ORGANOPHOSPHATE INSECTICIDE TOXICITY IN RAINBOW TROUT (*Salmo gairdneri*) : EFFECTS OF TEMPERATURE AND INVESTIGATIONS ON THE SITES OF ACTION

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by

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TABLE OF CONTENTS

TABLE OF CONTENTS (continued)

ABSTRACT

In order to protect fish from organophosphorus (OP) insecticide applications, field monitoring programs for assessing the effects of OP insecticides on fish have to be developed. Detection of OP insecticide pollution in natural water requires knowledge of the sites of action and the effects of environmental factors on the toxicity of these chemical in fish. Two OP insecticides; acephate (a phosphoramidothioate and a direct inhibitor of cholinesterase [ChE]) and fenitrothion (a phosphorothioate and an indirect inhibitor of ChE), were tested on rainbow trout (Salmo gairdneri) fingerlings to study the effects of temperature stress on acute lethality and ChE inhibition in brain and skeletal muscle. Physiological responses of cardiovascular and respiratory systems, ChE inhibition in various tissues and changes in serum electrolytes in adult fish exposed to each insecticide were observed to provide some more understanding on the sites of action of OP insecticide producing death in fish.

Temperature stress affected the acute lethality of each insecticide but was more pronounced with fenitrothion than with acephate during the first 24 hour period. The effects of temperature stress became less after 48 hours and no significant effects were observed after 96 hours of exposure. Expressed as LC_{50} values (the concentration that produced 50 percent mortality), the toxicity of fenitrothion was about 600 to 1000 times greater than acephate depending upon test temperature. There was no correlation between ChE inhibition levels in the brain and skeletal muscle of rainbow trout fingerlings and the concentration of acephate

and fenitrothion which produced mortality.

Acephate and fenitrothion both produced a decrease in heart rate, increase in ventilation rate and amplitude in adult rainbow trout. Fenitrothion produced an increase in cough frequency, but acephate did not. Acephate and fenitrothion produced differential patterns of ChE inhibition in various tissues of fish. The extent to which this enzyme was inhibited depends on the physicochemical properties and probably the distribution within the fish body of both insecticides. ChE activities in the tissues of cardiovascular and respiratory systems especially gills, heart and serum were inhibited to a greater extent than brain and skeletal muscle by each insecticide. It is suggested that these two systems are adversely affected by OP insecticides and therefore could be used to detect exposure to OP insecticides in fish. Acephate and fenitrothion produced changes in serum electrolytes characterized especially by an increase in serum potassium and a decrease in serum chloride concentrations. These changes were considered to be caused by the movement of electrolytes among fluid compartments to maintain electroneutrality.

This study indicates that the cardiovascular and respiratory systems in fish are very important sites of action for OP insecticide toxicity, and that this toxicity depends on physicochemical properties, eg. lipid solubility and degree of ionization of the insecticide, and on environmental factors, eg. temperature.

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LIST OF TABLES

Page

Table

LIST OF TABLES (continued)

LIST OF FIGURES

LIST OF FIGURES (continued)

INTRODUCTION

Organic compounds containing phosphorus are essential constituents of protoplasm and play important roles in the maintenance of life. On the other hand, many organophosphorus (OP) compounds are artificially produced for use as lubricants, oil additives, plasticizers, and pesticides (Eto, 1974). The discovery of the insecticidal action of these compounds was made in Germany during the Second World War from efforts directed toward development of chemical-warfare agents (O'Brien, 1967). In addition to insecticidal activity, a variety of other biological activities of OP compounds was discovered. For example, these compounds are used as acaricides, nematocides, anthelmintic agents and herbicides. It is surprising that such great varieties of chemical, physical and biological properties of these OP pesticides are governed by the selection of groups attached to the phosphorus atom.

Owing to the relatively low persistence and high effectiveness of OP pesticides, their application to agriculture, public health, and related fields has been growing rapidly in many countries. About 140 organophosphorus compounds are now used as pesticides and more than 60,000 tons a year of OP pesticides are produced in the United States alone (Eto, 1974).

Mechanism of action of OP insecticides in vertebrates

The insecticidal activity and mammalian toxicity of OP insecticides are generally believed to be due to the inhibition or inactivation of cholinesterases (ChE) which is a group of the hydrolytic enzymes for acetylcholine (ACh), a nerve transmitter, released in the process of cholinergic transmission. ACh is a neuro-transmitter operates in

cholinergic synapses which include synapses in the central nervous system eg. in brain and spinal cord. ACh also operates in the synapses of the peripheral somatic nervous system eg. the neuromuscular junction of the motor nerves, sensory nerve endings of skeletal muscle, and also in the autonomic nervous system eg. all preganglionic, postganglionic and a few postganglionic sympathetic synapses which consist of nerves, ganglia and plexuses that provide the innervation to the heart, blood vessels, glands, viscera and smooth muscle (Koelle, 1970a). The inhibition of ChE by OP insecticides, therefore, disturbs normal nervous function and finally results in the death of animals.

Knowledge of the involvement of ChE in the areas of neurophysiology (Ruch and Patton, 1965), neurobiochemistry (Silver, 1974) and neuropharmacology (Koelle, 1970a, 1970b) is extensive. These areas are well documented in the above text.

ChE inhibition in fish by OP insecticides

Experiments have shown that fish exposed to sublethal and lethal concentrations of several OP insecticides exhibited a reduced level of ChE activity in excised brain tissue and the surviving fish removed from exposure to OP insecticide demonstrated a capacity for regeneration of this enzyme (Weiss, 1958; 1959). However, brain ChE inhibition in fish exposed to OP insecticides showed a broad range of response. The brain ChE activity of fishes that died from exposure to OP insecticides ranged from zero activity to 98.6 percent of normal level (Weiss, 1961). Fish surviving such exposures had brain ChE activity as low as 5.4 to 10 percent of normal level (Weiss, 1961; Gibson *et al* 1969). These investigators suggested that death usually occurs when fish brain ChE

activity is 40 to 70 percent inhibited as compared to that of non-exposed, control fish of the same species. Coppage (1972) and Coppage and Mathews (1974) suggested 80 percent inhibition of the ChE activity of fish brain is the critical level in short term OP insecticide poisoning. These investigators also concluded that the degree of inhibition of brain ChE activity by OP insecticides is a function of concentrations of the insecticides, exposure time, specific chemical nature of insecticides, water chemistry conditions and fish species.

Inhibition of brain ChE in fish has been proposed as a means of detecting OP insecticide pollution in natural waters and has been used for monitoring purposes (William and Sova, 1966; Holland et al 1967; Coppage and Braidech, 1976). Nicholson (1967) suggested that a 10 percent depression of ChE concentration in fish brain should be used as an upper limit for evaluating water quality relative to OP insecticide contamination. Gibson et al (1969) reported that mortality and recovery from OP insecticide poisoning in fish are not necessarily related to the degree of ChE inhibition in the brain. The degree of ChE inhibition in brain has also been reported as not being correlated with the decline in behavioral and adaptive responses in fish (Rosic et al 1974).

Many studies have been done on the effect of anti-ChE agents on the neuromuscular system in other vertebrates, but only recently has the neuromuscular system in fish received attention. Pharmacological investigations of neuromuscular transmission in fish have been reported by Hidaka and Kuriyama (1969), Diamond and Mellanby (1971), and Mellanby and Thompson (1972). They reported that the major lethal action of

anti-ChE agents was a blocking of neuromuscular transmission by preventing both nerve stimulated and spontaneous release of ACh from presynaptic terminals. Schneider and Weber (1975) evaluated the significance of ChE to neuromuscular transmission in the pectoral fin abductor muscle of largemouth bass (Micropterus salmoides) and reported the occurrence of ChE inhibition by an OP insecticide, DFP (diisopropylfluorophosphate). They concluded, however, that the acute toxic effects of DFP to largemouth bass are not mediated by a collapse of neuromuscular function.

Fish skeletal muscle has also been suggested as a useful tissue as fish brain in ChE inhibition study in detecting exposure to OP insecticides and could facilitate sampling for enzyme assays especially with small fish where brain dissection may be difficult (Benke and Murphy, 1974).

Metabolism of OP insecticides in fish

OP insecticides can be separated into 2 major groups, according to whether they are activated, as direct inhibitors and indirect inhibitors (Loomis, 1974). The majority of OP insecticides are indirect inhibitors, eg. phosphorothioates, which have the sulphur atom linked to the central phosphorus atom (P=S) as the basic structure. These compounds have little, if any, direct inhibitory activity of ChE and are activated by the mixed-function oxidase (MFO) enzyme systems in liver to the more potent inhibitors, eg. the oxygen analogs (P=O). This enzyme system is one of the major metabolic systems available for eliminating foreign compounds such as drugs, petroleum products and insecticides (Chambers and Yarbrough, 1976). Although these metabolic reactions are most often detoxications, there are some reactions, especially epoxidation and

desulfuration, which can be activations to more toxic compounds (Chambers and Yarbrough, 1976).

The ability to metabolize OP insecticides and the fact that the toxicity of OP insecticides is related to hepatic metabolic activity has been demonstrated in a wide variety of fish species (Buhler and Rasmusson, 1968). The activation of the indirect inhibitors, eg. the phosphorothioate (P=S) compounds, to the active ChE inhibitor by liver preparations has been observed in brook trout (Salvelinus fontinalis), brown trout (Salmo trutta), pumpkinseed (Lepomis gibbosus), black bullhead (Ictalurus melas), winter flounder (Pseudopleuronectus americanus), and shorthorn sculpin (Myxocephalus scorpius) (Potter and O'Brien, 1964; Murphy, 1966). Liver of pumpkinseed sunfish can both activate and detoxify parathion and methyl parathion (phosphorothioate compounds), but at a slower rate than mouse liver (Benke et al. 1974). Sesamex, a MFO inhibitor, prevented the activation of parathion in mosquitofish (Gambusia affinis) and increased the 48 hr LC_{50} value (the concentration of insecticide that produced 50 percent mortality at 48 hours of exposure) by almost 11-fold (Ludke et al. 1972). A 57 percent inhibition of brain ChE in the sesamex-treated fish as compared to 89 percent inhibition in the non-treated groups of the same parathion concentrations was reported. Activation of parathion by MFO, as indicated by brain ChE inhibition, was also noted in golden shiners (Notemigonus crysoleucas), green sunfish (Lepomis cyanellus), and bluegill sunfish (Lepomis macrochirus) (Gibson and Ludke, 1973).

Effects of temperature on fish and OP insecticides

The effects of temperature on fish are profound. From enzymatic

reactions through hormonal and nervous control to digestion, respiration, osmoregulation and to all aspects of performance and behaviour, fish are influenced by temperature. Temperature always act as a controlling factor, and may at certain levels act as a directive or lethal factor for fish (Fry, 1967).

Temperature affects the Michaelis-Menten constant, Km (a substrate concentration for enzyme at which the velocity of reaction is half maximal), but the relationship between temperature and Km is quite complex. In general, over an upper temperature range, the Km varies directly with temperature; at lower thermal extremes the effects of temperature are often reversed (Somero, 1969; Fry and Hochachka, 1970). Brain ChE activity of killifish (Fundulus heteroclitus) varies inversely with the temperature of acclimation (Baslow and Nigrelli, 1964). Hazel (1969) however, reported that the specific activity of ChE in brains of goldfish (Carassius auratus) and killifish (Fundulus heteroclitus) acclimated to 25 C was significantly higher than in fish acclimated to 5 C. Increase in ChE activity with environmental temperature is also observed in brain tissue of bluegills (Lepomis macrochirus) (Hogan, 1970).

A rapid increase in temperature imposes a stress on fish which is exhibited by hyperglycemia, hypocholesterolemia, increased blood hemoglobin and decreased interrenal ascorbic acid (Wedemeyer, 1969; 1973). Acute and moderate thermal shock in rainbow trout (Salmo gairdneri) is accompanied by decreases in plasma sodium and chloride levels, a decrease in tissue water, and an increase in extracellular fluid (Reaves et al. 1968).

A temperature rise in an aquatic environment will cause the rates of many biological processes in fish including swimming activity (Brett, et al. 1958), digestion (Hathaway, 1927) and respiration (Hughes and Roberts, 1970) to increase. The term Q_{10} is a factor by which the reaction rate is increased by a temperature increase of 10 C (Warren, 1971). In general, Q_{10} values associated with physical processes, such as diffusion or conductivity, and those associated with photochemical reactions are less than 1.5, while Q_{10} for thermochemical (enzymatic) reactions range from 2 to 3 but can vary widely because the Q_{10} will depend on the thermal history and normal temperature range of a fish (Hoar, 1975).

With increases in temperature, toxicities of some substances are increased and the resistance to disease in fish is lowered (Jones, 1964). Toxicity of insecticides to fish is generally thought to be greater at higher temperatures. Macek et al.(1969) studied the effects of temperature on the susceptibility of bluegills (Lepomis macrochirus) and rainbow trout (Salmo gairdneri) to selected pesticides. They found an increase in the susceptibility of fish to most pesticides tested as temperature increased. They suggested that a probable mechanism involved is a higher rate of pesticide uptake at the higher temperature than at lower temperature by an indirect effect of temperature on metabolism. Increased temperature may also decrease toxicity of some pesticides to fish as observed with DDT and methoxychlor, chlorinated hydrocarbon insecticides, but the mechanism involved is still not clear (Johnson, 1968; Macek et al. 1969). Increasing temperature can accelerate not only the penetration and harmful action of insecticides but also

adaptive responses, including their elimination from the body (Wilber, 1969).

Temperature can also affect the metabolic processes of OP insecticides including biotransformation or activation by MFO enzyme system in fish liver (Chambers and Yarbrough, 1976). The increased susceptibility of fish to a pesticide could be related to an increased level of enzymatic activity at higher temperature than at lower temperature. Macek et al.(1969) suggested that the observed increase in the susceptibility of rainbow trout to Dursban, a phosphorothioate OP insecticide, as temperature increases is apparently related to an increase in the activation process of Dursban to its phosphate analog which is more toxic.

Sites of action of OP insecticides in fish

The target sites of action for OP insecticide acute lethal intoxication in mammals are well understood (Koelle and Gilman, 1949; Holmstedt, 1959; Koelle, 1970b). The cause of death in mammals is primarily respiratory failure, usually accompanied by a secondary effect on the cardiovascular system (Koelle, 1970b). Respiratory failure is caused by peripheral paralysis of the diaphragm owing to the blockage of neuromuscular transmission and by a disturbance of the respiratory center in the medulla oblongata of the brain resulting in hypoxia (Koelle, 1970). In poikilotherms, especially fish, the target organs and sites of action of OP insecticides are still not known.

Cardiovascular and respiratory systems of fish are controlled by the central and peripheral nervous systems as in other vertebrates (Campbell, 1970; Randall, 1970). The pacemaker of the fish heart is

normally located in the sinus venosus or at the junctional area between the sinus venosus and the atrium and is innervated by the cardiac branches of the vagus nerve (Randall, 1966). It was suggested that the vagal inhibitory effect on fish heart was essentially the same as that on the amphibian and mammalian hearts, and that the cardio-inhibitory effect is mediated by an cholinergic innervation (Cobb and Santer, 1973; Saito, 1973). ACh at low concentrations is reported to reduce the heart rate of the eel (Anguilla japonica) and the effect is abolished by atropine, a cholinergic blocking agent. The inhibition of ChE in the heart muscle by OP insecticides has been reported in mammals (Holmstedt, 1959; Sharma et al. 1973) but no data are available on the inhibition of ChE in fish heart muscle.

The heart must be supplied with sufficient oxygen and metabolic fuels to replace continuously the energy expended both as useful work and as energy lost because the heart is less than perfectly efficient as a pump (Nasser, 1970). The placement of the teleost heart far downstream of a single-loop circulation presents a problem of oxygen supply. Two sources of oxygen are available to it: a high volume, low concentration supply of venous blood going through the lumen; and a low volume, high-concentration arterial blood diverted from the dorsal aorta to the heart through the coronary arteries (Cameron, 1975). Myocardial ischemic conditions may occur whenever the coronary blood flow is insufficient in relation to oxygen requirement of the myocardium. The small quantity of oxygen contained within a given mass of water compared with the same mass of air clearly imposes a limit on the range of respiratory homeostasis in fish and therefore fish are in a greater danger

of hypoxia than land vertebrates (Satchell, 1971).

ChE activity in whole or fraction of human blood has been routinely used for a number of years as an indicator of exposure to anti-ChE agents (Gage, 1967). Significant inhibition of ChE activity within either plasma or red blood cells indicates exposure to an inhibitor of ChE (Witter, 1963; Wills, 1972). The preparation and purification of ChE in serum and erythrocyte of carp (Cyprinus carpio) and its chemical properties are reported by Kuwabara and Hayama (1961) and the inhibition of ChE in both serum and erythrocyte by OP insecticides is reported by Hayama and Kuwabara (1962). Investigation of the ChE characteristic in the blood to assess some of the effect of OP insecticides in other fish species is also reported in channel catfish (Ictalurus punctatus) (Hogan, 1971). Serum ChE activity of rainbow trout (Salmo gairdneri) is found to be more sensitive than brain ChE activity as an indicator of sublethal poisoning by fenitrothion, an OP insecticide (Lockhart et al. 1973).

Since the respiratory and circulatory systems of fish are intimately related, investigations on the effect of OP insecticides on physiological function of fish gills could provide useful information. The gills of freshwater teleosts function as the primary site for the active transport of ions or materials from the external media and for the respiratory exchange of gases. Therefore, any substances that interfere with gill functions will affect the homeostatic condition of the fish body. Gill functions in fish are underneural control and are regulated by autonomic nerve fibers in the gill (Campbell, 1970). The effects of cholinergic and adrenergic drugs on gill function and histological studies demonstrate the presence of nerve endings on the pillar cells and filamental vessels

of the gills (Ostlund and Fange, 1962; Rankin and Maetz, 1971; Randall et al. 1972). It is suggested that fish regulate vascular resistance as in higher vertebrates (Satchell, 1971) and ACh increases filamental sinus blood flow while epineprine increases secondary lamella blood flow (Richards and Fromm, 1969). The functional surface area of rainbow trout gills can be regulated by changing perfusion pathway of the blood flow and ACh decreases the functional gill surface area and increases the overall branchial vascular resistance (Bergman et al. 1974). Several insecticides, including OP, decrease the rate of fluid flow through isolated perfused rainbow trout gills which indicates that resistance to fluid flow through gills is increased (Fromm et al. 1971).

OP insecticides used in the study

Fenitrothion (O,O-dimethyl-O-(3-methyl-4-nitrophenyl) phosphorothioate) an indirect ChE inhibitor, is a broad spectrum OP insecticide used extensively throughout the world for control of agricultural and forest pests (NRCC, 1975). It has low toxicity to mammals and has been used as a replacement for DDT in Canadian forests to control the spruce budworm since 1969 (NRCC, 1975). In Canada, annual spray operations at the dosage of 2-4 ounces/acre range have involved millions of acres of forest (NRCC, 1975). Aerial spraying of fenitrothion causes the aquatic environment, existing within the forests, to be contaminated at levels which produce lethal and sub-lethal effects to fish species (NRCC, 1975). Fish mortalities, in and adjacent to areas after aerial spray of fenitrothion, have been reported (Hatfield and Riche, 1970; Kingsbury, 1973; Coté and Tétreault, 1973). Determination of ChE activity in the brain of atlantic salmon parr (Salmo salar) (Zitko et al. 1970) and in