CHAPTER I

INTRODUCTION

1.1 Statement and significance of the problem

Organophosphate (OP)

Organophosphate insecticides exhibit a high level of pest control ability combined with a low cost. Hence, they are used widely around the world in agriculture and industry, which has led to a variety of negative effects in nontarget species including humans. In Thailand, the insecticides have been imported in high quantities. It was reported that more than 20 millions kilogram (kg) of insecticides was imported in 2006, and costing more than 3 thousand millions bath (Office of agricultural Regulation, 2006).

Insecticides are classified into four groups: organophosphates, carbamates, organochlorines and pyrethroides. Data on insecticide used by 606 farmers in 6 provinces of Thailand during August 2003- July 2004 showed that 58% of the farmers used organophosphate insecticide. Almost all farmers have experienced signs and symptoms of insecticide poisoning. Only 6% of the farmers reported no signs and symptoms of poisoning (The IPM DANIDA project, 2004).

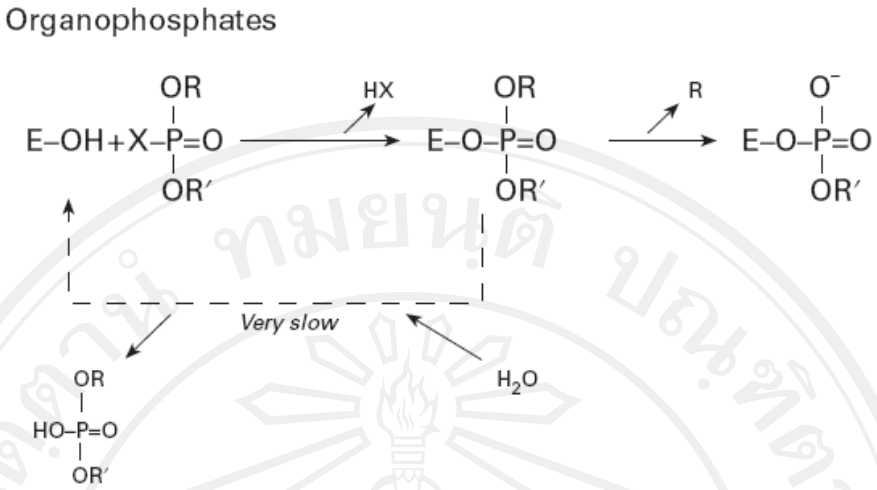

Figure 1 Inhibition of acetylcholinesterase by OP insecticide. E-OH is the AChE and $XPO(OR)_2$ is an OP (Glynn, 1999).

Toxicity from OP insecticides is primarily through inhibition of acetylcholinesterase (AChE) enzyme. The enzymatic inhibition reaction is shown in Figure 1. The first step is a complex formation between enzyme and OP, followed by covalent bonding between OP with AChE (abbreviated E) at hydroxyl group (OH) of the active-site serine. The second step involves the loss of a leaving group (X) which takes with it a hydrogen atom from the enzyme. The product of this second step is a phosphorylated enzyme. From here, the phosphorylated enzyme can be hydrolyzed by adding water to regenerate the original enzyme but this dephosphorylation with water (spontaneous reactivation) is very slow when the substrate is an OP. Thus phosphorylated enzyme can undergo dealkylation, which is termed ageing. This ageing involves covalent cleavage of a bond with in the phosphorylated enzyme, and the formation of a negative charge which stabilizes it and results in irreversible enzyme inhibition. Since AChE enzyme can't hydrolyze acetylcholine (ACh), the neurotransmitter saturates the receptors and accumulates in the synapses, causing

overstimulation at postsynaptic neuron or muscle cell (Reiner, 2001; Ray, 1998; Organophosphorus insecticides, 1998 [online]).

Symptoms of exposure to cholinesterase inhibitors include headaches, dizziness, confusion, blurred vision, nausea and vomiting, stomach cramps, shortness of breath, and tightness of the chest. Signs of overexposure include diarrhea, excessive salivation, sweating, muscle twitching, and miosis (Cholinesterase monitoring – A guide for the health professional, 2006 [online]).

The Thai Office of the Food and Pharmacy Commission and Department of Medical Sciences had investigated fresh vegetable samples from 1994 to 1999 and had astonishingly found that various insecticide residues were detected in 13.04% to 67.44% of total fresh vegetable samples, even in those that were claimed to be toxic free, as show in Table 1. It is noted that dicrotophos is one of the organophosphates oftenly detected in fresh vegetables. Despite its effects on nervous system, to date, it has not been banned (Katesomboon, 2003).

Dicrotophos was introduced in 1956 as a contact systemic insecticide with a wide range of applications. Eighty-five percent of the commercial grade consists of the E-isomer, which is amber in colour and more active to insects than the Z-isomer. It is effective against sucking, boring and chewing pests, and is recommended for use on coffee, cotton, rice, potato, vegetables, and other crops (Extoxnet pip-dicrotophos, 1995[online]).

Table 1 Chemical residues found in fresh vegetable sample (**)

Year	Numbers of samples (toxic-free and general fresh vegetable)	Chemical residues (% and %)	Chemical found
1994	38	39.5	**Dicrotophos** Profenophos Cypermethrin
1995	29 and 27	34.5 and 48.1	Cypermethrin Endosulfan(*) Methamidophos(*)
1996	22 and 49	54.55 and 61.22	Cypermethrin Endosulfan(*) Methamidophos(*) **Dicrotophos**
1997	36	22.0	Cypermethrin Methamidophos(*) **Dicrotophos**
1998	23 and 30	13.04 and 63.33	Cypermethrin Endosulfan(*) Methamidophos(*) Carbofuran
1999	47 and 43	63.83 and 67.44	Cypermethrin **Dicrotophos** Profenophos Monocrotophos(*)

* chemical banned (Banned pesticides, 2007)

** (Katesomboon, 2003)

The metabolic pathways of dicrotophos in animal studies are largely species independent. In mammals, including rats, mice, dogs, rabbits, and goats, dicrotophos undergoes demethylation to des-*O*-methyldicrotophos and hydrolysis to dimethyl phosphate and *N*-demethylacetoacetamide. Hydroxylation of the *N*-methyl group followed by N-demethylation is producing *N*-methyl-*N*-hydroxymethyl dicrotophos, monocrotophos, and *N*-hydroxymethyl monocrotophos (Bull and Lindquist, 1966; Menzer and Casida, 1965; Roberts and Hutson, 1999). These N-demethylated metabolites are better inhibitors of acetylcholinesterase than dicrotophos (Roberts and Hutson, 1999). Residues of dicrotophos are excreted 63-71% within 48 hours in urine (Menzer and Casida, 1965).

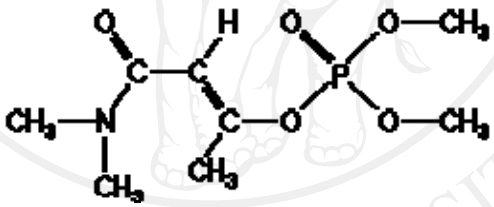

Figure 2 Structure of dicrotophos (Extoxnet pip-dicrotophos, 1995 [online]).

Dicrotophos belongs to the group of pesticides called organophosphates which affect the nervous system by inhibiting acetylcholinesterase. It can overstimulate the nervous system causing nausea, dizziness, confusion; and at very high exposures respiratory paralysis and death may occur. Delayed effect of dicrotophos is neurotoxicity including ataxia, sensory loss, and muscle weakness. These symptoms may be organophosphate-induced delayed neuropathy (OPIDN). This neuropathy is characterized by flaccid paralysis of the lower limbs, partial sensory loss,

degeneration of long axons in the spinal cords and peripheral nerves and becomes evident 1-3 weeks after exposure to OPs (Kim *et al*., 2005). Neuropathy target esterase (NTE), a neural protein with an esteratic activity, is believed to be the molecular target for OPIDN (Johnson, 1982). Several OPs can inhibit NTE by phosphorylation and aging of NTE which similar to that observed for AChE. NTE is localized to the cytoplasmic face of the endoplasmic reticulum (ER) and play a role in ER-associated phospholipids deacylation. Inhibition of NTE activity in neurons of susceptible animal to OPIDN will lead to disruption of phospholipids homeostasis in the ER. Thus axonal transport passes through the ER will be disturbed. In glial cells, inhibition of NTE activity by OPs will cause disturbance in membrane phospholipid metabolism and may impair glia-axon interaction (Glynn, 2006). In the central nervous system (CNS), there are three main types of glial cells including oligodendrocytes, astrocytes, and microglia. Analogous cells in the peripheral nervous system (PNS) are Schwann and Satellite cells. Oligodendrocytes and Schwann cells are involved in myelin formation. Myelin sheath is composed of 70% lipid and 30% protein that are wrapped around the axon. Myelin sheath is an effective insulator that increases resistance across the cell membrane and decreases membrane capacitance. Thus myelination also prevents the electrical current leakage from the axon. However, the myelin sheath does not cover the entire axon. The gaps are formed between myelin sheaths are called nodes of Ranvier. Myelinated axon has voltage-gated sodium channels only in the nodes of Ranvier. Action potential propagation along myelinated axon requires activation of voltage-gated sodium channels only in the nodes of Ranvier where depolarization occurs. Depolarization in myelinated axon jumps from one node of Ranvier to the next is called saltatory conduction. In

contrast, action potential propagation along unmyelinated axon requires activation of voltage-gated sodium channels throughout the entire length of the axon. Thus, action potential propagation in myelinated axon is much faster than unmyelinated axon (Giuliodori and DiCarlo, 2004). Since OP can cause disturbance in membrane phospholipid metabolism in glial cells thus myelin sheath may be damaged. In addition, nerve conduction deficits in peripheral nerve have been detected after exposure to some OPs (Dési and Nagymajtényi, 1999; Papp *et al*., 2004). These changes in nerve conduction may be reflecting the signs of nerve degeneration. However, no scientific research has reported the effect of dicrotophos on axon and nerve conduction velocity.

This study was to determine effects of dicrotophos on axon and nerve conduction velocity in rat, and aimed to verify the hypothesis that multiple exposures to dicrotophos induce axonopathy and decrease nerve conduction velocity in rats.

1.2 Literature review

Some organophosphorus-ester (OP) produced delayed neurotoxicity in man and many other species including hens. The initial biochemical event marking the production of lesion in axons is inhibition of at least 70% of the activity of NTE (Johnson, 1982). Human NTE is similar to a neuronal protein called Swiss cheese (SWS) of fruit fly, *Drosophila*. In wild-type flies, developing neurons are wrapped by a single layer of the plasma membrane of glial cells. SWS mutants show abnormality interaction between neurons and glial cells. These neurons are loosely wrapped with several layers of glial-cell membrane and then leads to apoptosis of both neurons and glial cells (Kretzschmar et al., 1997). In mice with brain-specific deletion of NTE, the brains of these mice show signs of neurodegenerative with loss of neurons and spongiform vacuolation (Akassoglou *et al*., 2004).

A rodent model of OPIDN has been developed using Long-Evans adult male rats exposed to tri-ortho-cresyl phosphate (TOCP) (Padilla and Veronesi, 1985). The inhibition of NTE activity in brain and spinal cord was correlated with the appearance of spinal cord pathology. It was observed that single dosages of TOCP ranging from 385-3480 mg/kg produced marked spinal cord pathology 14 days postexposure in most of similarly dosed animals. By electron microscopy, axonal swellings appeared to contain massive accumulations of smooth endoplasmic reticulum and aggregates of mitochondria, and were characteristically surrounded by a disproportionately thin myelin sheath. In contrast, no significant tibial nerve damage was noted in any of the TOCP-treated animals compared to controls at 14 days postexposure.

The effect of single injection of mipafox was administered to both Long-Evans hooded rats and White Leghorn hens (Dyer *et al*., 1992). All animals were monitored for clinical evidence of OPIDN for 21 days, and regions of the nervous system were histological evaluated. It was found that only hens manifested clinical signs of neuropathy; however, light and electron microscopic lesions were present in the nervous systems of both species. In rats, these lesions were well developed in only the highest dosage group which inhibited the activity of brain NTE greater than 80% of control values within 4 hr after intoxication. These lesions confined to the rostral level of the fasciculus gracilis in the medulla oblongata. Swollen axons containing a single vacuole filled with flocculent material were most prominent lesion in rats. Hens manifested more extensive and varied fiber breakdown in multiple spinal cord tracts, with the increased intensity of degeneration with increasing dosage of mipafox.

After 30 mg/kg mipafox ip injection, bilateral mipafox-induced neuropathy in the medulla and cervical spinal cord in both hens and rats were demonstrated with well-developed, vacuolar axonopathic lesions in the fasciculus gracilis by post-dosing day 7 in rats (Carboni *et al.*, 1992). Severely affected rats with lesions were noted through day 21, but not subsequently (days 28 and 35). However, the hen had a slower developing, but more severe, consistent and longer lasting neuropathy than the rat.

In another study, Adult male Long-Evans rats were exposed to 2 neurotoxic organophosphates in a setting of chronic stress, over a 63-day period (Jortner *et al.*, 2005). The organophosphates were tri-*ortho*-tolyl phosphate (TOTP) administered in 14 gavage doses of 75, 150 or 300 mg/kg, and chlopyrifos, given in two 60 mg/kg subcutaneous exposures. Corticosterone was added to the drinking water at 400 µg/ml, to model aspects of chronic stress. The major neuropathologic change was the

presence of axonal degeneration progressing to myelinated fiber degeneration, mainly in distal regions of selected fiber tracts and peripheral nerve, seen in animals sacrificed on experimental day 63. The cervical spinal cord and medulla level of the gracile fasciculus were most prominently affected. On day 90, after a 27-day period without exposure to the OP, the nerve fiber degeneration had progressed in all experimental groups administered the 300 mg/kg dose of TOTP. TOTP significantly inhibited hippocampal AChE and NTE activity on day 63. By day 90, AChE activity remained depressed, although to a lesser degree while NTE activity recovered.

Regarding the nerve conduction velocity, sheep farmers exposed to OP pesticides have a high incidence of neuropathy. The incidence of nerve conduction deficit increased from 7% in the non-neuropathic group to 35% in the probable or definite neuropathy group (Jamal *et al*., 2002). In addition, the nerve conduction deficit was observed in animal exposed to OP. Male Wistar rats were exposed to dichlorvos for 4, 8 or 12 wk in a 5 day per week schedule (Dési and Nagymajtényi, 1999). It was observed in that study that the drug decreased conduction velocity of the peripheral nerve (the tail nerve) in the groups receiving 3.92 mg/kg of dichlorvos for 8 or 12 wk. In another study, Male Fisher F344 rats exposed to chlorfenvinphos, diisopropyl fluorophosphates (DFP) or dimethoate for 4 wk were observed with a decrease in conduction velocity of the tail nerve and the velocity was most strongly reduced by chlorfenvinphos (Papp *et al*., 2004).

1.3 Objective of the study

1. To study the nerve conduction velocity after multiple dicrotophos exposure.
2. To study the histopathology of the cervical spinal cord and medulla level of the fasciculus gracillis and sciatic nerve after multiple dicrotophos exposure.