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The study and optimization of the Enzyme Inhibition Bioassay 96 for the detection of organophosphate and carbamate compounds

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THE STUDY AND OPTIMIZATION OF THE ENZYME INHIBITION BIOASSAY 96 FOR THE DETECTION OF ORGANOPHOSPHATE AND CARBAMATE COMPOUNDS

by

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ABSTRACT

The Study and Optimization of the Enzyme Inhibition Bioassay 96 For the Detection of Organophosphate and Carbamate Compounds

by

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The assessment of human exposure to hazardous compounds (such as pesticides) has been of great concern for decades. One method of reducing uncertainties in the assessment of human exposure is to better characterize the compounds that may be present in our immediate environment. A major limitation to this approach, however, has been the sampling and laboratory analysis of contaminated environmental and biological samples. Traditional sampling techniques can be slow and expensive, therefore, limiting the number of samples that may be analyzed within given time and budget constraints. Faster, simpler, and more cost-effective field screening tools can increase the amount of information available concerning the location, source, and/or concentration of pollutants present in the environment. The Enzyme Inhibition Bioassay 96 (EIB 96) is one such field-screening tool.

The EIB 96 is an assay that can detect the presence of organophosphate and carbamate compounds, providing scientists with valuable information to focus their future sampling efforts. The assay is based on the inhibition of the enzyme

acetylcholinesterase by organophosphate and carbamate compounds. The organophosphate and carbamate compounds that were used in this project were the pesticides carbaryl, chlorpyrifos, diazinon, dichlorvos, and parathion. The pesticides chlorpyrifos, diazinon, and parathion are organophosphorothioates, which are organophosphates with a P=S bond, and when these compounds are oxidized the compounds are converted to their oxygen analogs (P=O, substitution of a sulfur with an oxygen). Thus, this project was directed at determining the optimum oxidizing agent for use in the assay and then, to determine the oxidation products for the pesticide compounds used. The responses for the acetylcholinesterase in the presence of the oxidized and unoxidized pesticide compounds were measured using a V-max spectrophotometric kinetic plate reader. The analyses of the oxidation reactions were accomplished using an Agilent Technologies 6890 gas chromatograph/5973 mass-spectrometer (GC/MS).

The optimum oxidizing agent that was determined from this project was sodium hypochlorite (NaOCl), which oxidized the organophosphorothioates as well as bromine (Br_2). Bromine was the initial oxidizer choice but, because of the relative difficulty in obtaining Br_2 for use in the assay and its relatively higher cost as compared to NaOCl, NaOCl was determined to be the optimum choice. Also, the oxidative product for the model organophosphorothioates, parathion, was determined to be only paraoxon. Approximately 24±3% of the parathion was oxidized to paraoxon, and the non-organophosphorothioates, carbaryl and dichlorvos, were not damaged by the oxidation process using NaOCl and did not form any oxidation products.

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CHAPTER 1

INTRODUCTION

Pesticides have been used by humans to control myriad of nature's creatures for thousands of years. The term pesticide encompasses a group of chemical compounds that are used for the elimination of pests, and are grouped into classes based on their target organism, which include such groups as insecticides, fungicides, herbicides, rodenticides, and molluscicides (Britt, 2000). The earliest record of pesticide use was the Ebers Papyrus (circa 1550 BC), which lists preparations of pesticides to rid the home of fleas (Hayes, 1991). Homer (Greek poet) wrote of Greeks fumigating their domiciles with sulfur around 1000 BC (Ware, 1983), and in the *Odyssey* he wrote of how Odysseus burned sulfur "to purge the hall and the house and the court" (Hayes, 1991). Pliny the Elder's *Natural History* (circa 70 AD) has a summary of pest control practices extracted from the Greek literature of the preceding 200-300 years, although, most of the materials employed by the Romans were useless, based on superstition and folklore (Ware, 1983). The first successful use of chemicals to control pests came in the 19th Century. From 1800 to 1825 pyrethrums, lime and sulfur, arsenic, mercuric chloride, and soaps were used in and around homes and businesses (Ware, 1983). From 1826-1851 quassia, phosphorus paste, and rotenone were used (Ware, 1983). In Paris (1867-1868), the first scientific use of pesticides began with the use of arsenical Paris green and kerosene emulsions, which were used as a dormant spray for deciduous fruit trees (Ware, 1983).

The 20th Century saw a plethora of new pesticide compounds introduced into the arsenal of pest control with the intention of producing the chemical that would most closely resemble the ideal pesticide. Characteristics of the ideal pesticide as set forth by the 5th Edition of Truman's Scientific Guide to Pest Control Operations are that

> "...the pesticide will act rapidly on pests, yet be completely harmless to people, domestic animals, wildlife, and other aspects of the environment. Its residues would only last as long as was necessary to create the desired effect, usually for very short periods. It would also be inexpensive and readily available in necessary quantities, chemically stable (before application), non-flammable, and otherwise safe to use around homes or industrial sites. It would be easily prepared and applied, non-corrosive and non-staining, and it would have no undesirable odor" (Bennett et al., 1997).

However, no such synthetic pesticide exists, so any pesticide developed will be harmful, to some degree, to humans.

According to the US Environmental Protection Agency (US EPA) and the Pesticide Action Network (PAN) there are to date 2095 pesticides being used around the world (PAN, http://www.pesticideinfo.org). Out of the 2095 pesticides being used around the world 213 are organophosphates and 87 are carbamates, with 51 organophosphates and 16 carbamates registered in the US (PAN, http://www.pesticideinfo.org). Pesticide compounds are being used in a variety of formulations such as sprays, dusts, aerosols, and granulars (Ware, 1983). With so many of these chemicals (and none being the ideal pesticide previously mentioned) being used by commercial industries, agricultural communities, and consumers, concern as to their effects on humans and the environment has become an increasingly sensitive issue for the past few decades. Consequently, monitoring for the presence of pesticides in urban and agricultural centers has become necessary. There are a number of methods that are employed to monitor for the presence of pesticides; one of the most convenient and

inexpensive methods that is currently being employed is the use of biosensors. A biosensor (or bioassay) is a sensing device that incorporates a biological entity (enzyme, antibody, bacterial tissue, etc.) as a fundamental part of the sensing process (Diamond, 1998). Biosensors are tools that are particularly important for use in exposure research science since they can be used as a field screening method to increase the amount of information available concerning the location, source, and concentration of pollutants present in the environment (Rogers & Williams, 1995), which help researchers know where they should concentrate their efforts and their money.

In the United States (US) and around the world, a need has arisen for biosensors to specifically detect organophosphate and carbamate compounds in urban and agricultural environments. Organophosphate and carbamate compounds are two of the most widely used pesticide classes in the US. Organophosphates, alone, account for about half (by amount sold) of all pesticides used in the US (US EPA, http://www.epa.gov/pesticides/op/primer.htm). Approximately 60 million pounds of organophosphates are applied to approximately 60 million acres of U.S. agricultural crops annually with nonagricultural (urban) uses accounting for about 17 million pounds per year (US EPA, http://www.epa.gov/pesticides/op/primer.htm). Thus, the need to screen for potentially toxic amounts of organophosphates and carbamates in the environment has become increasingly important.

CHAPTER 2

REVIEW OF RELATED LITERATURE

Pesticide Regulations and Regulatory Levels

In 1947 the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) replaced the first United States federal pesticide act of 1910 (Briggs, 1992). FIFRA's primary purpose was to require the registration of pesticides to protect consumers from misbranding, adulteration, and ineffective pesticides (Trombley, 2000). In the early 1970's the US EPA assumed principal authority over pesticides, with some human health aspects under the Food and Drug Administration (FDA) and the Department of Human Health, Education, and Welfare (HEW) (Briggs, 1992). One important distinction between FIFRA and most of the other environmental protection laws is that it was originally a registration rather than reporting standard (Trombley, 2000). The main groups of people or businesses directly regulated by FIFRA fall into two basic categories: manufacturers and formulators. A manufacturer is a company that actually produces the chemical that is the active ingredient in a pesticide, and a formulator is a firm that mixes active ingredient(s) with various inert ingredients, such as diluents, carriers, propellants, etc., to create a product for an end user (Trombley, 2000).

The Food Quality Protection Act (FQPA) of 1996 amended the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food Drug, and Cosmetic Act (FFDCA), a law that is closely linked to FIFRA. These amendments

fundamentally changed the way EPA regulates pesticides. The requirements included a new safety standard-reasonable certainty of no harm-that must be applied to all pesticides used on foods (US EPA, http://www.epa.gov/oppfead1/fqpa). The US EPA, guided by the standards set forth by FIFRA, FFDCA, and the FQPA, has set regulatory levels for all pesticides used as well as any relevant information concerning pesticides including toxicological effects, environmental fate, and exposure guidelines.

Organophosphates

History and Background

The generic term organophosphate generally includes all of the pesticides that contain phosphorus (Ware, 2000). Other common names are: organic phosphates, phosphorus insecticides, nerve gas relatives, phosphates, phosphate insecticides, and phosphorus esters or phosphoric acid esters. The latter term is a more descriptive term since all organophosphate pesticides are derived from phosphoric acid esters (Ware, 2000).

The first preparations of phosphates synthesized in the field of organic chemistry were by Lassaigne in the early 19th Century to prepare phosphate esters (Eto, 1974). During the late 19th Century and the early 20th, the chemistry of organophosphorus compounds was developed extensively by Michaelis in Germany (Eto, 1974). Organophosphate pesticides for commercial value were developed from work on nerve agents, which was carried out during the Second World War by Saunders in Cambridge and Schrader in Germany (Alloway and Ayers, 1997). Before and during the Second World War Gerhard Schrader, of I. G. Farbenindustrie, was the key driving force behind

the development of organophosphates (Taylor, 1985). Schrader first synthesized organophosphates as agricultural insecticides, and later as potential chemical warfare agents (i.e. sarin, soman, and tabun) (Taylor, 1985). One of the first organophosphate insecticides that Schrader synthesized was parathion in 1944, which became one of the most widely employed insecticides of this class (Taylor, 1985). According to Timbrell (2000) organophosphorus pesticides are currently the most widely used anticholinesterase insecticides, and are applied to a number of habitats including agricultural crops, forests, wetlands, towns, and cities to control a variety of insects and other invertebrates, fungi, birds, mammals, and herbaceous plants (Hoffman et al., 1995). Organophosphorus pesticides were the first compounds of choice used to replace the more environmentally persistent organochlorine pesticides (Britt, 2000) since organophosphorus pesticides are more susceptible to biodegradation (Gallo and Lawryk, 1991).

General Structures

Organophosphates are usually esters, amides, or thiol derivatives of phosphoric acid (Woods, 1999). The general structure of organophosphates (those that have anticholinesterase properties) is represented by Figure 1. The R_1. and R_2. groups are difficult to displace (i.e. alkoxy, dialkylamino or alkyl) (Emsley and Hall, 1976), and the X is a fairly good leaving group placed in four main categories:

I. X contains a quaternary nitrogen (i.e. -S-CH_2-CH_2-N^+-$(CH_3)_3$)
II. X = F
III. X = CN, OCN, SCN, or a halogen other than F
IV. X = other moieties (i.e. –O-C_6H_4-CH_3) (Gallo & Lawryk, 1991)

The phosphate atom (P) of organophosphate pesticides is pentavalent and tetracoordinate (Mileson, et al., 1998). Three of the substituents are bound to the P by

single bonds, and the bond between the P and the fourth substituent is usually represented as a double bond (actually, a coordinate covalent bond) (Mileson, et al., 1998).

Typically organophosphates are divided into three groups and six subclasses. The three groups are aliphatic derivatives, phenyl derivatives, and heterocyclic derivatives (Ware, 2000). The six subclasses are phosphate, phosphonate, phosphorothioate, phosphorothiolate, phophrodithioate, and phosphoramidate (Ware, 2000).

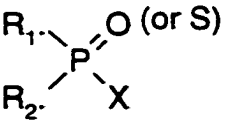


Figure 1. General structure of an organophosphate

The Groups

Aliphatic (literally "carbon chain") derivatives have a linear arrangement of carbon atoms (Ware 2000). Some examples of aliphatic organophosphates are tetraethyl pyrophosphate (TEPP), malathion (O,O-dimethyl-S-1,2-di(carboethoxy) ethyl phosphrodithioate), dichlorvos (O,O-dimethyl-O-2,2-dichloro-vinyl phosphate). TEPP was the first aliphatic organophosphate and was introduced into agricultural use in 1946 (Ware 2000). Malathion is the most heavily used aliphatic organophosphate, and dichlorvos (or DDVP) is an aliphatic organophosphate with strong fumigant properties (Ware, 2000).

Phenyl derivatives have a benzene ring attached to the phosphorus moiety of the organophosphate (Ware, 2000). This group of organophosphates is more stable and longer lasting than the aliphatic group. Some examples are parathion, which comes in two forms - ethyl (O,O-diethyl O-p-nitrophenyl phosphorothioate) and methyl (O,O-

dimethyl O-p-nitrophenyl phosphorothioate), crufomate (4-tert-butyl-2-chlorophenyl methyl-methylphosphoroamidate), and profenofos (O-[4-bromo-2-chlorophenyl]-O-ethyl-S-propyl phosphorothioate). Parathion is the most familiar of the phenyl organophosphates with the ethyl form introduced in 1947 and the methyl form introduced in 1949 for use in agriculture (Ware, 2000). Crufomate is a systemic insecticide used for the control of cattle grub, but is no longer available (Ware, 2000). Profenofos is a broad-spectrum insecticide used to control a variety of insects such as whitefly and pink bollworm (US EPA, http://www.epa.gov/oppsrrd1/op/profenofos/profbrief.htm).

Heterocyclic derivatives are those organophosphates that have ring structures composed of different or unlike atoms (i.e. carbons displaced by oxygen, nitrogen, or sulfur) with the ring having three, five, or six atoms (Ware, 2000). Some examples of these organophosphates are Diazinon (O,O-diethyl O-[2-isopropyl-4-methyl-6-pyrimidinyl] phophorothioate), Chlorpyrifos (O,O-diethyl O-[3,5,6-trichloro-2-pyridyl] phosphorothioate), Pirimiphos-methyl (O-[2-(diethylamino)-6-methyl-4-pyrimidinyl] O,O-dimethylphosphorothioate) (Ware, 2000). Diazinon is used for the control of insects in the home, lawn, garden, ornamentals, around pets, and for fly control in stables and pet quarters (Ware, 2000). Chlorpyrifos is the most frequently used pesticide of the organophosphorus class, and is used to control insects such as cockroaches around homes and in restaurants (Ware, 2000). Pirimiphos-methyl is a broad-sprectrum organophosphate used in agriculture for use in crops such as corn, sorghum, and wheat (Ware, 2000).

The Subclasses

The nuclei structures of the subclasses are shown in Figure 2. The nuclei structures are listed to give a better understanding of why organophosphorus compounds are given their unusual chemical names.

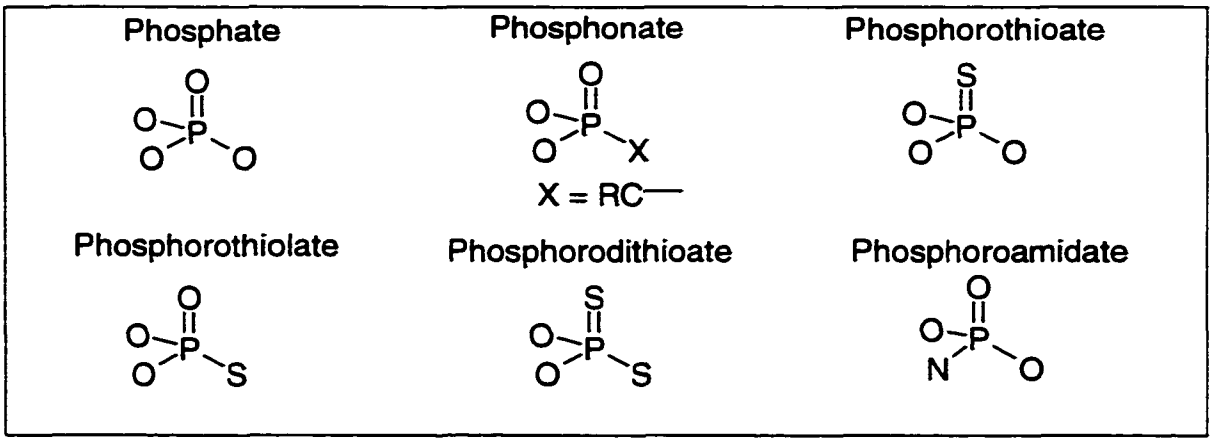


Figure 2. The six substructures of organophosphates. (Ware, 2000; and Scharpf, 1973)

General Exposures

Since the removal of organochlorine insecticides from use, organophosphate insecticides have become the most widely used insecticides available today, and all organophosphates run the risk of acute and subacute (repeated large or small doses or exposures over several hours and/or days to a toxic substance) toxicity (Reigert & Roberts, 1999). Chronic toxicities are not as common for organophosphates due to their high reactivity in the environment and thus, are less persistent in the environment. Individuals may be exposed to organophosphate compounds either occupationally, which may lead to cases of chronic exposure (e.g., from working in a pesticide formulating plant or from commercial pesticide application) or environmentally (e.g., from food

products such as fruits and vegetables treated for pests). Individuals may also be exposed to organophosphates at their residences (e.g., from use as home or garden pesticides) (Britt, 2000). Furthermore, organophosphates are used for therapeutic reasons such as in the treatment of myasthenia gravis and glaucoma (Britt, 2000).

Acute and Chronic Toxicities

Acute

Acute toxicity is an adverse or undesirable effect that is manifested within a relatively short time interval ranging from almost immediately to within several days following exposure (or dosing) (James, Roberts, and Williams, 2000). Organophosphate pesticides are cholinesterase inhibitors and among the most acutely toxic of all the pesticides in current use (Kamrin, 1997). Typically, the signs of acute exposure become noticeable when the normal activity of acetylcholinesterase (AChE) is reduced by about 50% (Kamrin, 1997). When inhaled, the first effects are usually respiratory and may include bloody or runny nose, coughing, chest discomfort, difficulty in breathing, and wheezing due to constriction or excess fluid in the bronchial tubes (Kamrin, 1997). Skin contact with organophosphates may cause localized sweating and involuntary muscle contractions (Kamrin, 1997). Following exposure by any route, other systemic effects may begin within a few minutes or be delayed up to 12 hours: these symptoms may include pallor, nausea, vomiting, diarrhea, abdominal cramps, headaches, dizziness, eye pain, and contraction or dilation of the pupils (Kamrin, 1997). Severe poisoning will affect the central nervous system and the peripheral nervous system (Kamrin, 1997). All of these symptoms may vary with the dose and the specific type of nerve cells that are

affected; generally, the acute toxic effects can be broken down into three broad categories: 1) effects on smooth muscles, including the heart and endocrine glands (muscarinic receptors), 2) effects on motor nerve endings in skeletal muscles and autonomic nervous system (nicotinic receptors), and 3) central nervous system effects (Timbrell, 1991). All of these symptoms may vary with the dose and the specific type of nerve cells that are affected (Kamrin, 1997). Information concerning acute toxicities for specific compounds can be found in Appendix A.

Chronic

Chronic toxicity is generally defined as repeated small doses over a long period of time, usually weeks, months, and/or years to a toxic substance. Repeated or prolonged exposure to some organophosphate compounds (e.g. leptophos and mipafox) may result in the same effects as acute exposure including some delayed symptoms (Kamrin, 1997) such as organophosphorus induced delayed neuropathy (OPIDN). OPIDN is a severe delayed distal axonopathy, which occurs when the degeneration of axons does not commence immediately after acute organophosphate exposure, but is delayed for 7 to 10 days (Hodgson and Levi, 1997). Generally, the toxic effects can be broken down into three broad categories: 1) effects on smooth muscles, including the heart and endocrine glands (muscarinic receptors), 2) effects on motor nerve endings in skeletal muscles and autonomic nervous system (nicotinic receptors), and 3) central nervous system effects (Timbrell, 1991). Information concerning chronic toxicities for specific compounds can be found in Appendix A.

Mechanism of Action and Toxicology

The main reason for the particular toxic effect of organophosphorus compounds is their cholinomimetic characteristic (mimicking the actions of acetylcholine), leading to the inhibition of acetylcholinesterase (Gallo and Lawryk, 1991). The inhibition of the enzyme by organophosphates will persist until hydrolysis of the phosphorylated enzyme occurs (Hodgson and Levi, 1997). The process by which acetylcholinesterase is inhibited by organophosphates is seen in Figure 3. The inhibition of the acetylcholinesterase leads to potentiation (an increase) or preservation of acetylcholine's activity within a synapse (Gallo and Lawryk, 1991). Acetylcholinesterase is a B-esterase, which is a family of esterases that becomes firmly, at times 'irreversibly', phosphorylated and, thus, inhibited in the central and peripheral nervous systems (Gallo and Lawryk, 1991). 'Irreversibly' is a term that delineates the rate of deacylation of the enzyme (breaking the phosphorus-enzyme bond requires an hour to >1000 hrs) (Gallo and Lawryk, 1991). If the enzyme does become irreversibly phosphorylated (which does happen with some organophosphate compounds such as paraoxon), then "aging" occurs. Aging refers to an additional reaction that stabilizes the phosphorylated enzyme (Hodgson and Levi, 1997), which occurs due to reactivation of the enzyme becoming progressively less efficient in proportion to the duration of inhibition (Gallo and Lawryk, 1991); basically, the rate of recovery (generally induced by reactivating agents) becomes less and less as the time of the inhibitor-enzyme contact becomes longer (Matsumura, 1985). Aging is also temperature and pH dependent, which is markedly accelerated at lower pHs and/or higher temperatures (Matsumura, 1985).

The inhibition of the enzyme prevents the enzyme from hydrolyzing acetylcholine (Gallo and Lawryk, 1991), and results in accumulation of endogenous acetylcholine within the nervous system and tissues (Arufe, Romero, Gamero, and Moreno, 2000). The build-up of acetylcholine leads to the failure of nervous transmission, convulsions, and eventually death (Alloway and Ayers 1997), due to a lack of brevity and unity of each normal propagated impulse (Gallo and Lawryk, 1991). Acetylcholine is the chemical mediator responsible for physiological transmission of nerve impulses from (a) preganglionic to postganglionic neurons of both the parasympathetic and sympathetic nervous systems, (b) postganglionic parasympathetic fibers to effector organs and postganglionic sympathetic fibers to sweat glands, (c) motor nerves to skeletal muscle, and (d) some nerve endings within the central nervous system (Gallo and Lawryk, 1991). The process by which the rapid destruction of acetylcholine by acetylcholinesterase takes place is seen in Figures 3 & 4.

Absorption, Distribution, Metabolism, and Excretion

Absorption

Organophosphates are absorbed by the skin (dermal absorption), respiratory tract (inhalation absorption), and gastrointestinal tract (oral absorption) (Gallo and Lawryk, 1991). Organophosphate compounds are mostly lipophilic compounds in order to facilitate penetration through insect chitin (Jokanovic, 2001), and since they are not ionized, they are absorbed rapidly following inhalation or ingestion (Vale, 1998). Human skin absorption is relatively slow; but, since the pesticides are difficult to remove from the surface of the skin, the process of absorption through the skin is prolonged (Gallo and

Lawryk, 1991). The degree of dermal absorption depends on the contact time with the skin, the lipophilicity of the agent involved, and the presence of solvents (e.g. xylene and

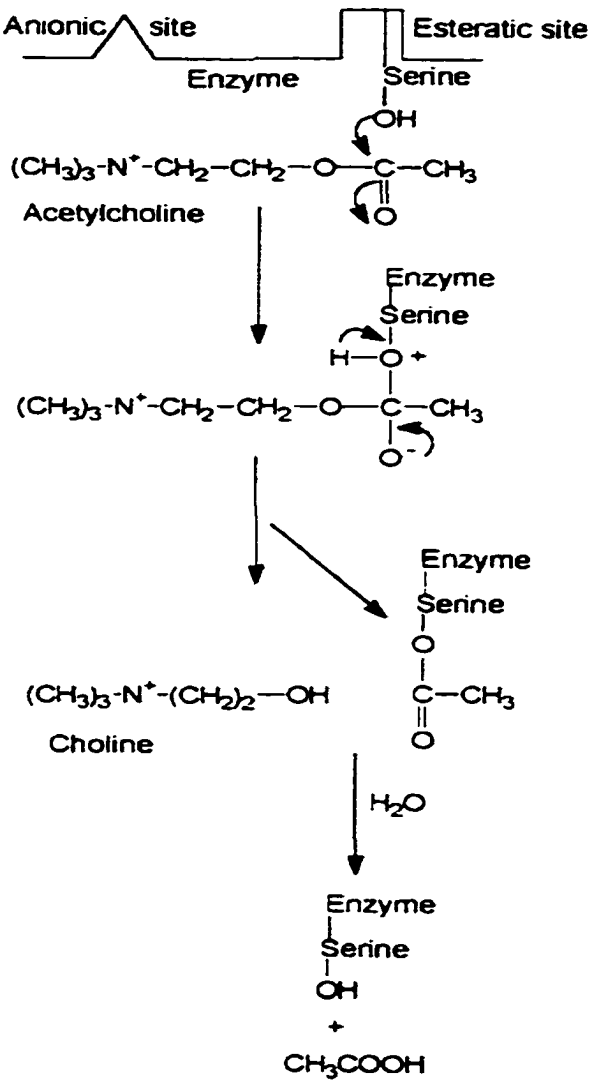


Figure 3. Interaction of acetylcholine and acetylcholinesterase. (Timbrell, 2000)

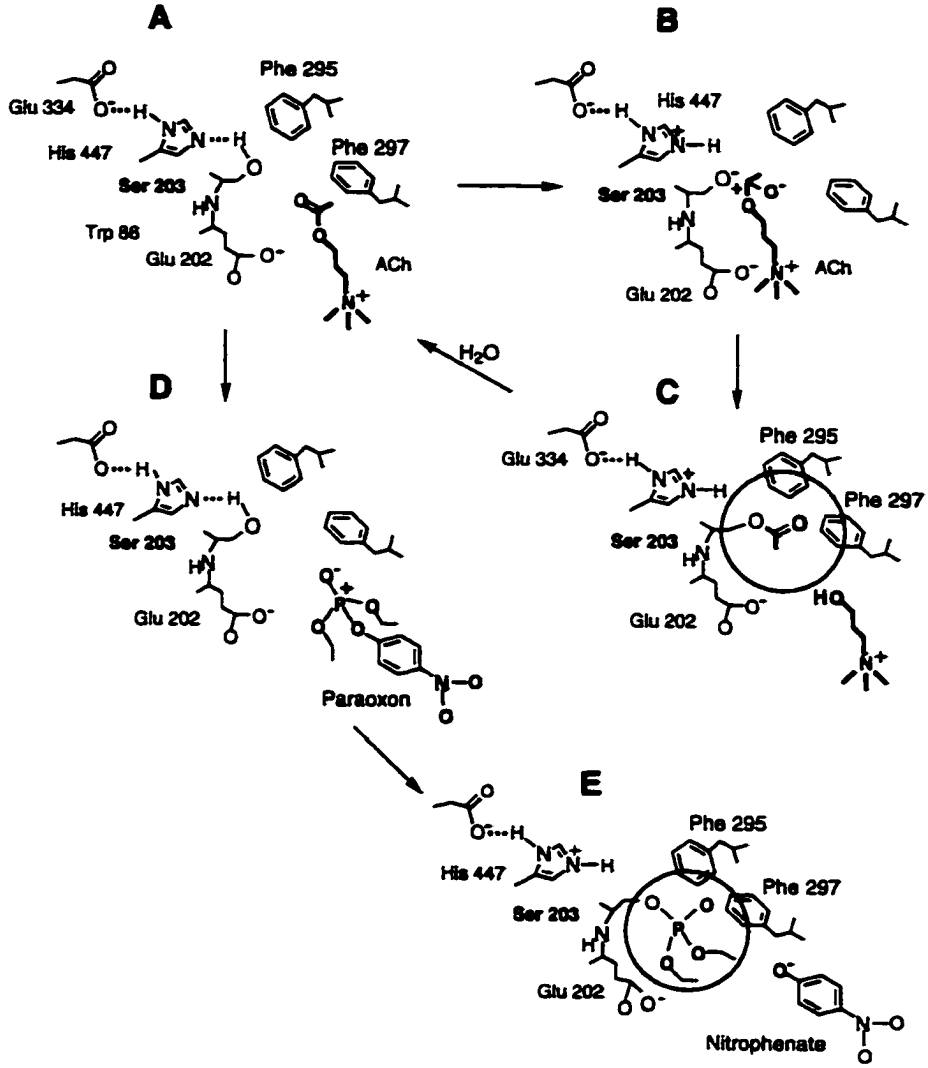


Figure 4. Mechanism of action of anticholinesterase pesticides. Vertical view of the active site of acetylcholinesterase as acetylcholine approaches it (A) and reacts with Serine 203 (Ser 203, B) to acetylate it (C), circled. The acetylserine is hydrolyzed to reactivate acetylcholinesterase (A). Paraoxon likewise can approach Ser 203 (D), but irreversibly phosphorylates it (E), circled (Crosby, 1998).

emulsifiers in the pesticide formulation can facilitate absorption) (Vale, 1998). Other factors include the volatility of the pesticide (e.g. dichlorvos is much more volatile than parathion), the permeability of clothing, the extent of coverage of the body surface, and personal hygiene (Vale, 1998). Also, absorption through the skin is increased at higher temperatures and humidity (Bonsall, 1985). Higher temperatures increase skin absorption by causing skin pores to open in relation to increases in body temperature, and cause dilation of blood vessels immediately beneath the skin, which then carry more blood, increasing the rate at which the pesticide is distributed (Bonsall, 1985). Humidity increases the skin absorption by hydrating the stratum corneum (a protective layer of hard dead cells on the surface of the skin), which makes the stratum corneum more permeable to materials that are soluble in water (Bonsall, 1985). Although most organophosphate compounds are lipophilic, some are less lipophilic (more soluble in water). Skin absorption also has the tendency to be greater in the presence of dermatitis (Hayes and Laws 1991), due to loss or damage of the stratum corneum. Although literature describing mammalian absorption of inhaled pesticide aerosols is limited (Ferguson, Jewell, Krieger, and Raabe, 1982), what is known is that absorption via the respiratory tract is relatively quick, and it is seen most often in cases of occupational exposure during either the manufacture or use of the organophosphate pesticides (Bonsall, 1985). Absorption via the gastrointestinal tract is also relatively quick where absorption occurs through smooth muscle, and is found most often in cases of ingestion of foods contaminated with organophosphates such as parathion (Timbrell, 2000).

Distribution

Organophosphate pesticides are distributed throughout the body (using parathion as the model compound) to the highest degree in the cervical brown fat and the salivary glands; to some degree in the liver, kidney, and ordinary adipose tissue; a fairly high degree in the gastric and intestinal walls, thyroid, spleen, and lungs; to a lower degree in the central nervous system, musculature, and bone marrow (Gallo and Lawryk, 1991). The phosphorothioates (e.g. diazinon and parathion) are more lipophilic than phosphates (i.e. dichlorvos), and are therefore stored extensively in fat, and since organophosphates are lipophilic, they readily cross the blood/brain barrier (Vale, 1998).

Metabolism (Biotransformation)

The biotransformation of organophosphates is rapid (Gallo and Lawryk, 1991). Both the routes and the rates of biotransformation are highly species specific, and dependent upon the substituent chemical groups attached to the basic backbone structure of the compounds (Klaassen and Watkins, 1999). Organophosphate pesticides undergo both Phase I and Phase II biotransformations via enzymes (microsomes, A-esterases, etc.) found within each phase (Klaassen and Watkins, 1999). In Phase I reactions, a polar group such as a *hydroxyl* (-OH), a *carboxyl* (-COOH), a *thiol* (-SH), and an *amino* ($-NH_2$) group, is introduced into the molecule through oxidation, reduction, and hydrolysis reactions (Jokanovic, 2001). Biotransformation products formed can be more toxic than the parent organophosphate compounds (i.e. parathion oxidized to paraoxon), but some less toxic biotransformation products are formed as well. In Phase II reactions, polar metabolites are conjugated with endogenous substrates such as glucuronides, sulfates,

acetates and amino acids, which form hydro-soluble products that can be readily excreted in urine (Jokanovic, 2001).

Organophosphate pesticides may undergo simultaneous enzymatic attacks at many different points in the molecule (Figure 5). However, the only reaction that significantly increases the toxicity of phosphorothioates is the oxidative desulfuration reaction (mechanism 1, Figure 5), which results in an oxygen analog of the original compound (Klaassen and Watkins, 1999), and even if the amount of metabolites formed in these reactions is low, it can be very significant from a toxicological standpoint (Jokanovic, 2001). Although the toxicity of the phosphorothioates increases with the production of the oxygen analog, the oxygen analog is readily hydrolyzed by aryl and aliphatic hydrolases found in mammalian tissues, but these hydrolases are deficient in insects (mechanism 8, Figure 5) (Klaassen and Watkins, 1999). This deficiency is the reason why insects are more susceptible than mammalian species such as humans to these types of compounds.

Excretion

Generally, organophosphate compounds (for measurement purposes) are almost entirely excreted in urine as hydrolysis products (Taylor, 1985). However, lesser amounts can be found in feces and expired air (Vale, 1998).

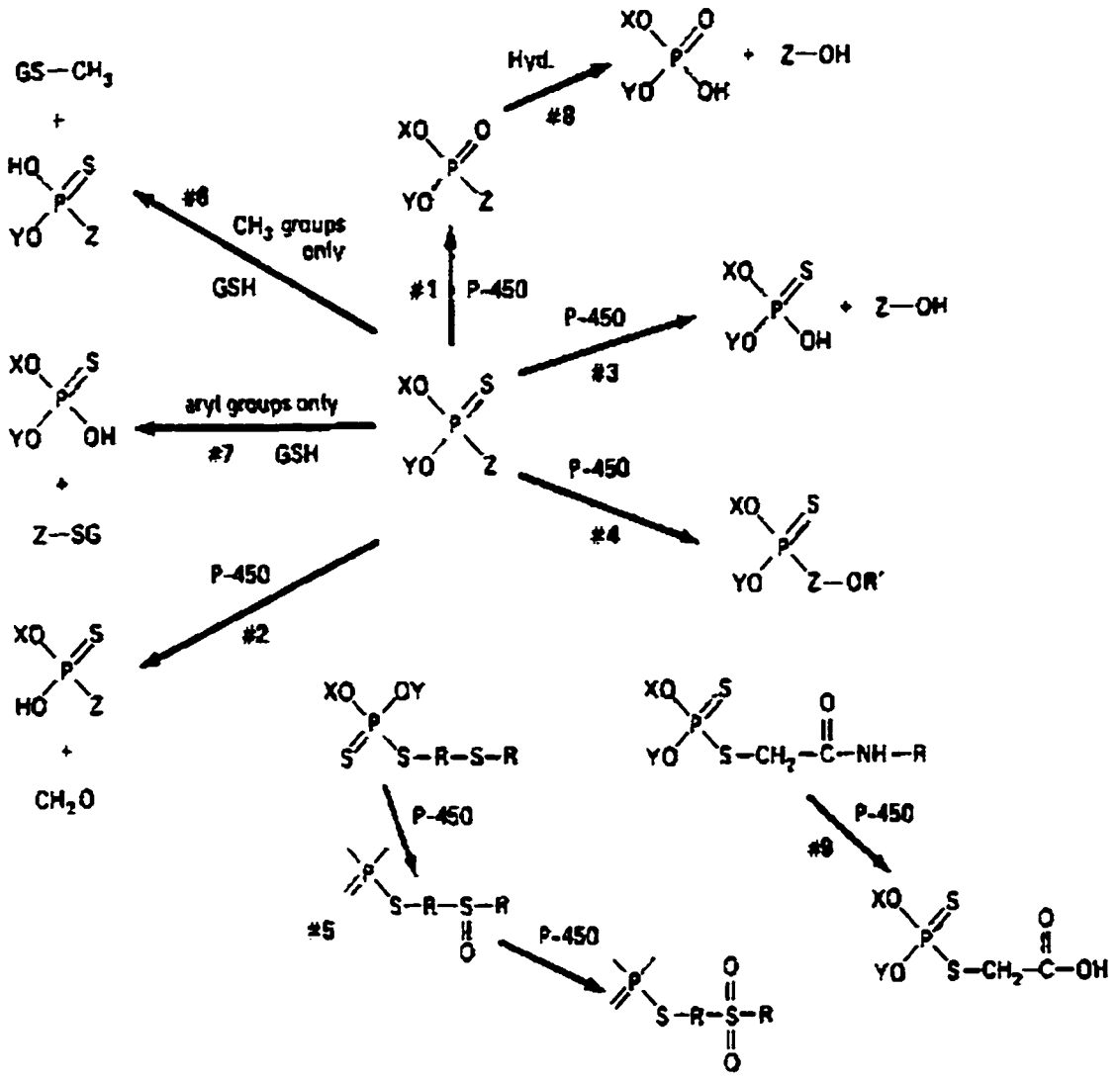


Figure 5. A schematic diagram depicting the various phase I and II biotransformation pathways of an organophosphorus ester and the nature of the products formed as a consequence of oxidative, hydrolytic, GSH-mediated transfer and conjugation of intermediate metabolites in mammals. (Klaassen and Watkins, 1999).

Carbamates

History and Background

The study of carbamates began with the discovery of physostigmine (Eserine), a natural carbamate, in Calabar, West Africa (Goodman and Gillman, 1985). The first use of Eserine was by native tribes of West Africa as a poison in witchcraft trials (Goodman and Gillman, 1985). It was brought to England in 1840, and was isolated as a pure alkaloid in 1864 by Jobst and Hesse (Goodman and Gillman, 1985). The first use of Eserine by Westerners was as a therapeutic drug for the treatment of glaucoma in 1877, one of its few clinical uses today (Goodman and Gillman, 1985). Although physostigmine is a potent AChE inhibitor, it is unsuitable as an insecticide since it is water soluble and therefore too polar to penetrate the insect cuticle (Perry, Yamamoto, Ishaaya, and Perry, 1998). In the late 1920's and early 1930's systematic investigations of a series of substituted phenyl esters of alkyl carbamic acids began with neostigmine, being the most promising member of the series, used therapeutically for the treatment of myasthenia gravis (Goodman and Gillman, 1985). In the 1950's, a series of heterocyclic, aromatic, and naphthyl carbamates was synthesized and found to have a high degree of selective toxicity against insects and to be potent anticholinesterase agents (Goodman and Gillman, 1985).

General Structure

Carbamate pesticides are derivatives of carbamic acid, which has the structure in Figure 6 (Ware, 2000). The carbamate esters that are derived have the common structure also shown in Figure 6, where the box identifies where attachment of an alcohol, oxime, or phenol would be (Baron, 1991). Most carbamates used as pesticides today are N-

methyl carbamates such as the pesticide carbaryl (1-naphthyl methylcarbamate) (Ware, 2000). Carbaryl is the first successful carbamate for pesticide use, introduced in 1956 (Ware, 2000), which is the first structure in subgroup 1 in Figure 7. There are two distinct qualities that have made Carbaryl a popular pesticide: (1) it has a very low oral and dermal mammalian toxicity and (2) it has a broad-spectrum of insect control (Ware 2000).

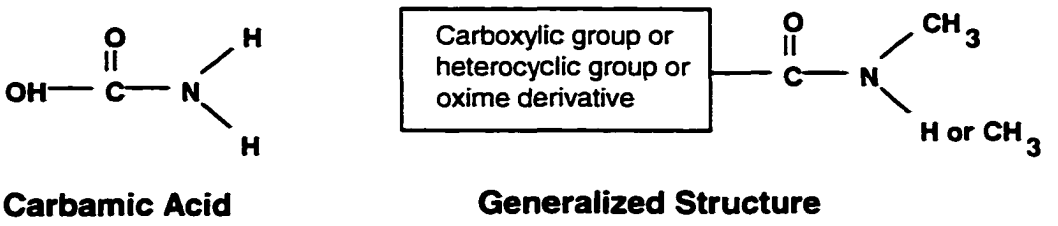


Figure 6. General structures of carbamates.

There are three general subgroups when identifying carbamate compounds for pesticide use (Figure 7). The three subgroups are as follows:

Subgroup 1. N-methyl carbamate esters of phenols, compounds with a hydroxyl group attached directly to a phenyl or naphthyl ring.

Subgroup 2. N-methyl- and N-dimethylcarbamate esters of heterocyclic phenols.

Subgroup 3. Oxime derivatives of aldehydes (hydroxylamine + carbamic acid)

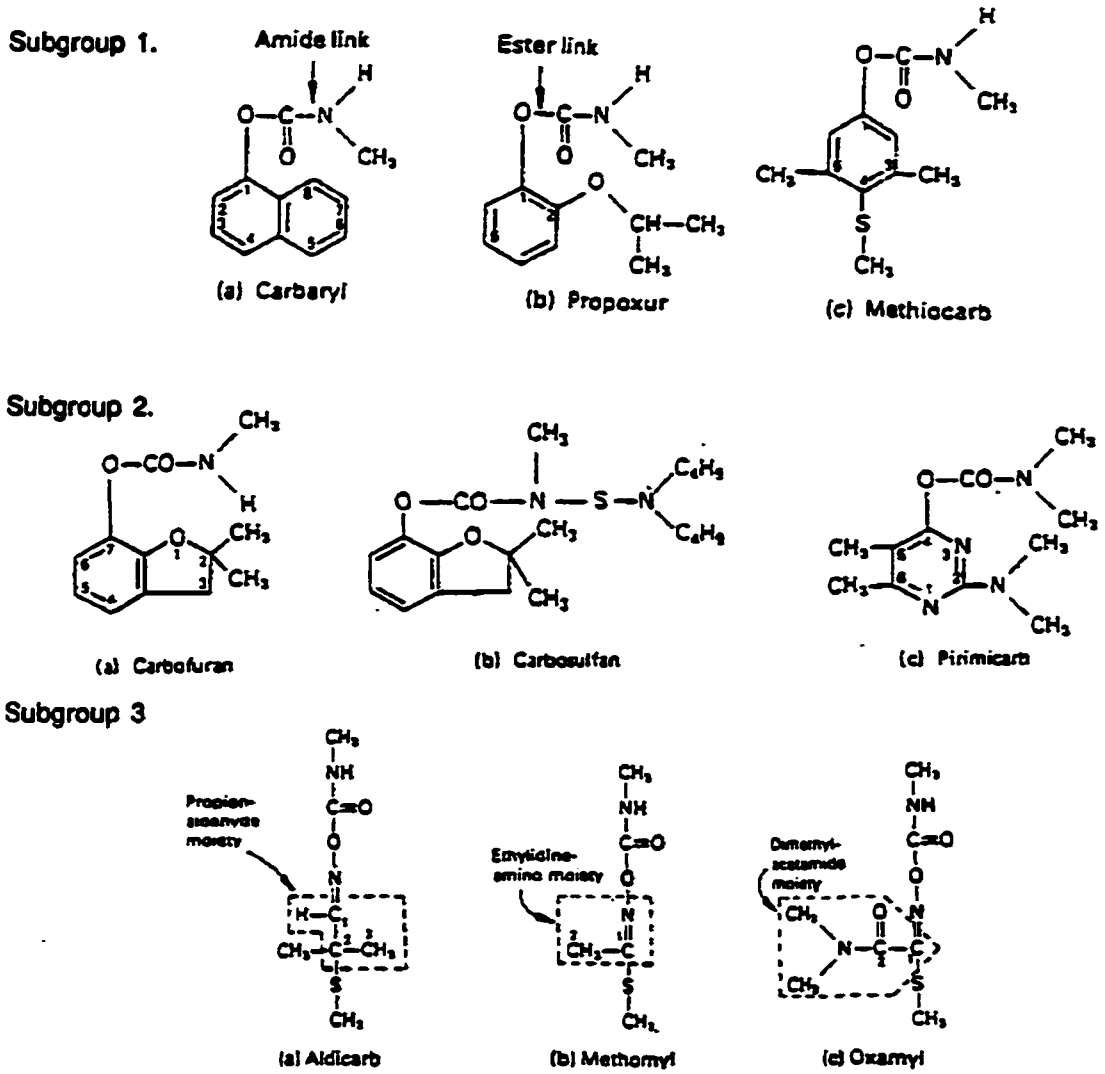


Figure 7. General subgroup structures (Perry, Yamamoto, Ishaaya, and Perry, 1998)

The structural diversity of the carbamate insecticides, as with the organophosphates, prevents broad generalizations regarding physical and chemical properties (Perry, Yamamoto, Ishaaya, and Perry, 1998). As a class, the carbamates are slightly to moderately soluble in water (10-500 ppm), moderately volatile, and readily biodegradable (Perry, Yamamoto, Ishaaya, and Perry, 1998). Because of the ester bonds, carbamates are susceptible to hydrolysis, but are more stable in neutral or slightly acidic

conditions but are readily hydrolyzed under alkaline conditions (Perry, Yamamoto, Ishaaya, and Perry, 1998).

Mechanism of Action and Toxicity

The mechanism of action and toxicity of carbamate pesticides (carbamates) is similar to that of the organophosphate pesticides. Carbamates are cholinomimetic, with the exception of the process of inhibition of acetylcholinesterase by carbamates; the process is rapidly and spontaneously 'reversible' rather than 'irreversible' as it is with the organophosphates. 'Reversible' is a term that refers to the rate of deacylation of the enzyme (Baron, 1991). The enzyme, after exposure to a carbamate compound, is reactivated in a matter of minutes rather than hours, days, or weeks (depending on the compound), which is observed in organophosphate exposure.

Absorption, Distribution, Metabolism, and Excretion

Absorption

Most carbamates are readily absorbed through the skin (dermal), respiratory tract (inhalation), and gastrointestinal tract (oral). The most important and likely human exposure route, for the general populace, is dermal, and the absorption rate is greatly influenced by the vehicle and environmental conditions (Baron, 1991). For example, the dermal absorption of methomyl is increased in conditions of high temperature and humidity (Baron, 1991), as with the organophosphates. Animal studies conducted with carbofuran indicated significant amounts were absorbed after oral exposure (Ferguson, et al., 1984) and aerosol inhalation (Ferguson et al., 1981). Carbamates are also readily absorbed through the gastrointestinal tract after exposure to residues in foods (Baron, 1991).

Distribution

Once absorbed, distribution of carbamates is rapid to the tissues and organs of the body (Baron, 1991). Oral absorption of carbofuran is characterized by distribution to the gastrointestinal tract, kidneys, lungs, and plasma (Ferguson et al., 1984), and the inhalation route is characterized by distribution to the head, trachea, lungs, and gastrointestinal tract (Ferguson et al., 1982). Tissues and organs that are responsible for xenobiotic metabolism tend to have the highest concentrations of carbamates (Baron, 1991). There is no evidence of bioaccumulation of carbamates since they are rapidly metabolized and eliminated (Baron, 1991).

Metabolism

The first step in carbamate metabolism is oxidation (N-demethylation, aromatic ring hydroxylation, O-dealkylation, alkyl hydroxylation, and sulfoxidation) that provides a site for a conjugation reaction (O- and N-glucuronides, sulfates, and mercapturic acid derivatives) yielding water-soluble products for excretion (Baron, 1991). Oxidative reactions can be classified into two groups:

1) Ring hydroxylation that may sometimes further oxidize to ketones or epoxidation followed by hydrolysis to the corresponding diol (Perry, Yamamoto, Ishaaya, and Perry, 1998).
2) Oxidation of side-chains. The side chain oxidations can take on many forms, and various aliphatic ring substitutions can be hydroxylated. Methyl groups can become hydroxymethyls and isopropyl moieties can become 1-hydroxyisopropyl groups. *N*-demethylation is extremely important for carbamates, since every carbamate posses an *N*-alkyl group. N-

hydroxymethylation may take place by stepwise reactions, but some carbamate molecules possess thioethers in side chains that can be oxidized to sulfoxides and sulfones (Perry, Yamamoto, Ishaaya, and Perry, 1998).

Regardless of the oxidative metabolic activities for carbamate insecticides, they can cause a variety of changes that will influence their toxicity and residual characteristics. Oxidized biotransformation products can be either more toxic or less toxic than the parent compound, but they usually do not become completely nontoxic. Generally, oxidation results in decreased stability of the molecule and provides sites for attack by conjugative enzymes so that although oxidation can result in activation, the net result is to increase overall levels of detoxification; however, whether there is activation or detoxification can be judged only by the stability, availability, and frequency of appearance of each metabolite in the animal (Perry, Yamamoto, Ishaaya, and Perry, 1998).

There are also two esterase-mediated routes of carbamate metabolism that have been shown: 1) esterases attack the bond on the side of the carbonyl group attached to the oxygen and 2) amidases attack the bond on the side attached to the nitrogen atom (Perry, Yamamoto, Ishaaya, and Perry, 1998). Esterase-catalyzed hydrolysis is probably less important for carbamates relative to oxidative metabolism, although the extent to which hydrolysis occurs depends on both the type of carbamate and organism (Perry, Yamamoto, Ishaaya, and Perry, 1998). For example, the oxidative biotransformation of carbaryl (Figure 8) occurs rapidly in a number of mammalian species but varies widely.

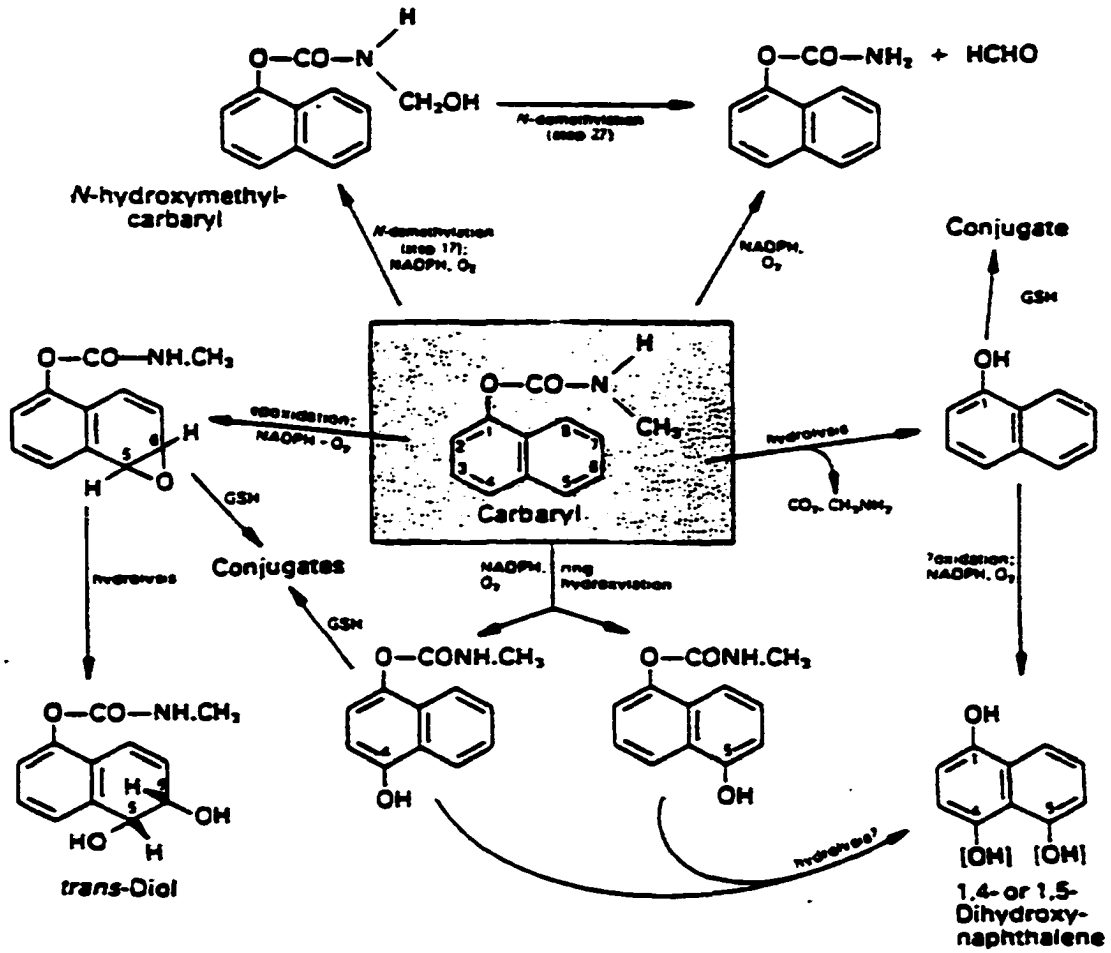


Figure 8. Metabolism of carbaryl by plants and animals.

Excretion

Excretion of carbamates is similar to that of organophosphates, which (for measurement purposes) is through the release of metabolites found in urine (Gallo and Lawryk, 1991), but may also be found in feces and expired air (IPCS, http://www.inchem.org/documents/ehc/ehc/ehc64.htm#SectionNumber:6.3).

Enzyme Inhibition Kinetics

The active site of acetylcholinesterase contains two sites of ligand binding, an acylation site and a peripheral site (Rosenberry, Mallender, Thomas, and Szegletes, 1999). If the enzyme (E) reacts with an inhibitor (AX) in an initially reversible manner, the reaction can be represented by

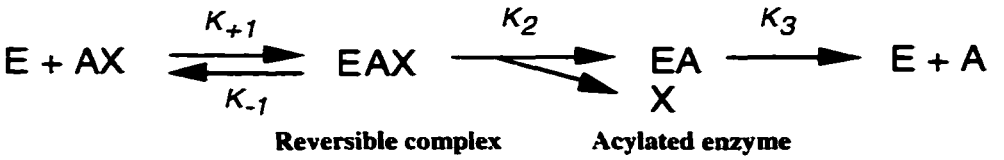


Figure 9. Representation of the enzyme-inhibitor complex.

The equation in Figure 9 is very similar to the traditional AChE catalytic pathway (Figure 10), which shows the initial enzyme-substrate complex ES (EAX, Figure 9) proceeding to an acylated enzyme intermediate EA (EAX, Figure 9) and then hydrolyzed to products P and E (E + A, Figure 9) (Rosenberry, Mallender, Thomas, and Szegletes, 1999).

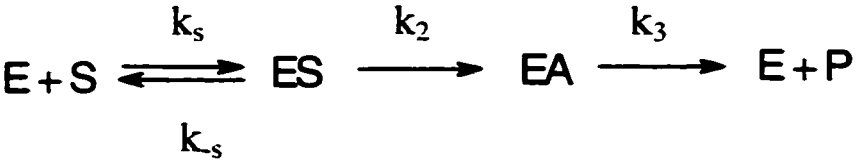


Figure 10. Traditional AChE catalytic pathway

In the equation (Figure 9), EAX represents the enzyme-inhibitor complex, X is the leaving group, A is that part of the inhibitor molecule that eventually blocks the enzyme, and EA is the inhibited enzyme or acylated enzyme intermediate (Perry,

Yamamoto, Ishaaya, and Perry, 1998). The affinity with which the substrate binds to the enzyme in the first step is often described by the dissociation constant.

$$K_d = k_{-1}/k_{+1} = \frac{[E][AX]}{[EAX]}$$

Figure 11. Equation describing the dissociation constant for the substrate-enzyme binding.

The smaller K_d is, the greater the affinity of the enzyme for the inhibitor (Perry, Yamamoto, Ishaaya, and Perry, 1998). In Figure 9, the rate constant k_2 defines the acylation step, and k_3 defines the rate of enzyme recovery (Perry, Yamamoto, Ishaaya, and Perry, 1998). The rate at which the enzyme is inhibited, or the rate at which EA is formed, is defined by two different processes: 1) affinity of the enzyme for the inhibitor or K_d; and 2) the rate at which the enzyme becomes acylated or k_2 (Perry, Yamamoto, Ishaaya, and Perry, 1998). A measure that takes into account the contributions of both K_d and k_2 is the inhibition constant, or bimolecular rate constant, K_i. This constant is defined as $K_i = K_2 / k_D$.

The bimolecular rate constant is particularly useful for comparing the inhibition potency of various compounds (i.e. comparing the sensitivity of AChE) to inhibition between resistant and susceptible strains of insects (Perry, Yamamoto, Ishaaya, and Perry, 1998). The inhibition constant (K_i) is also an accurate measure for predicting specific inhibitor activity in humans from animal data; specifically, it is useful for the extrapolation of rodent blood cholinesterase-anticholinesterase interactions to cholinesterase-anticholinesterase interactions in humans (Rao, Roberts, Pope, and Ferguson, 1994).

Differences between carbamates and organophosphates

Both carbamate and organophosphate pesticides produce toxic effects by inhibiting AChE (Perry, Yamamoto, Ishaaya, and Perry, 1998). Inhibition of AChE results from the reaction of an organophosphate pesticide with the esteratic site of AChE (phosphorylation of AChE) (Gallo and Lawryk, 1991). Inhibition results from the formation of a carbamate-AChE complex (carbamylation of AChE) (Baron, 1991). The differences between the action of organophosphate and carbamate pesticides arise from differences in the relative magnitudes of the rate constants associated with the overall reaction (Perry, Yamamoto, Ishaaya, and Perry, 1998).

With carbamates, the two rate constants of interest concern the processes of carbamylation (k_2) and reactivation (k_3) (Figure 9) (Perry, Yamamoto, Ishaaya, and Perry, 1998). In contrast to organophosphates, the rate constant for carbamylation is rather low, so that the complex, EAX (in which the enzyme and carbamate are physically aligned but not chemically bound), tends to accumulate (Perry, Yamamoto, Ishaaya, and Perry, 1998). Consequently, the reverse reaction governed by k_{-1} (Figure 9) becomes important (Perry, Yamamoto, Ishaaya, and Perry, 1998).

For organophosphates, this reaction is usually negligible since the concentration of the reactant is low (Perry, Yamamoto, Ishaaya, and Perry, 1998). The second key difference between organophosphates and carbamates is governed by the rate constant k_3 (Figure 9) (Perry, Yamamoto, Ishaaya, and Perry, 1998). The rate of hydrolysis of phosphorylated cholinesterase is defined by the rate constant k_3 (Perry, Yamamoto, Ishaaya, and Perry, 1998). For organophosphates, the recovery of active enzyme occurs much more slowly than the corresponding reaction with carbamates, breaking the

phosphorus-enzyme bond requires an hour to >1000 hrs (Gallo and Lawryk, 1991). Therefore, decarbamylation is rapid when compared with dephosphorylation, and it provides a second route for the functional free enzyme to be regenerated (Perry, Yamamoto, Ishaaya, and Perry, 1998).

The Bioassay

The bioassay that is the subject of this paper, the Enzyme Inhibition Bioassay 96 (EIB 96) (a basic diagram of the assay steps can be found in Appendix B), has been developed as a substitute for current bioassays such as Neogen's Agri-Screen Test Kits. The Neogen Agri-Screen Test Kit is a colorimetric-type assay that is used to identify whether there are potentially toxic amounts of organophosphates or carbamates in an area of concern (e.g. a kitchen countertop in a home). The kit contains ampules of reagent solutions and vials that provide the necessary components to screen for organophosphates and carbamates. These type of kits are expensive and less effective for screening organophosphates and carbamates at lower concentrations, which still may pose a threat to human health. The bioassays that have been tested in the past generally employed amperometric or fluorescent type biosensing elements to detect loss of activity of acetylcholinesterase, which have required expensive, messy, and time-consuming materials and methods to detect the activity loss. However, the EIB 96 has been developed in an attempt to minimize the use of expensive materials and to utilize relatively rapid methods for detecting the loss of acetylcholinesterase activity.

CHAPTER 3

EXPERIMENTAL METHODOLOGY

Introduction

The current anti-cholinesterase bioassay (EIB 96) has been developed to screen for the presence of organophosphate and carbamate compounds. It is not intended to be used as a tool to identify or quantify specific compounds. However, it is useful as a tool to indicate the presence of compounds that cause inhibition (or lack of activity) of acetylcholinesterase (AChE). The assay can also be used to determine whether a sample poses a potential risk to humans via enzyme inhibition, and warrants further study. The inhibition of the enzyme is determined based on IC_{50} (a measure of the inhibition of 50% of the enzyme) values. Similar to LD_{50} values, which are unique to the test animals, IC_{50} values are unique to an assay utilizing an enzyme as the biocomponent. There is, however, a relationship between the IC_{50} and the LD_{50} (lethal dose of a compound that kills 50% of an organism, usually rats) values. The compounds used in this project with their respective IC_{50} and LD_{50} values are listed in Table 1. The IC_{50} values and the LD_{50} values of the organophosphate and carbamate compounds are related by their potency to AChE. It is observed in Table 1 that as the IC_{50} values increase, the LD_{50} values increase and vice versa.

The protocol for the EIB 96 utilizes an oxidation step to produce the primary metabolites of the parent phosphorothioate compounds. The phosphorothioate

compounds that have been tested in the current assay are diazinon and parathion. It has been previously shown that the oxidation of these compounds result in a marked improvement (increased inhibition and reduced IC_{50} values) in inhibition of acetylcholinesterase. One of the questions that was addressed in this project was whether the improvement in inhibition was due to chemical reactivity of other compounds within the assay or due to the actual production of oxon (P=O bonding) metabolites from the parent organophosphorothioates. It was further determined how much metabolite was being produced by oxidation of the parent compound. Consequently, it was necessary to explore the chemical reactions that were taking place as a result of the oxidation protocol.

Table 1. Comparisons of IC_{50} values from the EIB 96 and rat LD_{50} values for the five compounds used in this project and two of the metabolites.

Anticholinesterase Compound	*IC_{50} (nM)*	*Oral LD_{50} (mg/kg, Rats)*
Parathion (oxon)	689 (0.15)	2-30
Dichlorvos	0.26	61-175
Carbaryl	8.7	250-850
Diazinon (oxon)	760 (140)	300-400

Experimental Approach

It is important to oxidize the organophosphorothioates because oxidation generally improves, as previously mentioned, the response of the assay to these compounds. The characterization of the chemical reactions resulting from the oxidation step of this assay was established by (1) optimizing the oxidation step (as measured by enzyme inhibition) to determine the most efficient, effective, and inexpensive oxidizer using parathion as the model compound; and (2) to analyze the oxidation products using

Gas Chromatography/Mass Spectroscopy (GC/MS) to ascertain what products are produced from the parathion reactions, and if necessary the dichlorvos and carbaryl reactions as well. The following experimental strategy was performed and specific details regarding the methodology will follow:

- Characterize the effects of the oxidizing agents bromine (Br_2), sodium hypochlorite (NaOCl), ferric chloride ($FeCl_3$), and rat liver microsomes on the performance of the EIB 96.
- Determine the primary oxidation product(s) from the oxidation of parathion, and if necessary, determine the primary oxidation products from the dichlorvos and carbaryl reactions.
- Quantitate the percent conversion of parathion to its oxidation product(s).
- Determine whether the oxidation of the assay will damage the non-phosphorothioate compounds' ability to inhibit acetylcholinesterase.

Characterizing the Effects of the Oxidizing Agents

Bromine (Br_2) activation is simple, effective, and efficient for converting phosphorothioates to their oxygen analogs (Barber et al., 1999). The oxidation of phosphorothioates (those organophosphates that have a P=S bond) is necessary since they are not as effective as their oxygen analogs (P=O) in the inhibition of acetylcholinesterase. Although Br_2 has been shown to be a good choice to oxidize the phosphorothioates, Br_2 effectiveness may be reduced in solutions that contain co-contaminants since it is a broad-spectrum oxidizing agent. Evidence strongly suggests that it will oxidize numerous compounds in addition to the target organophosphorothioate compounds. Previous experiments in the laboratory of Dr. Kim Rogers (Chemist and

Branch Chief for the Human Exposure Research Branch [HERB], US EPA, Las Vegas, Nevada) have also provided some evidence that supports the theory that Br_2 may disrupt and/or damage non-phosphorothioate organophosphate compounds and carbamate compounds that do not require oxidative activation (those compounds with a P=O bond). Thus, Br_2 may interfere with these compounds' ability to inhibit the target enzyme (AChE).

Sodium hypochlorite (NaOCl) has a reduction potential that is less than Br_2, but because its reduction potential is still relatively large, it was tested as possible oxidizer. Also, NaOCl is safer, and far less distressing for most people to use than is Br_2. Its actual effectiveness as an oxidizer for this assay was explored.

Ferric Chloride ($FeCl_3$) was chosen as a candidate because it may be safer to use than Br_2 due to ferric chloride's chemical characteristics (i.e. slight reactivity vs. the highly reactive Br_2). Ferric chloride's actual organophosphorothioate oxidizing effectiveness was explored.

The reduction potentials for the Br_2, NaOCl, and $FeCl_3$ are 1.0873 E^o/V, 0.81 E^o/V, and –0.037 E^o/V, respectively (Vanysek, 2001). The reduction potentials are standard reduction potentials with E^o values at 298.15 K (25.0 °C), and at a pressure of 101.325 kPa (1 atm) (Vanysek, 2001).

Rat liver microsomes (RLMs) were chosen as an oxidation candidate since they may be the most relevant oxidizer; RLM activation more closely mimics the biotransformation that would occur in the living animal (Barber et al., 1999). In the living animal RLMs are part of an important oxidation system, the cytochrome P450 mono-oxygenase system. The cytochrome P450 (CYP P450) system is the most

important of the mono-oxygenases, also called hydroxylases or mixed-function oxidases (MFOs); so called due to the nature of these systems to split atmospheric dioxygen that donates one O atom to a substrate and the other to water (Figure 12) (Crosby, 1999). The CYP P450 monooxygenase system is widely distributed throughout nature. In humans, it is present in most organs (in membranes of the endoplasmic reticulum - microsomal fraction of the cell, mitochondria, and nucleus), but the highest concentrations are found in the hepatic system (liver) (Bhavagan, 2002), and consist of a broad group of iron-containing isozymes (there are hundreds of isozymes) (Crosby, 1999). The CYP P450 system is part of family of P450 cytochromes that absorb light maximally at 450 nm when complexed in vitro with exogenous CO (Stryer, 1995). The isozymes in the CYP P450 system vary in their protein portion and substrate specificity, but utilize identical oxidizing systems. The desulfuration of phosphorothioates to phosphates is part of the many biochemical oxidations that are catalyzed by this system (Crosby, 1999). The chemical mechanism of CYP P450 oxidations is given in Figure 13. The hepatic microsomal cytochrome P450 (HM CYP P450) system is very well known, and it is from the system that the rat liver microsomes used in this project were obtained. The HM CYP P450 consists of a flavin reductase (NADPH-cytochrome P450 reductase) and one of at least six molecular species of cytochrome P450 (Bhagavan, 2002). The overall reaction begins by conjoining the substrate (XH) with the ferric form of the P450 to produce the ferrous form by accepting electrons from NADPH-cytochrome P450 reductase (steps a and b, Figure 14), and the reductase contains one molecule of FNM and one molecule of FAD (Bhagavan, 2002). The reduced P450 substrate complex binds molecular oxygen, which becomes activated upon acceptance of an electron form the

heme iron (steps c and d, Figure 14), followed by the substrate-oxygen complex accepting a second electron from either NADPH-cytochrome P450 reductase (step e, Figure 14) or cytochrome b_5 (step f, Figure 14). Finally, one oxygen atom accepts two protons to form a water molecule, and the other oxygen forms the hydroxyl group of the substrate (steps g-j, Figure 14), with the regenerated ferric form of P450 initiating the next cycle (Bhagavan, 2002).

$$R - H + O - O + H^+ \text{ NADPH} \rightarrow R - OH + NADP^+$$

Figure 12. The splitting of atmospheric dioxygen.

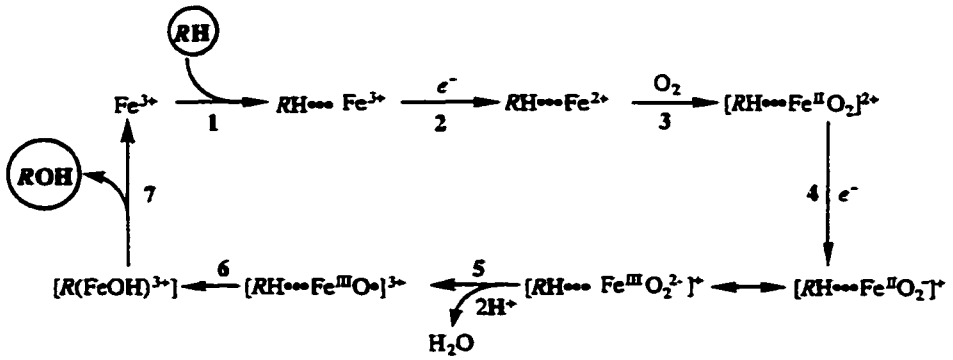


Figure 13. The chemical mechanism of P450 oxidations.

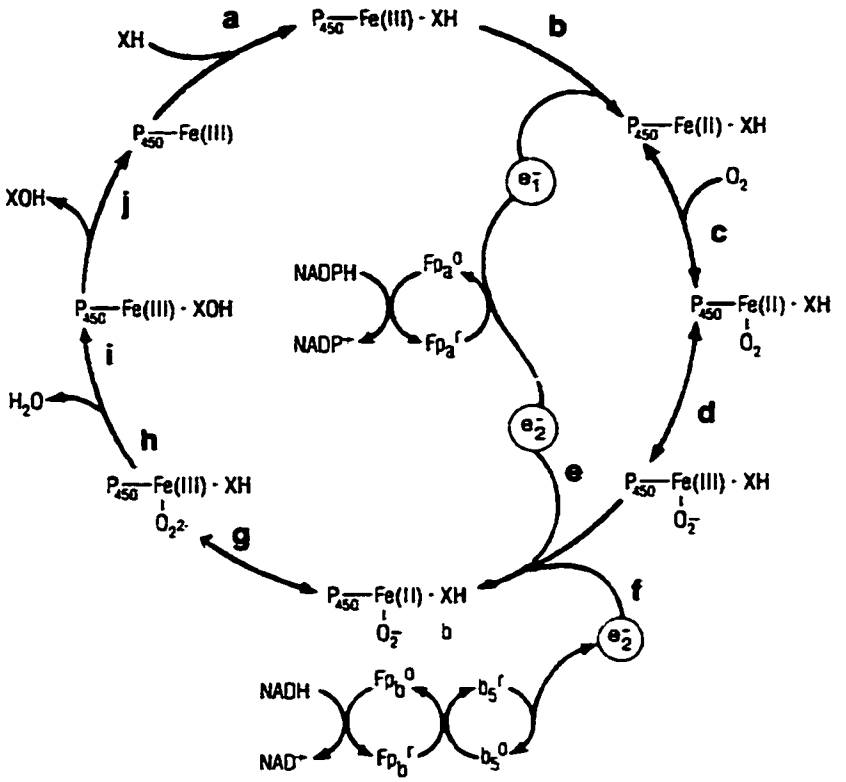


Figure 14. Cytochrome P450 monooxygenase system in the hepatic endoplasmic reticulum. P450-Fe(III) = ferricytochrome P450; P450-Fe(II) = ferrocytochrome P450; b_5 = ctyochrome b_5; Fp_a = NADPH-cytochrome P450 reductase; Fp_b = NADH-cytochrome b_5 reductase; XH = substrate. (Bhagavan, 2002)

Although RLMs closely mimic the biotransformation that would occur in vivo and may be safer to use than Br_2 due to their chemical and molecular make-up, RLM activation is more time-consuming and difficult to use as compared with Br_2 (Barber et al., 1999). But, the oxidizing effectiveness of the above compounds by RLMs was explored in this project.

The oxidation step was optimized by establishing the most efficient and effective oxidizing agent by the following experimental approach:

1. Each oxidizing agent was tested with each of the four pesticide compounds.

- Br_2 had a stock solution of 1.0% in solution with nanopure water, and the concentration was reduced through 1:10 serial dilutions to determine the effective concentration for the oxidation of the organophosphorothioates within the assay.
- Sodium hypochlorite concentrations were produced in the same manner as bromine.
- $FeCl_3$ had a stock solution of 1uM in solution with nanopure water and was reduced through 1:10 serial dilutions until the optimal concentration was found.
- Rat liver microsomes had a concentration of 1mg in solution with nanopure water and its concentration was reduced and increased by ½ until the optimal concentration was found.
    - Rat liver microsomes were mixed in 0.1 M phosphate buffer (pH 7.4) with the cofactors NADPH and EDTA at concentration levels of 1mM and 3mM, respectively (Barber, Correll, and Ehrich, 1999), in 1.5mL vials.
        - The first two incubations using parathion as the model compound were 20 min; this time was increased at 10 min and 30 min increments until the best incubation time was established.
        - Each incubation was performed in an oven at 37°C for each of the time periods described above (See Appendix II for details of this oxidation procedure).

2. A comparison of the data obtained from all of the trials was done to determine the optimal oxidizer from the group (primarily examining which oxidizing agent generates the most effective AChE inhibitor [Total AChE Inhibition]); this was done by examining the data qualitatively (i.e. examining the IC_{50} values from the graphs).
3. Oxidations with dichlorvos and carbaryl were performed with the oxidizing agent determined to be the most efficient, effective, and inexpensive. This was done to determine the effect the oxidizing agent would have on the non-phosphorothioate organophosphate and carbamate compounds that were used in this study. The IC_{50} values for dichlorvos and carbaryl were used as references and then compared to results from previous experiments.

The compounds that are to be used were studied for three primary reasons. First, the compounds were studied to determine how the oxidation step would affect the IC_{50} values of the compounds. Second, to determine the biotransformation products and quantities from the oxidation of the phosphorothioate compounds within the assay, and third, to determine the effect the oxidations have on non-phosphorothioate organophosphate and carbamate compounds, and if necessary to determine the biotransformation products and quantities from the oxidations of the non-phosphorothioate compounds.

Determining the Primary Biotransformation Products

The analysis of the biotransformation products from the oxidations were accomplished by the following experimental approach:

Compound standards for carbaryl, chlorpyrifos, diazinon, dichlorvos, and parathion were analyzed by the GC/MS to ensure that the compounds would be correctly identified by the instrument (Appendix III).

- The standards were mixed in HPLC-grade acetone and analyzed using GC/MS.
    - The standards were placed in GC/MS vials and sealed with a septum.
    - The concentrations of the standards began at 1mM and were serially diluted until the concentration most suited for use in the GC/MS was determined.
    - The temperature of the column was programmed from 60 to 300°C at 12°C/min, and a mass range of m/z 50 to 500 was scanned at ca 0.1 Hz

Quantitation of the Parathion Product

A calibration curve was developed to quantitate the metabolite production from the oxidation of parathion (an organophosphorothioate), which was used as the model compound for the phosphorothioates in this project.

A series of five solution mixtures was produced.

- The solutions mixtures contained parathion, paraoxon, and 4-bromophenyl ether (the internal standard) mixed in acetone.
- The parathion and paraoxon concentrations were varied, depending upon which concentrations were determined to be most suited for use in the calibration curve, and the

concentration of 4-bromophenyl ether was held constant in each solution mixer (i.e. the concentration of 4-bromophenyl ether was held at 2mM in each solution mixture).

- The reactions took place in a phosphate buffer system.
    - The oxidations were performed in a phosphate buffer system, at pH 7.4 and at room temperature, following incubation periods established by the initial oxidation protocol.
    - The reaction mixtures were then split into two samples. The first sample was used in the plate as established by the initial oxidation protocol, and the second sample was placed in a hood, under a nitrogen (N_2) blown-down system for drying to completeness.
        - After the second sample was dried, it was resolubilized in HPLC-grade acetone and analyzed by the GC/MS.

Determining the Effects of Oxidation on the Non-Phosphorothioate Compounds

Oxidations of dichlorvos and carbaryl were accomplished in the presence of the established oxidizing agent for the assay, NaOCl, to determine the amount of biotransformation product, if any, from the oxidations.

- The initial reactions were placed in conditions established by the organophosphate oxidation protocol in Appendix II.
- Any reactions for use in the GC/MS took place in a phosphate buffer system as stated in the above oxidation protocol.

Hypotheses

Given the above experimental approach the hypotheses for this study are as follows:

- Once optimized, the EIB 96 will be an effective and efficient method for screening for the presence of organophosphate and carbamate compounds. Further, this method will be faster, cleaner, and less costly than the current commercially available counterparts (e.g. Neogen's Agri-Screen Test Kits).
- Bromine (Br_2) will be the most efficient and most effective oxidizer of the organophosphorothioate compounds compared to sodium hypochlorite (NaOCl), rat liver microsomes (RLMs), and ferric chloride ($FeCl_3$).
- The oxidations of parathion will only produce the primary biotransformation product (metabolite) of parathion - paraoxon; no constituent metabolite compounds will be produced.
- The oxidations within the assay will not interfere with the ability of non-phosphorothioate compounds to effectively inhibit acetylcholinesterase.

CHAPTER 4

RESULTS AND DISCUSSION

Optimization of the Oxidation Step

The optimization of the oxidation step for the EIB 96 was accomplished by studying the oxidation characteristics of four different compounds. The compounds used as oxidizers were (1) bromine (Br_2), which has a standard reduction potential of 1.0873 E^o/V; (2) sodium hypochlorite (NaOCl), which has a standard reduction potential of 0.81 E^o/V; (3) ferric chloride ($FeCl_3$), which has a standard reduction potential of –0.037 E^o/V; and (4) rat liver microsomes (RLM). The curves for the graphs that are listed were generated from a four-parameter logistic equation (curve fitting equation) defined as

$$y = \frac{(a-d)}{1+\left(\frac{x}{c}\right)^b} + d$$

where y = response, a = response at high asymptote, b = slope factor, c = IC_{50}, d = response at low asymptote, and x = calibrator concentration (Howes, 1996). Figure 15 is a four-parameter logistic equation curve defining the four parameters and their influence on the slope of the curve. The scale of the x-axis is log-linear in the Figure 15; in the graphs from the project the scale of the x-axis is log_{10}, which reverses the curves generated by the four-parameter logistic equations that are typically observed. The four-

parameter equations are used to statistically analyze data that are obtained from bi-molecular binding events such as the binding events observed with organophosphates and carbamate to the enzyme acetylcholinesterase (Howes, 1996). The kinetics of the substrate-enzyme binding of organophosphates and carbamates to AChE is discussed in Chapter 2, Enzyme Inhibition Kinetics. The curves of the graphs generated during this project are fit by a B-spline fitting method, which traditional fits non-sigmoidal data to a sigmoidal curve (Howes, 1996).

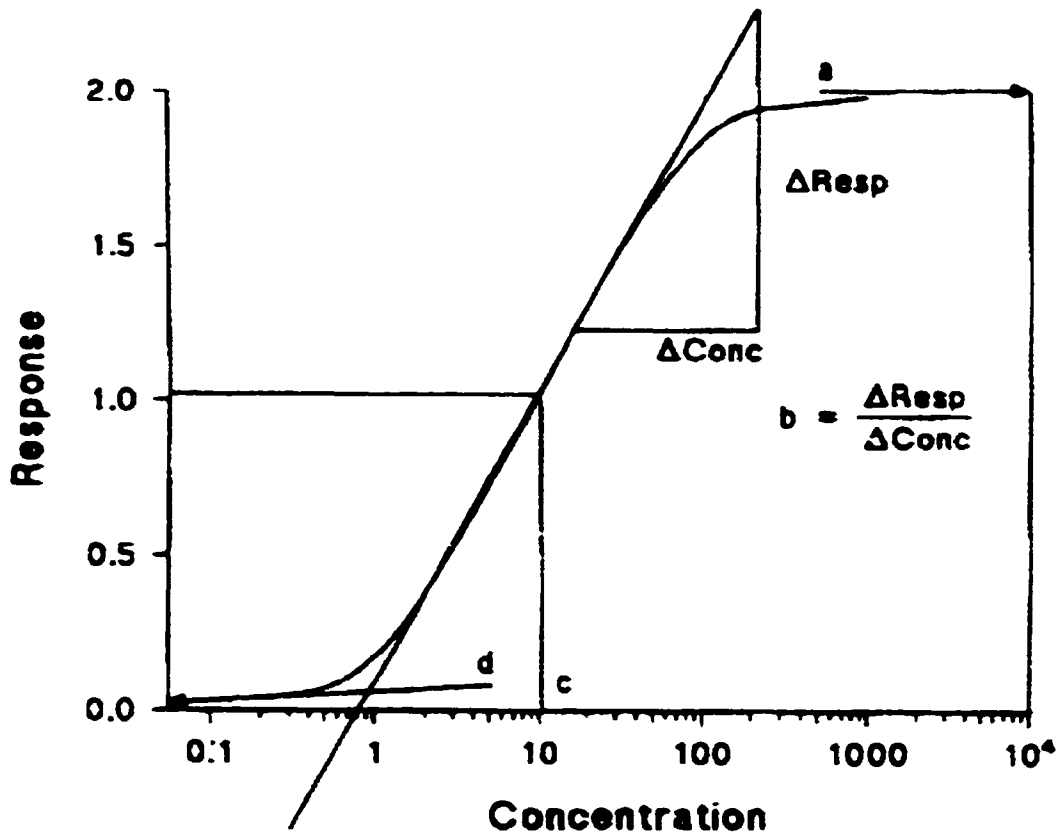


Figure 15. Example of a four-parameter logistics equation.

Bromine Oxidation

The assay was initially designed to use Br_2 as the oxidizing agent, and was studied using the organophosphorothioates diazinon and parathion. For each pesticide Br_2 concentrations were 0.1%, 0.01%, 0.001%, and 0.0001%. In Figures 16 and 17, the responses of each of the organophosphorothioates compounds are shown for each of the gr. The assay was more responsive to parathion treated by Br_2 oxidation than diazinon. A Br_2 concentration of 0.01% was the optimal concentration for reducing the IC_{50} value of parathion. Each point on each of the graphs is the mean of three measurements (n=3), and no error bars are evident at each point on each of the graphs due to the error bars being obscured by the symbols representing each of the points. The errors at each point were very small. See Appendix II for the oxidation protocol.

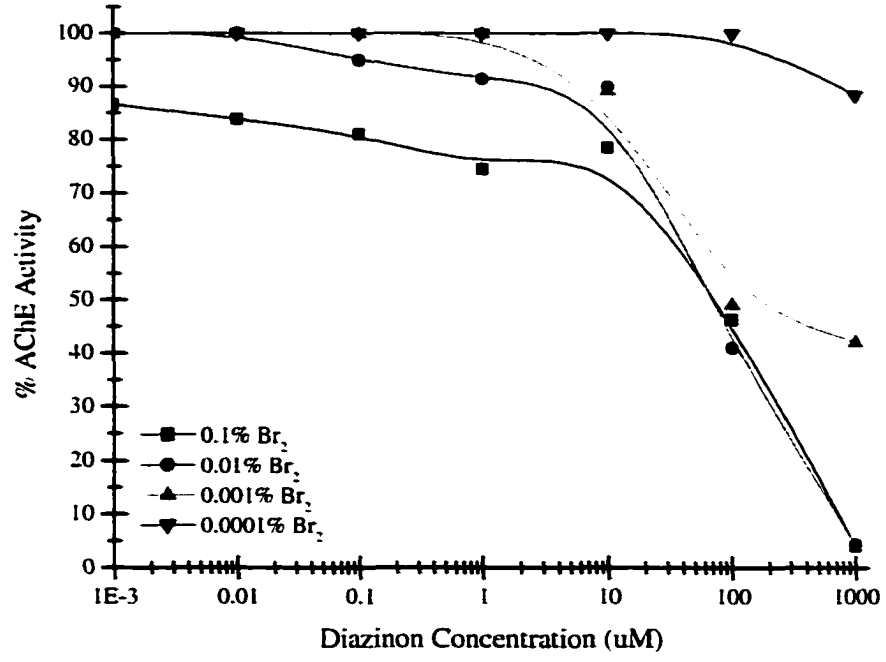


Figure 16. Plot of diazinon oxidized with varying concentrations of Br_2 (n=3).

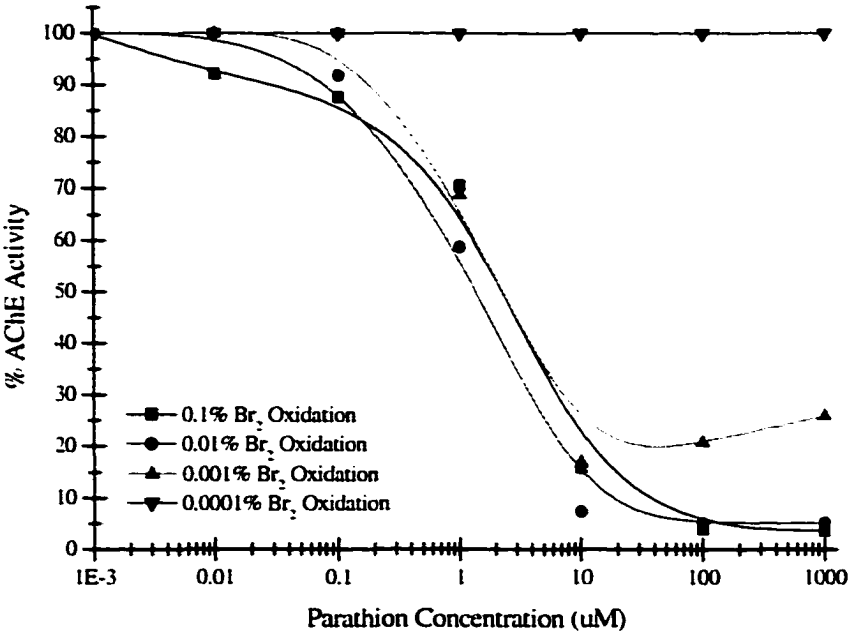


Figure 17. Plot of parathion oxidized with varying concentrations of Br_2 (n=3).

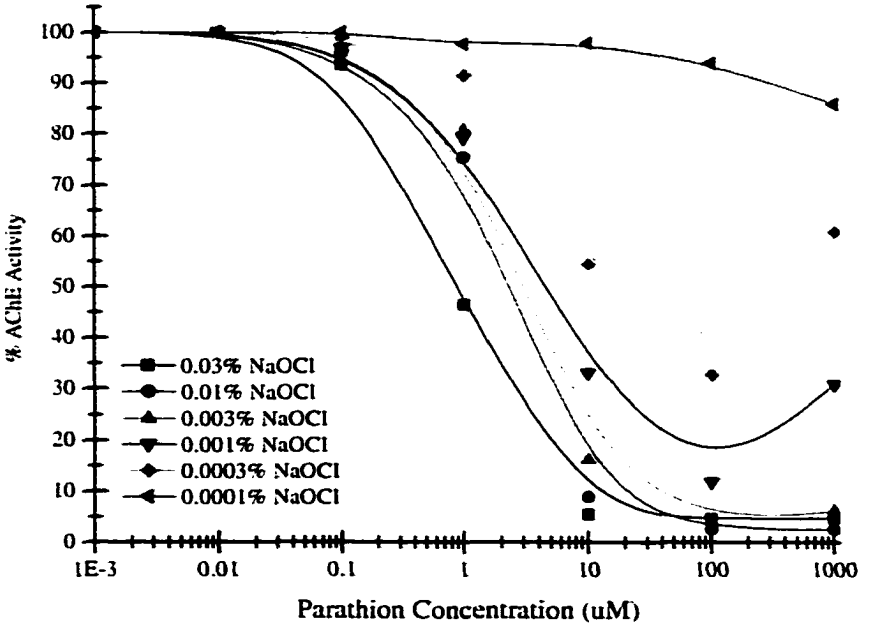


Figure 18. Comparison of various NaOCl concentrations using parathion (n=3).

Initially, the EIB 96 was designed to utilize 0.001% Br_2, however, as is evident in Figures 17 and 18, 0.01% Br_2 is slightly more effective regarding the reduction of the IC_{50} values for the organophosphorothioates. The assay responded more predictably than diazinon and resulted in a better fit of the curve.

Sodium Hypochlorite

Sodium hypochlorite was found to oxidize parathion relatively well as evidenced by its increase in anticholinesterase activity, and since parathion was chosen as the model compound a series of concentrations were established to determine the concentration that would be most suited for use within the assay. Figure 16 affords the results of the concentrations that ranged from 0.03% to 0.0001% for NaOCl.

Diazinon was also oxidized with 0.01% NaOCl with the results given in Figure 19. The diazinon oxidations yielded the characteristic smooth curve with a decrease in AChE activity above diazinon concentrations of 10 μM.

Carbaryl and dichlorvos were treated with 0.01% NaOCl to determine whether the oxidation within the assay would damage these compounds' ability to inhibit AChE. Theses compounds do not require oxidative activation, as do the phosphorothioate compounds, since carbaryl and dichlorvos are not phosphorothioate (P=S) compounds. The result of the treatment of carbaryl with 0.01% NaOCl can be found in Figure 20. As can be observed, there is no significant difference between the treated and untreated compound, which indicates that treatment with 0.01% NaOCl does not damage (or degrade) carbaryl's capacity to inhibit AChE. In Figure 21, the result of the treatment of dichlorvos with 0.01% NaOCl can be found, and there is minimal difference between the treated and untreated compound. The dichlorvos treatments were also analyzed by

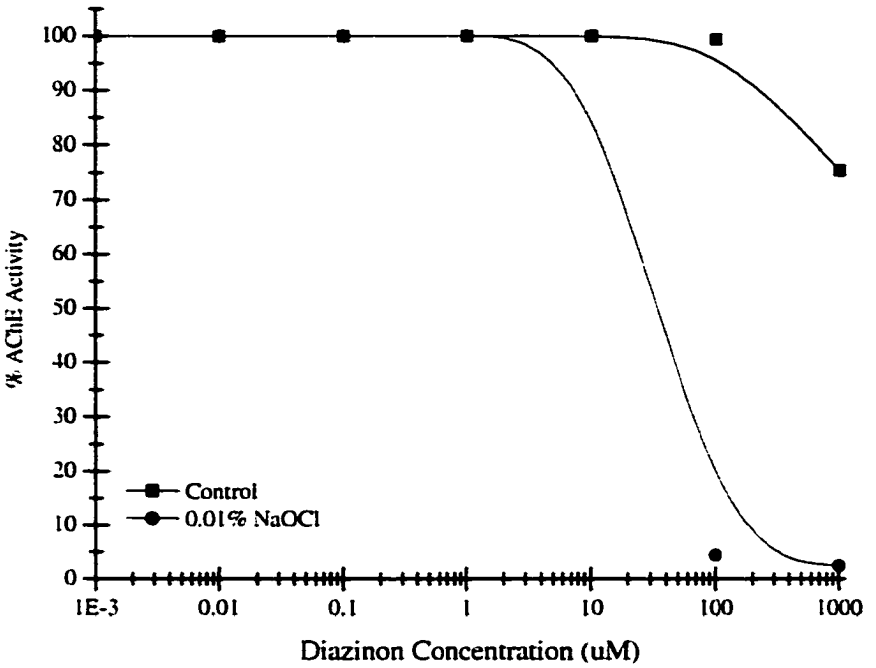


Figure 19. Graph of diazinon oxidized with 0.01% NaOCl (n=3).

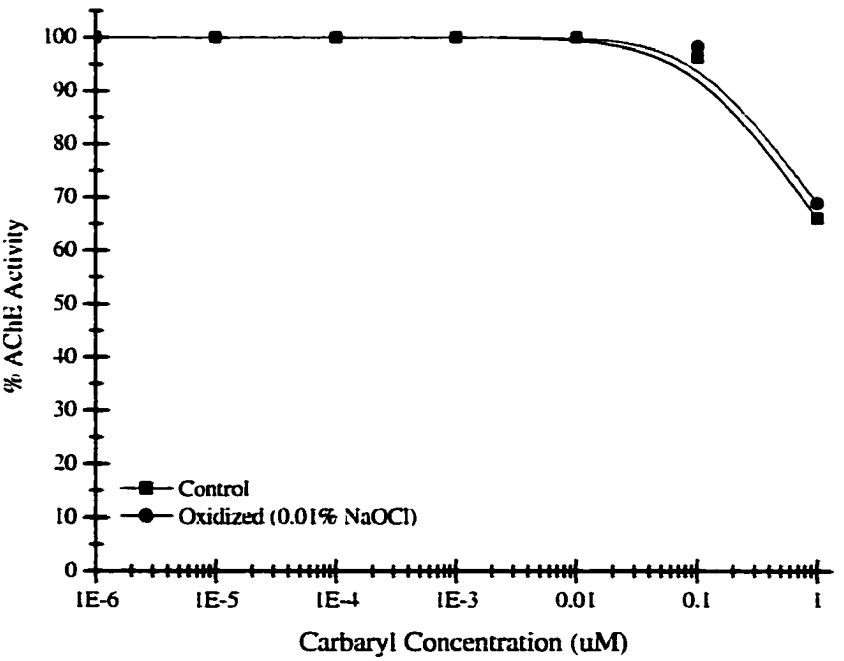


Figure 20. Graph of carbaryl oxidized with 0.01% NaOCl (n=3).

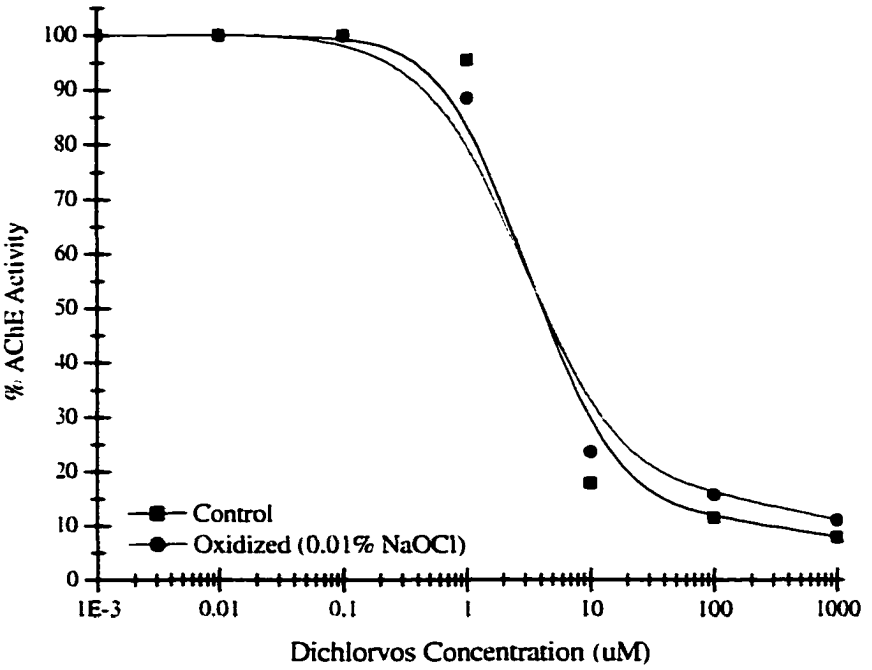


Figure 21. Graph of dichlorvos oxidized with 0.01% NaOCl (n=3).

GC/MS (data not given), and it was determined that no net loss of dichlorvos occurred during the treatment nor was there production of any metabolites. The IC_{50} values were also equivalent (4.17 μM).

Ferric Chloride

Diazinon and parathion were oxidized with $FeCl_3$ to determine the effectiveness of $FeCl_3$ as an oxidizing agent within the assay. Although the reduction potential for $FeCl_3$ is –0.037 E°/V, the relative ease with which one can obtain $FeCl_3$ and its chemical and physical properties, which are far less harmless than Br_2, made it a suitable candidate as an oxidizing agent for use in the assay. However, as is evident in Figures 22 and 23 $FeCl_3$ did not oxidize any of the organophosphorothioate compounds. $FeCl_3$ did, however, block the inhibiting characteristics of both diazinon (Figure 22) and parathion

(Figure 23). Any concentration of $FeCl_3$ above 5 μM produced a yellowish-orange color in each of the wells, which was a color darker than the bright yellow color produced from the reduction of DTNB in the presence of thiocholine. The darker color interfered with the absorption of DTNB.

The curve for diazinon was slightly below the curve for parathion after treatment with $FeCl_3$. The color of $FeCl_3$ did not interfere with diazinon's AChE inhibiting ability as much as it interfered with parathion's inhibiting ability. $FeCl_3$ was determined to not be suitable for use in the assay.

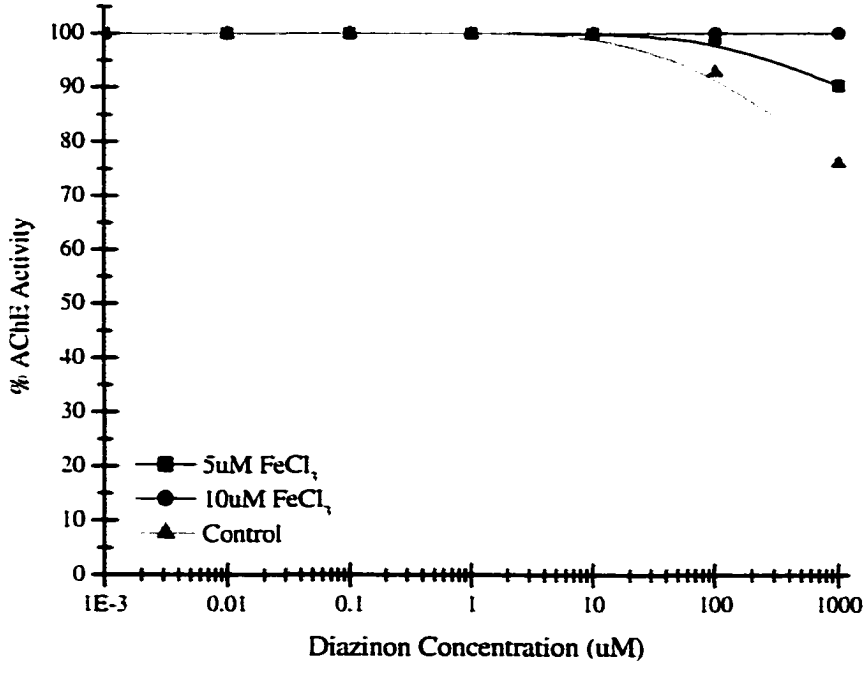


Figure 22. Oxidation of diazinon by 5 μM and 10 μM $FeCl_3$ (n=3).

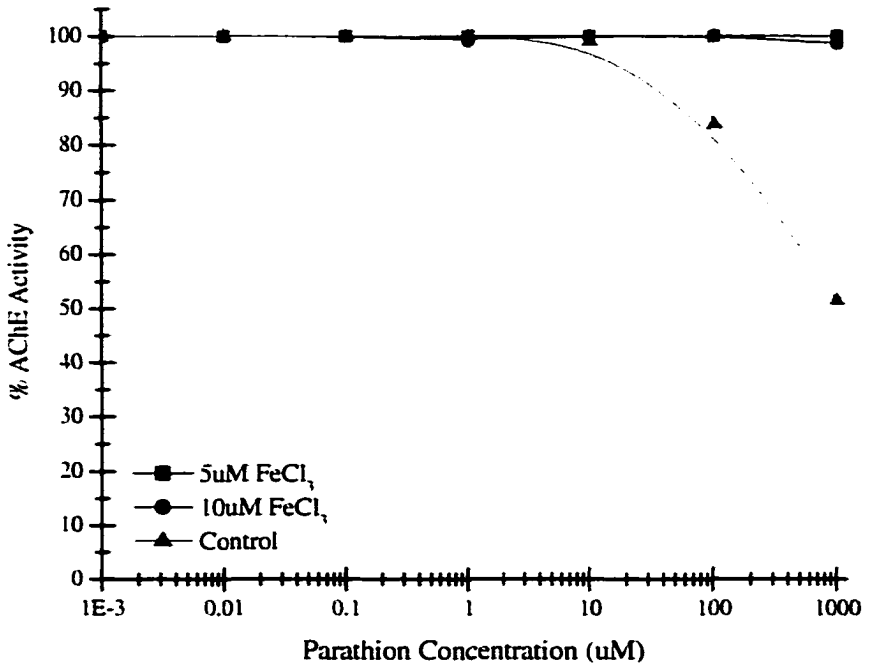


Figure 23. Oxidation of parathion by 5 μM and 10 μM $FeCl_3$ (n=3).

Rat Liver Microsomes

Since RLMs more closely mimic the biotransformation process in the living animal (Barber et al., 1999), it was believed that they would be an effective oxidizer within the assay. However, RLMs were not effective at oxidizing parathion (Figure 24), the model compound for the study as compared with the other oxidizing agents shown in the previous graphs. The reason for this result may be due to RLMs being only a part of a greater oxidizing system, the cytochrome P450 mono-oxygenase system, responsible for biotransformation of many organic compounds in vivo. To work more effectively, traditional standard in vitro techniques for microsomal oxidation will need to be incorporated into the assay oxidation process. The standard in vitro techniques used would require greater incubation times (far greater than would be useful for this assay),

and introduction of more components of the cytochrome P450 mono-oxygenase system, which would require more expense and time than using the oxidation protocol developed for the EIB 96. Therefore, RLMs are not suitable for use with the EIB 96.

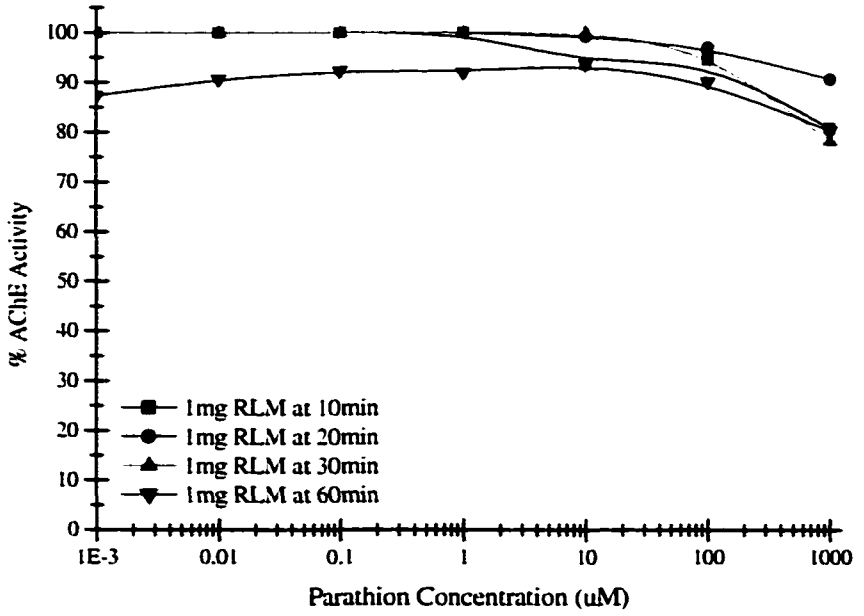


Figure 24. Graph of parathion oxidized with RLMs at various incubation times (n=3).

Bromine and Sodium Hypochlorite Comparisons

The oxidizing agents that were determined to be the most suitable choices for use within the assay were Br_2 and NaOCl. Although Br_2 oxidized parathion slightly more effectively than NaOCl (by reducing the IC_{50} value more effectively), NaOCl was determined to be the most suitable choice between the two compounds. In Figure 25, the IC_{50} values are 1.40μM and 2.33μM for Br_2 and NaOCl, respectively. The difference between the Br_2 and NaOCl IC_{50} values is marginal when compared with the IC_{50} values produced from the oxidation of parathion with the other oxidizing agents used in this project (Figure 26).

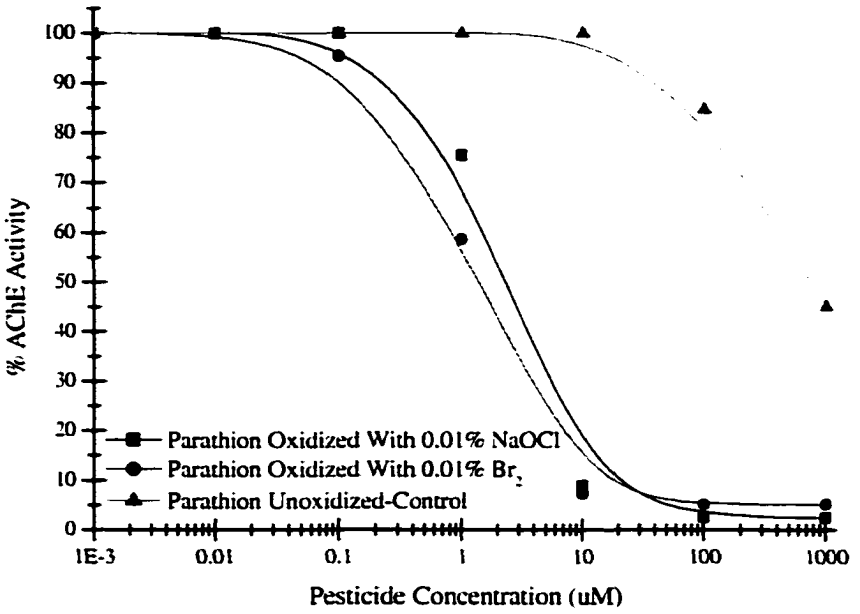


Figure 25. Comparison of the oxidation of parathion by 0.01% Br_2 and NaOCl (n=3).

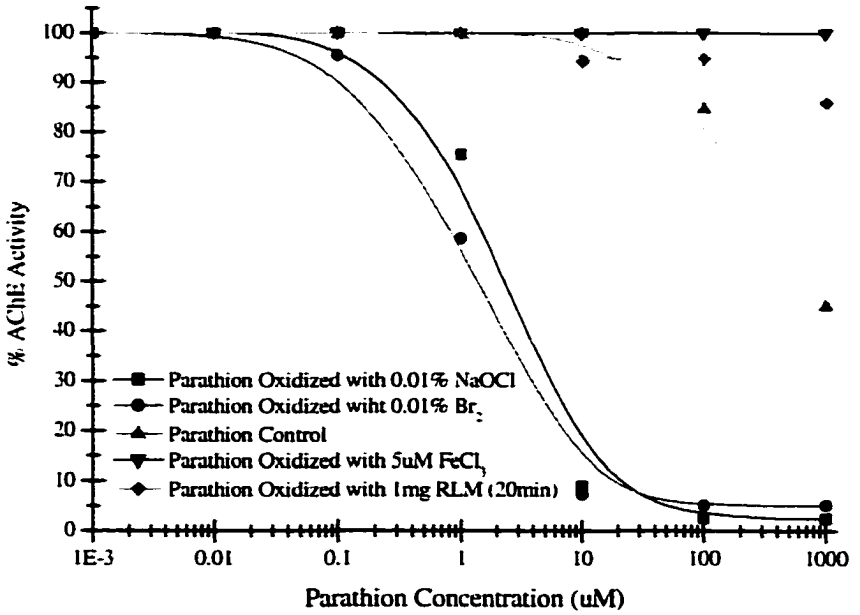


Figure 26. The oxidation of parathion with all of the oxidizing agents (n=3).

Analysis of Biotransformation Products Using GC/MS

Once the most suitable oxidizing agent was determined, an analysis of the oxidation reactions was necessary to establish the biotransformation products being generated during the oxidation step. An Agilent Technologies 6890 gas chromatograph/5973 mass-selective detector was used to both identify the biotransformation products and quantify the reactant and product(s). Parathion was selected as the compound to use for this part of the project for two reasons: (1) it was the model compound selected for use in this project; and (2) both the predicted product (paraoxon, primary metabolite) and reactant (parathion, parent compound) were available as standards for use in the calibration curve, which was necessary for quantitation of the product and reactant.

During the analysis of parathion by GC/MS, four separate oxidation reactions were completed (Table 2). The reactions were each placed in a buffer solution and allowed to incubate, as given in the oxidation protocol in Appendix III. The oxidation of parathion yielded only paraoxon as its biotransformation product, which was expected (Figure 27). In Figure 27, the parathion peak is on the right, and the paraoxon peak is on the left. Figure 28 is a simple representation of the oxidation reaction that took place in solution. Table 2 is a summary of the results from the four reactions with relative conversion and mass balance data listed, respectively. The mean relative conversion of parathion to paraoxon was $24.44 \pm 3.14\%$. Approximately 24% of the parathion was converted to paraoxon during the oxidations. Information for the method used for determining the quantities of parathion and paraoxon are in Appendix III.

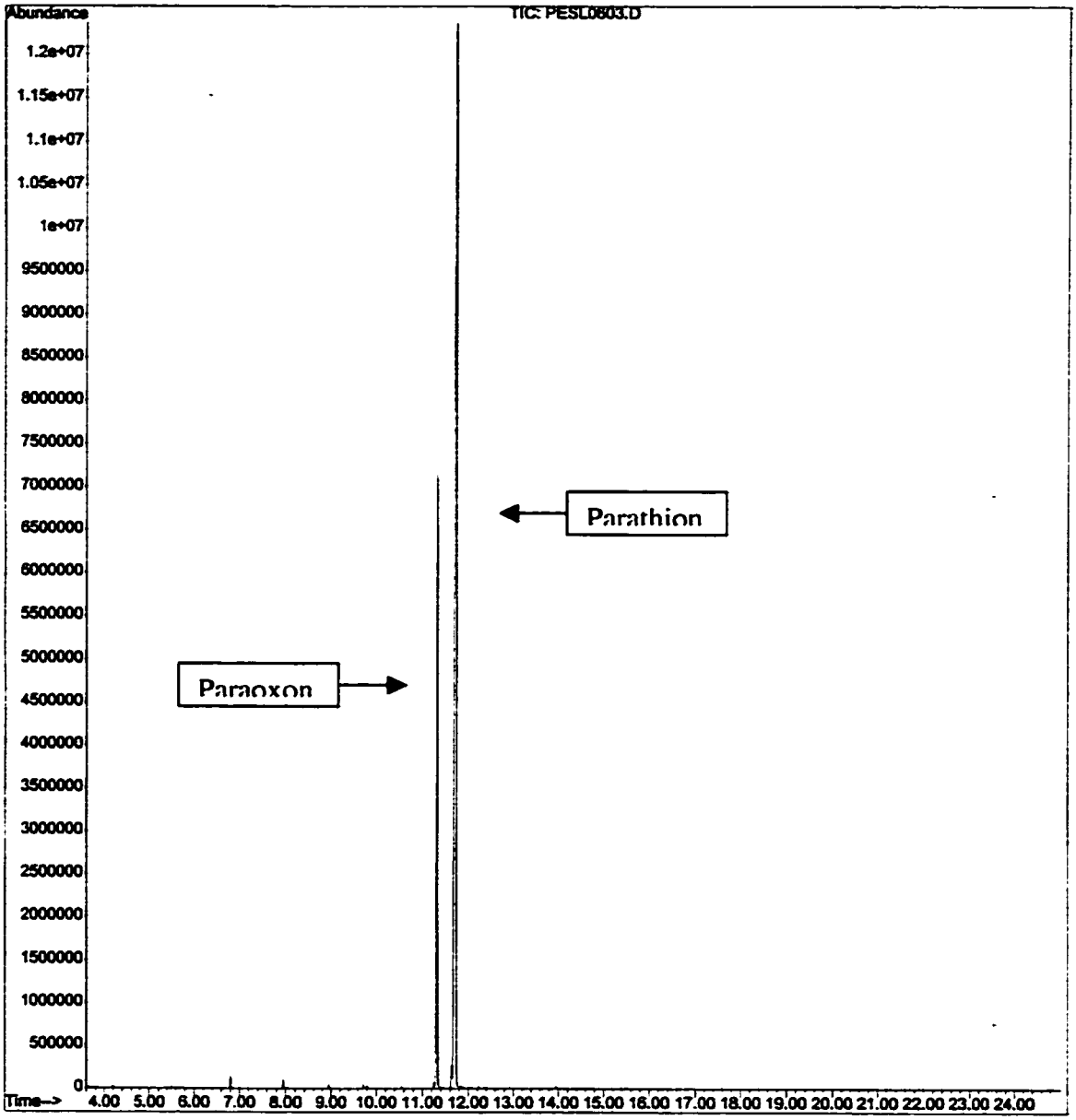


Figure 27. Mass spectrum of the oxidation product from parathion to paraoxon.

Table 2. Relative conversion and mass balance data for parathion.

Compound	Conversions (mM)	Relative Conversion (%)	Blanks (mM)
Parathion	0.76		1.24
Paraoxon	0.26	25.49	0.00
Relative Sum	1.02		
Parathion	1.01		1.11
Paraoxon	0.32	24.06	0.00
Relative Sum	1.33		
Parathion	0.90		1.06
Paraoxon	0.23	20.35	0.00
Relative Sum	1.13		
Parathion	1.01		1.22
Paraoxon	0.39	27.86	0.00
Relative Sum	1.40		
Sum (Σ)	4.88	97.76	4.63
Mean ($\bar{x}$)	1.22	24.44	0.86
SD (σ)	0.18	3.14	0.35

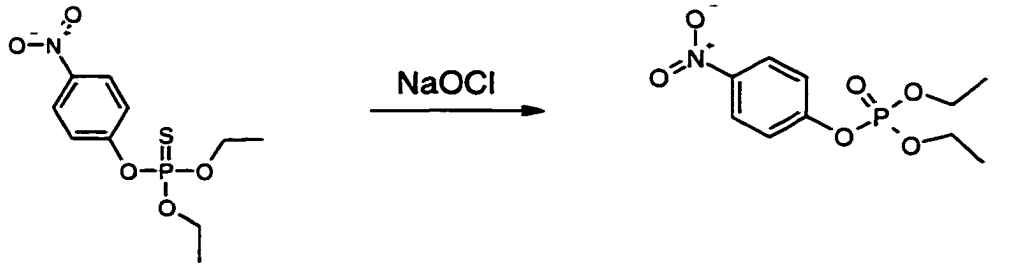


Figure 28. Production of paraoxon from NaOCl oxidation of parathion.

In Table 2, there is a discrepancy between the conversion amounts and the blank amounts, which are listed as 4.88 mM and 4.63 mM, respectively. The blank amounts are solutions that were worked-up as the reaction solutions were, minus the oxidizing agent NaOCl. Theoretically, the blanks should have a total concentration of compound(s) equal to the reaction solutions. However, three out four blanks that were quantitated had less total concentration of compound(s). This may be due to a greater loss of yield during transfer for the blank solutions than for the reaction solutions; since this portion of the analysis is key to producing accurate and precise results and great care needs to be taken during the transfer of any solutions. Also, there may have been systematic and/or indeterminate (random) errors introduced during the preparation of the solutions. A systematic error that may have occurred during the solution preparations may have come from the pipettes if they were consistently measuring volumes of the transfers above or below what was intended. An indeterminate error could have come from a misreading of the scale during the weighing of a pesticide compound.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

Introduction

This project studied and optimized a novel assay (the EIB 96) for the detection of the presence of organophosphate and carbamate compounds. The EIB 96 has been developed as a substitute for many of the current commercially available bioassays such as Neogen's Agri-Screen Test Kits, which are more expensive to use than the EIB 96 and do not have the detection sensitivity necessary to be as useful as a field screening tool. The EIB 96 is based on the use of colorimetric techniques to detect the presence of organophosphate and carbamate compounds by the loss in activity of the enzyme acetylcholinesterase. The assay is relatively rapid, simple, and inexpensive to use as a field screening tool by researchers who wish to reduce the need to perform unnecessary sample analyses, which consume valuable time and resources.

Conclusion

Project Goals

The primary goals of this project were to study, characterize, and optimize various strategies for improving the EIB 96 assay for pesticide screening through oxidative activation of phosphorothioate pesticides. The following questions were posed at the beginning of the project: (1) what compound would be the most efficient and effective

oxidizing agent for use in the EIB 96 assay; (2) what oxidative product(s) (metabolite(s)) would be produced from the oxidation of the organophosphorothioate compounds; (3) how much of the parent organophosphorothioate compound is being converted to its oxidative product(s); and (4) would the oxidation step of the assay interfere with the inhibiting characteristics of the compounds that did not require oxidative activation (the non-phosphorothioate compounds).

Oxidizing Agents

The oxidizing agents that were chosen to be studied during this project were bromine (Br_2), sodium hypochlorite (NaOCl), ferric chloride ($FeCl_3$), and rat liver microsomes (RLMs). Br_2 was determined to be the most efficient and effective oxidizing agent (initially the oxidizing agent of choice for use in this assay), specifically at the 0.01% concentration level. However, NaOCl was determined to be the most suitable choice for three reasons: (1) the differences in the IC_{50} values from the oxidation of parathion (Figure 25) were 1.40 μM and 2.33 μM for Br_2 and NaOCl, respectively. The difference between the two IC_{50} values is minimal; (2) since Br_2 is a lachrymator, highly volatile, and corrosive, and NaOCl is no more than a skin, eye, and lung irritant, the chemical and physical properties of NaOCl made it a much more suitable choice as an oxidizing agent for use in the assay; and (3) the availability and lower cost of NaOCl outweighed the oxidizing effectiveness of Br_2 within the assay.

The oxidizing agents that were not suitable for use in the EIB 96 were $FeCl_3$ and RLMs. $FeCl_3$ was not suitable for use in the assay for two reasons. First, the standard reduction potential is far too low (–0.037 E^{o}/V), and second, the color generated by the iron (Fe^{III}) in $FeCl_3$ was darker than the color generated by the reduction of DTNB and

interfered with the instrument measurement of AChE activity. RLMs were not suitable for use in the assay since they are only a part of the larger living system, the cytochrome P450 mono-oxygenase system.

Standard reduction potentials are potentials for electrodes in which all components are in a standard state at 25°C, with ion concentrations of 1 M and gas pressures of 1 atm. The values of electrode potentials are customarily reported for the reduction processes. The standard reduction potential for a metal electrode will be negative when referenced to the standard hydrogen electrode if the metal ion is more difficult to reduce than the hydrogen ion. A negative reduction potential for a metal ion (in this case Fe^{III}) means that it will be harder to reduce (is a worse oxidizing agent) than the hydrogen ion. Therefore, the reduction of $FeCl_3$ will not readily take place in aqueous solution systems such as the one the EIB 96. The color that $FeCl_3$ produced at the upper concentrations (5 μM and 10 μM) was far too dark (the greater the concentration the darker the color) to be of use in the assay. The color of $FeCl_3$ posed a problem for measurement in the assay because the Vmax spectrophotometric instrument measures the reduction of DTNB by thiocholine (see Appendix II for details of this reduction) beginning at an absorbance of 405 nm. However, in the presence of a compound that produces a darker color the absorbance will begin at a wavelength lower than 405 nm, and the reduction of DTNB will not be measured accurately, or at all. The concept of the absorbance of a compound in solution is based on the equation known as Beer's Law, defined as $A = \varepsilon lc$, where A is the absorbance, ε is the molar absorptivity, and c is the concentration of the compound in solution.

The rat liver microsomes (RLMs) were not suitable for use in the assay since, as mentioned previously, they were only one part of a larger system, the cytochrome P450 mono-oxygenase system. The protocol developed during this project for oxidation of parathion using RLMs did not optimize the RLM biological system, which is an effective endogenous oxidizing system. The use of RLMs may or may not be inferior as an oxidation system in vitro, but the system was not optimized during this project. In order for this system to be optimized more time and greater expense will need to be incurred, therefore, RLMs are not suitable for use in the EIB 96 assay.

Oxidative Products of the Organophosphorothioates

The oxidative activation of the organophosphorothioates was a key component of the optimization of the EIB 96, and identification of the biotransformation products of these compounds was necessary to determine what chemical reactions were taking place within the assay. The model compound, parathion, was utilized for this part of the project. The oxidative product of parathion within the assay was determined to be paraoxon, with a $24.44 \pm 3.14\%$ conversion of parathion to paraoxon (determined by GC/MS analysis, Appendix III). Although this is not a large conversion of parathion to its metabolite, only a conversion of a small amount of the parathion is required to render this assay useful for potential applications. This is primarily because the oxon derivative is a significantly more potent inhibitor of AChE. Theoretically, conversion of even a small percentage of any organophosphorothioate to its oxon derivative would be necessary for the assay to detect the presence of these compounds. If the percent conversion of the parent compound to its metabolite were improved, improvement in the response of the assay would be observed, but the improvement would only be minimal.

The response of the assay to 100% paraoxon gives an IC_{50} value of 0.15 μM (Mishra, 2001) as compared with 2.33 μM for parathion oxidized with NaOCl, and since the conversion of parathion will never be 100%, the observed improvement would be marginal.

Non-Phosphorothioate Compounds

Carbaryl (n-methyl carbamate) and dichlorvos (organophosphate) were the non-phosphorothioate compounds tested in this project. These compounds were selected since it was determined that they would be representative of compounds that did not require oxidative activation to inhibit AChE that would be tested in the assay. It was speculated that the potential effect (the degradation of the ability of carbamates and organophosphates to inhibit AChE) the oxidation step would have on these compounds would be minimal, if there was an effect at all. As expected, the oxidation step did not degrade the ability of the non-phosphorothioate compounds to inhibit AChE (Figures 20 and 21). Therefore, detecting these compounds after oxidation of a sample(s) that contain non-phosphorothioate compounds is expected.

Benefits and Limitations of the Enzyme Inhibition Bioassay 96 (EIB 96)

Benefits

The EIB 96 was developed as a substitute for many of the currently commercially available bioassays such as Neogen's Agri-screen Test Kits, in an attempt to minimize the use of expensive materials and to utilize relatively rapid methods to detect the loss of acetylcholinesterase activity. Also, the EIB 96 was developed as a screening tool to increase the amount of information available concerning the location, source, and

presence of pollutants in the environment (Rogers & Williams, 1995), which help researchers know where they should concentrate their efforts and their money.

The average cost per sample for bioassays currently available is about $22.00 - $25.00, while the cost per sample for the EIB 96 is $0.08. Since the assay plate has 96 wells, hence the name EIB 96, 96 samples can be tested for a total cost of $7.68. The assay throughput for the EIB 96 is approximately 45-50 minutes, the total time necessary to test 96 parallel samples using this assay. The traditional testing and analysis methods utilized by research scientists, GC/MS and HPLC methods using sequential sample testing, could cost hundreds to thousands of dollars and take days or weeks to get results from samples that were not necessarily contaminated with pollutants. Further, this assay provides ranges suitable for monitoring groundwater for compliance with US EPA standards for organophosphate and carbamate compound concentrations.

Limitations

During this project there were only two limitations identified for this assay. The first limitation concerned the oxidizing agent chosen as the most suitable for use in the assay. The concentration of 0.01% for NaOCl was determined to be optimum. Increasing the concentration of NaOCl would kill the enzyme because increasing the concentration of NaOCl above 0.01% (0.1%, 0.15%, 1.0%, etc.) would lead to necessary increases in concentrations of the oxidation neutralizer EtOH (20%, 30%, 40%, 50%, etc). However, the enzyme does not tolerate increases in concentration of either NaOCl and/or EtOH very well (the higher the concentration of either NaOCl and/or EtOH would lead to reduced activity of the enzyme before the introduction of pesticide). Therefore, increases in the amount of paraoxon produced by the oxidation of parathion treated with

NaOCl are limited to about 25%. The second limitation of this assay identified during this project is that the assay is that although the assay can be used for monitoring groundwater for compliance with US EPA standards, it is not adequate for use in monitoring drinking water for compliance with US EPA standards. The detection limit for the assay is not low enough for monitoring drinking water. For example, the maximum contaminant level (MCL) allowable, according to US EPA standards, for the carbamate carbofuran is 0.04 μM (Office of Water, http://www.epa.gov/safewater/mcl.html). The lowest detectable limit for the assay is about 0.1 μM.

Recommendations

Statistical Analysis

The data that were obtained during the optimization portion of this study were analyzed qualitatively with some statistical analysis of the data points (each point is a mean of triplicates, n = 3) performed by the four-parameter logistics equation as previously mentioned. Although a qualitative analysis with some statistical analysis of the data points was sufficient to answer all of the questions posed at the outset of the project, a statistical analysis using an analysis of variance (ANOVA) to determine the difference between the IC_{50} values would have bolstered the conclusions, added rigor to the analysis, and provided results with broader applicability. In as much as the data sets generated for the project were not large enough to support a quantitative analysis of this type, it is recommended that if the project were to be continued, more samples should be taken.

To determine the sample sizes for the data sets necessary to perform a statistical analysis, in this case an ANOVA, it may be prudent to estimate the required sample sizes for the ANOVA through the use of a power analysis (determination of the power of the test); how large do the sample sizes from the data sets need to be in order to achieve a desired power. A power can be specified that will detect a particular difference among the data set (population) means ($\bar{x}$). The estimation of the required sample sizes is done by iteration using the following equation

$$\phi = \sqrt{(((n)(\delta)^2)/((2)(k)(s^2)))}$$

where the n is the number of samples, δ is the minimum detectable difference, k is the number of data set means, and s^2 is the sample variance (Zar, 1999). Although the above equation requires an initial guess for a sample size, a simple calculation can aid in determining it. The equation for determining the initial guess is $n = ((t*s)/E)^{1/2}$, which is the sample variance equation, algebraically rewritten to determine the initial number of samples for the ANOVA. For example, choosing from a data set obtained from the optimization portion of the project that has a t value (t) = .05 (which is also α) with degrees of freedom (df) = 1, a standard deviation (s) = 0.1, and an acceptable error (E) = 20%, would result in the initial number of samples (n) being approximately 10 (9.97).

Now that an initial sample size has been determined, a power analysis can be performed using a power that is specified to have an 80%, 90%, 95%, etc. probability of detecting a difference between the data sets (80% is the generally accepted detection limit for a power analysis) with the degrees of freedom for the ANOVA, v_1 and v_2, defined as

k-1 and k(n-1), respectively. As an example of an estimation of required sample size for an ANOVA, the following values are given for each variable: $n = 10$, $\delta = 2$, $k = 4$, $s^2 = 1.6550$, and $v_2 = 36$, with an 80% probability of detecting a difference between the data set means and testing at the $\alpha = 0.05$ level of significance. From the given data $\phi = 1.74$ (1.738), which results in a power ≈ 0.80. The power is equal to what was specified, 80%, thus, it is estimated that using sample sizes of about 10 should result in an ANOVA with a power of about 0.80.

Once the power analysis is performed to determine the required sample sizes at the specified power, an ANOVA can be performed to test $\bar{x}_1 = \bar{x}_2 = \bar{x}_3 = \bar{x}_4$. Also, multiple comparison of data set means can be performed such as Tukey's HSD (Honestly Significantly Different). Tukey's HSD specifically tests all pairwise comparisons among means when the sample sizes of the data sets are equal (McClave and Sincich, 2000) and is defined as

$$t_s = \frac{M_i - M_j}{\sqrt{\frac{MSE}{n_h}}}$$

where $M_i - M_j$ is the difference between the i^{th} and j^{th} means, MSE is the Mean Square Error (the mean square error (MSE) is an estimate of the population variance in the analysis of variance, and is the denominator of the F ratio), and n_h is the harmonic mean of the sample sizes of groups i and j (Hyperstat Online, http://davidmlane.com/hyperstat/index.html). The harmonic mean is used to take the mean of the sample sizes (Hyperstat Online, http://davidmlane.com/hyperstat/index. html).

Gas Chromatography/Mass Spectroscopy (GC/MS) Analysis

The data obtained from the GC/MS analysis were only from the oxidation of parathion. Although this data can be extrapolated to answer questions concerning the biotransformation products of the other organophosphorothioates, were this project to continue, the other compounds in this group should be analyzed individually to determine the exact metabolites that may be generated from the oxidation of their parent compounds. Further, all organophosphates and carbamates that are to be detected by the EIB 96 should be tested individually to determine how well they will respond to the assay and be analyzed by GC/MS methods or HPLC (High Pressure Liquid Chromatography), whichever is more appropriate for the particular compound(s) being studied.

Enzyme Inhibition Bioassay (EIB) 96

During the study and optimization of the EIB 96, it was established that the assay is efficient and effective at detecting the presence of organophosphate and carbamate compounds in a laboratory setting. The potential uses of this assay will be for field screening and monitoring applications in all environmental matrices (air, water, and soil); also, the testing of complex mixtures such as those found in many environmental matrices, will be necessary with the EIB 96. Generally, environmental samples from any of the matrices will contain a mix of different compounds, not just one specific compound.

The ideal matrix to be tested with the EIB 96 would be water. This matrix would be relatively simple to test since most water could be transferred directly from its source into the assay. The only types of water that would pose a problem for the assay would be brines, highly acidic water such as those that are used for acid mine drainage, or water

contaminated with radioactive waste. Soil would also be a relatively simple matrix to test. The soil can be collected, placed in a container with an organic solvent such as hexane for washing any organic compounds out of the that may be present, and then filtered. The collected filtrate could be dried and the remaining solute resolubilized in an aqueous solution suitable for testing in the EIB 96. Most soils could be tested in this manner. Although soils with iron, copper, manganese, etc. may require precipitation of the metals, in order for the assay to accurately detect the presence of organophosphates and carbamates since these metals, depending upon there concentration in the soils, would produce a darker solution mixture like the solutions produced with $FeCl_3$. Air samples may pose the greatest challenge for testing with the assay since collection strips containing various compounds such as triolein (a triacylglycerol, used to capture particulate matter in air), would first need to be tested for potential measurement interference within the assay. If there is interference, then an extraction method and clean up procedure must be devised before testing could begin. This may be messy for the individual tasked with the extraction and clean up.

The efficacy of detecting the target carbamate and organophosphate compounds within complex (multi-compound) mixtures using the EIB 96 will need to be determined for the reason mentioned above. The feasibility of this assay to detect the target compounds within multi-compound mixtures could be established by (1) analyzing various compound mixture samples from stock neat solutions at varying concentrations, (2) comparing the compound mixture sample results with the results from the analysis of the separate compounds to determine the effects the mixtures will have on individual compounds, (3) establishing which compound will be the controlling factor (most potent

inhibitor) present within a solution mixture containing multiple organophosphate and carbamate compounds to determine what effect the potent inhibitor will have regarding the detection of the compounds within the mixture, and (4) comparing environmental samples with the pure compound mixtures to establish what the detection limit will be for environmental samples, especially in the presence of co-contaminants. Consequently, it is recommended that further study be conducted to determine the extent to which the assay could be utilized in different environmental matrices, and with multi-compound mixtures.

APPENDIX I

GENERAL AND REGULATORY PESTICIDE INFORMATION

Carbaryl*

Regulatory Status
Carbaryl is a General Use Pesticide (GUP). However, different formulations vary greatly in toxicity. For example, carbaryl is categorized as toxicity class I – highly toxic for Tercyl (Wettable Powder), toxicity class II – moderately toxic for Sevin 803 (granular), and toxicity class III – slightly toxic for some other products. Products containing carbaryl may bear the Signal Word Danger – Poison, Warning, or Caution, again, depending on the product formulation.

Chemical Class
Carbamate

Trade and Other Names
Product names include Adios, Bugmaster, Carbamec, Carbamine, Crunch, Denapon, Dicarbam, Hexavin, Karbaspray, Nac, Rayvon, Septene, Sevin, Tercyl, Torndao, Thinsec, Tricarnam, and Union Carbide 7744.

Uses
Carbaryl is a wide-spectrum carbamate pesticide which controls over 100 species of insects on citrus, fruit, cotton, forests, lawns, nuts, ornamentals, shade trees, and other crops, as well as on poultry, livestock, and pets. It is also used as a molluscicide and an acaricide. Carbaryl works whether it is ingested into the stomach of the pest or absorbed via direct contact. It is available as bait, dusts, wettable powders, granules, dispersions and suspensions.

Toxicities
Acute toxicity: Carbaryl is moderately to very toxic. It can produce adverse effects in humans by skin contact, inhalation, or ingestion. The symptoms of acute toxicity are typical of the other carbamates. Direct contact of the skin or eyes with moderate levels of this pesticide can cause burns. Inhalation or ingestion of very large amounts can be toxic to the nervous and respiratory systems resulting in nausea, stomach cramps, diarrhea, and excessive salivation. Other symptoms at high doses include sweating, blurring of vision, uncoordinated movement, and convulsions. The only documented fatality from carbaryl was through intentional ingestion.

The oral LD_{50} of carbaryl ranges from 250 mg/kg to 850 mg/kg in rats, and from 100 mg/kg to 650 mg/kg in mice. The inhalation LC_{50} in rats is greater than 200 mg/L. Low doses can cause minor skin and eye irritation in rabbits, a species in which carbaryl's dermal LD_{50} has been measured at greater than 2000 mg/kg (Kamrin, 1997).

Chronic toxicity: Information not available.

Environmental Fate
Breakdown in soil and groundwater: Carbaryl has a low persistence in soil. Degradation of carbaryl in the soil is mostly due to sunlight and bacterial action. It is

bound by organic matter and can be transported in soil runoff. Carbaryl has a half-life of 7 to 14 days in sandy loam soil and 14 to 28 days in clay loam soil. Carbaryl has been detected in groundwater in three separate cases in California.
Breakdown in water: In surface water, carbaryl is broken down by bacteria and through hydrolysis. Evaporation is very slow. Carbaryl has a half-life of about 10 days at neutral pH. The half-life varies greatly with water acidity.
Breakdown in vegetation: Degradation of carbaryl in crops occurs by hydrolysis inside the plants. It has a short residual life of less than 2 weeks. The metabolites of carbaryl have lower toxicity to humans than carbaryl itself. The breakdown of this substance is strongly dependent on acidity and temperature.

Environmental Concentrations[‡]
Surface Water: Carbaryl was detected but not quantified in organic extracts from British and West German river water samples. Carbaryl at a concentration of 0.003 ppm was detected in stream water adjacent to a land spraying area in Canada 5 days following an application rate of 280 g/ha. In a study conducted from April 1993 to April 1994, twenty-five water samples (one taken each month) were taken at the mouths of two tributary streams of the South Platte River in Colorado and studied for pesticide concentrations. The tributary that originated from an agricultural region contained carbaryl ranging from < 0.046-1.5 ug/L, while the tributary that originated from an urban setting contained carbaryl ranging from 0.15-2.5 ug/L. Carbaryl was only detected in the agricultural tributary from July to September, which corresponded to its use on beans in June and July for beetle control. Urban tributary samples consistently contained carbaryl probably as a result of repeated applications for residential and commercial insect control. Forty water samples collected between February and July 1992, from the rivers Canyoles, Albaida, Claria, Serpis, Polop, Belcaire, Turia, and Xuquer; the Lake Albufera; and the irrigation channels in Spain were analyzed for pesticide content. Carbaryl was detected in 6 of the samples ranging from 1.23-6.48 ug/ml.
Drinking Water: Detected but not quantified in US drinking water.
Ground Water: Carbaryl was not detected with a detection limit of 1 ug/L in 10 farm wells where the land was treated with the pesticide. Carbaryl was detected (concentrations not specified) in three groundwater samples in California. Carbaryl was detected in Solano and Ventura Counties in California at a concentration ranging from 10-55 ppb, taken from July 1, 1994 to June 30, 1995. In 1986 and 1987, 103 and 76 wells, respectively, were sampled in Southern Ontario, Canada for pesticide concentrations. Samples were collected in late November and in mid December in both 1986 and 1987. Carbaryl was detected (concentration not specified) in 10 out of 10 wells sampled in both 1986 and 1987 with a detection limit of 1.0 ug/L.
Rain/Snow/Fog: Coastal fog and air samples collected from three locations along the Pacific coast near Monterey, CA in September 1987 were analyzed for pesticide content. Carbaryl was detected ranging from 0.069-4.0 ug/L.
Effluent: Air emissions from carbaryl manufacturing has been reported to consist of 1.5 kg of hydrocarbons; 0.5 kg of carbaryl per metric ton of pesticide produced.
Sediment/Soil: A field study in India measuring soil persistence associated with heavier application rates of granular carbaryl showed a slower decline in residue levels. Reductions in soil residues of 62 to 81% after 15 days and 94-98% after 60 days were

measured for application rates of 13.4, 26.8, and 40.2 lb active ingredient per acre. Residues up to 6.3 ppm were still in the soil after 90 days. Soil samples were taken to a depth of 10 cm. A heavy application of 22.7 lb per acre of carbaryl to a sandy loam soil resulted in residues in the first 20 cm of soil after 4 months. The upper 1-meter soil layer, however, retained 6% of the initial carbaryl applied after 16 months. Carbaryl was detected in Southwestern Ontario farm soil in 1976 ranging from 0.03-0.08 ppm. Five days following a carbaryl application rate of 280 g/ha, carbaryl was detected ranging from 0.06-0.08 ppm in forest soil located in Canada. Two hours following a carbaryl application rate of 280 g/ha, carbaryl was detected in stream sediment located adjacent to the spraying area at a concentration of 0.03 ppm. Twenty-four hours later, no carbaryl was detected (detection limit: ≤0.005 ppm).
Atmospheric: Air samples taken after spraying for budworms at 91, 96, and 107 ng Carbaryl/m^3 in Maine, did not contain carbaryl (detection limit: 3.5 ng/m^3). The mean concentration of carbaryl in indoor air samples taken in Jacksonville, Florida for the summer (1986), spring (1987), and winter (1988) were 68.1, 0.4, and 0 ng/m^3, respectively. Carbaryl was detected in only outdoor air samples during the summer at 0.2 ng/m^3. Carbaryl was also detected in personal air samples during the summer and spring at 28.3 and 0.8 ng/m^3, respectively. Overall, an annual average daily concentration of carbaryl in Jacksonville, Florida air was determined to be 7.5 ng/m^3. Carbaryl was also detected in indoor and personal air samples taken from Springfield, Massachusetts in the spring (1987) at 0.3 and 0.1 ng/m^3. The annual average daily concentration of carbaryl in Springfield, Massachusetts's air was 0.1 ng/m^3.

Physical Properties
Appearance: Carbaryl is a solid that varies from colorless to white or gray, depending on the purity of the compound, and the crystals are odorless. Carbaryl is stable to heat, light, and acids. It is not stable under alkaline conditions, and it is noncorrosive to metals, packaging materials, and application equipment.
Chemical Name: 1-napthyl methylcarbamate
CAS Number: 63-25-2
Molecular Weight: 201.23
Water Solubility: 40 mg/L @ 30 °C
Solubility in Other Solvents: dimethylformaldehyde vs. acetone vs. dimethyl sulfoxide vs. cyclohexanone.
Melting Point: 142 °C
Vapor Pressure: <5.3 mPa @ 25 °C
Partition Coefficient: Not Available
Adsorption Coefficient: 300

Exposure Guidelines
ADI: 0.01 mg/kg/day
MCL: Not Available
RfD: 0.1 mg/kg/day
PEL: 5 mg/m3 (8-hour)
HA: 0.7 mg/L (lifetime)
TLV: Not Available

Diazinon*

Regulatory Status
Diazinon is classified as a Restricted Use Pesticide (RUP) and is for professional pest control operator use only. In 1988, the US EPA canceled registration of diazinon for use on golf courses and sod farms because of bird kills that often congregated in these areas. It is classified toxicity class II - moderately toxic, or toxicity class III - slightly toxic, depending on the formulation. Products containing diazinon bear the Signal Word WARNING or CAUTION.

Chemical Class
Organophosphate

Trade and Other Names
Trade names for this product include Basudin, Dazzel, Gardentox, Kayazol, Knox Out, Nucidol, and Spectracide. Diazinon may be found in formulations with a variety of other pesticides such as pyrethrins, lindane, and disulfoton.

Uses
Diazinon is a nonsystemic organophosphate insecticide used to control cockroaches, silverfish, ants, and fleas in residential, non-food buildings. Bait is used to control scavenger yellow jackets in the western U.S. It is used on home gardens and farms to control a wide variety of sucking and leaf eating insects. It is used on rice, fruit trees, sugarcane, corn, tobacco, and potatoes and on horticultural plants. It is also an ingredient in pest strips. Diazinon has veterinary uses for the fight against fleas and ticks. It is available in dust, granules, seed dressings, wettable powder, and emulsifiable solution formulations.

Toxicities
Acute toxicity: Acute toxic effects of diazinon are due to the inhibition of acetylcholinesterase, an enzyme needed for proper nervous system function. The range of doses that results in toxic effects varies widely with formulation and with the individual species being exposed. The toxicity of encapsulated formulations is relatively low because diazinon is not released readily while in the digestive tract. Some formulations of the compound can be degraded to more toxic forms. This transformation may occur in air, particularly in the presence of moisture, and by ultraviolet radiation. Most modern diazinon formulations in the U.S. are stable and do not degrade easily. The symptoms associated with diazinon poisoning in humans include weakness, headaches, tightness in the chest, blurred vision, nonreactive pinpoint pupils, salivation, sweating, nausea, vomiting, diarrhea, abdominal cramps, and slurred speech. Death has occurred in some instances from both dermal and oral exposures at very high levels.

The LD_{50} is 300 to 400mg/kg for technical grade diazinon in rats. The inhalation LC_{50} (4-hour) in rats is 3.5 mg/L. In rabbits, the dermal LD_{50} is 3600 mg/kg.

Chronic toxicity: Chronic effects have been observed at doses ranging from 10 mg/kg/day for swine to 1000 mg/kg/day for rats. Inhibition of red blood cell cholinesterase and enzyme response occurred at lower doses in the rats. Enzyme inhibition has been documented in red blood cells, in blood plasma, and in brain cells at varying doses and with different species.

Environmental Fate

Breakdown in soil and groundwater: Diazinon has a low persistence in soil. The $t_{1/2}$ is 2 to 4 weeks. Bacterial enzymes can speed the breakdown of diazinon and have been used in treating emergency situations such as spills. Diazinon seldom migrates below the top half inch in soil, but in some instances it may contaminate groundwater.

Breakdown in water: The breakdown rate is dependent on the acidity of water. At highly acidic levels, one half of the compound disappeared within 12 hours, while in a neutral solution, the pesticide took 6 months to degrade to one half of the original concentration.

Breakdown in vegetation: In plants, a low temperature and a high oil content tend to increase the persistence of diazinon. Generally the $t_{1/2}$ is rapid in leafy vegetables, forage crops, and grass; the range is from 2 to 14 days. In treated rice plants only 10% of the residue was present after 9 days. Diazinon is absorbed by plant roots when applied to the soil and translocated to other parts of the plant.

Environmental Concentrations[‡]

Surface Water: Diazinon (dissolved) was detected in estuary (2 of 2 stations), lake/reservoir (25 of 159 stations), spring (8 of 76 stations), and other surface water (3,213 of 12,034 stations) in the United States (US) at average concentrations of 0.045, 0.027, 0.044, and 0.085 ug/L, respectively. Diazinon was detected in 21,978 whole water samples from the USEPA STORET database with a range and average concentration of 0-33.4 ppm and 1.7 ppb, respectively. Diazinon was detected in 359 filtered water samples from the USEPA STORET database with a range and average concentration of 0-1.0 ppb and 0.031 ppb, respectively. In the National surface water-monitoring program from 1976 to 1980, diazinon was detected in 1.2% of the samples with a max concentration of 2.4 ppb. In selected streams in the US between 1968 to 1971, 1.6% of 448 samples had a range and average concentration of diazinon of 0.01-0.10 ppb and 0.04 ppb, respectively. The concentration of diazinon in the Beaver River (Beaver Falls, PA) was 0.09 ppb. In Ontario, Canada between 1975 to 1977, 0.1% of the samples collected from 11 agricultural watersheds had a range, max, and average concentration of diazinon of not detected-0.15 ppt, < 0.01ppt, and < 0.01 ppt, respectively. Diazinon was not detected (detection limit = 1 ppb) in a 1985-1987 study of drainage ditch water on farms in British Columbia, Canada in an area where the pesticide is known to be used even though the pesticide was sporadically found in the sediments of the ditches. Between 1983 and 1987, the highest concentration of diazinon in Sacramento-San Joaquin Delta water was 0.1 ug/L.

Drinking Water: Diazinon was not detected or quantified in 54 drinking water wells in California. Diazinon was not detected (< 1 ppt) in tap water from Ottawa, Ontario, Canada. Japanese tap water contained 0.9-4.7 ppt diazinon.

Ground Water: Diazinon (dissolved) was detected in groundwater in the US at 90 of 4,467 stations at an average concentration 0.071 ug/L.
Rain/Snow/Fog: The annual mean concentration of diazinon in precipitation (rain/snow) was 0.011 ug/L measured in Niigata City, Japan between April 1992 and March 1993; the highest concentration of diazinon (0.17 ug/L) was measured in August 1992. During the period between 1995-6, the diazinon concentration in rain and snow from the Sierra Nevada mountains ranged from < 0.057 to 19 ng/L. Fog-water collected from agricultural areas (Lodi, CA; Beltsville, MD; Monterey, CA; Parlier, PA) contained 22, 0.14, 3.6, 4.8, and 16.6 ug/L of diazinon, respectively.
Effluent: Diazinon was detected in the effluent of 1 of 10 wastewater treatment plants.
Sediment/Soil: Between 1976 and 1980, diazinon was detected in 0.5% of the sediment samples from the National surface water monitoring program, with a max concentration of 7.1 ppb. Diazinon was detected in the Scioto River (Highby, OH) at a concentration of 0.07 ppb. The concentration of diazinon in San Joaquin River sediment (in 1992) was < 0.5 ng/L.
As part of the US National Soils Monitoring Program in 1972, the concentration of diazinon, in 5.4% of the soil samples collected from 1,483 sites in 37 states, ranged from 0.07-0.17 ppm (dry wt) and averaged < 0.01 ppm. In 1972, diazinon was detected in soil from rice-growing areas of Texas - 7.8% of 99 samples had detectable levels of diazinon and 28% of 25 samples had a range and an average diazinon concentration of 0.01-0.06 ppm (dry wt) and 0.01 ppm, respectively. Diazinon was detected in 28% of 46 soil samples, with a max concentration of < 0.1 ppm, in 5 counties located in western Alabama. Diazinon was detected in 46% of soil samples taken from 28 farms in vegetable growing areas of Southwestern Ontario, Canada with a range and average concentration of trace-0.29 ppm and 8.9 ppm, respectively. Diazinon was sporadically found at a concentration up to 4.0 ug/kg in sediments, in drainage ditches, on farms in British Columbia in a study done between 1985 and 1987. The average and range of diazinon at agrochemical facilities in Illinois was 75 ug/kg and 2.3-17,000 ug/kg, respectively.

Atmospheric
Diazinon was measured in ambient air as part of a US National Monitoring Program in 1970, and the max and average concentration of diazinon in 61% of 787 samples taken in 14 states was 62.2 and 3.0 ng/m^3, respectively. In 1971, the max and average concentration of diazinon in 41% of 667 samples taken in 16 states was 27.9 and 2.0 ng/m^3, respectively. The concentration of diazinon was measured in ambient air at 10 locations in the US in 1980 and 48% of 123 samples had a max and average concentration of diazinon of 23 and 2.1 ng/m^3, respectively. In Pekin, IL between February and March (1980), the range and average concentration of diazinon in 73% of 11 samples was 1.4-10 and 3.3 ng/m^3, respectively.
Source Dominated: Air within 800 m of two formulation plants in Arkansas in 1970, from 66 samples with 62% positive, having a max concentration of 0.3-9.2 ng/m^3 and an average concentration of 1.8ng/m^3. In 1971, 60 samples were taken with 48% positive, having a max concentration of 0.3-5.8 ng/m^3 and an average concentration of 1.5 ng/m^3. In 1972, 64 samples were taken with 44% positive, having a max concentration of 0.5-18

ng/m^3 and an average of 3.2 ng/m^3. Air 275 m from a formulation plant in Tennessee in 1971, from 56 samples with 66% positive, having a max concentration of 0.5-27.9 ng/m3 and an average of 7.3 ng/m^3.

Indoor Air: As part of the National Human Exposure Assessment Survey (NHEXAS) in Arizona, the median concentration of diazinon in indoor air was 4.6 ng/m^3. In Jacksonville FL, Springfield MA, and Brownsville TX, the median concentration (ng/m^3) of diazinon in indoor air was [73 (summer) and 21 (winter)], [< 22 (spring) and <28 (winter)], and [3.5 (summer) and 1.4 (spring)], respectively. Also, as part of NHEXAS in Arizona, the median concentration of diazinon in house dust was 0.13 ng/m^3. In Jacksonville, FL and Brownsville, TX, the median concentrations (ng/m^3) of diazinon in house dust were [0.4 (winter)] and [0.07 (summer) and <22 (spring)], respectively. Between mid-September and mid-November 1991, diazinon was detected in household dust (in New Jersey) at a concentration of 90 ng/g.

Urban/Suburban: As part of the National Human Exposure Assessment Survey (NHEXAS) in Arizona, the median concentration of diazinon in outdoor air was < 2.1 ng/m^3. In Jacksonville FL and Springfield MA, the median concentration (ng/m^3) of diazinon in outdoor air was [<30 (summer) and <15 (winter)] and [< 22 (spring) and <28 (winter)], respectively. The estimated mean air concentration of diazinon in outdoor air for Springfield/Chicopee, MA in the spring 1987 and winter 1988 was 8.2 and 9.2 ng/m^3, respectively. The concentration of diazinon in ambient air near Kitakyushu City, Japan in 1992 was 0.05 ng/m^3.

Rural/Remote: The maximum and median concentrations, and percent detections of diazinon in air over the Mississippi River from New Orleans, LA to St. Paul, MN during June 1994 was 0.36 ng/m3, 0.08 ng/m3, and 100%, respectively.

Physical Properties

Appearance: Diazinon is a colorless to dark brown liquid.
Chemical Name: O,O-diethyl 0-2-isopropyl-6-methyl(pyrimidine-4-yl) phosphorothioate
CAS Number: 333-41-5
Molecular Weight: 304.35
Water Solubility: 40 mg/L @ 20 °C
Solubility in Other Solvents: petroleum ether vs. alcohol vs. benzene
Melting Point: Decomposes @ >120 °C
Vapor Pressure: 0.097 mPa @ 20 °C
Partition Coefficient: Not Available
Adsorption Coefficient: 1000 (estimated)

Exposure Guidelines

ADI: 0.002 mg/kg/day
MCL: Not Available
RfD: 9 x 10-5 mg/kg/day
PEL: Not Available
HA: 6 x 10-4 mg/L (lifetime)
TLV: 0.1 mg/m3 (8-hour)

Dichlorvos (DDVP)*

Regulatory Status
Dichlorvos is a Restricted Use Pesticide (RUP) and may be purchased and used only by certified applicators. The EPA has classified dichlorvos as toxicity class I - highly toxic, because it may cause cancer and there is only a small margin of safety for other effects. Products containing dichlorvos must bear the Signal Words DANGER - POISON.

Chemical Class
Organophosphate

Trade and Other Names
Trade names include Apavap, Benfos, Cekusan, Cypona, Derriban, Derribante, Devikol, Didivane, Duo-Kill, Duravos, Elastrel, Fly-Bate, Fly-Die, Fly-Fighter, Herkol, Marvex, No-Pest, Prentox, Vaponite, Vapona, Verdican, Verdipor, and Verdisol. Trade names used outside of the U.S. include Doom, Nogos, and Nuvan.

Uses
Dichlorvos is an organophosphate compound used to control household, public health, and stored product insects. It is effective against mushroom flies, aphids, spider mites, caterpillars, thrips, and white flies in greenhouse, outdoor fruit, and vegetable crops. Dichlorvos is used to treat a variety of parasitic worm infections in dogs, livestock, and humans. Dichlorvos can be fed to livestock to control botfly larvae in the manure. It acts against insects as both a contact and a stomach poison. It is used as a fumigant and has been used to make pet collars and pest strips. It is available as an aerosol and soluble concentrate.

Toxicities
Acute toxicity: Dichlorvos is highly toxic by inhalation, dermal absorption, and ingestion. Because dichlorvos is volatile, inhalation is the most common route of exposure. As with all organophosphates, dichlorvos is readily absorbed through the skin. Acute illness from dichlorvos is limited to the effects of cholinesterase inhibition. Compared to poisoning by other organophosphates, dichlorvos causes a more rapid onset of symptoms, which is often followed by a similarly rapid recovery. This occurs because dichlorvos is rapidly metabolized and eliminated from the body. Persons with reduced lung function, convulsive disorders, liver disorders, or recent exposure to cholinesterase inhibitors will be at increased risk from exposure to dichlorvos. Alcoholic beverages may enhance the toxic effects of dichlorvos. High environmental temperatures or exposures of dichlorvos to light may enhance its toxicity. Dichlorvos is mildly irritating to skin. Concentrates of dichlorvos may cause burning sensations, or actual burns. Application of 1.67 mg/kg dichlorvos in rabbits' eyes produced mild redness and swelling, but no injury to the cornea. Symptoms of acute exposure to organophosphate or cholinesterase-inhibiting compounds may include the following: numbness, tingling sensations, uncoordinated movement, headache, dizziness, tremor, nausea, abdominal cramps, sweating, blurred vision, difficulty breathing or respiratory depression, and/or slow heartbeat. Very high doses may result in unconsciousness, incontinence, and

convulsions or fatality. Some organophosphates may cause delayed symptoms beginning 1 to 4 weeks after an acute exposure that may or may not have produced immediate symptoms. In such cases, numbness, tingling, weakness, and cramping may appear in the lower limbs and progress to uncoordinated movement and paralysis. Improvement may occur over months or years, but some residual impairment may remain.

The oral LD_{50} for dichlorvos is 61 to 175 mg/kg in mice, 100 to 1090 mg/kg in dogs, 15 mg/kg in chickens, 25 to 80 mg/kg in rats, 157 mg/kg in pigs, and 11 to 12.5 mg/kg in rabbits. The dermal LD_{50} for dichlorvos is 70.4 to 250 mg/kg in rats, 206 mg/kg in mice, and 107 mg/kg in rabbits. The 4-hour LC_{50} for dichlorvos is greater than 0.2 mg/L in rats.

Chronic toxicity: Repeated or prolonged exposure to organophosphates may result in the same effects as acute exposure, including the delayed symptoms. Other effects reported in workers repeatedly exposed include impaired memory and concentration, disorientation, severe depressions, irritability, confusion, headache, speech difficulties, delayed reaction times, nightmares, sleepwalking, and drowsiness or insomnia. An influenza like condition with headache, nausea, weakness, loss of appetite, and malaise has also been reported. Repeated, small doses generally have no effect on treated animals. Doses of up to 4 mg/kg of a slow release formulation given to cows to reduce flies in their feces had no visibly adverse effects on the cows. But, blood tests of these cows indicated cholinesterase inhibition. Feeding studies indicate that a dosage of dichlorvos very much larger than doses that inhibit cholinesterase are needed to produce illness. Rats tolerated dietary doses as high as 62.5 mg/kg/day for 90 days with no visible signs of illness, while a dietary level of 0.25 mg/kg/day for only 4 days produced a reduction in cholinesterase levels. Rats exposed to air concentrations of 0.5 mg/L of dichlorvos over a 5-week period exhibited significantly decreased cholinesterase activity in the plasma, red blood cells, and brain. Dogs fed dietary doses of 1.6 or 12.5 mg/kg/day for 2 years showed decreased red blood cell cholinesterase activity, increased liver weights, and increased liver cell size. Chronic exposure to dichlorvos may also cause fluid to build up in the lungs (pulmonary edema). Liver enlargement has occurred in pigs maintained for long periods of time on high doses. Dichlorvos caused adverse liver effects, and lung hemorrhages may occur at high doses in dogs. In male rats, repeated high doses caused abnormalities in the tissues of the lungs, heart, thyroid, liver, and kidneys

Environmental Fate

Breakdown in soil and groundwater: Dichlorvos has low persistence in soil. Half-lives of 7 days were measured on clay, sandy clay, and loose sandy soil. In soil, dichlorvos is subject to hydrolysis and biodegradation. Volatilization from moist soils is expected to be slow, and the pH of the media determines the rate of breakdown. Breakdown is rapid in alkaline soils and water, but it is slow in acidic media. For instance, at pH 9.1 the $t_{1/2}$ of dichlorvos is about 4.5 hours. At pH 1 (very acidic), the $t_{1/2}$ is 50 hours. Dichlorvos does not adsorb to soil particles, and it is likely to contaminate groundwater. When spilled on soil, dichlorvos leached into the ground with 18 to 20% penetrating to a depth of 12 inches within 5 days.

Breakdown in water: In water, dichlorvos remains in solution and does not adsorb to sediments. It degrades primarily by hydrolysis, with a $t_{1/2}$ of approximately 4 days in lakes and rivers. This $t_{1/2}$ will vary from 20 to 80 hours between pH 4 and pH 9; hydrolysis is slow at pH 4 and rapid at pH 9. Biodegradation may occur under acidic conditions, which slow hydrolysis, or where populations of acclimated microorganisms exist, as in polluted waters. Volatilization from water is slow, and it has been estimated at 57 days from river water and over 400 days from ponds.
Breakdown in vegetation: Except for cucumbers, roses, and some chrysanthemums, plants tolerate dichlorvos very well.

Environmental Concentrations[‡]
Surface Water: Dichlorvos has been detected in a water reservoir and water supply-irrigation system in the USSR and in 4 polluted rivers. On September 9 to 11, 1988, dichlorvos was detected in marine waters off Beirtreach Bay, Ireland at concentrations up to 0.13 ug/L. Dichlorvos was found in the Yamaska River and its tributaries in Quebec, Canada in 1986-7. Its concentration at a site near the mouth of the river was 8.2 and 1.7 ng/L on two occasions, but it was not detected on two other occasions. Dichlorvos was detected in the Neya-gawa River in Osaka City and Osaka Bay, Japan during monitoring studies conducted in 1989-1990; concentration levels were not reported. Dichlorvos was not found in monthly samples of surface water taken at six sites between August 1989 and January 1990 in an area of the Netherlands used for bulb culture.
Drinking Water: No dichlorvos was detected in California well water performed between 7/1/1994 and 6/30/1995 as part of the state's well water inventory in which 46 wells in 7 counties were sampled.
Effluent: Dichlorvos was detected in wastewater from a dichlorvos production plant in Bulgaria, 16 g/L. As a result of a fire at Sandoz, Ltd. near Basel, Switzerland in November 1986, it was estimated that 1-3 kg was discharged into the Rhine River; the estimated water concentration at Village-Neuf was 0.15-0.65 ug/L.

Atmospheric
Indoor Air: As part of EPA's Non-Occupational Pesticide Exposure Study (NOPES) conducted in the summer 1986, spring 1987 and winter 1988 in Jacksonville, FL and Springfield/Chicopee, MA the estimated mean indoor air concentration of dichlorvos for Jacksonville residents was 134.5, 86.2, and 24.5 ng/m^3, respectively. The estimated spring and winter concentrations for Springfield/Chicopee residents were 4.3 and 1.5 ng/m^3, respectively. In Jacksonville, it was estimated that the percentage of residents with detectable levels of dichlorvos in indoor air was 33%, 14%, and 10% in summer, spring and winter, respectively. In Springfield/Chicopee only 2% and 1% of residents were exposed in spring and winter, respectively. A study comparing the concentration of indoor pesticides in air and household dust that determined dichlorvos levels in 7 representative New Jersey homes, found only one house with detectable air concentrations of dichlorvos; the average level in this house was 254.7 ng/m^3. No dichlorvos was found in household dust in this house. Concentration levels in households and food shops in which commercial pesticide strips were used were 0-26 ppb and < 1-3 ppb, respectively. Trials were conducted in the U.K., Australia and France between 1967 and 1970 to determine the concentration of dichlorvos in the air of homes

using "Vapona" strips. Results from more than 3000 air samples indicated that the great majority of values were 0.1 ug dichlorvos/liter of air or less; values ranged from < 0.01 to 0.24 ug/L, with the higher values being associated with closed-up homes or the use of multiple strips. In each trial the concentration of dichlorvos rose rapidly and then fell exponentially. In temperate area trials, the concentration was highest 1-2 weeks after placing the strips; the geometric mean of all values at this time was 0.04 ug/L, and 3 months after placement the mean concentration was 0.01 ug/L.
Urban/Suburban: As part of the US EPA's Non-Occupational Pesticide Exposure Study (NOPES) conducted in the summer 1986, spring 1987, and winter 1988, in Jacksonville, FL and Springfield/Chicopee, MA the estimated mean outdoor air concentration of dichlorvos in Jacksonville was 0, 0, and 3.2 ng/m^3 in the summer, spring, and winter, respectively. Dichlorvos was not detected in outdoor air in Springfield/Chicopee in the spring and winter. It was also not detected in the air of Kitakyushu City, Japan.

Physical Properties
Appearance: Dichlorvos is a colorless to amber liquid with a mild chemical odor.
Chemical Name: 2,2-dichlorovinyl dimethyl phosphate
CAS Number: 62-73-7
Molecular Weight: 220.98
Water Solubility: 10,000 mg/L (estimated)
Solubility in Other Solvents: dichloromethane vs. 2-propanol, vs. toluene vs. ethanol vs. chloroform vs. acetone vs. kerosene
Melting Point: Not Available
Vapor Pressure: 290 mPa @ 20 °C
Partition Coefficient: Not Available
Adsorption Coefficient: 30 (estimated)

Exposure Guidelines
ADI: 0.004 mg/kg/day
MCL: Not Available
RfD: 0.0005 mg/kg/day
PEL: 1.0 mg/m3 (8-hour) (skin)
HA: Not Available
TLV: Not Available

Parathion (Ethyl)†

Regulatory status
Because of its high toxicity and risks of exposure to agricultural workers and to birds, and in response to the manufacturers' request, the US EPA in January 1992 announced the cancellation of all uses of parathion on fruit, nut, and vegetable crops. The only uses retained are those on alfalfa, barley, corn, cotton, sorghum, soybeans, sunflowers, and wheat. Furthermore, to reduce exposure to agricultural workers, parathion may be applied to these crops only by commercially certified aerial applicators and treated crops may not be harvested by hand. The US EPA intends to cancel all uses of parathion in the near future.

Parathion is one of the most acutely toxic pesticides registered by the US EPA. Because of its highly toxic nature, parathion is classified as a Restricted Use Pesticide (RUP). RUP's may be purchased and used only by certified applicators. Products containing parathion must bear the Signal Word - Danger.

Chemical Class
Organophosphate

Trade or other names
Trade names include AC 3422, Alkron, Alerons, Aphamite, Corothion, E-605, ENT 15108, Ethyl parathion, Etilon, Fosferno 50, Niran, Orthophos, Panthion, Paramar, Paraphos, Parathene, Parawet, Phoskil, Rhodiatox, Soprathion, Stathion and Thiophos. The common name Thiophos is used in the former USSR.

Uses
Parathion is a broad spectrum, organophosphate pesticide used to control many insects and mites. It has non-systemic, contact, stomach, and fumigant actions. It has a wide range of applications on many crops against numerous insect species. Parathion is available in dust, emulsion concentrate, granular, ULV liquid, and wettable powder formulations.

Toxicities
Acute Toxicity: Parathion is highly toxic by all routes of exposure. Human fatalities have been caused by ingestion, dermal adsorption, and inhalation of parathion. As with all organophosphates, parathion is readily absorbed through the skin. Skin that has come into contact with this material should be washed immediately with soap and water and all contaminated clothing should be removed. Persons with cardiovascular, liver or kidney diseases, glaucoma, or central nervous system abnormalities may be at increased risk from exposure to parathion. High environmental temperatures or exposure of the chemical to visible or UV light may increase its toxicity. Parathion may cause thickening and roughening of the skin (hyperkeratinization). It does not cause sensitization (allergies). Parathion is not irritating to the eyes, but splashing parathion into an eye may cause constriction of the pupil, making it difficult to determine the path of moving

objects. Organophosphates are used to treat diseases of the eye, like glaucoma; however, it is possible that they cause cataracts to form. The organophosphate insecticides are cholinesterase inhibitors. When inhaled, the first effects are usually respiratory and may include bloody or runny nose, coughing, chest discomfort, difficult or short breath, and wheezing due to constriction or excess fluid in the bronchial tubes. Skin contact with organophosphates may cause localized sweating and involuntary muscle contractions. Eye contact will cause pain, bleeding, tears, pupil constriction, and blurred vision. Following exposure by any route, other systemic effects may begin within a few minutes or be delayed for up to 12 hours. These symptoms may include pallor, nausea, vomiting, diarrhea, abdominal cramps, headache, dizziness, eye pain, blurred vision, constriction or dilation of the eye pupils, tears, salivation, sweating, and confusion. Severe poisoning will affect the central nervous system, producing uncoordinated movement, slurred speech, loss of reflexes, weakness, fatigue, involuntary muscle contractions, twitching, tremors of the tongue or eyelids, and eventually paralysis of the body extremities and the respiratory muscles. In severe cases there may also be involuntary defecation or urination, psychosis, irregular heartbeats, unconsciousness, convulsions and coma. Death may be caused by respiratory failure or cardiac arrest.

The oral LD_{50} for parathion is 2 to 30 mg/kg in rats, 5 to 25 mg/kg in mice, 8 to 32 mg/kg in guinea pigs, 10 mg/kg in rabbits, 0.93 mg/kg in cats, and 3 to 5 mg/kg in dogs. The dermal LD_{50} in rats is 6.8 to 50 mg/kg, in mice 19 mg/kg, in guinea pigs 45 mg/kg, and in rabbits 15 mg/kg. The lowest dosage with toxic effects (TDlo) in humans is 240 ug/kg (less than 0.1 ounce). The LC_{50} (4-hour inhalation) for parathion in rats is 84 mg/m^3.

Chronic Toxicity: Repeated or prolonged exposure to organophosphates may result in the same effects as acute exposure including the delayed symptoms. Other effects reported in workers repeatedly exposed include impaired memory and concentration, disorientation, severe depressions, irritability, confusion, headache, speech difficulties, delayed reaction times, nightmares, sleepwalking and drowsiness or insomnia. An influenza-like condition with headache, nausea, weakness, loss of appetite, and malaise has also been reported. One study found that dietary doses of 50 ppm (about 2.5 mg/kg/day) produced toxic symptoms, growth retardation, and death in rats. In another feeding study, dietary doses of 2.5 mg/kg/day for 2-years had no effect on rats, while doses of 5 mg/kg/day produced only slight signs of toxicity and growth retardation, but no deaths.

Environmental Fate

Breakdown of Chemical in Soil and Groundwater: Parathion has little or no potential for groundwater contamination. It binds tightly to soil particles and is degraded by biological and chemical processes within several weeks. Degradation is faster in flooded soil. Residues of parathion can persist for many years, but usually remain in the upper 6 inches of soil. Photo-degradation may occur on soil surfaces, and sunlight can convert parathion into the active metabolite paraoxon, which is more toxic than parathion. The breakdown of parathion in soil or water increases with increasing (more alkaline) pH. Soil microorganisms, sunlight, plants, and water all breakdown parathion.

Breakdown of Chemical in Water: In open water, parathion will usually disappear within a week, mainly by adsorption to suspended particles and bottom sediments. Adsorbed parathion is subject to degradation by microorganisms and chemical hydrolysis. The $t_{1/2}$ for photo-degradation of parathion in water is 1 to 10 days. Increasing the pH (alkalinity) increases the rate of breakdown.
Breakdown of Chemical in Vegetation: Following spray applications, parathion residues on foliage will decay with a $t_{1/2}$ of 1 day, reaching low levels in a week or two. In orange groves, the $t_{1/2}$ of parathion is as long as one month, although, it is usually closer to one-two weeks. Most crops tolerate parathion very well; only at high application rates do apples, cucumbers, and tomatoes suffer from parathion usage.

Environmental Concentrations[‡]
Surface Water: Parathion was detected in the following samples collected during a USGS survey of western streams in which 20 stations were analyzed for parathion quarterly from January to June 1970 and monthly from July 1970 to September 1971: Gila River, AZ 40 ppt (2 samples), Sacramento River at Verna CA, 40 ppt, and 160 ppt (2 samples). Parathion was not detectable in any water sample in Little Miami River, OH above and below municipal wastewater outfall, July-September 1984. In a survey of surface waters in Germany 5 to 65 ppt were detected in 4 of 119 samples of unfiltered water from 28 locations, and 0.15 to 0.4 ppt in suspended solids in 3 samples from 20 sites. It was not detected in any water or suspended particulate matter samples in Lake Superior, and Lake Huron, including the Georgian Bay at quantitation limits of 5 ppt and 100 pg, respectively. The highest parathion concentration reported in surface water was 0.4 ppb. In the Erie River Basin, parathion was not detected in the more than 100 samples tested. Parathion was detected at 0.6% of 174 sampling stations of the nation's rivers. Parathion was detected in water samples collected from the Po River and the Adriatic Sea between April and August 1988, at concentrations ranging from < 1 to 8.9 ng/L; the highest concentration was detected in the Po River near the town of Ferrara, Italy in June 1988. Parathion was detected in a water sample collected from the Pinios River, Greece in February 1993, at a concentration of 0.15 ug/L. Parathion was detected in water from the Rhine River near Cologne at a concentration of 0.08 ug/L. 8 of 40 water samples collected from the Valencia Community, Spain, between February 1992 and July1992, contained parathion at concentrations ranging from 0.012 to 0.176 ug/ml. Parathion was also detected in ditch water draining cranberry bogs treated with parathion in the Lower Fraser Valley of British Columbia, Canada; concentrations ranged from not detected to 21 ug/L. Parathion was detected in farm ditch water collected from the Lower Fraser Valley of British Columbia, Canada, between July and December 1991, at mean concentrations of 0.13 and 0.20 ug/kg at Westham Island and Cloverdale, respectively.

Drinking Water: Parathion was not detected in samples of drinking water from 10 to 13 US cities collected between October 1975 and March 1982 for Infant and Toddler Total Diet samples. It was not detected in beverages (which included drinking water) in the adult Total Diet Studies from October 1965 to March 1982. No parathion was detected in 54 monitored wells in selected California communities. The wells selected for monitoring were primarily municipal supply systems near agricultural areas. Contaminated drinking water wells in CA contained 4.6 ppb. In unspecified drinking

water, parathion was detected at a level of 30 ppt. It was not detected in Ottawa tap water (detection limit: < 1 ppt).

Groundwater: Parathion was detected in well water in Florida (125-185 ft depth) at 1 ppb - agricultural source of contamination. Parathion was detected in a CA ground water aquifer at a concentration ranging from 4 to 6 ug/L. The highest concentration of parathion detected in Sacramento-San Joaquin Delta water was 0.035 ug/L.

Rain/Snow/Fog: Parathion was detected in atmospheric fog water from Parlier, Corcoran and Lodi, CA at concentrations of 9,000, 950 and 184,000 ng/L, respectively. It was detected in fog samples collected near Parlier, CA in January 1986 at concentrations of 3.6, 31, 30, 2.7, 39, and 23 ug/L. Parathion was detected in fog water samples collected in January 1989 in San Joaquin Valley, CA, during spraying near dormant orchards at concentrations ranging from 4.3 to 19.0 ppb. Parathion was detected in 11 rainfall, throughfall, and stemfall samples collected in two forests in Italy from May to October 1988, the concentrations ranged from 0.01 to 0.17 ug/L. Parathion was detected in wet deposition samples from Lindcove Field Station, elevation 114 m, in California's Sierra Nevada Mountains in December 1990, January, and February 1991, at concentrations ranging from 53 to 7,600 pg/ml. It was detected in wet deposition samples from Ash Mountain, elevation 533 m, at concentrations ranging from less than the quantification limit to 270 pg/ml. Parathion was detected in three wet deposition samples from Kaweah, elevation 1920 m, in February and March 1991, at concentrations of 7.7, 34, and 1.7 pg/ml.

Effluent: Ontario drainage ditch water contained 2 and 4 ppt (original soil vegetable growing area) from soil containing 0.6 and 2 ppm of parathion, respectively. Parathion was detected in lagoon water used to collect irrigation runoff from corn and sorghum fields in Kansas at a maximum concentration of 6.2 ppb.

Sediment/Soil: Parathion was detected in the organic soil of 12 of 28 farms in 6 vegetable growing regions of southwestern Ontario in 1976, at concentration ranging from 6 to 2500 ppb. Parathion was detected in 7 of 11 sediment sampling stations from Nissum Broad of Jutland, Denmark at concentrations of 1.4, 15.7, 19.9, 29.9, 43.1, 3198 and 3825 ug/kg. Parathion was under the detection limit of 0.1 ppb at all locations in the Delaware River estuary, an industrial and agricultural area (USGS survey - 11 samples). It was not detected in any sediment samples from Lake Superior or Lake Huron, including Georgian Bay at a quantitation limit of 20 ppb. It was not detected in urban soils from 5 US cities in the 1971 Urban Soils Monitoring Program. In a survey of rice growing soils in 5 states in 1972 (99 samples) parathion was detected in: Arkansas 4.2% positive, 0.01 ppm (dry weight) mean, 0.12 ppm max and California 10% positive, < 0.01 ppm (dry weight) mean, and 0.01 ppm max. In the National Soils Monitoring Program in 1972 (1246 samples of cropland soil in 37states) parathion was detected in 0.6% of the samples, < 0.01 ppm mean, and 0.19 ppm max. Only 1 sample of bottom sediment from the Lake Erie Basin contained parathion, 3.27 ppb in Huron River. Parathion was detected in Southern Ontario orchard soil, 5% of apple orchards contained parathion at 6 ppb. Parathion residues were found in the upper 15 cm, not in the 15-30 cm subsurface soil. It was not detected in sediment in irrigation water collection lagoons from corn and sorghum fields in Kansas in 1974.

Parathion was detected in bed sediments and suspended sediments collected from three locations in the Windrush River catchment in 1992 at concentrations of 0.3, 0.6, and 1.0

ug/kg in the bed sediments, and 13, 3.3, and 8.8 ug/kg in the suspended sediments. Parathion was detected in sediments in a ditch-draining cranberry bogs treated with parathion in the Lower Fraser Valley of British Columbia, Canada; concentrations ranged from not detected to 515 ug/kg. Parathion was detected in farm ditch sediments collected from the Lower Fraser Valley of British Columbia at a concentration of 8 ug/kg in Vancouver.

Parathion was detected in soil samples collected from an evaporation pit in California between May and September 1985 at concentrations ranging from 1,064 to 1,972 ppm 0 to 7.5 cm below the surface, 51 to 60 ppm 7.5 to 15 cm below the soil surface, 37 ppm 22.5 to 30 cm below the soil surface, and 18 ppm 60 to 67.5 cm below the soil surface. It was detected in soil from the center of the evaporation at a depth of 90 cm at a concentration of 143 ppm. Parathion was detected in crop soils collected from the Lower Fraser Valley of British Columbia, Canada, between July and December 1991, at mean concentrations of 10, 19, 15, and 1,419 ug/kg in Westham Island, Ladner, Burnaby, and Cloverdale, respectively. Parathion was detected in 4 of 822 soil samples collected from 49 agrochemical facilities located throughout Illinois at concentrations ranging from 69 to 5,540 ug/kg, and median concentration of 805 ug/kg. Parathion was detected in soil samples collected in the vicinity of an operating apple or pear orchard in eastern Washington State at a mean concentration of 46 ng/g, and range not detected to 932 ng/g.

Atmospheric

Source Dominated: Parathion concentrations in the air of a plum orchard were 3500 ng/m3 immediately after spraying and 4100, 394, 149, 21, and 16 ng/m^3 1, 2, 5, 14, and 21 days after spraying, respectively. 100 m downwind from the plum orchard parathion concentrations of 35, 9, 1.6, and 0.9 ng/m^3 were detected 2, 3, 6, and 21 days after spraying, respectively. 24-hour ambient air samples were collected near dormant orchards in the San Joaquin Valley, CA, for 17 days in Jan 1989, the most intensive period of dormant orchard spraying; parathion was detected at an average concentration of 63.5 ng/m^3. The average daytime and nighttime concentrations of parathion were 52.0 and 119.6 ng/m^3, respectively.

Urban/Suburban: Parathion was detected as follows in the National Air Pesticide Monitoring Program, 1970-1972 (selected sites in 14-16 states): 1970 - 3.2% of 787 samples positive, 64.2 ng/m^3 mean of positive samples, 834 ng/m^3 max; 1971 - 2.3% of 667 samples positive, 9.3 ng/m^3 mean of positive samples, 109 ng/m^3 max; 1972 - not detected in 1025 samples. Parathion was detected in suburban air in Miami, FL (60% positive, 2.8 ng/m^3 mean, 12.1 ng/m^3 max), but not in Jackson, MI or Fort Collins, CO. In a pilot study of pesticidal air contamination (4 sites in 9 locations throughout the US, 880 composite samples), 4 samples collected around Orlando, FL contained a maximum parathion concentration of 2.0 ng/m^3. Parathion was detected in air samples collected at Parlier, CA on January 12, 1986 at a total concentration of 9.4 ng/m^3, 78.5% was in the vapor phase.

Rural/Remote: Parathion was detected in 2 ambient air samples (detection limit: 0.009 ng/m3) collected between April and June 1989 at Vallombrosa forest, Italy, concentrations were 0.84 and 0.36 ng/m3. Parathion was detected in air samples from Lindcove Field Station, elevation 114 m, in California's Sierra Nevada Mountains in November 1990 and January to March 1991 at concentrations ranging from 26 to 13,000

pg/m3; air concentrations at Ash Mountain, elevation 533 m, ranged from less than the limit of quantification to 49 pg/m3.

PHYSICAL PROPERTIES AND GUIDELINES

Parathion hydrolyzes slowly at pH 7 or below, but is otherwise stable at normal temperatures. At temperatures above 120 degrees °C, parathion decomposes and may develop enough pressure to cause containers to explode. Thermal decomposition may release toxic gases such as diethylsulfide, sulfur dioxide, carbon monoxide, carbon dioxide, phosphorus pentoxide, and/or nitrogen oxides. Parathion poses a fire and explosion hazard in the presence of strong oxidizers, and it may attack plastics, rubber, and coatings.
The National Fire Protection Agency ratings for parathion include: A. Health: 4 = a few whiffs of the fumes could prove fatal; normal fire fighting gear is inadequate to protect against any exposure to the skin. B. Flammability: 1 = solids which must be preheated to burn, but which are combustible. It may be dangerous to use water to extinguish burning parathion. C. Reactivity: 2 = normally unstable materials which will react violently (with water.

Physical Properties

Appearance: Pure parathion is a pale yellow liquid with a faint odor of garlic at temperatures above 6 degrees oC. Technical parathion is a deep brown to yellow liquid.
Chemical Name: O,O-diethyl O-4-nitrophenyl phosphorothioate
CAS Number: 56-38-2
Molecular Weight: 291.3
Water Solubility: 12.4 mg/l at 25 degrees °C (24 ppm)
Solubility in Other Solvents: It is soluble in alcohols, animal & vegetable oils, aromatic hydrocarbons, esters, ethers, n-hexane, dichloromethane, 2-propanol, toluene, and ketones. It is insoluble in kerosene, petroleum ether, or spray oil.
Melting Point: 6 °C
Vapor Pressure: 8.9 x 10-6 mm Hg @ 20 °C
Partition Coefficient: 3.83
Adsorption Coefficient: Not Available

Exposure Guidelines

ADI: 0.004 mg/kg b.w.
MCL: Not Available
RfD: Not Available
PEL: Not Available
HA: Not Available
TLV: Not Available

Abbreviations and Exposure Guidelines Reference

Abbreviations

LD_{50} (oral or dermal): Lethal Dose - The amount of pesticide active ingredient (or formulated product) applied in milligrams per kilogram (mg/kg) of test animal body weight in which 50% of the test animals die.
LC_{50}: Lethal Concentration – The airborne concentration of the test substance that is lethal to 50% of the test animal over a specific exposure time (usually 4 hours). It is expressed in terms of the amount of pesticide or formulation (in mg) in a given volume of air (either in L or m3).

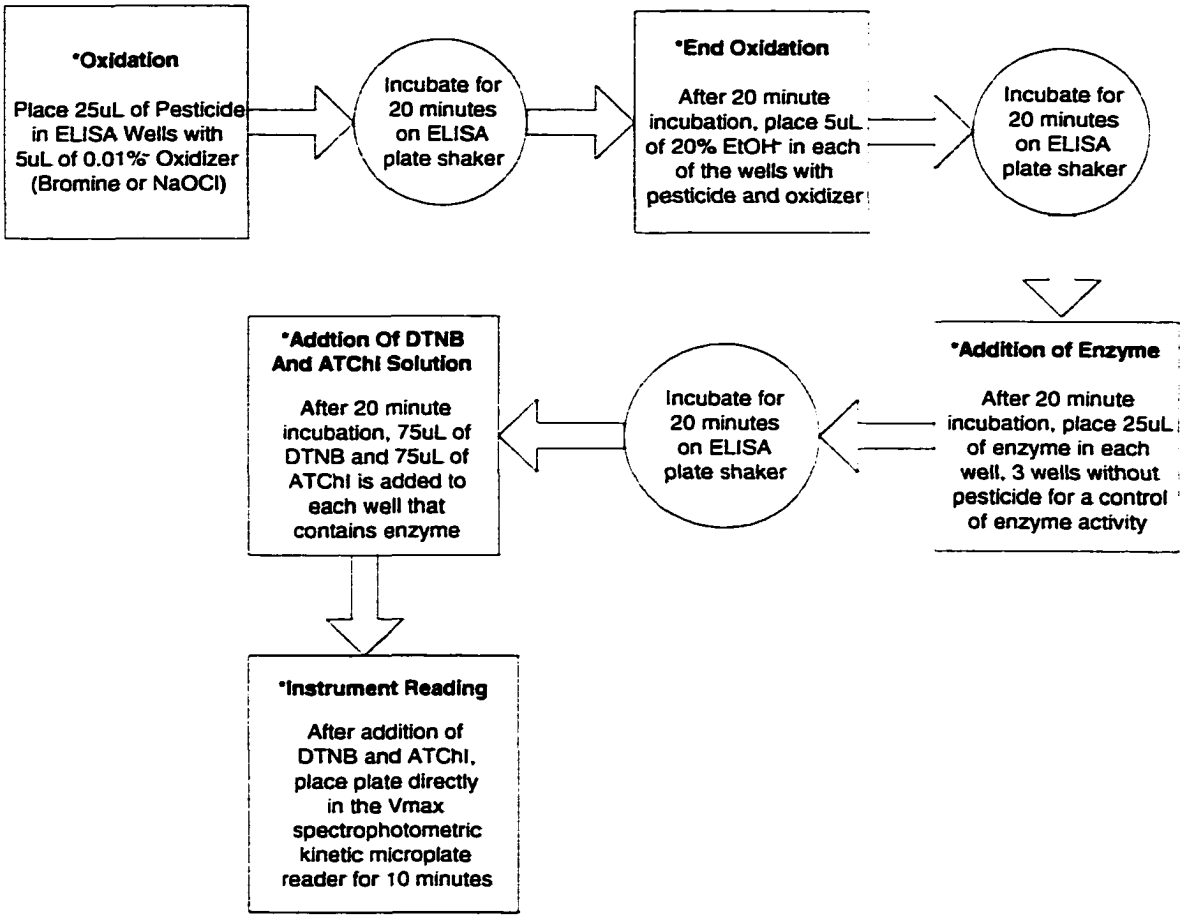
Exposure Guidelines

ADI: Acceptable Daily Intake – Obtained in a similar fashion to the RfD using animal study data, and incorporating scientific uncertainty.
MCL: Maximum Contaminant Level – The standard established under the Safe Drinking Water Act for the maximum permissible level of a contaminant in drinking water that is delivered to users of a public water system.
RfD: Reference Dose – The dose of the administered chemical that during an entire lifetime, appears to be without appreciable risk.
PEL: Permissible Exposure Limits – The acceptable levels of exposure to airborne contaminants in the workplace environment (established by OSHA).
HA: Health Advisory levels – Refer to drinking water contaminant levels that would no be anticipated to cause adverse health effects over a given exposure period.
TLV: Threshold Limit Value – The maximum workplace concentrations of air contaminants to which nearly all workers may be repeatedly exposed without adverse effects. Established by the American Conference of Governmental Industrial Hygienists (ACGIH), this value is used where the PEL is not available.

*Source: Kamrin, M. A. (Ed.) (1997). Pesticide Profiles: Toxicity, Environmental Impact, and Fate. Boca Raton: Lewis Publishers.
†Source: Extension Toxicology Network (EXTOXNET) (March 2002). *Pesticide Information Profiles: Parathion* [WWW document]. URL http://ace.orst.edu/info/extoxnet/pips/parathio.htm [4 March 2002].
‡Source: Toxicology Data Network (TOXNET) (March 2002). *Hazardous Substances Data Base* [WWW document]. URL http://www.toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB [5 March 5, 2002].

APPENDIX II

DIAGRAM OF EIB 96 ASSAY STEPS AND PROTOCOL



*** Details for each step of this assay (the oxidation steps have been modified as indicated in diagram with a [~]) can be found in the protocol.**

Figure 29. Basic diagram of EIB 96 assay steps.

PROTOCOL FOR ORGANOPHOSPHATES USING ELLMAN'S METHOD AND OXIDATIONS OF ORGANOPHOSPHATES WITH -P=S GROUP

Test Kit Protocol

Test Sample:

To make 1mM take (amount from vial) and dilute up to 1000uL of NP water
To make 1uM solution, take 1uL of 1mM and dilute up to 1000uL
To make 1nM solution, take 1uL of 1uM and dilute up to 1000uL

Serial dilution (1:10 dilution)
Take 9 sets of 1.5 mL tubes and place 900 uL of water in each tube, and then transfer 100uL from 1mM solution to 1st tube. Take 100uL (from 1st tube) → 2nd tube (take 100uL) → 3rd tube (take 100uL) → 4th tube (take 100uL) → 5th tube (take 100uL) → 6th tube and so on until 9th tube has been produced.

The concentration is

10^{-1} uM = 100nM		(1st tube)
10^{-2} uM = 10nM		(2nd tube)
10^{-3} uM = 1nM		(3rd tube)
10^{-4} uM = 0.1nM	= 100pM	(4th tube)
10^{-5} uM = 0.01nM	= 10pM	(5th tube)
10^{-6} uM = 0.001nM	= 1pM	(6th tube)

Ellman's Method

The EIB 96 assay is based on the measurement change in absorbance at 405 nM (the range set for the absorbance reading is 405 to 490 nM). The assay uses the thiol ester acetylthiocholine instead of the oxy ester acetylcholine (endogenous in organisms). AChE hydrolyses the acetylthiocholine to produce thiocholine and acetate. The thiocholine in turn reduces the 5,5-Dithiobis (2-Nitrobenzoic acid) (DTNB) freeing nitrobenzoate, which absorbs at 405 nM.

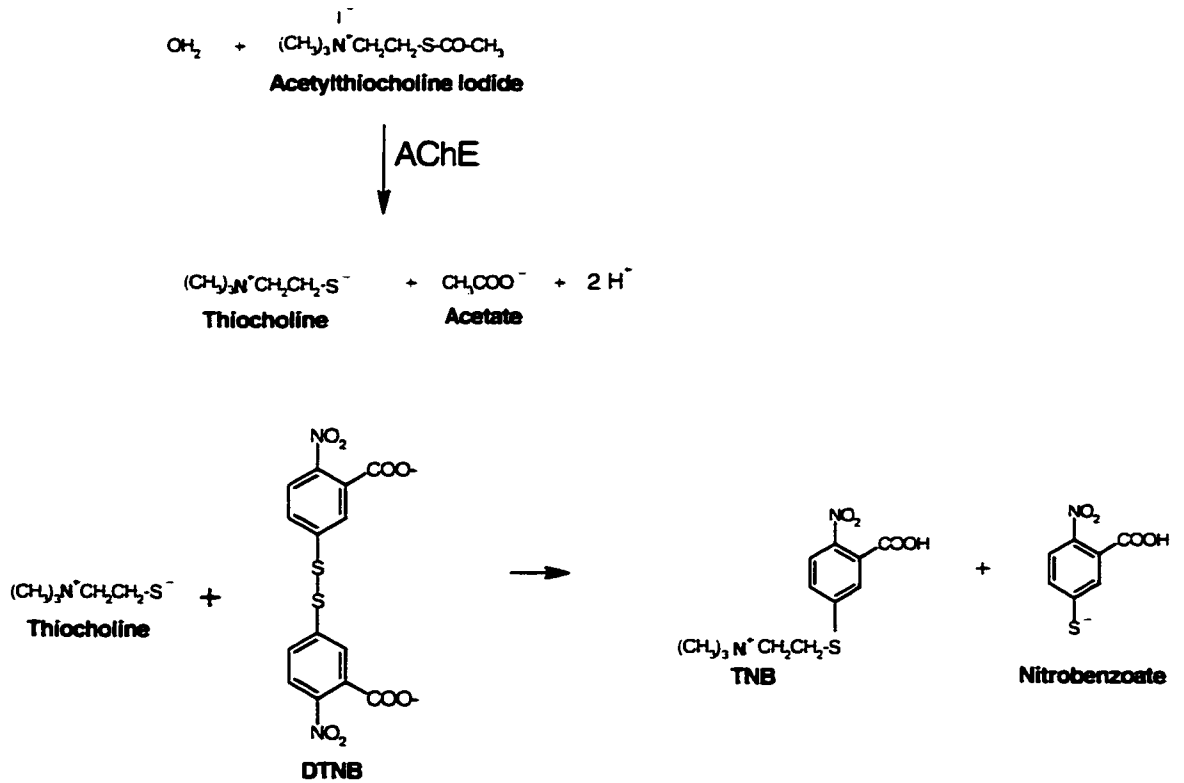


Figure 30. Reduction of DTNB by the hydrolysis of acetylthiocholine by AChE.

Preparation of Solutions:

1. To make (**TGGSASC**) Solution [D-(+)-Trehalose Dihydrate, Glucose, Gelatin, Sodium Azide, Sodium Chloride]

D-(+)-Trehalose Dihydrate
Molecular Formula: $C_{12}H_{22}O_{11}.\ 2H_2O$
Molecular Weight: 378.3
Storage Temp: Store at RT.

[A 1% solution is defined as 1 gram per 100 ml volume. To find the weight (in grams) needed for a particular volume (in ml) of solution, convert the desired percentage to a decimal (divide by 100) and multiply by the ml final volume desired.]

To make 5% of D-(+)-Trehalose Dihydrate in 5 mL

$$\frac{5}{100} \times 5\ mL = 0.25g$$

D-(+)-Glucose
Molecular Formula: $C_6H_{12}O_6$
Molecular Weight: 180.2
Storage Temp: Store at RT.

To make 5% of D-(+)- Glucose

$$\frac{5}{100} \times 5\ mL = 0.25g$$

Gelatin
Synonyms: Gelfoam, Puragel
High Average Mol. Weight (MW), Contain 25% glycine, 8.7% alanine, 2.5% valine, 3.2 % leucine,
1.4% isoleucine, 0.1 % cystine and cysteine, 1.0% methionine, 2.2% phenylalanine, 18.0% proline, 14% hydroxyproline, 0.4% serine, 1.9% threonine, 0.5% tyrosine, 6.6% aspartic acid,
11.4% glutamic acid, 8.1% arginine, 4.1% lysine, 0.8% histidine.
A protein stabilizer offered as an alternative to BSA and HSA.
Soluble in water, Store at RT.

To make 0.1% Gelatin in 5 mL

$$\frac{0.1}{100} \times 5\ mL = 0.005g$$

Sodium Azide

Molecular Formula: NaN3, Molecular Weight: 65.01
Store at RT. Highly Toxic

To make 0.02% Sodium Azide in 5 mL

$$\frac{0.02}{100} \times 5\ mL = 0.001g$$

Sodium Chloride

Molecular Formula: NaCl, Molecular Weight: 58.45
Store at RT.

To make 1% Sodium Azide in 5 mL

$$\frac{1}{100} \times 5\ mL = 0.05g$$

Dissolve 0.25 g Trehalose, 0.25g of glucose, 0.005 g of Gelatin, 0.001 g of Sodium Azide and 0.05g of sodium chloride in 5 mL of NP water. This is TGGSASC solution----------(A)

Acetylcholinesterase Enzyme (AchE), MW = 260,000

In Original Solution, the concentration of AChE is = 458 ug/mL = 0.458 ug/uL = 458 ng/uL
So one uL contain 458ng of Enzyme in original solution which is stored in freezer (white box) in
QAL-2.

Immobilization of Enzyme:

1. Take 99 uL of **solution A (*TGGSASC*)** in well and then place 1 uL (458ng) of enzyme
2. Mix it for 10 min.
3. Put this solution at room temperature for overnight drying
4. Next day you will find dry enzyme,
5. Dissolved dry enzyme in 100 uL of **solution A (*TGGSASC*)**

The AchE in solution is = 458ng/100 uL ---------- (B)

(1 uL = 04.58ng, 2 uL = 09.16ng, 3 uL = 13.74ng, 4 uL = 18.32ng, 5 uL = 22.90ng
10 uL = 45.80ng, 25 uL = 114.5ng)

Take 25uL(114.5ng) of B and dilute with **975 uL of solution A (*TGGSASC*) ---------(C)**
(This is = 114.5 ng/1000uL or 0.1145ng/uL)
Conc. of enzyme is
0.1145 ng/uL
---------- = 0.00000044 nmole/uL = 0.44 nM
260000ng/nmole

Ellman's reagent (5,5-dithio-2-bis-nitrobenzoic acid (DTNB)
MW: 396.4 MF: $C_{14}H_8O_8N_2S_2$
Store at room temperature
To make 1mM DTNB solution

Dissolve 3.96 mg (0.00396g) of DTNB in 10 mL of 0.05M SHP solution -----**(D)**
(To make 0.05 M Na_2HPO_4 (SHP), Dissolve 0.71g of SHP in 100mL Nanopure Water)

Acetylthiocholine Iodide (ATChI)
MW: 289.2 ; MF: $C_7H_{16}ONSI$
Store at 4°C
To make 1mM ATChI solution
Dissolve 2.89 mg (0.00289g) of ATChI in 10 mL of Nanopure Water ----------**(E)**

Vmax Reading Experiment

Take 25uL of enzyme (C) + 25uL of Pesticide – incubate 20min, then place 150 uL of (D) and (E) in the well of plate.

Click the Softmax icon in PC, then click the instrument, set dual kinetics (405-490) Set read time (10 min) then click OK

then click on 'read the plate'
After reading save the file and take printout of all necessary window.

Oxidation Protocol:

S
||

1. Incubate 25uL of pesticides (>P–) with 5uL of 0.001% Bromine in well — (20Min)
2. After 30 min add 5uL (5%) of ethanol ---- incubate 20 min
3. After 20 min add 25uL of enzyme (2.86ng)
4. Add 150uL mix solution of D and E, and read on Molecular Device

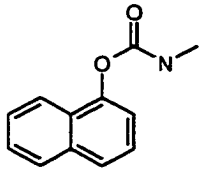
Carbaryl

Type: Carbamate

Synonyms: Carbamine, Denapon, Dicarbam, Hexavin, Karbaspray, Nac, Ravyon, Septene, Sevin, Tercyl, Tricarnam, and Union Carbide7744,OMS 29, UC 7744; Arylam; Carylderm; Clinicide; Derbac; Ravyon; Seffein; Padrin; Bug Master; Carbamec; Crunch; Denapon; Devicarb; Hexavin; Karbaspray; Murvin; NAC; Patrin; Savit; Thinsec; Tornado; Tricarnam; Carbaril; Carbomate;

Chemical Nomenclature: 1-Naphthyl N-methylcarbamate

Structure:



Molecular Formula: $C_{12}H_{11}NO_2$

Molecular Weight: 201.22

Soluble in water, benzene, alcohol, and other organic solvent

Hazard Symbol: Harmful, Dangerous for the environment

Standard Solution: 896ug/mL (in water) or 0.896ug/uL

To make stock solution: Take 1uL and dilute up to 1mL(1000uL) = 0.896ug/mL

$$\frac{0.896\text{ug/mL}}{201.22\text{ ug/umole}} = 0.00445\text{umole/mL} = \mathbf{4.45\ uM}$$

To make 1uM, take 224.72uL of Stock and dilute up to 1000uL of NP water

To make 1nM solution, take 1uL of 1uM and dilute up to 1000uL

To make 1pM solution, take 1uL of 1nM and dilute up to 1000uL

Serial dilution (1:10 dilution)

Take 6 set of 1.5 mL tube and place 900 uL of water in each tube, *then transfer 100uL from 1uM solution* to 1st tube. Take 100uL (from 1st tube) → 2nd tube (take 100uL) → 3rd tube (take 100uL) → 4th tube (take 100uL) → 5th tube (take 100uL) → 6th tube

The concentration is

10^{-1} uM = 100nM		(1st tube)
10^{-2} uM = 10nM		(2nd tube)
10^{-3} uM = 1nM		(3rd tube)
10^{-4} uM = 0.1nM	= 100pM	(4th tube)
10^{-5} uM = 0.01nM	= 10pM	(5th tube)
10^{-6} uM = 0.001nM	= 1pM	(6th tube)

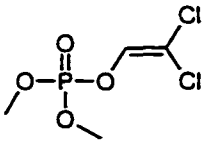
Dichlorvos

Type: Organophosphate

Other Names: Apavap, Benfos, Cekusan, Cypona, DDVP, Derriban, Derribante, Devikol, Didivane, Duo-Kill, Duravos, Elastrel, Fly-Die, Fly-Fighter, Herkol, Marvex, No-Pest, Prentox, Vapona, Vaponite, Verdican, Verdipor, Verdisol. Trade names used outside of the U.S. include Doom, Nogos, and Nuvan

Chemical Nomenclature: Phosphoric acid 2,2-dichloroethenyl dimethyl ester, O,O-dimethyl-O-(2,2 dichlorovinyl phosphate, 2,2-dichlorovinyl dimethyl phosphate DDVP

Structure:



Molecular Formula: $C_4H_7Cl_2O_4P$
Molecular Weight: 220.98
Density: 1.415
Hazard Symbol: Very toxic
Storage Temp: 4°C
Soluble benzene, alcohol, and other organic solvent
Standard Solution: 3uL/mL (In methanol)
Stock Solution: 3uL/mL (In 10% methanol)

$$\frac{3\text{uL/mL} \times 1.415\ \text{mg/uL}}{220.98\ \text{mg/mmole}} = 0.01921\ \text{mmole/mL} = \mathbf{19.21\ mM}$$

To make 1mM, take 52 uL of Stock and dilute up to 1000uL of water
To make 1uM solution, take 1uL of 1mM and dilute up to 1000uL
To make 1nM solution, take 1uL of 1uM and dilute up to 1000uL

Serial dilution (1:10 dilution)
Take 6 set of 1.5 mL tube and place 900 uL of water in each tube, *then transfer 100uL from 1uM solution* to 1st tube. Take 100uL (from 1st tube) → 2nd tube (take 100uL) → 3rd tube (take 100uL) → 4th tube (take 100uL) → 5th tube (take 100uL) → 6th tube

The concentration is

10^{-1} uM = 100nM		(1st tube)
10^{-2} uM = 10nM		(2nd tube)
10^{-3} uM = 1nM		(3rd tube)
10^{-4} uM = 0.1nM	= 100pM	(4th tube)
10^{-5} uM = 0.01nM	= 10pM	(5th tube)
10^{-6} uM = 0.001nM	= 1pM	(6th tube)

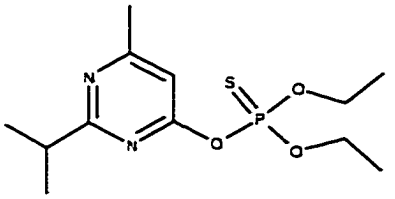
Diazinon

Type: Organophosphate

Synonyms: Dimpylate, Basudin, Garden Tox, SpectracideDimpylate; Basudin,Dipofene; Diazitol; AG-500; Antigal; Dacutox; Dassitox; Dazzel; Diagran; Diaterr-fos; Diazajet; Diazide; Diazol; Disyston, Trade names of this product include Knox Out, Spectracide and Basudin. Diazinon may be found in formulations with a variety of other pesticides such as pyrethrins, lindane and disulfoton.

Chemical Nomenclature: Phosphorothioic acid, O,O-diethyl O-(6-methyl-2-(1-methylethyl)-4-pyrimidinyl) ester

Structure:



Molecular Formula: $C_{12}H_{21}N_2O_3PS$
Molecular Weight: 304.34
Density: 1.116-1.118
Soluble in Water and miscible in common organic solvents
Hazard Symbol: Harmful, Dangerous for the environment
Storage Temp: 4°C
Standard Solution: 1uL/mL (in methanol)
Stock Solution: 1uL/mL (in 10% MeOH)

$$\frac{1\text{uL/mL} \times 1.116\ \text{mg/mL}}{304.34\ \text{mg/mmole}} = 0.00367\ \text{mmole/mL} = \mathbf{3.67\ mM}$$

To make 1mM, take 273uL of Standard and dilute up to 1000uL of water
To make 1uM solution, take 1uL of 1mM and dilute up to 1000uL
To make 1nM solution, take 1uL of 1uM and dilute up to 1000uL

Serial dilution (1:10 dilution)
Take 6 set of 1.5 mL tube and place 900 uL of water in each tube, *then transfer 100uL from 1uM solution* to 1st tube. Take 100uL (from 1st tube) → 2nd tube (take 100uL) → 3rd tube (take 100uL) → 4th tube (take 100uL) → 5th tube (take 100uL) → 6th tube

The concentration is

10^{-1} uM = 100nM		(1st tube)
10^{-2} uM = 10nM		(2nd tube)
10^{-3} uM = 1nM		(3rd tube)
10^{-4} uM = 0.1nM	= 100pM	(4th tube)
10^{-5} uM = 0.01nM	= 10pM	(5th tube)
10^{-6} uM = 0.001nM	= 1pM	(6th tube)

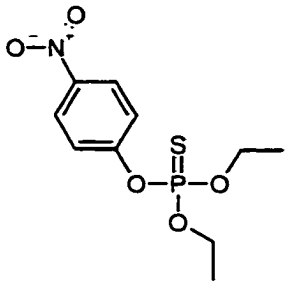
Parathion

Type: Organophosphate

Other name Ethyl parathion, ethylparathion, ethyl-parathion, E605; Thiophos 3422;DNTP; Niran; AAT; Etilon; DPP; Paraphos; Alkron

Chemical nomenclature: O,O'diethyl O'p-nitrophenylphosphorothionate (or thioate)

Structure:



Molecular Formula: $C_{10}H_{14}NO_5PS$

Molecular Weight: 291.27

Density: 1.26

Storage Temp: ROOM TEMPERATURE

Soluble in Alcohol

Hazard Symbol: Toxic, Dangerous for the environment

Standard Solution: 1uL/mL (in methanol)

Stock Solution: 1uL/mL (in 10% MeOH)

$$\frac{1\text{uL/mL} \times 1.26\text{ mg/uL}}{291.97\text{ mg/mmole}} = 0.00432\text{ mmole/mL} = \mathbf{4.32\ mM}$$

To make 1mM, take 231.5uL of Stock and dilute up to 1000uL of water

To make 1uM solution, take 1uL of 1mM and dilute up to 1000uL

To make 1nM solution, take 1uL of 1uM and dilute up to 1000uL

Serial dilution (1:10 dilution)

Take 6 set of 1.5 mL tube and place 900 uL of water in each tube, *then transfer 100uL from 1uM solution* to 1st tube. Take 100uL (from 1st tube) → 2nd tube (take 100uL) → 3rd tube (take 100uL) → 4th tube (take 100uL) → 5th tube (take 100uL) → 6th tube

The concentration is

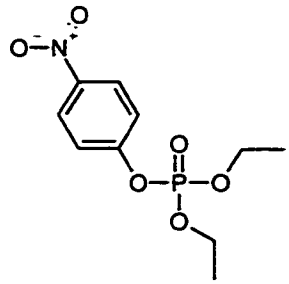
10^{-1} uM = 100nM		(1st tube)
10^{-2} uM = 10nM		(2nd tube)
10^{-3} uM = 1nM		(3rd tube)
10^{-4} uM = 0.1nM	= 100pM	(4th tube)
10^{-5} uM = 0.01nM	= 10pM	(5th tube)
10^{-6} uM = 0.001nM	= 1pM	(6th tube)

Paraoxon

Type: Organophosphate
Other name: phosphacol; E600; ester25; eticol; phosphakol; nintacol; miotisal A; solumlaucit

Chemical Nomenclature: diethyl p-nitrophenyl phosphate; phosphoric acid diethyl 4-nitrophenyl ester

Structure:



Molecular Formula: C10H14NO6P
Molecular Weight: 275.21
Storage Temp: 4°C
Density: 1.27
Soluble in water, Alcohol
Hazard Symbol: Very Toxic, Dangerous for the environment
Standard Solution: 1uL/mL (in methanol)
Stock Solution: 1uL/mL (in 10% MeOH)

$$\frac{1\text{uL/mL} \times 1.27\text{ mg/mL}}{275.21\text{ mg/mmole}} = 0.00461\text{ mmole/mL} = \mathbf{4.6\ mM}$$

To make 1mM, take 217.4 uL of Stock and dilute up to 1000uL of water
To make 1uM solution, take 1uL of 1mM and dilute up to 1000uL
To make 1nM solution, take 1uL of 1uM and dilute up to 1000uL

Serial dilution (1:10 dilution)
Take 6 set of 1.5 mL tube and place 900 uL of water in each tube. *then transfer 100uL from 1uM solution* to 1st tube. Take 100uL (from 1st tube) → 2nd tube (take 100uL) → 3rd tube (take 100uL) → 4th tube (take 100uL) → 5th tube (take 100uL) → 6th tube

The concentration is

10^{-1} uM = 100nM		(1st tube)
10^{-2} uM = 10nM		(2nd tube)
10^{-3} uM = 1nM		(3rd tube)
10^{-4} uM = 0.1nM	= 100pM	(4th tube)
10^{-5} uM = 0.01nM	= 10pM	(5th tube)
10^{-6} uM = 0.001nM	= 1pM	(6th tube)

Protocol For Oxidation of Phosphorothioates With Rat Liver Microsomes

Stock concentrations of each of the solutions used were 10x the concentrations of the diluted solutions.

Rat Liver Microsomes
Solution A

EDTA (Ethylenediamine Tetra-acetic Acid)
Solution B
Stock Concentration = 30mM
In Assay = 3mM

NADPH (Nicotinamide Adenine Dinucleotide Phosphate Hydrogen) –
Solution C
Stock Concentration = 10mM
In Assay = 1mM

Phosphate Buffer (pH 7.4)
Solution D
Stock Concentration = 0.1M
In Assay = 0.01M

Phosphorothioate Pesticide (Parathion)
Solution E
Stock Concentration = 10mM
In Assay = 1mM

The Incubations: 10uL of solution A, 20uL of solution B, 20uL of solution C, and 20uL of solution E placed in 150uL of solution D. Total volume of 200uL produced in a 1.5mL vial. Place vial in an oven at 37°C at various time periods, starting at 10min and increasing the time at 10min intervals. The oxidations are to be terminated with serial dilutions.

Serial Dilutions For Pesticide Concentrations: After incubation, take 25uL of incubated solution (1^{st} vial) → place in 2^{nd} vial containing 225uL of phosphate buffer (pH 7.4) → 3^{rd} vial (25uL from 2^{nd} vial) → 4^{th} vial (25uL from 3^{rd} vial) → to 6^{th} vial. There should be 7 different concentrations of pesticide from 1mM to 1uM.

Preparation For Instrument Reading: The dilutions are to be placed in an ELISA 96-well plate for measurement reading by the Vmax kinetic spectrophotometric reader as in the Ellman's method oxidation protocol. You are to begin at the point in the protocol that is after neutralization with EtOH (Add enzyme, incubate for 20min, then add DTNB & ATChI).

APPENDIX III

GC/MS ANALYSIS STEPS, PROTOCOL, AND METHODS

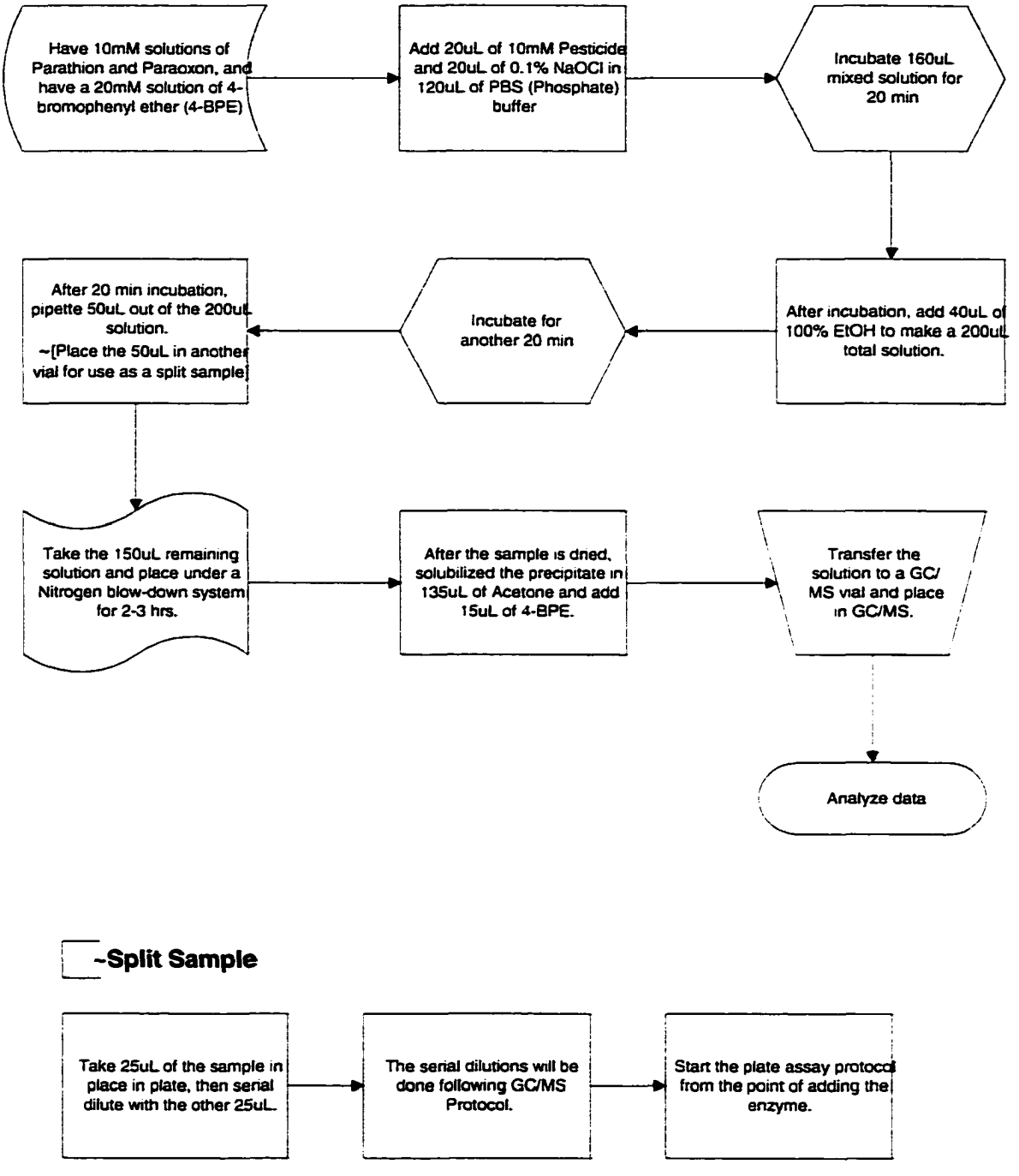


Figure 31. Diagram of GC/MS analysis steps.

PARATHION REACTION PROTOCOL FOR ANALYSIS BY GC/MS

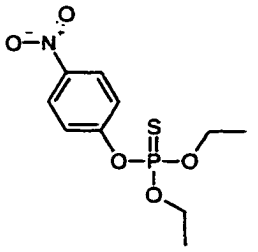
Parathion

Type: Organophosphate

Synonym: Ethyl parathion, ethylparathion, ethyl-parathion, E605; Thiophos 3422;DNTP; Niran; AAT; Etilon; DPP; Paraphos; Alkron

Chemical nomenclature: O,O'diethyl O'p-nitrophenylphosphorothioate

Structure:



Molecular Formula: $C_{10}H_{14}NO_5PS$

Molecular Weight: 291.27

Density: 1.26

Storage Temp: ROOM TEMPERATURE

Soluble in Alcohol, Organic solvents

Hazard Symbol: Toxic, Dangerous for the environment

Standard Solution: 1uL/mL (in methanol)

Stock Solution: 2.32 uL/mL (in 10% MeOH)

$$\frac{2.32\ \text{uL/mL} \times 1.26\ \text{mg/uL}}{291.97\ \text{mg/mmole}} = 0.00432\ \text{mmole/mL} = \mathbf{10\ mM}$$

TO MAKE REACTION SOLUTION

Take 20 uL of Stock Parathion and add to 120uL PBS Buffer (pH 7.4) in 1.5mL tube (vial) with 0.1% NaOCl (which will be 0.01% in PBS Buffer) and incubate for 20 min. After incubation, add 40uL of 100% EtOH and incubate for another 20 min. After the second incubation, take 50uL of solution mixture for the split sample.

SPLIT SAMPLE FOR PLATE ASSAY DETERMINATION

Serial dilution (1:10 dilution)

Take 6 set of 1.5 mL tube and place 225 uL of PBS Buffer in each tube, *then transfer 25 uL from the 50uL tube containing the 1mM solution* to 1st tube. Take 25uL (from 1st tube) → 2nd tube (take 25uL) → 3rd tube (take 25uL) → 4th tube (take 25uL) → 5th tube (take 25uL) → 6th tube (Add to plate as in previous organophosphate protocol).

The concentration is

10^{-1} mM = 100uM		(1st tube)
10^{-2} mM = 10uM		(2nd tube)
10^{-3} mM = 1uM		(3rd tube)
10^{-4} mM = 0.1uM	= 100nM	(4th tube)
10^{-5} mM = 0.01uM	= 10nM	(5th tube)
10^{-6} mM = 0.001uM	= 1nM	(6th tube)

Parathion Conversion

The parathion reactions were accomplished by adding 20 μL of parathion and 20 μL of 0.1% NaOCl (to make a 0.01% NaOCl) to 140 μL of PBS buffer (pH 7.4) for 20 min incubation. After the 20 min incubation, 20 μL of 100% EtOH was added to the solution mixture to make a 200 μL total solution mixture and then incubated for another 20 min.

After the second incubation, 50 μL of the solution was pipetted out for use in the plate assay to determine what effect, if any, the buffer would have on the response of the plate assay, and the remaining solution was placed under a N_2 blown-down system and allowed to dry. Dying time was between 2.5 and 3 hrs. Once the solution had dried, the precipitate was taken up in 135 μL of acetone, and 15 μL of 4-bromophenyl ether was added for use as the internal standard. This solution was then transferred to a GC/MS vial and placed on the GC/MS for reading.

Gas Chromatography/Mass Spectroscopy (GC/MS) Method

An Agilent Technologies 6890 gas chromatograph/5973 mass-selective detector was used. The gas chromatograph was fitted with a DB-XLB 30 cm x .18 mm id column with a 0.18 mm film thickness (J&W Scientific, Folsom, CA). The temperature of the column was programmed from 60 to 300°C at 12°C/min. The injection was on-column at 60°C by using an Agilent Technologies 7673A autosampler. The flow rate of He was 20 mL/min. The injection was in the pulsed-pressure splitless mode at 250°C and temperature programmed at 15°C/min from 60 to 300°C at a He flow rate of 1 mL/min. A mass range of m/z 50 to 500 was scanned at ca 0.1 Hz.

The following ions were monitored by the MS: m/z 109, 125, 149, 219, 235, 247, 263, 275, 291, 325, 328, and 330 for the compounds paraoxon, parathion, and 4-bromophenyl ether. Only the areas of the ions at m/z 275, 291, and 328 with appropriate scan ranges were integrated to determine response factors and to quantify the parathion reactions. The integration time was 25 ms for the ions. The ions were identified using the National Institute of Standards and Technology (NIST) Mass Spectroscopy (MS) database.

Quantitation of the reactions for parathion oxidized to paraoxon were accomplished by using areas obtained via the instrument ion chromatogram areas in retention time (R.T.) windows. The retention times for the ions were 11.380, 11.780, and 11.960 min for paraoxon, parathion, and 4-bromophenyl ether, respectively. A calibration curve was developed by plotting A_a/A_i (area of analyte/area of internal standard) on the y-axis and Am_a/Am_i (amount of analyte/amount of internal standard) on the x-axis using calibration solutions containing different concentrations of each analyte to be determined and the internal standard. For this curve the analytes are parathion and paraoxon with 4-bromophenyl ether as the internal standard. The two analytes were placed in 5 different solutions with concentrations ranging from 1mM to 10uM, and the internal standard was held at a concentration of 2mM for each solution. This gave 5 points for the line.

A linear regression was then applied to the curve to determine the concentration of each compound by using the linear equation ($y = mx + b$). For the parathion concentration the equation was: Response ratio = $1.75e^{0}$* Amount ratio + $5.03e^{-002}$ which, corresponded to the linear equation. In the parathion linear equation $1.75e^{0}$ is the linear

term (slope of the line) and $5.03e^{-002}$ is the constant term. The amounts were determined by using the following derivation of the linear equation:

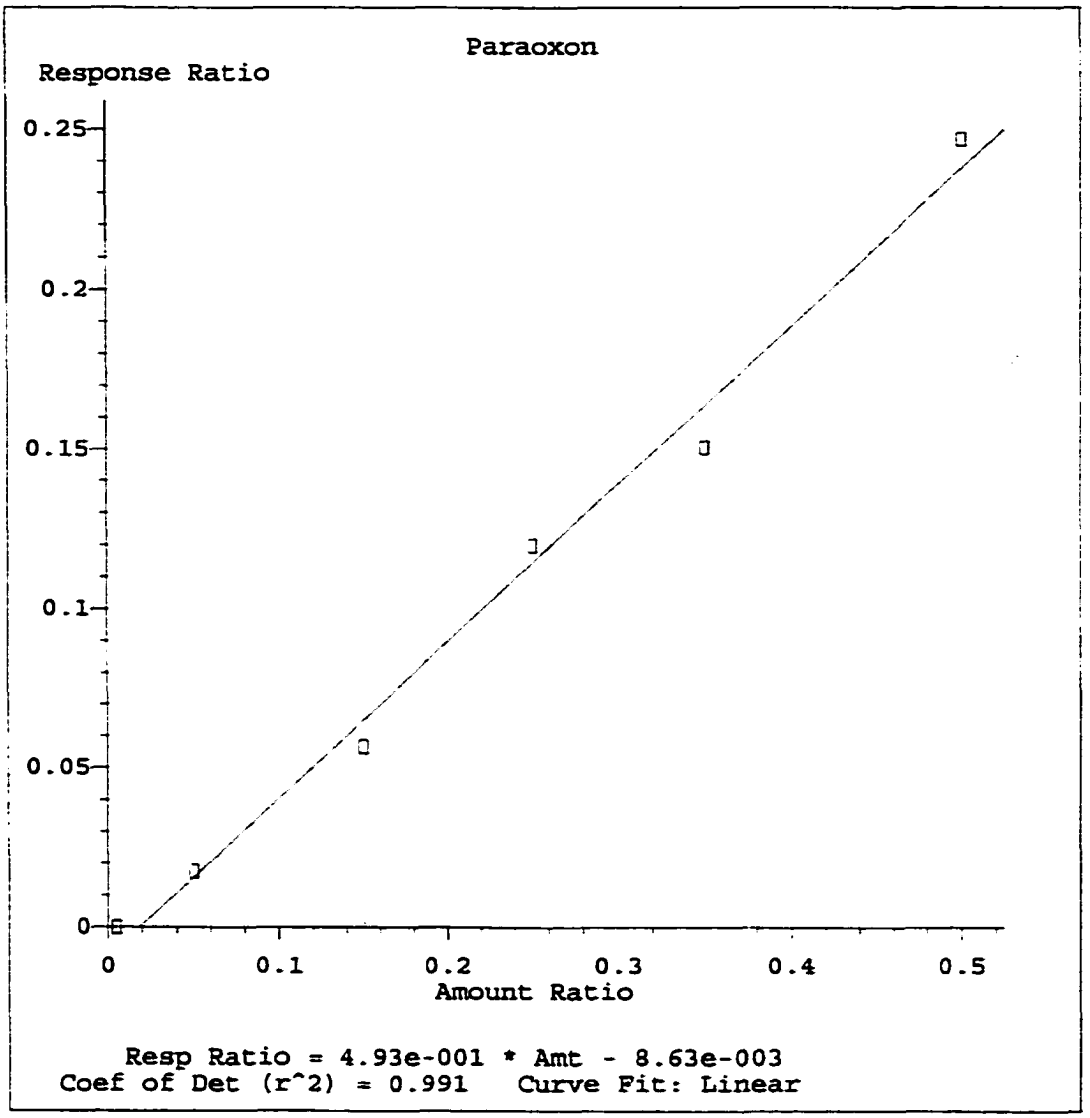
$$\mathbf{y_{rr} = m_{lt}x_{AmR} + b_{ct}}$$

$$\mathbf{x_{AmR} = (y_{rr} - b_{ct})/m_{lt} \rightarrow x_{AmR} = (amt_{unk}/amt_{ls})}$$

$$\mathbf{(amt_{unk}/amt_{ls}) = (y_{rr} - b_{ct})/m_{lt}}$$

$$\mathbf{amt_{unk} = amt_{ls} * (y_{rr} - b_{ct})/m_{lt}}$$

in the equations, $\mathbf{y_{rr}}$ is the response ratio (determined from the curve developed from calibration solutions and analyzed by the computer), $\mathbf{m_{lt}}$ is the linear term (slope of the line), $\mathbf{x_{AmR}}$ is the amount ratio, $\mathbf{b_{ct}}$ is the constant term (determined by the computer from the curve), $\mathbf{amt_{unk}}$ is the amount of the unknown analyte (in this case the parathion), and $\mathbf{amt_{ls}}$ is the internal standard amount. The curve was linear since the parameters (**y** and **m**) were linear.



Method Name: C:\HPCHEM\1\METHODS\EISCAN.M
Calibration Table Last Updated: Thu Feb 07 15:58:11 2002

Figure 32. Sample calibration curve representing paraoxon.

DETAILED GC/MS METHODS

INSTRUMENT CONTROL PARAMETERS

Sample Inlet: GC
Injection Source: GC ALS
Mass Spectrometer: Enabled

HP6890 GC METHOD

OVEN
Initial temp: 60 'C (On)
Initial time: 1.00 min
Ramps:

#	Rate	Final temp	Final time
1	20.00	300	12.00
2	0.0 (Off)		

Post temp: 0 'C
Post time: 0.00 min
Run time: 25.00 min

Maximum temp: 325 °C
Equilibration time: 0.50 min

FRONT INLET (HP PTV)
Mode: Split
Initial temp: 50 'C (Off)
Cryo: Off
Cryo use temp: 25 'C
Cryo Timeout: 30.00 min (On)
Cryo Fault: On
Pressure: 0.00 psi (Off)
Total flow: 45.0 mL/min
Gas saver: Off
Gas type: Helium

BACK INLET (SPLIT/SPLITLESS)

Mode: Pulsed Splitless
Initial temp: 250 °C (On)
Pressure: 8.22 psi (On)
Pulse pressure: 20.0 psi
Pulse time: 1.00 min
Purge flow: 50.0 mL/min
Purge time: 1.00 min
Total flow: 53.6 mL/min
Gas saver: On
Saver flow: 20.0 mL/min
Saver time: 3.00 min
Gas type: Helium

FRONT DETECTOR (NO DET)

SIGNAL 1
Data rate: 0.1 Hz
Type: test plot
Save Data: Off
Zero: 0.0 (Off)
Range: 0
Fast Peaks: Off
Attenuation: 0

BACK DETECTOR (NO DET)

SIGNAL 2
Data rate: 0.1 Hz
Type: test plot
Save Data: Off
Zero: 0.0 (Off)
Range: 0
Fast Peaks: Off
Attenuation: 0

COLUMN COMP 1
(No Detectors Installed)

COLUMN COMP 2
(No Detectors Installed)

COLUMN 2
Capillary Column
Model Number: HP 190918-433
HP-5MS DB-XLB
Max temperature: 325 °C
Nominal length: 30.0 cm
Nominal diameter: 180.00 um
Nominal film thickness: 0.18 um
Mode: constant flow
Initial flow: 1.0 mL/min
Nominal init pressure: 8.23 psi
Average velocity: 37 cm/sec
Inlet: Back
Inlet Outlet: MSD
Outlet pressure: vacuum

THERMAL AUX 2
Use: MSD Transfer Line Heater
Description: MSD XFR Line
Initial temp: 280 °C (On)
Initial time: 0.00 min

#	Rate	Final temp	Final time
1		0.0 (Off)	

POST RUN
Post Time: 0.00 min

7673 Injector

Front Injector: No parameters specified
Back Injector:

Sample Washes	3
Sample Pumps	3
Injection Volume	2.0 microliters
Syringe Size	10.0 microliters
Post Injection Solvent A Washes	3

Post Injection Solvent B Washes	3
Viscosity Delay	0 seconds
Plunger Speed	Fast
Pre Injection Dwell	0.00 minutes
Post Injection Dwell	0.00 minutes

MS ACQUISITION PARAMETERS

General Information

Tune File:	ATUNE.U
Acquisition Mode:	Scan

MS Information

Solvent Delay:	3.50 min	
EM Absolute:	False	
EM Offset:	506	
Resulting EM Voltage	1941.2	
[Scan Parameters]		
Low Mass:	50	
High Mass:	550	
Threshold:	125	
Sample #:	2	A/D Samples 4
Plot 2 low mass:	50	
Plot 2 high mass:	550	
[MS Zones]		
MS Quad:	150 °C	Maximum 200 °C
MS Source:	230 °C	Maximum 250 °C

END OF MS ACQUISITION PARAMETERS

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