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Inhibition of Acetylcholinesterase Activity in the Golden Apple Snail (*Pomacea canaliculata* Lamarck) Exposed to Chlorpyrifos, Dichlorvos or Carbaryl Insecticides

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Abstract

Long-acting inhibitors of acetylcholinesterase (AChE) are used in large amounts as insecticides in agriculture, and are thereby introduced into the evironment. By incubating golden apple snails in chlorpyrifos, dichlorvos, or carbaryl, we determined the LD_{50}'s to 3.7, 4.5 and 49 µM, respectively. AChE activities were measured in several organs; the highest activity was found in the gills. Following incubation in either of the three insecticides, the ED_{50}'s for AChE inhibition in the gills were 0.37 µM, 0.22 µM, and 14 µM, respectively; there were no sex differences, but AChE inhibition was more marked in larger snails than in smaller ones, and incubation at 32 °C brought about a higher AChE inhibition than at 27 °C. The time course of AChE inhibition was studied in snails incubated at ED_{50} with either of the inhibitors. There was a 50% enzyme inhibition after about 6 min, 5 min, and 7 min, respectively. After 96 h incubation, enzyme inhibiton reached about 90% in all three groups. Following this exposure to inhibitor, the snails were transferred to fresh water; after 30 days there was virtually no recovery of AChE actvity in the snails exposed to chlorpyrifos or dichlorovos, whereas about 20% was recovered in snails exposed to carbaryl.

Keywords: acetylcholinesterase; apple snail; carbaryl; chlorpyrifos; dichlorvos; enzyme inhibition; enzyme recovery; gills

1. Introduction

Most insecticides are used in agriculture, and the choice of insecticide depends to a great extent on their effectiveness, *i.e.,* their toxicity and their duration of action. Unfortunately, these characteristics make them harmful to the environment, as is wellknown from the debate on DDT in recent years. As a result, other insecticides have come in use which may be less toxic and have a shorter duration of action-characteristics which unfortunately also make them less desirable, not only in present-day, high-yield agriculture, but also for the eradiction of insect-borne diseases, such as malaria and dengue fever.

Several insecticides are potent, long-acting inhibitors of acetylcholinesterase (AChE). The duration of action of an insecticide depends, among other things, on how tight a bond it forms with the target molecule: in the present study we chose two organophosphates and one carbamide. The former are attached with tight bonds to AChE, with practically no recovery of enzyme activity, even after prolonged exposure in water free of insecticide (Chambers *et al*., 1989). The carbamides present other characteristics, which permit a slow, partial recovery of the enzyme activity when the test organism is exposed to fresh water. In the present study, we have investigated conditions for recovery of AChE activity after exposure to these two types of insecticides.

Following their release into the environment the insecticides remain active for considerable periods of time, and may thus act unfavourably on the biotope. Here we report an *in vivo* method for monitoring of AChE inhibition in a snail species which is abundantly present in areas of Thailand where these insecticides are used in large quantities.

2. Materials and Methods

2.1. Testing organisms

Adult (2.5 months old) golden apple snails *(Pomacea canaliculata* Lamarck) measuring 5.5-7.0 cm, and juveniles (1/2 month old) measuring 2.0-3.5 cm; were used throughout the study. They were acclimatized in an aerated water tank for 5 days, and fed with water fern, except for the last day before the experiment when they were fasted. Ambient water temperature was 27 ± 2 °C, pH 6.2-7.5, and dissolved

oxygen 6.5 ± 1.5 mg/l. The snails were held for at least 5 days before exposure to the insecticide.

2.2. Test conditions

The experiments were carried out in a continuous flow system in 20-litre test tanks with the same water characteristics as described above. Chlorpyrifos and dichlorvos (95% purity) were purchased from Gharda Chemicals Limited, Maharashtra, India. Carbaryl (99% purity) was purchased from Hunan Haili Chemical Industry Co.Ltd., Hunan, China. Aqueous solutions of the insecticides were prepared by dissolving each of them in acetone, diluted with an appropriate amount of water, and added to the aquaria. The concentration of acetone was 0.02 % in all test and control solutions. The AChE activity was not significantly influenced by the acetone (data not shown).

2.3. Acute toxicity

By probit analysis of the results from two replicates, the acute toxicity was calculated for each insecticide for each testing concentration. The 96 h LC_{50} for chlorpyrifos, dichlorvos and carbaryl were 6.1, 49, and 150 µM respectively. The sublethal concentrations of 3.7, 4.5, and 50 µM for chlorpyrifos, dichlorvos, and carbaryl, were estimated from probit regression and were selected as the sublethal concentrations for the study.

Variation in AChE activity in different target tissues was studied in gill, muscle, intestine, kidney and digestive gland of adult male snails.

Variation in AChE inhibition by sex was studied in adult male and female snails exposed to sublethal concentrations of the three insecticides in replicate aquaria. Control groups were kept in water. After 96 h exposure, the snails were sacrified and AChE activity was measured in gill tissues.

To investigate variation in AChE inhibition by snail size, two groups of snails, 5.5-7.0 cm and 2.0-3.5 cm, respectively, were used and exposed to the insecticides in the same manner as above. To avoid any potential effects from sex, only male snails were used. Following an exposure period of 96 h, the snails were sacrified and gill tissues excised for analyses of AChE activity.

To investigate variation in AChE inhibition due to different water temperature, adult male snails were used and treated at the sublethal concentration of insecticides at 27 °C and 32 °C. Following an exposure period of 96 h, the snails were sacrified and gill tissues excised for analyses of AChE activity.

The relationship between insecticide concentrations and AChE inhibition was examined over the sublethal ranges, 0-3.7 µM, 0-4.5 µM, and 0-50 µM for chlorpyrifos, dichlorvos and carbaryl, respectively, each for 96h. In the toxicokinetic study, snails were exposed to 3.7, 4.5 or 50 µM of chlorpyrifos, dichlorvos or carbaryl, respectively, in the continuous flow system for 96h. Snails were then moved to a tank supplied with a continuous flow of insecticide-free water for another 30 days. AChE activity in the gill tissues were measured during exposure and after transfer to insecticide-free water.

2.4. AChE activity analysis

Tissue samples were excised and homogenized (20 mg/ml) in 20 mM Tris/HCl, pH 7.5, and 0.5 mM EDTA. Aliquots of tissue homogenate were centrifuged and supernatant was taken for AChE analysis. This was carried out following the method of Ellman *et al*. (1961), which is based on a colorimetric assay employing the reaction of thiocholine with 5,5 –dithiobis-(2-nitrobenzoic acid) (DTNB). The reaction produces the compound 5-thio-2-nitrobenzoic acid and is effective for colorimetric measurement with absorbance at 412 nm.

2.5. Protein analysis

Total protein analysis was conducted with 100 µl of tissue homogenate from each snail using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Life Sciences Group, CA, USA). Colorimetric analysis was performed following the standard procedure outlined in the protein assay instructions. Bovine serum albumin (BSA) was used to obtain a standard curve (Bradford, 1976).

2.6. Data analysis

Results are expressed as means + SD. ANOVA were used to test for differences between each treatment. Results are expressed as AChE activity (nmoles/min/mg protein).

3. Results and Discussion

3.1. Variation in AChE activity in different target tissues

The specific AChE activity was highest ($p<0.05$) in gill followed by intestine, muscle, kidney and digestive gland, respectively (Fig. 1). For this reason, gill tissue was selected for the study of the effects of sex, size, water temperature, exposure concentrations, and duration of exposure.

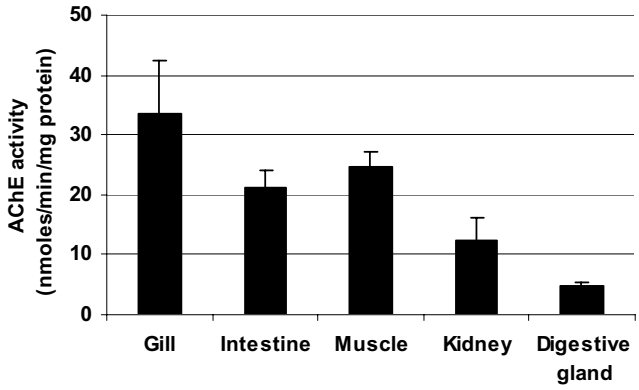

Figure 1. Acetylcholinesterase actvity in several organs of the golden apple snail (means + SD, n=5).

A high AChE activity in the gills has previously been observed in Spanish mussel (*Mytilus galloprovincialis*) (Mora *et al.,* 1999); it is presumably due to the presence of the gill ganglion, located in the branchial nerve which is responsible for the innervation of the gills.

3.2. Variation in AChE activity by sex, body size, and water temperature

AChE activity in controls and in snails exposed to the chlorpyrifos, dichlorvos and carbaryl did not differ significantly between male and female snails ($p>0.05$) as shown in Fig. 2, which is in agreement with observations by Beauvais *et al*. (2002) who studied AChE activity in brain from bluegill (*Lepomis macrochirus*).

In sharp contrast, AChE activity in gills of exposed snails differs significantly with body size ($p<0.05$), as small snails have higher AChE activity than large snails (Fig. 3).

Snails treated at ambient water temperature (27 °C) showed significantly higher AChE activity than those in the higher water temperature (32 °C) (Fig.4).

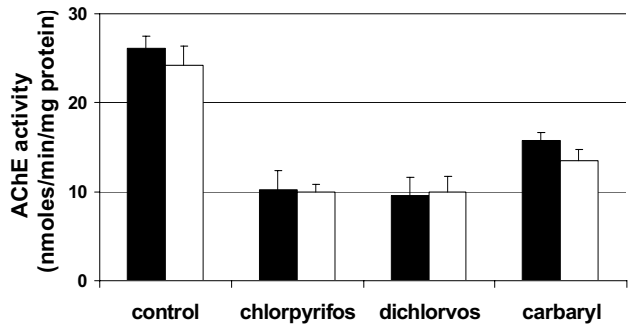

Figure 2. Acetylcholinesterase actvity in gills of male (black bars) and female (open bars) of the golden apple snail (means + SD, n=5).

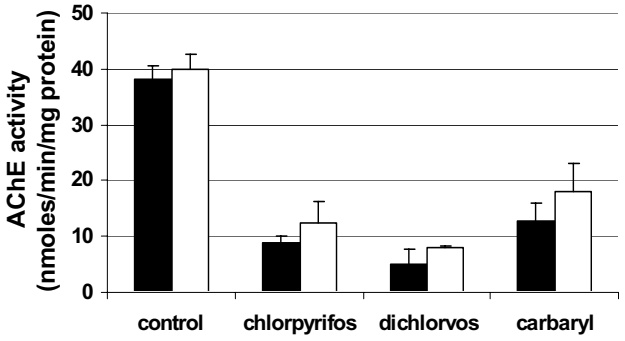

Figure 3. AChE activity (mean + SD, n=5) in gills from large snails (black bars) was significantly lower than from small snails (open bars). All three insecticides were significantly more effective in large snails than in small snails ($p<0.05$).

3.3. Variations in AChE activity during insecticide exposure at various concentrations

AChE activity in gills decreased significantly with increasing concentrations of insecticides as shown in Fig. 5. AChE activity in snails exposed to chlorpyrifos and dichlorvos, decreased more rapidly than those exposed to carbaryl. We calculated the ED_{50}s to 0.37, 0.22, and 14 μM for chlorpyrifos, dichlorvos and carbaryl, respectively. Thus, dichlorvos and chlorpyrifos are much more effective in inhibiting AChE than carbaryl. Table 1, which provides comparisons based on molar concentrations, demonstrates highly varying toxicities in different aquatic species. The organophosphates were always more toxic than carbaryl.

3.4. Time course of gill AChE inhibition and recovery

Time-course studies of gills from the snails exposed to sublethal concentration of chlorpyrifos revealed a 50% AChE inhibition already after about 6

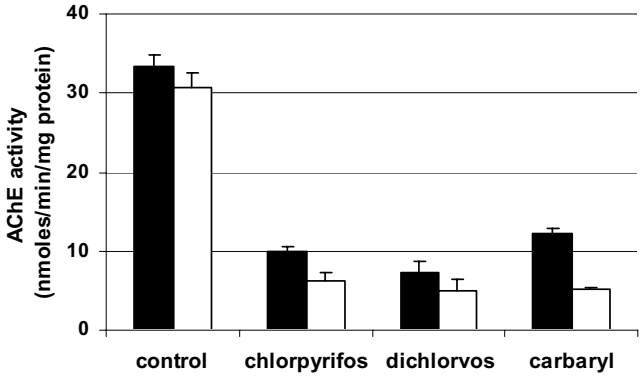

Figure 4. AChE activity (means+ SD, n=5) in gills from snails incubated at 27 °C (black bars) was significantly higher than from that snails incubated at 32 °C (open bars). All three insecticides were significantly more effective on snails incubated at 32 °C than on those incubated at 27 °C ($p<0.05$).

Table 1. LC_{50} for some pesticides in aquatic animals (μM)

Species	Incubation time, h	Chlorpyrifos	Dichlorvos	Carbaryl	Reference
Apple snail (*Pomacea canaliculata* Lamarck)	96	3.7	4.5	49	This study
Snail (*Pomacea patula)*	96			73*	Mora *et al*. 2000
Goldfish (*Carassius auratus*)	96			70*	Ferrari *et al*. 2004
Mussel (*Mytulis edulis*)	24		37 *		McHenery *et al.*1997
Lobster larvae (*Homarus gammarus*)	96		0.026*		Quoted from McHenery *et al.* 1997
Barnacle (*Balanus balanoides*)			20.4*		”
Herring larvae (*Clupea harengus*)			0.55*		”
Copepod (*Temora longicornis*)	24		0.64*		”
Bluegill (*Lepomis macrochirus*)	96	0.007*			Johnson and Finley 1980 quoted from Doran *et al.* 2001
Channel catfish (*Icalurus punctatus*)	96	0.80*			”
Mayfly (*Cloen dipterum*)	96	0.0007*			Barron and Woodburn 1995 quoted from Doran *et al.* 2001
Snail (*Aplexa hypnorum*)	96	>2.30*			”

* Calculated from published metric data.

min of incubation of the snails. The corresponding figures for dichlorvos and carbaryl were about 5 min and 7 min, respectively (Fig. 6).

In snails exposed to chlorpyrifos or dichlorovos for 96h, and then returned to fresh water for up to 30 days, there was virtually no recovery of AChE actvity. In snails exposed to carbaryl for 96h and then put into fresh water, about 11% of AChE activity was recovered already after 6 h, and 23 % was recovered after 30 days (Fig. 7).

The lack of recovery of AChE actvity (Table 2) after chlorpyrifos or dichlorvos treatment-even after 30 days in fresh water is similar to the results from a study of the anterior adductor muscle of the three-ridge mussel (*Amblema plicata*) (Doran *et al*. 2001). The marked recovery after treatment with carbaryl

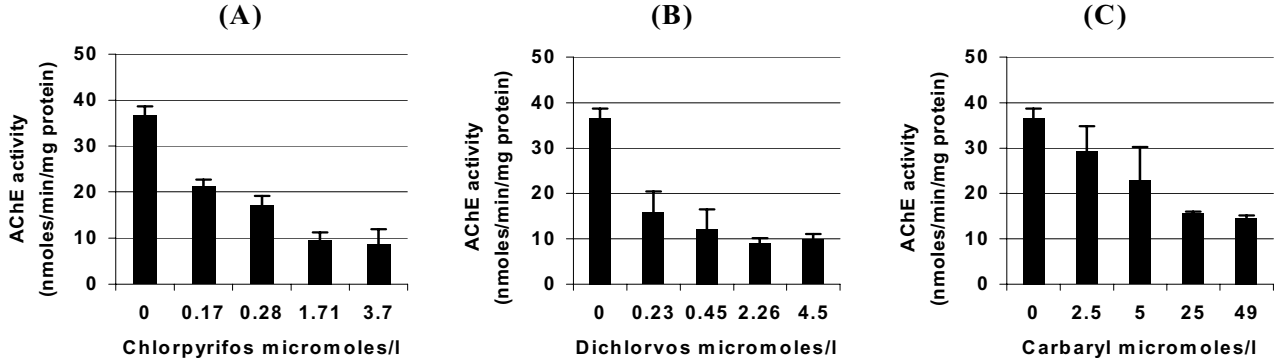

Figure 5. (A-C). AChE activity (means + SD, n=5) in gills from snails incubated in rising concentrations of chlorpyrifos, dichlorvos, carbaryl.

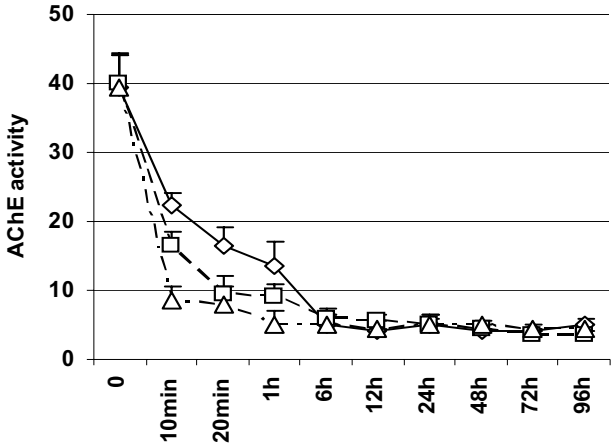

Figure 6. Time-course study of AChE activity,(moles ACTC/ min, mg protein; means, SD, n=5) in gills from snails during exposure to 0.22 µM dichlorvos (∆), 0.37 µM chlorpyrifos (□), or 14 µM carbaryl (◇).

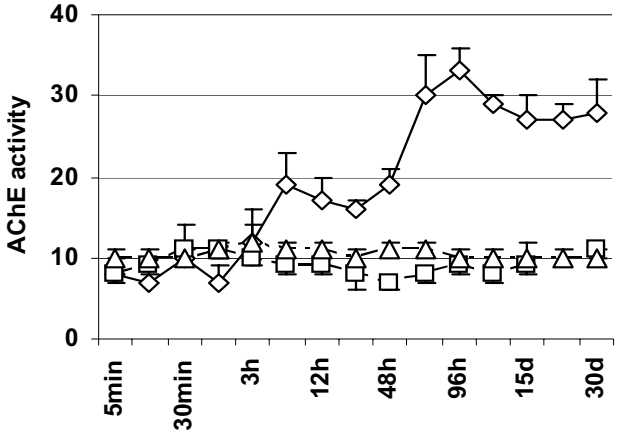

Figure 7. Time-course study of AChE activity (nmoles ACTC/min, mg protein; means, SD., n=5) in gills from snails during recovery from treatment with 0.22 µM dichlorvos (∆), 0.37 µM chlorpyrifos (□), or 14 µM carbaryl (◇).

already after 6 h is of interest when compared to findings by Ferrari et al in the gold fish brain (*Carassius auratus*) in which there was a 75% recovery after 96h.

Studies on the recovery of AChE activity have focused on the dissociation of the enzyme-inhibitor complex. This process depends on the degree of "aging" the enzyme: the longer the exposure to the inhibitor, the less reactivation takes place when the inhibitor is removed. In our study, where we used 96 h exposure to the inhibitors, carbaryl treated animals showed a robust enzyme recovery by some 20% after 72h, while the organophosphate treated animals had almost no recovery, even after 30 d. This would indicate less aging of carbaryl than of the organophosphates.

Studying rats, Chambers *et al.*(1989) reported that aging, estimated by the proportion of inhibition remaining after exposure to an organophosphate (paroxon), gradually increased from none on the day of treatment to virtually total after 4 days of treatment. In this case the recovery treatment included in vitro exposure to an oxime reactivator.

Two other processes may contribute to the return of AChE activity after inhibition with organophosphates or carabryl, *viz.,* a breakdown of the enzyme-inhibitor complex in the tissues, and a synthesis of new enzyme molecules. Whether or not any of these processes play any significant role for the return of AChE activity remains to be studied.

We conclude that the golden apple snail is well suited for monitoring contamination of insecticides which inhibit AChE activity. On a molar basis, the organophosphates chlorpyrifos and dichlorvos were at least 10 times more toxic than carbaryl. Moreover, the enzyme inhibition brought about by the two former compounds was virtually irreversible even after 30 days' exposure to fresh water, whereas carbaryl in-

Table 2. Recovery from AChE inhibition; mean percent activity recovered after incubation in fresh water

Species	Organ	Incubation time in fresh water	Chlorpyrifos	Dichlorvos	Carbaryl	Reference
Apple snail (*Pomacea canaliculata* Lamarck)	Gill	30 d	5	0	19	This study
Snail (*Pomacea patula)*	Digestive gland	1 h			50	Mora 2000
Goldfish (*Carassius auratus*)	Brain	96 h			75	Ferrari *et al*. 2004
Three-ridge mussel *(Amblema piccata*)	Muscle	21 d	No recovery			Doran *et al.* 2001

toxication showed clear signs of reversibility. Contamination by the organophosphates could therefore be monitored for considerable periods of time-perhaps several months after their release into the biotope.

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