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Effects of Organophosphorus Insecticides on *Dugesia tigrina*: Cholinesterase Activity and Head Regeneration

D. Villar,[1,2] M. González,[2] M. J. Gualda,[3] D. J. Schaeffer[1]

[1]Department of Veterinary Biosciences, University of Illinois, 2001 South Lincoln Avenue, Urbana, Illinois 61801, USA
[2]Departamento de Farmacología y Toxicología, Universidad de Córdoba, Avda, Medina Azahara, 9, 14071-Córdoba, Spain
[3]Servicio Andaluz de Salud, Córdoba, Spain

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Reliable values for the toxicity of organophosphorus insecticides [OPs; the main class of insecticides registered for use (Smith 1987)] to aquatic species, are needed to develop water quality standards and to estimate ecological risk. Planaria are inexpensive to use and sensitive bioassay organisms, and exhibit a range of acute, subchronic and chronic endpoints (Best 1983; Grebe and Schaeffer 1991; Schaeffer 1993) for monitoring aquatic ecosystem health. OPs exert teratogenic effects in mammals and birds (Smith 1987; Kitos and Suntornwat 1992), and we have shown that OPs produce abnormalities in *Dugesia dorotocephala* exposed during head regeneration (Villar et al. 1993). The present study examines the lethality and behavioral toxicity of OPs to intact *Dugesia tigrina*, and developmental effects during head regeneration. Changes in acetylcholinesterase (AChE) activity due to OP exposure were quantified.

MATERIALS AND METHODS

Asexual *D. tigrina*, a non-native species widely distributed in Spain, were collected from the Guadalbarbo stream (where it crosses Alcolea village) in southern Spain. Worms were maintained in a temperature-controlled room at 19 or 24°C using a 12:12 hr dark:light cycle. They were fed chicken liver once a week, but were not fed for at least 48 hr before the experiments.

Aliquots of technical-grade insecticides dissolved in acetone were pipetted into 400 ml beakers, 1-2 ml of acetone was added, and the acetone was evaporated under an air stream while rotating the beaker to coat the bottom surface and bottom 2 cm of the walls. To each beaker were added 100 ml of a standard test medium (STM; 7.5 g $CaCl_2$, 3 g $MgSO_4$, 1.5 g $NaHCO_3$, and 0.15 g KCl per 25 L distilled water) and 10 asexual *D. tigrina*. AChE assays used 40 planaria exposed to a given concentration of a specified OP or to STM alone.

p-Nitrophenol, the main metabolite of methyl parathion, was purchased from Sigma Chemical Co. (St. Louis, Missouri). Technical grade diazinon (38%), fenitrothion (95%), malathion (54.4%), and methyl parathion (48%) were a gift from Agrocros S.A. (Madrid). Methyl parathion and diazinon purities were assayed by gas chromatography at the Servicio Andaluz de Salud laboratory (Córdoba, Spain). Fenitrothion and malathion were analyzed by the veterinary diagnostic laboratory at the University of Illinois, and no impurities were detected.

Correspondence to: D. J. Schaeffer

After solubilization in STM, the concentrations of methyl parathion and fenitrothion were determined by high-performance liquid chromatography (HPLC). A 50 μL aliquot of the solution was injected into a Spectra-Physics IsoChrom LC pump fitted with a Rheodyne injector with a 10 μL loop. Analyses were carried out at 275 nm using a reverse-phase HPLC column (15 by 0.4 cm; Analytical-tracer column Spherisorb ODS-2; 5 μm particle diameter) connected to an SP8440 uv/vis detector and HP3390A integrator. The compounds were eluted using a mixture of methanol, water, acetic acid and triethylamine (50:50:0.6:0.008, by volume) at 1 ml/min.

Mortality (disintegration of animals) was recorded daily and survivors were placed in clean beakers at the same concentration. Most OP concentrations either killed most of the animals or none, so successive exposures were carried out in narrow concentration ranges in order to approximate the LC_{50} concentration. The LC_{50} at 4 d was calculated from all the data using the trimmed logit method (Sanathanan et al. 1987).

Head regeneration was observed between days 3-15 following decapitation behind the auricles, and scored at 16X magnification (Villar et al. 1993). The developmental stages scored were: (1) death, (2) acephalic head (completely absent), (3) anophthalmic head (rudimentary head without eyes and with or without a single median auricle), (4) both eyes present and normal, and (5) normally formed auricles. Typically, 6 days were enough to obtain fully regenerated heads, but because the number of control animals had almost doubled in 15 d at 24°C, the regenerating process was observed for 15 d.

Acetylcholinesterase activity was measured according to the procedure of Ellman et al. (1961). Forty animals were processed in an Ultra Turrax T25 homogenizer (Janke & Kunkel, Staufen, Germany) in 4 ml of 0.1 M potassium phosphate buffer (pH 7.9). Phosphate buffer (0.1 M, pH 7.9, 0.55 ml) and dithiobisnitrobenzoic acid (DTNB, 0.01 M, 0.2 ml) were added to the planarian homogenate (1/10 dilution, 0,1 ml) and the sample was incubated at 25°C for 5 min. A spectrophotometer (Beckman UV-VIS Model DU-70) was calibrated at 412 nm using a reference blank prepared in a similar way, except that the homogenate was omitted and 0.15 ml of the substrate acetylthiocholine iodide (AcT, 0.150 M in phosphate buffer) was added. The samples were then poured into cuvettes and 0.15 ml of the substrate was added. The reaction was followed over the next 6 min by recording the absorbance every 30 sec in thermostatted cuvette holders.

Protein determinations were performed according to Lowry et al. (1951) using bovine serum albumin as a standard. Due to the high affinity and efficiency of AChE under these conditions (Km $\approx$ 0.17 mM), substrate concentrations had to be increased to 150 mM to work at V_{max} conditions (usual concentration is 75 mM). No substrate inhibition was observed at this concentration. Supernatants were processed similarly, using 0.45 ml buffer, 0.2 ml DTNB, 0.2 ml sample, and 0.15 ml AcT. Results are reported as mU/mg protein.

RESULTS AND DISCUSSION

The solubilities of fenitrothion and methyl parathion in STM were similar to acetone dilutions of the same concentrations of those exposures listed in Table 1. Retention times were 20.5 and 24.0 min for methyl parathion and fenitrothion, respectively. The 96 hr acute toxicities (LC_{50}) are in Table 1.

Table 1. LC_{50} (96 hr) of OPs and *p*-nitrophenol to intact *D. tigrina*.

Chemical	Reported[1] solubility (mg/L)	Exposure Concentration (mg/L)	Experimental[2] LC_{50} (mg/L) 19°C	24°C
Diazinon	68.8	0.05-4	-	0.63±0.2
Fenitrothion	25.2	1-10	2.9±0.1	1.7±0.3
Malathion	145	4-12	-	4.4±0.8
Methylparathion	30-60	1-10	4.1±0.2	2.6±0.3
p-Nitrophenol	soluble	6-18	12.1±2.1	-

[1]Bowman and Sans (1983), Chapman and Cole (1982).
[2]In our study, most deaths occurred in the first two days of exposure for all the OP insecticides.

The tested OPs produced signs indicative of nervous interference (startle responses, pharynx protrusion, twitching, head shaking, abnormal contractions, depression). *p*-Nitrophenol produced startle responses in the animals and loss of surface-adherence capacity. Deaths during the first two days increased with concentration. Head loss increased with concentration and ranged from 15-100% of survivors. Head loss was followed by inactivity and loss of normal chemotactic, phototactic and other responses. Death rates and/or lengths of the inactive period depended on the insecticide and concentration. In addition to the lesions described below, the motor activity of planaria exposed to most OPs was noticeably impaired. Fenitrothion and methyl parathion caused hypersensitivity, and many animals exposed to fenitrothion exhibited spiraling motions at any concentration tested. In contrast, animals exposed to malathion or diazinon moved sluggishly, had difficulty moving along the glass surface, and adopted contracted body conformations.

Fenitrothion at concentrations above the LC_{50} produced white stripes along the dorsal surface starting behind the eyes or in the tail portion in the first day of exposure. Their length and the number of affected animals increased with time and steeply with concentration (2 and 7 animals at 2.5 and 3 mg/L, respectively). This was previously reported for *D. dorotocephala* exposed to methyl parathion (Villar et al. 1993). The lesions exposed the underepidermal tissue and progressed with head loss and possibly death, or, in some cases, abnormal growth of tissue at the head ending. Animals manifesting white stripes constantly contracted their head backwards. Another effect of fenitrothion on intact animals was depigmentation of the nose/head on all the animals surviving concentrations near the LC_{50} for 7-8 d.

Intact animals which had lost their heads during exposure to methyl parathion or fenitrothion at concentrations near the LC_{50} did not have normal head regrowth and were prone to develop nodules at the head ending. Animals fissioning during the exposure often developed rounded tail tips. The abnormal growths were usually followed by death or by sloughing followed by inactivity during the period of exposure to the insecticides.

The rate of fissioning in intact, unexposed, animals was 15 d at 24°C. Exposure to OPs often delayed the rate of fissioning, and was related to concentration (Table 2). Exposures near the LC_{50} ($\geq$3 mg/L methyl parathion, 5 mg/L malathion, $\geq$2.5 mg/L fenitrothion) completely inhibited head development

Table 2. Head abnormalities in regenerating *D. tigrina* on day 6 at 24°C.

Chemical (mg/L)	Number of Animals (n = 10)				
	Normal head	Abnormal head	Dead	Total Fissions Day 6	Day 15
Control	10	0	0	3	8
Malathion (MW 330.4)					
4.0	4	6	0	2	8
5.0	0	6	4	0	4
Methyl parathion (MW 263.2)					
1.0	10	0	0	2	6
2.0	9	1	0	1	4
2.5	9	1	0	0	1
3.0	0	6	4	0	1
4.0	0	5	5	0	3
Fenitrothion (MW 277.3)					
1.0	9	1	0	5	7
2.0	8	2	0	2	10
2.5	2	8	0	4	5
3.0	0	4	6	0	0
4.0	0	0	10	0	0

or prevented normal growth of the heads (Table 2), yielding animals with tiny heads, without normal auricles, and retracted (not extended) within the neck. Malathion (3 mg/L) and fenitrothion (2 mg/L) produced a single eye (teratomorphic) in one animal at each concentration and a third supplementary eye (with eye cavity) in another animal that was undergoing head regeneration. The same abnormal developmental effects were reproduced in animals regenerating their heads following normal fissioning. Control animals at 24°C developed normal heads 5-6 d after decapitation.

Mean AChE activity of the homogenates from controls was 312±35 mU/mg protein. The activity of the supernatant after centrifugation at 13,000 x g decreased to 49±15 mU/mg. Since AChE is not easily solubilized, tissue activity was determined on the whole homogenate (Table 3). *p*-Nitrophenol did not significantly alter the AChE activity over 9 d at 6 mg/L. Addition of 1 mg/L diazinon to homogenates from control animals caused total *in vitro* inhibition of AChE (and 55% inhibition with 0.1 mg/L), but identical concentrations of fenitrothion, malathion or methyl parathion produced little inhibition. All the insecticides inhibited AChE activity, with relative rates: diazinon > methyl parathion > malathion > fenitrothion. These relative rates of AChE inhibition corresponded to the LC_{50}s for all the insecticides, except for fenitrothion. Fenitrothion produced unusual signs not observed with the other OPs, such as increased spiraling and a white streak/line on the dorsal surface of the animals. None of these signs were observed with diazinon, the compound that produced the highest anticholinesterase action.

Concentrations above the LC_{50} produced rapid death and disintegration.

Table 3. AChE activity[1] in *D. tigrina* exposed to OPs (mg/L).

Hours	Methyl parathion		Malathion	Fenitrothion	Diazinon	
	1	0.5	1	1	0.2	0.1
2	-	100.6	-	-	-	53.7
2.5	-	-	-	-	16.7	-
4	-	99.7	-	-	-	32.9
17	96.5	-	94.6	95.0	-	-
20	87	90.6	-	-	11.3	-
40	93	92.7	80.9	92.5	-	-
45	-	-	-	-	6.5	7.6
65	54.8	94.6	80.0	92.5	-	-
70	-	-	-	-	4.1	4.9
90	65.8	-	71.5	72.5	3.9	-
144	-	83.3	-	-	-	3.1
216	-	72.9	-	-	-	2.1
336	-	56.3	-	-	-	1.9

[1]AChE activity is expressed as the percentage of the respective control group (312±35 mU/mg protein) for each time interval.

Below the LC_{50}, AChE inhibition increased with exposure duration, but the rates differed widely among the OPs tested (Table 3). Except for diazinon, large, dose-related decreases in AChE activity occurred in a few hours only at high concentrations. Thus, exposure to 15, 10, 6, 4, 2, or 0.5 mg/L methyl parathion for 2 hr decreased activity to 72%, 88.6%, 93.6%, 98% and 100.6% of control, respectively. A 3 hr exposure to 9, 6, or 3 mg/L malathion reduced activity to 60.5%, 69.4%, and 86.66% of the control. Exposure to 6 or 3 mg/L fenitrothion for 4.5 h reduced activity to 79% and 99%.

There was no clear correlation between AChE activity and behavioral, morphological and mortality effects. Behavioral responses to each insecticide differed during the first 4-5 d of exposure below the LC_{50} and then became indistinguishable, although enzyme activities differed between insecticides. Animals exposed to diazinon were able to move normally even at less than 10% of normal AChE activity. The apparent lack of correlation between AChE inhibition and behavior or mortality in planaria could be related to the presence of isoenzymes with overlapping function (or compensatory abilities) but different resistance to OP inhibition, as reported by Chang and Opperman (1991) in the root-knot nematode *Meloidogyne*.

The role of acetylcholine (ACh) in planarian regeneration is not well understood. Studies using atropine to competitively bind the ACh receptor concluded that ACh enhanced regeneration (Welsh 1946; Moses 1983). In contrast, others have reported that exposure to the ACh antagonists atropine, *d*-tubocurarine or decamethonium increased the rate of regeneration in *D. tigrina* (Lenicque and Jacobsen 1969). Furthermore, our results and those of Tiras (1978) and Lenicque and Feral (1976) found that inhibition of AChE (which increases endogenous ACh) retarded regeneration.

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