Quantitative and Qualitative Changes in the Skeletal Muscle Acetylcholinesterase Activity of *Oreochromis niloticus* Exposed to Methylparathion

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ABSTRACT

Spectrophotometric assays and histochemical tests for acetylcholinesterase activity in the epaxial skeletal muscle of maturing Oreochromis niloticus *after in-vivo exposure to 0.10 mg/L methylparathion showed significant inhibition of the enzyme by the pesticide. The assays manifested enzyme inhibition, after 48 and 96 hours of exposure, of 43.19% and 56.62%, respectively. These results were confirmed by the occurrences of decreased sites of acetylcholinesterase activity in the muscle fibers as exhibited upon performance of histochemical tests.*

INTRODUCTION

The ever-growing demand for increased food production to meet the requirements of the world's population has led to the use of different chemicals to destroy crop pests. One group of such chemical substances for this purpose is the organophosphate. This pesticide, widely used in pest control programs, however, has been proven to induce physiological and biochemical changes not only to target organisms but to non-target organisms as well. Thus, methylparathion (0.0 dimethyl, 0-4 nitrophenyl thiophosphate), an organophosphate which can be carried by run-off waters from agricultural farms to the natural aquatic environment, can affect fishes and other aquatic organisms.

The present work which employed laboratory experiments studied the effects of this pesticide on the neuromuscular system by employing both quan-

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titative and qualitative tests on *Oreochromis niloticus*, a fish species of commercial importance. It also experimented on the two tests for their possible application later on in the field as a monitoring procedure for environmental pollution.

Most of the studies done concerning organophosphates pertain to their capacity to inhibit acetylcholinesterase activity. Acetylcholinesterase is an important neurological marker being the inactivator of the neurotransmitter, acetylcholine. Enzyme inhibition in the central nervous system of different organisms including insects (1), fish (2-7), prawn (8), rats and rodents (9-10) and humans (11) have been worked on. Comparative anticholinesterase activity of different organophosphates have used malathion and parathion (1), guthion, phorate, parathion, and diazinon (2). Limited histochemical tests had been conducted and while previous studies had mainly used the enzyme bioassay technique, this was mostly on temperate animal species.

MATERIALS AND METHODS

Animals and Treatment

Maturing *Oreochromis niloticus* (2 ½ - 3 months old, 30-50 g) were obtained from the Bureau of Fisheries and Aquatic Resources Research Station in Tanay, Rizal. Prior to treatment with methylparathion, they were acclimated in continuously aerated dechlorinated tap water in 50-L glass aquaria for one week. A group of 40 fishes in one aquarium was then maintained without treatment with pesticide while another group of 40 fishes was treated with methylparathion (E.C. 50%) of sublethal concentration (0.10 mg/L). During acclimation and the experiments that followed, the fishes were fed with commercially available fish flakes.

Skeletal muscle tissue samples for the acetylcholinesterase bioassay and histochemical analysis were obtained at the 48th- and 96th- hour of exposure. Prior to actual experimentations, the 96 hr. LC_{50} was determined through static bioassays. Through graphical analysis (12), this was found to be 0.18 mg/L.

Assay for Acetylcholinesterase Activity

A piece of epaxial skeletal muscle tissue was homogenized in 0.65% NaCl (20 mg tissue/ml) with a ground glass homogenizer. The homogenates were then centrifuged for 5 minutes at 6,000 rpm. The supernatant was collected and the pellet discarded.

The acetylcholinesterase assay measured the colorimetric reaction between a hydrolysis product of the acetylcholine analogue (thiocholine), and the reagent 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) (13). The reaction mixture in a test tube contained the following: 2.6 ml phosphate buffer, pH 8.0; 100 μl 0.01M DTNB; 20 μl 0.075 M acetylthiocholine iodide and 0.4 ml supernatant as enzyme source. Absorbance readings were taken and recorded at 60s intervals using a Spectronic 21 spectrophotometer at 412 nm; as blank, a test tube containing buffer+DTNB and substrate. Levels of the enzyme were obtained following the recommended formula. Activity was expressed as μmol substrate hydrolyzed per minute per milligram protein of muscle tissue.

Protein Analysis

Protein concentrations in the tissues were determined using the Lowry method(14) with bovine serum albumin as standard.

Statistical Analysis

The results were analyzed using ANOVA and Least Significant Difference Test to compare the enzyme activities between the treatments (15).

Histochemical Analysis

The acetylthiocholine iodide method for cholinesterase in fish muscle(16) was used. For the histochemical tests, skeletal muscle tissue previously fixed in 10% formaldehyde was teased into 1-2 mm chunks. Then the tissues were incubated at 35 °C for one hour in the incubating medium consisting of 20 mg acetylthiocholine iodide and 10 ml of the recommended stock medium of pH 6.0. The tissues were then blotted, transferred to three successive baths of saturated sodium sulfate (1 min/bath), and immersed in diluted ammonium sulfide (0.4%) until brown crystals appeared. These were later rinsed in distilled water and placed in glycerine. They were mounted on a slide and covered with a glass slip. When examined under the light microscope, sites of AChE activity

were seen as deep brown areas while surrounding muscle showed a light yellow color. Photomicrographs were taken.

RESULTS AND DISCUSSION

The activity levels of acetylcholinesterase in both control and methylparathion-exposed *O. niloticus* are shown in Table 1. The fish exposed to a sublethal concentration of the organophosphate after 48 hours had reduced AChE activity by 43.19% compared to the control group while those exposed after 96 hours had reduction of 56.62%. It was also shown that the inhibition was greater with longer period of exposure to the pesticide.

The quantitative results matched the histochemical tests done for AChE activity (Figs. 1-4). Muscle fibers of the control group showed abundant patches of dark brown color signifying higher enzyme activity compared to those in 48-hour and 96 hour-exposed fish which showed decreased color development.

This study proved that methylparathion, like other organophosphates, is an effective acetylcholinesterase inhibitor. Organophosphates have been described as effectively interfering with synaptic transmission between neurons and muscle cells while other pesticides exert their effects on the nerve fiber itself(17).

In normal nervous and neuromuscular functions, neurotransmitters (e.g. acetylcholine) released from presynaptic sites diffuse across the synapses and trigger appropriate responses in the adjacent cell. In order to restore the sensitivity of the synapse to additional impulses, the neurotransmitter must be eliminated. Acetylcholine is promptly removed by acetylcholinesterase which hydrolyzes specifically the neurotransmitter(18). The inactivation process permits the repolarization of the membrane and its ability to respond to incoming impulses.

Methylparathion can be metabolically altered to an active AChE inhibitor by the conversion of the P=S compound to one containing P=O group. The resulting oxygen analogue (methylparaoxon) is several times more potent in inhibiting the enzyme through competition with acetylcholine. The strong electrophilic group (P=O) binds with the active site of the enzyme, thus, blocking ACh hydrolysis and inhibiting AChE activity (8, 19, 3).

Most studies now are focused on the capability to recover from enzyme inhibition after a high-dosage, short-term duration exposure, mostly after 96-hours. Hence, recovery period seems to depend on the amount of depression of enzyme activity, with a longer recovery period required for those fishes that have had larger amounts of depression (7,10). Possible compensatory mechanisms have been proposed(10).

The present study showed that exposure to sublethal concentration of methylparathion even for a short duration can inhibit AChE activity. This seems to indicate that the AChE assay can be used as a monitoring tool to detect contamination in aquatic systems with methylparathion and probably organo-phosphate pesticides and neurotoxins. More quantitative information, though, are needed for the application of the assay.

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Table 1. Activity levels of acetylcholinesterase in skeletal muscle of *Oreochromis niloticus* after short-term exposure to 0.10 mg/L methylparathion*

Group	Exposure Period (in hours)	
	48	96
Control	10.53 ± 0.15	11.41 ± 0.28
Experimental	6.00 ± 0.17	4.95 ± 0.07
(PDC)	(-43.19)**	(-56.62)**

* μmoles ACH hydrolyzed/min/mg protein. Each value is mean ± S.E.

PDC: Percent deviation over respective control

** Significant at 1% level

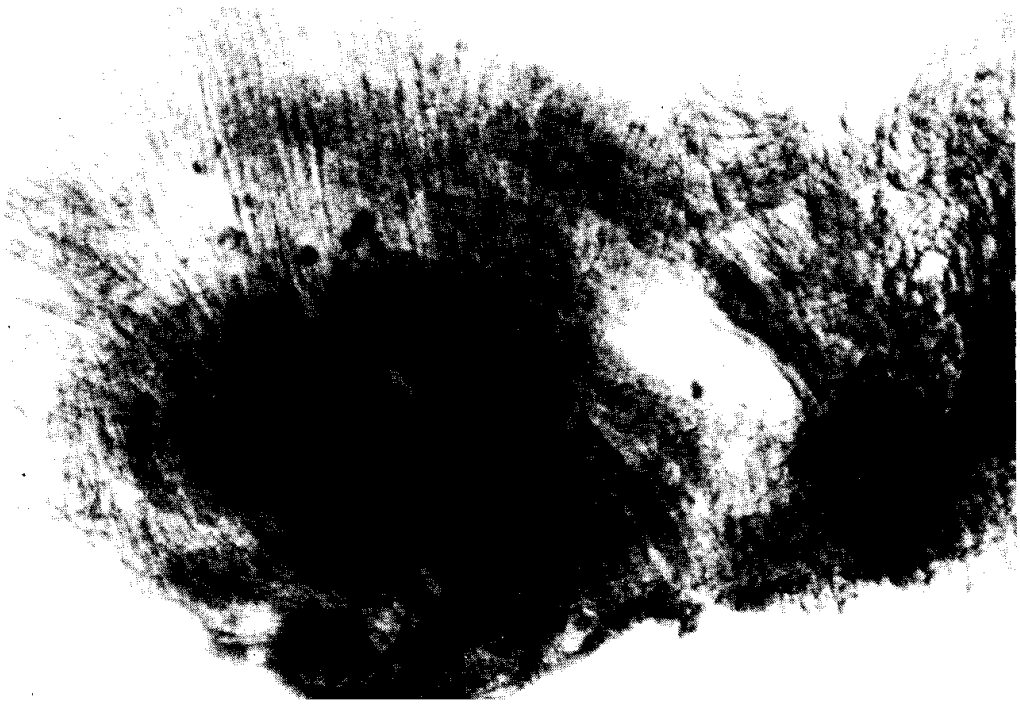

Figure 1. Photomicrograph of skeletal muscle of *O. niloticus* after 48-hour exposure to 0.10 mg/L methylparathion, x100.

Figure 2. Skeletal muscle of *O. niloticus* control fish at 48th hour of the experiment, x100. Muscle fibers of control fish show more abundant patches of dark brown color signifying higher level or activity of acetylcholinesterase.

Figure 3. Skeletal muscle of *O. niloticus* after 96-hour exposure to 0.10 mg/L methylparathion, x100.

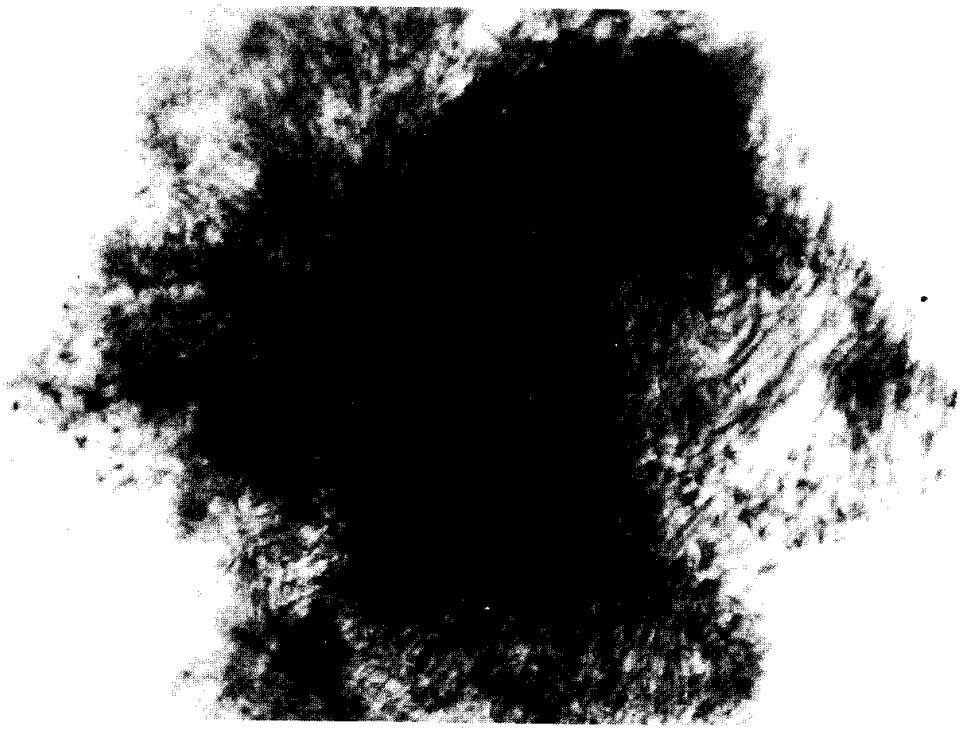

Figure 4. Skeletal muscle of *O. niloticus* control fish at 96th hour of the experiment, x100.