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EFFECT OF MALATHION ON *BUFO ARENARUM* HENSEL DEVELOPMENT—I

ESTERASE INHIBITION AND RECOVERY

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Abstract—Newly fertilized *Bufo arenarum* Hensel embryos were exposed continuously or for a brief period (72–120 hr) to malathion (44 ppm) and then resuspended in amphibian Ringer's solution. Continuous exposure depressed acetylcholinesterase (EC 3.1.1.7), butyrylcholinesterase (EC 3.1.1.8) and carboxylesterase (EC 3.1.1.1) activities. The activities of the three enzymes in embryos treated for 72 hr recovered after a delay of 24 hr, but these enzymes showed different rates of recovery in embryos treated for 120 hr. Acrylamide disc electrophoresis showed several bands of esterase activity in control embryos. Continuous exposure to malathion abolished all esterase activity within 48 hr, but if the exposure continued new bands of esterase activity appeared at 120 hr of exposure. The zymograms of embryos exposed for 72 or 120 hr to malathion and then transferred to uncontaminated medium for 120 hr were similar to that of control embryos.

The hypothesis that acetylcholine mediates intracellular communication during early embryogenesis has been discussed extensively [1–6]. *d*-Tubocurarine decreases and carbamylcholine increases the aggregation rate of cells from early embryos of the medaka *Oryzais latipes* [7]. Eserine produces an inhibition of muscle activity in ascidian (*Ciona intestinalis*) embryos [8], and cholinergic antagonists block the second phase of gastrulation in sea urchin embryos [9]. The consequences of interfering with the cholinergic balance have not been fully understood. The roles of acetylcholine and cholinesterase in early embryonic development need further demonstration.

Acetylcholinesterase has an important function in synapses and motor end-plates, preventing the accumulation of acetylcholine [10]. However, we have demonstrated a significant increase in acetylcholinesterase activity in young *Bufo arenarum* embryos prior to the onset of swimming [11]. Moreover, functional cholinesterase activity may be related to the morphological differentiation of the nervous system that begins soon after gastrulation [12].

Malathion is an organophosphorous insecticide widely used in Argentina. This pesticide inhibits cholinesterases, inducing the accumulation of acetylcholine. We report here that exposure to the insecticide produced a delay in development that was correlated to the duration of exposure.

MATERIALS AND METHODS

Adult *Bufo arenarum* Hensel toads were collected in the area of the city of Buenos Aires, Argentina. The animals were maintained in captivity, outdoors, in a small aquarium containing grass.

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Ovulation was induced by homogeneous hypophysis implantation both in summer and in winter. *In vitro* fertilization was accomplished with a testicular homogenate. Embryonic development took place in a 1:10 diluted amphibian Ringer's solution without bicarbonate (111 mM NaCl, 1.3 mM KCl and 2.7 mM $CaCl_2$) in glass containers. The experimental medium consisted of malathion (94% pure, Cyanamid Argentina) incorporated into Ringer's solution (as above). The malathion concentration was 44 mg/L, with 2.8% acetone. An equivalent volume of acetone was added to the control medium. Mediums were changed daily.

Sample embryos were taken by removing the jelly coat with 2% neutralized thioglycolic acid followed by extensive washing with Ringer's solution.

In vitro *experiments*

Embryos were continuously developed following fertilization in dishes containing Ringer's solution or Ringer's solution plus insecticide. Following 72 and 120 hr of malathion exposure, some embryos were transferred to insecticide-free Ringer's medium, where they continued their development (Fig. 1). At the stage indicated in Fig. 1, embryos or larvae were washed, counted, homogenized in SET buffer (250 mM sucrose, 1 mM EDTA, 50 mM Tris–HCl, pH 7.4), and centrifuged for 20 min at 10,000 *g*. The supernatant thus obtained was used for enzyme assay or electrophoretic analysis.

Enzyme assay

Cholinesterases. The cholinesterase activities were measured according to Ellman *et al.* [13] with some modifications. The 10,000 *g* supernatant (50 μL) was incorporated in a 1-mL final volume of an assay medium containing 1.5 mM acetylthiocholine iodide or 1.4 mM *S*-butyrylthiocholine iodide, 0.2 mM

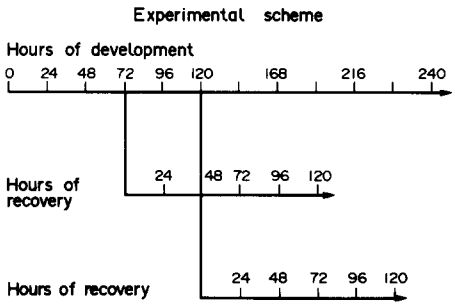

Fig. 1. Sequences of malathion exposure and recovery.

5,5-dithio-2-bis-nitrobenzoate (DTNB) in 100 mM phosphate buffer, pH 8.0. Activity was recorded automatically at 420 nm.

Carboxylesterase. The 10,000 *g* supernatant (50 μg) was raised to a 2.5-mL final volume with assay medium containing 1 mM *p*-nitrophenylbutyrate in 20 mM phosphate buffer, pH 7.0, containing 5% (v/v) acetone. Activity was recorded automatically at 400 nm.

All enzyme assays were measured in a double-beam UV spectrophotometer (Shimadzu UV-240).

Polyacrylamide gel electrophoresis.

The method of Davis [14] was used without modification. The gels were stained with α-naphthyl-acetate-Fast Blue B for carboxylesterase activity. A volume corresponding to five embryos was applied to each gel. The separating and stacking gels contained 7.5 and 4% acrylamide respectively.

Protein determination

The method of Lowry *et al.* [15] was used for protein determination with bovine serum albumin as standard.

Statistical analysis

Average values and standard errors are presented. Student's *t*-test was applied for data analysis.

RESULTS

The addition of eserine sulfate to the embryo homogenate abolished all cholinesterase activity; nevertheless, since cholinesterase activity could be caused by true cholinesterase as well as pseudocholinesterase, acetylthiocholine and butyryl-thiocholine iodide were used as substrates.

The measured pseudocholinesterase activity was 15% of the total acetylcholinesterase activity in 5-day embryos. Therefore, both true and pseudo-cholinesterase activities were present in early *Bufo arenarum* embryos with a ratio of true to pseudocholinesterase of 6.

Total acetylcholinesterase activity began to be detected in control embryos soon after gastrulation. At this stage a decrease in the rhythm of cell cleavage is evident, there are important metabolic changes, and the nuclear activity is increased in order to regulate the metabolism of the embryonic cells. A significant increase in enzyme activity was evidenced with a maximum on day 5 of development, followed by a steep decrease. Control embryos obtained during the summer and continuously exposed to 44 ppm of malathion showed negligible acetylcholine-hydrolyzing activity. On the other hand, embryos exposed for 72 or 120 hr to the same concentration of malathion recovered the activity but with a delay of 24–72 hr in the timing of development. The greatest activity achieved after the transient exposure was lower than in the control group in both cases. Figure 2 shows that total acetylcholinesterase activity was highly dependent on the season of ovulation. The developmental pattern of enzyme activity of winter embryos was slower than that of summer

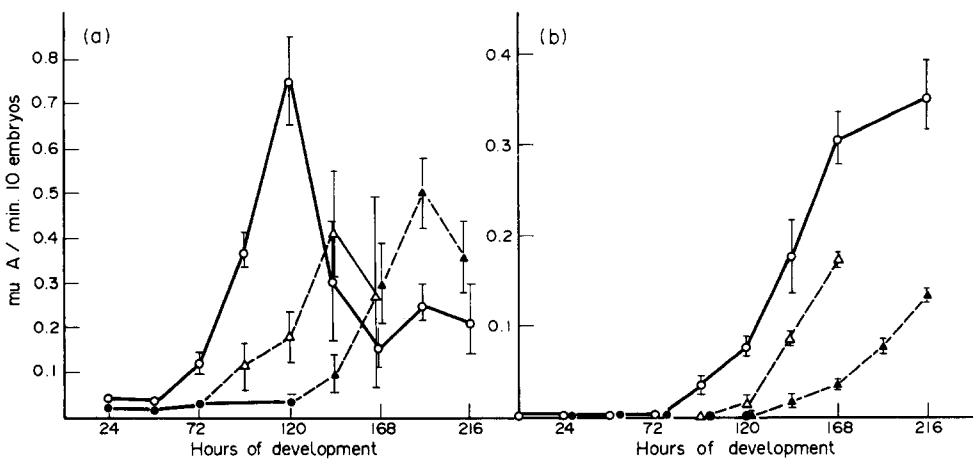

Fig. 2. Time course of inhibition and recovery of acetylcholinesterase activity after malathion treatment in toad embryos. (a) Summer ovulation. (b) Winter ovulation. Recently fertilized eggs were developed in control and malathion (44 mg/L) solutions and analyzed for AChE activity as described in Materials and Methods. Key: (○) control embryos, (●) embryos exposed continuously to 44 mg/L malathion, (△) embryos exposed for 72 hr, and (▲) embryos exposed for 120 hr. Average values and standard errors are presented.

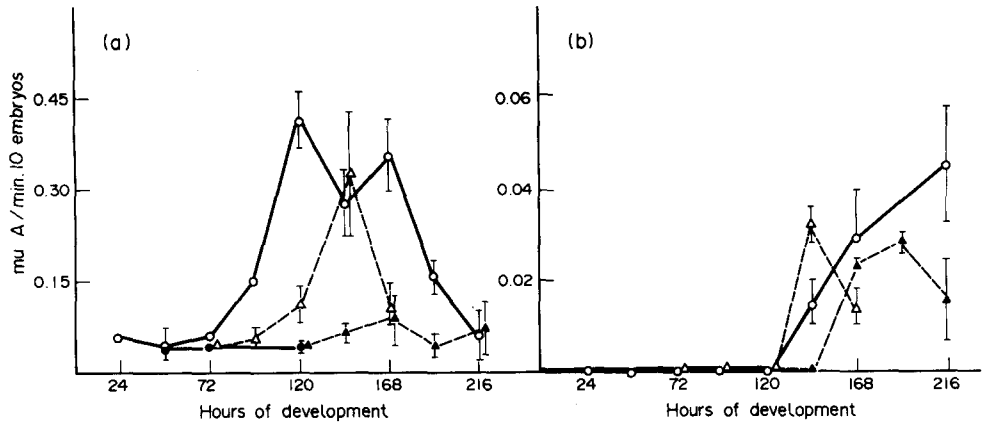

Fig. 3. Time course of inhibition and recovery of butyrylcholinesterase activity after malathion treatment in toad embryos. (a) Summer ovulation. (b) Winter ovulation. See the legends of Fig. 2 for experimental conditions and symbol key. In panel (b), embryos exposed continuously to 44 mg/L malathion did not show activity (●).

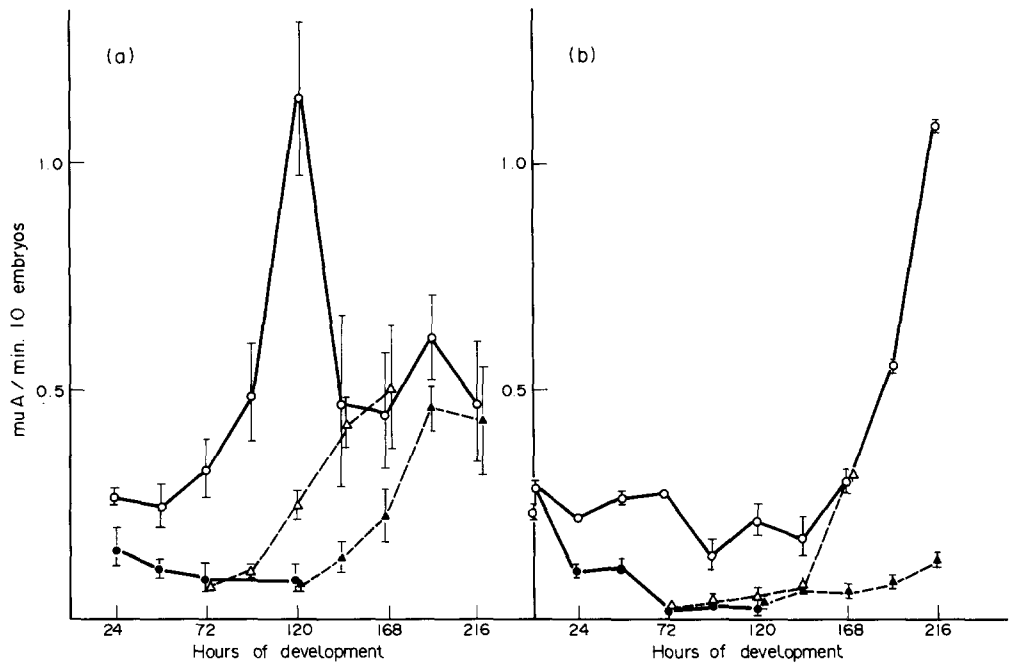

Fig. 4. Time course of inhibition and recovery of carboxylesterase activity after malathion treatment in toad embryos. (a) Summer ovulation. (b) Winter ovulation. See the legends of Fig. 2 for experimental conditions and symbol key.

embryos. These results were also reproduced for butyrylcholinesterase and carboxylesterase (Figs. 3 and 4). However, the recovery of acetylcholine-hydrolyzing activity after malathion exposure was similar in both seasons. The delay was 24 hr for the 72-hr exposed embryos and 48 hr for the 120-hr exposed embryos.

Acetylcholinesterase activity was paralleled by butyrylcholinesterase activity in summer and winter control embryos. The continuous exposure to 44 ppm malathion practically abolished all butyryl-cholinesterase activity in both seasons (Fig. 3 A and B). Summer embryos exposed to malathion for 72 hr recovered the enzyme activity with a delay of 24 hr, and 120-hr exposed embryos recovered it with a delay of 96 hr. In neither case did the recovered activity reach the control value. The recovery of butyrylcholinesterase in winter embryos exposed briefly seemed faster than in the summer ones (Fig. 3B).

The ratio of true to pseudocholinesterase after 120 hr of recovery was seen to decrease to 4.

p-Nitrophenylbutyrate-hydrolyzing activity (Fig. 4) was higher than that of the other reported enzymes. In both summer and winter ovulation, we detected enzyme activity in the first minutes after fertilization and also in the unfertilized oocyte (Fig. 4). Continuous exposure to 44 ppm malathion produced inhibition of the enzyme activity. At 120 hr of exposure, the percentage of inhibition in winter

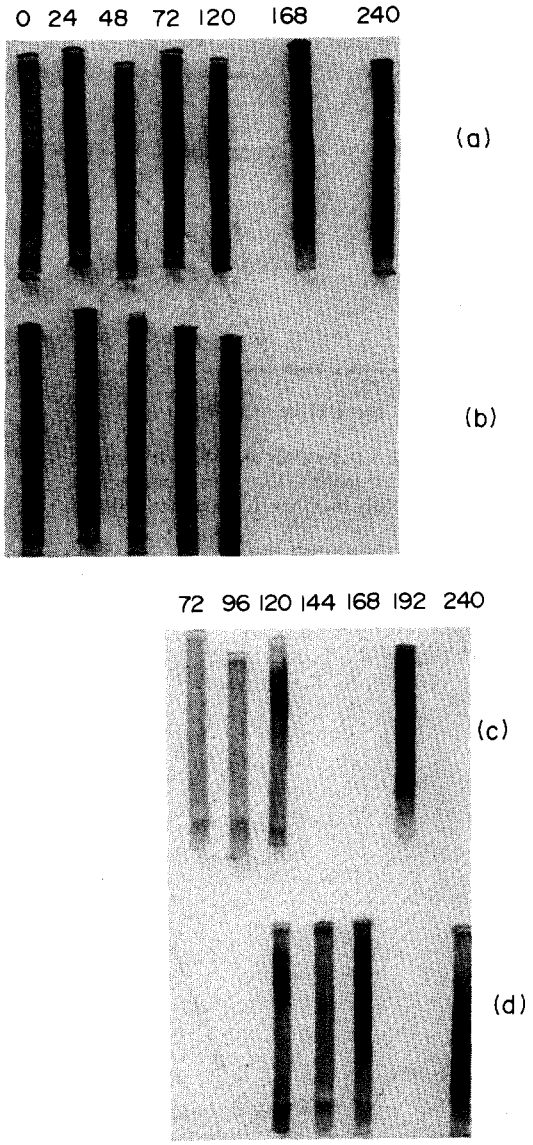

Fig. 5. Electrophoretic pattern of esterase activity in control and malathion-treated embryos. The gels shown in one line were preformed in the same run. An equal amount of sample was applied on each gel. The time (hr) since fertilization is indicated on the top. Line a: Zymograms of esterase activities during the development of control embryos. Line b: Esterase activities recovered after continuous malathion (44 mg/L) exposure. Line c: Esterase activities after 72 hr exposure to malathion (44 mg/L) and 24, 48, or 120 hr of recovery in Ringer's solution. Line d: Esterase activities after 120 hr of exposure to malathion (44 mg/L) and 24, 48 or 120 hr of recovery in Ringer's solution.

embryos (40%) was greater than that recorded in summer embryos (7%).

The recovered carboxylesterase activity obtained after a brief exposure to malathion resembled that depicted by total acetylcholinesterase activity in both summer and winter embryos.

Acrylamide disc gels stained with α-naphthyl-acetate-Fast Blue B showed several bands of esterase activity during early development (Fig. 5a). Malathion exposure abolished all esterase activity within 48 hr (Fig. 5b), but if the exposure was continued the appearance of two slight bands with mobilities similar to the one that appeared in 120-hr control embryos became evident.

In 72-hr exposed embryos (gel 3, Fig. 5c), it was confirmed that 72 hr of exposure abolished all α-naphthyl-acetate staining activity. At 48 hr of recovery in Ringer's solution some slow esterases appeared and at 120 hr of recovery the zymogram was similar to the one shown by 168-hr control embryos.

Figure 5d confirms the presence of α-naphthyl-acetate staining bands, even at 120 hr of malathion exposure. The activity disappeared then and was restored at 48 hr of development in Ringer's solution. After 120 hr of recovery, the pattern thus obtained was similar to the one found in control embryos at 240 hr of development.

DISCUSSION

Cell and tissue interactions may be mediated by chemical messengers [2]. Among the chemical transmitters acting during morphogenesis, neurotransmitters and correlated enzymes have been a subject of study. Their presence during embryogenesis has been reported in sea urchins [9, 16, 17], amphibians [11, 18], fish [7] and chicks [1].

Drews *et al.* [19] demonstrated that the appearance of cholinesterase activity is closely linked to formation of organs and they have postulated a functional relationship between cholinesterase activity and morphogenetic movement. This concept was extended by Schroder [20], who suggested that the cholinergic system is involved in the regulation of embryonic development. In addition, Falugi and Raineri [21] have postulated the hypothesis that plasma membrane-associated acetylcholinesterase may be part of the cholinergic system involved in the regulation of membrane functions during morphogenesis. The role of butyrylcholinesterase in morphogenesis is still being discussed [22].

We have found both acetyl- and butyryl-cholinesterase activities during the first days of development of the toad *Bufo arenarum* Hensel. Acetyl- and butyrylcholinesterase activities increased in a similar way, whereas carboxylesterase started earlier and showed a higher level of activity.

During the period of development studied, the ratio of true cholinesterase to pseudocholinesterase was quite similar. Important seasonal differences were evidenced in the three enzymes. These changes could not be ascribed to differences in the developmental temperature as a difference of only 2–3° between seasons was detected in the developmental vessels.

Cholinesterases, the primary target of organophosphates, were depleted by malathion, but if the exposure was interrupted, they returned to the normal level. However, the time required for complete recovery was variable. The patterns of recovery of acetylcholinesterase were similar for the different periods of exposure; however, the greatest activity reached by recovered embryos was smaller than the value obtained with control embryos. Malathion exposure for 120 hr strongly depressed

the recovery of butyrylcholinesterase activity and evidenced a selectivity in the recovery process. Michalek *et al.* [23] have described different patterns in the molecular forms of brain cholinesterases in rats intoxicated with diisopropyl fluorophosphate, and different rates of recovery.

Dephosphorylation and subsequent reactivation of inhibited enzyme usually occur at extremely slow rates [24]. We were unable to detect *in vitro* reactivation within 24 hr (data not shown); therefore, recovery of cholinesterase activity following malathion exposure may depend on *de novo* synthesis.

Nevertheless, the rapid initial recovery may be mediated by the combination of reactivation and new synthesis of the enzyme. In agreement with results previously reported by Koelle [10], our data show that the rate of cholinesterase recovery did not depend upon the duration of exposure. However, in recovered embryos, the ratio of acteyl- to butyrylcholinesterase was disturbed.

Embryos that were exposed for 72 and 120 hr showed similar electrophoretic patterns at 120 hr of recovery (Fig. 5, c and d). However, the intensity of the bands was stronger in the second case.

Since at 120 hr of continuous exposure to 44 mg/L malathion at least three zones of esterase activity appeared, this seems to be the time required for the *de novo* synthesis of protein and reactivation to replenish the active esterase pool. Recovery in malathion-free medium shortened this period.

The present results provide evidence that malathion exposure induces a lag in development directly related to the period of exposure, which is in agreement with the morphological pattern observed. If acetylcholine mediates intercellular communication in this system, we have no doubt that cell aggregation and movement must be affected. However, 120 hr of recovery in untreated medium restored the normal pattern of esterase activity and development.

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