

Increasing Uptake and Bioactivation with Development Positively Modulate Diazinon Toxicity in Early Life Stage Medaka (*Oryzias latipes*)

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Received November 9, 2000; accepted February 7, 2001

Diazinon, an organophosphate pesticide, becomes biotransformed to a more potent oxon metabolite that inhibits acetylcholinesterase (AChE). Early life stages (els) of medaka, *Oryzias latipes*, were used to determine how development of this teleost affects sensitivity to diazinon. With developmental progression, from day of fertilization to 7-day-old larvae, we found that the 96-h LC₅₀ and AChE IC₅₀ values decreased, indicating greater host sensitivity to diazinon upon continued development. We then examined changes in AChE activity, its inhibition by the active metabolite diazoxon, and uptake and bioactivation of the compound. AChE activity remained low during much of development but increased rapidly just prior to hatch. In addition, *in vitro* incubation of tissue homogenates from embryos or larvae showed no differences in the sensitivity of AChE to diazoxon. Uptake studies with ¹⁴C-diazinon revealed greater body burdens of ¹⁴C as medaka developed. In addition, AChE IC₅₀ values determined by *in vivo* exposure to diazoxon were greater in larvae than in embryos. Because diazinon is bioactivated by the P450 enzyme system, two P450 inhibitors were used *in vivo* to explore the role of metabolism in sensitivity. When exposure to diazinon occurred in the presence of increasing amounts of piperonyl butoxide (PBO), AChE inhibition decreased in a dose-response fashion and 2.0 × 10⁻⁵ M PBO alleviated any difference in inhibition between larvae and embryos. However, PBO did not alter total ¹⁴C uptake when exposed simultaneously with ¹⁴C-diazinon, nor did it affect AChE inhibition using diazoxon. Controls ruled out differential effects of PBO on uptake and inhibition. In addition, a second general P450 inhibitor, 1-aminobenzotriazole, also decreased AChE inhibition. Finally, using exogenous acetylcholinesterase as a trap for the oxon metabolite, larval microsomes displayed greater bioactivation of diazinon than did a microsomal preparation from embryos. Taken together, results suggest that uptake and bioactivation are working to enhance diazinon sensitivity in this developmental model of a teleost fish.

Key Words: organophosphate; developmental sensitivity; teleost.

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Chemical manipulation of organophosphorus esters (OPs) has resulted in selective pesticides with low environmental persistence (Ecobichon and Joy, 1994). However, under alkaline pH, certain OPs including diazinon, a phosphorothionate OP, persist for significant periods in aquatic environments. This persistence raises concern over the toxicity of OPs to nontarget species, including early life stage (els) fishes (Bailey *et al.*, 1994).

Diazinon is widely used to control pests in residential settings as well as commercial agriculture. Due to its varied and widespread use, diazinon release from nonpoint sources is significant (for a review see Larkin and Tjeerdema, 2000). Locally, diazinon has been detected in the Sacramento and San Joaquin Rivers, their delta, and the upper San Francisco Bay following dormant spray usage and at levels exceeding National Academy of Science guidelines as far down as the Upper San Francisco Bay (Kuivila and Foe, 1995). Our laboratory monitored the toxicity of these ambient waters using tests with the water flea, *Ceriodaphnia dubia* (Werner *et al.*, 2000), and toxicant identification evaluations indicated diazinon as the causative agent in assays with surface waters containing runoff from urban and agricultural areas in the watershed (Kuivila and Foe, 1995).

OP toxicity results from inhibition of acetylcholinesterase (AChE), and metabolic conversion of OPs to their oxon metabolite results in formation of potent AChE inhibitors. Metabolic conversion of diazinon to diazoxon is mediated by the cytochrome P450 monooxygenase system of fish (Fujii and Asaka, 1982; Hogan and Knowles, 1972). Limited studies to date demonstrate rapid increases in the activity of cytochrome P450-mediated enzyme activity in fish as hatching approaches (Binder and Stegeman, 1984; Binder *et al.*, 1985; Wisk and Cooper, 1992). Interestingly, the limited studies of phosphorothionate OPs, using parent compounds, indicate that embryos are less sensitive than larvae (Anguiano *et al.*, 1994; Takimoto *et al.*, 1984a). Studies of juvenile and adult fishes have concluded that sensitivity to OPs is determined by AChE sensitivity, as measured by *in vitro* AChE inhibition by oxon metabolites (see review, Chambers and Carr, 1995). However,

factors affecting sensitivity of elms fishes, including uptake, bioactivation, and the sensitivity of AChE, remain undefined.

Information on developmentally related changes in uptake, biotransformation, and resultant toxic effect of OPs is needed. Medaka (*Oryzias latipes*), a surrogate species in the present study, are small fish native to Japan and other countries of Southeast Asia. Because of their transparent chorion, precise staging of the developmental processes is possible without invasion, making medaka particularly advantageous for embryological and developmental investigations (Iwamatsu, 1994; Kirchen and West, 1976). Here we report on factors affecting the sensitivity of embryo-larval medaka to diazinon.

MATERIALS AND METHODS

Chemicals. Diazinon (*O,O*-diethyl-*O*-[2-isopropyl-4-methyl-6-pyrimidyl] phosphorothioate), purity 99%, was purchased from Chem Services (Philadelphia, PA). Diazoxon (0.89 mg/ml in hexane) and ¹⁴C-labeled diazinon (reported as 99.0% radiochemical purity, 99.8% chemical purity, and specific activity of 16 μ Ci/mg) were kind gifts from Ciba-Geigy Corporation (now Novartis; Greensboro, NC). Acetylthiocholine iodide, 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB), tetraisopropyl pyrophosphoramidate (iso-OMPA), and all other chemicals were purchased from Sigma Chemical Company (St. Louis, MO).

Stock preparation. Diazinon was weighed in a glass boat and transferred to a volumetric flask containing embryo-rearing medium (ERM) (Kirchen and West, 1976), and its concentration was confirmed by gas chromatographic analysis (Aston and Seiber, 1996). Measured concentration was within 5% of the nominal concentration. Dilutions of the defined stock solution were used for tests described below.

Embryo collection and culture. Culture conditions for medaka have been described (Hamm *et al.*, 1998). Briefly, broodstock were maintained in water reconditioned to U.S. Environmental Protection Agency moderately hard conditions (Horning and Weber, 1985) at 25°C under a 16L:8D photoperiod and fed a purified, casein-based diet (DeKoven *et al.*, 1992) supplemented with brine shrimp nauplii. Embryos were collected from females and individuals were separated by rolling clusters between fingertips to break connective filaments (Marty *et al.*, 1990). After cleaning, embryos were placed in ERM, aerated, and maintained at 25°C until exposure.

AChE measurement. Determination of AChE activity followed the method of Ellman *et al.* (1961) as modified for a 96-well microplate reader (see Hamm *et al.*, 1998 for details). Briefly, following homogenization in assay buffer (0.1 M sodium phosphate buffer, pH 8.0), samples were incubated for 15 min with 0.115 mM tetraisopropyl pyrophosphoramidate (iso-OMPA) to inhibit nonspecific cholinesterases (BChE). Following incubation, 30 μ l acetylthiocholine (10.7 mM) substrate was added, and with DTNB as chromogen, activity was determined. Substrate blanks and tissue blanks were used to standardize activity, and this value was normalized to protein concentration (see below).

Developmental pattern of AChE. Embryos and larvae were pooled by developmental stage (Kirchen and West, 1976), snap-frozen, and stored at -80°C until analysis. Numbers of animals used for a homogenate varied depending on amount of activity present, i.e., stage of development, ranging from 5 larvae to 20 early-stage embryos with three replicates per stage.

Sensitivity

LC₅₀ determination in embryos and larvae. Embryos at stages 11, 29, or 34 and 24-h-old larvae were used. These were placed in 20-ml borosilicate vials (Fisher Scientific, Pittsburgh, PA) containing 2 ml ERM at pH 7.2–7.3 or a solution of diazinon in ERM. Replicates ($n = 5$) per concentration with at least five concentrations of diazinon were tested. At 24-h intervals, vials were

monitored for dead organisms, solutions were withdrawn, and new solution was added. Dead embryos or larvae were removed daily. At 96 h, exposures were terminated, and the concentration required to kill 50% of the organisms was calculated.

Examination of developmental changes in the degree of AChE inhibition. Embryos at stages 31, 32, 33, 34, and 35, and larvae that were 24 h or 7 days old were exposed to diazinon for 24 h. Replicates ($n = 5$) of five individuals each were placed in 2 ml ERM for control or test concentrations of 0.088, 0.88, 3.53, 17.6, 44.1, and 88.2 μ molar diazinon. After 24 h, embryos were transferred to cryovials, snap-frozen in liquid nitrogen, and stored at -80°C until analysis of AChE activity (see above).

Determination of the in vitro sensitivity of AChE. A total of 100 stage 34 embryos and 100 24-h-old larvae were pooled and homogenized in 2 ml assay buffer using an ice-chilled teflon glass homogenizer and brief sonication as above. Homogenate (100 μ l) was placed into glass test tubes with 900 μ l assay buffer to yield three replicates per concentration. Sufficient diazinon in 1 μ l hexane was added to yield final μ molar concentrations of 0.1, 0.5, 1, or 10. Incubations with homogenate only and homogenate with 1 μ l hexane were run as controls. Incubations were run at 25°C for 15 min on a rotary shaker. Following incubation, AChE activity of homogenates was determined (see above).

Uptake

Uptake of diazinon. Embryos at stages 29, 33, and 35 or 24-h larvae were exposed to ERM or radiolabeled diazinon at 0.88, 3.53, or 17.6 μ molar concentrations. In addition, 24-h-old larvae were exposed to the above concentrations of labeled diazinon with 2.0×10^{-5} molar piperonyl butoxide (PBO) in order to determine if PBO affected uptake. At 24 h, embryos and larvae were removed from solution, rinsed twice, and homogenized in 500 μ l of 0.1 M sodium phosphate buffer. From the homogenate, 40 μ l was transferred to a 7-ml scintillation vial containing scintillation fluid (UniverSol™, ICN, Costa Mesa, CA) and counted on a Beckman LS-5801 scintillation counter (Beckman Instruments, Inc., Irvine, CA).

In vivo exposure to diazinon. Replicates ($n = 5$) of stage 34 and 24-h-old larvae were exposed to 0.01, 0.10, 1, 10, and 100 μ molar diazinon for 24 h, and AChE activity was analyzed.

Metabolism

Piperonyl butoxide (PBO) studies. PBO, an inhibitor of P450, was used to inhibit bioactivation of diazinon. A range-finding experiment was run using stage 34 embryos and 24-h-old larvae exposed to 0.88 μ molar diazinon in the presence or absence of 0, 2.7×10^{-7} , 2.0×10^{-6} , or 2.0×10^{-5} molar PBO (three replicates per treatment). AChE activity was determined after exposure for 24 h.

An additional P450 inhibitor, 1-aminobenzotriazole, was used to confirm findings with PBO. Replicates ($n = 3$) of five larvae (24 h old) were exposed to 0.88 μ molar diazinon for 24 h in the presence or absence of 0, 1×10^{-3} , 10^{-4} , or 10^{-5} M 1-ABT. Following exposure, larvae were handled as above for AChE analysis.

The role of metabolic differences accompanying degree of development was estimated, replicates ($n = 5$) of stage 31 or 34 embryos or five 24-h-old larvae were exposed to diazinon for 24 h in the presence or absence of 2.0×10^{-5} molar PBO, and AChE activity was determined. Diazinon concentrations were: 0.016, 0.088, 0.88, 3.53, 17.6, and 44.1 μ molar.

To ensure the effects of PBO were not due to changes in uptake or distribution of diazinon, 24-h-old larvae were exposed to the active metabolite diazinon with or without PBO. Replicates ($n = 4$) of five larvae (24 h old) were exposed to 0, 0.1, 0.5, 1, and 10 μ molar diazinon in the presence or absence of 2.0×10^{-5} molar PBO for 24 h and treated as above for AChE analysis.

In vitro bioactivation of diazinon. Bioactivation was measured by incubating diazinon with microsomes and indirectly measuring levels of oxon

produced by determining resultant AChE inhibition. The use of AChE inhibition to estimate levels of oxon metabolites gives comparable measures to gas chromatographic analysis (Mirer *et al.*, 1975) and has been used extensively (Forsyth and Chambers, 1989). Our methods followed this approach with the following exceptions: *a*) magnesium chloride (5 mM) was eliminated because it interfered with AChE determinations. As magnesium is a cofactor for P450, metabolism in our system should be lower than in others. *b*) The concentration of NADP was decreased from 7.5 mM to 375 μ M, with no recorded change in bioactivation. *c*) Incubation temperature was set at 35°C after initial tests showed little to no metabolism in incubations at 25 or 30°C. *d*) 2-methoxyethanol replaced ethanol as solvent for P450 inhibitors due to AChE inhibition with ethanol.

Using the above conditions, incubations ($n = 3$ per developmental stage) were prepared on ice in a final volume of 500 μ l using 0.1 M Tris-HCl (pH 7.4). Incubations consisted of microsomal protein (150 μ g) from either stage 31 embryos or 24-h-old larvae, 10 μ l of the exogenous AChE source (see below), an NADPH-generating system [NADP (375 μ M), glucose-6-phosphate (7.5 mM), glucose-6-phosphate dehydrogenase (0.5 I.U.)] and diazinon (50 μ M) in 5 μ l 2-methoxyethanol. A series of controls was incubated in conjunction with each experiment. All controls had the AChE, NADPH-generating system and one of the following: solvent, diazinon, or microsomes. Following set-up, incubation tubes were transferred to a shaking incubator for 30 min. After incubation, 30- μ l samples (in triplicate) were rapidly placed in individual wells of a 96-well plate for AChE determination.

Microsomal preparation. Microsomal preparation followed a modification of Buckpitt and Warren (1983). Adult medaka, 6 months old, were sacrificed by decapitation, and livers were quickly removed and transferred to ice-cold 0.02 M Tris-1.15% KCl, pH 7.4 buffer. All subsequent procedures were performed on ice. Pooled livers were removed from buffer, blotted, and weighed. Homogenization was in three volumes of ice-cold buffer (0.02 M Tris, 1.15% KCl, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 15% glycerol, pH 7.4), and resultant homogenate was centrifuged at $10,000 \times g$ for 30 min; the supernatant was then removed and centrifuged at $105,000 \times g$ for 1 h. The microsomal pellet was resuspended in Tris/KCl buffer in approximately half the original volume and repelleted at $105,000 \times g$ for 1 h. The final microsomal pellet was resuspended in 0.1 M sodium phosphate buffer, pH 7.4. Protein content was determined as described below.

For determination of developmental differences in *in vitro* bioactivation, 500 stage 31 embryos and 500 24-h-old larvae were separately pooled and microsomes were prepared as above, with the exception that microsomes were resuspended in a final volume of 100 μ l.

Preparation of exogenous AChE. Thirty adult medaka were decapitated and their brains were removed and pooled in ice-cold 0.1 M Tris-HCl (pH 7.4). Tissues were transferred to 3 ml 0.1 M Tris-HCl and homogenized using 5–7 passes of a chilled teflon glass homogenizer. Aliquots of 100 μ l were snap-frozen in liquid nitrogen and transferred to a -80°C freezer for storage until use.

Determination of protein concentration. Protein concentration was measured in homogenates using a simplified procedure of Smith *et al.* (1985), the bicinchoninic acid protein assay (Sigma Chemical Company; St. Louis, MO) with bovine serum albumin as standard.

Statistics. Levels of statistical significance were analyzed by ANOVA, followed by a Scheffé's F-test as a *post hoc* test to compare means between the different treatment groups. Differences were considered significant if $p < 0.05$. LC_{50} values at 96 h with 95% confidence intervals were calculated using probit analysis.

RESULTS

AChE Activity Rises Rapidly around the Time of Hatch

In staged, control embryos, AChE activity remained low until stage 31. From this point, enzyme activity gradually

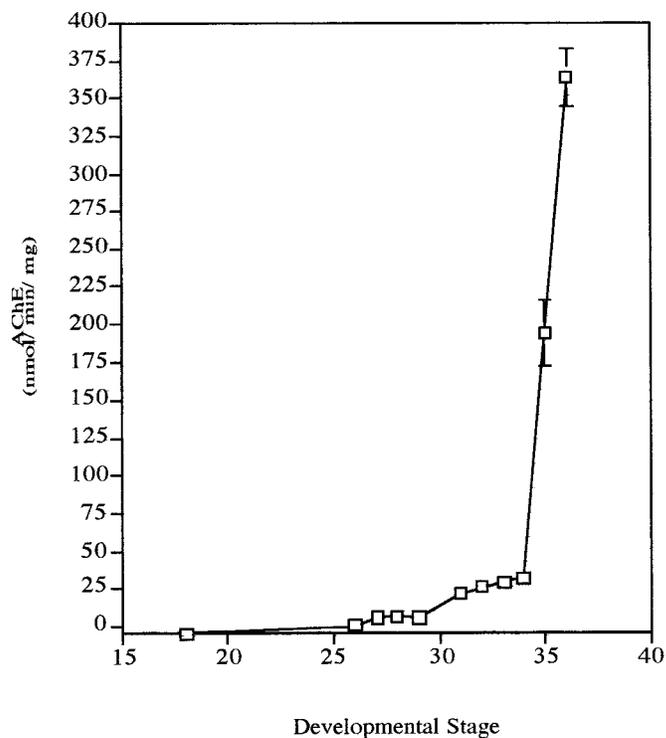


FIG. 1. Development of whole-body AChE activity in early life stage medaka. Embryos were staged according to Kirchen and West (1976) with 24-h-old larvae designated as stage 36. Embryos at each stage of development, the number varying depending on the degree of development, were pooled and homogenized, and AChE activity was determined. Values represent mean \pm SD for three pooled samples per developmental stage.

increased in later embryonic stages and rose rapidly immediately prior to and following hatch on day 10 of development (Fig. 1).

Medaka Become More Sensitive to Diazinon As Development Progresses

Determination of 96-h LC_{50} values showed a significant drop from 111 μ molar in stage 34 embryos to 31.5 μ molar in 24-h-old larvae (Table 1). In a similar fashion, AChE inhibition in whole-embryo homogenates exposed *in vivo* to diazinon for 24 h revealed lowered IC_{50} values as medaka embryos developed (Fig. 2). Embryos between stages 31 and 34 showed little difference in AChE sensitivity; however, in stage 35 embryos, just prior to hatch, the IC_{50} value decreased. This trend continued after hatch with 7-day-old larvae having the lowest IC_{50} .

The *in Vitro* Sensitivity of AChE to the Active Metabolite Diazoxon Does Not Change with Development

Determination of *in vitro* IC_{50} values using 15-min incubations of stage 34 or larval homogenates with a range of diazoxon concentrations resulted in 0 to near 100% inhibition of AChE over the concentrations tested. However, no difference

TABLE 1
Diazinon 96-h LC₅₀ Values for Embryo-Larval Medaka

Developmental stage ^a	96-h LC ₅₀ (μmolar)
11 (day 1)	102 ^b (90–110) ^c
29 (day 5)	103 (91–115)
34 (day 8)	111 (101–121)
24-h-old larvae (day 11)	32* (27–37)

^aThe developmental stage at which exposure was initiated; the number in parentheses represents the day of development.

^bValues represent mean ($n = 5$ replicates/concentration tested).

^cThe number in parentheses following LC₅₀ value represents the 95% confidence interval.

*Significantly different from other stages ($p < 0.05$).

in AChE sensitivity was noted between these two developmental stages, and the IC₅₀ values for both were approximately 18.8 μM (Table 2).

¹⁴C-Diazinon Reveals Developmental Changes in 24-Hour Bioaccumulation

¹⁴C-diazinon was used to examine possible differences in the uptake of the pesticide over a 24-h period. In our system, diazinon undergoes metabolism, and as the chemical form of the radioactivity was not determined, results are presented as counts per min (cpm) per animal. For all stages tested, analysis of whole-animal homogenates showed dose-dependent increases in the amount of radioactivity per animal. However, it was noted that with added development there was a gradual increase in uptake; hatchlings had a 1.5- to 2-fold greater level of total radioactivity than embryos just prior to hatch (Fig. 3).

Similarly, *in vivo* studies with the diazinon metabolite diazoxon suggested greater uptake with added development; larvae showed significantly greater inhibition of AChE than did similarly treated stage 34 embryos (Fig. 4).

P450 Inhibitors Decrease AChE Inhibition

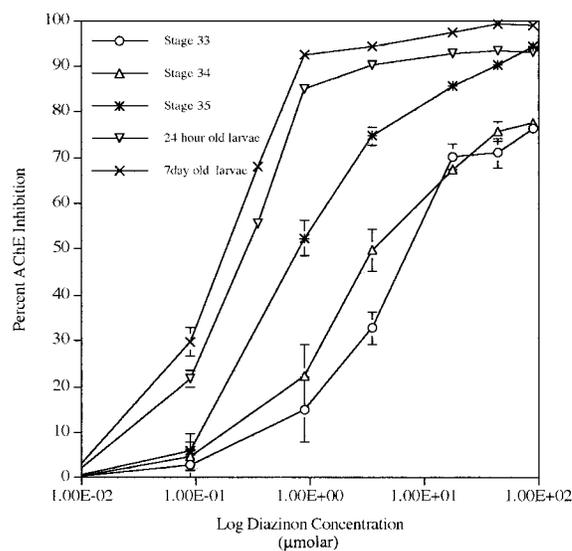
The P450 inhibitor PBO was used to determine if metabolic activation played a role in the observed increases in sensitivity. Exposure to 0.88 μmolar diazinon resulted in 35 and 75 % AChE inhibition in stage 34 embryos and 24-h-old larvae, respectively. However, over a range of PBO concentrations between 2.7×10^{-7} and 2.0×10^{-5} M, PBO decreased AChE inhibition in a dose-response fashion such that at 2.0×10^{-5} molar PBO, AChE activity was no longer statistically different from control animals (Fig. 5). A similar reduction in AChE inhibition was seen in larvae exposed to diazinon and the P450 inhibitor 1-aminobenzotriazole (Fig. 6).

Using 2.0×10^{-5} M PBO, AChE inhibition was examined over a range of diazinon concentrations in stage 31 and 34 embryos as well as in 24-h-old larvae (Fig. 7). PBO did not affect levels of AChE inhibition using stage 31 embryos. However, PBO caused a slight increase in the IC₅₀ value for

stage 34 embryos (Fig. 7) and increased the IC₅₀ value from 0.232 μmolar in diazinon-only treated larvae to 16 μmolar, an approximately 70-fold greater IC₅₀ with PBO (Fig. 7). In contrast to results obtained with diazinon, PBO did not affect IC₅₀ values determined in larvae exposed to diazoxon (Table 3). In addition, exposure of 24-h-old larvae to ¹⁴C-diazinon in the presence or absence of PBO confirmed that PBO did not affect its uptake (Table 4).

Developmental Increases in *In Vitro* Bioactivation of Diazinon

Using microsomal protein from adult medaka liver, we demonstrated that incubation of diazinon with microsomes resulted in significantly greater AChE inhibition than did reactions without microsomal protein, indicative of the metabolic



Diazinon Concentration	Stage of Development				
	33	34	35	1 Day Larva	7 Day Larva
8.82×10^{-8}	A	AB	AB	BC	C
8.82×10^{-7}	A	B	C	D	D
3.53×10^{-6}	A	B	C	D	D
1.76×10^{-5}	A	A	B	C	D
4.41×10^{-5}	A	A	B	B	C
8.82×10^{-5}	A	A	B	BC	C

FIG. 2. Comparison of AChE inhibition in early life stage medaka. Embryos at all stages were exposed to diazinon for 24 h. Following exposure, a whole-animal homogenate was prepared and AChE activity was determined as described in Materials and Methods. Values represent means \pm SEM ($n = 5$). (B) Statistical comparison of AChE inhibition. For a given diazinon concentration, developmental stages that do not share the same letter are significantly different ($p < 0.05$). Diazinon concentration given in moles.

TABLE 2
In Vitro Inhibition of Embryo and Larval AChE

Diazoxon concentration	AChE inhibition ^a	
	Stage 34	Larvae
Control	0.0 ± 5.7 ^b	0.0 ± 1.3
Solvent control ^c	3.3 ± 8.3	-0.6 ± 0.7
0.1 μM	-3.4 ± 3.8	-3.1 ± 0.8
0.5 μM	21.3 ± 4.6	24.8 ± 3.1
1 μM	37.3 ± 5.5	31.8 ± 0.8
10 μM	101.5 ± 3.8	101.4 ± 1.5

Note. No significant differences were determined between the age groups.

^aActivity is expressed in terms of a percent difference from the stage-specific control value.

^bValues represent the mean ± SD ($n = 3$).

^cSolvent control was hexane (1 μl/1 ml volume).

conversion of diazinon to the more potent AChE-inhibiting oxon metabolite (Forsyth and Chambers, 1989; Mirer *et al.*, 1975). In addition, boiling or carbon monoxide pretreatment of microsomal protein or removal of NADPH inhibited this reaction. Finally, using multiple microsomal samples, we demonstrated the same degree of bioactivation was achieved when

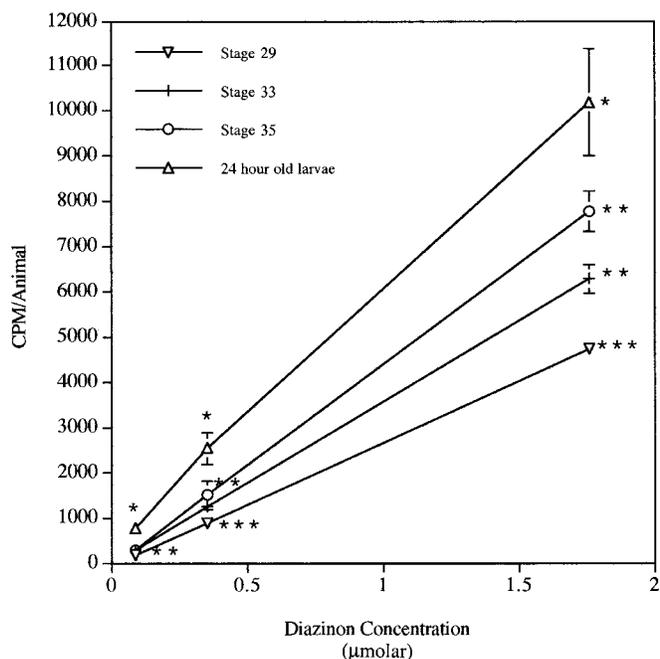


FIG. 3. Uptake of ¹⁴C-diazinon by early life stage medaka. Medaka at stage 29 (down triangle open), 33 (+), 35 (open circle) and 24-h-old larvae (open triangle) were exposed to ¹⁴C-diazinon for 24 h, and following exposure they were rinsed and homogenized in PBS. Aliquots of the homogenate ($n = 3$) were then placed in scintillation fluid, and total radioactivity was determined. Values represent mean cpm in the homogenate per animal ± SD ($n = 3$). Points followed by different numbers of asterisks are significantly different from other stages at the same diazinon concentration ($p < 0.05$).

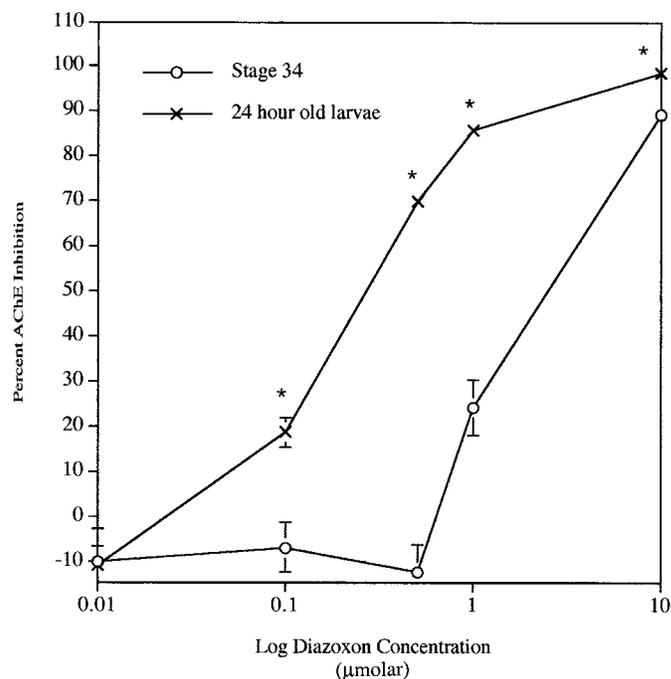


FIG. 4. *In vivo* sensitivity to diazoxon. Stage 34 embryos (open circle) and 24-h-old larvae (×) were exposed to diazoxon concentrations ranging from 0.1 to 100 μmolar for 24 h. Following exposure, AChE activity was determined and expressed as percent inhibition of control activity. Values are means ± SEM ($n = 5$). Asterisk indicates statistically significant difference from stage 34 ($p < 0.05$).

equivalent amounts of P450 were added to the reaction (data not shown).

Incubation of microsomes derived from early life stage medaka resulted in 7.3 ± 2.7 and $28.3 \pm 1.1\%$ inhibition, for stage 31 embryos and 24-h-old larva, respectively. These results indicate a greater ability of the larval preparation to generate the oxon metabolite *in vitro*.

DISCUSSION

This study demonstrates increased sensitivity of an embryo-larval fish to an OP, diazinon, as a function of development. Similarly, Anguiano *et al.* (1994) examined toxicity of parathion in the toad, *Bufo arenarum* Hensel, and reported higher AChE IC₅₀ and LC₅₀ values in embryos when compared to larvae. In addition, Takimoto *et al.* (1984a) reported that 96-h LC₅₀ values for fenitrothion decreased as medaka developed from embryos to 28-day-old larvae. However, a thorough literature search revealed a lack of studies aimed at understanding the factors affecting sensitivity to OPs during these life stages of teleosts. Although additional factors, including detoxification pathways, may determine overall sensitivity of embryo-larval medaka to diazinon, the work completed in this study demonstrates that increased uptake and bioactivation are

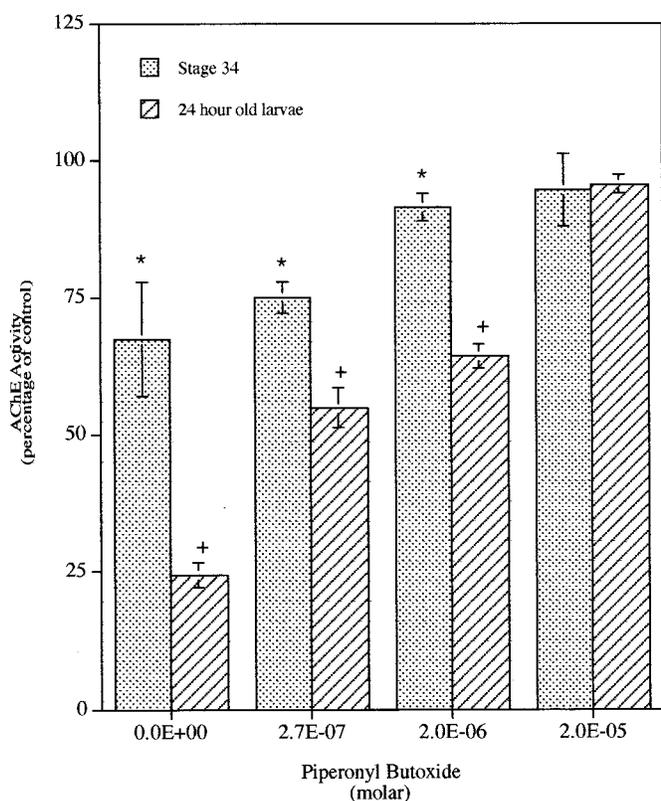


FIG. 5. Effect of piperonyl butoxide on AChE inhibition. For this experiment, groups of stage 34 embryos and of 24-h-old larvae were exposed to 0.88 μ M diazinon alone or in combination with varying concentrations of piperonyl butoxide for 24 h, and AChE activity was determined. Values represent means \pm SD ($n = 3$). Asterisk indicates statistically significant difference from the unexposed stage 34 control ($p < 0.05$). + indicates statistically significant difference from the unexposed larval control ($p < 0.05$).

associated with the increasing sensitivity observed with incremental development.

During the period of development examined, AChE activity rose rapidly, and this pattern appears similar to that reported for rainbow trout (Uesugi and Yamazoe, 1964). Previous results from this laboratory showed that histochemical staining of acetylcholinesterase activity appears in neural tissue along with heavy staining of skeletal musculature (Hamm *et al.*, 1998). Higher levels of cholinesterase activity have been reported to account for decreased sensitivity to OP pesticides in insects (Fournier *et al.*, 1993). However, in the current study, increasing levels of AChE were associated with greater sensitivity. It is possible that in early life stage teleosts the rapid development of the cholinergic system fosters a dependence on this system, for example neurotransmission controlling gill movement during respiration, that results in higher sensitivity to the lethal effects of anticholinesterase pesticides. We showed that with the development of the cholinergic system in the retina, cell death appeared at sites of cholinesterase activity following exposure to diazinon (Hamm *et al.*, 1998).

Comparison of vertebrates by species, sex, and age groups

reveals differences in sensitivity to OP compounds. These differences in sensitivity have been correlated to metabolic bioactivation and/or detoxification, A-esterase levels, and the sensitivity of AChE to inhibition (for a review see, Chambers and Carr, 1995). Benke and Murphy (1975) reported that age-dependent changes in OP sensitivity of rat pups was correlated to changes in P450-mediated detoxification, levels of glutathione, A-esterase levels, and binding to nontarget molecules.

In addition to the factors listed above, exposures of fish embryonated eggs must consider the role of uptake in toxicity. Because these life stages are surrounded by the chorion, a protective yet semipermeable barrier (for a review see Weis and Weis, 1989), the question of uptake is even more important. Helmstetter and Alden (1995) reported that the rate of uptake of agents topically applied to medaka embryonated eggs was proportional to their lipophilicity. OPs, including diazinon, with low water solubilities should be readily absorbed (Bowman and Sans, 1983). Medaka embryonated eggs exposed to radiolabeled fenitrothion showed rapid uptake, and subsequent autoradiography revealed distribution within internal organs and yolk sac of the embryo (Takimoto *et al.*, 1984b). Marty *et al.* (1990) studied the uptake of a series of radiolabelled com-

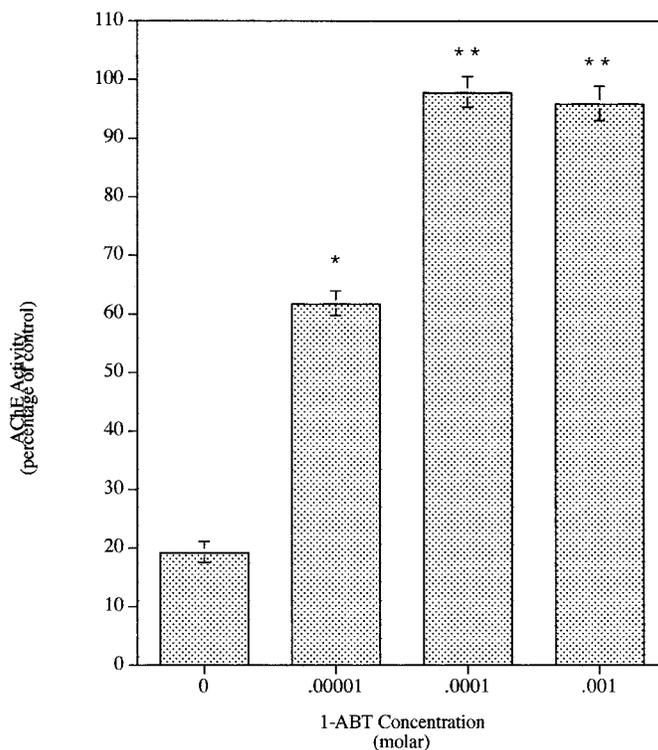


FIG. 6. Effect of the P450 inhibitor 1-aminobenzotriazole (1-ABT) on AChE inhibition in 24-h-old larvae. Larvae were exposed for 24 h to 0.88 μ M diazinon alone (0 1-ABT) or simultaneously with 1×10^{-7} to 10^{-5} M 1-ABT. Values represent means \pm SD ($n = 3$). Asterisk indicates statistically significant difference from diazinon-only treatment ($p < 0.05$). ** indicates statistically significant difference from 1×10^{-5} M 1-ABT ($p < 0.05$).

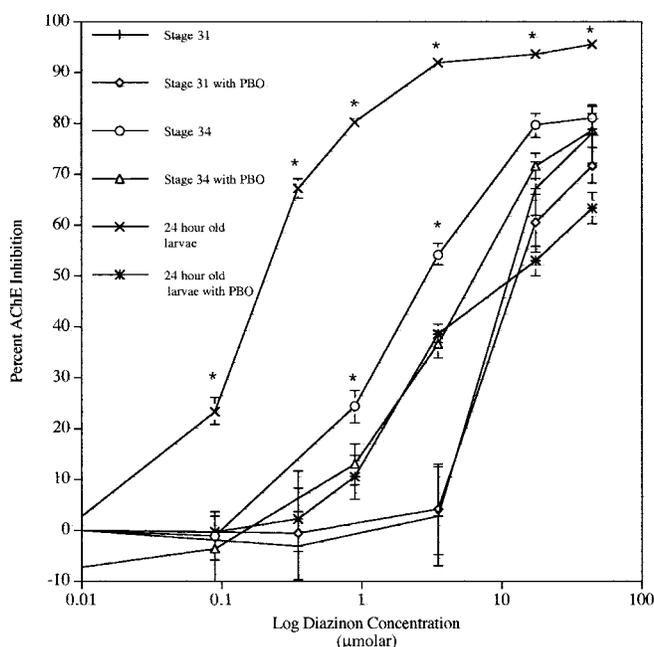


FIG. 7. Influence of PBO on diazinon-induced AChE inhibition. Medaka were exposed to diazinon alone (stages 31 [+], 34 [open circle] and 24-h-old larvae [×]) or diazinon and 2.0×10^{-5} M PBO (stages 31 [open diamond], 34 [open triangle], and 24-h-old larvae [*]; medaka treated with PBO are represented with dashed lines) for 24-h and AChE activity was measured. Values represent means \pm SEM ($n = 5$). Asterisk indicates statistically significant difference from diazinon-only treatment ($p < 0.05$).

pounds of varying hydrophobicity. No differences were seen until immediately prior to hatch, when uptake increased. In contrast to this observation, our exposures to ^{14}C -diazinon resulted in significantly greater uptake by more developed embryos (see Fig. 3; compare stage 29 to more developed embryos). In addition, these differences in uptake occurred at diazinon concentrations that resulted in substantial differences

TABLE 3
The Effect of Piperonyl Butoxide on AChE Inhibition in 24-h-old Larvae by Diazinon

Diazinon concentration	AChE inhibition ^a	
	Without PBO	With PBO ^b
Control	0 \pm 1.7 ^c	0.3 \pm 1.1
0.1 μM	22.7 \pm 3.8	23.6 \pm 1.3
0.5 μM	67.1 \pm 7.6	76.9 \pm 2.9
1 μM	89.7 \pm 2.9	89.4 \pm 0.5
10 μM	98.6 \pm 1.2	98.4 \pm 0.7

Note. No significant differences were determined to result from piperonyl butoxide treatment.

^aActivity is expressed as the percent difference from control activity.

^bTest solution contained 2.0×10^{-5} M PBO.

^cValues represent the mean \pm SEM ($n = 4$).

TABLE 4
Effect of Piperonyl Butoxide on Uptake of Diazinon in 24-h-old Larvae

Diazinon concentration (μmolar)	Larvae	Larvae with PBO ^a
0.882	782 \pm 42 ^b	815 \pm 18
3.53	2137 \pm 294	2109 \pm 220
17.6	9650 \pm 1360	9523 \pm 1376

Note. No significant differences were determined.

^aTest solution contained 2.0×10^{-5} M PBO.

^bAmounts reported as cpm/animal. Values represent the mean \pm SD ($n = 3$).

in AChE inhibition between the developmental stages tested. Finally, addition of 2.0×10^{-5} M PBO, which significantly decreases the toxicity of diazinon, to media containing ^{14}C -diazinon did not alter the total radioactivity in larvae. This lack of effect by PBO on uptake, demonstrated herein, is in contrast to earlier reports that PBO altered toxicity due to decreased uptake (Kuo *et al.*, 1983; Sriram *et al.*, 1995); however, in the previous cases, the investigators were not studying organophosphates but instead worked with ionized compounds.

Following uptake and distribution, a key component in the toxicity of phosphorothionates is bioactivation to oxon metabolites. Differences in the capacity to bioactivate these compounds affects toxicity (Chambers and Carr, 1995). Ma and Chambers (1995) demonstrated that, in rat tissues, parathion was readily bioactivated by desulfuration, whereas chlorpyrifos was readily detoxified by dearylation; these metabolic differences correspond to the lower toxicity of chlorpyrifos. Compared with mammalian species, fish are known to have a low ability to metabolize OPs. Hitchcock and Murphy (1971) studied bioactivation of parathion and guthion by rat and by two fish species, bullhead (*Ictalurus melas*) and winter flounder (*Pseudopleuronectes americanus*) and noted that rat tissues bioactivated both compounds 2- to 3-fold more than either fish species. However, when fish species are compared, data demonstrate that toxicity differences are related to host metabolism. Keizer *et al.* (1995) demonstrated that guppy (*Poecilia reticulata*), the most sensitive of several species they studied, had the highest capacity to bioactivate diazinon, whereas carp (*Cyprinus carpio*) were insensitive and had a low ability to bioactivate diazinon. In addition, alternative pathways of metabolism that produce more polar metabolites, presumably hydroxylated metabolites other than the oxon, were reported to account for the insensitivity to diazinon of adult medaka versus loach (*Misgurnus anguillicaudatus*) (Oh *et al.*, 1991).

Our work demonstrated that as early life stage medaka developed (from fertilization to larvae), equimolar concentrations of diazinon caused increasing amounts of AChE inhibition. However, when larvae were exposed to diazinon in combination with either of two P450 inhibitors, piperonyl butoxide (Anders, 1968) or 1-aminobenzotriazole (Knickle and Bend, 1992; Meschter *et al.*, 1994), levels of AChE inhibition were

greatly decreased. These data suggest that metabolism is present in these stages and, once formed, continues to be important in later stages. Further, PBO provided greater protection from AChE inhibition with further development. Ankley *et al.* (1991) reported that aqueous exposures with PBO decreased toxicity of four metabolically activated OPs, including diazinon, but did not alter the toxicity of three OPs not requiring bioactivation. Mirer *et al.* (1977) demonstrated that PBO inhibited bioactivation and P450-mediated detoxification of methyl parathion *in vitro* and resulted in a 40-fold decrease in toxicity following *in vivo* exposure. In contrast, PBO did not affect the *in vivo* toxicity of the active metabolite methyl paraoxon. In the present study, PBO had no effect on IC₅₀ values generated from *in vivo* exposures of early stage medaka to diazoxon (Table 3), suggesting that inhibition of P450-mediated detoxification steps has little influence on sensitivity changes. PBO's lack of effect on diazoxon was important because PBO is a nonselective inhibitor of P450 and inhibits the oxidative reactions that both bioactivate and detoxify diazinon (Smith *et al.*, 1974).

Further evidence for the role of metabolic activation in the sensitivity changes was obtained from *in vitro* metabolism studies. We used an incubation system in which inhibition of an exogenous AChE source is related to metabolic conversion of diazinon to the more potent AChE-inhibiting oxon metabolite. Using adult medaka hepatic microsomes, we showed that bioactivation in this system was inhibited by each of three well-established ways to deactivate cytochrome P450 activity: *a*) boiling, *b*) exposure of microsomes to carbon monoxide, or *c*) removal of NADPH. These results demonstrated the essential requirement of cytochrome P450 activity for conversion of diazinon to a form that inhibits AChE. Microsomes from medaka embryos were capable of bioactivating diazinon, and based on levels of AChE inhibition, 24-h-old larvae had significantly greater capacity for bioactivation than did stage 31 or earlier embryos. This apparent increase in metabolic capacity immediately after hatch mirrors increases in cytochrome P450-mediated enzyme activity occurring in early life stage teleosts at this time (Binder and Stegeman, 1984; Binder *et al.*, 1985; Wisk and Cooper, 1992). Similarly, our measurements of ethoxyresorufin-*O*-deethylase activity, associated with cytochrome P450 1A1 in teleosts (Stegeman, 1989), show a rapid increase around the time of hatch in medaka (unpublished observations in this laboratory).

Once bioactivated, organophosphate pesticides target AChE, and the sensitivity of this enzyme to *in vitro* inhibition has been used to explain species sensitivity differences. Combining substantial original research and a review of the literature, Chambers and Carr (1995) assert that *in vitro* sensitivity of AChE to oxon metabolites largely determines *in vivo* sensitivity of juvenile and adult fish. Johnson and Wallace (1987) compared species and noted that AChE from rats, a sensitive species, was more sensitive to inhibition by paraoxon than AChE derived from two insensitive species, fathead minnows and rainbow

trout. Murphy *et al.* (1968) compared sensitivity to two OPs and found that fish had higher *in vivo* sensitivity to gutoxon than paraoxon; this sensitivity paralleled the higher *in vitro* sensitivity of fish brain cholinesterase to gutoxon. Finally, Keizer *et al.* (1995) reported that *in vitro* sensitivity of AChE was a determinant of toxicity differences among four species of fish.

In addition to species-specific differences in AChE, some have suggested that developmental changes in the sensitivity of AChE explain differences in toxicity between age groups. Anguiano *et al.* (1994) demonstrated that toad embryos were less sensitive than larvae to parathion, and AChE from an embryo homogenate had a higher *in vitro* IC₅₀ value with paraoxon. AChE exists as several molecular forms, and the distribution of these forms varies with development (for a review see Massoulie and Bon, 1982). Therefore, developmental changes in AChE inhibition could result from differences in the sensitivity of these molecular forms. However, whereas Mortensen *et al.* (1997) found differences in IC₅₀ values between tissues and during development in crude homogenates incubated with chlorpyrifos-oxon, immunoprecipitation of AChE and subsequent *in vitro* incubation resulted in similar IC₅₀ values. The use of immunopurified AChE demonstrates that the apparent sensitivity of this enzyme may result from an interaction of oxon with nontarget molecules. Perhaps the tissue preparations used by Anguiano *et al.* (1994) resulted in a similar interaction with nontarget proteins. In this study, no difference between embryos and larvae in *in vitro* AChE inhibition by diazoxon was recorded (Table 2), further suggesting that *in vitro* AChE sensitivity is not a factor in developmental changes. Similarly, Benke and Murphy (1975) reported developmental changes in the sensitivity of rat pups to parathion and methylparathion, but did not detect changes in *in vitro* sensitivity of AChE.

In conclusion, the present study shows that toxicity of diazinon to a model early life stage teleost increases markedly around the time of hatch. Lower LC₅₀ values and greater AChE inhibition with added development were the evidence of this change. Over the period of development examined, both the uptake of ¹⁴C-diazinon and the AChE inhibition following *in vivo* exposure to diazoxon increased with added development, indicating at least some of the sensitivity was associated with greater uptake of the compound. In addition, further development was associated with enhanced metabolism of diazinon. As medaka developed, P450 inhibitors had an increasing protective effect. Finally, *in vitro* metabolism studies demonstrated a higher rate of bioactivation with added development. These mechanistic investigations provide an improved understanding of organophosphate toxicity in early life stages of fishes.

ACKNOWLEDGMENTS

Portions of this publication were made possible by grant 5 P42 ES04699 from the National Institutes of Environmental Health Sciences (NIEHS),

National Institutes of Health, with funding provided by the U.S. Environmental Protection Agency (U.S. EPA), by the U.S. EPA (R819658 and R825433) Center for Ecological Health Research at UC Davis, AFOSR-91-0226 U.S. Air Force grant, and P30-ES-05707 from the NIEHS. Contents of this publication are solely the responsibility of the authors and do not necessarily represent the official view of the NIEHS, NIH, or U.S. EPA. In addition, the authors would like to thank Jack Henderson for his technical assistance and Fumio Matsumura for use of the scintillation counter.

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