27 µM) for both reactions. This is the first publication deriving human P450 specific kinetic parameters for an OP compound that will help refine current PBPK/PD models that utilize kinetic values derived from rat liver microsomal metabolism that may not reflect human enzymes.

Buratti *et al* 2003 also observed variability in the kinetic parameters of the chlorpyrifos desulfration among single HLM and pooled HLM samples. The following plot illustrates (Figure 3) the chlorpyrifos oxon formation following incubation with pooled HLM samples.

Figure 3: Rates of oxon formation from chlorpyrifos illustrated with HLM pooled from five donors (Buratti *et al* 2003)



Incubation was carried out for 2 min with different chlorpyrifos concentrations. Each value represents the mean ± SD of at least triplicate determinations. The corresponding Eadie-Hosftee plot (V vs V/S) is shown in the inset

Oxon formation in the single individual HLM showed a significant correlation with CYP1A2-, 3A4-, and 2B6-related activities, at different levels of chlorpyrifos concentration. Anti-human CYP1A2, 2B6, and 3A4 antibodies inhibited oxon formation, showing the same chlorpyrifos concentration dependence. The Buratti *et al* 2003 data indicated that CYP1A2 is mainly involved in chlorpyrifos desulfuration at low concentrations, while the role of CYP3A4 is more prominent in the low-affinity (higher concentrations) component of chlorpyrifos bioactivation. The contribution of CYP2B6 to total hepatic oxon formation was relevant in a wide range of pesticide concentrations, being a very efficient catalyst of both the high- and low-affinity phase. Buratti *et al* 2003 suggested CYP1A2 and 2B6 as possible metabolic biomarkers of susceptibility to chlorpyrifos toxic effect at the actual human exposure levels.

Tang *et al* (2001) investigated the metabolism of chlorpyrifos by human CYP P450 isoforms and human, mouse, and rat liver microsomes (HLM, SLM, RLM, respectively). The kinetics of these reactions is presented in Table 8. Chlorpyrifos, chlorpyrifos oxon and TCP were assayed analytically using HPLC system with limit of detection for TCP and the oxon of 0.03 and 0.04 μM at an injection volume of 15 μl.

Table 8: Dearylation and desulfuration activities toward chlorpyrifos in HLM, RLM, or MLM (Tang *et al* 2001)

	Desulfuration			Dearlyation		
	K_m (μM)	V_{max} *nmol/mg protein/min*	K_m/V_{max}	K_m (μM)	V_{max} *nmol/mg protein/min*	K_m/V_{max}
HLM	30.2±1.7	0.4±0.1	0.01	14.2±2.2	0.7±0.1	0.05
RLM	6.1±1.1	1.0±0.2*	0.17	4.8±1.8*	2.1±0.4**	0.43
MLM	24.4±2.4	0.7±0.1	0.03	14.9±3.0	2.7±0.1**	0.18

Activities are expressed as mean ± S.E.M. (*n* = 3 determinations), means in RLM (pooled from three male rats) and MLM (pooled from three male mice) significantly different than HLM (pooled from 10 donors) are indicated by *p < 0.05 or **p< 0.01.

HLM displayed lower affinity (i.e., higher K_m) and lower reaction velocity toward chlorpyrifos for both desulfuration and dearylation than RLM. MLM exhibited similar affinities as the HLM but a higher reaction velocity toward chlorpyrifos. Both RLM and MLM have higher values of clearance terms (V_{max}/K_m) than HLM. Pooled female HLM showed significantly higher metabolic activity toward chlorpyrifos than pooled male HLM. A screen of several human CYP isoforms expressed in human lymphoblastoma cells demonstrated that CYP1A2, 2B6, 2C9*1, 2C19, and 3A4 were involved in chlorpyrifos metabolism, whereas no oxidation activity toward chlorpyrifos was detected using CYP1A1, 2A6, 2C8, 2C9*2, 2D6, 2E1, and 4A11. Desulfuration and dearylation activities were greatest for CYP2B6 and CYP2C19, respectively. Marked differences in metabolic activity toward chlorpyrifos were observed in five individuals examined representing contrasting activities of some important CYP isoforms as illustrated in Table 9. Individuals with high levels of CYP2B6 and 3A4 had high desulfuration activity; individuals with low levels of CYP2B6 and 3A4 had low desulfuration activity. The dearylation pathway was more predominant in the individual with high-CYP2C19 but low-3A4 levels. No particular increase in either metabolite was observed in the individual with high levels of CYP2D6. These individual differences suggest that differential sensitivities to chlorpyrifos may exist in the human population.

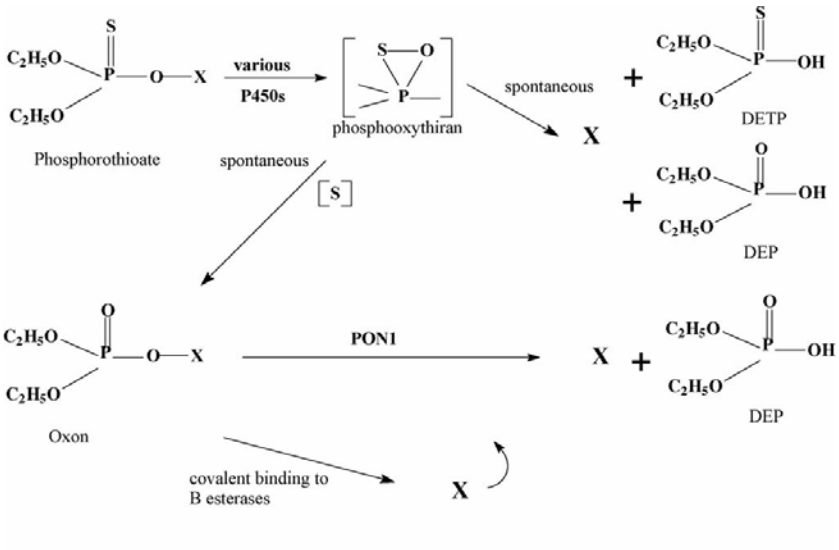
Table 9: Metabolic activities toward chlorpyrifos in individual human liver microsomes (Tang *et al* 2001)

Isozyme	Desulfuration* nmol/mg protein/minute	Dearylation* nmol/mg protein/minute
HG 006	0.09 ± 0.01 [a]	0.35 ± 0.03 [a]
HG023	0.16 ± 0.01 [a]	0.31 ± 0.04 [a]
HG042	0.74 ± 0.10 [b]	0.67 ± 0.07 [a b]
HG043	0.08 ± 0.01 [a]	0.61 ± 0.04 [a b]
HG112	0.67 ± 0.08 [b]	0.91 ± 0.10 [b]

Activities are expressed as mean ± S.E.M., (*n*= 3–4 determinations). Individual human liver microsomes (protein concentration, 20 mg/ml). * Means in the same column followed by the same letter are not significantly different, p<0.01

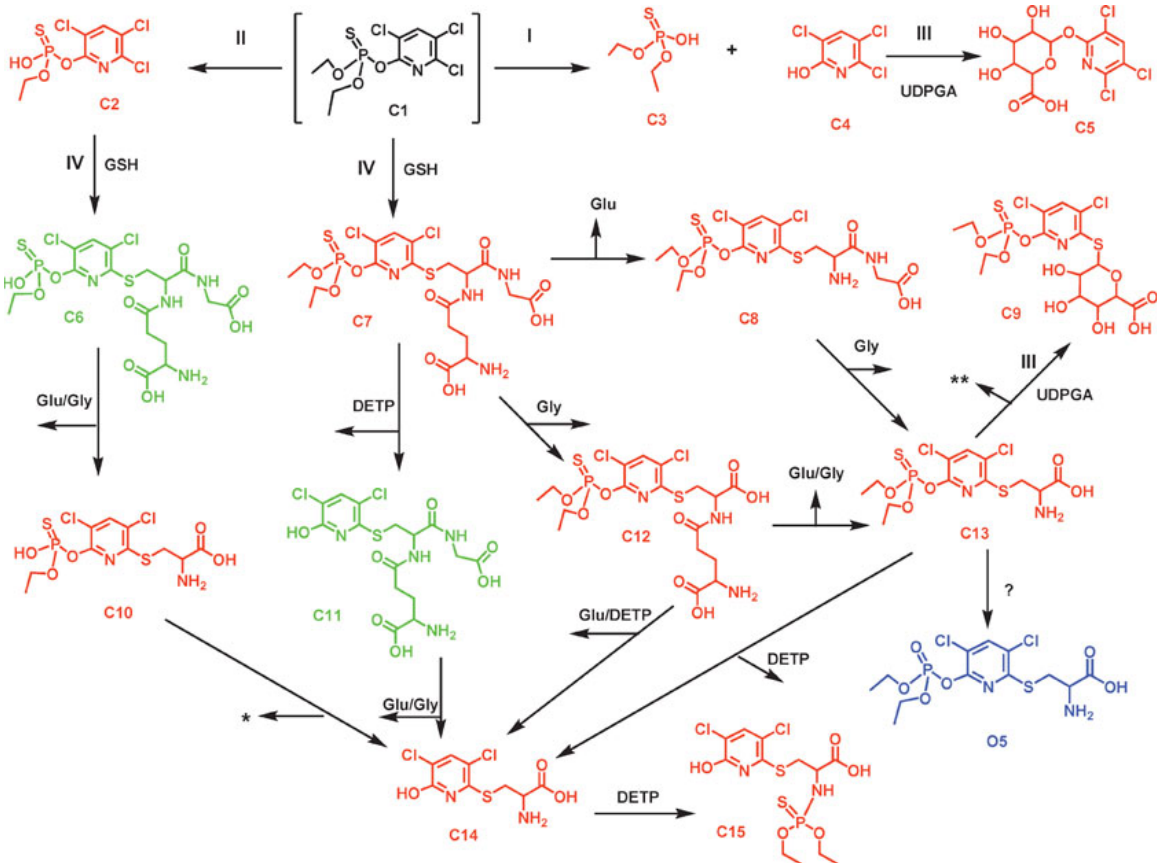
Mutch and Williams 2006 investigated the activation and detoxification of chlorpyrifos by recombinant P450 isozymes and by human liver microsomes. Wide variations in the activation of these OPs to their respective oxons and the production of their dearylated products were reported with data strongly suggesting that CYPs 3A4/5, 2C8, 1A2, 2C19 and 2D6 are primarily involved in the metabolism of chlorpyrifos. CYPs 2D6, 3A5, 2B6 and 3A4 were best at producing chlorpyrifos-oxon and CYPs 2C19, 2D6, 3A5 and 3A4 at producing TCP from chlorpyrifos (100 µM). The authors of this study concluded that the marked inter-individual variation in expression of the various P450 isozymes may result in sub-populations of individuals that produce higher systemic oxon levels with increased susceptibility to OP toxicity. The activation and detoxification metabolic pathways of chlorpyrifos and other OP compounds is illustrated in Figure 4.

Figure 4: The activation and detoxification metabolic pathways for diazinon, chlorpyrifos and parathion (Mutch and Williams 2006)



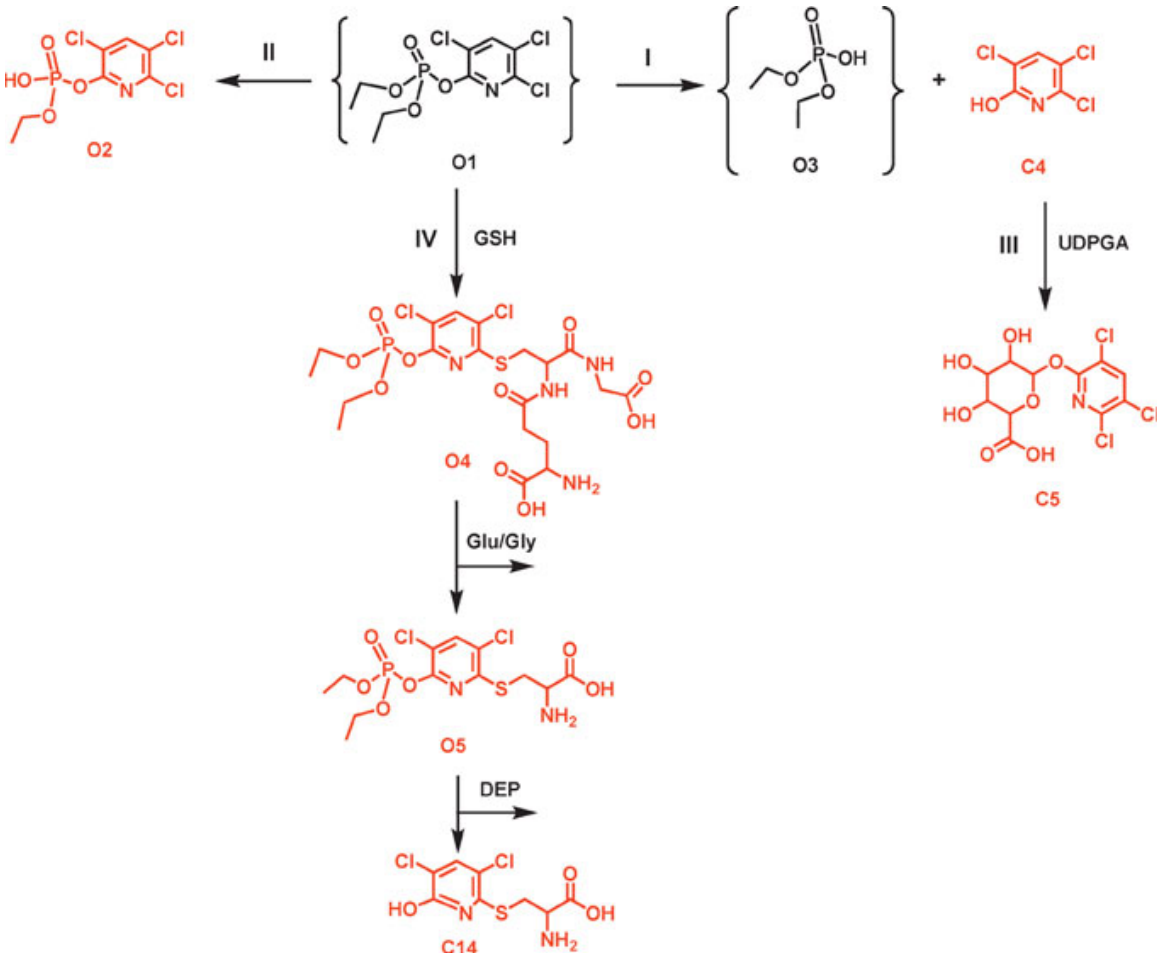
Choi *et al* 2006 investigated the metabolism of chlorpyrifos and its oxon in human hepatocytes and human liver S9 fractions using LC-MS/MS analytical techniques. Cytochrome P450 (CYP)-dependent and phase II-related products were determined following incubation with chlorpyrifos and the oxon. Pooled human liver S9 fraction (8 males and 7 females donors), was obtained from a commercial source. Primary human hepatocytes were also obtained through a commercial source representing 3 donors (2 males and 1 female). CYP-related products, TCP, diethyl thiophosphate, and dealkylated chlorpyrifos, were found following chlorpyrifos treatment and dealkylated chlorpyrifos oxon following chlorpyrifos oxon treatment. Diethyl phosphate was not identified because of its high polarity and lack of retention with the chromatographic conditions employed. Phase II related conjugates, including *O*- and *S*-glucuronides as well as 11 GSH-derived metabolites, were identified in chlorpyrifos-treated human hepatocytes, although the *O*-sulfate of TCP conjugate was found only when human liver S9 fractions were used as the enzyme source. *O*-Glucuronide of TCP was also identified in chlorpyrifos oxon-treated hepatocytes. Chlorpyrifos and chlorpyrifos oxon were identified using HPLC–UV. However, chlorpyrifos oxon was not found following treatment of human hepatocytes with either chlorpyrifos or chlorpyrifos oxon. Since chlorpyrifos oxon is itself readily metabolized by human hepatocytes, it was concluded "that it does not accumulate to be released into the bloodstream and transported to the nervous system. If that is the case, the activation of chlorpyrifos to its oxon within the nervous system, rather than the liver, may be responsible for the cholinergic effects of chlorpyrifos, whereas its metabolism in the liver will cause inhibition of CYP isoforms by the release of reactive sulfur." These results "suggest that human liver plays an important role in detoxification, rather than activation, of chlorpyrifos." This is the most detailed *in vitro* study demonstrating that chlorpyrifos undergoes not only CYP-dependent phase I biotransformation but also phase II conjugation pathways in human hepatocytes with extensive characterization of the various metabolic products. Based on these results, Choi *et al* proposed the following scheme of chlorpyrifos and its oxon biotransformation in human hepatocytes (Figures 5 & 6).

Figure 5: Proposed biotransformation scheme for chlorpyrifos in human hepatocytes. (Choi *et al* 2006)



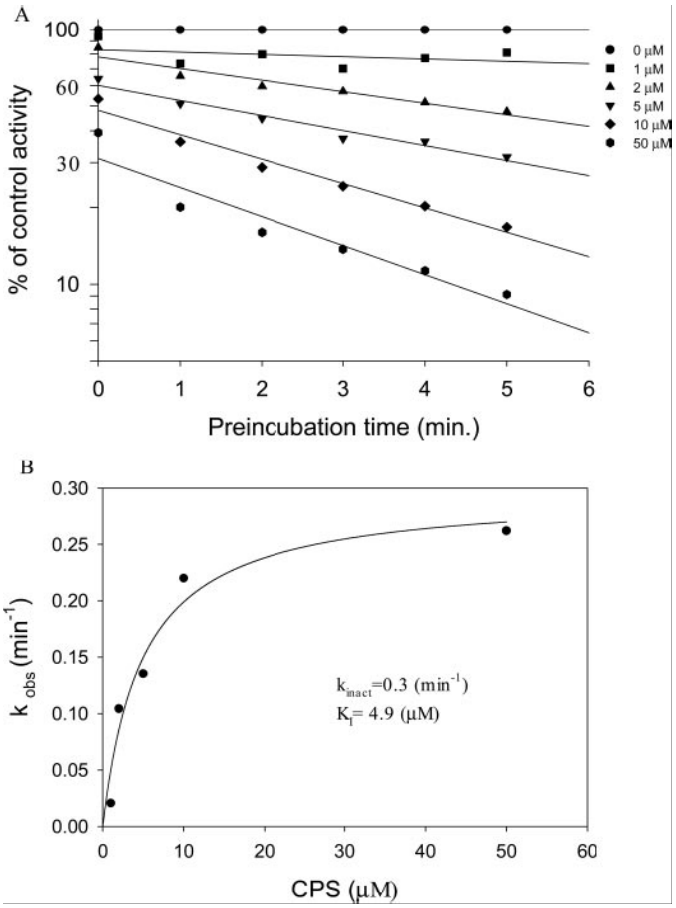
Four possible pathways: dearylation (I), dealkylation (II), glucuronide conjugation (III), and GSH-mediated conjugation (IV). ∗O-3,5-dichloro-6-mercaptopyridin-2-yl O,O-diethylphosphorothioate; ∗∗2-aminopropanoic acid

Figure 6: Proposed biotransformation scheme for chlorpyrifos oxon in human hepatocytes (Choi *et al*, 2006)



The *in vitro* hydroxylation of estradiol, an important female hormone, by human liver microsomes or CYP3A4 and CYP1A2 isoforms was markedly inhibited by chlorpyrifos (Usmani *et al*, 2006). Preincubation of CYP3A4, CYP1A2 or pooled human liver microsomes with chlorpyrifos (final concentration 50 μM) resulted in 94%, 96% and 80% inhibition, respectively of estradiol metabolism. To investigate the type of inhibition of CYP3A4 and CYP1A2 by chlorpyrifos in the production of 2-OHE_2, different concentrations of chlorpyrifos were preincubated for 5 min with CYP3A4 or CYP1A2 before adding varying concentrations of estradiol. Michaelis-Menten plots showed that the V_{max} values were significantly reduced without affecting K_m values, indicative of a noncompetitive inhibition of estradiol metabolism by chlorpyrifos. The production of 2-OHE_2 by CYP3A4 was inhibited by chlorpyrifos in a time- and concentration-dependent manner. Inactivation parameters (*k*inact and *K*I) determined for CYP3A4 metabolism of estradiol by chlorpyrifos were 0.3 min^{-1} and 4.9 μM, respectively (Figure 7). Even at a preincubation of zero time, the metabolic activity of CYP3A4 for estradiol was apparently decreased by chlorpyrifos in a dose dependent manner, indicating a very rapid initial rate of inhibition. This inhibition was shown to be irreversible.

Figure 7: Time- and concentration-dependent inactivation of CYP3A4 metabolism of E2 by chlorpyrifos (Usmani *et al* 2006)



A, correlation between preincubation time with various concentrations of chlorpyrifos and CYP3A4 activity. B, plot of inactivation rate constant as a function of chlorpyrifos concentration to determine kinact and KI values (reproduced from Usmani et al 2006; Fig. 8

3.2. The PON1 Enzyme

The oxon formed from the desulfuration of chlorpyrifos is detoxified to the dearylated metabolite by PON1, an A-esterase plasma enzyme tightly associated with high-density lipoprotein particles and also found in liver. The influence of PON1-192 genotype and phenotype on the capacity of human liver microsomes to detoxify chlorpyrifos oxon was investigated by Mutch *et al* 2007. Liver samples were from 27 unrelated Caucasian males (*n* = 13) and females (*n* = 10), and 4 of unknown sex. Their ages ranged 21 to 66 years (median, 47 years) and with known smoking and alcohol intake histories. Human liver microsomes were prepared using standard procedures. Microsomes were incubated with saturating concentrations of the test materials. For chlorpyrifos oxon it was 500 μM. Genomic DNA was extracted from about 500 mg of each human liver for the analysis of PON1-192 genotype. The frequency distribution of the PON1-192 genotype was: QQ = 0.48 (13 individuals), QR = 0.30 (8 individuals), and RR = 0.22 (6 individuals) with allele frequencies of Q = 0.63 and R = 0.37. This was consistent with published work using similar analytical methods. The rates of hydrolysis

of chlorpyrifos-oxon varied 16-fold among the different samples, regardless of PON1-192 genotype. Individuals with the PON1-192RR genotype preferentially hydrolyzed paraoxon ($p < 0.01$), and the R allele was associated with higher hydrolytic activity toward chlorpyrifos-oxon, but not diazoxon. There were strongly significant relationships between the activity of the PON1 hydrolysis of phenylacetate and chlorpyrifos-oxon hydrolysis ($p < 0.001$). The study investigators concluded that their data "highlight the importance of PON1 phenotype for efficient hydrolysis of chlorpyrifos-oxon".

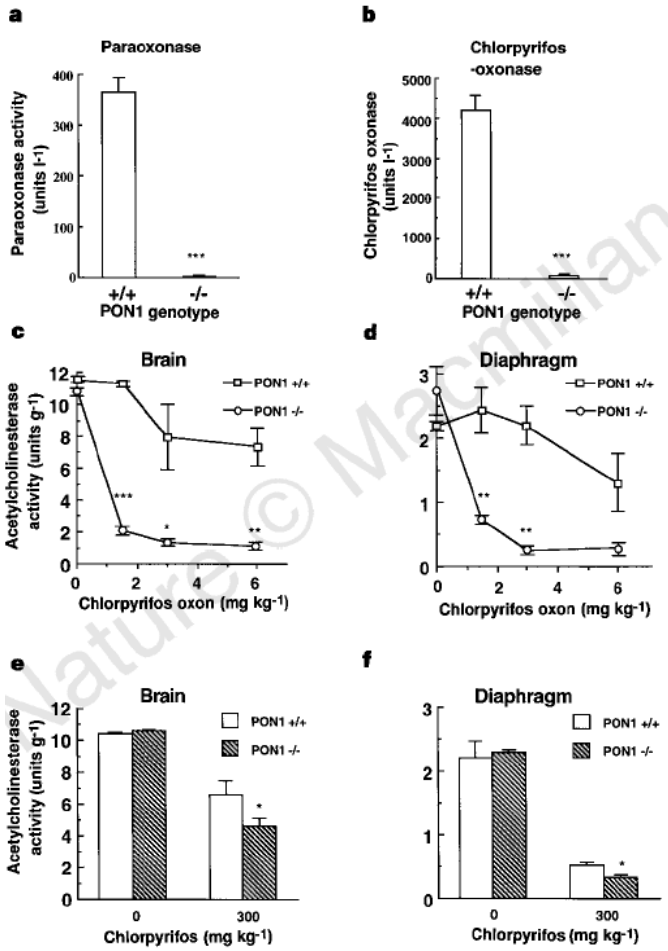
The PON1-mediated metabolism of chlorpyrifos oxon to TCP was measured in microsomes prepared from rat liver and intestinal enterocytes and the results are presented in Table 10 (Poet *et al*, 2003). The K_m for the metabolism of chlorpyrifos oxon was similar in both tissues, but the V_{max} was much greater (47 fold) in liver than enterocytes. This is in agreement with the findings of Pond *et al.* (1995). The parameters for PON1-mediated metabolism of chlorpyrifos 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 μM and 24 μmol/min/mg, respectively.

Table 10: Comparison of the Kinetic Parameters for PON1 Metabolism of chlorpyrifos oxon in Hepatic and Enterocyte Microsomes (Poet et al, 2003)

Sample	K_m	V_{max}	V_{max}/k_m
Hepatic	345	1280	3700
Enterocyte	328	4.60	14

Furlong, 2007 addressed the morphological variability of PON1 enzyme among the human population. In this regard PON1 is polymorphically distributed in human populations with an amino acid substitution (Glutamine/Arginine) at position 192 of this 354-amino acid protein that determines the catalytic efficiency of hydrolysis of some substrates. In addition to the variable catalytic efficiency determined by the position 192 amino acid, protein levels of PON1 vary by as much as 15-fold among individuals with the same PON1192 genotype (Q/Q; Q/R; R/R). The PON1-R192 allele was shown to be the high paraoxonase activity allele and the PON1-Q192 allele the low metabolizer allele. The creation of mice strain lacking PON1 activity (knockout mouse) in 1998 (Shih *et al*, 1998) accelerated the understanding and importance of this enzyme in the detoxification of OPs. The following figures from Shih *et al*, 1998 (Figure 8) illustrate the loss of PON1 activity in this mouse strain.

Figure 8: PON1 null mice are more sensitive to the toxic effects of chlorpyrifos oxon and chlorpyrifos (Shih *et al* 1998)



a, Plasma paraoxonase, and **b**, chlorpyrifos oxonase activities of wild-type and *PON1* null mice. There were 10 and 9 animals in the *PON1*+/+ and *PON1*−/−groups, respectively. **c, d**, Acetylcholinesterase activity in brain **(c)** and diaphragm **(d)** samples from *PON1*+/+ and *PON1*−/−mice treated with chlorpyrifos oxon. There were 3 animals in each treatment group. e, f, Acetylcholinesterase activity in brain **(e)** and diaphragm **(f)** samples from *PON1*+/+ and *PON1*−/−mice treated with chlorpyrifos. There were 4–6 mice in each group. For **a–f**, data are the means obtained from several animals. Error bars indicate standard errors; Student's *t*-test was used for statistical analysis. Asterisks indicate significant differences between *PON1*−/−and *PON1*+/+ groups at the significance level of **P* , 0:05, ***P* , 0:01, or ****P* , 0:0001, respectively

The influence of genetic variation on PON1 activity in neonates was investigated using cord blood and maternal blood from a group of 402 mothers in ongoing epidemiological study (Chen *et al*, 2003). In addition to the genetic variability, compared with adults, neonates had lower PON1 activity, implying reduced capacity to detoxify chlorpyrifos.

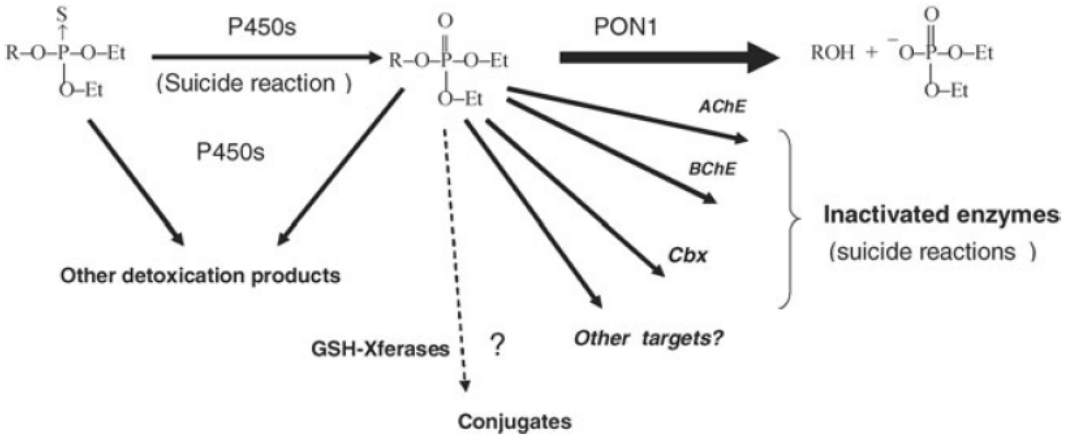
The OP insecticides are thus bioactivated by the CYP enzymes and are deactivated by both the CYP and PON1 enzymes. As Poet *et al* state "The balance between bioactivation and detoxification will drive the toxicity from OP exposures. At low

oral dose exposures, metabolism by CYPs and PON1 in intestine and liver may remove the active moiety from the circulation prior to systemic exposure".

3.3. B-Esterases (Carboxylesterases)

In addition to the PON1 status in chlorpyrifos detoxification, B-esterases (carboxylesterases) have been suggested to act as scavengers of OP compounds. These have strong affinity to specific OP compounds. Genetic variability of the B-esterases has also been demonstrated as reviewed by Furlong 2007. Significant individual variability in carboxylesterases in human liver microsomes, with activities varying from 5.3- to 44.7-fold, depending on the substrate used for assaying carboxylesterase activity was demonstrated (Hosokawa *et al.* 1995 as cited by Furlong 2007). They also noted the importance of regulation of carboxylesterase activities by exogenous inducers. In addition, a second B esterase, butyryl or serum cholinesterase (BuChE), plays a role in providing protection against OP exposure despite its ability to bind only a single OP molecule [Broomfield *et al* 1991 as cited by Furlong 2007) , leading to the prediction that individuals deficient in BuChE would exhibit increased sensitivity to OP exposure [Manoharan *et al* 2007 as cited by Furlong 2007]. The role of these various enzymes in the metabolism of OP compounds such as chlorpyrifos is presented in Figure 9. There is disagreement that BuChE can be a reasonable detoxification sink. According to Padilla (2008, personal communication) there aren't enough molecules of the BuChE enzyme noting that there are lots more CarbE molecules than BuChE molecules. Furthermore, no one has actually demonstrated that animals or people low in BuChE are more sensitive to organophosphate toxicity. To protect animals against nerve gases, they have to inject lots of BuChE.

Figure 9: Enzymes contributing to the metabolism of organophosphorothioate insecticides (Furlong 2007)



Carboxylesterase (CarbE) plays an important role in removing the chlorpyrifos oxon formed, thus reducing its toxicity. Due to its importance, it has been the subject of several investigations. Chanda *et al*, 1997 explored the tissue specific effects of

chlorpyrifos on CarbE and ChE activity in adult rats both *in vitro* and *in vivo.* Male and female Long-Evans rats were orally treated with chlorpyrifos and ChE and CarbE activities were assessed in the brain, liver and blood. There were some sex-related differences in the activity of basal CarbE and ChE in the brain, plasma, and liver of Long-Evans rats. The liver had the highest CarbE activity. Table 11 shows their activities in the control animals. *In vitro* tests showed that there were considerable differences in the sensitivity of both ChE and CarbE to chlorpyrifos oxon in the three tissues. Liver CarbE was about 90 times *more* sensitive to the inhibition by chlorpyrifos oxon compared to brain CarbE. Brain CarbE showed a definite biphasic response, suggesting that there may be two or more isoforms of the enzyme in the brain with different sensitivities to chlorpyrifos oxon. The rank order for CarbE sensitivity to chlorpyrifos-oxon between the tissues was liver > plasma >brain. In addition to the gender-related differences for CarbE activity, it was found that the *in vitro* sensitivity of CarbE to chlorpyrifos oxon is highly tissue specific. It was also demonstrated that the pattern of inhibition after *in vivo* dosing with chlorpyrifos was not necessarily predictable from the *in vitro* IC_{50} of these enzymes. Most importantly for the detoxification of chlorpyrifos, the number of CarbE molecules is very important in modifying its toxicity.

Table 11: Brain, Plasma, and Liver ChE and CarbE Activities in Control Male and Female Long-Evans Rats (Chanda *et al*, 1997)

	ChE activity[a] (µmol/min/g wet wt)		CarbE activity[a] (µmol/min/g wet wt)	
	Male	**Female**	**Male**	**Female**
Brain	7.45 ± 0.27	6.79 ± 0.31	14.62 ± 0.44	17.07 + 0.44*
Plasma	0.27 ± 0.02	0.67 ± 0.03*	1.36 ± 0.05	1.34 ± 0.05
Liver	0.43 + 0.01	0.31 ±0.01*	413.67 ±11.17	230.52+5.18*

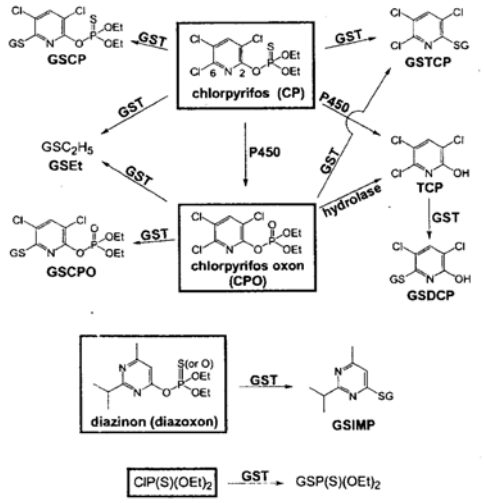
[a]Data represent activities of eight samples obtained from eight control groups containing five rats each (means ± SEM).
* Indicates significant differences between male and female activity ($p < 0.05$).

The binding of CarbE to chlorpyrifos can also affect protein/enzyme associations. A CarbE isozyme known as Egasyn (Fujikawa *et al* 2005) has been described as an accessory protein of ß-glucuronidase in the liver microsomes. The Egasyn- ß-glucuronidase complexes are located at the luminal sites of liver microsomal endoplasmic reticulum membrane. When OP insecticides are incorporated into the liver microsomes, the OP is tightly bound to egasyn, and subsequently, ß-glucuronidase is dissociated and released into the blood. A single administration of EPN, acephate or chlorpyrifos increased plasma BG activity to approximately over 100-fold the control level in rats. Thus, the increase of plasma BG activity after OP exposure can be considered as good biomarker of OP exposure and is a much more sensitive biomarker of acute OP exposure than acetylcholinesterase (AChE) inhibition (Fujikawa *et al* 2005).

3.4. Glutathione S-transferases

The role of glutathione S-transferases (GSTs) in the metabolic tranformations of chlopyrifos has been explored recently by Fujioka and Casida (2007). Chlorpyrifos, its oxon and TCP were each investigated by incubating with one or both of pooled human liver microsomes or human P450 3A4 and GST in the presence of glutathione (GSH) with or without NADPH under standard conditions. GSH and GSTs were obtained from equine liver, rat liver, and human placenta. Metabolites were analyzed by liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS). Five GSH conjugates were identified for chlorpyrifos and chlorpyrifos oxon. These were GS chlorpyrifos and GS chlorpyrifos oxon in which the 6-chloro substituent of chlorpyrifos and chlorpyrifos oxon, respectively, is displaced by GSH; *S*-(3, 5, 6-trichloropyridin-2-yl) glutathione; *S*-(3, 5-dichloro-6-hydroxypyridin-2-yl) glutathione; and *S*-ethylglutathione as shown in Figure 10.

Figure 10: GST- and P450-catalyzed metabolism of chlorpyrifos, chlorpyrifos oxon, diazinon, diazoxon, and ClP(S)(OEt)2

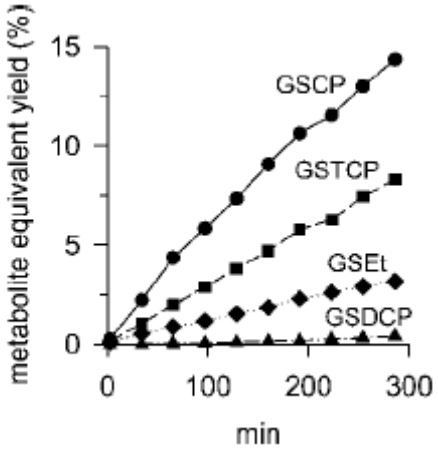


Although not shown, the desethyl derivatives of GSCP and GSCPO and the desglutamyl derivative of GSCP are also observed (Fujioka and Casida, 2007).

When chlorpyrifos, chlorpyrifos oxon, and TCP were incubated with one of both of human liver microsomes (P450) and GST in the presence of GSH with or without NADPH under standard conditions,chlorpyrifos gave three major metabolites [GSCP, GSTCP, and *S*-ethylglutathione (GSEt)] and one minor metabolite (GSDCP) on incubation with GST and GSH, with similar site specificity for equine and rat liver sources, and complete dependence in each case on added GSH. The GSCP: GSTCP:GSEt ratio with equine GST was 1.0:1.2:0.9 at 37 °C and 1.0:0.7:0.3 at 25 °C, indicating a possible temperature effect on metabolic formation or stability which was not studied further. When human liver microsomes or placental GST were used, chlorpyrifos gave only GSCP (47 or 1%, respectively) at 37°C. When incubated with human liver microsomes, NADPH, and the equine GST/GSH system, chlorpyrifos gave GSCP as the only significant metabolite (63%), presumably formed by an endogenous

GST in the microsomal preparation. Addition of microsomes to the equine GST/GSH system reduced the extent of chlorpyrifos loss and GSH conjugate formation except for GSCP, suggesting that this conjugate and its metabolites might inhibit GST. For a direct test of this hypothesis, chlorpyrifos (100 μM) and its metabolites marginally inhibited equine GST (8 ± 3%) on preincubation with GSH (5 mM) for 2 h at 37°C but not without preincubation. Under the preincubation condition, GSCP (0.4 μM), GSTCP (0.4 μM), GSEt (0.2 μM), and (EtO)2P(O)SH (0.3 μM) were formed, but GSDCP was not detected; i.e., these metabolite levels cause marginal or no GST inhibition. Chlorpyrifos oxon gave GSCPO, GSEt, GSTCP, GSDCP, and TCP with equine and rat liver enzymes. When human liver microsomes or placental GST was used, chlorpyrifos oxon gave TCP as a major metabolite and GSCPO as a minor metabolite. TCP was readily converted to GSDCP by equine GST, but not metabolized by microsomes. Figure 11 shows the time course of chlorpyrifos metabolism by equine GST and GSH at 25 °C.

Figure 11: Time course of chlorpyrifos metabolism by equine GST and GSH at 25°C (Fujioka and Casida, 2007)



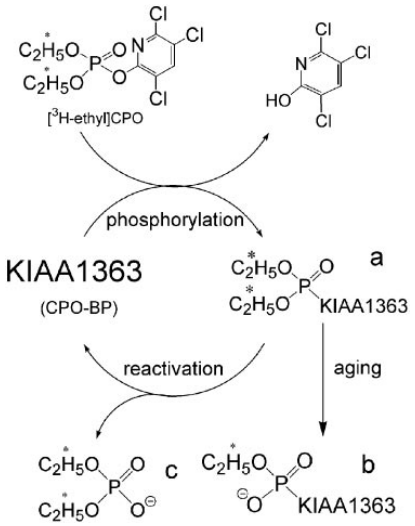
The *in vivo* relevance of the metabolites observed *in vitro* was examined by treating mice intraperitoneally with chlorpyrifos, ClP(S)(OEt)2, and tribufos at 100 mg/kg and analysis of the liver 15 min later by LC–ESI-MS with the positive and negative modes, assigning the GSH conjugates by comparison with those formed in enzymatic reactions. With CP, the parent compound, TCP, (EtO)2P(O)SH, (EtO)2P(O)OH, and one GSH conjugate, GSCP, were detected (each 7–13 μg/g of liver) but chlorpyrifos oxon and other GS conjugates (GSTCP, GSDCP, and GSEt) were not observed. There is evidence for the direct *in vivo* relevance of these findings in mice and humans. GSCP is the major GSH conjugate of chlorpyrifos in human hepatocytes (Choi *et al* 2006), in human liver microsomes, and in the liver of intraperitoneally treated mice. Cysteine and *N*-acetylcysteine conjugates of chlorpyrifos and chlorpyrifos oxon at the 6-position were found in the urine of a chlorpyrifos -intoxicated human (Bicker *et al*, 2005), suggesting *in vivo* GST-catalyzed GSH conjugation to form GSCP as an intermediate metabolite. The level of GSCP formed is comparable with that of TCP, (EtO)2P(O)SH, and (EtO)2P(O)OH, which are well-known metabolites produced by P450s and hydrolases. GST-catalyzed metabolism joins P450s and hydrolases as important factors

in vitro and possibly in vivo in OP detoxification. Twelve of 16 OPs were converted in the GST/GSH system to *S*-alkyl-GSH conjugates, and four produced *S*-aryl-GSH conjugates. CP and CPO underwent not only dealkylation and dearylation but also dehalogenation in the presence of GST. ClP(S)(OEt)2 yielded GSP(S)(OEt)2 with GST and GSH, and tribufos produced GSP(O)(SBu)2 in the P450/GST system. This is the first MS evidence of phosphorylated GSH derivatives formed both in vivo and in vitro. The toxicity of the phosphorylated conjugates and other GSH-derived metabolites of OP metabolites remain to be explored.

3.5. *KIAA1363* – A Mouse Brain Enzyme

Nomura *et al* 2005 identified a mouse brain enzyme (*KIAA1363)* as an enzyme for metabolizing low levels of chlorpyrifos-oxon in the brain which may play a role in the detoxification of the OPs. The predominant mouse brain protein labeled *in vitro* by [^{3}H-ethyl] chlorpyrifos oxon (1 nM) (designated chlorpyrifos-oxon-binding protein) was not one of the known OP toxicant targets. Chlorpyrifos oxon-BP is a 50-kDa membrane bound serine hydrolase measured by derivization with [^{3}H]C oxon and SDS/PAGE or filtration binding assay. It appears to undergo rapid diethylphosphorylation by [^{3}H] chlorpyrifos oxon followed by either dephosphorylation or reactivation or aging on loss of an ethyl group as shown in the figure below. Chlorpyrifos oxon and several other OP toxicants potently inhibit chlorpyrifos oxon-BP activity *in vivo* (i.p., 2 h) (50% inhibition at 2–25 mg/kg) and *in vitro* (50% inhibition at 8/68 nM). Using three chemical labeling reagents, i.e., [^{3}H] chlorpyrifos oxon and the activity-based proteomic probes fluorophosphonate-biotin and fluorophosphonate-rhodamine, mouse brain chlorpyrifos oxon-BP was identified as serine hydrolase KIAA1363 of unknown function. Brains from *KIAA1363* deficient mice show greatly reduced levels of chlorpyrifos-oxon labeling and hydrolytic metabolism compared to brains from wild-type mice. Figure 12 illustrates the role of this brain enzyme in the metabolic scheme of chlorpyrifos.

Figure 12: Reaction of KIAA1363 (also known as CPO-BP) with [3Hethyl] CPO involving phosphorylation



(a) followed by aging (b) (loss of one ethyl group and not reactivated) or reactivation (c) (accelerated by potassium fluoride). At 1 nM[3H]CPO, a ratio of 3:1 is observed for reactivation and aging (adjusted for loss of one labeled residue) (Nomura et al 2005).

3.6. Role of Butyrylcholinesterase in Chlorpyrifos Metabolism

Butyrylcholinesterase (BuChE), a non-specific cholinesterase, is present in all human and mouse tissues, and is more abundant than acetylcholinesterase (AChE) in all tissues except brain (Duysen *et al*, 2007). The use of human plasma-derived butyrylcholinesterase (HuBChE) to neutralize the toxic effects of nerve agents *in vivo* is well recognized and has been shown to both aid survival and protect against decreased cognitive function after nerve agent exposure (Lenz *et al* 2007). Recently, a commercially produced recombinant form of human HuBChE (r-HuBuChE) expressed in the milk of transgenic goats has become available. This material is biochemically similar to plasma-derived HuBuChE in *in vitro* assays. Its pharmacokinetic profile resembles that of plasma-derived HuBChE. Guinea pigs were injected with 140 mg/kg (i.m.) of pegylated r-HuBuChE 18 h prior to exposure (sc) to 5.5 × LD_{50} VX or soman. VX and soman were administered in a series of three injections of 1.5 × LD_{50}, 2.0 × LD_{50}, and 2.0 × LD_{50}, respectively, with injections separated by 2 h. Pretreatment with pegylated r-HuBuChE provided 100% survival against multiple lethal doses of VX and soman. Guinea pigs displayed no signs of nerve agent toxicity following exposure. Assessments of motor activity, coordination, and acquisition of spatial memory were performed for 2 weeks following nerve agent exposure. There were no measurable decreases in motor or cognitive function during this period. In contrast, animals receiving 1.5 × LD_{50} challenges of soman or VX and treated with standard atropine, 2-PAM, and diazepam therapy showed 50 and 100% survival, respectively, but exhibited marked decrements in motor function and, in the case of GD, impaired spatial memory acquisition. The

advances in this field have resulted in the decision to select both the plasma-derived and the recombinant form of BuChE for advanced development and transition to clinical trials.

The protective effects of BuChE were investigated in acetylcholinesterase deficient mice following exposure to chlorpyrifos oxon (Duysen *et* al, 2007). When adult female BChE+/+, −/− mice were treated transdermally on a shaved region between the shoulder blades with a non-lethal dose 14.0 mg/kg chlorpyrifos oxon dissolved in acetone, similar toxic symptoms were observed between the BChE genotypes. But when the same dose was administered to AChE−/− male and female mice, the cholinergic symptoms were more severe and all the AChE−/− mice died. Treatment with 14 mg/kg chlorpyrifos oxon resulted in almost total inhibition (99%) of blood plasma BuChE activity in the BuChE+/+ and AChE−/− mice. A significant but lesser degree of AChE inhibition was demonstrated in the BuChE+/+ (37% inhibition) and BuChE−/− (55% inhibition) mice treated with chlorpyrifos oxon. It was hoped that the BuChE−/−mouse would be a model for BChE deficiency in humans, to help define a population unusually sensitive to OP. BChE−/− mice were not more sensitive to OP toxicity than BuChE+/+ mice. This result can be explained by the high CarBE activity in mouse plasma. CarBE in mouse plasma consumes most of the OP, leaving little OP to react with BuChE or AChE. Human plasma, in contrast to mouse plasma, contains no CarBE (Li et al., 2005 as cited in Duysen *et* al, 2007). The predominant OP scavenger in human plasma is BuChE. It is predicted that BuChE deficient humans are unusually susceptible to OP toxicity. According to Padilla (2008, personal communication) there aren't enough molecules of the BuChE enzyme noting that there are lots more CarbE molecules than BuChE molecules. Furthermore, no one has actually demonstrated that animals or people low in BuChE are more sensitive to organophosphate toxicity. To protect animals against nerve gases, they have to inject lots of BuChE. Future studies with double knockout mice that have neither BuChE nor CarBE are expected to provide evidence for this issue.

4. Metabolism in Neonatal Animals

The differential sensitivity to chlorpyrifos toxicity may be attributed to the metabolic capacity of various age groups. As has been discussed previously, several enzymes act on chlorpyrifos resulting in its activation/detoxification and removal. These are the CYP450 family of enzymes, the PON-1 (A-esterase) and the B-esterases (acetyl- and BuChE and CarBE) as well as the phase 2 enzymes responsible for conjugation of the primary metabolites and their excretion from the body.

4.1. Metabolism and Toxicokinetic Studies in Pregnant Dams, Fetuses, and Post-Natal Pups

Concern for prenatal and post natal exposure to chlorpyrifos potential neurotoxicity has prompted many researchers to investigate the metabolism and toxicokinetics of chlorpyrifos and its metabolites in pregnant animals, fetuses, and post-natal pups. It is known that chlorpyrifos crosses the placenta and is distributed through

the fetal tissues including the brain. Additionally, chlorpyrifos from lactating animals partitones readily to the milk and thus is transferred to the young. A number of studies have addressed these issues and are discussed below.

4.2. Gestational Exposure (GD14-18): Measurement of TCP in rat maternal and fetal brain and liver tissues

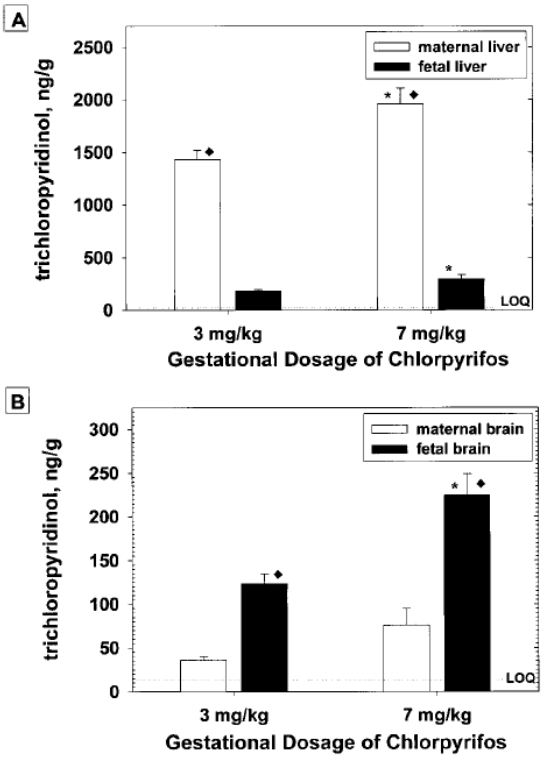
Hunter *et al*, (1999), compared the distribution of TCP in maternal and fetal brain and liver tissues following chlorpyrifos oral gavage of Long-Evans rats during late gestation (GD 14-18). In a time course study, groups of dams received 0 or 7 mg/kg of chlorpyrifos (dose volume: 1 ml/kg) on GD 14–18 and were euthanized at 2, 5, 10 (GD 18), 24 (GD 19), or 48 (GD 20) h after the last dose (*n*/dose/time point = 4). Additional dams and pups were euthanized 120 h (Postnatal Day 1, PND 1) after the last dose. The day of birth was considered PND 0. The only measurable metabolite was the TCP. The parent material and oxon metabolite were not detected (limits of quantitation: 59.2, 28.8, and 14.0 ng/g tissue for CPF, CPO and TCP, respectively). TCP maximal concentration in the maternal liver was approximately five-fold higher than the TCP concentration in fetal liver (1900 vs 400 ng/g liver weight); however, the maximal concentration of TCP in the fetal brain was two-fold higher than the TCP concentration in the maternal brain (260 vs 120 ng/g brain weight) at peak concentrations. The TCP concentration time course indicated that maximal concentrations of TCP were measured 5 h after the last dose for both maternal and fetal liver. TCP levels decreased sharply after the 5-h time point in both maternal and fetal liver and were below the LOQ 48 h postdosing in the fetus and by P1 in the dams. The half-life of the TCP was identical in the tissues examined (12–15 h).

In a dose response segment of the study by Hunter *et al*, the dams were dosed on GD14–18 by gavage with 0, 3, or 7 mg/kg chlorpyrifos in corn oil (*n* = 4/dose group). All dose response animals were euthanized 5 h after the last dose (time of peak concentration of TCP). As in the time course study TCP was the only metabolite present in measurable quantities. Maternal liver TCP concentrations were much higher than fetal liver levels for both the 3 and 7 mg/kg/day treatments: 8.0- and 6.6-fold, respectively. Conversely, brain TCP levels were much higher in the fetus than in the maternal brain (225 vs 76 ng/g in the 7 mg/kg/day group and 123 vs 36 ng/g in the 3 mg/kg/day group; see Figure 13)

The higher levels of TCP were considered not to be a result of increased accumulation within the fetal compartment, i.e., that the TCP was created in and was unable to leave the fetal compartment. If that were the case, the $T_{1/2}$ of TCP in the fetal tissues would be much longer than in the maternal tissues, but the $T_{1/2}$s for TCP in maternal and fetal tissues were not different. The study investigators theorized that because of the low levels of detoxification enzymes in the brain tissue, TCP in the brain probably arose from the binding of the oxon to cholinesterase, particularly the ChE inhibition and TCP levels in the brain peaked at the same time. The incomplete fetal brain development (fetal brain has more water and less lipid content than the maternal brain) limits partitioning of TCP from the blood. In the fetus there was no difference in

TCP concentration in the liver or brain tissue. Based on this finding, the study investigators proposed that the fetal liver does not play a significant role in detoxification and, therefore, TCP (and most likely also chlorpyrifos and chlorpyrifos-oxon) distributes evenly throughout the fetal compartment.

Figure 13: Dose response of TCP in the liver and brain (Hunter et al 1999)



In another po gestational exposure study Akhtar *et al* (2006) administered Wistar pregnant rats (20/dose) chlorpyrifos doses of 0.0, 9.6, 12 or 15 mg/kg/day from GD 0-20 and measured chlorpyrifos by gas chromatography in fetal and maternal tissues following ceasarean sectioning of fetuses on GD21. Cholrpyrifos residues were detected in both maternal and fetal tissues as reported in Table12. The doses tested varied by 1.5X maximum and it is difficult to make conclusions regarding dose relationships although the investigators saw some inconsistent dose relationships. Higher residues were found in the maternal brain than in the liver. Residue levels in the fetal tissues were highest in the liver >brain > placenta > amniotic fluid. Total residues were higher in the fetuses (0.0447 µg/g) than in the dams. There were no major malformations, but some minor anomalies such as reduced parietal ossification and absence of phalanges found significant in the high dose were not considered as compound-related effect.

Table 12: Residues levels (μg/g; mean ± SE of 3 samples) of chlorpyrifos in fetal and dam tissues (Akhtar *et al*, 2006)

Organs		Dose (mg/kg/day)			Total organ residue**
		9.6	12	15	
Dam	Brain	0.0030 ± 0.578#	0.035 ± 00.0051#	0.0603 ± 0.006#	0.0328# ± 0.333
	Liver	0.0025 ± 0.0005	0.0074 ± 0.0014	0.0115 ± 0.0003	0.0071 ± 0.001
	Amniotic fluid	0.002 ± 0.0001	0.0003 ± 0.00002	0.0003 ± 0.0001	0.0010 ± 0.0003
	Placenta	0.120 ± 0.011	0.0002 ± 0.0001	0.0006 ± 0.0001	0.040 ± 0.020
Fetus	Brain	0.0109 ± 0.011	0.0578 ± 0.0058#	0.0404 ± 0.0016#	0.0364 ± 0.333
	Liver	0.0147 ± 0.0004	0.0642 ± 0.0023#	0.0803 ± 0.0034#a	0.0531 ± 0.010
Dam	Brain + Liver	0.0028 ± 0.408	0.212 ± 0.0066	0.0359 ± 0.011	0.0120# ± 0.009
Fetus	Brain + Liver	0.0128 ± 0.001	0.061 ± 0.0031	0.0603 ± 0.009	0.0447# ± 0.016

** mean ± SE of 9 values each.
# Significant at the level of p<0.05 by three-way ANOVA.
#a p<0.05-0.01 by three-way ANOVA. Sample controls did not show chlorpyrifos residues.

4.3. Gestational & Lactational Exposure : Metabolites in milk and blood in dams, fetuses, and pups

Mattson *et al* 2000 (MRID 44648102) evaluated cholinesterase inhibition and determined chlorpyrifos and its principal metabolites in dams and pups. This study complements a developmental neurotoxicity study discussed in Section 4.0. Pregnant Sprague-Dawley CD® rats were administered chlorpyrifos by gavage at doses of 0, 0.3, 1.0, or 5.0 mg/kg/day in corn oil (1 ml/kg) beginning on gestation day (GD) 6 and continuing through lactation day 10. Five dams, as well as 5 male and 5 female pups/dose, were sacrificed on GD 20 and lactation days 1, 5, and 11 for chlorpyrifos and metabolite determinations. Milk samples were taken from the dams for chlorpyrifos and chlorpyrifos-oxon analyses. Blood samples were taken from dams and pups for chlorpyrifos, chlorpyrifos-oxon, and TCP analyses. Cholinesterase (ChE) was determined (plasma, RBC, brain, and heart) in an additional 5 dams/dose and 5 pups/sex/dose on GD 20 and lactation days 1, 5, 11, 22, and 65 (pups only). For all analyses, samples were taken from dams and fetuses 4 hours post dosing, and from pups 2 hours post dosing of the dams.

Mean concentrations of chlorpyrifos and TCP in blood as well as Chlorpyrifos concentrations in milk are given in Table 13. Mattson et al (2000) did not measure chlorpyrifos, chlorpyrifos oxon, or TCP in the brain.

As shown in Tables 13 and 14, chlorpyrifos was detected in the blood of high-dose dams at a mean concentration of 108.78 ng/g on GD 20. Levels of chlorpyrifos

then declined to 87% on lactation day 1, remained unchanged on lactation day 5, and were below the limit of detection by lactation day 11. Chlorpyrifos was detected at a low level (2.55 ng/g) in the blood of mid-dose dams only on GD 20 and was not detected at any time in the blood of low-dose dams. TCP was detected in the blood of all treated dams during the chlorpyrifos administration in a dose proportional manner, and declined to non-detectable levels in the low dose group and to very low levels upon cessation of treatment.

In milk, chlorpyrifos concentrations in the 0.3, 1.0, and 5.0 mg/kg/day groups were 20.57, 139.49, and 3022.00 ng/g, respectively, on lactation day 1 and were 13.54, 81.76, and 1533.98 ng/g, respectively on lactation day 5. By lactation day 11, chlorpyrifos was detected only in the high-dose group at a level of 19.79 ng/g. Chlorpyrifos-oxon was not detected in the blood or milk of any dams at any time point. Based on these results, Matson *et al* (2000) estimated that a pup nursing from the high dose group would consume at 0.5 mg chlorpyrifos/kg/day (assuming a rat consumes 1 mL of milk day a nd a weight of 6.1 g and a rate of growth of 0.6 g/day, and the maximum chlorpyrifos concentration in the milk of 3022 ng/g).

Blood concentrations of chlorpyrifos in male and female fetuses from high-dose dams were 52.81 and 39.40 ng/g, respectively on GD 20. Concentrations in the pups declined to less than half of the GD 20 levels by lactation day 1 and were below the limit of detection by lactation day 5. Levels of chlorpyrifos in the blood of male and female fetuses from the mid-dose dams were 0.99 and 1.19 ng/g, respectively, on GD 20, but were undetectable thereafter. Chlorpyrifos-oxon was detected in the blood of male and female fetuses from high-dose dams only on GD 20 at concentrations of 0.97 and 0.94 ng/g, respectively. TCP was detected in the blood of male and female fetuses from all dose groups in a dose dependent pattern on GD 20. Although levels of chlorpyrifos were higher in the dams on GD20 than fetuses, TCP levels in fetuses on GD20 and dams on GD20 are similar in all dose groups. TCP was essentially not detectable in the blood of low- and mid-dose pups by lactation day 5, but on lactation day 11, TCP was detected in the high-dose male and female pups at levels of 42.29 and 47.01 ng/g, respectively.

Table 13: Chlorpyrifos and TCP concentrations in blood of dams / Chlorpyrifos concentrations in milk of dams (Table 3 of Mattson *et al* 2000)

	0 mg/kg/day	0.3 mg/kg/day	1.0 mg/kg/day	5.0 mg/kg/day
Blood Chlorpyrifos (ng/g±sd)				
GD 20	NQ[2]	NQ	2.55±0.88	108.78±58.74
Lactation day 1	NQ	NQ	NQ	14.53±6.20
Lactation day 5	NQ	NQ	NQ	14.79±10.64
Lactation day 11	NQ	NQ	NQ	NQ
Blood TCP (ng/g±sd)				
GD 20	NQ	114.40±23.87	322.04±13.10	1974.00±472.26
Lactation day 1	NQ	111.46±26.07	394.54±65.23	2718.00±1509.00
Lactation day 5	NQ	142.93±37.37	536.5358.16	1449.92±95.27
Lactation day 11	NQ	NQ	9.87±5.08	71.40±22.60
Chlorpyrifos concentrations in milk of dams (ng/g±sd)				
GD 20	N/A[1]	N/A	N/A	N/A
Lactation day 1	NQ[2]	20.57±8.47	139.49±35.54	3022.00±1153.63
Lactation day 5	NQ	13.54±3.88	81.76±6.62	1533.98±192.24
Lactation day 11	NQ	NQ	NQ	19.79±6.52

[1]N/A = not applicable; milk samples not obtained on GD 20.
[2]NQ = not quantified; below limit of detection 0.7 ng/g.

Table 14: Chlorpyrifos, Oxon and TCP concentrations in blood of fetuses and pups (Mattson *et al* 2000)

	0 mg/kg/day	0.3 mg/kg/day	1.0 mg/kg/day	5.0 mg/kg/day	0 mg/kg/day	0.3 mg/kg/day	1.0 mg/kg/day	5.0 mg/kg/day
	Males				Females			
Chlorpyrifos (ng/g±sd)								
GD 20	NQ[1]	NQ	0.99±0.41	52.81±25.23	NQ	NQ	1.19±0.32	39.40±12.99
LD1	NQ	NQ	NQ	18.17±24.64	NQ	NQ	NQ	6.61±8.04
LD5	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
LD11	NQ	NQ	NQ	NQ	NQ	NQ	NQ	NQ
Chlorpyrifos Oxon (ng/g±sd)								
GD20	NQ	NQ	NQ	1.0 ± 0.6	NQ	NQ	NQ	0.9 ± 0.4
TCP (ng/g±sd)								
GD20	NQ	93.93±14.47	361.00±64.11	1680.00±241.35	NQ	99.49±13.67	339.13±93.75	1884.00±234.05
LD1	NQ	NQ	137.31±85.65	842.67±293.51	NQ	50.03±27.51	133.90±27.74	433.29±262.82
LD5	NQ	NQ	NQ	47.37±18.81	NQ	NQ	NQ	50.23±50.23
LD11	NQ	NQ	NQ	42.29±8.94	NQ	NQ	9.50±2.69	47.01±47.01

4.4. Gestational exposure: Effects on dams and the fetuses for ChE, CarbE and chlorpyrifos oxonase activity

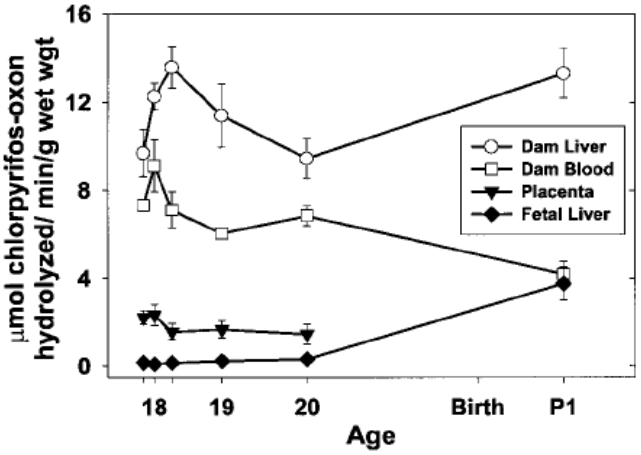
Two important groups of important enzymes in the detoxification of chlorpyrifos are the A-esterases, known as paraxonase (PON1) and the carboxylesterases (CarbE). PON1 are calcium activated enzymes and are distributed in various tissues including the liver, brain and blood. PON1 hydrolyzes the chlorpyrifos oxon. The design of a mouse strain lacking PON1 (knockout mouse) in 1998 accelerated the understanding of the role of this enzyme in chlorpyrifos toxicity. The extensive genetic variability among the human population in PON1 activity may have an impact on the metabolism and detoxification of chlorpyrifos. The population variability in PON1 is discussed extensively in Section 5 of this paper. Levels of PON1 have been shown to be lower in juvenile rats and newborn humans than adult levels.

It has also been demonstrated that chlorpyrifos oxon binds irreversibly to other non-target esterases, namely carboxylesterase (CarbE), a B-esterase. CarbEs are considered to act as a scavenger of the chlorpyrifos oxon. This binding has been described to be stoichometric. CarbEs are distributed among different issues (liver, blood (not in humans), lung, brain) with highest abundance in the liver. Significant individual variability in CarbEs in human liver microsomes with 5.3-44.7 fold difference depending on the substrate used for assaying its activity has been reported. There is also great variability in CarbEs between neonatal and adult animals. Thus, CarbE from fetal tissues was less sensitive (inhibited) to chlorpyrifos oxon than CarbE from adult tissues and the level of CarbEs in neonatal rats are significantly lower than in adult tissue. These differences in CarbE levels and sensitivity may impact the toxicity of chlorpyrifos on the growing fetus, since it has been also demonstrated that chlorpyrifos and its metabolites are carried through the palcenta to the fetus.

Lassiter *et al*, 1998 investigated selected toxicokinetic and toxicodynamic factors surrounding the toxicity of chlorpyrifos in pregnant rats dosed repeatedly or singly during late gestation. Dams were dosed daily by gavage with chlorpyrifos in corn oil (0 or 7 mg/kg; 1ml/kg) on GD 14 to 18. Animals were euthanized 2 to 120 h after the last dose and tissues were collected from dams, fetuses and pups for ChE, CarbE and chlorpyrifos oxonase (i.e., PON1) activity. Based on these analyses, it was found that (1) the maximal ChE inhibition time was the same (i.e., 5–10 h after dosing) for both maternal and fetal brain, (2) the degree of fetal brain ChE inhibition was 4.7 times less than maternal brain inhibition, and (3) the detoxification potential (i.e., carboxylesterase and chlorpyrifos-oxonase) of the fetal tissues was very low compared to the maternal tissues. Late gestational ontogeny of chlorpyrifos-oxonase activity is illustrated in Figure 2.3. The age-dependent changes in chlorpyrifos-oxonase activity in the maternal and fetal/neonatal tissues occurred primarily after birth. Maternal liver had the most activity, and the time course of this activity was variable during the late pregnancy, but ultimately demonstrated a slight postpartum increase. Maternal blood chlorpyrifos-oxonase activity was variable before birth, but decreased on the day after birth. The placenta had about 1/5 the activity of the liver which was consistent during late pregnancy. Fetal liver exhibited minimal activity during gestation, but the postnatal day 1 pup liver showed an eightfold increase in chlorpyrifos-oxonase activity. Maternal and fetal brains had no detectable chlorpyrifos-oxonase activity. The dosed animals showed marked inhibition of CarbE activity. Maternal liver CarbE activity was less than 18% of control 2 to 10 h after the last chlorpyrifos dose. Fetal liver CarbE activity was less than 50% of control values for the entire time course postdosing. Fetal liver CarbE did not recover to control levels, but the maternal liver CarbE activity was recovering by the 5-day time point *(i.e*., postnatal day one). Fetal brain CarbE activity was not inhibited in the chlorpyrifos-exposed subjects, but maternal brain activity was approximately 70% of control across the entire time course.

These are illustrated in Figures 14, 15 and 16. Pregnant dams receiving only one oral dose of 7 or 10 mg/kg chlorpyrifos on GD18, the degree of ChE inhibition in the fetal brain was comparable to the maternal brain ChE inhibition. Taking into consideration the net increase (more than fourfold) in fetal brain ChE activity from GD14 to 18 in control animals, and the fact that maternal brain ChE was inhibited more than fetal brain ChE only in a repeated dosing regimen, the study investigators concluded that the fetus is not genuinely protected from the toxic effects of a given dose of chlorpyrifos, but fetal brain ChE is simply able to recover more fully between each dose as compared to maternal brain ChE, giving the illusion that the fetal compartment is less affected than the maternal compartment. Liver carboxylesterase activity in chlorpyrifos exposed dams was less than 18% of control 2 to 10 h after the last dose. Fetal liver carboxylesterase was less than 50% of control values for the entire time course postdosing. Fetal liver carboxylesterase did not recover, but the maternal liver carboxylesterase activity was recovering by the 5-day time point (i.e., P1). Fetal brain carboxylesterase activity was not inhibited in the chlorpyrifos-exposed subjects, but maternal brain activity was approximately 70% of control activity across the entire time course.

Figure 14: Late gestational ontogeny of chlorpyrifos-oxonase activity



Brain, either maternal or fetal, had no detectable activity (not shown), Lassiter *et al*, 1998.

Fetal and maternal liver, blood and brain CarbE activity is presented in Figure 2.4A-C. In the liver, the major site of chlorpyrifos metabolism, fetal liver carboxylesterase was less than 50% of control values for the entire time course post dosing. Fetal liver carboxylesterase did not recover, but the maternal liver carboxylesterase activity was recovering by the 5-day time point (i.e., P 1). There was no inhibition of carboxylesterase in either placental or maternal blood, suggesting that no protective detoxification was provided by carboxylesterase in the placental or maternal blood. In the brain, fetal carboxylesterase activity was not inhibited in the chlorpyrifos-exposed subjects, but maternal brain activity was approximately 70% of control activity across the entire time course.

Figure 15: Fetal and maternal brain, placenta and liver cholinesterase inhibition after repeated dosing with 7 mg/kg chlorpyrifos (Lassiter *et al*, 1998)

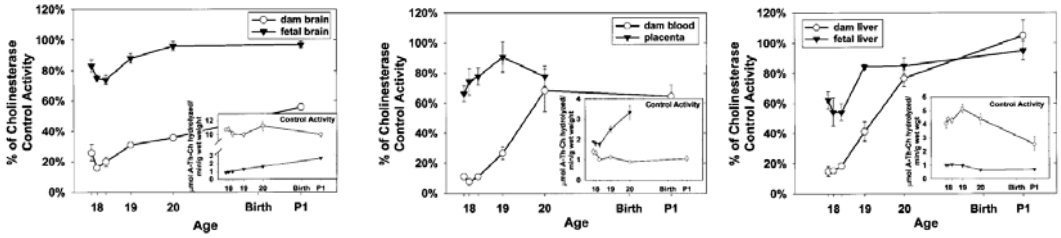
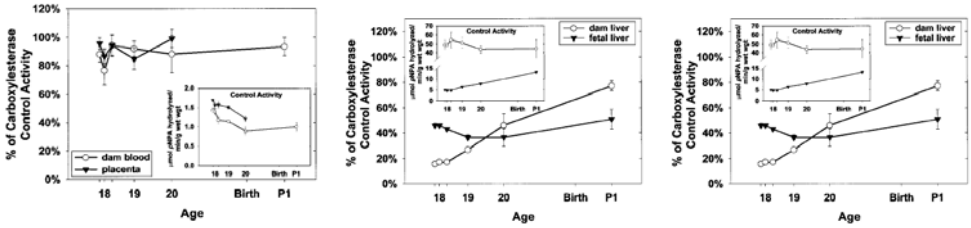


Figure 16: Fetal and maternal brain, placenta and liver carboxylesterase inhibition after repeated dosing with 7 mg/kg chlorpyrifos (Lassiter *et al* 1998)



Further work by Lassiter *et al* (1999), showed the importance of the enzyme CarbE for fetal exposure to chlorpyrifos. Using a similar experimental design as in their previous studies, Long-Evans pregnant rats were orally administered 0, 3, 5, 7 or 10 mg/kg of chlorpyrifos on GD 14 -18 and sacrificed 5 hours after the last dose. Following gestational exposure to chlorpyrifos, there was more CarbE inhibition in the maternal liver than in the fetal liver: maternal liver activity was less than 18% of the control activity at all doses; fetal liver CarbE activity was also maximally inhibited at all dosages, but with less severity than maternal liver (45-50% of control activity). Maternal brain CarbE was inhibited in a dose dependent manner (100, 85, 71 and 65% of control CarbE activity at 3, 5, 7 and 10 mg/kg of chlorpyrifos), where as fetal brain CarbE activity showed no inhibition at any dosage. Placental and maternal blood CarbE activities were not inhibited by repeated gestational exposures even at 10 mg/kg. The control levels of CarbE activities were age and tissue dependent as illustrated in table 2.6, below. The disparate pattern of CarbE inhibition related above may be partially explained by the IC_{50} and inhibitor resistant esterase (IRE) pattern related in Table 15. For example, fetal brain CarbE activity is not sensitive at all to inhibition by chlorpyrifos oxon with an IC_{50} approximately 1000 fold higher than either maternal liver or brain-this insensitivity explains the lack of inhibition in that compartment.

Table 15: CarbE activity in maternal and fetal tissues (Table 2. Lassiter *et al* 1999)

Tissue	Control activity[a]	IC_{50} ± S.E.	% IRE[b]
Maternal blood	1.8±0.1	Not determined	Not determined
Maternal liver	102.6±27.2	3.1 nM±0.2	16%
Maternal brain	4.6±0.3	26.5nM±0.8	49%
Placenta	2.4±0.1	19.9nM±0.8	53%
Fetal liver	4.4±0.4	0.9nM±0.2	38%
Fetal brain	2.2±0.1	19.1µM±2.1	Not determined

[a] micromoles of *p*-nitrophenol hydrolyzed/min/g of wet weight or mL
[b] inhibitor resistant esterase, i.e. percentage of CarbE activity resistant to inhibition to chlorpyrifos oxon, *in vitro.*

4.5. Post-natal TK and metabolism

As discussed above, the metabolism of chlorpyrifos involves CYP450 activation and detoxification to chlorpyrifos oxon and TCP, as well as AChE and BuChE, CaE, and A-esterase (PON-1) detoxification of the chlorpyrifos oxon to TCP. Newborn and juvenile rats are more sensitive to AChE inhibition caused by chlorpyrifos than adult rodents, not because of a difference in the affinity of chlorpyrifos oxon to AChE, but because the enzymes that detoxify chlorpyrifos oxon have not yet fully developed (Iyer, 2001). The activity levels of P450s and PON1 differ between the adult and infant or conceptus. For example, CYP3A7 makes up about 30% of all the P450s in the fetus, while the level of CYP3A4 is low (Hakkola *et al.*, 2001). In contrast, the level of CYP3A7 is extremely low in adults, while the level of CYP3A4 is very high (Hakkola *et al.*, 2001). In humans, the serum levels of PON1 are lower in infants up to 6 months old than levels in adults (Ecobichon and Stephens, 1973). Similarly, compared to adults, PON1 activity levels were lower in young rats up to 25 days old and in young mice up to 20 days old (Li *et al.*, 1997).

Aterrberry *et al* (1997) investigated several enzymatic activities that are important in the metabolism and toxicity of chlorpyrifos and parathion (as model compounds for the phosphorothionate insecticides) in male rats ranging in age from 1 to 80 days. These parameters included (1) activities of the target enzyme AChE in cerebral cortex and medulla oblongata/pons, (2) *in vitro* sensitivities of cortical and medullary AChE to inhibition by paraoxon or chlorpyrifos-oxon, (3) hepatic aliesterase activities and *in vitro* sensitivities to paraoxon and chlorpyrifos-oxon inhibition, (4) hepatic microsomal P450 levels and activation and detoxication of parathion and chlorpyrifos, (5) hepatic microsomal ethoxyresorufin *O*-deethylase (EROD) and pentoxyresorufin *O*-dealkylase (PROD) activities as indices of CYP1A and CYP2B enzymes, respectively, and (6) AChE and aliesterase inhibition following *in vivo* exposure of rats to parathion or chlorpyrifos. Brains or livers were removed from male rats 1, 3, 12, 33, or 80 days and processed for assaying the various enzymatic activities described. Results obtained from this investigation showed that specific activities of acetylcholinesterase in cerebral cortex, but not medulla oblongata and of liver aliesterases increased with age, indicating the presence of both more target esterases and more protective esterases, respectively, in the adult compared to the juvenile animal. Sensitivity of the brain acetylcholinesterase

to inhibition by paraoxon and chlorpyrifos oxon, as measured by IC50 values, did not change significantly with age, whereas the hepatic aliesterase sensitivity to inhibition decreased with age. Progressive increases in activities of P450-mediated activation (desulfuration) (6- to 14-fold) and detoxication (dearylation) (2- to 4-fold), as well as concentrations of P450 (7-fold) and protein (2-fold), were observed between neonate and adult hepatic microsomes. Microsomal pentoxyresorufin *O*-dealkylase activity followed a developmental pattern similar to desulfuration and dearylation, displaying a 16-fold increase between neonates and adults. However, microsomal ethoxyresorufin *O*-deethylase activity increased until 21 days of age, displaying a 16-fold increase, then decreased in adulthood to a level 10-fold higher than neonates. These results indicate that target enzyme sensitivity is not responsible for age-related toxicity differences, nor is the potential for hepatic bioactivation, whereas lower levels of hepatic aliesterase-mediated protection and P450-mediated dearylation probably contribute significantly to the greater sensitivity of juveniles to phosphorothionate toxicity.

In a second portion of study by Aterrberry *et al* (1997)**,** 3 rats of each age group were injected a parathion dose of 4 mg/kg (1/2 of the published LD_{50}) or a chlorpyrifos dose of 80 mg/kg (1/2 of the published LD_{50}) in 1 ml corn oil/kg body weight. Rats were sacrificed after 2 hours or if the animal died before the sacrifice time brain and liver were removed for brain AChE assay and liver aliesterase activities. A decreasing AChE inhibition was observed with increases in age following treatment with either compound. More than 90% inhibition of AChE activity occurred in the rats treated with either insecticide at 1, 3, and 12 days of age, only moderate inhibition (about 50%) in the 33 day old rats and little or no inhibition in the 80 day old rats. Despite the fact that the AChE activity in the cerebral cortex in insecticide-treated rats showed increases with age, all activities were still significantly lower than controls, except following parathion exposure at 80 days of age. Aliesterases were also substantially inhibited (more than 90%) following treatment with either insecticide in the 1, 3, 12 and 33 day old rats with no significant differences among these groups. The aliesterase activity at the 80 day age was significantly higher than the activities of the younger ages following treatment with both insecticides, but rats treated with chlorpyrifos still displayed substantial inhibition; aliesterase activity at 80 days was more inhibited than AChE activity. The times to death, also indicated an age-related difference. Following parathion treatment, the rats of ages 1, 3, and 12 days all died on average 55 min later. The rats of 33 and 80 days of age displayed no overt signs of toxicity. Rats of ages 1, 3, and 12 days treated with chlorpyrifos all died an average of 90 min after treatment and, like the parathion-treated rats, the two older ages displayed no overt signs of toxicity.

Timchalk *et al* (2006) investigated the toxicokinetics of chlorpyrifos, TCP, and the extent of blood (plasma/RBC), and brain ChE inhibition in rats on postnatal days (PND)-5, -12, and -17 following oral gavage administration of 1 or 10 mg chlorpyrifos/kg of body weight. In all preweanling ages the blood TCP exceeded the chlorpyrifos concentration, and within each age group there was no evidence of non-linear kinetics over the dose range evaluated. At both dose levels the pharmacokinetic data suggested that CPF was rapidly absorbed and metabolized since peak blood levels of both CPF and its major metabolite TCP were observed between 3 and 6 h postdosing at

all preweanling ages. Based on the blood conentrations of chlorpyrifos and TCP seen in Table16, the data suggest that older pups (PND 17) have slightly faster metabolism of the absorbed dose than the younger pups (PND 5) since TCP concentrations were higher. In addition, the increase in the blood TCP concentration (~3-fold) in PND-17 rats relative to the response in the younger rats, are consistent with an increase in CYP450 metabolic capacity with age. Younger animals demonstrated a greater sensitivity to ChE inhibition as evident by the age-dependent inhibition of plasma, RBC, and brain ChE. The brain sensitivity of younger animals (i.e. PND-5) was attributed to substantially lower levels of ChE activity relative to later preweanling stages and adults. The CYP450 metabolic capacity was apparently adequate to metabolize chlorpyrifos to both TCP and chlorpyrifos oxon based on the detection of TCP in blood and extensive ChE inhibition (biomarker of chlorpyrifos oxon) at all ages. The results of this study suggest that in the preweanling rat, chlorpyrifos was rapidly absorbed and metabolized, and the extent of metabolism and ChE inhibition was age-dependent.

Table 16: Chlorpyrifos and TCP concentrations (μmol/L) in the blood of PND 5, 12, and 17 pups (Timchalk *et al*, 2006)*

Chlorpyrifos				
Chemical	**Timepoint**	**PND5**	**PND12**	**PND17**
1 mg dose	3 hr 6 hr 24 hr	0.07 0.04 0.03	0.04 0.04 0.01	0.02 0.06 -
10 mg dose	3 hr 6 hr 24 hr	0.80 0.35 -	0.60 0.90 0.08	0.60 0.30 0.08
TCP				
1 mg dose	3 hr 6 hr 24 hr	3.5 4.0 3.5	3.5 2.2 2.0	1.9 2.6 0.8
10 mg dose	3 hr 6 hr 24 hr	9.0 30.0 4.0	20.0 20.0 9.0	37 58 10.0

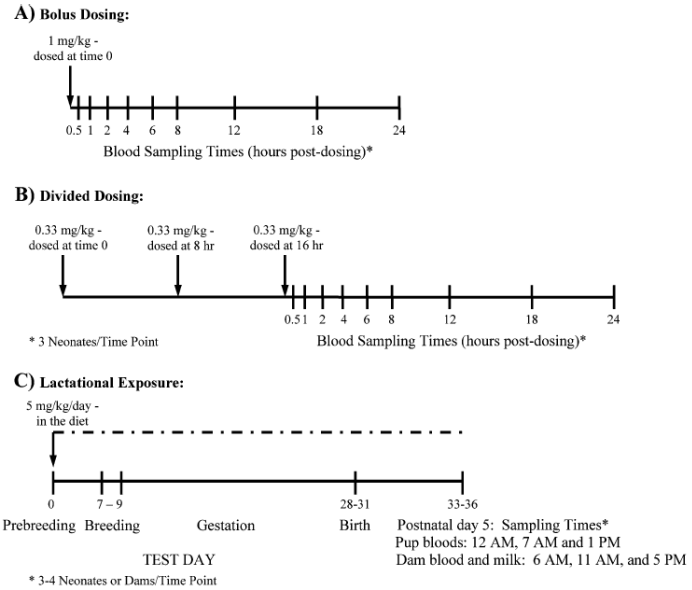
* The concentration values were obtained from Figures 2, 3 and 4 of Timchalk *et al,* 2006

Studies evaluating effects of chlorpyrifos in pregnant dams, fetuses or PND pups use a variety of routes of exposure. The two most often used are oral gavage, particularly in corn oil, and subcutaneous injection with DMSO. It is difficult to compare studies conducted by these different methods given that the toxicokinetics of each route may differ. Marty *et al* (2007) investigated the effect of route, vehicle, and divided doses on the pharmacokinetics of chlorpyrifos and its metabolite trichloropyridinol in neonatal rats. Male CD (Sprague-Dawley derived) rats at PND 5 were dosed with chlorpyrifos (1 mg/kg) using different routes of exposure, vehicles, and single versus divided doses. Groups included were:

- single gavage bolus versus divided gavage doses in corn oil (one vs. three times in 24h),
- single gavage bolus versus divided gavage doses in rat milk, and
- sc administration in dimethyl sulfoxide (DMSO)
- lactational exposure of PND 5 pups from dams exposed to CPF in the diet at 5 mg/kg/day for 4 weeks.

Blood concentrations of chlorpyrifos and TCP were measured at multiple times through 24 h. There were three neonates/time point for the direct dosing groups and 3-4 neonates for the indirect (lactational) dosing groups. Brain concentrations were not measured in the route comparison study. The experimental design of the study is depicted in Figure 17.

Figure 17: Diagram depicting the experimental designs used in this study (Marty *et al*, 2007)



PND 5 pups were directly exposed to CPF (1 mg/kg/day) as a bolus oral dose in milk or corn oil or as a bolus dose via sc injection (A) or as a divided dose (0.33 mg/kg three times per day) in milk or corn oil (B). Pup blood was collected at 0.5, 1, 2, 4, 6, 8, 12, 18, and 24 h after the last dose. In the lactational experiments (C), PND 5 pups were exposed to CPF via lactating dams receiving 5 mg/kg/day CPF in the diet. Dams were at steady state, and dam blood and milk samples were collected at 6:00 A.M., 11:00 A.M., and 5:00 P.M. Pup blood was collected at 12:00 A.M., 7:00 A.M., and 1:00 P.M. n ¼ 3-4 pups per time point per dosing scenario

Chlorpyrifos and TCP in the blood and milk of dams exposed to dietary chlorpyrifos (5 mg/kg/day for 6–7 weeks) and pup blood levels for lactationally exposed offspring are summarized in Table 17. Chlorpyrifos was not detected in the blood, but was detected in the milk at relatively steady amount. TCP was detected in the neonate and dam blood with higher values in the dams (6-23 fold). Dams used for the lactational exposures were exposed to dietary chlorpyrifos during the prebreeding, breeding, gestation, and lactation periods and, therefore, were considered to be at steady-state levels for chlorpyrifos. Table 18 summarizes the pharmacokinetics of chlorpyrifos and TCP following the different routes of exposure. An interesting observation shown in

Table 18 is that for PND5 rat pups, the kinetic properties for DMSO subcutaneous injection are more similar to exposure in milk than exposure from corn oil gavage for each property evaluated for chlorpyrifos and TCP (Cmax, AUC, ½ life, time to peak concentration). The time of maximum concentration for chlorpyrifos and TCP appeared to be similar among the administration methods (Table 18). The ½ life for chlorpyrifos was somewhat shorter for a single dose via corn oil compared to the other methods. For TCP, the ½ life was shortest after single oral administration in corn oil and almost twice through rat milk administration route. For a single dose of corn oil, the the maximum concentration (Cmax) of chlorpyrifos was approximately 7-fold higher than other methods but the AUC was only 2-fold higher. For chlorpyrifos, the Cmax values for the different routes of exposure were similar with very little variation except for corn oil gavage. For TCP, the area under the curve (AUC) and ½ lifes were somewhat higher for a single dose via corn oil but only 2-fold or less than the other methods.

Table 17: Chlorpyrifos and TCP Concentrations in the Blood and Milk of Dams Exposed to 5 mg/kg/day chlorpyrifos in the Diet and Pup Blood Levels Following Lactational Exposure (PND 5) (Marty *et al* 2007)

Sample time	Animal	Chlorpyrifos ng/g blood	TCP ng/g blood	Chlorpyrifos ng/g milk	Estimated chlorpyrifos ng/g blood[a]
12:00 A.M.	Neonates[b]	NQ[c]	48.63 ± 3.09	-	
6:00 A.M.	Dam[d]	NQ[e]	280.86	111.42	1.1
7:00 A.M.	Neonates	NQ	54.93 ± 2.10	-	
11:00 A.M.	Dam	NQ	1262.83	117.08	1.1
1:00 P.M.	Neonates	NQ	71.11 ± 8.00	-	
5:00 P.M.	Dam	NQ	566.40	127.28	1.1

[a]Estimated to be 1.1 ng chlorpyrifos /g blood assuming milk:blood partitioning of 104 from Mattsson et al. (2000).
[b]n =3–4 neonates per group; mean ± SD for TCP blood concentrations.
[c]Nonquantifiable; LOQ in neonatal blood ranged from 3.24 to 3.70 ng chlorpyrifos/g blood.
[d]n = 1 dam/group.
[e]Nonquantifiable; LOQ in dam blood ranged from 3.56 to 4.43 ng chlorpyrifos /g blood.

Table 18: Effect of Dose Rate, Vehicle, and Exposure Route on PK Parameters in PND 5 CD Pups Directly Exposed to 1 mg/kg/day chlorpyrifos (Marty *et al* 2007)

Vehicle	Dose route	Dose frequency	Chlorpyrifos					TCP				
			Tmax	Cmax		AUC	$T_{1/2}$	Tmax	Cmax		AUC	$T_{1/2}$
			h	Ng/ml	µmol/l	ng h/ml	h	h	Ng/ml	µmol/l	ng h/ml	h
Corn oil	oral	Single	2	48.7 ± 30.8	0.139	160	3.0	4	320 ± 112	1.53	2935	4.9
Rat milk	oral	Single	2		0.025	72	8.3	4	194 ± 120	0.93	2214	8.1
Corn oil	oral	Split	2	8.6 ± 1.0	0.020	ND	ND	4	112 ± NA[e]	0.54	ND	6.3
Rat milk	oral	Split	2	6.9 ± 0.7	0.016	ND	ND	4	100 ± 33	0.48	ND	5.0
DMSO	sc	single	2	5.6 ± 1.2 9.5 ± 2.7	0.03	82	8.3	6	171 ± 90	0.82	1754	6.7

Dose frequency: single dose of 1 mg chlorpyrifos/kg or three doses of 0.33 mg chlorpyrifos/kg administered 8 h apart.
Mean concentration ± SD.
AUC: Calculated from 0 to 24 h after last administered dose.
ND: Not determined.
NA : n=2

Marty *et al* (2007) also performed a limited time course study of rat pups injected subcutaneously with 1 mg/kg ^{3}H-chlorpyrifos in DMSO and euthanized at 5, 10, 15, 30, 60, or 120 min after injection. Over the 2-h monitoring period, > 90% of administered dose of radiolabel remained at the injection site and in the carcass. To illustrate the importance of kinetic data, including internal dosimetry, to put various dosing regimens and their toxicological outcome into perspective, pharmacokinetic data reported in this study was compared to data from previously published studies. This is illustrated in Table 19 where rows A–D illustrate chlorpyrifos and TCP levels measured in dams' blood, milk, and pup blood after dosing dams with 0.3–5 mg/kg/day chlorpyrifos by bolus gavage in corn oil or 5 mg/kg/day in the diet. Rows E–G illustrate that direct bolus dosing of neonatal rat pups with 1 mg/kg/day chlorpyrifos in any vehicle exceeded the chlorpyrifos and TCP blood levels achieved when pups were exposed lactationally following maternal exposures up to 5 mg/kg/day.

Table 19: PK Summaries from Various Studies Examining chlorpyrifos and TCP in Neonatal Rat Pups on PND 5 (reproduced from Marty *et al* 2007)

	End point	CPF in maternal blood (LD 5)	TCP in maternal blood	ChE inhibition[a] (dams on LD 5)	CPF in milk	Milk CPF ratio to 5 mg/kg gavage to dam	Estimated CPF exposures via milk	Pup exposure relative to dam	CPF in pup blood (Cmax)	CPF in pup blood (AUC)	TCP in pup blood (Cmax)	TCP in pup blood (AUC)	ChE inhibition[a] (pups on LD 5)
A	Gavage to dam: 0.3 mg/kg in corn oil	NQ at 4 h[b] (estimated 0.13 ng/g from milk data)	123 ng/g	Yes	13.5 ng/g[c]	0.009	0.003 mg/kg/day[d]	0.01	NQ[b]—2 h[e]	—	NQ[f]—2 h[e]	—	No
B	Gavage to dam: 1.0 mg/kg in corn oil	NQ at 4 h[b] (estimated 0.8 ng/g from milk data)	418 ng/g	Yes	81.8 ng/g[c]	0.05	0.017 mg/kg/day[d]	0.017	NQ[b]—2 h[e]	—	NQ[f]—2 h[e]	—	No
C	Diet given dam: 5.0 mg/kg/day	NQ (estimated 1.1 ng/g from milk data)	281–1263 ng/g	Yes[g]	111–127 ng/g	0.08	0.024 mg/kg/day[d]	0.005	NQ[h]	—	49–71 ng/g[i]	—	?[j]
D	Gavage to dam: 5 mg/kg/day in corn oil	14.8 ng/g at 4 h	2048 ng/g	Yes	1534 ng/g[c]	1	0.1 mg/kg/day[k]	0.02	NQ[b]—2 h[e]	—	49 ng/g – 2 h[e]	—	Residual from *in utero* exposure
E	Neonatal sc injection: 1 mg/kg in DMSO	NA	NA	NA	NA	NA	NA	NA	9.5 ng/ml—2 h	82 ng h/ml[l]	171.3 ng/ml—6 h	1754 ng h/ml[l]	Yes[m]
F	Neonatal gavage (bolus): 1 mg/kg in rat milk	NA	NA	NA	NA	NA	NA	NA	8.4 ng/ml—2 h	90.1 ng h/ml[l]	194 ng/ml—4 h	2577 ng h/ml[l]	Not evaluated
G	Neonatal gavage (bolus): 1 mg/kg in corn oil	NA	NA	NA	NA	NA	NA	NA	47.5 ng/ml—2 h	125.5 ng h/ml[l]	320 ng/ml—4 h	3087 ng h/ml[l]	Yes[n]

Note. LD, lactation day.
[a]Plasma and/or red blood cell cholinesterase (ChE) inhibition at 4 h postgavage dosing of dams; Mattsson *et al*. (2000).
[b]Nonquantifiable with LOQ = 0.7 ng/g; Mattsson *et al*. (2000).
[c]Milk samples collected 4 h after dosing the dam via gavage, the time of peak blood concentrations in treated dams.
[d]Calculated based on concentration of CPF in milk × 2.35 ml milk consumed on PND 5 × 1/bw (~11.5 g).
[e]Peak blood levels for CPF were estimated to be at 4 h postexposure in dams.
[f]Nonquantifiable with LOQ = 10 ng/g; Mattsson *et al*. (2000).
[g]Predicted response based on results from other studies cited in the table.
[h]Nonquantifiable with LOQ = 3.2–3.7 ng/g (current study).
[i]Peak time cannot be determined due to continuous exposure of pups via milk ingestion from CPF-exposed dams.
[j]ChE inhibition is uncertain; pups from dams given 5 mg/kg/day by gavage had ChE inhibition with lower TCP values, but TCP values were likely underestimated (samples were collected at 2 h; peak at 4 h).
[k]Mattsson *et al*. (2000).
[l]Calculated from zero to infinity.
[m]Song *et al*. (1997) with multiple doses.
[n]Timchalk *et al*. (2006).

Mortensen *et al* (1996) measured chlorpyrifos oxonase activity (PON1 activity) in the brain, plasma, and liver of male, postnatal day 4 (PND4) and adult (PND90) Long-Evans rats. No brain chlorpyrifos oxonase activity was detected in pups or adults. Plasma and liver chlorpyrifos oxonase activities were markedly lower at PND4 compared to adult: PND4 plasma and liver chlorpyrifos oxonase activities were 1/11 and 1/2 the adult plasma and liver activities, respectively. Because the K_m of chlorpyrifos oxonase activity was high (i.e., 210-380 µM), it was important to determine if this chlorpyrifos oxonase activity could hydrolyze physiologically relevant concentrations (i.e., nM to low µM) of chlorpyrifos oxon. This was accomplished by comparing the shifts in the tissue acetylcholinesterase (AChE) IC_{50} for chlorpyrifos oxon in the presence or absence of chlorpyrifos oxonase activity. An increase in the "apparent" IC_{50} is expected if chlorpyrifos oxonase hydrolyzes substantial amounts of chlorpyrifos oxon during the 30 minutes the tissue is preincubated with the chlorpyrifos oxonase. In the adult, both plasma and liver AChE apparent IC_{50} values were higher in the presence of chlorpyrifos oxonase activity, suggesting that the chlorpyrifos oxonase in those tissues was capable of hydrolyzing physiologically relevant concentrations of chlorpyrifos oxon within 30 minutes. In young animals, however, there was less of a shift in the IC_{50} curves compared to the adult, confirming that the young animal has less capacity than the adult to detoxify physiologically relevant concentrations of chlorpyrifos oxon via chlorpyrifos oxonase.

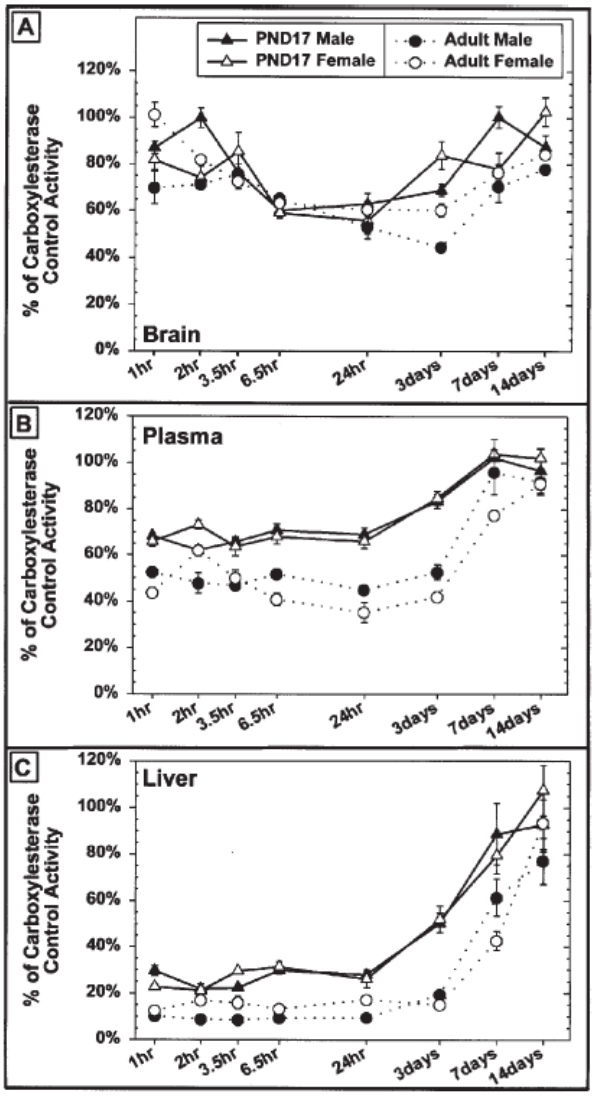
Chanda *et al*. (2002) studied the developmental profiles of CarbE. Liver, plasma and brain CarbE activities were measured in male and female Long Evans rats in the fetus (GD 18) and on postnatal days 17 pups and adults (~80 to 90 days). There was no plasma CarbE activity in the fetus, and it was 5 times lower in the pups compared to the adults. The liver CarbE activity was 13 times lower in the fetus compared to the pups and 50-90 times lower than in the adults. Similarly, the brain CarbE activity showed a similar trend as shown in Table 20. An interesting finding was that pregnant females had lower CarbE activity compared to non pregnant females. Enzyme levels were compared against the sensitivity of young rats to acute chlorpyrifos exposure at various ages; during development, an inverse relationship between the enzyme activities and sensitivity to chlorpyrifos toxicity was observed (Figure 18). It was concluded that a lack of these detoxifying enzymes in young rats could at least partially explain their increased sensitivity to chlorpyrifos.

Table 20: Carboxylesterase activity in control tissues

	Fetus	P17 Female	P17 Male	Pregnant Female	Adult female	Adult Male
Plasma	ND	0.3±0.01	0.3±0.01	ND	1.3±0.1	1.4±0.1
Liver	4.4±0.4	57.0±8.4	57.0±4.5	102.6±27.2	230.5±5.2	413.7±11.2
Brain	2.2±0.1	5.3±0.2	6.2±0.4	4.6±0.3	17.1±0.4	14.6±0.4

All data are expressed as µmoles substrate (*p*-nitrophenyl acetate) hydrolyzed/min/g wet weight (Chanda *et al*, 2002).

Figure 18: Time course of carboxylesterase activity in brain plasma and liver of female and male PND17 and adult rats following in vivo chlorpyrifos exposure



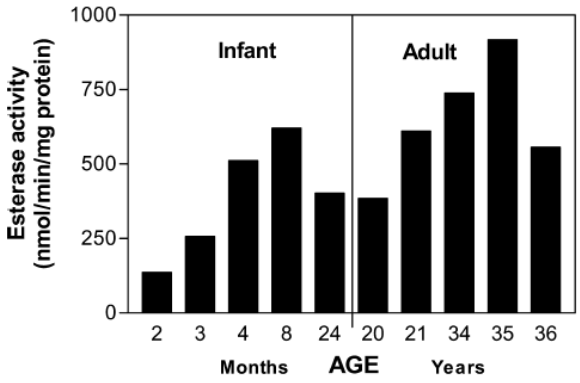
The rats received a single age-appropriate oral maximum tolerated dosage of chlorpyrifos: 15 or 80 mg/kg for PND17 or adult rats, respectively. There was no apparent effect of gender on the time course of carboxylesterase in these tissues. The carboxylesterase activity in the adult liver was more severely inhibited than any other tissue (Chanda et al, 2002).

The CarbE and A-esterase activities were also evaluated during maturation by Karanth and Pope (2000). Sprague Dawley rats of various ages were administered chlorpyrifos subcutaneously at the maximum tolerated doses (45, 127, 279, 279 mg CPF/kg bw for neonatal, juvenile, adult and aged, rats respectively). The CarbE and aliesterase (AE) activities in the liver, plasma and lung of neonatal (7days), juvenile (21 days), adult (3 months) and aged (24 months) were evaluated using *p*-nitrophenyl acetate as a substrate for CarbE and chlorpyrifos oxon or paraoxon as substrates for the AE activities. Neonatal and juvenile rats were more sensitive than adults to the acute lethality of chlorpyrifos. Levels of CarbEs and AEs in neonatal and juvenile rats were

significantly lower than in adult tissues. There were no significant age-related differences in *in vitro* sensitivity of CarbEs to either chlorpyrifos oxon in any tissues. In general, acute sensitivity (i.e, the maximum tolerated dose) was highly correlated with age-related differences in both esterase activities across all 3 tissues with chlorpyrifos. The authors conclued that both CarbE and A-esterase activities can be correlated with acute sensitivity to chlorpyrifos, but that age-related differences in CarbE activity are probably more important in differential toxicity.

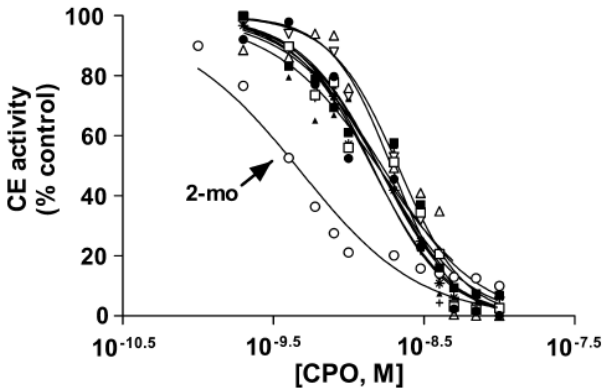
Recent work by Pope *et al* (2005) suggested that human liver CarbE expression changes relatively little in comparison to rodents. Human liver CarbE activity was measured in tissues from infants (2–24 months) and adults (20–36 years) using *p*-nitrophenyl acetate as a substrate. There was no significant difference between mean infant and adult CarbE activities with a rank order of: 2 months < 3 months < 20 years < 24 months < 4 months < 36 years < 21 years < 8 months < 34 years < 35 years (See Figure19). When proteins (3 µg) were separated and blotted using antibodies against rat hydrolase S (HS), human CarbE types 1 and 2, and CYP3A4, there were no significant differences in staining density between infant and adult tissues with any isozyme. When aliquots of each CarbE sample were pre-incubated (30 min, 37 °C) with chlorpyrifos oxon to evaluate in vitro sensitivity (Figure 20), no significant differences in IC_{50} values were obtained in 3-month to 36-year samples (range: 1.42–2.12 nM), while the IC_{50} was significantly lower in the 2-month sample (0.45 nM). CarbE activity across samples was correlated with cytochrome *b*5 content and HS immunosignal but not with other microsomal activities (total CYP450 content, testosterone hydroxylation, coumarin hydroxylation, and EROD). However the authors cautioned regarding conclusions from this study. This study is limited in scope, i.e., while tissues from adults were readily obtainable, only five tissue samples from individuals ≤2 years of age were available for study. Thus, the number of observations for comparing age-related differences in human liver CarbE was limited. In addition, information provided by the supplier indicated that some of the individuals were on medications (e.g., corticosteroids) that could have influenced CarbE expression. While limited in nature, the findings suggest that if maturational expression of liver CarbE contributes to age related sensitivity to chlorpyrifos or other OP insecticides in humans, it may only be important during very early postnatal maturation.

Figure 19: Carboxylesterase activity in liver S9 fractions from infants and adults



Carboxylesterase activity was assayed using *p*-nitrophenyl acetate as the substrate. S9 samples from five infants (2–24 months of age) and five adults (20–36 years of age) were pre-incubated at 37 °C for 10 min before adding *p*-nitrophenyl acetate (final concentration=0.5mM). Carboxylesterase activity was calculated using a *p*-nitrophenol standard curve and expressed as nmolmin^{-1} mg protein^{-1}. (Pope *et al* 2005)

Figure 20: In vitro inhibition of human liver carboxylesterase activity by chlorpyrifos oxon



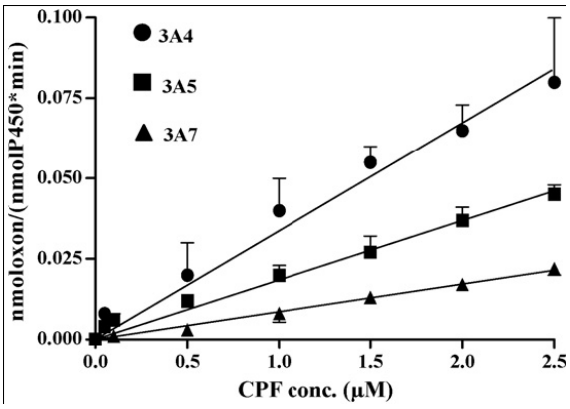
Aliquots of S9 samples from five infants and five adults were pre-incubated for 30 min at 37 °C with a range of concentrations of chlorpyrifos oxon (CPFO, dissolved in 100% ethanol) and residual carboxylesterase activity was measured. Data were plotted as percent of control using the GraphPad Prism software package. Note that the only significant difference in sensitivity to chlorpyrifos oxon (based on 95% confidence intervals) was noted in the tissue from the 2-month-old individual. (Pope *et al* 2005)

The cytochrome P450 family of microsomal enzymes (CYPs) is responsible for metabolic activation and deactivation. The liver is the richest organ in these enzymes which are important in the metabolism of many xenobiotics. Numerous isoforms of the CYPs have been identified and their enzymatic activity investigated demonstrating certain degree of specificity towards their substrates (Sams *et al* 2004). For example using human liver microsomes, the highest chlorpyrifos desulfuration occurred by CYP2B6 and the highest dearalyation activity by CYP2C19. Even in the same isoform of enzyme, there are several subforms distributed among the human population that exhibit various susceptibilities to drugs and xenobiotics. Thus CYP3A4 the most

abundant cytochrome P450 isoform in the adult human liver varies by 40 fold among the human population. The most metabolically active P450s for chlorpyrifos were CYP2B6, 2C19 and 3A4 while CYP1A2, 3A5, and 3A7 had limited metabolism of chlorpyrifos (Table 7).

Buratti *et al* 2006 assessed the catalytic activity of the fetal CYP3A7 toward chlorpyrifos by using recombinant enzymes. A comparison with the adult isoforms CYP3A4 and CYP3A5 has been also carried out. CYP3A7 produced significant levels of chlorpyrifos oxon in the range of tested concentrations (0.05–200 μM). Te reaction was linear up to the highest concentration tested as shown in Figure 21.

Figure 21: Rate of chlorpyrifos oxon formation vs. chlorpyrifos concentration (0.5–2.5 M) catalyzed by CYP3A4 (●), 3A5 (■), and 3A7 (▲)



Results are expressed as nmol oxon/(nmol P450 min) and represent mean ±S.E., calculated on at least three determinations. (Buratti et al 2006)

When the efficiencies of CYP3A isoforms were compared, the ranking efficiency order towards chlorpyrifos was 3A4 > 3A5 > 3A7; The CYP3A5 efficiency appeared to be more dependent on the single insecticide than its related isozyme CYP3A4. These results indicate that the levels of toxic metabolite formed *in situ* by CYP3A7 from chlorpyrifos have the chance to inhibit acetylcholinesterase, following prenatal exposure. However, due to the smaller weight of fetal liver, the contribution to total organophosphorothioate biotransformation is relatively low. CYP3A4 produced chlorpyrifos oxon and TCP at 10 and 50 μM chlorpyrifos, representative of low and high pesticide concentration, respectively. However, while at low pesticide concentration the detoxication reaction predominated, at 50 μM, chlorpyrifos oxon formation was higher, as expressed by the chlorpyrifos oxon/TCP ratio equal to 0.66 and 1.47 at 10 and 50 μM CPF, respectively. No oxon production was detectable for CYP3A5 and 3A7 at 10 μM of CPF, only the TCP was produced. At 50 μM chlorpyrifos, the chlorpyrifos oxon/TCP ratios were: 1.47, 0.43 and 0.42 for CYP3A4, CYP3A5 and 3A7, respectively. The authors suggested that these results "clearly indicate that at low chlorpyrifos concentrations, the formation of the non-toxic metabolites is highly favored in the fetus."

CYP3A7 makes up about 30% of all the P450s in the fetus, while the level of CYP3A4 is low (Hakkola *et al.*, 2001).

5. Chlorpyrifos Metabolism Studies in Humans

The metabolic profile of humans has been elucidated based on a combination of deliberate dosing studies in adults, poisoning cases, and *in vitro* studies. The following text focuses on those studies which provide toxicokinetic data relevant for risk assessment, such as animal to human extrapolation or within human variability, and for evaluating biomonitoring and epidemiology studies. There are several deliberate dosing studies in adults exposed to chlorpyrifos. According to the HS RULE, deliberate dosing human studies which include assessment of effect (e.g., ChE inhibition or clinical signs and symptoms) and are being relied on by OPP to develop risk assessments must be reviewed by the HSRB for scientific and ethical conduct. The studies described here have not yet been reviewed by the HSRB. The Agency has not yet developed a risk assessment for chlorpyrifos, and thus the Agency has not yet relied on these studies. The Agency will ask the SAP to provide comment on the way in which the Agency proposes to use these studies, namely for purposes of evaluating biomonitoring and epidemiology studies. Based on the feedback from the SAP at the September, 2008 meeting, the Agency will determine whether or not the Agency will indeed rely on these studies and will thus require review by the HSRB.

5.1. Human Volunteer Studies

There are three laboratory dosing studies with chlorpyrifos which include toxicokinetic data: Nolan, *et al* (1982), (MRID 00124144); Kisicki, *et al.,* 1999/2000; MRID 44811002/45144101); and Griffin, *et al.* 1999. These studies are summarized in this section. Quantitative comparisons of the blood and/or urine levels of TCP and chlorpyrifos are provided in Section 6 (Weight of the Evidence).

The first of these, Nolan *et al* (1982) investigated the kinetics of chlorpyrifos and TCP in a single-dose human toxicity study. Six healthy male subjects were given single oral doses of 0.5 mg/kg of chlorpyrifos analytical grade 99.8% purity (dissolved in food-grade methylene chloride and placed on a 0.5 g lactose tablet). Additionally, single dermal doses of 0.5 mg/kg (one subject) or 5.0 mg/kg (5 subjects) were administered (4 weeks after the oral dose) by spreading measured volumes (≈10 µL/kg) of chlorpyrifos dissolved in dipropylene glycol methyl ether (DPGME) on the volar surface (≈100 cm^2) of the forearm (not covered or occluded). Urine was collected 24-48 hours prior to dosing through 120 hours post dosing, with separate collections made for the intervals starting at 0, 6, 12, 24, 36, 48, 60, 72, and 96 hours post dose. Volume and creatinine concentrations of each collection were determined. Blood and urine chlorpyrifos and TCP concentrations were determined.

Blood chlorpyrifos concentrations following the oral and dermal doses were extremely low (<30 ng/mL; limit of detection: 5 ng/mL) and exhibited no temporal pattern and no chlorpryifos was detected in the urine indicating the complete metabolic

transformation of chlorpyrifos. TCP kinetic data showed a 1-2 hour delay in the absorption of the oral dose. The average blood TCP concentration (level of detection: 5 ng/mL) then increased rapidly ($t_{1/2}$ = 0.5 hr) and reached a maximum of 0.93 μg/mL (0.51- to 1.35 μg/mL) 6 hours after the dose ingestion. The elimination half-life following oral exposure was estimated to be 27 hours. Following the 5.0 mg/kg dermal dose, the average half life appearance of TCP in the blood was 22.5 hour, and the highest mean concentration was less than 10x the oral maximum: 0.063 μg/mL (0.029 to 0.122 μg/mL) after 24 hours of the dose application. The apparent volume of distribution was 181 ± 18 mL/kg and the mean half life of elimination of TCP from the blood was 26.9 hours following both the oral and dermal doses. Using assumptions of first-order absorption and elimination equations, it was estimated that 72 ± 11% of the ingested and 0.95 ±0.559% (based on the 5 subjects receiving 5 mg/kg dermal dose) and 3.18% (based on one subject receiving 0.5 mg/kg dermal dose) of the dermal dose were absorbed. These estimates of absorbed dose from Nolan *et al* (1982) were used by EPA in the 2000 RED to estimate exposure to individuals in worker and residential biomonitoring studies.

In another human oral/dermal dosing toxicity study, Griffin *et al* (1999) exposed adults to a single-dose with analytical grade chlorpyrifos. Five subjects (4 males and 1 female; age range 26-45 years; weight range 73-92 kg) were dosed orally with 1 mg chlorpyrifos applied to a sugar cube. Four weeks after the oral dose, 28.59 mg of chlorpyrifos was administered to the skin of the same subjects by spreading 100 µL of a commercial preparation of chlorpyrifos diluted in water, onto an area of 78 cm^2 of the inner forearm, which was then covered with a raised impermeable plastic container for 8 hours. The cover was then removed and swabbed, and the skin was washed with water and soap solution. Blood samples were collected over 24 hours and urine was collected over 100 hours at specified intervals. Plasma and erythrocyte (RBC) ChE activities were determined for each blood sample (ChE results reported in a separate chapter). The concentrations of two urinary metabolites of chlorpyrifos (diethylphosphate and diethylthiophosphate) were determined for each urine sample.

Results: Chlorpyrifos was rapidly absorbed, metabolized and eliminated following the oral ingestion as was seen by the rise to the maximum levels of excretion at 7 hours. This was followed by an exponential excretion profile. Most of the oral dose was excreted into the urine and recovered as dialkyl metabolites with a mean of 84.4±22.6% and a range of 55-115% of the administered dose. The apparent half life of elimination of the dialkyl metabolites corrected for creatinine was 15.5 hours. The dermal exposure resulted in 1% of the dose absorbed over the 8 hour exposure period as measured by the excretion of the dialkyl metabolites in the urine, the maximum concentration of metabolite in the urine corrected for creatinine occurred 24 hours after the start of the dermal exposure. The apparent elimination half-life of the urinary dialkyl phosphate metabolites for the dermal dose was nearly twice that of the oral dose.

In a single-dose human oral toxicity study (Kisicki *et al.,* 1999/2000; *MRID* 44811002/45144101), 6 human subjects/sex/group were dosed orally with chlorpyrifos (99.8%) in a gelatin capsule at dose levels of 0, 0.5, 1.0, or 2.0 mg/kg. Baseline

measurements of RBC ChE activity were obtained for each subject and were used for comparison to RBC ChE activity was monitored for 168 hours post dose (ChE results reported in Section 3). Plasma ChE was not assessed. Blood and urine were collected at selected intervals and analyzed for chlorpyrifos, chlorpyrifos oxon and TCP to define the pharmacokinetics of chlorpyrifos in humans. The paraoxonase (PON1) status was determined for each subject and is discussed in another chapter of this submission.

Results: Chlorpyrifos was detected in the blood of 4 of 12 subjects (2-8 hours post dose) at 1.0 mg/kg (range of 1.0-5.6 ng/g) and in 4 of 12 subjects (2-12 hours post dose) at 2.0 mg/kg (range 1.3-18 ng/g). Chlorpyrifos oxon was not found in any blood sample (LOQ 1 ng/g). TCP was detected in the blood of all subjects through 120 hours (0.5 and 1.0 mg/kg) and through 168 hours (2.0 mg/kg). The highest blood concentration of TCP reached ranged from 110-380 ng/g (0.5 mg/kg), 140-610 ng/g (1.0 mg/kg), and 370-1600 ng/g (2.0 mg/kg). The mean concentrations of TCP at each dose level remained relatively constant from 4-48 hours post dose after which the concentrations decreased in an apparent first-order manner. Half-lives describing the decrease in blood TCP levels were 30.4±5.9, 29.1±6.4, and 35.8±5.8 hours for the 0.5, 1.0, and 2.0 mg/kg dose levels, respectively. The average area under the blood concentration-time curve (AUC) for TCP were 14.6±4.7, 26.2±10.4, and 55.4±22.3 µg/g/hr with increasing dose. Both the peak TCP concentrations and AUC were proportional to dose.

Chlorpyrifos (LOQ 1.1 ng/g) and chlorpyrifos oxon (LOQ 1.2 ng/g) were not found in the urine of any subject. The average amount of TCP in the urine at each dose level was proportional to dose and displayed a consistent concentration-time profile. Following the ingestion of chlorpyrifos, the excretion of TCP increased rapidly and the amount of TCP excreted during the first four 12-hour collection intervals was relatively constant at each dose level. The greatest amount of TCP excreted during a 12-hour interval averaged about 0.7, 1.5, and 3.0 mg with increasing dose. After attaining peak concentrations, the amount of TCP excreted in the urine declined in a first-order manner with a mean half-life of 29.4±6.6, 31.0±7.3, and 35.7±6.7 hours with increasing dose. The average amount of TCP excreted was estimated to be 5.1±2.6, 9.2±4.0, and 17.7±5.7 mg with increasing dose. The lower levels of TCP and lack of ChE inhibition observed in Kisicki et al (1999/2000) indicate lower levels of absorption in this study. It has been suggested that the capsules used to administer the chlorpyrifos reduced the absorption compared to the other studies.

In another study, dermal application of cholrpyrifos to human volunteers resulted in higher dermal absorption as measured by the urinary TCP metabolite (Meuling *et al*, 2005). In this study 0.5 ml dilution of chlorpyrifos in ethanol was applied to an area of ~100 cm^2 of the volar side of the forearm of two groups of three volunteers each, at doses of either 5 mg or 15 mg of chlorpyrifos per study subject. The volunteers were males 20-42 years and 67-81 kg weight and 180-188 cm height. Duration of dermal exposure was 4 h, after which the non-absorbed fraction was washed off. Dosing solutions, wash-off fractions and urine samples collected up to 120 h after dosing were collected at pre-determined intervals for the determination of either CPF or its

metabolite TCP. Application of either 5 mg or 15 mg chlorpyrifos resulted in the total urinary excretion of 131.8 μg or 115.6 μg, respectively, of TCP 120 h after dosing. This indicated that 4.3% of the applied dose had been absorbed (5 mg), while at 15 mg dose no significant increase in urinary TCP (115.6 μg) was established with 1.2% of the applied dose was absorbed. The latter indicates that an increase in the dermal dose at a fixed area does not increase absorption, which suggests that the percutaneous penetration rate was constant. Urinary TCP levels peaked at 48–72 h after dermal application. Thereafter, the excreted amount per 24 h gradually decreased, but was still above background level for the 96–120 h period. Further, it was observed that the clearance of chlorpyrifos by the body was not completed within 120 h, suggesting that chlorpyrifos or TCP was retained by the skin and/or accumulated in the body. The skin washes accounted for 42 ± 11.6% of the applied dose in the low dose group and 66.8 ± 16.0% of the applied dose in the higher dose group. A mean elimination half-life of 41 h was established.

Overall, the human studies show that following oral exposures, that absorption is fairly complete and the maximum dose is approximately 4-7 hours post-exposure, the ½ life of elimination ranged from 15.5 to almost 36 hours. For dermal exposures, approximately 1% - 3% of the dermal dose was absorbed and the ½ life of elimination was approximately 30-40 hours.

5.2. Accidental Human Poisoning Studies

There are few published studies of accidental ingestion of toxic doses of chlorpyrifos in humans where chlorpyrifos and its metabolites were investigated. In a lethal human poisoning case where an individual accidentally ingested a pesticide formulation identified as a mixture of chlorpyrifos and malathion (diethyl mercaptosuccinate, S ester with O,O-dimethyl phosphorodithioate), a major metabolite of chlorpyrifos was extracted from the liver (Lores *et al*, 1978). Gas chromatography, MS and NMR analysis identified this metabolite as the O,O-diethyl phosphorothioate ester of a dichloro, methylthio, 2-pyridinol. The exact position of the methylthio (-SCH3) group on the pyridine ring could not be determined.

In a poisoning case of three individuals who ingested 20-100 ml of a liquid chlorpyrifos formulation containing 48-50% of the active ingredient resulted in the initial inhibition of RBC AChE activity by 80% (Vasilić *et al*, 1992). The kinetics of excretion of the urinary metabolites was investigated during the hospitalization of the affected individuals. No chlopyrifos was detected in the urine. The DETP and DEP metabolites were measured in the urine and their excretion followed a first order kinetic equation with a biphasic pattern with a first fast phase of excretion (half life of 3.5 – 5.5 hours) and a second slow phase of excretion (half life of 66.5 – 127.9 hours). Table 21 provides the kinetic data of urinary excretion of the DEP + DETP metabolites from the first order kinetic equation $y = y_o e^{-k1t} + y'_o e^{-k2t}$ in the chlorpyrifos poisoned individuals:

Table 21: Kinetic Data of urinary excretion of the DEP + DETP metabolites (Vasilić *et al*, 1992)

Person	Time Period	Number of samples	y_o nmol/mg creatinine	y'_o nmol/mg creatinine	$k1$ $(days)^{-1}$	$k2$ $(days)^{-1}$
M	0	1	784	Not calculated		
M	5-20	5	0	50	-	0.18
N	0-9	7	300	2	3.00	0.15
O	0-10	7	99	103	4.74	0.17

In another case where one male (25 years) and one female (28 years) ingested 30-60 ml of a liquid formulation containing 50% of chlorpyrifos and another 40 year old female ingested two spoons of a powdered formulation containing 4% of CPF, a significant depression of RBC ChE (78-82%) and serum ChE (84-89%) activities was noted upon hospital admission within 2-5 hours after ingestion of chlorpyrifos (Dervenkar *et al* 1993). The parent material was only detected in the serum and showed a biphasic log-linear decrease suggesting its fast metabolism and excretion. The duration of the initial faster K_1 elimination phase was 0.3-0.8 days for chlorpyrifos in serum and for the total DETP and DEP metabolites in serum and urine 0.5-1.3 days and 1.0-2.9 days, respectively. In that phase chlorpyrifos was eliminated from serum as twice as fast ($t_{1/2}$ = 2.2-5.5 hr). The elimination $t_{1/2}$ of total DEP+DETP metabolites from urine was about two times slower than that from serum and ranged from 5.1-9.5 hours. In the slower K_2 phase, the minimum elimination $t_{1/2}$ for chlorpyrifos in serum was 40.6 hours, for total DEP and DEPT metabolites in serum 21.9 hours and in the urine 38.8 hours. When the values from this study were combined with those reported from an earlier study from the same laboratory (Vasilić et al, 1992), the chlorpyrifos metabolites were excreted with an average elimination $t1_{/2}$ of 6±2 hours in the initial phase and 80±25 hours in the second slower phase. The presence of DETP in serum and urine samples suggests that chlorpyrifos was hydrolyzed before its oxidation to the oxon. The kinetics of the elimination of chlorpyrifos and its total DEP+DETP metabolites from serum is shown in Table 22.

Table 22: The elimination of chlorpyrifos and its total DEP+DETP metabolites from serum

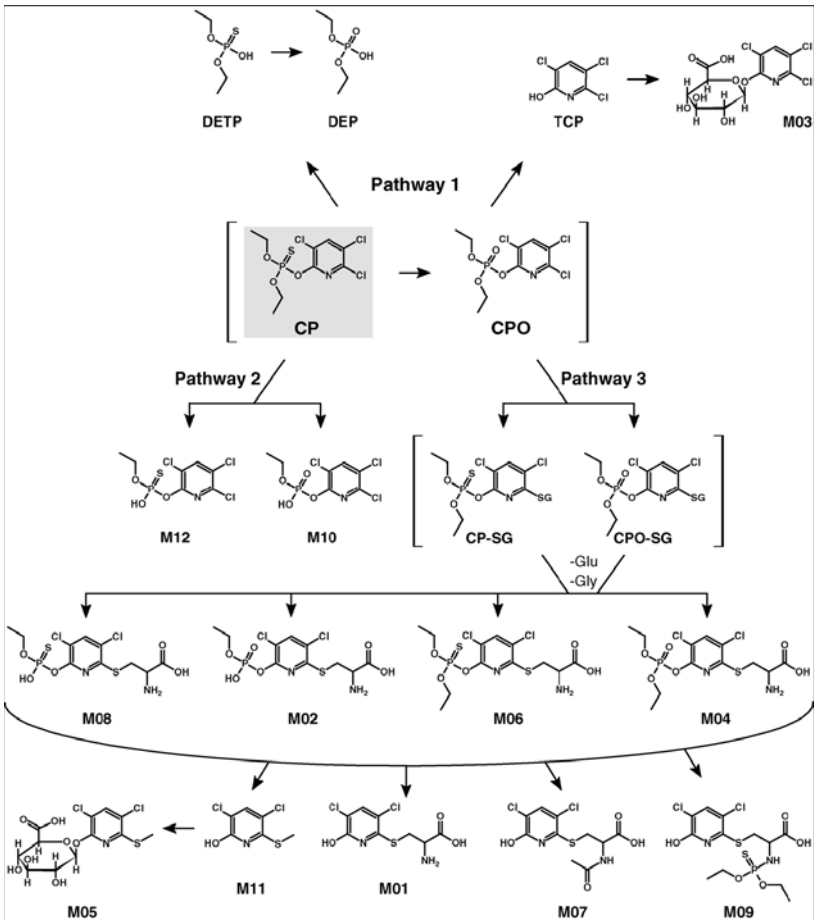
Person	Time Period[a] (days)	N	N	y_1 nmol/mL	Y_2 nmol/mL	*k1* $(days)^{-1}$	*k2* $(days)^{-1}$
Chlorpyrifos							
A	0-15	10	10	6.8	0.29	5.01	017
B	0-9	8	6	0.39	0.03	14.79	0.41
C	0-14	7	2	NC	NC	NC	NC
DEP+DETP							
A	0-15	10	10	23.94	2.53	3.06	0.21
B	0-9	8	6	17.79	1.45	7.66	0.76
C	0-14	7	2	4.92	0.55	3.03	0.01

a Days after poisoning when the metabolites were measured; N number of analyzed samples
N Number of analyzed samples; n number of positive samples; NC not calculated
Kinetic data from the sum of two first order equations y = y1e-k1t + y2e-k2t (Dervenkar *et al* 1993).

The most comprehensive and detailed study of the pharmacokinetics of chlorpyrifos in humans was investigated by Bicker *et al* (2005). In this study the metabolic fate of chlorpyrifos was investigated in an acutely intoxicated 59 years old female who ingested 25 mL of Dursban 2E formulation containing 20-25% chlorpyrifos (71-86 mg/kg based on a body weight of 70 kg). Urine samples were collected at least daily from the patient's admission to the hospital until the 14th day of clinical treatment (21 samples collected from 27-336 h post ingestion) when the patient was discharged with a serum ChE activity of 5% of the reference range. Urine samples were analyzed by liquid chromatography–electrospray ionization-tandem mass spectrometry (LC–ESI-MS/MS). Fifteen metabolites were identified. Neither chlorpyrifos nor its oxon were detected in the urine. The respective structures of the firmly identified degradation products are shown in Figure 22. Three distinct biotransformation routes of chlorpyrifos were proposed:

(1) cleavage reactions at the aromatic phosphoester bond,
(2) cleavage reactions at the alkyl phosphoester bonds, and
(3) glutathione (GSH) dependent nucleophilic substitution of the 6-chlorine at the aromatic moiety.

Figure 22: Proposed human biotransformation scheme of chlorpyrifos (CP) in case of acute poisoning



Brackets indicate the parent compound and tentative, not detected, intermediates (−SG. . .glutathione conjugates) (Bicker et al 2005).

Route 2 was not reported in humans before and route 3 is a previously unknown scheme of chlorpyrifos metabolism. Urinary markers of the latter were chiefly cysteine *S*-conjugates of the mono-dechlorinated chlorpyrifos and its oxon, as well as the 6-mercapturic acid conjugate of 3,5-dichloro-2-pyridinol. The presence of 3,5-dichloro-6-methylthio-2-pyridinol as well as its *O*-glucuronide suggests further a cysteine *S*-conjugate β-lyase mediated degradation. The excretion profile of the major metabolites is presented in Figure 23. Renal elimination profiles of DETP, DEP, and TCP normalized to the creatinine content were monitored simultaneously over 14 days using a validated LC–ESI-MS/MS assay (Figure 24). A biphasic first-order excretion mechanism with half-lives of 21.5 h (initial fast excretion phase) and 119.5 h (terminal phase) for the sum of free DETP and DEP was found. TCP was hardly eliminated in its free form (*O*-glucuronide identified as phase II conjugate) and half-lives calculated for

the total amount of TCP (acidic hydrolysis of urine samples) were 40.8 and 150.7 h. Free TCP was eliminated considerably faster ($t_{1/2}^{fast}$ = 12.4 h, $t_{1/2}^{slow}$ = 100.5 h). Toxicokinetic data for the urinary excretion of major chlorpyrifos metabolites (DETP, DEP, TCP) from this study are presented in Table 23.

Table 23: Toxicokinetic data for the urinary excretion of major chlorpyrifos metabolites (DETP, DEP, TCP)

	Fast elimination phase				
	Time interval[n] (h)	**k_1 (h-1)**	**y_1 (mmol mmol^{-1} crea**	**Half-life (h)**	**R^2**
Free amount (non-hydrolyzed samples)					
DETP	30-109	0.0404	0.2320	17.2	0.9555
DEP	33-120	0.0276	0.1619	25.1	0.9795
DEP+DETP	30-109	0.0323	0.3514	21.5	0.9534
TCP	59-109*	0.0561	0.4038	12.4	0.9517
Total amount (hydrolyzed samples)					
TCP	62-144**	0.0170	0.1256	40.8	0.9316

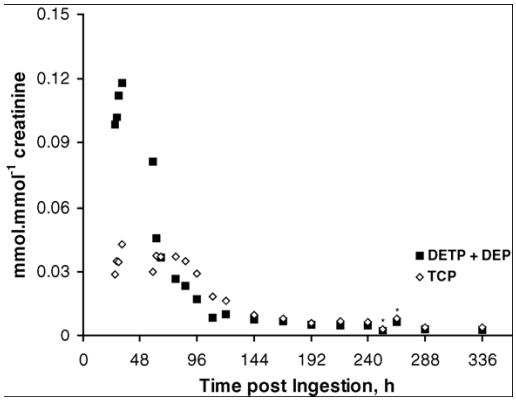
[n]: number of samples is 9 except if marked * is 7 or ** is 8.
Kinetic model: biphasic first-order elimination y = y1e-k1t + y2e-k2t (Bicker *et al* 2005)

Table 24: Toxicokinetic data for the urinary excretion of major chlorpyrifos metabolites (DETP, DEP, TCP)

	Slow elimination phase				
	Time interval [n] (h)	k_2 (h-1)	y_2 (mmol mmol^{-1} crea	Half-life (h)	R^2
DETP	109-336	0.0099	0.0085	70.0	0.9618
DEP	120-336**	0.0049	0.0119	141.5	0.9795
DEP+DETP	120-336**	0.0058	0.0185	119.5	0.9685
TCP	120-336	0.0069	0.0022	100.5	0.7552
Total amount (hydrolyzed samples)					
TCP	144-336*	0.0046	0.0177	150.7	0.8725

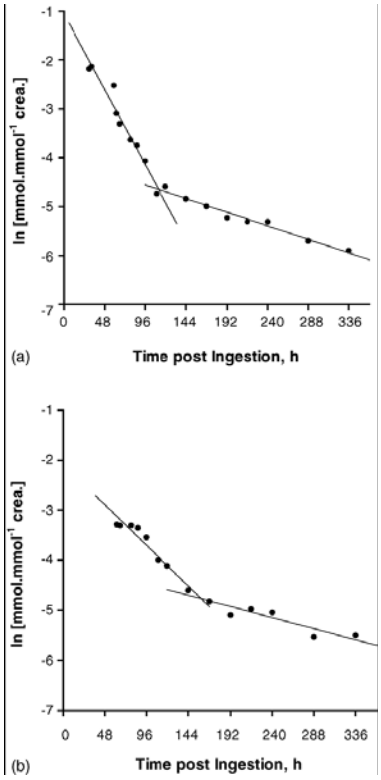
[n]: number of samples is 9 except if marked * is 7 or ** is 8.
Kinetic model: biphasic first-order elimination y = y1e-k1t + y2e-k2t (Bicker *et al* 2005)

Figure 23: Excretion profiles of major chlorpyrifos metabolites, DETP + DEP (non-hydrolysed samples) and the total amount of TCP (hydrolysed samples) over 14 days in urine of the patient.



Maximum 58.2 mg/L, DEP 61.3 mg/L, TCP 35.3 mg/L. Minimum concentrations (non-hydrolysed, 252 h): DETP 0.37 mg/L, DEP 2.27 mg/L, TCP 0.43 mg/L. (Bicker *et al* 2005)

Figure 24: Linearised elimination curves for (a) the free amount of the sum of DETP and DEP (determined in non-hydrolysed samples) and (b) the total amount of TCP (determined in hydrolysed samples) calculated with the data obtained from the analysis of chlorpyrifos

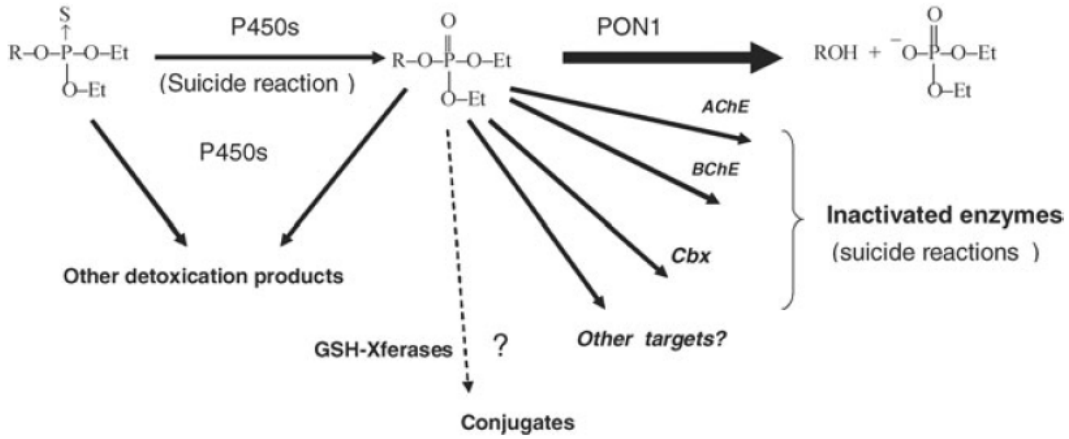


Data points before the maximum concentration and those with uncertainties in the collection time were eliminated (Bicker *et al* 2005).

6. Ontogeny of Metabolic Enzymes during Human Development

In addition to the PON1 (an A-esterase), status in chlorpyrifos detoxification, B-esterases (carboxyl esterases) act as scavengers of OP compounds thus providing protection against their toxicity. These have strong affinity to specific OP compounds. Genetic variability of the B-esterases has also been demonstrated by Furlong 2007. Significant individual variability in carboxylesterases in human liver microsomes, with activities varying from 5.3- to 44.7-fold, depending on the substrate used for assaying carboxylesterase activity was demonstrated (Hosokawa *et al.* 1995). They also noted the importance of regulation of carboxylesterase activities by exogenous inducers. In addition, a second B esterase, butyryl or serum cholinesterase (BChE), plays a role in providing protection against OP exposure despite its ability to bind only a single OP molecule (Broomfield *et al* 1991), leading to the prediction that individuals deficient in BChE would exhibit increased sensitivity to OP exposure (Manoharan *et al* 2007). The role of these various enzymes in the metabolism of OP compounds such as chlorpyrifos is presented in Figure 25.

Figure 25: Enzymes contributing to the metabolism of organophosphorothioate insecticides (Furlong, 2007)



Carboxylesterase (a B-esterase), plays an important role in removing the chlorpyrifos oxon formed, thus reducing its toxicity.

There are only a few studies in the older literature that have assessed A-esterase activity in children. Based on these studies, it appears that serum A-esterase levels are very low in human infants compared to adults (Augustinsson and Barr, 1963; Mueller *et al.,* 1983; Ecobichon and Stephens, 1973). After birth, there is a steady increase of this activity during the first six months to about one year (Augustinsson and Barr, 1963). In a related study of the age-dependence of total serum arylesterase activity (of which a large component is Aesterase activity), adult levels were achieved by two years-of-age (Burlina *et al.*, 1977). Although serum A-esterases are reported to achieve adult levels around six months to one year-of-age, there is uncertainty surrounding those values for the one-year-old due to the variability in the rate of maturation expected as these enzyme systems mature at different rates in a cross-section of one-year-old children.

Suggestive evidence of this is the large degree of variability seen in the six-month and one-year age groups in the limited serum esterase data available for children (Augustinsson and Barr, 1983). More recent studies by Holland *et al* (2006) and Chen *et al* (2003) have provided ARase and/or chlorpyrifos oxonase activities in newborns and their mothers. These studies have been analyzed by the Agency as part of consideration of data derived intraspecies extrapolation factor for chlorpyrifos. In addition, the Agency is aware of yet unpulished data of PON1 levels (A-esterase) in children up to age 5 from the laboratories of Drs. Nina Holland and Brenda Eskanazi with a much larger sample size (>200) than previous studies. After completion of the data analysis and ultimately publication, these data will substantially improve the overall understanding of the human ontogency of A-esterases.

Maturation of the cytochrome P450s to detoxify or activate chlorpyrifos to the oxon may also play a role in age-related differences in the young and adults. For example, oxidation by CYP3A4 plays a key role in the oxidation (activation) of 24 OP

pesticides including chlorpyrifos in humans (Butler and Murray, 1997). Ginsberg *et al.* (2002) using the children's pharmacokinetic data from the therapeutic drug literature showed that compared to adults, oxidation by CYP3A4 tends to be more active in children beginning as early as two to six months-of-age with this difference lasting until at least two years-of-age. While this may increase concern for greater oxidative bioactivation in the young, the CYP-mediated oxidative dearylation (detoxication) pathway, which may also be more active at these ages, is involved in the detoxication of these pesticides. Therefore, it is important to compare the maturation profiles for these two CYP pathways. Based on the ratio of Vmax/Km for humans and rats, the activation step is 2.5-fold faster and, the activation step has an 8.4-fold lower K_m than the dearylation step (Ma and Chambers, 1994; 1994; Timchalk *et al.* 2002). The significance of this is that at relatively low, environmental exposures, OP molecules reaching the liver may be much more likely to be activated to to the oxon rather than detoxified by the dearylation pathway. This evidence supports the potential concern that greater oxidative capacity in young children may lead to more OP activation than seen in adults. The enhanced ability of the young to bioactivate OP pesticides to their oxon form, however, has not been correlated with an increased sensitivity to ChE inhibition.

7. Metabolic Changes During Pregnancy

Metabolic activities can be altered during pregnancy. Regard PON1 activity, Ferre *et al* (2006) showed that the PON1 hydrolysis of paraoxon in serum decreased from a non-pregnant background of 146 U/L to 111 U/L in late gestation, indicating 76% of normal activity in late gestation pregnant women.

The most important P450s for cholrpyrifos metabolism in humans are 1A2, 2B6, 2C19, and 3A4. Bologa *et al* (1991) found that production of a marker substrate for 1A2 activity dropped to 41% of non-pregnant levels by the third trimester in epliptic women. While 1A2 and 2B activities decrease in pregnancy, 2C and 3A activities increase. In pregnant AIDS patients, endogenous cortisol metabolism (marker for 3A4 activity) increased by more than 2-fold (Homma *et al* 2000). Tsutsumi (2001) demonstrated that the mean metabolic ratios for CYP1A2 during pregnancy (6.80, 5.18, and 4.97 for the early phase, middle phase, and late phase, respectively) were significantly lower than the ratio after delivery (10.39) in healthy women. So the profile of CYP hepatic enzymes changes during pregnancy, but the data do not allow us to predict what the overall effect would be on the activation or detoxication of chlorpyrifos.

Weitman *et al* in 1986 examined the influence of pregnancy on the hepatic metabolism of parathion, an organophsphosphrothioate undergoing microsomal activation like chlopyrifos. They found that the *in vitro* rate of hepatic microsomal activation of parathion to paraoxon was significantly reduced in mice at 19 days of gestation when compared to nonpregnant controls. But *in situ* perfusion of livers from pregnant and nonpregnant mice with parathion did not result in any significant differnces on its total hepatic metabolism. Their results suggest that total hepatic matabolsim of

partathion, its oxon and the hydrloyxzed p-nitrophenol is not altered in pregnancy despite a decrease in specific activity for parathion activation. Pretreament of pregnant and non-prengant mice with phenobarbital had similar effects on parathion and paraoxon metabolism in perfused livers. Reductions in hepatic microsomal monooxygenase activity towards parathion on a per unit liver weight basis seems to be compensated for by increases in in liver weight during pregnancy.

In earlier studies Weitman *et al* (1983) found that pregnant mice treated with 5 mg/kg parathion demonstrated higher concentrations of both parathion and paraoxon in blood and brain than similarly treated virgin controls and it correlated with enhanced cholinesterase inhibition. Tissue concentrations of parathion and paraoxon were highest 1 hr after ip administration (in corn oil) of parathion (5 mg/kg) in both pregnant and virgin mice and were not detectable in any tissue examined after 12 hrs. Concentrations of parathion were significantly higher in blood and brain of pregnant mice compared to virgin animals. Maximum blood levels of parathion were 0.175±0.41 and 0.028±0.002 μg/mL in pregnant and virgin control mice, respectively.parathion brain levels were 0.149 ± 0.015 μg/mL in pregnant mice and 0.078 ± 0.029 μg/mL in virgin mice. Paraoxon was detected in pregnant mice (0.042 ± 0.007 μg/mL) but not in virgin control mice. Decreased paraoxonase activity was also demonstrated during pregnancy.

8. Discussion of Metabolism and Toxicokinetics - Conclusions

The metabolism and pharmackokinetics of chlorpyrifos has been extensively studied in animals and man as well as in *in vitro* systems.

There are several enzymes that play a role in the metabolism and toxicity of chlorpyrifos. The toxicity of chlorpyrifos has historically been attributed to the chlorpyrifos oxon. The oxon targets acetylcholinesterase (AchE) by binding to the serine active site and inactivating the ChE enzyme. The chlorpyrifos oxon also binds to other serine esterases such as butyrlycholinesterase.

The cytochrome P450 family of microsomal enzymes (CYPs) are responsible for the metabolic activation (producing chlorpyrifos oxon) and deactivation (producingTCP) of chlorpyrifos. Another group of important enzymes in the detoxification of chlorpyrifos is the A-esterases, also known as paraxonase or PON1. These are calcium activated enzymes and are distributed in various tissues including the liver, brain and blood. A-esterases hydrolyze chlorpyrifos oxon. It has also been demonstrated that chlorpyrifos oxon binds irreversibly to other non-target serine esterases, namely carboxylesterase (CarbE). Carboxylesterase is considered as a scavenger of the chlorpyrifos oxon removing it from reaching the target AchE enzyme. This binding has been described to be stoichometric. CarbEs are distributed among different tissues (liver, blood (not human), lung, brain) with highest abundance in the liver. The glutathione dependent enzymes also play an important role in the secondary metabolism of chlorpyrifos producing water soluble metabolites that are readily excreted into the urine.

Abundant studies have established that chlorpyrifos is readily absorbed following oral exposures by laboratory animals or humans and undergoes metabolic transformations mainly by the liver enzymes. Although lipophillic, chorpyrifos' extensive metabolism into water soluble metabolites does not lead to any accumulation of the parent material or its metabolites in the body tissues. The initial metabolic attack on the chlorpyrifos molecule is its desulfuration to the more toxic and potent cholinesterase inhibitor, the oxon form. Chlorpyrifos oxon is highly reactive and is rapidly deactivated through hydrolytic cleavage by a process called dearylation releasing TCP. Simultaneously during the desulfuration process, dearylation will be acting not only on the chlorpyrifos oxon metabolite, but also on the parent chlorpyrifos, leading to the release of TCP. TCP is further conjugated to form glycine or glucuronide conjugates and eliminated into the urine.

The metabolism of chlorpyrifos in humans is well understood through documented incidental poisoning cases as well as in human exposure laboratory studies. Three distinct biotransformation routes of chlorpyrifos were proposed in humans: (1) cleavage reactions at the aromatic phosphoester bond producing TCP, (2) cleavage reactions at the alkyl phosphoester bonds, and (3) glutathione (GSH) dependent nucleophilic substitution of the 6-chlorine at the aromatic moiety, chiefly cysteine *S*-conjugates of mono-dechlorinated chlorpyrifos, chlorpyrifos oxon, mono-*O*-deethyl chlorpyrifos, and mono-*O*-deethyl chlorpyrifos oxon as well as the 6-mercapturic acid conjugate of 3,5-dichloro-2-pyridinol. These routes yielded 15 metabolites excreted into the urine. Even at very high incidental ingestion of a chlorpyrifos dose, neither the parent material nor the oxon were found in the urine indicating their rapid metabolism and transformations.

Following both oral and dermal doses in adult humans, blood chlorpyrifos concentrations were extremely low and no chlorpryifos was detected in the urine indicating the complete metabolic transformation. TCP kinetic data showed a 1-2 hour delay in the absorption of the oral dose, and the average blood TCP concentration reached a maximum value 6 hours after the dose ingestion. Following the dermal dose, the highest mean concentration was observed after 24 hours. Approximately 72% of the ingested dose and 1%-3% of the dermal dose were absorbed. In another oral study in humans, no oxon was detected in the blood and only terminal metabolites were detected in the urine.

Studies by Dow (MRID 40458901: Nolan et al 1987) demonstrated that an orally administered ^{14}C-chlorpyrifos dose in rats was readily absorbed from the GI tract and rapidly excreted in the urine (>84%) and feces (>5%) with no detection of the parent in the excreted radioactivity. Residual ^{14}C-radioactivity in tissues (including blood) and carcass were less than 0.3% at animal sacrifice time (72 hours for males and 144 hours for females). Its elimination half life was estimated to be 8-9 hours in males and females following a single low oral dose of 0.5 mg/kg, but was longer following a larger single oral dose of 25 mg/kg (12.4 hours for males and 23.2 for females) indicating slower absorption and elimination with increased dose. There was no difference in its excretion half life if administered as a single dose or multiple daily doses (0.5 mg/kg/day

for 15 days). The urinary metabolites were investigated in this study by HPLC and MS analysis. Urinary metabolites were either the 3,5,6-TCP (hydrolysis product of chlorpyrifos) or its glucuronide (mainly) and the sulfate conjugates.

Peak chlorpyrifos blood concentrations in adult rats occurred within three hours of gavage treatment with single doses ranging from 1-100 mg/kg with a calculated blood half-lives of chlorpyrifos of 1.5 – 7.3 hours . Regardless of dose, the highest concentration of the oxon detected was 2.5 ng/g in the blood of rats treated with 50 mg/kg test material one hour post-treatment. Following treatment with 5 or 100 mg/kg labeled test material, ≥98% of the activity detected in the blood was identified as TCP metabolite with the remaining attributed to the parent compound. Since the oxon is an intermediate in the formation of TCP and none of the chlorpyrifos oxon was detected, these studies support that the half-life of the oxon metabolite is short (*in vitro* half life in rat blood of 10 seconds and 55 seconds in human blood (Brzak et al. 1998) .

Following ip injection of chlorpyrifos to adult rats, chlorpyrifos and TCP levels in blood reached maximum values at 5 h post-injection with TCP accounting for 83%of the total blood amount. Biological half-lives of the blood chlorpyrifos and TCP were estimated to 8.15 and 24.66 h, respectively. 1) about half of the chlorpyrifos administered was directly hydrolyzed to DETP and TCP, 2) 10 to 20% was hydrolyzed to DEP and TCP after the oxidation to chlorpyrifos oxon, and 3) about 30% was dealkylated to TCP-phosphate after the oxidation. TCP was excreted in the urine as conjugates.

The toxicokinetics (absorption, metabolism and elimination) of DEP, TCP and DETP following oral exposure to rats, all three metabolites were well absorbed with peak blood concentrations being attained between 1 and 3 h post-dosing. Peak amounts of these metabolites in urine were attained within 12 h post-dosing and the urinary elimination half lives ranged from 8 – 13 hours. These studies support the hypotheses that DEP, DETP and TCP present in the environment can be readily absorbed and eliminated in the urine of rats and potentially humans.

The liver has been identified as the site of chlopyrifos metabolism. It is thought that the majority of the conversion of chlorpyrifos to chlorpyrifos oxon occurs in the liver via cytochrome P450-dependent metabolism. Oxidative desulfuration as well as dearylation occur in the liver with the dearlyation predominating in chlorpyrifos. Hepatic microsomes from male rats were found to have higher capabilities of these reactions than female rats. The cytochrome P-450 monooxygenase system is implicated in these reactions as evidenced by the requirement for NADPH and inhibition by carbon monoxide. It has been found that in addition to the liver microsomes, other tissues such as brain mitochondria and brain microsomes have limited desulfuration activity. The enterocytes from the small intestine were very effective in the desulfuration/dearylaion reactions of chlorpyrifos; however their impact on the metabolism of chlopyrifos is not well understood. In liver, the production of TCP is the high affinity, high capacity path for chlorpyrifos. Whereas, in the enterocytes, the metabolism of chlorpyrifos to TCP has

a lower efficiency compared to the metabolism to chlorpyrifos oxon, although the capacity is still higher for the TCP pathway.

The in vitro metabolism of chlorpyrifos has been extensively investigated in liver microsomes from various animal sources and humans. Investigations by independent researchers identified the individual CYP enzymes involved in the activation and deactivation of chlorpyrifos. Chlorpyrifos, underwent desulfuration in human liver microsomes with the highest desulfuration activity occurred by CYP2B6, and the highest dearylation by CYP2C19. The CYP3A4 has been described as the most abundant isoform of cytochrome P450 in the adult human liver and is responsible for metabolism of approximately half the currently used drugs (Dai *et al 2001*). CYP3A4 varies 40-fold in individual human livers, and metabolism of CYP3A4 substrates varies at least 10 fold *in vivo.* CYP3A4 variants exist in the human population. The relative rates of activation and inactivation are critical to the toxicity of the compound.

Foxenberg *et al* (2007) investigated the human hepatic cytochrome P450 family of enzymes in the metabolism of chlorpyrifos and derived human P450 specific kinetic parameters that will help refine current PBPK/PD models that utilize kinetic values derived from rat liver microsomal metabolism that may not reflect human enzymes. They found that the most metabolically active P450s for chlorpyrifos were CYP2B6, 2C19, and 3A4 while CYP1A2, 3A5, and 3A7 had limited metabolism of chlorpyrifos. CYP2B6 also showed a higher affinity and activity for chlorpyrifos metabolism to chlorpyrifos-oxon. In contrast, CYP2C19 has a high affinity and activity for TCP formation (dearylation).

It has been demonstrated that chlorpyrifos undergoes not only CYP-dependent phase I biotransformation but also phase II conjugation pathways in human hepatocytes (Choi *et al* 2006). Phase II related conjugates, including *O*- and *S*-glucuronides as well as GSH-derived metabolites, were identified in chlorpyrifos-treated human hepatocytes. However, chlorpyrifos oxon was not found following treatment of human hepatocytes with either chlorpyrifos or chlorpyrifos oxon. Since chlorpyrifos oxon is itself readily metabolized by human hepatocytes, it was concluded “that it does not accumulate to be released into the bloodstream and transported to the nervous system. If that is the case, the activation of chlorpyrifos to its oxon within the nervous system, rather than the liver, may be responsible for the cholinergic effects of chlorpyrifos, whereas its metabolism in the liver will cause inhibition of CYP isoforms by the release of reactive sulfur.” These results “suggest that human liver plays an important role in detoxification, rather than activation, of chlorpyrifos.”

The chlorpyrifos oxon is detoxified to the dearylated metabolite by PON1, an A-esterase plasma enzyme tightly associated with high-density lipoprotein. There is considerable morphological variability of PON1 enzyme among the human population (Furlong, 2007). The creation of mice strain lacking PON1 activity (knockout mouse) in 1998 (Shih *et al*, 1998) accelerated the understanding and importance of this enzyme in the detoxification of Ops.

In addition to the genetic variability of PON1, neonates had lower PON1 activity than in adults, implying reduced capacity to detoxify chlorpyrifos.

The OP insecticides are thus bioactivated by the CYP enzymes and are deactivated by both the CYP and PON1 enzymes. The balance between bioactivation and detoxification will drive the toxicity from OP exposures.

Carboxylesterases (CarbE) play an important role in removing the chlorpyrifos oxon formed, thus reducing its toxicity. These have strong affinity to specific OP compounds. Genetic variability of the B-esterases has also been demonstrated (Furlong 2007). Due to its importance, CarbE has been the subject of several investigations such as (Chanda *et al*, 1997). The liver had the highest CarbE activity. The rank order for CarbE sensitivity to chlorpyrifos-oxon between different tissues was liver > plasma >brain. In addition to the gender-related differences for CarbE activity, it was found that the *in vitro* sensitivity of CarbE to chlorpyrifos oxon is highly tissue specific. It was also demonstrated that the pattern of inhibition after *in vivo* dosing with chlorpyrifos was not necessarily predictable from the *in vitro* IC_{50} of these enzymes. Most importantly for the detoxification of chlorpyrifos, the number of CarbE molecules is very important in modifying its toxicity.

A second B-esterase, (BuChE), plays a role in providing protection against OP exposure despite its ability to bind only a single OP molecule. There is disagreement that BuChE can be a reasonable detoxification sink.

Concern for prenatal and post natal exposure to chlorpyrifos potential neurotoxicity has prompted many researchers to investigate the metabolism and toxicokinetics of chlorpyrifos and its metabolites in pregnant animals, fetuses, and post-natal pups. It is known that chlorpyrifos crosses the placenta and is distributed through the fetal tissues including the brain. Additionally, chlorpyrifos from lactating animals partitoned readily to the milk and thus can be transferred to the young. Several studies have suggested that young animals are more sensitive than adults to the acute toxicity of chlorpyrifos (Atterberry *et al,* 1997; Moser and Padilla, 1998; Mortensen *et al*, 1996; Pope and Liu, 1997). Pope et al (1991) were the first to demonstrate that developing mammals are more sensitive than adults to a variety of OP insecticides. This differential sensitivity to chlorpyrifos toxicity may be attributed to the metabolic capacity of various age groups.

Based on the distribution of TCP in maternal and fetal brain and liver tissues following chlorpyrifos oral gavage of rats during late gestation (GD 14-18), Hunter *et al*, (1999) proposed that the fetal liver does not play a significant role in detoxification and, therefore, TCP (and most likely also chlorpyrifos and chlorpyrifos-oxon) distributes evenly throughout the fetal compartment.

It has been demonstrated that chlorpyrifos and its principal metabolite TCP are transferred to the fetuses and lactating pups from dams orally treated with chlorpyrifos from GD 6-20 and through lactation day 20 (Mattson *et al* 2000). However chlorpyrifos

is detected in the maternal and fetal blood at very low levels and declines upon exposure cessation and is proportional ot the exposure levels. Chlorpyrifos and TCP are also transferred to the growing pups through the milk during the lactation. Chlorpyrifos milk levels decline rapidly to non detectable after one day of cessation exposure to chlorpyrifos. Chlorpyrifos-oxon was not detected in the blood or milk of any dams at any time point.

Lassiter *et al*, 1998 reported that following daily dosing of rats by gavage with chlorpyrifos in corn oil at 7 mg/kg on GD 14 to 18, that (1) the maximal ChE inhibition time was the same (i.e., 5–10 h after dosing) for both maternal and fetal brain, (2) the degree of fetal brain ChE inhibition was 4.7 times less than maternal brain inhibition, and (3) the detoxification potential (i.e., carboxylesterase and chlorpyrifos-oxonase) of the fetal tissues was very low compared to the maternal tissues. Age-dependent changes in chlorpyrifos-oxonase activity in the maternal and fetal/neonatal tissues occurred primarily after birth. Maternal liver had the most activity, and the time course of this activity was variable during the late pregnancy, but ultimately demonstrated a slight postpartum increase. Maternal blood chlorpyrifos-oxonase activity was variable before birth, but decreased on the day after birth. The placenta had about 1/5 the activity of the liver which was consistent during late pregnancy. Fetal liver exhibited minimal activity during gestation, but the postnatal day 1 pup liver showed an eightfold increase in chlorpyrifos-oxonase activity. Maternal and fetal brains had no detectable chlorpyrifos-oxonase activity. The dosed animals showed marked inhibition of CarbE activity. Maternal liver CarbE activity was less than 18% of control 2 to 10 h after the last chlorpyrifos dose. Fetal liver CarbE activity was less than 50% of control values for the entire time course postdosing. Fetal liver CarbE did not recover to control levels, but the maternal liver CarbE activity was recovering by the 5-day time point *(i.e*., postnatal day 1). Fetal brain CarbE activity was not inhibited in the chlorpyrifos-exposed subjects, but maternal brain activity was approximately 70% of control across the entire time course.

Further work by Lassiter *et al* (1999), showed the importance of the enzyme CarbE for fetal exposure to chlorpyrifos. Using a similar experimental design as in their previous studies, Long-Evans pregnant rats were orally administered 0, 3, 5, 7 or 10 mg/kg of chlorpyrifos on GD 14 -18 and sacrificed 5 hours after the last dose. Following gestational exposure to chlorpyrifos, there was more CarbE inhibition in the maternal liver than in the fetal liver: maternal liver activity was less than 18% of the control activity at all doses; fetal liver CarbE activity was also maximally inhibited at all dosages, but with less severity than maternal liver (45-50% of control activity). Maternal brain CarbE was inhibited in a dose dependent manner (100, 85, 71 and 65% of control CarbE activity at 3, 5, 7 and 10 mg/kg of chlorpyrifos), where as fetal brain CarbE activity showed no inhibition at any dosage. Placental and maternal blood CarbE activities were not inhibited by repeated gestational exposures even at 10 mg/kg. The control levels of CarbE activities were age and tissue dependent as illustrated in table 2.6, below. The disparate pattern of CarbE inhibition related above may be partially explained by the IC_{50} and inhibitor resistant esterase (IRE) pattern related in Table 15. For example, fetal brain CarbE activity is not sensitive at all to inhibition by chlorpyrifos

oxon with an IC_{50} approximately 1000 fold higher than either maternal liver or brain-this insensitivity explains the lack of inhibition in that compartment.

Newborn and juvenile rats are more sensitive to AChE inhibition caused by chlorpyrifos than adult rodents, not because of a difference in the affinity of chlorpyrifos oxon to AChE, but because the enzymes that detoxify chlorpyrifos oxon have not yet fully developed (Iyer, 2001). The activity levels of P450s and PON1 differ between the adult and infant or conceptus. For example, CYP3A7 makes up about 30% of all the P450s in the fetus, while the level of CYP3A4 is low (Hakkola *et al.*, 2001). In contrast, the level of CYP3A7 is extremely low in adults, while the level of CYP3A4 is very high (Hakkola *et al.*, 2001). In humans, the serum levels of PON1 are lower in infants up to 6 months old than levels in adults (Ecobichon and Stephens, 1973). Similarly, compared to adults, PON1 activity levels were lower in young rats up to 25 days old and in young mice up to 20 days old (Li *et al.*, 1997).

It has been demonstrated that specific activities of acetylcholinesterase in cerebral cortex, but not medulla oblongata and of liver aliesterases increased with age in male rats ranging in age from 1- 80 days (Aterrberry *et al* (1997), indicating the presence of both more target esterases and more protective esterases, respectively, in the adult compared to the juvenile animal. Progressive increases in activities of P450-mediated activation (desulfuration) (6- to 14-fold) and detoxication (dearylation) (2- to 4-fold), as well as concentrations of P450 (7-fold) and protein (2-fold), were observed between neonate and adult hepatic microsomes. Microsomal pentoxyresorufin *O*-dealkylase activity followed a developmental pattern similar to desulfuration and dearylation, displaying a 16-fold increase between neonates and adults. However, microsomal ethoxyresorufin *O*-deethylase activity increased until 21 days of age, displaying a 16-fold increase, then decreased in adulthood to a level 10-fold higher than neonates. These results indicate that target enzyme sensitivity is not responsible for age-related toxicity differences, nor is the potential for hepatic bioactivation, whereas lower levels of hepatic aliesterase-mediated protection and P450-mediated dearylation probably contribute significantly to the greater sensitivity of juveniles to phosphorothionate toxicity.

It has been found that in postnatal day rats ranging in age from 5-17 days when administered oral doses of chlorpyrifos, older pups (PND 17) had slightly faster metabolism of the absorbed dose than the younger pups (PND 5) base on higher TCP concentrations in the older rats (Timchalk *et al* 2006). The increase in the blood TCP concentration (~3-fold) in PND-17 rats relative to the response in the younger rats was consistent with an increase in CYP450 metabolic capacity with age. Younger animals demonstrated a greater sensitivity to ChE inhibition as evident by the age-dependent inhibition of plasma, RBC, and brain ChE. The brain sensitivity of younger animals (i.e. PND-5) was attributed to substantially lower levels of ChE activity relative to later preweanling stages and adults.

Studies evaluating effects of chlorpyrifos in pregnant dams, fetuses or PND pups use a variety of routes of exposure. The two most often used are oral gavage,

particularly in corn oil, and subcutaneous injection with DMSO. It is difficult to compare studies conducted by these different methods given that the toxicokinetics of each route may differ. Marty *et al* (2007) investigated the effect of route, vehicle, and divided doses on the toxicokinetics of chlorpyrifos and its metabolite TCP in neonatal rats and demonstrated variable differences. It was observed that for PND5 rat pups, the kinetic properties for DMSO subcutaneous injection are more similar to exposure in milk than exposure from corn oil gavage for the various kinetic paramters chlorpyrifos and TCP parameters

It has been demonstrated that young animals such as 4 day old rats have less capacity than adult rats to detoxify physiologically relevant concentrations of chlorpyrifos oxon via chlorpyrifos oxonase due to much lower plasma and liver chlorpyrifos oxonase activities in the pups, Mortensen *et al* (1996). No brain chlorpyrifos oxonase activity was detected in pups or adults.

Lack or low levels of detoxifying enzymes in young rats partially explains their increased sensitivity to chlorpyrifos, Chanda *et al*. (2002). There was no plasma CarbE activity in the rat fetus, and it was 5 times lower in the pups compared to the adults. The liver CarbE activity was 13 times lower in the fetus compared to the pups and 50-90 times lower than in the adults. Similarly, the brain CarbE activity showed a similar trend. Pregnant females had lower CarbE activity compared to non pregnant females.

Neonatal and juvenile rats were more sensitive than adults to the acute lethality of chlorpyrifos (Karanth and Pope, 2000). Levels of CarbEs and AEs in neonatal and juvenile rats were significantly lower than in adult tissues.

It has been suggested that human liver CarbE expression changes relatively little in comparison to rodents (Pope *et al* (2005). There was no significant difference between mean infant and adult CarbE activities with a rank order of: 2 months < 3 months < 20 years < 24 months < 4 months < 36 years < 21 years < 8 months < 34 years < 35 years. However the authors cautioned regarding conclusions from this study. This study is limited in scope, i.e., while tissues from adults were readily obtainable, only five tissue samples from individuals ≤2 years of age were available for study. Thus, the number of observations for comparing age-related differences in human liver CarbE was limited. In addition, information provided by the supplier indicated that some of the individuals were on medications (e.g., corticosteroids) that could have influenced CarbE expression. While limited in nature, the findings suggest that if maturational expression of liver CarbE contributes to age related sensitivity to chlorpyrifos or other OP insecticides in humans, it may only be important during very early postnatal maturation.

Buratti *et al* 2006 assessed the catalytic activity of the fetal CYP3A7 toward chlorpyrifos by using recombinant enzymes and their results suggested that at low chlorpyrifos concentrations, the formation of the non-toxic metabolites is highly favored in the fetus.

Overall, rats and humans show similar patterns of metabolism for chlorpyrifos in adults and during pregnancy and maturation postnatally. The oxon metabolite has traditionally been considered the active toxic moiety. More recent studies (Section 4) have suggested that the chlorpyrifos itself may also have biological activity. TCP is the major excreted metabolite and used as the biomarker in toxicokinetics, biomonitoring, and epidemiology studies. TCP can be formed by two pathways, through the oxidative bioactivation first to the oxon then to TCP or through the detoxification, dearylation pathway. Absorption of chlorpyrifos via the oral routes is almost complete in rats and humans. Dermal absorption in humans appears to low, approximately 1 - 3%.

Rat fetuses and juveniles and newborn humans have lower capacity to detoxify than adults. This decreased capacity to detoxify has been associated with increased sensitivity in rats. Specifically, in rats, A-esterase activity is virtually nonexistent in the fetus (Lassiter *et al.*, 1998) and increases from birth to reach adult levels around PND21 (Mortensen *et al.*, 1996; Li *et al.*, 1997). The animal data regarding the role of carboxylesterase in mediating OP toxicity are also quite extensive (*e.g.*, Clement, 1984; Fonnum *et al.*, 1985; Maxwell, 1992 a,b). Fetal rats possess very little carboxylesterase activity (Lassiter *et al.*, 1998) with increasing activity as the postnatal rat matures, reaching adult values after puberty (50 days-of-age; Morgan *et al*., 1994; Moser *et al.*, 1998; Karanth and Pope, 2000). The temporal pattern of A-esterase activity (and carboxylesterases) correlates reasonably well with studies on OP sensitivity. Several studies have shown an increased sensitivity of newborn rats to OP compounds which are detoxified via the A-esterase and/or carboxylesterase pathways (Gagne and Brodeur, 1972; Benke and Murphy, 1975; Pope *et al.*, 1991; Chambers and Carr, 1993; Padilla *et al.*, 2000; 2002; Karanth and Pope, 2000).

In gestational studies, similar or higher levels of TCP in fetal brain and blood compared to dams suggests that chlorpyrifos and/or its metabolites reach the target tissue (brain) in the fetus (Hunter al, 1999; Mattson et al, 2000). Mattson et al (2000) provided data in rat milk which suggests that chlorpyrifos can reach milk at low doses (0.3 mg/kg/day). There are very little human breast milk data in the literature and none from U.S. exposures. During human pregnancy, key detoxification enzymes have somewhat lower levels than non-pregnant adults. Although the importance of these decreases is unknown at environmental exposures, this suggests a reduced capacity to detoxify chlorpyrifos during pregnancy.

In summary, toxicokinetic differences play an important role in the differential sensitivity of the young to ChE inhibition following treatment with OP pesticides, including chlorpyrifos. These toxicokinetic differences have been shown for fetuses, juvenile rats, and to a more limited extent in human newborns and young children. The following chapters will discuss the toxic effects of chlorpyrifos during gestation and post-natal exposures. The age-related sensitivity observed in the studies described can be attributed, at least in part, to the toxicokinetic differences described here.

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