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Organophosphate-Induced Acetylcholinesterase Inhibition and Embryonic Retinal Cell Necrosis *In Vivo* in the Teleost (Oryzias Latipes)

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Abstract: JONATHAN T. HAMM, BARRY W. WILSON AND DAVID E. HINTON. Organophosphate-Induced Acetylcholinesterase Inhibition and Embryonic Retinal Cell Necrosis *In Vivo* in the Teleost *(Oryzias Latipes). Neurotoxicology* **19**(6);853-870,1998. *Recent monitoring of the Sacramento- San Joaquin River system (CA) indicates that levels of the organophosphate pesticide, diazinon, exceed National Academy of Science guidelines and these levels result in toxicity in USEPA acute toxicity tests with Ceriodaphnia dubia. Since organophosphates (OPs) inhibit acetylcholinesterase (AChE), the present study examined the effects of diazinon on the embryonic nervous system of a model teleost, medaka, Oryzias latipes. Preliminary histological screens revealed limited retinal cell necrosis in control embryos with apparent increased necrosis in diazinon - exposed embryos. Subsequently, embryos were exposed to 1.8 x 10^{-5}, 4.4 x 10^{-5}, or to 8.8 x 10^{-5} M diazinon and replicates were frozen for biochemical analysis or were fixed for histopathological analysis at days 3, 5, and 7 of development. Diazinon exposure significantly inhibited AChE activity within whole embryos and in homogenates of retinas from treated animals. Histological examination of embryos indicated that as the retina underwent differentiation into distinct cell layers, between days 5 and 7, small foci of necrotic cells became apparent within the inner nuclear layer and isolated individual pyknotic cells were observed in the ganglion layer. Quantification of foci of necrotic cells revealed that 8.8 x 10^{-5} M diazinon increased number and area of these lesions. Enzyme histochemistry localized AChE activity to regions equivalent to sites of necrosis. Separate exposures of embryos to the OP, diisopropylphosphorofluoridate, produced large foci of necrotic cells at sites equivalent to those seen following diazinon exposure. © 1998, Intox Press, Inc.*

Key Words: Organophosphate, Acetylcholinesterase, Retinal Necrosis

INTRODUCTION

Acetylcholinesterase (AChE) (EC 3.1.1.7) plays a well- established role in the adult nervous system of a broad range of vertebrates by inactivating the neurotransmitter, acetylcholine (Katzung, 1992). However, prior to neural development and during early organ formation, embryos show temporal and spatial patterns of enzyme activity which may be more related to ontogeny of non-neural as well as neural tissues (for a review see Drews, 1975 or Layer and Willbold, 1994). Cholinesterase activity precedes nerve tract formation and may play a role in guiding the nerve growth cone (Layer and Kaulich, 1991; Layer and Willbold, 1994; Hanneman and Westerfield, 1989; Geula *et al.*, 1993). Studies of the cholinergic system in human fetal brain demonstrate that the expression of muscarinic receptors correlates to development of the system but that AChE shows an arbitrary pattern of activity suggesting a novel function (Egozi *et al.*, 1986). Additional studies, revealed that transfection of DNA encoding for AChE to glioma cells resulted in cell body enlargement and extension of processes (Karpel *et al.*, 1996).

While AChE is inhibited by organophosphate pesticides (OPs), the effects of such inhibition on the developing nervous system remain unclear due in part to

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contrasting observations. Some studies have reported behavioral change and morphological alterations within the brain (Chanda and Pope, 1996; Gupta *et al.*, 1985; Berge *et al.*, 1986) whereas others have only observed growth retardation (Deacon *et al.*, 1980).

In addition to reported effects on developing brain, ocular toxicity of OPs is being actively investigated following reports of related problems among the human population in the Saku region of Japan (see review by Dementi, 1994). The disease syndrome, so called Saku disease, is characterized by degenerative changes in the neural retina and has been proposed to result from chronic exposure to OPs, including diazinon, applied in and around this agricultural region.

The Central Valley of California, an extensive agricultural area, accounts for 10% of the national pesticide usage (Kuivila and Foe, 1995). United States Environmental Protection Agency (USEPA) aquatic toxicity tests have been used to analyze water quality of urban runoff and agricultural tailwaters (Kuivila and Foe, 1995; Bailey *et al.*, 1996) in these regions. Follow-up, toxicant identification procedures, on those water samples that proved toxic, identified diazinon and chlorpyrifos, another OP, as responsible agents (Bailey *et al.*, 1996).

Because of the widespread nature of surface water toxicity in the Sacramento and San Joaquin Rivers and their Delta in California, and following the above Japanese reports, we initiated investigations of a fish embryonic system designed to determine the possible role of cholinesterase inhibition in histogenesis of alterations of developing retina. Although these initial studies were not designed to mimic environmental concentrations, results describe the ontogeny of cholinesterase and illustrate importance of timing of exposure for overall toxic effect.

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). The glycol-methacrylate embedding kit (JB-4) was purchased from Polysciences (Worrington, PA). 3, 3'-Diaminobenzidine (DAB), diisopropylphosphorofluoridate (DFP) and all other reagents were purchased from Sigma Chemical Company (St. Louis, MO) and were of the highest quality available.

Stock Preparation and Analysis

Diazinon was weighed in a glass boat, transferred to a volumetric flask containing embryo rearing medium (ERM; 20mM NaCl, .40mM KCl, .25mM $CaCl_2$-$2H_2O$, .65mM $MgSO_4$) at pH 7.3 (Kirchen and West, 1976), stirred until dissolved, and stored at 4°C. Diazinon concentration was confirmed by gas chromatographic analysis (Aston and Seiber, 1996) and dilutions of the defined stock were used for tests as described below.

Embryo Collection and Culture

Our medaka colony, continuously maintained at UC Davis since 1986, were the source of broodstock. For details of diet and husbandry see DeKoven *et al.* (1992). Broodstock were housed in 40 L aquaria containing water reconditioned to USEPA moderately hard conditions (Horning and Weber, 1985) at 25°C under a 16L:8D photo period. Broodstock were fed a purified, casein-based diet (DeKoven *et al.*, 1992) supplemented with brine shrimp nauplii to stimulate continuous egg production. Embryos were collected within 6 hrs of fertilization by netting females and manually removing clusters of embryonated eggs. Clusters were rolled between moistened finger tips to break connective filaments and separate individuals (Marty *et al.* 1990). Under dissecting microscope, infertile eggs were identified and removed. Remaining viable embryos at multicellular stage 11, the early high gastrula phase (Kirchen and West, 1976) were then transferred to a culture chamber containing ERM, where they were aerated and maintained at 25°C until initiation of exposure.

Embryo Exposure and Analysis

Embryos (N=5) at the appropriate stage of development (see below) were transferred to individual 20 ml borosilicate vials (Fisher Scientific, Pittsburgh, PA) containing 2mls of test solution. Exposures were static, nonrenewal. The experimental set-up has been previously substantiated to provide adequate oxygen and to result in normal development of medaka embryos (Marty *et al.*, 1990; Villalobos *et al.*, 1996). All vials contained ERM at pH 7.2-7.3 and either 0.0 ppm diazinon or concentrations shown in pileot studies to produce sublethal toxicity. These were (in ppm): 1.8×10^{-5}, 4.4×10^{-5}, or 8.8×10^{-5} M diazinon. For histological examination (see below), replicates (N=3), comprising 15 embryos, were fixed by placement in 10 volumes of 10% neutral buffered formalin

at early afternoon of days 3 (46 hrs of development; stage 23), 5 (94 hrs of development; between stages 29 and 30); and 7 (142 hrs of development, stage 33). The Kirchen and West (1976) description of stages in medaka development was used and selected stages represented early, intermediate and late organogenesis respectively.

A complementary group of embryos was exposed beginning on day 1 and 5 replicates (N=5 in each) were rapidly transferred on days 3, 5, and 7 to cryovials and immediately frozen by placement in liquid N_2. After storage at -80 °C, these medaka were analyzed for AChE activity (see below).

A second set of exposures used the same procedure as above; however, only 3 replicates of 5 embryos each were exposed from day 5 to day 7. Diazinon concentrations were 4.4 $x10^{-5}$ and 8.8 $x10^{-5}$ M. On day 7, embryos were fixed in 10 volumes of 10% neutral buffered formalin for later histological examination (see below). In conjunction with this second exposure set, embryos exposed to 4.4 $x10^{-5}$ or 8.8 $x10^{-5}$ M diazinon between days 5 and 7, were processed for AChE analysis. On day 7, replicates (N=5) were snap frozen in liquid N_2 and stored for analysis (see below).

As a positive control, embryos were exposed to sublethal concentrations of the direct-acting anticholinesterase agent, DFP at a predetermined concentration of 2.5$x10^{-5}$ M. Embryos were exposed beginning day 1 and fixed (as above) for histological examination on day 7.

Histological Examination

Fixed embryos were manually dechorionated using fine tipped forceps. Following dehydration in a graded series of ethanol, they were infiltrated and embedded in the water -soluble, plastic resin, glycolmethacrylate (GMA). Embryos from a given replicate were embedded in a single block (5 embryos/block). To ensure uniform orientation (parasagittal), embryos were embedded with left eye facing downward and positioned so that both eyes were aligned when viewed from above. Entire eyes were serially sectioned at 10 µm thickness using a glass knife on a LKB Historange microtome. Sections were mounted on glass slides and stained with hematoxylin and eosin. For morphometric analysis, ten sections of equal interval per eye were randomly selected using the methods of Gundersen and Jensen (1987) and subjected to quantification of necrotic lesions. Estimating the necrotic lesion was accomplished by using the Stereology Toolbox (Morphometrix, Davis, CA) method by capturing digital images with NIH image program, version 1.60, (Wayne Rashland, author: available through National Technical Information Service), a video camera (Sony; DXC-151A, San Jose, California) and a frame grabber (Quick-Capture: Data Translation, Marlboro, MA) interfaced with a Macintosh Quadra 840AV computer. After images were imported into the stereology toolbox program, the total area of retina, the area and number of necrotic foci were determined.

AChE Determination

Optimal assay conditions for medaka tissues were studied, examining pH of assay buffer, substrate concentration, and specificity of activity with tetraisopropyl pyrophosphoramide (iso-OMPA) an inhibitor of butyrylcholinesterase (BChE) (Wilson and Henderson, 1992). Standard conditions of pH 8.0 and 1mM acetylthiocholine proved optimal for work with medaka. Incubation of horse serum, containing AChE and BChE, purified eel AChE, and homogenized medaka embryos with several concentrations of iso-OMPA demonstrated that 0.115 mM iso-OMPA (final concentration) inhibited BChE but not AChE in medaka preparations.

Control and exposed embryos were removed from the -80°C freezer, placed on ice, and allowed to warm to 0 to 4°C. Samples were homogenized in 0.1 M sodium phosphate buffer pH 8.0, using 5 to 7 passes of a chilled teflon-glass homogenizer, followed by brief sonication, using a setting of 25 to 30% of maximum on a Bronwill Scientific sonicator (Rochester, New York). Activity was determined using the method of Ellman et al. (1961). For analysis, 220 µl of assay buffer and 10 µl of DTNB (10.3 mM) were added to each well of a 96 well plate (96-well polystyrene, flat bottom microtiter plates, Dynatech Laboratories, Inc., Chantilly, VA); 30 µl of sample was added to each well and preincubated with 30 µl of 1.25 mM tetraisopropyl pyrophosphoramide (iso-OMPA) for 15 min at 25°C to inhibit nonspecific cholinesterases (BChE). Following preincubation, 30 µl acetylthiocholine (10.7 mM) was added and activity analyzed using an EL 340 Biokinetics reader (Bio-Tek Instruments) interfaced to a Zenith 248 computer running KinetiCalc software (Bio-Tek EIA Application Software, Winooski, VT). Substrate blanks, omitting sample, and tissue blanks, omitting substrate, were used to correct activity. In addition, activity was normalized to protein concentration (see below).

AChE Histochemistry

Embryos were freeze-dried using the method of Teh and Hinton (1993) to preserve enzyme activity. Briefly,

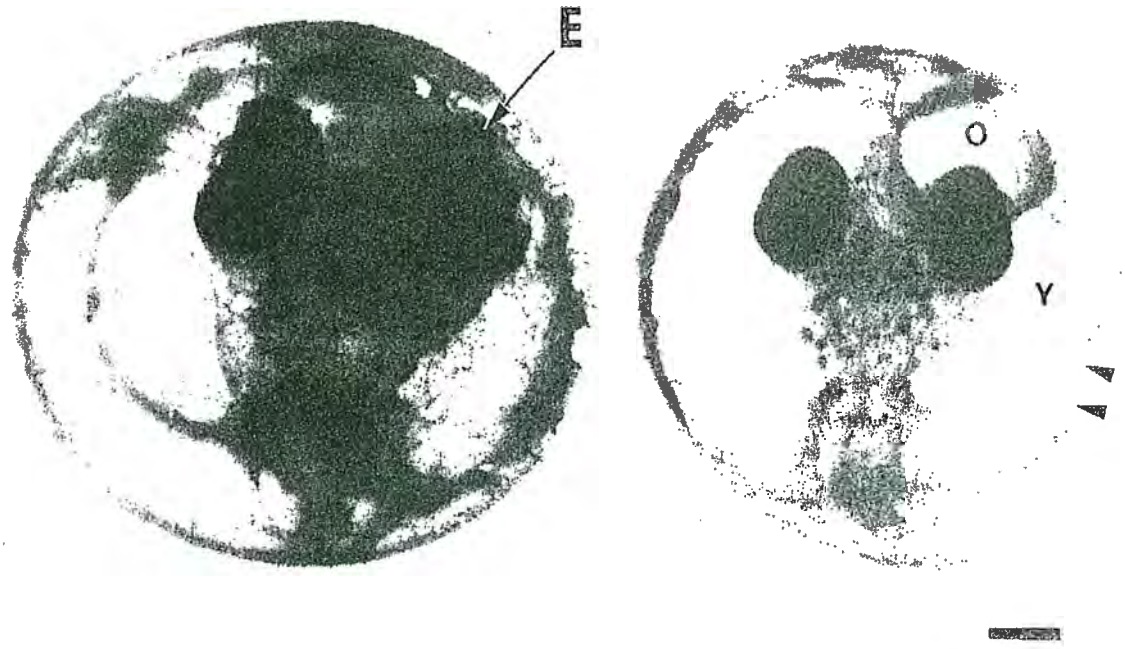

FIG. 1. Control embryo (left) and exposed embryo right. Exposure was from day 1 to day 7 to 8.82 x 10^{-5} M diazinon. Arrows on exposed embryo denote site where yolk edema displaces yolk. Eye (E), oil droplet (O) and yolk (Y) are shown. bar= 0.16 mm.

staged embryos were snap frozen in 2-methylbutane chilled in liquid nitrogen and transferred to a Tis-U-Dry freeze-dryer (FTS Systems INC., Stone Ridge, NY) where ice crystals within the tissue were sublimated under vacuum. Once samples were dried they were placed in imidazole-catalyzed GMA, placed under vacuum for 1 hr, stored overnight at 4°C. Infiltrated tissues were transferred to gelatin capsules and embedded in GMA.

Polymerized blocks were sectioned as above, collected on slides and allowed to air dry overnight at 4°C. Slides were removed from the refrigerator, allowed to warm to 20°C, and stained according to the methods of Karnovsky and Roots (1964) for demonstration of cholinesterase (ChE) activity. As a positive biological control, to ensure staining methods were working, sections of adult medaka brain were included with each group of slides stained. Butyrlcholinesterase (BChE) was eliminated by a 30 mins. preincubation of sections with 10^{-6}M iso-OMPA and inclusion of iso-OMPA in the color development solution. To demonstrate the specificity of staining, ChE activity was inhibited by a 30 mins. preincubation of sections with 10^{-5}M eserine sulfate and inclusion of eserine sulfate in the color development solution.

Localization of Apoptotic Cells

Embryos were exposed as above from day 1 to day 7 to 8.8x10^{-5} M diazinon, and following exposure, were fixed as above but embedded in paraffin. Sections from control and exposed embryos were stained according to the manufacturer's protocol in the ApopTag™ staining kit (Oncor, Inc., Gaithersburg, MD). The fragmented DNA of apoptotic cells was identified by incorporating digoxigenin-labeled nucleotides, reacted with a peroxidase labeled anti-digoxigenin antibody and visualized using DAB as chromogen. Slides were counterstained with methyl green to facilitate orientation.

Determination of Development of Retinal AChE Activity

Control embryos, replicates (N=3) of 10 embryos from stages 30, 31, 33, 35, and from larvae were used. Embryos were dechorionated and retinal tissue dissected and pooled in ice cold assay buffer. Tissues were homogenized, briefly sonicated, and AChE activity was normalized to protein content.

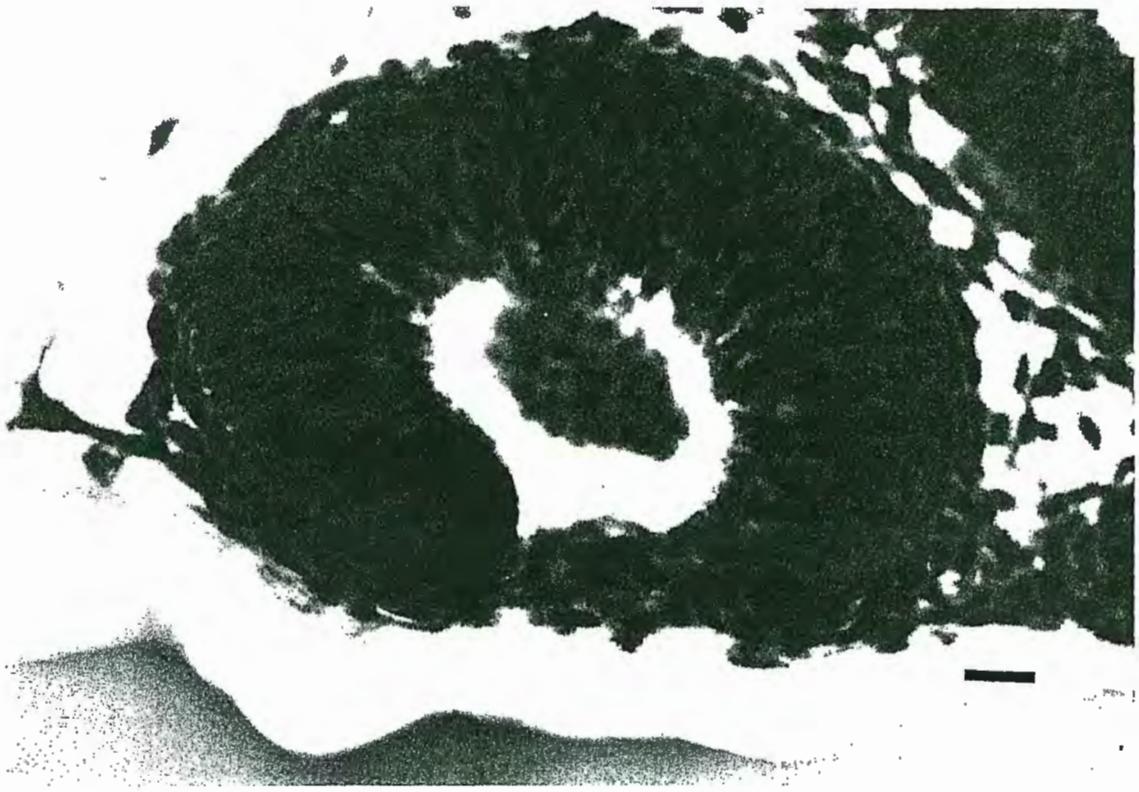

FIG. 2. Section through retina of control medaka embryo on day 3 at stage 23 of development. Cup-like retina shows no differentiation into layers. Cells in central space will be associated with future lens. H&E. bar= 14.6µm.

Determination of Retinal AChE Inhibition

Replicates (N=6) of 5 embryos each were exposed from day 1 to day 7 to 1.8×10^{-5}, 4.4×10^{-5}, or 8.8×10^{-5} M diazinon as above. On day 7, replicates were combined to give 3 groups of 10 embryos per treatment. All embryos were dechorionated, retinal tissue dissected, removed and placed in ice cold assay buffer. Following homogenization in chilled Potter Elvehgem tubes, resultant homogenates were briefly sonicated, and analyzed for AChE activity as above.

Determination of Protein Concentration

Protein in homogenates was estimated using a bicinchoninic acid protein assay (Smith *et al.*, 1985). Reagents were purchased from Sigma Chemical Company; St. Louis, MO) and bovine serum albumin was used as a standard.

Statistics

Levels of statistical significance were analyzed by ANOVA, followed by a Scheff F-test as a *post hoc* test comparing means between the different treatment groups. Differences were considered significant if $p \leq 0.05$.

RESULTS

Morphologic Studies

Under our culture conditions, medaka hatch at 9 days after fertilization. Larvae are capable of swimming and consuming exogenous food on the day of hatch. Using dissection microscope analysis through the intact, transparent chorion, the medaka nervous system, including eye, formed between days 1 and 3. Continuous exposure of embryos to 8.8×10^{-5} M diazinon from day 1 to day 7 resulted in changes which were detectable with the dissecting microscope. These included growth retardation and formation of edema in yolk sac adjacent to vitelline veins (Fig. 1).

Gross anatomical findings were extended to the tissue level by light microscopy and examination at day 3 revealed pigment epithelium and lens development but retina was comprised entirely of undifferentiated cells. At

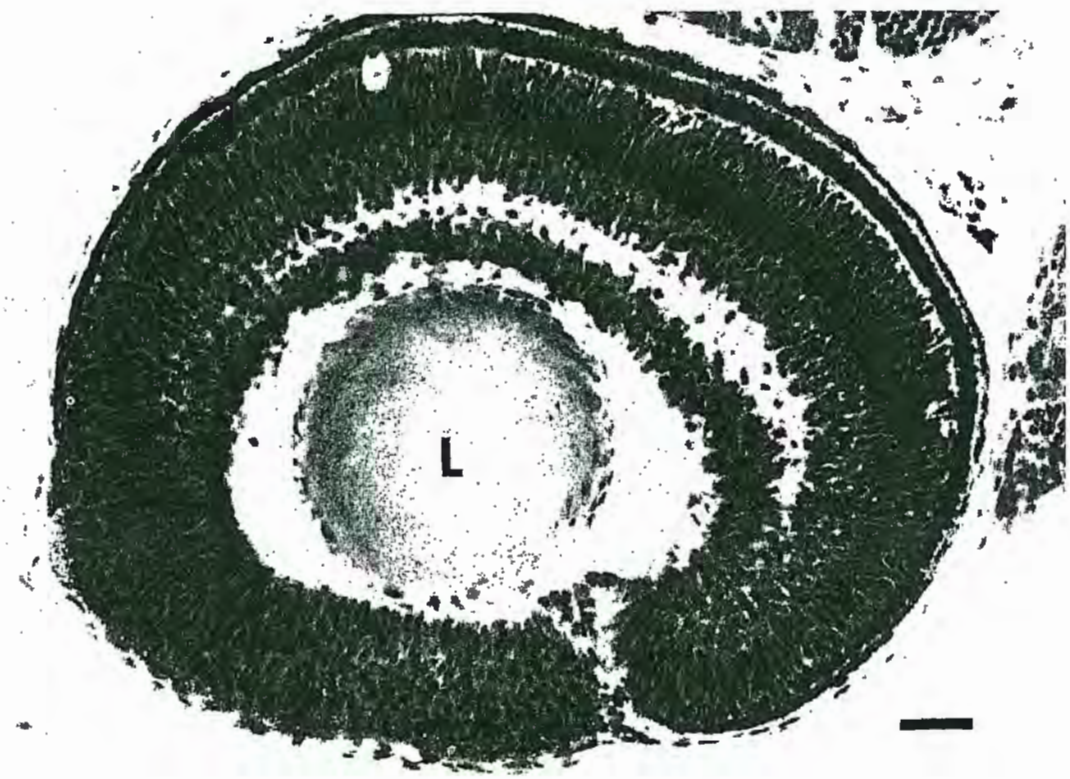

FIG. 3A. Section through retina of control medaka embryo on day 5 at stage 30 of development. Lens (L) is prominent and cell layers are forming in retina. H&E. bar= 21.0μm.

day 3, diazinon-exposed embryos were free of histological alteration (Fig. 2). By day 5, changes were apparent when control and exposed medaka were compared. Retinas of control fish had differentiated into pigment epithelium, inner- and outer- plexiform layers (Fig. 3A). However, retinas of exposed medaka were apparently developing more slowly. In contrast to retinas of control medaka, exposed medaka of the same age showed no differentiation of retina into distinct cell layers (Fig. 3B). No necrosis was observed on day 5.

Necrosis was a significant finding which was apparent at 7 days of development. Control retinas showed small numbers of cells with pyknotic nuclei localized in inner nuclear and ganglion cell layers adjacent to the inner plexiform layer (Fig. 4A). While control embryos in the day 1 to 7 exposure group had greater mean number of necrotic foci than controls of the day 5 to day 7 exposure group, means were not significantly different (Table 1, $p \leq 0.05$). Exposed animals had scattered, infrequent necrotic cells within brain and a dramatic increase in necrosis within inner nuclear layer of retina (Fig. 4B). In medaka exposed to 8.8×10^{-5} M diazinon, statistically significant increases in the number- and area- of necrotic foci (Table 1) were seen.

When the duration of the exposure was limited to 48 hours, i.e. from day 5 to day 7, no gross abnormalities were detected. External morphology was not altered, growth rates did not appear different from controls, and circulation of blood appeared normal. When analysis was extended to tissues and cells, embryos exposed to the highest diazinon concentration over this same duration revealed statistically significant increases in number- and area- of necrotic lesions when compared to controls (Table 1).

In initial range - finding studies, exposure to DFP (2.5×10^{-5} M) resulted in 10% mortality. Among embryo survivors, 30% showed growth retardation and edema. Histological analysis, directed at grossly normal embryos exposed to the same concentration revealed retinal cell necrosis of the inner nuclear and ganglion cell layers. This alteration was equivalent in severity to that seen in embryos exposed from day 1 to day 7 to the highest concentration of diazinon tested.

AChE Ontogeny, Localization and Inhibition

AChE activity was first detected in control embryo retinal homogenates between days 5 and 6; and, after day

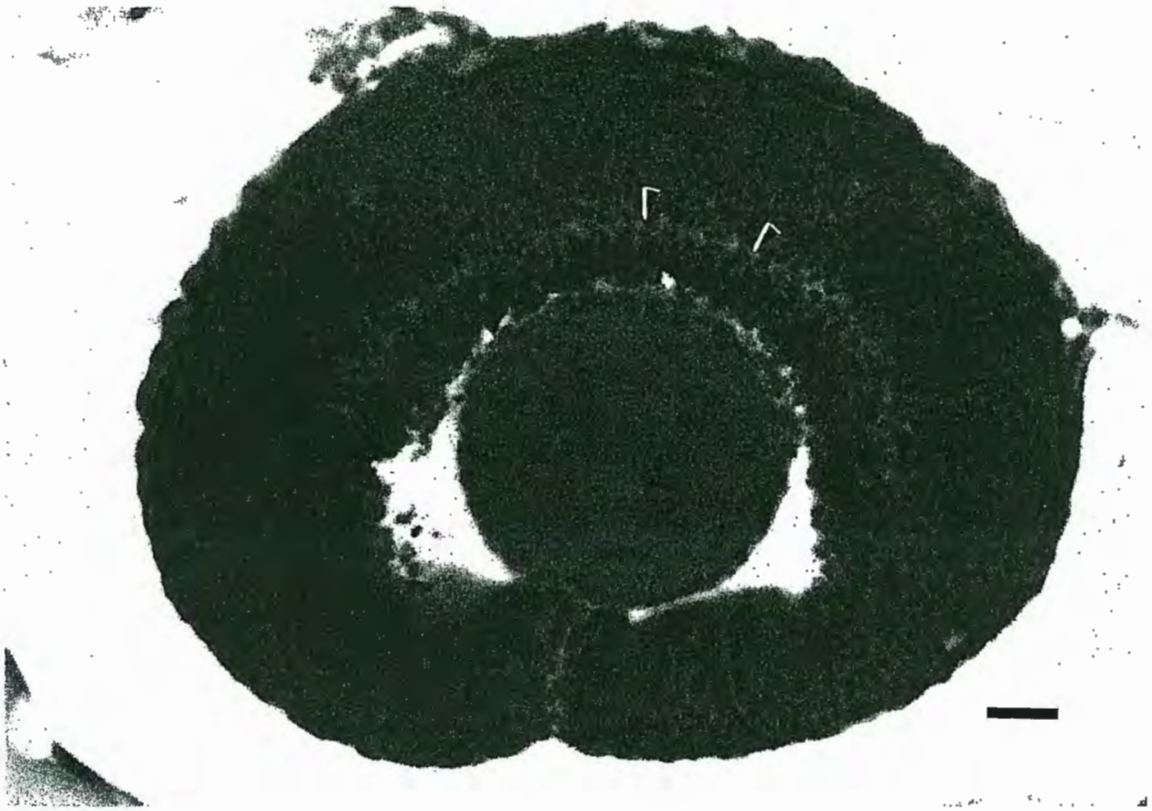

FIG. 3B. Section through retina of medaka embryo on day 5 at stage 30 of development following exposure (from day 1 to day 5) to 8.82×10^{-5} M diazinon. Lens is visible, however, cell layers show delayed differentiation. Inner plexiform layer is forming (arrows). H&E. bar= 21.0µm.

TABLE 1. Retinal Necrosis Following Diazinon Exposure.

	Mean Necrotic Foci per Retina	Percentage Lesion Area
Exposed Day 1- Day 7		
control	2.14 ± 0.26	0.5 ± 0.1
1.76×10^{-5}M	2.90 ± 0.36	0.9 ± 0.2
4.41×10^{-5}M	3.11 ± 0.29	0.9 ± 0.1
8.82×10^{-5}M	6.39 ± 0.47*	4.0 ± 0.6*
Exposed Day 5- Day 7		
control	1.47 ± 0.18	0.4 ± 0.1
4.41×10^{-5}M	2.06 ± 0.32	0.6 ± 0.1
8.82×10^{-5}M	2.65 ± 0.32*	0.7 ± 0.1*

Values are means ± SEM for 15 animals. See materials and methods for explanation of numbers of sections scored.

*Significantly different from control ($p < 0.05$)

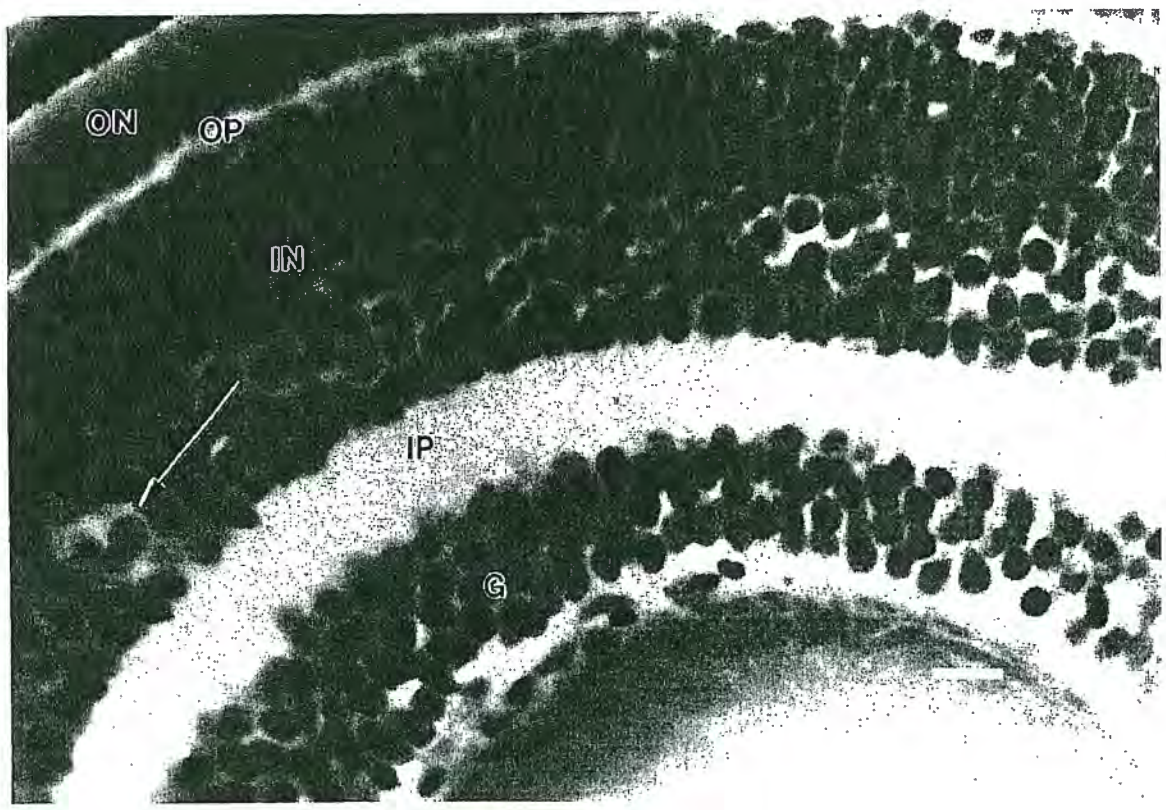

FIG. 4A. Section through retina of control medaka embryo on day 7 at stage 33 of development. Cell layers resolved are: ganglion (G), inner plexiform (IP), inner nuclear (IN), outer plexiform (OP), outer nuclear (ON). Note necrotic focus in inner nuclear layer (arrow). H&E. bar= 8.0µm.

7, AChE activity increased rapidly (Table 2). Similarly, there was no retinal cholinesterase (AChE or BChE) detected by enzyme-histochemical staining on day 5. However, AChE staining was apparent by day 6 and reaction product was localized to cells of the inner nuclear and ganglion layers as well as the cell processes from these two layers contained within the inner plexiform layer (Figs. 5A and 5B). In addition, abundant reaction product remained despite the use of the BChE inhibitor, iso-OMPA, indicating that staining was due primarily to AChE.

TABLE 2. Development of Retinal AChE Activity (nmoles/min/mg protein) in Medaka.

Stage	Retinal AChE Activity
30 (day 5)	ND
31 (day 6)	4.84 ± 0.94
33 (day 7)	15.21 ± 0.24
35 (day 9)	63.20 ± 1.22
Larval (day11)	125.60 ± 2.31

Values are means ± SEM for 3 homogenates. Each homogenate was a pool of retinal tissue from 10 individuals.
Stages 30-35 relate to embryos, with larger numbers equating to added age and development
Larval= 24 hours after hatch
Age in days from fertilization, see parentheses.
ND= not detected

Exposure of embryos to diazinon resulted in AChE inhibition of whole embryonal and retinal homogenates (Tables 3 and 4). Embryos exposed from day 1 to day 3 showed no discernible pattern of inhibition (results not shown). In contrast, embryos exposed from day 1 to day 5 showed moderate, equivalent levels of inhibition at all concentrations tested. A clear dose-response pattern of inhibition emerged when exposure was continued to day 7. Although AChE was significantly inhibited in embryos exposed between days 5 and 7, the inhibitory effect was even more pronounced after the longer exposure duration ($p \leq 0.05$).

Localization of Apoptotic Cells

When sections of retinas from control medaka embryos were reacted for the detection of apoptotic cells,

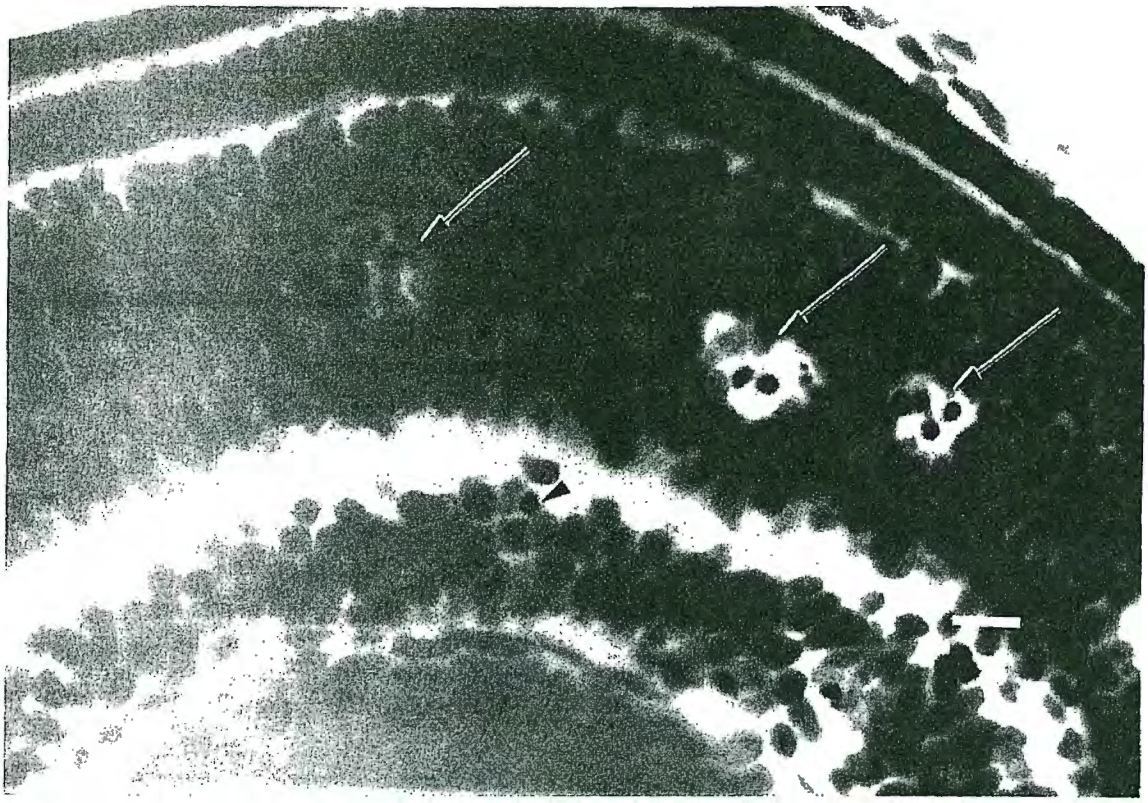

FIG. 4B. Section through retina of medaka embryo on day 7 at stage 33 of development following exposure (from day 1 to day 7) to 8.82x10^{-5} M diazinon. Cell layers are distinct, however, necrotic foci are visible within the inner nuclear layer (arrows) and single cell necrosis in the ganglion cell layer (short arrow). H&E. bar = 8.0μm.

small numbers of positive cells were seen in the inner nuclear layer (Fig. 6A). In contrast, large numbers of cells and focal clusters of cells in the inner nuclear and ganglion cell layers stained positively in retinal sections from those embryos exposed to 8.8 x 10^{-5} M diazinon from day 1 to day 7 (Fig. 6B).

DISCUSSION

This report shows that diazinon increases cell death during retinal development in a teleost fish. Fernald (1993) reviewed the physiology of vision in fishes and concluded that cell death (apoptosis) was not important in shaping teleost retinas; cell specification was sufficiently accurate to not require subsequent adjustment through pruning. Our findings in control medaka of this study support this view, sections through the eye revealed: two examples of necrotic cells at junction of outer nuclear and outer plexiform layers of a stage 30 embryo (Fig. 3A), a single focus of necrotic cells in inner nuclear- (Fig. 4A) and a single apoptotic cell in the ganglion- layer (Fig. 6A) of stage 33 embryos. By contrast, morphometric quantitation of foci of necrosis in medaka exposed to diazinon showed increased area and numerical density of these lesions (Table 1).

A temporal linkage between appearance of AChE, its inhibition by OP, and increased area and number of retinal necrotic lesions suggests they are related. Although BChE has been studied as a regulator of neuronal development (Layer and Willbold, 1994), preliminary experiments in this laboratory (data not shown) revealed complete inhibition of BChE at diazinon concentrations (8.82 X 10^{-7} M) well below those associated with retinal cell necrosis. Future investigations will examine BChE localization, its inhibition by OPs, and its possible role in the ultrastructural alterations of the developing retina. Like many other OP pesticides, diazinon must be converted to diazoxon before it becomes a potent cholinesterase inhibitor (Ecobichon, 1994). The extent of its conversion to its oxon is unknown and the possibility of a direct effect of the parent compound cannot be ruled out. Other studies (in preparation) show greater AChE inhibition with added time of development suggesting that the capacity for diazoxon formation also increases along a developmental gradient. While it is possible that

TABLE 3. AchE Inhibition in Whole Embryos.

	% Inhibition		
	Exposed Days 1 - 5	Exposed Days 1- 7	Exposed Days 5 - 7
Control	0 ± 2.3	0 ± 6.2	0 ± 4.0
	(10.7)	(40.6)	(41.3)
1.76X10^{-5}M	43.42 ± 5.7*	41.88 ± 4.4*	----
4.41X10^{-5}M	43.62 ± 3.4*	64.12 ± 0.7*+	48.2 ± 3.0*‡
8.82X10^{-5}M	39.18 ± 5.4*	76.12 ± 1.6*+	69.35 ± 1.9*+‡

Values are means ± SEM for 5 homogenates of 5 animals each
Control values indicated in parentheses below percentages; nmoles/min/mg
---- = not run in this exposure regime
*Significantly different from control ($p<0.05$)
+Significantly different from 1.76X10^{-5}M ($p<0.05$)
‡Significantly different from embryos exposed days 1-7 ($p<0.05$)

increased production of diazoxon leads to retinal toxicity, exposure of embryos throughout development to the direct-acting anticholinesterase, DFP, produced retinal lesions between days 5 and 7. When embryos were exposed to 8.82 X 10^{-5} M diazinon, between days 5 and 7, less necrosis resulted than when duration of exposure included the interval days 1-7. This result suggests that factors in addition to AChE inhibition are involved in retinal necrosis. Recently, chlorpyrifos has been shown to impair neural development apparently through a non-cholinergic mechanism involving alteration of cell signaling pathways (Whitney *et al.*, 1995; Song *et al.*, 1997). While this is a

FIG. 5A. Section through freeze-dried control medaka embryo on day 5 at stage 30 of development. Stained for AChE using the methods of Karnovsky and Roots. Note staining in muscle (bracket, lower right) and neurons of spinal cord (arrow). In contrast, no staining is present in the eye. bar= 41.7µm.

TABLE 4. AchE Inhibition in Retinal Homogenates.

Exposed Day 1- Day 7	% Inhibition
Control	0 ± 7.4
$1.76X10^{-5}M$	43.8 ± 2.9*
$4.41X10^{-5}M$	52.9 ± 3.6*
$8.82X10^{-5}M$	75.2 ± 6.4*+

Values are means ± SEM for 3 pools of 10 embryos
* Significantly different from control ($p < 0.05$)
+ Significantly different from $1.76X10^{-5}M$ ($p < 0.05$)

potential mechanism for diazinon toxicity, it remains to be seen whether diazinon or other OPs exert a similar effect. Indirect evidence suggests that medaka embryos may be able to produce the oxon. First, in reports in preparation, addition of piperonyl butoxide (PBO) an inhibitor of cytochrome P450, to the embryo rearing medium protected against AChE inhibition in medaka retinal preparations. We are currently investigating whether PBO addition protects against formation of retinal necrosis. Secondly, Stegeman and Hahn (1994) have shown that endothelium is a consistent extrahepatic site for cytochrome P450. Smolowitz *et al.* (1991; 1992) localized immunoreactive cytochrome P450 protein to neural endothelium. In fishes the retina lacks blood vessels (Fernald, 1993) and nutrients must pass by diffusion from vessels of the choroid rete. This same path could serve as the route of exposure of the developing retina to potentially toxic, endothelial-derived metabolites (Stegeman and Hahn, 1994). Finally, as a positive control, we used DFP, an accepted activated molecule in systems where OP-induced, delayed neuropathy is not expected. This compound caused 75.7 ± 3.3% inhibition of AChE (N=3, mean ± one standard error) and reproduced the necrotic foci in retinal layers previously shown to give rise to foci after exposure to diazinon. Although DFP is a protease inhibitor, this action of the compound is generally seen at much higher concentrations than those of this study. However, since DFP is a BChE inhibitor as well, both enzymes are implicated. This and the question of whether diazinon is a BChE inhibitor in the medaka embryo are currently under investigation.

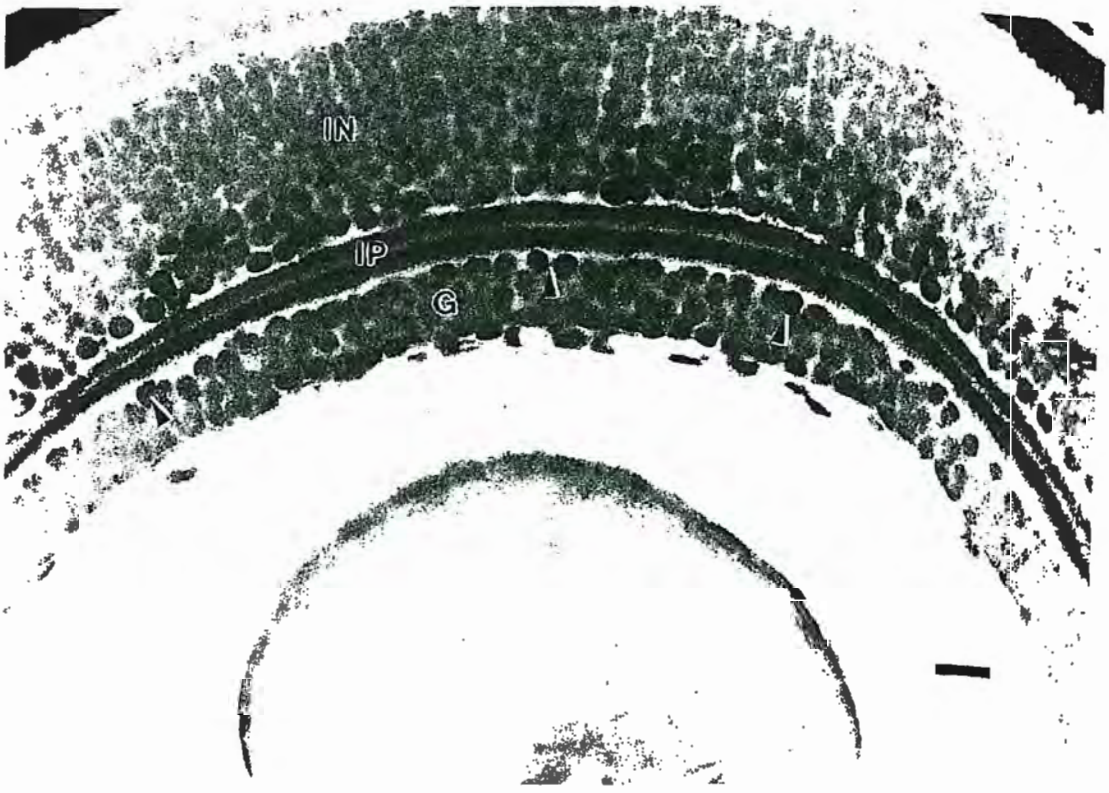

FIG. 5B. Section through freeze-dried retina from control medaka sampled on day 7 at stage 33 of development. AchE was localized using the method of Karnovsky and Roots. Note staining of cells in ganglion layer (G, arrows), two distinct bands within cell processes of inner plexiform layer(IP), and cells in inner nuclear layer (IN). bar= 13.3µm.

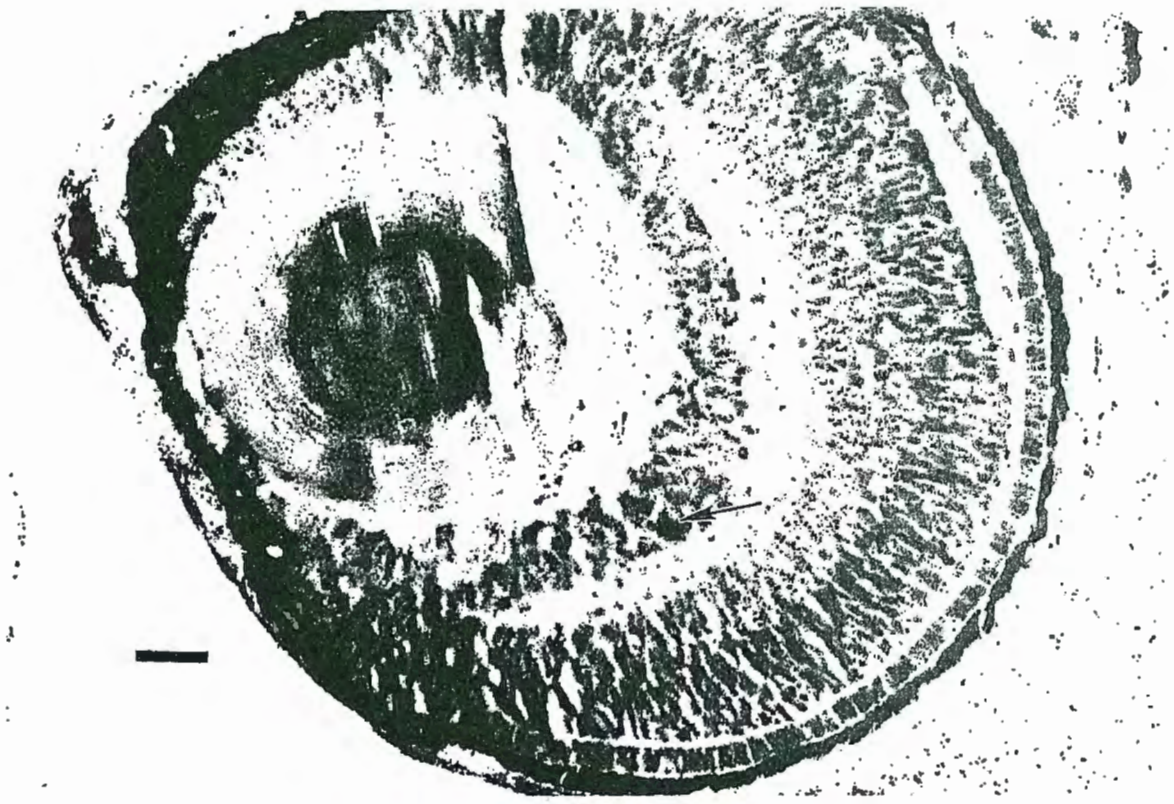

FIG. 6A. Section through paraffin-processed retina of control medaka embryo on day 7 stained for histochemical demonstration of apoptotic cells. Note single positive cell in ganglion layer (arrow). bar= 18.9µm.

Medaka embryos exposed to diazinon showed evidence of delayed development and differentiation of the retina (compare Figs. 3A and 3B) and we found supporting evidence for this response in other model systems. Layer and coworkers (Willbold and Layer, 1997; Robitzki and Layer, 1997; Robitzki *et al.*, 1997) used reaggregated chicken retinospheroids as model developmental systems and showed that overexpression of AChE protein was associated with an advanced degree of tissue differentiation. Further, inhibition of BChE gene expression induced apoptosis in a population of photoreceptor precursor cells. Neurite differentiation was enhanced or depressed in neuroblastoma cells which had previously been engineered for altered levels of AChE expression (Koenigsberger *et al.*, 1997).

Spatial linkage of AChE, foci of necrosis in hematoxylin and eosin-stained sections, and localization of cells showing apoptosis provides further evidence that inhibition of AChE in this model may be causally related to increased retinal cell death. Amacrine cells of the retina of a teleost, the roach (*Rutilus rutilus*), were localized using Golgi preparations (Wagner and Wagner, 1988) and shown to make contact with each type of retinal cell except photoreceptors (Dowling, 1979). Lasater (1990) investigated neurotransmitter types in teleost amacrine cells and found GABA, glycine, and acetylcholine in these cells. Our histochemical localization revealed reaction product for AChE in inner nuclear, inner plexiform, and ganglion layers. Additional work in the cat (Robinson, 1988), rat (Horsburgh and Sefton, 1987), and wallaby (Harman *et al.*, 1989) demonstrated the inner nuclear layer undergoes two periods of cell death. Furthermore, comparison of the timing and site of cell death with the timing of synaptogenesis, indicated that cells with pyknotic nuclei were amacrine cells of the inner nuclear layer and displaced amacrine cells in the ganglion cell layer that failed to establish synaptic connections (Horsburgh and Sefton, 1987; Robinson, 1988; Harman *et al.*, 1989). Therefore, if AChE plays a role in synaptogenesis, its inhibition by diazinon could interfere with formation of synaptic connections, uncovering a sensitive period of development. In support of this assertion, Elsas *et al.*, (1995) found that Ach caused axonal retraction in a dose-dependent fashion in the leech embryo. In addition, these investigators demonstrated a similar effect on nerve growth by physostigmine, a

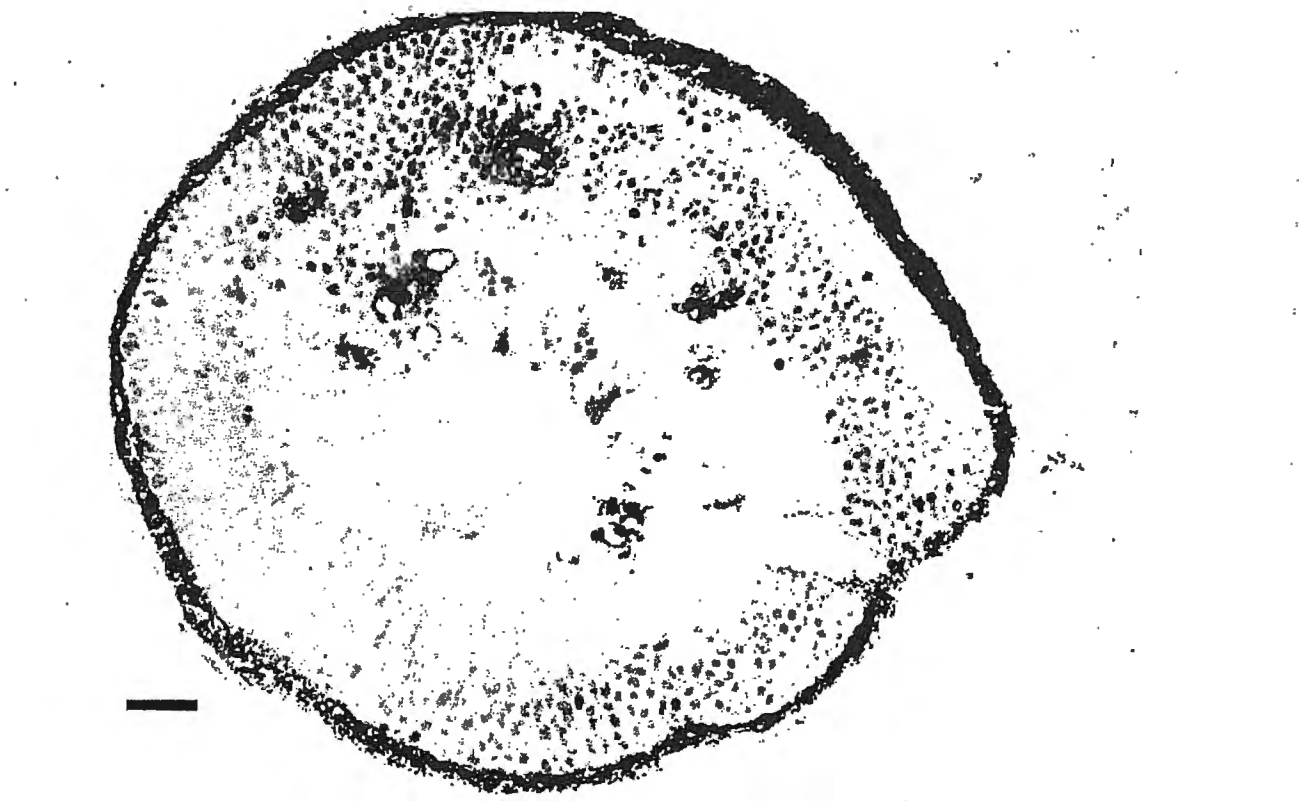

FIG. 6B. Section through paraffin-processed retina of medaka embryo on day 7 at stage 33 of development following exposure (from day 1 to day 7) to 8.82×10^{-5} M diazinon. Apoptotic cells are increased versus control (Fig. 6A) and are found in ganglion and inner nuclear layers. bar= 18.9µm.

cholinesterase inhibitor. Pugh and Berg (1994) demonstrated that 50 µM Ach caused axonal retraction *in vitro*. No such studies have been done in medaka. Investigations of this nature will be necessary to better characterize the mechanism by which diazinon and/or its active metabolite increases retinal cell necrosis.

In contrast to our study, most studies of OP effects on the developing nervous system have focused on the brain. Spyker and Avery (1977) reported that perinatal exposure of mice to diazinon caused aberrant neural cell growth and differentiation in forebrain but no cell death. However, two studies reported OP induced cell death (Berge *et al.*, 1986; Veronesi and Pope, 1990). Berge *et al.* (1986) demonstrated that *in utero* exposure of guinea pig pups to trichlorfon resulted in selective loss of Purkinje cells in the cerebellar cortex and outer granular layer thinning. In addition, Veronesi and Pope (1990) found necrotic changes within the hippocampus of rats exposed to parathion during early postnatal development in association with a high level (73%) of AChE inhibition. In addition to finding cell death, the level of AChE inhibition was similar to that required to cause a statistically significant degree of cell death in the present study. Unfortunately, only one dosage level was used preventing detection by these workers of a relationship between degree of AChE inhibition and extent of cell death.

In addition to studies on the brain, there is evidence in the literature of ocular effects on the human population of Saku, Japan (see review by Dementi, 1994). Following epidemiological findings, animal studies have and are being conducted in order to examine the role of OPs in ocular toxicity. Imai *et al.* (1983) chronically exposed rats to fenthion and reported development of neural degenerative changes including neural cell necrosis within retinal inner nuclear layer. This chronic administration of a different OP to a juvenile mammal produced retinal cell death within the same cell layer as that of the present study. In addition, testing by the US EPA and pesticide registrants of OPs suggests a link between OP exposure and ocular toxicity (Boyes *et al.*, 1994). Tandon *et al.* (1994) reported that fenthion caused a long- lasting decrease in inositol phosphate (IP) release; a measure of muscarinic cholinergic receptor (mChR) signal transduction. In contrast, despite down regulation of mChR and AChE inhibition, the cortex did not show changes in IP release

suggesting a specific effect on the retina. As a result, the United States Environmental Protection Agency (USEPA) requires testing of OPs for ocular toxicity (Hamernik, 1994).

Eye malformations have been reported in fish embryos following exposure to the OP, malathion (Weis and Weis, 1976). Dose-dependent gross malformations, microphthalmia and anopthalmia, occurred. Unfortunately histological examination was not conducted and therefore, it is unclear whether failure of the eye to form resulted from increased cell death and necrosis of optical tissue. Despite a clear dose-response relationship in the occurrence of gross abnormalities, Weis and Weis (1976) raised the possibility that an underlying weakness might be exacerbated by toxicant exposure since one control animal had an eye defect similar to those seen after toxicant exposure. The concept of an underlying weakness being manifested after toxicant exposure is a common finding in the study of teratogenic agents (Pratt and Taylor, 1990). Eye malformations have also been reported in embryo fishes after exposure to other toxicants including methyl mercury (Dial, 1978), oil dispersants (Wilson, 1976), colchicine (Waterman, 1940), and toluene (Devlin *et al.*, 1985). In perhaps the only study of those cited above, Dial (1978) used histological examination and reported pyknotic nuclei within retina of medaka embryos exposed to 80 ppb methyl mercury. While Dial (1978) did not describe the specific retinal cell layer which underwent alteration, retinal lesions first appeared between days 5 and 7 of development.

Examination of control medaka embryos demonstrates that the neural retina undergoes a period of remodeling and differentiation during which exposure to diazinon produces an increase in the number of pyknotic nuclei. Others have demonstrated that this period of embryonic development is characterized by intensive cell proliferation, tissue differentiation and remodeling in the teleost retina (Hagedorn and Fernald, 1992; Negishi *et al.*, 1990; Negishi and Wagner, 1995). Such a dynamic system might be inherently sensitive to toxicant exposure and this could serve as a basis for explanation of the temporal aspects of this developmental sensitivity. However, the enzyme must be present to be inhibited and the sensitivity may be more related to AChE ontogeny within the retina.

Alternatively, excessive levels of ACh following AChE inhibition could cause an excitotoxic effect. Activation of muscarinic or nicotinic receptors increases intracellular Ca^{2+} (Katzung, 1992; Castro and Albuquerque, 1995; Delbono *et al.*, 1997), and, in the retina, muscarinic and nicotinic agonists have been shown to cause calcium influx in ganglion cells (Baldridge, 1996). Excessive cholinergic stimulation in muscle, following AChE inhibition, has been shown to cause necrosis (Dettbarn, 1984; Gebbers *et al.*, 1986; Wecker *et al.*, 1986). Necrosis of muscle fibers appears to be associated with calcium accumulation at motor end plates (Inns *et al.*, 1990; Karalliede and Henry, 1993) and may involve calcium-dependent activation of proteases (Vanneste and Lison, 1993).

Finally, it is possible that culture conditions or greater generalized toxicity resulting from longer exposure may have interacted with AChE inhibition to result in more extensive cell death. The presence of edematous lesions in embryos exposed from day 1 to day 7 raised the question of whether circulatory abnormalities might be associated with adverse effects on retina. Veterinary surveillance of our broodstock cultures has shown a low incidence (1-2%) of embryos with edema. We examined these animals for the presence of retinal cell death. However, histological analysis of unexposed embryos with edema has failed to demonstrate an effect on retina. In addition, companion studies with thiobencarb, a carbamate and a reversible anticholinesterase agent, caused similar circulatory effects but did not elicit neural retina changes despite analysis of 200-300 individuals (Villalobos *et al.*, 1998). Interestingly, the concentrations of thiobencarb studied resulted in lower levels of AChE inhibition (Villalobos *et al.*, 1998). Finally, acute exposure to diazinon, between days 5 and 7, did result in increased retinal cell necrosis providing *in vivo* evidence for the embryo toxicity reported herein.

In addition to laboratory studies, field studies of anthropogenic impacts in the California Salton Sea, showed fish embryos with a high incidence of abnormalities (Matsui *et al.*, 1992). This inland sea receives agricultural drainage from the New and Alamo Rivers and recent, unpublished data from our laboratory has demonstrated that these waters contain chlorpyrifos. Among the malformations seen in feral embryos were retarded development of eye and degenerative changes including pyknosis of individual cells and necrotic foci, unfortunately localization to specific retinal layers was not reported (Matsui *et al.*, 1992).

The concentrations used in this work exceed those found in the environment. Our use of intact, chorionated embryos necessitated use of higher concentrations to overcome the protective nature of the chorion or egg shell membrane. Current investigations employ exposure of breeding pairs just prior to ovipositing of eggs. Under these conditions, compounds suspended in culture water of salinity identical to field conditions will be taken up immediately following fertilization, at the time of water hardening of the egg, and thereby be available for uptake

by the developing embryo. At this time, however, our studies are not intended for environmental relevance, but are designed to shed mechanistic information on toxic responses within developing vertebrate retina.

While the establishment of a mechanism for cell death is a difficult task, the correlation of retinal cell necrosis with the degree of AChE inhibition and the timing and location of necrotic cells in conjunction with the appearance of AChE staining suggest a linkage. Findings of the present study in combination with results of others demonstrating retinal damage in fish embryos from agricultural drains, the experiences in the Saku region of Japan, and observations of death of cells in inner nuclear layer of the retina in animal studies suggest the need for further research on the potential for OP- induced ocular toxicity particularly in the developing organism.

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