Project Title: **Complex Mixture Modeling Organophosphate Pesticides**

Project Dates: 9/30/2001 – 9/29/2005
Report: Final Report

Principle Investigator: Charles Timchalk, Ph.D., DABT
Co-Investigator: Torka S. Poet, Ph.D.

Research Institution: Battelle Memorial Institute
Center for Biological Monitoring and Modeling
902 Battelle Blvd., PO Box 999
Richland, WA 99352

Contact Information: Ph: (509) 376-0434
Email: charles.timchalk@pnl.gov

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1.0 List of Abbreviations

Name	Abbreviation
Acetylcholinesterase	AChE
Acetylthiocholine	ATC
Area under concentration curve	AUC
Bimolecular inhibition rate	Ki
Butyrylcholinesterase	BuChE
Butyrylthiocholine	BTC
Chlorpyrifos oxonase (arylesterase)	PON1
Chlorpyrifos	CPF
Chlorpyrifos-oxon	CPF-oxon
Cholinesterase	ChE
Cytochrome P450	CYP450
Diazinon	DZN
Diazinon-oxon	DZN-oxon
Diethylphosphate	DEP
Environmental Protection Agency	EPA
Intraperitoneal	ip
Isopropyl methyl hydroxypyrimidine	IMHP
Log likelihood function	LLF
Michaelis-menten affinity constant	Km
Michaelis-menten maximum velocity	Vmax
Physiologically based pharmacokinetic/ pharmacodynamic	PBPK/PD
Reactivation rate	Kr
Red blood cell	RBC
Skin permeability constant	Kp
Trichloropyridinol	TCP

2.0 Abstract

This project used a quantitative experimental and modeling approach to evaluate the potential impact that pesticide mixture exposures might have on agricultural workers, who are routinely exposed to insecticides. Pesticide interactions can share a common theme in which dosimetry and biological responses are altered when mixtures modify absorption rates, extent of metabolism, tissue distribution, clearance or pharmacological action. Organophosphorus insecticides are of particular concern since they are widely utilized, are neurotoxic, and a number of biomonitoring studies have documented both occupational and non-occupational exposures in adults and children to multiple pesticides. The current risk assessment paradigm focuses on individual chemicals; however, exposure is primarily to mixtures where there is limited understanding of the health effects. Occupationally exposed agricultural workers, handle concentrated pesticide formulations and therefore have higher exposures. Exposure to chemical mixtures can involve complex "chemical soups" which are poorly characterized in terms of components, concentrations, exposure duration and routes, and the overall toxicological effect of the mixture is most likely unknown. These uncertainties in terms of exposure, dose and biological response create difficulty in determining realistic risk from occupational exposure to mixtures. The overall approach was to develop an experimental/modeling strategy to understand the impact of complex chemical mixtures on the toxicological response of organophosphorus pesticides. The approach focused on understanding both the pharmacokinetic (absorption, distribution, metabolism and excretion) and pharmacodynamic (cholinesterase inhibition) responses associated with individual and binary mixtures of organophosphorus insecticides. The project strategy integrated the development of physiologically based pharmacokinetic and pharmacodynamic models with the acquisition of focused *in vitro* and *in vivo* experimental data for model development and validation. The strength of this approach is that it can be used to identify the most critical factors (i.e. exposure timing, routes of exposure, lack of protective equipment) that contribute to occupational exposure to insecticides, and allow researchers and regulators to assess internal dose and biological response with greater confidence. This project resulted in the development, validation and application of computational models assessing dosimetry and biological response for individual and binary mixtures of insecticides in animal models and humans. This experimentation/modeling strategy can now be expanded to assess the impact of lifestyle choices and in particular the impact of co-exposures to routinely consumed pharmacologically active agents (ex. pharmaceuticals, alcohol and nicotine) that have the demonstrated potential to likewise modify metabolism and biological response to insecticides. Since a comprehensive toxicological evaluation of chemical mixtures is not feasible, it is reasonable to develop strategies that can be applied to classes of chemical agents that have similar metabolic and mode of action profiles. In this regard, the Environmental Protection Agency (EPA) has developed a framework for conducting cumulative risk assessments for organophosphorus and other pesticides that have a common mode of toxicological action. They have indicated that physiologically based pharmacokinetic modeling approaches represent the future direction for conducting cumulative dose-response risk assessments. In this regard, the EPA has identified the organophosphorus insecticide models that have been developed as part of this project as representing key initial components in this process.

3.0 Highlights/Significant Findings

This project has resulted in the development, validation, and application of integrated PBPK/PD models to assess both dosimetry and dynamic response for individual and binary exposures to insecticides in animal models and humans. It is concluded that this modeling strategy can readily be extended to include other insecticides, and that limited *in vitro* experiments can be utilized to ascertain interactions both at the level of metabolism and for ChE inhibition. The following significant findings/results are particularly noteworthy.

Physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) models have been developed and validated for chlorpyrifos and diazinon in both rats and humans. These PBPK/PD models are capable of quantifying target tissue dosimetry and dynamic response in both rats and humans and can be used to link metabolism to ChE inhibition across species and strengthen the biological basis for individual chemical and mixture risk assessments (Poet *et al.*, 2004). For example, in the case of chlorpyrifos (CPF) and diazinon (DZN) the PBPK/PD models have enabled us to propose that metabolic interactions do occur, but at relatively high doses so that it is reasonable to assume that at occupational exposure levels the kinetics would be linear (no interaction), whereas the extent of ChE inhibition would follow a dose additive response. The models have also enabled us to evaluate susceptible subpopulations, such as children or individuals with genetic polymorphisms in key metabolic processes. For example, since the potential sensitivity of a given individual to the adverse health effects from exposure to chemicals may be related to differences in metabolic capacity, the PBPK/PD model for chlorpyrifos was used to further evaluate the potential importance of genetic polymorphisms in the arylesterase (PON1) detoxification of chlorpyrifos-oxon (Timchalk *et al.*, 2002). The results of the Monte Carlo analysis suggest that the polymorphism has the greatest impact on target tissue dosimetry at dose levels which overwhelm other detoxification pathways (i.e. non-target esterases). More recent efforts have extending the PBPK model to accommodate a transgenic mouse model of the human PON1 Q192R polymorphism (Cole *et. al.*, 2005). The rat PBPK/PD model was scaled to the mouse and the model used to reasonably simulate against experimental results in mice that express no PON1 activity ($PON1^{-/-}$ mice). These results in the transgenic mouse model provide an important starting point for refining the PBPK/PD model to take into account differences in detoxification efficacy due to PON1 polymorphisms.

In order to develop biologically based kinetic and dynamic models that are capable of predicting both target tissue dosimetry and response, there is a need to obtain metabolic and enzyme activity parameter estimates for the individual chemicals, their mixture interactions and physiological and biochemical parameter estimates for both rats and humans. Although some of these data are obtainable from the published literature, others are not and must be experimentally determined. Additionally, *in vivo* experiments that provide information on the dose-dependent kinetic and dynamic response are of critical importance for development and validation of the PBPK/PD models. In this regard, studies have been conducted to characterize the *in vitro* metabolism rates of CPF and DZN (Poet *et al.*, 2002). Likewise, *in vitro* experiments have helped characterize the BuChE interactions with oxon metabolite and further evaluated the potential role of an AChE peripheral binding site (Kousba *et al.*, 2003; 2004). In all cases these *in vitro*

determined parameter estimates have been used to help develop the PBPK/PD models. *In vivo* dosimetry and ChE inhibition data have been obtained for DZN and CPF alone or as a binary mixture (Poet *et al.*, 2002; Busby *et al.*, 2004; Timchalk *et al.*, 2005). These results demonstrate that co-exposure to CPF and DZN at equivalent low doses (≤ 15 mg/kg) did not alter the pharmacokinetics of either pesticide; however, a high binary dose (60 mg/kg) did increase the AUC and decrease the clearance of both parent pesticides, and is likely due to competition between CPF and DZN for CYP450 metabolism. A dose-dependent inhibition of ChE was also noted and the overall potency was CPF+DZN > CPF > DZN. For all chemicals and mixtures the overall ChE response appeared to be additive.

An additional unanticipated finding was the potential utility of saliva as a non-invasive biomonitoring matrix for evaluation of both dosimetry and dynamic response. Kousba *et al.* (2003) characterized rat saliva ChE as a nearly pure BuChE form. This finding was initially exploited to obtain needed *in vitro* parameter estimates for BuChE inhibition. However, it was also then recognized that saliva BuChE might represent a potential non-invasive matrix for biological monitoring. Additional investigation also indicated that the major metabolite of CPF, trichloropyridinol (TCP), was quantifiable in saliva following single oral exposures to CPF (Campbell *et al.*, 2005). To further establish the potential utility of saliva for organophosphorus insecticide biomonitoring, rats were exposed to a range of doses (1-50 mg/kg) and the time course of the major metabolite TCP and the extent of ChE inhibition were successfully determined in both blood and saliva (Timchalk *et al.*, 2004). These results have subsequently been used to develop additional research proposals that are focused on non-invasive biomonitoring and a revised grant application is currently pending review with NIOSH (ROH008173A).

We have also recently conducted *in vivo* pharmacokinetic and pharmacodynamic studies in neonatal rats to evaluate the age-dependent differences in metabolism and sensitivity to ChE inhibition (Timchalk *et al.*, 2006). This is the first reported study that evaluated both the pharmacokinetics of the parent pesticide, the major metabolite and the extent of ChE inhibition as a function of preweanling age. The results demonstrate that the extent of *in vivo* metabolism and ChE inhibition are age-dependent. These results provide important insight into the need to develop PBPK/PD models to address sensitive sub-populations such as children of agricultural workers, who are known to have higher pesticide exposure that the general population.

The most recent efforts have focused on extending the research to evaluate the impact of pharmacologically active agents on organophosphorus insecticide pharmacokinetic and pharmacodynamic responses. Initial experiments have evaluated the contribution of nicotine and ethanol. The rationale for this approach is based on the known high exposures associated with their routine use and the capability of nicotine and ethanol to induce metabolism potentially impacting insecticide dosimetry. Additionally, nicotine and ethanol are known to modify cholinergic receptor (nicotinic and muscarinic) function which is also a key pharmacodynamic target for organophosphorus insecticides. Preliminary results establish that both nicotine and ethanol can impact CPF metabolism; however, more extensive *in vitro* and *in vivo* studies are needed to characterize the metabolic interaction, to quantitate the impact on dosimetry, and for development of a PBPK/PD model. These results have subsequently been used as the primary focus of the

Competitive Renewal Application that was submitted to NIOSH (ROH003629B). A revised grant application based on reviewer comments is currently being prepared and will be submitted for review in March 2006.

4.0 Translation of Findings

There are a number of important ways that the research findings from this project can and should be used to prevent workplace disease and injury. It is important to recognize that occupational exposure to pesticides has the potential to occur during manufacturing, formulation, transportation, mixing, loading, application, and upon entering treated areas. Agricultural workers constitute a broad spectrum of the working population that has the potential to be exposed to higher concentrations of chemical mixtures, including pesticides, throughout the course of their working lives. Historically, the rate of occupational disease in agricultural workers has been as high as three times the rate for all other industries. Hence it is of critical importance to utilize the computational modeling approaches developed as part of this project to better understand the potential health implications of pesticide mixture exposures in agricultural workers.

Overall this project has made significantly contributions to a number of priority research areas that have been identified under NIOSH NORA. First, a major objective has been to develop an approach to address the impact of **mixed exposures** on agricultural chemical dosimetry and dynamic response. Secondly, the models that have been developed under this project represent a new approach for **quantitative exposure** and **risk assessment**. Finally, as demonstrated in this project it is also possible to use the models to quantitate risk to **special populations** (i.e. farm workers, children of farm workers; sub-populations with metabolic genetic polymorphisms).

Specifically, the research can be directly applied to several key areas that will directly impact agricultural workers. As suggested by the EPA, PBPK/PD modeling approaches, like the ones developed in this project represent the future direction for conducting cumulative dose-response risk assessments. These models can likewise be utilized to more fully evaluate sensitive sub-populations of workers such as those with pre-existing disease or genetic polymorphisms that might make them particularly sensitive to pesticide exposure. In addition, the models can be extended to better understand the potential greater sensitivity of children of agricultural workers who may be exposed to higher levels of pesticide than the general population.

Since PBPK/PD models accommodate all potential routes of human exposure, the models can also be integrated with exposure assessment approaches, to provide a quantitative assessment of anticipated dosimetry based on observed work practices. This has the potential to provide occupational health professionals with a tool to help them optimize safe work practices to continue to minimize worker exposure to the lowest levels possible.

New biomonitoring approaches, such as saliva analysis, have the potential to result in the development of novel non-invasive sensor technology that can be used for the onsite biomonitoring of agricultural workers. This is particularly relevant to pesticide applicators where routine biomonitoring (i.e. blood ChE determination) may be required as part of their ongoing health surveillance. In this regard, results from this project form

the foundation for the development of sensor based, field deployable biomonitoring platforms.

Finally, the mixture interaction studies provide a firm scientific framework for assessing cumulative occupational risk to pesticides. The findings also have the potential to be readily extended to understand the impact that lifestyle choices, may or may not, have on pesticide dosimetry in workers.

5.0 Outcomes/Relevance/Impact.

This project has resulted in the development, validation and application of integrated PBPK/PD models to assess both dosimetry and dynamic response for individual and binary exposures to insecticides in animal models and humans. It is concluded that this modeling strategy can readily be extended to include other insecticides, and that limited *in vitro* experiments can be utilized to ascertain interactions both at the level of metabolism and for ChE inhibition. These results have facilitated our understanding of complex chemical interactions as it relates to the occupational health implications of working with insecticides. This strategy of linking focused research with validated models is a significant step forward in developing an approach for the evaluation of complex chemical mixtures. The ultimate goal is to apply these models to assess the contribution that lifestyle choices, may or may not have on the risk associated with occupational exposure to insecticides.

6.0 Scientific Report.

6.1 Background. This is the final report, for a project that was initiated in September 2001 and completed in September 2005. The overall objective of the original project was to develop an experimental/modeling approach to understand the impact of complex chemical mixtures on the toxicological response to organophosphorus pesticides. The approach focused on understanding both the pharmacokinetic (absorption, distribution, metabolism and excretion) and pharmacodynamic response (ChE inhibition) associated with individual and binary mixtures of insecticides. The project strategy integrated the development of physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) models with the acquisition of focused *in vitro* and *in vivo* experimental data for model development and validation. The strength of this approach is that it can be used to identify the most critical factors (i.e. exposure timing, routes of exposure, lack of protective equipment) that contribute to occupational exposure to insecticides, and allow researchers and regulators to assess internal dose and biological response with greater confidence. Four **Specific Aims** were pursued:

1. To develop a PBPK/PD model for diazinon in the rat.
2. To access *in vitro* the metabolic interactions between chlorpyrifos and diazinon and obtain needed parameter estimates for PBPK/PD model development.
3. To conduct *in vivo* binary mixture studies with chlorpyrifos and diazinon in the rat to assess dosimetry and dynamic response interactions.
4. To develop a human diazinon PBPK/PD model and a binary chlorpyrifos and diazinon PBPK/PD model.

6.2 General Accomplishments. We have successfully developed PBPK/PD models for the insecticides chlorpyrifos and diazinon in rodents and humans. Both *in vivo* and *in vitro* studies have been conducted to obtain needed model parameter estimates and to validate the pharmacokinetic and pharmacodynamic response for individual and binary mixtures of organophosphorus insecticides. To date this project has produced 12 published manuscripts, contributed to 2 book chapters, and 21 invited talks/poster or platform presentations.

6.3 Specific Accomplishments. The detailed specific accomplishments have been categorized into 4 main areas: 1) *in vitro* metabolism and dynamic studies to determine model parameters; 2) *in vivo* dosimetry and dynamic studies for single insecticides or binary mixtures; 3) PBPK/PD model development and validation; and 4) preliminary *in vitro* data for nicotine/ethanol metabolic interactions with chlorpyrifos.

6.4 In Vitro Metabolism and Dynamic Studies.

6.4.1 Metabolism. *In vitro* metabolism studies were conducted to evaluate the role of hepatic and intestinal CYP450 and PON1 (A-esterase) metabolism of chlorpyrifos and diazinon (**Figures 1 and 2**) (Poet *et al.*, 2003). Parameter estimates from these experiments were used to further develop and refine the PBPK/PD models for chlorpyrifos, diazinon and the binary mixture model. Based on the overall hepatic metabolic efficiency (V_{max}/K_m), chlorpyrifos will undergo more hepatic metabolism *in vivo* than diazinon resulting in a greater amount of chlorpyrifos being metabolized to oxon than for diazinon, which is consistent with the observed toxic potency (chlorpyrifos>diazinon). This is also the first study to demonstrate similar CYP- and

PON1-mediated metabolic profiles in microsomes from liver or isolated intestinal enterocytes and suggests that intestinal metabolism may impact the dosimetry of chlorpyrifos and diazinon particularly at low-dose oral exposures. These experiments directly support **Specific Aims 1 & 2.**

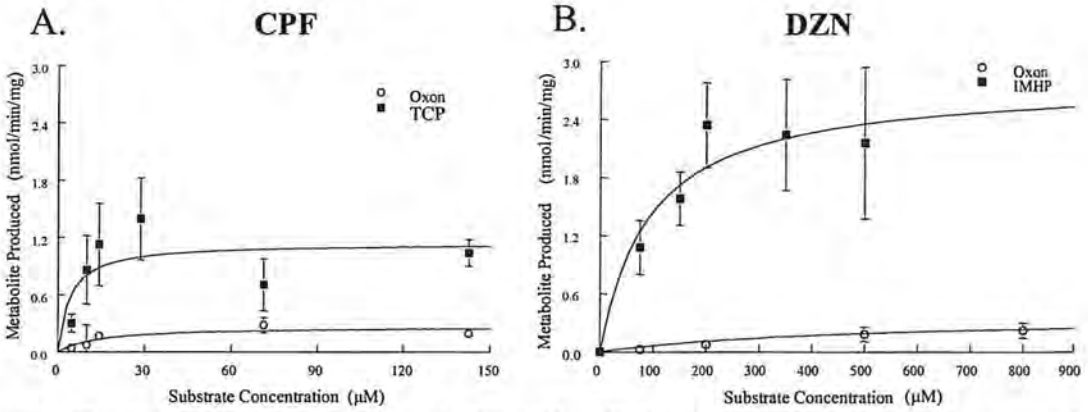

Figure 1. The CYP-mediated metabolism of chlorpyrifos (CPF) (A) and diazinon (DZN) (B) measured in microsomes prepared from livers of Sprague-Dawley rats. Values represent mean ± SD for 6 determinations per substrate concentration. Note the different x-axis scales (Poet *et al.*, 2003).

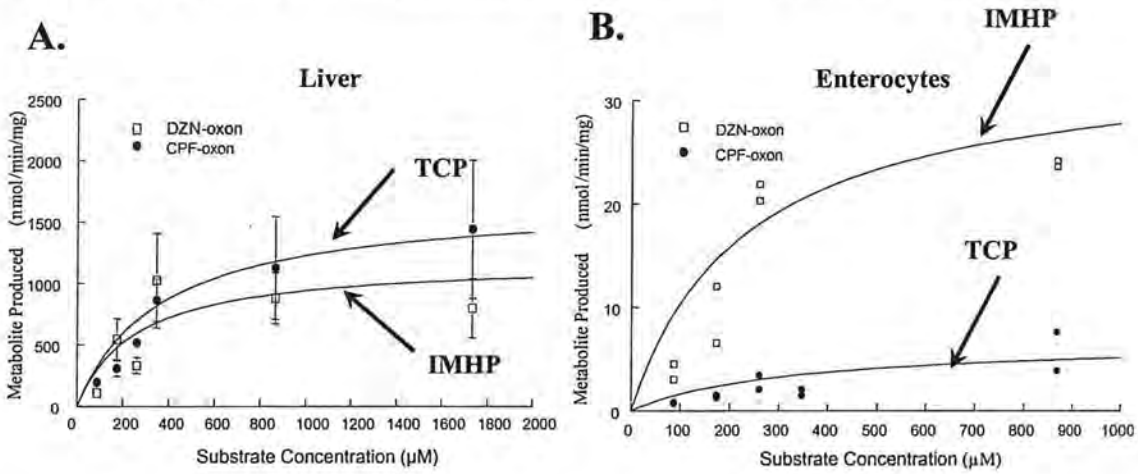

Figure 2. The PON1-mediated metabolism of chlorpyrifos-oxon (CPF-oxon) and diazinon-oxon (DZN-oxon) in liver (A) and enterocytes (B) prepared from Sprague-Dawley rats. Data represent mean ± SD for 6 determinations per substrate concentration for liver and individual determinations (2) per substrate concentration for enterocytes (Poet *et al.*, 2003).

In vitro CYP450 co-incubation studies were conducted to assess the impact of chlorpyrifos on diazinon metabolism to diazinon-oxon and isopropyl methyl hydroxypyrimidine (Wu *et al.*, 2004) and the results are presented in **Figure 3**. Results from these experiments were analyzed using Lineweaver-Burke (results not shown) as well as by a mathematical model (see equations 1 and 2). The metabolism of diazinon to isopropyl methyl hydroxypyrimidine appeared to be competitively inhibited by chlorpyrifos, whereas metabolism to the oxon was uncompetitively inhibited. However, the relatively high inhibitory binding constant (K_i) suggests that the importance of metabolic interactions on kinetics may be limited to high acute dose exposure to binary mixtures of chlorpyrifos and diazinon. This finding is consistent with the observed *in vivo*

kinetic response for a binary exposure in the rat (see **Figure 7**). The impact of diazinon on chlorpyrifos metabolism to chlorpyrifos-oxon and trichloropyridinol suggest that qualitatively similar metabolic interactions are observed. These experiments directly support **Specific Aims 3 & 4.**

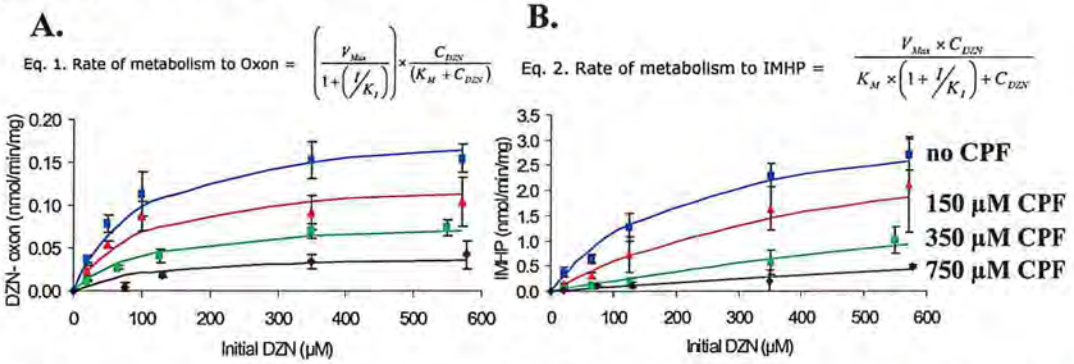

Figure 3. Experimental data and model fit for *in vitro* inhibition of CYP450 metabolism of (A) diazinon (DZN) to diazinon-oxon (DZN-oxon) non-competitive inhibition, and (B) diazinon (DZN) to isopropyl methyl hydroxypyrimidine (IMHP) competitive inhibition by chlorpyrifos at, 0 μM (**blue**), 150 μM (**red**), 350 μM (**green**), and 750 μM (**black**) in microsomes obtained from naïve Sprague-Dawley rats (Wu *et al.*, 2004).

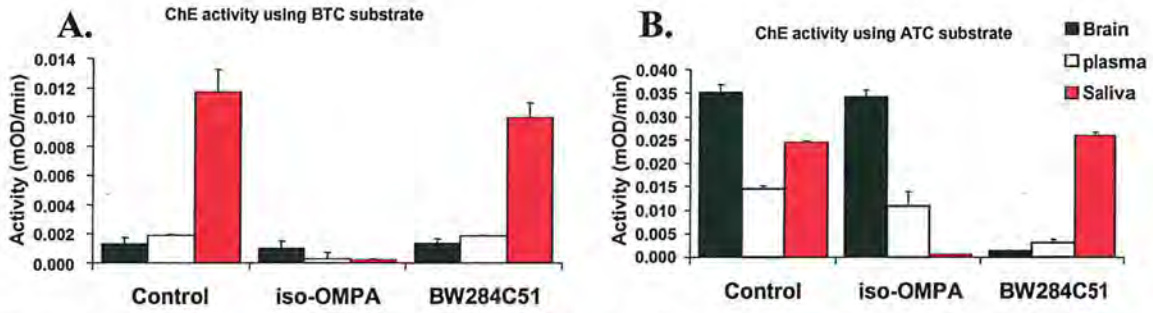

Figure 4. *In vitro* determination of total ChE activity described as (A) butyrylthiocholine (BTC) and (B) acetylthiocholine (ATC) hydrolysis rates in the controls and following iso-OMPA (BuChE inhibitor) or BW284C51 (AChE inhibitor) incubations with brain, plasma and saliva samples obtained from naïve adult male Sprague-Dawley rats. Each bar represents the mean ± S D for three determinations (Kousba *et al.*, 2003).

6.4.2 Dynamics. *In vitro* studies were conducted to characterize ChE activity and to obtain parameter estimates describing the dynamics of ChE (AChE and BuChE) inhibition (Kousba *et al.*, 2003). Experiments were conducted to characterize ChE activity with brain (nearly pure AChE), plasma (50:50; AChE:BuChE), and saliva (nearly pure BuChE), and these *in vitro* inhibition studies are illustrated in **Figure 4**. Characterization of AChE and BuChE in tissues is distinguished on the basis of substrate specificity. For example, AChE rapidly hydrolyzes acetylcholine, but shows no activity towards butyrylcholine, which is a preferred substrate for BuChE. Selective inhibitors for AChE (BW284C51) and BuChE (iso-OMPA) were also utilized to facilitate enzyme characterization. As is illustrated in **Figure 4B**, incubation of rat brain homogenates with iso-OMPA (BuChE inhibitor) using acetylthiocholine (ATC) had no impact on brain ChE; whereas, incubation with BW284C51 (AChE inhibitor) resulted in a marked decrease in activity (>95%). Likewise brain ChE activity is marginally active using

butyrylthiocholine (BTC) as a substrate (**Figure 4A**), and these results are consistent with brain ChE being predominantly AChE. In the case of saliva, iso-OMPA completely inhibited the enzyme activity using either butyrylthiocholine or acetylthiocholine as substrates, whereas BW284C51 (AChE inhibitor) had virtually no impact. These finding are consistent with >95% of the saliva ChE activity being due to BuChE, and based on these results efforts are underway to develop saliva BuChE as a biomarker for exposure to ChE inhibiting pesticides/nerve agents (Timchalk *et al.*, 2004). Further characterization of BuChE in rat tissue is illustrated in **Figure 5**, where the total number of BuChE active sites/tissue, reactivation rate (Kr) (data not shown), and the bimolecular inhibitory rate constant (Ki) were determined utilizing a pharmacodynamic model.

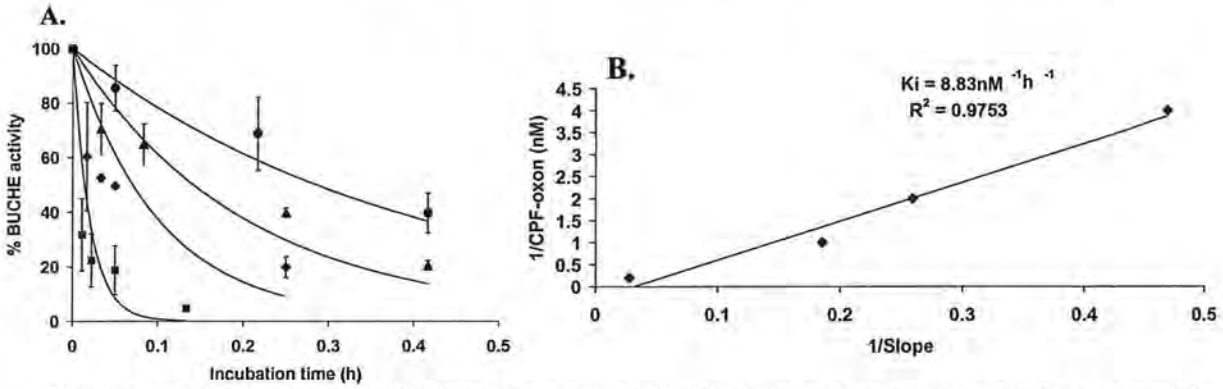

Figure 5. (A) Bimolecular inhibition rate constant (Ki) calculation using the pharmacodynamic model optimization against the experimental data. The data represent percentage of BuChE activity described as the rate of acetylthiocholine (ATC) substrate hydrolysis as a function of chlorpyrifos-oxon concentration for different incubation periods. Circles represent chlorpyrifos-oxon concentration of 0.25 nM; triangles 0.5 nM; diamonds 1.0 nM; and square 5 nM. Each line represents the model simulation for a given data set. (B) Final Ki determination plot. (Kousba *et al.*, 2003).

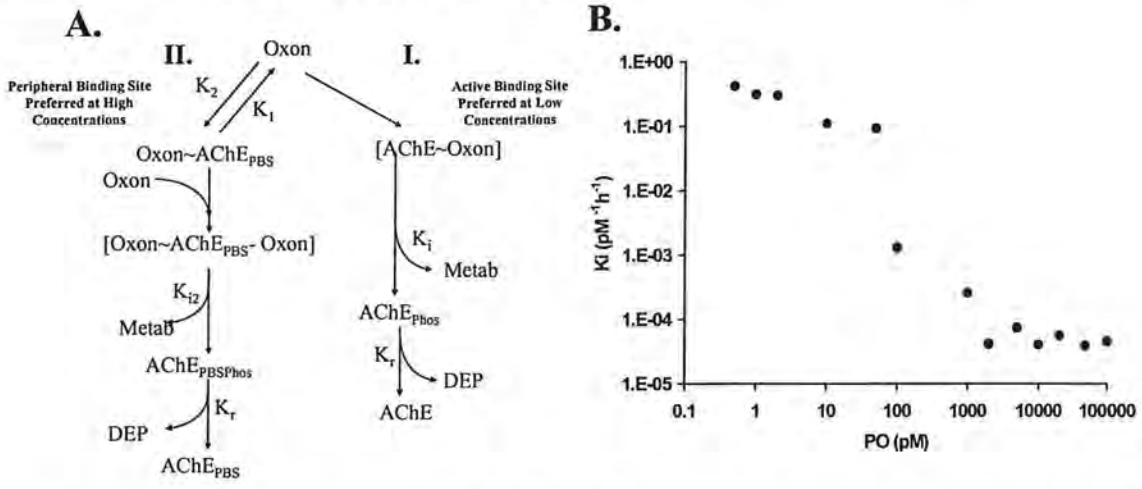

Figure 6. (A) Diagram of oxon interaction with the AChE binding site (**I**) and with the active binding site that includes a putative peripheral binding site (**II**); (B) relationship between Ki values and paraoxon (PO) concentrations where Ki values were determined using the model optimizations (Kousba *et al.*, 2004a).

The bimolecular inhibitory rate constant (Ki) describes the rate of ChE inhibition and the inhibitory potency for organophosphorus insecticides, therefore it is of critical importance for modeling the pharmacodynamics of ChE inhibition. Previous methods for

determining Ki utilized high concentrations of oxon and assumed that the reaction approximated first-order conditions. However, it has been suggested that the use of high oxon concentrations could be a source of error in determining a Ki, due to the presence of a peripheral binding site that when occupied reduces the capacity of other oxon molecules to phosphorylate the active site. A schematic diagram for the interaction of the oxon with the AChE active binding site (**I**) and with the active binding site that includes a putative peripheral binding site (**II**) is illustrated in **Figure 6A** and is more fully described in Kousba *et al.* (2004). Based on the peripheral binding site model, the relationship between Ki values and oxon concentration (paraoxon) is further illustrated in **Figure 6B**. At low oxon concentration ($5x10^{-4}$ to < 1 nM) the Ki ranges from 420 to 0.30 nM^{-1} h^{-1} and demonstrates a relatively linear change as a function of oxon concentration. Whereas, high oxon concentrations (1-100 nM) result in a substantially lower Ki (0.250 to 0.019 nM^{-1} h^{-1}) and were relatively insensitive to changes in oxon concentration. These data suggest that the presence of a peripheral binding site may play an important role in determining the biological interaction between AChE and oxon, and may be of particular relevance in understanding the dynamics associated with exposure at low environmentally relevant doses. These pharmacodynamic parameter estimates have been utilized in the development and refinement of the PBPK/PD models for chlorpyrifos, diazinon and a binary mixture. These experiments directly support **Specific Aims 1, 2 & 4.**

6.5 In Vivo Dosimetry and Dynamic Studies.

The pharmacokinetic and pharmacodynamic impact of acute binary exposures to chlorpyrifos and diazinon in rats was recently evaluated (Timchalk *et al.*, 2005). Co-exposure to chlorpyrifos:diazinon at 15:15 mg/kg, did not appreciably alter the pharmacokinetics of chlorpyrifos, diazinon or their metabolites in blood (data not shown); whereas, a 60:60 mg/kg dose resulted in a transient increase in the maximum blood concentration (C_{max}), area-under-the-concentration-curve (AUC), and decreased clearance of both compounds, likely due to competition between chlorpyrifos and diazinon for metabolism (see **Figure 7**).

The dynamics of ChE inhibition in brain, RBCs and plasma were likewise assessed following single or binary exposures to chlorpyrifos and diazinon over a range of doses and the results following the 30 mg/kg dose (Timchalk *et al.*, 2005) are presented in **Figure 8**. A dose-dependent inhibition of ChE was noted in tissues for both the single and co-exposures. The overall potency for ChE inhibition was greater for chlorpyrifos than diazinon and the binary mixture response appeared to be strongly influenced by chlorpyrifos. A comparison of the ChE binary response at the low dose (15 mg/kg), where there were no apparent pharmacokinetic interactions, suggested that the overall ChE response was additive (data not shown). These are the first reported experiments we are aware of that characterize both the pharmacokinetic and pharmacodynamic interactions between chlorpyrifos and diazinon in the rat, and are being used to further develop the binary physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for mixtures. These experiments directly support ***Specific Aim 3.***

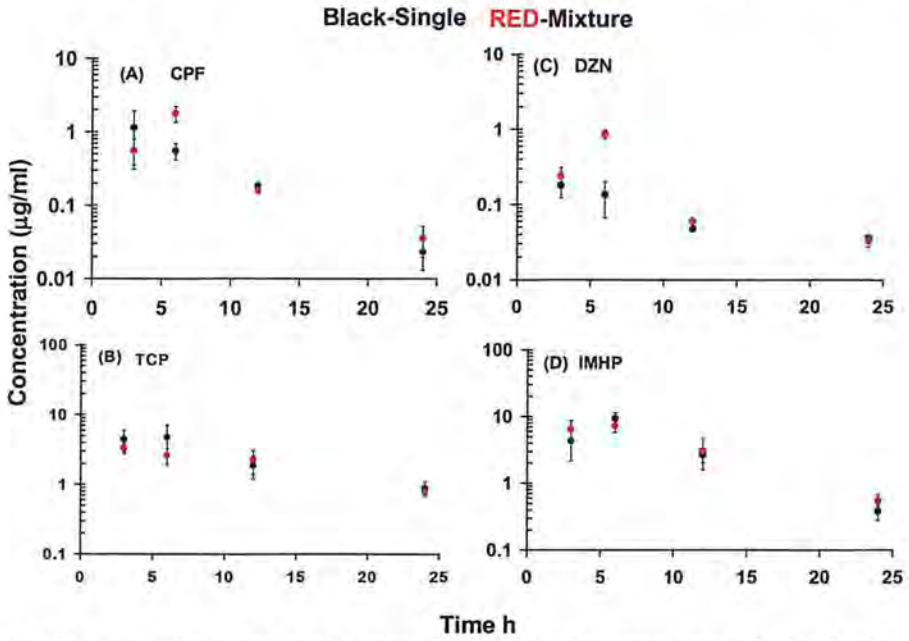

Figure 7. The concentration of chlorpyrifos (CPF) (A), trichloropyridinol (TCP) (B), diazinon (DZN) (C), and isopropyl methyl hydroxypyrimidine (IMHP) (D) in the blood of Sprague-Dawley rats following single 60 mg/kg oral gavage dose of chlorpyrifos or diazinon (**black** circles) and a binary mixture of chlorpyrifos : diazinon (**red** circles). The data are expressed as concentration (µg/ml) over time (h) and represent the mean ± S D of 4 animals per treatment time-point (Timchalk *et al.*, 2005).

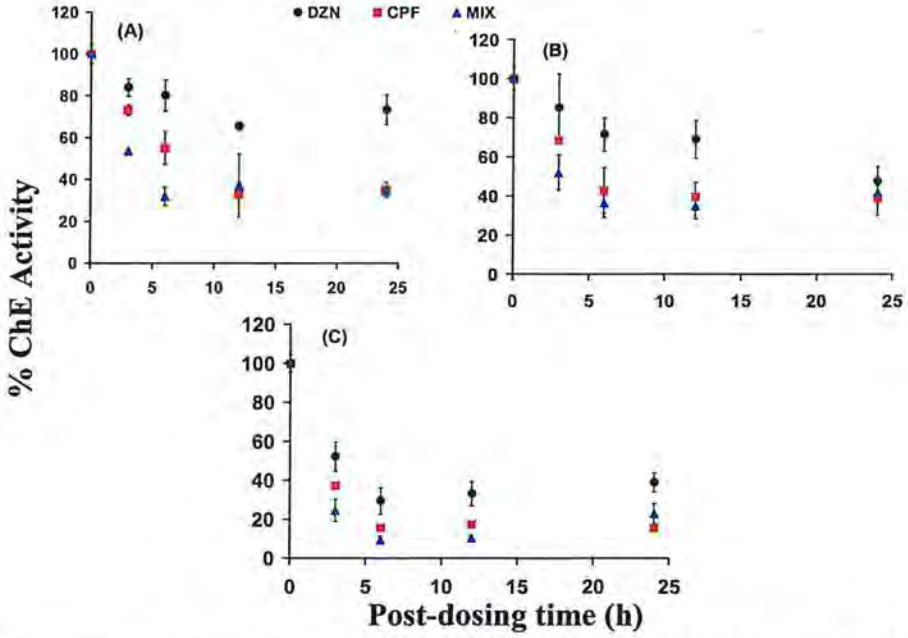

Figure 8. Experimental data for the brain (A), RBCs (B), and plasma (C) cholinesterase (ChE) activity in Sprague-Dawley rats following a 30 mg/kg oral gavage dose of diazinon (DZN) (**black**), chlorpyrifos (CPF) (**red**) and their binary mixture (**blue**). The data are expressed as % total ChE activity as a function of time (h) and represent the mean ± S D of 4 animals per treatment time-point (Timchalk *et al.*, 2005).

6.6 PBPK/PD Model Development and Validation.

A major objective for developing PBPK/PD models is to improve accuracy of human health risk assessments. Model sensitivity analysis has been used to critically characterize the distribution of model responses associated with variability and uncertainty in key model parameters. Initial sensitivity analysis of the chlorpyrifos PBPK/PD model suggested that the model was particularly sensitive to PON1 (arylesterase) metabolic capacity for the metabolism of chlorpyrifos-oxon to trichloropyridinol (Timchalk *et al.*, 2002). A genetic polymorphism in PON1 detoxification of organophosphorus insecticides, results in the expression of a range of enzyme activities within humans. Individuals with the QQ PON1 polymorphism are poor metabolizers of chlorpyrifos (most sensitive). A Monte Carlo analysis was conducted to investigate the impact of human PON1 status on the theoretical concentration of chlorpyrifos-oxon in the brain (Timchalk *et al.*, 2002). The distribution of the brain AUC for chlorpyrifos-oxon is presented in **Figure 9A** and the arrows indicate the lowest observed enzyme activity resulting in the highest theoretical levels of chlorpyrifos-oxon in the human brain. A dose-response for the inhibition of plasma BuChE in humans exposed to a broad range of chlorpyrifos doses is also presented in **Figure 9B**. Simulations of exposure to doses > 0.5 mg/kg results in nearly complete depletion of plasma BuChE and is consistent with the dose-dependent increase in brain oxon concentrations. These results suggest that the PON1 polymorphism has the greatest impact on target tissue dosimetry at dose levels (i.e. > 0.5 mg/kg) that overwhelm other detoxification pathways (i.e. non-target esterases). These results have been used to help determine the overall impact of parameter variability on the risk assessment prediction for organophosphorus insecticides.

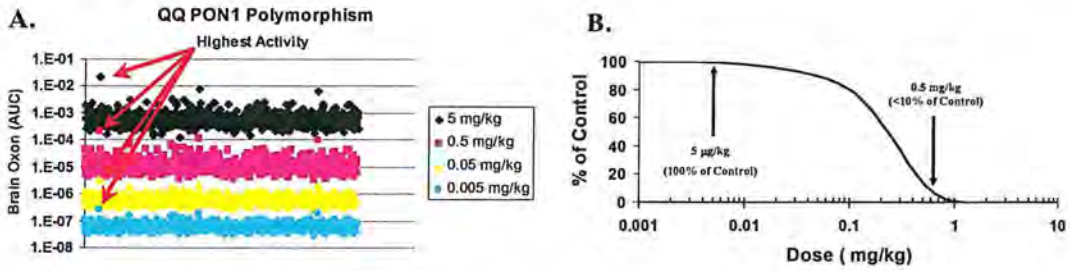

Figure 9. (A) Simulation of the distribution of brain chlorpyrifos-oxon (CPF-oxon) concentration for the distribution of enzyme activity in humans having a QQ polymorphism in PON1 over a range of chlorpyrifos doses. (B) Simulation of peak plasma butyrylcholinesterase (BuChE) inhibition dose-response in humans following an acute exposure to a broad range of chlorpyrifos doses (Timchalk *et al.*, 2002).

The PBPK/PD model developed for chlorpyrifos has been modified to incorporate diazinon specific parameters and *in vitro* and *in vivo* pharmacokinetic and pharmacodynamic studies have been conducted to support model development and validation. In addition, previously published rat and human dosimetry and ChE inhibition data have also been used to further validate the model (Poet *et al.*, 2004). The time-course of diazinon in plasma and AChE inhibition in brain of rats following acute oral administration of diazinon are presented in **Figure 10**. These data illustrate that the model accurately predicted both the plasma time-course of diazinon and the dose-dependent inhibition of brain AChE activity.

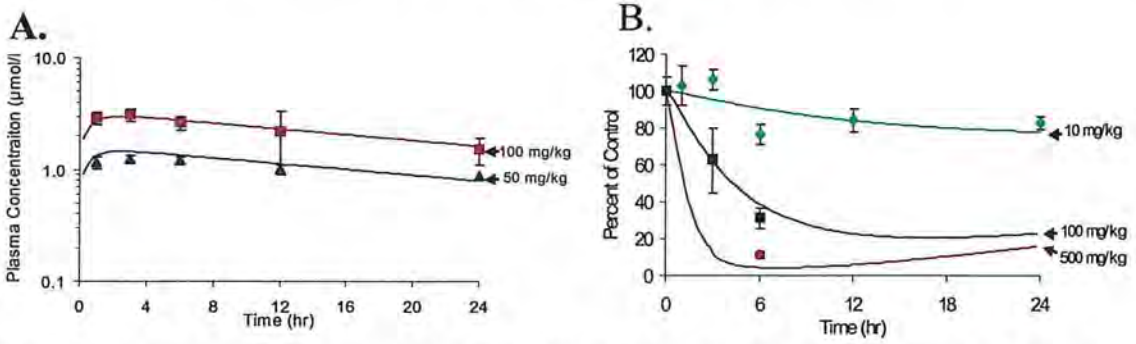

Figure 10. Experimental data (symbols) and simulations (lines) for (A) the concentration of diazinon in blood of rats, and (B) the inhibition of brain acetylcholinesterase (AChE) activity in rats administered diazinon by oral gavage at dose levels ranging from 10 -500 mg/kg. The data represent mean ± S D for 5 animals per treatment group (Poet *et al.*, 2004).

The model was also used to simulate available human oral and dermal dosimetry data obtained as part of a previously published controlled human exposure study. Human specific-physiological parameters were applied to the model and the resulting simulations and fits are presented in **Figure 11**. The fit of the PBPK/PD model to the oral and dermal absorption data was good, with more than 95% of the variability of the data explained by optimizing the dermal skin permeability coefficient (Kp) using the statistical Log Likelihood Function (LLF) within the simulation software (SIMUSOLV®). The LLF gives a percent variation explained, which is frequently used as a criteria to assess the quality of the model fit to the data, with 100% indicating a perfect fit.

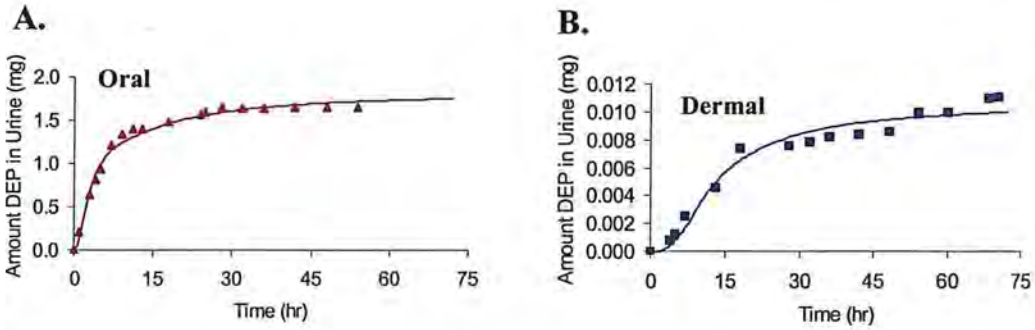

Figure 11. Experimental data from Garfitt *et al.* (2002) and simulations (lines) for the urinary elimination of diethylphosphates following oral (11 µg/kg) and dermal (3.5 mg/kg) exposures in human volunteers (Poet *et al.*, 2004a).

A binary PBPK/PD model for chlorpyrifos and diazinon has been developed. In this model, the CYP450 metabolism of both chlorpyrifos and diazinon to their oxon's and detoxification metabolites (trichloropyridinol & isopropyl methyl hydroxypyrimidine, respectively) is described by incorporating metabolic inhibition equations (see **Fig. 3 eq. 1 & 2**) and also assumes an additive response for ChE inhibition dynamics. This was based upon the *in vitro* and *in vivo* studies conducted as part of this project, and upon available data from the literature. Simulations of the binary interactions of chlorpyrifos and diazinon as equal doses and at doses that resulted in an equal pharmacodynamic

response are presented in **Figure 12**. As with the observed experimental results (see **Figure 8**) at equal doses (mg/kg) chlorpyrifos has a much greater impact on ChE inhibition in both plasma and brain, and the impact on dosimetry is only observed at very high acute doses (data not shown). This model development and validation directly supports **Specific Aims 1 & 4.**

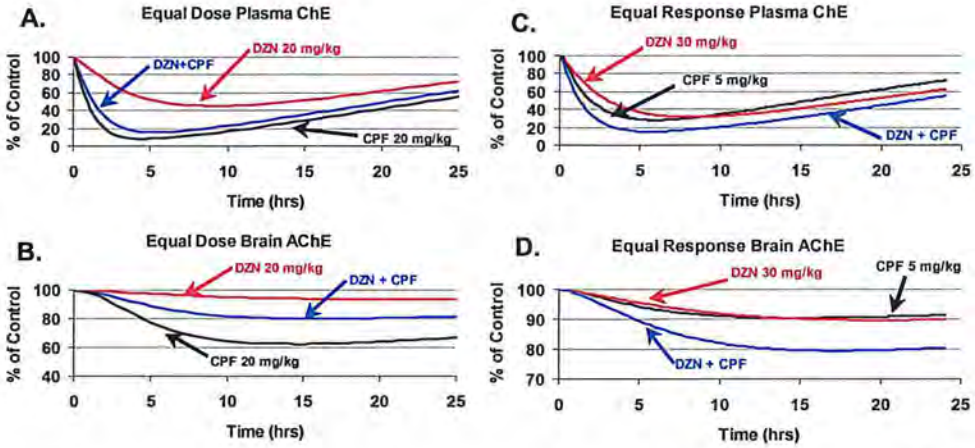

Figure 12. Binary PBPK/PD model simulation of pharmacodynamic response for plasma cholinesterase (ChE) and brain acetylcholinesterase (AChE) following equal individual doses (20 mg/kg) of chlorpyrifos (CPF) and diazinon (DZN) and CPF and DZN as a mixture (10 + 10 mg/kg) (A & B), or at equal response (C & D) doses (diazinon=30, chlorpyrifos=5 mg/kg).

6.7 Nicotine/Ethanol Metabolic Interactions with Chlorpyrifos.

To further establish the potential for metabolic interactions between chlorpyrifos and nicotine or ethanol, groups of rats were exposed to either nicotine (1 mg/kg, ip, 5 doses) or ethanol (1 g/kg, oral gavage, 5 doses), microsomes were then prepared and CYP450 metabolism of chlorpyrifos to chlorpyrifos-oxon and trichloropyridinol were compared for the nicotine and ethanol treatments against the response in microsomes from naïve rats and is illustrated in **Figure 13**. In these preliminary studies, nicotine appears to increase the V_{max} for metabolism of chlorpyrifos to both chlorpyrifos-oxon and trichloropyridinol; whereas, ethanol pretreatment may result in a decrease in the K_m for metabolism of chlorpyrifos to trichloropyridinol, but did not appear to have any impact on V_{max}. Although these preliminary results establish that both nicotine and ethanol can impact chlorpyrifos metabolism, more extensive *in vitro* and *in vivo* studies are needed to characterize the metabolic interaction, to quantitate the impact on dosimetry, and for development of a PBPK/PD model. Also, induction of at least CYP2E1 by nicotine is time-dependent and peak induction may not have been achieved for these samples, as evidenced by Western Blot analysis (data not shown). Secondly, based on previously published studies that established the complementary CYP induction from ethanol and nicotine co-exposure it is anticipated that a binary exposure to nicotine and ethanol may have an even greater impact on the metabolism of chlorpyrifos than either of these agents alone.

CPF-oxon	Nicotine	Ethanol	Naïve
V_{max}	1.76	1.10	0.929
K_m	63.2	41.0	41.0
TCP			
V_{max}	3.53	2.27	2.57
K_m	18.0	10.6	23.9

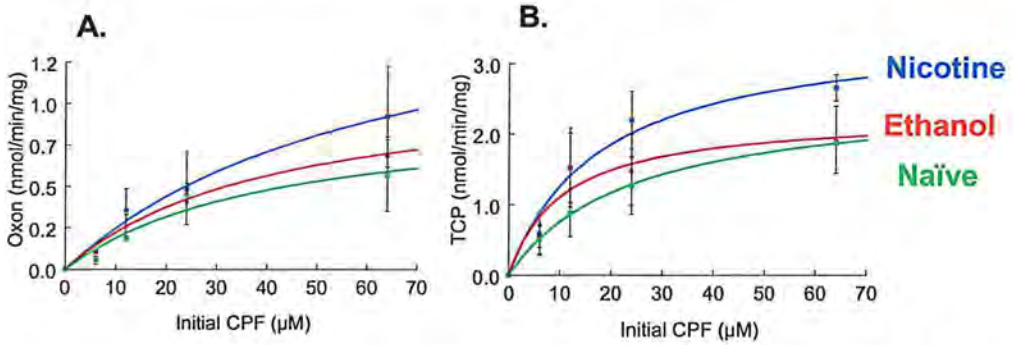

Figure 13. *In vitro* CYP450 metabolism of (A) chlorpyrifos (CPF) to chlorpyrifos-oxon (CPF-oxon) and (B) chlorpyrifos (CPF) to trichloropyridinol (TCP) in microsomes from livers of 4 Sprague-Dawley rats/group that were naive (**green**), exposed to nicotine (**blue**; ip 5 days) or ethanol (**red**; gavage 5 days). The data are expressed as mean ± S D for 4 samples. The K_m (μM) and V_{max} (nmol/min/mg) were determined by fitting the experimental data to the mathematical model, and the parameter estimates are presented in the table.

6.8 Summary Conclusion and Future Direction.

This project has resulted in the development, validation and application of integrated PBPK/PD models to assess both dosimetry and dynamic response for individual and binary exposures to insecticides in animal models and humans. It is concluded that this modeling strategy can readily be extended to include other insecticides, and that limited *in vitro* experiments can be utilized to ascertain interactions both at the level of metabolism and for ChE inhibition. For example, in the case of chlorpyrifos and diazinon metabolic interactions do occur, but at relatively high doses so that it is reasonable to assume that at occupational exposure levels the kinetics would be linear (no interaction), whereas the extent of ChE inhibition would follow a dose additive response. Future studies should extend the strategy to assess the impact of exposure to commonly used and abused drugs (i.e. ethanol and nicotine) that are routinely consumed at pharmacologically active doses and have the demonstrated potential to modify both the metabolism and dynamic response associated with acute and chronic exposure to insecticides.

7.0 Peer Reviewed Publications (Note: bullet points identify specific aim supported).

1. Timchalk C, Kousba A, Poet TS: [2002] Monte Carlo analysis of the human chlorpyrifos-oxon (PON1) polymorphism using a physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model. Toxicology Letter 135 (1): 51-59.
   - **Aims 1 & 4** (PBPK/PD model development).
2. Poet TS, Wu H, Kousba AA, Timchalk C: [2003] *In vitro* rat hepatic and enterocyte metabolism of the organophosphate pesticides chlorpyrifos and diazinon. Toxicological Science 72**:** 193-200.
   - **Aims 1, 2 & 4** (*in vitro* metabolic parameter estimates/model development)
3. Kousba AA, Poet TS, Timchalk C: [2003] Characterization of the *in vitro* kinetic interaction of chlorpyrifos-oxon with rat salivary cholinesterase: a potential biomonitoring matrix. Toxicology 188 (2): 219-232.
   - **Aims 1, 2, & 4** (*in vitro* metabolic parameter estimates/model development)
4. Timchalk C, Poet TS, Kousba AA, Campbell JA, Lin Y: [2004] Development of non-invasive biomonitoring approaches to determine dosimetry and risk following acute chemical exposure: Analysis of lead and organophosphate insecticide in saliva. Journal Toxicology Environmental Health, Part A 67: 635-650.
   - Review article highlights overall impact of research.
5. Poet TS, Kousba AA, Dennison S, Timchalk C: [2004] Physiologically based pharmacokinetic/ pharmacodynamic model for the organophosphate pesticide diazinon. Neurotoxicology 25 (6): 1013-1030.
   - **Aims 1 & 4** (PBPK/PD model development).
6. Kousba AA, Sultatos LG, Poet TS, Timchalk C: [2004] Comparison of chlorpyrifos-oxon and paraoxon acetylcholinesterase inhibition dynamics: potential role of a peripheral binding site. Toxicological Science 80**:** 239-248.
   - **Aims 1, 2, & 4** (*in vitro* inhibition parameter estimates/model development).
7. Busby A, Kousba A, Timchalk C: [2004] The *in vivo* quantitation of diazinon, chlorpyrifos and their metabolites in rat blood for the refinement of a physiologically–based pharmacokinetic/pharmacodynamic model. Journal of Undergraduate Research (Department of Energy) 4**:** 36 – 40.
   - **Aims 1, 3 & 4** (*in vivo* dosimetry and dynamic response/model development.
8. Timchalk C, Poet TS, Hinman MN, Busby AL, Kousba AA: [2005] Pharmacokinetic & pharmacodynamic interaction for a binary mixture of chlorpyrifos and diazinon in the rat. Toxicology Applied Pharmacology 205(1): 21-32.
   - **Aims 3 & 4** (*in vivo* dosimetry and dynamic response/model development).
9. Campbell JA, Timchalk C, Kousba AA, Wu H, Hoppe EW: [2005] Application of negative ion chemical ionization mass spectrometry for the analysis of trichloropyridinol in saliva of rats exposed to chlorpyrifos. Analytical Letter 38**:** 949-959.

- **Aims 3 & 4** (*in vivo* dosimetry and dynamic response/model development).

10. Cole TB, Walter BJ, Richter RJ, Shih, DM, Tward A, Lusis AJ, Timchalk C, Costa LG, Furlong CE: [2005] Toxicity of chlorpyrifos and chlorpyrifos oxon in a transgenic mouse model of the human paraoxonase (PON1) Q192R polymorphism. Pharmacogenetic Genomics 15(8): 589-598.
    - **Aim 4** (*in vivo* dynamic model development transgenic animal).
11. Furlong CE, Cole TB, Walter B J, Shih DM, Tward A, Lusis AJ, Timchalk C, Richter RJ, and Costa LC: [2005] Paraoxonase 1 (PON1) status and risk of insecticide exposure. Journal Biochemistry Molecular Toxicology 19(3): 182-183.
    - **Aims 1 & 4** (*in vivo* dynamic model development).
12. Timchalk C, Kousba AA, Poet TS: [2006] Age-dependent pharmacokinetic and pharmacodynamic response in neonatal rats following oral exposure to the organophosphorus insecticide chlorpyrifos. Toxicology, in press.
    - **Aim 1 & 4** (*in vivo* dynamic model development neonatal rat stage).

7.1 Book Chapters (Note: bullet points identify specific aim supported):

1. Timchalk C: [2001] Organophosphate Pharmacokinetics, Chapter 46 In: Hayes' Handbook of Pesticide Toxicology, Second Edition, (Ed. R. Krieger), Academic Press pp, 929 - 951.
    - Overview of organophosphate pharmacokinetics, highlight diazinon.
2. Timchalk C: [2005] Physiologically based pharmacokinetic and pharmacodynamic modeling of organophosphorus and carbamate pesticides. In: Toxicology of Organophosphate and Carbamate Pesticides. (Eds RC Gupta), Elsevier, in press.
    - Overview of PBPK/PD model development, highlights chlorpyrifos and diazinon.

7.2 Abstracts/Presentations:

1. Timchalk C, Kousba A, Poet TS: [2002] Assessing the impact of human PON1 polymorphisms: sensitivity and monte carlo analyses using a physiologically based pharmacokinetic/ pharmacodynamic (PBPK/PD) model for chlorpyrifos. Toxicological Science 66 (1-S): 1525.
2. Kousba A, Poet T, Timchalk C: [2002] Comparative study of the kinetic interaction of chlorpyrifos oxon with rat cholinesterase. Pacific Northwest Society of Toxicology Meeting, Richland, WA, Sept 19-20.
3. Wu H, Weitz K, Timchalk C, Poet TS: [2002] Hepatic and intestinal A-esterase metabolism of chlorpyrifos-oxon and diazinon-oxon. Pacific Northwest Society of Toxicology Meeting, Richland, WA, Sept 19-20.
4. Campbell JA, Wu H, Poet TS, Timchalk C: [2002] Determination of chlorpyrifos and the major metabolite 3,5,6-trichloro-2-pyridinol in blood and saliva of exposed rats. American Society Mass Spectrometry, Orlando, FL, June 2– 6.
5. Poet TS, Kousba A, Timchalk C: [2002] Physiologically based pharmacokinetic modeling of the organophosphate pesticide diazinon. International Society Exposure Assessment (ISEA), Vancouver, BC, August 11- 15.

6. Wu H, Kousba A, Timchalk C, Poet TS: [2002] Hepatic and intestinal CYP450 metabolism of chlorpyrifos. Toxicology Science 66 (1-S): LB134.
7. Campbell JA, Wu H, Poet TS, Kousba AA, Timchalk C: [2003] Determination of chlorpyrifos and the major metabolite 3,5,6-trichloro-2-pyridinol in blood and saliva of exposed rats. American Society of Mass Spectrometry and Allied Topics, Montreal, Canada, June 11.
8. Timchalk C, Lin Y: [2003] Development of non-invasive biomoitoring approaches to determine dosimetry and risk following acute chemical exposure: analysis of lead and organophosphate insecticide in saliva. Theories and Practices in Toxicology and Risk Assessment, Cinn., OH, April 28-30.
9. Timchalk C, Lin Y, Kousba A, Poet T: [2003] Development of pharmacokinetic and non-invasive biomonitoring approaches to determine dosimetry and assess risk in potentially sensitive sub-populations following exposure to individual chemicals and mixtures. CDC/NIOSH, 4th Annual NORA Symposium, Working Partnerships, Research to Practice, June 23-24.
10. Timchalk C, Kousba A, Poet TS: [2003] Development of a neonatal rat physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) model for chlorpyrifos. Toxicological Science 72 (S-1): 1483.
11. Kousba A, Poet TS, Timchalk C: [2003] Potential utility of saliva biomonitoring for assessing organophosphate insecticide dosimetry and esterase inhibition. Toxicological Science, 72 (S-1): 1484.
12. Wu H, Timchalk C, Kousba A, Poet TS: [2003] Intestinal metabolism of organophosphate insecticides: potential first-pass metabolism. Toxicological Science, 72 (S-1): 440.
13. Poet TS, Kousba A, Wu H, Dennison SL, Timchalk C: [2003] Development of a physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for the organophosphate pesticide, diazinon. Toxicological Science, 72 (S-1): 1485.
14. Wu H, Poet TS, Timchalk C: [2004] Inhibition of diazinon metabolism by chlorpyrifos in rat liver microsomes. Toxicological Science 78 (1-S).
15. Kousba A, Sultatos LG, Poet TS, Timchalk C: [2004] Comparison of chlorpyrifos-oxon and paraoxon acetylcholinesterase inhibition dynamics: potential role of a peripheral binding site. Toxicological Science, 78 (1-S).
16. Timchalk C, Poet TS, Hinman MN, Busby AL, Kousba A: [2004] Pharmacokinetic & pharmacodynamic interactions of a binary mixture of chlorpyrifos and diazinon in the rat. Toxicological Science, 78 (1-S).
17. Busby A, Kousba A, Timchalk C: [2004] The *in vivo* quantitation of diazinon, chlorpyrifos and their metabolites in rat blood for the refinement of a physiologically–based pharmacokinetic/pharmacodynamic model. American Association for the Advancement of Science (AAAS) Annual Meeting, Seattle, WA. Feb 12-16th.
18. Kousba A, Poet TS, Zangar R, Timchalk C: [2004] *In vitro* interactions between organophosphorus pesticides and consequence of pre-treatment with nicotine and alcohol on *in vitro* pesticide metabolism in rats. 7th International Meeting, International Society for the Study of Xeonbiotics (ISSX), Vancouver, Canada, Aug 29th – Sept. 2nd,

19. Timchalk C: [2004] Development of a physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model to quantitate biomarkers of exposure to organophosphorus insecticides. Environmental Protection Agency Science Forum, Washington, DC.
20. Timchalk C, Poet TS: [2005] Symposium: Pesticide neurotoxicity in adults: integrating contributions from epidemiology and toxicology: Presentation: Development of a physiologically based pharmacokinetic and pharmacodynamic model to determine dosimetry, dynamic response, and assess risk following exposure to organophosphorus insecticides. Society of Toxicology Annual Meeting, New Orleans, March.
21. Furlong CE, Cole TB, Pettan-Brewer C, Shih DM, Tward A, Lusis AJ, Timchalk C, Richter RJ, Costa LG: [2005] Paraoxonase 1 (PON1) status affects the metabolism of drugs and insecticides. Drug Metabolism Review, 37(2): 21.

FINANCIAL STATUS REPORT

(Short Form)

(Follow instructions on the back)

1. Federal Agency and Organizational Element to Which Report is Submitted	2. Federal Grant or Other Identifying Number Assigned By Federal Agency	OMB Approval No.	Page of
Centers for Disease Control and Prev.	R1OH03629A / 5 R01 OH003629-03	0348-0038	1 2 pages

3. Recipient Organization (Name and complete address, including ZIP code)
Batelle Memorial Institute, Pacific Northwest National Laboratory, 902 Battelle Blvd., Richland, WA 99352

4. Employer Identification Number	5. Recipient Account Number or Identifying Number	6. Final Report	7. Basis
31-4379427	29733A	☑ Yes ☐ No	☐ Cash ☑ Accrual

8. Funding/Grant Period *(See instructions)* From: (Month, Day, Year)	To: (Month, Day, Year)	9. Period Covered by this Report From: (Month, Day, Year)	To: (Month, Day, Year)

10. Transactions:	I Previously Reported	II This Period	III Cumulative
a. Total outlays	0.00	814,409.64	814,409.64
b. Recipient share of outlays			0.00
c. Federal share of outlays	0.00	814,409.64	814,409.64
d. Total unliquidated obligations			0.00
e. Recipient share of unliquidated obligations			0.00
f. Federal share of unliquidated obligations			0.00
g. Total Federal share *(Sum of lines c and f)*			814,409.64
h. Total Federal funds authorized for this funding period			819,124.00
i. Unobligated balance of Federal funds *(Line h minus line g)*			4,714.36

11. Indirect Expense	a. Type of Rate *(Place "X" in appropriate box)* ☑ Provisional ☐ Predetermined ☐ Final ☐ Fixed			
	b. Rate	c. Base	d. Total Amount	e. Federal Share
	See attached		367,242.14	367,242.14

12. *Remarks: Attach any explanations deemed necessary or information required by Federal sponsoring agency in compliance with governing legislation.*

13. Certification: **I certify to the best of my knowledge and belief that this report is correct and complete and that all outlays and unliquidated obligations are for the purposes set forth in the award documents.**

Typed or Printed Name and Title	Telephone (Area code, number and extension)
Jill Praznik / Accounts Receivable	509-373-7633
Signature of Authorized Certifying Official	**Date Report Submitted**
	December 19, 2005

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Centers for Disease Control
and Prevention

DATE: July 10, 2007

TO: William D. Bennett
Data Systems Team, Information Resources Branch, EID, NIOSH, P03/C18

THRU: W. Allen Robison, Ph.D., Program Lead
Office of Extramural Coordination and Special Projects, NIOSH, E74

FROM: Jim Newhall, Ph. D., Scientific Program Administrator
Office of Extramural Coordination and Special Projects, NIOSH, E74

SUBJECT: Final Report Submitted for Entry into NTIS, Grant Number: 5R01OH003629-03

The attached final report has been received from the principal investigator on the subject NIOSH grant. When the document is forwarded to the National Technical Information Service, please inform us of the document number. This will allow us to respond to inquiries received about the final report. Publications are highlighted on the attached final report summary.

Attachments:
Final Report Summary

cc: Sherri Diana, EID, P03/C18

Title: Complex Mixture Modeling Organophosphate Pesticides
Investigator: Charles Timchalk
Affiliation: Battelle Pacific Northwest Laboratories
State: WA
Telephone: (509) 376-0434
Award Number: 5R01OH003629-03
Start & End Dates: 9/30/2001-9/29/2005
Program Area: Exposure Assessment

NIOSH Scientific Administrator: Jim Newhall, Ph.D.

Final Report Abstract:
This project used a quantitative experimental and modeling approach to evaluate the potential impact that pesticide mixture exposures might have on agricultural workers, who are routinely exposed to insecticides. Pesticide interactions can share a common theme in which dosimetry and biological responses are altered when mixtures modify absorption rates, extent of metabolism, tissue distribution, clearance or pharmacological action.

Organophosphorus insecticides are of particular concern since they are widely utilized, are neurotoxic, and a number of biomonitoring studies have documented both occupational and non-occupational exposures in adults and children to multiple pesticides. The current risk assessment paradigm focuses on individual chemicals; however, exposure is primarily to mixtures where there is limited understanding of the health effects. Occupationally exposed agricultural workers, handle concentrated pesticide formulations and therefore have higher exposures. Exposure to chemical mixtures can involve complex "chemical soups" which are poorly characterized in terms of components, concentrations, exposure duration and routes, and the overall toxicological effect of the mixture is most likely unknown. These uncertainties in terms of exposure, dose and biological response create difficulty in determining realistic risk from occupational exposure to mixtures.

The overall approach was to develop an experimental/modeling strategy to understand the impact of complex chemical mixtures on the toxicological response of organophosphorus pesticides. The approach focused on understanding both the pharmacokinetic (absorption, distribution, metabolism and excretion) and pharmacodynamic (cholinesterase inhibition) responses associated with individual and binary mixtures of organophosphorus insecticides. The project strategy integrated the development of physiologically based pharmacokinetic and pharmacodynamic models with the acquisition of focused in vitro and in vivo experimental data for model development and validation. The strength of this approach is that it can be used to identify the most critical factors (i.e. exposure timing, routes of exposure, lack of protective equipment) that contribute to occupational exposure to insecticides, and allow researchers and regulators to assess internal dose and biological response with greater confidence. This project resulted in the development, validation and application of computational models assessing dosimetry and biological response for individual and binary mixtures of insecticides in animal models and humans. This experimentation/modeling strategy can now be expanded to assess the impact of lifestyle choices and in particular the impact of co-exposures to routinely consumed

pharmacologically active agents (ex. pharmaceuticals, alcohol and nicotine) that have the demonstrated potential to likewise modify metabolism and biological response to insecticides. Since a comprehensive toxicological evaluation of chemical mixtures is not feasible, it is reasonable to develop strategies that can be applied to classes of chemical agents that have similar metabolic and mode of action profiles. In this regard, the Environmental Protection Agency (EPA) has developed a framework for conducting cumulative risk assessments for organophosphorus and other pesticides that have a common mode of toxicological action. They have indicated that physiologically based pharmacokinetic modeling approaches represent the future direction for conducting cumulative dose-response risk assessments. In this regard, the EPA has identified the organophosphorus insecticide models that have been developed as part of this project as representing key initial components in this process.

Impact of the Project:
This project has resulted in the development, validation and application of integrated PBPK/PD models to assess both dosimetry and dynamic response for individual and binary exposures to insecticides in animal models of humans. It is concluded that this modeling strategy can readily be extended to include other insecticides, and that limited in vitro experiments can be utilized to ascertain interactions both at the level of metabolism and for ChE inhibition. These results have facilitated our understanding of complex chemical interactions as it relates to the occupational health implications of working with insecticides. This strategy of linking focused research with validated models is a significant step forward in developing an approach for the evaluation of complex chemical mixtures. The ultimate goal is to apply these models to assess the contribution that lifestyle choices, may or may not have on the risk associated with occupational exposure to insecticides

Publications:

Timchalk C, Kousba A, Poet TS: [2002] Monte Carlo analysis of the human chlorpyrifos-oxon (PON1) polymorphism using a physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model. Toxicology Letter 135 (1): 51-59.

Poet TS, Wu H, Kousba AA, Timchalk C: [2003] In vitro rat hepatic and enterocyte metabolism of the organophosphate pesticides chlorpyrifos and diazinon. Toxicological Science 72: 193-200.

Kousba AA, Poet TS, Timchalk C: [2003] Characterization of the in vitro kinetic interaction of chlorpyrifos-oxon with rat salivary cholinesterase: a potential biomonitoring matrix. Toxicology 188 (2): 219-232.

Timchalk C, Poet TS, Kousba AA, Campbell JA, Lin Y: [2004] Development of non-invasive biomonitoring approaches to determine dosimetry and risk following acute chemical exposure: Analysis of lead and organophosphate insecticide in saliva. Journal Toxicology Environmental Health, Part A 67: 635-650.

20029369 Poet TS, Kousba AA, Dennison S, Timchalk C: [2004] Physiologically based pharmacokinetic/ pharmacodynamic model for the organophosphate pesticide diazinon. Neurotoxicology 25 (6): 1013-1030.

TO LAD 12-5-07 Kousba AA, Sultatos LG, Poet TS, Timchalk C: [2004] Comparison of chlorpyrifos-oxon and paraoxon acetylcholinesterase inhibition dynamics: potential role of a peripheral binding site. Toxicological Science 80: 239-248.

TO LAD 12-5-07 Busby A, Kousba A, Timchalk C: [2004] The in vivo quantitation of diazinon, chlorpyrifos and their metabolites in rat blood for the refinement of a physiologically–based pharmacokinetic/pharmacodynamic model. Journal of Undergraduate Research (Department of Energy) 4: 36 – 40.

20029018 Timchalk C, Poet TS, Hinman MN, Busby AL, Kousba AA: [2005] Pharmacokinetic & pharmacodynamic interaction for a binary mixture of chlorpyrifos and diazinon in the rat. Toxicology Applied Pharmacology 205(1): 31-42. 31-42

TO LAD 12-5-07 Campbell JA, Timchalk C, Kousba AA, Wu H, Hoppe EW: [2005] Application of negative ion chemical ionization mass spectrometry for the analysis of trichloropyridinol in saliva of rats exposed to chlorpyrifos. Analytical Letter 38: 949-959.

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20029770 Timchalk C, Kousba AA, Poet TS: [2006] Age-dependent pharmacokinetic and pharmacodynamic response in neonatal rats following oral exposure to the organophosphorus insecticide chlorpyrifos. Toxicology, in press. 220(1); 13-25

7.0 Peer Reviewed Publications (Note: bullet points identify specific aim supported).

1. Timchalk C, Kousba A, Poet TS: [2002] Monte Carlo analysis of the human chlorpyrifos-oxon (PON1) polymorphism using a physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model. Toxicology Letter 135 (1): 51-59.
   - **Aims 1 & 4** (PBPK/PD model development).

2. Poet TS, Wu H, Kousba AA, Timchalk C: [2003] *In vitro* rat hepatic and enterocyte metabolism of the organophosphate pesticides chlorpyrifos and diazinon. Toxicological Science 72: 193-200.
   - **Aims 1, 2 & 4** (*in vitro* metabolic parameter estimates/model development)

3. Kousba AA, Poet TS, Timchalk C: [2003] Characterization of the *in vitro* kinetic interaction of chlorpyrifos-oxon with rat salivary cholinesterase: a potential biomonitoring matrix. Toxicology 188 (2): 219-232.
   - **Aims 1, 2, & 4** (*in vitro* metabolic parameter estimates/model development)

4. Timchalk C, Poet TS, Kousba AA, Campbell JA, Lin Y: [2004] Development of non-invasive biomonitoring approaches to determine dosimetry and risk following acute chemical exposure: Analysis of lead and organophosphate insecticide in saliva. Journal Toxicology Environmental Health, Part A 67: 635-650.
   - Review article highlights overall impact of research.

5. Poet TS, Kousba AA, Dennison S, Timchalk C: [2004] Physiologically based pharmacokinetic/ pharmacodynamic model for the organophosphate pesticide diazinon. Neurotoxicology 25 (6): 1013-1030.
   - **Aims 1 & 4** (PBPK/PD model development).

6. Kousba AA, Sultatos LG, Poet TS, Timchalk C: [2004] Comparison of chlorpyrifos-oxon and paraoxon acetylcholinesterase inhibition dynamics: potential role of a peripheral binding site. Toxicological Science 80: 239-248.
   - **Aims 1, 2, & 4** (*in vitro* inhibition parameter estimates/model development).

7. Busby A, Kousba A, Timchalk C: [2004] The *in vivo* quantitation of diazinon, chlorpyrifos and their metabolites in rat blood for the refinement of a physiologically–based pharmacokinetic/pharmacodynamic model. Journal of Undergraduate Research (Department of Energy) 4: 36 – 40.
   - **Aims 1, 3 & 4** (*in vivo* dosimetry and dynamic response/model development.

8. Timchalk C, Poet TS, Hinman MN, Busby AL, Kousba AA: [2005] Pharmacokinetic & pharmacodynamic interaction for a binary mixture of chlorpyrifos and diazinon in the rat. Toxicology Applied Pharmacology 205(1): ~~21-32.~~ 31-42.
   - **Aims 3 & 4** (*in vivo* dosimetry and dynamic response/model development).

9. Campbell JA, Timchalk C, Kousba AA, Wu H, Hoppe EW: [2005] ~~Application of~~ negative ion chemical ionization mass spectrometry for the analysis of trichloropyridinol in saliva of rats exposed to chlorpyrifos. Analytical Letter 38: 949-959.

- **Aims 3 & 4** (*in vivo* dosimetry and dynamic response/model development).

10. Cole TB, Walter BJ, Richter RJ, Shih, DM, Tward A, Lusis AJ, Timchalk C, Costa LG, Furlong CE: [2005] Toxicity of chlorpyrifos and chlorpyrifos oxon in a transgenic mouse model of the human paraoxonase (PON1) Q192R polymorphism. Pharmacogenetic Genomics 15(8): 589-598.
    - **Aim 4** (*in vivo* dynamic model development transgenic animal).
11. Furlong CE, Cole TB, Walter B J, Shih DM, Tward A, Lusis AJ, Timchalk C, Richter RJ, and Costa LC: [2005] Paraoxonase 1 (PON1) status and risk of insecticide exposure. Journal Biochemistry Molecular Toxicology 19(3): 182-183.
    - **Aims 1 & 4** (*in vivo* dynamic model development).
12. Timchalk C, Kousba AA, Poet TS: [2006] Age-dependent pharmacokinetic and pharmacodynamic response in neonatal rats following oral exposure to the organophosphorus insecticide chlorpyrifos. Toxicology, in press.
    - **Aim 1 & 4** (*in vivo* dynamic model development neonatal rat stage).

7.1 Book Chapters (Note: bullet points identify specific aim supported):

1. Timchalk C: [2001] Organophosphate Pharmacokinetics, Chapter 46 In: Hayes' Handbook of Pesticide Toxicology, Second Edition, (Ed. R. Krieger), Academic Press pp, 929 - 951.
    - Overview of organophosphate pharmacokinetics, highlight diazinon.
2. Timchalk C: [2005] Physiologically based pharmacokinetic and pharmacodynamic modeling of organophosphorus and carbamate pesticides. In: Toxicology of Organophosphate and Carbamate Pesticides. (Eds RC Gupta), Elsevier, in press.
    - Overview of PBPK/PD model development, highlights chlorpyrifos and diazinon.

7.2 Abstracts/Presentations:

1. Timchalk C, Kousba A, Poet TS: [2002] Assessing the impact of human PON1 polymorphisms: sensitivity and monte carlo analyses using a physiologically based pharmacokinetic/ pharmacodynamic (PBPK/PD) model for chlorpyrifos. Toxicological Science 66 (1-S): 1525.
2. Kousba A, Poet T, Timchalk C: [2002] Comparative study of the kinetic interaction of chlorpyrifos oxon with rat cholinesterase. Pacific Northwest Society of Toxicology Meeting, Richland, WA, Sept 19-20.
3. Wu H, Weitz K, Timchalk C, Poet TS: [2002] Hepatic and intestinal A-esterase metabolism of chlorpyrifos-oxon and diazinon-oxon. Pacific Northwest Society of Toxicology Meeting, Richland, WA, Sept 19-20.
4. Campbell JA, Wu H, Poet TS, Timchalk C: [2002] Determination of chlorpyrifos and the major metabolite 3,5,6-trichloro-2-pyridinol in blood and saliva of exposed rats. American Society Mass Spectrometry, Orlando, FL, June 2– 6.
5. Poet TS, Kousba A, Timchalk C: [2002] Physiologically based pharmacokinetic modeling of the organophosphate pesticide diazinon. International Society Exposure Assessment (ISEA), Vancouver, BC, August 11- 15.

Does not exist not found
6. Wu H, Kousba A, Timchalk C, Poet TS: [2002] Hepatic and intestinal CYP450 metabolism of chlorpyrifos. Toxicology Science 66 (1-S): LB134.
7. Campbell JA, Wu H, Poet TS, Kousba AA, Timchalk C: [2003] Determination of chlorpyrifos and the major metabolite 3,5,6-trichloro-2-pyridinol in blood and saliva of exposed rats. American Society of Mass Spectrometry and Allied Topics, Montreal, Canada, June 11.
8. Timchalk C, Lin Y: [2003] Development of non-invasive biomoitoring approaches to determine dosimetry and risk following acute chemical exposure: analysis of lead and organophosphate insecticide in saliva. Theories and Practices in Toxicology and Risk Assessment, Cinn., OH, April 28-30. not found
9. Timchalk C, Lin Y, Kousba A, Poet T: [2003] Development of pharmacokinetic and non-invasive biomonitoring approaches to determine dosimetry and assess risk in potentially sensitive sub-populations following exposure to individual chemicals and mixtures. CDC/NIOSH, 4th Annual NORA Symposium, Working Partnerships, Research to Practice, June 23-24. 20033138
10. Timchalk C, Kousba A, Poet TS: [2003] Development of a neonatal rat physiologically based pharmacokinetic/pharmacodynamic (PBPK/PD) model for chlorpyrifos. Toxicological Science 72 (S-1): 1483. 20033143
11. Kousba A, Poet TS, Timchalk C: [2003] Potential utility of saliva biomonitoring for assessing organophosphate insecticide dosimetry and esterase inhibition. Toxicological Science, 72 (S-1): 1484. 20022719
12. Wu H, Timchalk C, Kousba A, Poet TS: [2003] Intestinal metabolism of organophosphate insecticides: potential first-pass metabolism. Toxicological Science, 72 (S-1): 440. 20022560
13. Poet TS, Kousba A, Wu H, Dennison SL, Timchalk C: [2003] Development of a physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model for the organophosphate pesticide, diazinon. Toxicological Science, 72 (S-1): 1485. 20022717
14. Wu H, Poet TS, Timchalk C: [2004] Inhibition of diazinon metabolism by chlorpyrifos in rat liver microsomes. Toxicological Science 78 (1-S). 20025199
15. Kousba A, Sultatos LG, Poet TS, Timchalk C: [2004] Comparison of chlorpyrifos-oxon and paraoxon acetylcholinesterase inhibition dynamics: potential role of a peripheral binding site. Toxicological Science, 78 (1-S). 20025200
16. Timchalk C, Poet TS, Hinman MN, Busby AL, Kousba A: [2004] Pharmacokinetic & pharmacodynamic interactions of a binary mixture of chlorpyrifos and diazinon in the rat. Toxicological Science, 78 (1-S). 20025201
17. Busby A, Kousba A, Timchalk C: [2004] The *in vivo* quantitation of diazinon, chlorpyrifos and their metabolites in rat blood for the refinement of a physiologically–based pharmacokinetic/pharmacodynamic model. American Association for the Advancement of Science (AAAS) Annual Meeting, Seattle, WA. Feb 12-16th. Not Found
18. Kousba A, Poet TS, Zangar R, Timchalk C: [2004] *In vitro* interactions between organophosphorus pesticides and consequence of pre-treatment with nicotine and alcohol on *in vitro* pesticide metabolism in rats. 7th International Meeting, International Society for the Study of Xeonbiotics (ISSX), Vancouver, Canada, Aug 29th – Sept. 2nd, not found

20033144 19. Timchalk C: [2004] Development of a physiologically based pharmacokinetic and pharmacodynamic (PBPK/PD) model to quantitate biomarkers of exposure to organophosphorus insecticides. Environmental Protection Agency Science Forum, Washington, DC.

20026471 20. Timchalk C, Poet TS: [2005] Symposium: Pesticide neurotoxicity in adults: integrating contributions from epidemiology and toxicology: Presentation: Development of a physiologically based pharmacokinetic and pharmacodynamic model to determine dosimetry, dynamic response, and assess risk following exposure to organophosphorus insecticides. Society of Toxicology Annual Meeting, New Orleans, March.

20033145 21. Furlong CE, Cole TB, Pettan-Brewer C, Shih DM, Tward A, Lusis AJ, Timchalk C, Richter RJ, Costa LG: [2005] Paraoxonase 1 (PON1) status affects the metabolism of drugs and insecticides. Drug Metabolism Review, 37(2): 21.