

## Inhibition of acetylcholinesterase and aliesterases of fingerling channel catfish by chlorpyrifos, parathion, and *S,S,S*-tributyl phosphorotrithioate (DEF)

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### Abstract

Serine esterases can be inhibited by organophosphorus compounds. The *in vitro* potency of the organophosphorus pesticide DEF and oxons of the phosphorothionate insecticides chlorpyrifos (chlorpyrifos-oxon, Cpxn) and parathion (paraoxon, Pxn) were determined for brain, gill, liver, muscle, and plasma for acetylcholinesterase (AChE) and gill, liver, and plasma aliesterases (ALiE). AChE activity was inhibited less than 15% in all tissues in the presence of 1 mM DEF<sup>®</sup>. AChE  $I_{50}$  values for Cpxn were 28–33 nM, and for Pxn were 446–578 nM. ALiE  $I_{50}$  values for Cpxn were 0.1–0.2 nM, for DEF were 24–163 nM, and for Pxn were 6–46 nM. Fish were exposed to chlorpyrifos (Cp), parathion (Pth), DEF, and combinations of the phosphorothionates with DEF for 4 h followed by a 384-h recovery period. AChE inhibition following Cp and Pth exposures was rapid. Cp led to more persistent inhibition than Pth. DEF treatments yielded low levels of AChE inhibition in brain and muscle, and high levels of inhibition in gill, liver, and plasma. *In vitro* and *in vivo* results suggest that DEF's disposition and/or mode of action are different than those of Cp or Pth. Exposure to DEF resulted in persistent, high level inhibition of ALiE activity. Greater AChE inhibition in DEF plus Cp or Pth treatments was not evident, suggesting that ALiEs do not serve to appreciably protect AChE in channel catfish, even though ALiEs are inherently more sensitive to inhibition.

**Keywords:** *Ictalurus punctatus*; Organophosphates; Acetylcholinesterase; Aliesterase; Chlorpyrifos; DEF; Parathion

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### 1. Introduction

Culture of channel catfish, *Ictalurus punctatus*, is a rapidly growing industry in the

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Mississippi Delta region. Flat terrain, an abundant and inexpensive water supply, and soil having a clay composition make this an ideal location for such an industry. Agriculture is a well established enterprise in this area and is often located in close proximity to catfish culture ponds.

Insect control on agricultural land is commonly accomplished by aerial application of insecticides throughout the growing season. Wind drift and over-spray of insecticides are very real possibilities for unintentional contamination. Defoliants (herbicides), used at the end of the growing season to facilitate crop harvesting, are also aerially applied.

Two of the organophosphorus (OP) insecticides used presently or in the past in this region are chlorpyrifos (Cp; *O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphorothioate) and parathion (Pth; *O,O*-diethyl *O*-4-nitrophenyl phosphorothioate); the most frequently used OP herbicide is DEF<sup>®</sup> defoliant (*S,S,S*-tributyl phosphorotrithioate).

The phosphorothionate insecticides (Cp and Pth) are poor anticholinesterases and must be activated by cytochromes P450 to their oxon metabolites, chlorpyrifos-oxon (Cpxn; *O,O*-diethyl *O*-3,5,6-trichloro-2-pyridyl phosphate) and paraoxon (Pxn; *O,O*-diethyl *O*-4-nitrophenyl phosphate), to effectively inhibit acetylcholinesterase (AChE), their primary target. Murphy and DuBois (1959) suggested a possible cholinergic action for DEF in rats because of the display of typical signs of OP intoxication, i.e., salivation, lacrimation, urination, and occasional tremors. However, since atropine was an ineffective antidote, actions other than cholinesterase inhibition appeared to be important in poisoning by DEF. Casida et al. (1963) also found that actions other than cholinesterase inhibition were important in the toxicity of this phosphorotrithioate in mice, but did not identify these. Recent reports by Hur et al. (1992) implicated DEF sulfoxide and/or DEF sulfone as the metabolites possessing anticholinesterase activity.

There is a great range in acute toxicity levels of these OP pesticides among vertebrates. This diversity could result from differences in inhibitory potency for the target (presumably AChE) and non-target enzymes (such as aliesterases; ALiEs), disposition, and/or metabolism. ALiEs (also called carboxylesterases) are serine esterases and are assumed to provide protection against OP poisoning in mammals by their stoichiometric phosphorylation with resultant destruction of the oxon (Chambers and Chambers, 1991).

Previous studies have investigated the *in vitro* potencies of Pxn (Johnson and Wallace, 1987; Boone, 1991) and Cpxn (Boone, 1991) as anticholinesterases to teleosts, but there is a paucity of data on either DEF or channel catfish. Effects of Pth (Murphy et al., 1968; Benke et al., 1974; Benke and Murphy, 1974; Boone, 1991) and Cp (Jarvinen et al., 1983, 1988; Van der Wel and Welling, 1989; Boone, 1991) exposures on cholinesterase activity have been studied in several teleosts, but only limited information is available on channel catfish. Habig and DiGiulio (1988) studied the cholinergic effects of DEF on channel catfish; however, only effects on brain and muscle AChE were quantified. Chemical interaction has been previously studied in fish; Fabacher et al. (1976) observed the apparent potentiation of mortality of DEF plus methyl parathion in mosquitofish, but there is little information available on biochemical effects.

Thus, previous research has not sufficiently determined the effects on enzyme activity of the above compounds *in vitro* or *in vivo* on channel catfish or the possibility of interaction between the insecticides and DEF. In the present study, the *in vitro* inhibitory potencies of Cpxn, Pxn, and DEF on AChE and ALiE activity in channel catfish tissues were determined. Also, a study of esterase activity following exposure to the parent pesticides was conducted to determine the rate of enzyme inhibition and the speed of recovery. Rapid inhibition of target enzyme activity indicates less time is available for the fish to adapt to such an exposure. Recovery rates of these enzymes are important to assess susceptibility in future exposures to OP toxicant(s).

## 2. Materials and methods

### *Chemicals*

Phosphorothionates, oxons, and nitrophenyl valerate were provided by Dr. Howard Chambers (Department of Entomology, Mississippi State University). DEF defoliant was a gift from Dr. Robert L. Graney [Bayer (formerly Miles) Agriculture Division, Stilwell, KS]. All biochemicals and reagents were from Sigma Chemical Company (St. Louis, MO).

### *Animals and treatments*

Channel catfish fingerlings, average wet weight 31 g (*in vitro* study) and 35 g (*in vivo* study), of mixed sex were obtained from the College of Veterinary Medicine, Mississippi State University, MS. Fish were fed a commercial catfish feed at the rate of 3% of body weight/day. Fish were not fed 24 h prior to the *in vivo* experiment to prevent organic matter from affecting exposure concentration. Fish were held at  $30 \pm 1^\circ\text{C}$  and 12L/12D light cycle to minimize reported seasonal variation in cytochrome P450 monooxygenases, liver size, and other enzymes (Chambers and Yarbrough, 1976). Fish for both studies were acclimated for 2 weeks in a flow-through environment in 76-l glass aquaria containing 60 l of aerated, dechlorinated water (pH = 7.5 to 7.7, total alkalinity = 60 to 90 mg/l as  $\text{CaCO}_3$ , chloride = 20 to 30 mg/l); flow rate was 60 l/h.

The initial stocking rate for the *in vivo* study was 20 fish/aquarium. There were three aquaria per treatment, with each aquarium constituting a replication. Fish were exposed to the following pesticides in acetone as a vehicle for 4 h in a static environment: 0.25 mg/l Cp, 0.125 mg/l Cp, 5.0 mg/l DEF, 2.5 mg/l Pth, 1.25 mg/l Pth, 0.125 mg/l Cp + 2.0 mg/l DEF, 1.25 mg/l Pth + 2.0 mg/l DEF. Concentrations of Cp and Pth were designed to be sublethal but of sufficient magnitude to yield a relatively high degree of AChE inhibition as well as overt signs of intoxication without significant mortality. The concentration of DEF was selected to yield signs of poisoning without significant mortality. The system was then altered to allow for flow-through conditions. Fish were sacrificed and tissues sampled at 0, 4, 8, 12, 24, 48, 96, 144, 192, 240, 288, 336, or 384 h after the 4-h exposure period. A vehicle control was run for each replication.

### Tissue samples

Blood samples were taken without the use of anesthesia via caudal vein puncture. Fish were quickly decapitated and brain, gill, liver, and muscle were removed. Tissues were rinsed with 0.9% NaCl and quickly chilled. Blood was centrifuged at  $17\,500 \times g$  for 4 min to separate plasma. Tissue samples were stored at  $-70 \pm 2^\circ\text{C}$  until enzyme analysis.

### Enzyme assays

Activity of AChE was quantified using a modification of Ellman et al. (1961) using acetylthiocholine as the substrate and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) as the chromogen as previously described (Chambers and Chambers, 1989). Brain, gill, liver, and muscle were homogenized to give final assay concentrations of 0.2, 2.5, 1.8, and 1.0 mg/ml, respectively. Muscle samples (a source of peripheral AChE) were filtered through glass wool following homogenization. Plasma was diluted to give a final assay concentration of  $12.5 \mu\text{l/ml}$ .

ALiE activities were assayed spectrophotometrically in gill, liver, and plasma using 4-nitrophenyl valerate as the substrate as previously described (Carr and Chambers, 1991). Preliminary assays indicated that brain and muscle lacked measurable ALiE activity. Gill and liver were homogenized to give final concentrations of 2.5 and 0.5 mg/ml, respectively. Plasma was diluted to give a final assay concentration of  $2.8 \mu\text{l/ml}$ .

Analysis was carried out for the in vitro study on three fish (replicates). Data for each replication was derived from three subsamples. The  $I_{50}$  values were determined for AChE and ALiE by adding a series of at least five concentrations of the oxons or DEF to the enzyme sources 15 min prior to addition of the substrate; the concentra-

Table 1

Acetylcholinesterase (AChE) and alioesterase (ALiE)  $I_{50}$  values for chlorpyrifos-oxon (Cpxn), DEF, and paraoxon (Pxn) in tissues of fingerling channel catfish

Enzyme	Tissue	$I_{50}$ (nM)			Specific activity
		Cpxn	DEF	Pxn	
AChE	Brain	$28.4^C \pm 2.1$	— <sup>a</sup>	$446.1^B \pm 34.4$	$487.7 \pm 1.3$
	Gill	$33.1^C \pm 0.7$	—	$577.9^A \pm 15.6$	$58.2 \pm 4.2$
	Liver	$32.7^C \pm 2.5$	—	$498.4^{AB} \pm 20.0$	$45.4 \pm 5.2$
	Muscle	$31.6^C \pm 2.6$	—	$528.9^{AB} \pm 23.0$	$129.2 \pm 15.3$
	Plasma	$28.7^C \pm 1.5$	—	$481.0^B \pm 15.1$	$29.5 \pm 3.0$
ALiE	Gill	$0.2^{EF} \pm 0.0$	$163.4^A \pm 31.9$	$46.0^B \pm 10.4$	$30.6 \pm 1.3$
	Liver	$0.1^F \pm 0.0$	$34.3^B \pm 3.1$	$13.7^C \pm 0.5$	$44.4 \pm 3.7$
	Plasma	$0.2^E \pm 0.0$	$24.2^B \pm 1.6$	$5.7^D \pm 0.4$	$71.5 \pm 2.5$

$I_{50}$  values were calculated by log-probit analysis; means within either enzyme not followed by the same capital letter are significantly different ( $P < 0.05$ ) by the Student-Neuman-Keuls test. Data are expressed as mean  $\pm$  s.e.m., three replications.

Specific activity expressed as  $\text{nmol min}^{-1} \text{mg protein}^{-1}$ . Data are expressed as mean  $\pm$  s.e.m., three replications.

<sup>a</sup>DEF (1 mM) inhibited AChE < 15% in all tissues.

tions selected yielded between 10 and 90% inhibition. A vehicle control was run with each enzyme source. The amount of enzyme activity was assayed and the percent inhibition was calculated compared to the control.

### *Protein*

For all tissues, protein was quantified using the method of Lowry et al. (1951). Bovine serum albumin was used as the standard.

### *Statistics*

$I_{50}$  values were calculated using log-probit analysis (SAS Institute, Cary, NC) with three replications/data point;  $I_{50}$  value means were analyzed by analysis of variance followed by the Student-Neuman-Keuls means comparison test using SAS on a personal computer. Specific activities from the *in vivo* study were analyzed by the General Linear Model procedure followed by the means comparison test. A level of  $P < 0.05$  was used to conclude a significant difference among means.

## **3. Results**

### *In vitro study*

DEF was a weak inhibitor of AChE in all tissues, with less than 15% AChE inhibition observed at 1 mM (Table 1). AChE of all tissues exhibited similar sensitivities to Cpxn which yielded  $I_{50}$  values of about 30 nM, and to Pxn which yielded  $I_{50}$  values of about 500 nM. Specific activities of AChE in brain and muscle were appreciably higher than those in gill, liver, and plasma.

ALiEs were more sensitive to Cpxn than to Pxn by 287-, 105-, and 25-fold in gill, liver, and plasma, respectively. The  $I_{50}$  values for Cpxn were all within the same order of magnitude. Pxn was about 3- to 4-fold more potent as an ALiE inhibitor than DEF in all three tissues. With Pxn and DEF, plasma and liver ALiEs were more sensitive than gill ALiEs. Specific activities of ALiEs in the three tissues analyzed were within the same order of magnitude. ALiE activity in brain and muscle was negligible.

### *In vivo study*

Fish demonstrated signs of intoxication (i.e., torpor and lack of schooling behavior) in the DEF and Pth treatments by 1 h after exposure; piping at the surface was observed by 3 h. Cp-only treated fish displayed minor signs of stress during the static exposure period, but occasional mortalities occurred after the system was modified to flow-through conditions; infrequent mortality was also observed in Pth treatments. No mortality occurred in the DEF-only treatments.

Mean AChE specific activity, standard error, and statistical mean comparison for the first and the last time points only, 0 and 384 h after the 4-h exposure, are reported in Table 2 for the tissues studied; statistical outcomes for the other sampling times can be found in Straus (1994). Fig. 1 (A–E) displays percent inhibition of AChE activity compared to controls for all the sampling periods in this experiment. Mean control specific activities for AChE in this study were similar to those of the *in vitro* study.

Table 2  
Mean specific activity of channel catfish fingerling acetylcholinesterase 0 and 384 h following a 4-h static exposure to the test compound(s). Chlorpyrifos = Cp, parathion = Pth

Treatment	Brain		Gill		Liver		Muscle		Plasma	
	0 h	384 h	0 h	384 h	0 h	384 h	0 h	384 h	0 h	384 h
Control	454.0 <sup>1Aa</sup> ± 41.1	404.6 <sup>Aa</sup> ± 51.0	34.5 <sup>Aa</sup> ± 4.3	30.8 <sup>Aa</sup> ± 4.1	37.1 <sup>Aa</sup> ± 5.3	40.0 <sup>Aa</sup> ± 2.7	133.3 <sup>Aa</sup> ± 23.9	97.6 <sup>Aa</sup> ± 28.9	20.2 <sup>Aa</sup> ± 2.3	25.1 <sup>Aa</sup> ± 3.5
5.0 mg/l DEF	454.3 <sup>Aa</sup> ± 31.0	328.2 <sup>Bb</sup> ± 36.8	23.7 <sup>Ba</sup> ± 4.8	25.5 <sup>Ba</sup> ± 2.0	1.6 <sup>Ba</sup> ± 0.6	24.9 <sup>Bb</sup> ± 7.9	121.3 <sup>Aa</sup> ± 3.7	53.1 <sup>Bb</sup> ± 7.6	14.1 <sup>Ba</sup> ± 1.2	26.8 <sup>Aa</sup> ± 0.2
0.25 mg/l Cp	24.2 <sup>Ba</sup> ± 10.3	196.3 <sup>Bb</sup> ± 20.4	1.5 <sup>Ca</sup> ± 0.4	14.0 <sup>Bb</sup>						
	± 25.8	257.0 <sup>BCD</sup> ± 52.8	4.9 <sup>Ca</sup> ± 1.1	23.4 <sup>ABb</sup> ± 1.0	3.1 <sup>Ba</sup> ± 0.3	18.2 <sup>Bb</sup> ± 3.4	33.5 <sup>Ba</sup> ± 7.3	26.6 <sup>Ba</sup> ± 6.8	6.0 <sup>Ca</sup> ± 1.4	11.7 <sup>Cb</sup> ± 1.3
0.125 mg/l Cp										
+ 2.0 mg/l DEF	105.6 <sup>Ba</sup> ± 46.1	212.4 <sup>CD</sup> ± 10.9	8.3 <sup>Ca</sup> ± 2.5	18.4 <sup>Ba</sup> ± 4.4	1.8 <sup>Ba</sup> ± 0.2	18.0 <sup>Bb</sup> ± 3.5	51.1 <sup>Ba</sup> ± 12.6	26.1 <sup>Bb</sup> ± 7.8	7.0 <sup>Ca</sup> ± 1.5	10.0 <sup>Cb</sup> ± 1.0
2.5 mg/l Pth	27.2 <sup>Ba</sup> ± 25.7	303.6 <sup>BC</sup> ± 18.3	2.6 <sup>Ca</sup> ± 1.3	23.8 <sup>ABb</sup> ± 5.1	0.2 <sup>Ba</sup> ± 0.1	23.3 <sup>Bb</sup> ± 6.4	4.8 <sup>Ba</sup> ± 3.0	43.7 <sup>Bb</sup> ± 6.7	0.7 <sup>Ba</sup> ± 0.7	8.9 <sup>Cb</sup> ± 1.0
1.25 mg/l Pth	40.3 <sup>Ba</sup> ± 12.3	294.2 <sup>BC</sup> ± 33.7	1.3 <sup>Ca</sup> ± 0.8	23.6 <sup>ABb</sup> ± 2.4	0.9 <sup>Ba</sup> ± 0.3	26.8 <sup>Bb</sup> ± 3.7	4.6 <sup>Ba</sup> ± 3.0	32.6 <sup>Bb</sup> ± 8.7	0.8 <sup>Ba</sup> ± 0.2	8.8 <sup>Cb</sup> ± 0.8
1.25 mg/l Pth										
+ 2.0 mg/l DEF	76.5 <sup>Ba</sup> ± 29.6	301.9 <sup>BC</sup> ± 23.4	0.7 <sup>Ca</sup> ± 0.4	22.4 <sup>ABb</sup> ± 3.5	0.1 <sup>Ba</sup> ± 0.1	17.3 <sup>Bb</sup> ± 7.3	6.2 <sup>Ba</sup> ± 2.6	42.0 <sup>Bb</sup> ± 5.1	0.6 <sup>Ba</sup> ± 0.3	18.3 <sup>Bb</sup> ± 2.3

Specific activity expressed as nmol min<sup>-1</sup> mg protein<sup>-1</sup>. Means within a tissue/time (i.e., within vertical columns) not followed by the same capital letter are significantly different ( $P \leq 0.05$ ) by the Student-Neuman-Keuls test; different lower case letters indicate a significant difference between the 0 and 384 h means within a treatment/tissue ( $P \leq 0.05$ ). Data are expressed as mean ± s.e.m., three replications.

Specific activities of AChE at the earliest sampling points after the 4-h exposure to CP or Pth were greatly depressed in each tissue. By 384 h, specific activities were recovering, but still were significantly different from controls.

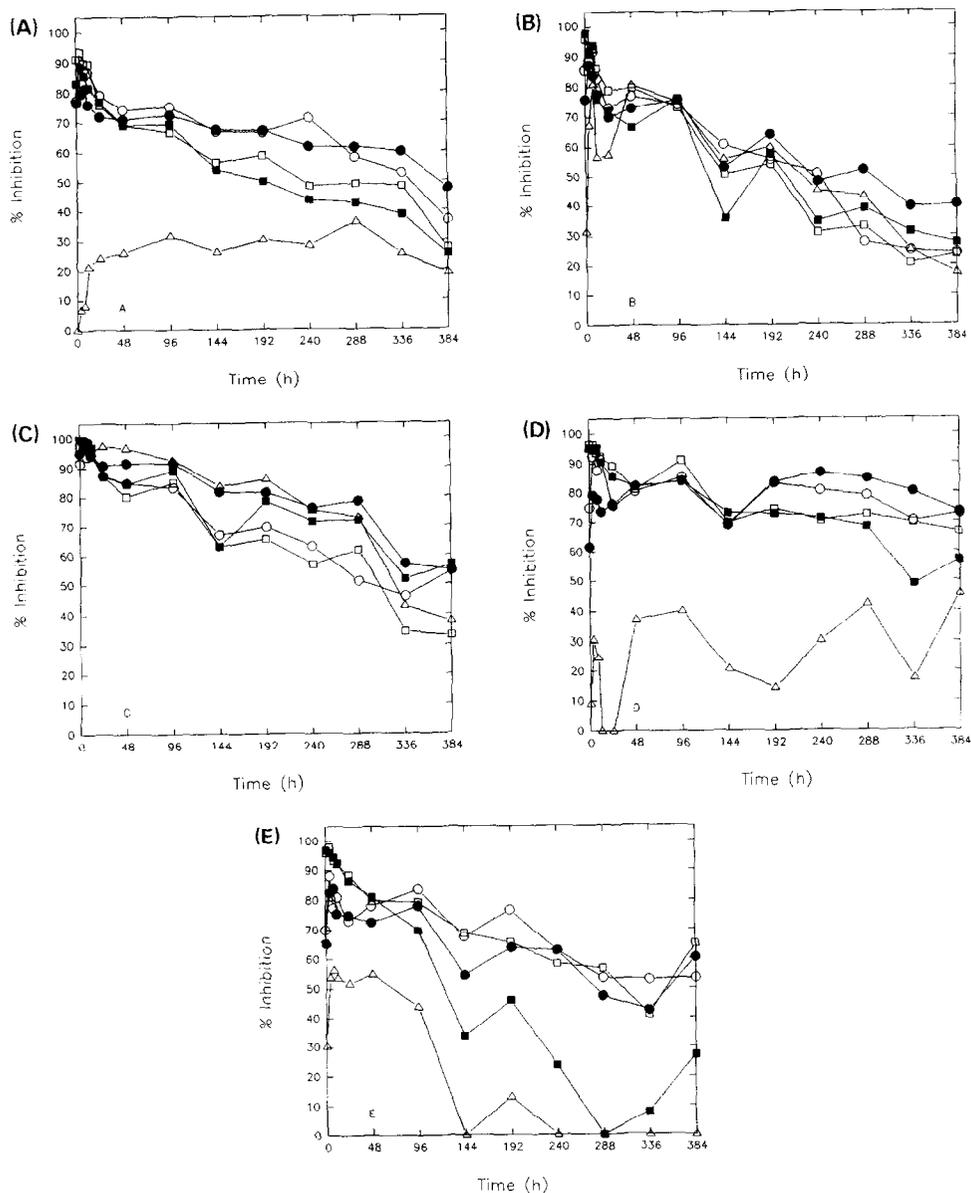


Fig. 1. Brain (A), gill (B), liver (C), muscle (D), and plasma (E) acetylcholinesterase inhibition in fingerling channel catfish after a 4-h static exposure to 0.125 mg/l chlorpyrifos (Cp) (open circle), 0.125 mg/l Cp + 2.0 mg/l DEF (closed circle), 5.0 mg/l DEF (open triangle), 1.25 mg/l parathion (Pth) (open square), and 1.25 mg/l Pth + 2.0 mg/l DEF (closed square); flow-through conditions were initiated at 0 h. There were usually three replications per data point.

DEF was a poor inhibitor of brain AChE at the earliest sampling times (Fig. 1A); specific activity was not significantly different from the control, but 20–35% inhibition occurred from 12 h on with little recovery observed. Brain AChE from all other treatment groups was inhibited greater than 81% by 8 h, and recovered significantly during the course of the experiment. Gill AChE also was inhibited greater than 81% (Fig. 1B) by 8 h in all treatments including the DEF-only treatment, and activities recovered significantly. Liver showed the highest percentage of AChE inhibition (Fig. 1C), greater than 96% by 8 h in all treatments; specific activity recovered significantly by 384 h.

The effect of DEF on AChE in muscle (Fig. 1D) was similar to its effect in brain in that maximal inhibition was delayed and there was no apparent recovery. The response of this parameter was less consistent than most. Muscle AChE from all other treatments showed in excess of 60% inhibition early in the experiment. Enzyme activities recovered slightly but significantly within all Pth treatments by 384 h.

Fish from all treatments displayed maximum plasma AChE inhibition by 8 h (Fig. 1E); this early inhibition was greater than 84% in treatments other than DEF. Fish from Cp treatments demonstrated similar specific activities; plasma AChE in Cp-only treated fish recovered significantly by 384 h. DEF-treated fish displayed maximum inhibition of AChE by 8 h (56%) and recovered completely by 240 h.

There were few suggestions of possible potentiation by DEF on the AChE inhibi-

Table 3

Least squares mean specific activity of channel catfish fingerling aliesterases 0 and 384 h following a 4-h static exposure to the test compound(s). Chlorpyrifos = Cp, parathion = Pth

Treatment	Gill		Liver		Plasma	
	0 h	384 h	0 h	384 h	0 h	384 h
Control	7.9 <sup>A,a</sup> ± 1.9	10.0 <sup>AB,a</sup> ± 1.8	68.2 <sup>A,a</sup> ± 5.6	100.2 <sup>A,a</sup> ± 22.2 <sup>1</sup>	62.5 <sup>A,a</sup> ± 25.1	96.6 <sup>A,a</sup> ± 34.8
5.0 mg/l DEF	0.2 <sup>B,a</sup> ± 0.2	1.7 <sup>C,a</sup> ± 0.6	2.2 <sup>B,a</sup> ± 0.2	5.0 <sup>B,a</sup> ± 0.0 <sup>2</sup>	0.0 <sup>B,a</sup> ± 0.0	0.2 <sup>B,a</sup> ± 0.2
0.25 mg/l Cp	2.2 <sup>B,a</sup> ± 0.1	4.2 <sup>BC,a</sup> ± 0.9	21.2 <sup>B,a</sup> ± 10.5	22.4 <sup>B,a</sup> ± 2.5 <sup>1</sup>	0.4 <sup>B,a</sup> ± 0.4	4.6 <sup>B,a</sup> ± 1.7
0.125 mg/l Cp	0.7 <sup>B,a</sup> ± 0.4	5.3 <sup>BC,a</sup> ± 1.8	23.7 <sup>B,a</sup> ± 12.5	22.6 <sup>B,a</sup> ± 0.0 <sup>2</sup>	1.7 <sup>B,a</sup> ± 1.7	27.9 <sup>B,a</sup> ± 18.6
0.125 mg/l Cp + 2.0 mg/l DEF	0.7 <sup>B,a</sup> ± 0.7	1.7 <sup>C,a</sup> ± 1.0	4.6 <sup>B,a</sup> ± 1.5	16.9 <sup>B,a</sup> ± 4.6 <sup>1</sup>	1.4 <sup>B,a</sup> ± 1.4	1.1 <sup>B,a</sup> ± 0.6
2.5 mg/l Pth	2.2 <sup>B,a</sup> ± 0.2	9.3 <sup>AB,b</sup> ± 1.6	28.6 <sup>B,a</sup> ± 5.6	51.3 <sup>AB,b</sup> ± 5.4 <sup>1</sup>	0.1 <sup>B,a</sup> ± 0.1	60.4 <sup>AB,b</sup> ± 7.4
1.25 mg/l Pth	2.6 <sup>B,a</sup> ± 0.8	12.2 <sup>A,b</sup> ± 2.4	21.9 <sup>B,a</sup> ± 2.7	50.3 <sup>AB,b</sup> ± 0.0 <sup>2</sup>	2.7 <sup>B,a</sup> ± 1.9	84.5 <sup>A,b</sup> ± 14.6
1.25 mg/l Pth + 2.0 mg/l DEF	1.1 <sup>B,a</sup> ± 1.1	2.2 <sup>C,a</sup> ± 0.3	2.8 <sup>B,a</sup> ± 0.9	10.8 <sup>B,a</sup> ± 3.5 <sup>1</sup>	0.6 <sup>B,a</sup> ± 0.6	0.0 <sup>B,a</sup> ± 0.0

Specific activity expressed as nmol min<sup>-1</sup> mg protein<sup>-1</sup>. Means within a tissue/time (i.e., within vertical columns) not followed by the same capital letter are significantly different ( $P \leq 0.05$ ) by the Student-Neuman-Keuls test; different lower case letters indicate a significant difference between the 0 and 384 h means within a treatment/tissue ( $P \leq 0.05$ ). Means adjusted for varying sample sizes by the General Linear Model procedure. Data are expressed as mean ± s.e.m., three replications unless indicated otherwise. Standard error based on three replications, unless indicated.

<sup>1</sup>  $n = 2$ .

<sup>2</sup>  $n = 1$ .

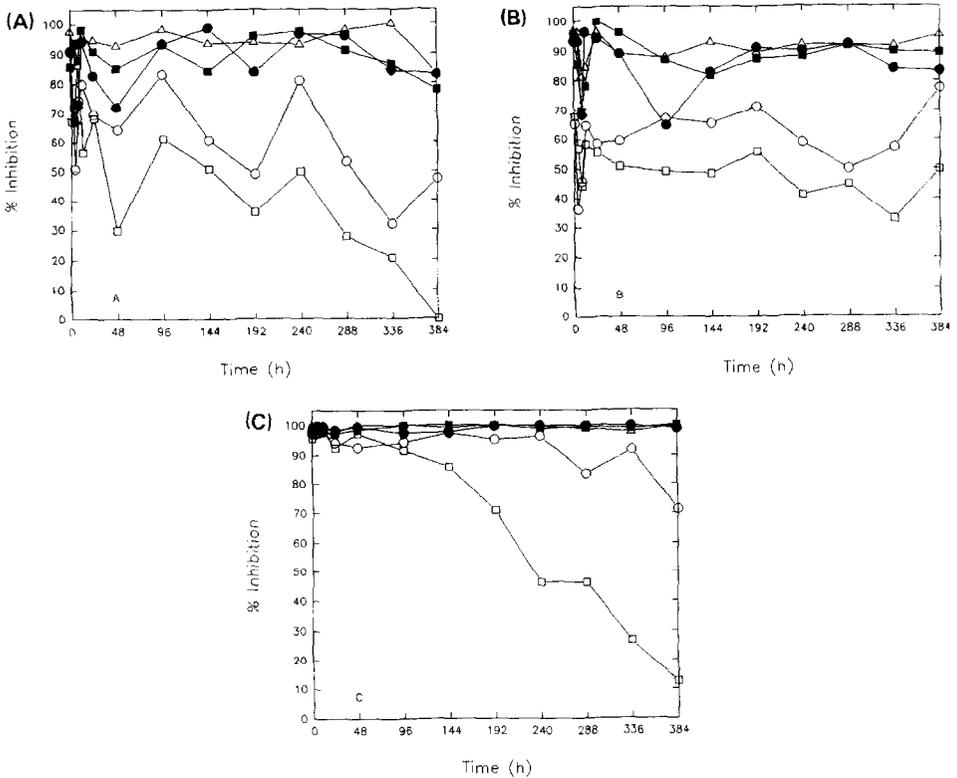


Fig. 2. Gill (A), liver (B), and plasma (C) aliesterase inhibition in fingerling channel catfish after a 4-h static exposure to 0.125 mg/l chlorpyrifos (Cp) (open circle), 0.125 mg/l Cp + 2.0 mg/l DEF (closed circle), 5.0 mg/l DEF (open triangle), 1.25 mg/l parathion (Pth) (open square), and 1.25 mg/l Pth + 2.0 mg/l DEF (closed square); flow-through conditions were initiated at 0 h. There were usually three replications per data point.

tion resulting from Cp or Pth exposures. An antagonistic effect of DEF on Pth-induced inhibition of AChE in plasma was evident, and these differences were significant at 144, 240, 288, and 384 h; there was no obvious explanation for any antagonism.

Mean ALiE specific activity, standard errors, and statistical means comparison are given in Table 3 for gill, liver, and plasma, respectively. Fig. 2 (A–C) displays percent inhibition of control ALiE activity by specific treatments at 0 through 384 h after the exposure period. Mean control specific activities for ALiEs in this study were similar to those of the *in vitro* study. This parameter displayed substantial individual variability, as can be seen by the standard errors (Table 3).

Initial ALiE specific activities of each tissue were significantly different from the controls in all treatments; specific activities were significantly different from those of control fish at 384 h in all treatments except the 2.5 mg/l Pth treatment in gill and the 1.25 mg/l Pth treatment in plasma.

Gill ALiE activities (Fig. 2A) were somewhat erratic and reached maximum inhibition greater than 73% in all treatments by 12 h except for the Cp + DEF and DEF-only treatments where maximum inhibition was reached by 144 and 336 h, respectively. Gill ALiEs were more persistently inhibited by treatment with Cp-only than by Pth-only.

Treatments containing DEF inhibited liver ALiEs (Fig. 2B) more than Cp- or Pth-only treatments; inhibition was persistent and was greater than 93% at 0 h.

Plasma ALiEs (Fig. 2C) were inhibited persistently by DEF; inhibition was greater than 98% at all time points. Cp- and Pth-only treatments yielded over 94% inhibition of plasma ALiE through 48 h. Activities from Cp-only treatments exhibited persistent inhibition; ALiE activities from the 0.125 mg/l treatment recovered slightly, but not significantly. Plasma ALiEs of fish from Pth-only treatments showed significant and substantial recovery.

Fig. 2 (A–C) demonstrates an ‘apparent’ additive effect between Cp and DEF or Pth and DEF in ALiE assays of all tissues. The greatest effect was evident in plasma ALiEs after exposure to Pth and DEF.

#### 4. Discussion

Cpxn was a much more potent anticholinesterase than Pxn in all tissues, which correlates with the acute toxicity levels of the parent insecticides. The *in vitro* data do not explain the relatively high acute toxicity of DEF since it is a poor anticholinesterase at a higher concentration (1 mM) than would be toxicologically relevant. Habig and DiGiulio (1988) also determined that DEF did not affect catfish brain AChE at concentrations of up to 2.5 mM. These results also agree with the findings of Casida et al. (1963) in the rat, and suggest an action other than direct inhibition of AChE. The putative oxidized metabolites of DEF were not available for study.

Results of the present study for AChE inhibition are different from those found by Boone (1991) in mosquitofish (*Gambusia affinis*);  $I_{50}$  values for Cpxn were 49.8 and 20.8 nM, and Pxn were 270.8 and 1.2 nM, in brain and muscle, respectively. While the brain  $I_{50}$  values of both compounds and the muscle  $I_{50}$  value of Cpxn were within the same order of magnitude, the mosquitofish muscle was over 400 times more sensitive to Pxn than channel catfish. The fact that Pth is 8-fold more toxic to mosquitofish than to channel catfish suggests that muscle AChE plays an important role in acute toxicity. The similarity in potencies of Cpxn or of Pxn to AChE in all tissues studied here suggests that channel catfish may have a similar isozyme(s) of AChE in these tissues.

While Cpxn had similar potency for ALiEs in each tissue, DEF and Pxn demonstrated differences in potencies to ALiEs among tissues. In all cases, the compounds were more potent ALiE inhibitors than AChE inhibitors and, therefore, ALiEs would be expected to protect AChE from inhibition by providing alternative phosphorylation sites, as is observed in mammals (Chambers and Chambers, 1990).

The structure of DEF incorporates a double-bonded oxygen, similar to the oxons

of the phosphorothionate. While DEF is not an anticholinesterase, its potency as an ALiE inhibitor suggests that it could interfere with some of the ALiE mediated protection occurring during phosphorothionate exposures. The *in vivo* experiments were designed to test the protective effect of ALiEs and the potential interaction of DEF with the phosphorothionates. Fish from all treatments, except the control, displayed similar signs of stress during the static exposure period. Surprisingly, the treatments containing 0.25 mg/l Cp and 2.5 mg/l Pth, the higher insecticide concentrations, resulted in similar very high inhibition of both enzymes as treatments containing half of their respective phosphorothionates, so a dose–response relationship was not observed. This was true considering data from all the sampling times not shown in tables or figures here (Straus, 1994). This suggests that saturation of the AChE may have been reached with the lower concentration and that the fish have some mechanisms for disposing of or adapting to higher exposure levels. A possible explanation is that the bioactivation enzymes, presumably cytochromes P450, are also saturated at the lower concentration so similar amounts of oxon were being produced at both concentrations.

Gill, liver, and plasma AChE exhibited high percentage inhibitions; fewer AChE molecules were probably present in these tissues as reflected by their low control specific activities, so a low concentration of oxon could have yielded an apparently large effect. However, the low specific activity could also have been the result of low affinity isozymes in these tissues. Muscle and brain, with their high AChE activities, displayed a proportionately lower degree of inhibition.

Cp and Pth appear to have been rapidly metabolized to very potent and effective cholinesterase inhibitors. A lower *in vivo* concentration of Cp than Pth was required to inhibit a similar amount of AChE, which can be explained by the greater potency of Cpxn than Pxn as an anticholinesterase.

Cp exposure led to more persistent cholinesterase inhibition than Pth exposure in all tissues except plasma at the higher exposure concentration, although persistence was similar for the lower concentrations for both compounds. Lipophilic compounds such as Cp are generally more slowly eliminated across the gill resulting from binding with blood proteins. The log  $K_{ow}$  for Cp and Pth (at 20°C) are 5.11 and 3.81, respectively (Verschuere, 1983). Lipophilic compounds may also be sequestered, and slowly released and bioactivated. Such persistent AChE inhibition could jeopardize the fish during future exposures to OP compounds.

Wallace and Herzberg (1988) suggested that *de novo* AChE synthesis is responsible for recovery in rainbow trout (*Oncorhynchus mykiss*) after exposure to malaoxon and Pxn; they found that some reactivation occurs in fathead minnows (*Pimephales promelas*). More similar to the trout, channel catfish displayed very slow spontaneous reactivation (Carr et al., 1995).

DEF yielded poor cholinesterase inhibition in brain and muscle; this coincides with results of the *in vitro* assays. Defense mechanisms and/or the blood:brain barrier may be preventing DEF from reaching these tissues as the active metabolite(s). Low levels of cytochromes P450 in brain and muscle would lead to little, if any, target site activation. An increase in inhibition with time during the *in vivo* study suggests slow bioactivation. Habig et al. (1986) reported delayed cholinesterase inhibition in chan-

nel catfish brain (approximately 58%) and muscle (approximately 63%) after a 4-day exposure to DEF; however, they did not analyze gill, liver, and plasma.

The catfish hepatic monooxygenases were probably responsible for activating DEF to a toxic metabolite(s). Several studies (Wing et al., 1983, 1984; Habig and DiGiulio, 1988) demonstrated that DEF was converted to a more potent inhibitor of AChE after incubation in the presence of liver microsomes; Hur et al. (1992) suggested that sulfoxidation catalyzed by liver microsomal oxidases may be responsible for the active metabolite(s), possibly a sulfone and/or a sulfoxide. The rapid inhibition of hepatic AChE observed here may reflect the close proximity to a major site of bioactivation.

In the present study, the presumed metabolite(s) of DEF was/were very effective inhibitor(s) of AChE in gill and liver, demonstrating a similar pattern of inhibition and persistence as the two insecticides. This inhibition and persistence may also be attributed to the lipophilicity of DEF; the  $K_{ow}$  for DEF (at 20°C) is 5.52 (Bayer Agriculture Division, personal communication). Plasma AChE activity was inhibited moderately by DEF (56% at 8 h) but recovered completely by 240 h; either plasma can detoxify DEF more rapidly than the other tissues, or AChE is replaced more rapidly in plasma than in other tissues.

DEF treatments (alone or combined with an insecticide) yielded the most effective and persistent inhibition of ALiEs in all tissues and treatments although the high level of inhibition in all treatment groups yielded few statistical differences among treatments; fish from Cp treatments displayed more persistent inhibition than Pth treatments in all tissues. ALiE activity did not recover in the DEF treatments as it did in the Cp and Pth treatments. If DEF is more stable *in vivo*, the greater persistence of its effects could have resulted in re-inhibition of ALiEs following its release from stores and bioactivation.

The high level and persistent ALiE inhibition induced by DEF was expected to yield a potentiation of the toxicity of Cp or Pth. Exposure to DEF could allow more of the metabolite(s) of Cp or Pth to inhibit AChE if ALiEs are present and if ALiEs serve a protective function. (Experiments indicated that exposure to 2.0 mg/l DEF inhibited greater than 95% ALiE activity by 4 h after the exposure period.) The inhibition of ALiEs by DEF in mosquitofish has been implicated in altering the *in vivo* toxicity of the herbicide 2,4-D and its butyl ester (Chambers et al., 1977). Therefore, the results suggest that ALiEs do not serve effectively as protective enzymes in channel catfish. Control specific activities in the rat for liver and plasma ALiEs are 614 and 94  $\mu\text{mol min}^{-1} \text{mg protein}^{-1}$ , respectively (Chambers and Chambers, 1990); hence, rats appear to have several orders of magnitude more ALiEs available to serve a protective function. The low ALiE levels in channel catfish do not appear to be sufficient to afford the fish appreciable protection.

In summary, the sensitivity of AChE to Cpxn and Pxn correlates with the toxicity of the parent insecticides. The inherent sensitivity of the catfish ALiEs to organophosphate inhibition suggested a possible protective role but the ALiEs did not demonstrate an appreciable protective role in the intact catfish, probably because of their low concentrations. Additionally, the slow inhibition of brain and muscle AChE following DEF exposure suggested slow bioactivation. Lastly, exposure to Cp yielded more persistent esterase inhibition than did exposure to Pth.

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