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Comparative Toxicity of Parathion in Early Embryos and Larvae of the Toad, *Bufo arenarum* Hensel

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Parathion (O,O-diethyl O-p-nitrophenyl phosphorothioate) is a widely used agricultural insecticide highly toxic to mammals; however, very little is known about the detoxication capacity in some natural enemies of insects such as amphibians (Harri et al 1979; Llamas et al. 1985; Caballero de Castro et al 1991; Gauna et al. 1991). The biota may be stressed by discharges of pesticides, and it is therefore essential to know its potential effect on non-target organisms before irreversible change occurs. Some monitoring techniques use amphibian larvae as an useful indicator for aquatic contamination (Dumpert and Zeitz 1984; Beiswenger 1988; Williams et al. 1989).

An ideal insecticide should be efficacious against pest species, but relatively safe for non-target organisms. To develop selective insecticides it is important to understand the differences and similarities of the defense systems between insects and the beneficial fauna such as predators and parasitoids. Thus, this report deals with a comparative study of parathion susceptibility on early embryonic and larval stages of the Argentinian toad *Bufo arenarum* Hensel in relation with its metabolism, and target enzyme inhibition.

MATERIALS AND METHODS

Adult *Bufo arenarum* Hensel toads were collected in the area of Buenos Aires city during winter, and kept in captivity outdoors in a small aquarium containing grass.

Ovulation was induced by homologous hypophisis implantation. Oocytes thus obtained were diseminated *in vitro* by the addition of a testicular suspension. Inmediately after fertilization, the embryos were separated in short strings, and allowed to develop in glass dishes with abundant Ringer's solution. Larvae were fed with boiled lettuce *ad libitum*.

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Toxicity testing consisted of five replicates of 20 embryos or larvae each exposed to at least five concentrations of technical grade parathion (E.P.A. standard Research Triangle Park) with 0.05% acetone as vehicle. The control medium consisted of diluted amphibian Ringer's solution with an equivalent volume of acetone. Mortality was determined 120 hr after exposure. The LC_{50} values were calculated by probit analysis.

Acetylcholinesterase (AChE) assay was performed according to the procedure described by Ellman et al. (1961) and samples were processed daily. Batches of 5-day-old embryos and 22-day-old larvae were exposed to 2mg/L of parathion (2 mg/L of parathion plus 0.05% of acetone in Ringer's solution), and controls were exposed to Ringer' solution plus 0.05% of acetone. After 7 days of exposure, several embryos and larvae were transferred to Ringer's solution in order to assay the rate of recovery. In all experiments, embryos and larvae were counted, extensively washed with Ringer's solution, and homogenized with 0.1 M sodium phosphate buffer of pH 8.0 using 2 to 3 embryos or larvae/mL. Adult toad brain and liver were homogenized in the same buffer at a final concentration of 0.15 to 0.30 mg of protein/mL. The homogenates were incubated 30 min with different concentrations of paraoxon (Sigma Chemical Co.) in order to determine the dose required for 50% of cholinesterase inhibition. The AChE reaction mixture consisted of acetylthiocholine (1.0 mM), 5,5'-dithiobis (2-nitrobenzoic acid) (0.15 mM), and 1.0 mL of 0.1 M phosphate buffer (pH 8.0). The change in absorbance was monitored at 412 nm against blanks. Results were expressed as percentage of control value. The dose producing 50% inhibition of cholinesterase in vitro (I_{50}) was determined from the linear part of curves obtained by plotting percent inhibition of cholinesterase against the log of paraoxon concentration.

Metabolism of parathion was analyzed on 100 embryos and larvae. Each was homogenized in 6 mL of 0.067 M phosphate buffer (pH 7.6). Each homogenate was centrifuged for 15 min at 16,000 sp gr, and the pellet was discarded. The incubation mixture (3 mL) contained 1,000 nM parathion, plus 2.5 µC ^{14}C-parathion (ICN Biochemicals, Inc.), and 3 mL of 16,000 sp gr supernatant (1 to 2.5 mg of protein). The mixture was incubated at 37°C for 1 hr with continous shaking, and the reaction was stopped by the addition of 3 mL of n-hexane. After partitioning, the aqueous phase was reextracted twice with 3 mL of n-hexane. Radioactivity was determined in aliquots from both the hexane extract and the aqueous phase using Aquasol II as the scintillation liquid. The remaining organic phase was analyzed by TLC in 250 nM Silica Gel G plates, and developed with benzene:acetone (19:1). Spots were visualized by iodine vapors, and scraped into vials

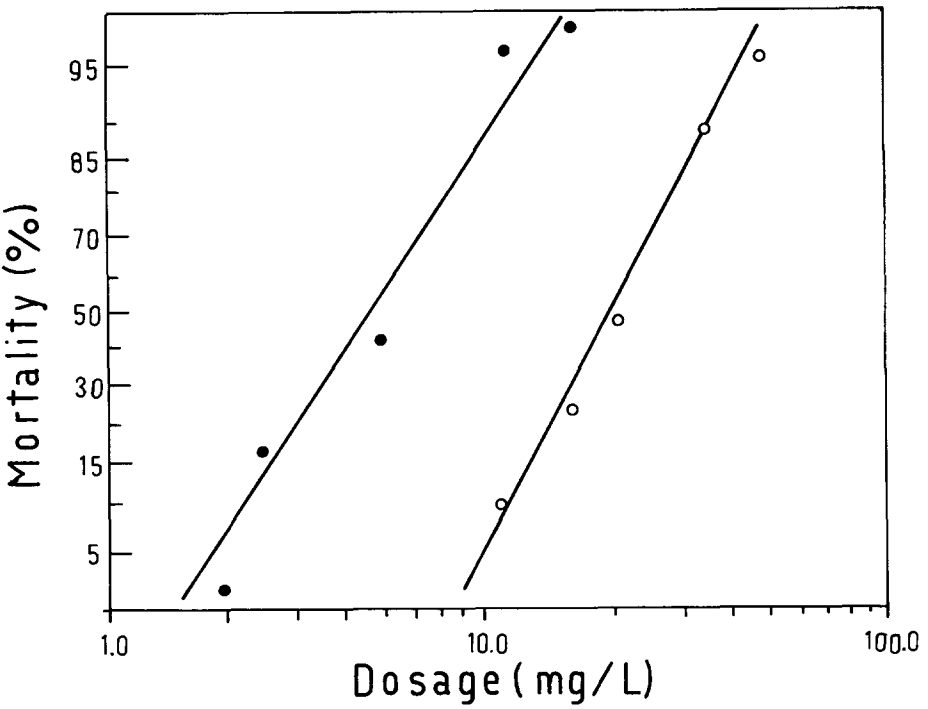

Figure 1. Mortality of *B. arenarum* embryos (o) or larvae (●) exposed to parathion. Each point represents an average of at least four samples.

containing 0.5 mL of ethyl acetate plus PPO/POPOP (0.40/0.02%) scintillation cocktail. Identification of metabolites was based on chromatography with standards which were visualized with 0.25% palladium chloride in 0.2 N HCl or iodine vapors.

RESULTS AND DISCUSSION

Mortality plots of bioassays on 5-day embryos and 22-day larvae are illustrated in Figure 1. Young embryos are 4.4 times more tolerant to the acute lethal effect of parathion than larvae. However, the LC_{50} values obtained with larvae are higher than those reported for other aquatic organisms (LeBlanc 1984; Clark et al. 1985; Borthwick et al. 1985).

The LC_{50} values observed for parathion in embryos and larvae were 20.2 and 4.5 mg/L, respectively. It was considered possible that these differences in LC_{50} values might be reflected in the kinetics of cholinesterase inhibition. As shown in Fig. 2, the activity of larvae cholinesterase declined faster than the embryonic enzyme after parathion exposure. In fact, a 50% reduction in the enzyme activity was achieved by parathion-treated embryos

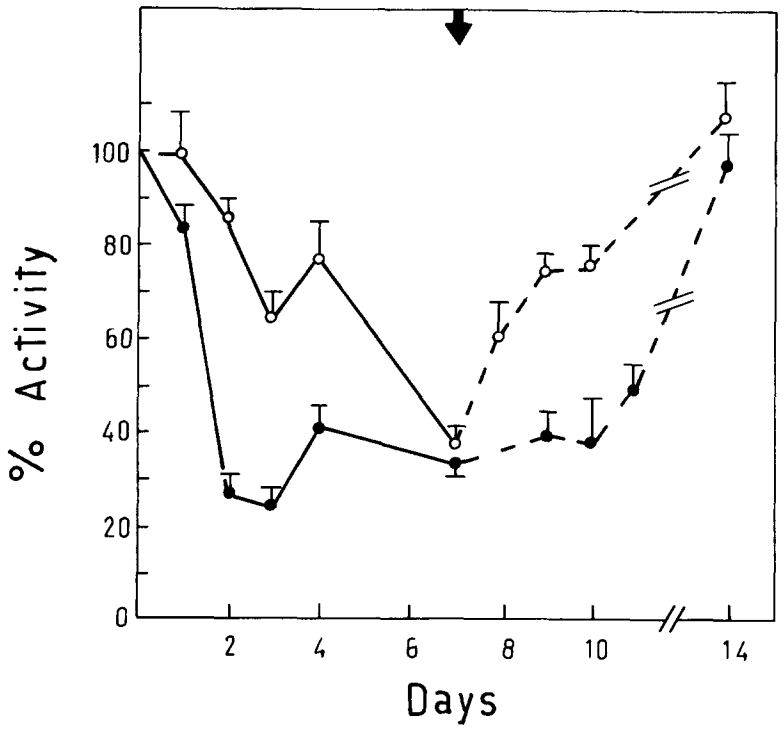

Figure 2. Progression of AChE inhibition with time in the presence of parathion (—) and recovery (--) using embryos (o) or larvae (•) homogenate. Each value is the mean of three experiments (± SD). The arrow indicates the time at which embryos or larvae were transferred to uncontaminated Ringer's solution.

after six days of exposure, while the same result was obtained in parathion-treated larvae in a two-day period. The selective tolerance of young *B. arenarum* embryos to parathion poisoning could not only be attributed to the differences observed in the kinetics of inhibition of the target enzyne, but also to a summation up of events. It is important to highlight that the enzyme recovery is faster from embryos, eventhough both stages achieved complete reactivation after 7 days in uncontaminated Ringer's solution.

Similar results were obtained with malathion-treated embryos, but in this case, the activity recovered was smaller than the control (Caballero de Castro et al. 1991). The differences in toxicity shown by early embryos and larvae also are associated to differences in cholinesterase sensitivity of both stages to paraoxon (Table 1). The embryonic AChE seems to have differences in the target site for the insecticide, making the early

stages of embryonic development less susceptible to organophosphate toxicity.

Table 1. *In vitro* acetylcholinesterase inhibition by paraoxon from different tissues of the toad *B. arenarum*.[a]

Tissue	I_{50} (μM)
5-7 day-old embryos	8.33 ± 2.30
17 day-old larvae	2.75 ± 1.85[b]
brain	3.33 ± 2.62[c]
liver	7.32 ± 2.00[c]

[a]Results are expressed as the concentration required to produced 50% inhibition (I_{50}) in vitro. I_{50} values were determined graphically by plotting percentage inhibition as a function of inhibitor concentration. At least five concentrations were used for each determination.
[b]$P < 0.05$ with respect to 5 to 7 day-old embryo.
[c]Non significant with respect to 5 to 7 day-old embryo.

Embryonic 16,000 sp gr supernatant degraded more ^{14}C-parathion to aqueous soluble metabolites than the larvae fraction (Table 2). This suggested that the cleavage of the aryl phosphate linkage is done more efficiently in embryonic supernatant than that from larvae. This point also supports the fact of the higher tolerance of embryos by a greater detoxication mechanism. The organic-soluble compounds identified by TLC were parathion and paraoxon. The amount of paraoxon formed by both homogenates was negligible. However, the sustained inhibition of the AChE

Table 2. *In vitro* metabolism of ^{14}C-parathion by embryos and larvae 10,000 xg supernatant.[a]

Days of development	% of dose recovered after treatment			
	Organic Phase		Aqueous Phase	Total % Recovered
	Parathion	Paraoxon		
5 day-embryos	88.08±0.9	0.10±0.09	9.30±0.2	97.48
13 day-larvae	94.04±1.5[b]	0.60±0.04[b]	1.65±0.4[c]	96.29

[a]Results are expressed as mean (± SD).
[b]$P < 0.01$ with respect to 5-day-old embryo.
[c]$P < 0.001$ with respect to 5-day-old embryo.

suggests a continous production of paraoxon that could not be explained by the small quantity of this metabolite.

The decreased rate of oxon production might facilitate the binding of paraoxon to other non-critical binding sites (Murphy 1969), like carboxylesterases, which are present in early developing embryos after fertilization occurred (Rosembaum et al. 1988). Paraoxon has a high affinity for carboxylesterase and this fact might induce a delay in the onset of toxic signs and death because of the greater opportunity of detoxication by binding.

We conclude that the mechanisms responsible for the increased tolerance of early embryos to parathion is related to the lower level of parathion activation, the increased cleavage of the aryl phosphate linkage, the slower kinetics of the acetylcholinesterase inhibition, the faster recovery of the target enzyme and the decreased sensitivity of early embryonic AChE to paraoxon.

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