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Determination of Age-Related Differences in Activation and Detoxication of Organophosphates in Rat and Human Tissues

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Determination of age-related differences in activation and detoxication of organophosphates in rat and human tissues

By

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The mechanism of toxic action for organophosphates (OPs), originally developed as insecticides, is the persistent inhibition of acetylcholinesterase (AChE) resulting in accumulation of acetylcholine and subsequent hyperstimulation of the nervous system. Many OPs require bioactivation via cytochromes P450 to oxon metabolites which are anticholinesterases. Organophosphates display a wide range of acute toxicities. Differences in the OPs' chemistries results in differences in the compounds' metabolism and toxicity. Acute toxicities of OPs appear to be principally dependent on compound specific efficiencies of detoxication, and less dependent upon efficiencies of bioactivation and sensitivity of AChE. Esterases, such as carboxylesterase (CaE) and butyrylcholinesterase (BChE), play a prominent role in OP detoxication. Organophosphates can stoichiometrically inhibit these enzymes, removing OPs from circulation thus providing protection for the target enzyme, AChE. This *in vitro* study investigated: 1) age-related sensitivity of AChE, BChE and CaE to structurally different OPs in rat tissues; 2) interspecies and intraspecies differences in bioactivation and detoxication of the OP insecticide malathion in rat and human hepatic microsomes; and

3) interspecies and intraspecies differences in sensitivity of AChE from erythrocyte ghost preparations to malaoxon. Sensitivities of esterases to 12 OPs was assessed by IC_{50}s. The OPs displayed a wide range of AChE IC_{50}s (low nM-μM) with no differences among ages; however, the CaE IC_{50}s generally increased with age (up to 100-fold) reflecting greater protection in adults. Kinetic analysis of the bioactivation of malathion to the anticholinesterase metabolite, malaoxon, was measured in hepatic microsomes from rats (adult) and humans (various ages) of both sexes. No statistical interspecies (rat and human) or intraspecies (among humans) differences were found. The CaE degradation of malathion and malaoxon was determined in the microsomal samples using indirect measurements. No interspecies or intraspecies differences were found; however, CaE activity in rat microsomes was significantly higher than in humans. Inhibition of AChE by malaoxon was analyzed kinetically in erythrocyte ghost preparations from rats (adults) and humans (three age groups) of both sexes. No statistical interspecies or intraspecies differences were found. These results suggest the age-related differences in acute toxicities of OPs in mammals is primarily a result of their detoxication capacity.

DEDICATION

I dedicate this work to my family. The love and support you have given me through the years have helped me become the person I am today and accomplish my educational goals. The sacrifices you have made for me are great and for that I am eternally grateful. To my parents, John and Louise Meek, I couldn't have asked for better a better mother and father and I miss you every day. You instilled in me faith, ethics, and hard work and I hope I made you proud. To my brother, the Meek boys, will always be best friends and I know you will always be there if I need anything. To my wife, Denise and step-son Chase, you are a blessing every day. Your love and support drive me to be a better man and I strive every day to be a better husband and father. I love you very much. To my grandparents and all my aunts and uncles that shaped my childhood, thank you. I wouldn't take anything for growing up in a rural area with all my family and the lessons they taught me. Finally, I would also like to dedicate this work to Jan Chambers for seeing a little potential in me and believing in me and Howard Chambers a great scientist and a better friend.

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LIST OF ABBREVIATIONS

2-PAM – 2-pyridine aldoxime methyl chloride

AAALAC – Association for Assessment and Accreditation of Laboratory Animal Care

ACh – acetylcholine

AChE – acetylcholine

ATCh – acetylthiocholine

BBB – blood brain barrier

BCh – butyrylcholine

BChE – butyrylcholinesterase

BNPP – bis-(4-nitrophenyl)phosphonate

BTCh – butyrylthiocholine

CaE – carboxylesterase

CBDP – 2-(*o*-cresyl)-4*H*-1,3,2-benzodioxaphosphorin-2-oxide

CNS – central nervous system

CYP – cytochrome P450

DFP – diisopropyl-fluorophosphate

DCA – dicarboxylic acid

DTNB – 5,5'-dithiobis(2-nitrobenzoic acid)

E – enzyme

EDTA – ethylenediaminetetraacetic acid

EI – enzyme-inhibitor complex

EPA – Environmental Protection Agency

EtOH – ethanol

FC – final concentration

FDA – Food and Drug Administration

FMO – FAD-containing mono-oxygenases

FQPA – Food Quality Protection Act

GABA – gamma-aminobutyric acid

HDL – high density lipoprotein

HPLC – high performance liquid chromatography

I – inhibitor

IACUC – Institutional Animal care and Use Committee

IC_{50} – fifty percent inhibitory concentration

iso-OMPA – tetraisopropyl pyrophosphoramide

K_I – dissociation constant for enzyme and inhibitor

k_i – bimolecular rate constant

K_m – Michaelis-Menten constant for enzyme and substrate

K_{mapp} – apparent Michaelis-Menten constant for enzyme and substrate

k_p – enzyme phosphorylation constant

LD_{50} – lethal dose at which fifty percent of animals expire

LDL – low density lipoprotein

mAChR – muscarinic acetylcholine receptors

MCA – monocarboxylic acid

NMR – nuclear magnetic resonance

NRC – National Research Council

OP – organophosphate

PB – pyridostigmine bromide

PBPD – physiologically based pharmacodynamic model

PBPK – physiologically based pharmacokinetic model

PND – post-natal day

PNS – peripheral nervous system

*p*NVA – *p*-nitrophenyl valerate

PON – paraoxonase

SCPP – saligenen cyclic phenylphosphonate

SEM – standard error of the mean

SLUD – salivation, lacrimation, urination, and defecation

TEPP – tetraethylpyrophosphate

TOTP – tri-*o*-tolyl phosphate

V_{max} – maximum enzyme velocity

WWII – World War II

CHAPTER I

INTRODUCTION

History and Usage of Organophosphates

Organophosphates (OPs) were first synthesized in the 1800s. A French chemist, Philippe de Clermont, reported the synthesis of the first known OP acetylcholinesterase inhibitor, tetraethylpyrophosphate, at a meeting of the French Academy of Sciences in 1854 (Fest and Schmidt, 1982). This class of compounds were named organophosphates for the central phosphorus element in their structure. In 1932, German chemists Willy Lange and Gerduh von Krueger were the first to describe the effects of OPs on the nervous system after observing that OP exposure resulted in pupil constriction, increased light sensitivity, and respiratory distress. Organophosphorus compounds were initially developed as insecticides by German chemists, led by Gerhard Schrader, in the late 1930s. Many of these OPs were found to be extremely toxic. According to German (Nazi) law at the time, all research discoveries with potential warfare applications were required to be reported to the government (Johnson *et al*., 2015). While working on developing insecticides for the chemical company, IG Farben, Schrader synthesized a highly toxic OP which he named "tabun" (Hersh, 1968; Hammon, 1994). Due to the observed toxicity and volatility of some of the OPs Schrader synthesized, he was ordered to develop them into chemical weapons in the lead up to WWII (Coleman, 2005; Tucker 2007). These so called "nerve gases" are more correctly termed "nerve agents" as they

are actually liquids that can be aerosolized and include the G agents and V agents. The G agents were developed in Germany and include sarin, soman, and tabun. The V agents, including VX, were initially developed by the British and later produced in the United States (VX) and the Soviet Union (VR). The G and V agents are both very potent anticholinesterases with the major difference being the G agents are faster acting while the V agents are much more persistent (Coleman, 2005; Johnson *et al*., 2015). Although these very potent OPs were stockpiled for chemical warfare during WWII, fortunately, the deployment of these toxic agents was limited.

In the 1940s and 1950s, the most heavily used insecticides were organochlorines (Ecobichon, 1991). These chlorinated compounds, though highly effective, were environmentally persistent and could bioaccumulate in wildlife and humans (Hickey and Anderson, 1968; Schmitt, 2002; Li *et al*., 2006). The deleterious effects of the organochlorines in wildlife populations as well as increasing insect resistance spurred the research and development of OP insecticides following WWII. The OPs, being potent insecticides and more environmentally labile than the organochlorines, began gaining popularity in the insecticide market during the late 1950s and early 1960s (Hayes and Laws, 1991). Tetraethylpyrophosphate (TEPP) was the first commercially available OP insecticide; however due to its extreme toxicity and chemical instability, it was not heavily used (Ecobichon, 1996). As the class of OP compounds began expanding, one of the most popular OPs, parathion (*O*,*O*-diethyl-*O*-4-nitrophenyl phosphorothionate), was developed and used as an insecticide for many years, despite its high mammalian toxicity (Hayes and Laws, 1991; US EPA, 2000; ATSDR, 2017). Several cases of poisoning of

factory and agricultural workers occupationally exposed to parathion stimulated the development of analogs that were less toxic to non-target species, wildlife and humans, and more selective for the target insect species (Hayes and Laws, 1991). The use of OPs in the United States became increasingly popular with the banning of organochlorines, such as DDT, in 1972 (Dunlap, 1981). Chlorpyrifos, [*O*,*O*-diethyl-*O*-(3,5,6-trichloro-2-pyridyl) phosphorothionate], an OP with moderate mammalian toxicity, was developed and registered in 1965 and has been used extensively in both agricultural and residential applications (US EPA, 2002; ATSDR, 2017). Malathion (*O,O*-dimethyl dithiophosphate of diethyl mercaptosuccinate), was developed in the early 1950s and is still one of the most heavily used OP insecticides due to its effectiveness as an insecticide and its very low mammalian toxicity (Meister, 1992; US EPA, 2006; ATSDR, 2017). Other OP insecticides that have been heavily used in the United States and throughout the world include diazinon, azinphos-methyl, dichlorvos, and methyl parathion (Hayes and Laws, 1991). The insecticidal applications for these and many other OPs include both agricultural and residential pest control (Matsumura, 1985; Hayes and Laws, 1991; Meister, 1992). In addition, OPs have been developed that have applications as fungicides, herbicides, defoliants, and flame retardants, which are not designed as anticholinesterases.

Due to the high usage of OP insecticides worldwide, the possibility of human exposure and subsequent intoxication remains a concern. Though both agricultural and residential applications have recently been restricted, especially in the United States (US EPA, 2011), the possibility of acute or chronic exposures still exist. Occupational

exposures to formulators and applicators offer the greatest likelihood of high acute exposures (Ware *et al*., 1973), while exposure to the general population is more likely to be lower in level, may be acute or chronic, and typically occur through ingestion of OP residues on food or from residential pest control (Simcox *et al*., 1995; Davis and Ahmed, 1998; Duggan *et al*., 2003). In 1993, the National Research Council produced a report titled "Pesticides in the Diets of Infants and Children" which raised concerns that children may be exposed to greater levels of pesticides due to their diet and greater food intake per unit body weight (NRC, 1993). This report was a driving force behind the passing of the Food Quality Protection Act (FQPA) in 1996. FQPA mandated that consideration must be given for vulnerable populations (children) in risk assessments for pesticides and, if necessary, an extra safety factor (up to 10x) must be included (Duggan *et al*., 2002). A child's behavior can also result in an increased risk of exposure via crawling on contaminated floors and placing fingers or hands in or around his or her mouth (Gurunathan, *et al*., 1998). The developmental process in humans is an elaborate highly coordinated cascade of processes that must occur in critical periods and is vulnerable to toxic insults. Any alteration or deviation from the normal processes of maturation can lead to pathological or behavioral aberrations immediately or later in life (Karczmar, 1984; West, 2002). The developing nervous system may be particularly vulnerable to toxic insults that may lead to long-term neurological damage (Selevan *et al*., 2000; West, 2002). Since the mode of action of many insecticides, including the OPs, is to attack the nervous system of an insect, the effects on the nervous system of non-target species is a logical concern, especially during developmental critical periods

(Karczmar, 1984). The sensitivity of immature animals to many OP insecticides has been well documented in rodents (Brodeur and DuBois, 1963; Benke and Murphy, 1975; Gaines and Linder, 1986; Pope *et al*., 1991, Moser *et al*., 1996; Atterberry *et al*., 1997).

Although the use of OP pesticides, especially in the United States, has been restricted, the relative low cost of these highly effective insecticides makes their use still prevalent in developing countries. In some cases, particularly in developing countries, OPs have been used to commit suicides (Eddleston, 2000, Buckley *et al*., 2004, Pandit *et al*., 2011). In addition, the possibility of exposure to OP nerve agents such as sarin, soman and VX still exists in warfare and terrorist attacks. The relative ease and low cost of synthesis of these types of nerve agents make them popular chemical weapons among rogue nations and terrorist groups (Tucker, 2007; Johnson *et al*., 2015). Although the Chemical Weapons Convention of 1993 outlawed the production and stockpiling of any chemical weapons, there are still stockpiles of these highly potent nerve agents in many countries, including the United States (Smart, 1997; Tucker, 2007; Johnson *et al*., 2015). During the Iran-Iraq War, Iraqi forces unleashed sarin on Iranian forces killing over 4000 soldiers and civilians (Rose and Baravi, 1988; Balali-Mood and Balali-Mood, 2008). In the late 1980s, Sadam Houssain ordered Iraqi troops to release sarin on Kurdish civilians in northern Iraq, killing and injuring thousands (Smart, 1997). More recently, sarin has been used several times by the Syrian regime killing several thousand rebels and civilians, including many children (Dolgin, 2013; Pita and Domingo, 2014; Rosman, Eisenkraft *et al*., 2014). Terrorist groups or individuals, so called "lone wolf" terrorists, who want to cause casualties as well as mass hysteria, may look to unconventional

methods, such as nerve agents. In 1995, a Japanese religious sect known as Aum Shinrikyo released sarin in the Tokyo subway system resulting in the deaths of 12 people and over 1000 injured. Some of the injured suffered long term neurological and behavioral deficits (Nagao *et al*., 1997; Yokoyama *et al*., 1998). In 2017, the nerve agent, VX, was used to assassinate Kim Jong Nam, the brother of North Korean leader Kim Jong Un, at Kuala Lumpur international airport (Bradley, 2017). Most recently, a former Russian spy for the United Kingdom along with his daughter were poisoned with Novichok, an extremely toxic OP nerve agent developed in Russia that is difficult to therapeutically treat (Peplow, 2018). The ability of terrorist groups, such as ISIS, to produce or procure these types of chemicals is of great concern. Even some of the more potent OP insecticides are included in the chemical threat agent category (Jett, 2012). Given the large quantities of OPs produced and the variety of applications, the potential for human exposure is still quite high.

Organophosphorus Compound Structure and Potency

Organophosphorus insecticides are a large family of compounds that are named for their central phosphorus atom. Most OPs are esters of pentavalent phosphorous acids that have a variety of side chains termed "leaving groups." This chemical family includes phosphates, phosphorothionates, and phosphorothionothiolates (Chambers, 1992). The phosphates (P=O) include compounds such as dichlorvos and naled. These compounds are active anticholinesterases and thus do not require bioactivation. The phosphorothionates (P=S) include the widely used insecticides chlorpyrifos, parathion, methyl parathion, and diazinon. The phosphorothionothiolates (P=S) include the

insecticides malathion and azinphosmethyl. The phosphorothionates and phosphorothionothiolates both require bioactivation by cytochromes P450 (CYP) to their "oxon" metabolites, which are the more potent anticholinesterases (Kulkarni and Hodgson, 1980; Sultatos and Murphy, 1983; Chambers and Forsyth, 1989; Chambers and Chambers, 1989; Fukuto, 1990; Hayes and Laws, 1991; Chambers, 1992). The P=S containing "parent" insecticides are generally not very potent anticholinesterases (Chambers and Chambers, 1989; Fukuto, 1990). The variety of structures of OPs produces a varied amount of potency both in the target species, insects, and non-target species, such as mammals (including humans) (Holmstedt, 1963; Fukuto, 1990; Hayes and Laws, 1991; Chambers, 1992). The US EPA human hazard classification consists of four levels (US EPA, 2004) and the OPs cover all four of these levels. Rat oral lethal dose 50 (LD_{50}) levels in mammals range from a very low mg/kg level (e.g., parathion, 3-13 mg/kg) to a high mg/kg level (e.g., malathion, 1,000-12,500 mg/kg) (Hayes and Laws, 1991; Meister, 1992). EPA class I compounds are classified as the greatest hazard and about one-half of the OPs fall into this classification (Meister, 1992).

There are several factors that affect the bioavailability and toxicity of an OP. These factors include absorption, distribution, metabolism, excretion and the sensitivity of the target enzyme, acetylcholinesterase (Mileson *et al.*, 1998; Timchalk *et al*, 2002). All of these factors are dependent on the structure of the OP. The more lipophilic OPs are often more easily absorbed and distributed (Garcia-Repetto *et al*., 1995; Chambers and Carr, 1993; Mileson *et al*., 1998). Once absorbed, the OPs can be transported to the liver where the phosphorothionates and phosphorothionothiolates can be bioactivated by

oxidation (desulfuration) via CYPs (O'Brien, 1062) to their oxon metabolites (anticholinesterase) (Murphy and DuBois, 1957; Nakatsugawa and Dahm, 1967, Sultatos *et al*., 1980; Forsyth and Chambers, 1989).

CYPs are heme-containing proteins with the heme iron usually in the ferric state. CYPs are found in most tissues with a high concentration located in the mitochondria and endoplasmic reticulum (microsomes) in livers of mammals (Guengerich, 1987). The CYP family consists of many different isoforms that have a variety of substrate specificities. CYPs are involved in the biosynthesis and catabolism of bile acids, steroid hormones, fatty acids, and fat soluble vitamins. In addition, CYPs mediate a variety of chemical reactions with xenobiotics including hydroxylations, dealkylations, epoxidations and oxidations (Guengerich, 1987). These reactions mediated by CYPs can result in both activation and detoxication of many xenobiotics. The activation of some xenobiotics, such as some OPs and environmental pollutants, to more biologically active metabolites, may result in toxicity (Kulkarni and Hodgson, 1980). CYPs are inducible by a variety of compounds including persistent organic pollutants (polychlorinated biphenyls and some organochlorine pesticides), pharmaceuticals (e.g., phenobarbital), and some carcinogens (Menzer and Best, 1968; Johnson, 1980; Guengerich, 1987). In addition to the CYP mono-oxygenase system, some OPs, such as disulfoton and phorate, can undergo oxidations via FAD-containing mono-oxygenases (FMOs) which are also present in substantial amounts in hepatic microsomes (Levi and Hodgson, 1988; Mileson *et al*., 1998).

The CYP mediated oxidations (desulfuration) reaction rates for OPs differ depending on CYP isozyme concentrations and OP structures (Ma and Chambers, 1995; Buratti *et al*., 2005). CYP mediated detoxication of some OPs, such as parathion, may also occur via oxidative cleavage (dearylation) yielding p-nitrophenol and diethylphoshorothioic acid (Ma and Chambers, 1994; Chambers and Carr, 1995; Timchalk, 2001). The desulfuration and dearylation are thought to result from the same phosphooxythiiran intermediate (Neal, 1980). The percentage of OP compound undergoing desulfuration (activation) compared to dearylation (detoxication) is dependent on the CYP isozymes present and will affect the amount of oxon produced resulting in toxicity (Levi and Hodgson, 1985; Timchalk, 2001; Buratti *et al*., 2005).

Organophosphate Mechanism of Toxic Action

Organophosphates can interact with a variety of enzymes, but their primary toxic action is due to inhibition of acetylcholinesterase (AChE) (Ecobichon, 1996; Mileson *et al*., 1998). The physiological function of AChE is to hydrolyze the neurotransmitter acetylcholine (ACh) in the cholinergic system (Watanabe *et al*., 1990). ACh is synthesized in a neuronal cell from a process in which pyruvate, from glycolysis, is converted to acetyl coenzyme A (acetyl CoA). Subsequently, acetyl CoA is combined with choline via the enzyme choline acetyltransferase, to form the neurotransmitter ACh. The resulting ACh is packaged and stored in vesicles for release into the synapse upon electrical stimulation by a nerve impulse (Taylor, 1990; Fukuto, 1990; Ecobichon, 1996). Once released into the synaptic cleft, ACh binds receptors on the adjacent nerve or effector cell triggering a subsequent electrical impulse. Stimulation of the cholinergic

system continues as long as ACh levels remain sufficient in the synapse or neuromuscular junction. To prevent overstimulation of the cholinergic system, the enzyme AChE cleaves ACh into acetate and choline. Choline is recycled back into the presynaptic nerve terminal via a choline transporter (Taylor, 1990; Fukuto, 1990; Purves, 2001; Thompson and Richardson, 2004).

OP oxons can inhibit AChE by phosphylating the serine hydroxyl group at the catalytic center or "gorge" of the enzyme; this persistent phosphorylation (inhibition) results in a loss of enzymatic activity (Fukuto, 1990; Chambers, 1992; Mileson *et al*., 1998). Inhibition of AChE results in the accumulation of ACh within the cholinergic synapses and neuromuscular junctions, leading to hyperactivity of the cholinergic system (Fukuto, 1990; Mileson *et al*., 1998). The elevation of ACh levels results in the excessive stimulation of both muscarinic and nicotinic ACh receptors (Wiener and Taylor, 1990; Fukuto, 1990). Muscarinic acetylcholine receptors (mAChR) function both pre- and post-synaptically and are involved in both the peripheral and central nervous systems. There are several subtypes of mAChRs and, depending upon the subtype, overstimulation from elevated ACh can produce a myriad of effects including involuntary salivation, lacrimation, urination, and defecation (SLUD). Overstimulation of nicotinic receptors in both the sympathetic and parasympathetic nervous systems in mammals can result in muscle fasciculation, tremors, convulsions, and paralysis. Bradycardia and tachycardia can both occur depending upon the balance between overstimulation of the parasympathetic and sympathetic cholinergic system (Taylor, 1990). Excessive secretion of mucus in the airway disrupts the flow of oxygen inducing respiratory distress. In the

CNS, overstimulation of the cholinergic system, involving both muscarinic and nicotinic receptors, can result in memory loss, restlessness, lethargy, confusion, and effects on respiratory control centers (Taylor, 1990; Ecobichon, 1996). In cases of severe OP intoxication, somatic muscles undergo fatigue and ultimately paralysis. The resulting paralysis of the diaphragm and intercostal muscles in conjunction with excessive mucus secretions lead to respiratory failure and ultimately death (Thompson and Richardson, 2004).

Organophosphate Toxicity Signs and Treatment

Exposure to organophosphates can result in a variety of toxic signs and symptoms. The severity of the toxicity depends on the OP and level of exposure. The cholinergic toxidrome associated with organophosphate exposure includes central nervous system responses such as miosis, headache, restlessness, convulsions, loss of consciousness, and coma. The respiratory system can be affected resulting in rhinorrhea, bronchorrhea, wheezing, dyspnea, chest tightness, hyperpnea, and bradypnea. Cardiac effects of OP exposure can result in tachycardia, bradycardia, other arrhythmias, hypertension and hypotension arrhythmias. OP exposure can also affect the GI tract causing abdominal pain, nausea, vomiting, and diarrhea. The musculoskeletal system can be affected resulting in weakness and fasciculation. Other signs and symptoms of OP exposure include lacrimation, excessive sweating, and urinary incontinence. If exposure is severe death can occur through respiratory depression (Taylor, 1990; Mileson *et al*., 1998; Bajgar, 2004).

If exposure to OPs is known or suspected, blood tests can be performed to assess cholinesterase activity. Erythrocyte AChE activity or plasma butyrylcholinesterase (BChE) activity can be measured and compared to a normal (average) activity range (Bajgar 2004, Worek *et al.*, 2005; 2016; Eddleson *et al*., 2008). AChE has been determined to be a single gene product (Taylor *et al*., 1993); therefore, erythrocyte AChE inhibition should reflect (biomarker) AChE inhibition in the CNS, the ultimate target of concern (Chen *et al*., 1999; Eddleston *et al*., 2008). Butyrylcholinesterase activity may also be used as a biomarker of OP exposure and is commonly measured by clinical chemistry analyzers (van Heel and Hachimi-Idrissi, 2011; Worek *et al.*, 2005; Schilha *et al*,. 2016). If AChE or BChE activity is decreased, suggesting OP exposure, testing for metabolites in the blood may be necessary to confirm OP exposure. Patient history, such as occupation or recent residential pesticide treatment, may help confirm the type of exposure. For example, an agricultural worker or exterminator, may provide information as to the insecticide they have been applying, thus the likely source of exposure. If signs of OP exposure are severe, especially in the case of nerve agents or industrial accidents, where death could occur quickly, immediate treatment may begin prior to confirmation of exposure (Bajgar, 2004; Eddleston *et al*., 2008).

The typical treatment following an exposure to an OP includes the administration of the muscarinic receptor antagonist, atropine, to temporarily block the effects of the accumulation of ACh and an oxime such as 2-pyridine aldoxime methyl chloride (commonly called 2-PAM or pralidoxime) to reactivate inhibited AChE (Kassa, 2002; Bajgar, 2004; Smythies and Golomb, 2004). Oximes, such as 2-PAM, reactivate AChE

by nucleophilically attacking the phosphorus of the OP covalently bound to the enzyme and removing the phosphyl moiety (Bajgar, 2004). Once reactivated, AChE is able to function normally and hydrolyze its endogenous substrate, ACh. Reactivation of OP inhibited AChE must occur prior to the enzyme "aging", a process in which the OP covalently bound to AChE is dealkylated (Beauregard *et al*., 1981; Wilson *et al*., 1992). The dealkylation of the bound OP stabilizes the enzyme-OP complex preventing spontaneous or chemically induced reactivation. The rate of aging primarily depends on the steric orientation of the OP in the active site of AChE and the side chains of the OP (Bošković *et al*., 1968; Carr and Chambers, 1996). Reactivation of OP inhibited AChE can spontaneously occur; however, this process is usually very slow and depends on the structure of the OP (Clothier *et al*., 1981; Wilson *et al*., 1992). Once the OP-enzyme complex has aged the only way to compensate for the loss of enzyme activity is *de novo* synthesis of AChE molecules (Thompson and Richardson, 2004), which has been shown to take 2-24 hours with complete recovery to normal activity taking 80-100 days (Rotundo and Fambrough 1980). The loss of AChE activity to aging may increase the susceptibility of the organism to subsequent OP exposures.

In cases of high level OP exposures such as industrial accidents or nerve agent attacks, CNS seizures may be induced. Treatment for OP induced seizures will typically include a benzodiazepine such as diazepam or midazolam (Marrs, 2003; Shih *et al*., 2003). The use of a benzodiazepine will bind gamma-aminobutyric acid (GABA) receptors producing a conformational change which activates chloride ion channels resulting in an influx of chloride ions to hyperpolarize the neuronal cell. The

hyperpolarization diminishes the electrical signals and thereby dampens seizure activity. OP induced seizures have been shown to produce neuroinflammation, neuropathology, and long term neurological behavioral deficits (Marrs, 2003; McDonough and Shih, 1997; Shih *et al*., 2003). A major limitation of the current treatment paradigm for severe OP exposure is the inability of the currently approved oxime therapeutics to cross the blood-brain barrier and reactivate OP inhibited AChE in the CNS. The inability to reactivate AChE in the brain may result in long term damage and cognitive dysfunction (Chambers *et al*., 2013). Ultimately, the toxicity of a given OP is determined by the sensitivity of the target enzyme, AChE, to the OP as well as the OP detoxication capacity of the organism (Mileson *et al*., 1998). The major detoxication enzymes for the OPs included both A-esterases and B-esterases. A-esterases are enzymes which can detoxify OP oxon molecules via catalytic hydrolysis. B-esterases, of which AChE is a member, include both carboxylesterases (CaE) and butyrylcholinesterase that stoichiometrically detoxify OP oxons (Aldridge, 1953; Maxwell, 1992; Chambers, 1992; Atterberry *et al*., 1997; Moser *et al*., 1998). These enzymes systems will be described in greater detail in the following sections.

Acetylcholinesterase

Acetylcholinesterase (AChE; EC 3.1.1.7) is a serine esterase that hydrolyzes its endogenous substrate acetylcholine (ACh) into its chemical components, acetic acid and choline. The hydrolysis of ACh terminates its function as a neurotransmitter (Taylor, 1990; Fukuto, 1990; Tripathi, 2008). AChE is one of the most efficient and fastest enzymes in the mammalian system, capable of hydrolyzing 10 to 25 thousand ACh

molecules of acetylcholine per second (Wilson and Harrison, 1961; Taylor and Radic, 1994). The catalytic (esteratic) gorge of AChE consists of a triad of residues; serine 203, glutamate 334 and histidine 447 (Tripathi, 2008). The enzyme also contains a secondary binding site (peripheral anionic site) which can alter catalytic activity through binding inhibitors preventing endogenous substrate binding in the catalytic center (Fukuto, 1990; Tripathi, 2008). The esteratic and anionic sites are both involved in the binding of ACh with the quaternary nitrogen of ACh interacting with the peripheral anionic site helping to orient the carbonyl group into the esteratic (active) site (Fukuto, 1990; Taylor and Brown, 1999). The enzyme AChE in mammals is believed to be encoded by a single gene product with alternative mRNA processing and post-translational modification giving rise to soluble and membrane-bound forms in various tissues (Taylor *et al*., 1993; Massoulié *et al*., 2008).

The three forms of AChE consist of a synaptic form (AChE-S, also known as the T (tail) form, found in brain and muscle) (Massoulié *et al*., 2008; Fisher and Wonnacott, 2012), a readthrough form (AChE-R found in embryonic and tumor cells), and an erythrocytic form (AChE-E, also known as the H (hydrophobic) form, found anchored to erythrocyte membranes) (Bartels *et al*., 1993; Massoulié *et al*., 2008). Synaptic AChE-S is multimeric and membrane-bound (Seidman et al., 1995). Readthrough AChE-R consists of soluble monomers (Karpel *et al*., 1994; 1996; Dori et al., 2007) and may be induced in response to psychological stress or AChE inhibition (Kaufer *et al*., 1998). Its endogenous function is not well understood, but recent work indicates that it may play an anti-apoptotic role following certain forms of cellular stress and its expression may be

increased in Alzheimer's disease (Campanari *et al*., 2016; Zimmermann, 2013). Erythrocytic AChE-E is dimeric and is anchored to the erythrocyte cell membrane by glycosylphosphatidyl inositol, a lipid moiety. Cysteine residues in synaptic and erythrocytic AChE allow them to exist as multimers and dimers, respectively, while the monomeric form of the readthrough AChE-R is devoid of cysteine residues (Li *et al*., 1991). Although the differences in endogenous function of the three different molecular forms is not completely understood, their catalytic properties are conserved (Schwarz *et al*., 1995; Taylor *et al*., 1993; Grisaru *et al*., 1999; Massoulié *et al*., 2008).

The fact that the catalytic domains of brain and erythrocyte AChE are identical within a given species suggests that their interactions with inhibitors would be similar (Herkert *et al*., 2012). This has given rise to the use of erythrocyte AChE activity as a surrogate for brain AChE inhibition and therefore a biomarker of OP exposure (Chen *et al*., 1999). The sequence homology (about 90%) of the catalytic domains of human and rat AChE (Wiesner *et al*., 2008; Uniprot, 2017a, 2017b), though not identical, makes the rat a logical choice for testing OP inhibition for extrapolation to humans. Studies have shown some small effects of both age and sex on AChE pharmacodynamics in rodents (Ecobichon and Stephens, 1972; Tang *et al*., 2003). AChE activity has been shown to increase from birth to weaning in rats with some small differences in sex (Tang *et al*., 2003; Timchalk *et al*., 2007). Differences in AChE sensitivities to OPs in brains of adult and juvenile rodents do not account for age-related differences in OP toxicity (Benke and Murphy, 1975; Pope *et al*., 1991; Mortensen *et al*., 1996; Moser *et al*., 1996; Atterberry

et al., 1997), providing more evidence that AChE activity matures quickly during development.

Drugs have been designed and approved to target AChE in the treatment of some diseases. Reversible AChE inhibitors have been used in the treatment of myasthenia gravis and glaucoma (Taylor, 1990). AChE inhibitors (reversible), such as tacrine, have been used in the treatment of Alzheimer's disease by increasing cholinergic neurotransmission and function (Camps *et al*., 2000). In addition, reversible inhibitors, such as the carbamate, pyridostigmine bromide, have been used by warfighters as prophylactics in anticipation of encounters with nerve agent (Marino *et al*., 1998). AChE also functions prominently in neural development in cell proliferation and differentiation (Layer and Willbold, 1995). AChE aids in the outgrowth of neurites (extensions of the axon at the ends of which are synapses), neuronal proliferation and migration, and axonal outgrowth (Sharma *et al*., 2001).

Butyrylcholinesterase

Butyrylcholinesterase (BChE; EC 3.1.1.8) is a serine esterase synthesized in the liver and is found in several other tissues including lung, heart, brain, and skeletal muscle but is ubiquitous in the serum (Chatonnet and Lockridge, 1989; Li *et al*., 2000; Kolarich *et al*., 2008). BChE is often referred to as pseudocholinesterase or plasma cholinesterase and interacts with many different choline esters making it a non-specific cholinesterase, but it does not appear to be necessary for survival (Primo-Parmo *et al*., 1996; Li *et al*., 2000). BChE is similar to AChE in that both are glycoproteins containing both an esteratic binding site (central catalytic site) consisting of the same catalytic triad or

residues and an anionic binding site (peripheral binging site) (Masson *et al*., 1993, 1997; Reiner *et al*., 1996; Lockridge *et al*., 1997; Simeon-Rudolf *et al*., 1999). The true endogenous function of BChE is not well understood; however, it does have the ability to hydrolyze acetylcholine and a variety of exogenous substrates including butyrylcholine, mivacurium, succinylcholine, aspirin, heroin, cocaine, and organophosphate oxons (Cook *et al*., 1989; Fukuto, 1990; Sparks *et al*., 1999; Li *et al*., 2000; Carmona *et al*., 2000; Hou *et al*., 2014).

Cholinesterase activity in human serum consists largely of BChE, approximately 90% (Li *et al*., 2000), giving rise to its use as a biomarker of OP exposure (Worek *et al*., 2005). Rodents typically have a more equivalent proportion of BChE and AChE in their serum (Li *et al*., 2000). This can be a confounder in extrapolating OP toxicities in animal studies to humans, due to differences in sensitivities of BChE and AChE. Similar to AChE, BChE is stoichiometrically inhibited by OP oxons; however, BChE inhibition serves as an OP detoxication mechanism by reducing the bioavailable levels of the OP, therefore reducing the amount of OP available to inhibit the target, AChE (Fukuto, 1990; Mileson *et al*., 1998). As with AChE, sensitivities of BChE to OPs depends on the structure of the OP (Worek *et al*., 2005).

Although BChE has been shown not to be necessary for survival, its ability to hydrolyze ACh has been suggested to be an important physiological function in both synapses and neuromuscular junctions in the CNS and the PNS, with BChE serving as a backup to AChE to hydrolyze excess ACh when AChE activity has been compromised (Li *et al*., 2000). BChE has been found in the neurofibrillary tangles and amyloid plaques

in brains of victims of Alzheimer's disease (Carson *et al*., 1991; Arendt *et al*., 1992; Moran *et al*., 1993), although the role BChE may play in these pathologies is not understood. More recently, BChE has been investigated as a potential medical countermeasure against nerve agents by acting as a bioscavenger of OPs (Masson and Lockridge, 2010; Nachon *et al*., 2013). Scavenging of nerve agent in the blood stream prior to reaching the target enzyme, AChE, could reduce the neurotoxicity following exposure (Raveh *et al*., 1993; Lenz *et al*., 2007). Researchers have created "pseudocatalytic" mutants of BChE to increase their efficiency as OP bioscavengers in the blood (Cerasoli *et al., 2005*).

A-esterases

A-esterases are hydrolases (Aldridge, 1953) synthesized predominantly in the liver and are associated with high-density lipoproteins (HDL) in the serum (Mackness *et al*., 1985; Zech *et al*., 1993). A-esterases can catalytically hydrolyze the oxons of some OP compounds and are typically divided into two groups. The first, paraoxonase (PON; EC 3.1.8.1), named for its ability to hydrolyze the OP, paraoxon, is a calcium dependent hydrolase. The second group, DFPases (EC 3.1.8.2), named for their ability to hydrolyze diisopropylfluorophosphate (DFP) are dependent on cofactors such as cobalt, magnesium, and manganese (Aldridge, 1953). Paraoxonases are the primary A-esterases involved in the catalytic destruction of the OP insecticides, though some are not hydrolyzed very efficiently by PON (e.g., paraoxon, despite being named for it), or not at all. Paraoxonase and A-esterase are often used interchangeably when referring to the catalytic destruction of OPs. The A-esterases in the serum and liver of rats were

determined to be the same protein using gel staining and site directed mutagenesis (Furlong *et al*., 1993; Sorenson *et al*., 1995). The catalytic activity of these enzymes is dependent on calcium ions, which is also involved in the stabilization of the enzyme structure (Vitarius and Sultatos, 1995; Kuo and La Du, 1998). Thus, the use of metal chelating agents, such as EDTA or EGTA, can inhibit the activity of paraoxonase.

Paraoxonases are a multigene family, including PON1, PON2, and PON3 (Primo-Parmo *et al*., 1996; Hegele, 1999). With a cysteine located in the catalytic center of the enzyme, these hydrolases are sensitive to inhibition by metallic salts (mercury, zinc, nickel, and copper) and mercurial compounds that have affinity for sulfhydryl groups (Aldridge, 1953; Gil *et al*., 1993; Gonzalvo *et al*., 1997).

Paraoxonases can affect the acute toxicity of several OP compounds (Costa *et al*., 1990; Chambers, 1992; Chambers *et al*., 1994; Pond *et al*., 1995; Mortensen *et al*., 1996). Mammals have higher levels of paraoxonase activity than most other species making species such as some birds and fish more sensitive to the acute toxicities of some OPs. (Machin *et al*., 1976; Brealey *et al*., 1980). Costa *et al*. (1990) reported that rats pre-treated (injected) with purified A-esterase were less sensitive (as evidenced by acetylcholinesterase inhibition) to paraoxon and chlorpyrifos-oxon. Li *et al*. (1993; 1995) reported similar findings of pre-treatment with A-esterase providing increased protection in mice exposed to chlorpyrifos and chlorpyrifos-oxon. The development of knockout mouse models has allowed for testing the detoxication potential of paraoxonase for certain OPs by "knocking" out the PON1 gene, therefore eliminating its catalytic activity (Shih *et al*., 1998). Furlong *et al*. (2000) demonstrated that the knockout

mutation of the gene eliminated PON1 catalytic activity in both liver and plasma of mice, further indicating that activities from these two tissues results from a single gene. Shih *et al*. (1998) demonstrated that PON1 wild type mice were much less sensitive (as evidenced by cholinesterase inhibition) to chlorpyrifos-oxon than PON1 knockout mice. In another study, knockout mice were also more sensitive to diazoxon (a good PON1 substrate) but did not show increased sensitivity to paraoxon (a poor PON1 substrate) (*Li et al*., 2000). These studies suggest that PON1 is an important detoxication pathway for some OPs and the catalytic efficiency of PON1 for a given OP is important in determining the level of protection provided for the target, brain acetylcholinesterase.

In humans, polymorphisms of paraoxonase have been determined that result in differences in PON1 concentrations and activities (Humbert *et al*., 1993; Davies *et al*., 1996). A PON1 polymorphism in an amino acid residue at position 192, a glutamate (Q) or an arginine (R), alter PON1 OP hydrolysis activity. An R isoform is more efficient at hydrolyzing paraoxon than a Q isoform (Smolen *et al*., 1991; Humbert *et al*., 1993; Richter and Furlong, 1999). Additional studies have shown the reverse of the isoform efficiency for diazoxon and the nerve agents soman and sarin (Davies *et al*., 1996). All isozymes have activity toward chlorpyrifos-oxon and the standard arylesterase substrate, phenyl acetate (Furlong *et al*., 1993; Davies *et al*., 1996). A substitution of a leucine or a methionine at residue 55 has been shown to result in differences in both enzyme activity and concentration (PON1 protein) (Adkins *et al*., 1993; Mackness *et al*., 1997). These studies and others demonstrate the role both genotype and concentration of PON1 play in an individual's ability to detoxify OPs (La Du and Eckerson, 1986; Geldmacher-von

Mallinkrodt and Diepgen, 1988; Furlong *et al*., 1993; Davies *et al*., 1996; Clendenning *et al*., 1996; Richter and Furlong, 1999; Akgur *et al*., 1999).

Associations of PON1 polymorphisms and disease states such as cardiovascular disease, type 2 diabetes, Parkinson's disease, and Alzheimer's disease have been increasingly studied over the last several years (Herrmann *et al*., 1996; Sanghera *et al*., 1997; Laplaud *et al*., 1998; Cascorbi *et al*., 1999; Aynacioglu and Kepekci, 2000; Gardemann *et al*., 2000; Kondo and Yamamoto, 1998; Akhmedova *et al*., 1999; Kalman *et al*., 1999; Sodeyama *et al*., 1999, Jarvik *et al*., 2000, Davis *et al*., 2009; Coombes *et al*., 2014; Crow *et al*., 2018). Studies have indicated that PON1 activities can be increased with exercise and diets rich in nuts and pomegranate juice (Costa *et al*., 2005). Other studies have shown that cigarette smoking and some medications (e.g., statins) appear to have an inhibitory effect on PON1 activity (Nishio and Watanabe, 1997; James *et al*., 2000; Mackness *et al*., 2012).

PON1 has been shown to hydrolyze many substrates, including phenyl acetate (arylesterase activity) and some organophosphates (Furlong *et al*., 1993). More recently, researchers have discovered PON1's ability to hydrolyze some lactones (Mackness *et al*., 1991; Billecke *et al*., 2000; Biggadike *et al*., 2000; Jakubowski, 2000). It is now speculated that PON1's endogenous function is as a lipolactonase (Jakubowski, 2000). PON1 has been shown to inactivate toxic products that result from the oxidation of lipid components of low-density lipoproteins (LDL). This action may play a role in preventing the development of atherosclerosis and ultimately cardiovascular disease (Mackness *et al*., 1993; Aviram *et al*., 1998).

PON2 is an intracellularly expressed enzyme found in the brain, liver, lung, kidney, heart, pancreas, small intestine, muscle, testis, endothelial cells, tracheal epithelial cells, and macrophages, but unlike PON1 and PON3, it is not present in serum (Ng *et al*., 2001). PON2 has very little hydrolytic activity towards OPs. PON2 functions primarily as a lactonase and may play a role in determining susceptibility to oxidative stress and neuroinflammation (Draganov *et al*., 2005; Levy *et al*., 2007)

PON3, similar to PON1, is synthesized in the liver and is associated with HDL in the serum. (Draganov *et al*., 2000). Like PON2, PON3 does not hydrolyze OPs but has been shown to hydrolyze lactones, such as statins. PON3 been shown to have both antioxidant and anti-inflammatory potential. Though the antioxidant mechanisms of PON3 in disease states are not well characterized, it may play a role in a variety of oxidative-stress related diseases such as atherosclerosis, metabolic syndrome, HIV, and chronic liver disease (Reddy *et al*., 2001; Draganov *et al*., 2005).

Carboxylesterase

Carboxylesterases (CaE; EC 3.1.1.1) are serine esterases (Aldridge, 1953) that can hydrolyze several different types of endogenous and exogenous compounds (Satoh and Hosokawa, 2006; Zou *et al*., 2018). CaEs are products of a multigene family that exist in several isozyme forms (Satoh and Hosokawa, 2006; Ross *et al*., 2012). CaEs hydrolyze aliphatic and aromatic esters and amides, and can activate some carcinogens (Satoh, 1987; Hosokawa *et al*., 1987; Maki *et al*., 1991; Zou *et al*., 2018). Substrates of carboxylesterases include steroids (Lund-Pero *et al*., 1994), salicylates (White *et al*., 1994), cocaine (Dean *et al*., 1995; Brzezinski *et al*., 1994), heroin (Brzezinski *et al*.,

1997; Pindel *et al*., 1997) as well as some organophosphates (Maxwell *et al*., 1988; Pond *et al*., 1990; Fukuto, 1990; Chambers and Carr, 1993; Satoh and Hosokawa, 2006; Zou *et al*., 2018).

Carboxylesterases, also termed aliesterases, are important in detoxication of organophosphate insecticides (Chambers, 1990; Fukuto, 1990; Satoh and Hosokawa; 2006). Like acetylcholinesterase, CaEs are members of the B-esterase family (Aldridge, 1953). CaEs can be stoichiometrically inhibited by many OP oxons, thus detoxifying OPs by removing them from circulation prior to reaching the target AChE (Pond *et al*., 1990; Moser *et al*., 1998). The inhibition of CaE by OPs does not directly affect survival but provides a non-catalytic detoxication pathway, in essence providing a "sink" to draw down circulating OP oxons. The degree of OP detoxication by CaEs depends both on the affinity for the OP and the concentration of the enzyme (Fukuto, 1990; Chambers *et al*., 1994; Ross *et al*., 2012). Some of the OPs that are good inhibitors for CaE include paraoxon, chlorpyrifos-oxon, and malaoxon. (Maxwell *et al*., 1988; Clement, 1984; Fonnum *et al*., 1985; Gupta *et al*., 1985; Gupta and Dettbarn, 1987; Jokanovic, 1989; Chambers *et al*., 1990; Gaustad *et al*., 1991; Maxwell, 1992; Chambers and Carr, 1993; Moser *et al*., 1998; Moser and Padilla, 2016). CaE can also hydrolyze parent OP insecticides containing carboxylic esters in their structure (e.g., malathion). This hydrolysis is catalytic and is an efficient detoxication pathway in mammals that results in low mammalian toxicity for malathion (Main and Braid, 1962; Talcott, 1979; Fukuto, 1990; Satoh and Hosokawa, 1998; Buratti and Testai, 2005).

CaEs are glycoproteins and are ubiquitous in a variety of tissues, including liver, kidney, intestine, skin, heart, muscle, lung, adipose, and blood with the liver containing the greatest activity, especially in the microsomal fraction (McCracken *et al*., 1993; Morgan *et al*., 1994; Zou *et al*., 2018). CaE isozymes have been divided into several families in mammals labeled CES followed by a number to reflect the isozyme family (Zou *et al*., 2018), with the CES1 family including the major CaE forms responsible for OP detoxication in both rats and humans (Yan *et al*., 1995; Crow *et al*., 2008; Ross *et al*., 2012; Zou *et al*., 2018). The CES1A1 family contains the major human isoforms, while CES1A2 contains the major isoforms of rat, mouse, dog, and rabbit carboxylesterase (Satoh and Hosokawa, 1998). CaEs have several conserved motifs with a catalytic triad of residues at the active center of the enzyme (serine 203, glutamate 335, and histidine 448) that are particularly conserved (Cygler *et al*., 1993).

CaEs have been shown to be induced by several different chemicals in rodents. Inducers of CaE include phenobarbital (Hosokawa *et al*., 1984), polycyclic aromatic hydrocarbons (Hosokawa *et al*., 1988), synthetic glucocorticoids, and clofibrate (Mentlein *et al*., 1986), many of which also induce CYP. Chemical inhibitors of CaE include tri-*o*-tolyl phosphate (TOTP), bis-(4-nitrophenyl)phosphonate (BNPP), tetraisopropyl pyrophosphoramide (iso-OMPA), or 2-(*o*-cresyl)-4*H*-1,3,2-benzodioxaphosphorin-2-oxide (CBDP) and saligenen cyclic phenylphosphonate (SCPP) (Casida, 2016; Zou *et al*., 2018). These inhibitors can be used experimentally to selectively inhibit the amount of functional CaE to determine the role of CaEs in detoxication pathways of other chemicals that are CaE substrates (Zou *et al*., 2018).

CaE has been shown to increase with age from birth to weaning in rodents, resulting in greater susceptibility to OP toxicity in younger animals (Clement, 1984; Peet *et al*., 1987; Chambers *et al*., 1990; Atterberry *et al*., 1997; Moser *et al*., 1998; Karanth and Pope, 2000; Padilla *et al*., 2000). Clement (1984) demonstrated that a developmental increase in CaE in rat liver and plasma correlated well with a decrease in the toxicity of the nerve agent soman. In another study in rats, an increase in liver CaE activity with age correlated with a decrease in the sensitivity to the insecticides parathion and chlorpyrifos (Burnett and Chambers, 1994). CaE expression in livers from humans has been shown to be low in children less than 3 weeks of age followed by a rapid increase by 6 years of age (Hinds *et al*., 2016). Sex differences in CaE activity have also been reported in rats. Moser *et al*. (1998) reported that adult female rats had lower CaE activities than adult males. Unlike rats, humans do not have CaE in their serum (Zou *et al*., 2018) which can be a confounder when extrapolating OP toxicity from rats to humans. The abundance of CaE in mammalian tissues and its ability to hydrolyze many OPs make it a very important in assessing the toxicity of OPs.

Research Summary

This research study was designed to determine the age-related differences for activation (cytochrome P450 mediated), detoxication and toxicity (target enzyme sensitivity) for several OP compounds that display a variety of chemistries and a common mechanism of toxicity. The overall hypothesis of this study is the lower vulnerability of adults to the toxic effects of some organophosphates is primarily the result of the maturation of enzymes capable of organophosphate detoxication and less likely the result

of differences in activation to their active metabolites (oxons) and/or the sensitivity of the target enzyme acetylcholinesterase.

The objectives of this research were to 1) determine the differences in AChE, BChE, and CaE sensitivity in several tissues to a series of OP oxons with a variety of leaving groups in three ages of rats; 2) investigate the differences in rates of CYP mediated activation (desulfuration) of the OP, malathion, in human (various ages) and rat liver microsomes and determine the major detoxication pathways of malathion and the active metabolite, malaoxon, using the same microsomes; and 3) investigate the differences in inhibitory rates for AChE by malaoxon in both human (various ages) and rat (adult) erythrocyte ghosts.

The OPs chosen for the initial portion of this study (chapter II) include the anticholinesterase metabolites (oxons) of some commercially available insecticides and some model compounds. The age-related sensitivity of the target enzyme, AChE, as well as two of the non-target (detoxication) enzymes, CaE and BChE, were determined *in vitro* by determining IC_{50}s for each of 12 OPs in a variety of tissues from rats of three ages (post-natal day 1, PND1; PND12; and PND70). IC_{50}s provide a good estimate of an inhibitor's potency; however, it does not describe the various steps in the enzymatic reaction. With the large number of compounds and the limited amount of tissue available from the young rats, IC_{50}s were chosen for this portion of the project to compare the potency of a given OP (12) among the 3 age groups. The inhibitory potency of OPs for the target enzyme AChE as well as the potency of OPs for the stoichiometric detoxication

enzymes, BChE and CaE, can provide information to help explain the differences in the toxicity of OPs with different structural characteristics.

The differences in OP toxicities in relation to structure has been investigated for many years in our laboratory and others. The widely used OP, malathion, has a very low mammalian toxicity (LD_{50} > 1 g/kg in rats); however, the active metabolite, malaoxon, is a reasonably good inhibitor of AChE *in vitro*. Differences in the activation and/or detoxication could provide an explanation for this discrepancy in the *in vitro* potency and the *in vivo* toxicity. The activation and detoxication potential for malathion were investigated using rat and human liver microsomes in chapter III.

The metabolism of malathion is complex and the pathways are difficult to separate kinetically due to several of the processes occurring essentially simultaneously including 1) the activation of malathion to malaoxon by CYP 2) the catalytic degradation of malathion by carboxylesterase and 3) the stoichiometric detoxication of malaoxon by carboxylesterase. Kinetic constants (V_{max} and K_m) for the CYP mediated activation of malathion to its anticholinesterase metabolite, malaoxon, were determined in rat and human (various ages) liver microsomes. In addition, the detoxication potential of the same microsomes for malathion and malaoxon was investigated. Carboxylesterase was determined to be important in the degradation of both malathion and malaoxon. Paraoxonase was determined to not be an important enzyme in the detoxication of malaoxon.

The determination of kinetic constants of activation for malathion and as well as identifying the enzymatic pathways involved in degradation of malathion and malaoxon

will be useful in the development of physiologically based pharmacokinetic (PBPK) models. These predictive models utilize a series of differential equations to mathematically describe the physiologic, biochemical, and physicochemical reactions that contribute to the pharmacokinetics of a compound. A well constructed and validated PBPK model can provide very accurate predictions for acute or chronic exposures for a given compound (Clewell and Anderson, 1985). PBPK models use engineering principles that are applied to biological systems. These models use mathematical descriptors for various compartments (organ systems) and the processes within these compartments. Each compartment can consist of diffusion rates, enzyme systems, and rates of those enzyme mediated processes. Physicochemical properties of the compound of interest must also be considered including structure, molecular weight, volatility, stability, and lipophilicity. For a PBPK model to accurately predict a response for a given exposure, it requires experimentally derived parameters including rates for a compound's absorption, distribution, metabolism, detoxication and elimination.

A PBPK model can estimate a tissue dose and subsequent response (forward dosimetry) for an administered dose of a compound. In addition, some PBPK models can use biomonitoring data (measured tissue dose) to estimate an exposure level (reverse dosimetry). PBPK models for toxic compounds in humans usually require data derived in animal models that are extrapolated to humans (interspecies) for a given dose and route of exposure. The interspecies variability in activation rates (human and rat microsomes) for malathion as well as the intraspecies (27 microsomes from humans of different ages) variability in activation rates were determined and may be valuable in developing a

human PBPK model for malathion. These kinetic parameters and models will be useful in determining the levels of safety factors to be applied in the risk assessment of malathion.

The kinetic rates for malathion activation (desulfuration) in rat and human microsomes were calculated according to concepts developed by Leonor Michaelis and Maud Menten. In 1913, Michaelis and Menten demonstrated that the rate of an enzyme-catalyzed reaction is proportional to the enzyme-substrate complex. The equation follows a mechanism in which substrate binds reversibly to an enzyme to form an enzyme-substrate complex, which then reacts irreversibly and generates a product and the free enzyme (Michaelis and Menten, 1913; Goody and Johnson, 2011). This widely used equation was used to calculate the maximum velocity (V_{max}) and substrate affinity (K_m) for the activation (desulfuration) of malathion to its anticholinesterase metabolite, malaoxon. These rates were obtained to determine differences in rat and human microsomes and differences with respect to age in human microsomes.

The kinetics of AChE inhibition by the active metabolite of malathion, malaoxon, were determined using human and rat erythrocyte ghosts (chapter IV). The difference in rates of AChE inhibition (bimolecular rate constants) will be valuable in the construction of PBPK models for human exposure to malathion based on both rat and human experimental data. The development and validation of these models is dependent on bimolecular rate constants calculated from measured AChE inhibition for increasing concentrations of inhibitor over a given time. The differences between rat and human bimolecular rate constants will be valuable in assessing interspecies safety factors in the

risk assessment for malathion. Double-reciprocal plots, commonly referred to as Lineweaver-Burk plots, were used to plot the best fit line for velocity versus the inhibitor concentration (1/ln (% AChE activity (k_{app}) versus 1/inhibitor concentration) (Lineweaver and Burk, 1934). A major concern with the Lineweaver-Burk model is the heavier weight of the lower concentrations and its effect on the slope of the line (Dowd *et al*., 1965); therefore, thanks to newer computer software, the rates of inhibition (*Vo*) were plotted versus substrate concentration producing a hyperbolic curve and using non-linear regression more accurate rate constants can be calculated. Bimolecular rate constants (*ki*) constants were calculated for malaoxon from both the double-reciprocal plots and hyperbolic plots. The knowledge of reaction rates is important for comparative analysis between different OP compounds and their interaction with the target enzyme as well as interspecies and intraspecies comparisons (Kitz and Wilson, 1962; Carr and Chambers, 1993; Worek *et al*., 2002; Coban *et al*., 2016). Our lab and others have previously reported inhibition rate constants for several OP insecticides, model OP compounds and nerve agents. This research focused on determining AChE inhibition rate constants for malaoxon in human and rat erythrocyte membranes to determine interspecies and intraspecies differences. These rate constants can be used in the development of a PBPK model for malathion and ultimately in the overall risk assessment for this heavily used insecticide.

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CHAPTER II

IN VITRO AGE-RELATED DIFFERENCES IN RATS TO ORGANOPHOSPHATES

Introduction

Organophosphate (OP) insecticides are widely used for a variety of agricultural applications throughout the United States and all over the world. Their major mechanism of toxic action is the inhibition of the serine hydrolase acetylcholinesterase (AChE) in synapses and neuromuscular junctions resulting in an accumulation of the neurotransmitter acetylcholine and ultimately hyperstimulation of the nervous system (Hayes and Laws, 1991; Ecobichon, 1996). The majority of OP insecticides undergo bioactivation via cytochrome P450 (CYP) to oxon metabolites, which are active anticholinesterases (Kulkarni and Hodgson, 1980; Sultatos *et al*., 1985; Chambers and Chambers, 1989; Fukuto, 1990).

Non-target esterases, such as carboxylesterases (CaEs) and butyrylcholinesterases (BChEs), can stoichiometrically bind oxon metabolites of OPs and limit the amount of OP available to target AChE, thus providing some protection to the organism (Aldridge, 1953; Russell and Overstreet, 1987; Chambers *et al*., 1990; Fukuto, 1990; Maxwell, 1992; Chambers and Carr, 1993; Chambers *et al*., 1994; Pond *et al*., 1995; Mileson *et al*., 1998; Moser and Padilla, 2016). Studies have shown that CaE activity in rats increases with age from birth to young adulthood, suggesting that neonates and juveniles would not have the same level of protection (detoxication) from an OP exposure as adult animals

(Clement, 1984; Maxwell, 1992; Atterberry *et al*., 1997; Tang *et al*., 1999). Hinds *et al*. (2016) showed an increase in hepatic CaE activity with age in human pediatric patients indicating a potential for greater OP toxicity in children.

In addition to CaEs and BChEs, some OPs can be hydrolyzed effectively by A-esterases (Aldridge, 1953; Furlong *et al*., 1989; Chambers *et al*., 1994; Pond *et al*., 1995). A-esterase, also termed paraoxonase (PON), is a calcium dependent enzyme synthesized in the liver and is associated with high density lipoproteins (HDLs) in the serum (Mackness *et al*., 1985; Furlong *et al*., 1989; Pond *et al*., 1995). Paraoxonase is a multigene family, including PON1, PON2, and PON3 (Primo-Parmo *et al*., 1996), with PON1 capable of catalytically hydrolyzing some OP compounds thus providing protection for the target enzyme AChE (Costa *et al*., 1990; Chambers *et al*., 1994; Le *et al*., 1995; Pond *et al*., 1995; Mortensen *et al*., 1996). Similar to CaE, PON1 has been shown to increase with age in rats from birth to young adulthood (Atterberry *et al*., 1997; Moser *et al*., 1998).

OP insecticides display a wide range of acute toxicities mostly due to the variety of their chemistries. Generally, the insecticidal OPs are either *O,O*-dimethyl or *O,O*-diethyl phosphates, phosphorothionates, and phosphorothionothiolates (Fukuto, 1990; Hayes and Laws, 1991; Chambers *et al*., 1992). The differences in the chemistries of the insecticides result in differences in the compounds' metabolism (activation and detoxication) (Aizawa, 1982; Chambers *et al*., 1990; Chambers *et al*., 1994; Atterberry *et al*., 1997; Mileson *et al*., 1998). Studies from our laboratory and others have demonstrated that the metabolic bioactivation of OPs to their active oxon metabolites can

increase their potency as AChE inhibitors by several orders of magnitude (Chambers and Chambers, 1989; Forsyth and Chambers, 1989; Fukuto, 1990; Mileson *et al*., 1998).

In adult mammals, the acute toxicity levels of individual OP insecticides appear to be principally dependent on the compound-specific efficiencies of detoxication, and less dependent upon the differences in CYP activation and/or sensitivity of the target enzyme, AChE (Chambers *et al*., 1990; Chambers *et al*., 1994; Atterberry *et al*., 1997; Pope *et al*., 2005). Previous studies suggested little age-related differences in *in vitro* rat brain AChE sensitivity to chlorpyrifos-oxon and malaoxon (Mortensen *et al*., 1996, 1998) or paraoxon and methyl paraoxon (Benke and Murphy, 1975, Atterberry *et al*.,1997). In addition, several studies have shown that adult animals are less susceptible to acute exposures of OP insecticides than younger animals (Brodeur and Dubois, 1963; Gagne and Brodeur, 1972; Benke and Murphy, 1975, Pope *et al.*, 1991; Atterberry *et al*., 1997). Mortensen *et al*. (1998) also compared the sensitivity of purified AChE from brain and plasma of neonate and adult rats to chlorpyrifos-oxon and found no age-related differences. Although age-related differences in CYP-mediated bioactivation (desulfuration) and detoxication (dearylation) of OPs has been documented (Atterberry *et al*., 1997), the maturation of OP detoxication enzymes appears to be the primary factor in the toxicity level for most OPs in young animals.

The current *in vitro* investigation was designed to determine the age-related sensitivity of acetylcholinesterase and carboxylesterase to 12 OP compounds that display a variety of chemistries. The OPs chosen for this study include 8 diethyl and 4 dimethyl oxons (the anticholinesterase metabolite of the OP) of which some are the active

metabolites of commercially available insecticides and some are model compounds. This work will support other studies that have investigated the age-related differences in acute toxicity of a select number of commercially available organophosphates and provide additional information about the structure/anticholinesterase activity relationship for OPs as well as the sensitivity of OP detoxication enzymes.

Materials and Methods

Chemicals

All organophosphates/oxons (Table 1) were synthesized by Dr. Howard Chambers at Mississippi State University using standard procedures from commercially available intermediates (Chambers and Chambers, 1989; Meek *et al*., 2011), except diazoxon which was purchased from ChemService (West Chester, PA) and azinphos-methyl-oxon which was a generous gift of Bayer Crop Protection (Stilwell, KS). All organophosphates were at least 95% pure. All other reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Figure 1 displays the chemical structures for the 12 OP compounds tested in this study.

Organophosphates

The 12 OPs tested in this study were selected for their differences in structure (Figure 1) which were predicted to demonstrate unique inhibitory and detoxication potential. By selecting the active metabolites "oxons" for testing, this strategy eliminates the involvement of CYP-mediated bioactivation (desulfuration) and detoxication (dearylation) and allows for the determination of the sensitivity of the target enzyme, AChE, as well as the non-target protective esterases. Data from our laboratory and others

suggests that esterase mediated hydrolyses are more important in the overall toxicity level of OPs than the differential efficiencies of CYP-mediated bioactivation and detoxication. Of the 12 compounds selected for study, four (chlorpyrifos-oxon, paraoxon, methyl-paraoxon and diazoxon) have an extensive literature database for comparing data generated in this investigation. The additional eight compounds are either commercial insecticides, metabolites (oxons) of commercial insecticides, or model OPs with unique chemistries. These compounds were selected for their biochemical characteristics that were predicted to result in a wide range of potencies.

Animals

Rats were the source of tissues for the *in vitro* testing of OP oxon potencies. Male Sprague Dawley-derived (Crl:CD(SD)BR) rats at postnatal days (PND) 1, 12, and 70 were obtained from breeding colonies derived from rats purchased from Charles River Laboratories and maintained at Mississippi State University (PND 1 and 12) or were purchased (PND 70) from Charles River Laboratories. Rats were housed in AAALAC accredited facilities within the College of Veterinary Medicine at Mississippi State University with temperature-controlled environments and 12 h dark-light cycle. Standard lab chow and tap water were provided *ad libitum*. All animal procedures received prior approval from the Mississippi State University Animal Care and Use Committee. Sex differences were not expected to be appreciable in neonates and juveniles; therefore, to reduce animal numbers and confounders such as time of estrus cycle in adult females, rats of only one sex (male) were chosen for study. The ages of rats were selected to represent a range of developmental stages and enzymatic activities, which have been

shown to mature with age in our laboratories and many others. PND 1 was chosen as a neonatal age in rats, with rats of this age reported to have low levels of protective (detoxication) esterases. PND 12 was selected because studies have reported significant increases in protective esterases at this age in rats (Gagne and Brodeur, 1972). PND 70 was selected as young adults with a full complement of protective esterases (Atterberry *et al*., 1997). The choice of ages was not intended to equate to any particular age in humans.

Tissue Collection and Preparation

Brain, heart, skeletal muscle, lung, liver and blood were collected from naïve PND 1, 12, and 70 male Sprague Dawley rats. The tissues were rinsed in ice cold 0.9% saline, immediately snap-frozen in liquid nitrogen and stored at -80ºC. The serum was prepared by centrifuging the blood at 10,000 *g* for 8 min, separated from sedimented erythrocytes, and was stored at -80ºC. Three different pools of each rat tissue (brain, heart, skeletal muscle, and lung) were utilized for experimental replication. Each pool consisted of six individual rat tissues and represented a single replication. Solid tissues were minced with scissors on ice and homogenized in 0.05 M Tris-HCl buffer (pH 7.4 at 37ºC) with a motorized homogenizer (Wheaton or Polytron). Serum was diluted with 0.05 M Tris-HCl buffer (pH 7.4 at 37ºC). Immediately prior to testing, tissues were diluted to the following final concentrations (FC; mg tissue/ml 0.05 M Tris-HCl buffer (pH 7.4 at 37ºC)): brain, 1 mg/ml; heart, 5 mg/ml; skeletal muscle, 5 mg/ml; lung, 2.5 mg/ml; serum, 10 µl/ml for cholinesterase assays and 0.5 mg/ml and 5 µl/ml for lung and serum carboxylesterase, respectively.

Esterase Activities

Enzymatic activity was determined for each tissue from PND 1, PND 12, and PND 70 rats. Acetylcholinesterase activity was determined for brain, heart, lung, skeletal muscle and serum using a discontinuous spectrophotometric assay (modification of Ellman *et al*., 1961) with acetylthiocholine as the substrate and 5,5'-dithio-bis(nitrobenzoic acid) (DTNB) as the chromogen (Chambers *et al*., 1988). Briefly, tissue homogenates were diluted (2 ml total assay volume) in buffer (0.05M Tris-HCl buffer (pH 7.4 at 37ºC)), vortexed, and placed in a shaking water bath preheated to 37ºC. Additional tubes (blanks) containing eserine sulfate (FC, 10 µM) were included in the assay to inhibit AChE and correct for non-enzymatic hydrolysis. Each assay contained triplicate subsamples for each tissue as well as duplicate eserine sulfate blanks. Following the initial incubation, 20 µl of acetylthiocholine (FC, 1 mM) was added as a substrate for AChE and was incubated for an additional 15 min. The reaction was terminated and color was developed using 250 µl of a 5% SDS/0.24 M DTNB mixture (4:1). Absorbance was measured at 412 nm using a spectrophotometer. Serum butyrylcholinesterase activity was determined by the same method as serum acetylcholinesterase activity with the substitution of butyrylthiocholine (FC, 1mM) for acetylthiocholine as the substrate. Liver and serum carboxylesterase activities were determined according to the method of Chambers *et al*. (1990) using *p*-nitrophenyl valerate (*p*NPV) as the substrate for CaE. Dilute homogenates of liver or serum (total assay volume: 2 ml for liver, 1 ml for serum), were vortexed and placed in a shaking water bath preheated to 37ºC. Tubes (blanks) containing paraoxon (FC, 10 µM) were

included in the assay to inhibit CaE and correct for non-enzymatic hydrolysis. Each assay contained triplicate subsamples for each tissue as well as duplicate paraoxon blanks. Following an initial incubation of 15 min, 20 µl of pNPV (FC, 500 µM) in ethanol vehicle was added as a substrate for the remaining uninhibited CaE and was incubated for an additional 15 min. The reaction was terminated with 500 µl of a 2% SDS/2% Tris-base mixture. Absorbance was measured at 405 nm using a spectrophotometer. The assays were run three times using a unique set of reagents for each assay. Protein content for each tissue was quantified by the method of Lowry *et al*. (1951) using bovine serum albumin as the standard. Specific activities were calculated as nmoles $min^{-1}mgP^{-1}$.

In Vitro Acetylcholinesterase IC_{50} Determination

The inhibitory concentration 50 (IC_{50}) of a compound represents the concentration at which 50% of a given enzyme's activity is inhibited. IC_{50}s are often determined as an index of potency for a chemical inhibitor. For this study, AChE IC_{50}s were determined for each of 12 organophosphates in each of four rat tissues using a discontinuous spectrophotometric assay (modification of Ellman *et al*., 1961) with acetylthiocholine as the substrate and 5,5'-dithio-bis(nitrobenzoic acid) (DTNB) as the chromogen (Chambers *et al*., 1988). Briefly, tissue homogenates were diluted (2 ml total assay volume) in buffer (0.05M Tris-HCl buffer (pH 7.4 at 37ºC)) and 20 µl of ethanol vehicle or one of five concentrations of each OP oxon in ethanol vehicle were added, vortexed, and placed in a shaking water bath preheated to 37ºC. Additional tubes (blanks) containing eserine sulfate (FC, 10 µM) were included in the assay to inhibit AChE and correct for non-

enzymatic hydrolysis. Each assay contained triplicate subsamples for vehicle controls and each inhibitor concentration as well as duplicate eserine sulfate blanks. Following the initial incubation, 20 µl of acetylthiocholine (FC, 1 mM) was added as a substrate for the remaining uninhibited AChE and was incubated for an additional 15 min. The reaction was terminated and color was developed using 250 µl of a 5% SDS/0.24 M DTNB mixture (4:1). Absorbance was measured at 412 nm using a spectrophotometer. Percent inhibition of control (EtOH vehicle) absorbance was calculated for each concentration. IC_{50} values were determined by linear regression analysis of the plot of the logit of percent inhibition versus $\log_{10}$ oxon concentration. The best-fit line was drawn using points corresponding to the 20-80 percent AChE inhibition range and the equation of the best-fit line was solved for the x-intercept to determine the IC_{50}. The procedure was used for brain, heart, lung, and skeletal muscle. The assays were run three times using a unique set of reagents for each assay.

In Vitro Serum AChE and BChE IC_{50} Determination

The determination of serum AChE and BChE IC_{50}s requires the usage of specific inhibitors to separate AChE and BChE activities. For the determination of both serum AChE and BChE IC_{50}s, 10 µl of serum was added to 990 µl of 0.05M Tris-HCl buffer (pH 7.4 at 37ºC) for a total assay volume of 1 ml. Ten microliters of 0.1 M EDTA was added to the dilute serum to inhibit A-esterase (paraoxonase) preventing any catalytic hydrolysis of oxons by paraoxonase. Ethopropazine (FC, 1 µM), a selective BChE inhibitor, was added to inhibit all BChE activity leaving AChE functional. In parallel samples, a selective AChE inhibitor (BW284C51) was added to inhibit all AChE activity

leaving BChE functional. Ten microliters of ethanol vehicle or one of five concentrations of each OP in ethanol vehicle were added, vortexed, and placed in a shaking water bath preheated to 37ºC. Additional tubes (blanks) containing eserine sulfate (FC, 10 µM) were included in the AChE assay to inhibit AChE and correct for non-enzymatic hydrolysis. Blank tubes for the BChE assay contained *iso*-OMPA (tetraisopropyl pyrophosphoramide, FC, 10 µM) to inhibit BChE and correct for non-enzymatic hydrolysis. Each assay contained triplicate subsamples for vehicle controls and each inhibitor concentration as well as duplicate blanks. Following an initial incubation of 15 min, 10 µl of acetylthiocholine (FC, 1 mM) or butyrylthiocholine (FC, 1 mM) was added as a substrate for the remaining uninhibited AChE or BChE, respectively, and was incubated for an additional 15 min. The reaction was terminated and color was developed using 125 µl of a 5% SDS/0.24 M DTNB mixture (4:1). Absorbance was measured at 412 nm using a spectrophotometer. Percent inhibition of control (EtOH vehicle) absorbance was calculated for each concentration. IC_{50} values were determined by linear regression analysis of the plot of the logit of percent inhibition versus $\log_{10}$ oxon concentration. The best-fit line was drawn using points corresponding to the 20-80 percent AChE or BChE inhibition range and the equation of the best-fit line was solved for the x-intercept to determine the IC_{50}. The assays were run three times using a unique set of reagents for each assay.

In Vitro Liver Carboxylesterase IC_{50} Determination

Liver tissue was homogenized in 0.05M Tris-HCl buffer (pH 7.4 at 37ºC). The liver homogenate was assayed according to the method of Chambers *et al*. (1990) using

p-nitrophenyl valerate (*p*NPV) as the substrate for CaE. To determine CaE IC_{50}s for each of the 12 OPs, 20 µl of ethanol vehicle or one of five concentrations of each OP in ethanol vehicle was added to dilute homogenates (2 ml total assay volume), vortexed, and placed in a shaking water bath preheated to 37ºC. Tubes (blanks) containing paraoxon (FC, 10 µM) were included in the assay to inhibit CaE and correct for non-enzymatic hydrolysis. Each assay contained triplicate subsamples for vehicle controls and each inhibitor concentration as well as duplicate paraoxon blanks. Following an initial incubation of 15 min, 20 µl of *p*NPV (FC, 500 µM) in ethanol vehicle was added as a substrate for the remaining uninhibited CaE and was incubated for an additional 15 min. The reaction was terminated with 500 µl of a 2% SDS/2% Tris-base mixture. Absorbance was measured at 405 nm using a spectrophotometer. Percent inhibition of control (EtOH vehicle) absorbance was calculated for each concentration. IC_{50} values were determined by linear regression analysis of the plot of the logit of percent inhibition versus $\log_{10}$ OP concentration. The best-fit line was drawn using points corresponding to the 20-80 percent CaE inhibition range and the equation of the best-fit line was solved for the x-intercept to determine the IC_{50}. The assays were run three times using a unique set of reagents for each assay.

In Vitro Serum Carboxylesterase IC_{50} Determination

Serum was diluted at a concentration of 5 µl/ml in 0.05M Tris-HCl buffer (pH 7.4 at 37ºC). The dilute serum (1 ml total assay volume) was assayed according to the method of Chambers *et al*. (1990) using *p*-nitrophenyl valerate (*p*NPV) as the substrate for CaE. To determine CaE IC_{50}s for each of the 12 OPs in this study, 10 µl of ethanol

vehicle or one of five concentrations of each OP in ethanol vehicle were added, vortexed, and placed in a shaking water bath preheated to 37ºC. Tubes (blanks) containing paraoxon (FC, 10 µM) were included in the assay to inhibit CaE and correct for non-enzymatic hydrolysis. Each assay contained triplicate subsamples for vehicle controls and each inhibitor concentration as well as duplicate paraoxon blanks. Following an initial incubation of 15 min, 10 µl of *p*NPV (FC, 500 µM) in ethanol vehicle was added as a substrate for the remaining uninhibited CaE and was incubated for an additional 15 min. The reaction was terminated with 250 µl of a 2% SDS/2% Tris-base mixture. Absorbance was measured at 405 nm using a spectrophotometer. Percent inhibition of control (EtOH vehicle) absorbance was calculated for each concentration. IC_{50} values were determined by linear regression analysis of the plot of the logit of percent inhibition versus $\log_{10}$ OP concentration. The best-fit line was drawn using points corresponding to the 20-80 percent CaE inhibition range and the equation of the best-fit line was solved for the x-intercept to determine the IC_{50}. The assays were run three times using a unique set of reagents for each assay.

In Vitro Serum AChE IC_{50} Determination Using Specific Inhibitors

Subsequently, additional experiments were conducted with adult serum to selectively inhibit non-target (detoxication) esterases (paraoxonase, carboxylesterase and butyrylcholinesterase). For these experiments, 10 µl of EDTA (1 mM), 10 µl of saligenin cyclic phenylphosphonate, SCPP, (FC, 50 nM) and 10 µl ethopropazine (FC, 1 µM) were added to adult rat diluted serum to inhibit paraoxonase, carboxylesterase, and butyrylcholinesterase, respectively, leaving AChE functional. Ten microliters of ethanol

vehicle or one of five concentrations of each OP in ethanol vehicle were added, vortexed, and placed in a shaking water bath preheated to 37ºC. Additional tubes (blanks) containing eserine sulfate (FC, 10 µM) were included in the assay to inhibit AChE and correct for non-enzymatic hydrolysis. Each assay contained triplicate subsamples for vehicle controls and each inhibitor concentration as well as duplicate eserine sulfate blanks. Following the initial incubation, 10 µl of acetylthiocholine (FC, 1 mM) was added as a substrate for the remaining uninhibited AChE and was incubated for an additional 15 min. The reaction was terminated and color was developed using 125 µl of a 5% SDS/0.24 M DTNB mixture (4:1). Absorbance was measured at 412 nm using a spectrophotometer. Percent inhibition of control (EtOH vehicle) absorbance was calculated for each concentration. IC_{50} values were determined by linear regression analysis of the plot of the logit of percent inhibition versus $\log_{10}$ OP concentration. The best-fit line was drawn using points corresponding to the 20-80 percent AChE inhibition range and the equation of the best-fit line was solved for the x-intercept to determine the IC_{50}. The assays were run three times using a unique set of reagents for each assay.

Statistics

Specific activities for each tissue were analyzed by an analysis of variance (ANOVA) using SAS software on a personal computer with mean separation by the Student-Newman-Keuls (SNK) post-hoc test. Significant difference among ages is reported for the $p < 0.05$ level. IC_{50}s were calculated as the mean of three independent linear regressions using Excel software and mean IC50s were subsequently analyzed

using SAS software on a PC with significant difference among ages determined by a lack of overlap of 95% Confidence Intervals for each compound.

Results

Acetylcholinesterase activities of brain significantly increased with age. Activity was 1.8-fold higher in juveniles than neonates and 2.2-fold higher in adults than neonates. The AChE activities of the peripheral tissues (heart, lung, and skeletal muscle) increased with age with PND 70 animals having significantly higher activities than PND 12s and PND 1s. Although activities were trending higher for PND 12s compared to PND 1s no significant differences were determined. Serum AChE activity was not significantly different among the three ages; however, serum BChE activities were significantly higher in PND 70 animals compared to PND 12 and PND 1 animals. Carboxylesterase activities for both liver and serum were significantly different among all three ages. The hepatic CaE activities of the PND 70 rats were about 2-fold higher than the PND 12 rats and about 8-fold higher than the PND 1 rats. Hepatic CaE activities increased 3.8-fold from PND 1 to PND 12. A similar increase was determined within serum among all three ages (Table 2). Adult rat tissues all had significantly higher protein levels than neonate or juvenile tissues (Table 3).

IC_{50}s were determined using equivalent amounts of tissue within a tissue for all three ages; therefore, the activities were different for some age groups. The diethyl insecticidal OPs were generally more potent AChE inhibitors than the dimethyl OPs as indicated by the IC_{50}s for brain, heart, lung, skeletal muscle and serum (Table 4-5). The two model diethyl OPs, ethyl ronnel-oxon and ethyl cyanophos-oxon, were not potent

inhibitors of AChE in any of the tissues. The more potent inhibitors of AChE, except for phoxim-oxon, contain a heterocyclic ring in their structure (Figure 1). The presence of the nitrogen in the ring and the difference in potency is evident when comparing chlorpyrifos-oxon and ethyl-ronnel-oxon, with chlorpyrifos-oxon (pyridine ring) about 70-fold more potent than ethyl-ronnel-oxon (aromatic ring) in brain and peripheral tissues (Table 4-5). Acetylcholinesterase IC_{50}s within brain, heart, lung and skeletal muscle for both the diethyl and dimethyl organophosphates were not different among the three ages (PND 1, PND 12, and PND 70). The OP IC_{50}s for AChE in the peripheral tissues typically exhibited greater variability than IC_{50}s in brain tissue with the more potent OPs usually having less variability (Table 4-5). IC_{50}s for serum AChE were generally equivalent in the neonates (PND 1) and juveniles (PND 12) but were significantly higher in adults (PND 70) for all OPs tested except azinphos-methyl-oxon, ethyl ronnel-oxon, and ethyl cyanophos (Table 6). The addition of a specific inhibitor for CaE in adult (PND 70) serum reduced the AChE IC_{50}s to values similar to those determined for the neonates and juveniles (Table 6). No significant differences were determined for azinphos-methyl-oxon, ethyl ronnel-oxon, and ethyl cyanophos among the three ages with or without CaE specific inhibitors. Three OPs, dicapthon-oxon, methyl coumaphos-oxon and nitropyrifos-oxon, exhibited IC_{50}s that were significantly lower in adults than the corresponding neonatal and juvenile IC_{50}s (Table 6). Serum BChE IC50s generally increased with age (Table 7). Butyrylcholinesterase IC50s were significantly higher for PND 70 serum than PND 12 and PND 1 IC_{50}s for all OPs except azinphos-methyl oxon and methyl paraoxon. Serum BChE was more sensitive to the diethyl OPs than the

dimethyl OPs, as evidenced by the greater potency of the diethyl OPs. Azinphos-methyl-oxon and methyl coumaphos-oxon were not good inhibitors of serum BChE (Table 7).

IC_{50}s for hepatic CaE generally increased with age (Table 8). The hepatic CaE IC_{50}s for methyl paraoxon, methyl coumaphos-oxon, chlorpyrifos-oxon and ethyl ronnel-oxon, were significantly higher for the PND 12 rats than the PND 1 rats. Hepatic IC50s were significantly higher for PND 70 rats compared to PND 1 rats for paraoxon, ethyl ronnel-oxon, diazoxon, and nitropyrifos-oxon. Additionally, CaE IC50s were significantly higher from livers of PND 70 rats compared to PND 12 rats for methyl paraoxon, methyl coumaphos-oxon, and chlorpyrifos-oxon. Because of low activity and limited sample volume, CaE IC_{50}s were not measured in serum from neonatal rats (PND 1). IC_{50}s for serum CaE generally increased with age from PND 12 to PND 70 with IC_{50}s for methyl paraoxon, paraoxon and chlorpyrifos-oxon from PND 70 rats significantly higher than those from PND 12s. Hepatic and serum CaEs were more sensitive to the diethyl OPs than the dimethyl OP oxons, as evidenced by the greater potency from the diethyl OPs in PND 70 (highest CaE activity) liver and serum. The two most potent CaE inhibitors were chlorpyrifos-oxon and ethyl ronnel-oxon both with chlorinated rings in their structures.

Discussion

Acetylcholinesterase IC_{50}s for the twelve organophosphates (OPs) in this study suggest that there is no age-related difference in the inhibitory potential toward AChE in the rat brain. Similar results were observed for the AChE IC_{50}s in heart, lung and skeletal muscle. These results were not surprising with AChE having been determined to be from

a single gene product (Massoulié *et al*., 2008). The decision to measure IC_{50}s in equivalent tissue concentrations and not activity levels within a tissue could mean the number of enzyme active sites were not the same among the ages, which could result in slightly different IC_{50}s. The diethyl OPs were generally more potent inhibitors of AChE than the dimethyl OPs, except for the two model compounds and diazoxon. Diazoxon was observed to be particularly unstable as well as it has a propensity to adhere to glass, which could account for the lower potency compared to the other diethyl insecticidal OPs. Acetylcholinesterase appears to be more sensitive to diethyl OPs containing heterocyclic rings in their structure compared to OPs containing aromatic rings, except for phoxim-oxon which is an equally good inhibitor. For most of the OP compounds, slight increases in IC_{50}s were observed with age but did not reach significance. These results are in agreement with previously reported data for chlorpyrifos-oxon (Mortensen *et al*., 1996; Atterberry *et al*., 1997) in rat brain. There were significant differences in serum AChE IC_{50}s between the adult (PND 70) and young rats (PND 1 or PND12) for 9 of the 12 OPs tested; however, these differences were negated when a specific CaE inhibitor was included in the assays, indicating that the higher IC_{50}s in the adult rat serum are most likely because of CaEs stoichiometrically binding circulating OP and preventing the inhibition of AChE. This correlates with the increase in CaE activity in the serum in adult rats compared to the juveniles and neonates.

Studies from our laboratory and others have shown that young rats have lower CaE activity than adults (Moser *et al*., 1998; Atterberry *et al*., 1997). Atterberry *et al*. (1997) reported hepatic CaE activities in young rats (PND 3) were about 5-fold lower

than adult rats (PND 70). Moser *et al*. (1998) also reported similar differences (6-fold increase) for serum CaE activity between the PND 3 and PND 70 rats. Atterberry *et al*. (1997) also showed a decrease in sensitivity *in vivo* to two OP insecticides, chlorpyrifos and parathion, paralleled the maturation of liver CaE with age in rats. This suggests that the age-related differences in acute toxicity levels that are associated with some organophosphate compounds are likely due to either a difference in the rate of CYP450-mediated metabolism to their bioactive metabolite or more likely to differences in the detoxication of the oxons by non-target esterases such as carboxylesterases and A-esterases (paraoxonases) (Gagne and Brodeur, 1972; Chambers *et al*., 1990; Forsyth and Chambers, 1989; Fukuto *et al*., 1990; Pope *et al*., 1991; Ma and Chambers, 1995; Pond *et al*., 1995; Atterberry *et al*., 1997; Moser *et al*., 1998). This hypothesis is supported by the data within this study.

The lower serum and hepatic CaE IC_{50}s, reported here, suggests that younger rats may be more sensitive to some OPs. This may be a result of lower concentrations of CaE and/or sensitivity of enzymes in younger ages. The age-related increase in serum AChE IC_{50}s without a specific inhibitor for carboxylesterases (CaE activity present) further exhibits the protection CaE can provide the target enzyme (AChE) to OPs. As with AChE, the structure of the OP can affect the inhibitory potential for CaE. Data within this study suggest that the diethyl OPs are much better inhibitors of CaE than the dimethyl OPs. The *in vitro* data from this study correlate with data from other studies comparing OP potencies for AChE *in vitro* and toxicities *in vivo* (Chambers *et al*., 1990; Chambers and Carr, 1993; Chambers *et al*., 1994). The greater detoxication of many

diethyl OPs helps explain the discrepancies between the AChE inhibitory potencies and acute toxicity. For example, chlorpyrifos-oxon (a diethyl OP) is a better inhibitor of AChE than methyl paraoxon or azinphos-methyl-oxon (dimethyl OPs); however, chlorpyrifos has a higher rat oral LD_{50} (96 mg/kg) than azinphos-methyl (12 mg/kg) or methyl parathion (6 mg/kg). Chlorpyrifos-oxon has a much lower CaE IC_{50} than azinphos-methyl-oxon or methyl paraoxon, indicating a higher affinity for the CaE and thus potentially scavenging a greater amount from circulation resulting in greater protection. All the diethyl OPs exhibited IC_{50}s in the low nanomolar range in both liver and serum indicating the high affinity for CaE and potential for greater detoxication. Pope *et al*. (1991) showed faster peak inhibition of brain cholinesterase activity in neonates compared to adults following chlorpyrifos exposure. This was most likely the result of lower detoxication (CaE and paraoxonase) enzymes in the neonates allowing more chlorpyrifos-oxon to reach the target brain AChE. Exposures to high acute levels or chronic lower levels of some OPs can saturate the detoxication enzymes resulting in substantial brain AChE inhibition and toxicity (Chambers and Chambers, 1990). While detoxication of some of the OPs by CaEs and paraoxonases provides substantial protection especially in mature animals, studies have shown that inhibition of the target enzyme, brain AChE, can occur prior to saturation of CaEs (Chambers and Chambers, 1990). Chambers *et al*. (1991) showed that CYP-mediated bioactivation for parathion can occur in the target site organ (brain). Although the CYP activity in the brain was reported to be low, the bioactivation to paraoxon was appreciable enough to produce brain AChE inhibition. Paraoxonase can hydrolyze some OPs very efficiently, such as

chlorpyrifos-oxon, but typically is more important in chronic lower level OP exposures (Pond *et al*., 1995; Furlong *et al*., 1989; Atterberry *et al*., 1997; Tang and Chambers, 1999). The lower levels of paraoxonase activity in juveniles can be important in the increased toxicity of some OPs in young animals (Atterberry *et al*., 1997; Li *et al*., 1997; Karanth and Pope, 2000).

In addition to CaE and paraoxonase detoxication of OPs, the stoichiometric inhibition of BChE by OPs can reduce OP toxicity by scavenging OP molecules prior to reaching the target enzyme, AChE. OPs with higher affinities for BChE than AChE could be detoxified more efficiently than OPs that have a higher affinity for AChE. Adult rat serum BChE IC_{50}s for the dimethyl OPs tested within this study were higher than serum AChE IC_{50}s indicating that the dimethyl compounds have a greater affinity for AChE than BChE. This suggests that BChE would not scavenge dimethyl OPs well; therefore, BChE would not afford much protection from OPs with these structural characteristics. The reverse was observed for the diethyl OPs with adult rat serum BChE IC_{50}s lower than adult rat serum AChE IC_{50}s indicating greater diethyl OP oxon affinity for BChE and subsequently the potential for increased detoxication. All of the diethyl OPs tested exhibited significantly higher BChE IC_{50}s in adults than neonates and juveniles. This decrease in potency is most likely because of the maturation of BChE with age. Only one of the dimethyl OPs, dicapthon-oxon, tested within this study exhibited significantly higher IC_{50}s in adult rat serum compared to neonates and juveniles, although the IC_{50}s for the other three dimethyl OPs were trending higher with increasing age. Similar to AChE, serum BChE appears to be slightly more sensitive to

diethyl oxons containing heterocyclic rings in their structure compared to OPs containing aromatic rings. Li *et al*. (2000) reported higher BChE concentration was higher in adult heart tissue compared to neonates and subsequently, Howard *et al*. (2007) showed that BChE activity in neonatal rat heart was more sensitive to inhibition by chlorpyrifos-oxon than activity in adult rat heart tissue. The stoichiometric inhibition of BChE by OPs can result in saturation of the enzyme which can lead to increased toxicity in high level exposures or chronic lower level exposures. Similarly, lower levels of BChE activity in the serum of younger animals may increase their susceptibility to OP toxicity.

Conclusion

The OP compounds investigated in this *in vitro* study displayed a wide range of inhibitory potential toward the target enzyme, AChE, as well as non-target detoxication enzymes, BChE and CaE, ranging over several orders of magnitude. Generally, the diethyl insecticidal oxons were more potent inhibitors than the dimethyl insecticidal oxons, which is in contrast to the acute toxicity of some of the parent insecticides. This discrepancy may be partially explained by the fact that many of the dimethyl compounds are poor inhibitors of the non-target esterases responsible for detoxication of OPs. This study and others have reported age-related differences in the *in vitro* potency of some OP compounds in tissues from rats (Mortensen *et al*., 1996, 1998; Moser *et al*., 2016). *In vitro* studies can provide valuable insights into the metabolism and detoxication pathways of OPs to help explain the age-related differences in acute OP toxicity *in vivo,* that have been reported for many years (Brodeur and DuBois, 1963; Benke and Murphy, 1975; Gaines and Linder, 1986; Pope *et al*., 1991; Atterberry *et al*., 1997; Moser *et al*., 1998).

Although the toxicological target for OPs is well described, the differences in bioactivation and detoxication with respect to age and sex must be considered when assessing risk. *In vivo* challenges of OPs are ultimately needed to address these differences; however, *in vitro* studies such as the one reported here can provide information (potency, kinetic rate constants, etc.) for the biochemical processes that can then be used in developing predictive models that, once validated, can reduce or replace the number of *in vivo* studies.

Table 2.1 Organophosphate compounds and their corresponding toxicities

Organophosphate Oxon (active metabolite)	Commercial Pesticide or Model Compound	Rat Oral LD_{50} (mg/kg)
Azinphos-methyl-oxon (AZMxn)	Azinphos-methyl	12 mg/kg
Methyl paraoxon (MPxn)	Methyl parathion	6 mg/kg
Dicapthon-oxon (DCxn)	Dicapthon	400 mg/kg
Methyl coumaphos-oxon (MCxn)	Model Compound	NA
Paraoxon (Pxn)	Parathion	2 mg/kg
Chlorpyrifos-oxon (CPxn)	Chlorpyrifos	96 mg/kg
Ethyl cyanophos-oxon (Ecxn)	Model Compound	NA
Ethyl ronnel-oxon (Erxn)	Model Compound	NA
Diazoxon (DZxn)	Diazinon	1250 mg/kg
Naftalophos (Nftf)	Naftalophos	140 mg/kg
Nitropyrifos-oxon (NPxn)	Model Compound	NA
Phoxim-oxon (Phxxn)	Phoxim	2000 mg/kg

LD_{50} values (rat oral) for the commercial organophosphates (parent compound) are presented as reported in (Meister *et al*., 1992 or EPA, 2006). NA (not available) = LD_{50} values have not been determined for model compounds (experimental use only).

Table 2.2 Specific activities for brain, heart, lung, skeletal muscle and serum acetylcholinesterase (AChE), serum butyrylcholinesterase (BChE), and liver and serum carboxylesterase (CaE) from rats of three ages (post-natal day, PND 1, 12 and 70).

	AChE Activities					**BChE Activities**	**CaE Activities**	
Age	**Brain**	**Heart**	**Lung**	**Skeletal Muscle**	**Serum**	**Serum**	**Liver**	**Serum**
PND 1	43.4 ±7.1^{A}	19.9 ± 0.2 ^{A}	17.9 ± 0.6^{A}	21.2 ± 0.3^{A}	10.4 ± 1.1^{A}	4.9 ± 0.2^{A}	147 ± 10^{A}	20 ± 6.2^{A}
PND 12	79.4 ± 4.8^{B}	22.8 ± 0.7^{A}	20.6 ± 2.0^{A}	22.9 ± 0.7^{A}	11.4 ± 0.6^{A}	6.0 ± 0.2^{A}	558 ± 11^{B}	70 ± 6.3^{B}
PND 70	98.8 ± 5.2^{C}	27.0 ± 0.9^{B}	25.4 ± 1.2^{B}	30.3 ± 1.2^{B}	12.9 ± 0.3^{A}	8.7 ± 0.4^{C}	1154 ± 22^{C}	160 ± 7.1^{C}

Specific activities expressed as nmoles min^{-1} mgP^{-1}, for AChE, BChE and CaE, means ± SEM of three independent replications. Means within a tissue not followed by the same letter are significantly different (p<0.05).

Table 2.3 Protein levels for brain, heart, lung, skeletal muscle, serum and liver from rats of three ages (post-natal day, PND 1, 12 and 70).

Age	Protein µg					
	Brain	**Heart**	**Lung**	**Skeletal Muscle**	**Serum**	**Liver**
PND 1	76 ± 0.9^{A}	137 ± 0.3^{A}	107 ± 0.1^{A}	121 ± 0.1^{A}	74 ± 1.1^{A}	135 ± 1.2^{A}
PND 12	96 ± 0.4^{A}	145 ± 0.1^{A}	126 ± 0.1^{A}	133 ± 0.9^{A}	96 ± 0.8^{B}	144 ± 0.7^{A}
PND 70	138 ± 0.5^{B}	176 ± 0.1^{B}	149 ± 0.1^{B}	150 ± 1.2^{A}	109 ± 0.4^{C}	189 ± 0.8^{C}

Protein levels expressed as µg P, for AChE, BChE and CaE, means ± SEM of three independent replications. Means within a tissue not followed by the same letter are significantly different ($p < 0.05$).

Table 2.4 Acetylcholinesterase inhibition by various organophosphates in rat cardiac, pulmonary, and muscle tissue.

	Heart			Lung			Skeletal Muscle		
	PND 1	**PND 12**	**PND 70**	**PND 1**	**PND 12**	**PND 70**	**PND 1**	**PND 12**	**PND 70**
Oxon	**IC_{50} (nM) (95%CI)**	**IC_{50} (nM) (95%CI)**	**IC_{50} (nM) (95%CI)**	**IC_{50} (nM) (95%CI)**	**IC_{50} (nM) (95%CI)**	**IC_{50} (nM) (95%CI)**	**IC_{50} (nM) (95%CI)**	**IC_{50} (nM) (95%CI)**	**IC_{50} (nM) (95%CI)**
AZMxn	430 (252,607)	495 (317,673)	212 (33,389)	407 (256,558)	281 (129,423)	279 (172,430)	154 (82,226)	159 (101,218)	83 (25,141)
MPxn	248 (36,461)	583 (410,757)	507 (334,681)	455 (307,503)	438 (290,586)	158 (8,307)	266 (119,413)	243 (123,363)	184 (64,305)
DCxn	67 (19,113)	84 (46,122)	34 (-4,73)	109 (95,123)	100 (86,115)	116 (101,131)	82 (64,101)	64 (49,78)	92 (78,107)
MCxn	955 (832,1077)	987 (846,1109)	894 (773,1017)	474 (377,571)	446 (350,543)	295 (198,391)	250 (162,348)	230 (161,299)	94 (25,163)
Pxn	156 (115,197)	167 (127,208)	103 (62,143)	69 (60,77)	65 (57,74)	54 (45,63)	34 (9,59)	47 (26,67)	50 (29,70)
CPxn	5 (3,8)	5 (3,7)	3 (1,5)	8 (6,10)	7 (5,8)	6 (5,8)	6 (4,9)	4 (2,6)	9 (6,10)
Ecxn	1313 (1116,1510)	1395 (1235,1556)	1040 (880,1241)	1163 (1038,1387)	1217 (993,1443)	812 (587,1036)	791 (645,937)	1022 (876,1169)	733 (587,879)
Erxn	717 (704,729)	721 (711,731)	708 (706,717)	652 (618,688)	687 (652,722)	699 (664,735)	744 (726,762)	752 (734,770)	773 (755,791)
DZxn	45 (32,57)	43 (32,53)	36 (26,47)	134 (91,177)	133 (90,177)	221 (177,264)	22 (7,37)	38 (26,51)	51 (37,62)
Nftf	17 (6,27)	11 (7,15)	4 (1,7)	43 (33,52)	30 (21,39)	44 (35,54)	20 (3,37)	17 (3,31)	41 (27,55)
NPxn	54 (44,64)	61 (38,54)	36 (28,44)	46 (29,63)	49 (32,66)	24 (7,41)	30 (17,43)	29 (15,42)	28 (15,42)
Phxxn	15 (9,20)	12 (7,16)	8 (4,13)	24 (19,29)	21 (16,27)	21 (15,26)	14 (6,21)	12 (5,18)	17 (11,24)

IC_{50}s are expressed as means with 95% confidence intervals of three independent replications for each age group. Means for each compound followed by an * are significantly different ($p < 0.05$) among ages within a tissue.

Table 2.5 Acetylcholinesterase inhibition by various organophosphates in rat brain.

	Brain Acetylcholinesterase		
	PND 1	**PND 12**	**PND 70**
Oxon	**IC_{50} nM (95%CI)**	**IC_{50} nM (95%CI)**	**IC_{50} nM (95%CI)**
AZMxn	115 (104,125)	98 (68,109)	96 (86,107)
MPxn	165 (132,198)	118 (85,151)	135 (102,168)
DCxn	175 (144,205)	131 (101,162)	139 (108,170)
MCxn	124 (89,160)	109 (73,144)	74 (38,109)
Pxn	32 (24,39)	36 (28,44)	25 (16,32)
CPxn	9 (6,12)	8 (5,11)	8 (5,11)
Ecxn	984 (864,1104)	925 (805,1045)	784 (664,904)
Erxn	622 (515,729)	716 (609,822)	691 (583,798)
DZxn	224 (185,264)	252 (212,291)	273 (233,312)
Nftf	62 (43,81)	75 (57,94)	64 (46,83)
NPxn	40 (29,52)	38 (26,49)	34 (23,46)
Phxxn	50 (38,63)	52 (39,64)	43 (30,55)

IC_{50}s are expressed as means with 95% confidence intervals of three independent replications for each age group. Means for each compound followed by an * are significantly different ($p < 0.05$) among ages within a tissue.

Table 2.6 Acetylcholinesterase inhibition by various organophosphates in rat serum.

	Serum Acetylcholinesterase			Serum + Selective Inhibitors
	PND 1	**PND 12**	**PND 70**	**PND 70**
Oxon	**IC_{50} nM (95%CI)**	**IC_{50} nM (95%CI)**	**IC_{50} nM (95%CI)**	**IC_{50} nM (95%CI)**
AZMxn	117 (58,175)	127 (68,185)	178 (118,236)	232 (160,304)
MPxn	137 (70,163)	114 (68,161)	219* (172,265)	75 (18,131)
DCxn	94 (86,101)	84 (77,92)	144* (137,152)	46** (37,55)
MCxn	217 (195,238)	212 (191,234)	295* (273,317)	138** (110,163)
Pxn	56 (46,64)	47 (38,56)	106* (97,116)	48 (36,60)
CPxn	10 (7,13)	10 (6,13)	54* (51,57)	7 (3,11)
ECxn	1140 (926,1354)	1359 (1144,1573)	1534 (1319,1748)	1234 (972,1496)
ERxn	462 (409,515)	477 (424,530)	490 (437,543)	399 (334,463)
DZxn	157 (127,186)	194 (164,223)	309* (280,338)	213 (177,248)
Nftf	39 (29,49)	34 (24,44)	67* (57,77)	32 (20,44)
NPxn	46 (43,49)	51 (47,54)	83* (80,86)	23** (19,27)
Phxxn	30 (25,35)	31 (26,36)	109* (103,114)	29 (23,35)

IC_{50}s are expressed as means with 95% confidence intervals of three independent replications for each age group. Means for each compound followed by an * are significantly different ($p < 0.05$) among ages within a tissue. Serum + selective inhibitors for CaE (SCPP) and BChE (ethopropazine).

Table 2.7 Butyrylcholinesterase inhibition by various organophosphates in rat serum.

	Serum Butyrylcholinesterase		
	PND 1	**PND 12**	**PND 70**
Oxon	**IC_{50} nM (95%CI)**	**IC_{50} nM (95%CI)**	**IC_{50} nM (95%CI)**
AZMxn	1051 (931,1382)	1259 (1181,1337)	1433 (1176,1691)
MPxn	389 (386,392)	381 (357,406)	394 (337,452)
DCxn	58 (55,62)	57 (53,61)	185* (179,192)
MCxn	1223 (1137,1308)	1212 (1191,1234)	1469 (1116,1821)
Pxn	47 (46,48)	52 (50,54)	76* (58,94)
CPxn	6 (5,7)	7 (6,7)	39* (30,49)
ECxn	208 (167,249)	224 (180,268)	339* (282,396)
ERxn	2 (1,3)	7 (3,8)	35* (23,48)
DZxn	8 (7,9)	10 (9,11)	31* (30,33)
Nftf	5 (4,6)	7 (6,7)	30* (37,43)
NPxn	15 (14,16)	14 (13,15)	64* (56,73)
Phxxn	2 (1,3)	31* (26,36)	60** (57,64)

IC_{50}s are expressed as means with 95% confidence intervals of three independent replications for each age group. Means for each compound followed by an * are significantly different ($p < 0.05$) among ages within a tissue.

Table 2.8 Carboxylesterase inhibition by various organophosphates in rat hepatic tissue or serum.

	Carboxylesterase				
	Liver IC_{50} (nM) (95%CI)			Serum IC_{50} (nM) (95%CI)	
Oxon	**PND 1**	**PND 12**	**PND 70**	**PND 12**	**PND 70**
AZMxn	182 (158,206)	190 (98,282)	337 (257,417)	213 (70,359)	282 (272,321)
MPxn	0.6 (0.4,0.9)	3.2* (2.7,3.6)	309** (260,357)	49 (37,61)	80* (73,87)
DCxn	55 (27,83)	61 (32,92)	66 (28,105)	61 (27,94)	81 (74,87)
MCxn	49 (36,62)	89* (64,113)	640** (550,730)	31 (27,34)	35 (25,44)
Pxn	0.13 (0.1,0.2)	0.14 (0.1,0.2)	1.6* (0.5,2.8)	2.5 (1.8,3.1)	4.3* (3.5,5.1)
CPxn	0.02 (0.01,0.02)	0.05* (0.04,0.05)	0.16** (0.06,0.27)	0.30 (0.2,0.3)	0.70* (0.6,0.8)
ECxn	0.2 (0.1,0.3)	0.2 (0.1,0.2)	0.45 (0.2,0.7)	0.17 (0.01,0.33)	0.21 (0.08,0.35)
ERxn	0.04 (0.03,0.06)	0.09* (0.08,0.1)	0.20* (0.1,0.3)	0.35 (0.24,0.46)	0.49 (0.39,0.6)
DZxn	0.2 (0.1,0.3)	0.3 (0.2,0.3)	1.1* (0.8,1.3)	0.3 (0.2,0.5)	0.5 (0.4,0.6)
Nftf	5.0 (3.7,6.3)	5.3 (4.9,5.6)	5.7 (5.0,6.5)	1.1 (0.7,1.5)	1.3 (1.1,1.6)
NPxn	10 (5,15)	17 (11,23)	30* (25,34)	11 (6,16)	16 (8,24)
Phxxn	0.2 (0.1,0.3)	0.2 (0.2,0.3)	0.4 (0.3,0.5)	0.9 (0.6,1.2)	1.1 (0.7,1.5)

IC_{50}s are expressed as means with 95% confidence intervals of three independent replications for each age group. Means for each compound followed by an * are significantly different (p<0.05) among ages within a tissue.

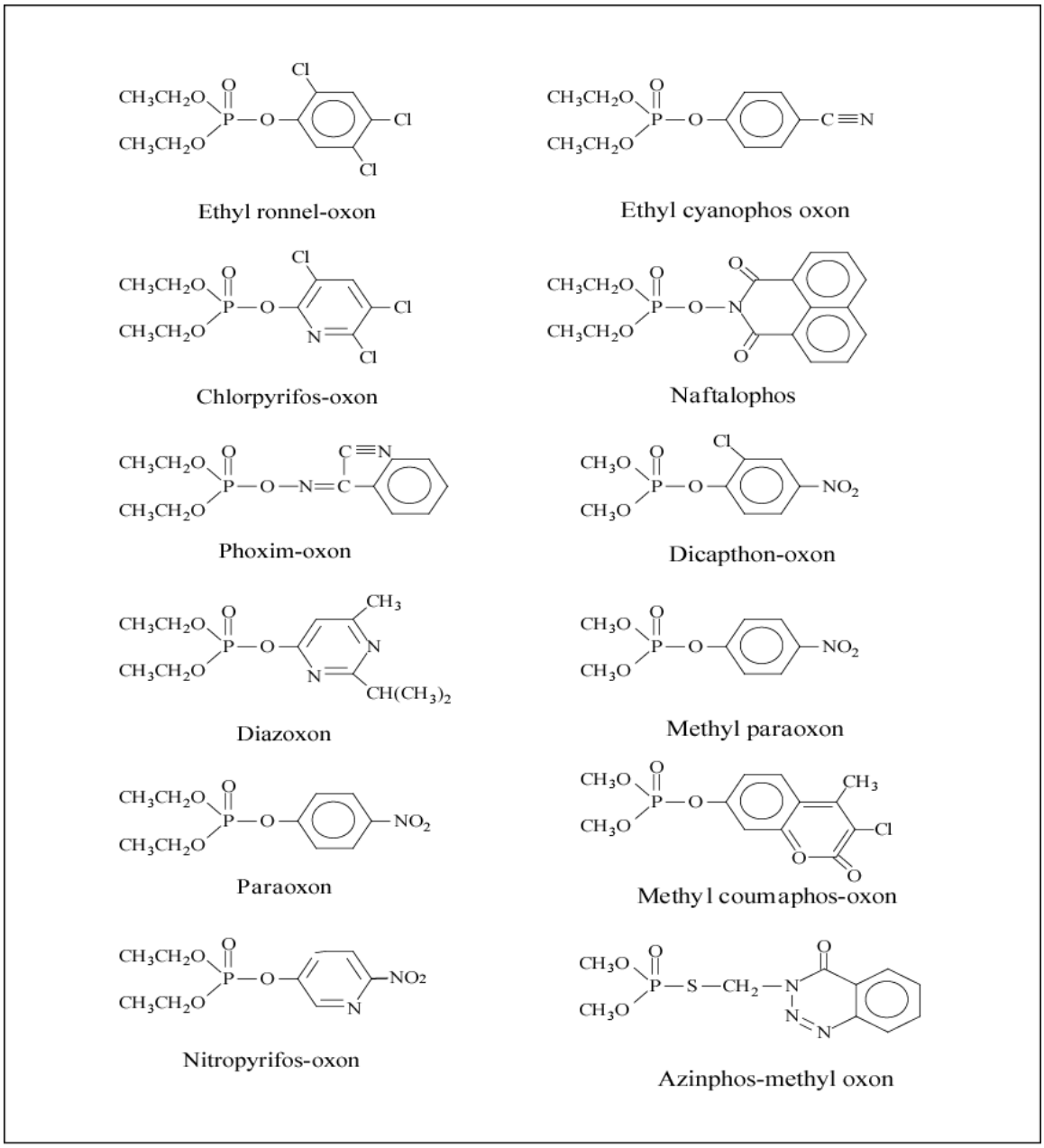

Figure 2.1 Chemical structures of 12 organophosphates/oxons.

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CHAPTER III

METABOLISM OF MALATHION BY HUMAN AND RAT HEPATIC MICROSOMES

Introduction

Malathion (*O,O*-dimethyl dithiophosphate of diethyl mercaptosuccinate; Figure 1) is one of the most extensively used organophosphate (OP) insecticides in the United States and throughout the world. First synthesized in 1956, malathion has continuously been heavily used because of its insecticidal properties combined with its low mammalian toxicity (rat oral LD_{50} > 1000 mg/kg) (Terrell, 1979; Fisher, 1991; Meister, 1992). Malathion is a non-systemic pesticide used to control a wide range of sucking and chewing insects in a variety of agricultural applications including crops and livestock. In addition, malathion is heavily used for public health in the control of vector-borne diseases such as the mosquito borne diseases malaria, zika, and dengue (ATSDR, 2017; US EPA, 2006). Depending on the application, malathion can be formulated as a dust, wettable powder, emulsifiable concentrate, or a liquid (US EPA, 2006). The heavy usage of this OP worldwide requires intensive risk assessment by regulatory agencies, such as the United States Environmental Protection Agency.

The primary toxic mechanism of action of malathion is via inhibition of the serine esterase, acetylcholinesterase (AChE, EC 3.1.1.7); this requires bioactivation of malathion by cytochrome P450 (CYP) to the active anticholinesterase metabolite,

malaoxon (Cohen, 1984; Forsyth and Chambers, 1989; Thompson *et al*., 1989; Fukuto, 1990; Buratti *et al*., 2005). The mammalian metabolism of malathion is very complex with bioactivation and detoxication processes occurring simultaneously (Dauterman and Main, 1966; Buratti *et al*., 2005). Talcott *et al*. (1979) reported that technical grade malathion can have impurities, such as isomalathion, that can affect its metabolism and toxicity. Malathion can be inactivated by glutathione *S*-transferases, although this is a minor pathway (Dauterman and Main, 1966; Talcott, 1979; Ketterman *et al*., 1987). The primary detoxication pathway involves the enzyme, carboxylesterase (CaE; EC 3.1.1.1), which is capable of degrading both the parent insecticide, malathion, and its anticholinesterase metabolite, malaoxon (Figure 1) (Casida, 1961; Main and Braid, 1962; Dauterman and Main; 1966). Malathion, containing carboxylic esters in its structure, can be catalytically hydrolyzed by CaE to a monocarboxylic acid (MCA) which can be further metabolized to a dicarboxylic acid (DCA) (Figure 2) (Seume and O'Brien, 1960; Main and Braid, 1962). The catalytic hydrolysis of malathion by CaE is very efficient and the MCA and DCA products are both considered non-toxic metabolites; therefore, this is considered an effective detoxication pathway (Main and Braid, 1962; Buratti *et al*., 2005).

Malaoxon, the anticholinesterase metabolite of malathion, can also stoichiometrically inhibit CaE decreasing the amount of malaoxon available to inhibit the target enzyme, AChE (Murphy and Dubois, 1957; Main and Braid, 1962). The detoxication potential via CaE of both malathion and malaoxon is a major factor in its low mammalian acute toxicity, rat oral $LD_{50} > 1$ g/kg (Casida, 1961; Main and Braid, 1962; Dauterman and Main; 1966). Main and Braid (1962) reported an increase of about

50-fold in the LD_{50} for malathion in rats pretreated with tri-*o*-tolyl phosphate, TOTP, a potent CaE inhibitor. This can be especially important in young animals that have been shown to have lower levels of CaE activity (Maxwell, 1992; Morgan *et al*., 1994; Atterberry *et al*., 1997). The abundance of CaE in adult rat liver and serum provides the potential for substantial degradation of many OPs. The absence of CaE activity in human serum (Main and Braid, 1962; Li *et al*., 2005) may reduce their overall detoxication capacity compared to the rat; however, humans possess large amounts of CaE in other tissues, especially the liver (Zhu *et a*l., 2009; Moser *et al*., 2016). Catalytic hydrolysis of some OP oxons by paraoxonase (PON), a calcium dependent hydrolase, can offer additional protection from OP intoxication (depending on the OP structure). Paraoxonase-mediated degradation of malaoxon has been proposed to be a potential mechanism in the detoxication of malaoxon; however, the validity of this detoxication pathway has not been confirmed. The maturation in OP detoxication enzymes (CaE and PON) with age has been well documented in animal models (Atterberry *et al*., 1997; Padilla *et al*., 2000) and humans, although activities in humans appear to be more variable with age (Cole *et al.*, 2003; Pope *et al*., 2005; Yang *et al*., 2005; Moser and Padilla, 2016).

The development of physiologically based pharmacokinetic (PBPK) models has become very valuable in evaluating risk for compounds with known toxicological targets (Reitz *et al*., 1988; Timchalk *et al*., 2002; Poet *et al*., 2004; Marino *et al*., 2006; Yang and Lu, 2006). These models use a series of differential equations to predict toxicity from a given exposure. Construction and validation of these mathematical models requires kinetic parameters generated from laboratory experiments for the target organs and

processes involved in the absorption, distribution, metabolism and excretion (ADME) of a given compound (Reddy, 2005). Kinetic constants for the bioactivation (desulfuration) and detoxication processes for malathion and its active metabolite, malaoxon, will be important for the construction of predictive pharmacokinetic models. Regulatory agencies have used PBPK models for other OPs in risk assessments for that compound (Timchalk *et al*., 2002; Knaak *et al*., 2004); however, the lack of a validated PBPK model for malathion is an impairment to the extrapolation from animal model data to human risk. The determination of kinetic rates for the degradation of malathion and malaoxon via carboxylesterases will be difficult with both detoxication processes occurring essentially simultaneously. This is further confounded by the stoichiometric inhibition of CaE by malaoxon, thus inactivating that CaE molecule preventing it from catalytically hydrolyzing malathion (Murphy and Dubois, 1957; Main and Braid, 1962). In addition, the maturation of enzymes responsible for activation (CYP) and detoxication (CaE and PON) with age must be considered when modeling the pharmacokinetics and pharmacodynamics of OPs, such as malathion (Timchalk *et al*., 2002; Clewell *et al*., 2004).

The majority of the risk assessment for OP compounds, such as malathion, has been based on data generated in animal models and extrapolated to humans (Timchalk *et al*., 2002; Knaak *et al*., 2004). In addition, human biomarker data following occupational or residential exposures has been used to refine and validate risk models and risk assessments (Bradman *et al*., 2003; Wessels *et al*., 2003; Barr and Angerer, 2006). The Federal Food, Drug, and Cosmetic Act (FFDCA), as amended by the Food Quality Protection Act of 1996, required the addition of uncertainty factors to add an additional

level of protection for infants and children which lowers the tolerances of exposure for these pesticides. Uncertainty factors are often included in risk assessments to account for differences in animals and humans (interspecies, 10x) and for differences in sensitive populations (intraspecies, 10x), such as children (US EPA, 1993, 1996; Dourson *et al*., 1996; Mileson *et al*., 1998; Yang and Lu, 2006). FQPA required an additional tenfold (10x) safety factor to account for differences with respect to exposure and toxicity in infants and children. FFDCA authorizes EPA to reduce the 10x FQPA safety factor only if sufficient data demonstrate that the margin of exposure is adequate (US EPA, 2009). The determination of kinetic parameters for the metabolism of malathion will be important in assessing if the additional levels of safety are necessary for interspecies and intraspecies differences.

The current investigation examined the metabolism of malathion using individual rat and human liver microsomes. Mammalian livers contain high levels of the enzymes responsible for both the bioactivation (CYP) and detoxication (CYP, CaE and PON) of OPs (Sultatos and Murphy, 1983; Chambers *et al*., 1990; Fukuto *et al.*, 1990; Pond *et al*., 1990; Chambers and Carr, 1993; Atterberry *et al*., 1997). The kinetic rate constants, V_{max} and K_{mapp}, for CYP-mediated bioactivation of malathion were determined *in vitro* using microsomes prepared from adult rats of both sexes and humans of both sexes and various ages and ethnicities. In addition, CaE activities and detoxication potential were determined for the same microsomes. Finally, the potential hydrolysis of malaoxon via paraoxonase was investigated as a detoxication pathway. The determination of kinetic rate constants and pathways of detoxication in both rat and human tissues will be useful

in the development of pharmacokinetic models for malathion and ultimately for risk assessment.

Materials and Methods

Chemicals

Malathion and its oxon metabolite, malaoxon, were supplied from FMC, Inc. Structures were confirmed by nuclear magnetic resonance (NMR) at FMC and the Department of Chemistry at Mississippi State University. All other reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or Thermo-Fisher Scientific (Waltham, MA). A selective carboxylesterase inhibitor saligenin cyclic phenylphosphonate (SCPP) was synthesized by Dr. Howard Chambers at Mississippi State University using standard procedures from commercially available intermediates (Chambers and Chambers, 1989) and purity was determined to be greater than 98% by NMR by the Department of Chemistry at Mississippi State University.

Human Microsomes

Human liver microsomes (archived frozen samples) were purchased from Sekisui Xenotech, LLC (Kansas City, KS). Twenty-seven individual microsomal samples were obtained that were from humans of both sexes, and of several races/ethnicities and span an age range of 0.04-75 years. In addition, a pooled microsomal sample prepared from 200 human livers was purchased to optimize assay conditions. All human liver microsomes were adjusted to 20 mg protein (P)/ml by the vendor.

Animals

Adult male (6) and female (6) Sprague Dawley-derived rats (post-natal day 70, PND 70; approximately 225-300 g) were purchased from Envigo, Inc. and housed in AAALAC accredited facilities in the College of Veterinary Medicine at Mississippi State University with temperature-controlled environments and 12 h dark-light cycle. Standard lab chow and tap water were provided *ad libitum*. All animal procedures received prior approval from the Mississippi State University Animal Care and Use Committee.

Rat Microsomes

Rat liver was collected from adult (PND 70) male and female rats, rinsed with 0.9% saline, immediately snap-frozen in liquid nitrogen and stored at -80ºC. Rat liver microsomes were prepared according to established procedures in our laboratories (Forsyth and Chambers, 1989). Briefly, a similar section of rat liver (0.5 g) was homogenized in 0.05M Tris-HCl buffer plus 0.15M KCl (pH 7.4 at 37ºC). Liver homogenates were centrifuged at 17,000 *g* for 15 min followed by ultracentrifugation of the resulting supernatant (110,000 *g* for 60 min). The supernatant was discarded and the microsomal pellet was suspended in 250 mM sucrose and stored at -80°C until assayed (suspension and storage conditions were equivalent to the purchased human microsomes).

Kinetics of Malathion Activation (Desulfuration)

Bioactivation (desulfuration) of malathion, which is CYP-mediated and occurs primarily in the liver, to malaoxon was quantified indirectly by the inhibition of an exogenous source of AChE (purified electric eel; FC, 0.2 U/ml) following incubation of

the microsomal fraction with malathion (Ma and Chambers, 1994; Buratti *et al*., 2005). The use of the AChE "trap" to capture the active metabolite, oxon, as it is being produced allows for detection at very low concentrations before appreciable degradation of the oxon can occur. Increasing concentrations of malathion were incubated with the dilute liver microsomes in order to establish curves to calculate the kinetic values, K_{mapp} and V_{max}. For this assay, microsomal pellets were thawed and resuspended in 0.05M Tris-HCl buffer plus 0.005M $MgCl_2$ (pH 7.4 at 37ºC). The resuspension was diluted to 1 mg equivalent/ml buffer. One hundred µl of the diluted microsomal resuspension was added to three borosilicate glass test tubes to which 100 µl of diluted electric eel AChE was also added and placed on ice. Eserine sulfate, 50 µl (FC, 0.2 mM), was added to the first tube to inhibit AChE and correct for non-enzymatic hydrolysis. To a second tube, a tissue blank, 50 µl of 0.05M Tris-HCl buffer plus 0.005M $MgCl_2$ (pH 7.4 at 37ºC) was added. To additional sample tubes (for each of eight concentrations of malathion), 50 µl of a NADPH generating system was added. The generating system consisted of 0.01 M glucose-6-phosphate, 0.001 M nicotinamide adenine dinucleotide phosphate) and 0.18 unit of glucose-6-phosphate dehydrogenase. Tubes were vortexed and placed in a shaking water bath pre-heated to 37ºC for 2 min. The reaction was initiated by the addition of 5 µl of each concentration of malathion in EtOH vehicle to all tubes. The tubes were vortexed and incubated in the water bath for 30 min. Following the incubation period, cholinesterase activity was monitored spectrophotometrically using a modified Ellman *et al*. (1961) assay (Chambers *et al*., 1988); 1.75 ml of 0.05M Tris-HCl buffer (pH 7.4 at 37ºC) plus acetylthiocholine iodide (ATCh, FC, 1 mM) was added, vortexed and incubated for an additional 10 min to react with any uninhibited AChE.

The reaction was terminated and color was developed using 250 µl of a 5% sodium dodecyl sulfate/0.024 M 5,5'-dithiobis(nitrobenzoic acid) (SDS/DTNB) mixture (4:1). Absorbance was measured at 412 nm using a spectrophotometer.

Malaoxon generated was quantified from a standard curve of malaoxon concentration versus electric eel AChE inhibition. A range of concentrations were used to result in AChE inhibition between 10% and 90%. Linear regression analysis was performed of the plot of the logit of percent inhibition versus $\log_{10}$ oxon concentration. The best-fit line was drawn using points corresponding to the 10-90% AChE inhibition range. The standard curve was run three independent times using a unique set of reagents and the data were averaged to produce a single standard curve to use for quantification of malathion bioactivation to malaoxon (Figure 3). This assay provides an advantage over some analytical chemistry techniques such as high-performance liquid chromatography, HPLC, that can be expensive, time consuming, require extraction of the metabolites increasing the likelihood of loss during extraction, and may not capture the oxon before degradation occurs.

Carboxylesterase Degradation of Malathion

In addition to the standard desulfuration assay, a single high concentration of malathion (200 µM) was incubated with each dilute microsome with and without pre-treatment with a selective CaE inhibitor, SCPP, to assess CaE-mediated degradation using AChE inhibition as an endpoint. The assay procedure was identical to the desulfuration assay previously described with the addition of a pre-incubation step (10 min in a shaking water bath at 37ºC) with SCPP (FC, 50 nM) or EtOH vehicle.

Carboxylesterase Degradation of Malaoxon

To assess the capacity of each rat and human hepatic microsome to degrade malaoxon by CaEs, an indirect measurement of the CaE-mediated degradation was employed. Parallel dilute hepatic microsomal preparations (0.25 ml, equivalent to the desulfuration assays) were pre-incubated with or without a specific CaE inhibitor, SCPP (FC, 50 nM in EtOH vehicle), for 10 min in a shaking water bath at 37ºC. Following pre-incubation, one of each of five concentrations of malaoxon in EtOH vehicle or EtOH vehicle were added, vortexed and incubated for 15 min. Malaoxon degradation was evaluated indirectly using the inhibition of an exogenous source of AChE (purified electric eel) as an endpoint. Eel AChE (FC, 0.2 U/mL) diluted in 1.75 ml of 0.05M Tris-HCl buffer (pH 7.4 at 37ºC), was added, vortexed and incubated for an additional 15 min to allow unbound malaoxon to inhibit AChE. Tubes (blanks) containing eserine sulfate (FC, 10 µM) were included in the assay to inhibit AChE and correct for non-enzymatic hydrolysis. The reaction was terminated and color was developed using 250 µl of a 5% sodium dodecyl sulfate/0.024M 5,5'-dithiobis(nitrobenzoic acid) (SDS/DTNB) mixture (4:1). Absorbance was measured at 412 nm using a spectrophotometer. Percent inhibition of control (EtOH vehicle) absorbance was calculated for each concentration. IC_{50} (AChE) values were determined by linear regression analysis of the plot of the logit of percent inhibition versus $\log_{10}$ oxon concentration. The best-fit line was drawn using points corresponding to the 10-90% AChE inhibition range and the equation of the best-fit line was solved for the x-intercept to determine the IC_{50}. The difference in the SCPP treated and the non-SCPP treated AChE IC_{50}s was determined as an index of CaE degradation of malaoxon.

Carboxylesterase Activities of Rat and Human Microsomes

Liver microsomes were diluted in 0.05M Tris-HCl buffer (pH 7.4 at 37ºC) and assayed according to the method of Chambers *et al*. (1990) using *p*-nitrophenyl valerate (*p*NPV) as the substrate for CaE. To determine CaE activity for each rat and human microsome, dilute homogenates (FC, 5 µg P/ml; 2 ml total assay volume) were vortexed and placed in a shaking water bath preheated to 37ºC. Tubes (blanks) containing paraoxon (FC, 10 µM) were included in the assay to inhibit CaE and correct for non-enzymatic hydrolysis. For each microsome, triplicate subsamples as well as duplicate paraoxon blanks were assayed. Following an initial incubation of 15 min, 20 µl of *p*NPV (FC, 500 µM) in ethanol vehicle was added as a substrate for the remaining uninhibited CaE and was incubated for an additional 15 min. The reaction was terminated with 500 µl of a 2% SDS/2% Tris-base mixture. Absorbance was measured at 405 nm using a spectrophotometer.

Paraoxonase Activities of Rat and Human Microsomes

The active metabolite malaoxon was not expected to be degraded (hydrolyzed) by paraoxonase (PON1), but this was investigated indirectly according to the method of Chambers *et al*. (1994). The indirect method involves the inhibition of an exogenous AChE source (electric eel) by oxon undegraded by paraoxonase. Individual rat or human liver microsomal suspensions (5 mg/ml) were added to two sets of tubes containing either 1 mM $CaCl_2$ or 1 mM EDTA (assay volume of 2 ml). SCPP (FC, 50 nM) was added to liver preparations to inhibit CaE. Malaoxon (FC, 500 nM) was incubated with dilute microsomes with and without calcium (stimulates paraoxonase activity) or EDTA

(chelates calcium, inhibiting paraoxonase activity). Eserine sulfate was added to two additional tubes to serve as a blank by inhibiting AChE activity and correcting for non-AChE hydrolysis. Reaction mixtures were incubated 15 min in a shaking water bath at 37°C followed by the addition of eel AChE (FC, 0.2 U/mL) diluted in 1.75 ml of 0.05M Tris-HCl buffer (pH 7.4 at 37ºC) + 1 mM EDTA to terminate any paraoxonase activity. Tubes were incubated for an additional 15 min. Acetylthiocholine iodide was added (as a substrate for the uninhibited AChE; 1 mM FC), and incubated for 15 min. The reaction was terminated and color was developed using 250 µl of a 5% sodium dodecyl sulfate/0.024M 5,5'-dithiobis(nitrobenzoic acid) (SDS/DTNB) mixture (4:1). Absorbance was measured at 412 nm using a spectrophotometer. The amount of malaoxon hydrolyzed was quantified through the inhibition of AChE by oxon not degraded by paraoxonase. The percent inhibitions for EDTA- and $CaCl_2$ -containing tubes were subtracted to determine the amount of oxon hydrolyzed by paraoxonase.

Calculations for Bioactivation of Malathion

The kinetic rates for malathion activation (desulfuration) were calculated according to concepts developed by Leonor Michaelis and Maud Menten, so called Michaelis-Menten kinetics. The widely used Michaelis-Menten equation derived from their work follows a mechanism in which substrate binds reversibly to an enzyme to form an enzyme-substrate complex, which then reacts irreversibly and generates a product and the free enzyme (Michaelis and Menten, 1913; Goody and Johnson, 2011). This equation was used to calculate the maximum velocity (V_{max}) and the apparent substrate affinity (K_{mapp}) for the activation (desulfuration) of malathion to its anticholinesterase metabolite,

malaoxon. The V_{max} and K_{mapp} for the CYP-mediated bioactivation of malathion was calculated from Lineweaver Burk plots of the reciprocals of the substrate (malathion) concentration versus velocity of malaoxon production (Figure 4), as previously performed for other OPs (Ma and Chambers, 1994, 1995). Data were plotted using SigmaPlot 13 software to calculate V_{max} and K_{mapp} for both rat and human microsomes.

For each rat and human liver microsome, the maximum velocity (*Vmax*) of CYP production of malaoxon was calculated as described in Equation 1 below:

$$v = \frac{Vmax[S]}{[Kmapp]+[S]} \quad (1)$$

Using the linear regression of the reciprocals of malathion concentration vs. malaoxon produced per min (Lineweaver Burk; double reciprocal plot, Figure 4) an equation for a line could be derived and used to determine the necessary kinetic values (V_{max} and K_{mapp}).

From the line equation (Equation 2) the kinetic values are determined with the slope equal to K_{mapp}/V_{max}, the y-intercept equal to $1/V_{max}$, (Kitz and Wilson, 1962; Ma and Chambers, 1995).

$$y = mx + b \quad (2)$$

$$Slope\ m = \frac{Kmapp}{Vmax}$$

$$y - intercept\ (b) = \frac{1}{Vmax}$$

$$x - intercept = -\frac{1}{Kmapp}$$

Statistical Analysis

Bioactivation data and carboxylesterase data were analyzed using SigmaPlot/SigmaStat 13 software on a PC. Differences in mean values for human and rat liver microsomes as well as differences in sexes within a species were determined using the Student's T-Test, with a level of $p \leq 0.05$ used to conclude a significant difference in means. Differences among ages for bioactivation and carboxylesterase activities were analyzed using Pearson Product Moment Correlation.

Results

To investigate interspecies differences in the bioactivation of malathion to malaoxon, twenty-seven individual human liver microsomes (8 females and 19 males) ranging in age from 2 weeks to 75 years were purchased (Xenotech, LLC) for comparison to microsomes prepared in house from male and female adult rats. The human liver microsomes were adjusted by the vendor to equivalent protein concentrations (20 mg/ml). For consistency, the rat liver microsomes were also adjusted to equivalent protein concentrations (20 mg/ml). Total CYP content for 23 of the human liver microsomes was determined and supplied by the vendor (Table 2) as well as individual CYP isozyme content for some of the microsomes. Total CYP content was not determined for the rat microsomes. Eight different concentrations of malathion (50 µM to 2000 µM) were tested for CYP-mediated bioactivation to the anticholinesterase metabolite malaoxon for both rat and human hepatic microsomes using an indirect

measurement of malaoxon produced (inhibition of an exogenous source of AChE). Malathion in its parent form did not inhibit the AChE trap at any of the concentrations tested. The maximum velocity (V_{max}) and an index of substrate affinity (K_{mapp}) for malathion desulfuration were calculated from Lineweaver Burk (double-reciprocal) plots that were plotted using SigmaPlot 13 software. The mean r^2 values for the Lineweaver Burk plots were 0.930 for the human microsomes and 0.955 for the rat microsomes (Table 1-2). The data were also plotted using a hyperbolic equation; however, the data for some of the microsomes did not fit the equation well, especially the lower concentrations. Therefore, the malathion bioactivation rates were calculated from Lineweaver Burk plots to determine differences in kinetic rates for rat and human microsomes (interspecies) and differences with respect to age and variability in human microsomes (intraspecies).

The V_{max} values (presented as nmol/min/mg P) for malathion desulfuration ranged from 3.44 to 59.24 for the human liver microsomes and 2.49 to 10.1 for the rat liver microsomes (Table 1-2). The K_{mapp} values for malathion ranged from 0.18 mM to 18.60 mM for the human liver microsomes and 0.39 mM to 8.19 mM for the rat liver microsomes (Table 1-2). The V_{max} values were significantly higher for the human microsomes than the rat microsomes ($p \leq 0.001$); however, there were no significant differences in the K_{mapp} values between the human and rat microsomes. The mean V_{max} values for both the humans and the rats were slightly higher for females than males but did not reach significance at the $p < 0.05$ level. No significant differences were determined for the K_{mapp} values between male and female rat microsomes or male and female human microsomes. No age-related statistical differences (Pearson Product

Moment Correlation) were determined among all of the human samples for V_{max} or K_{mapp}. In addition, the human liver microsomes were separated into two age groups, adults (> 12 years in age) and juveniles (< 12 years in age). No statistically significant differences in V_{max} or K_{mapp} were determined between the adults and juveniles.

The CaE activity of the individual human and rat liver microsomes was determined using the substrate *p*-nitrophenyl valerate (Figures 5-6). Mean activities for the rat liver microsomes were significantly higher than for the human liver microsomes ($p < 0.05$). Activities ranged from 228 to 1296 (nmoles/min^{-1} mg P^{-1}) and 5.4 to 36.4 (nmoles/min^{-1} mg P^{-1}) for the rat and human liver microsomes, respectively. No significant differences were determined between sexes for rats or humans. No age-related differences were determined for the human liver microsomes when analyzed as a complete data set.

Carboxylesterase mediated degradation of malathion was investigated by pre-incubating parallel microsomal samples with or without a specific inhibitor for CaE, SCPP, followed by the addition of a high concentration of malathion (200 μM). Malaoxon production, as measured using an exogenous AChE trap, was increased for 25 of 27 human liver microsomes and 9 of 12 rat liver microsomes pre-treated with the specific CaE inhibitor (SCPP). Fold increases in malaoxon production with SCPP pre-treatment ranged from 1.1 to 3.9 for the human liver microsomes and 1.2 to 6.5 for the rat liver microsomes (Table 3). Malaoxon production in rat and liver microsomes pre-treated with SCPP was significantly ($p < 0.01$) higher than those pre-treated with EtOH vehicle only.

Carboxylesterase degradation of malaoxon was investigated by pre-incubating parallel microsomal samples with or without a specific inhibitor for CaE, SCPP, followed

by the addition of one of five concentrations of malaoxon (FCs, 1 μM-10 nM). As an indirect measurement of CaE degradation of malaoxon, IC_{50}s for the exogenous (electric eel) AChE were calculated for comparison of the SCPP pre-treated samples to the EtOH vehicle pre-treated samples, with a decrease in IC_{50} indicating more malaoxon available to inhibit the AChE. IC_{50}s for all rat liver microsomes were significantly decreased (2.0-fold; $p > 0.0001$) with SCPP pre-treatment. IC_{50}s for 25 of 27 human liver microsomes were significantly decreased (2.3-fold; $p > 0.0001$) with SCPP pre-treatment (Table 4).

Finally, the proposed pathway of malaoxon hydrolysis by paraoxonase was investigated using an indirect assay with an exogenous source of AChE (electric eel) as measurement of remaining malaoxon following incubation with pooled rat or human liver microsomes. CaE activity was inhibited using the specific inhibitor, SCPP, and parallel samples with malaoxon were incubated with and without calcium (stimulates paraoxonase activity) and with and without EDTA (inhibits paraoxonase activity). No differences in AChE inhibition were determined indicating no paraoxonase mediated hydrolysis of malaoxon was occurring.

Discussion

This study was designed to investigate the differences between rat and human CYP-mediated metabolism of the organophosphate insecticide malathion. The kinetic rates for malathion activation (desulfuration) were determined for rat and human liver microsomes according to concepts developed by Leonor Michaelis and Maud Menten (Michaelis-Menten kinetics). The widely used Michaelis-Menten equation depicts an enzymatic reaction in which a substrate binds reversibly to an enzyme to form an

enzyme-substrate complex, which then reacts irreversibly and generates a product and the free enzyme (Michaelis and Menten, 1913; Goody and Johnson, 2011). This widely used equation was used to calculate the maximum velocity (V_{max}) and substrate affinity (K_{mapp}) of malathion bioactivation (desulfuration) to its anticholinesterase metabolite, malaoxon.

The higher V_{max} values for the human microsomes indicate human microsomal CYPs are more efficient than rat microsomes in the bioactivation of malathion to malaoxon. The K_{mapp} values for rat and human microsomes were not statistically different indicating that the affinity for CYPs from both species was similar. Differences in bioactivation (desulfuration) have been previously reported for several OPs (Sultatos, 1991; Chambers and Ma, 1994; 1995; Butler and Murray, 1997; Tang *et al*., 2001; Buratti *et al*., 2003). Some studies have shown an increase in rates of bioactivation with age for some OPs this could be a result of the calculation of data, whether it is normalized based on protein levels or wet weight (per gram of tissue). Total protein levels have been shown to increase with age, especially inducible proteins such as CYPs (Nims and Lubet, 1995; Atterberry *et al*., 2017). The representation of the data must be considered when analyzing maturing levels of enzymes. The purchased human samples were standardized by protein level (20 mg/ml) by the vendor; therefore, we adjusted the rat microsomes in a similar manner for comparison purposes. No information was provided as to the amount of tissue used to prepare the human hepatic microsomes, preventing the calculations on a wet weight basis.

The CYP family consists of many different isoenzymes which catalyze many of the same reactions but differ in their chemical properties or amino acid sequence (Wilkinson, 1970; Guengerich, 1987). The isozyme content varies greatly in rodents and

humans and given the differences in binding affinity (low, medium, and high) the overall K_{mapp} and V_{max} values can be quite different (Ma and Chambers, 1994; Tang *et al*., 2001; Buratti *et al*., 2003, 2005). Rat and human hepatic microsomes have exhibited two distinct straight lines for bioactivation of some OPs using Eadie-Hofstee plots, indicating that two components catalyzed by different isoforms with different affinities are involved. These different lines result in different kinetic parameters (Ma and Chambers, 1994; Buratti *et al*., 2005). In human liver microsomes, CYP2C19, 1A2, and 2B6 have been shown to be very efficient in the bioactivation of malathion to malaoxon, while CYP3A4 was shown to only be efficient at high malathion concentrations (Buratti *et al*., 2005). CYP1A2 was reported to be the most efficient isozyme for malathion bioactivation at lower malathion concentrations (more environmentally relevant levels) (Buratti *et al*., 2005). The difference in total CYP concentration and isozyme content may provide further variability between species. In addition, sex and age differences in bioactivation of some OPs have been reported (Chambers *et al*., 1994; Tang *et al*., 2001). These differences may be attributed to a difference in the total amount of CYP enzyme present, differences in the proportion of isozymes, differences in the isozymes affinity for the OP, or differences in normalization of the data.

The average K_{mapp} value for malathion desulfuration for adult male rats was 3.8 mM and 1.6 mM for adult female rats. The average K_{mapp} and V_{max} values for malathion desulfuration in human liver microsomes were 2.45 mM and 22.82 nmoles/min/mg P, respectively, which was similar to published data of Buratti *et al*. (2005) for three individual human liver microsomes. The affinity (K_{mapp}) of human and rat CYPs for OPs, such as malathion, and the efficiency (V_{max}) of bioactivation to the anticholinesterase

metabolites can be useful in defining the differences in acute toxicity levels for OPs (Ma and Chambers, 1994, 1995; Buratti *et al*., 2005) as well as constructing pharmacokinetic models (Timchalk, 2002).

The variability in the human samples could be attributed to the variability in CYP isozymes type and/or concentration present in the livers (Tang *et al*., 2001; Buratti *et al*., 2003, 2005). Buratti *et al*. (2005) reported a greater than 200-fold inter-individual variability in desulfuration rates for malathion among 16 human liver microsomes. In addition, it has been well documented that the CYP isozymes may be induced or inhibited by a variety of exogenous compounds (Guengerich, 1987; Nims and Lubet, 1995). The information supplied with the human samples did not include any information concerning medications the individual may have been taking or the overall health of the individuals, both of which could affect their metabolic capacities (Guengerich, 1987). In addition, the preparation of the human liver microsomes was performed by the vendor and the protocol used to prepare microsomes was not well defined. The isozyme distribution within the liver has also been shown to be zonal; therefore, the area of the liver used to prepare the microsomes may contribute to the variability of the isozyme content and thus the interindividual bioactivation variability (Buhler *et al*., 1992). Finally, 27 individual human microsomes is a relatively small sample size and may not be representative of the total human population.

In addition to the variability of the bioactivation, the detoxication potential of the rat and human microsomes were variable. The CaE variability among the human liver microsomes was 18-fold and 5-fold for the rat liver microsomes. This was not unexpected as many studies have shown variability in CaE activities in both laboratory animals and

humans (Pope *et al*., 2005; Maxwell, 1992; Hosokawa *et al*., 1995; Atterberry *et al*., 1997; Ross *et al*., 2012; Hinds *et al*., 2016; Moser *et al*., 2016). The increase in CaE activity with age in rats has been well documented and has been suggested to play a major role in the increase in susceptibility of immature animals to OP toxicity (Brodeur and Dubois, 1963; Gagne and Brodeur, 1972; Benke and Murphy, 1975; Pope *et al*., 1991; Atterberry *et al*., 1997). A study by Atterberry *et al*. (1997) demonstrated that lower detoxication capacity (lower levels of CaE, paraoxonase, and CYP-mediated dearylation) in the young animals contribute significantly to age-related differences in sensitivity to some OPs (Atterberry *et al*., 1997). Some studies have reported an age-related increase in CaE activity in human liver microsomes (Hosokawa *et al*., 1995; Pope *et al*., 2005; Moser *et al*., 2016); however, these studies all consisted of very few individuals and as reported in this study the intraspecies variability can be high. In addition, sex differences in CaE activities have been reported in rats (Chambers and Carr, 1993). The sex and age differences in CaE activities reported in some studies may be attributed to a difference in the total amount of enzyme present. Carboxylesterase levels can be induced by variety of exogenous compounds increasing the variability of a population (Zou *et al*., 2018).

Carboxylesterase, of which there is a finite number, is stoichiometrically inhibited by OP oxons, thus this detoxication pathway is saturable. In the case of a high exposure where the CaE is persistently phosphorylated by an OP oxon, the likelihood of the inhibition of the target brain AChE and toxicity increases. *In vivo* studies in rats and mice have used selective inhibitors of CaE, such as tri-*o*-tolyl phosphate (TOTP) and 2-(*o*-cresyl)-4*H*-1,3,2-benzodioxaphosphorin-2-oxide (CBDP), to potentiate the toxicity of

several OPs, including malathion (Casida, 1961; Dauterman and Main, 1966; Clement, 1984; Fonnum *et al*., 1985; Gupta and Dettbarn, 1987; Yang and Dettbarn, 1998). The more recent development of CaE knockout (KO) and transgenic mouse models provide an additional model to demonstrate the protective role of these detoxication enzymes.

Carboxylesterases are especially important for the detoxication of malathion given its structure. The carboxylic esters in malathion make it susceptible to catalytic hydrolysis by the promiscuous CaE to a non-toxic monocarboxylic acid metabolite that can be excreted. The CYP-mediated bioactivation to malaoxon can occur simultaneously. In addition, the malaoxon being produced can stoichiometrically inhibit CaE reducing the amount of anticholinesterase metabolite but also reducing the amount of CaE available to catalytically degrade malathion. This can be important in the case of a high acute exposure or chronic exposure that saturates the CaE pool. These types of exposures could result in increased toxicity to subsequent exposures of malathion or other OPs prior to the *de novo* synthesis of new CaE. The *in vitro* studies conducted using the specific CaE inhibitor, SCPP, exhibited the effectiveness of CaE to degrade both malathion and malaoxon. Similar CaE-mediated detoxication of chlorpyrifos-oxon and paraoxon has been reported for both rat liver and serum (Chambers and Carr, 1993, Atterberry *et al*., 1997). Dauterman and Main (1966) synthesized homologs of malathion and malaoxon and showed that increasing the carboxyalkyl chain length decreased the potency towards CaE and *in vivo* toxicity as compared to malathion in rats, further indicating the role of CaE in both malathion and malaoxon degradation. Impurities in technical malathion and storage conditions can result in increased CaE and AChE inhibition. Isomalathion, an impurity of malathion, is a more potent inhibitor of CaE and

can increase the toxicity of malathion by several fold (Talcott *et al*., 1979). Finally, the potential pathway of paraoxonase hydrolysis of malaoxon was determined to be inconsequential. The lack of difference in eel AChE inhibition (trap) between microsomes incubated with malaoxon with and without calcium indicates very little contribution, if any, of paraoxonase in the degradation of malaoxon. Similar results were reported by Moser *et al*. (2016).

Conclusion

The objective of this investigation was to determine the differences in bioactivation of malathion to its anticholinesterase metabolite malaoxon between rats and humans. Age-related differences in bioactivation of malathion were investigated using individual human liver microsomes of various ages. In addition, the detoxication potential of malathion and malaoxon were investigated using rat and human liver microsomes. The efficiency of the bioactivation of malathion was higher for the human liver microsomes than rats, but the apparent affinity for CYPs involved in the bioactivation was not different between the species. There were no age-related differences in the rates of bioactivation of malathion among the human liver microsomes; however, the relatively small sample size may not be sufficient to determine this type of difference for the human population. As expected, large variability was exhibited in CaE activities for both rat and human liver microsomes. Carboxylesterase activities in rat liver microsomes were higher than in the human microsomes. The detoxication of malathion was confirmed to be primarily CaE-mediated in both species. The amount of CaE-mediated degradation of both the parent insecticide, malathion, and the

anticholinesterase metabolite, malaoxon, was substantial. Therefore, the carboxylesterase-mediated detoxication of both malathion and malaoxon plays a major role in the low mammalian toxicity of this widely used insecticide.

The data presented here are consistent with other data in the literature indicating the importance of CaE in the detoxication of malathion. Other studies have investigated differences in individual human liver microsomes; however, most of these contained very small numbers of individual microsomes, did not include the wide age range that was investigated here, and/or did not measure both activation and detoxication in the same samples. Differences in the bioactivation of malathion may be more important in individuals with low intrinsic CaE activity or in individuals that may have multiple exposures to malathion or another OP that can reduce their CaE detoxication potential. The interspecies and intraspecies differences in sensitivity of the target enzyme, AChE, to malaoxon will be investigated in the next chapter. The metabolism of malathion is complex and presents challenges to creating a pharmacokinetic model that will accurately predict a toxicokinetic response for a given exposure. The interspecies and intraspecies variability of the bioactivation and detoxication must be considered when assessing risk to the human population from this widely used insecticide.

Table 3.1 Kinetics of malathion desulfuration in rat hepatic microsomes.

Subject #	Age (days)	Sex	K_{mapp} (mM)	V_{max} (nmoles/ min^{-1} mg P^{-1})	V_{max}/K_{mapp}	r^2
1	70	F	3.00	10.05	4.37	0.992
2	70	F	1.47	7.57	5.16	0.987
3	70	F	0.76	3.20	4.20	0.926
4	70	F	1.46	4.21	2.89	0.903
5	70	F	2.30	10.01	4.36	0.993
6	70	F	1.69	7.55	4.47	0.930
Mean	**70**	**F**	**1.66**	**7.10**	**4.24**	**0.960**
SEM	**70**	**F**	**0.34**	**1.66**	**0.43**	**0.02**
1	70	M	6.91	7.37	1.07	0.987
2	70	M	2.17	4.04	1.86	0.947
3	70	M	2.25	5.84	2.59	0.984
4	70	M	2.93	2.49	0.85	0.922
5	70	M	8.19	5.73	0.70	0.851
6	70	M	0.39	5.85	14.88	0.974
Mean	**70**	**M**	**3.81**	**5.22**	**3.66**	**0.940**
SEM	**70**	**M**	**1.76**	**0.98**	**3.20**	**0.03**

Kinetic values (V_{max} and K_{mapp}) for 12 adult rat liver microsomes of both sexes (6 each). Data were calculated from Lineweaver Burk plots of means of 3 subsamples of eight concentrations compared to vehicle controls. Differences in means between sexes were determined using the Student's t-test, with a level of $p \leq 0.05$. No statistical difference was determined for desulfuration with respect to sex.

Table 3.2 Kinetics of malathion desulfuration in human hepatic microsomes.

Vendor Sample #	Age (years)	Sex	K_{mapp} (mM)	V_{max} (nmoles/ min^{-1} mg P^{-1})	V_{max}/K_{mapp}	r^2	CYP (nmoles/ mg P)
354	0.04	F	8.15	46.42	5.70	0.942	NA
57	2	F	0.45	10.30	23.15	0.876	0.32
410	11	F	2.07	26.68	12.91	0.954	0.69
25	30	F	0.25	10.88	43.87	0.853	0.29
393	30	F	1.19	48.16	40.47	0.986	0.54
177	45	F	1.51	22.82	15.11	0.940	0.51
115	48	F	1.49	17.75	11.91	0.935	0.22
355	71	F	5.83	46.26	7.93	0.971	0.60
Mean	**29.6**	**F**	**2.62**	**28.66**	**20.13**	**0.930**	**0.45**
SEM	**14.3**	**F**	**1.63**	**9.30**	**8.42**	**0.026**	**0.10**
845	0.08	M	0.36	19.92	55.64	0.987	0.34
268	0.33	M	18.60	59.24	3.19	0.948	0.38
395	0.42	M	4.79	32.84	6.86	0.964	NA
270	0.42	M	0.66	5.69	8.57	0.887	0.29
825	0.92	M	0.44	13.66	31.26	0.930	0.49
322	1	M	5.36	24.71	4.61	0.880	NA
852	2	M	0.33	3.44	10.53	0.868	0.30
551	2	M	0.33	3.45	10.54	0.865	0.31
346	3	M	0.28	19.60	69.75	0.947	NA
792	4	M	0.76	9.10	12.04	0.971	0.34
215	6	M	2.10	13.21	6.29	0.938	0.33
59	9	M	0.18	4.23	24.17	0.956	0.14
485	10	M	0.38	21.43	56.25	0.979	0.21
133	17	M	1.80	15.40	8.56	0.970	0.34
236	17	M	0.54	12.19	22.79	0.897	0.45
36	37	M	1.15	25.12	21.84	0.904	0.79
420	42	M	2.29	30.99	13.53	0.979	0.20
201	58	M	4.72	57.90	12.27	0.960	0.54
203	75	M	0.24	14.85	62.13	0.897	0.40
Mean	**15.1**	**M**	**2.38**	**20.37**	**23.20**	**0.930**	**0.37**
SEM	**12.7**	**M**	**2.47**	**9.26**	**12.39**	**0.024**	**0.15**

Kinetic values (V_{max} and K_{mapp}) for 27 human liver microsomes of both sexes and various ages. Data were calculated from Lineweaver Burk plots of means of 3 subsamples of eight concentrations compared to vehicle controls. Differences in sexes were determined using the Student's t-test, with a level of $p \leq 0.05$. No statistical difference was determined for desulfuration with respect to sex. CYP activities were supplies by the vendor. NA = data not available.

Table 3.3 Indirect measurement of carboxylesterase degradation of malathion in rat and human liver microsomes.

		SCPP [malaoxon]	Ethanol [malaoxon]	Fold Change
Rat	Mean	13454^{A}	6657^{B}	2.0
	SEM	3326	2139	
Human	Mean	24128^{A}	11985^{B}	2.0
	SEM	7650	2840	

Data expressed as malaoxon (pmoles/min^{-1} mg P^{-1}produced from the CYP-mediated bioactivation of malathion (200 µM) means ± SEM of 12 rat microsomes or 27 human microsomes. Means within a species not followed by the same capital letter are significantly different ($p < 0.05$) and were determined using the Student's t-test. Higher concentrations of malaoxon for the SCPP pre-treated samples indicate greater degradation of malathion by carboxylesterase.

Table 3.4 Indirect measurement of carboxylesterase degradation of malaoxon in rat and human liver microsomes.

Liver Microsome	Pre-treatment	
	Ethanol (AChE IC_{50})	**SCPP (AChE IC_{50})**
Human	131 ± 33^{A}	80 ± 29^{B}
Rat	136 ± 25^{A}	68 ± 9^{B}

IC_{50}s expressed as nM, for electric eel AChE, means ± SEM of 12 rat microsomes or 27 human microsomes. Means within a species not followed by the same capital letter are significantly different ($p < 0.05$) and were determined using the Student's t-test. Higher AChE IC_{50} values for the ethanol vehicle pre-treated samples indicate less malaoxon available to inhibit the exogenous AChE and indirectly estimate degradation by carboxylesterase.

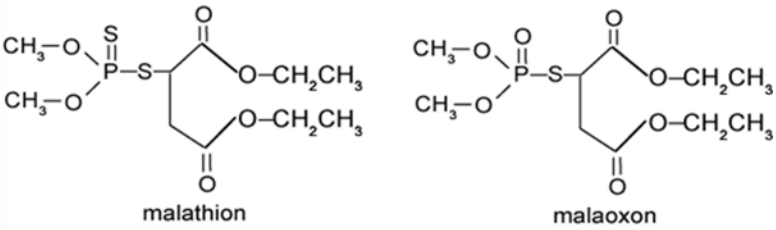

Figure 3.1 Chemical structures of malathion and its active metabolite malaoxon.

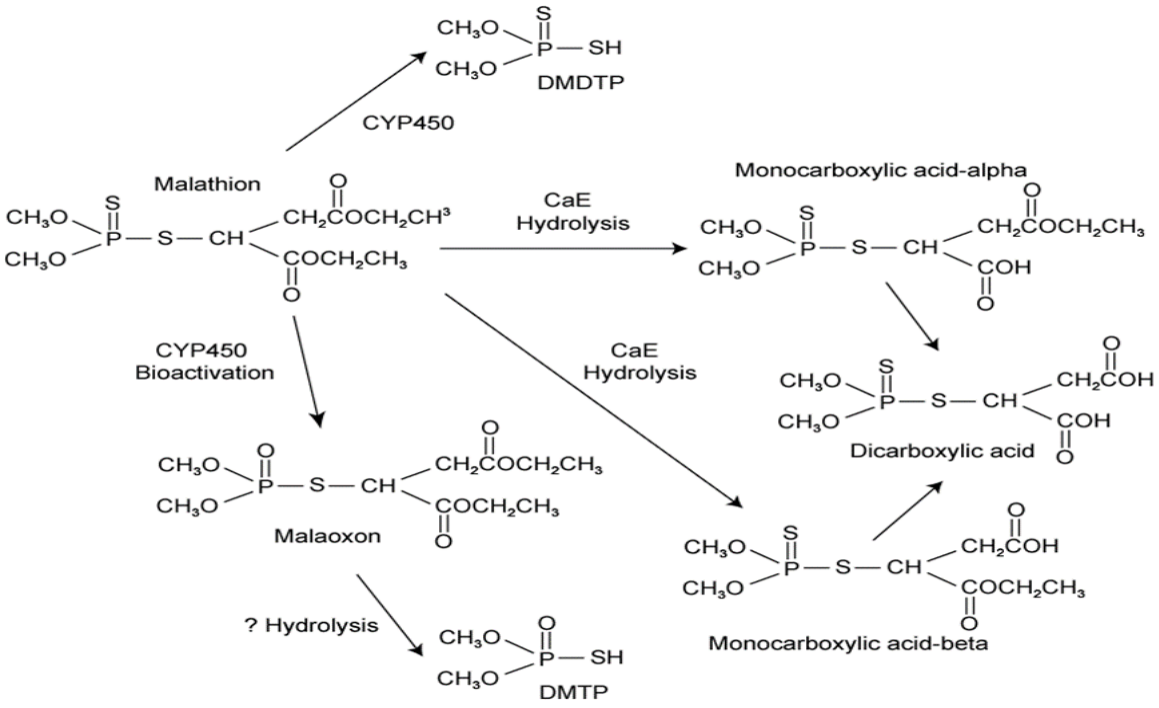

Figure 3.2 Proposed metabolic pathway of malathion in mammals. DMDTP = dimethyldithiophosphate; DMTP = dimethylthiophosphate; CaE = carboxylesterase.

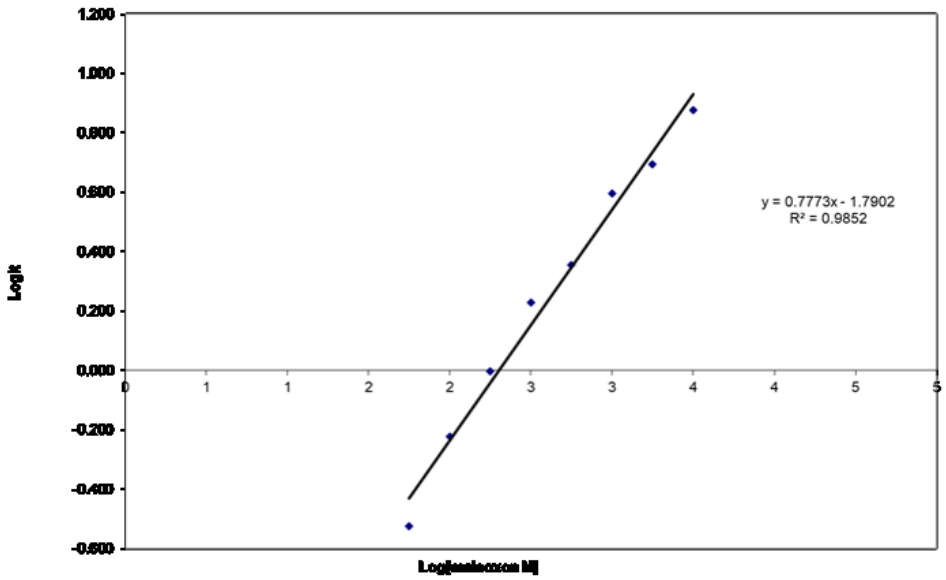

Figure 3.3 Standard curve for malaoxon inhibition of purified eel acetylcholinesterase.

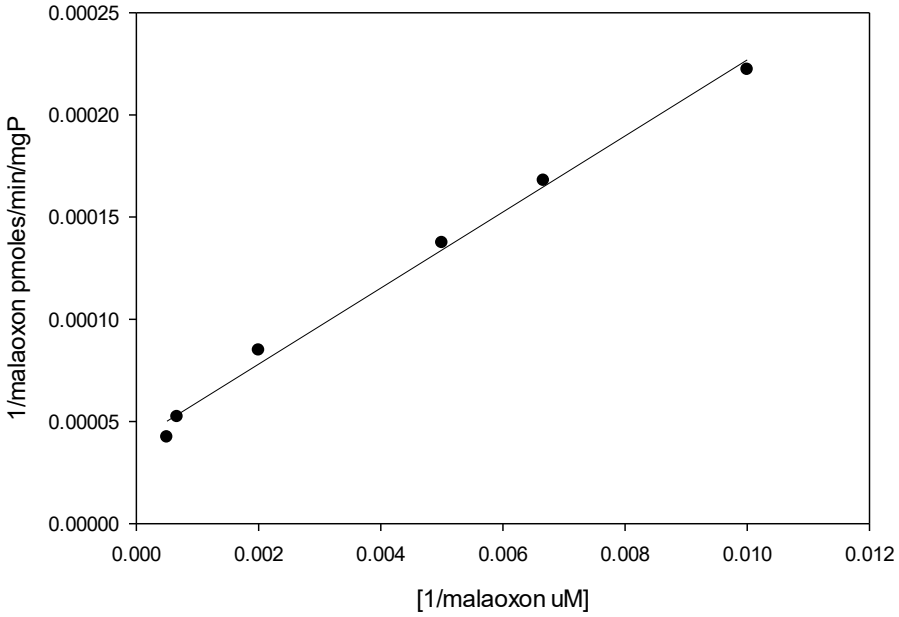

Figure 3.4 Lineweaver Burk plots for inhibition kinetics

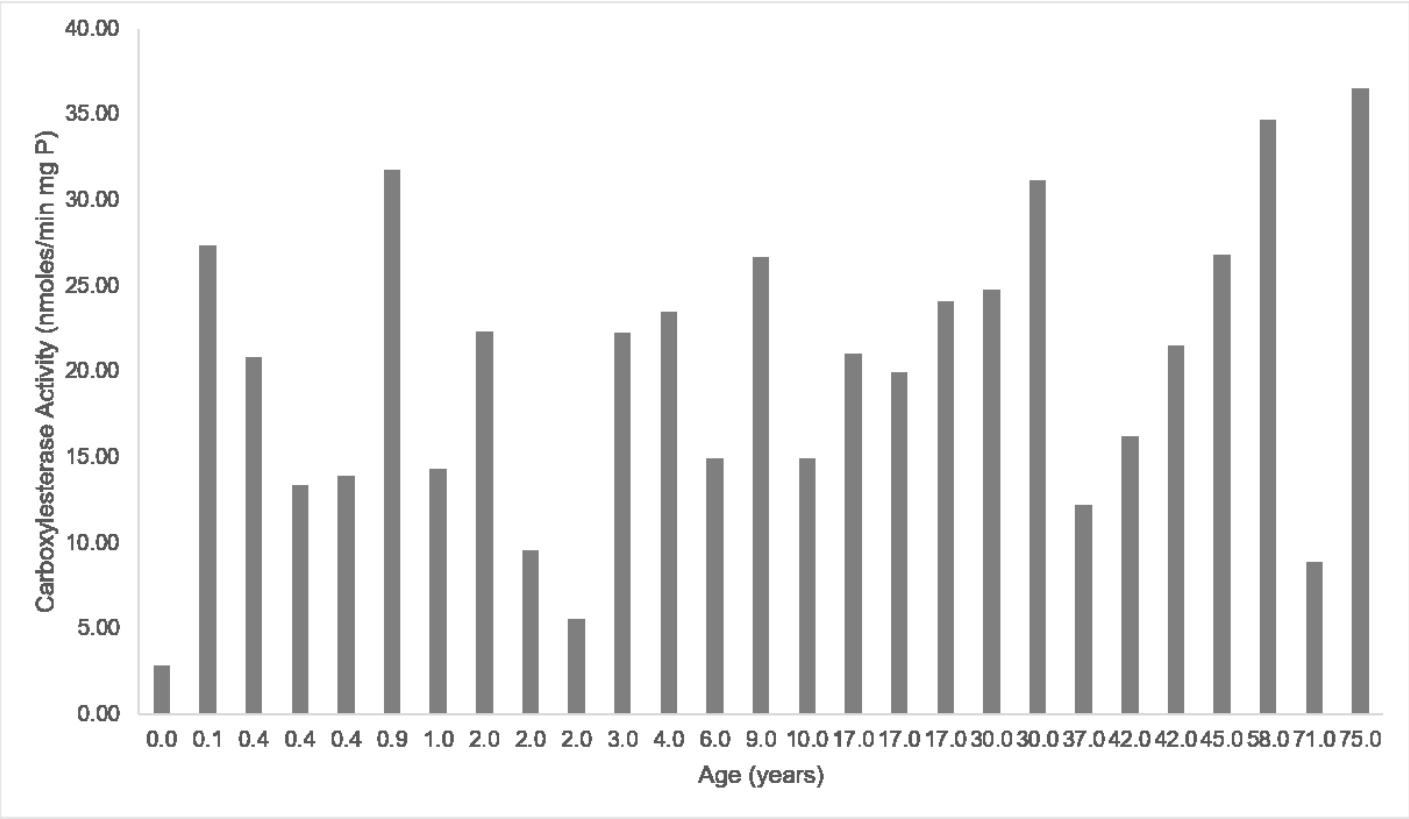

Figure 3.5 Individual human hepatic microsomal carboxylesterase activities.

Carboxylesterase activities for individual human liver microsomes n = 27. Data are expressed as nmoles/min^{-1} mg P^{-1} and are presented with increasing age in years. No statistical difference was determined for carboxylesterase with respect to age among the 27 samples, $p \leq 0.05$.

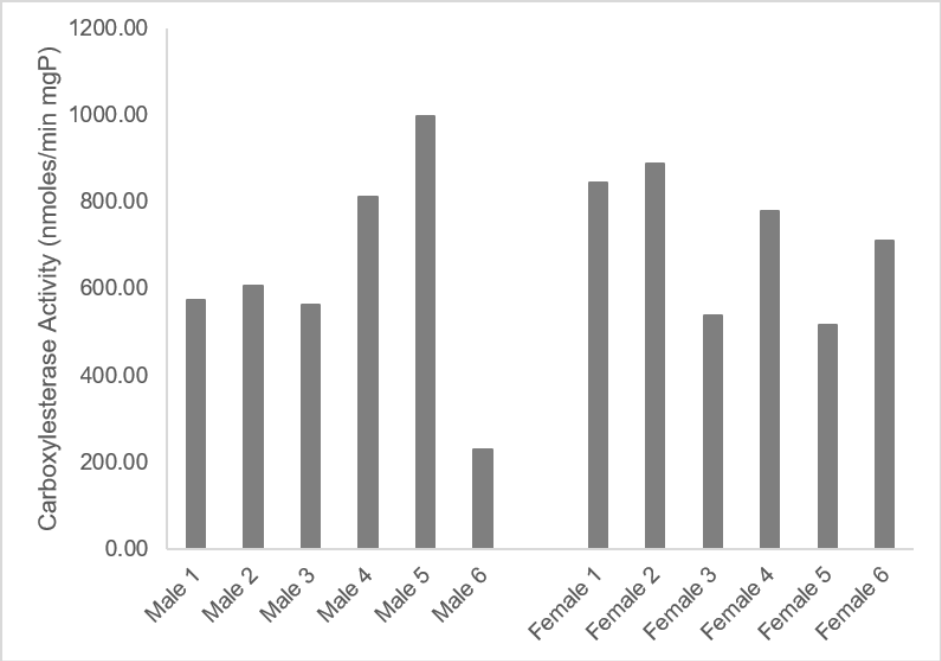

Figure 3.6 Rat hepatic microsomal carboxylesterase activities.

Carboxylesterase activities for individual adult rat liver microsomes n = 12. Data are expressed as nmoles/min^{-1} mg P^{-1} and are presented by sex. Differences in means for sexes were determined using the Student's t-test, with a level of $p \leq 0.05$. No statistical difference was determined for carboxylesterase with respect to sex.

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CHAPTER IV

KINETICS OF ACETYLCHOLINESTERASE INHIBITION BY MALAOXON

Introduction

Malaoxon is the anticholinesterase metabolite of one of the most widely used organophosphate (OP) insecticides, malathion (*O,O*-dimethyl dithiophosphate of diethyl mercaptosuccinate; Figure 1). Malathion is a multiuse insecticide that is approved for use in agriculture, both for crops and livestock, and in public health, for control of mosquitos that transmit diseases such as west nile, malaria and zika (US EPA, 2009). Malathion has a very low mammalian toxicity (male and female rat oral LD_{50}s > 1000 mg/kg) (Dauterman and Main, 1966; Terrell, 1979; Fisher, 1991; Meister, 1992); however, malaoxon has been shown to be a relatively potent acetylcholinesterase inhibitor (Dauterman and Main, 1966; Herzsprung, 1992).

The majority of the OP insecticides, such as malathion, must undergo bioactivation (desulfuration) by cytochrome P450s (CYPs) to their active oxon metabolites (e.g., malaoxon). The resulting oxons can inhibit the serine esterase, acetylcholinesterase (AChE), in cholinergic synapses and neuromuscular junctions. The inhibited AChE cannot inactivate the endogenous neurotransmitter ACh to terminate its action as a neurotransmitter resulting in excess ACh and hyperstimulation of the cholinergic system, followed by fatigue of ACh receptors (Mileson *et al*., 1998;

Thompson and Richardson, 2004). Depending on the level of OP exposure, cholinergic toxicity can occur followed by death, in cases of high level exposures (Taylor, 1990).

Acute toxicities from OP insecticides have been shown to vary among OPs and species. Species differences in acute toxicity to OPs have been suggested to be due to differences in the sensitivity of the target enzyme, acetylcholinesterase (AChE) (Murphy *et al*., 1968). In addition, age-related differences in OP toxicities in animal models have been demonstrated for many years (Benke and Murphy, 1975; Pope *et al*., 1991; Mortensen *et al*., 1996; Moser *et al*., 1996; Atterberry *et al*., 1997). These types of differences helped lead to the passing of the Food Quality Protection Act (FQPA) of 1996. FQPA mandated that pesticides with a common mechanism of toxic action were required to undergo cumulative risk assessments and uncertainty factors were added for interspecies and intraspecies variability (FQPA, 1996; Dourson *et al*., 1996; Mileson *et al*., 1998). The interspecies uncertainty factor for the pharmacodynamics for OP pesticides whose mechanism of toxic action is via the inhibition of AChE has been set to a default value of 10x (US EPA, 2014). This uncertainty factor can be modified if there is sufficient experimental data to support such a modification. In addition, there is an additional uncertainty factor (10x) for intraspecies variation in AChE inhibition (Dourson *et al*., 1996; US EPA, 2014). This *in vitro* study investigates both the interspecies (rat and human) and intraspecies (among humans) variability of the sensitivity of AChE from erythrocyte ghosts to the oxon metabolite of the OP insecticide malathion. These data could be useful in modifications of uncertainty factors for malathion exposures.

Erythrocytes or red blood cells (RBCs) are responsible for the transport of oxygen bound to hemoglobin molecules that are sequestered within the cells. Erythrocytes have

a high level of AChE activity which is subject to inhibition by OPs, such as malaoxon. Maulet *et al*. (1984) determined erythrocyte AChE to be a glycoprotein and, in 1985, this glycoprotein was shown to be located in the erythrocyte membrane (Ott, 1985). Acetylcholinesterase molecules occur in three forms that differ only in their C-terminal regions and are classified as R (readthrough), H (hydrophobic), or T (tail) (Massoulié *et al*., 2002). Erythrocytes contain the H-form that exists as a dimer attached to the membranes following posttranslational modification of the C-terminal region by glycerophosphatidylinositol (Massoulié *et al*., 2002; Bartels *et al*., 1993).

Erythrocyte membrane AChE activity has been implicated as a biomarker of membrane integrity with lower activity occurring in aged populations (Herz and Kaplan, 1973; Prall *et al*., 1998). In addition, sex differences have been determined with AChE activity higher in females than in males (Prall *et al*., 1998; Hilário *et al*., 2003). Humans with hemolytic anemia have been characterized by lower levels of erythrocyte AChE (Prall *et al*., 1998). Erythrocyte AChE is also used as a biomarker of hypertension, amyotrophic lateral sclerosis (ALS), Hirschsprung's disease and glaucoma (Saldanha, 2017). In addition, erythrocyte AChE has been determined to be a marker of inflammation (Saldanha, 2017) with the recruitment of leucocytes to the endothelial vessel wall and in the production of pro-inflammatory cytokines in disease states (Das, 1997; Saldanha, 2017). Sargento *et al*. (2003) reported lower erythrocyte activity levels in patients with diabetes mellitus. Acetylcholinesterase activity has also been shown to affect NO efflux from erythrocytes through the activation of a protein kinase C pathway (Saldanha, 2017). Erythrocyte AChE is used clinically as a diagnostic marker (biomarker) for OP exposure (Prall *et al*., 1998; Bajgar, 2004) and because the catalytic

domains are identical among tissues, erythrocyte AChE is used as surrogate biomarker of brain AChE inhibition (Herkert *et al*., 2012; Chen *et al*., 1999)

Kinetic rates and equilibrium constants associated with enzyme/OP inhibitor reactions can be measured using *in vitro* enzyme assays (Clothier *et al*., 1981; Johnson and Wallace, 1987; Chambers and Carr, 1996; Coban *et al*., 2016). The mathematical description of the inhibition of AChE by OP compounds has been described in depth by Kitz and Wilson (1961), Aldridge and Reiner (1972), and applied by many others (Clothier *et al*., 1981; Main, 1980; Richardson, 1992; Chambers and Carr, 1996; Johnson and Wallace, 1987). These calculated pharmacodynamic parameters for the inhibition, reactivation, and aging of AChE by an OP can be used in the construction and validation of pharmacodynamic models used in the prediction of OP induced toxicity and establishment of tolerance levels for OPs by regulatory agencies (Timchalk, 2001; Dourson *et al*., 1996). The rate constants for OP inhibition of AChE can differ by orders of magnitude depending on the differences in OP chemistries (Main, 1980). Therefore, determining inhibition rate constants for individual OPs, such as malaoxon, provide useful information that can help address differences in sensitivities to a OP both between species and within species.

Materials and Methods

Chemicals

Malaoxon was supplied from FMC, Inc. Structures were confirmed by nuclear magnetic resonance (NMR) at FMC and the Department of Chemistry at Mississippi State University. Purity of malaoxon was determined to be greater than 98%. All other

reagent grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA).

Animals

Adult male (6) and female (6) Sprague Dawley-derived rats (post-natal day 70, PND 70; approximately 225-300 g) were purchased from Envigo, Inc. and housed in AAALAC accredited facilities in the College of Veterinary Medicine at Mississippi State University with temperature-controlled environments and 12 h dark-light cycle. Standard lab chow and tap water were provided *ad libitum*. All animal procedures received prior approval from the Mississippi State University Animal Care and Use Committee. Blood from 6 rats of each sex was collected on ice and pooled for each of three replications for erythrocyte ghost preparations.

Human Acetylcholinesterase

Human blood samples from individual healthy humans (age range 16-60 for adults and 10-13 for juveniles) as well as cord blood samples were purchased from Innovative Research (adults), BioreclamationIVT (juveniles), or Zen-Bio (cord blood). Humans were of either sex and any race or ethnic group. All blood samples were collected and shipped with cold packs overnight to Mississippi State University. The samples were collected from 18 humans (9 adults, 5 juveniles and 4 cord bloods).

Preparation of Human and Rat Erythrocyte Ghosts

Erythrocyte ghosts (membrane preparations devoid of hemoglobin) were prepared by centrifugation of whole blood anticoagulated with K_2EDTA (3000 *g* for 10 min),

plasma and buffy coat were carefully removed and packed erythrocytes were washed 3 times (1000 *g* centrifugation) using 2 volumes of 100 mM phosphate buffer (pH 7.4). Erythrocytes were lysed using 20 volumes of hypotonic phosphate buffer (6.7 mM, pH 7.4) for 10 min on ice followed by centrifugation at 50,000 *g* for 30 min. The resulting pellet was washed 3 times with 2 volumes of 100 mM phosphate buffer (pH 7.4) and resuspended to original volume with 100 mM phosphate buffer (pH 7.4). The final ghost preparations were light pink in color indicating a minimal amount of hemoglobin. Ghost preparations from individual humans or rats were checked for AChE activity and diluted to equivalent AChE activities (absorbance values, 0.5-0.6 AU in 5 min). The diluted ghost preparations were aliquoted into individual cryogenic tubes and stored frozen at -80ºC until assay. A previously conducted pilot study provided evidence that the human ghost preparations did not lose appreciable AChE activity by freezing at -80ºC for several months but multiple freeze thaw cycles will decrease activity.

Determination of Erythrocyte AChE Inhibition Kinetics

Malaoxon inhibition kinetics studies were similar to previous kinetic studies from our laboratory for anticholinesterases (Carr and Chambers,1996; Coban *et al*., 2016). Inhibition of AChE in erythrocyte ghost preparations was conducted using a 96-well plate format with a final reaction volume of 250 µl per well. A continuous spectrophotometric assay was used to determine AChE activities (modification of Ellman *et al*., 1961) with acetylthiocholine (ATCh) as the substrate and 5,5'-dithiobis(nitrobenzoic acid) (DTNB) as the chromogen (Chambers *et al*., 1988; Carr and Chambers, 1996; Coban *et al*., 2016). Range finding studies were conducted using a pooled erythrocyte ghost preparation to

determine the effective concentration range of malaoxon that yielded about 10-90% AChE inhibition. For each individual rat or human sample, 198 µl of diluted erythrocyte ghost preparation was added to each well of a 96-well plate and warmed to 37ºC in a Jitterbug plate shaker for 5 min. To correct for non-enzymatic hydrolysis, 2 µl of eserine sulfate (FC, 60 µM) was added to wells in the first column to serve as blank samples to correct for non-AChE hydrolysis of substrate. The inhibition reaction was initiated by pre-incubation of the erythrocyte ghost preparation with EtOH vehicle or eight concentrations of malaoxon in EtOH. Additional wells were included that were devoid of malaoxon or EtOH vehicle to compare to EtOH vehicle wells to monitor any unexpected EtOH effects. Six pre-incubation periods were used (0-5 min at 1 min intervals). Malaoxon was dissolved in dry EtOH and was added in a volume of 2 µl per well. The inhibition reaction was terminated by addition of excess substrate, ATCh, which should out-compete any unbound malaoxon for the active site of AChE. The AChE reaction was initiated by the addition of a 40 µl mixture of ATCh and DTNB (to yield final concentrations of 1 mM ATCh and 5 mM DTNB). For the 0-min time point, the substrate/chromogen mixture was added simultaneously with malaoxon. Each reaction was monitored by recording the absorbance at 412 nm for 8 min at 50 second intervals (to yield 10 readings) and the velocity of each pre-incubation time was obtained for each concentration and control. The linearity of the reaction was verified using the substrate and chromogen alone to insure no substrate depletion (linearity was maintained beyond 10 min).

Calculations for Malaoxon Inhibition Kinetic Constants

For the determination of enzyme inhibition kinetic constants for malaoxon, the reaction of malaoxon with the enzyme was carried out without substrate, acetylthiocholine, present. The assumption is that the enzyme and inhibitor bind reversibly, followed quickly by the phosphorylation of the enzyme. The rate of dissociation of the enzyme inhibitor complex is assumed to be faster than the rate of phosphorylation. The persistence of the phosphorylation of the enzyme allows sufficient time to measure the residual enzyme activity prior to reactivation. The enzyme-inhibitor reaction can be quantified by the addition of substrate which out-competes the inhibitor for the residual non-phosphorylated enzyme and the resulting product can be measured spectrophotometrically over a given time. This procedure allows for the determination of enzyme inhibition as a function of both time and concentration. The process of this chemical reaction is described by the following equations:

Inhibition Equation $$[E] + [I] \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} [EI]_R \overset{k_2}{\rightarrow} [EI]_I \quad (1)$$

Simplified Inhibition Equation $$[E] + [I] \overset{k_i}{\rightarrow} [EI] \quad (2)$$

Where, k_i = bimolecular rate constant and $[EI]_I$ = enzyme-inhibitor complex

According to this equation, *[E]* is the concentration of free enzyme, [*I*] is the concentration of free inhibitor, $[EI]_R$ is the concentration of reversible enzyme-inhibitor complex, $[EI]_I$ is the concentration of irreversibly phosphorylated enzyme-inhibitor

complex. According to a method described by Kitz and Wilson (1962), kinetic constants can be calculated for this reaction including: the bimolecular rate constant, k_i, the phosphorylation constant of the enzyme, k_p, and the dissociation constant, K_I, for the enzyme-inhibitor complex. For each malaoxon incubation time, the enzyme (AChE) velocity following inhibition ($[E]_t$) was divided by the original uninhibited velocity ($[E]_o$) to obtain the fraction of AChE velocity remaining ($[E]_t/[E]_o$). The "apparent" rate of AChE phosphorylation (slope = $-k_{app}$) for each concentration was calculated by linear regression of the natural log (ln) of the $[E]_t/[E]_o$ as a function of time (Figure 2). Using a double reciprocal plot (Figure 3) of $1/k_{app}$ versus $1/[I]$, (SigmaPlot 13 software), the constants k_i (slope = $1/k_i$), k_p (y intercept = $1/k_p$), and K_I (x intercept= $-1/K_I$) were calculated (Wilkinson, 1961; Ritchie and Prvan, 1996). The data suggest that inhibition of AChE follows pseudo-first-order kinetics as described by Michaelis-Menten (Michaelis and Menten, 1913; Goody and Johnson, 2011) and better fit a hyperbolic curve. For comparison, the data were plotted as k_{app} versus $[I]$ which yielded a hyperbola (Figure 4). The data were fit to the equation for a hyperbola ($y = ax/(b + x)$) where $y = k_{app}$ and $x = [I]$ and the equation was solved for a and b ($a = k_p$ and $b = K_I$) (Wilkinson, 1961; Ritchie and Prvan, 1996). The data from the double reciprocal plot and the hyperbolic plot should be similar. In addition, IC_{50}s were calculated for the last time point (5 min) as a comparison because IC_{50}s are determined from the middle (50%) of an inhibition curve which should be the most accurate point (errors are greater at the upper and lower ends of inhibition curves). Statistical differences between mean kinetic values between rats and humans and between sexes within a species were determined by

Student's t-test. Statistical differences among human ghost preparations with respect to age were determined by linear regression.

Results

The erythrocyte ghost preparations were determined to be stable at -80°C. A final concentration range for malaoxon of 3.16 $x10^{-7}$ M to 1.77 $x10^{-5}$ M was determined to produce an inhibition range of about 10% to 90% of AChE in erythrocyte ghost preparations. This concentration range was used for each of the 24 ghost preparations (6 rats and 18 humans). Non-enzymatic hydrolysis of ATCh was determined to be minimal. The kinetic constants obtained for each individual human or rat erythrocyte ghost preparation incubated with increasing concentrations of malaoxon were initially calculated by analysis of a double reciprocal plot (e.g., Figure 3) for each erythrocyte ghost preparation with a mean r^2 for the lines of 0.947 for humans and 0.973 for rats (Table 1-2). Subsequently, kinetic constants were calculated from a hyperbolic plot (e.g., Figure 4) for malaoxon for each individual human or rat erythrocyte ghost preparation with a mean r^2 for the lines of 0.968 for humans and 0.992 for rats (Table 3-4).

The bimolecular rate constant (k_i) for malaoxon calculated from the double reciprocal plots from the 18 individual human erythrocyte ghost preparations ranged from 0.154 $x10^5$ to 3.722 $x10^5$ $L \cdot mol^{-1} \cdot min^{-1}$, while the k_i calculated from the double reciprocal plots for the rat erythrocyte ghost preparations ranged from 0.684 $x10^5$ to 1.780 $x10^5$ $L \cdot mol^{-1} \cdot min^{-1}$ for malaoxon. The phosphorylation constant (k_p) calculated from the double reciprocal plots for the 18 human erythrocyte ghost preparations ranged from 0.1408 to 1.061 min^{-1}. For the rat erythrocyte ghost preparations, the k_p calculated from

the double reciprocal plots ranged from 0.182 to 29.58 min^{-1}. The dissociation constant (K_I) calculated from the double reciprocal plots for the 18 human erythrocyte ghost preparations ranged from 0.900 $x10^{-6}$ to 12.70 $x10^{-6}$ $mol \cdot L^{-1}$, while the K_I calculated from double reciprocal plots for the rat samples ranged from 2.660 x 10^{-6} to 348.0 x 10^{-6} $mol \cdot L^{-1}$ (Table 1-2).

For comparison, the same data were fit to the more accurate hyperbolic equation. The k_is calculated by hyperbolic plot analysis using erythrocyte ghost preparations from the 18 individual human samples ranged from 0.078 $x10^5$ to 2.196 $x10^5$ $L \cdot mol^{-1} \cdot min^{-1}$ for malaoxon. In rats, the k_i calculated from the hyperbolic plots ranged from 0.419 $x10^5$ to 1.173 x 10^5 $L \cdot mol^{-1} \cdot min^{-1}$. The k_p calculated from the hyperbolic plots for the erythrocyte ghost preparations from the 18 individual human samples ranged from 0.259 to 1.581 min^{-1}. For the rat erythrocyte ghost preparations, k_p calculated from the hyperbolic plots ranged from 0.555 to 13.73 min^{-1}. Finally, the K_I calculated from the hyperbolic plots for the erythrocyte ghost preparations from the 18 individual human samples ranged from 2.032 $x10^{-6}$ to 15.88 x 10^{-6} $mol \cdot L^{-1}$ and 3.946 $x10^{-6}$ to 23.94 $x10^{-6}$ $mol \cdot L^{-1}$ for the rat erythrocyte ghost preparations (Table 3-4).

Kinetic rate constants were compared between the two methods of data representation (a linear double reciprocal plot or hyperbolic plot) and were not determined to be significantly different from each other for the human or rat erythrocyte ghost preparations ($p < 0.05$). Therefore, the results from this point forward will refer to the kinetic constants derived from more accurate hyperbolic analyses. The mean bimolecular rate constant (k_i), calculated from the hyperbolic plots, for the human erythrocyte ghost preparations was 1.07 x 10^5 $L \cdot mol^{-1} \cdot min^{-1}$, while the mean k_i for the rat

erythrocyte ghost preparations was 0.813 x 10^5 L·mol^{-1}·min^{-1}. The mean phosphorylation constant (k_p) calculated from the hyperbolic plot for the human erythrocyte ghost preparations was 0.594 min^{-1}, while the mean k_p for the rat erythrocyte ghost preparations was 0.830 min^{-1}. The mean dissociation constant (k_I), calculated from the hyperbolic plots, for the human erythrocyte ghost preparations was 5.75 x10^{-6} mol·L^{-1}, while the mean k_p for the rat erythrocyte ghost preparations was 1.08 x10^{-6} mol·L^{-1}.

Statistical differences were not found between the human and rat kinetic rate constants ($p < 0.05$). In addition, no statistical differences were determined for any of the three rate constants between sexes for the individual humans or rat erythrocyte ghost preparations. No age-related differences were determined for the three kinetic rate constants for the 18 individual human erythrocyte ghost preparations, when statistically analyzed as a complete population (linear regression) or when separated and compared as three separate age groups (cord blood, juveniles, and adults). Finally, no interspecies or intraspecies were found using IC50s from the five minute malaoxon incubation and the IC_{50}s correlated with the bimolecular rate constants (k_i).

Discussion

The kinetic data for malaoxon was calculated by the methods described by Kitz and Wilson (1962). The kinetic constants derived from these methods for malaoxon included: the bimolecular rate constant (k_i), phosphorylation constant (k_p), and dissociation constant (K_I). Initially, kinetic constants were calculated from a double reciprocal plot (Lineweaver Burk) as this has been widely used for many years to calculate enzyme kinetic constants (Wilkinson, 1961; Kitz and Wilson, 1962; Johnson

and Wallace, 1987; Ma and Chambers, 1995; Ritchie and Prvan, 1996; Coban *et al*., 2016). The Lineweaver Burk plots rely on transformed data which has been shown to magnify errors, especially at the lower and upper ends of the curve (Ritchie and Prvan, 1996). For comparison, data were fit to the equation for a hyperbola, which is the preferred form (Wilkinson, 1961; Ritchie and Prvan, 1996).

Acetylcholinesterase in mammals has been determined to be encoded by a single gene and the catalytic domains of the different molecular forms of AChE expressed in tissues of rat and humans have been determined to be identical (Taylor *et al*., 1993; Massoulié *et al*., 2008). The amino acid sequences of the catalytic domains of brain and erythrocyte AChE are identical within a given mammalian species (human or rat) (Basova and Rozengart, 2009; Carr and Ollis, 2009; Cygler *et al*., 1993; Herkert *et al*., 2012; Uniprot, 2017a,b; Wiesner *et al*., 2007). With catalytic domains for AChE molecules being identical, erythrocyte AChE activity and pharmacodynamic parameters have routinely been used as a surrogate for brain AChE activity and pharmacodynamic parameters. Rat erythrocyte AChE pharmacodynamic parameters have also been used as a surrogate for human AChE pharmacodynamic parameters. The catalytic domains of rat and human erythrocyte AChE have high sequence homology (88.6%) and therefore, a high degree of predicted 3D structural homology (Konagurthu *et al*., 2006; Massoulié *et al*., 2008; Uniprot 2017a,b). This gives rise to the hypothesis that rat and human AChE would be expected to interact similarly with OP inhibitors. The data from this study support this hypothesis that rat and human AChE are very similar in structure and function with no statistical differences determined in the erythrocyte ghost AChE inhibition kinetic constants between the two species. In addition, given the homology of

the catalytic site of AChE within tissues of the same species, one would not expect significant intrahuman variability in AChE inhibition from an OP. Variability in AChE activity with age has been reported in the literature, with some studies reporting increases or decreases but the majority reported no differences (Ecobichon and Stephens, 1972; Ramenjak, 2009). However, the differences in activity could be a result of the methodology of determining activity; i.e., is activity representing the total numbers of molecules or catalytic efficiency of a given molecule. Animal studies have indicated a decrease in sensitivity of AChE and toxicity to OP insecticides with increasing age (Benke and Murphy, 1975; Pope *et al*., 1991; Mortensen *et al*., 1996; Atterberry *et al*., 1997). However, these differences can be attributed to age related differences in pharmacokinetics and maturation in the OP detoxication enzymes, such as carboxylesterase and paraoxonase (Maxwell, 1992; Atterberry *et al*., 1997; Mortensen *et al*., 1998; Vidair, 2004).

The experimental data for malaoxon from this study support this hypothesis as no significant differences in inhibition kinetic constants were exhibited among the 18 human erythrocyte ghost preparations. The selection of 18 subjects represented a range of age as well as both sexes and several ethnicities. This is still a relatively small sample size and therein lies the concern of the sufficiency of 18 human samples to represent intrahuman variability for the whole population. However, the lack of differences in any of the inhibition rate constants combined with the complete homology of the catalytic site of AChE within a species suggests there is relatively small variability among the human population.

There have been differences in AChE activity within a species (intraspecies) reported in both rats and humans (Herz and Kaplan, 1973) which most likely results from differences in AChE concentration. The rate of inhibition of AChE for an OP would be expected to be proportional to the AChE mass and is determined by the activity level and the turnover rate (*de novo* synthesis). At environmentally relevant OP exposures levels where activity levels are not completely inhibited, the activity level probably does not greatly affect the percent inhibition level. Finally, the sites of post-translational modifications of AChE are peripheral to the catalytic site and have very little effect on the activity of AChE (Massoulié *et al*., 2008). With the lack of post translational modifications in the catalytic domain of AChE, genetic mutations within the catalytic domain would likely be the only factor to alter the pharmacodynamic parameters of the enzyme; however, these mutations are extremely rare (Lockridge *et al*., 2016).

The structure of OPs can affect the inhibitory potency for the target enzyme, AChE, and result in a wide range of toxicities (Main, 1980). Studies by Wang and Murphy (1982) demonstrated that differences in affinity and phosphorylation rates for OPs can result in differences in inhibition of AChE. Bimolecular rate constants (k^is) for the diethyl phosphates chlorpyrifos-oxon and paraoxon are different although the structures only differ in their leaving groups. Similar differences were reported with other OPs including insecticides, nerve agents and nerve agent surrogates (Bošković, 1968; Herzsprung *et al*., 1992; Coban *et al*., 2016). The differences in rates of phosphorylation (k_p) can contribute to the inhibitory potency of an OP, although these differences are suggested to be minimal while the association with the inhibitor (K_I) is suggested to be more important in inhibitor potency (k_i) (Carr and Chambers, 1996;

Wang and Murphy, 1982; Kemp and Wallace 1990). In addition, different potencies between OPs may be due to interactions at the anionic site that can produce conformational changes in the tertiary structure of the enzyme resulting in preferential binding of one inhibitor over another (Wallace, 1992). Finally, other factors can influence the potency of an OP for AChE such as pH and temperature (Main and Iverson, 1966; Carr and Chambers, 1996). All these factors must be considered when comparing interspecies and intraspecies *in vitro* potency values for AChE.

Conclusions

The kinetic constants derived for malaoxon may be used in pharmacodynamic models to address the toxicodynamics of malathion. The data determined in this study for malaoxon using erythrocyte ghost preparations are useful as a biomarker for the target, brain AChE, especially with the evidence that erythrocyte and brain AChE are the same gene product with identical catalytic domains. The kinetic parameters obtained for rats and humans from erythrocyte ghost preparations are intended to help address the interspecies uncertainty in risk assessment of malathion. Similarly, the kinetic parameters obtained from the individual human erythrocyte ghost preparations are intended to address the intraspecies uncertainty factors in the risk assessment of malathion. Malaoxon was shown to be a relatively potent inhibitor of AChE, but no significant differences were determined in the interspecies or intraspecies sensitivity of erythrocyte AChE to malaoxon, indicating the low mammalian toxicity for the parent insecticide, malathion, most likely results from the organisms' detoxication capacity.

Table 4.1 Inhibition kinetic constants for malaoxon in erythrocyte "ghosts" from humans (double reciprocal analysis).

Sample	Age (years)	Sex	k_i ($L \cdot mol^{-1} \cdot min^{-1}$)	k_p (min^{-1})	K_I ($mol \cdot L^{-1}$)	r^2	IC_{50}s (nM)
Human 1	0.691	M	0.359×10^5	0.1622	4.50×10^{-6}	0.867	293
Human 2	0.698	F	0.612×10^5	0.1408	2.30×10^{-6}	0.835	69
Human 3	0.70	M	0.837×10^5	0.6153	7.35×10^{-6}	0.983	393
Human 4	0.73	F	2.560×10^5	0.3987	1.56×10^{-6}	0.857	295
Human 5	10	F	1.378×10^5	0.7497	5.44×10^{-6}	0.962	555
Human 6	10	M	1.142×10^5	0.3349	2.93×10^{-6}	0.995	341
Human 7	10	F	0.194×10^5	0.1939	10.0×10^{-6}	0.942	90
Human 8	11	F	0.646×10^5	0.8319	12.9×10^{-6}	0.996	467
Human 9	13	M	0.994×10^5	0.4168	4.19×10^{-6}	0.956	129
Human 10	16	F	1.650×10^5	0.9378	5.60×10^{-6}	0.985	804
Human 11	23	M	0.154×10^5	0.1971	12.7×10^{-6}	0.958	50
Human 12	27	F	3.722×10^5	0.3212	0.90×10^{-6}	0.936	520
Human 13	30	F	1.299×10^5	0.5410	4.16×10^{-6}	0.988	509
Human 14	31	M	1.252×10^5	0.3231	2.58×10^{-6}	0.836	279
Human 15	35	F	0.735×10^5	0.8000	10.9×10^{-6}	0.979	372
Human 16	46	M	1.457×10^5	0.5485	3.77×10^{-6}	0.995	560
Human 17	51	F	1.560×10^5	1.061	6.80×10^{-6}	0.982	536
Human 18	60	M	1.273×10^5	0.5327	4.20×10^{-6}	0.999	482

Kinetic constants calculated from double reciprocal plots for 18 human erythrocyte ghost preparations. Data for k_i = bimolecular rate constant ($L \cdot mol^{-1} \cdot min^{-1}$), k_p = phosphorylation constant (min^{-1}), and K_I = dissociation constant ($mol \cdot L^{-1}$). IC_{50}s calculated from the 5 min time point. IC_{50} data expressed as nM.

Table 4.2 Inhibition kinetic constants for malaoxon in erythrocyte "ghosts" from rats (double reciprocal analysis).

Sample	**Age (days)**	**Sex**	k_i **(L·mol^{-1}·min^{-1})**	k_p **(min^{-1})**	K_I **(mol·L^{-1})**	r^2	**IC50s (nM)**
Rat 1	70	M	1.780×10^5	0.6729	3.80×10^{-6}	0.990	608
Rat 2	70	M	0.991×10^5	1.132	11.4×10^{-6}	0.991	404
Rat 3	70	M	1.117×10^5	0.8591	7.69×10^{-6}	0.966	392
Rat 4	70	F	0.850×10^5	29.580	348×10^{-6}	0.980	417
Rat 5	70	F	0.480×10^5	0.7250	15.1×10^{-6}	0.974	105
Rat 6	70	F	0.684×10^5	0.1820	2.66×10^{-6}	0.936	234

Kinetic constants calculated from double reciprocal plots for 6 pools of rat erythrocyte ghost preparations (3 males and 3 females). Data for k_i = bimolecular rate constant (L·mol^{-1}·min^{-1}), k_p = phosphorylation constant (min^{-1}), and K_I = dissociation constant (mol·L^{-1}). IC_{50}s calculated from the 5 min time point. IC_{50} data expressed as nM.

Table 4.3 Inhibition kinetic constants for malaoxon in erythrocyte "ghosts" from humans (hyperbolic analysis).

Sample	Age (years)	Sex	k_i ($L \cdot mol^{-1} \cdot min^{-1}$)	k_p (min^{-1})	K_I ($mol \cdot L^{-1}$)	r^2	IC_{50}s (nM)
Human 1	0.691	M	0.241×10^5	0.2588	10.75×10^{-6}	0.971	293
Human 2	0.698	F	0.353×10^5	0.3368	9.54×10^{-6}	0.978	69
Human 3	0.70	M	1.252×10^5	0.4039	3.23×10^{-6}	0.963	393
Human 4	0.73	F	1.736×10^5	0.4300	2.42×10^{-6}	0.831	295
Human 5	10	F	1.798×10^5	0.5433	3.02×10^{-6}	0.980	555
Human 6	10	M	0.880×10^5	0.6674	7.85×10^{-6}	0.992	341
Human 7	10	F	0.229×10^5	0.1150	5.01×10^{-6}	0.978	90
Human 8	11	F	0.943×10^5	0.4094	4.30×10^{-6}	0.987	467
Human 9	13	M	0.570×10^5	0.9057	15.9×10^{-6}	0.960	129
Human 10	16	F	2.196×10^5	0.7375	3.36×10^{-6}	0.984	804
Human 11	23	M	0.078×10^5	1.581	2.03×10^{-6}	0.958	50
Human 12	27	F	1.736×10^5	0.5131	2.96×10^{-6}	0.912	520
Human 13	30	F	1.072×10^5	0.7547	7.04×10^{-6}	0.994	509
Human 14	31	M	0.772×10^5	0.7203	9.33×10^{-6}	0.983	279
Human 15	35	F	1.188×10^5	0.3306	2.78×10^{-6}	0.979	372
Human 16	46	M	1.363×10^5	0.6756	4.96×10^{-6}	0.998	560
Human 17	51	F	1.670×10^5	0.7601	4.55×10^{-6}	0.982	536
Human 18	60	M	1.195×10^5	0.5536	4.48×10^{-6}	0.997	482

Kinetic constants calculated from hyperbolic plots for 18 human erythrocyte ghost preparations. Data for k_i = bimolecular rate constant ($L \cdot mol^{-1} \cdot min^{-1}$), k_p = phosphorylation constant (min^{-1}), and K_I = dissociation constant ($mol \cdot L^{-1}$). IC_{50}s calculated from the 5 min time point. IC_{50} data expressed as nM.

Table 4.4 Inhibition kinetic constants for malaoxon in erythrocyte "ghosts" adult rats (hyperbolic analysis).

Sample	Age (days)	Sex	k_i ($L \cdot mol^{-1} \cdot min^{-1}$)	k_p (min^{-1})	K_I ($mol \cdot L^{-1}$)	r^2	IC_{50}s (nM)
Rat 1	70	M	0.687×10^5	0.6842	3.95×10^{-6}	0.994	608
Rat 2	70	M	1.173×10^5	0.6494	5.54×10^{-6}	0.998	404
Rat 3	70	M	1.126×10^5	0.6652	5.91×10^{-6}	0.993	392
Rat 4	70	F	0.897×10^5	1.056	12.0×10^{-6}	0.990	417
Rat 5	70	F	0.574×10^5	1.373	23.9×10^{-6}	0.993	105
Rat 6	70	F	0.419×10^5	0.5548	13.3×10^{-6}	0.987	234

Kinetic constants calculated from hyperbolic plots for 6 pools of rat erythrocyte ghost preparations (3 males and 3 females). Data for k_i = bimolecular rate constant ($L \cdot mol^{-1} \cdot min^{-1}$), k_p = phosphorylation constant (min^{-1}), and K_I = dissociation constant ($mol \cdot L^{-1}$). IC_{50}s calculated from the 5 min time point. IC_{50} data expressed as nM.

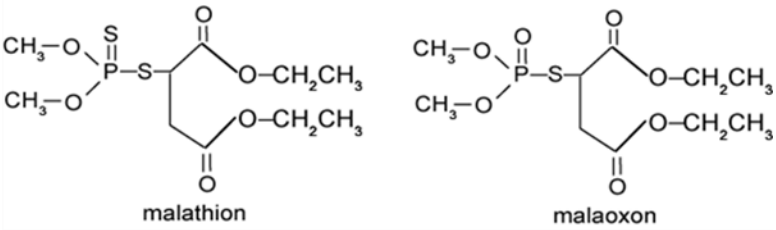

Figure 4.1 Chemical structures of malathion and its active metabolite malaoxon.

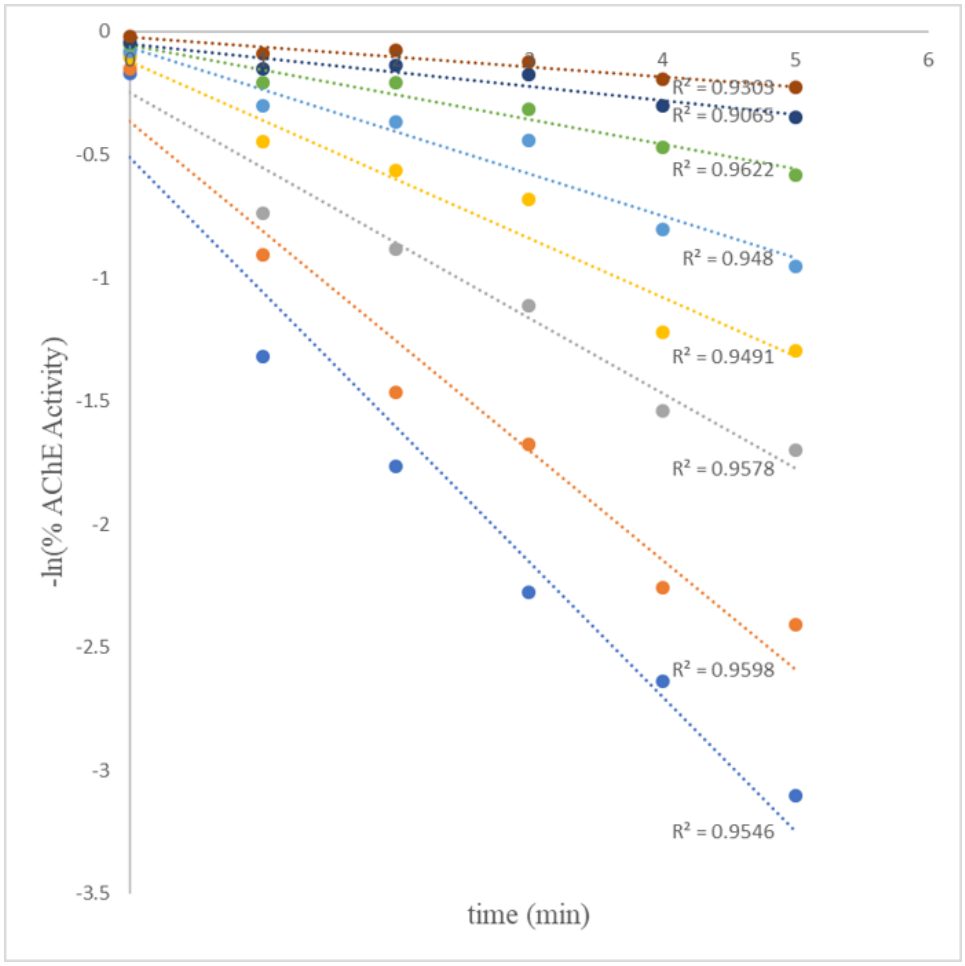

Figure 4.2 Inhibition curves for eight concentrations of malaoxon over six incubation periods.

Representative plot of -ln(% AChE activity) vs. time (min). One of 18 plots for human erythrocyte AChE incubated with 8 increasing concentrations of malaoxon compared to EtOH vehicle controls. Individual r^2 were determined for each line.

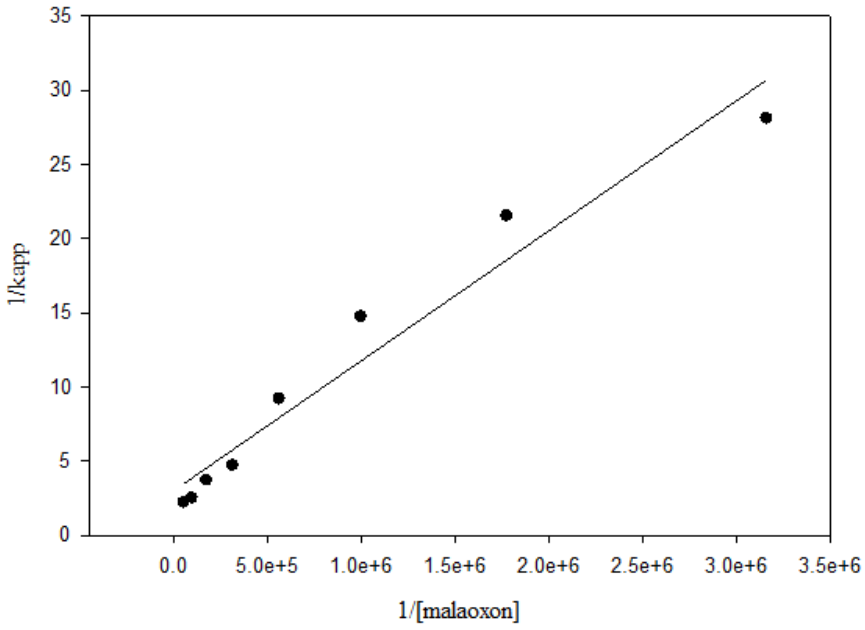

Figure 4.3 Double-reciprocal plot for malaoxon inhibition of erythrocyte acetylcholinesterase.

Representative double-reciprocal plot of 1/malaoxon concentration (M) vs. $1/k_{app}$. One of 18 hyperbolic plots for human erythrocyte AChE incubated with 8 increasing concentrations of malaoxon compared to EtOH vehicle controls.

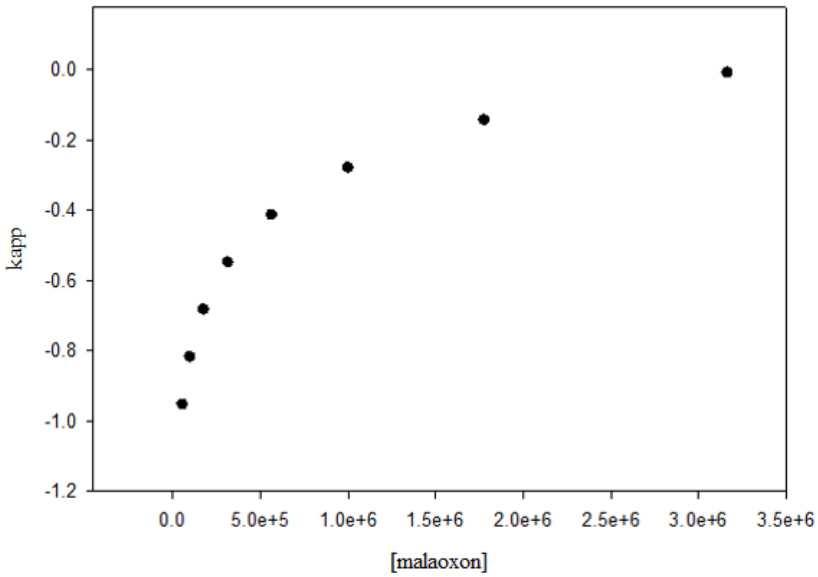

Figure 4.4 Hyperbolic plot for malaoxon inhibition of erythrocyte acetylcholinesterase.

Representative hyperbolic plot of malaoxon concentration (M) vs. k_{app}. One of 18 hyperbolic plots for human erythrocyte AChE incubated with 8 increasing concentrations of malaoxon compared to EtOH vehicle controls.

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CHAPTER V

CONCLUSION

Although the usage of organophosphate (OP) insecticides has declined over the last decade as insect resistance has developed and new insecticidal chemistries have been discovered with different modes of action, exposures to OPs are still a concern in the US and around the world. The wide variety of applications from agricultural to residential for OPs increases the potential of exposure to non-target species, such as animals and humans. Of particular concern is exposure to children, which are more susceptible to toxic insult from OPs because of their developing nervous systems as well as lower detoxication capacities. The passage of the Food Quality Protection Act (FQPA) in 1996 required additional consideration for the susceptibility of infants and children to pesticides. Under FQPA, the addition of 10x uncertainty (safety) factors for extrapolation of animal studies to humans (interspecies) and the protection of sensitive populations (intraspecies) was mandated unless sufficient experimental and epidemiological data allowed for the reduction of the safety factors. In addition, pesticides with a common mechanism of toxicity, such as the OPs, were required to undergo a cumulative risk assessment which addresses the risk associated with total exposure to multiple OPs from multiple routes. The reassessment of risk for all the registered OPs was a massive undertaking that lasted over a decade and required many additional *in vitro* and *in vivo* studies. Although the cumulative risk assessment for the OPs has been completed,

reassessments of individual compounds still occur when new data with toxicological implications is produced.

This research investigated the differences in activation and detoxication of OP insecticides in both rat and human tissues, with an overall hypothesis that the age-related differences in acute toxicity for many OPs are primarily the result of the maturation of detoxication enzymes and less likely the result of differences in bioactivation and sensitivity of the target enzyme, AChE, to the OP.

The first study was designed to determine age-related differences in sensitivity of the target enzyme AChE to a structurally diverse group of OPs *in vitro*. In addition, the age-related differences in sensitivity of OP detoxication enzymes, carboxylesterase (CaE) and butyrylcholinesterase, to the same diverse group of OPs was determined *in vitro* to assess the potential protection these enzymes may provide to the target enzyme AChE for a given OP structure. Log inhibitor concentration-logit percent inhibition regression lines were used to determine the IC_{50} concentrations for AChE, BChE and CaE in several tissues from neonatal, juvenile and adult male rats. The twelve OP compounds investigated displayed a wide range of inhibitory potential, as evidenced by their IC50s, toward the target enzyme, AChE, as well as non-target detoxication enzymes, BChE and CaE, ranging several orders of magnitude. Although IC50s do not provide as much information about an inhibitor as kinetic rate constants, they require less tissue, time and expense to determine and they provide a good way to compare potencies among compounds with similar chemistries in different tissues and species. The di-ethyl phosphate insecticidal OPs, such as chlorpyrifos-oxon and paraoxon, were generally more potent inhibitors of AChE than the di-methyl phosphate insecticidal OPs. This is in

contrast to the acute toxicities of some of the parent insecticides. The detoxication enzymes, CaE and BChE, were stoichiometrically inhibited at lower concentration of the diethyl phosphates (insecticidal) than the dimethyl phosphate inhibitors which could account for the greater acute toxicity for some of the dimethyl OPs. *In vitro* studies, such as these, can provide insights into the age-related differences in the acute toxicity reported for many OPs from *in vivo* challenges. *In vivo* challenges of OPs are ultimately needed to address the differences in toxicity for OPs differing in structure; however, *in vitro* studies such as the one reported here can provide information for the biochemical pathways that can then be used in developing predictive models that once validated can help address differences in species and age-related toxicities. Ultimately, these types of predictive models can reduce or replace the number of *in vivo* studies necessary to address risk to humans.

One of the most heavily used OPs still on the market is the phosphorothionothiolate insecticide malathion. The use of malathion in agriculture, such as the eradication of cotton boll weevil, and in public health, control of vector-borne (mosquito) diseases, increases the potential of exposure to humans. The popularity of malathion within the insecticide marketplace stems from its good insecticidal properties and its low mammalian toxicity (rat oral $LD_{50} > 1$ g/kg). Malathion requires cytochrome P450 (CYP) bioactivation (desulfuration) to its anticholinesterase metabolite, malaoxon. Malathion and malaoxon can both be efficiently degraded (detoxified) by CaE. The CaE-mediated degradation of malathion is catalytic, while the detoxication of malaoxon is stoichiometric, making malathion fairly unique among the OPs. The interspecies and intraspecies differences in bioactivation and detoxication of malathion were investigated

in the second part of this study using hepatic microsomes obtained from 6 male and 6 female adult rats and 27 humans of both sexes and various ages. The kinetics of malathion desulfuration in rat and human hepatic microsomes were determined using standard Michaelis-Menten equations. The efficiency of the bioactivation of malathion was higher for the human liver microsomes than rats, but the affinity of CYPs for malathion was not different between rats and humans. There were no age-related differences in the rates of malathion desulfuration among the human hepatic microsomes; however, the sample size may not be sufficient to determine an age-related difference for the human population. The detoxication of malathion by CaEs was investigated as the primary pathway in the detoxication of malathion in both rats and humans. CaE activities were variable in both rat and human hepatic microsomes, with CaE activities in rat hepatic microsomes higher than in the human hepatic microsomes. The catalytic degradation of malathion and the stoichiometric detoxication of malaoxon by hepatic microsomal CaE both contribute substantially to the reduction of circulating OP following exposure. This reduces the amount of OP available to inhibit the target enzyme, AChE, and is largely responsible for the low mammalian acute toxicity for malathion. The data in this study are consistent with data from the literature indicating the importance of CaE in the detoxication of malathion. Carboxylesterase concentrations and CYP isozyme content have been shown to vary among humans as well as be inducible or inhibited by a variety of xenobiotics, which could result in differences in bioactivation and detoxication of malathion and toxicity. In addition, multiple exposures to malathion or another potent OP could reduce a person's CaE detoxication capacity, thus increasing malathion toxicity. The kinetic rates for bioactivation as well as the

detoxication pathways reported here can be used in the development of physiologically-based pharmacokinetic and pharmacodynamic models (PBPK/PD). These models, once validated can be used to refine uncertainty factors and tolerance levels of humans to OPs, such as malathion.

In the final part of this study, the interspecies and intraspecies differences in sensitivity of the target enzyme, acetylcholinesterase, to malaoxon inhibition were investigated. Erythrocyte AChE derived from pools of 6 rats of both sexes (3 male pools and 3 female pools) and 18 individual humans from both sexes and a variety of ages and ethnicities was used to determine kinetic constants for the inhibition of AChE by malaoxon. Kinetic constants calculated from AChE inhibition measured over time with increasing concentrations of malaoxon included the bimolecular rate constant (k_i), phosphorylation constant (k_p), and dissociation constant (K_I). Kinetic analyses provide a quantitative means to evaluate the effects (potency) of inhibitors on enzymes. In addition, kinetic analyses allow the quantitation of differences between the same enzyme in different species or tissues from the same species. Acetylcholinesterase in mammals is encoded by a single gene that has identical catalytic domains; therefore, it was hypothesized that there would be no differences in interspecies and intraspecies malaoxon inhibition of AChE. The values for k_i, k_p, or K_I were not significantly different between rats and humans, this is important with the inability to test OPs in humans because of ethical limitations. Regulatory agencies are limited to using pharmacokinetic data from humans and toxicokinetic/dynamic data from animal models for risk assessments. In addition to the lack of species differences in AChE inhibition kinetic values, no significant differences were determined for kinetic values between sexes for

rats or humans. Finally, no age-related statistical differences were determined for any of the kinetic values. Given the literature evidence that erythrocyte and brain AChE are the same gene product with identical catalytic domains, the kinetic data determined in this study for malaoxon using erythrocyte ghost AChE preparations could be used as a surrogate for the target, brain AChE. The kinetic constants derived for malaoxon may be used in pharmacodynamic models to address the toxicodynamics of malathion. Malaoxon was shown to be a relatively potent inhibitor of AChE, but no significant differences were determined in the interspecies or intraspecies sensitivity of erythrocyte AChE to malaoxon, indicating the low mammalian toxicity for the parent insecticide, malathion, most likely results from the organisms' detoxication capacity. The absence of meaningful interspecies and intraspecies differences in erythrocyte AChE inhibition could provide sufficient evidence for the reduction of safety factors for malathion.

This study suggests that species and age-related differences in acute toxicities for OPs are primarily due to differences in detoxication capacities of mammals and less likely due to differences in bioactivation of OPs to their oxon metabolites and sensitivity of the target enzyme, AChE, to those oxon metabolites. Additionally, bioactivation, detoxication and inhibitory potential of OPs is largely dependent on the chemical structures of the OP. Future studies using software to predict structure activity relationships for OPs with human CYPs and CaEs could provide additional information aiding in predicting toxicity based on chemical structure. *In vivo* studies using knockout animal models for CaE could provide information on the role CaE plays in the detoxication of OPs. Finally, variability of bioactivation and detoxication enzymes in the human population must be investigated to ensure protection of sensitive populations.