

Chapter 1

INTRODUCTION

1.0 Introduction

The discovery that organochlorine pesticides, such as DDT, are highly persistent, bioconcentrate in food chains and can severely affect whole populations or species of wildlife has led to bans and use restrictions (Stickel, 1975). The decreased use of organochlorine pesticides has further expanded the market for less-persistent pesticides including organophosphate (OP) compounds.

Although research on OPs began as early as the nineteenth century, the insecticidal activity of these chemicals was only discovered in 1937. OPs were rapidly developed as insecticides in Germany during World War II and in the 50's the commercial use of OPs expanded markedly. Presently, OP pesticides are used as insecticides, herbicides, nematocides, acaricides, fungicides, rodenticides and bird repellents throughout the world.

The organophosphorus insecticides are neutral esters of phosphoric acid or its thio analogues. The phosphonates possess a carbon-phosphorus bond which appears to be biologically very stable. In other respects, however, they are very similar to other OP insecticides.

Most OP insecticides are regarded as being non-persistent, but some reports have indicated that residues of OP remain essentially unaltered for extended periods in organic soils and surrounding drainage systems (Harris and Miles, 1975). It has also been observed that more water-soluble, less persistent insecticides such as diazinon and parathion were not strongly absorbed by sediment while the less soluble, more persistent insecticides such as chlorpyrifos tended to be strongly absorbed by soil and

sediments. The tendency of an insecticide to associate with organic phases in the environment depends on its partition coefficient value. Octanol/water partition coefficients (K_{ow}) express the relative affinity of a compound for organic phases compared to aqueous and are considered to be the key parameters in the estimation of environmental partitioning (eg: sediment/water).

A study by Sharom *et al.* (1980) demonstrated that chlorpyrifos and diazinon are more persistent in natural waters compared to carbaryl and parathion. The study investigated the persistence of 12 insecticides representing organochlorine (OC), OP and carbamate chemicals in natural and distilled water. The persistence of the insecticide solutions in sterilized and unsterilized natural or distilled water was examined as a function of time in darkness at $21 \pm 1^\circ\text{C}$. Based on the percentage of the insecticide remaining after 8 weeks incubation, the most persistent insecticide in unsterilized natural water was found to be in the following order of decreasing persistence : ethion > leptophos > chlorpyrifos > diazinon > carbofuran > carbaryl > parathion.

In a more recent study, the persistent nature of diazinon was also demonstrated in natural water (Ferrando *et al.*, 1992). A $t_{1/2}$ of 70.5 hour for diazinon indicated that it was more persistent than OC such as lindane ($t_{1/2}$: 65hour) and endosulfan ($t_{1/2}$: 50.3hour).

Today, OP pesticide use is widespread on agricultural crops, rangelands, forests and wetlands and undoubtedly exposes many wildlife species to chemical hazards.

1.1 Mode of action of OP insecticides.

The toxic effects of OPs to mammals and insects are based on interference with the nervous system. The target enzyme is acetylcholinesterase (AChE), the normal function of which is to remove the neurotransmitter, acetylcholine (ACh), after it has performed its normal function in the synaptic transmission of a nerve impulse. There are two kinds of cholinesterases (ChE); acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Even though both kinds of ChE are common in vertebrates, AChE is found only in erythrocytes and nervous and muscle tissue. If the AChE in strategic locations are blocked, the animal dies. By contrast, BChE is found in numerous tissues, including blood plasma and liver as well as nervous tissue and it can be inhibited with no apparent ill effect to the animal (O'Brien, 1976). AChE is also referred to as "true cholinesterase" or "specific cholinesterase". It has a high specificity for ACh. A form of the enzyme has been purified from the electric organ of the electric eel (*Electrophorus electricus*) (Froede and Wilson, 1971) with a molecular weight of approximately 250,000 comprising of 4 subunits. The structure of AChE has been reviewed by Massoulie (Massoulie, 1980).

Acetylcholinesterase (AChE) belongs to a very large group of enzymes called "hydrolase", defined as enzymes that split substrates by the introduction of the elements of water. The hydrolases which split acetylcholine (ACh) are called acetylcholinesterase. Hence, AChE catalyzes the following reaction :

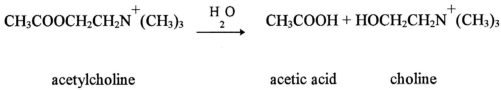


Figure 1.0: Hydrolysis of acetylcholine

In mammals, ACh is an excitatory transmitter for striated muscle, acting upon nicotinic receptors. It also serves as a transmitter for the autonomic system. ACh functions as both preganglionic (nicotinic receptors) and postganglionic (muscarinic receptors) transmitter for the parasympathetic system, while in the sympathetic system, ACh is commonly a preganglionic transmitter. It is also found as an excitatory transmitter in limited but critical regions of the central nervous system (Murphy, 1980; Namba, 1971). Since AChE is the enzyme responsible for the breakdown of ACh, it therefore terminates the electrochemical connection between two nerve cells.

In fish, the primary defense mechanism when exposed to insecticide is excessive production of mucus, since mucus contain antibodies (Murty, 1986). When homeostasis cannot be achieved, the fish exhibits increased respiration and nervous disorder due to damaged gill filaments and AChE inhibition in the central nervous system. In addition, the insecticide also causes damage to peripheral sense organs which delays fish reaction to mechanical disturbances.

Fish brain and muscle primarily possess AChE (Abou-Donia and Menzel, 1967; Coppage, 1971; Habig *et al.*, 1988). Fish brain is essentially nerve tissue and is rich in

AChE as compared to the BChE which is found in blood (Whittaker, 1951). A study by Szabo *et al.* (1992) on carp, a teleostean species, showed that the ratio of membrane-bound AChE to cytoplasmic-free AChE molecular forms increased in the following order : brain < trunk muscle < heart. In fish living in natural waters, even a relatively low concentration of organic phosphoric acid esters is capable of causing considerable AChE inhibition (Williams and Sova, 1966). This has been attributed to the enhanced accumulation of chemical pollutants in fish.

In most species of fish the brain is readily accessible and easily removed. The brain is supplied with oxygenated blood that comes directly from the gills. The transfer, therefore, of toxic materials from the water across the gill membranes and to the reaction site is quick and direct.

Experiments by Coppage (1971) showed that acetylcholine in fish brain homogenate underwent hydrolysis at a rate that varied linearly with increasing amount of enzyme (brain homogenate). It was also observed in the same study that maximal ACh hydrolysis took place between pH 7.0 and 7.5. AChE activity was found to be relatively constant between 20°C and 30°C . Since fish are poikilotherms, it is possible that warmer temperatures will denature and inactivate AChE which lead to reduced activity when temperature reached 40°C. AChE activity was also irreversibly destroyed at 50°C, hence complete loss of activity was reported when temperature was returned to 20°C (Coppage, 1971). However, within the brain there are differences in AChE activity in various regions of the brain. Gibson *et al.* (1969) studied various regions of the brain of bluegill sunfish i.e inferior lobe, optic lobes, cerebellum, cerebral hemisphere and medulla; and found that these regions differ in AChE activity. Thus it

is important that similar area of the brain be analyzed in exposed and control fish. Therefore, it is recommended that either the whole brain or half of the brain separated along the midline be used for enzyme analysis (Zinkl *et al.*, 1991).

Studies by Duangsawasdi and Klaverkamp (1979) showed that OP compounds affect fish in a number of ways, including actions on cardiovascular and respiratory systems and inhibition of ChE enzymes in brain, gill, heart, red blood cells, serum and skeletal muscle.

As stated above, AChE breaks down ACh to acetic acid and choline. A serine residue in the active site of the enzyme participates in the breakdown of ACh as shown in Figure 1. 1.



where

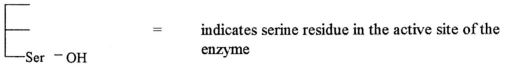
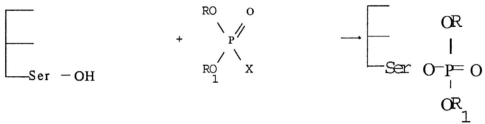


Figure 1. 1: Breakdown of the neurotransmitter acetylcholine to acetic acid and choline (Abdullah, A. R., 1986).

The enzyme is inhibited by phosphorylation of the serine hydroxyl group at its active site (Figure 1. 2).



where

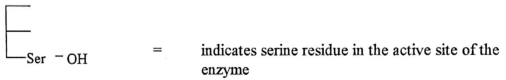
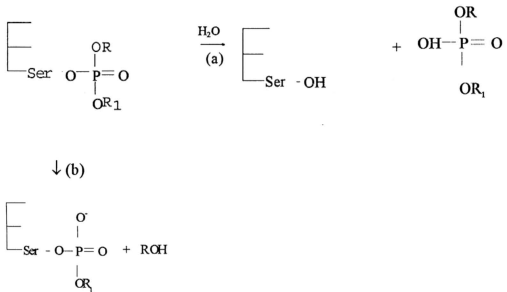


Figure 1.2: Phosphorylated enzyme (Abdullah, A. R., 1986)

Once the phosphoenzyme has been formed, it may undergo further reactions as presented in Figure 1. 3.



where

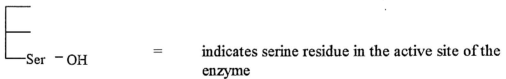


Figure 1. 3: Further reactions of phosphorylated serine esterases
(Abdullah, A.R., 1986)

The phosphoryl group may be hydrolyzed in a dephosphorylation process such that the active form of the enzyme is regenerated (path a, Figure 1.3) or an O-alkyl

group may be lost leading to a charged residue at the active site serine, a process known as ageing (path b, Figure 1.3). In the latter process, the aged enzyme can no longer be reactivated as in path (a).

1.2 Substrate/ inhibitor - AChE interaction.

AChE in the neuromuscular junction of the vertebrate is one of the important targets in poisoning by chemicals with anticholinesterase properties such as OPs and carbamates. The most conspicuous feature of all OP compounds is their structural complementarity with the target enzyme molecule. AChE has two active sites, the esteratic site and the anionic site (Wilson and Bergmann, 1950). Figure 1.4 illustrates the schematic structure of AChE.

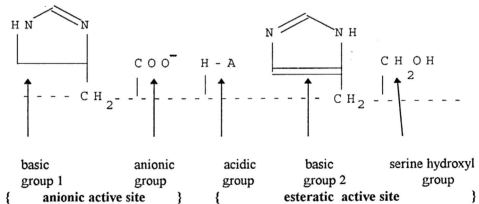


Figure 1.4: Schematic construction of AChE (Fest and Schmidt, 1973)

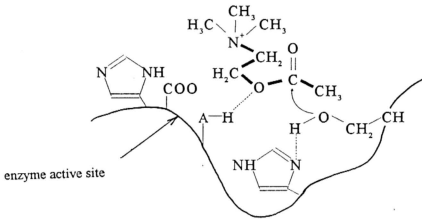


Figure 1.5: Enzyme substrate complex (schematic) (“Michaelis complex”) (Fest and Schmidt, 1973)

Hydrolysis of ACh by AChE commences with ACh binding to the enzyme to form an enzyme-substrate complex (Figure 1.5).

The amino acid serine is found at the enzyme’s active site. In this step, there is an overall interaction of substrate to the catalytic site on the enzyme in which the CH_3 group on the imidazole nitrogen of the ACh binds to the hydrophobic site and the acyl carbon or phosphorous atom binds to the esteratic site.

At this juncture, strong electrostatic interaction between the positively charged N of the choline moiety and the negatively charged COOH group at anionic group results in a change of conformation at cationic site whereby the serine OH group is now strongly ionized. A hydrogen atom is transferred from the enzyme (esteratic site) to the choline part of ACh (or O-X part for the OP compound) by hydrogen bond formation. The negatively charged oxygen of the serine moiety exert a nucleophilic

attack on the carbonyl at the acetyl moiety, the result of which is the release of choline moiety from the surface of the enzyme.

In the last step the acetylated enzyme rapidly undergoes further hydrolysis whereby H_2O exerts a nucleophilic attack on the acetyl carbonyl which is now attached to the enzyme, resulting in the release of the acetyl moiety from the enzyme molecule, and hence returning the catalytic site to normalcy (Figure 1.6).

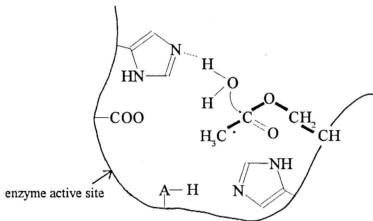
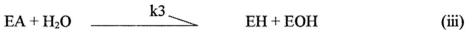
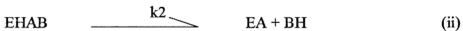


Figure 1.6: Hydrolysis of acetylated AChE (schematic) (Fest and Schmidt, 1973)

It is now recognised that OPs act as substrates for AChE and hence they have the ability to destroy its activity by reacting with the active serine within the same catalytic centre of the enzyme to produce O,O-dialkylphosphoserine in which form the enzyme is unable to hydrolyze choline esters (Smith and Usdin, 1966; Aldrich and Reiner, 1972). Within AChE, only the active site serine is phosphorylated by OPs.

Inhibition of AChE by OPs hence follow the general format (Aldridge and Reiner,1972) :



where

- E = Enzyme
- AB = Ester (inhibitor)
- EHAB = Michaelis complex
- EA = Acylated enzyme
- BH and EO = Breakdown products of inhibitors/ substrates

The affinity with which the substrate binds to the enzyme in the first step is often described by the dissociation constant, $K_d = k_{-1} / k_1$. The smaller K_d is, the greater the affinity. The complex formation is followed by an acetylation step, characterized by a rate constant k_2 , and ultimately a deacetylation step, defined by the rate constant k_3 , which regenerates the free enzyme. The rate of hydrolysis of enzyme-bound substrate (k_3) is very rapid for the natural substrate, ACh (less than 1 second), slower (several hours to days) for carbamates compounds, and extremely slow (months) for OP

chemicals (Matsumura, 1975). In the case of inhibition, the first step would be the formation of a reversible enzyme-inhibitor complex. This is achieved by the binding of the acyl carbon or phosphorus atom to the esteratic site, followed by phosphorylation. In step 3, the phosphorylated enzyme can undergo further rearrangement by the action of ambient water to form a hydroxylated complex. Any differences in the sensitivity of AChE to inhibitors such as OPs may depend on differences in both affinity (k_1/k_i) and phosphorylation (k_2) rates (Habig *et al.*, 1988; Wang and Murphy, 1982). These differences in affinity constants and phosphorylation rates explain varying *in vitro* sensitivities of mammalian, avian, fish and amphibians AChEs to several OPs (Wang and Murphy, 1982).

Although inhibition by some OPs is reversible, OPs are typically regarded as irreversible inhibitors because k_3 proceeds at a negligible rate (Habig and Di Giulio, 1991). Inhibitory effects of OPs may often be long lasting, suggesting that recovery in such cases arise primarily from the synthesis of new enzymes. For example, pumpkinseed sunfish given intraperitoneal injection of parathion at 12.5mg/kg showed very little, if any, early AChE recovery (Benke and Murphy, 1974). Similarly, channel catfish exposed to 3.6mg/l DEF (S, S, S -tri-n-butyl phosphorotrithioate) only regained 85% of control activity after 4 weeks following transfer to toxicant-free water (Habig *et al.*, 1986). This variation in the duration of inhibitory effects following such exposure to OPs appears to be not only dose dependent but also dependent on the chemical concerned. Therefore, recovery from the effects of some OPs can occur through dephosphorylation (k_3) or/and synthesis of new enzyme while recovery from

other OPs appears to primarily depend on synthesis of new enzyme (Habig and Giulio, 1991).

The mode of action of OP has been used to diagnose OP poisoning of mammals, birds and fish (Post, 1987; Hill and Fleming, 1982; Mount and Oehme, 1981; Coppage and Braidech, 1976; Coppage *et al.*, 1975; Williams and Sova, 1966). In addition, brain AChE activity has also been used to assist in the assessment of the effects of operational application of AChE-inhibiting insecticides on mammals, fish and birds (Haines, 1981; Lockhart *et al.*, 1973; Tagatz *et al.*, 1974; Thirugananam and Forgash, 1975; Rabeni and Stanley, 1979; Zinkl *et al.*, 1980). In an early study Weiss (1959) and later Weiss and Gakstatter (1964) suggested that very low concentrations of OP insecticides in natural waters could be detected by measuring the degree of inhibition of AChE activity in fish and invertebrates. Inhibition of brain AChE is generally regarded as a useful indicator of poisoning by OP pesticides (Martin *et al.*, 1981). Cook *et al.* (1976) reported that AChE measurement is the most sensitive indicator of organophosphorous poisoning in fish. Therefore, depression of AChE activity in brain is usually the most sensitive measure of toxicity. Sahib and Rao (1980) reported that among the various tissues of *Tilapia*, the highest level of AChE inhibition were noted in brain followed by muscle, gill, and liver.

There is some controversy in the literature as to the extent of AChE depression required to cause death in aquatic organisms (Abdullah *et al.*, 1993). In fish, most estimates lie in the range 70-85% AChE reduction (Richmond and Dutta, 1992). It has been accepted that a 20% or greater depression in AChE activity in either birds, fish or invertebrates indicates exposure to OP insecticides (Morgan *et al.*, 1990; Ludke *et*

al.,1975; Day and Scott, 1990); a 50% or greater depression is indicative of a life-threatening situation although some animals have been shown to survive higher levels of inhibition and others succumb at levels below 50% (Ludke *et al.*, 1975; Zinkl *et al.*, 1987; Busby *et al.*, 1989). A reduction in brain ChE activities in sheepshead minnows of 18% of normal levels was used to predict impending death due to poisoning by parathion, azinphosmethyl and phorate, while inhibition of < 17.7% of normal activity resulted in 40 to 60% fish mortality (Coppage, 1972). Weiss's (1959) suggestion that brain AChE inhibition could be used as evidence of exposure to antiChE compounds led to considerable controversy. For example Gibson *et al.*, (1969) reported that fish that become moribund in 750 µg/l parathion solution, showed 25% AChE inhibition, whereas those that became moribund in 20 µg/l parathion showed 50% AChE inhibition.

Although toxicities to fish (primarily in the form of 96-hour LC₅₀) of many cholinesterase inhibiting insecticides have been determined (Hartley and Kidds, 1987), acute lethality may not reflect the actual risk to an animal exposed to insecticide. Various factors which can affect the toxicity of pesticides include persistence in the environment, metabolism, interaction with other substances in addition to physiological variations.

Furthermore, fish can be exposed to insecticide concentrations that may range from acutely lethal, following accidental spillage, for instance, to sublethal as a result of spray drift and runoff.

1.3 Toxic effects of OPs to aquatic organisms.

In general, fish exposed to OP insecticides show signs of muscle paralysis, especially of the fins and respiratory apparatus, hyperactivity and loss of equilibrium. Other characteristics observed include hyperactivity, rigidity, stiffly flared pectoral fins, exaggerated opercular movements and mouth and opercular paralysis. Reports by McKim *et al.* (1987) indicated that fish poisoned by anticholinesterase lose equilibrium, swim in spiral pattern, overreact to stimuli, have increased amplitude of respiration and may have terminal tetany and convulsions. Sheephead minnow poisoned with diazinon had abnormal forward extension of the pectoral fins and were excessively reactive when startled (Goodman *et al.*, 1979). This forward position of the fins has been observed as a characteristic response to OP toxins (Henderson *et al.*, 1959). The fish subsequently suffered an apparent loss in balance and swam on their sides, finally settling in this position at the bottom of the tanks and showing only feeble opercular movements.

Sulaiman *et al.* (1989) reported that red tilapia exposed to malathion appeared hypersensitive to external stimuli. They appeared excited and swam erratically, contracted violently and tended to stay near the surface of water. Rapid movement of the opercula indicated significant increase in respiration rate. The rate of expansion and contraction of the buccal and opercular cavities also seemed to increase and this coughing-like behaviour may assist the fish in clearing the gills of the accumulated toxin as proposed by Carlson (1982).

75% of OP compounds are rather poor inhibitors of ChE *in vitro*, whereas the rest are potent and direct inhibitors (Murty, A. S , 1986). Yet, when the actual poisoning of the animals is considered, no such difference is noticeable, because of the *in vivo* conversion of the poor inhibitors to a more toxic form. This is more often seen in OP compounds with a P = S linkage (poor inhibitors of ChE) which are activated to the more toxic oxygen analog, P = O. *In vivo*, inhibition of AChE by a number of OP compounds was observed to be a function of the exposure concentration and duration of exposure suggesting that the *in vivo* response of fish brain AChE to low levels of OP compounds could be used to identify past and present exposure to AChE - inhibiting compounds.

1.4 Recovery from AChE inhibition.

Recovery is defined as a significant increase in AChE activity as a function of time that occurs following cessation of exposure to the anticholinesterase agent. Recovery of AChE activity following exposure to OP insecticides has been found to be a process that takes time, depending on factors such as type of OP insecticides, test species, and the extent of depression of AChE activity (Weiss, 1961; Post and Leasure, 1974; Morgan *et al.*, 1990). Furthermore, previous studies suggested that recovery of AChE in species exposed to OPs is due to synthesis of new enzymes (Benke and Murphy, 1974). It has also been shown that recovery of AChE activities was more rapid after exposure to carbamates than to OPs (Zinkl *et al.*, 1991).

Four weeks following transfer to toxicant-free water after 96 hour exposure to 2.0mg/L S, S, S - tri - n - butyl phosphorotrithioate (DEFTM), catfish brain tissue regained 85% of control AChE activity (Habig *et al.*, 1986). Kuhn and Streit (1994) reported that *Gammarus pulex* brain AChE exposed to 1µg/L fenitrothion was reduced to 24% - 27% activity after 5 days exposure but increased to 75% -85% activity after 16 days in clean water. After 15 days exposure to 1.25 mg/L dichlorvos, *Tilapia* brain AChE activity was 68% inhibited but only recovered up to 89% activity within a week in clean water (Rath and Misra, 1981). Sheepshead Minnow exposed to 6.5 µg/l diazinon for 108 days had normal brain AChE activity after 8 days in insecticide-free water (Goodman *et al.*, 1979). Guppies exposed to 1.13 and 3.25 µg chlorpyrifos/L for 14 days had about 56% brain AChE inhibition 14 days after being placed in clean water (Van der Wel and Welling, 1989). Therefore, it is apparent that brain AChE activity can be used to diagnose previous exposure to OP insecticides.

1.5 Xenobiotic biotransformation.

Organic compounds that do not belong in the category of fat, carbohydrate, protein, vitamin, steroid or mineral are considered to be foreign to the body. Such foreign compounds are utilised by man as drugs, pesticides, additives to food and fabric to name but a few examples. These compounds are referred to as xenobiotics and their metabolism is eventually a detoxification process. Xenobiotics have physical properties which allow them to cross the membranes of cells. Cell membranes are

predominantly comprised of lipid. Therefore lipophilic organic contaminants can cross the membrane. Xenobiotics are generally lipophilic in nature.

In the vertebrates, a primary metabolic pathway include biochemical reactions mediated by enzymes and result in the conversion of the parent chemical to more polar and readily excretable metabolites. These biotransformation reactions include conjugation, aromatic hydroxylation, deamination, epoxidation, dealkylation, desulfuration, nitro - and azo - reduction and ester and ether cleavage. Therefore, one of the results of biotransformation reactions is to facilitate the removal of toxic chemicals from an animal. Biotransformation can also result in the formation of products that are considerably more toxic than the parent chemical (Guengerich and Liebler, 1984; Mitchell and Horning, 1984; Nelson, 1982).

The duration, type and intensity of toxic response exhibited by a chemical in an animal is largely controlled by the types and extent of metabolism that the chemical undergoes *in vivo* (Mitchell and Horning, 1984; Nelson, 1982).

In addition, many xenobiotics and drugs are biotransformed to electrophilic, highly reactive metabolites (eg: epoxides) that can bind covalently to tissue macromolecules eg: DNA/protein to produce cytotoxic, teratogenic, mutagenic or carcinogenic effects.

However, the type of changes that occur depend on the chemical structure of the compound, but other factors such as species of animal, method of administration, and diet may also be involved. Certain compounds that are very polar or are insoluble in both water and lipids are not metabolized by the body and are excreted unchanged. Buhler and Williams (1988) reported that there are four types of chemical changes

which can occur. They are oxidation, reduction, hydrolysis and conjugation. Each group of reactions can be subdivided into different metabolic activities, depending on the type of substrate involved.

The liver is the main site for xenobiotic metabolism although other organs notably kidney, intestine and lung also contribute significantly. Some of the more important enzymes involved in this transformation are localized in the membranes of the endoplasmic reticulum, which is a lipoprotein tubular network extending from the cell wall throughout the cytoplasm. These enzymes associate with the microsomal fraction upon disruption of the cell and the endoplasmic reticulum (Buhler and Williams, 1988). The processing of xenobiotics in liver is called hepatic metabolism.

1.6 Microsomal mixed-function oxidase systems.

By far the most important biochemical reactions in the initial stages of insecticide metabolism are the ones involving microsomal oxidase enzyme system. Microsomal oxidases are characterized by the requirements of NADPH, microsomes, and *in vitro*-oxygen for degradation of their substrates. They are also characterized by their sensitivities toward methylenedioxyphenyl derivatives (pyrethrin synergists) such as sesamex and piperonylbutoxide.

Although there are redox reactions which are catalyzed by enzymes that are non-hepatic and non-microsomal, the highest concentrations of the xenobiotic metabolizing enzymes are found in the liver. It is the site for the major metabolic transformations. More specifically their transformation are associated with the

endoplasmic reticulum (Chambers and Yarbrough, 1976). As stated above, the ability to metabolize xenobiotics is not restricted to the liver. Thus, in low vertebrates such as fish, high levels of xenobiotic metabolizing enzyme activities have also been found in the kidney, gastrointestinal tract and gill, all of which play an important role in the uptake and excretion of compounds (Varanasi, 1989).

The NADPH-requiring general oxidation system, commonly referred to as the "mixed-function oxidase system" or MFO, is located in the microsomal portions of various tissues, particularly the liver (Brodie *et al.*, 1958; Gillette *et al.*, 1969). It is characterized as :

- 1) requiring NADPH as a cofactor.
- 2) involving an electron transport system with cytochrome P-450.
- 3) being capable of oxidizing many different kinds of substrates (i.e., substrate nonspecificity).

The MFO system processes foreign compounds via such reactions as hydroxylation, O - dealkylation, N - dealkylation and epoxidation. Although these reactions more often than not result in detoxication, there are implications that some reactions, especially epoxidation and desulfuration, can be activation in nature, resulting in more toxic compounds. As an example, this enzyme system has been identified to be responsible for the metabolic activation of P = S to P = O in parathion (Fukuto, 1990).

1.6.1 Cytochrome P-450

The key component of MFO system is a family of hemoproteins called cytochrome P-450. Cytochrome P-450 hemoprotein is the terminal oxidase component of an electron transfer system located within the microsomal fraction of the cell and is responsible for the metabolism of many xenobiotics. Each cytochrome P-450 -containing system is called a monooxygenase, and many monooxygenases comprise an MFO system.

Cytochrome P-450 is the substrate binding component and determines the specificity of the reaction. Cytochrome P-450 mediated oxidation include :

- 1) hydroxylation of aliphatic and aromatic compounds.
- 2) epoxidation of aliphatic and aromatic compounds.
- 3) N - dealkylation of amines.
- 4) O - dealkylation of ethers

Cytochrome P-450 binds with oxygen and the substrates. Another component of the oxidase system is a stable flavoprotein called NADPH-cytochrome-P-450 reductase. NADPH - cytochrome P-450 reductase is imbedded in the phospholipid matrix of the endoplasmic reticulum. In the scheme of oxidation, the substrate binds the oxidized form of cytochrome P-450. The flavoprotein transfers electrons from NADPH to cytochrome P-450 which then inserts one atom of oxygen into the substrate and reduces the second oxygen atom to form water (Goksoyr and Forlin, 1992).

In higher animals, cytochrome P-450 usually reside in microsomes, although a few exceptions exist (e.g., a specific monooxygenase to oxidize steroids exists in mitochondria of the adrenal cortex). It had been shown earlier that CO reacts with the reduced form of cytochrome P-450, giving a characteristic absorption spectrum with a peak at 450 nm (Omura and Sato, 1964).

1.6.2 Cytochrome P-450 mediated biotransformations.

There are two types of chemical reactions that are catalyzed by cytochrome P-450 dependent mixed function oxidases, also known as monooxygenases (MFOs). They are referred to as Phase I and Phase II reactions that convert contaminants to water soluble products. In Phase I, xenobiotics are altered or cleaved to produce a functional chemical group. This phase introduces polar groups into the molecule through oxidative, hydrolytic and reductive processes. Oxidative reactions are the most important category of phase I reactions (Buhler and Williams, 1988).

Phase II reactions involve conjugation of a chemical or its phase I metabolites with polar cellular constituents i.e glucuronic acid, sulfate or glutathione to form highly water-soluble metabolites, easily excreted by liver, kidney or gills. Phase I and phase II biotransformation reactions usually work together in a sequential manner to convert xenobiotics into more readily excretable metabolites. The different phase I and phase II enzymes compete with one another for the parent xenobiotics or its metabolites. Xenobiotic therefore undergo several types of biotransformation reactions, often

resulting in the formation of a large number of metabolites or conjugates (Buhler and Williams, 1988).

Fish generally possess most of the phase I and phase II (conjugation) pathways necessary for the biotransformation of insecticides and other xenobiotics, but the rate of metabolism in piscine species is often slower than that in mammals (Edwards and Millburn, 1985). However, in spite of low enzyme activities, fish appear to be able to metabolize agrochemicals reasonably effectively *in vivo*, provided that exposures are low (Edwards and Milburn, 1985). The major route of exposure, i.e chemicals present in the aqueous environment is of course, unique to fish and other aquatic organisms.

This must be considered in comparative metabolism and toxicological studies with terrestrial, arboreal and aerial species.

Fish and other aquatic animals are also exposed to a variety of pollutants in the sediment and water column as well as in their food. With respect to biotransformation reactions, the chemical might act as an inhibitor of the biotransformation enzymes such as GST. The chemical might also act as an inducer of cytochromes P-450 (Buhler and Williams, 1988).

Induction of cytochrome P-450 by xenobiotics is one of the most characteristic features of this enzyme system. Many compounds have the ability to induce specific patterns of cytochrome P-450 proteins. Inducers are classified according to the family or subfamily of cytochrome P-450 genes that they activate (Nebert and Gonzales, 1987; Nebert *et al.*, 1989).

Treatment of an animal with certain drugs or chemicals causes marked increases in the activities of one or more of the cytochrome P-450-dependent MFOs

and other xenobiotic metabolizing enzymes. These inducing agents include chemicals such as 3-methylcholanthrene (3-MC), β -naphthoflavone (BNF) and polychlorinated biphenyls (PCBs), all of which increase the activities of the microsomal MFOs benzo(α)pyrene BP hydroxylase (aryl hydrocarbon hydroxylase; AHH) and ethoxyresorufin-O-deethylase (EROD) (Buhler and Williams, 1988). 3-methylcholanthrene (3-MC) has been seen to induce cytochrome P-488-dependent MFO. The major involvement of cytochrome P-448 in the dealkylation of ethoxyresorufin was indicated in experiments by using purified preparations of cytochrome P-450 and P-448 (Burke and Mayer, 1975).

A study by Barron *et al.* (1993) on channel catfish exposed to chlorpyrifos, showed that chlorpyrifos was eliminated primarily after biotransformation. Evidence for this includes limited concentrations of parent chlorpyrifos in channel catfish urine, bile, and ambient water following oral, intravascular, or water routes of exposure (Barron *et al.*, 1991). The major metabolite of chlorpyrifos, which was excreted in both the urine and the bile of channel catfish, was the glucuronic acid conjugate of trichloropyridinol (TCP). TCP was formed by rapid dephosphorylation of chlorpyrifos. Minor metabolites included methoxytrichloropyridine and two polar urinary metabolites which were not identified due to the very low concentrations present. The insecticidal property of chlorpyrifos (AChE inhibition) is due to the toxic metabolite, chlorpyrifos oxon which was not detected in the channel fish. Lack of detection of the oxon in channel catfish is not surprising, since the oxon is undetectable in various mammalian species after administration of chlorpyrifos by various routes (Marshall and Roberts, 1978). Whitehouse and Ecobichon (1975) reported that the ability of liver to

hydrolyze paraoxon exceeds its capacity to activate parathion to paraoxon. Therefore, it was suggested that paraoxon formed in hepatic tissue is destroyed before it can be released into the circulation. Also, a large fraction of chlorpyrifos maybe metabolized prior to oxon formation (Sunaga *et al.*, 1989) by ester hydrolases (Soderlund *et al.*, 1982) and mixed-function oxidases (Sultatos and Murphy, 1983). The chlorpyrifos oxon was produced by phase I reaction in which the P=S linkage was oxidized to the more toxic P = O analogue. Chlorpyrifos oxon is generally not detected in the tissues of vertebrates because of rapid hydrolysis to TCP. Conjugation of the resultant TCP with glucuronic acid, which is a phase II reaction, subsequently produced trichloropyridinol - glucuronide. The metabolism of chlorpyrifos in channel catfish as demonstrated by Barron *et al.* (1993) is shown in Figure 1.7.

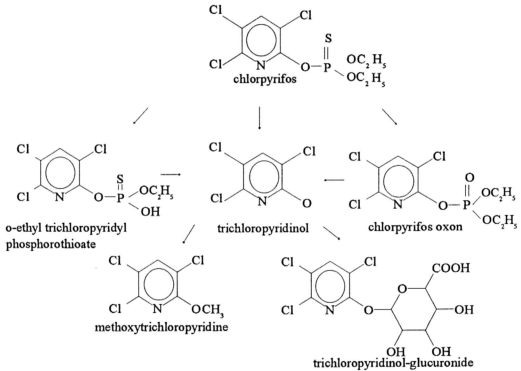


Figure 1.7: Metabolism of chlorpyrifos in channel catfish (Barron *et al.*, 1993)

Hogan and Knowles (1972) found that NADPH-cytochrome P-450 mixed function oxidase system in channel catfish liver microsomes was responsible for the oxidative desulfuration of diazinon to diazoxon and probably for cleavage of diazinon and diazoxon to diethyl phosphorothioic acid and diethyl phosphoric acid, respectively.

In mammals, inducers of the cytochrome P-450 system were initially divided into cyclic hydrocarbons (PAH)-type inducers and phenobarbital (PB)-type inducers.

These various P-450 mediated activities and differentiation demonstrate the existence of multiple enzymes catalyzing the same type of reaction. The development of molecular biology as a discipline has accelerated the speed at which the cytochrome P-450 proteins have been sequenced hence it has been possible to separate the cytochrome P-450 superfamily into families and subfamilies. At the mRNA and protein level the root symbol CYP, denoting cytochrome P-450, followed by the designation of the individual cytochrome P-450 form are recommended (i.e CYP1A1), but the term P4501A1 may also be used for the protein.

Multiple cytochromes P-450 have also been observed in various other species of freshwater and marine fishes. Cytochrome P-450 have been isolated and purified from the livers of the marine fish scup, *Stenotomus chrysops* (Klotz *et al.*, 1983, 1986) and the Atlantic cod, *Gadus morhua* (Goksoyr, 1985). From rainbow trout, *Salmo gairdneri* a major constitutive isozyme designated LM₂ and the principal BNF-inducible, cytochrome P-448 type isozyme, named LM₄ have also been purified (Williams and Buhler, 1982).

One P-450 form from fish, the rainbow trout P4501A1 has been completely sequenced by cDNA characterization (Heilmann *et al.* 1988). In addition immunological studies (William and Buhler, 1984; Goksoyr, *et al.*, 1991) showed that trout P450LM_{4b} is orthologous to mammalian CYP1A1. In common to mammalian CYP1A1 the orthologous fish enzyme is inducible by xenobiotics such as β -naphthoflavone (BNF) and polychlorinated biphenyls (PCB) and is of major toxicological significance because of its role in the metabolism and activation of aromatic hydrocarbons.

Although the induction process in fish is not fully characterized, there is a wealth of evidence for the induction of the enzyme activities of the mammalian P4501A1 ethoxyresorufin-O-deethylase (EROD) and aryl hydrocarbons hydroxylase, (AHH)) by PAH-type compounds in many fish species (Stegeman, 1981; Stegeman and Kloepper-Sams, 1987; Kleinow *et al.*, 1987). Cytochrome P-450 induction experiments showed that rainbow trout and other fish species did not respond to PB-type inducers (Lech *et al.*, 1982). However, chemicals of the cytochrome P-448 (3-MC)-inducing type, including PCBs, polybrominated biphenyls and BNF produced significant increases in the activities of the fish MFOs BP-hydroxylase, ethoxycoumarin O-deethylase (ECOD) and EROD. The induction response of some biotransformation enzymes in fish to certain classes of organic contaminants was the basis for early proposals that these biochemical responses may be used as biomarkers in monitoring environmental pollution (Payne, 1976). Payne *et al.* (1987) used the ability of 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) and crude petroleum in increasing cytochrome

P-448 concentrations to assess environmental exposure of fish and other aquatic species to petroleum and similar inducers. From various studies carried out it was clear that P-450 mediated monooxygenase activities; EROD activity and AHH activity are often seen to be elevated in fish found in polluted waters (Payne, 1984; Payne *et al.*, 1987; Vindimian and Garric, 1989). Hence it was proposed that assessing EROD and AHH activities may be the most sensitive means of determining the induction response in fish. These enzyme activities occur at low, sometimes even undetectable, levels in control or untreated fish.

P4501A1 levels may be determined by several biochemical procedures. The enzyme activity 7 - ethoxyresorufin O -deethylase (EROD) is used to determine the activity of P4501A1. Ethoxyresorufin (7-ethoxyphenoxazone), whose metabolic dealkylation to a single product, resorufin, by the cytochrome P-450 dependent monooxygenase system can be followed with direct fluorometry (Burke and Mayer, 1974). EROD is also the most convenient cytochrome P4501A1 activity determination for use in environmental studies as it is sensitive, cost effective and easy to measure in fish liver (Nebert *et al.*, 1989).

The reaction in hepatic microsomal fractions is dependent on NADPH and oxygen and is inhibited by CO, suggesting that a cytochrome P-450-mediated monooxygenase is involved.

1.7 Test species.

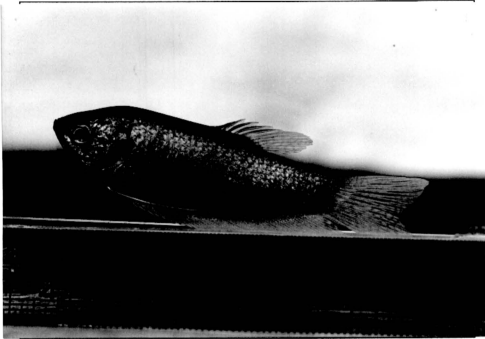


Figure 1.8: *Trichogaster trichopterus*

The test species used in this study is *Trichogaster trichopterus* or more commonly known as “sepat ronggeng” or Three-spot Gourami (Figure 1.8). In Malaysia, it is one of the most common freshwater fish. It favours shallow, stagnant water with a muddy substratum which is conducive to the feeding and air breathing requirements of the fish, hence it is usually found in areas such as swamps, paddy fields, ditches and drains (Tan *et al.* 1973). Its average length is 45mm and may reach 60mm at maturity. This species can be easily recognised by the pelvic fins which are reduced to a rudiment prolonged as a long filamentous ray. Fins greenish to grey with

numerous white to yellowish or orange spots, especially at the ends of the dorsal, caudal and anal fins. Two more or less distinct round, dark spots on the body, one underneath the dorsal fin, the other at the root of the tail (Sterba, 1962).

As an important economic species among the paddy field fishes in this country, it acts as a useful source of additional income to farmers. With the introduction of the double cropping pattern of paddy cultivation, accompanied by the greater and wider use of insecticides, a steady decline in fish productivity from paddy areas was noted as early as 1973 (Tan *et al.*, 1973).

1.8 Chlorpyrifos.

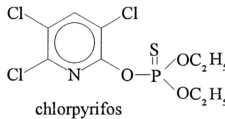


Figure 1.9

Chlorpyrifos is a broad-spectrum pesticide, displaying insecticidal activity against a wide range of insect and arthropod pests. Chlorpyrifos is formulated as a number of different commercial products. The most commonly available formulations include emulsifiable concentrates (EC), granulars (GR) and wettable powders (WP). It

can be found under trade names such as Bolt Roach Bait, Dursban, Lorsban and Pyrinex. In Malaysia, chlorpyrifos is one of the important products used on vegetables, rice and tobacco (Asian Dev. Bank, 1987).

To a great extent, the physical and chemical properties of a compound determine its behaviour in the environment. Although environmental variables are important in modulating transformation and transport processes, the basic pattern of persistence and partitioning is fundamentally derived from the chemical characteristics of the compound. The physicochemical characteristics of the chemical play an eventual role in the prediction of its environmental fate.

Chlorpyrifos is a nonsystemic insecticide applied at rates ranging from 0.0125 lb/acre for mosquito control to 4lb/acre for grub control on turf. It is considered to be of low to moderate environmental persistence, although when applied to soil, polluted water, and buildings it may retain its activity for several weeks (Thomson, 1982).

Chlorpyrifos has been determined to be of moderate volatility with a measured vapour pressure of between 1.8×10^{-5} and 2.0×10^{-5} mm Hg at 25°C. The rate of chlorpyrifos volatilization observed in the environment will be greatly influenced by the nature of the environmental matrix in which it is present and by other partitioning processes (e.g., adsorption and absorption).

Solvent solubility is an important secondary property that is dependent on the inherent polarity of a pesticide. The more nonpolar a compound is, the more soluble it will be in organic solvents and the less water soluble it will be. Chlorpyrifos is readily soluble in many organic solvents. The nonpolar nature of chlorpyrifos is also reflected

in its low water solubility. Water solubilities (23-25°C) have been reported as ranging from 0.94 to 2 µg/mL (Felsot and Dahm 1979).

The first study to examine its metabolism in fish was conducted by Smith *et al.* (1966) using goldfish, *Carrassius auratus*. Fish were placed in water containing 0.3 mg/l chlorpyrifos and assayed after 48 hr for residues. In addition to chlorpyrifos, several metabolites were tentatively identified, including a major one, TCP (3,5,6-Trichloro-2-pyridinol) and several minor ones (desethyl chlorpyrifos, desethyl chlorpyrifos oxon and TCP phosphate).

1.9 Diazinon

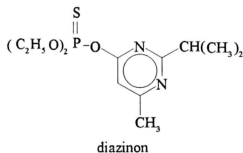


Figure 1.10

Diazinon or O,O-Diethyl O-(2-isopropyl-6-methyl-4-pyrimidinyl) phosphorothioate is an important insecticide which is widely used today. It has a relatively low mammalian toxicity, with an acute oral and dermal LD_{50} in rats of 150-220 mg/kg and 500-900 mg/kg, respectively. Geese and ducks, however, are very

susceptible to diazinon poisoning. Johnsen and Hanstbarger (1966) reported that diazinon has a wide spectrum of insect-killing power and can control various soil insects, DDT-resistant flies, household pests, and various vegetable and forage crop insects.

Diazinon is a colourless liquid with a boiling point of 83-84°C. The technical product is a brown liquid. It is sensitive to oxidation and heat, quickly degrading at temperatures above 100°C. It has a moderate volatility of 1.4×10^{-4} mm Hg at 20°C. Diazinon is relatively soluble in water (1 : 250 ; Gunther *et al.*, 1968) and is miscible with most organic solvents. It is relatively stable in dilute alkali but it is slowly hydrolyzed in water or dilute acid. Diazoxon, its oxygen analogue, has an I_{50} (against fly-head cholinesterase) of 3.5×10^{-9} M, making it one of the most potent cholinesterase inhibitors.

Matsumura and Hogendjik (1964) found that, like parathion, diazinon is largely degraded to diethylthiophosphoric acid.

1.10 Objectives.

The relatively persistent nature of both chlorpyrifos and diazinon in the aquatic environment gives rise to the possibility of prolonged toxic effects to non-target animals. The present study focuses on the effects of chlorpyrifos and diazinon on brain AChE and liver EROD₁ activity in *T.trichopterus* subjected to different exposure regimes of the insecticides.