Organophosphorus insecticides affect normal polyamine metabolism in amphibian embryogenesis

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A B S T R A C T

The objective of the present study was to evaluate the concentration- and time-dependent effects of the organophosphorus insecticides malathion and azinphos-methyl on polyamine metabolism, and relate them to normal and altered embryonic development of the common toad Rhinella arenarum. Control embryos showed that the higher polyamines spermidine and spermine acquired importance with respect to the diamine putrescine as embryonic development progressed. The activity of ornithine decarboxylase significantly decreased in complete operculum embryos. Continuous exposure to malathion caused a decrease in polyamine levels during embryonic development. However, there was an increase in putrescine levels in complete operculum embryos exposed to a sublethal concentration of the insecticide. Embryos exposed to malathion displayed a decrease in fresh weight and size, along with an increase in the number of malformed individuals. R. arenarum embryos exposed to a lethal concentration of azinphos-methyl showed an increase in putrescine levels and a decrease in spermidine and spermine levels, accompanied by an increase in ornithine decarboxylase activity. In conclusion, as the embryonic development of the toad R. arenarum progresses, polyamine metabolism shifts to higher polyamine levels with a more preponderant contribution of spermidine and spermine with respect to putrescine and involves a dramatic change in ornithine decarboxylase activity, one of the key regulatory enzymes of the pathway. Organophosphorus insecticides are capable of altering polyamine metabolism, slowing embryo development in parallel with a reduction in spermidine and spermine levels. An increase in the oxidative degradation of polyamines might be involved in the toxic action of organophosphorus insecticides and might also be related to other effects such as teratogenesis.

1. Introduction

Polyamines are aliphatic polycations essential for normal cell growth, differentiation and progression of developmental processes [1,2]. Due to their polycationic nature, they are capable of interacting with nucleic acids and proteins. Polyamines stimulate protein synthesis [3], regulate the activity of ion channels [4,5], the activity of G-proteins in signal transduction [6] and the expression of proto-oncogenes [7]. Polyamines also act as scavengers of reactive oxygen species [8]. Consequently, organisms have developed complex regulatory machinery to control intracellular levels of polyamines [9]. The first and key point of control in polyamine metabolism is the activity of ornithine decarboxylase (ODC), which catalyzes the decarboxylation of ornithine into the diamine putrescine (Put). Studies conducted in mice harboring a disrupted ODC gene and in pregnant mice exposed to the suicide ODC inhibitor α-difluoromethylornithine (DFMO), revealed that the enzyme ODC is essential for cell survival during early murine development [10,11].

Many studies, performed with different classes of pesticides, report both delay and arrest of embryonic development in several species [12–14]. In spite of the fact that polyamine metabolism is clearly related to normal and altered development, works connecting the effects of pesticides and polyamines are scarce. The herbicide paraquat has been the main compound employed in this kind of study, as its uptake into the cell is driven by the polyamine transport system. Paraquat’s effects on polyamine metabolism
have been studied on plants, mammals, and cultured cells [15–17]. Cochón et al. [18] performed a study in freshwater invertebrates, using a commercial formulation of paratraz, relating oxidative stress parameters to polyamine levels. Several authors have reported that aquatic invertebrates, such as amphibian embryos and larvae, exposed to organophosphorus (OP) and carbamate pesticides display diverse developmental alterations [19–22]. Some studies have been conducted to address the interaction between the toxicological effects of the anticholinesterasic OP pesticides and polyamines in aquatic organisms. These studies employed exogenously applied Put, spermidine (Spd) and spermine (Spm). Venturino et al. [23,24] determined that exogenously applied polyamines increased malathion (Mtn) toxicity and acetylcholinesterase inhibition, and decreased glutathione levels in R. arenaran larvae.

The objective of the present study was to evaluate the concentration- and time-dependent effects of the OP pesticides Mtn and azinphos-methyl (Azm) on polyamine metabolism, and relate them to normal and to altered embryonic development of the common toad R. arenarum.

2. Materials and methods

2.1. Chemicals

Azinphos-methyl (99.0% purity) was purchased from Chem Service (West Chester, PA, USA). Malathion (98% purity) was kindly provided by Cyanamid, Argentina, and purified by thin layer chromatography to eliminate possible storage contaminants. Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride, pyridoxal 5′-phosphate monohydrate, l-ornithine monohydrochloride and bovine serum albumin were purchased from Sigma Co. (St. Louis, MO, USA). l-[14C]-ornithine was purchased from New England Nuclear. Scintillation liquid Optiphase ‘Hisafe’ 3 was purchased from Perkin–Elmer (Shelton, CT, USA). All the other reagents used were of analytical grade.

2.2. Toad embryo development and insecticide exposure

Three independent experiments were performed for each pesticide, using three different sets of parents. Ovulation was induced by intraperitoneal injection of 2500 international units (IU) of human chorionic gonadotrophin. R. arenarum embryos were obtained by in vitro fertilization as previously described [25]. Groups of 500 newly fertilized embryos were transferred to glass recipients containing either amphibian Ringer’s solution (0.65 g/L NaCl; 0.01 g/L KCl; 0.02 g/L CaCl2)(control group) or pesticide solution, keeping a ratio of 1 embryo/mL solution. Pesticide solutions of 0.5 mg/L, 2 mg/L, and 9 mg/L Azm, and 22 mg/L and 44 mg/L Mtn were prepared by diluting an insecticide standard solution prepared in acetone, with an appropriate amount of amphibian Ringer’s solution, keeping acetone to 0.3% in the final solution. The exact concentration of the insecticide in the standard solutions was checked by gas chromatography with a nitrogen–phosphorus detector (GC-NPD). Controls of 0.3% acetone were also performed. The treatments were carried out in duplicate. The solutions were renewed every 48 h until embryos reached the stage of complete operculum (CO) [26]. The embryos were maintained at 18–20 °C in a 12 h light–12 h dark photoperiod without feeding. Viability of individuals and malformations were monitored with a stereoscopic microscope [27]. Embryonic development was assessed in samples of non-treated embryos and embryonic stages were determined according to [26]. During the continuous exposure, samples were taken at different embryonic stages to evaluate polyamine levels and ODC activity.

2.3. Ornithine decarboxylase activity

Fifty tail bud-(TB), twenty-five open mouth-(OM), and twenty-five CO embryos were sampled for determination of ODC activity and homogenized in 1 mL of 50 mM Hepes buffer pH 7.4 containing 0.1 mM EDTA, 0.04% Triton X-100, 1 mM dithiothreitol, 0.5 mM pyridoxal-5′-phosphate and protease inhibitors (0.05 mM phenylmethanesulfonyl fluoride and 0.001 mg/μL leupeptin, aprotinin and NaF). Homogenized samples were centrifuged at 20,000 g for 40 min at 4 °C and the supernatant was collected for determination of ODC enzymatic activity. ODC activity was assayed by measuring the release of 14CO2 from l-[14C]-ornithine according to [28], with slight modifications. The standard reaction mixture consisted of homogenization buffer plus l-ornithine and l-[14C]-ornithine (1 mM, 0.1–0.2 μCi) in a final volume of 50 μL. Enzyme reaction was initiated by the addition of 15 μL of samples. Blank solvent and sample controls were run in parallel, replacing sample with homogenization buffer and difluoromethylornithine (DFMO)-inactivated samples, respectively. The reaction was performed for 1 h at 30 °C in agitation and 14CO2 was trapped on a 2 cm × 2 cm piece of filter paper soaked with 2 N KOH. The reaction was stopped by the addition of 50 μL of 0.25 N HClO4 and maintained under the same conditions for 1 h. Filter papers were then transferred to scintillation vials and 0.5 mL 1% Triton X-100 were added along with 5 mL of scintillation liquid Optiphase ‘Hisafe’ 3. Radioactive CO2 was then measured in a liquid scintillation counter (Wallac Win-spectral 1414).

2.4. Polyamine content determination

Fifty embryos from early developmental stages were homogenized in 1 mL of 143 mM potassium phosphate buffer pH 7.5 with 6.3 mM EDTA and 0.2 N HClO4. Twenty-five embryos from OM stage on were also processed in this way. Samples were incubated on ice for 1 h and centrifuged 10 min at 3000 g. The supernatant was derivatized with 20 mg/mL dansyl chloride [29] and polyamine content was assessed by HPLC reverse-phase separation and fluorometric quantitation [30]. 1,7-Diamine heptane was used as an internal standard both for samples and calibration standards.

2.5. Evaluation of morphological parameters

Embryo morphology was evaluated with the aid of a stereoscopic microscope, and the type and number of malformations was registered. The malformations were typified according to [31]. Mortality was determined by the absence of blood circulation in the gills and caudal fin, or the lack of a heart beat. Dead embryos were removed and were not included in any morphological or biochemical analysis. Control embryos at the end of development displayed <5% mortality under laboratory conditions.

Embryo length was measured with a submillimetric scale under a stereoscopic microscope. Fresh weight was determined in groups of 20–40 embryos using an analytical balance, after a careful elimination of residual water with filter paper.

2.6. Protein determination

Protein content was determined according to [32] using bovine serum albumin as the standard.

2.7. Data analysis

Three independent experiments were performed for each pesticide and, within each assay, the different treatments were tested in duplicate. For the statistical analysis, data from the three experiments were pooled (n = 6 for each embryo stage and treatment)
as no significant differences between experiments were observed. Statistical differences between treatments were assessed by ANOVA and Fisher's lowest significant differences (LSD) post hoc test for polyamines and ODC data. Student-Newman–Keuls test was used for the statistical assessment of morphological parameters. Percentage mortality data were transformed by the arcsin of the square root of the probability value previous to the analysis.

The influence of the different biochemical and morphological parameters in the variability of response to OP treatments (concentration- and time-dependent) and possible correlations among them were assessed by principal component analysis (PCA) using the NTSYS program.

3. Results

Among the control embryos, the levels of the three polyamines studied markedly increased from oocyte to the last embryonic stage of CO (Fig. 1). Put was the major polyamine and showed a 15-fold increase at CO, reaching 30 nmol/mg protein. The increase in Spd levels throughout embryonic development was more significant, reaching 10 nmol/mg protein, which was about two orders of magnitude higher than Spd levels in the oocyte. A similar increase in Spm levels was also observed during development of *R. arena-rum*, which reached 1.2 nmol/mg protein at CO stage. Thus, the higher polyamines Spd and Spm gained importance with respect to the diamine Put as embryonic development progressed, since the Put:Spd:Spm ratio varied from 132.5:6.5:1 at fertilization to 24.5:8:1 at CO stage.

3.1. Effects of malathion on polyamine levels and embryonic development

The OP pesticide Mtn caused concentration- and stage-dependent alterations on polyamine levels. The first significant decrease in polyamine levels was observed at intermediate embryonic development, when embryos continuously exposed to Mtn reached the stage of gill circulation (GC; 6 days postfertilization) (Fig. 1). GC embryos exposed to 44 mg/L Mtn (close to LC50) displayed a significant decrease in Put, Spd and Spm levels (50%). Embryos continuously exposed to 22 mg/L Mtn (sublethal concentration) displayed a 50% reduction in Put levels and a 35% decrease in Spd levels, while Spm levels were not affected by insecticide exposure. In turn, at CO stage (10 days of exposure), exposure to 44 mg/L Mtn significantly diminished Put (35%), Spd (50%) and Spm (60%) levels. However, exposure to the sublethal concentration of 22 mg/L Mtn led to an increase in Put levels (65%) while the other two polyamines significantly decreased.

Corporal parameters, i.e. body length and fresh weight, as well as malformations caused by the exposure to Mtn, were also assessed to see if they were correlated with the effect on polyamine levels. Exposure to 44 mg/L Mtn caused a progressive shortening of the embryos, which became noticeable from GC stage on, but the sublethal concentration did not cause visible effects (Table 1). Fresh weight was a more sensitive parameter, since it decreased significantly from the early TB stage in embryos exposed to 44 mg/L Mtn and reached a 43% reduction by CO stage. Fresh weight in embryos exposed to 22 mg/L Mtn was significantly reduced from GC stage on. Teratogenic rates were also increased by exposure to 44 mg/L Mtn during embryonic development. At GC stage, 8% of the embryos displayed malformations when compared to control values (2%). At opercular fold stage (OF), 21% of embryos were malformed when compared to controls (8%). And at the end of embryonic development, 63% of embryos were malformed (control values 10%) (Table 1). The most common alterations registered at CO stage in embryos exposed to 44 mg/L Mtn were lateral flexure of the tail, generalized edema, axial shortening, neural tube closure impairment, organ displacement and deformed body axis. By the end of embryonic development, mortality increased in embryos exposed to 44 mg/L Mtn as a result of the alterations caused by this concentration, with a significant reduction in survival (30%) (p < 0.01). Exposure to the sublethal concentration of Mtn showed malformation rates similar to control embryos and no mortality was observed with this treatment.

3.2. Effects of azinphos-methyl on polyamine levels, ODC activity and embryonic development

The study of OP effects on polyamine levels was extended to Azm, another pesticide which is frequently used for pest control in fruit orchards in the northern Patagonic region. In these assays, two sublethal concentrations of Azm were tested (0.5 and 2 mg/L). A third concentration of 9 mg/L Azm was also included, which caused a significant mortality at the end of embryonic development after continuous exposure (18.9%; p < 0.05). None of the three concentrations of Azm were able to significantly affect polyamine levels in continuously exposed embryos by the stages of TB and OM (3 days and 7 days of exposure since fertilization, respectively) (Fig. 2A–C). However, after 10 days of exposure to 9 mg/L Azm, embryos reaching CO stage displayed a 61% increase in Put levels and a 42% decrease in the amount of Spd when compared to control values. Spm levels decreased 45% in CO embryos.
exposed to 9 mg/L Azm with respect to control values, although the difference was not statistically significant (p = 0.08).

ODC activity was analyzed in the same samples to determine if there was any kind of control at the level of this rate-limiting step of polyamine synthesis. Control ODC activity in *R. arenarum* embryos increased 2-fold between the stages of TB and OM, and subsequently decreased 22-fold when embryos reached the end of embryonic development (Fig. 2D). ODC activity in early TB embryos was not affected by Azm exposure. However, continuous exposure to 9 mg/L Azm caused a 2-fold and 10-fold increase in ODC activity at OM and CO stages, respectively, when compared to control values (Fig. 2D). The morphological abnormalities commonly found included gill atrophy, decreased body size, body blistering, dorsal tail flexure, wavy tail, abdominal edema and axial shortening. Embryos exposed to 9 mg/L AM suffered significant mortality (18%, p < 0.05) at the end of embryonic development.

### 3.3. Correlation analysis

We analyzed the contribution of the different variables and their covariance to the variability of response to different OP treatments, concentration and time of exposure, employing principal component analysis (PCA). The PCA including corporal parameters clearly showed a covariance of polyamines and body weight and length on the main component explaining 71.0% of the variability in embryonic development as a result of OP exposure. The rate of malformations was the single variable of influence in the second component in order of importance (15.8%) (Fig. 3A). The projection of the combined treatments and embryo stages in the main plane of the principal components resulted in two clusters defined by early (TB–GC) and intermediate embryonic stages (OM–OF) (Fig. 3B). This suggests that there were no major effects due to the applied treatments. At CO stage, treatments caused an important separation in the first component (defined by alterations in polyamine levels and body parameters) and the second component (defined by the increase in the number of malformations).

Finally, the correlation of ODC activity and polyamine levels was analyzed. For this purpose, each polyamine/ODC ratio was calculated from data for each embryonic stage and Azm treatment. To compare the three polyamines, ratios were in turn referred to TB stage values, and significant differences between control values among different embryonic stages (p < 0.05), determined by ANOVA and Fisher’s LSD test. CO: complete operculum; ND: not detected; OM: open mouth; TB: tail bud.

### Table 1

<table>
<thead>
<tr>
<th>Stage (exposure duration)</th>
<th>Control 22 mg/L</th>
<th>Malathion 44 mg/L</th>
<th>Malathion 22 mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB (3 days)</td>
<td>3.4 ± 0.2</td>
<td>3.4 ± 0.2</td>
<td>3.2 ± 0.3</td>
</tr>
<tr>
<td>GC (6 days)</td>
<td>5.5 ± 0.2</td>
<td>5.5 ± 0.1</td>
<td>4.8 ± 0.1**</td>
</tr>
<tr>
<td>OF (8 days)</td>
<td>7.1 ± 0.3</td>
<td>7.1 ± 0.0</td>
<td>6.2 ± 0.2**</td>
</tr>
<tr>
<td>CO (10 days)</td>
<td>7.9 ± 0.2</td>
<td>7.5 ± 0.2</td>
<td>6.5 ± 0.0**</td>
</tr>
</tbody>
</table>

Mean ± SEM are shown. Treatments were performed in duplicate and data from three independent assays were pooled for statistical analysis. Total length was measured in 20 embryos of each treatment. Fresh weight was determined in groups of 40 (TB stage) or 20 (GC–CO stages) embryos. Malformations were analyzed in all embryos of each experimental unit. Statistical differences were assessed by ANOVA-Student-Newman–Keuls test: *p < 0.05, **p < 0.01, ***p < 0.001 vs. controls; "p < 0.05, ""p < 0.001 vs. Mtn 22 mg/L. TB: tail bud; GC: gill circulation; OF: opercular fold; CO: complete operculum.
were observed between polyamine/ODC ratios at TB with respect to OM stage and no significant effects were caused by Azm at these stages (Fig. 4A). At CO stage, the control polyamine/ODC ratio increased noticeably, being 28 times higher than the Put/ODC ratio, 87 times higher than the Spd/ODC ratio and 66 times higher than the Spm/ODC ratio with respect to TB stage (Fig. 4B). These increased ratios resulted from a rise in the three polyamines and a subsequent downregulation of ODC activity at CO stage in non-exposed embryos (Fig. 2). Azm exposure prevented, in a concentration-dependent manner, the increase in polyamine/ODC ratios at CO stage, with a maximal effect leading to an approximately 4-fold increase for the three polyamines at the 9 mg/L treatment.

4. Discussion

Polyamine levels continuously increased during the embryonic development of the toad *R. arenarum* (Figs. 1 and 2). Polyamines are involved in different biological processes and their participation in embryonic development has been documented in organisms such as sea urchin [33], fish [34], frogs [35,2] and mice [10,36]. It has been shown that a coordinated regulation of polyamine metabolism is indispensable for the operation of the cell cycle. In human dermal fibroblasts, an increase in polyamine content is necessary for DNA replication, and the subsequent relative polyamine depletion due to polyamine acetylation by Spermidine/spermine N\(^{1-}\)acetyltransferase (SSAT) is necessary for mitosis to reach completion [37]. Disruption of the cell cycle, as well as DNA damage, has also been reported in kidney epithelial cells which overexpress SSAT [38]. The first rate limiting step in the polyamine biosynthetic pathway is catalyzed by the enzyme ODC. In our study, an increase in ODC activity was accompanied by an increase in Put and the higher polyamines during the early and intermediate stages in non-exposed embryos (Fig. 2). Azm exposure prevented, in a concentration-dependent manner, the increase in polyamine/ODC ratios at CO stage, with a maximal effect leading to an approximately 4-fold increase for the three polyamines at the 9 mg/L treatment.
Osborne et al. [39] reported that ODC activity in X. laevis increased from the 2-cell stage up to the mid blastula transition and then decreased rapidly. The reason for the decrease in ODC activity observed in CO R. arenarum embryos is not clear; nevertheless, the sustained increase in Put, Spd and Spm could suggest a regulatory effect of polyamines on ODC activity. The enzyme ODC is regulated by polyamines at the levels of transcription and translation as well as transcript and ODC protein stability [40]. Both the transcription of the ODC gene and the translation of ODC mRNA are stimulated by low levels of polyamines. On the contrary, high levels of polyamines exert a feedback inhibition on ODC through stimulation of a frameshifting event on antizyme (AZ) mRNA. The AZ protein binds to the ODC monomer thus inactivating ODC and targeting it for degradation [41,40]. The pattern of polyamine levels determined in control R. arenarum embryos in the present study, where the main polyamine is Put, followed in order of abundance by Spd and Spm, coincides with that reported for organisms such as X. laevis and the freshwater gastropod Biomphalaria glabrata [19,2]. In some cases, however, the polyamines Spd and/or Spm become more abundant than Put, as occurs in the freshwater oligochaete Lumbricus variegatus [19] as well as in cultured cells [42]. According to Igarashi and Kashiwagi [43], Put and Spd predominate in prokaryotic cells, which grow rapidly, whereas Spd and Spm predominate in eukaryotic cells, which proliferate relatively slowly. Osborne et al. [2] reported that the intracellular Spd concentration must be retained within certain limits with respect to Put to allow normal development in X. laevis. The maintenance of high Put levels with respect to Spd and Spm is a necessary factor for correct embryonic development in X. laevis and can be explained by the low levels of both S-adenosylmethionine decarboxylase (SAMDC) mRNA and SAMDC protein as reported by Shinga et al. [44]. Also, the fact that Put was the most abundant polyamine detected in R. arenarum embryos in this work could point to the high rate of cell division that embryos experience during their development. Another feature observed in R. arenarum development was the increase in Spd and Spm levels with respect to Put as embryos progressed to CO stage. Studies performed by Ogawara et al. [45] determined that Spm was the most potent polyamine with regards to protein synthesis stimulation, followed by Spd and Put in order of potency. Thus, Igarashi and Kashiwagi [43] suggest that the existence of Spm in slowly growing eukaryote cells may be biochemically and energetically economical.

In spite of the fact that polyamines are recognized as essential polycations for normal development and that their levels are as a consequence highly regulated, almost no studies have been undertaken to analyze the influence of pesticides on polyamine metabolism. Intracellular levels of polyamines must be maintained within narrow limits, since a decrease of polyamine levels interferes with cell growth, whereas an excess appears to be toxic [46,47]. In the present study, we demonstrate that two OP pesticides, Azm and Mtn, are able to affect in a concentration- and time-dependent manner the levels of polyamines during the embryonic development of the amphibian R. arenarum. The reduction in polyamines, mainly Spd and Spm, is highly correlated to a decrease in embryo development as a consequence of OP exposure, measured using body parameters such as fresh weight and length (Fig. 3A). The depletion of polyamines impairs several processes such as cell cycle progression and cellular proliferation and growth [48,38], which may explain the reduction in the developmental rate observed in embryos exposed to OP pesticides in the present study. Some of the malformations elicited by OP exposure in tadpoles may be related to tissue-specific alterations in polyamine metabolism. Nevertheless, polyamine levels and the number of alterations in development were not correlated in the present work (Fig. 3A).

An increase in Put levels was detected at the end of embryonic development in R. arenarum embryos exposed to a sublethal concentration of Mtn and those exposed to a lethal concentration of Azm (Figs. 1 and 2). Increasing levels of Put have also been detected in Xenopus laevis tadpoles exposed to a convulsant agent, suggesting a neuroprotective role for polyamines due to Put conversion into γ-amino butyric acid (GABA) neurotransmitter [49]. We suggest that the increase of Put observed in CO embryos exposed to OP in the present study is an attempt to overcome the depletion of higher polyamines (Spd and Spm) and to recover normal growth. In fact, ODC activity was induced in CO embryos exposed to 9 mg/L Azm, which may in part justify the increase in Put levels. An increase in ODC activity along with a massive increase in Put levels, produced by electroshock-induced seizures in transgenic mice, have been previously described as neuroprotective effects rather than the cause of damage [50]. Also, increases in Put levels and ODC activity have been reported in a rat model of acute porphyria [30] and in rats exposed to hexachlorobenzene [51]. The rise in Put content observed in the present study could also occur if the oxidative degradation of Spd and Spm through the enzymes SSAT and acetyl-polyamine oxidase (APAO) was active [52]. Similarly, a decrease in SAMDC activity would explain these results. As the embryos progressed from intermediate developmental stages to the CO stage, increasing concentrations of Azm decreased the Put/ODC ratio. This result suggests that Put levels were controlled not only through synthesis but also via degradation by diamine oxidase (DAO) or due to an inhibition of SAMDC activity. Besides the increase in Put levels, R. arenarum embryos exposed to OP experienced a decrease in Spd and Spm levels. Once more, an increase in their oxidative degradation through SSAT-APAO enzymes as well as a decrease in SAMDC activity would account for these results. As Spd and Spm participate in the regulation of ODC activity [41], their decrease in embryos exposed to Azm would explain the effects of increasing concentrations of the insecticide on Spd/ODC and Spm/ODC ratios (Fig. 4). An increase in the oxidative metabolism of polyamines and a resulting oxidative stress situation was previously suggested in R. arenarum embryos exposed to Mtn and exogenously applied polyamines [26]. During oxidative catabolism of polyamines, the reactive aldehyde acrolein is generated along with other reactive species such as hydrogen peroxide. It has been reported in cultured cells that the

Fig. 4. Effect of Azm on PA/ODC ratios. (A) PA/ODC ratios were calculated dividing polyamine concentrations by ODC activities at the different embryonic stages. (B) PA/ODC ratios were normalized with respect to TB control ratios. A: azinphos methyl; CO: complete operculum; TB: tail bud.
aldehyde acrolein triggers the expression of multiple phase 2 genes, including glutathione transferase, in response to the addition of exogenous polyamines [42], denoting an impact on the antioxidant defense system. This is particularly relevant when the exposure to contaminants alters not only polyamine metabolism but also redox status, leading to additive effects. Also it has been suggested that the injury produced by polyamines during early development may be attributable to the hydrogen peroxide formed during the interconversion of spermine to spermidine and putrescine [53].

In conclusion, as embryonic development of the toad *R. arenarum* progresses, polyamine metabolism shifts to higher polyamine levels with a more preponderant contribution of Spd and Spm with respect to Put and involves a dramatic change in ODC activity, one of the key regulatory enzymes of the pathway. OP insecticides are capable of altering polyamine metabolism, thereby slowing embryo development in parallel with a reduction in Spd and Spm levels. An increase in the oxidative degradation of polyamines may be involved in the toxic action of OP pesticides and might also be related to other effects such as teratogenesis.

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**References**


[22] R. arena-


